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DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ESTIMATION OF URSOLIC ACID AND OLEANOLIC ACID IN METHANOLIC EXTRACT OF *DRAGEA VOLUBILIS*

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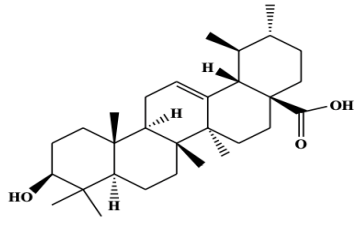
Abstract

The Aim of the present study is to develop and validate a HPTLC method for estimation of ursolic acid and oleanolic acid in methanolic extract of *Dragea volubilis (DV)* by using TLC densitometry technique. Shade dried leaves obtained from *Dragea volubilis* were sequentially extracted in a Soxhlet extractor with petroleum ether and methanol. The extract was further studied using TLC and HPTLC for quantitative analysis. A mixture of Toluene: ethyl acetate (4:1) for ursolic acid and Toluene: ethyl acetate (3:2) for oleanolic acid were used as a mobile phase. The chromatography was performed on a TLC plate precoated with silica gel GF_{254} and the developed TLC plate were visualized and quantified at 580 nm. The quantity of ursolic acid and oleanolic acid in the methanolic extract was found to be 0.0512 ± 0.0001 ng and 0.0306 ± 0.0000 ng respectively. R_f value 0.25 and 0.3 were observed respectively for ursolic acid and oleanolic acid respectively .The method was validated for Instrumental precision, repeatability, coefficient of determination (r^2), linearity range (ng), LOD (ng), LOQ (ng), intra-day and inter-day precision for both ursolic acid and oleanolic acid and all the parameters were found to be within the acceptable range as per the ICH guidelines. The newly developed HPTLC method was found to be prompt, cost effective, precise accurate and reproducible for the qualitative as well as quantitative analysis of ursolic acid and oleanolic acid in methanolic extract of *Dragea volubilis*.

Keywords: *Dragea volubilis,* Oleanolic acid, Ursolic acid, HPTLC, Validation.

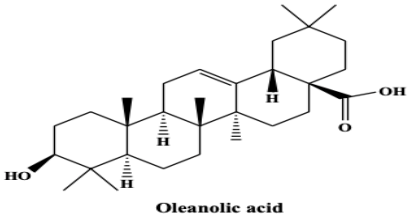
Introduction

Phytochemical, Phytopharmacological and Phyto-molecular studies had an important role in drug development. (Khanna *et al*., 2018) Advancement of chromatographic techniques and spectral analysis has changed the entire perspective of herbal drugs in drug discovery. *Dragea volubilis* (L.f.) Benth is a fairly large woody plant having smaller size than a tree with several main stems arising at or near the ground. It is extensively distributed in southern parts of India and is also found in other Asian countries like Srilanka, Myanmar, Indonesia, Thailand and China (Hossain *et al*., 2012). Many phytoconstituents have been isolated from this plant that has shown promising results in some studies. The plant is plentiful source of phytoconstituents. Various studies have been reported the presence of Drevogenin A, D and P, drebbysogenin Dregeosides, hyperoside, kaempferol, polyhyroxy pregnane glycosides volubilioside A, B, C (Biswas *et al*., 2010). A wide variety of pharmacological activities were reported with phenolic acids like chlorogenic, hydroxycinnamic acids, flavonol glucosides, rutin, kaempferol 3-rutin obtained from *Dragea volubilis* (Hossain *et al*., 2010). The plant is used as traditional medicine in ayurveda to cure many ailments like inflammation, pain and wound healing and it is considered to be a wonder herb (Kirtikar *et al*., 1935; Vyas *et al*., 2016; Vyas *et al*., 2018). An active fraction obtained from leaves of *Wattakaka volubilis* was found to possess anti-leukemic activity and effect was comparable to standard drugs (Nandi *et al*., 2012). Extracts obtained from leaves from *wattakaka volubilis* has shown potential to improve dyslipidemia and diabetes in rats (Mohan *et al*., 2010). Polypregnane glycosides extracted from *Dragea volubilis* has shown potential neuroprotective activities in cerebral ischemic rats (Jhadav *et al*., 2013). The extracts of *Dragea volubilis* and polypregnane glycosides isolated from it have shown chondroprotective properties. It has also shown inhibitory effect of pro-inflammatory cytokine, IL-1β on the cartilage explants (Sanyacharernkul *et al*., 2009). Comprehensive chemical exploration of *Dragea volubilis* revealed presence of phenolic acids like chlorogenic acid, ursolic acid, flavonol glucosides like Kaempferol and triterpenoids (Sahu *et al*., 2002). Most of the pharmacological activities of *Dragea volubilis* can be attributed to its strong anti-oxidant properties. Moreover, phytochemical analysis reported the presence of terpenoids, steroids, glycosides alkaloids, tannins, flavonoids, phytosterol, anthocyanidins etc. (Panigrahi and Vyas, 2015; Chauhan *et al*., 2017) However still a little is known about pharmacological activities of isolated compounds. Ursolic acid and oleanolic acid derivatives have potential acetylcholinesterase inhibition activity and have shown potential anticancer agents (Csuk *et al*., 2019). *In vivo* studies exhibiting analgesic and anti-inflammatory activities were also reported for ursolic acid and oleanolic acid (Wilson *et al*., 2006).



