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Phytochemical and Antimicrobial Screening of Bark Extract of *Shorea robusta* (Sal)

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Abstract

Different parts of *Shorea robusta* (Sal) are being used in ancestral and ayurvedic medicines and are known to cure health ailments. The different phytochemicals present in *S. robusta* is known to possess antimicrobial property. The different botanical parts of this plant have been used in ayurvedic medicines to cure certain infectious diseases. The main aim of this study was to screen phytochemicals and antimicrobial activity of bark extract of *S. robusta*. Literatures were collected through books, journals and further additional information were collected from residents and traditional ayurvedic practitioners. The ethanolic bark extract of *S. robusta* was obtained through 70% ethanol in rotatory shaker for 72 hours at 37 °C and then the crude extract was dried, preserved and analyzed for phytochemical analysis and antimicrobial activity. The phytochemical screening of ethanolic extract of bark of *S. robusta* indicated presence of phytochemicals like, alkaloids, flavonoids, tannins, steroids, anthraquinone and absence of phlobatannins, terpenoids, starch and proteins. The extract of *S. robusta* on *Staphylococcus aureus* exhibited clear zone of inhibition of 21mm at minimum inhibitory concentration (MIC) of 2 mg/mL while on *Escherichia coli* exhibited clear zone of inhibition of 9 mm at MIC of 4 mg/mL. The antimicrobial activity may be conferred due to the presence of plant phytochemicals. *S. robusta* bark extract exhibiting significant minimum inhibitory concentration and antimicrobial activity indicates the efficacy of this plant to be considered for discovering and extracting new antimicrobial products against the pathogens. These findings need further support for appropriate formulation of the drug and its therapeutic use in clinical settings.

Keywords: Antimicrobial screening, Bark extract, Minimum Inhibitory concentration, pathogens, phytochemical profile, *Shorea robusta*

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Introduction

Shorea robusta is a deciduous large tree, exceptionally reaching a height of 50 m. [1]. *S. robusta* has always been a tree of medical, cultural and economic importance and is known by common name 'Sal'. *S. robusta* belongs to family Dipterocarpaceae and is reported to possess antimicrobial properties [2]. The entire tree of *S. robusta* is used for different purposes such as timber in house construction, firewood, leaves for making leaf-plates and cups [3]. The different chemical composition of *S. robusta* plant such as Asiatic acid, triterpenic acid, tannic acid and phenol is known to possess antimicrobial property [4]. The different parts of *S. robusta* like bark, flower, resins are known to possess pharmacological action to treat diarrheal diseases, Diabetes mellitus and bacterial infections etc. [5].

Staphylococcus aureus is a pathogenic superbug that causes bloodstream infection, tissue infections and often associated with infective endocarditis [6]. Whereas, *Escherichia coli* is prominent cause of gastroenteritis, urine infection and bloodstream infections [7]. These two are the most prevalent and common cause of human infections that induce clinical mastitis and need to be correctly cured by appropriate antimicrobials for preventing and controlling emerging drug resistance and nosocomial infections [8].

Since the pathogens are emerging with drug resistance, the need for research in antimicrobial products from medicinal plants can address the issue brought by drug resistant strains in clinical settings [9, 10]. The number of infections caused by emerging drug resistant pathogens grow daily and the hospitalized patients with immunocompromised condition are more prone to severe infections [11]. There is a line of antibiotics on market, but bacterial species shows resistance to most antimicrobials used in the clinical treatment [12].

It has been documented that the bark of *S. robusta* is traditionally used as astringent, acrid, cooling, anthelminthic, anodyne, constipating, and urinary astringent, union promoter depurative and tonic [13]. They are useful in vitiated conditions of cough, ulcers, wounds, bacterial infections diarrhea, dysentery,

gonorrhea, leucorrhea, pruritus, leprosy, cough, and anemia [14]. Therefore, present study was aimed to screen and evaluate phytochemicals and antibacterial activity of crude ethanolic extracts of *S. robusta* bark extract against *S. aureus* and *E. coli*.

Material and Methodology

Research Design

This study was conducted from February 2017 to April 2017 at Microbiology Laboratory of Central Campus of Technology, Dharan. Bark of *S. robusta* was collected from Bijaypur hill forest of Dharan-14 which extends at an altitude of 390 meters (Latitude 26° 49′ 12″ N and Longitude: 87° 18′ 0″ E). The selected plants were firstly identified from herbarium collection of Postgraduate Campus Biratnagar, Nepal. The different botanical information was collected by field study, research articles and books. Medicinal, cultural and economic information about the plants was obtained from experienced traditional ayurvedic practitioners and local individuals. All the information about plants, medicinal values and uses were documented. About 500 grams bark samples were collected from the forest and were brought in Microbiology lab of Central Campus of Technology, Dharan.

