

Antibacterial activity of nutmeg (*Myristica fragrans* Houtt.) extract against foodborne pathogens on raw beef during chilled and frozen storage

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Abstract

In recent years, demands for minimal processing and free-synthetic preservatives are increasing because of growing concern among consumers regarding the safety issues of additives. Nutmeg (*Myristica fragrans* Houtt.) has been used as a spice and traditional medicine in Asian countries. This study aimed to determine the antibacterial activity of nutmeg extract against foodborne pathogens on raw beef during storage. Nutmeg seeds were extracted using a maceration method with methanol as a solvent. The extract was assessed for antibacterial activity against a range of microorganisms using the disc diffusion assay, minimum inhibitory concentration and minimum bactericidal concentration. The effect of heat and different pH of the extract on its antibacterial activity was also conducted to evaluate the stability of the extract. The effects of the extract at different concentrations on the microbial population of the raw beef during chilled (4.0±0.2°C) and frozen (-18.0±0.2°C) storage for 21 days were then evaluated. The nutmeg extract exhibited antimicrobial activity against the range of microorganisms tested, which was stable at high temperature (80.0±2.0°C) and in the pH range tested (3.0, 6.8, and 11). Furthermore, the extract significantly inhibited microbial growth on raw beef stored for 21 days in chilled or frozen conditions, indicating that the nutmeg extract has the potential to be developed as a natural antimicrobial preservative in beef.

1. Introduction

Microbial activity by food spoilage bacteria is one of the primary causes of deterioration of many foods and is often responsible for food quality, spoilage, and economic loss. In addition, food industries have many safety concerns regarding particular foodborne pathogens, such as *Salmonella* spp, *Escherichia coli*, and *Staphylococcus aureus*, which are responsible for most foodborne diseases (Acheson, 2003). The problem of food preservation has become more complicated as new food products are frequently introduced on the market requiring long-term shelf-life protection from microbial spoilage (Patal and Kamble, 2011). Several preservation techniques have been used to improve beef freshness, including heat treatment, salting, and acidification (Davidson and Taylor, 2007). However, these techniques can cause deterioration in nutrient value and food safety (Annalisa *et al.*, 2012). Furthermore, due to negative consumer perceptions of artificial preservatives, attention

is shifting towards natural preservatives. Recently, interest has focused on the utilisation of plants that have the antimicrobial activity to control pathogens in foods. Consequently, alternative preservatives possessing antimicrobial activity with no harmful effects on human health are in high demand (Patal and Kamble, 2011).

Plant parts including leaves, flower, seed, stem, bark, root or whole plant have been used in foods since ancient times, not only as folk medicine, but also as flavouring agents and food preservatives (Dillon, 1994; Culter, 1995) due to their antimicrobial activity against specific pathogens (Erasto *et al.*, 2004). Moreover, the plant part does not exhibit toxicity at consumed levels and are considered as GRAS (generally recognised as safe) substances (Souza *et al.*, 2005). Nutmeg, *Myristica fragrans* Houtt., locally named as “pala” is an evergreen tree that produces drupe type fruits, belonging to the Myristicaceae family. Nutmeg is widely used as a spice in cooking and numerous traditional medicines (Dorman

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et al., 2000). Various studies have established the therapeutic value of *M. fragrans*; mace and seed of nutmeg possess antioxidant and antimicrobial effect (Ashish *et al.*, 2013), thus nutmeg may be a potential natural preservative to inhibit the growth of pathogenic and spoilage microorganisms. There are several ways to use natural preservatives in the food, they can be directly added to the product formulation such as coating, spraying or dipping on the surface of the food (Velasco and Williams, 2011).

The present study was conducted to determine the antibacterial activity of *M. fragrans* Houtt. extract against selected foodborne pathogens on raw beef during storage at 4°C (chiller) and -18°C (freezer). It is expected that the study findings will be used as primary evidence of the potential of nutmeg extract to be used as natural preservative to replace chemical preservatives.

