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Research Article

Inhibition of rat liver mitochondrial membrane permeability transition and lipid peroxidation by seeds extracts of *Myristica fragrans* Houtt.

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ABSTRACT

Myristica fragrans Houtt. seed commonly known as "nutmeg" is used in ethnomedical of practice as phytotherapy in the management of degenerative diseases. This study was designed to investigate the effects aqueous and dichloromethane extracts of *M. fragrans* seeds on calcium ion-induced rat liver mitochondrial membrane permeability transition (MMPT) and lipid peroxidation. Results indicated that 50, 150 and 300 µg/mL aqueous extract of *M. fragrans* seeds had a significantly ($P<0.05$) higher inhibition property of Ca^{2+}-induced MMPT than dichloromethane extract in a concentration-dependent manner. Aqueous extract inhibited Ca^{2+}-induced MMPT by 60.18±2.51% and 75±0.01% while dichloromethane inhibited Ca^{2+}-induced MMPT by 51.85±1.27% and 72.68±0.01% as minimal and maximal effects, respectively. Furthermore, 1 mg/mL aqueous extract of *M. fragrans* seed (92.19%) exhibited a significantly ($P<0.01$) higher inhibition of Fe^{2+}-H_2O_2 EDTA-induced mitochondrial membrane lipid peroxidation than 1 mg/mL dichloromethane extract of *M. fragrans* seed (15.95%). Our results indicated that the aqueous extract of *M. fragrans* seeds exhibited a higher mitochondrial membrane protection effect against free radical elicited damage than dichloromethane extract of *M. fragrans* seeds. In addition, these findings provide scientific insight for the ethnomedical use of *M. fragrans* seed decoction as phytotherapy in the management of degenerative diseases.

Keywords: *Myrista fragrans*, seed, mitochondria, extract, lipid peroxidation

INTRODUCTION

Plants are primary sources of medicinal compounds used as therapy for a number of debilitating diseases that plagues humanity. Sundry parts of the plant have been proven scientifically as rich sources of bioactive compounds that could be channeled towards pharmaceutical drug development (Joseph and Priya, 2011; Cragg and Newman, 2013). In addition, selected plant seeds possess nutritive, culinary or medicinal values which make it use essential for optimal functioning of the body systems (Oedemelam, 2005).

Myristica fragrans Houtt. seeds commonly called "nutmeg" belongs to the family of myristiceae. It is a dried kernel seed located inside the fruit (Barceloux, 2008; Bamidele *et al.,* 2011). *M. fragrans* tree is native to the Moluccas-East Indonesia also known as Spice Islands and it is cultivated throughout Malaysia. The genus *Myristica* is distributed from India and South-East Asia to North Australia and the Pacific Islands (Tripathi *et al.,* 2016). *M. fragrans* seed is a choice spice known for its aromatic and curative properties (Tripathi *et al.,* 2016). Furthermore, *M. fragrans* seed is used traditionally to improve blood circulation, stimulate cardiovascular system and aids food digestion. It is also used for the treatment of rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhea, flatulence, anxiety, aphrodisiac, and as an abortifacient (Barceloux, 2008).

The essential oil content in *M. fragrans* seeds has been reported to range from 3.9 to 16.5% (Maya *et al.,* 2004). Furthermore, *M. fragrans* seed kernel consists 30 - 55% oil

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and 45 - 60% solid matter, while volatile oil from *M. fragrans* seeds accounts for 5-15%, and fixed oil accounts for 24-40% (Leela *et al.,* 2008). Previous findings from our laboratory had shown that the aqueous extract of *M. fragrans* seeds had higher phenolic (0.10 mg gallic acid equivalent (GAE g^{-1}) and flavonoid (1.10 mg quercetin equivalent (QE) g^{-1}) contents than dichloromethane extracts of *M. fragrans* seeds' phenolic (0.03 mg GAE g^{-1}) and flavonoid (0.10 QE mg g^{-1}) contents. Aqueous and dichloromethane extracts of *M. fragrans* seeds contained 6 and 18 bioactive compounds respectively (Asika *et al.,* 2016). Some of these bioactive compounds are natural free radical scavengers that act to terminate the formation of reactive species or break the deleterious chain reactions generated by reactive oxygen species. Reactive oxygen species are noxious chemicals that could offset the chemical balance of the body system through abstraction of electron(s), causing alteration of macromolecular structures which could induce mitochondrial membrane permeability transition (MMPT) and eventually diseases may ensue (Katerina and Gonzalo, 2016).

