Original Research Article

Exploration of Chemical Composition and Unveiling the phytopharmaceutical potentials of Essential Oil from fossilized resin of *Pinus succinefera*

Abstract

Background: Most of the plant natural flora contributes significantly to medicinal benefits. To address the short coming usage of synthetic drugs, natural products are in demand which offers broad spectrum of activities with negligible side effects. *Pinus succinefera* is a fossilized resin from coniferous woods that belongs to the genus Pinus of the *Pinaceae* family and is widely known as amber. The present study explores the physiochemical properties and broad spectrum of biological activities of the essential oil.

Method: The essential oil obtained using the hydro-distillation process, and chemical composition was examined by GC-MS analysis as well as the functional group present in the essential oil were determined using FTIR. Thermal characterisation was performed using the TGA and DSC methodologies. The antimicrobial potential of the essential oil was evaluated against different microorganisms meanwhile antimalarial activity against *Plasmodium falciparum* was examined. The free radical scavenging capability of the essential oil was assessed using DPPH reagent. HPTLC was used to examine the qualitative components of the essential oil.

Result: The GC-MS profile indicated 5 major compounds in the essential oil, which consisted of 99% of the total oil. The thermal analysis of the essential oil established the thermal stability. The essential oil showed moderate antioxidant and a moderate to significant potential was observed against microbial species including *Plasmodium falciparum*. In HPTLC five bands of essential oil components with Rf values ranging from 0.2 to 0.7. At 254nm were represented.

Conclusion: The results indicated that the essential oil obtained from *Pinus succinefera* has the potential to be used in the pharmaceutical industry to manufacture medicinal substances, as well as it can show a significant capability in other domains.

Keywords: Antimicrobial, antioxidant, HPTLC, FTIR, antimalarial, DSC, essential oil, GC-MS, *Pinus succinefera*

UNDER PEER REVIEW

Introduction

Medicinal and aromatic plants constitute a vast segment of natural flora. They are the source of raw materials used in various fields such as pharmaceuticals, cosmetics, food industries, fragrance and perfumery industries.[1] Currently, more than 80% of the world's population relies on traditional plant-based medicines to solve various human health problems. Despite significant advancements in synthetic drug development, plants that produce high diversity of secondary metabolites are still considered the principal sources of medicaments and have extensive use in the pharmaceutical industry.[2][3]

Essential oils are volatile, complex, natural compounds with low molecular weight and strong odours. Their vapour pressure is sufficiently high at atmospheric pressure and room temperature, so found mainly in the vapour state.[4] Essential oils are soluble in ether, fixed oil and alcohol and insoluble in

water.[3] These oils have a refractive index and a very high optical property.[5] They are widely used in phytomedicine and aromatherapy.[6] Moreover, these oils are also used in the food and pharmaceuticals industry to increase the shelf life due to their various biological properties.[7]
The essential oil obtained from medicinal and aromatic plants belongs to various chemical classes such as alcohols, aldehydes, ketones, ethers or oxides, esters, amides, amines, phenols, heterocycles, and mainly the terpenes.[5][8][9] Terpenes are made up of isoprene units and can be classified into several groups such as monoterpenes, sesquiterpenes, diterpenes and triterpenes. [10] Monoterpenes constitute approx. 90% of essential bioactive compounds. α-pinene and β-pinene belong to monoterpenes which exhibit diverse biological properties and can be used as a fungicide, antiviral, antimicrobial and anticancer agent.[11] Polymeric products such as phenolic acids, flavonoids, stilbenes, and lignans have redox properties and, thus, play a significant role in neutralising free radicals and peroxide decomposition.[12][13] Therefore, they are strongly associated with antioxidants properties along with anti-inflammatory, anti-allergic, anti-atherosclerotic, anticoagulant and antimutagenic effects. [14][15][16] Polyphenols also help reduce the progression of cardiovascular, neurogenerative and cancer disease.[17][18]
Amber is the fossilized resin belonging to the genus *Pinus* of the *Pinaceae* family derived from coniferous forests.[19] (24), (25) oleoresins are extracted from this species to extract rosins and turpentine. Rosins are composed of resin acids, terpenes, and the mixture of isomeric organic acids from abietic acid whereas, turpentine mainly consist of α-pinene, β-pinene and δ-3-carene.[20][21] The most critical applications for rosin include adhesives, printing ink, insulating materials for electronics, synthetic rubber, chewing gum, soaps, detergent, waxes, paints, soaps, adhesives, and pharmaceutical products.[22] Turpentine is used in paint thinner and varnishes, cleaning agents, and disinfectants, among others, with social and economic importance.[23]
To the best of our knowledge, this is the first report wherein different analytical techniques are used to characterise essential oil extracted from *Pinus succinefera*. GC-MS characterised the chemical composition of the oil. Other analytical procedures such as FTIR, TG, and DSC were also performed. Furthermore, to comprehend the potential of the essential oil, several biological activities such as antimicrobial, antioxidant, antimalarial, and anticancer were analysed.

Material and methods

Sample collection and extraction of essential oil

The fossilized resin of *Pinus Succinefera* was obtained from a regional market in Mumbai, Maharashtra. It was then transported to the research facility. The essential oil was extracted from *Pinus succinefera* using a Clevenger hydro-distillation technique. [24] In a 2-liter flask, 100 g of fossilized resin was added to 800 ml of distilled water. To assure the condensation of essential oil, the set was placed in a balloon warmer connected to a refrigerator for a duration of three hours. Two phases—an aqueous phase (aromatic water) and an organic phase (essential oil), which is less dense than water—were obtained after the distillation. Following extraction, the oil was dried over magnesium sulphate and kept at 4 °C in a dark brown container.

GC-MS analysis of essential oil

The chemical compounds of *Pinus succinefera* essential oil were identified by using Clarus GC-MS 600C system with 30 m GsBP 5-MS capillary column (50% diphenyl/95 % dimethyl polysiloxane), 0.25 mm internal diameter, and 0.25 μm film thickness. This approach was used to look at the sample both quantitatively and qualitatively. The electron ionization system (EI, which stands for "electron impact ionization technique to convert neutral molecules in the gas phase to ionized molecules acceptable for detection") was used to disclose sample components. The GC-MS technique is used operates in electron impact mode with an ionisation energy of 70 eV. In this approach, helium gas (99.999 percent purity) is used as a carrier gas. At a steady flow rate of 1.20 mL/min and an injection volume of 1 μL, this carrier gas exerts a consistent effect on the sample

UNDER PEER REVIEW

component (a split ratio of 150:1). The injector temperature was kept constant at 250°C, while the ion-source temperature was 220°C and the oven temperature was set from 40°C (isothermal for 3 minutes) to 230°C with a 10°C/min rise. The programme lasted 25 minutes in total. The sample components were determined by comparing them to the Mass Spectral Libraries of Wiley 6.0 and the National Institute of Standards and Technology (NIST), as well as retention indices literature data.

FTIR analysis of essential oil

The functional groups of the essential oil were examined using a Carry 630 Fourier transform infrared spectrometer. The system was pre-heated and stabilized at first. In a clean KCl pellet, a drop of substance was inserted. The oil sample was then placed evenly and vesicularly between two KCl pellets after the salt pellet was inserted into the KCl pellet. Initially, KCl pellets were gently rotated to establish a homogenous liquid membrane followed by attaching and inserting them in the infrared spectrometer sample holders. The infrared spectrometer was calibrated to absorbance with a resolution of 8 cm^{-1}. The given oil samples were analyzed under the under range of 4000-650 cm^{-1}

Thermo-Gravimetric analysis of essential oil

TGA analysis of the sample oil was performed using universal V4.5A TA instrument. The experiment was carried out in a nitrogen gas environment with a flow rate of 300 µl/min. The samples were weighed at 29.9170 mg and deposited in aluminum crucibles. The sample was heated to 500 °C at a variety of ambient temperatures at a constant flow rate of 20°C/min throughout the operation.

Differential scanning calorimetry of essential oil

DTA instrument type DSC Q20 V24.11 was used to develop a DSC profile of an essential oil. To conduct the experiment, 14 mg of sample was put in aluminum crucibles. A nitrogen gas flow rate of 40 µl/min was used to evaluate the samples. A dynamic scan was also performed over a temperature gradient of 25°C to 400°C at a constant heating rate of 10°C/min.

