Saudi Journal of Biological Sciences 30 (2023) 103659

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Saudi Journal of Biological Sciences

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Original article

Soluble laticifer proteins from *Calotropis procera* as an effective candidates for antimicrobial therapeutics

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ARTICLE INFO

Article history:
Received 6 March 2023
Revised 24 March 2023
Accepted 16 April 2023
Available online 23 April 2023

Keywords:
Calotropis procera
Antibacterial activity
Antifungal activity
Soluble laticifer proteins
Cysteine proteases
Antimicrobial peptides
Gel filtration chromatography

ABSTRACT

Calotropis procera is a latex-producing plant with plenty of pharmacologically active compounds. The principal motivation behind this study was to separate and characterize laticifer proteins to check their antimicrobial potential. Laticifer proteins were separated by gel filtration chromatography (GFC) and investigated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE assay detected proteins of molecular weights of 10 to 30 kDa but most of them were in the range of 25 to 30 kDa. The soluble laticifer proteins (SLPs) were tested against Gram-positive bacteria i.e., *Streptococcus pyogenes* and *Staphylococcus aureus* whereas *Escherichia coli* and *Pseudomonas aeruginosa* were tested as Gram-negative bacteria, we determined a profound anti-bacterial activity of these proteins. In addition, SLPs were also investigated against *Candida albicans* via the agar disc diffusion method which also showed significant anti-fungal activity. SLP exhibited antibacterial activity against *P. aeruginosa*, *E. coli,* and *S. aureus* with a minimum inhibitory concentration (MIC) of 2.5 mg/mL for each, while MIC was found at 0.625 mg/mL for *S. pyogenes* and 1.25 mg/mL for *C. albicans*. Moreover, enzymatic activity evaluation of SLP showed the proteolytic nature of these proteins, and this proteolytic activity was greatly enhanced after reduction which might be due to the presence of cysteine residues in the protein structure. The activity of the SLPs obtained from the latex of *C. procera* can be associated with the involvement of enzymes either proteases or, protease inhibitors and/or peptides.

1. Introduction

Bioactive phytochemicals produce definite physiological actions in the human body. There are two types of metabolites, primary and secondary metabolites found in plants. Primary metabolites consist of carbohydrates, proteins, and chlorophyll, among them the biologically active peptides are attracted many researchers' attention because of their remarkable antimicrobial potential (Indarmawan et al., 2016). On the other hand, secondary metabolites comprising terpenoids, alkaloids, flavonoids, tannins, and phenolic compounds also grab a lot of attention due to their pharmacological importance. Both the primary and secondary constituents of plants have therapeutic potential, they may serve as anticancer agents, nervous system activators or suppressors, cardiovascular agents, antimicrobial compounds, immunomodulators, and anti-inflammatory agents (Shobowale et al., 2013).

It is believed that the treatment by the plants is less toxic and more curative than the synthetic drugs because they are easily biodegradable (Singh et al., 2013). Secondary metabolites of plants are mainly responsible for the therapeutic tendencies of medicinal plants. Medicinal plants are so beneficial in the field of health that

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2023.103659
1319-562X/

most of them are proposed to be utilized as food components to get benefit from their pharmacological properties. Consequently, extracts from plants are widely used by various researchers from all over the world to abolish infections and their causative agents (Al Akeel et al., 2014).

Almost all parts of plants like seeds, leaves, stems, and roots possess bioactive peptides and they are mostly involved in cell signaling processes, so they are pharmacologically active for curing various diseases including microbial ailments. If compared, to bioactive peptides obtained either from plants or animals, most of them are structurally quite similar, thus depicting that these bioactive peptides can kill both the animal and/or human pathogenic microbes (Salas et al., 2015). Antimicrobial peptides (AMPs) are not only constitutive in nature but can also be produced in response to attack from microbes and serve as defensive secondary metabolites. Natural AMPs can be divided into various classes on a structural basis, among them some AMPs are stabilized by disulfide bridges and, the molecular weight of such AMPs fall in the range of 2 to 10 kDa (Datta et al., 2016). By various studies, AMPs of plant origin are recognized to possess antibacterial, antifungal and antiviral characteristics (Carlos Ruiz-Ruiz et al., 2017).

Latex-containing plants are widely spread and their major constituent of them is cis polyisoprene which is used in the production of rubber. More than 12,000 species of plants contain latex, however, the soluble proteins present in these plants are not only involved in production of rubber but also played pivotal role in plant's defense system and metabolism. Such plants are well known traditionally for pharmacologically active constituents. Similarly, *C. procera* is also famous among latex containing plants, it is traditionally used for different skin diseases like for treating leprosy, inflammation, eczema, infections, syphilis and others may include low hectic fever, malaria and as an abortifacient (Olaitan et al., 2013).. Different studies confirm significant pharmacological characteristics of *C. procera* like anti-inflammatory, antioxidant (Mohamed et al., 2011), anti-nociceptive, anti-diabetic properties (de Oliveira et al., 2007) antiviral (Saher et al., 2018) and anticancer (Rabelo et al., 2021).

Latex is produced by all parts of *C. procera* that oozes out enormously on cutting any part of the plant and it is found by various studies that latex is full of large number of biologically active constituents including steroids, flavonoids, alkaloids, resins and terpenes. These phytoconstituents are responsible for various therapeutic activities (Rabelo et al., 2021) such as they serve as anti-inflammatory agents, antidiarrheal compounds, antimicrobial, antioxidant, antiviral, and hepatoprotective agents (Mohamed et al., 2015). Among various constituents protein components of *C. procera* exhibit anti-tumor activity (Samy et al., 2012), anti-inflammatory actions (Alencar et al., 2006) and anti-malarial activity (Abdulkadir et al., 2016).