MMPT is an important step in the instigation of apoptotic and necrotic cascades which could proceed to cell death. MMPT has been characterized as one of the central molecular event in many disease conditions (Wong, 2011; Izzo *et al.,* 2016). Overload of calcium ion (Ca^{2+}) and reactive oxygen species-induced oxidative stress within the mitochondria are known to induce MMPT. This results into massive swelling and bioenergetics depolarization of the mitochondria triggering apoptotic cascades and ultimately cell death (Halestrap and Richardson, 2015). Several biological agents that target the mitochondria-mediated pathways leading to apoptosis or necrosis are currently being developed (Bachurin, 2016). In addition, plant extracts that could confer mitochondria-protection against oxidative stress-induced MMPT would serve as an important source of therapeutic agent(s) against mitochondrial-mediated diseases (Kavita and Setty, 2012; Simona *et al.,* 2015). Therefore, this study was designed to investigate the inhibitory effects of *M. fragrans* seeds extracts on calcium ion-induced mitochondrial membrane permeability transition and mitochondrial membrane lipid peroxidation.

MATERIALS AND METHODS

Plant collection

Seeds of *M. fragrans* were purchased from a local market in Lagos State, Nigeria and identified in the Department of Basic Sciences, Babcock University, Ilisan Remo, Ogun State.

Plant processing and extraction

Aqueous extraction: *M. fragrans* seeds were oven-dried using SANFA DHG-9202 thermostatic hot air oven (Gulfex Medical and Scientific, England) at 35°C for 7 d. Dried *M. fragrans* seeds were crushed and ground with an electrical blender. Ground *M. fragrans* seeds (20g) were dissolved in 800 mL of distilled water (1:8 v/v). The resultant solution was placed on a heater (Stuart Heater) at 80°C, stirred intermittently for 30 min and allowed cool. This was followed by filtration of the solution using Whatman No.1 filter paper. The obtained filtrate was subsequently stored at 4°C in a refrigerator until further use.

Dichloromethane extraction: Ground *M. fragrans* seeds (50g) were refluxed in 400 mL dichloromethane using a Soxhlet apparatus for 8 h to obtain an extract. The obtained extract was subsequently concentrated in a rotatory evaporator (Buchi Rotavapor RE; Switzerland) and further dried in a SANFA DHG-9202 thermostatic hot air oven (Gulfex Medical and Scientific, England) at 45°C and subsequently stored at 4°C in a refrigerator until further use.

Animals: Five male albino rats (Wistar strain) weighing between 120–250g were obtained from the Preclinical Animal House, Physiology Department, University of Ibadan, Ibadan, Nigeria. The animals were acclimatized for 14 d in the Animal Facility, Benjamin S. Carson, School of Medicine. The animals were given water and rat chow *ad libitum* and were kept under standard conditions of temperature and 12-hour dark/light cycle in accordance with the policy of humane use and care for laboratory animal guidelines (NIH, 2010).

Isolation of rat liver mitochondrial membrane fraction: Mitochondrial membrane fraction from rat liver was isolated by conventional differential centrifugation technique in a buffer solution containing 210 mM mannitol, 70 mM sucrose, 5 mM 2-(4-[2-hydroxyethyl] piperazin-1-yl) ethane sulfonic acid (HEPES) at pH 7.4 and 1 mM ethylene glycol tetraacetic acid (EGTA). In the final wash solution, EGTA was omitted (Hogeboom *et al.,* 1948). Mitochondrial protein content was determined using Folin-Ciocateau method using bovine serum albumin as standard protein (Lowry *et al.,* 1951).

Mitochondrial membrane permeability transition assay: Mitochondrial membrane permeability transition assay was carried out according to the method of Lapidus and Sokolove (1993). Change in absorbance of mitochondrial fraction was monitored at 540 nm in a double beam UV-visible spectrophotometer (T80 model, PG instrument) every 30s for 12 min. Mitochondrial fraction (0.4 mg/ml) were suspended in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-potassium hydroxide (pH 7.4),

0.8 µM rotenone and 5 mM succinate. Preloaded mitochondrial fraction (0.4 mg/ml) were incubated in Eppendorf tubes in the presence 50, 150 and 300 µg/µl aqueous or dichloromethane extracts of *M. fragrans* seeds respectively. Mitochondrial membrane permeability transition was triggered using Ca^{2+} while spermine served as a standard inhibitor of mitochondrial membrane permeability transition. Percentage inhibition of mitochondrial membrane permeability transition was calculated using the formula below:

$$\text{Percentage inhibition} = \frac{\Delta\text{Absorbances (control)} - \Delta\text{absorbance(test)}}{\Delta\text{Absorbances (control)}} \times 100$$

