The utilization of nutmeg seed (*Myristica fragrans* Houtt) extract as an antimicrobial on tempeh sausage

K A Panggabean¹, H Rusmarilin¹* and D Suryanto²

¹Department of Food Science, Faculty of Agriculture, Universitas Sumatera Utara, Medan, North Sumatera, Indonesia
²Department of Biology, Faculty of Mathematics and Natural Science, Universitas Sumatera Utara, Medan, North Sumatera, Indonesia

E-mail: *herla_surabaya@yahoo.com or kartika_panggabean@yahoo.com

Abstract. Nutmeg seed is an herb containing antimicrobial compounds and can be applied as an antimicrobial on foodstuffs. This study was aimed to know antimicrobial compounds of nutmeg seed extracting in water, methanol, ethyl acetate and hexane. Assay on the extract to inhibit pathogenic microbes (*Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*) was conducted using dilution method. Phytochemical test was conducted to know the extract compounds. Minimum Inhibitor Concentration (MIC) test was measured to know minimum concentration of the extract to be applied in tempeh sausage. The results showed that nutmeg seed extract contained alkaloids, flavonoids, steroids, saponins, tannins and phenolics. Methanol extract was shown to have higher inhibition to the tested microbes compared to the other extracts. Minimum Inhibitor concentration (MIC) occurred at concentrations of 0.1% and 0.25% of nutmeg seed extract on *Staphylococcus aureus* and *Escherichia coli*. Nutmeg seed extract added on the tempeh sausage was able to reduce total microbial cell up to 5 days of storage compared to that of the control, i.e. 561x10⁴ CFU/g for control, 61x10⁴ CFU/g for 0.1% nutmeg seed extract and 54x10⁴ CFU/g for 0.25% nutmeg seed extract.

1. Introduction

Indonesia is known as a country rich in various types of spices, one of which is nutmeg [1]. Nutmeg is widely used as a natural preservative, food component and drug formula because it acts as an antioxidant and antimicrobial [2-3]. Antimicrobials are often used to prevent the growth of pathogenic bacteria as the main cause of damage and reduce food shelf life and cause various types of disease [4-5]. Pathogenic bacteria commonly found in foods are *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* [6]. The antimicrobial ability of nutmeg is caused by its phytochemical compound [3]. Extraction of phytochemical compounds of nutmeg can be done by maceration, percolation and soxhlet extraction [7-8]. Tempeh is made from fermentation of soybean seeds using several species of Rhizopus, such as *Rhizopus oligosporus* and *Rhizopus stolonifer* which are rich in dietary fibre, B vitamins, calcium and iron [9-10]. Tempeh cannot be stored for longer than 2 x 24 hours, because the fungus Rhizopus will die and the other fungi and bacteria will grow that can remodel the protein in tempeh causing bad odour [9, 11]. Tempeh can be processed into various forms of processed foods such as sausage [12]. The addition of nutmeg extract on making tempeh sausage is expected to extend...
its shelf life. The purpose of this study was to know the antimicrobial compounds in nutmeg extract qualitatively, to know the most potential solvent to extract the antimicrobial compound and to find the potential doses of nutmeg extract in inhibiting the growth of *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus* by taking into account the clear zone formed.

2. Materials and methods

2.1. Materials

Nutmeg obtained from Aceh Selatan, Indonesia. Bacterial cultures used in this study were *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8938) and *Bacillus cereus* (KCCM 40152). Chemicals used were nutrient agar, nutrient broth, mueller hinton agar, dimethyl sulfoxide, NaCl 0.9%, distilled water, ethyl acetate, methanol, hexane. The material used for bacterial test was Plate Count Agar (PCA) medium. Equipment used in this study were laminar air flow cabinet (Astec HLF 1200L), autoclave (Express), micrometer pipettes (Eppendorf), analytical balance (Mettler Toledo), oven (Memmert), refrigerator (Toshiba), incubator, rotary evaporator (Stuart), oxoid paper and colony counter.

