Research Article

In-Vitro Antimicrobial Analysis of Aqueous Methanolic Extracts and Crude Saponins Isolated from Leaves and Roots of *Sarcococca Saligna*

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Abstract | In order to overcome antibiotic drugs resistance, plants provide source for discovery of new antimicrobial drugs. This study was carried out, include *in-vitro* antibacterial and antifungal screening of aqueous methanolic extracts as well as crude saponins isolated from leaves and roots of *Sarcococca saligna*, using disc diffusion method. The tested bacterial strains include, *B. subtilis, E. coli, S. aureus, P. fluorescens* and fungal strains were *Aspergillus. niger, A. flavus, and D. turcica.* The maximum zones of inhibition, 23.00±0.56 mm and 25.00±0.50 mm was given by ethyl acetate fraction of leaves against *P. fluorescens and A. niger* respectively. The crude saponins isolated from leaves did not give significant results against the tested bacterial strains where as it was significantly active against fungal strains. The results were compared to standard drug, Ciprofloxacin and Fluconazole which gave 30.0±0.0 mm and 28.0±0.0 mm zone of inhibition respectively. The phytochemical analysis of leaves and roots extracts revealed the presence of alkaloids, saponins, cardiac glycosides, tannins, phenols and flavonoids etc. It is concluded that *S. saligna* crude extracts as well as crude saponins exhibit broad antimicrobial spectrum against various disease-causing microbes.

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Introduction

Medicinal plants and their secondary metabolites have been use since ages for the ailment of different diseases. Furthermore, they also provide us shelter, food and oxygen. The medicinal use of plants has greatly increased throughout the world since last decade (Cimanga Kanyanga et al., 2018). Plants synthesize secondary metabolites for the purpose of different functions including protection against predators and growth hormones as source of energy etc. hence provide us as a source of chemotherapeutic chemical constituents (Rosenthal and Berenbaum, 2012). Use of plants as medicine both for humans and veterinary ailments is well known practice in rural areas since ages (Aziz, Khan, Adnan and Ullah, 2018). Since the past decade traditional medicines are in demand by local people as well as local practitioners. People in developing countries greatly depend upon plants for their health care against infectious diseases

(Edziri et al., 2018). Their popularity is mainly due to fewer side effects, easily availability and cost effectiveness as compared to modern synthetic medicines. According to WHO, there is about 80% of world population rely on plant medicines for their health care (Aziz, Adnan et al., 2018). Out of 2,50,000 medicinal plant species worldwide only seventeen percent of these have been medicinally examined so far (Mamedov, 2012). Among these, 8000 medicinally active plant species are widely distributed in South Asia and about 2000 species are found in Pakistan. There are almost 50.000 herbal practitioners in Pakistan. Almost 60 to 85% of Pakistanis depends upon alternative medicines rather than allopathic medicines (Gill, 2003; Murad, Ahmad, Gilani and Khan, 2011; Shinwari, Khan, Naz and Hussain, 2009; Zaidi, 1998). *Sarcococca saligna* (D.Don) Muell is a dicotyledonous and an evergreen shrub with scaly buds, belongs to genus *Sarcococca* of family Buxaceae (having 4 genera and approximately 100 species), largely found in Himalaya region, also present in Swat, Northern areas of Khyber Pakhtunkhwa, Murree Hills and Kashmir (Srinagar), at an altitude of 5000-9000 feet (Atta-Ur-Rahman, Choudhary, Khan and Iqbal, 1998; Feroz et al., 2004). This specie is also widely located throughout the Himalyian region extended towards Afghanistan (Khalid, Ghayur, Feroz, Gilani and Choudhary, 2004). In local language of Hazara division i-e Hindko it is known as "Seela". In English it is called "Sweet box or Christmas box" (Gilani, Ghayur, Khalid and Choudhary, 2005). Traditionally this specie is used for several disorders including bacterial infections, ulcer, G.I.T disorders, ulcer, fever, malaria, rheumatism and possesses potent immunosuppressive and antidiabetic activities; Ali et al., 2015; Khalid, Anjum, Khan and Choudhary, 2002; Shazia, Afgan and Khan, 1997; Yousuf, Musharraf, Iqbal, Adhikari and Choudhary, 2011). Its arial parts are boiled and applied on swollen joints (Flora.o.Pak., 1972). The extracts from its roots are used in the treatment of gonorrhea (Devkota, Lenta, Fokou and Sewald, 2008). The dichloromethane (DCM) fraction of *S. saligna* bears cytotoxicity potential, ethanolic extracts is antifungal, Petroleum ether and Ethyl acetate fraction is anti-hyperglycemic effect whereas the crude extracts are antibacterial and acetylcholinesterase (AChE) and butyryl cholinesterase (BChE) inhibitors. It is a rich source of steroidal alkaloids having antileishmanial, antimicrobial and anticholinesterase activity which have been reported by number of phytochemical isolation (Iqbal et al., 2015). Medicinal plants are the rich source of antimicrobial and antiamoebic agents (Cimanga et al., 2018). *S. saligna* is a well-known Chinese medicine used for Gastrointestinal tract (GIT) abnormalities (Yan et al., 2011).

