

Chemical composition and biological potential of *Pinus roxburghii* oleoresin essential oils extracted by steam distillation, superheated steam, and supercritical fluid CO₂ extraction

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Abstract

Pinus roxburghii is a rich source of high-quality oleoresin composed of resin acids and essential oil. The main purpose of the present study was to compare the yield, biological activities and chemical composition of *Pinus roxburghii* oleoresin essential oil. Steam distillation, supercritical fluid extraction, and superheated steam extraction at different temperatures (120 C, 140 C, and 160 C) were employed to extract essential oil from *Pinus roxburghii* oleoresin. It was observed that extraction methods significantly affected the yield, biological activity, and chemical composition of essential oils. The maximum yield (19.92%) was found in the essential oil extracted by SHSE at 160 C. Antioxidant activity was determined by total antioxidant content/ FRAP, DPPH-FRSA, hydrogen peroxide scavenging assays and percentage inhibition in linoleic acid oxidation. The essential oil extracted by SHSE at 160 C showed the highest DPPH-FRSA (63.33%), linoleic acid oxidation inhibition (96.55%), hydrogen peroxide scavenging activity (59.42%), and total antioxidant contents/FRAP (134.49 mg/L of gallic acid equivalent). The antimicrobial activity of EOs was determined by resazurin microtitre-plate, disc diffusion, and micro-dilution broth susceptibility assays. The antimicrobial activity results showed that superheated steam-extracted essential oil of 120°C and 140°C revealed the highest antifungal and antibacterial activity, respectively. GC-MS results showed that 3-carene, α -pinene, and β -pinene were the major chemical compounds present in essential oils. It is concluded that superheated steam extraction is an alternative and effective technique for extraction of oleoresins essential oil which improves the essential oil yield and biological activities.

1. Introduction

Food borne illness is major health concern of people in both developed and developing countries (Abunna, 2016). According to World Health Organization, the infectious diseases that occur by consuming contaminated water or food are called foodborne diseases. (Abebe et al., 2020). Micro-organisms are food borne pathogens i.e. bacteria, fungi, viruses. Particularly food borne bacteria i.e. *Salmonella enterica*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* spread food borne ailments (Zhao, 2014). Food borne diseases affect 30% of population in the developed countries whereas up to 2 million fatalities are estimated per year in the developing countries (Abunna, 2016). Oxidation reactions during production, distribution and storage of food products lower the food quality (Mirian Pateiro, 2018). Synthetic preservatives are used as antimicrobial and antioxidants in food products that may lead to allergies, intoxication, and degenerative diseases (Laranjo, 2017). So, scientists are searching for antimicrobial agents and antioxidants of natural origin that may help attenuate oxidative damage. Essential oils (EOs) may be used as alternative to synthetic preservatives due to their resistance against pathogens (Myszka et al., 2019).

The compounds which neutralize free radicals and reactive oxygen species (ROS) in the cells are called antioxidants. The antioxidants protect organisms from free radicals that are responsible for chronic diseases such as cardiovascular disease, cancer, aging, and anemia etc. (Zehiroglu & Ozturk Sarikaya, 2019). Free radicals are produced by uncoupled flow of electrons during metabolic processes. They may be harmful to lipids, nucleic acids, proteins, carbohydrates (Gulcin, 2020). Antioxidants reduce the oxidative stress related diseases by counteracting the deleterious effects of free radicals (Neha et al., 2019). Using EOs as natural antioxidants help avoid degenerative diseases (Bhalla et al., 2013). In a study 1% (v/w) EO of *T. Capitata* to minced beef significantly reduced the population of *L. monocytogenes* (El Abed et al., 2014). Therefore, EOs can be used for the preservation of meat products to increase the shelf life against *L. monocytogenes*. EOs are volatile, secondary metabolites of medicinal plants. They are aromatic oily liquids obtained from different parts of plants e.g. roots, leaves, herbs, flowers, barks, and buds (Burt, 2004). Essential oils possess antimicrobial and anti-inflammatory properties so they are used as analgesics, sedatives, anesthetics, and spasmolytic materials (Bakkali et al., 2008).

There are various techniques used for extraction of EOs from plants. The most common methods of extraction are hydro distillation (HD), steam distillation (SD), supercritical fluid CO₂ extraction (SCF-CO₂), subcritical water extraction (Reyes-Jurado et al., 2015). The hydro distillation process (HD), devised by Avicenna; is the oldest method of essential oil extraction. HD is the simplest, oldest method for extraction of EOs from plant materials. However, this technique have some disadvantages like low extraction efficiency, high fuel and water consumption, hydrolysis of compounds, prolong extraction time and decomposition of heat sensitive compounds. SD is simple, comfortable, more reproducible, and has high energy efficiency (Akdağ & Öztürk, 2019).

SD has disadvantages just like HD such as prolonged extraction time, degradation of ester or unsaturated compounds, and low extraction efficiency (Reyes-Jurado et al., 2015).

SCF-CO₂ involves the use of recycling fluid in periodic steps of compression or decompression. carbon dioxide is mainly used to extract essential oils by SCFE (Fornari et al., 2012). This process provides several properties such as lower density, lower viscosity, and high diffusivity. These properties enable fast extraction of essential oil at low temperatures. It is an environment friendly, cost-effective, and simple process (El Asbahani et al., 2015). This method requires less time as compared to conventional techniques. The major demerit of this technique lies in the non-polar nature of CO₂. Due to the non-polar nature of CO₂, the total content of bioactive phenolic compounds in essential oils cannot be extracted (Tyskiewicz et al., 2018). (Xiong & Chen, 2020) used this technique for the extraction of tangerine peel EO. Similarly, (Wu et al., 2019) extracted *Cymbopogon citronella* leaves EO using SCF- CO₂. (Ayub et al., 2018) used SCF- CO₂ to extract EO from *Oleogum resin* of *Boswellia serrata* Roxb

Superheated steam extraction (SHSE) is an alternative extraction technique in which superheated steam is used for extraction of EO. Superheated steam has temperature higher than saturation temperature at normal pressure. In the form of latent heat of vaporization, it carries a lot of thermodynamic energy. It has a high thermal conductivity, extraction capacity, and low oxygen conditions, preventing oxidation of extracted components. (Ayub et al., 2022). (Rouatbi et al., 2007) employed SHSE for the extraction of thyme and black pepper EO. Similarly, (Ayub et al., 2022) used this technique for the extraction of EO from oleogum resin of *Boswellia serrata*

