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Studies on Essential Oils and DNA Extraction from *Ocimum* species

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Summary

Asia has one of the largest biodiversity regions in the world, holding abundant medicinal and aromatic plant species. The Genus *Ocimum* is ranked highest among some of the astonishing herbs for having enormous medicinal potentialities. The "Queen of Herbs" Tulsi is used as a medicinal plant in India since about five thousand years. The *Ocimum* essential oils are valued because of their main component, methylchavicol and methyleugenol which possess therapeutic properties. Thus, its major use is in the pharmaceutical industry. In the present paper, extraction of essential oils from three different *Ocimum* species *viz*. *O. gratissimum*, *O. sanctum* and *O. americanum* were carried out with oil yield of 1.0%, 1.5% and 1.25%, respectively. GC analysis of essential oil indicates the presence of important constituents. TLC of leaf pigments represents the most important pigments followed by TLC of essential oils for determining the presence of alcohols, esters and carbonyl compounds. The plant DNA were isolated with concentration of 200ng/µl (*O. gratissimum*), 210ng/µl (*O. sanctum*) and 280 ng/µl (*O. americanum*), based on spectrophotometric reading.

Key Words: *Ocimum*, Essential oil, GC analysis, TLC, DNA

Introduction

Essential oils are the volatile materials derived by a physical process from odorous plant material of a single botanical form and species with which it agrees in name and odour. The essential oils are mixtures of up to 200 organic compounds, many of which are either terpenes (with 10 carbon atoms) or sesquiterpenes (with 15 carbon atoms) (Ming, 2006). Essential oils are fragrant, highly concentrated essences of plants which are considered to exemplify the soul or life-source of the plant. Essential oils are approximately 75-100 times more concentrated than dried herbs (Harborne and Baxter, 1993). Nowadays, there are many efficient and economical methods of extraction of essential oils being developed. These methods include- Cold pressing, Hydro distillation, Steam distillation, Solvent extraction, Supercritical CO_2 extraction and Enfleurage. Essential oils find wide applications in a number of consumer goods such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes, confectionery food products, soft drinks, distilled alcoholic beverages (hard drinks) and insecticides.

Ocimum sanctum L. (Tulsi) is a widely grown, sacred plant of India belonging to the Lamiaceae family. It is also called by names like Manjari / Krishna Tulsi (Sanskrit), Trittava (Malayalam), Tulshi (Marathi), Thulsi (Tamil and Telegu) and Holy Basil (English). Tulsi is one of roughly 60 species of the genus *Ocimum,* the basil genus. Within the Tulsi family, there are several kinds grown in India. Most varieties are native to India, including the foothills of the Himalayas; they are also found throughout the Middle East.

The essential oils of *Ocimum* extracted via steam distillation from the leaves and flavouring tops are used to flavour foods, dental and oral products, in fragrances, and in traditional rituals and medicines. Essential oils extracted from fresh leaves and flowers can be used as aroma additives in food, Pharmaceuticals and cosmetics. Extracted essential oils have also been shown to contain biologically-active constituents that are insecticidal (Chavan and Nikam, 1982; Chogo and Crank, 1981), nematicidal (Chatterjee *et al*., 1982), fungistatic (Reuveni *et al*., 1984) or which have antimicrobial properties (Ntezurubanza *et al*., 1984). These properties can frequently be attributed to predominant essential oil constituents, such as methyl chavicol, eugenol, linalool, camphor and methyl cinnamate. Methyl eugenol is the main component of *O. sanctum* from India.

The present paper deals with extraction of essential oils, their GC analysis, TLC profiling of pigments and essential oils and DNA extraction and purification from leaves of three *Ocimum* species *viz*. *O. gratissimum*, *O. sanctum* and *O. americanum*.

