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Isolation, Purification and Structural Elucidation of Novel Bioactive Phytoconstituents from *Azima tetracantha*

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Abstract

The objective of the study was to isolate and characterize the bioactive phytoconstituents from *Azima tetracantha* leaf extract. For isolation of the compounds, ethanolic extract of *Azima tetracantha* leaf extract was fractionated for conventional column chromatography. Three compounds (COMPOUND- I, COMPOUND- II, COMPOUND-III) were isolated by gradient elution technique and purified with methanol. The subsequent structures of isolated compounds were elucidated by various spectrophotometric analysis. The subsequent structures of isolated compounds were elucidated by various Spectrophotometric analysis. The mass spectrum of compound **I** is displayed signal at m/z 427.91, compound **II** is signal at m/z 625.526 and compound **III** is displayed signal at m/z 319.714. In compound **I** ^{13}C NMR spectrum the signal appeared in the region 14.13- 29.12 ppm is due to the methyl carbons. In compound **II** the signal appeared in the region 128.07-129.44 ppm is due to the aromatic carbons. The methyl carbon was appeared at 14.11 ppm. In compound **III** the signal appeared in the region 123.22-145.99 ppm is due to the aromatic carbons. The carbonyl carbon was appeared at 177.71 ppm. From the physical, chemical and spectral characters were concluded that COMPOUND- I, COMPOUND- II, COMPOUND-III were concluded as Friedelin (Triterpenoids), Isorhamnetin 3-O-rutinoside (Triterpenoids) and Myricetin (Flavanoids).

Keywords: *Azima tetracantha* ,Isolation, Friedelin, Isorhamnetin 3-O-rutinoside, Myricetin

INTRODUCTION

India has rich ancient heritage of traditional medicine [1]. From last two decades, the utility of medicinal plants have been phenomenally increased due to their vast chemical biodiversity as World Health Organization advocated traditional medicines as safe remedies [2]. The conventional therapeutic experiences of an array of bioactive phytoconstituents from those species, over hundreds years areconsidered as valuable remedial recipe to treat various acute and chronic disorders.

Among them *Azima tetracantha* (Salvadoraceae) is a well known medicinal herb, termed '*Mulsangu*' in Tamil and '*Kundali*' in Sanskrit. Root, root bark and leaves of *Azima tetracantha* (lam) are used with food as a remedy for rheumatism, diuretic and as stimulant [4]. Traditionally Indian medical practitioners use *Azima tetracantha* (lam) in inflammatory conditions, cough, asthma, small pox and diarrhoea [5,6]. The major phyto-constituents reported in *Azima tetracantha* (lam) are azimine, azecarpin, carpine, isorhamnitine-3-O-rutinoside, friedelin, lupeol, glutinol and β-sitosterol [7,8]. *Azima tetracantha* (lam) is reported to have antifungal[9] antitumour [10], antidiabetic [11] , antidiarrhoeal [12] and hepatoprotective [13,14] activities.

Azima tetracantha (lam) is a low, spinouts, highly branched bush, woody below but with pale green, herbaceous, almost quadrangular young branches. The leaves are in opposite to sub-opposite, decussate pairs. They are shortly petiolate, about 2x4cm long, entire, elliptic, acute, sharply mucronate, rigid, pale green with an acute base. Usually, there are two laterally placed spines in the axil of a leaf. The spines which morphologically represent the first pair of leaves of the auxiliary shoot are about three cm long, more or less, triangular in cross section, very sharp and with an indurate apex. The plant is dioeciously. The flowers are borne in the axils of leaves. Generally, there is cymes of three flowers in the axil of a leaf which is the upper branches, especially of the male plants become greatly reduced or even completely suppressed. Since, the plant posses diverse medicinal properties, the present work had been designed to isolate and characterize novel bioactive phytoconstituents from *Azima tetracantha* (lam).