Pinus roxburghii, referred to as Chir Pine, belongs to the Pinaceae family of gymnosperms. It is located in the Himalayas range of Pakistan, China, Afghanistan, India, and Nepal (Shuaib, 2014). Five species of pines including *Pinus Roxburghii* cover an area of 1928 thousand hectares in Pakistan. These species are mainly found in rangelands of northwest Frontier, Punjab, and Baluchistan (Hassan & Amjid, 2009). The plant is bitter, pungent, sweet, heating, oleaginous, intestinal antiseptic, antidyslipidemic and is used in treatment of the ear, throat, eye, skin, blood, bronchitis, diaphoresis, ulcer, inflammations, and itching (Abbasi et al., 2010). Wood is stimulant, diaphoretic and used in cough, fainting, and ulceration (Chopra et al., 2002). *Pinus roxburghii* EO has been used for the treatment of bronchitis, typhoid. (Arshad & Ahmad, 2004). *Pinus Roxburghii* is the rich source of high-quality oleoresin. Oleoresin, on distillation, produces an essential oil generally known as turpentine oil and rosin is left behind as a non-volatile solid residue (Shuaib et al., 2018). Diterpene resin acids are major constituents of rosin. Oleoresin is also composed of terpenes which are complex mixtures of monoterpenes, diterpenes, sesquiterpenes, oxygenated monoterpenes, oxygenated diterpenes, and oxygenated sesquiterpenes (da Silva Rodrigues-Corrêa et al., 2013). Turpentine oil is a complex mixture of monoterpenes and sesquiterpenes. Pinus oleoresins have role in pharmaceuticals and cosmetics (da Silva Rodrigues-Corrêa et al., 2013). It is also used for scorpion and snakebite stings (Sinha & Tandon, 2019). Turpentine oil is used in disinfectants and denaturants (Sood, 2018).

A thorough evaluation of previously published research studies manifested that SHSE is still not practiced for extraction of EO from *Pinus roxburghii* oleoresin. Therefore, the research work was performed to extract EO from *Pinus roxburghii* oleoresin by SD, SCF-CO₂ and SHSE at different temperatures. Moreover, the effect of extraction methods and extraction temperatures on the yield, antioxidant and antimicrobial activities, and chemical composition of EOs was evaluated.

2. Materials And Method

The *Pinus roxburghii* oleoresin was collected from fully grown wild pine trees of Shimla hills of Abbottabad District, Khyber Pakhtunkhwa Province, Pakistan. Shimla hills are located 10 km away from Abbottabad city. Shimla hills are 4100 feet high and lies at 34.1584° N, and 73.1962° E in Pakistan. The average annual temperature of Abbottabad is 15.9°C. The annual rainfall in Abbottabad is 60.3 inch. Borehole drilling method was used for collection of oleoresin as described by (Sharma & Lekha, 2013). One hole was drilled in the tree about 10 cm above the ground. Holes were drilled using hand-driven drill bits. The holes were drilled with a slight slope toward the opening to allow for free drainage of the oleoresin. Plant material was identified, verified by taxonomist, Dr. Fahim Arshad, Associate Professor, Department of Botany, University of Okara, Pakistan.. A voucher specimen ABT 004 was submitted in herbarium Department of Botany, University of Okara, Pakistan.

All methods were performed in accordance with the relevant guidelines and regulations.

2.1 Extraction of Essential Oil

Pinus roxburghii oleoresin EO was extracted by steam distillation (SD), supercritical fluid extraction- CO₂ (SCF- CO₂), and superheated steam extraction SHSE (at 120°C, 140°C and 160°C).

2.1.1 Steam distillation

Steam distillation apparatus consisted of a round bottom flask, a biomass flask, condenser, a heating mantle, thermometer, and collecting vessel. The oleoresin sample (100g oleoresin soaked in 100g cotton wool) was placed in biomass flask which was adjusted on the top of round bottom flask. Steam was generated by boiling water using heating mantle. The steam was allowed to enter biomass flask where it interacted with oleoresin sample. The EO vapors along with water vapors were passed through a condenser which cooled them to liquid droplets. The EO and water layers were separated on the basis of density in collecting vessel. EO layer was separated and collected in amber glass vial. Steam distillation was carried out for 3 hours. The EO was dried using anhyd. Na₂SO₄, followed by filtration using a micro filter, and was stored at + 4 C until further process. The extraction procedure was repeated five times to ensure that results were reproducible.

2.1.2 Supercritical fluid CO₂ Extraction

Liquid CO₂ chilled to 10°C was charged into the extraction tank (which was already kept at 45°C) using a high-pressure pump. The pressure was adjusted to an accuracy of 1% over the measuring range. To enable the easy effusion of supercritical CO₂ through extraction vessel, it was filled with raw materials and glass beads. After passing through a heated micrometer valve, the SC-CO₂ with dissolved compounds was inflated to ambient pressure. The extract was precipitated in a collection vessel at room temperature and pressure. At a known temperature and pressure, the total amount of CO₂ was measured using a calibrated wet-test meter. For each extraction test, the extractor was stuffed with about 500 g of oleoresin soaked in 500 g cotton wool. Flow rate of CO₂ was 2 L/min. An analytical balance was used to measure the weight of oil (Guan et al., 2007).

2.1.3 Superheated Steam Extraction

Superheated steam extraction was carried out in a custom sized superheated steam extractor apparatus consisting of a stainless steel extraction vessel connected to superheated steam generator, condensers and hydrosol collection vessel. Temperature of superheated steam was continuously monitored by thermocouples attached at the entry and exit of stainless steel extraction vessel. The oleoresin (100 g soaked in 100g cotton wool) was packed in the extraction chamber and subjected to superheated steam at 120 °C, 140 °C, and 160 °C for 1 hour. Moisture from EOs (10g) was removed by adding 0.25–0.50 g of anhy. Na₂SO₄, filtered using a micro filter and was stored in amber glass vials till further analysis. The extraction procedure was repeated five times to ensure that results were reproducible (Ayub et al., 2022).

