Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Antioxidant and antiangiogenic activities of the essential oils of *Myristica fragrans* and *Morinda citrifolia*

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ARTICLE INFO

Article history:
Received 14 September 2011
Received in revised form 15 January 2012
Accepted 15 March 2012
Available online 20 April 2012

Keywords:
Essential oil
Antiangiogenic
Antioxidant
Myristica fragrans
Morinda citrifolia

ABSTRACT

Objective: Toinvestigate the anti-angiogenic activity and antioxidant properties of *Myristica fragrans (M. fragrans)* (nutmeg) and *Morinda citrifolia* (*M. citrifolia*)(mengkudu) oils. **Methods:** The nutmeg and megkudu essential oils were obtained by steam distillation. The antioxidant activities of both essential oils were determined by beta-carotene/linoleic acid bleaching assay and reducing power while the anti-angiogenic activity was investigated using rat aortic ring assay using various concentrations. **Results:** The results showed that nutmeg oil has higher antioxidant activity than mengkudu oil. The nutmeg oil effectively inhibited the oxidation of linoleic acid with (88.68±0.1)% while the inhibition percentage of oxidation of linoleic acid of the mengkudu oil is (69.44±0.4)%. The nutmeg oil and mengkudu oil showed reducing power with an EC_{50} value of 181.4 μg/mL and 3 043.0 μg/mL, respectively. The antiangiogenic activity of nutmeg oil showed significant antiangiogenic activity with IC_{50} of 77.64 μg/mL comparing to mengkudu oil which exhibits IC_{50} of 109.30 μg/mL. **Conclusion:** Bioactive compound(s) will be isolated from the nutmeg essential oil to be developed as antiangiogenic drugs.

1. Introduction

Essential oils are volatile aromatic oily liquids which are identified by a strong smell and are produced by aromatic plants as secondary metabolites. Essential oil can be isolated using a number of isolation methods. The aromatic plants and spices are commonly used in phytotherapy and mostly related to various activities of their essential oils, such as antimicrobial, antioxidant, antifungal spasmolytic, carminative, hepatoprotective, antiviral and anticarcinogenic activities[1].

The increased discoveries in herbs and spices as sources of natural antioxidants have initiated researchers to look for natural antioxidants with low cytotoxicity[2]. The free radicals which cause oxidative stress are believed to play an essential role in human health. They are produced in body system in the form of reactive oxygen species (ROS) which can be obtained from the environment. These free radicals cause harm to the components of the cell resulting in cellular and metabolic disruption such as cardiovascular diseases, inflammation and cancer[3].

Nutmeg is the seed of *Myristica fragrans (M. fragrans)* Houttuyn, a medium-sized tree cultivated in tropical regions and native to the Maluku Province of Indonesia. The essential oil of nutmeg was able to block lipid peroxidation in chicken tissue homogenates and egg yolk fat and showed hepatoprotective activity against liver damage[4]. Pepper and nutmeg are one of the most widely used spices in preparation of ayurvedic drugs[5]. Nutmeg is a widely used spice and flavouring ingredient in food products, with possible health beneficial effects, such as anti-inflammatory and antimicrobial activities[6,7].

Morinda citrifolia (*M. citrifolia*) L. (Rubiaceae), known as "Noni" or Indian mulberry, "mengkudu" in Malaysia,

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Foundation project: This project was funded by Universiti Sains Malaysia Research University (RU) Grant (1001/PFARMASI/813021).

"painkiller bush" in the Caribbean, or "cheese fruit" in Australia is a shrub that grows in tropical Asia and Polynesia[8]. *M. citrifolia* has been used traditionally for a broad range of therapeutic uses such as anti-microbial, anti-cancer, anti-inflammatory, antioxidant agents since decades[9]. About 51 volatile compounds have been identified in the ripe fruit[10], including organic acids (mainly octanoic and hexanoic acids), alcohols (3-methyl-3-buten-1-ol), esters (methyl octanoate, methyl decanoate), ketones (2-heptanone), and lactones [(E)-6-dodeceno-glactone][11].

