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PHARMACOGNOSTIC STUDIES AND ANTI-MICROBIAL INVESTIGATION OF BARK, ROOTS, AND CONES OF *PINUS ROXBURGHII SARGENT*

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A B S T R A C T

The present study was aimed at the assessment of the anti-microbial potential of extracts of *Pinus roxburghii* bark, roots, cones, and a combination of these three parts in different solvents such as dist. water, methanol, chloroform, and petroleum-ether prepared by maceration. *Pinus roxburghii* sargent a medicinal plant belonging to Pinaceae is the world's oldest terrestrial ornamental plant known as "Chir pine." Anti-microbial activities were determined against bacteria i.e; *Pseudomonas alcaligenes* and *Xanthomoas campestris* and fungi i.e; *Alternaria alternata and Fusarium solani* by disc and well diffusion methods. Standard discs were Ampicillin and Fluconazole. Petroleum ether and dist. water extracts of roots and cones were selected for antimicrobial investigation due to their maximum inhibitory effects. MIC was determined by serial dilutions. Minimum inhibitory concentrations of roots and cones in petroleum ether and dist. water were 3.125, 6.25, 12.5, 25, 50 and 100mg/ml for bacteria and 150, 175, 200 and 250mg/ml for fungi. Minimum bactericidal and minimum fungicidal concentrations were 100, 125, and 150 mg/ml for petroleum ether roots and cones extracts and 200, 250, and 300 mg/ml for aqueous roots and cones extracts respectively. Roots and cones in petroleum ether showed zone of inhibition i.e., (22 ± 1^{a}) and (20 ± 1^{b}) against *P. alcaligenes* and *X. campestris* respectively by well diffusion. The same plant parts in aqueous extracts showed ZOI i.e., (23 ± 1^{a}) and (14 ± 1^{d}) by well and disc diffusion respectively against *A. alternata*. Ampicillin showed ZOI i.e., (7 ± 2^{c}) against *P. alcaligenes* and (26 ± 1^{a}) against *X. campestris*. Fluconazole showed ZOI (22 ± 1^{b}) against *A. alternata*. Fluconazole and extracts showed no inhibitory effect on *F. solani*. From the findings it has been determined that maximum antimicrobial potential was exhibited against *Pseudomonas alcaligence, Xanthomonas campestris,* and *Alternaria alternata* by roots and cones in petroleum ether and aqueous extracts. The result showed that *P. roxburghii* is a promising phytomedicine having antifungal and antibacterial properties.

Keywords: *Pinus roxburghii,* Maceration, Antibacterial, and antifungal activity, MIC, MBC, MFC, ZOI.

INTRODUCTION

Nature has rewarded our country with an abundance of medicinal plants. The advancement of knowledge to cure diseases continued at a rapid rate, and several new plant-derived drugs were developed. Herbal plants are thought to be rich in phytochemical ingredients, which give them medicinal value and have the potential to be a source of new herbal drugs. The pharmacological effects of medicinal plants have been regarded as a promising future medicine for the management of health care in the twenty-first century (Shakya, 2016). Aromatic plant species are thought to be valuable with antimicrobial agents against common pathogenic microorganisms. According to the World Health Organization (WHO), antibiotic resistance is raised due to the misuse of pharmaceutical antibiotics which is a major cause of illness prolongation with a higher risk of death (El-Said *et al.*, 2021). *P. roxburghii* is the world's oldest terrestrial artistic plant having therapeutic prospects and belongs to the family Pinaceae. This plant is a gymnosperm pine

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cone-producing (naked seeded) perennial coniferous species. Botanical Synonym for this plant referred to as *Pinus longifolia* and known by the common name "Chir pine". This plant occurred in North Western Himalayas, Kashmir to Bhutan and Siwalik hills, and Himachal Pradesh (Sharma *et al.*, 2018). In Pakistan, this plant was found in Swat and Hindukush (Khan *et al.*, 2021). The growth temperature for *P. roxburghii* in Pakistan ranges from 5-15°C at an elevation of 1200-1850m (Khalid *et al.*, 2016). Economically, this plant's rosin can be used as a binder, toner, and in paper manufacturing. The tree's lumber is utilized in home construction and furnishings, tea chests, sporting items, and musical instruments. The green needles are trilateral, thin, and about 20-30cm tall (Sharma *et al.*, 2018). Pine bark has flaky layers of scales. Male flowers are 1.5 cm long and grouped in cone shapes and female cones are seen as much bigger than male cones and typically present on the top branches (Kumari *et al.*, 2017). Phytoconstituents present in the essential oil extracted from the bark, needles, and cones of *P. roxburghii* include alkaloids, vitamin C and tannins. The bark contains about 7-10% of tannins and flavonoids including gallocatechin, rhamnetin, and myricetin. Needles and bark also contain flavonoid glycosides, phenolic acids, and terpenyl alcohols (Kumari *et al.*, 2017), (Sinha and Tandon, 2018). Turpentine oil of the plant is employed as a solvent in formulations of the drug, the perfume industry, the production of synthetic pine oil, pesticides, and disinfectants. Antibacterial, diaphoretic, rubefacient, fragrant, and carminative properties have also been found in wood oil (Sharma *et al.*, 2018). Approximately 25%–45% of in-patients in hospitetals are prescribed antimicrobials. There is a need to develop pertinent programs to address concerns related to antimicrobials not only in Punjab but across Pakistan where they exist (Saleem *et al.*, 2019). Nowadays, microbes appear to be resistant due to the inappropriate use of antibiotics which has caught the attention of international and national organizations. According to the WHO, infectious diseases are spreading and emerging faster. So that the discovery and development of new antimicrobial compounds with diverse structures and action mechanisms are critical in the last decade. As a result, a global effort has been undertaken to develop novel antibiotics (Cota *et al.*, 2019). *Alternaria* can be caused infection in the leaves and stems of the plant. This fungus produced black necrotic lesions surrounded by chlorotic halos that appeared as spots on the leaf. *Alternaria* is also frequently associated with human airway disorders such as asthma, allergy, and chronic rhino sinusitis. *Alternaria spp.* are also being recognized as human invasive pathogens (Puvača *et al.*, 2020). *F. solani* is a plant pathogen, endophyte, detrivores, pretentious human fungi, and insect nutritional symbiont. This strain causes dry rot in potatoes (Schroers *et al.*, 2016). *X. campestris* is a gram-negative rod-shaped bacteria. Yellow spots are caused by *Xanthomonas species*, which cause serious plant diseases in over 400 different plant species. The bacterial leaf spot disease caused by *X. campestris* on pepper and tomato is one of the most damaging diseases to these hosts (Park and Han, 2017). *P. alcaligenes* is an aerobic gram-negative rod-shaped bacterium found in soil and water. These microbes are both non-fermenting environmental microorganisms and rare opportunistic human pathogens (Suzuki *et al.*, 2013). Current study suggested antibacterial and antifungal efficacy of bark, roots and cones of *Pinus roxburghii* against two selected bacterial and fungal pathogens.

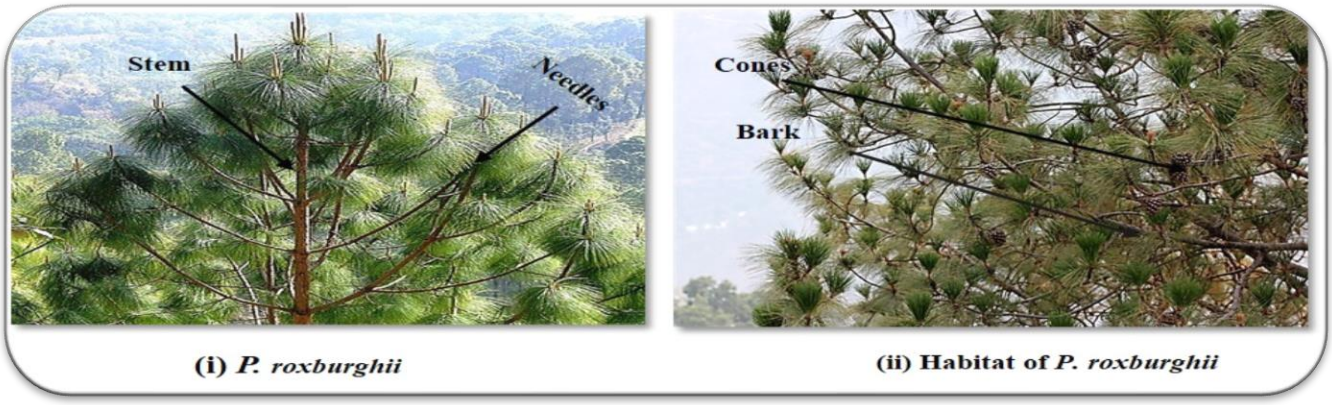

Figure 1. (i) and (ii) represented the whole plant with habitat.

