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Bark extracts of *C. fistula* and *M. indica* inhibit the human salivary metalloproteinases

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Abstract
Human salivary host derived matrix metalloproteinases (MMPs) are involved in dentin caries pathogenesis. Previous studies suggest that by the inhibition of those MMPs, we can control the dental carries progression. To study the inhibition of human salivary proteinases by bark methanolic extracts of *C. fistula* and *M. indica*, we used the gelatin as substrate incorporated with polyacrylamide gel. The total phenolics were determined by Folin-ciocalteau assay and C. *fistula* bark was found to have more phenolic content than *M. indica* bark. The inhibition of human salivary proteinases was observed by the treatment of bark extracts of *C. fistula* and *M. indica* at 100µg/ml phenolics concentration. Bark extract of *C. fistula* and *M. indica* may be used for prevention of dental caries progression.

Keywords: dentin caries, metalloproteinase, *C. fistula, M. indica*, gelatin zymography

1. Introduction

Matrix metalloproteinases (MMPs) are a family of metal dependent endoproteinases, capable for degradation of all kinds of extracellular matrix (ECM) including native and denatured forms of collagens [1]. The oral bacteria (*Streptococcus* mutans and others) produce acids which demineralize inorganic minerals that result initiation of dental caries progression [2]. The demineralization is a favourable for degradation of the collagenous organic matrix of dentin. It was hypothesized that bacterial proteases are responsible for dental caries progression by degradation of collagenous matrix. It was investigated that cariogenic bacteria are only responsible for demineralization of the dentin and proteases produced from these bacteria are not capable for degradation of collagens in vitro [3, 4]. The host salivary MMPs are activated by acids produced from bacteria and involved in degradation of demineralized dentin organic collagenous matrix [5]. Therefore, inhibition of acid activated MMPs is a probable way to control dental caries progression. The plant *Cassia fistula* Linn (*Leguminosae*-Caesalpinoideae) is grown throughout tropical countries of the world, used as medicine for the treatment of a variety of diseases [6]. It possesses the various pharmacological activities like antioxidants, antifungal, antimicrobial, anti-inflammatory and anti-tumour [7]. *Maduca indica* is an Indian tropical tree, used as antiulcer, anti-diabetic, anti-pyretic, hepato-protective, anti-oxidant, wound healing and controlling health associated problems [8]. Plant phenolic compounds are known to possess health benefiting properties like antioxidant, anti-inflammatory, anti-cancer, anti-arthritis and antimicrobial, it indicates that they deserve for consideration as natural medicine [9]. In present study, the phenolic extracts of C. *fistula* bark and *M. indica* bark have been tested for inhibitory activity against human salivary metalloproteinases by using gelatin zymography.

2. Materials and Methods

2.1 Chemicals and Reagents

Gelatin skin porcine was procured from Sigma Aldrich. Triton X-100, Tris-hydroxymethyl amine, Acrylamide, Bisacrylamide, Calcium chloride, Zinc chloride, EDTA, 1,10-Phenanthroline, Glycine, Glycerol, Bromophenol blue, Coomassie brilliant blue R-250, Acetic acid, and Methanol were purchased from RANKEM.

2.2 Collection of human saliva

The saliva (10ml) was collected in ice cold glass tube from healthy human volunteer, who was prior subjected to 12 hours of fasting condition. Before collection of saliva, the volunteer was instructed to rinse their mouth with water. After collection the saliva sample was immediately centrifuged at 10,000 r. p. m. for 15 minutes in cold condition (5°C). The supernatant was preserved at -20°C temperature for further study. The protein concentration of supernatant was determined by using Lowry method [10].

2.3 Collection of plant sample

The barks of *C. fistula* and *M. indica* were obtained from the campus of Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (M.S.) India. After collection, barks were thoroughly washed with distilled water and completely dried with incubating in oven at 50 ^{0}C for overnight. The dried barks were pulverized into fine powder by grinder and mixer and fine powder preserved at room temperature.

2.4 Preparation of extract

Extraction of total phenolics content from fine powders was performed by the earlier utilized procedure of Esmaeili (2015) [11]. Fine powder of each sample (2g) was soaked in 50ml of methanol and stirred at room temperature for 2 hrs by magnetic stirrer. The extracts were filtrated through

Whatman filter paper and the methanol from extract was evaporated at room temperature. The obtained residues were preserved in refrigerator at 4^0C.

