

Institute for Environmental and Animal Hygiene
and Veterinary Medicine (with Animal Clinic)
Faculty of Agricultural Sciences
University of Hohenheim

Department: Environmental and Animal Health
Prof. Dr. Reinhard Böhm

**Antimicrobial Activity of Thai Traditional Medicinal Plants Extract
Incorporated Alginate-Tapioca Starch Based Edible Films
against Food Related Bacteria Including Foodborne Pathogens**

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Presented by
Racha Tepsorn
B.Sc., M.Sc. Food Science and Technology
Pattani, Thailand
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Examination Committee

Supervisor and Review Prof. Dr. Reinhard Böhm

Co-Reviewer Prof. Dr. Herbert Schmidt

Additional examiners Prof. Dr. Werner Bessei

Vice-Dean and Head of Committee Prof. Dr. Werner Bessei

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meines Bruders, für mein geliebtes Heimatland und
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แต่ แผ่นดินไทย บ้านเกิดอันเป็นที่รัก และชาวไทยทุกท่าน
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LIST OF USED ABBREVIATIONS

% (v/v)	= percentage volume by volume
°C	= degree Celsius
A549	= Carciomic human alvedar basal epithelial cells
<i>approx.</i>	= approximately
ATCC	= American Type Culture Collection
BGM	= Buffalo-green-monkey cells
BSA	= Bovine Serumalbumin
CFU/g	= Colony Forming Unite/gram
CFU/ml	= Colony Forming Unite/milliliter
cm	= centimetre
cm ²	= square centimetre
DIN	= Deutsche Institut für Normung e.V.
DMEM	= Dulbeccos minimum essential medium
DMST	= Department of Medical Science Thailand
DPPH	= 1,1-Diphenyl-2-picrylhydrazyl
DSMZ	= Deutsche Sammlung von Mikroorganismen und Zellkulturen
DVG	= Deutsche Veterinärmedizinische Gesellschaft
EDTA	= Ethylendiamine tetraacetic acid
EN	= Europäische Norm
FIC	= Fraction Inhibitory Concentration
FKS	= Fetal caft serum
g/100 ml	= gram/100 milliliter
IC ₅₀	= half maximal Inhibitory Concentration
L	= Litter
MBC	= Minimum Bactericidal Concentration
MDCK	= Madin-Darby canine kidney cells
mg	= milligram
mg/ml	= milligram/millilitre
MIC	= Minimum Inhibitory Concentration
ml	= millilitre

MTCC	= Microbial Type Culture Collection and Gene Bank
Na-EDTA	= Ethylenediamine tetraacetic acid-Sodium salt
ppm	= part per million
rpm	= round per minute
TH	= Terapene heart cells
v/v	= volume by volume
WSH	= Wasser standardisierten Härtegrades
µg/ml	= microgram / millilitre
µl	= micro litter

1 INTRODUCTION AND OBJECTIVE

1.1 Introduction

The increased consumer demand for high quality, long shelf-life, and ready-to-eat foods has initiated the development of only mildly preserved products that keep their natural and fresh appearance as far as possible. Microbiological growth commonly induces undesirable organoleptic and appearance change during the storage of food products. If bacterial growth in food products could be delayed, or halted, large gains in products shelf-life would be possible.

Avoidance of pathogenic and spoilage microorganisms in food is usually achieved by using chemical preservatives. These chemicals act as antimicrobial compounds which inhibit the growth of undesirable microorganisms. While a number of traditional or regulatory approved antimicrobials, they have many limitations. There is a currently strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogen attributes as well as residual toxicity. For these situations, consumers have a propensity to be suspicious of chemical additives and thus the exploration of naturally occurring antimicrobials for food preservations receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Skandamis *et al.*, 2001; Schuenzel and Harrison, 2002). That has led to search for novel antimicrobial compounds from natural sources. Naturally derived compounds and other natural products may have applications in controlling bacteria in foods (Deloguis and Mazza, 1995, Bowles and Juneja, 1998). The primary incentive for indentifying effective antimicrobials among naturally occurring compounds is to expand the spectrum of antimicrobial activity over that of the regulatory-approved substances. Traditional antimicrobials are generally limited to high acid, low fat food products because of interactions with pH and food components. Interest in natural antimicrobials is also driven by the fact that international regulatory agencies are generally very strict as to requirements for toxicological evaluation of novel direct food antimicrobial. One group of naturally derived antimicrobial compounds is

medicinal plants and their essential oils. These compounds have been safe, have been shown to have varying degree of antimicrobial activity, and could provide another hurdle to growth of foodborne pathogens and spoilage bacteria, thereby improving the shelf-life of food products. Numerous studies have reported that medicinal plants produce a large number of secondary metabolites with antimicrobial effects on pathogens (Mari *et al.*, 2003; Obagwu and Korsten, 2003). Medicinal plant extracts, therefore, for the control of the growth of foodborne pathogens and food spoilage bacteria are emerging as alternatives to conventional natural preservatives as they are generally safe to humans, and environmentally friendly (Thangavelu *et al.*, 2004).

However, natural antimicrobial activity of medicinal plants and their essential oils is often variable. Most research on medicinal plants as natural antimicrobial has been conducted *in Vitro* in microbiological media. Applications of these substances to food are limited and the activity in food is very different because of the microenvironment in food. There exists a need to improve the activity of these antimicrobial compounds in foods.

Antimicrobial packaging is a promising form of active food packaging, and come to be the one of the approaches to prevent contamination of bacteria on the surface of food products and delay spoilage. An ideal solution for the food industry to overcome the food safety and environment problems is to incorporate antimicrobial substances in to edible films (Padgett *et al.*, 1998). Several antimicrobial agents were incorporated into edible films and were shown to inhibit the food spoilage bacteria. Soy protein can be used to produce edible antimicrobial film to apply antimicrobial agents on the surface of food products.

The increase of the demand for fresh, convenient, and long shelf-life products presages a bright future for antimicrobial packaging. The effectiveness of natural plant extracts is needed to be evaluated to specify their antimicrobial activity and potential side effects in package food. Therefore, the study of the antimicrobial activity of some Thai traditional medicinal plants and the application in edible film has become an important research interest and a big challenge.

1.2 Objective

The objectives of this study were to investigate

1. The efficacy test of Thai traditional medicinal plants included dried leaves of King of Bitter (*Andrographis paniculata* (Burm.f.) Wall. ex Nees), dried rhizomes of Zedoary (*Curcuma zedoaria* (Christm.) Roscoe), dried fruit shells of Mangosteen (*Garcinia mangostana* Linn.), dried flower of Roselle (*Hibiscus sabdarifa* Linn.), dried blossom of Banana (*Musa sapientum* Linn.), dried rhizomes of Lotus (*Nelumbo nucifera* Gaetn.), dried leaves of Betel leaf (*Piper bettle* Linn.), dried fruit shells of Pomegranate (*Punica granatum* Linn.), and dried leaves of Guava (*Psidium guajava* Linn.) The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and suspension test (Time-killing analysis) were carried out against foodborne pathogens and food spoilage bacteria
2. The effect of different extraction method on the antimicrobial activity obtained from Thai traditional medicinal plants were determined by measuring the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) against foodborne pathogens and food spoilage bacteria
3. The effect of interfering substances, representing the microenvironment in food matrix on the antimicrobial properties of Thai traditional medicinal plant extracts
4. Evaluation of antimicrobial activities of Thai traditional medicinal plant extracts-incorporated alginate-tapioca based edible film against foodborne pathogens and food spoilage bacteria
5. The antimicrobial properties of alginate-tapioca based edible film and coating in model food system against foodborne pathogens and food spoilage bacteria

2 REVIEW OF LITERATURE

2.1 Medicinal Plants (Herbs)

The word “herbs” comes from the Latin ‘*herba*’. It means a medicinal plant. The meaning of herb in a narrow sense is a non-lasting plant that withers after blooming without its stems becoming woody. Perennial herbs are widely used for the purpose of dyeing and gardening utilized for medical purpose. Some edible herbs belong to the category of spice. Even some herbs containing poisonous components can be categorized as spices if the poisonous element can somehow be naturalized with heating or other cooking procedure. An herb is botanically classified as a perennial plant, but the meaning of spice comes from its use in cooking, not any plant classification. A medicinal plant should, therefore, be edible. In fact, no spice definitions distinguish clearly between a spice and an herb. The term spice can be broadly defined as a compound that has a pungent flavour or colouring activity or one that increases appetite or enhances digestion. Herbs contain some complicated mixtures of organic chemicals that may vary depending upon many factors related to the growth, production, and processing of the herbal product. A herb is obtained from seed, berries, buds, leaves, bark and roots of plants growing mainly in the tropical, the subtropical and the temperate zone. Culinary herbs and their essential oils have been used extensively for many years in food products, perfumery, dental and oral products due to their different medicinal properties (Suppakul *et al.*, 2003a; 2003b).

Nowadays, food professionals continually search for “new” and unique medicinal plants because of the growing global demand for authentic ethnic and cross-cultural cuisines. Consumers are also seeking natural foods and natural preservatives for healthier lifestyles and natural ways of preventing ailments. So, medicinal plants are also being sought for their medicinal value, as antioxidants and as antimicrobials.

2.1.1 *Andrographis paniculata* (Burm.f.) Wall. ex Nees (King of bitters)

2.1.1.1 General information

Andrographis paniculata (Burm.f.) Wall. ex Nees is a well known plant with many names. Scientific synonyms are *Andrographis paniculata* Wall ex. Nees (DMPRD, 1990) and *Justicia paniculata* Burm. f. English name is King of Bitters and other names are Chiretta, The Creat, Creyat Root, Halviva, Kariyat, Green Chiretta and Kreat (Manjunath, 1948; Huang, 1993; Ruengrungsri and Tuntiwat, 1994; Maunwongyathi, 1994). The local Thai names of *Andrographis paniculata* (Burm.f.) Wall. ex Nees are varying, depending on the region. In central plain region in Thailand, it is called Num-Lai-Pung-Porn, Fah-Tha-Laai-Joan or Kei-Tai-Yai-Klum. In southern region it is called Ya-Gun-Ngoo, Fah-Sa-Tan or Mek-Tha-Laai. In northeast region it is called Sam-Sib-Dee (Thai Pharmacopoeia, 1997; Maunwongyathi, 1994).



Figure 1 *Andrographis paniculata* (Burm.f.) Wall. ex Nees (King of bitter)

Andrographis paniculata (Burm.f.) Wall. ex Nees is the member of *Acanthaceae*. This family includes many species, expected to have medical properties (Hanchanlerd

et al., 1994; MPRI, 1999). *Andrographis paniculata* (Burm.f.) Wall. ex Nees is an annual-branched, erect-running 0.5-1.0 m in height. Its stem is dark green, 2-6 mm in diameter, quadrangular with longitudinal furrows and wings at angles of the younger parts (Figure 1). The leaves are opposite, decussate, lanceolate, up to 8 cm long and broad, glabrous, margin entire, and venation pinnate; the petiole is very short. The flowers are small with bilabial corollas. The fruits are small 2-celled odourless capsules which taste intensely bitter. *Andrographis paniculata* (Burm.f.) Wall. ex Nees grows wild and abundantly in Southeast Asia. In cultivation, it is cultivated extensively in China and Thailand. It is found in evergreen, pine and deciduous forests and along roadsides. The plant has been observed to grow luxuriously in mild humid locations with tropical temperature and high rainfall (DMPRD, 1990; MPRI, 1999).

The harvesting should be performed from the beginning of the flowering period until around 50% blooming (Dechatiwongse na Ayudhya *et al.*, 1988) and when have an age of 110-150 days (Suwanbareerak and Chaichantipyuth, 1991). This period will give highest percentage of active ingredients in *Andrographis paniculata* (Burm.f.) Wall. ex Nees. The plants will bloom fast or slow depending on the environmental situation (Promoto *et al.*, 1997). By harvesting the stem should be cut 5-10 cm above ground level to allow for renewed growth for the next harvest.

The leaves of *Andrographis paniculata* (Burm.f.) Wall. ex Nees can be found on markets in every region of Thailand. A farmer in Kanjanaburi Province, the centre region of Thailand, has cultivated *Andrographis paniculata* (Burm.f.) Wall. ex Nees plants for 3 years. The useable leaves were harvested up to 1000 kg dried per 1,600 m² per year. The cost can be up to 100,000 Bath or 2,000 Euro (Chaiwongkeart, 1997).

2.1.1.2 The uses of *Andrographis paniculata* (Burm.f.) Wall. ex Nees

Andrographis paniculata (Burm.f.) Wall. ex Nees is a traditional medicinal plant. It has been extensively studied, most of it in the last half 20th century, especially

concentrating on pharmacological, composition, safety, efficacy and mechanism of action (Tang and Eisenbrandt, 1992). It has been frequently used for centuries to successfully treat upper respiratory tract infections, fever, sore throat, herpes and also to reduce inflammation and stop diarrhoea (Techadmrogsin *et al.*, 1999). In ancient Chinese medicine writing, *Andrographis paniculata* (Burm.f.) Wall. ex Nees is an important “cold property” herb, it is used to rid the body of heat, as in fevers and to dispel toxins from the body (Bensky and Gamble, 1993; OMPD, 1989). Moreover, pharmacological properties of *Andrographis paniculata* (Burm.f.) Wall. ex Nees were reported including anti-inflammatory, antipyretic, diabetes, hypertension, antibacterial, immunological, antivenin, antithrombotic, hepatoprotective, antifertility, counteract, induce subsidence of swelling, healing influenza with fever, sore throat, ulcers in the mouth, acute or chronic cough, colitis, dysentery, urinary infections with difficult painful urination, carbuncles, and venomous snake bite (Deng *et al.*, 1982; Yeung *et al.*, 1987; Akbarsha *et al.*, 1990; Handa and Sharma, 1990; Ahmad and Asmawi, 1993; ACACMSP, 1995).

Andrographis paniculata (Burm.f.) Wall. ex Nees is also well known in the Indian Pharmacopoeia. It is prominent in at least 26 Ayurvedic formulas. In Scandinavian countries, *Andrographis paniculata* (Burm.f.) Wall. ex Nees is also used extensively to prevent and treat common colds (Research Review, 1997). In Thailand, the utilization of *Andrographis paniculata* (Burm.f.) Wall. ex Nees as a medicinal herb has a long history of successful use in treatment of numerous conditions. Thai Traditional herbalists use *Andrographis paniculata* (Burm.f.) Wall. ex Nees leaves powder to reduce diarrhoea, sore throat, cough, inflammations from injuries. It is also used as internal and external drug. It can be used for dysentery, intestinal inflammation, common cold, tonsillitis, influenza, healing fire burns, healing itching (OMPD, 1989; DMPRD, 1990; Maunwongyathi, 1994; PMPPHC, 1996). An extract of boiled *Andrographis paniculata* (Burm.f.) Wall. ex Nees was suggested to be effective against *Staphylococcus aureus*, the methanolic extract can be effective against *Proteus vulgaris* and *Shigella* bacteria but it is not effective against cholera. Also an ethanol extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees is

effective against *Staphylococcus aureus*, while hot water extract is not effective. An ethanol extract made by boiling is effective to *E. coli* (Bunyapraphatsara, 2000).

Andrographolide is a major compound isolated from *Andrographis paniculata* (Burm.f.) Wall. ex Nees. It is of colourless crystalline appearance with a very bitter taste and this compound was identified as lactone (SCHRI, 1973). There are four lactones isolated from *Andrographis paniculata* (Burm.f.) Wall. ex Nees, including deoxyandrographolide (Andrographis A), andrographolide (Andrographis B), neoandrographolide (Andrographis C), and deoxydidehydroandrographolide (Andrographis D) (Garcia *et al.*, 1980; Deng *et al.*, 1982; Sangalungarn *et al.*, 1990; Dhamma-Upakorn *et al.*, 1992).

The other medicinal chemicals are also bitter principles which are deoxyandrographolide, 19 β -D-glucoside and neo-andrographolide. These compounds were identified as diterpenoids (Cava *et al.*, 1965; Chem and Liang, 1982; Techadamrongsin *et al.*, 1999).

Diterpene lactones and flavonoids are also the active chemical constituents of *Andrographis paniculata* (Burm.f.) Wall. ex Nees. The main diterpenoids are 14-deoxyandrographis and 14-deoxy-11, 12-didehydroandrographolide (Balmain and Connolly, 1973; Zhu and Liu, 1984; Kuroyanagi *et al.*, 1987; Tang and Eisenbrandt, 1992; Matsuda *et al.*, 1994; Kongkathip, 1995).

The concentration of these diterpene lactones depend both on growing region and season. The leaves contain the highest amount of the active components and the stems contain the lower (MPRI, 1999), while the seeds contain the lowest amount (Sharma *et al.*, 1992). Several studies have focussed at the disposition of andrographolide in various organs of the body. For the antimicrobial activity of *Andrographis paniculata* (Burm.f.) Wall. ex Nees, the results obtained for antimicrobial and antifungal activity of aqueous extracts showed that andrographolide and arabinogalactan proteins had significant antibacterial and antifungal activities in comparison to some known antibiotics (streptomycin, gentamicin and nystatin). The investigation revealed the

biological value of the cumulative effects of arabinogalactan proteins and andrographolide resulting in enhanced antimicrobial activities (Singha *et al.*, 2003).

In the case of antidiarrheal effect, extracts of *Andrographis paniculata* (Burm.f.) Wall. ex Nees have been shown to have significant effect against the diarrhoea caused by an *Escherichia coli* infection. The components of *Andrographis paniculata* (Burm.f.) Wall. ex Nees extract, andrographolide deoxyandrographolide, and neoandrographolide, showed analogous activity to loperamide (Imodium), the most common antidiarrheal drug (Gupta *et al.*, 1990).

2.1.2 *Curcuma zedoaria* (Christm.) Roscoe (Zedoary)

2.1.2.1 General Information

The Zingiberaceous plant *Curcuma zedoaria* (Christm.) Roscoe, is commonly called zedoary in Thai as Khamin Oi. Its common names in various countries are Zedoary (English); Zedoarwurzel, Zittwer (Germany); Zedaire, Zedaire bulbeux (France); Zedoaria (Italian); Kachura, Kalihaladi (Hindi); Temoelawa (Java); Gajusutsu (Japan); Er-Chu (China) (Kirtikar and Basu, 1980; Saralamp *et al.*, 2000; Matsuda, *et al.*, 2001a; Mau *et al.*, 2003).

Curcuma zedoaria (Christm.) Roscoe (Figure 2) is considered to be a native of the North-Eastern India and spread in cultivation throughout the Indian subcontinent and Malaysia. It grows mainly on South and Southeast Asia countries including China, Vietnam, India and Japan. This plant is a perennial herb. It is a rhizome, or underground stem, like turmeric and ginger.

The rhizome is large and tuberous with many branches. The interior of the rhizome is yellow and when dried, has an agreeable musky odour with a slight smell of camphor and a pungent bitter taste. The main tubers, known as bulb, are about 8 × 5 cm, with many short, thick branches and tuberous roots, known as finger (Purseglove *et al.*, 1981; Hong *et al.*, 2002; Morikawa *et al.*, 2002).

The rhizome contains many types of sesquiterpenoids and yellow pigment called curcuminoids (Evan, 2002). The leafy shoots are up to 1 metre tall with about 4-6 leaves with long green petioles. Its leaves are oblonglanceolate, finely acuminate, glabrous on both surfaces, clouded with purple down the middle, 30-60 cm long. The inflorescences, about 22 cm tall, are separate from the leaf shoots. The spikes are about 16 cm tall, with the lowest green bracts, the middle bracts tipped with purple and the uppermost bracts entirely purple. The flowers, about five to each bract, are pale yellow in spikes. The flowering bracts, 3-8 cm long, are ovate, recurved, cymbiform, green tinged with red. The bracts of the coma are crimson or purple about 5 cm long. The calyx, about 8 mm long is obtuse, 3-toothed. The corolla is tube twice as long as the calyx, funnel-shaped. The lip is broad, sub-orbicular, deflexed, and deep yellow. The capsule is ovoid, thin, smooth, bursting irregularly. Seed are ellipsoid with a white lacerate aril (Hooker, 1894; Kirtikar and Basu, 1980).



Figure 2 *Curcuma zedoaria* (Christm.) Roscoe (Zedoary)

2.1.2.2 The uses of *Curcuma zedoaria* (Christm.) Roscoe

Curcuma zedoaria (Christm.) Roscoe, has been widely cultivated as a vegetable or spice in East, South and Southeast Asian countries including China, Vietnam, India, Japan and Thailand (Saralamp *et al.*, 2000; Hong *et al.*, 2002). In Japan it is listed in the Japanese Pharmacopeia XIII and there and in the Chinese traditional medicine, *Curcuma zedoaria* (Christm.) Roscoe has been prescribed as a stomachic, emmenagogue, or for the treatment of “Oketsu” syndrome, which is presumed to be caused by blood stagnation and for promoting menstruation in various preparations (Shiobara *et al.*, 1985; Matsuda *et al.*, 1998; Yoshikawa *et al.*, 1998; Matsuda *et al.*, 2001c). Furthermore, *Curcuma zedoaria* (Christm.) Roscoe also has been used as an important fragrance and spice (Matsuda *et al.*, 2001a).

In Thailand, *Curcuma zedoaria* (Christm.) Roscoe has long been used in many preparations to relieve stomach discomfort, antidiarrhoeal, antiemetic and antipyretic. It is also used externally as an astringent for wounds (Saralamp *et al.*, 2000).

Curcuma zedoaria (Christm.) Roscoe contains curcuminoids, including curcumin, demethoxycurcumin and bisdemethoxycurcumin and volatile oils including sesquiterpenes, and monoterpenes. The major sesquiterpene compounds, including dehydrocurdione, furanodiene, germacrone, curdione, curcumenol, neocurdione, isocurcumenol, aerugidiol, zedoarondiol and curcumenone were found to show biological activities (Syu *et al.*, 1998; Yoshioka *et al.*, 1998; Mau *et al.*, 2003).

These biological activities of *Curcuma zedoaria* (Christm.) Roscoe include analgesic, anti-cancer, antihepatotoxic, anti-inflammatory, antioxidant, antimicrobial, antifungal, anti-mutagenic as well as vasorelaxant activities (Gupta *et al.*, 1976; Lee and Lin, 1988; Matsuda *et al.*, 1998; Syu *et al.*, 1998; Yoshioka *et al.*, 1998; Matsuda *et al.*, 2001a; Matsuda *et al.*, 2001b; Hong *et al.*, 2002; Lee *et al.*, 2002; Morikawa *et al.*, 2002; Navarro *et al.*, 2002; Mau *et al.*, 2003).

2.1.3 *Garcinia mangostana* L. (Mangosteen)

2.1.3.1 General information

Mangosteen (*Garcinia mangostana* L.) (Figure 3) belongs to the family Guttiferae (Morton, 1987). It is known as mangosteen (English), mangostanier (French), mangostan (Spanish), manggis (Malaysian), manggustan (Philippine), mongkhut (Cambodian), mangkhut (Thai) and can cut (Vietnamese) (Nakasone and Paull, 1998). The fruit is 4-7 cm across covered by the smooth and hard pericarp about 6-10 mm thick and end with the persistent calyx. The pericarp turns purple at ripening and exudes latex with bitter yellowish and purple-staining juice if damaged (Nakasone and Paull, 1998). When compared with other tropical fruits, mangosteen has a comparatively small edible partition or aril as the pericarp is thick and tough. The edible aril, which makes up 30% of total fruit weight is of pearly white colour, slightly translucent and consists of 4 or 8 segments with one or two large segments containing apomictic seeds and have no limiting membrane (Martin, 1980).



Figure 3 *Garcinia mangostana* L. (Mangosteen)

2.1.3.2 The uses of *Garcinia mangostana* L.

Fresh fruit of mangosteen is widely consumed in south Asia and South East Asia. The round dark purple-brown fruit looks rather like a smooth small oddly coloured cricket ball. The juicy flesh of the mangosteen is similar to that of a lychee.

The antifungal activity of xanthone isolated from fruit hulls of *Garcinia mangostana* L. and some derivatives of mangostin against *Fusarium oxysporum* f. sp. *vasinfectum*, *Alternaria tenuis* and *Drechslera oryzae* was evaluated. The natural xanthones inhibited the growth of all fungi (Gopalakrishnan *et al.*, 1997; Gopalakrishnan and Banumathi, 2000).

An extract of *Garcinia mangostana* L. showed inhibitory effects against the growth of *Staphylococcus aureus* and some components also had activity against methicillin-resistant *Staphylococcus aureus* (MRSA). One active isolate, α -mangostin, a xanthone derivative, had a minimum inhibitory concentration (MIC) of 1.57-12.5 $\mu\text{g/ml}$ against MRSA. Other related xanthones were also examined to determine their anti-MRSA activity. The strong *in-vitro* antibacterial activity of xanthone derivatives against both methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* suggested the compounds might find wide pharmaceutical use (Inuma *et al.*, 1996).

Garcinia mangostana L. fruit hulls are used as an anti-inflammatory agent (Chairungrilerd *et al.*, 1996), astringent and to treat diarrhoea and has been used as Thai indigenous medicine for many years. The 40% ethanol extract of mangosteen has potent inhibitory activities of both histamine release and prostaglandin E2 synthesis (Nakatani *et al.*, 2002).

In the course of a search for antioxidants, the methanol extract of the fruit hulls of *Garcinia mangostana* L. was found to exhibit a potent radical scavenging effect. By monitoring this radical scavenging effect, two xanthones, α -mangostin and χ -mangostin, were isolated, together with (-)-epicatechin and procyanidins A-2 and B-2, as active principles. The antioxidant activity of the two xanthones was measured by

the ferric thiocyanate method; χ -mangostin was more active than butylhydroxyanisol and α -tocopherol (Yoshikawa, 1994).

As mentioned before *Garcinia mangostana* L. is used to prepare astringent medicines for use in dysentery, enteritis (Burkill, 1994). The rind or fruit, which contains resin which may act as a stimulant to the intestines, the bark and young leaves are used for this purpose and for ailments of the genito-urinary tracts (Jayaweera, 1981). The pericarp is also regarded as very efficacious in curing chronic intestinal catarrh and the fleshy pericarp is a valuable astringent and has been successfully employed in the advance stages of dysentery and in chronic diarrhoea as well as for a strong decoction as an external astringent application in dysentery (Drury and Colonel, 1973).

Garcinia mangostana L. also has anti-tubercular action with α -mangostin, β -mangostin and garcinone B which exhibited strong inhibitory effect against *Mycobacterium tuberculosis* with the minimum inhibitory concentration (MIC) value of 6.25 μ g/ml (Suksamrarn, 2002a; 2002b). The rind of partially ripe fruits yields a polyhydroxy-xanthone derivative termed mangostin, also β -mangostin. That of fully ripe fruits contains the xanthone, gartanin, 8-disoxygartanin, and normangostin. A derivative of mangostin, mangostine-6-di-*O*-glucoside, is a central nervous system depressant and causes a rise in blood pressure (Morton, 1987).

2.1.4 *Hibiscus sabdariffa* L. (Roselle)

2.1.4.1 General information

Hibiscus sabdariffa L. (Figure 4) belongs to the family Malvaceae where it is known by different synonyms and vernacular names including roselle, sorrel, red sorrel, Jamaica sorrel and karkade. *Hibiscus sabdariffa* L. is an annual herb with reddish, cylindrical stem, nearly or quite glabrous. The leaves are simple, having petiole, blade 3-5 lobed or parted the lobes serrate or obtusely toothed. The flowers are solitary,

axillary, nearly sessile, 5-7 cm in diameter and consist of 8-12 epicalyxes-segments. (Morton, 1987; Farnsworth and Bunyapraphatsara, 1992).



Figure 4 *Hibiscus sabdariffa* L. (Roselle)

2.1.4.2 The uses of *Hibiscus sabdariffa* L.

Hibiscus sabdariffa L. is a folk remedy for abscesses, heart ailments and hypertension (Akindahunsi and Olaleye, 2003). In Thailand, its red calyxes are used for making cold drinks and jam and also traditional medicines including astringent, antihypercholesterolemic, anti-diabetic, diuretic, digestive, expectorant, stomachic, antihypertensive, and treatment of gallstones (Farnsworth and Bunyapraphatsara, 1992; Cheeptham and Tower, 2002).

Since the phenolic compounds such as anthocyanins, flavonoids and phenolic acids are attributed with antioxidant activity and chelating ability (Heim *et al.*, 2002), the significance of phenolic compounds in Roselle has been of considerable interest. There were many reports on pharmacological activities of these components including antihypertensive activity (Haji-Faraji and Haji Tarkhani, 1999; Onyenekwe *et al.*, 1999; Odigie *et al.*, 2003), anti-atherosclerotic (Lee *et al.*, 2002; Chen *et al.*, 2003 and Hirunpanich *et al.*, 2005), angioprotective (Jonadet *et al.*, 1990), antihyperlipidemic activity (Aboutabl *et al.*, 1999; Chen *et al.*, 2004; Hirunpanich *et al.*, 2005), antileukemia (Chang *et al.*, 2005), antitumor promotion effect (Tseng *et al.*, 1998),

anti-mutagenic activity (Chewonarin *et al.*, 1999), anti-inflammatory activity (Mounnissamy *et al.*, 2002), antimicrobial activity (Cheeptham and Tower, 2002), hepatoprotective in rat (Amin and Hamza, 2005) and protective effect on CCl₄-induced rat liver fibrosis (Liu *et al.*, 2005).

A beverage containing *Hibiscus sabdariffa* L. was bactericidal against *Escherichia coli*, *Bacillus subtilis*, *Salmonella Typhosa* and *Klebsiella pneumoniae* (Alian *et al.*, 1983). An inhibitory effect of *Hibiscus sabdariffa* L. flower heads on the growth and aflatoxin production of toxigenic strain of *Aspergillus flavus* has also been reported (El-Shayeb and Mabrouk, 1984).

The chemical compositions and bioactivity of *Hibiscus sabdariffa* L. have been investigated and evaluated for the potential application (Nhung *et al.*, 1998). The flavonoid constituents showed good effect on peroxidase and protease activity in human blood which opens perspective for using *Hibiscus sabdariffa* L. as antioxidant and anti-aging. Furthermore, *in vitro* and *in vivo* studies suggest the potential use of the flavonoid for anticancer (Nhung *et al.*, 1998).

The aqueous extract of *Hibiscus sabdariffa* L., given orally, was found to significantly prevent the ulcer formation in experimental gastric ulcers induced in rat by indomethacin, water immersion restraint stress and pylorus-ligation and the effect was comparable to those of pirenzepine and cimetidine (Rujjanawate *et al.*, 1992).

In an acute oral toxicity study of an aqueous extract of *Hibiscus sabdariffa* L., Kanjanapothi *et al.*, (1997) concluded that a concentration of 5 g/kg appeared to be virtually nontoxic. In subacute and subchronic oral toxicity studies *Hibiscus sabdariffa* L. in varying doses 200, 400 and 800 mg/kg were administered for 28 and 90 days. Practically no serious toxic changes in body and tissue weights, hematology, histopathology and serum chemistries were found. It revealed from the tests for reproductive performance that did not markedly affect the reproductive capacity of male and female rats. In addition, *Hibiscus sabdariffa* L. devoided of teratogenic effect, since the administration of *Hibiscus sabdariffa* L. to pregnant rats did not

significantly affect fetal viability and subsequent normal growth. According to the test for mutagenic potential, using Mouse micronucleus test and Bacterial mutation assay, it is indicated that an aqueous extract of *Hibiscus sabdariffa* L. was not mutagenic.

2.1.5 *Musa sapientum* L. (Banana)

2.1.5.1 General information

Musa sapientum L. (Banana) are tropical, fruit crop grown in many countries of the world (Rastrelli *et al.*, 2004). They are the world's fourth most important food crop after rice, wheat and maize in terms of gross value of production. *Musa sapientum* L. belongs to the genus *Musa*, the family Musaceae and the order Zingerberales. The Musaceae family is made up of three genera, *Musa*, *Ensete* and *Musella*. *Musa* is the largest group, with about 35 species (Weaver, 1974). Banana is a climacteric fruit made up of peel and edible pulp that has a high nutritional value. Edible bananas are vegetatively parthenocarpic berries; i.e., they develop a mass of edible pulp without pollination (Diaz Romero *et al.*, 2003).

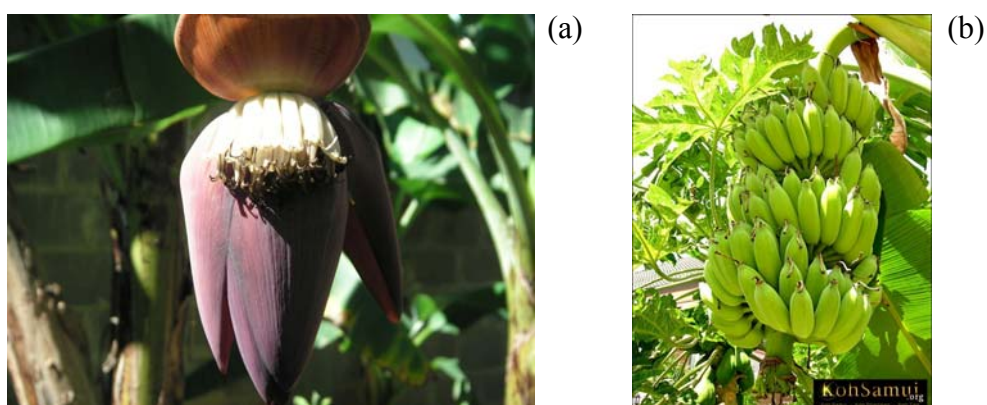


Figure 5 Flower (a) and fruit (b) of *Musa sapientum* L. (Banana)

Musa sapientum L. (Banana) is a perennial herbaceous plant, which grows to 5 - 9 m in height. It has tuberous subterranean rhizome, from which the leaves are folded

within each other producing false stem, from which the long, narrow blades protrude and spread out. In the centre of the folded leaf sheaths, a growing point forms the top of rhizomes, grows up and emerges as an overhanging inflorescence with a succession of reddish brown bracts. The bracts unfold from the base to the tip and fall off. Within the lower 1-12 bracts arise 14 -18 female flowers in double rows these develop into fruits. The roots are adventitious. The leaves are large from 1.5 - 3.5 m and 0.6 meter wide (Eddy and Ebenso, 2008).

Banana is an excellent tropical fruit, has an agreeable flavour and a high nutritional value. Bananas contain about 74% water, 23% carbohydrates, 1% proteins, 0.5% fat, and 2.6% fibre (these values vary between different banana cultivars, degree of ripeness and growing conditions). In an unripe banana the carbohydrates are mostly starches. In the process of ripening, the starches are converted to sugars; a fully ripe banana has only 1-2% starch (Skidmore and Smith, 2001). The contribution to the intake of sugars, fibre, vitamins, and mineral from the consumption of banana is high, with a very low contribution to the intake of fat (Rastrelli *et al.*, 2004).

2.1.5.2 The uses of *Musa sapientum* L.

As a staple, bananas (including plantains and other types of cooking bananas) contribute to the food security of millions of people in much of the developing world. They are perennial crops that grow quickly and can be harvested all year round (Arias *et al.*, 2003). Almost half of the bananas produced in the world are eaten raw as a dessert fruit; the other half is cooked, usually by frying, boiling, roasting or baking (Diaz Romero *et al.*, 2003).

Musa sapientum Linn (*Musaceae*) is extensively cultivated in northern part of India and many parts of Asia and Africa. It possesses many curative properties and prevents many kinds of illnesses and conditions. Different parts of the plant are used very frequently in different worship ceremonies by the Indians. Every part of the tree is being used for some purposes like food, fuel or timber (Eddy and Ebenso, 2008).

Several studies have been carried out on *Musa sapientum* L. peels for the production of biogas (Ilori *et al.*, 2007), ethanol production by hydrolysis and fermentation (Sirkar *et al.*, 2008), antacid and diuretic activity of the ash and extracts of the peel (Jain *et al.*, 2007), antibacterial and antioxidant activities (Mokbel and Hashinaga, 2005) and biomass production (Essien *et al.*, 2005).

In India hot water extracts of dried banana fruits, flowers and roots are used orally for diabetes, the dried flower along with dried fruits of *Coccinia indica* is used to prevent conception orally. The roots are used as anthelmintic, aphrodisiac, laxative and tonic. The fresh fruit is used for peptic and duodenal ulcers. The leaf ash is mixed with honey and taken orally for cough. Besides being a good source of energy, banana is a rich source of potassium, and hence is highly recommended for patients suffering from high blood pressure (Skidmore and Smith, 2001).

2.1.6 *Nelumbo nucifera* Gaertn. (Lotus)

2.1.6.1 General information

Nobuko (2001) stated that lotus is any of various water lilies, especially the white lotus (*Nymphaea*) once scapes in Egypt, or the pink or with Asian lotus (*Nelumbo nucifera*), use as a religious symbol in Hinduism and Buddhism.



Figure 6 *Nelumbo nucifera* Gaertn. (Lotus)

Lotus, an unusual plant by any measure, the Indian or sacred Lotus (*Nelumbo nucifera* Gaertn., syn *Nelumbo speciosum* Willd., *Nelumbium nelumbo* Druce, *Nymphaea nelumbo* L.) is a perennial herbaceous plant confined to an aquatic environment (Hicks and Haigh, 2001, Sainty and Jacobs, 2003). It is one of the oldest cultivated plants known to mankind (Swarup, 1989).

It is a plant regarded as sacred in Chinese and Indian cultures and is associated with Buddhism and Hindu religions (Herklots, 1972). Hindus believe that the white lotus is the seat of Saraswati (goddess of learning, knowledge, and wisdom), while Lakshmi (goddess of wealth) is always portrayed seated on a red lotus. Buddhists believe about the symbolization of lotus as vitality, the enlightenment of Lord Buddha, and total detachment from worldly qualities of greed, anger, lust, passion, jealousy and ego. Lord Buddha used lotus as symbolic of the different type of all living creature in the universe. Its flowers are often metaphorically depicted in religious symbolism, representing the spiritually pure soul rising from the turgid miasma of existence (Lovelock *et al.*, 1986).

Botanists originally assigned lotus to the family Nelumbonaceae, within the order Nymphaeales (Sculthorpe, 1967). There are 8 genera, 50 species of lotus based on flower colour (pink, red, or white), flower size (single or double flowers), fragrance, and leaf colour (green or variegated) (Lawan, 1990). Lotus is roughly classified into two botanical types (Nobunkyo, 1983). One is *Nelumbo lutea* Pers., generally called 'American lotus' or 'American nelumbo', originated from North America. The flower colour is mainly yellow. The other is *Nelumbo nucifera* Gaertn., generally called 'East Indian lotus', original from east Asia. The flower colours are mainly white, pink and red.

Nelumbo lutea Pers. is native to North America (Conard, 1907). It is quickly crowded out by its cousin, the pink or Indian lotus, *Nelumbo nucifera* Gaertn. *Nelumbo nucifera* Gaertn. resembles the former in all respects, except for colour of flower and vigor of growth. The flowers are larger than those of the *Nelumbo lutea* Pers., and each petal is tipped with rosy pink. At the centre of the flower is a big yellow

receptacle like an inverted cone, surrounded by innumerable yellow stamens. *Nelumbo nucifera* Gaertn. is vernacularly known as *Nelumbo*, *Nymphaea*, *lotus*, *sacred lotus*, etc. They are also *bua-luang*, *sattabut*, *patum*, and *ubon* in Thailand. Lotus is an aquatic plant and is cultivated in paddy fields or in shallow swamps, and prefers high temperature climates (Ong, 1996).

According to Jittavigul and Photithitirat (1990), the three well-known genera of lotus in Thailand are genus *Nelumbo*, called 'bua-lung' in Thai; genus *Nymphaea*, called 'bua-saai', 'bua-pan', 'bua-puean' in Thai; genus *Victoria lindl*, called 'bua-victoria' in Thai.

Nelumbo nucifera Gaertn. is distributed across Asia from Japan to North-East Africa (Borsch and Barthlott, 1996). It covers a broad latitudinal span from Beijing 40° N as far south as Northern Australia 20° S. It has been transported to most of these locations and speculates that its true centre of origin is India.

Nelumbo nucifera Gaertn. has a strikingly distinct morphology. It is a large plant, which apart from dwarf varieties, grows up to 15 m in stolon length (Liu, 1994). Most of the *Nelumbo nucifera* Gaertn. plants are unseen as the stoloniferous stems are anchored in the pond substrate. It is only the large peltate leaves (50-70 mm diameter) and distinct flowers which are visible to the observer. The initial leaves produced from a young stolon are floating before emergent leaves borne on spiny 1-2 m petioles dominate (Sainty and Jacobs, 2003) The leaf epidermis is covered by epicuticular wax crystalloids which prevent surface contamination due to the water repellency known as the 'Lotus -Effect' (Barthlott and Neinhuis, 1977). Flowers are solitary (10-20 cm diameter) and rise from leaf axils above emergent leaves (Sainty and Jacobs, 2003) and open and close depending upon ambient temperature (Seymour *et al.*, 1998). The reproductive structures in *Nelumbo nucifera* Gaertn. consist of an obconical receptacle housing a multi-carpelly gynoecium, which looks like an inverted watering can rose, and develops into a head of nutlike achenes (Swarup, 1989). Numerous 15-20 mm linear stamens subtend the receptacle (Sculthorpe, 1967). The below-ground structure, consist of stolons, internodes with fibrous feeder roots and the

overwintering rhizome storage organs. Stolon segments can reach 1 m in length and be up to 30 mm diameter (Ni, 1987). Stolons tend to grow horizontally about 20 cm below the surface of the substrate allowing feeder roots to penetrate into free water. Rhizomes develop as a response to stress in the environment. Rhizome resemble a chain of sausage approximately 50 cm long divided into 3 to 4 segments, 50 to 150 mm in diameter, with distinct internodes (Nguyen, 2001).

2.1.6.2 The uses of *Nelumbo nucifera* Gaertn.

Nelumbo nucifera Gaertn. provides a food source, floricultural products and is used as an ornamental feature plant in landscapes (Ni, 1983). It also has potential for use in filtering nutrients from water in recycling systems (Nguyen, 2001).

All parts of the *Nelumbo nucifera* Gaertn. plant are useful to man. Generally, the whole plant is considered as a coolant and most of the parts are used for the treatment of diarrhoea and haemostasis (Yu and Hu, 1997).

As a fresh cut flower, *Nelumbo nucifera* Gaertn. has a very limited vase life. Flowers have to be harvested prior to opening. Green and dried pods are also marketed as products of the flower industry. Green pods have a longer vase life than flowers, and dried pods last for years are very brittle and easily damaged (Nguyen, 2001).

The large circular leaves are used to wrap food preparations of rice and tapioca desserts. In South and South-East Asia, the leaves are dried and used as disposable plates, sewn together to make cups used in temple as containers to distribute food. In traditional medicine, they are used with other herbs to treat fever, sunstroke, diarrhoea, dysentery, dizziness, and stomach problems (Usha, 2008).

Seeds of *Nelumbo nucifera* Gaertn. are eaten raw or cooked, unripe or ripe and are popular ingredients in desserts (Usha, 2008). Seeds are also consumed raw and cooked with the testa and bitter embryo removes (Flach and Rumawas, 1996). Seeds are reported to be rich in protein and adequate in the amounts of essential mineral

(Ibrahim and El-Eraqy, 1996). These seeds are regarded as popular health food and an alkaloid (liensinine) extracted from lotus serves as an effective drug to treat arrhythmia (Ling *et al.*, 2005). The seeds are also used in indigenous (e.g., Ayurveda, Chinese) and folk medicines to treat tissue inflammation, cancer, diuretics and skin diseases (e.g. leprosy) (Chopra *et al.*, 1956; Liu *et al.*, 2004).

Nelumbo nucifera Gaertn. rhizome (Figure 7) is a creeping stem lying, usually horizontally, at or under the surface of the soil and differs from a root in having scaled leaves, bearing leaves or aerial shoots near its tips, and produces roots from its under surface.



Figure 7 Rhizome of *Nelumbo nucifera* Gaertn.

In an increasingly complex food supply, consumers are increasingly interested in herbal remedies, dietary supplements, alternative medicine and foods with associated health benefit. *Nelumbo nucifera* Gaertn. rhizome is recognized as one of these foodstuffs in many Asian and Oceania countries. It contains abundant amount of protein, amino acid, dietary fibre, carbohydrates and vitamins C, B₁ and B₂ (Wu, 1987). It is also rich in Vitamin B₁₂, rare among vegetables, which supports liver function. This makes *Nelumbo nucifera* Gaertn. rhizome a famous of dietary cure foodstuff (David, 2005). It is widely favoured by Asian people because of its hard and crispy texture, special aroma and mouth feel. It is often used to make different dishes, such as salads, pickled vegetable, stir-fired food and confections. It is often used to

3-*O*-glucoside and Luteolin 7-*O*-glucoside showed significant activity against 6 bacteria and *Candida albicans* and exhibited anti-inflammatory and anapalgesic activities. Lotusine, Dimethylcoclaurine, Liensinine, Isoliensinine, Neferine, Nornuciferine, Pronuciferine, Methylcorypalline, Norarmepavine, Liriodenine, and Nelumboside were also found in lotus rhizome extract (Anonymous, 1997).

2.1.7 *Piper betle* L. (Betel leaf or Betelvine)

2.1.7.1 General information

Piper betle L. (Figure 9) belongs to the dicotyledonous family Piperaceae, and is a twining plant widely cultivated in the warm and moist parts of South India and Ceylon for its leaves (Nadkarni, 1976). *Piper betle* L. require a cool climate and high humidity during their life span of 2-3 years. If the plants are exposed to extreme heat that leaves become dark green and brittle. If a cool climate and shade are created in the garden, the leaves will be light green. The leaf is simple, alternate, cordate, 8-12 cm wide, 12-16 cm long, and with description odour and spicy taste. The inflorescence is in auxiliary spike, with unisexual, white flowers, and fruit globosely berry. It is often chewed in combination with the betel nut (*Areca catechu*) as a stimulatory.

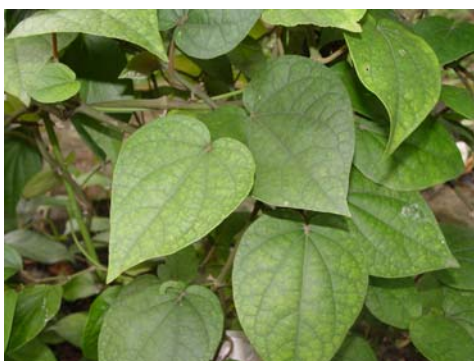


Figure 9 *Piper betle* L. (Betel leaf or Betelvine)

2.1.7.2 The uses of *Piper betle* L.

Piper betle L. has been intimately linked with ancient history, region and culture (Balasubramanyam *et al.*, 1994). Reference to it are found in the early Sanskrit literature (3000 BC), Pali treaties (2000 BC), Tamil literature (2nd century BC) and the travelogues (4th century BC) of Herodotus and Megasthenes. Its ethnomedicinal aspects have also been long known. The tribal population and aborigines of India chew the leaves as a narcotic which causes swooning and profuse sweating and also helps to give warmth to the body during winter. Fresh leaves of *Piper betle* L. are widely used in the Indo-Pak subcontinent in the form of packets known as 'Betel quid', which consist of *Piper betle* L. leaves painted with burnt lime and catechu, containing pieces of areca nut, with or without tobacco and flavours. Sometimes, cardamom and/or cloves are also added. Approximately 200 million persons chew betel regularly throughout the western Pacific basin and south Asia (Norton, 1998). The chewing of 'betel quid' is well known to produce copious salivary secretion that improves voice and digestion and this action is possibly due to the presence of arecoline, the cholinomimetic constituent of Areca nut (Bowman and Rand, 1980) and some uncharacterized constituents of betel leaves (Nadkarni, 1976). The users believe that chewing *Piper betle* L. and combination with those ingredients can improve their efficiency and stamina. *Piper betle* L. leaves are also considered useful in treating bronchitis, difficulty in breathing and cough (Nadkarni, 1976).

The liquid extract of the plant has been used traditionally in curing inflammation and infections of the respiratory tract, cough, dyspnoea, indigestion, diphtheria, hysteria, as well as general and sexual debility (Nadkarni, 1976). The roots are used as a contraceptive and are chewed by singers to improve their voice (Usmanghani *et al.*, 1997).

The crude extract of the plant and its constituents have been studied and found to have diverse biological activities, which include antimicrobial (Prasad *et al.*, 1992; Garg and Jian, 1992; Baby *et al.*, 1993), antifungal (Misra and Dixit, 1979; Evans *et al.*, 1984), antioxidant (Wang and Wu, 1996), wound healing (Santhanam and Nagaraiam,

1990), and enzyme inhibitory activity against succinic dehydrogenase (Shilaskar and Parasar, 1985). Similarly, antifertility (Ratnasooriya *et al.*, 1990; Adhikary *et al.*, 1989), platelet activating factor antagonistic (Yin *et al.*, 1991; Zeng *et al.*, 1997), antiplatelet and anti-inflammatory (Saeed *et al.*, 1993), anthelmintic (Evans *et al.*, 1984), and antihypertensive activities (Chen *et al.*, 1995) have also been reported. *Piper betle* L. is used in the treatment of bronchitis, cough, cold and chills (Chopra *et al.*, 1954), as well as in dyspepsia, anoxia, and sleeplessness due to racking dry cough and in pulmonary infection.

The leaf juice is used in eye-drops and as an indelible ink for marking and labelling garments (Kharkongar and Joseph, 1984). The leaves are also used as a masticatory which acts as a gentle stimulant (Borthakur, 1981) and the people (smear with mustard or castor oil) to promote bowel movement. The roots with rice beer or bark pepper are given to prevent conception. The leaf juice is added to alcoholic beverages to enhance the intoxicating effect and leaves fried in oaken oil are used against earache (Balasubramanyam *et al.*, 1994).

The application of warm leaves on swelling, particularly on the ear and throat, is a common practice in the villages of India. The medicinal properties as a carminative, aromatic, digestive and stimulant are described in the Susruta Samhita (600 AD) a medico-scientific treatise on the indigenous Ayurvedic system of medicine (Balasubramanyam *et al.*, 1994).

2.1.8 *Punica granatum* L. (Pomegranate)

2.1.8.1 General information

Pomegranate (Figure 10) belongs to the family Punicaceae. The plant is an erect shrub up to 3 m high, much branched from the base, having branchlets slender, often ending in a spine. The trunk is covered by a red-brown bark which later becomes gray. The leaves are simple, and consisting of obtuse or emarginated apex, acute base, shiny and glabrous texture. Flowers are showy, orange red, about 3 cm in diameter, others

solitary in highest leaf-axils, sessile or subsessile; consisting of calyx 2-3 cm long. Fruits are globose berries, crowded by persistent calyx-lobes, having pericarp leathery filled with numerous seeds, which are surrounded by pink and red transparent, juicy, acid, pleasant-tasting pulp. In each sac there is one angular, soft or hard seed (Farnsworth and Bunyaphatsara, 1992).

Punica granatum L. is also long-lived. There are specimens in Europe that are known to be over 200 years of age. The vigour of *Punica granatum* L. declines after about 15 years, however. High temperatures are essential during the fruiting period to get best flavour. *Punica granatum* L. may begin to bear in 1 year after planting out, but 2 to 3 years is more common. Under suitable conditions the fruit should mature some 5 to 7 months after bloom.



Figure 10 *Punica granatum* L. (Pomegranate)

2.1.8.2 The uses of *Punica granatum* L.

Many researchers have focused on antioxidant actions *in vitro*, *ex vivo* and *in vivo*, while other work has elaborated on the ability of juice, seed oil, peel or flower

extracts, and their derivatives to kill bacteria and viruses, or to fight vascular disease, diabetes and cancer. In everything from improving erectile insufficiency in rabbits to healing ethanol induced stomach ulcers in rat, antioxidant action and root of the observed beneficial effect. Chemical studies of *Punica granatum* L. have reported on many compounds isolated from different parts of the plant (Tanaka *et al.*, 1986; Neuhofer *et al.*, 1993; Satomi *et al.*, 1993; Nawwar *et al.*, 1994; Artik, 1998; Amakura *et al.*, 2000a; Amakura *et al.*, 2000b; de Pascual-Teresa *et al.*, 2000; Gil *et al.*, 2000; Kim *et al.*, 2002; Noda *et al.*, 2002; Vidal *et al.*, 2003; van Elswijk *et al.*, 2004; Wang *et al.*, 2004; Huang *et al.*, 2005). In addition to the more common anthocyanins, pentose glycosides of malvidine and pentunidin have been described in pericarp and juice (Sharma and Seshadri, 1995).

The root bark as well as stem barks of the plant is astringent and antihelminthic. The dried flowers are used in haematuria, haemorrhoids, haemoptysis and dysentery. The powdered flowerbuds are used in bronchitis. The seed are considered to be stomachic and the pulp as cardiac and stomachic. The fruit rind is valued as an astringent and green leaves are made into a paste and applied in conjunctivitis (Ross *et al.*, 2001).

It has been reported that the aqueous extract of the fruit peel showed antioxidant activity when evaluated by DPPH radical scavenging assay, 5-lipoxygenase assay and chemiluminescence assay with IC₅₀ value of 0.094, 0.198 and 0.944 mg/ml, respectively. In addition the ethyl acetate extract of *Punica granatum* L. fruit peel also showed antioxidant activity with IC₅₀ value of 8.492, 0.245 and 6.93 mg/ml, respectively (Ricci *et al.*, 2006).

The extract of *Punica granatum* L. also showed antibacterial activity against *Shigella flexneri* with a minimum inhibitory concentration (MIC) of 1-4 mg/ml (Alantís *et al.*, 2005). A methanolic extract of *Punica granatum* L. exhibited inhibitory effect against *Staphylococcus aureus* and processed strong *in vitro* antibacterial activities against *Escherichia coli* (Braga *et al.*, 2005; Meléndez and Capriles, 2005; Meléndez and Capriles, 2006). This extract had an inhibitory effect against *Proteus vulgaris* and *Bacillus subtilis* with minimum inhibitory concentration (MIC) values of 1.5 and 6.0

mg/ml, respectively (Prashanth *et al.*, 2001). Both aqueous and ethanolic extracts of *Punica granatum* L. were highly effective against *Escherichia coli* O157:H7 with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 0.09, 0.78 and 0.19, 0.39 mg/ml, respectively (Voravuthikunchai *et al.*, 2004).

Punica granatum L. seed oil, juice, peel and seed cake, were shown to stimulate keratinocyte proliferation in monolayer culture. In parallel, a mild thickening of the epidermis was observed in skin organ culture. The same *Punica granatum* L. seed oil that stimulated keratinocyte proliferation was without effect on fibroblast function (Aslam *et al.*, 2006). It has been reported that administration of a 70% methanolic extract of *Punica granatum* L. fruit peel (250 mg/kg and 500 mg/kg) shows percentage of inhibition of 22.37, 74.21, 21.95 and 63.41 in aspirin-and ethanol-induced gastric ulceration, respectively (Ajaikumar *et al.*, 2005).

Punica granatum L. fruit peel powder at the dose of 100 mg/kg orally as aqueous suspension was found to stimulate the cell-mediated and humeral components of the immune system in rabbits. It elicited an increase in antibody titer to lymphoid-H antigen. It also enhanced the inhibition of leucocyte migration in the leucocyte migration inhibition test and induration of skin in a delayed hypersensitivity test with purified protein derivative (Ross *et al.*, 2001). A methanolic extract of *Punica granatum* L. flower inhibited glucose loading-induced increase of plasma glucose levels in *Zucker diabetic fatty* rat, but not in *Zucker lean* rats (Huang *et al.*, 2005). Whole fruit hydroalcoholic extracts of *Punica granatum* L. at a dose of less than 0.1 mg per embryo are not toxic in the chicken embryo model. The LD₅₀ of the extract, determined in OF-1 mice of both sexes after intraperitoneal administration, was 731 mg/kg. The confidence limits were 565-945 mg/kg (Vidal *et al.*, 2003).

2.1.9 *Psidium guajava* L. (Guava)

2.1.9.1 General information

Psidium guajava L. (Figure 11) commonly known as guava, belonging to the family Myrtaceae, is a native of tropical America and has long been naturalized in southeast Asia (Levitt, 1980; Begum *et al.*, 2002). The tree, commonly named guayabo in Spanish and guava in English, is frequently cultivated as food for its pleasant fruit that also is used in the manufacture of the jam. Today, the tree can be found cultivated or growing wild in nearly all the countries of the Tropical World Belt, from the West Coast of Africa to the Pacific Region, including India, China, Thailand, Malaysia, Indonesia and Japan with varieties originally introduced from America over the past 300 years (Lozoya *et al.*, 2002). The fruit is commercially important in India, South Africa, Florida, Hawaii, Egypt, Brazil, Colombia, the West Indies, Cuba, Venezuela, New Zealand, the Philippines (Yadava, 1996), Vietnam (Le *et al.*, 1998) and Thailand (Tate, 2000). It was introduced to Thailand in early 1700's. The fruit is a berry, round or oval in shape, with vary thin skin and many small, hard seeds embedded in the centre of fruit pulp.

The fruit is an excellent source of vitamin C, but has a low energy value (275kJ/100 g) and 1% protein content, containing about 17% dry matter and 83% moisture. The dry matter is made up mostly of structural and non-structural carbohydrate (Verhij and Coronel, 1991). *Psidium guajava* L. fruit growth follows a simple sigmoid curve (Rathore, 1976). The days from anthesis to harvest vary from about 120-200 days depending upon temperature during fruit development and cultivars (Paull and Goo, 1983). *Psidium guajava* L. is a climacteric fruit, exhibiting a typical increase in respiration and ethylene production during ripening (Akamine and Goo, 1979; Wills *et al.*, 1983). *Psidium guajava* L. fruit are harvested at the mature-green stage (colour change from dark-green to light-green). Ripening of the *Psidium guajava* L. fruit is characterized by softening of the fresh which is associated with cell wall disassembly (Lim and Khoo, 1990). Low temperature storage is used to preserve fruit quality, fruit at the mature-green stage storage at 8-10°C can prolong shelf life to 14-21 days.

Storage below 5°C causes the chilling injury (Vazquez-Ochoa and Colinas-Leon, 1990).



Figure 11 *Psidium guajava* L. (Guava)

2.1.9.2 The uses of *Psidium guajava* L.

Psidium guajava L. is used for prevention and treatment of scurvy in Asia and Africa (Council of Scientific and Industrial Research, 1969; Watt and Branehwizk, 1969). Different parts of the plant are used in the indigenous system of medicine for the treatment of various human aliment such as wounds, ulcers, bowels and cholera. *Psidium guajava* L. juice has been reported to process hypoglycaemic activity in both mice and humans (Cheng and Yang, 1983). The fruit is a tonic and laxative and is good in bleeding gum. The bark is valued as an astringent and as an anti-diarrhoeatic in children. The flowers are said too cool the body and are used for the treatment of bronchitis and eye sore (Krishnamurthi, 1969; Dymock *et al.*, 1972; Perry, 1980).

Scientific studies on the medicinal properties of *Psidium guajava* L. leaves products began in the decade of the 1940's. Three main tracks are followed for validation of the curative properties of *Psidium guajava* L. leaves: (a) the anti-microbial capacity of the watery and alcoholic extracts evaluated by *in vitro* studies, confirming its growth inhibiting effect particularly on *Staphylococcus aureus*, *Escherichia coli*, and other

common entero-pathogenic cultures (Colliere, 1949; Cáceres *et al.*, 1993a; 1993b; Jaiarj *et al.*, 1999; Gnan and Demello, 1999; Coutino-Rodríguez *et al.*, 2001); (b) the anti-diarrheic measured as an effect on motility property of intestine of the methanolic extract using *in vitro* and *in vivo* animal models also demonstrating anti-spasmodic effects (Lutterodt, 1989; Maikere *et al.*, 1989; Lozoya *et al.*, 1990; Lutterodt, 1992; Lozoya *et al.*, 1994; Morón *et al.*, 1999; Tona and Kambu, 2000), and (c) the sedative effect of some *Psidium guajava* L. extracts measured in animals on locomotor and nervous activity (Lutterodt and Maleque, 1988; Lutterodt, 1993; Meckes *et al.*, 1996; Shaheen *et al.*, 2000).

The young leaves are used as a tonic in diseases of digestive function. The decoction of young leaves and shoot is prescribed as a febrifuge and spasmolytic (Krishnamurthi, 1969; Perry, 1980; Dymock *et al.*, 1972). In addition, *Psidium guajava* L. leaves have been used to treat many ailments including cough and pulmonary diseases in Bolivia and Egypt (Batick, 1984). Young *Psidium guajava* L. leaves are used in India as a remedy against coughs (Khan and Ahmad, 1985). People in China use *Psidium guajava* L. leaves as an anti-inflammatory agent (Hon-Ning, 1988). A decoction of leaves is used for the treatment of cholera, to reduce vomiting and diarrhoea (Council of Scientific and Industrial Research, 1969; Watt and Branchwizk, 1969; Watt, 1972). In Mexico, *Psidium guajava* L. leaves are extensively used to stop diarrhea, with quercetin and its glycosides thought to be the active ingredients (Lozoya *et al.*, 1994). Water, alcohol and chloroform extracts of fresh *Psidium guajava* L. leaves were effective against *Aeromonas hydrophila*, *Shigella* spp. and *Vibrio* spp. (Chulasiri *et al.*, 1986). Water extract of dried leaves also processes bactericidal activity against *Staphylococcus phlei* (Malcolm and Sofowora, 1969). However, an ethanol extract of *Psidium guajava* L. was not effective against eight microbial strains isolated from stool of patients with infectious diarrhoea (Gritsanapan and Chulasiri, 1983). According to modern ethnomedical information, no side effects are observed in the use of *Psidium guajava* L. leaves remedy and traditional healers consider it efficacious and safe (Aguilar *et al.*, 1996).

Phytochemical studies undertaken by different groups of workers on different parts of the plant have resulted in the isolation and identification of various terpenoids, flavonoids and tannins (Seshadri and Vasishta, 1965; Seshadri and Tanaka *et al.*, 1992; Lozaya *et al.*, 1994; Meckes *et al.*, 1996).

2.2 Antimicrobial Activity of Medicinal Plants, Spices, and their Essential Oil

2.2.1 *In Vitro* Test of Antimicrobial Activity

2.2.1.1 Medicinal Plants Extract, Spices Extract, and their Essential Oil

At the end of the last century, antimicrobial activities of medicinal plants and spices had already been examined. Ground mustard, clove and cinnamon and their essential oils were known to retard microbial spoilage in food (Nakatani, 1994). Up to the 1940s, many reports were published on the inhibitory activity of spices against pathogenic foodborne organisms. Dold and Knapp (1949) examined the antimicrobial activity of 27 plants extracts against 8 strains of tested organisms, such as *Escherichia coli*, *Salmonella Typhosa*, *Shigella paradysenteria* and so on, and showed garlic to be active against all organisms tested. Onion, nutmeg and clove inhibited the growth of all tested organisms, except *Bacillus subtilis*. From the view point of plant taxonomy, the Liliaceae family showed the highest activity, followed by the Myrtaceae, Cruciferae and Labiatae family. Ueda *et al.* (1982) also tested the ethanolic extracts of medicinal plants and spices for inhibition of bacteria and fungi in culture media at different pHs. Clove exhibited remarkable antibacterial activity against all organisms tested including *Bacillus subtilis* PCI, *Staphylococcus aureus* 209P, *Escherichia coli*, *Salmonella Typhimurium*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Proteus morgani*. Oregano and cinnamon extracts showed a wide inhibitory spectrum against Gram-positive bacteria while they were less potent against Gram-negative bacteria. Higher activity was exhibited at a lower pH. Galli *et al.* (1985) reported excellent activity of mustard oil against both Gram-positive and negative bacteria, and good activity of cinnamon, marjoram, oregano and thyme. Shelef *et al.* (1984) studied the sensitivity of common foodborne bacteria (24 Gram-

positive and 22 Gram-negative bacteria) to sage, rosemary and allspice. Here also, Gram-positive bacteria were more sensitive than Gram-negative bacteria. At a concentration of 0.3%, either sage or rosemary was bacteriostatic and at 0.5% the effect was bactericidal. A combination of sage and rosemary displayed enhances antimicrobial activity. Shelef (1983) compiled the studies on *in vitro* antimicrobial activity of ground medicinal plants and their extracts. Shelef (1984) reported the susceptibility of strains of *Staphylococcus aureus*, *Pseudomonas sp.*, and *Salmonella* Typhimurium to sage in nutrient broth. Except for *Salmonella* Typhimurium, MIC of sage against the microorganism was 0.1-0.5% in nutrient broth. *Samonella* Typhimurium required 10 times more the sage for inhibition in broth than *Bacillus cereus*.

The ability of commercially available medicinal plants to prevent growth of *Listeria monocytogenes* in tryptose broth also was investigated by Bahk *et al.* (1989). With the exception of an increase lag phase, behaviour of Listeriae during extended incubation at 35 and 4°C was relatively unaffected by the presence of 0.5% ginger, onion, garlic or mustard as well as ginseng saponin or mulberry extract at concentration <0.3%. However, unlike the previous study, addition of 0.5% cinnamon was somewhat inhibitory to *Listeria monocytogenes* with the pathogen attaining maximum population that were 1.5-2.6 orders of magnitude lower than in controls. Growth of Listeriae at 35°C also was partly suppressed by presence of 0.5% clove, with the pathogen attaining the maximum population 1.6 orders of magnitude lower than that observed in the control. However, *Listeria* populations decreased steadily in tryptose broth containing 0.5% cloves during extended incubation at 4°C. The germicidal action of cinnamic aldehyde in cinnamon and eugenol in clove is well recognized and so one can speculate that these compounds also may be at least partly responsible for the inhibitory activity of these plants toward *Listeria*.

Ting and Deibel (1992) reported on a survey of medicinal plants antimicrobial activity in which the concentration gradient method was used to study the effect of 14 test organisms in the presence of 3% (w/v) black pepper, chilli pepper, cinnamon, garlic, mustard, paprika, parsley, and red pepper during extended storage at 4 and 25°C,

Listeria monocytogenes was sensitive at 25°C to oregano, clove, sage, rosemary, nutmeg, and thyme with calculated MICs of 0.70, 0.72, 1.29, 1.54, 2.72 and 2.75%, respectively. The latter six medicinal plants also were inhibitory to *Listeria monocytogenes* at 4°C. When added to culture media, growth of the pathogen at both incubation temperatures was prevented by as little as 0.5% oregano. However, when exposed to 0.5% clove, *Listeria* populations decreased only 2.4 orders of magnitude after 1 and 14 days of incubation at 25 and 4°C, respectively. Unfortunately, further experiments showed that cloves and oregano were not active against *Listeria* when in a meat slurry at 1.0%. According to Aureli *et al.* (1992), essential oils of pimento, clove, origano and thyme effectively inhibited the growth of *Listeria monocytogenes* on microbiological media using a paper disc diffusion method and an inhibition curve. This pathogen was completely killed within 2-4 hours at the essential oils concentration 5µl/ml in a saline solution system. Garlic also has antimicrobial activity against several pathogenic bacteria. For example, Arora and Kaur (1999) reported garlic had bactericidal effect against *Staphylococcus aureus* and *Salmonella* Typhi with a 1-2 Log₁₀ decrease in 2 hours. However, cells recovered after 3 hours leading the author to hypothesize that resistant bacteria started the growth. Elgayyar *et al.* (2001) demonstrated that oil of oregano completely inhibited growth of *Salmonella* Typhimurium and strongly inhibited *Listeria monocytogenes*. Additionally, angelica, celery, coriander and parsley had moderate antilisterial activity. Friedman *et al.* (2002) used a microplate assay to evaluate the bactericidal activity level of essential oils and oil components against pathogenic bacteria, cedar-wood, bay leaf, clove bud, oregano, cinnamon, allspice, thyme, and patchouli oil. Those were most active against *Salmonella enterica* were thyme, oregano, cinnamon, clove bud, allspice, bay leaf, palmarosa, and majoran oils.

2.2.1.2 Components of Medicinal Plants Extract, Spices Extract, and their Essential Oils

The components of medicinal plants, spices, and their essential oils have been studied for their antimicrobial properties. Katayama and Nagai (1960) examined the activity of 43 compounds in essential oils of medicinal plants. Eugenol, thymol,

carvacrol, isoborneol, vanillin, and salicylaldehyde showed inhibitory effect against *Bacillus subtilis*, *Salmonella* Enteritidis, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus morganii* and *Escherichia coli*. Kurita *et al.* (1979) reported against fungi growth inhibition by the volatile aldehydes obtained from spices. The proposed inhibitory mechanism of the antifungal action of aldehydes was due to the ability to form charge transfer complexes with electron donors and reactivity with the SH group in cysteine or glutathione moieties. Thompson (1990) reported the influence of pH on the inhibitory activity of thymol and carvacrol against 8 strains of *Aspergillus*. Mycelial growth was completely inhibited by thymol at pH 4, 6 and 8 while inhibited by carvacrol only at pH 4 and 8. Hydroxycinnamic acids, commonly found on herbs, were investigated for antimicrobial activity against *Pseudomonas fluorescens* and *Saccharomyces cerevisiae*. *p*-Coumaric acid increased the lag phase at 100 ppm, and ferulic acid increased the lag time at 50 ppm and completely inhibited growth at 250 ppm (Baranowski *et al.*, 1980). Gingerenone A isolated from ginger exhibited a moderate anticoccidium activity and a strong antifungal effect against *Pyricularia aryzae*, a plant pathogen (Endo *et al.*, 1990). Masuda *et al.* (1991) found four antibacterial phenylpropanoids from *Piper sarmentosum*. Compound 48 showed complete inhibition against *Bacillus subtilis* and *Escherichia coli* at an MIC of 100 µg/ml measured by agar dilution method. Friedman *et al.* (2002) found the compounds in essential oils most active against *Listeria monocytogenes* included cinnamaldehyde, eugenol, thymol, carvacrol, citrol, geraniol, perillaldehyde, carvone S, estragole, and salicylaldehyde. Those most active against *Salmonella enterica* were thymol, cinnamaldehyde, carvacrol, eugenol, salicylaldehyde, geraniol, isoeugenol, terpineol, perillaldehyde, and estragole.

2.2.2 Tests of Antibacterial Activity of Medicinal Plants, Spices and their Essential Oils in Food System

Medicinal plants, spices and their essential oils have been used for millennia to provide distinctive flavours for food and beverages around the world. In addition to contributing flavour to foods, many medicinal plants, spices and their essential oils also exhibit antibacterial activity and could provide another hurdle to the growth of

foodborne pathogens, thereby improving food safety. Sample of using the antibacterial of medicinal plants, spices and their essential oils or components in food are shown in Table 1.

In many instances, concentrations of compounds in medicinal plants, spices and their essential oils necessary for inhibiting microorganisms exceed the normal usage levels in food. Nevertheless, the preservation effect of these agents should not be discounted. When an extract is mixed into food, the antimicrobial effect is reduced by reaction or interaction with food components. A greater concentration of medicinal plants, spices extract or their essential oils is need to achieve the same effect in foods as in microbiological media (Shelef, 1983; Smid and Gorris, 1999). The ratio has been shown to be approximately twofold in semi-skimmed milk (Karatzas *et al.*, 2001), 10-fold in pork liver sausage (Pandit and Shelef, 1994), 25-fold in soft cheese (Mendoza Yepes *et al.*, 1997), and 50-fold in soup (Ultee and Smid, 2001). Several studies have been reported on the effect of foodstuffs on microbial resistance to medicinal plants, spices and their essential oils, none appears to have quantified it or have explained the mechanism, although suggestions have been made as to the possible causes. The greater availability of nutrients in foods compared to laboratory media may enable bacteria to repair damaged cell faster (Gill *et al.*, 2002). Not only are the intrinsic properties of the food (fat/protein/water content, antioxidants, preservatives, pH, salts and other additives) relevant, the extrinsic determinants (temperature, packaging in vacuum/gas/air, characteristics of microorganisms) can also influence bacteria sensitivity (Shelef, 1983; Shelef *et al.*, 1984; Tassou *et al.*, 1995).

2.2.2.1 Dairy and Dairy Products

Addition of garlic to butter (1:4 w/w) enhanced the rates of inactivation of *Salmonella* sp., *Escherichia coli* O157:H7 and *Listeria monocytogenes* at 21°C or 37°C (Adler and Beuchat, 2002). Mint oil at 5-25µl/g is effective against *Salmonella* Enteritidis in low fat yoghurt and cucumber salad (Tassou *et al.*, 1995). Also, vanillin had an inhibitory effect on *Listeria monocytogenes* in yoghurt (Tipparaju *et al.*, 1995).

