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Separation and identification of phytosteroles and alkaloids in *Withania somnifera* (Ashwagandha) naturally grown in Iraq

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Keywords:
Withania somnifera, Beta-sitosterol, Stigmasterol, Alkaloids, phytosteroles.

ABSTRACT

Withania somnifera is interest plant. It's a distinguished medicinal plants in Indian systems of medicine, and many other places in the world this important plant used against many type of diseases, due to a many of different classes of chemical constituents that contains in Ashwagandha such as: steroidal lactones, alkaloids, flavonoids, saponine, somniferine and Iron. Due to these important chemical constituent Ashwagandha have different pharmacological activity such as anti-inflammatory, anticancer, antidiabetic, ant stress, antimicrobial, neuroprotective, cardio protective, modulate mitochondrial activity and reinforce endothelial function. The plant used single or with other medicine plants as combination to treat many type of humane diseases. Phytochemical screening of Iraqi *Withania somnifera* with the isolation and quantitative identification of some secondary metabolites. Using the root of plant, the extraction stage was preceded by a defatting step using n-hexane for 48 hours , followed by typical technique of extraction that were used in this work for plant material, Cold technique by maceration with 85 percent methanol followed by further fractionation by using different solvent according to increase of polarity, petroleum ether, chloroform, ethyl acetate, n-butanol and separation using high-performance liquid chromatography (HPLC),the Hexane, chloroform fractions was analyzed by thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and the identification of isolated compounds by using ultraviolet (UV), high-performance thin layer chromatography (HPTLC), thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) gas chromatography-mass spectrometry (GC-MS) was used for further analysis for the hexane fractions. Preliminary chemical testing revealed the presence of alkaloids, steroids, terpenoids, in the extracted substance. The separation of three phytoconstituents found by various chromatographic methods resulted in the confirmation of the non-polar chemicals separated from the hexane fraction, which included the phytosterol: Stigmasterol, Beta sitosterol with Caffeine was separated from the chloroform fraction. The secondary metabolites contained in the Iraqi *Withania somnifera* plant have a wide range of therapeutic effects. Caffeine will be the first isolation from the Withania plant, and beta sitosterol and stigmasterol is also the first

isolation from the species near Iraq.

1. INTRODUCTION

Plants have played a major role in the introduction of new therapeutic agents because they help alleviate human diseases. Despite the considerable development in the field of homeopathic medicine in the 20th century, plants are still one of the main sources of medicines in modern and traditional medical systems [1]. As plant-derived medicines continue to play an important role in the healthcare system of large populations of the world, herbal medicines are generally accepted. In fact, there are several medicinal plants in the world that are traditionally used to prevent and treat a variety of diseases. Secondary metabolites found in herbs include alkaloids, glycosides, steroids, and flavonoids, all of which could be used as medicinal ingredients [2]. Herbal products are complex mixtures of organic chemicals, which may come from any original or processed part of the plant, including leaves, stems, flowers, roots and seeds [3]. *Withania somnifera* is interest plant. It's a distinguished medicinal plants in Indian systems of medicine, and many other places in the world. this important plant used against many type of diseases, due to a many of different classes of chemical constituents that contains in Ashwagandha such as: steroidal lactones, alkaloids, flavonoids, saponine, somniferine and Iron. due to these important chemical constituent Ashwagandha have different pharmacological activity such as anti-inflammatory, anticancer, antidiabetic, ant stress, antimicrobial, neuroprotective, cardio protective, modulate mitochondrial activity and reinforce endothelial function. The plant used single or with other medicine plants as combination to treat many type of humane diseases [4], [21- 41].

Different parts of plant have be used to treat various type of diseases that affect humane health. Dried root for example is used widely to treat nervous and sexual diseases. withanolides, which is the major active constituent in plant and its give plant various biological activities [5].

Taxonomical classification of Ashwagandha:
Kingdom: Plantae, Plants;
Subkingdom: Tracheobionta, Vascular plants;
Super division: Spermatophyta, Seeds plants;
Division: Angiosperma
Class: Dicotyledons
Order: Tubiflorae
Family: Solanaceae
Genus: Withania
Species: Somnifera Dunal (*Withania somnifera* (L.) Dunal) [6].

