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Antimicrobial and Antioxidant Activities of Betel Oil

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ABSTRACT

Betel oil has been studied for its antimicrobial and antioxidant activities against ten pathogenic and spoilage bacteria and three strains of yeast using an agar well diffusion assay and against oxidative bleaching using a β-carotene agar well diffusion assay, respectively. The minimum inhibitory concentration (MIC) and minimum oxidative bleaching inhibitory concentration (MOBIC) of betel oil were determined using an agar dilution method. At the concentration of 50 μL mL^{-1}, betel oil showed a zone of inhibition, ranging from 9.15 to 17.30 mm in diameter. The MICs of betel oil in a range of 12.5-100 μL mL^{-1} could inhibit the growth of all test microorganisms except *Pseudomonas aeruginosa*, which was not sensitive to this oil even at the highest concentration (200 μL mL^{-1}). The most sensitive bacteria to betel oil were *Listeria monocytogenes* and *Salmonella* Enteritidis. Betel oil (100 μL mL^{-1}) also revealed ability to inhibit the oxidation of β-carotene, yielding a yellow zone surrounding the well with a 8.40 mm in diameter. The MOBIC of betel oil was 100 μL mL^{-1}. Betel oil might have a potential application in controlled and released food packaging technology as antimicrobial and antioxidant agents.

Key words: betel; antimicrobial; antioxidant; MIC; MOBIC

INTRODUCTION

The appearance of foods is one of the major determinants of its sensory appeal to consumers and consequently, sales of the product. Microbial contamination and lipid oxidation are the main factors that determine food quality loss and shelf-life reduction. Therefore, preventing microbial contamination and delaying lipid oxidation are highly relevant to food processors. The growth of microorganisms in food products may cause spoilage or foodborne diseases. Oxidative processes in food products lead to the degradation of lipids and proteins which, in turn, contribute to the deterioration in flavour, texture and colour of the products (Decker *et al.*, 1995).

Prevention of pathogenic and spoilage microorganisms in foods is usually achieved by using chemical preservatives. These chemical preservatives act as antimicrobial compounds which inhibit the growth of undesirable microorganisms. However, the onset of increasing demand for minimally-processed, extended shelf-life foods and reports of chemical preservatives as having potential toxicity demand food manufacturers to find alternative means (Conner, 1993; Nychas, 1995). There is a currently strong debate about the safety aspects of chemical preservative since they are considered responsible for many carcinogen and teratogenic attributes as well as residual toxicity. For these reasons, consumers tend to be suspicious of chemical

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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).

Oxidative deterioration of fat components in food products is responsible for off-flavours and rancidity which decrease nutritional and sensory qualities. An addition of antioxidants is required to preserve product quality. Synthetic antioxidants (e.g. butylate hydroxytoluene (BHT), butylate hydroxyanisole (BHA), tert-butylhydroxyhydroquinone (TBHQ) and propyl gallate (PG)) are widely used as antioxidants in the food industry. Their safety, however, has been questioned. BHA was revealed to be carcinogenic in animal experiments. At high doses, BHT may cause internal and external hemorrhaging, which leads to death in some strain of mice and guinea pigs (Ito *et al.*, 1986). There is much interest among food manufacturers in natural antioxidants, to act as replacements for synthetic antioxidants currently used (Plumb *et al.*, 1996).

Piper betel Linn. is a tropical plant closely related to the common pepper. It is extensively grown in Sri Lanka, India, Malaysia, Thailand, Taiwan and other Southeast Asian countries. Its common names are betel (in English), pan (in Indian), phlu (in Thai) and sirih (in Bahasa Indonesian). It has been historically known as traditional herb used as mouth wash, dental medicine, cough medicine, astringent, tonic and others (Farnsworth and Bunyapraphatsara, 1992). Recently, Bhattacharya *et al.* (2006) have found that betel ethanolic extract appears to be a promising formulation for further investigation as a new natural photo-protector. Several researchers have reported that betel extract and betel oil showed antimicrobial and antioxidant activities in model systems (Salleh *et al.*, 2002; Lei *et al.*, 2003; Dilokkunanant *et al.*, 2004; Suliantari *et al.*, 2005; Bhattacharya *et al.*, 2006).

