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EVALUATION OF HUMAN SALIVARY METALLOPROTEINASE INHIBITORS IN PLANT LEAVES

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ABSTRACT: Previous studies investigated the host-derived salivary matrix metalloproteinases (MMPs) are responsible for dentin caries progression. The utilization of the MMP inhibitors can become a successful strategy to control dentin caries. In this study, we have tested twenty-four different medicinal plant leaf extracts against human salivary MMPs for investigation of inhibitors. Total phenolics were extracted in methanol, and their assessment was performed by Folin-ciocalteau assay. The presence of salivary MMP inhibitors in all plant leaf extracts was tested by using gelatin zymography. *S. cumini* leaf extract was found to be containing the highest total phenolics content. Leaf extracts of *S. cumini*, *T. catappa* and *C. fistula* exhibited strong inhibitor activity against salivary MMPs. Presence of inhibitor activity was found to be depending on the amount of phenolics concentration in extracts. The inhibition of salivary MMPs due to the binding action of phenolics with salivary proteins was concluded. As per the data of image J gel analysis software, *T. catappa* leaf exhibited prominent inhibitor activity as compared to other plants. The IC_{50} value of *T. catappa* against P3 proteinase band was determined between 25 and 50 µg/ml phenolics. Inhibitory activity exhibiting plants found in this study could be used for controlling the dental carries progression.

INTRODUCTION: Phenolic compounds are secondary metabolites found in plants, which are originated biogenetically from either the shikimate and phenylpropanoid pathways [1]. They have benzene rings, with one or more hydroxyl substituents and ranging from simple phenolic molecules to highly polymerized compounds such as lignins [2].

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These phenolic compounds or polyphenols are consisting different compounds such as phenolic acids, flavonoids, complex flavonoids, and colored anthocyanins [3]. They are synthesized in the plant as a defensive system in response to invading pathogens and environmental conditions.

However, they are involved in other processes like accelerating pollination by incorporating attractive substances, coloring for concealment and defense against herbivores, antibacterial and antifungal activities [4, 5]. Phenolics rich vegetables and fruits contribute to delay the aging process and decrease the oxidative stress risk related to chronic diseases such as atherosclerosis, cardiovascular diseases, diabetes, cancer, neurological diseases, and

disorder of cognitive function. They possess different biological properties such as anti-oxidant, anti-inflammatory, anti-arthritis, anti-microbial, and anti-proliferative [6]. Matrix metalloproteinases (MMPs) are a family of metal-dependent endo-proteinases capable of degrading all kinds of extracellular matrix (ECM), including native and denatured forms of collagens [7]. They play a vital role in physiological and pathological conditions; their secretion and activity under physiological conditions are balanced by endogenous activators, tissue inhibitors (TIMPs), and serine protease inhibitors [8]. Salivary MMPs, especially MMP-2,-8, and 9 degrade the collagen matrix of dentin [9, 10]. Demineralization of inorganic minerals is caused by acids secreted from oral bacteria that result from initiation of dental caries progression [11]. The demineralization is favorable for the degradation of the collagenous organic matrix of dentin.

Salivary MMPs are activated by acids produced from oral bacteria, and activated MMPs degrade demineralized dentin collagenous matrix after pH neutralization by salivary buffers that cause dental caries progression [12, 13]. Prevention of degradation of dentin matrix could be achieved by employing MMP inhibitors. Researchers have studied the use of chlorhexidine and chemically modified tetracyclines (CMTs) as MMP inhibitors for reduction of the degradation of the dentin *in-vivo* and *in-vitro*, which could prevent dental caries progression [14, 15, 16]. It has been investigated that green tea polyphenols, especially epigallocatechin-3-gallate (EGCG) as MMP inhibitor, reduce dentin wear under erosive/abrasive conditions [17]. Therefore, in the present study, the effort has been made to screen twenty-four different plant leaf phenolics extracts against human salivary metalloproteinase for the investigation of novel inhibitors. Phenolic compounds from different leaves of the plant were extracted in methanol, and their evaluation was done by using Folin-ciocalteau assay. The presence and assessment of human salivary metalloproteinase inhibitors in different extracts were performed by using the gelatin zymography technique.

MATERIALS AND METHODS:

Chemicals and Reagents: Triton X-100, Tris-hydroxymethyl amine, Gallic acid, Acrylamide, Bisacrylamide, Calcium chloride, Zinc chloride, EDTA, Folin-ciocalteau reagent, 1,10-Phenan-throline, Glycine, Glycerol, Sodium carbonate, Bromophenol blue, Sodium hydroxide, Coomassie brilliant blue R-250, Acetic acid, and Methanol were purchased from RANKEM. Gelatin skin porcine was purchased from Sigma Aldrich. All chemicals used in this study were of analytical grade.