MATERIALS AND METHODS

Collection of plant material: Bark, roots, and cones of locally available medicinal plant ***P. roxburghii sarg***. were collected from Bagh-e-Jinnah, Lahore, Pakistan. To remove moisture, plant parts including bark, roots, and cones were dried under shade at room temperature for extraction and powdered with the help of a grinder. The powdered material of bark, roots, and cones was routed individually through sieve No.40 to obtain fine powder which was preserved in airtight amber-colored containers and labeled.

Solvent extraction: The solvent extraction process was performed by the maceration method. Sixteen plant extracts were prepared. All these extracts were tested for antimicrobial activity against four microorganisms (two bacteria and two fungi) as well as pharmacognostic investigations. Four solvents were selected based on different polarities, such as dist. water and methanol were considered polar solvents and chloroform and petroleum ether were referred to as non-polar solvents. For preparation of extracts, about 50g of each powder of *P. roxburghii* bark, roots, cones, and a mixture of these three parts weighed and soaked in around 300ml of solvents including dist. water, methanol, chloroform, and petroleum ether for eight days with intermittent shaking. After eight days, filtration was done by using Whatman No.1 filter paper. Each filtrate was collected in an empty glass conical flask and the powder was air dried for future use. All extracts were air dried and stored in a fridge (SVC-1000AY) at 4°C. (Shamim *et al.*, 2016).

Experimental Design: Antimicrobial Screening: The antimicrobial activity of the plant extracts including bark, roots, cones, and a mixture of these three components was tested against bacterial species such as *X. campestris* (003) and *P. alcaligence* (372) as well as fungal species including *A. alternata* (1174) and *F. solani* (1199).

These pure cultures were purchased from the First Fungal Culture Bank, Faculty of Agricultural Sciences, University of the Punjab, Lahore. The antibacterial and antifungal activities were investigated using well diffusion and disc diffusion techniques. The broth dilution technique was used to establish the MIC of extracts for bacteria and fungi. The MBC and MFC of extracts were then evaluated by using the disc diffusion technique at concentrations greater than MIC for bacteria and fungi.

Preparation of Microbial Plates: Bacterial species including *X. campestris* and *P. alcaligenes* were grown on Petri plates (9.5cm×1.2cm) containing Nutrient Agar (NA) medium overnight in an incubator for 2-days and fungal species including *A. alternata* and *F. solani* were grown on Potato Dextrose Agar (PDA) plates for 7-days by contaminating the plates with sterile inoculated loop for 7-days (C *et al.*, 2019).

Preparation of inoculum: After culturing, the bacterial inoculum was prepared from both bacterial isolates containing approximately a concentration of 10^8 cells/ml suspended in 10 ml normal saline solution instead of using 0.5 McFarland standard. The fungal inoculum was prepared from fungal isolates containing spores which were scraped off from Petri dishes using a sterile spatula and suspended in 10ml normal saline solution. The spore density of the fungus was approximately 10^6 spores/ml. Shake each test tube vigorously until turbid solutions were produced (Balouiri *et al.*, 2016; Gonelimali *et al.*, 2018)

Preparation of Media: NA was used for bacterial growth and PDA for fungal regeneration. 23g/L of NA and 39g/L of PDA were dissolved in 1 liter of distilled water. Both media were mixed well and heated. After boiling, media were sterilized in an autoclave at 121°C for 15min. It was cooled to about 45°C, then about 15-25 ml of each media was poured into sterile petri-dish under aseptic conditions, allowed to set the plates, and stored in the refrigerator (Sanusi *et al.*, 2015; Yanto *et al.*, 2019).

Preparation of Plant Extracts: Sixteen (16) plant extracts were prepared comprised of bark, roots, cones, and a mixture of these three parts at a concentration of 100mg/ml in distilled water, methanol, chloroform, and petroleum ether. Only four extracts including petroleum ether and aqueous extracts of roots and cones were selected based on maximum inhibitory effects (Sanusi *et al.*, 2015).

Screening methods for the determination of anti-microbial activity: Agar well diffusion method: In the agar, well diffusion method, 16 agar plates were inoculated aseptically by spreading a standard volume of microbial inoculum over the entire agar surface. Each agar plate was divided into four portions (bark, roots, cones, and a mixture of three parts) and labeled. Four NA plates were inoculated with *X. campestris,* and four NA plates with *P. alcaligenes.* Four PDA plates were inoculated with *A. alternata,* and the other four with *F. solani*. A hole with a diameter of 6 to 8 mm was punched aseptically using a sterile cork borer No.2 and wells were made and filled with a uniform volume of

each plant extract of about 100mg/ml (Massoud *et al*., 2019; Gonelimali *et al.*, 2018). All plates were incubated in an incubator (37°C for bacterial growth for 24 hours and 28°C for fungus for 7 days) (Shamim *et al.*, 2016)

Disc diffusion method: Sixteen agar plates (8 with NA and 8 with PDA) were prepared and inoculated aseptically with bacterial and fungal isolates by contaminating the plates with a sterile inoculated loop. Filter paper discs about 5mm in diameter were made with the help of paper puncture and placed in a 100ml conical flask. Then placed this flask in autoclave for sterilization. The sterilized discs were placed in each plant extract having a concentration of 100mg/ml. Label each Petri plate with plant extracts and microbial names. Now place all the discs impregnated with plant extracts at the center of each portion of agar plates (Sanusi *et al.*, 2015). Standard AMP discs (10µl) were placed on NA plates for the detection of antimicrobial activity against bacterial cultures such as *X.compestris and P.alcaligenes* whereas FCA (25µl) discs were placed on two PDA plates for fungal cultures *A. alternata* and *F.solani* (Massoud *et al.,* 2019). The antimicrobial agent diffuses into the agar medium and inhibited the germination and growth of microorganisms in a circular region. All plates were examined using a colony counter and the size of restricted growth zones was measured by using a measuring scale (Balouiri *et al.*, 2016).

Broth dilution method for Minimum Inhibitory Concentration: Dilution methods are more appropriate and quantitative methods to determine MIC values. MIC value is usually expressed in µg/mL or mg/L (Sanusi *et al.*, 2015). Broth dilution methods include micro and macro-dilution methods. Different concentrations of 1, 2, 4, 8, 16, and 32 µg/mL of four plant extracts were prepared by two-fold serial dilution. Take 24 test tubes, 12 test tubes were autoclaved with 2ml of NB and 12 with YMEB liquid growth media. Each test tube was inoculated with 1ml microbial inoculum suspension including *X. campestris*, *P. alcaligence*, *A. alternata* and *F. solani* in normal saline solution. In each tube, add 1ml of an antimicrobial agent such as extracts of roots and cones in dist. water and petroleum ether respectively at concentrations 3.125, 6.25, 12.5, 25, 50, and 100 mg/ml and were tested against all four pathogens. In the case of fungal species, concentrations for the determination of MIC were raised at 150, 175, 200, and 250 mg/ml. MIC was detected in the unaided eye (Balouiri *et al.*, 2016)

Preparation of Nutrient broth and Yeast Malt Extract Broth: NB media consisted of 28g of powder NB while YMEB media composed of yeast extract 1.5 g, malt extract 1.5 g, glucose 5 g, and peptone 2.5 g. NB and YMEB were separately dissolved in 1000 ml and 500 ml of distilled water respectively, stirred, heated, and sterilized by autoclave at 121°C for 15min (Sulistyaningtyas *et al.*, 2019)

Disc diffusion technique for MBC and MFC: To assess minimum bactericidal and fungicidal activities, three concentrations greater than MIC were used. For bactericidal effect 100, 125, and 150mg/ml concentrations and for fungicidal action, 200, 250, and 300mg/ml were chosen and the ZOI was measured by disc diffusion technique. Following the application of the disc, the inoculated plates were incubated under the conditions specified in the disc diffusion technique. Compare the zone of varied concentrations and observed conclusions (Balouiri *et al.*, 2016; Owuama, 2017)

Solvents: For the comparison, zones of pure solvent and the standard discs (Ampicillin and fluconazole) were also recorded (Shamim *et al.*, 2016).

