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Chemical composition analysis and biological activities of essential oil from *Eucalyptus polybractea* (L.), growing in plains of Punjab, Northern India, by two different hydro-distillation methods

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Abstract

Eucalyptus essential oil poses various biological activities due to the presence of various bioactive compounds. The objective of this study was to evaluate the effect of direct heat-based hydro distillation (DHBH) and in-direct heat-based hydro-distillation (IDHBH) methods on chemical profile, extraction, and biological activities of essential oils from *Eucalyptus polybractea*. Eucalyptus Essential Oil (EEO) was extracted from green leaves of eucalyptus plant using DHBH and IDHBH methods. Aroma profile was evaluated by GC-FID technique. Content of phenolics, flavonoids and tannins accompanied by antioxidant activities like iron-chelation and DPPH were studied. Other biological activities like anti-inflammatory and anti-microbial were also analysed. GC-FID chemical profiling revealed a greater number of bioactive compounds in IDHBH extract than DHBH. Relationship between antioxidant activity and chemical composition of extracted oils was investigated. Significant amount of β- pinene, 1,8 cineole, eugenol, Pentadecanoic acid are responsible for antioxidative capacity of both extracts. The concentration of all compounds, especially Pentadecanoic acid and other unique bioactives were higher in IDHBH extract. The level of antioxidants like phenolics, flavonoids and tannins by was high also high in IDHBH extracted oil. This corresponds to higher antioxidant activities like DPPH and iron-chelating activity of IDHBH oil compared with DHBH extract. Similarly, anti-inflammatory, and anti-microbial potential was more in oil extracted with IDHBH than DHBH. The present paper reported that different extraction methods lead to different biochemical composition of essential oils and the choice of a suitable method is extremely important to obtain more preferred compounds with more potent biological activities and this method can be the choice for essential oil-based companies.

Keywords: aroma profile; biological activities; direct heat; essential oil; indirect heat

Introduction

In the area of herbal biotechnology, underutilized aromatic plants are used as a source of traditional and complementary medicines (Newman and Cragg, 2016). So, detailed phytochemical studies of these underutilized plants are pre-requisite especially, wherever primary healthcare system utterly depends upon traditional medicines. These plants are utilized for development of novel, biodegradable, and highly effective drugs as a better alternative to contemporary or western medicine as these synthetic drugs have led to increase

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resistance in pathogenic microbes (Moradi *et al.*, 2020). The contribution of medicinal plants to phytomedicine production and utilization has attracted interest of scientific community around the globe (Gao *et al.*, 2012; Medini *et al.*, 2014).

Plant essential oils, high value, low volume commodities and represent essence of odor and flavor of the plant, are classified as secondary metabolites that are produced by aromatic plants and are generally defined as complex mixture of various bioactive components like hydrocarbons, terpenes, aromatic derivatives, alcohols, esters, and other aromatic compounds (Swamy *et al.*, 2016). These oils can be obtained from different tissue like: leaves, stems, roots, seeds, barks, and flowers etc. (Elaissi *et al.*, 2011). Due to these complex and diverse bio-structures plant essential oils poses various kind of biological activities like: antimicrobial, fungicidal, herbicidal, anti-parasitic and antiviral, anti-allergic and anti-carcinogenic activities (Tyagi *et al.*, 2014). As natural produce from herbal plants, essential oils are used as raw materials in sectors including perfumes, cosmetics, aromatherapy, botanical medicine, spices and nutrition, pesticides. Consequently, a great interest has been devoted to essential oils day by day as raw material in industries and represent alternative therapeutic medicines (Wissal *et al.*, 2016).

Eucalyptus, is a member of Myrtaceae family, native from Australia, and comprises about 900 species. It is a fast-growing tree that can attain height 25-50 meters and can sustain various adverse climatic conditions with temperature range from 0-47 °C (Silva *et al.*, 2003). Literature survey revealed that more than 300 species of this Eucalyptus are naturally rich in essential oil content and many are known for high content various bioactives particularly 1,8 cineole (about 70%) in their leaves. Essential oil from this plant is often used in traditional and complementary alternative medicine to cure various respiratory disorders like bronchitis, sinusitis, pharyngitis (Cermelli *et al.*, 2008). Some studies have highlighted its key role to combat the infections of various bacterial *Streptococcus pneumonia, Streptococcus agalactiae, Streptococcus pyogenes, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Hemophilis influenzae* and fungal infections like *Candida albicans* (Sartorelli *et al.*, 2007; Elaissi *et al.*, 2011). Some studies have highlighted its role as anti-viral agent against: herpes simplex mumps and Adenovirus viruses (Elaissi *et al.*, 2011). Consequently, its role as therapeutic is increasing day by day in various sectors like: cosmetic, food, pharmaceutical, aromatherapy and phototherapy (Raho and Benali, 2012). So, the quality of essential oils is often judged by the presence of type and abundance of phytochemicals in it. It was reported that variability in chemical composition of essential oils is often governed by many factors like: plant species, seasonal, geographical, and climatic conditions, harvest time and extraction techniques (Dhifi *et al.*, 2016). Among all, extraction or distillation method is the major factor that affects chemical profile of essential oil. Extraction of essential oil is traditionally carried out by using various techniques like: solvent extraction, steam distillation and hydro distillation, mechanical expression *etc* (Karakaya *et al.*, 2014). Among all, hydro-distillation (based on direct heat) and steam distillation (based in in-direct heat) is the most commonly used approach to extract plant essential oils as compared to other techniques due to various factors like cost effectiveness, cost of solvent in comparison to other solvents (P′erino-Issartier *et al.*, 2013). Some previous studies for example Bahmini *et al.* (2018) documented the effect of ultrasonic pre-treatment during hydro-distillation on quality of essential oil of *Artemisia dracunculus*. Elyemni *et al.* (2019) studied microwave assisted hydro distillation in the extraction of essential oils of *Rosmarinus officinalis*. Microwave-assisted hydro distillation has been used for the extraction of laurel essential oils (Kosar *et al.*, 2005), lavender (Périno-Issartier *et al.*, 2013), and thyme (Golmakani and Rezaei, 2008), and rosemary has also been studied (Okoh *et al.*, 2010; Moradi *et al.*, 2018). Elyemni *et al.* (2019) also cited that these techniques cause the loss of certain volatile compounds due to long extraction times and degradation of unsaturated or esterified compounds by thermal or hydrolytic effect. However, how direct- and in-direct heat-based sources affect quality of oil in terms of bio-constituents and biological activities of is still not well known. Thus, this study aimed to evaluate the effect of direct- heat based hydro distillation (DHBH) and in-direct heat based (IDHBH) hydro distillation on chemical profile and biological activities of essential oil from *Eucalyptus polybractea.* For a complete understanding of EEOs function further analyses of antioxidant and anti-microbial activities of EEOs obtained by two different extraction methods were evaluated. This study will

