

The Isolation and Structural Determination of Flavonoids from *Justicia Gendarussa*

Raghu.M.G¹, Pushpa Agrawal^{2*}

¹ Department of Biotechnology; Tumkur University; Tumkur – 572106; Karnataka, INDIA

² Department of Biotechnology; C.M.R.T.U; R.V.College of Engineering Campus; Bangalore – 560059; Karnataka, INDIA

Abstract: The current study was intended for isolation of bioactive polyphenolic compounds from methanol extract of *Justicia Gendarussa* and their subsequent characterization. The flavonoid compounds were isolated and identified by using thin layer chromatography (TLC), were qualified by using reverse phase high performance chromatography (RP-HPLC). Their structures and chemical bonds were analyzed using Ultraviolet-Visible spectrophotometer (UV-VIS spectrophotometer), Fourier Transform-Infra Red spectroscopy (FTIR), Nuclear magnetic resonance NMR (13C and 1H), LC-MS, DSC, SEM and EDX techniques. Two flavonoids were identified as Apigenin and Vitexin. For the first time Apigenin and Vitexin have been isolated successfully from the *Justicia Gendarussa*. The isolation of the above considered flavonoids would be helpful to practise plant-based pharmaceutical research to treat various difficulties linked with human diseases.

Keywords: *Justicia gendarussa*, TLC, HPLC, UV, FTIR, SEM, EDX, DSC, NMR, LC-MS, Apigenin, Vitexin

I. Introduction

Justicia Gendarussa has been suggested in the Indian system of medicine for huge number of diseases. The plant is used in traditional folk medicinal practice for chronic rheumatism, bronchitis, dyspepsia, eye diseases, arthritis, jaundice, cephalgia, hemiplegia, eczema, fever etc. The leaves and roots are bitter, thermogenic, anti-periodic and acrid. In old times decoction of *Justicia Gendarussa* is used for the treatment of fever, rheumatic disorders, dysuria and diarrhea. Medicinal uses- *Gendarussa* is reputed for its beneficial effects properties such as antioxidant and free radical scavenging, anti-inflammatory, analgesic, anti-arthritis, antifertility, hepatoprotective and anticancerous properties. Alkaloids, flavonoids, phenolics, saturated steroidal saponins, aromatic amines, amino acids and are rich in potassium salts contains in the leaves of *Justicia Gendarussa*. Medicinal plants show the confirmation of anti-fungal, anti-oxidant and anti-bacterial activity. Microbiologists have conducted experiments that involve frequent demanding food-borne yeast, fungi, and bacteria with phytochemicals extract of plants. Many methods have been used to examine antimicrobial activity, and the most important data vary significantly in quantity and quality among different spices. However, at the moment it's clear that lots of spices have effective antimicrobial properties [1-5]. More than 4000 chemically unique flavonoids have been isolated and identified in plant extracts obtained through solvent extraction processes [6-7]. More recently, a few flavonoids have also been isolated from microorganisms as their secondary metabolites [8]. Flavonoids are of great importance for the bioactivities, related to their antioxidant activities and many enzymatic reactions, resulting in a decrease of platelet activation and aggregation, against cardiovascular diseases, cancer chemoprevention and anti-inflammatory activity [9-15].

However, to the best of our knowledge, flavonoids are useful in pharmaceutical applications for drug discovery and development. Hence we have isolated, identified and reported the flavonoids from methanolic extracts of leaves. Furthermore, we have been characterized the flavonoids through UV, FTIR, (13C and 1H), LC-MS, DSC, SEM and EDX techniques.

II. Materials And Methods

2.1 Preparation of extracts:

The fresh leaves of *Justicia Gendarussa* were washed with running water followed by double distilled water. Shade dried for a period of one month. The dried leaves were powdered coarsely. Shade dried leaf powder of the plant was weighed and extracted with methanol using Soxhlet method, for 24 hours at 50°C. Decant the methanol extract and the extract was filtered through Whatman filter paper No 1. Concentrated the solvent extract in an air circulating oven at 45°C until total dryness. Dried extract was stored at 4°C for further analysis.

2.2 Extraction of Flavonoid:

To separate the lead molecule from methanol extract of *Justicia Gendarussa*, the residue was dissolved in 500mL of distilled water. The filtrate was partitioned with petroleum ether, ethyl ether and ethyl acetate.

