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POLARITY GUIDED EXTRACTION AND CHROMATOGRAPHIC IDENTIFICATION OF ACETOGENINS FROM *Annona glabra* L. LEAVES

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ABSTRACT

***Annona glabra* L. (family: Annonaceae) is used as a traditional medicine against several human infirmities, including cancer. Most of the members of family annonaceae possess a unique class of natural product called acetogenins. The present study was aimed to extract and fractionate the acetogenins from the leaves of *Annona glabra* using the solvents of increasing polarity, such as hexane, chloroform, ethyl acetate ethanol, and water. Solvents with higher polarity such as ethyl acetate were found more effective in extracting lactone-containing compounds as compared to the less polar solvents. The quantity of acetogenins isolated from the *A. glabra* leaves was estimated by lactone concentration test. LC-MS/MS screening showed the presence of acetogenins in the samples with m/z values 596.9, 606.9, 679.8, 566.8, 550.85, 672.15, 672.15, 624.9 and 565.35. These findings open up effective methods for separation and quantification of bioactive compounds. Further studies are to be focused on evaluating the efficacy of these compounds in pre-clinical and clinical trials, which may potentially contribute in developing the novel therapeutic formulations.**

Keywords: Acetogenin, column chromatography, lactone concentration test, LC-MS/MS, polarity guided extraction

INTRODUCTION

Natural products are widely used in drug discovery (Hamann, 2006). A large number of modern drugs have been isolated from natural sources (Hamann, 2006; Gavamukulya *et al.*, 2017). Compounds derived from natural sources have shown high biological activity. Recently, a novel class of bioactive neutral compounds called annonaceous acetogenins has aroused tremendous interest. Various reports showed that acetogenins were widely distributed in the Annonaceae family (Kojima and Tanaka, 2009). Acetogenins constitute a series of natural products.

Acetogenins possess dynamic biological properties such as immune-suppressive, antimicrobial, cytotoxic, antiparasitic, antitumoral and pesticidal properties. Acetogenins were anticipated as possible additives for future anticancer drugs. Annonaceous acetogenins constitute a unique class of natural products with linear carbon chains, C_{35} or C_{37}, derived from polyketide pathways (Kojima and Tanaka, 2009). The general structure was characterized by a long aliphatic chain bearing a terminal methyl-substituted α, β-unsaturated γ-lactone ring. Usually, one to three tetrahydrofuran (THF) rings occur in the middle hydrocarbon chain, which often contains a number of oxygenated moieties (Kojima and Tanaka, 2009; Liaw *et al*., 2016).

Annona glabra L. commonly known as pond apple, is a tropical wild fruit tree native to Southeast Asia and America (Liu *et al*., 1998). *A. glabra*, belonging to the family Annonaceae, is a small, woody

tree with ovate to oblong leaves. Leaves are glossy green and hairless, with a distinctive scent like green apples. The flowers are pale yellow and leathery textured. The pinkish-orange fruit pulp is edible. Fruits contain with 6-8 light yellowish brown seeds (Dev and Joseph, 2021). The bark and leaves of *A. glabra* are used in Chinese medicine against cancer and other human ailments (Biba *et al*., 2014). The family Annonaceae is a rich source of acetogenins, which possess potential anticancer activity and are one of the most potent restrains of mitochondrial complex I (Cochrane *et al*., 2008).

Chromatographic techniques have been used to separate novel drugs of biomedical importance. Column chromatography has been widely used for separating, isolating, and purifying compounds from complex mixtures. This technique involved the separation of a mixture of compounds based on their differential interactions with the stationary phase (typically a solid support) and the mobile phase (typically a liquid solvent) (Yang *et al*., 2010). Compound separation using column chromatography involves the following key steps: sample loading, elution, fraction collection and analysis. The fractions collected are further analyzed using various analytical techniques, such as LC-MS to identify the presence of specific compound, and assess the purity of the isolated fractions (Mulia *et al*., 2015). Column chromatography was vital method for isolating the acetogenin-rich fraction in the polarity-guided extraction and purification of acetogenins from *A. glabra* leaves.

