



Isolation and Charecterization of Phytoconstituents from *Annona reticulata* L. Leaf Extracts

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Authors' contributions

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

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ABSTRACT

Aims: The present work was designed to isolate the chemical constituents from the leaf extracts of the plant *Annona reticulata* L. most commonly known as Ramphal or Bullock's heart or Custard apple belongs to the family *Annonaceae*.

Methods: The extraction of leaf powder was done using various solvents. The preliminary phytochemical tests were carried out using standard chemical tests. Phytochemical constituents were isolated using column chromatography and characterization of the compounds were carried out using IR, ¹H NMR, and mass spectroscopy data.

Results: The results from the preliminary phytochemical studies showed the presence of alkaloids, triterpenoids, carbohydrates, saponins and flavonoids. Isolation of leaf extracts revealed the presence of a steroid, alkaloid and flavonoid which were confirmed by spectral analysis to be α – amyryn caprylate, xylopine and fisetin.

Conclusion: The study confirmed the presence of α – Amyryn caprylate, Fisetin and Xylopine as its main chemical constituents.

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1. INTRODUCTION

Herbal medicine also known as phytotherapy is the science of using herbal remedies to treat various sicknesses. It covers all the aspects of herbal medicine, that is, plants with powerful actions to those with gentle actions. Currently, people have come to know of the side effects of synthetic drugs, thus leading to an increased development of natural products as treatment for ailments [1]. This led to an increase in the curiosity of the scientists and various pharmaceutical industries to develop new herbal medicine for various diseases with decreased side effects [2]. India, a country with highest number of plants with medicinal and therapeutic importance became a target for these pharmaceutical industries to export these plants for their plan development. Thus, leading to an increase in the export business of India [3].

The isolation of the active compounds from the medicinal plants has been very time consuming, hence the developing of new methodology and strategy has been a necessity to improve the process of this plant isolation. Currently, the identification of the active constituents has been done using nuclear magnetic resonance (NMR) and mass spectrometry (MS), but the easy isolation of these active constituents has been a drawback. Natural products have been collected in very small quantities that it has been found to be insufficient for lead optimisation, lead development and clinical trials. However, even if the drug discovery from the natural products faces challenges, natural product isolation from the medicinal plants is still of high importance in the search of new medicines [4].

Annona reticulata Linn (AR). belongs to family *Annonaceae*. The plant is most commonly also known as Ramphal, Bullock's heart and Custard apple. Nearly about 119 different species of the *Annona* genus (*Annonaceae*) are identified, most of them being shrubs and trees [5]. In India, it is widely cultivated and naturalized as a fruit consuming plant and deciduous tree. In old system of medicines, this plant was reported to have pharmacological activities such as antifungal, anticancer, spasmolytic, anticonvulsant, antimalarial, anthelmintic and anti-syphilitic [6]. The present work is to isolate the chemical constituents from the leaf extracts

of the plant AR and characterize them by using IR, NMR and mass spectroscopical data.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The leaves of AR were collected in the month of April-May from Mangalore, Karnataka. The botanical identity of the leaves of the plant was verified and authenticated by Dr. K.V. Nagalakshamma, HOD, Department of Botany, St. Aloysius College, Mangalore. A voucher specimen (No. 16PC007) was deposited in NGSM Institute of Pharmaceutical Sciences, Paneer, Deralakatte, Mangalore.

2.2 Preparation of Extracts

The leaves were collected from Mangalore, Karnataka, During May 2017. The leaves were cleaned from adhering soil, dust and other material [7] and then it was dried under the shade for 30 days. The dried leaves were cut into small pieces and finely powdered (5 Kg) by using a dry grinder. The powdered leaves were subjected to cold maceration extraction using ethanol as the solvent [8]. They were exhaustively extracted by maceration with ethanol for three days, after three days, the ethanol layer was decanted off. The process was repeated thrice. The solvent obtained after decantation was distilled off and the concentrate was evaporated to syrup like consistency by maintaining the required temperature conditions and then was evaporated to dryness (545 g) on an open water bath at room temperature to a dark green solid mass. It was stored in a dessicator.