provide insights about the impact of oil extraction methods on aroma profile and biological activity and thus will be detrimental to design appropriate extraction method to extract EEOs with desired composition. This study will help industrialists and other stake holders to select type of heat source and design extraction methods appropriately for best quality oil.

Materials and Methods

Extraction of essential oil

Fresh leaves of *Eucalyptus polybractea* were collected from naturally growing plants locally in the vicinity of study area located at 30°33 north longitude and 71°31 east latitude east latitude Jalandhar, Punjab India. The area experiences humid subtropical climate with hot and cold months. The climate is dry overall. The experiment was conducted on February 2020. The plants were authenticated and voucher specimen (BT-1) was deposited in herbarium. Fresh leaves were plucked and stored in dark envelops at 4 °C. The essential oil was extracted from fresh cleaned leaves through two different modes of distillation by pilot-plant following Sharma *et al.* (2021) with modifications. In first set of experiment, wire grid containing leaves (about 20 kg) were completely submerged in water (about 20 Liters) which is boiled for 2 hr, hence termed as DHBH. After distillation, upper phase having essential oil (organic) was collected. In second set of experiment, wire grid containing leaves (about 20 kg) was maintained well above the water level. The water (about 20 Liters) is boiled below the wire grid containing leaves and "wet" steam passes through the plant material hence termed as IDHBH. After distillation for 2 hr, upper phase (organic layer having essential oil) was collected after condensation. After both methods oil was separated from by-product water and stored in dark bottles in refrigerator till further use. Color of essential oil was noted immediately after distillation of oil by both methods. The extraction yield of the essential oils obtained from all methods was calculated as Extraction yield (%) = Mass of extracted oil/ Mass of Fresh Leaves x 100. 1 mL of pure oils were diluted in pure methanol and used for various estimations.

UV-VIS, FT-IR and fluorescence analysis

The UV-VIS spectral analysis of essential oil was studied following Sharma *et al.* (2021) using UV-VIS spectrophotometer (Labtronics) with slit width of 2 nm, using 10 mm cell at room temperature in the range of 200 nm to 400nm. The FT-IR (Fourier Transfer Infra-Red) Spectroscopy was used for identification of different functional groups in bioactive compounds in oil. For this about 10 µl of oil was taken and analyzed and using FT-IR Spectrophotometer (Perkin Elmer, USA spectrophotometer) in the region 4000-400 cm^{-1}. The fluorescence spectrum of oils (2 mL) was recorded using Perkin Elmer Spectrophotometer (FL6500).

Gas chromatography analysis

Gas chromatography (GC-FID) analysis was performed following Sharma *et al.* (2021) using a Chemtron 2045 gas chromatograph coupled with FID (flame ionization detector). A 2 m long column of stainless steel filed with 10% OV-17 on 80-100% mesh Chromosorb W (HP) was used. Carrier gas was Nitrogen at flow rate of 30 ml/min. The detector and injector temperatures were kept at 210 °C and 260 °C and 0.2 µl sample was injected. Ramping conditions for oven were: 110°C (initially maintained) ramped to 200 °C at 2 °C/min. Bioactives were identified by comparison of their relative retention times with either those of known standards (euginol, citral, eucalyptol, geraneol, sigma Aldrich analytical grade) or with published data in the literature or NIST spectral libraries spectra.

DPPH scavenging activity

The DPPH (1, 1-diphenyl-2- picryl-hydrazyl) assay was used for determination of free radical scavenging ability of oils following Sharma *et al.* (2021). To different aliquot of both type of oils (10-50 µl), 2.8 mL of

DPPH solution was added. The mixture was then incubated in dark at room temperature (~30 °C) for 1hr. After incubation the absorbance of each sample was recorded at 517nm against 82% methanol as blank. 3 mL DPPH solution was taken as control. Ascorbic acid was taken as positive control. The test was performed in triplicates. The DPPH scavenging activity of oils was expressed in term of % scavenging activity by using the given formula i.e., % scavenging = Abs. of control – Abs. of sample × 100/ Abs. of control.

Iron chelating activity

In this assay (Sharma et al., 2021), different aliquot of oils (10-50µl) was mixed with 900 µl of 500 mM $FeSO_4$ was added followed by addition of 78 µl of 0.25% 1, 10-Phenanthroline. The absorbance of resulting solution recorded at 510nm against distilled water as blank. Solution with $FeSO_4$ was taken as control. EDTA was taken as standard. % Chelating ability = (Abs (c) –Abs (s)/Abs (c) x100; Abs (c) = OD of the control. Abs (s) =OD of the reaction mixture.

