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Full Length Research Paper

Differential Antioxidant Properties of Ethanol and Water Soluble Phytochemicals of False Nutmeg (*Monodora myristica*) Seeds

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In traditional medicine, several solvents like water, ethanol, limewater, coconut water and honey are used for extraction of phytochemicals. However, information on the solvent differential of these commonly used solvents in relation to their therapeutic effect is still poorly understood. Hence, the present study sought to evaluate the differential effect of water and ethanol which are commonly used for extraction in traditional medicine. The amount of some antioxidant agents such as phenols, flavonoids and vitamin C were determined in the ethanolic and aqueous extracts of the seed. In addition, the possible antioxidant mechanisms of the extracts were assessed by measuring their reducing property, iron (II) chelating ability and free radical scavenging properties. Furthermore, the ability of both extracts to inhibit lipid peroxidation and deoxyribose degradation was also measured. Generally, results showed that the phytochemical content of the ethanolic extract was significantly (P <0.05) higher than the aqueous. In a similar fashion, ethanolic extract exhibited stronger antioxidant potency than aqueous extract in all antioxidant indices determined. Taken together, ethanol exhibited a stronger solvent differential than water in the extraction of *Monodora myristica* seed. Hence, ethanol is better than water when full exploitation of the therapeutic potentials of *Monodora myristica* seed is intended.

Key words: Antioxidant, *Monodora myristica*, lipid peroxidation, deoxyribose degradation, ethanol, aqueous.

INTRODUCTION

The onset of degenerative pathologies has been linked to oxidative stress which results when the rate of production of free radicals exceeds the body's antioxidant capacity to mop them consequently causing havoc to critical biological macromolecules. Hence, research efforts have been tailored towards the discovery of plants with potent antioxidant properties that could be used as food or introduced into foods as remedy for degenerative diseases (Fattouch et al., 2007). Interestingly, the antioxidant properties of plants have been attributed to their secondary metabolites (phytochemicals) primarily synthesized to offer protective shield from attack by invaders. These phytochemicals, such as phenolics, and flavonoids have attracted a great deal of attention recently due to their role in disease prevention (Fattouch et al., 2007). For instance, phenolics play a major role in the defense mechanism of plants and contribute to the development of color, taste and palatability (Tarnai et al., 1994). Reports abound on the multiple biological effects of phenolics, flavonoids, including their antimicrobial activity (Payne et al., 1989; Ejechi et al., 1998; Angioni et al., 2004). However, the solvents for the extraction of these phytochemicals could affect the level of these bioactive ingredients commonly found in both edible and inedible parts of these plants (Wojdylo et al., 2007). The effects of solvent differential in the extraction of plants photochemical becomes more obvious when such plant is intended to be used for therapeutic purposes

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(Mulualem et al., 2011; Jianhui and Hui, 2011). Unfortunately, there is little or no awareness in this regard especially among traditional medical practitioners who habitually use solvents especially water and ethanol for extraction. Hence, there is a dire need to investigate the solvent differential of the two solvents as it affects the therapeutic potency of medicinal plants.

Meanwhile, African nutmeg (*M. myristica),* a perennial edible plant of the Annanacea family is a berry that grows wild in the evergreen forests of Africa (Burubai, 2008). It has a characteristic pleasant fragrance, slightly warm taste and used to flavor many kinds of baked goods, confections, puddings, meats, sausages, sauces, vegetables and beverages. The economic relevance of *M. myristica* are enormous as almost all its parts are economically beneficial with special emphasis on its seeds which are embedded in a white sweet-smelling pulp (Okigbo, 1977; Okafor, 1987) which are subjected to a series of unit operations such as fermentation, washing, drying and cracking (Ejiofor et al., 1998) to obtain the kernel. When ground to powder, the kernel (seed) is a popular condiment used to prepare pepper soup believed to act as a stimulant to relieve constipation and to control passive uterine hemorrhage in women immediately after childbirth (Okafor, 1987; Iwu et al., 1987; Udeala et al., 2000). *M. myristica* seed has also been reported to exhibit diuretic properties and could also be administered for quick relief from mild fever and headache (Iwu et al., 1987; Gill, 1992). Its pulp has been found to contain essential oil including pinene, camphene, and dipentene which are used as condiments and carminatives and to scent soaps and perfumes while its ointment has been used as a counter-irritant and in the treatment of rheumatism. Traditionally, *M. myristica* is used in the treatment of arthritis, cutaneous and subcutaneous parasitic infection, eye infection and diabetes mellitus (Shafran et al., 1977).

Despite the widespread usage of solvents for the extraction of phytochemicals in folkloric medicine, little is known about their effect on the amount of phytochemicals and antioxidant properties of *M. myristica*. Meanwhile, the amount of phytochemicals extracted is largely dependent on the type of solvent used for extraction. Hence, this study is geared towards unraveling the solvent differential of ethanol and water as it affects the antioxidant properties and phytochemical content of *M. myristica*. Our intention is to identify which of ethanol and water would guarantee a better extraction of phytochemicals in *M. myristica* seed which could be harnessed for the exploitation of its therapeutic potentials.

