IJPSR (2011), Vol. 2, Issue 11 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 19 July, 2011; received in revised form 13 September, 2011; accepted 27 October, 2011

HISTOCHEMICAL AND PHYTOCHEMICAL MARKERS FOR THE AUTHENTICATION OF AYURVEDIC RAW DRUG HALLAKAM (*KAEMPFERIA ROTUNDA*) AND ITS MARKETED ADULTERANT

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Keywords:
Hallakam,
Kaempferia rotunda,
Lagenandra toxicaria,
TLC,
GCMS

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ABSTRACT

Comparative pharmacognostic and phytochemical studies are the reliable source to identify the genuine raw drug from its adulterants. This paper deals with the characterization of the repute ayurvedic drug *hallakam* from its substitute/adulterants. Ayurvedic experts equated rhizomes of *Kaempferia rotunda* L. of Zingiberaceae as *hallakam* and in certain market samples *Lagenandra toxicaria* Dalz. is also sold as *hallakam*. The distinguishing pharmacognostic and phytochemical characters evolved from the study help to detect the genuine and market adulterant of *hallakam*.

INTRODUCTION: *Hallakam* is mentioned in *Amarakosam* [1] and given several synonyms like *Sauganthika* (aromatic), *hallakam* (which attracts bee), *Raktasandhyakam* (reddish), *Kalhara* (water plant) etc. The drug is stomachic, anti-inflammatory to wounds and bruices, improve complexion, cure burning sensation, mental disorders and insomnia [2]. The rhizome is used for the preparation of many Ayurvedic formulations. From the reported synonyms, *hallakam* is equated with *Kaempferia rotunda* by Nadkarni [2], Chopra *et al* [3].

Moosad, (*Amarakosam*[1]) equated *hallakam* with *chengazhinirkizhangu* (*K. rotunda*) of Kerala physicians. However Rheede [4] equated this with *Malankua* (*Zingiber zerumbet*). Nicolson [5] also equated it with *Zingiber zerumbet*. According to Sivarajan and Balachandran[6], *hallakam* is equated with *K. rotunda* and they reported that in practice *L. toxicaria* of Araceae is also used in Kerala. The controversies do exist in the case of *hallakam* by physicians.

Hence the present author made a thorough classical literature survey to identify the genuine plant. According to Dalhan a famous commentator of *Sushrutasamhita* [7], the synonym *kalhara* is one of the varieties of *utpala* (water lilly). *Kalhara* has been mentioned in another famous lexicon *Bhavaprakashnighantu* [8] written around 16 AD being included in the pushpavargha. The Ayurvedic expert Thakur Balwant Singh in his *Glossary of vegetable drugs* [9] has suggested that *Sougandhika* is considered to be a variety of *Utpala*.

So, *Hallakam* evidently denotes water lilly, even though there has been different opinion on this. Some of the Ayurvedic experts of Kerala have considered '*Chengazhi*' (*K. rotunda*) and another water plant *L. toxicaria* as *Hallakam*. This paper deals with the characterization of the repute ayurvedic drug *Hallakam* from its substitute/adulterants. The distinguishing characters evolved from the study help to detect the genuine and substitute of *Hallakam.*

MATERIALS AND METHODS:

Anatomical studies: Plant materials for the present study were collected from Herb Garden, Arya Vaidya Sala, Kottakkal. The materials for anatomical study were fixed in Formalin: Acetic acid: Alcohol mixture (FAA). Histological and histochemical staining was carried out according to Johansen [10].

Photomicrographs were taken using Canon G3 camera attached to Zeiss microscope. Polarization microscopic studies were highly useful to locate and distinguish the types of crystals and minerals present in the useful parts. The characters were observed under Motic BA 400 polarization microscope. Fluorescent microscopic studies of the useful parts were done with the help of UV light. Observations were done under Leica DM 1000 LED fluorescent microscope and photographs were taken with the help of a digital camera. For examining the cell structure in powder form, material were powdered and sieved and mounted under glycerol and saffranin to study the nature and identification of particles.

Determination of Quantitative Data: Physicochemical parameters such as water soluble extractive, alcohol soluble extractive, percentage of total ash and acid insoluble ash were estimated according to the standard procedures [11].

