Chemical Composition, Antioxidant, and Antibacterial Activity of the Essential Oil from the Leaves of *Pinus sylvestris*

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ABSTRACT

The essential oil of air-dried leaves of *Pinus sylvestris* obtained through hydro-distillation was characterized by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry analyses (GC-MS). The oil yielded 0.78% per dry weight of sample. The oil was composed majorly of mono- and sesquiterpenoids. The major components of the essential oil of *P. sylvestris* were humulene (13.24%), α-guaiene (11.57%), allo-ocimene (9.40%), terpinolene (8.84%), caryophyllene (8.84%), and terpineol (5.02%). The air-dried leaf oil showed strong activity against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*, as well as a promising antioxidant potency. To our knowledge, this is the first report concerning chemical composition and antimicrobial activities of the essential oil from *Pinus sylvestris.*

(Keywords: essential oil, *Pinus sylvestris, Scots pine, Scotch pine, Baltic pine, GC-MS, GC-FID, antibacterial, humulene, antioxidants*)

INTRODUCTION

Reactive oxygen species (ROS) are formed as a natural byproduct of the normal metabolism of oxygen and environmental stress can increase their level dramatically resulting in significant damage to cell structures[1]. This is cumulatively known as oxidative stress. They are chemically reactive chemical species containing oxygen. Examples include peroxides, superoxide, hydroxyl radical, and singlet oxygen[2].

Molecular oxygen (O_2) is the premier biological electron acceptor that serves vital roles in fundamental cellular functions[3]. However, its reduction in the cells produces superoxide, which is the precursor of most other reactive oxygen species[4]. The damaging effect of these oxygen species include apostasies, cardiovascular diseases, oxidative damage, cellular ageing, and cancer [5,6,7,8,9].

The damaging effects of these reactive oxygen species can be mitigated through the positive effects of antioxidants, both natural and synthetic. Natural antioxidants are the secondary metabolites of phytochemicals and are preferred over synthetic antioxidants, which are found to impose side effects[10].

Pinus sylvestris is of the genus *pinus* belonging to the family *Pinaceae,* is a species widely distributed from Europe, including Britain, from Scandanavia South and East to Spain, Albania, temperate Asia, and tropical locations[11]. The medicinal properties of various parts of *P. sylvestris* have been studied by many researchers. It has been used as an antiseptic agent and is known to have beneficial effect on the respiratory system[12].

It is a valuable remedy in the treatment of kidney, bladder, and rheumatic affections, and also in diseases of the mucous membranes and the treatment of respiratory complaints. Externally it is used in the form of liniment plasters and inhalers[13]. The leaves and young shoots are antiseptic, diuretic, and expectorant. They are harvested in the spring and dried for later use. They can be added to the bath water for treating

fatigue, nervous exhaustion, sleeplessness, and skin irritations. They can also be used as an inhalant in the treatment of various chest complaints[14]. The essential oil from the leaves is used in the treatment of asthma, bronchitis, and other respiratory infections, and also for digestive disorders such as excessive flatulence. An essential oil obtained from the seed has diuretic and respiratory-stimulant properties[12].

Although some earlier studies revealed the medicinal attributes of *P. sylvestris*, very little work has been done on the antioxidant and antimicrobial activities of the essential oil of this plant. It is on this backdrop that this research was undertaken.

EXPERIMENTAL

Plant Material and Essential Oil Extraction Technique

The healthy leaves of *Pinus sylvestris* were collected from the botanical garden of the University of Lagos, Akoka, Yaba Area of Lagos State, Nigeria in August 2020. The botanical identification and authentication was done in the Herbarium of the Department of Botany, University of Lagos, Nigeria. The fresh leaves of *P. sylvestris* were air-dried for a period of one week and pulverized using a mechanical grinder prior to extraction. The essential oils from the air-dried leaves were obtained by hydro-distillation of 300g each of the plant material using the modified Clevenger-type apparatus[15]. The oil was dried over anhydrous sodium sulphate and stored in a refrigerator prior to analysis.

