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Author: Brankica G. Raskovic Natalija Dj. Polovic

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Title

# Collegenase activity in fig latex could contribute to its efficacy in ethnomedicinal preparations

Author names and affiliations

Brankica G. Raskovic, and Natalija Dj. Polovic*

[1]Department of Biochemistry, Faculty of Chemistry – University of Belgrade, Studentski trg 16, 11 000 Belgrade, Republic of Serbia

e-mail: braskovic@chem.bg.ac.rs

polovicn@chem.bg.ac.rs

*Corresponding author:

Natalija Polovic, PhD

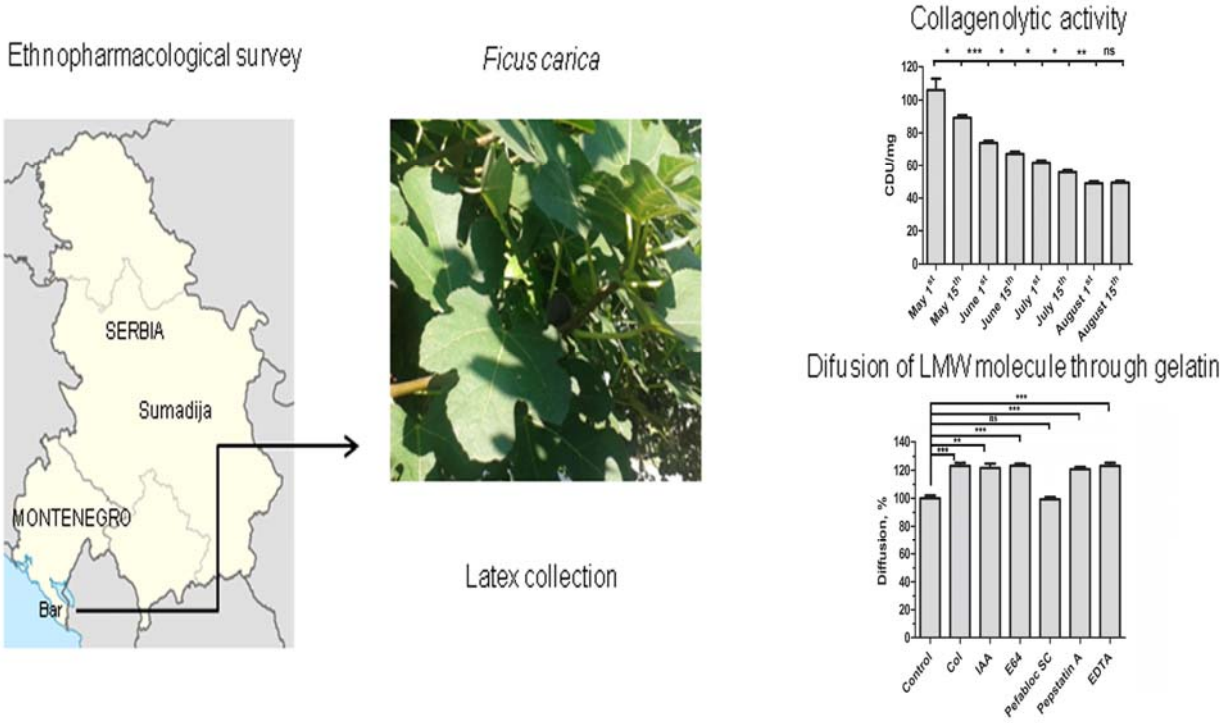
Department of Biochemistry, Faculty of Chemistry – University of Belgrade

Studentski trg 16, 11 000 Belgrade, Serbia

Phone: +381113336721, Fax: +381112184330

e-mail: polovicn@chem.bg.ac.rs

Graphical abstract




Highlights:

- Collagenolytic activity in fig latex was highest in the spring.
- Fig latex collagenase improved diffusion of low molecular weight molecule through the gelatin hydrogel.
- Fig latex collagenase was stabile during boiling.
- Fig latex collagenase was stabile in the simulated gastric conditions.

