Investigation of Hyaluronidase, Collagenase and Elastase Inhibitory Potentials and Comparative Evaluation of the Antimicrobial, Antioxidant and Homeostatic Activities of Two Natural Polysaccharides

Rukiye BORAN*[1], Aysel UGUR[2], Nurdan SARAC[3]

[1]Aksaray University, Vocational School of Health Service, Department of Medical Services and Techniques, 68100, Aksaray, Turkey
[2]Gazi University, Faculty of Dentistry, Section of Medical Microbiology, Department of Basic Sciences, 06510, Ankara, Turkey
[3]Mugla Sitki Kocman University, Faculty of Science, Department of Biology, 48000, Mugla, Turkey

Abstract: The aim of this study was to investigate the hyaluronidase, collagenase and elastase inhibitory effects, which play important role for wound healing, together with the antibacterial, antioxidant and homeostatic activities of tragacanth gum (TG) and locust bean gum (LBG). The antimicrobial activities were tested against four bacteria and the antioxidant activities were estimated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2) radical scavenging and β-carotene bleaching assays. Homeostatic effect was evaluated with the Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) test parameters. The wound healing potentials were determined with the inhibition of hyaluronidase, collagenase and elastase enzymes. The TG showed antibacterial activity against *Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922. The results showed that TG and LBG possessed antioxidant properties including DPPH scavenging (21.0% and 17.6%, respectively) and H_2O_2 radical scavenging (59.4% and 79.0%, respectively) activities. The polysaccharides displayed significantly reducing PT and aPTT results. Between the two tested polysaccharides LBG showed significant hyaluronidase and collagenase inhibition activity at 10 mg/mL concentration. These findings show that these natural polysaccharides can be used to support of wound healing.

İki Doğal Polisakkaritin Hiyalüronidaz, Kollajenaz ve Elastaz İnhibitör Potansiyellerinin Araştırılması ve Antimikrobiyal, Antioksidan ve Homeostatik Aktivitelerinin Karşılaştırmalı Olarak Değerlendirmesi

Özet: Bu çalışmanın amacı, kitre zamkı (TG) ve keçiboynuzu zamkı (LBG)'nin antibakteriyel, antioksidan ve homeostatik aktiviteleri ile birlikte yara iyileşmesi için önemli olan hiyalüronidaz, kollajenaz ve elastaz inhibitör etkilerini araştırmaktır. Antimikrobiyal aktiviteleri dört bakteriye karşı test edilerek, antioksidan aktiviteleri 1,1-difenil-2-pikrilhidrazil (DPPH), hidrojen peroksit (H_2O_2) radikal temizleme ve β-karoten ağartma deneyleri ile tespit edilmiştir. Homeostatik etki Protrombin Zamanı (PT) ve Aktive Edilen Kısmi Tromboplastin Zamanı (aPTT) test parametreleri ile değerlendirilmiştir. Yara iyileştirme potansiyelleri ise, hiyalüronidaz, kollajenaz ve elastaz inhibisyonu ile belirlenmiştir. TG, *Pseudomonas aeruginosa* ATCC27853 ve *Escherichia coli* ATCC25922'ye karşı antibakteriyel aktivite göstermiştir. Sonuçlar TG ve LBG'nin DPPH temizleme (sırasıyla %21.0 ve %17.6) ve H_2O_2 radikal temizleme (sırasıyla %59.4 ve %79.0) aktiviteleri de dahil olmak üzere antioksidan özelliklere sahip olduğunu göstermiştir. Polisakkaritler, PT ve aPTT'de önemli azalma göstermiştir. Test edilen iki polisakkarit arasından LBG, 10 mg/mL konsantrasyonda, önemli hiyalüronidaz ve kollajenaz inhibisyon aktivitesi göstermiştir. Bu bulgular, bu doğal polisakkaritlerin yara iyileşmesini desteklemek için kullanılabileceğini göstermektedir.

