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PHYTOCHEMICAL CHARACTERIZATION, *IN-VITRO* ANTIOXIDANT AND ANTIMICROBIAL EFFICACY OF *PICEA SMITHIANA* (HIMALAYAN SPRUCE) NEEDLES FROM KUMAUN HIMALAYA

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ABSTRACT
The use of pharmacologically active substances found in medicinal plants in preventing diseases caused by microbial attack and oxidative stress, have received a great deal of attention. The present study aimed to evaluate the biochemical properties of Himalayan spruce *Picea smithiana* needles using four different extracts (hexane and aqueous) by qualitative and quantitative phytochemical methods along with their antibacterial activity against some gram-positive and gram-negative bacteria responsible for many diseases including human. From the observed results it is clear that ethanol extract was found more suitable to extract out the primary as well as secondary metabolites and also showed good amount of total phenolic and total flavonoid content ranged from 254- 43 µg/ gm dry extract weight of the plant with low IC_{50} value for free radical scavenging activity. In antimicrobial screening the ethanol extracts was found effective against all tested bacteria except *Pseudomonas aeruginosa* with zone of inhibition (ZOI) upto 11.5 mm at 1000 µg/ml concentration and MIC 31.25 µg/ml. These results reveal that the extracts of *P. smithiana* are possible good source of phenolic and flavonoid content as well as good free radical scavenger along with having good antimicrobial potential and new antibiotics.

KEYWORDS: Picea Smithiana, Himalaya Antioxidant, Antimicrobial, Phenols, Flavonoids.

1. INTRODUCTION
From the very beginning of human existence, man has familiarized himself with plants and used them in a variety of ways throughout the ages. Therapeutic properties of herbal drugs are present in plant which varies according to their age and maturity.[1] Traditional medicine is the oldest method of curing diseases and infections and various plants have been used in different parts of the world to treat human diseases and infections.[2-4]

Phytochemical are the chemical compounds produced in the plants naturally. These phytochemicals sometimes works as antioxidants with nutrients and fibers to form an integrated part of defense system against various diseases and stress conditions. Plants are rich in a variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosoids, and volatil oils[5] which have been found to be responsible for pharmacological effects.[6] Antioxidants are the compounds that have the ability to capture, deactivate or repair the damage caused by the reactive oxygen species, such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynitrite which results in oxidative stress leading to cellular damage.[7,8] Thus compounds or antioxidants that can scavenge free radicals have vital role in the improvement of various diseased conditions.[9] Natural antioxidant tends to be safer also possess antimicrobial, anti-inflammatory, anticancer and hepato-protective properties.[10] Therefore tracing out the various phytochemicals and antioxidants properties of various plants is an important step in the identification of their ability to develop in bioactive compounds.

Picea is the third largest genera of family Pineaceae with about 38 species all over the world.[11] It ranges from Arctic Circle to Mexico and Taiwan, America, Europe (including Turkey and the Caucasus region), to the Ural Mountains and Asia.[12-13] The genus *Picea* is described as native to Asia in recent molecular phylogenetic study.[13]

P. smithiana is a tall, evergreen tree, reaches upto 55-60 m in height, having drooping branches with conical crown. The tree trunk diameter is up to 1.5 to 2 m. Shoots are pale-brown and glabrous. Leaves are needle-like, about 3-5 cm long. Cones are cylinder in shape

about 9-16 cm long and green at juvenile stage but turn to brown at maturity. It grows at altitude range 2200 to 3600 m, from Afghanistan to Nepal, mixed in forests of deodar, kail, and oak.

Wood of *P. smithiana* is used for construction, planking, tea and apple boxed, shingles, packing cases etc., as wood is rich in long fiber, suitable for paper industry, matchwood and treated wood is used in construction of railway sleeper.[14] Its essential oil is also reported to be used in bath salts, room sprays, deodorants and antiseptic (IUCN, 2006). It also has anti-proliferative and free radical scavenging activity (Shah and Dar, 2014). Therefore in the continuation of the research in exploring the biochemical potentiality of the Himalayan spruce, the phytochemical characterization, antioxidant and antimicrobial activity was evaluated.

2. MATERIALS AND METHODS

2.1. Sample collection

Leaves (needles) of selected plant was collected during the month of March 2016, from district Nainital (Uttarakhand), India and authenticated by Prof. Y. P. S. Pangtey, Department of Botany of the university. A voucher specimen was deposited in the departmental herbarium.

2.2. Processing of plant material

The plant material washed under tap water and shade dried at room temperature in the laboratory. The dried plant material was then grinded into fine powder by using electrical grinder. The obtained powder of the plant material then stored into self seal air tight polythene bags for use. Exposure to sunlight was avoided to prevent the loss of active components.[15]

2.3. Preparation of the extract

The solvent used for extract preparation were in increasing order of polarity *i.e*. Hexane, Chloroform, Ethanol and double distilled water. Fine powdered plant material was soaked into solvents (w/v 1: 5) separately in conical flasks and kept in electrical shaker at 120 rpm and 25ºC for 48 hours. After 48 hours, the mixture was filtered by using Whatman's filter paper no. 1. Supernatant was taken and solvent was evaporated by using vacuum evaporator at 40ºC and stored at 4ºC for further use.

