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Evaluation of antioxidant potentials of *Monodora myristica* (Gaertn) dunel seeds

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The antioxidant activity of the flavonoid fraction of the seed extract of *Monodora myristica* (Gaertn) Dunel was evaluated. The *M. myristica* seed was extracted with aqueous ethanol (70%) to produce the crude extract. Phytochemical analysis of *M. myristica* ethanolic extract revealed the presence of secondary metabolites, such as alkaloids, flavonoids, saponins, cardiac glycosides and tanins. Fractionation of the crude extract by solvent-solvent extraction using n-hexane, dichloromethane, ethylacetate and butanol yielded the EtOAC fraction which tested positive for flavonoid as the flavonoid fraction (FF). The FF exhibited potent and appreciable anti-oxidant potentials with maximum DPPH-radical scavenging activity (41.20 ± 0.12 %), hydroxyl radical scavenging activity (46.34 ± 0.24%). It also exhibited a significant $p < 0.05$ reduction of Fe^{3+} to Fe^{2+} (64.64 ± 0.11%). It exhibited a dose dependent protective effect against free radical induced haemolysis of red blood cells with maximum protection of 37.00 ± 0.13 and 46.23 ± 0.41% inhibition of lipid peroxidation and free radical generation in liver homogenate. The result suggests that flavonoid fraction of *M. myristica* seed possessed significant antioxidant properties.

Key words: *Monodora myristica,* haemolysis, 1,1-dipheny-2-pycryl hydrazine (DPPH), flavonoid fraction.

INTRODUCTION

It is ironic that oxygen, an element indispensible for life can, under certain situations have several deleterious effects on the human body. Most of the potentially harmful effects of oxygen are due to the formation and activity of a number of chemical compounds known as reactive oxygen species. The radicals derived from oxygen represent the most important class of radical species in living systems (Grassi et al., 2009). Reactive oxygen species includes hydrogen peroxide (H_2O_2), superoxide radical anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), nitric oxide ($NO^{\cdot}$), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl). These species are commonly called “free radicals” because they contained one or more unpaired electrons in their atomic or molecular orbital and thus have the tendency to donate oxygen to other substances (Bagchi and Puri, 1998; Halliwell, 2006). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical.

Reactive oxygen species are formed under normal physiological conditions or from external sources such as X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals (Bagchi and Puri, 1998; Jae-young et al., 2005; De Groot and Rauen, 1998). These reactive oxygen species are considered to contribute decisively to a wide variety of degenerative processes and diseases such as cancer, atherosclerosis, neurodegenerative diseases (Parkinson’s disease, Alzhemeir), hypertension, diabetes mellitus, dementia and reperfusion injury of the brain, liver as well as protein oxidation, DNA damage and lipid peroxidation (Valko et al., 2007; Finkel, 2005). However, controlled production of these radicals has important physiological roles and also serves useful

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purposes in human body. Several observations indicate that the oxygen radicals in living systems are probably necessary compounds in the maturation processes of cellular structures, white blood cells release free radicals to destroy invading pathogenic microbes as part of the body's defense mechanism against diseases (Halliwell, 1996; Morrissey and O'Brien, 1998; Miguel, 2009).

The biological systems have developed several effective antioxidant mechanisms to counteract the harmful effects of reactive oxygen species. Antioxidants are a group of substances which when present at low concentrations in relation to oxidisable substrates significantly inhibit or delay the oxidation and oxidative processes, while often being oxidized themselves (Kanner et al., 1999; Halliwell, 1990). The anti-oxidative principles include enzymes such as superoxide dismutase, catalase and GSH peroxidase (which degrades O_2^- and H_2O_2, respectively), but also not enzymatic compounds such as water soluble glutathione (GSH), polyphenols, proteins (Ferritin), trace elements (e.g. selenium, zinc), ascorbic acids (vitamin C) and the membrane-bound α-tocopherol (vitamin E) (Malterud et al., 1993, Abdou et al., 2010).