Total phenolic content

The total phenolic content of plant was determined by the method described by Sharma *et al.* (2021). Gallic acid was taken as standard (calibration curve). Results were expressed as mg Gallic Acid Equivalent (GAE)/ mL using standard equation $y = 0.022x + 0.290$, $R^2 = 0.832$. All samples were analyzed in triplicates.

Determination of flavonoid content

The total flavonoid content of plant was determined by the method described by Sharma *et al.* (2021). Determination was calibrated with standard curve of prepared Rutin solution. Results were expressed as mg rutin equivalent (RUE)/ mL using standard equation $y = 0.020x + 0.270$, $R^2 = 0.88$. All samples were analyzed in triplicates.

Total tannins

The total tannins (proanthocyanidins) in oils were determined according Sharma *et al.* (2021). Tannic acid was used in calibration curve. The results were expressed as mg of tannic acid equivalent (TAE)/mL using standard curve equation $y=0.0001x+0.001$ $R^2 = 1$. All samples were analyzed in triplicates.

Anti-inflammatory activity

The anti-inflammatory assay of both types of oils was performed according to the method described by Sharma *et al* (2021) by using fluorescent assay. To 0.4mL 1% BSA, 4.80 mL phosphate buffered saline (PBS, pH 6.4) and aliquot of oil (50 µl) was added and incubated for 15 min at 37 °C. Then mixture was heated at 70 °C for 5 min and allowed to cool down. 1 mL of mixture was subjected to fluorescent spectroscopy analysis on Perkin Elmer Spectrophotometer (FL6500) using excitation wavelength was 280 nm and fluorescence emission spectrum was recorded over wavelength range from 300-400 nm. All experiments were done at room temperature (~30 °C).

In-vitro anti-bacterial activity

The in-vitro antimicrobial activity of dark and light oils was determined through agar disc diffusion method against four test organisms, gram-negative *Escherichia coli* (MTCC 40), *Pseudomonas aeruginosa* (MTCC 424), and Gram-positive *Staphylococcus aureus* (MTCC 3160), following Sharma *et al.* (2021). Pathogens were purchased from Institute of Microbial Technology, Chandigarh. Sterile paper discs (10 mm in diameter) were impregnated with 50 µl oil. 12-h cultures were used Inoculums and OD of suspensions was adjusted to 0.6. A swab of bacteria suspension was spread on to LB-Agar plates and allowed to dry for 30 min. The discs with essential oil were then applied and plates were left for 20 min at room temperature to allow to diffusion of oil followed by incubation at 37 °C for 24 hours. Zone of inhibition was measured. Streptomycin (30 mg) was used as positive control.

Statistical analysis

All the data are expressed as mean± SD (n=3) which were computed with the help of MS Office (2007). Experimental results were further subjected to Pearson's correlation analysis of phenolics and tannins with different antioxidant assays and tested for significance by student's test ($P<0.05$) using micro soft excel software (ver 2010)

Results and Discussion

Yield of EEO obtained by DHBH and IDHBH extraction methods was almost equal i.e., 0.81 and 0.83%, respectively. The color of essential oil obtained after both methods was recorded (Figure 1). It was found that color of EEO obtained after DHBH method was dark brown while with IDHBH, color of EEO was light yellow, hence, termed as "dark oil" and "light oil" for further analysis. The dark color of essential oil obtained after DHBH method may be due to burning of charge or thermal degradation of hydrocarbons esters or aldehydes at high temperatures. This observation was in consonance with reports of earlier studies of Öztürka *et al.* (2009) in *Salvia potentillifolia*. Kusuma *et al.* (2016) and Moradalizadeh *et al.* (2013) also cited that high uncontrolled temperature not only affect color but also affect essential oil composition or degradation of esterified or unsaturated compounds thus loss of highly volatile bioactives.

Figure 1. Photographic view of color of aromatic oil, DH: direct heat, IH: indirect heat

Changes on antioxidants

Polyphenolics rich herbal extracts are effectively involved in the mitigation of diseases such as cardiovascular and cancer diseases, have antioxidant, anti-inflammatory properties, and anticoagulant effects (Cory *et al.*, 2018). Eucalyptus essential oil is rich in bioactive molecules like phenolics, terpenoids etc. that have demonstrated to have potential antioxidant activities (Goodger *et al.* 2016). However, maximum abundance of bioactive molecules in EEO depends on the type of extraction method used (Li *et al.*, 2021). In this study, effect of two different methods: DHBH and IDHBH were employed to extract eucalyptus essential oil. As indicated in Table 1, as compared to EEO extracted by DHBH method (dark oil), the level of tannins, phenolics and flavonoids compounds were substantially higher in IDHBH extracted oil (light oil). For instance, in the light oil the phenolics content was about 35 mg GAE as compared to dark oil which contained about 17 mg GAE. A nearly similar trend was noticed in case of total condensed tannins. For example: in light oil the tannin content was about 3.5 mg TAE as compared to dark oil which contained about 2.5 mg TAE. Flavonoid's content was 2-fold higher in light oil than dark oil. According to this result, IDHBH method seems to be the best extraction technique for obtaining total phenolic content, flavonoid, and tannins for this plant.

In this context, it was reported in several investigations that content of polyphenolics is modulated by type of extraction method used. For instance: Elyemni *et al.* (2019) studied microwave assisted hydro distillation in the extraction of essential oils of *Rosmarinus officinalis* and observed higher number of bioactives. Sandhu *et al.* (2021) in *Citrus indica* and Bahmini *et al.* (2018) in *Artemisia dracunculus* also documented higher content of phenolics upon ultrasonic pre-treatment during hydro-distillation on quality of essential oil.

