IJBPAS, August, 2013, 2(8): 1691-1698

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF *BOSWELLIA SERRATA* AGAINST MULTI-DRUG RESISTANT BACTERIAL CLINICAL ISOLATES

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ABSTRACT

The present study was undertaken to assess the antibacterial potential of various fractions of methanolic extract of *Boswellia serrata* against multi-drug resistant (MDR) clinical isolates. Methanolic extract of *B. serrata* was prepared and subsequently partitioned with various solvents in increasing order of polarity. The extracts and fractions were evaluated for antibacterial potential to identify the most active fractions. The bioactive fractions were then subjected by thin layer chromatography and direct bioautography to validate the observed antibacterial activity. Phytochemical analysis of the various fractions demonstrated the presence of alkaloids, flavonoids, steroids and triterpenoids. The dichloromethane (DCM) fraction was identified as the most active fraction against MDR *Enterococcus* sp. The results were confirmed by direct bioautography. The antibacterial activity of *B. serrata* might be attributed due to the presence of alkaloids, flavanoids, steroids and triterpenoids. The bioautography of these n- hexane and DCM fractions showed the presence of few important chemical constituents which could serve as a promising lead against MDR bacteria.

Keywords: Antibacterial, Bioautography, *Boswellia serrata,* Multi-Drug Resistant, Phytochemical

INTRODUCTION

The emergence of multi-drug resistant (MDR) bacterial pathogens has become the focus of recent concern in human medicine **[1]**. Infections caused by resistant microbes fail to respond to treatment, resulting in prolonged illness and greater risk of death. The increasing incidence of antibiotic resistance among bacterial pathogens necessitates medicinal plants as an alternative approach for development of novel antimicrobial agents **[2]**.

Boswellia serrata (Family: Burseraceae) is a deciduous middle sized tree, which is mostly concentrated in tropical; parts of Asia and Africa. In India it occurs in dry hilly forests of Rajasthan, Madhya Pradesh, Gujarat, Bihar, Assam, Orrisa as well as central penisular regions of Andhra Pradesh, Assam etc. *B. serrata* gum resin has been reported to have analgesic, anti-inflammatory, antiarthritic and anti-pyretic activity. Boswellic acids are the major constituents of the gum derived from the plant *B. serrata*. The gum resin comprises of β-boswellic acids as the main triterpenic acid along with 11-keto-β-boswellic acids and their acetates. Raja et al. reported the antibacterial activity of acetyl-11-keto-β-boswellic acid and its inhibitory effect on biofilms generated by *S. aureus* and *Staphylococcus epidermidis* **[3]**. Methanol and aqueous extracts of *B. dalziellii* stem bark have been found to possess wide spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. The extracts of *Boswellia* species have been reported to exert anticarcinogenic, antiproliferative, antitumor, apoptotic, and cytostatic activities **[4, 5]**. The present study was undertaken to assess the *in vitro* antibacterial activity of *B. serrata* against MDR bacterial clinical isolates. The potential bioactive fractions against MDR bacteria were also identified by direct bioautography method.

MATERIALS AND METHODS

Plant Material Collection and Extraction

Oleo gum resin of *B. serrata* was procured from authorized vendor of Delhi. The plant material was authenticated by Dr. M.P. Sharma, Department of Botany, Hamdard University, New Delhi and voucher specimen was deposited in the herbarium of Amity Institute of Biotechnology, Amity University, Uttar Pradesh, Noida, India. 500 g of the plant material was extracted with MeOH:Water (9:1) at room temperature. The concentrated methanol extract of the plant was partitioned subsequently into *n*-hexane (*n*-Hex), dichloromethane (DCM), ethylacetate (EtOAc) and aqueous (Aq) fractions **[6]**. The fractions were evaporated under reduced pressure and dried using rotary evaporator below 50°C. The fractions were then assayed for antibacterial activity.

