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Antimicrobial Screening and HPLC-DAD-MS Characterization of the Flavonoid–Rich Fractions of the Methanol Leaf-Extract of *Lawsonia inermis* Linn

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ABSTRACT

Flavonoids are known for their wide biological activities, including antimicrobial activity. This study was aimed at investigating the antimicrobial potential of the flavonoid-rich fractions of *Lawsonia inermis* leaf extract. The ethylacetate fraction from the methanol extract of *L. inermis* leaves was subjected to Vacuum Liquid Chromatography (VLC) using binary combinations of Hexane: Ethylacetate and Dichloromethane:Methanol to obtain fractions F_1-F_{14}. Fractions F_{11} and F_{12} which contain flavonoids were further subjected to High Performance Liquid Chromatography–Diode Array Detector (HPLC-DAD) and Liquid Chromatography-Mass Spectrometry (LC-MS) analysis, and subsequently subjected to *in vitro* test for antimicrobial activity against laboratory strains of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger, Candida albicans* using Agar well diffusion method at concentrations of 0.0625-1 mg/mL. Erythromycin (50 µg/mL) and Miconazole (50 µg/mL) were used as positive controls for bacteria and fungi, respectively. HPLC-DAD and LC-MS analysis of the flavonoid-rich fraction (F_{12}) led to the detection and identification of four flavone pigments namely; vitexin, luteolin 4-glucoside, apigenin monoglycoside and isoorientin (luteolin 6C-*β*-glucoside). Fraction F_{11} (constituents not identified) and fraction F_{12} (the flavone-rich fraction) showed moderate antibacterial activity, with *E. coli* being the most susceptible bacteria with Minimum Inhibition Concentrations (MIC) of 62.5 µg/mL for F_{11} and 500 µg/mL for F_{12}. Very mild anti-fungal activity was only observed against *C. albicans* but not against *A. niger*. The unidentified polar compounds (the major components of F_{11}) and luteolin 6C-*β*-glucoside (the major flavones detected in F_{12}) may contribute to the observed antimicrobial activity of *L. inermis* leaf extract.

***Keywords*:** Flavones, *Lawsonia inermis* , antimicrobial activity, HPLC-DAD, LC-MS.

Introduction

Over the years, plants have been used in ethnomedicine for the management of disease states associated with microbial infections. The activity of these plants can be traceable to the presence of one or more of the phytoconstituents which have been a source of inspiration for the development of novel antimicrobial agents.[1] Traditionally, investigation of plants or plant parts have followed the conventional activity guided fractionation and isolation of the bioactive constituents.[2] This approach has frequently led to the isolation of the easily assessable phytoconstituents (the low hanging fruits), and in most cases the phenolics[2]. In recent times, however, the acceptable practice has been to employ one or two hyphenation techniques to quickly identify the phytoconstituents present in plant extracts or fractions as a way of prioritising such extracts or fractions for further detailed chemical and pharmacological investigations.[3] In this way, scarce manpower or resources is saved for investigation of samples with greater potential of generating novel bioactive agents.

Lawsonia inermis (family *Lythraceae*), also known as Hina, the Henna tree or mignonette tree,[4,5] Alcanna, Egyptian Privet, Hennae Folium, Henne, Henné, Jamaica Mignonette, Lawsonia alba, Mehndi,[6] Mendee, Plante du Paradis, Reseda, Smooth Lawsonia etc. is an indigenous plant used in many countries of the world, including Nigeria for over 9,000 years. Its leaves have been used to treat a variety of ailments such as rheumatoid arthritis, headache, ulcers, diarrhoea, leprosy, fever, leucorrhoea, diabetes, cardiac disease and liver problems[7] suggesting that the leaf extract may possess strong anti-microbial and anti-inflammatory properties. This folkloric use is supported by the review report by Babu and Subhasree[8] that *L. inermis* has a broad spectrum of antimicrobial activity including antibacterial, antiviral, antimycotic and antiparasitic activities. Also supporting this folkloric use are pharmacological reports on its antibacterial activity.[9] The plant, Henna, is also known to be useful as a cosmetic agent.

