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RESEARCH PAPER

Effect of the Crude Saponin Extract from *Gypsophila pilulifera* Boiss. & Heldr. on Protease from *Bacillus subtilis* ATCC 6633 and Antioxidant Properties of the Extract

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Abstract
The study was carried out to purify protease produced by *Bacillus subtilis* ATCC 6633 strain and to examine the effect of the crude saponin extract (CSE) obtained from *Gypsophila pilulifera* Boiss. & Heldr. on the purified enzyme. In addition, the present study dealt with the evaluation of antioxidant and DNA cleavage potentials of the extract. Protease from *B. subtilis* ATCC 6633 was produced and purified using ammonium sulfate precipitation, gel filtration chromatography with 27.2-fold. The purified enzyme showed a single band on SDS-PAGE, and it was determined that its molecular weight was 30.1 kDa. In addition, to examine the effect of saponin as natural surfactant on the protease activity, crude saponin from *G. pilulifera* was extracted in methanol and the purified protease was incubated with the extract. It was observed that the activity of enzyme increased in the presence of extract. In the in vitro assays, while total phenolic content of the extract was determined by Folin–Ciocalteau method, antioxidant properties were evaluated by DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical scavenging activity, FRAP (ferric-ion reducing antioxidant parameter), CUPRAC (cupric ion reducing antioxidant capacity) and β-caroten-linoleic acid method. The results obtained in this study showed that the extract possesses low antioxidant activity for studied all in vitro models. Also, effect of the extract on plasmid DNA was determined with agarose gel electrophoresis. The extract caused conversion of supercoiled DNA structure to other forms. As a result, the extract exhibited DNA cleavage activity as dose-dependent.

Keywords Antioxidant activity · DNA cleavage activity · Enzyme purification · *Gypsophila pilulifera* Boiss. & Heldr. · Protease · Saponin

1 Introduction

Proteases are one of the commercially important groups of enzymes and used in leather, detergent, food, chemical, and pharmaceutical industries (Johnvesly and Naik 2001). Proteases can be produced from plants, animals and microbial sources (bacteria, fungi). The genus *Bacillus* is frequently used for the production of various enzymes (Dias et al. 2008).

Microbial enzymes are favored at various industrial applications due to their stability, greater catalytic activity, and ease of production than plant and animal enzymes. Also, the use of the enzymes has become widespread due to reduced processing time, cost effectiveness, and eco-friendly characteristics (Singh et al. 2016). Nearly half of the enzymes produced commercially are obtained from *Bacillus* sp. strains. Microbial proteases from *Bacillus* sp. are the most widely used industrial enzymes in detergent formulations. The performance of enzymes in detergents is influenced various factors such as the pH of the detergent, washing temperature, and detergent compounds (surfactants and oxidizing agents etc.). For this reason, the major problem for the use enzymes in detergents is the stability

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of the enzyme. A good protease for detergent formulations should be stable and active. There is considerable interest in new enzymes that can enhance the washing performance of detergents (Beg and Gupta 2003; Oberoi et al. 2001; Nadeem et al. 2013).

In detergent formulations, one of the other important components used is surfactants. Generally surfactants are surface active agents dissolved in water. Surfactants in modern detergents are generally synthesized chemically. Natural surfactants offer advantages in terms of low or non-toxicity, biodegradability, and high specificity compared to synthetic surfactants. Plants are an important source of many surface-active compounds (Xu et al. 2011).

Saponins are natural surfactants that can be found in many plant species. Saponins possess emulsifying and foaming properties, pharmacological properties (Cheeke 2000; Xu et al. 2011). *Gypsophila pilulifera* Boiss. & Heldr. (Caryophyllaceae) are perennial herb found in the south western region of Turkey. Roots from *Gypsophila* species are an extremely rich source of triterpenoid saponins (Arslan et al. 2012). They are used commercially in medicines, detergents, adjuvants, and cosmetics (Gevrenova et al. 2010).

Phytochemicals are plant-derived chemical compounds namely phenols, flavonoids, alkaloids, cardiac glycosides, saponins, terpenoids, steroids. These compounds have biological activity against various diseases such as cancer (Mujeeb et al. 2014). Saponin-containing plants have been used for medical purposes. In previous studies, saponins have been found to have biological activities (Arslan et al. 2012).

