Academic Sciences

International Journal of Current Pharmaceutical Research

ISSN- 0975-7066 Vol 6, Issue 4, 2014

Research Article

ISOLATION AND IDENTIFICATION OF A FLAVONE FROM FRUIT PULP OF *FERONIA LIMONIA*

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Received: 13 July 2014, Revised and Accepted: 08 August 2014

ABSTRACT

Phenolic constituents are the principle bioactivity compounds exist in *Feronia limonia*; not much of the phenolic compounds are reported from the plant previously. The present paper describes the isolation, identification of flavonoid from medicinal plant *Feronia limonia*. Fruit pulp was defatted using petroleum ether and further extracted with methanol by Soxhlet extraction. Shinoda test and ferric chloride tests were carried out for preliminary confirmation of flavonoids; thin layer chromatography was employed for the separation of flavonoids. Isolated flavonoid was purified using silica gel column chromatography and further subjected to spectral characterization using FT-IR, ^{1}H-NMR and LC-MS studies. Pure compound was identified as luteolin having molecular formula 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one, chemical formula $C_{15}H_{10}O_7$ and molecular mass 283.35 by comparing their retention time (RT) and MS spectrum values with those that had been identified and the published data. With the help of the obtained phytochemical and spectral data, structure of the flavonoid was drawn using the software Chem Draw ultra version 12. Moreover, the compound 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one has been isolated for the first time from the plant *Feronia limonia*.

Keywords: Flavonoid, Luteolin, Fruit pulp, *Feronia limonia*, Methanolic extract.

INTRODUCTION

Plants produce a diverse range of bioactive molecules making them rich source of different types of medicines. There are a number of plants with powerful therapeutic effects, and, as a result, an extensive investigation of their content is necessary. The natural extracts of plants are an important source for the identification of new biologically active compounds with possible applications in the pharmaceutical field. Phytotherapy embraces especially the isolation from herbs, of compounds with unique chemical structures, which are considered to be pharmacologically active. There are many classes of compounds that can be found in an alcoholic, natural extracts: amino acids, peptides, small proteins, phenols, polyphenols, saponins, flavonoids and sugars. Compounds of great interest are the flavonoids which show important pharmacological activities [1]. Flavonoids are a subclass of the polyphenols, which are characterized as containing two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge. Flavonoids are among the most ubiquitous groups of plant secondary metabolites distributed in various foods and medicinal plants. Flavonoid family is divided into different sub families namely- Flavones, Isoflavones, Flavonols, Flavanols, Anthocyanidins, Flavanones [2]. However, exact mechanisms for many of the actions attributed to flavonoids have not yet been established, but the relationship between their activity and the presence of specific functional groups in the molecules is undeniable. The process of structure elucidation of a natural product involves the determination of many physical-chemical properties: melting point, optical rotation, solubility, absorption, infrared spectroscopy, mass and nuclear magnetic resonance spectroscopy. The chemistry of natural products includes three main areas: isolation, structure elucidation, and synthetic methods. The isolation step is considered to be a part of structure elucidation, and therefore analysis and characterization methods, such as the UV-Vis and infrared spectroscopy, mass spectrometry and various chromatographic techniques, are all important tools for a proper identification of the components of an extract [3]. This article herein describes the isolation and structure elucidation of the flavanol from the methanolic extract of *Feronia limonia* fruit pulp by FTIR, NMR, LC-MS spectroscopy methods.

MATERIALS AND METHODS

Plant Material and Extraction

Fruits from the plant *Feronia limonia* were obtained from a tree growing in Bellary, Karnataka. Samples were collected at the brown maturation period (March 2012). Fruits (200 g) were selected randomly from the tree and immediately blended into a homogeneous paste, stored at 4 °C stored in air tight container. Fruit powder was defatted with petroleum ether for 3 cycles and extracted with 70% methanol. Further methanol extract was spanned at 10,000 rpm; supernatant was collected, concentrated and used for the present study.

Preliminary screening for flavonoids

Methanolic extract was used for preliminary screening of flavonoids using Shinoda test and ferric chloride test [4].

