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Original Article

Study of Catalase, Protease, Antioxidant and Antimicrobial Activities of *Tabernaemontana divaricata* Latex

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Abstract

The latex was collected from an Indian native species, *Tabernaemontana divaricata* (L.) R.Br. ex Roem. & Schult. The latex was analysed for different bioactivities, i.e., catalase, protease, antioxidant and antimicrobial activities. Catalase activity was determined by the catalytic decomposition of H_2O_2, which was monitored by the decrease in the absorbance at 240 nm using a UV-Vis spectrophotometer. Protease activity was determined by the hydrolysis of milk casein proteins, the resultant hydrolysate was colorimetrically analysed using Folin-Cioclateu reagent at 700 nm, in a UV-Vis spectrophotometer. The antioxidant activity was analysed by the reduction of DPPH (1,1-diphenyl-2-picrylhydrazyl) reagent at 518 nm in a UV-Vis spectrophotometer. The antibacterial activity was determined by well diffusion method using the agar medium. The antifungal activity was determined by well diffusion method using the potato dextrose agar medium. Based on the results, latex of *T. divaricata* possessed mild catalase activity (15.2±3.93 U/ml), high protease activity (4461.55±230 µg/ml), and moderate antioxidant activity (28±3.0%). Latex of *T. divaricata* inhibited *Enterococcus faecalis* bacteria growth, but did not affect *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria. The fungal species *Aspergillus flavus* showed no susceptibility, but *A. niger* was inhibited by latex of *T. divaricata*.

Keywords: *Tabernaemontana divaricata*, Catalase activity, Protease activity, Antioxidant activity, Antimicrobial activity

Introduction

Plants are rich in various kinds of molecules. Plants and products of the plants exhibit different bioactivities. Hence several researchers are working on the plants in identifying and isolating different types of molecules that can be exhibit certain activity, or might be useful for other purposes which can be applicable for the use of mankind for various purposes such as pharmaceuticals and other industries. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found *in vitro* to have antimicrobial properties [1,2].

Plants have developed diverse morphological, functional and chemical adaptations to ward off pathogens and pests [3]. Latex is a sticky sap that flows out of the points of laticiferous tissue upon mechanical wounding or insect herbivory [4] and contains several classes of secondary metabolites and proteins [5-7]. Latex, as found in nature, is a milky fluid in 10% of all flowering plants [8]. It is a complex emulsion containing of protein, alkaloids, starch, sugar, oils, tannins, resins, gums that coagulate on exposure in air. It serves mainly as defence against herbivorous insects [8]. The proteins in latex are peptidases, peptidase inhibitors, chitinases, and anti-oxidative enzymes. Recently, it was demonstrated that latex proteins have various biological functions including transcription, translation, protein degradation and response to environmental stimuli [9], and some of them play roles in protecting plants against insects and fungi [4,6,7,10].

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With this background we have assayed the latex of *Tabernaemontana divaricata* for different bioactivities, i.e., catalase activity, protease activity, antioxidant activity, antibacterial and antifungal activities.

Material and Methods

Sample Collection

A specimen of the plant located at our Sriniketan institute campus was submitted to the Department of Botany, Visva-Bharati (A Central University), Santiniketan, (India), which was identified as *Tabernaemontana divaricata*. Latex was freshly collected from *T. divaricata* between 09:30 – 11:00 AM. The twig of the plant was thoroughly cleaned with wet cotton and then tissue paper, a small scratch was made with a blade and the latex was collected in a micro tube. After the collection the samples were diluted 1:10 with distilled water, and the diluted latex was centrifuged at 10,000xg for 15 minutes. The precipitate was discorded and the supernatant was collected and used in the experiments. Laboratory experiments were carried out at Department of Soil Science & Ag. Chemistry, Institute of Agriculture (Palli Siksha Bhavana), Visva-Bharati (A Central University), Sriniketan.

Determination of Catalase Activity

Catalase activity was determined as previously described [11]. Catalase molecule catalyse the decomposition of H_2O_2, which was monitored by the decrease in the absorbance at 240 nm over a period of 3 min using a UV-Vis spectrophotometer. 1 ml of latex (diluted 1:10 in distilled water) and 1 ml of distilled water is taken in a quartz cuvette and 1 ml of H_2O_2 was added. Absorbance readings of the initial (i.e., 0 minute) and three consecutive 1-minute intervals (i.e., 1st, 2nd & 3rd minute readings) were taken at 240 nm and the catalase activity was calculated.