Materials and Methods

Collection and extraction of plant material

Sarcococca saligna was collected from Ayubia National Park, Pakistan (location coordinates = 34°03′20″N 73°25′0″E) on September 2017. Leaves and roots of *S. saligna* were thoroughly washed with water. Both roots and leaves were separately shade dried, grinded into fine powder and separately macerated for 21 days using aqueous methanol (30:70). The crude extracts were filtered and concentrated using vacuum rotary evaporator (Rota vapor, R.210-BUCHI) at a temperature of 45 °C under vacuum.

Phytochemical screening

The protocol given by Wadood et al., 2013 (Wadood et al., 2013) and Harborne, 1998 (Harborne, 1998) was adopted for qualitative analysis of different secondary metabolites present in *S. saligna* crude aqueous methanolic extracts of leaves and roots.

Isolation of crude saponins

Crude extracts of *S. saligna* leaves and roots were separately dissolved in 300ml Methanol and then concentrated to 100 ml. 150ml n-Butanol (already saturated with distilled water) was added to each of the extracts, making the total volume 250ml. The mixture was shaken well in a separating funnel, allowed to stand until the water and n-Butanol layer got separated. The lower layer was discarded and took the upper (n-Butanol) layer. The n-Butanol fraction was then concentrated and dissolved in the Methanol (concentrated mixture). Pre–cooled Diethyl ether was added drop wise, as result light yellow color precipitate was formed which was filtered and dissolved in Methanol. The solvent was evaporated and precipitates were collected in dry form (Alam, Saqib, Waheed, 2015). The extraction of crude saponins is given in Figure 1 schematically.

Growth and preservation of bacterial strains

Bacterial strains, *Bacillus subtilis, E. coli, Staphylococcus aureus, Pseudomonas fluorescens* and fungal strains, *Aspergillus niger, Aspergillus flavus,* and *Dreschlera turcica* were collected from Veterinary Research Institute (VRI) Abbottabad, Pakistan. These bacterial and fungal strains were used for antibacterial and antifungal

assay. Nutrient agar media was used for bacterial species at 37 °C whereas Potato dextrose agar was used for maintaining fungal strains at 28 °C.

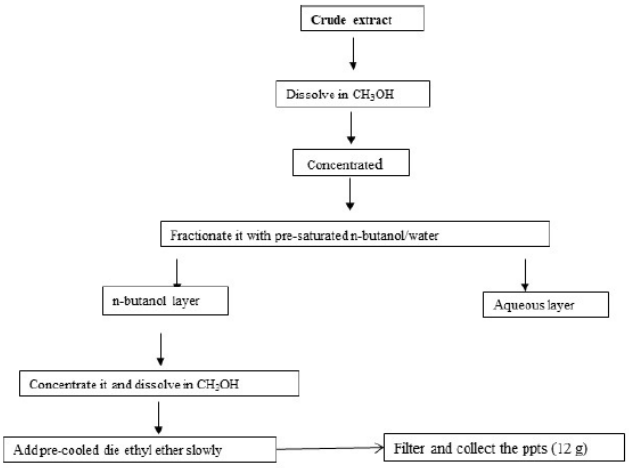

Figure 1: *Flow chart for separation of crude saponins from crude aqueous methanolic extracts of S. saligna leaves and roots.*