MATERIALS AND METHODS

Collection of plants

The aerial part (leaves) of *Azima tetracantha* (lam) was collected from the Panayur area of Madurai, Tamilnadu as raw material, during the second week of February 2015 and a voucher specimen is stored in C.L. Baid Mehta College of Pharmacy (001/ATL/CLBP) and the plant material was authenticated by a renowned botanist. About 500 g of coarse powdered leaf in 2.5 L water is boiled, cooled and filtered. The filtrate is evaporated to dryness in desiccator and stored in refrigerator (Yield- 26.5% w/w). The aqueous extract of *Azima tetracantha* (lam) (AEAT) was subjected to preliminary phytochemical analysis [15]

Column chromatography of ethanol extract

Among the various extracts obtained from 800 gm of coarsely powdered leaf of the plant, ethanol and ethyl acetate extract were found to be promising. The ethanol extract was brown residue (12gm). The ethyl acetate extract was dark brown residue (16gm).The ethanol extract was chromatographed over about 300 gm of silica gel (100-120 mesh) using petroleum ether, ethyl acetate, chloroform, methanol and their mixtures in various proportions in order of their increasing polarities. The column was packed by using the suspension of silica gel in petroleum ether. Each 100 ml of the elute was collected and concentrated. The obtained fractions were tested for the presence of various constituents and nature of the compounds.

Table No: 1
Data showing the column chromatography of Ethanol Extract

S.No.	Number of Fractions	% of Solvent	Volume of Solvent (ml)	Compounds
1	1-50	98%pet ether: 2% ETOAc	600	
2	51-90	95% pet ether : 5% ETOAc	500	
3	91-120	90% pet ether :10% ETOAc	500	
4	121-160	85%chloroform: 15% ETOAc	600	Compound I
5	161-180	80% chloroform: 20% ETOAc	800	
6	181-200	75%choloroform:25% ETOAc	800	Compound II
7	201-220	100% chloroform	400	
8	221- 238	98% chloroform: 2% MeOH	350	Compound III
9	239 - 250	90% chloroform: 10% MeOH	250	

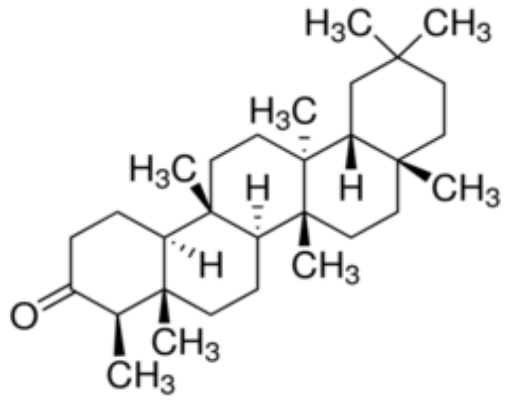
EtOAc - Ethyl Acetate, Pet ether- Petroleum ether.

Phytochemical Screening
The extract was subjected to phytochemical analysis to test the presence of carbohydrates, glycosides, alkaloids, flavonoids, tannins, sterols, and saponins in leaf extracts.

Structural characterization of compounds
The structures of the isolated compounds were elucidated by spectroscopic methods viz. UV (Shimadzu UV-1700 Pharmac-spec UV-Vis spectrophotometer) and IR (Alpha-Bruker IR spectrophotometer), 1H NMR & 13C NMR (Bruker Advance II 400 NMR spectrophotometer), mass (TOF MS ES - 3.26e3 spectrophotometer). 1H & 13C NMR were recorded using CDCl3 as solvent and with tetramethylsilane (TMS) as standard.

RESULTS AND DISCUSSION

SPECTROSCOPY ANALYSIS OF COMPOUND- I, COMPOUND- II, COMPOUND-III
These compounds eluted with solvents of increasing polarity like petroleum ether, ethyl acetate, chloroform and methanol. In the course of isolation procedure, the selected 3 compounds were further processed and characterized.



Structure of Friedelin

COMPOUND-I is amorphous dark green powder which after crystallization with methanol provides colourless crystalline substance (120mg) and the melting point is 83°C and soluble in ethyl acetate and methanol. The IR, ^{1}H NMR, ^{13}CNMR, MASS Spectral data showed the characteristic feature of triterpenoids and which was also confirmed by chemical test.

