RESEARCH ARTICLE

Phytochemical Investigation of some Chemical Compounds Present in *Vicia villosa L*. Widely Grown in Iraq

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ABSTRACT

Vicia villosa is one of the wild plants in Iraq that is wildly distributed in northern areas of the country; it belongs to the Fabaceae family, and it's preferred common name is hiary vetch. The plant contains many active constituents that have pharmacological activities. Flavonoids and phenolic acids are the most important chemical constituents of *V. villosa;* thus, they have antioxidant and antiproliferative activity.

The whole plant (*V. villosa*) was collected from Khalow Bazian near Kirkuk city, north of Iraq, in April 2019. After that, the whole plant dried at room temperature in the shade, then pulverized by mechanical mills and weighed. Then the Pulverized whole plant was defatted by maceration with n-hexane for 24 hours, then allowed to dry at room temperature. The defatted plant materials were extracted Soxhlet apparatus using absolute methanol 99.8% as solvent extraction. The crude methanol extract was suspended in 300 mL water and partitioned successively with n-hexane, chloroform, ethyl acetate, and n-butanol (3x300 mL) for each fraction. High-performance liquid chromatography (HPLC) examination was done for n-butanol fraction revealed the presence of flavonoids and phenolic acids.

Five compounds were isolated from n-butanol fraction, symbolized as MH1, MH2, MH3, MH4, and MH5. Two of these compounds (MH1 and MH2) were isolated by preparative layer chromatography using one mobile phase, while other compounds (MH3, MH4, and MH5) were isolated by Preparative high-pressure liquid chromatography. All of the five isolated compounds were identified by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), UV-diode array spectra, and Fourier transform infrared (FT-IR) spectra.

The results show that the isolated compounds MH1, MH2, MH3, MH4, and MH5 could be identified as gallic acid, caffeic acid, genistein, apigenin, and silybin, respectively, two phenolic acids (gallic acid and caffeic acid), and three flavonoids (genistein, apigenin, and silybin).

Keywords: *Vicia villosa*, Flavonoids, Phenolic acid, Thin layer chromatography, High-performance liquid chromatography.

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INTRODUCTION

V. Villosa belongs to the family Leguminosae or Fabacea. With about 700 genera and 19,000 species, Leguminosae is the third largest flowering plant family in the world.[1] The genus Vicia L. belongs to the Fabaceae family. These plants originate in East Asia, North and South America, Europe, and tropical Africa. There are many tax studies regarding the Vicia type, including molecular, karyotype, and genetic research. Vicia includes between 130 and 240 species, divided into six categories and 15 series.[2-5] More than 40 species of Vicia have economic value and are cultivated.[2] Hairy vetch (Vicia villosa Roth) is an annual or biannual viny legume with a woolly appearance thanks to long soft hairs borne on the stems and leaves.[6-8] hairy vetch includes a scrambling and prostrate habit when young. It's 30 to 70 cm tall in mature swards.[7] When it has furnished support for upright growth, hairy vetch may climb and reach a height of 1 to 2 m. it is a shallow taproot that goes no deeper than 1-m and powerful lateral secondary roots. The plant produces 3 to 10 slender stems, which are finely ridged and carinate to angled in shape. The stems are rather weak, and while they would reach a length of about 4 m, they can not grow upright without support. The leaves are alternate, pinnately compound, bearing up to 10 pairs of leaflets. They bear a terminal tendril, essential for his or her climbing habit. The leaflets are narrowly oblong, 10–25 mm long. Inflorescences are 7 cm long, spike-like racemes that bear 10 to 20–30 flowers.[9] The flowers are papilionaceous, purple to blue, and sometimes white, up to 1 cm long and 7 mm in

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diameter.[10] Hairy vetch fruits are elongated, finely-haired, brown-colored dehiscent pods. Each pod contains 4–8 globular seeds, blackish-brown in color and 3–5 mm in diameter.[7]

Several types of flavonoids and phenolic compounds had been detected in the plant, such as Daidzein, Quercetin, Genistein, Luteolin, Kaempferol, Formononetin, Diosmetin, Biochanin A,[11] myricetin,[12] petunidin, delphinidin, malvidin,[13] Gallic acids,[11] and P-hydroxy benzoic acid.[14]