Disc diffusion antibacterial assays

The dried extracts/fractions of plant were dissolved in aqueous methanol to make a final concentration up to 30 mg/ml and filtered via Millipore filters having size of 0.45µm. Disc-diffusion method was adopted for antimicrobial screening as previously reported by Khalid et al., (Essawi and Srour, 2000). Both media (Nutrient agar and Potato dextrose agar) were prepared according to the instructions of manufactures. Pre-sterilized media (30ml) was added into Petri-plates. Approximately 10µl test microorganisms (10^6cells/ml) were seeded into culture media. The discs (6 mm in diameter) were impregnated with 10µl of the extracts (300µg/disc) at the concentration of 30 mg/ml and placed on the inoculated media. Ciprofloxacin (30 µl) and fluconazole (30 µl) were used as control. Zone off Inhibitions were measured after incubations (37°C for antibacterial 28°C for antifungal, up to 24hrs).

Results and Discussion

In past few decades diverse approaches have been made to elucidate new antimicrobial moieties from medicinally active plants (Moghaddam et al., 2010). *S. saligna* is traditionally used for different disorders including skin infections, gonorrhea and syphilis. It also shows significant antibacterial activity against *Shigella*, *Pseudomonas* as well as *Carnebacterium* species (Ghayur and Gilani, 2006). In order to justify the local uses of this species, its crude aqueous methanolic extracts of leaves and roots as well as crude saponins isolated from it were examined against human pathogenic bacterial strains e.g. *Bacillus subtilis, E. coli, Staphylococcus aureus, Pseudomonas fluorescens* and fungal strains including *Aspergillus niger, Aspergillus flavus, and Dreschlera turcica.* The current study also justified the presence of several secondary metabolites in both leaves and roots aqueous methanolic extracts of *S. saligna* given in Table 1.

Table 1. *Qualitative phytochemical analysis of aqueous methanolic extracts of Sarcococca saligna leaves and roots.*

Secondary Metabolites	Test/Reagent	Leaves	Roots	Ethyl Acetate Leaves
Alkaloid Test	Dragendorff's reagent	+++	+++	+++
	Mayer's reagent	+++	+++	+++
	Wagener's reagent	+++	+++	+++
Flavonoid Test	Alkaline reagent	+++	+++	_
Saponin Test	Froath test	+++	+++	_
	Lead acetate	+++	+++	_
Steroids	H2SO4	-	-	-
Cumarins	NaOH	-	-	-
Tannins	10 % Gelatin	++	++	++
Reducing sugars	Fehling's reagent	_	++	_
Proteins	Xanthoprotic acid	+	_	-
Cardiac Glycosides	Keller- Killiani"s test	++	++	++
Phenols	FeCl3 test	+++	+++	++
Triterpenes	Salkowski"s test	-	+	_

The maximum zone of inhibition was recorded by Ciprofloxacin as 30.0±0.0 mm against the tested bacterial strains whereas 28.0±0.0 mm by Fluconazole was recorded against fungal species. Previous studies of *S. saligna* have confirmed its antimicrobial potential against various human pathogenic bacterial as well as fungal strains e.g. its ethanolic extract has antifungal potential (Iqbal et al., 2015). It is a rich source of steroidal alkaloids; some bacterial steroidal alkaloids have been isolated from its roots and stem i.e. saligcinnamide, Na-methyl epipachysamine-D, and epipachysamine D. These compounds justified significant activity against human pathogenic organisms including *Klebsilla pneumonia*, *Streptococcus areus*, *St. pyrogenus, Pseudomonas aeruginosa, S. typhii, Shigella boydii* (*Atta-ur-Rahman et al., 1998). Bioassay