Abstract

*Ficus carica*, the common fig has been widely used for centuries for medicine as well as food. Fig latex is used for the treatments of cancer, inflammatory diseases, bacterial and

gastrointestinal nematode infections, warts and skin diseases. The aim of the study was to investigate if some of the fig latex applications could be attributed to collagenase activity. The usage of figs in the Western Balkans indicated that the latex of unripe fruits is often used for treatments that could involve remodeling of connective tissue. Collagenase activity in fig latices collected in spring was twice as high as the one detected in summer. Collagenase improved diffusion of low-molecular-weight model molecule through the gelatin hydrogel. Fig latex collagenase was stable during boiling and in the simulated gastric conditions for up to 1 hour. The presence of fig latex collagenase in traditional medicine preparations could increase treatment efficacy by hydrolyzing collagen present in extracellular matrix and facilitating the penetration of active molecules through the connective tissue.

*Keywords:* West Balkan, fig latex, collagenase, *Ficus carica*, extracellular matrix

## 1. Introduction

The fig tree (*Ficus carica*) has been cultivated in southern parts of temperate zones to be used as food and medicine. Fig latex is produced by laticiferous cells and it has been suggested that the latex secretion is a defense mechanism against wounding or pests such as insects and microorganisms (Oliveira et al., 2010). It has been extensively investigated for its proteolytic enzymes, LMW (low molecular weight) compounds and rubber (Kang et al., 2000). The use of fig fruit and latex for the treatment of cancer, inflammatory diseases, bacterial and gastrointestinal nematode infections, as well as warts and skin diseases, has been part of traditional medicine worldwide, especially in developing countries (Lansky et al., 2008) Representing a rich source of proteolytic enzymes, many of the ethnopharmaceutical uses of fig latex has been traditionally attributed to ficin activity (Lansky and Paavilainen, 2011). Ficin is a common name for endoproteolytic activity in latex of the genus *Ficus*. Ficin (EC 3.4.22.3) is a proteolytic enzyme present in the latex of fig trees (*F. glabrata* and *F. carica* species) (Azarkan et al., 2011; Devaraj et al., 2008). Ficin forms are recognized as sulfhydryl enzymes which contain cysteine residue in their active site and have molecular weight of about 24 kDa.
The activities of fresh latex usually attributed to proteases (e.g. ficin) include treatment of various skin diseases involving itching and eruptions, as well as treatment of rheumatism and wart removal (Lansky et al., 2008). The usage of fig latex in wart removal is at least as effective as conventional cryotherapy (Bohlooli et al., 2007). It is proven, as well, that proteases derived from fig activate human factor X and affect haemostasis (Richter et al., 2002). Recent studies have shown that the effects of papaya, fig and pineapple latices or fruit extracts against rodent

gastrointestinal nematodes are the consequences of the proteolytic activity leading to the complete digestion of nematodal cuticle (Stepek et al., 2007).

Furthermore, latex of *Ficus carica* is used in traditional medicine for the softening of solid tumors and debridement and healing of ulcers. Fig latex is usually administered combined with other botanical ingredients such as blue flag (*Iris versicolor* L.), barley, fenugreek, ginger and hot pepper. The most common route of administration has been external, but it is also given orally (boiled or fresh, mixed with flour, starch or dried milk) (Lansky et al., 2008).

A novel protease expressed in fig latex has been recently described (Raskovic et al., 2014). The protease belongs to serine protease family and cleaves preferentially collagen and gelatin. The enzyme has molecular weight of about 45 kDa. Additionally, this enzyme exhibits a very broad range of pH and temperature stability.

Since some of the ethnomedicinal activities of fig latex are clearly dependent of fig proteases (Lansky and Paavilainen, 2011), we postulated a hypothesis that collagenolytic fig latex protease, rather than ficin, could enable rearrangements of connective tissue and facilitate diffusion of active LMW (low molecular weight) molecules through the skin and internal connective tissues. The list of the use of fig latex in ancient and contemporary ethnomedicine that could have collagenase activity involved is presented in Table 1.