HPTLC

A solvent system consisting of Toluene: Ethyl acetate in a ratio of 9.7: 0.3 was utilized for identification and separation of the essential oil extracted from *Pinus succinefera* by HPTLC. The essential oil was brought about on a 100.0x 100.0 mm silica gel 60 F 254 HPTLC plate (Merck). 50 µL of sample solution was administered in 1 mL solvent (methanol) were applied as 8 mm wide bands at a delivery speed 150 nL/s. The plate was then developed at room temperature in a CAMAG twin-trough vertical development chamber that had been pre-saturated with the solvent solution indicated above for 20 minutes. The migration distance was maintained at 85 mm. Following this, the plate was subjected to densitometric scanning at a wavelength of 254 nm and 366 nm, utilizing a scanning speed of 20 mm/s and slit dimension of 5 mm x 0.2 mm, with deuterium and tungsten as the light sources.

Anti-microbial Screening of essential oil

MIC of Anti-Microbial Activity

The potential of the essential oil was evaluated using the microdilution technique using 96-well microtiter plates in order to determine the least inhibitory concentration against the selected 9 resistant bacterial strains such as, Carbapenem-Resistant Acinetobacter (CRA), Carbapenem-Resistant *Pseudomonas aeruginosa* (CRP), Carbapenem-Resistant *Escherichia coli* (CRE), Carbapenem-Resistant *Klebsiella pneumonie* (CRK), Extended Spectrum beta-lactamase *Escherichia*

UNDER PEER REVIEW

coli (ESBL), Quinolone resistant Salmonella (QRS), and Vancomycin-resistant Enterococci (VRE), Methicillin-resistant *Staphylococcus aureus* (MRSA), Erythromycin resistant Streptococci (ERS) and 5 test fungal strains *Trichophyton rubrum, Microsporum gypseum, Aspergillus niger*, *Aspergillus clavatus* and *Candida auris*. The microbial suspensions were regulated until they attained a concentration of 1.0 × 105 CFU/mL. Later, the essential oil was dissolved in a combination of 5% DMSO, 0.1% of polysorbate-80 (1 mg/mL), and then to reach the desired concentrations, it was added to a Luria-Bertani medium (100 µL) possessing a bacterial inoculum of 1.0 × 104 CFU/mL. Then the inoculated plates were incubated at a temperature of 37°C for about 24 hours at 180 rpm. Following the incubation period, each well was given 5 µL of Resazurin dye (2mg/mL) to detect microbial proliferation by a pink coloration.[25]

In-vitro Antimalarial screening

In-vitro Antimalarial assay was carried out in order to assess the efficacy of the essential oil against *Plasmodium falciparum* using 96 well microtiter plate. The *Plasmodium falciparum* and its drug resistant variant were maintained in enriched RPMI-1640 growth medium [26]. Both the parasites were treated with 5% D-sorbitol to attain the ring stage cells which was followed by synchronization of *P. falciparum* and drug resistant *P. falciparum* as described by [27]. In this assay a parasite suspension containing mainly of the ring stage, was adjusted to 0.8 to 1.5% parasitaemia and 3% haematocrit and introduced in 200 µL of RPMI-1640 medium. [28] This suspension was subjected to JSB (Jaswant Singh Bhattacharya) staining method [29] for measuring the percentile parasitaemia (rings) and was evenly maintained with 50% RBCs (O+). A stock solution of the essential oil was prepared in 5mg/mL DMSO with subsequent dilutions ranging from 0.1 µg/mL to 2.0 µg/mL. 20 µL of the diluted samples were pipetted in test wells and duplicate wells containing both the parasitized cell suspensions respectively. The plates containing the preparation were incubated at 37℃ for 36 to 40 hours in a candle jar. [28]. Followed by incubation, a JSB staining was carried out for thin blood smears prepared from each well. Chloroquine and Quinine were used as the control drugs for the experiment. In a Microscopic examination of the slides, the maturation of ring stage parasites into schizonts and trophozoites was observed when different concentrations of the test samples were used. The concentration which demonstrated the complete maturation of both the *P. falciparum* strains into schizonts was noted as the Minimum Inhibitory Concentrations (MIC). The recorded IC_{50} value for each later compared with the standard values.

Antioxidant activity of essential oil

Using the 2,2′- diphenyl-1-picrylhydrazyl (DPPH) reagent, the essential oil's free radical scavenging activity was evaluated in vitro.[30] To a 2.0 mL sample, 1 mL of a 0.5 mM methanol solution of the DPPH radical was added, along with 2.0 mL of 0.1 M sodium acetate buffer (pH 5.5). The solution was thoroughly mixed and kept at room temperature for 30 minutes in the dark. A double beam UV-VIS spectrophotometer was used to detect the absorbance at 517 nm. As a negative control, methanol was employed. At various concentrations (100-1000 µg/mL), a 3 mL aliquot of this solution was mixed with 100 µL of the sample. The reaction mixture was thoroughly mixed before being incubated at room temperature for 15 minutes in the dark. In addition, the absorbance was measured at 517 nm, and the control was made in the same way as the sample. The scavenging activity was calculated using the following equation based on the percentage of DPPH radicals scavenged. The formula used to determine the percentage activity-

$$\text{Scavenging effect (\%)} = \frac{\text{(Absorbance of control - Absorbance sample)}}{\text{(Absorbance of control)}} \times 100$$

Plotting percentage free radical scavenging activity against sample concentrations yielded the sample concentration that provided 50% inhibition (IC50).

UNDER PEER REVIEW

Results and Discussion

GC-MS characterization of essential oil

The essential oil is a rare oil that is extracted from the fossilized tree resin of ancient trees known as *Pinus succinefera* (pine trees). The characteristics of essential oil and their physiologically active constituents were evaluated by using Gas Chromatography-Mass Spectroscopy. The analysis discovered 5 chemical compounds in *P. succinefera* essential oil, which included the oil's entire composition, as indicated in **Table no. 1.** Volatile components like (E)-Cinnamic acid (2.21%), Benzyl benzoate (2.20%), Benzyl cinnamate (6.75%), Bis (2-ethylhexyl) adipate (3.07%) and Diethyl phthalate (87.98%) are major compounds in the essential oil with retention time of 15.38, 18.49, 21.74, 25.15 and 16.75 respectively, as shown in the **Figure no. 1**. Benzyl cinnamate and cinnamic acid can be employed as flavoring agent which are classified as cinnamate ester and monocarboxylic acid and having various biological properties like antimicrobial, antioxidant and anticancer. [31] Whereas, benzyl benzoate is an aromatic ester which depicts efficient antibacterial property[32], and Bis (2-ethylhexyl) adipate is a diester, utilized as a plasticizer.[33] The presence of a high proportion of phthalate ester in the essential oil indicated as a hazardous compound that is also utilized as a plasticizer in the chemical and pharmaceutical industries.[34] Diethyl phthalate has potent antimicrobial property[35] however, it is toxic to mankind.[34] In accordance with our result, the essential oil can be vastly applied to areas like food, cosmeceutical and pharmaceutical industries.

