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In-vitro antioxidant and anti-microbial potential of the pollen extracts of *Pinus roxburghii* Sarg

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Abstract

Pollen grains of *Pinus roxburghii* Sarg. Possess antioxidant and antibacterial potential. To test this hypothesis, pollen dust was collected from the *P. roxburghii* trees of the University of Malakand, Dir Lower Campus and its methanolic crude extracts at various concentrations (i.e. 1000, 500, 250, 125 and 62.50 µg/mL) were prepared and tested for antioxidant activity using DPPH and ABTS methods. The possible antimicrobial potential of the pollens was also tested using Ager Disc Diffusion method against seven different pathogenic bacterial strains i.e. *Proteus mirabilis, Staphylococcus aurous, Escherichia coli, Bacillus cereus, Salmonella typhi, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Results obtained for antioxidant activity show that in the free radical scavenging activity the DPPH value for 1000µg/mL was the highest with the value of 81.70±0.74, While the ABTS value was 78.30±0.15 for the same concentration. The IC_{50} values of the pollen extract of the ABTS was 23.46 µg/mL while the DPPH was 15.98µg/mL. The most sensitive bacteria recorded in the antimicrobial activity for the concentration of 100µg/mL of the pollen extract was *Pseudomonas aeruginosa* (8.666± 0.71) using the mean± standard deviation value as a measure followed by *Bacillus cereus*(5.666± 4.24), *Escherichia coli* (5.333± 0.71) and *Staphylococcus aurous* (4.333± 0.71). The ANOVA results obtained for the 50µg/mL of methanolic pollen extract in comparison with the controlled 50mg/mL of antibiotic (Ciprofloxacin),show a significant difference between their action of inhibition with the calculated P value being much smaller (i.e. 0.00) than the alpha level of 0.5. We conclude by reporting it for the first time that *P. roxburghii* pollens have antioxidant and antibacterial potential and can possibly be exploited as a food and medicine source.

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Introduction

Pinus roxburghii Sarg. is commonly known as the long needle pine, long-leaved Indian pine or Chir pine, belongs to the family Coniferae. It is an indigenous tree species of the outer Himalayas and prevalent in the Hindukush and Himalayan ranges. In Pakistan, trees of Chir pine are found in Murree, Hazara, Khyber, Orakzai and Malakand Agencies (Ali *et al.*, 2014). In Khyber Pakhtunkhwa (KP) and Punjab's Northern areas, Chir pine trees are easily cultivated. In different areas of KP like Mansehra, Balakot, and Abbottabad, plantations of these trees have been carried out on a huge scale in the reforested and afforested lads (Khan *et al.*, 2014). Pines germinate undomesticated species but in nurseries, it is also cultivated for plantation and propagation due to its relative adoptability features (Ishtiaq *et al.*, 2015).

Several parts of the plants have been used in traditional system of medicine for coughs, colds, influenza, tuberculosis, bronchitis, antiseptic, diaphoretic, diuretic, rubefacient, stimulant, and vermifuge (Chopra *et al.*, 1986). A bark paste is used in burns and scalds. Resin is applied on boils and gastric troubles (Manandhar, 2002). Wood oil is used as a nerve tonic, hemostatic, expectorant, and diuretic. Bark is used for skin diseases, burns, and cracks (Bajracharya, 1979; Dash and Gupta, 1994).

In gymnosperm and angiosperms, the pollen gains are small structure soften referred as the dust (Arruda *et al.*, 2013), in plants pollen grains are present in the male gametes (Basim *et al.*, 2006), and the pollens are transmitted to female sex organ of the plants (i.e. flower) for their purpose (Arruda *et al.*, 2013).

Pollen is farinaceous to rough fine-grained material produced in the male cones in abundance during spring and early summer. These pollen grains are the male gametophytes of the seeded plants. Pollination occurs when the pollen grains move to the female reproductive structure pollen grains of *Pinus* trees are very minute and fine. Balloon-like air sacs are present which help the pollen grains in buoyancy (Chary and Reddy, 2003). It is very easy to identify the pollen grains of Pine because they have sacs which make them big in size. Non-enzymatic anti-oxidants as pro-vitamin A, B complex, C, D, and E plus which provide minerals and amino acids are present in a large amount in these pollen grains. Seemingly, there is an extreme protection of pine pollen against radioactive caesium which is becoming visible in dairy and other foods in the U.S (Dixit *et al.*, 2016).