2.2. Moisture content determination

Moisture content of nutmeg seed was determined by the method of Divekar [13]. Five grams of sample was dried in gravity air oven at 105 °C for 24 hours till constant weight was obtained. Final moisture content was counted by the formula:

\[ M(\%) = \frac{W_1 - W_2}{W_2} \times 100\% \tag{1} \]

Note: \( M = \) moisture content, \( W_1 = \) weight of wet sample (g), \( W_2 = \) weight of bone dry sample (g)

2.3. Preparation of nutmeg seed extract

Nutmeg seed were washed and dried in the oven at 40°C for 48 hours, then powdered by using electrical blender and ready to extract by maceration methods. The dried powder of nutmeg seed was divided into 4 groups of 25 grams powder and dissolved with 150 ml solvent (water, methanol, ethyl acetate and hexane) in a erlenmeyer then macerated for 72 hours and shaking periodically. The precipitated residue was separated from the solvent by filtration and concentrated by using vacuum evaporator at 50°C to produce a crude extract. The crude extract extracted again twice with 150 ml solvent to obtain a clear colour residue. Qualitative phytochemical test were carried out on the extracts obtained to determine the presence of several chemical compounds by the method of Indonesian Ministry of Health [14] and Fransworth [15] included alkaloids, flavonoids, steroids, saponins, tannins and phenols.

2.4. Evaluation of antimicrobial activity

Preparation of stocks solution (100% extract) was done by dissolved 2 g of each extract into 2 ml DMSO (Dimethyl Sulfoxide). Furthermore, the stock solution was reconstituted with DMSO to obtain 75%, 50% and 25% of extract concentration. The tested organisms used (*E. coli*, *S. aureus* and *B. cereus*) were obtained from Biology Laboratory, Universitas Sumatera Utara and were performed by agar disc-diffusion method by Mahesh and Satish [16]. Filter paper disc containing nutmeg seed extracts from different solvents (25%, 50%, 75% and 100%) were placed on the agar surfaces (Mueller-Hinton agar). The petri dishes were incubated at 37°C for 24 hours. The bacterial inhibition zone around the disk shown the antimicrobial susceptibility [17]. The minimum inhibitory concentrations (MIC) was performed by a serial dilution technique (0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0 and 3.0%) of the concentrates [17-18]. As much 10 µL of bacteria test culture were mixed with extract of nutmeg (the best extract of several solvent) and shaken using shaker incubator at speed 150 rpm for
24 hours. The diameters of the inhibition zones were measured in mm and the minimum extract concentration that inhibits 90% of the growth of bacteria known as MIC [17-18].

2.5. Application on tempeh sausage
Application on tempeh sausage was done by adding the extract of nutmeg seed into tempeh sausage dough. The ingredients were used to make tempeh sausage that were 62% of tempeh porridge that has been steamed at 80°C for 10 minutes as much as, 7% of skim milk, 5% of vegetable oil, 2% of carrageenan, 0.5% of sugar, 2.5% of salt, 0.5% of pepper, 2% of red onion, 1.5% of garlic and 17% of tapioca flour. Sausage dough was divided into three treatments that were the addition 0%, 0.1 and 0.25 of nutmeg seed extract. After the nutmeg seed extracts were blended, then the dough was inserted into a 12 cm long sleeve / baling cord (Devro), fastened and steamed for 20 minutes. Once cooked the sausage was lifted and then cooled and packed. Then the sausage was stored in the refrigerator at 4°C for 5 days for observation. The tempeh sausage then analysed for moisture content [13], total microbial determination by Total Plate Count (TPC) method [19] and organoleptic test [20].

3. Results and discussions

3.1. Moisture content of nutmeg
Moisture content of nutmeg seeds were used in this study were 7.37%. This value is lower than result of research. that is 14.3% and has met the Indonesian National Standard requirements which is a maximum of 10% [21].

3.2 Yield of nutmeg seed extraction
The result of extraction of nutmeg seed with several type of solvents are shown in Table 1.

| Solvent   | Yield (%) |
|-----------|-----------|
| Methanol  | 4.22^A    |
| Ethyl acetate | 2.64^B |
| Hexane    | 1.84^c    |
| Water     | 2.93^b    |

Table 1 shows that the highest yield of nutmeg seed extract was found in methanol extract and followed by water, ethyl acetate and hexane. The highest yield in methanol is caused by the characteristic of methanol that can dissolve almost all components, both polar, semi-polar and non-polar [22].

