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ANTIMICROBIAL POTENTIAL OF FEMALE CONES AND NEEDLES OF CHIR PINE *(PINUS ROXBURGHII SARGENT)*

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ABSTRACT

The present study was undertaken to evaluate the antimicrobial potential of the plant *Pinus roxburghii* Sarg. The aerial parts of *Pinus*(needles and female cones) were extracted using various solvents *viz*. petroleum ether, chloroform, methanol and distilled water. These extracts were used for preliminary phytochemical screening by using disc diffusion method and were tested against two fungi *viz. Alternaria alternata&Fusarium solani* and two bacteria *viz*. *Xanthomona soryzae&Pseudomon asalcaligenes*. Results revealed that female cones of *P. Roxburghii* have more antimicrobial activity as compared to needles therefore, female cones extract in chloroform showed maximum inhibitory zones against fungus *A. Alternate* (22 ± 2^{a}) and methanolic extract against *F. solani* (20 ± 2^{a}). When comparing with *P. alcaligenes, X.oryzae*growth was more inhibited by cones and needle extracts. From results it may concluded that methanolic extract of cones and needles created maximum zones of inhibition against *X.oryzae*i.e.,23.3 ± 2^{a} and16 ± 4^{a} respectively and 22 ± 2^{a}. In case of *P. alcaligenes,* female cones methanolic extract showed maximum value of zone of inhibition i.e., 22 ± 2^{a} while needles showed maximum value in chloroform extract i.e.,15 ± 0.35^{b}. Therefore it can be declared that*P. roxburghii* is promising phyto medicine having strong antifungal and antibacterial activities.

Keywords: Chir Pine; Maceration; Disc diffusion method; Antifungal; Antibacterial

INTRODUCTION

Earth is endowed with a rich wealth of herbal and medicinal plants containing active compounds and constituents serving as a blessing to mankind. The community's wide spread belief has generated considerable interest in use of green medicines, which are safer and healthier as compared to synthetic one. Hence herbs and medicinal plants can be attributed as first medicines. Considerable research efforts have been made since last two decades to explore antibacterial properties of different plants. It has been reported that aromatic and herbal plants have rich antibacterial substanceswhilecertain bacteria are developing resistant against certain synthetic medicines (Parihar*et al*., *2006;*Bissa*et al*., 2008; Siddiqui *et al*., 2009; *Kaushiket al*., *2010;*Kaushik*et al*., 2013;Qadir*et al*., 2014; Salem*et al*., 2014; Chaudhary*et al*., 2014; Ishtiaq *et al*., 2015 and Nakagawa*et al*., 2016).*Pinus roxburghii* Sarg. (*Pinaceae*) colloquially known as "Chir pine" oldest terrestrial ornamental plant in the world, is only tree having diverse medicinal potential (Figure 1).Its range extends from Nepal to Bhutan, across Northern India (Punjab, Sikkim, Himachal Pradesh, Uttrakhand, Jammu and Kashmir) including Pakistan (Khyber Pakhtunkhwa and Azad Kashmir). However, in Pakistan it grows well at mean coldest temperature ranging between 5-15ºC at an altitude of 1200-1850m (Nasir and Nasir, 1987; Sidiqui *et al*., 1999; Shah, 2006).Economically pines play a pivotal role as a source of woody and timber material which also endow with turpentine oil and non-woody material i.e. pine needles (14-16 cm long). Needles of pines have rich source of alkaloids, vitamin C, tannins, pulp, essential oils, resin and nuts (Siddiqui, 1991; Sehgal*et al*., 1995; Vallejo *et al*., 1994: Sidiqui*et al*., 1999; Asta *et al*.,2006; Chaudhary *et al*., 2014). Turpentine obtained from resin of all pines has been documented as antiseptic, diuretic, rubifacient and vermifuge. It has

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been documented that every year in The United States almost 2 million people acquired bacterial infections but pressing problem is that these bacterial strains are resistant to at least one drug, hence study of antimicrobial agents has become increasingly significant (Alexandria, 2005). Consequently pharmaceutical companies tend to concentrate their research efforts in this area (Taylor *et al*., 2002; Ishtiaq *et al*., 2015 and Nakagawa*et al*., 2016). Hence current investigation was undertaken to evaluate antifungal and antibacterial efficacy of aerial parts of *Pinus roxburghii* against selected two fungal and two bacterial pathogens.

