(International Print/Online Journal)

SJIF IMPACT FACTOR: 5.565
PUBMED-National Library of Medicine ID-101739732

ISSN (Print): 2209-2870
ISSN (Online): 2209-2862

International Journal of Medical Science and Current Research (IJMSCR)
Available online at: www.ijmscr.com
Volume 4, Issue 5, Page No: 1429-1439
September-October 2021

IJMSCR

Evaluation of Anti-Inflammatory Activity and Effect of Myristica Fragrans as Pulpotomy Medicament in Primary Teeth

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Type of Publication: Original Research Paper
Conflicts of Interest: Nil

Abstract

Background: Maintenance of Primary teeth until its physiological exfoliation is a very important consideration for normal development of facio-skeletal structures. There is a constant search for an ideal pulp medicament to save infected primary teeth. Many materials have been tried with each having certain draw-backs. *Myristica fragrans* (Nutmeg) is a wonder drug from nature with excellent beneficial biological properties. The aim of this study was to assess the effect of *Myristica fragrans* as a pulpotomy medicament in primary teeth clinically and histologically.

Methods: Essential oil of *Myristica fragrans* was evaluated for anti-inflammatory activity using In-vitro methods. A clinical and histological assessment of 20 pulpotomized primary teeth indicated for orthodontic extraction was done using essential oil of Nutmeg. These teeth were divided into two groups of equal size depending on follow up period. All the teeth were evaluated clinically and group1 teeth were extracted after 7 days and teeth in group 2 were extracted after 45 (6 Weeks) days. Extracted teeth were subjected for histological evaluation.

Results: Essential oil of *Myristica fragrans* has effective anti-inflammatory activity as shown by protein denaturation and Human red blood cell membrane stabilization methods. All the pulpotomized teeth showed no signs of pain, abscess or mobility. Histological sections of the extracted teeth showed signs of healing tissue.

Conclusion: *Myristica fragrans* exhibited excellent anti-inflammatory activity. It demonstrated good healing effect on primary tooth pulp. Thus, *Myristica fragrans* can be used in pulpotomy of the Primary teeth.

Keywords: Anti-inflammatory**,** Myristica fragrans, Pulp medicaments

INTRODUCTION

Presence of healthy primary teeth in the dental arch until their normal exfoliation is very important for proper growth and development of jaws and facio-skeletal complex with good functional occlusion and aesthetics. Thus, endodontic treatment of pulpally infected primary teeth is essential to maintain these teeth in function. Any product from mother nature basket is source of attraction in treating disease. In recent years use of natural products in dentistry is increasing. One such natures gift is *Myristica fragrans*, which is popularly known as “nutmeg”.

M. fragrans is an aromatic evergreen tree usually growing to about 5m to13m high, occasionally 20m. It produces two spices: mace and nutmeg. Nutmeg is the seed kernel inside the fruit and mace is the red lacy covering (aril) on the kernel. Nutmeg is popular as a spice and also possesses various therapeutic properties.[1] Nutmeg has a characteristic pleasant fragrance and a slightly warm taste, used for flavouring many kinds of baked foods, confections, puddings, meats, sausages, tea, vegetable dishes and beverages. *Myristica fragrans* has been investigated for a variety of biological properties. In traditional Ayurvedic, Chinese and Thai medicine nutmeg is used in the treatment of rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhoea, flatulence and anxiety in addition to use as aphrodisiac and abortifacient. The constituents of *Myristica fragrans* are found to have antimicrobial, anti-inflammatory, antioxidant, antidiabetic, antidepressant, aphrodisiac, memory enhancing, hepatoprotective, pesticidal and anti-cancerous pharmacological activity [2-10]. Nutmeg possesses medicinal properties that are anti-bacterial, anti-inflammatory, anti-oxidant and anti-collagenolytic in effect. As these are the properties very much required for a successful treatment of pulpitis, the present study was undertaken to find out the effect of essential oil of Nutmeg on the pulp of primary teeth [2-12].

MATERIALS AND METHODS

It is a preliminary interventional clinical and histological study. After obtaining clearance from Institutional Ethics committee and review board, a total of 20 children, aged between 4 to 10 years were recruited based on inclusion and exclusion criteria. A written informed consent was taken from the parent/legal guardian before the start of the procedure and a documented assent from each child was recorded.