Ursolic acid

(a)



(b)

Fig. 1: Chemical Structure of (a) Ursolic acid; (b) Oleanolic acid

Hence there is a need to develop and standardize a chromatographic method for quantification of ursolic acid and oleanolic acid, for standardization of *Dragea volubilis.* There are no such methods reported for quantification of ursolic acid and oleanolic acid in *Dragea volubilis* so far. Hence, the present work focuses on development and validation of high-performance thin layer chromatographic method for the estimation of ursolic acid and oleanolic acid in methanolic extract of *Dragea volubilis.*

Materials and Methods

Materials

The sample of fresh leaves of *Dragea volubilis (Asclepiadaceae)* was collected from Nilgiri hills, Tamil Nadu and authenticated from Punjabi university, Patiala vide: specimen no: 115. The standard marker compounds used for the study i.e. ursolic acid and oleanolic acid was procured from Sigma Aldrich. Other chemicals used during the study were of analytical grade and were used as received from different sources.

Extraction procedure

Shade dried leaves were crushed to powder with grinder and defatted with petroleum ether (60-80°C) to detach the adhesive materials and then extracted with hot methanol in a Soxhlet apparatus (Biju *et al*., 2007). Extraction with methanol was carried until the solution in the side tube of the apparatus became almost clear. This methanol extract was concentrated with vacuum rotary evaporator (Vyas *et al*., 2017; Prabhu *et al*., 2007).

Thin layer chromatography (TLC)

TLC plates (10cm x 20cm; E. Merck) precoated with silica gel GF_{254} (stationary phase) were used for the analysis. The plates were eluted in Toluene: ethyl acetate: (4:1) for ursolic acid and Toluene: ethyl acetate (3:2) for oleanolic acid. The methanolic extract was applied on the TLC plate by using capillary tube and elution was carried out with above mentioned solvent system. Then the TLC plates were sprayed with sulphuric acid (H_2SO_4) (20%) in methanol and heated in oven at 105ºC. Accordingly, R_f values were computed by measuring the distance travelled by sample and solvent front. (Anuradha *et al*., 2018; Vyas, 2018).

High Performance Thin Layer Chromatography (HPTLC)

The optimized solvent system was Toluene: ethyl acetate: (4:1) for ursolic acid and for Toluene: ethyl acetate (3:2) for oleanolic acid. Methanol was used as solvent for sample application. Chromatographic separation was achieved on precoated TLC plates with silica gel GF_{254} as the stationary phase using above mentioned solvent systems. (Di *et al*., 2003) Continuous radiation of UV spectrum between 190 and 600 nm was emitted from a deuterium lamp. All determinations were carefully done at room temperature with detection wavelength at 580 nm. Peak areas were plotted against the corresponding values of concentration to get the linear calibration regression (Amir *et al*., 2013; Vyas *et al*., 2019).

Spraying agent

Development of plates was performed using a Camag glass tank and developed dried plates were immersed in 0.5 % anisaldehyde-sulphuric acid reagent. Plates were heated at 110°C for 2 min till the spots were distinguishably visible and was then visualized in TLC scanner at 580 nm respectively for ursolic acid and oleanolic acid.

Assay

Standard and test solutions were observed on a precoated TLC plate. The percentage quantity of ursolic acid and oleanolic acid present in *Dragea volubilis* methanolic extract were estimated by comparing the areas computed for the test and standard solution.

Detection of Linearity

Evaluation of linearity of different concentrations of standard solution of ursolic acid and oleanolic acid was done. The calibration curves of both the standard were observed to be linear over a range from 150 to 900 ng/spot on TLC plates in the form of sharp bands. The response for detection of ursolic acid and oleanolic acid was measured for each band at wavelength of 580 nm, using Camag TLC Scanner equipped with win CAT software. The peak areas were observed for all concentrations of ursolic acid and oleanolic acid. A graph depicting peak areas of ursolic acid and oleanolic acid for linearity was plotted against applied concentration of ursolic acid and oleanolic acid in ng respectively (Mridula *et al*.,2009).

