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Original Article

Phytochemical Screening, Antibacterial-Guided Fractionation, and Thin-Layer Chromatographic Pattern of the Extract Obtained from *Diploknema butyracea*

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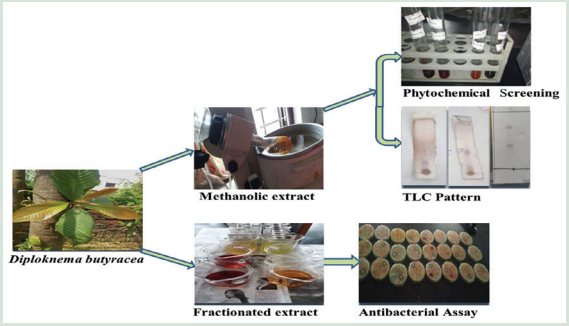
ABSTRACT

Objective: *Diploknema butyracea* (family: *Sapotaceae*), commonly known as the Indian butter tree or *Chyuri*, is habitat to Nepal and distributed from Garhwal to Sikkim up to Bhutan. This study was aimed to investigate and examine the qualitative phytochemical screening, antibacterial-guided fractionation, and thin-layer chromatographic (TLC) pattern of the *D. butyracea* bark extracts. **Materials and Methods:** Phytochemical screening of *D. butyracea* extract was carried out by different chemical tests. Normal-phase TLC was conducted by using different solvent system to investigate the presence of phytochemicals. Antibacterial activity on different fractions of methanolic bark extract was determined by the agar disc diffusion method. **Results:** Phytochemical screening revealed the presence of alkaloid, tannin, and flavonoid in barks; alkaloid and flavonoid in flowers; and alkaloid, tannin, and steroid in leaves. Among the different fractions of bark extracts, the acetone fraction was found to be most effective against Gram-positive bacteria *Staphylococcus aureus*, whereas the highest sensitivity against Gram-negative bacteria *Escherichia coli* was shown by n-butanol fraction. Both fractions showed a zone of inhibition of 15 mm at the concentration of 1 mg extract per disc. TLC of the bark extract confirmed the presence of prominently visible compounds at ethyl acetate fraction. **Conclusion:** Isolated compounds from dried barks of *D. butyracea* could be the cradle of the new useful drug.

Key words: *Diploknema butyracea*, antibacterial, phytochemicals, thin-layer chromatography

SUMMARY

- This study was aimed to investigate the antibacterial activity of *Diploknema butyracea* bark extract against both gram -positive and gram-negative bacteria along with preliminary phytochemical screening of leaves, bark and flower of the plant. Furthermore TLC profile of bark extract was observed via bioassay guided fractionation. The antibacterial activities of the bark extract from butanol and acetone fraction revealed positive results for both bacterial strains. The TLC pattern of the extracts in different solvents also revealed the presence of major compounds in ethyl acetate fraction of bark extract. We suggest the further study in this plant regarding isolation of antibacterial compounds.



Abbreviations Used: TLC: Thin Layer Chromatography; MHA: Mueller Hinton Agar; H_2SO_4: Sulphuric acid; NaOH: Sodium hydroxide; °C: Celsius; h: hour; ml: mililitre.

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INTRODUCTION

An excellent repository of cultural heritage and the use of plants as folklore medicines have been practiced since the beginning of human civilization. Natural product such as plant extracts, either as unalloyed compounds or as standardized extracts, provides unlimited prospects for new drug discoveries because of the unmatched availability of chemical diversities.[1] According to the World Health Organization, more than 80% of the world's population relies on traditional medicines for their primary healthcare needs. The primary steps to extract the biologically active compound from plant resources are extraction, pharmacological screening, isolation, and characterization of bioactive compounds.[1] Approximately 1700 species of flowering plants are currently being utilized as medicines in Nepal, and the number is expected to grow as infrastructure allows greater access to unexplored parts of the country.

Diploknema butyracea (*Chyuri*) belonging to a family *Sapotaceae* is a deciduous tree (height around 20 m).[2] It is distributed through the Sub-Himalayan tract of 300–1500 m and is also known as the Indian butter tree as the main product of tree is vegetable ghee, a fat extracted from seeds. Various parts of *Chyuri* plants are used by different ethnic groups of Nepal for medicinal and other purposes.