MATERIALS AND METHODS

Chemical reagents

Thiobarbituric (TBA), 2-deoxyribose sugar, DPPH (2, 2 - diphenyl-1- picrylhydrazyl) and 1,10 phenanthroline were obtained from Sigma (St. Louis, MO). All other chemical were obtained from standard chemical suppliers and were of analytical grade.

Plant material

Seeds of *M. myristica* were bought from the main market in Akure (Nigeria) and were identified at the crop soil and pest Management Department of the Federal University of Technology, Akure. The seeds were pulverized using Marlex blender and the powdered seed were stored in polythene bags and placed at room temperature until they were used.

Preparation of plant extracts

Five grams of powdered seeds were weighed in separate extraction bottles and one hundred milliliters of solvent (ethanol and water) was added to the bottle containing the powdered seeds and left for 24 h in the dark to allow for extraction. Thereafter, the solutions were filtered separately using a Whatman filter paper. The extracts were stored air tight in a refrigerator until required for use. This served as the stock solution for all determinations.

Animals

Male adult Wistar rats (200 to 250 g) from our own breeding colony were used. Animals were kept in separate animal cages, on a 12-h light:12-h dark cycle, at a room temperature of 22 to 24°C, and with free access to food and water. The animals were used according to standard guidelines of the Committee on Care and Use of Experimental Animal Resources.

Determination of total phenol content

The total phenol content of the aqueous and ethanolic extracts of seeds of *Monodora myristica* were determined by mixing (0 to 1.0 ml) of seed extract with equal volume of water according to the modified method of Singleton et al. (1999). 2.5 ml Folin-Ciocalteau's reagent and 2 ml of 7.5% sodium carbonate were subsequently added, and the absorbance was measured at 765 nm after incubating at 45°C for 40 min. The amount of phenols in seed extract was expressed as gallic acid equivalent (GAE).

Determination of total flavonoid content

The flavonoid content of *M. myristica* seed extract was determined using quercetin as a reference compound. Briefly, (0 to 500 µl) of stock solution of ethanolic seed extract was mixed with 50 µl of aluminium trichloride and potassium acetate according to the method of Meda et al. (2005). The absorption at 415 nm was read after 30 min at room temperature. Standard quercetin solution was

prepared from 0.01g quercetin dissolved in 20 ml of ethanol. All determinations were carried out in triplicate. The amount of flavonoids in the seed extract was expressed as quercetin equivalent (QE).

Vitamin C content

The level of vitamin C in aqueous and ethanolic extracts of *M. myristica* was determined colorimetrically as described by Jacques-Silva et al. (2001). Briefly, 1 ml H_2SO_4 65% (v/v) was added. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and CuSO4 (0.075 mg/ml), and the absorbance of the colored product was measured at 520 nm. The content of ascorbic acid is related per gram of dried sample.

Free radical scavenging ability

The free radical scavenging ability of the aqueous and ethanolic extracts seeds of *M. myristica* against DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radicals were evaluated according to Gyamfi et al. (1999). Briefly, 600 µl of either aqueous or ethanolic extract was mixed with 600 µl, 0.3 mM methanolic solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance was measured at 516 nm.

Reducing property

The reducing property was determined by accessing the ability of the aqueous and ethanolic extract of seeds of *M. myristica* to reduce $FeCl_3$ solution as described by Pulido et al. (2000). Briefly seed extract (0 – 250 µl) was mixed with 250 µl, 200 mM sodium phosphate sodium phosphate buffer (pH 6.6) and 250 µl of 1% potassium ferrocyanide, the mixture was incubated at 50°C for 20 min, thereafter 250 µl, 10% trichloroacetic acid was added, and subsequently centrifuged in 650 rpm for 10 min, 1000 µl of the supernatant was mixed equal volume of water and 100 µl of 0.1 g/100 ml ferric chloride, the absorbance was later measured at 700 nm, at higher absorbance indicates a higher reducing power.

Fe^{2+} chelating property

The Fe^{2+} chelating ability of aqueous and ethanolic extract of *M. myristica* seed was determined using a modified method described by Puntel et al. (2005). Freshly prepared 500 µmol/L FeSO4 (150 µl) was added to reaction mixture containing 168 µl of 0.1mol/L Tris-HCl (pH 7.4), 218 µl saline and extract (0 to 100 µl). The reaction mixture was incubated for 5min before the addition of 13 µL of 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe (ii) chelating ability was subsequently calculated with respect to the reference (which contains all the reagents without seed extract).

Deoxyribose degradation

Deoxyribose degradation was determined by Halliwell et al. (1987). Deoxyribose is degraded by hydroxyl radicals with the release of thiobarbituric acid (TBA) reactive materials. Deoxyribose (6 mM) was incubated at 37°C for 30 min with 50 mM potassium phosphate pH 7.4 plus Fe^{2+} (0.1 mM) and/or H_2O_2 (1 mM) to induce deoxyribose degradation, and seed extracts (0 to 80 µl) of stock. After incubation, 0.4 ml of TBA (0.8%) and 0.8 ml of TCA (2.8%) were added, and the tubes were heated for 20 min at 100°C and spectrophotometrically measured at 532 nm after cooling.

