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EFFECT OF *ASPARAGUS CURILLUS* ON LEVELS OF MATRIX METALLO PROTEINASES IN GCF AND SALIVA OF PERIODONTAL PATIENTS

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ABSTRACT: This study was planned to evaluate the anti-inflammatory effect of roots of *Asparagus curillus* in periodontal inflammation *via* assessing gingival crevicular fluid (GCF) and salivary inflammatory markers namely MMP-8, -9, 13, TIMP-1 and IL-6. Samples of GCF and saliva were obtained from patients with chronic periodontitis (n = 98) and healthy controls (n = 27). Study population (n = 98) was divided into two groups - Group A: patients with periodontitis receiving only conservative periodontal treatment and Group B: patients with periodontitis receiving both the conservative periodontal treatment and Ayurvedic therapy in combination. Concentrations of periodontal inflammatory markers *i.e.* MMP-8, -9,-13, TIMP-1 and IL-6 were estimated by Enzyme linked immuno absorbent assay and were analysed at two different time intervals *i.e.* at baseline and 3 months after therapy. There was statistically significant decrease in the concentrations of MMP-8, -9, -13 in GCF and saliva in both the groups at 3 months after therapy. ($p < 0.05$) However, there was found a statistically significant lower concentration of the markers in Group B subjects than in Group A subjects at three months after therapy ($p < 0.05$). This data indicate that *Asparagus curillus* may show a promise as an anti- inflammatory drug and combined use of this plant with conservative periodontal therapy is more effective treatment for chronic periodontitis.

INTRODUCTION: Genus *Asparagus* (Asparagaceae) is called "King of vegetables" and is used in salads, vegetable dishes and soups [1]. It is mainly cultivated in temperate and tropical regions of the world [2]. There are about 300 varieties of this genus among which only 20 are edibles.

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Various compounds like saponins, flavonoids, oligosaccharides, amino acid derivatives, lignans and phenolic derivatives are isolated from this genus [3]. These compounds have numerous beneficial effects on human body like anti-inflammatory [4], anti-diarrhoea and anti-tuberculosis [5], anticancer [3], anticytotoxic [6], antimutagenic [7], antinociceptive and anti-inflammatory [8, 9]. For maintaining proper dental health, the ancient Indian system of medicine (Ayurveda) advises different methods for regular practice [5]. Ayurvedic medicine to treat chronic periodontitis (PD) is not new in India and China.

Thus, it is not surprising to see asparagus being heralded as an anti inflammatory food because it provides a truly unique combination of anti-inflammatory nutritients [10]. However, there is no reference in literature to our knowledge regarding the anti-inflammatory action of roots of *Asparagus curillus* (AC) on periodontal inflammation.

Periodontitis is a biofilm-induced inflammatory disease affecting the periodontium [11]. Cause of inflammation in chronic periodontitis is formation of bacterial biofilm of specific Gram negative species that results in weakening of periodontal attachment [12] which mainly happens through the degradation of type 1 collagen [13]. This bacterial biofilm also produces local or systemic responses that contribute to a systemic disease [14]. Articles in recent decade confirms that the periodontal pathogens or complex microbial biofilm has a potential association between periodontitis [15] and the occurrence of atherosclerosis, cardiovascular disease [16], coronary artery ectasia [17], diabetes [18], [19] and a higher risk of preterm low birth-weight babies [20]. Therefore, the treatment of periodontitis includes the removal of bacterial biofilm by different type of therapies.