The study was aimed to purify protease produced by *Bacillus subtilis* ATCC 6633 strain and to examine effect of the crude saponin extract (CSE) obtained from *G. pilulifera* Boiss. & Heldr. on the purified enzyme. In addition, the study dealt with the evaluation of antioxidant and DNA cleavage potentials of the extract.

2 Experimental

2.1 Materials

Folin–Ciocalteau reagent, Gallic acid, Trolox, β-carotene, Neocuproine and DPPH$^{\cdot}$ from Sigma-Adrich and Linoleik acid from Alfa Aesar were purchased. Plasmid DNA pBR322 was purchased from Fermantas.

Casein from Carlo Erbaa, pepton and meat extract from Merck, and yeast extract and Sephadex G-100 from Sigma were purchased. *B. subtilis* ATCC 6633 were obtained from Refik Saydam National Public Health Agency.

2.2 Production of Protease

Protease was produced from *B. subtilis* ATCC 6633 in the medium composed of 1 g meat, 2 g yeast extract, 5 g peptone, 5 g NaCl, 1000 mL distilled water (pH 74). The medium was inoculated with *B. subtilis* ATCC 6633 and was maintained in a shaker incubator (150 rpm) at 37 °C for 48 h. After that, it was centrifuged to separate cells and insoluble materials for 10 min (4000 rpm). The obtained supernatant was used in subsequent analyses.

2.3 Purification of Protease

The supernatant was fractionated by precipitation with ammonium sulfate between 0 and 70% of saturation. After centrifugation (4000 rpm, 20 min), the precipitate was dissolved in minimal volume of 0.05 M phosphate buffer (37 °C, pH 7.5) and loaded into Sephadex G-100 column previously equilibrated in with 0.05 M phosphate buffer (37 °C, pH 7.5). The column was drained at a flow rate of 1 mL/min in buffer. The fractions were collected as 1 mL. The protease activity and protein concentration were determined after the active fractions were pooled.

2.4 Protease Activity Assay

Proteolytic activity of the enzyme was evaluated based on method described by Cupp-Enyard (2008). Firstly, casein was dissolved in phosphate buffer (0.05 M, pH 7.5) as substrate. 1 mL of the purified enzyme was mixed with 5 mL of casein solution (0.65% w/v). The reaction was stopped by addition of 5 mL of TCA after the mixture was incubated at 37 °C for 10 min in bath water. The tubes were allowed to stand for 30 min 37 °C. The reaction mixture was centrifuged for 10 min (14000 rpm) and then, to 2 mL of the above centrifuged mixture was added 5 mL of 0.5 M sodium carbonate and 1 mL of fivefold-diluted Folin–Ciocalteau reagent. The mixture was incubated (30 min, 37 °C). The amount of tyrosine released was determined spectrophotometrically at 660 nm against the enzyme blank.

2.5 Protein Assay

The protein content of the sample was assayed as described by Lowry et al. (1951) Standard protein used in the study was BSA (Bovine serum albumin).

2.6 SDS-PAGE Method

Molecular weight of protease and its purity were estimated using sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) based on the method of Laemmli (1970). An equal volume of sample buffer (20% glycerol, 10% β-mercaptoethanol, 4% SDS, and 0.02% bromophenol blue in 0.125 M Tris–HCL buffer) (pH 6.8) was mixed with the enzyme and the mixture was incubated at boiling water for 5 min. The samples were then subjected to SDS-PAGE (16% resolving gel and 5% stacking gel). The gel was stained with Coomassie Brilliant Blue R-250 after electrophoresis.

2.7 Plant Material and Extraction

Gypsophila pilulifera Boiss. & Heldr. was collected from former Lara road, Antalya Province of Turkey. It has been deposited at the Herbarium of the Department of Biology, Suleyman Demirel University.

Roots of *G. pilulifera* Boiss. & Heldr. were dried in the shade and then pulverized. Extract of plant materials was prepared by using solvent and the extraction protocol which is given below: 64 g of pulverized the plant material was firstly defatted with petroleum ether in soxhlet apparatus (8 h). The defatted material was re-extracted with 80% methanol in soxhlet apparatus (8 h, 3 times). In order to get the saponin rich fraction extract was again dissolved in methanol. To precipitate the saponins, acetone was added. The precipitate was dried under vacuum (Yan et al. 1996). The extract was stored at 4 °C.

