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Discriminating the leaves of *Ocimum sanctum* Linn. conferring to their age by gas chromatographic fingerprint analysis assisted with multivariate analysis

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Abstract

Ocimum species is having massive variations in their chemical composition that includes day time, terrestrial and cyclical variation according to the season etc. In this study, variation in overall essential oil constituents among matured, intermediate and young leaf samples of O. *sanctum* was studied. The leaf samples according to their age- matured, intermediate and young leaves were collected from three different plants at 11 a.m. each. Extract of collected leaf samples were then scrutinized using gas chromatographic technique having flame ionization detector (GC-FID). Result reveals that the noteworthy variance was observed in overall essential oil constituents of the leaf samples according to their age. The Average chromatographic fingerprint data of triplicate run for different leaf samples were then treated to multivariate methods such as hierarchical cluster analysis and principal component analysis**.** In this study, instead of considering one or two biomarkers, we have exasperated to obtain as many constituents in chloroform extracts of chosen leaf samples. Scree plot reveals that nine components were responsible for the whole variation. Score plot and dendogram efficiently discriminated all the three leaf types according to their age in different clusters. The outcome as an inference was, *O. sanctum* leaves according to their age exhibit variation in its chemical composition and developed tool can be used for routine quality control.

Keywords

Ocimum sanctum leaves; Gas chromatography, Fingerprint, Age, principal component analysis, hierarchical cluster analysis

Introduction

The genus *Ocimum* (Lamiaceae) has massive importance as having the aromatic values and medicinal uses of several species belonging to it (1-5). Yet, enormous intra-specific disparities have been observed in various species, among their morphologies and chemical configuration. Dire vicissitudes in the constituents like eugenol and 1,8-cineole were experiential through the day time in a study performed to evaluate the amount of day time variation in the constituents of *O. gratissimum* (1). Likewise, noteworthy day time variations were also witnessed in the composition and total yield of essential oil in varius *Ocimum* species *O. basilicum, O. kilimandscharicum,* and *O. americanum* (2). Momentous geographical and season wise variations were found in the content of essential oils of *O. sanctum* and *O. basilicum*, and these differences will help to choose the suitable season as well geographical region for collection of these samples (3,4). Substantial variances were observed in overall essential oil constituents among the mature and young

leaves of *O. basilicum* (4). However, variation in essential oil composition of *O. sanctum* for young, intermediate, and mature leaves has not been studied so far. In the Indian ayurvedic system *O. sanctum*, has been considered as a high valued and consecrated plant. It is having vast therapeutic impact and can be used for various purpose, i.e. anti-carcinogenic, anti-bacterial, anti- oxidant, anti-stress, neuro-protective, cardioprotective etc. (5).

This study would help in collection of leaves on the basis of their age to obtain the maximum of its potential-both medicinal as well aromatic. Our study is suggestively diverse from the studies where only single biomarker is taken in consideration for standardization. In this study, a robust and steadfast GC method was developed and from the developed chromatographic fingerprinting profile, we have tried to obtain as many essential oil constituents in chloroform extracts of different leaf samples according to their age. The data that was gained by GC fingerprint profiling was then coupled with chemometrics methods, like PCA and HCA (6, 7). Based on the treatment of fingerprint data with multivariate techniques; these leaves were discriminated efficiently according to their age whether they are belonging to which age group - young, intermediate, and mature. So, the developed method can be used for routine quality control.

Materials and methods

Leaf samples collection

The leaf samples of *O. sanctum* were collected from plants budding in Gandhinagar (Lat: 23°22'; Long: 72°63') Gujarat, India, located at ~78.93 m above sea level. The leaf samples were collected in the May month for three consecutive days from Gandhinagar, Gujarat. Three different types of leaf sample as shown in fig 1- Young, Intermediate, and Matured from three different plants were collected. They were labelled as Y-1, Y-2, Y-3, I-1, I-2, I -3, M-1, M-2, and M-3. As previous study suggested no diurnal variation in *O. sanctum;* Devoid of specific time period all the samples were collected at 11 a.m. Plant was validated as a part of authentication by Director of Rahi botanical garden, Dr. Sandip Patel, Valsad, vide sample voucher number NDP-07. Thus, collected specimens were dried in shade, ground to a course powder and stowed in air-tight containers in a unruffled and gasping place.

Extraction of plant material

In a 100 mL conical flask, 1 g powder of dried leaf samples of *O. sanctum* was soddening overnight for all the nine samples with 25 mL of. Thus, prepared chloroform extract was then filtered using Whatman filter paper No. 1. It was then refluxed and final volume was concentrated on a water bath and temperature was kept 60°C. Concentrated extract was adjusted to 5 mL as a final volume which was then injected for GC analysis.

