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Simultaneous Determination of Oleanolic acid and Lupeol from *Triumfetta rhomboidea* using High-Performance Liquid Chromatography

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Abstract

The present study was carried out to develop a reversed phase high performance liquid chromatography (RP-HPLC) method for simultaneous estimation of Oleanolic acid and Lupeol in *Triumfetta Rhomboidea*. The chromatographic separation was achieved using analytical C18 (4.6mm × 25cm), 5µm column under isocratic elution of acetonitrile and 2-propanol (90:10) with a flow rate of 1.0ml/min and the detection wavelength was set at 210nm. The column temperature was maintained at 25^{0}C and the run time was set at 20 minutes. The developed method was validated for linearity, specificity, system suitability, accuracy, limit of detection, limit of quantification and precision as per the ICH guidelines. The calibration curve was found to be linear between the concentration ranges of 0.009-0.108mg/ml for Oleanolic acid and 0.006-0.072mg/ml for Lupeol. The LOD and LOQ of Oleanolic acid were 0.001105mg/ml and 0.003349mg/ml respectively. The LOD and LOQ of Lupeol were 0.001317mg/ml and 0.003992mg/ml respectively. The mean recovery values were in the range of 99.45% and 99.44% for Oleanolic acid and Lupeol. The % RSD values of intra- and inter-day precision analysis were lower than 2%. System suitability parameters were found to be within the acceptance limit.

***Key words*:** *Triumfetta rhomboidea*, RP-HPLC, Simultaneous estimation, Oleanolic acid, Lupeol

Standardization is a significant tool for ensuring the quality of herbal drugs. The drug identity, purity, content, chemical and other biological properties determine the quality of the products. Quantitative estimation of chemical markers within the crude drugs is usually troublesome. Separation of markers using optimal separation techniques offer high resolution of the compounds and least interferences [1]. Now days traditional systems of medicine have been explored in current global drug market. Quality control and Standardization both are most important aspect for the herbal drug formulation. Generally herbal formulations are based on polyherbal formulation. Plant based drugs are extracted, isolated and purified for their therapeutic utility based on their selective pharmacological activity. Standard markers are used quantitative and qualitative analysis for herbal drug formulation. Lack of proper standard parameters and methods for the standardization of herbal formulation and preparation has led to several instances of substandard herbs and adulterated herbs coming into existence [2]. The plant *Triumfetta rhomboidea* (Family: Tiliaceae) is commonly known as Chinese bur or Zinjurdi, is a shrub generally found in tropical and subtropical regions of India, Ceylon, Malay Peninsula, China, Africa and in America. TR contains carbohydrate glycosides, alkaloid glycosides, phytosterol, steroids, flavonoids, tannin and phenolic compounds. Various Parts of the plant used for medicinal purpose which are fruit, flower, leaves, bark and root. Root is used as tonic styptic, galactagogue, aphrodisiac, cooling, useful in dysentery and as diuretic. TR is also used as anti-snake bite. Pounded roots are used to treat Intestinal ulcer. Leaves, Flowers and Fruit are mucilaginous, demulcent, astringent, and also used in gonorrhea and against leprosy. Fruits, flowers and leaves of this plant is used as demulcent and astringent. Bark and fresh leaves are used to treat diarrhea, dysentery like disease [3]. *Triumfetta rhomboidea* contains Lupeol and Oleanolic acid along with various other chemical markers [4]. Oleanolic acid (OA) is a natural product that has been isolated from several food and medicinal plants. It is a pentacyclic triterpenoid which is abundant in plants of the Oleaceae family such as the olive plant. In these plants, OA is often found in the epicuticular waxes where they act as a barrier against pathogens and water loss. Apart from its ecological roles in plants, some pharmacological activities such as anti-oxidant, anti-tumour, anti-inflammatory, anti-diabetic, anti-microbial effects have been attributed to OA in different models of diseases. Oleanolic acid has been used as a hepatic drug for over 20 years in China because of its hepatoprotective effect. The exploration of the other biological activities of OA and its synthetic derivatives can lead to the development of potent drugs for the treatment or management of human diseases [5]. Lupeol is reported to

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exhibit a spectrum of pharmacological activities against various disease conditions. These include conditions such as inflammation, arthritis, diabetes, cardiovascular ailments, renal disorder, hepatic toxicity, microbial infections and cancer [6]. The present research work analysis the simultaneous determination of oleanolic acid and lupeol from the medicinal plant, *Triumfetta Rhomboidea.*

MATERIALS AND METHODS

Chemicals

HPLC grade 2-propanol and Acetonitrile was procured from Merck Life Science Private limited, Vikhroli, India. Reference standard Oleanolic acid and Lupeol was purchased from the Sigma Aldrich.