Determination of Protease Activity

Protease activity was determined as previously described [12]. Protease enzymes hydrolyse proteins into peptide fragments and amino acids. Protease enzyme reaction was carried out in micro Eppendorf tubes. 20 µl of the latex sample was taken into each Eppendorf tube and diluted to 100 µl with buffer, then 900 µl of casein (1% dissolved in the phosphate buffer, pH=7) was added to the tube. The mixture was incubated at room temperature for 15 minutes and at the end of this incubation, 1 ml of trichloro acetic acid (10% TCA in distilled water) was added to stop the reaction. Then the tubes were shaken thoroughly and centrifuged at 5000xg for 15 minutes. After centrifugation, 0.5 ml of the supernatant was taken and transferred into a clean and dry glass test tube. To this 5 ml of 0.5M NaOH solution was added and mixed, then 0.5 ml of Folin–Ciocalteu reagent was added to the tube and mixed thoroughly. The intensity if the blue colour developed was measured in a UV-Vis Spectrophotometer at 700 nm. The proteolytic activity of trypsin is calculated. The enzyme activity is expressed as µg of tyrosine liberated in 15min/ml of enzyme. One unit of protease activity was defined as the amount of enzyme liberating one micromole of tyrosine equivalent under the assay conditions.

Control: The control reaction was carried out similarly except that the TCA was added to the latex prior to the addition of the substrate casein.

Standard: Instead of 0.5 ml of aliquot of centrifuged assay tubes, 0.5 ml of Standard Tyrosine solution (100 µg/ml) was taken and 5 ml of NaOH and 0.5 ml of FC reagent was added.

Blank: Instead of sample aliquot or Tyrosine standard, 0.5 ml of buffer was taken and 5 ml of NaOH and 0.5 ml of FC reagent was added.

Protease activity of plant sample=T–C/S–B×50×50×2, (where, T = Test; C = Control; S = Standard; B = Blank).

Determination of Antioxidant Activity

The scavenging ability of the natural antioxidants of the plant sample towards the stable free radical DPPH (1-1, dipenyl-2- picrylhydrazyl) was measured by the method of DPPH assay [13]. 10 µl of latex was taken into a clean test tube and diluted by addition of 1 ml of phosphate buffer (pH=7.0), and to this 1 ml of DPPH (0.4 mM in ethanol) was added. The mixture was allowed to react at room temperature for 10 minutes. In the control tube no plant source was added, only 1010 µl of buffer and 1 ml of DPPH were taken. The discoloration of the purple colour was measured at 518 nm in a UV-Vis Spectrophotometer. The Anti-oxidant activity was calculated.

Determination of Antibacterial Activity

The following bacterial strains *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 779), *Staphylococcus aureus* (MTCC 96), *Enterococcus faecalis* (MTCC 2128) were used for in vitro antibacterial activity. These bacterial strains were obtained from microbial Type culture collection (MTCC), Chandigarh, India.

The antibacterial activity was determined by well diffusion method [14]. About 20 ml of molten Muller Hinton agar was poured into sterile petri plates (Himedia, Mumbai, India). The agar was allowed to solidification. The plates were incubated with freshly prepared inoculums which were swabbed over the entire

surface of the medium, rotating the plate 60 degrees after each application by using a sterile cotton swab, to ensure the spread of the tested microbes on the surface of the plate completely. Inoculums were 10^8 CFU/ml of bacteria. Wells of 6 mm diameter were bored in the medium of each plate with the help of sterile cork-borer. They were sterilized by autoclaving and subsequently dried at 80 °C for an hour. The wells were filled with different volumes of plant latex sample, i.e., 25µl, 50µl, 75µl, and 100µl. Buffer was used as control. The wells were made far enough to avoid both reflection waves from the edges of the petri dishes and overlapping rings of inhibition. Finally, the Petri dishes were incubated for 18 h at 37 °C in an incubator. After incubation, the diameter of the inhibition zone around each disc was measured (mm) and recorded. Clear zone of growth inhibition around the well indicates the antibacterial activity.