Collection of Plants and Preparation of Extracts: The matured leaves of different plants were obtained from the campus of (BAMU) Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (M.S.) India. All plant leaves were identified and authenticated by taxonomist, Department of Botany, BAMU. The obtained leaves were sorted out, washed thoroughly with warm distilled water, and kept in an oven at 40 ºC until complete drying. Thereafter, all leaves were crushed into fine powder by using a grinder and mixer, and fine powders were preserved at room temperature. The extraction of phenolics available in leaves was performed by using the procedure of Jaiwal and Tak [18] with slight modifications. Fine powders of all leaves were soaked in methanol with the proportion of 1:10 w/v and stirred at room temperature for 2 h by a magnetic stirrer. The obtained suspensions were centrifuged at 10000 rpm for 15 min at 4 ºC, and supernatants were collected. Methanol from each extract was evaporated at room temperature; obtained residues were dissolved in double distilled water (10 ml) and preserved in the refrigerator at 4 ºC.

Determination of Total Phenolics: The amount of total phenolics presence in leaf aqueous extracts was determined by using the procedure of Jaiwal *et al.*, [19] Equal volume of each leaf extract (50 µl) was mixed with Folin-Ciocalteau reagent (0.5 ml) and incubated at room temperature for 3 min. After incubation, 1ml of Na_2CO_3 (20% w/v) was added in each test tube and incubated in a boiling water bath for 1 min. All aliquots were cooled under tap water, and absorbance of developed blue-black color was recorded at 650 nm. Simultaneously, different concentrations of gallic acid were reserved in other aliquots as standard. The concentrations of phenolics were determined by using the standard graph of gallic acid, and the concentration of phenolics were expressed as gallic acid equivalents (GAE mg/ml).

Collection of Human Saliva: The human saliva was collected in an ice-cold glass tube from a healthy volunteer who was prior exposed to 12 h of fasting condition. Before collection of saliva, the volunteer was instructed to rinse their mouth with distilled water. After collection, the saliva sample was immediately centrifuged at 10,000 rpm for 15 min at 4 ºC. The supernatant was preserved at -20 °C in the deep freezer, and the protein concentration was determined by using the method of Lowry *et al.*, [20]

Detection of Proteinases Activity in Human Saliva (Gelatin Zymography): The gelatinase activity of human salivary proteinases was detected by using the same procedure mentioned in earlier research paper [21]. The gelatin zymography was prepared by incorporating 0.1% porcine gelatin type A (Sigma) into the 10% SDS-PAGE. The supernatant of human saliva was mixed with an appropriate volume of non-reducing loading sample buffer. A hundred microliters (600 µg protein) of the sample was loaded onto each well of electrophoresis, and electrophoresis was carried out at room temperature with providing 25 mA of constant current supply. After electrophoresis, the gel was removed, washed with distilled water, and cut into strips. The gel strips were incubated for 1 h at room temperature in 100 ml of renaturation buffer (2% Triton X-100) on a rotary shaker. Thereafter, one gel-strip was incubated overnight at 37 °C in activation buffer (50 mM Tris-HCL pH 7.5, 10 mM $CaCl_2$). Another gel strip was incubated separately in the same buffer containing standard metalloproteinase inhibitors (30 Mm EDTA and 10Mm 1, 10-phenanthrolin). After incubation, each gel strip was washed with distilled water and then stained with 0.5% Coomassie blue R-250 prepared in 30% methanol and 10% acetic acid for 2 h. After staining, gel strips were de-stained with 30% methanol and 10% acetic acid. Photographs of gels were recorded by gel-document system (Alpha Innotech (HP).

Screening of Plant Leaf Extracts for Anti-metalloproteinase Activity: Screening of anti-metalloproteinase activity in plant leaf extracts was carried out by using gelatin zymography. Human saliva supernatant (100 µl) was mixed with 50 µl of each plant extract and incubated at 37 ºC for 10 min. After incubation, the same mixed sample was mixed with an appropriate volume of non-reducing sample buffer and loaded onto gelatin zymography. After electrophoresis gel was incubated for 1 hr at room temperature in 100ml of renaturating buffer (2% Triton X-100) on a rotary shaker and incubated overnight at 37 ºC in activation buffer (50Mm Tris-HCL pH 7.5, 10 Mm $CaCl_2$). The rest of the procedure was carried out the same as discussed in the methodology section of gelatin zymography.

Dose-Dependent Inhibitory Activity of Inhibitory Activity Exhibiting Plant Extract: The extracts of inhibitory activity showing plants (*T. catappa*, *C. fistula,* and *S. cumini*) containing various phenolics concentrations (25, 50, 75, 100, 125 and 150 µg/ml) were treated with human saliva supernatant separately and incubated at 37 ºC for 10 min. An aliquot (Human saliva supernatant) was made without plant extract as control and incubated at the same temperature for 10 min. After incubation, the same mixtures were mixed with sample buffer and allowed for separation on gelatin zymography. The rest of the procedure was carried out the same as discussed above method section. After completion of the total electrophoretic process, gels were visually assessed, and images were taken under a gel-documentation system (Alpha Innotech (HP). Scanned images were exported for analysis as tiff files and analyzed using Image J image analysis software. Proteinase activity is generally observed as white colour bands against dark gray/black background. Therefore, the images were opened in Image J software and inverted so that the bands were black. The control lane of each image/gel was selected using a rectangle tool as a first-line and compared the selected rectangular area with sample lanes. The band intensity peaks were plotted by using the line tool option in analysis of image J software [22]. The area under each peak was measured by selecting each band containing inhibitors as compared with active control band (protease activity). The size of the active enzyme control band or peak was indicated as a hundred percent of the total size of corresponding peaks. This means control band area was considered as a zero-percentage inhibition. On the basis of this statistical analysis *i.e.*, subtraction of area under peak decreased due to increase in the amount of extract or phenolic concentration, the percentage of inhibition of enzyme was calculated.