The use of fig latex in the Western Balkans as a folk medicine in several pathologies and conditions (such as skin diseases: hardening of skin, acne, psoriasis; scars and wart removal; treatment of ulcers and tumors), was investigated with a particular emphasis on its possible contribution to collagenolytic activity as a part of its molecular mechanism of action.

## 2. Material and Methods

### *2.1 Materials*

Collagen from rat tail; IAA; E-64 (*N*-[*N*-(L-3-*trans*-carboxyirane-2-carbonyl)-L-leucyl]-agmatine); Pefabloc SC; Pepstatin A; EDTA (ethylenediaminetetraacetic acid disodium salt dehydrate); sodium azide, bovine serum albumin; pepsin A (3.64 U/mg of protein); BPB (bromphenol blue) were purchased from Sigma–Aldrich (Steinheim, Germany). Gelatin was purchased from Merck (Darmstadt, Germany). Unstained protein molecular weight marker was bought from Thermo Scientific (Rockford, IL, USA). All other chemicals were commercial products of analytical grade and were used without further purification.

### *2.2 Ethnopharmacological survey*

Ethnopharmacological survey was carried out in the period 2012-2014 in Adriatic cost-side Montenegro (Bar region) and central Serbia (Sumadija region). Seventeen subjects (8 in Montenegro and 9 in Serbia) who had practiced traditional medicine were enrolled in the survey. Written consent was obtained from every study subject prior to the survey after which they were asked for their knowledge of the usage of figs (fruits, milk and leaves) in the treatment of any health condition. The specific questions included the part of the plant used; medicinal uses; and route of administration.

### *2.3 Latex collection*

Fresh latex was collected by mechanical incision every 15 days in the period May-August 2013 from the fig trees (*F. carica* var. Brown Turkey) in Bar, Montenegro. Each time, 10 mL of the

latex fluid was collected from the same fig trees (three in total, each time approximately 10 fruits were used). The latex was immediately stored at -20°C until used.

*2.4 Protein Concentration Determination*

The protein concentration was quantified by the dye binding method (Bradford, 1976) using bovine serum albumin as a standard.

*2.5 Proteolytic Assay*

Proteolytic activity of latex samples was estimated using BAPNA as substrate. BAPNA hydrolyzing activity was quantified according to Raskovic et al. (Raskovic et al., 2014). The assay was run in triplicate. Proteolytic activity was calculated as the amount of enzyme that catalyzed the release of 1 micromole of product per minute under standard conditions (International Unit of enzyme activity – 1IU). The specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in mU/mg.

*2.6 Collagenolytic activity*

Collagenolytic activity of latex samples was assayed as previously described (Raskovic et al., 2014). One unit of collagen digestion activity (CDU) was defined as the amount of enzyme that releases peptides from collagen equivalent in ninhydrin colour to 1 mmol of leucine in 5 h. The specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in CDU/mg. The assay was run in triplicate.

*2.7 Diffusion Assay*

In order to check if fig latex collagenases could facilitate diffusion of LMW molecules through the extracellular matrix, the authors applied a modified protocol, the same one as used for determining migration of cells through the collagen hydrogel (Shin et al., 2012). The experiment was conducted in glass capillary tubes (2 mm inner diameter, 5 cm high). Fig latex (sample collected on May 1$^{st}$ 2013) was added to 1% gelatin solution in PBS buffer and specific inhibitors were added. Final concentration of latex proteins was 0.5 mg/mL. Final concentrations of inhibitors were: 2 mM each: IAA; Pefabloc SC and EDTA; 2 µM E-64 and 1.46 µM Pepstatin A. Controls were set by adding PBS instead of latex or inhibitors. Gelatin hydrolysis was allowed to proceed for 30 minutes at room temperature. The tubes were chilled at 10°C to allow gelatin to form hydrogel for 15 minutes. After formation of hydrogel, 0.05 mL of 10 µg/mL BPB solution in PBS was carefully added to the top of the gel. Diffusion proceeded for 30 min at room temperature, and then the diffusion front was measured. The experiment was run in triplicates. The results are presented as relative diffusion in the absence of fig latex.

