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COMPARATIVE EVALUATION IN CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF *PINUS WALLICHIANA* NEEDLES ESSENTIAL OILS OBTAINED THROUGH STEAM AND HYDRO-DISTILLATION

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ABSTRACT

The present study was planned to compare the chemical composition and biological activities of *Pinus wallichiana* (*P. wallichiana*) needles essential oils extracted through steam and hydro-distillation. It was found that the average yield of *P. wallichiana* needles essential oils (PWNSEO) was higher when extracted through steam-distillation as compared to hydro-distillation essential oil (PWNHEO). The chemical composition of PWNSEO and PWNHEO showed that main compounds (>5.0%) α-pinene (13.65-14.14%), limonene (2.67-8.21%), α-terpinolene (5.21-6.26%), 4-terpineol (1.09-9.12%) and caryophyllene (5.94-6.56%) were present in both essential oils whereas, α-terpineol (8.58%), α-terpinyl acetate (8.41%) were present in PWNSEO and β-myrcene (10.89%), β- Terpineol (6.98 %), were present in PWNHEO. Biological activities were evaluated by using different bioactivity assays on essential oils. The anti-bacterial activity of essential oils was estimated by well diffusion as well as through MIC assays. PWNHEO showed higher activity for Gram positive bacteria (Inhibition zone, 29.15-31.02 mm) than Gram negative bacteria (Inhibition zone IZ, 14.56-22.68 mm). PWNSEO also exhibits stronger antibacterial activity against Gram-positive bacteria, as indicated by larger inhibition zones (18.02–27.19 mm). In contrast, its activity against Gram-negative bacteria is comparatively weaker, with inhibition zones ranging from 12.86 to 16.49 mm. Also, PWNHEO showed high antibacterial activity against different bacterial strains as compared to PWNSEO.

Keywords: Pinene, limonene, GC-MS, *E. coli*, DPPH

1. INTRODUCTION

Oxidative stress and its detrimental effects on the health of humans have emerged as a major problem. In stressful conditions, there is enhance production of reactive species such as, hydroxide radical (OH), hydrogen peroxide (H_2O_2) superoxide ion (O_2) in cellular respiration as compared to enzymatic antioxidants (e.g., catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) and antioxidants which are non-enzymatic (e.g., flavonoids, glutathione, ascorbic acid (vitamin C), carotenoids, α-tocopherol (vitamin E). The imbalance between the production of reactive oxygen species (ROS) and the antioxidant status could hurt the cellular biomolecules such as lipids, proteins,

carbohydrates, and deoxyribonucleic acid (DNA) that may lead toward genetic and metabolic changes[1].

Antioxidants are very important with reference to human health which protects the human body from the effect of ROS. Several artificially prepared antioxidants are being used such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) but gradually a concerned about numerous side effects caused by them and their carcinogenicity reduces their uses[2]. Therefore, people are searching for natural antioxidants and the increasing trend of use of natural antioxidants was observed due to their nutritional and therapeutic values and presumed safety[3]. Different parts of plant materials showed the availability of naturally occurring antioxidants as reported[4, 5].

On the other hand, globally infectious diseases majorly causing death. Due to rise in deadly bacterial infections and decrease susceptibility to antimicrobic drugs, the number of multidrug-resistant strains of bacteria increases. The pathogenic microbes have increased the occurrence of lethal infections worldwide, especially in developing countries, it has become vital cause of death and disease in immunosuppressed patients[6]. The growing prevalence of multidrug-resistant bacterial strains, along with the emergence of those with reduced antibiotic susceptibility, has brought the threat of "untreatable" infections into focus and intensified the need for novel approaches to combating infections[7]. Although effective antibiotics and antifungals are available, resistant and multidrug-resistant strains continue to emerge, highlighting the ongoing need for the discovery and development of new medications[8]. Therefore, it is crucial that the search for novel antibiotic sources be a continuous effort. Plants represent a cost-effective and safer alternative for antimicrobial agents.

