Indian Journal of Traditional Knowledge
Vol. 13 (3), July 2014, pp. 535-542

Standardization of an Ayurvedic Formulation- *Kalyanavleha* and estimantion of Curcumin using HPTLC

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Received 24.06.13, revised 31.03.14

Standardization of a compound ayurvedic formulation is essential for establishing the authenticity, quality and efficacy of Ayurvedic medicines/finished herbal product. *Kalyanavleha* is an polyherbal ayurvedic formulation used in aphasia and hoarseness containing eight medicinal plants, viz. *Curcuma longa* L., *Cyclospermum leptophyllum* (Pers.) Sprague, *Zingiber officinale* Roscoe, *Cuminum cyminum* L., *Glycyrrhiza glabra* L., *Saussurea costus* (Falc.) Lipsch., *Acorus calamus* L., *Piper longum* L. To achieve the desired aim three batches of *Kalyanavleha* were prepared in the lab as per standard protocol of Ayurvedic Formulary and were investigated by microscopy, physicochemical parameters and high performance thin layer chromatography (HPTLC). Curcumin was evaluated qualitatively and quantitatively as the biomarker of *Kalyanavleha* and it was estimated 0.25-0.26 % in the formulation.

Keywords: Curcumin, HPTLC, *Kalyanavleha*, Microscopy, Polyherbal formulation, Standardization

IPC Int. Cl.[8]: A36K 36/00, A61P 15/00, A61P 15/02, A61P 31/00, A61P 31/04

Ayurveda is the oldest healing system of medicine. The drug formulation in Ayurveda is based on two principles: (i) used as single drug and (ii) use of more than two drugs. When two or more herbs are used in a formulation, it is known as polyherbal formulation. Different forms of ayurvedic formulations are decoctions, infusions, tinctures, solid dosage forms (pills, powders), liquid dosage forms (*asavas, aristhas*) and semisolid dosage forms (*ghritas, avlehas*)[1]. *Avlehas* are polyherbal medicinal preparations made by addition of jaggery, sugar or sugar candy and boiled with prescribed juices or decoction. These preparations generally have ghee, drug powder and honey. India's ancient system of plant based medicine, Ayurveda is gaining recognition throughout the world and many ayurvedic drugs are now clinically tested and accepted for manufacture[2,3]. The World Health Organization has also considered phytotherapy in its health programs and suggested basic guidelines and procedures for the validation of drugs from plant origin both for developed western countries and developing countries like India and China[2]. Despite, various efforts by WHO[4], there is lack of supporting studies regarding the scientific evaluation of formulation and preparation related parameters. As most of the tests described in ancient literature appear to be based on observation and seem subjective without valid scientific backing. Since, polyherbal formulations are difficult to standardize due to more than one markers coming from different plant species. Hence, standardization and development of reliable quality protocols for polyherbal ayurvedic formulations using modern techniques of analysis is extremely important[5].

Kalyanavleha (KA), a compound preparation, composed of eight Indian medicinal herbs (*Curcuma longa* L., *Cyclospermum leptophyllum* (Pers.) Sprague, *Zingiber officinale* Roscoe, *Cuminum cyminum* L., *Glycyrrhiza glabra* L., *Saussurea costus* (Falc.) Lipsch., *Acorus calamus* L., *Piper longum* L.) commonly used for treating rheumatic and arthritic diseases and also used clinically for the treatment of *Svarbheda* and *Mukata*[1]. The Pharmacopoeial Standards for Ayurvedic Formulations published by the Government of India, Ministry of Health and Family Welfare give certain physical parameters as standards for *KA*[6]. However, these are not sufficient and there is a need to develop other additional methods for quality control based on

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modern analytical methods. Efforts are being made by our group to develop analytical protocols both for single herbal drugs[7-11] as well as for compound herbal formulations[12-15] that can be used as valuable analytical tools in the routine standardization of Ayurvedic drugs and formulations.