2.2 Antioxidant Activity

2.2.1 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was used to determine total antioxidant content (Chan et al., 2007). 1 mL of each essential oil extracted by different extraction methods was mixed with 2.5 mL of phosphate buffer (pH 6.6, 0.2 M) and 2.5 mL of 1% w/v potassium ferricyanide solution. The test tubes were incubated in water at 50 C for 25 minutes. 2.5 mL of 10%w/v solution of trichloroacetic acid was added to each test tube. 2.5 mL of the reaction mixture from each test tube was transferred to a separate test tube, and diluted with 2.5 mL of deionized water. 500 µL of 0.1% w/v solution of ferric chloride was added to each test tube. Test tubes were incubated at room temperature for 30 minutes. A UV-Visible spectrophotometer was used to measure the absorbance of each solution at 710 nm. Total antioxidant activity is expressed in terms of mg/mL of Gallic acid equivalents. Total antioxidant activity was calculated by the Gallic acid calibration curve (0-100 mg/mL).

2.2.2 DPPH free radical scavenging activity (DPPH-FRSA)

DPPH assay was carried out in accordance with the literature (Bozin et al., 2006). 1 mL of DPPH solution (0.09 mM) was added to 2.5 mL of EO, and the final volume was made up to 4 mL using MeOH (95%). A spectrophotometer was used to measure the absorbance of the solution after 1 hour at room temperature in the dark at 515 nm. A positive control was butylated hydroxytoluene (BHT) at a concentration of 100 ppm. Eq. 1 was used to calculate the percentage of free radical inhibition by DPPH:

$$\%I = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (1)$$

2.2.3 Percentage inhibition in linoleic acid system

The percentage inhibition in linoleic acid peroxide formation was estimated based on the previous literature (Singh et al., 2006). 50 μ L EOs dissolved in 1 mL EtOH was mixed with 1 mL of 2.5% v/v solution of Linoleic acid, 4 mL of 99.5% EtOH, and 4 mL of 0.05 M sodium phosphate buffer (pH 7). The solution was incubated at 40 C for 175 hours. The colorimetric method was employed to estimate the extent of oxidation based on the peroxide value (Yen et al., 2000). To 0.2 mL sample, 10 mL of 75% of EtOH, 0.2 mL of 30% aqueous solution of ammonium thiocyanate, and 0.2 mL of ferrous chloride solution (20mM in 3.5% HCl) were sequentially added. The solution was stirred for 3 minutes. The absorbance was measured at 500 nm using a spectrophotometer. A spectrophotometer was used to measure absorbance at 500 nm. The same procedure was used to run a control group. A positive control was butylated hydroxytoluene (BHT). Eq. 2 gives the percent inhibition of linoleic acid.

$$\%Inhibitionlinoleicacidoxidation = 100 - \left[\left(\frac{Abs.increaseofsampleat175h}{Abs.increaseofcontrolat175h} \right) \times 100 \right] \quad (2)$$

2.2.4 Hydrogen Peroxide scavenging activity

The spectrophotometric estimation of hydrogen peroxide scavenging activity was described by (Bakkali et al., 2008). In phosphate buffer (pH 7.4) a 2 mM solution of hydrogen peroxide was prepared. 600 μ L EO was mixed with 600 μ L of hydrogen peroxide (2 mM). The resulting mixture was incubated at room temperature for 10 minutes. The absorbance was measured at 230 nm. Same procedure was carried out for Ascorbic acid (100 mg/L). Eq. 3 was used to compute the percentage of hydrogen peroxide scavenging activity:

$$\%Hydrogenperoxidescavengingactivity = \left(\frac{A_o - A_1}{A_o} \right) \times 100 \quad (3)$$

2.3 Antimicrobial Activity

2.3.1 Microbial Strains

EOs extracted by different extraction techniques were tested against *Escherichia coli* (*E. coli*), *Aspergillus flavus* (*A. Flavus*), *Staphylococcus aureus* (*S. aureus*), *Alternaria alternate* (*A. Alternata*), *Bacillus subtilis* (*B. subtilis*), *Fusarium solani* (*F. Solani*), *Pasteurella multocida* (*P. multocida*) and *Aspergillus niger* (*A. Niger*) collected from Institute of Microbiology, University of Agriculture Faisalabad, Pakistan.

2.3.2 Agar well diffusion method

Agar well diffusion was used to test the antibacterial activity of *Pinus roxburghii* oleoresin EO (Rashid et al., 2013). The overnight cultures of indicator strains were transferred to flasks containing 25 mL nutrient agar. The mixtures of flasks was transferred to 6 inches petri dishes to solidify at room temperature. A sterilized cork borber was used to create wells. These wells were filled with 10 μ L EOs and standard drug i.e. ampicillin (1 mg/mL) for evaluation of antibacterial activity. The petri plates were incubated at 37 C for 24 hours. The width of zones of inhibition (mm) was measured after incubation in order to determine the antimicrobial activity.

2.3.3 Resazurin microtitre-plate assay

A modified resazurin microtitre-plate test was used to determine the minimum inhibitory concentration (MIC) of EOs against various bacterial strains, as mentioned in the literature (Sarker et al., 2007). 10 mL of EO was dissolved in 1 mL of 10% DMSO to make the sample solution. 27 mg of resazurin was dissolved in 4 mL of sterilized deionized water to make resazurin indicator solution. In the first row of 96 well plates, about 100 μ L of sample solution and standard antibiotic Ampicillin (1 mg/mL in 10% DMSO) were pipetted. After that, with the exception of first row; 50 mL nutrient broth was added in all wells and two fold serial dilutions were conducted until each well had 50 μ L of the sample mixture. In each well, 30 μ L of 3.3x strength isosensitised broth, 10 μ L of resazurin solution and 10 μ L (5×10^5 colony forming units per mL) were added. Plates were incubated at 37 °C for 24 hours. After incubation process, MIC values measured visually. The bacterial growth was shown by the colour change from purple to colourless or pink, and the MIC value was determined by the lowest concentration at which the colour change occurred.

2.3.4 Micro-dilution broth susceptibility assay

The micro-dilution broth susceptibility assay was used to determine the MIC of EOs extracted by different extraction techniques against various fungal strains, as previously described in the literature (Wayne, 2008). 10 mg of EO and 1 mL of standard antibiotic in 1 mL of 10% DMSO solution were mixed to prepare sample solution of EOs and standard antibiotic (Fluconazole). Into the first row of the 96 well plates, about 100 μ L of samples and standard solutions were pipetted. After that, with the exception of the first row, 50 μ L of Sabouraud dextrose broth was added in all wells and two fold serial dilutions were conducted until each well had 50 μ L of the sample mixture. Then, 130 μ L Sabouraud dextrose broth and 20 μ L of microorganism suspension (5×10^5 colony forming units per mL) were added in all wells and well plate was incubated at 30°C for 48 h. The MIC value was visually determined. It is the lowest concentration at which fungal growth is completely inhibited.