Synthetic compounds or drugs are being consider or band because they have numerous side effects, so now a days interest is shifting towards naturally occurring compounds to use in food, pharmaceutical products and even cosmetic to replace synthetic compounds[9]. Essential oils are concentrated plant extracts which are volatile and aromatic oily liquids. These are composed of complex mixtures constituents having with high volatility and low boiling point[10]. Composition of essential oils based on two major groups of volatile compounds are aromatic substances and terpenoids. Major proportion is of terpenoids, which are further categorized into mono, di and sesquiterpene based on the isoprene units numbers[11]. Among essential oils, the aromatic constituents, contribute to essence of oils are mainly aldehyde, phenols, alcohols, and methoxy derivative[12]. All components of the essential oils are very important and each of them contribute to the useful or adverse effects[13]. Essential oils along with their constituents are used as multi-functional purpose, and their relatively safe status, are reasons to gaining more interest and wide acceptance by consumers[14]. Another important usage of these extracts of plant and their essential oils is preservation of food and being used from thousands of years[10]. They are also used in aromatherapy[15], natural therapies, alternative medicine and pharmaceuticals[16]. Essential oils are used in perfumes and cosmetics due to having unique odour and fragrance[17]. Therefore, investigation of such plants scientifically is urgent necessity, which have been used in traditional medicine to provide healthcare and to improve its quality.

Blue pine (*Pinus wallichiana*) is one of the main species of coniferous biome in Pakistan[18]. *Pinus wallichiana,* a coniferous evergreen tree commonly known as (Peuch/Nakhtar, Shunty), is native species of Himalaya, Hindu-Kush, and Eastern Afghanistan. It has an altitude ranging from 1800-4000 meters[19]. *Pinus wallichiana* essential oil is employed for therapeutic purposes[1]. In the past, attempts were made to transform leaves into fiber, which was then woven into therapeutic undergarments, used to make rough matting similar to coconut matting, and applied in the production of surgical bandages. In recent years, in several rural regions of Kashmir, wood pieces of blue pine were used to extract a thick sticky substance dark brown in color known as Killam. Traditionally, farmers used this substance on their legs and arms to shield themselves from insects known as (Khase) when they were working in flooded rice fields. Killam adhered strongly, safeguarding exposed skin from insect bites (Khase) etc[20].

Many researchers have worked to determine or explore the chemical composition and biological activities of essential oil of *Pinus wallichiana*[21, 22]. But no reports are present on comparison of

composition and biological activities of essential oil of *Pinus wallichiana* needles obtained by steam and hydro-distillation. Therefore, the purpose of this investigation was to compare the chemical composition of the *Pinus wallichiana* essential oils extracted through steam and hydro-distillation and their antioxidant and antibacterial actions.

2. Materials and Methods

2.1 Collection and Pretreatment of Plant Materials

Pinus wallichiana needles were obtained from Patriata (7500 feet high, 33.877538° N and 73.448622°) Pakistan. All samples are collected from June to August. Specimens were authenticated by Institute of Horticultural Sciences, University of Agriculture Faisalabad, Pakistan and voucher specimens 23-N-002 was submitted in the Herbarium of the Department.

2.2 Isolation of Essential Oil

Essential oils were obtained from 10-15 kg of fresh samples using steam and hydro-distillation as reported[23]. The isolated oils sample were kept in glass vials at –20ºC after drying over anhydrous sodium sulphate and prior to analysis.

2.3 Gas chromatography-mass spectrometric (GC-MS) analysis of essential oils

The chemical constituents of PWNSEO and PWNHEO essential oils were identified through gas chromatography-mass spectrometry (GC-MS). GC-MS analysis was carried out on an Agilent Technologies 6890N Network GC system, equipped with a 5975 inert XL mass selective detector and a 7683B series auto injector, provided by Agilent Technologies (Little Falls, California, USA). Separation of compounds was carried out on an HP-5 MS capillary column (30 m x 0.25 mm, 0.25 µm film thickness; Little Falls, CA, USA). A 1.0 µL sample was introduced in split mode with a 1:100 split ratio. An electron ionization system operating at 70 eV ionization energy was used for GC/MS detection. The temperature program for the column oven was the same as that used in the GC analysis. Helium was used as the carrier gas with a flow rate of 1.5 mL min^{-1}. The mass range was set from 50 to 550 m/z, with the injector and MS transfer line temperatures adjusted to 220°C and 290°C, respectively. The identification of the essential oil components was carried out on the basis of their retention indices relative to (C_9-C_{24}) n-alkanes with published data or with authentic compounds [23, 24]. Compounds were also identified by using their MS data and by comparing it to those from the NIST mass spectral library and those published mass spectra [25, 26].