Determination of Antifungal Activity

The fungal strains *Aspergillus niger* (MTCC 872), *Aspergillus flavus* (MTCC 873) were used for in vitro antifungal activity. These fungal strains were obtained from microbial Type culture collection (MTCC), Chandigarh, India.

The antifungal activity was determined by well diffusion method [15]. About 20ml of Potato Dextrose Agar medium (PDA) was poured into sterile plates (Himedia, Mumbai, India). The agar was allowed to solidify, the sterilized medium taken in the sterilized petri plates was inoculated with the fungal spore suspension. Wells of 6 mm diameter were bored in the medium of each plate with the help of sterile cork-borer. They were sterilized by autoclaving and subsequently dried at 80 °C for an hour. The wells were filled with different volumes of plant latex sample, i.e., 25µl, 50µl, 75µl and 100µl. Buffer was used as controls. The wells were made far enough to avoid both reflection waves from the edges of the petri dishes and overlapping rings of inhibition. Finally, the Petri dishes were incubated for 4 days at 37 °C in an incubator. After incubation, the diameter of the inhibition zone around each disc was measured (mm) and recorded. Clear zone of growth inhibition around the well indicates the antifungal activity.

Results and Discussion

Catalase Activity

Several harmful chemicals with the oxidizing capacity may be generated within the individual due to metabolism of the individual or microbes and also may be ingested through various sources such as diet. These chemicals may be detrimental to the health of the individual. So, these harmful chemicals should be neutralized or modified to harmless or comparatively less harmful compounds. Catalase plays very important role in performing this job, whereby confirming the safety of the living organism. Catalase scavenges H_2O_2 generated during β-oxidation of the fatty acids, electron transport in mitochondria and photorespiratory oxidation [16,17]. Catalase is the most efficient enzyme as an antioxidative enzyme which lowers hydrogen peroxide or superoxide to accumulate to toxic levels in plant growth [18]. Catalase also neutralize the toxins by oxidizing them using hydrogen peroxide, such as phenols, formic acid, formaldehyde and alcohols [19-21]. Catalase is an important enzyme of cell defence mechanisms against oxidative stress in living organisms. This antioxidant enzyme widely distributed in a variety of life forms, including microorganisms, plants and animals [22]. In plants, multiple isoforms of the enzyme are usually present, and they are expressed in different tissues and developmental stages. In green leaves majority of catalase activity is found in peroxisomes [19,23]. Catalase activity has been reported in several plants, such as spinach, maize, cotton, sunflower, tobacco, van apple, and parsley [24-27]. However, in *T. divaricata*, catalase activity was neither studied nor reported so far. In the present study, we have analysed the catalase activity in the latex of *T. divaricata*. From the results it was observed that *T. divaricata* has showed negligible catalase activities, i.e., 10 µl of latex (undiluted) showed the activity in terms of ΔOD_{240nm} of 0.02/min, i.e., 15.2 U/ml of catalase activity was observed.

Protease Activity

Proteases occur naturally in living organisms. Growth and development in all organisms occur as a result of an overall balance between protein synthesis and proteolysis. Commercially, proteolytic enzymes from the plant sources have received special attention because of their broad substrate specificity as well as activity in wide range of pH and temperature [28]. Proteases are effective in removing damaged and infected tissues from wounds and thus play an important role in the wound healing process. Proteases from various sources such as plant, microbes, maggots and animals were found to be useful in wound debridement [29]. In this present study we have observed that the proteases activity was also found in the latex of *T. divericata* latex (4461.55±230 µg/ml). Similarly, Gonçalves *et al*. [30] were also studied and reported proteases in plant source. In their study, extracts of leaves, seeds, roots, and stem from a tropical legume, *Canavalia ensiformis* (L.) DC., were prepared employing buffers and detergent in aqueous solution. Leaf extracts had the highest protein content and the most pronounced peptidase activity with optimal pH in the neutral to alkaline range [30].

