HPTLC and HPLC Methods to Calculate Oleanolic Acid in Subsequent Leaf Extracts of*Leucas aspera and Tridaxprocumbens* andtheir *In vitro* Antiinflammatory Activity

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ABSTRACT

Aims: The study was started with the goal of quantifying the oleanolic acid from consecutive leaf extracts of *Leucas aspera* and*Tridax procumbens*using a marker oleanolic acid by utilizing HPTLC and HPLC techniques, as well as to perform an 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. Petroleum ether, ethyl acetate and methanol fractions for which the phytochemical analysis were conducted. Standardization of oleanolic acid was performed by using HPTLC and HPLC techniques. Quantification of oleanolic acid in the two plants sequential leaf extracts were done. *In vitro study* of antiinflammatory activity was performed by Xanthine oxidase inhibitory activity in the ethyl acetate fraction.

Results: Alkaloids, glycosides, terpenoids, steroids, flavonoids, saponins, carbohydrates, and proteins were all found in the two plant extracts by using phytochemical screening.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: Standardization of oleanolic acid was conducted 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 conducted. *In vitro* study using xanthine oxidase inhibitory activity showed that combined extracts of ethyl acetate fractions exhibited better antiinflammatoryproperty than the individual extracts of the selected plants.

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

1.INTRODUCTION

All around the world, traditional medical systems have been slowly increasing in significance and recognition. Consequently, a sizable component of the current global medicine business is made up of plant materials and herbal-based drugs that are derived from them. In this

situation, it is important to guarantee that herbal medications and preparations containing them are of the highest possible standard of quality. Consequently, a sizable share of the current global drug business is made up of plant-based substances and medications made from them. [1] Plants contain therapeutically significant elements, frequently in conjunction with several inert chemicals, according to chemical analyses. The medicinally useful components are separated from the plants and processed based on their distinct pharmacological activity. Strong therapeutic substances are developed with the help of medicinal plants. Because of the toxicity and side effects connected to allopathic drugs, the usage of herbal remedies is growing in popularity. [2]

The use of markers to standardize plant extracts is a crucial step in maintaining the quality control of herbal medicines, which will boost their acceptance on a global scale. The term "formulation of standards for a substance or for a procedure" refers to standardization. The procedure of standardization is crucial for assuring the quality control of herbal medicines.

"Standardization refers to the body of knowledge and procedures required to create materials with a tolerable degree of uniformity'' by the American Herbal Products Association (AHPA) states. This is accomplished by using quality assurance techniques in both the manufacturing and agricultural processes to reduce the inherent diversity in the composition of natural products. [3]

Chromatographic fingerprinting techniques are playing an vital role in the standardization of herbal products. These are employed in both quantifying marker chemicals and verifying the presence of various herbs in polyherbal compositions. Plant elements can be separated using the incredibly straightforward HPTLC approach. It is a qualitative tool for the quick, inexpensive, and straightforward component separation of simple mixes, as well as a quantitative analytical tool with a high throughput of simple operations. The most popular use of the very sensitive HPLC analytical technology is the quantitative and qualitative analysis of herbal medicines. [4]

*Leucas aspera*belongs toLamiaceae family, also called "Thumbai." Because it has multiple therapeutic benefits, the entire plant has historically been valued. Flowers from the *L.aspera*plants are employed as insecticides, expectorants, diaphoretics, and stimulants. The fluids from the leaves are thought to be a treatment for chronic rheumatism, psoriasis, and other skin eruptions. The leaves are also used as a pesticide and mosquito repellent. Glucosides, tannins, saponins, sterols, oleic, linoleic, palmitic, stearic, oleanolic, ursolic, and nicotine were discovered in the various portions of L.aspera. [5,6]

Tridaxprocumbens, belongs to Asteraceae family, has a long history of use. In traditional medicine, T.procumbens has been used for centuries to treat wounds, skin conditions, and to prevent blood clotting. It has antileishmanial, antioxidant, anticancer, immunomodulatory, insecticidal, anthelmintic, cardiovascular, antiseptic, antibacterial, and insecticidal effects. It also has anticoagulant, antileishmanial, and anticancer characteristics. [6]

Oleanolic acid, also known as 3b-hydroxyolean-12-en-28-oic acid, is a pentacyclic triterpenoid molecule that is found in many different types of plants. The substance can be found in nature either as a free acid or as the precursor to the triterpenoid saponins known as aglycones, in which it is joined to more than one sugar chains. Oleanolic acid and its derivatives have a number of pharmacological properties that are promising, including hepato-protective benefits and anti-inflammatory, antioxidant, or anticancer properties. [9,10]

The enzyme xanthine oxidoreductase, which is also known as xanthine oxidase and xanthine dehydrogenase, has interconvertible forms. It is a member of the molybdenum iron-sulfur flavin hydroxylase enzyme subclass. Xanthine oxidase is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, and brain as well as the plasma.