Common name: Indian Winter Cherry, *Ashwagandha* [7], "Indian Ginseng" due to its rejuvenating effects [8], *Rasayana* in Ayurveda, certain herbal formulae known as *Rasayana* are considered to be rejuvenating [9], Poison gooseberry [10].

Botanical description of the plant:

Withania somnifera is a small shrub with a width of 1 meter and a height of 2 meters. The stem is brown, tapering and standing upright, sometimes without leaves.

Almost the entire plant is covered with short, thin silver-gray branch hairs. 1-7 inconspicuous bisexual flowers appear on leaf nodes on stems 2-5 mm long 5-lobed calyx ± 5 mm; fruit length ± 20 mm, spherical or pot-shaped, membranous and 5-10 polygonal. Corolla 5-lobed, 5-8 mm long, pale yellow to yellow-green. The five stamens are orange-yellow and are somewhat stressed.

The leaves are alternate (opposite to the flower buds), single leaves, whole to slightly wavy, Broad ovate, oblique or oblong, 30-80 mm long, 20-50 mm wide, petiole narrow 5-20 mm long, upper part is almost glabrous green, and lower part is densely hairy.

The fruit is a spherical, hairless berry, 5-8 mm in diameter, orange-red to red when mature, and is surrounded by enlarged calyxes. Many seeds are dark brown, 2.5 mm wide, kidney-shaped, rough and granular.

Collectors describe it as a smelly shrub with a particularly strong root, and they also commented on the smell of the leaves, which has a strong smell of green tomatoes. *Withania somnifera* is known because its red fruit is covered with brown, leafy and enlarged calyxes [11], [12].

2. Materials and Method

2.1 Collection of the plant material

The whole plant of *Withania somnifera* (L.) Dunal (solanaceae) family was collected from many places of Baghdad city during month of April (2020), the plant was notarized by Assistant Professor Dr. Khansaa Rashid Majid / Iraq natural history research center and museum / plant and environment department / university of Baghdad. The plant was cleaned, dried at room temperature in the shade then pulverized by mechanical mills and weighed.

2.2 Extraction of plant material

First step defatting of root by soaked with hexane for 48 hours then filtrated and dried for the next step for Extraction, plant material was soaked in 85 % methanol in umber closed container, with occasional shaking, at room temperature. After 3 days, the extract was filtered off. The filtrate was evaporated to get rid from

organic solvent under vacuum using rotary evaporator. A dark orange to brownish residue for root was obtained. The residue was suspended in water and partitioned successively with equal amount for each of petroleum ether (B.p. 40-60) (3times), chloroform (3 times), ethyl acetate (3times), and n-butanol (3times) until reach clear layer for each fraction. The first three fractions dried over anhydrous sodium sulfate, filtered, and evaporated to dryness [13].

Preliminary phytochemical examination of crude extracts and Different fractions: [14], [15]
Test for alkaloids:
(5 ml) of Alcoholic extract was stirred with (2.5 ml) of 1%HCL on a steam bath.
Mayer's reagent: (5gm of potassium iodide in 10 ml water + 1.35gm mercuric chloride in 60 ml of water) were added, the formation of a white color precipitate indicates the presence of alkaloids.
Dragendroff's reagent: (8gm potassium iodide in 20 ml water + 0.8gm bismuth nitrate in 40 ml distilled water + 10 ml glacial acetic acid, boiled for few min), then be added, appearance of a reddish-brown color precipitate indicates the presence of nitrogenous compounds this test is general.

Test for steroid (libermann - Burchard test):
0.5 ml of ethanolic extract + 0.5 ml chloroform + 1-2 ml acetic anhydride + 1-2 drops of sulphoric acid were added carefully, an array of a color change indicate the presence phytosterol.

Thin layer chromatography (TLC) examination of fractions:
Thin layer chromatography examination of hexane fraction of root:
Suspend a few milligrams of hexane in about 1 mL of chloroform, place them on an analytical TLC plate pre-coated with GF254 silica gel, and expand into the following mobile phase:
S1: Toluene: Ethyl acetate (90:10)
S2: Toluene: Ethyl acetate (93:7)
S3: Dichloromethane: Hexane (50:50)
S4: Methanol: Hexane (30:70)
S5: Hexane: Ethanol (70:30)
S6: Toluene: Ethyl acetate: Chloroform (50:10:40)
After rising the mobile phase to solvent front line through the plate was allowed to dry at room temperature, and the separated spots were identify by the Lieberman-Borchard reagent for identification of steroids. Spray this reagent onto the developing plate and heat it in an oven at 105 °C for 5 minutes. The doped spots (β-sitosterol& stigmasitosterol) are red to pink and purple to torture the acid [16].