*2.8 Determination of digestion stability of fig latex collagenase*

Gastric fluid was prepared in accordance with US Pharmacopeia (3.2 g/L pepsin A in 0.1M HCl containing 2g/L NaCl). Twenty microliters of crude latex (sample collected on May 1$^{st}$) or thermally treated crude latex (boiled for 5 minutes) were pre-warmed and mixed with 20 µL of previously pre-warmed (37°C) 0.2 M HCl containing 4 g/L of NaCl and 6.4% of pepsin A, 3.64 U/mg of protein (Sigma-Aldrich, Taufkirchen, Germany). Digestion proceeded at 37°C with continuous shaking. The digestions were stopped after 1; 5; 15; 30 and 60 minutes with 12 µL of 0.2 M $Na_2CO_3$, and samples were diluted by adding 52 µL of deionized water (Polovic et al.,

2007). For SDS-PAGE analysis samples were denatured by mixing with 26 µL of 60 mM Tris buffer pH 6.8 containing 5% 2-mercaptoethanol; 25% glycerol; 2% SDS and 0.1% bromphenol blue followed by boiling for 5 minutes. The effect of starch and milk on fig latex protease digestion by pepsin was checked as published (Polovic et al., 2009). Briefly, 10 mg of starch or milk powder were mixed with 20 µL of crude latex before addition of simulated gastric fluid. The digestion was run for 1 hour and analyzed by SDS-PAGE and zymography as described in 2.9.

*2.9 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and activity staining*

SDS-PAGE was run in a discontinuous buffer system according to the method of Laemmli (Laemmli, 1970) using 12% polyacrylamide gel. To obtain zymography detection by negative staining, 0.2% of gelatin as a substrate was added to the gel before polymerization. Ten microliters of 200 fold diluted fig latex previously digested with pepsin was applied per lane. Electrophoresis was performed in reducing conditions; zymogram was developed according to previously published procedure (Gavrovic-Jankulovic et al., 2005) with minor modifications. Briefly, after electrophoresis, gel was extensively washed with MilliQ water and PBS buffer containing 10 mM L-cysteine. After incubation in the same buffer for 16 hours, gel was stained with CBB R-250 (Coomassie Brilliant Blue R-250) according to previously published procedure (Gogly et al., 1998).

*2.10 Data analysis and presentation*

Data evaluation and statistical analysis were performed using GraphPad Prism software version 5.00 (San Diego, California, USA). One way analysis of variance (ANOVA) test was performed to establish significance of all data sets. Two-tailed t-test analyses were used to establish the rassociation between: 1) enzymes' activities and ripening period and 2) facilitation of diffusion of LMW molecules through the simulated extracellular matrix and collagenase activity. The significance levels were $p<0.05$, $p<0.01$ and $p<0.001$. All data are expressed as mean ± standard error of the mean (SEM).

## 3. Results and Discussion

*3.1 Ethnopharmacological use of figs in West Balkan region*

The knowledge of ethnopharmacological usage of figs in Adriatic coast-side Montenegro (Mediterranean) and Central Serbia (Continental) is presented in Table 2. To the best of the authors knowledge, this is the first report of the usage of figs in folk medicine in West Balkan. A detailed presentation of both ancient and contemporary usage of figs worldwide (including fruit; leaves and latex, milk) can be found in (Lansky and Paavilainen, 2011) and (Lansky et al., 2008).

*3.2 Collagenolytic and proteolytic activity in fig latex during fruit ripening*

Our study participants insisted that for the effective treatment of some of the medical conditions mentioned the latex of unripe fruits had to be used (Table 2). It prompted the authors to monitor whether if there were different proteolytic profiles in fig latex collected in May-August period. Collagenolytic and general proteolytic activity were assayed. The results are presented in Fig. 1.

Since fig latex ficin can hydrolyze BAPNA and cannot hydrolyze collagen, while collagenolytic serine protease does not contribute to BAPNA hydrolysis (Devaraj et al., 2008; Raskovic et al., 2014), it was possible to monitor the changes in activities of these enzymes by using different substrates.