2. Materials and methods

2.1 Nutmeg extraction

Nutmegs were collected at Taman Pertanian, Bukit Ekspo, UPM, Selangor, Malaysia and dried in the oven at 50°C for three days. The dried nutmeg (100 g) was ground and extracted with 400 mL of 99.8% (w/v) absolute methanol (Sigma-Aldrich, Saint Louis, MO, USA) for seven days at room temperature according to the method of Rukayadi *et al.* (2008) with some modification. After seven days, the plant material was filtered using Whatman No. 1 filter paper (Whatman International Ltd., Middlesex, England) and concentrated using a rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) at 50°C with a speed of 150 rpm for 3–4 hrs. The temperature was increased to 80°C for 2 × 30 s to obtain methanol-free extracts. The active compounds in the extract were unaffected by this temperature (Rukayadi *et al.*, 2008). The crude extract was then stored at 4°C.

2.2 Antibacterial activity of *Myristica fragrans* Houtt. extract against foodborne pathogens

2.2.1 Preparation of nutmeg extract and chlorhexidine solutions

A 10% (w/v) extract of *M. fragrans* Houtt. was prepared by dissolving 100 mg of crude extract in 1 mL of dimethyl sulfoxide (DMSO) (99.9%) (R and M Marketing, Essex, UK). This was further diluted in 1:10 sterile distilled water to produce 1% (w/v) extract, with a final DMSO concentration of 10%, which does not kill bacteria (Rukayadi *et al.*, 2008). A 1% (w/v) solution of chlorhexidine (Sigma, St. Louis, MO, USA) was prepared by dissolving 1 mg in 1 mL of double-distilled water (ddH₂O).

2.2.2 Bacterial strains

B. cereus ATCC33019, *B. subtilis* ATCC6633, *E. coli* ATCC25922, *K. pneumoniae* ATCC13733, *L. monocytogenes* ATCC19112, *P. aeruginosa* ATCC9027 and *P. mirabilis* ATCC21100 were obtained from the American Type Culture Collection (Rockville, Maryland, USA). *S. aureus* KCCM12255 was obtained from the Korean Culture Centre of Microorganisms (Seoul, South Korea). All bacteria were cultured and maintained statistically in nutrient agar (NA: Difco, Sparks, Maryland, USA).

2.2.3 Disc diffusion assay

M. fragrans Houtt. extract was tested for antibacterial activity using a standard paper disc diffusion assay (CLSI, 2012). Briefly, 100 µL of microbial inoculum was spread on Mueller Hinton Agar (MHA: Difco, Franklin Lakes, NJ, USA) using a sterile cotton swab. Sterile paper discs (6 mm) (Schleicher and Schuell, Dassel, Germany) were impregnated with 10 µL of 1% of *M. fragrans* Houtt. extract, with 1% chlorhexidine included as a positive control and 10% DMSO as a negative control. The plates were incubated at 37°C for 24 hrs and the inhibition zones surrounding the paper discs were measured. The test was performed in triplicate to verify the results.

2.2.4 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Determination of MIC and MBC values was performed using a method described in the CLSI (2012). MIC was conducted in 96-well round-shaped bottom microtiter plate (Brandon™, Malaysia), using two-fold standard broth microdilution methods with an inoculum of approximately 10⁶ CFU/mL. Nutmeg extract or positive control 1% chlorhexidine were two-fold diluted in the respective medium containing inoculum. Column 12 of the microtiter plate contained the highest concentration of extract (5 mg/mL), while column 3 contained the lowest concentration of extract (0.0097 mg/mL). Column 1 served as negative growth control (only medium, no inoculum, and no antimicrobial agent), while column 2 served as positive growth control for all samples (only medium and inoculum or antimicrobial agent-free well). The plates were then incubated at 37°C for 24 hrs. The MIC was defined as the lowest concentration of antimicrobial agent that resulted in the complete inhibition of visible growth (CLSI, 2012), that is, the well with no visible growth compared to the positive and negative growth wells.

The MBC was defined as the lowest concentration of antimicrobial agent at which no growth occurred on the

agar plates and was determined by sub-culturing the suspension (10 μ L) from each well on MHA. The plates were then incubated at 37°C for 24 hrs or until growth was observed in the positive growth control. The lowest concentration that did not produce visible growth after the incubation period was considered as the MBC (Andrews, 2001).

