Original Research Article

HPTLC and HPLC Methods for the Estimation of Oleanolic Acid in Successive Leaf Extracts of *Leucas aspera and Tridaxprocumbens* andtheir *In vitro* Antiinflammatory Activity

ABSTRACT

Aims: The study was undertaken with the objective to quantify the oleanolic acid and estimate from successive leaf extracts of *Leucas aspera* and*Tridax procumbens* with the help of standard marker oleanolic acid by using HPTLC and HPLC methods and to carry out the evaluation of their antiinflammatory activity.

Place and Duration of Study: The study was carried out between October 2022 and June 2023 in the Department of Pharmaceutical Analysis and Pharmacognosy, Sri Ramakrishna Institute ofParamedical Sciences, College of Pharmacy, Coimbatore-44, Tamil nadu, India

Methodology: The extraction of leaves is done using successive extractions by Continuous hot percolation method using soxhlet extractor. The phytochemical analysis of petroleum ether, ethyl acetate and methanol fractions were carried out. Standardization of oleanolic acid was carried out by HPTLC and HPLC methods. Quantification of oleanolic acid in successive leaf extracts of the two plants were carried out. *In vitro study* of antiinflammatory activity was performed by Xanthine oxidase inhibitory activity in the ethyl acetate fraction.

Results: The phytochemical screening of the two plant extracts revealed the presence of alkaloids, glycosides,terpenoids, steroids, flavonoids, saponins, carbohydrates and proteins. In HPTLC method, petroleum ether, ethyl acetate and methanol leaf extracts of *Leucas aspera* and *Tridax procumbens* were developed in suitable mobile phase of toluene: ethyl acetate: formic acid (7:3:0.2%v/v/v)followed by derivatizing with anisaldehyde sulphuric acid derivatizing agent and scanned under 530nm.HPLC of standard marker and successive leaf extracts of *Leucas aspera* and *Tridax procumbens* were carried out using methanol: 25mM phosphate buffer (pH-3) in the ratio of 90:10% v/v at flow rate of 1ml/min and chromatograms were recorded at 202nm.Xanthine oxidase inhibitory activity of combined leaf extracts of ethyl acetate showed IC_{50}value of 0.026µg/ml.

Conclusion: Standardisation of oleanolic acid was carried out by HPTLC and HPLC methods and linearity were found to be 0.9964 and 0.9998 respectively. Quantification of oleanolic acid in successive leaf extracts of the two plants were carried out. *In vitro* study using xanthine oxidase inhibitory activity showed that combined extracts of ethyl acetate fractions produced better antiinflammatory activity than the individual extracts of the respective plants.

Keywords: Leucas aspera, Tridax procumbens, oleanolic acid, Xanthine oxidase inhibitory activity, HPTLC, HPLC.

1.INTRODUCTION

Traditional systems of medicine have been steadily gaining importance and acceptance all over the world. Consequently, plant materials and herbal based drugs derived from them

represent a substantial proportion of the current global drug market. In this scenario, there is a need to ensure that herbal drugs and preparations containing them possess optimum and consistent quality. Hence, there is a need to create and maintain a comprehensive quality assurance system. Quality control and standardization of herbal drugs are considered to be one of the major issues in herbal drug development.[1]

Chemical analyses of plants show the presence of therapeutically important constituents usually in combination with many inert substances. The active principles are extracted from the plants and purified for therapeutic utility based on their selective pharmacological activity. Medicinal plants play an important role in the development of potent therapeutic agents. The use of herbal medicines is becoming popular due to the toxicities and side effects associated with allopathic medicines. [2]

Standardization of plant extracts with the help of markers is an essential procedure for ensuring the quality control of the herbal drugs which would lead to increased global acceptance of them. Standardisation is defined as "formulation of standards for a substance or for a procedure". Standardization is an essential process for ensuring the quality control of the herbal drugs. In the development of botanical drugs, standardisation refers to a set of technique or standards that are applied to the manufacture of herbal formulations. According to the American Herbal Products Association (AHPA), "Standardisation refers to the body of information and controls necessary to produce materials of reasonable consistency. This is achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing processes". [3]

Chromatographic fingerprinting techniques are playing an ever more important role in the standardisation of herbal products. They are being used for confirming the presence of different herbs in poly herbal formulations as well as the quantification of marker compounds. HPTLC is a very simple technique used for the separation of plant constituents. It is a qualitative tool for separation of the components of simple mixtures where speed, low cost and simplicity are required and it is also a tool for quantitative analysis with high simple throughput. HPLC is a very sensitive analytical technique most widely used for quantitative and qualitative analysis of pharmaceuticals.[4]

Leucas aspera belongs to the family Lamiaceae commonly known as ‘Thumbai’. The whole plant is traditionally important because it has many therapeutic values. *Leucas aspera* flowers are used as stimulant, expectorant, asperient, diaphoretic and insecticide. The leaves are used as insecticide and mosquito repellent and the leaf juices are considered as a remedy for chronic rheumatism, psoriasis and other chronic skin eruptions. From the various parts of *Leucas aspera*, glucosides, tannins, saponins, sterols, oleic acid, linoleic acid, palmitic acid, stearic acid, oleanolic acid, ursolic acid and nicotine were identified.[5]

Tridax procumbens belongs to the Asteraceae family and is an ayurvedic herb of Asia with a history of traditional use. *Tridax procumbens* has been used from ancient times to treat wounds, skin diseases and to stop blood clotting in folk medicine. It possesses anticoagulant, antileishmanial, antioxidant, anticancer, immunomodulatory, insecticidal, anthelmintic, cardiovascular, antiseptic, antimicrobial, and insecticidal properties.[6]

Oleanolic acid (3b-hydroxyolean-12-en-28-oic acid) is a pentacyclic triterpenoid compound with a widespread occurrence throughout the plant kingdom. In nature, the compound exists either as a free acid or as an aglycone precursor for triterpenoid saponins, in which it is linked to one or more sugar chains. Oleanolic acid and its derivatives possess several promising pharmacological activities, such as hepato protective effects, and antiinflammatory, antioxidant, or anticancer activities.[7]

Xanthine oxidase and xanthine dehydrogenase are interconvertible forms of the same enzyme, known as xanthine oxidoreductase. It belongs to a class of enzymes known as molybdenum iron - sulphur flavin hydroxylases. Xanthine oxidase is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, and brain as well as the plasma.