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The bark is used as fish poison,[3] while other parts are used as food and medicine.[4]

Phytochemicals are regarded as secondary metabolites which occur naturally in plants and can be derived from any part of the plant such as bark, leaves, fruits, flower, and seeds.[5] The presence of phytochemicals may lead to its further isolation, purification, and characterization.[6] Phytochemical screening refers to extraction, screening, and identification of the medicinal active substances that can be derived from plants. It is important as it is considered effective in discovering the bioactive profile of plants of therapeutic importance. The extracts can then be made with various solvents isolate and purify the active compounds that are responsible for the bioactivity.

Although human medicine is making good progress, infectious diseases caused by pathogenic microbes are still a threat to human health. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance.[7] Owing to side effects and the resistance of pathogenic microorganisms against antibacterial agents, recent attention has been given to extracts and biologically active compounds isolated from plant species.[8]

Thin-layer chromatography (TLC) is a quick, delicate, and inexpensive technique used as a qualitative tool to determine the chemical constituents present in the extract, to determine the solvent composition for preparative separations, and also to analyze the fractions obtained from column chromatography. TLC techniques can be used to accelerate the process of purification of the bioactive molecules.[9] Such technique permits identification of the compounds in both samples by direct comparison. It is also used for the analysis of natural medicines and also for the qualitative determination of a small amount of impurities.

The present study focused on a stepwise method of phytochemical screening, antibacterial-guided fractionation, and TLC pattern of the extracts obtained from *D. butyracea*. *D. butyracea* (*Chyuri*) samples were collected from the Mid-Western Himalayan region. Methanolic extracts of the plant were screened for the nature of its chemical constituents and potential antibacterial activity. TLC fingerprinting was used to determine the biologically active secondary metabolites.

MATERIALS AND METHODS

Standard drug

Norfloxacin, 10 µg/disc, obtained from HiMedia Laboratories Private Limited, India, was used as the standard control.

Solvents

Methanol, ethyl acetate (Thermo Fisher Scientific India Pvt. Ltd.), chloroform, acetone, butanol (Merck Specialties Pvt. Ltd.), and hexane (Loba Chemie Pvt. Ltd.) were solvents used.

Chemicals

Mercuric chloride, sodium hydroxide, hydrochloric acid, sulfuric acid (H_2SO_4), potassium iodide, acetic acid (Thermo Fisher Scientific India Pvt. Ltd), ferric chloride (Lova Chemie, Mumbai), Mueller–Hinton agar (MHA), nutrient broth (HiMedia Laboratories Pvt. Ltd.), dimethyl sufoxide (DMSO) (Thermo Fischer Scientific India Pvt. Ltd.).

Instruments

Digital balance (ATX224, Shimadzu Corporation, Philippines), hot air oven (S.M. Scientific Instruments (P) Ltd., Delhi), sonicator (Indo Sati Scientific instrument (P) Ltd., Delhi), autoclave (S.M. Scientific Instruments (P) Ltd., Delhi), refrigerator (LG), incubator (S.M. Scientific Instruments (P) Ltd., Delhi), and rotatory evaporator (R-210/215, Buchi Labortechnik AG, Switzerland).

Plant materials

The plant materials for *D. butyracea* were collected from Bagdula, Pyuthan, in April 2019 and identified by the National Herbarium and Plant Laboratories, Nepal. The herbarium voucher specimen of the plant has been deposited in the National Herbarium and Plant Laboratories Herbarium (Voucher Specimen number: LN: 075/076) and in Pharmacognosy Laboratory of Crimson College of Technology (Voucher Specimen number: CCT-HRB-075-184).

Extract preparation

The plant materials were cleaned and shade-dried at normal room temperature followed by comminution. A total of 500 g dried powdered plant materials was extracted using 2500 ml methanol for bark, and flower and leaves were extracted in water. Extraction was performed by triple cold maceration in which plant materials were soaked with solvent for 72 h with changing of menstruum at every 24 h. The filtrate obtained from triple extraction was mixed and subjected for drying with the help of a rotary vacuum evaporator at 40°C. The dried extract was stored in air tight vial at 4°C before use.

Fractionation of methanolic extract of the bark

Methanolic extract of the bark was further fractionated by using hexane, chloroform, ethyl acetate, n-butanol, acetone, and ethanol guided by polarity-based solvent fractionation to ensure the extraction of chemical constituents in accordance with the polarity of solvent. Dried extract from all the fractions was investigated for antibacterial assay.

