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Potential of Nutmeg (*Myristica fragrans* Houtt) Leaf Extracts as a Source of Functional Ingredients with Antibacterial, Antifungal and Antioxidant Activities

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ABSTRACT

Purpose: *The objectives of this study were to evaluate inhibition activities of acetone, chloroform, ethanol and hot water extracts of nutmeg (Myristica fragrans Houtt.) leaves against Salmonella enterica, Listeria monocytogenes, Shigella dysenteriae, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Aspergillus niger and, to assess the in vitro antioxidant activities and to discover the active compound profile of chloroform extract of nutmeg leaf.*

Research Method: *Agar well diffusion and agar disc diffusion methods were used to evaluate the antibacterial activity and the antifungal activity of the extracts respectively. Antioxidant activities of the acetone extract were evaluated through DPPH, FRAP, ABTS+ radical scavenging and Ferrous Iron Chelating assays. The total phenolic content (TPC) of the acetone extract was determined using the Folin-Ciocalteu assay.*

Findings: *All the extracts showed inhibitory activity for the tested bacteria at different intensities and it was found that the selection of the extraction solvent is important to get the maximum inhibition against different bacterium. Acetone and chloroform extract (20%) showed the highest inhibition against A. niger (49.42% and 51.52% respectively). Acetone extract showed strong antioxidant activities, 715.78 ±51.09 in FRAP (mg of Trolox /g of leaves), 65.56±0.93 in DPPH (mg of Ascorbic acid /g of leaves), 31.67± 0.49 in ABTS^{+} (mg of Trolox equivalent/g of leaves), 10.87±1.85 in Ferrous Ion chelating (mg of EDTA/g of leaves) and, higher percentages of total phenolic content of 895.12 ±44.24 TPC (mg GAE /g of leaves). Myristic acid was detected as the major compound (57.36%) in GC-MS analysis and that may be the major compound responsible that offers a great potential to use as antibacterial, antifungal and antioxidant agent of nutmeg leaf.*

Originality/ Value: *Results revealed that the nutmeg leaf possesses marked biological activities and showed a great potential to use for the management of human pathogenic bacteria, plant pathogenic fungi, and oxidative stress-associated chronic diseases.*

Keywords: *Antibacterial, antifungal, antioxidant, functional ingredients, Myristica fragrans leaves*

INTRODUCTION

Spices have played a remarkable role in the human history. Their flavor and pungency make them very important in the food and pharmaceutical industries and used in the preparation of many foods and medicines all over the world (Bhagya and Raveendra, 2017; Peter and Babu, 2012). Besides the primary use of nutmeg and mace as a spice, oleoresins and essential oils are also derived. Those oils and extracts are standardized to increase consistency and hygiene, hence there is a big potential in their use for new product developments in the food and pharmaceutical industries (Parthasarathy *et al*., 2008).

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The spice nutmeg is the seed of a type of evergreen tree (*Myristica fragrans* Houtt) and mace is the red colour aril around the seed. Pharmacological research disclosed various bioactivities such as antioxidant, antibacterial, antidiabetic, hypolipidemic, hepatoprotective (Gupta *et al*., 2013; Warsito, 2021), and therefore, nutmeg seed and mace have been identified as a good treatment for cancers, rheumatism, diarrhea, asthma, and atherosclerosis (Ha *et al*., 2020; Piaru *et al*., 2012). The essential oils extracted from mace give medicinal benefits such as stimulating the heart system, stomachache, rheumatism, muscle pain, toothache, eliminating toxins in the liver (Nurhayati *et al*., 2022). Further, (Nikolic *et al*., 2021) reported that seeds showed potent antibacterial activity against both gram-positive and gram-negative bacteria.

Further, the leaves of some spice trees are also used in fresh or dried form as spices for cooking and medicinal purposes (Matthews and Jack, 2011). For example, dried cinnamon leaves are added to cooked rice and curries (Parthasarathy *et al*., 2008) and use to treat rheumatism, colic, and diarrhea (Sharma and Nautiyal, 2011). Abeysekera *et al*. (2013 and 2019) reported that extracts of Ceylon cinnamon leaves show noticeable activities as antioxidant and leaves showed higher activities than the bark. Further, leaf extracts of pepper showed strong antioxidant activities due to high amount of phenolic compounds (Shanmugapriya *et al*., 2012). Hence, oil and extracts of cardamom leaves are suggested as a novel antimicrobial agent to treat for different bacterial infections (Jebur *et al*. (2014). Therefore, leaves of many spices have been identified as rich sources of different bioactivities to develop different functional foods, and especially to recover drugs in the pharmaceutical industry.