Therapy use for the treatment of choronic periodontitis patient is conventional therapy includes various mechanical, non surgical and surgical methods like scaling and root planning (SRP) and various adjunctive anti-infectious methods like photo-dynamic therapy, antibiotic therapy, disinfectant therapy, and low dose doxycycline therapy. The major purposes of the therapies are to remove bacterial biofilms from the root surface of periodontium. But SRP is not able to remove these pathogenic bacteria from multi-rooted tooth, concavities, interproximal areas and deep pockets. Low dose doxycycline therapy in combination with SRP can reduce GCF-MMP-8 levels and improve clinical periodontal parameters in CP patients [21]. Dilsiz A *et al.*, 2013 [22] observed that Potassium-Titanyl phosphate laser and photodynamic adjunctive therapy did not provide additional clinical benefits than conventional scaling and root planning therapy. A evidence based assessment by Sgolastra *et al*., 2013 [23] on current available literature suggested that the adjective use of photodynamic therapy could provide additional short term benefits to SRP.

However, these beneficial effects seemed to be modest and not stable over the time. So, it is our necessary to improve conventional therapy. Bacterial biofilm causes degradation of collagen fibers and release of MMPs by resident cells of periodontal ligament [24, 25]. The most common type of MMPs are mainly of collagenases family and includes: MMP-8 [18, 26, 27, 28], MMP-13 [29], MMP-9 [15], MMP-14 [30]. Therefore, the assessment of these MMPs and their inhibitor in GCF and saliva is considered as important tool in diagnosis and prognosis of periodontal diseases [31].

In India, many herbs are used for cleaning teeth, so the use of plant material for maintenance of oral health and for the treatment of periodontal problems is more common and safer way. It is necessary to check the efficacy of herbs by clinical trials. So the purpose of the present study is to evaluate the effect of AC roots on serum concentrations of inflammatory markers present in patients with chronic periodontitis. The study aimed at comparing the levels of MMP-8, MMP-9, MMP-13, TIMP-1, IL-6 in GCF and saliva of CP patients and controls at baseline and 3 months after conventional therapy.

MATERIALS AND METHODS: This study has been conducted to evaluate the anti-inflammatory action of dry roots of *Asparagus curillus* (AC) on the periodontal inflammation. The study has been approved by local ethical committee. The written informed consent was obtained from each patient before their enrolment in the study.

Selection of Plant Material: The plant material "*Asparagus curillus*" (BSD 112754) used in the study was collected from hilly area of Srinagar, Uttarakhand, India. This plant was identified and authenticated by Botanical survey of India, Dehradun, India. A specimen voucher was made and deposited at the same institute. For the study purpose, 5g of stored refrigerated roots were used as chewing material by the subjects after every meal (*i.e.* three times a day for 5 min) for 3 weeks and advised, not to drink water for 1 h afterwards.

Patient Selection: Respective saliva and ginigival crevicular fluid (GCF) sample were collected from patients with periodontitis (n = 98) and healthy control subjects (n = 27). These periodontitis (PD)

patients were selected from the J. N. Kapoor D.A.V.© Dental college, Yamuna Nagar and consecutively enrolled with a diagnosis of chronic periodontitis. Subjects who had not received any kind of periodontal treatment, smoking, systemic metabolic diseases, aggressive periodontitis, platelet and coagulation disorder, unwillingness to participate in the study were considered under exclusive criteria. Criteria for diagnoses of chronic periodontitis was assigned according to the International Workshop for a Classification of Periodontal Diseases and Condition [32]. Subjects with >14 natural teeth, excluding third molar and including >10 posterior teeth with at least 5 - 6 teeth with sites of probing depth (>5 mm), attachment loss (>5 mm) detectable bone loss in radiographs (>50%) of supporting tissue were involved. Study population (n = 98) was divided into two periodontal groups 1) Group A include 49 patients with periodontitis (24 men and 25 women, mean age: 45.56 ± 12.3 year), 2) Group B include 49 patients with periodontitis (25 men and 24 women, mean age: 48.12 ± 13.6 year). The patients under Group A received only conservative periodontal management while patients under Group B received both the conservative management and dry root of AC in combination.

