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GC Analysis of Essential Oils, TLC Profiling of Pigments and DNA Extraction from *Eucalyptus* Species

Sadaf Quereshi[1*], Ankita Upadhyay[1], Rupal Singh[1], Noor Afshan Khan[2], Abin Mani[1], Jaswant Patel[1]

[1]*Centre for Scientific Research and Development, People's Group, Bhopal, Madhya Pradesh, India*
[2]*Agricultural University, Jabalpur, Madhya Pradesh, India*

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*Corresponding Author

Tel : +91-9827-283300
Fax : --

Email: sadaf2577@gmail.com

Abstract

Medicinal plants, herbs, spices & herbal remedies are integral components of alternative system of medicine since times immemorial. *Eucalyptus* essential oils are highly valued in therapeutic and pharmaceutical preparations because of their main component 1, 8-cineole. In this study, extraction of essential oils from three different varieties of *Eucalyptus* species *viz*. *E. saligna*, *E. tereticornis* and *E. tetragona* were carried out with oil yield of 2.0%, 1.0% and 1.5%, respectively, along with their GC analysis. Major peaks from GC study indicate the presence of important constituents. TLC analysis of leaf pigments represents the most important pigments followed by DNA extraction and purification which reveal spectrophotometric data of DNA concentration-250ng/µl (*E. saligna*), 220ng/µl (*E. tereticornis*) and 200ng/µl (*E. tetragona*).

Key Words: *Eucalyptus*, essential oil, GC analysis, TLC, DNA extraction

Introduction

Plants are good sources for new safe, biodegradable and renewable drugs (1). The genus *Eucalyptus* (Myrtaceae) comprises well-known plants of over 600 species of trees. *Eucalyptus* is a tall evergreen tree and important species include: *E. saligna*, *E. tereticornis, E. tetragona, E. abdita, E. acies, E. apiculata, E. albopurpurea, E. alligatrix, E. ammophila, E. amplifolia, E. andrewsii* etc. *Eucalyptus* essential oil is typically a blend of compounds, principally terpenoids, which represent the dominant class in *Eucalyptus* leaves (2). Various extracts of plant parts and essential oils are of great interest due to their potential uses as alternative remedies for the treatment of many infectious diseases (3). The essential oils obtained from *Eucalyptus* have many medicinal and commercial uses. The essential oils possess many bioactivities (antimicrobial, antiviral, fungicidal, insecticidal and herbicidal activities) and these bioactivities are highly associated with their unique chemical composition. The novel biological functions of *Eucalyptus* essential oils suggest research on all *Eucalyptus* species to fully exploit their commercial benefits (4). There exist some species of the genus *Eucalyptus* which exhibit high essential oil contents in their leaves while others have medium or low contents (5). Aromatic plants are a source of fragrances, flavors, cosmeceuticals, health beverages and chemical terpenes. An essential oil is a concentrated, hydrophobic liquid containing volatile aroma compound from plants. Essential oils are known as volatile, ethereal oils or aetherolea (6).

Eucalyptus is known for its use either as an essential oil or leaf tea for its ability to relieve congestion and ease breathing in colds. Its oil is used as the pain reliever for sore and overextended muscles. The essential oil of *Eucalyptus* contains cineole, a potent antiseptic that helps in killing the bacteria and fungi (7). It brings relief to the patients of asthma and bronchitis and an excellent topical remedy for aching joints and rheumatism. It helps in increasing cardiac action and is taken in all types of fever. It also aids in purifying the blood, lowers the blood sugar as well as helps in improving the blood circulation (8).

The present investigation deals with extraction of essential oils, their GC analysis, TLC profiling of pigments, DNA extraction and purification from leaves of three *Eucalyptus* species *viz*. *E. saligna*, *E. tereticornis* and *E. tetragona*.

Materials and Methods

Extraction of oil

Fresh leaves of the three varieties of *Eucalyptus* were collected on May, 2010 from Bhopal city, Madhya Pradesh, India. About 100g leaves of each species were collected, air-dried and subjected to hydrodistillation for 3-4 hours using a Clevenger-type apparatus for essential oil extraction. The extracted oils were separated from water by decantation and were dried by filtration over anhydrous sodium sulfate.

GC Analysis

GC analyses were performed using a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector (FID). The analysis was carried out using a DB-5 fused-silica column (30 m × 0.25 mm, film thickness 0.25 µm, J & W Scientific Inc., Rancho Cordova, CA, USA). The operating conditions were as follows: injector and detector temperature-250°C; carrier gas-Nitrogen; oven temperature programme-100°C for 2 minutes; temperature was increased at the rate of 15°C/minute up to 240°C for 4 minutes.