To the best of the author's knowledge, although a detailed investigation of mengkudu and nutmeg species was done, studies on the volatile oils of both species were less reported. No studies were conducted on the essential oil of fruits of mengkudu and only studies on the essential oil of the mengkudu leaves were done to date. Therefore, the present work is to determine the anti-angiogenic activity of nutmeg and mengkudu oils obtained from the fruit parts by rat aortic ring assay, to evaluate their antioxidant properties by beta-carotene/linoleic acid bleaching assay and reducing power.

2. Materials and methods

2.1. Plant material

The fresh fruits of *M. fragrans* and *M. citrifolia* were obtained from Balik Pulau, Penang, Malaysia.

2.2. 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 11 round bottom flask fitted with a condenser. The extracted essential oils were dried over anhydrous sodium sulphate and after filtration, stored at 4 ℃ until tested and analyzed. The samples were weighed and the essential oil concentration (%) in the plant tissue samples was calculated:

$$\text{Essential oil concentration (\%)} = \frac{\text{amount of essential oil recovered (g)}}{\text{amount of crop biomass distilled (g)}} \times 100$$

2.3. Antioxidant activity

2.3.1. β-Carotene/linoleic acid bleaching assay

In this assay, antioxidant activity was determined by measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by Miraliakbari and Shahidi (2008)[12] was used with slight modifications. A stock solution of β-carotene and linoleic acid was prepared with 0.5 mg of β-carotene in 1 mL chloroform, 25 μL of linoleic acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 mL of aerated distilled water was then added to the residue. The samples (2 g/L) were dissolved in DMSO and 350 μL of each sample solution was added to 2.5 mL of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 ℃ for 1 h, together with two blanks, one contained the antioxidant BHT as a positive control and the other contained the same volume of DMSO instead of the extracts. The test tube with BHT maintained its yellow colour during the incubation period. The absorbance was measured at 470 nm on an ultraviolet spectrophotometer. Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

I% = (A β-carotene after 1 h assay/Ainitial β-carotene) × 100

Where A β-carotene after 1 h assay is the absorbance of β-carotene after 1 h assay remaining in the samples and Ainitial β-carotene is the absorbance of β-carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

2.3.2. Estimation of reducing power (RP)

The reducing power was determined according to the method of Oyaizu (1986)[13]. Each extract (31.25 - 500 μg/mL) in methanol (1 mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium, and the mixture was incubated at 50 ℃ for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added, the mixture was centrifuged at 200 g for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of de-ionized water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm against blank. A higher absorbance indicates a higher reducing power. EC_{50} value (μg/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. Ascorbic acid was used as a positive control.

2.4. Antiangiogenic assay

2.4.1. Experimental animals

Four (12-18 weeks old) Sprague Dawley male rats were obtained from the animal unit facility of University Science Malaysia. The animals were kept for one week in the transit animal unit to acclimatize with the new environment. The rats were kept in ventilated cages at 12 h light cycle with continuous supply of food and water. After euthanizing the animals by CO_2, a midline incision was made into the abdominal and thoracic cavities and the thoracic aortas

were collected. The experiment was performed according to guidelines of USM Animal Ethics Committee and had their approval, reference USM/ Animal Ethics Approval/ 2011/(66) (301)

2.4.2. Antiangiogenic effects on rat aortic rings

Antiangiogenic effect of the essential oils was investigated ex vivo on rat aortic rings according to the protocol reported by Brown *et al*[14]. Briefly, the cleansed thoracic aortas were cross sectioned into thin rings of about one millimeter thickness. One ring was placed in the center of each well of 48-well plate containing 500 μL of M199 basal medium supplied with fibrinogen, aprotinin and L-glutamine at 3 mg/mL, 5 μg/mL and 1% wt/v, respectively. Then, 10 μL thrombin was added to each well. After 90 min incubation at 37 ℃, another 500 μL M199 medium supplied with; fetal bovine serum (FBS) at 20% v/v, L-glutamine at 2 mM, aminocaproic acid at 1 mg/mL, amphotericin B at 2.5 μg/mL and gentamicin at 60 μg/mL were added on top of the solidified bottom layer. Essential oils (6.25-100 μg/mL range) were also included in the top layer medium. The top layer medium was replaced with fresh one containing the extracts after 4 d incubation at 37 ℃ in 5% CO_2. The magnitude of the growth of sprouting microvessels was quantified on day 5 according Nicosia *et al*[15] using a Leica inverted phase-contrast microscope (Leica, Germany) equipped with the quick imaging system to take photos. Briefly, the distance of growth of at least thirty points was measured for each ring. The results were presented as percent of inhibition by 50% (n = 5). Suramine was used as a positive control.

