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In-Vitro Inhibitory Effect of Standard Phenolic Compounds against Human Salivary Metalloproteinases

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ABSTRACT

Previous studies recommended that salivary and pulp-derived host matrix metalloproteinases (MMPs) participate in dentin caries pathogenesis. In this study we assessed in-vitro inhibitory effect and mechanism of action of standard phenolic compounds such as caffeic, p-hydroxybenzoic, coumaric, gallic and syringic acid against human salivary metalloproteinases.Gelatin zymography revealed six prominent gelatinalytic bands (P1, P2, P3, P4, P5, and P6) from human concentrated saliva out which P3, P4, P5 and P6 were confirmed as metalloproteinases. All standard phenolics exhibited dose-dependent inhibition against salivary metalloproteinases as well as metal chelating activity.Phenolic acids range 1-2 mM concentration required for complete inhibition of metalloproteinase on zymography. Caffeic acid exhibited maximum inhibition and chelating activity (93.75 IC_{50} µg/ml) as compared to other phenolics. Inhibitory effect of phenolic compound may be due to metal chelating action was concluded. Results of this study suggested that further study is needed to see the inhibitory effect of phenolic compounds against individual salivary MMP.

Key word: Gelatin zymography, Standard phenolics, Caffeic acid, Chelating activity.

INTRODUCTION

Phenolic compounds are an important group of secondary metabolites in plants and are divided into polyphenols and phenolic acids. They are synthesized in plants as chemical defense system against predators,ultra violet (UV) lights and pathogens and also they are involved in plant reproduction as well as plant-plant interference [1]. An aromatic rings with attached several hydroxyl groups are present in phenolic compounds. They are grouped on the basis of

function and number of aromatic rings that they contain and the radicals that bind these rings to one another [2]. Among phenolic compounds, the flavonoids, phenolic acids and tannins are considered as important dietary components because of their preventive action against chronic degenerative diseases such as cancer, diabetes, Alzheimer's disease, chronic diseases and cardiovascular diseases [3, 4]. Phenolics rich vegetables and fruits contribute to the delay aging process and decrease the oxidative stress risk related with chronic diseases such as atherosclerosis, cardiovascular diseases, diabetes, cancer, neurological diseases and disorder of cognitive function[5]. They possess different biological properties such as antioxidant, anti-inflammatory, anti-arthritis, antimicrobial and anti-proliferative [5]. Matrix metalloproteinases (MMPs) are family of zinc-dependent proteolytic enzymes,involved in the degradation of extracellular matrix (ECM) including native and denatured forms of collagens [6]. They are involved in physiological and in pathological conditions, their secretion and activity under physiological conditions is regulated by endogenous activators, tissue inhibitors (TIMPs) and serine protease inhibitors[7]. Human salivary MMPs particularly MMPs 2, 8, and 9 destroy the collagen matrix of dentin[8, 9]. The dental caries process encompasses the demineralization of inorganic minerals, mostly hydroxyapatite by acids produced by oral bacteria (Streptococcus mutans and others) [10]. The demineralization exposes the degradation of the collagenous organic matrix of dentin. Acids produced from oral bacteria activate the salivary MMPs which involved in the degradation of demineralized dentin collagenous matrix after pH neutralization by salivary buffers that cause dental caries progression[11, 12]. MMP inhibitors are capable for prevention of dentin matrix degradation that avoidsdental caries progression.Researchers have studied that use of chlorhexidine and chemically modified tetracyclines (CMTs) as MMP inhibitorsfor reduction of the degradation of the dentinin vivo and in vitro could reduce the dental caries progression [13, 14, 15]. It has been investigated that green tea polyphenols, especially epigallocatechin-3-gallate (EGCG) as MMP inhibitor reduces the dentin wear under erosive/abrasive conditions [16].As the health benefiting effects of phenolic compounds studied in earlier research, we aimed to study the inhibitory effect of standard phenolic compounds and their mechanism of action against human salivary metalloproteinases.