Ethyl acetate extract was concentrated by vacuum oven. Ethyl acetate extract was subjected to column chromatography on silica gel eluted with chloroform-methanol gradient to afford Compound-A (CHCl₃: Methanol 9:1). Further the same ethyl acetate extract was subjected to column chromatography on Sephadex LH-20 eluted with methanol-water gradient to obtain Compound-B (Methanol: Water 1:1).

2.3 Physical and Chemical Characteristics of isolated compounds

The Thin layer chromatography silica gel 60 F254 aluminum plates which are 20 x 20cm with 0.25mm thickness were procured and the extract was separated by TLC with the following mobile phases: ethyl acetate: formic acid: water (50:4:10) for Compound-A and ethyl acetate: acetic acid: formic acid: water (100:11:11:26) for Compound-B. The plates were developed using ammonia fumes and visualized under UV lamp. The R_f values of separated bands were calculated. The isolated compounds were dissolved in methanol, filtered and were analysed in HPLC (Dionex Ultimate 3000), by injecting 20µl and 10µl through the Thermo scientific Synchronis C18, 5µm, 4.6mm*250mm and Inertsil ODS-3V, 5µm, 4.6mm*250mm at column temperature 25°C and 30°C with the flow rate of 1 mL/min for Apigenin and Vitexin. For Apigenin, the mobile phase consists of two different solutions, solution A is 100% acetonitrile and solution B is 0.1% trifluoroacetic acid. Both the solutions are mixed in the ratio of 30:70v/v, filtered with 0.45µ pore size filter and sonicated to degas for 5 minutes and the run time was 40 minutes. For Vitexin, the mobile phase consists of two different solutions, solution A is 100% acetonitrile and solution B is 0.1% orthophosphoric acid. Both the solutions are mixed in the ratio of 20:80v/v, filtered with 0.45µ pore size filter and sonicated to degas for 5 minutes and the run time was 30 minutes. Peaks are monitored at wavelength 337nm and 335 nm for Apigenin and Vitexin respectively by UV detection. Wavelength is fixed as per lambda maxima obtained from UV spectra.

The isolated compounds likely Apigenin and Vitexin were dissolved in methanol and their maximum UV absorption ranges were recorded using the UV Visible is double-beam spectrophotometer. FTIR spectra of the compounds were measured using IR grade potassium bromide (KBr). The compounds were separately mixed with 200mg KBr to obtain round disc with the help of hydraulic press. Round disc was later subjected to FTIR in the range of 4000-400cm⁻¹ at a resolution of 4cm⁻¹. The compounds were dissolved in CDCl₃ for H¹ and C¹³ NMR spectra using NMR spectroscopy with TCI Cyroprobe were recorded. The chemical shift values were reported in ppm unit and the coupling constants (J) are in Hz. Molecular mass was identified by LC-MS. The melting point is determined by DSC. The compounds were sealed in the aluminium crimp cell and heated at the speed of 10 °C/min from 0 to 500 °C in the atmosphere of nitrogen using Perkin Elmer Differential Scanning Calorimeter. The data were recorded and processed by Pyris Manager Software.

The isolated compounds derived from plant extracts were subjected to the elemental analysis using Scanning Electron Microscope (SEM) with an energy dispersive x-ray spectrometer (EDX). Energy dispersive X-ray spectrometers take advantage of the photon nature of light. In the X-ray range the energy of a single photon is just sufficient to produce a measurable voltage pulse X-ray, the output of an ultra low noise preamplifier connected to the low noise are a statistical measure of the corresponding quantum energy. By digitally recording and counting a great number of such pulses with in a so called Multi Channel Analyzer, a complete image of the X-ray spectrum is building up almost simultaneously. This digital quantum counting technique makes the energy dispersive spectrometry exceedingly reliable. A semiconductor material is used to detect the x-rays together with processing electronics to analyses the spectrum. The data were recorded and processed by Noran System 7.

III. Results and Discussion

3.1 Physical and Chemical Characteristics of isolated compounds

The methanol extract of *Justicia Gendarussa* on phytochemical screening showed the presence of large number of compounds. The methanolic extract filtrate was partitioned with petroleum ether, ethyl ether and ethyl acetate. Ethyl acetate extract was concentrated by vacuum oven. Ethyl acetate extract was subjected to column chromatography. TLC was done for the compounds obtained by column chromatography and the R_f values shown 0.81 for Compound-A and 0.77 for Compound-B.

Table 1: Showing the TLC R_f values for the compounds isolated

Compound	R _f Value	Color
Apigenin	0.81	Orange
Vitexin	0.77	Green

The Chromatographic analysis using HPLC indicated that compound-1 and compound-2 had the same retention time (R_t: 27.3 and 11.0) as the standard flavonoids, namely Apigenin and Vitexin respectively. The peaks of Apigenin and Vitexin are pure and the match factor is 999.