2.5. Statistical analysis

The statistical analysis was performed by one-way ANOVA in GraphPad Prism Version 5 Software. The results were expressed as mean±SEM and mean to show differences between two samples. The differences are considered significant when $P < 0.01$.

3. Results

3.1. Extraction yield

The steam distillation of nutmeg and mengkudu yields 0.12% (w/w) and 0.06% (w/w) on wet weight basis, respectively.

3.2. Antioxidant assay

3.2.1. β-Carotene/linoleic acid bleaching assay

At 1 mg/mL concentration, the oxidation of linoleic acid was effectively inhibited by nutmeg essential oil (88.68±0.1)%, while the mengkudu essential oil exhibited (69.44±0.4)% of inhibition. The positive control BHT showed the highest activity at (93.20±0.1)%. Nutmeg essential oil showed significant inhibiting activity (P<0.01) against control.

3.2.2. Reducing power

Reducing power of the essential oils is presented in Figure 2. As can be seen from the table nutmeg oil and mengkudu oil showed antioxidant activity with an EC_{50} value of 181.4 μg/mL and 3 043.0 μg/mL, respectively. Ascorbic acid as a positive control showed EC_{50} value of 36.1 μg/mL. The nutmeg essential oil showed significant antioxidant activity (P<0.01) against control.

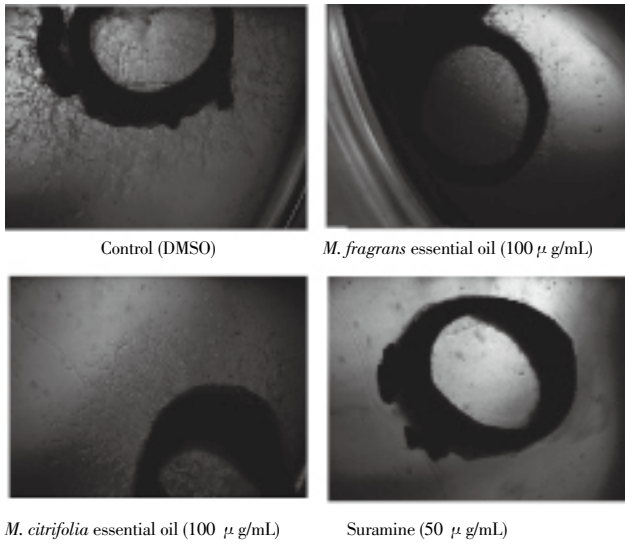

Figure 1. Effect of essential oil of *M. fragrans* and *M. citrifolia* on sprouting vessel of rat aortic ring. Vascular vessels sprouting from each ring was photographed at a 100× magnification using a Leica inverted phase-contrast microscope equipped with the quick imaging system. Representative photos were indicated from six replicates.

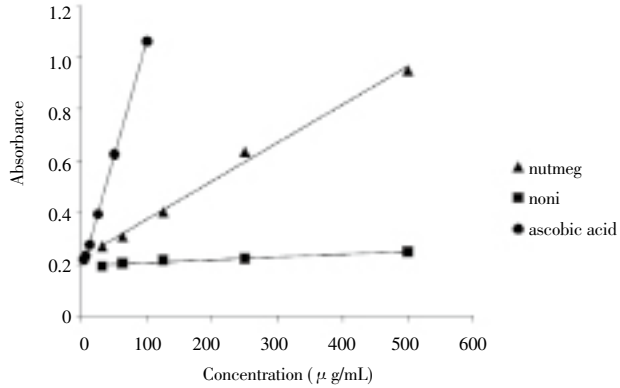

Figure 2. Reducing power essential oil of *M. fragrans* and *M. citrifolia*.

3.3. Antiangiogenic activity

The inhibition of the growing blood vessels from the rat

aortic ring by the essential oils was determined as IC_{50} in a dose-dependent manner and shown in Figure 1. The antiangiogenic activity of nutmeg oil was determined at IC_{50} of 77.64 μg/mL(Y=1.938 2X-100.5, R^2=0.946) while mengkudu oil exhibited IC_{50} of 109.3 μg/mL(Y=1.015 X-60.885, R^2=0.8925). The suramine, a commercial drug showed IC_{50} of 16.6 μg/mL.