Phytochemical Analysis of the Fractions

Conventional standard protocols were performed with *n*-Hex, DCM, EtOAc and Aq fractions of methanolic extract of *B. serrata* to detect the presence of various phytochemicals as described by Rajesh *et al* **[7]**. Flavanoids, steroids, alkaloids and tannins were detected by NaOH/HCl test, Salkowsi's reaction, Dragendroff's reaction and ferric chloride test respectively. Additional tests were carried out for check the presence of reducing sugars, cardiac glycosides, anthraquinones, triterpenoids and phlobatannins.

Bacterial Strains Used and Growth Condition

The MDR clinical isolates *Escherichia coli, Staphylococcus aureus, Enterococcus sp., Acinetobacter sp*. and *Serratia sp.* along with their respective antibiotic resistance profiles **(Table 1)** were procured from Department of Microbiology, Rajiv Gandhi Cancer Research Institute, New Delhi, India**.** Standard isolates *Staphylococcus aureus* (MTCC 96) and *Escherichia coli* (MTCC 443) were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. All bacterial strains were maintained on nutrient agar slants at 4°C, and sub-cultured on to nutrient broth for 24 h prior to antimicrobial testing.

Determination of Antibacterial Activity

Antibacterial activity of plant fractions was determined by agar well diffusion method as described by Jhon J Rojas *et al* **[8]**. Nutrient agar plates were inoculated with 0.1 ml of each bacterial organism (1×10^8 CFU/ml) and spread well with sterile glass spreader. Subsequently, wells of 7 mm size were bored into the agar set plates containing the bacterial culture and filled with 50µL of the plant extract prepared from a stock solution of 1mg/mL of DMSO and water, where the proportion of DMSO was not more than 2 %. Sterilized distilled water was taken as the negative control where as standard antibiotic disc of gentamicin (30 µg) was used as positive control. The plates were incubated at 37ºC for 24 h. All tests were performed in triplicate and the antibacterial activity was expressed as the mean diameter of inhibition zones (mm) with standard deviation produced by the tested fractions.

Thin Layer Chromatography and Direct Bioautography (TLC-DB)

The *n*-Hex and DCM fractions exhibiting maximum antibacterial potential against *Enterococcus* sp. were analyzed using TLC-DB. TLC profiling was performed in accordance with the methods described by Das Talukdar *et al* **[9]**. 10 µL of 1mg/mL of these fractions were loaded on pre coated silica gel plates (TLC-grade; Merck India; 60 F_{254}). The plates for *n*-Hex and DCM fractions were developed with 70:30 *n*-Hex: EtOAc solvent system. TLC plate of each fraction was run in triplicate. TLC chromatogram A (lane 1) for *n*-Hex and TLC chromatogram A (lane 2) for DCM fractions were visualized in UV light at 254 nm and the fluorescent bands were marked. TLC chromatograms B was subjected to *p*-anisaldehyde-sulphuric acid spray reagent for detection of steroids, glycosides and phenolic compounds. Individual R_f for each spots on all TLC plates were measured.

The bacterial suspension of *Enterococcus* sp. was sprayed on TLC chromatogram C until wet. The plate was kept for incubation at 37°C for 24 h in a humid environment for the bacteria to multiply on the plate. Subsequently, the plate was sprayed with 2.5 mg/mL 2, 3, 5 – triphenyl tetrazolium chloride (TTC) and kept in incubation at 37^0C again for 4-5 h. White zones against pink background indicated the presence of antibacterial compounds in the particular zone of the chromatogram C. The R_f of the inhibition zones on plate C were compared with the R_f of reference chromatograms (plates A and B).

RESULTS

Phytochemical Analysis

Phytoconstituents of methanolic extract and its various fractions of *B. serrata* are presented in **Table 2**. The bioactive fractions, *n*-Hex and DCM showed the presence of flavonoids, alkaloids, triterpenoids and steroids. However, anthraquinones, phlobatanins, saponins, tannins, cardiac glycosides and reducing sugars were not observed in any of the tested fractions.