Separation and purification of flavonoids

In the first step, flavonoids were separated using the preparative thin layer chromatography technique [5]. The analysis was performed on precoated 20 ×20 cm (0.25 mm thick) TLC plates K6F silica gel 60 A purchased from Whatman, USA. Methanolic extract was applied as spot onto TLC sheets along with the standard. Five different mobile phases were used to optimize the TLC conditions. The plates were developed at room temperature in a previously saturated vertical separating chamber. After drying, visualization was performed in two ways: First in short UV light (254 nm) and next in spraying with 1 % ferric chloride solution. Purification of the separated flavonoid was done using Silica gel column chromatography technique [6]. Borosil glass column with standard laboratory size was washed and dried. Column was tightly packed with silica gel of mesh size 60. Using the eluting buffer column was washed and checked for presence of air bubbles and cracks. 5 gm of methanolic extract was loaded onto the column. Elution buffer (methanol: water: : 70: 30) was used for the elution. Fractions were collected at a regular interval in 2 ml tubes. Collected fractions were again run on TLC; fractions with similar Rf values were pooled together and concentrated.

Table 1: Shows the solvent system used for optimization of flavonoids using TLC

Solvent system	Ratio (V/V/V)
Ethyl acetate: Formic acid: Water	65:15:20
Acetic acid: Chloroform	1:9
Ethyl acetate: Benzene	9:11
Toluene: Chloroform: Acetic acid	45:25:35
Chloroform: Ethyl acetate: Formic acid	5:4:1

Characterization of flavonoid

FTIR, NMR and LC MS spectroscopic techniques were used for the characterization of the flavonoids. Functional group present in the compound was studied by using Fourier–transformation– Infra Red spectroscopy ((FT– IR– Bruker, Japan). Sample was prepared by fixing in potassium bromide discs and 600-4000 cm-1 spectrum range was scanned [7]. Chemical shifts of purified compound dissolved in Dimethyl sulphoxide was observed using the sophisticated multinuclear NMR Spectrometer model Advance II (Bruker) with 400 MHz frequency, with a cryo magnetic strength 9.4 Tesla [8]. Molecular mass and mass by charge ratio of purified compound was calculated using Liquid Chromatography Mass Spectroscopy instrument (Waters Micromass Q-Tof Micro) equipped with electrospray ionization was used in a mass range of 4000 – 20000 atomic mass units [9,10].

RESULTS AND DISCUSSION

Preliminary screening of flavonoids

Preliminary phytochemical screenings are the basic bricks for any drug discovery [11]. Unless the preliminary tests respond positively, it is difficult to proceed for the separation and purification techniques of any compound. Methanolic extract of *Feronia limonia* was screened for the flavonoids using two preliminary tests. The result of the preliminary screening is tabulated in Table 2.

Table 2: Shows the qualitative examination of flavonoids

Test	Observation
Shinoda test	Flavonoids present. (Pink Colour)
Ferric chloride test	phenol hydroxyl group present (Violet Colour)

Separation and Purification

Thin layer chromatographic (TLC) is the simplest technique used to separate and identify natural products of interest. This method readily provides qualitative information and possibly quantitative data. Bioassay separation of any plant metabolite resembles the process of separating a desired compound from chemical machinery. Hence, we need to optimize the separation conditions like Concentration, Solvent system, Visualizing methods. In the present study five different solvent systems were used for the separation of flavonoids from methanolic extract of *Feronia limonia*. Solvent system (Chloroform: Ethyl acetate: Formic acid**;** 5:4:1; v/v/v) has supported for a clear separation with good resolution. Purified fractions were screened for their Rf value and pooled together for characterization.

Characterization

FTIR Spectroscopy

Fourier transformation – infra – red (FT – IR) spectrum result of the isolated flavonoid is shown in Fig. 1. Several absorption peaks belonging to functional and / or structural groups were recorded. Appearance of broad band at 3396.3 cm-1 represents presence of stretching vibration of phenolic groups containing hydrogen bonding. The weak band at 2936.15 cm-1 indicates stretching vibration of aromatic (C-H) group and also the medium band at 2038.65 cm-1 represents bending vibration of aliphatic (C-H) group. Presence of weak peak at 2864.09 cm-1 indicates stretching aliphatic (C-H) group whereas the sharp and strong band at 1654.81 cm-1 represents stretching vibration of aromatic(C=O) group. Appearance of medium band at 1535.23 cm-1 indicates stretching vibration of aromatic (C=C) group.