Lipid peroxidation

Rats were decapitated under mild cervical dislocation and the cerebral (whole brain), hepatic (liver) were rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 50 mM Tris-HCl, pH 7.4. The homogenates were centrifuged for 10 min at 4000 g to yield a pellet that was discarded and a low speed supernatant (S1). An aliquot of 100 µl of S1 was incubated for 1h at 37°C in the presence of both seed extracts, with and without prooxidants, iron (final concentration 10 µM) and sodium nitroprusside (SNP) (final concentration 30 µM). This was then used for lipid peroxidation determination. Production of thiobarbituric acid reactive species (TBARS) was determined as described by Ohkawa et al. (1979), expecting that the buffer of the colored reaction has a pH of 3.4. The color reaction was developed by adding 300 µl of sodium dodecyl sulphate (SDS, 8.1%) to S1, followed by sequential addition of 500 µl of 0.8% thiobarbituric acid (TBA). This mixture was incubated at 95°C for 1 h. TBARS produced were measured at 532 nm and the absorbance was compared to that of the controls.

Statistical analysis

The results were expressed as mean ± SD of four independent experiments performed in triplicate and were analyzed by appropriate analysis of variance (ANOVA), followed by Duncan's multiple range test. Differences between groups were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical constituents of *M. myristica*

The antioxidant constituents of *M. myristica* that was determined in the present study as shown in Table 1 include total phenols, flavonoids, vitamin C. The phenolic content of the aqueous and ethanolic extract of seeds of

Table 1. Phytochemical content of *M. myristica*

	Aqueous	Ethanolic
Total phenolics	14.5 ± 0.20 mg/g(GAE)	22.2 ± 0.42 * mg/g (GAE)
Total flavonoid	5.2 ± 0.33 mg/g(QE)	10.5 ± 0.12* mg/g (QE)
Vitamin C	35.4 ± 0.15mg/g	40.2 ± 0.22 mg/g

Each observation is a mean ± SD of 3 to 4 independent experiments.* indicates a statistically significant difference at P<0.05. GAE- Gallic acid equivalent, QE- quercetin equivalent

M. myristica (22.2 ± 0.42 and 14.5 ± 0.20 mg gallic acid equivalent) for ethanolic and aqueous extracts respectively, while the total flavonoids for ethanolic and aqueous extracts were 10.5 ± 0.12 and 5.2 ± 0.33 mg quercetin equivalent) respectively and vitamin C content were estimated to be 40.2 ± 0.22 and 35.4 ± 0.15 mg/g of seed respectively for ethanolic and aqueous extracts. In the present study, we discovered that both aqueous and ethanolic extract contains phenols, flavonoids and vitamin C. However, the amount of these plant phytochemicals was higher in the ethanolic than aqueous extracts. Since phenols and flavonoids are generic names given to class of compounds with great structural diversities and activities, it is expected that the antioxidant constituents present in *M. myristica* may have different antioxidant mechanisms.

Meanwhile, the onset of oxidative stress can be complex and involve several processes. In fact, free radicals induced oxidative stress is thought to play a central role on the development of many complications (Flechner et al., 1990; Gille et al., 2002). In this regard, potential antioxidant agents have been tested for their *in vitro* effect in our laboratory (Kade et al., 2008a, b). Since the seeds of *M. myristica* have been reported to be promising candidate in mopping oxidative species, we therefore sought to investigate the effect of solvent differential between ethanol and water on the antioxidant properties of the seed.

Antioxidant properties of *M. myristica*

Generally, *M. myristica* exhibited potent dose - dependent antioxidant properties such as ferric reducing, metal chelating, free radical scavenging and inhibition of both lipid and deoxyribose oxidation. However, ethanolic extract displayed stronger antioxidant properties than the aqueous extract.

DPPH radical scavenging properties *of M. myristica*

Figure 1 shows the free radical scavenging property of the seeds of *M. myristica.* Apparently, both extracts exhibited potent DPPH free radical scavenging activity which was significant ($P < 0.05$) even at a dilution of 20 fold of the stock solution than the aqueous extract.

DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidants induce a reduction of diphenylpicrylhydrazyl (DPPH) radicals causing a decrease in the absorbance as a result of a visual discoloration from purple to yellow. Hence, DPPH is usually used as a substrate, to evaluate antioxidative activity of antioxidants since such antioxidant have the ability to readily donate their hydrogen to DPPH. Interestingly, Figure 1 shows that both ethanolic and aqueous extracts of *M. myristica* are potent radical scavengers suggesting that they could act as chain breaking antioxidants. However, Figure 1 also revealed that the ethanolic extract exhibited a significantly ($P < 0.05$) higher scavenging effect than the aqueous extract. While the reason for this observation is not completely understood, it could suggest that, since the phytochemical constituents, which are responsible for scavenging free radicals are higher in the ethanolic extract (Table 1), logically, that may account for the higher free radical scavenging effect observed in the ethanolic extract than the aqueous. Furthermore, the higher phytochemical content observed in the ethanolic extract could be attributed to the solubility of the individual phytochemical in different solvent. Most of the phenols which might not be soluble in water may become soluble in ethanol; hence they get extracted leading to the observed increase in the phytochemical content.