Table1. Phytochemical analysis of *Eucalyptus globulus* essential dark and light oil

Type	Dark (DHBH)	Light (IDHBH)
Total flavonoids ug/gRE	2.1±0.02	4.2^{a}±0.03
Total phenolics ug/gGAE	20.1±0.03	35.3^{b}±0.04
Total tannins mg/gTAE	2.5±0.02	3.5^{c}±0.02

Values are mean of triplicate determination (n = 3) ± standard deviation; a,b,c indicates significant difference of light oil at P≤0.05 vs dark oil

Next, activities of EEO were determined by DPPH, and the Ferric reducing antioxidant power assay. The DPPH scavenging activity of the tested EEO was observed at concentrations of 10-50 µL/mL. All of the EEOs, regardless of extraction method, showed DPPH scavenging capacity that increased in a concentration dependent manner (Figure 2). Using 50 µl of oil, IDHBH extracted oil was more potent (85% scavenging) than DHBH oil (78% scavenging). As shown in Figure 2, EEOs exhibited iron cheating power ability, with EEO from IDHBH demonstrating higher scavenging activity than those extracted by DHBH. At 50ul/mL, IDHBH EEO demonstrated highest activity (60%) than DHBH extract (50%). Earlier studies have cited that antioxidant capacity of essential oils extracted from plants depends mainly upon the abundance of phenolic components of the oils which further regulates scavenging of free radicals, metal ion chelation and inhibition of lipid peroxidation of cell membranes (Yosr *et al.*, 2013). Taken all data together, it was observed that higher antioxidant potential of IDHBH than DHBH oil could be attributed to the differences in the main chemical compositions of the EEOs. Similar extraction-based variations in level of antioxidants and corresponding biological activities have been reported by various researchers (Özek *et al.*, 2008; Chenni *et al.*, 2016; Kusuma *et al.*, 2019; Lawal *et al.*, 2019; Abifarin *et al.*, 2020). To correlate these finding data with the different methods, a regression analysis was carried out (r^2, Table 2). The greater correlation between assays was found in DSA and ICA (r^2 = 0.94-0.97) in IDHBH extract than DHBH (r^2 = 0.87-0.93). Our correlation studies validated this observation in which high values of r^2 depicting tight association between level of polyphenolics and antioxidant activities was detected. Previous works have reported that the antioxidant activities are strongly correlated with the quality and quantity of the phenolic compounds (Shrestha *et al.*, 2006; Marina *et al.*, 2009; Nguyen *et al.*, 2015; Aryal *et al.*, 2019). Therefore, the obtained differences between IDHBH extract and DHBH extracts in phenolic compounds and antioxidant activities can be explained by the relationship between phenolic content in sample extracts and their DAA and ICA capacities. Based on these results, it can be concluded that this plant possesses an important antioxidant activity. Moreover, the IDHBH method seems to be the appropriate extraction method compared to DHBH.

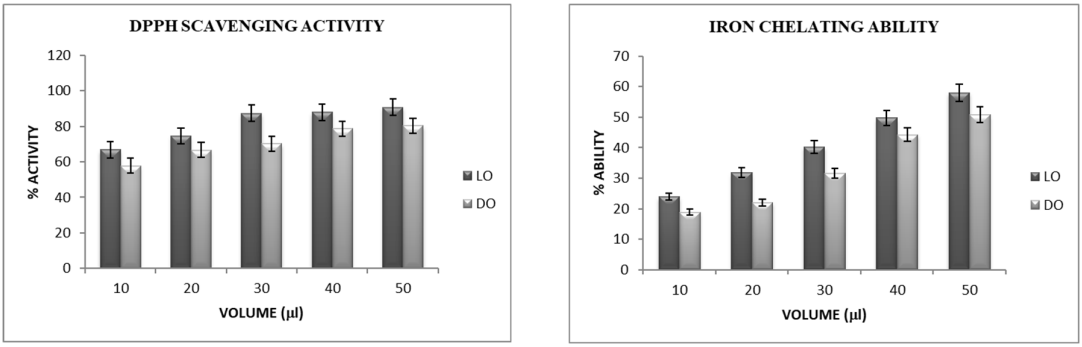


Figure 2. Antioxidant activities and correlation study of Dark essential oil (DHBH) and light essential oil (IDHBH)

Symbols used: TPC: total phenolic content, TTC: total tannin content, DSA: DPPH scavenging activity, ICA: iron cheating activity. Values on bar are mean ± SD (n=3). * Indicates significant difference of light oil vs dark oil at P≤0.05

Dark oil (DHBH)			Light oil (IDHBH)		
R-square values	DSA	ICA	R-square values	DSA	ICA
TPC	0.915671	0.939785	TPC	0.92691	0.979375
TTC	0.920843	0.87801	TTC	0.966421	0.945207

