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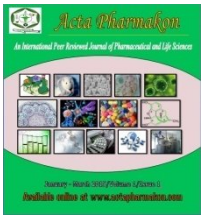

Research Article

WHO Standardization and HPTLC Estimation of Oleanolic acid in different samples of *Leucas cephalotes* (Roth) Spreng.

Shikhar Verma[*1], Abhishek Gupta[1] and Antariksh katara[2]

[1]Department of Phamacognosy and Ethnopharmacology, National Botanical Research Institute, CSIR-NBRI, Rana Pratap Marg, Hazratganj, Lucknow-226001, Uttar Pradesh, India
[2]Herbal Research Division, Ayushraj Enterprises Pvt. Ltd., Gram Mansinghpura, Dehmi-Begus road, Ajmer Road, Jaipur-303007, Rajasthan, India

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Corresponding Author:
Shikhar Verma
Email Id:
vermashikhar001@gmail.com

ABSTRACT

Standardization is a prime requirement for the basic assessment of herbal medicine. World Health Organization (WHO) has purely suggested that each herbal raw material should be assessed as per test procedures after that it would be considered as a herbal ingredient and use for therapeutic potential. In present study standardization were carried out on whole plant of *Leucas cephalotes* (Dronpushpi), which is a well-known herb found in Himalayas and an upland rainy season weed in the plains of central to south India. For proper quality assessment, three different samples LC 1-3 were collected from different zones of North to East India and tested for various WHO prescribed quality control parameters i.e. physico-chemical, biological and toxicological. For biomarker standardization, quantitative estimation of triterpenoidal compound Oleanolic acid was also performed in all samples by HPTLC. Authentic sample (LC1) was found superior on all quality parameters and Oleanolic acid content from the other two (LC 2&3) market samples.

1. INTRODUCTION

The World Health Organization currently encourages, recommend and promotes traditional herbal remedies in national health care programs because such drugs are easily available at low cost, are comparatively safe and the people have faith in such remedies. Plant material and herbal remedies derived from them represent a substantial proportion of the global drug market

and in this respect internationally recognized guidelines for their quality assessment are necessary. The WHO emphasizes the need to ensure quality control of medicinal plant products by using modern techniques and applying suitable standards.[1]

Raw material can be defined as starting material or any intermediate which will be utilized for further processing. Before finished pharmaceutical dosage forms are produced, the identity, purity and quality of raw materials as per specifications for impurities and other related substances present must be established with use of suitable test methods. Pharmacopoeias and formularies of various countries provide standardized test methods for the most common and widely used materials in their monographs.[2] Analytical techniques have been developed for quality control of herbal raw material is performed by selecting a marker compound and known active constituent for qualitative and quantitative target to assess authenticity and inherent quality. Quantification of the marker compound is done by HPTLC (High performance Thin Layer Chromatography), a major tool for analyzing the active marker component in herbal raw material and finish products.

Leucas cephalotes (Roth) Spreng. (LC) family- Lamiaceae, is an important medicinal plant commonly known as Dronpushpi and Guma found throughout India.[3] Though whole herb is used in Ayurvedic and Modern systems of medicine, leaves and inflorescence are the most important part in the field of medicine.[4,5] This plant is found throughout the greater part of India ascending up to 600 - 1,800 m. in Himalayas and in East Asia.[6,7] Whole herb of LC reported for new labdane-, norlabdane and abietane type diterpenes, triterpene, oleanolic acid, sterols.[8] A rarely found Laballenic acid (octadeca-5,6-dienoic acid), luaric acid, glutaric acid, adipic acid and tridecanoic acid.[9] A major compound β-sitosterol and its glucoside have been isolated from this plant [10]. LC was found to be hepatoprotective, antidiabetic, antibacterial, antihelminthic, antipyretic action, antioxidant, antifungal and antiviral.[11,12] The whole plant powder in the proportion of 70% in the herbal composition is patented to cure epileptic convulsions and cerebral function disorders [13].
In present study Dronpushpi, an Ayurvedic drug widely used throughout India for its therapeutic properties and also exploited commercially because of ingredient in various Ayurvedic formulations. At this stage, competent study is required for proper standardization of LC, to avoid inferior quality and drug adulteration.

2. MATERIALS AND METHODS

Oleanolic acid was obtained from Merck. All the reagents and chemicals used for the study were of analytical grade.

