Fractional Separation Assisted Ultra Violet Spectrophotometric Determination of Oleanolic Acid in *Eugenia caryophyllus* (Spreng.)

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Ultraviolet spectrophotometry has been successfully used for the qualitative as well as quantitative determination of various phytoconstituents. For the quantitative determination of phytoconstituents in the extract by ultraviolet spectrophotometry, the absorbance at specific wavelength is measured. As extract is a mixture of number of constituents, the absorbance of different constituents at the same wavelength along with the compound of interest can occur giving wrong interpretation of observed data and subsequently the results. So, in order to overcome said problem, the technique of fractional separation was used to remove the interfering chemical constituents at the wavelength of compound of interest. In this study, we quantitatively determined the oleanolic acid as a compound of interest in *Eugenia caryophyllus* flower buds using ultraviolet spectrophotometry.

Key words: Ultraviolet spectrophotometry, cloves, oleanolic acid, fractional separation

Oleanolic Acid (OA) is a pentacyclic triterpenoid molecule distributed in huge variety of plants[1,2]. It shows several pharmacological activities of interest like antiviral, anti-inflammatory, hepatoprotective and antidiabetic[3-9]. Now-a-days, OA pays attention of the whole world as an emerging future medicine for the treatment of various ailments[10-18]. Because of its wide distribution, various plant sources can be utilized for its isolation on commercial scale. One of such rich and economically isolable source is clove[19]. Clove is an unopened dried flower bud of a tree, *Eugenia caryophyllus* (*E. caryophyllus*) (synonym *Syzygium aromaticum*), belonging to family Myrtaceae with crimson to dark brown in color, aromatic odour and taste produces numbness of tongue. It is about 10-17 mm in length, 4 mm in width and 2 mm thickness[20]. It is indigenous to Molucca, also called as 'Clove Island'[21]. Traditionally, cloves have been used in various parts of the world to get relief from nausea, vomiting, stomach disorders and various protozoan infections like malaria. In dentistry, clove oil is very useful as dental analgesic[22-24]. Now-a-days, various countries like India, China, Indonesia, Sri Lanka, Tanzania and Madagascar are commercially producing the cloves[25]. The quantitative determination of Phytoconstituents in the extract holds an important aspect to determine the quality of crude drugs to be utilized for their isolation. In Ultraviolet (UV) spectrophotometry, for the quantitative determination, the absorbance of phytoconstituents at specific wavelength (λ_{max}) is considered. As extract is a mixture of variety of different complex chemical constituents, their absorbance may occur at the same wavelength to that of compound of interest giving false interpretation of observed data and subsequently the results as explained in some studies by Li *et al.*[26] and Khanchi *et al.*[27]. Fractional separation is a chemical method use for the separation of constituents from the mixtures based on specific property of individual component to be separated like solubility. It is commonly use technique for