2.4 Gas chromatography/mass spectrometry (GC-MS) analysis

Gas Chromatography-Mass Spectrometry (GC-MS) (Shimadzu gas chromatograph GC-2010 system) equipped with QP-2010 plus mass detector and DB-5 capillary column (50 m $\times$ 0.25 mm, film thickness of 0.25 μ m) was used to quantify the volatile components of EOs extracted by different extraction techniques. Temperature of Injector was set at 200 °C. An injection volume of 1 μ L EO was injected in to injection port using an injection syringe. Column was heated at 60 °C for 3 min and then gradually its temperature was increased up to 240 °C at the heating rate of 24°C/min and kept constant for next 10 min. Carrier gas was Nitrogen gas which flowed through the system at the flow rate of 1.5 mL/min. The temperature of MS transfer line was kept to 240 °C. For MS detection, an electron ionization mode (70 eV) was used (Ayub et al., 2018). By running n-alkanes (C₉-C₂₄) standards under same conditions, retention indices of identified compounds were determined. These retention indices and mass spectrum data were also compared to previously published data, the NIST mass spectral database library and pherobase mass spectral database library (Ayub et al., 2018; Hussain et al., 2013).

2.5 Statistical analysis

All the experiments were carried out in triplicate and data was statistically analyzed using analysis of variance (ANOVA) with Post-Hoc Tukey HSD test using STATISTICA 5.5 (Stat Soft Inc., Tulsa, OK, USA) software. A probability value at $p \leq 0.05$ was considered statistically significant. The results are presented as mean values $\pm$ standard deviation calculated from triplicate determinations.

3. Results And Discussion

3.1 Yield of EOs

Pinus roxburghii oleoresin EO was extracted by SD, SCF-CO₂, and SHSE (carried out at 120 °C, 140 °C, and 160 °C temperatures) and results are shown in Fig. 1. It was inferred from the results that extraction methods affected the yield of EO. The highest EO yield was observed by SHSE (19.92%) at 160 C. The higher temperature of SHSE may increase the yield of EO. High temperature of SHSE causes more vaporization of EO from oleoresin which leads to high yield. These results are similar to previous study in which EO yield of thyme leaves and black pepper extracted by SHSE was higher than HD (Rouatbi et al., 2007). It was observed that EO yield was increased with increasing the temperature of superheated steam. A much similar pattern was reported in previous literature in which EO yield of *Boswellia serrata* oleo-gum resin was increased with increasing temperature of

superheated steam (8.50–10.75%) (Ayub et al., 2022). Similarly, It was reported that *Citrus hystrix* leaves EO yield was increased by increasing extraction temperature of subcritical water extraction (Halim et al., 2021). It was also mentioned that high temperature decreased the hydrogen bonding, dielectric constant, polarity, viscosity, density and surface tension of water molecule and increased the diffusing power, mass transfer rate and EO yield (Halim et al., 2021). In another study, it was reported that anethole extraction efficiency of *Pimpinella anisum* seeds was increased by increasing temperature up to 170 C. Moreover, it has been reported that decrease in dielectric constant, surface tension, viscosity, and density of water with increasing the temperature, increased the extraction ability of water for polar molecules (Khajenoori et al., 2015). According to (Mottahedin et al., 2017), EO yield of *Curcuma longa* was increased with increasing temperature of SWE up to 120 C. Extraction techniques comparison showed that SHSE revealed higher EO yield followed by SD and SCF-CO₂, respectively. Similarly, (Badrkhani et al., 2013) reported that EO yield of *Trachyspermum ammi* seeds extracted by superheated water extraction (SWE) (5.68%) was more as compared to HD (2.95%) and Soxhlet extraction (3.65%). EO yield of *Pinus roxburghii* oleoresin was much higher than other parts of its plant extracted by HD i.e. bark 0.022% (Labib et al., 2017), needles 0.11% (Zafar et al., 2010), wood 0.5% (Khan et al., 2012), leaves 0.50% (Arfa sajid, 2015), flowers 0.08% (Kaushik et al., 2013) stem 0.24% (Hassan & Amjid, 2009). Similarly, SD of *Pinus roxburghii* needles produced 0.26% EO (Shuaib et al., 2013). So, it can be concluded that SHSE is a better extraction technique than SD and SCF-CO₂ with high EO yield.

3.2 Antioxidant Activity of EO

Antioxidant activity of EOs was performed by DPPH-FRSA, FRAP assay, H₂O₂ scavenging activity and % inhibition in linoleic acid system. DPPH is a reliable and simple assay used for evaluation of antioxidant activity of plant extracts. The antioxidant activity of EOs is because of hydrogen donating ability, resulting in free radical scavenging. This leads to reduction of DPPH and discoloration of violet color (Sharma et al., 2020). Antioxidant activity of *Pinus roxburghii* oleoresin EO obtained by different extraction techniques is shown in Table 1. The experimental results showed that EOs exhibited less DPPH free radical scavenging activity as compared to standard BHT. It was noticed that isolation techniques significantly affected the antioxidant activity of EOs of *Pinus roxburghii* oleoresin. EO extracted by the SHSE (160 C) showed maximum ability of DPPH free radical scavenging activity (63.33%). The lowest DPPH free radical scavenging activity was observed by SCF-CO₂ (49.53%), while SD showed (62.4%) DPPH-FRSA. The difference in FRSA might be due to the difference in concentration of chemical compounds present in EOs. It was previously reported that EOs from fresh fruits of *Pinus roxburghii* showed negligible scavenging activity (Qadir and shah et al., 2014). Similarly, it was mentioned that *Pinus roxburghii* needles EO exhibited lower antioxidant activity (50%) as compared to tannic acid (80%) while bark (85%) and wood (82%) were higher than tannic acid (Salem et al., 2014). In another study, it was reported that resin extracts and turpentine oils of *P. oocarpa*, *P. insulari*, *A. loranthifolia*, and *P. merkusii* showed less antioxidant potential as compared to control (ascorbic acid) (Tillah et al., 2016). The results of percentage inhibition of linoleic acid oxidation shown by *Pinus roxburghii* oleoresin EOs is given in Table 1.