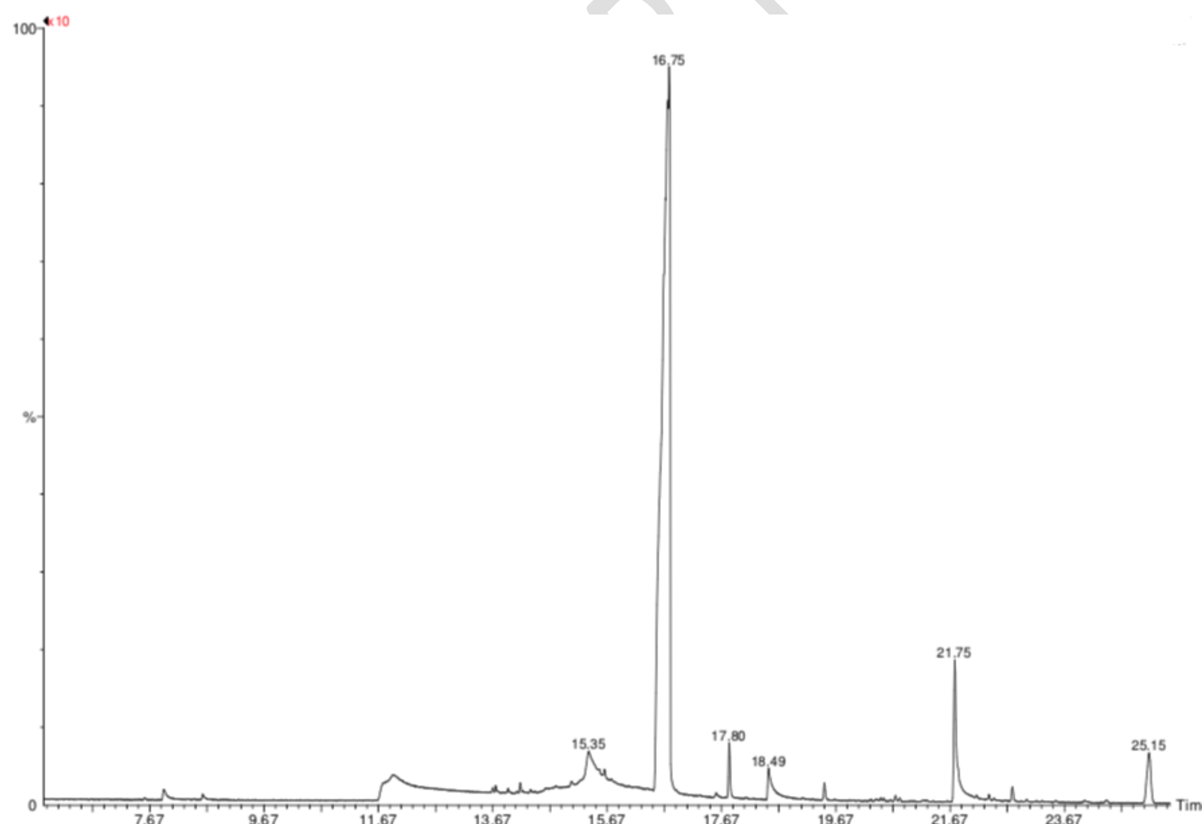

Figure No. 1- GC-MS analysis of essential oil of Pinus succinefera

SR.NO	Component Name	Molecular Formula	Molecular-weight (g/mol)	Classification	Component Composition (%)	Retention time (mins)
1	(E)-Cinnamic Acid	$C_9H_8O_2$	148.16	monocarboxylic acid	2.21	15.384
2	Diethyl Phthalate	$C_{12}H_{14}O_4$	222.24	phthalate ester	87.98	16.75
3	Benzyl Benzoate	$C_{14}H_{12}O_2$	212	Aromatic ester	2.2	18.492
4	Benzyl Cinnamate	$C_{16}H_{14}O_4$	238.28	Cinnamate ester	6.75	21.747
5	Bis(2-Ethylhexyl) adipate	$C_{22}H_{42}O_4$	370.6	Diester	3.07	25.15

Table No. 1 Chemical composition of essential oil extracted from Pinus succinefera.

UNDER PEER REVIEW

FT-IR analysis of essential oil

FTIR (Fourier-transform infrared spectroscopy) is a technique used to study the physical properties of solids, liquids, and gases. More precisely, it enables the investigation of material's absorptive and emissive capabilities. The choice of IR wavelengths employed in FTIR method is beneficial for analyzing chemical bonds between atoms.[36] The IR characteristic peaks for the essential oil sample were analysed **Figure no. 2.** The spectral graph illustrated the different functional groups that are present in the essential oil. broad area of absorption between 2000 and 3500 cm -1 are allotted to stretching vibrations due to O-H stretching observed at 2929.7 and 2955.8 corresponding to alcohol group. Similarly, N-H stretching was observed at 3436.6, and 2862.2 peaks associated with heterocyclic amine and amine salt, respectively. The fingerprint region was observed within a range of 2000 to 1000 cm -1. It showed presence of 1461.1, 1379.1, 957.9 peaks which represented carbonate, nitrate and silicate ions, respectively with an exception of 3067.6 peak present in broad stretching region corresponding to the ammonium ion. 864.7 and 823.7 peaks depicted peroxide functional group with C-O-O stretching whereas 741.7 and 704.5 corresponded to aliphatic chloro

compounds with C-Cl stretching. A peak at 1722.0 represents a ketone group with peaks 1118.2, 1069.7 and 1267.3 depicting C-O stretching associated with secondary alcohol, primary alcohol and alkyl aryl ether, respectively. C-N stretching was observed at 1039.9 which is associated with primary amine whereas peak 1636.3 with a C=C stretching showed presence of alkenyl group. Furthermore, C≡C, stretching was observed at 2109.7 corresponding to monosubstituted terminal alkyne, C=C=C stretch was illustrated at 1982.9 with allene group and C=C-C stretch was observed at peak 1599.0 representing aromatic ring stretch. This study indicates the importance of FTIR technique in identifying the components in the given essential oil sample.

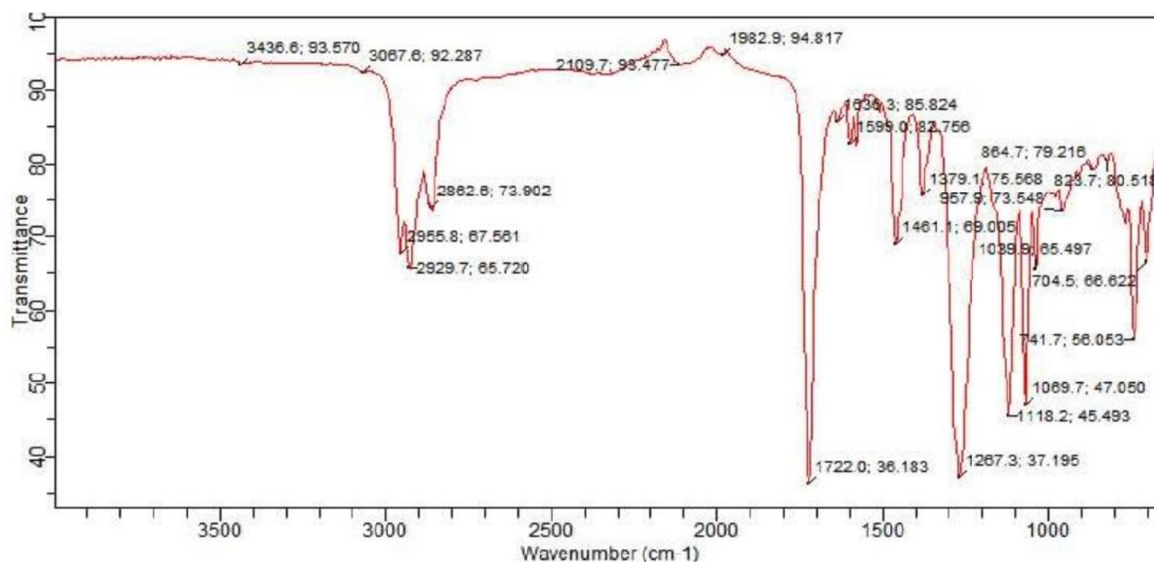

Figure No. 2- FT-IR analysis of essential oil of Pinus succinefera

Thermo-gravimetric Analysis

TGA is a type of thermal analysis in which the mass of a sample is monitored in relation to time or temperature while the sample's temperature is regulated in a specific environment.[37] The temperature-dependence of the reaction rate, as stated by the Arrhenius equation, causes chemical reactions to accelerate with rising heat. Based on this, the van't Hoff law indicates that a temperature increase of 10°C roughly doubles chemical reaction rates, a relationship that can be used to estimate stability at various temperatures.[38] Thermogravimetry was performed to assess the thermal stability of essential oil from *Pinus succinefera*. **Figure no. 3** depicts the TG curve, which shows the changes in the mass of the *Pinus Succinefera* essential oil while heating under oxygen till 500 °C as well as the fact that the weight loss happened mostly in two stages of decomposition. The essential oil demonstrated the first and second thermal degradation events, with Tonset and Tmax of 225°C to 375°C and 400°C to 475°C, respectively, and the curve then stabilizes beyond this temperature, remaining with 0.46% residue after a total weight loss of 99.54%, which could be due to the presence of inorganic compounds with high stability.[39] This demonstrates that the chosen essential oil from has a higher temperature for mass loss, indicating its strong stability.

