Article

by Nabeela Zahid

In vitro and *in silico* validation of antibacterial potential of *Pinus roxburghii* and *Cedrus deodara* leaves' extract against human pathogenic bacteria

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Abstract

The emergence of resistant pathogenic bacterial strains has threatened the human beings and already developed remedial measures. Based on the traditional herbal therapeutic history, present study is aimed to assess *in vitro* and *in silico* inhibition potential of leaves extracts of *Pinus roxburghii* and *Cedrus deodara* against human pathogenic bacteria *Staphylococcus aureus*, *Salmonella typhi* and *Pseudomonas aeruginosa*. Hexane, methanol and acetone extracts of both plants were evaluated against above mentioned bacterial strains employing agar well diffusion technique. While docking analyses were performed to analyze the interaction of vital bioactive compounds and bacterial virulence proteins to get an idea about potential candidates for drug discovery. Both plant extracts exhibited greater antibacterial activities against *S. aureus* as compared to *S. typhi* and *P. aeruginosa*. The activity of different extracts also portrayed the role of polarity of solvent and compound to be extracted in each solvent i.e., activity of hexane extract > methanol > acetone with some variations. MIC (minimum inhibitory concentration) values of *P. roxburghii* extracts were less than that of *C. deodara* against tested strains, while variation was observed in MBCs (minimum bactericidal concentrations). Furthermore, molecular docking of studied plants bioactive compounds and bacterial proteins showed strong interactions (binding affinity) i.e., taxifolin > nortrachelogenin > bisabolene > valencene > caryophyllene. Antibacterial efficiencies of *P. roxburghii* and *C. deodara* suggested their application as effective therapeutic agents against diseases caused by mentioned bacterial strains. *In silico* analysis suggests the isolation and usage of bioactive components as potential antibacterial agents/drugs after further experimentations on animals.

Key words: Gymnosperm, antibacterial, *P. roxburghii, C. deodara,* MIC, MBC

1. Introduction

The emergence and spread of antibiotic resistance in pathogenic bacterial strains has substantially threatened the present-day remedial measures (Manandhar et al., 2019). Multidrug resistant bacterial infections mainly increase cost of treatments and mortality rate. There are limited and expensive therapeutic preferences for these infectious agents with significant adverse effects (Nakagawa et al., 2016). The present-day exigence persuaded human to evaluate novel natural antimicrobial drugs with less side effects and greater efficiency (Salem et al., 2014). Therefore, current study was designed to employ plant extracts (*P. roxburghii* and *C. deodara*) for their antibacterial activity.

P. roxburghii (Chir pine) is one of the substantial pine species of Indo-Pakistan coniferous forests (Sadeghi et al., 2016). *P. roxburghii* is reported to have anti-inflammatory, hepato-protective antibacterial and anticonvulsant (Kumari et al., 2017). The beneficial properties of pine needles have also been portrayed in individuals with diabetes, rheumatism, obesity, cardiovascular diseases, liver and stomach infections, chronic bronchitis and cancer (Saad et al., 2017). Bark extracts of pines portrayed anti-mutagenic, anti-carcinogenic, anti-aging, anti-inflammatory and high antioxidant properties (Sood, 2018).

C. deodara (deodar), member of family *Pinaceae* is of immense ethnobotanical and therapeutic importance (Kumar et al., 2013). In China, *C. deodara* is one of the extensively exploited traditional medicinal herbs having anticancer effects with additional therapeutic capacities in relieving itches, removing dampness and destroying parasites (Shi et al., 2016). Needles have anti-inflammatory, anti-rheumatic and antimicrobial activities (Buneri et al., 2019).

Based on above findings *in vitro* antibacterial activity analyses of *P. roxburghii* and *C. deodara* crude extracts were evaluated against human pathogenic bacterial strains. In addition, to support results of this study, *in silico* interaction between bioactive compounds (already identified in crude extracts of selected plants) and virulent proteins of selected pathogenic bacterial strains was conducted to evaluate bioactive compounds that could be responsible for inhibition of pathogenic strains.

