International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 8 (6): 572-576, 2012
ISSN 1811-7775 / DOI: 10.3923/ijp.2012.572.576

Determination of Antibacterial Activity of Essential Oil of *Myristica fragrans* Houtt. using Tetrazolium Microplate Assay and its Cytotoxic Activity Against Vero Cell Line

Suthagar Pillai Piaru, Roziahanim Mahmud and Shanmugapriya Perumal
School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

Abstract: Essential oils and volatile constituents of *Myristica fragrans* (nutmeg) are widely used as antioxidants, antidiabetic agents and for the prevention and treatment of different human diseases such as cancer, cardiovascular diseases and bacterial and viral infections. The aim of the study is to determine the antimicrobial activity and *in vitro* cytotoxicity of essential oil of nutmeg on normal cell line (Vero cell line). The Minimum Inhibitory Concentration (MIC) of the antimicrobial activity was determined using tetrazolium microplate assay. Eight gram negative and 2 g positive species of test microorganism were selected to determine the MIC exhibited by the essential oil. Cytotoxicity assay of the essential oil against normal cell line was conducted using Vero cell line. The percentage of inhibition was calculated and expressed as IC_{50}. Essential oil exhibited variant antimicrobial activity against the tested strains. The MIC values of nutmeg oil ranged from 0.031 to 1 mg mL^{-1} and showed higher activity against *Shigella dysenteriae* with MIC value of 0.031 mg mL^{-1}. The nutmeg oil possessed low cytotoxicity against Vero cell line with IC_{50} at 24.83 μg mL^{-1}. Nutmeg essential oil exhibited strong antibacterial activity against the *Shigella dysenteriae* with low cytotoxic effects. Thus, the essential oil can be further studied on its action of mechanism and fractionation will be carried out to determine the strength of its antibacterial activity.

Key words: Nutmeg, antibacterial, cytotoxicity, Vero cell line, essential oil

INTRODUCTION

Since long time back, aromatic herbs and spices have been added to various foods as ingredients to improve the taste, flavor and organoleptic properties. Essential oils are secondary metabolites which derived from plants, herbs and spices are made up of volatile compounds comprising volatile constituents like lipids, terpenoids, ketones, phenols and oxygenated derivatives. They are characterized by a strong odor and are formed by aromatic plants (Bakkali *et al*., 2008).

The antimicrobial activities of plant oils and extracts have formed the foundation of many applications such as in raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans, 1997).

Nutmeg (*Myristica fragrans* Houttuyn) is the seed kernel of inside the fruit and mace is the lacy covering (aril) on the kernel (Nadkarni, 1988) and cultivated in tropical regions and indigenous to the Maluku Province of Indonesia. It has been studied to having anthelmintic, hepatoprotective anti-inflammatory, aphrodisiac, insecticide properties and a treatment for rheumatism, diarrhea, asthma, atherosclerosis and flatulence (Burkill, 1966; Ozaki *et al*., 1989; Pooja *et al*., 2012). In addition to that nutmeg mace was found to exhibit strong antifungal and antibacterial activities (Singh *et al*., 2005). Eugenol, a component of nutmeg, is widely used in dentistry as root canal sealers reported to show antibacterial activity against oral bacteria (Lai *et al*., 2001).

To our knowledge, though extensive studies on nutmeg have been conducted but less research has been studied on the volatile mixtures of the plant species. Therefore, the aim of this study is to investigate the antibacterial activity and *in vitro* cytotoxicity of essential oil of nutmeg on normal cell line. The cytotoxic study is important to determine the safety of the essential oil for therapeutic application since few compounds especially myristicin, one of chemical compounds of nutmeg is reported to be toxic (Pooja *et al*., 2012).

MATERIALS AND METHODS

Plant material: The fresh fruits of nutmeg (*Myristica fragrans*) were obtained from Balik Pulau, Penang, Malaysia. Voucher specimens (11253) *Myristica fragrans*

Corresponding Author: Roziahanim Mahmud, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia Tel: +6046532209/+6046534701

was authenticated by botanist, Mr. Shunmugam and deposited at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia.