2.1 Plant Material, Procurement and Preparation

The fresh plant material was collected from Haridwar, Uttarakhand (LC1) and two more samples of the same plant was purchased from Khari- Bawoli market, Old Delhi (LC2) and local market of Lucknow, UP (LC3).The plant was authenticated as *Leucas cephalotes*(Roth) Spreng. F- Lamiaceae by the Dr. Anjula Pandey (Taxonomist), National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi. A voucher specimen (Specimen No: NHCP/NBPGR 2009-9/900) is preserved in

herbarium section of taxonomic department of NBPGR, New Delhi.

The collected fresh plant material was thoroughly washed with water to remove all debris and then shade dried. After drying plant material samples were powdered by using electric grinder and sieved from 20#size.

2.2 Physico-chemical parameters

Physicochemical parameters such as water soluble and methanol soluble extractive (by both cold maceration and hot percolation process), pH of 1% and 5% aqueous solution, loss on drying at 110°C, total ash value, acid insoluble ash, water soluble ash and sulphated ash were performed on powdered plant material.[3, 16-19]

2.3 Biological parameters

Evaluation of bitterness value, swelling Index, foaming index and total tannin content tested on dried powder.[18,19]

2.4 Toxicological parameters

Estimation of Heavy metals, multi residue pesticides, total viable aerobic count and total microbial load were performed on crude drug.[18]

2.5 Quantitative Estimation of Oleanolic acid by HPTLC

2.5.1 Sample preparation

Powdered plant material (1.0gm) in triplicate was extracted separately with 3X10 ml methanol. Extracts were concentrated under vacuum and re-dissolved in methanol, filtered and finally made up to 50 ml with methanol prior to HPTLC analysis.

2.5.2 Chromatographic Conditions

Chromatography was performed on Merck HPTLC pre-coated silica gel $60GF_{254}$ (10X10 cm) plates. Methanol solutions of samples and standard compound oleanolic acid of known concentrations were applied to the layers as 6 mm-wide bands positioned 15 mm from the bottom and 15 mm from side of the plate, using Camag Linomat V automated TLC applicator with nitrogen flow providing a delivery speed of 150nl/s from application syringe. These conditions were kept constant throughout the analysis of samples.

2.5.3 Detection and Quantification of Oleanolic acid

Following sample application, layers were developed in a Camag twin trough glass chamber which was pre-saturated with mobile phase of Toluene: ethyl acetate: formic acid (8:2:0.1 v/v) till proper separation of bands up to 8 cm height. After development, layers were dried with an air dryer and derivetise by dipping method with freshly prepared anisaldehyde sulphuric acid reagent post drying in oven at 105^0C for 5-10 minutes to visualize under white day light. Oleanolic acid was simultaneously quantified using Camag TLC scanner model 3 equipped with Camag Wincats IV software. Following scan conditions were applied: slit width, 5 mm x 0.45 mm; wavelength, 650 nm; and absorption-reflection mode.

In order to prepare calibration curves, stock solution of oleanolic acid (0.1 mg/ml) was prepared and various volumes of these solutions were analyzed through HPTLC, calibration curves of peak area vs. concentration were also prepared.