Conventional periodontal therapy (non-surgical cause related periodontal therapy) involves regular monitoring of probing depth, reinforcement of oral hygiene and re-motivation of patients with further scaling and root planning (SRP) over three to four week period using Gracey curettes (Hu-friedy Manufacturing Inc. Chicago). Subjects were followed up to 3 months after completion of therapy. In each visit, oral hygiene instructions were reinforced and supra gingival prophylaxis was carried out. From initial therapy to follow up visits, the patients were supervised by same clinicians.

In Control Group: 14 men and 13 women were selected (Mean age 51.6 ± 8.2 year). Subjects of this group were the staff of J. N. Kapoor D.A.V. © Dental College, Yamuna Nagar. These staff members were systemically and periodontally healthy and had neither history nor sign of periodontal disease. Levels of the inflammatory markers in saliva and GCF were analyzed at base line and three months after therapy in both the periodontitis groups as well as in healthy non periodontitis control group (n = 27).

Gingival Crevicular Fluid Sample Collection: GCF sample was collected with paper strips (Periopaper, Proflow Inc., Amity-ville, NY, USA), placed in the orifices of gingival sulcus pocket for 30 sec. Strips contaminated by saliva or blood were discarded. GCF was extracted from the strips by centrifugation by at 12,000 rpm (Remi R8C) for 5 min in 40 µl elution buffer containing 0.9 % NaCl. The elution procedure was repeated twice and eluted samples were stored at -80 ºC for further analysis [21].

Saliva Sample Collection: Non-stimulated whole saliva sample was collected as described by (app. 3 ml) [30, 33]. These samples were frozen at -80 ºC in sterile tubes for further use. For analysis frozen saliva samples were thawed and centrifuged at 9300 g for 3 min.

Estimation of Inflammatory Markers: In GCF and saliva, concentrations of MMP-8, MMP-9, MMP-13, TIMP-1, TIMP-2 and IL-6 were determined in duplicates for each subjects using an enzyme linked immune sorbent assay (ELISA kit, DuoSet R and D Systems Inc., Minneapolis, MN USA). These results were expressed in ng/ml, then being multiplied by the initial sample volume (0.1ml buffer + GCF volume) to obtain results as ng/sample [30, 33, 34]. For whole procedure, highly purified analytical reagents were used and obtained from Sigma Chemical Co. (UK). The analysis of the collected samples was done at two different time intervals *i.e.* baseline and after 3 months.

Statistical Analysis: Statistical analysis was done with the statistical package for the social science (Version 15, SPSS Chicago, III). GCF volume and periodontitis marker in GCF and saliva were expressed in mean ± S.D. The comparisons between two related groups were analyzed by using the paired t- test or Wilcoxon test depending on data distribution and the comparisons between two unrelated groups were analyzed by the unpaired t-test (normal distribution) and Mann Whitney test.

Spearman correlation was applied to determine association between variables. $p<0.05$ was considered statistically significant.

RESULTS: This study was performed to evaluate the anti-inflammatory action of dry roots of *Asparagus curillus* (AC) in periodontal inflammation. In this present study, the concentrations of different inflammatory markers like MMP-8, MMP-13, TIMP-1 in saliva and GCF were measured. In addition to this, saliva and GCF were also assessed for MMP-9 and IL-6 levels respectively. The characterization of study population by age and periodontal status is presented in **Table 1**.

TABLE 1: DEMOGRAPHIC DATA AND CLINICAL PERIODONTAL CHARACTERISTICS OF STUDY GROUP (MEANS ± S.D)

Variables	Healthy individuals	Patients with Periodontitis (n = 98)		
	Control Group (n = 27)	Group A (n = 49)	Group B (n = 49)	*p-value
Age (years)	51.6 ± 8.2 [a]	45.56 ± 6.1[a]	48.56 ± 8.6 [a]	0.002*
Gender: Men / Women	14/13	24/25	25/24	--
No. of remaining teeth	29.2 ± 2.6 [a]	25.4 ± 3.1 [a,b]	24.9 ± 4.6 [a,b]	<0.001*
Probing depth (PD) < 3mm	0[a]	68.9 ± 8.1[b]	66.2 ± 7.9 [b]	<0.001*
Probing depth (PD) > 3mm	0[a]	25.6 ± 6.9 [b]	27.1 ± 5.2 [b]	<0.001*
Clinical attachment lose (Cal) (mm)	2.14 ± 0.07 [a]	4.24 ± 0.16 [b]	4.37 ± 0.18[b]	<0.001*

Data are shown in Mean ± S.D

[a,b] For the Mann- Whitney test groups with a different superscript letters were statistically significant ($p<0.05$).