Table 2. Comparison of FT-IR peak values in dark and light oil

Light oil			Dark oil			Peak features		
Peak Number	X (cm-1)	Y (cm-1)	Peak Number	X (cm-1)	Y (cm-1)	Functional group	Compound	Frequency Range
1	2923.18	74.45	1	2922.20	77.55			
2	2854.97	86.08	2	1643.58	94.77	O-H stretch	Alcohol	3550-3200
3	1745.89	83.69	3	1515.21	94.65	C-H stretch	Alkane	3000-2840
4	1464.21	87.10	4	1456.36	88.06	C ≡ N, C ≡ C	alkyne or nitrile	2260-2100
5	1375.38	85.90	5	1376.68	86.34	C=O stretch	Aldehydes, Ketones, Esters, Carboxylic acids, Anhydrides	1830-1650
6	1361.43	88.41	6	1214.68	87.84	C=C stretch	Alkene	1662-1626
7	1306.01	93.09	7	1166.46	89.06	C=C stretch	α, β ketone	1620-1610
8	1234.16	88.65	8	1079.69	87.25	O-H bending	Alcohols	1420-1330
9	1214.49	83.43	9	1053.78	89.40	C-H bending	Alkanes	1450-1465
10	1165.73	81.96	10	1016.17	91.81	O-H Bending	Phenol	1390-1310
11	1079.60	82.13	11	985.35	83.57	C-N stretch	Aliphatic amines	1250-1020
12	1053.42	87.22	12	920.07	91.94	S=O stretch	Sulfoxide	1070-1030
13	1015.89	91.66	13	885.53	86.72	C-H Bending	1,2,3-tridistributed	780±20
14	984.80	76.08	14	843.02	90.71			
15	920.04	92.47	15	814.61	84.33			
16	886.78	89.21	16	787.66	90.31			
17	843.10	87.94						
18	814.61	93.08						

Red color text indicates differentiated peaks

Fingerprint analysis

UV-VIS, fluorescent and FT-IR spectroscopy-based methods offer a very broad understanding about the quantitative and qualitative formulations of bioactive molecules of plant origin (Chen *et al.*, 2016). The comparative qualitative UV Spectroscopy profile of essential oils obtained of both IDHBH and DHBH methods revealed sharp peaks at about 250nm and 350 nm, exhibiting accumulation of secondary metabolites (Parekh *et al.*, 2007). However, quantitative differences were apparent, as peak absorbance was drastically less

in DHBH extracted essential oil (dark oil) (Figure-3A) compared with IDHBH light oil. Fluorescent spectroscopy is another more reliable and sensible method for detection of bioactive compounds (Sharma *et al.*, 2021). The fluorescent emission spectrum of both kinds of IDHBH and DHBH essential oils is given in Figure-3B. All spectra are grouped together as per excitation wavelengths. Notably, only one major peak at 450-500 nm (Green fluorescent, GF) region was detected in both types of essential oils. Surprisingly, peaks with different intensities were detected in both kinds of oils. In IDHBH extracted oil, INT units were 1.8 as compared to DHBH oil having INT units 0.4. Differences in the intensities of fluorescence may be related to chemical composition of essential oils extracted by IDHBH and DHBH techniques. It was reported that the bioactive compounds for GF emission (lambda near 500nm) are polyphenolics including phenolics, flavonoids and tannins (Mylle *et al.*, 2013).

Functional group analysis of bioactives present in dark and light oils was studied by Fourier Transform Infrared Spectrophotometer (FT-IR) which is posed to be powerful, rapid, non-destructive method for fingerprint analysis plant extracts (Chen *et al.*, 2016). FT-IR peak profile of both types of essential oils extracted by IDHBH and DHBH methods are given in Figure-3C, D. Both types of essential oils revealed the presence of different functional groups in bioactive compounds. Surprising part of this study was there were marked differences in the number of FT-IR peaks and their absorbance values. Notably in IDHBH generated light oil, number of FT-IR peaks were 19 whereas in DHBH generated dark oil number of FT-IR peaks was 17, indicating difference in presence of functional groups. Further, from superimposed form of FT-IR spectra of both IDHBH and DHBH oils (Figure 3C, Table 2), it was evident that there were drastic differences in absorbance values of peaks. Notably, in IDHBH oil there were some peaks (2854.97,1745.89,1464.21,1375.38,1361.43,1306.01,1234.16, 1015.16 cm^{-1}) which were absent in DHBH based dark oil.