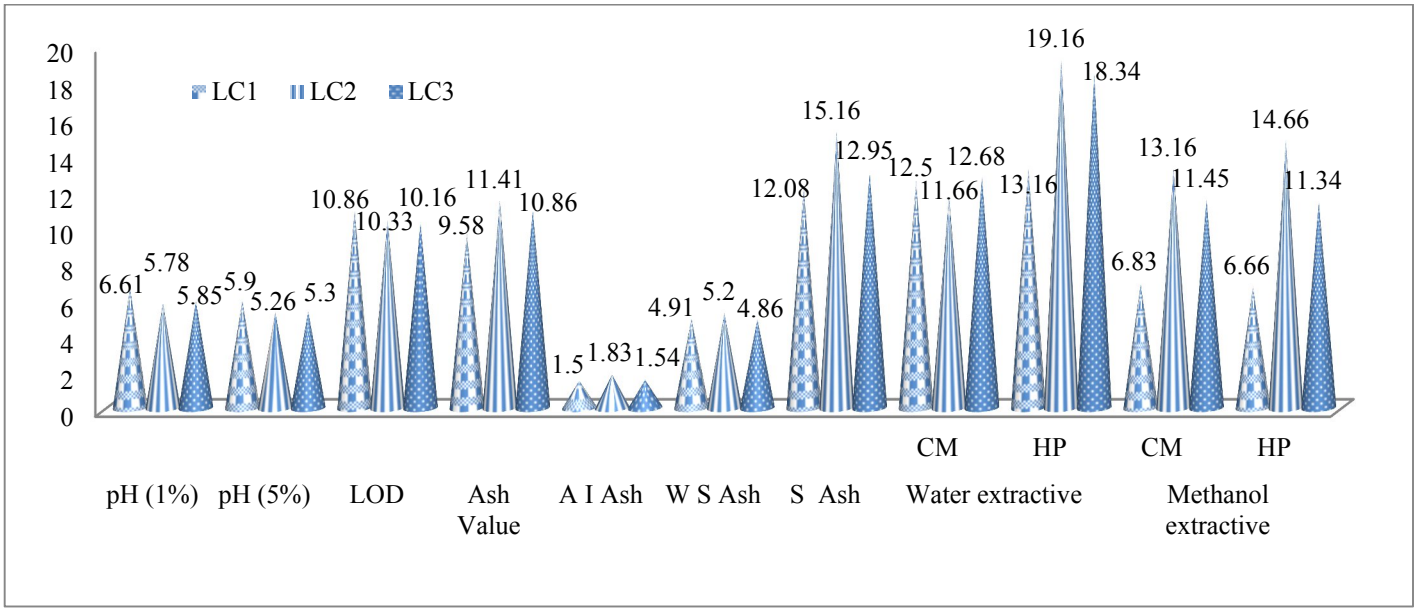

Figure 01: Figure 1- Values of different Physiochemical parameters (pH value at 1 & 5 %, LOD; Loss on drying, AI Ash; Acid insoluble ash, WS Ash; Water soluble ash, S Ash; Sulphated Ash, CM; Cold maceration and HP; Hot percolation.)

Table 1- Organoleptic parameters of LC herbs powder

	LC1	LC2	LC3
Colour	Green	Greenish brown	Greenish Brown
Taste	Characteristic	Slightly pungent	Slightly bitter
Odor	No	No	No

Table 2- Biological parameters of LC herb samples

Parameters	LC1	LC2	LC3
Bitterness value (units/gm)	14.5	14.5	14.5
Swelling index (ml)	6.8	7	6.6
Foaming index	≤ 100	≤ 100	≤ 100
Tannin content (%)	0.01	0.012	0.014

3. RESULTS

For comparative standardization on different samples of LC, physiochemical parameters including extractive values (aqueous and alcoholic), ash values (total ash, acid insoluble ash, water soluble and sulphated ash), pH determination (1 & 5%) and loss on drying have been performed in triplicates and results are shown in Figure-1.

Table 3- Heavy metal analysis

Heavy metals	Wavelength (nm)	Limit (ppm)	Result (mg/kg) ppm		
			LC1	LC2	LC3
Arsenic (As)	192.696	3	0.059	0.102	0.086
Lead (Pb)	220.353	10	0.657	0.952	0.736
Mercury (Hg)	253.652	1	0.010	0.012	ND
Cadmium (Cd)	228.802	0.3	0.047	0.069	ND

ND- Not detected

Organoleptic parameters are shown in comparison with other samples in Table 1. Other biological parameters bitterness value, swelling Index, foaming index and total tannin content tested on different samples of LC and results are shown in

Table 2. The main parameters for toxicity evaluation of different samples including heavy metal analysis, pesticide residue value, total viable aerobic count and microbial load were also performed and results with their limits are shown in Table 3, 4 & 5.