STATISTICAL ANALYSIS

The findings were presented as the mean of three replicates Mean ± S.D. (M ± standard deviation). All outcomes were contrasted by (Snedecor, 1980) and significant differences among replicates were reported as Duncan's multiple range tests (Steel and Torrie, 1997) in the form of probability *<p>* values (0.05) using the computer software STATIX 8.1.

RESULTS AND DISCUSSION

Isolation of microbes (bacteria and fungi): Microbial isolates were detected on NA for bacteria and PDA for fungi as shown in (Table 1 and Figure 2) below.

Table 1. Physical appearance and morphology of desired microbes.

Sr. no.	Species	Morphology	Issuance code
1.	*X. campestris*	Small colonies and white	003
2.	*P. alcaligenes*	Small colonies and white	372
3.	*A. alternata*	Dark green hyphae in groups	1174
4.	*F. solani*	Hyphae of light pink to red	1199

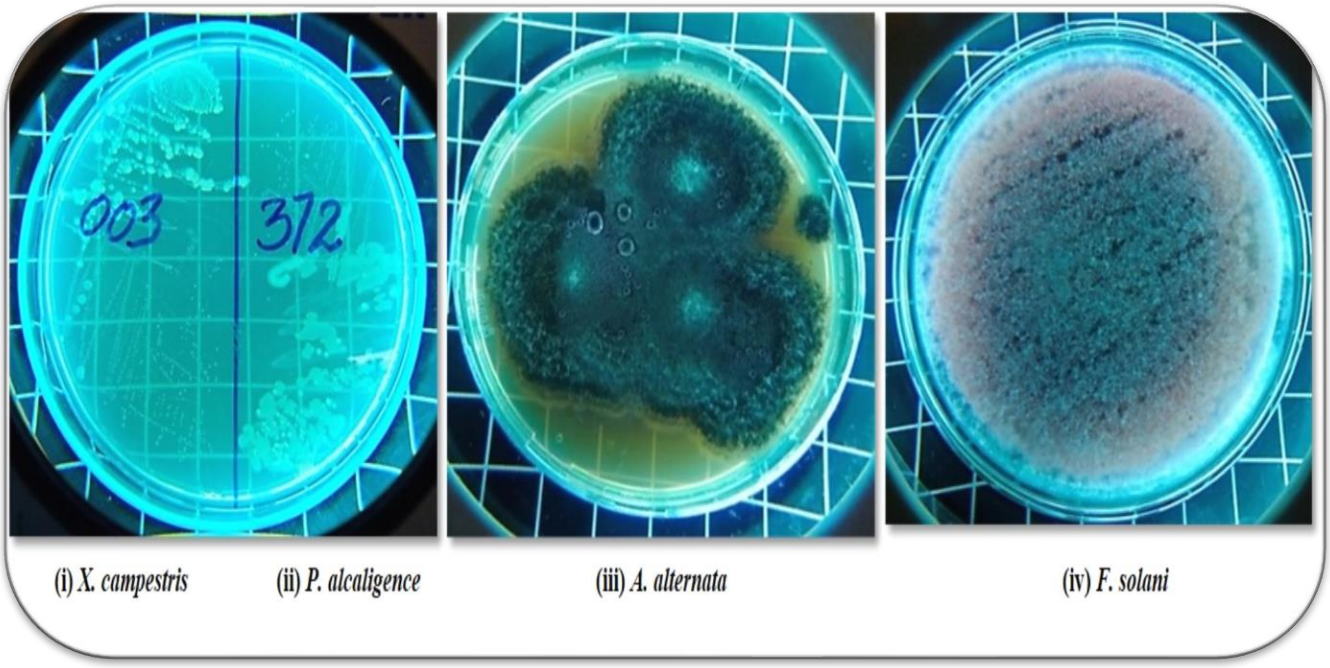

Figure 2. Representation of bacterial species such as *X. campestris* and *P. alcaligence* and fungal species including *A. alternata* and *F. solani*

Antibacterial Activity: The antimicrobial activity of plant extracts was assessed by agar well and disc diffusion methods. Among all extracts only petroleum ether extract of *P.roxburghii* roots and cones showed greatest value of ZOI, i.e., 22±1^{a}, 18±1^{bc} and 18±1^{bcd} and 20±1^{b} against *P. alcaligenes* and *X. campestris* respectively by well diffusion technique as shown in (Figure 3,4,5 and Table 2). Cones methanolic extract also effective to kill *X. campestris* and manifest ZOI; 19±1^{bc}. The preliminary phytochemical screening of the successive extracts prepared from *P. roxburghii* bark, roots, cones and a mixture of these three parts indicated the presence of carbohydrates, alkaloids, glycosides, flavonoids, steroids, terpenoids, phenolic compounds and tannins, fixed oils and fats, volatile oils and saponins by which the broad spectrum of antibacterial activity recorded. It was confirmed by the subsequent extracts by petroleum ether, chloroform, methanol and dist. water. The bioactive compounds of petroleum ether extracts showed more efficacy against pathogenic microbes. It means petroleum ether was best solvent for extraction of phytopharmaceuticals including alkaloids, steroids, and terpenoids, as compared to other solvents. Because these compounds strongly indicated in case of petroleum ether roots and cones extracts by phytochemical testing that may exhibited cytotoxic, and antimicrobial actions (Abdel-Raouf *et al.*, 2015). Alkaloids considered as heterocyclic nitrogen compounds that showed putative antibacterial mechanism by inhibition of cell division protein, the cell function of bacteria through damaging the cell structure, as well as protein and DNA synthesis inhibitors that result in bacterial death. Phenolic compounds revealed different mode of action against bacterial species by binding with the cell membrane and caused inhibition of cell wall biosynthesis. This compounds also cause inhibition of certain critical enzymes including urease, sortase A and dihydrofolate reductase. Terpenoids taken as an important compounds of herbal resins showed antibacterial action by increasing its fluidity and permeability, changing the molecular structure of bacterial proteins and produced abnormality across the respiration chain (Khameneh *et al.*, 2019). Petroleum ether extract of *P.roxburghii* roots has strong antibacterial action 22±1^{ab} against *P.alcaligenes* than cones do 20±1^{c} against *X.campestris*. All extracts were tested against bacterial species but four extracts showed the highest values of ZOI while the remaining extracts showed less or no ZOI against bacterial cultures (Rubab *et al.*, 2022; Shamim *et al.*, 2016) as shown in (Figure 3 and Table 2).

Table 2. Antibacterial activity of plant parts including bark, roots, cones, and a mixture of these parts in various solvents (distilled water, methanol, chloroform, and petroleum ether) was measured by well diffusion and disc diffusion techniques.

Sr. No.	Plant parts	Solvents	Zone of inhibition (mm)			
			Well diffusion method		Disc diffusion method	
			X.campestris	*P.alcaligenes*	*X.campestris*	*P.alcaligenes*
1.	Bark	Dist. water	13±1	×	×	×
		Methanol	×	×	×	×
		Chloroform	×	×	×	×
		Petroleum ether	11±1	11±2	×	×
2.	Roots	Dist. water	×	×	×	×
		Methanol	×	×	×	8±2
		Chloroform	12±1	10±1	×	7±2
		Petroleum ether	18±1^{bc}	22±1^{a}	16±1	×
3.	Cones	Dist. water	×	×	×	×
		Methanol	19±1^{bc}	12±1	×	×
		Chloroform	16±1	11±2	×	9±1
		Petroleum ether	20±1^{b}	18±1^{bcd}	×	×
4.	Mixture	Dist. water	×	×	×	×
		Methanol	×	×	×	×
		Chloroform	13±2	12±1	×	×
		Petroleum ether	18±2	13±2	×	×

Values in columns followed by the letters ^{a} and ^{b} are significantly different at $P<0.05$, analyzed by DMRT test; values are means of three replicates.