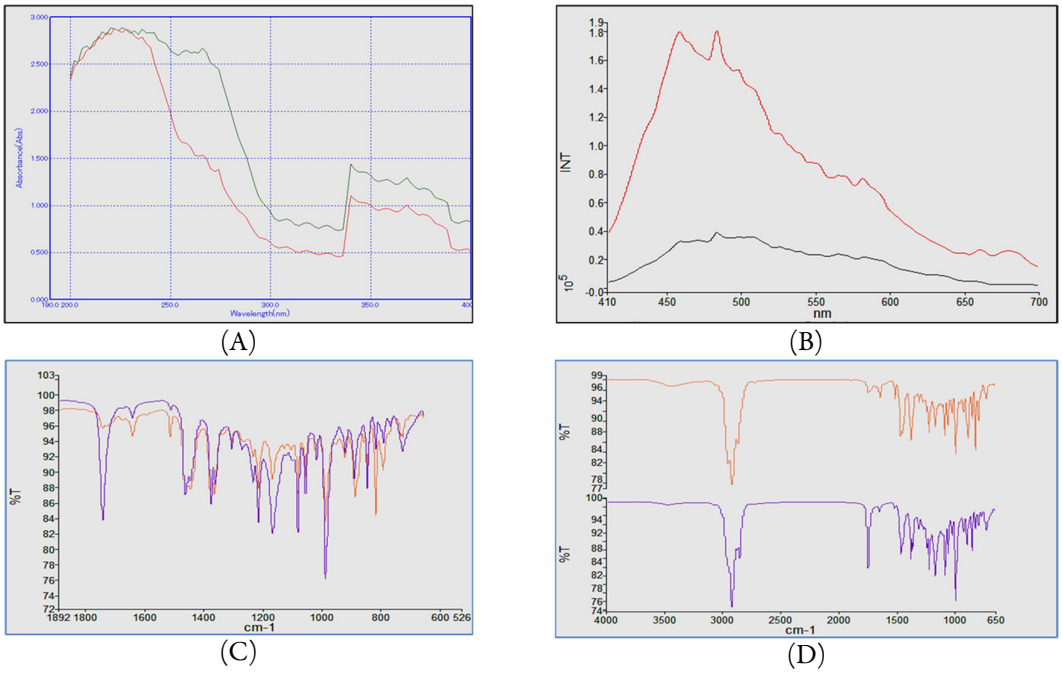


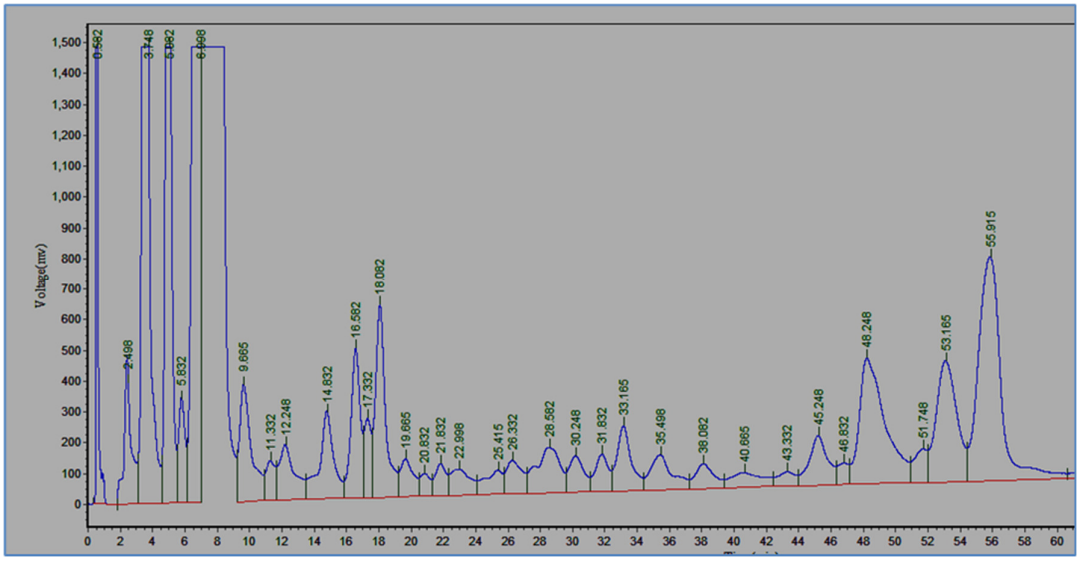
Figure 3. UV (A), Fluorescent (B) and FT-IR analysis (C: superimposed; D: split form) of light (IDHBH) and dark oil (DHBH)

From all these observations, it was apparent that EEO which was extracted by DHBH, number of bioactives was less as compared with IDHBH oil. In FT-IR spectrum, a broad peak at 2923 cm^{-1} indicated C-H stretching due to alkenes, peak at 1456 cm^{-1} attributed to C-H bending due to alkanes and peak at 1377 cm^{-}

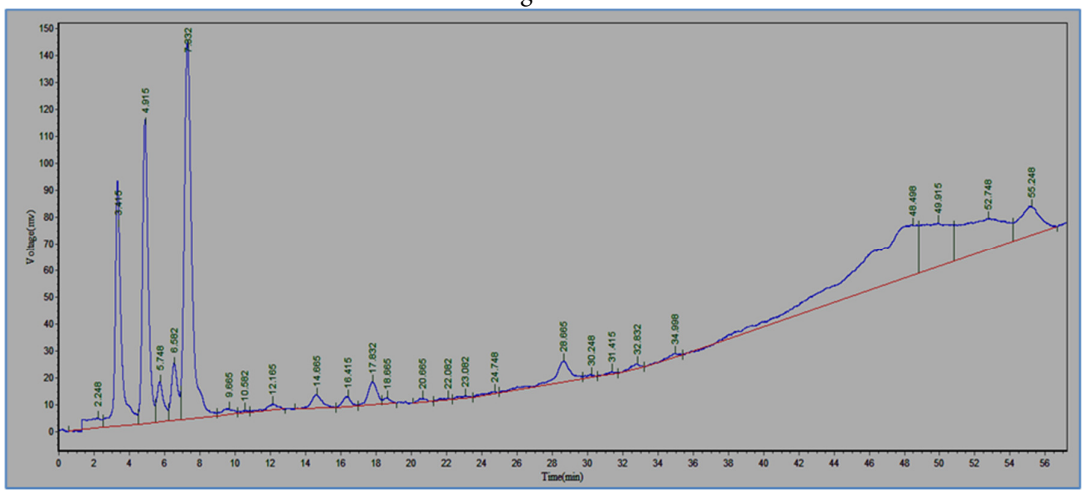
[1] shows S=O bending due to sulphonamides, peaks around 1158 cm^{-1} and 1119 cm^{-1} were due to aliphatic ether, ketones and alcohols respectively (Deepashree *et al.*, 2013; Mauro *et al.*, 2011). FT-IR analysis confirmed the presence of aromatic compounds, alcohols, phenols, alkanes, alkynes, and amines in essential oils extracted from eucalyptus. All these bioactives are classified as plant secondary metabolites (Paulraj *et al.*, 2011), which could be the possible reason behind various bioactivities and medicinal properties of these oils.