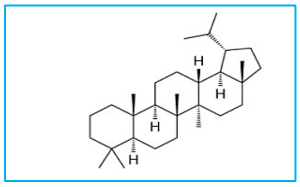

Fig 1 Lupeol

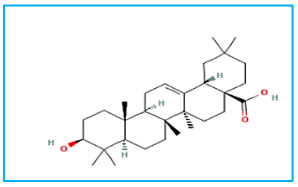

Fig 2 Oleanolic acid

Plant material

Triumfetta rhomboidea was collected from the Saphale, Palghar region, Maharashtra, India. The selected plant was authenticated at VIVA College, Virar. The leaves were washed with water to remove any dust particles and dried in shade. The whole plant was dried and powdered, and then sieved through mesh size 0.22 micron and stored at 25 °C in an airtight ambered coloured container.

Preparation of stock solutions

Standard stock solutions of pure compounds were prepared separately by dissolving 10 mg of each compound in 10 mL of Methanol to get the concentration of 1000 µg/mL. For the calibration curve, solutions from 9-108 µg/mL and 6-72 µg/mL were prepared from the above stocks for Oleanolic acid and Lupeol respectively.

Sample preparation

About 0.1 gm of dried whole plant powder of *Triumfetta rhomboidea* was accurately weighed in a round bottom flask. 10 ml of methanol was added to the flask and the mixture was kept on the shaker for overnight. The extract was then filtered through Whatman filter paper no. 41 (Merck, Mumbai, India). This extract was used for further analysis.

Instrumentation

Jasco HPLC system was utilized for the development and validation of liquid chromatography, facilitated with a pump (model: PU 2080 plus), an autosampler (ALS) (model: AS2055 plus), and C18 (250 cm × 4.6 mm), 5µm column (Crest Pak), and the detector included UV/VIS (model: UV 2075 plus) operated at 210nm. Chrom Nav Software (version) was used in order to process and evaluate the obtained results. Additionally, an analytical balance demonstrating four digits was used (Contech) for weighing purposes and a sonicator (PCI analytics, model: USB1.5L50H, India) was used prior to dissolve the reagents.

Chromatographic conditions

The solvent mixture of 2-propanol: Acetonitrile (10:90%v/v) at a flow rate of 1.00ml/min, the column was maintained at room temperature and the detector was set at 210nm.The mobile phase was filtered through 0.45µm membrane filters and degassing was done by sonication for 20 min. The injection volume and flow rate were 10µL and 1.0 mL/min, respectively, in addition to the runtime of 20 minutes.

Validation of the method

The developed LC method was validated according to the ICH guidelines [7]. The validation parameters evaluated include specificity, linearity, precision, accuracy and robustness of the method.

Specificity: Selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers, and known (or likely) degradation products that may be expected to be present in the sample matrix[8].The peaks from sample solutions were confirmed by comparing the Rt of the peaks to those of the standards.

Linearity: The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure[7]. Working standard solutions of Oleanolic acid 81µg/ml – 135µg/ml and Lupeol 30µg/ml – 66µg/ml were injected in triplicate. The peaks areas of Oleanolic acid and Lupeol were plotted against concentrations. Subsequently, the linearity was examined with the help of a calibration curve to assess slope, correlation coefficient, and intercept on the Y axis.

Precision: The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation of series of measurements [8]. Variability of the method was studied by analysing quality control samples of Oleanolic acid 81-135µg/ml and Lupeol 30-66 µg/ml on same day (intra-day precision) and on different days (inter day precision) and the results were expressed in % RSD.

Accuracy / Recovery: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists [8]. The tests were carried out to check the accuracy of the method by adding two concentration levels (50% and 100%) of the mixed standard solutions to known amounts of *Triumfetta rhomboidea* samples. The resultant samples were then analyzed with the standard method. The average percentage recoveries were calculated by ratio of the detected amount against added amount.