V. villosa extract has antioxidant activity that was confirmed by previous studies.[15,16] The study of antiproliferative action of the *V. Villosa seed* extract incubation using Caco-2 cells was carried out to determine the possible effect of these extracts on cell proliferation.[16] *V. villosa* extract also has Allelolopathy activity.[17,18]

V. villosa can cause poisoning in mammals, including cattle and horses, and poultry.[19,20] In mammals, symptoms include dermatitis, and mortality has been recorded in cattle and poultry.[20]

The present study was designed for screening the phytochemicals and their properties present in *(V. villosa L.),* grown widely in Iraq; since there is no phytochemical study had been done previously in Iraq.

MATERIALS AND METHOD

Collection of Plant Materials

The whole plant (*V. Villosa*) was collected from Khalow Bazian near Kirkuk city, in the north of Iraq, in April 2019. The plant was identified and authenticated by Prof. Dr. Sukaena Abass, Department of Biology, College of Sciences, University of Baghdad. After that, the whole plant dried at room temperature in the shade, then pulverized by mechanical mills and weighed.

Equipment and Chemical

The instruments utilized in this study were rotary evaporator (Heidolph Germany), HPLC (Knauer Germany), fourier transform infrared spectroscopy (FTIR) from SHIMADZU/ Japan, and UV–detector (Desaga /Germany). All solvents used were of analytical grade and obtained from Central Drug House, (p) Ltd, India, except methanol that is HPLC. The standards Quercetin, Kaempferol, Myricetin, Gallic acid, luteolin, caffiec acid, apigenin, genistein, chlorogenic acid, syringic acid, silybin, vanillic acid, and catechol were purchased from Chengdu Biopurify Phytochemicals, China (purity > 97). TLC aluminum plates pre-coated with silica gel 60 F 254 (20 cm×20 cm, 0.2 mm thick) used were obtained from Macherey–Nagel Germany.

Extraction and Fractionation of Plant Material

Four hundred grams of shade-dried pulverized whole plant were defatted by maceration with n-hexane for 24 hours, then allowed to dry at room temperature. The defatted plant materials were extracted using Soxhlet apparatus in which the powder was packed in the thimbles and extracted with 6L of absolute methanol 99.8% as a solvent extraction for 24 hours. The extract was filtered, and the solvent was evaporated under reduced pressure using a rotary evaporator to get a dry extract (about 86 gm). The crude methanol extract was divided into two halves, one half was kept in the refrigerator, and the other half was suspended in 300 mL water and partitioned successively with n-hexane, chloroform, ethyl acetate, and n-butanol (3x300 mL) for each fraction. Each fraction was filtered and evaporated by a rotary evaporator to dryness. Each fraction was weighted and assigned for further analysis.[21,22]

Preliminary Phytochemical Examination of Crude Extract and Different Fractions

A-Test of Flavonoids

Few drops of 1 % potassium hydroxide solution were added to 1 mL of crude extract and 1mL of each fraction solution. Formation of intense yellow color, which became colorless on the addition of dilute acid, indicates the presence of flavonoids.[23,24]

B-Test for phenols

2-3 drops of ferric chloride were added to 1 mL of crude extract and 1mL of each fraction. The formation of dark greenish-blue color indicated the presence of phenols.[23,24]

Preparation of standards and samples for HPLC

Standard solutions for HPLC: Quercetin, Kaempferol, Myricetin, Gallic acid, luteolin, caffiec acid, apigenin, genistein, chlorogenic acid, syringic acid, silybin, vanillic acid, and catechol were prepared by dissolving a few milligrams in 1 mL of HPLC grade methanol. Dried samples were suspended in 1 mL HPLC grade methanol. The standard and sample solutions were filtered through 0.45 µm disposable. The injection volume was 20 µL of the sample injected into HPLC.

High-performance Liquid Chromatography (HPLC) Examination of n-butanol Fraction.