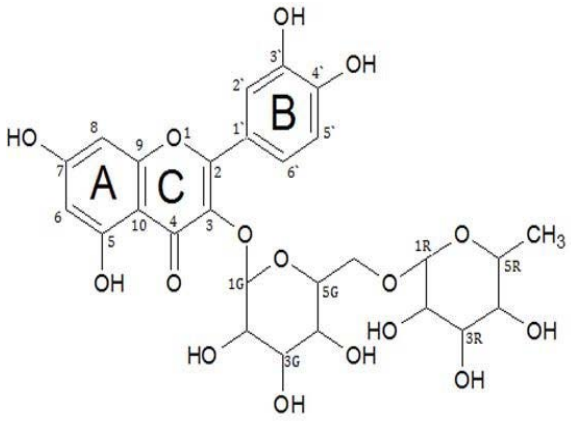
IR spectral analysis of compound- I
The FT-IR spectra were recorded in the region of 4000-400 cm^{-1}. A collection of medium bands observed in the region of 3106-2673 cm^{-1} is attributed to C-H stretching vibration of aromatic and aliphatic groups. Strong absorption bands in the region of 1707 cm^{-1} are characteristic for carbonyl group stretching vibrations.

^{1}H NMR analysis of compound I
^{1}H NMR signals are assigned based on their positions multiplicity, integral values and comparison with that of their parent compound signals. In compound **I**, the six methyl protons are appeared in the region 0.730 – 1.250 ppm as a singlet. The eleven methylene protons are appeared in the region 1.216 – 2.444 ppm.

^{13}C NMR analysis of compound I
^{13}C NMR spectrum of compound **I** the signal appeared in the region 14.13- 29.12 ppm is due to the methyl carbons. The methylene carbons were appeared in the region 29.12 – 60.29 ppm.

Mass Spectral Analysis of compound I
The mass spectrum of compound **I** is displayed signal at m/z 427.91, corresponding to $[M+H]^{+}$ is in agreement with calculated value (m/z 426.729). Mass spectral studies strongly confirmed the formation of compounds and their results are in strong agreement with the proposed molecular formula of the respective compounds.

COMPOUND-II is amorphous yellow powder which crystallization with methanol provides colourless crystalline substance (105mg) and the melting point is 84°C and soluble in ethyl acetate and methanol. The IR, ^{1}H NMR, ^{13}CNMR, MASS Spectral data showed the characteristic feature of flavanoids and which was also confirmed by chemical test.



Structure of Isorhamnetin 3-O-rutinoside

IR spectral analysis of compounds II

The FT-IR spectra were recorded in the region of 4000-400 cm^{-1}. A collection of medium bands observed in the region of 3063-2805 cm^{-1} is attributed to C-H stretching vibration of aromatic and aliphatic groups. Strong absorption bands in the region of 1637 cm^{-1} are characteristic for carbonyl group stretching vibrations. Bands at 3232 cm^{-1} are characteristic for OH stretching vibrations of the synthesized compounds.

^{1}H NMR analysis of compound II

^{1}H NMR analysis of compound **II** ^{1}H NMR signals are assigned based on their positions multiplicity, integral values and comparison with that of their parent compound signals. In general, the aromatic protons are absorbed in the higher frequency region around at 7 ppm due to their magnetic anisotropic effect. In compound **II**, the signals observed in the region of 7.26-7.38 ppm with an expected proton integral values. Therefore, these signals are unambiguously assigned to aromatic protons. The methoxy proton signal observed at 3.866 ppm as a doublet. A methyl proton observed at 2.413 ppm, oxygen connected methylene protons are observed at 3.610 -3.526 ppm as a triplet. All the hydroxyl protons are appeared in the region 1.10-2.12 ppm as a multiplet.