Liquid chromatography-mass spectrometry (LC-MS) is a powerful analytical technique used to identify and quantify chemical compounds present in a sample (Mulia *et al*., 2015). In LC-MS analysis, compounds in sample are separated by liquid chromatography and identified by mass spectrometry. The resulting mass spectrum reveals the information on molecular weight and fragmentation pattern, which helps in identification and quantification of bioactive compounds (Yang *et al*., 2010).

Polarity-guided extraction was a method of extracting specific compounds from a mixture based on their polarity (Fatima *et al*., 2022). Solvents with varying polarities were used to extract the compounds of interest. Compounds dissolved in the solvent based on their polarity. It was possible to extract compounds of specific polarities using solvents with increasing or decreasing polarities (Mulia *et al*., 2015). The objective of this study was to develop an unsophisticated separation technique for acetogenins from the leaves of *A. glabra* Solvents with increasing polarity such as hexane, chloroform, ethyl acetate, ethanol, water and their mixtures were used for the extraction, fractionation and isolation. The lactone concentration referred to the quantity of lactone-containing compounds in a sample. Kedde's reagent was engrossed in determining the amount of acetogenins present in each fraction. Higher lactone concentrations indicated the presence of a high amount of acetogenins. Andrographolide was used as the standard compound to determine the total concentration of lactone present in the sample. LC-MS/MS technique was used to validate the presence of acetogenin compounds in *Annona glabra* L. leaves.

MATERIALS AND METHODS

Plant material

The leaves of *Annona glabra* L. were collected from Kumarakom (Kerala). The identity of plants was authenticated at Kerala Forest Research Institute, Peechi, Thrissur, India. The leaves were thoroughly washed with distilled water, dried in shade, milled to a fine powder, and stored in air-tight containers.

Extraction and isolation of acetogenins using column chromatography

Dried leaf material of *A. glabra* (50 g) was extracted with 95% ethanol at 20-22°C for 5 days. The ethanol was evaporated using a rotary evaporator and the sludge was solubilised in acetone. The resulting mixture was evaporated and placed in a Buchner funnel over a layer of silica gel 60. Polarity guided elution was performed stepwise with 75 mL of each solvent according to the increasing polarity. Solvents such as H_2O, ethanol and ethyl acetate were highly polar solvents. Chloroform was

considered a moderately polar solvent (Mulia *et al*., 2015; Growther, 2018). The extraction, fractionation and isolation of acetogenins from *A. glabra* leaves involved a step-wise process. The fractions were combined on the basis of their similarity, into five groups such as F1 (water), F2 [water + ethanol (7:3)], F3 [water + ethanol (1:1)], F4 [ethanol + (ethanol-ethyl acetate 1:1) + ethyl acetate] and F5 (chloroform). F4 fraction was further subjected to column chromatography. The column chromatography was performed using a 20 mm x 300 mm cylindrical glass column. Silica gel of particle size 60 -120 mesh served as stationary phase. The silica gel was made into a slurry using 100 mL hexane and packed in column. Then 3 g silica gel was pre-adsorbed with 1 mL of F4 fraction and introduced at the top of column. The solvents such as F4.1 (hexane), F4.2 [hexane-chloroform (8:2)], F4.3 [hexane-chloroform (1:1)], F4.4 (ethyl acetate) were used as mobile phase. The eluted fractions were collected separately and evaporated to dryness for further studies.

Identification of acetogenin by using Kedde's reagent

The dried ethyl acetate fraction (F4.4) was further analysed by the modified method of acetogenin isolation process (Luna *et al*., 2006; Sheba *et al*., 2022). The separation was carried out by thin layer chromatography method using silica gel as stationary phase and chloroform-methanol (9:1) as mobile phase. The chromatography plates were sprayed with Kedde's reagent to develop characteristic colour of acetogenin. Kedde's reagent was prepared by mixing equal volumes of 2% solution of 3,5-dinitrobenzoic acid in ethanol and 5.7% solution of potassium hydroxide in ethanol. A pinkish purple coloured spot developed in the TLC plate indicated the presence of acetogenin (Sheba *et al*., 2022).