GC-FID analysis

Further, the chemical composition of essential oil obtained by IDHBH was compared with that DHBH. GC-FID chemical profile is illustrated in Figure 4. Chemical composition of both types of essential oils is tabulated in Table 3. Figure 5 has shown the comparative diagram of the main essential oil constituents of eucalyptus oil by IDHBH and DHBH methods of this study. In dark essential oil, obtained after DHBH, a total of 11 peaks (both major and minor) were detected. Notably, in light oil, obtained after IDHBH, a total of 21 peaks (both major and minor peaks) were detected. These observations indicated that number of bioactive compounds were more in essential oil which was obtained by IDHBH. Less number of bioactives molecules detected in dark oil obtained by DHBH may be due to degradation of bioactive molecules upon high temperature. Authors claimed that primary reason for degradation of bioactive molecules in above temperatures is hydrolysis and oxidation hence prime origin for loss of quality of any herbal product (Chaaban *et al.*, 2017; Jiménez-Zamora *et al.*, 2016). Table 2 has shown that major constituents observed in essential oil extracted by IDHBH were: β- pinene (10%), 1,8 cineole (10%), eugenol (7%), and pentadecanoic acid (11%). However, in DHBH method, essential oil was majorly composed of β- pinene (10%), eugenol (22%). Notably, the percentage of bioactive pentadecanoic acid in IDHBH distilled essential oil was high (11%), while the abundance of this compound was quite low in DHBH extracted oil (4%). It is fascinating to note that 14 compounds were unique to only IDHBH extracted light oil, while 7 were common. The results have demonstrated that the components in IDHBH and DHBH methods were different because of the reactions on the essential oil components of this herb under the high temperature of the DHBH in hydro distillation method. The different components in the essential oil of eucalyptus due to the heat effect (baked form of the herb in DHBH hydro distillation method) have made different medicinal properties for this herb. So, the medicinal herb Eucalyptus would be changed during hydro distillation method by chemical changes on the essential oil of this herb. The components like 1,8 cineole (10%), and Pentadecanoic acid (11%) in the essential oil of this herb extracted by IDHBH method have the main roles in the medicinal properties of Eucalyptus plant hence immensely used in food, pharmaceutical, and aromatherapy sectors (Raho and Benali, 2012). By comparing the results of the two methods (IDHBH and DHBH) on the essential oil components, IDHBH hydro distillation method exhibited high contents of 1,8 cineole, β-Cymene, β-myrcene, Carvacrol, and Pentadecanoic acid, thus adding medicinal properties to Eucalyptus plant. These observations were consonance with earlier reports citing that the chemical composition of essential oils is dependent on the extraction methods (Ghasemi *et al.*, 2011; Kusuma *et al.*, 2016, Taherpour *et al.*, 2017). Higher number of bioactive compounds in essential oil observed in the IDHBH method indicates the superiority of the method over DHBH. It is postulated that higher and uncontrolled temperature during DHBH could be attributed thermal and hydrolytic degradation of bioactive compounds and loss of highly volatile compounds when compared with the water-steam method. Earlier studies also reported that thermal degradation of unsaturated or esterified compounds of oxygenated compounds is higher in hydro-distillation as compare to other methods (Öztürka *et al.*, 2009; Ghasemi *et al.*, 2011; Moradalizadeh *et al.*, 2013; Elyemni *et al.*, 2019)



Light oil



Dark oil

Figure 4. GC-FID profile of dark (DHBH) and light (IDHBH) colored eucalyptus essential oils

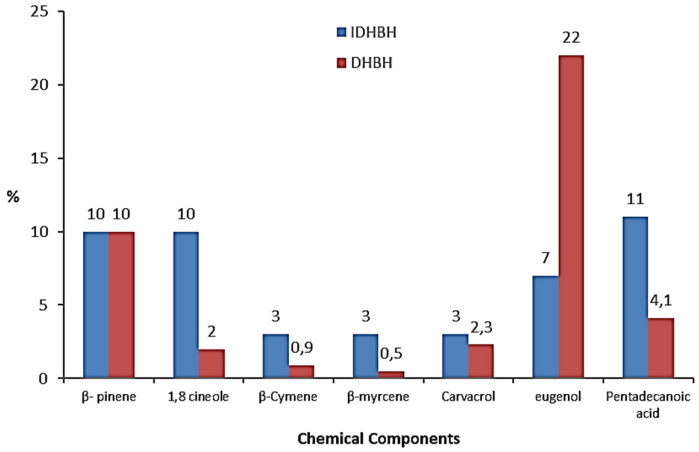


Figure 5. The comparative diagram of the main essential oil constituents of *Eucalyptus polybractea (L.)* by IDHBH and DHBH methods of this study

Anti-inflammatory activity

Major reason behind tissue inflammation is the protein denaturation that results in pain, function loss and swelling at affected areas along with redness and exertion of heat (Khan *et al.*, 2017). Eventually it was suggested that bioactive compounds that poses ability to inhibit heat induced protein-denaturation can possibly be used as therapeutic anti-inflammatory drugs. In the present study, comparative-protein denaturation inhibitory ability of EEOs extracted by IDHBH and DHBH methods was investigated by fluorescent based denaturation assay (Sharma *et al.*, 2021). Typical fluorescent spectra for denatured bovine serum albumin (BSA) and BSA plus EEOs are shown the Figure 6. BSA showed fluorescent INT units near to 5.0. Although both oils depicted considerable inhibition of protein denaturation, as fluorescence intensity decreased substantially (INT units near to 4.5 with DHBH and 3.0 with IDHBH) after the addition of oils to BSA. However, rate of decrease was more by using IDHBH based light oil as compared to DHBH dark oil. These results provide strong evidence for the more potential of IDHBH based oil as a strong anti-Inflammatory agent. The high protein denaturation inhibition capability of IDHBH oil may be ascribed to more content of complex mixtures of bioactive compounds. Previously, the effect of plant extracts on protein denaturation has been evaluated by different researchers, for example: ethanolic extract of *Wdelia trilobataon* on bovine albumin, *Albuscas etosaon* on egg albumin (Umapathy *et al.*, 2010; Govindappa *et al.*, 2011). Literature review on epidemiological studies also indicated that inflammatory activities of herbal derivatives in directly correlated with content of bioactives like polyphenolics present in them (Pan *et al.*, 2010; Gunathilake *et al.*, 2018)