GC–FID and GC/MS Analyses of Volatile Oils

The essential oil samples were analyzed using a Varian CP-3800 gas chromatograph fitted with a flame ionization detector (FID) and dimethylpolysiloxane (100%) column (CP Sil-5 CB: 50 m length × 0.25 mm i.d. × 0.4 μm film thickness) (Varian, Netherlands). Nitrogen was the carrier gas with a 16-psi inlet pressure. Samples (0.2 μL) were injected in split mode with a ratio of 1:100. The column was initially held at 60°C for 5 minutes then heated to 220°C at a 5°C/minute ramp rate and was held for 3 minutes at that temperature. The temperature was further raised to 250°C at a 5°C/minute ramp rate and was held at this temperature for 4 minutes. The injector and detector temperatures were maintained at 250° and 300°C, respectively.

The gas chromatography/mass spectrometry (GC/MS) analyses performed on a Perkin Elmer Turbo mass Clarus 600 Instrument at 70 eV ionization energy with a mass range of 40–500 amu, employing an Elite-5 column (5 % phenyl and 95 % dimethylpolysiloxane) of 30 m length, 0.25 mm internal diameter and 0.25 μm film thickness (PerkinElmer, USA). Helium (1 mL/min) was used as a carrier gas. The initial temperature was 60 °C (1 min), this was increased to 240 °C at rate of 6 °C/min, and remained at 240 °C for 6 min, and then continued to increase to 250 °C at rate of 10 °C/min, with a final stage of 10 min at 250 °C. The oven temperature was programmed from 50 °C to 250 °C at a 5 °C/min dynamic rate and remained for 15 min at 250 °C. Sample (0.1 uL) were injected with a split less mode.

Identification of Volatile Oil Constituents

Component identification was accomplished by comparison of the retention indices (RI) of the GC peaks with those obtained using saturated n-alkanes (C8–C30) (Aldrich, USA), those reported in the literature[16,17,18,19] and by comparison of the mass spectra of the peaks with those reported in the literature[20,21] and stored in the NIST library. Peak area percentages were calculated from GC–FID response without employing correction factors. RI values were calculated for all components using a homologous series of *n*-alkane. Mixtures (C7-C30) injected under conditions similar to those of the samples and computer matched with the NIST libraries.

Antioxidant Assay

DPPH Radical Scavenging Assay: The free radical scavenging capacity of the compounds was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method[22,23] with modifications. The essential oil was allowed to react with stable free radical, DPPH for half an hour at 37 ^{O}C. The concentration of DPPH was 1 mM. The oils (10, 20, 30, 40 and 50 μL) were mixed with DPPH prepared in methanol. Ascorbic acid (4 mg/mL in methanol) was used as positive control. DPPH solutions at the same concentration without the tested oil was used as negative control. Each sample, as well as each control was analyzed in triplicate. The end volume for each sample was

100 µL in each well of the 96 well plate. After incubation, decrease in absorbance was measured at 517 nm using microplate reader (BMG Labtech Fluostar Omega UV-VIS microplate reader Instrument, Inc., USA). Percentage radical scavenging activity was calculated using the formula:

$$Inhibition\ \% = \frac{AC - AS}{AC} \times 100$$

AC = Absorbance of control.
AS = Absorbance of Sample.

In order to calculate the IC_{50}, the essential oil was prepared in a series of concentrations of 1, 10, 20, 40, 60, 80, 200, 400, 800, and 2000 µg/mL. The test was repeated as described above for all concentration of each oil in triplicates. Inhibition % was plotted against concentration and the IC_{50} was calculated graphically.

FRAP - Ferric Reducing Antioxidant Power Assay: Ferric ion reducing capacity of the essential oil of *P. sylvestris* was conducted using the method described by[24]. The ability of the essential oil to reduce ferric tripyridyltriazine (Fe(III)-TPTZ) complex to its ferrous colored form (Fe(II)-TPTZ) at low pH was determined using a spectrophotometer. 1.5 mL of FRAP reagent (2.5 mL of 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6) was added to 50 µL of each sample (100 µg/mL). After incubation at 37 °C for 10 min, the absorbance was measured at 593 nm. FRAP reagent without the sample was as blank and the experiment was performed in triplicate. Different concentration of aqueous solution of $FeSO_4.7H_2O$ (in a range of 125-1000 µmol/L) was used for calibration curve. The relative antioxidant activities of samples were reported as mmole Fe^{2+}/100 g of fractions.