The chemical composition of pollen can vary because of their organic compounds and their environmental origin (Abarca *et al.*, 2004). Most of the pollen part contain carbohydrate, protein, starches, and lipids are amount are successfully among55%,13%,40%,20%,0.3%, 10% and 1% individually (Villanueva *et al.*, 2002). Different minor component and minerals are found i.e. phenolic compound, sterols, vitamins, carotenoids, terpenes and flavonoids (Bogdanov, 2011). Aspartic acid, proline, phenylalanine and glutamic corrosive are the essential amino acids in pollen grain (Roldan *et al.*, 2011).

In the present investigation, the primary objective of the study was to evaluate antioxidant and antibacterial potential of *Pinus roxburghii* pollens and to open a new venture for further research in this area which could potentially open ways for its use in the future medicine and food industry.

Material and methods

Pollens grains were collected from the main campus of the University of Malakand, Dir lower Khyber Pakhtunkhwa Pakistan during spring and early summer 2017.

The collected powder material (200gm) was added to 80% methanol and 20% distal water in a glass container and was constantly stirred for 7 to 9 days to extract the pollen contents. Then the extract was added to more solvent to get precise concentrations and were stored at 5°C for further analysis.

DPPH radical scavenging assay

The DPPH (organic chemical compound 2,2-diphenyl-1-picrylhydrazyl) scavenging activity was carried out after Kamal *et al.* (2015). DPPH (2 mg) was dissolved in 100 mL of methanol to get DPPH solution. The stock solutions were prepared to the concentrations of 1000, 500, 250, 125, 62.5µg/mL. The various concentrations of the methanolic pollen solution were mixed with 3 mL of DPPH solution and left for incubation at 23°C for 30 minutes and afterwards the absorbance was measured with the help of a spectrophotometer at 517 nm. For positive control, ascorbic acid was used and was processed through the same spectrophotometer. Every trial was taken in triplicates and the information acquired was assessed as mean ± S.E.M. The percent radical scavenging action was computed utilizing the following formula:

$$\text{Percent DPPH scavenging Potential} - \frac{\text{Control absorbance} \quad \text{sample absorbance}}{\text{control absorbace}} \times 100$$

ABTS free radical scavenging assay

Antioxidant potential of the pollen extracts was also evaluated using free radical scavenging activity of 2, 2-azinobis [3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) following standard procedure (Ullah *et al.*, 2015). ABTS 7 mm and potassium persulfate 2.45 m Msolution were mixed thoroughly. For producing free radicals the solution were placed in dark all night. 50% methanol was added to ABTS solution to adjust the absorbance 0.7 to 745nm after incubation. 3 mL of ABTS solution was added to test samples (300 µl) and solution was transferred to cuvette. By using a double beam spectrophotometer for six minutes to measure the absorbance. Ascorbic acid was used for positive control. The data was recorded in triplicate and percent ABTS free radicals scavenging activity was calculated as follows:

$$\text{percent ABTS scavenging effect} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

Estimation of IC_{50} values

IC_{50} values of DPPH and ABTS were calculated from those response ratios using Microsoft excel program.

Anti-Bacterial assay

Standard nutrient media was used for the stock bacterial strains to grow. The average viable count per ml of the stock cultures having Bactria *Proteus mirabilis, Staphylococcus aureus, Escherichia coli, Bacillus cereus, Salmonella typhi, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was carried out using surface viable count technique. Aliquots of approximately 108-109 colony-forming units (CFU) per ml were used and each time fresh stock suspension was made (Sofowara, 1993). Using Agar disc diffusion method (Sofowara, 1993), extracts were evaluated for antimicrobial potentials. Calibrated bacterial inoculums (108-109 CFU/ml) were mixed with 60ml sterile nutrient Agar maintained at 55 C° and then transferred aseptically to sterilized petri plates that were left for a while to solidify in laminar flow hood. Next, using a sterile cork borer approximately 7 mm wells were punched equidistantly from each other. The disc was placed in plats with concentration 50 mg/ml and 100mg/ml of pollen extract. Plates were kept in refrigerator for 45 minutes to allow the extracts to diffuse in seeded nutrient agar plates. The plates were incubated at 37C° overnight in upright position. Controls included standard antibiotics as well as the solvent used for extraction. The tests were done in triplicates and average zones of inhibitions were measured in millimeters (Chouhan *et al.*, 2002).