*Corresponding author: rukiyeboran_@hotmail.com

1. Introduction

Tragacanth gum (TG) and locust bean gum (LBG) are natural polysaccharides that are obtained from stems and branches of *Astragalus* plant and *Ceratonia siliqua* seeds, respectively [1, 2], and generally classified as safe (GRAS) materials in foodstuffs [3, 4]. Tragacanth is acidic polysaccharide with a high molecular weight (about 8.4 105 Da). It consist two major fractions; tragacanthin and bassorin representing 60–70% of total gum and includes D-galacturonic acid, D-galactopyranose, D-xylopyranose, L-fucose, L-arabinofuranose and L-rhamnose. The gum also contains amino acids, their derivatives, calcium, magnesium and potassium salts [2]. It has been used medicinally for thousands of years for a persistent cough, laxative, diarrhea, and as an aphrodesiac. The modern pharmaceutical use of tragacanth is an adhesive agent for tablets and pills, and for emulsifying oil droplets in pastes, creams and lotions [5]. It also could act as a stabilizer, emulsifier, thickener and suspending agent in food products [3]. LBG is a neutral galactomannan polysaccharides comprised of a high molecular weight (about 50000-3000 Da), consists of a β-(1-4)-D-mannopyranosyl backbone with α-D-galactopyranosyl substituted on C_6 of mannose [6]. It has high viscosity of the solution in water and traditionally used in the food industry as thickener, stabilizer, emulsifier, and gelling agent. It is also used in the pharmaceutical industry as excipient in drug formulations, and in biomedical applications [4].

TG and LBG are non-toxic, renewable, usually biodegradable, economical, readily available and biocompatible. In recent years, various studies have been reported to use TG such for burns or wound dressings [7], hydro gel membranes [8], matrix for cell immobilization [9] and drug encapsulation [10]. Similarly LBG can be used as hydro gel beads for controlled oral [1]; buccal, colonic, ocular and topical drug delivery [11].

The wound healing process has multi stage phenomenon of physiological events, such as coagulation, formation of granulation tissue, re-epithelization and remodelling of the extracellular matrix (ECM) [12]. New research shows that the only function of the ECM is not passive physical support for cells and that the ECM is also involved in tissue repair. It is now clear that undistorted ECM molecules have the ability to transform signals important for cellular processes in wound healing in connection with growth factor activation [13]. ECM molecules in dermis are the collagens, elastin, proteoglycans, and glycosaminoglycans. Glycosaminoglycans interact with proteins in the ECM. Hyaluronic acid is the predominant glycosaminoglycan in the skin. Under normal conditions, the ECM is composed of collagen, the most abundant protein in the body. Collagen plays a significant role in all phases of wound healing process. Elastin, another protein found in the ECM, gives skin and other tissue elasticity [14].

However inflammatory response accelerates the synthesis of dermal enzymes leading to degradation of ECM. Hyaluronic acid depolymerised by hyaluronidase, elastase hydrolyzes fibrin and elastin fibers, and matrix metalloproteinases-1 particularly breaks the type I collagen. Suggesting that the expression of dermal enzymes and the down-regulation of fiber synthesis play a major role in the process of skin wound [15].

In particular, chronic wounds show a long-standing inflammatory response and this provides an ideal environment for bacterial infiltration and bacterial growth [16]. The presence of infection prolongs inflammation, damages tissue, inhibit collagen production and cause healing to be delayed [17]. The healing process may be also hampered by the presence of reactive oxygen radicals. In fact, injured tissues affect the healing process by increasing the formation of reactive oxygen products and reducing various enzymatic and non enzymatic free radical scavengers [18]. However, the reactive oxygen species (ROS) excess transcends the beneficial effect, and causes inflammation, death of cells, tissue damage and decreasing the healing process, so antioxidant substances will reduce the possibility of these adverse events occurring. For this reason, they appear to be important for the successful management of wounds [19].

The presence of drugs that can trigger the wound healing process in the positive direction is still limited to in the modern medicine. Although there has been much progress in this area, there is still a shortage of safe and effective wound healing treatments [19]. Lately, many useful applications of natural polysaccharides have been reported in the management of wound care [8, 20, 21].

The topics of this study was to research the hyaluronidase, collagenase and elastase inhibitory effects, important for wound healing, together with the antibacterial, antioxidant and homeostatic activities of TG and LBG. To our knowledge, this is the first report showing the enzyme inhibitory, homeostatic and antioxidant activities of TG and LBG.

2. Materials and Method

2.1. Materials

The TG, LBG, H_2O_2, DPPH, β-carotene, linoleic acid, bovine hyaluronidase, **citrated plasma**, *Clostridium histolyticum* collagenase, N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Ala (FALGPA), porcine pancreatic elastase, sodium hyaluronate, epigallocatechin gallate (EGCG) and N-Succinyl-Ala-Ala-Ala-p-nitroanilide were purchased from Sigma-Aldrich

Chemicals Company (St. Louis, USA). *p*-dimethyl amino benzaldehyde, culture mediums were purchased from Merck Chemical Co. (Darmstadt, Germany).