Limit of detection (LOD): Limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value [8]. The calibration curve was analyzed for seven times and Standard Deviation (SD) of the intercept was evaluated using the given formula to calculate LOD.

$$LOD = (3.3 \times SD) / Slope$$

Limit of quantification (LOQ): The limit of Quantitation (LOQ) or Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy [8]. The calibration curve was analyzed for seven times and Standard Deviation (SD) of the intercept was evaluated using the given formula to calculate LOQ.

$$LOQ = (10 \times SD) / Slope$$

Robustness: Robustness is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage. Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples [8].

RESULTS AND DISCUSSION

Method development and optimization

The various physicochemical characteristics of Oleanolic acid and Lupeol were acquired from the previously published literature. A suitable analytical method was developed prior to selecting preliminary reverse phase HPLC-UV chromatographic conditions such as stationary phase, mobile phase, determining wavelength and procedure of sample preparation. In order to achieve the goal, many mobile phase combinations were tried from which solvent mixture of 2-propanol and Acetonitrile gave good resolution. This mobile phase gave the resolution of Oleanolic acid and Lupeol at 4.79 and 14.88 respectively [9-10]. These standards were also resolved from other components present in the sample extract enabling simultaneous quantification.

Method validation

Specificity: The developed method was tested to determine specificity to make sure that no other interference from the solvent and matrix are present in the chromatograms of the three standards. The blank (mobile phase and extracting solvent), reference of the three standards and sample were injected and their chromatograms were compared. No interference was observed due to the blank in the main peaks of the reference standard [11-13]. Hence, it can be concluded that the developed method is specific for the simultaneous determination of oleanolic acid and lupeol.