Ferric reducing properties of *M. myristica*

The reducing property of *M. myristica* is presented in Figure 2. One-way ANOVA revealed that the ethanolic extract of *M. myristica* is rich in free electrons and readily supplies such electrons to Fe^{3+} than the aqueous thereby reducing Fe^{3+} to Fe^{2+}. This reductive ability of *M. myristica* was significant ($P < 0.05$) at the least volume of extract tested.

Meanwhile, transition metals such as iron are known to be important in the proper functioning of most biological systems. However, in disease states and normal cell recycling, the oxidation state of iron could change from Fe^{2+} to Fe^{3+} as in the case of methaemoglobin, consequently generating free electrons ultimately resulting in oxidative stress and damage to macromolecular structures such as lipids, proteins, carbohydrates and nucleic acids. Hence, effective reduction of Fe^{3+} - Fe^{2+} may be considered an antioxidant

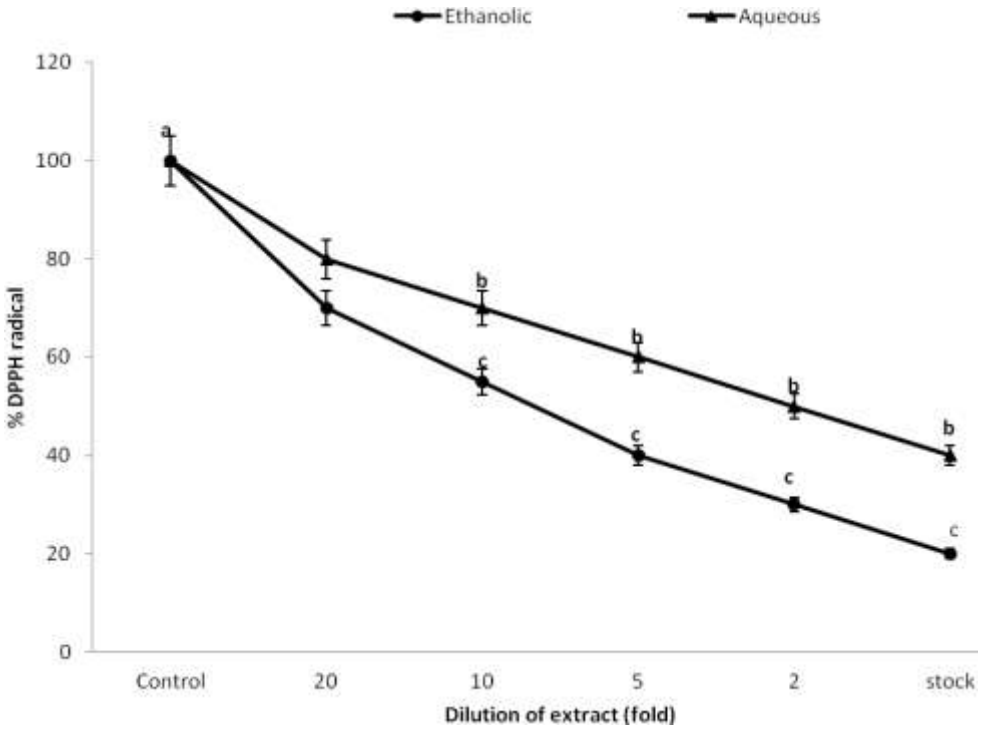

Figure 1. Free radical scavenging ability of aqueous and ethanolic extract of *M. myristica* seed. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

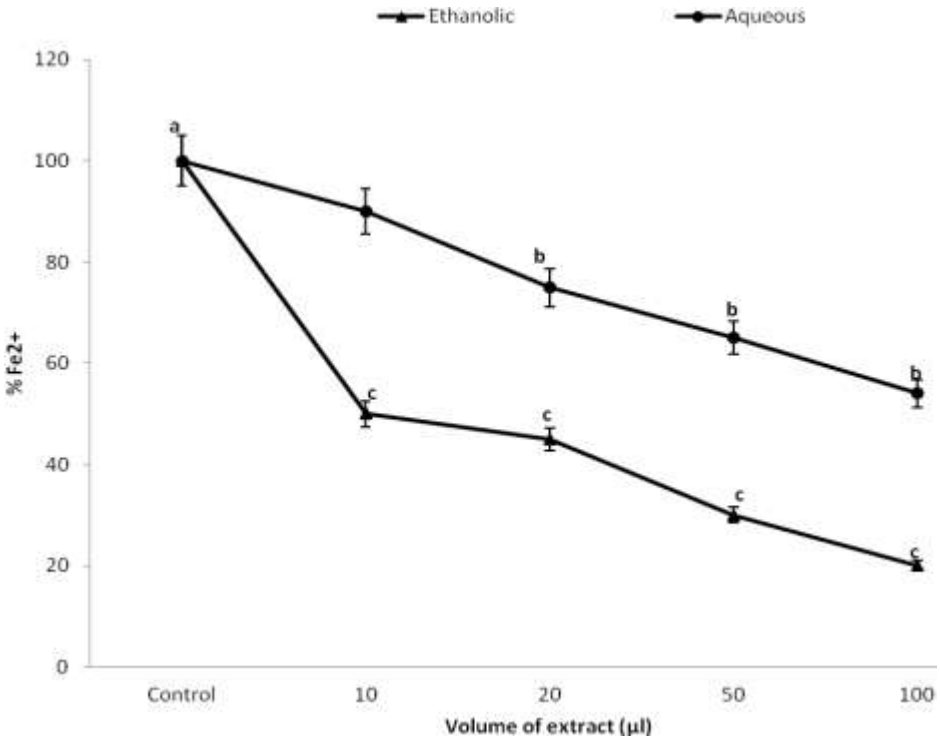

Figure 2. Iron (II) - chelating properties of aqueous and ethanolic extracts of *M. myristica* seed. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