2.2.5 Stability of *Myristica fragrans* Houtt. extract to heat and different pH

The stability of the extract to heat and different pH was assessed by the method reported by Lee *et al.* (2008). Briefly, 1% (w/v) crude extract was heated to 80 \pm 2°C for 30 mins using a water bath heater, then cooled. The original pH of the extract was 6.8 \pm 0.2, so the pH was adjusted to 3.0 \pm 0.2 and 11.0 \pm 0.2 using 1 M HCl and NaOH, respectively. The antibacterial activity of all treated extracts was then assessed by the disc diffusion assay as described in section 2.2.3.

2.3 Effect of *Myristica fragrans* Houtt. extract on microbial growth on raw beef during chilled and frozen storage

The crude nutmeg extract was diluted using sterile deionised water (DIW) (B Braun Medical Industries, Penang, Malaysia) to prepare concentrations of 0.00% (only DIW), 0.25%, 0.50%, 1.25%, 2.50% and 5.00% (w/v). Beef samples were purchased at Pusat Sembelihan Lembu Daging, Shah Alam, Selangor and transported to the Food Science Laboratory, UPM on ice within 40 mins. The beef was cut into 2.5 \times 2.5 \times 2.0 cm samples, weighing an average of 3.0 \pm 0.1 g, before randomly divided into three groups: control group, chilled group and frozen group. The beef samples were then soaked in 10 mL of different concentrations for 15 mins, before packaged with overwrapped trays with stretched polyethylene permeable film (Polypack Enterprises, Selangor, Malaysia) and stored in the chiller (4.0 \pm 0.2°C) for 21 days and freezer (-18.0 \pm 0.2°C) for 14 days. The experiment was repeated two times with duplicates (n = 2 \times 2).

On day zero, 1st, 4th, 7th, 10th, 14th and 21st, the microbial populations in the beef samples were evaluated by homogenising a sample using a stomacher. Serial dilutions using 10 mL M phosphate buffer were prepared, of which, 0.02 mL was spread on plate count agar (PCA: Difco, Spark, MD, USA), chromocult coliform agar (Merck, Darmstadt, Germany), Listeria selective agar (Merck, Darmstadt, Germany) and *E. coli* chromagar (Merck, Darmstadt, Germany). The petri dishes were incubated at 37°C for 24 hr, then plate counts were performed to calculate the logarithm numbers of colony-forming unit per gram (Log₁₀ CFU/g)

of samples.

2.4 Statistical analysis

Data were analysed using MINITAB v16.1 Windows (Minitab Inc) by the analysis of variance (ANOVA), one-way, unstacked, where Tukey's test was used to determine the significance of difference (P = 0.05) between treatments.

3. Results and discussion

3.1 Antibacterial activity of the *Myristica fragrans* Houtt. extract and its stability

M. fragrans Houtt. extract inhibited the growth of the eight foodborne pathogens tested as shown by the inhibition zones presented in Table 1. The disc diffusion assay is a semi-quantitative analysis and according to Gangoué-Piéboji *et al.* (2009), some active compounds may be trapped in the disc pores and unable to pass through into the inoculated media, hence the results do not truly represent their complete antimicrobial activity. Furthermore, the inability of hydrophobic compounds to diffuse into the media agar could provide non-accurate results (Othman *et al.*, 2011). Nonetheless, as a screening technique, the disc diffusion assay allows the detection of active compounds in plant extracts, as the size of the inhibition zone indicates the degree of antibacterial activity. Hence, *M. fragrans* Houtt. extract was more effective against *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. mirabilis* and *S. aureus* strains, being less effective against the Gram-negative *P. aeruginosa*. Generally, in gram negative bacteria, their outer membranes serve as permeability barrier which allow only small hydrophilic molecules to pass through into the cell, restricting their rate of penetration for certain antimicrobial compounds and excluding larger molecules. Furthermore, they possess multidrug-resistant pumps which also exclude some antibacterial compounds; thus, they are more tolerant to exposure to foreign compounds (Lambert, 2002).