2.8 Effect of the CSE on Protease Activity

The CSE in various concentrations (1 and 5%) employed to determine its effect on the protease activity. To 0.5 mL of casein solution (0.65% w/v) were added 0.2 mL of enzyme solution and 0.2 mL of extract solution. After incubation (37 °C, 10 min), to tubes was added 0.5 mL of TCA and tubes were incubated (30 min, 37 °C). The mixture was centrifuged to separate the supernatant (14000 rpm, 10 min). To 1 mL of supernatant, 350 µL of Folin–Ciocalteau reagent and 2.5 mL of Na_2CO_3 solution were added. After incubation (37 °C, 30 min), the amount of tyrosine released was determined spectrophotometrically at 660 nm. The enzyme activity without the extract was taken as control (100%).

2.9 Total Phenolic Content Assay

Total phenolic content of the extract was determined by Folin–Ciocalteau method (Singleton et al. 1999); 100 µL of extract solution containing 100 µg of the extract was mixed with 50 µL Folin–Ciocalteau reagent and 2300 µL methanol. The mixture was shaken in a thorough manner for 3 min. After that, the mixture was added 150 µL of the sodium carbonate solution (2% w/v) and left to stand for 2 h with shaking intermittently. Absorbance at 760 nm of the sample was read against a methanol blank. All of the experiments were repeated three times. The same procedure was performed for all standard gallic acid solutions. The total phenolic content was expressed as µg of garlic acid equivalents (GAE) per mg of extract (dry weight).

2.10 Antioxidant Activity Assays

2.10.1 Radical Scavenging Assay

Determination of free radical scavenging capacity ($DPPH^{\cdot}$) of the extract was performed based on the method of Sanchez-Moreno et al. (1998). The extract dissolved in methanol was prepared in various concentrations (50–400 µg/mL). 500 µL of the extract solutions mixed with 3 mL of $DPPH^{\cdot}$ solution (6×10^{-5} M) dissolved in methanol. After incubation at room temperature for 30 min, absorbance of samples at 517 nm was read against a methanol blank. All of the experiments were conducted in triplicate. Inhibition of free radical, $DPPH^{\cdot}$, in percent (I %) was calculated:

$$I\% = \left(A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}\right) \times 100.$$

The same test was performed for the butylated hydroxyanisole (BHA) solutions which were used as standard. IC_{50} (50% inhibition) values for the extract and BHA were calculated.

2.10.2 Fe^{3+} Reducing Power (FRAP) Assay

The method of Oyaizu (1986) was used to evaluate Fe(III) reducing ability of the extract. Firstly, the extract solutions in different concentrations in methanol (0.08–2.4 mg/mL) were prepared. The extract solution (2.5 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer and 2.5 mL of 1% potassium ferricyanide. After incubation in water bath for 20 min (45 °C), 2.5 mL of 10% TCA was added to the mixture. The tube was centrifuged at room temperature (4000 rpm, 10 min). 5 mL of the obtained supernatant was mixed with 5 mL of deionized water and 0.5 mL of $FeCl_3$ (0.1%). The blank was prepared with the buffer without extract solution. The absorbance was measured at 700 nm. Trolox was used as standard. The ferric ions (Fe^{3+}) reducing power of the extract was expressed as trolox equivalent antioxidant capacity (TEAC) ($\mu g_{trolox}/g_{extract}$).

2.10.3 CUPRAC (Cupric Ion Reducing Antioxidant Capacity) Assay

The cupric ion (Cu^{2+}) reducing power of the extract was determined using the method described by Apak et al. (2004). To this end, various milliliters (10–1100 µL) of the

extracts (10,000 μg/mL) were mixed with 1 mL $CuCl_2$ (10 mM), 1 mL of neocuproine (7.5 mM) and 1 mL of NH_4Ac buffer solution (1.0 M) in tubes. Finally, H_2O was added to the tubes to make total volume 4.1 mL. After being mixed, the tubes were left to stand (30 min). The absorbance was measured at 450 nm. Trolox was used as standard. The result was expressed as TEAC ($\mu mol_{trolox}/g_{extract}$).