Hence, it may be worth carrying out studies to evaluate the bioactivities of leaves of spice trees for pharmaceutical product developments. Nurmilasari *et al*., (2017) reported that methanol extract of nutmeg leaves shows better antioxidant activities in DPPH experiment. Compositional variation in the leaf, mace, kernel, and seed essential oil of nutmeg grown in India has been investigated (Ashokkumar *et al*., 2021) and suggested that the major constituents of leaf, mace, kernel and seed of nutmeg can be utilised in the food, perfumery, aroma and pharmaceutical industries. Therefore, in the present research, antimicrobial, antifungal and antioxidant activities of *M. fragrans* Houtt leaves collected from Matale, Sri Lanka were evaluated to find their potential in food, agriculture and pharmaceutical industries.

MATERIALS AND METHODS

Materials

Freshly harvested mature healthy nutmeg leaves were collected from local cultivations in Matale, Sri Lanka.

Acetone, chloroform and ethanol were purchased from Sigma-Aldrich, USA. All solvents used were analytical grade with 99% purity. Four different solvents (having different polarities) were used for the extraction purpose in order to separate constituents according to their polarity. Hot water extraction is an attractive and environmentally sustainable alternative for the extraction of compounds from plant materials even though non-polar bioactive compounds cannot be extracted with hot water (Zhang *et al*., 2020).

Reference bacterial strains of *Salmonella enterica, Listeria monocytogenes, Shigella dysenteriae, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were kindly gifted by Medical Research Institute, Sri Lanka. Reference fungi of *Aspergillus* niger was isolated from infected onion (*Allium cepa* L.) and authenticated at the Institute of Postharvest Technology (IPHT), Anuradhapura, Sri Lanka.

Preparation of Nutmeg Leaf Extracts

Fresh nutmeg leaves were cleaned with chlorinated water and then with deionized water and allowed to air dry until the leaves were crushable (about three days at ambient conditions) and grounded using a laboratory grinder to reduce the particle size. Leaf sample was stored in air tight glass containers at 4°C in a refrigerator until tested. Soxhlet apparatus was used to prepare the extracts for the antibacterial experiments. Ethanol, acetone and chloroform were used as the solvents at their boiling points (ethanol: 78°C, acetone: 56°C and chloroform: 61°C) for 4 hours in Soxhlet apparatus. Rotary evaporator was used to concentrate the crude extracts for experiments (at temperature ~ 40°C, under vacuum). From the leaf samples, 15 g was extracted with 250mL of each solvent. All extractions were conducted in triplicates. The same weight (15 g) of plant material was extracted with 250mL of distilled water to get the hot water extracts by keeping in a hot water bath (80°C) for 4 hours. The filtered extracts (Whatman No.1 filter papers) were stored in a freezer condition until further experiments.

In antifungal studies, a 30 g of grounded sample was extracted with 100mL of distilled water, in a water bath at 80°C for 30 minutes to get hot water extracts. Further, 30 g of leaf samples were extracted in 100mL of each solvent keeping them in dark for 48 hours at 25°C ± 1. Then the filtrates (Whatman No.1) were directly tested.

Preparation of Reference Bacteria and Fungi

The surface viable counting technique described by Miles *et al*. (1938) was used to calculate the average number of viable bacteria in a milliliter of the stock suspensions. Inoculums of 10^8 -10^9 colony forming units (CFU) in milliliter were prepared. Dilutions of bacteria suspensions were prepared to at least 10^{-8}, diluting with phosphate buffered saline. Equally divided nutrient agar (HiMedia, India) plates were labelled according to the dilutions. Surface of each sector of the plate was dropped with 20µL volume of the appropriate dilution. The plates were allowed to dry and incubated at 37°C for 24 hours in an inverted position. Colonies on each sector were counted and the number of CFU in milliliter of the original aliquot was calculated as below.

CFU per milliliter = Average number of colonies for a dilution x 50 x dilution factor.

Reference fungal strain, *A*. niger was isolated from black molds in onions. Surfaces were sterilized in 60% alcohol solution. Infected onion skins were cut into small segments with a sterilized blade. The surface-sterilized parts were placed on Potato Dextrose Agar (PDA) (HiMedia, India) aseptically and incubated at 25°C for 7 days. The isolated colonies were sub-cultured repeatedly on PDA to obtain pure cultures. Isolated fungi were identified according to the colony morphology at IPHT, Anuradhapura, Sri Lanka.