2.2.2.2 Meat and Meat Products

Certain oils stand out as better antimicrobials than others for meat applications. Eugenol, clove, oregano and thyme oils were found to be effective at levels of 5-10 µl/g in inhibiting *Listeria monocytogenes* in meat products (Aureli *et al.*, 1992; Hao *et al.*, 1998a, 1998b; Tsigarida *et al.*, 2000; Vrinda Menon and Garg, 2001) whilst sage, mint, cilantro were less effective or ineffective (Shelef *et al.*, 1984; Tassou *et al.*, 1995; Tassou *et al.*, 1996; Gill *et al.*, 2002). Fat content appears to markedly reduce the action of essential oils in meat products. For example, mint and cilantro essential oils were not effective in products with a high level of fat, such as pâté (which generally contains 30-40% fat) and a coating for ham containing canola oil (Tassou *et al.*, 1995; Gill *et al.*, 2002). Immobilising cilantro essential oil in a gelatine gel, improved the antimicrobial activity against *Listeria monocytogenes* in ham (Gill *et al.*, 2002). One study found that encapsulated rosemary oil was much more effective than standard rosemary essential oil against *Listeria monocytogenes* in pork liver sausage, although whether the effect was due to the encapsulation or the greater percentage level used was not further elucidated (Pandit and Sherel, 1994).

2.2.2.3 Fish Products

In fish, oregano and carvacrol had an antibacterial effect against *Salmonella* sp. (Kim *et al.*, 1995a; 1995b; Koutsoumanis *et al.*, 1999) whereas mint oil was ineffective (Tassou *et al.*, 1995; 1996).

2.2.2.4 Fresh Produce

Salmonella sp., contaminated on alfalfa seed, was reduced after fumigation with 200 mg/l_{air} cinnamaldehyde or 600 mg/L_{air} thymol at 50°C (Weissinger *et al.*, 2001).

2.2.2.5 Rice

Sage oil at 200-500 ppm when used against *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella* Typhimurium in rice was ineffective, whereas carvacrol at 150-750 ppm was very effective at extending the lag phase and reducing the final population compared to a control (Shelef *et al.*, 1984).

2.2.2.6 Beverages

In apple juice (pH 3.7), 0.1-0.3% cinnamon inactivated 4 logs of *Listeria monocytogenes* at 5 and 20°C within one hour. *Salmonella* Typhimurium was less sensitive to cinnamon than *Listeria monocytogenes*. It survived in apple juice combined with 0.3% cinnamon stored at 5°C more than seven days. Cells were more suppressed when the temperature was increased (Yuste and Fung, 2002; Yuste and Fung, 2003). Moreover, synergistic effects were reported between cinnamon and the preservatives, sodium benzoate or potassium sorbate (Ceylan *et al.*, 2004). In addition, a combination of cinnamon and nisin accelerated the death of *Salmonella* Typhimurium and *Escherichia coli* O157:H7 in this product (Yuste and Fung, 2004).

2.2.2.7 Food Models

Extraction of clove, cinnamon, bay and thyme were tested against *Listeria monocytogenes* and *Salmonella* Enteritidis in soft cheese diluted 1:10 in buffer. The former medicinal plant extracts were less easily inhibited in diluted full-fat cheese than in a low-fat version, indicating the protective action of fat. The level of fat in the cheese protected the bacteria cells to a different extent depending on which oil was used; clove oil was in fact more effective against *Salmonella* Enteritidis in full-fat cheese slurry (Smith-Palmer *et al.*, 2001).

Table 1 Overview studies testing the antibacterial activity of spices, essential oils or their components in food

Food	Medicinal Plants, Spices, Essential oils or component	Concentration applied	Note on experimental Set-up ¹	Observations ²		References
				Extension of lag phase of growth	Reduction in final population	
Dairy Products						
Butter	Garlic	20% w/w	Type of garlic Three storage temperatures	Yes	37°C:+++ 37°C:+++	Adler and Beuchat, 2002
Mozzarella cheese	Clove oil	0.5-1.0%		Yes	+	Vrinda Menon and Garg, 2001
Semi skimmed milk	Carvacrol	2-3 mmol/l	Two storage temperatures	ND	+	Karatzas <i>et al.</i> , 2001
Soft cheese	'DMC Base Natural' preservative comprising 50% essential oils of rosemary, sage and citrus	250-2500 ppm		From 1.0µl/g	+	Mendoza-Yepes <i>et al.</i> , 1997
Tzatziki (yoghurt and cucumber salad)	Mint oil	0.5-2.0% (v/w)	Two storage temperatures Gram negative bacteria	From 1.5% v/w	++	Tassou <i>et al.</i> , 1995
			Two storage temperatures Gram positive bacteria	Increase in growth	--	Tassou <i>et al.</i> , 1995

Table 1 (Continued)

Food	Medicinal Plants, Spices, Essential oils or component	Concentration applied	Note on experimental Set-up ¹	Observations ²		References
				Extension of lag phase of growth	Reduction in final population	
Vanilla-flavored yoghurt	Vanilla		Two levels of fat	No	+	Tipparaju <i>et al.</i> , 2004
Meat Products						
Minced mutton	Clove oil	0.5-1.0%	Two storage temperatures	Yes	+	Vrinda Menon and Garg, 2001
Roast beef sirloin sliced	Eugenol	0.1 ml spread over surface of 25 g slice	Two levels of inoculums and two storage temperatures	Yes	++	Hao <i>et al.</i> , 1998b
Cooked chicken breast pieces	Eugenol	0.1 ml spread over surface of 25 g piece chicken	Two levels of inoculums and two storage temperatures	Yes	++	Hao <i>et al.</i> , 1998b
Pork liver sausage	Rosemary oil, encapsulated rosemary oil	1% and 5% respectively		Yes	Rosemary oil: + Encapsulated oil: +++	Pandit and Shelef, 1994
Chicken noodles, beef	Sage oli	200-500 ppm		No	Chicken noodles: 0 Beef: 0	Shelef <i>et al.</i> , 1984
Beef fillets	Oregano oil	0.8% v/w	Modified atmosphere packaging (40% CO ₂ , 30% N ₂ , 30% O ₂)	In MAP but not in ambient air	MAP: +++ Air: +	Tsigarida <i>et al.</i> , 2000

Table 1 (Continued)

Food	Medicinal Plants, Spices, Essential oils or component	Concentration applied	Note on experimental Set-up ¹	Observations ²		References
				Extension of lag phase of growth	Reduction in final population	
Pâté	Mint oil	0.5-2.0% v/w	Two storage temperatures	No	0	Tassou <i>et al.</i> , 1995
Minced pork	Thyme oil	0.02 ml mixed with 25 g meat		Yes	+	Aureli <i>et al.</i> , 1992
Vacuum packed ham	Cilantro oil	0.1-6.0% v/v in coating	Coating: gelatine gel or canola oil	No	0	Gill <i>et al.</i> , 2002
Fish Products						
Red grouper	Carvacrol, citral, geraniol	0.5-3.0% w/v		Carvacrol at 30 µl/ml killed all cells	Carvacrol: ++ Citral and geraniol: + Surface: ++ Flesh: +	Kim <i>et al.</i> , 1995a; 1995b
Taramasalad (cod's roe salad)	Oregano oil	0.5-2.0% v/w	Various pHs and storage temperatures tested	Yes	+++	Koutsoumanis <i>et al.</i> , 1999
Taramasalad (fish roe salad)	Mint oil	0.5-2.0% v/w	Various pHs and storage temperatures tested	No	0	Tassou <i>et al.</i> , 1996

Table 1 (Continued)

Food	Medicinal Plants, Spices, Essential oils or component	Concentration applied	Note on experimental Set-up ¹	Observations ²		References
				Extension of lag phase of growth	Reduction in final population	
Produce						
Alfalfa seeds	Cinnamaldehyde	200, 600 mg/l	Fumigation at 50°C or 70°C	ND	50°C: + 70°C: 0	Weissinger <i>et al.</i> , 2001
Rice						
Boiled rice	Sage oil	200-500 ppm		No	0	Shelef <i>et al.</i> , 1984

Table 1 (Continued)

Food	Medicinal Plants, Spices, Essential oils or component	Concentration applied	Note on experimental Set-up ¹	Observations ²		References
				Extension of lag phase of growth	Reduction in final population	
Beverage						
Apple juice	Cinnamon	0.1-0.3%	Two different pH Two storage temperatures Gram positive bacteria	Yes	+++	Yuste and Fung, 2002
		0.3%	Two storage temperatures Gram negative bacteria	No	+	Yuste and Fung, 2003, 2004

In paper where the combined effect of an essential oil or an essential oil component has been studied in combination with another preservation method, only the results for essential tested alone are cited.

¹ Products were mostly stored under refrigeration, but temperature used range from 2 to 30°C

² The following classification has been used: +++ Large reduction compared to control ($> 3 \log_{10}$ CFU/g or CFU/ml fewer)
 ++ Medium reduction compared to control ($1.5-3.0 \log_{10}$ CFU/g or CFU/ml fewer)
 + Slight reduction compared to control ($\leq 1.5 \log_{10}$ CFU/g or CFU/ml more)

These classifications apply to the end point of the experiment, which varies between references from 15 min to 33 days.

³ ND = Not determined

2.2.3 The Studies of Antimicrobial Activity of Medicinal Plants, Spices and their Extracts in Thailand against Foodborne Pathogens

Stonsaovapak *et al.* (1995) studied the effect of nine dried Thai medicinal plants, including cinnamon (*Cinnamomum zeylanicum*), black pepper (*Piper nigrum*), white pepper (*Piper nigrum*), clove (*Eugenia caryophyllata*), cardamom (*Amomum krervanh*), coriander (*Coriandrum sativum*), star anise (*Illicium verum*), nutmeg (*Myristica fragrans*) and cumin seed (*Cuminum cyminum*) against *Listeria monocytogenes* in microbiological media. The results showed that clove had the most significant antimicrobial activity, followed by nutmeg and star anise. Moreover, effectiveness at 35°C was better than at 4°C. Klayrung (1998) tested the antimicrobial activity of crude extracts of 39 Thai medicinal plants by well assay against *Listeria monocytogenes* isolated from Thai food. The results showed that crude extract from finger-root (*Boesenbergia pandurata* Holtt.), jackfruit heartwood (*Artocarpus indica* Lam.), nutmeg (*Myristica fragrans* Holtt.), leadwort (*Plumbaago indica* L.), licorice (*Glycyrrhiza glabra* L.), sappan (*Caesalpinia sappan* L.) and Indian senna (*Cassia angustifolia* Vahl.) caused listerial growth inhibition. The MIC of jackfruit heartwood of *Listeria monocytogenes* in broth was 500 µg/ml, whereas the MIC values of mace and licorice were 2000 µg/ml. The extracts from finger-root, leadwort and sappan could not completely inhibit listerial growth even at 5000 µg/ml. Furthermore, their effectiveness depended upon pH and temperature. For example, the potential of inhibition of *Listeria monocytogenes* was reduced when the pH of the medium was increased. The greatest antilisterial activity of jackfruit heartwood, mace and licorice was in a range of pH 4.5-5.5. In addition, at refrigeration temperature (4°C), inhibition efficacy was lower than at higher temperatures (35°C or 45°C). These results supported the report by Stonsaovapak *et al.* (1996). Kongbangkerd (1995) investigated the efficacy of garlic and clove on inhibiting *Salmonella* Typhimurium and *Staphylococcus aureus* in frozen chicken meat. The results indicated that 3% powdered garlic and 0.1% clove oil could inhibit the growth of the bacteriae; however, their numbers were not reduced. Suwannarat (2001) determined the efficiency of garlic for inhibiting foodborne pathogens in frozen black tiger prawns by dipping the prawns into a crude water extract of garlic, at a ratio of fresh garlic to

water 1:3 (w/v). On dipped prawns lower numbers of *Salmonella* sp., *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio parahaemolyticus* were detected than on non-dipped.

2.3 Methods for Evaluation the Efficacy of Food Antimicrobial

Methods which are used to evaluate the activity of food antimicrobial may be divided into *in vitro* and application tests. The former may be termed “screening methods” and might include any test in which the compound is not applied directly to the product under use conditions. Generally, these tests provide preliminary information to determined potential usefulness of the test compound. The second type includes those tests in which an antimicrobial is applied directly to a food product.

2.3.1 *In vitro* Methods

In vitro methods or screening methods may be subdivided into endpoint and descriptive tests. Endpoint tests are those in which a microorganism is challenged for an arbitrary period. The results reflect the inhibitory power of a compound only for the time specified. In descriptive test, the microorganism is also challenged, but periodic sampling is carried out to determine changes in viable cell number over time.

2.3.1.1 Endpoint Screening Methods

2.3.1.1.1 Agar Diffusion

Of the endpoint test, the agar diffusion test has probably been the most widely used throughout history. It has often been referred to as the disk assay; however, this terminology is probably too narrow. There are many variations of the test method, including the use of cylinder, well, the ditch plate, and agar overlays. Other variations such as the flip-overlay may be used for the evaluation of the antimicrobial activity of bacteriocins.

In the most common variations of the assay, the antimicrobial compound is applied to an agar plate, using an impregnated filter paper disk, or placed in a well. The compound diffuses through the agar, setting up a concentration gradient. The concentration is inversely proportional to the distance from disk or area. Inhibition, which is the measure of activity, is indicated by a zone of no growth around the disk or well. The size of the zone is dependent upon the rates of diffusion and cell growth.

The most widely used screening methods to measure the antimicrobial efficacy of medicinal plants, spice, their essential oil, and their constituents have been agar diffusion method (Kivanc and Anguel, 1986; Deans and Ritchie, 1987; Farag *et al.*, 1989; Aureli *et al.*, 1992; Hefnawy *et al.*, 1994; Qamar *et al.*, 1994; Bara and Vanetti, 1995; Domokos *et al.*, 1997; Lis-Balchin and Deans, 1997; Firouzi *et al.*, 1998; Ilcim *et al.*, 1998; Lachowicz *et al.*, 1998; Wei *et al.*, 1998; Sun Kyung *et al.*, 1999; Rauha *et al.*, 2000; Yildirim *et al.*, 2000; Young Seon *et al.*, 2000; Elgayyar *et al.*, 2001; Martins *et al.*, 2001; Miniya and Thoppil, 2001; Okeke *et al.*, 2001; Unal *et al.*, 2001; Iscan *et al.*, 2002; Rasooli *et al.*, 2002; Velickovic *et al.*, 2002; Venturini *et al.*, 2002; Aureli *et al.*, 2003; Faleiro *et al.*, 2003; Safak *et al.*, 2003; Vilijoen *et al.*, 2003; Thiem and Goslinska, 2004). One of the most commonly used is the disk diffusion method, where a paper disk soaked with medicinal plant extract is laid on top of an inoculated agar plate. Factors such as the volume of extract placed on the paper disk, the thickness of the agar layer and whether a solvent is used vary considerable between studies. This means that this method is useful for selection between extracts but comparison of published data is not feasible. The agar well test in which the extract is deposited into well cut into the agar can be used when large number of extracts and/or large number of bacterial isolates are to be screened (Dorman and Deans, 2000). To make bacterial growth easier to visualize, triphenyl tetrazolium chloride may be added to the growth medium (Elgayyar *et al.*, 2001; Mourey and Canillac, 2002).

The results of agar diffusion method are generally quantitative. Microorganisms are generally termed susceptible, intermediate, or resistance, depending upon the diameter of the inhibition zone. Quantitative results are possible with a high degree of

standardization, but better methods are available. A requirement of the test is that all strains used must grow rapidly and uniformly, or little or no repeatability will be achieved. The test also must be appropriate for the compound and test microorganism. For example, the agar diffusion test cannot be used for a highly hydrophobic antimicrobial, since the compound will not diffuse and no inhibition can be detected. This method should also not be used for anaerobic microorganisms.

2.3.1.1.2 Agar and Broth Dilution

The strength of the antimicrobial activity can be determined by dilution of compound in agar or broth. In the agar dilution method, the compound is serially diluted in bottles of melted agar and plates poured. The plates are then inoculated with a single microorganism or with multiple strains. This may be done manually with a loop or with a multiple inoculators. The approximate initial cell concentration is 1×10^4 CFU/spot. Results for the assay are determined after 24 or 48 hours of incubation. A single colony, a haze, or no growth is considered negative. Advantages of the method include the following: a large number of strains may be tested at once, contamination is easily detected, and the medium may contain opaque materials.

To evaluate the antimicrobial activity of medicinal plant extracts or their essential oils in different literatures, the published studies using agar dilution have used different solvents to incorporate the medicinal plant extracts in the medium (Prudent *et al.*, 1995; Pintore *et al.*, 2002), different volumes of inoculums (1-100 μ l) (Juven *et al.*, 1994; Prudent *et al.*, 1995), and inoculation techniques, e.g. dotting (Pintore *et al.*, 2002) or streaking (Farag *et al.*, 1989). Despite these variations, the MICs of medicinal plant extracts determined by agar dilution generally are in approximately the same order of magnitude (Farag *et al.*, 1989; Prudent *et al.*, 1995; Pintore *et al.*, 2002).

In the broth dilution assay, a compound is serially dilute and distributed in a nutrient broth, which then is inoculated with a single strain of microorganism. A generally recommended initial cell concentration is 5×10^5 CFU/tube. For the broth assay,

absence of turbidity is considered a negative test. The broth assay may be used in both the macro-and microdilution versions. The microtube version allows an increase in productivity through the use of microtiter plates. Critical control points or standardization of the assay may be achieved at the following stages, medium type, pH, stock solutions, concentration range, inoculum density, and incubation conditions.

With both the agar and broth dilution assays, the objective is to generate a single statistic to describe the inhibition of a microorganism at a specific endpoint in time. The measurement of inhibition at a specific time is termed the minimum inhibitory concentration (MIC). The MIC may be defined as the lowest concentration at which no growth occurs in a nutrient medium. However, the definition of the MIC differs between publications. In some cases, the minimum bactericidal concentration (MBC) or the bacteriostatic concentration is stated, both terms agreeing closely with the MIC. A list of the most frequently used terms in antimicrobial activity testing of crude medicinal plants extract, and essential oils are presented in Table 2.

What is often found in antimicrobial assays is that concentrations at and above the MIC cause reversible inhibition. Removing the inhibitory pressure of the antimicrobial will result in growth. This can best be demonstrated with the broth dilution assay. An aliquot of medium from any tube which demonstrates no growth in the MIC assay is transferred to fresh medium which contains no antimicrobial. If no growth occurs in the fresh medium, lethality has occurred. This is defined as the Minimum Lethal Concentration, MLC.

In general, dilution tests should be used when quantitative data are desired, when the strains tested have variable growth rates, when it is desirable to determine lethality, to measure the effects of combinations of antimicrobials quantitatively, and when the test should be performed with anaerobic or microaerophilic microorganisms.

Table 2 Terms used in antibacterial activity testing

Term	Definition, with reference to concentration of crude medicinal plants extract and essential oil	Reference
Minimum inhibitory concentration (MIC)	Lowest concentration resulting in maintenance or reduction of inoculums viability	Carson <i>et al.</i> , 1995a
	Lowest concentration required for complete inhibition of test organism up to 48 hours incubation	Wan <i>et al.</i> , 1998; Canillac and Mourey, 2001
	Lowest concentration inhibiting visible growth of test organisms	Barry, 1976; Hammer <i>et al.</i> , 1999a; Delaquis <i>et al.</i> , 2002
	Lowest concentration resulting in significant decrease in inoculums viability (>90%)	Cosentino <i>et al.</i> , 1999
Minimum bactericidal concentration (MBC)	Concentration where 99% or more of the initial inoculums are killed	Carson <i>et al.</i> , 1995b; Cosentino <i>et al.</i> , 1999; Canillac and Mourey, 2001
	Lowest concentration at which no growth is observed after sub-culturing into fresh broth	Barry, 1976; Onawunmi, 1989
Bacteriostatic concentration	Lowest concentration at which bacterial fail to grow in broth, but are grow when broth is plated onto agar in the absence of the inhibitor	Smith-Palmer <i>et al.</i> , 1998
Bactericidal concentration	Lowest concentration at which bacteria fail to grow in broth and fail to grow when broth is plated onto agar in the absence of the inhibitor	Smith-Palmer <i>et al.</i> , 1998

The agar dilution method was used to determine the strength of the antimicrobial activity of medicinal plants extract, spices, and essential oils by Banerjee *et al.*, (1982), Kivac and Akguel (1986), Bara and Vanetti (1995), Sekiyama *et al.*, (1996), Firouzi *et al.*, (1998), Ward *et al.*, (1998), Hammer *et al.*, (1999a, 1999b), Elgayyar *et al.*, (2001), Unal *et al.*, (2001), Iscan *et al.*, (2002), Velickovic *et al.*, (2002), Venturini *et al.*, (2002), Alzoreky and Nakahara, (2003), Aureli *et al.*, (2003), Burt and Reinders (2003), Nguefack *et al.*, (2004), Rajenda Prasad *et al.*, (2004), and Tepe *et al.*, (2004). The broth dilution method was used by Bara *et al.*, (1995), Carson (1995b), Cosentino *et al.*, (1999), Hammer *et al.*, (1999a, 1999b), Jeong Jun *et al.*, (1999), Unal *et al.*, (2001), Delaquis *et al.*, (2002), Jee Young *et al.*, (2002), Velickovic *et al.*, (2002), Venturini *et al.*, (2002), Araujo *et al.*, (2003), Cosentino *et al.*, (2003), Tepe *et al.*, (2004), Thiem and Goslinska (2004) and Yu *et al.*, (2004).

An alternation determination of the end point of broth dilution assays is the measurement of conductance/conductivity or impedance (Tassou *et al.*, 1995; Wan *et al.*, 1998; Marino *et al.*, 1999, 2001). A new microdilution method for determining the MIC of oil based compounds uses the redox indicator resazurin as a visual indicator of the MIC. The results compare favourably with those obtained by viable count and OD measurement and the method is more sensitive than the agar dilution method (Mann and Markham, 1998). A patented colour indicator based on resazurin has been used to determine the MICs for methanolic extracts of plant material (Salvat *et al.*, 2001) and essential oils (Burt and Reinders, 2003) and the method can be automated by measuring the end point by fluorescence instead of visual means (Burt, 2004).

2.3.1.1.3 Gradient Plates

A method similar to the agar dilution assay is the gradient plate technique. In this method, melted agar is dispensed into a Petri dish with one edge elevated. The agar is allowed to solidify and form a wedge. A second, overlay wedge which contains the antimicrobial is then poured. The result is a plate which contains a gradient of antimicrobial concentrations from near 0 to the maximum in the overlay. The plate

may then be inoculated using a spread plate method or by parallel streaks of several strains of microorganisms. The advantage of the analysis is that the microorganism is exposed to a continuous gradient of concentrations, as opposed to the 2-fold or 10-fold dilutions used in the previous tests. This technique was used to test the antimicrobial activity of medicinal plants, spices, and essential oils by Thing and Deibel (1992) to study the sensitivity of *Listeria monocytogenes* to various extracts such as clove, oregano and black pepper.

A variation of the gradient plate technique utilizes a spiral plating system. This assay uses the spiral plate to deposit a radial gradient of antimicrobial on an agar plate. Because the volume of liquid deposited on any section of the plate is known, the concentration of antimicrobial may then be determined as well. A bacterial suspension is inoculated onto the surface of the plate to produce a radial streak. The MIC of the compound against the microorganism is determined by a zone of no growth on the streak.

2.3.1.2 Descriptive Screening Method

While endpoint methods are excellent for screening compounds, they give little information concerning the effect of the compound on dynamic growth of microorganisms. In food products, the using of the minimum antimicrobial concentration needed to cause inhibition of potential spoilage or pathogenic microorganisms is often studied. Even extension of the lag phase of a microorganism can be important under conditions of abuse. Therefore, one information has been determined about the MIC for the microorganism, it is important to look at the microorganism's growth over time. There are several possible methods for evaluating the effect of an antimicrobial on the growth of a microorganism over time. Two of the most popular are the turbidimetric assay and the inhibition curve.

2.3.1.2.1 Turbidimetric Assay

The simplest, most economical and productive growth measurement system is probably the turbidimetric assay. It simply involves following the growth of a microorganism with a spectrophotometer.

One of the major problems with turbidimetric analysis is the range of detection. Spectrophotometers generally require 10^6 - 10^7 CFU/ml for detection, thereby limiting cell concentrations which can be successfully assayed with this method. This may create a situation in which no growth (i.e., no absorbance increase) is observed, when, in fact, undetectable growth is occurring at levels below 10^5 CFU/ml. An erroneous interpretation of 'lethality' could result (Davidson and Parish, 1989).

There have been many publications using this method to study the antimicrobial activity of medicinal plants, spices, and their essential oils including Shelef *et al.*, (1984), Meena *et al.*, (1986), Syed *et al.*, (1986a, 1986b), Ismaiel and Pierson (1990), Kanemaru and Miyamoto (1990), Rajashekhara *et al.*, (1990), Kim *et al.*, (1995a, 1995b), Sivropoulou *et al.*, (1995, 1996), Chaibi *et al.*, (1997), Wilson *et al.*, (1997), Smith-Palmet *et al.*, (1998), Ultee *et al.*, (1998), Jeong Jun *et al.*, (1999), Pol and Smid (1999), Kokoska and Rada (2001), Lambert *et al.*, (2001), Skandamis *et al.*, (2001), Ultee and Smid (2001), and Mejlholm and Dalgaard (2002).

2.3.1.2.2 Inhibition or Time Killing Curve

In addition to turbidimetric analyses, there is the inhibition curve, also known as the 'killing curve' in clinical research. This test simply involves inoculation of a microorganism into a medium, addition of an antimicrobial, followed by incubation and periodic sampling to determine growth of survival. It is a more accurate analysis than the turbidimetric assay because of the wide detection range. Some of the resulting curves are easy to interpret, other are not.

Figure 12 shows some of possible situations one might encounter in running this type of test. First (A) is growth level suppression. This is sometimes used with a term called ‘percentage growth inhibition’ which may be misleading because it is a function of time. Second (B) is a lag phase increase. Third (C) is a decrease in the growth rate with little effect on lag time. Forth (D) is lethal effect, the time killing-curve is the only test which will show this effect. Following an antimicrobial test in which lethality occurs, every small percentage of the original population will often remain viable.

This method is versatile but has several disadvantages. No simple statistical method is available to detect differences, no single statistic is produced to compare treatment such as MIC and it is labour intensive and expensive.

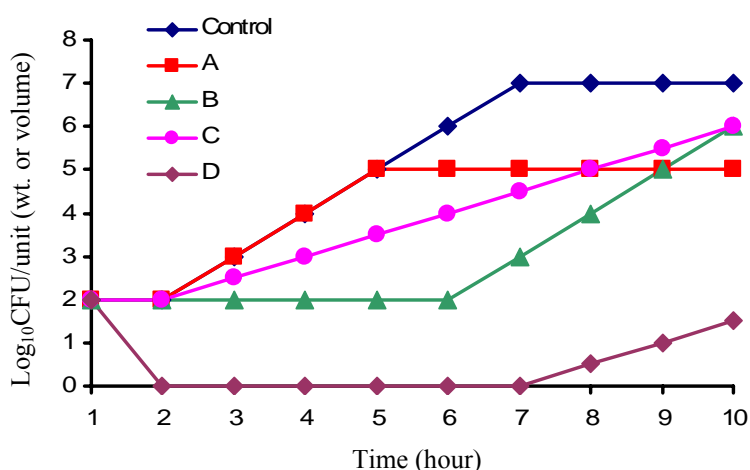


Figure 12 Type of growth and inhibition curves possible when a microorganism is exposed to an antimicrobial over time

Source: Davidson and Parish (1989)

The time kill curve was used to evaluate the antimicrobial activity of medicinal plants, spices, and essential oils by Beuchat (1976), Shelef *et al.* (1984), Ting and Deibel (1992), Aureli *et al.* (1992), Stecchini *et al.* (1993), Tassou *et al.* (1995),

Sivoropoulou *et al.* (1996), Ultee *et al.* (1998), Wan *et al.* (1998), Pol and Smid (1999), Sun Kyung *et al.* (1999), Periago and Moezelaar (2001), Skandamis *et al.* (2001), Sung Hwang and Young Rok (2001), Ulte and Smid (2001), Mejlholm and Dalgaard (2002), Pintore *et al.* (2002), Young Rok and Sung Hwan (2002), Burt and Reinders (2003), Cressy *et al.* (2003), and Nakamura *et al.* (2004).

Screening methods should be used together, one endpoint and one descriptive test. The endpoint test helps to determine the approximate effective concentration, and the descriptive test evaluates the effect of a compound on growth over time.

2.3.2 Application Methods

While *in vitro* tests can give a good deal of information antimicrobial performance, they cannot necessarily duplicate all the variability which might exist in a food. Therefore, once it has been determined that the antimicrobial performs well in an *in vitro* situation, it should be applied to a food system. In many cases, researchers simply start with the food product and apply an antimicrobial without conducting initial background testing. Without the screening method data, it is difficult to determine starting concentrations.

It may also be useful to evaluate the effect of some components in a food system, such as lipids, proteins, and divalent cations, which might influence the effectiveness of the compounds. Lipids may cause a decrease in activity of lipophilic compounds, and since many food antimicrobials have hydrophobic character, there will invariably be some reduction. Divalent cations may affect the activity of some compounds by affecting the microorganisms itself or interacting with the antimicrobial.

Applying the antimicrobial to a food involves either model food system or the actual food. The microorganism used should be natural contaminants (bio-burden) or pathogens of interest, and incubation condition should reflect use and abuse. Success of the test may be determined by increase shelf life or prevention of problems due to abuse. As with the screening test, there are no standardized methods available, however, a modification of the endpoint test or inhibition curve is possible.

2.3.3 Combination Studies

While combinations of antimicrobials are currently utilized in the food industry, e.g., potassium sorbate and sulphur dioxide in sparkling wines, their interactions are often poorly characterized. Combined antimicrobial agents have been extensively studied in the pharmaceutical industry, and methods have been developed to determine types of interactions between two antimicrobials (Barry, 1976; Krogstad and Moellering, 1986; Squires and Cleeland, 1985). However, the applicability of such procedures to food preservative combinations has not been extensively explored.

When two antimicrobials are used in combination, three things may occur. First, there may be an additive effect. As defined by Barry (1976), 'the combined effect is equal to the sum of the effects observed with the two agents tested separately or equal to that of the most active agent in combination.' Additive effects occur when the antimicrobial activity of a compound is neither enhanced nor reduced while in the presence of another agent. The second occurrence can be synergistic. Again as defined by Barry (1976), 'the effect observed with a combination is greater than the sum of the effects observed with the two agents independently.' Synergism refers to an enhancement of overall antimicrobial activity of a compound when in the presence of a second antimicrobial agent. Finally, one may have antagonism between the pair. Antagonism occurs when the antimicrobial activity of one compound is reduced in the presence of a second agent.

According to Krogstad and Moellering (1986), synergistic interactions are generally due to sequential inhibition of a common biochemical pathway e.g., use of compounds which inhibit enzyme that interactive antimicrobials, combinations of cell wall active agent, and use of cell wall active compounds to increase uptake of other compounds. The mechanisms for antagonism are much more complex and less well studied. Examples of causes for antagonism are combinations of bacteriostatic and bactericidal agents, use of agents with same active site (e.g., the 50S submit of the ribosome), and a chemical interaction (direct or indirect) between two agents (Krogstsad and Moellering, 1986).

Perhaps the most misused of the above term is synergism. This term has been used to describe relationships between agents without regard to the overall concentration of antimicrobial in combination. In other words, increased antimicrobial activity in a combination which contains 100 mmol of compound A and 100 mmol of A or B alone and would not necessarily constitute synergy. Many reports exist where the activity of a single agent is reported along with the activity of a combination and synergism is claimed by the investigator.

A report of synergism requires that the antimicrobial activity of each agent be reported with the combined effect and that the combined effect be greater than the expected, based on the activity of the individual compounds.

Two methods are available which specifically address the measurement of interaction—agar dilution and broth dilution tests. A more useful and quantitative test is the broth dilution assay. A typical broth dilution assay makes use of an array of culture tubes or a microtiter plate in which serial dilutions of two antimicrobial agents are dispensed. The result is a checkerboard type agreement. Once diluted, each tube or well is inoculated and incubated for a prescribe period, after which growth or survival of the organism is determined. If desired, this test may be conducted using agar medium as well.

Results of the tests produce MIC data as described earlier for individual antimicrobials. Since day-to-day biological variability of MICs makes it difficult to compare confidently results from different tests, the data may be transformed to produce what is called the fractional inhibitory concentration, FIC (Barry, 1976). The FIC for an individual antimicrobial agent is the ratio of the concentration of the antimicrobial in an inhibitory combination with a second agent to the concentration of the antimicrobial by itself.

In other words, the concentration of a given compound needed to inhibit growth is given a value of 1, and the amount of the compound needed to inhibit growth when combined with another antimicrobial agent at a given concentration is expressed as a

fraction. Most researchers add FICs for individual agents in a combination to produce a FIC_{Index} (Barry, 1976).

Calculation of a combined FIC yields a single number which can be indicative of additive, synergistic, or antagonistic effects. Theoretically, a combined FIC near 1 indicates additivity, <1 indicates synergy, and >1 indicates antagonism. However, the degree to which a value must be greater than or less than 1 to suggest antagonism or synergism is not clearly established.

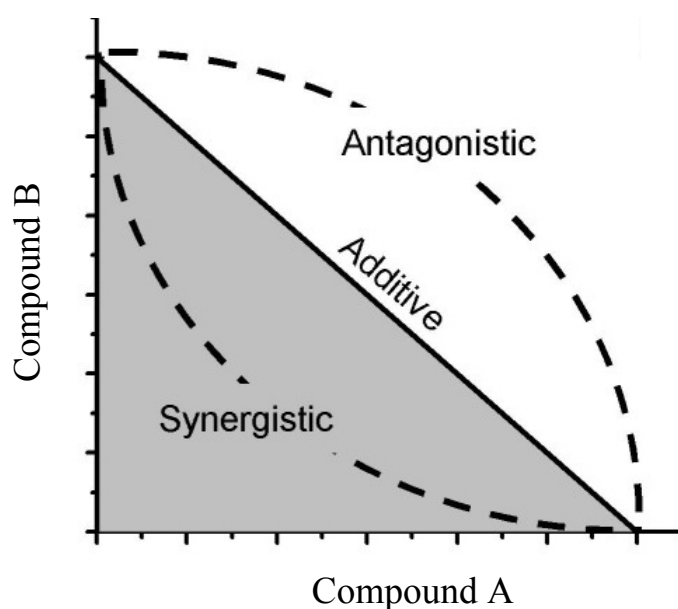


Figure 13 Isobolograms displaying the three types of results possible with combination of antimicrobials

A method to visualize the interactions of antimicrobial combinations is the isobologram (Figure 13). This can be constructed using MIC data directly or calculating FICs. Using MICs, all that is done is to plot the results of the checkerboard assay as the first points at which no growth occurred. If the two compounds are additive, the results will fall on a straight line between the X and Y

axes. Synergism is indicated by a curved deviation to the left of the additive line, while antagonism is a curved deviation to the right of the additive line.

Example of research in which FIC was used to determine the interaction of medicinal plant extract, spices extract, and essential oil components were Kang *et al.* (1992), Matamoros Leon *et al.* (1999) and Jee Young *et al.* (2002). Matamoros Leon *et al.* (1999) used FIC isobologram curve to evaluate the interaction between spices extract and potassium sorbate on inhibition *Penicillium digitatum*, *Penicillium glabrum*, and *Penicillium italicum*.

2.4 Active Packaging Technologies and its Applications

Active packaging is one of the innovative food packaging concepts that have been introduced as a response to the continuous changes in current consumer demands and market trends. It can be defined as a mode of packaging in which the package, the product, and the environment interact to prolong shelf life or enhance safety or sensory properties, while maintaining the quality of the products. This definition of active packaging was chosen for the European FAIR-project CT 98-4170 (Vermeiren *et al.*, 1999). In general, active food packaging can provide several functions that do not exist in conventional packaging systems. The active functions may include scavenging of oxygen, moisture or ethylene, emission of ethanol and flavours, and antimicrobial activity.

2.4.1 Oxygen Scavenging System

The presence of oxygen in a packaged food is often a key factor that limits the shelf life of products. Oxidation can cause change in flavour, colour, and odour, as well as destroy nutrients and facilitate the growth of aerobic bacteria, moulds, and insects. Oxygen scavenging is an effective way to prevent growth of aerobic bacteria and moulds in dairy and bakery products. Packaging of crusty rolls in a combination of carbon dioxide and nitrogen has shown to be an effective measure against mould growth (Smith *et al.*, 1987). Lyver *et al.* (1998) monitored the physical, chemical,

microbiological, texture, and sensory change in surimi nuggets inoculated with *Listeria monocytogenes*, packaged in either air or carbon dioxide with and without oxygen scavenger. The results indicated that these conditions were not effective in controlling the growth of the pathogen in either raw or cooked nuggets and also that the pathogen overcame competitive inhibition and pH reduction caused by lactic acid bacteria.

2.4.2 Moisture-Absorbing and Controlling System

In solid foods, a certain amount of moisture may be trapped during packaging or may develop inside the package due to generation or penetration. The minimization via packaging can be achieved either by liquid water absorption or humidity buffering.

2.4.2.1 Liquid water absorption

The main purpose of liquid water control is to lower the water activity (a_w) of the product, thereby suppressing the growth of microorganisms on the foodstuff (Vermerien *et al.*, 1999). Basically, this system consists of a superabsorbent polymer located between 2 layers of a micro-porous or non-woven polymer. Such sheets are used as drip-absorbing pads placed under food. The preferred polymers used for this purpose are polyacrylate salts and graft copolymers of starch (Rooney 1995).

2.4.2.2 Humidity buffering

This approach involves interception of moisture in the vapour phase by reducing the in-pack relative humidity and thereby the surface-water content of the food. Desiccants, silica gel, molecular sieves, calcium oxide, sodium chloride and natural clays have been successfully used for moisture control in a wide range of food (Rooney 1995).

2.4.2.3 Carbon Dioxide Generating Systems

Carbon dioxide is known to suppress microbial activity. Relatively high CO₂ levels (60%-80%) inhibit microbial growth on surfaces and, in turn, prolong shelf life. Therefore, a complementary approach to O₂ scavenging is the impregnation of a packaging structure with a CO₂ generating system or the addition of the latter in the form of a sachet. High CO₂ levels may, however, cause changes in taste of products and the development of undesirable anaerobic glycolysis in fruits. Consequently, a CO₂ generator is only useful in certain applications such as fresh meat, poultry, fish and cheese packing (Floros *et al.*, 1997). Nakamura and Hoshino (1983) reported that an oxygen-free environment alone is insufficient to retard the growth of *Staphylococcus aureus*, *Vibrio* sp., *Escherichia coli*, *Bacillus cereus*, and *Enterococcus faecalis* at ambient temperature. A combined treatment involving O₂ scavenging with thermal processing, or storage under refrigeration, or using a CO₂ enriched atmosphere was recommended for complete inhibition of these microorganisms.

2.4.3 Ethanol Generating Systems

Ethanol is used routinely in medical and pharmaceutical packaging applications, indicating its potential as a vapour phase inhibitor (Smith *et al.*, 1987). It prevents microbial spoilage of intermediate moisture foods such as cheese, and bakery products. It also reduces the rate of staling and oxidative changes (Seiler, 1989). Ethanol has been shown to extend the shelf life of bread, cake and pizza when sprayed onto product surface prior to packaging. Sachets containing encapsulated ethanol release its vapour into the packaging headspace thus maintaining the preservative effect (Labuza and Breene, 1989).

Smith *et al.* (1987) demonstrated the usefulness of ethanol vapour in extending the shelf life of apple turnovers. The shelf life was found to be 14 days for the product packaged in air or in a CO₂/N₂ gas mixture (60% CO₂) and stored at ambient temperature. Afterwards, visible swelling occurred as a result of *Saccharomyces cerevisiae* growth and additional CO₂ production. When encapsulated ethanol was

incorporated in the package, yeast growth was totally suppressed and the shelf life was extended to 21 days.

2.4.4 Antimicrobial Migrating and Non-migrating Systems

Antimicrobial food packaging materials have to extend the lag phase and reduce the growth rate of microorganisms in order to extend shelf life and to maintain product quality and safety (Han, 2000). Alternatives to direct additives for minimizing the microbial load are canning, aseptic processing and modified atmosphere packaging. However, canned foods cannot be marketed as fresh produces. Aseptic processing may be expensive and hydrogen peroxide, which is restricted in level by regulatory agencies, is often used a sterilizing agent. In certain cases, modified atmosphere packaging can promote the growth of photogenic anaerobes and the germination of spores, or prevent the growth of spoilage organisms which indicate the presence of pathogens (Farber, 1991). If packaging materials have self-sterilizing abilities due to their own antimicrobial effectiveness, the need for chemical sterilization of the packages may be obviated and the aseptic packaging process simplified (Hotchkiss, 1997).

2.4.4.1 Gas Emission or Flushing

Gas emission or flushing controls the growth of mould. Typical spoilage moulds include *Botrytis cinerea*, *Penicillium*, *Aspergillus*, and *Rhizopus* species commonly found in citrus and berry fruits. Sulphur dioxide (SO₂) is known to be the most effective material in controlling the decay of grapes and is superior to the gamma irradiation and heat-radiation combination methods (Smilanick *et al.*, 1990). Another volatile compound exhibiting antimicrobial effects is allyl isothiocyanate, the major pungent component of black mustard (*Brassica nigra*), brown mustard (*Brassica juncea*), and wasabi (*Eutrema wasabi* Maxim.) (Isshiki *et al.*, 1992).

2.4.4.2 Coating with Antimicrobial Agents

Appropriate coating can sometimes impart antimicrobial effectiveness. A polymer based solution coating would be the most desirable method in terms of stability and adhesiveness of attaching a bacteriocin to a plastic film. It was found that the low density polyethylene film coated with a mixture of polyamide resin in *i*-propanol/*n*-propanol and a bacteriocin solution provided antimicrobial activity against *Micrococcus flavus* (An *et al.*, 2000). Low density polyethylene coating was successfully coated with nisin using methylcellulose or hydroxypropyl methylcellulose as a carrier, it was found to be effective in suppressing *Staphylococcus aureus* and *Listeria monocytogenes* (Cooksey, 2000).

2.4.4.3 Incorporation of Antimicrobial Additives

The direct incorporation of antimicrobial additives in packaging coating is a convenient means by which antimicrobial activity can be achieved. Several compounds have been proposed and tested for antimicrobial packaging using this method (Weng and Hotchkiss, 1993; Ishitani, 1995; Chen *et al.*, 1996; Han and Flores, 1997; Huang *et al.*, 1997; Luck and Jager, 1997; Weng and Chen, 1997; Begin and Calsteren, 1999; Siragusa *et al.*, 1999; Weng *et al.*, 1999; Devlieghere *et al.*, 2000a; 2000b; Dobias *et al.*, 2000; Ouattara *et al.*, 2000a; Ouattara *et al.*, 2000b; Vermeiren *et al.*, 2002).

Natural plant extracts are also selected as an incorporated additive in antimicrobial packaging systems. These compounds are perceived to be safe and were claimed to alleviate safety concerns (Davidson and Parish, 1989; An *et al.*, 1998; Chung *et al.*, 1998; Lee *et al.*, 1998; Hong *et al.*, 2000; Rodrigues and Han, 2000; Coma *et al.*, 2001; Ha *et al.*, 2001; Suppakul *et al.*, 2002;).

2.4.4.4 Immobilization

Besides diffusion and sorption, some antimicrobial packaging systems utilize covalently immobilized antimicrobial substances that suppress microbial growth. It is known that cellulose triacetate containing lysozyme or polyamide bonded with nisin yields high antimicrobial activity (Appendini and Hotchkiss, 1977; Scannell *et al.*, 2000).

2.5 Antimicrobial Biodegradable Films and Coating

Antimicrobial packaging is a promising form of active food packaging. Since microbial contamination of foods occurs primarily at the surface, due to post-processing handling, attempts have been made to improve safety and delay spoilage by use of antimicrobial sprays or dips. However, direct surface application of antibacterial substances onto food have limited benefits because the active substances are neutralized on contact or diffuse rapidly from the surface into the food mass (Stefania and Loredana, 2002).

2.5.1 Edible Films and Coating History

The concept of employing edible films as protective coating for foods is not at all novel, the earliest document was in the 1800s (Allen *et al.*, 1963). Waxes have also been used for a long time to coat fruits and vegetables to limit physiological deterioration processes and microbial spoilage and to control gas exchange (Mack and Janer, 1942; Kraght, 1966; Waks *et al.*, 1985). Biopolymer films and coating are generally designed using biological material such as polysaccharides, polyester, protein, lipid and also their derivatives (Aranyi *et al.*, 1970; Friedman, 1970; Robey *et al.*, 1989; Mueller *et al.*, 1991).

2.5.2 Developing the Antimicrobial Packaging Systems

Antimicrobial substances incorporated into packaging materials can control microbial contamination by reducing the growth rate and maximum growth population and/or extending the lag-phase of the target microorganism or by inactivating microorganisms by contact (Stefania and Loredana, 2002).

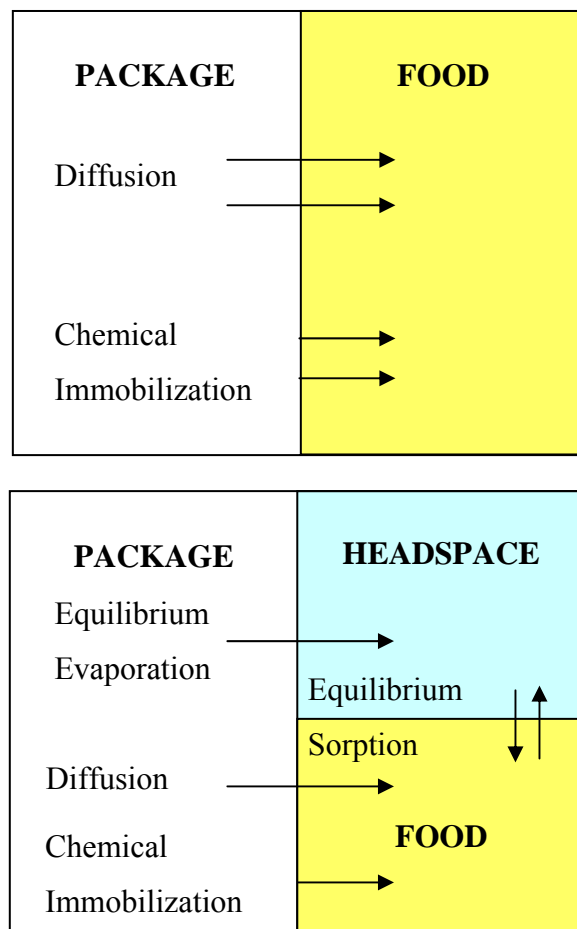


Figure 14 Food packaging systems and relative behaviour of active substances

Source: Han (2000)

Most food packaging systems represent either a package or food system or a package/headspace/food system (Figure 14). A package/food system is a solid food product in contact with the packaging material, or a low-viscosity or liquid food without headspace. Diffusion between the packaging material and the food and partitioning at the interface are the main migration phenomena involved in this system. Antimicrobial agents may be incorporated into the packaging material initially and migrate into the food through diffusion and partitioning (Han, 2000).

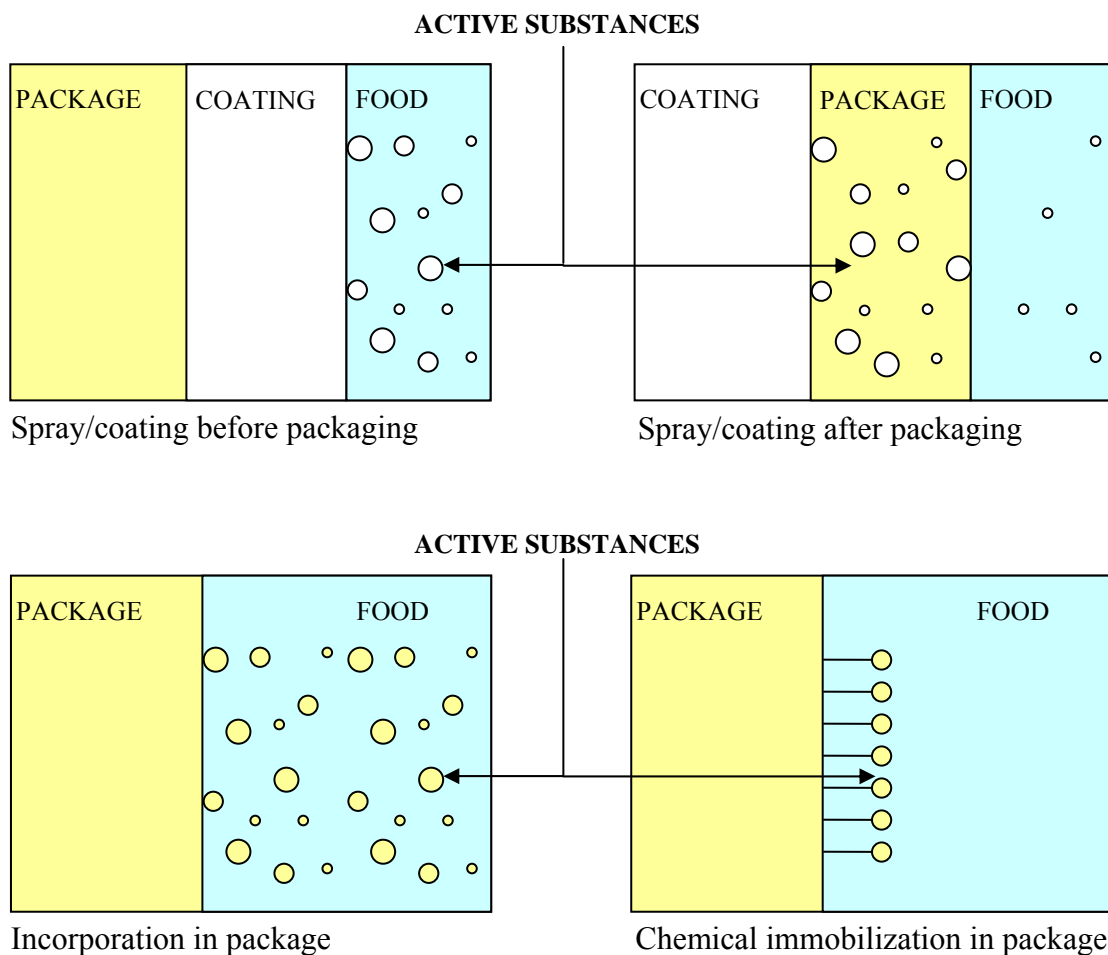


Figure 15 Migration of active substance in different applications of antimicrobial packaging systems

Source: Han (2000)

The incorporation of an antimicrobial substance into food packaging system can take several approaches. One is to put the antimicrobial into the film by adding it in the extruder when the film or the co-extruded film is produced. An alternative to extrusion is to apply the antimicrobial additive in a controlled matter where the material is needed and not lost; it can be incorporated into the food-contact layer of a multilayer packaging material (Figure 15).

3 MATERIALS AND METHODS

3.1 Material

3.1.1 Medicinal Plants

- *Andrographis paniculata* (Burm.f.) Wall. ex Nees
- *Curcuma zedoaria* (Christm.) Roscoe
- *Garcinia mangostana* Linn.
- *Hibiscus sabdarifa* Linn.
- *Musa sapientum* Linn.
- *Nelumbo nucifera* Gaertn.
- *Piper betle* Linn.
- *Punica granatum* Linn.
- *Psidium guajava* Linn.

3.1.2 Bacterial Strains

3.1.2.1 Bacterial strain provided by the laboratory of Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand

- *Escherichia coli* O157:H7 (VTEC) DMST 12743

3.1.2.2 Bacterial strains provided by the laboratory of Food Microbiology and Food Safety, Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkhan Campus, Bangkok, Thailand

- *Bacillus cereus* ATCC 11778
- *Escherichia coli* ATCC 8739
- *Listeria monocytogenes* 101
- *Pseudomonas fluorescens* ATCC 13525

- *Salmonella* Typhimurium ATCC 13311
- *Staphylococcus aureus* ATCC 13565
- *Vibrio parahaemolyticus* ATCC 20502
- *Yersinia enterocolitica* ATCC 2779

3.1.2.3 Bacterial strains obtained from the Institute of Environmental and Animal Hygiene, University of Hohenheim, Stuttgart, Germany

- *Bacillus cereus* ATCC 128263
- *Bacillus subtilis* ATCC 6633
- *Escherichia coli* DSMZ 632
- *Listeria monocytogenes* DSMZ 20600
- *Pseudomonas aeruginosa* DSMZ 939
- *Salmonella* Typhimurium DSMZ 5569
- *Staphylococcus aureus* DSMZ 799

3.1.3 Culture Media

- Trypticase Soy Broth with neutralizer
- Trypticase Soy Agar (Oxoid, Hampshire, England)
- Trypticase Soy Broth (Oxoid, Hampshire, England)

3.1.4 Chemical Agents

- Albumin (Albumin bovine Fraction V, Serva, Heidelberg, Germany)
- EDTA (Ethylenediamine-tetraacetic acid -2Na dihydrate; $C_{10}H_{14}N_2Na_2O_2 \cdot 2H_2O$, Carl Roth GmbH & Co., Karlsruhe, Germany)
- Ethanol (Alkoholvertrieb Süd GmbH, Langenenslingen, Germany)
- Glycerol (Glycerin; $C_3H_8O_3$, Merck, Darmstadt, Germany)
- Lecitin (L- α -Phosphatidylcholine from egg yolk, Fluka Analytical, Sigma-Aldrich Chemie GmbH, Steinheim, Germany)

- L-Histidin (L-2-amino-3-(3H-imidazol-4-yl)propanoic acid, Applichem, Darmstadt, Germany)
- Saponin (Saponins from Quillaja Bark, Applichem, Darmstadt, Germany)
- Sodium Alginate ($\text{NaC}_6\text{H}_7\text{O}_6$, Carl Roth GmbH & Co., Karlsruhe, Germany)
- Sodium Chloride (NaCl , Merck, Darmstadt, Germany)
- Sodium hypochlorite (NaOCl ; Carlo Erba Reagent, Rodano, Michigan, USA.)
- TWEEN[®] 80 (Polyoxyethylene (20) sorbitan monooleate; $\text{C}_{64}\text{H}_{124}\text{O}_{26}$, Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- WSH (Wasser standardisierter Härte, 300 ppm, distilled water, containing 2.42 mM CaCl_2 and 0.61 mM MgSO_4)
- Yeast extract (Oxoid, Hampshire, England)

3.1.5 Equipment

- 0.2 μm filter cup (FP 30/0.2 CA-S, Schleicher & Schuell, MicroScience GmbH, Dassel, Germany)
- 0.45 μm filter cup (FP 30/0.45 CA-S, Whatman GmbH, Dassel, Germany)
- 0.45 μm filter cup (Minisart, Sartorius, Hannover, Germany)
- 47 mm glass microfibre filters (GF/D, Whatman, Whatman International Ltd., Maidstone, England)
- Autoclave (Federgari Autoclavi S.p.A, Italy)
- Autoclave (Typ 135S, H+P Labortechnik GmbH, Oberschleißheim, Germany)
- Balance 1 digit (Precisa 6000D, PAG ORERLIKON AG, Zürich, Switzerland)
- Balance 1 digit (Sartorius universal, Sartorius, Hannover, Germany)
- Balance 2 digits (Mettler PC 440, Delta Range, Mettler waagen GmbH, Switzerland)
- Blender (VB 100 model 15BL21, Waring, New Hartford, Connecticut, USA.)
- Blender bag (Standard 400, 180 mm×300 mm×70 μm , Neolab, Heidelberg, Germany)
- Centrifuge (Suprafuge 22, Heraeus Sepatech GmbH, Osterode/Harz, Germany)

- Centrifuge (Varifuge 3.2RS, Heraeus Sepatech GmbH, Osterode/Harz, W. Germany)
- Freezer (-86C FREEZER Model 958, Forma Scientific, Ohio, USA.)
- Hot plate stirrer (Heidolph MR 3001 K, Heidolph Instruments GmbH & Co., KG, Schwabach, Germany)
- Hot plate stirrer (IKA-COMIMAG RCT, Janke and Kukel GmbH and Co KG, IKA Labortechnik, Staufen, Germany)
- Hot plate stirrer (IKAMAG[®] RCT, Janke and Kukel GmbH and Co KG, IKA Labortechnik, Staufen, Germany)
- Laminar air flow chamber (BDK, Luft- und Reinraumtechnik GmbH, Sonnenbühl-Genkingen, Germany)
- Orbital shaker (Pilot-Shake[®], Adolf Kühner AG, Basel, Switzerland)
- Shaking water bath (Kottermann, Hänigsen, W. Germany)
- Stomacher (BagMixer[®] Model 400P, Interscience, St-Nom, France)
- Ultrasonic bath (Bandelin Sonorex RK 510 Transistor, BANDELIN electronic GmbH & Co., KG Berlin, Germany)

3.2 Methods

3.2.1 Stock Culture Preparation and Condition

All test organisms were maintained in Trypticase Soy Broth with cryoprotectant (Glycerol, Merck, Germany), and stored at -80°C. Preparation of working stock culture, test organisms were thawed at 4°C and grown separately in 50 ml Trypticase Soy Broth on an orbital shaker at 37°C for 24 hours. Cultures were streaked out on Trypticase Soy Agar (Oxoid, England) and incubated at 37°C for 18-24 hours. All cultures were maintained on Trypticase Soy Agar at 4°C and transferred monthly to maintain viability. For *Vibrio parahaemolyticus* ATCC 20502, culture was kept separately on Trypticase Soy Agar at 20 ± 5°C and transferred weekly to maintain viability.

3.2.2 Preparation of Medicinal Plants

Big amount of fresh and semi-dried samples used in this study (Table 3) were purchased in the local markets of Thai traditional spices and medicinal plants in Bangkok, Songkhla, Pattani, and Narathiwat, Thailand. The samples were prepared in Thailand using some facility of Thai traditional medicinal manufactory in Songkhla, the southern province of Thailand.

All samples were transferred to the manufactory and started to be processed. They were washed in 50µg/mL hypochlorite solution, sliced and air-dried at 50°C in a hot air oven. The final moisture content determined by gravimetrically method was 5-8% (dry basis). Dried samples were ground to powder using a mechanical grinder, and kept separately in plastic bags in dry condition until use.

Table 3 List of Thai traditional medicinal plants used in the antibacterial assay

Botanical species	Family	Plants part
<i>Andrographis paniculata</i> (Burm.f.) Wall. ex Nees	Acanthaceae	Leaf
<i>Curcuma zedoaria</i> (Christm.) Roscoe	Zingiberaceae	Rhizome
<i>Garcinia mangostana</i> L.	Clusiaceae	Fruit shell
<i>Hibiscus sabdariffa</i> L.	Malvaceae	Flower
<i>Musa sapientum</i> L.	Musa	Blossom
<i>Nelumbo nucifera</i> Gaertn.	Nymphaeaceae	Rhizome
<i>Piper betle</i> L.	Piperaceae	Leaf
<i>Psidium guajava</i> L.	Myrtaceae	Leaf
<i>Punica granatum</i> L.	Punicaceae	Fruit shell

3.2.3 Preparation of Crude Extracts

Because of the limitation of solubility of fine particles of the 9 Thai traditional medicinal plants, the highest preparable concentration was 30 g/100 ml.

3.2.3.1 Hot Water Extraction

30 g of the sample were batch extracted by dissolving it in 100 ml boiled distilled water, and shaken for 30 minutes at 100°C in a shaking water bath. Crude extracts were centrifuged at 10000×g for 30 minutes (Suprafuge 22, Germany) and then vacuum filtered using a 47 mm glass microfibre filter to obtain the clear extracts. The extracts were filtered again using 0.45 µm filter to obtain the sterile extracts. The hot water extracts were kept in sterile glass bottle and stored frozen at -40°C.

3.2.3.2 Ultra-Sonication Extraction

Ultra-sonication extraction was performed in an ultrasonic bath (Bandelin Sonorex RK 510 Transistor, Germany) by the mode of indirect sonication at a fix-frequency of 35 MHz. The temperature of the ultrasonic bath was 30°C. 30 g of dried medicinal plants were dissolved in 100 ml distilled water and sonicated for 30 minutes. Crude extracts were centrifuged at 10000×g for 30 minutes and then vacuum filtered using a 47 mm glass microfibre filters to obtain the clear extracts. The extracts were filtered again using 0.45 µm filter to obtain the sterile extracts. The ultra-sonication extracts were kept in sterile glass bottle and stored frozen at -40°C.

3.2.3.3 Ethanolic Extraction

The plants powder (30 g) was extracted using 50% ethanol (100 ml) for 24 hours at 4°C. The ethanolic extracts were centrifuged at 10000×g for 30 minutes and vacuum filtered using a 47 mm filter paper to obtain the clear extracts. Remaining ethanol in the crude extract was removed by heating the extract on a hot plate stirrer at 50°C for 10 hours. The extracts were centrifuged again at 10000×g for 30 minutes. Ethanolic

extracts were vacuum filtered using a 47 mm glass microfibre filter to obtain the clear extracts. The extracts were filtered again using a 0.45 µm filter to obtain the sterile extracts. The ethanolic extracts were stored at -40°C.

3.2.4 Determination of the Antimicrobial Activity

3.2.4.1 Determination of the Minimum Inhibitory Concentrations (MIC)

3.2.4.1.1 Preparation of the Inoculums

For the preparation of the inocula, test organisms were taken from the stock culture and were grown separately in 50 ml Trypticase Soy Broth and incubated at 37°C for 24 hours on an orbital shaker at 200 rpm. Each test organism suspension was subsequently streaked out on Trypticase Soy Agar and incubated at 37°C for 24 hours. A single colony was transferred to Trypticase Soy Agar slants and incubated at 37 °C for 24 hours. These stock cultures were kept at 4°C. For use in experiments a loop of each test organism was transferred in 50 ml Trypticase Soy Broth, and incubated separately at 37°C for 18-20 hours. This culture was used for the antibacterial assays.

3.2.4.1.2 Determination of the Minimum Inhibitory Concentrations (MIC)

A broth dilution susceptibility assay was performed using the method described in the DVG guidelines (DVG, 2000) for the determination of the MIC-values. Briefly, all Thai traditional Medicinal plant extracts were dissolved in sterile water of standardized hardness (WSH; Wasser standardisierter Härte, 300 ppm). 5.0 ml of each geometric dilution of the extract ranging from 30 to 0.2 g/100 ml was transferred to separate, sterile tubes containing 5.0 ml of a double concentrated Trypticase Soy Broth resulting in final concentration of 15.0 to 0.1 g/100 ml. To each tube, 0.1 ml of a 1:10-diluted suspension of the test organism (equivalent to 1.0×10^7 to 1.0×10^8 CFU/ml) was added. The tubes were incubated for 72 hours at 37°C. Growth controls were performed using sterile WSH instead of the extract. In a negative control 0.1 ml WSH was used instead of the bacterial test suspension. For the determination of

suitable neutralizers 0.3% Tween 80, 0.3% Lecithin, 3.0% Saponin, and 0.1% Histidin were added to the double concentrated Trypticase Soy Broth. Each test was carried out in duplicate and in three repetitions. Growth of the test organisms resulted in a visible clouding of the culture medium. The lowest concentration of each extract that inhibited visible growth was determined as the Minimum Inhibitory Concentration (MIC).

3.2.4.2 Determination of the Minimum Bactericidal Concentrations (MBC)

Referring to the results of the MIC assay, all tubes showing complete absence of growth were identified. One loop full of each tube was transferred on a Trypticase Soy Agar plate. All plates were incubated at 37°C for 24 hours. The lowest concentration of the extract with complete absence of growth was considered as the Minimum Bactericidal Concentration (MBC).

3.2.4.3 Determination of the Antimicrobial Activity by Studying the Time Killing Analysis

The ethanolic extracts were used for the determination of the antibacterial activity by determining the Time Killing Analysis in a suspension test. The test method was performed modified according the methods described in DIN EN 1040 and DIN EN 1276 published by the CEN/TC 216 (European Committee for Standardization, Technical Committee “Disinfectants and Antiseptics”). As representative test organisms *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Escherichia coli* O157:H7 (VTEC) DMST 12743, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas areruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* Typhimurium ATCC 13311, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779 were chosen.

3.2.4.3.1 Studying the Time Killing Analysis without Interfering Substances

3.2.4.3.1.1 Preparation of the Working Cultures

A working culture was prepared by inoculating a loopful of the stock culture into 10.0 ml of Trypticase Soy Broth. Then 2 subcultures in Trypticase Soy Broth were prepared before use. A third subculture was inoculated in 50.0 ml Trypticase Soy Broth and incubated for 18 hours at 37°C. To avoid the excess nutrient, 40.0 ml of this 18-hour culture was centrifuged at 5000×g for 10 minutes (Varifuge 3.2RS) at a controlled temperature of 4°C. The pellet was washed twice in 40 ml sterile normal saline solution. The washed cells were then re-suspended in 10 ml sterile normal saline solution to *approx.* 8.00-9.00 log₁₀ CFU/ml for use as an inoculum.

3.2.4.3.1.2 Determination of the Antimicrobial Activity of Thai Traditional Medicinal Ethanolic Extracts by Studying the Time Killing Analysis without Interfering Substances

The test method was performed modified according to the method described by DIN EN 1040 (Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics). 1.0 ml of bacterial test suspension was mixed with 1.0 ml sterile distilled water. After 2 minutes exposure time 9.0 ml of a 1.25 times pre-concentrated dilution of the ethanolic extracts to obtain the desired concentration, 15.0, 10.0, 5.0, and 1.0 g/100 ml, was added and the exposure time started. All tubes were worked at room temperature (20 ± 5°C). Survivors were monitored at intervals of 0_A, 0_B, 1, 3, 6, and 9 hours. 0_A was set conditions as initial population and 0_B as contact time within 15 seconds, by withdrawing a sample, serially dilution and plating on Trypticase Soy Agar. Plates were incubated at 37°C for 24 hours before enumeration.

3.2.4.3.2 Studying the Time Killing Analysis with Interfering Substances

3.2.4.3.2.1 Preparation of the Interfering Substances

High level soiling, described in the CEN method, was used as interfering substance in this study. It was the mixture of bovine albumin fraction V and yeast extract. The final concentration in the test procedure was 10 g/L yeast extract and 10 g/L bovine albumin.

10 g yeast extract powder was dissolved in 150 ml distilled water in a 250 ml volumetric flask and the foam was allowed to collapse. The volume was made up to 250 ml with distilled water. The prepared yeast extract solution was transferred to a clean dry glass bottle and sterilized at 121°C for 15 minutes. The sterile solution was allowed to cool to 20°C.

25 ml of this sterile yeast extract solution was pipetted into a 50 ml volumetric flask containing 10 ml of distilled water. 1 g of bovine albumin fraction V was dissolved in the solution, shaken and the foam was allowed to collapse. The volume was made up to 50 ml with distilled water. The solution was sterilized by using a 0.2µm filter. The interfering substance solution was kept in a refrigerator at 2-8°C and used within 1 month.

3.2.4.3.2.2 Determination of the Antimicrobial Activity of Thai Traditional Medicinal Ethanolic Extracts by Studying the Time Killing Analysis with Interfering Substances

The test method was performed modified according to the method described by DIN EN 1276 (Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas). Ethanolic extracts of *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* L., *Hibiscus sabdariffa* L., *Piper bettle* L., and *Punica granatum* L. were selected for to study. Test organisms were *Bacillus cereus* ATCC

128263, *Pseudomonas aeruginosa* DSMZ 939, and *Staphylococcus aureus* DSMZ 799.

1.0 ml of the interfering substance was mixed with 1.0 ml of the bacterial test suspension for 2 minutes, and then 8.0 ml of the pre-concentrated ethanolic extracts were added. The final concentrations in the test were 15.0, 10.0, 5.0, and 1.0 g/100 ml. Survivors were monitored at intervals of 0_A, 0_B, 1, 3, 6, and 24 hours, 0_A was set conditions as initial population and 0_B after a contact time of 15 seconds, by withdrawing a sample, serially dilution and plating on Trypticase Soy Agar. Plates were incubated at 37°C for 24 hours before counting.

3.2.5 Application Study

3.2.5.1 The Application of Thai Traditional Medicinal Plant Ethanolic Extracts on the Coating Surface

This application was tested with ethanolic extracts of *Piper betle* L. against *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Escherichia coli* O157:H7 DMST 12743, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* Typhimurium ATCC 13311, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779.

A working culture was prepared by inoculating a loopful of the stock culture into 10.0 ml of Trypticase Soy Broth. Two subcultures were prepared in the same way. Then a third subculture of the test organisms was prepared in 50.0 ml Trypticase Soy Broth and incubated for 18 hours at 37°C. To avoid the excess nutrient, 40.0 ml of 18-hours culture was centrifuged at 5000×g for 10 minutes at controlled temperature of 4°C. The pellet was washed twice in 40 ml sterile normal saline solution. The washed cells were then re-suspended in sterile normal saline solution to *approx.* 8.00-9.00 log₁₀ CFU/ml for use as an inoculum. High level soiling, described in DIN EN 1276 (see

2.4.3.2.1), was used as interfering substance. The final concentration of the interfering substance in the test procedure was 10 g/L yeast extract and 10 g/L bovine albumin.

Prior starting the test, 1.0 ml of the interfering substance was mixed with 1.0 ml bacterial test suspension for 2 minutes. Then 0.05 ml of the mixture was inoculated on a sterilized metal surface (diameter 2.0 cm, 2B finish). The contaminated surfaces were dried at 37°C for approximately 30 minutes.

For the preparation of the antimicrobial surfaces, 0.5 ml of the ethanolic extract of *Piper betle* L., in concentrations ranging from 30.0-10.0 g/100 ml, were coated on a second sterilized metal surface. These surfaces were dried at 37°C for *approx.* 45 minutes. After drying, both contaminated metal surfaces and antimicrobial coated surfaces were put together and kept at room temperature. Survivors on the both contaminated metal surfaces were monitored at intervals of 0, 1, 6, 24, and 48 hours. To recover surviving test organisms a contaminated metal surface was placed in 10 ml normal saline solution containing neutralizer; 0.3% Tween 80, 0.3% Lecithin, 3.0% Saponin, and 0.1% Histidin; and glass beads. They were shaken for 2 minutes, the serial tenfold dilution was prepared and out of the neutralized mixture and each dilution step samples of 0.1 ml were plated out on Trypticase Soy Agar. The plates were incubated at 37°C for 24 hours before counting.

3.2.5.2 Application by Incorporating the Extracts in Antimicrobial Edible Films

3.2.5.2.1 Preparation of Alginate-Tapioca-based Antimicrobial Edible Films

Commercial tapioca starch was modified by autoclaving at 130°C for 30 minutes. 4 g of this modified tapioca starch and 1 g of sodium alginate were diluted in 200 ml distilled water, 0.1 g of Na-EDTA was added as additive, and glycerol was added as plasticizer with an amount of 20% of film based material. In the gelatinization process, the film based solution was heated and continuously stirred at 85°C for 45 min. The antimicrobial film based solution was prepared by adding ethanolic extracts of Thai traditional medicinal plants into the gelatinized film based solution with

concentrations of 20.0%, 15.0%, 5.0% and 1.0%. For the incorporating process the antimicrobial film based solution was continuously stirred at 45°C for 45 minutes. The incorporated antimicrobial film solution was cast by pipetting 20 ml of solution into sterile plastic plate (8 cm diameter). The solutions were dried for approximately 24 hours at 40°C, after which the film were peeled from the plastic plates and store at room temperature until use.

3.2.5.2.2 Determination of the Antimicrobial Activity of Thai Traditional Medicinal Plants Ethanolic Extracts Incorporated in Alginate-Tapioca-based Edible Films

The antimicrobial test on films was carried out using the agar diffusion test method and the suspension test. The zone of inhibition assay on solid media was used for the determination of the antimicrobial effects of the films against the test organism, including *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Escherichia coli* DSMZ 632, *Escherichia coli* O157:H7 (VTEC) DMSZ 12743, *Listeria monocytogenes* 101, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* Typhimurium ATCC 13311, *Salmonella* Typhimurium DSMZ 5569, *Staphylococcus aureus* ATCC 13565, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779.

The edible films were cut into 1 cm² and then placed on Trypticase Soy Agar plates, which had been previously seeded with 0.1 ml of inoculum containing approximately 10⁶-10⁸ CFU/ml of the test organisms. The plates were then incubated at 37°C for 24 hours. After that, the plates were examined for “zone of inhibition” around the film discs. The contact area was used to evaluate the growth inhibition underneath the film disc in direct contact with target microorganisms on the agar. The area of the whole zone was calculated and then subtracted from the film disc area, and this difference area was reported as zone of inhibition.