3.3. Phytochemical compound of nutmeg extracts
The results of the qualitative phytochemical compounds analysis of nutmeg seed extract is showed in Table 2. Table 2 shows that extraction of nutmeg seed using methanol has more phytochemical compounds that are alkaloids, flavonoids, steroids, tannins and phenolics. Methanol has capability to dissolve polar compounds, such as phenolic compounds with medium polarity and low-medium molecular weights, sugar, glycoside compounds, amino acid, aglycon flavonoid, anthocyanin, tannin, terpenoid, saponin, xanthoxilin, polyphenol, totarol, lacton, phenone, flavone and quacinoid [23].
Table 2. Analysis of qualitative phytochemicals compounds of nutmeg seed extract with several solvents

| Phytochemical | Solvent Extract [(+] means present, (-) means absent] |
|---------------|-----------------------------------------------------|
|               | Methanol    | Ethyl Acetate | Hexane | Water     |
| Alkaloids     | +           | -            | -      | +         |
| Flavonoids    | +           | +            | +      | -         |
| Steroids      | +           | -            | +      | -         |
| Saponins      | -           | +            | -      | +         |
| Tannins       | +           | -            | -      | +         |
| Phenolics     | +           | -            | -      | +         |

3.4. Antimicrobial activity of nutmeg seed extracts

Antimicrobial activity were tested on 3 microbes i.e. *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* by measuring the inhibition zone as shown in Table 3. The minimum inhibitory concentrations (MIC) of ethyl acetate extracts of nutmeg seed against the tested organisms showed on Table 4.

Table 3. The inhibition zone (antimicrobial activity) of nutmeg extracts

| Test Bacteria       | Type of solvent | Inhibition zone (mm) of nutmeg seed extracts | Concentration of solvent (%) |
|---------------------|-----------------|----------------------------------------------|------------------------------|
|                     |                 |                                              | 25  | 50  | 75  | 100 |
| *Escherichia coli*  | Water           | 8.250                                        | 8.500| 8.625| 8.375|
|                     | Methanol        | 10.750                                       | 11.500| 13.125| 11.500|
|                     | Ethyl acetate   | 10.625                                       | 10.750| 11.375| 10.875|
|                     | Hexane          | 9.750                                        | 9.875| 11.13| 11.125|
| *Staphylococcus aureus* | Water       | 8.250                                        | 8.750| 9.000| 9.875|
|                     | Methanol        | 10.750                                       | 10.375| 10.750| 11.750|
|                     | Ethyl acetate   | 10.300                                       | 10.475| 11.275| 10.725|
|                     | Hexane          | 9.625                                        | 10.000| 10.125| 10.375|
| *Bacillus cereus*   | Water           | 8.500                                        | 8.875| 8.875| 9.000|
|                     | Methanol        | 10.875                                       | 12.250| 12.125| 13.125|
|                     | Ethyl acetate   | 9.750                                        | 10.000| 11.250| 9.125|
|                     | Hexane          | 10.750                                       | 9.250| 11.125| 9.875|

Table 4. MIC determination of ethyl acetate extract of nutmeg seeds against tested organisms

| Extract concentration (%) | Tested organisms |
|---------------------------|------------------|
|                           | *Escherichia coli* | *Staphylococcus aureus* |
| 3.000                     | +                | +                        |
| 2.000                     | +                | +                        |
| 1.000                     | +                | +                        |
| 0.500                     | +                | +                        |
| 0.250                     | +                | +                        |
| 0.100                     | +                | +                        |
| 0.050                     | -                | -                        |
| 0.025                     | -                | -                        |