The plant *Monodora myristica* belongs to the custard apple family of flowering plants called Annonecea. It is widely distributed from Africa to Asia, Central and South America and Australia (Omobuwajo et al., 2003). It is native to West, Central and East Africa extending from Sierra Leone to Uganda, Kenya, Kongo and Angola (Keay, 1989). It grows well in the ever green forest zone of West Africa and most prevalent in the Sothern part of Nigeria (Adegoke and Akinsanya, 1970). Its local names include: Ehuru or Ehiri (Igbo), Ariwo (Yoruba), Jamaica nutmeg, African nutmeg, Calabash nutmeg, Airama. Ethnomedically, *M. myristica* is used in Ivory Coast to treat hemorrhoids, stomach ache and fibrie pain. The seeds are aromatic and are employed after grinding to a powder as condiments in food providing a flavour resembling that of nutmeg (Ekeanyanwu et al., 2010). The seeds are also used as an aromatic and stimulating addition to medicines and to snuff (Burkill, 1985; Ekeanyawu et al., 2010). When pulverized, the kernel is used to prepare pepper soup as stimulant to relieve constipation and control passive uterine hemorrhage in women immediately after child birth (Okafor, 1987; Udeala, 2000; Iwu, 2002).

Phytochemical studies of *M. myristica* seeds have shown that it is rich in alkaloids, glycosides, flavonoids, tannins, saponin and steroids (Adegoke et al*.,* 1968; Uheghu et al., 2011; Ekeanyanwu et al., 2012). This study therefore sorts to evaluate the antioxidant potentials of *M. myristica* seeds.

MATERIALS AND METHODS

Plant materials collection and identification

Dried seeds of *M. myristica* were purchased from a local market in Ile-Ife, Nigeria. The seeds were identified and authenticated at IFE Herbarium, Obafemi Awolowo University, Ile-Ife, Nigeria with voucher specimen reference number 4734. The seeds were decocted to release the kernels which were later ground to fine powder with electronic blending machine.

Reagents and chemicals

All the reagents used were of analytical grade, obtained from the following Chemical Manufacturing Companies: British Drug House (BDH), Poole, U.K., Sigma Chemical Company, Louis, U.S.A., Fluka Chemical Company and Pharmacia Fine Chemicals, Uppsala, Sweden. Aluminium Oxide was from Associated Chemical Enterprises, South Africa.

Preparation of ethanolic extract of *M. myristica*

The ethanolic extract of seed of *M. myristica* was prepared according to the procedure earlier described by Oyedapo et al. (2004). Typically, 400 g of defatted seeds of *M. myristica* was suspended in aqueous ethanol (70% v/v, 1000 ml). The suspension was stirred occasionally and kept at room temperature for 24 h. The supernatant was collected by filtration through two layers of cheese cloth followed by filtration with filter paper Whatman number 2. The residue was washed with 70% (v/v) ethanol until the supernatant was clear. The filtrates were combined and concentrated under reduced pressure on rotatory evaporator at 40°C to give a brown residue termed ethanolic extract (EE).

Phytochemical screening of ethanolic extract of *M. myristica*

The ethanolic extract was subjected to phytochemical screening for the presence of secondary metabolites using standard procedure that was based on the methods described by Sofowora (2006), Evans (2002) and Oyedapo et al. (1999).

Estimation of flavonoid concentration

The concentration of flavonoid in the ethanolic extract of *M. myristica* was estimated spectrophotometrically according to the method of Sun et al. (1999). Typically, 0.5 ml of working solution of EE (5 mg/ml) was pipetted into test tubes in triplicates and diluted to 5.0 ml with distilled water after which 0.3 ml of 5% (w/v) $NaNO_2$, 0.3 ml of 10% $AlCl_3$ and 4.0 ml of 4% (w/v) NaOH were added to each of the test tubes. The reaction mixtures were incubated at room temperature for 15 min and the absorbance of the product was read at 500 nm against reagent blank. Rutin was used as standard flavonoid.

