ISOLATION AND CHARECTERIZATION OF PHYTOCONSTITUENTS FROM *ANNONA RETICULATA* L. LEAF EXTRACTS

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, ^{1}H 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 – amyrin caprylate, xylopine and fisetin.

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

Keywords: *Annona reticulata, Annonaceae, epilepsy, α – Amyrin caprylate, Fisetin and Xylopine*

1. INTRODUCTION

Herbal medicine also known as phytotherapy is the science of using herbal remedies to treat various sickness. 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 lead 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].

Epilepsy is a major neurological disorder in which a person suffers from recurrent unprovoked seizures. Epilepsy being a common disorder of the CNS was found to affect 5% of the world population [1]. Seizure is the characteristic feature in epilepsy and is associated with disordered and rhythmic high frequency discharge of impulses by a group of neurons in the brain. The current therapy for epilepsy with the modern synthetic antiepileptic drugs were found to be having certain dose-related side effects and toxicity [5]. Herbs were claimed on having better antiepileptic effects. Hence phytochemicals were identified from various traditional medicinal plants, thus presenting an exciting opportunity for the development of new types of therapeutics.

Annona reticulata Linn. 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 [6]. 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 [7].

2. MATERIAL AND METHODS

2.1. Collection of Plant Material

The leaves of *Annona reticulata* 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 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 [5]. 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 a 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 *Annona reticulata* was carried out to detect the presence of various phytoconstituents. The extracts were screened for these contistuents using standard procedure [8].

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. The above fraction was then evapourated 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^{0}c), 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 110^0c) [9]. 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 Ac_2O 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(156-160^0c).

2.5. Isolation of Compound from methanol 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 aqeous liquid phase while many impurities remain in the organic phase. The aqeous phase was collected and treated with NH_3, the liberated free base was extracted with chloroform and finally the chloroform was evapourated to leave behind the pure compound. The compound was subjected to TLC using Ethyl acetate and Benzene which upon spraying with Dragendroff's reagent gave yellowish orange colouration. The product obtained was a yellow crystalline powder of 35g, designated as Compound II. The compound II gave positive identification for the Mayer's, Wagner's, Dragendorff's and Hager's Test. The melting point of Compound II was 218-221^oc.

2.6. Isolation of compound 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/NH_3 . 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 coloured 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 100^0c for 2hr and then evapourated to dryness under reduced pressure. After the addition of the distilled water (6ml) it was extracted with ether and concentrated. Then dried over anhydrous Na_2SO_4 to deposit a yellow solid with melting point of 325-382^0c.

3. RESULTS AND DISCUSSION

3.1. Preliminary Phytochemical Investigation

On undergoing the preliminary phytochemical screening, the leaves of *Annona reticulata* 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 Hager's Test Wagner's Test Mayer's Test	 +ve +ve +ve +ve
2	Carbohydrates Molisch's Test Benedict's Test Fehling's Test Tollen's Test	 +ve +ve +ve +ve
3	Flavanoids Shinoda's Test	 +ve
4	Triterpenoids Liebermann Burchard's Test	 +ve
5	Proteins Biuret Test Millon's Test	 -ve -ve
6	Resins	-ve
7	Saponins	+ve
8	Steroids Liebermann Burchard Test Salkowski Test	 +ve +ve
9	Tannins	-ve

3.2. Characterization of compound I

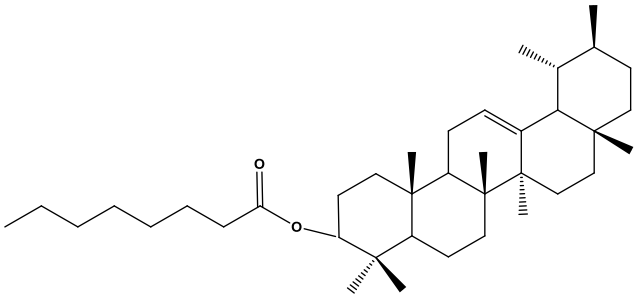
3.2.1. Analysis of Compound I: Physical state : Off white Crystal,Rf value : 0.81 (Petroleum ether: Ethyl acetate, 98:2)Melting Point: 160^0C