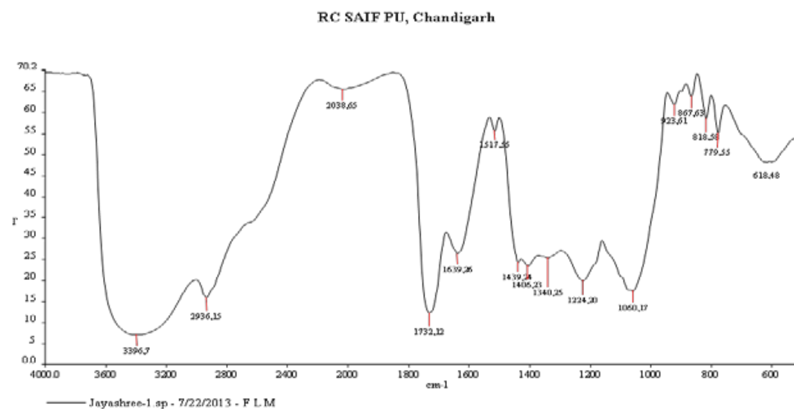

Fig. 1: It shows the ft-ir spectra of isolated luteolin

NMR Spectroscopy

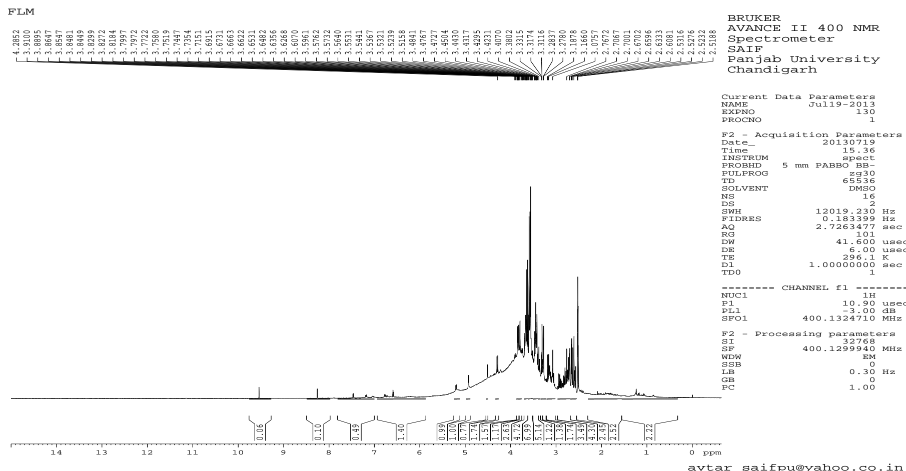

Fig. 2: It shows the nmr spectra of isolated luteolin

NMR spectroscopy offers the most useful and valuable information about the structure of perhaps any natural product. The method has the advantage of excellent reproducibility. Even though it is considered to be one of the more expensive techniques, NMR is relatively cheap, fast sensitive and easily used as a routine application for amino acid analysis. The[1] H-NMR spectrum showed 2 peaks at δ 4.21 (1H, d, *J* =1.8 Hz) and 4.14 ppm (1H, d, *J* =1.8 Hz) consistent with the meta protons H-6 and H-8 on A-ring and an AA'BB' system at 4.54 (2H, d, *J* =8.9 Hz, H-2', 6') and 3.89 (2H, d, *J* =8.9 Hz, H-3', 5') corresponding to the protons on B-ring. Compound presented the aglycone signal patterns.

By comparison of proton up field shift values with the literature data, the ^{1}H NMR data of isolated compound (Fig. 2) is compatible with the literature libraries of standard flavonoids.

Mass spectrometry

Mass spectrometry represents one of most efficient techniques for natural product, structure elucidation. It functions by a separation of the ions formed in the ionization source of the mass spectrometer, according to their mass-to-charge (m/z) ratios. Over the past 2 decades, mass spectrometry has proved to be one of the most effective techniques in biomedical research, particularly for the analysis of complex mixtures in biological samples. A particular advantage of liquid chromatography mass spectrometry (LC-MS) is its capability to determine both free and conjugated forms of flavonoids [12]. The ESI-MS yielded a quasi-molecular ion peak at *m/z* 283.05. Molecular mass of the isolated flavonoid was found to be 283.35 g/mol which is the nearest value to luteolin having molecular mass 286.24 g/mol as shown in Fig. 3.

