In vitro Anti-inflammatory Evaluation of African Nutmeg (*Monodora myristica*) Seeds

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Author's contributions

This work was carried out in collaboration between both authors. Author OOO designed the work, author KFA analyzed, interpreted and prepared the manuscript. Both authors read and approved the final manuscript.

| *Original Research Article* |

ABSTRACT

Aim: To determine the anti-inflammatory potential of flavonoid rich fraction of *Monodora myristica* seeds.
Study Design: *In vitro* evaluation of anti-inflammatory assays: Membrane stability, Inhibition of denaturation of albumin and lipoxygenase inhibition.
Place and Duration: Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria (May–November, 2014).
Methodology: Flavonoid rich fraction of the seeds of *M. myristica* was obtained from the ethanol extract of *M. myristica* seeds by solvent partitioning. Standard methods were employed in the anti-inflammatory assays (Membrane stability, Inhibition of denaturation of albumin and lipoxygenase inhibition).
Results: Flavonoid rich fraction of *M. myristica* exhibited significant *in vitro* anti-inflammatory potentials by stabilizing red blood cell membrane exposed to hypotonic and heat induced lyses with maximum percentage stability of 88±0.45% in a biphasic mode of response that is comparable with Ibuprofen a standard anti-inflammatory drug. It also inhibits heat induced albumin denaturation with maximum inhibition of 75.38±0.56% in a concentration dependent manner that is comparable with

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aspirin. Flavonoid rich fraction of *M. myristica* showed an anti-lipoxygenase activity range from 19±1.28% to 71±2.13% which is comparable to that produced by indomethacin.

Conclusion: The result obtained in the present investigation indicates that flavonoid rich fraction of *M. myristica* could be a potential source of anti-inflammatory agent. This substantiates the role of *M. myristica* seeds as a natural source of anti-inflammatory agent.

1. INTRODUCTION

Monodora myristica seed is a popular condiment used as a spicing agent in both African and continental cuisines in Nigeria. They are the most economically important part of *M. myristica* tree. *M. myristica* seeds are contained in a hard kernel with a thin seed coat. Both the seeds and seed coats of the plant are used as spices. The seeds once dried have an aroma reminiscent of nutmeg and are sold whole to be grated as a nutmeg substitute [1]. When the seeds are roasted and ground, they are rubbed on the skin for skin diseases [2]. The seeds are also chewed and used as insecticides [3]. In traditional medicine the seeds are used as a stimulant, stomachache and treatment of headaches. They are also used as rosary beads and are considered by some to have magical properties.

M. myristica seed extracts contained important pharmacological compounds such as alkaloids, flavonoids, glycosides, tannins, saponins, lipids, vitamin A, vitamin E and steroids [4-6]. The seed extract of *M. myristica* possesses antibacterial, antimicrobial, antioxidant, and anti amylase activities [7-10].

Inflammation is defined as a part of complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants [11]. It is characterized by redness, edema, fever, pain, and loss of function [12]. Inflammation may be associated with general "flu-like" symptoms such as fever, fatigue, cold, loss of appetite and muscular stiffness [13]. The migration of leukocytes from the venous systems to the site of damage and the release of cytokines are known to play a crucial role in the inflammatory response. These chemicals released increase blood flow to the area, resulting in redness and warmth. Some of the chemicals cause leakage of fluid into the tissues resulting in swelling. Hence, the inflammatory process may stimulate nerves, cause pain, swelling and redness [14].

Inflammation can be classified as either acute or chronic. Acute inflammation is a short-term process, usually appearing within a few minutes or hours and ceasing upon the removal of injurious stimulus. Chronic inflammation ensues when the persistence (due to non-degradable pathogens) of injurious agents (foreign bodies) leads to a progressive shift in the type of cells present at the site of inflammation and it may last for many days, months or even years [11]. Chronic inflammation is characterized by dominating presence of macrophages in the injured tissue. Although, these cells are powerful defensive agents in the body, the toxins they release (including reactive oxygen species) are injurious to the organisms' own tissues as well as invading agents. Consequently, chronic inflammation is always accompanied by tissue destruction.