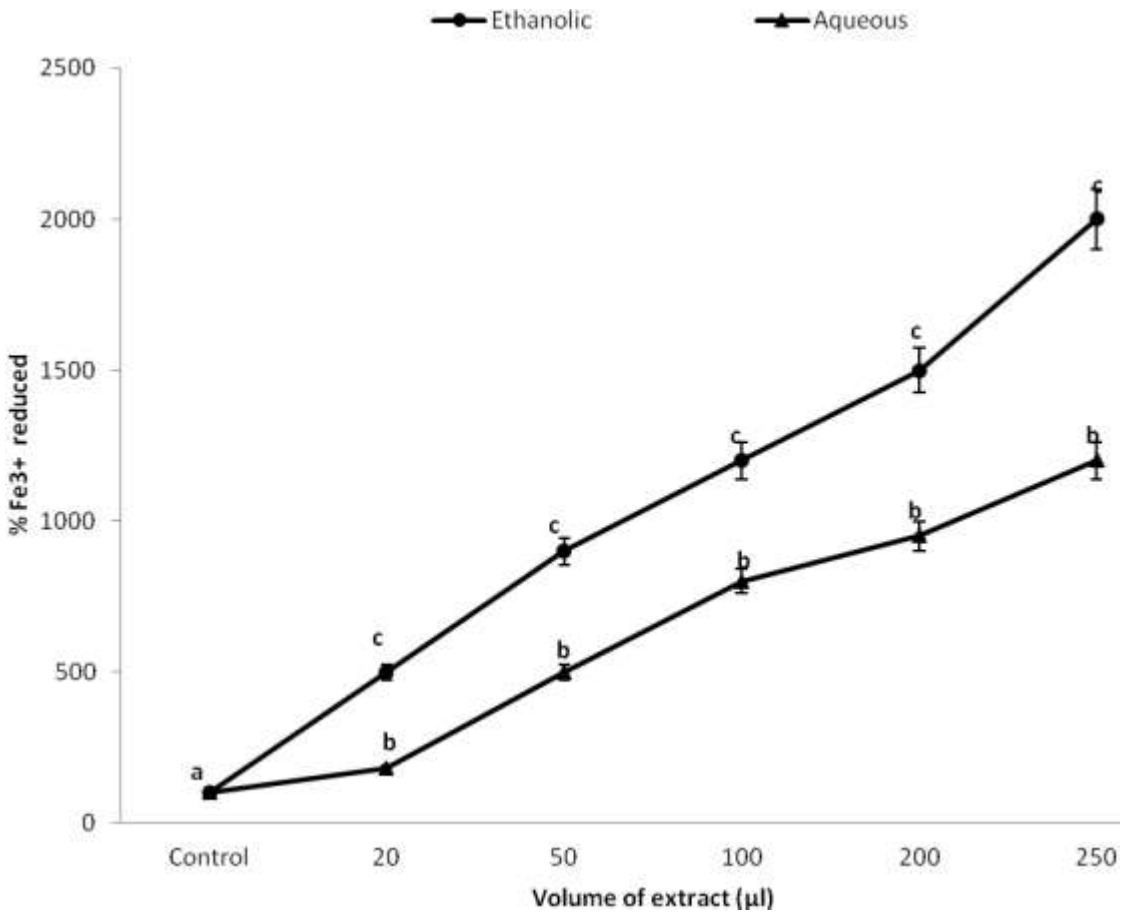

Figure 3. Ferric reducing properties of aqueous and ethanolic extracts of *M. myristica* seed. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

mechanism. Figure 2 shows that both aqueous and ethanolic extract of *M. myristica* exhibited potent reductive effect on Fe^{3+} which is significant ($P < 0.05$) even at the least volume of extract used. This may explain in part the wide usage of *M. myristica* in folk medicine as panacea to oxidative stress - related disorders. The fact that ethanolic extract exhibited a significantly ($P< 0.05$) higher reducing power than the aqueous extract could be due to the differences in level of the phenols and flavonoids (Table1).

Fe^{2+}- chelating property of *M. myristica*

Figure 3 shows the Fe^{2+}- chelating properties of *M. myristica..* One-way ANOVA followed by Duncan's test showed that the ethanolic extracts of seeds of *M. myristica* greatly chelated Fe^{2+} than the aqueous extract when compared to control ($P < 0.05$). Generally, the ability of an agent to chelate or deactivate transition metals that are intrinsically linked to crucial stages of free radicals induced macromolecular damage has been regarded as an antioxidant mechanism. In this regard, *M. myristica* showed marked metal chelating ability. Figure 3 revealed that while both extracts exhibited marked chelating effect, the ethanolic extract was a stronger iron chelator probably due to its higher phytochemical content.