Xanthine oxidase is the enzyme which catalyses the hydroxylation of purines, particularly conversion of xanthine into uric acid. It is one of the major enzymes which are involved in the catabolism of purine nucleotides. It converts hypoxanthine to xanthine and xanthine into uric acid. Uric acid is excreted in urine. Xanthine oxidase inhibitor is much useful, since it possess lesser side effects compared to uricosuric and antiinflammatory agents.[8, 9]

2.MATERIAL AND METHODS

Material and Methods

Materials

Chemicals and solvents used

AR/HPLC grade methanol, water, petroleum ether, ethyl acetate, toluene, formic acid, potassium dihydrogen phosphate, anisaldehyde, glacial acetic acid and concentrated sulphuric acid, were supplied by Thermo FisherScientificIndiaLimited,Mumbai,IndiaandMerckPvt.Ltd.,Mumbai,India. Xanthine oxidase enzyme is purchased from S.D.FinechemicalsLtd.,Mumbai,India. Oleanolic acid is purchased from Sigma Aldrich Pvt.Ltd.,Mumbai,India.

Plant material

The leaves of *Leucas aspera* were collected from Salas- Coonoor, Nilgiris, Tamilnadu and the leaves of *Tridax procumbens* were collected from Sri Ramakrishna Institute of Paramedical Sciences-College of pharmacy campus, Coimbatore, Tamilnadu. The collected leaves of *Leucas aspera* and *Tridax procumbens* were identified and authenticated by Dr.S.P.Subramanian, Scientist, Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamilnadu.

Methods

Preparation of powder for extraction

The leaves of the plants were collected, separated from extraneous materials, cleaned,washedand welldried atroomtemperatureto avoidthedegradationofphytoconstituents.The driedleaves were groundwellforgettingsemicoarsepowder.

Process of extraction

The crude drug powders were extracted by successive extraction methods using organic solvents viz., Petroleum ether, ethyl acetate and methanol. The solvents were selected based on the solubility of the selected marker oleanolic acid. The extraction process was carried out by continuous hot percolation method using soxhlet apparatus. The extraction process was carried out for 3 days for each solvent so as to effect complete extraction of the plant material. After extraction the extracts were collected and dried at room temperature to get well dried extract.

Extraction

Method : Successive extraction
Principle : Continuous hot percolation
Apparatus : Soxhlet extractor
Temperature maintained :30-45˚C
Quantity of leaves powder used : 100g
Volume of solvent used : 1000ml
Duration of each extraction process :3 days

The percentage yields of the extracts are mentioned in Table: 1a, b.The extracts were stored in the refrigerator at 4˚ C until further analysis. The collected extracts were subjected to preliminary phytochemical screening, TLC, HPTLC, HPLC analyses and *in vitro* antiinflammatory studies.

Phytochemical screening of plant extracts

The quantitative chemical analysis of the crude extracts were carried out for the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, saponins, carbohydrates and proteins using the standard methods previously described.[10, 11, 12]

Thin layer chromatographic analysis of successive leaf extracts of *Leucas aspera* and *Tridax procumbens*.[13]

The basic thin layer chromatography was done in order to compare R_f value of the marker with the plant extract. The collected successive plant extracts of *Leucas aspera*, *Tridax procumbens* and marker were applied as a spot 1cm from the edge of the plate using capillary tube. The plates were then kept aside for the evaporation of the solvent. Then plates were placed in a closed container previously saturated with vapors of developing solvents toluene: ethyl acetate: formic acid (7:3:0.2%v/v/v) with care being taken to avoid contact between the sample and the developer. After developing the plates up to two-third of the length of the plate, they were removed from the chamber and dried. Then the plates were sprayed with derivatizing agent anisaldehyde-sulphuric acid reagent and then examined in ultra-violet chamber under fluorescence light in order to identify the various spots.

Development of validated HPTLC method for the estimation of oleanolic acid in successive leaf extracts of *Leucas aspera* and *Tridax procumbens* as per ICH guidelines.[13]

Preparation of standard stock solution of marker

A quantity of 5mg of oleanolic acid was transferred into 10 ml standard flask, dissolved in a small quantity of methanol and made up with methanol to 10ml to get a concentration of 500µg/ml.

Preparation of derivatizing agent

0.5 ml anisaldehyde is mixed with 10ml of glacial acetic acid followed by 85 ml methanol and 5ml ofconcentrated H_2SO_4 acid.

Fixed experimental conditions

Stationary phase : Pre-coated Silica gel $60F_{254}$ on aluminium sheets
Mobile phase : Toluene: Ethyl acetate: Formic acid (7:3:0.2%v/v/v)
Chamber saturation time : 20 minutes
Migration distance : 80 mm
Band width : 6 mm
Slit dimension : 5 x 0.35 mm
Source of radiation : Tungsten lamp
Derivatizing agent : Anisaldehyde-Sulphuric acid
Detection wavelength : 530 nm
R_f value : 0.54

Validation of the method[19]

Validation of the developed method was carried out in accordance with ICH guidelines in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), interday and intraday precision, repeatability of sample application, repeatability of measurement and stability study.