UNDER PEER

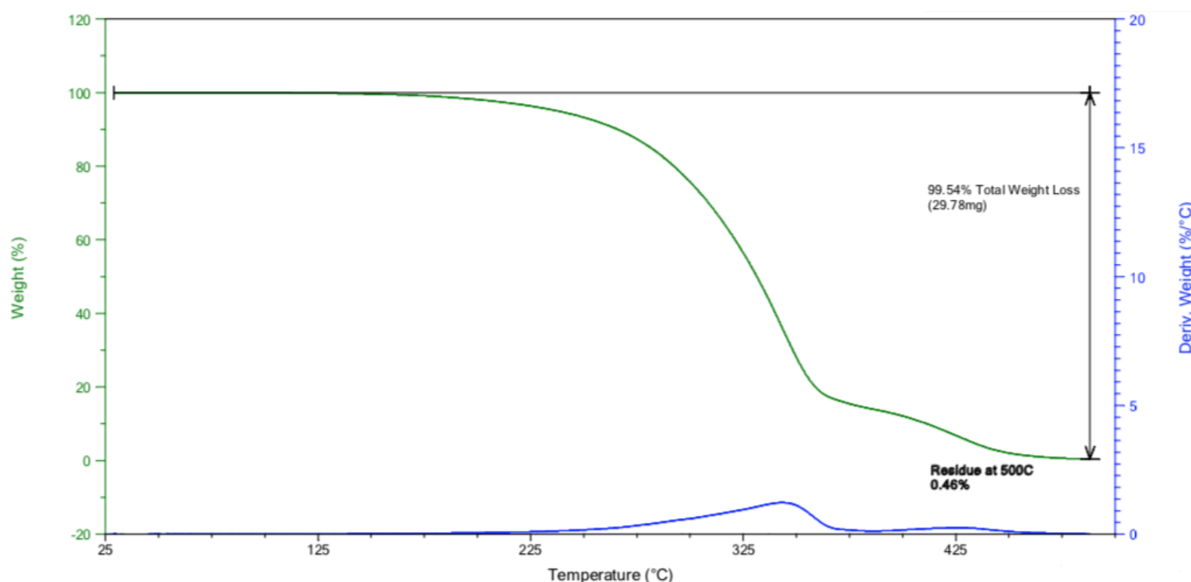

Figure No. 3- Thermo-Gravimetric Analysis of essential oil of Pinus succinefera

DSC Characterization

Heat is released or absorbed when a material changes physical state or performs a chemical reaction. The DSC detects the change in thermal energy of the sample and monitors the heat effects associated with physical transitions and chemical reactions as a function of temperature during the thermal event. The DSC principle is based on the fact that under constant pressure conditions, the change in heat in any transition is equivalent to the change in enthalpy.[40] The DSC curve profile of *Pinus Succinefera* essential oil **Figure no. 4** revealed an endothermic peak associated with the evaporation process at 34.97°C, with a decrease in heat flow of -0.6484 W/g. Furthermore, it demonstrated only one phase shift, vaporisation, and displayed an exothermic peak at 293.21°C attributable to a change into the solid structure, which was associated to melting.[41] At 226.19°C, melting enthalpy of 232.1 J/g was measured due to solid restructuration. However, the peaks were generally smooth, with the exception of some roughness detected locally between the vaporisation and melting curves as seen in the DSC chart, which could be owing to the presence of likely impurities, such as Diethyl Phthalate, as proven in our GC-MS analysis.

UNDER PEER REVIEW

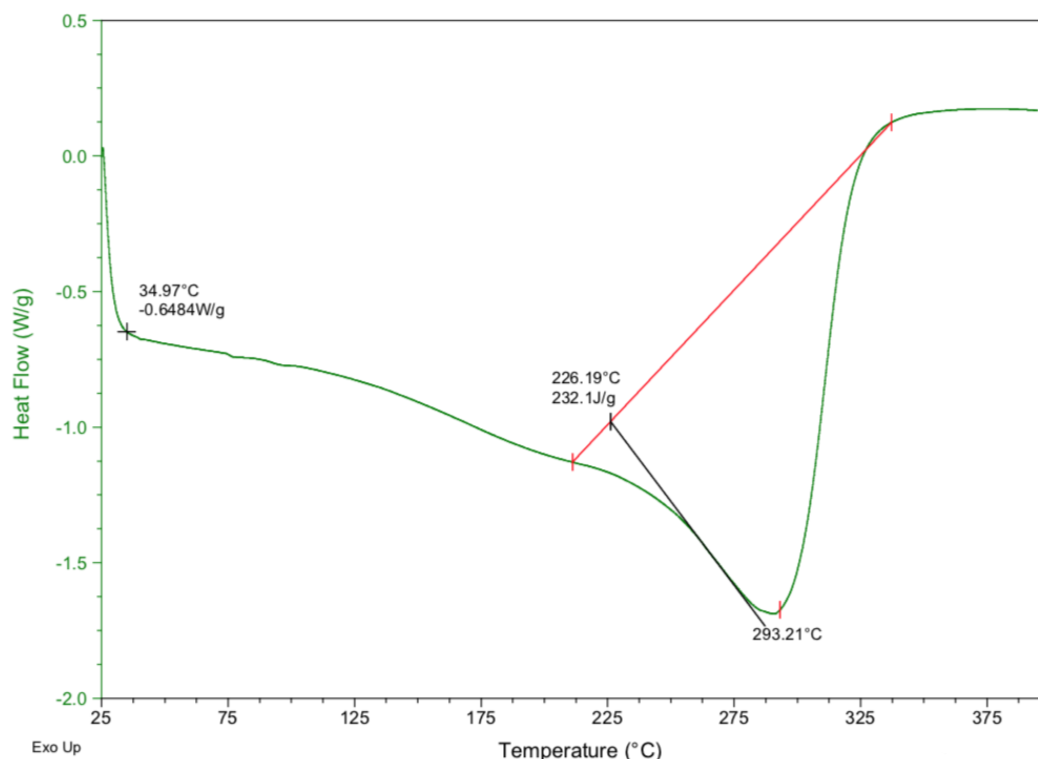

Figure No. 4- Differential Screening Calorimetry analysis essential oil of Pinus succinefera

High Performance Thin Layer Chromatography Analysis

HPTLC is an analytical technique based on TLC, but with modifications aimed at improving the separation resolution of the compound to be separated and allowing qualitative analysis of the component.[42] It is a strong tool that combines biological instruments for detecting potentially adverse effects, physicochemical fractionation techniques, and chemical analytical methods for elucidating compound structures. [43] HPTLC is also well-known as a phytochemical marker and more efficient in the field of plant taxonomy.[44] Special advantages of HPTLC include high sample throughput and low cost per analysis since numerous samples and standards can be separated simultaneously, and sample preparation is frequently minimal because of the stationary phase is disposable.[45] HPTLC has been proved to be a linear, precise, and accurate approach for identifying herbal components.[44] The present study aimed to analyze the HPTLC fingerprint profile of secondary metabolites from *Pinus succinefera* essential oil, which was subjected to the solvent system Toluene: Ethyl acetate (9.3:0.7 v/v). After derivatization of the chromatogram with vanillin-sulphuric acid reagent, many coloured bands were visible in daylight. The coloured bands include blue, purple, and light brown, indicating the presence of various essential oil compounds as shown in **Figure no. 6**. It represented five bands of essential oil components with Rf values ranging from 0.2 to 0.7. At 254nm, approximately five such dark bands were seen, however no distinctive fluorescence was observed at 366nm. Under UV illumination at 254 nm, compounds containing at least two conjugated double bonds quenched fluorescence and appeared as black zones against the light-green fluorescent background of the TLC plate from the middle to the bottom of the plate, corresponding to diethyl phthalate (Rf =0.6) and other constituents such as benzyl benzonate, benzyl cinnamate, cinnamic acid, and bis(2-ethylhexyl) adipate having Rf values ranging from 0.1 to 0.3 as seen in the densitogram of the essential oil revealing six distinct peaks **Figure no. 5.** Based on the findings, the HPTLC approach paired with our GC-MS study can be introduced as acceptable quality controls for sample identification and recognition of plant components utilised for the extraction of essential oil via the percentage of the compounds observed.