Plant sources play an important role due to secondary metabolites and responsible for several biological activities in humans as well as animals. It is also used to treat variety of diseases. These medicinal plants are not only providing traditional and ethnic medicine but it promising for highly effective bioactive molecules. Plant latex contains hazardous chemical substances that cause allergic reactions and induce immediate-type hypersensitivity in them. In addition, plant latex contains wide diversity of bioactive chemicals which showed different biological activities such as anti-carcinogenic, anti-proliferative, anti-inflammatory, vasodilatory, anti-oxidant, anti-microbial, anti-parasitic and insecticidal [31]. The present study showed that latex of the *T. divericata* plant was a rich source of proteases molecules.

Antioxidant Activity

The antioxidant activity of the latex of *T. divericata* was determined using ethanoic solution of diphenylpicryl hydrazine (DPPH) reagent. DPPH is a stable free radical. The effect of an antioxidant on DPPH radical scavenging is due to their hydrogen donating ability or free radical scavenging activity [32]. When a solution of DPPH is mixed to the substance that can donate a hydrogen atom, then this gives rise to the reduced form diphenylpicryl hydrazine which loss of its violet colour [33]. The DPPH test gives the information on the reactivity of the test compounds with a stable free radical scavenging activity. DPPH gives a strong absorption band at 517 nm in visible region [34]. When the odd electron becomes paired and free radical scavenger absorb, it reduces the DPPH solution and is decolorized. The colour changes from deep violet to pale yellow.

From the results, it was observed that the latex of *T. divericata* has showed moderate, i.e., 28±3.0% antioxidant activity. This indicates that the latex of this plant does have moderate and measurable amounts of antioxidant molecules. The aqueous leaf extract of *T. divaricata* was analysed for antioxidant activity by different methods, namely, DPPH, Hydroxyl radicals, Hydrogen peroxide radical, Lipid peroxidation and Ferrozine-Fe^{2+} complex methods. *T. divaricata* shown possess 56.40±4.439%, 45.83±7.216%, 37.10±1.450%, 63.88±4.809% and 32.63±1.322% of antioxidant activity, respectively [35]. Though aqueous leaf extract *T. divaricata* has showed 56.40% antioxidant activity by DPPH method according to Padmajaa and Hemalathaa [35], in our study we have observed only 28% antioxidant activity in the latex of the same plant source. This variation may be due to the environmental conditions of the plant and/or the sample/part of the plant analysed. In our study antioxidant activity was analysed in the latex, whereas others have analysed in the leaf of the same plant [35].

Antibacterial & Antifungal Activities

For the treatment of diseases, inhibitory chemicals are employed to kill microorganisms or prevented their growth, these are called antimicrobial agents. These are classified according to their application and spectrum of activity, as germicides that kill micro-organisms, whereas micro-biostatic agents inhibit the growth of pathogens and enable the leucocytes and other defence mechanism of the host to cope up with static invaders. The germicides may exhibit selective toxicity depending on their spectrum of activity. They may act as viricides (killing viruses), bactericides (killing bacteria), algicides (killing algae) or fungicides (killing fungi).

Antibiotics are produced by microorganisms or they might be fully or partly prepared by chemical synthesis. They inhibit the growth of microorganisms in minimum concentration. Antibiotics may be of microbial origin of purely synthetic or semisynthetic [36]. The multidrug resistant of bacterial and fungal strains of clinically important pathogens fetches the interest of scientist to develop newer broad-spectrum antimicrobial agent [37]. Antimicrobial drugs may either kill microorganisms outright or simply prevent their growth. There are various ways in which these agents exhibit their antimicrobial activity [38]. The less availability and high cost and greater side effects of new generation antibiotics necessities looking for the substances from alternative medicines which claimed antimicrobial activity have been reported in literature [39-41].

Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found *in vitro* to have antimicrobial properties [1, 2]. Plant extract has been used traditionally to treat a number of infectious diseases including those caused by bacteria and fungi [42]. Plant based antimicrobial compounds have enormous therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials [43]. Antimicrobial peptides/proteins have been reported to kill Gram negative and Gran positive bacteria, enveloped viruses, fungi, and even transformed/cancerous cell. In contrast to many conventional antibiotics these peptides appear to be bactericidal instead of bacteriostatic [44]. A number of reports are available which states the in-vitro or in-vivo efficacy of plant extracts against plant and human pathogens causing infections, but more study and screening is required because there has been an alarming increase in the incidence of new and re-emerging of infectious diseases [45].