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the isolation of natural products from the extract in pure form[28-30]. In order to overcome the problem of false interpretations of the observed data and results in UV spectrophotometry as discussed above, we have used the fractional separation technique to remove undesired constituents of extract showing absorbance at similar wavelength to that of OA as a compound of interest. To study the concept, we quantitatively analyzed the OA present in clove. For this study, cloves (ATC® Spices) were purchased from the market of Drug city of state Chhattisgarh, India. These cloves were authenticated from the Botanical Survey of India, Central National Herbarium and Howrah, India as *E. caryophyllus* (Spreng.) Bullock and S.G. Harrison, family Myrtaceae. Pure OA (>98 %) was purchased from Yucca Enterprises, Mumbai, India. Methanol of HPLC grade was purchased from Loba Chemie, Mumbai, India. All the solvents for extraction and Thin Layer Chromatography (TLC) study were of High-Performance Liquid Chromatography (HPLC) grade. Throughout the experiment, double distilled water was used. PC based double-beam UV spectrophotometer, spectral bandwidth 1 nm and wavelength accuracy ±0.5 nm (Systronic, India) was used to determine the λ_{max} and absorbance of the samples. Fourier Transform-Infrared (FT-IR) study was carried out using FT-IR spectrophotometer model RZX (Perkin Elmer, United States of America (USA)) by Potassium bromide (KBr) disk method while Electrospray Ionisation Mass Spectrometry (ESI-MS) study on mass spectrometer, Model-Impact HDTM (Bruker Daltonik GmbH, Germany). Nitrogen was used as a drying gas at 200° with flow rate of 0.120 ml/min and a charging voltage of 2000 V. For the determination of λ_{max} of standard OA, methanol was used. Here, methanol was the choice of the solvent due to lower UV cutoff wavelength[31], determined in the range of 190-600 nm. The absorbance values corresponding to the wavelength was noted down. Accurately weighed 1 g of clove powder was refluxed with 50 ml methanol using reflux assembly at 70° for 30 min. After 30 min, the extracted solution allowed to cool at room temperature and filtered using Whatman filter paper no.1. The solution was then transferred to 50 ml volumetric flask and volume adjusted up to the mark. The UV spectrum of the solution was studied for the marked separation of OA peak with other constituents along with TLC studies. Further, accurately weighed 1 g of clove powder was refluxed at 100° in 50 ml water using reflux assembly for 30 min. After 30 min, cooled the solution at room temperature and filtered using Whatman filter paper no.1. The clove powder was washed thrice with distilled water to remove any adhering constituents of extract. Dried the powder in the hot air oven at 35°-40°. Care was taken to avoid any loss of powder which may affect the accurate quantitative determination of OA. The water extract obtained here as a filtrate was dried and dissolved in methanol. The UV spectrum of said water extract dissolved in methanol was studied for the presence of OA as well as other constituents in it along with TLC studies like previous step. Now in this step we used the technique of fractional separation based on solubility of constituents, in which the water exhausted dried clove powder obtained was further refluxed with 50 ml methanol using reflux assembly at 70° for 30 min. After 30 min, the extracted solution was cooled at room temperature and filtered using Whatman filter paper no. 1. The solution was then transferred to 50 ml volumetric flask and adjusted the volume up to the mark. The concentration of the said solution will be 20 000 µg/ml representing the clove powder. Similar to previous steps, the UV spectrum of this solution was also studied for the marked separation of OA peak with other constituents along with TLC studies. For the TLC study, analytical precoated TLC plate (Merck, 1 mm thick, silica gel 60 F_{254}) was used. The plate was pre washed with methanol and dried before use. Spot of standard OA in methanol, water extract of cloves in methanol, methanolic extract of cloves previously exhausted with water and methanolic extract of cloves without exhaustion with water were applied on the plate. The plate was developed in a mobile phase, petroleum ether:ethyl acetate (8.2:1.8). After complete development, the plate was dried for 10 min and sprayed with 10 % (v/v) ethanol solution of sulphuric acid and heated to 120° for 3 min in hot air oven. The visualized spots were documented in UV at 366 nm. For the preparation of standard solutions, 50 mg of OA was dissolved in 50 ml methanol to obtain 1 mg/ml (1000 µg/ml) of stock solution. Further, to study the linearity of standard solution, the serial dilutions were made in the range of 5-80 µg/ml using stock solution. For the

quantitative determination of OA, methanolic extract solution of cloves previously exhausted with water (i.e. 20 000 µg/ml) was used to prepare 1000 µg/ml and analyzed. In the present study, λ_{max} for standard OA in methanol was found to be 210.3 nm as shown in fig. 1A. When the UV spectrum of cloves extracted with methanol was studied, we found that, the absorbance peak of OA was neither sharp nor clear and it was merged with the other neighboring peaks as shown in fig.1B). The UV spectrum of dried water extract of cloves dissolved in methanol showed that, it was free from OA but contain other component having λ_{max} 217.6 nm with some absorbance at 210.3 nm too as shown in fig. 2. While, in case of UV spectrum of cloves extracted with methanol previously exhausted with water clearly indicated that, the interfering merged peaks being lost and sharp OA peak could be observed (fig. 1C). In addition, the OA was also confirmed by FT-IR (–OH (3431.2 cm^{-1}), –OH of carboxylic group (2924.2 cm^{-1}), C=C (1692.7 cm^{-1}), and –CH3 (1463.4 cm^{-1}) (fig. 3) and Electrospray Ionization High-Resolution Mass Spectrometry (ESI-HRMS) ($[M+Na]^+$ at m/z 479.349541 and $[M+H]^+$ at m/z 457.366982 (cald. For Oleanoic acid ($C_{30}H_{48}O_3$), 456.3) (fig. 4) studies. It means that, on exhaustion of cloves with water, some polar fraction components of cloves solubilized in it get lost but not the OA and hence we used this solution previously exhausted with water for quantitative determination of OA in the cloves. In the TLC study, Spot C and Spot D represented the presence of OA visualized as pink spot under 366 nm in UV corresponding to the R_f value 0.23 (fig. 5) along with other constituents as different spots of different R_f values. These different spots other than OA are due to complex mixtures of components in the extracts as explained earlier. While OA was not reported corresponding to the spot B most probably because OA is insoluble in water. The said confirmation about complete absence of OA in water extract was made by UV spectroscopy (fig. 2). Hence, the TLC study indicated that, OA was not lost during boiling the clove powder with water. The absorbance values of standard solutions of OA in the range of 5-80 µg/ml were noted down at 210.3 nm (fig. 6 and fig. 7). The plot of concentration of standard OA *vs.* absorbance was found to be linear (fig. 8). According to the linear regressive relation between concentration and absorbance, the calibration curve could be expressed using following equation.