Lactone concentration test

Acetogenins were quantified as the concentration of lactone present in the fractionated residue using andrographolide as the standard compound. Acetogenins and andrographolide developed a pinkish purple spot on spraying Kedde's reagent. The sample was mixed with Kedde's reagent in a ratio of 2:1 and the absorbance were read using UV spectrophotometer (Shimadzu's Model UV-VIS Mini-1240) at 536nm wavelength using quartz cuvettes. The quantity of acetogenins was expressed as total lactone concentration equivalent to the standard andrographolide (Sigma-Aldrich).

LC-MS/MS analysis

LC-MS/MS analysis was performed by using LC-MS system (Shimadzu model LC-MS-8045). The system accomplishes high sensitivity and ultra-high-speed detection. It was equipped with a heated electrospray ionization (ESI) probe and ion transfer optics. It had Shimpack GISS C18 column and 1.9 um particle size with 2.1 mm x 150 mm dimension. The mobile phase used was 0.1% formic acid in water (A) and methanol (B) at a flow rate of 0.3 mL min^{-1}. High-definition mass spectrometry was carried out using a single Quadrupole Mass system detector and run in ESI positive resolution mode (Gavamukulya *et al*., 2019). With a scan mass range of 100-800 m/z, the mass spectrometer was run in an extended dynamic range. Spectra were stored in mass spectrometer for further identification. Compounds were identified by using m/z value, retention time and fragmentation pattern based on the matching of MS/MS data against National Library of Medicine (*pub-chem.ncbi.nlm.nih.gov*) and literature (Anaya-Esparza *et al*., 2020; Kaur *et al*., 2022; Teoh *et al*., 2023).

The experiments were conducted in a completely randomized block design and each treatment replicated three times. The data were analysed by using Origin software (version 7.0383, OriginLab Corporation, Northampton, USA) and results were expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Identification of acetogenin by using Kedde's test and column chromatography

For the isolation of acetogenin, F4 fraction was further separated by column chromatography with hexane, chloroform, ethyl acetate as eluents and silica gel as stationary phase. Solvents such as

hexane, hexane-chloroform, and ethyl acetate were used as eluents during column chromatography to separate and purify the compounds of interest.

The identification of acetogenin by TLC involved the use of silica gel as stationary phase and chloroform-methanol (9:1) as mobile phase. The sample was spotted on TLC plate and allowed to run in solvent until the solvent front reached the top of plate. It was sprayed with Kedde's reagent to develop chromatogram. The formation of a pinkish-purple spot on TLC plate indicated the presence of acetogenin. Growther (2018) reported the presence of acetogenin as a positive reaction with Kedde's reagent in *Annona muricata* leaves. The fractions obtained from column chromatography were spotted on a TLC plate (Fig. 1). The F4.4 (ethyl acetate) fraction showed a pinkish purple spot in thin layer chromatogram on spraying Kedde's reagent, which indicates the presence of acetogenin. The other fractions eluted showed no colour change on reaction with the reagent. Even though all the fractions obtained from column chromatography were spotted on a TLC plate, the ethyl acetate fraction only showed a positive reaction with Kedde's reagent, other fractions did not show any pinkish purple spot. (Growther, 2018; Sheba *et al*., 2022) reported the formation of pinkish purple spot in the ethyl acetate fraction of *A. glabra* leaves on spraying Kedde's reagent. The results confirmed that thin layer chromatography method is a reliable procedure for identifying the presence of acetogenin.