2.4. Chemicals and reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), Quercetin, sodium nitrite ($NaNO_2$), ascorbic acid, Ferric chloride ($FeCl_3$), gallic acid, Potassium di-hydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), TPTZ (2,4,6- tripyridyl 1,3,5 triazine), Sodium acetate (CH_3COONa), Ferrozine, Ferrous chloride, Ethylene diamine tetra acetic acid (EDTA), Riboflavin and Nitro-blue tetrazolium (NBT) were obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. Folin-Ciocalteu's reagent, Molisch's reagent, conc. H_2SO_4, Fehling's reagent, glacial aceti acid, conc. HCl, NH_4OH, Meyer's reagent (potassiomercuric iodide solution), 2,2 [azinobis (3ethyl benothiazoline- 6sulphonic acid) diammonium salt] (ABTS), hexane, chloroform, ethanol and. methanol were obtained from Merck, Mumbai, India. All chemicals used were of analytical grade.

2.5. Phytochemical Analysis

2.5.1. Qualitative Phytochemical Analysis

For the qualitative assessment of chemical composition of various extracts, a preliminary qualitative phytochemical analysis was conducted according to the standard methods.[16]

2.5.2. Quantitative Phytochemical Assays

2.5.2.1. Determination of Total Phenolic Content (TPC)

The total phenolic content of the sample extract was determined by Folin-Ciocalteu's colorimetric method given by Singleton and Rossi (1965)[17] with certain modifications. Absorbance of the sample was measured spectrophotometrically (UV-VIS) at 765nm. Quantification of total phenolic content was based on standard curve of Gallic acid. The results were expressed in mg gallic acid equivalent (GAE)/gm dry extract weight of the sample.

2.5.2.2. Determination of Total Flavonoid Content (TFC)

Content of flavonoids of the sample extract were determined by $AlCl_3$ colorimetric method given by Chang *et al*, 2002[18] with certain modifications. The absorbance was recorded at 415 nm using UV-VIS spectrophotometer. Quantification of total flavonoid content was done on the basis of standard curve of Quercetin. Results were expressed in mg quercetin equivalent (QE)/gm dry extract weight of the sample.

2.6. Determination of Antioxidant Activity

The antioxidant potential of *P. smithiana* was evaluated by using four different methods.

2.6.1. FRAP (Ferric Reducing Antioxidant Potential) Assay

Ferric reducing antioxidant power (FRAP) assay was performed method given by Faria *et al.* (2005)[19] with minor modification. Results were expressed in mg ascorbic acid equivalent (AAE), Butylated hydroxylanisole equivalent (BHAE), and Butylated hydroxyltoluene equivalent (BHTE) per gm dry weight of the sample, respectively.

2.6.2. DPPH Antioxidant Activity Assay

The DPPH assay was done according to the method of Brand-Williams *et al.* (1995)[20] with certain modifications. The reduction in absorbance was recorded at 517 nm in UV-VIS spectrophotometer. Ascorbic acid (AA), Butylated hydroxyl anisole (BHA) and Butylated hydroxyl toluene (BHT) was used as standard and for

control absorbance of DPPH cations was taken without adding sample extract.

2.6.3. ABTS Antioxidant Activity Assay

The ABTS (2,2' -Azinobis-3-ethylbenzotiazoline-6-sulphonic acid) assay was conducted according to Miller *et al*. (1993)[21] and Re *et al.* (1999)[22] with minor modifications. The antioxidant activity of tested sample was calculated by determining the decrease in the absorbance at 734nm at different concentrations.

2.6.4. Superoxide radical Scavenging Activity (SSA) Assay

Superoxide scavenging assay was performed by following Gülçin (2009)[23] with certain modifications. Absorbance was recorded at 590nm in UV-VIS spectrophotometer. Ascorbic acid, BHA & BHT was used as standard and phosphate buffer saline as blank. For control, absorbance of reagent was taken without adding sample extract.

% scavenging /Inhibition for DPPH, and ABTS were calculated as.

$$\% \text{ scavenging} = \frac{\text{absorbance of control} - \text{absorbance of test sample}}{\text{absorbance of control}} X100$$

2.7. Antimicrobial Activity

2.7.1. Microorganisms used and maintenance of culture media

The bacterial culture used were obtained from IMTECH, Chandigarh. Five microorganisms *Pseudomonas aeruginosa* (MTCC 3542), *Klebsiella pneumoniae* (MTCC 7028), *Proteus mirabilis* (MTCC 3310), *Staphylococcus aureus* (MTCC 737) and *Listeria monocytogenes* (MTCC 657) were used to test the sensitivity against plant extracts. Bacterial stains were revived and grown on nutrient agar plates at 37ºC and maintained by periodic subculture on nutrient agar plates.

2.7.2. Preparation of standard culture inoculums of test organism

Three or four isolated colonies were inoculated in the 25 ml nutrient broth and brain heart infusion broth and incubated for 24 hour at 37ºC in shaking condition for even suspension of bacterial colonies and maintained equivalent with Mac-Farland standard (0.5%) as recommended by WHO.

2.7.3. Determination of zone of inhibition (ZOI) and minimum inhibitory concentration (MIC)

Antibacterial tests of selected microorganisms were carried out by using disc diffusion method.[24] The freshly prepared inoculums was swabbed on Muller Hinton Agar (MHA) plates using sterile cotton swab and left the plates in laminar for 1 hour. Sterile discs were soaked with 40 µl of different concentrations of the different extracts of selected plants with the help of micropipette. Plates were incubated with closed lid at 37ºC and 30ºC for 24 hour and measure the zone of inhibition with the help of zone meter. All the tests were performed in triplicates and mean of triplicates was considered as MIC of the extract tested. Gentamicin (10 microgram), Ampicillin (10 microgram) and Kanamycin (30 microgram) were used as positive control and 5% DMSO was used as negative control.