Table 4- Total microbial count and pathogens

Test parameters	Results (cfu/g)			Limits
	LC1	LC2	LC3	
Total microbial count				
Total bacterial count	7500	8280	7950	100000 cfu per g
Total fungal count	275	290	315	1000 cfu per g
Pathogens				
Escherichia coli	Ab	Ab	Ab	Should be absent per 10 g
Salmonella spp.	Ab	Ab	Ab	
Peudomonas aeruginosa	Ab	Ab	Ab	
S. aureus	Ab	Ab	Ab	

Table 5- Pesticide residual value

Pesticide	Limit (mg/kg) ppm	Result		
		LC1	LC2	LC3
Organo Chlorine	Min. 0.1	Not detected		
Organo Phosphorus	Min. 0.1	Not detected		

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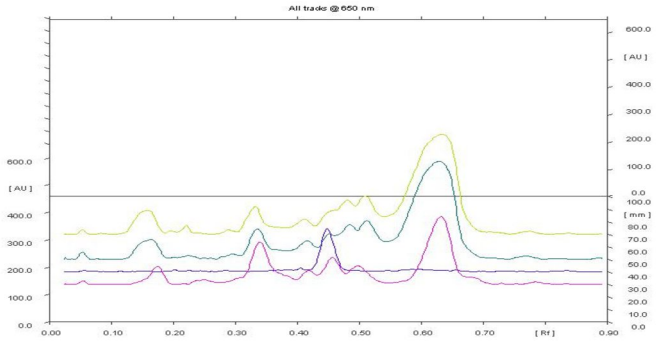

Figure 2: HPTLC chromatogram of standard with comparison of LC samples.

HPTLC of alcoholic extract of different LC samples were performed with oleanolic acid at five point calibration curve in which oleanolic acid was observed and quantified.

A densitogram and banding pattern obtained from extract shows oleanolic acid presence. Obtained data are shown in Figure 2 to 7.