Extraction of essential oil: The fresh fruits of the plants collected were submitted to water distillation for 3 h using a modified Clevenger's apparatus. The ground samples (200 g) were boiled with water (200 mL) for 3 h in a 1 L round bottom flask fitted with a condenser. The extracted essential oils were dried over anhydrous sodium sulphate and after filtration, stored at 4°C until tested and analyzed.

Antibacterial assay

Microbial strains, culture medium and inocula preparation: Thirteen species of test microorganism which selected to study were originally clinical isolates obtained from Department of Medical Microbiology and Parasitology (JTMP), School of Medical Sciences, University Science Malaysia, Kelantan. Gram-negative species which involved in this study were *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella dysenteriae* and Gram-positive species were *Staphylococcus aureus* and *Bacillus subtilis*. Their identities were confirmed by culturing on the specific media followed by biochemical test using API system as previously reported (Mbaveng *et al*., 2008). Bacterial strains stock cultures were kept on Nutrient Agar (Difco, USA) at 4°C. All the microbial strains were sub-cultured on a fresh appropriate agar plate 24 h prior to antibacterial test. Inocula were prepared by transferring several single colonies of microbes to a sterile broth. The microbial cell suspension was mixed to homogeneity to give a final density of 5×10^5 CFU mL^{-1} and these were confirmed by viable counts. The infective dose for most microorganisms is 10^5 CFU mL^{-1}.

Preparation of crude extracts and antibiotics: The essential oil was dissolved in 50% Dimethyl Sulfoxide (DMSO) in sterile Mueller Hinton Broth (MHB) for bacterial isolates in order to obtain a stock concentration of 10 mg mL^{-1}. The stock solution was further diluted using respective broth in five-folds to obtain working concentration of 2 mg mL^{-1}. The final concentration of DMSO in the well was ensured to be less than 2%. Preliminary analyses with 2% (v/v) DMSO affected neither the growth of the test organisms nor the change of tetrazolium color due to this growth. Gentamicin, amoxycillin, vancomycin, chloramphenicol and penicillin were prepared to a final concentration of 0.1 mg mL^{-1} and served as the positive drug control against bacterial strains.

Tetrazolium microplate assay: The Minimum Inhibitory Concentration (MIC) of test microorganisms and reference antibiotics were determined by using tetrazolium microplate assay according to Eloff (1998) with slight modification. This assay was performed using 96-well clear microtitre plates. The wells in column A of each row were left blank and the last seven wells from column B to H were filled with 100 μL of sterilized MHB (bacterial isolates). Working solution of plant extracts were added to the wells in column A and B of each row and an identical two-fold serial dilution were made from column B to the column G. The last wells in column H was served as drug-free controls. An appropriate solvent blanks (DMSO) were included as negative control. Then, 100 μL of bacterial inoculum were added in all the wells from column A to H and mixed thoroughly to give final concentrations ranging from 1-0.015 mg mL^{-1}. Tests were carried out in triplicates. The cultured microplates were sealed with parafilm and incubated at 37°C for 24 h for bacterial were incubated at 28°C for 48 h. The MIC of sample was detected following addition (50 μL) of 0.2 mg mL^{-1} MTT in all the wells (MTT, Sigma-Aldrich, USA) and incubated at 37°C for 30 min. Microbial growth were determined by observing the change of color tetrazolium (MTT) in the microplate wells (purple formazan when there is growth and clear solution when there is no growth). MIC was defined as the lowest sample concentration showing no color change (clear) and exhibited complete inhibition of bacterial growth. MIC value <0.5 mg mL^{-1} was defined as potential strong activity.

Cytotoxicity screening Vero cell line: The Vero cell line was obtained from kidney of a normal adult African green monkey on March 27th, 1962, by Yasummura and Kawakita at the Chiba University, Japan (APHA, 1992). Vero cells was maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine (2 raM), penicillin (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}). The cells were cultured at 37°C in a humidified 5% CO_2 incubator. Vero cells were cultured and maintained in RPMI 1640 medium supplemented with 10% FBS. The cells were cultured at 37°C in a humidified 5% CO_2 incubator.