2.8. STATISTICAL ANALYSIS

All the measurements were taken in triplicates and the results obtained were expressed as mean ± standard error (SE). The results were further analyzed by ANOVA (analysis of variance) and Duncan test using SPSS 20.0 software. At $p<0.05$ and $p<0.01$ the values were considered to be significant.

3. RESULTS

The Preliminary phytochemical analysis, antioxidant potential and antimicrobial activity of *P. smithiana* needles were conducted using hexane (PS_H), chloroform (PS_C), ethanol (PS_E) and aqueous (PS_A) extracts.

3.1. Phytochemical Analysis

3.1.1. Qualitative Phytochemical Analysis

Phytochemical analysis of four extracts was tested by standard methods given by Herborne, 1998, and presented in table 1, perusal of which reveals the presence of phenols, flavonoids, saponins, tannins, resins, and gallotannins. It was observed that resin and saponin were found present in all four extracts whereas terpenoids and volatile oils did show their presence in PS_E. Protein, flavonoids and phenols were tested positive only in polar extracts *i.e.* PS_E and PS_A extracts.

Table. 1: Qualitative Phytochemical Analysis of different extracts *Picea smithiana* needles

S. N.	Phytochemicals	Tests performed	Solvent extracts			
			PS_H	PS_C	PS_E	PS_A
1.	Alkaloids	Mayer test	-	-	-	-
		Wagner test	-	-	-	-
2.	Carbohydrates	Molish's test	+	+	-	-
		Fehling's test	-	-	-	-
3.	Protein	Biuret test	-	-	+	-
		Millon's test	-	-		+
		Xanthoprotic test	-	-		
4.	Flavonoids	Ferric chloride test	-	-	+	+
		NaOH Test	-	-		
5.	Phenols	$FeCl_3$ Test	-	-	+	+
6.	Tannin	$Fecl_3$ Test	-	+	+	-
7.	Gallo-tannin	$FeCl_3$ Test	-	-	+	-
8.	Resin	Turbidity Test	+	+	+	+
9.	Saponin	Foam Test	+	+	+	+
10.	Quinones	H_2SO_4 Test	-	+	+	+
11.	Volatile oils	NaOH-HCl Test	-	-	+	-
12.	Glycosides	Keller-Kiliani Test	+	+	-	-
13.	Terpenoids	Salkowski's Test	-	-	+	+
	Total		**4**	**6**	**10**	**7**

+ = Present; - = absent; PS_H – *P. smithiana* hexane extract; PS_C – *P. smithiana* chloroform extract; PS_E – *P. smithiana* ethanol extract; PS_A – *P. smithiana* aqueous extract

3.1.2. Quantitative Phytochemical Analysis

3.1.2.1. Determination of Total Phenolic Content (TPC): Total phenolic content of different samples were calculated from the regression equation of calibration curve for GA, BHA and BHT gm^{-1} of sample in their dry extract respectively. Results revealed that the phenolic content was highest in the PS_E *i.e.* 254.76±20.65 mg GAE gm^{-1} dry extract followed by PS_A (238.10±4.29 mg GAE gm^{-1} dry extract), PS_C (202.38±4.29 mg GAE gm^{-1} dry extract), and PS_H (185.71±4.12 mg GAE gm^{-1} dry extract). TPC in different extract followed the same pattern, irrespective to the standards used (Table 2).

Table. 2: Total phenolic content (TPC) in different extracts of *P. smithiana* needles with respect to GA, BHA and BHT.

S.N.	Extracts	TPC (mg GAE gm^{-1} dry extract)	TPC (mg BHAE gm^{-1} dry extract)	TPC (mg BHTE gm^{-1} dry extract)
1.	PS_H	185.71±4.12	68.75±7.22	52.38±5.50
2.	PS_C	202.38±4.29	97.92±7.51	74.60±5.72
3.	PS_E	254.76±20.65	227.76±17.05	173.02±12.99
4.	PS_A	238.10±4.29	160.42±7.51	122.22±5.72

Values are mean±SE of three independent observations, each in triplicate; GAE- Gallic Acid Equivalent; BHAE- Butylated Hydroxy anisole Equivalent; BHTE - Butylated Hydroxy toluene Equivalent

3.1.2.2. Determination of Total Flavonoids Contents (TFC): Total flavonoids content of the sample extracts was calculated from the regression equation of calibration curve of Quercetin, BHA and BHT and expressed as mg gm^{-1} of sample in their dry extract weight respectively. Total flavonoid in *P. smithiana* needles was highest in PS_E (108.05±6.99 mg QE gm^{-1}; 162.96±11.26 mg BHAE gm^{-1}; 182.22±13.52 mg BHTE gm^{-1} dry extract), and lowest in PS_H (43.68±3.04 mg QE gm^{-1}; 59.26±4.90 mg BHAE gm^{-1}; 57.78±5.88 mg BHTE gm^{-1} dry extract) (Table 3).

Table. 3: Total flavonoid content (TFC) in different extracts of *P. smithiana* needles with respect to Quercetin, BHA and BHT.

S.N.	Extracts	TFC (mg QE gm^{-1} dry extract)	TFC (mg BHAE gm^{-1} dry extract)	TFC (mg BHTE gm^{-1} dry extract)
1.	PS_H	43.68±3.04	59.26±4.90	57.78±5.88
2.	PS_C	62.07±3.98	88.89±6.41	93.33±7.70
3.	PS_E	108.05±6.99	162.96±11.26	182.22±13.52
4.	PS_A	80.46±4.14	118.52±6.68	128.89±8.01

Values are mean±SE of three independent observations, each in triplicate;
QE- Quercetin Equivalent; BHAE - Butylated Hydroxy anisole Equivalent; BHTE - Butylated Hydroxy toluene Equivalent.