Linearity

The marker was prepared in various concentrations and analysed by HPTLC method in order to establish that the linear regression data shows a good linear relationship over the concentration under study. From the standard stock solution of marker 100 to 600 ng/band of oleanolic acid were applied using CAMAG semi applicators. The R_f value was found to be 0.54 ± 0.03. The slope, intercept and correlation co-efficient values were found from the calibration graph (Fig: 1). Calibration data are presented in Table: 2

Limit of detection and limit of quantification

The limit of detection and limit of quantification of the standard were determined by the application on the plate of decreasing amounts of the drug in triplicate. The lowest concentration at which the peak is detected is referred to as the "Limit of Detection" and the lowest concentration at which the peak is quantified is referred to as the "Limit of Quantification". Table: 3 shows the LOD and LOQ values of marker. The detection limit (DL) may be expressed as:

DL= 3.3 σ/S

Where, σ= the standard deviation of the response and S = the slope of the calibration curve.

The quantitation limit (QL) may be expressed as:

QL = 10 σ/S

Where, σ = the standard deviation of the response and S = the slope of the calibration curve.

Precision

The precision of the analytical procedure indicates the closeness of the agreement between a series of multiple sampling measurements of the same homogeneous sample under the prescribed conditions.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Precision of the method adopted in the present work was demonstrated by

a) Intraday precision
b) Inter day precision
c) Repeatability

i. Repeatability of sample application
ii. Repeatability of sample measurements

(a) **Intraday precision**

Intraday precision was studied by performing analyses of the standard drug at two different concentrations in the linearity range of marker three times on the same day and the %RSD was calculated (Table: 3)

(b)**Interday precision**

Interday precision was studied by carrying out the analyses of the standard drug at different concentrations in the linearity range of marker for three days over a week and %RSD was calculated (Table: 3)

(c) **Repeatability**

i. Repeatability of sample application

Repeatability of sample application was assessed by spotting 300 ng concentration of standard marker solutions six times on pre-coated TLC plate. Plate was then developed, scanned and %RSD was calculated (Table: 3)

ii. Repeatability of sample measurement

Repeatability of sample measurement of peak area was assessed by spotting 300 ng concentration of standard marker solution on pre-coated TLC plate. After development of the plate, the separated spots were scanned six times without changing position of the plate and %RSD was calculated (Table: 3)

Stability studies

The analyte are may be prone to decompose when developed chromatographic plate is exposed to the atmosphere. Therefore, after development, the stability of the plates must be confirmed.

The stability of the plate was studied at different time intervals and the peak areas were compared with the peak area of the freshly scanned plate. The developed plate was found to be stable for 30 minutes under room temperature (Table: 3)

HPTLC analysis of successive leaf extracts of *Leucas aspera* and *Tridax procumbens*.

Preparation of stock solution of the petroleum ether extracts of *Leucas aspera* and *Tridax procumbens*:

The petroleum ether extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000 µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10 µl/band of the solution was applied on the TLC plate.

Preparation of stock solution of the ethyl acetate extracts *Leucas aspera* and *Tridax procumbens*:

The ethyl acetate extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000 µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10 µl/band of the solution was applied on the TLC plate.

Preparation of stock solution of the methanol extracts of *Leucas aspera* and *Tridax procumbens*:

The methanol extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000 µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10 µl/band of the solution was applied on the TLC plate.

Recording of the chromatogram

The peak areas of the chromatogram of the two leaf extracts were compared with standard chromatogram and the amounts of oleanolic acid present in the extracts werecalculated from the calibration graph (Fig: 1)

Development of validated RP-HPLC method for the estimation of oleanolic acid in successive leaf extracts of *Leucas aspera* and *Tridax procumbens* as per ICH guidelines.[14]

Preparation of standard stock solution of marker

An accurately weighed quantity of 10mg of oleanolic acid was transferred into 10 ml standard flask, dissolved in methanol and made up with methanol to 10ml to get a concentration of 1000µg/ml.

Fixed chromatographic conditions

Stationary phase : Shim-pack Solar C (250 ×4.6 mm, 5 µm particle size)
Mobile phase : Methanol: 25mM phosphate buffer (pH-3)
Mobile phase ratio : 90:10%v/v
Flow rate : 1ml/min
Injection volume : 20 µl
Detection wavelength : 202 nm
Operating temperature : 25˚ C
Operating pressure : 139 kgf

VALIDATION OF RP-HPLC METHOD[19]

Validation of the developed method was carried out in accordance with the ICH guidelines in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), interday and intraday precision, repeatability of sample injection and measurement and stability studies.

Linearity

Different concentration of marker were prepared by making the final volume with methanol and injected into HPLC system. Linear regression data showed a good linear relationship over a concentration range of 1to100 µg/ml for oleanolic acid. The peak areas were noted and a linear graph was plotted between concentrations (x) versus peak area (y). Calibration graphs are given in (Fig: 8) Calibration data are presented (Table: 4).

Limit of detection and limit of quantification

The limit of detection and limit of quantification of the standard were determined by the application on the plate of decreasing amounts of the drug in triplicate. The lowest concentration at which the peak is detected is referred to as the "Limit of Detection" and the lowest concentration at which the peak is quantified is referred to as the "Limit of Quantification". Table:5 shows the LOD and LOQ values of marker. The detection limit (DL) may be expressed as:

DL= 3.3 σ/S

Where, σ= the standard deviation of the response and S = the slope of the calibration curve.

