



Pharmacological Study

Effect of *Azadirachta indica* (Neem) and Aloe vera as compared to subantimicrobial dose doxycycline on matrix metalloproteinases (MMP)-2 and MMP-9: An *in-vitro* study

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Abstract

Background: A critical outcome of periodontal diseases is degradation of collagen in the periodontal tissues, by enzymes such as Matrix Metallo-Proteinases (MMPs). Doxycycline is known to down-regulate the activity of MMPs. *Azadirachta indica* (Neem) and Aloe vera are herbs known to have an anti-inflammatory effect. The present study was designed to evaluate the anti-inflammatory effect of Neem and Aloe vera by way of its inhibitory effect on MMP-2 and MMP-9 activity in cases of chronic periodontitis and compare it with doxycycline. **Materials and Methods:** A total of 30 subjects were enrolled in this study. Gingival tissue samples were obtained from patients diagnosed with the chronic periodontitis. The tissue extracts were treated with the said drug solutions and inhibition of MMP-2 and MMP-9 was analyzed. Enzymatic activity was detected by electrophoresis. The data was subjected to Student's paired *t*-test. **Results:** The results showed that the activity of MMP-2 and MMP-9 was significantly decreased by the use of doxycycline, Neem and Aloe vera. A 53.5% reduction in the MMP-2 and 52.5% reduction in the MMP-9 activity was seen when samples were subjected to Neem treatment at the concentration of 1500 µg/ml. Tissues treated with Aloe vera in the concentration of 2000 µg/ml showed a 20.09% reduction in the MMP-2 and 20.4% reduction in the MMP-9 activity. Doxycycline in the concentration of 300 µg/ml, showed an 82.1% reduction in the MMP-2 and 82.6% reduction in the MMP-9 activity. **Conclusion:** The present study demonstrated an inhibitory effect of Neem and Aloe vera on MMP-2 and MMP-9, which are involved in the extracellular matrix degradation during periodontitis.

Key words: Aloe, *Azadirachta*, doxycycline, matrix metallo-proteinases, periodontitis

Introduction

The periodontal diseases are specific mixed infections, which cause periodontal destruction in the susceptible host.^[1] The host reacts to the bacterial challenge by activating its defense mechanisms in an attempt to localize and eventually eliminate the pathogens.^[2] Matrix Metallo-Proteinases (MMPs) are believed to play a key role in tissue destruction and are capable of degrading denatured interstitial collagens. Periodontal treatment through the ages has focused on the reduction of bacterial infection by mechanical removal of infectious agents. Scaling and Root Planning (SRP) still remains the "gold

standard".^[3] Host modulatory therapy combined with traditional periodontal therapies like SRP and risk factor modification like smoking cessation therapy constitute a comprehensive treatment strategy for periodontitis. To date, Subantimicrobial Dose Doxycycline (SDD) is the only approved systemic therapy prescribed as a host response modifier.^[4] Medicinal plants such as *Azadirachta indica* A. Juss. (Neem) and Aloe vera are known to have a wide spectrum of biological activity.^[5] They are known to have an anti-inflammatory effect as well.^[6]

In view of the above facts, this study was designed to evaluate the anti-inflammatory effect of Neem and Aloe vera by their inhibitory effect on MMP-2 and MMP-9 activities and compare them with SDD.

Materials and Methods

Source of data

A total of 30 subjects both males and females, visiting the Department of Periodontology, Maratha Mandal's N.G.H.

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Institute of Dental Sciences and Research Center, Belgaum were enrolled for the purpose of the study. Prior to the initiation of the study, ethical clearance was obtained from the Institutional Ethics Committee. A written informed consent, in a language known to them, was obtained from all patients prior to the study.

Inclusion criteria

1. Probing depth and or clinical attachment loss of 5 mm or more
2. Presence of bleeding on probing.

Exclusion criteria

1. Patients with any systemic diseases/condition
2. Pregnant women
3. Smokers
4. Patients with a history of dental treatment in the last 3 months
5. Patients on any antibiotics or anti-inflammatory agents 3 months prior to the study.

Sample collection

Gingival tissue samples of 2-3 mm were obtained from patients diagnosed clinically as having chronic periodontitis with a probing depth and/or clinical attachment loss of 5 mm or more, in teeth indicated for extraction. Tissue samples were collected from marginal tissues before the tooth was extracted. All gingival samples following excision were briefly washed under saline and then transferred into small sterile plastic vials [Figure 1] containing the transport medium-Tris buffer and then was stored at -70°C until extraction of MMP-2 and MMP-9 was done.