Highest inhibition zone on *S. aureus* bacteria was found in nutmeg seed extracted by methanol solvent with a concentration of 100%, because phenolic and flavonoid compound in nutmeg seed
extracted by methanol acts as an anti-bacterial against S. Aureus [24-25]. Highest inhibition zone on E. coli bacteria was found in nutmeg seed extracted by methanol solvent with a concentration of 75%. Nutmeg seed extracted by methanol contained steroids. Steroid has antimicrobial characteristics can inhibits gram-positive and gram-negative bacteria such as E.coli. The peroxide and vinyl bonds in steroids structures have a role as antibacterial [26]. Highest inhibition zone on B. cereus bacteria was found in nutmeg seed extracted by methanol solvent with a concentration of 100%. The phytochemical components act as antibacterial against B. cereus were tannin and phenolic compounds [27]. In nutmeg extract using methanol as a solvent, there are many bioactive compounds compared to extraction using other solvents. Phenolic compounds are compounds capable of inhibiting the growth of S. Aureus [28]. The alkaloid and flavonoid compounds contained in the extract have good inhibitiability of E. coli and B. cereus bacteria [29-31]. Phenolic compounds and tannins play a role inhibiting the growth of B. cereus bacteria [27].

Nutmeg seed extract used in MIC testing was extracted by ethyl acetate because ethyl acetate has been known as a good solvent for phytochemical extraction and is safe for human consumption because ethyl acetate is neither phototoxic nor photo allergenic in human tests [32]. Methanol solvent is not recommended for human, because it can cause toxicity which causes various health problems such as irritation, coughing, dizziness, vomiting, brain disorders, unconsciousness and even the risk of death [33]. To determine the MIC value of E. coli and S. aureus, further testing was needed by applying nutmeg seed extract directly on tempeh sausage. The value of MIC of nutmeg seed extract ranged from 0.1 – 3%, with E. coli and S. aureus showing MIC of 0.1% respectively.

3.5. Application of nutmeg seed extract on tempeh sausage
The addition of 0.10% and 0.25% nutmeg seed extract to tempeh sausage able to reduce the value of total plate count during storage (Table 5). Total count plate (TPC) of tempeh sausage without added nutmeg seed extract (control) reach the level of 2.82 x 10⁶ after five days storage, but the TPC of tempeh sausage with added 0.25% of nutmeg seed extract was 2.69 x 10⁵, which was lower than control tempeh sausage. This value shows that addition of nutmeg seed extract can inhibit the growth of microbe during storage.

Table 5. The TPC value of tempeh sausage with addition of nutmeg seed extract during storage in 4°C

| Days | Concentration of extract | Moisture content (%) | Total plate count (CFU/ml) | Colour | Flavour | Taste | Texture |
|------|-------------------------|----------------------|----------------------------|--------|---------|-------|---------|
| 1    | Control                 | 45.90                | 9.33x10⁵                   | 4.30   | 4.17    | 4.45  | 4.25    |
|      | 0.10%                   | 45.30                | 8.13x10⁵                   | 4.30   | 4.05    | 4.30  | 4.20    |
|      | 0.25%                   | 44.50                | 6.17x10⁵                   | 4.37   | 3.90    | 4.25  | 4.32    |
| 3    | Control                 | 45.65                | 1.82x10⁶                   | 3.87   | 3.60    | 3.90  | 3.40    |
|      | 0.10%                   | 45.40                | 5.13x10⁵                   | 4.00   | 3.73    | 3.95  | 3.47    |
|      | 0.25%                   | 44.55                | 3.39x10⁵                   | 4.23   | 3.85    | 3.87  | 3.43    |
| 5    | Control                 | 45.80                | 2.82x10⁶                   | 3.62   | 3.30    | 3.62  | 3.00    |
|      | 0.10%                   | 45.10                | 3.09x10⁵                   | 3.76   | 3.39    | 3.52  | 3.24    |
|      | 0.25%                   | 44.26                | 2.69x10⁵                   | 3.89   | 3.43    | 3.57  | 3.33    |

Table 5 shows that the addition of nutmeg seed extract can inhibit the growth of spoilage microbial, maintain the moisture content of sausages, slow the change in colour, flavour and texture due to the decomposition process. The highest inhibition was obtained at a concentration of 0.25% nutmeg seed extract. It was happened because the extract contain phytochemical compounds that acts as antimicrobial and antioxidant [34].
4. Conclusions
Extraction of nutmeg seed by using methanol produced the best quality of extract. Nutmeg seed extract contains various bioactive compound such as flavonoids, alkaloids, steroids, saponins, tannins and phenolics. The nutmeg seed extract has antimicrobial activity against the *S. aureus*, *E. coli* and *B. cereus*. The ethyl acetate extract has the higher antimicrobial activity with the MIC value of 0.1% and can applied as a preservative to inhibit the growth of spoilage microbial in tempeh sausage during storage.