2.10.4 β-Carotene/Linoleic Acid Assay

β-Carotene/linoleic acid method was performed as described by Tepe et al. (2007). β-Carotene (0.2 mg) was dissolved in 1 mL of chloroform and 25 μL linoleic acid and 200 mg tween 40 were added. To mixture, oxygenated distilled water (100 mL) was added with shaking vigorously after the chloroform in the mixture was removed by evaporation. 0.2 mL of the extract (2 mg/mL) was added to 5 mL of emulsion solution. Finally, the mixture was incubated in the water bath at 40 °C. The absorbance of samples was measured at 490 nm by taking measurements at 20 min intervals for 120 min. The same procedure was repeated with standard antioxidant, butylated hydroxyanisole (BHA).

2.11 Interaction of Plasmid DNA with the CSE

Agarose gel electrophoresis was employed to monitor the changes on plasmid DNA. In the experiment, 0.5 μL supercoiled pBR322 DNA was treated with 1.5 μL of the extract solutions (2, 10, 20, 30, 40, 50 μg/mL extracts which was dissolved in water) and incubated at 37 °C for 2 h. Finally, 1 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in H_2O) and 8.5 μL distilled water was added to each tube. The mixed solution was loaded on 1% agarose gel. The electrophoresis was performed for 80 min at 100 V in TBE buffer (89 mM Tris–borate, pH 8.0, 2.5 mmol/L EDTA). The gel was placed in ethidium bromide solution (1 mg/mL) for 10 min. The DNA bands were visualized on a UV-transilluminator.

3 Results and Discussion

3.1 Protease Purification

Bacillus subtilis is one of the intensively investigated microbial groups for enzyme production. In the study, protease production from *B. subtilis* ATCC 6633 was carried out culture medium for 48 h. Adinarayana et al. (2003) reported that enzyme production from *B. subtilis* PE-11 reached maximum at 48th h. In present study, ammonium sulfate precipitation was resulted in a 6.56-fold purification with a 67.73% yield. After Sephadex G-100 column, the enzyme was purified 27.2-fold with 7.79% yield. The results of purification steps of protease are given in Table 1.

In earlier studies performed using same purification steps with our study, protease purified from *B. subtilis* PE-11 with 21-fold increase in specific activity and 7.5% recovery (Adinarayana et al. 2003), *B. subtilis* M-11 with 15.8-fold increase in specific activity and 1.5% recovery (Sahin et al. 2015) and *B. licheniformis* UV-9 with 36.8-fold increase in specific activity and 11% recovery (Nadeem et al. 2013).

In the study, a good purification fold was obtained. This result showed that the steps chosen for purification were efficient. Also, the high purification fold indicates that there are no undesirable interactions with chromatographic substances or other substances in the solution during purification process.

The molecular mass of proteases varies in the range of 45–80 kDa (Sinha and Khare 2013). However, Adinarayana et al. (2003) reported lower molecular weight protease with 15 kDa in *B. subtilis* PE-11. In the study, the molecular weight of the purified protease was 30.1 kDa by comparison of standard marker proteins (Fig. 1). The single band observed in SDS-PAGE confirmed the homogeneity of the purified enzyme. The result is similar to molecular weight of proteases from other *Bacillus* species such as *B. subtilis* M-11 (32 kDa) (Sahin et al. 2015), alkaliphilic *Bacillus* sp. (30 kDa) (Fujiwara et al. 1993), *B. Mojavensis* (30 kDa) (Beg and Gupta 2003).

3.2 Effect of the CSE on Protease Activity

The effect of the CSE on protease activity was evaluated by incubating the enzyme with various concentrations of the extract (Table 2). It was found that the purified protease activity increased by 80% in the presence of CSE (5% w/v).

Proteases which retain their activity in presence of surfactants, oxidants and detergents are desirable for possible application in commercial detergents. The compatibility of proteases with different agents has been tested by researchers (Gupta et al. 1999; Oberoi et al. 2001; Rajput et al. 2010). There is no study examining effect of natural crude saponin from plant on protease activity according to our knowledge. Previous studies found that surfactants stimulated activity of protease with almost twofold enhancement in the presence of saponin (Gupta et al. 1999; Rajput et al. 2010). Also, Moreira et al. (2002) reported that activity of protease from *Nocardiopsis* sp. was almost stable or stimulated in presence of detergents and surfactants tested. On the other hand, other studies showed that protease activity decreased after incubation with saponin and other surfactants (Oberoi et al. 2001; Espósito et al.