2.4 Antioxidant activity

2.4.1 DPPH radical scavenging activity

The 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was performed spectrophotometrically as mentioned by[23].

2.4.2 Percentage inhibition in linoleic acid system

The antioxidant activity of PWNSEO and PWNHEO was determined by measuring percentage inhibition of peroxidation in the linoleic acid system, following the method described by[27].

2.4.3 Reducing Power Assay

The reducing power assay of PWNSEO and PWNHEO was determined by method as described by [28].

2.5 Antibacterial activity

2.5.1 Collection of Bacterial Strains

PWNSEO and PWNHEO were tested against five Gram-negative bacteria namely *Escherichia coli (E. coli), Acinetobactor, Pseudomonas aeruginosa (P. aeruginosa), Klebsiella pneumonia (K. pneumonia), Salmonella enterica (S. enterica) and* two Gram-positive bacteria namely *Bacillus*

cereus (B. cereus), *Staphylococcus aureus (S. aureus)* collected from local hospitals and identified from the Institute of Microbiology, Government College University Faisalabad, Pakistan.

2.6.2 Agar well diffusion assay

The antibacterial activity of PWNSEO and PWNHEO were determined by Agar well diffusion assay techniques as reported by [29] with partial modification by using 15 µL instead of 100 µL of the essential oils. The diameter of zones of inhibition was measured in millimeter including well zones. Ciprofloxacin was taken as a standard drug.

2.4.3 Measurement of minimum inhibitory concentration (MIC)

For minimum inhibitory concentration (MIC) measurement, for PWNSEO and PWNHEO, a modified resazurin microtiter-plate assay was applied as already reported [30]. Ciprofloxacin was used as a standard drug.

2.7 Statistical Analysis

From each plant material three samples were collected and examined separately in triplicate while data was statistically studied by using analysis of variance (ANOVA) along with Post-Hoc Tukey HSD test using STATISTICA 5.5 (Stat Soft Inc., Tulsa, OK, USA) software and the probability value of $p \leq 0.05$ showed a statistical significance difference. These results were mentioned as mean values ± standard deviation.

3. Results and Discussion

3.1 Yields of the Essential Oils

The percentage yields of PWNSEO and PWNHEO were found to be 0.35% and 0.28 %, respectively. The percentage yield obtained in our study is same in case of PWNSEO and very close in case of PWNHEO to the previously reported study in which Maurya et al.[31] extracted essential oil of needles of *Pinus wallchiana* and yield was found to be 0.35%. Dambolena et al.[22] and Sharma et al.[1] revealed a greater yield of oils as compared to our study which were 0.97% and 1.2% respectively.

3.2 Chemical Composition of Essential Oils

Chemical composition of essential oil of *Pinus wallchiana* needles extracted by steam and hydro-distillation was determined by GC-MS analysis and results of chemical composition of these analyzed oils are shown in table 1. The 92.4 % essential oil of *Pinus wallchiana* needles extracted by steam-distillation was composed of 16 identified components including the major components such as α-pinene (13.65 %), 4-terpineol (9.12 %), α- terpineol (8.58 %) and α-terpinyl acetate (8.41%), caryophyllene (6.56 %) and α-terpinolene (5.21 %). It was also observed from results that analyzed essential oil contained oxygenated monoterpenes hydrocarbons (38.1 %) as major class of compounds followed by monoterpenes hydrocarbons (23.68 %), sesquiterpenes hydrocarbons (17.17 %) and oxygenated sesquiterpenes hydrocarbons (8.27 %). In essential oil of *Pinus wallchiana* needles extracted by hydro-distillation, 25 components were identified including the major components α-Pinene (14.14 %), β-myrcene (10.89 %), D-limonene (8.21 %), β- terpineol (6.98 %) and caryophyllene (5.94 %) which represented 93.93 % of total oil. This analyzed essential oil also showed that monoterpenes hydrocarbons (41.63%) were the major class of compounds followed by oxygenated monoterpenes hydrocarbons (24.85 %), sesquiterpenes hydrocarbons (19.56 %) and oxygenated sesquiterpenes hydrocarbons (7.89 %).