The MICs of nutmeg extract are presented in Table 1. The extract exhibited satisfactory MBCs of 0.31 mg/mL, 1.25 mg/mL, 0.63 mg/mL, 0.63 mg/mL, 1.25 mg/mL, 2.50 mg/mL, 0.63 mg/mL and 0.63 mg/mL for *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *L. monocytogenes*, *P. aeruginosa*, *P. mirabilis* and *S. aureus*, respectively. Taken together, these results show that the extract was bacteriostatic to *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *P. mirabilis* and had a bactericidal effect on *L. monocytogenes* and *S. aureus*. The nutmeg extract was most effective against *B. cereus*, as evidenced by the largest inhibition zone (12.00 \pm 0.00 mm), and lowest MIC (0.16 mg/mL) and MBC (0.31 mg/mL) values.

Table 1. Inhibition zone, MICs and MBCs of *M. fragrans* Houtt. extract against foodborne pathogens without treatment, after heat and pH treatments

Bacterial strain	MIC (mg/mL)	MBC (mg/mL)	Inhibition zone (mm) of 1% extract without treatment	Inhibition zone (mm) of 1% extract after heating at 80.0 ± 2.0°C	Inhibition zone (mm) of 1% extract at pH		
					3	6.8	11
<i>B. cereus</i> ATCC33019	0.16	0.31	12.00 ± 0.00	10.00 ± 0.00	12.00 ± 0.00	12.00 ± 0.00	12.00 ± 0.00
<i>B. subtilis</i> ATCC6633	0.63	1.25	11.33 ± 0.67	11.33 ± 0.67	11.00 ± 0.00	11.33 ± 0.67	10.00 ± 0.00
<i>E. coli</i> ATCC25922	0.31	0.63	10.67 ± 0.33	8.67 ± 0.33	11.33 ± 0.67	10.67 ± 0.33	9.67 ± 0.33
<i>K. pneumoniae</i> ATCC13733	0.31	0.63	11.00 ± 0.00	10.00 ± 0.00	11.00 ± 0.00	11.00 ± 0.00	10.33 ± 0.67
<i>L. monocytogenes</i> ATCC19112	1.25	1.25	9.50 ± 0.50	8.00 ± 0.00	9.00 ± 0.00	9.50 ± 0.50	8.00 ± 0.00
<i>P. aeruginosa</i> ATCC21100	1.25	2.5	8.00 ± 0.00	7.00 ± 0.00	8.00 ± 8.00	8.00 ± 0.00	7.50 ± 0.50
<i>P. mirabilis</i> ATCC9027	0.31	0.63	10.00 ± 0.00	10.00 ± 0.00	10.33 ± 0.67	10.00 ± 0.00	8.67 ± 0.33
<i>S. aureus</i> KCCM12255	0.63	0.63	11.33 ± 0.67	11.00 ± 0.00	10.00 ± 0.00	11.33 ± 0.67	10.33 ± 0.67

These results agree with previous studies (Bin *et al.*, 2007; El Malti *et al.*, 2008; Ababutain, 2011), that nutmeg extract displayed antibacterial activity against a range of gram positive and Gram-negative bacteria including different serotypes of *E. coli*, *Salmonella* spp., *L. monocytogenes*, and *Aeromonas hydrophila*.

The stability of the extract was tested at three different pH (3.0, 6.8 and 11.0) and a high cooking temperature (80.0±2.0°C) and the results are shown in Table 1 column 5, 6, 7 and 8. The antimicrobial activity of the nutmeg extract was heat and pH stable, as there were no significant differences in the inhibition zones between the different conditions tested.

Numerous active compounds have been isolated from nutmeg seed, such as β -pinene, α -pinene, sabinene, safrole, terpinene-t-ol, myristicin, α -terpineol, α -terpinene (Dorman *et al.*, 2000; Chatterjee *et al.*, 2007), eugenol, isoeugenol (Janssens *et al.*, 1990), lignans (diarylbutane, aryltetraline) (Kwon *et al.*, 2008), macelignan (Chung *et al.*, 2006), which may be responsible for the antimicrobial effects. Previously, β -pinene, isolated from the *M. fragrans* Houtt, was shown to possess antioxidant and antimicrobial effects (Takikawa *et al.*, 2002). These compounds which are responsible for antibacterial activity are stable to the change of temperature and pH.