In this background, we, herein report identification and quantification of bioactive marker curcumin in the polyherbal formulation KA, containing *Curcuma longa* as a chief ingredient. Curcumin is the principal curcuminoid of the popular Indian spice turmeric- *Curcuma longa*, which is a member of the ginger family Zingiberaceae. The curcuminoids are polyphenols and are responsible for the yellow colour of turmeric. It has been used to relieve pain and inflammation since ancient times in traditional medicine. Studies have shown that curcumin is not toxic to humans. Curcumin exerts anti-inflammatory activity by inhibition of a number of different molecules that play an important role in inflammation[16-18]. It is reported as anticancer against colon[19-20], breast[21-23], and prostate[24-25] cancers, as well as melanoma[26]. Curcumin appears to block synthesis of certain prostaglandins[27]. Extensive researches have revealed Curcumin as antioxidant[28-30], hepatoprotective[31-35], used for cardiovascular protection and lipid lowering[36-37], gastrointestinal disorders[38] and chronic anterior uveitis- treatment[39].

Therefore to achieve the desired aim, the objectives were: (i) to prepare KA according to Ayurvedic formulary of India (AFI)[6], (ii) to develop the physicochemical standards of KA, (iii) to develop fingerprint profiles of KA for identification using HPTLC and (iv) to identify and quantify curcumin in KA methanolic extracts by measuring λ_{max} using high-performance thin layer chromatography (HPTLC).

Methodology

Chemicals and reagents

All solvents of analytical grade and pre-coated HPTLC plates with 0.2 mm nano silica and fluorescent indicator were purchased from SD fine Chemicals, Mumbai. Whatman No.1 filter paper was used for filtration of the samples. Curcumin standard was procured from CDH Laboratory Reagents, Mumbai.

Preparation of formulation

Three batches of KA were prepared according to Ayurvedic Formulary. Properly authenticated eight plant materials, viz. *Curcuma longa* L., *Cyclospermum leptophyllum* (Pers.) Sprague, *Zingiber officinale* Roscoe, *Cuminum cyminum* L., *Glycyrrhiza glabra* L., *Saussurea costus* (Falc.) Lipsch., *Acorus calamus* L., *Piper longum* L. and rock salt were dried, powdered separately and passed through 85 sieve mesh. All the ingredients were mixed thoroughly in equal quantity and *Sarpi* (*Goghṛta*) was added at ~ 50^0 in the mixture by rigorously stirring to form *Avaleha*[6].

Standardization using physicochemical parameters

The samples of KA were analyzed for various physicochemical parameters such as *p*H, total ash, acid insoluble ash, ethanol soluble extractives and water soluble extractives according to the methods given in the API[6].

Sample preparation for microscopic evaluation

For microscopic analysis a small quantity of *avleha* was washed with hexane to remove the *Sarpi* and then with hot water, dried, clarified with chloral hydrate and mounted in 50% glycerol to observe the microscopic identification markers[6]. To examine the starch grains a small quantity of *avaleha* was also washed thoroughly with water, dried, stained with iodine and mounted in 50% glycerol.

Sample preparation for TLC finger printing

For TLC each sample of KA (5 gm) was extracted successively with n-hexane (50 ml x 3) and chloroform (50 mL × 3) by continuous stirring with a magnetic stirrer for 30 min at 40°C. The extracts were pooled, filtered through Whatman No. 1 filter paper, concentrated at reduced temperature (below 50°C) by rotary evaporation (Büchi, USA) to yield the respective samples. Each sample (10 mg) was re-dissolved in 1mL solvent to give test solutions (10 mg mL^{-1}) for TLC analysis.

TLC fingerprinting

TLC fingerprinting of all the extracts were carried out using precoated silica gel 60 F_{254} plates (E. Merck) as stationary phase and toluene : ethyl acetate (8:2) and toluene: ethyl acetate: methanol (9: 1: 1) as mobile phase for hexane soluble and chloroform soluble fractions respectively. Spots were observed under UV and visible light after spraying with anisaldehyde sulphuric acid followed by heating at 105°C for 5-10 min.

HPTLC analysis

Accurately weighed 10 mg chloroform fraction of KA was dissolved in 1mL of methanol, and filtered through a 0.45µm filter membrane; the filtrate was used as sample solution. A standard solution of

curcumin (0.05 mg/ mL) was prepared in methanol. Different volumes of standard solution, 4, 8, 12, 16, 20, µL were applied onto silica gel 60 F_{254} HPTLC plates using a Linomat 5 Automatic Sample Applicator to obtain concentrations of 200, 400, 600, 800, 1000 ng per spot of curcumin, respectively. The plates were developed in a CAMAG glass twin trough chamber up to a distance of 8.0 cm using toulene: ethylacetate (7:3 v/v) as mobile phase. After development, the plates were dried in air. The documentation was done by Camag Reprostar 3 video documentation unit under UV 366nm. The densitometry was performed in Camag TLC Scanner III with Wincats 3.2.1 software in absorbance mode at 400 nm.