2.3 Preliminary Phytochemical Screening of Plant Extract

The preliminary qualitative phytochemical investigation of the ethanolic extract of AR was carried out to detect the presence of various phytoconstituents. The extracts were screened for these constituents using standard procedure [9,10].

2.4 Isolation of Compound from the Petroleum Ether Extract

The brown coloured petroleum ether fraction (31g) was saponified using the 20% ethanolic

KOH (350ml) for 2hr [11]. The above fraction was then evaporated to remove traces of ethanol. The unsaponified portion was extracted with ether (500ml). The ethereal fraction obtained were washed with distilled water (20ml) and dried to give a yellow residue (3.2 g).

The yellow residue (3.2 g) was dissolved in chloroform and absorbed on to silica gel (20 g), which was loaded onto a silica gel column (150 g) prepared in petroleum ether. The column was eluted initially with 100% petroleum ether (60-80^oc), 10%, 20%, 40% and 80% benzene in petroleum ether in gradient manner, then finally with 100% benzene. The eluted fractions were further monitored by TLC (Benzene: ethyl acetate, 90:10; aluminium coated silica gel, visualization; vanillin-sulphuric acid reagent and heated at 110^oc) [12]. The eluates from 90:10 of benzene: ethyl acetate gave one spot on the TLC. This eluate was concentrated to obtain a white colour compound. The residue obtained was recrystallized with benzene to yield a white crystalline powder (50mg). The product obtained was designated as Compound I. The compound I gave green colour for Liebermann-Burchard test and Salkowski test for steroids.

2.4.1 Acetylation of compound I

The compound I (10 mg) was taken along with dry pyridine (0.5ml), to which freshly distilled Ac₂O was added. This mixture was kept at room temperature overnight, after which was added to crushed ice, stirred and kept for 2 hrs. The mixture was filtered and then dried, the remaining was recrystallized using benzene. As a result, white flakes were obtained with melting point of compound I (156-160^oc).

2.5 Isolation from Methanolic Extract

The powdered material was defatted using the petroleum ether and the defatted crude material was extracted using methanol. The methanol extract was concentrated and moistened with water containing dilute acid like acetic acid. The above extract was subjected to steam distillation to remove methanol and filter to remove traces of methanol. The above mass was treated with sufficient chloroform and shaken well for 15 mins. Filtered and the filtrate was rejected. The same process was repeated thrice. The compound salts were in the aqueous liquid phase while many impurities remain in the organic phase. The aqueous phase was collected and treated with NH₃, the liberated free

base was extracted with chloroform and finally the chloroform was evaporated to leave behind the pure compound. The compound was subjected to TLC using Ethyl acetate and Benzene which upon spraying with Dragendorff's reagent gave yellowish orange coloration. The product obtained was a yellow crystalline powder of 35g, designated as Compound II. The compound II gave positive identification test for Mayer's, Wagner's, Dragendorff's and Hager's Test. The melting point of Compound II was 218-221^oc.

2.6 Isolation from Chloroform Extract

The dark green chloroform fraction (28g) was dissolved in chloroform; this was loaded on a silica gel column (150g) which was prepared initially with 100% ethyl acetate, followed by gradual addition of Methanol and Water in ethyl acetate. The eluted fractions were further monitored by TLC (Ethyl acetate: Methanol: Water, 100:13.5:10) visualized with UV/NH₃. Eluates with ethyl acetate and 1% Methanol in Ethyl acetate showed the presence of similar compounds on TLC. These were then concentrated to yield a yellow crystalline solid. This was then washed, filtered and then recrystallized from methanol. It was obtained as an yellow colored compound (40mg). The eluates of the 2% and 5% methanol in ethyl acetate gave a resinous mass on concentration, they were not processed further. The product obtained was designated as Compound III. The compound III gave orange colour with Shinoda's test .

2.6.1 Hydrolysis of compound III

To the solution of the compound III (15mg) an equal volume of 2N HCl was added. The mixture was refluxed at 100^oc for 2hr and then evaporated to dryness under reduced pressure. After the addition of the distilled water (6ml) it was extracted with ether and concentrated. Then dried over anhydrous Na₂SO₄ to deposit a yellow solid with melting point of 325-382^oc.