The quantitation limit (QL) may be expressed as:

QL = 10 σ/S

Where, σ = the standard deviation of the response and S = the slope of the calibration curve.

Precision

The precision of the analytical procedure indicates the closeness of the agreement between a series of multiple sampling measurements of the same homogeneous sample under the prescribed conditions.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Precision of the method adopted in the present work was demonstrated by

a) Intraday precision
b) Interday precision
c) Repeatability
    i. Repeatability of sample injection

(a) Intraday precision

Intraday precision was studied by performing an analysis of the standard drug at two different concentrations in the linearity range of marker three times on the same day and the %RSD was calculated (Table: 5)

(b) Interday precision

Interday precision was studied by carrying out the analysis of the standard drug at different concentrations in the linearity range of marker for three days over a week and %RSD was calculated (Table: 5)

(c)Repeatability of sample injection

Repeatability of sample application was assessed by injecting same concentration of standard marker solution six times and %RSD was calculated (Table: 5)

Stability

The marker solutions were subjected to stability studies under refrigeration and at room conditions. Stability was assessed by looking for any changes in retention time, resolution,

peak shape. when compared to chromatogram of freshly prepared solution. The marker was found to be stable at room temperature for one day and under refrigeration condition up to 5 days.

System suitability studies

System suitability parameters like number of theoretical plates (N), tailing factor, resolution (Rs) etc. were studied. The results were shown in (Table: 5).

Robustness

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

±1 in ratio of methanol: phosphate buffer

± 0.1 in units of flow rate

The slight changes obtained with the variation in conditions were found almost same with the standard solution under optimized conditions. So the developed method was found to be robust.

Analysis of successive leaf extracts of *Leucas aspera* and *Tridax procumbens* by RP-HPLC

Preparation of stock solution of the petroleum ether extracts of *Leucas aspera* and *Tridax procumbens*:

The petroleum ether extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000 µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10 µg was injected.

Preparation of stock solution of the ethyl acetate extracts *Leucas aspera* and *Tridax procumbens*:

The ethyl acetate extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000 µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10 µg was injected.

Preparation of stock solution of the methanol extracts of *Leucas aspera* and *Tridax procumbens*:

The methanol extract (10mg) of each plant was weighed and made up to 10ml with methanol as the solvent to get a concentration of 1000 µg/ml solution and the solution was filtered through whatmann filter paper. A quantity of 10 µg was injected.

Recording of the chromatogram

The peak areas of the chromatogram of the two leaf extracts were compared with standard chromatogram and the amounts of oleanolic acid present in the extracts werecalculated from the calibration graph (Fig: 8)

In vitro antiinflammatory study[15, 16]

The study was carried out with xanthine oxidase inhibitor with xanthine as substrate. Allopurinol was used as the standard drug and the two plant extracts were prepared in ethyl acetate.

Principle

Xanthine oxidase is the enzyme which catalyses the hydroxylation of purines, particularly conversion of xanthine into uric acid. It is one of the major enzymes which are involved in the catabolism of purine nucleotides. It converts hypoxanthine to xanthine and xanthine in to uric acid. Uric acid is excreted in urine.In smaller mammals presence of the enzyme uricase further oxidizes uric acid to allantoin. But in higher mammals the uricase enzyme is not present which parallels a similar loss of ability to synthesize ascorbic acid. It is also

presumed that uric acid may act as antioxidant as do ascorbic acid in such species. Both uric acid and ascorbic acid are strong reducing agents and potent antioxidants. The uric acid produced from xanthine oxidase contributes to the antioxidant capacity of the blood. The reduction of oxygen (0_2) and hydrogen peroxide (H_2O_2) Xanthine oxidase catalysis by xanthine has been proposed as a central mechanism of oxidative injury in some situations.

Reagents

- Phosphate buffer of pH 7.5 in distilled water (prepared with dihydrogen phosphate and disodium phosphate)
- Enzyme solution (0.01 units/ml in phosphate buffer, pH 7.5)
- 150Mm xanthine in the phosphate buffer
- 1N hydrochloric acid.

Procedure[9]

The assay mixture consisted of 1ml of the test compound (0.01-0.4 µg/ml), 2.9 ml of phosphate buffer (pH 7.5) and 0.1 ml of xanthine oxidase enzyme solution (0.1 units/ml in phosphate buffer, pH 7.5), which was prepared immediately before use. After preincubation at 25° C for 15 minutes the reaction was initiated by the addition of various concentrations of the substrate solution (2 ml). The assay mixture was incubated at 25° C for 30 min. The reaction was stopped by adding 1 ml of 1N HCl and the absorbance was measured at 290 nm using UV spectrophotometer. Allopurinol was used as the standard. The percentage inhibition was calculated by,

$$\text{Percentage inhibition} = \frac{(A-B)-(C-D)}{(A-B)} X\ 100$$

Where A is the activity of the enzyme without the compound, B is the control of A without the compound and enzyme, C and D are the activities of the compound with and without the enzyme respectively. The assay was done in triplicate and IC_{50} values were calculated from the percentage inhibition.

Statistical analysis

All experiments were performed in triplicate (n=3) and the results were expressed as mean ± SEM.

3. RESULTS AND DISCUSSION

The leaves of *Leucas aspera* contain significant amounts of pentacyclic triterpenoids likeoleanolic acid, phytosterol like β-sitosterol, glycoside, diterpenes and phenolic compoundsof which oleanolic acid has pharmacological actions like antiinflammatory, antidiabetic,antioxidant activities.Hence,thisplantwasselectedforthepresentstudy.