Microorganisms used

The bacterial strains used in this research were *S. aureus* (25923) and *E. coli* (CFT073) strain. These microbial cultures were requested and received from Microbiology Department of Central Campus of Technology, Dharan. The growth media used were Mannitol Salt agar (MSA) (HiMedia, India) for *S. aureus* and MacConkey agar (MAC) (HiMedia, India) for *E. coli*. These selective growth medias were used for recovering the bacterial strains from preserved culture for further study. The isolated bacterial strains from selective media were further subcultured in brainheart infusion broth media (HiMedia, India).

Extract Preparation

The extraction methodology was carried out according to Agrawal and Paridhavi (2012) [15]. The bark of *S. robusta* was chopped and dried under shade at room temperature of 25 °C for two weeks. The dried bark of *S. robusta* was powdered using mortar and pestle at room temperature. About 20 grams of the powdered plants was extracted with 400 mL of 70% ethanol in rotatory shaker for 72 hours at 37 °C. The obtained extract was concentrated and dried by evaporation in hot air oven at 60 °C. Stock solutions of 32 mg/ mL was prepared in 10% sterile dimethyl sulfoxide (DMSO). The stock solution was stored at 4 °C until use. Crude powder extract or aqueous suspension of ethanolic dried extract was used in phytochemical screening.

Phytochemical Assays

Screening of the phytochemical constituents was carried out for detection of documented chemical constituents of bark extract as described by Thilagavathi et al., (2015) [17] and Harborne (1998) [18].

Test for Alkaloids

In a test tube, 2 mL ethanolic bark extract was inoculated with 2-3 drops of HCl (dilute hydrochloric acid). To this suspension about 1 mL of Dragendorff's reagent was added. The presence of alkaloids is indicated by the appearance of orange to red precipitate.

Test for flavonoids

In a test tube, 4 mL ethanolic bark extract was suspended with 1.5 mL methanol. The solution was gently heated to warm with addition of magnesium with 4 drops of Conc. HCL (Concentrated Hydrochloric acid). Development of color change is indicative for presence of flavonoids.

Test for Phlobatannins

Bark extract sample was boiled with 1% aqueous hydrochloric acid. Suspension of red precipitate is evidence for the Phlobatannins.

Test for tannins

About 2 mL of ethanolic bark extract was treated with 2-3 drops of 10% lead acetate. Tannins are indicated by the development of white precipitate.

Test for steroids

About 10 mL of chloroform was taken in a test tube and 2 mL of ethanolic bark extract was added to it. This suspension was treated with 1 mL of acetic anhydride and then 2 mL of concentrated sulphuric acid. The presence of steroids is indicated by the development of blue green color at the junction.

Test for Anthraquinone

In a test tube, dilute Sulphuric acid and 1 mL of diluted ammonia were added to 5 mL of the ethanolic bark extract. Development of pink color indicates the existence of anthraquinone.

Test for terpenoids

About 10 mL of chloroform was added to 2 mL of ethanolic bark extract. The resulting suspension was inoculated with 1 mL of acetic anhydride and 2 mL of concentrated sulphuric acid. The existence of terpenoids is indicated by development of red, pink or violet color at the junction.

Test for Starch
Benedict's test: One litre of Benedict's solution was be prepared from 100 gm of anhydrous sodium carbonate, 173 gm of sodium citrate and 17.3 gm of copper (II) sulfate pentahydrate. In a test tube, 0.5 mL of Benedict's reagent was added to 0.5 mL of the ethanolic bark extract. The suspension was heated on water bath at 100 °C for 2 minutes. The existence of starch is indicated by the development of red color precipitate.

Test for proteins
Ninhydrin Test: About 1 mL of the ethanolic bark extract was treated with 2-3 drops of Ninhydrin agent and heated in a boiling water bath. The presence of proteins is indicated by the appearance of purple blue color.