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Investigation

On undergoing the preliminary phytochemical screening, the leaves of AR showed and confirmed the presence of steroids, flavanoids, glycosides, triterpenoids and alkaloids.

Table 1. Results of qualitative tests for phytoconstituents

Sl. No	Tests	Inference
1	Alkaloids	
	Dragendorff's Test	+ve
	Hager's Test	+ve
	Wagner's Test	+ve
	Mayer's Test	+ve
2	Carbohydrates	
	Molisch's Test	+ve
	Benedict's Test	+ve
	Fehling's Test	+ve
	Tollen's Test	+ve
3	Flavanoids	
	Shinoda's Test	+ve
4	Triterpenoids	
	Liebermann Burchard's Test	+ve
5	Proteins	
	Biuret Test	-ve
	Millon's Test	-ve
6	Resins	
	Ferric chloride test	-ve
7	Saponins	
	Froth test	+ve
8	Steroids	
	Liebermann Burchard Test	+ve
	Salkowski Test	+ve
	Tannins	
9	Lead acetate test	-ve

3.2 Characterization of Compound I

3.2.1 Analysis of compound I

Physical state: Off white Crystal, Rf value : 0.81 (Petroleum ether:

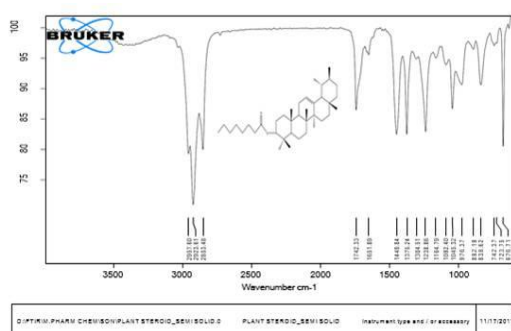
Ethyl acetate, 98:2) Melting Point: 160^oC

3.2.2 Spectral characterization of compound I

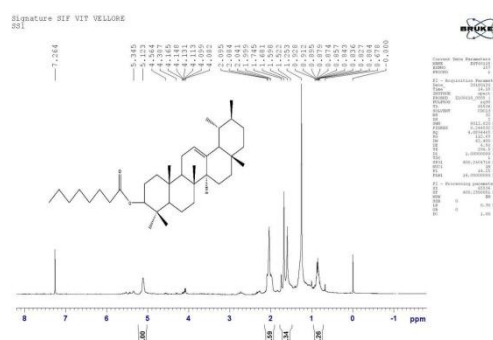
IR (KBr):2957 cm⁻¹ (C – H stretching in CH₃), 2853 cm⁻¹ (C – H stretching in CH₂), 1742 cm⁻¹

(C=O stretching), 1449 cm⁻¹ (C-H deformation in CH₃), 1375 cm⁻¹ (C – H deformation in gem dimethyl), 742 cm⁻¹ (=C – H out plane bending). ¹HNMR (CDCl₃) δ 5.33 (m, 1H, vinylic proton at C – 12), δ 1.25 (m, 6H, 2XCH₃), δ 0.67 (m, 3H, 1XCH₃), δ 1.68 to δ 1.74 (m, 6H, 3XCH₂), δ 0.85 to δ 1.20 (m, 18H, 9XCH₂), δ 0.9 to δ 1.5 (m, 5H, 5XCH), δ 2.04 to δ 2.09 (m, 3H, 1XCH₂). Molecular Formula: C₃₂H₅₂O₂, Molecular Weight is 468, EIMS (m/z): 465 (M⁺, 50%), 414 (M⁺, CH₃, 20%), 381 (18%), 329 (12%), 281 (20%), 207 (50%), 133 (48%), 43 (100%).