The enzyme xanthine oxidase is responsible for hydroxylating purines, specifically for turning xanthine into uric acid. It is one of the primary enzymes responsible for the breakdown of purine nucleotides. It converts hypoxanthine into xanthine and xanthine intouric acid.Uric acid is excreted in Urine. Xanthine oxidase inhibitor is much more beneficial, since it has lesserside effects than uricosuric and anti-inflammatory drugs. [11-13]

2.MATERIAL AND METHODS

Material and Methods

Materials

Chemicals and solvents utilized

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 procured from S.D.FinechemicalsLtd.,Mumbai,India. Oleanolic acid is procured from Sigma Aldrich Private.Limited.,Mumbai,India.

Plant material

The leaves of T.procumbens were obtained at the Sri Ramakrishna Institute of Paramedical Sciences-College of Pharmacy campus in Coimbatore, Tamil Nadu, and the leaves of L. aspera were collected in Salas-Coonoor, Nilgiris. Dr. S.P. Subramanian, Scientist, Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, as recognized and verified the gathered leaves of L. aspera and T. procumbens.

Methods

Preparation of powder for extraction

To prevent the deterioration of phytoconstituents, the plant leaves were collected, removed from unwanted components, cleaned, rinsed, and thoroughly dried at room temperature. The dried leaves were thoroughly pulverized and to produce a semi-coarse powder.

Process of extraction

The organic solvents petroleum ether, ethyl acetate, and methanol were used in a series of extraction procedures to separate the crude drug powders. The solubility of the chosen marker oleanolic acid was taken into consideration when choosing the solvents. Utilizing a Soxhlet apparatus, the extraction procedure was conducted in the continuous hot percolation method. For each solvent, the extraction procedure was run for three days in order to effective extraction from the plant material. Following extraction, the extracts were collected and properly dried at room temperature.

Extraction

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

Table 1a and 1b lists the % yields of the extracts. Until further investigation, the extracts were preserved in the cool place like refrigerator at a temperature of 4 °C. Preliminary phytochemical screening, TLC, HPTLC, and HPLC analyses, as well as in vitro anti-inflammatory research, were performed on the obtained extracts.

Phytochemical screening of plant extracts

The quantitative chemical analyses 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.[14, 15, 16]

Thin layer chromatographic analysis of successive leaf extracts of *L.aspera* and *T.procumbens*.[17]

The R_f value of the marker was compared with the plant extract using basic thin layer chromatography. Using a capillary tube, the collected plant extracts of L. aspera, T. procumbens, and marker were placed as a spot 1 cm from the plate's edge. The plates were then set aside to allow the solvent to evaporate. Plates were then put into a container that had been sealed and previously filled with the vapors of the developing solvents toluene, ethyl acetate, and formic acid (7:3:0.2%v/v/v), taking care to keep the sample away from the developer. The plates were taken out of the chamber and dried after being developed up to two-thirds of their length. Derivatizing agent anisaldehyde-sulphuric acid reagent was then sprayed onto the plates.

Development of validated HPTLC method for the estimation of oleanolic acid in successive leaf extracts of *L.aspera* and *T.procumbens* as per ICH guidelines.[17]

Preparation of a marker's typical stock solution

5mg of oleanolic acid was put into a 10 ml standard flask, dissolved in a tiny amount of methanol, and then brought up to 10 ml with methanol to produce a concentration of 500 µg/ml.

Preparation of derivatizing agent

A mixture of 0.5ml anisaldehyde, 10ml of glacial acid followed by 85ml methanol and 5ml of concentrated H_2SO_4.

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

Method Validation [22]

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, the developed techniques were validated in compliance with ICH criteria.