Estimation of total phenol concentration

The total phenol in the ethanolic extract of *M. myristica* was estimated using the Folin Ciocalteau's phenol reagent reaction as described by Yoshikana et al. (2002). Typically, 0.5 ml of 5 mg/ml of EE was pipetted into clean dried test tubes in triplicate and the volumes were adjusted to 1.0 ml with distilled water. Into each of the test tubes, 1.5 ml of Folin Ciocalteau's phenol reagent (1:10) was added. The reaction mixtures were incubated at room temperature for 5 min after which 1.5 ml of 10% (w/v) $NaHCO_3$ was added and the reaction mixture was further incubated for additional $1^1/_2$ hours. The absorbance was read at 725 nm against the reagent blank. Gallic acid was used as standard phenol.

Partitioning of ethanolic extract of *M. myristica*

Ethanolic extracts of *M. myristica* was partitioned by solvent-solvent extraction according to a procedure that was based on those reported by Aderogba et al. (2007) and Owoyele et al. (2008). Typically, EE (20 g) was taken up in hot distilled water (0.2 L). This was successively extracted with 1 L of n-hexane, dichloromethane, ethylacetate and n-butanol in a separating funnel (1 L) to afford four fractions namely n-Hex, DCM, EA and nB, respectively. The fractions were screened for flavonoids. Phytochemical screening, thin layer chromatography, staining with specific detection reagents together with chemical tests with 2,4-dinitrophenyl hydrazine revealed that ethyl acetate fraction contained the bulk of flavonoid of the seed of *M. myristica*. The fraction was evaporated to dryness and termed flavonoid fraction (FF).

Biochemical analysis

Assay of DPPH- radical scavenging activity

The DPPH-radical scavenging activity of flavonoids of *M. myristica* was assessed using a modified method of Blois (1985) as reported by Cakir et al. (2003). Typically, varying concentrations (0 to 350 µg/ml) of the extract of *M. myristica* was pipetted into clean dried test tubes in triplicates and the volumes adjusted to 1 ml with 10 mM acetate buffer, pH 4.5. This was followed by the addition of 2 ml of 0.2 mM DPPH solution in methanol. The reaction mixture was mixed thoroughly by inversion and then incubated in the dark for 30 min. The absorbance was read at 517 nm against the blank that contained 1 ml of 10 mM acetate buffer, pH 4.5 and 2 ml of 0.2 mM DPPH solution in methanol. The above procedure was followed for ascorbic acid (1 mg/ml) and rutin (1 mg/ml) standard. The percentage scavenging activity was evaluated using the expression:

$$\text{Percentage scavenging activity} = \frac{\text{(Absorbance of control- Absorbance of sample)}}{\text{Absorbance of control}} \times 100$$

Assay of hydroxyl-radical scavenging activity

The ability of flavonoid fraction of *M. myristica* to scavenge hydroxyl radical was investigated by method of Halliwell et al. (1988) with slight modification. The varying concentrations of flavonoid fraction of *M. myristica* were pipetted into clean dried test tubes in triplicates and the volume in each tube was adjusted to 1 ml with distilled water.

The substrate (1 ml) consisting of 3 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM EDTA, 0.1 mM ascorbic acid and 2 mM H_2O_2 was added and followed by incubation at 37°C for 1 h. The reaction was terminated by the addition of 1.0 ml of TCA (1%) and TBA (1%) was later added. The mixture was incubated in boiling water at 100°C for 20 min. The tubes were removed, cooled and the absorbance was read at 532 nm against reagent blank. The percentage hydroxyl radical scavenging activity was calculated using the expression:

$$\text{Percentage inhibition} = \frac{A - B}{A} \times 100$$

Where, A = absorbance without the fraction and B = absorbance with fraction.