Figure 1.Female cone (right) and male cone (left) of*Pinusroxburghaii*Sarg.

MATERIALS AND METHODS

Plant Collection and Preservation: Female cones and needles of locally available plant, *P.roxburghii* (Chir pine)were collected from Bagh-e-jinnah, Lahore.Plant material including cones and needles were dried at room temperaturefor extraction and powder them with the help of grinder. Powders were sealed in the amber colored specimen jars, and labelled appropriately until required.

Solvent Extraction: Solvent extraction was done by using maceration method (Aftab and Khan, 2016). For this cones and needles (50g each) were powdered and soaked using 250 ml of non-polar and polar solvents e.g., Petroleum ether, chloroform, methanol and distilled water in sequential manner. For maceration method, the powders were flooded using petroleum ether in shaker at room temperature for the period of eight days. After shaking, filtration done by using filter paper (Whatman No. 1). Residue was further drenched in the next sequential solvent i.e., chloroform and the filtrate was potted in the amber colored glass jars (labeled) and stored in fridge at 4^{o}C. The same procedure was done by using methanol and distilled water. At the end, we prepared four extracts both for female cone and four for needle of *P.roxburghii*. Antimicrobial activity was determined by following the method described by Ferreira *et al*. (1996) and Ortega *et al*. (1996).

Experimental Design: The Petri plates containing the solidified and inoculated medium (potato dextrose agar medium for fungi and Lauria Bertani (LB) medium for bacteria) were prepared for the measurement of antimicrobial activity (antifungal and antibacterial respectively) of crude extract of plant parts i.e., needles and cones.In order to make uniform hole at the middle of each petri plate cork borer No. 2 was used and the extract was poured into this hole with the help of sterilized glass dropper. Plates were incubated in incubator (28^{o}C for fungus for 7 days: 37^{o}C for bacterial growth for 24 hours).Clear zone of inhibition became prominent after the required incubation time. For the comparison, zones of pure solvent and the standard discs were also recorded.Actual measurement of zone of inhibition of plant extract was obtained after subtracting the value produced by pure solvent.

STATISTICAL ANALYSIS

The data was presented as Mean ± S.D. (M± standard deviation). The treatment effects were compared as described by Snedecor and Cochran (1980) and significant differences among replicates was presented as Duncan's multiple range tests (Steel and Torrie, 1984) in the formof probability <p> values, using the computer software Costat, cs6204 W.exe. Standard error was calculated by using the formulae as describe by Alfonso *et al*. (1985).

RESULTS AND DISCUSSION

Antifungal Activity: The data presented for antifungal activity clearly depicts the inhibitory zones of *Alternaria alternata* and *Fusarium solani* created by plant material extracts in petroleum ether, chloroform methanol and distilled water (Figure 2). It is clearly showed from the results that antifungal activity against *A. alternata, P.roxburghii*female cones extract in petroleum ether showed maximum value of zone of inhibition i.e., 22±2^{a} when compared with remaining three solvents. Whilst in case of *Pinus* needles extract, chloroform and methanol extract showed maximum value of zone of inhibition i.e., 9±2^{a} and 9±3^{a} against *A. alternata*. It is concluded that *P.roxburghii*female cones extract in petroleum ether has naturally maximum antifungal activity than needles against *A.alternata*(Table 1). Generally those secondary