Laboratory procedure

Preparation of *Neem* extract

Neem extract was prepared from the dried leaves of *Neem* by macerating 15.0 g of dry powder of *Neem* leaves with 100 ml of 70% (w/v) ethyl alcohol for a week in a round bottom flask with occasional shaking. The flask was kept in the dark to avoid the effect of light on the active ingredients of the *Neem*. The extract was then filtered through a muslin cloth for coarse residue and finally through Whatman No. 1 filter paper, measured and kept in an airtight amber color container.^[7]



Figure 1: Obtained gingival tissue sample

Aloe vera powder of 99% purity was procured from M/s Shanbag Ayur Products, Yallapur, Uttar Kannada district, Karnataka.

Preparation of drug solutions

Pure extract of *Neem*, *Aloe vera* and doxycycline was used for the purpose of the study. The *Neem* solution was prepared by dissolving 15 mg of *Neem* extract in 10 ml of distilled water (1500 $\mu\text{g/ml}$). The *Aloe vera* solution was prepared by dissolving 20 mg of *Aloe vera* in 10 ml of distilled water (2000 $\mu\text{g/ml}$). The doxycycline solution was prepared by dissolving 3 mg of doxycycline in 10 ml of distilled water (300 $\mu\text{g/ml}$).^[8]

Extraction of MMP-2 and MMP-9

For extraction of MMP-2 and MMP-9, frozen gingival tissue was allowed to thaw to room temperature. Then the tissue was mixed with Tris buffer, homogenized with Triton X-100 2.5% and centrifuged at 6000 rpm for 30 min at 4°C . The resultant supernatant was separated and used for analysis.

Pretreatment of gingival tissue extract with *Neem*, *Aloe vera* and doxycycline

To compare the inhibition of MMP-2 and MMP-9 activity, 50 μl of gingival tissue extract was preincubated with freshly prepared solution of 50 μl of *Neem* (1500 $\mu\text{g/ml}$), 50 μl of *Aloe vera* (2000 $\mu\text{g/ml}$) and 50 μl of doxycycline (300 $\mu\text{g/ml}$) solution separately, for 60 min at room temperature in separate vials. In addition, 50 μl of gingival tissue extract was also incubated with 50 μl of distilled water, which was used as the control [Figure 2].

Gel electrophoresis

The presence of MMP-2 and MMP-9 was studied by gelatin zymography. Pretreated tissue extracts were subjected to Sodium Dodecyl Sulfate (SDS)-Poly acrylamide Gel electrophoresis on 10% polyacrylamide containing 10% SDS and 1 g/l gelatin under non-reducing conditions without prior boiling. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 h to remove SDS and allow the protein to denature. The gels were subsequently immersed in a mixture containing Tris HCl 50 m M/l (pH 7.6) and CaCl₂ 20 mM/l for 16 h at 37°C . The gels were then stained with 0.25% Coomassie Brilliant Blue R250/40% methanol/10% acetic acid and destained with 30% methanol/10% acetic acid and 60% water. Enzymatic activities were detected as clear bands of gelatin lysis against the grey background [Figure 3]. To measure the relative MMP-2 and MMP-9 levels, clear zones were scanned and the percentages of inhibitors were analyzed by multi gel documentation system (Total Lab Phoretic software, UK).

Statistical analysis

The data were subjected to Student's paired *t*-test by using computer software (IBM-SPSS no. 12 trial version) to enable us to determine whether the inhibition by the test drugs is significant in comparison to the control group. A $P < 0.05$ was considered as significant.