Inhibition of free radical induced oxidative heamolysis of red blood cells with $CuSO_4.5H_2O$ and H_2O_2

The ability of flavonoid fraction of *M. myristica* to inhibit free radical induced heamolysis of red blood cell was determined by a modified method of Amzal et al. (2008) as reported by Bode and Oyedapo (2011). The assay involved pipetting varying concentrations (0 to 350 µg / ml) of flavonoid fraction into clean dried test tubes in triplicate. The volumes in each test tube was made up to 1.5 ml with normal saline and was followed by the addition of 1 ml of 10 mM $CuSO_4.5H_2O$ and 1 ml of H_2O_2 (30% v/v) to each test tube. Freshly prepared 2% red blood cell (1 ml) was then added to the reaction mixture, mixed thoroughly and incubated at 37°C for 1 h. The tubes were cooled and centrifuged at 3000 rpm for 10 min and supernatants were carefully collected. The absorbance of released haemoglobin was read at 540 nm against the reagent blank. This procedure was repeated with rutin (1 mg/ml) as standard. The percentage inhibition was evaluated using the expression:

$$\text{Percentage inhibition} = \frac{A - B}{A} \times 100$$

Where, A is the absorbance without flavonoid fraction and B is the absorbance with flavonoid fraction

Assay of the reducing power

The ability of flavonoid fraction of *M. myristica* to reduce iron from the form (III) to the form (II) was assessed with the method of Hinneburg et al. *(*2006). Aliquots of the flavonoid fraction (0.5 ml) was mixed with 1.25 ml of phosphate buffer (0.2 M, pH 6.6) and 1.25 ml of a 1% (w/v) aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. After 30 min incubation at 50°C, 1.25 ml of 10% (w/v) trichloroacetic acid was added, mixed and shaken thoroughly. The suspension was centrifuged at 2000 rpm for 10 min. Then, 1.25 ml of the upper layer was mixed with 1.25 ml of distilled water and 0.25 ml of aqueous $FeCl_3$ (0.1% w/v). The absorbance was read at 700 nm against the reagent blank. The percentage reducing power was calculated by this expression:

Reducing power (RP) = (A_m/ A_b - 1) x 100

where A_m = Absorbance of reaction mixture; A_b = absorbance of blank mixture.

Assay of inhibition of lipid peroxidation

The inhibitory activity of flavonoid fraction of *M. myristica* on lipid peroxidation (LPO) was determined by thiobarbituric acid method. The mixture of $FeCl_2$-H_2O_2 (ratio 1:1) was used to induce peroxidation according to the method of Su et al. (2009) with slight modification. Typically, 0.2 ml of flavonoid fraction of *M. myristica* (1.25 mg/ml) was mixed with 1.0 ml of 1% (w/v) liver homogenate (1 g rat liver was homogenized to give 100 ml filtrate), then 50 µl of $FeCl_2$ (0.5 mM) and 50 µL of H_2O_2 (0.5 mM) were added. The reaction mixtures were incubated at 37°C for 1 h. This was followed by the addition of 1.0 ml of trichloroacetic acid (15%) and 1.0 ml of 2-thiobarbituric acid (0.67%) and the mixtures were heated in a boiling water bath for 15 min. The absorbance was read at 532 nm against the reagent blank. Quercetin and gallic acid were used as the positive controls. The percentage inhibition was calculated according to following equation:

Inhibition rate (%) = {1- (A_1 - A_2)/A_0} x 100

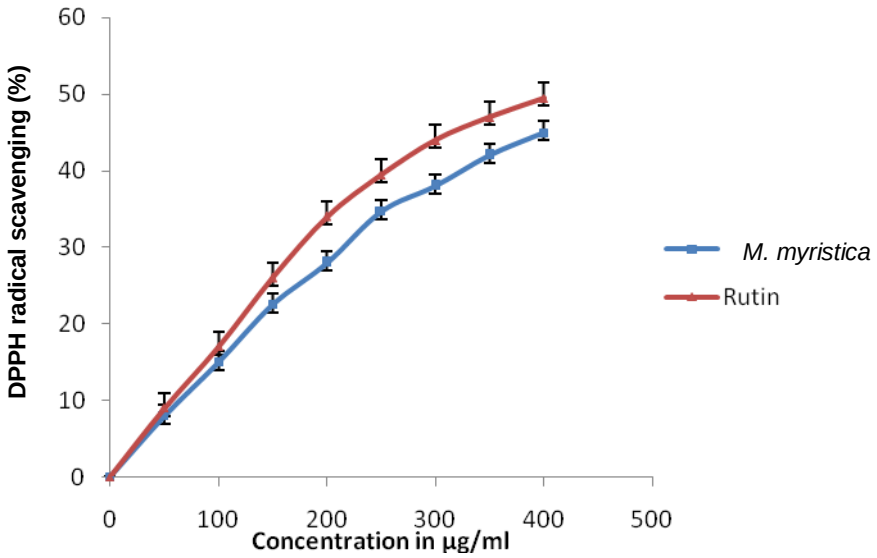

Figure 1. DPPH-radical scavenging activity of flavonoid fraction of *M. myristica* and rutin. Each value represents the mean ± SD of three parallel measurements.