In the suspension test, 0.1 ml of the bacterial test suspension (10^6 - 10^8 CFU/ml), consisting of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Escherichia coli* O157:H7 (VTEC) DMST 12743, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* Typhimurium ATCC 13311, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779, was placed on incorporated antimicrobial edible film discs (1 cm²) with prepared 20.0% ethanolic extract of all medicinal plants and without ethanolic extracts as control. The films were incubated for 24 hours at 20°C. After incubation, the film discs were placed into 10 ml normal saline solution containing neutralizer; 0.3% Tween 80, 0.3% Lecithin, 3.0% Saponin, and 0.1% Histidin; and glass beads. The film disc was agitated for 2 minutes. The solution was decimally diluted in sterile normal saline solution and samples out of the neutralized mixture and every dilution step were plated in duplicate onto Trypticase Soy Agar plates. The plates were incubated at 37°C for 24 hours, counted, and CFU/ml was calculated.

3.2.6 Studying the Cytotoxicity of the Ethanolic Extracts

Dilutions of the product were inoculated on different cell lines and incubated for 8 days. Cytotoxicity was determined in the cell lines after incubation. Tenfold dilution (10^{-1} - 10^{-5}) of the product was prepared in water of standardized hardness. 0.25 ml of each product dilution was inoculated on 4 wells containing the cell lines, including TH1 (Terrapene heart cells), A549 (Carcinomic human alveolar basal epithelial cells), BGM (Buffalo-green-monkey cells), and MDCK (Madin-Darby canine kidney cells) to be tested. Micro plates were incubated at 37 °C for 1 hour. After the incubation period, 100 µl of DMEM (Dulbeccos minimum essential medium) plus 2% FKS (Fetal calf serum) and antibiotics, including Streptomycin, Gentamycin, Amphotericin B, and Penicillin were added to each well. The cytotoxicity was determined after 8 days of incubation at 37°C. The lowest concentration (highest dilution) of the product which was toxic to the tested cell lines was determined

3.2.7 Studying the Antimicrobial Properties of Alginate-Tapioca Based Edible Films and Coating Incorporated Thai Traditional Medicinal Plant Extracts in a Model Food System

3.2.7.1 Determination of the Antimicrobial Activity of Alginate-Tapioca based Edible Films incorporated Thai Traditional Medicinal Plant Extracts against a pre-contaminated Model Food System

The pre-contaminated model food system represented the contamination of food related bacterial on food before processing. The experiments were performed on the contaminated model food system and covered with antimicrobial edible films. The numbers of bacterial survivors were determined after different storage times at different temperatures.

3.2.7.1.1 Preparation of the Test Organisms

Bacillus cereus ATCC 128263 and *Salmonella* Typhimurium ATCC 13311 were selected as test organisms in this experiment. A working culture was prepared by inoculating a loopful of the culture prepared according 2.1 into 10 ml of Trypticase Soy broth. Two subcultures were prepared in the same way and a third subculture was prepared in 50 ml Trypticase Soy Broth.

The working culture was prepared by pipetting 20 ml of this third 18-hour subculture in 180 ml sterile normal saline solution, mixed thoroughly on a magnetic stirrer at 100 rpm for 10 minutes, and kept for use according 2.5.4.2 in a laminar air flow chamber.

3.2.7.1.2 Preparation of the Alginate-Tapioca based Antimicrobial Edible Films incorporated Thai Traditional Medicinal Plant Extract

The ethanolic extracts of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. were selected for the study in the model food system. The antimicrobial edible films were prepared as described in 2.5.2.1 in a concentration of 20% v/v of the

ethanolic extracts. The casted films were peeled off and cut into pieces of 4×4 cm² size. The prepared films were kept in dry condition.

3.2.7.1.3 Preparation of the Contaminated Model Food System

Carrots and chicken meat were selected as model food systems for studying the antimicrobial activity of Alginate-Tapioca based edible films and coating incorporated ethanolic extract of Thai traditional medicinal plants.

Carrots were purchased from the supermarket in Stuttgart, Germany one day before use. The carrots were peeled off and cut in to circle pieces of approx. 10 g per piece, diameter *approx.* 2 cm. Each piece was disinfected in 70% ethanol for 5 minutes, and dried in a laminar air flow chamber for 10 minutes. Carrot samples were immersed in the working test organism culture prepared according 2.7.1.1, for 10 minutes by stirring, allowed to drip free of excess inoculum and dried for 20 minutes in a laminar air flow chamber. The initial population contaminated on each piece was *approx.* 1.0×10^4 CFU/g.

Chicken meat was also purchased from the supermarket in Stuttgart, Germany one day before use. The samples were cut into pieces of *approx.* 10 g each, and prepared according the same method as described for the preparation of the contaminated carrots. The initial population contaminated on each piece was *approx.* 1.0×10^4 CFU/g.

3.2.7.1.4 Application of Alginate-Tapioca based Antimicrobial Edible Films on the Pre-contaminated Model Food Systems

Alginate-Tapioca antimicrobial films with 20% v/v ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. were placed to both side of the contaminated carrots and chicken meat. Each sample was placed in a sterile Petri dish. The Petri dishes were sealed with parafilm and separately stored at room temperature ($20 \pm 5^\circ\text{C}$) or at 4°C . The numbers of bacterial survivors were monitored after 0, 24

and 48 hours storage time. Contaminated samples without application of the antimicrobial films and samples with Alginate-Tapioca edible films without medicinal plant extracts served as controls. After the storage times, the sample was homogenized with 90 ml Tryptone-sodium chloride with neutralizer in a stomacher at the highest speed for 3 minutes. The solution was serially diluted. Appropriate dilutions were surface-plated on Trypticase Soy Agar. Plates were incubated at 37°C for 24 hours before enumeration.

3.2.7.2 Determination of the Antimicrobial Activity of Alginate-Tapioca based Edible Coating incorporated Thai Traditional Medicinal Plant Extracts against Pre-contaminated Model Food Systems

The antimicrobial edible coating method could determine the antimicrobial ability of thin coated material, coated on the food surface with the purpose of elimination the contaminated bacteria on food surfaces. The experiments were applied on the contaminated model food systems coated with antimicrobial edible coated material. The numbers of survivors were determined after different storage times at different temperatures.

3.2.7.2.1 Preparation of the Test Organisms

The test organisms were prepared as described in 2.7.1.1

3.2.7.2.2 Preparation of Alginate-Tapioca based Antimicrobial Edible Coating Incorporated Thai Traditional Medicinal Plant Extract

Ethanollic extracts of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. were selected for the experiments in the model food systems. The antimicrobial edible films were prepared as described in 2.5.2.1 with the concentration of 20% v/v of ethanolic extracts. These antimicrobial film based solutions were used as antimicrobial coating.

3.2.7.2.3 Preparation of the Contaminated Model Food Systems

The test organisms were prepared according the description in 2.7.1.3.

3.2.7.2.4 Application of Alginate-Tapioca based Antimicrobial Edible Coating on Pre-contaminated Model Food Systems

The samples contaminated with the test organisms (2.7.2.3) were dipped into an edible film coating. They were allowed to dry under a laminar air flow chamber for 20 minutes before putting them into sterile Petri dishes. The Petri dishes were sealed with parafilm and separately stored at room temperature ($20 \pm 5^\circ\text{C}$) or at 4°C . The numbers of surviving bacteria were monitored after 0, 24 and 48 hours storage time. Contaminated samples without application of the antimicrobial coating and samples with Alginate-Tapioca edible coating without medicinal plant extracts served as controls. After the different storage times, the samples were homogenized with 90 ml Tryptone-sodium chloride with neutralizer in a stomacher at the highest speed for 3 minutes. The solution was serially diluted. Appropriate dilutions were surface-plated on Trypticase Soy Agar. Plates were incubated at 37°C for 24 hours before enumeration.

3.2.7.3 Determination of the Antimicrobial Activity of Alginate-Tapioca based Edible Films Incorporated Thai Traditional Medicinal Plant Extracts against Post-contaminated Model Food Systems

Post-contaminated model food systems represented the contamination of food related bacterial into food after processing. The application of antimicrobial edible films on those referred to the antimicrobial ability of the edible films with the purpose of prevention the “re-contamination” with bacteria of the environment. The experiments were conducted on the model food systems covered with antimicrobial edible films and contaminated with the selected test organisms. The numbers of survivors were determined after different storage times at different temperatures.

3.2.7.3.1 Preparation of the Test Organisms

Bacillus cereus ATCC 128263 and *Salmonella* Typhimurium ATCC 13311 were selected as test organisms in this experiment. A working culture was prepared by inoculating a loopful of culture prepared as described in 2.1 into 10 ml of Trypticase Soy broth. Two subcultures were prepared in the same way and a third subculture was prepared by transferring a sample into 50 ml Trypticase Soy Broth and inoculated for 18-20 hours at 37°C.

3.2.7.4 Preparation of Alginate-Tapioca based Antimicrobial Edible Coating Incorporated Thai Traditional Medicinal Plant Extracts

The preparation of the Alginate-Tapioca based edible films is described in 2.7.1.2.

3.2.7.4.1 Preparation of the Model Food Systems

For the preparation of the model food systems the procedure described in 2.7.1.3 without contaminated with test organisms was followed.

3.2.7.4.2 Application of Alginate-Tapioca based Antimicrobial Edible Films on Post-contaminated Model Food Systems

Alginate-Tapioca antimicrobial films with 20% v/v ethanolic extracts of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. were placed to both side of carrots and chicken meat. To each sample, 0.1 ml of a 1:10-diluted suspension of the test organisms (equivalent to 1.0×10^6 CFU/ml) was added. Each sample was placed in separate sterile Petri dishes. The Petri dishes were sealed with parafilm and separately stored at room temperature ($20 \pm 5^\circ\text{C}$) or at 4°C. The numbers of bacterial survivors were monitored after 0, 24 and 48 hours storage time. Contaminated samples without application of the antimicrobial films and samples with Alginate-Tapioca edible films without medicinal plant extracts served as controls. After the different storage times, the samples were homogenized with 90 ml Tryptone-sodium chloride with neutralizer

in a stomacher at the highest speed for 3 minutes. The solution was serially diluted. Appropriate dilutions were surface-plated on Trypticase Soy Agar. Plates were incubated at 37°C for 24 hours before enumeration.

3.2.7.5 Determination of the Antimicrobial Activity of Alginate-Tapioca based Edible Coating Incorporated Thai Traditional Medicinal Plant Extracts against Post-contaminated Model Food Systems

As described above, the edible coating method could mimicry the antimicrobial ability of thin layer coating material, coated on the food surface. In post-contaminated model food systems, the application of antimicrobial thin coated material may provide the purpose to prevent the contamination with bacteria on the food surface. The experiments were done on the model food systems coated with antimicrobial edible coated material and contaminated with selected test organisms. The numbers of survivors were determined after different storage times at different temperature.

3.2.7.5.1 Preparation of the Test Organisms

The preparation of test organisms followed the procedure as described in 2.7.3.1.

3.2.7.5.2 Preparation of Alginate-Tapioca based Antimicrobial Edible Coating Incorporated Thai Traditional Medicinal Plant Extracts

The preparation of the test organisms is described in 2.7.2.2.

3.2.7.5.3 Preparation of the Contaminated Model Food Systems

The model food systems were contaminated according 2.7.3.3.

3.2.7.5.4 Application of Alginate-Tapioca based Antimicrobial Edible Coating on Pre-contaminated Model Food Systems

The samples were dipped into an edible films coating. They were allowed to dry in a laminar air flow chamber for 20 minutes before putting them into sterile Petri dishes. To each sample, 0.1 ml of a 1:10-diluted suspension of the test organisms (equivalent to 1.0×10^6 CFU/ml) was added. The Petri dishes were sealed with parafilm and separated stored at room temperature ($20 \pm 5^\circ\text{C}$) or at 4°C . The numbers of bacterial survivors were monitored after 0, 24 and 48 hours storage time. Contaminated samples without application of the antimicrobial coating and samples with Alginate-Tapioca edible coating without medicinal plant extracts served as controls. After the different storage times, the samples were homogenized with 90 ml Tryptone-sodium chloride with neutralizer in a stomacher at the highest speed for 3 minutes. The solution was serially diluted. Appropriate dilutions were surface-plated on Trypticase Soy Agar. Plates were incubated at 37°C for 24 hours before enumeration.

4 RESULTS

4.1 Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of Thai Traditional Medicinal Plants

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of nine Thai traditional medicinal plants were determined by a broth dilution susceptibility assay.

The highest concentration of medicinal plant extracts possible to test was 30.0 g/100 ml because of the limitation of solubility activity of fine particles of medicinal plant samples. According to this reason and the test method described by DVG, the highest final concentration presented in the assay was 15.0 g/100 ml.

The Minimum Inhibitory Concentration (MIC) could not be determined because of the interaction between extracts and culture media. The solution became turbid appearance.

4.1.1 Antimicrobial properties of *Andrographis paniculata* (Burm.f.) Wall. ex Nees

The antimicrobial activity of dried leaves of *Andrographis paniculata* (Burm.f.) Wall. ex Nees is shown in Figure 16. The hot water extract had activity against only *Bacillus cereus* ATCC 11778 at a concentration of 8.0 g/100 ml. Ultrasonication extract had no effect on all test organisms. Ethanolic extract of this medicinal plant showed antimicrobial activity against several strains of the test organisms. At a concentration of 3.0 g/100 ml, the ethanolic extract demonstrated an antimicrobial property determined by MBC-value against *Bacillus cereus* ATCC 11778. At concentration < 10.0 g/100 ml, this extract showed a bactericidal activity against *Bacillus cereus* ATCC 128263, *Listeria monocytogenes* 101, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779 with MBC-values of 4.0, 6.0,

7.0, and 7.0, respectively. Among test organisms, *Escherichia coli* O157:H7 DMST 12743, *Escherichia coli* ATCC 8738, DSMZ 632, and *Pseudomonas aeruginosa* DSMZ 939 exhibited the highest resistance to the ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees against those test organisms the MBC-value was at the concentration of 15.0 g/100 ml.

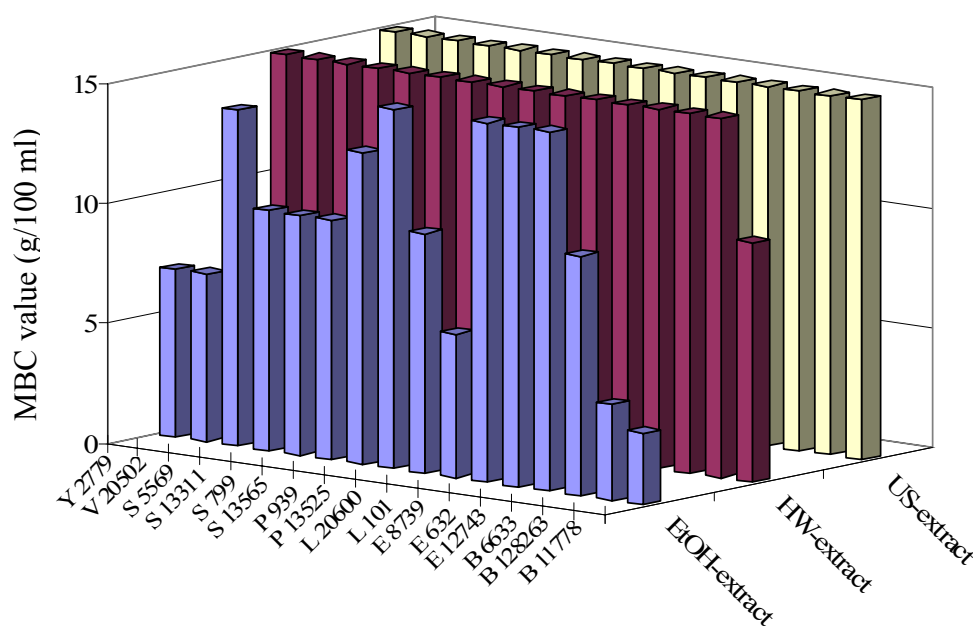


Figure 16 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Andrographis paniculata* (Burm.f.) Wall. ex Nees against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

4.1.2 Antimicrobial properties of *Curcuma zedoaria* (Christm.) Roscoe

Hot water extraction and ultrasonication of the dried rhizome of *Curcuma zedoaria* (Christm.) Roscoe at concentration up to 15.0 g/100 ml had no significant antimicrobial effect on all test organisms (Figure 17). An antibacterial activity of this plant was only found when using an ethanolic extract. The lowest MBC-value of the ethanolic extract was detected against *Vibrio parahaemolyticus* ATCC 20502 at a concentration of 0.1 g/100 ml. Comparing the results with the different test organisms, they indicated that gram negative test organisms almost had a higher resistance against the ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe with the exception of *Vibrio parahaemolyticus* ATCC 20502. The MBC-value of gram positive test organisms was less than 3.0 g/100 ml. The lowest was 0.5 g/100 ml, which was found in the tests with *Bacillus cereus* ATCC 11778, and ATCC 128263, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 13565, and *Listeria monocytogenes* 101, and DSMZ 20600. The limiting test organism was *Pseudomonas aeruginosa* DMZ 939 with the highest MBC-value of 11.0 g/100 ml.

4.1.3 Antimicrobial properties of *Garcinia mangostana* L.

The MBC-values of hot water extract, ultrasonication extract and ethanolic extract of the dried pericarp of *Garcinia mangostana* L. are given in Figure 18. Ultrasonication extract of *Garcinia mangostana* L. showed antibacterial activity against gram positive test organisms, not including *Staphylococcus aureus* ATCC 13565 and *Staphylococcus aureus* DSMZ 799 which provided the high resistance against the ultrasonication extract of this plant. The gram negative test organisms also demonstrated resistance to the ultrasonication extract. The result with the hot water extract indicated that this extract of *Garcinia mangostana* L. expressed the better antibacterial activity against gram positive test organisms than the ultrasonication extract. MBC-values of *Staphylococcus aureus* ATCC 13565 and *Staphylococcus aureus* DSMZ 799 were 5.0 g/100 ml and 10.0 g/ml, respectively. On the other hand, gram negative test organism still showed a strong resistance to the hot water extract of *Garcinia mangostana* L. The best antibacterial activity of *Garcinia mangostana* L.

was obtained with the ethanolic extract. Gram positive test organisms were highly sensitive to this extract, but the spore former *Bacillus subtilis* ATCC 6633, had a high MBC-value > 15.0 g/100 ml.

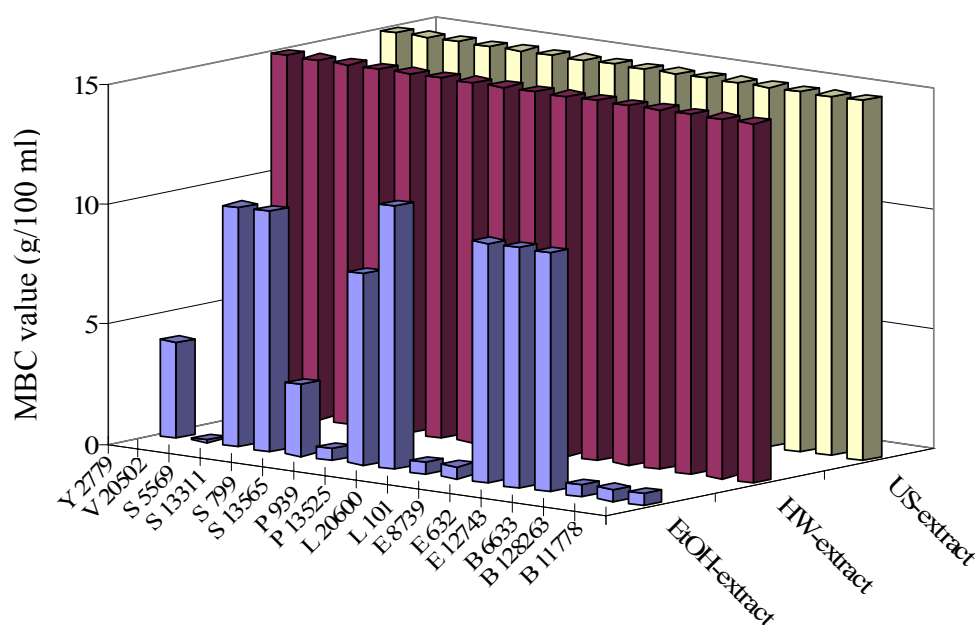


Figure 17 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Curcuma zedoaria* (Christm.) Roscoe against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

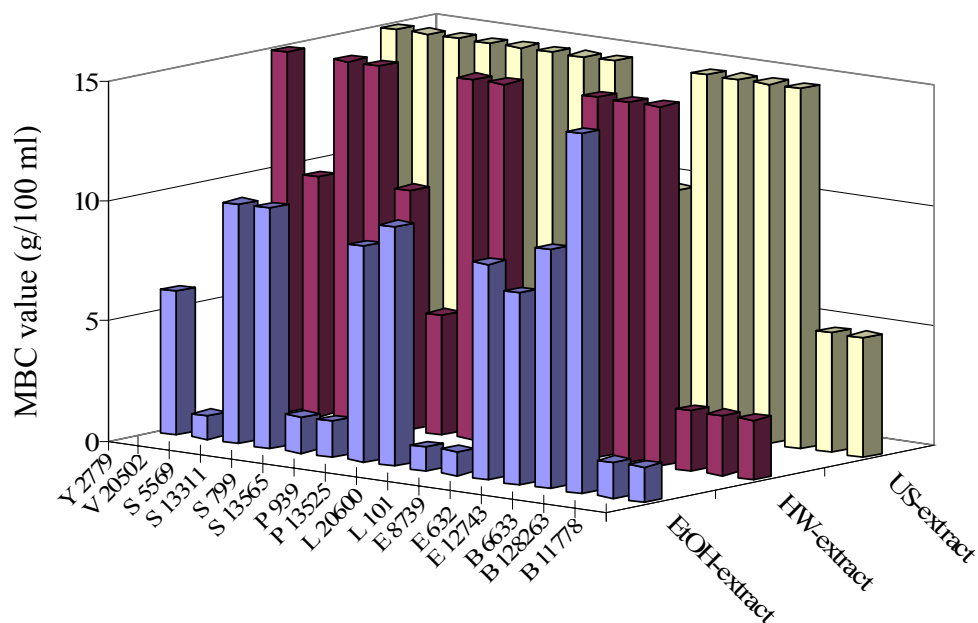


Figure 18 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Garcinia mangostana* L. against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

4.1.4 Antimicrobial properties of *Hibiscus sabdariffa* L.

The ultrasonication extract of the dried flower of *Hibiscus sabdariffa* L. showed good antimicrobial activity against all test organisms with MBC-values of ≤ 10.0 g/100 ml. The highest resistance against this extract showed *Bacillus subtilis* ATCC 6633, where 10.0 g/100 ml was determined as MBC-value. The lowest MBC-value of the ultrasonication extract of *Hibiscus sabdariffa* L. was 2.5 g/100 ml, obtained with *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Listeria monocytogenes* 101, *Listeria monocytogenes* DSMZ 20600, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779 as test organisms.

The hot water extract of this medicinal plant exhibited a greater antimicrobial activity than the ultrasonication extract. The MBC-values ranged from 2.5 g/100 ml to 5.0 g/100 ml, while gram negative test organisms expressed stronger resistance to the hot water extract with exception of *Pseudomonas aeruginosa* DSMZ 939, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779. These test organisms demonstrated MBC-value of 2.5 g/100 ml which was comparable with those of the gram positive test organisms. The highest measurable MBC-value was 5.0 g/100 ml, obtained when using the other gram negative test organisms.

The ethanolic extract of *Hibiscus sabdariffa* L. demonstrated the outstanding antimicrobial property. The MBC-values of this extract ranged from 0.1 to 5.0 g/100 ml. The antimicrobial activity against *Vibrio parahaemolyticus* ATCC 20502 showed the lowest MBC-value with 0.1 g/100 ml. The highest MBC-value was 5.0 g/100 ml obtained in tests with *Bacillus subtilis* ATCC 6633. The gram negative test organisms still had higher resistance to this extract than the gram positive test organism. Only 2 strains exhibited stronger resistance, including *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* DSMZ 799 with the MBC-value of 5.0 and 3.0 g/100 ml, respectively. *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779 with MBC-values of 0.1 and 0.3 g/100 ml, respectively were highly susceptible against this extract (Figure 19).

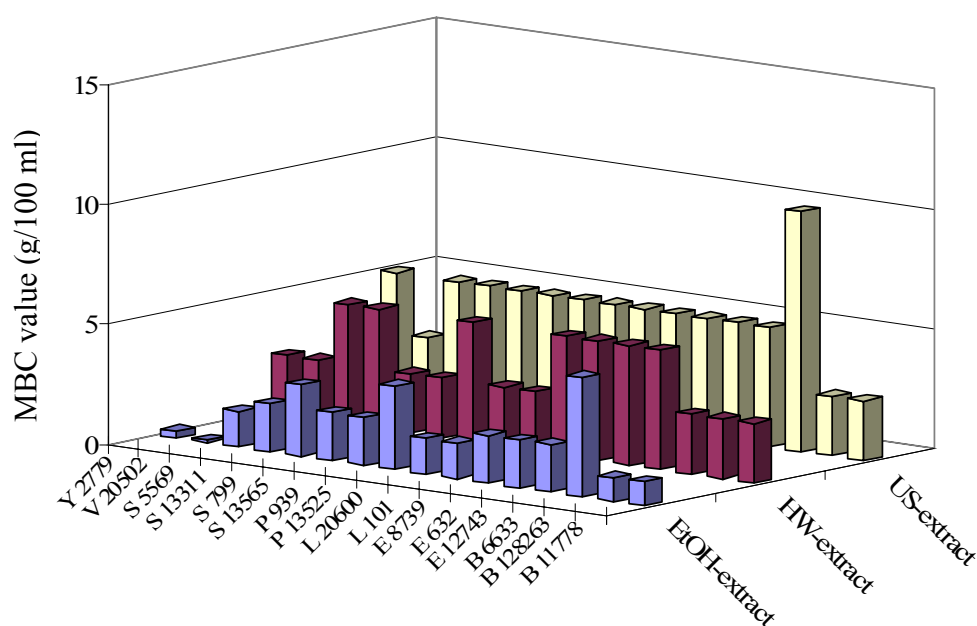


Figure 19 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Hibiscus sabdariffa* L. against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

4.1.5 Antimicrobial properties of *Musa sapientum* L.

The ultrasonication extract and the hot water extract of dried blossom of *Musa sapientum* L. had no antimicrobial effect against all tested organisms. Antimicrobial activity was found only in the ethanolic extract. This extract provided good antimicrobial activity against *Bacillus cereus* ATCC 128263 at a MBC-value of 0.5 g/100 ml. The vegetative cells of the spore forming bacteria *Bacillus subtilis* ATCC 6633 showed a high resistance with a MBC-value of 11.0 g/100 ml. A MBC-value of 1.0 g/100 ml was determined when using *Vibrio parahaemolyticus* ATCC 20502 as test organism, while >15.0 g/100 ml were necessary to inactivate *Pseudomonas aeruginosa* DSMZ 939, *Escherichia coli* ATCC 8739, *Escherichia coli* DSMZ 632, *Salmonella* Typhimurium ATCC 13311, and *Salmonella* Typhimurium ATCC 5569. Generally gram negative test organisms demonstrated a higher resistance to the ethanolic extract of dried blossom of *Musa sapientum* L. (Figure 20)

4.1.6 Antimicrobial properties of *Nelumbo nucifera* Gaertn.

The antimicrobial activity of dried rhizome of *Nelumbo nucifera* Gaertn. is given in Figure 6. A hot water extract of dried rhizome of *Nelumbo nucifera* Gaertn. could not be prepared, due to physical changes during the heating process. Ultrasonication extract of dried rhizome of *Nelumbo nucifera* Gaertn., presented no antimicrobial activity against all test organisms.

The antimicrobial property of dried rhizome of *Nelumbo nucifera* Gaertn. was only found in the ethanolic extract. The lowest MBC-value was 0.5 g/100 ml when using *Vibrio parahaemolyticus* ATCC 20502 as test organism. The highest MBC-value provided *Bacillus subtilis* ATCC 6633, *Pseudomonas fluorescens* ATCC 1325, and *Salmonella* Typhimurium ATCC 13311 with > 15.0 g/100 ml.

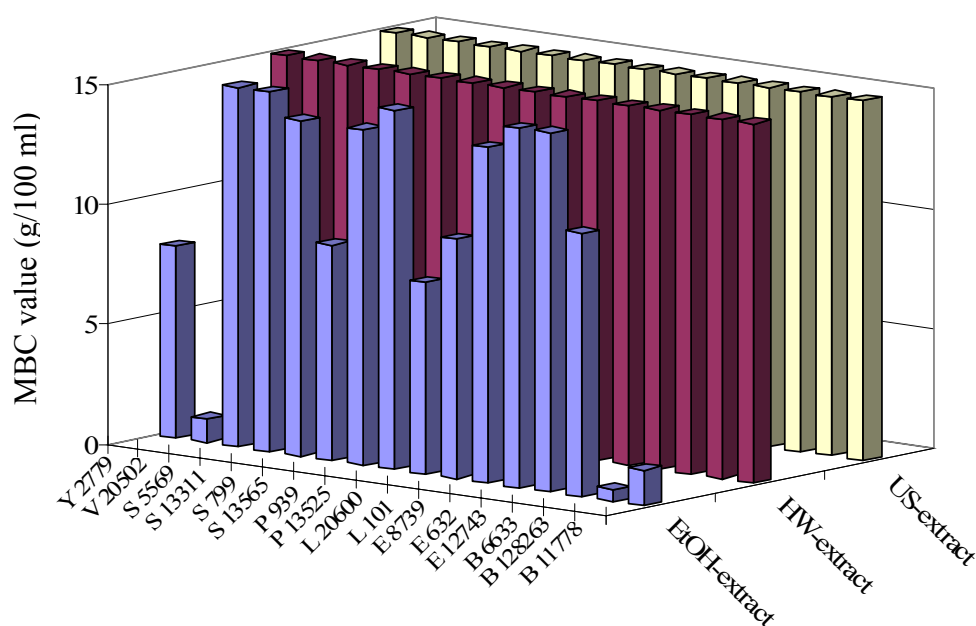


Figure 20 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Musa sapientum* L. against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

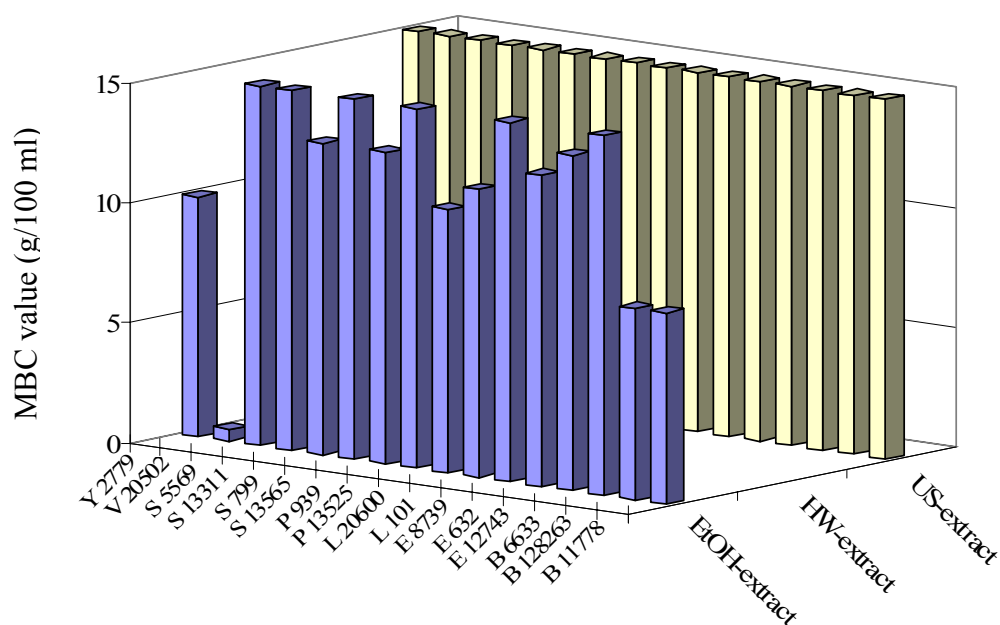


Figure 21 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Nelumbo nucifera* Gaertn. against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

4.1.7 Antimicrobial properties of *Piper betle* L.

The ultrasonication extract of dried leaf of *Piper betle* L. showed a good antimicrobial activity against all test organisms with concentrations ≤ 10.0 g/100 ml. One exception was *Bacillus subtilis* ATCC 6633, where 15.0 g/100 ml was determined as MBC-value. 62.5 % (10 strains) of the test organisms showed MBC-values ≤ 5.0 g/100 ml. When using *Staphylococcus aureus* DSMZ 779, *Pseudomonas aeruginosa* DSMZ 939, *Listeria monocytogenes* 101, *Listeria monocytogenes* DSMZ 20600 as test organisms a MBC-value of 5.0 g/100 ml was determined.

The hot water extract of *Piper betle* L. also had an antimicrobial activity. The MBC-values ranged from 5.0 g/100 ml to 15.0 g/100 ml. The highest MBC-value was 15.0 g/100 ml when using *Bacillus subtilis* ATCC 6633 as test organism. *Pseudomonas aeruginosa* DSMZ 939, *Listeria monocytogenes* 101, and *Escherichia coli* O157:H7 DMSZ 12743 showed a MBC-value of 10.0 g/100 ml. The lowest measurable MBC-value was 5.0 g/100 ml, obtained from others gram negative test organisms.

Among the different extracts of the dried leaf of *Piper betle* L. the ethanolic extract demonstrated the greatest antimicrobial activity. The MBC-value of this extract ranged from 0.2 to 6.0 g/100 ml. *Yersinia enterocolitica* ATCC 2779 was the most susceptible test organism with a MBC-value of 0.2 g/100 ml. The highest MBC-value was 6.0 g/100 ml obtained in the trials with *Bacillus subtilis* ATCC 6633. The ethanolic extract demonstrated strong antimicrobial activity against both gram positive test organism and test gram negative organisms. Highly resistant against this extract were *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* DMZ 939, and *Vibrio parahaemolyticus* ATCC 20502 with the MBC-values of 6.0, 1.5 and 1.0 g/100 ml, respectively (Figure 22).

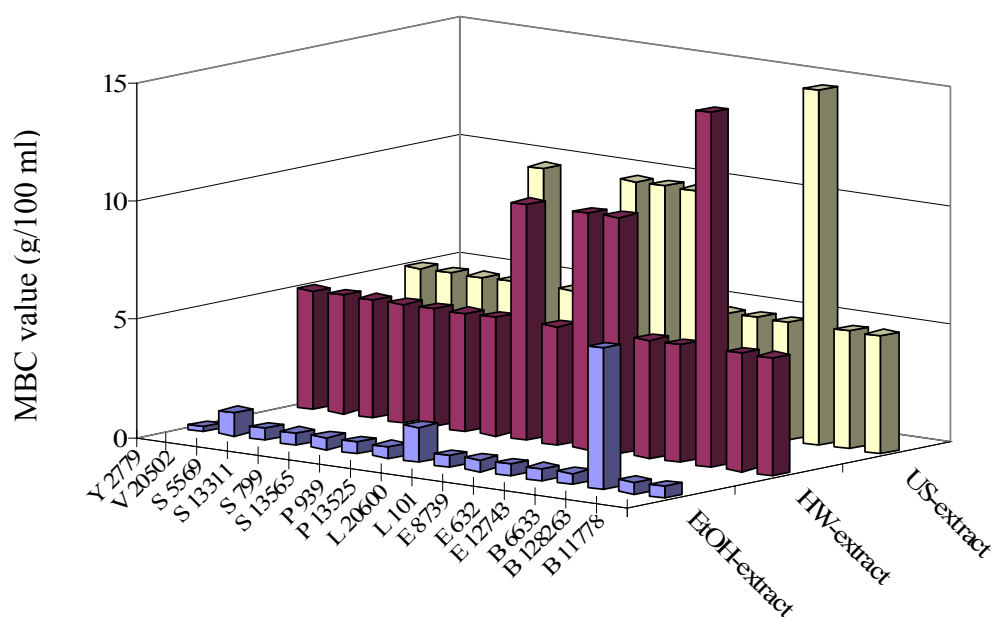


Figure 22 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Piper betle* L. against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

4.1.8 Antimicrobial properties of *Psidium guajava* L.

The antimicrobial activity of dried leaf of *Psidium guajava* L. is presented in Figure 23. The ultrasonication extraction expressed antimicrobial activity against *Bacillus cereus* ATCC 11778 at a MBC-value of 10.0 g/100 ml. The other test organisms showed a strong resistance to this extract. From the results with the hot water extract of *Psidium guajava* L. indicated that this extract had an antimicrobial activity against *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 13565, and *Listeria monocytogenes* DSMZ 20600 with MBC-values of 2.5, 5.0, 5.0, 5.0, and 10.0 g/100 ml, respectively. For the other test organisms, the MBC-value was > 15.0 g/100 ml. The ethanolic extract of this medicinal plant exhibited a better antimicrobial activity. The measurable MBC-value ranged from 0.1 to 11.0 g/100 ml. *Vibrio parahaemolyticus* ATCC 20502 was highly susceptible against the ethanolic extract. The highest MBC-value was determined against *Bacillus subtilis* ATCC 6633 and *Salmonella* Typhimurium with a concentration of 11.0 g/100 ml.

4.1.9 Antimicrobial properties of *Punica granatum* L.

The antimicrobial activity of dried pericarb of *Punica granatum* L. is shown in Figure 24. The ultrasonication extract had no effect against ten of the tested strains (62.5%), against 6 strains MBC-values \leq 10.0 g/100 ml were determined. The ultrasonication extract of *Punica granatum* L. showed a good activity against *Vibrio parahaemolyticus* ATCC 20502 with a MBC-value of 2.5 g/100 ml. The hot water extract of *Punica granatum* L. showed better activity, especially, against gram positive test organisms. The lowest MBC-value was 2.5 g/100 ml, determined against *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 13565, *Staphylococcus aureus* DSMZ 799, and *Vibrio parahaemolyticus* ATCC 20502. The outstanding antimicrobial activity of *Punica granatum* L. was exhibited with the ethanolic extract. The MBC-value with this extract ranged from 0.2 to 8.0 g/100 ml. The most resistant test organism was *Escherichia coli* ATCC 8739,

and the most susceptible were *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, and *Vibrio parahaemolyticus* ATCC 20502.

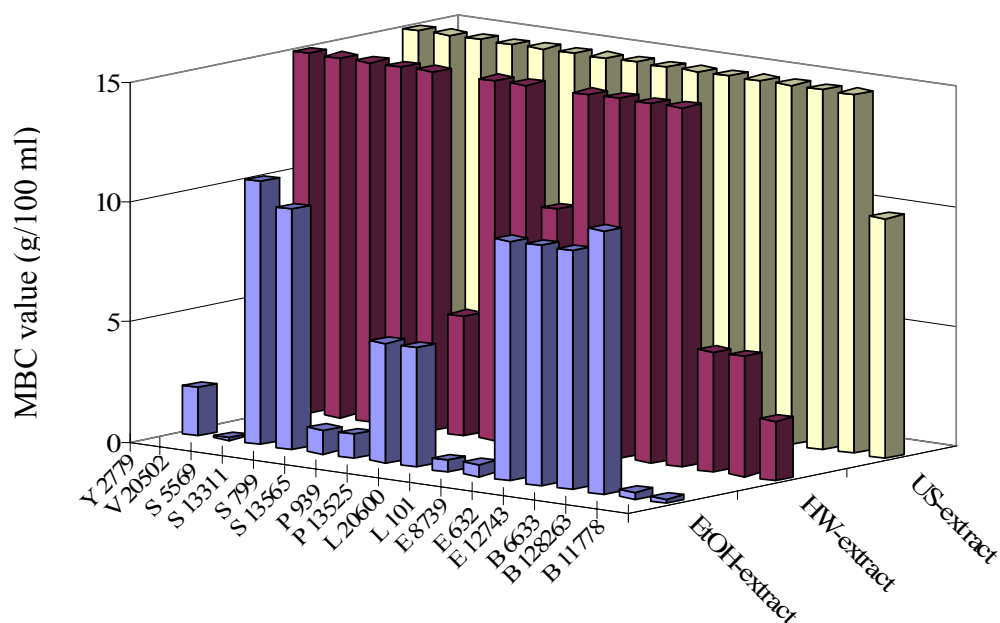


Figure 23 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Psidium guajava* L. against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

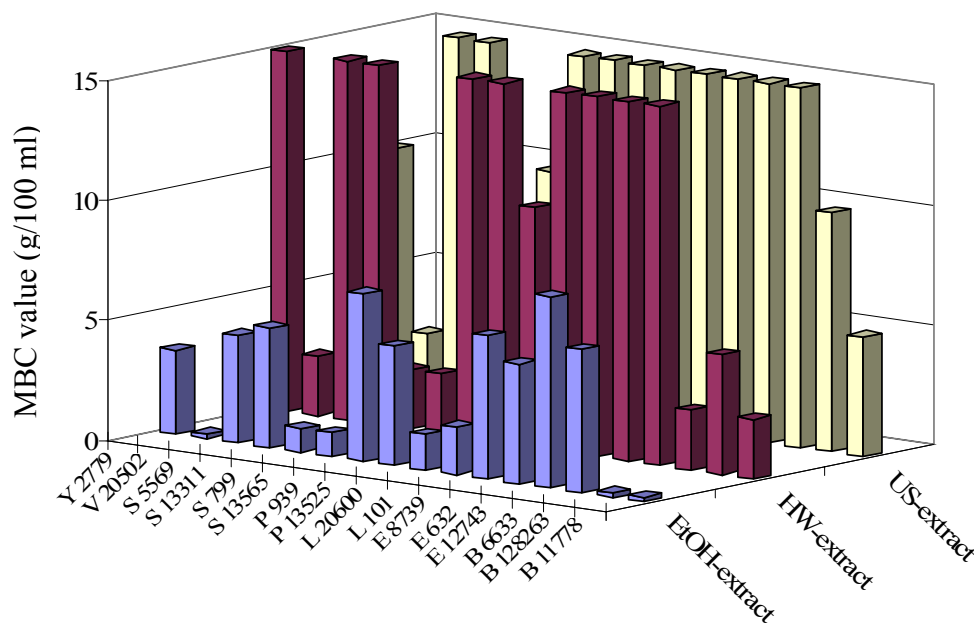


Figure 24 MBC-values (g/100 ml) of hot water extract (HW-extract), ultrasonication extract (US-extract) and ethanolic extract (EtOH-extract) of *Punica granatum* L. against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 5569: *Salmonella* Typhimurium DSMZ 5569; S 13311: *Salmonella* Typhimurium ATCC 13311; S 799: *Staphylococcus aureus* DSMZ 799; S 13565: *Staphylococcus aureus* ATCC 13565; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778)

4.2 Determination of the Antimicrobial Effect of Thai Traditional Medicinal Ethanolic Extracts by Studying the Time Killing Analysis without Interfering Substances by Time-Killing Analysis

The time killing analysis (survivor curve plot) is the method whereby the number of viable cells remaining in suspension after addition of antimicrobial substances is plotted against time.

According to the results of the determination of MIC- and MBC-value of nine Thai traditional medicinal plants, the ethanolic extract of those plants which demonstrated an outstanding antimicrobial activity were selected for the suspension test. The slightly modified test method described by DIN EN 1040 (Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics). The concentrations of the extracts were 15.0, 10.0, 5.0, and 1.0 g/100 ml.

From the MBCs results, 10 out of 16 strains, including *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Escherichia coli* O157:H7 DMST 12473, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* Typhimurium ATCC 13311, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 were selected as test organisms.

4.2.1 Time Killing Analysis of *Andrographis paniculata* (Burm.f.) Wall. ex Nees

The Time Killing Analysis of *Andrographis paniculata* (Burm.f.) Wall. ex Nees is shown in Figure 25-27. The ethanolic extract of this plant expressed a good antimicrobial activity against *Vibrio parahaemolyticus* ATCC 20502. At the concentration of 15.0 g/100 ml, the complete destructive (100% reduction) was investigated in 1 hour. Decreasing the concentration to 1.0 g/100 ml, the complete the destructive was obtained in 9 hours. The test bacteria suspension of *Listeria monocytogenes* DSMZ 20600 was the one which expressed sessitivity to the ethanolic extract of this plant.

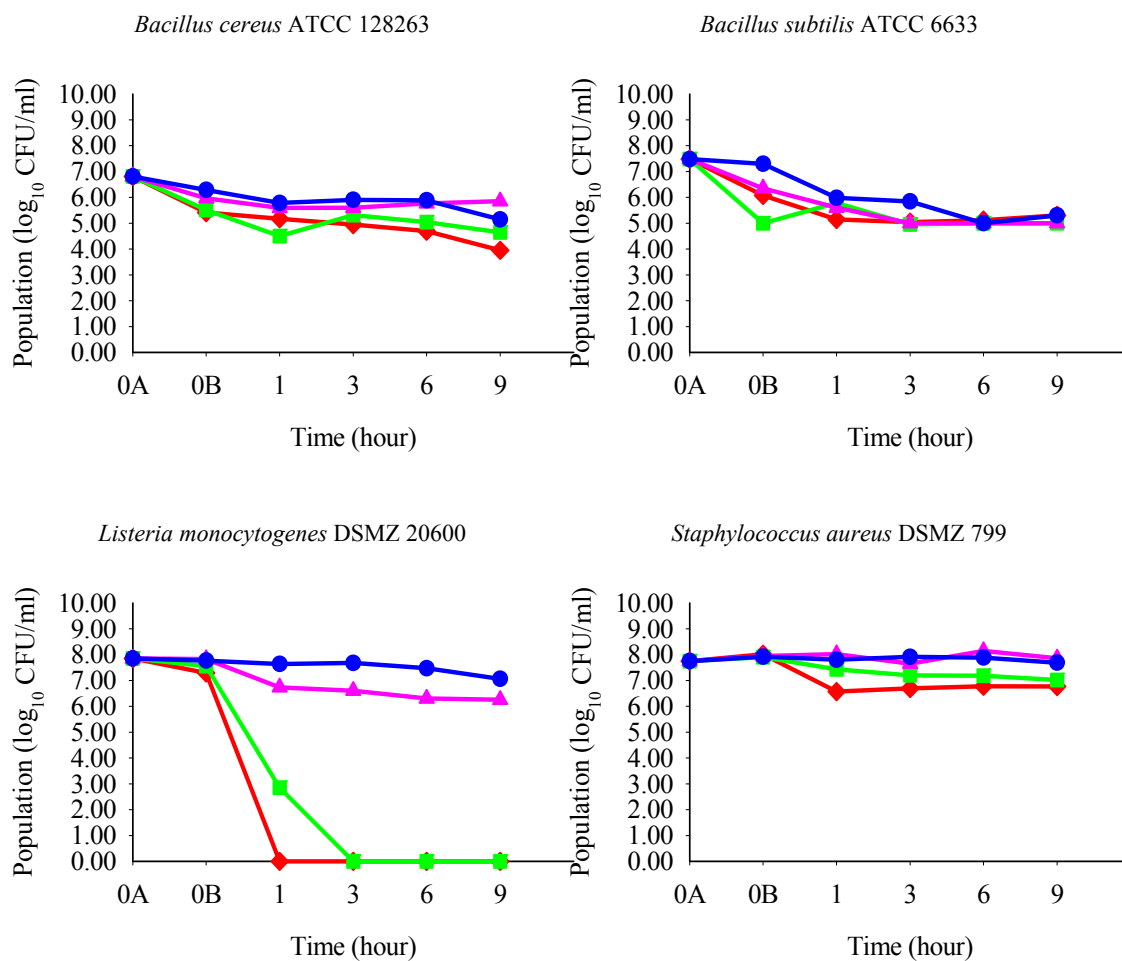


Figure 25 Effect of *Andrographis paniculata* (Burm.f.) Wall. ex Nees ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

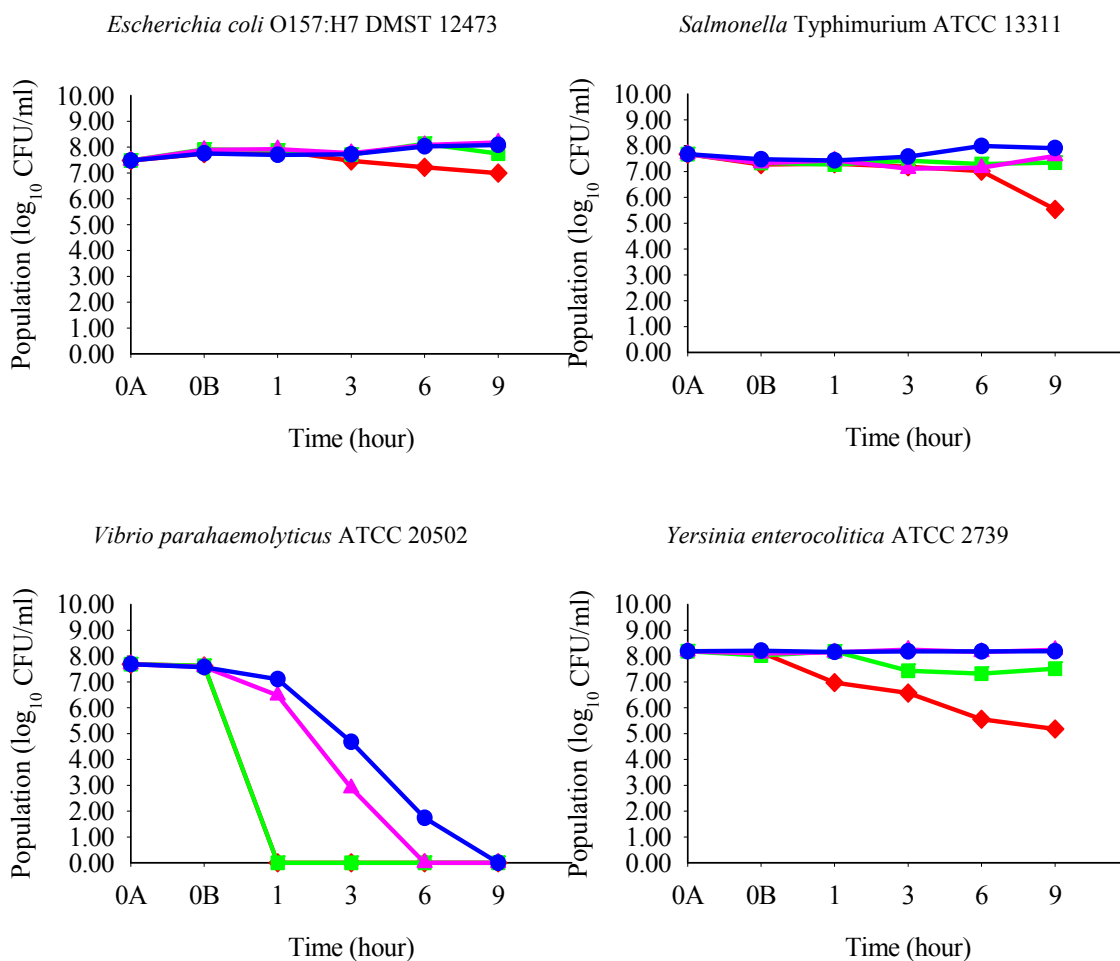


Figure 26 Effect of *Andrographis paniculata* (Burm.f.) Wall. ex Nees ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

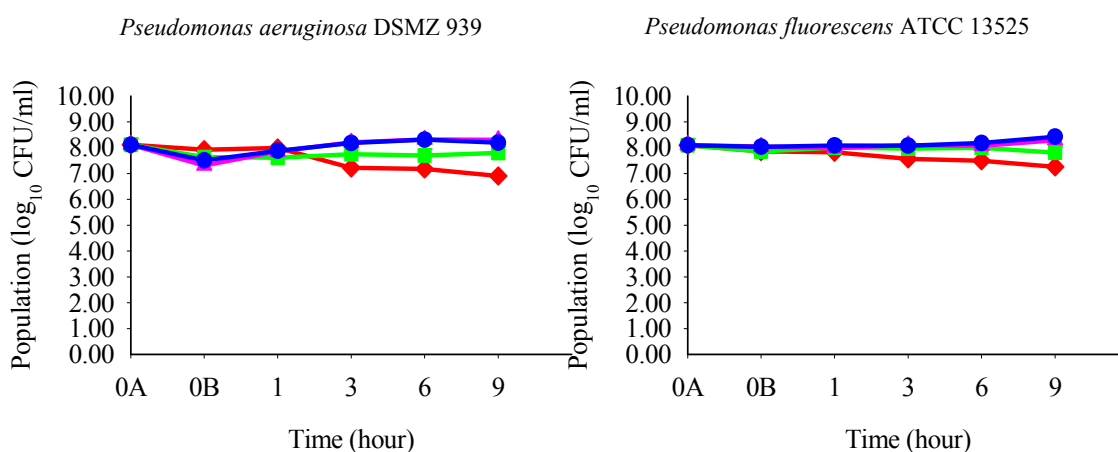


Figure 27 Effect of *Andrographis paniculata* (Burm.f.) Wall. ex Nees ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

The ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees at concentration of 15.0 g/100 ml demonstrated 100% reduction of the test organism within 1 hour, and showed the same effect in 3 hours at concentration of 10.0 g/100 ml. At the highest concentration, 15.0 g/100 ml, *Yersinia enterocolitica* ATCC 2779 was reduced to 5.18.

The other test organisms showed resistance to this extract. *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* DSMZ 799, *Escherichia coli* O157:H7 DMST 12743, and *Salmonella* Typhimurium ATCC 13311 stayed in the constant stage through 9 hours of contact time at all concentration levels.

4.2.2 Time Killing Analysis of *Curcuma zedoaria* (Christm.) Roscoe

Different concentrations of ethanolic extract of dried rhizome of *Curcuma zedoaria* (Christm.) Roscoe were added into the cells suspension of different test organisms and the Time Killing Curves were given in Figure 23-28. During 9 hours of contact time, *Bacillus cereus* ATCC 128263 and *Bacillus subtilis* ATCC 6633 demonstrated high resistance to this extract, 2.28 and 2.43 log-reductions were observed in 9 hours of contact period with the concentration of 15.0 g/100 ml, respectively.

This extract presented a good bactericidal effect against *Listeria monocytogenes* DSMZ 20600. The initial population at 7.85 log₁₀ CFU/ml decreased slightly in 6 hours in the presence of 1.0 g/100 ml ethanolic extract of this plant and decreased to undetectable levels in 9 hours. Similarly, at the concentrations of 10.0, and 5.0 g/100 ml, the extract showed bactericidal effect and reduced the populations to undetectable level in 1 hour. The bactericidal effect increased when the presence concentration in cell suspension increased. At the highest test concentration, 15.0 g/100 ml, the bactericidal effect occurred within 15 seconds. *Vibrio parahaemolyticus* ATCC 20502 also presented the sensitivity to this extract. At the concentration of 15.0 and 10.0 g/100 ml the initial population, was reduced to 0.00 log₁₀ CFU/ml within 15 seconds. When *Vibrio parahaemolyticus* ATCC 20502 was treated with 5.0 g/100 ml of this extract, the numbers of bacteria were reduced from 7.69 log₁₀ CFU/ml to 3.02 log₁₀ CFU/ml within 15 seconds, and the lethality effect was occurred in 1 hour of contact time. At the lowest working concentration, 1.0 g/100 ml, the lethal effect was detected in 1 hour. The initial populations at 8.19 log₁₀ CFU/ml of *Yersinia enterocolitica* ATCC 2279 were inactivated within 15 seconds after applied the ethanolic extract this plant at the concentration of 15.0 g/100 ml into the cell suspension. At the concentration of 10.0 g/100 ml, the bacteria was completely destroyed (100% reduction) in 1 hour.

The bactericidal effect of this extract at the concentration of 15.0 g/100 ml was investigated also against *Staphylococcus aureus* DSMZ 799, *Escherichia coli* O157:H7 DMST 12743, *Salmonella Typhimurium* ATCC 13311, *Pseudomonas*

aeruginosa DSMZ 939, and *Pseudomonas fluorescens* ATCC 13525 in the 9th, 1st, 6th, 6th, and 1st hour of the contact period, respectively.

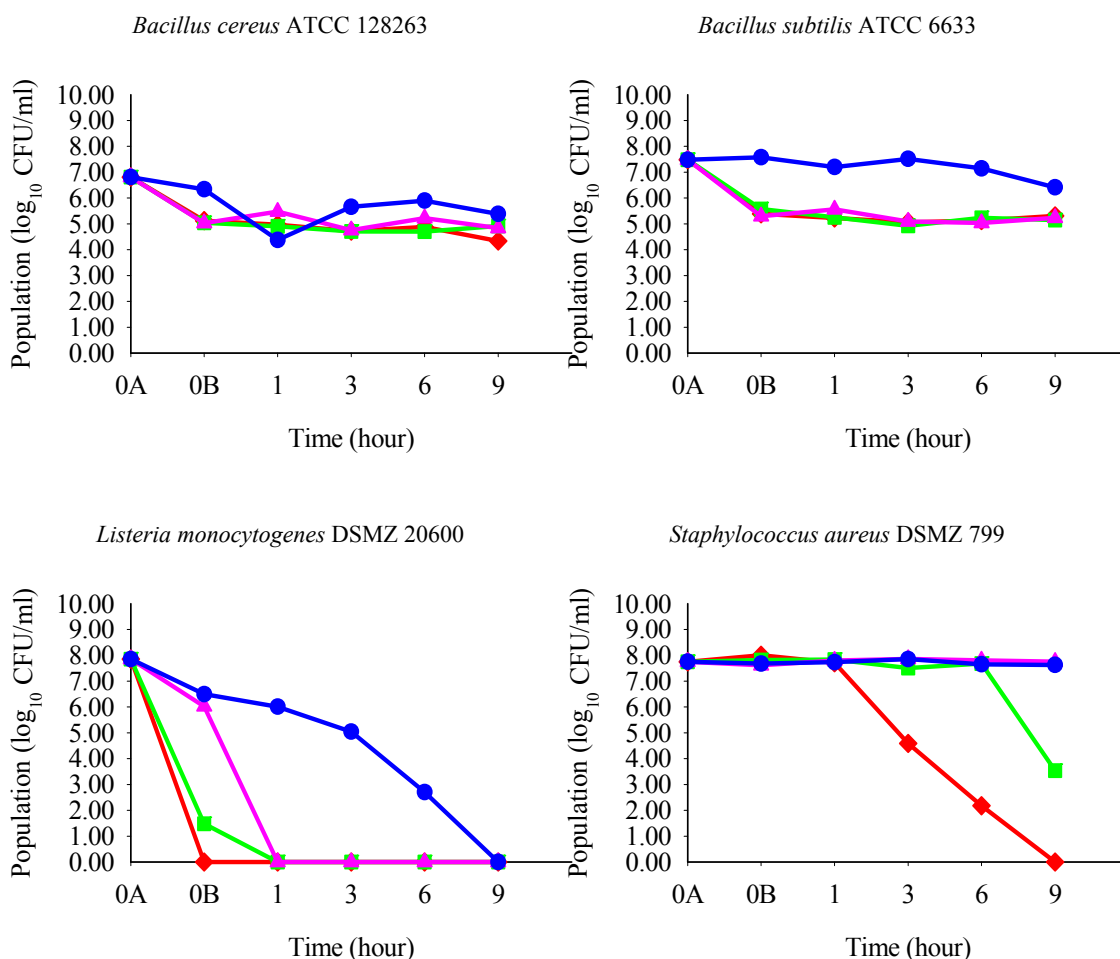


Figure 28 Effect of *Curcuma zedoaria* (Christm.) Roscoe ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

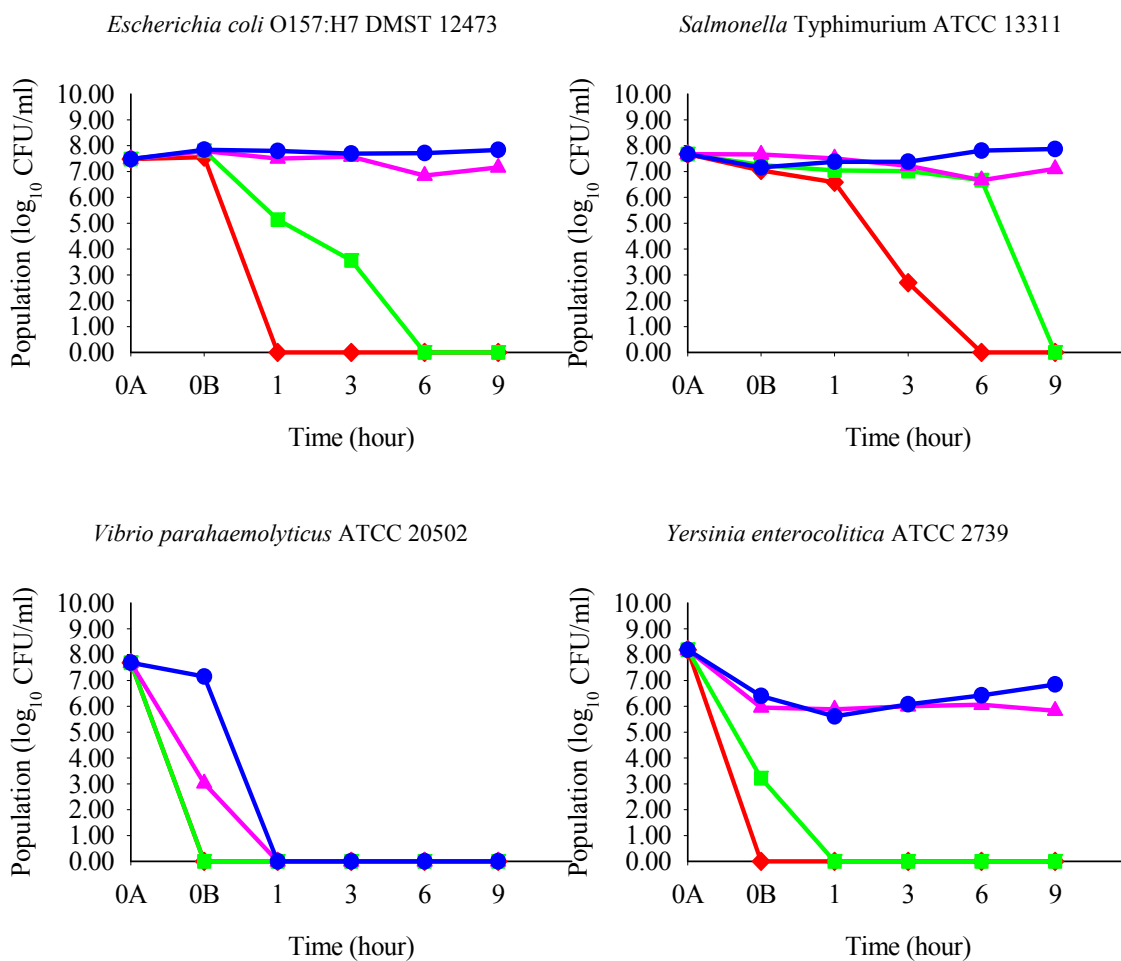


Figure 29 Effect of *Curcuma zedoaria* (Christm.) Roscoe ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

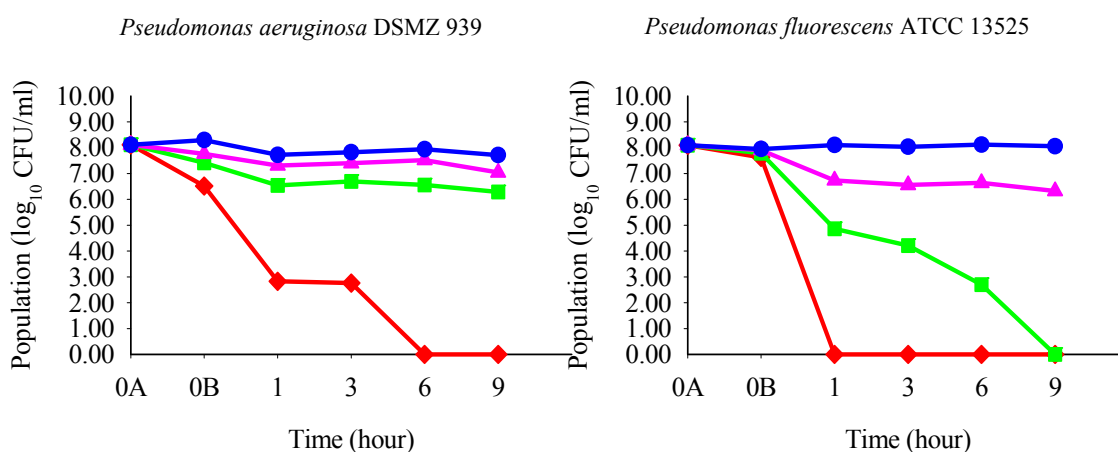


Figure 30 Effect of *Curcuma zedoaria* (Christm.) Roscoe ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

4.2.3 Time Killing Analysis of *Garcinia mangostana* L.

Antimicrobial properties of ethanolic extract of dried pericarb of *Garcinia mangostana* L. determined by Time Killing Analysis were presented in Figure 31-33. *Bacillus cereus* ATCC 128263 and *Bacillus subtilis* ATCC 6633 demonstrated the high resistance to ethanolic extract of *Garcinia mangostana* L. at all concentration levels. The bacteria test suspensions were reduced only in the first 1 hour of contact period and after that they remained constant through 9 hours. The total reduction was approx. 3.00 log₁₀ CFU/ml. *Listeria monocytogenes* DSMZ 20600 was sensible this extract. The initial population at 7.85 log₁₀ CFU/ml were reduced to undetectable level within 15 seconds at the concentration of 15.0, 10.0 and 5.0 g/100 ml. The presence of this extract at the concentration of 1.0 g/100 ml rapidly decreased to undetectable level in 1 hour. This extract demonstrated the strong bactericidal activity against *Vibrio parahaemolyticus* ATCC 20502.

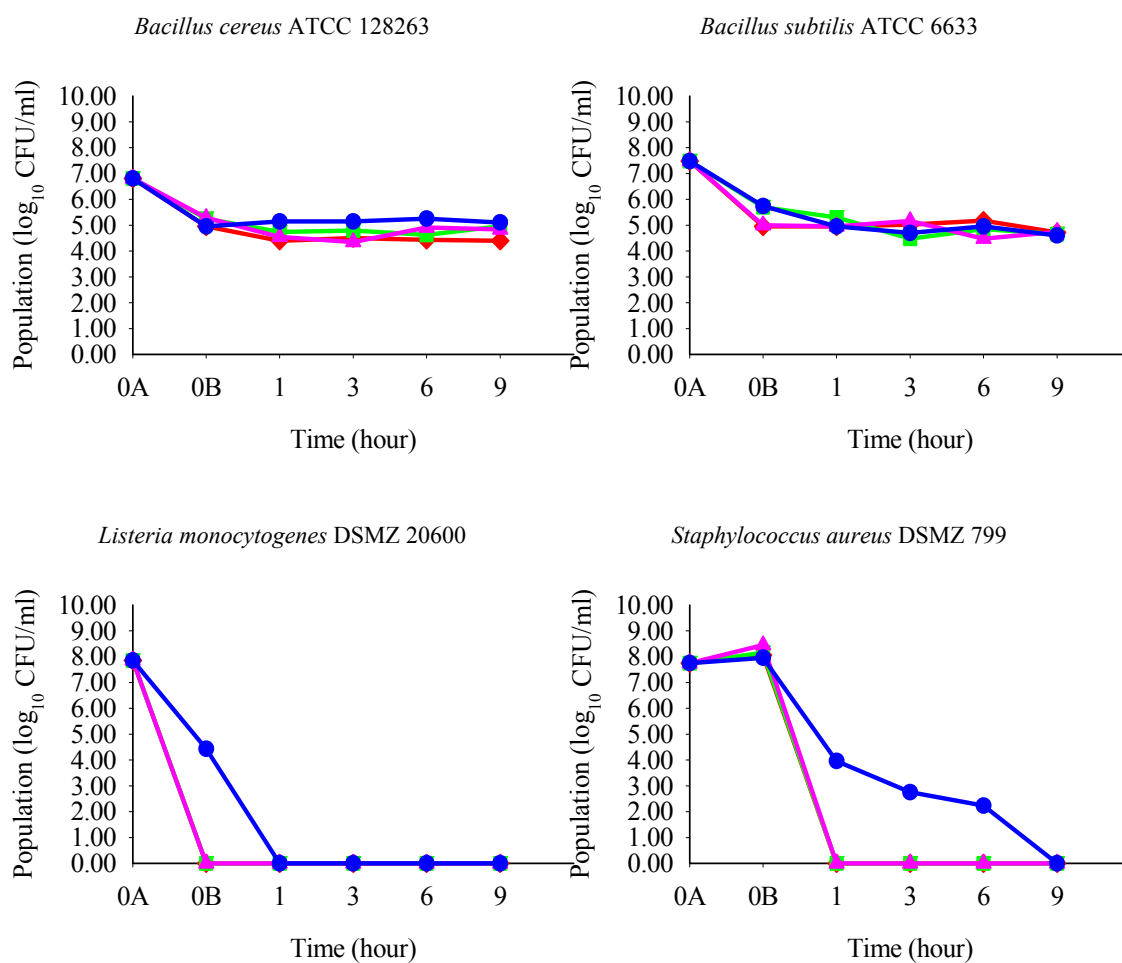


Figure 31 Effect of *Garcinia mangostana* L. ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

The initial population, 7.69 log₁₀ CFU/ml, was abated to undetectable level within 15 seconds at the concentration or 15.0, 10.0 and 5.0 g/100 ml. At the concentration of 1.30 g/100 ml, the population was also instantaneously reduced by 3.99 logs within 15 seconds and to the undetectable level in 1 hour. *Staphylococcus aureus* DSMZ 799

was also sensitive to this extract. The lethal effect was obtained within 1 hour when the ethanolic extract of *Garcinia mangostana* L. at the concentration of 15.0, 10.0, and 5.0 g/100 ml presented in cells suspension.

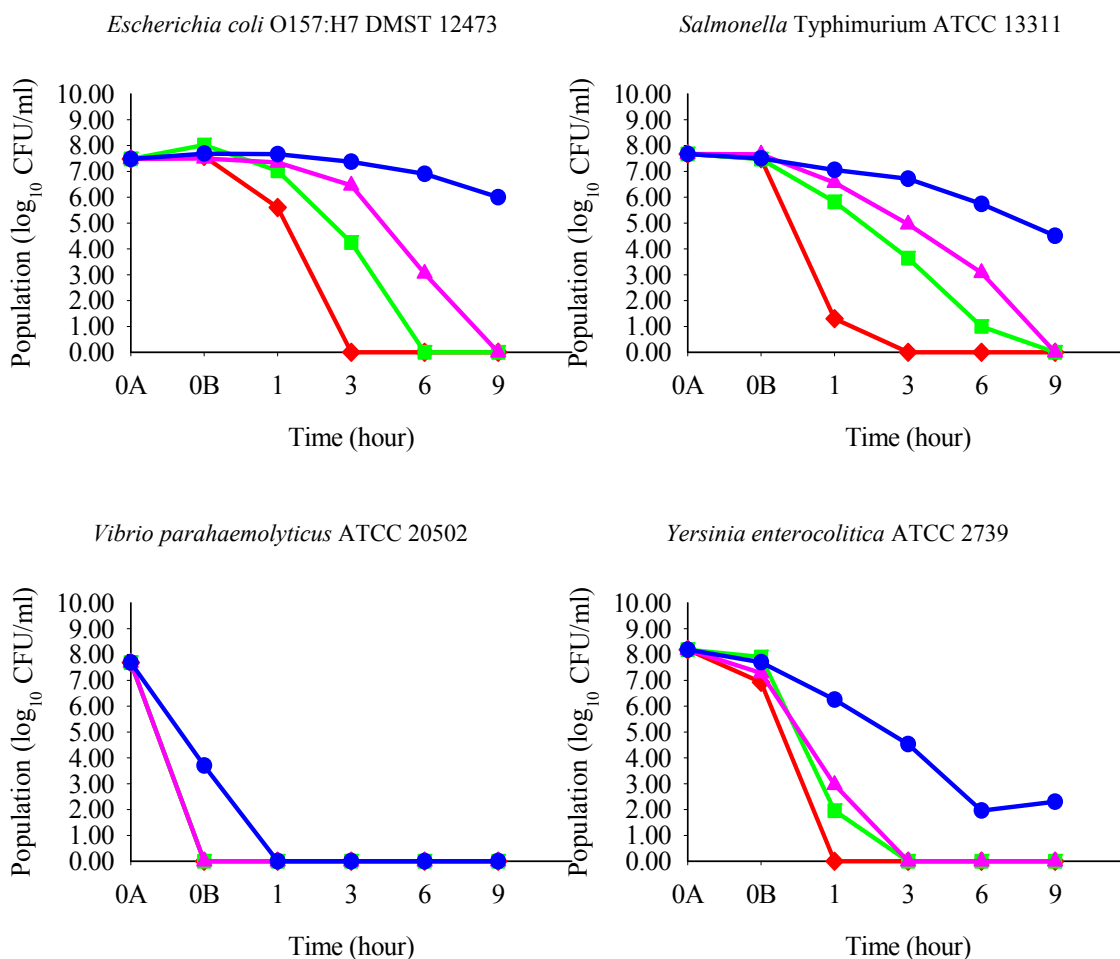


Figure 32 Effect of *Garcinia mangostana* L. ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials) At the concentration of 1.0 g/100 ml, the bacteria

suspension was rapidly decreased, the bactericidal effect was found in 9 hours, the cells population decreased to undetectable level.