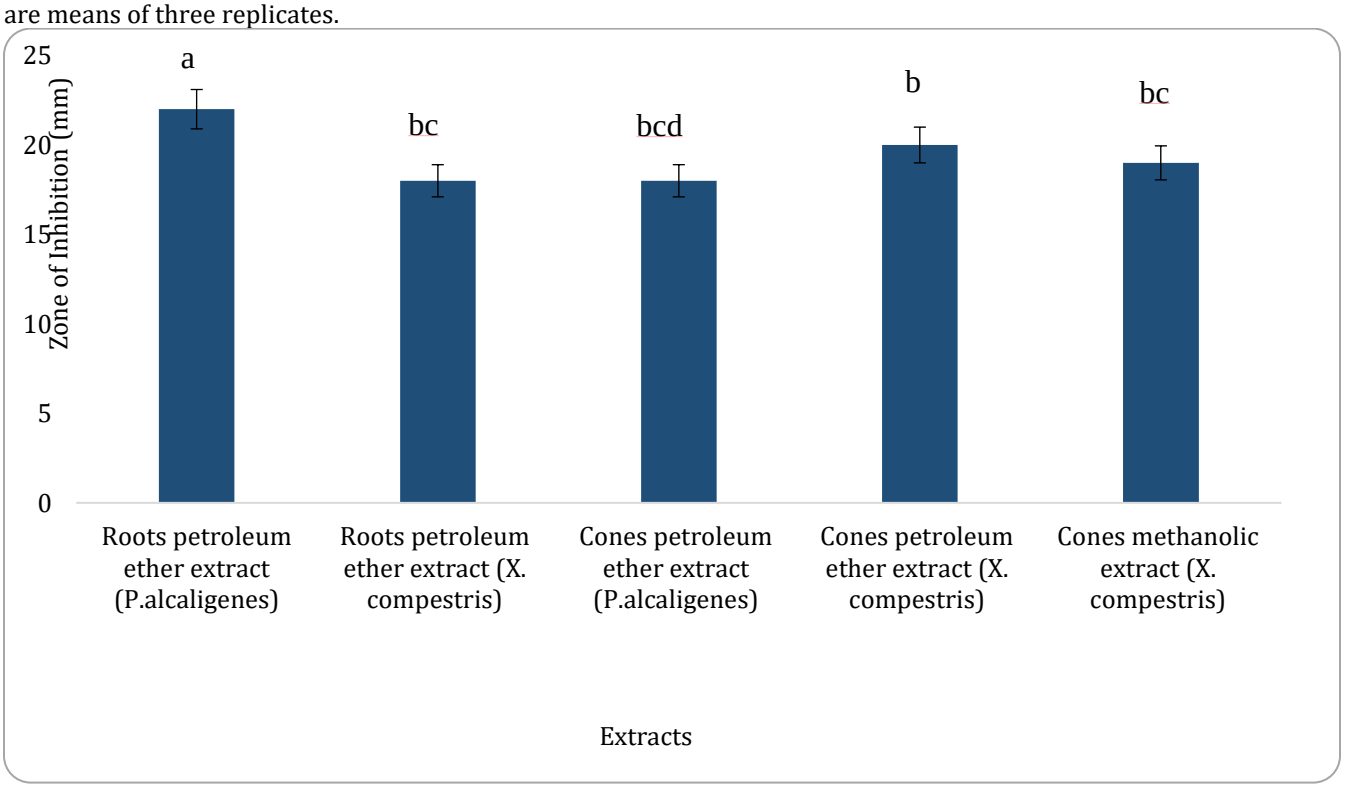

Figure 3. Graphical presentation of ZOI produced by roots and cones extracts against *P. alcaligenes*, *X. campestris* respectively and cones methanolic extract against *X. campestris*.

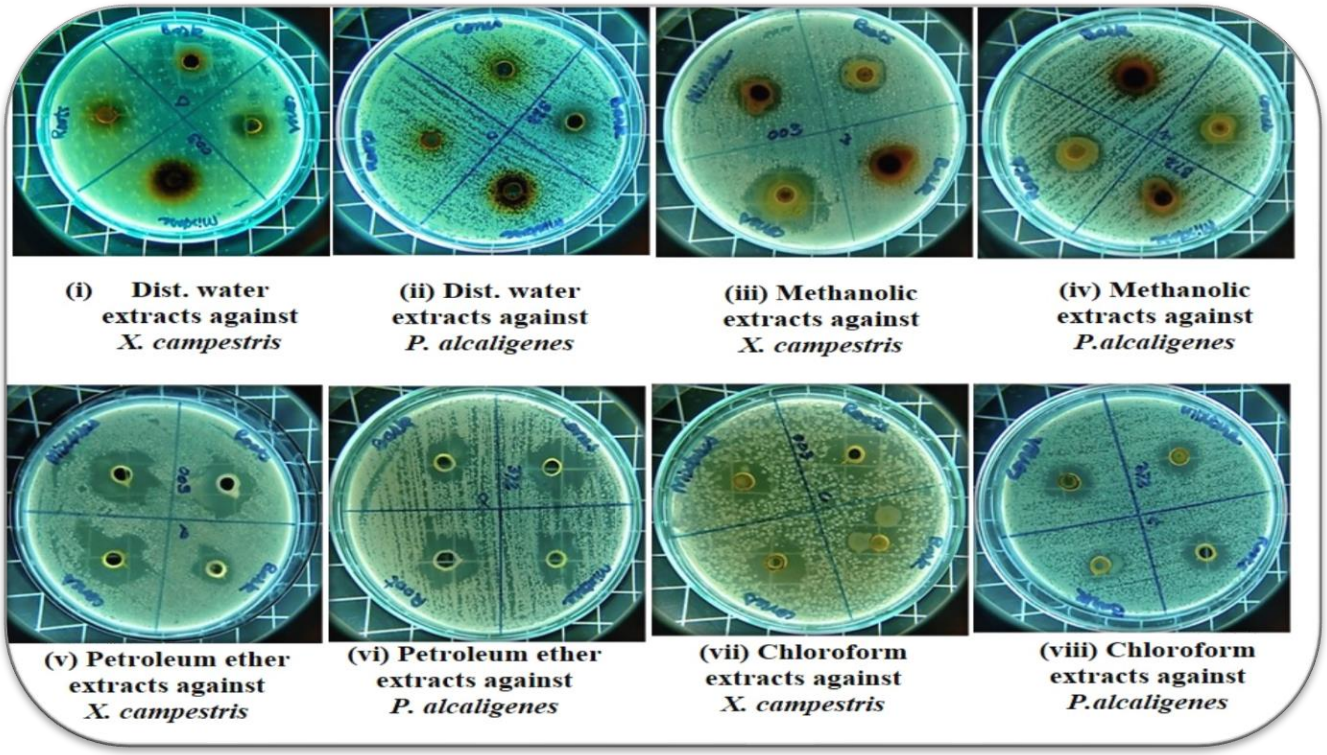

Figure 4. Antibacterial activity of plant extracts in (i), (ii) dist. Water, (iii) and (iv) methanolic, (v) and (vi) petroleum ether, (vii) and (viii) chloroform extracts against *X. campestris* and *P. alcaligenes* respectively by agar well method.