Phytochemical Studies: The dried root tuber and rhizomes were powdered and 5 g each *K. rotunda* and *L. toxicaria* were kept in 100 ml each of petroleum ether for overnight. The extract was then filtered and the solvent was evaporated under reduced pressure in a rotary evaporator. The residue was then dissolved in 10 ml of petroleum ether and subjected to TLC and GCMS profiling. Essential oils were distilled using Clevenger apparatus to perform GCMS.

GCMS analysis: GCMS is one of the widely used methods for analysis of volatile compounds present in herbal drugs Gas chromatography-mass spectrometry was carried out on an Agilent GC-MS 6850 under electron impact ionization (70 eV). The interface temperature was 230°C, and the MS scan range was 50-800 atomic mass units (AMU). The separation of constituents was done on HP5 - MS capillary column (30 m x 0.25 mm internal diameter). The carrier gas used was helium at a flow rate of 1 ml/min. The oven temperature was 60°C to 250°C with a constant increase of 5°C. The injection was performed in split mode (1:100) at 250°C.

RESULTS AND DISCUSSION:

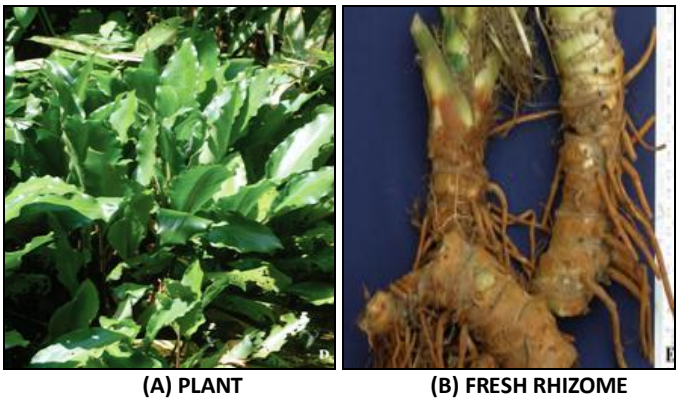
Morphological characters: Morphological characters of these two species are entirely different. In *K. rotunda* rhizome is very fleshy and having 3 - 3.5 cm in length and 1.5 - 1.75 cm in diameter. Each tuber has the shape of club with a bulged lower portion and a stalk like cylindrical upper portion. But in the market, it is obtaining in dried transversely cut pieces (**Fig. 1 A-C**).

(A) PLANT (B) FRESH RHIZOME

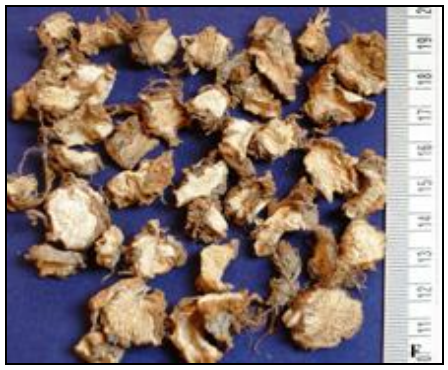
(C) DRIED PIECES OF RHIZOME

FIG. 1 A-C: *KAEMPFERIA ROTUNDA*

In *L. toxicaria* rhizomes are long simple or branched, very thick, 2 - 2.5 cm in diameter, spongy and on drying dark brown in color; nodes and internodes are very prominent and internodes are very short; alternately arranged leaf scars are seen at the nodes (**Fig. 1 D-F**).



(A) PLANT (B) FRESH RHIZOME



(C) DRIED PIECES OF RHIZOME
FIG. 1 D-F: *LAGENANDRA TOXICARA*

From the thorough classical literature survey to identify the genuine *hallakam* using the synonyms, it is aggreable in the case of *K. rotunda* ie., one of the characteristic feature of *hallakam* is *souganthika*. It is applicable only to *K. rotunda* and no aromatic smell is observed in *L. toxicaria.*

Anatomical characters: Preliminary pharmacognostic studies of *K. rotunda*, was carried out by Nambiar *et al* [12]. There are no reports regarding the comparative pharmacognostic characterization of *K. rotunda* and *L. toxicaria*. From the present study both the rhizomes showed significant anatomical characters. Both the rhizomes are almost circular in outline (**Fig. 2 A & F**). Periderm is well developed with broad cork in *K. rotunda* when compared to that of *L. toxicaria* (**Fig. 2 B & G**). Though *K. rotunda* belongs to monocot a correct demarcation between the outer and inner zone by endodermis like layer is present and it is termed as endodermoidal layer.