HPLC was carried out to examine some chemical compounds present in n-butanol fractions. The retention times of analyzed samples were compared to retention times of standard materials under the same conditions. HPLC system from Knauer Germany. HPLC conditions for analyzing the fraction were the separation on C18 column (Knauer, Germany) (250_ 46 mm i.d., 5-µm particle size, 80 Å pore size),[25] the mobile phase contains mobile A (0.05% TFA in HPLC grade water) and mobile B (acetonitrile). Gradient elution was performed. The flow rate was adjusted to 3 mL/min. The column was thermostatically controlled at 28°C. The injection volume was kept at 20 µL. HPLC chromatograms were detected using a photodiode array UV detector at three different wavelengths (272, 280, and 310 nm). The detection of each compound was performed by matching the retention time and absorbance spectrum of the standards.[25]

Isolation of Flavonoids and Phenolic Acids from N-Butanol Fraction by Preparative Layer Chromatography (PLC)

Preparative layer chromatography was done for n-butanol fraction by using Readymade preparative plates of silica gel GF_{254nm} (20x20 cm) of 1 mm thickness (Sanpont, China) as

Table 1: Qualitative analysis of phytochemical constituents in crude extract and different fractions of the plant.

Phytochemicals	*Crude extract*	*Hexane fraction*	*Chloroform fraction*	*Ethyl acetate fraction*	*n-butanol fraction*
Flavonoids	+	+	+	+	+
Phenols	+	+	+	+	+

(+), (-) represent the presence or absence of phytochemicals, respectively.

Table 2: Retention times in minutes of flavonoids & phenoles in n-butanol fraction

Compound	*Retention time of standard*	*Retention time in n-butanol fraction*
Gallic acid	5.5	5.5
Genistein	18	18.1
Apigenin	21	20.9
Caffeic acid	23	23
Silybin	26.1	26.1

stationary phase, and the mobile phase used for separation of compounds was S_{2n}: Chloroform: methanol: formic acid (70: 25: 5). A needle under UV light allocated the separated bands using a wavelength of 254 nm and 366 nm. Two bands were separated from n-butanaol fraction and symbolized as compound MH1 and compound MH2.

Isolation of Flavonoids and Phenolic Acids from the N-Butanol Fraction by Preparative High-pressure Liquid Chromatography (HPLC)

Preparative HPLC was performed using HPLC system from Knauer, Germany.

The sample was prepared by dissolving 1g in 3 mL of HPLC grade methanol. Three compounds were isolated from the n-butanaol fraction and symbolized as compound MH3, compound MH4, and compound MH5. The mobile phase used was Gradient of mobile A (0.05% TFA in HPLC grade water) and mobile B (acetonitrile).

Identification of the Isolated Phenolic Acids and Flavonoids (compound MH1, MH2, MH3, MH4, and MH5)

The isolated Compounds (MH1, MH2, MH3, MH4, and MH5) from the n-butanol fraction were identified by several methods, including chromatographic and spectral methods as High-performance liquid chromatography (HPLC), UV-diode array, and FT-IR (Fourier transform infrared spectroscopy).

Quantitative HPLC Analysis of the Isolated Compounds

Serial concentrations of standard external materials calculated the concentration of each isolated compound to build a calibration curve between concentration and its equivalent peak area.[25]

RESULTS

The results of the preliminary phytochemical analysis are given in Table 1.

Isolation of Flavonoids and Phenolic Acids from the n-butanol Fraction

Preparative layer chromatography was used to isolate and purify flavonoid and phenolic acid compounds (MH1 and MH2), as shown in Figure 1. Three compounds were isolated from the n-butanol fraction (MH3, MH4, and MH5) by Preparative high-pressure liquid chromatography