Where, A_0 is the absorbance of the control (without extract), A_1 is the absorbance of the extract addition and A_2 is the absorbance without liver homogenate.

Statistical analysis

Data for the biochemical assays were expressed as mean ± SEM. Analysis was performed using GraphPad Instant 3 (version1.1, 2007).

RESULTS AND DISCUSSION

Numerous natural products are effective antioxidants and many medicinal plants with long history of use in folk medicine in different countries against a variety of diseases have turned out to be rich sources of antioxidant (Mantle et al., 2000; Pereira da Silva et al., 2000; Cho et al., 2003; Lee et al., 2002). Recently, great interest has been given to naturally occurring antioxidants which played prominent roles in inhibiting both free radicals and oxidative chain reactions within tissues and membranes (Nsimba et al., 2008). Antioxidant mechanisms are diverse and a variety of *in vitro* techniques has been developed. The proposed mechanisms of action of antioxidants include direct radical scavenging, inhibition of enzymes such as NO-synthase, xanthine oxidase, cyclooxygenase and lipooxygenase; iron chelation and direct inhibition of lipid peroxidation (Xanthopoulou et al., 2009). Therefore, it is better to employ different assays based on different mechanisms to evaluate the antioxidant capacity. In this study, DPPH radical scavenging, hydroxyl radical scavenging, the reducing power, inhibition of lipid peroxidation and inhibition of free radical induced haemolysis of red blood cell were employed to evaluate the antioxidant potential of flavonoid fraction of *M. myrisica.*

The scavenging of 1.1-diphenyl-2-pycrylhydrazine (DPPH) radical is a commonly used procedure which has been validated against several other assays for antioxidant activities (Malterud et al., 1993; Tilak et al., 2004). The procedure has been employed by various researchers to evaluate the free radical scavenging activity of molecules and plant extracts despite the fact that this chromogen does not solubilize easily, albeit it does generate a strongly coloured solution with methanol and ethanol as solvent (Han et al., 1999; Yan et al., 1998; Matsukawa et al., 1997). DPPH is a stable free radical because of delocalization of the spare electron over the molecule so that the molecules do not dimerise. DPPH radical are capable of accepting electrons or hydrogens to become diamagnetic molecule converting it to 1,1-diphenyl-2-pycrylhydrazine (Fargere et al., 1995). The reduction of DPPH radical was evident by the loss of deep violet colour to light yellow and concomitant decrease in absorbance at 517 nm by the antioxidant that donate hydrogen or electron (Kulisic et al., 2004). The degree of reduction in absorbance measurement was indicative of the radical scavenging power of the antioxidant. The flavonoid fraction (FF) of *M. myristica* was able to scavenge DPPH radical with an IC_{50} value of 412 µg/ml ($r = 0.9825$, $p < 0.0001$). Rutin used as positive control was able to scavenge DPPH with an IC_{50} values of 361 µg/ml ($r = 0.9808$, $p < 0.001$) (Figure 1). Evident from this study, a good linear relationship was observed between the decrease in absorbance of DPPH and different concentrations of flavonoids fraction of *M.*