β-Carotene Bleaching Test: The β-carotene bleaching capacity of the essential oil of *P. sylvestris* was conducted using the method of Kelvin *et al*[25] with slight modification; 10 mg of β-carotene was dissolved in 10 mL of chloroform. The carotene-chloroform solution, 0.2 mL, was pipetted into a boiling flask containing 20 mg linoleic acid and 200 mg Tween 40. Chloroform was removed using a rotary evaporator and 50 mL of distilled water were added slowly with vigorous agitation to the residue, to form an emulsion. 5 mL of the emulsion were added to a tube containing 2 mg of essential oils and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50 °C and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60 min period. Control samples contained 10 µL of water instead of essential oils. Butylated hydroxy anisole (BHA) was used as a reference.

The antioxidant activity was expressed as inhibition percentage with reference to the control after a 60 min incubation using the following equation:

$$AA = 100(DRC - DRS)/DRC$$

where AA is the antioxidant activity, DRC is the degradation rate of the control = [log (a/b)/60], DRS is the degradation rate in presence of the sample = [log (a/b)/60]; a is the absorbance at time 0; b is the absorbance at 60 min.

Antibacterial Assay

The essential oil of *P. sylvestris* was tested on three different bacterial strains. The strains were maintained at 4 °C and they are, *P. aeruginosa* ATCC 21234, *K. pneumoniae* ATCC 15522 and *E. coli* ATCC 25922. The bacterial strains were cultured in a Thermo Scientific Oxoid Nutrient agar (NA) at 37 °C for 24 hours. The disc diffusion method[26] was used to determine the antimicrobial activities of the essential oils. Petri plates were prepared by pouring 20 mL Thermo Scientific Oxoid Nutrient agar (NA) and the solution was allowed to solidify. The plates were then dried, and 0.1 mL of the standardized inoculum containing 106-107 colony-forming units/mL of the bacterial suspension was poured, uniformly spread, and allowed to dry for 5 minutes.

The essential oil was prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL. 100 µL was taken from this stock solution and was added to respective wells. The control well received only 100 µL DMSO. Gentamycin (positive control) was used as the reference antibiotics. The plates were left at room temperature to allow diffusion and then incubated at 37 °C for 24 hours for bacterial growth. The antimicrobial activity was evaluated by measuring the diameter of the zones of inhibition against the test organisms. The experiments were repeated

in triplicate and the results are expressed as average values.

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method using 96-well microplates. The inoculum of the microbial strains was prepared from 24 to 48 hours broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Serial concentrations (500, 250, 125, 62.5, 31.3, 15.6, 7.81, 3.9, 1.95, 0.98, and 0.49 µg/mL) of essential oil were prepared. 100 µL from culture broth was mixed with 100 µL of different concentration of the essential oils of *P. sylvestris* in the corresponding well and plates were incubated either at 37 °C for 24 hours for antibacterial activity. The lowest concentration of the tested oil showing no microbial growth was defined as the MIC.

Minimum bactericidal concentration (MBC) values were determined by taking a part of the liquid from each well that showed no growth and incubating on agar plates at 37 °C for another 24 hours. The lowest concentration that disclosed no visible growth of bacteria or fungi was confirmed as MBC.

RESULTS AND DISCUSSION

The yield of leaf essential oil obtained by hydro-distillation was 0.65 % (w/w relative to dry material weight). The analysis by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) identified 35 volatile compounds, accounting for 98.86 % of the total extracted oil, which were identified by matching retention times of available authentic standards, retention indices (RIs), and mass spectra in the NIST 17 database (Table 1).