Bacterial strains

Standard human isolates of *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) were identified, isolated, and kindly provided by National Path Lab, Butwal.

Phytochemical screening

The methanolic extracts of the flower and bark were subjected to phytochemical screening according to the procedures described below.

Screening for alkaloids (Mayer's test)

One milliliter of the extract was measured in a watch glass, and little amount of diluted hydrochloric acid and Mayer's reagents were added to the solution; the formation of a white precipitate indicated the presence of alkaloids.[10]

Detection of glycosides (Liebermann's test)

The presence of glycoside was done using "Liebermann's test" for leaves extract in which 2 ml of the organic extract was dissolved in 2 ml of chloroform and 2 ml of acetic acid was added. The solution was cooled in ice, and after some time, H_2SO_4 was added carefully and the color change from violet to blue to green indicates the presence of steroidal nucleus (i.e., aglycon portion of glycoside).[11]

For bark, the extract was treated with diluted H_2SO_4 and warmed on a water bath, and then, it was filtered and neutralized with 5% NaOH solution. After neutralization, 0.1 ml of Fehling's solution was added and alkalinity was tested using pH paper.[11]

Detection of tannins

Tannins were detected by treating 5 mg of extract residues that were dissolved in 5 ml of distilled water with few drops of neutral 5% ferric chloride solution. The formation of a dark green color indicated the presence of tannins.[12]

Detection of flavonoids (alkaline reagent test)

Alkaline reagent tests were done for bark and flower extract, in which 2% of NaOH was added drop by drop in the extract, and then, the color changes to intense yellow; then, again after the addition of diluted hydrochloric acid, the color disappears after few seconds.[13]

Detection of terpenoids (Salkowski test)

The extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 (3 ml) is carefully added to form a layer. A reddish-brown coloration of the interface is formed to show a positive result of the presence of terpenoids.[14]

Detection of steroid

The extract was treated with chloroform and H_2SO_4. The presence of yellow with green fluorescence indicates the presence of steroids.[15]

Screening for antibacterial activity

The antimicrobial activity was investigated by using the disc diffusion method.[16] Initially, MHA of pH 7 was prepared and stored in sterilized condition for 15 min at 15 lb (pound) pressure using an autoclave. In aseptic conditions, 25 ml of medium was poured for solidification followed by storing at 50°C. The media culture was dried at 50°C using hot air oven and cooled in aseptic condition in sterilized hood for antimicrobial assay. For the preparation of bacterial suspension, each 5 ml of sterilized nutrient broth was poured in the sterilized tube; *E. coli* and *S. aureus* strains were placed in the separate tube using an inoculating loop. The suspension culture was left for 24 h at 37°C. 0.5 McFarland standard was used to optimize the density of bacterial suspension. Finally, culture of microorganism was inoculated into the sterile MHA. The filter disc having a diameter of 4 mm was impregnated with 10 µl from each extract solution of 100 mg/ml concentration in DMSO to load the 1 mg of plant extract per disc. After placing the disc in the inoculated agar, 10 µg/disc of broad-spectrum antibiotic norfloxacin was impregnated. Each plate represents four regions, i.e., one as a standard drug, second for methanolic extract as a positive control, third for DMSO as a negative control, and fourth for different fractions of extract. The impregnated disc was incubated for 24 h at 37°C. The antibacterial potential of the sample was expressed as the diameter of the zone of inhibition by extract and antibiotic against test organisms. Each assay was replicated three times, and the mean zone of inhibition was calculated.

Fractionation of the extract for thin-layer chromatographic analysis

Fifteen grams of methanolic extract was dissolved in 200 ml of distilled water and shaken vigorously for 15 min in a separating funnel. Then, 200 ml of hexane was added, shaken vigorously for 15 min, and allowed to settle in a stand for 24 h. After 24 h, both fractions were separated in two layers, i.e., hexane fraction at the top and water fraction at the lower part of the funnel. Both fractions were collected separately and labeled. Now, the same process was repeated for water fraction and 200 ml ethyl acetate. The collected fractions were evaporated to get dried extract. Process of fractionation is given in Figure 1.