2.5

2.6 Estimation of Total phenolics

Polyphenolic compounds in extracts were determined by using Folin-ciocalteau assay with slight modification of method used by Ainsworth and Gillespie, (2007) [12]. Ten miligram of crude extract was individually dissolved in 1ml of corresponding extracting solvent. Gallic acid was used as a reference standard for plotting of calibration curve. Methanolic extract (10 µl) was diluted up to 1.5 ml with distilled water. To this extract 0.5 ml Folin-ciocalteau reagent was added and incubated at room temperature for 3 min. Thereafter, each aliquot was neutralized by adding 1 ml sodium carbonate (20% w/v). Reaction mixtures were incubated at room temperature for few min with intermittent shaking for color development. Absorbance of resulting blue color was measured at 650 nm using double beam UV-VIS spectrophotometer. Amount of total phenolics was estimated from standard graph and expressed as gallic acid equivalent GAE (mg/ml).

2.7 Gelatin zymography

The detection of human salivary metalloproteinases was performed by using the procedure of Barbara (2004) [13]. The gelatin zymography was prepared by incorporating 0.1% porcine gelatin type A into the 10% SDS-PAGE. The saliva sample was mixed with appropriate volume of zymography sample buffer. The 100µl (600 µg protein) sample was loaded to each well for electrophoresis. The electrophoresis was carried out at room temperature and 25mA of constant current supply was provided. After electrophoresis gel was removed, washed with water and cut into strips. The gel strips were incubated for 1 h at room temperature in 100ml of renaturating buffer (2% Triton X-100) on a rotary shaker. Thereafter one gel-strip was incubated overnight at 37°C in activation buffer (50Mm Tris-HCL pH 7.5, 10 Mm $CaCl_2$). Another gel strips were incubated separately in same buffer containing standard metalloproteinase inhibitors (30 Mm EDTA and 10Mm 1, 10-phenanthrolin) and plant extract containing 100µg/ml phenolics concentration. 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 hrs. After staining, gel strips were de-stained with 30% methanol and 10% acetic acid. The photograph of gels was taken by gel-document system (Alpha Innotech (HP).

3. Results & Discussion

3.1 Total phenolic content

Phenolic compound are secondary metabolites commonly synthesized by plant in defensive mechanism in various parts such as bark, leaves, fruits, seeds, flowers etc [14]. It was observed that barks of *C. fistula* and *M. indica* contain substantial amount of total phenolics and *C. fistula* bark consisting more phenolics (195.25 µg/mg residue) than the phenolic (130.46 µg/mg residue) of *M. indica* bark. It was observed that the phenolic content in *C. fistula* bark is approximately equal to the phenolics content observed by the researchers Lai and Liew (2013) [15] who estimated bark of this plant has highest phenolics content compared to stem, leaf and root extracts. The phenolics content in *M. indica* bark was slightly more than the phenolic content estimated by earlier reported study [16].

3.2 In-gel detection of metalloproteinase activity

Gelatin zymography is the widely spread most sensitive and reliable technique used for detection and expression of gelatinases. Human saliva was applied on 10% SDS-PAGE for separation in presence of non-reducing sample buffer. Figure 1 lane (a) shows that detection of proteinases activity with six prominent bands on black background of gel. The same proteinases activity was inhibited due to treatment of metal chelating compounds (EDTA and 1, 10-phenanthrolin) this indicates that metalloproteinases in human saliva are responsible for gelatinases activity (Lane b). The pattern of metalloproteinases activity is comparatively similar to the pattern of MMPs activity detected on gel zymography in human demineralized dentinal lesions studied by previous report [5]. Therefore, it is possibility that gelatinases activity detected in our study was the activity of human salivary MMPs. It has been reported that human saliva consists various MMPs such as MMP-2 (72-kDa gelatinase /type IV collagenase; Gelatinase A), MMP-9 (92-kDagelatinase/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 [17, 18].