Determination of Antibacterial Activity

Agar well diffusion method described by Irshad *et al*. (2012) was used to evaluate antibacterial activity of nutmeg leaves. A 25mL of autoclaved nutrient agar (HiMedia, India) was added into 100 mm petri- plates. Nutrient agar was seeded with 100µL reference bacterial strains. Wells were punched on the agar plates using a sterile cork borer (4 mm). Each well was treated with 100µL volume from the stock solutions (10 mg of extract/ mL of solvent) and incubated at 37°C and readings were taken after every 24 hours for 3 days. The stock solutions were prepared by adding 40µL of DMSO 960µL of relevant solvent. The diameters of the clear inhibition zones formed around the wells were measured. Wells treated only with solvents were used as the negative control. Thereby antimicrobial activities of different extracts of nutmeg leaves against the reference pathogenic bacteria were evaluated. Three replications were done for each experiment. Amoxicillin (10 mg of powder/ mL of sterilized water), the antibiotic was used as the positive control.

Determination of Antifungal Activity

The agar dilution technique (Neela *et al*., 2014) was used to evaluate antifungal activities. A 5mL of filtered extract and 95mL of PDA were mixed to prepare a 5% concentration of extract in PDA. In this way, 10% and 20% extract in PDA were prepared. After solidification of the extract and PDA mixture (25mL per perti plate), mycelia block (4 mm) from week-old colonies of fungi (*A. niger*) was placed in the middle on the surface of each plate and incubated at 25 ± 1°C. The colony diameters were measured after 10 days of incubation period. Each experiment was done with three replicates. PDA plates amended separately with acetone, ethanol, chloroform and sterilized distilled water were used as negative controls. Captan (2 table spoons/ 3.7L of sterilized water), the commercially available fungicide was used as the positive control. The inhibition percentage was calculated using the following formula.

$$\text{Inhibition } \% = \frac{(C - T)}{C} \times 100$$

C - Colony diameter (negative control)

T - Colony diameter (treatment)

Determination of Total Phenolic Content and Antioxidant Activities of Leaf Extracts

Total phenolic content and antioxidant activities were evaluated for acetone leaf extract as it showed higher activities in antibacterial and antifungal experiments. All the analyses were carried out using High-throughput 96-well micro-plate readers (SpectraMax Plus384, Molecular Devices, USA and SPECTRA max-Gemini EM, Molecular Devices Inc, USA).

Determination of Total Phenolic Content

TPC of the extracts was measured by the Folin-Ciocalteu method (Singleton *et al*., 1999) using 96-well micro-plate readers. Freshly prepared Folin-Ciocalteu reagent (Sigma-Aldrich, USA) was used after diluting10 times for the test. The assay was prepared by adding 20µL of 1 mg/ mL of nutmeg leaf extract, 110µL of reagent and 70µL of 10% sodium carbonate. The mixture was allowed to incubate at 25°C for half an hour and the absorbance was taken at 765 nm. Different concentrations (0.06-1 mg/mL) of gallic acid were tested to get the standard curve for the experiment. Results were calculated as gallic acid equivalents (mg) in a gram of leaves (dry weight).

DPPH Radical Scavenging Assay

The protocol explained by (Blois, 1958) was followed for 1,1-Diphenyl-2-picrylhydrazyl radical scavenging assay. Results were calculated as ascorbic acid equivalents antioxidant capacity in mg of ascorbic acid in a gram of leaves (dry weight). One milligram of leaf extract was initially dissolved in 20µL DMSO and then diluted in 980µL of methanol. For the assay, 90µL of methanol, 50µL of extract/ standard/ methanol as the control and 60µL of DPPH were used. After 10 minutes (incubation period) at 25°C, absorbance was taken at 517 nm. Then percent radical scavenging activities of samples and standard were calculated.

A- Absorbance of sample at concentration N

B- Absorbance of control

% Radical scavenging activity = (B-A) / B x 100

Ferric Reducing Antioxidant Power (FRAP) Assay

The protocol described by Benzie and Szeto (1999) was followed in a 96-well micro-plate readers. To prepare the FRAP reagent for the assay 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-Tripyridyl-S-triazine (TPTZ)

solution (dissolved in 40 mM HCl) and 20 mM $FeCl_3.6H_2O$ were mixed to the ration of 10:1:1 (v/v/v) and incubated at 37°C for 10 minutes. Volume of a well included with 150µL of FRAP reagent, 30µL of buffer and 20µL of extract (1 mg/mL). After mixing, the plates were incubated 25°C for 8 minutes and absorbance was recorded at 600 nm. Results were expressed as milligrams of Trolox equivalents in a gram of plant extract were calculated.