One of the features of inflammation is increased oxygenation of arachidonic acid which is metabolized by two enzymic pathways: The 5-lipooxygenase and the cyclooxygenase (CO) pathways leading to production of mediators of inflammation; leukotrienes and prostaglandins respectively [15].

Flavonoids are known to have anti inflammatory properties [16]. Several mechanisms have been proposed for the cellular action of *in vivo* anti-inflammatory activities of flavonoids. Certain flavonoids modulate the activities of enzymes such as cyclooxygenase (COX), phospholipase A2 (PLA2), lipoxygenase (LOX), nitric oxide (NO) producing enzymes and nitric oxide synthase (NOS) which are involved in metabolizing arachidonic acid (AA). Inhibition of these enzymes by flavonoids reduces the production of crucial mediators of inflammation such as AA, prostaglandins (PG), leukotrienes (LT) and NO. Thus, the inhibition of these enzymes exerted by flavonoids is definitely one of the important cellular mechanisms of anti-inflammation [17].

Anti-inflammatory agents exert their effects through a variety of mechanisms, including

inhibition of cotton pellet granulation, uncoupling of oxidative phosphorylation, inhibition of denaturation of protein, stimulation and inactivation of adenosine triphosphate phosphatase, erythrocyte membrane stabilization, lysosomal membrane stabilization, fibrinolytic assay [18-20], proteinase inhibition [21] and inhibition of some enzymes that are involved in inflammation. This study however, employed Membrane stabilization, lipoxygenase inhibition and inhibition of denaturation of albumin to evaluate the anti-inflammatory mechanism of a flavonoid rich fraction of *M. myristica* seeds.

2. MATERIALS AND METHODS

2.1 Sample Collection and Extraction

Dried seeds of *M. myristica* were purchased from Sabo 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 the electronic blending machine. Powdered seeds of *M. myristica* (312.14 g) were extracted with aqueous ethanol (70%) as described by [22] to obtain the ethanol extract (41.03 g). The flavonoid rich fraction of *M. myristica* was obtained from the ethanol extract as previously described by [23].

2.2 Reagents and Chemicals

All the reagents and chemicals used were of analytical grades and were obtained from different sources such as Merck (Darmstadt, Germany), Sigma Aldrich (St. Lous, MO), British Drug House (BDH) England. Solutions, buffers and reagents used were prepared with glass distilled water and stored in the refrigerator.

2.3 Investigation of Anti-inflammatory Activities

2.3.1 Membrane stabilizing potential

2.3.1.1 Preparation of red blood cells

The red blood cell was prepared as described by [22]. Typically, fresh bovine blood was collected into an anticoagulant (3.8% trisodium citrate) in a clean sterile bottle, mixed by invasion and brought to the laboratory in an ice bucket. Bovine blood was washed with normal saline by centrifugation at 4000 rpm for 10 min at room temperature on a Galemkamp Junior Table centrifuge and the supernatant was carefully decanted. The process of washing and centrifuging was repeated until the supernatants became clear. The clear supernatant was decanted and 2% (w/v) red blood cells was prepared from the packed cell with normal saline and kept in the refrigerator.

2.3.1.2 Membrane stabilizing activity

The ability of flavonoid rich fraction of *M. myristica* to stabilize membrane was determined by a procedure of [19] as modified by [24] and [22]. The assay mixture consisted of 1 mL of hyposaline, 0.5 mL of 0.5 M phosphate buffer pH 7.4, varying concentrations (0–350 µg/mL) of flavonoid rich fractions of *M. myristica* and 2% erythrocyte suspension (0.5 mL). The volumes were adjusted to a total assay volume of 3.0 mL with normal saline. The blood control was prepared as above without the flavonoid while the drug control contained all other reagents except the erythrocytes suspension. The reaction mixture was incubated at 56ºC for 30 min. The tubes were cooled and centrifuged at 3,500 rpm for 10 min. The supernatant was collected and the absorbance was read at 560 nm against the reagent blank. This same procedure was employed with standard anti inflammatory drug Ibuprofen (1 mg/mL). The percentage membrane stability was determined using the expression;