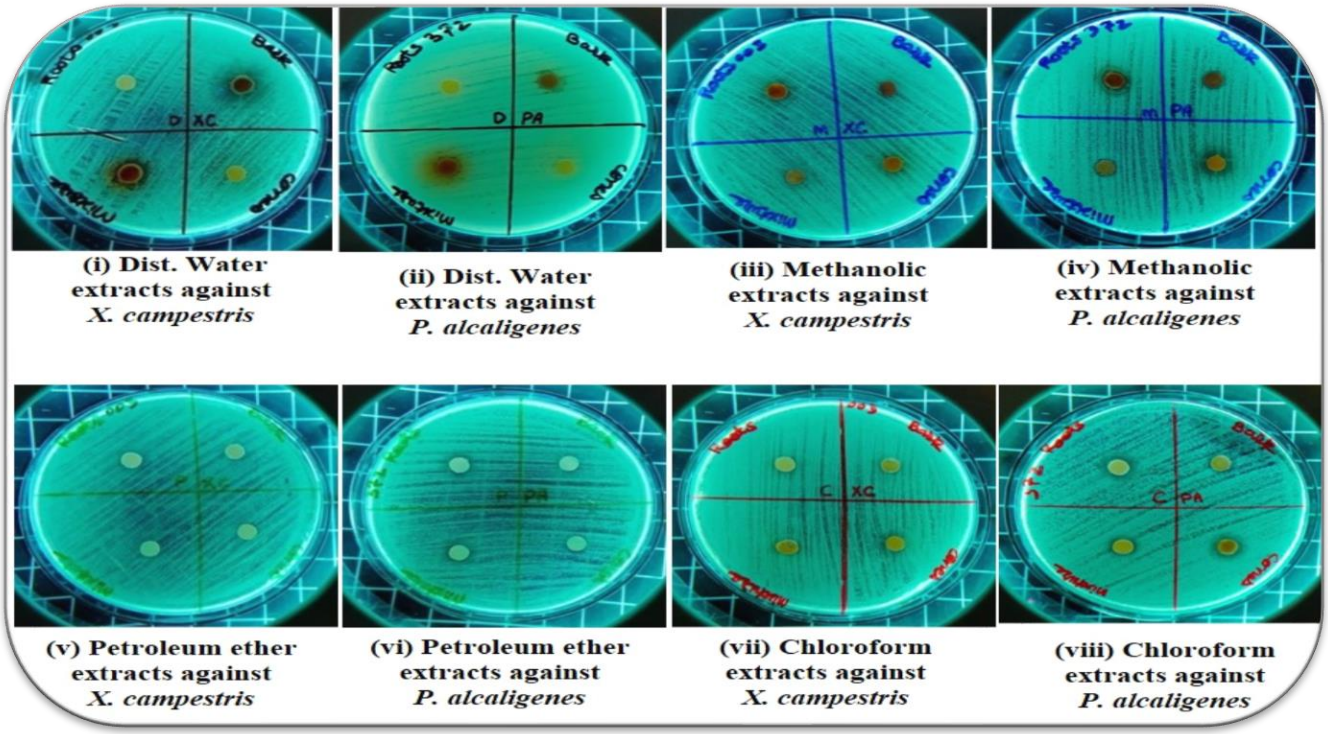

Figure 5. Antibacterial activity of plant extracts in (i), (ii) dist. Water, (iii) and (iv) methanolic, (v) and (vi) petroleum ether, (vii) and (viii) chloroform extracts against *X. campestris* and *P. alcaligenes* respectively by agar disc method.

Antifungal Activity: This activity of plant extracts was assessed by agar well and disc diffusion methods. Among all extracts only dist. water extract of *P.roxburghii* roots and cones created the maximum value of ZOI, i.e., 23±1[a] and 14±1[d] against *A. alternata* by well diffusion and disc diffusion technique as shown in (Figures 6,7,8 and Table 3). The antifungal activity of *P. roxburghii* was studied against *A. alternata* and *F. solani*. The aqueous roots and cones extract showed maximum activity against *A. alternata*. Alkaloids were taken as naturally occurring chemical compounds containing basic nitrogen atoms. Flavonoids enhanced the effects of Vitamin C and function as antioxidants. They are also known to be biologically active against liver toxins, tumors, viruses, and other microbes (bacteria and fungi). Plant terpenoids have been used extensively for their aromatic qualities. They play a role in traditional herbal medicines and are now under investigation for pharmaceutical, antineoplastic and antibacterial properties. Tannins have shown potential antiparasitic, antibacterial, and antiviral effects. The antifungal activity was screened because of its great medicinal properties for pathogenic organisms. The medicinal plant *P. roxburghii* showed good antifungal potential (Patel *et al.*, 2014). *P. roxburghii* predominantly showed antimicrobial activity due to presence of essential oils containing Oleoresin, α -pinenes, β-pinenes, terpenes, alkaloids, and phenols. Oleoresin further composed of a complex mixtures of sesquiterpenes, diterpenes, monoterpenes, and also oxygenated form of these three compounds. α -pinenes, and β-pinenes are active against fungi due to their capability to induce toxic effects on membrane structure and function. These compounds caused abnormality in the transportation of ions including K^+, H^+, Ca^{+2} and Na^+ etc that damaged the fungal cells due to outflow of ions and other cell components. A significant loss of cell contents or essential molecules and ions will result in cell death. Monoterpenes due to their lipophilic nature, easily cross the membrane structures and mitochondria, and caused membrane expansion, enhanced membrane fluidity, and inhibition of membrane-embedded enzyme that inhibited the fungal cell growth (Ayub *et al.*, 2022). Cones with methanol and a mixture of bark, roots, and cones with dist. water showed fewer values of ZOI like 12±1 and 11±1 against *A.alternata* respectively. Dist. water was the best solvent due to its greater affinity with polar constituents that showed the greatest antifungal action. However, bark and mixture of bark, roots, and cones also showed maximum % age yield in dist. water but didn't show an antimicrobial effect considerably. *F.solani* also has no or less inhibitory effect by extracts. Only petroleum ether cones extract created ZOI 7±1 and mixture dist. water extract showed ZOI 10±1 against *F.solani* (Rubab *et al.*, 2022).

Table 3. Antifungal activity of plant parts including bark, roots, cones, and a mixture of these parts in various solvents (distilled water, methanol, chloroform, and petroleum ether) was measured using disc diffusion techniques.

Sr. No.	Plant parts	Solvents	Zone of inhibition (mm)			
			Well diffusion method		Disc diffusion method	
			F.solani	*A.alternata*	*F.solani*	*A.alternata*
1.	Bark	Dist. water	×	×	×	8±2
		Methanol	×	×	×	×
		Chloroform	×	×	×	×
		Petroleum ether	×	×	×	×
2.	Roots	Dist. water	×	23±1[a]	×	×
		Methanol	×	×	×	×
		Chloroform	×	×	×	×
		Petroleum ether	×	×	×	×
3.	Cones	Dist. water	×	×	×	14±1[b]
		Methanol	×	12±1	×	×
		Chloroform	×	×	×	×
		Petroleum ether	×	×	7±1	×
4.	Mixture	Dist. water	×	11±1	10±1	×
		Methanol	×	×	×	×
		Chloroform	×	×	×	×
		Petroleum ether	×	×	×	×

Values in columns followed by the letters [a] and [b] are significantly different at $P< 0.05$, analyzed by DMRT test; values are means of three replicates.

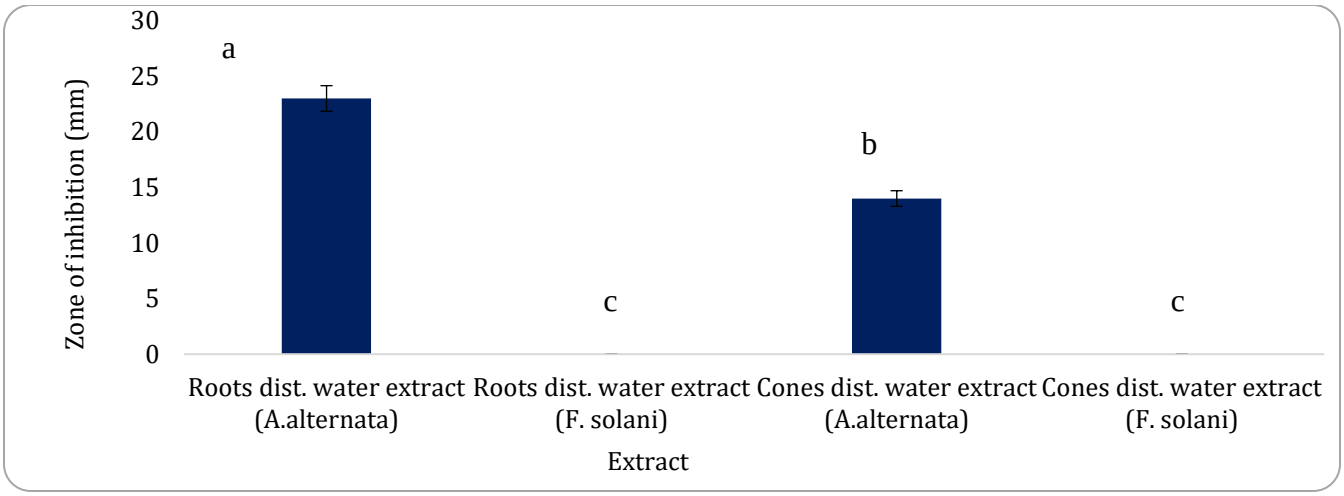

Figure 6. Graphical presentation of ZOI produced by roots and cones dist. water extracts against *A.alternata*.