metabolites in the plants showing the elevated polarity that have the ability to extract voluntarily in solvents. The presence of such secondary metabolites may be the reason behind the putative antifungal value of the extracts and vice versa (Ray and Majumdar, 1976).Moreover, comparable work was done by Hassan and Amjid (2009) and examined that the essential oil extracted from the needles of *P.roxburghaii* have very strong antifungal abilityagainst *Aspergillus flavus, Aspergillusniger, Aspergillus vessicolor, Trichoderma viride, Aspergillus terrus* and *Aspergillus candidus*. Furthermore, maximum antifungal activity of *Pinus roxburghii* female cones and needles against *Fusarium solani*gave 20±2[a] in methanolic extract and 20±5[a] in petroleum ether extract respectively. As well as minimum antifungal activity against same fungus was given by *P.roxburghii* female cones extract in distilled water that is 4±2[o]C(Table 1). This result indicates that *P.roxburghii* female cones extract in distilled water is naturally most susceptible of *F.solani* as compared to needles as well as other solvents. It means that the compounds that show strong antifungal activity are either insoluble in the *P.* roxburghii female cones distilled water extract and in methanol extract in case of needles.

Table 1. Antifungal activity of *Pinus roxburghii* (female cone and needles) extracts in various solvents(petroleum ether, chloroform, methanol and distilled water).

Plant Parts	Plant Extracts (solvents)	Inhibition zones (mm)	
		Antifungal activity	
		Alternaria alternata	*Fusarium solani*
Cone (♀)	Petroleum Ether	16.6 (±3.51[ba])	7.66 (±2.51[c])
	Chloroform	22 (±2[a])	12 (±2[b])
	Methanol	7 (±2c)	20 (±2[a])
	Distilled water	7.66 (±2.51[c])	4 (±2[c])
Needles	Petroleum Ether	6.33 (±1.52[a])	20 (±5[a])
	Chloroform	9 (±2[a])	9 (±3[b])
	Methanol	9(±3[a])	8.66 (±3.05[b])
	Distilled water	7.66 (±3.05[a])	12.33 (±2.51[b])

[a]Values in columns followed by the same letters are not significantly different at *P*<0.05, analyzed by DMRT test; values are means of 3 replicates.

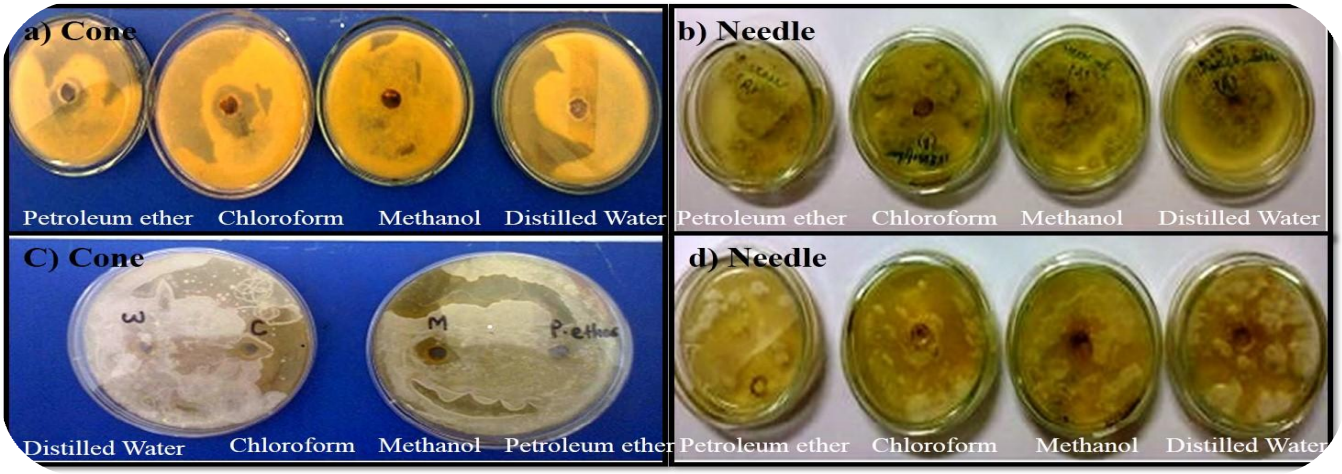

Figure 2. Antifungal activity of Pinusroxburghii(female ones and needles) against (a,b) *Alternaria alternata* and (c,d) Fusariumsolani in various solvents polar viz. petroleum ether, chloroform, methanol and non-polar i.e., water with plant extract.