4. Discussion

In β-carotene/linoleic acid bleaching assay, β-carotene undergoes fast discoloration in the absence of an antioxidant. This is due to the generation of free radicals by coupled oxidation of β-carotene and linoleic acid. The free radicals of linoleic acid formed upon the extraction of a hydrogen atom from one of its diallylic methylene group attacks the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidized and broken down. Subsequently, the system loses its colour (orange color), which is monitored spectrophotometrically. The presence of an antioxidant can deter the extent of β-carotene destruction by "neutralizing" the linoleate free radical formed within the system[16].

According to Gurdip Singh 2005[17], the nutmeg essential oil is reported to consisting major components such as sabinene, terpinen-4-ol, safrole, α-pinene, β-phellandrene, γ-terpinene, β-pinene, α-terpinene, terpinolene and α-thujene.

Hence the inhibitory effect of the nutmeg oil in the linoleic acid system might be due to the presence of above components[18].

The other way of defense mechanism in preventing body against the harmful effects of free radicals is by reducing these radicals by the antioxidant substances. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Reducing powers of nutmeg essential oil was strong and increased with increasing concentration, and it showed good reducing ability in comparison with ascorbic acid. The reducing powers of nutmeg essential oil might be due to their hydrogen-donating ability. The components present in the essential oil could act as good reductants, which would lead to stabilization and termination of free radical chain reactions.

Phenolic groups are reported to playing a vital role in antioxidant activity. Hence, the presence of phenolic compounds such as methyl eugenol in essential oil and anisole (p-pentyl), eugenol, methyl eugenol, transisoeugenol, trans-methyl eugenol in mace extract are responsible for the antioxidative activity showed in all antioxidant assays studied suggesting the significant antioxidant results of the nutmeg oil from this studies[19,20].

The antiangiogenic properties in nutmeg and mengkudu oils were investigated using *ex vivo* rat aortic assay. The rat aortic assay is regarded to mimic the *in vivo* environment because it involves nonendothelial cells besides the endothelial cells and the endothelial cells themselves are not preselected by sub-culturing and thus are not in the proliferation state at the time of explanation and more representative of the real life[21]. The assay is based on the capability of the aortic wall to produce neo-vessels in bio-matrix gels after mechanical wound or angiogenic factor stimulation. Angiogenesis is considered as a critical process in various physiological and pathological mechanisms such as wound healing, growth and metastasis of solid tumors and chronic inflammatory diseases[22].

The antiangiogenic activity of the nutmeg essential oil is more significant than that of mengkudu essential oil maybe due to the presence of some potential antiangiogenic compounds such as myristicin, limonene, eugenol and terpinen-4-ol. Myristicin have been reported to induce cytotoxicity in human neuroblastoma SK-N-SH cells by mean of apoptotic mechanism[23]. D-limonene exhibits as a potential chemopreventive agent in hepatocellular carcinoma models[24-27]. Apart from that, studies on terpinen-4-ol showed that it can induce apoptosis in human melanoma cells[28]. Eugenol; one of the components in the nutmeg essential oil also has been studied to show apoptosis-inducing effects[29].

Formation of reactive oxygen species (ROS) have been reported as the important mediators for angiogenesis. Hydrogen peroxide, one of the vital endogenous ROS involves in stimulating angiogenesis *in vitro*[30,31]. Thus, antioxidant activities can be correlated with anti-angiogenic activity. From the studies conducted, nutmeg oil showed significant activity in both antioxidant activities and anti-angiogenesis assay compared to mengkudu oil which showed less significant activities in both assays.

In conclusion, essential oils generally showed strong antioxidant properties, which are useful in daily life as preventive and treatment agents from various diseases. In the case of antiangiogenic activity, the nutmeg oil showed potent antioxidant and antiangiogenic activities. Thus, further studies concerning the antiangiogenic activities of fractions of nutmeg essential oil and isolate the compound(s) which is responsible to the antiangiogenic activity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This project was funded by Universiti Sains Malaysia Research University (RU) Grant (1001/PFARMASI/813021).

One of the authors Suthagar Pillai Piaru would like to thank Norshirin Idris, Nahdzatul Syima and Shanmugapriya Perumal for their contribution to the research.

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