2. Materials & Methods

2.1. Sampling and extract preparation

The fresh leaves of *P. roxburghii* and *C. deodara* plants were collected from Khyber Pakhtunkhwa Forest Department and Billion Tree Tsunami Afforestation Project Nursery Abbottabad, respectively. Selected plants were identified by Dr. Arshad Mehmood Abbasi who is a plant taxonomist at Department of Environmental Sciences, CUI Abbottabad Campus, Pakistan. Voucher specimens of *P. roxburghii* and *C. deodara* were CUHA-346 and CUHA-21 respectively. Collected leaves were washed properly with distilled water followed by shade drying for 4-5 weeks (25°C) to prepare their crude extract using standard methodologies with some modifications (Bhattacharjee et al., 2006). Dried leaves were ground into fine powder and soaked in hexane, methanol and acetone solvents. 10 g of fine powder from both plants was soaked in 200 ml of each solvent in separate glass bottle placed on shaking incubator for overnight and finally filtered with Whatman No. 1 filter paper. The process of soaking the residues in distinct solvents, overnight incubation, and filtration to attain a clear filtrate was repeated 2 times and the resultant filtrates were evaporated and dried under reduced pressure at 30-40°C using rotary vacuum evaporator (Büchi® rotary evaporator Model R-200). Extracts were dried further using lypholizer and their yields were weighed and placed in air tight vials for future use. Percentage yields were calculated with following formula: Extract yield = R/S × 100 R = Weight of extract, S = Weight of plant raw material (Mostafa et al., 2018)

Sample dilutions were prepared by dissolving different required quantities of dried powder in 1 ml of dimethyle sulphoxide (DMSO).

2.2. Determination of antibacterial activity

To assess the effectiveness of selected plant extracts, *S. typhi* (ATCC 6539), *P. aeruginosa* (ATCC 9027) and *S. aureus* (KX262679) were collected from National University of Sciences & Technology, Pakistan. Antibacterial activity was evaluated employing agar well diffusion assay (Sen and Batra, 2012). Autoclaved nutrient agar media was poured in petri plates and placed in incubator (25°C) for 3-4 hours. Bacterial suspensions were prepared in autoclaved dH_2O to get 0.5 OD at 600 nm (107-108 CFU/ml). 20 μl of each bacterial suspension was spread in petri plates and then wells were made with 6 mm cork borer, 30 μl of plant extract was poured in each well and plates were incubated at 37°C for 18 h. Streptomycin (30 μg/well) and DMSO were also used as positive and negative control respectively. The diameter of clear zones of inhibition were measured as a sign of antibacterial activity (Chauhan et al., 2013).

2.2.1. Determination of MICs and MBCs

MICs were evaluated for those effective plant extracts which displayed antimicrobial activity at concentration of 50 mg/ml. While MBCs were determined for the lowest concentrations of plant extracts which did not exhibit any visible growth by streaking them on fresh media (Rehman et al., 2018).

2.3. Molecular docking analysis

To authenticate the outcome of *in vitro* antibacterial activity of crude extracts, *in silico* study was conducted to investigate the interaction of bioactive compounds in the targeted plant species with virulent proteins i.e., cysteine and serine proteases of selected bacterial strains. Corresponding 3D structures of the protein targets were obtained from RCSB Protein Data Bank. Bioactive compounds were actually selected from already available GC-MS data of studied extracts using aforementioned solvents. Ligand molecules were obtained from online database ZINC15 *http://pubs.acs.org/doi/abs/10.1021/acs.jcim.5b00559*. Docking analysis was done using online tool CB-Dock and ligand-protein binding features were analyzed in Discovery Studio 4.1 (Dassault Systems Biovia) (Sampangi-Ramaiah et al., 2020).

2.4. Statistical analysis

Antibacterial activity was done in triplicates and the data was presented as Mean ± Standard deviation. Tukey's HSD post hoc test following One-way ANOVA was carried out to investigate the significant difference in antibacterial activity of different concentrations of both plant extracts.