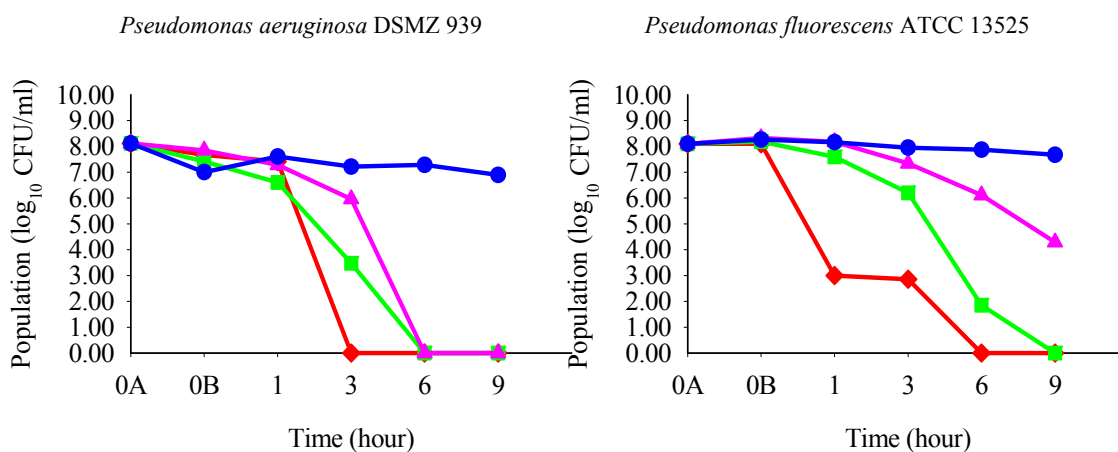


Figure 33 Effect of *Garcinia mangostana* L. ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

The other test organism also demonstrated sensitivity to the ethanolic extract of *Garcinia mangostana* L. *Escherichia coli* O157:H7 DMST 12743 was reduced from 7.48 log₁₀ CFU/ml to undetectable level in 3 hours at the concentration of 15.0 g/100 ml. The lowest concentration which demonstrated the bactericidal activity was 5.0 g/100 ml with the requirement contact time as 9 hours. *Salmonella* Typhimurium ATCC 13311 presented the same results at concentration of 1.0 g/100 ml. For *Yersinia enterocolitica* ATCC 2779, the initial population, 8.19 log₁₀ CFU/ml decreased to undetectable stage in 1 hour at the concentration of 15.0 g/100 ml, and at the concentration of 10.0 and 5.0 g/100 ml, the undetectable level was observed in 3 hours. *Pseudomonas aeruginosa* DSMZ 939 decreased to the undetectable state in 1, 3, and 3 hours with the concentration of this extract of 15.0, 10.0, and 5.0 g/100 ml,

respectively. The initial population *Pseudomonas fluorescens* ATCC 13525, decreased to undetectable stage in 6 and 9 hours at the concentration of 15.0 and 10.0 g/100 ml, respectively.

4.2.4 Time Killing Analysis of *Hibiscus sabdariffa* L.

In Figure 34-36, the Time Killing Curve, represented the antimicrobial activity of ethanolic extract of dried flower of *Hibiscus sabdariffa* L. against deferent test organisms varied with the concentration and contact time were shown.

During 9 hours of contact time, the initial population, 6.81 log₁₀ CFU/ml, of *Bacillus cereus* ATCC 128263 and *Bacillus subtilis* ATCC 6633 provided the strong stablet when treated with the ethanolic extract of dried flower of *Hibiscus sabdariffa* L. The bacteria suspension of *Bacillus cereus* ATCC 128263 was reduced to 4.48, 4.46, 4.74 and 5.96 log₁₀ CFU/ml in the presence concentration of 15.0, 10.0, 5.0, and 1.0 g/100 ml *Hibiscus sabdariffa* L.-ethanolic extract, respectively. The initial population of *Bacillus subtilis* ATCC 6633 at , 7.48 log₁₀ CFU/ml, was decreased *approx.* 1.00 log-cycles within 1 hour and then stayed constant at around 6.00 log₁₀ CFU/ml through 9 hours of contact period.

All concentrations of *Hibiscus sabdariffa* L.-ethanolic extract exhibited a good bactericidal activity against *Vibrio parahaemolyticus* ATCC 20502. The initial population of the organism at 7.69 log₁₀ CFU/ml was reduced to undetectable stage within 15 seconds.

At all concentrations of *Hibiscus sabdariffa* L.-ethanolic extract demonstrated the bactericidal effect against *Listeria monocytogenes* DSMZ 20600, *Salmonella* Typhimurium ATCC 13311, and *Yersinia enterocolitica* ATCC 2779. At the lowest concentration, 1.0 g/100 ml, the required contact time to reduce these test organisms from *approx.* 8.00 log₁₀ CFU/ml to undetectable was 6 hours for *Listeria monocytogenes* DSMZ 20600 and *Salmonella* Typhimurium ATCC 13311. In case of *Yersinia enterocolitica* ATCC 2779, 1 hour was the longest required contact time.

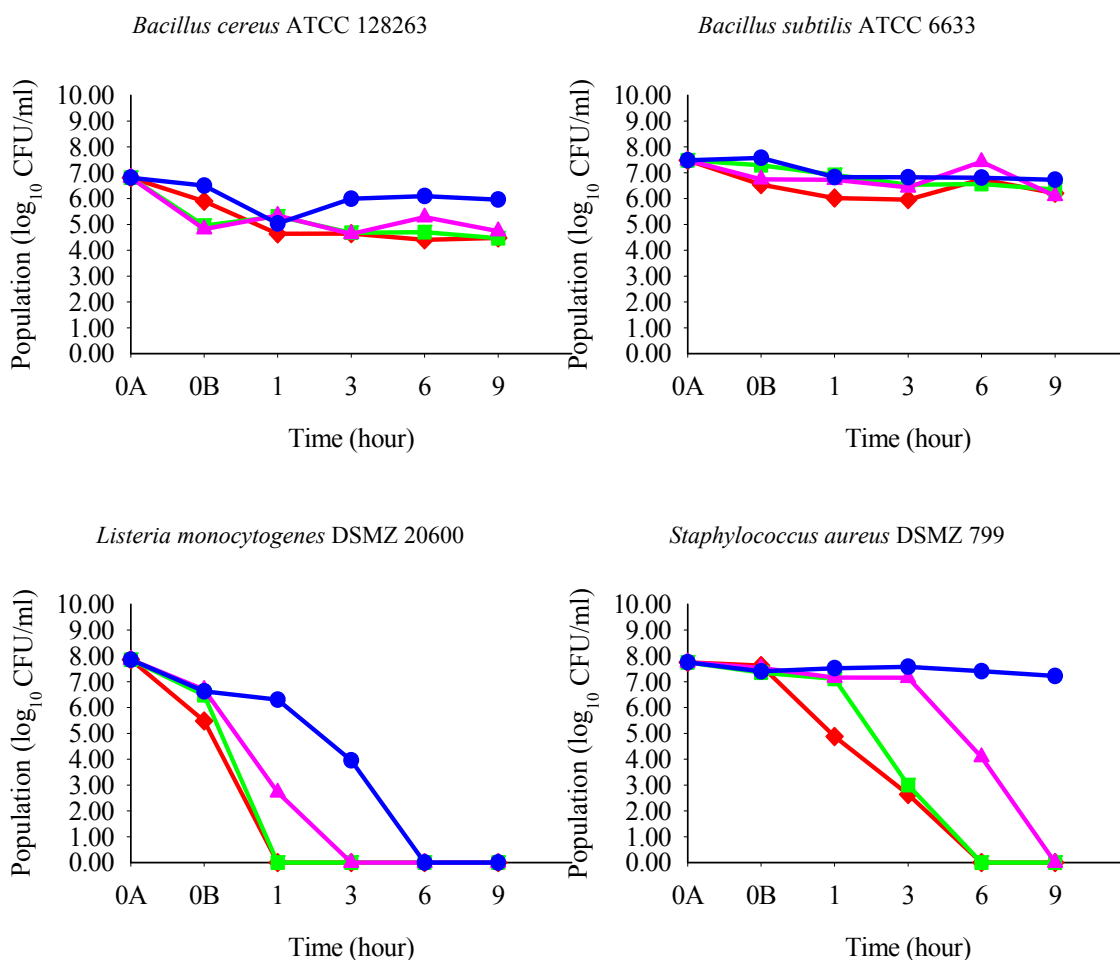


Figure 34 Effect of *Hibiscus sabdariffa* L. ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

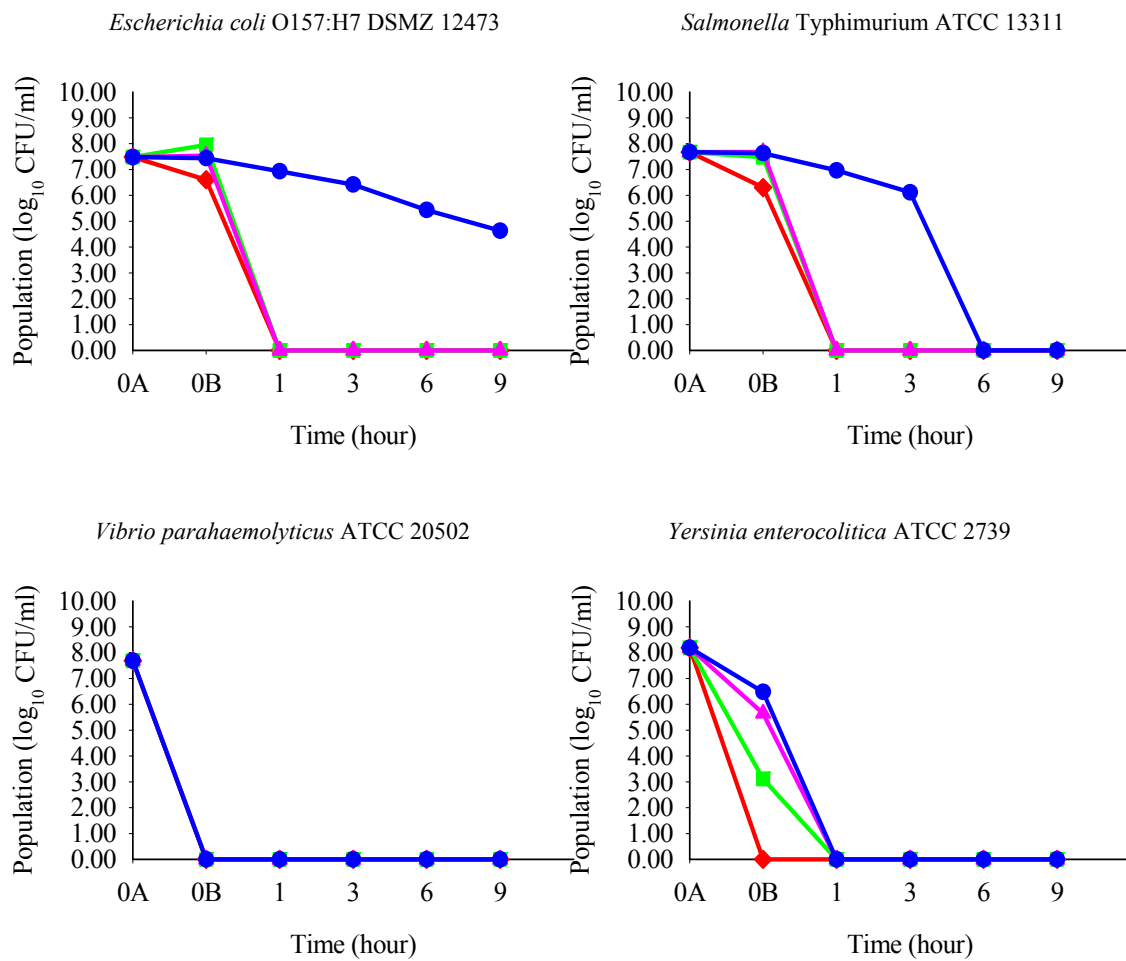


Figure 35 Effect of *Hibiscus sabdariffa* L. ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

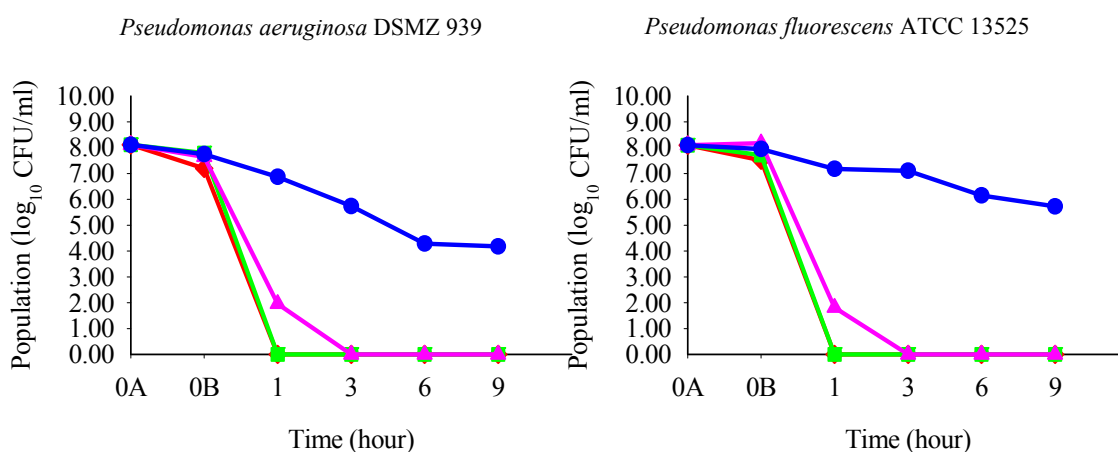


Figure 36 Effect of *Hibiscus sabdariffa* L. ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

The ethanolic extract of *Hibiscus sabdariffa* L. at the concentration of 1.0 g/100 ml had no ability to provide the lethal effect against *Staphylococcus aureus* DSMZ 799, *Escherichia coli* O157:H7 DMST 12743, *Pseudomonas aeruginosa* DSMZ 799, and *Pseudomonas fluorescens* ATCC 13525 from approx. 8.00 log₁₀ CFU/ml within 9 hours. However, *Escherichia coli* O157:H7 DMST 12743 was reduced to undetectable level within 1 hour at the concentration of 15.0, 10.0, and 5.0 g/100 ml. *Pseudomonas aeruginosa* DSMZ 799, and *Pseudomonas fluorescens* ATCC 13525 required at least 3 hours at concentration of 5.0 g/100 ml and 1 hour at concentration of 15.0 and 10.0 g/100 ml. Within 9 hours of contact time, *Staphylococcus aureus* DSMZ 799 was reduced to undetectable level at concentration of 15.0, 10.0, and 5.0 g/100 ml.

4.2.5 Time Killing Analysis of *Musa sapientum* L.

The Time Killing Curves of dried blossoms of *Musa sapientum* L.-ethanolic extract against different test organisms varied with the concentration and contact time were presented in Figure 37-39,

The ethanolic extract of *Musa sapientum* L. had no antimicrobial activity against *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, *Escherichia coli* O157:H7 DMST, *Salmonella* Typhimurium ATCC 13311, *Yersinia enterocolitica* ATCC 2779, *Pseudomonas aeruginosa* DSMZ 799, and *Pseudomona fluorescens* ATCC 13525 at all concentrations. The population stay in steady stage through 9 hours of contact time.

This extract showed an ambiguous antimicrobial activity against *Bacillus subtilis* ATCC 6633. After the extract was applied to the cell suspensions for 9 hours, the bacterial population was reduced by 2.62, 2.48, 1.3, and 0.59 log-reductions at the concentration of 15.0, 10.0, 5.0, and 1.0 g/100 ml, respectively.

All concentration of *Musa sapientum* L. -ethanolic extract exhibited good bactericidal activity against *Vibrio parahaemolyticus* ATCC 20502. At the concentration of 15.0 g/100 ml, the initial population of the organism at 7.69 log₁₀ CFU/ml was reduced to undetectable stage within 1 hour and in 3, 6, and 9 hours at the concentration of 10.0, 5.0, and 1.0 g/100 ml, respectively. At the highest concentration, 15.0 g/100 ml, this extract provided the antilisterial. The initial population of *Listeria monocytogenes* DSMZ 20600 at 7.85 log₁₀ CFU/ml decreased to undetectable in 1 hour. At the other concentrations, the antimicrobial activity was ambiguous.

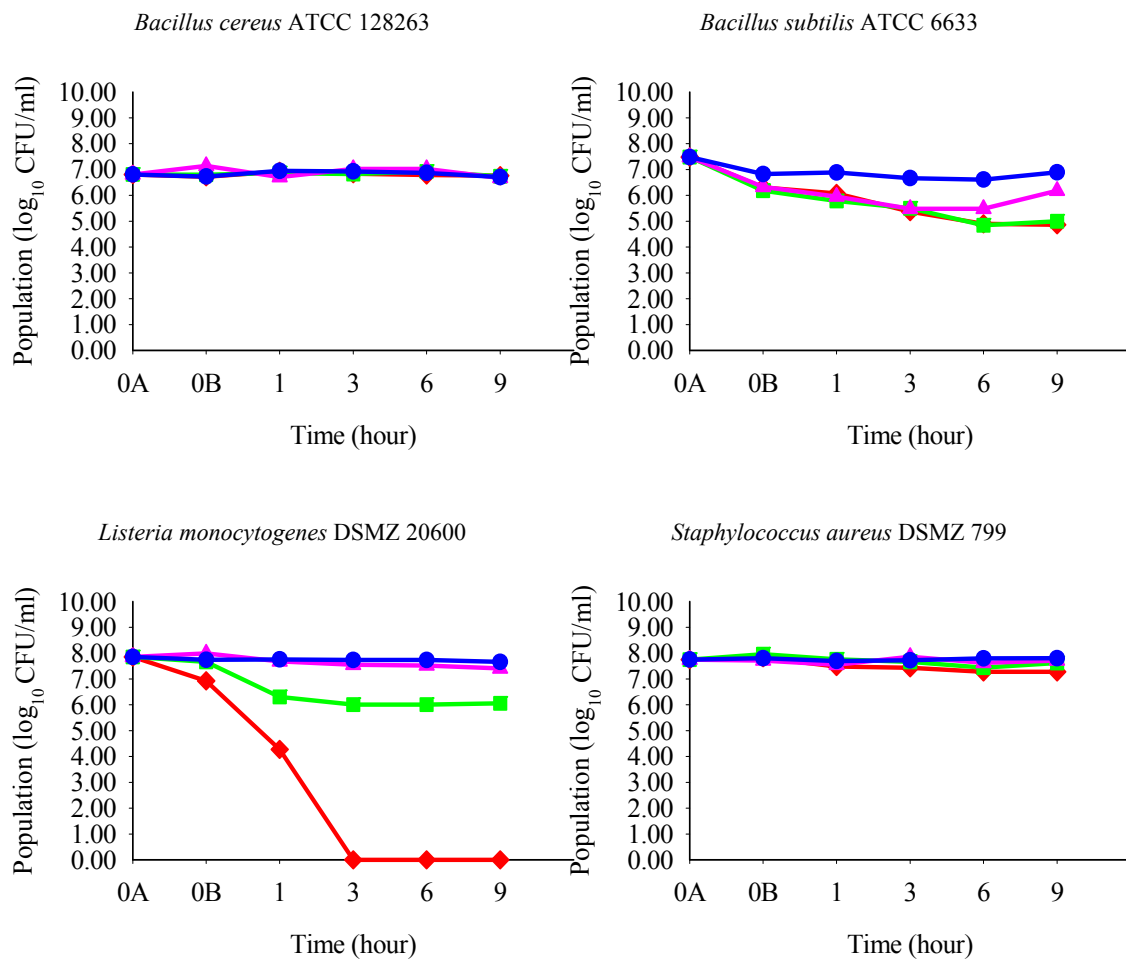


Figure 37 Effect of *Musa sapientum* L. ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

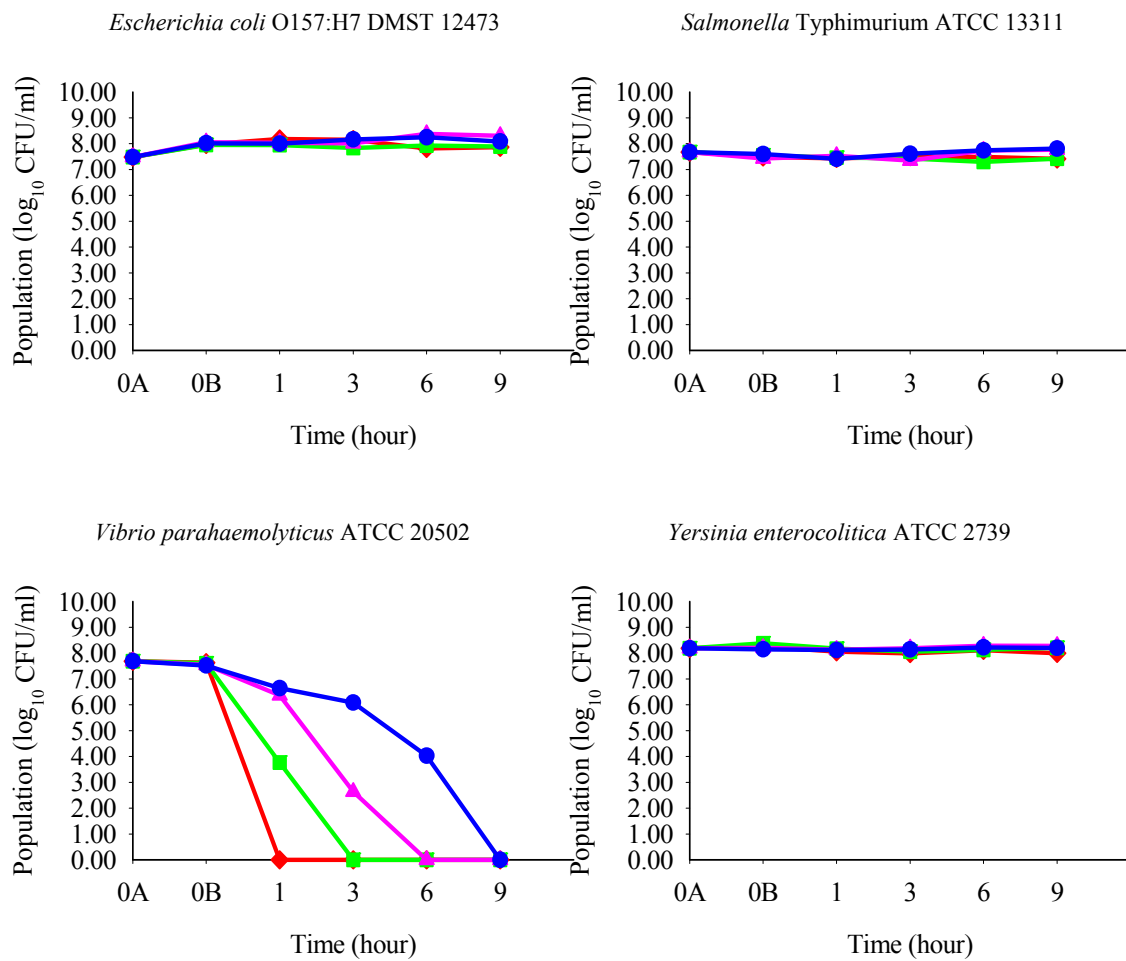


Figure 38 Effect of *Musa sapientum* L. ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

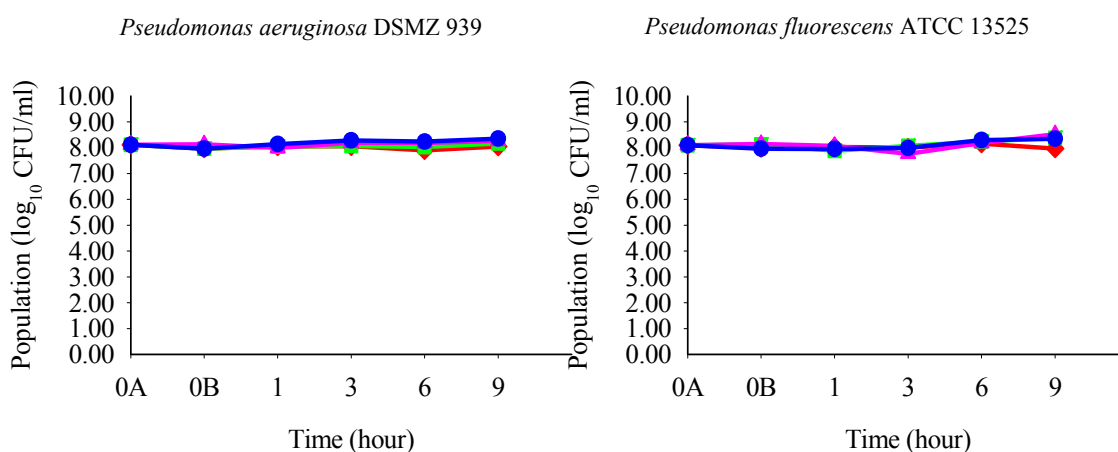


Figure 39 Effect of *Musa sapientum* L. ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

4.2.6 Time Killing Analysis of *Nelumbo nucifera* Gaertn.

The antimicrobial properties of ethanolic extract of dried rhizomes of *Nelumbo nucifera* Gaertn. against different test organisms determined by The Time Killing Analysis were given in Figure 40-42

The ethanolic extract of *Nelumbo nucifera* Gaertn. at all concentration had no antimicrobial activity against *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, *Staphylococcus aureus* DSMZ 799, *Escherichia coli* O157:H7 DMST 12743, *Salmonella* Typhimurium ATCC 1331, *Pseudomonas aeruginosa* DSMZ 799 and *Pseudomona fluorescens* ATCC 13525.. The population stayed in steady stage through 9 hours of contact time.

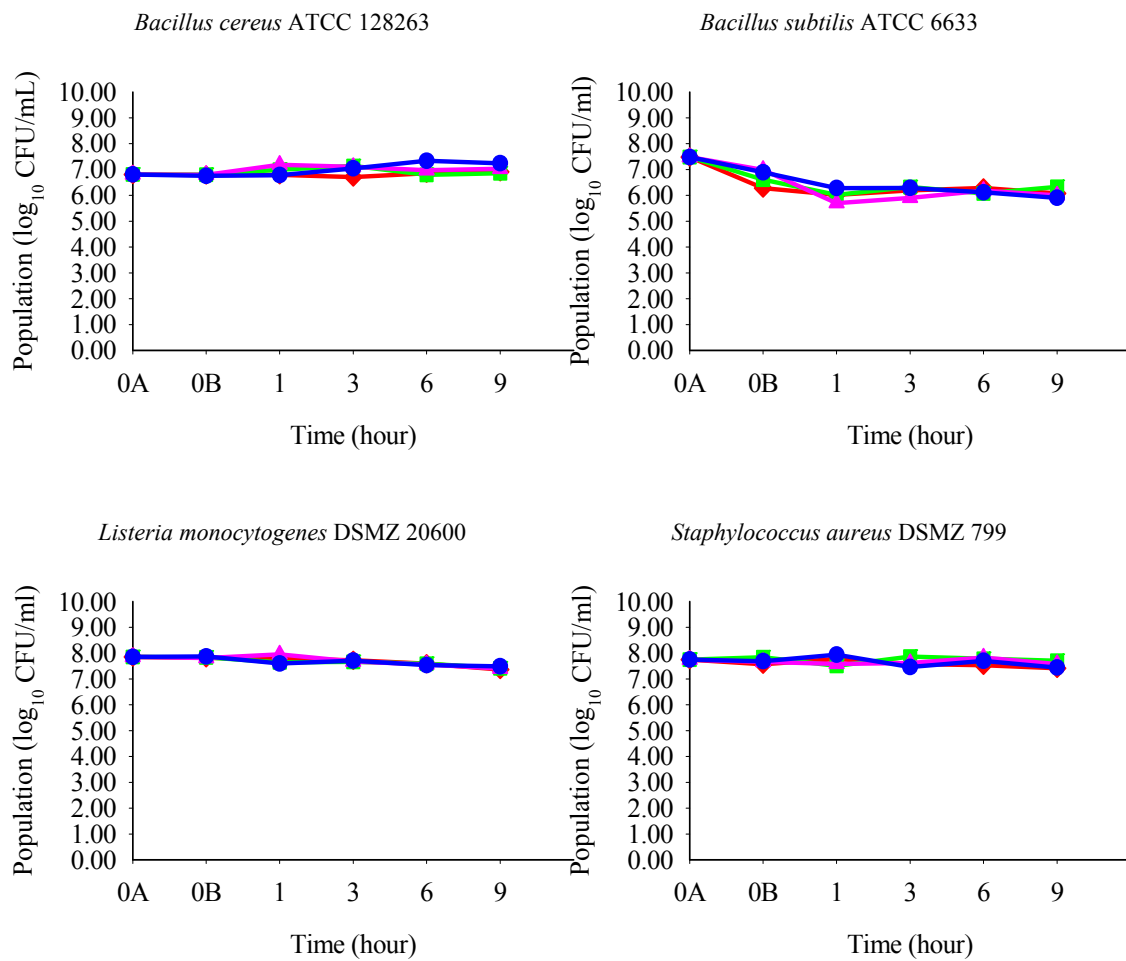


Figure 40 Effect of *Nelumbo nucifera* Gaertn. ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

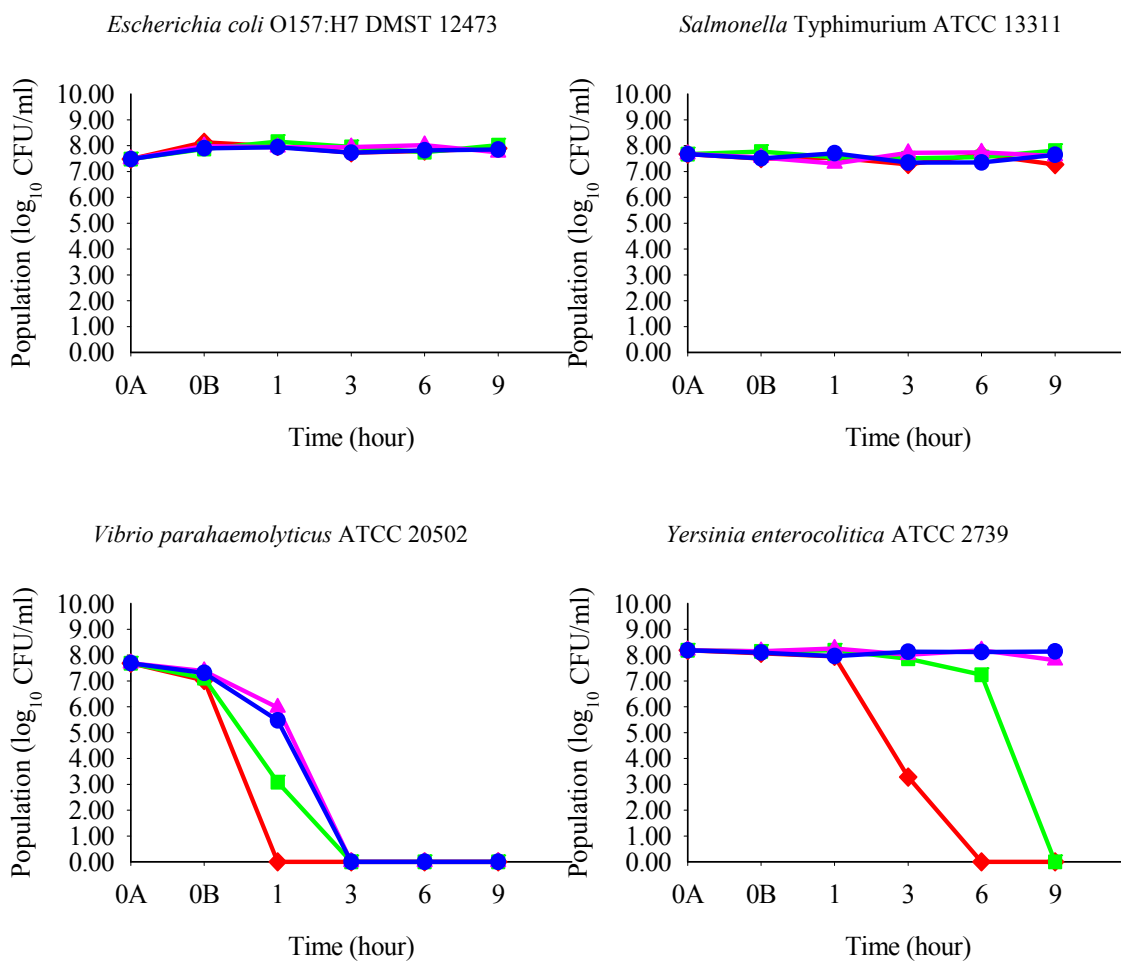


Figure 41 Effect of *Nelumbo nucifera* Gaertn. ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

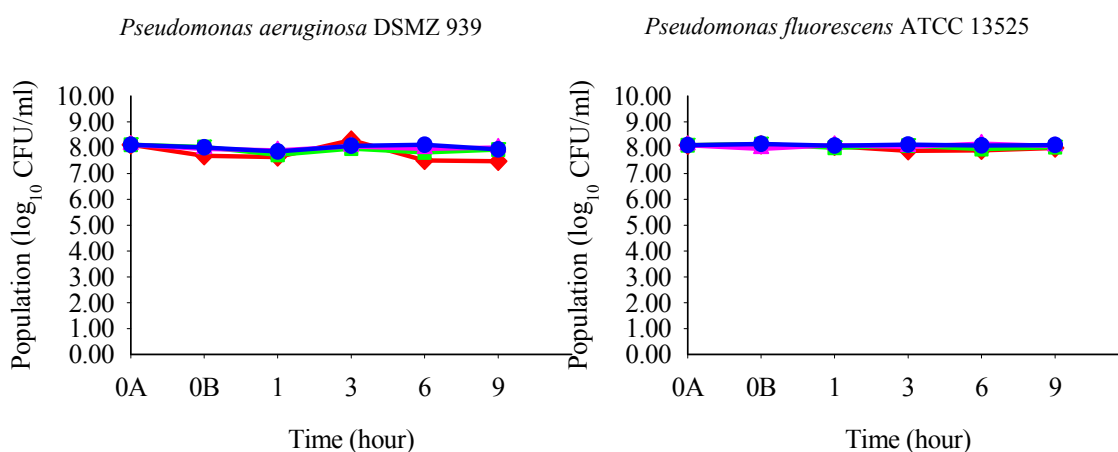


Figure 42 Effect of *Nelumbo nucifera* Gaertn. ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

In contrast, this extract exhibited an antimicrobial activity against *Vibrio parahaemolyticus* ATCC 20502. At the concentration of 15.0 g/100 ml, the numbers of initial population, 7.69 log₁₀ CFU/ml, decreased to undetectable level in 1 hour, and in 3 hours for the concentration of 10.0, 5.0, and 1.0 g/100 ml. This extract also presented the bactericidal effect against *Yersinia enterocolitica* ATCC 2779. At the highest concentration, 15.0 g/100 ml, bactericidal effect occurred at the 6th of contact period, and showed the same effect in 9 hours with the concentration of 10.0 g/100 ml. For the lower concentrations of the extract, 5.0 and 1.0 g/100 ml, the antimicrobial activity could not be detected.

4.2.7 Time Killing Analysis of *Piper betle* L.

The Time Killing Curves of ethanolic extract of dried leaves of *Piper betle* L. against different test organisms were shown in Figure 43-45. The initial population at 6.81 log₁₀ CFU/ml of *Bacillus cereus* ATCC 128263 and *Bacillus subtilis* ATCC 6633 demonstrated the strong resistance to this extract. At the presence of 15.0 g/100 ml *Piper betle* L.-ethanolic extract, bacteria suspension of *Bacillus cereus* ATCC 128263 was reduced to 4.13 log₁₀ CFU/ml and to 6.05 log₁₀ CFU/ml for *Bacillus subtilis* ATCC 6633 in 9 hours.

At the presence of 15.0 g/100 ml *Piper betle* L.-ethanolic extract. The population was reduced by 2.12, 2.06, and 1.55 log-reductions when they contacted with the extract at the concentration of 10.0, 5.0, and 1.0 g/100 ml, respectively. The insignificant antimicrobial was observed against *Bacillus subtilis* ATCC 6633. The cells population decreased from 7.48 log₁₀ CFU/ml at the initial stage to 6.05, 6.26, 6.52, and 6.36 log₁₀ CFU/ml in 9 hours at the concentration of 15.0, 10.0, 5.0, and 1.0 g/100 ml, respectively.

The bacteria test suspension of *Vibrio parahaemolyticus* ATCC 20502 demonstrated the high susceptibility against ethanolic extract of *Piper betle* L. At all the concentrations of the extract demonstrated the bactericidal effect. At the concentration of 15.0, 10.0, and 5.0 g/100 ml, the initial population, 7.69 log₁₀ CFU/ml, decreased to undetectable level in 15 seconds, and in 1 hour for the concentration of 1.0 g/100 ml. Moreover, the ethanolic extract of this medicinal plant also provided high antimicrobial activity against *Escherichia coli* O157:H7 DMST 12743 and *Yersinia enterocolitica* ATCC 2779. At the lowest concentration, 1.0 g/100 ml, these bacteria test suspension were reduced to undetectable level in 9 and 6 hours, respectively.

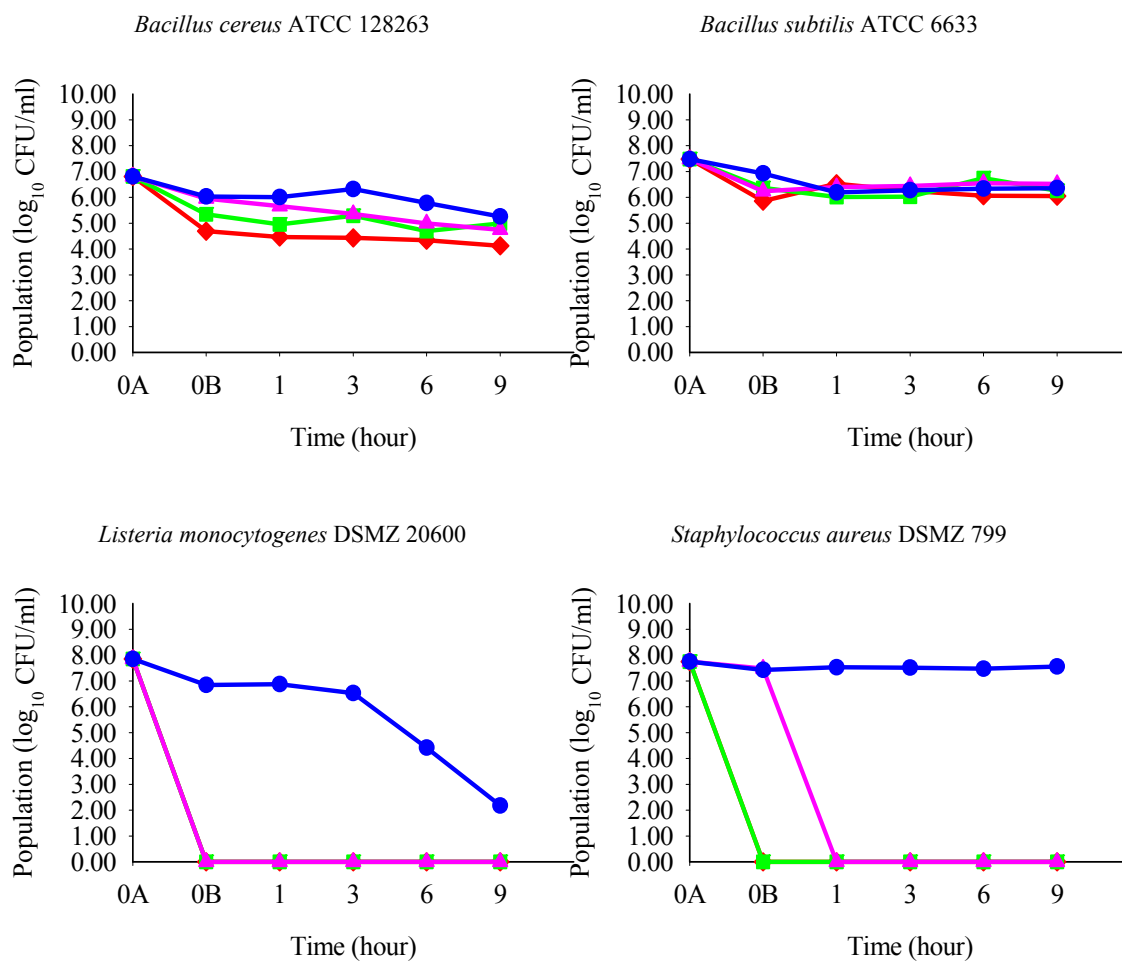


Figure 43 Effect of *Piper betle* L. ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

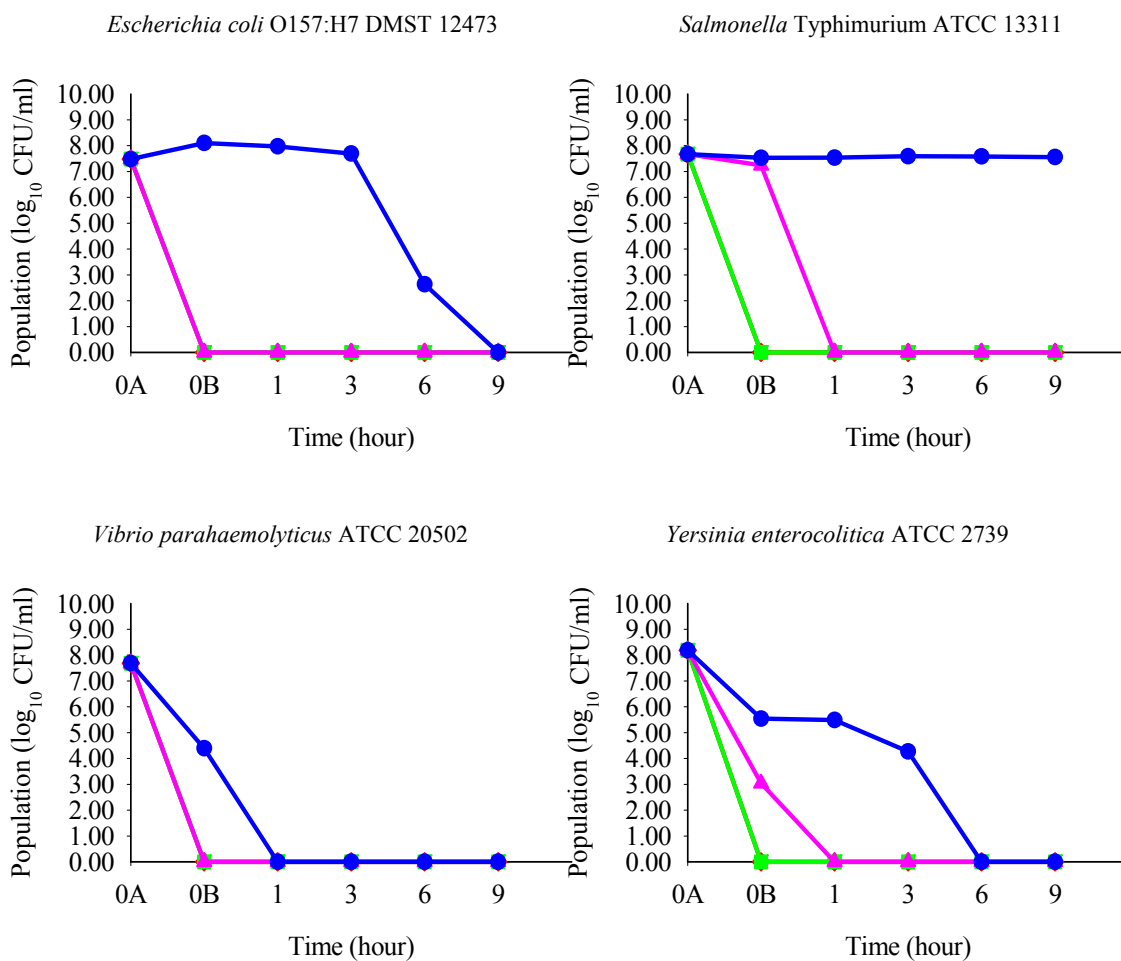


Figure 44 Effect of *Piper betle* L. ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

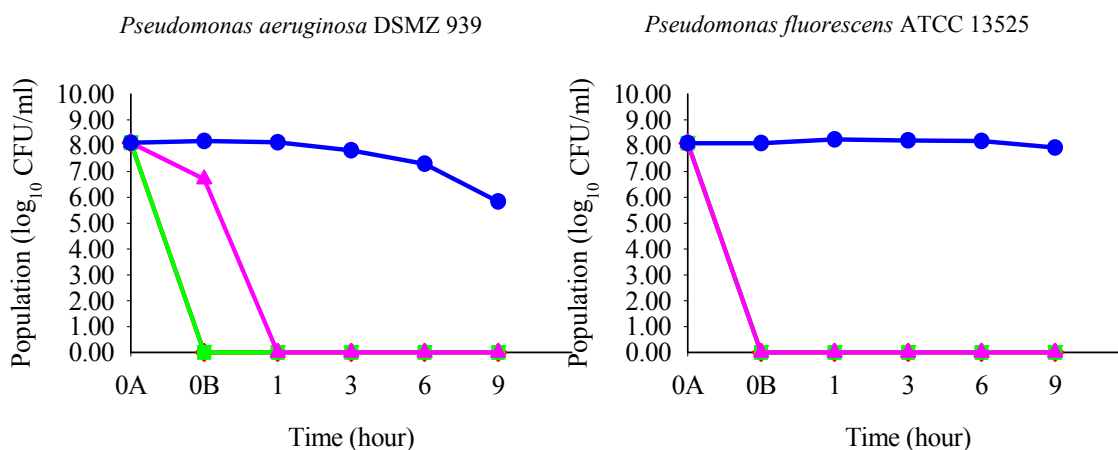


Figure 45 Effect of *Piper betle* L. ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

At the highest concentration of ethanolic extract of *Piper betle* L., 15.0 g/100 ml, the bacteria suspension of *Listeria monocytogenes* DSMZ 20600, *Staphylococcus aureus* DSMZ 799, *Escherichia coli* O157:H7 DMST 12743, *Salmonella* Typhimurium ATCC 1331, *Vibrio parahaemolyticus* ATCC 20502, *Yersinia enterocolitica* ATCC 2779, *Pseudomonas aeruginosa* DSMZ 799, and *Pseudomonas fluorescens* ATCC 13525 were reduced from approx. 8.00 log₁₀ CFU/ml to undetectable level within 15 seconds. Similarly, at the concentration of 10.0 g/100 ml, this extract provided the lethal effect within 15 seconds. However, at the lowest concentration, 1.0 g/100 ml had no ability to reduce bacteria suspension of *Listeria monocytogenes* DSMZ 20600, *Staphylococcus aureus* DSMZ 799, *Salmonella* Typhimurium ATCC 1331, *Pseudomonas aeruginosa* DSMZ 799, and *Pseudomonas fluorescens* ATCC 13525 to undetectable level within 9 hours.

4.2.8 Time Killing Analysis of *Psidium guajava* L.

In Figure 46-48, the antimicrobial determined by Time Killing Analysis of ethanolic extract of dried leaves of *Psidium guajava* L. against deferent test organisms at several contact time were presented.

Listeria monocytogenes DSMZ 20600 and *Vibrio parahaemolyticus* ATCC 20502 expressed the high sensitivity to the ethanolic extract of *Psidium guajava* L. At the concentration of 15.0 and 10.0 g/100 ml, the initial population of *Listeria monocytogenes* DSMZ 20600, 7.85 log₁₀ CFU/ml, decreased to the undetectable stage within 15 seconds. The bacteria test suspension was rapidly decreased from 7.85 log₁₀ CFU/ml to 3.77 and 3.94 log₁₀ CFU/ml within 15 seconds at the concentration of 5.0 and 1.0 g/100 ml, respectively. At the both level of concentration, the cells decreased to undetectable level in 1 hour. For *Vibrio parahaemolyticus* ATCC 20502, the initial population at 7.69 log₁₀ CFU/ml decreased to undetectable level in 1 hour at all concentrations.

This extract exhibited non-significant antimicrobial activity against *Pseudomonas aeruginosa* DSMZ 799 in 9 hours. The initial population, 8.11 log₁₀ CFU/ml, was reduced by 2.36, 1.37, and 1.09 logs in 9 hours at the concentration of 15.0, 10.0, and 5.0 g/100 ml, respectively. The bacteria survived in the presence concentration of *Psidium guajava* L.-ethanolic extract at 1.0 g/100 ml for 9 hours. Regarding, the activity against *Pseudomona fluorescens* ATCC 13525, the initial population of 8.10 log₁₀ CFU/ml was reduced by 2.35, 1.33, and 0.89 logs in 9 hours at the concentration of 15.0, 10.0, and 5.0 g/100 ml, respectively. The cells showed survivability for 9 hours at 1.0 g/100 ml concentration of the extract. *Bacillus cereus* ATCC 128263 and *Bacillus subtilis* ATCC 6633 were the other two test organisms which expressed the resistance to this extract. The bacteria test suspension of both strains decreased by approx. 2.00 log-reductions within the 1st hour of contact time and then stayed in constant through 9 hours.

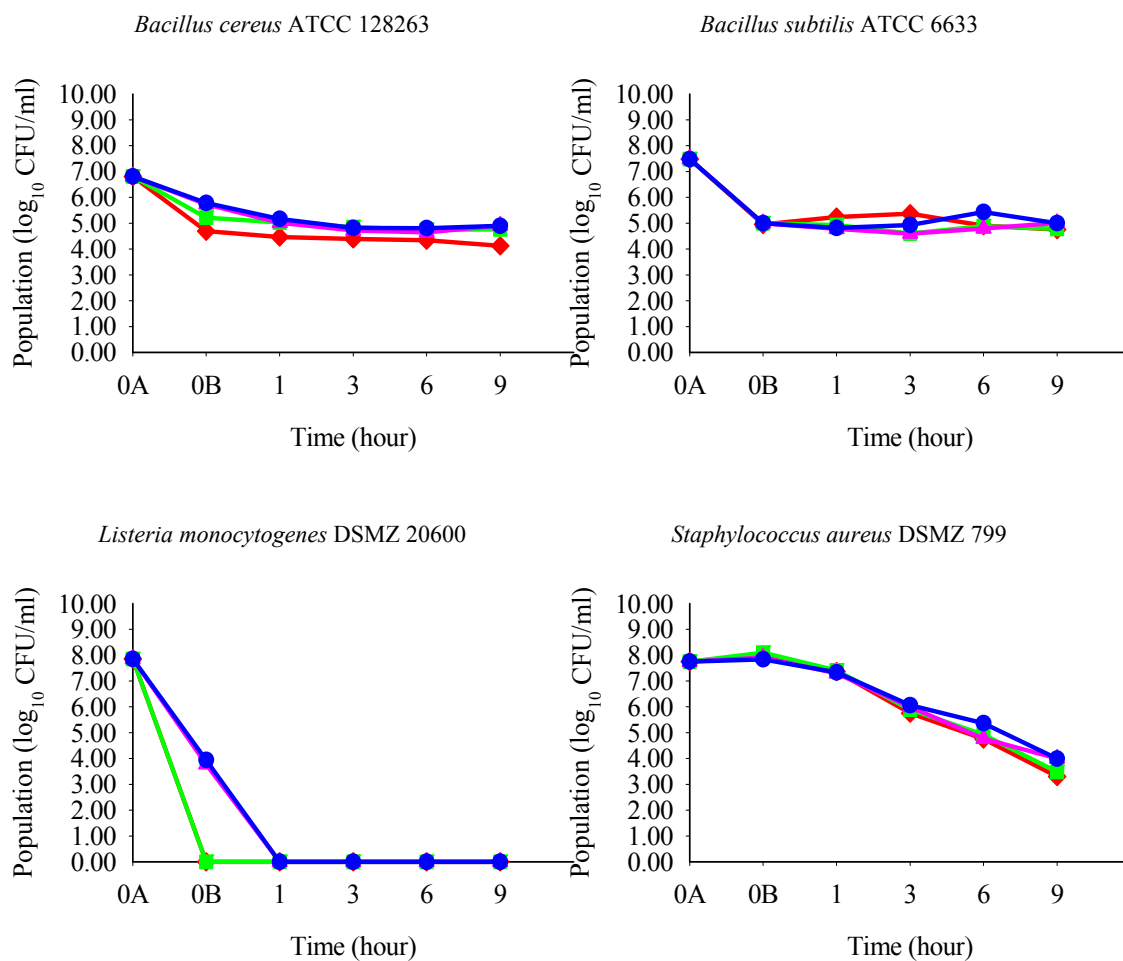


Figure 46 Effect of *Psidium guajava* L. ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

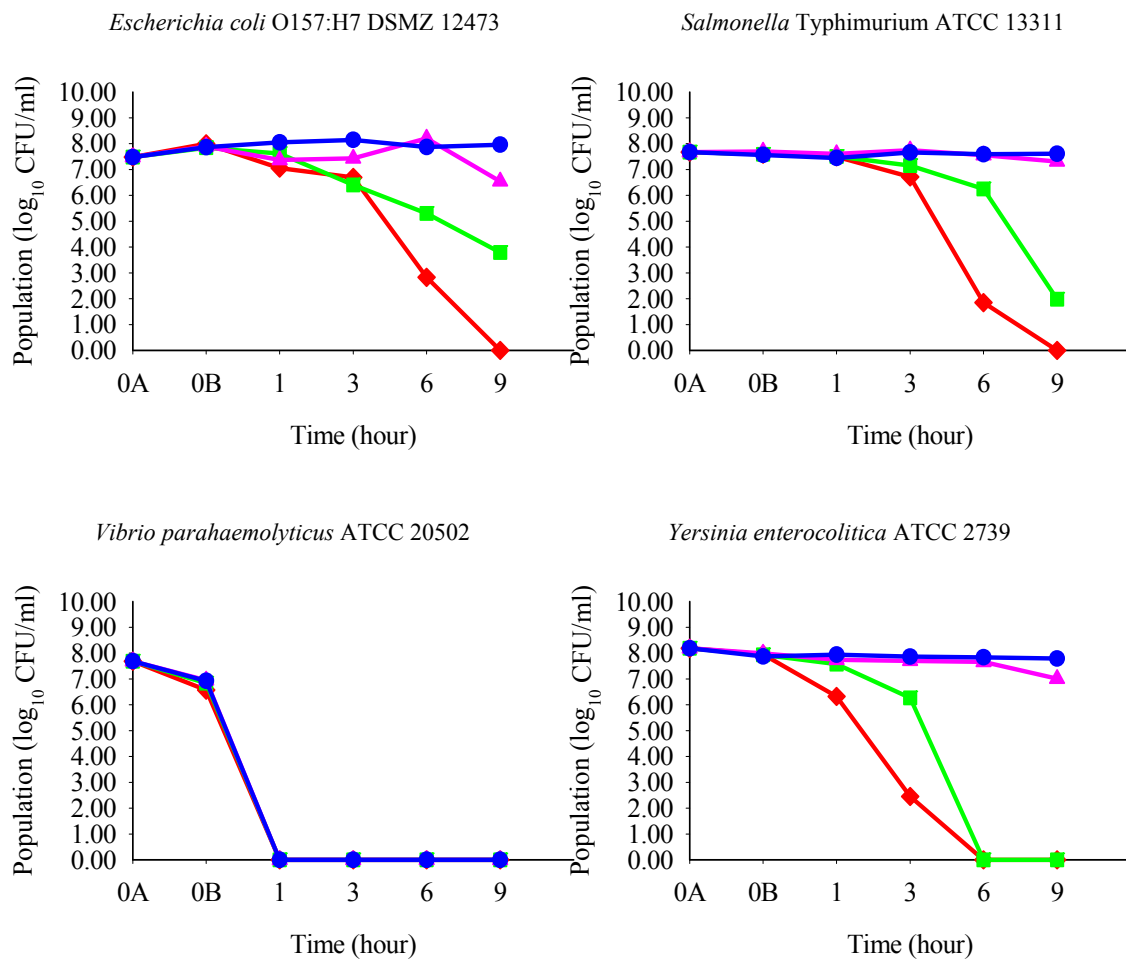


Figure 47 Effect of *Psidium guajava* L. ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

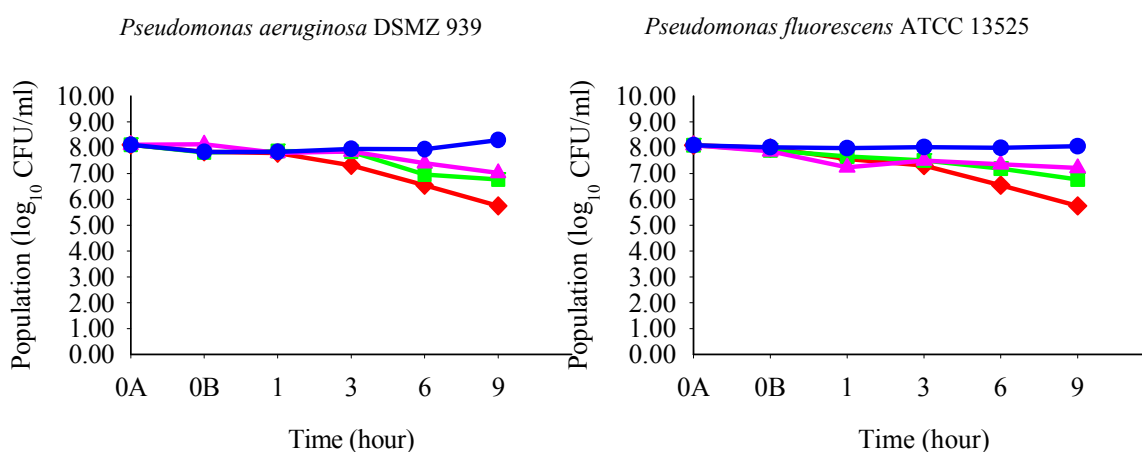


Figure 48 Effect of *Psidium guajava* L. ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

Staphylococcus aureus DSMZ 799 demonstrated the sensitivity. Although the bacteria test suspension was not reduced to undetectable level at all concentration levels, the survival tended to decrease during 9 hours of contact period. At the end of the contact period, the population was *approx.* 4.00 log₁₀ CFU/ml

Escherichia coli O157:H7 DMST 12743, *Salmonella* Typhimurium ATCC 1331, and *Yersinia enterocolitica* ATCC 2779 presented the sensitivity to this extract only in the presence of high concentration level. *Escherichia coli* O157:H7 DMST 12743 and *Salmonella* Typhimurium ATCC 1331 decreased to undetectable level at the 9th hour contact period in the presence of 15.0 g/100 ml ethanolic extract of *Psidium guajava* L. *Yersinia enterocolitica* ATCC 2779 demonstrated the same effect at the 6th hour contact period in the presence of 15.0 and 10.0 g/100 ml ethanolic extract of this plant.

4.2.9 Time Killing Analysis of *Punica granatum* L.

The Time Killing Plot, corresponding to the antimicrobial activity of ethanolic extract of dried pericarp of *Punica granatum* L. against different test organisms varied with the concentration and contact time were presented in Figure 49-51.

The ethanolic extract of *Punica granatum* L. at the concentration of 15.0 g/100 ml presented the bactericidal effect to all test organisms with the exception of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* DSMZ 799. In the presence of ethanolic extract of *Punica granatum* L. at 15.0, 10.0, 5.0, and 1.0 g/100 ml, the population of *Bacillus cereus* ATCC 128263 decreased from 6.81 log₁₀ CFU/ml to 4.90, 4.32, 4.74, and 4.79 log₁₀ CFU/ml, respectively, in 9 hours. *Bacillus subtilis* ATCC 6633 at the initial population of 7.48 log₁₀ CFU/ml was reduced by approx. 3.00 log-cycles in 9 hours at the concentration of 15.0, 10.0, 5.0, and 1.0 g/100 ml.

The ethanolic extract of *Punica granatum* L. at the concentration of 15.0 g/100 ml demonstrated the antimicrobial effect against the cells of *Staphylococcus aureus* DSMZ 799. The initial population of *Staphylococcus aureus* DSMZ 799, 7.75 log₁₀ CFU/ml decreased to 3.63 log₁₀ CFU/ml in 3 hours and stayed constant through 9 hours of contact time at the concentration of 15.0 g/100 ml. The non-significant antimicrobial effect occurred when the extract was used at the concentration of 10.0, 5.0 and 1.0 g/100 ml.

Listeria monocytogenes DSMZ 20600 and *Vibrio parahaemolyticus* ATCC 20502 showed the high sensitivity to this extract. The initial population, approx. 8.00 log₁₀ CFU/ml, decreased to undetected level within 1 hour at the concentration of 15.0, 10.0, 5.0, and 1.0 g/100 ml.

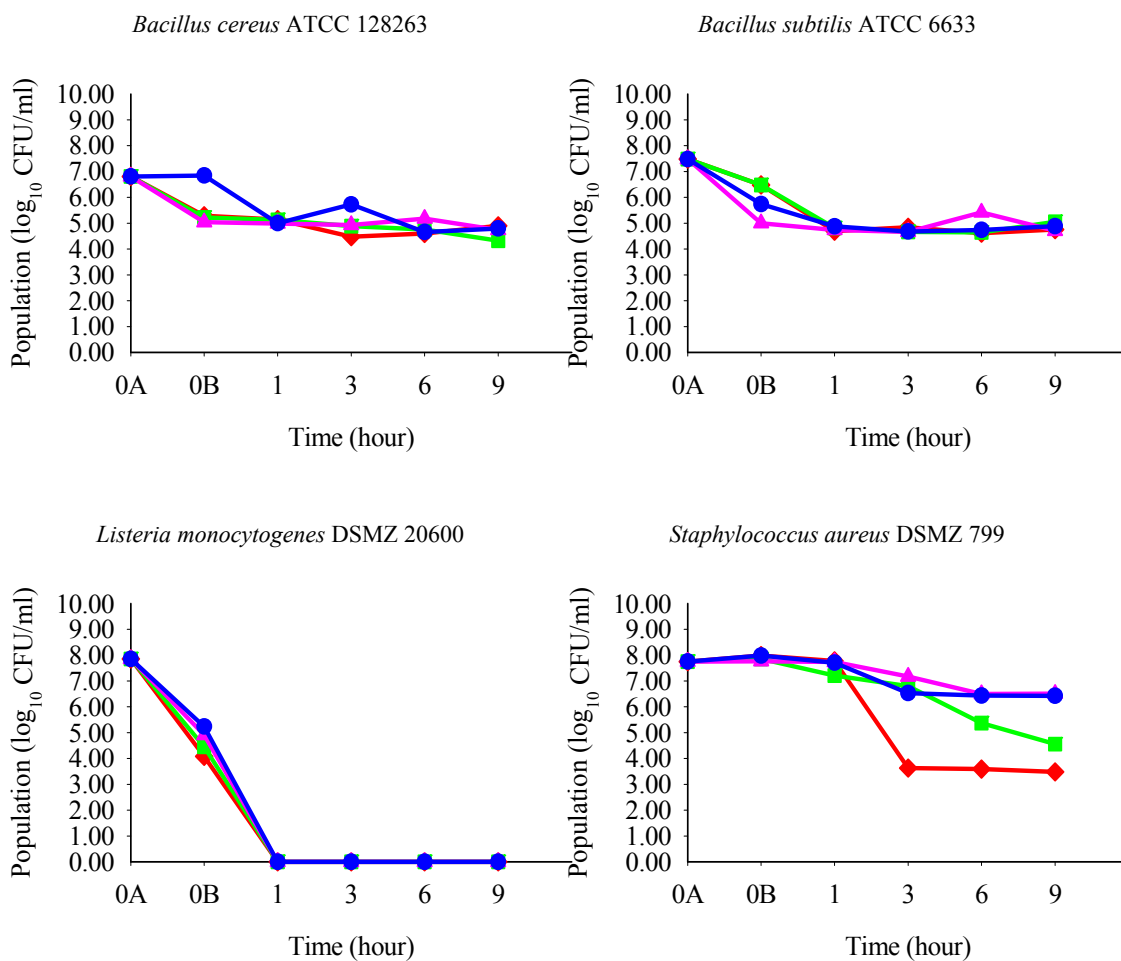


Figure 49 Effect of *Punica granatum* L. ethanolic extract without interfering substances on the number of *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Staphylococcus aureus* DSMZ 799 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

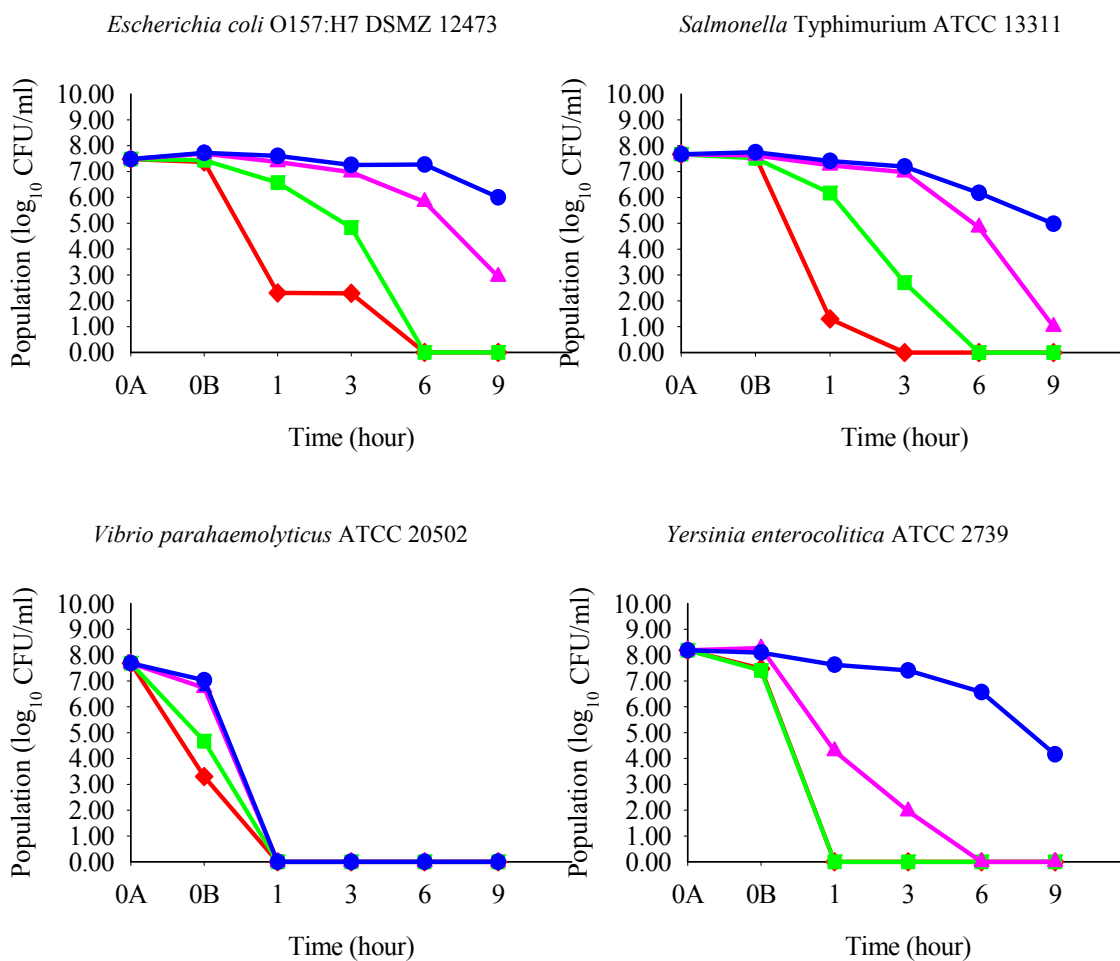


Figure 50 Effect of *Punica granatum* L. ethanolic extract without interfering substances on the number of *Escherichia coli* O157:H7 DMST 12473, *Salmonella* Typhimurium ATCC 13311, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

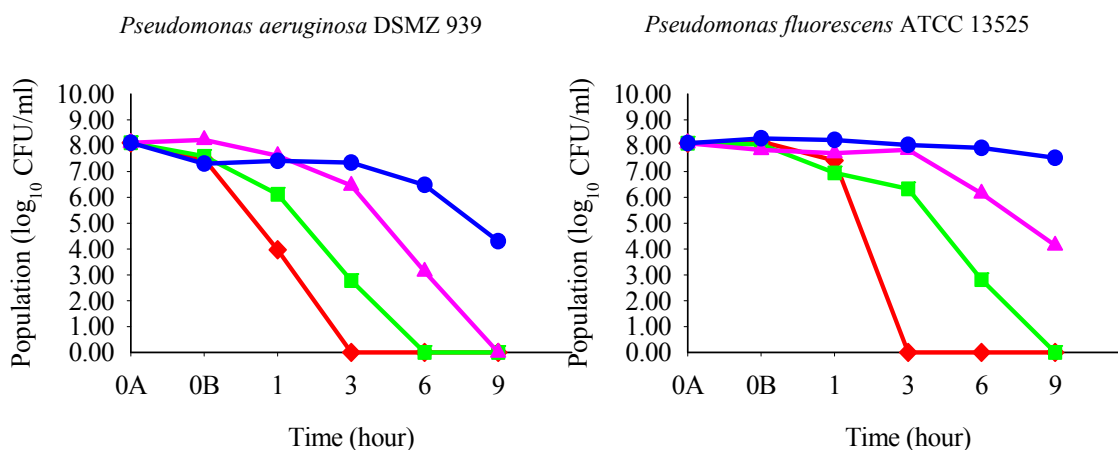


Figure 51 Effect of *Punica granatum* L. ethanolic extract without interfering substances on the number of *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 at room temperature according to the DIN EN 1040 method; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

In addition, at the concentration of 15.0 g/100 ml, the ethanolic extract of *Punica granatum* L. exhibited the bactericidal effect against *Escherichia coli* O157:H7 DMST 12743, *Salmonella* Typhimurium ATCC 1331, *Yersinia enterocolitica* ATCC 2779, *Pseudomonas aeruginosa* DSMZ 939 and *Pseudomonas fluorescens* ATCC 13525 within the 6th, 3rd, 1st, 3rd, and 3rd hour of contact period, respectively. Similarly, the same effect was observed against those test organisms in 6, 6, 1, 3, and 3 hours, respectively at the concentration of 10.0 g/100 ml.

4.3 Determination of the Antimicrobial Activity of Thai Traditional Medicinal Ethanolic Extracts by Studying the Time Killing Analysis with Interfering Substances

While the *in vitro* determination of antimicrobial activity without adding interfering substances can give a good deal information on antimicrobial performance, they cannot necessarily duplicate all the variability which might exist in environmental system. Therefore, once it has been determined that the antimicrobial activity performs well in an *in vitro* without adding interfering substances situation, it should be applied with interfering substances, which represent the environmental system.

According to the previous experiment, ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* L., *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L., presented the good antimicrobial effect against several test organisms. They were selected for the determination of their antimicrobial properties in the presence of interfering substances. Three stable strains of test organisms, including *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 739, were selected as test organisms.

The test method was modified by the method described by DIN EN 1276 (Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas). High level soiling, described by the above methods, was used as interfering substances in the study, containing a mixture of bovine albumin fraction V and yeast extract. The final concentration in test procedure was 10 g/l yeast extract and 10 g/l bovine albumin. The working concentrations were 15.0, 10.0, 5.0, and 1.0 g/100 ml. Survivors were monitored at intervals of 0_A , 0_B , 1, 3, 6, and 24 hours, 0_A was set conditions as initial population and 0_B as contact time within 15 seconds, by withdrawing a sample

4.3.1 Antimicrobial property of *Curcuma zedoaria* (Christm.) Roscoe-ethanolic extract in the presence of interfering substances

In Figure 52, the time kill curve represented the antimicrobial properties of *Curcuma zedoaria* (Christm.) Roscoe-ethanolic extract in the presence of interfering substances against different test organisms was given.