AMPs are produced in all organs of plants, sometimes constitutively and sometimes in response to microbial infection. They are not crucially the first line of defense in plants because plants also produce many other secondary metabolites for their protection (Ningappa et al., 2010). However, these AMPs act as host defense system and are active against different types of microorganisms either by direct antimicrobial action or by immunomodulatory effects (Manoury et al., 2011). AMPs have been studied against various Gram-positive and Gram-negative bacteria in which these proteins were separated from *C. procera* bark (Perumal Samy and Chow 2012). In present study, we isolated the pharmacologically active SLPs from latex of this plant, these SLPs are not yet evaluated for antimicrobial activity. The day by day increasing resistance of bacteria against available antimicrobial agents has prompted us to explore more potential bioactive compounds with antimicrobial activity. *C. procera* is found abundantly in South Punjab, Pakistan. Keeping in view, the traditional uses of this plant we were motivated to isolate the proteins from latex and to examine their antimicrobial potential.

2. Materials and methods

2.1. Chemicals and reagents

Media used for antimicrobial activity experiment include MacConkey agar media, eosin methylene blue (EMB) agar media, nutrient agar, nutrient broth, mannitol salt agar and sabouraud dextrose agar (Oxoid, Thermo Fisher Scientific, UK). A centrifugal filter device (Amicon Ultra-15 10 K dialysis tube) was utilized for dialysis. Chemicals for SDS-PAGE analysis, gel filtration chromatography and proteolytic activity include Tris-HCl, acrylamide, bis-acrylamide, tris base, sodium dodecyl sulphate, ammonium per sulphate solution, coomassie blue stain (R250), glycine, glacial acetic acid, sephadex G-100, 50 mM tris Cl buffer, azocasein, dithiothreitol and sodium hydroxide. All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Latex collection and processing for protein content separation

Latex was collected from *C. procera* (Asclepiadaceae) located in the District Bahawal Nagar, Punjab. The plant material was identified from the Herbarium of Department of Botany, Government College University, Lahore, Pakistan (voucher No:3658). Latex was collected from aerial parts in falcon tubes with 1:1(v/v) ratio in distilled water. Natural coagulation phenomena of the latex was avoided by continuous agitation during collection. The samples were then centrifuged at 5000 g for 10 min at 4 °C and the supernatant was further processed in two ways, one portion was studied as such with the name of rubber free latex (RFL) and the second portion of RFL was further processed to get soluble laticifer proteins. RFL was dialyzed by dialysis tube (Amicon Ultra-15) having cut off of 10 kDa as described by various studies (Alencar et al., 2006, Lima-Filho et al., 2010) and this fraction was named as soluble laticifer protein (SLP). Centrifugation and then lyophilization was done to prepare sample (Alencar et al., 2006). The acetone precipitation method was also followed to get proteins (Niu et al., 2018). RFL was precipitated by mixing with a 1:6 vol ration of cold acetone and placed at −20 °C overnight; pellet obtained was washed two times with cold acetone. Centrifugation was done at 10,000 g at 4 °C for 30 min; the final pellet was obtained and redissolved in PBS for further testing (Abdulkadir et al., 2016) this fraction was named as acetone precipitated protein (APP).

2.3. Protein profiling and molecular weight determination

Protein content in SLP and APP was determined following Nano-Drop Protein quantification method(Desjardins et al., 2009). Denaturing SDS-PAGE was performed as given previously (Bizani et al., 2005). Acrylamide gel (12%) in Tris-HCl buffer (1.5 M, pH 8.8) were run at 100 V for 2 h at room temperature. Well defined bands were obtained by Coomassie brilliant blue staining. Standard curve was obtained between log molecular weight (MW) and relative flow (Rf) for known samples (ladder) to get relative molecular weight.

2.4. Gel filtration chromatography and isolation of protein

Electrophoresis of sample was done and molecular weight was estimated by comparing sample protein bands with known molecular weight unstained ladder (Invitrogen Mark12 Unstained Standard) having 12 bands when run on gel in the range of 2.5 to 200 kDa. Isolation or separation of different molecular weight proteins was done by gel filtration chromatography (GFC). It separated

proteins truly on the basis of molecular weight (Hagel 1998). Sephadex G-100 was ideal for 4 kDa to 150 kDa molecular weight protein and protein fractions to be separated were found to fall in this selected range.

2.5. Column and sample preparation

Hydrated gel suspension of Sephadex G-100 was carefully poured in column and allowed to settle. Sample after dialysis having protein content of 10 mg/mL was passed through the column. After the complete casting of the sample in the column, the proteins started eluting using 50 mM Tris Cl buffer pH 8.0, with a flow rate of 1 mL per minute. Forty fractions, each of 1.5 mL from the column were collected in Eppendorf tubes and assayed for protein content by Nanodrop method at 280 nm in a UV visible spectrophotometer. The MWs of the separated proteins were calculated by running on SDS-PAGE by running with ladder. These separated proteins or fractions were named as soluble laticifer protein-I (SLP-I) and soluble laticifer protein-II (SLP-II).