$ABTS^+$ Radical Scavenging Assay

The protocol reported by Re *et al*., (1999) was used in 96 well micro plates. From the leaf extract, 1 mg was initially dissolved in 20µL DMSO and then diluted in 980µL of distilled water. ABTS was incubated in potassium persulfate at 25°C for 16 hours in the dark to prepare the radical solution. The volume of a well in the plate (200µL) was completed with 40µL of $ABTS^+$ radical and 50µL of extracts. The leaf extract was tested in five different concentrations of 50, 25, 12.5, 6.25 and 3.125 µg/mL. The filled plates were kept at 25°C for 10 minutes before recording the absorbance at 734 nm. Trolox was used to make the standard curve (50, 25, 12.5, 6.25 and 3.12 µg/mL). Results were expressed as milligrams of Trolox in a gram of dry weight of leaves were calculated. Percentage radical scavenging activities of samples were calculated as described in DPPH assay.

Ferrous Iron Chelating Assay

The protocol mentioned by (Dinis *et al.*, 1994) was pursued with slight modifications to evaluate ferrous iron-chelating activity. From the extract, 1 mg was initially dissolved in 40µL of DMSO and then diluted in 960µL of distilled water. For the assay, 100µL of the sample, 20µL of ferrous sulphate solution, 40µL of distilled water mixed and pre-plate readings were taken. Water was used as the blank. Then, 40µL of ferrozine solution was added to the assay to complete 200µL and incubated for 10 minutes. Absorbance readings were taken at 562 nm.

Milligrams of EDTA equivalents in a gram of leaves were calculated. Percentage radical scavenging activities of samples were calculated as described DPPH assay.

GC-MS Analysis of Nutmeg Leaf Extract

GC-MS analysis was performed for acetone leaf extract as it showed higher activities in antibacterial and antifungal experiments. GC-MS studies were carried out using Agilent 7890B GC system and MS of 5977A Mass Selective Detector (MSD). The separations were carried out on Agilent HP-5 ms ultra inert column (30 m × 250 µm × 0.25 µm). Initial temperature for the GC analysis was 50°C (held for 2 min), which was raised to 240°C (ramp 5°C/min). The 240°C temperature was maintained for 2 min. The sample (1µL) was injected using split-less mode. Helium was used as the carrier gas with a flow rate of 4.8 mL/min. MS transfer line temperature was maintained at 260°C. The assignment of the peaks is based on the National Institute of Standards and Technology (NIST) reference database. Total run time was 50 minutes.

Statistical Analysis

Data were reported as mean ± SD for triplicate (n=3) determinations. Statistical analysis was done with descriptive and inferential statistics by using Minitab Version 17 (Minitab Inc., State College, PA, USA). The one-way ANOVA (analysis of variance) and Tukey's test were used to compare the mean values and find the significant differences of the means ($p<0.05$).

RESULTS AND DISCUSSION

Antibacterial Activity

P. aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium. It has been identified as an opportunistic pathogen of both humans and plants. *L. monocytogenes* gram-positive rod that is responsible for causing the infection listeriosis. *S. enteritica* is a rod-headed, flagellate, facultative anaerobic, Gram-negative bacterium. *E. coli* is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium. *S. dysenteriae* is a rod-shaped bacterial genus *Shigella*. *Shigella* species can cause shigellosis (bacillary dysentery). *Shigellae* are Gram-negative, non-spore-forming, facultative anaerobic, non-motile bacteria (Centers for Disease Control and Prevention, 2022).

As shown in the Table 01, Amoxicillin the positive control showed the highest inhibition against every bacterium. Among the solvents, the chloroform extract showed the highest inhibition zone (0.97 cm) against *P. aeruginosa*. There was no significant difference ($p>0.05$) of inhibitions between ethanol, acetone and hot water against *P. aeruginosa*. The highest inhibition (1.61 cm) against *S. dysenteriae* was also detected by the acetone leaf extract. Both ethanol and acetone extracts showed the highest inhibition against *E-coli* (1.27 cm) and the mean inhibitions were equal. The highest inhibition against *S. enterica* was detected by ethanol extract (0.98 cm). Acetone extracts showed the highest inhibition against *L. monocytogenes.* When consider the entire results of the current study, the lowest inhibition was given by hot water extracts. This may be due to the presence of less amounts of bioactive compounds in the hot water extracts. According to the results of the antibacterial assay, it was clear that the inhibitory activities of the leaf extracts against the reference bacteria greatly depend on the solvent used to prepare the extract. The same or the highest inhibition was not shown by the solvent extracts against each bacterium used in this study. Different leaf extracts inhibited the bacteria at different percentages. Therefore, the selection of the solvent used to prepare the extract is important to get maximum inhibition.

