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Anti-Protease activity of *Myristica fragrans* compared to doxycycline on Periodontal Tissues: An Ex-Vivo study

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ABSTRACT

To evaluate the anti-protease activity of *Myristica fragrans* compared to doxycycline on periodontal tissues. Tissue sample from a patient of chronic periodontitis was taken and used for evaluation of the anti-protease activity. A protease assay was set up to evaluate the anti-protease ability of *Myristica fragrans* and doxycycline. *Myristica fragrans* when added to the tissue sample showed no zone of clearance when compared to a big zone of clearance of tissue sample alone. Doxycycline shows a small zone of clearance. *Myristica fragrans* possesses a better anti-protease activity as compared to doxycycline in-vitro.

Keywords: *Myristica fragrans*, nutmeg, periodontitis, anti-collagenolytic, protease assay

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INTRODUCTION

The supporting structures around the teeth or the periodontium consist of two soft tissues- the gingiva and periodontal ligament and two hard tissues- the cementum and alveolar bone. The cells along with the extracellular matrix work as an integrated structure that serves to anchor the teeth. The periodontium is constantly subjected to various physical and bacterial assaults. Yet it is able to maintain its integrity as it is continuously able to re-model its connective tissue components. The main structural component of the periodontium is proteins. Collagen forms 60% of the protein content of the periodontium. Ninteen types of collagens have been described so far which are broadly divided into 3 categories bases on their abilities to form fibrils- Fibril forming collagens, fibril associated collagens with interrupted triple helices and network forming collagens. [1] Apart from collagens, various non-collagenous proteins have been associated not only to provide structural architecture but also regulate cellular activities and tissue functions. The non-collagenous proteins associated with the periodontium are elastin, fibronectin, laminin, nidogen, tenacin, thrombospondins, vitronectin, osteopontin, osteonectin, osteocalcin and bone sialoprotein. Additionally, receptors for extracellular matrix- the integrins provide a valuable link between the extracellular matrix and the cytoskeleton.

Remodelling of the proteins associated with the extracellular matrix is essential to maintain the architecture of healthy periodontium. Regulation of collagen synthesis can take place at the gene transcriptional level or can be regulated post translationally by the extent of prolyl hydroxylation as under hydroxylated collagen is unstable at physiologic temperature and is degraded rapidly. Once the proteins are produced, their abundance is regulated by specific proteases called Matrix metalloproteinases (MMP). Based on the substrate specificity, they are broadly divided as collagenases, gelatinases, stromelysins and matrilysins. MMP-1 or the fibroblast collagenase is responsible with collagen degradation in a healthy periodontium.

Periodontitis is a chronic infectious inflammatory disease, the prime cause of which is bacterial insult on the periodontium. Six bacteria are considered mainly to be of periodontopathic nature. They are *Porphyromonasginigvalis (Pg), Treponemadenticola (Td), Tannerella forsythia (Tf), Prevotellaintermedia (Pi), Aggregatibacteractinomycetemcomitans (Aa), Capnocytophaga*. [2,3] However, the presence of bacteria is not enough to cause this complex nature of disease. The body in response to these bacteria produces various molecules to kill the bacteria but in turn it leads to destruction of the host itself. As soon as the microbe attacks the periodontium, there is a complex interplay of immune inflammatory reactions that begins with the release of chemokine interleukin-8 (IL-8). Chemokines are a class of chemotactic cytokines that stimulate the recruitment of relatively specific leukocyte subsets. IL-8 is secreted by epithelial cells, macrophage, monocytes and fibroblasts and it is chemo-attractant to neutrophils. The polymorphoneclear leukocytes, macrophages, kerationocytes and fibroblasts in turn lead to the production of MMP. The destruction of periodontal connective tissue and extracellular matrix by these proteases is the hallmark of periodontitis.

Dental pulpitis, which when left untreated leads to the formation of apical periodontitis. The prime pathogen associated with apical periodontitis is *Enterobacter foecalis* which leads to a cascade of host-immune interaction, following which the neutrophils are produced. Neutrophils in turn produce neutrophil type of collagenases that cause the peri-apical bone to resorb thereby causing subsequent pain and mobility of the tooth involved. The collagenases most commonly seen to be associated with peri-apical lesions are MMP-2, MMP-9 (gelatinases) and MMP-8, MMP-13 (collagenases) [4-6].