3.2 Effect of *Myristica fragrans* Houtt. extract on microbial population in raw beef

The effects of different concentrations of nutmeg extract on the microbial population including total plate count (TPC), coliform, *E. coli* and *L. monocytogenes* of raw beef in two storage conditions 4.0±0.2°C and -18.0±0.2°C were evaluated and the results are presented in Figure 1 to Figure 4. Typically, during 21 days of storage at 4°C, beef samples are spoiled, producing

unpleasant by the 13th day. The North American meat industry recommends 2°C as the appropriate temperature for meat storage, nonetheless, even if 2°C is maintained, there is still a probability of 50% product loss (Jeremiah and Gibson, 2001).

The effect of nutmeg extract on the TPC of beef during storage at 4°C is presented in Figure 1a, showing that the number of TPC increased at low extract concentrations (0.00%, 0.25% and 0.65%) as conditions were still favourable for microbial growth (Tan and Chen, 2005). In contrast, there were significant reduction of TPC at higher extract concentrations (1.25%, 2.50% and 5.00%) from 5.02 log₁₀CFU/g to 4.00 log₁₀ CFU/g, 3.70 log₁₀ CFU/g and 3.42 log₁₀ CFU/g, respectively. The initial number of TPC for freezer temperature (-18.0±0.2°C) was 5.11 log₁₀ CFU/g as shown in Figure 1b. The numbers of TPC at all concentrations were relatively constant, with no significant differences in TPC until the 4th day. However, from 5th day onwards, the TPC reduced significantly from 5.11 to 4.11, 3.92, 3.59 and 3.03 with concentrations of 0.25%, 0.65%, 1.25%, 2.50% and 5.00%, respectively (Figure 1b), suggesting that the extract can reduce the survival of TPC during freezer temperature storage.

The initial number of coliform at chiller storage temperature (4.0±0.2°C) was 3.08 log₁₀ CFU/g, which increased in the control sample, 0.00% and 0.25% extract conditions by the 14th day (Figure 2a). This low antimicrobial activity may be due to the formation of protective biofilms on the meat through excretion of extracellular polysaccharides by coliform (Doyle, 2005). Hence, the presence of biofilms on different beef may cause the non-uniform survival of the microbial and bacterial load during storage. At frozen storage temperature (-18.0±0.2°C), the initial coliform count was 3.12 log₁₀ CFU/g (Figure 2b). After treatment with

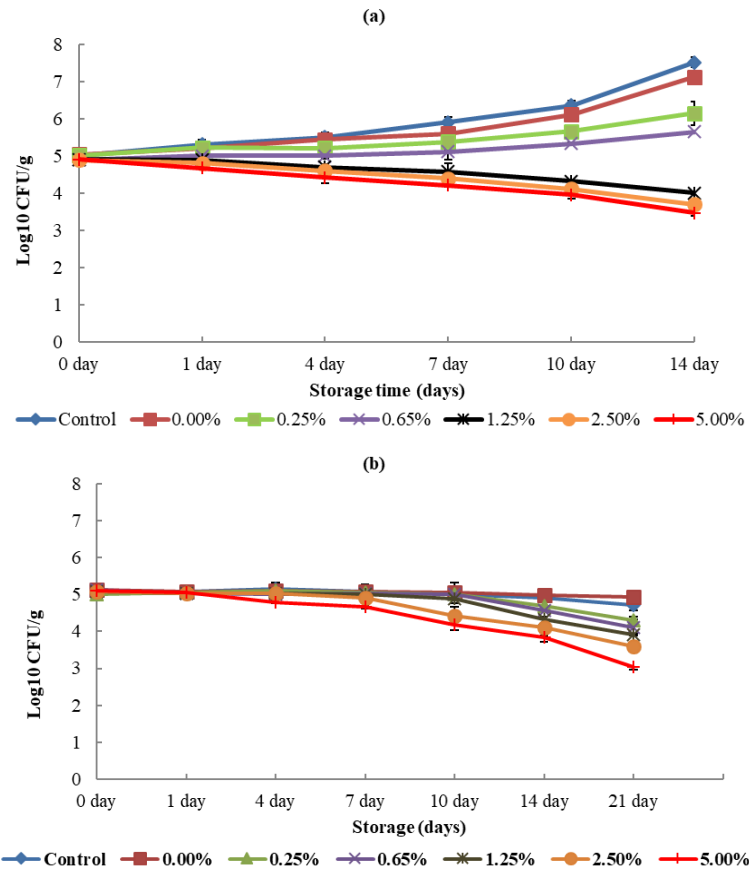


Figure 1. Effect of *M. fragrans* Houtt. extract treatment at concentration of 0.00% (DW, distilled water), 0.25%, 0.65%, 1.25%, 2.50% and 5.00% on the total plate count (TPC) of beef during storage at 4±2°C (a) and -18±2°C (b). Control was untreated raw beef sample.