Hitherto, there are no recent reports associating the antimicrobial activity of this plant to known chemical entities. This study is therefore aimed at assessing the antimicrobial properties of the flavonoid-rich fractions of *Lawsonia inermis* leaf extract as well as identifying some major compounds from these fractions which are most likely responsible for the antimicrobial and anti-inflammatory activities using HPLC-DAD-MS. HPLC-DAD and HPLC-MS are readily available and accessible hyphenation techniques used for online detection and dereplication studies of plants phenolics. We have, therefore, employed a combination of both for the detection and identification of the constituents of the flavonoid rich fractions of the plant in the current study.

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Materials and Methods

Collection of plant materials

The leaves of *L. inermis* were collected from Benin-City, Edo State, Nigeria in March, 2016 and authenticated by Mrs Amaka R. Onwunyili, a taxonomist in the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State of Nigeria. Samples of the leaf were thereafter deposited at the herbarium (PCG/474/A/072)

Extraction, Fractionation and Vacuum Liquid Chromatography (Preparation of the Flavone-Rich Fraction)

The ethyl acetate fraction of the methanol leaf-extract of dried and pulverised *L. inermis* leaves was subjected to vacuum liquid chromatographic separation for the purpose of obtaining the flavonoid rich fractions as has been previously reported.[10.] Briefly, 800 g of the ethyl acetate fraction was triturated with 25 g of silica gel after which it was introduced into the VLC column (5 L sintered column) packed with 100 g of Silica gel (200 – 400 mesh size) to a 10 cm bed size and covered with a small amount of silica gel up to 1 cm. The column was thereafter covered with cotton wool and eluted with 500 mL each of combinations of hexane: ethyl acetate (10:0, 9:1, 7:3, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10) and thereafter 500 mL each of the combinations-dichloromethane: methanol (9:1, 7:3, 5:5, 2:8 and 0:10)

Each of the solvent fractions was collected separately in a flask and evaporated using rotary evaporator to obtain fractions F_1-F_{14}. Preliminary results showed that the flavonoids were concentrated in the fractions F_{11} (eluted with dichloromethane: methanol, 7:3) and F_{12} (eluted with dichloromethane: methanol, 5:5). These two fractions were selected for HPLC-DAD-MS analysis and antimicrobial screening.

High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) Analysis

About 2 mg each of the fractions were reconstituted with 2 mL of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter centrifuged at 3000 rpm for 5 min. 100 µL of the dissolved samples was transferred into HPLC vial containing 500 µL of HPLC grade methanol. HPLC analysis was carried out on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nano pure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The compounds were detected by comparing the retention times and UV-spectra with inbuilt library.

Liquid Chromatography-Electrospray Ionization Mass Spectroscopy (LC-ESIMS) analysis

The fractions were further subjected to Liquid Chromatography-Electrospray Ionization Mass Spectroscopy (LC-ESIMS) using a Thermo-Finnigan LCQ-Deca Mass Spectrometer (Germany) connected to an UV-detector. The samples were dissolved in MeOH and injected into the HPLC/ESI-MS set up. A solution of the sample was then sprayed at atmospheric pressure through a 2-5 kV potential. HPLC was run on a Eurospher C-18 (6 x 2 mm, i.d.) reversed phase column. The mobile phase was 0.1% Formic acid solution in nano pure water (A), to which MeOH (B) was added by a linear gradient. The flow rate was at 400 µL/min and the absorbance detected at 254 nm. ESI (electrospray ionization) was performed at a capillary temperature of 200ºC and drift voltage of 20 eV. MS/MS experiments were also carried out on the molecular ion to obtain daughter ions which are diagnostic of the compounds. The constituents were determined by comparison of the molecular and fragments ions with literature values.