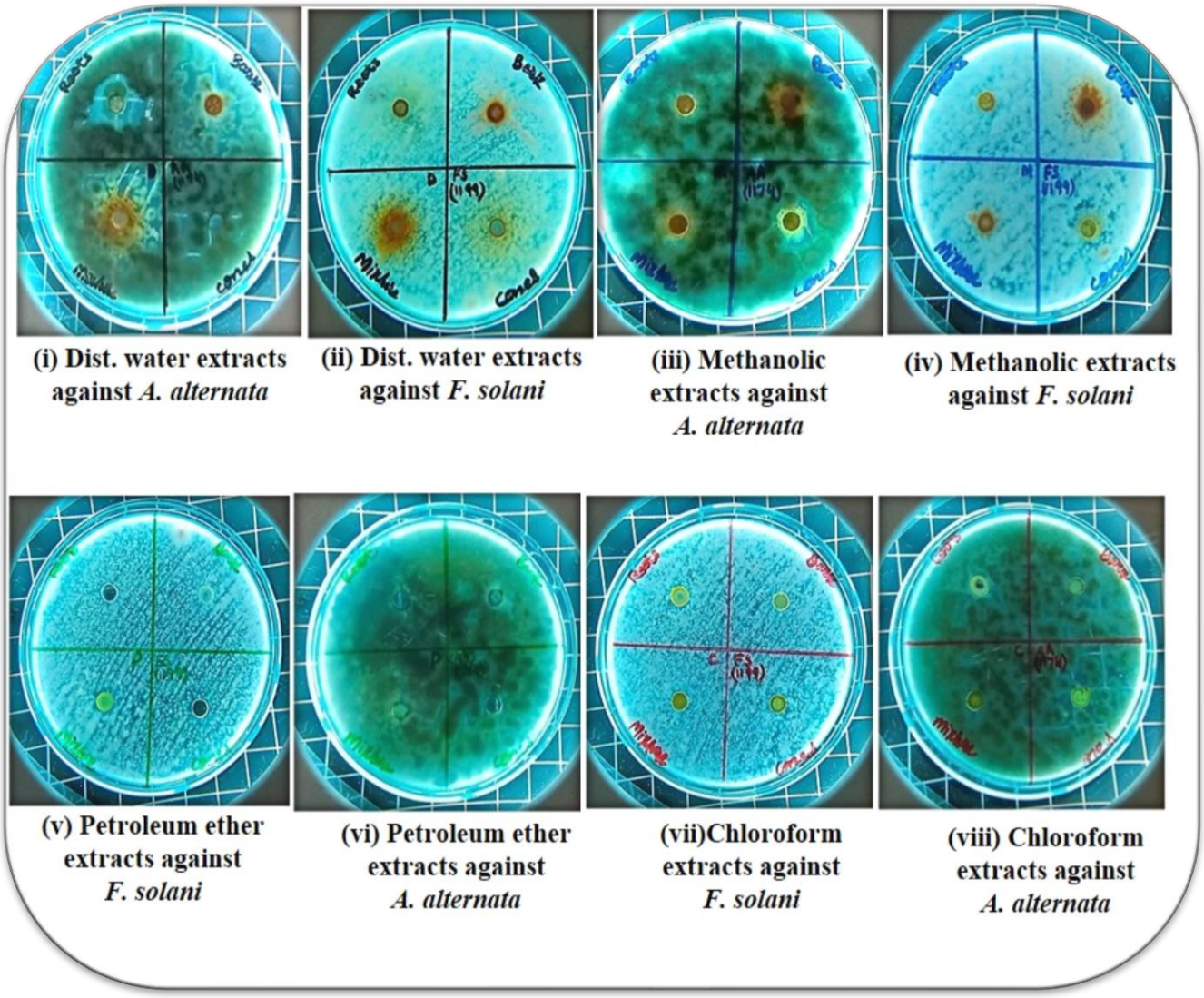

Figure 7. Antifungal activity of plant extracts in (i), (ii) dist. Water, (iii) and (iv) methanolic, (v) and (vi) petroleum ether, (vii) and (viii) chloroform extracts against *A.alternata* and *F.solani* respectively by agar well method.

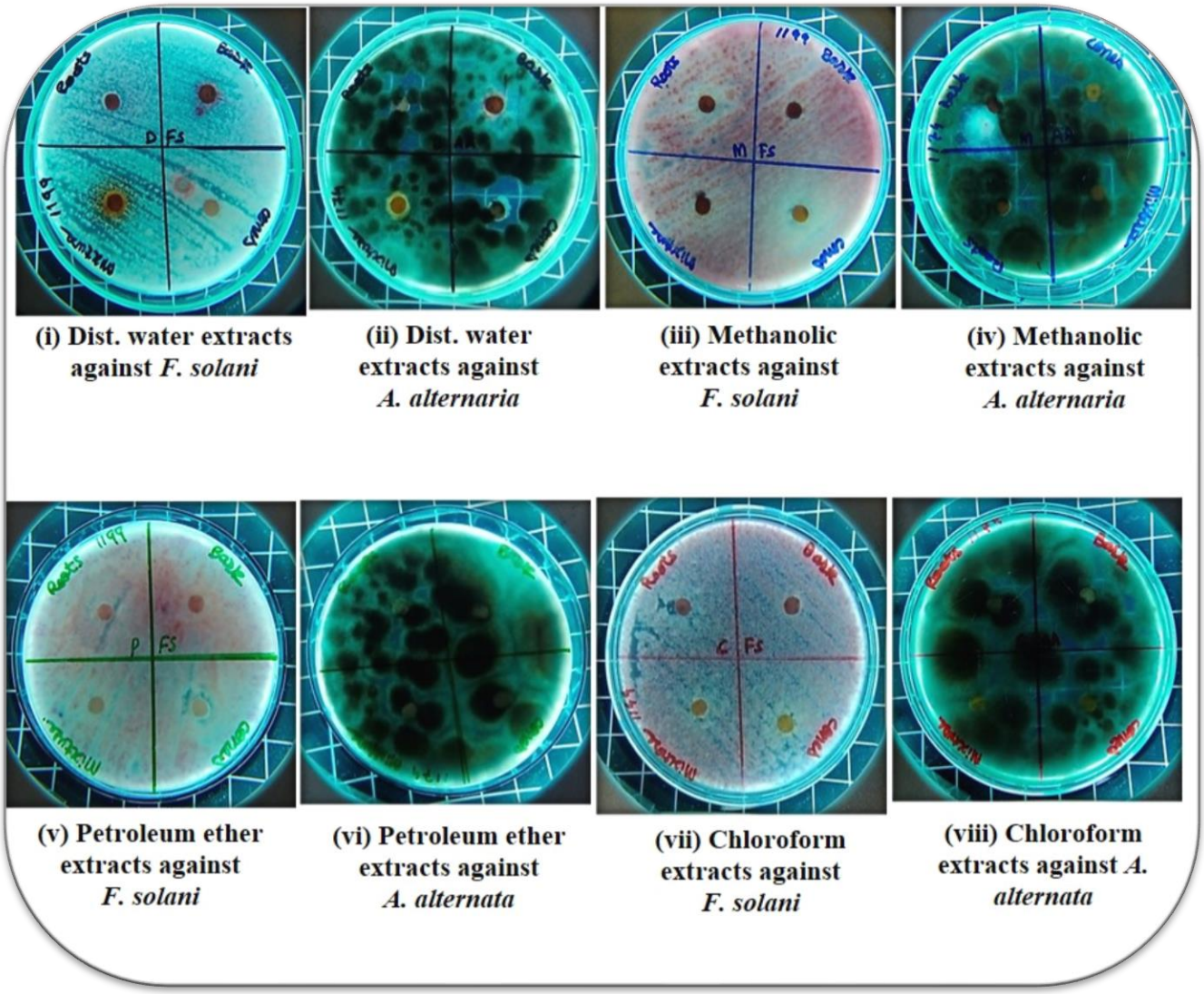

Figure 8. Antifungal activity of plant extracts in (i), (ii) dist. Water, (iii) and (iv) methanolic, (v) and (vi) petroleum ether, (vii) and (viii) chloroform extracts against *A.alternata* and *F.solani* respectively by agar disc method.