The objectives of this study are aimed at investigating for antimicrobial activity of betel leaves oil against several pathogenic and spoilage bacteria and yeasts and at assessing for its antioxidant activity against oxidative bleaching of β-carotene.

MATERIALS AND METHODS

Materials

Betel oil from fresh leaves was purchased from Thai-China Flavours and Fragrances Industry Co.,Ltd. (Bangkok, Thailand). This oil is obtained by distillation of the fresh leaf of *Piper betel* Linn. The company claimed that major constituents of betel oil were chavibetol, chavibetol acetate and caryophyllene.

Dimethyl sulfoxide (DMSO), sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$), di-sodium hydrogen orthophosphate (Na_2HPO_4), butylate hydroxyanisole (BHA), butylate hydroxytoluene (BHT) and sodium chloride (NaCl) were purchased from Fluka Chemie (Buchs, Switzerland). Glycerol, linoleic acid and β-carotene were purchased from Sigma-Aldrich (Singapore). Ethanol was supplied by Liquor Distillery Organization, Excise Department, Ministry of Finance (Chachoengsao, Thailand).

The media used in the present studies were nutrient broth, yeast malt broth and bacteriological agar were obtained from Hi-media (India). The count plates used in the experiments were 3M Petrifilm™ aerobic count plates and 3M Petrifilm™ yeast and mould count plates. All these count plates were supplied by 3M Microbiology Products, USA.

Microbial strains and inoculum preparation

The microorganisms used in this study were *Aeromonas hydrophila* DMST 2798, *Bacillus cereus* DMST 5040, *Escherichia coli* DMST 4212, *Escherichia coli* 0157:H7 DMST 12743, *Listeria*

monocytogenes DMST 17303, *Micrococcus luteus* DMST 15503, *Pseudomonas aeruginosa* DMST 4739, *Salmonella enterica* serotype Enteritidis DMST 15676 and *Staphylococcus aureus* DMST 8840 were obtained from the Depatment of Medical Sciences (DMSC), Thailand whereas *Enterococcus faecalis* TISTR 379, *Candida albicans* TISTR 5779, *Saccharomyces cerevisiae* TISTR 5240 and *Zygosaccharomyces rouxii* TISTR 5044 were obtained from the culture collection at Thailand Institute of Scientific and Technological Research (TISTR) , Thailand. Stock cultures were stored at frozen temperature in 40 % (v/v) glycerol-either nutrient or yeast malt broth. Working bacterial culture and yeast culture were grown at 37 °C for 24 h on nutrient agar and at 30 °C for 48 h in yeast malt agar, respectively. To obtain cells in the stationary growth phase, bacterial culture and yeast culture were subcultured twice at 37 °C for 24 h on nutrient broth and at 30 °C for 48 h in yeast malt broth, respectively. Cells were harvested by centrifugation at 6,000 × g for 2 min and washed once with a 5 mM NaCl solution. The supernatant was discarded and the cells were washed again. Bacterial cells and yeast cells were re-harvested and suspended in fresh nutrient broth and yeast malt broth, respectively. Cell densities of approximately 1×10^6 CFU mL^{-1} were calculated and prepared from cultures by dilution with 0.1 M sodium phosphate buffer (pH 7.0). Cell densities were also confirmed by 3M Petrifilm™ aerobic count plates for bacteria and 3M Petrifilm™ yeast and mould count plates for yeasts.