Method Validation

The newly developed method was checked for accuracy, perfection and meticulousness. Repeated scanning (n=5) of the same spot of ursolic acid and oleanolic acid respectively was done to confirm the reproducibility of the method. Solutions of standard ursolic acid and oleanolic acid were analyzed at a concentration of 300 ng and 450 ng/spot five times on the same day and on the different days to check inter-day and intra-day precision. Results confirmed the accuracy of the method. To analyze the accuracy of the method the recovery studies were executed by the standard addition method.

The percentage recovery and average percentage were calculated. Recovery trial was done for the added quantities of standards and were studied at three variant levels in a similar manner to that of reported assay (Rashmin *et al*., 2012). Additions were repeated for three times on three different days. Recovery of the added amount of standard was calculated for the same. Evaluation of Limit of detection and limit of quantization were also calculated by the proposed method.

Results and Discussion

The percentage yield was found to be 0.0512 ± 0.0001 for ursolic acid and 0.0306 ± 0.0000 for oleanolic acid.

Thin Layer Chromatography (TLC)

R_f value = Distance travelled by sample/Distance travelled by solvent. The R_f value was calculated as 0.25 and 0.3 for ursolic acid and oleanolic acid and which was in accordance with the reported value of ursolic acid and oleanolic acid marker confirming the content of ursolic acid and oleanolic acid in extract (Vyas *et al*., 2018).

High Performance Thin Layer Chromatography (HPTLC)

Silica gel GF_{254} HPTLC plates were used as stationary phase in this method and mobile phase as Toluene: ethyl acetate: (4:1) for ursolic acid Toluene: ethyl acetate (3:2) for oleanolic acid respectively (Larsen *et al*., 2004). Ursolic acid and oleanolic acid in *Dragea volubilis* leaves extract were quantified at the at a R_f value of 0.25 and 0.3 respectively. The outcomes of method validation parameters are shown in table 1 below.

Calibration curve

The calibration curve showed linearity in the range of 300 to 700 ng, 200-1300 ng for ursolic acid and oleanolic acid respectively and the correlation coefficient was found to be 0.997 and 0.998 (Fig. 3). The identity of the band of ursolic acid and oleanolic acid in the sample extract was observed by overlaying the tracks showing peak values of sample with that of marker (Fig. 2 a & b). Limit of detection was 146 ng/spot and the limit of quantification was found to be 125 ng. The method was substantiated in terms of accuracy, perfection and meticulousness expressed as % CV (coefficient of variation) which were found to be less than 1.11%. and 0.97%. The recovery values obtained were 95.85 to 99.28% with an average percentage recovery of 99.46% showing the accuracy of the method.