Where, ΔAbsorbance (control) indicates change in absorbance of control at 540 nm

ΔAbsorbance (test) indicates change in absorbance of test extract at 540 nm

Inhibition of mitochondrial lipid peroxidation assay

Mitochondrial lipid peroxidation was induced using Fe^{2+}-ethylene diamine tetra acetic acid (EDTA) system to form thiobarbituric acid reactive substances (TBARS). The concentration of TBARS formed was determined by the method of Ohkawa *et al.* (1979). Briefly, the reaction mixture contained 0.1 ml mitochondrial fraction in Mannitol-Sucrose-HEPES buffer (pH 7.4), 0.1 ml EDTA, 0.1 ml $FeSO_4$, 0.1 ml H_2O_2, and 0.5 ml of 0.25, 0.5 and 1.0 mg/ml of aqueous or dichloromethane extract in a final volume of 1 ml. The reaction mixture was incubated for 1 h at 37°C. After the incubation, 0.5 ml of the reaction mixture was treated with 0.2 ml sodium dodecylsulfate, 1.5 ml thiobarbituric acid, and 1.5 ml acetic acid. The total volume was made up to 4 ml by distilled water and kept in a water bath at 95-100°C for 1 h. The reaction mixture (2 ml) was then mixed with 3 ml n-butanol, vortexed and centrifuged at 3000 rpm for 10 min. The organic layer was removed and its absorbance was measured at 532 nm in a UV- visible spectrophotometer. Test tubes without Fenton's reaction mixture or *M. fragrans* extracts containing rat mitochondria were included as blank, while those without *M. fragrans* extracts alone were taken as control. Inhibition of lipid peroxidation was determined by comparing the mean absorbance of the *M. fragrans* extracts treated tubes with that of Fenton reaction alone.

Statistical analysis

Statistical analysis was carried out with the aid of SPSS for Windows; SPSS Inc., Chicago, Standard version 17.0 to determine the difference between mean using analysis of variance. P <0.01 and 0.05 were considered significant. Graphical analysis and presentation were performed using Microsoft Excel, version 2010. All studies were conducted in triplicate and reported as a mean ± standard error of the mean.

RESULTS AND DISCUSSION

Data presented in Figure 1 and 2 showed that in the absence of Ca^{2+} (Δ 540 nm = -0.005) no observable inductions of MMPT were recorded. However, mitochondrial fraction incubated in the presence of Ca^{2+} alone (Δ 540 nm = -0.216) showed a large amplitude in the induction of MMPT. However, the preloaded mitochondrial fraction with Ca^{2+} incubated in the presence of spermine (Δ 540 nm = -0.039) showed a substantial decline in Ca^{2+}-induced MMPT. This suggested that the isolated mitochondrial fraction was respiring before

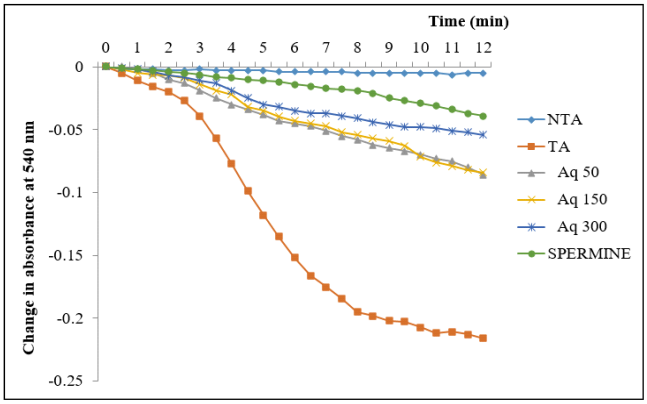

Figure 1: Inhibition of Ca^{2+}-induced MMPT byaqueous extract of *M. fragrans* seed at Δ540nm for 12 min energized by sodium succinate

NTA-Non-Triggering agent; TA- Triggering agent; Aq- aqueous extract of *M. fragrans* seed

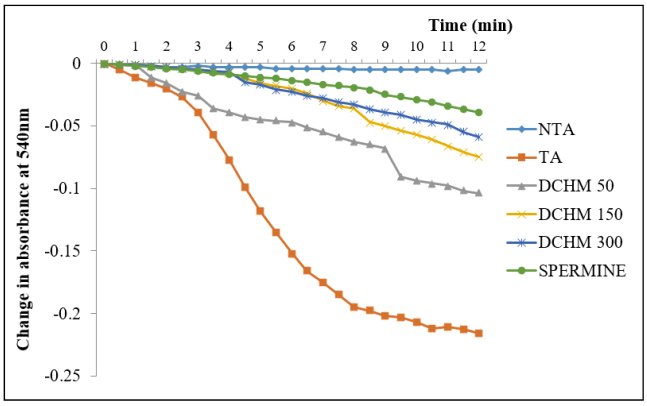

Figure 2: Inhibition of Ca^{2+}-induced MMPT by dichloromethane extract of *M. fragrans* seed at Δ540nm for 12 min energized by sodium succinate

NTA-Non-Triggering agent; TA- Triggering agent; DCHM-Dichloromethane extract of *M. fragrans* seed

the study was carried out. Previous work had shown that increase in the concentration of intracellular Ca^{2+} elicits the induction of rat liver MMPT while spermine which is a polyamine is known to inhibit Ca^{2+}-induced MMPT (Lapidus and Sokolove, 1993; Rasola and Bernadi, 2014).