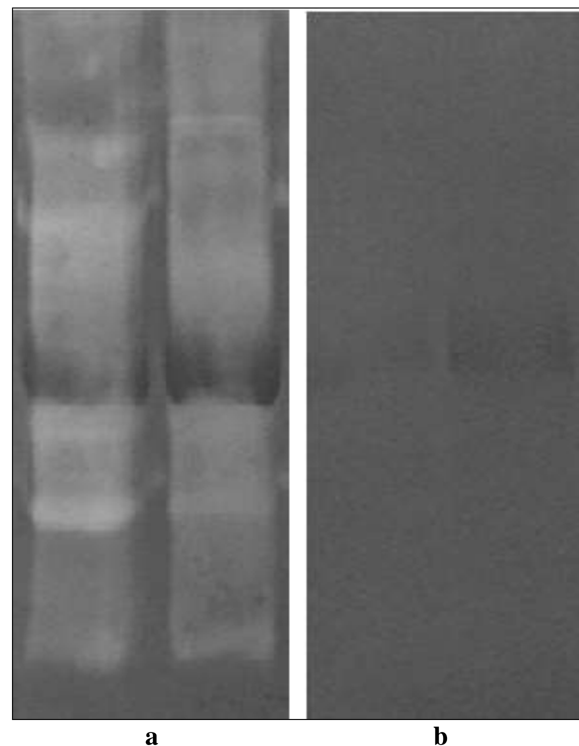

Fig 1: Zymograms represent the detection of gelatinase activity from human saliva (a) Zymogram which was incubated in activation buffer at 37°C for overnight. (b) Zymogram which was incubated t in 30Mm EDTA and 10Mm 1, 10-phenathralin solution at 37°C for overnight.

3.3 In gel inhibition of human salivary metalloproteinases

The most of phenolic compounds from plants have been known to have anti-MMPs activity. The 5-caffeoyl quinic acid isolated in methanolic extract from Euonymus *alatus*

bark, exhibits strong inhibitory activity against MMP-9 in concentration-dependent manner on zymography [19]. Figure 2 lanes d and e showed that barks methanolic extracts of *C. fistula* and *M. indica* exhibited inhibitory activity against human salivary gelatinases. This indicates that barks extract of these plants have human salivary MMP inhibitors. The extracts containing 100µg/ml concentration of phenolics was sufficient for inhibitory activity. Our study is similar to previous reported study of Longatti *et al*., 2011 who investigated the inhibition of phenolic extract of *T. guianensis against* Matrix metalloproteinases (gelatinases) in human cancers by using gelatin zymography [20]. *C. fistula* has been well known for therapeutics importance, consisting many bioactive compounds like dihydroxy anthraquinone, Oxy anthraquinone, (-) epiafzelechin, (-) epiafzelechin-3-Oglucoside, (-) epicatech in Kaempferol, biflavonoids, triflavonoids etc [21]. The *M. indica* bark has been reported to have phenolics such as flavonoids, triterpene and sterol [8]. The synergistic action of these active metabolites of these plants may be responsible for inhibitory effect.

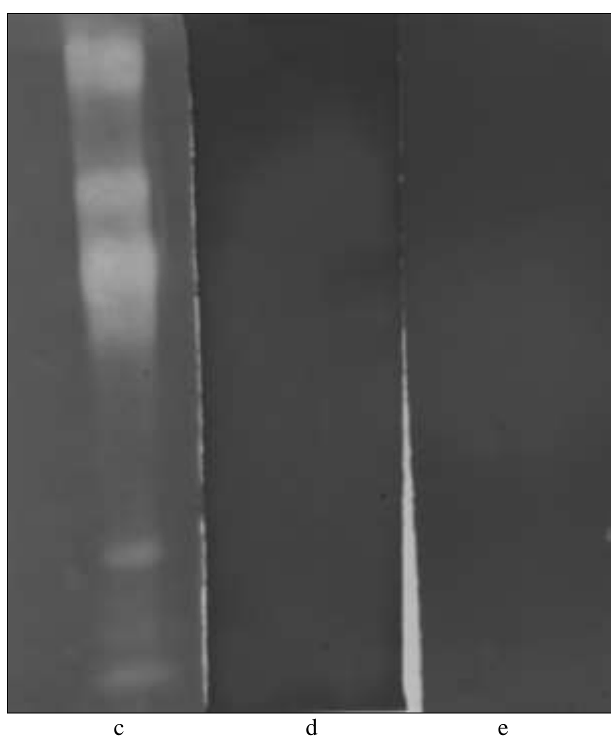

Fig 2: Zymograms represent inhibitory effect of plant extracts on human salivary metalloproteinases (c) Human salivary metalloproteinase activity (d) Zymogram was incubated with methanolic leaf extract of *C. fistula* at 37^{0}C for overnight and (e) Zymogram was incubated with methanolic leaf extract of *M. indica* at 37^{0}C for overnight.

4. Conclusions

From the result of present study it was concluded that bark extracts *C. fistula* and *M. indica* are sources of human salivary proteinase inhibitors. Extract of these plants may be useful as therapeutic agents for prevention of dental carries progression and other MMPs associated disorders.

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