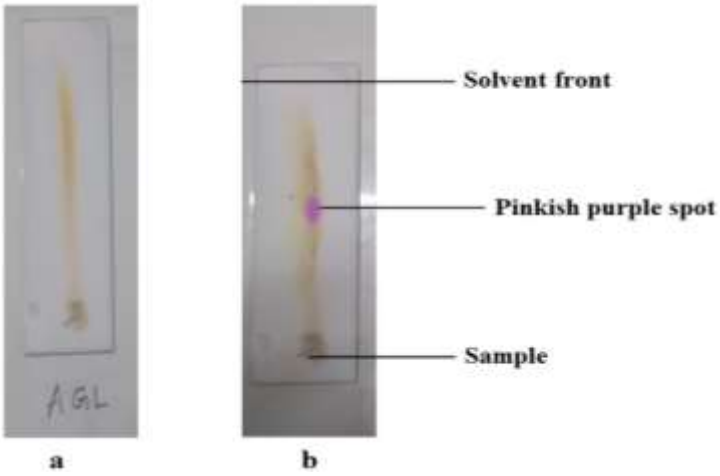

Fig. 1: Chromatogram of *Annona glabra* leaf extract using F4.4 (ethyl acetate) fraction; a) Before the spray of Kedde's reagent, b) After the spray of Kedde's reagent showed a pinkish purple spot at Rf 5.2

Assay of total lactone concentration using Kedde's reagent

The total lactone content of the isolated fractions was quantified using UV spectrophotometric analysis. The quantity of acetogenins in the sample was expressed as total lactone equivalent to andrographolide. The Kedde's reagent was added to the sample solution and the absorbance was measured at 536 nm using a UV spectrophotometer. The optical density value obtained was used to develop a model for predicting the lactone content of the isolated fractions. The study conducted by *(*Aromdee *et al.,* 2005) provides valuable information on the spectrophotometric determination of total lactones in *Andrographis paniculate* and supports the analytical method as well as its comparison with the standardized method. The total lactone content was calculated using andrographolide as standard in the spectrophotometric method.

The F4.4 (ethyl acetate) fraction and standard solution showed a pinkish purple colour on reaction with the Kedde's reagent. The result showed that more polar solvents have high lactone concentration compared to less polar solvents. In addition, the total lactone in the F4.4 (ethyl acetate) fraction was found to be higher than that in F4.0 fraction as shown in Table 1.

Table 1: Lactone concentration in F4 fractions of *Annona glabra* leaf extract

S. No.	Fraction	Eluents	Lactone concentration (µg/g) (mean ± standard deviation)
1.	F4.4	Ethyl acetate	251.33 ± 1.52
2.	F4.0	Ethanol extract and ethyl acetate fractionatior without column separation	191 ± 2.08

The presence of acetogenin in the F4.4 fraction underlines the reliability of the separation procedure. Similar results were reported earlier (Mulia *et al*., 2015) which showed that the total lactone concentration varies among the fractions depending upon its polarity. Fractions containing higher lactone concentrations were obtained using polar solvents. The results emphasize the importance of solvent polarity in the extraction and isolation of lactone-containing compounds. The open-column chromatography approach increases lactone yield in the ethyl acetate fraction compared to extraction methods other than column chromatography.

LC-MS/MS analysis

In LCMS, the compounds were separated based on their retention time. The separated compounds got ionized in the mass spectrometer. The m/z value of ions were measured. The detected ions m/z values were plotted on the x-axis and their relative intensity was represented on y-axis of mass spectrum. The m/z values helped to identify the molecular weight and structure of the compound. The parent ion got fragmented into smaller ions. The pattern of these fragments was used in structural elucidation by interpreting the m/z values and relative intensity. Compounds were tentatively identified by comparing retention time and mass spectra with reference to standard. Ion chromatograms were used in confirming the presence of compounds by the specific m/z values. Database searching was done to match and identify compounds based on the observed mass spectra against libraries of known spectra (Hartler *et al*., 2017).