Antimicrobial Assay
Antibacterial tests were carried out by well diffusion method as described by Aneja (2009) [16]. For antibacterial bioassay, the fresh bacterial inoculum with standard turbidity of 0.5 McFarland standard for microorganism was arranged in Mueller Hinton broth (HiMedia, India) and about 100 µL culture was seeded over the Mueller Hinton agar (HiMedia, India). Mueller Hinton agar (MHA) with 2% NaCl was seeded by *S. aureus* culture and MHA without 2% NaCl was seeded by *E. coli* culture. With the cork borer no. 6, the wells of about 6mm were created in the media plates. The different test concentrations ranging from, 0.0625-16 mg/mL of bark extract was developed in 10% DMSO solution. About 50 µL aliquot of extracts with different concentrations were inoculated into the wells of MHA plates seeded by the *S. aureus* strains. Similarly, about 50 µL aliquot of extract with different concentrations were inoculated into the wells of MHA plates seeded by the *E. coli* strains. The inoculated culture medias were allowed to incubate at 37 °C for 24 hours. About 50 µL of 10% sterile DMSO solution was used for the negative control and penicillin (10 µg) and Gentamicin (10 µg) was used as the positive control. After the incubation, the plates were observed for the halo zone around the well. The halo zone around the well represented zone of inhibition which was measured and documented. The experiments were performed for three times and the mean zone of inhibition was computed.

Minimum Inhibitory Concentration
The minimum inhibitory concentration (MIC) of plant extracts was studied using 96-well microtitre plates as explained by CLSI (2012) [19]. The 96-well plates were prepared from 95 µL aliquot of Mueller Hinton Broth (MHB) (HiMedia, India) suspended in wells. In each well, 5 µL bacterial culture of 0.5 McFarland standard, prepared in MHB medium was inoculated. About 100 µL of stock extract was suspended in first well and so was the same volume of suspension serially diluted to achieve two-fold dilution ranging from 16-0.0625 mg/mL. For negative control DMSO solution was used. The microtitre plates were covered with sterile lid and incubated at 37 °C exactly for 24 hours. The minimum concentration of the extract sample, that inhibited growth of tested organism after overnight incubation, was determined as MIC.

Quality Control
Complete aseptic condition was maintained during media preparation, sample collection, sample processing. Reagents and culture media were regularly monitored for their manufacture and expiry date and proper storage. Laboratory equipment like incubator, refrigerator, autoclave and hot air oven were regularly monitored for their efficiency. The temperature of the incubator and refrigerator was monitored every day. Contamination of biological samples was prevented by performing the work in laminar flow cabinet.

Data analysis
The information collected was documented and the data would be analyzed by Microsoft Excel 2010.

Results
Phytochemical Screening
The phytochemical screening of ethanolic extract of bark of *Shorea robusta* indicated presence of phytochemicals like, alkaloids, flavonoids, tannins, steroids, anthraquinone and absence of phlobatannins terpenoids, starch and proteins **(Table 1)**.

Antimicrobial assay and MIC
The ethanolic bark extract was tested for antimicrobial activity against both bacteria *S. aureus* (25923) and *E. coli* (CFT073). The antimicrobial assay with zone of inhibition with positive controls are shown in **Table 2**.

Table 1. Phytochemicals screening of bark of *Shorea robusta*

Phytochemicals	Results	Intensity of color
Alkaloids	Present	+++
Flavonoids	Present	++
Phlobatannins	Absent	-
Tannins	Present	++
Steroids	Present	+
Anthraquinone	Present	++
Terpenoids	Absent	-
Starch	Absent	-
Proteins	Absent	-

The ethanolic bark extract of *S. robusta* on *S. aureus* exhibited clear zone of inhibition of 21 mm at MIC of 2 mg/mL. The ethanolic bark extract of *S. robusta* on *E. coli* exhibited clear zone of inhibition of 9 mm at MIC of 4 mg/mL **(Figure 1 and 2)**.

Table 2. Antimicrobial assay of positive controls

Microorganisms	Type of Bacteria	Antibiotic Zone of inhibition in mm
S. aureus	Gram +	Penicillin G (10µg)- 30 mm
E. coli	Gram -	Gentamicin (10 µg) – 17 mm

The ethanolic bark extract of *S. robusta* expressed suppressive activity on both *S. aureus* (25923) and *E. coli* (CFT073). The findings of this study provide significant in vitro antimicrobial activity of ethanolic extract of bark of *S. robusta* against *S. aureus* and *E. coli*.