Collagenolytic activity of fig latex was the highest in the beginning of fruit ripening period while general proteolytic activity attributed to ficin reached maximum when the fruits were ripe and decreased with over ripening (Fig. 1). Furthermore, seasonal chemistry of fig latex showed an increase in total protein concentration (Raskovic et al., 2016). The content of major protein bands did not change significantly during ripening but more importantly, the activities of specific enzymes' varied, e.g. chitinolytic activity decreased over time, as well as total caseinolytic activity while milk clotting activity (digestion of κ-casein) usually attributed to one form of ficin increased during the same period (Raskovic et al., 2016). Taken altogether, the changes in collagenolytic and BAPNA hydrolyzing activity (Fig. 1) and in general caseinolytic and specific κ-caseinolytic activity (Raskovic et al., 2016) suggested that different forms of proteases were expressed in fig latex during flowering and fruit ripening. The results indicated that collagenolytic serine protease, rather than ficin, could be considered as the major proteolytic enzyme of latex collected in spring, that contributed to some of the effect of fig latex in ethnopharmacological preparations.

*3.3 Ethnopharmacological use of fig latex that could involve connective tissue remodeling*

The uses of fig latex that involve connective tissue remodeling most probably by action of fig latex collagenolytic protease are listed in Tables 1 and 2.

Scar tissue and wound repair are among the most usual pathological conditions which have been traditionally cured with fig latex (Table 1 and 2). Furthermore, conventional treatment of these conditions, as well as fibroproliferative diseases (reduction in burn scar formation, Dupuytren's contracture, wound repair), involves usage of commercial bacterial collagenase (Frye and Luterman, 2005; Onesti et al., 2013; Hurst et al., 2009). In our previous work (Raskovic et al., 2014), it has been shown that fig latex serine protease was capable of hydrolyzing the firm triple helical structure of native collagen. Recently described by the authors this fig latex collagenase that has high activity and broad thermal and pH stability (Raskovic et al., 2014), represents a potential candidate that can replace commercial collagenase from microbial sources in many fields of industry and biomedicine, such as fibroproliferative diseases.

*3.4 Fig latex collagenase improve diffusion of low molecular weight dye through the gelatin hydrogel*

Another interesting usage of fig latex in ethnomedicine that can be attributed to some extent to the action of collagenase, is the treatment of solid tumors and ulcers (Table 1).

Cancer therapy may often be affected by failure in drug delivery targeted to tumor tissue. This is mainly caused by elevated interstitial fluid pressure that indirectly reduces the uptake of molecules by solid tumors. Modulation of the tumor extracellular matrix may reduce interstitial fluid pressure and subsequently improve transport of macromolecules (Boucher et al., 1990). Recent studies showed that the co-usage of commercial bacterial collagenase improved cancer therapy by increasing the uptake of therapeutics by solid tumors (Eikenes et al., 2004) causing remodeling of extracellular matrix mainly composed of collagen. Co-usage of commercial collagenase improved delivery of both low molecular weight drugs and relatively large

assemblies such as nanoparticles to solid tumors (Goodman et al., 2007). Thus, there is a possibility that the presence of collagenase in fig latex could improve penetration of anti-proliferative LMW compounds from remedies to the tissue embedded tumors.

The diffusion of colored LMW model molecule (bromphenol blue - BPB) through the gelatin hydrogel previously treated with fig latex was presented in Fig. 2.

It could be observed that BPB migration through gelatin hydrogel was increased by approximately 25% if collagenase was present. The statistically significant positive effect of collagenase on BPB diffusion was ended in the presence of specific serine protease inhibitor Pefabloc SC. Since it has been recently shown that fig latex collagenase belongs to the serine protease family (Raskovic et al., 2014) it is clear that the enhancement of BPB diffusion can be completely attributed to the action of collagenase. To rule out any possibility that the well known fig latex protease ficin contributed, specific inhibitors of other proteases classes were used as well. Specific cysteine, aspartic and metallo protease inhibitors IAA and E-64; Pepstatin A and EDTA, respectively, did not show any statistically significant effect (Fig. 2). It is noteworthy that dozens of molecules with proven anti-proliferative and anti-inflammatory activities had been identified in fig latex and summarized in the literature (Lansky and Paavilainen, 2011; Lansky et al., 2008; Rubnov et al., 2001). Furthermore, in ancient remedies, fig latex was often used mixed with other active extracts or molecules such as ginger, hot pepper, blue flag, hydrated sodium carbonate, quicklime, vitriol, table sugar, starch and table salt (Lansky et al., 2008). The presence of fig latex collagenase in remedies could provide improved delivery of anti-proliferative LMW compounds originated from other extracts to the solid tumors and ulcers, leading to the increased treatment efficacy.