Table 2: *Antibacterial screening of S. saligna aqueous methanolic extracts of leaves and roots as well as crude saponins isolated from it.*

Sample	Zone of Inhibition (mm)			
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas fluorescens*
Crude Aq:MeOH Ext. of Leaves	15.00±0.04	9.00±0.00	18.6±0.60	15.30±1.3
Crude Aq:MeOH Ext. of Roots	16.00±0.06	13.12±0.20	10.00±0.80	09.00±0.00
Ethyl acetate frct. of leaves	09.00±0.47	13 ±0.57	09.00±0.47	23.00 ±0.56
Crude saponins of Leaves	09.00±0.00	09.00±0.00	09.00±0.00	09.00±0.00
Crude saponins of roots	14.00±0.00	14.84±0.2	16.73±0.30	09.00±0.00
Ciprofloxacin	30.00±0.00	30.00±0.00	30.00±0.00	30.00±0.00

Values are the mean inhibition zone (mm) ± S.D of three replicates; zone of inhibition <9.00 mm: inactive

Table 3: *Antifungal screening of S. saligna aqueous methanolic extracts of leaves and roots as well as crude saponins isolated from it.*

Sample	Zone of Inhibition (mm)		
	Aspergillus niger	*Aspergillus flavus*	*Dreshlera turcica*
Crude Aq:MeOH Ext. of Leaves	9.00±0.00 mm14	14.00±0.04 mm	9.00±0.00 mm
Crude Aq:MeOH Ext. of Roots	12.00±0.06 mm	13.12±0.20 mm	9.00±0.00 mm
Ethyl acetate frct. of leaves	25.00 ± 0.5 mm	13.00 ±1.00 mm	12.00 ±1.15 mm
Crude saponisns of Leaves	09.00±0.00 mm	09.00±0.00 mm	14.00±1.02 mm
Crude saponisns of roots	14.00±0.00 mm	12.84±0.2 mm	16.73±0.30 mm
Fluconazole	28.00±0.00 mm	28.00±0.00 mm	28.00±0.00 mm

Values are the mean inhibition zone (mm) ± S.D of three replicates, zone of inhibition <9.00 mm : inactive.

guided isolation of antimicrobial steroidal alkaloids from other species of same genus have confirmed 5 α-pregnene type steroidal alkaloids from *Sarcococca hokoriana* which include two new hookerianamides J and K as well as eight known compounds including hookerianamides H and I, chonemorphine,, *N*-methypachysamine A, epipachysamine-*E*, 2, 3-dehydrosarsalignone, vagenine A and sarcovagine C. all these compounds are known to be active against *Leishmania major* as well. Besides these active isolated moieties, the crude extracts, n-hexane, $CHCl_3$, Ethyl acetate and aqueous fraction of arial parts of *S. saligna,* have confirmed significant antibacterial potential i.e. 76.9, 50, 80.7, 65.3, and 56.3 % inhibition respectively against *S. aureus* and 48.1, 55.5, 25.9, 44.4 and 14.8 % inhibition against *P. aeruginosa* respectively. All the mentioned above tested samples were also active against *E. coli*, *S. typhii, S. pneumonia, B. pumalis* and *S. epidermidis* as well as against *F. oxysporium* fungal strain. According to Ashoke (2014), Ethyl acetate, methanolic and Petroleum ether fractions of *S. saligna* are also potent against *Proteus vulgaris, P. aurigino-sa, E. coli* and *S. aureus* respectively. The same fractions were also investigated against fungal strain *Aspergillus flavus* and results showed that crude and methanolis extracts were more active than Petroleum ether and Ethyl acetate fraction (Ashok Kumar*1, 2014). A significant antifungal effect of *S. saligna* ethanol extract against, *A. treus and A. flavus* was also reported in a previous study (Moghaddam et al., 2010). The chloroform fraction of *S. saligna* exhibit significant antibacterial activity (80%) against *Staphylococcus aureus*, whereas its methanolic extracts possess significant (77%) accomplishment. This research study revealed that crude aqueous methanolic extracts of *S. saligna* leaves and roots as well as crude saponins isolated from it, showed significant antibacterial and antifungal activities as shown in Table 2 and 3. The highest zones was 18.6±0.60 mm shown by crude aqueous methanolic extracts of leaves against *E. coli* whereas 16.73±0.30 mm was measured against *D. turcica* fungal strain by crude saponins isolated from roots of *S. saligna*. This study revealed that the tested *S.saligna* crude aq: MeOH extracts as well as crude saponins isolated from its leaves and roots possess significant antibacterial as well as antifungal potential. Zones of inhibitions were measured for both tested samples as well as standard drugs i.e. Zones of inhibitions for bacterial strains are given in Table 2 whereas for fungal strains it is given in Table 3.