The results obtained by the research of Indu *et al*. (2006) have reported that hot water extract of nutmeg seed had inhibition ability against different serotypes of *E. coli, Salmonella* spp. and *Listeria*, and higher inhibition activities against *Listeria* but, poor inhibition against *Salmonella* spp. However, the results obtained from this study showed nutmeg leaves had strong inhibition activities against both *Listeria* and *Salmonella* spp. Further, Agoramoorthy *et al*. (2007) have reported antibacterial activities of leaves of some spices and herbs like cumin, cinnamon, fennel and anise against *S. aureus, P, aeruginosa, Enterococcus,* and *Mycobacterium smegmatis*. The diameters of the inhibition zones created by the cumin, cinnamon, fennel and anise leaf extracts were very close or had small deviations from the diameters of nutmeg leaf extracts at 10 mg/mL concentration in this study. Cumin, clove and fennel leaves did not show inhibition against *P. aeruginosa and E. coli* in that study. Agoramoorthy *et al*. (2007) used ether extracts of leaves and without dilution. Therefore, the present study strongly supported that nutmeg leaves showed strong antimicrobial activity against selected pathogenic bacteria with higher inhibition activities compared to previous studies reported for cumin, clove and fennel leaves.

Antifungal Activity

Five types of nutmeg leaf extracts at three concentrations (5%, 10%, 20%) were evaluated for antimicrobial activity against pathogenic fungi (*A. niger*) using disc diffusion method. Methanol extracts of plant materials are reported to show high inhibition in many antifungal experiments (Gupta *et al.*, 2013). Therefore, antifungal experiments methanol has also been used as an additional solvent.

Table 01: Antibacterial activities of nutmeg leaf extracts.

Extract	Mean diameters of the inhibition zones by the different extracts against reference bacteria (cm) ± SD (n=3)				
	P. aeruginosa	*L. monocytogenes*	*S. enterica*	*E. coli*	*S. dysenteriae*
Ethanol	0.56±0.04^{c}	1.26±0.06^{c}	0.98±0.05^{b}	1.27±0.05^{b}	1.56±0.05^{c}
Acetone	0.57±0.02^{c}	1.37±0.11^{b}	0.61±0.07^{e}	1.27±0.04^{b}	1.61±0.04^{b}
$CHCl_3$	0.97±0.04^{b}	1.04±0.02^{d}	0.83±0.04^{c}	1.00±0.07^{c}	0.90±0.07^{d}
Hot Water	0.57±0.02^{c}	0.61±0.01^{e}	0.72±0.05^{d}	0.61±0.02^{d}	0.52±0.03^{e}
Amoxicillin	2.73±0.01^{a}	3.1±0.01^{a}	3.5±0.01^{a}	3.3±0.01^{a}	3.7±0.02^{a}

In each column, different letters are significantly different by the Tukey's test ($p<0.05$); Data expressed as mean ± standard deviation (n =3)

Table 02: Antifungal activities of nutmeg leaf extracts.

Extract	Mean inhibition % against *A. niger* ± SD (n=3)		
	5%	10%	20%
Methanol	6.38±3.98^{d}	28.89±3.6^{b}	38.10±7.72^{c}
Ethanol	10.48±4.21^{c}	26.09±8.37^{b}	40.00±9.43^{b}
Acetone	12.50±4.42^{b}	27.78±7.86^{b}	49.92±9.82^{a}
$CHCl_3$	19.76±3.86^{a}	42.86±10.1^{a}	51.52±3.71^{a}
Hot Water	9.44±2.97^{c}	24.67±3.56^{c}	33.75±4.42^{d}

As shown in Table 02, the antifungal activities of the extracts increased linearly with increase in percentage of extracts against *A. niger*. Complete inhibition was not detected by any of the solvent extracts. However, complete inhibition of *A. niger* was detected with Captan. The highest inhibition of 51.52% was shown by the chloroform extract. Hot water extracts showed the lowest inhibition against *A.niger* while chloroform extract showed the highest antifungal activity followed by acetone extract. However, the mean inhibition% shown by the acetone extracts (49.42%) was not significantly different to the inhibition% shown by the chloroform extract. The inhibition% shown by methanol and ethanol extracts was not statistically significant at the highest percentage of extract (20%). Mvuemba *et al*. (2009) have also reported that nutmeg seed water extract showed very poor and negligible antifungal activity against *A. niger*. However, hot water extract of leaf showed 34% inhibition against *A. niger* in present study. Thus, our results indicated the possible use of water extract of nutmeg leaves than nutmeg seeds to control *A. niger*. Rahman *et al*. (1999) reported 15% inhibition of *A. niger* by DMSO diluted nutmeg seed essential oil. In this study, more than 15% inhibition was reported (19%) even at a 5% concentration of chloroform leaf extract. Therefore, the results obtained by the present study showed the possibility of antifungal product developments from nutmeg against *A. niger*.