The main treatment strategy of periodontitis aims to reduce the bacterial load of the periodontium. Dental pulpitis demands endodontic invasion like root canal therapy to eliminate the bacterial load. Chronic periodontitis on the other hand is primarily treated by scaling and/or surgically by pocket elimination procedures. However, anti-proteases have been used to prevent the destruction of the protein component by proteases like MMP present in the extra-cellular matrix. The only FDA approved drug for the anti-protease activity till date is sub-antimicrobial dose of Doxycycline i.e. 20mg doxycycline hyclate that has shown to be effective in reducing excessive proteases during periodontitis. [7]

Myristica fragrans commonly known as nutmeg belongs to the kingdom: Plantae as Angiosperms, order: Magnoliales, family: Myristicaceae and genus: Myrstica.Myristica has been used traditionally in the ancient Ayurveda, chinese and Thai medicine. *Myristicafragrans* has four parts - The skin, the fruit, the seed and the mace. Fruit is a pendulous, succulent pericarp, the mace arillus covering the hard endocarp, and a wrinkled kernel with ruminated endosperm. When the arillus is fresh it is a brilliant scarlet, when dry it is more

of a horny, brittle texture, and a yellowish brown colour. Literature reports various therapeutic potentials of nutmeg like anti-microbial, anti-oxidant and anti-inflammatory effect. [8]

In this article we present the anti-protease activity of *Myristica fragrans* as compared to doxycycline in periodontitis. This is a preliminary experimental research on *Myristica fragrans* and it has not been reported in the literature before to the best of our knowledge.

SUBJECTS AND METHOD

The study has been approved by the Scientific Review Board of Saveetha University, Chennai. Ethical approval was taken prior to the collection of tissue sample from the patient.

Preparation of the doxycycline solution

Commercially available doxycycline (Vibramycin® by Pfizer Pharmaceutical Company) was used in the study. The doxycycline solution was prepared by adding 20mg doxycycline in 10ml distilled water. It was kept at room temperature for further use.

Preparation of Nutmeg solution

Dried mace of nutmeg was used for our study and ground into fine powder. Decoction of nutmeg was done by adding 2g of nutmeg powder in 50ml distilled water, boiling it for 10 minutes and filtered by a Whatman filter paper. This was stored at 4°C for further use.

Collection of gingival tissue

Before collection of the tissue sample, informed consent from the patient was taken after explaining the study to the patient. Chronic Periodontitis was diagnosed clinically as well as radiographically. The patient had no history of dental/ periodontal treatment previously. He has not been on any antibiotic, anti-inflammatory or hormonal treatment therapy for the past one year. The patient has no history of any other systemic diseases like diabetes, cardiovascular problems, hypertension or hormonal imbalances. Periodontal flap surgery for the treatment of periodontitis was performed under local anaesthesia. During the surgery, granulation tissue sample from the deepest periodontal pocket was taken for the study purpose. The tissue was rinsed in phosphate buffer saline (PBS) to remove any blood stains and was stored at -20°C in 1ml PBS for further use.

Preparation of tissue solution

The stored tissue was thawed to room temperature. 100mg wet weight of tissue sample was taken and a homogenate was made using mortar and pestle. The tissue homogenate was transferred in an eppendorf containing 1ml PBS and sonicated in a vibra cell sonicator for 1 minute at 4°C following which it was kept in a micro centrifuge at 10,000 revolutions per minute for 10 minutes at 5°C. The resultant solution was further filtered using a sterile microfilter. This is the final tissue sample solution that was used for the protease assay. It was stored at -20°C for further use.

Protease Assay

To qualitatively estimate the anti- protease activity of *Myristica fragrans* and doxycycline, we prepared a protease assay. The assay was prepared by boiling 0.5g Agar in 25ml of PBS till the Agar dissolves. To this, we added 0.25g of gelatin and mixed it well. This solution was poured in a petridish and kept undisturbed. After the solidification of this solution, four holes were made in the petridish and labelled as A (Nutmeg), B (Tissue Sample), C (Tissue Sample plus Nutmeg), D (Tissue Sample plus doxycycline). 50 µL of each A, B, C, D were added in the holes and left undisturbed for 48 hours and coomassie brilliant blue stain was added to observe the result.