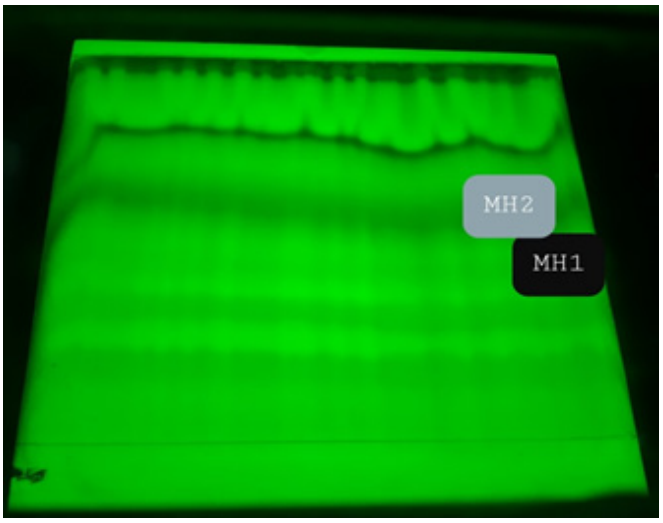

Figure 1: Preparative layer chromatography for n-butanol fraction developed in S_{2n} & detect under UV light at 254 nm (MH1, MH2).

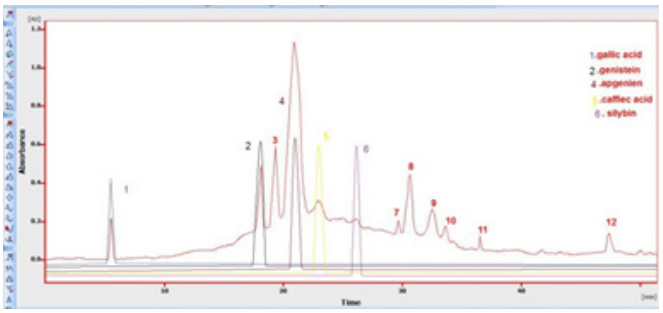

Figure 2: HPLC chromatogram of n-butanol fraction.

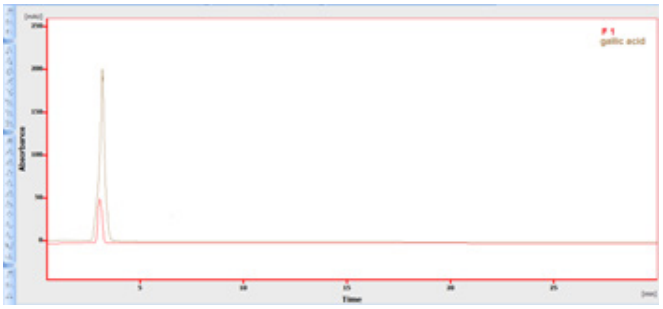

Figure 3: HPLC chromatogram of the isolated MH1 compound & gallic acid standard.

Result of High-performance liquid chromatography (HPLC)

In HPLC, qualitative identifications have been made by comparison of the retention times obtained at identical chromatographic conditions of analyzed samples with

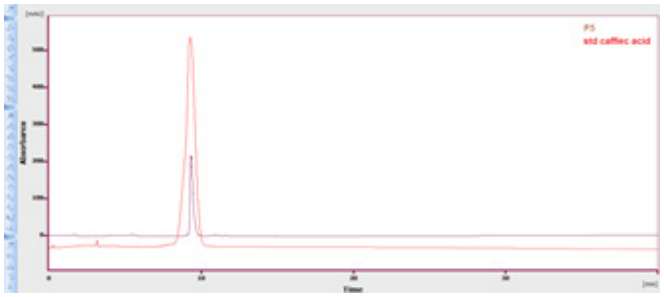

Figure 4: HPLC chromatogram of the isolated MH2 compound & caffeic acid standard.

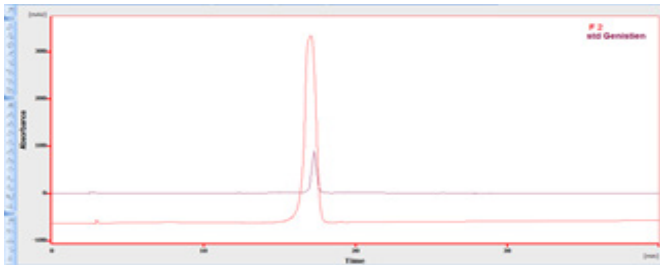

Figure 5: HPLC chromatogram of the isolated MH3 compound & of genistein standard.