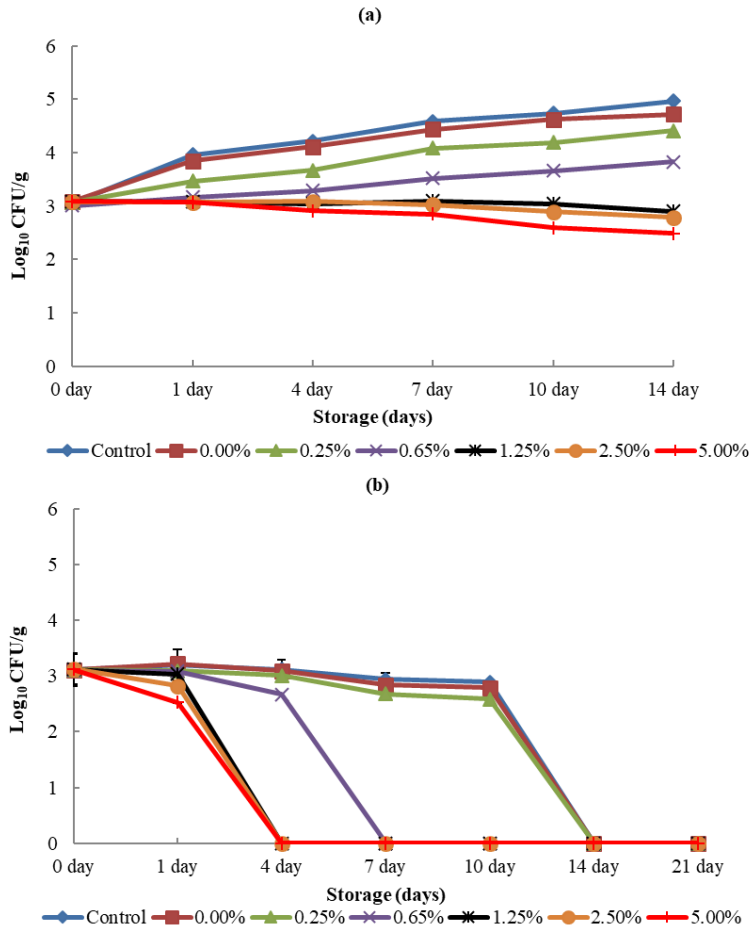


Figure 2. Effect of *M. fragrans* Houtt. extract treatment at concentration of 0.00% (DW, distilled water), 0.25%, 0.65%, 1.25%, 2.50% and 5.00% on coliform on beef during storage at 4±2°C (a) and -18±2°C (b). Control was untreated raw beef sample.