Successive extractions were carried out by continuous hot percolation method for extraction of powdered leaves of *Leucas aspera* and *Tridax procumbens*. Petroleum ether, ethyl acetate and methanol have been chosen as appropriate solvents for drug extraction. The percentage yield of the successive extracts of *Leucas aspera* and *Tridax procumbens* petroleum ether, ethyl acetate and methanol extracts were found out (Table 1a,b)

Table 1a: The amount and percentage yield of extracts obtained

S.NO	Successiveextractsof *LeucasAspera*(500gm)	Amountofextractobtained(g)	% of extract obtained(%w/w)
1	Petroleumether extract	17.5	3.5
2	Ethylacetate extract	34	6.8
3	Methanolextract	49	9.8

Table 1b: The amount and percentage yield of extracts obtained

S.NO	Successive leaf extracts of *Tridax procumbens*(500gm)	Amount of extract obtained (g)	% of extract obtained (%w/w)
1	Petroleum ether extract	6	1.2
2	Ethyl acetate extract	26	5.2
3	Methanol extract	30	8.2

For the successive extracts, preliminary phytochemical tests were done which confirmed the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids and carbohydrates.The preliminary TLC studies showed that *Leucas aspera* and *Tridax procumbens* in ethyl acetate extract spots were near to the standard oleanolic acid spot.Hence, further analysis was carried out based on these comparisons.

HPTLC method

A mobile phase system consisting of toluene: ethyl acetate: formic acid (7:3:0.2%v/v/v) was selected for the determination of oleanolic acid by HPTLC method. The system gave symmetric peaks with R_f value 0.54 for oleanolic acid at the selected wavelength of 530nm. Calibration graph was plotted (Fig: 1).The linearity was found to be in the concentration range of 100-600 ng/band for oleanolic acid (r=0.9964). From the calibration graph slope and intercept values were found to be 1.1774 and 52.733 respectively. The validation parameters were carried out for marker and they are tabulated (Table-3). HPTLC fingerprinting were obtained for successive leaf extracts of *Leucas aspera* and *Tridax procumbens* (Fig: 2-7). The amount of oleanolic acid(10 mg) present in the two leaf extracts were 0.4155 mg in ethyl acetate extract of *Leucas aspera* and 0.5671 mg, 0.1190 mg in ethyl acetate extract and methanol extract of Tridax procumbens respectively.

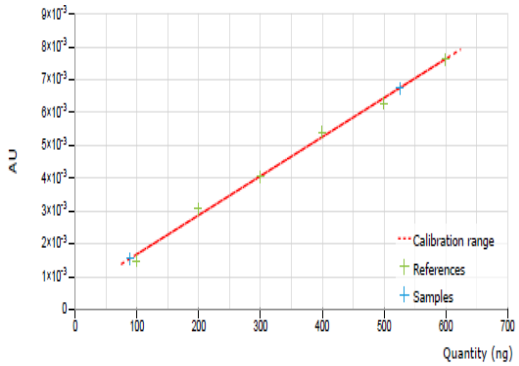

Fig 1: Calibration graph of oleanolic acid (100-600ng/band)

Table 2: Calibration data for oleanolic acid (100-600 ng/band)

CONCENTRATION (ng/band)	Peak Area
100	0.00157
200	0.00304
300	0.00403
400	0.00538
500	0.00626
600	0.00761

