



HISTOCHEMICAL LOCALIZATION AND CHROMATOGRAPHIC ANALYSIS OF LEAF ESSENTIAL OILS OF SELECTED SPECIES OF *CURCUMA* L.

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Abstract

The genus *Curcuma* L. have been used in traditional medicine from ancient times. The essential oil content of the genus is of great importance and has demonstrated a wide variety of properties. Moreover, studies on the chemotaxonomical and characterization of *Curcuma* species have a significant role in the selection of genotype with higher yield and better quality. This study gives the histochemical localization and quantification of the oil cells, and a chromatographic fingerprinting of essential oil from the leaves of six species of *Curcuma*, validated using HPLC techniques. Further, correlation analysis of the histochemical parameters exhibited high positive correlation (0.886), statistically significant at * $p \leq 0.05$ and ** $p \leq 0.01$, respectively, between the oil content and oil index. The result obtained gives a relative description on the histochemical parameters along with the chromatographic fingerprinting which might be relevantly utilized in species identification.

Key words: Chemotaxonomy, *Curcuma*, Essential oil, Histochemical, HPLC.

Introduction

Several species of *Curcuma* (Family- Zingiberaceae) are aromatic and possess essential oil in various parts of the plant such as leaves, rhizome and tuber are of great importance as medicinal herbs and spices. The use of anatomical features as a marker is now very significant and popular in systematic botany due to its medicinal and commercial value (Sherlija 1989 and Choudhury 1996). Although the biological significance, pharmacognostic, phytochemical, essential oil studies of various *Curcuma* species has been highlighted in the underground rhizomes, there is no specific documentation of anatomical feature and variation in the chemical constituents in the leaves which might be relevantly utilised in species identification. In our own previous work we studied the histochemical localization of curcumin in the rhizomes and its significance in chemotypic characterization of selected species of *Curcuma* L. validated by HPTLC (Seema and Lavania, 2015). For the enhanced understanding of the role and function of secondary metabolites, knowledge

of chemical composition along with anatomical description of the secreted substances is of utmost importance (Fahn, 1979). This approach will serve as an important tool for taxonomic demarcation (Metcalf and Chalk 1979) and shall be helpful in determining drug adulteration (Solereeder 1908, Linga and Savithamma, 2011). Moreover, Histochemical characters are commonly used to deduce taxonomic conclusions (Serrato –Valenti G *et al.*, 1997, Edeoga and Okoli 1995 and Mbagwu *et al.*, 2009). In this present study a validated HPLC method for the analysis and comparative fingerprinting of leaf essential oil of six *Curcuma* species has been analysed along with the histochemical localization and quantification of the oil cells which may be used as a chemotaxonomical tool. A comparative study of oil content of wild and market sample of *Curcuma zedoaria* L. was also quantified, due to its commercial importance.

Materials and Methods

Plant material

The plant specimens of the same age (belonging to

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third rhizome generation) were collected in the month of November 2010, 2011 and 2012 from the natural habitats of Kannur, Kasaragod and Thiruvananthapuram districts of Kerala, Lucknow and Shillong, India and were identified and determined by comparison with the authentic herbarium specimens deposited at the herbarium of Jawaharlal Nehru Tropical Botanic Garden & Research Institute, Palode, Thiruvananthapuram, Kerala (JNTBGRI) by the taxonomists of JNTBGRI, Thiruvananthapuram. The voucher numbers are *C. longa* (36252, Pathanamthitta, 9-10-1999), *C. zedoaria* (14575, Palode, 30-05-1994), *C. aromatica* (60664, Palode, 10-01-2013), *C. haritha* (14576, Thiruvananthapuram, 30-5-1994), *C. caesia*, (51817, Achankovil, 21-10-2004) and *C. raktakantha* (60668, Thiruvananthapuram, 08-5-2013).

Oil isolation

The air dried leaves of each *Curcuma* species were cut into small pieces after collection and subjected to hydrodistillation in a Clevenger-type apparatus for 5 hours. The oil separated and dried with anhydrous sodium sulphate. The volume of oil (in cc) obtained was expressed and tabulated as the yield as a percentage of volume/weight.