The LC-MS analysis of ethyl acetate fraction (F4.4) provides information about the specific compounds, particularly the type of acetogenins present. The extraction and isolation of acetogenins from F4.4 fraction worked quite effectively. Acetogenins were identified based on m/z values and fragmentation patterns of the mass spectrum. The resulting MS spectrum showed the mass-to-charge ratio plotted against the peak intensity. The ESI-MS/MS was operated with a scanning range of m/z 100-800. MS spectrum of the fraction with retention time 7.5 m gave a molecular ion peak at m/z value of values 107.25, 113.20, 151.20, 205.05, 218.50, 245.50, 248.95, 262.25, 264.70, 293.25, 306.30, 329.25, 463.30, 487.60, 497.60, 565.35, 579.35, 595.40 and 677.50. The identification of the chemical constituents was carried out using available services and websites along with literature data comparison. The LCMS/MS graph of F4.4 (ethyl acetate) fraction of *Annona glabra* leaf extract is shown in Fig. 2. The significant lines appearing in this graph represented the compounds present in the F4.4 fraction. A total of 10 compounds of acetogenins were tentatively identified. Relative intensity and m/z value of each identified peak were summarized in Table 2. From the mass spectrum, the measured molecular mass was 596.90, 596.87 and 596.46 which depicts a molecular composition of $C_{35}H_{64}O_{7.}$ Based on this data the compounds were tentatively identified as Annonacin A, Isoannonacin A, Glacin A, B. These compounds showed the characteristic features of acetogenin. Luna *et al*. (2006) reported the molecular mass of annonacin as 596.466 and molecular formula $C_{35}H_{64}O_7$ from the mass spectroscopy analysis as the characteristic value of acetogenin. Mulia *et al*. (2015) reported that LCMS analysis confirms the presence of annonacin in the F4.4 fraction of *Annona muricata,* indicating the successful separation and enrichment of this compound from the

Table 2: List of acetogenins identified during LCMS/MS analysis of *Annona glabra* leaf extract

S. No.	Predicted compounds	M/z	Molecular formula	Relative intensity
1	cis-Reticulactin-10-one	606.90	$C_{37}H_{66}O_6$	5.54
2	Annonacin A	596.90	$C_{35}H_{64}O_7$	12.30
3	Isoannonacin A	596.87	$C_{35}H_{64}O_7$	11.30
4	Glacin A, B	596.46	$C_{35}H_{64}O_7$	5.30
5	Plagioneurin C, D, E	679.80	$C_{39}H_{68}O_8$	10.00
6	Longanin	566.80	$C_{35}H_{66}O_5$	15.74
7	Artemoin	550.85	$C_{35}H_{66}O_5$	6.02
8	Annoheptocin-A, B	672.15	$C_{37}H_{68}O_{10}$	5.22
9	Annoglacin- A, B	624.90	$C_{37}H_{68}O_7$	7.21
10	Solamin	565.35	$C_{35}H_{64}O_5$	7.72

other compounds existing in the soursop extract. The measured molecular mass of annonacin reported from the LC-MS analysis is consistent with previous findings, indicating the reliability and accuracy of the analysis. The identified compounds were also discussed in a review done by (Al Kazman *et al*., 2022). Glacin A and B were reported in *Annona glabra* seeds and had been studied for their potential pharmacological activities, particularly in cancer. They also discussed the therapeutic potential of cis-Reticulactin-10-one, Annonacin A, and Isoannonacin A. The results of LC-MS analysis revealed the unique properties of a wide range of compounds, which will contribute to developing novel pharmaceuticals (Lee and Kerns, 1999).

Conclusion: Acetogenins in *A. glabra* leaves was fractionated and isolated using open column chromatography. Total lactone concentration assays conducted using Kedde's reagent confirmed the presence of acetogenin, which strongly suggested that the separation procedure worked effectively. Screening of bioactive compounds using LCMS/MS analysis resulted in the successful identification of acetogenins in the ethyl acetate fraction. Further studies could be directed to explore the biological properties of acetogenins, such as cytotoxic and antitumoral effects, which may, in turn, contribute to the pharmaceutical industry.