3. Results

3.1. Antibacterial activity of plant extracts

Investigated extracts of *P. roxburghii* and *C. deodara* displayed potential effectiveness in suppressing pathogenic bacterial growth. Variation in activity was observed which might be due to different type of pathogenic organisms and types of extracts. Overall, zones of inhibition for all the selected extracts ranged from 10.3-17.7 mm, 9.7-19.3 mm and 9.7-18.7 mm against *P. aeruginosa*, *S. aureus* and *S. typhi* respectively.

P. aeruginosa was revealed as the most resistant strain to plant extracts followed by *S. typhi* while *S. aureus* was the most susceptible strain to the plants extracts. *P. roxburghii* extracts showed more activity than *C. deodara* extracts against *P. aeruginosa, S. aureus* and *S. typhi* while a little variation in zones of inhibition was observed by *C. deodara* hexane extract against *S. typhi* which showed larger zones of inhibition. Maximum zone of inhibition (17.7 mm) against *P. aeruginosa* was shown by *P. roxburghii* acetone extract at concentration of 100 mg/ml, while hexane and methanolic extracts (100 mg/ml) of *P. roxburghii* depicted maximum activity against *S. aureus* by showing zones of inhibition of 19.3 mm. Similarly, *S. typhi* was greatly inhibited by *P. roxburghii* methanol extract (100 mg/ml) with zones of inhibition of 18.7 mm. Streptomycin displayed zone of inhibition of 19.7±0.6 mm against *P. aeruginosa* and *S. typhi* each, while 26.7±0.6 mm of zone of inhibition against *S. aureus*. DMSO did not show any zone of inhibition (Fig. 1-3).

Hexane extracts (100 mg/ml) of *P. roxburghii* and *C. deodara* depicted greater activity against *S. aureus* followed by *P. aeruginosa* and *S. typhi* (Fig. 1 a & b). Methanol extracts (100 mg/ml) of both plants showed different trend of antimicrobial activity. *P roxburghii* methanol extract inhibited *S. aureus* greater than *S. typhi* and *P. aeruginosa,* while *C. deodara* methanol extract portrayed larger zones of inhibition against *S. typhi* than other studied strains (Fig. 2 a & b). Acetone extract (100 mg/ml) of *P. roxburghii* inhibited *P. aeruginosa* followed by *S. typhi* and *S. aureus,* while *C. deodara* acetone extract (100 mg/ml) showed opposite trend of activity with maximum inhibition against *S. aureus* followed by *S. typhi* and *P. aeruginosa* (Fig. 3 a & b).

3.1.1. MICs and MBCs of the effective plant extracts

The inhibitory effects of *P. roxburghii* hexane, methanol and acetone extracts were started to be visualized at 25, 30 and 30 mg/ml with inhibition zones of 12.3±0.6, 13.7±0.6 and 15.3±0.6 mm against *P. aeruginosa* strain respectively. Likewise, *P. roxburghii* hexane extract showed MIC of

1 mg/ml against *S. aureus*. The growth of *S. typhi* was suppressed by all tested strains of *P. roxburghii* at minimum concentrations of 10 mg/ml (Table 1, Fig. 1-3).

C. deodara extracts showed potentially less bacteriostatic activity against *P. aeruginosa* which was proved to be more resistant, and its MIC in hexane, methanol and acetone extracts reached to 30, 50 and 50 mg/ml respectively. *C. deodara* hexane extract suppressed growth of *S. aureus* and *S. typhi* at MIC of 1 and 10 mg/ml correspondingly (Table 1).

P. roxburghii hexane, methanol and acetone extracts showed potential bactericidal activity against *P. aeruginosa* with MBC value of 30 mg/ml for each extract while their MBC against *S. aureus* reached to 1, 20 and 20 mg/ml individually. *P. roxburghii* hexane, methanol and acetone extracts showed MBCs of 20, 20 and 10 mg/ml against *S. typhi*. Likewise, MBCs of *C. deodara* hexane, methanol and acetone extracts were 30, 50 and 50 mg/ml respectively against *P. aeruginosa* which was proved to be more resistant. *C. deodara* hexane, methanol and acetone extracts against *S. aureus* showed MBC of 10, 10 and 50 mg/ml respectively. MBCs of *C. deodara* extracts against *S. typhi* were observed to be 20, 30 and 50 mg/ml.