Antibacterial Activity

Results of antibacterial activity of various fractions of methanolic extract of *B. serrata* revealed that *n*-Hex, DCM and EtOAc fractions inhibited the growth of *S. aureus* and *Enterococcus* sp. **(Table 3)**. Furthermore, only EtOAc fraction was the active fraction against *Acinetobacter* sp. However, *Serratia* sp. was resistant to all the fractions. The negative control plate containing sterilized distilled water did not exhibit inhibition on the tested bacteria where as standard gentamicin antibiotic discs produced significantly larger inhibition zones.

Thin Layer Chromatography and Direct Bioautography (TLC-DB)

Plate A is the chromatogram showing separation of the components of *n*-Hex and

DCM fractions of *B. serrata* developed with 70:30 *n*-Hex: EtOAc visualized under UV at 254 nm. The components of both these fractions separated into one major spot and several minor spots. Plate B is the chromatogram sprayed with *p*-anisaldehyde-sulphuric acid reagent for the detection of steroids, glycosides and phenolic compounds. All of the spots stained positive for phenolic compounds. During TLC-DB, both the *n*-Hex and DCM fractions showed the presence of one clear zone with R_f value 0.67 and one large inhibition zone with R_f value ranging from 0.15- 0.37 against *Enterococcus* sp. **(Figure 1)**. The R_f values of the clear zones of inhibition in The TLC bioautography plate C corresponds to the spots with the same R_f values on the TLC plate B being sprayed by *p*-anisaldehyde-sulphuric acid reagent.

DISCUSSION

The results in the present study are in agreement with the findings of Abdallah *et al*., **[10]** that prove the presence of saponins and alkaloids in the EtOAc and MeOH extracts of oleo-gum resin of *B. papyrifera*. Preliminary phytochemical analysis also showed the presence of flavonoids, steroids, cardiac glycosides and triterpenoids in the various fractions of *B. serrata* **(Table 2)**. The presence of these phytoconstituents is thought to be responsible for the observed antibacterial activity. Our study has demonstrated varied antibacterial activity of one or more fractions of *B. serrata* against all the tested organisms except *Serratia* sps. which was found to be resistant to all the fractions **(Table 3)**. This correlates with the observation of Camarda *et al* [11] who reported that *Boswellia* species oleo-gum resins demonstrated antibacterial activitiy against methicillin resistant *S. aureus* (MRSA), *P. aeruginosa* and *E. coli*. In another study, the methanolic extracts of *B. elongate* and *B. ameero* have been reported to possess antibacterial activity against gram-positive bacteria including MDR *Staphylococcus* strains **[12]**. Further, the antibacterial activity demonstrated by agar well diffusion method is in line with the TLC-DB results **(Figure 1)**. Most of the data available for this plant are from essential oils extracted from this plant or from crude acetone and methanol based extracts. However, the present study has been carried out with the various fractions derived from methanolic extract.

CONCLUSION

The results of our present study are helpful in identifying potentially bioactive fractions from *B. serrata* which can be used as therapeutic agents against MDR bacteria. The antibacterial activity of the fractions might be attributed due to the presence of alkaloids, flavanoids, steroids and triterpenoids. The bioautography of these n- hexane and DCM fractions showed the presence of few important chemical constituents which could serve as a promising lead against MDR bacteria.

ACKNOWLEDGEMENTS

The authors express their sincere gratitude to Dr. Sabari Ghosal, Professor, Amity Institute of Biotechnology, Amity University, Noida for her constant guidance and encouragement.