The bacteria test suspension of *Bacillus cereus* ATCC 128263 showed the strong resistance to the ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe in the presences of yeast extract and bovine albumin fraction V. Expected results should decrease the initial population of bacteria suspension by *approx.* 4.00 log-reductions. In the presence of interfering substances the antimicrobial activity decreased, this extract had no antimicrobial activity against the cells of *Bacillus cereus* ATCC 128263 during 24 hours.

The antimicrobial activity of *Curcuma zedoaria* (Christm.) Roscoe-ethanolic extract at the concentration of 15.0 g/100 ml presented the bactericidal effect against the bacteria test suspension of *Staphylococcus aureus* DSMZ 799. The initial population at 8.53 log₁₀ CFU/ml was reduced to 7.24 log₁₀ CFU/ml in 1 hour. At the 3rd hour of contact time, the numbers of survivors were 5.34 log₁₀ CFU/ml and decreased to 3.22 log₁₀ CFU/ml at the 6th hour period of contact. The bacteria test suspension decreased to the undetectable level in 24 hours. At the concentration of 10.0 g/100 ml, the numbers of bacteria was reduced to 7.69 log₁₀ CFU/ml in 6 hours, and then to 5.87 log₁₀ CFU/ml in 24 hours.

For the activity against *Pseudomonas aruginosa* DSMZ 739, the population was reduced from 8.46 log₁₀ CFU/ml to 6.22 log₁₀ CFU/ml in 1 hour at the concentration of 15.0 g/100 ml, and then to 4.75 log₁₀ CFU/ml in 3 hours. At the 24 hours of contact time, the bactericidal effect was detected. At the concentration of 5.0 g/100 ml, the initial population decreased to 5.88 log₁₀ CFU/ml in 6 hours, and the undetectable level was observed in 24 hours. At the lower concentration, the bacteria test

suspension showed survivability in the extract, and less than 1.00 log-reductions were detected after 24 hours.

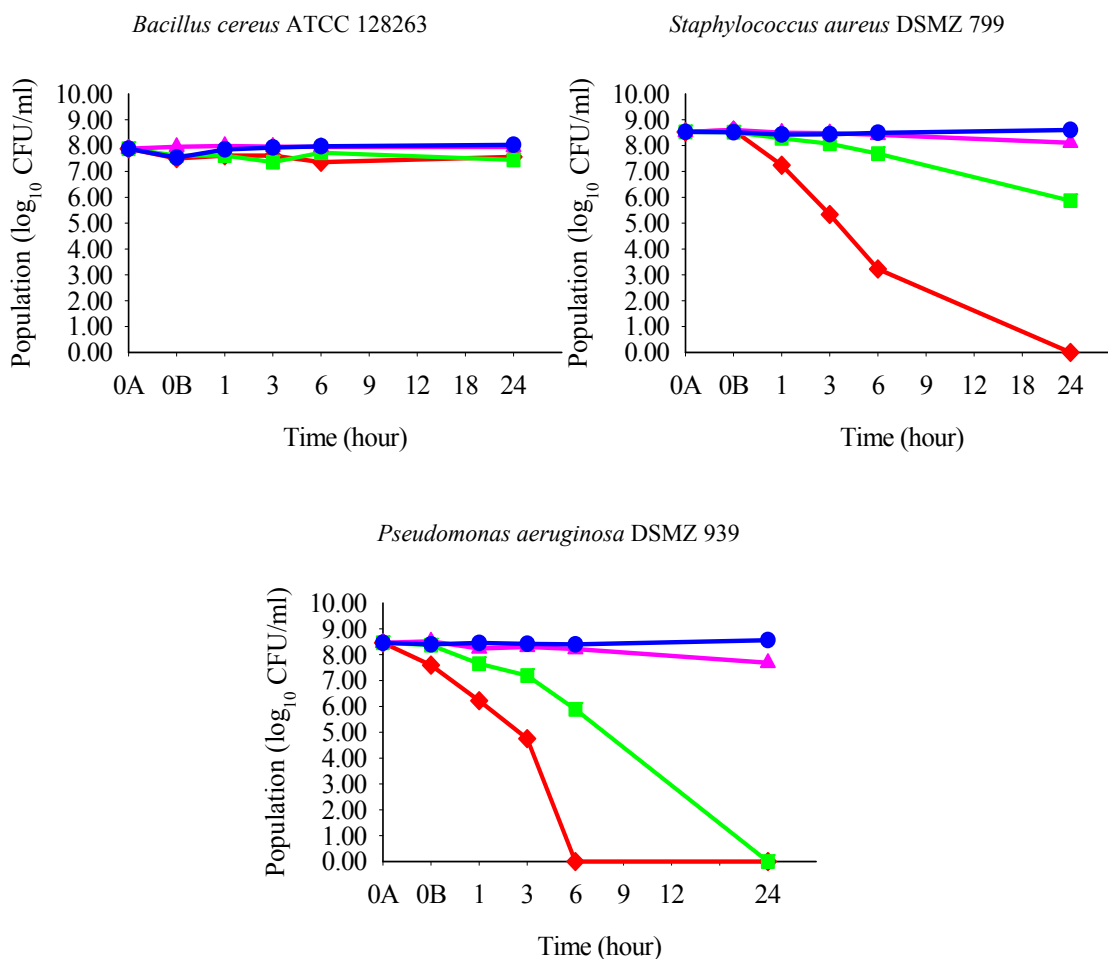


Figure 52 Population of *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 939 after treated with *Curcuma zedoaria* (Christm.) Roscoe ethanolic extract in the presence of interfering substances according to the DIN EN 1276 method at room temperature; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

4.3.2 Antimicrobial property of *Garcinia mangostana* L.-ethanolic extract in the presence of interfering substances

The antimicrobial properties of the ethanolic extract of *Garcinia mangostana* L. in the presence of interfering substances against *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 393 were presented in Figure 53.

The ethanolic extract of *Garcinia mangostana* L. at all concentration showed no antimicrobial effect against bacteria test suspension of *Bacillus cereus* ATCC 128263 in 24 hours. The cells showed survivability property in the extract solution added with interfering substances.

For the results of *Staphylococcus aureus* DSMZ 799, the initial population at 8.53 log₁₀ CFU/ml decreased to 6.20 log₁₀ CFU/ml in 1 hour at the concentration of 15.0 g/100 ml. The abatement was investigated through the period of contact time. The population decreased 3.63 log₁₀ CFU/ml in 3 hours. The undetectable level was observed in 6 hours. At the concentration of 5.0 g/100 ml, the initial population was reduced to 7.64 log₁₀ CFU/ml in 1 hour and to 6.09 and 3.72 log₁₀ CFU/ml in 3 and 6 hours, respectively. The undetectable level was observed at 24 hours. The antimicrobial activity of *Garcinia mangostana* L.-ethanolic extract could not be determined in 24 hours at the concentration of 5.0 and 1.0 g/100 ml.

The *Garcinia mangostana* L.-ethanolic extract at the concentration of 15.0 g/100 ml in the presence of interfering substances presented the bactericidal effect against *Pseudomonas aeruginosa* DSMZ 799. The initial population decreased from 8.46 log₁₀ CFU/ml to 3.21 log₁₀ CFU/ml in 6 hours and to the undetectable level in 24 hours. At the concentration of 10.0 g/100 ml, the initial population was reduced to 7.64 log₁₀ CFU/ml in 6 hours and at the 24th hour contact period, the population was 5.26 log₁₀ CFU/ml. At the lower concentration, 5.0 and 1.0 g/100 ml, the antimicrobial activity could not be detected.

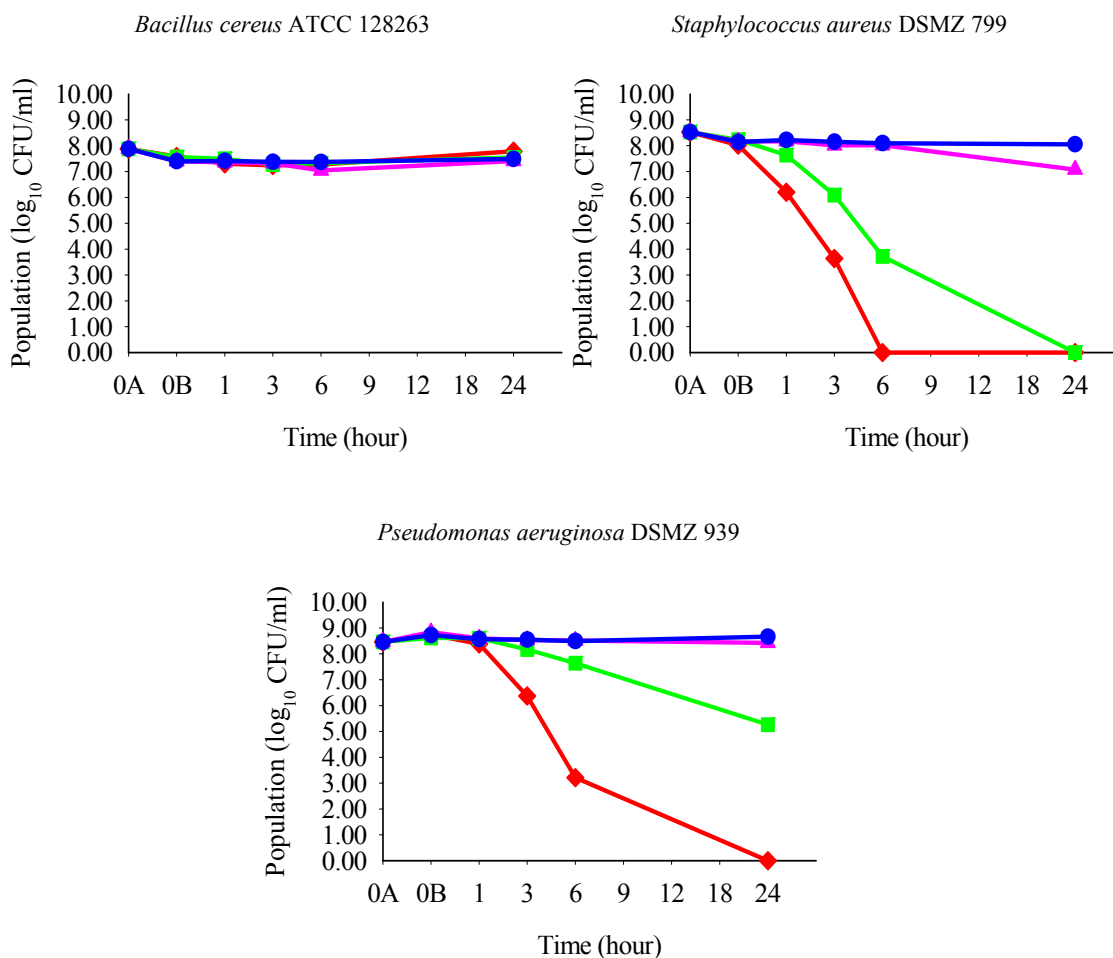


Figure 53 Population of *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 939 after treated with *Garcinia mangostana* L. ethanolic extract in the presence of interfering substances according to the DIN EN 1276 method at room temperature; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

4.3.3 Antimicrobial property of *Hibiscus sabdariffa* L.-ethanolic extract in the presence of interfering substances

In Figure 54, the time kill curve represented the antimicrobial activity of the ethanolic extract of *Hibiscus sabdariffa* L. in the presence of interfering substances against different test organisms was presented.

This extract in the presence of yeast extract and bovine albumin fraction V as interfering substances, showed few antimicrobial properties against the bacteria test suspension of *Bacillus cereus* ATCC 128263. At the concentration of 15.0 and 10.0 g/100 ml, the initial population at 7.83 log₁₀ CFU/ml decreased in 24 hours to 6.75 and 6.82 log₁₀ CFU/ml, respectively. At the lower concentration the antimicrobial activity could not be detected.

The ethanolic extract of *Hibiscus sabdariffa* L. in the presence of interfering substances exhibited the antimicrobial effect against *Staphylococcus aureus* DSMZ 799. The initial population at 8.53 log₁₀ CFU/ml was reduced to 6.56 log₁₀ CFU/ml in 1 hour at the concentration of 15.0 g/100 ml. At the 3rd hour of contact period, the population was 3.73 log₁₀ CFU/ml and decreased to undetectable level in 6 hours. At the concentration of 10.0 g/100 ml the initial population was rapidly reduced from 8.53 log₁₀ CFU/ml to 5.77 log₁₀ CFU/ml in 3 hours and to 2.70 log₁₀ CFU/ml in 6 hours. At the 24th hour of contact period, the undetectable stage was observed. At the concentration of 5.0 g/100 ml, the population was rapidly reduced to undetectable stage between the 6th and the 24th hour of contact period. Around log 1.00 reductions were obtained in 24 hours at the concentration of 1.0 g/100 ml.

For *Pseudomonas aeruginosa* DSMZ 939, this extract demonstrated the good antimicrobial properties. The initial population at 8.46 log₁₀ CFU/ml decreased to undetectable stage in the 1st, 3rd, and 3rd hour of contact period at the concentration of 15.0, 10.0, and 5.0 g/100 ml, respectively. At the concentration of 1.0 g/100 ml, the initial population decreased from 8.46 log₁₀ CFU/ml to 7.95 log₁₀ CFU/ml in 3 hours, and decreased to 6.70 and 2.73 log₁₀ CFU/ml in 6 and 24 hours, respectively.

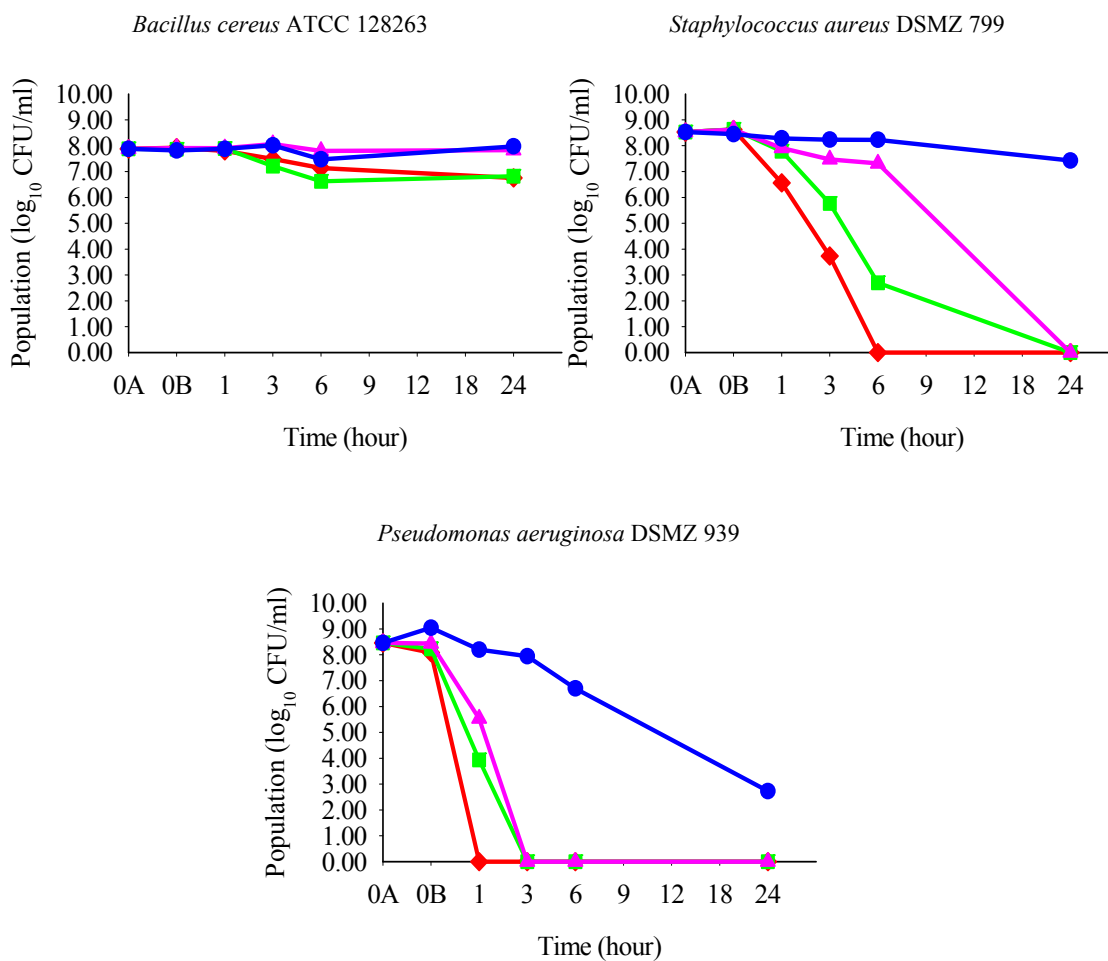


Figure 54 Population of *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 939 after treated with *Hibiscus sabdariffa* L. ethanolic extract in the presence of interfering substances according to the DIN EN 1276 method at room temperature; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

4.3.4 Antimicrobial property of *Piper betle* L.-ethanolic extract in the presence of interfering substances

The antimicrobial properties of the ethanolic extract of *Piper betle* L. in the presence of interfering substances, the mixture of yeast extract and bovine albumin fraction V, against *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 393 were presented in Figure 55.

The cells of *Bacillus cereus* ATCC 128263 exhibited the strong resistance to the ethanolic extract of *Piper betle* L. in the presence of interfering substances. The bacterial test suspension contacted with all concentration presented the survivability against this extract in 24 hours.

For the results of *Staphylococcus aureus* DSMZ 799, the ethanolic extract of *Piper betle* L. in the presence of yeast extract and bovine albumin fraction V as the interfering substances showed a good antimicrobial activity against this test organism. At the concentration of 15.0 g/100 ml, the initial population at 8.53 log₁₀ CFU/ml was destroyed to the undetectable stage within 15 seconds. At the lower concentration, 10.0 g/100 ml, the population was rapidly decreased to 5.21 log₁₀ CFU/ml within 15 seconds, and was reduced to undetectable stage in 1 hour. At the concentration of 5.0 g/100 ml, the population decreased rapidly to 4.37 log₁₀ CFU/ml in 3 hours and to undetectable level at the 3rd hour of contact period. At the lowest concentration, 1.0 g/100 ml, the measurable antimicrobial activity was log 1.41 reduction in 24 hours.

This extract in the presence of interfering substances also showed the good antimicrobial property against *Pseudomonas aeruginosa* DSMZ 799. At the concentration of 15.0 and 10.0 g/100 ml, the numbers of bacteria were reduced from 8.46 log₁₀ CFU/ml to undetectable level within seconds. At the lower concentration, 5.0 g/100 ml, the number of bacteria test suspension decreased from 8.46 to 6.42 log₁₀ CFU/ml within 15 seconds and to the undetectable level in 1 hour. At the lowest test concentration, 1.0 g/100 ml, the initial population was reduced by log 1.66 and 4.56 reductions in 6 and 24 hours, respectively.

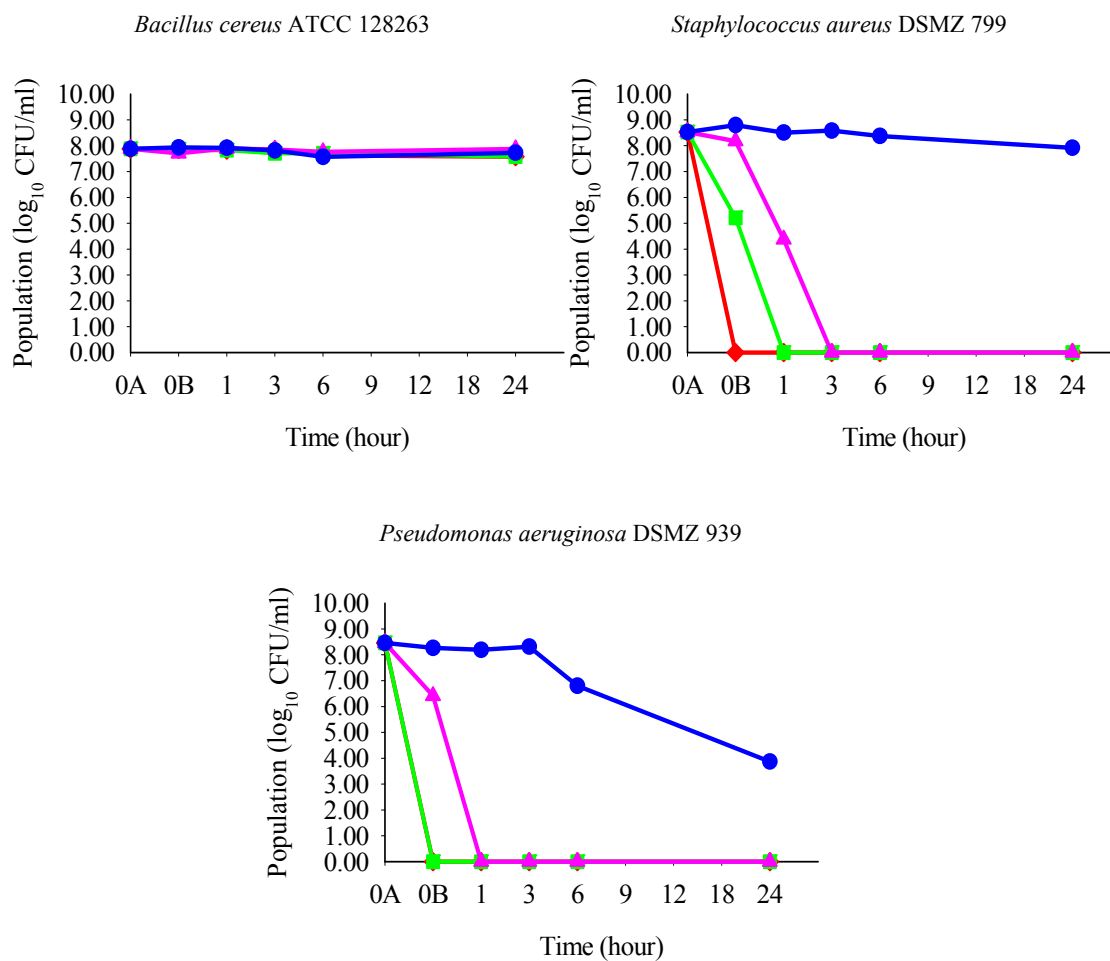


Figure 55 Population of *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 939 after treated with *Piper betle* L. ethanolic extract in the presence of interfering substances according to the DIN EN 1276 method at room temperature; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

4.3.5 Antimicrobial property of *Punica granatum* L.-ethanolic extract in the presence of interfering substances

In Figure 56, the time kill curve represented the antimicrobial activity of the ethanolic extract of *Punica granatum* L. in the presence of interfering substances, yeast extract and bovine albumin fraction V, against different test organisms was presented.

The ethanolic extract of *Punica granatum* L. at all concentration in the presence of interfering substances had no ability to destroy *Bacillus cereus* ATCC 128263 at the initial population, 7.88 log₁₀ CFU/ml. The final population survived in the extract was 7.50, 7.44, 7.38, and 7.39 Log₁₀ CFU/ml at the 24th hour of contact period.

This extract also presented no capability to destroy *Staphylococcus aureus* DSMZ 799 with the exception at the highest test concentration, 15.0 g/100 ml. The initial population at 8.53 log₁₀ CFU/ml was reduced to 5.60 log₁₀ CFU/ml in 24 hours. The final populations survived in the other concentrations were 7.55, 7.55, and 8.06 log₁₀ CFU/ml at the concentration of 10.0, 5.0, and 1.0 g/100 ml, respectively.

For the Gram-negative *Pseudomonas aeruginosa* DSMZ 939, the ethanolic extract of *Punica granatum* L. presented the antimicrobial activity at high concentration. At the concentration of 15.0 g/100 ml, the initial population at 8.46 log₁₀ CFU/ml decreased to 6.67 log₁₀ CFU/ml in 1 hour and to 2.75 log₁₀ CFU/ml in 3 hours. The bactericidal effect occurred in the 6th hour of contact period, the bacteria were completely destroyed to undetectable stage at this period of contraction. At the concentration of 10.0 g/100 ml, the numbers of bacteria were slightly decreased to 7.64 log₁₀ CFU/ml in 3 hours, and then to 5.19 log₁₀ CFU/ml in 6 hours. The abatement to the undetectable level was investigated in 24 hours. At the concentration of 5.0 g/100 ml, the initial population, 8.46 log₁₀ CFU/ml, decreased to 6.31 log₁₀ CFU/ml in 24 hours. At the lowest test concentration, 1.0 g/100 ml, the antimicrobial activity could not be determined.

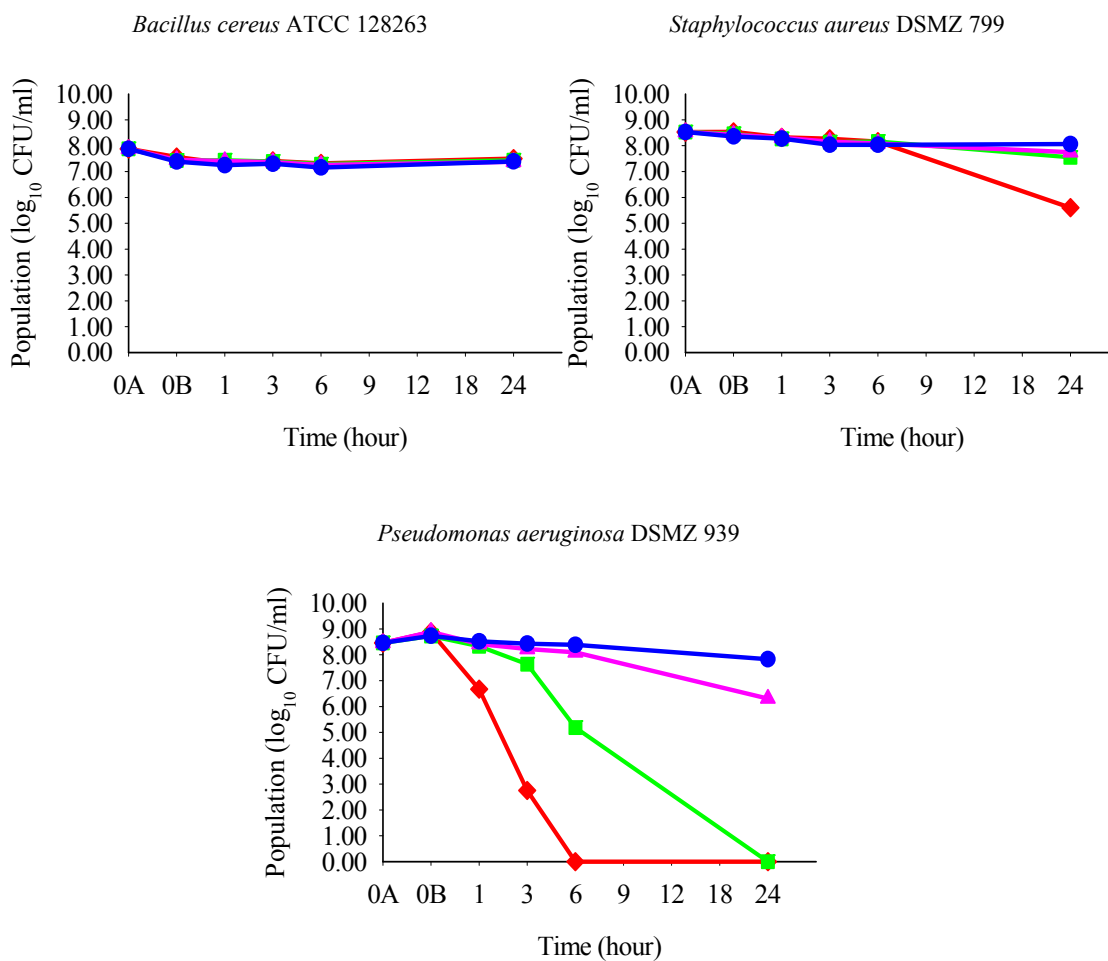


Figure 56 Population of *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 939 after treated with *Punica granatum* L. ethanolic extract in the presence of interfering substances according to the DIN EN 1276 method at room temperature; 15.0 g/100 ml (♦), 10.0 g/100 ml (■), 5.0 g/100 ml (▲), and 1.0 g/100 ml (●) (Values represent mean of 3 independent trials)

4.3.6 The effect of interfering substances on the growth of test organisms

All test organisms, *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 939 grew well in the solution of interfering substance, 10g/L yeast extract and 10g/L bovine albumin fraction V. Interfering substances exhibited no effect against all test organisms and had capability to maintain the numbers of bacteria suspension in the solution (Figure 57).

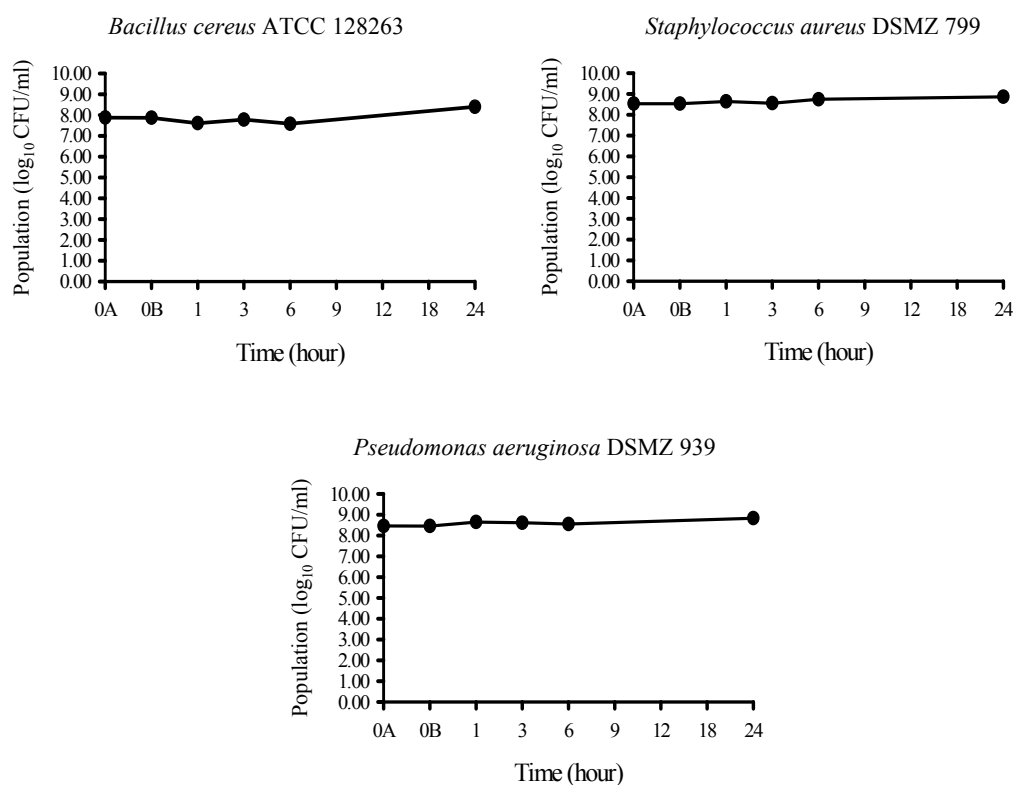


Figure 57 The population growth of *Bacillus cereus* ATCC 128263, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 939 in interfering substances solution at room temperature (Values represent mean of 3 independent trials)

4.4 Application Study: Antimicrobial Activity of the Artificial Coated Surface

The ethanolic extract of *Piper betle* L. demonstrated an excellent antimicrobial activity against several strains of test organisms. It provided low MBC-values and the antimicrobial activity occurred in short contact time. Although the presence of interfering substances occurred in the environment, this extract still provided the good antimicrobial effect. According to this reasons, the ethanolic extract of *Piper betle* L. was selected to the study of antimicrobial property by coating the extract on the surface.

The ethanolic extract of *Piper betle* L. at different concentrations were coated on the sterile metal coin, dried and use as the artificial antimicrobial material surface. The simulated test surface was the metal surface placed with the mixture of cells suspension of test organisms and interfering substances, kept until dry under the flowed air condition to produce the biofilm phase. The test organisms, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Escherichia coli* O157:H7 DMST 12473, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* Typhimurium ATCC 13311, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20500, and *Yersinia enterocolitica* ATCC 2779 were selected. The stimulated test surface was contacted with the artificial antimicrobial material surface. The numbers of survivors were determined at 0, 1, 6, 24, and 48 hours.

Bacillus cereus showed survivability on the metal surface throughout the contact period of 24 hours, against the artificial metal surface coated with sterile distilled water (control). The numbers of survivors of *Bacillus cereus* decreased from 5.76 log₁₀ CFU/ml to 3.88 log₁₀ CFU/ml after contact with the antimicrobial coated surface coated with the extract at the concentration of 30.0 g/100 ml in 1 hour, and the staying in constant level was observed during 48 hours. For the lower level of concentration, 15.0 and 10.0 g/100 ml, the population was reduced by ca. log 1.00 reductions in 48 hours (Figure 58).

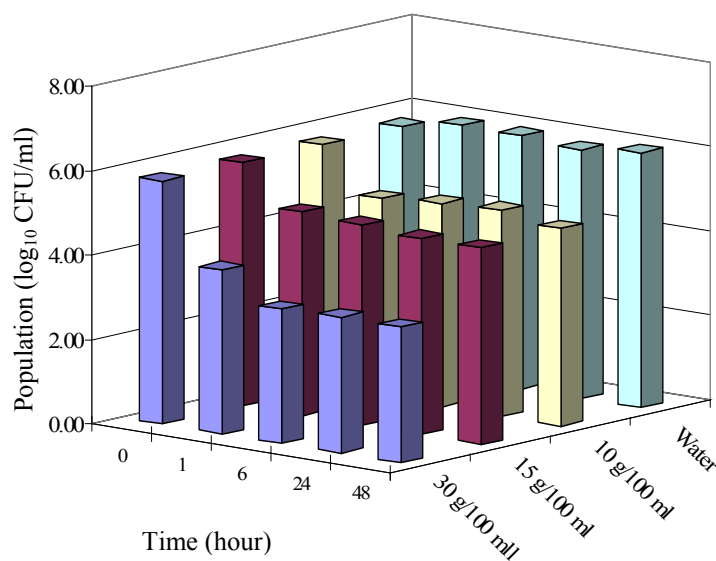


Figure 58 Survivors population (\log_{10} CFU/ml) of *Bacillus cereus* ATCC 128263 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

The initial population of *Bacillus subtilis* at 6.54 \log_{10} CFU/ml survived on the surface after contacted with control coated surface. With the coated surface at the concentration of 30.0 g/100 ml, the initial population decreased from 6.54 \log_{10} CFU/ml to 2.04 \log_{10} CFU/ml in 6 hours. At the 24th hour of contact period, the numbers of bacteria were 1.29 \log_{10} CFU/ml and decreased to 0.43 \log_{10} CFU/ml in 48 hours. The initial population was reduced to 1.66 and 2.50 \log_{10} CFU/ml at the concentration of coated surface at 15.0 and 10.0 g/100 ml, respectively, in 48 hours (Figure 59).

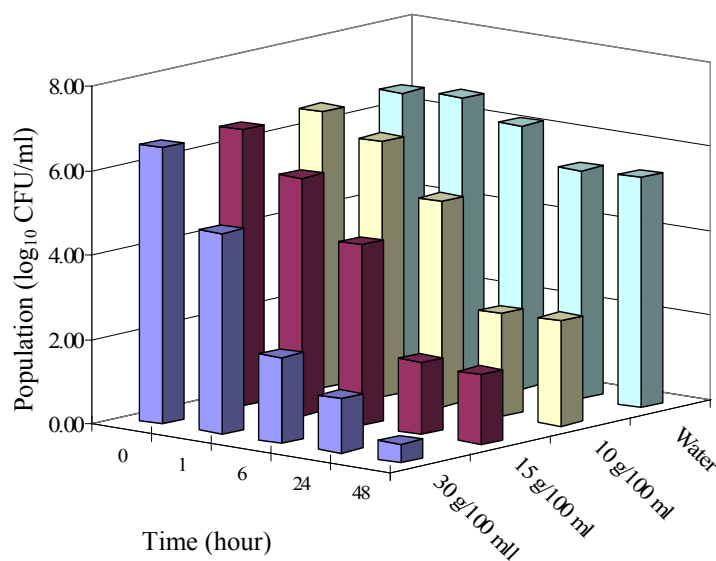


Figure 59 Survivors population (log₁₀ CFU/ml) of *Bacillus subtilis* ATCC 6633 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

As the results of antimicrobial activity of *Piper betle* L.-ethanolic extract coated metal against *Escherichia coli* O157:H7, the survived population was stated in Figure 60. *Escherichia coli* O157:H7 survived on the surface after contact with uncoated extract metal surface. The population contaminated on the surface, however, was eliminated from 7.00 log₁₀ CFU/ml to 4.59 log₁₀ CFU/ml in 1 hour after the coated metal at 30.0 g/100 ml in concentration of the extract was applied against the contaminated surface. Along with the same procedure, the population decreased from the initial to 3.69 and 3.81 at the concentration of 15.0 and 10.0 g/100 ml, respectively,

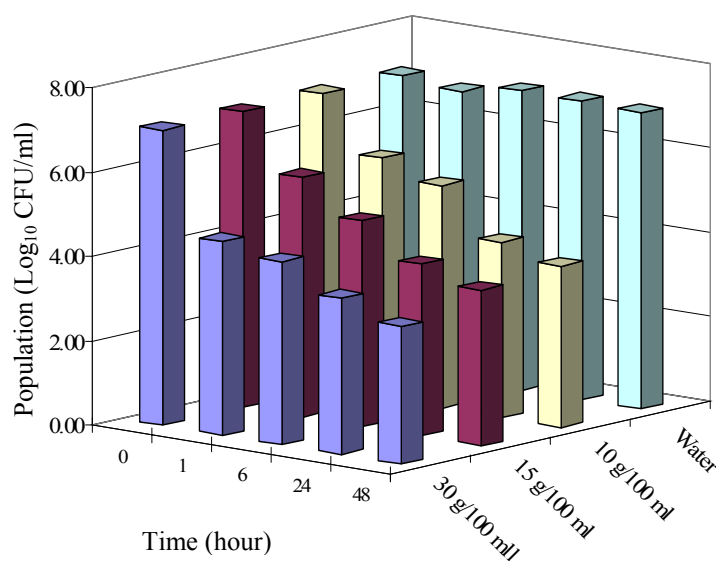


Figure 60 Survivors population (\log_{10} CFU/ml) of *Escherichia coli* O157:H7 DMST 12743 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

Listeria monocytogenes demonstrated around $\log 2.00$ reduction on the metal surface contacted with control metal surface coated with sterile distilled water in 48 hours. At the concentration of extract on the surface at 10.0 g/100 ml, the initial population decreased in the same amount compared with control coated surface. It indicated that no antimicrobial activity measured by the coated surface at this concentration. At the concentration of coated extract at 15.0 g/100 ml, the initial population was reduced from 7.40 \log_{10} CFU/ml to 4.63 \log_{10} CFU/ml in 48 hours. The higher concentration of the extract coated on the metal surface, the higher antimicrobial could be determined. At the concentration of 30.0 g/100 ml, the initial population decreased to 3.73 \log_{10} CFU/ml in 24 hours and to 0.33 \log_{10} CFU/ml in 48 hours (Figure 61).

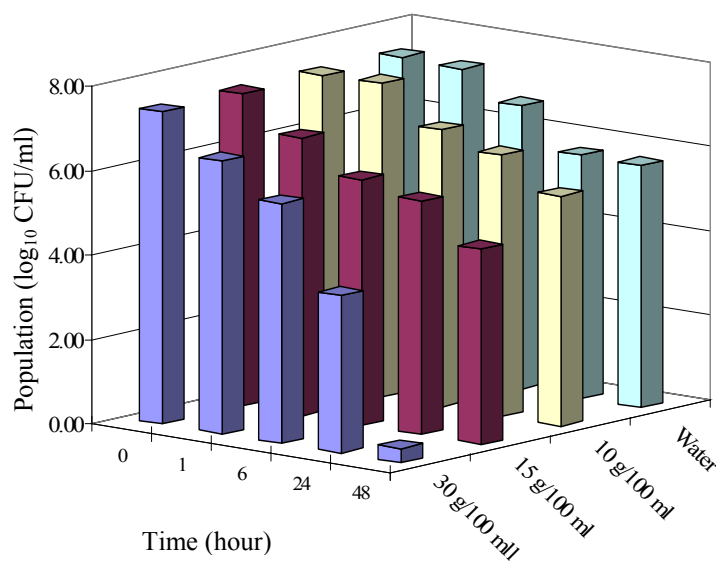


Figure 61 Survivors population (\log_{10} CFU/ml) of *Listeria monocytogenes* DSMZ 20600 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

In Figure 62, the antimicrobial property of *Piper betle* L.-ethanolic extract coated surface against Gram-negative food related *Pseudomonas aeruginosa* was presented. As expected, the bacteria showed the survivability on the metal surface during 48 hours. However, the population decreased after the antimicrobial coated metal surface was applied. At the concentration of coating at 10.0 g/100 ml, the population was reduced from 7.50 \log_{10} CFU/ml to 6.37 \log_{10} CFU/ml in 6 hours and to the undetectable level in 24 hours. As the same procedure, the cells population contacted with the coated metal surface at the concentration of coated extract of 15.0 g/100 ml, the undetectable was observed in 24 hours. At the highest concentration, the population decreased rapidly to 2.01 \log_{10} CFU/ml in 6 hours, to the undetectable level in 24 hours.

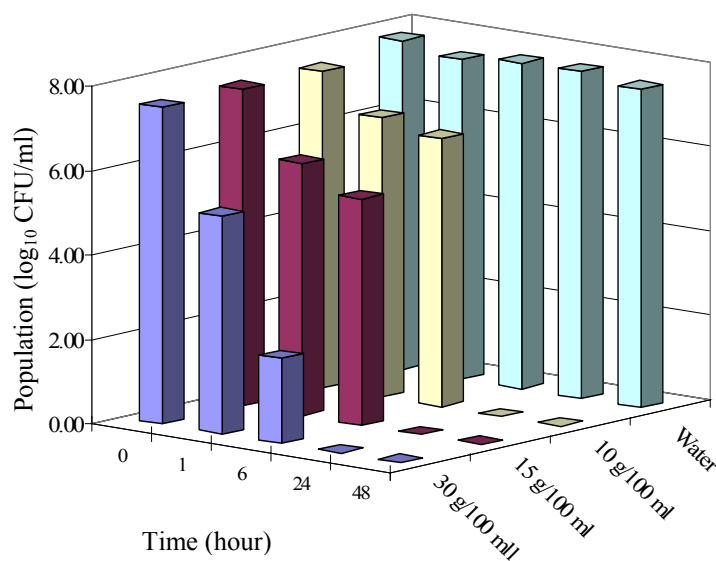


Figure 62 Survivors population (\log_{10} CFU/ml) of *Pseudomonas aeruginosa* DSMZ 939 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

Pseudomonas fluorescens exhibited approx. \log_{10} 2.00 reductions on the metal surface contact with control surface; surface coated with sterile distilled water. Although the abatement was detected at the control stage, the artificial coated surface at the concentration of coated extract of 10.0 g/100 ml exhibited more levels of reduction. After the contact of the inoculated surface with the artificial antimicrobial surface, the initial population of *Pseudomonas fluorescens* decreased to 4.31 \log_{10} CFU/ml in 24 hours and to the undetectable level in 48 hours. In comparison, at the concentration of 30.0 and 15.0 g/100 ml, the undetectable level occurred rapidly. The undetectable levels were occurred in 6 hours (Figure 63).

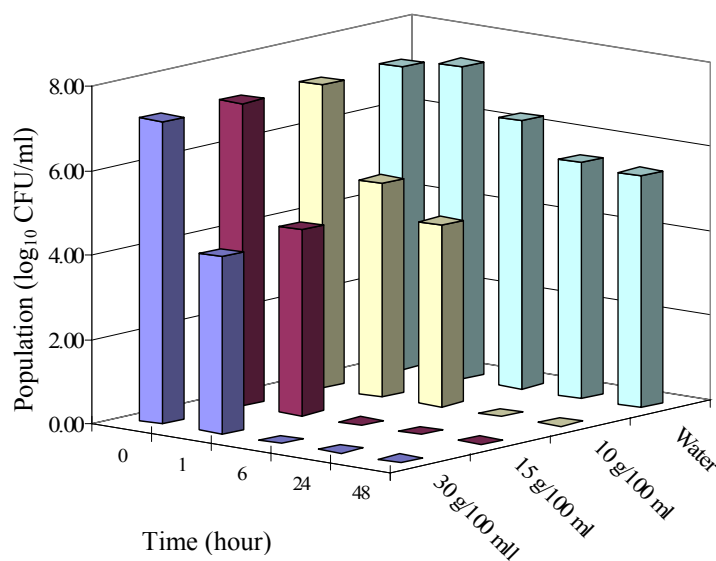


Figure 63 Survivors population (\log_{10} CFU/ml) of *Pseudomonas fluorescens* ATCC 13525 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

In Figure 64, the antimicrobial property of coated metal surface with the ethanolic extract of *Piper betle* L. against *Salmonella* Typhimurium 1 was presented. The bacterial survived on the metal surface through 48 hours. For the results of coated surface at the concentration of coated extract of 10.0 g/100 ml, the initial population, 7.14 \log_{10} CFU/ml, was reduced by log 2.00 reductions in 48 hours. At the concentration of coated extract at 15.0 g/100 ml, the population was reduced by 3.0 \log_{10} CFU/ml in 48 hours. At the highest coated concentration level, 30.0 g/100 ml, the numbers of cells decreased from 7.14 \log_{10} CFU/ml to 4.92 \log_{10} CFU/ml in 24 hours and to 4.38 \log_{10} CFU/ml in 48 hours.

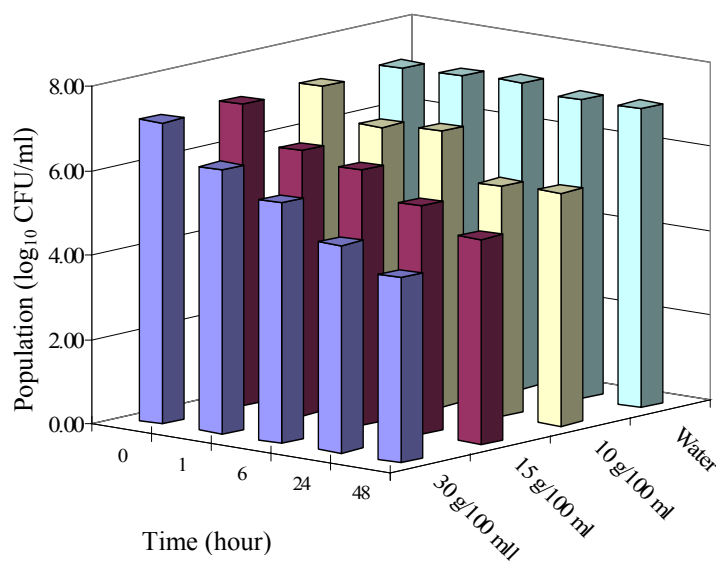


Figure 64 Survivors population (log₁₀ CFU/ml) of *Salmonella* Typhimurium ATCC 13311 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

Staphylococcus aureus showed more resistance to the artificial antimicrobial coated surface. The bacteria survived well on the metal surface during 48 hours in the control condition. Around 1.00 log-reductions was observed in 48 hours after the contaminated surface contact directly with the artificial antimicrobial metal surface coated with the ethanolic extract of *Piper betle* L. at the concentration of 10.0 and 15.0 g/100 ml. At the highest coated concentration, 30.0 g/100 ml, *Staphylococcus aureus* exhibited the strong resistance. The initial population at 7.54 log₁₀ CFU/ml was slowly reduced to 5.91 log₁₀ CFU/ml in 6 hours. The survivor population at the 24th hour of contact period was 5.85 log₁₀ CFU/ml and 5.13 Log₁₀ CFU/ml at the 48th hour of contact period (Figure 65).

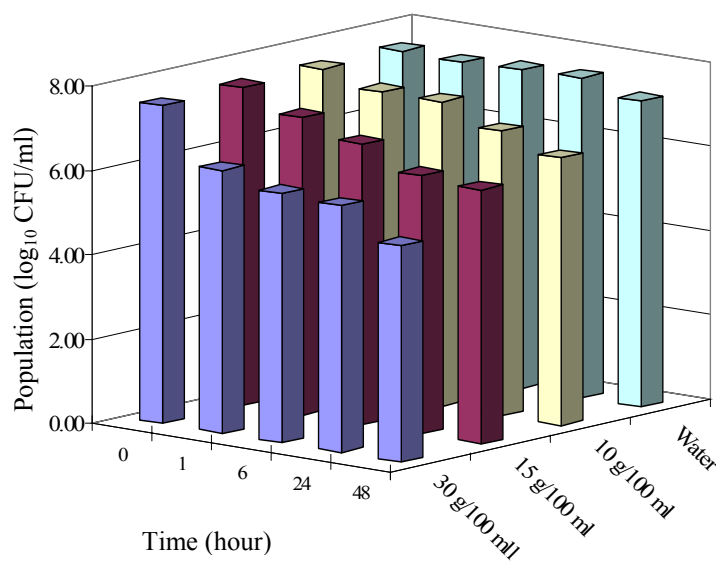


Figure 65 Survivors population (\log_{10} CFU/ml) of *Staphylococcus aureus* DSMZ 799 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

Vibrio parahaemolyticus showed the less survivability on the metal surface. In the control condition, the numbers of bacteria were decreased from 5.39 \log_{10} CFU/ml to 3.64 \log_{10} CFU/ml in 48 hours. However, the population reduction related to the survivability of this organism, the population to the undetectable level was detected at all concentration coated on the metal surface. At the concentration of extract coated on metal surface at 10.0 g/100 ml, the initial population at 5.39 \log_{10} CFU/ml was reduced to 2.46 and 1.42 \log_{10} CFU/ml at the 1st and the 6th hour of contact period. The undetectable level occurred in 24 hours. At the concentration of extract on metal surface at 15.0 and 30.0 g/100 ml, the undetectable level occurred in 1 hour (Figure 66).

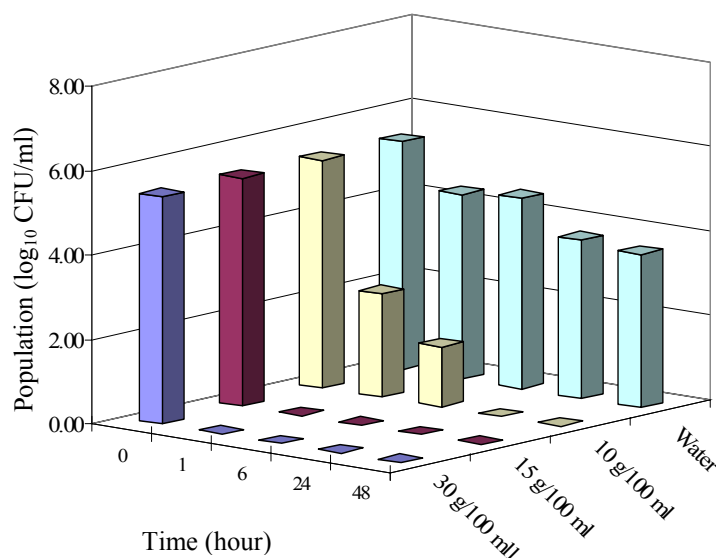


Figure 66 Survivors population (\log_{10} CFU/ml) of *Vibrio parahaemolyticus* ATCC 20502 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

Yesinia enterocolitica presented the good survivability on the metal surface in the control condition (Figure 67). The artificial coated metal surface coated with ethanolic extract of *Piper betle* L. at the concentration of 10.0 g/100 ml presented the antimicrobial activity. The initial population decreased from 6.77 \log_{10} CFU/ml to 5.51 \log_{10} CFU/ml in 1 hour. The population decreased to 4.86, 4.49, and 3.64 \log_{10} CFU/ml in 6, 12, and 48 hours, respectively. At the concentration of coated metal surface at 15.0 g/100 ml, the population was reduced to 4.74 \log_{10} CFU/ml in 1 hour, and the to 4.09 and 0.39 \log_{10} CFU/ml in 6 and 24 hours, respectively. The undetectable was detected in 48 hours. At the highest concentration, 30.0 g/100 ml, the population decreased to undetectable level within 1 hour.

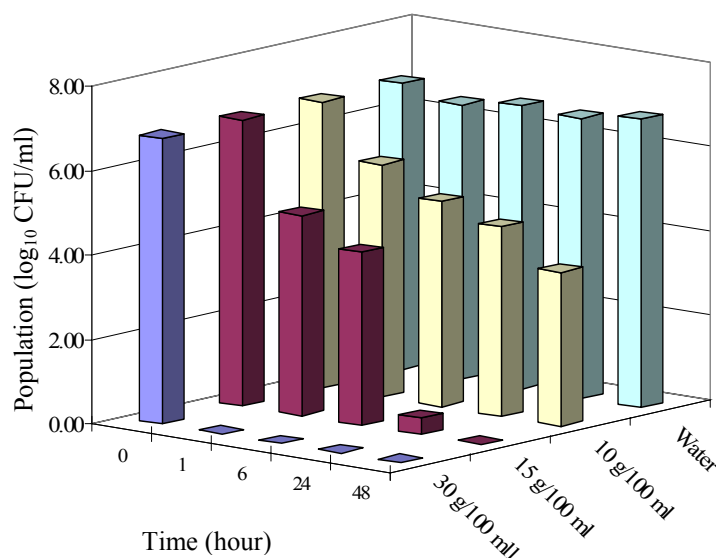


Figure 67 Survivors population (log₁₀ CFU/ml) of *Yersinia enterocolitica* ATCC 2739 on the metallic surface contacted with ethanolic extract of *Piper betle* L. coated on metal surface. (Values represent mean of 3 independent trials)

4.5 Application Study: Antimicrobial Activity of Thai Traditional Medicinal Plant Ethanolic Extract-Incorporated Alginate-Tapioca Starch based edible films

All ethanolic extracts of Thai traditional medicinal plants, presented the outstanding antimicrobial properties when those were compared among the extract procedure, and all were selected to study the antimicrobial properties of incorporated Alginate-Tapioca starch based edible films against different test organisms. The antimicrobial activity test of edible films was carried out using the agar diffusion method and the total count of survivors. The antimicrobial activity determined by agar diffusion method was used to measure the “zone of inhibition” of the film discs. The area of the whole zone was calculated and then subtracted from the film disk area, and the difference area was “zone of inhibition”. The zone of inhibition was measured based on the clear zone surrounding a circular film disk. If there is no clear zone, it is assumed that there is no inhibition. The contact area was used to evaluate growth

inhibition underneath the film disk in direct contact with target microorganisms in the agar. The total plate count of survivors was tested to quantify the killing effect of edible films.

The test organisms including, *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Escherichia coli* O157:H7 DMST 12743, *Escherichia coli* DSMZ 632, *Escherichia coli* ATCC 8739, *Listeria monocytogenes* 101, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas aeruginosa* DSMZ 939, *Salmonella* Typhimurium DSMZ 5569, *Salmonella* Typhimurium ATCC 13311, *Staphylococcus aureus* ATCC 13565, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739, were selected to determine the antimicrobial activity of edible film incorporated with different concentration of ethanolic extract of those Thai traditional medicinal plants by determination of zone of inhibition and contact area underneath of the film. The concentrations of ethanolic extract incorporated Alginate-Tapioca starch based edible film were 20.0, 10.0, 5.0, and 1.0 % (v/v), sterile distilled water incorporated edible film was used as control film

Ten of sixteen test organisms including, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Escherichia coli* O157:H7 DMST 12743, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas aeruginosa* DSMZ 939, *Salmonella* Typhimurium ATCC 13311, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2739 were selected as the test organisms for the determination of antimicrobial activity by total plate count of survivors. The test concentration was 20.0 % (v/v), the contact period was 24 hours.

4.5.1 Antimicrobial activity of *Andrographis paniculata* (Burm.f.) Wall. ex Nees-ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.1.1 The zone of inhibition

Antimicrobial activity of the Alginate-Tapioca starch based edible film incorporated with the ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees was expressed in the terms of inhibition zone was presented in Figure 68. The edible film incorporated with this extract at concentration 1.0, 5.0, and 10. % (v/v) had no antimicrobial property against all test organisms. The inhibition was observed in Alginate-Tapioca starch based edible film containing 20.0% (v/v) ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees. The maximum zone of inhibition was 0.84 cm determined against *Listeria monocytogenes* 101. Moreover, the Alginate-Tapioca starch based edible film incorporated with ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees exhibited the antimicrobial property against several test organisms including, *Bacillus cereus* ATCC 1176, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, and *Yersinia enterocolitica* ATCC 2779 with the determined zone of inhibition at 0.42, 0.23, 0.53, 0.47, 0.53, and 0.73 cm, respectively. The other test organisms presented the resistance to the Alginate-Tapioca starch based edible film incorporated with *Andrographis paniculata* (Burm.f.) Wall. ex Nees.

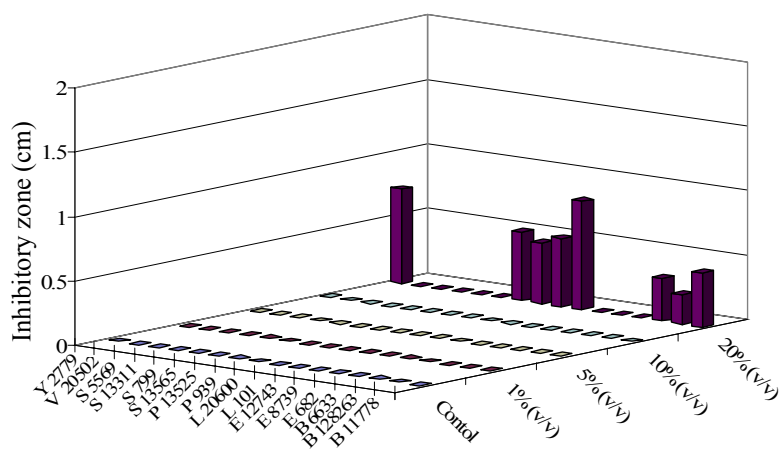


Figure 68 Antibacterial activity of *Andrographis paniculata* (Burm.f.) Wall. ex Nees ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

Table 4 Antibacterial activity of *Andrographis paniculata* Burm f. Nees. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	-	-	-	-	+
<i>Bacillus cereus</i> ATCC 11778	-	-	-	-	+
<i>Bacillus subtilis</i> ATCC 6633	-	-	-	-	+
<i>Escherichia coli</i> ATCC 632	-	-	-	+	+
<i>Escherichia coli</i> ATCC 8739	-	-	-	+	+
<i>Escherichia coli</i> O157:H7 DMST 12743	-	-	-	+	+
<i>Listeria monocytogenes</i> 101	-	-	+	+	+
<i>Listeria monocytogenes</i> DSMZ 20600	-	-	+	+	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	-	-	+	+	+
<i>Pseudomonas fluorescens</i> ATCC 13525	-	-	+	+	+
<i>Salmonella</i> Typhimurium ATCC 13311	-	-	-	-	+
<i>Salmonella</i> Typhimurium DSMZ 5569	-	-	-	-	+

Table 4 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanollic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	–	–	+	+
<i>Staphylococcus aureus</i> DSMZ 799	–	–	–	+	+
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	–	–	–	+
<i>Yersinia enterocolitica</i> ATCC 2779	–	–	+	+	+

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

4.5.1.2 Contact area underneath the film

The Alginate-Tapioca starch based edible film incorporated with ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees demonstrated an inhibitory effect determined by the inhibitory underneath of the film or contact area. At the incorporated concentration of 20.0 % (v/v) of the extract, the growth of all test organisms was inhibited. At 10.0 % (v/v) incorporated concentration, the growth at the contact area of *Listeria monocytogenes* DSMZ 20600, *Listeria monocytogenes* 101, *Staphylococcus aureus* DSMZ 799, *Staphylococcus aureus* ATCC 13565, *Escherichia coli* DSMZ 632, *Escherichia coli* ATCC 8739, *Escherichia coli* O157:H7 DMST 12743, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, and *Yersinia enterocolitica* ATCC 2779 was inhibited. At 5.0 % (v/v), the growth of *Listeria monocytogenes* DSMZ 20600, *Listeria monocytogenes* 101, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, and *Yersinia enterocolitica* ATCC 2779 was inhibited (Table 4).

4.5.1.3 Total count of survivors

Alginate-Tapioca starch based edible film incorporated with 20.0 % (v/v) ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees was used to determine the antimicrobial property by determining the total of survivors. The high antimicrobial activity was observed against *Bacillus subtilis* ATCC 6633 and *Pseudomonas fluorescens* ATCC 13525, the initial population decreased by 4.34 and 4.04 log-reductions, respectively. The low inhibition effect (< 2.00 log-reductions) was observed against *Escherichia coli* O157:H7 DMST 12743, *Salmonella* Typhimurium ATCC 13311, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779 with 1.69, 1.05, 0.59, 1.44, and 1.86 log-reductions, respectively (Figure 69).

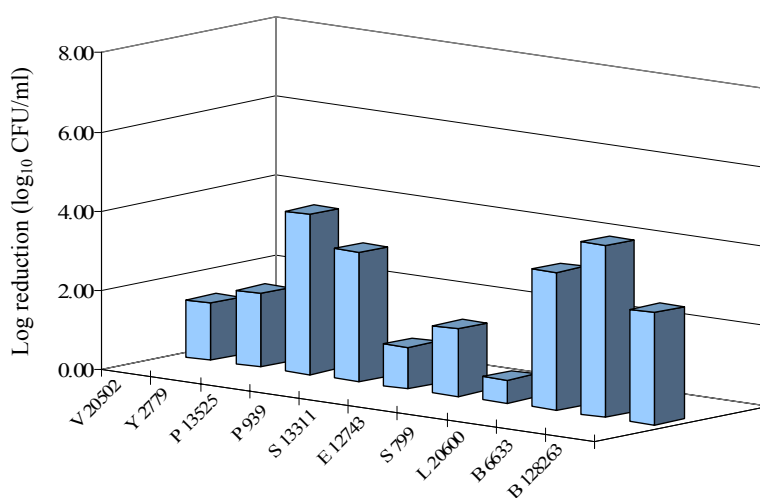


Figure 69 Population decrease (log₁₀ CFU/ml) of test organisms (V 20502: *Vibrio parahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) after contact with an with Alginate-Tapioca starch based edible film containing 20% (v/v) *Andrographis paniculata* (Burm.f.) Wall. ex Nees-ethanolic extract after 24 hours of contact time (Values represent mean of 3 independent trials)

4.5.2 Antimicrobial activity of *Curcuma zedoaria* (Christm.) Roscoe -ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.2.1 The zone of inhibition

Antimicrobial activity of the Alginate-Tapioca starch based edible film incorporated with the ethanolic extract of *Curma zedoaria* (Christm.) Roscoe was interpreted in the terms of inhibition zone and was presented in Figure 70. The edible film incorporated

with this extract at all concentrations presented no zone of inhibition against all test organisms.

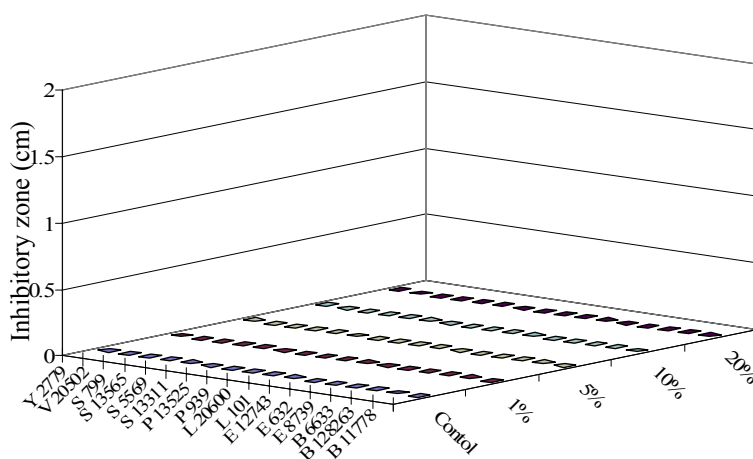


Figure 70 Antibacterial activity of *Curcuma zedoaria* (Christm.) Roscoe ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

Table 5 Antibacterial activity of *Curcuma zedoaria* (Christm.) Roscoe ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	–	–	+	+	+
<i>Bacillus cereus</i> ATTC 11778	–	–	+	+	+
<i>Bacillus subtilis</i> ATCC 6633	–	–	+	+	+
<i>Escherichia coli</i> ATCC 632	–	–	–	–	+
<i>Escherichia coli</i> ATCC 8739	–	–	–	–	+
<i>Escherichia coli</i> O157:H7 DMST 12743	–	–	–	–	+
<i>Listeria monocytogenes</i> 101	–	–	+	+	+
<i>Listeria monocytogenes</i> DSMZ 20600	–	–	+	+	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	–	–	–	+	+
<i>Pseudomonas fluorescens</i> ATCC 13525	–	–	–	+	+
<i>Salmonella</i> Typhimurium ATCC 13311	–	–	–	–	+
<i>Salmonella</i> Typhimurium DSMZ 5569	–	–	–	–	+

Table 5 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanollic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	–	–	–	+
<i>Staphylococcus aureus</i> DSMZ 799	–	–	–	–	+
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	–	+	+	+
<i>Yersinia enterocolitica</i> ATCC 2779	–	–	–	+	+

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

4.5.2.2 Contact area underneath the film

As in Table 5, the Alginate-Tapioca starch based edible film incorporated with the ethanolic extract of *Curma zedoaria* (Christm.) Roscoe expressed the inhibitory effect under the contact area. At the highest incorporated concentration, 20.0% (v/v), the edible film expressed the inhibitory effect against all test organisms. The inhibitory effect underneath the film decreased when the incorporated concentration decreased. At 10.0% (v/v), 9 of 16 test organism showed the sensitivity. At 5.0% (v/v), test organisms including *Bacillis cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* 101, *Listeria monocytogenes* DSMZ 20600, and *Vibrio parahaemolyticus* ATCC 20502 presented the sensitivity against antimicrobial Alginate-Tapioca starch based edible film.

4.5.2.3 Total count of survivors

Through 24 hours, Alginate-Tapioca starch based antimicrobial edible film incorporated with 20.0 % (v/v) ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe exhibited the good inhibitory effect against *Bacillus subtills* ATCC 6633, *Listeria monocytogenes* DSMZ 20600, and *Vibrio parahaemolyticus* ATCC 20502, the populatuions decreased to 4.04, 3.81, and 5.35 log₁₀ CFU/ml, respectively. *Bacillus cereus* ATCC 128263, *Pseudomonas fluorescens* ATCC 13525, *Yersinia enterocolitica* ATCC 2779 was reduced by 2.20, 2.38, 2.13 log-reductions, respectively. The cells population of *Escherichia coli* O157:H7 DMST 12743, *Salmonella* Typhimurium ATCC 13311, and *Pseudomonas aeruginosa* DSMZ 939 was decreased from 6.60, 6.93, 6.66 log₁₀ CFU/ml to 5.36, 5.54, and 4.67 log₁₀ CFU/ml in 24 hours, respectively. The cells of *Staphylococcus aureus* DSMZ 799 presented the resistance against the incorporated edible film; the measured decreased population was 0.28 log₁₀ CFU/ml (Figure 71).

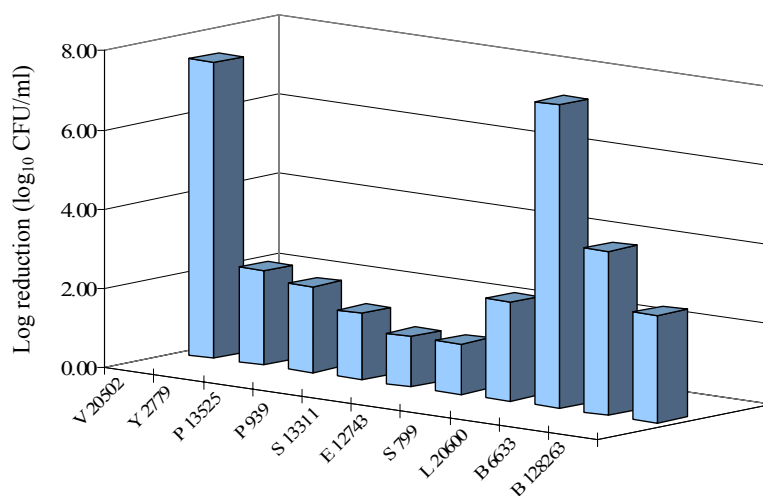


Figure 71 Population decrease (log₁₀ CFU/ml) of test organisms (V 20502: *Vibriob paraahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) after contact with an with Alginate-Tapioca starch based edible film containing 20% (v/v) *Curcuma zedoaria* (Christm.) Roscoe-ethanolic extract after 24 hours of contact time (Values represent mean of 3 independent trials)

4.5.3 Antimicrobial activity of *Garcinia mangostana* L. -ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.3.1 The zone of inhibition

Very little inhibitory effect was observed in Alginate-Tapioca starch based antimicrobial edible film incorporated with 5.0 % (v/v) ethanolic extract of *Garcinia mangostana* L. The inhibitory zone determined around the edible film observed against *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Pseudomonas aeruginosa* DSMZ 939, *Staphylococcus aureus* ATCC 13565, and *Staphylococcus aureus* DSMZ 799 was 0.23, 0.20, 0.40, 0.07, and 0.03 cm, respectively. When the incorporated concentration in the edible film increased, the zone of inhibition and the numbers of sensitive test organisms against antimicrobial edible film also increased. Besides, the above sensitive test organisms, *Escherichia coli* DSMZ 636, *Escherichia coli* ATCC 8739, *Escherichia coli* O157:H7 DMST 12743, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* Typhimurium ATCC 13311, *Salmonella* Typhimurium DSMZ 5569 and *Yersinia enterocolitica* ATCC 2779 provided the zone of inhibition after the use of Alginate-Tapioca starch based edible film incorporated with 10.0 % (v/v) ethanolic extract of *Garcinia mangostana* L. was applied. At the highest incorporated concentration, 20.0 % (v/v), all test organisms presented the sensitivity. *Salmonella* Typhimurium ATCC 13311 showed the highest sensitivity, the determined zone of inhibition was 1.27 cm. Little inhibition was observed against *Listeria monocytogenes* 101 and *Listeria monocytogenes* DSMZ 20600 with the determined zone of inhibition at 0.27 and 0.33 cm, respectively (Figure 72).

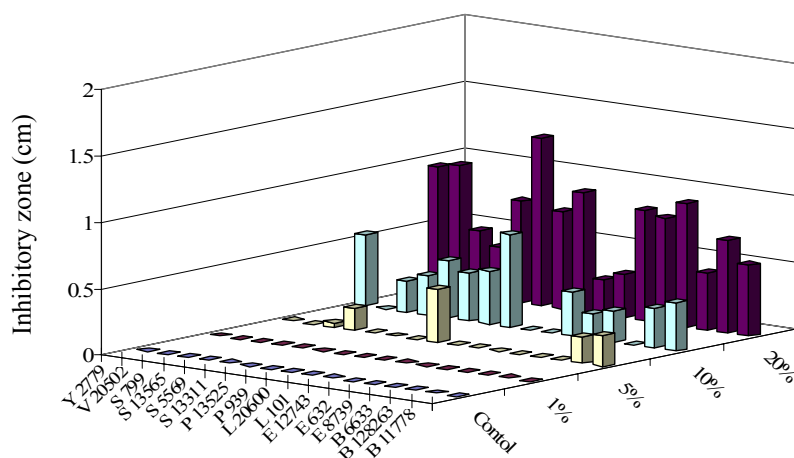


Figure 72 Antibacterial activity of *Garcinia mangostana* L. ethanolic extract-incorporated Algininate-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

Table 6 Antibacterial activity of *Garcinia mangostana* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	-	+	+	+	+
<i>Bacillus cereus</i> ATTC 11778	-	+	+	+	+
<i>Bacillus subtilis</i> ATCC 6633	-	+	+	+	+
<i>Escherichia coli</i> ATCC 632	-	-	-	+	+
<i>Escherichia coli</i> ATCC 8739	-	-	-	+	+
<i>Escherichia coli</i> O157:H7 DMST 12743	-	-	-	+	+
<i>Listeria monocytogenes</i> 101	-	-	+	+	+
<i>Listeria monocytogenes</i> DSMZ 20600	-	-	+	+	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	-	+	+	+	+
<i>Pseudomonas fluorescens</i> ATCC 13525	-	+	+	+	+
<i>Salmonella</i> Typhimurium ATCC 13311	-	-	-	+	+
<i>Salmonella</i> Typhimurium DSMZ 5569	-	-	-	+	+

Table 6 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanollic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	–	+	+	+
<i>Staphylococcus aureus</i> DSMZ 799	–	–	+	+	+
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	+	+	+	+
<i>Yersinia enterocolitica</i> ATCC 2779	–	+	+	+	+

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

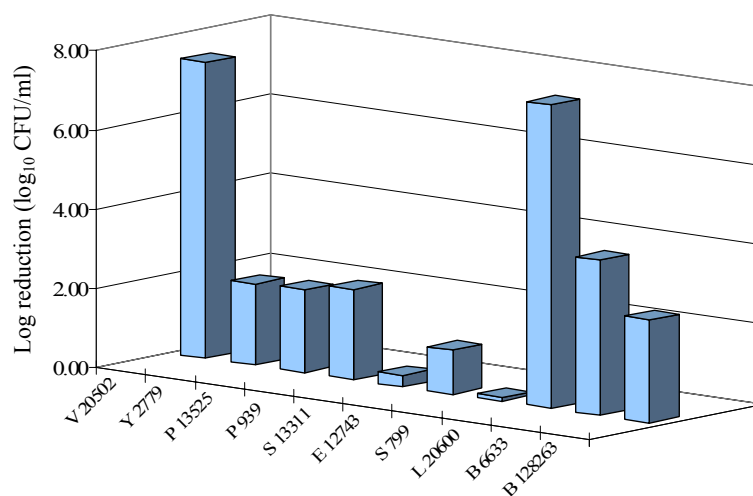


Figure 73 Population decrease (log₁₀ CFU/ml) of test organisms (V 20502: *Vibriob paraahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) after contact with an with Alginate-Tapioca starch based edible film containing 20% (v/v) *Garcinia mangostana* L.-ethanolic extract after 24 hours of contact time (Values represent mean of 3 independent trials)

4.5.3.2 Contact area underneath the film

The lowest incorporated concentration that performed the inhibitory effect was 1.0 % (v/v). The test organisms that showed the sensitivity included *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Vibrio paraahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779. The above test organisms that presented the sensitivity against Alginate-Tapioca starch based

edible film incorporated with ethanolic extract of *Garcinia mangostana* L. at concentration of 10.0 % (v/v), besides *Listeria monocytogenes* 101, *Listeria monocytogenes* DSMZ 20600, *Staphylococcus aureus* ATCC 13565, and *Staphylococcus aureus* DSMZ 799 also exhibited the sensitivity. Antimicrobial edible film at the incorporated concentration 10.0 and 20.0 % (v/v) provided inhibitory effect against all test organisms (Table 6).