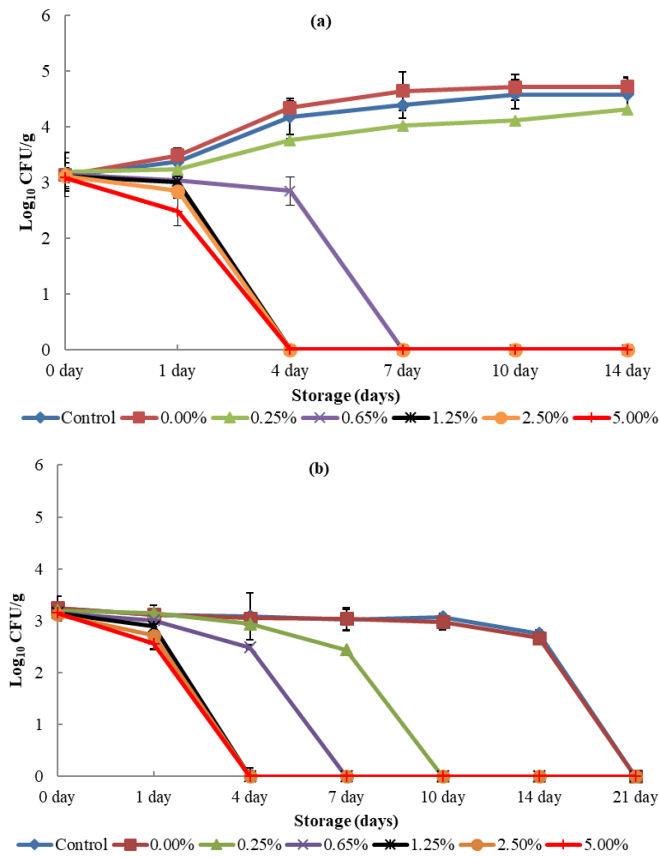


Figure 3. Effect of *M. fragrans* Houtt. extract treatment with concentration of 0.00% (DW, distilled water), 0.25%, 0.65%, 1.25%, 2.50% and 5.00% on *E. coli* on beef during storage at $4 \pm 2^\circ\text{C}$ (a) and $-18 \pm 2^\circ\text{C}$ (b). Control was untreated raw beef sample.

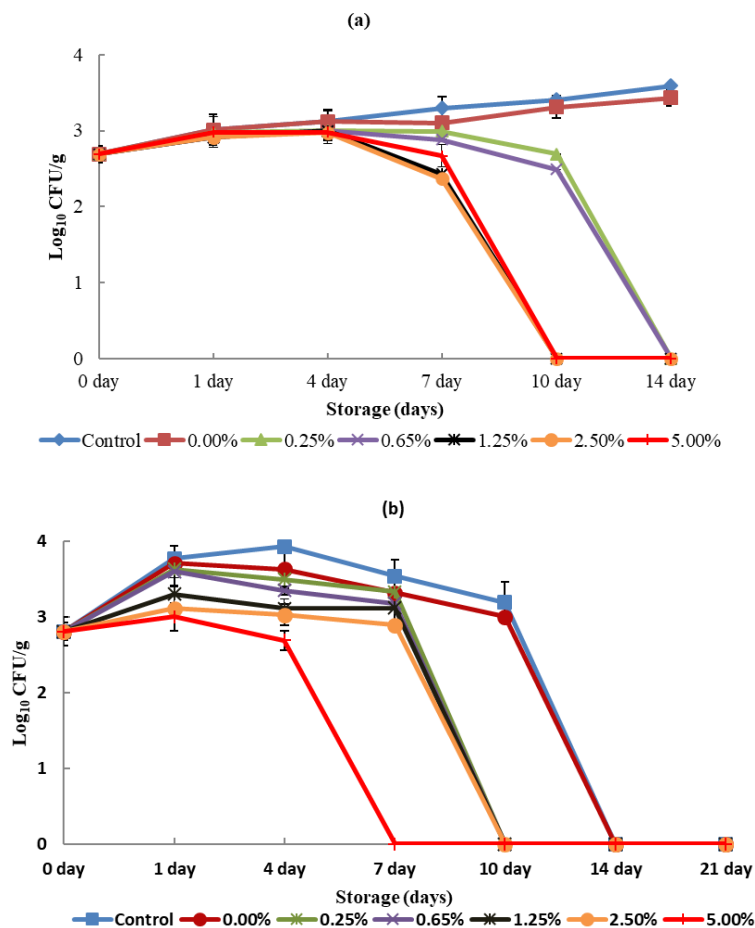


Figure 4. Effect of *M. fragrans* Houtt. extract treatment with concentration of 0.00% (DW, distilled water), 0.25%, 0.65%, 1.25%, 2.50% and 5.00% on *L. monocytogenes* on beef during storage at $4 \pm 2^\circ\text{C}$ (a) and $-18 \pm 2^\circ\text{C}$ (b). Control was untreated raw beef sample.

1.25% extract, coliform was inhibited by the 4th day, whereas treatment with 0.65%, 0.25%, 0.00% and control inhibited the growth of coliform after the 7th day. These differences between the antimicrobial activity at different storage temperatures suggest that the antimicrobial activity of the nutmeg extract was enhanced at the lower temperature of -18°C.