3.2. Determination of Antioxidant Activity

3.2.1. Determination of Ferric Reducing Antioxidant Potential (FRAP): The ferric reducing antioxidant potential was calculated by using regression equation of calibration curve of three standards (AA, BHA and BHT). The highest FRAP value stands for PS_E (98.25±1.75 mg AAE gm^{-1}; 128.21±2.56 mg BHAE gm^{-1}; 208.33±4.17 mg BHTE gm^{-1} dry extract), and lowest value stands for PS_H (26.32±6.08 mg AAE gm^{-1}; 23.08±8.88 mg BHAE gm^{-1}; 37.50±14.43 mg BHTE gm^{-1} dry extract) (Table 4).

Table. 4: FRAP content in different extracts of *P. smithiana* needles with respect to AA, BHA and BHT.

S.N.	Extracts	FRAP (mg AAE gm^{-1} dry extract)	FRAP (mg BHAE gm^{-1} of dry extract)	FRAP (mg BHTE gm^{-1} dry extract)
1.	PS_H	26.32±6.08	23.08±8.88	37.50±14.43
2.	PS_C	36.84±3.04	38.46±4.44	62.50±7.22
3.	PS_E	98.25±1.75	128.21±2.56	208.33±4.17
4.	PS_A	68.42±6.08	84.62±8.88	137.50±14.43

Values are mean±SE of three independent observations, each in triplicate.
AAE- Ascorbic Acid Equivalent; BHAE- Butylated Hydroxy anisole Equivalent; BHTE- Butylated Hydroxy toluene Equivalent.

3.2.2. DPPH Free Radical Scavenging Activity Assay
DPPH free radical scavenging activity of different plant extracts was analyzed at different concentrations (1-50 µg ml^{-1}). At higher concentration (50 µg ml^{-1}), PS_E presented maximum inhibition (43%; IC_{50}- 64.21±1.77 µg ml^{-1}), followed by PS_A (30%, IC_{50}- 81.74±1.78 µg ml^{-1}), PS_C (26%, IC_{50}- 134.16±19.57 µg ml^{-1}), and PS_H (22%, IC_{50}- 136.19±13.44 µg ml^{-1}). At same concentration different standards (AA, BHA, BHT) exhibited 72.98%, 79.05% and 68.57% inhibition with IC_{50} - 6.42±0.61, 3.48±0.74, 8.40±1.31 µg ml^{-1}, respectively (Table 5).

Table. 5: % Inhibition of DPPH radicals at 50 µg ml^{-1} and IC_{50} values of different extracts of *P. smithiana* needles and Standards

S.N.	Samples	% Inhibition	IC_{50} (µg ml^{-1})
1.	AA	72.98	6.42±0.61
2.	BHA	79.05	3.48±0.74
3.	BHT	68.57	8.40±1.31
4.	PS_H	22.57	136.19±13.44
5.	PS_C	26.93	134.16±19.57
6.	PS_E	43.23	64.21±1.77
7.	PS_A	30.48	81.74±1.78

Values are mean±SE of three independent observations, each in triplicate. AA- Ascorbic Acid; BHA- Butylated Hydroxy anisole; BHT - Butylated Hydroxy toluene

3.2.3. ABTS Free Radical Scavenging Assay
The ABTS scavenging activity of different extracts was measured using three different standards (AA, BHA, and BHT). Highest ABTS scavenging was recorded in PS_E (61% inhibition; IC_{50}- 39.15±0.32 µg ml^{-1}), followed by PS_A (50 % inhibition; IC_{50}- 49.40±1.93 µg ml^{-1}), PS_C (46% inhibition; IC_{50}- 59.69±3.80 µg ml^{-1}), and PS_H (42% inhibition, IC_{50}- 74.97±9.28 µg ml^{-1}) (Table 6).

Table. 6: % Inhibition of ABTS radicals at 50 µg ml^{-1} and IC_{50} values of different extracts of *P. smithiana* needles and standards.

S. N.	Samples	% Inhibition	IC_{50} (µg ml^{-1})
1.	AA	99.12	9.08±2.12
2.	BHA	96.64	12.77±0.83
3.	BHT	98.94	6.05±0.92
4.	PS_H	51.63	55.12±2.98
5.	PS_C	46.76	74.97±9.28
6.	PS_E	61.70	39.15±0.32
7.	PS_A	50.10	49.40±1.93

Values are mean±SE of three independent observations, each in triplicate. AA- Ascorbic Acid; BHA- Butylated Hydroxy anisole; BHT - Butylated Hydroxy toluene.

3.2.4. Superoxide Scavenging Activity (SSA)

The superoxide scavenging activity results (Table 7) presented, PS_E as a good superoxide radical scavenger with 52% inhibition at 50 µg ml^{-1} concentration with IC_{50} value 47.64±2.12 µg ml^{-1} while lowest value for superoxide radical inhibition (31%; IC_{50}- 142.28±33.73 µg ml^{-1}) was observed by PS_H.