Cytotoxicity assay: The essential oil of nutmeg tested for *in vitro* cytotoxicity, using Vero cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) with slight modifications. Briefly, after being harvested from culture flasks, the cells were seeded at 1×10^6 cells in each well of 96-well plate containing 100 μL of fresh growth medium per well and cells were permitted to adhere for 24 h. The cells were

treated with the nutmeg essential oil which were serially diluted with growth medium to obtain various concentration (100, 50, 25, 12.5, 6.25 μg mL^{-1}). One hundred microliter of each concentration was added to each well. After 24 h of treatment the medium was aspirated and the cells were washed once with sterile Phosphate Buffered Saline (PBS). 5 mg mL^{-1} of MTT in PBS was added to each well and the plate was incubated at 37°C in 5% CO_2 for 2-4 h, until a purple precipitate was clearly visible under a microscope. The medium was discarded and 200 μL of Dimethyl Sulfoxide (DMSO) was added to each well to dissolve the dark blue crystals of formazan salt and the plates shaken for 5 min. After incubation at 37°C for 10 min, the absorbance was measured at 540 nm using a Multiskan Ascent microplate reader. Each plate contained the essential oil, negative control (0.1% DMSO) and blank. Cytotoxicity is expressed as the percentage of inhibition against various concentrations and concentration of oil inhibiting cell growth by 50% (IC_{50}). All tests and analyses were run in triplicate.

Statistical analysis: All values are expressed as Mean±standard deviation. The MIC data for each microorganism were analyzed using One-way Analysis of Variance (ANOVA). The p-value <0.05 was considered as significant. The software SPSS Version 16 was used for the statistical analysis.

RESULTS AND DISCUSSION

Antibacterial assay: The antibacterial activity of the nutmeg essential oil was studied using the tetrazolium microplate assay and the results were shown in Table 1. This colorimetric assay represents an alternative approach to determine MIC economically and yields greater reproducible result. The application of tetrazolium salt in the assay as colorimetric indicator have enhanced the sensitivity and accuracy of MIC determination since the formazan derivatives produced by bacteria or fungi can be quantified (Masoko *et al*., 2007).

Essential oil exhibited antibacterial activity against the tested strains but in variable degree. The synergistic and/or antagonistic effects of essential oil might be taken into consideration for the variation of antibacterial effects observed. The variation in the degree of sensitivity of the bacterial strains towards the extract may be due to the intrinsic tolerance of the bacterial and the nature and combinations of phytochemical compounds present in the extract as reported by Nanasombat and Lohasupthawee (2005).

The results obtained from this studies support the antimicrobial activity in the previous reports by Dorman *et al*. (2000). The MIC values ranged from 0.031 to 1 mg mL^{-1}. The essential oil showed higher activity against *Shigella dysenteriae* with MIC value of 0.031 mg mL^{-1} followed by *Staphylococcus aureus*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus mirabilis* with MIC value of 1 mg mL^{-1}. *Escherichia coli* and *Pseudomonas aeruginosa* have been known to be multi-drug resistant and is very difficult to control by therapeutic means, respectively. The oil exhibited the lowest MIC values against *Klebsiella pneumonia*, *Salmonella typhi*, *Bacillus subtilis* and *Proteus vulgaris* with value above 1 mg mL^{-1}. The resistance *Bacillus subtilis* is the ability of the bacteria to form resting spores and are more resistant to environmental conditions than any other tested bacteria while *Salmonella typhi* is a multi-drug resistant bacterium. Nutmeg essential oil showed lower MIC value compared to chloramphenicol, a commercial antibiotic against *Shigella dysenteriae*. This indicates that the essential oil shows significant and potent antibacterial activity against *Shigella dysenteriae*. Control treatment (DMSO) did not show an inhibitory effect on any of the tested bacteria. Sensitivity of the microorgamsms against amoxycillin, gentamicin, chloramphenicol and vancomycin was presented in Table 1.