Thin layer chromatography

TLC analysis of all extract samples was carried out in normal phase TLC plate, according to the previous method with slight modification.[17] Commercial aluminum oxide plate coated with silica gel Si 60 F_{254} (Merck) was purchased from the market and cut into 5 cm × 5 cm size, and the analysis was carried out in the following manner.

Thin layer chromatographic analysis

Initially, 1 mg/ml solutions of hexane, ethyl acetate, and aqueous fraction of bark extract were prepared in suitable solvent. A straight horizontal line was made 1 cm above the bottom and 1 cm below the top of the plate; sample was loaded at appropriate position over the TLC plate using fine capillary tube. Suitable solvent for the TLC analysis was optimized on the basis of changing the polarity of eluent starting from very non-polar solvent n-hexane and then slightly increasing the polarity by adding ethyl acetate and chloroform. Hence, hexane, ethyl acetate, and chloroform were mixed at optimized ratio to ensure the appropriate polarity of the eluent for the better chromatographic separation. 5 ml of eluent was pipetted into 10 cm × 10 cm TLC tank and allowed for saturation using a small piece of filter paper. A sample-loaded TLC plate is dipped carefully inside the tank and allowed for the movement of eluent until it reached to the upper horizontal line. After the completion, TLC plate was removed and dried using air dryer. Finally, dried TLC plates were dipped into the 10% H_2SO_4 solution and kept on a hot plate heated to 100°C until the dark visible spots appeared.

RESULTS

Phytochemical screening

This study confirmed the presence of diverse bioactive phytoconstituents in the whole plant. Alkaloids, tannins, and

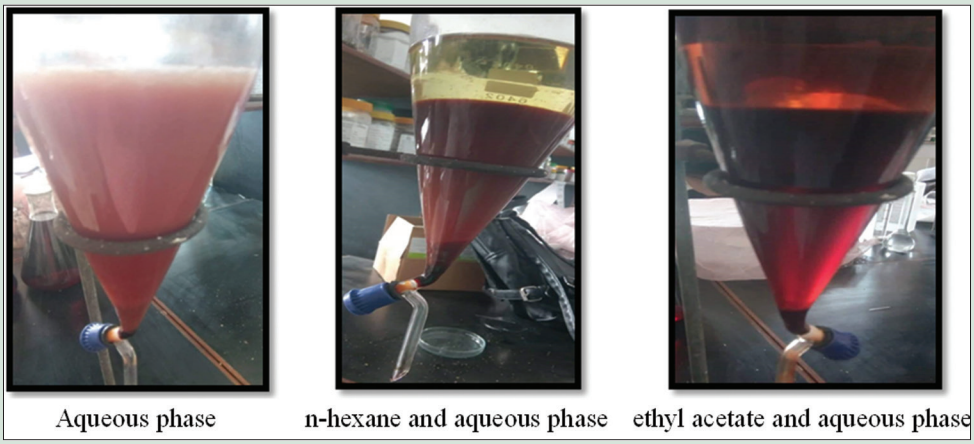

Figure 1: Fractionation of bark extract in n-hexane and ethyl acetate through separating funnel for thin-layer chromatographic analysis

flavonoids were present in bark. The leaves extract exhibited the presence of tannins, alkaloids, and steroids. In addition, the presence of flavonoids and alkaloids was detected in the flower extract. The results for the phytochemical investigation of bark, leaves, and flower are shown in Tables 1-3.

Table 1: Phytochemical screening of methanol extract of the bark

Tests	Results
Alkaloids	+
Tannins	+
Flavonoids	+
Steroids	–
Glycosides	–
Terpenoids	–