Results

Table 1 shows the range, mean values and the standard deviation (SD) values for *Neem*, *Aloe vera* and doxycycline for inhibition of MMP-2. The mean values showed an 82.1% reduction in the MMP-2 activity with the addition of doxycycline, 53.5% reduction in the MMP-2 activity with the

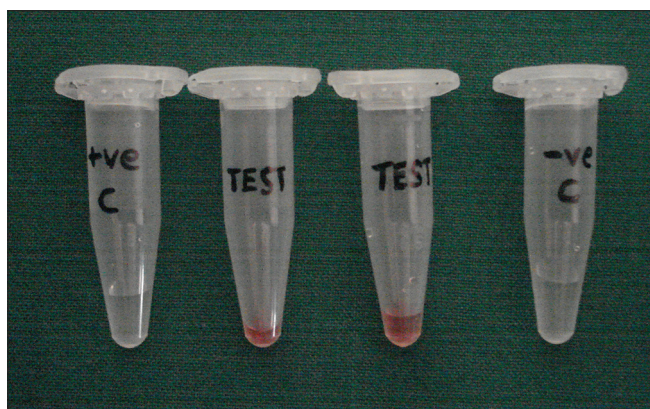


Figure 2: Pretreatment of gingival tissue extract with doxycycline (+ve c), *Neem* (test), *Aloe vera* (test) and distilled water (-ve c)

addition of *Neem* and 20.9% reduction in the MMP-2 activity with the addition of *Aloe vera* to the gingival tissue extract respectively under identical conditions.

Table 2 shows the range, mean values and the SD values for *Neem*, *Aloe vera* and doxycycline for inhibition of MMP-9. The mean values show 82.6% reduction in the MMP-2 activity with the addition of doxycycline, 52.5% reduction in the MMP-9 activity with the addition of *Neem* and 20.4% reduction in the MMP-9 activity with the addition of *Aloe vera* to the gingival tissue extract respectively under identical conditions.

Discussion

MMP-2 and MMP-9 (gelatinases) are widely distributed and have been identified in fibroblasts, keratinocytes, endothelial cells, monocytes/macrophages, osteoblasts and chondrocytes. Numerous studies have substantiated the relationship between these MMPs and periodontal diseases.^[3,9,10] In another study it is suggested that MMP-2 and MMP-9 could participate in tissue destruction in periodontitis.^[11]

The activity of MMPs is regulated by several types of inhibitors like tissue inhibitors of metalloproteinases, tetracyclines, doxycycline. Tetracycline's have traditionally been considered useful adjuncts in the treatment of periodontal diseases. Its inhibitory effect on collagenase might involve the drug's well-known ability to bind metal ions like Ca^{2+} and Zn^{2+} which are required by the enzyme to maintain its proper conformation and hydrolytic activity. This concept is supported by the observation that Ca^{2+} can overcome the inhibition of the enzyme by the tetracycline.^[12] In one of the recent report,^[13] it has been stated that they exert their anti-proteolytic effects by both direct inhibition of MMPs and by inhibiting their expression. Direct inhibition of MMPs appears to be mediated by an interaction between the tetracycline molecule and metal ions within the MMP; it appears that the mechanism of inhibition is dependent on chelation of structural metals rather than chelation of the active site Zn^{2+} . Doxycycline has a high affinity toward Zn^{2+} when compared with tetracycline, which is relatively much lower. Hence, it has been shown that doxycycline is more potent than tetracycline's.

Various clinical trials have tested the sub antimicrobial dose as a host modulating agent. Golub *et al.*^[14] reported that the

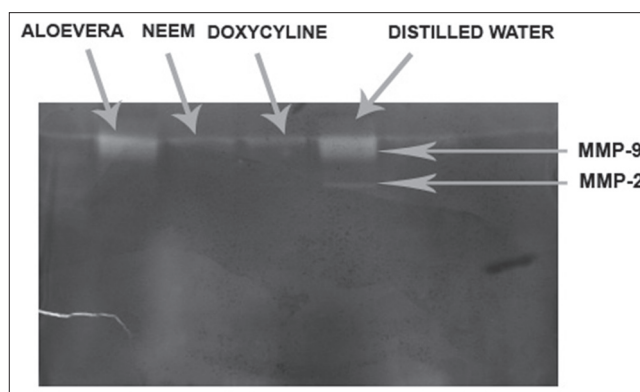


Figure 3: Gelatin zymography revealing bands of gelatin lysis showing matrix metalloproteinases (MMP-2) and MMP-9 inhibition by doxycycline, *Neem* and *Aloe vera*

Table 1: Range, mean value and standard deviations for doxycycline, *Neem* and *Aloe vera* for inhibition of MMP-2 in percentage

Particulars	Doxycycline	<i>Neem</i>	<i>Aloe vera</i>
Range	78-86	25-80	0-65
Mean	82.1	53.5	20.9
Standard deviation	2.08	15.80	16.20

MMP: Matrix metalloproteinase

Table 2: Range, mean value and standard deviations for doxycycline, *Neem* and *Aloe vera* for inhibition of MMP-9 in percentage

Particulars	Doxycycline	<i>Neem</i>	<i>Aloe vera</i>
Range	77-87	30-78	0-48
Mean	82.6	52.5	20.4
Standard deviation	2.53	11.89	13.79

MMP: Matrix metalloproteinase

semisynthetic compound doxycycline was more effective than the parent compound tetracycline in reducing excessive collagenase activity in the gingival crevicular fluid of chronic periodontitis.