Extraction of Essential oil of *Myristica fragrans*:

The hydro distillation method had been used for the extraction of essential oils from nutmeg using Clevenger's Apparatus. Nutmeg, the kernel of *Myristica fragrans* were grounded into fine powder. The distillation flask of 500 ml contained distilled water about 2/3rd of its volume and 50 gm of the powder. Heat was applied to the flask and the volatile oil was carried with the steam to a cold condenser, the lighter oil rises to the top of the separator. This process was carried out for about 12 hours until the oil inside the water runs out and stops collecting. The volume of essential oils was determined from a calibrated trap. The essential oils collected was dried over anhydrous sodium sulphate, weighed and stored in a sealed dark coloured vial at 4° C. [13-15]

Determination of anti-inflammatory activity:

Anti-inflammatory activity by protein denaturation method:

Principle:

Steroidal and nonsteroidal anti-inflammatory agents share a large number of different pharmacological actions. This multiplicity may result from independent interactions with various cellular sites or it may represent multiple secondary indications of a fundamental biochemical effect. The inherent attractiveness of the second possibilities calls for care in viewing an effect as fundamental; nevertheless, the possibility of reconciling some of the numerous actions of anti- inflammatory drugs requires that promising leads be examined. One such lead is the inhibition of protein denaturation by anti-inflammatory drugs reported by Mizushima et al. [16] This effect seems to be related to the reversal of enhanced plasma protein coagulability in drug treated animals. Denaturation may be broadly defined as any modification of the secondary, tertiary or quaternary structure of the protein molecules which does not involve the breaking of covalent bonds.' Nearly all modifications of a protein's environment can be shown to produce some measurable alteration of its native structure and, since such structural changes may be reversible and quite subtle (for example, the behaviour of allosteric enzymes), it is not necessary to envisage drastic pathological counterparts to changes imposed in vitro. Denaturation affects nearly all physico-chemical properties of protein molecules. To approach an understanding of the behaviour of anti- inflammatory agents, several kinds of denaturing conditions should be imposed and several different parameters measured.[17] In the present study we examined the influence of anti-inflammatory effect of *Myristica fragrans* on the denaturation of egg albumin induced by heat.

Methodology:

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Protein denaturation assay was done according to the method described by Gambhire et al[18], with some modifications as described in Gunathilake et al. The reaction mixture (5 mL) consisted of 0.2 mL of 1% egg albumin, 4.78 mL of phosphate buffered saline (PBS, pH 6.4), and 0.02 mL of extract, and the mixture was mixed, and was incubated in a water bath (37^0 C) for 15 min, and then the reaction mixture was heated at 70^0 C for 5 min. After cooling, the turbidity was measured at 660 nm using a UV/VIS spectrometer (Optima, SP-3000, Tokyo, Japan). Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:[19]

% inhibition of denaturation = 100 × (1− A2/A1),

where A1 = absorption of the control sample, and A2 = absorption of the test sample.

Anti-inflammatory activity by HRBC (Human red blood cells) membrane stabilization method:

Principle:

Inflammation is the reaction of living tissues to injury, infection or irritation. Lysosomal enzymes released during inflammation produce a variety of disorders which leads to the tissue injury by damaging the macromolecules and lipid peroxidation of membranes which are assumed to be responsible for certain pathological conditions as heart attacks, septic shocks and rheumatoid arthritis etc. The extra cellular activity of these enzymes is said to be related to acute or chronic inflammation. Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane.[26] HRBC or erythrocyte membrane is analogous to the lysosomal membrane[27] and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an in vitro measure of anti- inflammatory activity of the drugs or plant extracts.

Procedure for HRBC Membrane Stabilization Assay: [12,28,29,30]

The human red blood cell (HRBC) membrane stabilization method has been used as a method to study the in vitro anti-inflammatory activity. The blood was collected from healthy human volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment and mixed with equal volume of Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid and 0.42% NaCl). All the blood samples were stored at 4 °C for 24 h before use. It was centrifuged at 2500 rpm for 5 min and the supernatant was removed. The cell suspension was washed with sterile saline solution (0.9 % w/v NaCl) and centrifuged at 2500 rpm for 5 min. This was repeated three times till the supernatant was clear and colourless and the packed cell volume was measured. The cellular component was reconstituted to a 40 % suspension (v/v) with phosphate buffered saline (10 mm, pH 7.4) and was used in the assay. Hypotonicity Induced Human Red Blood Cell (HRBC) Membrane Stabilization Method, 1.0 mL of test sample of different concentrations (20µg – 200 µg) in 1 ml of 0.2 M phosphate buffer and 0.5 mL of 10% HRBC suspension, 0.5 ml of 0.25 % hyposaline were incubated at 37°C for 30 min and centrifuged at 3,000 rpm for 20 min. and the haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac was used as standard and a control was prepared by distilled water instead of hypo saline to produce 100 % haemolysis without plant extracts. The percentage of HRBC haemolysis and membrane stabilization or protection was calculated by using the following Formula:

% Of Hemolysis = Optical density of test/ Optical density of control × 100 sample

% Protection = 1 – (Optical density of test sample/ Optical density of control × 100)

Pulpotomy Clinical Procedure:

Criteria for selection: [31-34]

The following were the inclusion criteria for the study:

1.Tooth should be vital with healthy periodontium.

2.Pain if present should neither be spontaneous nor persistent.

3.Tooth should be restorable.

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4.Tooth should possess at least 2/3rd of the root length.

5.Haemorrhage from the amputation site should be pale red and easy to control.

6.Children who were to go for serial extraction procedure or with over retained deciduous teeth

7.Antibiotics should not be received by patient at least one week prior to the treatment.

8.Child patient who gave assent.

9.Parents consents for the procedure and willing to be the part of the study.

The following were the exclusion criteria for the study:

1.Evidences of internal resorption.

2.Presence of any interradicular bone loss.

3.Existence of abscess or fistula in relation to the tooth.

4.Radiographic signs of calcific globules seen in pulp chamber.

5.Caries penetrating floor of pulp chamber.

6.Tooth close to natural exfoliation.

Total number of Sample (n-20) were divided into 2 groups of 10 subjects each depending on follow-up time period:

Group 1: 10 teeth for evaluation after 1 week (7 days) followed by extraction.

Group 2: 10 teeth for evaluation after 6 weeks (45 days) followed by extraction.

Pre-operative radiographs were taken to assess the tooth before the beginning the procedure. Routine pulpotomy procedure was done on the indicated teeth using essential oil of *Myristica fragrans*. Then coronal pulp was removed using spoon-excavator and the sample was sent for histopathological examination. The pulp chamber was cleaned with normal saline, followed by haemostasis with wet cotton pellets and then essential oil of *Myristica fragrans* was applied over the root stump. This was followed by placement of non-eugenol containing temporary restorative material Provipast and then final restoration of the cavity was done with GIC. All the Patients were recalled after 7 days for checking-up any clinical symptoms of pain, mobility, abscess. For group 1 extraction was done on day 7 for group 2 patients were again recalled after 6 weeks, teeth were clinically evaluated followed by extraction of the treated tooth. Extractions of all the teeth was performed and the extracted tooth was kept in formalin containing container and sent for histopathological evaluation [**Figure 1**].

Histologic Evaluation:[31-36]

Decalcification of the tooth samples was done by using week acid, buffered formic acid. The sample was then processed by routine tissue processing methods and embedded in paraffin wax block. Sections of 4µm -5µm thick in bucco-lingual directions were obtained and these sections were further stained using routine Haematoxylin and Eosin staining procedure and were observed under 40x and 100x magnifications in optical microscope.

RESULTS

The results are summarized in Table 1. The present findings exhibited a concentration dependent inhibition of protein (albumin) denaturation by the test extract throughout the concentration range of 50 to 5000µg/ml. Diclofenac sodium was used as the reference drug which also exhibited concentration dependence.

In **Table 2** the values of the sample are compared to a positive control to check the percentage of haemolysis of HRB cells and percentage of protection **(Figure 2**). Minimal concentration of 200µg the sample showed a haemolysis of 55.09% while the positive control's activity on haemolysis is 17.02 %.

All the pulpotomized teeth in both the groups showed no signs of abscess, mobility, pain after 2 months from the date of completion of procedure.