4.5.3.3 Total count of survivors

Population decrease (\log_{10} CFU/ml) of test organisms after contact with an Alginate-Tapioca starch based edible film containing 20 % (v/v) *Garcinia mangostana* L.-ethanolic extract after 24 hours was given in Figure 73. The edible film expressed the outstanding antimicrobial activity against *Listeria monocytogenes* DSMZ 20600 and *Vibrio parahaemolyticus* ATCC 20502, the numbers of cells were reduced to undetectable level and \log 7.65 and 7.46 reductions were observed. A decrease in the population between \log 4.00 to 2.00 reductions was observed against *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, and *Yersinia enterocolitica* ATCC 2779. The most resistance strain was *Staphylococcus aureus* DSMZ 799 which population decreased only in \log 0.09 reduction.

4.5.4 Antimicrobial activity of *Hibiscus sabdariffa* L. -ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.4.1 The zone of inhibition

Alginate-Tapioca starch based edible film incorporated with *Hibiscus sabdariffa* L.-ethanolic extract at concentration 1.0 % (v/v) presented the inhibitory effect against *Bacillus subtilis* ATCC 6633, the zone of inhibition was 0.13 cm. Inhibition increased when the incorporated concentration increased. At the higher incorporated concentration, 5.0 % (v/v), the sensitive test organisms increased from 1 strain to 6 strains including *Bacillus cereus* ATCC 6633, *Pseudomonas aeruginosa* DSMZ 939,

Salmonella Typhimurium ATCC 13311, *Salmonella* Typhimurium DSMZ 5569, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779, the inhibition zone was 0.37, 0.53, 0.60, 0.48, 0.47, and 0.57 cm, respectively.

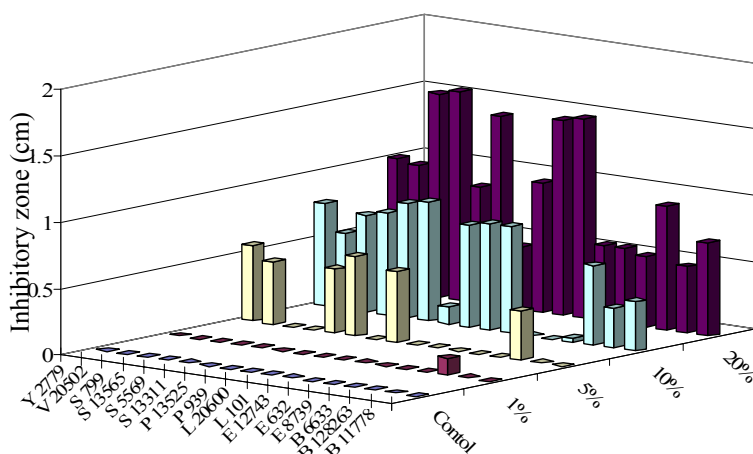


Figure 74 Antibacterial activity of *Hibiscus sabdariffa* L. ethanolic extract-incorporated Alginata-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

All test organisms exhibited the sensitivity against Alginate-Tapioca starch based antimicrobial edible film incorporated with 10.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. with the exception of *Escherichia coli* DSMZ 632 and *Escherichia coli* O157:H7 DMST 12743. The edible film at the incorporated concentration at 20.0 % (v/v) exhibited the inhibitory effect against all test organisms (Figure 74).

4.5.4.2 Contact area underneath the film

The lowest incorporated concentration into the Alginate-Tapioca starch based edible film which presented the inhibitory effect underneath the film against all test organisms was 5.0 % (v/v). Thirteen out of sixteen strains, including *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Escherichia coli* DSMZ 632, *Escherichia coli* O157:H7 DMST 12743, *Listeria monocytogenes* 101, *Listeria monocytogenes* 101, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* Typhimurium ATCC 13311, *Salmonella* Typhimurium DSMZ 5569, *Staphylococcus aureus* ATCC 13565, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779, demonstrated the sensitivity effect against Alginate-Tapioca starch based edible film incorporated with 1.0% (v/v) ethanolic extract of *Hibiscus sabdariffa* L. (Table 7).

4.5.4.3 Total count of survivors

More than 6.00 log-reductions were determined after the contact of test organisms, excluding *Bacillus cereus* ATCC 128263 and *Bacillus subtilis* ATCC 6633, contacted with Alginate-Tapioca starch based antimicrobial edible film incorporated with 20.0 % (v/v) ethanolic extract of *Hibiscus subdariffa* L. in 24 hours. In addition, the test organisms which presented the high level of population decrease were reduced to the undetectable levels in 24 hours. In case of the resistance strains, the cells population of *Bacillus cereus* ATCC 128263 was reduced from 7.17 log₁₀ CFU/ml to 4.61 log₁₀ CFU/ml and *Bacillus subtilis* ATCC 6633 was reduced from 7.47 log₁₀ CFU/ml to 3.33 log₁₀ CFU/ml in 24 hours (Figure 75).

Table 7 Antibacterial activity of *Hibiscus sabdariffa* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	–	–	+	+	+
<i>Bacillus cereus</i> ATTC 11778	–	–	+	+	+
<i>Bacillus subtilis</i> ATCC 6633	–	+	+	+	+
<i>Escherichia coli</i> ATCC 632	–	+	+	+	+
<i>Escherichia coli</i> ATCC 8739	–	+	+	+	+
<i>Escherichia coli</i> O157:H7 DMST 12743	–	+	+	+	+
<i>Listeria monocytogenes</i> 101	–	+	+	+	+
<i>Listeria monocytogenes</i> DSMZ 20600	–	+	+	+	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	–	+	+	+	+
<i>Pseudomonas fluorescens</i> ATCC 13525	–	+	+	+	+
<i>Salmonella</i> Typhimurium ATCC 13311	–	+	+	+	+
<i>Salmonella</i> Typhimurium DSMZ 5569	–	+	+	+	+

Table 7 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanollic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	+	+	+	+
<i>Staphylococcus aureus</i> DSMZ 799	–	+	+	+	+
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	–	+	+	+
<i>Yersinia enterocolitica</i> ATCC 2779	–	+	+	+	+

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

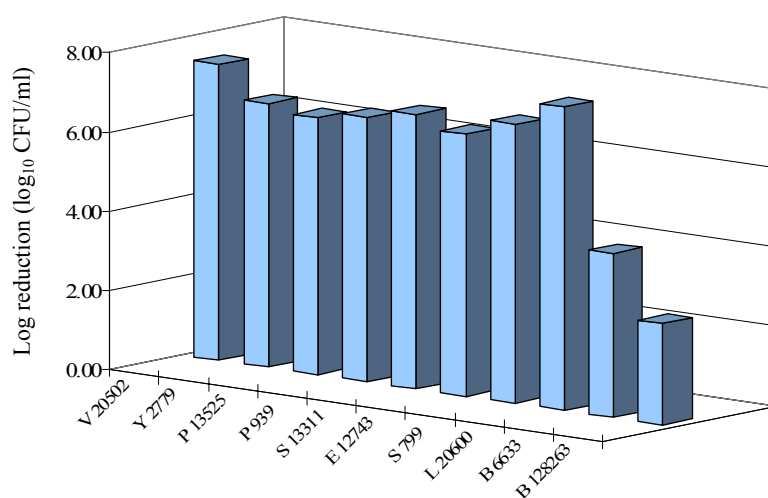


Figure 75 Population decrease (log₁₀ CFU/ml) of test organisms (V 20502: *Vibriob paraahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) with Alginate-Tapioca starch based edible film containing 20 % (v/v) *Hibiscus sabdariffa* L.-ethanolic extract after 24 hours of contact time (Values represent mean of 3 independent trials)

4.5.5 Antimicrobial activity of *Musa sapientum* L. -ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.5.1 The zone of inhibition

As in Figure 76, the antimicrobial activity of Alginate-Tapioca starch based incorporated with 20.0 % (v/v) ethanolic extract of *Musa sapientum* L. was given. The edible film presented the inhibitory effect only against *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* 101, and *Listeria monocytogenes* DSMZ 20600, the determined zone of inhibition was 0.17, 0.23, 0.33, 0.22, and 0.43 cm, respectively. The others test organisms had no sensitivity against Alginate-Tapioca starch based edible film incorporated with ethanolic extract of *Musa sapientum* L. at this concentration. At the lower concentration than 20.0 % (v/v), the zone of inhibition could not be detected.

4.5.5.2 Contact area underneath the film

At the highest incorporated concentration into the Alginate-Tapioca starch based edible film, 20.0 % (v/v) was the only incorporated concentration which presented the inhibitory effect at the contact area. The test organisms which provided the sensitivity including, *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* 101, *Listeria monocytogenes* DSMZ 20600, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Staphylococcus aureus* ATCC 13565, *Staphylococcus aureus* DSMZ 799, and *Yersinia enterocolitica* ATCC 2739 (Table 8). The others test organisms showed no inhibitory effect.

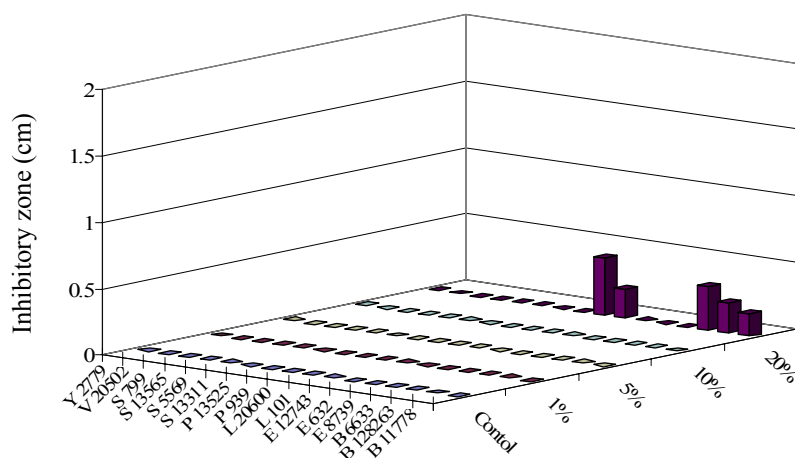


Figure 76 Antibacterial activity of *Musa sapientum* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

Table 8 Antibacterial activity of *Musa sapientum* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	-	-	-	-	+
<i>Bacillus cereus</i> ATTC 11778	-	-	-	-	+
<i>Bacillus subtilis</i> ATCC 6633	-	-	-	-	+
<i>Escherichia coli</i> ATCC 632	-	-	-	-	-
<i>Escherichia coli</i> ATCC 8739	-	-	-	-	-
<i>Escherichia coli</i> O157:H7 DMST 12743	-	-	-	-	-
<i>Listeria monocytogenes</i> 101	-	-	-	-	+
<i>Listeria monocytogenes</i> DSMZ 20600	-	-	-	-	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	-	-	-	-	+
<i>Pseudomonas fluorescens</i> ATCC 13525	-	-	-	-	+
<i>Salmonella</i> Typhimurium ATCC 13311	-	-	-	-	-
<i>Salmonella</i> Typhimurium DSMZ 5569	-	-	-	-	-

Table 8 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanollic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	–	–	–	+
<i>Staphylococcus aureus</i> DSMZ 799	–	–	–	–	+
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	–	–	–	–
<i>Yersinia enterocolitica</i> ATCC 2779	–	–	–	–	+

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

4.5.5.3 Total count of survivors

Bacillus cereus ATCC 128263, *Bacillus subtilis* ATCC 6633, and *Listeria monocytogenes* DSMZ 20600 expressed the sensitivity to Alginate-Tapioca based edible film incorporated with 20.0 % (v/v) ethanolic extract of *Musa sapientum* L. with a population decreased by 2.57, 3.44, and 3.23 log-reductions, respectively. In the other hand, the others test organisms presented resistance properties against this antimicrobial edible film. The measured population decrease was less than 0.50 log-reductions (Figure 77).

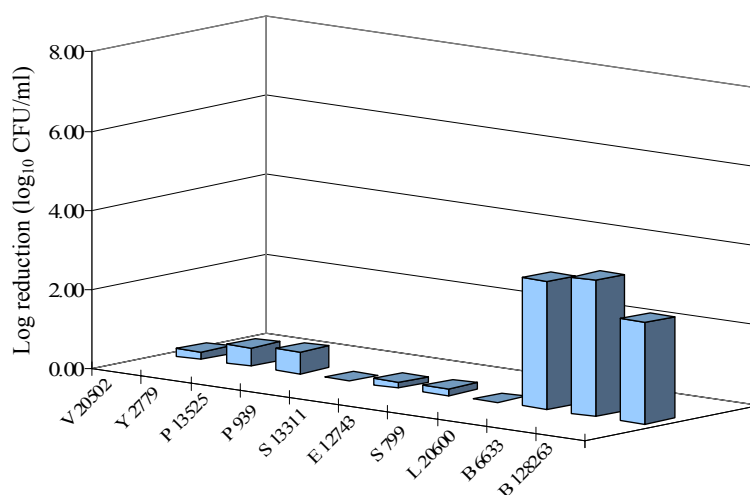


Figure 77 Population decrease (log₁₀ CFU/ml) of test organisms (V 20502: *Vibriob paraahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) with Alginate-Tapioca starch edible film containing 20% (v/v) *Musa sapientum* L.-ethanolic extract after 24 hrs of contact time (Values represent mean of 3 independent trials)

4.5.6 Antimicrobial activity of *Nelumbo nucifera* Gaertn. -ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.6.1 The zone of inhibition

Alginate-Tapioca starch based edible film incorporated with ethanolic extract of *Nelumbo nucifera* Gaertn. at all incorporated concentration presented no zone of inhibition against all test organisms (Figure 78).

4.5.6.2 Contact area underneath the film

At the incorporated concentration of 10.0 % (v/v), incorporated into Alginate-Tapioca starch based edible film showed the inhibitory effect at the contact area against *Listeria monocytogenes* 101 and *Listeria monocytogenes* DSMZ 2006. When the incorporated concentration increased, the numbers of sensitivity test organism increased. At the incorporated concentration at 20.0 % (v/v), *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* 101, and *Listeria monocytogenes* DSMZ 20600 presented the sensitivity to Alginate-Tapioca starch based edible film incorporated with ethanolic extract of *Musa sapientum* L. (Table 9).

4.5.6.3 Total count of survivors

As shown in Figure 79, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, and *Listeria monocytogenes* DSMZ 20600 demonstrated the high level of population decrease (more than 2.50 log-reductions), being 2.53, 4.03, and 4.12 log-reductions, respectively. The others test organisms presented the very little inhibition (less than 0.50 log-reductions).

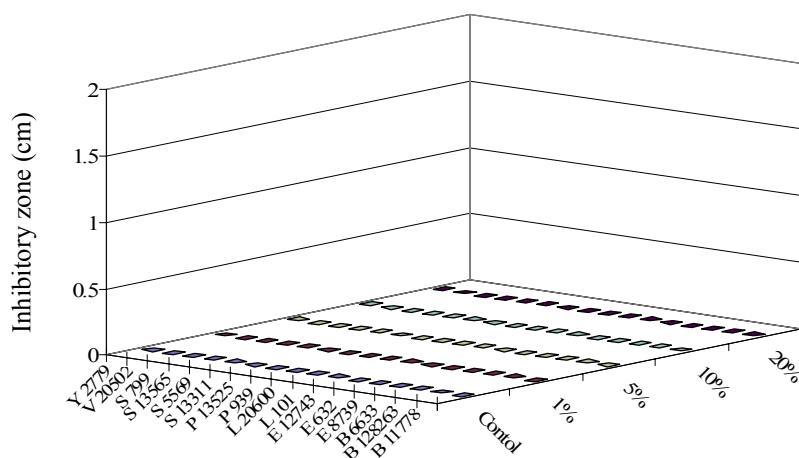


Figure 78 Antibacterial activity of *Nelumbo nucifera* Gaertn. ethanolic extract-incorporated Alginata-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

Table 9 Antibacterial activity of *Nelumbo nucifera* Gaertn. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	-	-	-	-	+
<i>Bacillus cereus</i> ATTC 11778	-	-	-	-	+
<i>Bacillus subtilis</i> ATCC 6633	-	-	-	-	+
<i>Escherichia coli</i> ATCC 632	-	-	-	-	-
<i>Escherichia coli</i> ATCC 8739	-	-	-	-	-
<i>Escherichia coli</i> O157:H7 DMST 12743	-	-	-	-	-
<i>Listeria monocytogenes</i> 101	-	-	-	+	+
<i>Listeria monocytogenes</i> DSMZ 20600	-	-	-	+	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	-	-	-	-	-
<i>Pseudomonas fluorescens</i> ATCC 13525	-	-	-	-	-
<i>Salmonella</i> Typhimurium ATCC 13311	-	-	-	-	-
<i>Salmonella</i> Typhimurium DSMZ 5569	-	-	-	-	-

Table 9 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	–	–	–	–
<i>Staphylococcus aureus</i> DSMZ 799	–	–	–	–	–
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	–	–	–	–
<i>Yersinia enterocolitica</i> ATCC 2779	–	–	–	–	–

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

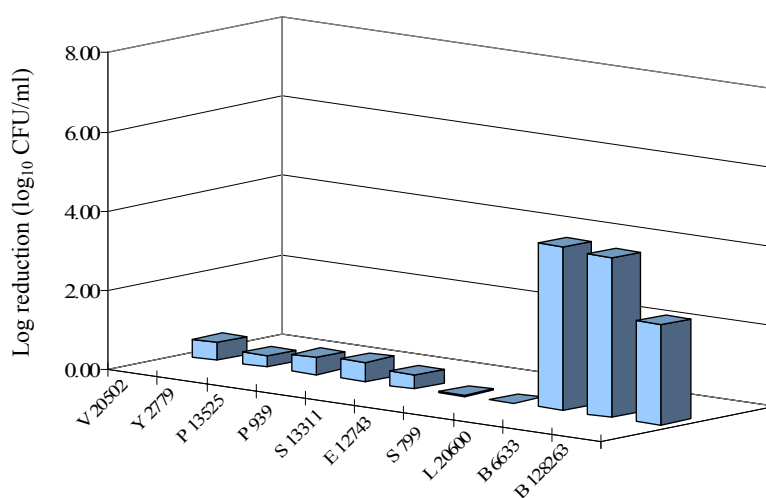


Figure 79 Population decrease (log₁₀ CFU/ml) of test organisms (V 20502: *Vibriob paraahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) with Alginate-Tapioca starch edible film containing 20% (v/v) *Nelumbo nucifera* Gaertn.-ethanolic extract after 24 hrs of contact time (Values represent mean of 3 independent trials)

4.5.7 Antimicrobial activity of *Piper betle* L. -ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.7.1 The zone of inhibition

The zone of inhibition was observed in Alginate-Tapioca starch based edible film incorporated with 1.0 % (v/v) ethanolic extract of *Piper betle* L. against *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* DSMZ 799, the determined inhibition

zone was 0.30 and 0.03 cm. However, inhibition of test organisms in the numbers of strains and inhibition zone increased in the presence of ethanolic extract of *Piper betle* L. 5.0 % (v/v) incorporated in to Alginate-Tapioca starch based edible film. At 5.0 % (v/v), the zone of inhibition determined against *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* DSMZ 939, *Staphylococcus aureus* DSMZ 799, *Salmonella* Typhimurium DSMZ 5569, and *Yersinia enterocolitica* ATCC 2779 was 0.53, 0.70, 0.17, 0.03, and 0.33 cm, respectively. With the exception with *Listeria monocytogenes* 101 and *Listeria monocytogenes* DSMZ 20660, all test organisms demonstrated the sensitivity against Alginate-Tapioca starch based edible film incorporated with 10.0 % (v/v) ethanolic extract of *Piper betle* L. In addition, Alginate-Tapioca starch based edible film incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. had an ability to inhibit all of test organism with the zone of inhibition more than 0.6 cm (Figure 80). The most resistance strains was *Salmonella* Typhimurium ATCC 13311 which presented zone of inhibition at 0.63 cm, the most sensitive strains was *Pseudomonas aeruginosa* DSMZ 939 with the determined zone of inhibition at 1.63 cm.

4.5.7.2 Contact area underneath the film

The results of antimicrobial properties of Alginate-Tapioca starch based edible film incorporated with the ethanolic extract of *Piper betle* L. determined the effect underneath the film was summarized in Table 10. Alginate-Tapioca starch based edible film incorporated with 20.0 and 10.0 % (v/v) ethanolic extract of *Piper betle* L. had ability to inhibit the growth of all test organisms underneath the film. The cells of *Listeria monocytogenes* 101 and *Listeria monocytogenes* DSMZ 20600 showed the resistance against Alginate-Tapioca starch based edible film incorporated 5.0 % (v/v) ethanolic extract of *Piper betle* L. Seven strains of test organisms including *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Staphylococcus aureus* ATCC 13565, and *Staphylococcus aureus* DSMZ 799, exhibited the sensitivity against Alginate-Tapioca starch based edible film incorporated with 1.0 % (v/v) ethanolic extract of *Piper betle* L.

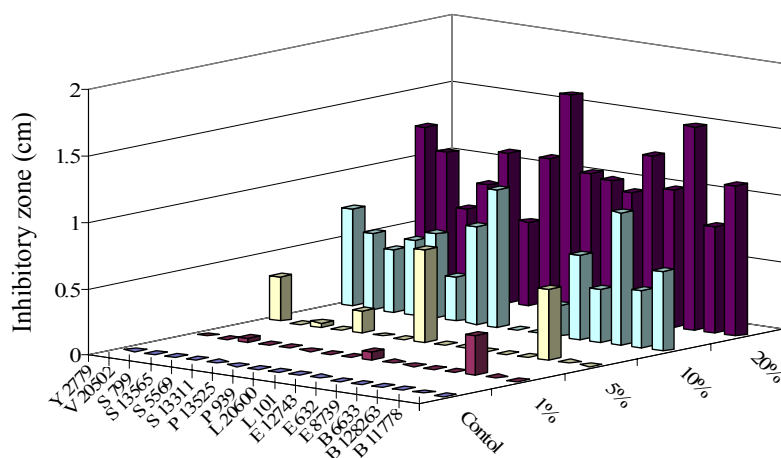


Figure 80 Antibacterial activity of *Piper betle* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

Table 10 Antibacterial activity of *Piper betle* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	–	–	+	+	+
<i>Bacillus cereus</i> ATTC 11778	–	+	+	+	+
<i>Bacillus subtilis</i> ATCC 6633	–	+	+	+	+
<i>Escherichia coli</i> ATCC 632	–	–	+	+	+
<i>Escherichia coli</i> ATCC 8739	–	–	+	+	+
<i>Escherichia coli</i> O157:H7 DMST 12743	–	–	+	+	+
<i>Listeria monocytogenes</i> 101	–	–	–	+	+
<i>Listeria monocytogenes</i> DSMZ 20600	–	–	–	+	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	–	+	+	+	+
<i>Pseudomonas fluorescens</i> ATCC 13525	–	+	+	+	+
<i>Salmonella</i> Typhimurium ATCC 13311	–	–	+	+	+
<i>Salmonella</i> Typhimurium DSMZ 5569	–	–	+	+	+

Table 10 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	+	+	+	+
<i>Staphylococcus aureus</i> DSMZ 799	–	+	+	+	+
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	–	+	+	+
<i>Yersinia enterocolitica</i> ATCC 2779	–	+	+	+	+

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

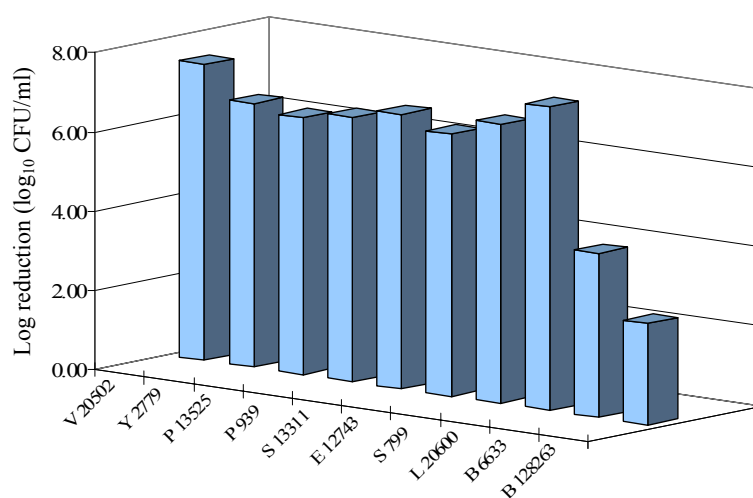


Figure 81 Population decrease (log₁₀ CFU/ml) of test organisms (V 20502: *Vibriob paraahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) with Alginate-Tapioca starch edible film containing 20% (v/v) *Piper betle* L.-ethanolic extract after 24 hrs of contact time (Values represent mean of 3 independent trials)

4.5.7.3 Total count of survivors

Bacillus cereus ATCC 128263 showed the high resistance to Alginate-Tapioca starch based edible film incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. after 24 hours. The population reduction was 2.55 log₁₀ CFU/ml. Moreover, 4.15 log₁₀ CFU/ml was determined against *Bacillus subtilis* ATCC 6633. The others test organisms exhibited the high levels of reduction in the population. In addition, the initial population of *Listeria monocytogenes* DSMZ 20600, *Staphylococcus aureus* DSMZ 799, *Escherichia coli* O157:H7 DMST 12743, *Salmonella* Typhimurium

ATCC 13311, *Pseudomonas aeruginosa* DSMZ 799, *Pseudomonas fluorescens* ATCC 13525, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779 were also reduced to undetectable in 24 hours after contact with Alginate-Tapioca starch based edible film incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. (Figure 81).

4.5.8 Antimicrobial activity of *Psidium guajava* L. -ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.8.1 The zone of inhibition

Antimicrobial activity of Alginate-Tapioca starch based edible film incorporated with ethanolic extract of *Psidium guajava* L. was first detected at the incorporated concentration of 5.0 % (v/v). *Bacillus cereus* ATCC 11778 and *Bacillus cereus* ATCC 128263 were the sensitive test organisms against antimicrobial edible film. The measurable zone of inhibition was 0.10 and 0.13 cm for *Bacillus cereus* ATCC 11778 and *Bacillus cereus* ATCC 128263, respectively. At 10.0 % (v/v) incorporated concentration, eleven strains of test organisms presented the sensitivity against Alginate-Tapioca starch based edible film. Among the sensitive test organisms, *Salmonella* Typhimurium DSMZ 5569 exhibited the highest sensitivity with the zone of inhibition at 0.53 cm. 13 strains of test organisms exhibited the sensitivity against Alginate-Tapioca starch based edible film incorporated with 20.0 % (v/v) ethanolic extract of *Psidium guajava* L. The highest sensitive strain was *Yersinia enterocolitica* ATCC 2779, the inhibition zone was 1.07 cm. However, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* 101, and *Listeria monocytogenes* DSMZ 20660 staged the resistance to Alginate-Tapioca starch based antimicrobial edible film (Figure 82).

4.5.8.2 Contact area underneath the film

Regarding the antimicrobial activity underneath the film, the growth of all test organisms were inhibited when the Alginate-Tapioca starch based edible films incorporated at 10.0 and 20.0 % (v/v) were applied. Eleven strains were inhibited after

the edible film contained 5.0 % (v/v) ethanolic extract of *Psidium guajava* L. was applied. At the lowest incorporated concentration, 1.0 % (v/v), the growth underneath the film *Listeria monocytogenes* 101, *Listeria monocytogenes* DSMZ 20600, and *Vibrio parahaemolyticus* ATCC 20502 was inhibited (Table 11).

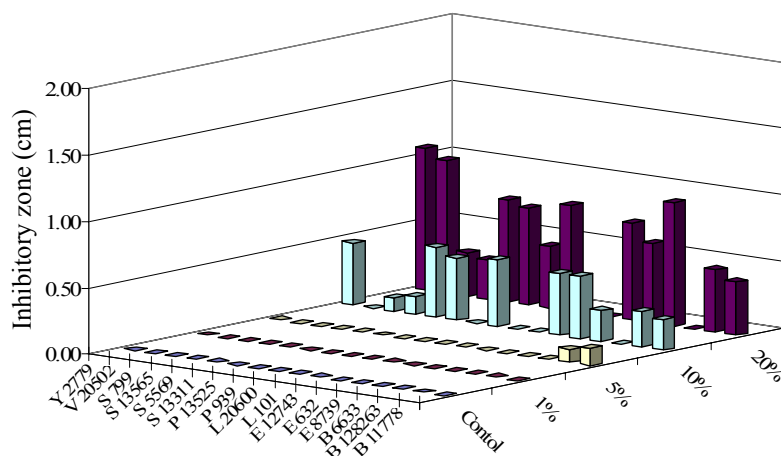


Figure 82 Antibacterial activity of *Psidium guajava* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

Table 11 Antibacterial activity of *Psidium guajava* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	–	–	+	+	+
<i>Bacillus cereus</i> ATTC 11778	–	–	+	+	+
<i>Bacillus subtilis</i> ATCC 6633	–	–	+	+	+
<i>Escherichia coli</i> ATCC 632	–	–	–	+	+
<i>Escherichia coli</i> ATCC 8739	–	–	–	+	+
<i>Escherichia coli</i> O157:H7 DMST 12743	–	–	–	+	+
<i>Listeria monocytogenes</i> 101	–	+	+	+	+
<i>Listeria monocytogenes</i> DSMZ 20600	–	+	+	+	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	–	–	+	+	+
<i>Pseudomonas fluorescens</i> ATCC 13525	–	–	+	+	+
<i>Salmonella</i> Typhimurium ATCC 13311	–	–	–	+	+
<i>Salmonella</i> Typhimurium DSMZ 5569	–	–	–	+	+

Table 11 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanollic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	–	–	+	+
<i>Staphylococcus aureus</i> DSMZ 799	–	–	+	+	+
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	+	+	+	+
<i>Yersinia enterocolitica</i> ATCC 2779	–	–	+	+	+

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

4.5.8.3 Total count of survivors

The number of cells of *Listeria monocytogenes* DSMZ 20600 and *Vibrio parahaemolyticus* ATCC 20502 were reduced to undetectable level or exhibited the population reduction at 7.65 and 7.46 \log_{10} CFU/ml, respectively, after contact with 20.0 % (v/v) ethanolic extract of *Psidium guajava* L. incorporated into Alginate-Tapioca starch based edible film. The cells of *Bacillus subtilis* ATCC 6633 was reduced by 4.15 log-reductions, the others test organisms was reduced by less than 2.5 log-reductions (Figure 83).

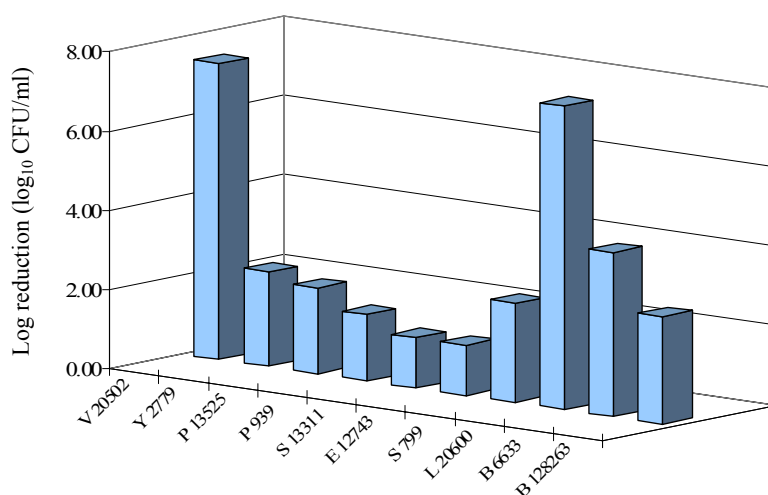


Figure 83 Population decrease (\log_{10} CFU/ml) of test organisms (V 20502: *Vibriob parahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) with Alginate-Tapioca starch edible film containing 20% (v/v) *Psidium guajava* L.-ethanolic extract after 24 hrs of contact time (Values represent mean of 3 independent trials)

4.5.9 Antimicrobial activity of *Punica granatum* L. -ethanolic extract-incorporated Alginate-Tapioca starch based edible film

4.5.9.1 The zone of inhibition

Alginate-Tapioca starch based edible film incorporated with the ethanolic extract of *Punica granatum* L. exhibited the antimicrobial activity against several test organisms at 1.0 % (v/v) incorporated concentration. The zone inhibition determined against *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Staphylococcus aureus* ATCC 13565, *Staphylococcus aureus* DSMZ 799, *Vibrio parahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779 was 0.43, 0.33, 0.93, 0.67, 0.20, 0.30, 0.93, and 0.85 cm, respectively. Fourteen strains out of sixteen strains of test organisms presented the sensitivity against Alginate-Tapioca starch based edible film incorporated with 5.0 % (v/v) ethanolic extract of *Punica granatum* L. *Vibrio parahaemolyticus* ATCC 20502 exhibited the biggest zone of inhibition (1.0 cm), *Bacillus subtilis* expressed the smallest zone of inhibition at 0.33 cm. The zone of inhibition was observed in Alginate-Tapioca starch based edible film incorporated with 10.0 and 20.0 % (v/v) ethanolic extract of *Punica granatum* L. against all test organisms. At 20 % (v/v) incorporated concentration, *Escherichia coli* DSMZ 632 presented the highest sensitivity, the zone of inhibition was 1.73 cm, *Bacillus subtilis* ATCC 6633 was the most resistance strain, the determined zone of inhibition was 0.63 cm (Figure 84).

4.5.9.2 Contact area underneath the film

The results as presented in Table 9 showed the antimicrobial effect underneath the Alginate-Tapioca starch based edible film incorporated with ethanolic extract of *Punica granatum* L. The growth underneath the film at all incorporated concentration was inhibited (Table 12).

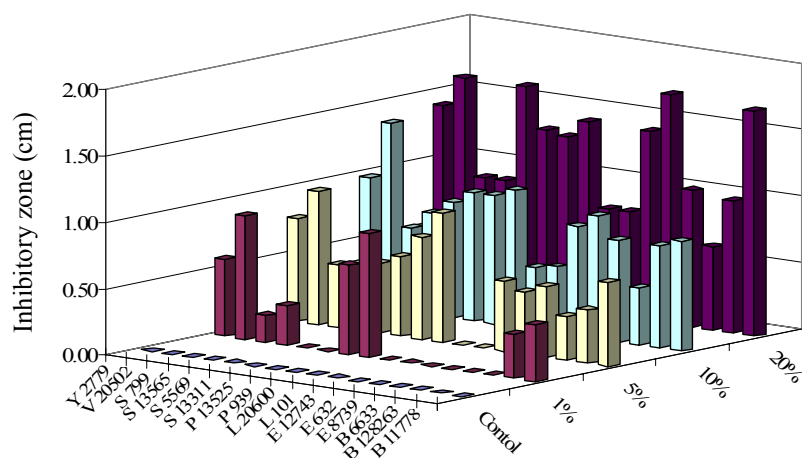


Figure 84 Antibacterial activity of *Punica granatum* L. ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms (Y 2739: *Yersinia enterocolitica* ATCC 2739; V 20502: *Vibrio parahaemolyticus* ATCC 20502; S 13565: *Staphylococcus aureus* ATCC 13565; S 799: *Staphylococcus aureus* DSMZ 799; S 13311: *Salmonella* Typhimurium ATCC 13311; S 5569: *Salmonella* Typhimurium DSMZ 5569; P 939: *Pseudomonas aeruginosa* DSMZ 939; P 13525: *Pseudomonas fluorescens* ATCC 13525; L 20600: *Listeria monocytogenes* DSMZ 20600; L 101: *Listeria monocytogenes* 101; E 8739: *Escherichia coli* ATCC 8739; E 632: *Escherichia coli* DSMZ 632; E 12743: *Escherichia coli* O157:H7 DMST 12743; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263; B 11778: *Bacillus cereus* ATCC 11778) (Values represent mean of 3 independent trials)

Table 12 Antibacterial activity of *Punica granatum L.* ethanolic extract-incorporated Alginate-Tapioca starch based edible film against different test organisms determined the underneath contact area in 24 hours

Test organisms	Contact area underneath the edible film				
	Ethanolic extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Bacillus cereus</i> ATCC 128263	–	+	+	+	+
<i>Bacillus cereus</i> ATTC 11778	–	+	+	+	+
<i>Bacillus subtilis</i> ATCC 6633	–	+	+	+	+
<i>Escherichia coli</i> ATCC 632	–	+	+	+	+
<i>Escherichia coli</i> ATCC 8739	–	+	+	+	+
<i>Escherichia coli</i> O157:H7 DMST 12743	–	+	+	+	+
<i>Listeria monocytogenes</i> 101	–	+	+	+	+
<i>Listeria monocytogenes</i> DSMZ 20600	–	+	+	+	+
<i>Pseudomonas aeruginosa</i> DSMZ 939	–	+	+	+	+
<i>Pseudomonas fluorescens</i> ATCC 13525	–	+	+	+	+
<i>Salmonella</i> Typhimurium ATCC 13311	–	+	+	+	+
<i>Salmonella</i> Typhimurium DSMZ 5569	–	+	+	+	+

Table 12 (Continue)

Test organisms	Contact area underneath the edible film				
	Ethanol extract concentrations (% v/v)				
	Control	1.0	5.0	10.0	20.0
<i>Staphylococcus aureus</i> ATCC 13565	–	+	+	+	+
<i>Staphylococcus aureus</i> DSMZ 799	–	+	+	+	+
<i>Vibrio parahaemolyticus</i> ATCC 20502	–	+	+	+	+
<i>Yersinia enterocolitica</i> ATCC 2779	–	+	+	+	+

+ : Represents an inhibitory effect

– : Represents no inhibitory effect

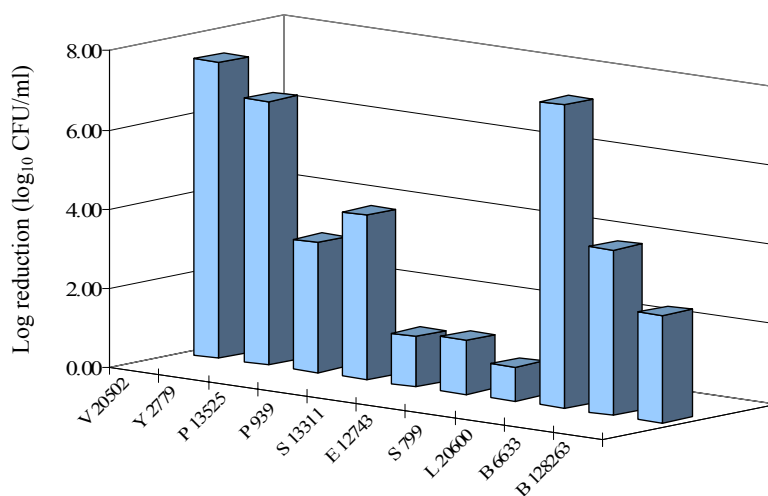


Figure 85 Population decrease (log₁₀ CFU/ml) of test organisms (V 20502: *Vibriob paraahaemolyticus* ATCC 20502; Y 2739: *Yersinia enterocolitica* ATCC 2739; P 13525: *Pseudomonas fluorescens* ATCC 13525; P 939: *Pseudomonas aeruginosa* DSMZ 939, S 5569: S 13311: *Salmonella* Typhimurium ATCC 13311; E 12743: *Escherichia coli* O157:H7 DMST 12743; S 799: *Staphylococcus aureus* DSMZ 799; L 20600: *Listeria monocytogenes* DSMZ 20600; B 6633: *Bacillus subtilis* ATCC 6633; B 128263: *Bacillus cereus* ATCC 128263) with Alginate-Tapioca starch edible film containing 20% (v/v) *Punica granatum* L.-ethanolic extract after 24 hrs of contact time (Values represent mean of 3 independent trials)

4.5.9.3 Total count of survivors

At 20.0 % (v/v) incorporated concentration of ethanolic extract of *Punica granatum* L, in to Alginate-Tapioca starch based edible film had the ability to reduce the initial population of *Listeria monocytogenes* DSMZ 20600, *Vibrio paraahaemolyticus* ATCC 20502, and *Yersinia enterocolitica* ATCC 2779 to undetectable levels or by 7.65, 7.46, and 6.66 log₁₀ CFU/ml, respectively, in 24 hours. The population of *Bacillus ssubtilis* ATCC 6633, *Pseudomonas aeruginosa* DSMZ 939, and *Pseudomonas fluorescens* ATCC 13525 decreased by 4.18, 4.14, and 3.28 log₁₀ CFU/ml,

respectively. *Bacillus cereus* ATCC 128263, *Escherichia coli* O157:H7 DMST 12743, and *Salmonella* Typhimurium ATCC 13311 was reduced by 2.71, 1.36, and 1.28 log₁₀ CFU/ml, respectively. The cells of *Staphylococcus aureus* DSMZ 799 expressed the highest resistance; the population decrease was 0.87 log₁₀ CFU/ml (Figure 85).

4.6 Application Study: Cytotoxicity Test of Thai Traditional Ethanolic Extract

Over the last few of decades several *in vitro* assays using mammalian cell cultures have been developed thus avoiding the excessive use of laboratory animals which is expensive, time consuming, and often involves ethical problems.

The cytotoxicity of ethanolic extract of Thai traditional medicinal plants were evaluated in TH1 (Terrapene heart cells), A549 (Carcinomic human alveolar basal epithelial cells), BGM (Buffalo-Green-Monkey cells), and MDCK (Madin-Darby canine kidney cells).

Ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees at the concentration of 20.0 % (v/v) exhibited the toxicity activity against all test cell line at 10⁰ and 10⁻¹ level of dilution. When the concentration of this extract decreased to 10.0 % (v/v), the toxicity activity at 10⁻¹ level of dilution was observed against TH₁ and BGM and at 10⁰ level of dilution the toxicity activity against all test cells line was monitored. The lower concentration level at 5.0 and 1.0 % (v/v) stated the toxicity against all test cells line at 10⁰ level of dilution (Table 13).

Table 13 Cytotoxicity activity of ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH₁						
	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-
A549						
	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	+	-	-	-
BGM						
	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-
MDCK						
	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	+	-	-	-

+ : represented toxicity activity

- : represented non toxicity activity

As shown in Table 14, ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe demonstrated the toxicity activity against all tested cells lines. At the concentration of 1.0 % (v/v), this extract exhibited the toxicity at only 1:1 level of dilution. When the concentration of the extract increased to 5.0 % (v/v), the toxicity activity at 1:10 level of dilution was observed only against MDCK, the toxicity activity against others test

cells line was observed at 1:1 level of dilution. At the concentration of 10.0 % (v/v), toxicity against all test cells line was monitored at 1:1 dilution level and at 1:10 level of dilution against MDCK. The highest tested concentration, 20.0 % (v/v), this extract showed toxicity activity at 1:1 level of dilution against all tested cell lines and given the same activity at 1:10 level of dilution against A549 and MDCK.

Table 14 Cytotoxicity activity of ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH ₁	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	-	-	-	-
A549	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	+	-	-	-
BGM	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	-	-	-	-
MDCK	1.0	+	-	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-

+ : represented toxicity activity

- : represented non toxicity activity

The ethanolic extract of *Garcinia mangostana* L. at the concentration of 20.0 % (v/v) expressed the cytotoxicity against all test cells line at 10^0 , 10^{-1} , and 10^{-2} level of dilution. The concentration decreased to 10.0 % (v/v), the toxicity at 1:100 level of dilution was observed against A549 and MDCK, the level of dilution at 1:1 and 1:10 showed toxicity activity against all test cells line. At the concentration of 1.0 and 5.0 % (v/v), the toxicity activity was monitored at 10^0 and 10^{-1} level of dilution against TH₁, A549, BGM, and MDCK (Table 15).

Table 15 Cytotoxicity activity of ethanolic extract of *Garcinia mangostana* L.

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH ₁	1.0	+	+	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	+	-	-
A549	1.0	+	+	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-
BGM	1.0	+	+	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	+	-	-
MDCK	1.0	+	+	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-

+ : represented toxicity activity

- : represented non toxicity activity

Table 16 Cytotoxicity activity of ethanolic extract of *Hibiscus sabdariffa* L.

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH ₁	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-
A549	1.0	+	+	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-
BGM	1.0	+	-	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-
MDCK	1.0	+	-	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-

+ : represented toxicity activity

- : represented non toxicity activity

Considering the cytotoxicity activity of ethanolic extract of *Hibiscus sabdariffa* L. it showed that the toxicity activity at 10^{-2} level of dilution observed against A549 at the concentration of 20.0 and 10.0 % (v/v). For TH₁, BGM, and MDCK, toxicity activity at the concentration of 20.0 % (v/v) was monitored at 10^0 , and 10^{-1} level of dilution. When the concentration of ethanolic extract of *Hibiscus sabdariffa* L. decreased to 5.0 % (v/v), toxicity was detected at 1:1 level of dilution for all test cells line, and at

1:10 level of dilution the activity was detected against A549, BGM, and MDCK. At the lowest concentration, 1.0 % (v/v), the extract at 1:10 level of dilution expressed the toxicity activity against A549, the toxicity activity at this concentration against other test cells line occurred at 1:1 level of dilution (Table 16).

Table 17 Cytotoxicity activity of ethanolic extract of *Musa sapientum* L.

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH ₁	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	-	-	-	-
A549	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	-	-	-	-
BGM	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	-	-	-	-
MDCK	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	-	-	-	-

+ : represented toxicity activity

- : represented non toxicity activity

In Table 17, the cytotoxicity activity of ethanolic extract of *Musa sapientum* L. against TH₁, A549, BGM, and MDCK was presented. The extract showed low toxicity activity against all tested cell lines. At the concentration of 20.0 % (v/v), the toxicity activity was presented only at the 1:1 level of dilution monitored against all test cells line. At the lower concentration, 10.0, 5.0, and 1.0 % (v/v), the toxicity activity was also presented against at 1:1 level of dilution for all test cells line.

Table 18 Cytotoxicity activity of ethanolic extract of *Nelumbo nucifera* Gaertn.

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH ₁	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-
A549	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	+	-	-	-
BGM	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	-	-	-	-
	20.0	+	+	-	-	-
MDCK	1.0	+	-	-	-	-
	5.0	+	-	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-

+ : represented toxicity activity

- : represented non toxicity activity

Ethanollic extract of *Nelumbo nucifera* Gaertn. expressed also low toxicity activity against all tested cell lines. At the concentration of 20.0 % (v/v) the toxicity activity against TH₁, A549, BGM, and MDCK was detected at 1:1 and 1:10 level of dilution. When the concentration of ethanollic extract of *Nelumbo nucifera* Gaertn. decreased to 10.0 % (v/v), the toxicity at 1:10 level of dilution was detected against TH₁ and MDCK, cytotoxicity activity at 1:1 level of dilution was monitored against TH₁, A549, BGM, and MDCK. At the concentration of 20.0 % (v/v), cytotoxicity activity was detected against all test cells line (Table 18).

Cytotoxicity activity of ethanollic extract of *Piper betle* L. was presented in Table 19. At the lowest concentration, 1.0 % (v/v), cytotoxicity activity was observed against all test cells line at 1:1 level of dilution and at 1:10 level of dilution monitored by A459, BGM, and MDCK. The ethanollic extract of *Piper betle* L. increased to 5.0 % (v/v), cytotoxicity against A459, BGM, and MDCK was observed up to 1:100 level of dilution while cytotoxicity activity against TH₁ was observed up to 1:10 level of dilution. Toxicity activity against TH₁ was measured up to 1:10 level of dilution at the concentration of this extract at 10.0 and 20.0 % (v/v). For A459, BGM, and MDCK, toxicity activity was monitored up to 1:100 level of dilution at the concentration of 10.0 and 20.0 % (v/v).

Table 19 Cytotoxicity activity of ethanolic extract of *Piper betle* L.

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH ₁	1.0	+	-	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	-	-	-
	20.0	+	+	-	-	-
A549	1.0	+	+	-	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-
BGM	1.0	+	+	-	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-
MDCK	1.0	+	+	-	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-

+ : represented toxicity activity

- : represented non toxicity activity

Ethanolic extract of *Psidium guajava* L. presented the extreme cytotoxicity activity. At 1.0 % (v/v) concentration, toxicity activity was measured up to the dilution ratio at 1:10 against all test cells line. When the concentration increased to 5.0 % (v/v), the dilution ratio that presented the toxicity activity also increased. The ethanolic extract of *Psidium guajava* L. at this concentration demonstrated the toxicity activity against all test cells line at 1:100 level of dilution. For the cytotoxicity test at the

concentration of 10.0 % (v/v), the presented results were same as results of 5.0 % (v/v) concentration. At the highest tested concentration, 20.0 % (v/v), toxicity activity presented up to the dilution ratio at 1:1000 against TH₁, A459, and BGM. In case of MDCK, toxicity activity presented at 1:100 level of dilution (Table 20).

Table 20 Cytotoxicity activity of ethanolic extract of *Psidium guajava* L.

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH ₁	1.0	+	+	-	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	+	-
A549	1.0	+	+	-	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	+	-
BGM	1.0	+	+	-	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	+	-
MDCK	1.0	+	+	-	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-

+ : represented toxicity activity

- : represented non toxicity activity

Table 21 Cytotoxicity activity of ethanolic extract of *Punica granatum* L.

Cell Culture	Extract concentration (% v/v)	Cytotoxicity activity				
		Dilution level				
		1:1	1:10	1:100	1:1000	1:10000
TH ₁	1.0	+	-	-	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-
A549	1.0	+	+	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-
BGM	1.0	+	+	-	-	-
	5.0	+	+	-	-	-
	10.0	+	+	+	-	-
	20.0	+	+	+	-	-
MDCK	1.0	+	+	+	-	-
	5.0	+	+	+	-	-
	10.0	+	+	+	+	-
	20.0	+	+	+	+	-

+ : represented toxicity activity

- : represented non toxicity activity

The toxicity activity of ethanolic extract of *Punica granatum* L. was given in Table 21. Against TH₁, this extract demonstrated the toxicity activity at all concentration. At 1.0 % (v/v), the toxicity activity was monitored at 10⁰ level of dilution. The higher concentration increased the higher level of dilution that showed the toxicity activity was detected. At the concentration of 5.0, 10.0, and 20.0 % (v/v), cytotoxicity activity determined against TH₁ increased up to 10² level of dilution. Consideration against

A549 indicated that toxicity activity was detected up to 10^3 level of dilution at the concentration of the extract at 10.0 and 20.0 % (v/v). The detectable toxicity activity at 1.0 and 5.0 % (v/v) was observed at the level of dilution up to 10^3 . At the concentration of 10.0 and 20.0 % (v/v), the toxicity activity was detected up to 10^1 level of dilution. BGM presented the same results as determined against A549. The extreme toxicity activity was determined against MDCK. At 10.0 and 20.0 % (v/v) concentration, cytotoxicity activity determined against this cell line was observed at the level of dilution up to 10^3 . At the concentration of 1.0 and 5.0 % (v/v), toxicity activity determined against MDCK presented at the level of dilution up to 10^2 .

4.7 Application Study: Antimicrobial Activity of Alginate-Tapioca Starch Based Edible Films and Coating Incorporated Ethanolic Extract of Thai Traditional Medicinal Plants in Model Food System

Although the *in vitro* antimicrobial activity determination of Alginate-Tapioca starch based edible films incorporated with ethanolic extract of Thai traditional medicinal plants provided good information on antimicrobial performances, they cannot necessarily duplicate all the variability which might exist in a food. Once it has been determined that the antimicrobial performs well in model food system. It is also useful to evaluate the effect of antimicrobial substances in food system, such as lipids, proteins and divalent cations, which might be influence the effectiveness of the antimicrobial substances.

According to the previous experiment, Alginate-Tapioca starch based edible films and coating solution contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. were selected to use as antimicrobial edible material in food model system.

Carrot (*Daucus carota* L.) and chicken meat were selected as the model food systems. *Bacillus cereus* ATCC 128263 and *Salmonella* Typhimurium ATCC 13311 were chosen as test organisms. Both model food systems were divided into 2 stages of microbial protecting procedures; coating by the antimicrobial incorporated edible

coating solution and wrapping by antimicrobial incorporated edible films. The artificial contamination was performed into pre-contamination for determination of the antimicrobial property of coating material and edible films to eliminate the contaminated microorganism and post-contamination for the determination of contaminated avoidance. All contaminated model food systems were stored at room temperature (22°C) or cold condition (4°C). The microbial survivors were examined at 0, 24, and 48 hours.

4.7.1 Antimicrobial activity of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L.-ethanolic extract incorporated Alginate-Tapioca starch based edible coating against pre-contaminated *Bacillus cereus* ATCC 128263 and *Salmonella* Typhimurium ATCC 13311 contaminated on carrot (*Daucus carota* L.)

The Number of pre-contaminated *B. cereus* (\log_{10} CFU/g) survived on pieces of carrot coated with edible films based solution contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., *Punica granatum* L. ethanolic extract and control solution (without plants extract) stored at room temperature (22°C) or cold condition (4°C) were given in Figure 86 and Figure 87.

Bacillus cereus pre-contaminated on carrot coated with edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. exhibited the survivability during 48 hours at room temperature (22°C) and at cold condition (4°C). The total log-reductions determined at 48 hours were less than 0.5 log-reductions. As expected, the numbers of bacteria increased on carrot coated with control edible coating stored at room temperature (22°C). The initial population of *Salmonella* Typhimurium pre-contaminated on carrot at *approx.* 4.00 \log_{10} CFU/g stayed steady until 24 hours after coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. and stored in room temperature (22°C), and then increased to *approx.* 6.00 \log_{10} CFU/g in 48 hours.

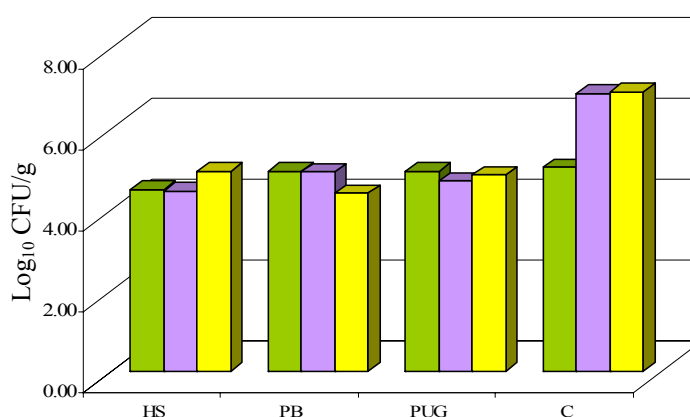


Figure 86 Number of pre-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of carrot coated with Alginate-Tapioca starch based edible coating contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) stored at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

In cold condition (4°C), the numbers of bacteria were in a constant stage. The reduction was monitored when Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract *Piper betle* L. was applied. At room temperature (22°C), the initial population was immediately reduced to 2.94 log₁₀ CFU/g in 0 hour and to 1.00 log₁₀ CFU/g in 24 hours, and few increased to 1.87 log₁₀ CFU/g at 48 hours of storage time. At cold condition (4°C), the initial population at approx. 4.00 log₁₀ CFU/g decreased directly to 2.94 log₁₀ CFU/g in 0 hour and decreased by log 1.94 reductions in 24 hours. The complete contamination elimination was observed in 48 hours.

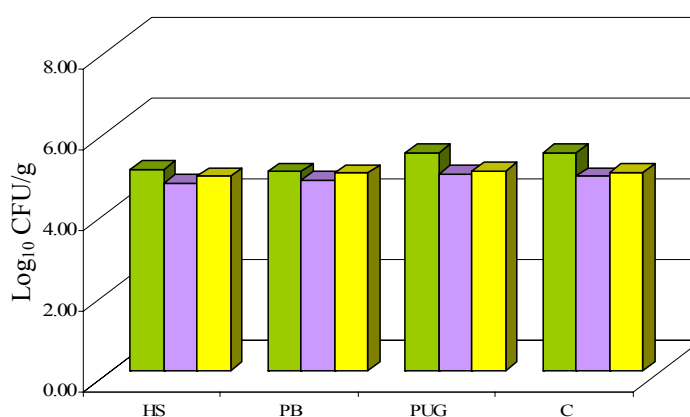


Figure 87 Number of pre-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of carrot coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) stored at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Punica granatum* L. had no ability to eliminate pre-contaminated *Salmonella* Typhimurium on carrot during 48 hours. The numbers of bacteria increased to 7.69 Log₁₀ CFU/g in 48 hours at room temperature (22°C). At cold condition (4°C), the numbers of bacteria stayed in steady stage (Figure 88 and Figure 89)

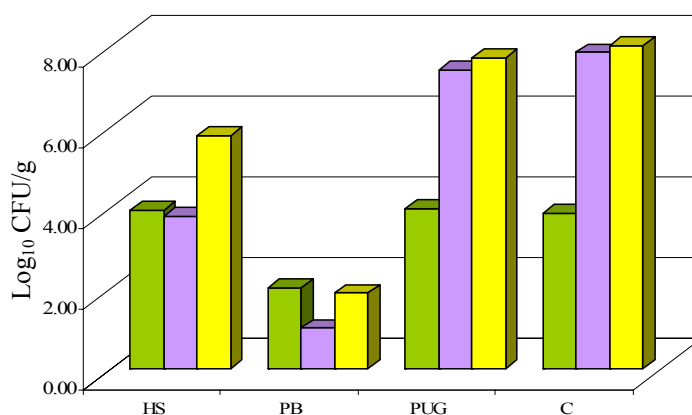


Figure 88 Number of pre-contaminated *Salmonella Typhimurium* (\log_{10} CFU/g) survived on pieces of carrot coated with Alginata-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

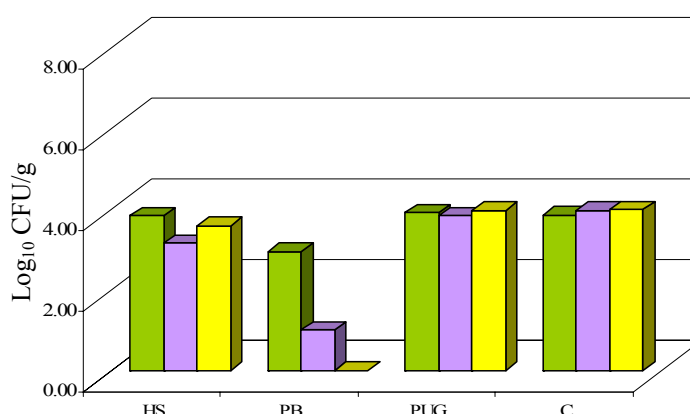


Figure 89 Number of pre-contaminated *Salmonella Typhimurium* (log₁₀ CFU/g) survived on pieces of carrot coated with Algininate-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

4.7.2 Antimicrobial activity of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L.-ethanolic extract incorporated Algininate-Tapioca starch based edible coating against post-contaminated *Bacillus cereus* ATCC 128263 and *Salmonella Typhimurium* ATCC 13311 contaminated on carrot (*Daucus carota* L.)

The number of pre-contaminated *B. cereus* (log₁₀ CFU/g) survived on pieces of carrot coated with edible films based solution contained 20.0 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., *Punica granatum* L. ethanolic extract and control solution (without plants extract) stored at room temperature (22°C) or cold condition (4°C) were showed in Figure 90 and Figure 91.

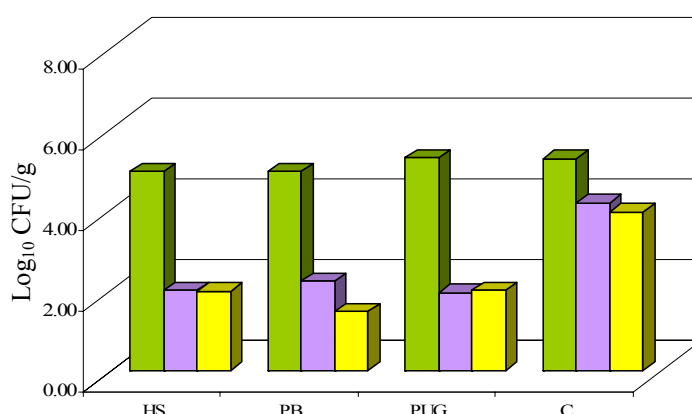


Figure 90 Number of post-contaminated *Bacillus cereus* (\log_{10} CFU/g) survived on pieces of carrot coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

At room temperature (22°C), *Bacillus cereus* was reduced to 1.99 \log_{10} CFU/g in 24 hours after contacted with carrot coated by Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., and then stayed stable until 48 hours. For carrot coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Piper betle* L., the initial population as *approx.* 5.00 \log_{10} CFU/g decreased to 2.21 and 1.47 \log_{10} CFU/g in 24 and 48 hours, respectively. Carrot coated with Alginde-Tapioca starch based edible coating containig ethanolic extract of *Punica granatum* L. presented the same activity as previous. The numbers of bacteria decreased to 1.94 and 1.98 \log_{10} CFU/g in 24 and 48 hours, respectively (Figure 90). At cold condition (4°C), Alginde-Tapioca starch based edible coating incorporated with ethanolic extract of *Hisbiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. presented good antimicrobial activity against post-contaminated *Bacillus cereus* on coated carrot. The initial population of

Bacillus cereus at approx. 5.00 log₁₀ CFU/g was reduced to 1.99, 2.04, and 2.26 log₁₀ CFU/g after contacted with carrot coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L., respectively, in 48 hours.

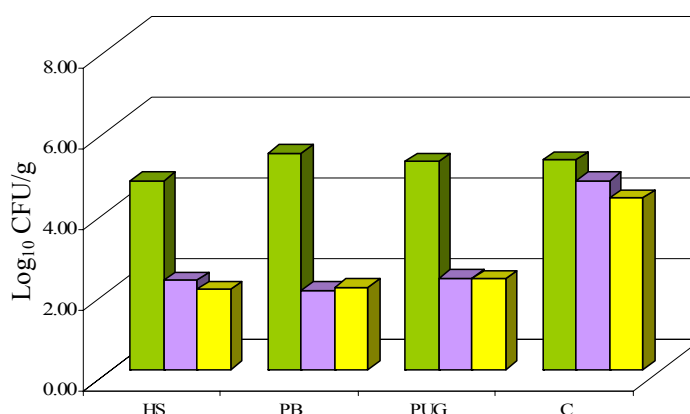


Figure 91 Number of post-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of carrot coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

In Figure 92, the survivors number of post-contaminated *Salmonella* Typhimurium (log₁₀ CFU/g) on carrot coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., *Punica granatum* L. ethanolic extract at room temperature (22°C) was presented.

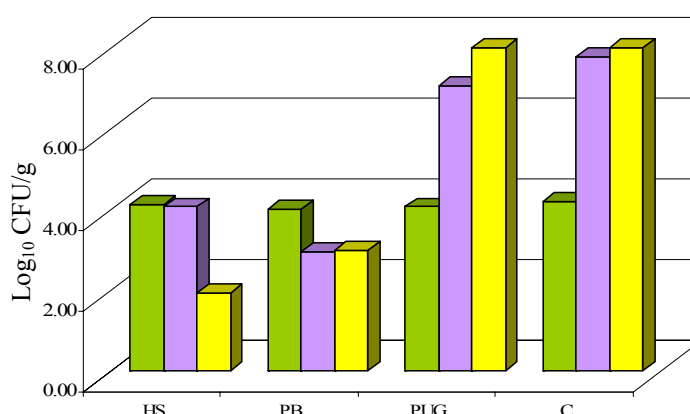


Figure 92 Number of post-contaminated *Salmonella* Typhimurium (\log_{10} CFU/g) survived on pieces of carrot coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

At room temperature (22°C), *Salmonella* Typhimurium contacted with carrot coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. was reduced from 4.11 \log_{10} CFU/g to 1.92 \log_{10} CFU/g in 48 hours. When Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Piper betle* L. was applied, bacteria were reduced from 3.99 \log_{10} CFU/g to 2.97 \log_{10} CFU/g in 48 hours. Comparison with control Alginde-Tapioca starch based edible coating stated that ethanolic extract coating of *Punica granatum* L. had no ability to reduce the post-contaminated *Salmonella* Typhimurium in 48 hours.

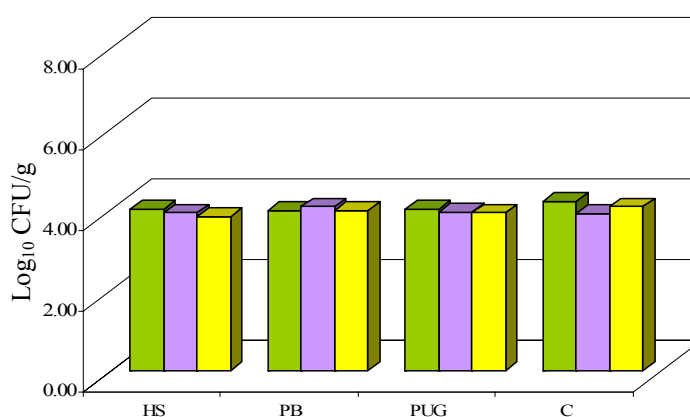


Figure 93 Number of post-contaminated *Salmonella* Typhimurium (\log_{10} CFU/g) survived on pieces of carrot coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

At cold condition (4°C), the population of *Salmonella* Typhimurium presented constant stage during 48 hours storage period after contacted with carrot coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. (Figure 93).

4.7.3 Antimicrobial activity of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L.-ethanolic extract incorporated Alginate-Tapioca starch based edible coating against pre-contaminated *Bacillus cereus* ATCC 128263 and *Salmonella* Typhimurium ATCC 13311 contaminated on chicken meat

The population of pre-contaminated *Bacillus cereus* at approx. 5.00 log₁₀ CFU/g on chicken meat coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., *Punica granatum* L. stored at room temperature (22°C) stayed in constant stage during 48 hours (Figure 94).

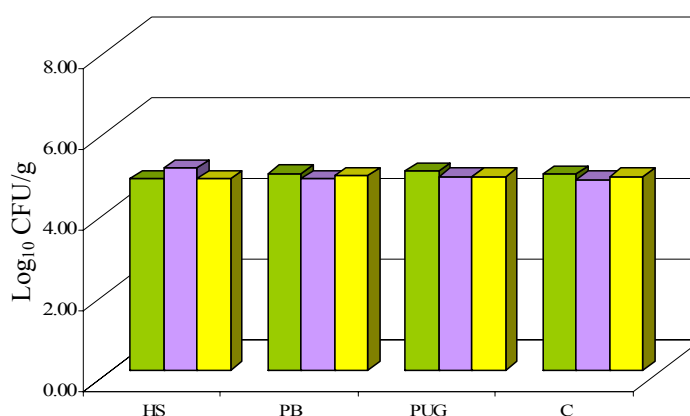


Figure 94 Number of pre-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of chicken meat coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

The antimicrobial activity of Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., *Punica*

granatum L. at cold storage condition (4°C) was given in Figure 95. Non significant changed in the numbers of bacteria could be observed during 48 hours storage time of all chicken meats coated with Alginate-Tapioca starch based edible coating.

The initial population of *Salmonella* Typhimurium pre-contaminated on chicken meat was reduced from 3.79 log₁₀ CFU/g to 3.37 log₁₀ CFU/g in 48 hours after coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Piper betle* L. also affected the population of bacteria on chicken meat. Bacteria were reduced from 4.10 log₁₀ CFU/g to 3.34 log₁₀ CFU/g in 48 hours. Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Punica granatum* L. gave log 0.36 reduction in 48 hours (Figure 96).

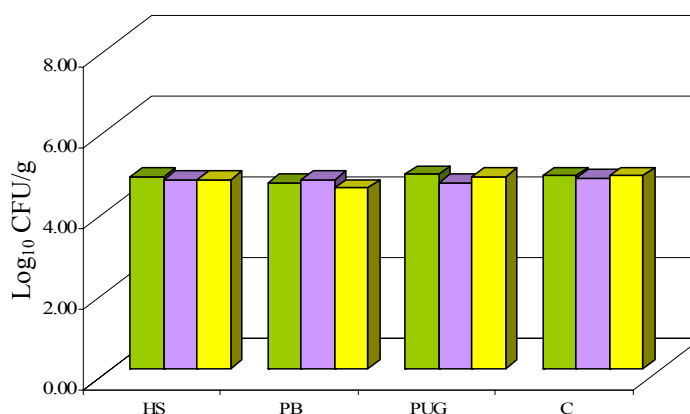


Figure 95 Number of pre-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of chicken meat coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

In Figure 97, the number of pre-contaminated *Salmonella* Typhimurium survived on pieces of chicken meat coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., *Punica granatum* L. ethanolic extract at cold condition (4°C) was presented. The initial population of *Salmonella* Typhimurium decreased after contacted with chicken meat coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract by 0.33 log-reductions and 0.66 log-reductions in 24 and 48 hours, respectively. In case of Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Piper betle* L., the detected log-reductions were 0.12 and 0.27 at 24 and 48 hours, respectively. At 20.0 % (v/v) ethanolic extract of *Punica granatum* L. incorporated in Alginate-Tapioca starch based edible coating demonstrated no activity during 48 hours of storage time.

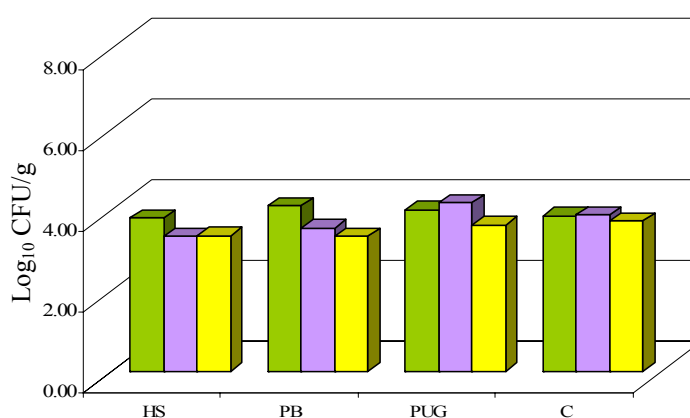


Figure 96 Number of pre-contaminated *Salmonella* Typhimurium (log₁₀ CFU/g) survived on pieces of chicken meat coated with Alginate-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

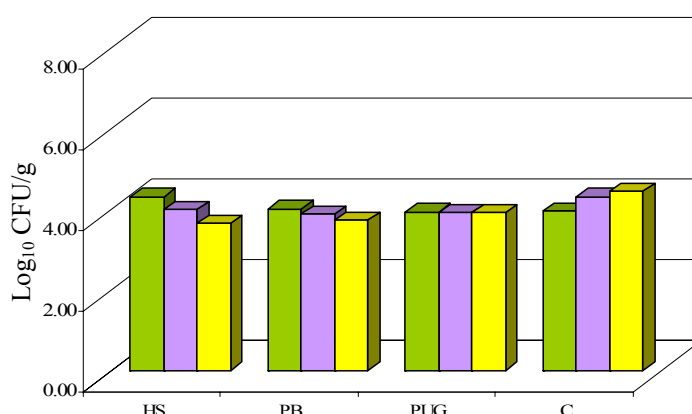


Figure 97 Number of pre-contaminated *Salmonella Typhimurium* (log₁₀ CFU/g) survived on pieces of chicken meat coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

4.7.4 Antimicrobial activity of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L.-ethanolic extract incorporated Alginde-Tapioca starch based edible coating against post-contaminated *Bacillus cereus* ATCC 128263 and *Salmonella Typhimurium* ATCC 13311 contaminated on chicken meat

The coated chicken meat with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. were contaminated with approx. 5.00 log₁₀ CFU/g *Bacillus cereus* and stored at room temperature (22°C), followed by the determination of survivors. For Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., the antimicrobial activity could not be determined during 48 hours. Similarly, Alginde-Tapioca starch based edible coating containing 20.0 %

(v/v) ethanolic extract of *Piper betle* L., and *Punica granatum* L. also showed no antimicrobial activity (Figure 98).