2.6. Total proteolytic activity

SLP was examined for total proteolytic activity by using Azocasein as a non-specific substrate (Hosseininaveh et al., 2009). SLP was preincubated with 3 mM DTT for period of 10 min in order to reduce cysteine proteases present in SLP (Rinschen 2019). Reaction mixture containing SLP and azocasein was tested in a way that activity was checked on gradually increasing volume starting from 50 μL to 200 μL of sample made from 1 mg/mL of the SLP while 1% azocasein in buffer (50 mM PBS buffer of pH 6.0) was added in amount of 200 μL in each aliquot of test sample. The reaction was proceeded in two forms i.e., SLP with or without DTT at 37 °C and it was stopped after 60 min by adding 300 μL of 20% trichloroacetic acid. Centrifugation was done at 5000 g for period of 10 min at 25 °C. Supernatants were alkalinized by adding 400 μL of 0.2 N NaOH solutions. Optical density (OD) was checked at 420 nm (Freitas et al., 2007). One unit of activity of enzyme was defined as the amount of enzyme capable of increasing absorbance at 420 nm by 0.01.

2.7. Antibacterial and antifungal activity

Bacterial and fungal strains were used for the bioassay studies. The bacterial isolates including *S. aureus* ATCC 25923, *S. pyogenes* ATCC 19615 as Gram-positive bacteria, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 as Gram-negative bacteria and *Candida albicans* (clinical isolate separated from oral cavity and confirmed by germ tube test) were obtained from the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore. All the media were weighed and mixed in water according to the protocol given on the label and after complete dissolution, sterilized by autoclaving at 121 °C for 15–20 min.

2.8. Identification and confirmation of bacteria

Confirmation of bacteria was done by using standard and specific biochemical tests (catalase, citrate utilization, oxidase, and coagulase) for each strain as described by Bergey's manual of determinative bacteriology (Bergey et al., 1994). After confirmation (data provided in supplementary materials) all the bacterial isolates were kept in a refrigerator at 4 °C until required for further processing in experimentation.

2.9. Antimicrobial susceptibility testing

Antibacterial and antifungal activity of SLP, SLP-I and SLP-II against bacterial and fungal isolates were examined by using agar disc diffusion method and MIC in a 96 well microtiter plate by broth micro dilution method (Kawo et al., 2009, Ishnava et al., 2012). Different concentrations of test samples were evaluated for antibacterial activity against microbes by using disc diffusion and microbroth dilution assays as described previously by other researchers (Ishnava et al., 2012, Shobowale et al., 2013). Selective medium agar plates were marked and labeled for specific organism. Glass spreader was used to spread 100 μL of fresh bacterial culture (10^8 CFU/mL) over agar plates for bacteria and 100 μL of fresh fungal culture having 10^5 CFU/mL for fungi.

2.10. Disc diffusion method

Antibacterial activity was examined by agar disc diffusion method (Wesierska et al., 2005, Sharifi-Rad et al., 2022). Whatman No. 1 filter paper was used to form 6 mm diameter paper discs and sterilized by placing them in ultraviolet light radiations. Lyophilized RFL, SLP, SLP-I and SLP-II (10 mg/mL for each test sample) after dissolving in distilled water were soaked on discs. All bacterial strains were brought to their log phase on their specific media by placing in incubator at 37 °C for 24 h. Each bacterial suspension was spread over the surface of media as a thin film that was poured in plates previously. RFL, SLP, SLP-I and SLP-II discs were applied to the bacterial-cultured plates. Each plate was incubated for 24 h at 37 °C. Results were measured in the form of zones of inhibition (ZOIs). Experiment was performed in triplicate.

2.11. Minimum inhibitory concentration

To check MIC of RFL, SLP, SLP-I and SLP-II, following steps were performed as given in previous studies (Aref et al., 2010, Raghavendra and Mahadevan 2011). The stock solutions with concentration of 10 mg/mL for all test samples were prepared in Eppendorf tubes by using distilled water. Ten different concentrations were tested made by two-fold serial dilutions starting from 10 mg/mL. In 96-well plate, all wells were filled by 100 μL of nutrient broth, and then 100 μL of RFL was poured in 1st well of row-A and 2-fold dilutions were made upto 10th well. At the end 100 μL of standardized inoculum was poured in all wells. OD was checked at 625 nm wave length at zero time i.e., just after mixing of drug, media, and inoculum. Plates were placed in incubator for 24 h at 37 °C. OD was again checked after 24 h of incubation period (Sharifi-Rad et al., 2020). Similar procedure was performed for SLP, SLP-I and SLP-II. All test samples were tested in same way for all four bacteria and fungi. Bacteria and fungi alone were considered as positive controls and broth was considered as negative control.

2.12. Statistical analysis

To perform statistical analysis, we used raw data from three to five independent experiments and analyzed by GraphPad Prism 8.0.1. Data was assumed to be derived from either normal or log normal distributions. One way analysis of variance (ANOVA) was used to compare three or more groups while student's *t*-test was used to compare two groups. *t*-test was also used in multiple comparisons and multiple comparisons were corrected by false discovery rate (FDR).

3. Results

3.1. Soluble laticifer proteins were obtained by SDS-PAGE

After collection of latex, it was centrifuged to separate rubber part and this fraction was named as RFL. RFL was processed to get SLP and APP. The protein profile obtained after APP and SLP on SDS-PAGE was shown in Fig. 1. Many studies have used APP method for protein precipitation; however, we got significantly higher amount of protein in SLP where we used aqueous medium (Fig. 1).