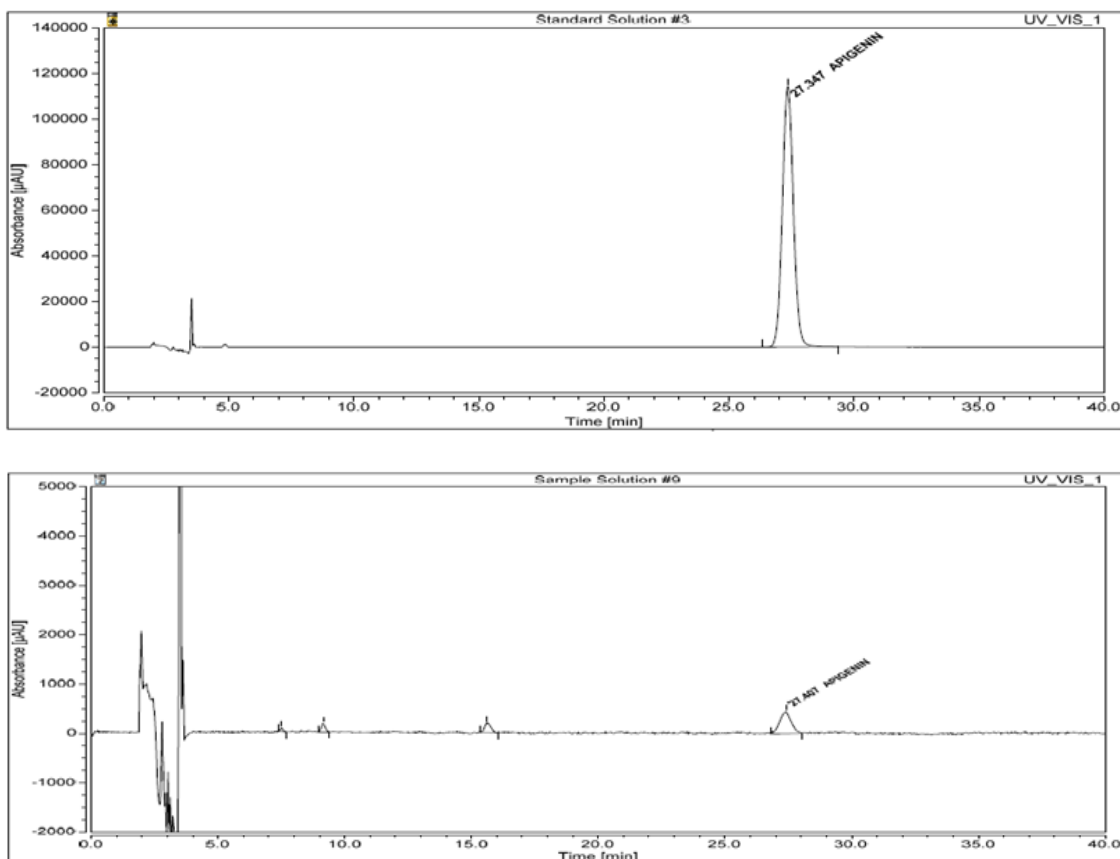


Figure 1 – HPLC Chromatogram for the Apigenin standard and sample

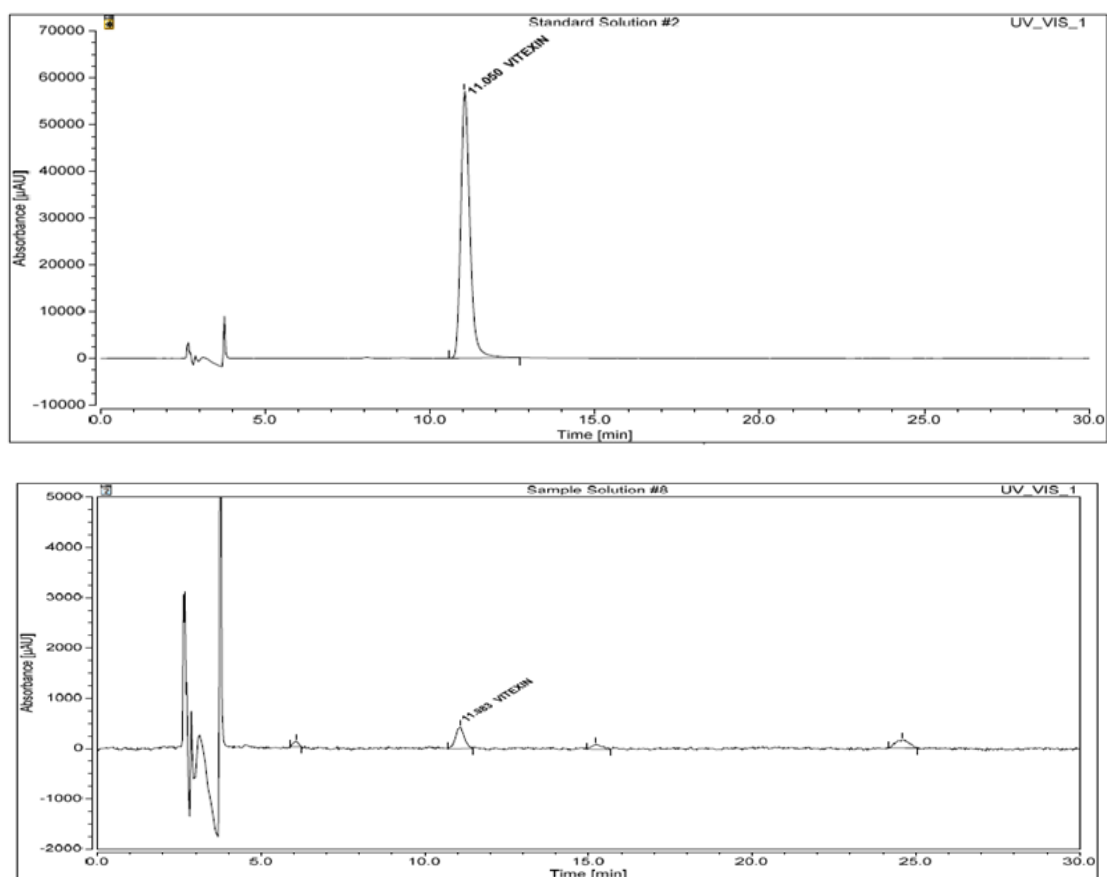
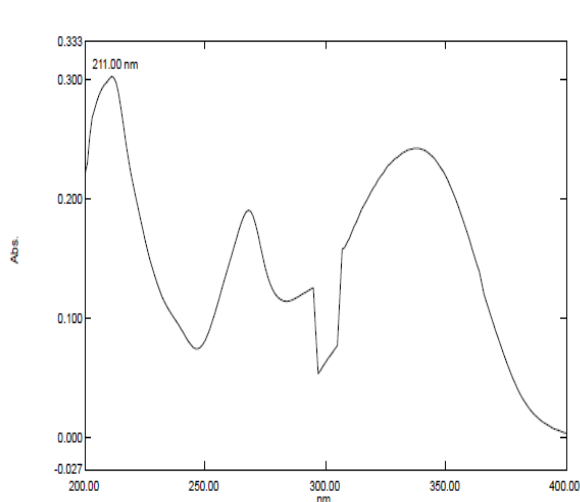


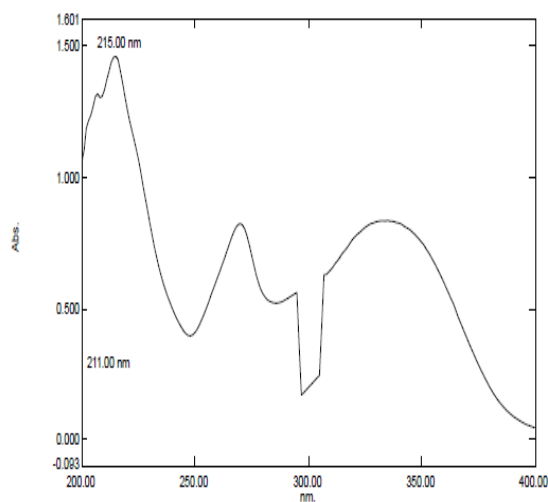
Figure 2 – HPLC Chromatogram for the Vitexin standard and sample

The UV spectrum of the methanol solution of compounds has characteristic bands at λ_{max} = 337 nm, 268 nm, 211 nm and λ_{max} = 335 nm, 270 nm and 215 nm which leads to the conclusion that the compound belongs to the group of flavonoids and quotes Apigenin and Vitexin respectively as comparison with the literatures.