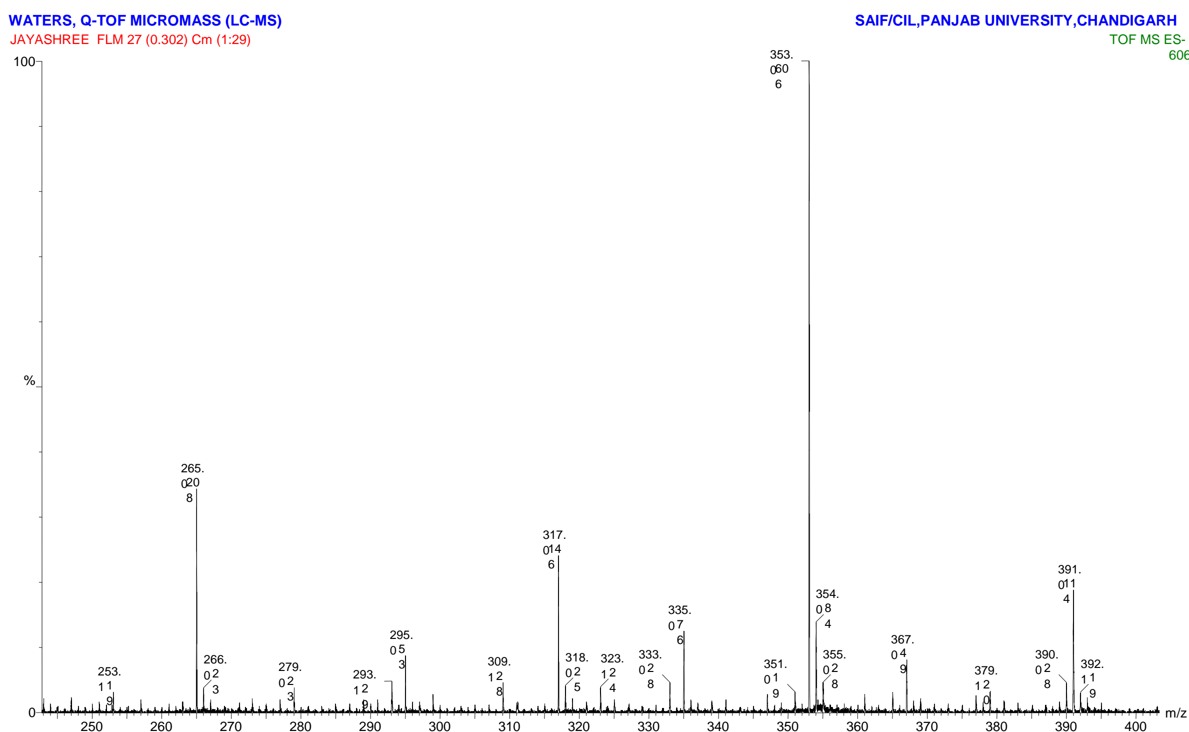

Fig. 3: It shows the mass spectra of isolated luteolin

Structural elucidation of pure compound luteolin

The spectroscopic studies FTIR, NMR and LC-MS revealed the functional group, chemical shifts and mass of the compound. Using these spectral details, the compound was identified as luteolin. Molecular formula and elemental analysis of isolated compound is $C_{15}H_{10}O_{6}$ and C, 62.94; H, 3.52; O, 33.54 respectively. Molecular weight is 286.24 with m/z of 286.05 (100.0%), 287.05 (16.6%), 288.05 (2.5%). The two dimensional structure of the isolated flavonoid was drawn using Chem Draw, Ultra, Version 12 software. The structure of the isolated compound is shown in Fig. 4.

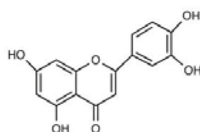

Fig. 4: It shows the structure of isolated flavonoid luteolin from *feronia limonia*

Flavonoids such as luteolin are ubiquitous plant secondary metabolites and have a variety of biological effects, including antioxidant, anti-inflammatory activities [13-15]. Belonging to the flavone group of flavonoids, luteolin has a C6-C3-C6 structure and possesses two benzene rings (A, B), a third, oxygen-containing (C) ring, and a‒32 carbon double bond. Luteolin also possesses hydroxyl groups at carbons 5, 7, 3', and 4' positions (Fig.4) [16]. The hydroxyl moieties and ‒23 double bond are important structure features in luteolin that are associated with its biochemical and biological activities [17]. As in other flavonoids, luteolin is often glycosylated in plants, and the glycoside is hydrolyzed to free luteolin during absorption [18]. To our best knowledge, the present study is the first report on the isolation and identification of flavonoid luteolin from the fruit pulp of *F. limonia*. We also developed a solvent extraction and TLC method with optimized conditions to separate and identify the constituents present in the methanolic extract.

Using preliminary phytochemical screening, preparative thin layer chromatography and silica gel column chromatography a pure flavonoid 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one was obtained from fruit pulp of *Feronia limonia* Linn. Data of spectral studies reveals the elemental, structural and molecular details of the isolated compound. The isolated compound is known

to exert many medicinal plants. Hence further research is requisite to be carried out to identify its biological activities.

ACKNOWLDGEMENT

Authors are thankful to the sophisticated analytical instrument facility centre (SAIF), Punjab University, Chandigarh, Punjab, India for providing the instrumentation facility for spectroscopic studies of the compound.

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