Materials and Methods

Chemicals and Reagents

Triton X-100, Tris-hydroxymethyl amine, Acrylamide, Bisacrylamide, Calcium chloride, Zinc chloride, EDTA, 1,10-Phenanthrolin, Glycine, Glycerol, Bromophenol blue, Coomassie brilliant blue R-250, Sodium phosphate, Sodium hydroxide, Sodium chloride, Sodium acetate, Acetic acid, Iron chloride ($FeCl_3$) andMethanol were purchased from RANKEM. P-Hydroxybenzoic acid, Gallic acid, Caffeic acid, Coumaric acid and Syringic acid were purchased from HIMEDIA. All chemicals used in this study were of analytical grade.

Collection and Concentration Human Saliva

The saliva (20ml) was brought 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). Supernatant of saliva was concentrated up to $1/10^{th}$ volume of original saliva using dialysis bag (cut of size

12kDa) which was kept in sugar at 15 ^{0}C for overnight. The protein concentration in concentrated saliva was determined by using Lowry method and preserved at -20°C temperature for further study.

Gelatin zymography

The detection of human salivary metalloproteinases was performed by using the gelatin zymography technique [17]. 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. Fifteen microliter of concentrated saliva 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-100) on a rotary shaker. Thereafter one gel-strip was incubated overnight at 37°C in activation buffer containing 50Mm Tris-HCL, pH 7.5, 10Mm $CaCl_2$. Rest of gel strips were incubated in buffer containing standard metalloproteinase inhibitors (30 Mm EDTA and 10Mm 1, 10-phenanthrolin). After incubation, each gel-strip was washed with distilled water and stained with 0.5% Coomassie blue R-250. After staining, gel strips were de-stained with 30% methanol and 10% acetic acid. The photograph of gel trips was taken by gel-documentation system (Alpha Innotech HP).

Effect of standard phenolic compounds on human salivary metalloproteinases

Effect of standard phenolic compounds on human salivary metalloproteinases was assessed by using the gelatin zymography [17]. Standard phenolics (p-Hydroxybenzoic, Gallic acid, Caffeic acid, Coumaric acid and Syringic acid) were dissolved in warm activation buffer with increasing concentrations (0.5, 1, 1.5 and 2 mM). Concentrated saliva was used for detection of metalloproteinase activities on gelatin zymography (Procedure of zymography discussed in above section). After electrophoresis gel was cut into slices and washed by Triton X-100 for 1 h followed by distilled water. Gel strips were incubated at 37 ^{0}C for overnight in activation buffer containing different concentrations of each standard phenolic compound. Thereafter, each gel strip was vigorously washed by distilled water. Staining and de-staining were performed same as discussed in zymography section. Gels were visually assessed and images were taken under gel-documentation system (Alpha Innotech).

Metal chelating activity of standard phenolic compounds

Metal chelating activities of standard phenolic compounds were determined using the procedure of Benzie and strain [18]. A typical reaction contains 0.5 ml (0.5 mg/ml) O-phenanthrolin, 1 ml $FeCl_3$ (200 M) and 2 ml each of phenolic compound with different concentrations (75, 150….1500 µg/ml) were incubated at ambient temperature for 15 min. After incubation the optical density was recorded at 510 nm. Blank was performed without phenolic compounds. The percentage of iron chelating activity of each phenolic compound was calculated by using following formula.

$$\text{Percent chelating activity} = \left[\frac{\text{Test absorbance} - \text{Control}}{\text{Test absorbance}}\right] \times 100$$