No.	P/V	Wavelength	Abs.
1	⬆	337.00	0.243
2	⬆	268.00	0.191
3	⬆	211.00	0.303
4	⬇	297.00	0.053
5	⬇	246.00	0.074

Figure 3: UV Absorbance of Apigenin



No.	P/V	Wavelength	Abs.
1	⬆	335.00	0.835
2	⬆	270.00	0.823
3	⬆	215.00	1.460
4	⬇	297.00	0.172
5	⬇	248.00	0.397

Figure 4: UV Absorbance of Vitexin

The intensive bands between 1600 and 1580 cm^{-1} are corresponds to aromatic ring vibrations. The bands at 3287 cm^{-1} and 3097 cm^{-1} is probably the result of Hydrogen bonded O=H stretch and C-H vibrations. The bands between 1300 and 1000 cm^{-1} are corresponds to C-O stretch vibrations. The intensive bands between 1440 and 1500 cm^{-1} assigned to the C-H vibrations and the peak at 2697 cm^{-1} assigned to the C-H symmetric stretching.

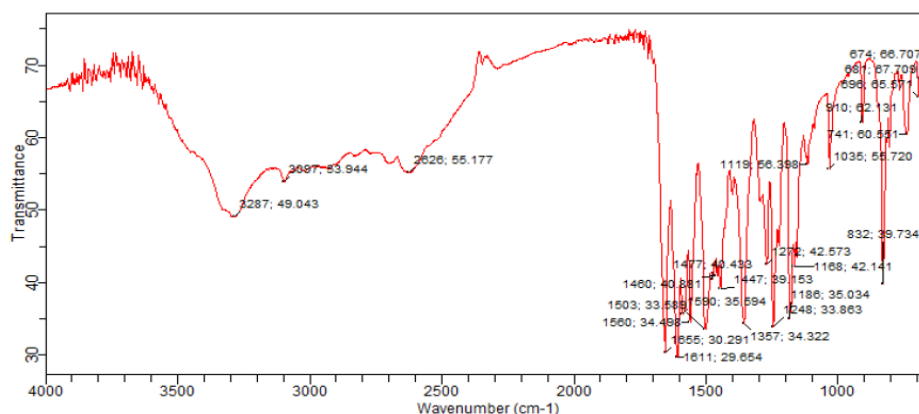


Figure 5: Apigenin sample FTIR chromatogram

The intensive bands between 1600 and 1580 cm^{-1} are corresponds to aromatic ring vibrations. The bands at 3254 cm^{-1} and 3254 cm^{-1} is probably the result of Hydrogen bonded O=H stretch. The strong band at 2911 cm^{-1} assigned to the C-H asymmetric stretching vibration and the peak at 2874 cm^{-1} assigned to the C-H symmetric stretching. The bands between 2874 cm^{-1} , 2911 cm^{-1} , 1110 cm^{-1} , 1095 cm^{-1} represents C-H stretching vibrations.