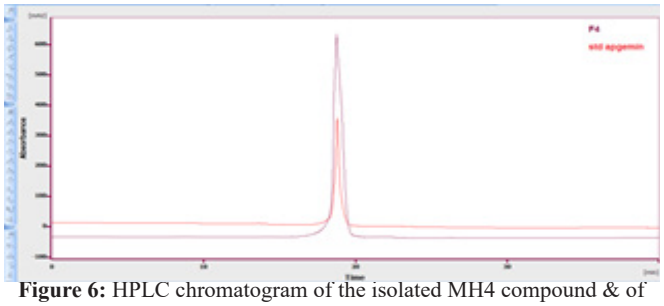

Figure 6: HPLC chromatogram of the isolated MH4 compound & of apigenin standard.

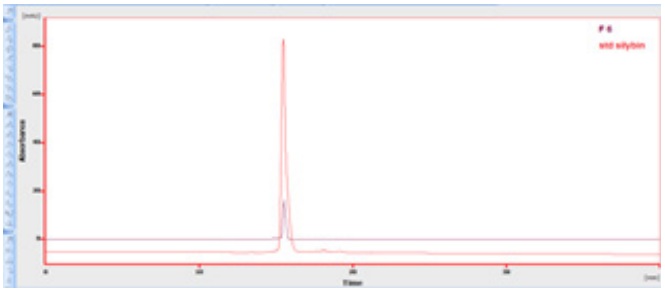

Figure 7: HPLC chromatogram of the isolated MH5 compound & of silybin standard.

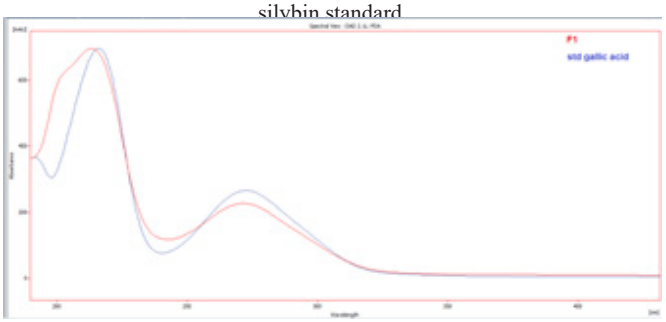

Figure 8: UV spectrum of the isolated MH1 compound & gallic acid standard.

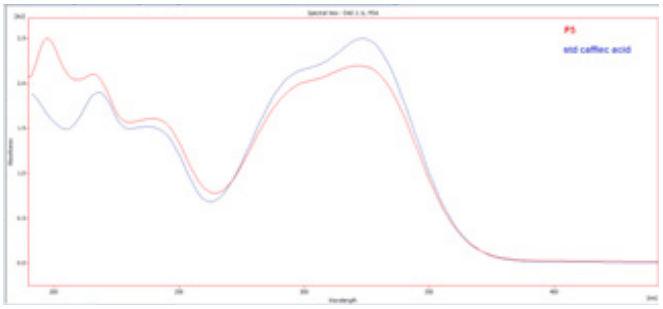

Figure 9: UV spectrum of the isolated MH2 compound & caffeic acid standard.

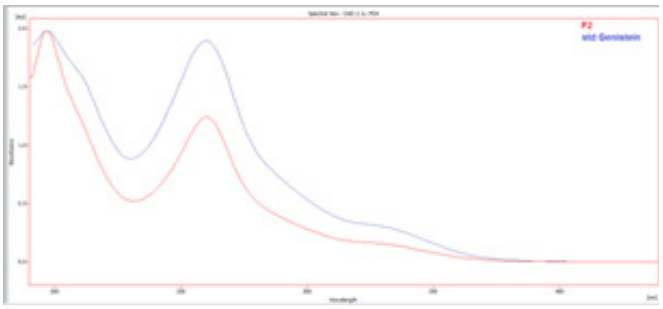

Figure 10: UV spectrum of the isolated MH3 compound & genistein standard.