Histochemical analysis of Oil cells

For Oil localization under light microscopy, free hand cut sections of fresh leaves were stained with Sudan III. For oil localization under fluorescent microscopy, fresh leaf sections were cut, washed and dried by keeping it on a Whatman filter paper and then transferred to a vial containing 0.75% Schiff's reagent, incubated for 45min kept for staining under room temperature. Sections were then transferred to a filter paper, dried and then mounted in 1N HCl solution.

Microscopic analysis

The light microscopy study was carried out using a compound Olympus microscope. The measurements were recorded with a calibrated eyepiece micrometer at 400X resolution. Observations were done and photographs were taken under Tx red (Ex 540-580), FITC (Ex 465-495) and DAPI (Ex 340-380) using Nikon-HC00L fluorescent microscope.

HPLC analysis

HPLC experiment was performed using an Agilent system equipped with a vacuum degasser, quaternary solvent mixing, auto-sampler and a UV detector. UV spectra were collected at 254 nm for chromatograms. Empower software was utilized for instrument control, data collection and data

processing. The column used was an ACE C₁₈ (4.6 × 250 mm, 5 μm). The mobile phase was an isocratic combination of acetonitrile: isopropanol (1:1) with a flow rate of 0.8 ml/min. Injection volume for all samples (1 mg/ml, in acetonitrile) solutions was 8 μl.

Results and Discussion

Histochemical analysis

The sections mounted in Sudan III showed oil cells, secretary in function, distributed in the undifferentiated mesophyll tissue, easily distinguishable as small, spherical cells with suberized walls (Plates 5-8). Each oil cell appeared as a small irregular, refractive body, cluster of globular drop like structures when localized under fluorescent microscope (Plates 1-4). According to the species examined variations was observed in the oil content. Leaves of *C. longa* showed significantly higher content compared with other species. Difference was also observed in the wild and market sample of *C. zedoaria*. The oil content in the wild sample was 69% higher than the market sample. Moreover, the oil cell diameter and the oil cell index showed 27% and 20% increase in the the wild sample. Oil cell diameter was found similar and maximum in size both in *C. caesia* (65 ± 2.04 μm) and *C. aromatica* (65 ± 1.5 μm) followed by *C. haritha* (53 ± 0.83μm) and *C. raktakantha* (49 ± 2.14μm). However, similarities were found in the sizes in *C. longa* (45 ± 2.3 μm), and *C. zedoaria* (45 ± 2.2 μm), and minimum value was seen in the market sample of *C. zedoaria* (33 ± 2.2 μm). The oil cell index was found maximum in *C. longa*, (18.86 ± 0.42), as anticipated from the high yield of the essential oil (2.2 cc, v/w) and minimum in *C. haritha*. (Table 1). Oil cells in *C. caesia* under fluorescent microscopy of three different wavelengths are shown in Plates 1-4. Further, correlation analysis was performed among the oil content, oil index and oil cell diameter to find the relationship among the parameters. High positive correlation (0.886) was obtained between the oil content and oil index statistically significant at *p ≤ 0.05 and **p ≤ 0.01. The localization and quantification

Table 1: Leaf oil cell features of different *Curcuma* species. cc (cubic centimetre) μm (micro metre), % (percentage).