The essential oil was mainly composed of 13 monoterpene hydrocarbon (37.14%), 2 oxygenated monoterpenes (5.71%), 13 sesquiterpene hydrocarbon (37.14%), 5 oxygenated sesquiterpenes (17.14%) and 1 diterpenes (2.85%). As shown in Table 1, the major compounds are humulene (13.24%), α-guaiene (11.57%), allo ocimene (9.42%), terpinolene (8.84%), caryophyllene (8.27%), γ-terpinene and terpineol (5.02%).

The essential oil *P. sylvestris* from Turkey contained α-pinene, camphene, and β-pinene as major constituents[27] while those from Lithuania had significantly higher concentration of γ-Terpinene, Caryophyllene oxide, δ-3-Carene, α-Terpinene, γ-Terpinene and Terpinolene. Sabinene + β-Pinene, 1-epi-Cubenol, Camphene, Sabinene + β-Pinene, Myrcene, α-Cadinene and 1-epi-Cubenol[28]. In another research, the essential oil of from the twigs of *P.sylvestris* was composed 49·2% α-pinene, 30·1% sabinene, 14·9 % β-pinene and 7·9 % limonene while the needles had 69·5 % α-pinene, 14·9 % camphene, 9·1 % β-pinene, 3·6 % sabinene and 2·8 % limonene[29].

The essential oil from this research (Nigeria) had some components common to those from Lithuania, Turkey and Spain. These components include: α-terpinene, γ-terpinene and terpinolene. However, this research reports the presence of some major components not found in or present in very low composition in the essential oil *P. sylvestris* earlier reported.

These components include humulene, allo ocimene, α-guaiene, caryophyllene, Isoaromadendrene epoxide and longiverbinone. Worthy of note is the presence of diterpene hydrocarbon, cembrene in the essential oil from this research, which has never been reported in earlier reports. These variations observed in the chemical composition of the essential oil may be due to geographical location. A different chemotype is suggested.

Antimicrobial Activity

The *in vitro* antimicrobial activities of essential oil against 3 pathogenic microorganisms (*Klebsialla pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa*) were evaluated using the disc diffusion method. The disc diameters of the zone of inhibition and the MIC of the essential oil for the tested microorganisms are shown in Table 2.

The essential oil was effective against *K. pneumoniae*, *E. coli* and *P. aeruginosa* with inhibition zones of 21.50, 18.90, and 20.20 mm respectively. In the broth microdilution assay, the essential oil showed the highest sensitivity to *K. pneumoniae* with MIC and MBC values of 65.5 and 135.6 µg/mL, followed by *E. coli* (MIC and MBC values of 105.2 and 215.50 µg/mL), and *P. aeruginosa* (MIC and MBC values of 78.3 and 156.70 µg/mL).

Table 1: Chemical Composition of the Essential Oil from *Pinus sylvestris.*

Compounds	aRI	bRI	%
α-Pinene	931	932	1.25
Fenchene	946	945	0.38
Camphene	942	946	1.47
Sabinene	961	967	0.24
β-Pinene	980	974	1.66
2,4,6-Octatriene, 3,4-dimethyl-	998	993	0.78
3-Carene	1014	1008	0.05
Benzene, 1-methyl-3-propyl-	1038	1042	1.85
γ-Terpinene	1048	1054	7.87
Terpinolene	1086	1086	8.84
Cycloheptene, 5-ethylidene-1-methyl-	1090	1095	1.55
Allo-Ocimene	1122	1128	9.42
Terpinen-4-ol	1165	1174	0.55
Terpineol	1174	1186	5.02
α-Terpinyl acetate	1342	1346	0.92
Bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl-	1411	1407	0.43
Isocaryophyllene	1416	1409	2.83
Cedrene	1405	1410	0.81
Caryophyllene	1416	1417	8.27
γ-Elemene	1440	1434	0.12
α-Guaiene	1437	1437	11.57
cis-β-Farnesene	1444	1440	0.52
Humulene	1453	1452	13.24
cis-Muurola-4(15),5-diene	1384	1465	3.23
Cadina-1(10),4-diene	1467	1469	3.44
α-Muurolene	1504	1500	1.72
γ-Cadinene	1514	1513	0.49
(Z)-γ-Bisabolene	1511	1514	0.09
Caryophyllene oxide	1583	1582	0.48
trans-Z-α-Bisabolene epoxide	1590	1586	0.10
Humulene epoxide 2	1603	1608	0.14
Longiverbenone	1637	1632	4.75
Isoaromadendrene epoxide	1645	1639	4.77
11,11-Dimethyl-4,8-dimethylenebicyclo[7.2.0]undecan-3-ol	1641	1646	0.18
Cembrene	1938	1937	0.93