Linearity

In order to confirm that the data from the linear regression demonstrate a good linear connection over the concentration under examination, the marker was produced in various concentrations and examined by HPTLC technique. Using CAMAG semi-applicators, 100 to 600 ng/band of oleanolic acid were added to the standard stock solution of the marker. It was discovered that the Rf value was 0.54 0.03. The calibration graph (Fig. 1) was used to determine the values for the slope, intercept, and correlation coefficient. Data from calibration are shown in Table: 2.

Limit of detection and limit of quantification

By applying progressively smaller amounts of the drug in triplicate on the plate, the limit of detection and the limit of quantification of the standard were established. The "Limit of Detection" refers to the lowest concentration at which the peak can be identified, while the

"Limit of Quantification" refers to the lowest concentration at which the peak can be quantified. The LOD and LOQ values for the marker are shown in (Table: 3). The detection limit (DL) can be stated as follows:

$$DL = 3.3\ \sigma/S$$

Where S is the slope of the calibration curve and is the response's standard deviation.
The quantitation limit (QL) could be written as follows:

$$QL = 10\ \sigma/S$$

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**

By analyzing the standard marker at two different concentrations in the linearity range of the marker three times on the same day,intraday precision was investigated and %RSD was computed.(Table: 3)

(b)**Interday precision**

By analyzing the reference drug at various concentrations within the linearity range of the marker for three days over the course of a week, interday precision was examinedand %RSD was computed. (Table: 3)

(c) **Reproducibility**

i.Repetition of the test application

By dropping 300 ng concentration of standard marker solution six times on a pre-coated TLC plate, the replicability of sample application was evaluated. Following plate development, scanning, and %RSD was calculated.(Table: 3)

ii.Repeatability of measurement

By dropping 300 ng of a standard marker solution on a TLC plate that has already been coated, the replicability of the sample measurement of peak area was evaluated. Following plate development, the separated locations were scanned six times without moving the plate, and the %RSD was computed. (Table: 3)

Stability studies When the created chromatographic plate is exposed to the environment, the analyte may be susceptible to decomposition. Therefore, it is necessary to confirm the stability of the plates following development.
At various time intervals, the stability of the plate was examined, and the peak regions were compared to the peak regions of newly scanned plates. It was observed that thecreated plate was stable for 30 minutes at room temperature. (Table: 3)

HPTLC analysis of successive leaf extracts of *L.aspera* and *T.procumbens*.

Preparation of stock solution of the petroleum ether extracts of *L.aspera* and *T.procumbens*:

Each plant's 10 mg of petroleum ether extract was weighed, made up to 10ml with methanol as the solvent, and then filtered with whatmann filter paper to achieve a concentration of 1000µg/ml. The solution was added to the TLC plate in amounts of 10 µl/band.

Preparation of stock solution of the ethyl acetate extracts *L.aspera* and *T.procumbens*:

Each plant's 10mg of ethyl acetate extract was weighed, made up to 10ml with methanol as the solvent, and then the solution was filtered with whatmann filter paper to achieve a concentration of 1000µg/ml. The solution was added to the TLC plate in amounts of 10µl/band.

Preparation of stock solution of the methanol extracts of *L.aspera* and *T.procumbens*:

The methanol extract (10mg) of each plant was weighed, produced up to 10ml with methanol as the solvent, and filtered through whatmann filter paper to achieve a concentration of 1000µg/ml. The solution was added to the TLC plate in amounts of 10µl/band.

Recording of the chromatogram

The amounts of oleanolic acid present in the extracts were determined from the calibration graph by comparing the peak regions of the chromatograms for the two leaf extracts with the reference chromatogram. (Fig: 1)

Development of validated RP-HPLC method for the estimation of oleanolicacid in successive leaf extracts of *L.aspera* and *T.procumbens* as per ICH guidelines.[18]

Preparation of standard stock solution of marker

10mg of oleanolic acid that had been precisely weighed was put into a 10ml standard flask, dissolved in methanol, and made up to 10ml with methanol to obtain 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[22]

In terms of linearity, limit of detection (LOD), limit of quantification (LOQ), interday and intraday precision, repeatability of sample injection and measurement, and stability tests, the developed technique was validated in compliance with the ICH criteria.

Linearity

Various concentration of reference samples was prepared by making the final volume with methanol and injected into HPLC system. Linear regression data exhibited good linear relationship over a concentration range of 1 to 100µg/ml for oleanolic acid. The peak regions were noted and a linear graph was plotted between concentrations (x) versus peak region (y). Calibration graphs are given in (Fig: 8) Calibration data are presented (Table: 4).