The constituents present in nutmeg essential oil such as p-cymene, α-pinene, β-pinene, limonene, α-terpinene, α-terpinolene, caryophyllene oxide and camphene reported by Piaru *et al*. (2011) have also been studied for their antimicrobial activity (Sokmen *et al*., 2004). Based on a report, pinene-type monoterpene hydrocarbons

Table 1: Minimum inhibitory concentration (mg mL^{-1}) of nutmeg essential oil and antibiotics

Microorganism	Nutmeg	Amoxycillin	Gentamicin	Chloramphenicol	Vancomycin
Enterobacter aerogenes	1	-	0.062	0.015	-
Escherichia coli	1	-	0.015	0.015	-
Klebsiella pneumonia	>1	-	0.015	0.015	-
Proteus mirabilis	1	-	0.015	0.031	-
Proteus vulgaris	>1	-	0.500	0.5	-
Pseudomonas aeruginosa	1	-	0.015	-	-
Salmonella typhi	>1	0.062	0.015	0.062	-
Shigella dysenteriae	0.031	-	0.015	0.1	-
Staphylococcus aureus	1	0.1	-	-	0.015
Bacillus subtilis	>1	0.1	-	-	0.015

All the samples were run in triplicate (n = 3), -: Not tested

Table 2: *In vitro* cytotoxic activity of nutmeg essential oil against Vero cell line

Concentration (μg mL^{-1})	Percentage of inhibition±SD
100	74.54±2.9*
50	75.57±0.8
25	55.60±2.3
12.5	28.97±0.8
6.25	16.09±1.1*

Values are Mean±SD, the sample was run in triplicate (n = 3), *p<0.05, evaluated by one-way analysis of variance (ANOVA)

(α-pinene and β-pinene) had slight activity against number of microorganisms (Dorman *et al*., 2000).

Gram-negative *Pseudomonas aeruginosa* is known to have a high level of intrinsic resistance against all known antimicrobials and antibiotics, due to a very restrictive outer membrane barrier which is highly resistant even to synthetic drugs (Al-Howiriny, 2002). However, nutmeg essential oil was able to inhibit growth of this bacterium. In most literature, Gram-positive organisms appear to be more sensitive than Gram negative to essential oil. However, according to Seyyednejad *et al.* (2008) gram-positive bacteria have been found to be less or equally sensitive to gram-negative bacteria. The studies are sound scientifically since our studies showed the same pattern of sensitivity of nutmeg oil against gram positive bacteria.

Cytotoxicity assay: Essential oil of nutmeg was evaluated *in vitro* for its cytotoxicity activity against Vero cells using MTT assay. The results of cytotoxicity evaluation of the essential oil are shown in Table 2. The results showed that nutmeg essential oil possessed very low cytotoxicity against Vero cell line with IC_{50} at 24.83 μg mL^{-1}. Even though in the presence of myristicin and elemicin (Pooja *et al*., 2012), is often related to intoxication of nutmeg while safrole has been studied to show carcinogenic effects (Zhou *et al*., 2007), the lower cytotoxicity effect of nutmeg oil against the Vero cell line (normal cell line) maybe due to the synergistic effect of presence of other compounds in the extract. The interaction of the compounds would have inhibited the cytotoxic effects of myristicin, elemicin and safrole against the normal cell line. This suggests the antibacterial activity is not due to the cytotoxic effects of the nutmeg essential oil.

CONCLUSION

Summarizing these results, it can be concluded that nutmeg essential oil exhibited broad spectrum of antibacterial activity against the tested microorganisms with low cytotoxic effects against normal cell line. Thus, the essential oil can be further studied on its action of mechanism and fractionation will be done to determine the strength of its antibacterial activity.

ACKNOWLEDGMENT

This project was funded by Universiti Sains Malaysia Research University (RU) Grant (1001/PFARMASI/813021). The authors report no conflict of interest.

REFERENCES

APHA, 1992. Compendium of Methods for the Microbiological Examination of Foods. 3rd Edn., American Public Health Association, Washington, DC., USA., ISBN-13: 9780875531731, Pages: 1219.

Al-Howiriny, T.A., 2002. Chemical composition and antimicrobial activity of essential oil of *Salvia verbenaca*. Biotechnology, 1: 45-48.