Screening of the samples for Antibacterial Activity

The antimicrobial activity of the samples was carried out against laboratory strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtillis*, *Aspergillus niger and Candida albicans*, which were obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. The antimicrobial assay was determined using the agar well diffusion method.[11] The samples labelled 'F_{11}' and 'F_{12}' were dissolved in DMSO to obtain a final concentration of 1 mg/mL. Dilutions of 500, 250, 125 and 62.5 µg/mL were prepared from the 1 mg/mL stock solution of each sample in a 2-fold dilution process. A 0.5 McFarland standard bacterial and fungi suspensions of each of the test isolates was prepared and these formed the bacterial and fungi stock solutions used in this assay. The media, i.e., Mueller-Hinton Agar (Titan. Biotech) (MHA) and Sabouraud Dextrose agar (SDA) were prepared and treated according to the manufacturer's specification. The sterile MHA and SDA plates were inoculated with the test culture by seeding method. Here, 0.1 mL of each of the previously standardized culture was transferred into the sterilized MHA and SDA plates and 20 mL of the molten agar that has been cooled to 50ºC was added and then mixed thoroughly by swirling in the clockwise and anticlockwise direction to obtain uniformity of the inoculums and to ensure even growth of the organisms. A sterile cork-borer was used to make five wells (8 mm in diameter) on each of the MHA and SDA plates. Aliquots of 80 µL of each extract dilutions previously prepared, were put in each of the wells in the culture plates previously seeded with the test organisms. Erythromycin (50 µg), Miconazole (50 µg) and DMSO served as the positive and negative controls respectively. The cultures were incubated at 37ºC for 24 h and room temperature (25-27ºC) for 48 h for bacteria and fungi respectively to allow the growth of microorganism. The inhibition zone diameters (IZDs) were measured and recorded. Each extract was tested against all the bacterial and fungi isolates. The size of the cork borer (8 mm) was deducted from the values recorded for the IZDs to get the actual diameter. The procedure was conducted in triplicates and the mean IZDs were calculated and recorded.

Results and Discussion

The results of the HPLC-DAD analysis of samples F_{12} and F_{11} are shown in Figures 1 and 2 respectively, while the result of LC-ESI-MS analysis of sample F_{12} is shown in Figure 3 and Table 1.

Dereplication using hyphenated techniques has been recently used for prioritization of plant extracts to improve the speed and chances of discovering novel therapeutic molecules. In the present study, the flavonoid rich fractions from *L. inermis* were subjected to HPLC-DAD-(ESI)MS analysis for the purpose of rapid online detection and identification of the compounds present. Previous analysis of the UV-Vis spectra of F_{11} and F_{12} showed that both fractions are flavonoid-rich as evidenced by the two prominent maxima (250-270 and 330-370 nm) present in each of the compounds (A, B, C, D, G and H). HPLC-DAD analysis, thus showed that the compounds are Vitexin (A), Luteolin 4'- glucoside (B), Apigenin monoglycoside (C), Luteolin-6C- **β** -glucoside (D), quercetin derivative (G) and Kaempferol derivative (H). In other to substantiate the results from HPLC-DAD analysis, we have also extended the hyphenation to ESI-MS analysis. Compound A was confirmed to be Vitexin based on the observed *m/z* 433.2 $[M+1]^+$ in the negative mode and 431.3 $[M-1]^-$ in the negative mode, supporting the molar mass of 432 g/mol. The molar mass of compound B was confirmed as 448 g/mol based on the pseudo molecular ion peaks at *m/z* 449.2 $[M+1]^+$ and 447.3 $[M-1]^-$ in the positive and negative modes respectively. Similarly, the molar mass of compound D was confirmed as 448 g/mol based on the pseudo molecular ion peak at *m/z* 449.1 $[M+1]^+$ and 447.2 $[M-1]^-$ in the positive and negative modes respectively. The fragment peak at *m/z* 287.3 $[M-162+1]^+$ observed in the MS spectra of both B and D is attributed to the presence of the flavonoid aglycone, luteolin (molar mass 286 g/mol). It is important to note that this fragment peak was formed from the molecular ion via a loss of 162 amu, which represents the loss of hexose (glucose) unit. The two compounds B and D were thus confirmed as Luteolin-4'-glucoside and isoorientin (Luteolin-6C-β– glucoside) respectively and this was also corroborated by the library suggestions. It is also important to note that Luteolin-4'O-glucoside has a lower retention time (22.50 min), thus higher polarity,

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than that of Luteolin-6C-β– glucoside (25.08 min), which was the basis for their differentiation by the HPLC-DAD. The chemical structures of compounds A, B and D which were confirmed by HPLC-DAD-(ESI)-MS analysis are shown in Figure 4.

The major components of fraction F_{11} which are Compounds E and F, (Fig 2), could not be fully characterised using HPLC-DAD and LC-ESIMS analysis. It was, however observed that both E and F are not flavonoids, considering their observed UV maxima. Furthermore, comparison of the UV maxima of compounds G and H with earlier reported data[11] provided evidence that both are Quercetin and Kaempferol derivatives respectively. This was also corroborated by the library suggestion.