Statistical analysis revealed that there was no significant difference between petroleum ether root extract[a] against *P.alcaligenes* and petroleum ether cones extract[b] against *X.campestris*. Whereas significant antifungal difference was found between dist. water roots extract[a] and cones extract[b] against *A.alternaria* at P-value *P*<0.05 analyzed by DMRT test which considered as means of three replicates.

Standard discs Ampicillin and fluconazole were employed to compare petroleum ether roots and cone extracts against *P. alcaligenes* and *X. campestris* and aqueous extracts of roots and cones against *A. alternata* respectively. Fluconazole disc was also applied against *F. solani,* it showed no inhibitory action as shown in (Table 4).

Table 4. Ampicillin and Fluconazole showed inhibitory zones (mm) against selected pathogenic species.

Standards	Concentration	Pathogens	ZOI (mm)
Ampicillin	AMP10	*X. campestris*	26±1[a]
		P. alcaligenes	7±2[c]
Fluconazole	FCA25	*A.alternata*	22±1[b]
		F.solani	×[d]

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

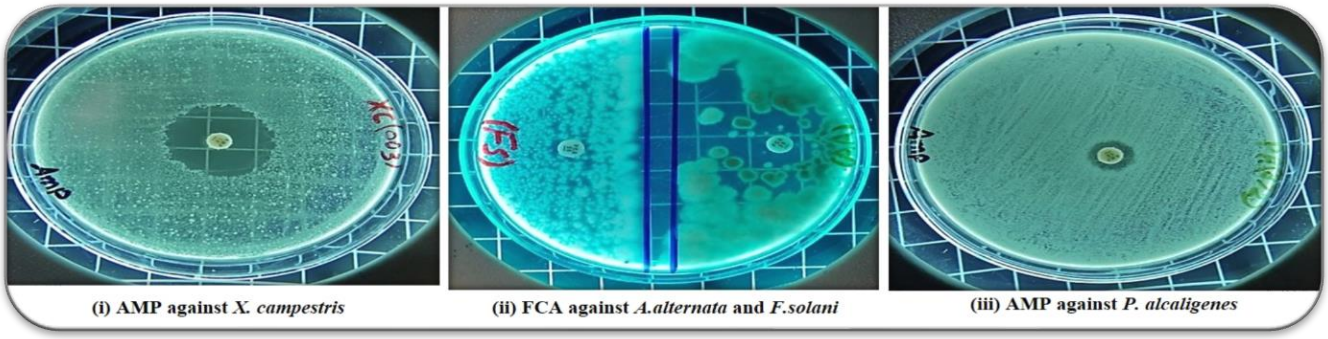

Figure 9. ZOI (mm) showed by standard discs AMP and FCA against bacterial species such as (i) *X. campestris,* (iii) *P. alcaligenes* and fungal species such as (ii) *A. alternata* and *F. solani*.

The statistical graphical presentation of above mentioned standard discs is represented as given below.

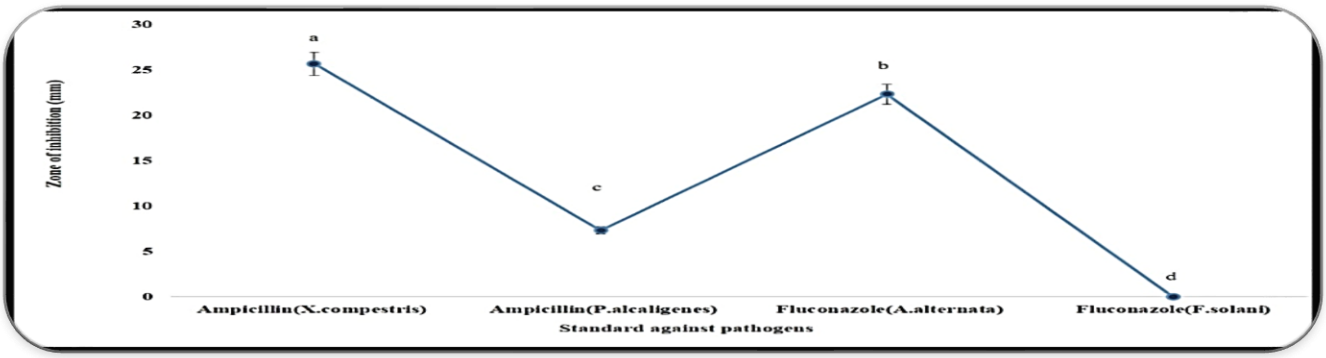

Figure 10. Graphical presentation of statistical analysis of AMP and FCA against desirable microbes.

DMRT- statistical analysis for standard demonstrated that there was significant difference between anti-bacterial including AMP discs against *X. campestris*[a] and *P. alcaligenes*[c] and anti-fungal discs including FCA against *A. alternata*[b] and *F. solani*[d]. There was no significant difference between AMP discs against *X. campestris*[a] and FCA against *A. alternata*[b] (Shamim *et al.*, 2016)

Firstly, we determined MIC at 1, 2, 4, 8, 16, and 32µg/ml but bacterial and fungal growth was not inhibited at these concentrations. As a result, we increased doses in mg via successive dilutions of extracts such as 3.125, 6.25, 12.5, 25, 50, and 100mg/ml. Petroleum ether roots and cones extracts were potent against *P.alcaligenes* and *X.campestris* at 50mg/ml respectively. However, fungal growth was not prevented at these doses. Then, we further increased the concentrations to 150, 175, 200, and 250 mg/ml. At 200mg/ml, both root and cone aqueous extracts showed a clear solution with no fungal growth against *A.alternata* as shown in (Table 5 & 6). Similarly, MIC elaborated by serial dilutions was the lowest concentration of an antimicrobial agent that inhibited the growth of any micro-organism in tubes or microdilution wells as detected by the unassisted eye (Balouiri *et al.*, 2016). The development of DTM made microbes dormant (but not killed) by a particular concentration of plant extracts. Interestingly, the new DTM and DM give the same results, but DTM has the advantage that it is less expensive, less time-consuming, and less stressful as no nutrient agar plates are required (Chikezie, 2017).

Table 5. Minimum inhibitory concentration of *P. roxburghii* petroleum ether and aqueous extracts of roots and cones.

Sr.No.	Solvent	Sample	Pathogens	MIC (mg)					
				100	50	25	12.5	6.25	3.125
1.	Petroleum ether	Roots	*P.alcaligenes*	×	×	✓	✓	✓	✓
2.		Cones	*X.campestris*	×	×	✓	✓	✓	✓
3.	Dist. Water	Roots	*A.alternata*	✓	✓	✓	✓	✓	✓
4.		Cones	*A.alternata*	✓	✓	✓	✓	✓	✓

✓ and × Showed the existence of turbidity (presence of microbes) and non-turbidity (absence of microbes) respectively.

Table 6. Minimum inhibitory concentration of *P. roxburghii* petroleum ether and aqueous extracts of roots and cones.

Sr.No.	Solvent	Sample	Pathogens	MIC(mg)			
				250	200	175	150
1.	Dist. water	Roots	*A.alternata*	×	×	✓	✓
2.		Cones	*A.alternata*	×	×	✓	✓

✓ and × Showed the existence of turbidity (presence of microbes) and non-turbidity (absence of microbes) respectively.

At these doses, fungal growth was not suppressed. Then, concentrations were increased up to 150, 175, 200, and 250mg/ml. A clear solution with no fungal growth was obtained at 200mg/ml. It was assumed that the bacterial MIC was concluded as 50mg/ml and the fungal MIC was 200mg/ml.