Statistical analysis

Results were obtained using statistical mean and ± standard deviation (SD), mean ± standard error (mean± SEM) of three replicates to determine using SPSS and MS Excel. We also used ANOVA to find any significant difference between t the mean resultant values of the antioxidant and antimicrobial activities between the strains and concentration.

Results

DPPH scavenging assay

DPPH free radical scavenging potential of methanolic extract were 81.70±0.74, 75.00±1.15, 47.85±0.17, 67.91±0.55, 64.60±0.21and 57.76±0.42with their IC_{50}

values29.74 µg/mL at concentration range of 1000, 500, 250, 125 and 62.5 µg/mL (Table1). Percent DPPH inhibition of methanolic extract was compared with positive control (Ascorbic acid) which showed concentration dependent response. Ascorbic acid had91.64±0.90inhibitions at 1000 µg/mL against DPPH with IC_{50} value 15.98µg/mL.

ABTS Scavenging Assay

In ABTS free radical scavenging assay, methanolic extract showed % ABTS inhibition the % inhibition of methanolic extract 78.30±0.15, 71.68±0.76, 65.73±1.15,61.70±0.74, and 54.44±0.77 with their IC_{50} value 38.66µg/mL at concentration 1000, 500, 250, 125 and 62.5 µg/mL result shown in (Table 2).

Percent ABTS inhibition of methanolic extract was compare with positive control (Ascorbic acid) shown concentration dependent response. Ascorbic acid has shown 88.25±0.81inhibitions at 1000 µg/mL against ABTS with IC_{50} value 23.46 µg/mL.

Table 1. (Values are the average of triplicate experiments and represented as mean ± S.E.M) shows ABTS and DPPH (DPPH — 2,2-diphenylpicryl hydrazyl) of *Pinus roxburghii* pollen.

Conc. Of extract (µg/mL)	% ABTS activity of pollen extract	%ABTS Ascorbic acid	% DPPH activity of pollen extract	% DPPH Ascorbic acid
1000	78.30±0.15	88.25±0.81	81.70±0.74	91.64±0.90
500	71.68±0.76	83.06±0.06	75.00±1.15	85.96±0.36
250	65.73±1.15	75.91±0.55	67.91±0.55	79.61±0.32
125	61.70±0.74	68.60±0.21	64.60±0.21	75.33±0.20
62.50	54.44±0.77	63.25±0.81	57.76±0.42	67.23±0.78

Table 2. The inhibitory result of *Pinus roxburghii* pollen extracts at different concentration levels against the bacterial strains (inhibition zones diameters are measured in mm), (Mean± SD*).

Test bacteria	Concentrations of the pollen extracts			
	pollen extract (mg/ml) (Mean± SD*)		Ciprofloxacin (mg/ml) (Mean± SD*)	
	50	100	50	100
Proteus mirabilis	2.666±0.00	5.333± 1.41	27.130± 2.900	30.130± 5.900
Staphylococcus aurous	3.333±0.71	4.333± 0.71	20.000± 0.000	25.000± 1.000
Escherichia coli	2.333±0.00	5.333± 0.71	19.000± 0.000	27.130± 2.900
Bacillus cereus	3.333±1.41	5.666± 4.24	23.000± 3.000	27.130± 6.900
Salmonella typhi	6.333±0.00	3.00± 3.54	27.130± 2.900	4.104± 0.000
Klebsiella pneumoniae	3.333±1.41	3.00± 3.54	19.333± 1.155	4.104± 0.000
Pseudomonas aeruginosa	3.666±0.71	8.666± 0.71	20.000± 0.000	30.130± 5.900