Table 1

Antioxidant potential of *Pinus roxburghii* oleoresin essential oils extracted by steam distillation, supercritical fluid CO₂ extraction and superheated steam extraction

Extraction Method	Inhibition in linoleic acid system (%)	DPPH free radical scavenging activity (%)	Hydrogen peroxide scavenging (%)	Total antioxidant contents/FRAP ^A
Steam distillation	80.46 ± 1.42 ^f	62.4 ± 0.41 ^c	55.32 ± 0.29 ^c	67.44 ± 0.95 ^e
Supercritical fluid extraction (40 °C, 80 Bar)	88.37 ± 1.92 ^e	49.53 ± 0.11 ^f	52.49 ± 0.26 ^e	99.92 ± 1.06 ^d
Superheated steam (120 °C)	92.18 ± 1.33 ^d	54.67 ± 0.38 ^e	53.76 ± 0.16 ^d	102.07 ± 1.12 ^c
Superheated steam (140 °C)	93.16 ± 1.53 ^c	60.68 ± 0.34 ^d	50.43 ± 0.19 ^f	127.12 ± 1.21 ^b
Superheated steam (160 °C)	96.55 ± 1.71 ^b	63.33 ± 0.47 ^b	59.42 ± 0.32 ^b	134.49 ± 1.34 ^a
BHT	98.85 ± 1.89 ^a	97.92 ± 1.82 ^a	—	—
Ascorbic acid	—	—	85.57 ± 1.14 ^a	—
Values are mean ± Standard Deviations of three separate determinations.				
Different letters in superscript represent significant differences among <i>Pinus roxburghii</i> oleoresin essential oils extracted by different extraction methods.				
^A Total antioxidant contents/FRAP (mg/L of essential oil, measured as Gallic acid equivalent).				

The inhibition in linoleic acid activity of *Pinus roxburghii* oleoresin EOs was lower than control (BHT). The results are in agreement with (Karapandzova et al., 2019) in which EO of *Pinus mugo* needles showed less inhibition in linoleic acid as compared to control BHA and Ascorbic acid. EO extracted by SHSE at 160 °C showed maximum linoleic acid oxidation inhibition (96.55%), while EO extracted by SD showed minimum linoleic acid oxidation inhibition (80.46%). Results of hydrogen peroxide free radical scavenging activity are shown in Table 1. EO extracted by SHSE at 160 °C showed maximum hydrogen peroxide scavenging activity (59.42%), while EO extracted by SHSE at 140 °C showed minimum hydrogen peroxide scavenging activity (50.43%). Total antioxidant contents are represented as mg/L of GAE are given in Table 1. Total antioxidant contents in EO range from 67.44 mg/L to 134.49 mg/L. EO extracted by SHSE at 160 °C contained highest amount of total antioxidant contents, while EO extracted by SD contained the lowest amount of total antioxidant contents. These results are in accordance with previous literature study in which scavenging activity of *Citrus pomace* extract processed by superheated steam was increased by increasing extraction temperature (Wang et al., 2018).

Overall, SHSE showed higher antioxidant activity than SD and SCF-CO₂. EOs are complex mixture of variety of compounds. It is difficult to assess specific compound responsible for their antioxidant activity (Ayub et al., 2018). It was reported that scavenging activity of *Pinus roxburghii*, *P. wallichiana*, and *P. gerardiana* extracts might be due to the presence of phenolic compounds, that can donate electrons to H₂O₂ converting it to H₂O (Sharma et al., 2015). According to (Tsvetkov et al., 2019), the antioxidant activity of crude acetone extracts of *Pinus roxburghii* knot wood compounds was less than Gallic acid. Moreover, it was also mentioned that antioxidant activity was related to number of hydroxyl groups present on benzene ring, the degree of shielding of the hydroxyl groups by other functional groups, and the nature of other substituents present at the benzene ring capable of increasing and decreasing the electron density of the ring and thus modifying the oxidizability of the hydroxyl groups (Tsvetkov et al., 2019).

3.3 Antimicrobial Activity of EOs

Antimicrobial activity of EOs of *Pinus roxburghii* oleoresin extracted by different extraction techniques against bacteria and fungi was evaluated by well diffusion assay, micro dilution broth assay, and resazurin micro titer plate assays and results are given in Table 2. The inhibition zone and MIC values of EOs extracted by various extraction techniques were in range of 10.02 mm to 24.74 mm and 54.27 to 281.47 µg/mL, respectively. The values for positive control (Amoxicillin and Fluconazole) were 21.11 mm to 28.53 mm and 2.92 µg/mL to 13.33 µg/mL, respectively. The lowest antibacterial activity was shown by SCF-CO₂ EO with inhibition zone (10.14 mm to 16.30 mm) and MIC values (112.58 µg/mL to 281.47 µg/mL). The highest antibacterial activity was shown by EO extracted by SHSE at 140 C with inhibition zones (14.77 mm to 24.74 mm) and MIC values (56.29 µg/mL to 112.58 µg/mL). Variation in chemical constituents of EOs might be responsible for this difference in the antimicrobial activity. It was observed that *Pinus roxburghii* oleoresin EO was more effective against *Staphylococcus aureus* (a gram positive strain of bacteria). These results are in accordance with (Hassan & Amjid, 2009; Kaushik et al., 2013; Salem et al., 2014; Zafar et al., 2010) in which it was reported that EO of *Pinus roxburghii* extracted from stems, needles, wood, and bark was effective against *Staphylococcus aureus*. According to (Shuaib et al., 2013), the methanol resin extract of *P. roxburghii* showed better activity against Gram-positive bacteria than Gram-negative bacterial strains. In another study, it was reported that n-hexane resin extract of *P. oocarpa* was most effective against *Staphylococcus aureus* (Tillah et al., 2017). The results of antifungal activity are given in Table 2. The inhibition zone and MIC values of EO extracted by different extraction techniques were in range of 7 mm to 24.03 mm and 42.22 to 675.54 µg/mL, respectively and the values for positive control (Amoxicillin and Fluconazole) were mm 22.61 to 24.82 mm and 5 to 8.42 µg/mL, respectively. The highest antifungal activity was exhibited by EO extracted by superheated steam at 120 C with larger inhibition zone 17.84 mm to 24.03 mm and lower MIC values 70.36 µg/mL to 253.32 µg/mL. The lowest antifungal activity was exhibited by EO extracted by SHSE at 160 C with lowest inhibition zone 7 mm to 10 mm and highest MIC values 422.8 µg/mL to 675.54 µg/mL. It was noted that *Pinus roxburghii* oleoresin EO was more effective against *Fusarium solani* and *Alternaria alternata* with maximum inhibition zone (9–24.03 mm), (10–22 mm) and minimum MIC values (42.22- 506.65 µg/mL), (70.36- 337.76 µg/mL) respectively. Similar observations were made by (Khalid et al, 2016) in which it was reported that chloroform extract of *Pinus roxburghii* female cones showed maximum inhibition zone (22mm) against *Alternaria Alternata*, while methanol extract of *Pinus roxburghii* female cones showed maximum inhibition zone (20mm) against *Fusarium Solani*. Similarly, chloroform and methanol extract of *Pinus roxburghii* needles showed maximum inhibition zone (9 mm) against *Alternaria Alternata*, while distilled water extract of *Pinus roxburghii* needles showed maximum inhibition zone (12.33 mm) against *Fusarium Solani*. Antimicrobial activity of *Pinus* species is related to compounds such as terpenes, alkaloids, and phenols (Hajdari et al., 2016). Similarly, (Salem et al., 2014) reported that terpenes and phenolic compounds of *Pinus roxburghii* act as antibacterial and antifungal agents. α -pinenes, and β -pinenes *Pinus roxburghii* were the essential compounds of oleoresin EOs. Because of their capability to induce toxic effects on membrane structure and function, α -pinenes, and β -pinenes are active against bacteria and fungi. Cytoplasmic membranes of bacteria and fungi provide a barrier to ions i.e. K⁺, H⁺, Ca⁺² and Na⁺ etc. This property of semi-permeability of membrane is essential for many cellular functions. Monoterpenes, partition from aqueous phase into membrane structures, due to their lipophilic character. This causes membrane expansion, enhanced membrane fluidity, and inhibition of membrane-embedded enzymes (Mercier et al., 2009). In another study, it was reported that the hydrophobic character of EOs and their constituents is responsible for their interaction with lipids in the microbial plasma membrane and mitochondria, rendering them more permeable. This enhanced permeability causes the outflow of ions and other cell components. A certain amount of outflow from microbial cells can be endured without causing cell death but a significant loss of cell contents or essential molecules and ions will result in cell death (Ju et al., 2019).