Rema Shree *et al.,* [13] reported endodermidal layer in *Zingiber* species whereas these layer is not observed in *L. toxicaria*. Nature of vascular bundles shows much difference in these two species. It is typical monocot type in *K. rotunda* and amphivasal type in *L. toxicaria* (**Fig. 2 C & H**). Its number and distribution also shows differences. Difference is observed in the case of nature of crystals *i.e*., large number of raphides of calcium oxalate are observed in *L. toxicaria* but in *K. rotunda*, it is sandy crystal type. Though starch grains and oleoresin cells are present in both the species, difference is seen in their size, shape and number. Oleoresins and starch grains are more in *K. rotunda*. Starch grains oval in shape in *K. rotunda* whereas it is elongated and finger like in *L. toxicaria* (**Fig. 2 D, E, I & J**). Comparative anatomical features of these two plants listed in **Table 1**.

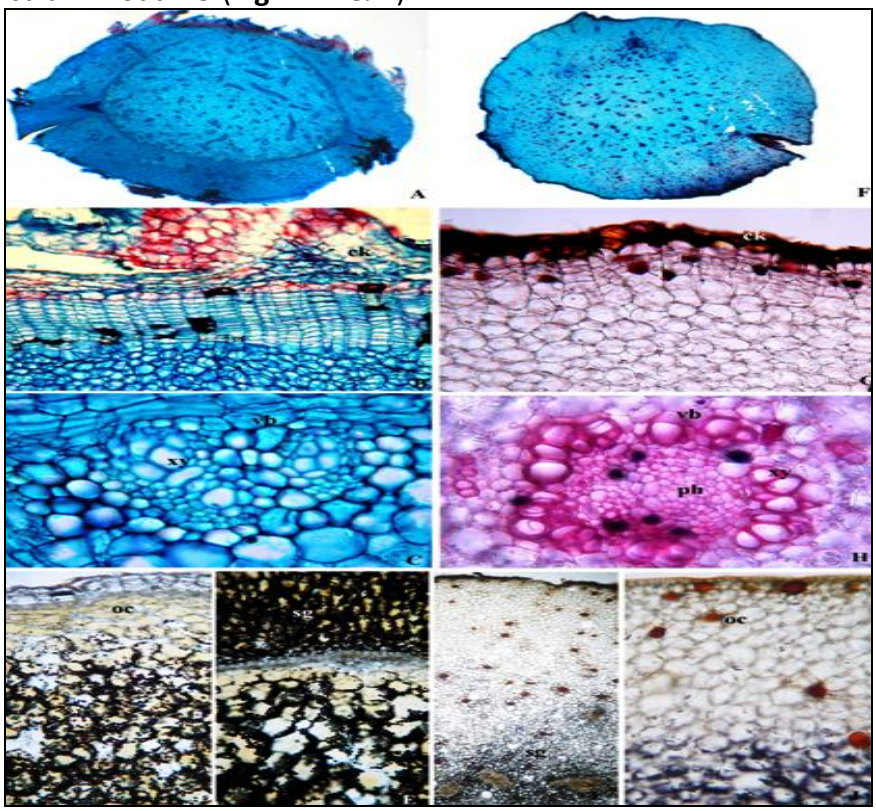


FIG. 2 A-E: MICROSCOPY OF *K. ROTUNDA* RHIZOME. A: CS OF GROUND PLANE X 40. B: CORK REGION ENLARGED X 400. C: VASCULAR BUNDLE ENLARGED X 400. D & E: HSTOCHEMICAL LOCALIZATION OF STARCH & OLEORESIN CELLS X 200& 400. F-J: MICROSCOPY OF *L. TOXICARIA*. F: CS OF GROUND PLANE X 40. G: CORK REGION ENLARGED X 400. H: VASCULAR BUNDLE ENLARGED X 400. I & J: HSTOCHEMICAL STAINING OF STARCH & OLEORESIN CELLS X 200& 400. ck: Cork, vb: Vascular bundle, ph: Phloem, sg: Starch grain, oc: Oleoresin cells, xy: Xylem