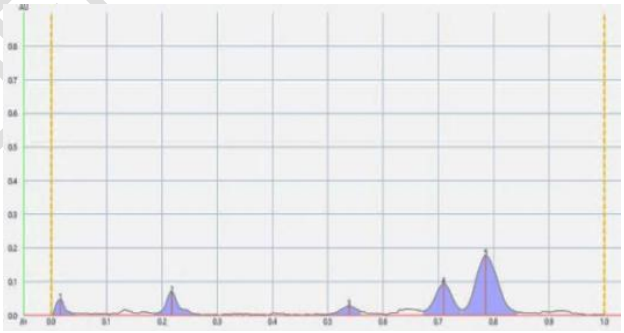

Fig 2: HPTLC fingerprint of *Leucas aspera* petroleum ether extract at 530nm

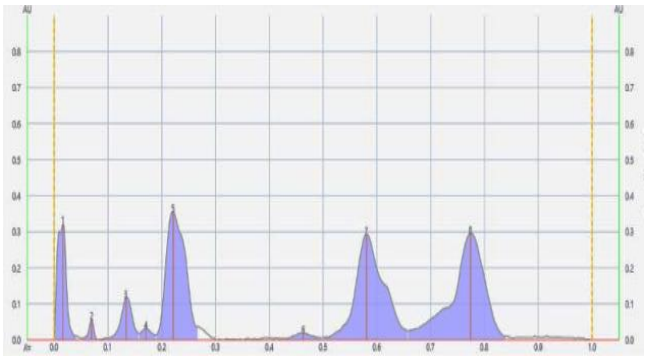

Fig 3: HPTLC fingerprint of *Leucas aspera* ethyl acetate extract at 530nm

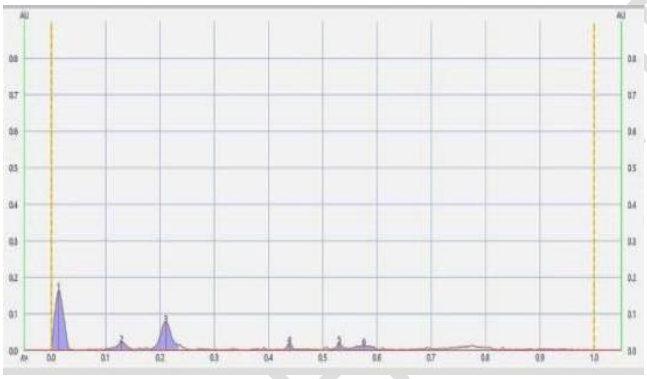

Fig 4: HPTLC fingerprint of *Leucas aspera m*ethanol extract at 530nm

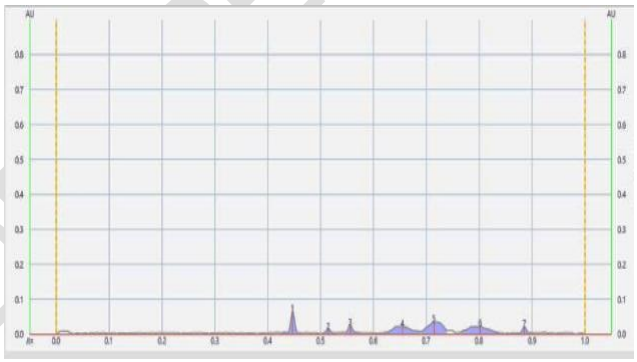

Fig 5: HPTLC fingerprint of *Tridax procumbens*petroleum ether extract at 530nm

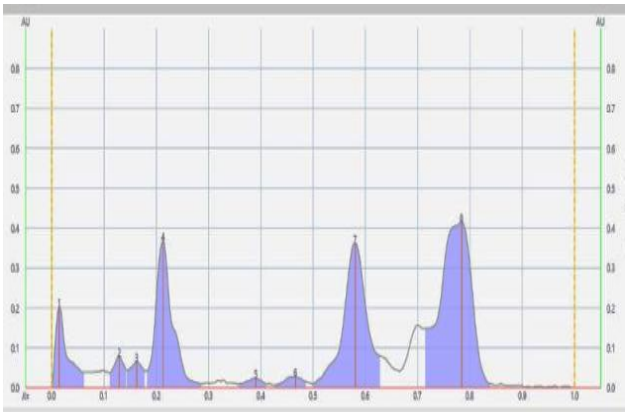

Fig 6: HPTLC fingerprint of *Tridax procumbens* ethyl acetate extract at 530nm

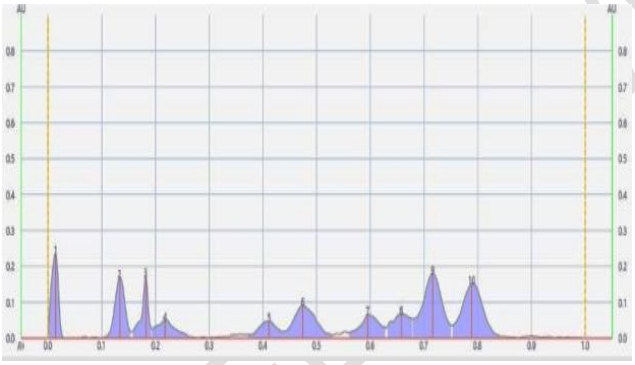

Fig 7: HPTLC fingerprint of *Tridax procumbens*methanol extract at 530nm

Table 3: Validation parameters for oleanolic acid by HPTLC method

PARAMETERS			**HPTLC**	
Linearity			100-600 ng/band	
Correlation coefficient			0.9964	
Regression equation			y=1.1774x+52.733	
LOD			10.52006 ng/band	
LOQ			48.0325 ng/band	
Precision	Intraday		%RSD*	0.5959
	Interday			1.0126
	Repeatability	Sample application		1.2400
		Sample measurement		1.3619
Plate stability			30 minutes	

RP-HPLC method

In RP-HPLC methods, a mobile phase system containing methanol: 25mM phosphate buffer (pH-3) in the ratio of 90:10% v/v at flow rate of 1ml/min was employed for the determination of oleanolic acid because this system gave symmetric peak shape and minimum of tailing with a retention time of 15.7minutes at 202 nm. Linearity of oleanolic acid was found to be over the range of 1-100 µg/ml and correlation coefficient values for the oleanolic acid were found to be 0.9998 (Fig:8). From the calibration graph slope and intercept values were found to be 10043 and 3847.8 respectively showing good correlation between concentration and peak area response. The validation parameters were carried out for the marker oleanolic acid and are tabulated (Table-5). HPLC fingerprinting of successive leaf extracts of *Leucas aspera* and *Tridax procumbens*wereperformed (Fig: 9-14).The amount of oleanolic acid (10 mg) present in the two leaf extracts were 0.0589 mg, 0.0815 mg, 0.0298 mg in petroleum ether extract, ethyl acetate extract and methanol extract of *Leucas aspera* and 0.2068 mg, 0.0972mg, 0.0606 mg in petroleum ether extract, ethyl acetate extract and methanol extract of *Tridax procumbens*respectively.