Materials and Methods

Extraction of oil

Fresh leaves of three varieties of *Ocimum* were collected on May, 2010 from Bhopal city, Madhya Pradesh, India. About 100g leaves from each species were washed, air-dried and subjected to hydrodistillation for 3-4 hours using a Clevenger-type apparatus for extraction of essential oils. 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 0.25µm, 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: (Griffin et al., 2004)

Sample Preparation: To 0.5 g of fresh *Ocimum* leaves, 0.5 g of anhydrous magnesium sulphate and 1 g of sand were added. The mixture was grinded using a mortar and pestle until it becomes fine, light green powder. To this, 2 ml of acetone was thereby added and the solution was stirred using a stir bar for 2 minutes. Centrifugation was done at 5000 rpm for 30 minutes. The green top layer collected was used as sample for 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). After complete run, R_f values of different pigments were determined.

TLC profiling of oils (Nigam et al., 1965)

Extracted oils were subjected to TLC for determining the presence of alcohols, esters and carbonyl compounds. Different solvent systems employed were as follows:

- Determination of Alcohols- Benzene: Methanol (10:1)
- Determination of Esters- Benzene
- Determination of Carbonyl Compounds: nHexane: Ether (20:3)

Location Reagent: Localization of separated compounds was achieved by placing the TLC plate in chamber saturated with Iodine Vapours.

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 65^0C for an hour. After incubation, the homogenate was cooled to 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 4^0C 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 supernatant (aqueous phase), half the volume of NaCl was added, gently mixed and finally 0.8 volume of cold propanol was added to precipitate the genomic DNA. The mixture was incubated for 30 minutes at -20^0C to accentuate the precipitation. The precipitated DNA solution was spun at 8000 rpm for 20 minutes at 4^0C 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 -20^0C 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 -20^0C to accentuate precipitation. The precipitated DNA solution was spun at 8000 rpm for 20 minutes at 4^0C to pellet the DNA. The DNA pellet was washed with 70% ethanol, air dried and stored in TE (Tris-EDTA) buffer at -20^0C until further use.

Electrophoresis of DNA Samples

DNA Electrophoresis was carried out in 0.8% agarose containing Ethidium Bromide (EtBr) for separating the particular size fragments expected in the DNA samples, extracted from the leaves of three *Ocimum* species. Just enough Electrophoresis buffer (1X Tris Borate EDTA) 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 and Discussion

Essential Oil Yield

The yields of extracted essential oil were 1.0% for *O. gratissimum*, 1.5% for *O. sanctum* and 1.25% for *O. americanum*. Jeba and Vaidyanathan (2011) extracted essential oils of *O. santum* and *O. basilicum* using Clevenger apparatus with oil yield of 1.45% (w/w) and 0.98% (w/w), respectively. Similarly, essential oil yield from *Eucalyptus globulus* Labill was 0.18% (Song *et al*., 2009) and from *E. gillii* and *E. microcarpa* were 2.45% and 1.40%, respectively (Jaimand *et al*., 2006). Chowdhury *et al*. (2008) calculated oil yield as per weight of dried material and the yield were 0.5% for *Murraya paniculate* and 1.0% for *M. koenigii*. The oil was extracted from the aerial parts of *Adenosma capitatum* and *Limnophila aromatica* by Bhuiyan *et al*. (2010) with yields of 1.1% and 1.0% respectively.

Gas Chromatography (Ocimum spectrum interpretation)

The chromatogram of GC analysis of oil obtained from the leaves of *O. gratissimum* show the major peaks at Rt. 6.539, 7.163, 7.710. Similarly, in *O. sanctum* the total no.55 peaks were obtained out of which the peaks at Rt 6.507, 6.932, 7.165, 12.562, 12.610 are the major ones. However, in *O. americanum* out of 13 peaks obtained, 9 peaks are major that indicate the presence of main compounds. The qualitative and quantitative determination of the important constituents will be further done on comparison with standard chromatograms of the chemical compound.