UNDER PEER REVIEW

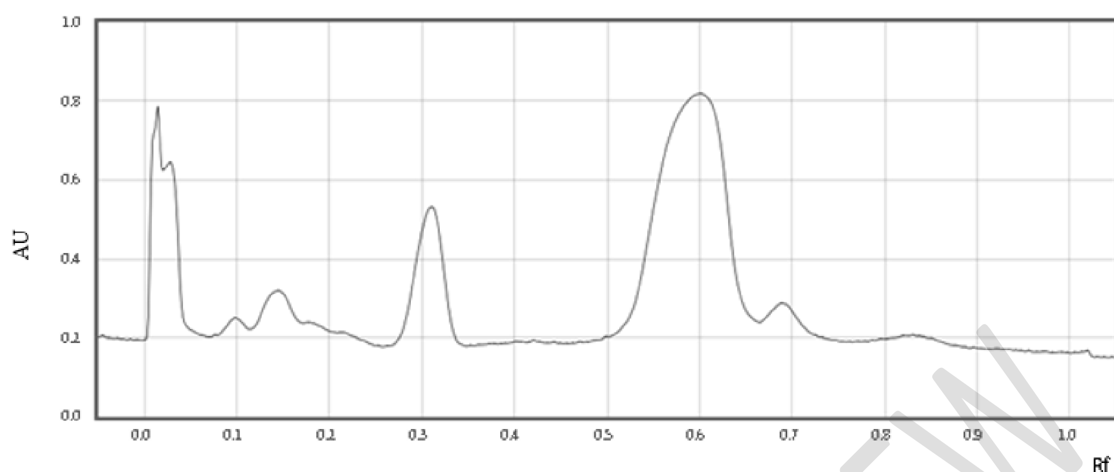

Figure No. 5- HPTLC analysis of essential oil of Pinus succinefera

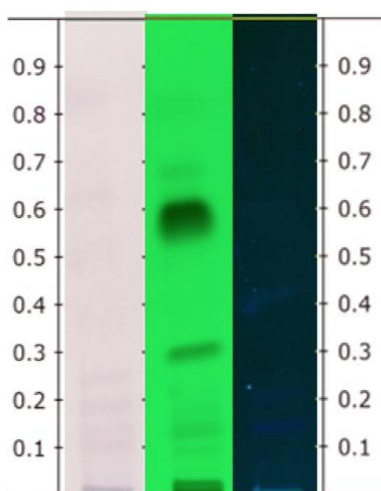

Figure No. 6 Representation of HPTLC fingerprint of essential oil Pinus succinefera

Antimicrobial activity of essential oil

Evaluation of Minimum Inhibitory Concentration (MIC):

The current study's MIC data is summarised in **Table no. 2**. Based on the results obtained against nine drug-resistant bacterial strains, the essential oil showed the greatest potential against beta-lactamase *Escherichia coli* and Erythromycin Resistant Streptococci, both with the same MIC value of 100mg/mL. This oil, on the other hand, has the lowest efficacy against Carbapenem-resistant *Pseudomonas aeruginosa* and Vancomycin-resistant Enterococci, with a MIC value of 500mg/Ml **Figure no.7**. However, overall antibacterial activity screening findings demonstrated that the essential oil has the potential to be efficient antibiotics against drug-resistant bacteria. The higher quantity of Phthalic Acids in the essential oil was found to be responsible for its antibacterial action in the current investigation. Antioxidant enzymes that protect organisms include glutathione peroxidase, superoxide

dismutase, and glutathione reductase (1,2). Oxidative Diethyl phthalate can inhibit the action of antioxidant enzymes by antagonising ions such as Cu^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}, resulting in a decrease in superoxide dismutase activity and the production of reactive oxidation species such as superoxide, H_2O_2, and OH. (3) The formation of superoxide anions in cells exposed to diethyl phthalate causes oxidative stress, which destroys nucleic acids, proteins, and lipids (4). Diethyl phthalate causes cells to create superoxide anion, oxygen, and hydrogen peroxide, leading in a build-up of lipid peroxides that attack polyunsaturated fatty acids in cell membranes, culminating in cell death (5). Previous research has shown that carboxylic acids found in this essential oil, such as (E)-Cinnamic Acid, Benzyl Benzoate, Benzyl Cinnamate, and Bis(2-Ethylhexyl) adipate, have considerable antibacterial action. These chemicals have a variety of targets; specifically, they attack the cell membrane and cytoplasm, which can lead to changes in cell shape (6,7). Cinnamic acid and benzyl cinnamate are phenolic components that work synergistically with other medicines to effectively inhibit *Escherichia coli* and *Staphylococcus aureus* (8, 9). Cinnamic acid has also been shown to suppress TB-causing bacteria such as *Mycobacterium tuberculosis* and *Mycobacterium avium* (10,11). Dicarboxylic acid, such as bis(2-Ethylhexyl) adipate, is responsible for inhibiting cell wall production by preventing Gram-positive bacteria from incorporating N-acetyl glucosamine and N-acetyl muramic acid (12). The presence of components like Cinnamic Acid and Benzyl Cinnamate in the essential oil were confirmed by GC-MS analysis. Benzyl Cinnamate has been shown to have promising antifungal efficacy against *Candida albicans* and *Aspergillus niger* in previous research.[46] Cinnamic acids inhibited fungal growth by interacting with benzoate 4-hydroxylase, an enzyme responsible for aromatic detoxification found in fungus, according to a study by Koroec et al..[47]. It can be seen that *Trichophyton rubrum, Microsporum gypseum, Aspergillus niger* and *Aspergillus clavatus* exhibited antifungal activity at a concentration ranging from 1000 µg/mL to >1000 µg/mL with an exception of *Candida auris* showing antifungal activity at 500 µg/mL **Figure no.8**. These findings indicate that the essential oil has potent antifungal action against the test cultures in the study

Serial No.	Strain Name	MIC (µg/mL)
1	Carbapenem Resistant *Acinetobacter species*	250 ± 0.82
2	Carbapenem Resistant *Pseudomonas aeruginosa*	500 ± 0.01
3	Carbapenem Resistant *E. coli*	125 ± 0.01
4	Carbapenem Resistant *Klebsiella pneumonie*	125 ± 0.81
5	Extended Spectrum beta lactamase *E. coli*	100 ± 0.82
6	Vancomycin resistant *Enterococci*	500 ± 0.82

UNDER PEER REVIEW

7	Quinolone resistant *Salmonella*	125 ± 0.85
8	Methicillin resistant *Staphylococcus aureus*	250 ± 0.05
9	Methicillin resistant *Staphylococcus aureus*	100 ± 0.82
10	*Candida auris*	500 ± 0.49
11	*Aspergillus niger*	1000 ± 0.08
12	*Aspergillus clavatus*	1000 ± 0.82
13	*Trichophyton rubrum*	1000 ± 0.80
14	*Microsporum gypseum*	1000 ± 0.79

Table No. 2 Examination of Antimicrobial properties of essential oil isolated from Pinus succinefera