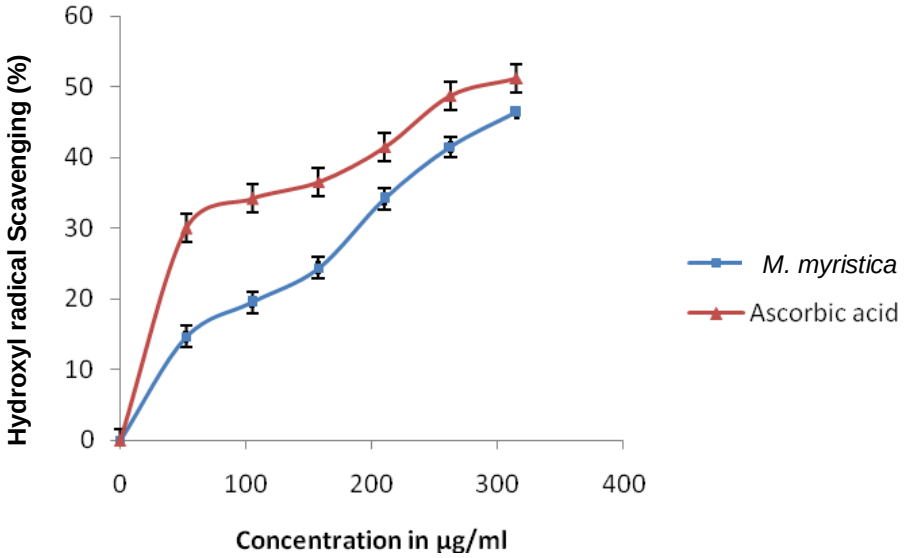

Figure 2. Hydroxyl radical scavenging activities of flavonoid fraction of *M. myristica* and ascorbic acid. Each value represents the mean ± SD of three parallel measurements.

myristica. The increased concentration of *M. myristica* showed increased DPPH radical scavenging activity. The DPPH-radical scavenging activity was concentration dependent and compared favorably with standard compound. Hydroxyl radical is a highly potent oxidant that reacts with almost all biological molecules found in living cells (Tanasait et al., 2010). The chemical activity of hydroxyl radical is the strongest among reactive oxygen species (Jae-Young et al., 2005).

Therefore, the removal of hydroxyl radical is probably one of the most effective defenses of a living body against various diseases. Fe^{2+} can react with hydrogen peroxide (H_2O_2) to produce the hydroxyl radical (OH) via the fenton reaction. In this study, the formation of hydroxyl radicals (OH) from Fenton reaction was quantified using 2-deoxyribose oxidative degradation. The ability of *M. myristica* to scavenge the generated hydroxyl radical was investigated by the inhibition of deoxyribose degradation.

The results reveal that the flavonoid fraction was able to inhibit deoxyribose degradation at maximum percentage inhibition of 46.34% at concentration of 315 µg/ml. The flavonoid concentration that caused 50% inhibition (IC_{50}) was estimated to be 329 µg/ml. Ascorbic acid used as standard antioxidant gave an IC_{50} value of 271 µg/ml (Figure 2). The flavonoids of *M. myristica* was able to scavenge hydroxyl radicals generated by Fenton reaction in a concentration dependent manner. The results of this study are in agreement with earlier reports of Abdou et al. (2010), Adefega and Oboh (2011) that extracts of *M. myristica* inhibited the decomposition of deoxyribose.

Ferric reducing antioxidant power is a novel antioxidant defense mechanism. It is used to assess the metal (iron exclusively) ions binding ability (Nsimba et al., 2008). The two mechanisms that are available and affect this property are electron transfer and hydrogen atom transfer (Dastmalchi et al., 2007).

The reducing power is generally associated with the presence of reductones which exerts antioxidant actions by breaking free radical chain by donating hydrogen atom (Prasad et al., 2010). FRAP assay measures the ability of antioxidant components to reduce Fe^{3+} / ferricyanide complex to ferrous form which has an intense bluish colour. The measurement of the formation of Perl's Prussian blue at 700 nm was used to monitor the Fe^{2+} concentration.

It was observed that flavonoid of *M. myristica* gave a maximum percentage reduction of Fe^{3+} to Fe^{2+} of 68.68% at 400 µg/ml. The ability of flavonoids of *M. myristica* to reduce Fe^{3+} to Fe^{2+} was found to increase as the concentration increases and compared favourably with 2,6-di-tert-butyl-4-methyl phenol BHT (Figure 3), a standard phenol.