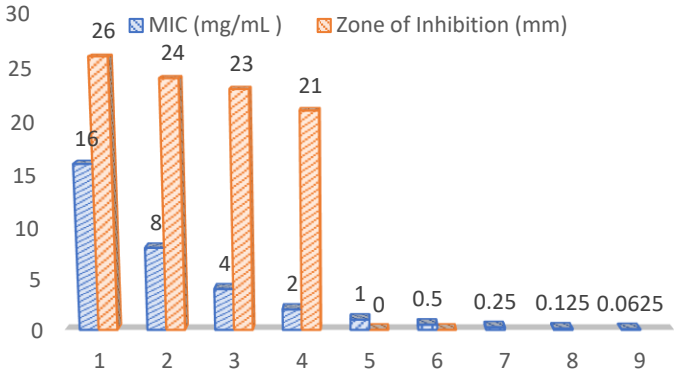

Figure 1. Antimicrobial Screening of bark extract of *S. robusta* against *S. aureus*

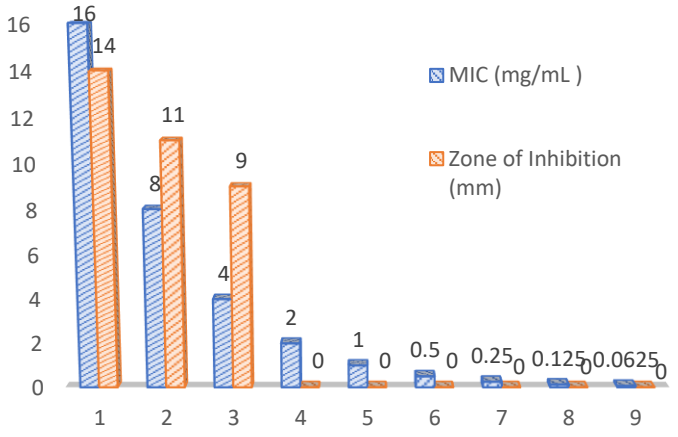

Figure 2. Antimicrobial Screening of bark extract of *S. robusta* against *E. coli*

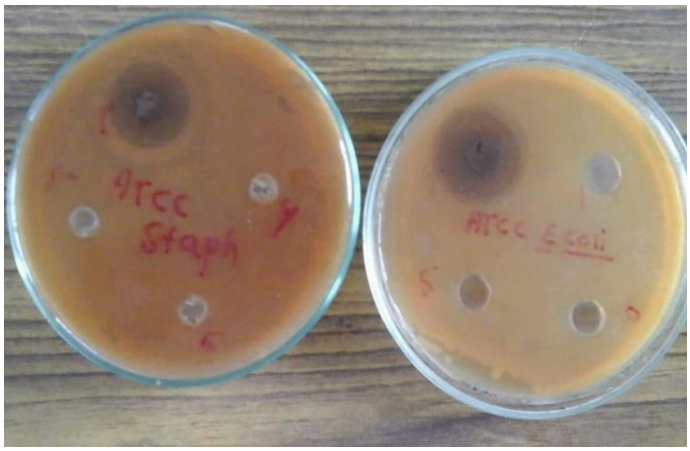

Figure 3: Antimicrobial susceptibility test by *S. robusta* extract.

Medicinal information

The medicinal, cultural, and economic information was collected from Ayurvedic practitioners and locals are included in **Table 3**.

Table 3. The medicinal, cultural, and economic information of *S. robusta*

Plant	Parts	Medicinal use	Other cultural and economic use
S. robusta	Bark	Enhance immunity power, treat typhoid, diarrhea, ulcer.	Used in incense stick
	Leaves	Reduce obesity, inhibit pain.	Biodegradable leaf plates and cups
	Resins	Lower Fever, skin disorder, diarrhea.	Used in incense stick

Discussion

Nepal has always stood a nation of natural biodiversity rich in natural vegetation that includes herbs, shrubs at different climate and altitude [20]. Many of such herbs have been used as traditional medicines by local people including some even been market as ayurvedic medicine [21]. Nowadays, pharmacology industries are also seemed interested in incorporating natural drugs since because of modern drugs imposing many health side effects with growing incidence of antibiotic resistance. The *S. robusta* which is one of the species of plant found in Nepal has been known to be best timber producing tree [22]. Its many parts have been used by local people for medicinal and cultural purposes [23].