Anti- bacterial assay

Result shows that the pollen Methanolic extracts have antibacterial potential. Disc diffusion method was used for antibacterial assay of the pollen extract.The50mg/mL and 100mg/ml concentration of the pollen solution produce zone of inhibition *Pseudomonas aeruginosa* which is 3.666mm and 8.666mm,*salmonella typhi* 6.333 and concentration 100mg/mL 3.00 mm respectively, likewise, the breadths for zone of inhibitions for ciprofloxacin were 20.000 and 30.130mm at concentration 50 and 100, while in concentration 100mg/ml against *Pseudomonas aeruginosa* high inhibition zones the extracts is successful in low concentration to

Klebsiella pneumoniae zone for limit about 3.333 and 3.00 mm individually to the same concentrations and the standard antibiotic indicated 19.333 Also 4.104mm inhibition zones for same result (Table 2).In order affectability of the microscopic organisms accompanied the grouping *E. coli*> *Proteus mirabilis*>*Klebsiella pneumoniae, staph aurous, bacillus cereus*> *Pseudomonas aeruginosa*> *salmonella typhi*. The highest active concentration toward the tested bacteria wasfor100mg/mL of the pollen extract. That the extract is effective at high concentration but in low concentration the efficacy decreases. Data is provided for in the type of mean of two values Furthermore standard deviation determined alongside (Table 2, Figure 3).

Table 3. ANOVA table for assessing variation between bacterial strains and various concentrations.

Source of Variation	SS	df	MS	F	P-value	F crit
Strain	57.694	6	9.615	1.932	0.22	4.283
50mg/mL	1218.236	1	1218.237	244.883	0.000	5.987
Error	29.848	6	4.974			
Total	1305.780	13				
Source of Variation	SS	df	MS	F	P-value	F crit
Strain	476.794	5	95.358	1.714	0.284	5.050
100mg/mL	634.627	1	634.627	11.410	0.019	6.607
Error	278.099	5	55.619			
Total	1389.521	11				

Note: F crit means, critical value. DF means "the degrees of freedom in the source." (3) SS means "the sum of squares due to the source." (4) MS means "the mean sum of squares due to the source." (5) F means "the F-statistic." (6) P means "the P-value."

The ANOVA results obtained for the 50mg/mL of methanolic pollen extract in comparison with the controlled 50mg/mL of antibiotic (Ciprofloxacin), there is no significant difference between the various strains as all strains are affected by the extracts. There is no difference between the mean zones of inhibition among the strains the F-critical value is higher than the F-calculated value. On the other hand there is a significance difference recorded among the standard antibiotic solution with that of 50mg/mL pollen extracts. The P value is much smaller (i.e. 0.00) than the alpha level of 0.5 and the calculated F value (is greater than the F –critical value (5.987). The data result shown in (Table 3).

The ANOVA results obtained for the 100mg/mL of methanolic pollen extract in comparison with the controlled 50mg/mL of antibiotic (Ciprofloxacin), there is no significant difference between the various strains as all strains are affected by the extracts. There is no deference between the mean zones of inhibition among the strains the F-critical value is more than the F-calculated value. On the other hand there is a significance difference recorded among the standard antibiotic solution with that of 100mg/mL pollen extracts. The P value is much smaller (i.e. 0.01) than the alpha level of 0.5 and the calculated F value (is greater than the F –critical value (6.607). The data result shown in (Table 3).

Discussion

In the current investigation, the antioxidant activity, in terms of scavenging of radical DPPH, decreasing power and antioxidant activity of the methanolic extracts of *Pinus roxburghii* was determined and compared. The response of DPPH with various antioxidants activity has been distributed and the stoichiometry (Cuvelier *et al.*, 1992).