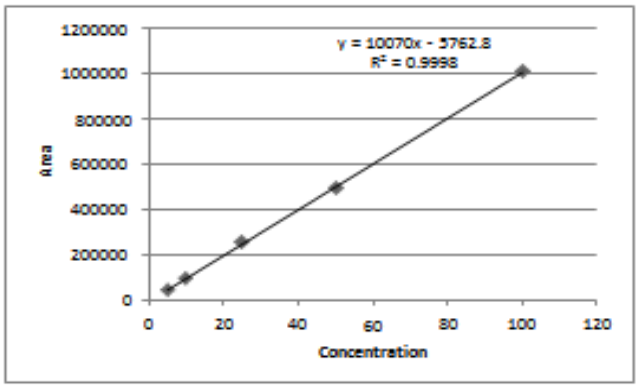

Fig 8: Calibration Graph of Oleanolic acid

Table 4: Calibration data of Oleanolic acid

Concentration (µg/ml)	Peak Area
5	44009
10	95293
25	251401
50	490102
100	1003726

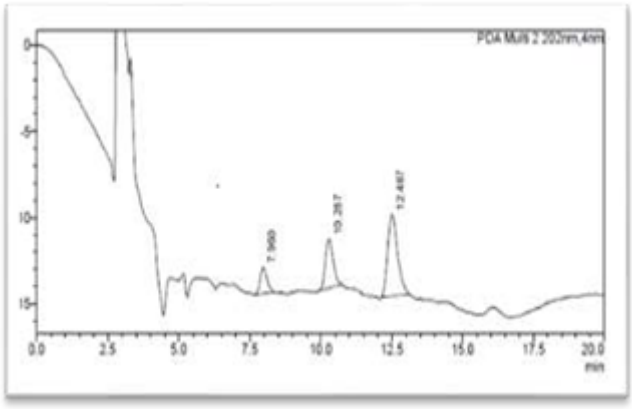

Fig 9: Chromatogram of Petroleum ether extract of *Leucas aspera* at 202nm

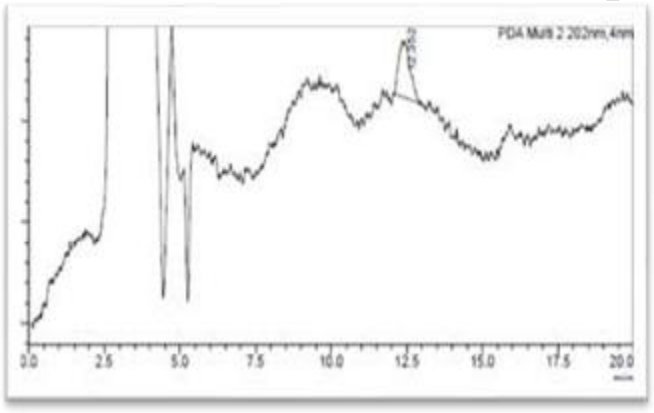

Fig 10: Chromatogram of Ethyl acetate extract of *Leucas aspera* at 202nm

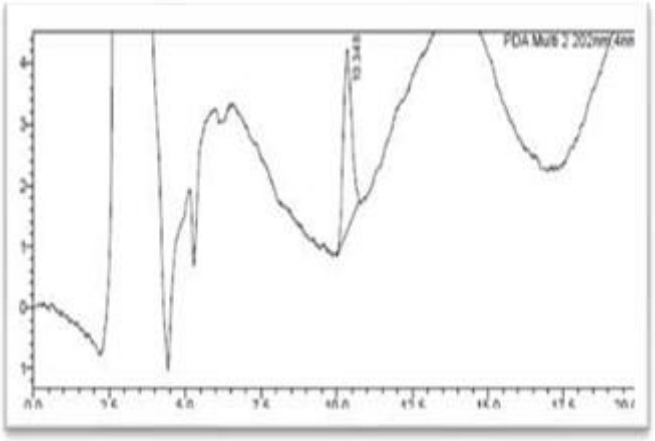

Fig 11: Chromatogram of Methanolextract of *Leucas aspera* at 202nm

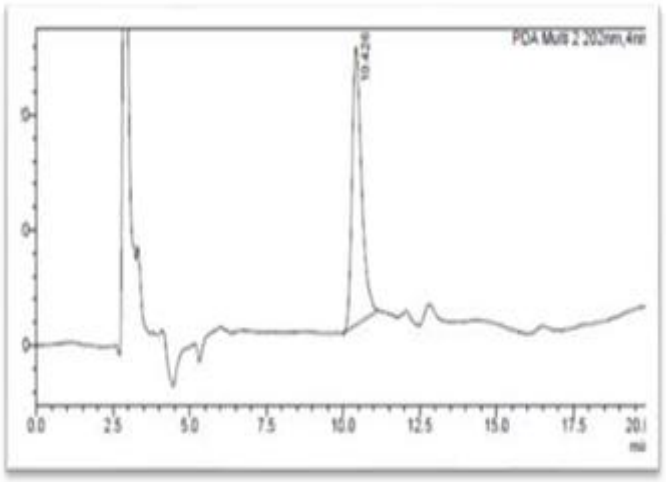

Fig 12: Chromatogram of Petroleum ether extract of *Tridax procumbens* at 202nm

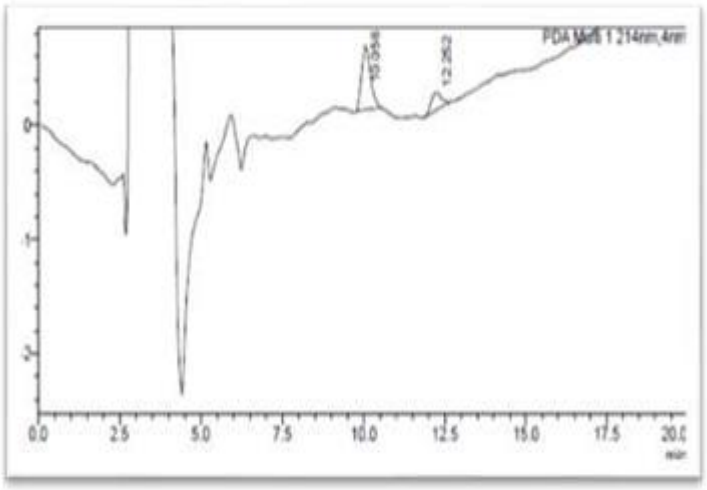

Fig 13: Chromatogram of Ethyl acetate extract of *Tridax procumbens* at 202nm

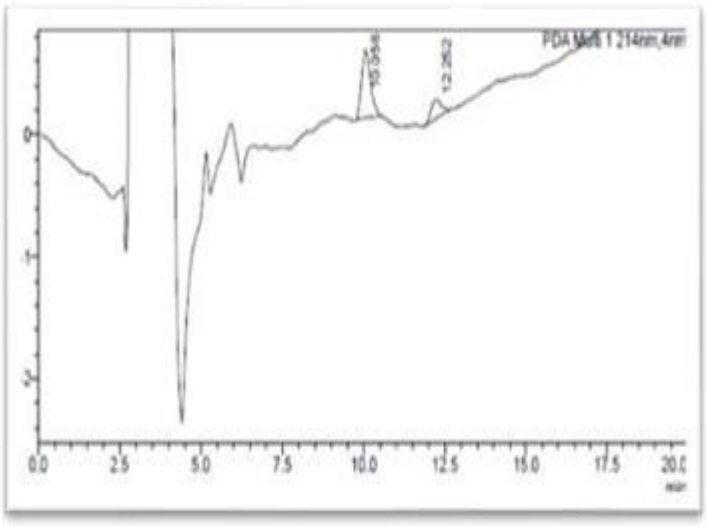

Fig 14: Chromatogram of Methanol extract of *Tridax procumbens* at 202nm

Table 5: Validation parameters for oleanolic acid by HPLC method

PARAMETERS		**HPLC**	
Linearity		1-100 µg/ml	
Correlation coefficient		0.9998	
Regression equation		y=10043x-3847.8	
LOD		0.4113 µg/ml	
LOQ		0.8466 µg/ml	
Precision	Intraday	%RSD*	0.0438
	Interday		0.9657
	Repeatability in sample injection		0.5718
Stability in solution	Room temperature	One day	
	Refrigeration	Five days	
Number of theoretical plates		5438	

Tailing factor	1.310

Biological activity of the extract

The biological activities of the extracts were confirmed by carrying out *invitro* antiinflammatory activity. The antiinflammatory activity was confirmed by xanthine oxidase inhibitory method. The plant extracts were compared with a standard allopurinol for inhibition of xanthine and good activities were observed (Table: 6).Individual ethyl acetate extracts of *Leucas aspera* and *Tridaxprocumbens* have shown inhibitory activity of 24.14% and 50.18% respectively at the concentration of 0.2µg/ml. It was seenthat with increasing concentration of the combined ethyl acetate extracts the activity considerably increased when compared to individual extracts. The combined ethyl acetate extracts of *Leucas aspera* and *Tridax procumbens*exhibited higher inhibition activity of87.86% at the concentration of 0.2µg/ml. Ithas shown synergistic activity.
The inhibitory activity of allopurinol was shown (Table: 6) which gave the IC_{50} value of 29.0±0.2µg/ml, in the concentration range of 5µg/ml to 80µg/ml whose % inhibition was found to be 23.3±0.7 to 92.3±0.92 respectively.