2.2. Antimicrobial activity

Staphylococcus aureus ATCC25923, *Streptococcus pyogenes* ATCC12344, *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853 were purchased from American Type Culture Collection (ATCC). *S. pyogenes* was cultured in Brain Heart Infusion Broth and other strains were cultured in Nutrient Broth at 37ºC. Suitable soluble solutions of TG and LBG with concentrations of 20 mg/mL and 10 mg/mL were prepared by dissolving in water at 60ºC, respectively. In accordance with the National Committee for Clinical Laboratory Standards, the antibacterial activities of the TG and LBG against microorganisms were determined with disc diffusion method and broth dilution method [22].

Minimum inhibitory concentration (MIC) assays were performed to determine the antibacterial activity of the polysaccharides against pathogenic bacteria by serial dilution of the TG and LBG. An inoculum containing 5×10^5 cfu/mL bacterial suspension, fresh broth medium containing different concentrations of TG (2 μg/mL-2 mg/mL) or LBG (1 μg/mL-1 mg/mL) were inoculated into tubes and were incubated under appropriate conditions for 16-20 h. Optical density was measured at 550 nm.

2.3. Antioxidant activity

DPPH scavenging activities of the polysaccharides and synthetic antioxidant substances, used as a positive control, were determined in accordance with the Ebrahimabadi et al. [23] with a little modification [24], which is based on the principle of scavenging the DPPH radical.

H_2O_2 radical scavenging activities of TG and LBG on hydroxyl radical were performed by the method of Zhang et al. [25]. The colour change of the mixture was observed by measuring the absorbance at 510 nm after incubation for 30 min.

DPPH scavenging and H_2O_2 radical activities were calculated as follows:

Scavenging rate (%)= [(Abs$_c$-Abs$_s$)/Abs$_c$] x 100   (1)

Abs$_c$ is the absorbance of the control, and Abs$_s$ is the absorbance of the TG and LBG mixed with reaction solution.

β-carotene bleaching assay was performed according to Rauter et al. [26]. The oxidation of the reaction was determined at 470 nm. The activity was calculated using the equations below:

Ln (Abs) = ln (Abs0) + R × t   (2)

R: the bleaching rate is the slope of ln (Abs) vs. time line, which can be determined by linear regression, being t the time in minutes.

Antioxidant activity(%)=[(Abs$_c$-Abs$_s$)/Abs$_c$] x100   (3)

Abs$_c$ is the absorbance of the control, and Abs$_s$ is the absorbance of the TG and LBG mixed with reaction solution.

2.4. Prothrombin time and activated partial thromboplasmatin time assays

For *in vitro* PT assay, 50 μL citrated plasma was incubated with 50 μL polysaccharide for 3 min at 37ºC and 100 μL PT reagents were added. After that clotting time was immediately recorded. For *in vitro* aPTT assay, 50 μL citrated plasma was incubated with 50 μL polysaccharide and 50 μL aPTT reagents at 37ºC for 3 min. aPTT clotting time was recorded after the addition of 100 μL calcium chloride (CaCl$_2$) (20 mM) [27].

2.5. Enzyme inhibitory activity

50 μL of bovine hyaluronidase (in 100 mM acetate buffer, pH 3.5) was added to 100 μL polysaccharide samples in various concentrations and incubated at 37ºC for 20 min. 100 μL of CaCl$_2$ was put in to the reaction and incubated at same temperature for 20 min. The mixture was supplemented with 250 μL sodium hyaluronate and again incubated at 37ºC for 40 min. After that, 50 μL of 200 mM NaOH and 100 μL of 200 mM sodium borate was put in the emulsion and incubated in a bath of boiled water for 3 min. After waiting to cool to room temperature *p*-dimethyl amino benzaldehyde solution was put in to the reaction and again incubated at 37ºC for 20 min [28]. The absorbance was measured at 585 nm. Tannic acid and deionized water was used as the reference and negative control, respectively.

25 μL of collagenase (0.8 U/mL) were mixed in 25 μL tricine buffer (with 400 mM NaCl and 10 mM CaCl$_2$), pH 7.5, and 25 μL parts of the test material in having various concentrations. After 20 min of preincubation at 37ºC, 50 μL of FALGPA (1.6 mM) was added. EGCG was used for reference and deionized water was used as a negative control. Absorbance was observed at 335 nm quickly and at 2-min intervals [29].