In this present study, the antimicrobial activity of *T. divaricata* was determined against gram-negative bacteria and gram-positive bacteria. The antimicrobial activity of *T. divaricata* was studied against gram-negative organisms (*Escherichia coli, Pseudomonas aeruginosa*) and gram-positive organisms (*Staphylococcus aureus, Enterococcus faecalis*) at four different concentration range of 25µl, 50µl, 75µl and 100µl and the results obtained were shown in Table-1 and Fig-1. The bacterial species *Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus* were showed no susceptibility to the latex of *T. divaricata*. However, *Enterococcus faecalis* was shown to be inhibited by the latex of *T. divaricata*.

The antifungal activity of the latex of *T. divaricata* was evaluated using agar diffusion method. The latex of *T. divaricata* was tested against *Aspergillus niger* and *Aspergillus flavus* and the results obtained were shown in Table-2, Fig-2. The latex of *T. divaricata* exhibited significant antifungal activity. Among the fungi tested *T. divaricata* exhibited higher zone of inhibition on *Aspergillus niger* and no zone of inhibition on *Aspergillus flavus*.

Several other researchers have reported the presence of antimicrobial activity in the *Tabernemontana divaricata* [46-51]. It was reported that the ethanolic extract of flowers and stems of the double flower variety of *T. divaricata* showed more inhibition against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*. Ethanolic extract of leaves showed inhibitory action against *Staphylococcus aureus* but no inhibition activity against *E. coli* and *B. subtilis* [46].

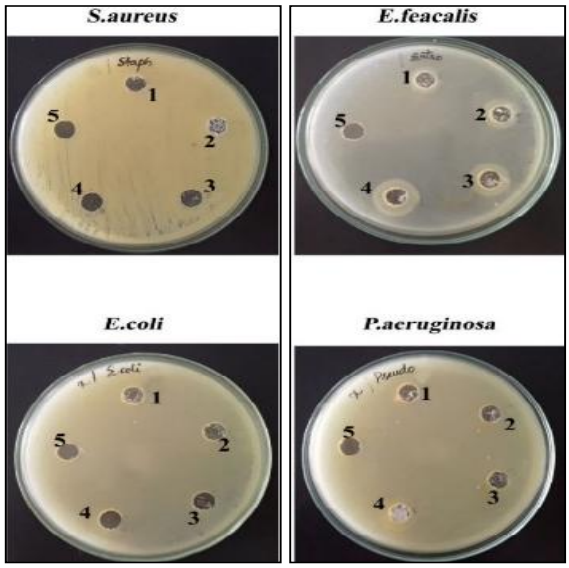

Fig. 1 Antibacterial activity of *T. divaricata.* 1 = 25 µl; 2 = 50 µl; 3 = 75 µl; 4 = 100 µl; 5 = Buffer control.

It was observed that ethanolic, methanolic and acetone extracts of *T. divaricata* L. was found to be effective against almost all the bacteria. However, the aqueous extract of *T. divaricata* showed no antibacterial activity [47]. Silver nano particles (SNPs) and chloroauric nano particles (AuNPs) were synthesized from the aqueous extract of leaves of *T. divaricata* and these SNPs and AuNPs inhibited Gram-positive and Gram-negative bacteria in various bacterial cultures [48].

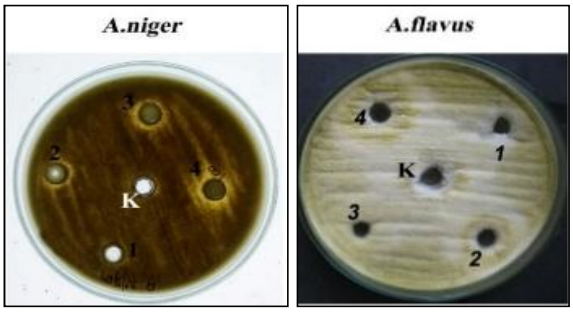

Fig. 2 Antifungal activity of *T. divaricata.* 1 = 25 µl; 2 = 50 µl; 3 = 75 µl; 4 = 100 µl; K = Buffer control.

T. divaricata was tested against Gram positive and Gram-negative bacteria including *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella* sp. The flower extract of *T. divaricata* was tested against standard bacterial strains by disc diffusion method and found that the methanolic extract of the flower inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* [49].