Hydroxyl radical scavenging property of *M. myristica*

Another critical macromolecule susceptible to attack is deoxyribonucleic acid (DNA). Hence, the protective effect of antioxidants against attack from hydroxyl radicals ($OH^{\bullet}$) generated between the interaction of Fe^{2+} and H_2O_2 via the Fenton reaction is often used as a measure of their antioxidative power. Hydroxyl radical have been reported to attack deoxyribose component of the DNA structure causing a loss of structural integrity and a compromise of its cellular function. Interestingly, Figure 4 clearly showed that *M. myristica* offered optimal protection against deoxyribose degradation. One – way ANOVA showed that the ethanolic extract offered a significantly ($P< 0.05$) higher protection than the aqueous extract presumably due to its elevated phenolics and

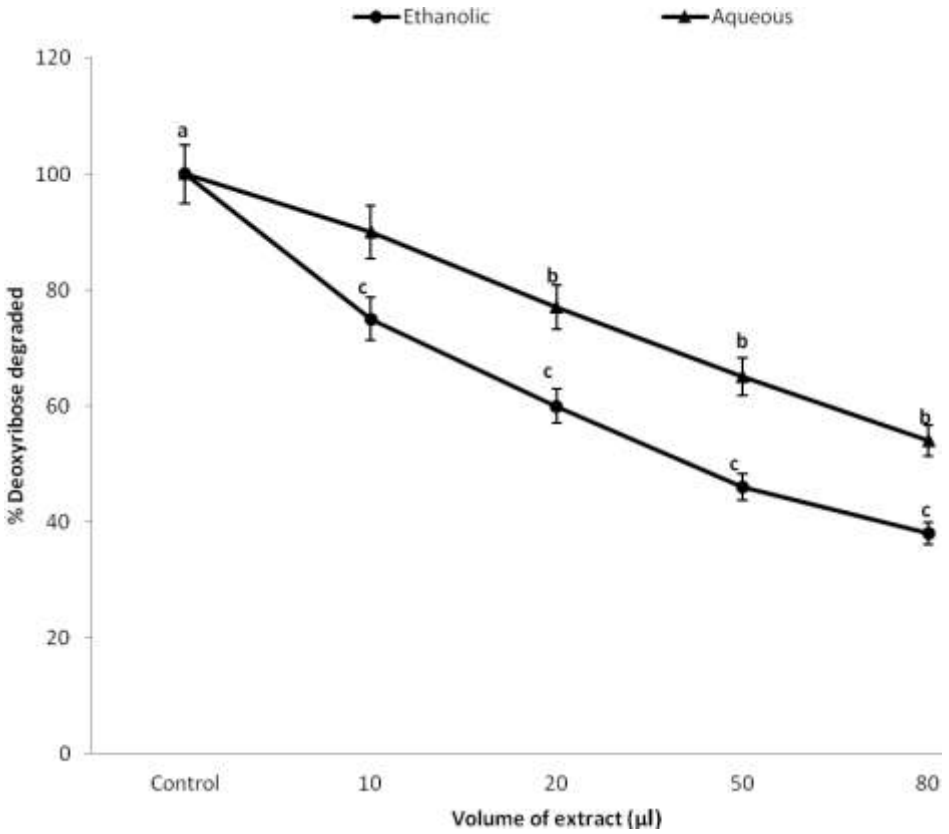

Figure 4. Hydroxyl radical scavenging properties of aqueous and ethanolic extracts of *M. myristica* seed. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

flavonoid content (Table 1) which are known hydroxyl radicals scavengers.

Inhibitory effect of M. myristica on lipid peroxidation

Apart from deoxyribose, lipids are another class of macromolecule that is prone to oxidative damage in biological system. Interestingly *M. myristica* also inhibits *in vitro* lipid peroxidation in the brain and liver irrespective of the prooxidant employed for oxidative assault. Figures 5 to 8 show the effect of ethanolic and aqueous extracts of *M. myristica* on lipids subjected to various oxidants assaults. One - way ANOVA revealed that irrespective of the prooxidant or lipid types, the inhibitory effect of *M. myristica* was significant at the lowest volume of extract tested ($P < 0.05$). However, ethanolic extract exhibited a significantly higher inhibitory effect than the aqueous extract.

Meanwhile, it has been postulated that iron causes its harmful effect on critical biological macromolecules by reacting with superoxide anion (O_2) and hydrogen peroxide (H_2O_2) to produce the $OH^{\bullet}$ via the Fenton reaction (Graf et al., 1984). These radicals can also lead to formation of other reactive oxygen species (ROS) (Klebanoff et al., 1992). The overproduction of ROS can directly attack the polyunsaturated fatty acid of the cell membrane and induce lipid peroxidation. Figures 5 and 6 revealed that the ethanolic extracts demonstrated a stronger inhibitory effect against iron (II) – induced lipid peroxidation than the aqueous extract. This observation could be due to the higher phytochemical content which presumably conferred a better inhibitory effect on the ethanolic possibly through its higher iron (II) - chelating effect.