% Membrane stability =

$$100 - \frac{(\text{Abs of test drug} - \text{Abs of drug control})}{\text{Abs of blood control}} \times 100$$

2.3.2 Inhibition of denaturation of albumin

The ability of the flavonoid rich fractions of *M. myristica* to inhibit the denaturation of albumin was investigated by method of [25] as reported by [21] with minor modifications. Typically, varying concentrations (0-350 µg/mL) of flavonoid fraction of *M. myristica* were prepared and the volumes were adjusted to 2.5 mL with 0.85% NaCl. After which 0.5 mL of albumin (1.5 mg/mL) was added. The mixture was incubated at 37ºC for 20 min and further incubated at 57ºC for 20 min. The tubes were cooled and 2.5 mL of 0.5 M Sodium phosphate buffer pH 6.3 was added. The turbidity was measured spectrophotometrically at 660 nm. The experiment was carried out in triplicates and the

standard was used in place of the extract. Percentage inhibition of albumin denaturation was evaluated as follows:

$$\text{Percentage inhibition: } \frac{Abs_{control} - Abs_{Test}}{Abs_{control}} \times 100$$

2.3.3 Lipoxygenase inhibitory activity

Inhibition of lipoxygenase activity was studied using linoleic acid as substrate and lipoxidase as enzyme [26]. Various concentrations of flavonoid rich fractions were dissolved in 0.25 mL of 2 M borate buffer pH 9.0 and 0.25 mL of lipoxidase enzyme solution (20,000 U/mL) was added and incubated for 5 min at 25ºC. After which, 1.0 mL of linoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234 nm. Indomethacin was used as reference standard. The percent inhibition was calculated from the expression:

%inhibition= [{Abs control- Abs sample}/Abscontrol] x 100

2.4 Statistical Analyses

Data obtained from this study were expressed as mean±SEM. P values less than 0.05 and 0.01 were considered to be statistically significant.

3. RESULTS AND DISCUSSION

Fig. 1 shows the result of membrane stabilizing activities of flavonoid rich fraction of *M. myristica* seeds. Flavonoid rich fraction of *M. myristica* seeds exhibited a biphasic mode of response with maximum percentage stability of 88±1.43% at a concentration of 200 μg/mL while Ibuprofen a standard anti-inflammatory drug gave a maximum percentage stability of 70±1.57% at 300 μg/mL.

The viability of cells depends on the integrity of their membranes [27]. Compounds with membrane stabilizing potentials are well known for their ability to interfere with the early phase of inflammatory reactions; prevent the release of phospholipase that triggers the formation of inflammatory mediators [28]. The exposure of erythrocytes to injurious substances such as hypotonic medium and heat resulted in lyses of its membrane which is accompanied by haemolysis and oxidation of hemoglobin [29]. The haemolytic effect of hypotonic solution was due to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane.

Such injury to red blood cell membrane would further render the cell more susceptible to secondary damage through free radical–induced lipid peroxidation [27,29]. This notion was consistent with the observation that the breakdown of biomolecules lead to the formation of free radicals which in turn enhanced cellular damage [30,31]. The stabilization of erythrocyte membrane exposed to both heat and hypotonic induced lyses by flavonoids of *M. myristica* as investigated in this study revealed that *M. myristica* showed a dose dependent membrane stabilizing activity over the concentration ranges tested. This result compared favourably with the activity of ibuprofen, a non- steroidal anti-inflammatory drug. Although information is scarce on the ability of flavonoid of *M. myristica* to stabilize red blood cell membrane, but it has been speculated that the ability of plants to stabilize membrane might be due to the presence of some naturally occurring phytochemicals including flavonoids [24]. Several herbal derived drugs have been demonstrated to contain principles that possess ability to facilitate the stability of biological membranes when exposed to induced lyses [19]. Some plant extracts have been reported to protect and stabilize red blood cells exposed to heat induced stress [21,32] or both hypotonic and heat induced stress [24,22,33].