Gas chromatographic analysis

Perkin Elmer GC Clarus 500 instrument was used for the analysis. The method was developed and optimized by means of ped with a fused silica capillary column ZB-5 having dimensions: 30 m × 0.25 mm; and film thickness 0.25 µm. Detection was done using a flame ionization detector. Nitrogen was the flowing gas as a career with flow rate of 1.0 mL/min. Temperature of the oven was raised to 225°C from the initial temperature 70°C where ramp rate was 8.0 °C/min and holding time for 5 mins at that temperature. The injector temperature was 245°C and temperature of the detector was 255°C, respectively. 2.0 µL was the volume for the injection of the sample with a split ratio of 20:1.

Data analysis

Average chromatographic fingerprint data of three different plants; plant-1, 2 and 3 for their three different samples according to their age; young, intermediate and matured for three successive days was coupled to Minitab 17 software for their statistical analysis. Each sample was treated and examined in triplicate. To assess the variation in the chemical composition of leaves based on their age. For further classification of the examined specimens, principal component analysis (PCA) (8, 9), was performed grounded on the alterations in the samples. With the aim of gauge, the similarity and variations in the leaf samples according to their age, hierarchical cluster analysis (HCA) (10, 11) was accomplished on the data of areas of peaks in the fingerprints of GC.

Result and Discussion

The three different types of *O. sanctum* leaves- young, intermediate, and matured leaves as shown in Fig 1, according to their age were poised for three sequential days in the May month from three different plants; plant-1, 2, and 3 at 11 a.m.

Since the solar strength and temperature are uppermost through this time as likened to other time of the year, it was believed to be the idyllic time of the season to study the chemical variation in *O. sanctum* leaves according to their age. As the essential oil components are highly soluble in Chloroform, it was used as a solvent for extraction of leaf samples. Another solvent with high polarity would also dissolve non-volatile oil components, but there are all chances that it may hinder with the GC fingerprint analysis of volatile constituents. Extraction process was performed in a controlled condition especially it was taken care that the temperature not surpasses the value of 60°C, because it may lead to loss of essential oil components. A robust and reliable method was established for fingerprint analysis leaf sample extracts using GC.The percentage contents in their relative aspects for each component in the extracts were strong-minded, where for all the resolved peaks- relative areas in the gas chromatogram were taken in consideration.

The GC data for fingerprint profiling of *O. sanctum* leaf samples-extract showed many peaks. GC fingerprint profile of Plant -1 for their young, intermediate and matured leaf samples is shown in fig 2. Instead of considering a single peak of marker compound all the peaks were taken in consideration for additional analysis of the fingerprint data by chemometric means. For statistical analysis, peak areas

for their respective retention times of chromatographic fingerprint by considering the average data of leaf samples poised for three sequential days were used. The GC fingerprint data obtained from analysis of leaf samples can be easily classified by applying a dimensionality reduction method such as PCA and HCA [12, 13]. Correlation type of matrix was used in PCA on the GC fingerprint data of different leaf samples according to their age. All obtained data were presented as scores, as well loadings in a synchronize system of principal component analysis, in which data resulted from dimensionality reduction. From the scree plot, it was concluded that the first nine principal components (PC 1 to PC 9) accounted for total variation, as shown in the scree plot (Fig 3 B). Different leaf samples were successfully discriminated according to their age and that can be witnessed in the score plot (Fig 3 A). Thus, chemometric analysis by means of PCA for fingerprint data of *O. sanctum* leaf samples has classified the leaves according to their age in different groups. A hierarchical agglomerative clustering analysis (HCA) of nine specimen of *O. sanctum* leaf samples collected from three diverse plants according to their age was achieved and that too after normalization process to the data, to envisage the alterations and/or resemblances amid samples. In HCA dendrogram (Fig 3 C), the nine leaf samples got disseminated into three groups as shown in figure; Cluster-I contained samples Y-1, Y-2, Y-3; cluster-II contained samples I-1, I-2, I-3 and cluster-III contained M-1, M-2, and M-3.

Conclusion

The genus *Ocimum* has massive importance as having the high aromatic values and medicinal uses and hence variation in the essential oil constituents of the essential oil from *O. sanctum,* can create a huge impact on its medicinal activities. We have developed chemometric assisted GC fingerprint profile for *O. sanctum* samples to distinguish them according to their age and the established method was precise, stable, and simple. As the settled method is non-targeted and can be used to distinguish the leaves according to their age this method can be further useful for targeted analysis by GC/MS and extensive yet another elaborative studies. This quality control tool can also be used as a routine analysis for the evaluation of crude *O. sanctum* samples.