Table 2 shows the result of the antimicrobial assay for each of the samples (F_{11} and F_{12}) carried out on strains of *S. aureus, B. subtilis, E. coli, P. aeruginosa, A. niger,* and *C. albicans.* F_{11} showed activity against *S aureus* at 1000 and 500 µg/mL, against *B. subtilis* at 1000 µg/mL, against *E. coli* at all the tested concentrations as low as 62.5 µg/mL and against *C. albicans* at 1000 and 500 µg/mL. Similarly, F_{12} showed activity against *S aureus* and *B. subtilis* at 1000 µg/mL, against *E. coli* at 1000 and 500 µg/mL and against *C. albicans* at 1000 µg/mL. None of the fractions showed activity against *P. aeruginosa* and *A. niger* at all the tested concentrations.

Fraction $F_{12,}$ is a mixture of flavone (apigenin and luteolin) glycosides with the major component as Luteolin-6C-*β*– glucoside (isoorientin). This fraction showed mild antimicrobial activity against *E. coli, S. aureus* and *C. albicans,* which is supported by earlier reports that plants containing luteolin or its glycosides or both possess antibacterial activity [12-15] and significant antifungal activities similar to that of the antifungal agent, ketoconazole[14].

Fraction F_{11}, on the other hand, contains a kaempferol and a quercetin derivative, and two unidentified isomeric compounds. This fraction showed a stronger, dose-dependent antimicrobial activity against *E. coli* (at all tested concentrations), *S. aureus* and *C. albicans* than fraction F_{12}.Quercetin and kaempferol derivatives have been previously shown to exhibit antimicrobial activity[11] and would have contributed to the antimicrobial activity of *L inermis* although they are not the major components of the fraction. We speculate that the antimicrobial activity would be largely contributed by the major components E and F, which were not identified in this study by our dereplication techniques.

The observed strong activity of F_{11} against *E. coli* justifies the traditional use of *L. inermis* leaves as an antimicrobial in the management of diarrhoea, since the majority of diarrhoea cases are caused by *E. coli* infection. A previous report has shown that apigenin, quercetin and kaempferol glycosides exhibited synergistic antimicrobial activity against strains of bacteria.[16] It is possible that these compounds, which were also detected in both fractions used in this study may interact synergistically to support the earlier reported strong antimicrobial activity of the extracts of *L. inermis* against *E. coli, Psuedomonas spp* and *Proteus spp*.[17,18]

In this research work, we present the antimicrobial activity demonstrated by the plant extract which could be as a result of the flavones present: Vitexin (apigenin-8-C-glucoside), Luteolin-4'-glucoside and Isoorientin. These molecules, also present in other plants, have been widely reported to show antimicrobial activity.[1]