Antibacterial Activity: It is clearly shown in Figure 3 that *P.roxburghii* female cones methanol extract of plant formed the greatest value of zone of inhibition i.e., 23.3±2[a] and Pinus needles methanol extract showed maximum value of zone of inhibition i.e., 16±4[a] against *Xanthomonas oryzae*.These values indicated the

presence of strong antibacterial compounds having substantial antibiotic potential.Besides from these results it maysuggested that *P.roxburghii*female cones have more antibacterial activity against *X.oryzae*instead of needles.However, for the evaluation of antimicrobial ability, stereo-configuration is a sensitive technique as well as reproducible. Savluchinske*et al*. (1997) used this technique and identified compounds which are responsible for possible synergistic effect in order to elaborate their antibiotic activity. When it comes to antibacterial activity against *Pseudomonas alcaligenes,* again *P.roxburghii*female cones has more antifungal activity than needles. *P.roxburghii*female cones extract in methanol showed 22±2[a] value of zone of inhibitionwhich is the highest most reading among all the extracts of *Pinus* cones and needles (Table 2).Needle showed 15±0.35[b] maximum value in chloroform extract that is less than *P. roxburghii*female cones extract in methanol.Sometimes, essential oils extracted from the needles of *P.roxburghii*do not inhibit the growth of bacteria as studied by Zafar *et al*. (2010) where oil inhibited the growth of *Bacillus subtilis*and *Staphylococcus aureus*but has no inhibitory effect o*n Enterobacteraerogenes, E. coli* and *Salmonella typhi*.Needle showed 15±0.35[b] maximum value in chloroform extract that is less than *P. roxburghii*female cones extract in methanol (Figure 4).Sometimes, essential oils extracted from the needles of *P.roxburghii*do not inhibit the growth of bacteria.