Results and discussion

The diagnostic cellular structures and cell contents for all powdered ingredients in the polyherbal formulation were described with appropriate diagrams (Figs.1A-H)

Curcuma longa: Yellow coloured parenchymatous cells of different shapes and sizes, either solitary or in groups; filled with curcumin and starch grains; greenish yellow fluorescent, thick walled oil cells; pitted stone cells with narrow boundary and large lumen; some unicellular, non-grandular hairs (Fig. 1A).

Cuminum cyminum: Groups of yellow coloured, suberized, angular parenchymatous cells, patches of pitted parenchyma with beaded cell walls, pits simple, patches of thick walled, angular cells filled with very small simple and compound, starch grains, multicellular, multiseriate trichomes, fragments of vittae (Fig. 1B).

Cyclospermum leptophyllum: Patches of thick walled angular or slightly wavy parenchyma, pitted parenchyma, parenchymatous cells with reticulate thickenings, oil cells, unicellular, simple and glandular trichomes and fragments of vittae showing large polygonal epitheial cells (Fig. 1C)**.**

Zingiber officinale: Groups of suberized cork cells, parenchymatous cells, densely packed with starch grains, isolated starch grains, simple, oval to rod shaped, measuring 15 to 70 µ in length, hilum eccentric, lamellae distinct; yellow coloured oleo resin cells, non-lignified, simple trichome, sepatate fibres some of them bearing marks of adjacent cells pressing against them, 30 to 50 µ broad (Fig. 1D).

Piper longum: Groups of large perisperm cells packed with minute starch grains, stone cells measuring 130 to 190 µ in dia. with broad lumen in groups of 2 to 8 (Fig. 1E).

Saussurea costus: Groups of polygonal and elongated parenchymatous cells; orange or brownish resin cells; branched tracheids; inulin crystals (Fig. 1F).

Acorus calamus : Groups of large parenchymatous cells with intercellular spaces and filled with spheroidal starch grains which are mostly single, rarely in 2 or 3 groups, 2 or 10 µ in diam.; oil cells with suberized walls (Fig. 1G).

Glycyrrhiza glabra: Crystal fibres and pitted vessels showing honey comb structure (Fig. 1H).

The results of the microscopical examination indicated that the characteristics of all eight herbal ingredients viz. *Curcuma longa*, *Cyclospermum leptophyllum*, *Zingiber officinale*, *Cuminum cyminum*, *Glycyrrhiza glabra*, *Saussurea costus*, *Acorus calamus* and *Piper longum* were present in all three batches of KA.

Standardization of KA was also carried out based on the physicochemical evaluation and results are represented in histograms (Fig. 2). The *p*H of 1% KA was 5.2 to 5.5 and moisture content ranges 4.10- 4.65. The alcohol, water and hexane soluble extracts were found 46.67-50.00, 11.25- 15.95 and 50.12-53.50 %, respectively. Likewise, total ash, acid insoluble ash and sulphated ash were quantified 9.78-11.56, 1.69-1.84 and 14.1-14.9 %, respectively.

TLC fingerprint profiles of hexane and chloroform fractions of three batches of KA were developed along with the ingredients *Curcuma longa*, *Cyclospermum leptophyllum*, *Zingiber officinale*, *Cuminum cyminum*, *Glycyrrhiza glabra*, *Saussurea costus*, *Acorus calamus* and *Piper longum* and are presented in Figs. 3 & 4. Hexane fraction of all three batches showed six blue bands at Rf 0.14, 0.28, 0.36, 0.46, 0.56 and 0.64 under UV light at 366 nm (Fig. 3, Table 1). These bands were present in more than one ingredient i.e. at Rf 0.14 in all eight plants; at Rf 0.28 in *Curcuma longa* and *Acorus calamus* and *Cyclospermum leptophyllum;* at Rf 0.36 in *Acorus calamus, Curcuma longa*; at Rf 0.46 in *Acorus calamus, Curcuma longa, Cyclospermum leptophyllum, Glycyrrhiza glabra* and *Piper longum;* at Rf 0.56 in *Curcuma longa, Cuminum cyminum, Cyclospermum leptophyllum, Saussurea costus,* and *Zingiber officinale;* at Rf 0.64 in *Acorus calamus, Cyclospermum leptophyllum, Glycyrrhiza glabra, Saussurea costus* and *Zingiber officinale* under UV 366nm. However, the chloroform fraction of all three batches showed ten bands under UV 366 nm (Fig. 4, Table 2). After comparing the