Elastase inhibition activity was assayed by the method of Lee et al. [28]. The reaction sample contained 50 μL Tris–HCl buffer (200 mM, pH 8.0) and 25 μL of elastase enzyme. The samples were preincubated at 25ºC for 20 min and 125 μL substrate solutions were supplemented to initiate the reaction. The absorbance was determined at 410 nm

for 20 min. EGCG and deionized water was used as the reference and negative control, respectively.

The inhibition percentage of collagenase, hyaluronidase and elastase of polysaccharides were determined by the following formula:

$$\text{Inhibition (\%)} = [(A - B) – (T-D)] / (A-B) \times 100 \quad (4)$$

Here, A: absorbance without the test sample, B: absorbance without the test sample and enzyme, T: absorbance with the test sample, and D: absorbance with the test sample without enzyme.

2.6. Statistical analysis

The study results were presented as the average and standard deviation. The data was analyzed for statistical significance using analysis of variance (ANOVA).

3. Results

In this study, the antimicrobial potential of TG and LBG were evaluated according to their inhibition zone against various pathogens and the results were compared with the activity of the standard antibiotics (Table 1). The results revealed that TG has potent antimicrobial properties against *E. coli* and *P. aeruginosa.* The MIC values of the polysaccharides for the test microorganisms have not been determined at used concentrations. However, Figure 1 illustrates the inhibition of cell growth by the TG and LBG. The TG exhibited an inhibitory effect on *S. aureus*, *S. pyogenes*, *E. coli* and *P. aeruginosa* cell growth (39.5, 47.7, 68.7 and 63.5%, respectively) at 2 mg/mL concentration. The results of the present study showed that doses below the 1 mg/mL of LBG did not inhibit the cell growth, whereas 1 mg/mL dose exhibited bacterial cell growth inhibition.

The antioxidant activities of the TG and LBG were investigated using the DPPH, H_2O_2 radical scavenging and β-carotene bleaching assays. TG and LBG showed *in vitro* antioxidant activity in both H_2O_2 radical scavenging and in DPPH scavenging activity in *concentration* dependant manner (Table 2). The antioxidant activity of the LBG was better than that of the TG. At 10 mg/mL concentration LBG, TG, ascorbic acid and alpha- tocopherol showed 17.6, 10.4, 83.0 and 16.8% inhibition in DPPH assay, respectively. LBG indicated a remarkable effect by inhibiting hydroxyl radical formations (79.0%) which are twofold of that provided of TG at 10 mg/mL (41.7%). LBG showed better H_2O_2 radical scavenging activity than the standard ascorbic acid and alpha-tocopherol (Table 2).

Total antioxidant effect was observed by monitoring the bleaching effects of TG and LBG to β-carotene (Table 2). TG and LBG exhibited low β-carotene bleaching activity (13.0 and 19.3%, respectively).

**Table 1.** Antibacterial activities of TG and LBG against bacterial test organisms

	Test microorganisms			
	Inhibition zone (mm)			
	S. aureus ATCC25923	*E. coli* ATCC25922	*P. aeruginosa* ATCC27853	*S. pyogenes* ATCC12344
TG (20 mg/mL)	- [d]	10.0±0.6[a]	9.0±0.5	-
LBG (10 mg/mL)	-	-	-	-
Vancomycin (30 μg)[b]	16.0±0.4	Ns	Ns	Ns
Gentamicin (10 μg)[b]	17.0±1.0	16.0±0.2	14.0±0.2	Ns
Penicillin (10 U)[b]	Ns	Ns	-	22.0±1.0
Erythromycin (15 μg)[b]	Ns	-	Ns	24.0±0.6
Imipenem (10 μg)[b]	Ns	21.0±0.3	20.0±1.3	Ns