RESULTS AND DISCUSSION:

Total Phenolics Content in Plant Leaves: Plant phenolics are key elements of plants and are highly evaluated for commercial purposes as therapeutic agents due to their valuable properties and different roles in plants, including growth, development, and defense [23]. Total phenolics concentrations in aqueous extracts of twenty-four different medicinal plant leaves were evaluated to see the inhibitory effect on human salivary proteinases by standard Folin-ciocalteau assay, as shown in **Table 1**. On basis of phenolics concentration, plant leaf extracts (Twenty four) were sorted and grouped into five categories a) very low < 1 mg/ml (6 plants) b) low 1-2 mg/ml (7 plants) c) moderate 2-5 mg/ml (6 plants) d) high 5-10 mg/ml (3 plants) and e) very high >10 mg/ml (2 plants) as shown in table 1. Among all plants, the highest concentration of phenolics was found in *S. cumini* leaf aqueous extract (14.5 ± 0.28 mg/ml), while the lowest concentration of phenolics was found in *O. elatior* leaf aqueous extract (0.42 ± 0.020 mg/ml). Previous study has reported that methanolic extract of S. cumini leaf exhibits different biological activities and estimates the rich level of total phenolics (369.75 ± 17.9 mg GAE/g) in crude extract [24].

In the case of the very low phenolics concentration group, the maximum phenolics were observed in *F. religiosa* (0.98 ± 0.003 mg/ml) followed by in *G. sepium* (0.89 ± 0.02 mg/ml). In the low phenolics concentration group, the concentration of the maximum phenolic was found in *T. procumbens* (1.95 ± 0.02 mg/ml) followed by in *H. suaveolens* (1.89 ± 0.03 mg/ml) while minimum phenolics concentration was found in *C. tora* (1.16 ± 0.41 mg/ml) followed by in *J. sambac* (1.50 ± 0.49 mg/ml). The amount of phenolic concentrations in moderate group were in order of *D. wrightii* (3.23 ± 1.49 mg/ml) > *O. sanctum* (3.14 ± 1.49 mg/ml) > *A. lebbeck* (2.90 ± 0.02 mg/ml) > *F. carica* (2.86 ± 0.49 mg/ml) > *N. arbortristis* (2.80 ± 0.75 mg/ml) > *A. squamosal* (2.48 ± 0.08 mg/ml). The maximum phenolic concentrations were found in plants such as *T. catappa* (10.73 ± 0.37 mg/ml), *C. fistula* (8.13 ± 0.06 mg/ml), *F. benghalensis* (5.66 ± 0.18 mg/ml) and *P. pinnata* (5.13 ± 0.09 mg/ml) as shown in **Table 1**. Abdulaziz [25] has reported the presence of the highest total phenolic content (285.7727 mg GAE/g) in 95% ethanol extract of *T. catappa* leaf. It has been reported that the accumulation of phenolic components depends upon the type of plant tissue, maturity at harvest, growing conditions, soil conditions, and post-harvest treatment [26]. Different parts of the same plant can synthesize and store different compounds or different amount of a specific compound due to their differential gene expression, which in turn affects the different biological and antioxidant activities [27, 28]. Plant phenolic compounds are powerful antioxidant agents and utilized commercially for a different purpose. In this study, total phenolics estimated from leaves of medicinal plants could be comparatively similar to phenolic compounds estimated from 26 medicinal plants [29].

TABLE 1: LIST OF MEDICINAL PLANT LEAVES BELONGING TO DIFFERENT FAMILY SELECTED FOR SEARCHING INHIBITOR ACTIVITY AGAINST HUMAN SALIVARY METALLOPROTEINASES

S. no.	Name of plant	Family	Phenolics content mg/ml (Mean ± S.D.)	Presence of Inhibitor activity
1	*Opuntia elatior*	Cactaceae	0.42 ± 0.020	-
2	*Morus rubra*	*Moraceae*	0.48 ± 0.020	-
3	*Mimosa pudica*	*Fabaceae*	0.66 ± 0.03	-
4	*Achyranthes aspera*	*Amaranthaceae*	0.75 ±0.05	-
5	*Gliricidia sepium*	*Fabaceae*	0.89 ± 0.02	-
6	*Ficus religiosa*	Moraceae	0.98 ± 0.003	-
7	*Cassia tora*	*Fabaceae*	1.16 ± 0.41	-
8	*Jasminum sambac*	*Oleaceae*	1.50 ± 0.49	-
9	*Ficus racemosa*	Moraceae	1.54 ± 0.04	-
10	*Madhuca longifolia*	*Sapotaceae*	1.58 ± 0.062	-
11	*Allium sativum*	*Amaryllidaceae*	1.83 ± 0.06	-
12	*Hyptis suaveolens*	*Lamiaceae*	1.89 ± 0.03	-
13	*Tridax procumbens*	Asteraceae	1.95 ± 0.02	-
14	*Annona squamosa*	*Annonaceae*	2.48 ± 0.08	-
15	*Nyctanthes arbour tristis*	Oleaceae	2.80 ± 0.75	-
16	*Ficus carica*	*Moraceae*	2.86 ± 0.49	-