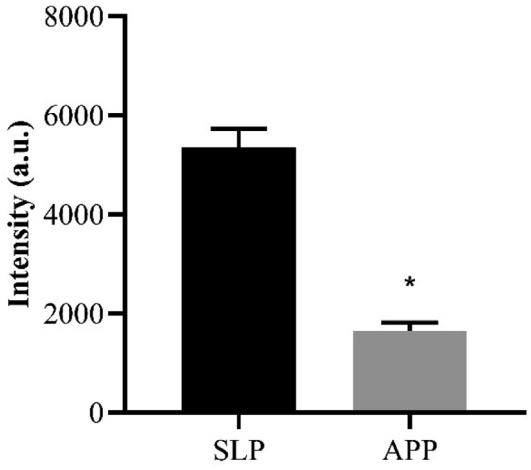

Fig. 1. Comparison of latex proteins of *C. procera* obtained by APP and SLP methods. Latex was obtained from *C. procera* and proteins were purified using two methods, acetone precipitation method (APP) and soluble laticifer protein method (SLP). Both samples were run on a gel with Coomassie blue staining. The data are presented as densitometric quantification using ImageJ app. Data are presented in the form of mean ± SD where n number is 3–4. * represents $p < 0.05$.

3.2. Gel filtration chromatography separated SLP into SLP-I and SLP-II

After purifying SLP, it was run through GFC column and two major peaks were obtained (Fig. 2a). For all the 40 filtration aliquots (1.5 mL each) obtained from GFC, absorbance was quantified at wavelength of 280 nm (NanoDrop 1000, Spectrophotometer, Thermo Scientific, USA). The major first peak (SLP-I) consisted of filtration aliquot no 13–16 and the second one (SLP-II) consisted of filtration aliquots no 16–19. SLP-I exhibited protein content of 1.627 ± 0.003 mg/mL while SLP-II exhibited protein content of 2.2 ± 0.139 mg/mL.

Subsequently, total SLP and both fractions were run on SDS-PAGE along with ladder and the molecular weight of the proteins was calculated from the standard curve (Fig. 2b-c). Molecular weight of SLP-I and SLP-II were found almost 29.5kDa and 27.4 kDa respectively. From the Rf values, the molecular weight of each band was calculated as shown in Table 1.

3.3. Proteolytic activity of SLP was enhanced by DTT

Some studies have demonstrated that these latex proteins are proteolytically active (Abdulkadir et al., 2016, Bezerra et al., 2017). Hence, to find out the nature of SLP, proteolytic action of laticifer proteins was examined by substrate digestion method using azocasein. SLP showed an intense activity against the substrate. The activity increased as the volume of the SLP was increased from 50 µL to 200 µL. The comparison of these two volumes is represented by # ($p < 0.05$). Furthermore, when SLP was

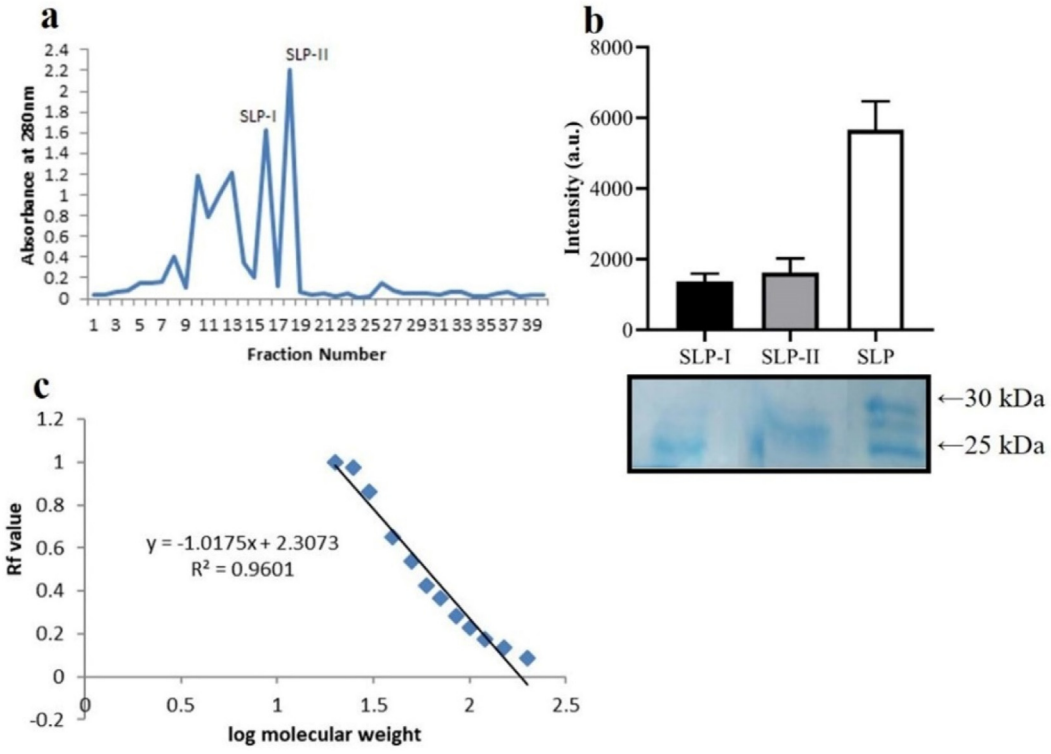

Fig. 2. Isolation of two fractions of SLP by gel filtration chromatography. a) Two fractions of SLP were obtained after gel filtration chromatography (GFC). Two sharp peaks obtained were named as SLP-I and SLP-II. b) The total SLP and its two fractions named SLP-I and SLP-II were run on gel using SDS-PAGE. The data comprise of densitometric quantification of signals and a representative gel image. Data are shown as mean ± SD. c) Relative flow values of these protein fractions were measured with the help of standard curve. The data are representative of 3–4 independent experiments.

Table 1
Distances travelled (mm) by proteins in polyacrylamide resolving gel (12%) by SDS-PAGE analysis.