TABLE 1: COMPARATIVE ANATOMICAL CHARACTERS

Characters	*Kaempferia rotunda*	*Lagenandra toxicaria*
Shape of rhizome in CS	CS is nearly circular in outline (Fig. 2. A).	CS is circular in outline (Fig. 2. F).
Nature of cork	Broad cork, 8-10 layered; cells thin walled, rectangular and slightly tangentially elongated (Fig. 2. B).	Cork consists of 2-3 layered; cells thin-walled, radially elongated with dark contents (Fig. 2. G)
Nature of phellogen	3-4 layered	1-2 layered
Nature of outer cortical zone	Outer cortical zone is very broad, and composed of oval to thin walled cells with inter cellular spaces.	Ground tissue composed of oval or round thin-walled parenchyma with intercellular spaces.
Nature of endodermis/endodermoidal layer	Endodermoidal layer is conspicuous, single layered with tangentially elongated cells.	No endodermis/endodermoidal layer.
Nature of vascular bundle	Typical monocot bundle with 2-3 small vessels and phloem tissues. Vascular bundles are scattered in the cortex and in the ground tissue inner to the endodermoidal layer. Small bundles are embedded in the endodermoidal layer (Fig.2. C).	Vascular bundles are amphivasal in nature with central phloem tissue is surrounded by xylem elements. Vascular bundles are smaller towards the periphery and larger towards the centre (Fig. 2. H).
Nature of xylem	Xylem consists of tracheids, xylem parenchyma and fibers. Tracheids with spiral and reticulate thickenings are seen.	Xylem consists of tracheids, xylem parenchyma and fibers. Tracheids with spiral and reticulate thickenings are seen.
Nature of inclusions	A few number of sandy crystal containing cells are present. Oleoresin containing cells are scattered throughout the section. Tannin content absent (Fig. 2. D & E).	Large number of bundles of raphide crystals containing cells is present in the ground tissue. Large numbers of reddish brown deposits are seen scattered throughout the ground tissue. Tannin content absent (Fig. 2. I & J).
Nature of starch grains	All the cells in the ground tissue are fully filled with starch grains. Starch grains are large and oval in shape (Fig. 4. E).	Elongated and finger like starch grains are seen in the cells towards the centre leaving a few layers empty towards the periphery (Fig. 4. K).

Polarization microscopy: Polarization microscopic studies revealed the presence, position and shape of sandy crystals and lignified cells. In *K. rotunda* presence of sandy crystals observed in cork, outer zone and inner zone and lignified layer under endodermal layer; show polarization (**Fig. 3 A**), whereas in *L. toxicaria* raphide crystals of calcium oxalate in the ground tissue and tracheids with lignified walls also showed polarization (**Fig. 3 D**).

Fluorescent microscopy: Fluorescent microscopic studies showed auto fluorescent with yellow color. In *K. rotunda* sandy crystals in the outer cortical region, endodermoidal layer were showed yellow fluorescence (**Fig. 3 B & C**), whereas in *L. toxicaria* outer cork layer and the xylem tracheids showed yellow fluorescence (**Fig. 3 E & F**).

Powder microscopy: In *K. rotunda* powder microscopy shows trachieds with spiral and reticulate thickening and large and oval shaped starch grains, groups of longitudinally cut xylem parenchyma and oleoresin containing cells and sandy crystals (**Fig. 4 A-F**). Whereas in *L. toxicaria* powder shows tracheids with spiral and reticulate thickenings, bundle of raphide crystals and scattered needles of calcium oxalate crystals, fragments of parenchyma with finger like starch grains and masses of reddish brown oleoresin depositions (**Fig. 4. G-L**).