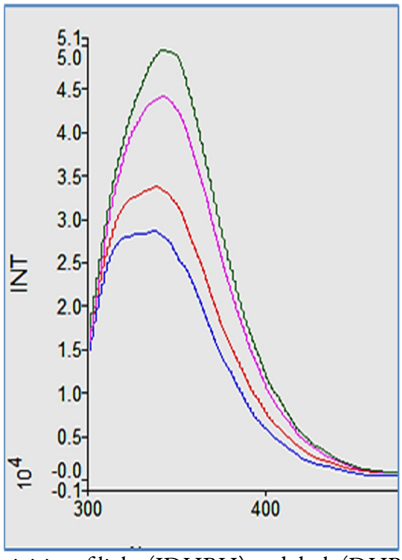


Figure 6. Anti-inflammatory activities of light (IDHBH) and dark (DHBH) essential oils
Green: BSA; Pink: Dark oil; Red: Light oil; Blue: Ibugesic
Ibugesic (+ control)

Antibacterial properties

The antibacterial activity of both type of essential oils were measured against drug resistant microbial strains of *Escherichia coli* (MTCC-40), *Pseudomonas aeruginosa* (MTCC-424) and *Staphylococcus aureus* (MTCC-3160), the results of which are depicted in Table 4. The present study shows that both type of oils depicted bacterial inhibition, however rate of inhibition was more in IDHBH oil than DHBH oil against *Escherichia coli* (MTCC-40), and *Staphylococcus aureus* (MTCC-3160). Both type of oils exhibited total inhibition against gram negative *Pseudomonas aeruginosa* (MTCC-424). The high antimicrobial activity of light oil than dark oil might occur due to high content of bioactives. Similar correlation studies on tight association between polyphenolics content and microbial inhibition have been reported in literature (Chen *et al.*, 2016; Moradi *et al.*, 2019). Our results demonstrated the antibacterial activities of the EEOs, which could

be attributed to the volatile oil compounds present in the EEOs. The inhibitory effects of volatile compounds include adsorption to cell membranes, interaction with enzymes and substrates, and deprivation of metal ions (Tajik *et al.*, 2017).

Table 3. Chemical profile of bioactives in dark and light oils of eucalyptus

No.	IDHBH method			DHBH method		
	RT (min)	Compound	Percentage	RT (min)	Compound	Percentage
1	0.5	-	2	3.7	β- pinene	10
2	2.4	-	2	4.9	-	11
3	3.7	β- pinene	10	5.7	-	1
4	5.0	-	8	6.9	1,8 cineole	2
5	5.8	-	1	7.3	-	18
6	6.9	1,8 cineole	10	14.6	β-Cymene	0.9
7	9.6	Limonene	3	16.4	β-myrcene	0.5
8	12.2	α-Pinene	2	17.8	Camphor	1.3
9	14.8	β-Cymene	3	28.6	Carvacrol	2.3
10	16.5	β-myrcene	3	48.2	eugenol	22.7
11	18.0	(2E)-Octenal	5	55.9	Pentadecanoic acid	4.1
12	28.5	Carvacrol	3			
13	30.2	Longipinene	3			
14	31.8	α-Copaene	3			
15	33.1	Longifolene	2			
16	35.4	β-Guaiene	2			
17	38.0	α-Calacorene	1			
18	45.2	Tetradecanoic acid, ethyl ester	2			
19	48.2	eugenol	7			
20	53.1	Octadecane	5			
21	55.9	Pentadecanoic acid	11			

Text in red color indicate common bioactives

Table 4. Anti-microbial activity analysis of dark and light oil from Eucalyptus

Sample/ Cultures	ZOI (cm)	ZOI (cm)	ZOI (cm)
	MTCC-40	MTCC-424	MTCC-3160
BLANK	NI	NI	NI
Streptomycin (30 mg + control)	2±0.1	3.5±0.2	1.5±0.2
Light oil (IDHBH)	[a] 4±0.2	TI	[a] 3.2±0.3
Dark oil (DHBH)	3.1±0.1	TI	2.2±0.2

Values are mean of triplicate determination (n = 3) ± standard deviation. ZOI: zone of inhibition, NI: no inhibition; TI: total Inhibition, a: indicates significant difference of light oil at P≤0.05 vs. dark oil.

Conclusions

To conclude, the present study highlighted the fact that in-direct heat source during essential oil extraction process maintained higher number of chemical bioactive compounds, polyphenolics, and associated-antioxidant activities of the oils. It was found that IDHBH process is the best way to extract essential oil from plants with potent bioactives. In addition, our results suggested that the extraction technology significantly affects chemical profile and biological activities of EEOs. Among the tested EEOs, IDHBH was the most effective extraction method for generating EEO with greater antioxidant and antibacterial activity. The efficacy of these EEOs against bacteria could in part be attributed to their volatile bioactive oil components. This research may be highly useful to industrialists or pharmaceutical and food-based companies to design experiments to extract important industrial products from plants like essential oils. EEOs established inordinate medicinal, economic, and nutritional values due to their wide-spectrum biological activities. For instance, EEOs could be employed in the pharmaceutical and food industries, or as a therapeutic and preventive agent against a variety of diseases. However, the complexity of the aroma compositions of EEOs might have contributed to the distinctions in biological activities we observed. Therefore, further investigations are needed to reconnoitre the biological activities of EEOs *in vivo* and to identify the molecular mechanisms behind these biological activities.

Authors' Contributions

ADS and IJK: designed study; SA: wet lab.
All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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