UNDER PEER REVIEW

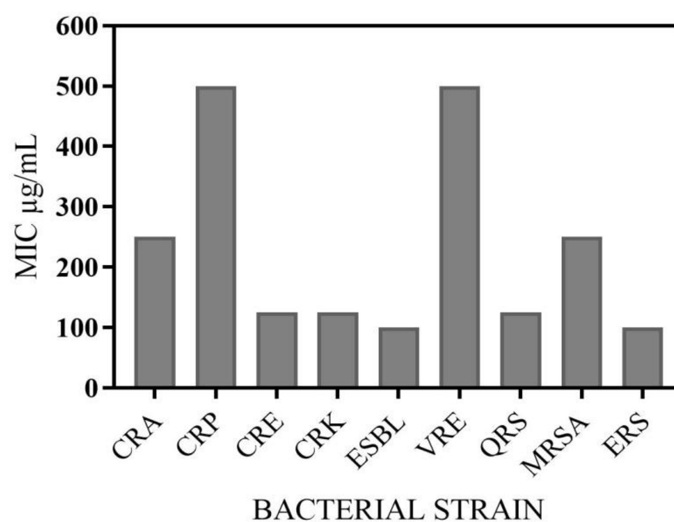

Figure No. 7- Antibacterial activity of essential oil of Pinus succinefera

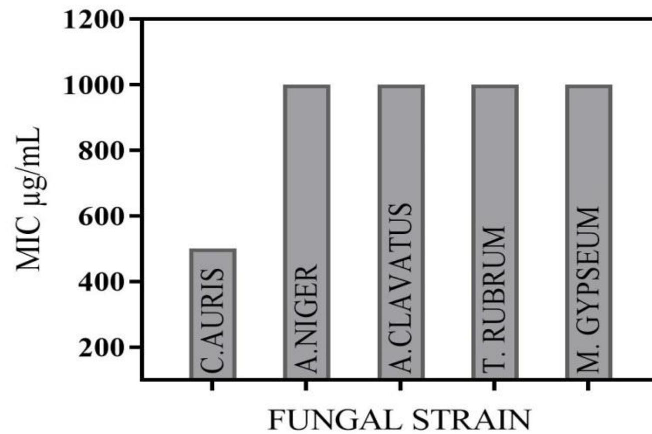

Figure No. 8- Antifungal activity of essential oil of Pinus succinefera

Antimalarial activity of essential oil

The antimalarial activity of the essential oil was evaluated by determining its minimal inhibitory concentration against the parasite, *Plasmodium falciparum* **Table no.3**. The mean IC_{50} values of the essential oil was estimated to be 0.85 µg/mL and the minimum inhibitory concentration against the drug sensitive *Plasmodium falciparum* was estimated to be 1.12 µg/mL, which was higher as compared to the standard drugs. Chloroquine and Quinine showed IC_{50} values 0.020µg/mL and 0.268µg/mL, respectively **Figure no.9.** The essential oil has greater IC50 values against both *Plasmodium falciparum* strains than the standard drugs, which could be due to the presence of additional components in the essential oil. The GCMS analysis did not reveal the existence of an antimalarial component, but it did confirm the presence of diethyl phthalate, which has been shown to have modest inhibitory effect against *P. falciparum*. Dibutyl phthalate (DBP) generated by Streptomyces sp. H11809 has previously been proven to suppress *Plasmodium falciparum* 3D7 (Pf 3D7) malaria parasites. [48]

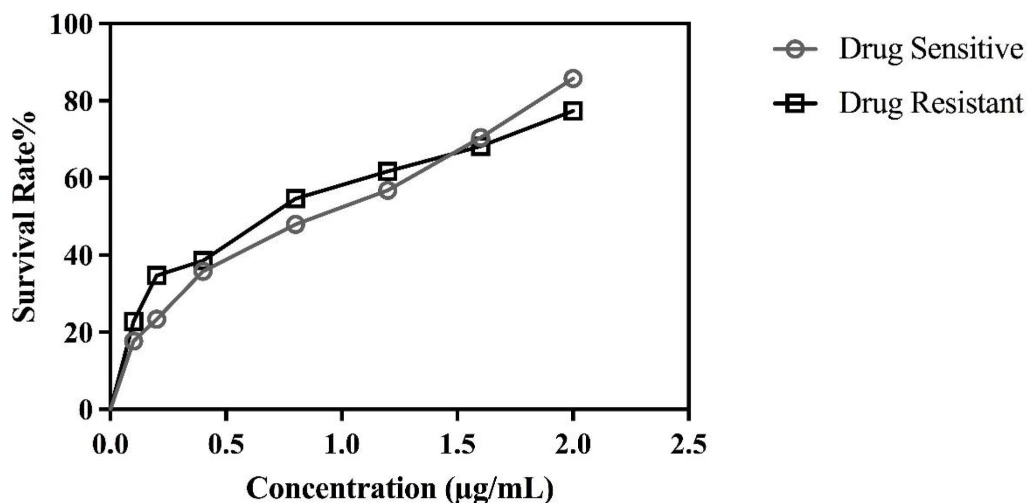

Figure No. 9- Anti-malarial activity of essential oil of Pinus succinefera

UNDER PEER REVIEW

Concentration (μg/ml)		**Drug sensitive** ***Plasmodium falciparum***		**Drug resistant** ***Plasmodium falciparum***		**Standard Drug (control)**	
		Inhibition %	**IC_{50} value**	**Inhibition %**	**IC_{50} value**		**IC_{50} value**
0.1	Amber Essential oil	17.67	0.85 μg/ml	22.8	1.127 μg/ml	Chloroquine	0.020 μg/ml
0.2		23.39		34.7		Quinine	0.268 μg/ml
0.4		35.78		38.5			
0.8		47.98		54.6			
1.2		56.79		61.7			
1.6		70.45		68.2			
2		84.78		77.4			

Table No. 3 Evaluation of Anti-malarial activity of Pinus succinefera essential oil

UNDER PEER REVIEW

Antioxidant activity of essential oil

The DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical is extremely stable, interacts with hydrogen-donating chemicals, and has a UV–vis absorption maximum of 515 nm. Here antioxidants react with the stable free radical 1,1-diphenyl-2-picrylhydrazyl and convert it to 1,1-diphenyl-2-picrylhydrazine which gives a colour change from deep violet color to pale yellow. The intensity of the discolouration obtained indicates the free antioxidant's radical scavenging activities.[49] In this study, antioxidant activity of the essential oil was assessed using DPPH assay **Table no.4**. The highest percentage inhibition of free radical DPPH was observed at a concentration of 1000 μg, with a value of 95.17 %, while the lowest percentage inhibition activity was observed at 100 μg, with a value of 71.45 % **Figure No. 10**. The antioxidant activity exhibited by the essential oil can be associated to the presence of Cinnamic Acid which was confirmed using GC-MS. Cinnamic acid derivatives, particularly those that combine the cinnamoyl moiety with hydroxyl groups, have potent free radical scavenging abilities. 4-hydroxy-trans-cinnamic acid has antioxidant properties, acting as a direct scavenger of reactive oxygen species (ROS) and preventing low-density lipoprotein oxidation.[50]

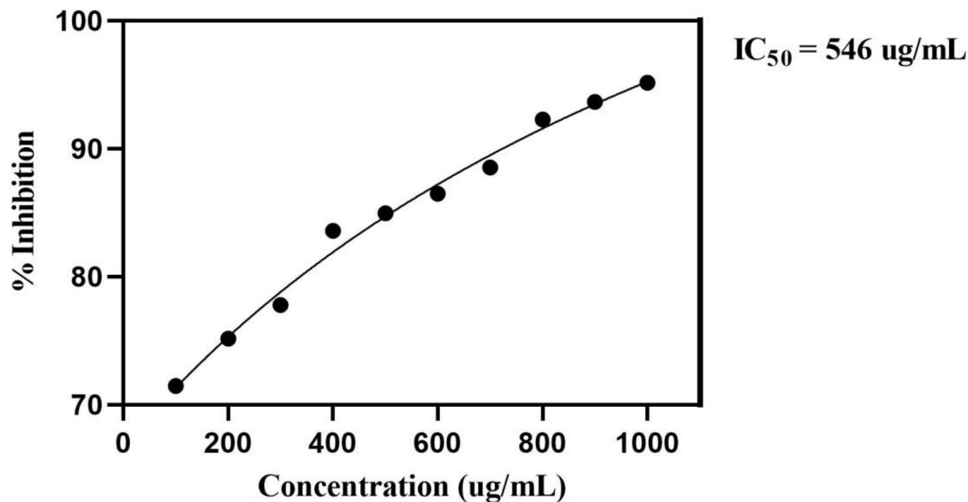

Figure No. 10- Antioxidant activity of essential oil of Pinus succinefera

Conc.(ug/mL)	OD (515 nm)	% Inhibition
100	0.207	71.45
200	0.18	75.17
300	0.161	77.79
400	0.119	83.59
500	0.109	84.97
600	0.098	86.48
700	0.083	88.55
800	0.056	92.28
900	0.046	93.66
1000	0.035	95.17