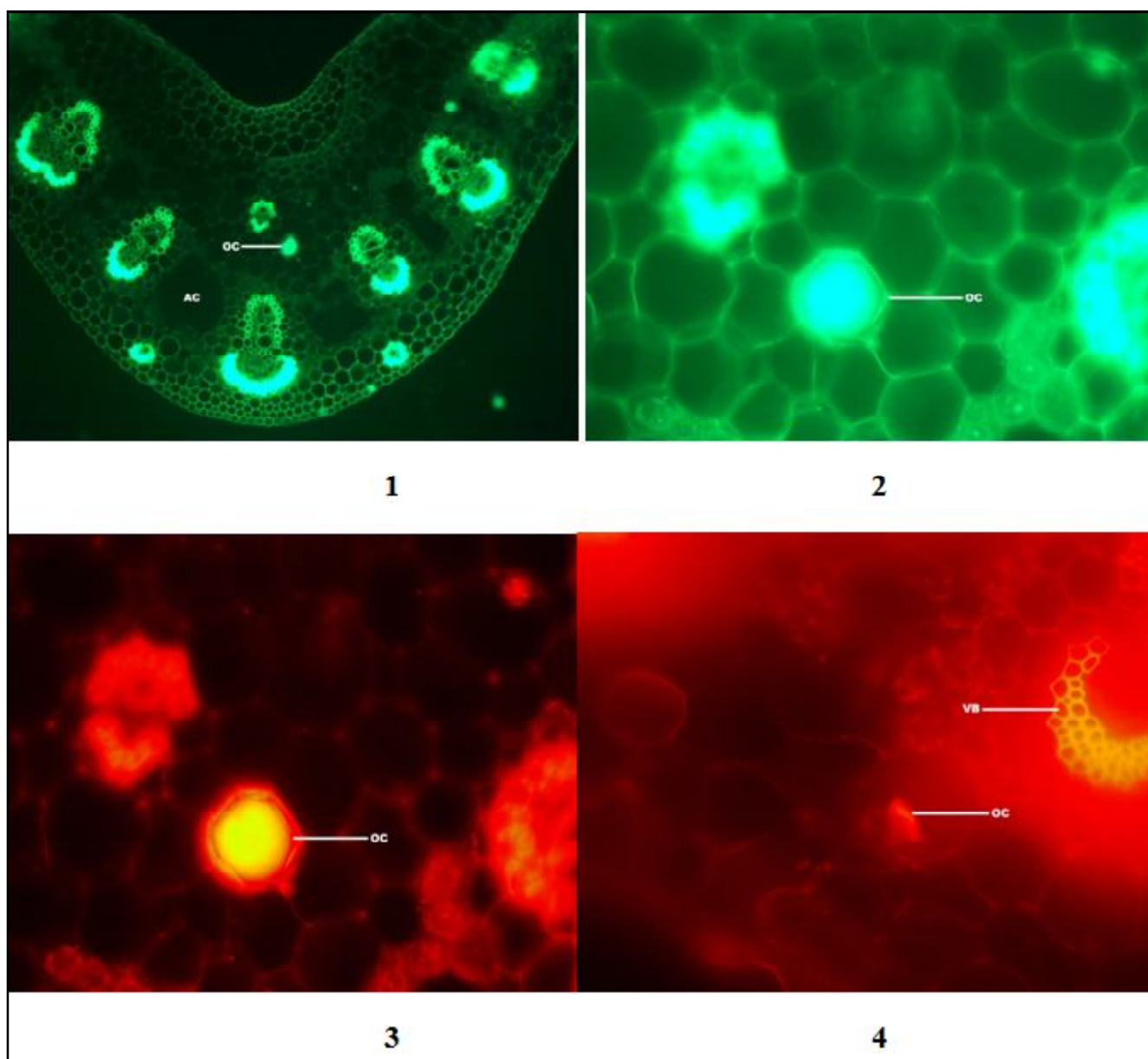
| S. No. | Name of Species | % yield (cc) | Oil cell Diameter (μm) | Oil Cell Index (%) |
|--------|-----------------------------|--------------|------------------------|--------------------|
| 1 | <i>C. longa</i> | 2.2 | 45 ± 2.3 | 18.86 ± 0.42 |
| 2 | <i>C. haritha</i> | 0.5 | 53 ± 0.83 | 3.31 ± 0.46 |
| 3 | <i>C. zedoaria</i> (wild) | 1.3 | 45 ± 2.2 | 10.1 ± 0.58 |
| 4 | <i>C. zedoaria</i> (market) | 0.4 | 33 ± 2.2 | 8.06 ± 0.68 |
| 5 | <i>C. aromatica</i> | 1.8 | 65 ± 1.5 | 11.2 ± 0.50 |
| 6 | <i>C. caesia</i> | 0.5 | 65 ± 2.04 | 4.50 ± 0.32 |
| 7 | <i>C. raktakantha</i> | 0.4 | 49 ± 2.14 | 7.22 ± 0.26 |

of leaf oil cells confirms previous studies restricted to the rhizomes of different species and now this study expands the knowledge on the presence and significance of leaf oil cells in the selected *Curcuma* species. However, no correlation existed among the other parameters.