Furthermore, mitochondrial membrane fraction pretreated with 50, 150 and 300 µg/µL aqueous or dichloromethane extracts of *M. fragrans* seeds significantly ($P<0.05$) inhibited Ca^{2+} induced MMPT in a concentration-dependent manner. Aqueous extract of *M. fragrans* seeds exhibited a significantly ($P<0.05$) minimal (60.18±2.51%; Δ540nm = -0.086) and maximal (75±0.01%; Δ540nm = -0.054) inhibitions of Ca^{2+}-induced MMPT at 50 and 300 µg/µl respectively when compared with dichloromethane extract of *M. fragrans* seeds with minimal (51.85±1.27%; Δ540nm = -0.104) and maximal (72.68±0.01%; Δ540nm = -0.059) inhibitions of Ca^{2+}-induced MMPT at 50 and 300 µg/µl, respectively. This indicates that aqueous extract of *M. fragrans* seeds exhibited a higher mitochondrial membrane protective effect against Ca^{2+}-induced MMPT than dichloromethane extract. Previous study had shown that Ca^{2+} overload causes a collapse of the inner mitochondrial membrane potential as a result of reactive oxygen species generation which could degenerate into oxidative stress (Halestrap and Richardson, 2015). However, evidence has shown that antioxidants are potent scavengers of reactive oxygen species (Katerina and Gonzalo, 2016). It has also been reported that the aqueous extract of *M. fragrans* seeds possesses higher phenolic and flavonoid contents than dichloromethane extract of *M. fragrans* seeds (Asika *et al.,* 2016). Phenol and flavonoid compounds are known scavengers of reactive oxygen species in the body system (Ghasemzadeh and Ghasemzadeh, 2011). Hence, the presence of higher phenols and flavonoids in aqueous extract of *M. fragrans* seeds than dichloromethane extract could account for the recorded higher mitochondrial membrane protective effect by the aqueous extract of seeds of *M. fragrans*.

Table 1: Percentage inhibition of rat liver mitochondrial lipid peroxidation by varying concentrations of aqueous and dichloromethane extracts of *M. fragrans* seeds

Concentration (mg/ml)	Percentage inhibition (n=3)	
	Aqueous extract of *M. fragrans* seed	**Dichloromethane extract of *M. fragrans* seed**
0.25	36.93*	10.43
0.5	44.72*	12.88
1	92.19*	15.95

***** indicates statistically significant at $P<0.01$ when compared with the equivalent concentration of dichloromethane extract of *M. fragrans* seeds.

Table 1 showed that aqueous or dichloromethane extracts of *M. fragrans* seeds significantly ($P<0.01$) inhibited Fe^{2+}-H_2O_2 EGTA-induced rat liver mitochondrial lipid peroxidation in a concentration-dependent manner. More so, aqueous extract of *M. fragrans* seeds at 0.25 and 1 mg/mL exhibited a significantly ($P<0.01$) higher percentage inhibition of mitochondrial membrane lipid peroxidation by 36.93% and 92.19% respectively than dichloromethane extract of *M. fragrans* seeds which inhibited mitochondrial lipid peroxidation by 10.43% and 15.95%, respectively. This further supports the previously observed protection of mitochondrial fraction by the aqueous extract of *M. fragrans* seeds against free radical elicited mitochondrial membrane damage. Previous work had shown that iron-mediated mitochondrial lipid peroxidation was associated with intense mitochondrial DNA damage derived from the direct damage by reactive oxygen species (Almeida *et al.,* 2006). Furthermore, it was reported that the aril part of *M. fragrans* had significant antioxidant activity due to its capacity to inhibit lipid peroxidation and superoxide radical scavenging activity in rat (Yadav and Bhatnagar, 2007).

Therefore, this study shows that the *M. fragrans* seed extracts contain inhibitory agent(s) against lipid peroxidation on Ca^{2+}-induced mitochondrial membrane permeability transition which is a prelude to induction of apoptotic cascade. In addition, the aqueous extract of *M. fragrans* seeds exhibited a higher percentage inhibition of Ca^{2+}-induced mitochondrial membrane permeability transition and lipid peroxidation. Hence, it is recommended that further study should be carried out on the aqueous extract of *M. fragrans* seeds to elucidate its anti-apoptotic property against several disorders.

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