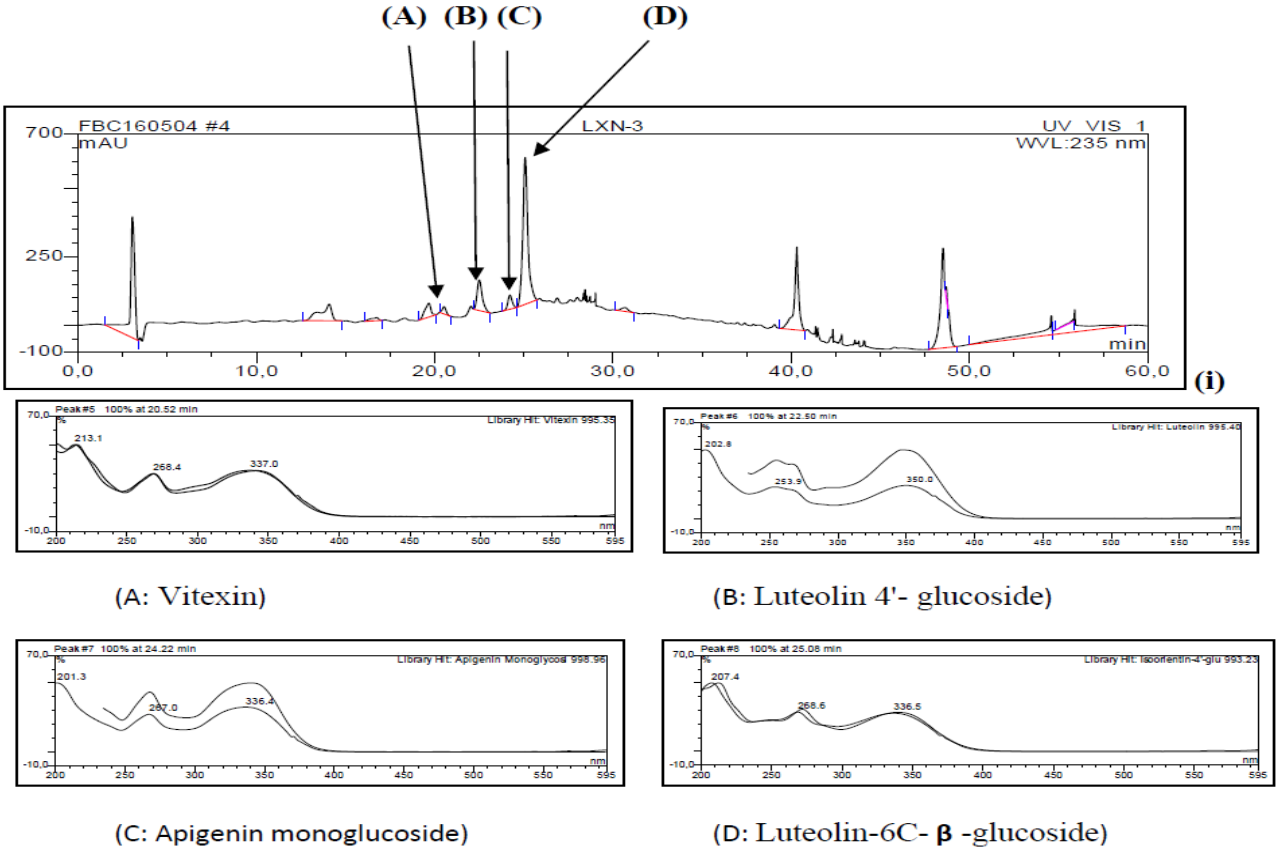

Figure 1: HPLC Chromatogram of Fraction 12 showing the detected and identified flavones: Vitexin (A), Luteolin-4'–glucoside (B), Apigenin monoglycoside (C), Luteolin-6C-*β*– glucoside (D), and their UV (DAD) spectra.

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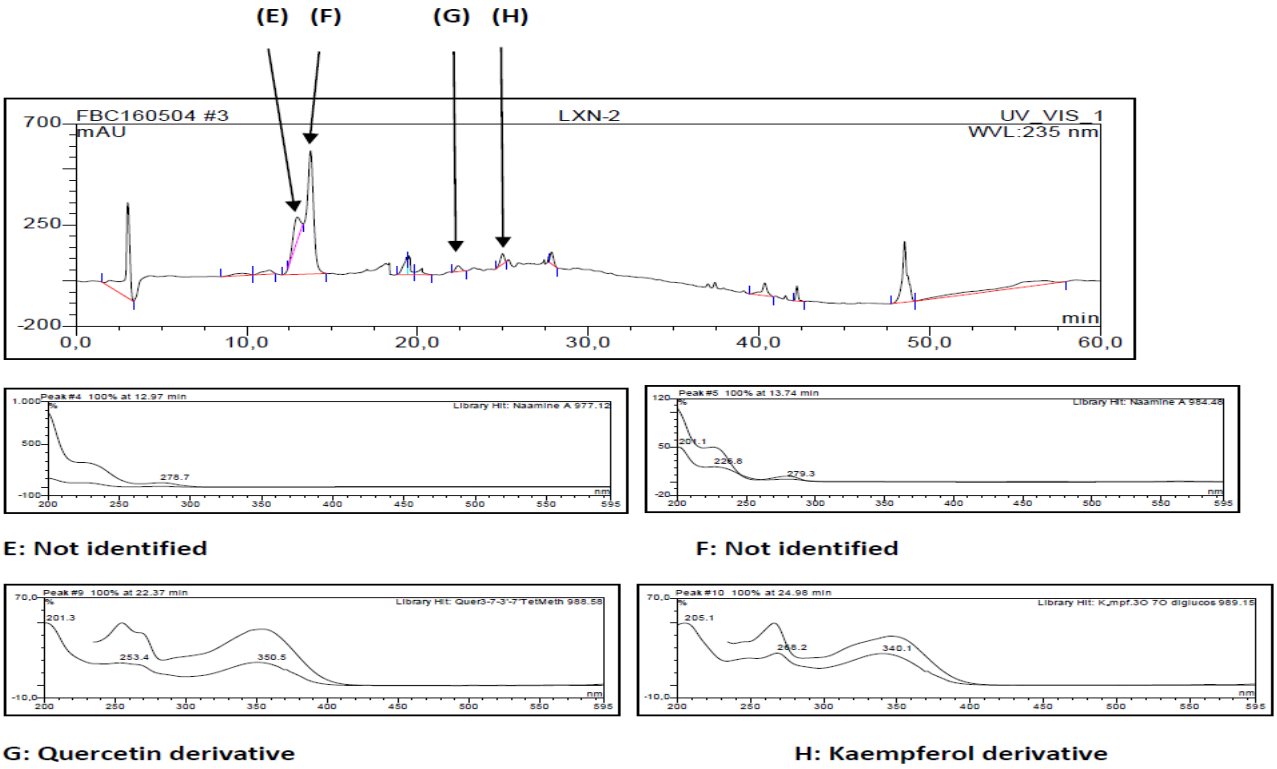