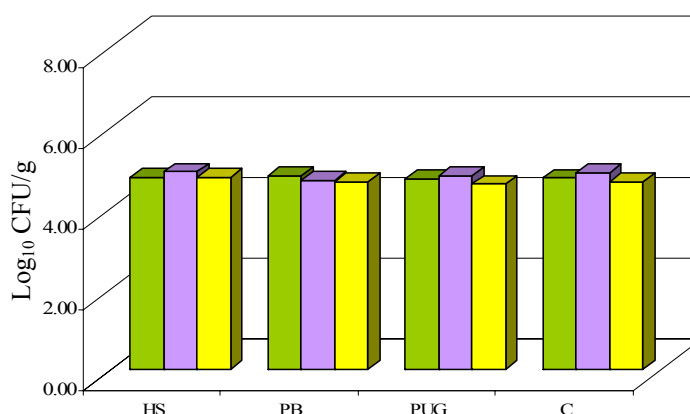


Figure 98 Number of pst-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of chicken meat coated with Alginde-Tapioca starch based edible coating contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

In figure 99, the numbers of post-contaminated *Bacillus cereus* ATCC 128263 survived on chicken meat coated with Alginde-Tapioca starch based edible coating contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., *Punica granatum* L. ethanolic extract stored at cold condition (4°C) were presented. The initial population of bacteria at approx. 5.00 log₁₀ CFU/g was maintained in constant level during 48 hours. Comparison between control coating and Alginde-Tapioca starch based edible coating contained ethanolic extract of 3 medicinal plants indicated that the incorporated edible coating had no antimicrobial effect.

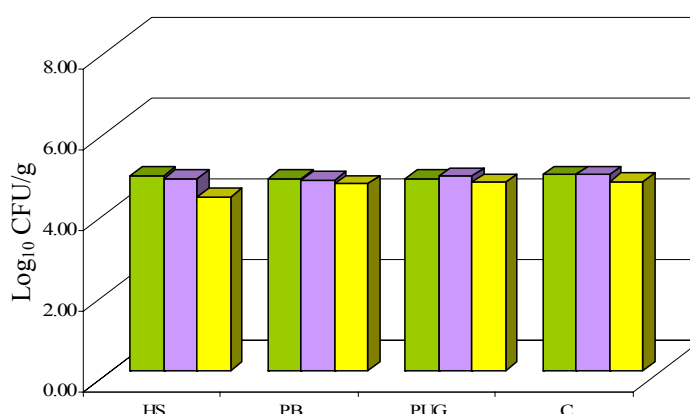


Figure 99 Number of post-contaminated *Bacillus cereus* (\log_{10} CFU/g) survived on pieces of chicken meat coated with Alginde-Tapioca starch based edible coating contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

Salmonella Typhimurium contaminated on coated chicken meat coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. and stored at room temperature (22°C) was reduced from approx. 4.00 \log_{10} CFU/g to undetectable level in 24 hours, and stayed in undetectable during 48 hours. Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Piper betle* L. showed the same effect. The initial population at approx. 4.00 \log_{10} CFU/g was reduced to undetectable level in 24 hours and stayed in the same stage during 48 hours. Bacteria were reduced to 3.59 \log_{10} CFU/g in 24 hours after contaminated on coated chicken meat coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Punica granatum* L., and the reduced to undetectable stage in 48 hours (Figure 100).

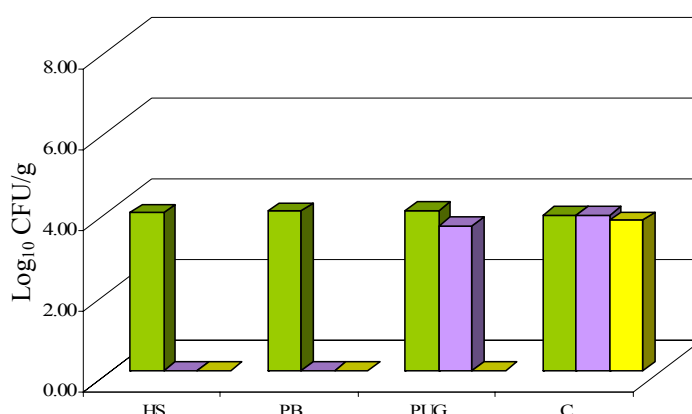


Figure 100 Number of post-contaminated *Salmonella Typhimurium* (log₁₀ CFU/g) survived on pieces of chicken meat coated with Alginde-Tapioca starch based edible coating contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

At cold condition (4°C), a few reduction was observed when *Salmonella Typhimurium* at approx. 4.00 log₁₀ CFU/g was contaminated on chicken meat coated with Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., log 0.38 reductions were measured at 48 hours storage time. For Alginde-Tapioca starch based edible coating 20.0 % (v/v) ethanolic extract of *Piper betle* L., and *Punica granatum* L., the measured log-reductions in 24 hours were approx. 0.25 for both medicinal plants extract (Figure 101).

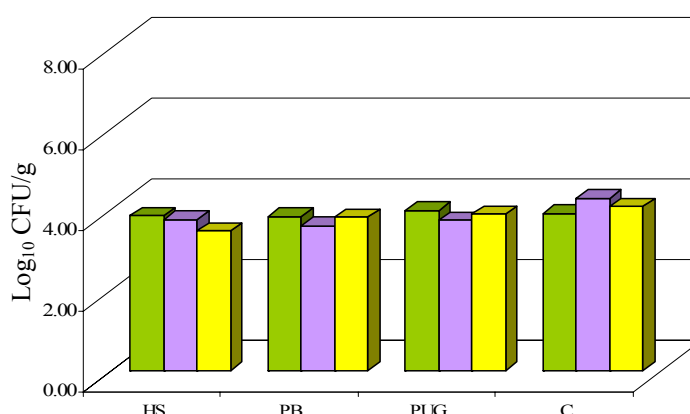


Figure 101 Number of post-contaminated *Salmonella Typhimurium* (log₁₀ CFU/g) survived on pieces of chicken meat coated with Alginate-Tapioca starch based edible coating contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

4.7.5 Antimicrobial activity of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L.-ethanolic extract incorporated Alginate-Tapioca starch based edible films against pre-contaminated *Bacillus cereus* ATCC 128263 and *Salmonella Typhimurium* ATCC 13311 contaminated on carrot (*Daucus carota* L.)

In Figure 102, the behavior of *Bacillus cereus* pre-contaminated on carrot after contacted with Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. stored at room temperature (22°C) was presented.

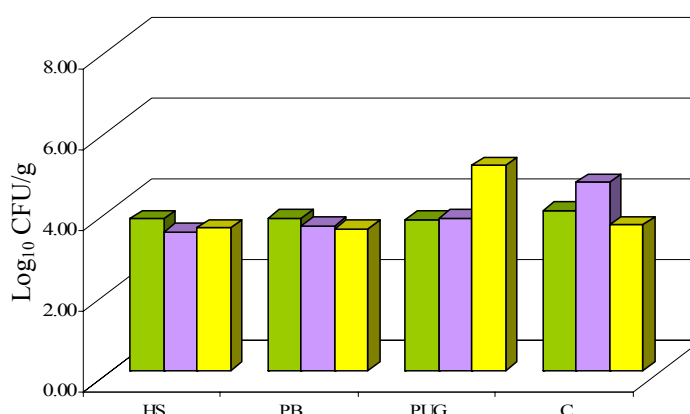


Figure 102 Number of pre-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of carrot wrapped with Alginde-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) stored at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

In the contacted with Alginde-Tapioca starch based edible films incorporated with *Hibiscus sabdariffa* L. the population was reduced only by 0.21 log-reduction in 48 hours. Alginde-Tapioca starch based edible films incorporated with ethanolic extract of *Piper betle* L. had the ability to reduce the population by 0.27 log-reductions in 48 hours. Ethanolic extract of *Punica granatum* L. had no effect against *Bacillus cereus* pre-contaminated on carrot, at the 48th hour of storage time the population decreased to 5.07 log₁₀ CFU/g from 3.73 log₁₀ CFU/g.

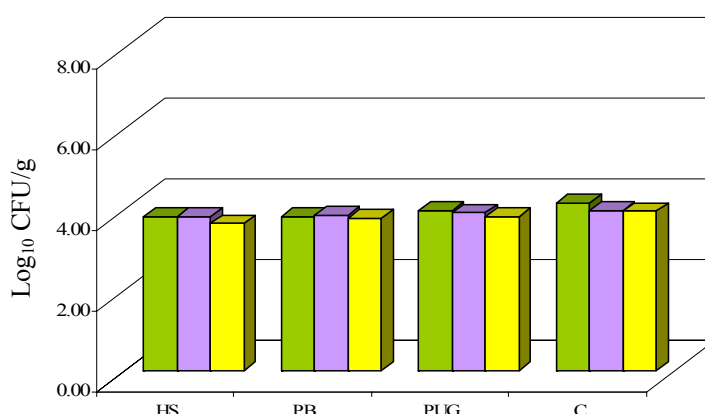


Figure 103 Number of pre-contaminated *Bacillus cereus* (\log_{10} CFU/g) survived on pieces of carrot wrapped with Alginde-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) stored at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

The population of *Bacillus cereus* pre-contaminated on carrot had no significant change during 48 hours of storage time at cold condition (4°C) after wrapped with Alginde-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. (Figure 103).

As shown in Figure 104, *Salmonella* Typhimurium survived on pieces of carrot wrapped with Alginde-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., *Punica granatum* L. ethanolic extract at room temperature (22°C) was presented. Alginde-Tapioca starch based edible coating contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. demonstrated good antimicrobial activity. The initial population as 3.97 \log_{10} CFU/g was reduced to 1.00 \log_{10} CFU/g in 24 hours and was reduced to undetectable level in 48 hours. The excellent activity was observed by Alginde-Tapioca starch based edible films

incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. The numbers of bacteria were reduced from 3.72 log₁₀ CFU/g to undetectable level in 24 hours and stayed in this stage during 48 hours. Log 1.05 reductions were found in 24 hours when the pre-contaminated carrot with *Salmonella* Typhimurium was wrapped with Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L. The numbers of cells increased again at the 48th storage time. The total reduction numbers were 0.50 log-reductions in 48 hours.

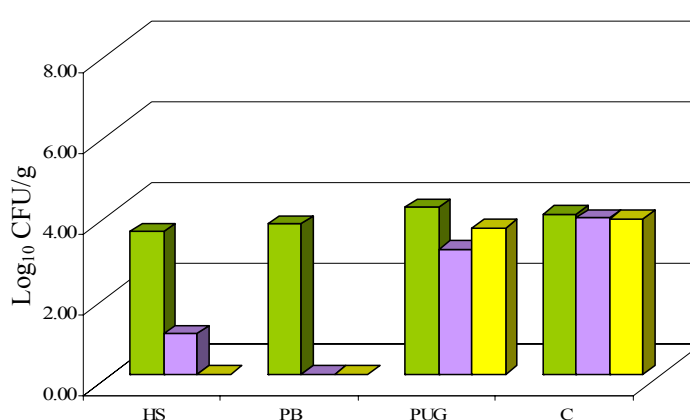


Figure 104 Number of pre-contaminated *Salmonella* Typhimurium (log₁₀ CFU/g) survived on pieces of carrot wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

At cold condition (4°C), the initial population of *Salmonella* Typhimurium pre-contaminated on carrot at 4.08 log₁₀ CFU/g was reduced to 2.97 log₁₀ CFU/g after short contacted with Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscis sabdariffa* L. At the end of storage period, 48 hours,

bacteria were reduced to 1.00 log₁₀ CFU/g. The total population reductions were 1.97 log₁₀ CFU/g.

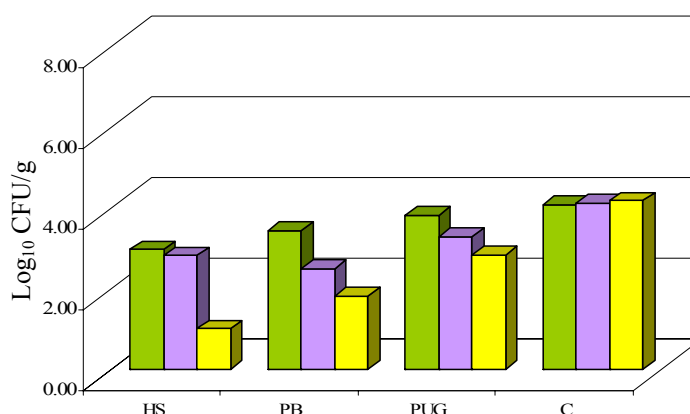


Figure 105 Number of pre-contaminated *Salmonella Typhimurium* (log₁₀ CFU/g) survived on pieces of carrot wrapped with Alginde-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

Alginde-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Piper betle* L. had ability to reduce pre-contaminated *Salmonella Typhimurium* on carrot from 3.43 log₁₀ CFU/g to 2.49 and 1.81 log₁₀ CFU/g after 24 and 48 hours storage time, respectively. The summary reductions were 1.62 log₁₀ CFU/g. The numbers of population of *Salmonella Typhimurium* on carrot decreased from 3.82 log₁₀ CFU/g to 3.28 log₁₀ CFU/g in 24 hours and at the end of storage period, the population decreased to 2.83 log₁₀ CFU/g after Alginde-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Punica granatum* L. was applied. Total reductions were 0.98 log₁₀ CFU/g (Figure 105).

4.7.6 Antimicrobial activity of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L.-ethanolic extract incorporated Alginate-Tapioca starch based edible films against post-contaminated *Bacillus cereus* ATCC 128263 and *Salmonella* Typhimurium ATCC 13311 contaminated on carrot (*Daucus carota* L.)

The number of post-contaminated *Bacillus cereus* survived on pieces of carrot wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., *Punica granatum* L. ethanolic extract stored at room temperature (22°C) was presented in Figure 21. *Bacillus cereus* was reduced by 0.24 log-reductions at the 48th hour of storage period after contaminated on carrot wrapped by Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L.

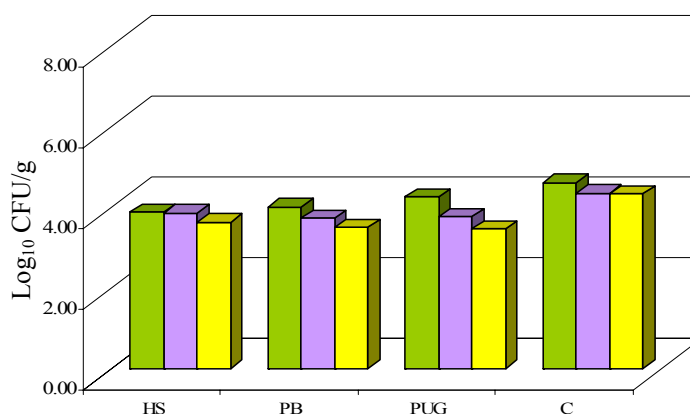


Figure 106 Number of post-contaminated *Bacillus cereus* (\log_{10} CFU/g) survived on pieces of carrot wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) stored at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

Log 0.51 reductions were observed against Alginate-Tapioca starch based edible films contained 20.0 % (v/v) *Piper betle* L. when *Bacillus cereus* were contaminated onto wrapped carrot. The initial populations of *Bacillus cereus* at 4.24 log₁₀ CFU/g decreased to 3.75 log₁₀ CFU/g in 24 hours and then decreased to 3.46 log₁₀ CFU/g in 48 hours after the bacteria were contaminated onto carrot wrapped with Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L.

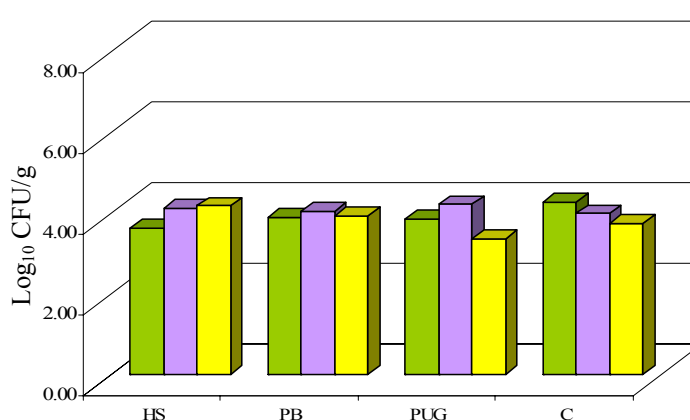


Figure 107 Number of post-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of carrot wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) stored at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

At cold condition (4°C), the population of *Bacillus cereus* increased first from 3.81 log₁₀ CFU/g to 4.11 log₁₀ CFU/g in 24 hours and then again to 4.16 log₁₀ CFU/g in 48 hours after contaminated onto wrapped carrot with Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic

extract of *Piper betle* L. caused the change in the population of *Bacillus cereus* contaminated onto the wrapped carrot and stored in cold condition. The bacteria numbers changed from 3.88 log₁₀ CFU/g to 4.05 log₁₀ CFU/g in 24 hours and then expressed a few abetments, the population changed to 3.92 log₁₀ CFU/g in 48 hours. Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Punica granatum* L. wrapped on carrot caused the population change of *Bacillus cereus*. The population changed from 3.85 log₁₀ CFU/g at the initial stage to 4.20 log₁₀ CFU/g in 24 hours and then decreased to 3.36 Log₁₀ CFU/g in 48 hours (Figure 107).

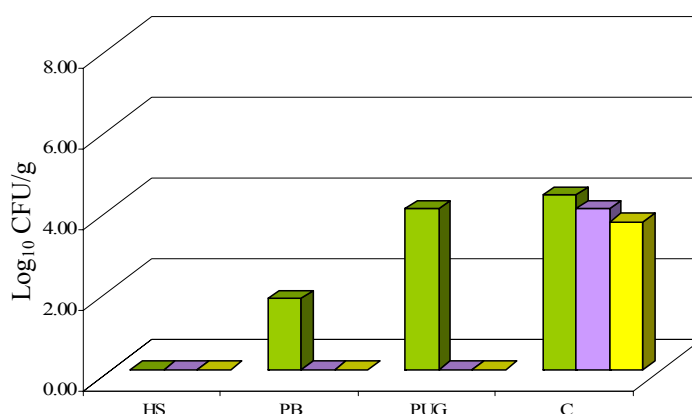


Figure 108 Number of post-contaminated *Salmonella* Typhimurium (log₁₀ CFU/g) survived on pieces of carrot wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

The number of post-contaminated *Salmonella* Typhimurium survived on pieces of carrot wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. ethanolic extract at

room temperature (22°C) were presented in Figure 108. The initial population of *Salmonella* Typhimurium 1 at 4.32 log₁₀ CFU/g decreased immediately to the undetectable level in short time after contaminated onto carrot wrapped by Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. The undetectable level was still observed during 48 hours of storage time. The population of *Salmonella* Typhimurium contaminated on carrot wrapped by Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. was reduced instantaneously to 1.75 Log₁₀ CFU/g in the short period of contact. The undetectable level was detected at 24 hours. At 48 hours, the same results were obtained. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L., also caused the change of contaminated population of *Salmonella* Typhimurium on wrapped carrot. The initial population at 3.98 log₁₀ CFU/g was immediately decreased to undetectable level in 24 hours and the same results were detected at the 48th hour storage period.

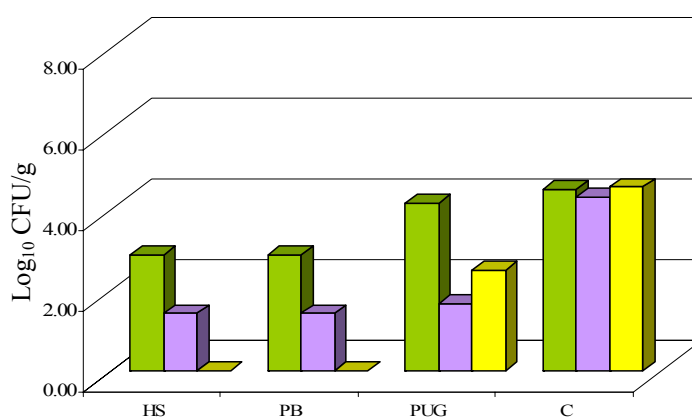


Figure 109 Number of post-contaminated *Salmonella* Typhimurium (log₁₀ CFU/g) survived on pieces of carrot wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

In Figure 109, the number of post-contaminated *Salmonella* Typhimurium survived on pieces of carrot wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. ethanolic extract at cold condition (4°C) were presented.

The initial population of *Salmonella* Typhimurium at 4.49 log₁₀ CFU/g contaminated on wrapped carrot with Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. was reduced in the short contact period to 2.88 log₁₀ CFU/g. After the storage time at 24 hours, the population was reduced to 1.43 log₁₀ CFU/g and the undetectable level occurred at 48 hours. The same effect was also detected against Alginate-Tapioca starch based edible films containing 20.0 % (v/v) ethanolic extract of *Piper betle* L. The population of *Salmonella* Typhimurium decreased to 2.88 log₁₀ CFU/g in the short period of contraction and then to 1.43 log₁₀ CFU/g in 24 hours. The population decreased to undetectable level in 48 hours. The initial population of *Salmonella* Typhimurium at 4.16 log₁₀ CFU/g decreased to 1.67 log₁₀ CFU/g after contaminated on carrot wrapped by Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L. in 24 hours. The population was increased again to 2.49 log₁₀ CFU/g in 48 hours.

4.7.7 Antimicrobial activity of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L.-ethanolic extract incorporated Alginate-Tapioca starch based edible films against pre-contaminated *Bacillus cereus* ATCC 128263 and *Salmonella* Typhimurium ATCC 13311 contaminated on chicken meat

The pre-contaminated chicken meats with *Bacillus cereus* were wrapped with Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. and stored in room temperature (22°C). The survivors were examined at different time. There was no antimicrobial activity of Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic

extract of *Hibiscus sabdarifa* L. during 48 hours of storage period. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. expressed the same effect. The population of *Bacillus cereus* pre-contaminated on chicken meat had no significant change during 48 hours of storage period. The population of *Salmonella* Typhimurium pre-contaminated on chicken meat presented also no significant change after Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L. was applied (Figure 110).

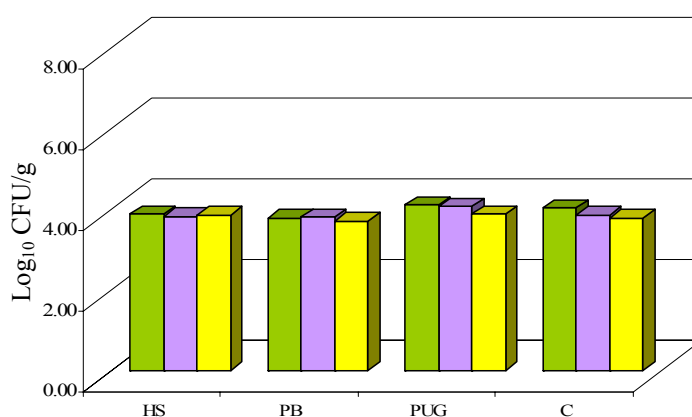


Figure 110 Number of pre-contaminated *Bacillus cereus* (\log_{10} CFU/g) survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

In Figure 111, the number of pre-contaminated *Bacillus cereus* survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. ethanolic extract stored at cold condition (4°C) was presented. The initial population of *Bacillus*

Bacillus cereus contaminated on chicken meat changed from 4.07 log₁₀ CFU/g to 3.79 log₁₀ CFU/g in 24 hours and changed to 3.74 log₁₀ CFU/g in 48 hours after Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Hibiscus sabdarifa* L. was wrapped on the contaminated chicken meat. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Piper bettle* L. also presented the effect in population change. The population of *Bacillus cereus* at 3.97 log₁₀ CFU/g pre-contaminated on chicken meat changed to 3.74 log₁₀ CFU/g in 24 hours and changed to 3.98 log₁₀ CFU/g in 48 hours. In effect of Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L., the population decreased from 4.11 log₁₀ CFU/g to 3.57 log₁₀ CFU/g in 24 hours and increased again to 4.03 log₁₀ CFU/g in 48 hours.

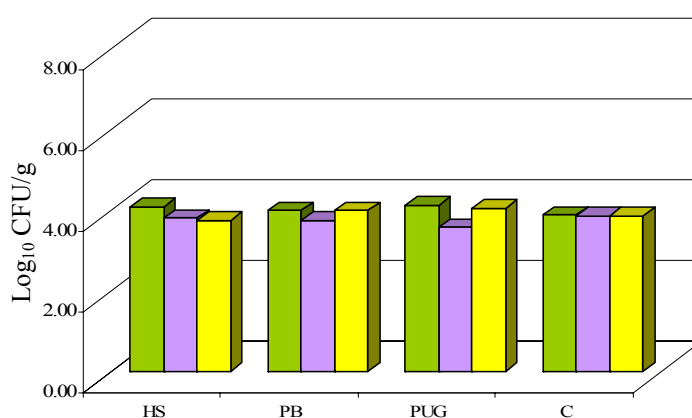


Figure 111 Number of pre-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper bettle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

The number of pre-contaminated *Salmonella* Typhimurium survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. ethanolic extract stored at room temperature (22°C) was given in Figure 112.

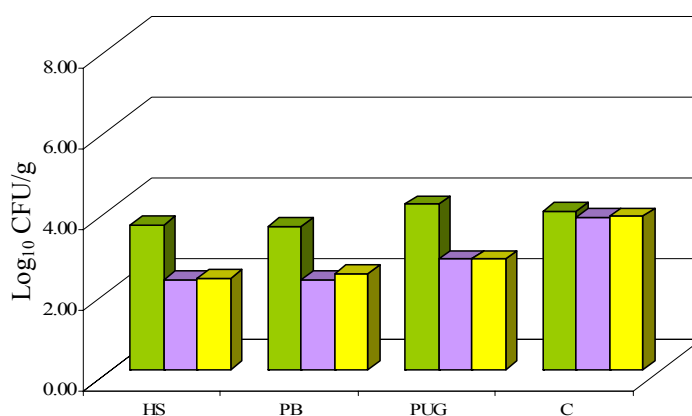


Figure 112 Number of pre-contaminated *Salmonella* Typhimurium (\log_{10} CFU/g) survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

The population of *Salmonella* Typhimurium contaminated on chicken meat was reduced from 3.58 \log_{10} CFU/g to 2.24 \log_{10} CFU/g in 24 hours and stayed stable during 48 hours of storage period after the use of Alginate-Tapioca starch based edible films containing 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. also showed the antimicrobial activity against the population of *Salmonella* Typhimurium contaminated on chicken meat. The population was reduced from 3.54 \log_{10} CFU/g to 2.23 \log_{10} CFU/g in 24 hours and stayed in the

constant stage during 48 hours storage time. The initial population of *Salmonella* Typhimurium at 4.09 log₁₀ CFU/g was reduced to 2.74 log₁₀ CFU/g in 24 hours after Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L. wrapped on the contaminated chicken meat.

At cold condition (4°C), the initial population of pre-contaminated *Salmonella* Typhimurium decreased from 4.14 log₁₀ CFU/g 3.40 log₁₀ CFU/g in the first period of storage time and decreased to 3.09 log₁₀ CFU/g in 24 hours after contacted with Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. Bacteria stayed in constant stage until 48 hours of storage time.

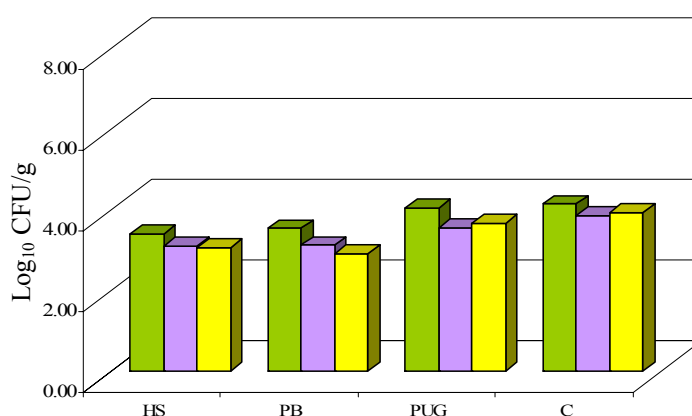


Figure 113 Number of pre-contaminated *Salmonella* Typhimurium (log₁₀ CFU/g) survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

The antimicrobial activity of Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. against *Salmonella* Typhimurium contaminated on chicken meat was also detected. The population of *Salmonella* Typhimurium was reduced from 4.14 log₁₀ CFU/g to 3.53 log₁₀ CFU/g in the first period of storage time. The population was also reduced to 3.11 log₁₀ CFU/g and 2.91 log₁₀ CFU/g in 24 and 48 hours, respectively. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L. subjected to the population change of *Salmonella* Typhimurium. The initial population at 4.04 log₁₀ CFU/g decreased to 3.56 log₁₀ CFU/g in 24 hours and then the population was reduced to 3.66 Log₁₀ CFU/g in 48 hours (Figure 113).

4.7.8 Antimicrobial activity of *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L.-ethanolic extract incorporated Alginate-Tapioca starch based edible films against post-contaminated *Bacillus cereus* ATCC 128263 and *Salmonella* Typhimurium ATCC 13311 contaminated on chicken meat

In Figure 114, the number of post-contaminated *Bacillus cereus* survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. stored at room temperature (22°C) was presented.

The population of *Bacillus cereus* changed from 3.71 log₁₀ CFU/g to 4.38 log₁₀ CFU/g in 24 hours after contact with Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. stored in room temperature (22°C). The population changed again to 3.79 log₁₀ CFU/g after the wrapped chicken meat was stored for 48 hours. When the Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Piper betle* L. was applied, the population decreased from 4.63 log₁₀ CFU/g to 4.18 log₁₀ CFU/g in 24 hours and then decreased to 3.70 log₁₀ CFU/g in 48 hours. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L. expressed the reduction effect. The population of *Bacillus cereus* at 4.32

Log_{10} CFU/g was reduced to 3.76 log_{10} CFU/g in 24 hours. The population showed a few increase to 3.94 log_{10} CFU/g in 48 hours. The total log-reductions were 0.37 log cycle.

Chicken meat was wrapped with Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Hibiscus sabdarifa* L. and the suspension of *Bacillus cereus* was contaminated and storage at cold condition (4°C). The population was reduced from 3.713 log_{10} CFU/g to 3.72 log_{10} CFU/g in 24 hours and increased again to 4.03 log_{10} CFU/g in 48 hours.

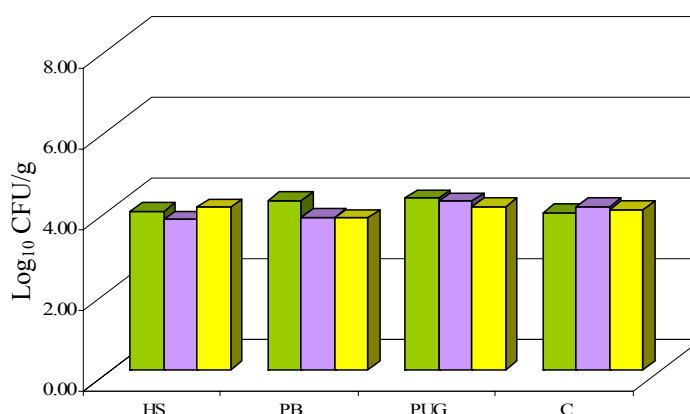


Figure 114 Number of post-contaminated *Bacillus cereus* (log_{10} CFU/g) survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

The initial population of *Bacillus cereus* at 4.18 log_{10} CFU/g was reduced to 3.77 log_{10} CFU/g in 24 hours and still stayed in constant stage until 48 hours after contact with Alginate-Tapioca starch based edible films incorporated 20.0 % (v/v) ethanolic

extract of *Piper betle* L. The population of *Bacillus cereus* decreased from 4.25 log₁₀ CFU/g to 4.17 and 4.04 log₁₀ CFU/g in 24 and 48 hours, respectively, when the Alginate-Tapioca starch based edible films contained with 20.0 % (v/v) ethanolic extract of *Punica granatum* L was applied (Figure 115).

For the results of *Salmonella* Typhimurium, the number of post-contaminated *Salmonella* Typhimurium survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L., *Piper betle* L., and *Punica granatum* L. stored at room temperature (22°C) was showed in Figure 116.

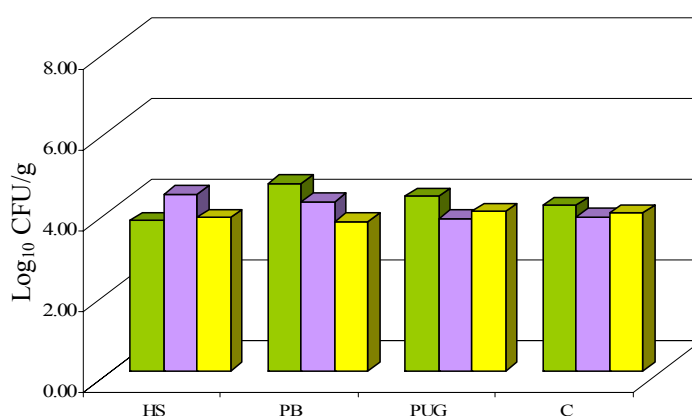


Figure 115 Number of post-contaminated *Bacillus cereus* (log₁₀ CFU/g) survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

Chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdariffa* L. and suspension of *Salmonella*

Typhimurium at *approx.* 4.00 log₁₀ CFU/g was contaminated on the wrapped chicken meat. The population was reduced to 3.20 at the short period on storage time, and decreased to undetectable level in 24 hours. At 48 hours, the undetectable level occurred. The initial populations of *Salmonella* Typhimurium decreased to 2.51 log₁₀ CFU/g in the short period of storage time after contact with chicken meat wrapped with Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Piper bettle* L. The reduction to undetectable level was observed at the 24th hour of storage period. The undetectable level also determined during 48 hours of storage period. Antimicrobial effect was also observed against Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Punica granatum* L. The initial populations were reduced from *approx.* 4.00 log₁₀ CFU/g to 2.69 log₁₀ CFU/g in 24 hours, and then the population decreased to the undetectable level in 48 hours.

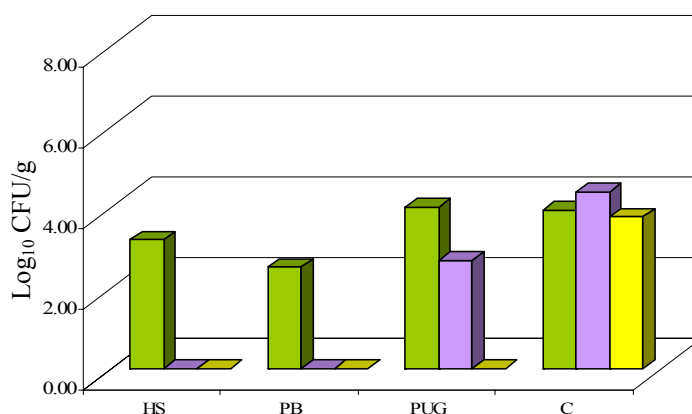


Figure 116 Number of post-contaminated *Salmonella* Typhimurium (log₁₀ CFU/g) survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at room temperature (22°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

In cold condition (4°C), the initial number of cells of *Salmonella* Typhimurium contaminated on wrapped chicken meat by Alginate-Tapioca starch based edible films contained 20.0 % (v/v) ethanolic extract of *Hibiscus sabdarifa* L. at 4.10 log₁₀ CFU/g decreased to 2.01 log₁₀ CFU/g in 24 hours and expressed the reduction to undetectable level in 48 hours. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Piper bettle* L. also showed good effect. The population decreased from approx. 4.00 log₁₀ CFU/g to 3.27 log₁₀ CFU/g in the short period of storage and decreased to 2.38 log₁₀ CFU/g in 24 hours. At 48 hours, the undetectable stage was observed. Among the Alginate-Tapioca starch based edible films, Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Punica granatum* L. expressed weak ability. The population was reduced from 4.15 log₁₀ CFU/g to 3.69 and 3.05 log₁₀ CFU/g in 24 and 48 hours of storage time, respectively (Figure 117).

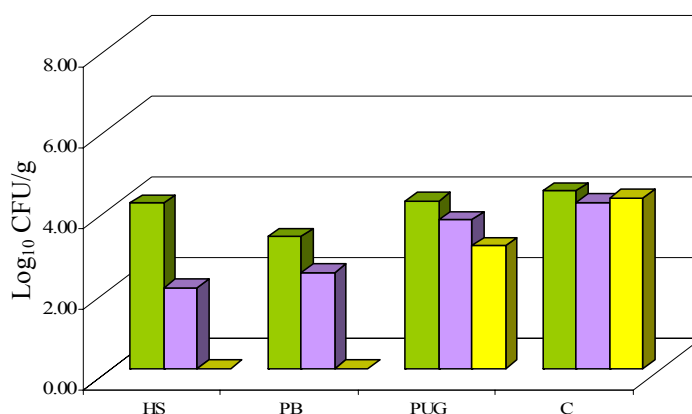


Figure 117 Number of post-contaminated *Salmonella* Typhimurium (log₁₀ CFU/g) survived on pieces of chicken meat wrapped with Alginate-Tapioca starch based edible films contained 20 % (v/v) *Hibiscus sabdariffa* L. (HS), *Piper betle* L. (PB), *Punica granatum* L. (PUG) ethanolic extract and control solution (C) (without plants extract) at cold condition (4°C) for 0 hour (■), 24 hours (■), and 48 hours (■). (Values represent mean of 3 independent trials)

5 DISCUSSION

5.1 Antimicrobial Properties of Nine Thai Traditional Medicinal Plants Determined by the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC)

The results of the determinations of the antimicrobial activity of all Thai traditional medicinal plants extracts and tested bacterial strains are presented as MIC- and MBC-values in Figure 16-24. The values of the MIC are those obtained after 72 hours incubation at 37°C, MBC were determined by streaking out a sample on TSA and incubation for further 24 hours at 37°C.

The results demonstrated a wide range of activities of the different Thai traditional medicinal plants and extracts against the tested organisms. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal concentration (MBC) of nine Thai traditional medicinal Plants were determined using the broth dilution assay cited by the DVG. The MIC value was defined as the lowest concentration that completely inhibited the growth (Hammer *et al.*, 1999; Delaquis *et al.*, 2002) and was determined by visual observation. As the same concept, the MBC value was defined as the lowest concentration at which 99.9% of the test organisms were killed and no growth was observed after incubating.

The Minimum Inhibitory Concentration and the Minimum Bactericidal Concentration are the accepted and well used criterion for measuring the susceptibility of microorganisms to inhibitors. Many factors affected the MIC-value and MBC-value obtained, including temperature, inoculum size and type of organism (Lambert, 2000). For instance, if an inoculum size was reduced by half but remained the same level of inhibitor, there was now twice as much inhibitor per cell. If inhibitor was not in a vast excess over the cellular contents, then this might have had an effect on the level of inhibitory observation. Parallel to the determination of the MIC- and MBC-values suitable neutralizer combinations were checked in the broth dilution assay.

According to the presented results, the Minimum Inhibitory Concentration was not detectable in many cases because of the reaction between plants extract and microbiological media. The effect was seen when using extracts of the tested plant extracts. Due to the fact that the MIC-values should be determined by visual examination of growth in a liquid medium and the mixed solution became cloudy as soon as the plant extract was added into the broth, MBC-values were determined in parallel. The same problem occurred when checking suitable neutralizers.

The inactivation or neutralization of the disinfectant activity is an important part of effectiveness testing methods. In the practice of activity and effectiveness tests, the contact time has to be measured carefully. Therefore, an active has to be inactivated, neutralised or removed at the end of its exposure to the test organism. This can be done by using a form of neutralization solution. The neutralization solution which is used in this tests should be “neutral” in other word it should neither have any influence on the work of the disinfectant activity (not supporting the disinfectant activity) nor have a negative, toxic or inhibiting effect on the test organisms. If a bactericidal activity is not inactivated or removed it may exert a bacteriostatic effect on the test organisms in the recovery medium (Hunsinger, 2005).

A substance selected as a neutraliser ideally should not have an inhibitory effect on the test organisms; it should totally neutralise the active against which it is being used and the resultant product should be non-toxic to microorganisms or cell cultures (MacKinnon, 1974).

Therefore a well proven combined neutralizers consisting of 0.3% polysorbate 80, 0.3% Lecithin, 3.0% Saponin, and 0.1% Histidin was chosen according the laboratory experience. This neutralizer was able to inactivate the extracts up to 15.0 g/100 ml, and was used for the further testing.

5.1.1 Dried leaves of *Andrographis paniculata* (Burm.f.) Wall. ex Nees

The ultrasonication extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees had no activity against any type of test organisms. However, the hot water extract presented an activity against *Bacillus cereus* ATCC 11778. In the other hand, Prajjal *et al.* (2003) reported that the water extract of *Andrographis paniculata* (Burm. f) Nees had an antibacterial activity against several microorganisms. The report stated that the water extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees at a concentration of 10 mg/disc determined by antibacterial disc diffusion assay method had antimicrobial property against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*.

The ethanolic extract showed greater antibacterial activity, especially against all strains of the tested Gram-positive organisms. The lowest MBC-value was 3.0 g/100 ml when using *Bacillus cereus* ATCC 11778 as test organism. As described by Pluemjai (1992), the 70% and 80% alcoholic extracts from *Andrographis paniculata* (Burm.f.) Wall. ex Nees leaf was tested against diarrhoea and respiratory tract bacteria. The results indicated that both alcoholic extracts showed high activity. However, at the same value of the Minimum Inhibitory Concentration (MIC), the 85% alcoholic extract which contained 8.30 mg of andrographolide showed significant antibacterial activity against *Escherichia coli*, *Salmonella* Krefeld, *Salmonella* Typhimurium, *Vibrio cholerae* 01 and *Shigella dysenteriae*, whereas the 70% alcoholic extract showed less antibacterial activity.

A reason for these different results compared with this present study may be due to the different methods that were used. Another possible reason could be the different content of active constituents of *Andrographis paniculata* (Burm.f.) Wall. ex Nees leaf in the studies. According to Pluemjai (1992), the plants material in her studies contained 6.77% andrographolide in 70% alcoholic extract and 8.30% andrographolide in 85% alcoholic extract. There was no quantitative chemical experiment in the actual study, so the contents of the active compounds were not determined.

In the clinical experiment, 1 gram of *Andrographis paniculata* (Burm.f.) Wall. ex Nees leaf powder in capsule was given to the diarrhoea group of patients in the hospital. Chturvedi *et al.* (1983) found an overall effectiveness of *Andrographis paniculata* (Burm.f.) Wall. ex Nees leaves on bacterial dysentery and diarrhoea and Thanangkul and Chaichantipyuth (1985) found that *Andrographis paniculata* (Burm.f.) Wall. ex Nees had effect on curing diarrhoea and bacillary dysentery in the group of patients in Ramatipbodee Hospital in Bangkok, Thailand. Dhamma-Upakorn *et al.* (1992) and Sawasdimongkol *et al.* (1990) found that the *Andrographis paniculata* (Burm.f.) Wall. ex Nees extract had an effect to reduce the movements of the smooth muscle in stomach and intestinal tract in human.

A possible reason for antimicrobial activity could be the effect of bioactive compounds found in *Andrographis paniculata* (Burm.f.) Wall. ex Nees, andrographolide and neoandrographolide. There are some studies which reported the bioactive compounds in *Andrographis paniculata* (Burm. f) Nees., neoandrographolide, which are possibly responsible for the antibacterial activity (Chan *et al.*, 1968). Yin and Guo (1993) found that a dose of 500 mg per day for six day of andrographolide extract from *Andrographis paniculata* (Burm.f.) Wall. ex Nees was effective on acute bacterial diarrhoea in human patients.

Andrographolide is the major compound isolated from *Andrographis paniculata* (Burm.f.) Wall. ex Nees It is of colourless crystalline appearance with a very bitter taste and this compound was identified as lactone (SCHRI, 1973). There are four different lactones isolated from *Andrographis paniculata* (Burm.f.) Wall. ex Nees, including deoxyandrographolide (Andrographis A), andrographolide (Andrographis B), neoandrographolide (Andrographis C), and deoxydidehydroandrographolide (Andrographis D) (Dhamma-Upakorn *et al.*, 1992; Sangalungarn *et al.*, 1990; Deng *et al.*, 1982; Garcia *et al.*, 1980).

Beside the lactones other medicinal chemicals, also bitter principles which are deoxyandrographolide, 19 β -D-glucoside and neoandrographolide were isolated from

Andrographis paniculata (Burm.f.) Wall. ex Nees and were identified as diterpenoids (Techadamrongsin *et al.*, 1999; Chem and Liang, 1982; Cava *et al.*, 1965).

Diterpene lactones and flavonoids are also the active chemical constituents of *Andrographis paniculata* (Burm.f.) Wall. ex Nees. The main diterpenoids are 14-deoxyandrographis and 14-deoxy-11,12-didehydroandrographolide (Kongkathip, 1995; Matsuda *et al.*, 1994, Tang and Eisenbrandt, 1992; Kuroyanagi *et al.*, 1987; Zhu and Liu, 1984; Balmain and Connolly, 1973). Those compounds seem to be eluted by the alcohol so that more of them are in the extract.

5.1.2 Dried rhizomes *Curcuma zedoaria* (Christm.) Roscoe

Hot water and ultrasonication extracts of *Curcuma zedoaria* (Christm.) Roscoe had no significant effect against all tested microorganisms. An antibacterial activity of this plant was only found when using an ethanolic extract. The lowest MBC value was 0.5 g/100 ml (5.0 mg/ml), which was obtained against *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 13565, *Listeria monocytogenes* DSMZ 20600, and *Listeria monocytogenes* 101. The antimicrobial activity of extracts of *Curcuma zedoaria* (Christm.) Roscoe was also studied by Wilson *et al.* (2005). They studied the activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 9144, *Micrococcus luteus* ATCC 9341, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 2091 and *Aspergillus niger* ATCC 6275 using the agar well diffusion and broth dilution methods. The achieved results indicated that ethanol, petroleum ether, hexane, chloroform, and acetone extracts exhibited antibacterial as well as antifungal activity. The MIC values for the different strains and extracts ranged from 0.01 to 0.15 mg/ml.

Many *Curcuma* species are traditionally used for medicinal properties. Antifungal, antibacterial and anti-inflammatory activity has been reported for different species such as *Curcuma longa*, *Curcuma zedoaria*, *Curcuma aromatica* and *Curcuma amada* (Apisariyakul *et al.*, 1995; Yoshioka *et al.*, 1998; Negi *et al.*, 1999; Mujumdar *et al.*, 2000). These *Curcuma* tubers are utilized for their starch, which is comparable

to arrowroot starch (Jyothi *et al.*, 2003) and is highly valued as diet for infants and convalescents due to its cooling and demulcent properties. Wongseri (1995) reported the antimicrobial activity of *Curcuma zedoaria* (Christm.) Roscoe against 100 strains of clinical isolated bacteria from cancer patients. The extract showed antimicrobial activity against 18 strains of *Staphylococcus spp.* And 11 strains of *Streptococcus spp.* The chemical analysis of the extract resulted in the isolation of 3 curcuminoid compounds classified as curcumin, 4-hydroxycinamol (feruloyl) methane and bis (4-hydroxycinamoyl) methane. The results stated that curcumin, the major curcuminoid, was the most effective antibacterial agent. Moreover, *Curcuma zedoaria* (Christm.) Roscoe contained curcuminoids, including curcumin, demethoxycurcumin and bisdemethoxycurcumin and volatile oil including sesquiterpenes and monoterpenes. The major sesquiterpene compounds, including dehydrocurdione, furanodiene, germacrone, curdione, curcumenol, neocurdione, isocurcumenol, aerugidiol, zedoarondiol and curmenone were found to exhibit biological activities (Syu *et al.*, 1998; Yoshioka *et al.*, 1998; Mau *et al.*, 2003).

5.1.3 Dried pericarp of *Garcinia mangostana* L.

Ultrasonication extract of *Garcinia mangostana* L. showed an antibacterial activity against the tested *Bacillus* strains and Gram-positive test organisms, not including *Staphylococcus aureus* ATCC 13565 and *Staphylococcus aureus* DSMZ 799. Hot water extract of *Garcinia mangostana* L. seems to possess higher antibacterial activity against Gram-positive test organisms than the ultrasonication extract. On the other hand, Gram-negative test organism still showed a strong resistance to hot water extract of *Garcinia mangostana* L. The best antibacterial activity of *Garcinia mangostana* L. was obtained from the ethanolic extract. The same result was described by Inuma *et al.* (1996). In their studies the extract of *Garcinia mangostana* L. showed inhibitory effects against the growth of *Staphylococcus aureus* and some of components had also activity against methicillin-resistant *Staphylococcus aureus* (MRSA).

The ethanolic extract of *Garcinia mangostana* L. possessed antibacterial activity due to the biological compounds in this plant. Xanthose, terpenoids and sugar have been reported from the fruit hulls, and some of them have shown a variety of biological activities (Mahabusakam and Wiriyachitra, 1987; Praveen *et al.*, 1991; Suksamran *et al.*, 2002a; 2002b). Sundaram *et al.* (1983) reported that mangostin, xanthone, and 4 of its derivatives isolated from fruit of *Garcinia mangostana* L. were tested for their *in vitro* antibacterial and antifungal properties. Mangostin (C₂₀H₂₂O₅) was highly effective against both bacteria and fungi. Isomangostin and 3-O-methyl mangostin were highly effective against bacteria and fairly effective against fungi, while 3,6-di-O-methyl mangostin and mangostin triacetate had little effect on either bacteria or fungi. Furthermore, tannin was observed as a biological compound of pericarp of *Garcinia mangostana* L. The pericarp contained 7-13% tannin, aresin and a bitter principle called mangostin. A new xanthone with geranyl group, named mangostione, and 7 known xanthones (α -, β -, γ -mangostins, gartanin, garcinone E, 1,5-dihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone and 1,7-dihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone), were isolated from pericarp of *Garcinia mangostana* L. (Asai *et al.*, 1995; Nilar and Harrison, 2002). In addition, polyoxygenated xanthone, mangostanol, 8-deoxygartanin, 5,9-dihydroxy-2,2-dimethyl-8-methoxy-7-(3-methylbut-2-enyl)-2H,6H-pyranof[3,2-b]xanthen-6-one, 2-(γ , γ -dimethylallyl)-1,7-dihydroxy-3-methoxyxanthone, epicatechin, mangostenol (1), mangostenone A (2), mangostenone B (3), trapezifolixanthone, tovophyllin B (4), garcinone B, garcimangosone A, garcimangosone B, garcimangosone C, garcimangosone D, and benzophenone glucoside were isolated as new biological active compounds of *Garcinia mangostana* L. (Chairungrilerd *et al.*, 1996; Gopalakrishnan and Balaganesan, 2000; Huang *et al.*, 2001; Suksamran *et al.*, 2002a; 2002b).

Mangostin was obtained by boiling the parts of *Garcinia mangostana* L. in water, and tannin was removed by exhausting by boiling in alcohol and evaporating, the resulting product was mangostin and resin. Resin was precipitated by redissolving it in alcohol and water, and evaporating water. It occurred in small yellow scales, tasteless neutral, insoluble in water, but readily soluble in alcohol and ether (Nadkarni and Nadkarni, 1999). Among the biological compounds presented in *Garcinia mangostana* L.,

α -mangostin provides significant antibacterial activity (Iinuma *et al.*, 1996), α -mangostin is able to extract with alcoholic solvent (Sakami *et al.*, 2005), that could indicate that ethanolic extract of *Garcinia mangostana* L. would provide an outstanding antibacterial activity.

5.1.4 Dried flowers of *Hibiscus sabdariffa* L.

According to the presented results, the ultrasonication extract and the hot water extract of *Hibiscus sabdariffa* L. showed good antibacterial activity against all of the tested microorganisms at concentrations <5.0 g/100 ml (50 mg/ml) with exception of *Bacillus subtilis* ATCC 6633. Comparing the extraction methods, the ethanolic extract of *Hibiscus sabdariffa* L. demonstrated higher antibacterial activity than the hot water and the ultrasonication extracts. Similar to the report of Alian *et al.* (1983) and El-Shayeb and Mabrouk (1984), the results of the actual study indicated that a beverage containing *Hibiscus sabdariffa* L. was bactericidal against *Escherichia coli*, *Bacillus subtilis*, *Salmonella* Typhosa and *Klebsiella pneumoniae*. Additionally, the authors found an inhibitory effect of *Hibiscus sabdariffa* L. flower heads on the growth and aflatoxin production of toxigenic strain of *Aspergillus flavus*.

The chemical compositions and bioactivity of *Hibiscus sabdariffa* L. have been investigated and evaluated for a potential application (Nhung *et al.*, 1998). Several bioactive compounds; alkaloid, anthoxanthin, anthocyanins, L-ascorbic acid, aspartic acid, chrysanthemins, citric acid, cyaniding-3(2(G)-glycosyl)-rutinoside, cyaniding-3,5-diglucoside, cynidin-3- β -D-glucoside, cyaniding-3-sambubioside, cyanin, delphinidin, delphinidin-3-O- β -glucoside, delphinidin-3-monoglucoside, delphinidin-3-sambubioside, galactose, galacturonic acid, garcinia acid, glycolic acid, gossypetin, gossypetin-3-O- β -glucoside, gossypetin-7-glucoside, gossypetin-8-glucoside, gossypin, gossypitrin, gossytrin, heteoside, hibiscetin, hibiscic acid, hibiscin, hibiscitrin, hibiscus acid, malic acid, malvin, myrtillin, oxalic acid, pectin, , quercetin, resin, sabdaretin, sabdaretrin, β -sitosterol, tartaric acid; were obtained from the extract of flowers of *Hibiscus sabdariffa* L. (Seshadri and Thakur, 1961; Duke, 1992;

Fransworth and Bunyapraphatsara, 1992; Mueller and Franz, 1992; Tseng *et al.*, 1996; Ahmad *et al.*, 2000; Mounnissamy *et al.*, 2002).

The antimicrobial property of the extract of *Hibiscus sabdarifa* L. could be caused by the presenting of phenolic compounds such as protocatechuic acid, anthoxanthin, and anthocyanins, flavonoid glycoside such as gossypetin, gossypetin-3-O- β -glucoside, gossypetin-7-glucoside, gossypetin-8-glucoside, gossypin, gossypitrin, and gossytrin and organic acid such as L-ascorbic acid, aspartic acid, citric acid, galacturonic acid, garcinia acid, glycolic acid, hibiscic acid, hibiscus acid, malic acid, oxalic acid, and tartaric acid in the extract. Since the phenolic compounds, flavonoids and organic acids are attributed with antioxidant activity and chelating ability (Heim *et al.*, 2002). There were many reports on activities of these components (Tseng *et al.*, 1998; Jonadet *et al.*, 1990; Aboutabl *et al.*, 1999; Chewonarin *et al.*, 1999; Haji-Faraji and Haji Tarkhani, 1999; Onyenekwe *et al.*, 1999; Cheeptham and Tower, 2002; Lee *et al.*, 2002; Mounnissamy *et al.*, 2002; Chen *et al.*, 2003; Odigie *et al.*, 2003; Chen *et al.*, 2004; Amin and Hamza, 2005; Chang *et al.*, 2005; Hirunpanich *et al.*, 2005; Liu *et al.*, 2005) which correspond to the findings in the present study.

5.1.5 Dried blossom of *Musa sapientum* L.

The results obtained in the MIC- and MBC-value determination indicated that hot water and ultrasonication extracts of *Musa sapientum* L. had no bactericidal effect but the ethanolic extract showed antibacterial activity against the vegetative cells of the spore-forming bacteria *Bacillus cereus* ATCC 128263.

β -sitosterol, 12-hydroxystearic acid, palmitic acid and d-malic acid were bioactive compounds isolated from *Musa sapientum* L. (Mokbel and Hashinaga, 2005). An ethanolic extract of *Musa sapientum* L. was tested in the cited publication and significant antimicrobial activities were reported, while a water extract showed no activities. The bioactive compounds were tested against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Samonella* Enteritidis, and *Escherichia coli*. β -sitosterol and malic acid were active against all tested Gram-negative and Gram-

positive bacterial species, while palmitic acid was had less effect against all tested bacteria species. This study indicated that malic acid exhibited a stronger antibacterial activity compared to β -sistosterol and palmitic acid, while, 12-hydroxystearic acid recorded weak antimicrobial activity when measured by paper disk method. According to this investigation, it could be indicated that antimicrobial activity of the ethanolic extract of *Musa sapientum* L. is due to the present of those bioactive compounds.

5.1.6 Dried rhizome of *Nelumbo nucifera* Gaertn.

For *Nelumbo nucifera* Gaertn. (Figure 21), the hot water extract could not be collected, due to physical changes during the heating process. As described by Chiang and Luo (2007), the results pointed out that heating decomposes intercellular mucilage, resulting in a weakened cell wall, softened texture, and decreased fracturability. It was, therefore, speculated that heating would cause the loss of water-soluble polysaccharides, leading to decrease of fracturability. The decrease in relative hardness had an important relationship with the percentage of solid loss. From the experimental results of Rehman *et al.* (2003), it appeared that, due to the effects of heat during the heating process, the dietary fibre in the cell wall changes. Due to the pectin loss, damaged carbohydrates and soluble contents, solid loss heating process increased, causing a rapid decrease in the texture of the lotus rhizome. The contents of cellulose and hemi-cellulose are reduced as a result of boiling. Therefore, during the heating process, decrease in dietary fibre is one of the factors that cause the changes in texture of the lotus rhizome.

The ultrasonication extract of *Nelumbo nucifera* Gaertn., presented no antibacterial activity against all tested microorganisms. 8.0 g/100 ml (80 mg/ml) of the ethanolic extract was the lowest concentration that showed activity against *Bacillus cereus* ATCC 11778 and *Bacillus cereus* ATCC 128263 strains, and 0.5 g/100 (5.0 mg/ml) ml was necessary to exhibited antimicrobial activity against the Gram-negative bacteria *Vibrio parahaemolyticus* ATCC 20502. There was only few previous research carried out on the antimicrobial ability of the rhizome of *Nelumbo nucifera*

Gaertn. One was described by Mukherjee *et al.* (1995a; 1995b; 1995c). The authors stated that a chloroform extract of *Nelumbo nucifera* Gaertn. exhibited antimicrobial activity against *Staphylococcus aureus* ATCC 29737, *Escherichia coli* ATCC 10536, *Bacillus subtilis* ATCC 6633, *Bacillus pumilis* ATCC 14884, and *Pseudomonas aeruginosa* ATCC 25619. Furthermore, bioactive compounds including betulinic acid, demethylcoclaurine, kaempferol 3-*O*-glucoside, liensinine, liriodenine, lotusine, luteolin 7-*O*-glucoside, isoliensinine, neferine, nornuciferine, pronuciferine, methylcorypalline, norarmepavine, and nelumboside showed significant activity against 6 bacteria and *Candida albicans* (Anonymous, 1997).

5.1.7 Dried leaves of *Piper betle* L.

According to the results, ultrasonication and hot water extracts of *Piper betle* L. showed antibacterial activity against most of the tested microorganisms at concentrations < 5.0 g/100 ml (50 mg/ml). In comparison with the other tested medicinal plants the ethanolic extract of *Piper betle* L. possessed an outstanding antibacterial activity. The minimal MBC-values determined for *Yersinia enterocolitica* ATCC 2779 was 0.2 g/100 ml (2 mg/ml), whereas the limiting test organism was *Bacillus subtilis* ATCC 6633 where a MBC-value of 6.0 g/100 ml (60 mg/ml) was determined. The MBC-values of the other tested bacterial strains lay in between.

The *Piper betle* L. leaf extract had a chemical composition in accordance with the study of Rimando (1986) who reported the constituents of extract being chavibetol, chavibetol acetate, caryophyllene, allylpyrocatechol diacetate, campene, methyl chavibetol, eugenol, pinene, limonene, safrole, 1,8-cineole and allylpyrocatechol monoacetate. In addition, Atal *et al.* (1975) showed that extracts of *Piper betle* L. contain chavicol, allylpyrocatechol, chavibetol, methyl chavicol, methyl eugenol, 1,8-cineole, eugenol, caryophyllene and cadinene.

Different studies on the antimicrobial activity of *Piper betle* L. and its principal constituents have been reported. Most bioactive compounds are highly volatile and

show poor solubility in the aqueous phase (Friedman *et al.*, 2002). Carvacrol, eugenol and chavibetol, an isomer of eugenol, were among the most bioactive components isolated from *Piper betle* L. (Dorman and Deans, 2000; Friedman *et al.*, 2002). Mechanism of action of monoterpenes (e.g. 1,8-cineole, pinene and limonene), sesquiterpene (e.g. caryophyllene and cadinene), phenylpropanes (e.g. chavibetol, eugenol, methyl eugenol, chavicol, methyl chavicol) and phenol (e.g. carvacrol) (Pauli, 2001) in *Piper betle* L. should be similar to other terpenes and phenolic compounds as indicated an involvement in disruption of the cytoplasmic membrane and coagulation of cell content. Veldhuizen *et al.* (2006) have investigated the structural requirement for the antimicrobial activity of carvacrol. It was found that hydroxyl group and aliphatic side chains in carvacrol featured amphipathicity of this molecule, affecting the initial interaction with the bacterial membrane. The hydrophilic part of the molecule interacted with the polar part of the membrane, whereas the hydrophobic benzene ring and the aliphatic side chains were buried in the hydrophobic inner part of the bacterial membrane. Moreover, as a weakly acidic compound, carvacrol had a capacity to donate proton, involved in antimicrobial mode of action. It might diffuse back and forth through the bacterial membrane, while exchanging the acidic proton for another cation on the cytosolic side of the membrane and the opposite cation exchange at the exterior.

Ultee *et al.* (1999) studied the mechanisms of action of carvacrol on the foodborne pathogen *Bacillus cereus*. Carvacrol made the cell membrane permeable for K^+ and H^+ and, consequently, inhibited ATP synthesis by dissipating the proton motive force. Based on these findings, Ultee and Smid (2001) hypothesized that, during exposure to carvacrol, the driving force for optimal secretion of the toxin is not sufficient, resulting in accumulation of the toxin inside the cell. Hence, intracellular toxin might destroy its own synthesis, so called feedback inhibition.

Chavibetol acetate is an ester of acetic acid which acted as other organic acid, having membrane gradient neutralization and denaturing of proteins inside the cell (Freese *et al.*, 1973; Burt, 2004; Oonmetta-aree *et al.*, 2006). Bennis *et al.* (2004) revealed that eugenol led to *Saccharomyces cerevisiae* cell lysis. With scanning electron

microscopic observation, it showed that the surface of the treated cells was significantly damaged.

5.1.8 Dried leaves of *Psidium guajava* L.

An, ultra sonicated extract of *Psidium guajava* L. showed antibacterial activity against *Bacillus cereus* ATCC 11778 in a concentration of 8.0 g/100 ml (80 mg/ml), but had no effect on the other tested microorganisms. For hot water extract an antibacterial activity was determined against Gram-positive test organisms and the tested *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, and *Bacillus subtilis* ATCC 6633. The results of the MBC-value determination of the ethanolic extract of *Psidium guajava* L. showed higher activity compared with those extracts. The MBC-value ranged from 0.1 to 11.0 g/100 ml (1.0 – 110 mg/ml). The lowest MBC-value was determined against *Vibrio parahaemolyticus* ATCC 20502.

This result corresponded with the results of Jaiarj *et al.* (1999). The growth inhibition of *Staphylococcus aureus* strains was observed when these were diluted in a water-, methanol- and chloroform- *Psidium guajava* L. leaf extract. Gnan and Demello (1999) also reported a complete inhibition of a hot water (80°C) extract of *Psidium guajava* L. leaves against *Staphylococcus epidermidis* and *Salmonella* Typhimurium. Cáceres *et al.* (1993ab) tested *Psidium guajava* L. leaf extracts with three solvents of different polarities (n-hexane, acetone and ethanol) and discovered that the ethanol extract was the most efficient against the pathogenic enterobacteria tested. In addition, Colliere (1949) and Coutino-Rodríguez *et al.* (2001) stated the antimicrobial capacity of the water and alcoholic extracts of *Psidium guajava* L. evaluated by *in vitro* studies, confirming its growth inhibition effect particularly on *Staphylococcus aureus*, *Escherichia coli*, and other common entero-pathogenic cultures.

In view of the attributed medicinal properties, studies were undertaken on fresh and uncrushed *Psidium guajava* L. leaves, which resulted in the isolation and structure elucidation of two new triterpenoids, named guavanoic acid (20 β -acetoxy-2 α ,3 β -dihydroxyurs-12-en-28-oic acid) and guavacoumaric acid (2 α ,3 β -dihydroxy-24-*p*-z-

coumaroyloxyurs-12-en-28-oic acid) along with six known compounds 2 α -hydroxyursolic acid, jacoumaric acid, isoneriu coumaric acid, asiatic acid, ilelatifol D, and β -sitosterol-3-*O*- β -D-glucopyranoside. (Ogura *et al.*, 1977; Furuya *et al.*, 1987; Siddiqui *et al.*, 1987; Yamagishi *et al.*, 1988; Numata *et al.*, 1989; Kitajima and Tanaka, 1993; Nishimura *et al.*, 2000). The other antimicrobial compounds were described by Arima and Danno (2002). The obtained result indicated that two flavonoid glycosides; morin-3-*O*- α -L-lyxopyranoside and morin-3-*O*- α -L-arabopyranoside; and two known flavonoids; guaijavarin and quercetin; isolated from leaves of *Psidium guajava* L. presented the antimicrobial properties. The minimum inhibitory concentration of morin-3-*O*- α -L-lyxopyranoside and morin-3-*O*- α -L-arabopyranoside was 200 μ g/ml for each against *Salmonella* Enteritidis, and 250 μ g/ml and 300 μ g/ml against *Bacillus cereus*, respectively. Morin, quercetin and quercetin-3-*O*-arabinoside, bioactive compounds isolated in alcoholic extracts of *Psidium guajava* L. leaves also had antimicrobial activity (Lutterodt, 1989; Raucha *et al.*, 2000).

The microbicidal activity of *Psidium guajava* L. was attributable to guajaverine and to psydiolic acid. Furthermore, the leaves contain large amounts of tannin, triterpenoids (cateogolics, guaijavolic, oleanolics and ursolic acid) and essential oils containing β -sitosterol, β -bisabolene, β -cariophyllene, aromadendrene, β -salinene, guajaverine, nerolidiol and sel-11-en-4 α -ol (Morton, 1981). The water, ethanol (20%, 50%, 60%, 80%) and acetone (20%, 50%, 60%, 80%) extract of *Psidium guajava* L. was also tested with pathogenic virus including Infectious Haematopoietic necrosis Virus (IHNV), Infectious Pancreatic necrosis Virus (IPNV), *Oncorhynchus masou* Virus (OMV), and YellowHead Virus (YHV). The 60% ethanolic extract and 60% acetone extract demonstrated the antiviral activity against all three. More over, the extract was tested against *Vibrio harveyi*, *Vibrio splendidus*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Vibrio vulnificus*, *Vibrio cholerae*, *Vibrio alginoliticus* and *Aeromonas hydrophila*, the extract showed MIC-value ranging from 625-5,000 μ g/ml against all pathogenic tested bacteria (Direkbusarakom *et al.*, 1997). This results correspond to the findings in the actual study where the MBC-value of ethanolic extract against *Vibrio parahaemolyticus* was 1.0 mg/ml, however, differing MBC-

values with other test organisms might be due to varying extract solvent concentrations.

5.1.9 Dried pericarp of *Punica granatum* L.

The MBC-values of *Punica granatum* L. extract showed that all Gram-positive test organisms were sensitive against the ultrasonication extract of this plant, except the foodborne pathogens *Listeria monocytogenes* 101 and *Listeria monocytogenes* DSMZ 20600. Hot water extracts provided higher effectivity, and the ethanolic extract demonstrated the highest activity. The MBC-values ranged from 2.0 to 80 mg/ml. The results of this investigation had been confirmed by several studies. Navarro *et al.* (1996) reported that a methanolic extract of *Punica granatum* L. showed a general antimicrobial effect against *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8937, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 10231 at concentrations of 10 mg/ml or below. Prashanth *et al.* (2001) also reported the petroleum ether, chloroform, methanol, and water extracts of *Punica granatum* L. fruit rind to be active against *Staphylococcus aureus* MTCC 737, *Escherichia coli* MTCC 732, *Klebsiella pneumoniae* MTCC 109, *Proteus vulgaris* MTCC 1771, *Bacillus subtilis* MTCC 441, and *Salmonella* Typhi MTCC 537 at concentration of 50 mg/ml or below. Results obtained in this study on antimicrobial activity of *Punica granatum* L., seem also to agree with those obtained by Ahmad and Beg (2001) who reported that alcohol extracts of *Punica granatum* L. fruits at concentration of 150 mg/ml exhibited antimicrobial activity when tested against *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* IOA 106, *Escherichia coli* UP 2566, *Salmonella* Paratyphi IOA 107, *Shigella dysentery* IOA 108, and *Candida albicans* IOA 109. Alanís *et al.* (2005) stated that an alcoholic extract and an aqueous extract of *Punica granatum* L. at a concentration of 8.0 mg/ml exhibited antimicrobial activity against *Escherichia coli* ATCC 25922, *Escherichia coli* O157:H7, *Shigella sonnei*, *Shigella flexneri*, and *Salmonella* sp. Likewise, there was evidence of the antibacterial properties of *Punica granatum* L. (Braga *et al.*, 2005; Mathabe *et al.*, 2006; Meléndez and Capriles, 2006).

Flavonoids, tannins, sterols, triterpene, alkaloids, and glycosides were isolated from *Punica granatum* L. (Husain *et al.*, 1992). This indicated that *Punica granatum* L. contains large amounts of tannin (25%). Phytochemical screening of the ethanolic extract yield positive results for sterols, flavonoids, triteroenes, phenols, and tannin. The antimicrobial property of tannins is well established. A number of interesting novel secondary metabolites such as gallotannins, ellagitannins and punicalagin have been isolated. The large group of bioactive compound of *Punica granatum* L. extract was also indentified, classified as ellagitannins, including punicalin, punicalagin, corilagin, casuarinin, gallagyldilacton, pedunculagin, tellimagrandin, granatin A, and granatin B (Tanaka *et al.*, 1986; Tanaka *et al.*, 1990; Scalbert, 1991; Crockett *et al.*, 1992; Satomi *et al.*, 1993; Nawwar *et al.*, 1994; Honda, 1977; Hussein *et al.*, 1997; Ito *et al.*, 1997; Matsumoto *et al.*, 1997; Cowan, 199p; Sugita-Konishi *et al.*, 1999; Djipa *et al.*, 2000; Gil *et al.*, 2000; Prashanth *et al.*, 2001; Sakagami *et al.*, 2001; Machado *et al.*, 2002; Machado *et al.*, 2003).

5.2 Effect of the Extraction Method on the Antimicrobial Activity

Comparing all results the hot water extracts and ultrasonication extracts of all Thai traditional medicinal plants generally showed no or only a low antibacterial activity against the tested microorganism. According to the high MBC-values determined when using ultrasonication extract of all Thai traditional medicinal plants, it indicated that this extraction procedure provided the weakest ability to extract the antimicrobial compounds or bioactive components from the medicinal plants. In contrast, Thongson *et al.*, (2004) reported that ultrasonic extraction took only 5 minutes to obtain the bioactive components that exhibited the antimicrobial activity from three different spices, including *Zingiber officinale* Rose, *Bosenbergia pandurata* Holtt, and *Curcuma longa* Linn, while the classical extraction required 24 hours to obtain the same results. The different results could depend on the extract solvent, sonication time and ultrasonic intensity. According to Tongson *et al.*, (2004), the high intensity ultrasound assisted extraction was performed using isopropanol, hexane or a mixture of isopropanol-hexane at a ratio of 7:3 (v/v) as solvent extract. The extraction process was performed in ice-bath using 20 kHz ultrasonic generator connected to a 1.25 cm

transducer at an ultrasonic intensity of 6.8 W/cm². The extraction process took 5 minutes. It is known that the efficiency of the ultrasonic extraction generally increases with sonication time and ultrasonic intensity. While the study of Thongson *et al.*, (2004) used higher ultrasonic intensity and solvent extract to prepare an extract, this present study used only ultrasonic bath no high energy ultrasonic transducer or solvent extract. It is therefore conceivable that either longer sonication time or higher ultrasonic intensity levels might result in extracts containing higher amounts of active compounds and achieve lower MIC-value compared with the “classical extraction”. In addition, a suitable solvent extract has to be selected depending on the active compounds expected in the plants (Panchev *et al.*, 1988; Hromadkova *et al.*, 1999; Vinatoru, 2001; Wu *et al.*, 2001; Hromadkova *et al.*, 2002; Palma and Barroso, 2002). Clearly, more in-depth studies on the influence of sonication time and intensity on yield, composition and activity of medicinal plant extracts should be performed to optimize the extraction process to obtain spice extracts with the highest antimicrobial activity.