Table. 7: % Inhibition of Superoxide radicals at 50 µg ml^{-1} and IC_{50} values of different extracts of *P. smithiana* needles and standards

S.N.	Samples	% Inhibition	IC_{50} (µg ml^{-1})
1.	AA	93.10	12.38±3.01
2.	BHA	96.30	5.87±0.96
3.	BHT	83.00	11.01±2.17
4.	PS_H	31.88	142.28±33.73
5.	PS_C	35.14	107.56±22.30
6.	PS_E	52.96	47.64±2.12
7.	PS_A	47.31	55.49±3.55

Values are mean±SE of three independent observations, each in triplicate. AA- Ascorbic Acid; BHA- Butylated Hydroxy anisole; BHT - Butylated Hydroxy toluene.

3.3. Determination of Antimicrobial Potential

3.3.1. Antimicrobial Activity Screening: In the qualitative screening for the antimicrobial activity testing, four extract (PS_H, PS_C, PS_E and PS_A) of *P. smithiana* needles were used. The antimicrobial activity was noticed for different extracts, exhibiting zones of inhibition against all five pathological bacterial strains, tested by using disc-diffusion method. The results revealed that the all the extract except aqueous extract, showed significant to moderate antimicrobial activity towards all the bacteria tested**.** The aqueous extract was found to be completely inert towards all the bacteria tested.

Results (Table 8) showed that *L. monocytogenes* was the most susceptible bacteria which show the maximum zone of inhibition (11.5 mm) for PS_E, followed by PS_C (11mm), and for PS_H (10 mm). The second susceptible bacteria was *S. aureus*, whose growth was inhibited by all three active extracts with maximum ZOI 11 mm for PS_E. The growth of *P. mirabilis* was inhibited by PS_E and PS_C only with ZOI 10.5 mm and 8.5 mm, respectively, while *K. pneumoniae* was inhibited by PS_E (ZOI 10.5 mm) only. It is worth mentioning here that out of three standards used, at four instances Ampicillin (10 mcg) was found less potent than extracts used in various tests performed (Fig 1).

Table. 8: Antibacterial screening of different extracts of *P. smithiana* needles and different standards.

Bacteria strain	ZOI (mm)						
	1000 µg ml^{-1} concentration				10 mcg	10 mcg	30 mcg
	PS_H	PS_C	PS_E	PS_A	G	A	K
P. aeruginosa	NA	NA	NA	NA	16	8	12
K. pneumoniae	NA	NA	10.5	NA	20	09	14
P. mirabilis	NA	8.5	10.5	NA	20	17	15
S. aureus	8.5	10	11	NA	25	18	12
L. monocytogenes	9.5	11	11.5	NA	22	00	20

NA- Not active; G- Gentamicin; A- Ampicillin; K- Kanamycin; mcg- microgram.

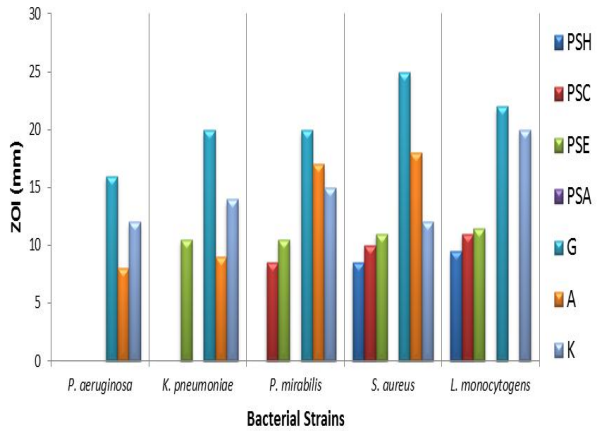


Fig. 1: Comparative account of activity of different extracts of *P. smithiana* needles on tested pathogens with three standard antibiotics (Gentamicin, Ampicillin and Kanamycin).

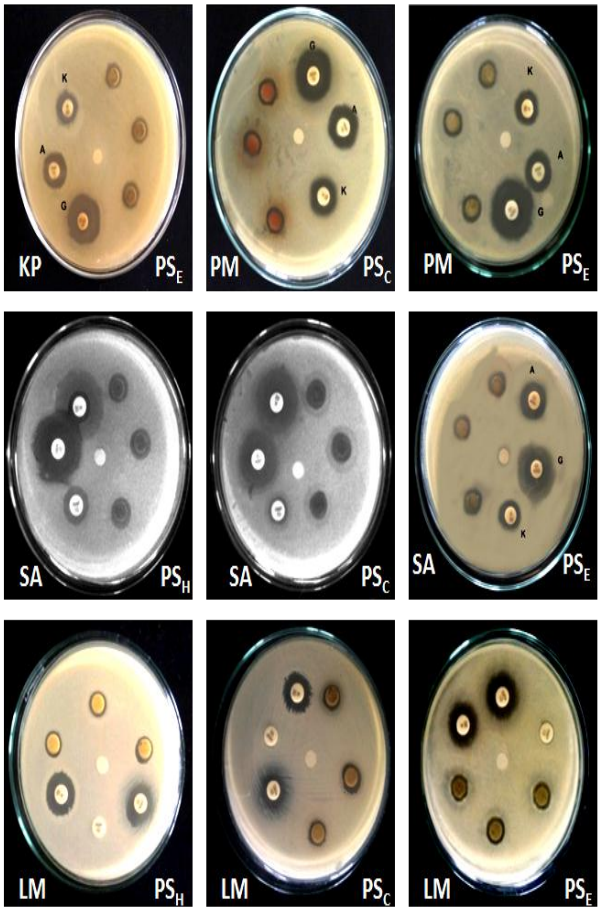
3.3.2. Minimum Inhibitory Concentration (MIC)

Total 9 tests were found positive, out of 20 screening test performed of four extract of *P. smithiana* needles. These positive screening results were further elaborated for evaluating the minimum inhibitory concentration against each tested bacterial strain tested, along with zone of inhibition at MIC. The range of MIC of different extracts for different bacterial strains was 250 µg ml^{-1} to 31.25 µg ml^{-1} with ZOI range 6.7mm to 8.7mm. The lowest MIC was 31.25 µg ml^{-1} of PS_E against *P. mirabilis* and highest MIC was 250 µg ml^{-1} of PS_C for *P. mirabilis* (Table 9).