Table 1 Summary of the protease from *Bacillus subtilis* ATCC 6633

Purification steps	Total protein (mg/mL)	Total activity (EU/mL)	Specific activity (EU/mg)	Yield (%)	Purification (fold)
Culture supernatant	191.4	2213.2	11.50	100	1
Ammonium sulfate precipitation	21.15	1499.1	70.87	67.73	6.56
Column chromatography	0.55	172.5	313.63	7.79	27.20

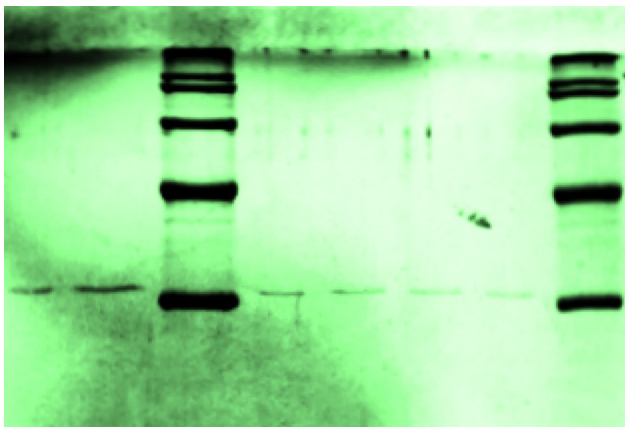


Fig. 1 SDS-PAGE of protease from *B. subtilis* ATTC 6633. Molecular mass marker; 200, 116, 97, 66, 45, and 29 kDa

Table 2 Effect of the CSE on protease activity

Saponin	Activity (EU/mL)	Activity (%)
Control E[a]	25.6	100
E + (1% w/v) saponin	34.9	136
E + (5% w/v) saponin	46.1	180

[a]Enzyme (E)

2009). Surfactants have been used to enhance enzymatic hydrolysis of cellulosic materials in biogas and industrial glucose production bioreactors (Kamande et al. 2000). In previous studies, enhancement of enzyme activity with surfactants was based on increased enzyme stabilization and the ability of the surfactant to prevent inactivation of adsorbed enzymes (Castanon and Wilke 1981; Helle et al. 1993)

The study showed that the activity of protease from *B. subtilis* ATCC 6633 increased in presence of the CSE which is a natural surfactant. The result shows that the purified protease is a potential candidate as commercial detergent enzyme (additive). Also, the study provides important data in terms of the usability of the saponin from *G. pilulifera* Boiss. & Heldr. as natural surfactant for the purpose of increasing the enzyme activity in industrial processes where protease is used.

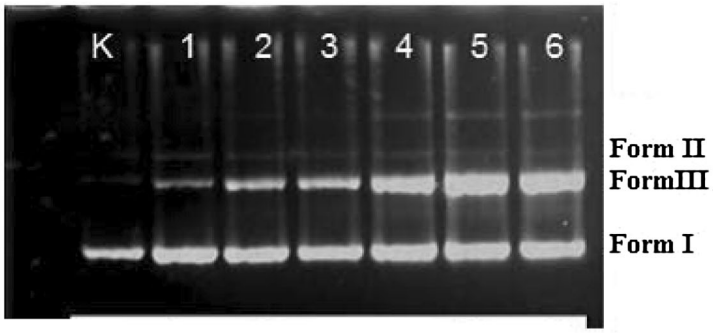
3.3 Antioxidant Properties of the CSE from *Gypsophila pilulifera* Boiss. & Heldr.

Most plants contain compounds with antioxidant activity, such as flavonoids, anthocyanins, carotenoids, tannins, saponins, steroids, terpenoids and rotenoids (Otang et al. 2012). Antioxidants are compounds which prevent the oxidation of lipids or other molecules and protect against various disorders (Zheng and Wang 2001). There has been intensive research on natural antioxidants from plants as alternatives to synthetic antioxidants.

In the study, the total phenolic content in the extract was found as 6.5 μg (GAE)/mg (extract). Antioxidant activity of the extract was evaluated using four methods. The ability of the extract to scavenge DPPH radical was measured. DPPH scavenging activity assay is extensively preferred to measure antioxidant activity. The amount of the extract required to scavenge 50% of DPPH, IC_{50} was 4.56 mg/mL and the amount was 19.01 μg/mL for BHA. Also, TEAC value of the extract was found to be 23.5 $\mu g_{trolox}/g_{extract}$ by using FRAP assay, while TEAC value was determined as 0.083 $\mu mol_{trolox}/g_{extract}$ by Cuprac assay. However, the study showed that the extract was quite insufficient to inhibit the linoleic acid oxidation according to the β-carotene/linoleic acid assay.