+: Indicate presence; –: Indicate absence

Table 2: Phytochemical screening of aqueous leaves extract

Tests	Results
Alkaloids	+
Tannins	+
Steroids	+
Glycosides	–
Terpenoids	–

+: Indicate presence; –: Indicate absence

Table 3: Phytochemical screening of methanolic flower extract

Tests	Results
Alkaloids	+
Flavonoids	+
Steroids	–
Terpenoids	–

+: Indicate presence; –: Indicate absence

Antibacterial activity

Different fractions (hexane, chloroform, ethyl acetate, butanol, acetone, and ethanol) of *D. butyracea* bark were screened for antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative *(E. coli)* bacteria. Significant inhibition of Gram-positive bacterial growth was observed by the entire fraction even at low concentration (1 mg). Among them, the most effective inhibition was shown by acetone fraction with a 15 ± 0 mm zone of inhibition, whereas the lowest activity was shown by ethyl acetate fraction with a 10.66 ± 0.57 mm zone of inhibition. The zone of inhibition for standard drugs at the same concentration was found to be 29 ± 0 mm. Furthermore, n-butanol fraction was found to be most effective against Gram-negative bacteria with 15 ± 1.41 mm of zone of inhibition. In contrast, hexane fraction was found to be ineffective for either Gram-positive bacterial strain. The data for standard and test samples against different bacteria strains are presented in Tables 4 and 5. Measurement of the zone of inhibition for all fractions against both strains is represented in Figures 2 and 3. The graphical representations of the zone of inhibition of different fractions against Gram-negative and Gram-positive bacteria are described in Figures 4 and 5, respectively.

Thin-layer chromatography analysis of different fractions

Hexane, ethyl acetate, and aqueous fraction from methanolic extract of *D. butyracea* bark were used for TLC analysis using different solvent systems. Major compounds were appeared in ethyl acetate fraction with many visible spots in the eluent composition hexane:ethyl acetate of 1:3

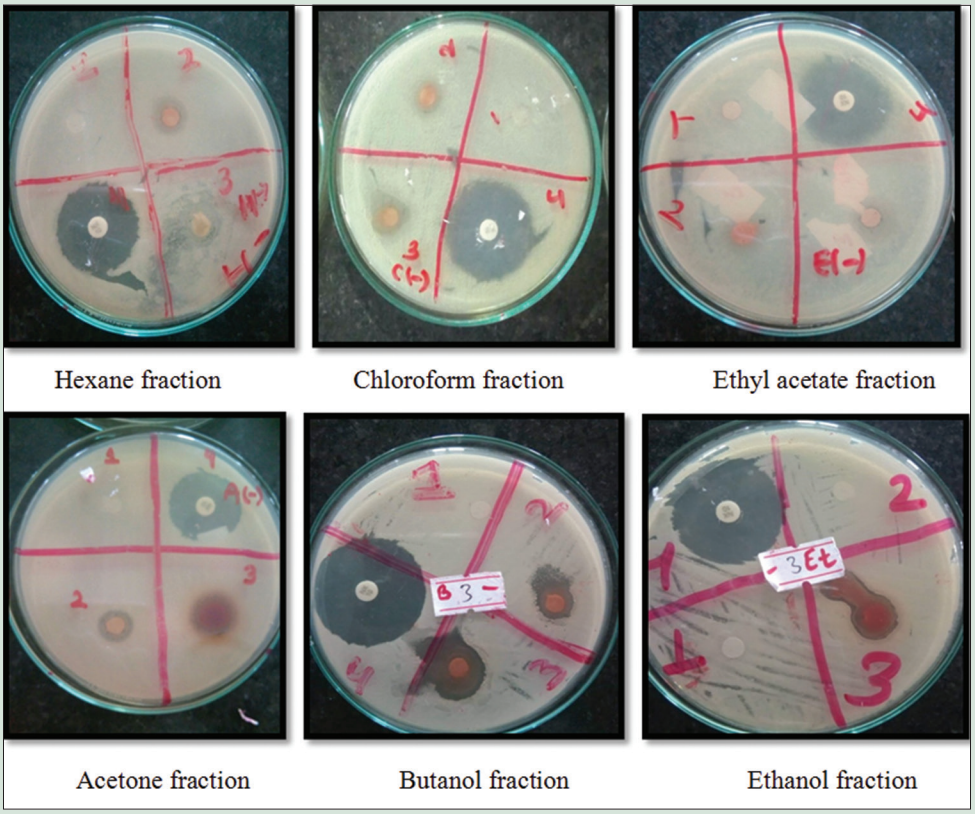

Figure 2: Measurement of the zone of inhibition from the different fraction of bark extract against Gram-negative bacteria *Escherichia coli*