17	*Albizia lebbeck*	*Fabaceae*	2.90 ± 0.02	+
18	*Ocimum sanctum*	*Lamiaceae*	3.14 ± 1.49	-
19	*Datura wrightii*	*Solanaceae*	3.23 ± 1.49	-
20	*Pongamia pinnata*	*Fabaceae*	5.13 ± 0.09	+
21	*Ficus benghalensis*	*Moraceae*	5.66 ± 0.18	+
22	*Cassia fistulla*	Fabaceae	8.13 ± 0.06	++
23	*Terminalia catappa*	*Combretaceae*	10.73 ± 0.37	++
24	*Sigium cumini*	*Myrtaceae*	14.5 ± 0.28	++

The phenolics content in leaf extracts of these plants was determined by using the folin-ciocalteau method and expressed as mg/ml gae. + sign indicates the presence of low inhibitor activity while, ++ sign indicates the presence of strong inhibitor activity and – sign indicates the absence of inhibitor activity in the extract

In-gel Detection of Metalloproteinase Activity from Human Saliva: Gelatin zymography is the most sensitive technique used for the detection of a wide spectrum of metalloproteinase activity from the biological sample at a very minute level [30].

The supernatant of human saliva display gelatinolytic and collagenolytic activities, and these activities are partially inhibited by EDTA [31]. The proteases with high molecular weight complex form (>300 and 120 kDa) and a latent form (92 kDa)) of matrix metalloproteinase- 9 (MMP-9) are present in saliva, and these proteinases are autoactivated at 37° C and condensed to a 42-kDa protein through 100, 67, and 50 kDa proteins [31].

For the accession of proteinase activity, the human saliva supernatant was applied on 10% SDS-PAGE copolymerized with gelatin (Skin porcine) with using of a non-reducing sample buffer. Figure 1 lane (A) shows that the detection of six proteinases major/prominent band (P1, P2, P3, P4, P5, and P6) and six non-prominent/minor proteinase bands (not mentioned in the figure) from human saliva.

The same proteinase activity on gelatin zymography was inhibited due to overnight treatment with metal-chelating compounds (EDTA and 1, 10-phenanthrolin), indicating these proteinases were metalloproteinases **Fig. 1** lane B.

The pattern of detected metalloproteinases on gelatin zymography is comparatively similar to the pattern of MMPs activity detected from human demineralized dentinal lesions studied in previous report [32].

Among six prominent protease bands, protease (P3) was found to be broad with highly intensive, followed by band P5 and P6 as compare to others. From these results, it seems that human saliva contains more than twelve proteases which could be known if long length gelatin zymography and 2D electrophoresis are used for its analysis. Few studies have reported the presence of MMPs in human saliva; therefore, based on the result, it was assumed that metalloprotease activity detected on gelatin zymography was the activity of MMPs.

It has been reported that human saliva contains host MMPs such as MMP-2 (72-kDa gelatinase /type IV collagenase; Gelatinase A), MMP-9 (92-kDa gelatinase/type IV collagenase; Gelatinase B), and MMP-8 (human neutrophil collagenase; PMN-MMP-8; collagenase-2), and most of MMPs are originated from the gingival crevices surrounding the teeth [33, 34]. Agnieszka *et al.,* [35] have reported the presence of MMP-2, MMP-8, MMP-9, TIMP-1, and TIMP-2 in human saliva.

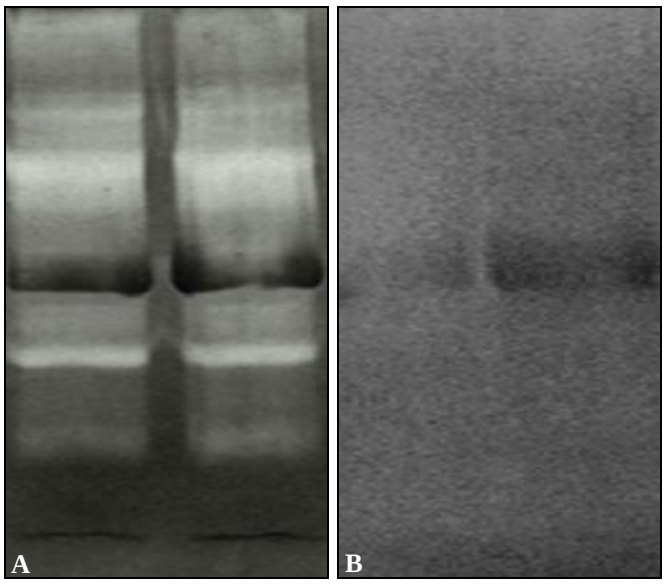

FIG. 1: ZYMOGRAM REPRESENTS THE DETECTION OF GELATINASE ACTIVITY FROM HUMAN SALIVA SUPERNATANT. Lane (a) shows gelatinase activity bands (p1, p2, p3, p4, p5, and p6) when incubated in activation buffer at 37°c for overnight and lane (b) shows inhibition of same gelatinase activity when incubated in 30 mm EDTA and 10 mm 1, 10-phenathralin solution at 37 °C for overnight indicating metalloproteinases exist in human saliva.