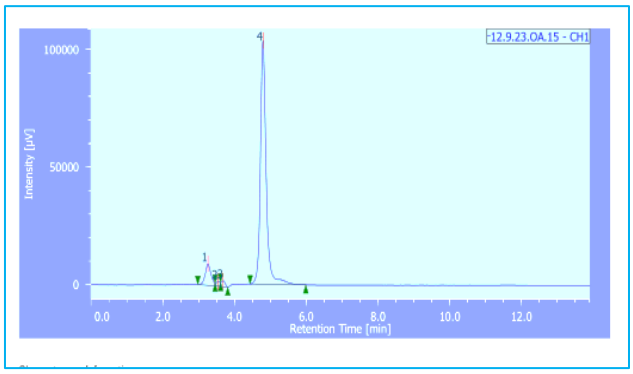

Fig 3 Chromatogram of oleanolic acid

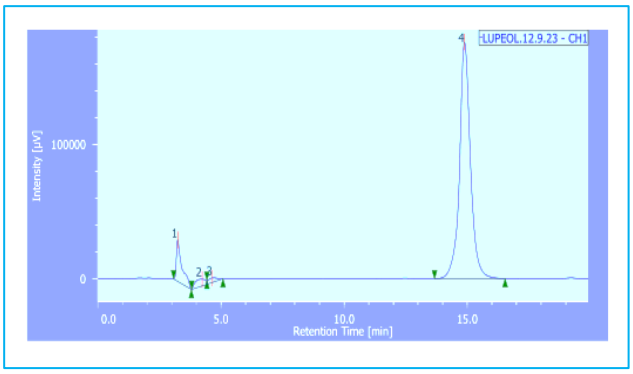

Fig 4 Chromatogram of lupeol

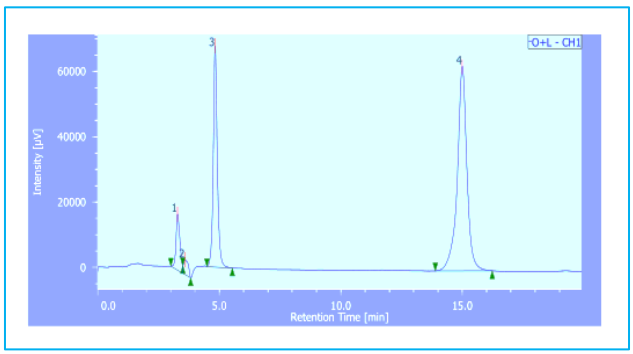

Fig 5 Chromatogram of oleanolic acid and lupeol

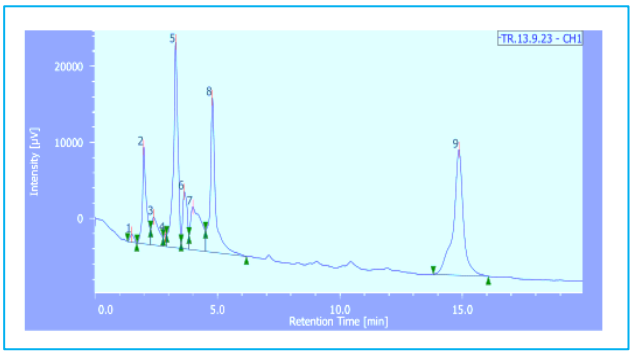

Fig 6 Chromatogram of *Triumfetta rhomboidea*

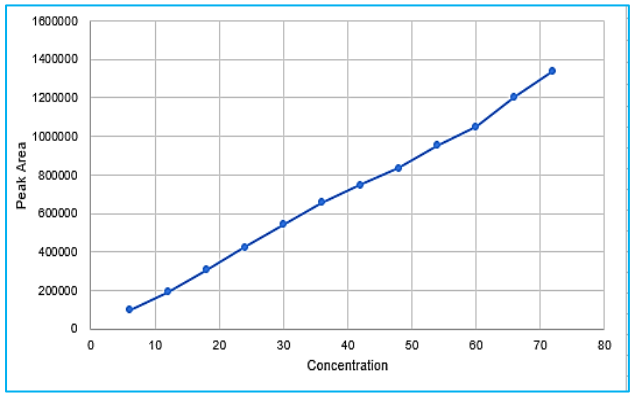

Fig 7 Calibration curve of lupeol

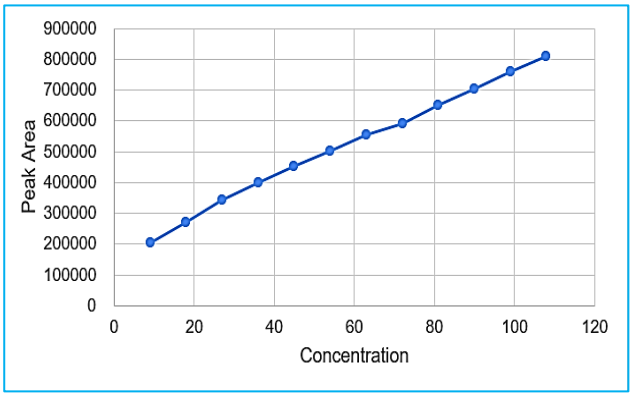

Fig 8 Calibration curve of oleanolic acid

Linearity: The linearity of the response (peak area) of the drugs was determined at six concentration levels ranging from 25% to 150% of the assay concentration for each of the three drugs. The assay concentration (100%) was 0.108mg/ml for Oleanolic acid and 0.048mg/ml for Lupeol. The seven concentration levels were in the range of 0.081 – 0.135mg/ml for Oleanolic acid and 0.030 – 0.066mg/ml for Lupeol.

Precision: Precision was determined as % RSDs of the peak areas of the drugs. The % RSD for the repeatability, intra- and inter- day precisions were less than 2% for the respective drugs at the assay concentration of 0.108 and 0.048 mg/ml for oleanolic acid and lupeol respectively. Results of the % RSD values of repeatability and intermediate precision studies showed that the method is precise for simultaneous determination of the three standards [14].

LOD and LOQ: These parameters were assessed by determining Oleanolic acid and Lupeol as per the formula. The LOD and LOQ of Oleanolic acid were 0.001105mg/ml and 0.003349mg/ml respectively. The LOD and LOQ of Lupeol were 0.001317mg/ml and 0.003992mg/ml respectively.

Accuracy / recovery: Accuracy was evaluated as percent recovery of the added standards of the three drugs at the concentration levels of 50% and 100% of the assay concentration of each standard to the equivalent weights in the whole plant powder. The percentage recovery of Oleanolic acid and Lupeol were 99.45% and 99.44% respectively. The percentage recoveries of the three standards were in the range of 98–102% indicating the good accuracy of the optimized method for simultaneous determination of Oleanolic acid and Lupeol [15].

Robustness: This testing parameter was analyzed by assessing the impact of slight alteration in chromatographic conditions. The data of robustness evaluation demonstrated that a minor modification of method conditions including flow rate and pH of mobile phase was found to be robust within the desired range [16].

CONCLUSION

Standardization of medicinal plants is an important aspect and therefore it requires proper identification and estimation of chemical markers. This research work provides a simple reverse phase HPLC method for simultaneous determination of Oleanolic acid and Lupeol from *Triumfetta rhomboidea*. The developed method is validated for various parameters and was found to be accurate, specific and precise. The developed method will be helpful in various herbal industries manufacturing polyherbal formulations.

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