Table 2

Antimicrobial activity of *Pinus roxburghii* oleoresin essential oils extracted by steam distillation, supercritical fluid CO₂ extraction and superheated steam extraction

Microorganism	Essential oils extracted by different extraction methods					Positive control ^a
	Steam distillation	Supercritical fluid extraction	Superheated steam (120 °C)	Superheated steam (140 °C)	Superheated steam (160 °C)	
	Inhibition zone (mm)					
<i>Escherichia coli</i>	13.90 ± 0.09 ^d	14.44 ± 0.04 ^c	12.86 ± 0.06 ^f	14.77 ± 0.08 ^b	13.12 ± 0.05 ^e	21.11 ± 0.08 ^a
<i>Staphylococcus aureus</i>	21.12 ± 0.05 ^d	16.30 ± 0.06 ^e	14.22 ± 0.03 ^f	24.74 ± 0.08 ^b	22.00 ± 0.07 ^c	25.38 ± 0.09 ^a
<i>Pastreulla multocida</i>	16.12 ± 0.04 ^c	10.14 ± 0.05 ^e	10.02 ± 0.03 ^f	16.80 ± 0.07 ^b	15.90 ± 0.05 ^d	28.53 ± 0.06 ^a
<i>Bacillus subtilis</i>	14.70 ± 0.03 ^c	10.44 ± 0.07 ^f	13.20 ± 0.08 ^e	15.45 ± 0.06 ^b	14.15 ± 0.04 ^d	22.32 ± 0.07 ^a
<i>Fusarium solani</i>	10.50 ± 0.06 ^e	12.42 ± 0.05 ^c	14.03 ± 0.06 ^b	12.13 ± 0.04 ^d	9.00 ± 0.07 ^f	24.36 ± 0.08 ^a
<i>Aspergillus niger</i>	9.00 ± 0.04 ^e	10.58 ± 0.03 ^c	17.84 ± 0.08 ^b	10.02 ± 0.03 ^d	7.00 ± 0.05 ^f	22.61 ± 0.06 ^a
<i>Alternaria alternata</i>	11.00 ± 0.05 ^e	15.02 ± 0.05 ^c	22 ± 0.09 ^b	14.50 ± 0.06 ^d	10.00 ± 0.04 ^f	24.82 ± 0.05 ^a
<i>Aspergillus flavus</i>	8.06 ± 0.03 ^f	11.46 ± 0.05 ^c	19.80 ± 0.04 ^b	11.00 ± 0.06 ^d	8.97 ± 0.03 ^e	23.32 ± 0.07 ^a
Minimum inhibition concentration (µg/mL)						
<i>Escherichia coli</i>	126.64 ± 1.28 ^c	112.58 ± 1.18 ^e	168.88 ± 1.52 ^a	119.60 ± 1.08 ^d	140.73 ± 1.22 ^b	5.88 ± 0.06 ^f
<i>Staphylococcus aureus</i>	168.88 ± 1.24 ^c	140.73 ± 1.16 ^b	42.22 ± 0.74 ^a	56.29 ± 0.88 ^d	54.27 ± 0.46 ^e	2.92 ± 0.04 ^f
<i>Pastreulla multocida</i>	140.73 ± 1.20 ^d	274.43 ± 1.86 ^b	281.47 ± 1.76 ^a	112.58 ± 1.02 ^e	168.88 ± 1.34 ^c	8.33 ± 0.08 ^f
<i>Bacillus subtilis</i>	140.73 ± 1.44 ^c	168.88 ± 1.32 ^b	281.47 ± 1.72 ^a	56.29 ± 0.96 ^e	70.36 ± 0.48 ^d	13.33 ± 0.07 ^f
<i>Fusarium solani</i>	253.32 ± 1.18 ^b	337.76 ± 1.56 ^d	168.88 ± 1.24 ^e	245.22 ± 1.72 ^c	506.65 ± 2.56 ^a	5.88 ± 0.05 ^f
<i>Aspergillus niger</i>	478.49 ± 2.42 ^b	329.66 ± 1.86 ^e	112.58 ± 1.06 ^d	337.76 ± 1.86 ^c	675.54 ± 3.42 ^a	8.42 ± 0.06 ^f
<i>Alternaria alternata</i>	281.46 ± 1.14 ^b	160.78 ± 1.42 ^e	70.36 ± 0.82 ^d	168.88 ± 1.28 ^c	337.76 ± 1.56 ^a	5.12 ± 0.03 ^f
<i>Aspergillus flavus</i>	562.94 ± 2.56 ^a	273.34 ± 1.86 ^e	84.44 ± 1.04 ^d	281.46 ± 1.22 ^e	422.80 ± 2.24 ^b	6.66 ± 0.03 ^f
Values are mean ± Standard Deviations of three separate determinations. Different superscript letters represent significant difference among <i>Pinus roxburghii</i> oleoresin essential oils extracted by different extraction methods.						
^a Positive control for bacteria and fungi was Amoxicillin and Fluconazole (25 µg/disc), respectively.						