profiles with the ingredients five yellow colour bands at Rf 0.08, 0.24, 0.29, 0.37 and 0.41 resembled to *Curcuma longa,* blue bands at Rf 0.10, 0.50, resembled to *Glycyrrhiza glabra;* blue bands at Rf 0.60 and 0.68 represented the *Cyclospermum leptophyllum* under UV 366 nm.

After preliminary studies of the hexane and chloroform fractions *Curcuma longa* was found to be the most prominent ingredient of KA for its quality evaluation using modern analysis technique, i.e. HPTLC. Hence, it was decided to quantify curcumin in three batches of KA as curcumin is

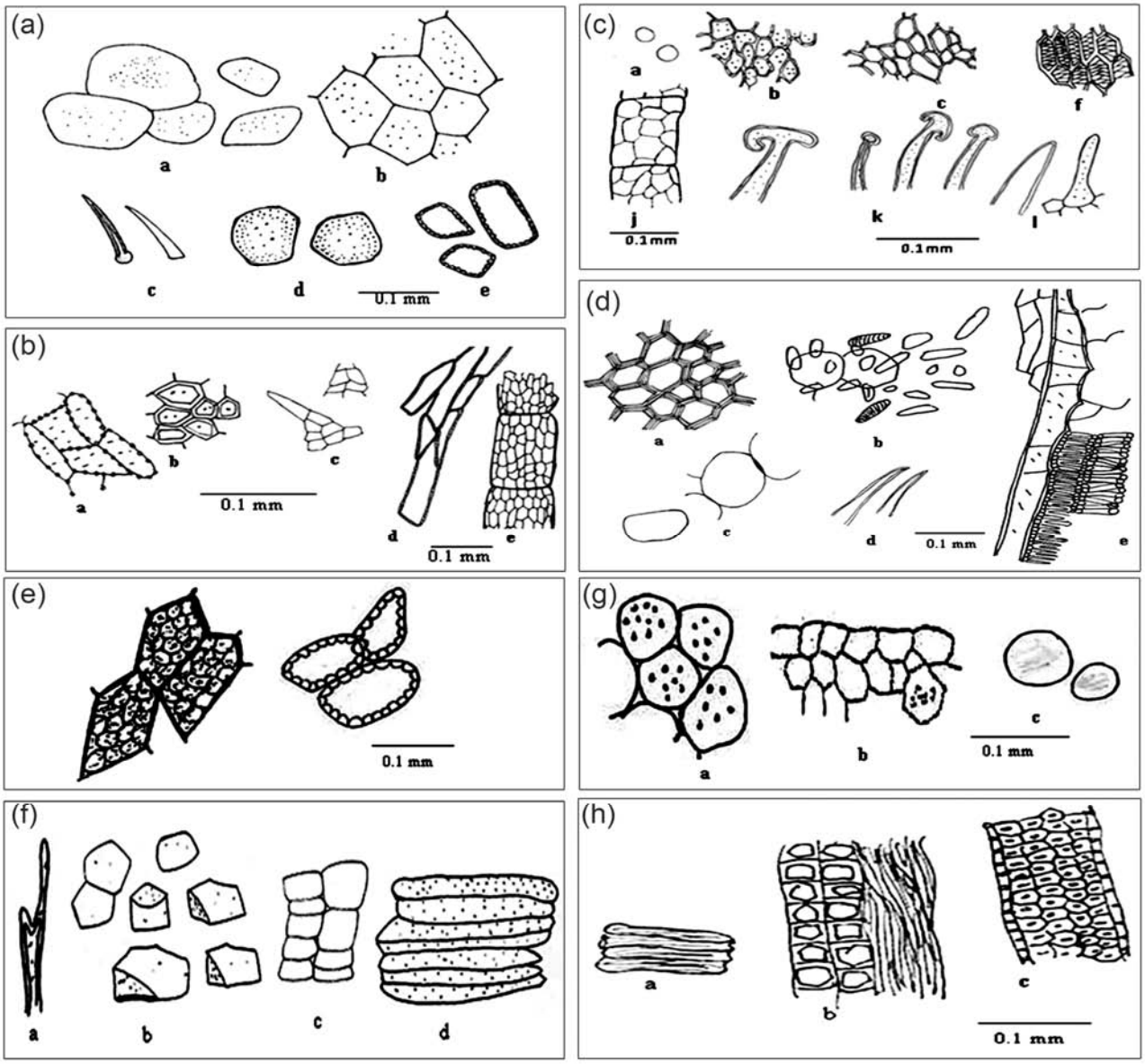

Fig. 1A—Microscopic characters of *Curcuma longa,* Abbreviations: a-b, Parenchymatous cells with starch grains and curcumin content; c, hair; d, oil cells; e, stone; Fig. 1B—Microscopic characters of *Cuminum cyminum* , Abbreviations: a, pitted cells; b, thick walled cells; c, multicellular appendages; d, stone cells; e, vittae in surface; Fig. 1C—Microscopic characters of *Cyclospermum leptophyllum,* Abbreviations**:** a, oil cells; b, pitted parenchyma; c, thick walled cells; d, reticulate parenchyma; e, vittae; f, glandular trichomes; g, simple trichomes; Fig. 1D—Microscopic characters of *Zingiber officinale,* Abbreviations: a, suberized cells; b, parenchyma and starch grains; c, oleo-resin cells; d, simple trichome; septate fibre with adjacent cells and vessels; Fig. 1E—Microscopic characters of *Piper longum,* Abbreviations: a, perisperm cells; b, stone cells; Fig. 1F—Microscopic characters of *Saussurea costus,* Abbreviations: a, branched tracheid; b, inulin