Determination of antimicrobial activity

The antimicrobial activity of betel oil was investigated using a modified agar well diffusion technique (Chung *et al.*, 1990). Petri dishes contained 15 ml of nutrient agar for bacteria or yeast malt agar for yeasts, supplemented by test strains at a density of 1×10^6 CFU mL^{-1}. Four wells (diameter 5 mm) were made in each agar plate using a sterile cork borer. Fourty microlitres of betel oil in DMSO to give final concentration of 50 μL mL^{-1} was added in each well and DMSO blank was used as control. The plates of bacteria and yeasts were incubated at 37 °C for 24 h and at 30 °C for 48 h, respectively. The zone of inhibition surrounding the tested sample well was measured as diameter (mm) using Vernier calipers. The antimicrobial index of betel oil was expressed as: (diameter of clear zone – diameter of well)/ diameter of well (Villaseñor *et al.*, 2004).

Determination of minimum inhibitory concentrations

The agar dilution method of the European Society of Clinical Microbiology and Infectious Diseases (2000) was adopted for determination of minimum inhibitory concentrations (MICs). Petri dishes contained 15 ml of nutrient agar for bacteria or yeast malt agar for yeasts, supplemented by test strains at a density of 1×10^6 CFU mL^{-1}. Four wells (diameter 5 mm) were made in each agar plate using a sterile cork borer. Betel oil was dissolved in DMSO in two-fold serial dilutions from 0.5 to 200 μL mL^{-1}. Fourty microlitres of betel oil dilutions were individually added in wells and DMSO blank was used as control. The plates of bacteria and yeasts were incubated at 37 °C for 24 h and at 30 °C for 48 h, respectively. Minimum inhibitory concentration was defined as the lowest concentration of betel oil that resulted in a zone of inhibition.

Determination of antioxidant activity

The antioxidant activity of betel oil was investigated using a β-carotene agar well diffusion technique (Dorman *et al.*, 2000). Two grams of bacteriological agar was slowly added to 100 mL of boiling water and stirred until it completely dissolved. The agar solution was allowed to cool to 50 °C, then 2.0 mL of linoleic acid in ethanol (2.0 mg mL^{-1}) and 10 mL of β-carotene in acetone

(2.0 mg mL^{-1}) were flushed into the agar. The agar was pored into Petri dishes and allowed to set for 30 min. As acetone is used to solubilize the β-carotene, the agar does not completely set hard but remains jelly-like. Two wells (4 mm diameter) were punched into the agar of each Petri dish. Fifteen microlitres of betel oil in ethanol to give final concentration of 100 μL mL^{-1} was added in each well and ethanol blank was used as control. At a concentration of 100 μL mL^{-1}, butylated hydroxyanisole and butylated hydroxytoluene were used as synthetic references. Plates with test samples were incubated at 45 °C overnight until the background colour had bleached. The zones of yellow colour surrounding the tested sample wells were marked by pen, and measured in diameter using Vernier calipers. The antioxidant index of betel oil was expressed as: (diameter of yellow zone – diameter of well)/diameter of well, as adapted from Villaseñor *et al.* (2004).

Determination of minimum oxidative bleaching inhibitory concentrations

According to the European Society of Clinical Microbiology and Infectious Diseases (2000), the agar dilution method was adopted for determination of MICs. Consequently, minimum oxidative bleaching inhibitory concentrations (MOBICs) could be approached with this MICs concept. Petri dishes contained 15 mL β-carotene agar. Two wells (4 mm diameter) were punched into the agar of each Petri dish. Betel oil was dissolved in ethanol in two-fold serial dilutions from 0.195 to 200 μL mL^{-1}. Fifteen microlitres of betel oil dilutions was individually added in wells and ethanol blank was used as control. Butylated hydroxyanisole and butylated hydroxytoluene were used as synthetic references. Plates with test samples were incubated at 45 °C overnight until the background colour had bleached. Minimum oxidative bleaching inhibitory concentration was defined as the lowest concentration of betel oil that resulted in a yellow zone.

Statistical analysis

All experiments were carried out in triplicate. Data points were represented by the mean of the measured values. The data were subjected to an analysis of variance (ANOVA) and the Tukey test at the 0.05 level of significance using KyPlot 2.0 for Windows (Kyence Inc., Japan).