Band #	Lane-1		Lane-2		Lane-3		Log MW from Rf value	Antilog of MW (kDa)
	Distance travelled	Rf	Distance Travelled	Rf	Distance Travelled	Rf	-	-
1	–	–	–	–	150.2	0.824	1.442541	27.09
2	155.7	0.854	–	–	156.58	0.859	1.463396	29.4
dye front	182.2	–	170.6	–	182.2	–	–	–

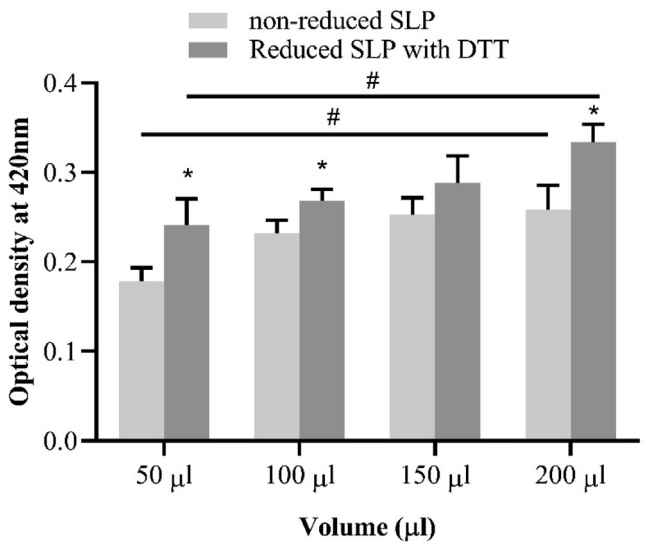

Fig. 3. Proteolytic activity of SLP. The total SLP were used to digest azocasein to observe its proteolytic activity. The Dithiothreitol (DTT) was added to further activate the SLP. Optical density of SLP is measured at 420 nm in non-reducing (SLP alone) and reducing (SLP + DTT) conditions. * represents the comparison between non-reduced SLP and reduced SLP at each volume and # shows the comparison between the same group at different dilutions (volumes) where $p < 0.05$.

reduced by Dithiothreitol (DTT) prior to the digestion of substrate, the total proteolytic action was profoundly increased (Fig. 3). The comparison of SLP non-reduced and reduced forms were made for each volume. The digestive activity was increased significantly at 50 µL, 100 µL and 200 µL. * is used to represent the comparisons between SLP non-reduced and reduced forms ($p < 0.05$). This finding approves the presence of a robust proteolytic activity in SLP which is due to cysteine protease activation by DTT (Freitas et al., 2007).

3.4. SLP showed in vitro antibacterial activity

Antibacterial activity of RFL, SLP and its fractions was determined against of both, Gram-positive and Gram-negative bacterial strains, *E. coli, P. aeruginosa*, *S. aureus* and *S. pyogenes*. SLP and SLP-II were found effective against bacteria with mean diameter of zone of inhibitions as given in Table 2 and Fig. 4. RFL gave very small zone of inhibition (data not given) thus did not further tested for MIC.

After confirmation of antibacterial activity, total SLP and its fractions were further analyzed for MIC. The results showed that SLP significantly inhibited the growth of both, Gram-positive and Gram-negative bacterial strains, *E. coli, P. aeruginosa*, *S. aureus* and *S. pyogenes*. However, SLP-II inhibited the growth of both strains of Gram positive and Gram-negative bacteria more efficiently even at low concentrations. Difference in OD noted for SLP and SLP-II at 24 h was compared as shown in Fig. 5a-d. MIC of all test samples is given in Table 3.

3.5. SLP showed invitro antifungal activity

RFL, SLP and two fractions of SLP were then analyzed for antifungal activity which was determined against *C. albicans*. The zone of inhibition was observed after application of different doses of all the protein components. The results of the assay showed that all fractions of laticifer proteins were found to be active against fungi. However, SLP-I was found highly effective against the tested strain of fungi (Fig. 6). Mean MIC of RFL, SLP and SLP-I was calculated (Table 4).

4. Discussion

In the present study, latex was obtained and laticifer proteins were separated from *C. procera* and studied for their antimicrobial potential. Plants have plenty of antimicrobial secondary metabolites like proteins that work as antimicrobial compounds. These antimicrobial peptides play significant roles in the defense mechanisms of living organisms not only for plants themselves but also for insects, amphibians and mammals that feed on them (Kim et al., 2009). These antimicrobial peptides are widely studied as promising therapeutic agents against bacteria, fungi, and certain viruses that cause diseases both in plants and animals. Rapid emergence of resistant microbial pathogens to currently available antibiotics has triggered us to find and isolate potent antimicrobial proteins (peptides) of plant origin as an excellent candidate for antimicrobial activity.

All parts of *C. procera* have been examined for its therapeutic capability against various diseases including bacterial and fungal infections (Shobowale et al., 2013). Among various parts, latex have been found to be more effective against these microbes (Kareem et al., 2008). Latex is rich in proteins and these proteins could be the reason for therapeutic activity of the latex (Saher et al., 2023). In current study, we aimed to find out the antimicrobial potential of these proteins which has not yet been studied. Of note, our work strongly evidences the antibacterial activity of laticifer proteins examined against various strains of bacteria and fungi.

Soluble laticifer proteins (SLP) were obtained by using previously described methods (de Freitas et al., 2010, Lima-Filho et al., 2010). Latex of *C. procera* is found to be rich in protein

Table 2
Mean diameter of Zone of inhibition (ZOI) around filter papers with samples in mm.