crystals; c, resin cells; d, elongated parenchymatous cells; Fig. 1G—Microscopic characters of *Acorus calamus*, Abbreviations: a, parenchyma with starch grains; b, parenchyma with oil cells; c, oil cells; Fig. 1H—Microscopic characters of *Glycyrrhiza glabra*, Abbreviations: a, sclerenchymatous fibre; b, crystal fibre; c, pitted vessels

Table 1—Rf values of the components present in hexane fractions and ingredients of Kalyaṇavleha under UV 366 nm

Rf	Colour	1	2	3	4	5	6	7	8	9	10	11
0.14	Blue	+	+	+	+	+	+	+	+	+	+	+
0.28	Blue	+	+	-	-	-	-	+	-	+	+	+
0.36	Blue	+	+	-	-	-	-	-	-	+	+	+
0.46	Blue	+	+	-	+	-	-	+	+	+	+	+
0.56	Blue	+	-	+	-	+	+	+	-	+	+	+
0.58	Green	-	-	+	-	+	-	-	+	-	-	-
0.64	Blue	-	+	+	-	+	-	+	+	+	+	+
0.70	Red	-	+	-	-	-	-	-	-	-	-	-

1-Curcuma longa, 2-Acorus calamus, 3-Saussurea costus, 4-Piper longum, 5-Zingiber officinale, 6-Cuminum cyminum,7 -Cyclospermum leptophyllum,, 8-Glycyrrhiza glabra, 9,10,11 - Hexane fraction of batch I, II and III

Table 2—Rf values of the components present in chloroform fractions and ingredients of Kalyaṇavleha under UV 366 nm

Rf	Colour	1	2	3	4	5	6	7	8	9	10	11
0.08	Yellow	-	-	+	-	-	-	-	-	+	+	+
0.10	Blue	+	-	-	+	+	-	-	-	+	+	+
0.22	Blue	+	-	-	-	-	-	-	+	+	+	+
0.24	Yellow	-	-	+	-	-	-	-	-	+	+	+
0.29	Yellow	-	-	+	-	-	-	-	-	+	+	+
0.29	Blue	+	+	-	-	-	-	-	-	-	-	-
0.37	Yellow	+	-	+	-	-	-	-	-	+	+	+
0.38	Green	-	-	-	-	-	-	-	-	-	-	-
0.41	Yellow	+	-	+	-	-	-	-	-	+	+	+
0.50	Blue	+	-	-	-	-	-	-	-	+	+	+
0.58	Purple	+	-	-	+	-	-	-	-	-	-	-
0.60	Blue	-	+	-	-	-	-	-	+	+	+	+
0.68	Blue	-	+	-	-	-	-	-	-	+	+	+
0.74	Purple	+	-	-	+	-	-	-	-	-	-	-