Apart from iron, sodium nitroprusside (SNP) has been suggested to exert its cytotoxic effect through the release of cyanide and/or nitric acid ($NO^{\bullet}$) (Rauhala *et al*., 1998). Meanwhile, $NO^{\bullet}$ has been implicated in the Pathophysiology of several neurologic disorders including strokes, traumas, seizures, Alzheimer's and Parkinson's diseases (Castill et al., 2000; Prast and Philippou, 2001). It is now known that light exposure promotes the release of $NO^{\bullet}$ from SNP through a photodegradation process (Arnold et al., 1984; Singh et al., 1995), and data from the literature have demonstrated that after the release of NO, SNP or $[NO\text{-}Fe\text{-}(CN)5]^{2-}$ is converted to iron containing $[(CN)5\text{-}Fe]^{3-}$ and $[(CN)4\text{-}Fe]^{2-}$ species (Loiacono and Beart, 1992). After the release of NO, the iron moiety

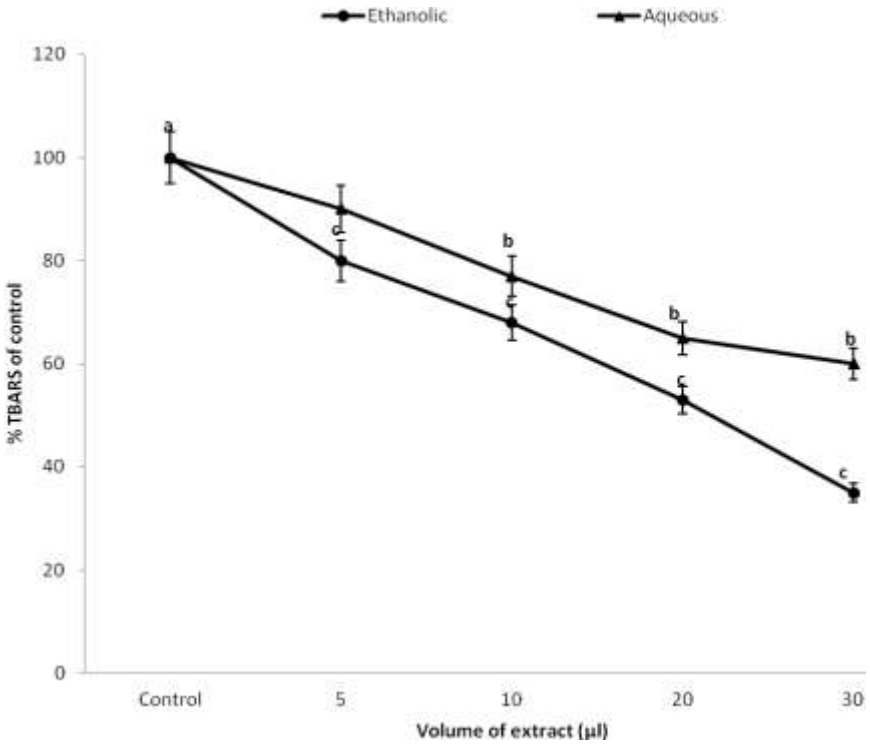

Figure 5. Inhibitory effects of aqueous and ethanolic extracts of *M. myristica* seed on iron (II)-induced hepatic lipid peroxidation. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

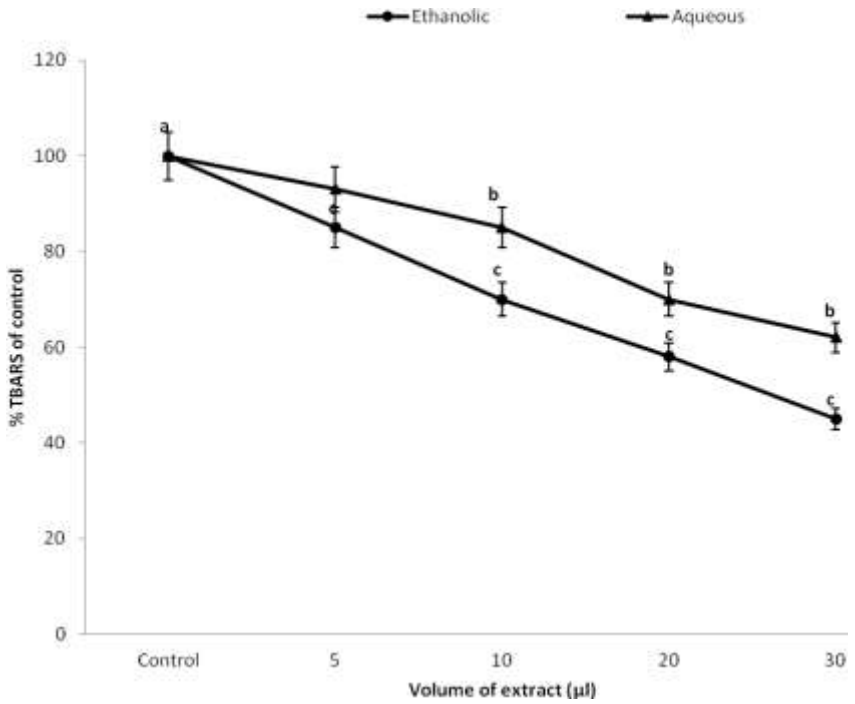

Figure 6. Inhibitory effects of aqueous and ethanolic extracts of *M. myristica* seed on iron (II) - induced cerebral lipid peroxidation. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