Dambolena et al[22] extracted the essential oil of young needles of *Pinus wallchiana* through hydro-distillation and analyzed the chemical composition of this essential oil which reported that β-pinene (34.0 %), α-pinene (14.8 %), limonene (17.8 %) and β-bisabolene (4.8 %) were the major **Table 1: Quantitative and qualitative analysis of *Pinus wallchiana* needles essential oils (PWNSEO and PWNHEO) by GCMS.**

Sr. No.	Compound	RT	PWNSEO	PWNHEO	Method of identification
Monoterpene hydrocarbons					
1.	α-pinene	2.269	13.65 ± 0.48[b]	14.14 ± 0.42[a]	RT, MS
2.	β-myrcene	2.369	-	10.89 ± 0.32	RT, MS
3.	3-carene	2.903	2.82 ± 0.67	-	RT, MS
4.	Limonene	2.94	2.67 ± 0.13[b]	8.21 ± 0.24[a]	RT, MS
5.	Camphene	3.13	-	2.13 ± 0.10	RT, MS
6.	α-Terpinolene	3.365	5.21 ± 0.26[b]	6.26 ± 0.25[a]	RT, MS
7.	1,3,8-p-Menthatriene	3.657	3.33 ± 0.16	-	RI, MS
Oxygenated monoterpene					
8.	Fenchol	3.679	-	2.94 ± 0.06	RT, MS
9.	Isopinocarveol	4.012	-	4.34 ± 0.08	RI, MS
10.	Borneol	4.4	-	3.86 ± 0.09	RT, MS
11.	4-Terpineol	4.557	9.12 ± 0.32[a]	1.09 ± 0.05[b]	RT, MS
12.	α- Terpineol	4.912	8.58 ± 0.31	-	RT, MS
13.	β- Terpineol	5.154	-	6.98 ± 0.28	RI, MS
14.	bornyl ester	6.792	4.12 ± 0.16	-	RI, MS
15.	1,2-Oxolinalool	7.678	3.87 ± 0.14	-	RI, MS
16.	Myrcenol	7.89	-	3.36 ± 0.03	RI, MS
17.	α-Terpinyl acetate	8.01	8.41 ± 0.29	-	RT, MS
18.	Nerol acetate	8.551	3.73 ± 0.06[a]	0.61 ± 0.03[b]	RT, MS
Sesquiterpene hydrocarbons					
19.	Longifolene	9.929	3.20 ± 0.06[b]	2.89 ±0.11[a]	RT, MS
20.	Caryophyllene	10.317	6.56 ± 0.26[a]	5.94 ± 0.23[b]	RT, MS
21.	β-Farnesene	10.883	3.16 ± 0.11[a]	2.64 ± 0.03[b]	RT, MS
22.	α-Amorphene	12.228	-	1.74 ± 0.09	RT, MS
23.	δ-Cadinene	12.904	4.25 ± 0.16[b]	4.01 ± 0.20[a]	RT, MS
24.	α-Muurolene	13.171	-	2.34 ± 0.02	RT, MS
Oxygenated Sesquiterpene hydrocarbons					
25.	Caryophyllene oxide	14.44	4.54 ± 0.09[b]	2.37 ± 0.12[a]	RT, MS
26.	Ledol	14.615	-	0.11 ± 0.01	RT, MS
27.	Longiborneol	14.772	-	1.16 ± 0.058	RI, MS
28.	α-Humulene epoxide II	15.041	-	0.33 ±0.02	RI, MS
29.	α -Cadinol	15.88	-	3.12 ± 0.16	RT, MS
30.	α-Bisabolol	16.74	-	0.19 ± 0.01	RT, MS
Monoterpene hydrocarbons			23.68	41.63	
Oxygenated monoterpenes			38.10	24.85	
Sesquiterpene hydrocarbons			17.17	19.56	
Oxygenated Sesquiterpenes			8.27	7.89	
			87.22	91.65	

Values are reported as mean ± standard deviation of triplicate determinations.