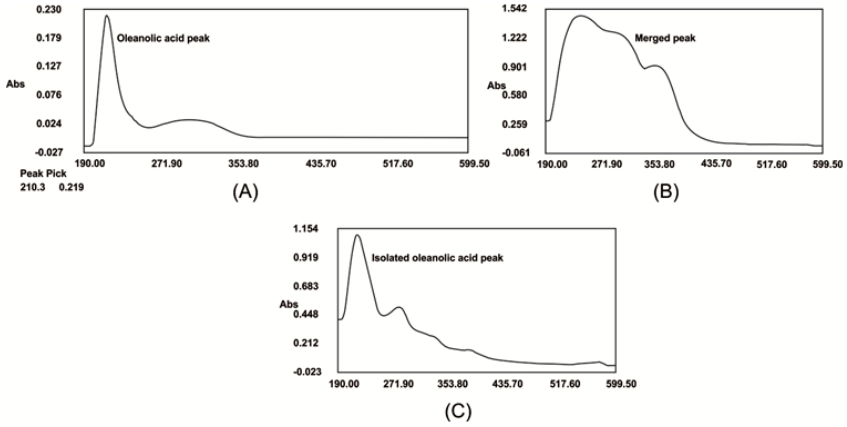

Fig. 1: UV spectrum of (A): Standard OA in methanol λmax=210.3 nm; (B): Methanolic extract of cloves and (C): Methanolic extract of cloves previously exhausted with water