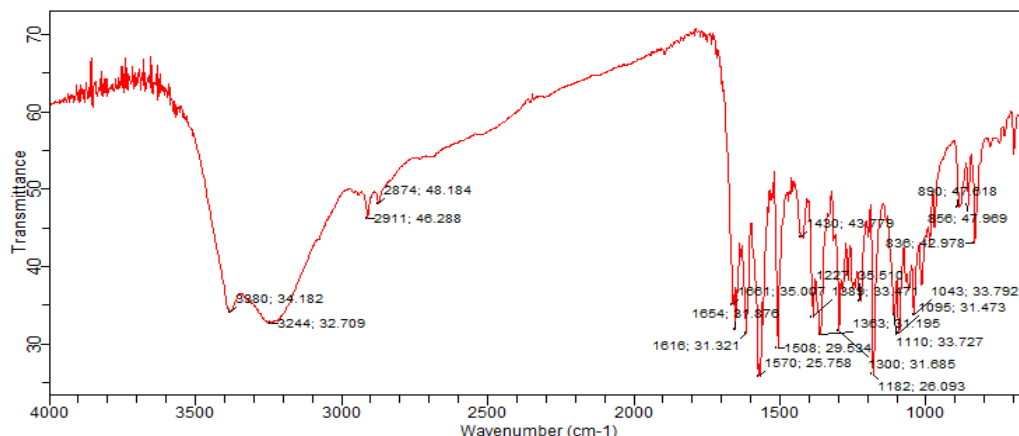


Figure 6: Vitexin sample FTIR chromatogram

Sample is detected yellow amorphous powder, EI-MS m/z : [M+H]⁺ + 268.8, which match up to the molecular formula C₁₅H₁₀O₅; ¹H-NMR (DMSO-d₆,400MH_Z): δ 13.0 (1-H,s,H-17), 10.801(1-H,s,H-18), 10.338 (1-H,s,H-19), 7.9 (2H,d,H-5,H-3), 6.9 (2H,d,H-2,H-6), 6.7 (1H,s,H-15), 6.46 (1-H,s,H-13), 16.186 (1H,s,H-16); ¹³C-NMR (DMSO-d₆,125MH_Z): δ 181.7 (s,C-4),164.1 (s,C-5),163.7 (s,C2), 161.5 (s,C-4), 161.2 (s,C-9), 157.3 (s,C-7), 128.4 (d,C-2' and C-6'), 121.2 (s,C1), 116.0 (d,C-3' and C-5'), 103.7 (s,C-10), 102.8 (d,C-3), 98.8 (d,C-6), 94.0 (d,C-8).Thus, according to the mentioned data and by comparison with the literature, compound identified as Apigenin.

Sample is detected light yellow amorphous powder; EI-MS m/z : [M+H]⁺ + 431.0, which match up to the molecular formula C₁₂H₂₀O₁₀; ¹H-NMR (DMSO-d₆,400MH_Z): δ 13.10 (1H,s,H-23), 10.8 (1H,s,H-24), 10.3 (1H,s,H-25), 7.9 (2H,d,H-5,H-3), 6.9 (2H,d,H-2,H-6), 6.7 (1H,s,H-13), 6.259 (1H,s,H-22), 5.0 (1H,s,H-26), 4.6 (3H,m,H-27,H-28,H-29), 3.7 (2H,m,H-21), 3.6 (1H,d,H-16), 3.5 (2H,m,H-17,H-18), 3.4 (2H,m,H-19,H-20); ¹³C-NMR (DMSO-d₆,125MH_Z): δ 182.1 (s,C-4), 164.0 (s,C-2), 162.6 (s,C-7), 161.1 (s,C-4'), 160.4 (s,C-5), 156.0 (s,C-9), 129.0 (d,C-2'and C-6'), 121.6 (s,C-1'), 115.8 (d,C-3'and C-5'), 104.6 (s,C-6), 104.1 (s,C-10), 102.5 (d,C-3), 98.2 (d,C-8), 81.9 (d,C-5''), 78.7 (d,C-3''), 73.4 (d,C-1''), 70.9 (d,C-2''), 70.6 (d,C-4''), 61.3 (t,C-6'').Thus, according to the mentioned data and by comparison with the literature, compound identified as Vitexin.

