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

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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. 561×10^4 CFU/g for control, 61×10^4 CFU/g for 0,1% nutmeg seed extract and 54×10^4 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 (Mettler), 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:

$$M (\%) = \frac{W_1 - W_2}{W_2} \times 100\% \quad (1)$$

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.

Table 1. The yield of nutmeg seed extract

Solvent	Yield (%)
Methanol	4,22 ^{aA}
Ethyl acetate	2,64 ^{cB}
Hexane	1,84 ^{dC}
Water	2,93 ^{bB}

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 quacinoind [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 ie. *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
<i>Escherichia coli</i>	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
<i>Staphylococcus aureus</i>	Water	8.250	8.750	9.000	9.875
	Methanol	10.750	10.375	10.750	11.750
	Ethyl acetate	10.3000	10.475	11.275	10.725
	Hexane	9.625	10.000	10.125	10.375
<i>Bacillus cereus</i>	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	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
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 inhibitability 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 2.82×10^6 after five days storage, but the TPC of tempeh sausage with added 0.25% of nutmeg seed extract was 2.69×10^5 , 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)	Sensory value			
				Colour	Flavour	Taste	Texture
1	Control	45.90	9.33×10^5	4.30	4.17	4.45	4.25
	0.10%	45.30	8.13×10^5	4.30	4.05	4.30	4.20
	0.25%	44.50	6.17×10^5	4.37	3.90	4.25	4.32
3	Control	45.65	1.82×10^6	3.87	3.60	3.90	3.40
	0.10%	45.40	5.13×10^5	4.00	3.73	3.95	3.47
	0.25%	44.55	3.39×10^5	4.23	3.85	3.87	3.43
5	Control	45.80	2.82×10^6	3.62	3.30	3.62	3.00
	0.10%	45.10	3.09×10^5	3.76	3.39	3.52	3.24
	0.25%	44.26	2.69×10^5	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.

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