Denaturation of protein is a well documented cause, of inflammation [21]. Therefore as part of the investigation to determine the anti-inflammatory mechanism of flavonoid rich fraction of *M. myristica,* its ability to inhibit protein denaturation was evaluated. It was observed from this study, that flavonoid rich fraction of *M. myristica* showed a dose-dependent maximum inhibition of denaturation of albumin of 75.38± 0.56% at 350 μg/mL: IC_{50} value of 258 μg/mL at correlation coefficient value (r) = 0.9796 while a standard anti inflammatory drug (aspirin) showed maximum inhibition of 98.41±0.13% at the same concentration Fig. 2.

Plant extracts have been reported to inhibit protein denaturation. Although, the precise mechanism of this membrane stabilization was yet to be elucidated, but it has been proposed that the extract might possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation [32]. These neutrophils lysosomal constituents include bactericidal enzymes and proteinases which upon extracellular release cause further tissue inflammation and damage [34].

In Fig. 3 is the result of lipoxygenase inhibition by flavonoid rich fraction of *M. myristica*. Flavonoid rich fraction of *M. myristica* was able to inhibit lipoxygenase enzyme in a concentration dependent manner with maximum percentage inhibition of 71.00±2.13%, which is comparable to that produced by indomethacin a standard anti inflammatory drug (87.00±1.32%).
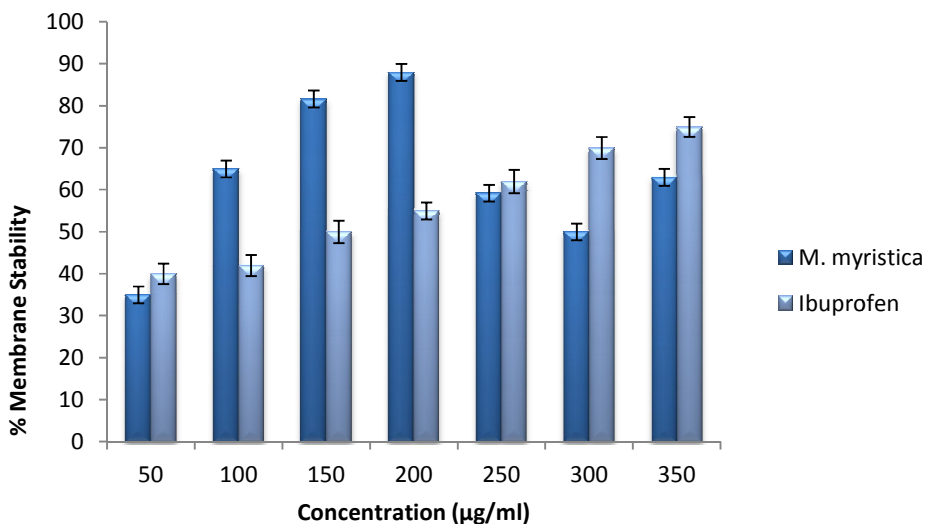