Figure 2: HPLC Chromatogram of Fraction 11 showing the detected compounds and their UV (DAD) spectra

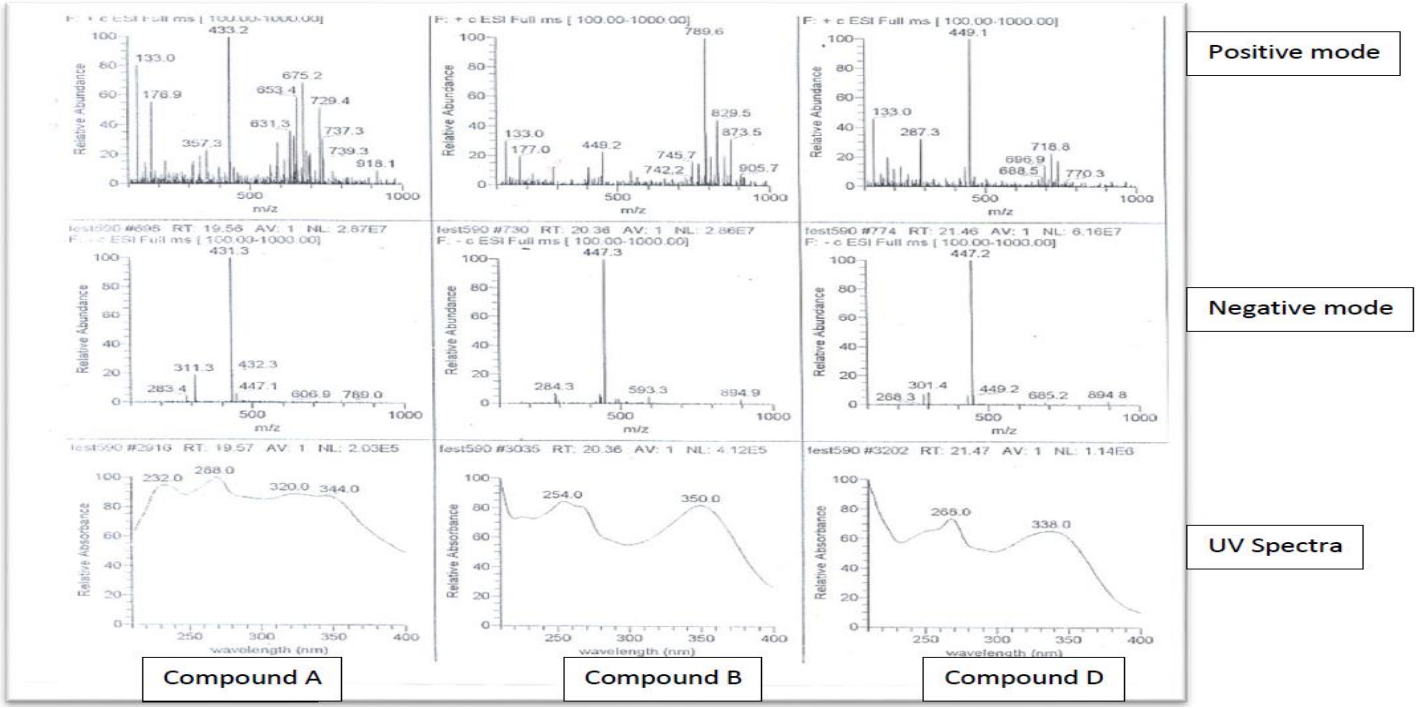

Figure 3: LC-MS spectra of F12 showing ionizations (pseudomolecular and fragment ions) in both the positive and negative modes and the accompanying UV spectra of the detected compounds A, B and D.

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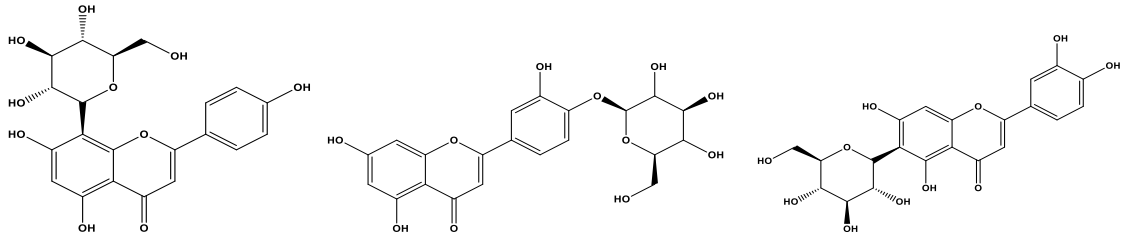

Vitexin (A) Luteolin 4'- glucoside (B) Luteolin-6C-1-glucoside (D)

Figure 4: Chemical Structures of the flavones detected and identified in F_{12}.

Table 1: HPLC-DAD-ESIMS analysis of sample F_{12}

S/N	Label	Retention time (RT) (min)	UV λ_{max} (nm)	M+1 (*m/z*)	Fragment ions (+)	M-1 (*m/z*)	Fragment ions (-)	Name of detected and Identified Compound
1	A	20.50	213.1 268.4 337.0	433.2	-	431.3	-	Vitexin (Apigenin-8C-β-glucoside)
2	B	22.50	202.8 253.9 350.0	449.2	287.3 (M-162)	447.3	284	Luteolin-4'-glucoside
3	C	24.22	201.3 267.0 336.4	-	-	-	-	Apigenin monoglucoside
4	D	25.08	207.4 268.6 336.5	449.1	287.3 t(M-162)	447.2	-	Isoorientin (Luteolin-6C-β-glucoside)

Table 2: Result of the antimicrobial activity of $F_{11,}$ and F_{12} showing the Mean Inhibition Zone Diameters (IZDs) produced against test organisms