Antioxidant Properties

Antioxidant experiments were performed for acetone leaf extract as it showed higher activities in both antibacterial and antifungal experiments.

Table 03: Antioxidant activities of chloroform extract of nutmeg leaves.

Antioxidant activities of acetone extract ± SE				
TPC (mg GAE /g of leaves)	FRAP (mg of Trolox /g of leaves)	DPPH (mg of Ascorbic acid /g of leaves)	$ABTS^{+}$ (mg of Trolox equivalent/g of leaves)	Ferrous Ion chelating (mg of EDTA/g of leaves)
895.12±44.24	715.78±51.09	65.56±0.93	31.67±0.49	10.87±1.85

TPC- Total Phenolic Content, FRAP - Ferric Reducing Antioxidant Power; $ABTS^{+}$ - $ABTS^{+}$ radical scavenging activity, DPPH - 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

Table 03 showed antioxidant activities of acetone extracts of nutmeg leaves. Tan *et al*. (2013) reported antioxidant properties of different parts of nutmeg fruit and reported that the TPC were 114, 18.8, 37.39 and 26.26 mg of GAE/g dry weight for skin, seed, mace and pulp of the fruit respectively. The findings of this study also showed that nutmeg leaf contains a high amount of phenolic compounds (895.12±44.24 mg GAE /g of leaves) than seed, mace, pulp, or skin of the fruit. Nurmilasari *et al*. (2017) have also reported that nutmeg leaves show higher antioxidant activities in antioxidant experiments due to high phenolic compounds.

Ferrous reducing powers of the compounds are linked with and increase the antioxidant activities of foods. Further, there is a link between the presence of reductones and reducing properties of a compound. The breaking of the free radical chain, or preventing peroxide formation can be mentioned as the antioxidant action of reductones (Duh, 1998). Therefore, presence of reductones, and compounds with reducing properties of the leaf extracts may be some reasons to show a higher value in FRAP assay like in TPC. Tan *et al*. (2013) reported FRAP value for methanol extract of nutmeg kernel as 190.83 µmol Trolox equivalent in a gram of dry weight which was lower than the values shown in this study for nutmeg leaves as 715 mg Trolox equivalent in a gram of leaves dry weight. Therefore, it can be concluded that nutmeg leaf showed a strong value in FRAP study than kernel when Trolox is considered as the standard.

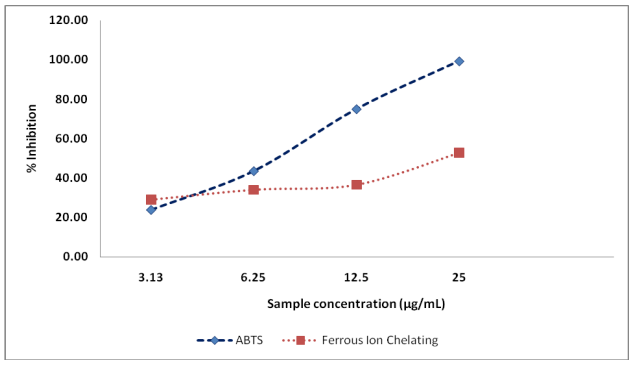

Figure 01: ABTS radical scavenging and ferrous ion chelating activity of acetone extract of nutmeg leaves.

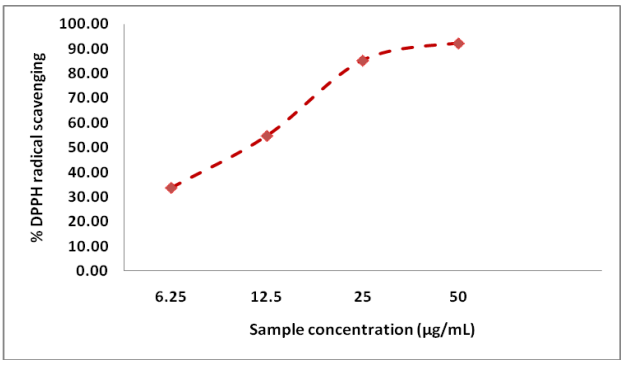

Figure 02: DPPH radical scavenging activity of acetone extract of nutmeg leaves.

The radical scavenging activities (ABTS and DPPH) and ferrous ion chelating of acetone nutmeg leaf extract was dose dependant (Figure 01 and Figure 02). The nutmeg leaf extracts demonstrated notably higher DPPH radical scavenging activity (65.56 mg of Ascorbic acid /g of leaves) than the ABTS radical scavenging activity (31.67 µg of Trolox equivalent/g of leaves). This may be due to the fact that the presence of compounds with DPPH scavenging activities, but does not get the ABTS scavenging activities. Further, stereo selectivity of the radicals and solubility differences of the extract may also result the above mentioned pattern (Wang *et al*., 1998; Yu *et al*., 2002). Further, compounds such as polyphenols may be more efficient reducing agents for ferric iron but may not scavenge the DPPH free radicals efficiently due to steric hindrance (Wong, *et al.,* 2006).