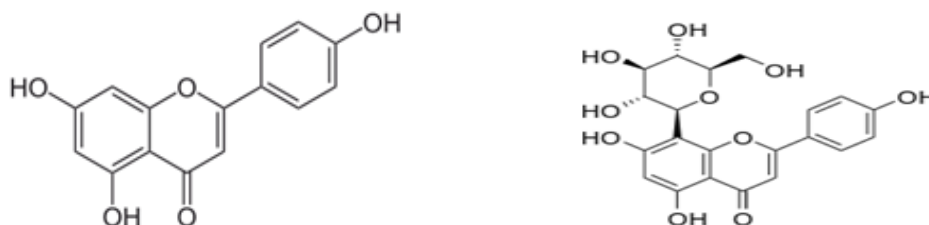


Figure 8: Apigenin structure and Vitexin Structure

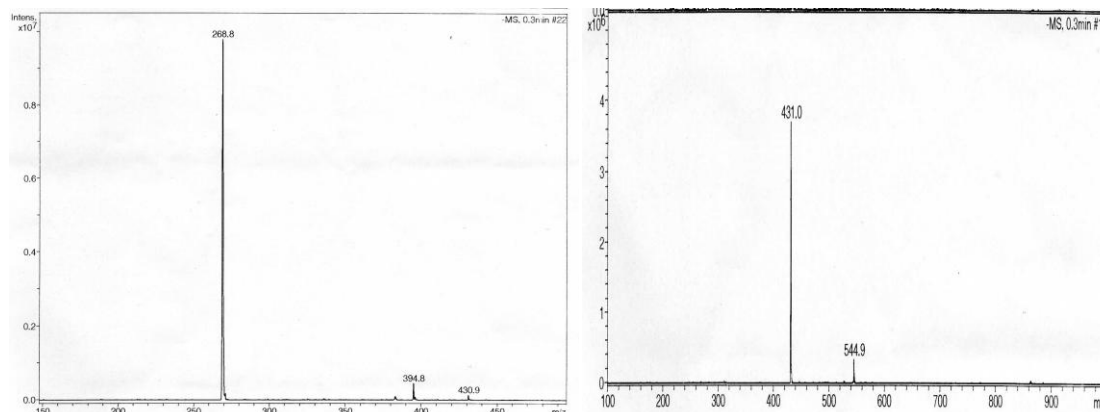


Figure 9: LCMS Spectra showing for Apigenin and Vitexin

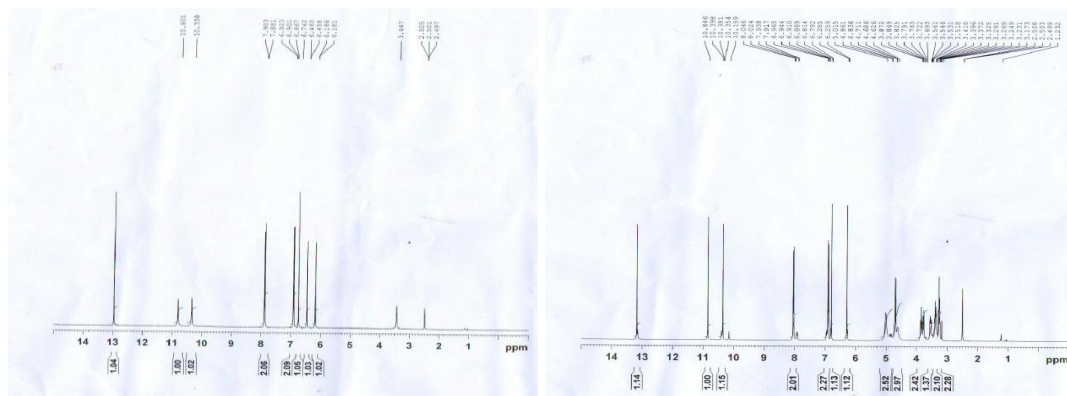


Figure 10: Proton ¹H-NMR showing for Apigenin and Vitexin

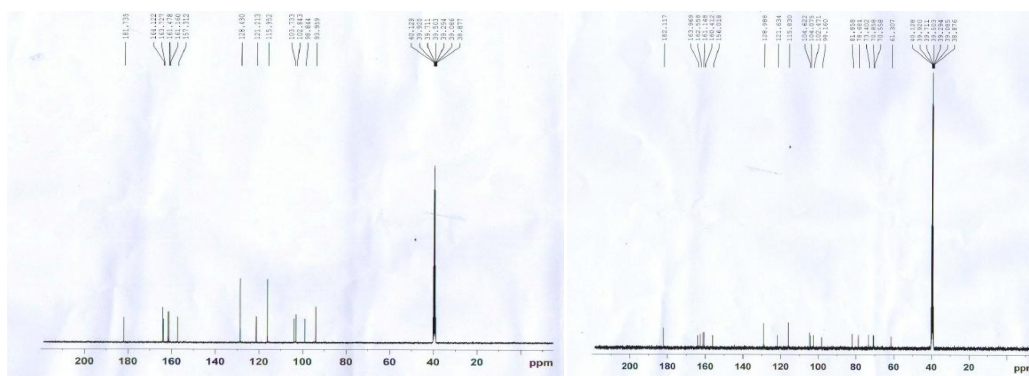


Figure 11: Carbon ¹³C-NMR showing for Apigenin and Vitexin

The DSC curve of samples showed an endothermic peak with an onset temperature at about 346.23°C and 203.82°C for the Apigenin and Vitexin respectively as shown in the figure 12 below.