(c)Repeatability of sample injection

By injecting the same concentration of the standard marker solution six times, the repeatability of sample administration was evaluated and the %RSD was computed. (Table: 5)

Stability

Studies on the stability of the marker solutions were conducted both at room temperature and while refrigerated. By examining for any changes in retention time, resolution, or peak form, stability was evaluated. as compared to a freshly made solution's chromatogram. The marker was observed to be stable for one day at room temperature and for up to five days under refrigeration.

System suitability studies

Number of theoretical plates (N), tailing factor, resolution (Rs), and other system appropriateness characteristics were investigated. The outcomes were displayed in (Table: 5).

Robustness

The following optimum conditions were slightly changed in order to show the method's resilience.

±1 in ratio of methanol: phosphate buffer

± 0.1 in units of flow rate

Under idealized settings, the modest changes resulting from the difference in conditions were discovered to be nearly identical to the standard answer. So, it was determined that the developed procedure was reliable.

Analysis of successive leaf extracts of *L.aspera* and *T.procumbens* by RP-HPLC

Preparation of stock solution of the petroleum ether extracts of *L.aspera* and *T.procumbens*:

Each plant's 10 mg of petroleum ether extract was weighed, made up to 10 ml with methanol as the solvent, and then filtered with whatmann filter paper to achieve a concentration of 1000 µg/ml. A dose of 10µg was administered.

Preparation of stock solution of the ethyl acetate extracts *L.aspera* and *T.procumbens*:

Each plant's 10 mg of ethyl acetate extract was weighed, made up to 10ml with methanol as the solvent, and then the solution was filtered with whatmann filter paper to achieve a concentration of 1000µg/ml. A dose of 10µg was administered.

Preparation of stock solution of the methanol extracts of *L.aspera* and *T.procumbens*:

The methanol extract (10mg) of each plant was weighed, produced up to 10mg with methanol as the solvent, and filtered through whatmann filter paper to achieve a concentration of 1000µg/ml. A dose of 10µg was administered.

Recording of the chromatogram

The peak regions of the two leaf extracts' chromatograms were compared to a reference chromatogram, and the calibration graph was used to quantify the amounts of oleanolic acid present in the extracts. (Fig: 8)

*In vitro*antiinflammatory study[19, 20]

The study was carried out with xanthine oxidase inhibitor with xanthine as a substratum. The two plant extracts were made in ethyl acetate, Allopurinol was utilized as the reference drug.

Principle

The enzyme known as xanthine oxidase is responsible for hydroxylating purines, specifically for turning xanthine into uric acid. It is one of the primary enzymes responsible for the breakdown of purine nucleotides. It turns xanthine into uric acid and hypoxanthine into xanthine. Uric acid is eliminated in urine. The enzyme uricase further oxidizes uric acid to allantoin in smaller animals. But in higher mammals the uricase enzyme is not present which parallels a comparable decline in ascorbic acid synthesis. It is also presumed that uric acid may act as antioxidant as do ascorbic acid in such species. Ascorbic acid and uric acid are

both powerful reducing agents and antioxidants. The blood's antioxidant capacity is enhanced by the uric acid that xanthine oxidase produces. Oxygen (O_2) and hydrogen peroxide (H_2O_2) reduction in some circumstances, it has been suggested that the primary mechanism of oxidative damage is the catalysis of xanthine oxidase by xanthine.

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[13]

The 1ml of the test substance (0.01-0.4 µg/ml), 2.9 ml of phosphate buffer (pH 7.5), and 0.1 ml of freshly prepared xanthine oxidase enzyme solution (0.1 units/ml in phosphate buffer, pH 7.5) made up the assay combination. The reaction was started by adding varied amounts of the substrate solution (2 ml) following a preincubation at 25° C for 15 minutes. For 30 minutes, the assay mixture was incubated at 25° C. A UV spectrophotometer was used to detect the absorbance at 290 nm after adding 1 ml of 1N HCl to stop the process. Standardization was done with respect to allopurinol. % inhibition was obtained using,

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

Where A represents the activity of the enzyme without the compound, B represents the control of A without the compound and enzyme, and C and D represent, respectively, the activities of the compound with and without the enzyme. The experiment was conducted in triplicate, and the percentage inhibition was used to calculate the IC_{50} values.