Qualitative Analysis of High Performance Liquid Chromatography (HPLC) Profiling

A chromatographic fingerprint is practically a chromatographic pattern of some common kinds of pharmacologically active and chemically characteristic components in the plant sample (extract or essential oil). This chromatographic profile should feature the fundamental attributions of “reliability” and “uncertainty”,

in other words, “sameness” and “differences”. With the help of chromatographic fingerprints, the authentication and identification of herbal medicines can be accurately conducted, even if the quantity of the chemically characteristic constituents is not exactly the same among different samples which belong to the same kind of species. The chromatographic fingerprints could reveal both the “similarity” and “dis-similarity” between various samples successfully. In the present work, 6 batches of essential oil samples of *Curcuma* species were analyzed under the optimum chromatographic conditions. Peaks existing in all chromatograms were considered as “common peaks”, indicating the sameness among various samples. The volatile oils from leaves of *curcuma* species

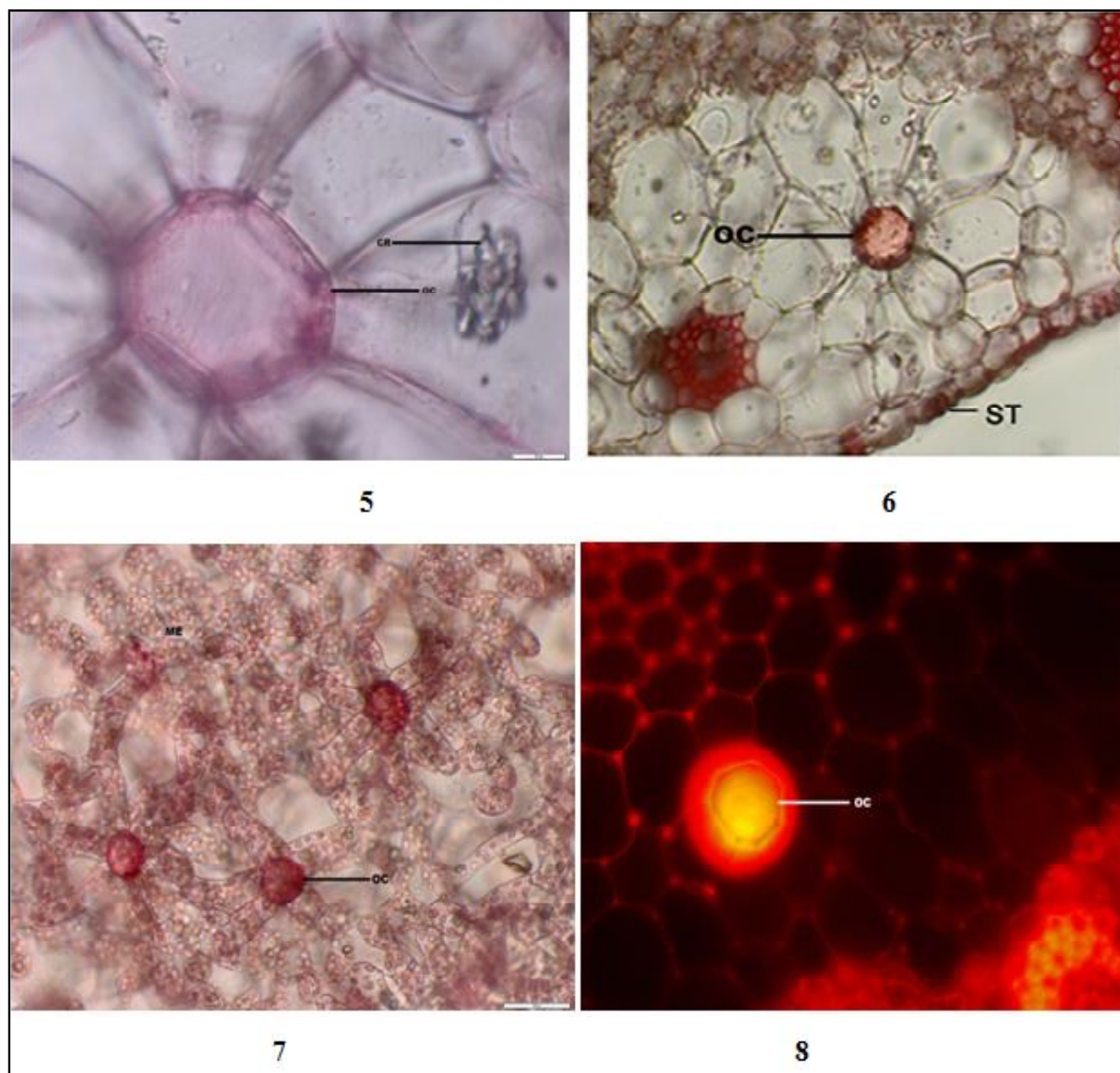


Plates 1-4: T.S. of leaf showing oil cells 1-3: *C. caesia* under fluorescent microscopy. 1&2 DAPI (Ex 340-380); 3&4 Tx red (Ex 540-580). 4. *C. raktanta*. (under fluorescent microscopy) OC, oil cell. AC, air canal.

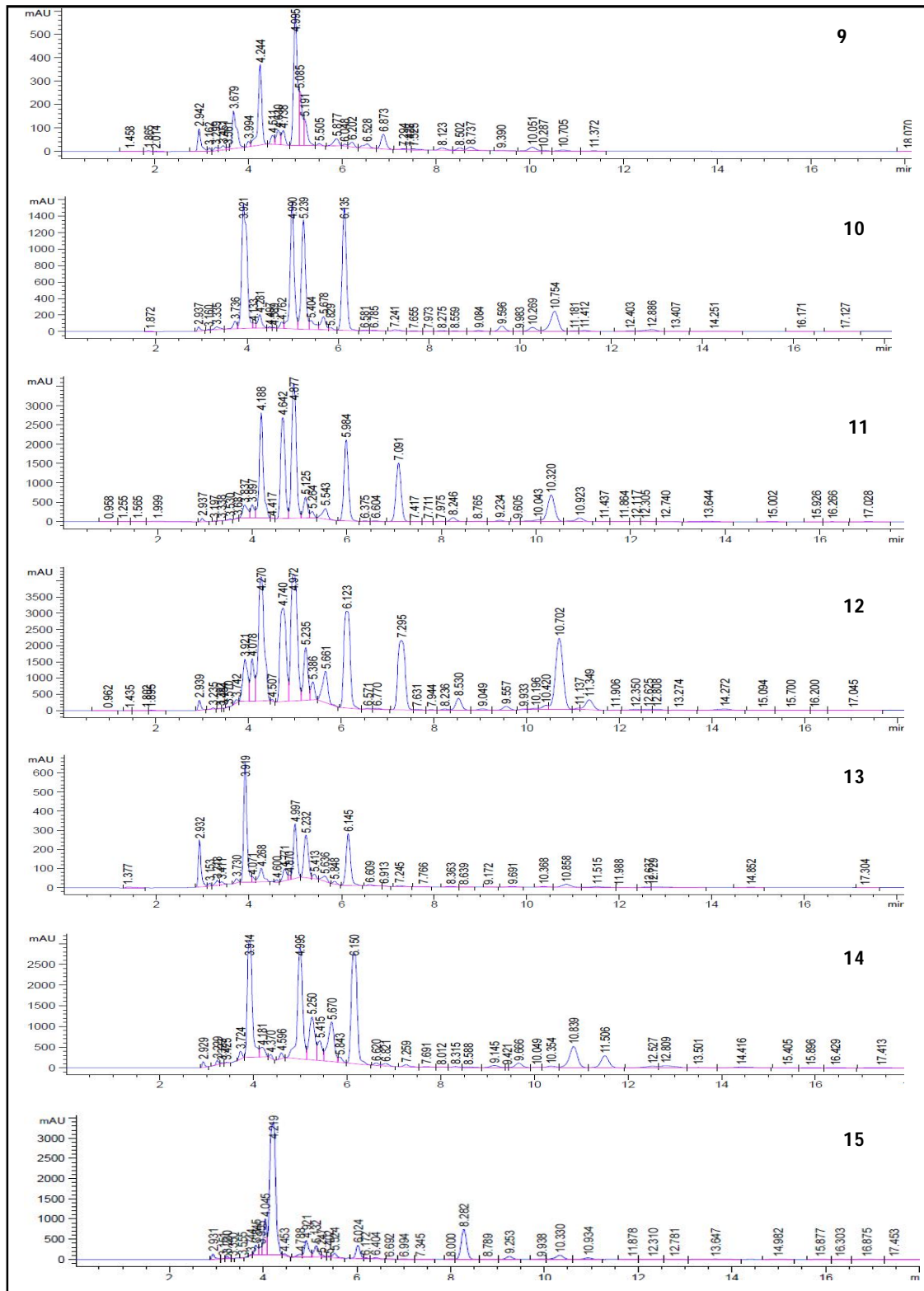
were usually dominated by mono and sesquiterpenes and among the common components (Behura and Srivastava 2004, Garg *et al.*, 2002 and McCarron, *et al.*, 1995). The non-common peaks in each chromatogram, which represents the uncertainty among the same kind of *Curcuma* sp. along with the different content of the same component existing in the samples, were examined. The results from HPLC essential profiles for leaves extract of *C. haritha*, *C. longa*, *C. aeruginosa*, *C. aromatica*, *C. caesia*, *C. zedoaria* and *C. raktantha* showed a number of peaks at the retention time between 0 to 18 minutes (Plate 9-15). The pattern of peak spectrum which