References
[1] Kareem M A, Gadhamsetty S K, Shaik A H and Kodidhela L D 2009 Effect of aqueous extract of nutmeg on hyperglycaemia, hyperlipidaemia and cardiac histology associated with isoproterenol-induced myocardial infarction in rats *Trop. J. Pharm. Res.* 8 pp 491-552
[2] Gottardi D, Bukvicki D, Prasad S and Tyagi A K 2016 Beneficial effects of spices in food preservation and safety *Front. Microbiol.* 7 pp 1-20
[3] Gupta A D, Bansal V K, Babu V and Maithil N 2013 Chemistry, antioxidant and antimicrobial potential of nutmeg (*Myristica fragrans Houtt*) *Journal of Genetic Engineering and Biotechnology* 1 pp 25-31
[4] Ahvenainen R 2003 *Novel Food Packaging Techniques* (Cambridge: Woodhead Publishing Limited)
[5] Wu V C H 2008 Microbial injury and recovery methods in food: A review *Food Microbiol.* 25 pp 735-44
[6] Behling R G, Eifert J, Erickson M C, Gurtler J B, Kornacki J L, Line E, Radcliff R, Ryser E T, Stawick B and Yan Z 2010 *Principles of Microbiological Troubleshooting in the Industrial Food Processing Environment*, ed J L Kornacki (USA: Springer)
[7] Assa, J R, Widjanarko S B, Kusnadi J and Berhimpon S 2014 Antioxidant potential of fles, seed and mace of nutmeg (*Myristica fragrans Houtt*) *Int. J. ChemTech Res.* 6 pp 2460-8
[8] Adewole E, Ajiboye B O, Idris O O, Ojo O A, Onikan A, Ogumnodede O T and Adewumi D F 2013 Phytochemical, antimicrobial and GC-MS of African nutmeg (*Monodora myristica*) *International Journal of Pharmaceutical Science Invention* 2 pp 25-32
[9] Indonesian National Standard 2012 *Tempeh: Indonesian offerings for the world* (Jakarta: BSN)
[10] Nout M J R and Kiers J L 2005 Tempe fermentation, innovation and functionality: update into the third millenium *J. Appl. Microbiol.* 98 pp 789-805
[11] Muslihhah S, Anam C and Andriani M A M 2013 Tempeh Storage with Modified Atmosphere Method (Modified Atmosphere) to Maintain the Quality and Power of Tempeh *Journal of Teknosains Pangan* 2 pp 51-60
[12] Moedjiharto T J 2003 Evaluasi fisikokimia sosis tempeh-dumbo *Jurnal Teknologi dan Industri Pangan* 14 pp 164-8
[13] Divekar S P, Thakor N J, Mulla H Y and Sawant M V 2011 Effect on drying on physical properties of nutmeg *Engineering and Technology India* 2 pp 18-23
[14] Indonesian Ministry of Health 1995 *Materia Medika Indonesia* vol VI (Jakarta: Direktorat Pengawasan Obat dan Makanan) pp 247-51
[15] Fransworth N R 1996 Biological and phytochemical screening of plants *Journal of Pharmaceutical Sciences* 55 pp 257-9
[16] Mahesh B and Satish S 2008 Antimicrobial activity of some important medicinal plant against plant and human patogens *World Journal of Agricultural Sciences* 4 pp 839-43
[17] Hombach M, Zbinden R and Bottger E C 2013 Standardisation of disk diffusion result for antibiotic susceptibility testing using the sircan automated zone reader *BMC Microbiology* 13 pp 1-8
[18] Kubo A, Lunde, CS and Kubo I 1995 Antimicrobial activity of the olive oil flavour compounds *J. Agric. Food Chem.* 40 pp 1629-33
[19] AOAC 2002 *Official Methods of Analysis of AOAC International* 17th eds (USA: Association of Official Analytical Chemistry)