Tonzibo *et al*. (2009) extracted essential oil from *Hyptis suaveolens* by hydrodistillation and analyzed by GC and GC/MS. The oil of leaves was predominated by sabinene, β-caryophyllene, benzyl benzoate and a diterpene hydrocarbon II and palustrol. The chemotype of leaves was constituted by 1, 8-cineole and β-caryophyllene. The oil of leaves from *H. suaveolens* was constituted manly by β-caryophyllene and germacrene D. Oxygenated compounds were present in relatively small proportion, particularly monoterpenes and oxygenated diterpenes. On the other hand, sesquiterpene hydrocarbon showed the most important amount.

O. basilicum is an aromatic plant. The oil of *O. basilicum* is categorized as high essential oil which means the aroma will evaporate within 24hours after it is applied to the body. The oil

can be used for aroma therapeutic massage by lightening and refreshing the body. It can also reduce the intensity of digestion problem, headaches, strained muscles and nervous breakdowns.

O. gratissimum and other species of the plant have been shown to yield essential oils, which are rich in eugenol, thymol, camphor and citral. These components are however not all present in all the species and the composition of the essential oil varies according to the origin of the plant (Dokosi, 1998).

In East Africa, the essential oil of *O. canum* Sims contains 16 – 25% camphor whilst in Central Africa, methyl cinnamate predominates. Also the oil of *O. basilicum*, which is botanically similar to *O. canum* can contain up to 75% estragol i.e. methyl chavicol. The essential oil of *O. Viride* contains mainly thymol (32 – 65%) and eugenol (Oliver-Bever, 1986). Work done on the Nigerian species of *O. viride*, thought to contain both thymol and eugenol showed only the presence of thymol (El-Said *et al*., 1969).

TLC Analysis of Pigments

TLC method used for the separation of a number of colourful pigments from leaf extracts is depicted in Fig.1 and their respective R_f values are enlisted in Table 1.

Fig. 1. TLC of leaf extracts (pigments).
TLC 1: *O. gratissimum*
TLC 2: *O. sanctum*
TLC 3: *O. americanum*

Table 1:- R_f values of Different fractions from leaf extracts of *Ocimum* (Bottom to top)

S. No	Name of Pigments	R_f values			Colour of Pigments
		O. gratissimum	*O. sanctum*	*O. americanum*	
1.	Lutein	-	-	0.513	Light Yellow bands
2.	Chlorophyll a	0.431	0.445	0.40	Dark Green bands
3.	Pheophytin a	0.573	0.59	0.58	Light Yellow bands
4.	Xanthophyll	-	0.74	-	Dark Yellow bands
5.	Oil	-	-	-	Greyish spot
6.	Chlorophyll b	-	-	0.38	Green Band
7.	β- carotene	0.78	0.934	0.82	Dark yellow band

TLC Analysis of essential oils

TLC has been applied successfully to characterize essential oils for the presence of alcohols, esters and carbonyl compounds. These classes of compounds with different polarity necessitate the use of different developing systems (Nigam *et al*., 1965). TLC analysis of extracted essential oils of *Ocimum* species reveals the presence of such compounds.