Sample	ZOI (mm) of *S. aureus*	ZOI (mm) of *S. pyogenes*	ZOI (mm) of *P. aeruginosa*	ZOI (mm) of *E. coli*	ZOI (mm) of Neg Control
SLP	11 ± 0.2	11.5 ± 0.1	10.5 ± 0.1	10.2 ± 0.10	–
SLP-I	–	–	–	–	–
SLP-II	14 ± 0.1	14.2 ± 0.2	12 ± 0.2	14 ± 0.00	–

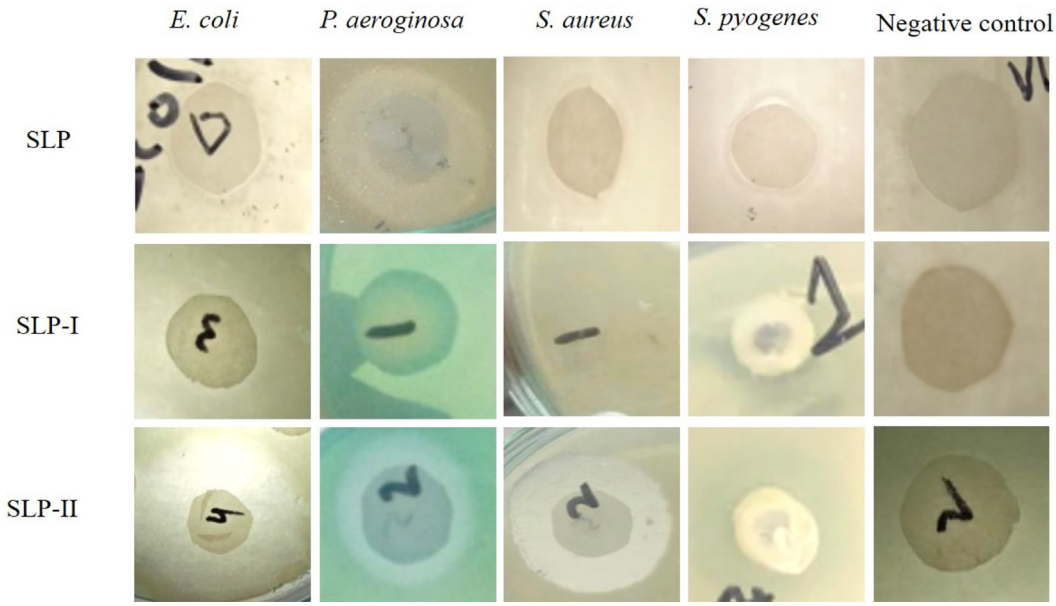

Fig. 4. Zone of inhibition observed for SLP and its fractions against **different bacteria.** Zone of inhibition was calculated by disc diffusion method using different bacteria. Two Gram-negative (*E. coli* and *P. aeruginosa)* and two Gram-positive bacteria (*S. aureus* and *S. pyogenes*) were used in the experiment. SLP, SLP-I and SLP-II were incubated with bacteria. Zone of inhibition was measured using a mm scale. Distilled water was used as negative control. The representative images are shown.