Ability to chelate transition Fe^{2+} ions results the antioxidant properties of plant extracts (Yusof *et al*., 2013). Nutmeg leaf extract should be rich with a complexing agent like phenolic compounds since it showed a higher TPC value. Therefore, the presence of complexing agents may be the reason to get a lower value for ferrous ion chelation activity for nutmeg leaf extract in this study.

(Gupta *et al*., 2013a) reported 48% of Fe^{+2} chelating power of acetone extract of nutmeg seed when compared to EDTA (99.8%). However, in the presence study chlororform extract of nutmeg exhibited 10.87 mg of EDTA/g of leaves activity.

Active Compound Profile of Leaf Extract

GC-MS analysis was carried out for acetone leaf extract prepared for antibacterial studies, as it showed higher activities in antibacterial and antifungal experiments. GC-MS analysis is an analytical method that combines the features of gas chromatography and mass spectrometry to identify different substances within a sample component matrix. GC-MS analysis is generally considered one of the most accurate analyses available to detect active compounds in plant extracts (Beale *et al*., 2018).

Table 04: Major compounds detected in GC-MS analysis of nutmeg leaf extracted with acetone with their biological activities.

Compound	% Content	Retention time (minutes)	Bioactivity	Reference
Gamma-terpinene	0.312	26.272	Antibacterial	(Owolabi *et al*., 2009)
Caryophyllene	0.288	41.822	Antibacterial	(Kim *et al*., 2008)
Naphthalene	2.004	44.214	Antimicrobial	Rokade and Sayyed (2009)
Myristic acid	57.36	48.259	Antimicrobial	Chen *et al*. (2019)
Hexanoic acid, Myristic acid, Sucrose	14.14	50.842	Antimicrobial	Alva-Murillo *et al*., (2012); Huang *et al*., (2011); Liu and Huang (2012); Shulgin and Sargent (1967)
Gamma & alpha eudesmol	9.114	51.535	Antimicrobial	Asakura *et al*. (2000)
Phenol, 2,6-dimethoxy-4-(2-propenyl)-	0.401	52.976	Antimicrobial	Hameed, *et al.*(2018)
Elemicin	0.652	56.165	Antibacterial	(Rossi *et al*., 2007)
Isomallotochromanol, 2-(1-Butoxyvinyl)-4,4-dimethyl-2-cyclohexene-1-one	7.689	58.790	Anti-inflammatory	Ishii *et al*. (2003)

Compounds detected as a single compound in the GC-MS are presented in Table 04. Some compounds were detected one in a mixture of several compounds. Myristic acid was detected in the highest percentage (57.36%) and it can act as a natural antibacterial agent (Chen *et al*., 2019). Naphthalene was detected in 2.004% in the present study. Rokade and Sayyed (2009) reported that naphthalene has an antimicrobial effect against a wide range of human pathogens. Naphthalene has several medicine uses due to various and interesting antibiotic properties. Nafacillin, naftifine, tolnaftate, terbinafine are some drugs that contain naphthalene. These drugs play a crucial role in the control of microbial infections (Rokade and Sayyed, 2009). Among many derivatives, beta-naphthol possesses a very good antimicrobial property. Beta eudesmol has a role as a volatile oil component. (Miyazawa, *et al.*, 1996) reported the antimutagenic activity of beta-Eudesmol. Gamma and alpha eudesmol were detected in 9.114% in this study. Further, Tsuneki *et al*. (2005) reported that beta-eudesmol block the extracellular-signal-regulated kinase signaling pathway and inhibits angiogenesis. beta-eudesmol has also been suggested to use the development of drugs to treat angiogenic diseases. 2-Naphthalenemethanol is a synonym for alpha eudesmol and it is an inactive compound (PubChem, 2019). However, alpha-eudesmol has anti-neurogenic inflammation action (Asakura *et al*., 2000). Therefore, alpha-eudesmol can be identified as a valuable compound that can be isolated from nutmeg leaves to develop drugs. Zachariah *et al*. (2008) reported leaf profiles of three species of nutmeg; *M. beddomeii* (King), *M. fragrans* (Houtt.) and *M. malabarica* (Lamk.), while α-eudesmol has only been detected in 0.14% in *M. fragrans* (Houtt.) and beta-eudesmol had been only detected in 0.22% in *M. beddomeii* (King). (Ishii *et al*., 2003) isolated isomallotochromanol from the pericarp of *Mallotus japonicus* as an active ingredient. As mentioned in their study, isomallotochromanol and isomallotochromene were the most potent in inhibiting cytokine production and human blood monocytes. Ishii *et al*. (2003) reported detection of isomallotochromanol in *Mallotus japonicus.* Isomallotochromanol and isomallotochromene have been isolated from the pericarps of *M. japonicus,* as active compounds. They are phloroglucinol derivatives. And in this study too, isomallotochromanol was detected in nutmeg leaves. However, no literature was found about the detection of isomallotochromanol in nutmeg. Therefore, the present study may be the first time that reported isomallotochromanol detection in *M. fragrans* (Houtt).