showed the similar pattern is between the highest peak spectrum at 2 to 6 minutes and the moderate peak spectrum at 6 to 18 minutes. The wild variety of *C. zedoaria* (Plate 12) found to have better profile than its market counterpart (Plate 11) in both compound peak abundance and yield (Table 1).

All the samples except *C. caesia* contained many distinct common peaks within 18 min in varying percentage. This shows the significant variation of phytochemical constituents in *C. caesia* compared to other species. Thus, by analysing the HPLC fingerprints, it is possible to distinguish the various bioactive compounds



Plates 5-8: T.S. of leaf showing oil cells in 5: *C. longa*, 6: *C. aromatica*, 7: *C. haritha*, 8: *C. zedoaria*, OC, oil cell; CR, Crystal; ST, Stomata; VB, Vascular Bundle; ME, mesophyll.



Plates 9-15: HPLC Chromatogram: 9: *C. longa*, 10: *C. haritha*, 11: *C. zedoaria* (market sample), 12: *C. zedoaria* (wild), 13: *C. aromatica*, 14: *C. raktantha* and 15: *C. caesia*.

from among the set of chromatographic signals and their variations among the same and different species. In certain cases, where the identification of the correct species may be challenging, the HPLC fingerprints of

the species provide a better tool to distinguish from each other. The utility of HPLC fingerprinting illustrated in this study shows the difference in the phytochemical profiles of selected *Curcuma* species.

Jayasree (2005) reported a comparative anatomy of 15 species of *Curcuma* on the dermal morphology, petiole, midrib, leaf margin and also included venation pattern of bracts for taxonomic purposes. In the present study, which includes the leaf samples of six species of *Curcuma* L., oil cell size was observed smallest in the market sample of *C. zedoaria* and largest in *C. aromatica* and *C. caesia*. The significant variations in the histochemical and HPLC estimation technique in the results shows that it can be used in quantifying secondary metabolites including essential oils in different medicinal plants for chemotyping. Plant diversity studies based on leaf epidermal characteristics under optical microscopy has also shown significant variations among several monocotyledons and dicotyledons and proved valuable in species identification (Cutler, 1982). Histochemical characters are commonly used to deduce taxonomic conclusions (Edeoga and Okoli 1995; Shrelija *et al.*, 1998; Ramasree *et al.*, 2006; Srivastava *et al.*, 2007; Mbagwu *et al.*, 2009). Sherlija *et al.*, (1998), Remashree and Indira (2006) prepared a key for species identification based on the rhizome anatomical features and histochemical localization of oil cells, curcumin cells and starch in selected *Curcuma* species. Moreover, histochemical analysis is an important tool for the botanical identification and standardisation of crude drugs. The differences observed in the present study are of taxonomic importance and can be used in species identification. The results also suggest the need of morphological and anatomical study combined with the chemo-profiling of species which may contribute to the taxonomy of the genus.

In conclusion, we analysed the volatile essential oil profiles of the leaves of six *Curcuma* sp. by HPLC along with histochemical localization and quantification of the oil cells. Volatile profiles yielded from HPLC analysis provide abundant information not only for metabolism-related research, but also for chemotaxonomy. Results showed several compounds in the essential oil content in varying concentration and may be used for qualitative chemotaxonomic investigations to establish relations between the morphological and histochemical characteristics of selected *Curcuma* species.

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