RESULTS

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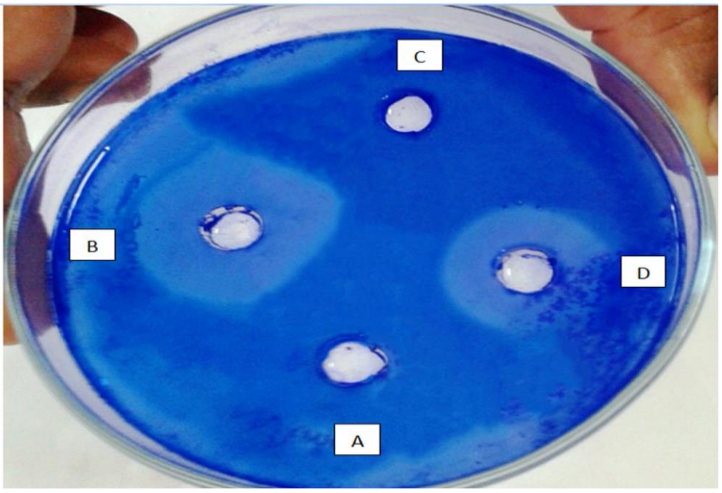
The result of the protease assay observed after 48 hours is shown in figure 1. Label A or nutmeg shows no clear zone. Label B or tissue sample shows the maximum zone of clearance (14mm). Label C or tissue sample with nutmeg shows no zone of clearance. Label D or tissue sample with doxycycline shows a little zone of clearance (9mm).



DISCUSSION

The destruction of extracellular matrix followed by breakdown of the periodontal tissues including the periodontal ligament and the alveolar bone is the main feature of periodontal disease. Elevated levels of collagenases (MMP-1, MMP-8, MMP-13) and gelatinases (MMP-2, MMP-9) have been reported in peri-apical lesions and periodontal diseases. The activity of these proteases has been shown to correlate with the severity of the disease and the depth of the pocket. [9] It has been suggested that the main source of these proteinases that mediate connective tissue breakdown is the polymorphonuclear leukocytes secreted in response to the infectious burden to the periodontal tissues. [10] Hence, inhibition of these proteases is extremely useful to treat periodontal disease [11].

Tetracyclines have been used as an adjunct to periodontal therapy for not only its antimicrobial effect but also for its anti-protease activity at low doses. Of these, doxycycline hyclate at 20mg has shown the best results as anti-collagenase agent. The pharmacological activity of tetracyclines lies in its ability to bind to the Zn^{2+} or Ca^{2+} which is the active binding sits of MMP, hence blocks its active sites. Tetracycline has however shown to possess various side effects like drug resistance, tooth discolouration, axorexia, nausea and fatty liver. Due to the adverse side effects, the use of herbal remedies as adjunct treatments to periodontitis has been extensively studied.

Myristica fragrans, a common Indian spice has been used since ancient times; however the lack of its scientific background and systematic literature has failed to bring it to clinical practice. In traditional medical applications, nutmeg was considered the king of spices when it came to oral health. The active antibacterial components of nutmeg mean that it helps to fight conditions like halitosis and boosts the immunity of gums and teeth. This is why nutmeg and its extracts are commonly found in toothpastes and mouthwashes, particularly in organic or herbal varieties. In the present study, we analysed the anti-protease efficacy of nutmeg in periodontal tissues using protease assay.

The prepared assay contains the protein gelatin. On addition of any substrate, if there is a zone of clearance, it implies that the substrate has a protease effect. However, no zone of clearance implies that there is no protease effect. In our experiment, nutmeg (A) shows no zone of clearance. This signifies that nutmeg does not have any protease ability hence does not contribute to the destruction of protein by itself. When the tissue sample solution (B) is added to the assay, it shows the maximum zone of clearance (14mm). This signifies that the tissue contains proteases and has cleaved the protein content present in the assay showing a big zone of clearance. When nutmeg is added to the tissue sample solution (C), there is a complete absence of any zone of clearance. This implies that nutmeg has completely nullified the protease ability of the tissue

sample and acts as a protease inhibitor or anti-protease agent. On adding doxycycline to the tissue sample solution (D), the zone of clearance has reduced when compared to the zone of clearance observed in the tissue sample solution alone. This signifies that doxycycline inhibits the protease ability present in the tissue sample but not to an extent nutmeg does.

Our present study is the first attempt in evaluating the anti-protease property of nutmeg that can be put to use in the management of periodontitis. It has also shown to have a better efficacy than the known standard drug doxycycline.

CONCLUSION

The present experimental research confirms *Myristica fragrans* to be a potent anti-protease drug. Further in-vivo studies need to be carried out to evaluate its toxicological profile before it can be put to use in periodontal therapeutics.

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