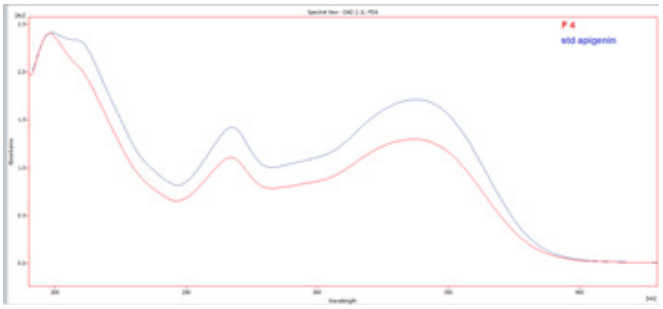

Figure 11: UV spectrum of the isolated MH4 compound & apigenin standard.

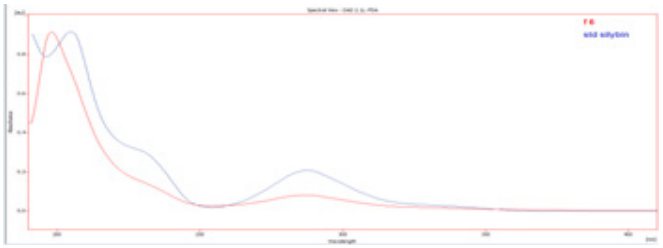

Figure 12: UV spectrum of the isolated MH5 compound & silybin standard.

the authenticated reference standards as in Figure 2 and Table 2.[26]

HPLC was carried out to identify isolated compounds (compound MH1, MH2, MH3, MH4, and MH5). The retention times of isolated compounds were compared to retention times of standard materials under the same conditions, as shown in Figures 3-7 and Table 3.

UV-diode array: The UV spectrum of each isolated compound with its authentic standards has been shown in Figures 8-12.

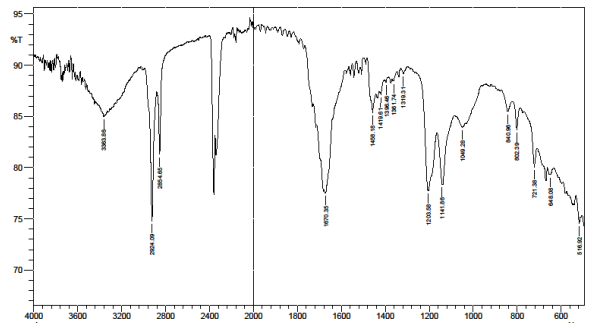

Figure 13: FTIR spectrum of isolated MH1 compound.

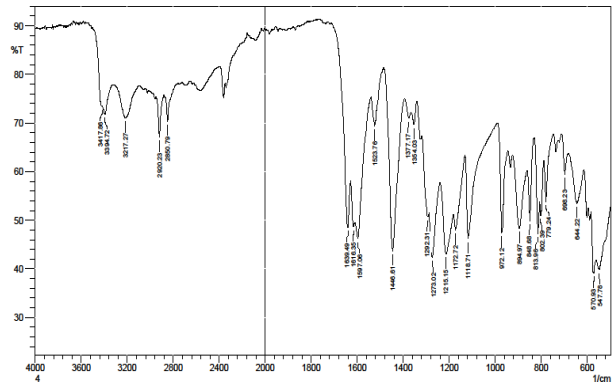

Figure 14: FT-IR spectrum of isolated MH2 compound.

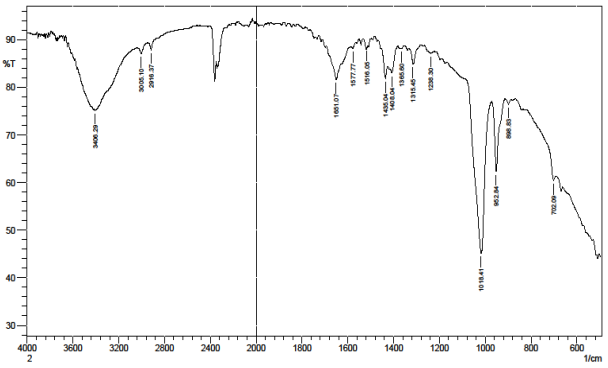

Figure 15: FT-IR spectrum of isolated MH3 compound.