Statistical analysis

Each experiment was run in triplicate (n=3), the findings of each experiment was expressed as mean ±SEM.

3. RESULTS AND DISCUSSION

The leaves of *L.aspera*and*T.procumbens*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,these plants were chosen for the current investigation.

For successive extractions of powdered leaves of *L.aspera* and *T.procumbens*, the continuous hot percolation method was used. For the purpose of extracting drugs, petroleum ether, ethyl acetate, and methanol have been selected as suitable solvents. The percentage yield of the sequential petroleum ether, ethyl acetate, and methanol extracts of *L. aspera* and *T. procumbens* was determined. (Table 1a,b)

Table 1a: The quantity and yield percentage of the extracted materials

S.NO	Successiveextractsof *L.Aspera*(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 *T.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, the results of preliminary phytochemical analyses showed that alkaloids, glycosides, terpenoids, steroids, flavonoids, and carbohydrates were present. L. aspera and T. procumbens in ethyl acetate extract spots were close to the typical oleanolic acid spot, according to preliminary TLC experiments. As a result, additional analysis was done using these comparisons.

HPTLC method

A mobile phase system toluene: formic acid: ethyl acetate (7:3:0.2%v/v/v) was chosen to use the HPTLC method to measure oleanolic acid.The system produced R_f-valued symmetric peaks. The system produced R_f-valued symmetric peaks0.54 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). The calibration graph revealed values for the slope and intercept of 1.1774 and 52.733, respectively. For the marker, the validation parameters were run, and the results are summarized (Table- 3). Leaf extracts of L. aspera and T. procumbens were successively collected using HPTLC to produce fingerprints. (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 *L.aspera* and 0.5671 mg, 0.1190 mg in ethyl acetate extract and methanol extract of T.procumbens respectively.

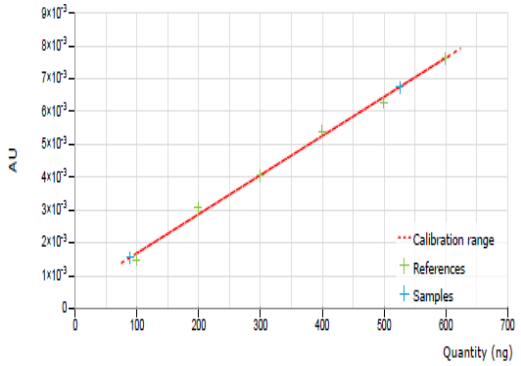

Fig 1: Calibration graph of oleanolic acid (100-600ng/band)