RESULTS

Determination of antimicrobial activity

Betel oil showed antimicrobial activity against all test strains except *Ps. aeruginosa* (Table 1). At the concentration of 50 μL mL^{-1}, betel oil showed a zone of inhibition, ranging from 9.15 to 17.30 mm in diameter. Betel oil yielded the biggest zone of inhibition on *Micrococcus luteus* and exhibited a wide spectrum of antimicrobial activity against 9 out of 10 bacteria including Gram-positive and Gram-negative and against all yeast strains used in this study. In addition, *Escherichia coli* O157:H7 (Figure 1), a pathogen, was more sensitive to betel oil than the non-pathogenic strain (*E. coli*). According to Elgayyar *et al.* (2001), the results showed that betel oil could be qualitatively characterized as having "moderate inhibitory" characteristics. Betel oil was found to be very strong antimicrobial agent against *M. luteus* (antimicrobial index = 2.46). *Saccharomyces cerevisiae* and *S.* Enteritidis were also prone to growth inhibition with the index of 1.81 and 1.68, respectively.

Determination of minimum inhibitory concentrations

The MICs of betel oil in a range of 12.5-100 μL mL^{-1} could inhibit the growth of all test microorganisms except *Ps. aeroginosa*, which was not sensitive to this oil at the highest concentration used (200 μL mL^{-1}). It is interesting to note that among the test microorganisms, *L. monocytogenes* and *S.* Enteritidis were the most sensitive to betel

Table 1 Antimicrobial activity of betel oil using an agar well diffusion assay[a].

Microorganisms	Zone of inhibition[b] /mm	Scale of inhibition[c]	Antimicrobial index
Gram Positive Bacteria			
Bacillus cereus	11.84±0.82 ^{CD}	++	1.37
Enterococcus faecalis	10.28±0.17 ^{AB}	++	1.06
Listeria monocytogenes	10.47±0.46 ^{B}	++	1.06
Micrococcus luteus	17.30±0.29 ^{G}	+++	2.46
Staphylococcus aureus	10.37±0.13 ^{AB}	++	1.07
Gram Negative Bacteria			
Aeromonas hydrophila	12.40±0.28^{DE}	++	1.48
Esherichia coli	9.15±0.12^{A}	+	0.83
Escherichia coli O157: H7	10.93±0.25^{BC}	++	1.19
Pseudomonas aeruginosa	ND	-	0
Salmonella Enteritidis	13.41±0.15^{EF}	++	1.68
Yeast			
Candida albicans	11.31±0.49^{BCD}	++	1.26
Saccharomyces cerevisiae	14.07±0.64^{F}	++	1.81
Zygosaccharomyces rouxii	11.47±0.46^{BCD}	++	1.29

[a] Betel oil with a concentration of 50 µL mL^{-1}

[b] Data (mean ± standard deviation) having different superscripts were significant different ($P < 0.05$).

[c] - no inhibitory (<5 mm), + mild inhibitory (5-10 mm), ++ moderate inhibitory (10-15 mm), +++ strong inhibitory (>15 mm) (Elgayyar *et al.*, 2001)

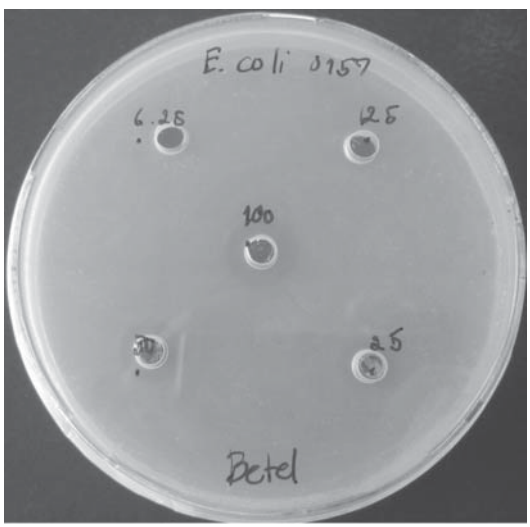

Figure 1 Clear zone of betel oil against *E. coli* O157: H7.

oil which only required 12.5 µL mL^{-1} followed by *E. faecalis*, *M. luteus*, *A. hydrophila*, *E. coli* O157: H7, *C. albicans* and *S. cerevisiae* which were inhibited by 25 µL mL^{-1} (Table 2).