The shift of focus today is towards nature cures and use of natural adjunct medicaments and therapies that can be termed as alternative therapies. Ayurvedic drugs such as *Neem*, *Triphala*, *Bakul* to name a few, have been used therapeutically since ancient times to treat diseases, including periodontal diseases. As these drugs are widely acclaimed for their minimal side-effects and cost-effectiveness, their use has increased in recent times. In an *in vitro* study,^[10] the ability of a blackcurrant extract and its major anthocyanins to inhibit the activity of MMPs was investigated. MMP-1 and MMP-9 were significantly inhibited by pure anthocyanins. This suggests that such natural compounds may represent promising agents for use in adjunctive treatments for periodontitis as well.

Like doxycycline, certain herbal extracts have also been shown to have the host modulation effect. One study, which evaluated the effect of *Triphala* on the activity of MMP-9 enzyme and authors found a significant inhibition of the enzyme activity.^[8]

Neem is known for its anti-inflammatory, astringent, antiseptic, antiviral, antihyperglycemic and immunostimulant properties. It has shown to have antiplaque activity as well.^[7]

This prompted us to under-take the present study, to evaluate the anti-inflammatory property of *Neem* on MMP-2 and MMP-9 in patients with chronic periodontitis. In the present study, we compared the anti-collagenase activity of *Neem* with that of doxycycline for both MMP-2 and -9. The results show mean inhibition of MMP-2 by doxycycline as 82.1% and of *Neem* as 53.5% [Table 1]. When the mean difference of inhibition of MMP-2 by doxycycline and *Neem* were compared by using Student's paired *t*-test, the value obtained was $P < 0.0001$ for doxycycline showing that doxycycline had significantly better inhibition of MMP-2 than *Neem*. The percentage inhibition of MMP-9 by doxycycline, *Neem* and *Aloe vera*, the results show mean inhibition of MMP-9 by doxycycline as 82.6% and of *Neem* as 52.5% [Table 2]. When the mean difference of inhibition of MMP-9 by doxycycline was compared with that of *Neem* by using Student's paired *t*-test the value obtained was $P < 0.0001$ for doxycycline showing that doxycycline had significantly better inhibition of MMP-9 than *Neem*.

Furthermore, the test of significance between doxycycline and *Neem* proved that doxycycline was significantly better than *Neem* in inhibition of MMP-2 and MMP-9 at the concentrations used in this study.

Elevated activity of MMP-2 and MMP-9 has been found in inflamed tissues from adult periodontitis patients.^[15] The results of the present study suggest that *Neem* does have an inhibitory effect on MMP-2 and MMP-9. This study can open avenues for furthering research in the area of use of *Neem* in the treatment of periodontal diseases with its specific action on MMP-2 and MMP-9 inhibition.

The current study also evaluated the inhibitory effect of *Aloe vera* on MMP-2 and MMP-9. *Aloe vera*, which has aloin as its active component, has shown to have anti-inflammatory, antimicrobial, immunostimulatory properties and is an antioxidant as well. Barrantes and Guinea^[6] showed that *aloe gels* and *aloin*s are effective inhibitors of stimulated granulocyte MMPs. *Aloe vera* has various therapeutic uses. The anti-inflammatory activities of *Aloe vera* in periodontal therapy has not been researched adequately yet. In a recent study, Bhat *et al.*^[16] evaluated the effect of *Aloe vera* in periodontal conditions. The results showed improvement in plaque index and probing depth.

The results of our trial, show mean inhibition of MMP-2 by doxycycline as 82.1% and of *Aloe vera* as 20.9% [Table 1]. When the mean difference of inhibition of MMP2 by doxycycline and *Aloe vera* were compared by using Student's paired *t*-test. The value obtained was $P < 0.0001$ for doxycycline showing that doxycycline was significantly better than *Aloe vera*.