I-a (IR spectral data)



I-b (¹H-NMR spectral data)



I-d (Mass spectral data)

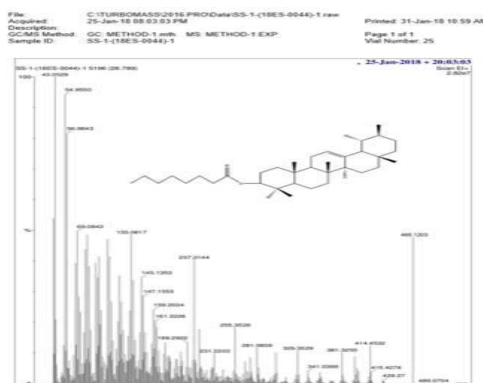
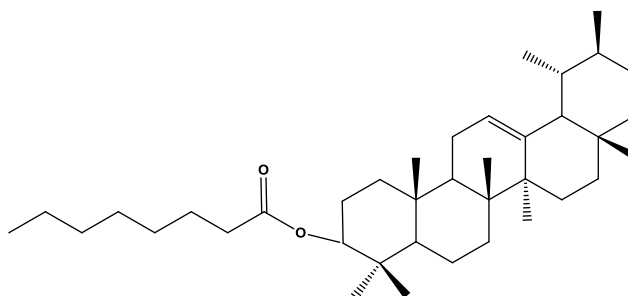


Fig. 1. Spectral data of compound 1

Melting point was observed at 160⁰C. It gave a characteristic colour reaction for the triterpenes, i.e green colour with the Liebermann Burchard Test. The mass fragmentation was that of α – amyryn skeleton. An ester linkage from the characteristic IR absorption was obtained at 1742cm⁻¹.The CH₂COO- of the ester was indicated by the singlet at δ 2.04 to δ 2.09. In the obtained mass spectra of the compound, the molecular ion peak of the compound of molecular weight 468 was found at 465. The number of vinylic proton was obtained from the ¹H-NMR [13].



alpha - Amyrin caprylate

3.3 Characterization of Compound II

3.3.1 Analysis of compound II

Physical state :White solid powder, Rf value : 0.58 (Ethyl acetate: Benzene, 1:1) Melting Point: 221⁰c

3.3.2 Spectral characterization of compound II

IR (KBr):3393 cm⁻¹ (N-H),2925 cm⁻¹ (C-H),1512 cm⁻¹ (C=C),1028 cm⁻¹ (C-O-C),1260 cm⁻¹ (O-CH₂); ¹HNMR (CDCl₃): δ 3.864 (s, 3H, OCH₃), δ 5.05 (m, 2H, OCH₂), δ 7.21 (m, 1H, NH), δ 3.404 (m, 4H, Ar-H), δ 2.42 (m, 2H, CH₂), δ 2.06 (m, 2H, CH₂), δ 1.61 (m, 2H, CH₂); Mass Spectra (EI-

MS);Molecular Formula :C₁₈ H₁₇ N₀₃,Molecular Weight :295,EIMS (m/z):293 (M⁺, C₁₈H₁₇NO₃, 69%), 281 (25%), 207 (100%), 117(40%), 97 (30%), 73 (55%), 43 (97%).

This compound gave a positive result for all the standard alkaloid tests like the dragendroff's test and the melting point was observed at 221⁰C. From the IR spectra obtained for this compound, it was interpreted that there was absorption of the N-H group at 3393cm⁻¹ and the C=C group at 1512 cm⁻¹. In the ¹HNMR the N-H group was indicated at δ 7.21. The aliphatic group containing the carbon atom was indicated at δ 5.05. The molecular ion peak of the compound of molecular weight 295 was found at 293 [14].