Results and Discussion

Detection of human salivary metalloproteinase activities on gelatin zymography

Gelatin zymography is the most profound technique widely applied for detection of gelatinase activity from various biological samples [19]. Human saliva comprises gelatinolytic and collagenolytic activities and these activities could be partially inhibited by the treatment of EDTA[20]. The proteinases with high molecular weight complex (>300 and 120 kDa) and a latent form (92 kDa)) of matrix metalloproteinase- 9 (MMP-9) are present in human saliva, that proteinases are auto activated at 37° C and condensed to a 42-kDa protein through 100, 67, and 50 kDa[20]. For the detection 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 prominent bands (P1, P2, P3, P4, P5, and P6) as gelatinase activity from human saliva. Out of six gelatinolytic bands, four bands (P3, P4, P5, and P6) were completely inhibited due to overnight treatment with metal-chelating compounds (EDTA and 1, 10-phenanthrolin), indicating these bands were metalloproteinases and two bands (P1 and P2) were not inhibited by same treatment, that indicating bands P1 and P2 were not metalloproteinases (Figure 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 [21]. Among six prominent proteinase bands, proteinase (P3) was found to be broad with highly intensive, followed by band P5 and P6 as compared to others. Few studies have reported the presence of MMPs in human saliva therefore, based on the result, it was assumed that metalloproteinase activities detected on gelatin zymography was the activities 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 [22, 23]. Kobus*et al*have reported the presence of MMP-2, MMP-8, MMP-9, TIMP-1, and TIMP-2 in human saliva[24].

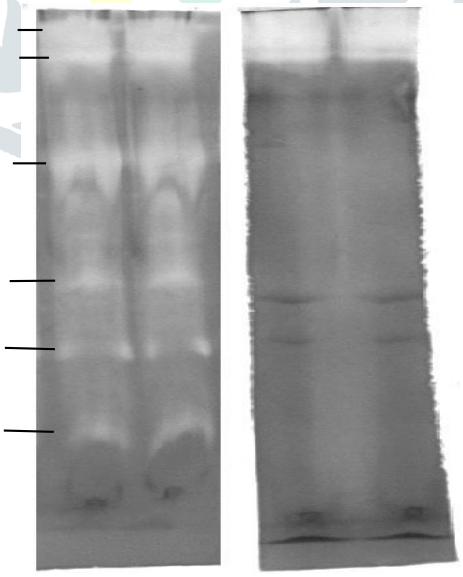


Figure 1: Zymogram represents the detection of gelatinase activity from human saliva. Lane (**A**) shows gelatinase activity bands (P1, P2, P3, P4, P5 and P6) when gel zymogram was incubated in activation buffer (50mM Tris-HCL pH 7.5, 10mM $CaCl_2$) at 37°C for overnight and lane (**B**) shows inhibition of human salivary gelatinase activities

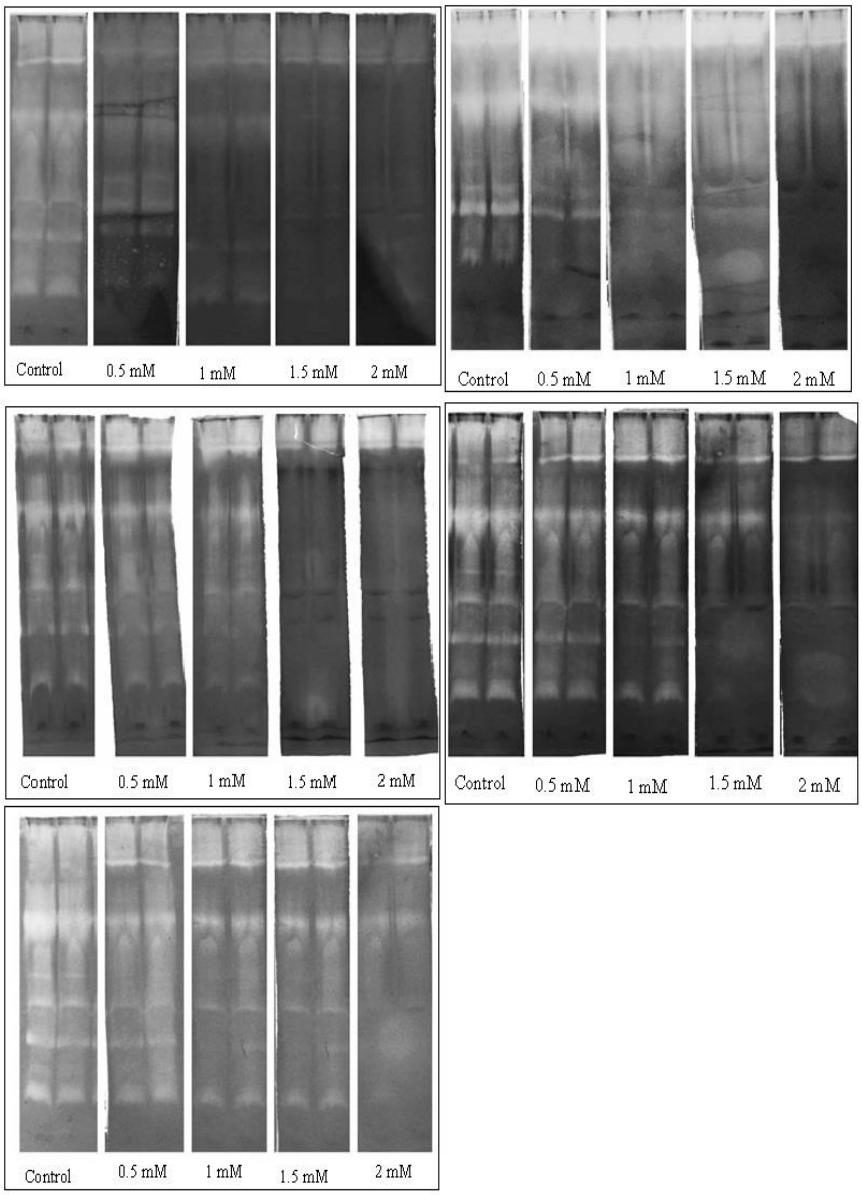
when incubated in 30mM EDTA and 10mM 1, 10-phenathralin solution at 37°C for overnight that confirmed the metalloproteinases exist in human saliva.