Table 1 Antibacterial activity of *T. divaricata*

Volume of Latex	25 µl	50 µl	75 µl	100 µl
	Zone of Inhibition (mm)			
Staphylococcus aureus	-	-	-	-
Enterococcus feacalis	8	10	12	14
Escherichia coli	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-

Methanol, Dichloromethane and Ethyl acetate extracts of *T. divaricata* petals of flowers were screened for antibacterial activity against pathogenic microbes. Among the three extracts, Dichloromethane extract of *T. divaricata* exhibited significant antibacterial activity on nine gram-positive bacteria viz., *Staphylococcus aureus*, *S. epidermidis*, *S. agalactiae*, *Gardnerella vaginalis*, *Enterococcus faecalis*, *Propionibacterium acnes*, *Corynebacterium macbinleys*, *Bacillus serus*, *B. subtilis* & a gram negative bacteria viz., *E. coli* [50]. Petroleum ether and ethanolic leaf extracts of *T. divaricata* was found to be having antibacterial activity against bacterial pathogens such as *Pseudomonas* sp., *Proteus* sp.,

Staphylococcus aureus, *Escherichia coli*, *Klebsiella* sp. and *Salmonella* sp. Simultaneously these extracts of *T. divaricata* was found to be having antifungal activity against fungal pathogens such as *Aspergillus niger*, *Mucor* sp., *Candida* sp. [52]. These results concluded that *T. divaricata* have antibacterial activity. Similarly, another species of the same *Tabernaemontana* has also been reported to having antimicrobial activity. Leaf extracts of different solvents (i.e., aqueous, methanol, ethanol, chloroform and acetone) of *Tabernaemontana heyneana* Wall. were tested for antibacterial and antifungal activities.

Table 2 Antifungal activity of *T. divaricata*

Volume of latex	25 µl	50 µl	75 µl	100 µl
	Zone of Inhibition (mm)			
Aspergillus niger	-	8	10	12
Aspergillus flavus	-	-	-	-

These extracts were tested against bacterial pathogens (i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhii*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Bacillus subtilis*) and fungal pathogens (i.e., *Aspergillus niger*, *Giberella fujikori*, *Pencillium chrysogenum*, *Aspergillus terreus*, *Candidda albicans*, *Rhizopus mucor* and *Trichoderma viridians*). Methanol and acetone leaf extracts showed good antibacterial activity and ethanol leaf extract showed good antifungal activity [51]. It was observed that the extracts of different organic solvents have shown to possess the antimicrobial activity, but the aqueous extracts and the latex found to be poor in antimicrobial activity against the tested organisms.

Plants with antibacterial and antifungal activities are rich in polyphenolic substance such as tannins, catechins, alkaloids, steroids and polyphenolic acids. Various flavonoids, sesquiterpenoid alcohols, triterpenoids from plants may also be useful as antimicrobial agents [53]. The activity may be due to their ability to form a complex with soluble proteins, which then binds to bacterial cell wall and ruptures the cell wall [54]. Phenols are also toxic to microorganisms because of the number of hydroxyl moiety in the phenol groups. There is evidence that highly oxidized phenols possess more inhibitory action. The presence of various phytochemicals such as flavonoids, alkaloids and phenols in *T. divaricata* were revealed in the preliminary phytochemical investigation, which might be responsible for their antimicrobial and antifungal activities.

Conclusion

Catalase activity, Protease activity, Antioxidant activity, Antibacterial activity and Antifungal activities were observed in the latex of *T. divaricate*

Author contributions

Conceptualization: [Y. Vasudeva Rao] & [Merrine Raju]; Methodology: [Y. Vasudeva Rao] & [Merrine Raju]; Formal analysis and investigation: [Y. Vasudeva Rao] & [Merrine Raju]; Writing - original draft preparation: [Y. Vasudeva Rao]; Writing – review and editing: [Y. Vasudeva Rao] & [Merrine Raju]; Funding acquisition: [NA]; Resources: [Department of Soil Science & Ag. Chemistry, Institute of Agriculture, Visva-Bharati]; Supervision: [Y. Vasudeva Rao]. All authors read and approved the final manuscript. The authors declare that there are no conflicts of interest.

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