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

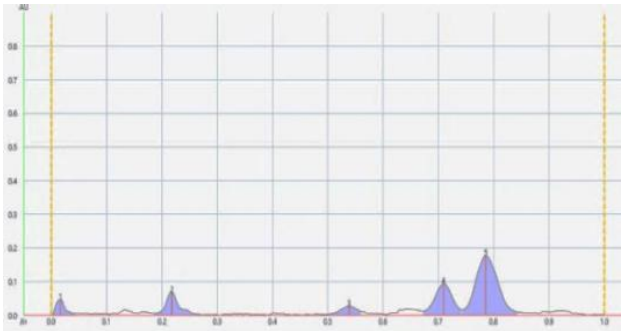

Fig 2: HPTLC fingerprint of *L.aspera*petroleum ether extract at 530nm

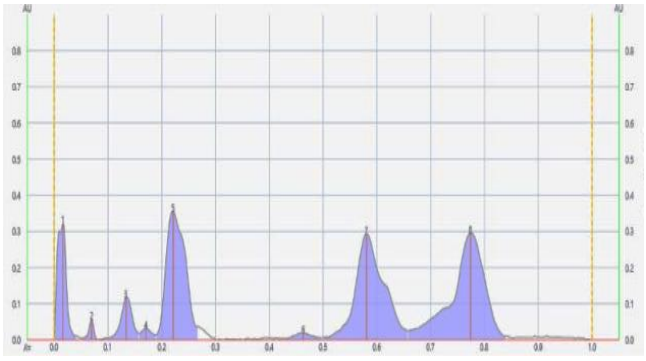

Fig 3: HPTLC fingerprint of *L.aspera*ethyl acetate extract at 530nm

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

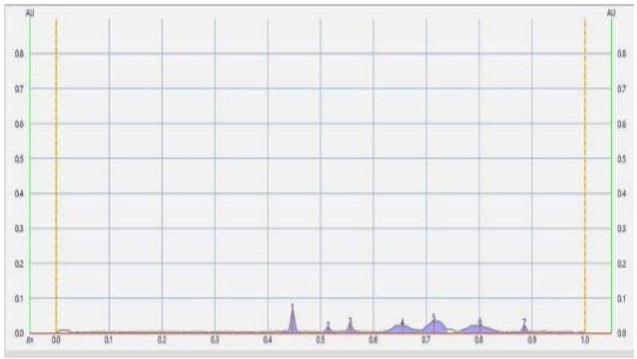

Fig 5: HPTLC fingerprint of *T.procumbens*petroleum ether extract at 530nm

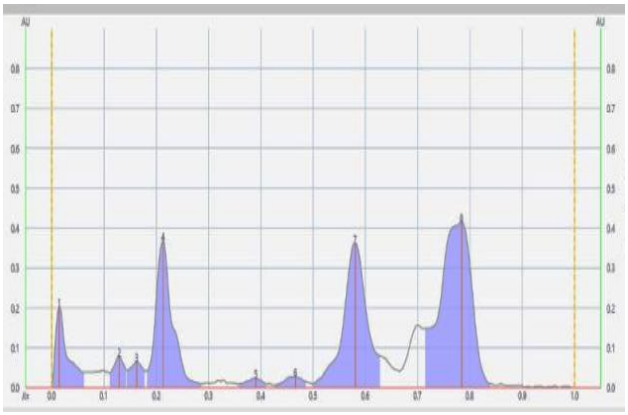

Fig 6: HPTLC fingerprint of *T.procumbens*ethyl acetate extract at 530nm

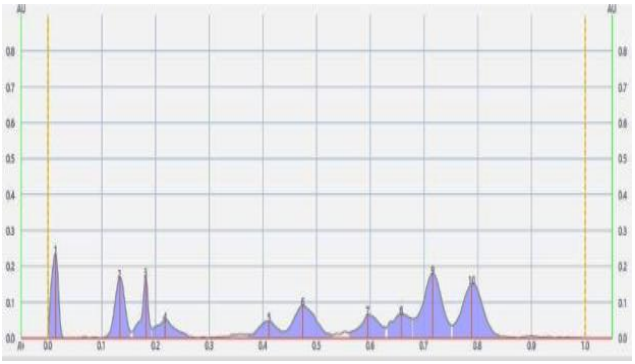

Fig 7: HPTLC fingerprint of *T.procumbens*methanol extract at 530nm

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

Oleanolic acid was determined using RP-HPLC methods, and a mobile phase system with methanol:25mM phosphate buffer (pH-3) in the ratio of 90:10% v/v at flow rate of 1ml/min was used because it produced a symmetric peak shape and minimal tailing with a retention time of 15.7 minutes at 202 nm. Oleanolic acid was discovered to be linear over the concentration range of 1-100 µg/ml, and correlation coefficient values for the substance were discovered to be 0.9998 (Fig: 8). Slope and intercept values from the calibration graph were determined to be 10043 and 3847.8, respectively, demonstrating a strong correlation between concentration and peak area response. For the marker oleanolic acid, the validation parameters were completed and summarized (Table- 5). Characterization of consecutive leaf extracts of L.aspera and T.procumbens were performedby HPLC(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 *L.aspera* and 0.2068 mg, 0.0972mg, 0.0606 mg in petroleum ether extract, ethyl acetate extract and methanol extract of *T.procumbens*respectively.