Previous studies on the other medicinal plants, water extracts associated with hot water or hydrosol showed antibacterial activity of medicinal plants to be limited but activity associated with alcoholic solvent to be high, especially associated with essential oils (Hayes and Markovic, 2002; Wilkinson *et al.*, 2003; Burke *et al.*, 2004). Water had limited ability to extract some components from medicinal plants such as oil-based components. Alcohol extraction therefore is recommended to obtain extracts with higher antibacterial activity.

5.3 Influence of the Gram type on the Antimicrobial Activity

High MBC-values correlating with higher resistance were often seen when Gram-negative microorganisms were tested. Three strains of *Escherichia coli* and *Pseudomonas aeruginosa* presented the highest MBC-values at 15.0 g/100 ml (150 mg/ml) of ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees, and also with the ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe, *Pseudomonas aeruginosa* demonstrated the highest MBC-value of 11.0 g/100 ml (110 mg/ml).

Higher resistance of Gram-negative bacteria to external substances had been reported (Negi, *et al.*, 2005). It is attributed to the presence of lipopolysaccharides, making them naturally resistant to certain antibacterial agents, in their outer membrane (Nikaio and Vaara, 1985). On the other hand, Gram-positive test organisms showed higher sensitivity against the tested medicinal plants than the Gram-negative bacteria. The reason could be attributed to the differences between their cell wall compositions. Gram-positive bacteria contain an outer peptidoglycone layer, which is an infective permeability barrier (Scherrer and Gerhardt, 1971).

The selective bactericidal activity against Gram-negative bacteria of several extracts of Thai traditional medicinal plants could be contributed to the fact that a number of antimicrobial compounds could not inhibit the growth of Gram-negative bacteria due to a failure of outer membrane penetration. As mentioned before the resistance of Gram-negative bacteria towards antimicrobial substances is related to lipopolysaccharides in its outer membrane (Gao *et al.*, 1999). Recently, Pasqua *et al.* (2006) have studied the changes in membrane fatty acids composition of microbial cells in the presence of a sublethal concentration of antimicrobial compounds (e.g. thymol, carvacrol, limonene, cinnamaldehyde and eugenol) in response to a stress condition. It was found that Gram-negative bacteria did not show substantial changes in its fatty acid compositions. This is an indication of the high resistance of Gram-negative bacteria to the tested compounds. According to Cosentino *et al.* (1999), Gram-negative bacteria were also resistant to pinene, cymene, carvacrol at the highest concentration (900 µg/ml) tested, but were found to be sensitive to pulegone, isopulegone, and piperitone, as well as to oils rich in these compounds (such as mint oil and nepitella oil) (Panizzi *et al.*, 1993; Sivropoulou *et al.*, 1995).

Due to the fact that data concerning the chemical components in the tested medicinal plants were missing, only a vague comparison with reported bioactive compounds in those plants extracts and their single effect on the penetration through the outer membrane of bacteria was possible.

Beside that each medicinal plant extract tested in the actual study, contained not only a single compound, but also mixture of several compounds. Therefore the bactericidal activity was the result of a mixture of several components. Effects of single components were not detectable. The extract of *Punica granatum* L. showed high activity against Gram-positive and low effect against Gram-negative, The major bioactive compounds in this plants were flavonoids, tannins etc. There was no publication available about the penetration of these compounds into cell, but the results indicated that they also fail to penetrate the outer membrane. As another example *Piper betle* L. containing big amounts of oil based compounds such as carvacrol, eugenol and chavibetol showed good antimicrobial activity against both Gram-negative and Gram-positive test organisms while a higher resistance of Gram-negative bacteria due to the components was expected. The major bioactive compounds and their amount in each medicinal plant extract were varying and the bactericidal effect was a result of the mixture in the actual extracts.

5.4 Antimicrobial Activity of the Ethanolic Extract of the Medicinal Plants Determined by Time Killing Analysis

The rapidity of the bactericidal effect or the duration of a bacteriostatic effect can be determined by time kill analysis (survivor curve plot) whereby the number of viable cells remaining in cell suspension after the addition of antimicrobial substances is plotted against time. The time killing analysis is equal the inhibition curve, also known as the 'killing curve' in clinical research. More over this assay is known as suspension test especially in the effectiveness testing of microbicides as disinfectants, antibiotics etc.

The test simply involves inoculation of a microorganism into a media, addition of antimicrobial substances, followed by incubation and periodic sampling to determine growth of survival. This method is widely used in effectiveness testing i.e. of disinfectants.

As mentioned before in a suspension test, defined microorganism suspension are mixed with the disinfectant dilutions to be tested, and the samples taken after different contact times are checked for the presence of viable test organisms (Kleiner and Trenner, 1990). This check can be qualitative with +/- results (end point method) or quantitative, by determining the number of surviving microorganisms (Hunsinger, 2005).

Suspension tests have a number of benefits. They are relatively simple and do not require specialized or expensive laboratory equipment and other than labour cost. They are also well defined and are thus, with normal microbiological limits, repeatable and reproducible. Within this methodology it is also possible to test a wide range of variables including contact time, temperature, microorganism type, and interfering substances. The major limitation of suspension test, however, is that they do not necessarily reflect in-use conditions (Holah *et al.*, 1998).

Schönemann (1987) stated that the qualitative test method had far higher requirement to a microbicidal active than the quantitative, he referred to the end-point method as being more informative while being less complex in terms of experiment. Schliesser (1980) requires a reduction in the initial microbial count by at least 5log i.e. 99.999% irrespective of whether the result was obtained by the end point method (qualitative) or with the reduction method (quantitative).

According to Borneff *et al.* (1975), due to their structure, suspension tests are used solely for the determination of the effectiveness of disinfectants and their impairment through different factors. For varying contact times, the test mainly provides information with respect to the dynamic of the effectiveness of the active compounds (Werner and Reybrouck, 1976). In addition, depending on the requirement, potential interfering substances such as protein or cleaning agents can be added, or the effect of different dilution waters on the effectiveness can be determined (Reybrouck, 1998).

In the actual study the quantitative analysis was carried out because of the more accurate analysis than the qualitative assay due to the wide detection range. Time

killing analysis can be used for monitoring the number of survival change at different time. In addition, the data from this assay can be helpful for recommending the optimum concentration and contact time for each antimicrobial substance to eliminate or destroy the contaminated microorganism when they are in suspension. However, some of the resulting curves are easy to interpret, other are not and recommendations for use concentrations under practical conditions are only limited possible, because the results of suspension tests do not take into account the effectiveness on surfaces.

The advantage of suspension test is that, in liquid medium with agitation, the bacterium was exposed to the antimicrobial substances better than on a solid medium. Another possible reason is the physical structure of the medium limit the antibacterial activity of antimicrobial substances. According to Skandamis *et al.*, (2000), the antimicrobial of oregano oil against *Salmonella* Typhimurium in liquid medium was greater than in gelatine gel mix. The gel matrix dramatically reduced the inhibitory effect of the oil. This was theorized to be the limitation of diffusion by the structure of the gel matrix. Davidson and Parish (1989) stated that time killing analysis was the only method which will show 'lethality effect.' Following an antimicrobial test in which lethality occurs, a very small percentage of the original population remained viable. This population will generally remain static for a period and then, under ideal growth condition, begin to increase in cell concentration.

From the MBC-value results, those ethanolic extracts of the medicinal plant which exhibited an outstanding antimicrobial activity were selected to study further in Time Killing Analysis without interfering substance as described in material and methods; no. 3.2.4.3.1, page 77.

The results obtained with the ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees indicated that a concentration of 15.0 g/100 ml (150 mg/ml), had a potential to completely reduce cells of *Listeria monocytogenes* and *Vibrio parahaemolyticus* within 1 hour contact time, and had the same effect against *Salmonella* Typhimurium within 9 hours. As expected the reducing of the concentration of the extract caused longer contact times to exhibit the complete

reduction. For the other test organisms, ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees at the highest concentration (15.0 g/100 ml) was not able to inactivate the microorganisms within 9 hours contact time.

The ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe demonstrated greater antibacterial activity. With a concentration of the ethanolic extract of 15.0 g/100 ml (150 mg/ml) *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Vibrio parahaemolyticus* was completely destroyed within 15 seconds, and this concentration showed the same ability against *Escherichia coli* O157:H7 and *Pseudomonas fluorescens* within 1 hour, against *Salmonella* Typhimurium in 3 hours, *Pseudomonas aeruginosa* in 6 hours, and *Staphylococcus aureus* in 9 hours. At a concentration of 10.0 g/100 ml (100 mg/ml), *Vibrio parahaemolyticus* was inactivated within 15 seconds, the other test organisms required longer contact times.

For the ethanolic extract of *Garcinia mangostana* L., the results indicated that the outstanding antibacterial activity was present against *Listeria monocytogenes* and *Vibrio parahaemolyticus*. A concentration of 5.0 g/100 ml (50 mg/ml) could give 'lethality effect' within 15 seconds and at a concentration of 1.0 g/100 ml (10 mg/ml) required 1 hour contact time.

In the case of the ethanolic extract of *Hibiscus sabdariffa* L., the extract demonstrated a good antibacterial activity against several test organisms. When a concentration of 15.0 g/100 ml was used, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella* Typhimurium, and *Vibrio parahaemolyticus* were completely inactivated within 1 hour contact time, while *Staphylococcus aureus* required 6 hours for complete reduction, but *Yersinia enterocolitica* required only 15 seconds for the same results. At lower concentrations, 100% reduction was also detected, but a longer contact time had to be applied.

According to the presented results, the ethanolic extract of *Musa sapientum* L. provided an antibacterial activity only against *Listeria monocytogenes* and *Vibrio parahaemolyticus*. For *Listeria monocytogenes*, ethanolic extract of *Musa sapientum*

L. at the highest concentration (15.0 g/100 ml or 150 mg/ml) presented reduction of $>5\log$ in 3 hours, and in case of *Vibrio parahaemolyticus* the population was inactivated within 1 hour contact time.

The results of the suspension tests of the ethanol extract of *Nelumbo nucifera* Gaertn indicated that the antimicrobial activity of this extract presented only against 2 strains of the tested microorganisms, including *Yersinia enterocolitica* and *Vibrio parahaemolyticus*. At concentration of 15.0 g/100 ml, it had the ability to reduce *Yersinia enterocolitica* below the detectable level within 6 hours contact time, and provided the same action against *Vibrio parahaemolyticus* in 1 hour. A concentration of 1.0 % (v/v) of this extract demonstrated a reduction of $> 5\log$ of *Vibrio parahaemolyticus* within 3 hours contact time.

The ethanolic extract of *Piper betle* L., showed an outstanding bactericidal activity against the test organisms, with the exception of *Bacillus cereus* and *Bacillus subtilis*. Inactivation of the other microorganisms was detected within 15 seconds contact time when this extract was added to the test suspensions at concentrations of 10.0 g/100 ml or higher. As expected, longer contact times were required to obtain the same effect when the concentration was reduced.

The results of the suspension tests with the ethanolic extract of *Psidium guajava* L. indicated that *Listeria monocytogenes* was the most sensitive test organism. The initial population of *Listeria monocytogenes* was completely reduced within 15 seconds when using 10.0 g/100 ml or higher concentrations of the ethanolic extract of this medicinal plant. The lethality effect occurred in 1 hour at concentrations of at least 1.0 g/100 ml (10 mg/ml). A bactericidal effect was also monitored against *Vibrio parahaemolyticus* within 1 hour contact time at all concentrations. 15.0 g/100 ml of this extract could provide the complete reduction against *Salmonella* Typhimurium and *Escherichia coli* O157:H7 in 9 hours. At the same concentration, it proved a weak potential to reduce *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*.

All tested concentrations of the ethanolic extract of *Punica granatum* L. completely inactivated *Vibrio parahaemolyticus* and *Listeria monocytogenes* within 1 hour contact time. Concentrations of 10.0 g/100 ml and higher of the extract showed a bactericidal effect against *Yersinia enterocolitica* in 1 hour. In case of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Salmonella* Typhimurium these test organisms required 3 hours contact time for a reduction $> 5\log$ and a concentration of 15.0 g/100 ml.

Generally the results indicated, that bioactives in the extracts with higher amounts/concentrations of phenolic compounds, such as carvacrol, eugenol, and thymol processing the antimicrobial properties against foodborne pathogens. They exhibit a wide range of biological effects, including antioxidant and antimicrobial properties. The mode of action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electrolyte flow, and active transport, and coagulation of bacterial cell contents (Burt, 2004).

Consideration for all results, it could be investigated that *Bacillus cereus* and *Bacillus subtilis* showed the highest resistance to all extract of medicinal plants. This was expected because these organisms have the ability to produce spores, which are very stable against bactericidal agents in medicinal plants as well as against disinfectant agents. The remaining resistant spores in suspension can germinate after enumeration on agar media. It is generally known that dormant bacterial spores are highly resistant to many physical treatment including heat, drying, radiation, and also chemical agents (Gould, 1983). It has been shown that spores form clumps in suspensions and that these clumps decrease the spore inactivation rate during disinfection treatment (Sakaguchi and Amaha, 1951; Stumbo, 1965; Aiba and Toda, 1966; Toda and Aiba, 1966; Chef, 1977; Pflug *et al.*, 2001). It was also reported that the formation of spore clumps through physical and chemical treatment and the presence of various resistance spore populations in the cell suspension introduced tailing death curves (Stumbo, 1965; Chef, 1977; Pflug *et al.*, 2001). Furukawa *et al.* (2005) reported that the diameters of spores clumps which were smaller than 5 μm contained approximately 90 to 10^2 spores. More over, it was indicated that these spores were

five time more resistant than a single spore. It was considered that the formation of spore clumps was dependent on the increase of the hydrophobicity of the spore surface. The increase of the hydrophobicity was considered to be induced by the denaturation of the surface protein of the spore coat. It also was considered that the decrease of the inactivation ratio, tailing of the surviving curves, was partly due to the formation of those spore clumps. The addition of the surface-active agent to the bacterial suspensions prevented the formation of the spore clumps through physical and chemical treatments. It could increase the rate of inactivation (Furukawa *et al.*, 2005).

In generally, the studies to determine the action of the ethanolic extract of those medicinal plants on test organisms at different concentration showed that the survivors of these test organisms were decreased by increasing concentration of the extract, and completely inhibited at the concentration of 15.0 g/100 ml. This result indicated that mostly vegetative cells were present in the bacterial test suspension of *Bacillus cereus* and *Bacillus subtilis*, because much higher concentrations of the ethanolic extract would be expected to be effective against a spore suspension of this test organisms.

Moreover, it could be indicated that the longer the contact time the lower the necessary concentration of the extract. Prolongation of the contact times allows the reduction of the concentration. Due to the fact that in the intended field of application long contact times are expected (wrapping, coating and then storage) a reduction of the concentration of the extracts might be possible. The lower the concentration the less the possible negative effect on the food, especially influence on the taste or smell could be reduced.

5.5 Influence of Interfering Substances on the Antimicrobial Activity of the Ethanolic Extracts of Thai Traditional Medicinal Plants Determined by the Time Killing Analysis

In the food to be preserved contains high load of protein, fat, and other nutrient components, they may interfere with the activity of the plant extracts. The studying on the influence of interfering substances will be an important step to decide the optimum concentration and contact time. High level soiling, the mixture of 10 g/L bovine albumin fraction V and 10 g/L yeast extract was used as interfering substances. From the result it could be specified that the activity of all tested ethanolic extracts was decreased when this interfering substance was added to the suspension test system. The required contact times which were necessary to reduce the viable counts of the test organisms more than 5 log was increased, especially when the low concentrations of ethanolic extract were used. In case of *Staphylococcus aureus*, the concentration necessary to reduce the test organisms by >5log had to be increased or the contact time prolonged, respectively. None of the extracts were able to reduce *Bacillus cereus* in presence of the interfering substance. For *Pseudomonas aeruginosa*, the antibacterial activity of the ethanolic extract of Betel leaves was not affected by addition of the interfering substances. The antibacterial activity of other ethanolic extracts was decreased by addition of the high level soiling at the concentration below 15.0 g/100 ml.

According to the presented results, it could be indicated that high level soiling, the mixture of 10 g/L bovine albumin fraction V and 10 g/L yeast extract affected the antimicrobial activity of ethanolic extracts of the selected medicinal plants. When adding the interfering substance to the suspension test the concentration of ethanolic extracts had at least to be doubled to achieve the same effect than without interfering substance. The ethanolic extract of *Piper betle* L. at a concentration of 5.0 g/100 ml could reduce *Staphylococcus aureus* to undetectable level within 1 hour, while this extracts in the presence of interfering substances required 10.0 g/100 ml concentration step to reduce this test organism > 5 log within the same contact time.

When using the same concentrations as in the tests without interfering substance the contact time had to be prolonged up to 8 times. The ethanolic extract of *Garcinia mangostana* L. at the concentration of 15.0 g/100 ml completely reduced *Pseudomonas aeruginosa* within 3 hours, while the suspension test in the presence of interfering substances required 24 hours at the same concentration to obtain the complete reduction. In this study, the ethanolic extract of *Punica granatum* L., and *Garcinia mangostana* L. were more affected by the addition of the interfering substance than the ethanolic extracts of *Piper betle* L. and *Hibiscus sabdarifa* L.

One of the most important environmental factors to influence disinfectant activity is undoubtedly the presence of organic matter (Böhm, 2002). And the phenomenon of failure of active agents due to addition of interfering substances (protein failure) is well known from the disinfectant testing (Hunsinger, 2005). This can be present in various forms, such as soil, food residues and dried residues of milk. The hygienic condition of the intended application area can be simulated during the effectiveness test using interfering substance. The concentration and sort of interfering substance which is used in the test depends on the application area which is wanted to be simulated. For instance, based on DIN EN standards in the actual tests the mixture of 10 g/L BSA and 10 g/L yeast extract was chosen simulate the intended use of the extracts on food.

Different substance commonly used for disinfection will be affected differently in the presence of interfering substances. Generally, the quantity of disinfectant needed to kill bacteria under dirty conditions is higher than under clean conditions (Böhm, 1984). This was also confirmed for the medicinal plant extracts in the actual study.

It is also widely accepted that higher concentration of medicinal plants extracts are required in present of protein and fat than in the distilled water (Farbood *et al.*, 1976). Moreover, it is supposed that the high level of fat and/or protein protect the bacteria from action of medicinal plant extracts in some way (Aureli *et al.*, 1992; Pandit and Shelef, 1994; Tassou *et al.*, 1995). For instance, if the medicinal plant extracts

dissolves in the lipid phase, there will be relatively less available to act on bacteria present in the aqueous phase (Mejlholm and Dalgaard, 2002).

A reaction between carvacrol, a phenolic component of various plant extracts, and proteins have been suggested as a limiting factor in the antimicrobial activity against *Bacillus cereus* in the presence of milk protein (Pol and Smid, 1999). Similarly, protein interaction has been suggested a factor reducing the action of clove oil against *Salmonella* Enteritidis in diluted low-fat cheese (Smith-Palmer *et al.*, 2001). Carbohydrates do not appear to protect bacteria from the action of medicinal plant extracts as much as fat and protein do (Shelef *et al.*, 1984). Moreover, the presence of metal ions such as monovalent cations (Na^+ and K^+) or divalent ions (Mg^{2+} and Ca^{2+}) presented the inactivation effect against most of antimicrobial substances (Anderson and Yu, 2005).

In the intended field of application of the tested medicinal plant extracts such as in the food industries and on food products a high amount of interfering substances were expected. The extracts of *Piper betle* L. and *Hibiscus sabdarifa* L. which showed a good antimicrobial activity in the presence of interfering substances provided more advantages for the application in food products.

5.6 Artificial Antimicrobial Coated Surface

The aim for this application was to investigate the antimicrobial activity of an ethanolic extract of Thai traditional medicinal plants on artificial coated surfaces. The ethanolic extract of *Piper betle* L. which provided the outstanding antimicrobial activity and the smallest protein failure against several test organisms was selected for this application study.

With the ethanolic extract of *Piper betle* L. coated stainless steel discs demonstrated an antimicrobial property against the test organisms. Most susceptible was *Vibrio parahaemolyticus*. At a coating concentration of 30.0 % (v/v) and 20.0 % (v/v), this organism was completely reduced within 1 hour contact time. With decreased

concentration the contact time had to be prolonged to achieve the same effect. In case of both *Pseudomonas* sp., ethanolic extract of *Piper betle* L. at a concentration of 10.0 % (v/v) and higher coated on the metal discs showed antimicrobial activity. A completely reduction of these Gram-negative test organisms was detected within at least 48 hours. *Yersinia enterocolitica* was inactivated within 1 hour and a concentration of 30.0 % (v/v). However, with ethanolic extract of *Piper betle* L. coated steel discs presented only a limited surface activity against the other test organisms within the longest tested contact time of 48 hours.

The application of coating on stainless steel discs to simulate and test the surface activity of the ethanolic extract of *Piper betle* L., which showed outstanding antimicrobial activity in the suspension tests, was not appropriable. It might be indicated that other ethanolic extracts of Thai traditional medicinal plants coated on metal pieces would exhibit no effect against the microorganisms.

An artificial coated surface is one of possible antimicrobial packaging ideas. The coating of the packaging with a matrix acts as a carrier for the antimicrobial agent. These categories of materials can release the antimicrobial agents onto the surface of the food. The system is more efficient than a direct application of the antimicrobial agent, because it slows the migration of the agents away from the surface, and thus helps to maintain high concentrations where they are needed (Appendini and Hotchkiss, 2002).

According to the presented experiment, the results, however, indicated that the artificial metal coated surface is unsustainable material to use as antimicrobial packaging due to the weak antimicrobial adsorption and releasing to target microorganisms. Polymer surfaces were recommended for the adsorbed material. Bacteriocins could be coated or adsorbed to polymer surfaces. Examples include methylcellulose coatings for polyethylene films, ethylene vinyl acetate, polypropylene, polyamide, polyester, acrylics and polyvinyl chloride (Chen and Williams, 2005). *et al.* (1997) applied bacteriocins to the inner surface of plastic vacuum-packaging bags. They reported, using the coated materials with nisin and

pediocin, inhibition of *Listeria monocytogenes* growth. Polyvinyl chloride or polyethylene films coated with nisin were effective in reducing *Salmonella* Typhimurium growth.

5.7 Antimicrobial Activity of Medicinal Plant Ethanolic Extract-Incorporated Alginate-Tapioca Starch Based Edible Films

Antimicrobial packaging, an innovative concept, can be defined as a version of active packaging in which the package, the product and the environment interact to extend the lag phase and/or reduce the growth rate of microorganisms. By this action, the shelf life of the product is prolonged and its quality and safety are better preserved (Suppakul *et al.*, 2003a; 2003b).

Bacterial growth occurs mainly at the surface; attempts have been made to solve this by using antibacterial sprays or dips (Ouattara *et al.*, 2000a; 2000b). However, direct surface application of antibacterial substances has limited benefits, because the active substances are neutralized on contact or diffused rapidly (Torres *et al.*, 1985; Siragusa and Dickson, 1992). Potential properties and applications of edible films and coatings have been extensively reviewed (Pena and Torres, 1991; Han, 2001; Bravin, *et al.*, 2006; Jagannath *et al.*, 2006; Min *et al.*, 2005; Serrano *et al.*, 2006). The method is different from direct application, as the incorporation of antimicrobial agents into edible film or edible coating localizes the functional effect at the surface. The antimicrobial agents are slowly released to the surface, and therefore, they remain at high concentrations for extended periods of time (Ouattara *et al.*, 2000a; 2000b; Coma *et al.*, 2001).

In the present study, inhibitory activity was measured based on the clear zone surrounding a circular film disk. If there was no clear zone, it was assumed that there was no inhibition.

A mixture of sodium alginate and tapioca starch was used as the film-based material. A mixture of starch and Na-alginate to form edible films has been studied by Wu *et*

al. (2001). Na-alginate is the sodium salt of the alginic acid and it is a polyelectrolyte. It is a copolymer consisting of D-mannuronic and L-guluronic acid monomers (Borchard *et al.*, 2005). Edible films prepared from alginate form strong films and exhibit poor water resistance because of their hydrophilic nature (Guilbert, 1986; Kester and Fennema 1986). Alginate has a potential to form biopolymer film or coating component because of its unique colloidal properties, which include thickening, stabilizing, suspending, film forming, gel producing, and emulsion stabilizing (King, 1982; Guilbert, 1986; Kester and Fennema, 1986).

Starch is a mixture of linear D-glucan amylose and the highly branched amylopectin. These polymers exhibit different behaviours with regard to gelation and development of crystallinity. In gel formation, amylose and amylopectin form inter- and intramolecular physical crosslink to produce a macromolecular network (Miles *et al.*, 1985a, 1985b). Physical crosslinkages contributes to the higher tensile strength of films (Rindlav-Westling *et al.*, 1998). Several studies have been done to analyze starch-based film properties (Arvanitoyannis and Biliaderis 1998, 1999; Rindlav-Westling *et al.*, 1998; Mali *et al.*, 2002; Ryu *et al.*, 2002; Rodriguez *et al.*, 2006; Talja *et al.*, 2007). Starch produces films that have good mechanical properties and coverings that are efficient barriers against low-polarity compounds (Kester and Fennema 1986); therefore, the mixture of these 2 biopolymers is desirable to improve the mechanical properties of film.

The results showed that the Alginate-Tapioca starch based edible films containing the ethanolic extracts of *Psidium guajava* L., *Piper betle* L., *Andrographis paniculata* Burm. f. Nees., *Garcinia mangostana* L., *Punica granatum* L., and *Hibiscus sabdariffa* L. were effective against the test organisms. With increasing concentrations of the ethanolic extract, the zone of inhibition also increased at all levels. *Psidium guajava* L. ethanolic extract film at a concentration of 20.0 % (v/v) showed the best inhibitory effect. The lowest concentration of *Psidium guajava* L. ethanolic extract incorporated into edible film which demonstrated the inhibitory effect was 5.0 % (v/v) and it was also achieved with the extracts of *Garcinia mangostana* L., *Piper betle* L., and *Punica granatum* L.

The inhibitory zone of Alginate-Tapioca starch based edible films containing ethanolic extract of *Musa sapientum* L. showed the activity only against *Bacillus cereus* ATCC 11778, *Bacillus cereus* ATCC 128263, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* 101, and *Listeria monocytogenes* DSMZ 20600.

Alginate-Tapioca starch based edible films containing ethanolic extract of *Piper Betle* L. showed the best effectiveness at a concentration of 20.0 % (v/v). At the lowest concentration, 1.0 % (v/v), incorporated into edible film demonstrated this activity only against *Bacillus subtilis* ATCC 6633. The size of zone of inhibition was depending on the concentration incorporated into the films. In case of *Bacillus subtilis* ATCC 6633, the area was 1.53 cm at the concentration of 20.0 % (v/v) and decreased to 0.30 cm when the concentration was reduced to 1.0 % (v/v). *Andrographis paniculata* Burm. f. Nees ethanolic extract-incorporated edible film presented the activity only at 20.0 % (v/v) concentration. Edible film containing ethanolic extract of *Nelumbo nucifera* Gaetrn., and *Curcuma zedoaria* (Christm.) Roscoe presented no inhibitory zone against all test organisms. However, an inhibition at direct contact area of the film with the test organism was also detected.

In case of *Garcinia mangostana* L. ethanolic extract incorporated film, the outstanding antimicrobial activity was presented against *Salmonella* Typhimurium ATCC 13311 at 20.0 % (v/v) incorporated in the film with 1.27 cm zone of inhibition. The lowest incorporated concentration which showed the effect was 5.0 % (v/v) against *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* DSMZ 799, and *Pseudomonas aeruginosa* DSMZ 939.

Punica granatum L. ethanolic extract-incorporated in an edible film was the one which showed good antimicrobial activity. The lowest incorporated concentration which provided the antimicrobial effect against all test organisms was 10.0 % (v/v). The incorporation of only 1.0 % (v/v) was necessary to demonstrate the effect against *Bacillus cereus* ATCC 128263, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* DSMZ 799, *Staphylococcus aureus* ATCC 13565, *Pseudomonas aeruginosa* DSMZ 939, *Pseudomonas fluorescens* ATCC 13525, *Yersinia enterocolitica* ATCC 2779,

and *Vibrio parahaemolyticus* ATCC 20505. Edible film incorporated with *Hibiscus sabdariffa* L. ethanolic extract also showed an inhibitory effect against all test organisms. But a concentration of 20.0 % (v/v) was necessary to provide the antimicrobial activity against all test organisms.

Observation on the contact area, it revealed that incorporating ethanolic extracts of those medicinal plants into Alginate-Tapioca starch based edible film revealed inhibitory effect shown by limited growth underneath films for all bacteria.

The agar diffusion test is a method commonly used to examine antimicrobial activity regarding the diffusion of the compound tested through water-containing agar plate. The diffusion itself is dependent on the size, shape and polarity of the diffusing material. The chemical structure and the cross-linking level of the films also affect this phenomenon (Cagri *et al.*, 2001). When antimicrobial agents are incorporated, there will be diffusing material through agar gel, and furthermore, resulting clearing zone on the bacterial growth.

In the survivor count of test organisms after contact with the Alginate-Tapioca starch based edible films incorporated with ethanolic extract of Thai traditional medicinal plants extracts, it was indicated that the complete reduction counts of test organisms occurred by edible films containing *Piper betle* L., *Hibiscus sabdariffa* L., *Psidium guajava* L., *Garcinia mangostana* L., *Punica granatum* L. ethanolic extract. The most effective extracts incorporated in the edible films were *Piper betle* L. *Hibiscus sabdariffa* L., and *Punica granatum* L. A concentration of 20.0 % (v/v) showed the highest activity. The most susceptible organisms against the extract/extracts were *Listeria monocytogenes* and *Vibrio parahaemolyticus* . The most stable test organisms were *Bacillus cereus* and *Bacillus subtilis*.

The extracts of most of medicinal plant are rich in phenolic compounds, such as flavonoid and phenolic acids, which exhibit a wide range of biological effects, including antioxidant and antimicrobial properties. Direct incorporation of extracts to food will result in the immediate reduction of bacterial population but alter the

sensory characteristics of the added food. This incorporation of extract to edible films were particularly interesting.

The edible film incorporated with ethanolic extract of those medicinal plants provided antimicrobial activity due to the antimicrobial activity of those plant extracts. As expected, ethanolic extracts of those plants which presented good antimicrobial activity in previous experiments (suspension tests) also provided the antimicrobial activity when incorporated with Alginate-Tapioca starch based edible film. However, it could be investigated that the inhibition effects of medicinal plants extract incorporated into Alginate-Tapioca starch based edible film were lower than those of pure extracts. One possible reason for the decrease in activity of the plant extracts incorporated in the Alginate-Tapioca starch based edible film compared with activity of pure extracts due to potential partial loss of highly volatile compounds of the extracts during film preparation. Another possible reason due to the slower or controlled release of active compounds from the Alginate-Tapioca starch based edible film (Zivanovic *et al.*, 2005). And a third reason might be the loss of activity of the extracts due to the protein failure after adding interfering substance, which was detected in the suspension test with interfering substance and was discussed before.

5.8 Cytotoxicity of the Ethanolic Extracts of Thai Traditional Medicinal Plants

The present study was undertaken to evaluate the cytotoxic activity of some Thai traditional medicinal plants that were used in folk medicine as antimicrobial substances. The information based on the medicinal traditional use of plants, ethnopharmacological data, has been one of the common useful ways for the discovery of biologically active compounds from plants (Cordell *et al.*, 1991; Cragg *et al.*, 1994). The big advantage of the ethnopharmacological information was that the extensive literature already allowed for some rationalization with respect to the biological potential of a reputed use (Cordell *et al.*, 1991).

In this study, ethanolic extracts were used. Since it was known that different cell lines might exhibit different sensitivities towards a cytotoxic compound, the use of more

than one cell line was therefore considered necessary in the detection of cytotoxic compounds.

As can be seen in table 13-21, cell type cytotoxic specificity was observed in all plant extracts and was likely to be due to the presence of different classes of compounds in the extract, as it had been documented in the case of known classes of compounds (Cragg *et al.*, 1994). The presented results indicated the presence of cytotoxic activity in all of the plant extracts against all of the test cell line, but was only detected in the undiluted or lightly diluted extracts.

The ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees at all concentrations showed toxicity against all tested cell lines at 10^0 and 10^{-1} level of dilution. Ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe also demonstrated cytotoxicity against the tested cell lines. At high concentrations, the extract showed toxicity at 10^0 - 10^{-1} level of dilution against MDCK and A549 cells. Consideration about ethanolic extract of *Garcinia mangostana* L., it can be investigated that this extract processed higher cytotoxicity. At all concentrations it showed toxicity against all tested cell lines at 10^0 - 10^{-2} level of dilution.

There was cytotoxicity against all tested cell lines detected for ethanolic extract of *Hibiscus sabdarifa* L. At a concentration of 20.0% toxicity of extract was monitored at 10^0 - 10^{-2} level of dilution. The ethanolic extract of *Musa sapientum* L., and *Nelumbo nucifera* Gaertn. showed lower cytotoxicity values, but the ethanolic extract of *Piper betle* L. also provided high cytotoxicity. At high concentration, 20.0 g/100 ml, cytotoxicity was monitored at 10^0 - 10^{-3} level of dilution. The cytotoxicity against the tested cell lines was also given for the ethanolic extracts of *Psidium guajava* L., and *Punica granatum* L.

The results of the present study indicated the presence of cytotoxic compounds in all of ethanolic extracts of the medicinal plants traditionally used in Thailand as folk medicine. However, it is interesting to note that the tested plant extracts, which showed substantial cytotoxic activity in this study, contain potential bioactive

compounds, such as alkaloids, and phenolic compounds (flavonoids, lignan, tannin) (Sonoda *et al.*, 1991; Hirano *et al.*, 1994; Chan *et al.*, 2000).

Although the tested products showed cytotoxicity, this correlates more with their effect on pathogenic organisms on the food than with their possible effect on the mucous membranes of human. The oral mucous membranes are better protected against toxic substances than cell culture monolayers. Together with the dilution of the substances through the food products they are applied to, no toxic effect can be expected on the final user. This present results give addition evidence that those plant extracts are potential sources of biologically active compounds. Further investigation of the active extracts and isolated compounds in animal models for the safety data and toxicity would provide evidence to determine whether those medicinal plants could be beneficial for new antimicrobial discovery.

5.9 Antimicrobial Properties of Alginate-Tapioca based Edible Films and Coating incorporated Traditional Thai Medicinal Plant Extracts in a Model Food System

Edible films and coating prepared from polysaccharides, proteins, and lipids have a variety of advantages such as biodegradability, edibility, biocompatibility, aesthetic appearance, and barrier properties against oxygen and physical stress. With regard to biodegradable packaging, starch is the commonly used agricultural raw material because it is a renewable source, widely available, relatively easy to handle, and inexpensive (Lourdin *et al.*, 1995).

Alginate-Tapioca starch based edible film incorporated with 20.0 % (v/v) ethanolic extract of *Hibiscus sabdarifa* L., *Piper betle* L., and *Punica granatum* L. which provided good antimicrobial activity were selected for studying in model food.

5.9.1 Antimicrobial Efficacy of Alginate-Tapioca Starch Based Edible Film and Coating Incorporated with Ethanolic Extracts of *Hibiscus sabdarifa* L., *Piper betle* L., and *Punica granatum* L. in Carrots (*Daucus carota* L.)

Carrot is a major vegetable in commerce and is consumed largely i.e. as a salad vegetable or in curries. This is because of its rich carotenoid contents (precursor of vitamin A) and its good content of fibre and carbohydrates. It has about 85% moisture content, which can be preserved by use of preservation and processing technologies. Emulsion coating is an option that is used to extend the shelf-life of vegetables after harvesting.

In the coating process, the Alginate-Tapioca starch based edible coating incorporated with those ethanolic extracts demonstrated an antimicrobial activity against *Bacillus cereus* ATCC 128263 and *Salmonella* Typhimurium ATCC 13311. The strongest bactericidal effects of those were investigated against post-contaminated pieces of carrot with exception of *Salmonella* Typhimurium ATCC 13311 at the cold storage. In case of pre-contaminated carrot, the antimicrobial activities were investigated against *Salmonella* Typhimurium ATCC 13311 and had no activities against pre-contaminated carrot with *Bacillus cereus* ATCC 128263.

This presented results indicated that Alginate-Tapioca starch based edible coating incorporated with ethanolic extract of *Hibiscus sabdarifa* L., *Piper betle* L., and *Punica granatum* L. have the potential to protect carrot against bacteria contaminating the environment more than eliminate them directly on food. The others possible reasons due to the diffusion properties of those extracts into food matrix and the natural properties of food matrix. It is, generally, known that food matrix contains not only organic substances but also many pores. The organic substances present in food matrices act like the interfering substances that can cause the decrease of the antimicrobial activity of bactericidal substances. Moreover, the pores in the food surface take an action as the physical protection of bacteria. When the antimicrobial substances are applied to the food matrices, they cannot penetrate through the pores where the bacteria are protected. This causes the survival detection of bacteria

contaminated on food matrices. It is similar to the results of the wrapping process. Alginate-Tapioca starch based edible films incorporated with ethanolic extract of *Hibiscus sabdarifa* L., *Piper betle* L., and *Punica granatum* L. were previously produced and used as the films to wrap the pieces of carrot. The results showed that there were no antimicrobial activities of Alginate-Tapioca starch based edible film against carrot which were pre- and post-contaminated with *Bacillus cereus*. However, the antimicrobial activities against carrot contaminated with *Salmonella* Typhimurium were detected. The outstanding antimicrobial activity was investigated when the Alginate-Tapioca starch based edible films were applied with post-contaminated carrot with *Salmonella* Typhimurium at room temperature.

It could be investigated that *Bacillus cereus* showed the highest resistance to all extracts of medicinal plants. This was expected because of the ability to produce spores, which are very stable against bactericidal agents in medicinal plants as well as against disinfectant agents. It is generally known that dormant bacterial spores are highly resistant to many physical treatments. It also was considered that the decrease of the inactivation ratio, tailing of the surviving curves, was partly due to the formation of spore. Furukawa *et al*, (2005) suggested that the addition of the surface-active agent to the bacterial suspensions prevented the formation of the spore clumps through physical and chemical treatments. It could increase the rate of inactivation.

The antimicrobial activities of those decreased in cold condition. Correlation between temperature and lethal effect of antibacterial compounds and disinfectant can be clearly proven. Temperature is a sensitive factor which gives either positive or negative effect to the lethal ability. Generally, most of disinfectants work optimally at room temperature (20°C), but there are some exceptions (Thiel, 1977). When the temperature is decreased to 10° C from 20° C, the required time to destroy the organism is longer for many of the bactericidal actives (Herre, 1985). Due to the fact that, the extracts of most of the medicinal plants are rich in phenolic compounds such as flavonoid and phenolic acids, but also contain a variety of other active compounds the decreased activity of the extracts can be explained. Additionally several researchers found that the protective action of antimicrobial films accelerated at

higher temperature, due to high diffusion rates in the polymer (Vojdani and Torres, 1989a; 1989b; Wong *et al.*, 1996). Generally, increasing storage temperature could accelerate the migration of the active agent in the film/coating layers, while refrigeration slowed down the migration rate. The temperature conditions during production and distribution have to be predicted to determine their effect on the residual antimicrobial activity of the active compounds.

Although the antimicrobial activities against pre-contaminated carrot were detected, the post-contaminated *Salmonella* Typhimurium on wrapped carrot was more sensitive than the pre-contaminated one. On the food surface, there are both organic substances and pores. The pre-contaminated bacteria can have reaction against these. Organic substances act as interfering substances. They cause the decreasing antimicrobial activity and result in the surviving of bacteria. Pores of food matrices can protect the bacteria against the environmental impacts such as antimicrobial activity. In contrast, food surface that were coated or wrapped with antimicrobial coating or antimicrobial films provided the protection effect against the contamination of microorganism contaminated in the environment. Although the organic substances naturally occurring in food matrices can act as interfering substances, the pores on food surface were coated and wrapped. Therefore the physical protections were reduced and bacteria had no longer physical protective from the food surface. They were destroyed because of the direct contact with those antimicrobial materials.

5.9.2 Antimicrobial Efficacy of Alginate-Tapioca Starch Based Edible Film and Coating Incorporated with the Ethanolic Extracts of *Hibiscus sabdarifa* L., *Piper betle* L., and *Punica granatum* L. in Chicken Meat

Incorporation of bactericidal agents or growth inhibitors into meat formulations results in partial inactivation of the active substances by product constituents which act as interfering substance and is therefore expected to have only limited effect on the surface flora. Also, direct application of antimicrobial agents onto meat surface, by dipping or spraying, has been shown to be inefficient, due to the rapid diffusion of

the active substances within the bulk of food (Torres *et al.*, 1985; Siragusa and Dickson, 1992).

In the coating process, the Alginate-Tapioca starch based edible coating incorporated with those ethanolic extract of *Hibiscus sabdarifa* L., *Piper betle* L., and *Punica granatum* L. showed no activities against all contaminated bacterial both pre- and post-contamination with exception of post-contaminate *Salmonella* Typhimurium at room temperature. At this condition the strong antimicrobial effect of Alginate-Tapioca starch based edible coating incorporated with those ethanolic extracts was detected. They had abilities to protect the chicken meat from the post-contaminated bacteria. The post-contaminated bacteria were destroyed and reduced to an undetectable level within 24-48 hours contact time.

The outstanding antimicrobial activity was obtained when using the wrapping method. Antimicrobial activity of the Alginate-Tapioca starch based edible films incorporated with ethanolic extracts of *Hibiscus sabdarifa* L., *Piper betle* L., and *Punica granatum* L. was found against *Salmonella* Typhimurium in both contamination processes. Especially, Alginate-Tapioca starch based edible films incorporated with those three ethanolic plant extracts had abilities to inactivate *Salmonella* Typhimurium ATCC 13311 on post-contaminated chicken meat to undetectable level in 24-48 hours at room temperature and at cold storage only Alginate-Tapioca starch based edible films incorporated with *Hibiscus sabdarifa* L. and *Piper betle* L. presented the complete reduction ability.

As already discussed the storage temperature affects the activity of antimicrobial edible films. Several researchers found that the protective action of antimicrobial films accelerated at higher temperature, due to high diffusion rates in the polymer (Vojdani and Torres, 1989a; 1989b; Wong *et al.*, 1996). Generally, increasing storage temperature could accelerate the migration of the active agent in the film/coating layers, while refrigeration slowed down the migration rate. The temperature conditions during production and distribution have to be predicted to determine their effect on the residual antimicrobial activity of the active compounds.

Food components significantly affect the effectiveness of the antimicrobial substances and their release. Physico-chemical characteristics of food could alter the activity of antimicrobial substances. Foods with different biological and chemical characteristics are stored under different environment condition, which, in turn, cause different patterns of microflora growth. Aerobic microorganisms can exploit headspace O_2 for their growth. The pH of a product affects the growth rate of target microorganisms and changes the degree of ionization of the most active chemicals, as well as the activity of the antimicrobial substances (Han 2000). Weng and Hotchkiss (1993) reported that low density polyethylene (LDPE) films containing benzoic anhydride was more effective in inhibiting moulds at low pH values. Rico-Pena and Torres (1991) found that the diffusion of sorbic acid decreased with an increase in pH. The food a_w altered the microflora, antimicrobial activity, and chemical stability of active ingredients applied by impregnation. Vojdani and Torres (1989a) showed that the diffusion of potassium sorbate through polysaccharide films increased with a_w ; this had a negative impact on the amount available for protection. Rico-Pena and Torres (1991) found that potassium sorbate diffusion rates in Methylcellulose (MC) or Hydroxypropyl methylcellulose (HPMC) films containing palmitic acid were much higher at higher values of a_w .

The literature is more meagre and recent on the subject of adding functional extracts to edible films. Seydim and Sarikus (2006) reported that prepared various whey protein based edible films enriched with essential oils obtained from oregano, rosemary or garlic, the oregano-enriched film exhibited the strongest antibacterial properties, followed by the garlic enriched film. In opposition, the rosemary-enriched film had no antibacterial attributes at all. Oussalah *et al.* (2004) tested the antibacterial effects of milk protein-based edible films containing extract from oregano or pimento against foodborne pathogens and food spoilage bacteria in preserved whole beef muscle. The results indicated that the film containing oregano extract to be the most effective, achieving reductions of around 1 log unit for each of these bacterial species at the end of storage compared with uncoated samples.

Kim *et al.* (2006) tested the antimicrobial activity of edible soy protein isolate films enriched with green tea extract against several types of bacteria, including food pathogens, on agar media and found *Staphylococcus aureus* to be inhibited. Other workers combined the properties of a chitosan film with the extract of oregano and observed it to be efficacious against both *Listeria monocytogenes* and *Escherichia coli* O157:H7. In that study, of several film formulations used to coat the surface of inoculated bologna slices, pure chitosan by itself displayed bactericidal activity, but higher activity was achieved by chitosan film enriched with oregano extract (Zivanovic *et al.*, 2005).

In this present study, Alginate-Tapioca starch based edible films and coating incorporated with 20.0 % (v/v) crude ethanolic extract of *Hibiscus sabdarifa* L., *Piper betle* L. , and *Punica granatum* L. demonstrated the antimicrobial activity against contaminated *Bacillus cereus* and *Salmonella* Typhimurium on carrot and chicken meat. They provided the protective ability better than an eliminating ability. The edible films provided the greater protective ability than edible coating.

This is the report discuss about the incorporation of crude extract of those medicinal plant into the Alginate-Tapioca starch based edible films, there were only few researchers dealing with this. The effectiveness is one point but the taste is the second limitation. Alginate-Tapioca starch based edible films incorporated with 20.0 % (v/v) ethanolic extract of *Hibiscus sabdarifa* L., *Piper betle* L., and *Punica granatum* L. which provided good antimicrobial activity also had a sour, peppery and bitter taste, which could make them unacceptable. Further research should focus on qualification and quantification of active compounds in extracts and the determination of antimicrobial activity of those and the application as antimicrobial edible films. Moreover, the determination of potential losses during edible films preparation is important. Full characterization of stability, diffusion of the active compounds from the edible films into the product, and resulting antimicrobial effects will assist in optimizing the application of the active edible films on food products.

6 CONCLUSIONS

The determination of the antimicrobial activity of Thai traditional medicinal plants included plant extracts of *Andrographis paniculata* (Burm.f.) Wall. ex Nees, *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* L., *Hibiscus sabdarifa* L., *Musa sapientum* L., *Nelumbo nucifera* Gaertn., *Piper betle* L., *Psidium guajava* L., and *Punica granatum* L. Based on the results of the broth dilution susceptibility assay according the method described by the DVG (2000) to determine the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentrations (MBC) it can be concluded that: all Thai traditional medicinal plant extracts tested showed antibacterial activity against at least one of the test organisms. In general, ethanolic extracts showed higher activity than hot water extracts and ultrasonicated extracts. The appropriate extraction process with an outstanding antimicrobial activity of the extract was the alcoholic extraction with 50% ethanol for 24 hours. Among all Thai traditional medicinal plants used in this study, *Piper betle* L. and *Hibiscus sabdariffa* L. showed an outstanding antibacterial activity against the tested microorganisms. The most susceptible test organisms to these two extracts were *Vibrio parahaemolyticus* and *Yersinia enterocolitica*, while *Bacillus subtilis* was the most stable ones.

In the suspension test or Time Killing Analysis modified according DIN EN 1040, ethanolic extracts of those Thai traditional medicinal plants which presented the outstanding antimicrobial properties were tested without addition of interfering substance to determine their basic bactericidal activity. Ethanolic extract of *Piper betle* L. presented the greatest antimicrobial activity followed by ethanolic extract of *Hibiscus sabdarifa* L. Ethanolic extract of *Piper betle* L. at concentration of 10.0 g/100 ml showed the 'letal effect' within 15 seconds against 8 of 10 strains of test microorganisms, while ethanolic extract of *Hibiscus sabdarifa* L. at 10.0 g/100 ml presented the same effect within 15 seconds only against *Vibrio paraheamolyticus*. The other test microorganisms required at least 1 hour contact time.

The other ethanolic extract of Thai traditional medicinal plants showed the bactericidal effect at concentration of 15.0 g/100 ml against *Vibrio paraheamolyticus*

and *Listeria monocytogenes*. Using a concentration of 15.0 g/100 ml of ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe and *Garcinia mangostana* L. in the suspension test, those test organisms were completely inactivated within 15 seconds contact time. *Punica granatum* L. and *Psidium guajava* L. presented completely destroyed those test organisms within 1 hour. Ethanolic extract of *Andrographis paniculata* (Burm.f.) Wall. ex Nees required more than 1 hour. At the lower concentration, ethanolic extract of *Piper betle* L. and *Hibiscus sabdarifa* L. presented the outstanding antimicrobial activity.

As mentioned before the highest bactericidal activity were *Piper betle* L. and *Hibiscus sabdarifa* L., followed by *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* L., *Punica granatum* L., *Psidium guajava* L., *Andrographis paniculata* (Burm.f.) Wall. ex Nees, and *Musa sapientum* L., the weakest activity exhibited by *Nelumbo nucifera* Gaertn. The limiting test organisms were *Bacillus cereus*, *Bacillus subtilis*; representing the Gram-positive bacteria and *Pseudomas aeruginosa*; representing the Gram-negative bacteria. The most susceptible organism was *Vibrio paraheamolyticus*, followed by *Listeria monocytogenes*.

Due to the fact that the expected contact time in the intended field of application is relatively long during the storage time, all extracts can be recommended which inactivate the most test organisms within the longest contact time. The expected contact time in the field of application is relatively long and based on the results of the suspension test without interfering substances it can be concluded that all of Thai traditional ethanolic extracts showed bactericidal activity against the tested organisms. They could be recommended as bactericidal in food industries in a field of application where the extracts are in suspension but without presence of interfering substance.

Based on the results of the suspension tests with interfering substance modified according DIN EN 1276 for the Time Killing Analysis it can be concluded that the addition of interfering substances inhibits the activity of all ethanolic extracts. High level soiling, the mixture of 10 g/L bovine albumin fraction V and 10 g/L yeast extract was used as interfering substances to represent the environmental food system.

Higher concentrations and/or longer contact times were necessary to reduce the test organisms.

The limiting test organisms were *Bacillus cereus*. This bacteria presented the higher resistance to the ethanolic extract in the presence of interfering substances compared with in the absence one. *Pseudomonas aeruginosa*, the representing resistance Gram-negative test organism was inactivated when using 15.0 g/100 ml concentration of the extracts within 6 hours.

The effect on the different extracts was different, while the extract of *Piper betle* L. and *Hibiscus sabdarifa* L. were less affected by the presence of interfering substance, the extracts of *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* L., and *Punica granatum* L. showed decreased bactericidal activity.

For the application in fields at which soiling is expected, the extract of *Piper betle* L. and *Hibiscus sabdarifa* L. should be recommended. However, ethanolic extract of all Thai traditional medicinal plant extracts could be used because of the fact that long contact times are generally used. These extracts show activity against most of test organisms within 24 hours contact time

Artificial coated stainless steel surface coated with the ethanolic extract of *Piper betle* L., which showed outstanding antimicrobial activity in previous tests was used as artificial antimicrobial surface and tested against several test organisms. At the coated concentration of 30.0 g/100 ml, *Pseudomonas* sp., *Vibrio parahaemolyticus*, and *Yersinia enterocolitica* contaminated on a second metal surface were inhibited and showed the lethal effect in at least 24 hours contact time. A lower coated concentration resulted in the decreasing of antimicrobial activity. As discover from the results, the artificial coated surface is not successful application method to simulate the antimicrobial packaging. The stainless steel surface lacks the property to absorb and release the natural active substances. Additional especially Gram-negative test organisms are killed by long drying and/or contact times. The bactericidal activity

of the extracts coated on the surfaces can not be clearly distinguished from the natural dying effect of those test organisms. Therefore, this application is not suitable.

The finding of the present study demonstrate that the natural antimicrobial compounds of those Thai traditional medicinal plants can be successfully incorporated into Alginate-Tapioca starch based edible film and retain their inhibitory effect against microbial growth in model media based on the inhibitory zone, inhibitory underneath the films, and suspension test. The outstanding antimicrobial activity was obtained from Alginate-Tapioca starch based edible film incorporated with ethanolic extract of *Piper betle* L., *Hibiscus sabdarifa* L., and *Punica granatum* L.

The cytotoxic activity of those ethanolic extracts of medicinal plant were determined, because in the intended field of application a contact to mucous membranes will occur. The presented results indicate the presence of cytotoxic activity in all of the plant extracts against all of the tested cell line. This correlates more with their effect on microorganisms than with their possible effect on the mucous membranes of human. Together with the dilution of the substances through the food products they are applied to, no toxic effect can be expected on the final user. This present results give addition evidence that those plant extracts are potential sources of biologically active compounds.

Finally, both Alginate-Tapioca starch based edible coating and Alginate-Tapioca starch based edible films incorporated with ethanolic extract of *Piper betle* L., *Hibiscus sabdarifa* L., and *Punica granatum* L. had the ability to control *Bacillus cereus* and *Salmonella* Typhimurium pre- and post-contaminated on carrot and chicken meat. Alginate-Tapioca starch based edible coatings incorporated with those ethanolic extracts provide a protectable effect against the post-contaminated microorganisms better than bactericidal effect against pre-contaminated microorganisms. Alginate-Tapioca starch based edible coating incorporated with ethanolic extract of *Piper betle* L. showed the outstanding antimicrobial activity followed by ethanolic extract of *Hibiscus sabdarifa* L. and *Punica granatum* L.

The Alginate-Tapioca starch based edible films provide the better antimicrobial properties than the coating one. They express not only protective properties against post-contaminated microorganisms but also have a bactericidal effect against pre-contaminated microorganisms. They also present good antimicrobial activities in both carrot and chicken meat. Alginate-Tapioca starch based edible films incorporated with ethanolic extract of *Hibiscus sabdarifa* L. present the outstanding antimicrobial properties followed by *Piper betle* L. and *Punica granatum* L. Moreover, the great antimicrobial activity of Alginate-Tapioca starch based edible films incorporated with those ethanolic extracts are noticed in the room temperature condition.

The bactericidal effect of both application forms is affected by the storage temperature. The activity is reduced at lower temperature (4 °C), so application at room temperature (20 ± 5 °C) should be recommended. However, growth of bacteria is also reduced at lower temperatures, the recommended field of application is wrapping method and stored at cold condition. The wrapping antimicrobial edible films presented the bactericidal activity and the low temperature condition exhibited the growth reduction.

Concluding, those Thai traditional medicinal plants by itself or incorporated into Alginate-Tapioca starch based edible films and coating has potential for inhibiting or inactivating foodborne pathogens and food related bacteria in microbiological media and model food system; therefore, they could be useful as a food additive for controlling growth of foodborne pathogens or food related bacteria. The results also supported the healing potency of some Thai traditional medicinal plants used in Thai practitioner traditional medicine. This study demonstrated that simple extracts of nine Thai traditional medicinal plants have activity against food related bacteria. Their extracts, therefore, might have potential to extend shelf-life or improve safety of foods. Furthermore the interaction of the extracts on foods flavour should be evaluated. These factors may influence the applicability of medicinal plants extracts in certain food products.

7 SUMMARY

In Thai traditional medicine, different plant extracts are known to have a bactericidal or at least a bacteriostatic effect on bacteria and/or fungi. In Thailand, medicinal plants have been used safely since ancient times as herbal medicines and also as food colouring and flavouring agents. The application of selected plant extracts to foods could prevent foodborne diseases and food spoilage. In this study the antimicrobial activities of *Andrographis paniculata* (Burm.f.) Nees, *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* Linn., *Hibiscus sabdariffa* Linn., *Musa sapientum* Linn., *Nelumbo nucifera* Gaertn., *Piper betle* Linn., *Psidium guajava* Linn., and *Punica granatum* Linn. used by Thai practitioners of traditional medicine were investigated against different strains of food related bacteria, including 3 strains of *Escherichia coli* 2 strains of *Salmonella* Typhimurium, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, 1 strain of *Bacillus subtilis*, *Vibrio parahaemolyticus*, *Yersinia enterocolitica*, and 2 strains of *Pseudomonas* sp. Minimum Inhibitory Concentration (MIC-value) and Minimum Bactericidal Concentration (MBC-value) of hot water extracts, ultra sonicated extracts, and ethanolic extracts were determined by using the method described by the DVG (Deutsche Veterinärmedizinische Gesellschaft). From the results, it can be investigated that all crude extracts exhibited antimicrobial activity at least against one of the test organisms at concentration of 15.0 g/100 ml or lower. The results also indicated that the antibacterial activity of hot water extracts was better than ultra sonicated extracts and the ethanolic extracts exhibited the best antibacterial activity. Among the ethanolic extracts, the highest inhibitory activity was caused by *Hibiscus sabdariffa* Linn. and *Piper betle* Linn. The antimicrobial activity of both extracts against the test organisms was determined as Minimum Inhibitory Concentrations (MIC-value) ranging from 0.1 to 3.0 g/100 ml and 0.1 to 2.5 g/100 ml, as well as Minimum Bactericidal Concentrations (MBC-value) ranging from 0.2 to 3.5 g/100 ml and 0.2 to 1.5 g/100 ml for the ethanolic extracts of *Hibiscus sabdariffa* Linn. and *Piper betle* Linn., respectively.

Bactericidal activity of the extracts was determined using a modified suspension test method according to DIN EN 1040 (Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics) and DIN EN 1276 (Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and institutional areas). Concentrations of 15.0 g/100 ml of the pure ethanolic extract of *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* Linn., *Hibiscus sabdarifa* Linn., and *Piper betle* Linn., demonstrated a lethal effect within 15 seconds contact time. The others required more than 1 hour contact time to inactivate the test organisms. Among the test organisms, spore forming *Bacillus* spp. exhibited the highest resistance. The bactericidal activity of Thai traditional medicinal ethanolic extracts was decreased when interfering substances were added.

To determine the bactericidal activity on surfaces the ethanolic extract of *Piper betle* Linn. was coated on stainless steel discs, but the results indicated that stainless steel discs were not the suitable surfaces to use as an artificial antimicrobial surface.

To simulate the application under use conditions the antimicrobial activity of Alginate-Tapioca starch-based edible films were studied by incorporation of those ethanolic extracts as a natural antimicrobial agent. The edible films exhibited antimicrobial activity against bacteria tested by using agar diffusion assay. The presence of 20.0 % v/v of those extracts in the edible films had the ability to inactivate the test organisms within 24 hours incubation time. These results revealed that ethanolic extracts of those medicinal plants had a good potential to be incorporated to make antimicrobial edible film or coating for various food applications. The cytotoxic effect of those ethanolic medicinal plants extract were also determined using 4 different cell lines. The results of the cytotoxicity screening showed that the activity of the extracts correlates more with their effect on microorganisms than with their possible effect on the mucous membranes of human.

The antimicrobial edible films and coating were studied in model food. The results demonstrated that the edible film wrapping method had advantages and showed more

excellent benefit in the application with food than the edible coating method. In addition, the storage temperature was an important factor. Antimicrobial edible films presented higher activity at room temperature than under cold conditions (4°C).

In summary, the extract of those Thai traditional medicinal plants by itself or incorporated into edible films had potential for inhibiting or inactivating foodborne pathogens or food related bacteria in microbiological media and model food system; therefore, they could be useful as a method for controlling growth of foodborne pathogens and food related bacteria.

8 ZUSAMMENFASSUNG

In Thailand ist bei Ärzten der traditionellen Heilkunst seit langem die inaktivierende oder wenigstens statischen Wirkung verschiedener Pflanzenextrakte auf Bakterien und/oder Pilze bekannt. In Thailand, wurden Heilpflanzen von alters her sicher als Medizinkräuter und zum Würzen und Färben von Lebensmitteln eingesetzt. Die Anwendung ausgewählter Pflanzenextrakte auf Lebensmitteln könnte zur Vermeidung von Lebensmittelverderbnis und -infektionen beitragen. In der vorliegenden Studie wurde die bakterizide Wirkung von *Andrographis paniculata* (Burm.f.) Nees, *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* Linn., *Hibiscus sabdariffa* Linn., *Musa sapientum* Linn., *Nelumbo nucifera* Gaertn., *Piper betle* Linn., *Psidium guajava* Linn., and *Punica granatum* Linn., die in der traditionellen thailändischen Heilkunde angewendet werden, auf verschiedene Bakterien getestet, die mit Lebensmittelverderbnis und -infektionen im Zusammenhang stehen. Hierzu zählten 3 *Escherichia coli*-Stämme, 2 Stämme von *Salmonella* Typhimurium, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas sp* und jeweils 1 repräsentativer Stamm von *Bacillus subtilis*, *Vibrio parahaemolyticus* und *Yersinia enterocolitica*. Von allen Heilpflanzen wurde jeweils ein Heißwasser- Ultraschall- und Alkohol-Extrakt hergestellt, von denen dann zunächst mittels der von der Deutschen Veterinärmedizinischen Gesellschaft (DVG) beschriebenen Methode die Minimalen Hemmkonzentration (MHK) und die Minimalen Bakteriziden Konzentration (MBK) bestimmt wurde. Die Ergebnisse zeigten, dass die reinen Extrakte in Konzentrationen von 15.0 g/100 ml oder weniger bakterizide Wirkung gegen mindestens einen der getesteten Mikroorganismen entwickelten. Die beste Wirksamkeit zeigte dabei das Alkohol-, die geringste Aktivität das Ultraschall-Extrakt. Unter den Alkohol-Extrakten besaßen die von *Hibiscus sabdariffa* Linn. und *Piper betle* Linn. die stärkste inhibitorische Wirkung. Dabei lag die MHK für das Extrakt von *Hibiscus sabdariffa* Linn. in Abhängigkeit vom Testorganismus zwischen 0.1 und 3.0 g/100 ml und die MBK bei 0.2 bis 1.5 g/100 ml. Mit dem Alkohol-Extrakt von *Piper betle* Linn. wurden MHK-Werte zwischen 0.1 und 2.5 g/100 ml, bzw. MBK-Werte von 0.2 bis 3.5 g/100 ml ermittelt.

Zur Bestimmung der Inaktivierungskinetik und des Eiweißfehlers der Alkohol-Extrakte wurden Suspensionsversuche modifiziert nach DIN EN 1040 (Quantitativer Suspensionsversuch zur Bestimmung der bakteriziden Wirkung (Basistest) chemischer Desinfektionsmittel und Antiseptika) und DIN EN 1276 (Quantitativer Suspensionsversuch zur Bestimmung der bakteriziden Wirkung chemischer Desinfektionsmittel und Antiseptika in den Bereichen Lebensmittel, Industrie, Haushalt und öffentliche Einrichtungen). Ohne Zusatz von Belastungssubstanz wirkten die Extrakte von *Curcuma zedoaria* (Christm.) Roscoe, *Garcinia mangostana* Linn., *Hibiscus sabdarifa* Linn., and *Piper betle* Linn., in einer Konzentration von 15.0 g/100 ml innerhalb von 15 Sekunden Einwirkungszeit bakterizid auf die Testkeime. Bei gleicher Konzentration mussten die übrigen Extrakte mindestens 1 Stunde einwirken, um die Testkeime zu inaktivieren. Unter den Testkeimen zeigten die sporenbildenden *Bacillus* spp. die größte Stabilität gegenüber den Extrakten. Alle Heilpflanzenextrakte wiesen bei Zusatz einer repräsentativen Belastungssubstanz einen mehr oder weniger ausgeprägten Eiweißfehler auf.

Zur Bestimmung der Wirksamkeit auf Oberflächen wurden zunächst rostfreie Stahlplättchen mit dem Alkohol-Extrakt von *Piper betle* Linn. beschichtet. Die Ergebnisse zeigten jedoch, dass dieses Trägermaterial nicht zur Simulation des späteren Anwendungsgebietes der Pflanzenextrakte geeignet war.

Zur Simulation der praktischen Anwendungsbedingungen wurde die bakterizide Wirksamkeit einer eßbaren, auf Alginat-Tapioka-Stärke basierenden Beschichtung, die die jeweiligen Alkohol-Extrakte als natürliche, antibakteriell wirksame Substanzen enthielten, getestet. Im Agar-Diffusionstest wurde die bakterizide Wirksamkeit der eßbaren Folien nachgewiesen. Dabei war ein Volumenanteil von 20.0 % v/v der Extrakte in der Beschichtung ausreichend, um die Testkeime innerhalb von 24 Stunden zu inaktivieren. Diese Ergebnisse ließen erkennen, dass gute Einsatzmöglichkeiten für in eßbare Beschichtungen inkorporierte Alkohol-Extrakte der getesteten Heilpflanzen im Lebensmittelbereich bestehen könnten. Dies wurde auch durch die Ergebnisse der Zytotoxizitätsversuche an 4 Zelllinien untermauert. Hierbei wurde festgestellt, dass der ermittelte, geringe zytotoxische Effekt sich nicht

negativ auf die menschliche Schleimhaut, die mit den eßbaren Beschichtungen in Kontakt kommt, auswirken, sondern auf Mikroorganismen beschränkt sein dürfte.

Die bakterizide Wirksamkeit von eßbaren Folien und Beschichtungen wurde an Modell-Lebensmitteln getestet. Die Ergebnisse zeigten, dass die Verpackung mit einer eßbaren Folie wesentliche Vorteile gegenüber einer eßbaren Beschichtung aufwies. Daneben stellte die Lagerungstemperatur einen wesentlichen Faktor dar. So war die bakterizide Wirksamkeit der eßbaren Beschichtungen bei Raumtemperatur deutlich höher als bei Kühlschranktemperatur (4°C).

Zusammenfassend kann festgestellt werden, dass die Extrakte der getesteten thailändischen traditionellen Heilpflanzenextrakte als pure Extrakte oder aber inkorporiert in eßbare Beschichtungen das Potential besitzen durch Lebensmittel übertragbare Pathogene und Verderbniserreger in Suspensions- und Modell-Lebensmittel-Versuchen zu Inaktivieren oder zumindest zu Hemmen. Daher könnte ihre Anwendung dazu beitragen Lebensmittelinfektionen und – verderbnis zu verhindern.

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10 ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Alle Stellen, die dem Wortlaut oder dem Sinn nach anderen Werken entnommen wurden, sind durch Angabe der Quellen kenntlich gemacht

Die Arbeit wurde noch bei keiner anderen Prüfungsbehörde als Prüfungsleistung vorglegt.

Stuttgart-Hohenheim, Januar 2009

.....

(Racha Tepsorn)

11 CURRICULUM VITAE

Name: Mr. Racha Tepsorn

Date of Birth: December 27, 1977

Place of Birth: Pattani, Thailand

Education Background:

Primary School: 1983-1987
Anuban Pattani School, Pattani, Thailand

High School: 1990-1996
Demonstration School
Faculty of Education
Prince of Songkhla University, Pattani Campus, Pattani, Thailand,

Bachelor's Degree: 1996-2000
Bachelor's Degree of Science (Food Science and Technology) 2nd class honors
Department of Food Science and Technology
Faculty of Agro-Industry
Kasetsart University, Thailand,

Master's Degree: 2000-2003
Master's Degree of Science (Food Science)
Department of Food Science and Technology
School of Graduate study
Faculty of Agro-Industry
Kasetsart University, Thailand

Scholarship:

2000-2003:
Academic Lecturer Development Program from the Ministry of University Affair,
Thailand

2005-present
German Academic Exchange Service (Deutscher Akademischer Austausch Dienst:
(DAAD) Ref. 422, PKZ: A/05/14827

The academic project and presentation:

B.Sc. Project:

Technique for the determination of D-Value of *Listeria monocytogenes* in Surimi

B.Sc. Seminar:

The Used of HACCP plan in Frozen Chicken Process

First M.Sc. Seminar:

Elimination of Pathogenic Bacteria Contaminated on Chicken Meat before Frozen Process

Second M.Sc. Seminar:

Effect of Peroxyacetic acid on the Contamination Reduction in Frozen Chicken Process and Cell Injury of Multi-Antimicrobial Resistance of *Salmonella* sp

M.Sc. Thesis:

Effect of Peroxyacetic acid on the Contamination Reduction in Frozen Chicken Process and Cell Injury of Multi-Antimicrobial Resistant *Salmonella* sp. Isolated from Chicken Meat and Chicken Product in Thailand

Award:

1. The Outstanding Graduated published Thesis in Agro-Industry field. The award provided by the Graduate School, Kasetsart University, 2004
2. Poster competition award 2008 for the best Poster in the Food Safety category, in 41st Annual AIFST Convention 2008. The award provided by the Australian Institute of Food Science and Technology.

International Presentation:

Sudsai Trevanich., **Racha Tepsorn.**, Preeya Vibulsresth., and Takahisa Miyamoto. 2003. Use of Peroxyacetic acid to Reduce the Multi-Antimicrobial Resistant *Salmonella* Virchow Contaminated on Chicken Meat. 41st Kasetsart University Annual Conference. 3-7 February 2003. Kasetsart University, Bangkok, Thailand. **(Poster Presentation)**

Racha Tepsorn, Birgit Hunsinger, and Reinhard Böhm. 2006. Antibacterial Activity of Some traditional Thai Medicinal Plants against Foodborne Pathogens: Determination of Minimum Inhibition Concentration and Minimum Bactericidal Concentration. The International Conference on Innovations in Food and Bioprocesses Technologies. 12-14 December 2006. Asian Institute of Technology, Bangkok, Thailand. **(Oral Presentation)**

Racha Tepsorn, Birgit Hunsinger, and Reinhard Böhm. 2007. Antibacterial Activity of Nine Traditional Thai Medicinal Plants against Foodborne Pathogens: Minimum Inhibition Concentration and Minimum Bactericidal Concentration Determination. Annual Meeting and Food ExpoSM 2007. 28 July-1 August 2007. Institute of Food Technologist, Chicago, Illinois, United State of America. **(Poster Presentation)**

Racha Tepsorn, Birgit Hunsinger, and Reinhard Böhm. 2007. Antimicrobial Effects of Roselle (*Hibiscus sabdarifa* L.) and Betel Leaves (*Piper betle* L.) on the Growth of Food-Related Bacteria Including Foodborne Pathogens **The International Agricultural Engineering Conference, section 441**. 3-6 December 2007. Asian Institute of Technology, Bangkok, Thailand. **(Oral Presentation)**

Racha Tepsorn, Birgit Hunsinger, and Reinhard Böhm. 2008. Bactericidal Properties of Traditional Thai Medicinal Plants (*Hibiscus sabdariffa* L, and *Piper betle* L.) Extracts on the Growth of Food Related Bacteria Including Foodborne Pathogens. Annual Meeting and Food ExpoSM 2008. 28 June-1 July 2008. Institute of Food Technologist, New Orleans, Louisiana State, United State of America. **(Poster Presentation)**

Racha Tepsorn, Birgit Hunsinger, and Reinhard Böhm. 2008. Antimicrobial Effect of Roselle (*Hibiscus sabdariffa* L.) and Betel Leaf (*Piper betle* L.) Extract-Incorporated Alginate-Tapioca-Based Edible Film against Food Related Bacteria Including Foodborne Pathogens. 41st Annual AIFST Convention 2008. 21-24 July 2008. The Australian Institute of Food Science and Technology, Sydney, Australia. **(Poster Presentation)**

Publication:

Racha Tepsorn, 2003. Effect of Peroxyacetic acid on the Contamination Reduction in Frozen Chicken Process and Cell Injury of Multi-Antimicrobial Resistant *Salmonella* sp. Isolate from Chicken Meat and Chicken Product in Thailand. **M.Sc. Thesis**, Kasetsart University, Bangkok.

Sudsai Trevanich., **Racha Tepsorn**., Preeya Vibulsresth. and Takahisa Miyamoto. 2003. Use of Peroxyacetic acid to Reduce the Multi-Antimicrobial Resistant *Salmonella* Virchow Contaminated on Chicken Meat. **In The Proceeding of 41st Kasetsart University Annual Conference**. 3-7 February 2003. Kasetsart University, Bangkok, Thailand.

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