Figure 3a shows the log₁₀ CFU/g reduction of *E. coli* in beef stored at 4.0°C, which was reduced to undetectable limits with the treatment of 1.25%, 2.50% and 5.00% extract by the 4th day. In contrast, the lower concentration extracts were not as effective, with 0.65% inhibiting *E. coli* growth by the 7th day, whereas 0.25% and 0.00% had no inhibitory effects on the growth of *E. coli*. The effects of different concentrations and exposure time of *M. fragrans* Houtt. extract on *E. coli* in beef stored at -18°C are shown in Figure 3b, showing that treatment with 1.25%, 2.50% and 5.00% extracts inhibited *E. coli* growth by the 4th day, with 0.65% and 0.25% extracts inhibiting *E. coli* growth by the 7th day and 10th day, respectively. Taken together, these results demonstrated that the increasing concentration of nutmeg extract and the storage at -18°C enhanced the inhibition of *E. coli*.

Figure 4a shows the effect of nutmeg extract on *L. monocytogenes* in treated beef stored at 4°C, showing that there was no growth inhibition activity until 10th day, which may be due to the adaptation of the microbe itself as well as the biological adaptations of the active compound in the nutmeg. The unstable phase of microbial growth is attributed to microbial adaptation to the changing conditions, such as chill temperatures and surface desiccation (Raftari et al., 2009). The pattern of logarithmic growth occurs after the microorganism accommodates to a new environmental setting and their metabolism. Treatment with 1.25% extract and above exhibited the highest antimicrobial activity by the 10th day of storage, while 0.65% and 0.25% extract inhibited bacterial growth by the 14th day (Figure 4a).

The *L. monocytogenes* growth count of treated beef stored at -18°C is shown in Figure 4b, indicating that all treatments inhibited *L. monocytogenes* growth. The 5.00% extract was most effective, showing inhibitory effects by the 7th day, followed by treatment with 2.50%, 1.25%, 0.65%, and 0.25% extracts showing inhibitory effects by the 10th day, whereas control samples and treatment 0.00% extract showed microbial inhibition on the 14th day. These findings indicate that higher concentrations of extract can inhibit the growth of *L. monocytogenes* at low temperature storage conditions.

In summary, these results showed the concentration-dependent antimicrobial effects of the nutmeg extract,

with a maximal reduction average of 2.66 log₁₀ CFU/g and minimal reduction of 0.28 log₁₀ CFU/g of total bacterial count in beef in the samples treated with 5.00% and 0.25% *M. fragrans* Houtt. extract, respectively. In general, treatment with 1.25% extract showed significant antimicrobial activity (P<0.05) against all tested microorganisms.

The antimicrobial activity of nutmeg extracts mainly depends on their constituents, with inhibitory action being more related to the major than the minor active compounds. It has been demonstrated that several components are involved in the fixation on cell walls and cellular membrane. Significant antimicrobials and antioxidants have been reported in nutmeg seeds extracts including α -pinene, β -pinene, *p*-cymene, carvacrol, and β -caryophyllene (Ogunwande et al., 2003; Dorman et al., 2004), oestrogenic activities, spasmolytic activity (Olajinde et al., 1999), and inhibitory activity of membrane functions by localisation to membranes (Panizzi et al., 2002). The generated volatile compounds by microorganisms during refrigerated storage of the meat at 4°C are a significant source of undesirable odours that are considered as an indication of shelf-life expiration. Therefore, it is crucial to reduce or inhibit microbial growth to prevent rancid odours, since it is an indicator of the freshness or the spoilage of the beef (Lee and Shin, 2019).

4. Conclusion

Nutmeg (*M. fragrans* Houtt.) extract exhibited antibacterial activity against selected foodborne pathogens including *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *L. monocytogenes*, *P. aeruginosa*, *P. mirabilis* and *S. aureus*. In general, the antibacterial activity of the nutmeg extract was not affected by heating or changes in pH. Furthermore, 1.25% nutmeg extract exhibited antibacterial activity on beef during storage at 4°C and -18°C, suggesting that *M. fragrans* Houtt.) extract could be used as a natural preservative in beef.

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