Saponins are natural compounds which are found in a lot of plant species. A wide range of plants contain saponins in their leaves, fruit, and roots. Also, saponin has been use for biological effects in traditional medicine. Previous studies have reported that saponins have properties such as antioxidant, antiviral, antifungal, antibacterial, hypocholesterolaemic and immunostimulant (Fidan and Dundar 2008).

There are some studies determining the antioxidant activity of saponins from different plant sources. Chen et al. (2011) reported a significant correlation between antioxidant activity and total phenolic and saponin contents of the extracts. However, Huong et al. (1998) suggested that protective effect against radicals of Vietnamese ginseng is attributable to minor components rather than the main saponin components. Lee et al. (2011) showed that the antioxidant activities of the saponins were marginal when compared with phenolic compounds. There are not many studies that examine the total phenolic amount of



K: Plasmid DNA, 1: Plasmid DNA + 2 μg CSE, 2: Plasmid DNA +10 μg CSE, 3: Plasmid DNA+ 20μg CSE, 4: Plasmid DNA+ 30 μg CSE, 5: Plasmid DNA +40 μg CSE, 6: Plasmid DNA + 50 μg CSE

Fig. 2 Gel electrophoresis diagram showing pBR322 plasmid DNA–the CSE interaction. K: plasmid DNA, 1: plasmid DNA + 2 μg CSE, 2: plasmid DNA + 10 μg CSE, 3: plasmid DNA + 20 μg CSE, 4: plasmid DNA + 30 μg CSE, 5: plasmid DNA + 40 μg CSE, 6: plasmid DNA + 50 μg CSE

Gypsophila sp. and its antioxidant activity. Serteser et al. (2009) found that the *Gypsophila* species showed lower antioxidant activity than other plant extracts analyzed.

As a conclusion, the extract exhibited low antioxidant activity for studied all in vitro models. Phenolic compounds are bioactive phytochemicals and good antioxidant agents. It has suggested a close relationship between total phenolic content and antioxidant activity of plant extracts (Serteser et al. 2009; Chen et al. 2011; Lee et al. 2011). So, this low antioxidant activity may be linked to phenolic contents of the extract.

3.4 Interaction of Plasmid DNA with the CSE

To evaluate DNA cleavage activity of the extract, the plasmid DNA (pBR322) was incubated with various concentrations of the CSE. The extract was found to cause conversion of supercoiled plasmid DNA (Form I) to other forms as dose dependent (Fig. 2).

Biosurfactants have major functions such as solubilization, emulsification, foaming, and detergent capacity and antimicrobial activity (Xu et al. 2011). Saponins protect plants against pathogen microorganisms and are toxic for some animals. Many pharmacological activities have been reported about saponins such as antifungal, antiviral (Cheeke 2000). Arslan et al. (2012) found that triterpenoid saponin, which was obtained from *G. pilulifera*, was extremely cytotoxic for lung carcinoma cell and they suggested that the saponin could be beneficial in anticancer treatment.

DNA is the pharmacologic target of many drugs in clinical use/trials. Today, there is growing interest in organic molecules that are capable of binding to DNA and cleaving DNA, to develop new drugs and synthetic restriction enzymes (Subramanian et al. 2006). Khanam et al. (2015) found that *Averrhoa carambola* L. fruit extracts, which is a rich source of polyphenols, exhibited DNA cleavage activity. Yernale and Bennikallu Hire Mathada (2014) have suggested that all the metal complexes exhibiting DNA cleavage activity are good pathogenic microorganism inhibitor. To our knowledge, no report is available related to DNA cleavage activity of extracts from *G. pilulifera*. Thus, the present study evaluated plasmid DNA pBR322 cleavage potential of the CSE from *G. pilulifera.*

Consequently, the extract showed DNA cleavage activity as dose-dependent. Therefore, it can be suggested that the extract may inhibit the growth of the pathogenic organism by cleaving DNA. Also, the extract may be used in therapeutic approaches. At the same time, the use of the CSE may provide antibacterial properties to detergent as well as increases protease activity.

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