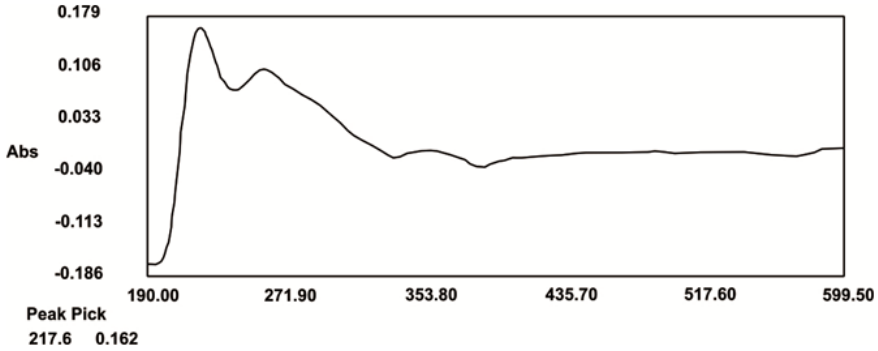

Fig. 2: UV spectrum of water extract of cloves in methanol

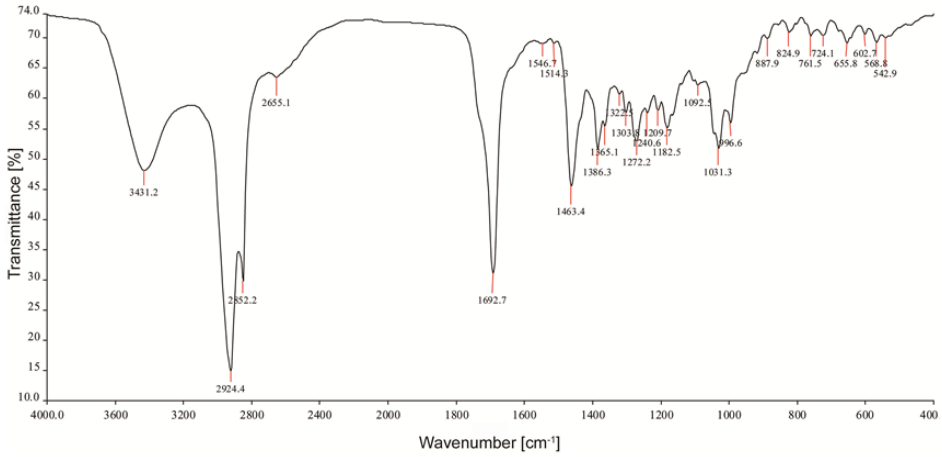

Fig. 3: FT-IR spectrum of OA

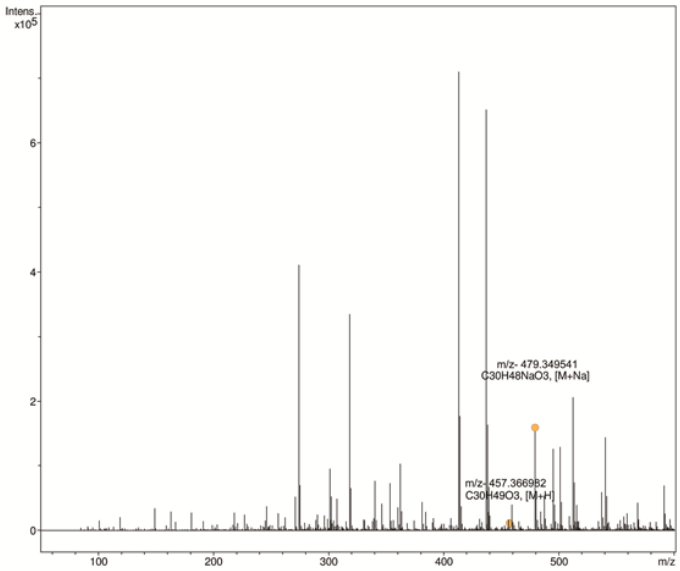

Fig. 4: ESI-HRMS of OA

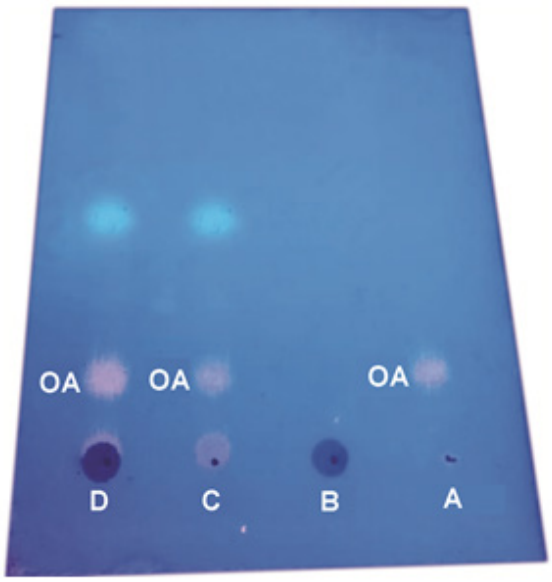

Fig. 5: TLC study of (A): Standard OA; (B): Water extract of cloves; (C): Methanolic extract of cloves previously exhausted with water and (D): Methanolic extract of cloves without exhaustion with water

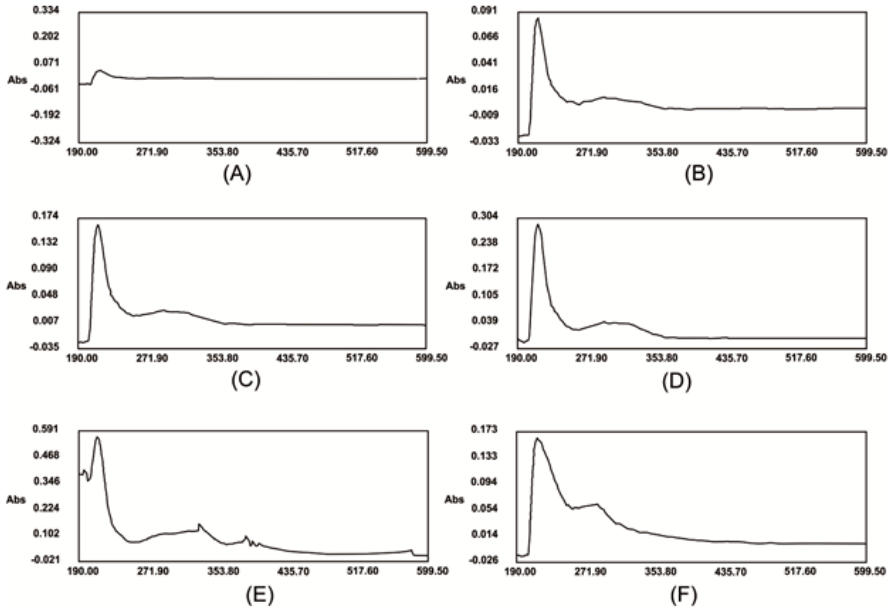

Fig. 6: UV absorbance spectra of standard OA in concentration of 5, 10, 20, 40 and 80 µg/ml (A-E): Respectively and (F): OA in methanolic extract of cloves previously exhausted with water