components. This study reported the same content of α-pinene compared to our results but high content of limonene. Also, β-pinene and bisabolene were absent in our results. In another study, Sharma et al[1] also extracted the essential oil of needles of *Pinus wallchiana* through hydro-distillation and reported that the α-pinene (48.6%), β-pinene (45.6%) and limonene (5.6%) were the major components of *Pinus wallchiana* needles essential oil. In our results, α-pinene and limonene were also major components but with different percentages. These results showed greater content of α-pinene, and limonene as compared to our results. In another study, Maurya et al[31] determined the

chemical composition of *Pinus wallchiana* needles essential oil extracted through hydro-distillation and found that α-pinene (31.5%), β-pinene (42.7%), limonene (8.1%) and β-Myrcene (7.2 %). Again α-pinene and limonene were major components in this study as shown by our results but with different percentage. The variation in the percentage composition in present study results and in already published results might be due to the variation in seasonal condition in which they are grown, geographical and agro-climatic may also affect[32].

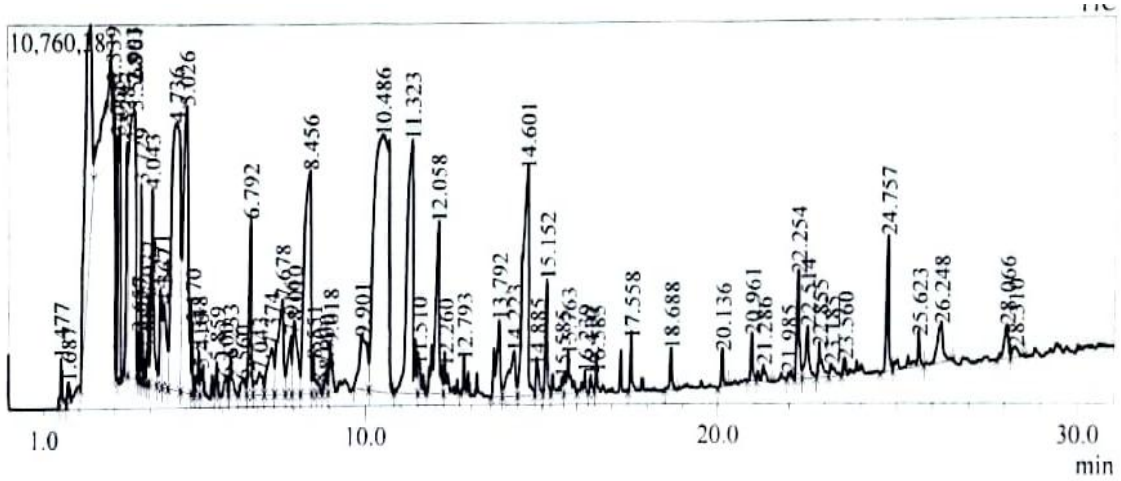

Figure 1: Chromatogram *Pinus wallchiana* needles essential oils extracted by steam-distillation.