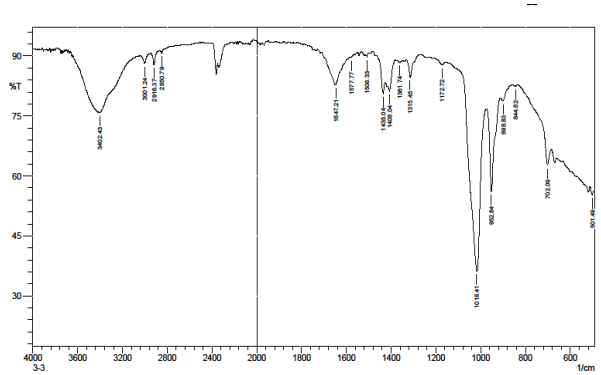

Figure 16: FT-IR spectrum of isolated MH4 compound.

Fourier Transform Infrared Spectroscopy (FTIR): The FTIR spectrum of each isolated compound has been shown in Figures 13-17. The explanations for each isolated compound's obtained FTIR spectral analysis are shown in Tables 3-8.

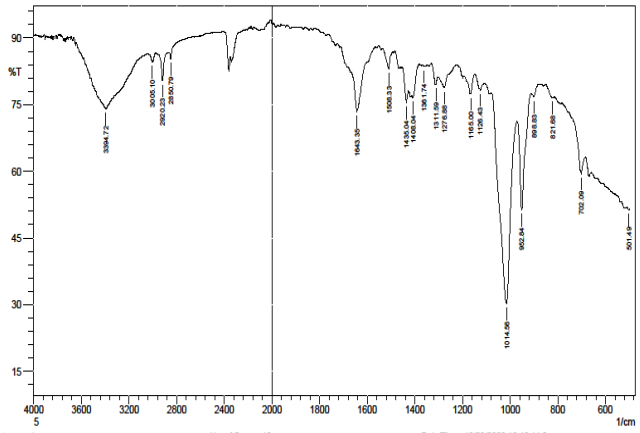

Figure 17: FT-IR spectrum of isolated MH5 compound.

Table 3: Retention times in minutes of flavonoids & phenoles in n-butanol fraction

Compound	*Retention time of standard*	*isolated compound*	*Retention time in n-butanol fraction*
Gallic acid	3	MH1	2.9
Caffeic acid	9.3	MH2	9.3
Genistein	17.2	MH3	17.4
Apigenin	18.8	MH4	18.9
Silybin	15.4	MH5	15.4

Table 4: FTIR spectral data for isolated MH1 compound.

IR bands of isolated MH1 compound	*Interpretation*
3363.86	O-H broad stretch band of carboxylic acid
2924.09,2854.65	Asymmetric and symmetric C-H stretching of CH
1670.35	C=O str. of conjugated carboxylic acid
1458.18	O-H bending of carboxylic acid
1419.18,1396.46	C=C Aromatic stretching
1319.31	C-O stretching of carboxylic acid
1203.58,1141.86,1049.28	C-H bending fingerprint of aromatic (in plane)
840.96,802.39,721.38, 648.08	C-H bending fingerprint of aromatic (out plane)

Quantitative HPLC analysis of the isolated compounds: Results of concentration calculation of each isolated compound are shown in Table 9.

DISCUSSION

The preliminary phytochemical analysis confirmed the presence of phenols and flavonoids in the crude extract and each fraction.

The results HPLC analysis of n-butanol fraction shows the presence of gallic acid, caffeic acid, genistein, apigenin, and silybin.

Table 5: FT-IR spectral data for isolated MH2 compound.

IR bands of isolated MH2 compound	*Interpretation*
3417.86,3394.72	O-H stretching of phenol
3217.27	O-H broad stretch band of carboxylic acid
2920.23,2850.79	Asymmetric and symmetric C-H stretching of CH2
1639.49	C=O str. of conjugated carboxylic acid
1616.35,1597.06,1523.76	C=C Aromatic stretching
1446.61	O-H bending of carboxylic acid
1377.17,1354.03	O-H bending of phenol
1292.31	C-O stretching of carboxylic acid
1273.02,1215.15,1172.72, 1118.71	C-H bending fingerprint of aromatic (in plane)
894.97,848.68,779.24, 644.22	C-H bending finger print of aromatic (out plane)

Table 6: FT-IR spectral data for isolated MH3 compound.