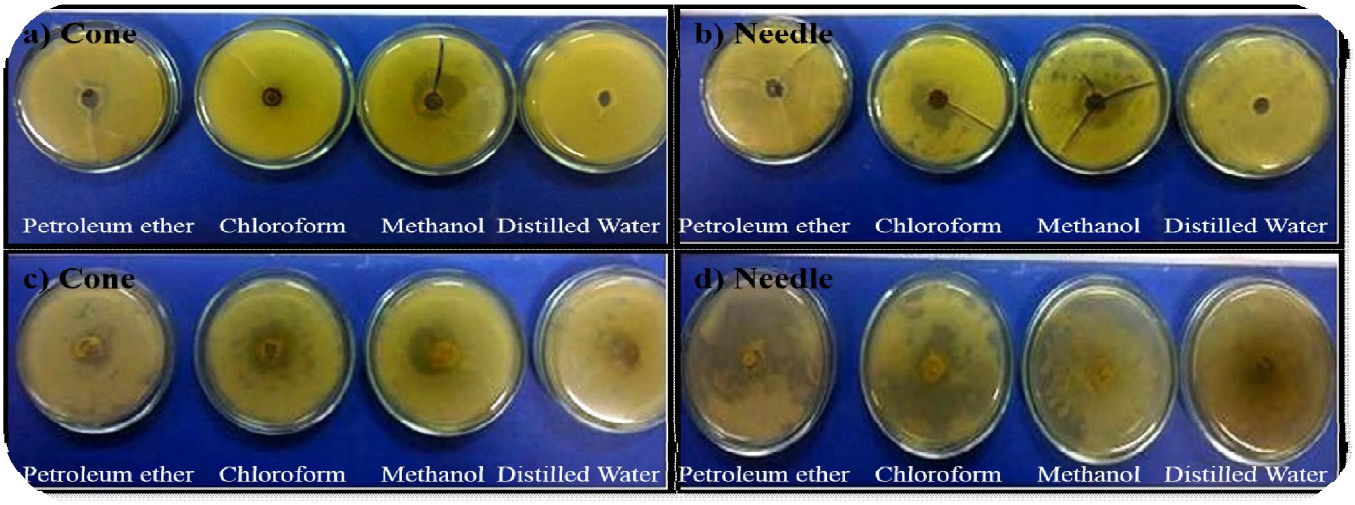

Figure 3.Antibacterial activity of *Pinus roxburghii* (female cones and needles) against (a,b)*Xanthomonas oryzae* and (c,d) *Pseudomonas alcaligenes* in various solvents (petroleum ether, chloroform, methanol and distilled water).

Table 2. Antibacterial activity of *Pinus roxburghii*(female cone and needles) extracts in different polar (petroleum ether, chloroform, methanol) and non-polar (distilled water) solvents.

Plant Parts	Plant Extracts (solvents)	Inhibition zones (mm)	
		Antibacterial activity	
		Xanthomonas oryzae	*Pseudomonas alcaligenes*
Cone (♀)	Petroleum Ether	17.66 (±2.51[b])	9.66 (±2.51[c])
	Chloroform	16.33 (±3.05[b])	17.33 (±2.51[b])
	Methanol	23.3 (±2[a])	22 (±2[a])
	Distilled water	9 (±2[c])	9 (±2[c])
Needles	Petroleum Ether	6 (±2[b])	6.66 (±3.05[b])
	Chloroform	15 (±2[a])	15 (±2[a])
	Methanol	16 (±4[a])	6 (±2[b])
	Distilled water	6 (±2[b])	5.33 (±2.51[b])

[a]Values in columns followed by the same letters are not significantly different at $P<0.05$, analyzed by DMRT test; values are means of 3 replicates.

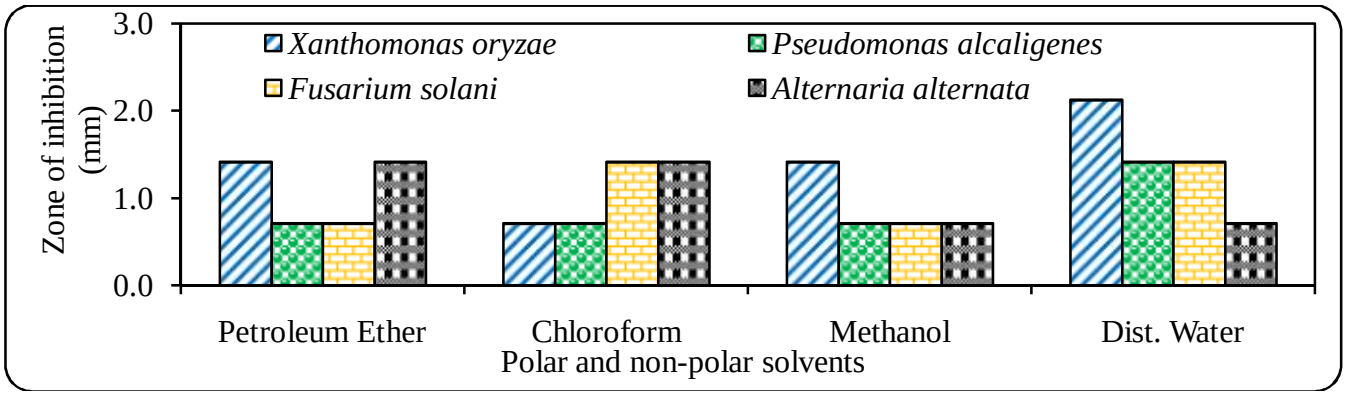

Figure 4. Graphical presentation of inhibition zones (mm) produced by various solvents (polar and non-polar) without plant extract against fungi (*Alternaria alternata&Fusariumsolani*) and bacterial (*Xanthomonas oryzae&Pseudomonas alcaligenes*) pathogens.