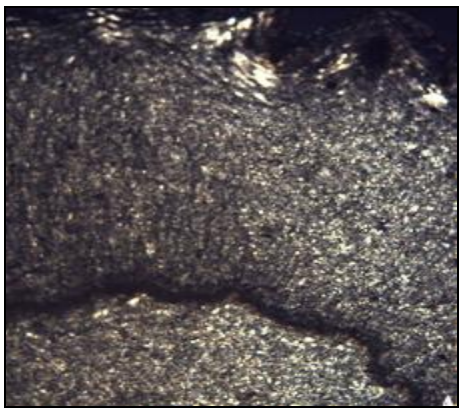


FIG. 3 A: POLARIZATION MICROSCOPY OF *K. ROTUNDA RHIZOME* X 40

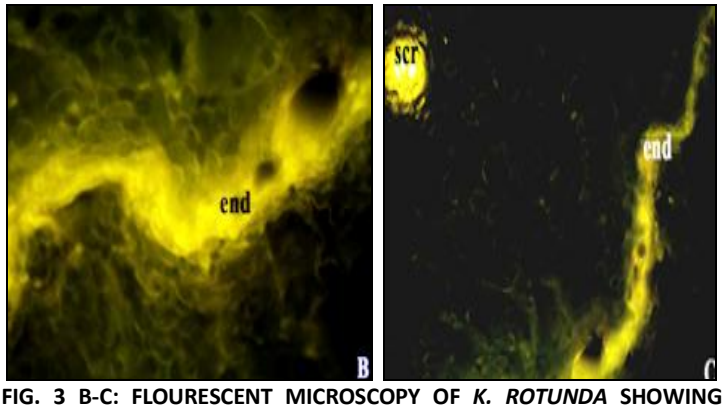


FIG. 3 B-C: FLOURESCENT MICROSCOPY OF *K. ROTUNDA* SHOWING ENDODERMOIDAL LAYER AND SANDY CRYTAL X 400. ct: Cortex, end: Endodermoidal layer, sc: Sandy crystal

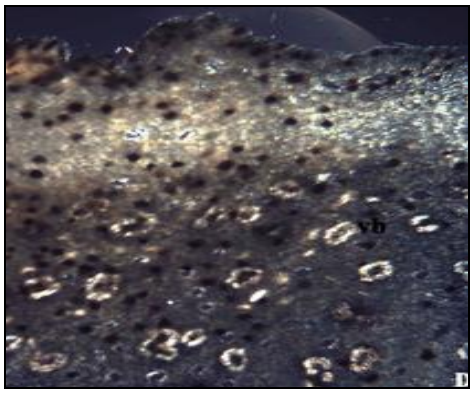


FIG. 3 E: POLARIZATION MICROSCOPY OF *L. TOXICARIA* RHIZOME x 40

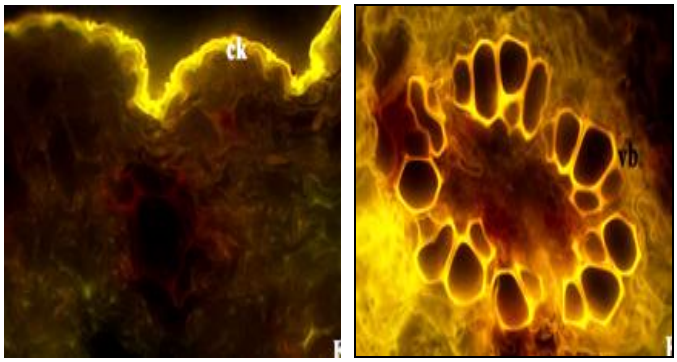


FIG. 3 E-F: FLOURESCENT MICROSCOPY OF *L. TOXICARIA* SHOWING CORK AND VASCULASR BUNDLE x400. ck: cork, vb, vascular bundle