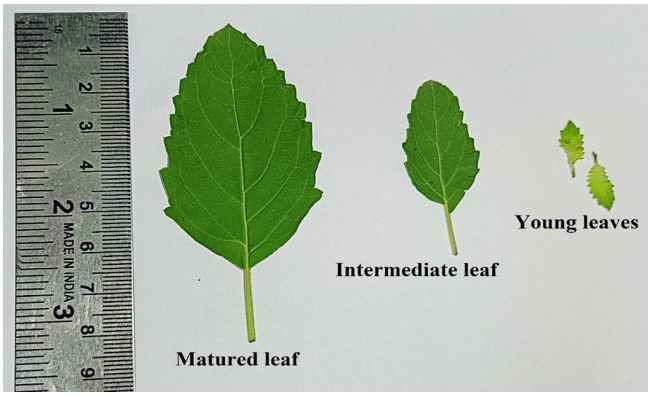

Fig-1: Leaves of *O. sanctum* plant according to their age- Matured, Intermediate, and young leaves

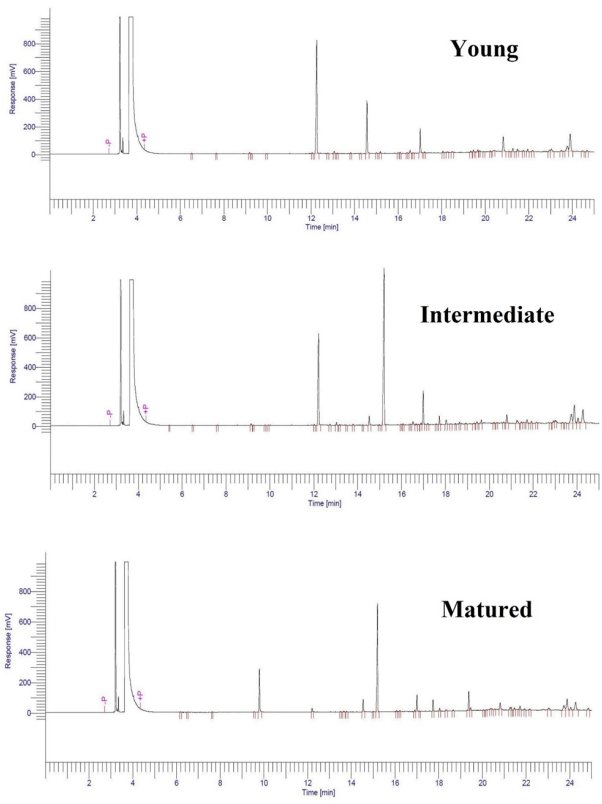

Fig. 2. Gas chromatographic fingerprint profile of O. sanctum young, intermediate and matured leaf samples plant-1.

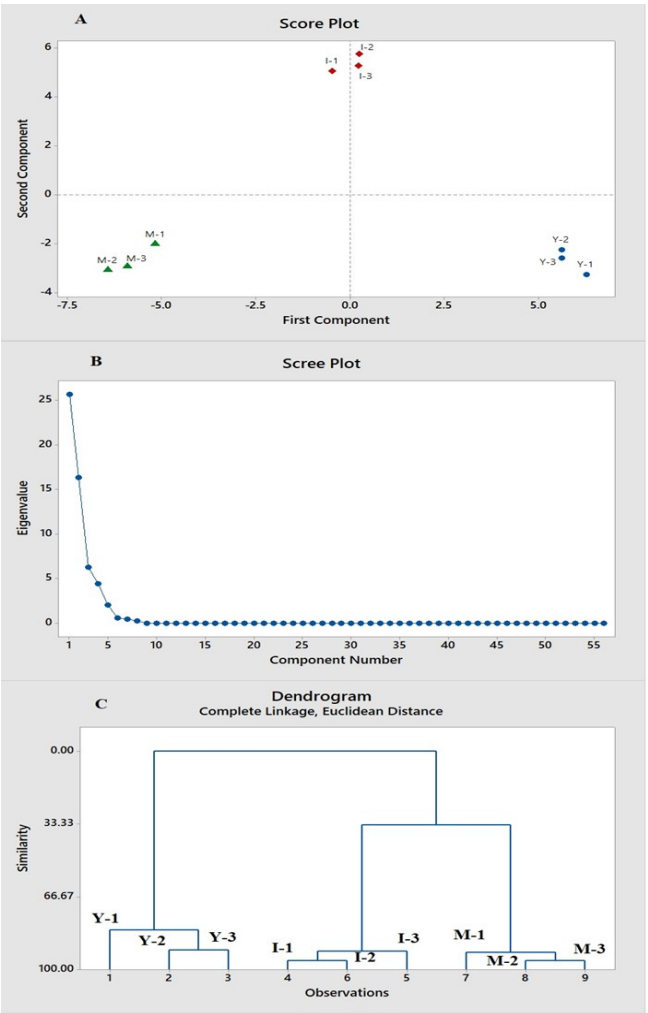

Fig. 3. Score plot (A) and Scree plot (B) obtained by principal component analysis and Dendrogram obtained by hierarchical cluster analysis of chromatographic fingerprint data of *O. sanctum* leaf samples

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