GC-MS results showed that high percentage of monoterpenes (3-carene, α -pinene, and β -pinene) in *Pinus roxburghii* oleoresin EO might be responsible for antimicrobial activity. Similar findings were mentioned by (Zafar et al., 2010), that terpenes of *Pinus roxburghii* needles EO were highly active against microbes. In another study, antifungal activity of *Pinus roxburghii* wood treated with *P. halepensis* branch n-hexane extract and *S. terebinthifolius* branch EO was found to be active against the growth of *R. solani*, *F. oxysporum*, and *B. oryzae*. It was also reported that monoterpenoids, the most prevalent compounds found in the extract, might be responsible for this effect (Mohamed et al., 2020). According to (Gündüz et al., 2012), the major components present in EO of plants were monoterpenes, sesquiterpenes and their derivatives, which act as antifungal and antibacterial substances. The most well-known of these components were terpenes and phenolic compounds. It was also reported by that *Pinus roxburghii* needles EO was effective against Gram-positive bacteria (Bhagat et al., 2018). It was also mentioned that the antimicrobial activity could be attributed to the higher concentration of α -phellandrene, α -pinene and α -terpinolene in *P. roxburghii* needles EO.

3.4 Chemical Composition

The results of GC-MS analysis of *Pinus roxburghii* oleoresin EOs extracted by different extraction techniques are shown in Table 3. Monoterpene hydrocarbons were the major compounds present in *Pinus roxburghii* oleoresin EO (92.44 to 97.93%). Oxygenated monoterpenes were (0.19 to 0.78%) while, sesquiterpenes were (0.59 to 5.74%). Overall, 19 compounds were quantified with 3-carene as the major compound (47.31 to 34.93%) followed by α -pinene (33.14 to 28.36%) and β -pinene (24.46 to 15.83%). These results are in accordance with (Shuaib et al., 2013) in which it was stated that *Pinus roxburghii* oleo-gum-resin EO contained higher percentage of 3-carene followed by α -pinene and β -pinene. Similarly, in another study it was reported that GC-MS analysis of *Pinus roxburghii* leaves EO contained 3-carene as major component (Kaushik et al., 2013). Similar findings were observed by (Sharma et al., 2020) in which *Pinus roxburghii* bark essential oil contained 3-carene as major component. EO of *Pinus mugo* needles extracted by SD contained 3-carene as a major compound (Karapandzova et al., 2019). There is no previous literature available on chemical composition of *P. roxburghii* oleoresin EO extracted by SHSE. So, the results of current study were compared to EO extracted using other extraction methods. (Satyal et al., 2013) reported that *P. roxburghii* cone EO contained caryophyllene, terpinen-4-ol, δ -3-carene, α -humulene, while needle EO of *P. roxburghii* contained caryophyllene, terpinen-4-ol, α -humulene, α -terpineol as main compounds. Similarly, (Zafar et al., 2010) reported that *Pinus roxburghii* needles EO contained α -pinene, β -myrcene, 3-carene, α -terpineol and caryophyllene as major compounds. In another study, (Hassan & Amjid, 2009) reported that stem EO of *P. roxburghii* contained α -pinene, 3-carene, limonene and caryophyllene as major compounds. The compounds that varied with extraction techniques were β -pinene, cis-limonene oxide, trans-limonene oxide, trans-verbenol, pinocarvone, myrtenal, verbenone, and longicyclene (Highest percentage composition in EO extracted by SHSE: (24.46%, 0.28%, 0.07%, 0.15%, 0.31%, 0.02%, 0.16%, 4.53% respectively); 3-carene (Highest percentage composition in EO extracted by SD: (47.3%); α -thujene, α -pinene, camphene, m-cymene, p-cymene, limonene, terpinolene, α -longipinene, sativene, trans-caryophyllene (Highest percentage composition in EO extracted by SCF-CO₂ : (0.41%, 33.14%, 0.67%, 0.22%, 0.93%, 0.15%, 1.81%, 0.16%, 0.11%, 5.47% respectively).

Table 3

GC-MS analysis of *Pinus roxburghii* oleoresin essential oils extracted by steam distillation, supercritical fluid CO₂ extraction and superheated steam extraction

Components ^A	RT ^B	RI ^C	% Composition of essential oils					Method of identification
			Steam distillation	Superheated steam (120°C)	Superheated steam (140°C)	Superheated steam (160°C)	Supercritical fluid extraction (40°C, 80 Bar)	
Monoterpene Hydrocarbon								
α-Thujene	5.09	923	0.11 ± 0.00 ^b	0.09 ± 0.00 ^c	0.11 ± 0.00 ^b	0.09 ± 0.00 ^c	0.41 ± 0.02 ^a	a, b
α-Pinene	5.33	933	30.91 ± 0.56 ^c	43.00 ± 0.84 ^a	29.53 ± 0.34 ^d	28.36 ± 0.18 ^e	33.14 ± 0.16 ^b	a, b
Camphene	5.75	952	0.18 ± 0.01 ^b	–	–	–	0.67 ± 0.04 ^a	a, b
β-Pinene	6.80	988	16.76 ± 0.04 ^d	19.32 ± 0.08 ^b	16.99 ± 0.02 ^c	24.46 ± 0.02 ^a	15.83 ± 0.05 ^e	a, b
3- Carene	7.45	1011	47.31 ± 0.42 ^a	34.93 ± 0.29 ^e	45.78 ± 0.34 ^b	40.09 ± 0.31 ^d	41.09 ± 0.37 ^c	a, b
m-Cymene	7.97	1023	0.19 ± 0.01 ^b	0.17 ± 0.00 ^c	0.07 ± 0.00 ^e	0.15 ± 0.00 ^d	0.22 ± 0.01 ^a	a, b
p-Cymene	8.12	1027	0.42 ± 0.01 ^b	0.42 ± 0.00 ^b	0.08 ± 0.00 ^d	0.63 ± 0.01 ^c	0.93 ± 0.02 ^a	a, b
Limonene	8.18	1031	0.04 ± 0.00 ^c	–	0.09 ± 0.00 ^b	–	0.15 ± 0.00 ^a	a, b
α-Terpinolene	10.19	1088	0.51 ± 0.01 ^c	–	1.39 ± 0.04 ^b	0.26 ± 0.01 ^d	1.81 ± 0.02 ^a	a, b
Oxygenatd Monoterpene Hydrocarbons								
Cis-limonene oxide	12.36	1138	–	0.07 ± 0.00 ^c	0.55 ± 0.00 ^a	0.28 ± 0.00 ^b	–	a, b
Trans-limonene oxide	12.44	1139	–	0.07 ± 0.01 ^a	–	0.06 ± 0.00 ^a	–	a, b
Trans-verbenol	12.53	1144	0.06 ± 0.02 ^c	0.15 ± 0.01 ^a	–	0.11 ± 0.00 ^b	–	a, b
Pinocarvone	13.33	1162	0.10 ± 0.00 ^c	0.16 ± 0.01 ^b	–	0.31 ± 0.02 ^a	–	a, b