The decalcified sections of the extracted teeth at day 7 showed intact radicular pulp with features of vital pulp like disrupted odontoblastic layer, delicate fibro-cellular connective tissue stroma with blood vessels and few chronic inflammatory cells and extravasated RBC's (**Figure 3).** Specimens after 6 weeks showed disorganized odontoblastic layer along the dentine, connective tissue with sparse inflammatory cells **(Figure 4).**

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DISCUSSION

There are certain problems associated with use of animals in experimental pharmacological research such as ethical issues and the lack of rationale for their use when other suitable methods are available, or could be investigated. Hence, in the present study the protein denaturation bioassay was selected for in vitro assessment of anti-inflammatory property of essential oil of *Myristica fragrans*. Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto-antigens in certain arthritic diseases may be due to denaturation of tissue proteins in vivo. [21,22] Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development. In the present study the in vitro anti-inflammatory effect of *Myristica fragrans* was evaluated against the denaturation of egg albumin (protein denaturation); however, the effect of *Myristica fragrans* was comparable with diclofenac sodium at higher concentration as shown in the **Table1**. The increments in absorbance of test sample in our study with respect to control indicated stabilization of protein i.e., inhibition of protein (albumin) denaturation or anti-denaturation effect by the test extract and the reference drug diclofenac sodium.[23] In the present study, the in vitro anti-inflammatory effect of *Myristica fragrans* may be due to its polyphenol contents.[24] The effect may be due to synergistic effect rather than single constituent. It has been reported that one of the features of several non- steroidal anti-inflammatory drugs is their ability to stabilize (prevent denaturation) heat treated albumin at the physiological pH.[25] Therefore, from the findings of the present preliminary experiment it can be concluded that the *Myristica fragrans* has marked anti-inflammatory effect against the denaturation of protein in vitro.

The lysosomal membrane is similar to the erythrocyte membrane and its stabilisation implies that the extract may as well stabilise lysosomal membranes. The importance of stabilising the lysosomal membrane is in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as protease and bactericidal enzymes which cause cellular release. Though the exact mechanism of the membrane stabilization by the extract is not known yet, hypotonicity induced haemolysis may rise from shrinkage of the cells due to osmotic loss of intracellular electrolytes and fluid components. The percentage of haemolysis is also shown in **Table 2**. As and when the concentrations of the extract increase, the haemolysis activity decreases. When the concentration was 1000µg, the samples activity on haemolysis was 24.12% while the positive control activity was 2.55%. Thus, lower the percentage of haemolytic activity, greater the anti-inflammatory. In the verified results, both the sample and positive control showed anti-inflammatory activity, but positive control being more efficient.

The sample is compared with a positive control to check the percentage of protection of HRBC cells. As seen in **Table 2,** when started with a minimal concentration of 200µg the sample showed a protection of 44.90% while the positive control activity on protection was 82.97%. As and when the concentrations increase, the protective ability of both the sample and the positive control were increased. When the concentration was 1000µg, the samples protective activity was 75.87% while the positive control activity on protection was 97.44%. This is depicted in the form of graph in **Figure 2**.

In the above results, obtained from the performed experiments, the positive control or standard (Diclofenac) has more efficient anti-inflammatory effect compared to Nutmeg extract. But the standard being a synthetic drug, is associated with many side effects such as abdominal pain, stomach bloating, burning, cramping, belching, tarry stools, cloudy urine, constipation, decrease in urine output or decrease in urine concentrating ability, diarrhea, dizziness, feeling of indigestion, headache, increased bleeding time, itching, skin or rash, loss of appetite, nausea and vomiting, pain in the chest below the breast bone, pale skin, severe stomach pain, swelling, troubled breathing with exertion, unusual bleeding or bruising, unusual tiredness or weakness, vomiting of blood or material that looks like coffee grounds and weight loss.[36] Comparatively, Nutmeg spice is a good source of minerals like copper, potassium etc. being a natural compound has very less side effects and can be used in combination with other compounds for better anti- inflammatory activity in the future.[37]

Inflammation, which is the host response to tissue injury or insult, plays a central role in the