Bakkali, F., S. Averbeck, D. Averbeck and M. Idaomar, 2008. Biological effects of essential oils: A review. Food Chem. Toxicol., 46: 446-475.

Burkill, I.H., 1966. A Dictionary of the Economic Products of Malay Peninsular. Ministry of Agriculture, Kuala Lumpur, Malaysia, pp: 1547-1556.

Dorman, H.J.D., A.C. Figueiredo, J.G. Barroso and S.G. Deans, 2000. *In vitro* evaluation of antioxidant activity of essential oils and their components. Flavour Fragrance J., 15: 12-16.

Eloff, J.N., 1998. A sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extracts for bacteria. Planta Med., 64: 711-713.

Lai, C.C., F.M. Huang, H.W. Yang, Y. Chan, M.S. Huang, M.Y. Chou, Y.C. Chang, 2001. Antimicrobial activity of four root canal sealers against endodontic pathogens. Clin. Oral Invest., 5: 236-239.

Lis-Balchin, M. and S.G. Deans, 1997. Bioactivity of selected plant essential oils against *Listeria monocytogenes*. J. Applied Bacteriol., 82: 759-762.

Masoko, P., J. Picard and J.N. Eloff, 2007. The antifungal activity of twenty-four Southern African *Combretum* species (Combretaceae). S. Afr. J. Bot., 73: 173-183.

Mbaveng, A.T., B. Ngameni, V. Kuete, I.K. Simo and P. Ambassa *et al*., 2008. Antimicrobial activity of the crude extracts and five flavonoids from the twigs of *Dorstenia barteri* (Moraceae). J. Ethnopharmacol., 116: 483-489.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Method, 65: 55-63.

Nadkarni, K.M., 1988. Myristica Fragrance. In: Indian Materia Medica, Nadkarni, A.K. (Ed.). 3rd Edn., Bombay Popular Parkashan, Bombay, pp: 830-834.

Nanasombat, S. and P. Lohasupthawee, 2005. Antibacterial activity of crude ethanolic extracts and essential oils of spices against *Salmonellae* and other *Enterobacteria*. KMITL Sci. Technol. J., 5: 527-538.

Ozaki, Y., S. Soedigdo, Y.R. Wattimena, A.G. Suganda, 1989. Antiinflanımatory effect of mace, aril of *Myristica fragrans* Houtt. and its active principles. Jpn. J. Pharmacol., 49: 155-163.

Piaru, S.P., R. Mahmud, A.M.S. Abdul Majid, S. Ismail, C.N. Man, 2011. Chemical composition, antioxidant and cytotoxicity activities of the essential oils of *Myristica fragrans* and *Morinda citrifolia*. J. Sci. Food Agric., 92: 593-597.

Pooja, V., H. Sanwal, S. Bhatnagar and A.K. Srivastava, 2012. Targeting virulence: Novel effect of *Myristica fragrans* on melanisation and conidiation of *Aspergillus niger*. Am. J. Drug Discovery Dev., 2: 32-39.

Seyyednejad, S.M., S. Maleki, N. Mirzaei Danıabi and H. Motanıedi, 2008. Antibacterial activity of *Prunus mahaleb* and Parsley (*Petroselinum crispum*) against some pathogen. Asian. J. Biol. Sci., 1: 51-55.

Singh, G., P. Marimuthu, C.S. de Heluani and C. Catalan, 2005. Antimicrobial and antioxidant potentials of essential oil and acetone extract of *Myristica fragrans* Houtt. J. Food Sci., 70: M141-M148.

Sokmen, A., M. Gulluce, H.A. Akpulat, D. Daferera, B. Tepe and M. Polissiou, 2004. The *in vitro* antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic *Thymus spathulifolius*. Food Contry, 15: 627-634.

Zhou, G.D., B. Moorthy, J. Bi, K.C. Donnelly, K. Randerath, 2007. DNA adducts from alkoxyallylbenzene herb and spice constituents in cultured human (HepG2) cells. Environ. Mol. Mutagen., 48: 715-721.