*Statistically significant for Kruskal –Wallis one-way analysis when compared among the groups ($p<0.05$).

PD-Probing depth, Cal = Clinical attachment lose.

Analysis of Salivary Sample: Table 2 shows levels of inflammatory makers in control, Group A and Group B patients before and three months of after therapy. Of the tested salivary markers, MMP-8, MMP-9, MMP-13 and IL-6 were present in higher concentrations but TIMP-1 was present in lower concentration in periodontitis (PD) group in comparisons to healthy control. Analysis of levels of inflammatory markers showed a statistically significant decrease in the mean values of MMP- 8, -9 and -13 at three months after therapy in both the periodontitis groups in comparison to their baseline values ($p<0.05$). However, at three months after therapy, there was found a statistically significant lower concentrations of MMP -8, - 9, -13 and IL-6 but higher concentration of TIMP-1 in Group B subjects than in Group A subjects ($p<0.05$).

TABLE 2: SALIVARY CONCENTRATIONS (MEAN ± S.D) OF PERIODONTITIS (PD) MARKERS AT BASELINE AND 3 MONTHS AFTER THERAPY IN DIFFERENT GROUPS

Variables	Healthy individuals (n=27)		Group A (n=49)			Group B (n=49)		
	Baseline	3 month	Baseline	3 months	[#] *p*-Value healthy individuals *vs.* Group A	Baseline	3 months	*p*-Value healthy individuals *vs.* Group B
MMP-8 (ng/µL)	62.8 ±41.5[β]	60.8 ±47.2[β]	99.8 ±65.2[α]	85.5 ±40.8[α]	>0.001	96.7 ±42.9*	74.3 ±25.8*	>0.002
MMP-9(ng/µL)	65.1 ±24.9[β]	67.3 ±27.8[β]	95.2 ±38.5[α]	83.4 ±35.9[α]	>0.001	92.8 ±39.1*	73.1 ±29.7*	>0.002
MMP-13(ng/µL)	0.11 ±0.07[β]	0.12 ±0.09[β]	0.4 ±0.23 [α]	0.28 ±0.15[α]	>0.002	0.38 ±0.26*	0.20 ±0.11*	>0.002
TIMP-1 (ng/µL)	3.17 ±0.99[β]	3.26 ±0.91[β]	1.81 ±1.12[α]	2.01 ±0.91[α]	>0.001	1.94 ±1.23*	2.46 ±0.85*	>0.001
IL-6 (pg/ µL)	8.78 ±2.14 [β]	8.90 ±2.49 [β]	20.16 ±5.11[α]	12.71 ±2.23[α]	>0.001	24.31 ±5.81*	13.83 ±2.42*	>0.001

Data are shown as Mean ± S.D

[α]$p<0.05$-Statistically significant value for comparison within Group A between baseline and 3 months (paired t-test)

*$p<0.05$-Statistically significant value for comparison within Group B between baseline and 3 months (paired t-test)

[#]$p<0.05$-Statistically significant value for comparison between healthy individuals and Group A at 3 months (unpaired t-test and Mann-Whitney test)

$p<0.05$-Statistically significant value for comparison between healthy individuals and Group B at 3 months (unpaired t-test and Mann-Whitney test)

[β]$p=0.02$ value for comparison within healthy individuals between baseline and 3 months (paired *t*-test)

MMP- Matrix metalloproteinase, TIMP-Tissue inhibitor of Metalloproteinase.