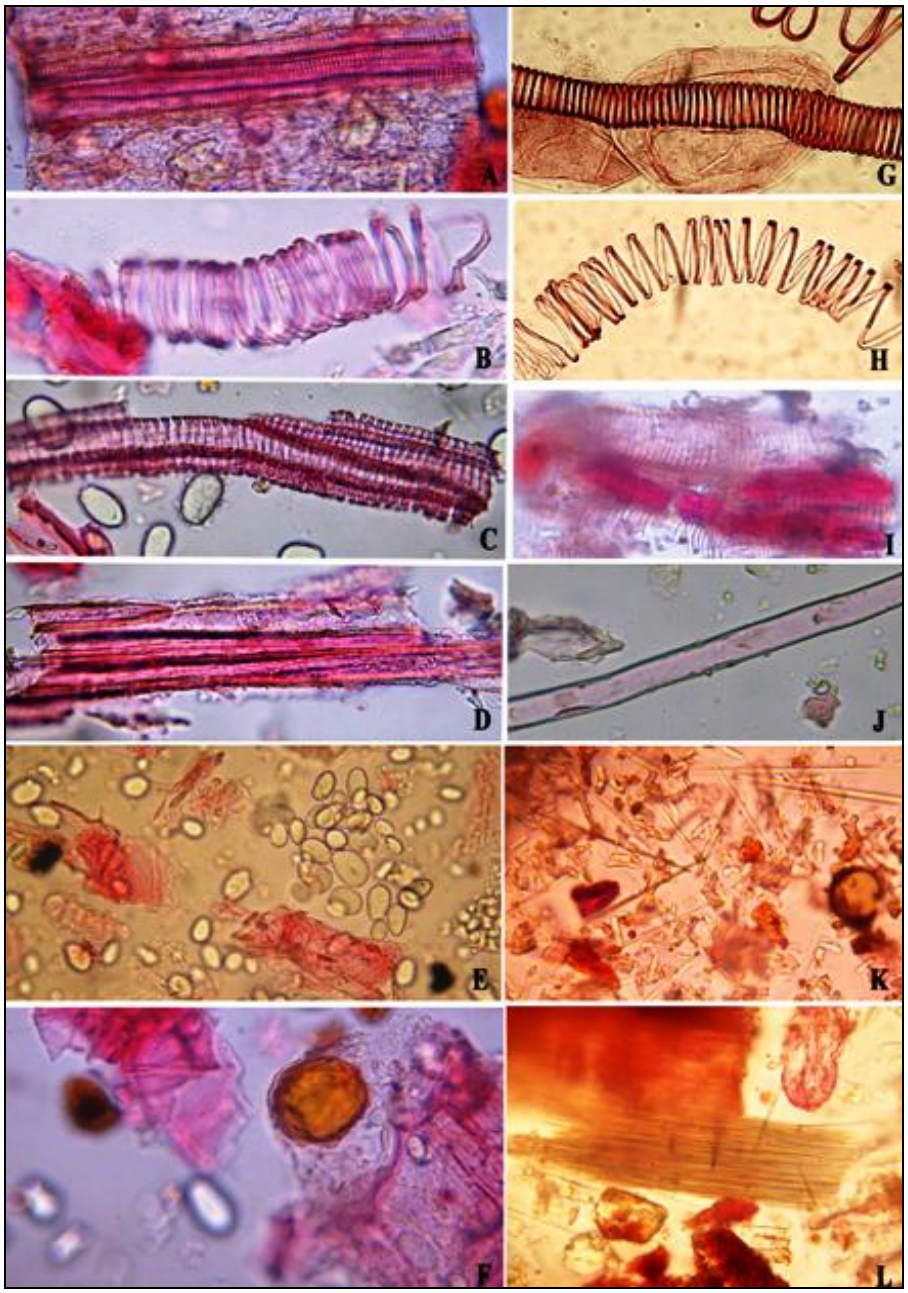


FIG. 4 A-F: POWDER MICROSCOPY OF *K. ROTUNDA* RHIZOME. A-C: GROUPS OF TRACHIEDS WITH SPIRAL THICKENING X 40. D: FRAGMENTS OF LONGITUDINALLY CUT FIBERS WITH TRACHIEDS X 400. E: STARCH GRAINS X 600. F: OLEORESIN CONTAINING CELLS X 400.

FIG. 4 G-L: POWDER MICROSCOPY OF *L. TOXICARIA* RHIZOME. G-I: GROUPS OF TRACHIEDS WITH SPIRAL THICKENING X 40. J: FRAGMENTS OF LONGITUDINALLY CUT FIBERS WITH TRACHIEDS X 400. K: STARCH GRAINS AND NEEDLES OF RAPHIDE CRYTSALS X 600. L: BUNDLES OF RAPHIDE CRYSTALS OF CALCIUM OXALATE X 600.