HPTLC Densitograms

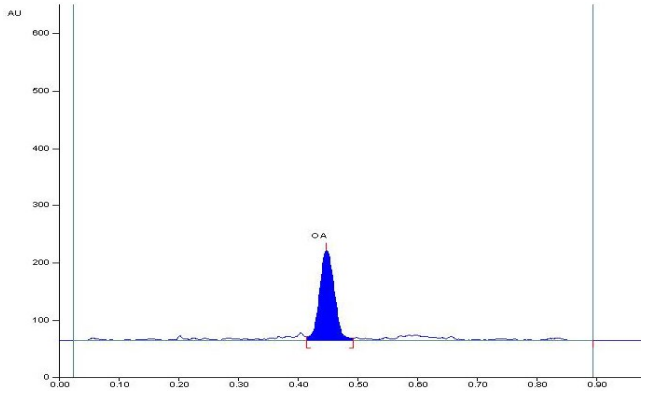

Figure 3- Oleanolic Acid

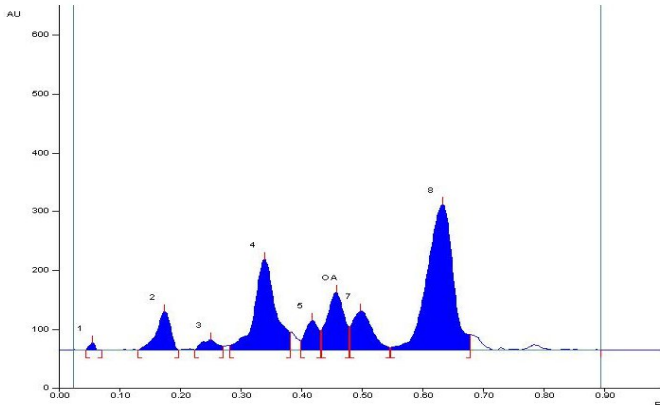

Figure 4- Sample LC1

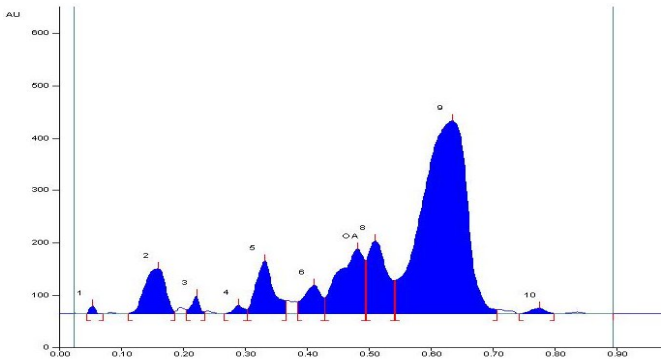

Figure 5- Sample LC2

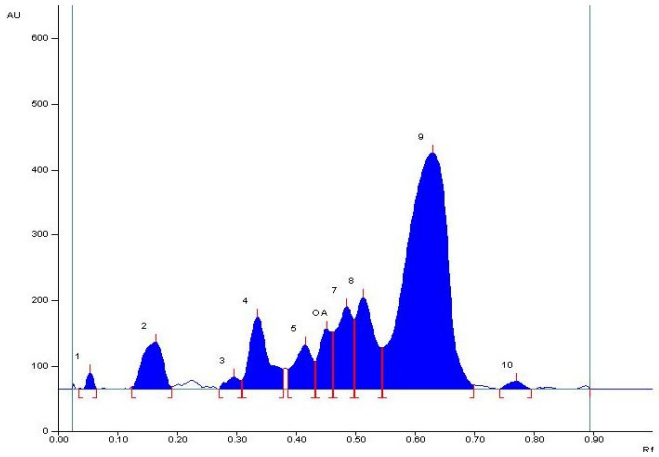

Figure 6- Sample LC3

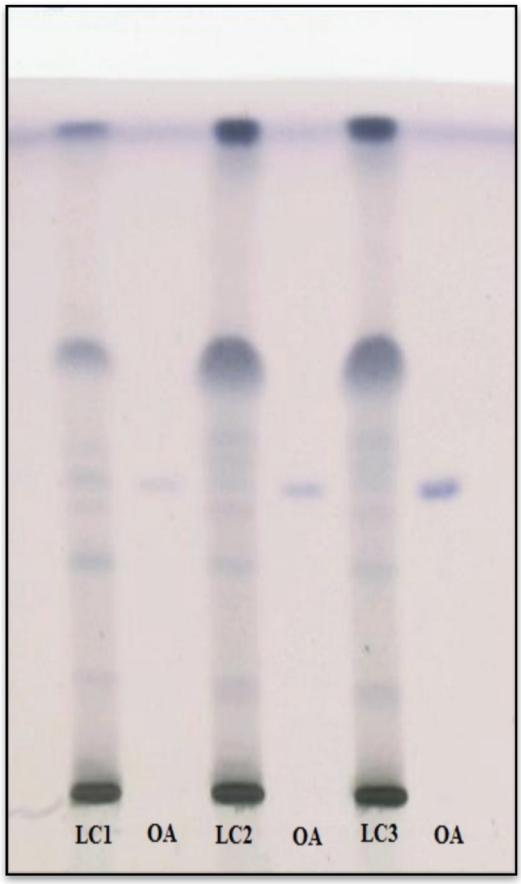

Figure 7- Band pattern of LC1, LC2 & LC3 samples and standard Oleanolic acid (OA) in different concentrations (in visible light).

Oleanolic acid in different samples of LC have been observed and quantified; results are shown in Table 06 .Oleanolic acid showed R_f 0.45±0.01; and r^2 of 0.981±0.02.

Table 6- Quantitative determination of Oleanolic acid by HPTLC in LC herb samples.

	LC1[a]	LC2[a]	LC[a]
Oleanolic acid (%w/w)	0.087±0.02	0.026±0.01	0.029±0.01
Rf value	0.45±0.01	0.45±0.01	0.45±0.01

[a]In triplicate

4. DISCUSSION

Standardization is a system to establish the quality standards of every plant medicine in the market because the scope for variation in different batches of medicine is enormous. Therefore, with reference to the quality control of natural products it is clear that an intense need exists for standardization of the plant material used as drug in raw form, as semi processed form or as final product. Standardization can prove useful in improving quality and efficacy of herbal drugs and herbal based products.

The WHO has developed guidelines for the assessment of crude drugs as well as herbal medicines on the basis of *quality assessment*, *stability*, *safety* and *efficacy*. Raw herbs from the market are main source of herbal medicine but their quality is quite inferior when compared to the original collected herbs. This shows the degree of adulteration and substitution in the place of original plant material.

Oleanoilc acid is already reported in the plant.[8] This triterpenoidal compound can be an important biomarker for standardization, though oleanolic acid has been evaluated for several pharmacological activities such as hepatoprotection, antihyperlipidemic, anti-inflammatory and antitumor effect.[20]

5. CONCLUSION

Three different samples of *L. cephalotes* from different areas of North to East India are standardized and evaluated using physiochemical, biological, toxicological parameters and chromatographic quantification. All the samples are screened for the presence of oleanolic acid which is found to be positive and quantified. This study provides data for complete standardization and comparative profile of LC and also explores it as a source of biomarker oleanolic acid.

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