Table. 9: MIC of different extracts of *P. smithiana* needles along with corresponding ZOI.

Bacteria strain	PS_H		PS_C		PS_E	
	MIC (µg ml^{-1})	ZOI (mm)	MIC (µg ml^{-1})	ZOI (mm)	MIC (µg ml^{-1})	ZOI (mm)
P. aeruginosa	NT	NT	NT	NT	NT	NT
K. pneumoniae	NT	NT	NT	NT	125	8.7±0.44
P. mirabilis	NT	NT	250	6.7±0.17	31.25	7.3±0.17
S. aureus	125	7.2±0.17	125	7±0.29	62.5	8±0.29
L. monocytogenes	125	6.7±0.33	125	7.7±0.44	62.5	8±0.29

NT- not tested due to lake of observable inhibition at 1000 µg ml^{-1};
Values are mean±SE of three independent observations, each in triplicate.



PHOTOPLATE :**Antibacterial activity of different extracts of *Picea smithiana* needlesagainst pathogenic bacteria: Representing ZOI (mm) at MIC (µg ml^{-1})**

PA- *Pseudomonas aeruginosa*, **KP**- *Klebsiella pneumoniae*, **PM**- *Proteus mirabilis*,
SA- *Staphylococcus aureus*, **LM**- *Listeria monocytogenes*;
PS_H – *P. smithiana* hexane extract, **PS_C** - *P. smithiana* chloroform extract,
PS_E- *P. smithiana* ethanol extract;

G – Gentamicin, **A**- Ampicillin, **K**- Kanamycin;
In centre negative control (solvent);
Rest three are triplicates of minimum inhibitory concentration (MIC) (µg ml^{-1}) of each extract (which were found active at screening test) showing corresponding ZOI (mm).

4. DISCUSSION

It is evident from broad literature survey of genus *Picea* that out of 38 species found worldwide, only four species namely *P. abies*, *P. sitchensis* and *P. excesla* and *P. smithiana* have been evaluated for their essential oil composition as well as bioactive potential.

Various studies of chemical composition of *Picea* species reveals that δ-3-carene, β-pinene, α- pinene, α-terpinolene, *p*-cymene, β-myrcene, limonene, camphene, δ-cadinene and β-phelledrene are the major component and these components are also associated with antimicrobial properties of the plants.[25-28]

P. smithiana is less explored species for its phytochemical characterization as well as broad spectrum bioactive potential. So, in this study, different extracts of *P. smithiana* (PS_H, PS_C, PS_E and PS_A) were targeted for the presence of different phytochemicals, antioxidant potential and antimicrobial properties.

For the preliminary phytochemical analysis 13 phytochemical classes including primary and secondary phytoconstituents was tested. Out of these, highest number of phytochemicals were present in PS_E (10), followed by PS_A (7), PS_C (6) and PS_H (4).

Polyphenols are a class of secondary metabolites of the plant and also referred as glycones and extracted with solvents based on polarity[29] which play an important role in antimicrobial and antioxidant potential of the plant. Among the polyphenols, total phenolic content and total flavonoid content plays a key role for various biological effects such as antimicrobial, anti-insecticidal, anti-inflammatory, anti-carcinogenic etc.[30, 31] In this regard *P. smithiana* leaves extracts could serve as possible new source of these valuable chemicals as no previous record found about its polyphenolic profile. While compared different standard, the highest TPC and TFC was found in ethanolic extract (PS_E) which suggests that ethanol is good solvent to extract out the phenolic and flavonoid content from the leaves of *P. smithiana* (Fig 2 : A&B). In addition polyphenols are found to be associated with scavenging activity of free radical[22,32,33] which are produced in the living organisms due to various metabolic reaction. Phenolic compounds in addition to reducing agents, hydrogen donators, and singlet oxygen quenchers also reported as metal chelators.[32] It is of interest to note that

FRAP content followed the same trend as that of TPC and TFC *i.e.* $PS_E > PS_A > PS_C > PS_H$ (Fig 2: C).