Such results were documented by Zafar *et al*. (2010) that oil inhibited the growth of *Bacillus subtilis*and *Staphylococcus aureus*but has no inhibitory effect o*n Enterobacteraerogenes*, *E. coli* and *Salmonella typhi*.Standard antimicrobial discs were used to compare the results and they showed significant values for the zone of inhibition. Fuconazole showed the value i.e., 44.3±2.51^{a} against *A.alternata* and Kenamycin showed the value (36.3±2.08^{b}) against *F.solani*. Ampicilin showed the value (36±3.60^{b})against*X.oryzae*whereasAmikacin showed the value (28.3±2.08^{c}) against *P.alcaligenes*. All these results were compared with the standard antimicrobial discs that are commercially available e.g., Fuconazole, Kenamycin and Ampicillin, Amikacin etc. As some of the extracts showed very comparable results with the standards so these can be used as alternatives for the standard ones e.g., Methanolic Cone extract against *Pseudomonas alcaligenes*was 22±3.05^{a} which gave almost same value, given by the standard Amikacin i.e., 28.3±2.08^{c}(Figure 5).

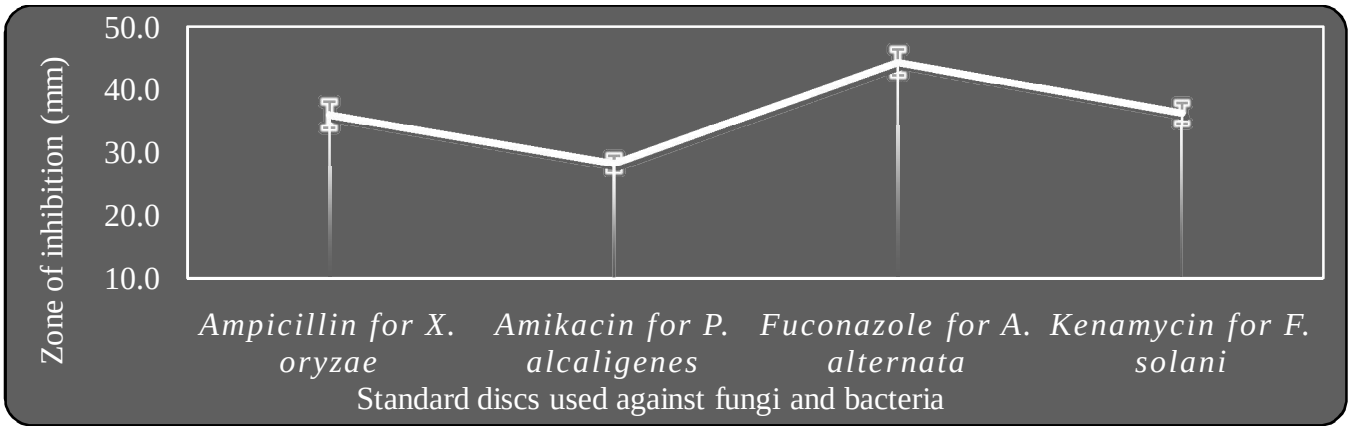

Figure 5. Graphical presentation of inhibition zones (mm) produced by various commercially available standard discs without plant extract against bacteria (*Xanthomonas oryzae&Pseudomonasalcaligenes*) and fungi (*Alternaria alternata&Fusarium solani*).

CONCLUSION

The results of this study suggest that *P.roxburghii* cones and needles have medical usage. They naturally possess antifungal as well as antibacterial potential that can be used to treat contagious diseases.Further investigation is needed to open new avenues for the use of this plant while focusing on isolation of different compounds for therapeutic purpose.

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