*3.5 Fig latex collagenase is stable in the simulated gastric conditions*

Survival of fig latex collagenase in gastric conditions was explored to prove its activity after oral intake since fig latex preparations are often orally administrated, alone or combined with flour, starch or milk, and sometimes boiled (Table 1 and 2).

Fig latex proteins (regardless of thermal treatment) were resistant to pepsin digestion for up to 1 h (Fig. 3A). Collagenase was identified at molecular weight of 45 kDa. Fig latex collagenase remained active if incubated with pepsin at low pH value (Fig. 3B) for up to 1 hour. In the case of previously boiled latex samples, decrease in collagenolytic activity could be observed. Apparently, thermal treatment did not enhance digestibility of fig latex collagenase but led to protein denaturation and subsequent partial loss of the activity.

Starch and milk, the common ingredients of traditional remedies containing fig latex, did not influence either digestion in simulated gastric fluid nor collagenolytic activity of fig latex protease (Fig. 4).

Even if administered orally, fig latex collagenase could survive harsh acidic and proteolytic conditions present in the stomach. However, limited digestibility in simulated gastric fluid could increase possibilities of developing allergic sensitizations and reactions (Polovic et al., 2007), thus the latex usage in traditional medicine should be avoided in the case of atopic individuals.

## 4. Conclusions

The ethnopharmacological use of fig latex (often collected in the spring) in the Western Balkans is predominantly limited to external treatments of skin conditions. Nevertheless, internal administration has been practised as well, especially for indications such as tissue embedded tumor treatment, infertility, haemorrhoids, obesity, gastritis, insomnia and bronchitis/respiratory

problems. Collagenolytic activity was the highest at the beginning of the fruit ripening period, while general proteolytic activity assigned to ficin reached its maximum when fruits were completely ripe, thus fig latex collagenase, rather than ficin, may contribute to some of the effects of fig latex in ethnopharmacological preparations.

Fig latex collagenolytic serine protease can cleave triple helical native collagen chains. Since native collagen represents the main constituent of extracellular matrices of the tissues, collagenase treatment could promote the remodeling of connective tissue. This finding is very important, since it reveals a mechanism of fig collagenase action in debridement of ulcers and wounds and the treatment of fibroproliferative skin conditions (e.g. scar tissue repair). Furthermore, fig latex collagenase, with its broad pH and temperature stability range, has a potential for numerous applications in biomedicine and biotechnology.

Fig latex collagenase facilitated migration of model LMW molecule through the gelatin hydrogel indicating that collagenase could improve availability of LMW anti-proliferative molecules in cancer treatments by enhancing their delivery to tissue embedded tumors.

Even if administered orally, fig latex collagenase could survive harsh acidic and proteolytic conditions present in the stomach, regardless of the presence of common ingredients of traditional remedies.

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Conflict of Interest Statement
The authors declare no conflict of interest.

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Figure captions

Fig.1. Collagenolytic (CDU/mg – collagen digestion units, CDU, per mg of protein content; black) and proteolytic activity (BAPNA hydrolyzing activity – mU per mg of protein content; grey) of fig latices collected in the period May-August 2013. Error bars represent standard error of the mean (SEM). The significance levels were $p<0.05$*, $p<0.01$* and $p<0.001$***.

Fig. 2. Diffusion of bromphenol blue through the gelatin hydrogel. Control – non-treated hydrogel; Col; IAA; E-64; Pefabloc SC; Pepstatin A and EDTA – collagenase treated hydrogel without or with the inhibitor added. Error bars represent standard error of the mean (SEM). The significance levels were $p<0.05$*, $p<0.01$** and $p<0.001$***.