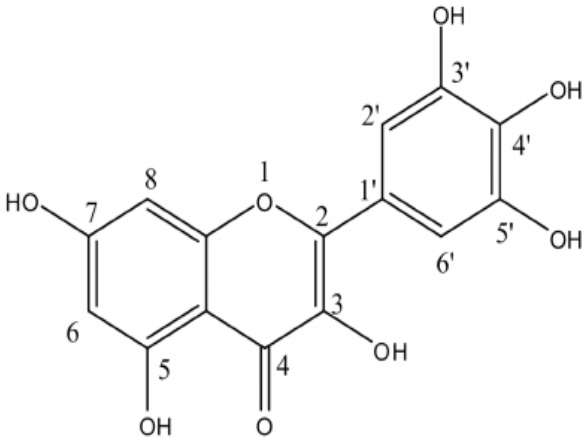
^{13}C NMR analysis of compound II

^{13}C NMR analysis of compound **II**^{13}C NMR signals are assigned based on their positions multiplicity, integral values and comparison with that of their parent compound signals. In general, the aromatic carbons could be readily distinguished from the other carbons due to their characteristic absorption around 120.00 ppm. The *ipso* carbons are absorbed in the higher frequency region compared to the aromatic carbons. In compound **II** the signal appeared in the region 128.07-129.44 ppm is due to the aromatic carbons. The methyl carbon was appeared at 14.11 ppm. A methoxy carbon signal appeared at 22.65 ppm. Methylene carbons are appeared in the region 22.65-60.18 ppm.

Mass Spectral Analysis of compound II

The mass spectrum of compound **II** is signal at m/z 625.526, corresponding to $[M+H]^{+}$ is in agreement with calculated value (m/z 624.548). Mass spectral studies strongly confirmed the formation of compounds and their results are in strong agreement with the proposed molecular formula of the respective compounds.

COMPOUND-III is amorphous green powder which after crystallization with methanol provides colourless crystalline substance (114mg) and the melting point is 82-83°C and soluble in ethyl acetate and methanol. The IR, ^{1}H NMR, ^{13}CNMR, MASS Spectral data showed the characteristic feature of flavanoids and which was also confirmed by chemical test.



Structure of Myricetin

IR spectral analysis of compounds III

The FT-IR spectra were recorded in the region of 4000-400 cm^{-1}. A collection of medium bands observed in the region of 3084-2810 cm^{-1} is attributed to C-H stretching vibration of aromatic and aliphatic groups. Strong absorption bands in the region of 1716 cm^{-1} are characteristic for carbonyl group stretching vibrations. Bands at 3408 cm^{-1} are characteristic for OH stretching vibrations of the synthesized compounds.

^{1}H NMR analysis of compound III

^{1}H NMR analysis of compound **III** ^{1}H NMR signals are assigned based on their positions multiplicity, integral values and comparison with that of their parent compound signals. In general, the aromatic protons are absorbed in the higher frequency region around at 7 ppm due to their magnetic anisotropic effect. In compound **III**, the signals observed in the region of 7.33-7.50 ppm with an expected proton integral values. Therefore, these signals are unambiguously assigned to aromatic protons. The hydroxyl protons signal observed at 2.012 ppm as a doublet and 3.943 ppm as a singlet.

^{13}C NMR analysis of compound III

^{13}C NMR analysis of compound **III**^{13}C NMR signals are assigned based on their positions multiplicity, integral values and comparison with that of their parent compound signals. In general, the aromatic carbons could be readily distinguished from the other carbons due to their characteristic absorption around 120.00 ppm. The *ipso* carbons are absorbed in the higher frequency region

compared to the aromatic carbons. In compound **III** the signal appeared in the region 123.22-145.99 ppm is due to the aromatic carbons. The carbonyl carbon was appeared at 177.71 ppm.

Mass Spectral Analysis of compound III

The mass spectrum of compound **III** is signal at m/z 319.714, corresponding to $[M+H]^+$ is in agreement with calculated value (m/z 318.23). Mass spectral studies strongly confirmed the formation of compounds and their results are in strong agreement with the proposed molecular formula of the respective compounds.

CONCLUSION

In the present study, 3 compounds were isolated by column chromatography have been identified from the ethanolic leaf extract of *Azima tetracantha*. The ethanol extract is mainly composed of terpenoids and flavanoids. Thus, *Azima tetracantha* is found to possess significant phytonutrients, which attribute to its medicinal worth.

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