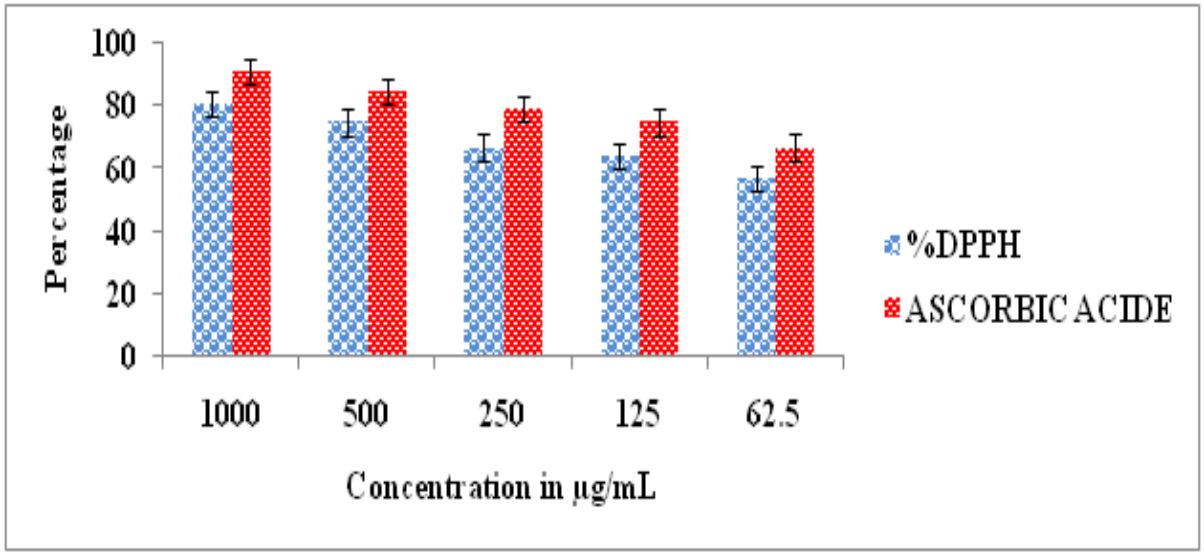

Fig. 1. Graphical representation of percentage scavenging of DPPH radical by *Pinusroxburghii*(Values are the average of triplicate experiment and represent as mean ±S.E.M).

The high amount of phenolic and flavonoid substance of *Pinus roxburghii* may found to its potential cancer prevention agent property and remedial capacity adsorbing and killing free radicals. Numerous specialists have reported that pollen possesses antioxidative activities (Campos *et al.*,2003; Silva *et al.*, 2006; Abarca *et al.*, 2007; Carpes *et al.*, 2007; Moraisa *et al.*, 2011). The DPPH cancer prevention agent measure depends on the rule that 2,2-diphenyl-1-picryl-hydrazyl (DPPH) is ready to decolorize within the presences of free radical scavenging (antioxidant).

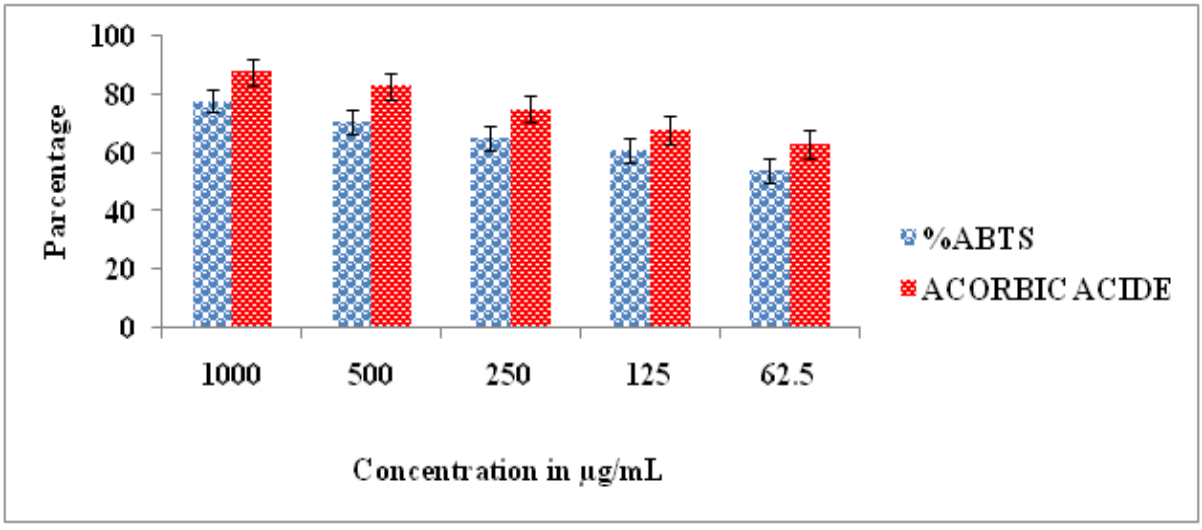

Fig. 2. Graphical representation of percentage scavenging of ABTS radical by *Pinus roxburghii* Sarg. (Values are the average of triplicate experiment and represent as mean ±S.E.M).