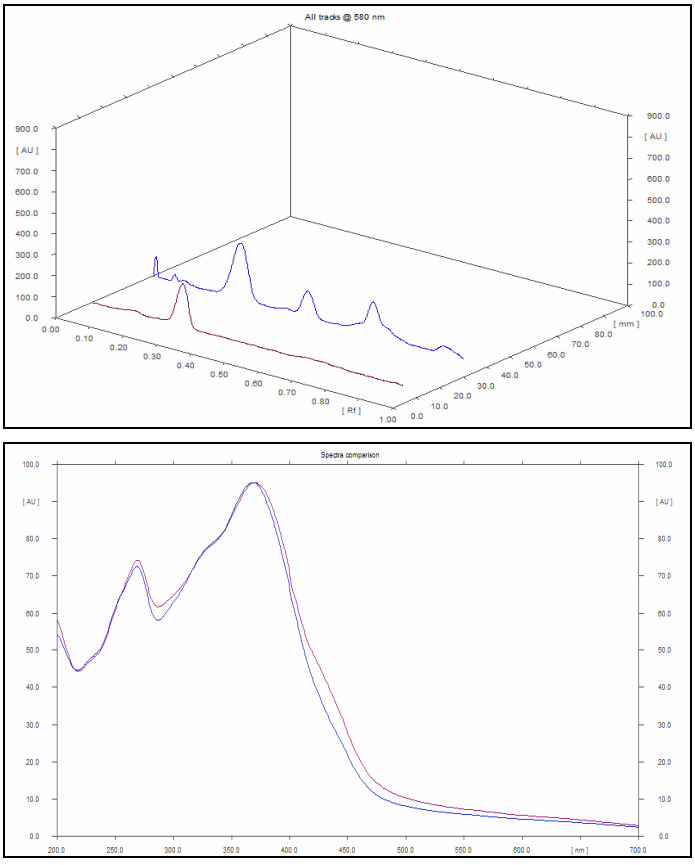


Fig. 2 : (a) Spectra overlay of ursolic acid;
(b) Peak overlay of ursolic acid

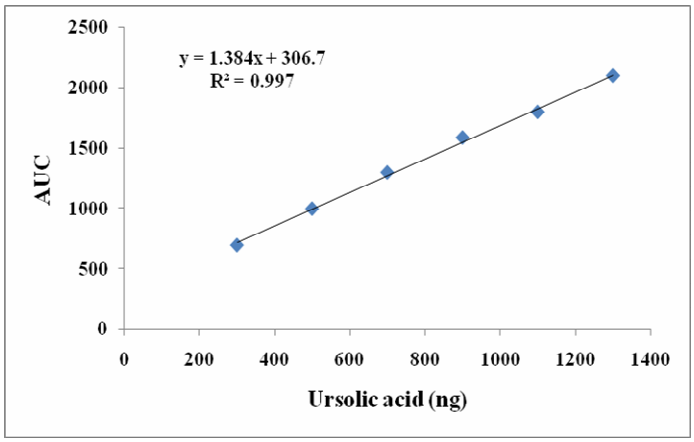
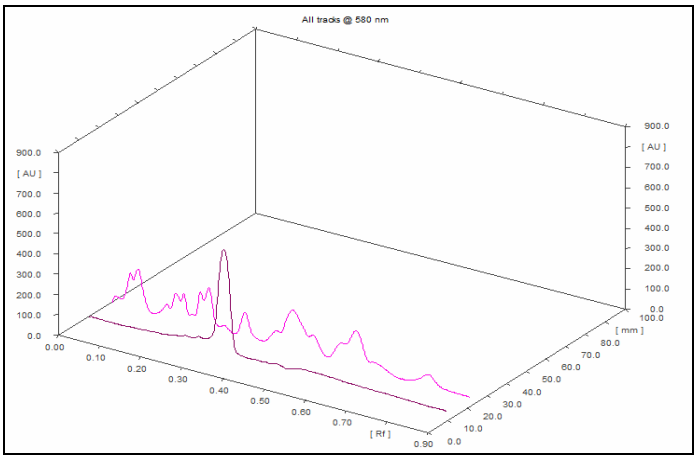


Fig. 3 : Calibration curve for ursolic acid



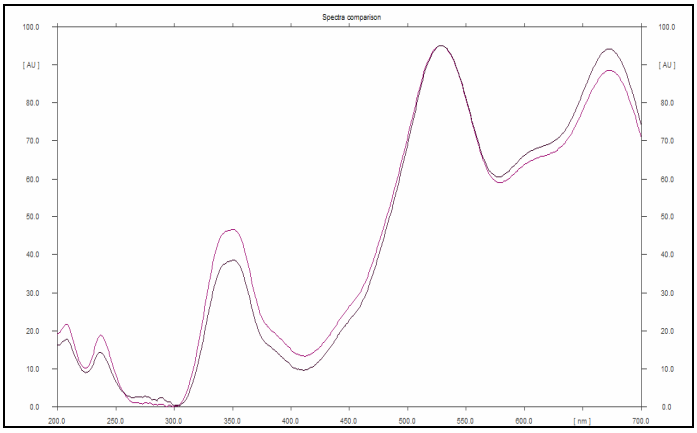


Fig. 4 : (a) Spectra overlay of oleanolic acid;
(b) Peak overlay of oleanolic acid

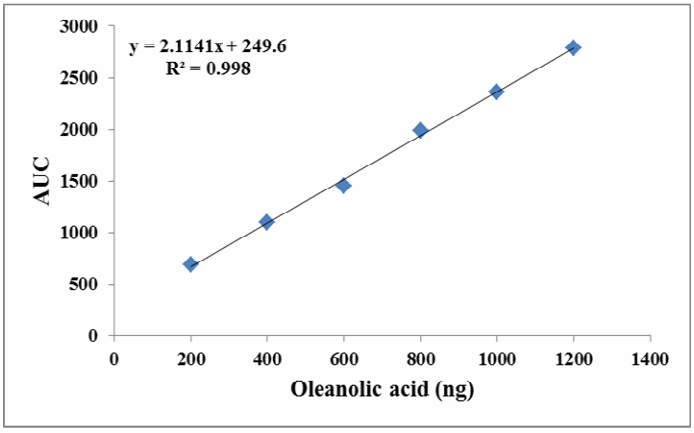


Fig. 5 : Calibration curve of oleanolic acid

Table 1 summarizes the observed values for validation parameters.

Table 1 : Summary of validation parameters

Sr. No.	Parameter	Ursolic acid	Oleanolic acid
1	Instrumental precision (% CV, n=7)	1.11	0.97
2	Repeatability (%CV, n=5)	1.46	1.56
3	Coefficient of determination (r^2)	0.997	0.998
4	Linearity range (ng)	300-1300	200-1200
5	LOD (ng)	41	46
6	LOQ (ng)	125	146
7	Intra-day precision (% CV, n=9)	1.45	1.25
8	Inter-day precision (% CV, n=9)	1.91	1.56
9	Specificity	Specific	Specific

Conclusion

The developed HPTLC method was found to be swift, remunerative, precise, and can be utilized for the quantitative as well as qualitative estimation of ursolic acid and oleanolic acid

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