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pathogenesis of diseases.[31] Neutrophils act as the first line of defence followed by the monocytes/ macrophages, T cells and B cells. Once they are activated, they produce inflammatory mediators such as anaphylatoxins of the complement cascade, kinins of the coagulation system, leukotrienes, prostaglandins and neuropeptides.[38,39] Various pro-inflammatory cytokines have been implicated in the destruction of the tissue. Cytokines responsible for early responses are IL1α, IL-1β, IL- 6 and TNF-α.[40] Other pro-inflammatory mediators include leukaemia inhibiting factor (LIF), IFN-γ, oncostatin M (OCM), ciliary neurotrophic factor (CNTF), transforming growth factor beta (TGF-β), IL-11, IL-12, IL-17, IL-18, IL-8 and a variety of other chemokines that attract inflammatory cells.[41-46] In the inflammatory process there is also the expression of cellular adhesion molecules, such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM).[47] The essential oil of nutmeg contains limonene, β-pinene, α-pinene and sabinene which are known 5-lipoxygenase inhibitors.[48] Limonene also is a COX-2 selective inhibitor having significant inhibitory effects on PGE2 production.[43] Terpene-4-ol which is 7.2% in seed oil and 23.6% in mace oil suppresses the production of TNF-α and IL-1β, IL-8, IL-10 and PGE2.[38,50] α-pinene was able to reduce pro-inflammatory IL-6 production in mouse colon but does not suppress IL-1β45. Sabinene, eugenol, α-pinene inhibits TNF-α production.[47] Sabinene also inhibits IL-1β and IL-6.[42] A study by Morita et al, among 21 spices, only myristicin - the major component of nutmeg oil - presented a potent hepatoprotective activity due to the inhibition of TNF-α release from macrophages.[52]

Pulp samples of the teeth at day 7 and day 45 in our study exhibited good healing properties on the tissue. As *Myristica fragrans* has shown very good anti-bacterial properties against endodontic pathogens [53] and our present study establishes its anti-inflammatory activities and has shown good healing on the pulp tissue of primary teeth *M fragrans* can be used as an effective alternative pulp medicament in primary teeth.

CONCLUSION

Myristica fragrans, a popular spice and a traditional medicine with amazing beneficial biological properties has shown positive effects on primary tooth pulp. *M fragrans* being a natural product it is very economical and affordable, so can be considered as an effective alternative in pulp therapies.

LIMITATIONS

The present study is a preliminary interventional histological study showing positive effect of *Myristica fragrans* as Pulp medicament. long-term clinical studies with higher number of study participants will be more beneficial.

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Figure: **1**

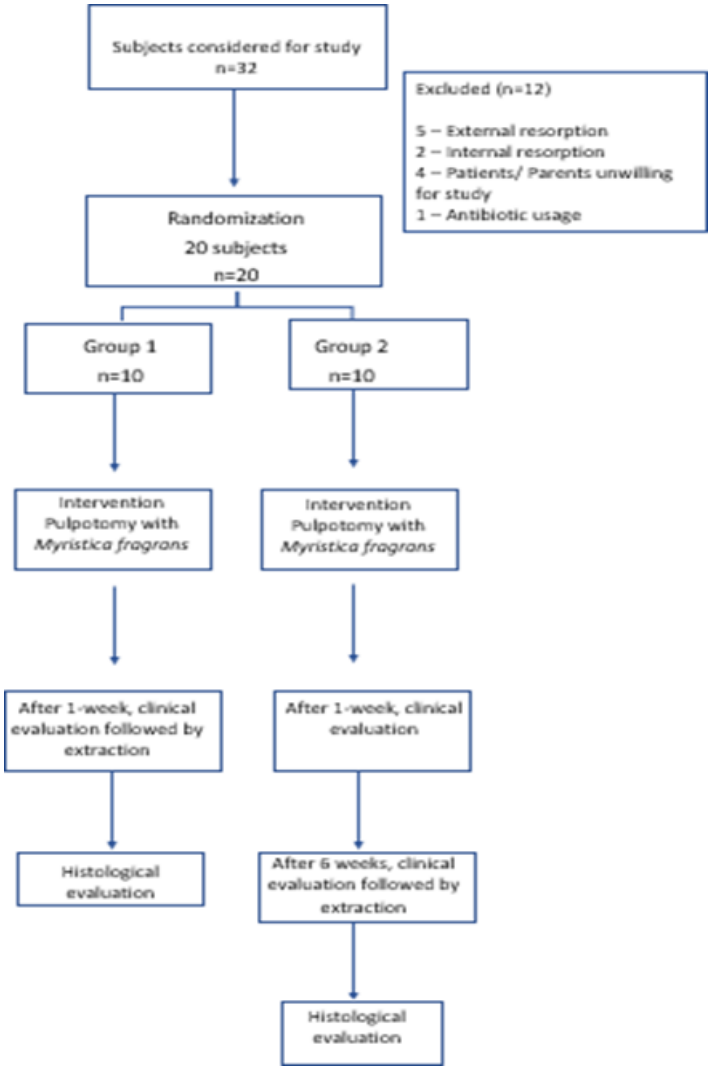

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Figure: 2 Percentage protections on HRBC