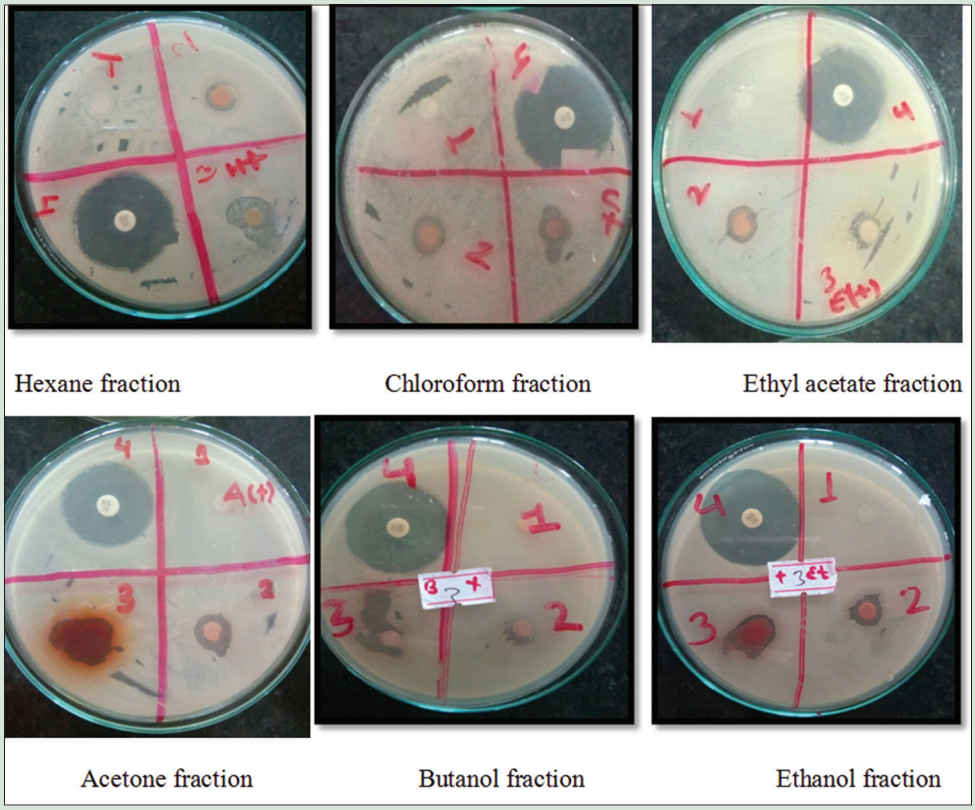

Figure 3: Measurement of the zone of inhibition of different fraction of the bark extract against Gram-positive bacteria *Staphylococcus aureus*

Table 4: Zone of inhibition of different fraction from bark extract of *Diploknema butyracea* against Gram-negative bacteria *Escherichia coli*

Solvent fraction	Standard	Test	Positive control	Negative control
Hexane	28.33±0.577	12±0.577	9.66±0.57	0±0
Chloroform	28±0	11±2.645	10.66±0.57	0±0
Acetone	29±0	15±0	10±1	0±0
Ethyl acetate	28.33±0.57	10.66±0.57	10±0	0±0
Ethanol	29.66±0.57	11.66±1.15	10.33±0.57	0±0
Butanol	29.33±0.57	13±0	10.33±1.15	0±0

Data are expressed as mean±SEM, experiment was done in triplicate.
SEM: Standard error of mean

Table 5: Zone of inhibition of different fraction from bark extract of *Diploknema butyracea* against Gram-positive bacteria *Staphylococcus aureus*

Solvent fraction	Standard	Test	Positive control	Negative control
Hexane	29±0	0±0	9±1	0±0
Chloroform	28.66±0.57	6.66±6.50	3.66±5.50	0±0
Acetone	28.66±0.57	14±1	9.66±0.57	0±0
Ethyl acetate	28±0	5±4.58	6.66±5.85	0±0
Ethanol	28.66±0.57	13.66±0.57	0±0	0±0
Butanol	28.5±0.70	15±1.41	11.5±0.70	0±0

Data are expressed as mean±SEM, experiment was done in triplicate.
SEM: Standard error of mean

after spraying with 10% v/v H_2SO_4. In the case of aqueous fraction, one major spot was detected when 1:1 ratio of chloroform and methanol was used as eluent system. However, no significant spot was detected in hexane fraction. Results for the TLC analysis of different fractions are given in Table 6 and Figure 6.