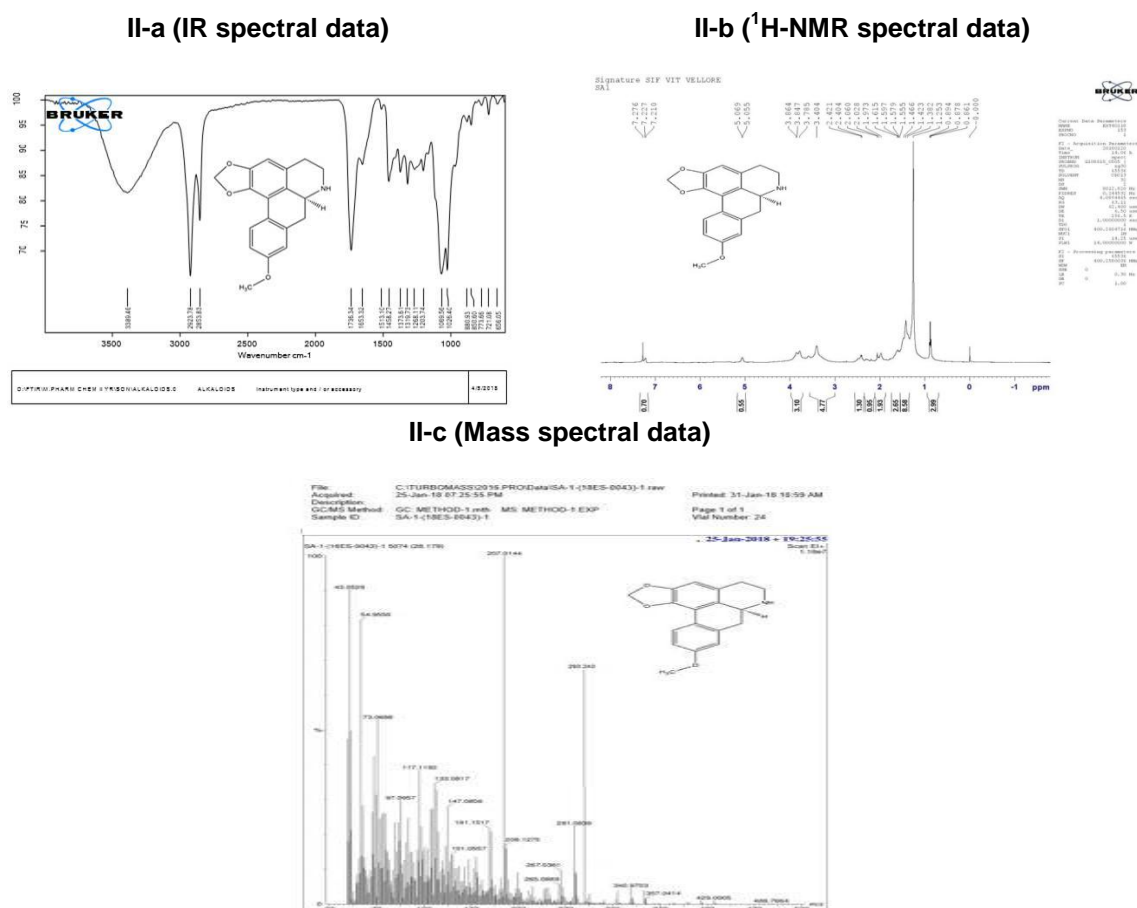
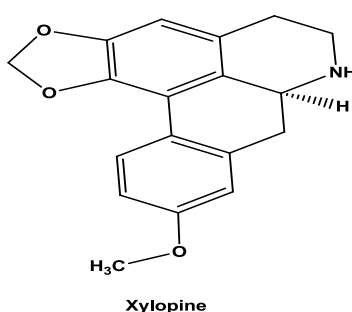


Fig. 2. Spectral data of compound II



3.4 Characterization of Compound III

3.4.1 Analysis of compound III

Physical state : Yellow Crystalline powder, R_f value : 0.6 (Ethyl acetate: Methanol: water, 75:15:10) Melting Point: 328^oC

3.4.2 Spectral characterization of compound III

IR (KBr):3385 cm⁻¹ (OH stretching), 2923 cm⁻¹ (Ar C-H stretching), 1513 cm⁻¹ (C=C stretching),