Fig. 3. A) Digestibility of fig latex proteins in simulated gastric fluid. B) Zymography detection of collagenase activity after incubation in simulated gastric fluid. P - pepsin; C - control; Mw - molecular weight markers; kinetics of digestion: 1 – 1 minute, 2 – 5 minutes, 3 – 15 minutes, 4 – 30 minutes and 5 – 60 minutes; * - previously boiled latex samples.

Fig. 4. The influence of starch and milk on the digestion of fig latex proteases in simulated gastric fluid. Digestion was run for 1 hour. (A) SDS PAGE and (B) zymogram. L - latex; LS - latex and starch; LM - latex and milk; P - pepsin; Mw - molecular weight markers.

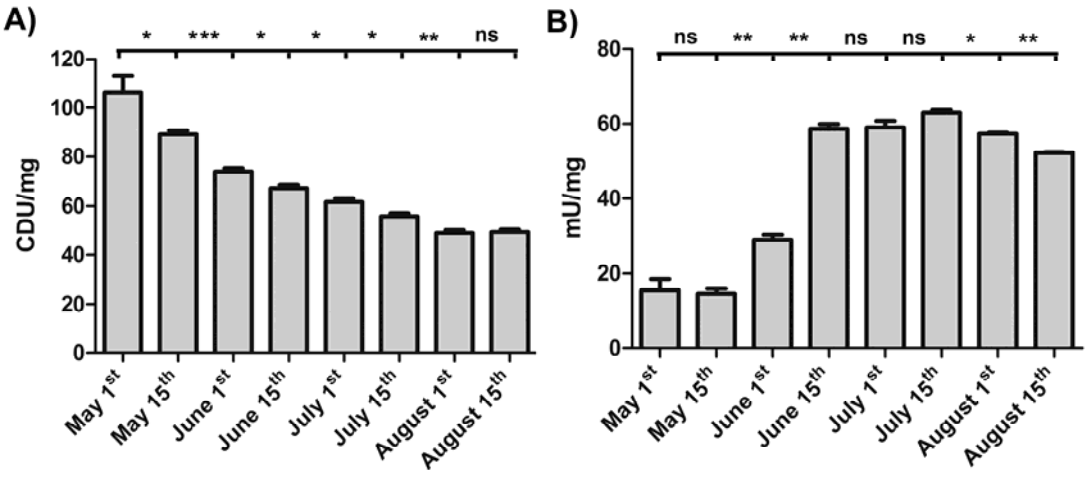


Fig 1

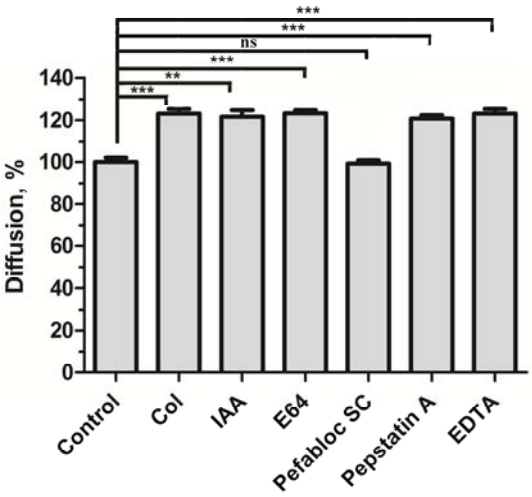


Fig 2

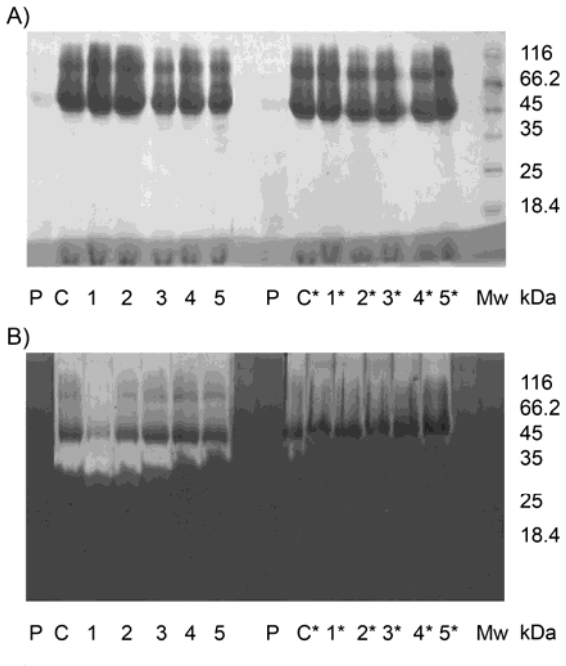


Fig 3

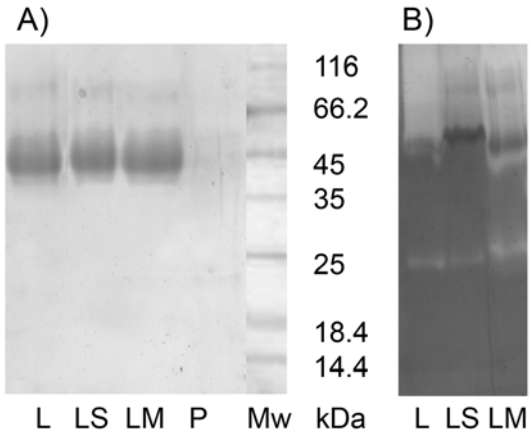


Fig 4

Table 1. The use of fig latex in ancient and contemporary ethnomedicine that could involve collagenase activity.

| **Use** | **Medicament** | **Place** | **Route of administration** | **References** |
|---|---|---|---|---|
| **Scar removal** | Latex | Persia | External application | (Lansky and Paavilainen, 2011) |
| **Warts** | Fresh latex | Iran | External application | (Bohlooli et al., 2007) |
| | Latex | Italy | External application | (De Feo et al., 1992) |
| | Fresh latex | Turkey | External application | (Yeşilada et al., 1995) |
| **Liver cirrhosis** | Latex (collected in the beginning of spring) | Syria, Persia, Turkey, England | Poultice, drink | (Lansky et al., 2008) |
| **Head wounds** | Latex (collected in the beginning of spring) | Turkey | Ointment | (Lansky et al., 2008) |