Anti-metalloproteinase Activity of Plant Leaf Extracts: Besides the dynamic role in host plants, phenolic compounds exhibit a broad spectrum of biological properties that prevent the cause of various disorders and promote the persistence of health in human exhibit inhibitor activity against matrix metalloproteinase [36].

The aqueous extracts from different plant leaf extracted residues were prepared to search inhibitor activity, all these extracts (Twenty four) were treated with human saliva supernatant, and the inhibitory effect against metalloproteinase activity was tested on gelatin zymography **Fig. 2**.

The inhibition of the complete gelatinolytic activity of human saliva on gelatin zymography was observed by the treatment of *C. fistula*, *T. catappa* and *S. cumini* leaf extracts, indicates this plant leaves exhibit strong inhibitor activity against human salivary metalloproteinases **Fig. 2**, lane 12, 16 and 23.

Partial gelatinolytic activity or few proteolytic bands inhibition was observed in the case of *A. lebbeck*, *P. pinnata*, and *F. benghalensis* leaf extracts treatments indicates these plant leaf extracts exhibit weak inhibition against human salivary metalloproteinases as compared to the above-mentioned plants **Fig. 2**, lane 1, 3, and 7.

Rest of plant leaf extracts (mentioned in **Table 1** with an indication of - sign) not exhibited inhibitory activity against salivary metallo-proteinases on zymography.

Inhibitory activity of plants was found to be depending on the amount of total phenolics present in leaf extracts except *A. lebbeck* leaf extract, this plant was found to be containing a moderate amount of total phenolics **Table 1** even though this plant exhibits inhibitor activity thus it is the possibility that structurally different types of phenolics or non-phenolic compounds of this plant exhibit inhibitory activity.

It has been reported that *A. lebbeck* consisting of valuable phytoconstituents such as D-catechin, melacacidin, albiziahexoside, β-sitosterolbetulnic acid, and echinocystic acid glycosides are responsible for various biological properties, and traditionally this plant is known to be used as an anti-inflammatory, anti-asthmatic anti-diarrhoeal anti-fertility antiseptic, anti-dysenteric and anti-tubercular [37].

Phenolic compounds from complexes with protein molecules affect the migration and mobility of protein molecules on gel electrophoresis under the influence of electric current [38].

During electrophoresis under electric current, the mobility of low molecular weight molecules like phenolic compounds and others move faster than the salivary protein molecules that affect change in mobility and release the binding between enzyme and inhibitors [39] in such case phenolic compounds are unable to inhibit the enzyme.

In this study, plants such as *C. fistula*, *T. catappa*, and *S. cumini* were capable of inhibiting the complete gelatinase activity on electrophoresis; therefore the phenolic compounds of these plants may have the ability to make strong association with salivary metallo proteinases. Phenolic compounds have metal chelating properties as well as protein binding ability [40, 41].

Therefore, inhibition of human salivary metalloproteinase by binding action of phenolic compounds with enzyme and not by chelation of calcium and zinc metals has been confirmed.

The previous study has reflected that the property of phenolic compounds such as gallic acid, ferulic acid, chlorogenic acid, quercetin, apigenin, and catechin and phenolics from plant extracts (green tea and green coffee) to form complex with protein fractions of white bean (albumins and globulins) [41].

Screening procedure we utilized in this study to test salivary metalloprotease inhibitor activity in plant extract was reliable and reproducible and could be used to search MMP inhibitors in different biological samples and synthetic compounds.

Dentin carries progression can be prevented by applying the strategy of the utilization of salivary MMP inhibitors.

Chlorhexidine and green tea extract have been reported to prevent dentine wear caused by erosion and abrasion by the inhibition of salivary MMP inhibition [42].