[a]Values expressed are means ± S.D. of three replications (p < 0.05), [b]Reference compounds, [c]Not studied, [d]No activity
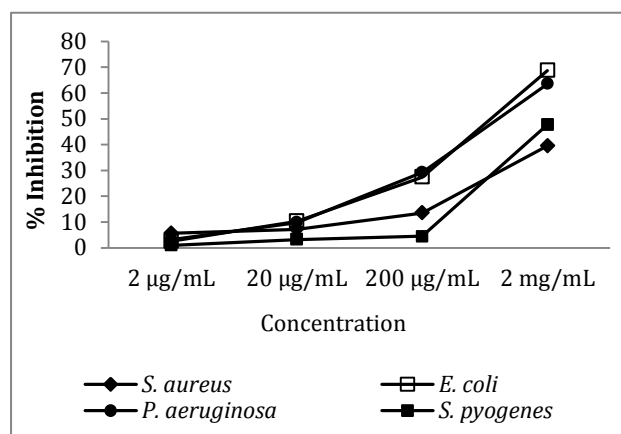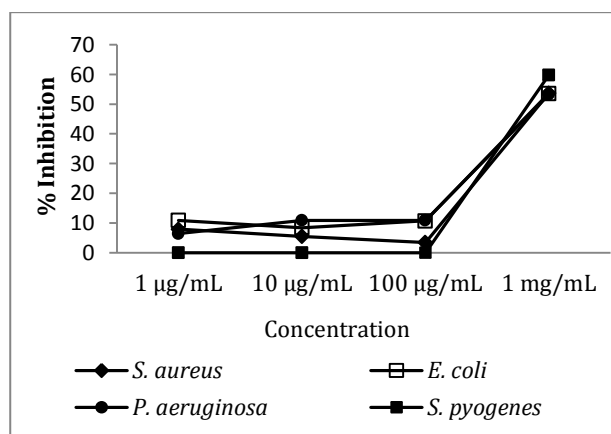
(A)   (B)

Figure 1. The inhibition effect of increasing TG (A) and LBG (B) concentrations on *S .aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. pyogenes* ATCC 12344 cell growth

Table 2. DPPH scavenging activity, H_2O_2 radical scavenging activity and β-carotene bleaching activity of the TG and LBG

		Test Test Systems		
	Concentration	DPPH scavenging rate (%)	H_2O_2 scavenging rate (%)	β-carotene bleaching activity (%)
TG	20 mg/mL	21.0±2.8[a]	59.4±0.1	13±0.1
	10 mg/mL	10.4±0.7	41.7±0.1	10.0±0.0
	1 mg/mL	1.2±0.1	6.3±0.9	4.0±0.8
LBG	10 mg/mL	17.6±0.4	79.0±1.4	19.3±0.1
	1 mg/mL	1.0±0.0	67±0.1	2.3±0.8
Ascorbic acid[b]	10 mg/mL	83.0±0.0	72.5±0.4	66.4±0.2
	1 mg/mL	72.0±5.3	11.3±0.2	48.0±0.5
Alpha-tocopherol[b]	10 mg/mL	16.8±0.9	- [c]	19.7±0.7
	1 mg/mL	15.0±0.0	6.0±0.4	-

[a]Values expressed are means ± S.D. of three replications (p < 0.05), [b]Positive control, [c]No activity

In the homeostatic activity study, the results of PT and aPTT assays (*in vitro)* showed that TG and LBG significantly shortened PT and aPTT. TG and LBG had the ability to shorten the PT clot time from 16.3 s to 15.0 s and 15.9 s and, aPTT clot time from 33.7 s to 29.9 s and 28.3 s, respectively, at 10 mg/mL concentration (Table 3).

In the present study, *in vitro* inhibitory activities of the TG and LBG on hyaluronidase, collagenase and elastase enzymes were investigated (Table 4). TG displayed inhibitory effect on hyaluronidase enzyme with the inhibition value of 19.0%. Similarly LBG was determined to have hyaluronidase and collagenase inhibitory activities with the values of 20.0% and 34.7%, respectively. The results of elastase inhibitory activity assay showed that neither the TG nor LBG have a significant inhibitory activity on elastase enzyme at 10 and 1 mg/mL concentrations.

4. Discussion and Conclusion

In this study, we found that TG had potential antimicrobial activity at 20 mg/mL, whereas LBG did not have any antimicrobial effect at 10 mg/mL (Table 1). In addition, at 2 mg/mL concentration, TG inhibited cell growth of *E. coli* and *P. aeruginosa* by 68.7 and 63.5%, respectively, while LBG showed approximately 50.0% bacterial cell growth inhibition at 1 mg/mL concentration (Figure 1). *Astragalus* polysaccharides were reported as antimicrobial agents in an earlier study [30]. 20 mg/L and 40 mg/L contents of *Astragalus* polysaccharides could significantly inhibit the proliferation of *Salmonella typhimurium, E. coli* and *S. aureus* [31]. In another study, LBG (0.5%) led to the growth inhibition of *Salmonella enterica*, *E. coli* and *S. aureus* [32].

Many aerobic and anaerobic bacteria such as *S. pyogenes, E. coli, S. aureus* and *P. aeruginosa* are involved in the infections *of wounds* [21]*.* In fact; it is known that the use of a high concentration of local antibiotics may reduce the biological burden of the wound [33]. Wound healing based formulations using polysaccharide matrices are particularly studied because their biological performance is good, their toxicity is low, and provide stability and appropriate wound physiological environment [11, 20].