3.2.2. Spectral characterization of Compound I

IR (KBr):2957 cm^{-1} (C – H stretching in CH_3), 2853 cm^{-1} (C – H stretching in CH_2),1742 cm^{-1} (C=O stretching),1449 cm^{-1} (C-H deformation in CH_3),1375 cm^{-1} (C – H deformation in gem dimethyl),742 cm^{-1} (=C – H out plane bending). ^{1}HNMR ($CDCl_3$) δ 5.33 (m, 1H, Vinylic proton at C – 12),δ 1.25 (m, 6H, 2XCH_3),δ 0.67 (m, 3H, 1XCH_3), δ 1.68 to δ 1.74 (m, 6H, 3XCH_2), δ 0.85 to δ 1.20 (m, 18H, 9XCH_2),δ 0.9 to δ 1.5 (m,5H, 5XCH),δ 2.04 to δ 2.09 (m, 3H, 1XCH_2). Molecular Formula:$C_{32}H_{52}O_2$,Molecular Weight 468,EIMS (m/z):465 (M^+,,50%), 414 (M^+, CH_3, 20%), 381 (18%), 329 (12%), 281 (20%), 207 (50%), 133 (48%), 43 (100%).

3.2.3.Compound I : α – Amyrin caprylate

Melting point was observed at 160^0c. 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 – Amyrin skeleton. An ester linkage from the characteristic IR absorption was obtained at 1742cm^{-1} . The CH_2COO- 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 ans singlet proton was obtained from the ^{1}H-NMR[10].



alpha - Amyrin caprylate

3.3. Characterization of compound II

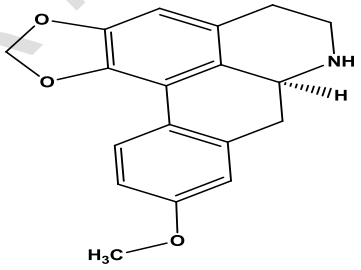
3.3.1. Analysis of Compound II:Physical state :White solid powder,Rf value : 0.58 (Ethyl acetate:Benzene, 1:1)Melting Point: 221^{0}c

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} (0-CH_2); ^{1}HNMR ($CDCl_3$):δ 3.864 (s, 3H, OCH_3),δ 5.05 (m, 2H, OCH_2),δ 7.21 (m, 1H, NH),δ 3.404 (m, 4H, Ar-H),δ 2.42 (m, 2H, CH_2),δ 2.06 (m, 2H, CH_2),δ 1.61 (m, 2H, CH_2); Mass Spectra (EI-MS);Molecular Formula :C_{18} H_{17} $N0_3$,Molecular Weight :295,EIMS (m/z):293 (M^+, C_{18} H_{17} $N0_3$, 69%), 281 (25%), 207 (100%), 117(40%), 97 (30%), 73 (55%), 43 (97%)

3.3.3.Compound II : Xylopine

m.p. 221^{0}c. This compound gave a positive result for all the standard alkaloid tests like the dragendroff's test. 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[11].



Xylopine

3.4. Characterization of compound III

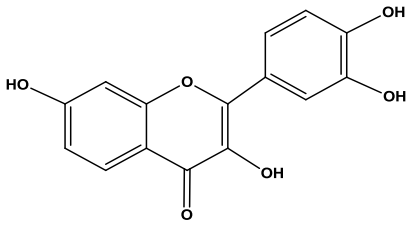
3.4.1. Analysis of Compound III :Physical state : Yellow Crystalline powder,Rf value : 0.6 (Ethyl acetate: Methanol: water, 75:15:10)Melting Point: 328^{0}c

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).^{1}HNMR ($CDCl_3$):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:$C_{15}H_{10}O_6$,Molecular Weight:286,EIMS (m/z)281 (M^+, $C_{15}H_{10}O_6$,86%), 207 (30%), 117 (33%), 71(45%), 56 (100%), 43 (97%).

3.4.3. Compound III : Fisetin

The compound III gave a positive result for the Shinoda's Test and the melting point was found to be 328^{o}C . 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 1HNMR 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[12].



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, Xylopine and Fisetin.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT
Not Applicable

ETHICAL APPROVAL
Not Applicable

NOTE:

The study highlights the efficacy of "Herbal medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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