Chemical studies: Phytochemical studies of *K. rotunda* has been attributed to contain flavonoids, crotepoxide, chalcones, quercetin, flavonols, *β*- sitosterol, stigmasterol, syringic acid, protocatechuic acid and some hydrocarbons [14]. Woerdenbag *et al.,* [15] reported the presence of volatile constituents in *K. rotunda* like benzyl benzoate (69.7%), n-pentadecane (22.9%) and camphene (9.1%). Methanol extract of the rhizome oil of *L. toxicaria* was subjected to GCMS analysis and the chemical constituents identified as Methyl ester of 2-hydroxy benzoic acid, Diethyl phathalate, Oleic acid, Palmitic acid ethyl ester and Dioctyl phthalate. Diethyl phathalate was found to be the major constituent (89.461%) [16].

In the present study comparing the physicochemical parameters like Moisture content, Water soluble extractive, Alcohol soluble extractive, Ash value and Acid insoluble ash, except the acid insoluble ash all other characters are higher in *L. toxicaria* than in *K. rotunda* (**Table 2 & Fig. 5**).

TABLE 2: PHYSICOCHEMICAL PARAMETRES (%)

Physicochemical characters	*K. rotunda*	*L. toxicaria*
Moisture content	10.413	13.666
Water soluble extractive	27.218	34.625
Alcohol soluble extractive	7.788	19.7843
Ash value	5.261	7.4212
Acid insoluble ash	0.0053	0.0051

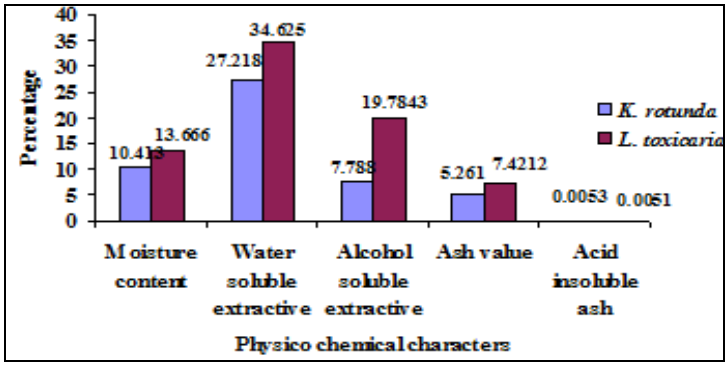


FIG. 5: PERCENTAGE OF PHYSICOCHEMICAL CHARACTERS OF *K. ROTUNDA* AND *L. TOXICARIA*

From the GCMS analysis carried out by the author using petroleum ether extracts by cold maceration of dried and powdered samples of *K. rotunda* and *L. toxicaria* showed entirely different compounds in their essential oil. The major compounds identified in K. rotunda are n-dodecane, hexadecane, stearaldehyde, dodecanoic acid, kauren-ol whereas in *L. toxicaria*, we were able to identify a single compound 3-eicosyne from their GCMS analysis (**Table 3 & Fig. 6 & 7**). Essential oils were distilled using Clevenger apparatus to perform GCMS, and found that *K. rotunda* having fairly good amount of oil. But in the case of *L. toxicaria* the quantity was very less to perform GCMS analysis. Hence, GCMS performed only in volatile oil of *K. rotunda*. In the present study, we could able to quantify and identify 13 compounds from the essential oil of fresh sample of *K. rotunda* using GCMS. Among the compounds bornyl acetate and benzyl benzoate was found to be major constituents in the oil (**Table 4 & Fig. 8**).

TABLE 3: COMPOUNDS IDENTIFIED USING MASS SPECTROSCOPIC STUDIES

Plant names	Retention Time	% of compounds	Name of compounds
K. rotunda	13.719	33.1	n-dodecane
	17.712	6.32	hexadecane
	20.224	37.9	stearaldehyde
	22.624	9.48	dodecanoic acid
	25.419	12.6	kauren-ol
L. toxicaria	20.252	68.5	3-eicosyne