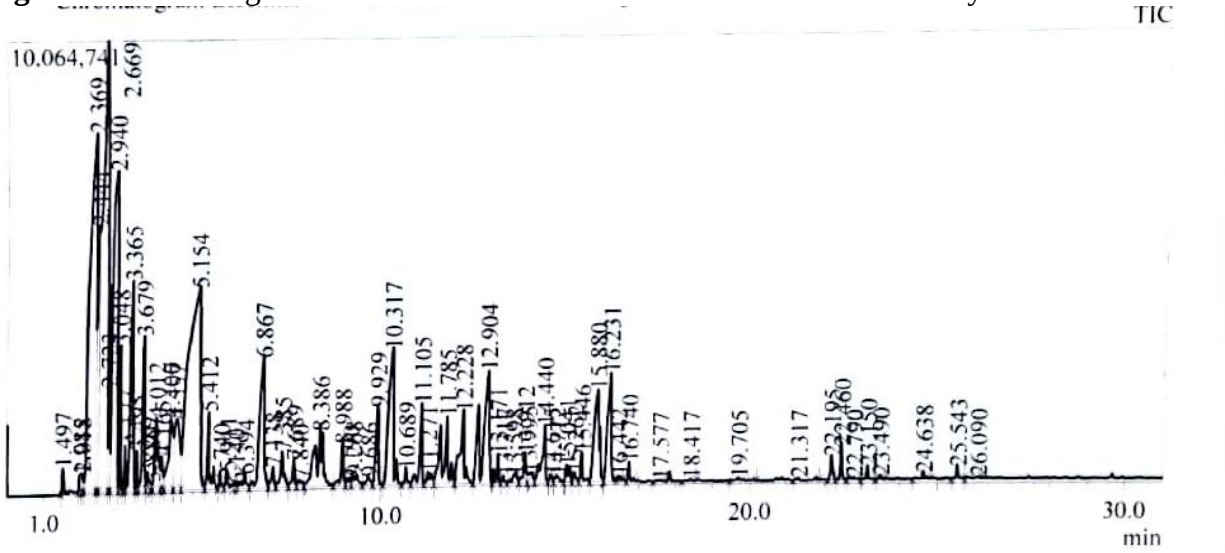

Figure 2: Chromatogram of *Pinus wallchiana* needles essential oil extracted by hydro-distillation.

3.2 Antioxidant activity

3.2.1 DPPH radical scavenging activity

Free radical scavenging activity of essential oil of *Pinus wallchiana* needles extracted by steam and hydro-distillation was evaluated by DPPH assay and results are shown in table 2. The results showed that IC_{50} value of PWNSPO and PWNHEO are 41.74 µg /mL and 33.15 µg /mL respectively. BHT is a synthetic antioxidant which was used as standard and its IC_{50} value was recorded as 0.12. Both PWNSEO and PWNHEO have high IC_{50} value but low free radical scavenging activity than BHT. Also, PWNSEO showed higher IC_{50} value but low free radical scavenging activity than PWNHPO. The order of free radical scavenging activity was as follows: BHT > PWNHEO > PWNSEO. The variations in the radical scavenging activity of PWNSEO and PWNHEO was statistically significant ($p \leq 0.05$). The major components in PWNHEO, α-pinene and limonene showed IC_{50} values of 11.98 µg/mL and 13.35 µg/mL and outstanding radical scavenging activity. Relatively higher percentage of

these constituents may one of the reasons to mention the correlated DPPH radical scavenging activity of PWNHEO. Also, essential oils are complicated mixture of various chemical constituent. However, it is difficult to study the characteristics like antioxidant activity /radical scavenging of complete essential oil to one or more main components[33].

Present results are comparable with the findings of Sharma et al.[21]who reported the radical scavenging activity of essential of bark of *Pinus wallchiana* with IC_{50} value 58.4 µg/mL. Shrama et al.[1] also reported the radical scavenging activity of essential oil of *Pinus wallchiana* isolated from leaf part with IC_{50} value 514.4 µg/mL and this IC_{50} value is very much high than our findings for PWNSEO (41.74 µg /mL) and PWNHEO (33.15 µg /mL).

Table 2. Antioxidant and Antiproliferative activities of *Pinus wallchiana* needles essential oil (PWNSEO and PWNHPO).

Antioxidant assays	PWNSEO	PWNHEO	α-pinene	*Longifolene*	BHT
DPPH, IC_{50} (µg /ml)	41.74 ± 0.83^{e}	33.15 ± 0.66^{d}	11.98 ± 0.34^{b}	14.47 ± 0.72^{c}	0.12 ± 0.01^{a}
Inhibition in linoleic acid system (%)	55.41 ± 1.65^{b}	68.21 ± 2.04^{b}	57.89 ± 3.54^{c}	52.34 ± 4.01	93.48 ± 3.74^{a}

Values are mean ± standard deviations of triplicate determinations. Letters in superscript show the significant ($p \leq 0.05$) difference among the PWNSEO and PWNHEO essential oils.

3.2.2 Percentage inhibition of linoleic acid oxidation

Table 2 shows the results of the percentage inhibition of linoleic acid oxidation by PWNSEO and PWNHEO. The values of percentage inhibition of PWNSEO and PWNHEO are 55.41% and 68.21%. PWNHEO showed higher inhibition value in linoleic acid than PWNSEO. The inhibition value in linoleic acid of both PWNSEO and PWNHEO was lower as compared to BHT i.e. 93.48%. The order of inhibition value of linoleic acid oxidation was as follows: BHT > PWNHEO > PWNSEO. The percentage inhibition of PWNSEO and PWNHEO determined in our study was lower than published by Ayub et al.[34] who described the linoleic acid peroxidation for *Pinus roxburghii* oleoresin essential oils as being in the range 80.46–96.55%. The variation in the percentage inhibition in present study results and in already published results might be due to different composition of essential oils which depends upon extraction techniques, seasonal condition, agro-climatic, geographical variation where these are grown up[34].