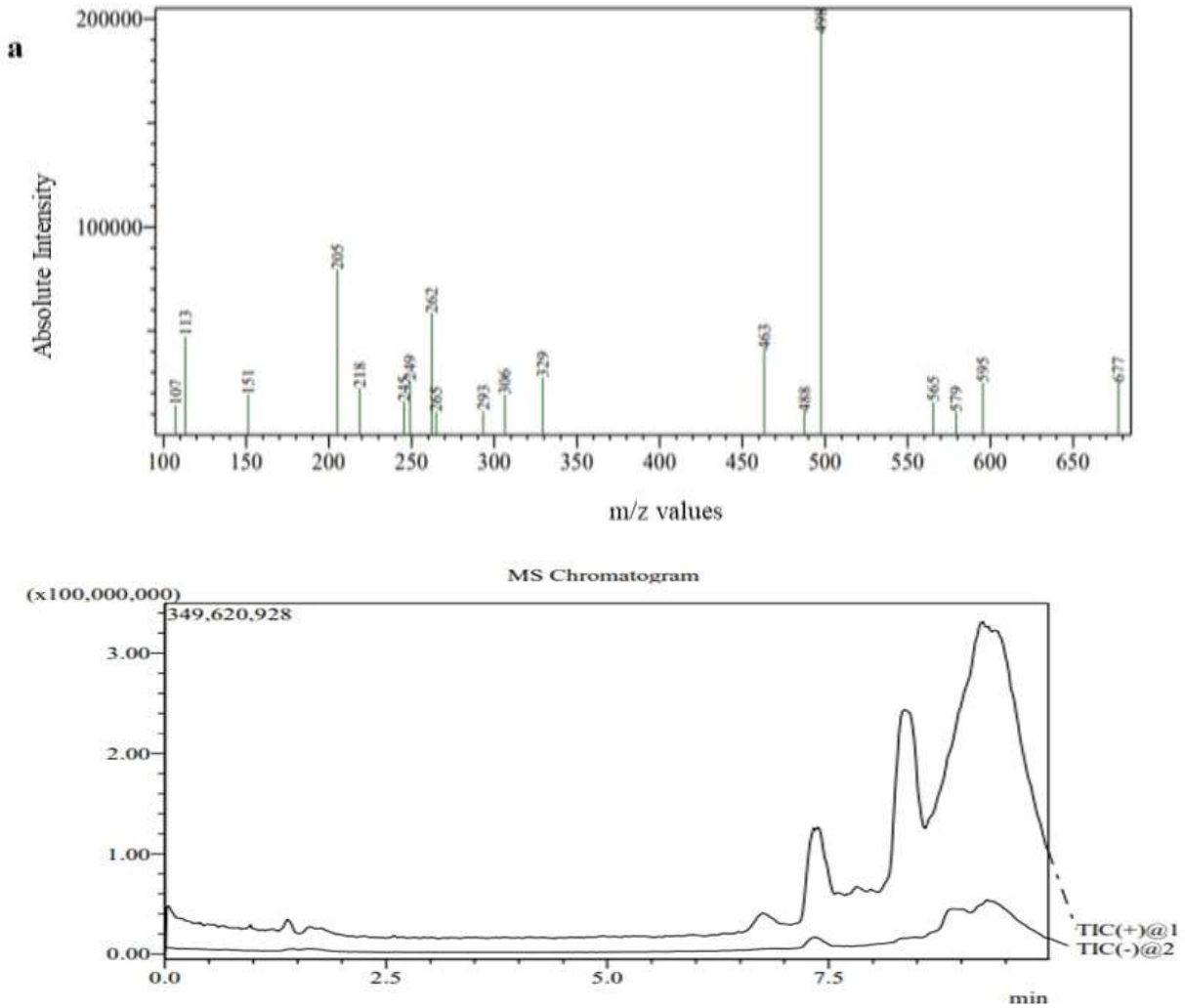

Fig. 2: Mass spectrometry graph from F4.4 (ethyl acetate) fraction of *Annona glabra* leaf extract; Mass spectrum of singly charged ion peak representing m/z value on the x axis and absolute intensity on the y axis (above) and MS chromatogram of chromatographic peak with retention time 7.5 m (below).

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Conflict of interest: The authors declare that they have no conflict of interest.

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