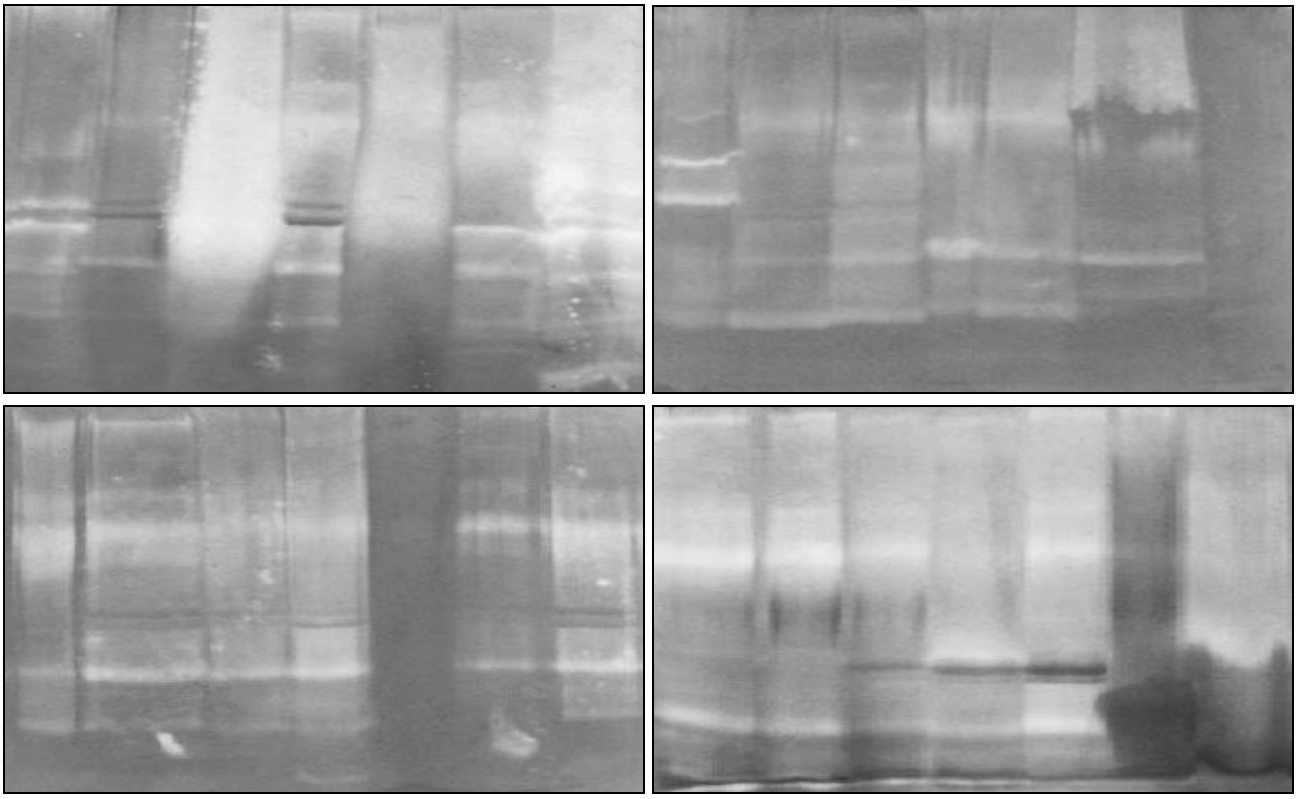

FIG. 2: SCREENING OF PLANT LEAF EXTRACTS AGAINST HUMAN SALIVARY METALLOPROTEINASES. LEAVES EXTRACTS OF DIFFERENT PLANTS WERE TREATED WITH HUMAN SALIVA AND PROTEINASE ACTIVITY WAS ACCESSED ON GELATIN ZYMOGRAPHY. Lane (1) *A. Lebbeck* (2) *O. Elatior* (3) *P. Pinnata*(4) *M. Rubra* (5) *M. Pudica* (6) *A. Aspera* (7) *F. Benghalensis* (8) *G. Sepium* (9) *F. Religiosa* (10) *C. Tora* (11) *J. Sambac* (12) *T. Catappa* (13) *F. Racemosa* (14) *M. Longifolia* (15) *A. Sativum* (16) *S. Cumini*(17) *H. Suaveolens* (18) *T. Procumbens* (19) *A. Squamosa* (20) *N. Arbortristis* (21) *F. Carica*(22) *O. Sanctum*(23) *C. Fistula*(24) *D. Wrightii*.

TABLE 2: PROTEINASE ACTIVITY AND % INHIBITION OF P3 PROTEINASE BAND DETERMINED AFTER TREATMENT OF *S. CUMINI, T. CATAPPA* AND *C. FISTULA* LEAF EXTRACTS WITH HUMAN SALIVA

Lane No.	Phenolics (µg/ml)	*S. cumini*			*T. catappa*			*C. fistula*		
		Area	Protease activity	% Inhibition	Area	Protease activity	% Inhibition	Area	Protease activity	% Inhibition
1	0	19772	100	0	6436	100	0	22760	100	0
2	25	17724	89.64	10.35	4372	67.93	32.06	22750	99.95	0.043
3	50	16853	85.23	14.76	2156	33.49	66.50	18587	81.66	18.33
4	75	14111	71.36	28.63	1318	20.47	79.52	15447	67.86	32.13
5	100	13615	68.86	31.14	357	5.54	94.45	13587	59.69	40.30
6	125	68	0.34	99.65	326	5.06	94.93	9505	41.76	58.23
7	150	-	-	-	311	4.83	95.16	6279	27.58	72.41

Leaf extracts of plants were treated with human saliva, these mixtures were incubated at 37 ºC for 10 min, and proteinase activity was accessed from each mixture on gelatin zymography. After completion of electrophoresis, the photograph of zymography was processed by using image j gel analysis software and determined area of p3 band with corresponding protease activity and % inhibition was calculated.