3.2.3 Reducing Power Assay

The antioxidant activity of PWNSEO and PWNHEO was also evaluated by reducing power assay. Figure 2 represented the reducing potential of PWNSEO and PWNHEO. The reducing potential of PWNSEO and PWNHEO was up to 0–10 mg/mL concentrations. The comparison of results showed that reducing potential of PWNHEO was higher than PWNSEO but on comparing with positive control, the reducing potential of both PWNSEO and PWNHEO was negligible. The reducing capacity assessments of essential oils and positive control were in this order: BHA> PWNHEO> PWNSEO. These results are well supported with reported findings i.e., *pinus wallichiana* essential oil has low or negligible reducing potential[1].

3.4 Antibacterial Activity

Antibacterial activity of PWNSEO and PWNHEO against seven bacterial strains, i.e., *Escherichia coli, Acinetobactor, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella enterica* (Gram-negative) and *Staphylococcus aureus, Bacillus cereus, (*Gram-positive) is presented in table 3.

Inhibition zones and MIC values of PWNSEO and PWNHEO were in range of 12.86 mm to 31.02 mm and 58.1 to 256.4 µg/mL, respectively. The values for positive control (Ciprofloxin) were 20.30 mm to 49.00 mm and 28.8 µg/mL to 63.9 µg/mL, respectively. The lesser antibacterial activity was

presented by PWNSEO with inhibition zone (12.86 mm to 27.19 mm) and MIC values (72.2 µg/mL

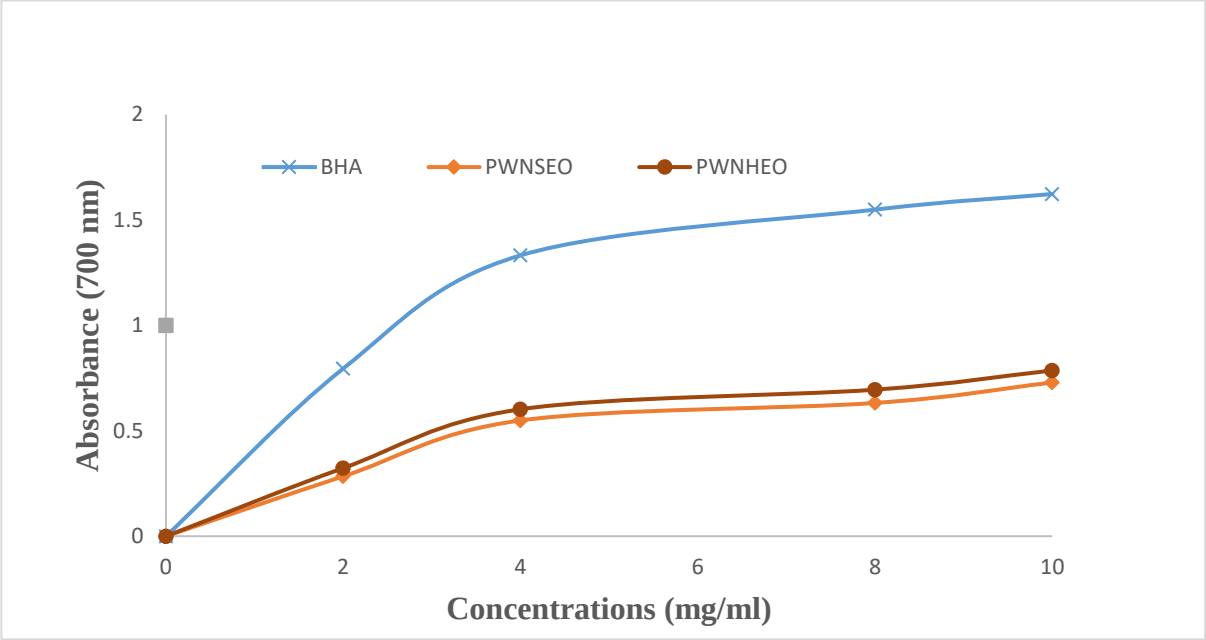

Figure 3. Reducing potential of *Pinus wallchiana* needles essential oil (PWNSEO and PWNHPO)