With the results of GC-MS analysis, it can be concluded that the presence of myristic acid exceeding 50% and the presence of some other active compounds shown in Table 4 in considerable amounts may be responsible for the higher antimicrobial and antifungal properties of nutmeg leaves. Further with the GC-MS analysis, phenolic compounds such as elemicin, phenol, 2,6-dimethoxy-4-(2-propenyl)- were detected in nutmeg leaf extract. These phenolic compounds contain dihydroxy groups and those groups can conjugate transition metals, by preventing the metal-induced free radical formation. Therefore, this can be the reason for the lower value of ferrous ion chelating activity and the higher value for TPC.

In this study, only 18 compounds were identified (Figure 03) in GC-MS analysis. Therefore, it is suggested to use some other columns for GC-MS analysis to separate more compounds in nutmeg leaves.

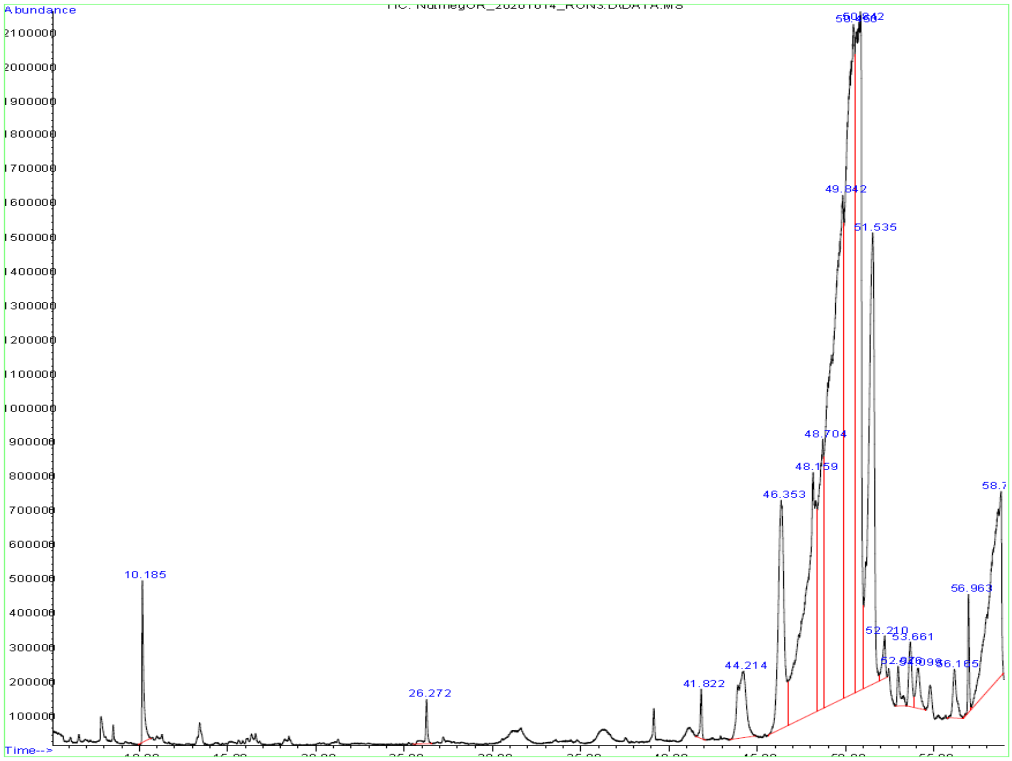

Figure 03: GC-MS chromatogram of nutmeg leaf extracted by acetone.

CONCLUSION

Nutmeg leaf extracts showed marked antibacterial, antifungal, and antioxidant properties. Acetone extract of nutmeg leaves showed comparatively higher activities in both antifungal and antibacterial experiments when compared with other extracts. The results indicate the possible use of nutmeg leaves to control the diseases caused by human pathogenic bacteria, to manage plant fungal pathogens, to reduce oxidative stress-associated chronic diseases, to design and develop drugs and to isolate myristic acid as the principal compound detected.

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Declaration of conflicting interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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