1-Glycyrrhiza glabra; 2-Cyclospermum leptophyllum, 3-Curcuma longa, 4-Acorus calamus, 5-Saussurea costus, 6-Piper longum, 7-Zingiber officinale, 8-Cuminum cyminum, 9,10,11-Chloroform fraction of batch I, II and III

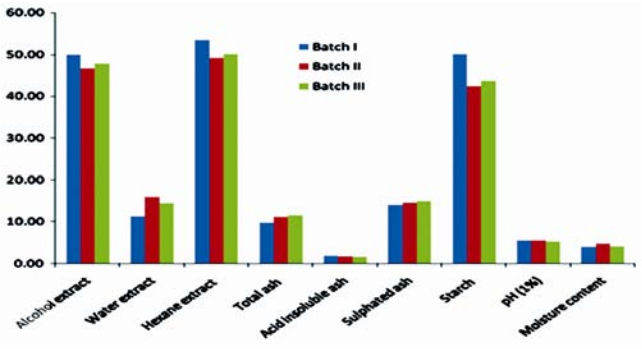

Fig. 2—Physicochemical parameters of *Kalyanavleha*

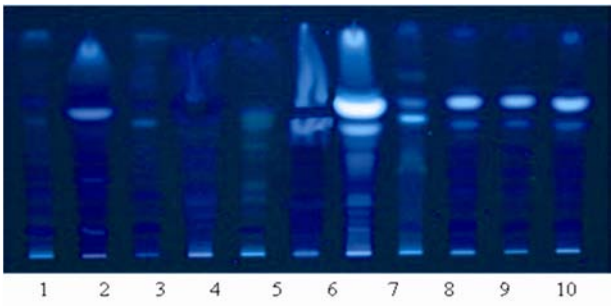

Fig. 3—TLC finger print profile of hexane fractions and ingredients of Kalyaṇavleha Under UV 366 nm. 1- *Curcuma longa*, 2- *Acorus calamus*, 3-*Saussurea costus*, 4-*Piper longum* , 5-*Zingiber officinalis* 6-*Cuminum cyminum*, 7-*Cyclospermum leptophyllum*, 8- *Glycyrrhiza glabra*, 9,10,11-Hexane fraction of batch I, II and III.

the best known biomarker of *Curcuma longa*. The HPTLC procedure was optimized. Initially chloroform – methanol and toluene – ethyl acetate – methanol in varying ratios were tried. Finally, the mobile phase toluene – ethyl acetate (7:3, v/v) gave a sharp and well-defined peak at Rf 0.54 (Fig. 5). The spots were more pronounced when the chamber was saturated with mobile phase for 30 min at room temperature (24°C). Different concentration of the standard curcumin was applied and the calibration plot was prepared by plotting peak area against amount of curcumin applied and the linear range was determined. The linear range was found to be 200-1000 ng per spot. The linear regression equation

obtained was y=1822.273+17.899x with a correlation coefficient (r) of 0.9912. This correlation coefficient indicated good linearity between the concentration and peak area in the applied concentration range.

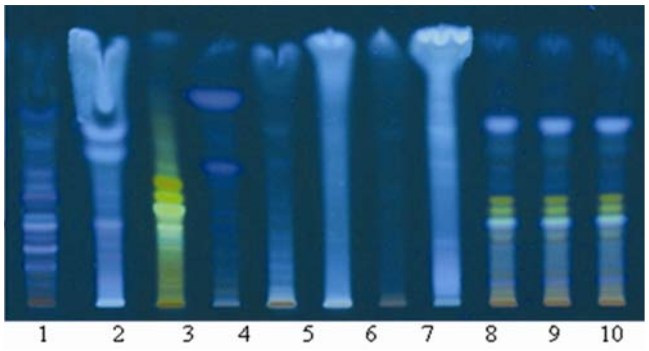

Fig. 4—TLC finger print profile of chloroform fractions and ingredients of Kalyaṇavleha under UV 366 nm. 1-Glycyrrhiza glabra; 2-Cyclospermum leptophyllum, 3-Curcuma longa, 4-Acorus calamus, 5-Saussurea costus, 6-Piper longum, 7-Zingiber officinalis 8-Cuminum cyminum, 9,10,11-Chloroform fraction of batch I, II and III

Curcumin content was determined quantitatively in the different samples and was found ranged from 0.252 - 0.265 %. The presence of curcumin was confirmed by comparing the Rf values and the spectra of the standard with corresponding bands in the fraction. The overlay spectra of identified peaks in the standards as well as the corresponding peak in the KA samples showed total superimposition at peak start, peak maximum and peak end, thus confirming the purity of the peaks in the sample solutions.