to 256.4 µg/mL). The highest antibacterial bacterial activity was shown by PWNHEO with inhibition zones (14.56 mm to 31.02 mm) and MIC values (58.1 µg/mL to 216.6 µg/mL). Both PWNSEO and PWNHEO were found to be more active against Gram positive strains in comparison to Gram-negative strains. The inhibition zones ranged from 18.09-31.02 mm and 12.86-22.68 mm against Gram-positive as well as Gram-negative bacterial strains in respective manner by PWNSEO and PWNHEO. Sharma et al.[21] evaluated the antibacterial activity of essential oil of *Pinus wallchiana* and reported that this essential oil inhibited the growth of *E. coli*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These findings are in accordance with our results. Parihar at al.[35] reported that leaf extract of *P. roxburghii* inhibit growth of *E. coli* and *Salmonella sp*. The results reported by Parihar showed close agreement with our findings. Gram-positive bacteria have been frequently reported as more sensitive to essential oil and its components as compare to Gram-negative bacteria[36]. Both PWNSEO and PWNHEO contain high content of α-pinene, α-terpinolene and caryophyllene which may be responsible for their antibacterial activity. Similarly antibacterial activity of essential oil varies with concentration of α-pinene and type of bacteria[36].

Table 3. Antibacterial activity of *Pinus wallchiana* needles essential oils (PWNSEO and PWNHEO).

Microorganism	PWNSEO		PWNHEO		Standard (Ciprofloxin)	
	IZ (mm)	MIC (µg/mL)	IZ (mm)	MIC (µg/mL)	IZ(mm)	MIC(µg/mL)
Acinetobactor	14.84±0.74	228.7±4.57	18.56±0.92	185.23±5.55	49.00±2.45	28.8±1.40
B. cereus	18.09±0.91	138.4±2.76	29.15±1.45	72.18±2.88	46.40±2.32	37.1±1.48
E. coli	16.46±0.82	158.5±4.75	22.48±0.89	126.28±3.78	20.30±0.81	63.9±3.78
K. pneumoniae	16.49±0.98	179.6±5.38	22.68±1.13	131.7±3.95	30.90±1.85	55.5±2.75
P. aeruginosa	13.18±0.79	233.8±4.67	17.66±0.70	196.3±3.92	41.70±2.08	45.8±2.70
S. enterica	12.86±0.77	256.4±5.12	14.56±0.72	216.6±6.49	34.00±2.04	51.9±2.04
S. aureus	27.19±1.08	72.12±2.6	31.02±1.24	58.1±2.32	39.30±1.96	49.1±2.94

PWNSEO and PWNHEO samples and their mean values ± standard deviation, analyzed in triplicate individually.
IZ, inhibition zones diameter in (mm) along with 6 mm. of disc diameter
MIC, minimum concentration of inhibiton (mg mL^{-1}).

3.5 Conclusion

The results of the present study suggest that *Pinus wallichiana* needle essential oil extracted through hydro-distillation (PWNHEO) contains significantly higher levels of monoterpene hydrocarbons and sesquiterpene hydrocarbons compared to Pinus wallichiana needle steam-extracted oil (PWNSEO). This difference in chemical composition may contribute to the observed superior antioxidant and antibacterial activities of PWNHEO, indicating that it holds greater potential for use in therapeutic applications.

The essential oils extracted from *Pinus wallichiana* needles, whether by steam distillation or hydro-distillation, exhibit notable biological properties, which align with their traditional use in medicine. This suggests that these oils could play a supportive role in various treatments or wellness products.

To further unlock their therapeutic potential, future studies should focus on:

1. Fractionation of the essential oils from both extraction methods to isolate specific compounds, helping to better understand their individual contributions to the biological activities.
2. Exploring the pharmaceutical and nutraceutical applications of these essential oils and their fractions to determine how they could be integrated into modern health products.

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Conflict of Interest: The authors declare no conflict of interest.

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