Table No. 4 Evaluation of DPPH assay of essential oil of Pinus succinefera

UNDER PEER REVIEW

Conclusion:

From single celled to complex species, including plants, the planet Earth has a diverse variety of life forms. Because of their low negative effects, many medicinal properties of plants are exploited as natural products or raw materials in many industrial aspects. For this reason, we evaluated various properties of the essential oil extracted from *Pinus succinefera* to explore its pharmaceutic potential. The essential oil has substantial antibacterial action, including against plasmodium falciparum, as well as considerable antioxidant activity, as evidenced by GC-MS fingerprints due to the presence of certain bioactive components. They are especially intriguing since they are natural and have higher bioactivities than synthetic drugs. Therefore, due to its diverse properties, the essential oil of *Pinus Succinefera* has, if not enormous, then specific oriented potential to be used as a strong pharmacological agent, and it may also be further explored and used in a variety of industries. Researchers are investigating the therapeutic potential of this plant since it possesses additional therapeutic characteristics that are unknown. However, there are certain limitations to the study

including low bioavailability of plant extracts as well as the need to carry out toxicological analysis in order to ensure their safe application. Furthermore, in vivo studies are necessary to reveal the mechanism and side effects of the essential oil for their optimization as therapeutic agents.

Abbreviations

GC-MS, Gas Chromatography- Mass Spectroscopy; FT-IR, Fourier Transform Infrared Spectroscopy; DSC, Differential Scanning Calorimetry; TGA, Thermogravimetric Analysis; MIC, Minimum Inhibitory Concentrations; HPTLC; High-Performance Thin-Layer Chromatography, DPPH Assay, 2,2′-diphenyl-1-picrylhydrazyl Assay; LB, Luria-Bertani; RT, Room temperature.

References

[1] M. Ekor, “The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety,” *Front. Neurol.*, vol. 4 JAN, no. January, pp. 1–10, 2014, doi: 10.3389/fphar.2013.00177.
[2] D. A. Dias, S. Urban, and U. Roessner, “A Historical overview of natural products in drug discovery,” *Metabolites*, vol. 2, no. 2, pp. 303–336, 2012, doi: 10.3390/metabo2020303.
[3] J. B. Sharmeen, F. M. Mahomoodally, G. Zengin, and F. Maggi, “Essential oils as natural sources of fragrance compounds for cosmetics and cosmeceuticals,” *Molecules*, vol. 26, no. 3, 2021, doi: 10.3390/molecules26030666.
[4] S. Bhavaniramya, S. Vishnupriya, M. S. Al-Aboody, R. Vijayakumar, and D. Baskaran, “Role of essential oils in food safety: Antimicrobial and antioxidant applications,” *Grain Oil Sci. Technol.*, vol. 2, no. 2, pp. 49–55, 2019, doi: 10.1016/j.gaost.2019.03.001.
[5] W. Dhifi, S. Bellili, S. Jazi, N. Bahloul, and W. Mnif, “Essential Oils’ Chemical Characterization and Investigation of Some Biological Activities: A Critical Review,” *Medicines*, vol. 3, no. 4, p. 25, 2016, doi: 10.3390/medicines3040025.
[6] B. Ali, N. A. Al-Wabel, S. Shams, A. Ahamad, S. A. Khan, and F. Anwar, “Essential oils used in aromatherapy: A systemic review,” *Asian Pac. J. Trop. Biomed.*, vol. 5, no. 8, pp. 601–611, 2015, doi: 10.1016/j.apjtb.2015.05.007.
[7] S. Chouhan, K. Sharma, and S. Guleria, “Antimicrobial Activity of Some Essential Oils—Present Status and Future Perspectives,” *Medicines*, vol. 4, no. 3, p. 58, 2017, doi: 10.3390/medicines4030058.
[8] M. Moghaddam and L. Mehdizadeh, *Chemistry of Essential Oils and Factors Influencing Their Constituents*, no. October. 2017.
[9] D. Cox-Georgian, N. Ramadoss, C. Dona, and C. Basu, “Therapeutic and medicinal uses of terpenes,” *Med. Plants From Farm to Pharm.*, pp. 333–359, 2019, doi: 10.1007/978-3-030-31269-5_15.
[10] S. Koyama and T. Heinbockel, “The effects of essential oils and terpenes in relation to their routes of intake and application,” *Int. J. Mol. Sci.*, vol. 21, no. 5, 2020, doi: 10.3390/ijms21051558.
[11] M. Zielińska-Błajet and J. Feder-Kubis, “Monoterpenes and their derivatives—recent development in biological and medical applications,” *Int. J. Mol. Sci.*, vol. 21, no. 19, pp. 1–38, 2020, doi: 10.3390/ijms21197078.
[12] J. Dai and R. J. Mumper, “Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties,” *Molecules*, vol. 15, no. 10, pp. 7313–7352, 2010, doi: 10.3390/molecules15107313.
[13] S. Suriyaprom, P. Mosoni, S. Leroy, T. Kaewkod, M. Desvaux, and Y. Tragoolpua, “Antioxidants of Fruit Extracts as Antimicrobial Agents against Pathogenic Bacteria,” 2022.
[14] M. G. Miguel, “Antioxidant and anti-inflammatory activities of essential oils: A short review,” *Molecules*, vol. 15, no. 12, pp. 9252–9287, 2010, doi: 10.3390/molecules15129252.
[15] M. Dziedziński, J. Kobus-Cisowska, and B. Stachowiak, “Pinus species as prospective reserves of bioactive compounds with potential use in functional food—current state of knowledge,” *Plants*, vol. 10, no. 7, 2021, doi: 10.3390/plants10071306.
[16] D. Vauzour, A. Rodriguez-Mateos, G. Corona, M. J. Oruna-Concha, and J. P. E. Spencer, “Polyphenols and human health: Prevention of disease and mechanisms of action,” *Nutrients*, vol. 2, no. 11, pp. 1106–1131, 2010, doi: 10.3390/nu2111106.
[17] K. B. Pandey and S. I. Rizvi, “Plant polyphenols as dietary antioxidants in human health and disease,” *Oxid. Med. Cell. Longev.*, vol. 2, no. 5, pp. 270–278, 2009, doi:

UNDER PEER REVIEW

10.4161/oxim.2.5.9498.
[18] H. Cory, S. Passarelli, J. Szeto, M. Tamez, and J. Mattei, “The Role of Polyphenols in Human Health and Food Systems: A Mini-Review,” *Front. Nutr.*, vol. 5, no. September, pp. 1–9, 2018, doi: 10.3389/fnut.2018.00087.
[19] A. P. Wolfe *et al.*, “A new proposal concerning the botanical origin of Baltic amber,” *Proc. R. Soc. B Biol. Sci.*, vol. 276, no. 1672, pp. 3403–3412, 2009, doi: 10.1098/rspb.2009.0806.
[20] B. Wiyono, S. Tachibana, and D. Tinambunan, “Chemical composition of Indonesian Pinus merkusii turpentine oils, gum oleoresins and rosins from Sumatra and Java,” *Pakistan J. Biol. Sci.*, vol. 9, no. 1, pp. 7–14, Jan. 2006, doi: 10.3923/PJBS.2006.7.14.
[21] H. J. Lee, M. M. Ravn, and R. M. Coates, “Synthesis and characterization of abietadiene, levopimaradiene, palustradiene, and neoabietadiene: Hydrocarbon precursors of the abietane diterpene resin acids,” *Tetrahedron*, vol. 57, no. 29, pp. 6155–6167, Jul. 2001, doi: 10.1016/S0040-4020(01)00605-6.
[22] R. A. Sarria-Villa, J. A. Gallo-Corredor, and R. Benítez-Benítez, “Characterization and determination of the quality of rosins and turpentines extracted from Pinus oocarpa and Pinus patula resin,” *Heliyon*, vol. 7, no. 8, 2021, doi: 10.1016/j.heliyon.2021.e07834.
[23] K. G. Ramawat and J. M. Mérillon, “Natural products: Phytochemistry, botany and metabolism of alkaloids, phenolics and terpenes,” *Nat. Prod. Phytochem. Bot. Metab. Alkaloids, Phenolics Terpenes*, no. June 2014, pp. 1–4242, 2013, doi: 10.1007/978-3-642-22144-6.
[24] M. Elyemni *et al.*, “Extraction of Essential Oils of Rosmarinus officinalis L. by Two Different Methods: Hydrodistillation and Microwave Assisted Hydrodistillation,” *Sci. World J.*, vol. 2019, 2019, doi: 10.1155/2019/3659432.
[25] C. M. Mann and J. L. Markham, “A new method for determining the minimum inhibitory concentration of essential oils,” *J. Appl. Microbiol.*, vol. 84, no. 4, pp. 538–544, 1998, doi: 10.1046/j.1365-2672.1998.00379.x.
[26] L. K. Basco, “Field Application of In Vitro Assays for The Sensitivity of Human Malaria Parasites to Antimalarial Drugs,” *World Heal. Organ.*, p. 191, 2007.
[27] C. Lambros and J. P. Vanderberg, “Falciparum of Plasmodium Synch Stages in Culture,” *Society*, vol. 65, no. 3, pp. 418–420, 2012.
[28] R. L. Van Zyl, S. T. Seatlholo, and A. M. Viljoen, “Pharmacological interactions of essential oil constituents on the in vitro growth of Plasmodium falciparum,” *South African Journal of Botany*, vol. 76, no. 4. pp. 662–667, 2010, doi: 10.1016/j.sajb.2010.08.002.
[29] J. Singh and L. M. Bhattacharji, “Rapid Staining of Malarial Parasites by a Water Soluble Stain,” *Ind. Med. Gaz.*, vol. 79, no. 3, p. 102, Mar. 1944.
[30] S. Thusoo *et al.*, “Antioxidant activity of essential oil and extracts of Valeriana jatamansi Roots,” *Biomed Res. Int.*, vol. 2014, 2014, doi: 10.1155/2014/614187.
[31] Y. P. Cao, G. Y. Zhi, L. Han, Q. Chen, and D. H. Zhang, “Biosynthesis of benzyl cinnamate using an efficient immobilized lipase entrapped in nano-molecular cages,” *Food Chem.*, vol. 364, no. April, p. 130428, 2021, doi: 10.1016/j.foodchem.2021.130428.
[32] H. Diastuti, M. Chasani, and Suwandri, “Antibacterial activity of benzyl benzoate and crotepoxide from Kaempferia rotunda L. Rhizome,” *Indones. J. Chem.*, vol. 20, no. 1, pp. 9–15, 2020, doi: 10.22146/ijc.37526.
[33] C. Name, R. T. K. Substance, C. A. S. Number, D. O. T. Number, and T. Chloride, “THALLOUS CHLORIDE HOW TO DETERMINE IF YOU ARE BEING HEALTH HAZARD INFORMATION Acute Health Effects Cancer Hazard Reproductive Hazard Other Long-Term Effects Medical Testing,” no. November, 2000.
[34] A. Manayi, M. Kurepaz-Mahmoodabadi, A. R. Gohari, Y. Ajani, and S. Saeidnia, “Presence of phthalate derivatives in the essential oils of a medicinal plant Achillea tenuifolia,” *DARU, J. Pharm. Sci.*, vol. 22, no. 1, pp. 1–6, 2014, doi: 10.1186/s40199-014-0078-1.
[35] D. Dubey, R. Patnaik, G. Ghosh, and R. N. Padhy, “InVitro Antibacterial Activity, Gas Chromatography-Mass Spectrometry Analysis of Woodfordia fruticosa Kurz. Leaf Extract and Host Toxicity Testing With InVitro Cultured Lymphocytes From Human Umbilical Cord Blood,” *Osong Public Heal. Res. Perspect.*, vol. 5, no. 5, pp. 298–312, 2014, doi: 10.1016/j.phrp.2014.08.001.
[36] “FTIR: Fourier-Transform Infrared Spectroscopy Principles and Applications.” https://www.findlight.net/blog/2019/03/27/ftir-principles-applications/ (accessed Mar. 31, 2022).
[37] M. V. K. Kök, M. A. Varfolomeev, and D. K. Nurgaliev, “Crude oil characterization using TGA-DTA, TGA-FTIR and TGA-MS techniques,” *J. Pet. Sci. Eng.*, vol. 154, pp. 537–542, 2017, doi: 10.1016/j.petrol.2016.12.018.
[38] C. Turek and F. C. Stintzing, “Stability of essential oils: A review,” *Compr. Rev. Food Sci. Food*
UNDER PEER REVIEW

Saf., vol. 12, no. 1, pp. 40–53, 2013, doi: 10.1111/1541-4337.12006.
[39] A. Górska, R. Brzezińska, M. Wirkowska-Wojdyła, J. Bryś, E. Domian, and E. Ostrowska-Ligęza, “Application of thermal methods to analyze the properties of coffee silverskin and oil extracted from the studied roasting by-product,” *Appl. Sci.*, vol. 10, no. 24, pp. 1–15, 2020, doi: 10.3390/app10248790.
[40] D. Mishra, K. Glover, S. Gade, R. Sonawane, and T. R. Raj Singh, “Safety, biodegradability, and biocompatibility considerations of long-acting drug delivery systems,” *Long-Acting Drug Deliv. Syst.*, pp. 289–317, Jan. 2022, doi: 10.1016/B978-0-12-821749-8.00008-2.
[41] Ö. Süfer and F. Bozok, “Characterization of essential oil from Matricaria sevanensis by microwave-assisted distillation,” *J. Therm. Anal. Calorim.*, vol. 140, no. 1, pp. 253–261, 2020, doi: 10.1007/s10973-019-08829-x.
[42] M. Attimarad, K. K. Mueen Ahmed, B. E. Aldhubaib, and S. Harsha, “High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery,” *Pharm. Methods*, vol. 2, no. 2, pp. 71–75, 2011, doi: 10.4103/2229-4708.84436.
[43] M. Stan, I. Lung, O. Opriş, and M. L. Soran, “High-performance thin-layer chromatographic quantification of some essential oils from anethum graveolens extracts,” *J. Planar Chromatogr. - Mod. TLC*, vol. 27, no. 1, pp. 33–37, 2014, doi: 10.1556/JPC.27.2014.1.6.
[44] J. Senguttuvan and P. Subramaniam, “HPTLC Fingerprints of Various Secondary Metabolites in the Traditional Medicinal Herb Hypochaeris radicata L.,” *J. Bot.*, vol. 2016, 2016, doi: 10.1155/2016/5429625.
[45] M. Srivastava, *High-performance thin-layer chromatography (HPTLC)*, no. May 2014. 2011.
[46] I. Bin Jantan, B. A. Karim Moharam, J. Santhanam, and J. A. Jamal, “Correlation between chemical composition and antifungal activity of the essential oils of eight Cinnamomum species,” *Pharm. Biol.*, vol. 46, no. 6, pp. 406–412, 2008, doi: 10.1080/13880200802055859.
[47] B. Korošec *et al.*, “Antifungal activity of cinnamic acid derivatives involves inhibition of benzoate 4-hydroxylase (CYP53),” *J. Appl. Microbiol.*, vol. 116, no. 4, pp. 955–966, 2014, doi: 10.1111/jam.12417.
[48] K. Mohd, F. Mustaffa, C. H. Leow, H. Osman, and H. M. Sidek, “Bioactivities and Mode of Actions of Dibutyl Phthalates,” pp. 1–16, 2022.
[49] S. Škrovánková, L. Mišurcová, and L. Machů, “Antioxidant Activity and Protecting Health Effects of Common Medicinal Plants,” *Adv. Food Nutr. Res.*, vol. 67, pp. 75–139, Jan. 2012, doi: 10.1016/B978-0-12-394598-3.00003-4.
[50] E. Pontiki, D. Hadjipavlou-Litina, K. Litinas, and G. Geromichalos, “Novel cinnamic acid derivatives as antioxidant and anticancer agents: Design, synthesis and modeling studies,” *Molecules*, vol. 19, no. 7, pp. 9655–9674, 2014, doi: 10.3390/molecules19079655.
UNDER PEER REVIEW