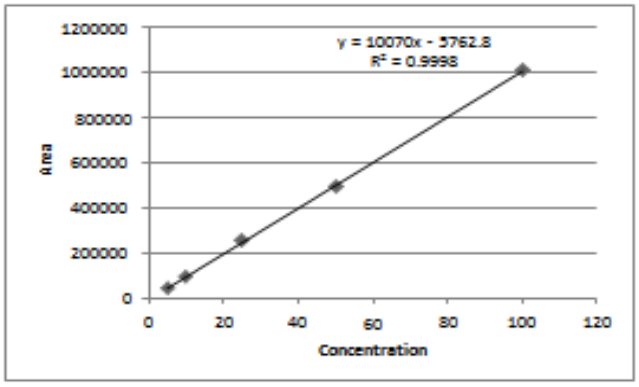

Fig 8: Calibration Graph of Oleanolic acid

Table 4: Calibration data of Oleanolic acid

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

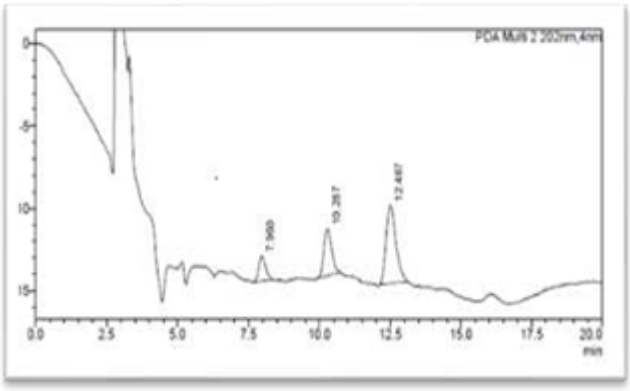

Fig 9: Chromatogram of Petroleum ether extract of *L.aspera*at 202nm

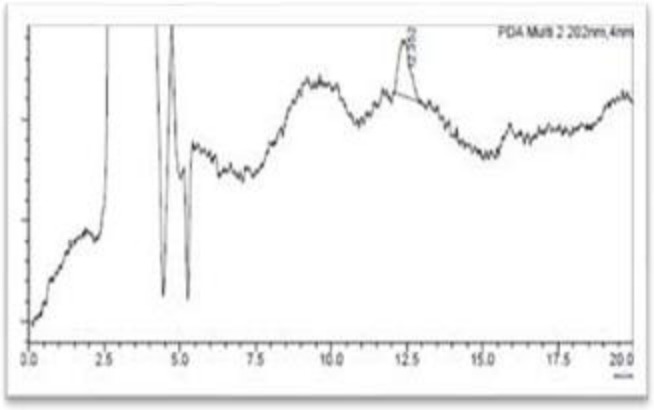

Fig 10: Chromatogram of Ethyl acetate extract of *L.aspera*at 202nm

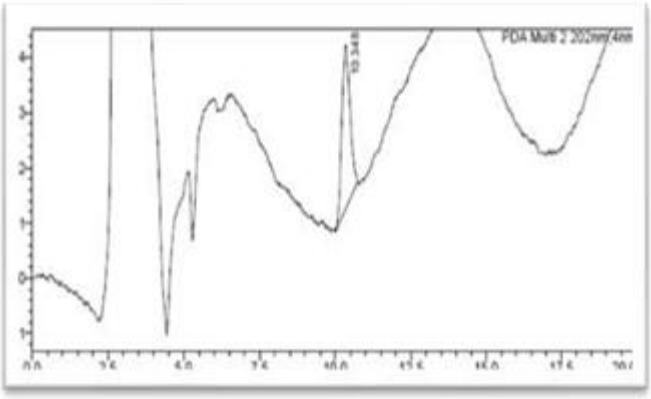

Fig 11: Chromatogram of Methanolextract of *L.aspera*at 202nm

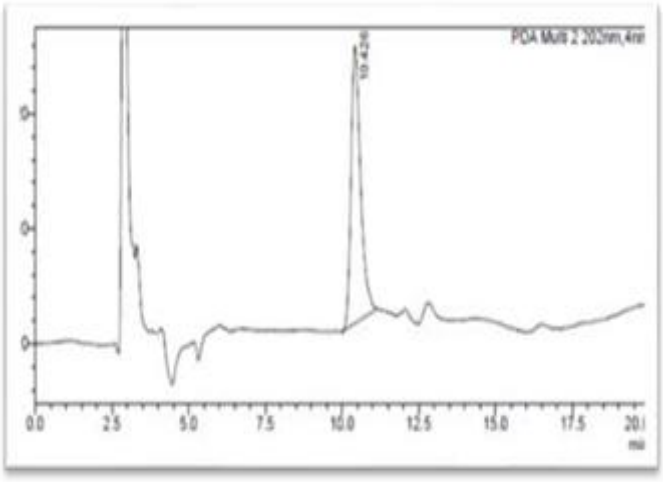

Fig 12: Chromatogram of Petroleum ether extract of *T.procumbens*at 202nm

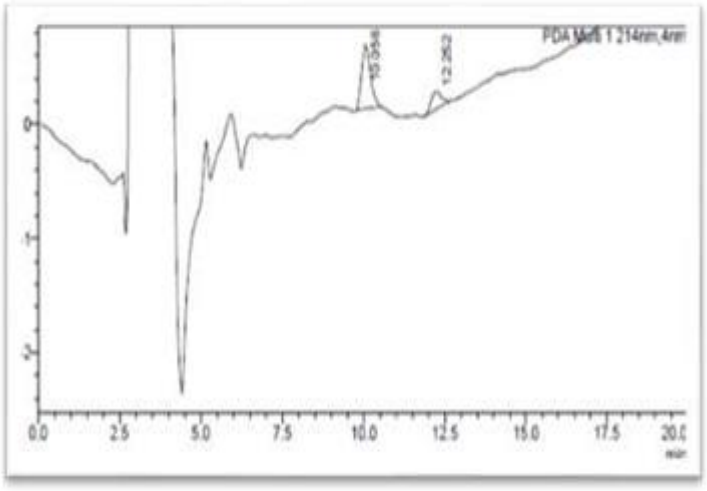

Fig 13: Chromatogram of Ethyl acetate extract of *T.procumbens*at 202nm

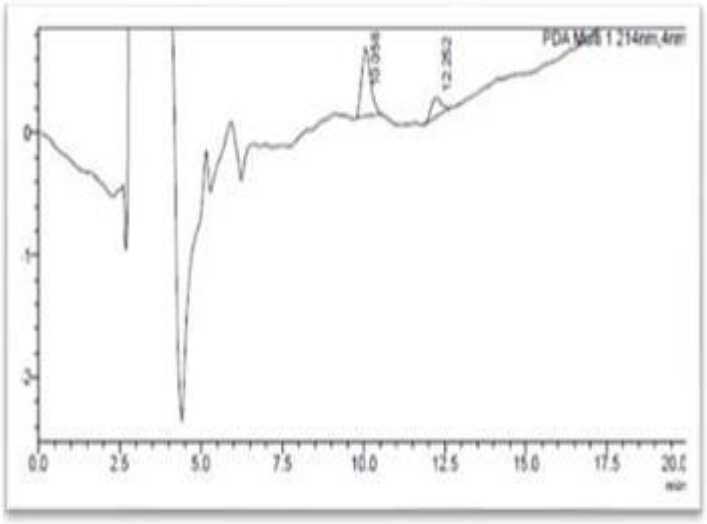

Fig 14: Chromatogram of Methanol extract of *T.procumbens*at 202nm

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 extracts

The *in vitro*antiinflammatory activity was used to confirm the biological effects of the extracts. The xanthine oxidase inhibitory technique was used to confirm the anti-inflammatory efficacy. 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 *L.aspera* and *T.procumbens* 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 *L.aspera* and *T.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 *L.aspera* and *T.procumbens.*

Ethyl acetate extract	Concentration (µg/ml)	% Inhibition concentration (µg/ml)	IC_{50} (µg/ml)
L.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	
T.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 *L.asperaand**T.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.

Conclusion