Fig. 1. Red blood cell membrane stabilizing activities of flavonoid rich fraction of *M. myristica* seeds
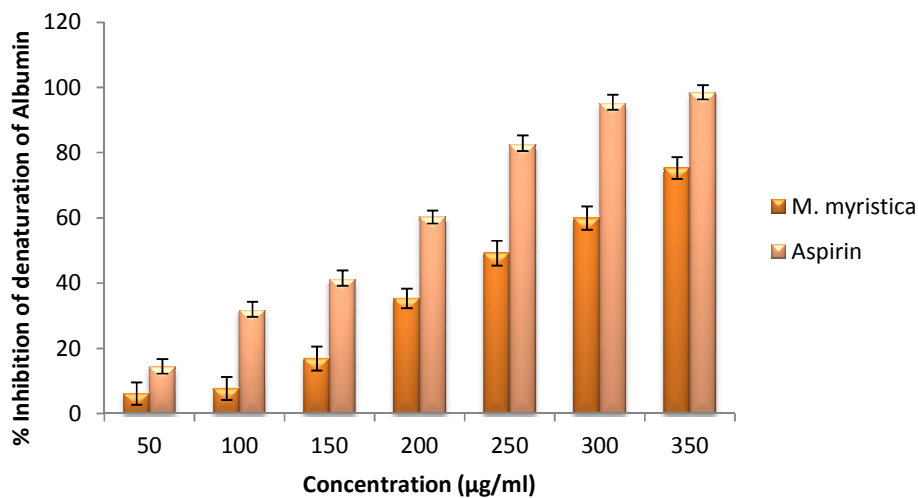

Fig. 2. Inhibition of denaturation of albumin by flavonoid rich fraction of *M. myristica* and Aspirin
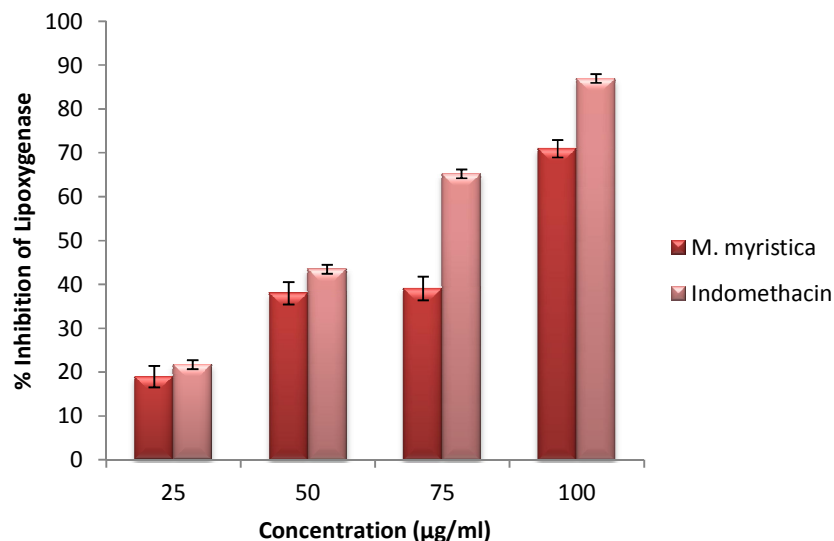
Fig. 3. Anti-lipoxygenase activities of flavonoid rich fraction of *M. myristica* and Indomethacin

The mechanism of anti inflammation involves a series of events in which metabolism of Arachidonic acid plays an important role. Arachidonic acid is cleaved from the membrane phospholipids upon appropriate stimulation of neutrophils, and can be converted to leukotrienes and prostaglandins through lipoxygenase and cyclooxygenase pathways respectively. Lipoxygenase catalyze the oxidation of Arachidonic acid (linoleic acid) to produce leukotrienes which are important mediators in a variety of inflammatory events [35]. Therefore, *in vitro* inhibition of lipoxygenase serves as a good model for the screening of plants with anti-inflammatory potentials. The inhibitory effect of flavonoid rich fraction of *M. myristica*on lipoxygenase activity could be attributed to its high phenolic content (flavonoid). Studies have shown that phenolic compounds may block the cascade process of arachidonic acid metabolism by inhibiting lipoxygenase activity and may serve as scavengers of reactive free radicals which are produced during arachidonic acid metabolism [36,37].

4. CONCLUSION

The results of this study showed that flavonoid rich fraction of *M. myristica* has anti-inflammatory properties. This provides the rationale for the employment of *M. myristica* as a medicinal plant used in the treatment and management of pain.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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