The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm decreases from 9660 to 1640 when the odd electron of DPPH radical winds up noticeably combined with hydrogen from a free radical antioxidant scavenging to decreased DPPH-H.

The odd electron in the DPPH radical is in control of the absorbance at 517 nm and furthermore for the obvious deep purple color (Kumarasamy *et al.*, 2007). Antioxidant in the extract of *Pinus* pollen responded with DPPH which is diminished to the DPPH-H.

Therefore, the absorbencies diminished from the DPPH radical to the DPPH-H formed. The level of staining showed the scavenging capability of the extract in the terms of hydrogen giving capacity. The scavenging ability of this plant was critical and related to the presence of high amount of phenolic compound. It is, in this manner, reason that high antioxidant in the distinctive concentrates of *Pinus roxburghii* have brought about the abnormal state of DPPH radical scavenged in this examination. Medicinal plants having flavonoids display numerous pharmacological characteristics (Janbaz *et al*., 2002).

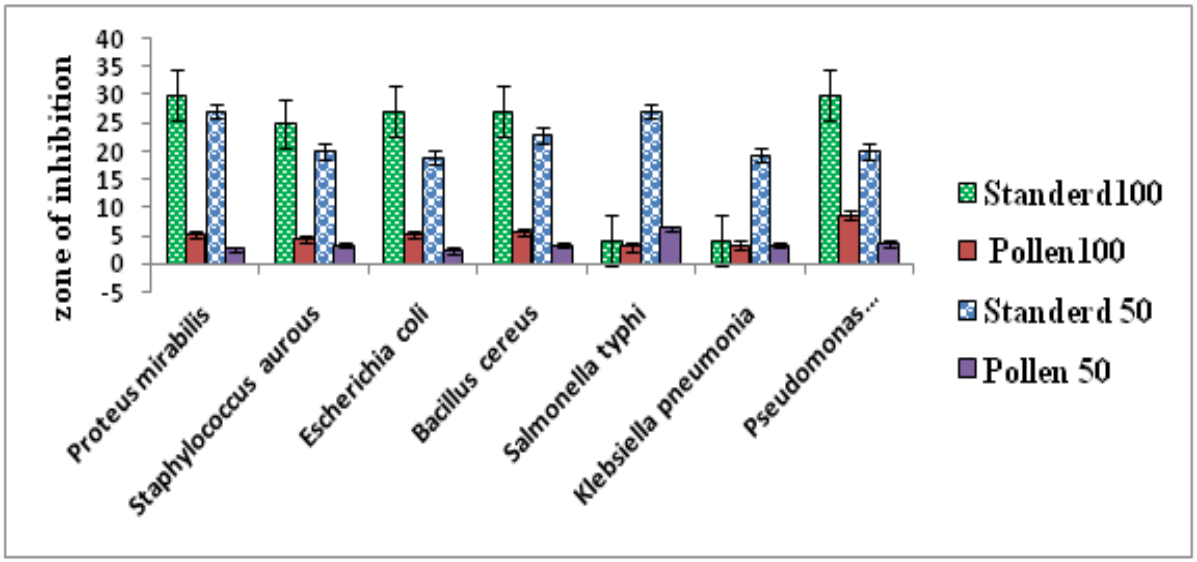

Fig. 3. Antibacterial activity of *Pinus roxburghii* pollen extract against different pathogenic bacterial strain.