In the current effort, L. aspera and T. procumbens were authenticated, investigated, and standardized. The consecutive leaf extracts of L. aspera and T. procumbens were the subjects of the study. All of the extracts underwent preliminary phytochemical testing for the presence of terpenoids, steroids, alkaloids, glycosides, flavonoids, and carbohydrates. The goal of the ongoing research is to establish chromatographic methods, such as HPTLC and RP-HPLC, for the standardization and quantification of oleanolic acid found in plant extracts from L. aspera and T. procumbens. Herbal extracts and the biomarker oleanolic acid will both yield chromatograms and fingerprints that can be used to identify and standardize the bioactive ingredients. The procedures were created and confirmed in accordance with ICH regulations, which are of prime importance for importers or manufacturers of herbal drugs. The combined ethyl acetate extracts of L.aspera and T.procumbens provide high inhibition of

87.86% at 0.02 mcg/ml according to the Xanthine oxidase inhibitory activity that was carried out for the *in vitro*antiinflammatory activity. T.procumbens and L. aspera combined ethyl acetate extracts have demonstrated synergistic efficacy. Since, there are currently no such studies published in the literature for these two plants, L. aspera and T. procumbens, the results of this project would support herbal enterprises and phytochemical investigations to use this as primary reference materials.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

CONSENT AND ETHICAL APPROVAL

It is not applicable

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