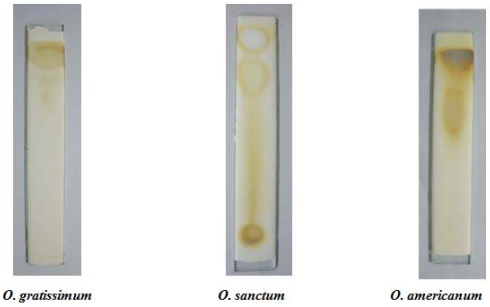


Fig. 2.For Alcohols: TLC of essential oil showing presence of Alcohols

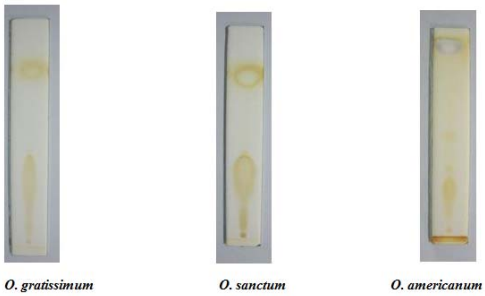


Fig. 3.For Esters: TLC of essential oil showing presence of Esters

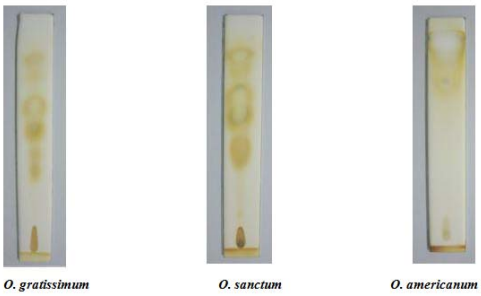


Fig. 4.For carbonyl compounds: TLC of essential oil showing presence of Carbonyl compounds

Remote sensing of chl a, chl b, chl a + chl b and total carotenoid content in *Eucalyptus* leaves- characterization and applications is being effectively done by Datt (1998).

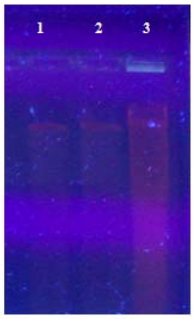
Similarly, Griffin *et al*. (2004) carried out experiments that focus on extraction and thin layer chromatography of chlorophyll a and b from spinach. Several solvent systems were tried before the five solvent systems was finally employed. Petroleum ether (6 ml), Cyclohexane (1.6 ml), Ethyl acetate (1 ml), Acetone (1 ml) and Methanol (0.4 ml) comprised of the mobile phase yielding 5 pigment lines.

Quach *et al*. (2004) extracted and separated the leaf pigments by thin layer chromatography. They used TLC techniques for the separation of a number of colourful pigments from the leaves. They isolated pigments such as chlorophyll-a, chlorophyll-b, xanthophylls, pheophytin-a, pheophytin-b and carotenoids. R_f value was then calculated for the isolated pigments.

The essential oils extracted from three varieties of *Ocimum* using Clevenger type apparatus were later subjected to Gas Chromatographic analysis.

DNA electrophoresis

Genomic DNA was isolated from all the three species of *Ocimum* and they exhibited the A260 / A280 ratio 1.7 to 1.8 after purification. The quality of the genomic DNA isolated was checked by electrophoresis on 0.8% agarose gel. Based on spectrophotometric reading the DNA samples were 200ng/µl (*O. gratissimum*), 210 ng/µl (*O. sanctum*) and 280 ng/µl (*O. americanum*).



Lane 1: *O. gratissimum*
Lane 2: *O. sanctum*
Lane 3: *O. americanum*

Fig. 5.Genomic DNA of three species of *Ocimum*

Thus the three *Ocimum* species shows marked difference with respect to their pigment composition, essential oil yield and DNA electrophoresis studies.

Khanuja *et al*. (1999) carried out rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant species belonging to the same or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus the biochemical composition in plant tissues of different species is expected to vary considerably. They extracted DNA fron *O. kilmandolens* and carried out PCR amplification.

Harisaranraj *et al*. (2008) analyzed inter-species relationship of 7 *Ocimum* species using RAPD markers. Labra *et al*. (2004) carried out studies on morphological

characterization, essential oil composition and DNA genotyping of *O. basilicum* L. cultivars. They concluded that the combined analysis of morphological traits, volatile oil composition and molecular markers represent the optimal approach to verify taxonomy and to correlate it with agronomic traits.

For developing phytomedicines or phytotherapeutics as a major area of concern, it would be essential to adopt a holistic interdisciplinary approach, have a scientific basis of the understanding of the plant systems, new innovations and their conservation for utilization in future on a sustainable basis.

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