Test drugs	*Conc (µg/mL)*	*S. aureus (IZD)*	*B. subtilis (IZD)*	*E. coli (IZD)*	*P. aeruginosa (IZD)*	*A. niger (IZD)*	*C. albicans (IZD)*
F11	*1000*	*7*	1	7	0	0	2
	500	2	0	6	0	0	1
	250	0	0	5.5	0	0	0
	125	0	0	5	0	0	0
	62.5	0	0	4	0	0	0
F12	*1000*	3	1	5	0	0	2
	500	0	0	4	0	0	0
	250	0	0	0	0	0	0
	125	0	0	0	0	0	0
	62.5	0	0	0	0	0	0
ERYT	50	12	16	11	0	ND	ND
MICO	50	ND	ND	ND	ND	23	21

F11 = Flavone rich fraction 11, F_{11} = Flavone rich fraction 11, ERYT = Erythromycin, MICO = Miconazole, IZD = Inhibition zone diameter, ND = Not determined.

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Conclusion

This research work has shown that *Lawsonia inermis* possesses moderate antibacterial and very mild antifungal activity most likely due to the presence of the detected phenolic compounds and further substantiates the ethno-medicinal use of *Lawsonia inermis* leaves as an antimicrobial agent. Work is currently ongoing in our laboratory to properly harness these compounds as potential sources of antimicrobial agents.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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