Table 3. Effects of TG and LBG on clotting times

Test items	Concentration	PT(s)	aPTT(s)
Control		16.3±0.1[a]	33.7±0.8
TG	10 mg/mL	15.0±0.1	29.9±0.0
	1 mg/mL	15.4±0.2	29.9±0.3
	0.1 mg/mL	15.8±0.1	29.4±0.1
LBG	10 mg/mL	15.9±0.9	28.3±0.3
	1 mg/mL	15.9±0.3	28.2±0.0
	0.1 mg/mL	16.0±0.4	28.3±0.1

[a]Values expressed are means ± S.D. of three replications (p < 0.05)

The free radicals cause damage to skin lipids, fibroblasts and other cells due to lipid peroxidation, so that antioxidant substances are known to stimulate the healing of the wound. In the study, the antioxidant activities of the TG and LBG were researched using the DPPH, H_2O_2 scavenging and β-carotene bleaching assays. The study results show that TG and LBG exhibit antioxidant activity against DPPH and hydroxyl radicals in a concentration-dependent approach (Table 2). Similarly, *Astragalus* polysaccharides also showed strong antioxidant activity in some studies [34, 35].

The healing of a wound is a natural response of tissue. At the first stage of healing, homeostasis occurs, and inflammatory cells migrate to this injured area [36]. In this study, the homeostatic activities of TG and LBG were measured by PT and aPTT analyses (Table 3). PT is used to figure out the coagulation factors V, VII, and X in extrinsic pathway while aPTT is used to evaluate the VIII, IX, XI, XII, and prekallikrein in intrinsic pathway [37]. The use of TG and LBG shortened the PT and aPTT clotting times (Table 3).

The matrix metalloproteinase enzymes have degenerative effects on the structural proteins but metalloproteinase inhibitors may reduce the severity of the injury and contribute the healing process. Thus, inhibition of hyaluronidase, collagenase and elastase enzymes which break down these components could be useful for the wound healing process. Indeed, it was previously reported that it is

Table 4. Hyaluronidase, collagenase and elastase inhibition of TG and LBG

Test items	Concentration	Inhibition (%)		
		Hyaluronidase	Collagenase	Elastase
TG	10 mg/mL	19.0±0.6[a]	16.0±0.1	-[d]
	1 mg/mL	16.5±0.2	13.7±0.1	-
LBG	10 mg/mL	20.0±0.5	34.7±1.2	17.9±0.0
	1 mg/mL	21.0±1.0	11.5±0.2	-
Tannic acid[b]	10 mg/mL	94.0±0.7	Ns[c]	Ns
	1 mg/mL	61.6±0.0	Ns	Ns
Epigallocatechin gallate[b]	0.1 mg/mL	Ns	24.6±4.5	47.0±0.1

[a]Values expressed are means ± S.D. of three replications ($p < 0.05$), [b]Reference compounds, [c]Not studied, [d]No activity

vital to minimize the level of these breakdown enzymes [38]. In the present study, LBG was found to have both hyaluronidase and collagenase inhibitory activities which could clearly explain its wound healing potential (Table 4).

Recently, it has been reported that the TG has inherent wound healing potential which fasten the wound healing. Tragacanth is also effective in the proliferation and remodeling phases of wound healing [39]. In a study, Fayazzadeh et al. [2] examined the effects of topical implementation of TG on healing in rats. When the percentage of wound closure was examined on the 7th day of the study, it was determined that the percentage of wound closure in the tragacanth treated specimens was higher than the control group. On the 10th day, the differentiation in the healing index determined by histologically was found to be statistically significant between the two groups.

Various therapeutic interventions and medical approaches can affect different processes involved in the wound healing cascade. In this study, the wound healing mechanisms of the TG and LBG, natural polysaccharides, were the first time screened according to hyaluronidase, collagenase and elastase enzyme inhibitory activities. The *in vitro* assays demonstrated that polysaccharides can promote the wound healing process by inhibiting the enzymes involving wound healing. The inhibition of these enzymes that lead to ECM matrix degradation during wound repair can provide support of healing of wounds. In addition, the homeostatic activity of the polysaccharides can accelerate the first stage of wound healing, and antimicrobial and antioxidant activity can promote the wound healing process. Consequently, their consumption in routine diet or application to wounds may accelerate the wound healing with enzyme inhibition, homeostatic, antimicrobial and antioxidant effects.

Acknowledgments

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