Effect of standard phenolic compounds on human salivary proteinases and their chelating activity

Human saliva consists several MMPs like collagenases and gelatinases derived from either the gingival crevicular fluid or the secretion of salivary glands. MMPs are strongly involved in dentin carries progression by the degradation of cellular matrix. The use of MMP inhibitors could be a successful strategy in the prevention of caries progression [25]. Figure 2 zymograms show that all standard phenolics exhibited dose-dependent inhibitory effect against human salivary metalloproteinases. Caffeic acid exhibited maximum while syringic acid exhibited minimum inhibitory activity as compared to other phenolics. 1mM caffeic acid was sufficient for complete inhibition of gelatinolytic activity of salivary metalloproteinases. Visual observation of zymograms revealed (A, B, C, D and E), the inhibitory potentials of phenolics were in order of caffeic acid > p-hydroxybenzoic acid >coumaric acid >gallic acid >syringic acid (Table 1 and figure 2). All phenolic compounds not exhibited inhibitory activity against gelatinolytic activity of P1 and P2 proteinase bands indicating these are non-metalloproteinases present in human saliva. The highestchelating activity (IC_{50} 93.75 µg/ml) was observed in caffeic acid while the lowest chelating activity (IC_{50} 780 µg/ml) was observed in syringic acid (Table 1). The chelating activities of remaining phenolic compounds were observed in order of gallic acid (IC_{50} 136.25 µg/ml) > p-hydroxybenzoic acid (IC_{50} 555 µg/ml) >coumaric acid (IC_{50} 690 µg/ml) (Table 1 and Figure 3). These results indicated that chelating property of standard phenolics may be responsible for inhibition of human salivary metalloproteinases by the chelation of calcium and zinc. Mechanisms of MMPs inhibition by phenolics may be Zn^{2+} and Ca^{2+} chelation because it has been investigated that phenolics groups exhibit promising chelating property [26]. Previous study investigated the caffeic acid isolated from methanol extract of *Euonymus alatus* exhibits strong MMP-9 inhibitor activity[27]. P-Hydroxybenzoic acid has been investigated to exhibits antifungal, antimutagenic, antisickling, estrogenic[28]and antimicrobial [29]activities. Wang*et al*have reported that gallic acid inhibits keloid fibroblast (KF) proliferation, migration and invasion with the downregulation of MMP-1 and MMP-3 and upregulation of tissue inhibitors of metalloproteinase-1[30].