Table 6: %Inhibition of ethyl acetate extracts of *Leucas aspera* and *Tridax procumbens*.

Ethyl acetate extract	Concentration (µg/ml)	% Inhibition concentration (µg/ml)	IC_{50} (µg/ml)
Leucas aspera	0.01	0.35 ± 0.1	-
	0.02	0.72 ± 0.01	
	0.04	5.53 ± 0.22	
	0.08	8.27 ± 0.52	
	0.1	8.92 ± 0.48	
	0.2	24.14 ± 0.76	
Tridax procumbens	0.01	5.45 ± 0.69	0.13 ± 0.36
	0.02	10.25 ± 0.12	
	0.04	34.02 ± 0.39	
	0.08	44.63 ± 0.64	
	0.1	50.08 ± 0.35	
	0.2	50.18 ± 0.41	
Combined extracts of *Leucas aspera* and *Tridax procumbens*	0.01	35.42 ± 0.27	0.02 ± 0.006
	0.02	45.20 ± 0.32	

	0.04	56.94 ± 0.14	
	0.08	78.47 ± 0.44	
	0.1	83.95 ± 0.76	
	0.2	87.86 ± 0.91	
Allopurinol (standard)	5	23.3 ± 0.7	29.0 ± 0.2
	10	38.0 ± 0.9	
	20	46.0 ± 1.2	
	40	66.7 ± 1.42	
	80	92.3 ± 0.92	

*Values are expressed as mean ± SEM of three parallel measurements.

1. Conclusion

Authentication, investigation and standardization of *Leucas aspera and Tridax procumbens* were carried out in the current work. The study was conducted on the successive leaf extracts of *Leucas aspera* and *Tridax procumbens*. The preliminary phytochemical tests for the presence of terpenoids, steroids,alkaloids, glycosides, flavonoids and carbohydrates were carried out for all the extracts. The current research work marches to lay down chromatographic techniques like HPTLC and RP-HPLC methods for the standardization and quantification of oleanolic acid present in *Leucas aspera and Tridax procumbens* plant extracts. The fingerprint and chromatogram of HPTLC and HPLC analysis of biomarker oleanolic acid and herbal extracts will provide a data for identification and standardization of bioactive constituents. The methods were developed and validated conforming with ICH regulation which of paramount importance for the herbal drug manufacturers or the importers. The Xanthine oxidase inhibitory activity was carried out for the *in vitro* antiinflammatory activity has proven that combined ethyl acetate extracts of *Leucas aspera and Tridax procumbens* produces high inhibition of 87.86% at 0.02mcg/ml. Combined ethyl acetate extracts of *Leucas aspera* and *Tridax procumbens* have shown synergistic activity. The result outcomes of this project would support herbal industries and phytochemical researches to use this as primary reference documents and since, till date no such studies are available in the literature for these two plants viz., *Leucas aspera* and *Tridax procumbens*.

CONSENT AND ETHICAL APPROVAL

It is not applicable

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