a = Identification based on retention index

b = Identification based on comparison of mass spectra

^A Compound listed in order of elution from a DB-5 capillary column.

^B Retention time

^C Retention indices on the DB-5 capillary column

Components ^A	RT ^B	RI ^C	% Composition of essential oils					Method of identification
			Steam distillation	Superheated steam (120°C)	Superheated steam (140°C)	Superheated steam (160°C)	Supercritical fluid extraction (40°C, 80 Bar)	
Myrtenal	14.71	1193	0.01 ± 0.00 ^b	–	–	0.02 ± 0.00 ^a	–	a, b
Verbenone	15.16	1204	0.02 ± 0.00 ^c	0.16 ± 0.01 ^a	–	0.03 ± 0.00 ^b	–	a, b
Sesquiterpene Hydrocarbons								
α-longipinene	21.67	1351	0.07 ± 0.00 ^c	–	0.09 ± 0.00 ^b	0.09 ± 0.00 ^b	0.16 ± 0.01 ^a	a, b
Longicyclene	22.64	1373	2.89 ± 0.04 ^b	0.68 ± 0.03 ^c	–	4.53 ± 0.08 ^a	–	a, b
Sativene	23.51	1393	0.05 ± 0.00 ^c	–	0.07 ± 0.00 ^b	–	0.11 ± 0.01 ^a	a, b
Trans caryophyllene	24.55	1416	0.17 ± 0.00 ^c	–	0.43 ± 0.02 ^b	0.03 ± 0.00 ^d	5.47 ± 0.12 ^a	a, b
Total			99.81	99.22	95.18	99.5	99.99	
a = Identification based on retention index								
b = Identification based on comparison of mass spectra								
^A Compound listed in order of elution from a DB-5 capillary column.								
^B Retention time								
^C Retention indices on the DB-5 capillary column								

3.5 Conclusion

It can be concluded from the results of this study that the superheated steam extraction had a significant effect on yield, chemical composition, antioxidant and antimicrobial activities of *Pinus roxburghii* oleo resin EO. The highest EO yield and antioxidant activity was shown by EO extracted by SHSE at 160°C. EO extracted by SHSE at 140 °C demonstrated the maximum antibacterial activity and the EO extracted by SHSE at 120 °C exhibited highest antifungal activity. According to GC-MS results the major chemical components of EO of *Pinus roxburghii* oleoresin were 3-carene, α-pinene, and β-pinene that might be responsible for their antimicrobial activity.

Declarations

Data availability

All data generated or analyzed during this study are included in this published article. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Figures

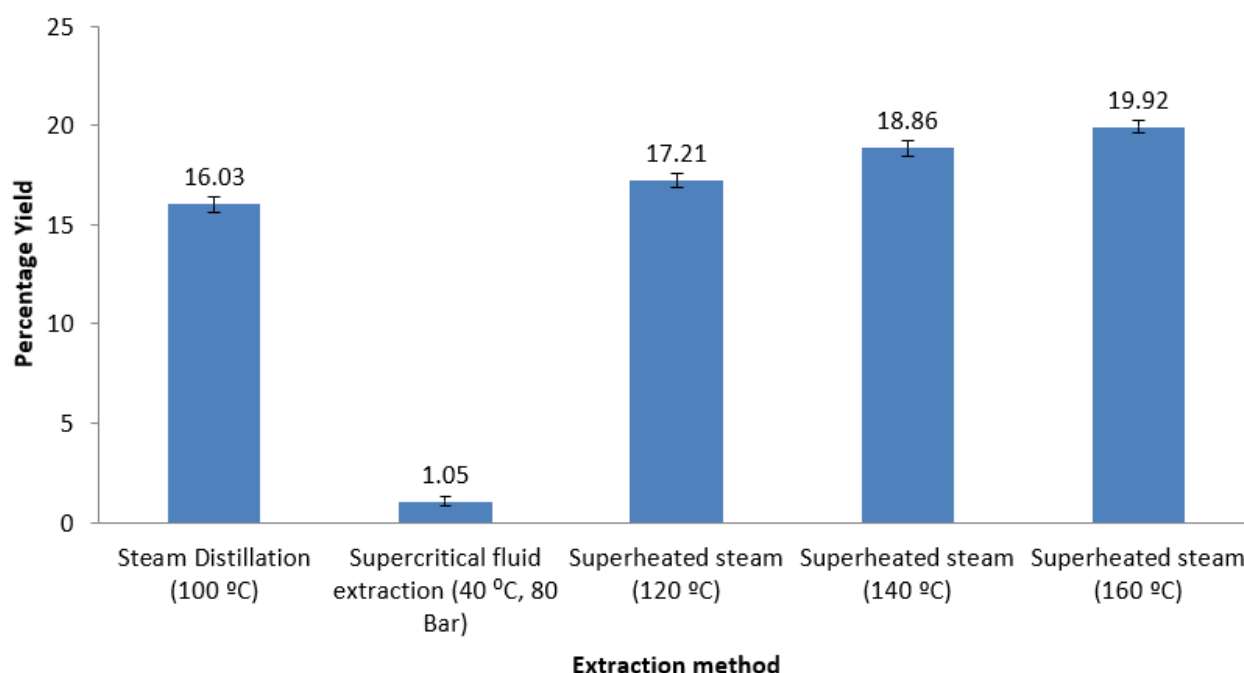


Figure 1

Yield (g/100g) of *Pinus roxburghii* oleoresin essential oils extracted by steam distillation, supercritical fluid CO₂ extraction and superheated steam extraction

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