S. saligna leaves

Crude aqueous methanolic extracts of *S. saligna* leaves showed maximum inhibition i.e. 15.0±0.04 mm, 9.0±0.0 mm, 18.6±0.6 mm and 15.30±1.3 mm against *St. aureous, B. subtilitis, E. coli, P. fluorescens* respectively as shown in Table 1. Whereas against fungal strains 14.0±0.04 mm zone of inhibition was recorded against *Aspergillus flavus* and it was inactive against other two tested fungal strains (Table 2).

S. saligna roots

Crude aqueous methanolic extracts of roots showed 16.0±0.06 mm 13.12±0.2 mm and 10.0±0.8 mm zones against the tested bacterial strains i.e. *St. aureus, B. subtilitis, E. coli,* respectively (Table 1). Whereas 12.0±0.6 mm and 13.12±0.2 mm zones were measured against *Aspergillus niger and Aspergillus flavus* species. It was inactive against *P. fluorescens* and *D. turcica* (Table 2).

Ethyl acetate fraction of leaves

Ethyl acetate fraction of leaves gave maximum inhibition of 23.0±0.56 mm against *P. fluorescens,* 13.0±0.57 mm against *B. subtilitis* but have no significant results against *E. coli and St. aureus* (Table 1) on the other hand, maximum result (25.0±0.5 mm) was recorded against *A. niger,* 13.0±1.0 mm and 12.0±1.15 mm zones was recorded against A. flavus and D. turcica respectively.

Crude saponins isolated from S. saligna leaves and roots

Crude saponins isolated from *S. saligna* leaves were moderately active against bacterial and fungal strains except *D. turcica* (14.0±1.02 mm), whereas crude saponins from roots were considerably active against bacterial and fungal strains i.e. 14.0±0.0 and 14.84±0.2 mm zones were measured against *S. aureous and B. subtilitis* respectively, 16.73±0.30 mm was recorded against *E. coli* and it was inactive against *P. fluorescens.* In case of antifungal studies 14.0±0.0 mm, 12.84±0.2 mm and 16.73±0.30 mm was shown against *Aspergillus niger, Aspergillus flavus* and *Dreshlera turcica* respectively as shown in Table1 and Table 2.

Conclusions and Recommendations

Our investigations of screening *S. saligna* confirmed its therapeutic potential, used in traditional medicines by local people of Pakistan. From this study it is concluded that *S. saligna* leaves and roots can be used as natural source of antibiotics and antifungal drugs at different extents against certain infections and pathogenic disorders, claimed by local people of Northern KPK, Kashmir like skin infections, GIT diseases, UTIs and sexually transmitted diseases like syphilis and gonorrhea. The study provides us a base for selecting this medicinal plant specie for further phytochemical and pharmacological evaluation. *S. saligna* is under way for further biological evaluation in our research laboratories.

Author's Contribution

Sabi-Ur-Rehman, Neelam Zaman and Ayeza Mehmood: Performed the experimentation and draft writing.

Anwar Khalid: Verified the analytical methods and developed the theory.

Qazi Najam Us Saqib: Supervised the project.

Farooq Ahmad and Shaheed-Ur-Rehman: Performed the calculation and result interpretation.

Abdul Samad: Supervised the findings and developed the theory.

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