3.2. Molecular docking analysis

Docking analysis of monoterpenoids in hexane extract of both plants showed same pattern of inhibition potential as that of plants crude extracts i.e., *P. aeruginosa*>*S. typhi*>*S. aureus* by showing greater binding affinity. While sesquiterpenoids showed greater interaction with *S. aureus* with binding affinity ranged between -5.1 to -7.7 kcal/mol. Bioactive compounds of methanol extracts showed some variation in interaction and binding affinity with corresponding crude extracts activity. However, components of acetone extracts followed *in vitro* antibacterial activity pattern by showing greater binding affinity with *P. aeruginosa* and *S. aureus* respectively (Fig. 4-6)

4. Discussion

Microbial infections always posed a threat with high morbidity and mortality in immune-compromised individuals, but the discovery of alternate traditional medicines is of prime importance to eliminate microbial infections and limit the use of toxic synthetic antibiotics. *S. aureus* is causative agent of skin infections and food borne diseases while gastroenteritis diseases in humans are caused by metabolites and toxins produced by *S. typhi* and *P. aeruginosa* (Siddiqui et al., 2009). Selected extracts portrayed potential antimicrobial activity against studied strains signified the occurance of maximum bioactive components in extracts of both plants.

Zafar et al. (2010) reported that oil extracted from pinus species inhibited the growth of *S. aureus* but has no inhibitory effect on *S. typhi,* like our current findings in which greater antimicrobial activity of *P. roxburghii* leaf extracts was observed against *S. aureus* than *S. typhi*. The constituents in respective crude extracts cause disruption of microbial cell membrane by interacting with its proteins and enzymes. A flux of protons disperse towards cell exterior might obstruct enzymes essential for amino acid synthesis or induce cell death (Burt, 2004).

Hexane extract of both plants showed greater inhibition against *S. aureus* and *P. aeruginosa* than *S. typhi* while variable potency was observed by *C. deodara* extract to inhibit *P. aeruginosa* (Fig. 1). These extracts comprised of monoterpenoids, diterpenoids and sesquiterpenoids which play key part in antimicrobial activity by posing toxic effects on structure and functions of bacterial membrane (Tsvetkov et al., 2019). The possible reason of showing a little variation in activity of both extracts could be the absence/less quantity of specific components in hexane extract of *C. deodara* due to which *P. aeruginosa* showed resistance at low concentrations and its high concentrations are required to inhibit aforementioned bacterial strain. Concentration of bioactive compounds might be different in both extracts due to two different plant species, their age and growth environment as *C. deodara* plants were younger than *P. roxburghii* and both were collected from different locations (Yadav et al., 2017). Likewise, methanolic extracts of *P. roxburghii* inhibited *S. aureus* and *S. typhi* greater than *P. aeruginosa*. Whereas *C. deodara* methanolic extract inhibited *S. typhi* followed by *S. aureus* and *P. aeruginosa* (Fig. 2). Previous study showed the presence of α-terpineol, linalool, limonene, anethole, caryophyllene and eugenol as bioactive components in methanolic extracts which are involved in dysfunction and disruption of the membrane, outflow of cytoplasmic constituents which lead to bacterial cell death (Gupta et al., 2011). *P. roxburghii* acetone extract prominently suppressed *P. aeruginosa and S. typhi* than *S. aureus* against which *C. deodara* extract showed action to a greater extent (Fig. 3). Literature presented 64.3% of the acetone extract of *P. roxburghii* constituents secoisolariciresionol and nortrachelogenin, while *C. deodara* acetone extract consists of sesquiterpene, flavanoids, alkaloids, tannins, ferulic acid and beta-glucoside which are mainly involved in destruction of bacterial cytoplasmic membrane (Thapa et al., 2018). Greater activity of extracts against gram negative bacteria may be due to the presence of porin channels in their outer membrane, which facilitate transport of low-molecular-weight constituents, and lipophilic drugs have trouble to cross these channels (Guimarães et al., 2019).