Table 1: Metal chelating and salivary metalloproteinase inhibitory activities of standard phenolic compounds.

Sr. No.	Phenolic compound	Chelating activity IC_{50} (µg/ml)	Concentration of phenolic compound at which complete inhibition of metalloproteinases
1.	Caffeic acid	93.75	1 mM
2.	Coumaric acid	690	1.5 mM
3.	p-Hydroxybenzoic acid	555	1.5 mM
4.	Gallic acid	136.25	2 mM
5.	Syringic acid	780	2 mM


Control
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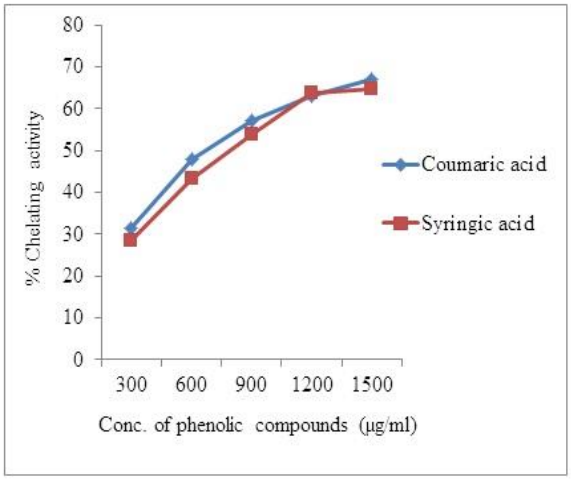

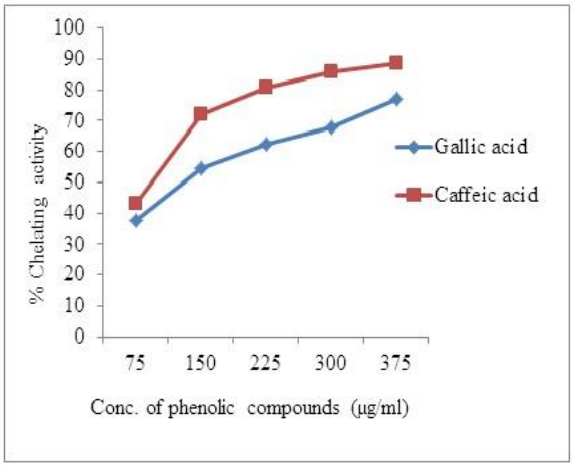

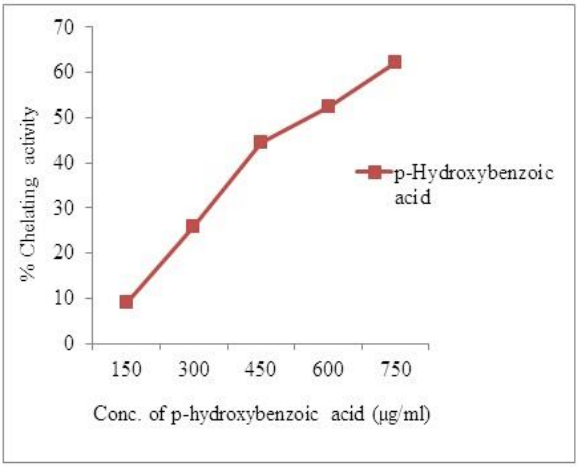

Conclusion

From the result it was concluded that standard phenolics compounds such as caffeic, p-hydroxybenzoic, coumaric, gallic and syringic acid exhibit dose-dependent inhibitory effect against human salivary metalloproteinases. All tested phenolic compounds also exhibit metal chelating activities. Among them caffeic acid exhibits prominent inhibitory as well as chelating effect while Syringic acid exhibits minimum inhibitory and chelating effect. Inhibition of metalloproteinases may be due to metal chelating action of standard phenolic compounds were concluded. Phenolic compounds can be useful as therapeutic candidates for prevention of dental carries progression through the inhibition of salivary MMPs.

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