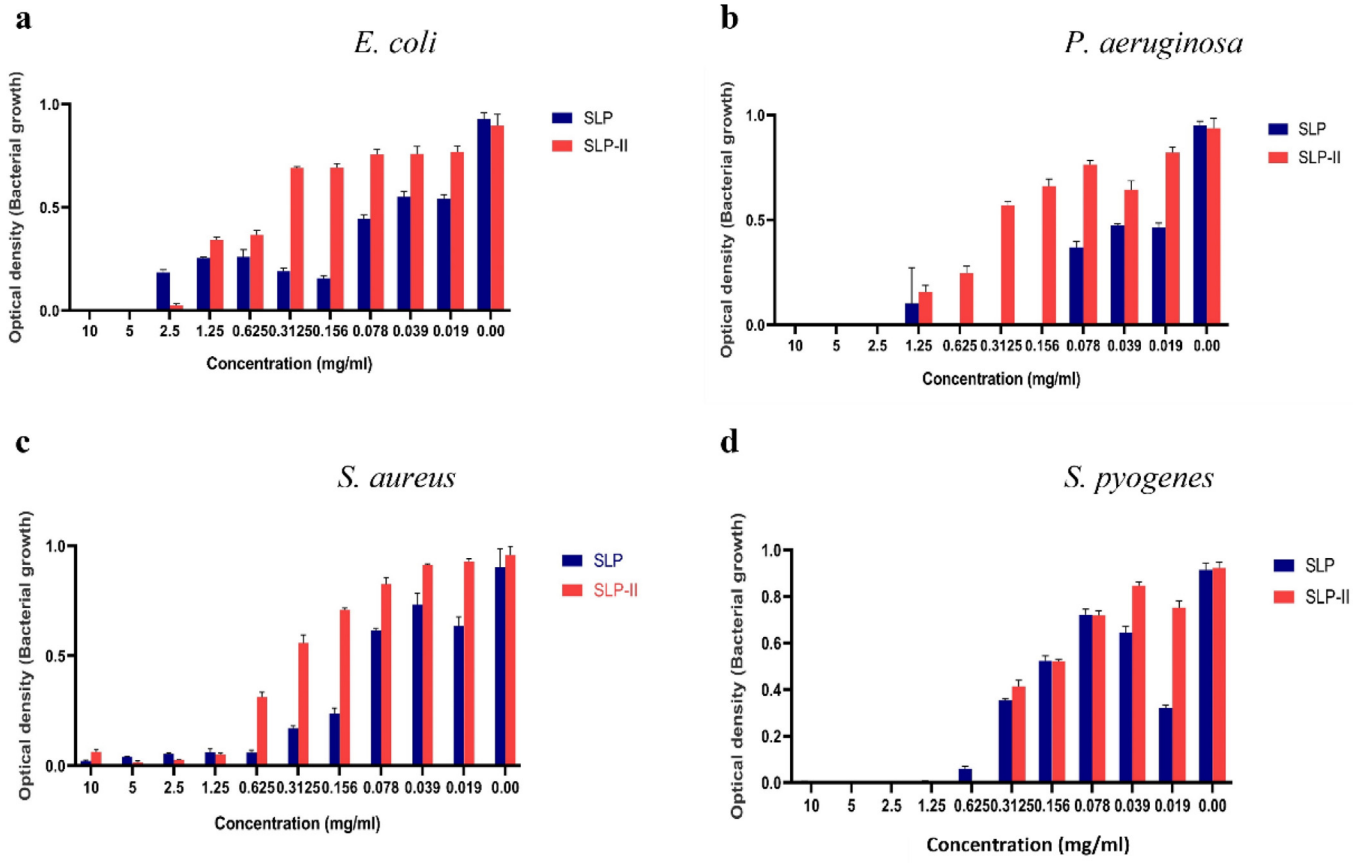

Fig. 5. Comparison of antibacterial activity of SLP and SLP-II at different doses. The optical density of SLP, SLP-II and negative control was measured at different concentrations for above mentioned bacteria in Fig. 4. The concentration 0.00 mg/ml represents the negative control. The optical density was measured after 24 h of bacterial incubation at 625 nm. It showed a significant antibacterial activity of SLP and SLP-II at various doses especially higher doses.

(Khanzada et al., 2008). In our study the concentration of SLP was found significantly higher (6.992 mg ± 0.208) which is quite comparable to another study where the enzymatic activities of laticifer proteins were reported (Freitas et al., 2007). To get purified proteins from plant extracts, we have to remove other unnecessary compounds (small organic metabolites, ions and salts etc). For this

Table 3
Minimum inhibitory concentration (MIC) in mg/mL of laticifer proteins against bacteria.

Sample	MIC against *S. aureus*	MIC against *S. pyogenes*	MIC against *P. aeruginosa*	MIC against *E. coli*
SLP	5	1.25	0.156	5
SLP-II	2.5	0.625	2.5	2.5

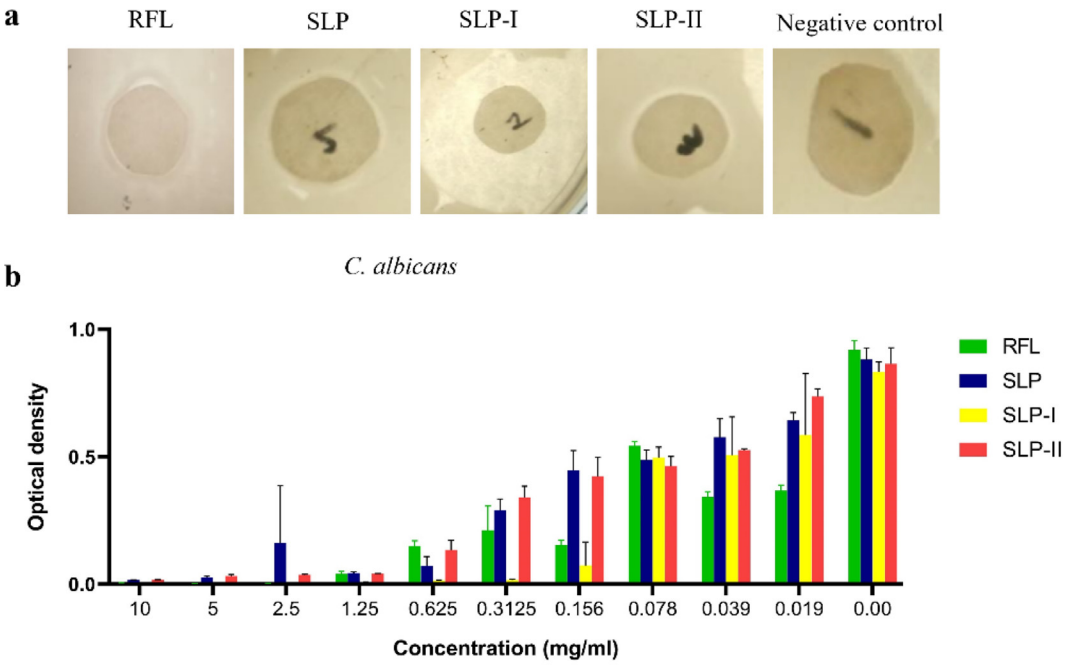

Fig. 6. Comparison of antifungal activity of RFL, SLP, SLP-I and SLP-II at different doses. a) Zone of inhibition for *C. albicans* was calculated by disc diffusion method. The representative images are shown. Broth was used as negative control. b) The optical density of RFL, SLP, SLP-I SLP-II, and negative control was measured at 625 nm for different concentrations against *C. albicans*. The optical density was measured after 48 h of fungal incubation. It showed a significant antifungal activity of SLP, SLP-I and SLP-II at various doses.

Table 4
Zone of inhibition and Minimum inhibitory concentration of laticifer proteins against C. albicans.

Sample	ZOI (mm)	MIC (mg/mL)
RFL	10 ± 0.2	1.25
SLP	12 ± 0.1	1.25
SLP-I	20 ± 0.3	0.312
SLP-II	8 ± 0.6	1.25

purpose, different methods are used one of which is acetone precipitation method. This was a reliable and most commonly used method but absolute pulverization, repeated rinsing, and prolonged incubation in TCA/acetone cause the precipitated proteins difficult to re-dissolve in this method (Niu et al., 2018). There is another simple way (SLP method) to get solubilized protein from the latex of *C. procera* (de Freitas et al., 2010). Both methods were followed in our work and yield was calculated. APP and SLP both generated comparable yields and proteome profiling, but SLP was found more efficient.