In this study the phytochemical screening of ethanolic extract of *S. robusta* bark showed the presence of alkaloids, flavonoids, anthraquinone tannins and steroids whereas phytochemicals like terpenoids, starch, phlobatannins and proteins were absent. This result

coincides with the study performed by [2, 24] which showed the presence of common phytochemicals. Similarly, the study performed by [3] showed the absence of anthraquinone which has shown similarity with this present study. There are other many factors that affect phytochemical composition such as geographical condition, climatic condition, collection procedure, storage condition, etc. [25, 26, 27]. The effectiveness of the plant extract against selected bacterial strains may be due to the collective antimicrobial action of different phytochemical constituents [28]. The botanical biomolecules like flavonoids, alkaloids and variety of other phenolic constituents have been identified with antimicrobial properties [29].

The phytochemical screening of ethanolic extract of bark of *S. robusta* showed the presence of flavonoids, which is an antioxidant compound having antimicrobial property to suppress both certain species of Gram-positive and Gram-negative bacteria [30]. Studies have forwarded different antimicrobial mechanism of phytochemicals. Some phytochemicals are supposed to suppress growth and development of microorganism, bacterial cell membrane disarrangement, halting microbial metabolism and modifying microbial genetic expression [31]. Investigators have proved antibacterial activity of *S. robusta* extracts against bacterial pathogens [32]. In one study, *S. robusta* extract exhibited antibacterial activity against different clinical pathogens [2]. In agreement with this study even in the present study the ethanolic extract of bark of *S. robusta* exhibited significant antimicrobial activity with significant level of Minimum inhibitory concentration against both bacterial pathogens. These results clearly suggest its antimicrobial efficacy in treating the infection caused by those pathogens.

Plant extract for natural antibiotic could be a strong potent drug against many pathogenic species and could be drug of choice against emerging drug resistant strains [33]. The extended study for purification, activation and therapeutic uses of plant extracts should be conducted to examine its effective antimicrobial role against pathogenic microorganisms. If being effective the nature's best metabolite could be effective in treating infections caused by antibiotic drug resistance pathogens that have become one of the major therapeutic challenges in clinical settings.

The indigenous community of developing nations are still using the plant based traditional drugs for treating many diseases. Resins, leaves, flower and bark extracts of *S. robusta* has been identified with rich medicinal importance [34]. Potent antimicrobial drugs extracted from *S. robusta* may be safe for treating many infections and inflammations. In some Asian nations the research on antimicrobial and immunomodulatory effect of the plant has been carried out and even they have come up with excellent results on its antimicrobial and immunological property [35]. These findings will support for scientific research on this plant for pharmacological importance against nosocomial and community acquired pathogens. With new emerging infectious diseases people have been depending upon allopathic, homeopathic and ayurvedic medicines. If proper research could be carried out, then this botanical species could be used for excellent antimicrobial and immunomodulatory medicine.

In Nepal, the study has been limited to Taxonomic study and conservation. The bark of *S. robusta* is not available like that of other plant products. It is the tree which requires long tenure for its proper growth and development [36]. Therefore, scientific tissue culture, conservation and promotion can be achieved through community forest and commercial forestry. Where in context of Nepal the scientific screening and study of *S. robusta* based drugs are not adequately performed and analyzed, thus this study will help to provide lead to explore more about the pharmacological importance of *S. robusta.*

Conclusion

Ethanolic extract of *S. robusta* bark extract displayed acceptable inhibitory activity against both *S. aureus* and *E. coli*. The antimicrobial activity may be conferred due to the presence of plant phytochemicals like alkaloids, tannins, anthraquinone etc. Further development of the plant-based drugs could bring sustained drug release and would help to reduce side effects of synthetic drugs. In-vivo studies of these plant-based extracts are required for therapeutic application.

Authors Contribution

BKS and BD designed the study, participated in sample collection, extraction and processing. BKS and JS participated in literature review, sample extraction, sample processing quality control, data analysis and result interpretation. RS and SC participated in drafting and proofreading the manuscript. All five authors participated in drafting the manuscript and approved for publication.

Competing Interests

There is not any competing interest.

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Ethical Approval and Consent
Not applicable.

Abbreviations
MSA: Mannitol Salt Agar
MAC: MacConkey agar
MHA: Mueller Hinton Agar
MHB: Mueller Hinton Broth
CLSI: Clinical & Laboratory Standards Institute
DMSO: Dimethyl sulfoxide
MIC: Minimum Inhibitory Concentration
CFU/mL: Colony Forming Unit
NaCl: Sodium Chloride

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