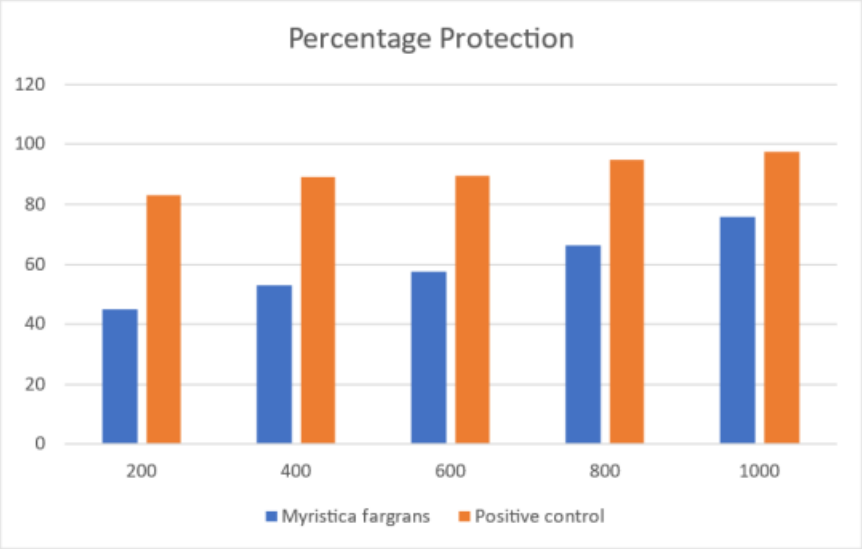

Fig: 3 H& E-stained histological section.

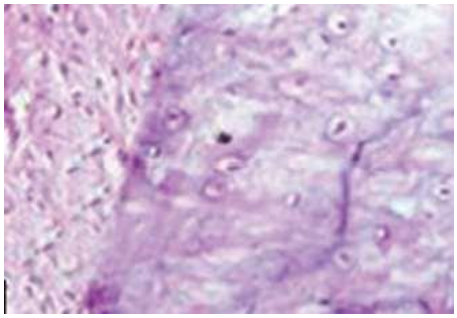

Fig 4 H & E-stained histological sections at 6 weeks

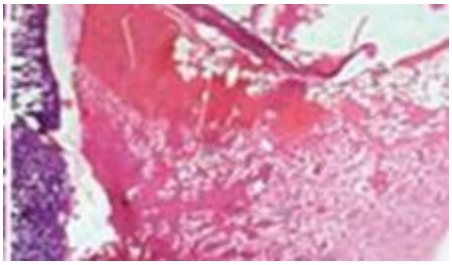

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Table 1: Percentage of Inhibition of Protein denaturation

Inhibition of protein denaturation				
Conc. Of drug (µg/ml)	Myristica fragrans			Diclofenac Sodium
50 µg	28.1	27.7	26.9	31.111
100 µg	34.3	33.7	34.1	36.213
200 µg	42.4	42.5	42.1	44.01
400 µg	51.2	50.7	50.7	52.19
500 µg	58.1	57.2	57.2	59.7

Table 2 - Anti - inflammatory activity by HRBC method-Percentage of haemolysis and percentage of protection of HRBC by *Myristica fragrans* and Diclofenac Sodium

	Percentage haemolysis		Percentage Protection	
Concentration (µg/ml)	Myristica fragrans (sample)	Positive Control (Diclofenac Sodium)	Myristica fragrans (sample)	Positive Control (Diclofenac Sodium)
200	55.09766	17.02082	44.90234	82.97918
400	46.91994	10.968019	53.08006	89.031981
600	42.584246	10.58167	57.415754	89.41833
800	33.784074	5.022537	66.215926	94.977463
1000	24.125349	2.5541962	75.874651	97.445804

LEGENDS

Figure 1: Methodology – Flow chart.

Figure 2: Percentage protection on HRBC.

Figure 3: H& E-stained histological section.

Figure 4: H & E-stained histological sections at 6 weeks.

Table 1: Percentage of Inhibition of Protein denaturation.

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