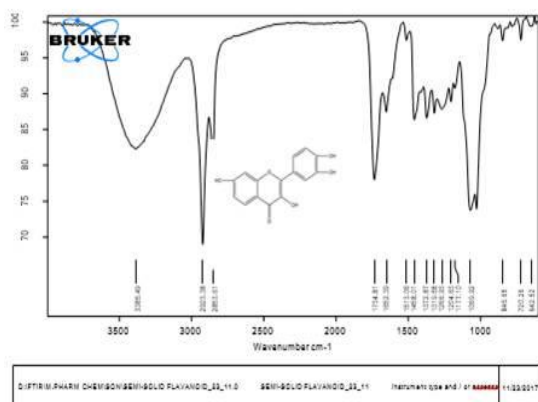
1652 cm⁻¹ (C=O stretching),1069 cm⁻¹ (C-O-C stretching).¹HNMR (CDCl₃): δ 7.27 (d, 2H),δ 3.40- 3.85 (m, 4H, Ar-H, Phenyl ring),δ 1.33 – 2.49 (m, Ar-H, 5H), δ 1.44 (m, 4H). Mass Spectra (EI-MS):Molecular formula: C₁₅H₁₀O₆, Molecular Weight: 286, EIMS (m/z)281 (M⁺, C₁₅H₁₀O₆, 86%),207(30%),117(33%), 71(45%, 56 (100%), 43 (97%).

The compound III gave a positive result for the Shinoda's Test and the melting point was found to be 328^oC. The IR spectra showed a absorption band for the hydroxyl group at

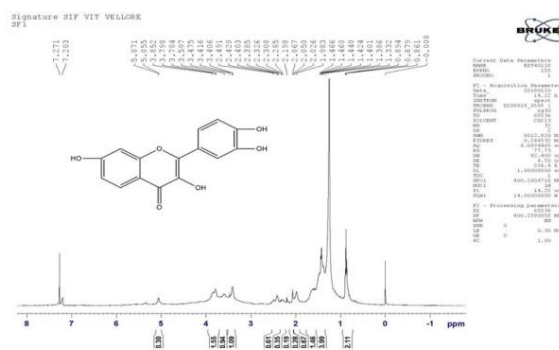
3385cm⁻¹ and the characteristic C=O group at 1652cm⁻¹ and the C-O-C group at 1069cm⁻¹. In the ¹H-NMR data of this compound, the phenyl ring was indicated at δ 3.40 – δ 3.85. The mass

spectra of the compound indicated that molecular ion peak of the compound of molecular weight 286 was found at 281. The vinylic proton of the compound was found at δ 1.44 [15].

III-a (IR spectral data)



III-b(¹H-NMR spectral data)



III-c (Mass spectral data)

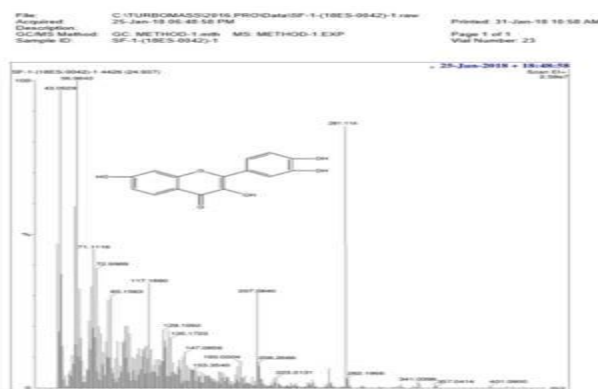
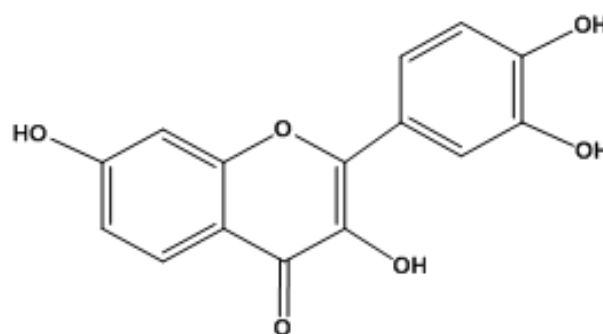


Fig. 3. Spectral data of compound III



Fisetin

4. CONCLUSION

The Phytochemical screening of the extract revealed the presence of secondary metabolites such as Flavanoids, Steroids, Glycosides, Alkaloids and Triterpenoids on preliminary phytochemical investigation. Further, the detailed investigation of the leaves of the plant, upon fractionation of the plant extract and subjecting to characterization using various methods revealed the presence of an Steroid, Alkaloid and Flavanoid which were confirmed by spectral analysis to be α – Amyrin caprylate, Xylopinine and Fisetin.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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