Analysis of GCF Sample: Similar results were observed for the levels of MMP-8, -9 and TIMP-1 in GCF samples ($p<0.05$). It was found that the levels of IL-6 showed a statistically significant reduction in both the periodontitis groups at three month after therapy in comparisons to their base line value ($p<0.05$).

However, Group B shows a statistically significant lower concentration of IL-6 with respect to Group A, three months after therapy shown in **Table 3** ($p<0.05$). In patients of Group A and B, concentration of MMP-13 did not show a statistically significant reduction as compare to healthy individuals (data not shown).

TABLE 3: CONCENTRATIONS OF PERIODONTITIS MARKERS (MEAN ± S.D) IN GCF AMONG DIFFERENT GROUPS

Variables	Healthy individuals (n=27)		Group A (n=49)		*#p-value*	Group B (n=49)		*p-value*
	Baseline	3 month	Baseline	3 months	Control *vs.* Group A	Baseline	3 month	Control *vs.* Group B
MMP-8(ng/ml)	70.8 ±18.6$^{\beta}$	68.6 ±39.1$^{\beta}$	95.6 ±19.1$^{\alpha}$	87.2 ±18.5$^{\alpha}$	>0.001	99.7 ±20.1*	78.7 ±11.5*	>0.005
MMP-9(ng/ml)	63.6 ±8.1$^{\beta}$	61.1 ±25.2$^{\beta}$	88.6 ±11.8$^{\alpha}$	81.9 ±14.9$^{\alpha}$	>0.002	90.3 ±17.3*	72.1 ±11.7*	>0.005
TIMP-1(ng/ml)	105.1 ±30.1$^{\beta}$	99.1 ±21.9$^{\beta}$	61.2 ±15.4$^{\alpha}$	75.1 ±13.7$^{\alpha}$	>0.002	64.3 ±12.8*	83.2 ±10.1*	>0.004
IL-6 (pg/ml)	11.4 ±0.5$^{\beta}$	13.2 ±1.25$^{\beta}$	34.2 ±7.6$^{\alpha}$	25.4 ±9.3$^{\alpha}$	>0.002	31.5 ±6.8*	16.7 ±5.1*	>0.0001
Volume of GCF (µl)	0.25 ±0.15$^{\beta}$	0.27 ±0.17$^{\beta}$	1.08 ±0.21$^{\alpha}$	0.65 ±0.25$^{\alpha}$	>0.001	1.25 ±0.15*	0.59 ±0.22*	>0.001

Data are shown as Mean ± S.D

$^{\alpha}p<0.05$-Statistically significant value for comparison within Group-A between baseline and 3 months (paired t-test)

*$p<0.05$-Statistically significant value for comparison within Group-B between baseline and 3 months (paired t-test)

#$p<0.05$-Statistically significant value for comparison between healthy individuals and Group A at 3 months (unpaired t-test and Mann-Whitney test)

$p<0.05$-Statistically significant value for comparison between healthy individuals and Group B at 3 months (unpaired t-test and Mann-Whitney test)

$^{\beta}p=0.01$ value for comparison within healthy individuals between baseline and 3 months (paired t-test)

MMP- Matrix metalloproteinase, TIMP-Tissue inhibitor of Metalloproteinase, IL-6- Interleukin-6.

DISCUSSION: Results of this study showed that there is a significant decrease in the concentration levels of the inflammatory markers in gingival crevicular fluid (GCF) and saliva three months after conservative periodontal treatment and Ayurvedic supplementation. However, it was observed that the level of the markers was even much lower in patients receiving scaling and root planning (SRP) and dry roots of (*Asparagus curillus*) AC the treatment in combination.