Proteomic investigations can be best performed by using an electrophoretic technique. Therefore, we used SDS–PAGE for the determination of molecular weights of proteins after separation of proteins from latex. Two distinctive bands of proteins named SLP-I and SLP-II were obtained at molecular weight 29.5 and 27.4 kDa respectively.

GFC is a versatile method that is famous for effectively separating bioactive molecules in high yield. It was used to separate proteins on the basis of their molecular weight by using Sephadex G 100 column that is appropriate for separating proteins of molecular weight ranging from 4 to 150 kDa (Ó'Fágáin et al., 2017). According to previous studies on laticifer proteins of *C. procera*, it is found that most of the proteins obtained from its latex also fall with in this range (Freitas et al., 2007). According to elution profile, we gained in our work after passing through GFC, consequently two peaks; SLP-I and SLP-II were obtained This result further confirmed the presence of two fractions of SLP as we obtained via SDS-PAGE analysis. Subsequently, we used spectrophotometric quantification of these proteins, where we used NanoDrop protein estimation method (Desjardins et al., 2009). All the three fractions; SLP, SLP-I and SLP-II were run on gel along with ladder and by using standard curve, molecular weight of SLP-I and SLP-II were estimated, and it was found 27 kDa and 29 kDa that is comparable to other studies previously done and similar molecular weight proteins were separated. These proteins possess various pharmacological activities, as was described in a study where enzymatic activities of laticifer proteins were analyzed. Protein profile obtained in our study was quite comparable to Freitas et al. (2007) work and similar pattern was also seen on gel. Another study was performed to check invitro cytotoxic activity of laticifer proteins and similar protein profile patterns were also traced in their work (de Oliveira et al., 2007).

The ability of SLP to digest substrates like azocasein that is endoproteinases in nature was determined. Cysteine protease mediated proteolytic activity was found in SLP as the proteolytic behavior was enhanced in the presence of azocasein. In addition,

when SLP was activated by some reducing agent i.e., DTT, the proteolytic action was greatly increased. This result strengthens the concept that latex contains DTT activated cysteine proteases. Other researchers confirmed presence of different types of cysteine proteases in *C. procera* latex, but in our study the latex was first time studied for such activities in this region (Freitas et al., 2007). A study conducted previously (Dubey and Jagannadham 2003) recommended the presence of different proteases in *C. procera* latex and thus confirmed the findings of this study. Different authors have their positive opinions about defensive role of latex and its correlation with the presence of cysteine protease (Konno et al., 2004, Martínez et al., 2012).

SLP and its fractions were tested for their antibacterial activity after lyophilization against Gram positive and Gram negative bacteria. Latex obtained from *C. procera* was traditionally used to cure different skin infections either caused by bacteria or fungi. We have investigated latex and its proteins against skin diseases-causing bacterial and fungal strains. Bacteria investigated in our study were *S aureus*, *S pyogenes*, *E coli*, and *P aeruginosa*, all of them are involved in different types of skin and soft tissue infections (Ki and Rotstein 2008). SLP-II among all others gave widest ZOIs that ranges from 12 to 14 mm, while SLP has smaller ZOI as compared to SLP-II. The results showed that SLP reduced the growth of both Gram-positive and Gram-negative bacterial strains; *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenes* with 5 mg/mL, 0.156 mg/mL, 5 mg/mL and 1.25 mg/mL respectively. In the case of SLP-II, it inhibited the growth of both strains of Gram-positive and Gram-negative bacteria having MIC in the range of 0.625 to 2.5 mg/mL. The possible mechanism of action of laticifer proteins is membrane permeabilization, according to previous studies it is confirmed that AMPs have ability to form pores in membrane of bacteria (Datta et al., 2016) that causes membrane destabilization of microbes resulting cell death (Bhunia et al., 2010).

It was the first study in which laticifer proteins were tested for antimicrobial activity. In previous studies, different extracts were analyzed against these microbes and latex were found effective. A study was performed on the protein separated from stem bark named Cal-Protein and tested against various Gram negative and Gram positive bacteria and it was found effective comparable to chloramphenicol (Perumal Samy and Chow 2012). Similarly, another study was designed in which bioactive proteins were isolated and characterized from fruits of *B. pinguin* and further this protein was fractionated by gel filtration chromatography. They checked the antibacterial activity against *E. coli* and *S. aureus* and MIC for both bacteria which was found positive (Carlos Ruiz-Ruiz et al., 2017). Similarly, proteins obtained in our study were not only effective against fungus but also against both Gram-positive and Gram-negative bacteria.

5. Conclusions

The present study concluded that latex is a rich source of pharmacologically active proteins, so can be used in the future for different ailments. Keeping in view increase in resistance among currently available antibiotics, bioactive peptides were screened for anti-microbial effects against Gram-positive bacteria, Gram-negative bacteria and fungi. Laticifer proteins are soluble proteins in nature and having lot of other pharmacological activities and our study reveals that these proteins can be used for treatment of many bacterial diseases while there is a need of time that *in vivo* clinical trials are also important for further effects analysis. As far as the yield is concerned the yield of laticifer proteins obtained in our study is comparable to previous ones. Upon qualitative analysis of antibacterial activity of all gel filtration chromatographic fractions possessed maximum activity followed by pure soluble laticifer protein and rubber free latex.

CRediT authorship contribution statement

Uzma Saher: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Muhammad Ovais Omer:** Conceptualization, Methodology. **Aqeel Javeed:** Methodology. **Aftab Ahmad Anjum:** Methodology. **Kanwal Rehman:** . **Tanzeela Awan:** .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103659.

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