ABTS radical scavenging activity

The result that phenolic antioxidant have been described for to search ABTS through hydrogen atom (Evans *et al*., 1996), electron exchange or even with the assortment of the two systems (Tyrakowska *et al* .,1999) may clarify a present attention for the applicability of the ABTS+ examine in deciding the radical scavenging activity of pollen extract. Like the DPPH examine, ABTS test measured the aggregate antioxidant action of the extract. The various antioxidant activities of the extract and the capacity of methanol to display more antioxidant activity. The pollen samples in our study demonstrates a high activity. Additionally,(Abarca *et al*., 2007)discovered EC_{50} found in the range of 6.4± 0.3, 14.0 ±0.9 µg/mL in their pollen tests from the In many plants the actual constituents like phenolic and flavonoids respond to free radical scavenging and antioxidant activity (Larson,1988: Johnston *et al*., 2006;). The effective compound is phenolic for the free radical scavenging activity.(Williams *et al*., 2007) displaying antioxidant activity by neutralizing free radical lipid, before through keeping the decay of hydroperoxides into free radicals (Maisuthisakul *et al*., 2007). taxa *Zea mays* L. (Poaceae), *Tagetes* sp. (Compositae), *Amaranthus hybridus* L. (Amaranthaceae), *Solanum rostratum* Dun. (Solanaceae), *Bidens odorata* Cav. (Compositae) and *Ranunculus petiolaris* HBK. (Ranunculaceae), (Dorea *et al*., 2010). These results demonstrate incredible similarities with our results. Contrasted with these results, the specimen of pollen in our examination demonstrates a high activity. Likewise Abarca *et al*. (2007),discovered EC_{50} value in the rang of 6.4 ± 0.3, 14.0 ± 0.9 µg/mL in their pollen tests from the taxa *Zea mays* L. (Poaceae), *Tagetes* sp. (Compositae), *Amaranthus hybridus* L. (Amaranthaceae), *Solanum rostratum* Dun. (Solanaceae), *Bidens odorata* Cav. (Compositae) and *Ranunculus petiolaris* HBK. (Ranunculaceae) (Dorea *et al*., 2010).these comes about show extraordinary likenesses with our results. Our pollen samples and the standard antioxidant material, hydrogen peroxide clearance activities were resolved by standard method (Ullah *et al*., 2015).

Determination of Anti-Bacterial activity

The pollen extract were used for *in vitro* antibacterial activity against seven strains of bacteria, According to the results, among these bacterial strains, *Salmonella typhi* was the most sensitive strain at 50 mg/mL while at the concentration of 100mg/mL the most sensitive bacteria was recorded to be *Pseudomonas aeruginosa*, and the sensitivity of the bacteria decreased in the following order: *E coli*>*Proteus mirabilis*>*Klebsiella pneumoniae*> *Staph aurous* > *Bacillus cereus*. This evident that *Salmonella typhi* bacterial strain are very susceptible to concentration 50mg/mL which means that there is a highly bioactive compound controlling *Salmonella typhi* (Table 2). Distinctive examples of affectability determined alongside pollen loads are because of various phenolic compound of pollen (Muradian *et al*., 2005; Carpes *et al*., 2007).

The best extraction conditions, identifying with living properties, likewise different extracts exhibited diverse cell support and antibacterial activities, there might make distinctive sorts from appealing phenolic content in distinctive pollen focus. As stated by the result got in these investigations and additionally for our study, pollen appears to have interesting biochemical properties, what more could be acknowledged of this freely available resource is its use as food and medicine. Because of the extraordinary diversity of the pollen-producing plants, this investigation is just the tip of the iceberg and needs further aid for understanding of the practical properties of pollens (Kacaniova *et al*., 2012). We did not evaluate the amount of the active compounds present in the pollens but rather indicated that there are those compounds present which are of extreme importance to our future needs (Carpes *et al.,* 2007).

Conclusion

We conclude that the pollens of Pine tree have antioxidant and antimicrobial potential and can be used in the future medicine and food industries warranting further studies.

As Pakistan's northern areas are rich in natural forests, mostly in *P. roxburghii* Sarg., the country has the greatest potential for wild harvesting of these therapeutic products without even harming the plants or spending more on artificial production. As indicated by the results, pollen appears to have interesting natural properties, and can be exploited as practical nourishment or medicine.

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