Dose-Dependent Inhibitory Activity of Inhibitory Activity: The plant extracts that showed complete inhibition against salivary proteinase on gelatin zymography were considered for the dose-dependent inhibition analysis study. For evaluation of dose-dependent inhibitor activity, the extract containing various concentrations of plant phenolics were treated with supernatant of saliva, and inhibition of proteolytic activity was assessed on gelatin zymography. After completion of the zymography process, the photographs of gelatin zymography were analyzed by image J gel analysis software [22]. In the analysis data, different intensity peaks respective to gelatinase activity on the gel were obtained. The areas of peaks given by software were used for the calculation of gelatinase activity and percent inhibition. The percent inhibitions of all gelatinase bands that appeared on

zymography were calculated, but in this study, we have shown percent inhibition only prominent P3proteinase band. **Fig. 3**, **4,** and **5** show that various concentrations of phenolic compounds from *S. cumini, C. fistula,* and *T. catappa* leaf extracts were able to exhibit dose-dependent inhibition against salivary metalloproteinases on gelatin zymography. It was observed that intensities of obtained peaks declined as the concentration of phenolics increased. Assessment of MMPs activity and their inhibition on gelatin zymography have been performed similarly by densitometer associated with image gel software in previous studies [43, 44]. Percent inhibitions of P3 proteinase band by these plant leaf extracts as shown in **Table 2**.

Among three plants, *T. catappa* leaf extract showed strong inhibition against human salivary metalloproteinases as compared to *C. fistula* and *S. cumini* leaf extracts. *T. catappa* has been well documented for its biological properties such as antidiabetic, anti-inflammatory, hepato-protective, antioxidant, and anticancer activities, and a leaf of this plant contains medicinally essential phytoconstituents such as punicalagin, kaempferol, punicalin, tercatain, quercetin, tergallagin, terflavin A, and terflavin B, cyanidin-3-glucoside, β-carotene, gallic acid, ellagic acid and tannins [45]. The actual percent inhibition with corresponding to various concentrations of phenolics of P3 proteinase band is shown in **Table 2**.

As per the percent inhibition values given in **Table 2**, the IC_{50} value of *T. catappa* against P3 proteinase band was determined between 25 and 50 µg/ml phenolics while IC50 values of *S. cumini, C. fistula* were determined between 100 and 125 µg/ml phenolics concentration. Figures of inhibitions indicate that the inhibition mechanism of these plants' phenolics is varying among them. Gradual increment in percent inhibition by *S. cumini* leaf phenolics was observed upto 100 µg/ml phenolics while suddenly increased higher at 125 µg/ml phenolics. *S. cuminiis* a traditional medicinal plant consisting of pharmaco-logically important bioactive compounds such as gallic acid, oxalic acid, tannins, cynidin, glycoside, oleanolic acid and flavonoids and these components have been elucidated for their anticancer, anti-inflammatory, antimicrobial, anti-oxidant, free radical scavenging (ROS), gastro-protective and piles curing properties [46].

A pattern of inhibition on gelatin zymography was found to be quite similar in the case of *T. catappa* and *C. fistula* leaf phenolics; this indicates similar components of both these plants may be responsible for acting as inhibitors.

The fruits, stem bark, and leaves *C. fistula* contain biologically active compounds flavonoids, anthraquinones, flavon-3-ol derivatives, tannin, terpenoids, glycosides, and saponin, and extract of these tissue exhibit various activities like anti-inflammatory, antidiabetic, antioxidant, antimicrobial, antitumor, antiulcer and antipyretic [47].

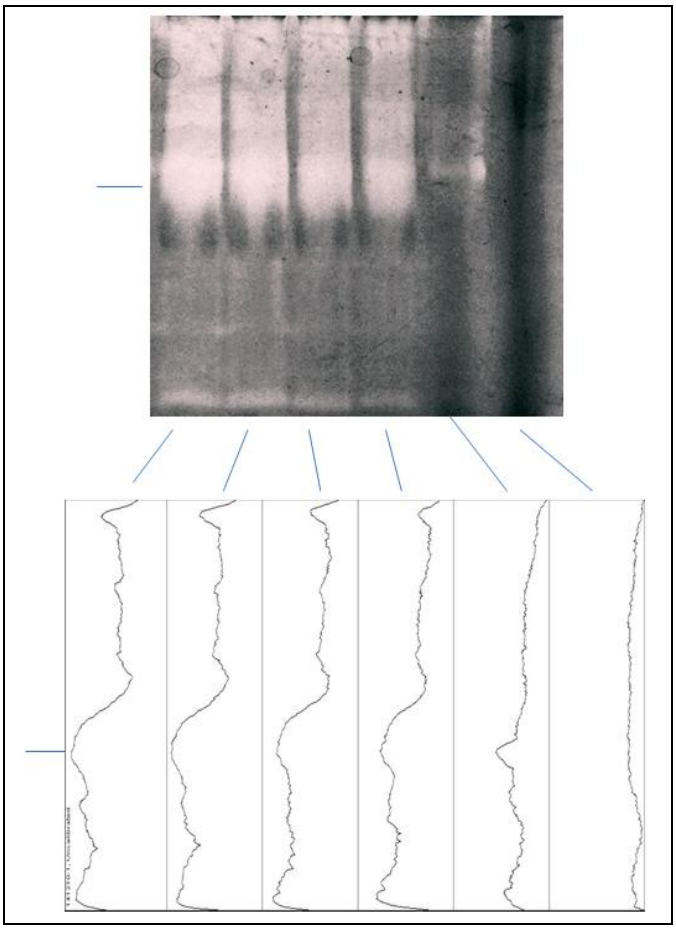

FIG. 3: DOSE-DEPENDENT INHIBITORY EFFECT OF VARIOUS CONCENTRATIONS OF *S. CUMINI* LEAF PHENOLICS AGAINST HUMAN SALIVARY METALLO-PROTEINASES. The various concentrations of phenolics from the same plant leaf extract were treated with human saliva, and the proteinase activity was accessed on gelatin zymography as mentioned in the methodology section. The photograph of gelatin zymography was processed by image j gel analysis software for determination of % inhibition. Decline in peak area indicates inhibition of proteinases by leaf extract.