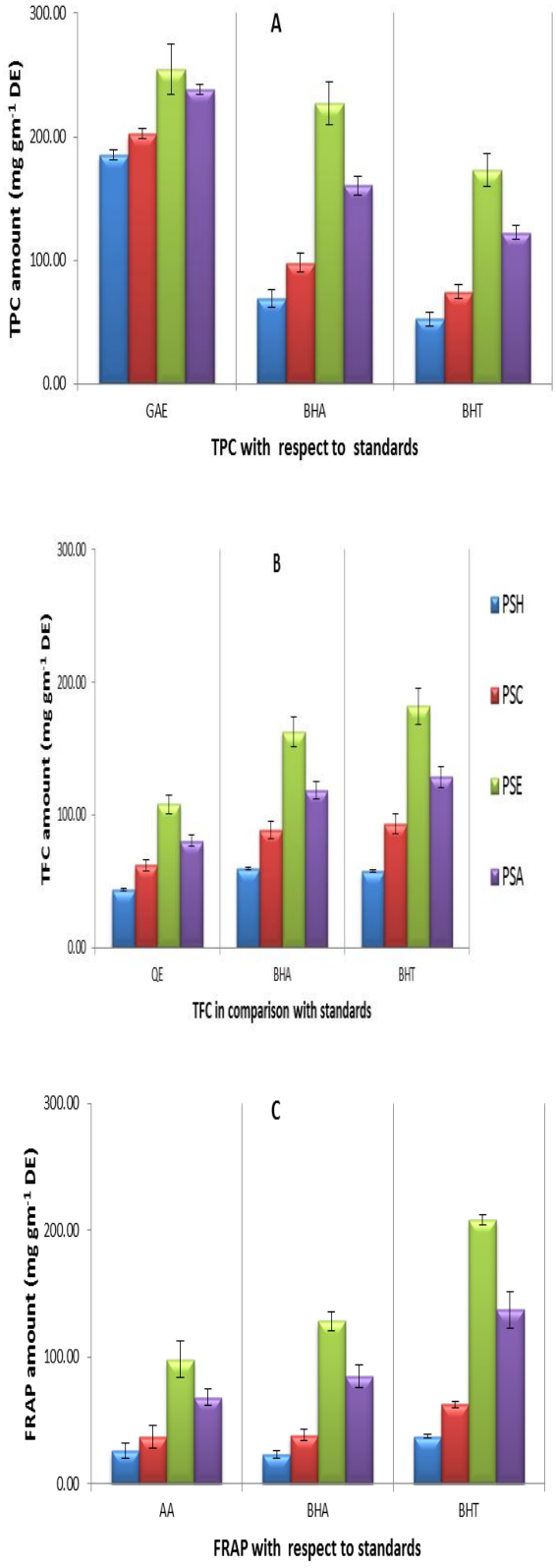


Fig 2: (A) Total phenolic content (TPC) (B) Total flavonoid content (TFC) (C) FRAP content in different extracts of *P. smithiana* with respect to different standards

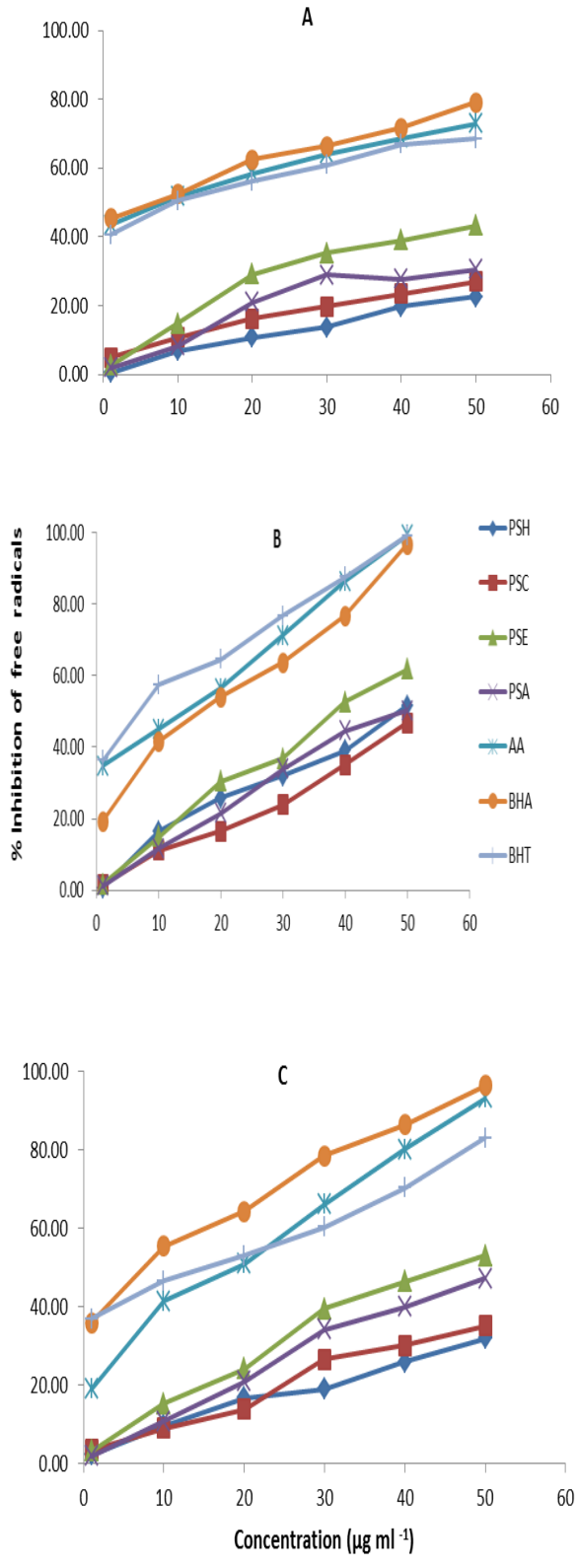


Fig 3: Free radical Scavenging activities of different extracts of *P. smithiana* needles and standards using (A) DPPH Assay (B) ABTS Assay (C) SSA Assay.

It is evident from the antioxidant potentiality testing, evaluated by using FRAP, DPPH, ABTS and SSA assays, that PS_E exhibited very low IC_{50} value than other

corresponding extracts; the exception was the SSA, in which PS_A had almost similar activity as that of PS_E (Fig 4). The plant extract provides electron and hydrogen ion to stabilize the compounds responsible for free radical scavenging activity and work as antioxidant compound. In present study ethanol extract (PS_E) showed high level of antioxidant activity which are comparable to three known standards Ascorbic acid, BHA and BHT (Fig 3).