| **Softening of solid tumors** | Boiled latex, mixed with other ingredients or fresh latex only (collected in the beginning of | Syria, Persia, Turkey | Poultice, drink often together | (Lansky et al., 2008) |

| | | | | |
|---|---|---|---|---|
| | spring) | | | |
| **Debridement and healing of ulcers** | Latex with egg yolk or vegetable oil | Germany, Italy | Poultice, liniment | (Lansky et al., 2008) |

Table 2. Ethnopharmacological use of figs in the Western Balkans.

| **Use** | Medicament | Route of administration |
|---|---|---|
| **Scars** | Latex[#1] | External |
| **Freckles, moles, toothache** | Latex[#2] | External |
| **Gastric ulcers** | Latex[#1,2], usually diluted with water | Drink |
| **Cutaneous ulcers** | Latex or fruit puree[1,2] | External, poultice |
| **Warts, insect bite** | Latex[#1,2] | External |
| **Thorn, sea urchin** | Latex[1,2] | External |
| **Hardening of skin, dry callus** | Latex[1,2] | External |
| **Psoriasis** | Latex[2] | External |
| **Internal tumors** | Fruits with red wine[1] | Eat |
| **External tumors** | Roasted fruit, sometimes mixed with yeast and garlic[2] | External |
| **Acne** | Fresh fruit puree[1,2] | External |
| **Infertility** | Dry fruits in olive oil[1] | Eat |
| **Laxative, Haemorrhoids, Obesity, Gastritis, Insomnia** | Fruits[1,2] | Eat |

| | | |
|---|---|---|
| **Bronchitis/respiratory problems** | Dry fruits boiled in milk[2] | Eat |

[1]Adriatic cost-side Montenegro (Mediterranean)

[2]Central Serbia (Continental)

[#]To achieve better effects use latex of unripe fruits