Hexane extracts of both plants showed less MICs against tested strains than methanol and acetone extracts, which might be due to the polarity of solvent. Other causes of variation in current MICs could be extracted constituents, extraction techniques and bacterial strains (Chaudhary et al., 2014).

Docking analysis of monoterpenoids showed more binding affinity with *P. aeruginosa* while sesquiterpenoids displayed greater interaction with *S. aureus* (Fig. 4, Table 2). The most common interacting amino acids in docked complexes of monoterpenoids and sesquiterpenoids were ALA, GLY, SER and LYS, ALA, VAL and PRO respectively (Fig. 4). Docking analysis of some of the components of methanol extracts showed similar binding pattern with bacterial proteins as that of crude extracts, but others showed some variation. Common interacting aminoacids in docked complexes were ALA, VAL, LYS, ARG and LEU with conventional hydrogen bonds, Van der waals and Pi-alkyl interactions (Fig. 5, Table 2). *In silico* activity confirmed greater interaction of nortrachelogenin with *P. aeruginosa* and Taxifolin with *S. aureus* which make them ideal inhibitory components (Fig. 6, Table 2). Taxifolin from *C. deodara* acetone extract exhibited strong interaction with maximum binding affinity of -8.3 kcal/mol with *S. aureus* proteases. Interacting aminoacids involved in this docking complex were GLU, THR, ASN and LYS with conventional hydrogen bonds, Van der waals and Pi-alkyl interactions (Fig. 6B). Thus, flavonoids mainly taxifolin followed by Nortrachelogenin, bisabolene, valencene and caryophyllene proved to be potential candidates for possessing antibacterial activity as documented by Brown et al., 2015. Active compounds having antimicrobial activities restrain pathogenic bacterial strains by targeting significant constituents of bacterial metabolism including protein synthesis, cell wall, RNA polymerases. DNA gyrase & proteases. These bacterial constituents have direct impact in virulence by degrading virulence regulators and resisting adverse conditions in host (Human). In view of this, *in silico* analysis was done to identify the binding interaction of bioactive compounds present in studied crude extracts with the pocket of bacterial proteases. *In silico* analysis gave an idea to isolate and purify those components from crude extracts that showed strong interaction with bacterial virulence proteins and their usage as natural drugs against antibiotic resistant bacteria after more experimentation on animals.

5. Conclusion

Based on current findings, it could be concluded that all the tested extracts of selected plants possess the potential antibacterial activity which can be enhanced by increasing extracts concentration. *P. roxburghii* extracts having more antimicrobial potential than *C. deodara* extracts could be used as effective therapeutic agents against all tested strains and diseases instigated by them, limiting the use of health hazardous chemically synthesized antibacterial agents. Docking analyses further suggested the possible usage of selected natural compounds of *P. roxburghii* and *C. deodara* that showed strong interaction with bacterial virulence proteins could be isolated in purified form for potential drugs synthesis in future after *in vivo* experimentation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure and table legends

Figure 1: Antibacterial activity of (a) *P. roxburghii* and (b) *C. deodara* hexane extracts

Figure 2: Antibacterial activity of (a) *P. roxburghii* and (b) *C. deodara* methanol extracts

Figure 3: Antibacterial activity of (a) *P. roxburghii* and (b) *C. deodara* acetone extracts

Figure 4: Docking analysis of bacterial proteases with A) monoterpenoids - Linalool (a, b, c), Ocimene (d, e, f) and B) sesquiterpenoids - Bisabolene (a, b, c), Valencene (d, e, f).

Figure 5: Docking analysis of bacterial proteases with A) Limolene (a, b, c) Eugenol (d, e, f) and B) Anethole (a, b, c), Caryophyllene (d, e, f).

Figure 6: Docking analysis of bacterial proteases with A) Nortrachelogenin (a, b, c) and B) Taxifolin (a, b, c).

Table 1: MICs of the plant extracts against human pathogens

Table 2: Binding affinity of bioactive compounds with bacterial proteases

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