TLC profiling of pigments from Eucalyptus leaves

Sample Preparation: 0.5 g of fresh *Eucalyptus* leaves were weighed. To this was added 0.5 g of anhydrous magnesium

sulphate and 1 g of sand. Using a mortar and pestle, the mixture was grinded until it becomes fine, light green powder. This was transferred into a test tube and 2 ml of acetone was thereby added and stirred using a stir bar for 2 minutes. The mixture was allowed to settle for 10 minutes. The solid settled to the bottom, leaving a green liquid layer on top. Centrifugation was done at 5000 rpm for 30 minutes. The green layer was transferred to another test tube using a pipette, subsequently it was subjected to TLC.

TLC analysis: Silica Gel-G was used for TLC analysis. Solvent System employed consisted of: Petroleum ether: cyclohexane: Ethyl acetate: Acetone: Methanol (6:1.6:1:1:0.4). R_f values of different pigments were determined after complete run.

Extraction of Genomic DNA:

5 g of powdered leaf samples (using liquid nitrogen) were mixed with 20 ml of DNA extraction buffer, 2% PVP and 2% β-mercaptoethanol and were incubated at 65^{0}C for one hour. After incubation, the homogenate was cooled at room temperature and equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1 v/v) solution was added and emulsified gently for 15 minutes. The mixture was centrifuged at 15000 rpm for 20 minutes at 4^{0}C and the supernatant was collected. This step was repeated with equal volume of Chloroform: Isoamyl alcohol (24:1 v/v) and emulsified for 15 minutes till a clear supernatant was obtained. To this aqueous phase collected, half the volume of NaCl was added and gently mixed to which 0.8 volume of cold propanol was added to precipitate the genomic DNA. The mixture was incubated for 30 minutes at -20^{0}C to accentuate the precipitation. The precipitated DNA solution was spun at 8000 rpm for 20 minutes at 4^{0}C to pellet the DNA. The DNA pellet was washed with 70% ethanol and air dried. The dried DNA pellet was dissolved in TE (Tris-EDTA) buffer [10 mM Tris HCl (pH 8.0), 1mM EDTA (pH 8.0)] and stored at -20^{0}C until further use.

Purification of Genomic DNA:

Accurate amount of enzyme RNase (20µg/ml) was added to isolated DNA samples and were incubated at room temperature for about 20 minutes. The DNA samples were extracted with equal volume of Chloroform: Isoamyl alcohol (24:1 v/v) and emulsified for 15 minutes till a clear supernatant was obtained. To this aqueous phase collected, 0.8 volume of cold propanol was added to precipitate the DNA. The mixture was incubated for 30 minutes at -20^{0}C to accentuate precipitation. The precipitated DNA solution was spun at 8000 rpm for 20 minutes at 4^{0}C to pellet the DNA. The DNA pellet was washed with 70% ethanol and air dried. The dried DNA pellet was dissolved in TE (Tris-EDTA) buffer and stored at -20^{0}C until further use.

Electrophoresis of DNA Samples

Electrophoresis buffer used was 1X TBE (Tris Borate + EDTA). 0.8% agarose in 1X TBE buffer containing Ethidium Bromide (EtBr) was prepared for separating the particular size fragments expected in the DNA samples, extracted from the leaves of three *Eucalyptus* species. Just enough Electrophoresis buffer was added to cover the gel to a depth of less than 1mm. The samples of DNA were mixed with gel loading buffer and allowed to run. Voltage of 1 to 5V/cm was applied. After complete electrophoretic run, the gel was examined under UV-light.

Results

Essential oil yield

Extracted essential oil yields were 2.0% for *E. saligna*, 1.0% for *E. tereticornis* and 1.5% for *E. tetragona.*

Gas Chromatographic Analysis (Eucalyptus spectrum interpretation)

In the GC analysis of oil obtained from the leaves of *E. saligna*, the total numbers of peaks are 36, out of which the peaks at Rt 2.419, 2.769, 3.269 are the major ones. Similarly, *E. tereticornis* shows a total of 42 peaks, out of which the major peaks were obtained at Rt 2.378, 2.722, 3.095, 3.208, 3.404, 4.809; the major peaks indicate the presence of important compounds present in oil including eugenol. However in *E. tetragona*, a large number of peaks are obtained indicating the presence of noise and impurity. The peaks at Rt 2.456, 2.816 and 3.329 are the major ones that indicate the presence of important chemical compounds.

TLC analysis

R_f values of different fractions from leaf extracts of *E. saligna*, *E. terticornis* and *E. tetragona* are enlisted in Table 1. While TLC profiling of separated pigments are depicted in Fig.1.