The reaction of $CuSO_4.5H_2O$ with hydrogen peroxide generates hydroxyl radical. This generated hydroxyl radical is capable of oxidizing membrane lipids of the red blood cells resulting in haemolysis which is evident by the release of haemoglobin. Flavonoid of *M. myristica* was able to inhibit the action of free radical generated as a result of the reaction of $CuSO_4$ with H_2O_2 by causing a decrease in the amount of haemoglobin released. The result showed a concentration-dependent mode of inhibition which is comparable with the mode of inhibition of rutin, a standard flavonoid. The flavonoid fraction gave maximum percentage protection of 37% at concentration of 290 µg/ml while rutin gave a maximum percentage protection of 40% at a concentration of 300 µg/ml (Figure

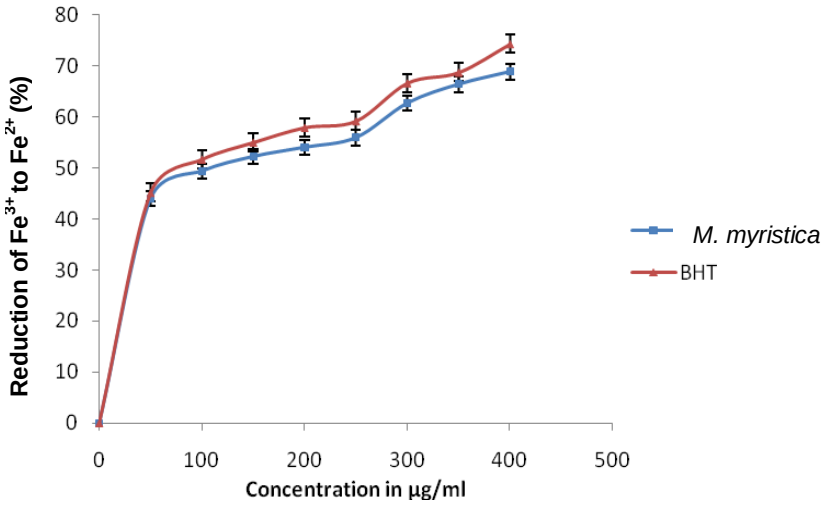

Figure 3. Ability of flavonoid of *M. myristica* and BHT to reduce Fe^{3+} to Fe^{2+}. Each value represented mean ± SD of n = 3.

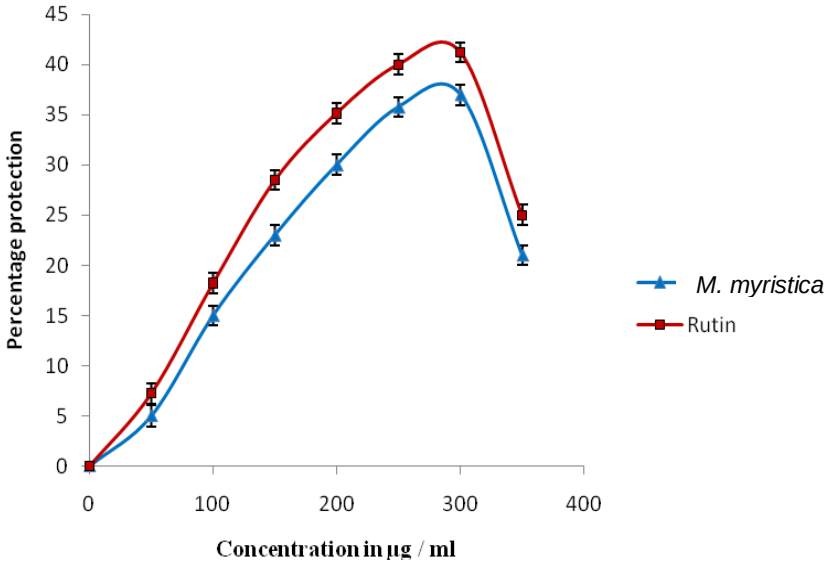

Figure 4. Ability of flavonoid fraction of *M. myristica* and rutin to inhibit free radical induced haemolysis of red blood cells. Each value represents the mean ± SD of three parallel measurements.