aRI: Retention index determined relative to *n*-alkanes (C7-C30) on the HP-5ms column.
bRI: literature retention indices[16-19]

Table 2: Zones of Growth Inhibition (mm), MICs, and MBCs of Essential Oil from *Pinus sylvestris* Against the Growth of Microorganisms. [a]

Microorganisms	Diameters of Zones of Inhibition		MICs	MCBs
	Essential oil	Antibiotic	(µg/mL)	(µg/mL)
Klebsiella pneumonia	21.50±0.2	23.00±0.2	65.50	135.60
Escherichia coli	18.90±0.1	23.00±0.3	105.20	215.50
Pseudomonas aeruginosa	20.20±0.1	21.00±0.2	78.30	156.70

Abbreviations: MBC, minimal bactericidal concentration; MIC, minimum inhibitory concentration. Results were mean ± SD of triplicate values. Antibiotics used was, gentamicin

Table 3: DPPH, FRAP, and β-Carotene Bleaching Antioxidant Assay of the Essential Oil From *Pinus sylvestris.*

Antioxidant Assay	*Pinus Sylvestris* Essential Oil	Ascorbic Acid (Positive control)
DPPH Assay (µg/mL)	45.12 ± 3.48	40.24 ±. 3.22
FRAP Assay (µg/g)	3.70 ± 0.04	5.55 ± 0.04
β-Carotene Bleaching Assay (%)	80.66 ± 4.34	84.82 ± 4.36

Results of antioxidant capability were reported in mean ± SD of triplicate values.

The promising antibacterial activity of the essential oil could be attributed to the presence of major chemical components in the oil. The antimicrobial activity of humulene and caryophyllene and terpinene has been reported[30,31,32,33,34].

The activity shown by the essential oil of this plant could also be attributed to the synergistic effect of some of its minor components. Earlier reports have shown that components such isocaryophyllene, which in minor composition could also elicit good antimicrobial activities[35,36,37]

Antioxidant Activity

The antioxidant activity of the essential oil of *P. sylvestries* was evaluated using three different methods. These are, the FRAP, DPPH and β-carotene bleaching assays (Table 3). The essential oil of *P. sylvestris* showed good antioxidant activity with 45.12 µg/mL, 3.70 µg/g and 80.66 % for DPPH, FRAP and β-carotene bleaching assays. The antioxidant activity of the essential oil of this plant could be attributed to the presence of some major components of its essential oil.

The antioxidant property of caryophyllene has been earlier reported[38].The antioxidant effect of β-caryophyllene protects rat liver from carbon tetrachloride-induced fibrosis by inhibiting hepatic stellate cell activation[39].

The antioxidant activity of the essential oil of *Photinia serrulata and senecio nudicaulis* was attributed to the presence of α-humulene, caryophyllene oxide, γ-elemene, β-caryophyllene, epi-α-cadinol, epi-α-muurolol and δ-elemene[40,41]. In the 3 Assays carried out, the essential oil of *P. sylvestris* showed antioxidant potency that was almost similar to that of the standard drug (Ascorbic Acid) used (Table 3).

CONCLUSION

The essential oil of *P. sylvestris* was composed mainly of mono and sesquiterpenoids. The essential oil showed promising antibacterial and antioxidant capacity, which is suggested to be due to the presence of the major and minor components in the oil and their synergistic effect. The results indicate that the essential oil of *Pinus sylvestris* might be suitable for use as a natural antibacterial and antioxidant agent.

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