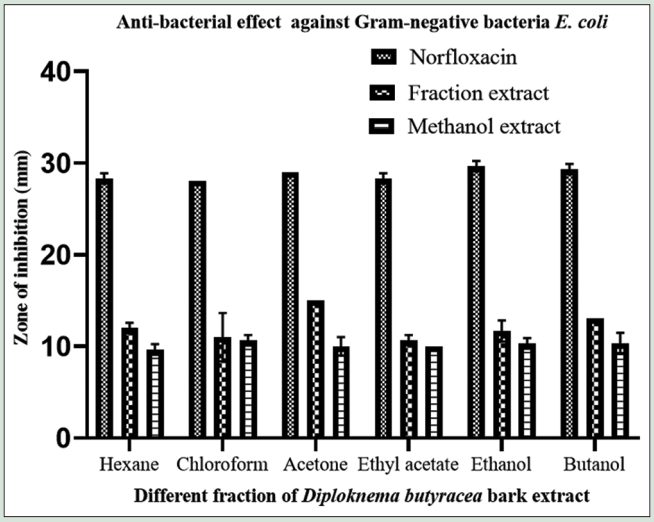

Figure 4: Zone of inhibition (mm) of *Diploknema butyracea* bark extract fractions (1 mg/disc) in the different solvent systems against Gram-negative bacteria *Escherichia coli* compared with standard antibiotic norfloxacin (10 µg/disc)

DISCUSSION

Phytochemical screening

Phytochemical screening of plants gives preliminary information about diverse classes of active secondary metabolites having a crucial role toward the beneficial physiological and medicinal activities, such as antidiabetic, antimicrobial, antioxidant, and anticarcinogenic.[18]

Phytochemical investigation of *D. butyracea* leaves, bark, and flower has not been studied till date. Our present study revealed the presence of alkaloid, tannin, flavonoids, and phenolic compounds in methanolic extracts of bark; alkaloid and flavonoid in methanolic extracts of flower; and alkaloid, tannin, steroid, and phenolic compounds on aqueous extract of leaves upon phytochemical investigation of the plant *D. butyracea*. A similar type of result was reported regarding phytochemical screening of its fruit, but fruit extract exhibited negative results for alkaloid test.[17] The secondary metabolites in plants are synthesized in plants for physiological function, and they have different useful biological activity in humans and animals. Hence, plant source has been used as herbal medicine since ancient times.[19] Secondary metabolites in plants confer them protection next to bacterial, fungal, and pesticide attacks and thus are responsible for the exertion of antimicrobial activity against some microorganisms. For example, tannins have been reported to hasten the healing of wound and inflamed mucus membrane and to seize bleeding. The tannin-containing plant extracts were used as astringents, against diarrhea; as diuretics, against stomach and duodenal tumors; and as anti-inflammatory, antiseptic, antioxidant, and hemostatic pharmaceuticals. Hence, phytochemical investigation of this plant concluded that this plant can be used as a potential source for the isolation of diverse bioactive compounds.

Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. Among the different fractions extracted from *D. butyracea*, butanol fraction (15 mm) showed maximum antibacterial activity against *E. coli* and acetone fraction (15 mm) against *S. aureus* at the concentration of 1 mg extract per disc. The fractionated extract showed more promising activity as compared to methanolic extract of *D. butyracea*. The antibacterial activity of *D. butyracea* might be due to the presence of alkaloids, tannins, saponins, flavonoids, steroids, etc.

TLC profiling of extracts gives an impressive result in directing toward the presence of several phytochemicals. Various phytochemicals give different R_f values in different solvent systems. This variation in R_f values of the phytochemicals provides a very significant clue in understanding their polarity and also helps in the selection of an appropriate solvent system for separation of pure compounds by column chromatography. Bioassay-guided isolation of compounds from ethyl acetate fraction of bark should be performed for the isolation of major bioactive compounds. This information will assist in an assortment of appropriate solvent system for further separation of the compound from these plant extracts.[20]

Table 6: R_f values for major compounds in different fraction of *Diploknema butyracea* bark extract under different thin-layer chromatographic solvent systems

Fractions	Eluent composition	Spot	R_f value
Ethyl acetate	Hex: EA: 1:1	1	0.22
	Hex: EA: 1:2	1	0.44
	Hex: EA: 1:3	1	0.75
Hexane	Hex: EA: 1:1	-	-
	Hex: EA: 1:2	-	-
	Hex: EA: 1:3	-	-
Aqueous	$CHCl_3$:MeOH: 1:1	1	0.77
	$CHCl_3$:MeOH: 1:2	-	-