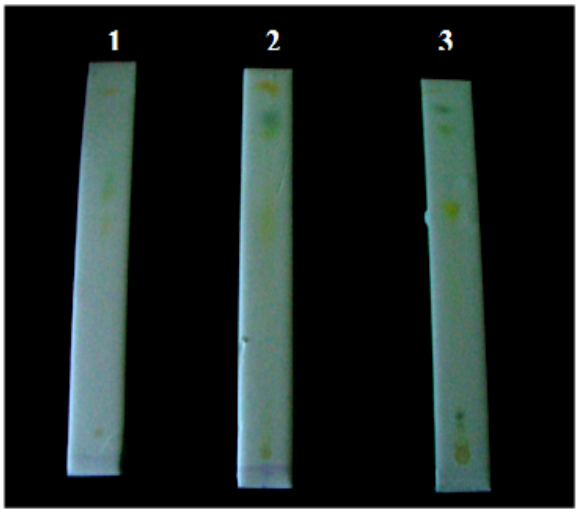

Fig.1. TLC profile of pigments

TLC 1: *Eucalyptus saligna*

TLC 2: *Eucalyptus tereticornis*

TLC 3: *Eucalyptus tetragona*

Table 1: R_f values of different fractions from leaf extracts of *E. saligna*, *E. terticornis* and *E. tetragona*. (Bottom to top)

S.No.	Name of Pigments	R_f values			Color
		E. saligna	*E. tereticornis*	*E. tetragona*	
1.	Lutein	0.29	0.09	0.07	Light Yellow bands
2.	Chlorophyll a	0.42	0.54	0.50	Dark Green bands
3.	Pheophytin a	0.60	-	0.81	Light Yellow bands
4.	Xanthophyll	-	0.13	0.11	Dark Yellow bands
5.	Oil	0.73	0.81	0.87	Greyish spot
6.	Chlorophyll b	-	-	0.38	Green Band
7.	β- carotene	0.78	0.91	0.93	Dark yellow band

DNA electrophoresis

Fig. 2.Genomic DNA of three species of *Eucalyptus*
Lane 1: *Eucalyptus saligna*
Lane 2: *Eucalyptus tereticornis*
Lane 3: *Eucalyptus tetragona*

Genomic DNA was isolated from all the three species of *Eucalyptus* and they exhibited the A260 / A280 ratio between 1.7 to 1.8 after purification. The quality of the genomic DNA isolated was checked by electrophoresis on 0.8% agarose gel and based on spectrophotometric reading the DNA samples were 250ng/µl (*E. saligna*), 220ng/µl (*E. tereticornis*) and 200ng/µl (*E. tetragona*).

Discussion

GC and GC/MS analysis was done in two species of *Eucalyptus*; results indicate high content of 1, 8-cineole in *E. microcarpa* and *E. gilli*. Therefore, the oil was classified as "eucalyptol or medicinal type" (9).

Studies were carried out on two *Eucalyptus* species- *E. saligna and E. camaldulensis* in which essential oils were extracted employing hydrodistillation using a Clevenger-apparatus. The oil was analyzed using Flame Ionization Detector (FID) (30 m x 0.25 mm coated with DB-5, film thickness-0.25µm). The chemical composition carried out by GC/MS revealed 1,8-cineole as the major component of these two oils (10). Remote sensing of chl a, chl b, chl a + chl b and total carotenoid content in *Eucalyptus* leaves, characterization and applications was being effectively achieved (11). Similarly, experiments were carried out that focus on extraction and thin layer chromatography of chlorophyll a and b from spinach (12). Several solvent systems were tried before the five solvent systems were finally employed. Petroleum ether (6 ml), Cyclohexane (1.6 ml), Ethyl acetate (1ml), Acetone (1ml) and Methanol (0.4 ml) comprised of the mobile phase yielding 5 pigment lines depicting five pigments (Bottom to top) *viz*., Lutein, chlorophyll b, chlorophyll a, Pheophytin a and β-carotene. TLC analysis of spinach extract reveals four pigment lines. From the bottom of the plate up, a yellow line for xanthophylls was observed, a green line for chlorophyll-b was observed a brighter green line was visible which corresponds to chlorophyll-a and for β-carotenes a yellow line was observed.

Development of improved *Eucalyptus* genotypes involves the routine identification of breeding stock and superior clones. Currently, microsatellites and random amplified polymorphic DNA markers are the most widely used DNA-based techniques for fingerprinting of these trees. While these techniques have provided rapid and powerful fingerprinting assays, they are constrained by their reliance on gel or capillary electrophoresis, and therefore, relatively low throughput of fragment analysis. In contrast, recently developed microarray technology holds the promise of parallel analysis of thousands of markers in plant genomes. A study was undertaken to develop a DNA fingerprinting chip for *Eucalyptus grandis* and to investigate its usefulness for fingerprinting of *Eucalyptus* trees (13). High molecular weight DNA was isolated from fresh and dry leaves of *T. arjuna*. Isolated DNA was free from contamination and color and could be used for further studies like restriction digestion and PCR amplification. The main aim of the protocol was to provide a simple method of DNA isolation using in house prepared reagents (14).

Conclusion

Eucalyptus oil has potential therapeutic value. Its constituents are indicated by the number of peaks in chromatogram. Major peaks from GC-analysis indicate the presence of compound eugenol. The qualitative and quantitative determination of the important constituents can be further done on comparison with standard chromatogram of the

chemical compound. The quality of the genomic DNA isolated was checked by electrophoresis and intra specific variation of the three species can be determined by employing RAPD and RFLP.

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