4). The result obtained in this study was in agreement with those of Liu et al. (2002), Amzal et al. (2008) and Bode and Oyedapo (2011) that certain plant metabolites protected erythrocytes from free radical induced haemolysis of red blood cells. However, information was limited on the ability of flavonoid of *M. myristica* to inhibit $CuSO_4$ induced haemolysis of red blood cells from the literature.

The role of peroxidative processes in disease is a subject of intense research interest. Lipid peroxidation of cell membranes is associated with various pathological events such as atherosclerosis, inflammation and liver injury (Roome et al., 2008). In this study, the percent inhibition of lipid peroxidation was quantified by measuring the reduction of TBARS in the presence of flavonoid fraction of *M. myristica*. The flavonoid fraction of *M. myristica* caused a decrease in the malondialdehyde produced. The production of MDA was used as a biomarker to measure the level of oxidative stress in organism (Cheeseman and Holley, 1993). MDA reacts

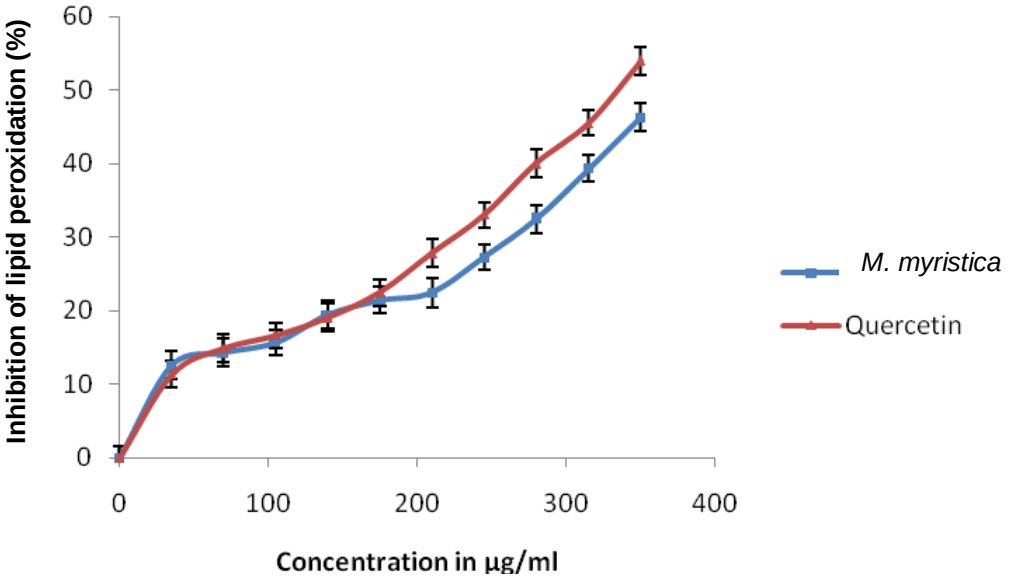

Figure 5. Percentage inhibition of lipid peroxidation in rat liver homogenate by flavonoid fraction of *M. myristica* and quercetin. Each value represented mean ± SD of n = 3.

with thiobabituric acid to produce a fluorescent product (TBARS). Flavonoid fraction of *M. myristica* was able to reduce TBARS produced thereby inhibiting lipid peroxidation with a maximum percentage inhibition of 46.23% at 350 µg/ml. This mode of inhibition compared favourably with quercetin, a standard flavonoid (Figure 5). This result was in agreement with that of Adefega and Oboh (2011) that extracts of *M. myristica* showed inhibitory effects on Fe^{2+}- induced lipid peroxidation in rat liver homogenates.

Conclusion

It was evident from this study that *M. myristica* seeds contained secondary metabolites with antioxidant activity which appeared to be significant in the reduction of free radicals in a dose dependent manner. Its phenolic and flavonoid contents could be a plausible explanation for the antioxidant activities exhibited by this spices. Therefore, its consumption could possibly boost the antioxidant defense system; thereby reduce the free radical status in humans.

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