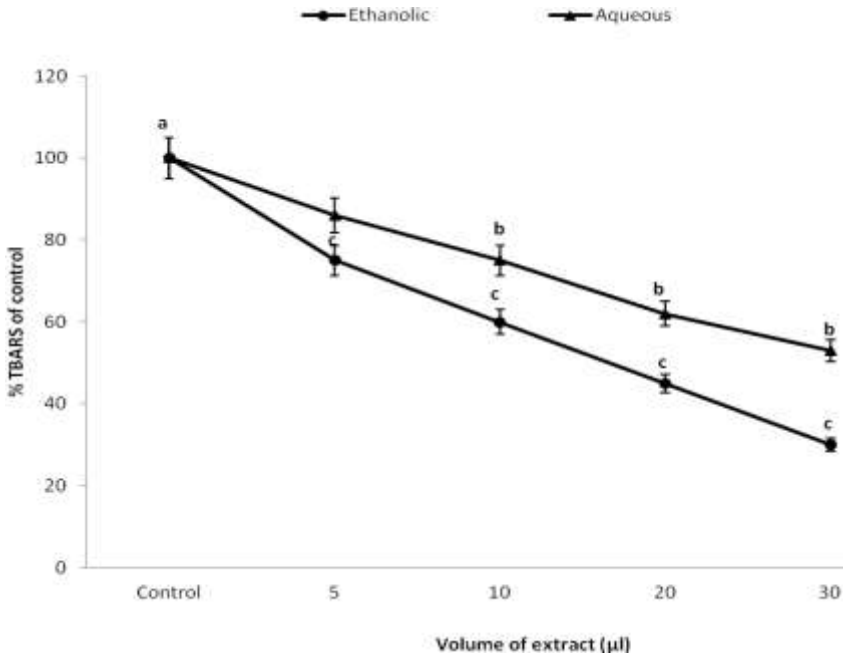

Figure 7. Inhibitory effects of aqueous and ethanolic extracts of *M. myristica* seed on SNP - induced hepatic lipid peroxidation. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

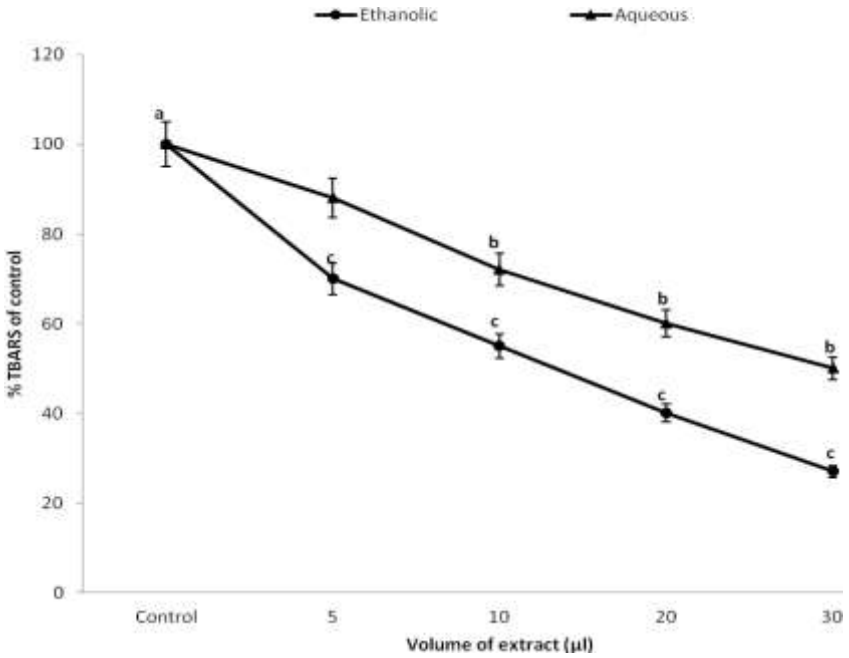

Figure 8. Inhibitory effects of aqueous and ethanolic extracts of *M. myristica* seed on SNP - induced cerebral lipid peroxidation. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

may react with SNP which could lead to formation of highly reactive oxygen species, such as hydroxyl radicals via Fenton reaction (Graf et al., 1984). Interestingly, Figures 7 and 8 showed that ethanolic extract of *M. myristica* seed exhibited a significantly higher ($P < 0.05$) inhibitory effect than the aqueous extract. Although the

reason behind this observation is still an enigma, we could logically attribute it to its higher phenolics and flavonoid content (Table 1).

In conclusion, these data suggest that ethanol is a better solvent for the extraction of phytochemicals present in the seed of *M. myristica* especially when intended to be used for therapeutic purposes. Moreover, the phytochemicals in the ethanolic extract utilizes various antioxidant mechanisms including free radical scavenging, reduction and deactivation (possibly by chelation) of transition metals involved in initiation of free radical induced macromolecular damage among others.

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