[20] Setyaningsih D, Apriyantono A and Sari M P 2010 *Sensory Analysis for Food and Agro Industry* (Bogor: IPB Press) pp 59-67

[21] Indonesian National Standart 1993 *Biji Pala SNI 01-0006-1993* (Jakarta: Badan Standarisasi Nasional)

[22] Al-Ash’ary M N, Titin S F M and Zackiyah 2010 Penentuan pelarut terbaik dalam mengekstrakski senyawa biokatif dari kulit batang Artocarpus heterophyllus (Determining the best solvent in extracting bioactive compounds from Artocarpus heterophyllus bark) *Jurnal Sains dan Teknologi Kimia* 1 pp 150-8

[23] Widyawati P S, Budianta T D W, Kusuma F A and Wijaya E L 2014 Difference of solvent polarity to phytochemical content and antioxidant activity of Pluchea indicia less leaves extracts *Journal of Pharmacognosy and Phytochemical Research* 6 pp 850-5

[24] Nurjanah S, Putri I L and Sugianti D P 2017 Antibacterial activity of nutmeg oil 2nd Proc. Int. Conf. on Sustainable Agriculture and Food Security : A Comprehensive Approach, KnE Life Science pp 563-9

[25] Elmasri WA, Zhu R, Peng W, Al-Hariri M, Kobeissy F, Tran P, Hamood A N, Hegazy M F, Pare P W and Mechref Y 2017 Multitargeted flavonoid inhibition of the pathogenic bacterium Staphylococcus aureus: a proteomic characterization *J. Proteome Res.* 40 pp 1-8

[26] Dogan A, Otlu S, Celebi O, Kilicle P A, Saglam A G, Dogan A N C and Mutlu N 2017 An investigation of antibacterial effects of steroids *Turkish Journal of Veterinary and Animal Sciences* 41 pp 302-5

[27] Hamdan M, Ismail K A and Delaimy K A 2007 The antibacterial activity of selected edible plant extracts against Bacillus cereus *Jordan Journal of Agricultural Science* 3 pp 148 – 55

[28] Kwon Y I, Apostolidis E, Labbe R G and Shetty K 2007 Inhibition of Staphylococcus aureus by phenolic phytochemicals of selected clonal herbs species of lamiaceae family and likely mode of action through proline oxidation *Food Biotechnology Journal* 21 pp 71-89

[29] Wu T, Zang X, He M, Pan S and Xu X 2013 Structure–activity relationship of flavonoids on their anti-Escherichia coli activity and inhibition of DNA gyrase *Journal of Agricultural and Food Chemistry* 61 pp 8185-90

[30] Dusane D H, Hosseinidoust Z, Asadishad B and N Tufenkji 2014 Alkaloids modulate motility, biofilm formation and antibiotic susceptibility of uropathogenic Escherichia coli *Open Access Plos One Journal* 9 pp 1-9

[31] Manosalva L, Mutis A, Urzua A, Fajardo V and Quiroz A 2016 Antibacterial activity of alkaloid fractions from *Berberis microphylla* G. Forst and study of synergism with ampicillin and cephalothin *Journal Molecules* 21 pp 1-10

[32] Liebert M A 1989 Final report on the safety assessment of ethyl acetate and butyl acetate *Journal of the American College of Toxicology* 8 pp 681-705

[33] National Agency of Drug and Food Control of Republic Indonesia 2014 *Methanol Methyl Alcohol* http://ik.pom.go.id [April 20th 2018]

[34] Gupta A D and Rajpurohit 2011 *Nuts and Seeds In Health and Disease Prevention* Chapter 98 Antioxidant and Antimicrobial of Nutmeg (*Myristica fragrans*) (London: Elsevier Inc. Academic Press) pp 831-938