Determination of antioxidant activity and minimum oxidative bleaching inhibitory concentrations

Betel oil revealed ability to inhibit the oxidation of β-carotene (Figure 2), yielding a yellow zone surrounding the well with a 8.40 mm in diameter, as shown in Table 3. The MOBIC of betel oil was 100 µL mL^{-1}. In comparison with synthetic antioxidant references (e.g. BHA and BHT), betel oil yielded slightly antioxidant activity with this assay.

DISCUSSION

Betel oil used in this study had a chemical composition in accordance with the study of Rimando (1986) which reported the constituents of Philippine betel oil being chavibetol, chavibetol acetate, caryophyllene, allylpyrocatechol diacetate, carvacrol, campene, methyl chavibetol, eugenol, pinene, limonene, safrole, 1,8-cineole and allylpyrocatechol monoacetate. In addition, Atal *et al.* (1975) reported that betel oil contains chavicol, allylpyrocatechol, chavibetol, methyl chavicol, methyl eugenol, 1,8-cineole, eugenol, caryophyllene and cadinene.

Different studies on the antimicrobial activity of betel oil and its principal constituents have been reported. However, it is difficult to compare the results of these studies because of variations in betel oil, test microorganisms and test methods. Most essential oils and their active compounds are highly volatile and show poor solubility in the aqueous phase (Friedman *et al.*,

Table 2 Minimum inhibitory concentration of betel oil against test microorganisms.

Microorganisms	MIC /μL mL^{-1}
Gram Positive Bacteria	
Bacillus cereus	50
Enterococcus faecalis	25
Listeria monocytogenes	12.5
Micrococcus luteus	25
Staphylococcus aureus	100
Gram Negative Bacteria	
Aeromonas hydrophila	25
Esherichia coli	50
Escherichia coli O157: H7	25
Pseudomonas aeruginosa	>200
Salmonella Enteritidis	12.5
Yeast	
Candida albicans	25
Saccharomyces cerevisiae	25
Zygosaccharomyces rouxii	50

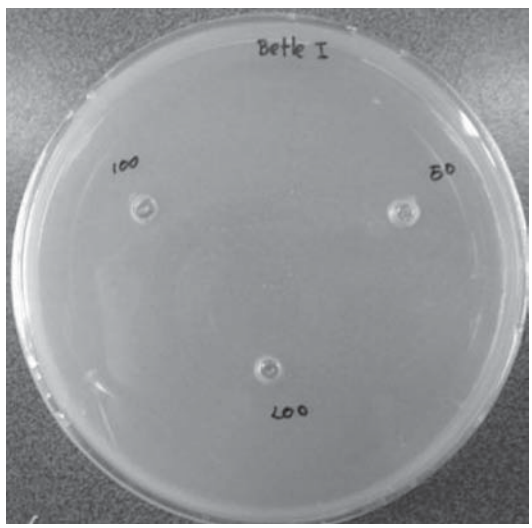

Figure 2 Yellow zone of betel oil against oxidative bleaching of β-carotene.

Table 3 Antioxidant activity[a] and minimum oxidative bleaching inhibitory concentration of betel oil using a β-carotene agar well diffusion assay.

Substances	Yellow zone[b] /mm inhibition[c]	Scale of oxidative	Antioxidant index	MOBIC/ μL mL^{-1}
BHA	24.74±1.46^{B}	++++	5.18	0.195
BHT	8.67±1.08^{A}	+	1.17	6.25
Betel oil	8.40±0.70^{A}	+	1.10	100

[a] Substance with a concentration of 100 μL mL^{-1}

[b] Data (mean ± standard deviation) having different superscripts were significant different ($P < 0.05$).