After that, we determined MBC and MFC at higher doses than MIC. We take 100, 125, and 150mg/ml petroleum ether roots and cones extract against *P.alcaligenes* and *X.campestris,* respectively, to determine MBC. By increasing concentrations, the value of the ZOI also increased. Petroleum ether roots and cones extracts had ZOI (9±1, 10±1, and 15±1) against *P.alcaligenes* and (9±1, 10±1, and 11±2) against *X.campestris* respectively. For MFC, the ZOI at doses of 200, 250 and 300mg/ml was (9±1, 22±2, and 22±2) and (6±1, 15±1, and 22±1) for roots and cones aqueous extract against *A.alternata* was observed respectively as shown in (Figure 9, Table 7). A similar work conducted in 2020 demonstrated that MBC and MFC are considered as the lowest concentration of an antimicrobial agent required to kill 99.9% of the final inoculum with no observable growth under standardized circumstances (Siddique *et al.*, 2020)

Table 7. Minimum bactericidal and Minimum fungicidal concentration of four distinct extracts against desirable microorganisms.

Parameters	Petroleum ether extracts for MBC (mg)						Dist. water extracts for MFC (mg)					
	Roots			Cones			Roots			Cones		
	P.alcaligenes			*X.campestris*			*A.alternaria*			*A.alternaria*		
Conc.(s)	100	125	150	100	125	150	200	250	300	200	250	300
ZOI (mm)	9±1	10±1	15±1	9±1	10±1	11±2	9±1	22±2	23±2	6±1	15±1	22±1

MBC and MFC showed inhibitory zones by *P. roxburghii* petroleum ether and aqueous extracts of roots and cones

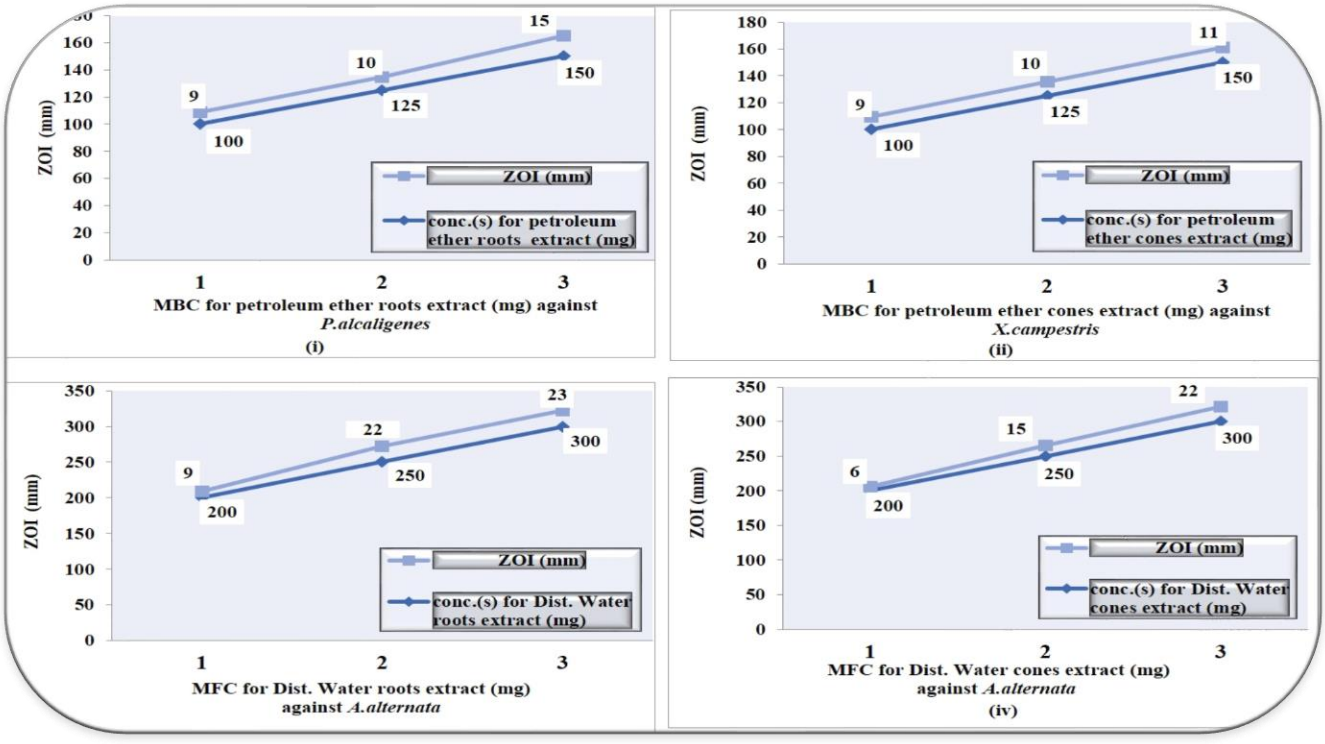

Figure 11. Graphical representation of the concentration-dependent killing effect of selected extracts against microbes.

Solvents:

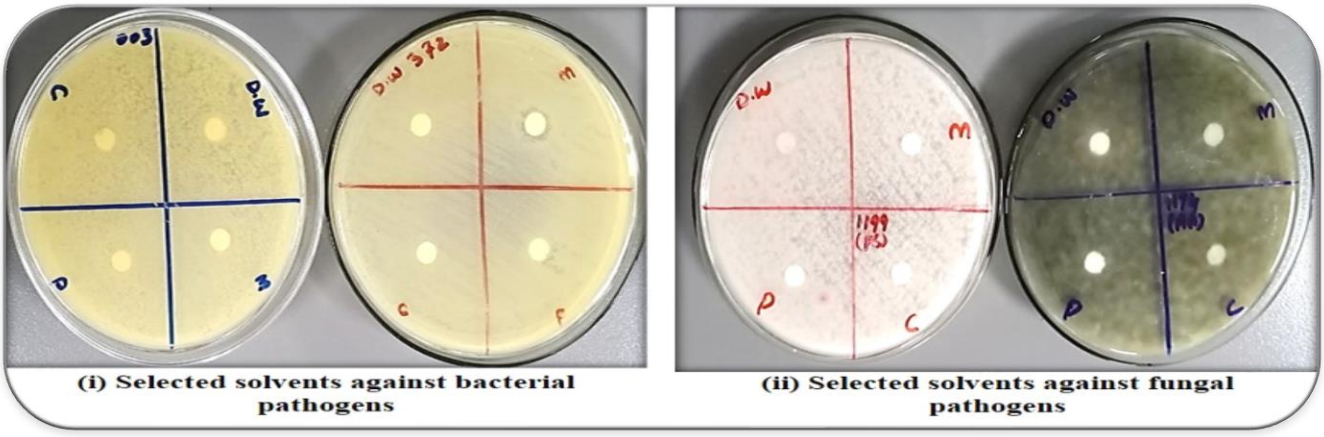

Figure 12. (i) and (ii) represented the effect of pure solvents including distilled water, methanol, chloroform, and petroleum ether against four pathogens.

CONCLUSION

The results of the present study showed that the roots and cones of *P. roxburghii* possess antimicrobial potential that can be attributed in treating many infectious diseases. The objective of the current study was pharmacognostic studies and assessment of antimicrobial activity of naturally occurring medicinal plant. The pharmacognostic study of *P. roxburghii* bark, roots, cones, and a combination of these three parts in different solvents (polar and non-polar) as well as FTIR analysis provided may give important information for its identification. So that it may be scientifically shown to access the plant's pharmacological reactions to determine its folkloric applications. More research is needed to open up new paths for the usage of this plant, with an emphasis on the separation of various components for medicinal purposes.

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Zeenat Aman	:	conceived the idea, planed the research design, responsible for conducting the antimicrobial assays and interpreted the results.
Atika Afzal	:	conceived the idea, planed the research design, responsible for conducting the antimicrobial assays and interpreted the results.
Fozia Masood	:	contributed to the development the research design and assisted to conduct the research work
Ayesha Mahmood	:	assisted in manuscript draft and proofreading
Waheed Anwar	:	helped in data analysis