Inhibition of MMP-9 by doxycycline in our study was 82.6% and of *Aloe vera* was 20.4% [Table 2]. When the mean difference of inhibition of MMP-9 by doxycycline was compared with that of *Aloe vera* by using Student's paired *t*-test the value obtained was $P < 0.0001$ for doxycycline showing that doxycycline had significantly better inhibition of MMP-9 than *Aloe vera*.

The test of significance between doxycycline and *Aloe vera* proves that doxycycline is significantly better than *Aloe vera* in inhibition of MMP-2 and MMP-9 at the concentrations used in

this study. However, the concentration of *Aloe vera* used in this study is only 2000 µg/ml. An increase in concentration of *Aloe vera* could probably render an even better inhibitory action on MMP-2 and MMP-9. The present study has established, that *Aloe vera* does have an inhibitory effect on MMP-2 and MMP-9.

We compared the percentage inhibitory effect of *Neem* with that of *Aloe vera* for both MMP-2 and MMP-9. The mean inhibition of MMP-2 by *Neem* was 53.5% and of *Aloe vera* was 20.9% [Table 1]. When the mean difference of inhibition of MMP-2 by *Neem* was compared with that of *Aloe vera* by using Student's paired *t*-test the value obtained was $P < 0.0001$ for *Neem*, showing that *Neem* had significantly better inhibition of MMP-2 than *Aloe vera*. For MMP-9, the mean inhibition by *Neem* was 52.5% and of *Aloe vera* was 20.4% [Table 2]. When the mean difference of inhibition of MMP-2 by *Neem* was compared with that of *Aloe vera* by using Student's paired *t*-test the value obtained was $P < 0.0001$ for *Neem*, showing that *Neem* had significantly better inhibition of MMP-9 than *Aloe vera*.

The present study was structured similar to the study performed by Abraham *et al.*^[8] where they evaluated the inhibitory effect of *Triphala* in percentage against MMP-9 along with that of doxycycline and kamillosan. The mean percentage inhibition of MMP-9 was 76%, 58.7% and 47.36% for *Triphala*, doxycycline and kamillosan respectively.

Conclusion

On the basis of the current trial, it can be stated that *Neem* achieved inhibition of MMP-2 and MMP-9 at 1500 µg/ml concentration, *Aloe vera* had an inhibitory effect on MMP-2 and MMP-9 at 2000 µg/ml concentration and doxycycline had an inhibitory effect on MMP-2 and MMP-9 at 300 µg/ml concentration.

This was a pioneering *in vitro* study to evaluate the efficacy of *Neem* and *Aloe vera* on MMP-2 and MMP-9 in periodontitis. The results of this study have been very encouraging and have established a fact that both *Neem* and *Aloe vera* have inhibiting effects on MMP-2 and MMP-9. Having established the efficacy of these agents *in vitro*, it would only be fair in the interest of science, to further research their efficacy in clinical settings.

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हिन्दी सारांश

मेट्रिक्स मेटॅलोप्रोटीनेजेस, एम.एम.पी.-२ एवं एम.एम.पी.-९ पर डॉक्सीसाइक्लिन की सब एण्टीमाइक्रोबियल मात्रा की तुलना में नीम एवं घृत कुमारी का प्रभाव

मिथुन डी. कुडाळकर, आरती नायक, किशोर एस. भट, रंगनाथ एन. नायक

पेरीओडोन्टल व्याधि में पेरीओडोन्टल उत्तकों का क्षय कुछ एन्जाइम जैसे मेटॅलोप्रोटीनेजेस के कारण होता है। डॉक्सीसाइक्लिन, एम.एम.पी. के प्रभाव को कम करने के लिए ज्ञात है। नीम एवं घृत कुमारी अपने शोधहर कर्म हेतु जाने जाते हैं। इस अध्ययन में नीम एवं घृतकुमारी के शोधहर कर्म का प्रभाव एम.एम.पी.-२, एम.एम.पी.-९ पर पेरीओडोन्टिस में डॉक्सीसाइक्लिन की तुलना में किया गया है। शोध के परिणाम में यह पाया गया कि डॉक्सीसाइक्लिन नीम एवं घृत कुमारी, एम.एम.पी.-२ एवं एम.एम.पी.-९ के प्रक्रिया को कम करने में अत्यंत प्रभावी हैं।