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Table 1: Antibiotic Resistance Profiles of Multi-Drug Resistant Clinical Isolates

Antibiotics	*E. coli*	*S. aureus*	*Enterrococcus* sp.	*Serratia* sp.	*Acinetobacter* sp.
Amikacin	S	S	R	S	R
Ampicillin	R	-	-	R	-
Ciprofloxacin	R	S	R	R	R
Ceftriaxone	R	S	R	R	-
Chloramphenicol	R	-	-	R	-
Gentamicin	S	S	R	R	R
Imepenem	S	S	R	S	R
Levofloxacin	R	S	R	R	-
Meropenem	S	S	R	S	R
Nalidixic acid	R	-	-	-	-
Nitrofurantoin	S	-	-	-	-
Norfloxacin	R	-	-	-	-
Ofloxacin	R	S	R	R	-
Piperacillin	R	S	R	S	R
Vancomycin	-	S	R	-	-
Tobramycin	R	-	-	R	R

NOTE: (R): Resistant; (S): Sensitive

Table 2: Phytochemical Screening of Methanol Extract and Fractions of the Plant *Boswellia serrata* as Described by Rajesh *et al* [7]

Phytoconstituents	*n*-Hex	DCM	EtOAc	MeOH	Aq.
Flavonoids	-	+	+	+	+
Alkaloids	+	+	+	+	-
Reducing Sugars	-	-	-	-	-
Steroids	+	+	+	+	+
Tannins	-	-	-	-	-
Anthraquinones	-	-	-	-	-
Cardiac Glycosides	-	-	+	+	+
Triterpenoids	+	+	+	+	+
Phlobatanins	-	-	-	-	-
Saponins	-	-	-	-	-

NOTE: (+): Presence; (-): Absence

Table 3: Antibacterial Activity of the Methanolic Extract and Plant Fractions of *Boswellia serrata* Expressed as Zone of Inhibition in mm (Mean ± SD of three Assays)

Bacterial Strains	*n*-Hex	DCM	EtOAc	Aqueous
Enterococcus sp.	12± 0.8	15± 0.2	10± 0.6	8±0.5
E. coli	12± 0.9	10± 0.8	-	-
Acinetobacter sp.	-	-	9± 0.6	-
S. aureus	10± 0.3	8± 0.2	8± 0.7	-
Serratia sp.	-	-	-	-

NOTE: Inhibition zone in mm includes diameter of the borer (7mm); * 50 µL of 1 mg/mL of the extracts were poured into 7 mm diameter agar wells and zone of inhibition diameter was noted after incubation at 37º C for 24 hours; (-): No inhibition

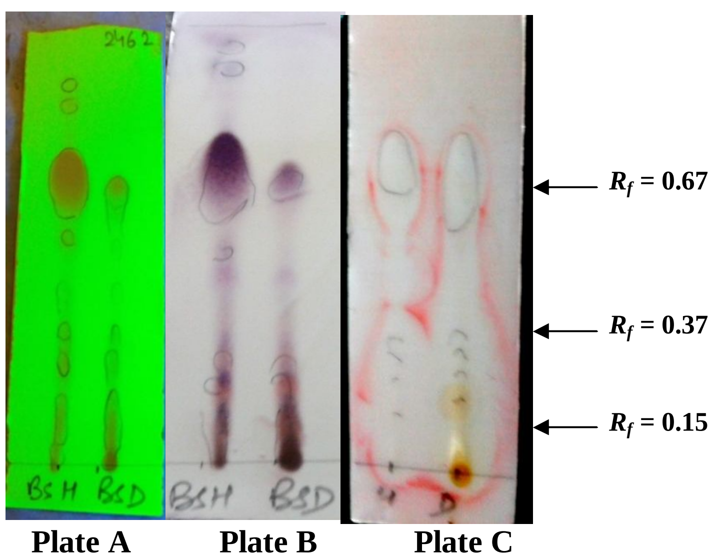

Figure 1: TLC Chromatogram as Visualized Under UV (Plate A), TLC Chromatogram Sprayed with *p*-anisaldehyde- Sulphuric Acid (Plate B) and Bioautogram for *n*-Hex and DCM Fraction Against *Enterococcus sp.*(Plate C). 10µL of Sample was Loaded on TLC-Grade; Merck India; 60 F_{254} and Developed with 70:30 *n*-Hex: EtOAc Solvent System. White Zones Against Pink Background Indicated the Presence of Antibacterial Compounds in the Particular Zone of the Bioautogram