While analyzing antioxidant activity using DPPH assay, Shah and Dar[34] reported a maximum inhibition of only 35.08 % inhibition at 100 µg ml^{-1} concentration. Our results which agreed the report of Shah and Dar, also present *P. smithiana* as good source of antioxidant compound. The present investigation which reports a much higher DPPH scavenging activity highlights the importance of medicinal plants of Kumaun Himalaya. Previous reports on bioactivity testing of the other species of genus *Picea*, have mainly focused on its essential oil composition and antimicrobial activity using some bacterial and fungal strains. Earlier researches have reported the essential oil composition and antimicrobial activity of *P. abies*[35-37] and *P. exelsa*.[38]

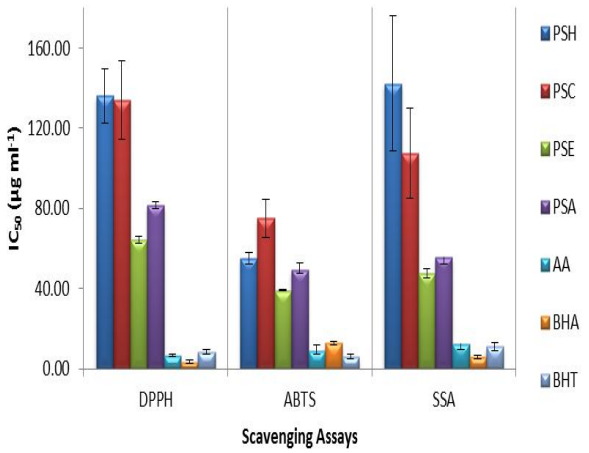


Fig. 4: Comparison of IC_{50} of three free radical scavenging activity (DPPH, ABTS and SSA) of different extracts of *P. smithiana* needles with standards.

The different extracts of *P. smithiana* showed good activity against tested pathogen. PS_E was found significantly active against *L. monocytogenes* and *S. aureus*, and moderately active against *P. mirabilis*. Some previous studies on antimicrobial activity of *P. smithiana* needles extracts support our finding[39] tested *P. smithiana* needles extracts against *B. subtilis*, *E. coli* (animal pathogen) and *A. tumefaciens*, *E. chrysanthemi* and *X. phaseoli* (plant pathogen), and reported their significant activity[40] tested *P. smithiana* bark extracts against some plant and animal pathogen and also observed significant results. In case of *K. pneumoniae*, PS_E was found more effective than Ampicillin, this could be due to the some phytochemical components present in the extract that may have checked the bacterial growth more effectively than a standard antibiotic and this could be correlated with the different extraction of the components in the solvents. *P. aeruginosa* and *K. pneumoniae* are gram negative bacteria, having some 'intrinsic resistance' to some antibiotics due to the activity of efflux systems present in their bacterial outer membrane that helps them to survive in hostile environment.[41,42]

Observation regarding effectiveness of different plant extracts in term of antibacterial equivalence with different standards are presented in Table 10. ABEq table suggests that antibacterial potential of different extracts is comparable with only Ampicillin at two cases *i.e. K. pneumoniae* and *L. monocytogenes*. In this regard *P. smithiana* can be used as antimicrobial agent in selected cases.

Results of the phytochemical investigation, antioxidant potential and antimicrobial activity of the different extract of the *P. smithiana* reveals the potentiality of the plant. The ethanol extract of plant showed good results in all instances. Though some work on antimicrobial potential of the plant extracts has been done previously by some worker but the phytochemical investigation and total phenolic content, total flavonoid content and free radical scavenging activity of the plant is evaluated for the first time from Kumaun Himalaya.

The plant posses a good amount of TPC and TFC and also a good free radical scavenger along with good antimicrobial activity against animal pathogen supports the ethno-medicinal use of the plant by local healers.

Table. 10: Antibacterial Equivalence (ABEq) and Antibacterial Equivalence Index [AEI] of different extracts of *P. smithiana* with respect to different standards for different bacterial strains

Bacterial Strains	Antibacterial Equivalence (ABEq)	Antibacterial Equivalence Index [AEI]
P. aeruginosa	-	00
K. pneumoniae	$(\geq E)^{A10}$	01
P. mirabilis	-	00
S. aureus	-	00
L. monocytogenes	$(\geq E)^{A10}$ $(\geq C)^{A10}$ $(\geq H)^{A10}$	03

E – Ethanol extract; C- Chloroform extract; H- Hexane extract; A- Ampicillin

Detailed information about phytochemical profile of bioactive fractions obtained from leaves of *P. smithiana* are still lacking and addressing the need to work out the functional aspects of health-protecting components. Like scavenging and detoxification of specific oxygen–based radicals that requires better understanding and in-depth investigation of antioxidant and antimicrobial active component present in them, for canalizing their use as functional ingredient in pharmaceutical and neutraceutical industry.

5. CONCLUSION

The results of the study showed the leaves of the *P. smithiana* contains appreciable amount of alkaloids, flavonoids, phenols, resins, saponins, glycosides, volatile oils and also reducing power as well as good antimicrobial activity. Thus this can be concluded that the plant could be a rich source for the development of new therapeutic medicines for future.

CONFLICT OF INTEREST

It is declared that there is no conflict of interest.

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