Hex: Hexane; $CHCl_3$: Chloroform; EA: Ethyl acetate; MeOH: Methanol

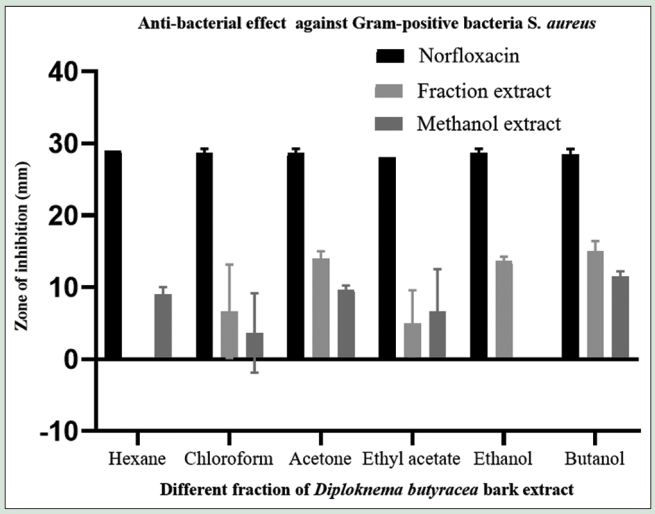

Figure 5: Zone of inhibition (mm) of *Diploknema butyracea* bark extract fractions (1 mg/disc) in the different solvent systems against Gram-positive bacteria *Staphylococcus aureus* compared with standard antibiotic norfloxacin (10 µg/disc)

CONCLUSION

The present study is a preliminary investigation for the screening of bioactive compounds and antibacterial potency of the plant, which revealed that methanolic extract of the *D. butyracea* bark can be strongly recommended for antibacterial use. The antibacterial activities of the extract from butanol and acetone fraction showed positive results for Gram-positive and Gram-negative bacteria. The TLC of the extracts

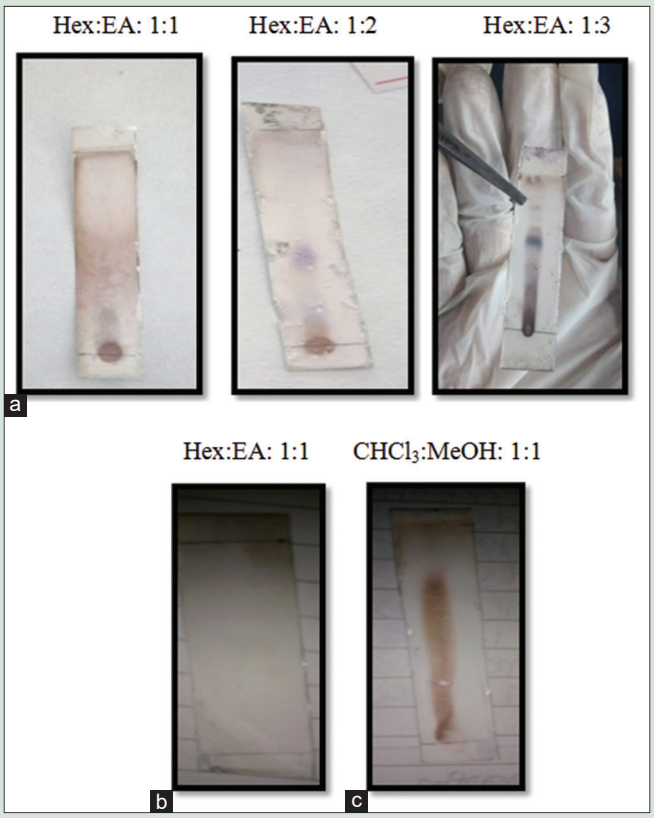

Figure 6: Thin-layer chromatographic pattern of (a) ethyl acetate, (b) n-Hexane, and (c) aqueous fraction from bark extract of *Diploknema butyracea* in different solvent systems. Hex: Hexane; $CHCl_3$: Chloroform; EA: Ethyl acetate; MeOH: Methanol

in different solvents also revealed the presence of major compounds in ethyl acetate fraction of bark extract.

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Conflicts of interest

There are no conflicts of interest.

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