[c] - no inhibitory (<5 mm), + mild inhibitory (5-10 mm), ++ moderate inhibitory (10-15 mm), +++ strong inhibitory (15-20 mm), ++++ highly strong inhibitory (>20 mm) (Adapted from Elgayyar *et al.* (2001))

2002). Factors such as rate of vapourization and solubility of the oils are difficult to monitor and may result in erroneous findings. Consequently, with a standardized test method of agar well diffusion assay with an application of DMSO as an oil solubilizer, it is possible to use this method as a quantitative screening method. The concentration of DMSO used in this assay was kept below 30 μL mL^{-1}, as recommended by Hili *et al.* (1997), to ensure that its effect on bacterial and yeast growth was minimal. In addition, MIC is an accepted and well used criterion for measuring the susceptibility of microorganisms to inhibitors. Many factors affected the MIC value obtained, including temperature, inoculum size and type of organism (Lambert, 2000). For instance, if an inoculum size is 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 an effect on the level of inhibitory observed.

Carvacrol, eugenol and chavibetol, an isomer of eugenol, were among the most active components against Gram-positive and Gram-negative bacteria (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 betel oil 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) have been investigated the mechanisms of action of carvacrol on the foodborne pathogen *B. cereus*. Carvacrol made the cell membrane permeable for K^+ and H^+ and, consequently, inhibited ATP systhesis 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 *S. cerevisiae* cell lysis. With scanning electron microscopic observation, it showed that the surface of the treated cells was significantly damaged.

Betel oil could not inhibit the growth of *Ps. aeruginosa* (Gram-negative bacteria) due to a failure of outer membrane penetration. The resistance of this Gram-negative bacteria towards betel oil 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 compound (e.g. thymol, carvacrol, limonene, cinnamaldehyde and eugenol) in response to a stress condition. It was found that

Pseudomonas sp. did not show substantial changes in its fatty acid compositions. This is an indication of the high resistance of *Pseudomonas* sp. to the tested compounds. According to Cosentino *et al.* (1999), *Ps. aeruginosa* was resistant to pinene, cymene, carvacrol at the highest concentration (900 $\mu g\ mL^{-1}$) tested, but was 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).

Betel oil was able to inhibit the oxidative bleaching of β-carotene. In comparison with the study of Dorman *et al.* (2000), it was found that undiluted betel oil which yielded a 13.66 mm in diameter of yellow zone, showed higher antioxidant activity than that of nutmeg oil (12.64 mm) and lesser than that of clove oil (15.55 mm). According to Duke (1996) and Dorman *et al.* (2000), chavibetol, eugenol, methyl eugenol, and carvacrol played an important role in antioxidant effectiveness. Caryophyllene did not show antioxidant effect (Dorman *et al.*, 2000), conversely it was prone to autoxidation (Sköld *et al.*, 2006). Choudhary and Kale (2002) reported that betel ethanolic extract inhibited the radiation induced lipid peroxidation process effectively. This could be attributed to its ability to scavenge free radicals involved in initiation and propagation steps. In addition, Bhattacharya *et al.* (2005) have found that betel ethanolic extract, containing major constituents of chavibetol and allpyrocatechol, acts as an excellent radioprotectant, exerting the activity through its superior radical scavenging properties.

CONCLUSION

This study revealed that betel oil could inhibit a wide spectrum of food pathogenic and spoilage microorganisms. It is interesting to note that *L. monocytogenes* and *S.* Enteritidis, foodborne pathogens, were the most sensitive towards the inhibitory effect of betel oil. Betel oil showed slightly antioxidant activity, compared to synthetic antioxidants. Betel oil showed a promising potential application in controlled and released food packaging technology as antimicrobial and antioxidant agents. However, further research is required to investigate comprehensively in the areas of antioxidant, for instance, radical scavenging activity against free radical using a 2,2-diphenyl-1-picryhydrazyl (DPPH) assay.

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