IR bands of isolated MH3 compound	*Interpretation*
3406.29	O-H stretching of phenol
3005.10,2916.37	Asymmetric and symmetric C-H stretching of CH2 andCH3
1651.07	C=O stretching
1577.77,1516.05	C=C Aromatic stretching
1435.03,1408.04	C-H bending of CH2 andCH3
1365.60,1315.45	O-H bending of phenol
1238.30	C-O-C stretch of ether
1018.41,952.84	C-H bending of aromatic (in plane)
898.84,702.09	C-H of aromatic (out plane)

Table 7: FT-IR spectral data for isolated MH4 compound.

IR bands of isolated MH4 compound	*Interpretation*
3402.43	O-H stretching of phenol
3001.24,2916.37,2850.79	Asymmetric and symmetric C-H stretching of CH2 andCH3
1647.21	C=O stretching
1577.77,1508.33	C=C Aromatic stretching
1435.04,1408.04	C-H bending of CH2 and CH3
1361.74,1315.45	O-H bending of phenol
1172.72	C-O-C stretch of ether
1018.41,952.84	C-H bending of aromatic (in plane)
898.84,844.82,702.09	C-H of aromatic (out plane)

The results of HPLC analysis of isolated compounds (compound MH1, MH2, MH3, MH4, and MH5) show that isolated compounds have the same retention time of the following standards, respectively: gallic acid, caffeic acid, genistein, apigenin, and silybin.

The UV-diode array results of isolated compounds show the following points:

Table 8: FT-IR spectral data for isolated MH5 compound.

Isolated compound	*Gallic acid µg/mL*	*Caffiec acid µg/mL*	*genistein µg/mL*	*apgenin µg/mL*	*silybin µg/mL*
MH1	3.05				
MH2		100.57			
MH3			8.97		
MH4				198.25	
MH5					25.84

Table 9: The concentration of each isolated compound

IR bands of isolated MH5 compound	*Interpretation*
3394.72	O-H stretching of phenol
3005.10,2920.23,2850.79	Asymmetric and symmetric C-H stretching of CH2 andCH3
1643.35	C=O stretching
1508.33	C=C Aromatic stretching
1435.04,1408.04	C-H bending of CH2 andCH3
1361.74,1311.59	O-H bending of phenol
1276.88	C-O-C stretch of ether
1014.56,952.84	C-H bending of aromatic (in plane)
898.83,821.68,702.09	C-H of aromatic (out plane)

- In the UV spectrum, the isolated MH1 compound absorbed and gave peaks at (220 nm and 275 nm) at UV region that was the same region of gallic acid standard peaks.
- In the UV spectrum, the isolated MH2 compound absorbed and gave peaks at (215 nm and 325 nm) at UV region that was the same region of caffeic acid standard peaks.
- In the UV spectrum, the isolated MH3 compound absorbed and gave peaks at (200 nm and 260 nm) at UV region that was the same region of genistein standard peaks.
- In the UV spectrum, the isolated MH4 compound absorbed and gave peaks at (270 nm and 340 nm) at UV region that was the same region of apigenin standard peaks.
- In the UV spectrum, the isolated MH5 compound absorbed and gave peaks at (205 nm and 285 nm) at UV region that was the same region of silybin standard peaks.

According to FT-IR spectral explanations, the suspected structure of isolated compounds (compound MH1, MH2, MH3, MH4, and MH5) could be the same as the structure of gallic acid, caffeic acid, genistein, apigenin, and silybin, respectively.

Finally, from all these data obtained from HPLC, UV-diode array, and FT-IR of isolated compounds (compound MH1, MH2, MH3, MH4, and MH5), the isolated compounds could be identified as gallic acid, caffeic acid, genistein, apigenin, and silybin, respectively.

CONCLUSION

- Five compounds were isolated from n-butanol fraction, two phenolic acids (gallic acid and caffeic acid), and three flavonoids (genistein, apigenin, and silybin).
- Caffeic acid, apigenin, and silybin were isolated from *V. Villosa* plant for the first time.

- The higher yield of the isolated compounds was apigenin, while the lower yield of the isolated compounds was gallic acid.

ACKNOWLEDGMENT

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