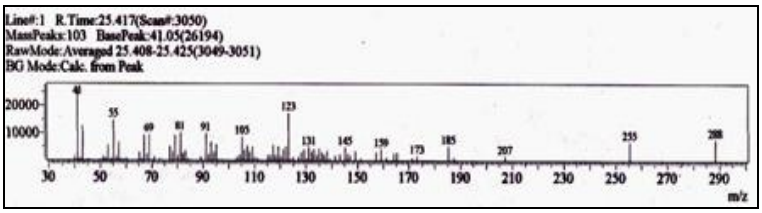


FIG. 6: FULL SCAN GCMS SPECTRUM OF *K. ROTUNDA*

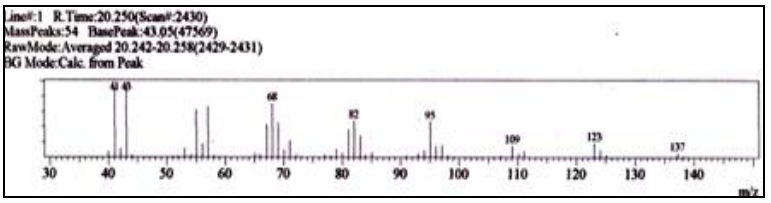


FIG. 6: FULL SCAN GCMS SPECTRUM OF *L. TOXICARIA*

TABLE 4: COMPOUNDS IDENTIFIED IN *K. ROTUNDA* USING MASS SPECTROSCOPIC STUDIES

Retention Time	% of total	Name of compounds
3.975	1.349	α-Pinene
4.172	7.539	Camphene
5.159	1.133	*β*-Pinene
5.224	4.255	Cineole
6.079	2.599	Linalool
6.819	7.180	Camphor
7.099	5.929	Borneol
8.743	30.121	Bornyl acetate
10.585	3.047	Caryophyllene
10.881	1.308	Aromadendrene
11.374	2.177	n- tetra decane
12.624	0.944	Caryophyllene oxide
14.581	16.595	Benzyl benzoate

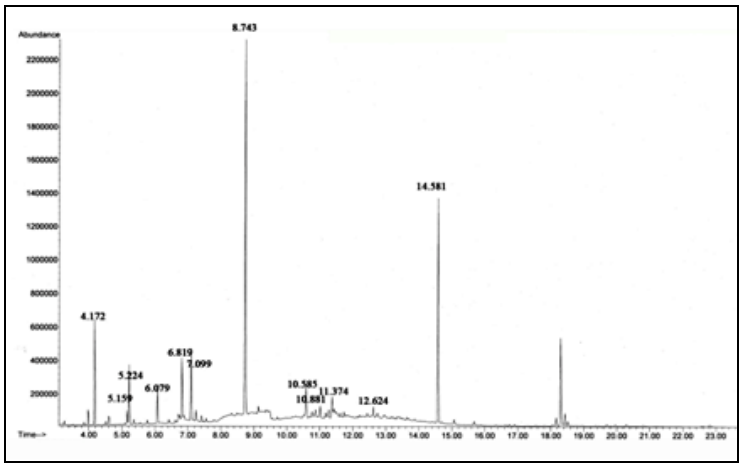


FIG. 8: GCMS PROFILE OF ESSENTIAL OIL OF *K. ROTUNDA*

From the available literature and present study shows that *L. toxicaria* don't have any similar therapeutic activity mentioned for *hallakam* in classical texts and moreover it is reported to be a poisonous plant [17], though it is having similarities with the synonym i.e., Water lilly (*kalhara*) and *hallakam* (powerful odour which attracts bee) etc, it is not the genuine source plant of *hallakam*.

CONCLUSION: From the present study the anatomical and histochemical characters observed between these two plants shows differences in their nature of cells and cell inclusions. Phytochemical studies between these two plants shows much difference in their chemical constituents. So, it is a very helpful tool to distinguish the genuine dried raw drug from the adulterants and it is concluded that *L. toxicaria* is a clear case of adulterant.

ACKNOWLEDGEMENT: The authors are grateful to Kerala State Council for Science Technology & Environment (KSCSTE), Govt. of Kerala and Arya Vaidya Sala, Kottakkal for providing support to carry out this work. We are thankful to Dr. Indira Balachandran, Project Director, CMPR, Arya Vaidya Sala, Kottakkal for the necessary guidance.

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