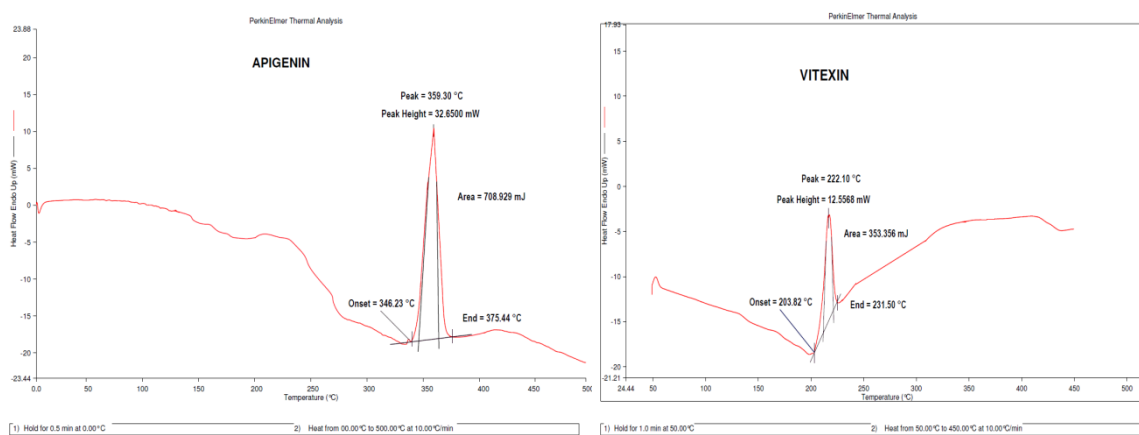


Figure 12: Showing the DSC curves of Apigenin and Vitexin

The results of the elemental composition of compounds using SEM and EDX technique showed in table 2. The SEM EDX spectra of the compounds are shown in the figure 13 and 14.

Table 2: The percentage of trace elements present in the samples

Position	Compound	C-K [wt% / wt % error (± 1 sigma)]	O-K [wt% / wt % error (± 1 sigma)]
Point 1	Apigenin	80.92+/-1.91	19.08+/-1.61
Point 1	Vitexin	74.23+/-1.32	25.77+/-1.49

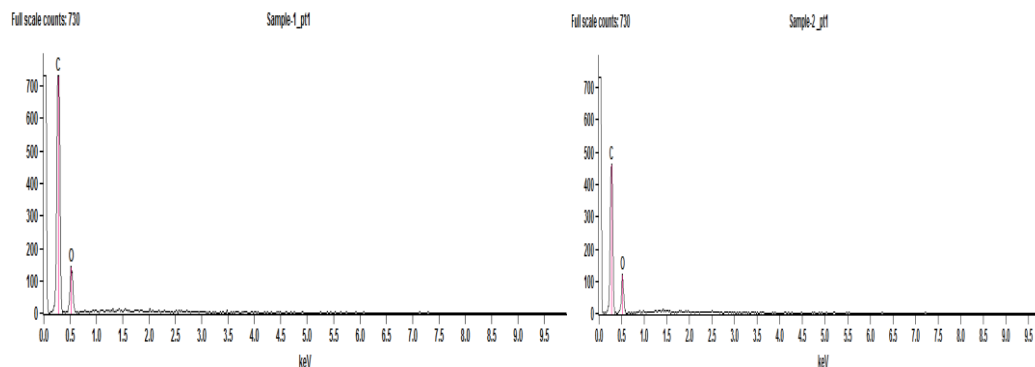


Figure 13: EDX of the Apigenin and Vitexin Sample

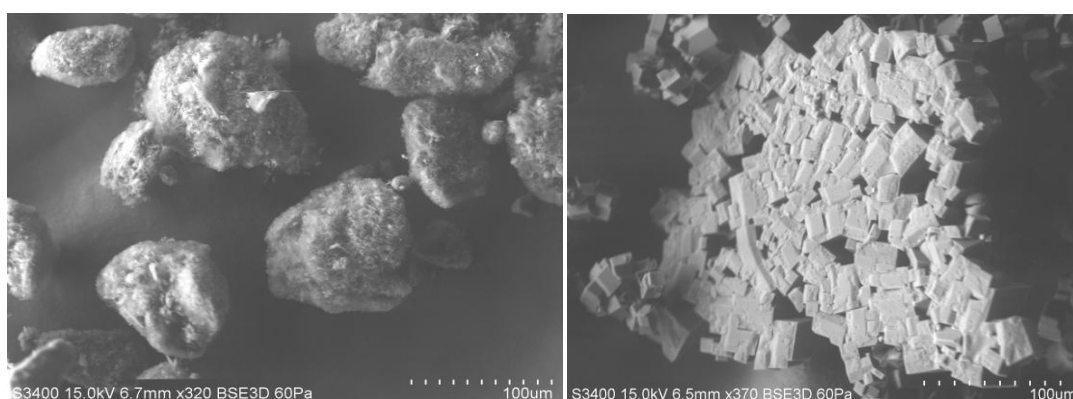


Figure 14: Figure showing SEM of Apigenin and Vitexin sample

IV. Conclusion

Finally, two bioactive flavonoids have been successfully isolated from *Justicia Gendarussa* under the present study. On the basis of the spectral data and the compounds were identified as Apigenin and Vitexin.

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