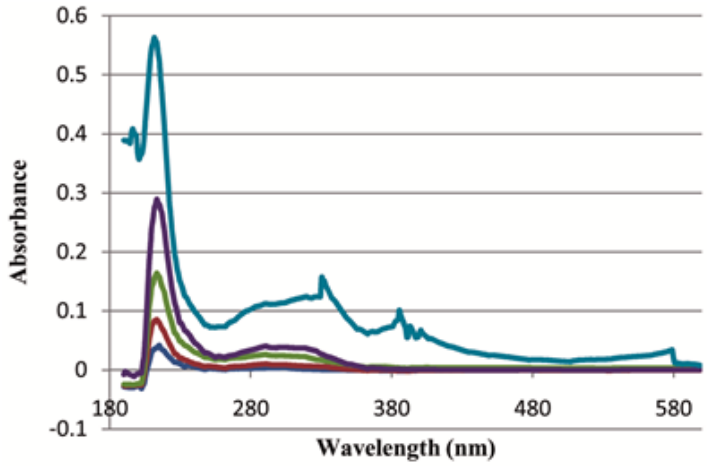

Fig. 7: Overlay UV absorbance spectra of standard OA in concentration of 5, 10, 20, 40, 80 µg/ml respectively
Note: ( ) 5 µg/ml; ( ) 10 µg/ml; ( ): 20 µg/ml; ( ): 40 µg/ml and ( ): 80 µg/ml

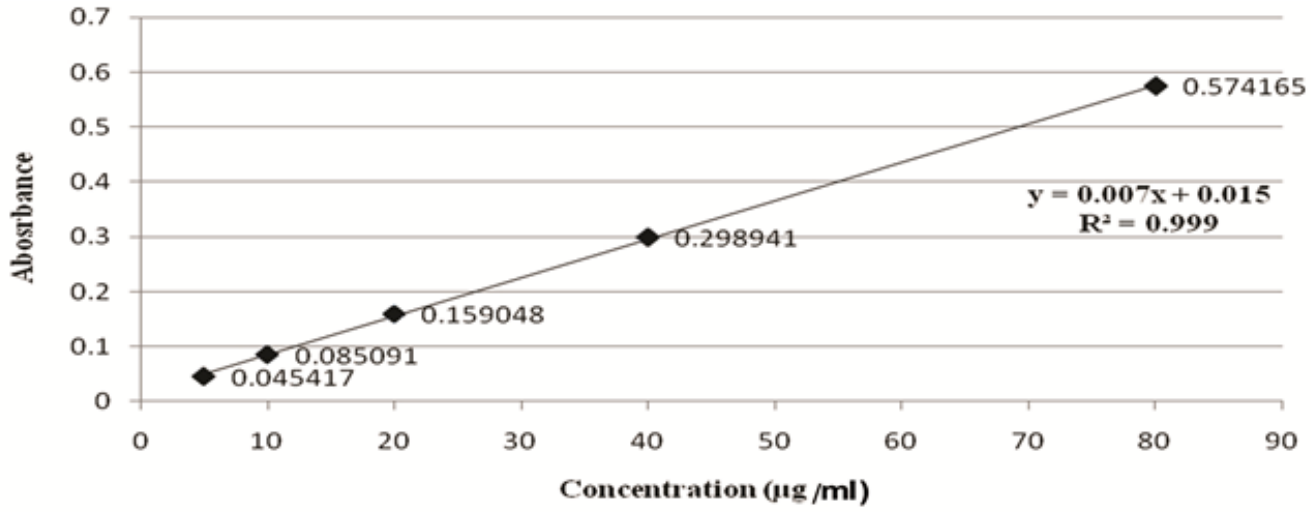

Fig. 8: Plot of concentration of standard OA *vs.* absorbance

$y=0.007x+0.015$ ($R^2=0.999$)

Where, x is concentration (µg/ml) of OA; y is corresponding absorbance of OA. By using the above equation, the concentration of OA in 1000 µg/ml of solution of cloves previously exhausted with water corresponding to absorbance 0.156032 was found to be 20.1474 µg/ml. So, the percentage of OA in cloves was calculated as 2.01 %. Concluding the above mentioned study, the quantitative determination of OA in *E. caryophyllus* (Spreng.) flower buds also called as cloves was carried out. In this study we found that, in UV spectrum, some unwanted constituents of extract formed the interfering merged peak with OA to be quantified. We found that this method is suitable for the quantitative determination of the constituent of the extract by using the technique of fractional separation in which the unwanted interfering constituents were removed and the actual percentage can be calculated. In this study, the percentage of OA in cloves was estimated as 2.01 %.

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Conflict of interests:

The authors declared no conflict of interests.

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