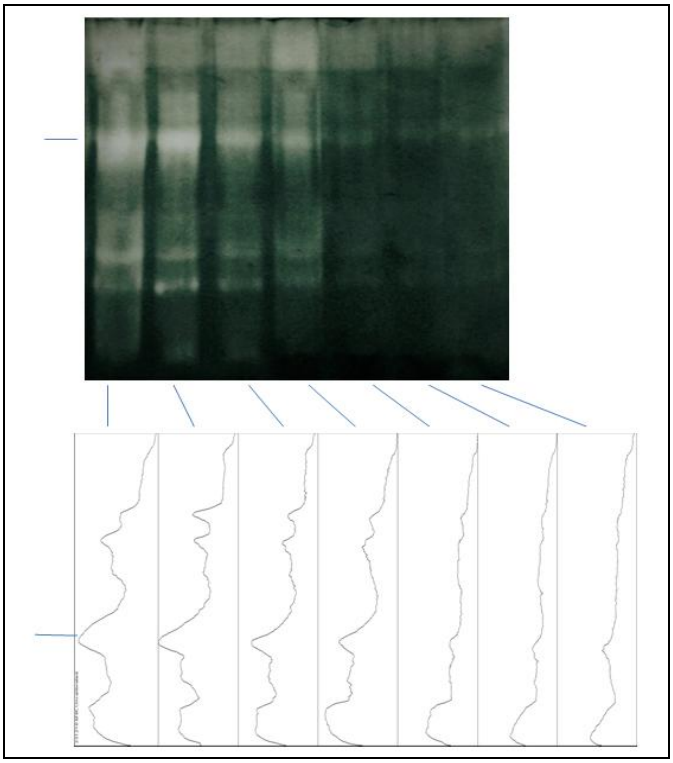

FIG. 4: DOSE-DEPENDENT INHIBITORY EFFECT OF VARIOUS CONCENTRATIONS OF *T. CATAPPA* LEAF PHENOLICS AGAINST HUMAN SALIVARY METALLO-PROTEINASES. Peaks showing the activity of proteinases declined by the inhibitory effect of plant extract.

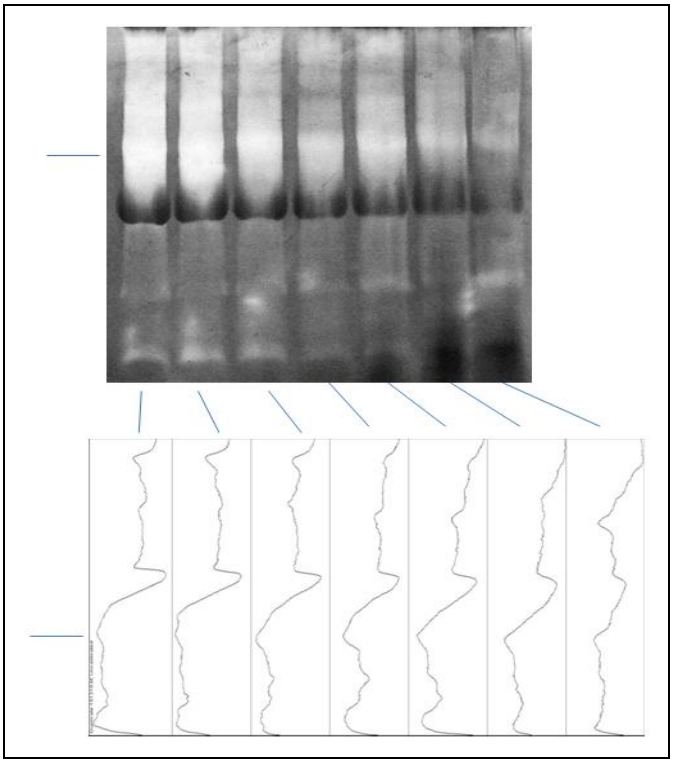

FIG. 5: DOSE-DEPENDENT INHIBITORY EFFECT OF VARIOUS CONCENTRATIONS OF *C. FISTULA* LEAF PHENOLICS AGAINST HUMAN SALIVARY METALLO-PROTEINASES. Peaks showing the activity of proteinases declined by the inhibitory effect of various concentrations of leaf phenolics.

CONCLUSION: Based on results, it was concluded that leaf extracts of *S. cumini*, *C. fistula*, and *T. catappa* exhibit human salivary metallo-proteinase inhibitor activity. Phenolic compounds of these plants are responsible for inhibitor activity by their binding ability with protein molecules. Inhibitor activity may depend on amount of total phenolics present in plant leaf extracts. Extracts of *S. cumini*, *C. fistula*, and *T. catappa* could be utilized for the prevention of dental caries progression and other MMPs associated diseases. This study suggests that purification and characterization of novel and specific MMP inhibitors from these plants could be beneficial for designing drugs.

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