Ayurveda advised different herbs and different herbal methods for regular practice to maintain optimal dental health [10]. Plant food supplements have received a great acceptance by European consumers. Genus Asparagus has a long history of traditional medicinal use. Various compounds have been isolated from this genus and have been shown to exhibit anti- neuro- inflammatory activity [4]. Thus it is not surprising to see Asparagus being heralded as an anti-inflammatory food because it provides a truly unique combination of anti-inflammatory nutritients.

A study done by Paul A *et al*., in 2003 [8], reported the anti inflammatory action of roots of *A. pubuscens* in rodents. Similar kind of anti-inflammatory action of *A. cochinchinensis* in mice has also been observed by Lee DY *et al*., in 2009 [9]. However, there is no reference in literature to our knowledge as to the possible anti-inflammatory efficacy of the roots of AC in periodontal inflammation. Periodontitis (PD) is inflammatory response in the periodontium induced by micro-organisms in dental plaque, which contribute to tissue destruction, bone loss and eventually tooth loss [35].

This set of inflammatory reactions involves the migration and activation of neutrophils, lymphocyte and macrophages to disease site [36]. Activated neutrophils degranulate and results in the secretion of certain endopeptidases *i.e.* MMP-8 and -9 [25, 37]. Thus assessment of MMPs in GCF and saliva may serve as an important biomarker in diagnosis of periodontal disease also for prognostic follow up.

Several studies on matrix metalloproteinase showed that MMP-8, -9 and -13 levels are statistically significant elevated as periodontitis progresses [25, 28, 29, 38, 39, 37]. Moreover, Hernandez M in 2007 [38] found decrease TIMP-1 levels during disease progression in association with MMP-13 elevation. The results of this study support our results that MMP-13 has an important role in tissue destruction associated with periodontitis progression. We demonstrated that MMP-13 expression is significantly elevated in periodontitis.

In the present study, the value of these inflammatory markers decreased after three months of therapy in both the periodontitis groups. The results of the present study are in concordance with the finding observed by [25]. They found a significant reduction in the levels of MMP-8 and- 9 in GCF of the patients three months after periodontal therapy [35, 36]. Osteoclaset activity and bone resorption is directly correlated with IL-6 levels in periodontitis patients.

A study on periodontitis and diabetes patients by Priscila PC *et al*., 2010 [34] showed increase in IL-6 and MMP-8 levels in periodontitis patients with diabetes. Similar finding were also observed that in periodontitis groups but IL-6 levels was significantly decreased after combined use of conservative periodontal management and Ayurvedic treatment.

It is well known that primary objective of scaling and root planning is to remove the elements that provoke gingival inflammation (plaque and calculus) from the tooth surface. As we know calculus is porous and harbours bacteria and endo-toxins so it can be justified that removal of plaque and calculus results in eradication of inflammation[12].

When the rationale of scaling and root planning is thoroughly understood it becomes apparent that this kind of treatment is essential to the ultimate success of any kind of periodontal therapy. Moreover, chemical control of the plaque is always considered in adjunct to the mechanical control as former is qualitative control of the factors responsible for plaque formation. So we considered the Group B as patients receiving both the mechanical as well as chemical modalities of periodontal therapy.

This is probably the first study using *Asparagus curillus* as an anti-inflammatory herb in periodontitis patients. Till now several anti-inflammatory compounds like aspacochinosides N(1), O(2), and P(3), active gradients or secondary metabolites such as saponins, alkaloids, flavonoids or glycosides [4] have been isolated from various Asparagus species. Results observed in Group B might be explained on the basis of these anti-inflammatory compounds. However, this study conclude the effectiveness of combination therapy of conservative periodontal management and Ayurvedic therapy in periodontitis but the effect has not been evaluated against allopathic medication in periodontitis. So, further study is required to compare the Ayurvedic therapy with allopathic medication in periodontitis.

CONCLUSION: Although the mechanisms of action of conservative periodontal therapy and Ayurvedic therapy are different, their combined impact on periodontal inflammatory markers in GCF and saliva showed increase in the efficacy of treatment of periodontitis.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest.

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