Standardization and development of reliable quality protocols for Ayurvedic formulations are important for keeping a check on the batch to batch consistency. The results obtained from the study could be utilized as a reference for setting limits in the routine standardization for the quality control and quality assurance of the compound formulation *Kalyanavleha* and curcumin can be used as an appropriate bio-marker for standardization of this Ayurvedic compound formulation.

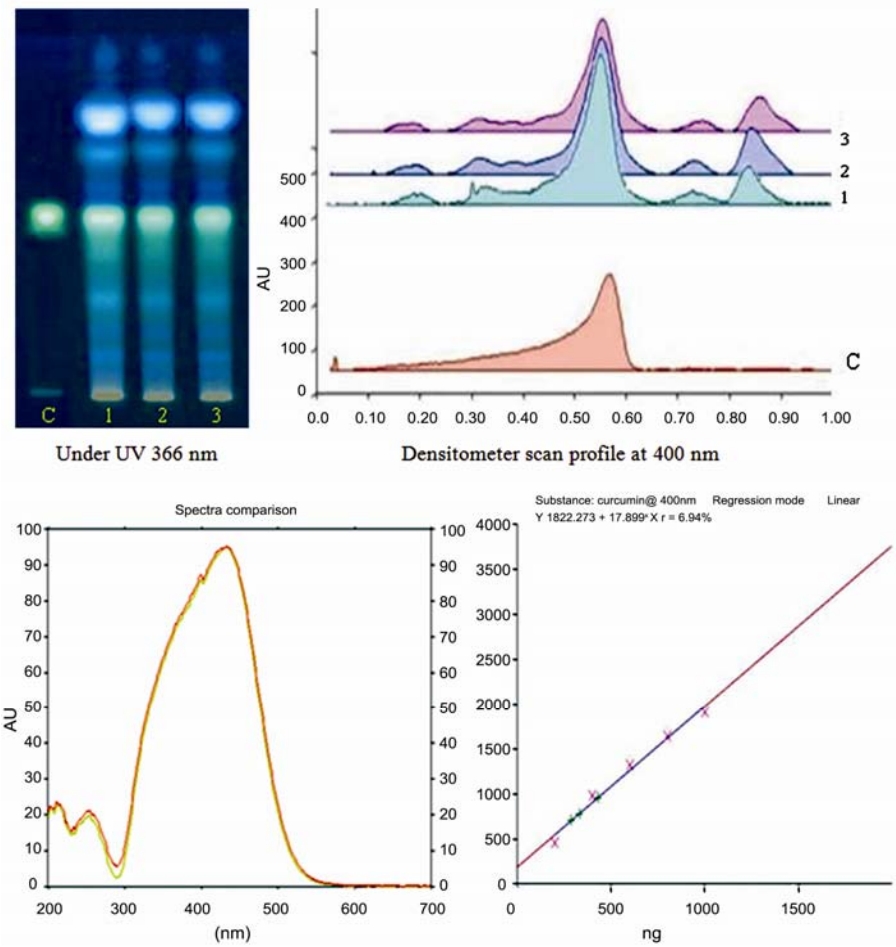

Fig. 5—HPTLC analysis of Chloroform fraction of Kalyaṇavleha for estimation of curcumin biomarker C, curcumin; 1-3, three batches of Kalyanavleha

Conclusion

From the present studies, it can be concluded that the characteristic microscopical features, curcumin and other distinguishing bands in the HPTLC profiles are very important for monitoring the quality of *Kalyanavleha* formulation as well as for establishing the presence of all ingredients in the formulation. Hence, the microscopic characters, physicochemical parameters and HPTLC profiles together may be used for quality evaluation and the standardization of compound formulations and maintaining their quality, purity and efficacy.

Acknowledgement

The authors are thankful to director CSIR-NBRI for providing adequate lab facilities and encouragement and Department of AYUSH, Ministry of Health and Family Welfare for financial support.

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