Thin layer chromatography examination of Chloroform fraction of root.
Suspend a few milligrams of chloroform fraction in approximately 1 mL of ethanol, apply it to a ready-made analytical TLC plate pre-coated with GF254 silicone gel, and develop the following mobile phase:
S7: Acetone: Water: Ammonia (90:7:3)
S8: Chloroform: Ethyl acetate: Formic acid (60:40:10) (modify) [17].

HPLC analysis and separation of several compounds from the hexane fraction: by tow method
1- HPLC system from Knauer Germany, The separation was achieved on C18
(250X4.6) particles size 5 µm (Knuaer, Germany).in this condition Stigmasterol and β-sitosterol standards compared to the sample of the fraction prepared as a diluted solution of ethanol.
The mobile phase was composed of 0.1% TFA (trifluoroacetic acid) aqueous solution (a) and ACN (b) at a flow rate of 1.0 mL/min. The HPLC gradient profile was as follows: 10% b at 0–5 min, 10–90% b at 5–45 min, 100% b at 45–65 min.

The sample injection volume was 20 µL. 210 nm wave length was selected to determination of each standard compounds and each chromatographic peaks of compounds were confirmed by comparing their retention time and UV spectrum patterns.

The retention period of each compound was used to identify it, and uv/vis spectrum matching with its respected standard material [18].

For quantitative measurements, use the area under the curve (AUC) on the y-axis to draw a calibration curve against the four standard concentration levels on the x axis. Obtain a linear equation (y = mx + b) from which the concentration of the analyzer is calculated [19], [20].

Where:
y is the response factor (%area)
m is the slope of the curve
x is the concentration in part per million (ppm)
b is the y-intercept
2- Hplc condition for analysis of Hexane fraction as shown in Table (1).

Table (1) Hplc condition for analysis of Hexane fraction

HPLC condition	model SYKAMN (Germany)
Mobile phase	Acetonitrile: DW: acetic acid (60: 25: 5).
Column	C18-ODS (25 cm * 4.6 mm)
Detector	UV- 280 nm (S 2340)
Column oven model	S 4115
Pump model	S 2100 quaternary gradient pump
Fraction collector model	FOXY R1
Mode of operation	isocratic elution
Flow rate	1 ml/min.
Injection volume	200 µL for isolation and 100 µL for analysis

HPLC analysis of chloroform fraction: by tow method
1- The mobile phase was made up of 1 percent aq. acetic acid solution (Solvent A) and acetonitrile (Solvent B), with a flow rate of 0.7 ml/min, a column temperature of 280°C, and an injection volume of 20 l. The percentage of solvent B to solvent A was varied to provide a gradient elution. For 28 minutes, the gradient elution was altered in a linear fashion from 10% to 40% B, then from 40% to 60% B in 39 minutes, and finally from 60% to 90% B in 50 minutes. In 55 minutes, the mobile phase composition was restored to its original state (solvent B: solvent A: 10: 90) and the system was let to operate for another 10 minutes before injecting another sample. Time spent on each sample in total was 65 min. HPLC chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) according to absorption maxima of analyzed compounds.

The gradient as the below table:

Table (2) HPLC for analysis of chloroform fraction:

Time	Mobile A concentration %	Mobile B concentration %	Flow rate ml/min
0	90	10	0.7
5	90	10	0.7
28	60	40	0.7
39	40	60	0.7
50	10	90	0.7
55	90	10	0.7

Table (3) HPLC condition for analysis of chloroform fraction & isolation of compound from chloroform fraction:

Instrument model	SYKAM, Germany
Stationary Phase	Column C18- Octadecyl silyl (25cm* 4.6mm)
Mobile Phase	Methanol: Distilled water: Formic acid (70:25:5 v/v/v)
Detector	UV- detector (S-2340)
Wavelength	280 nm
Flow Rate	1.3 ml / min.
Total Run time	10 minutes
Injection Volume	100 µL
Mode of Operation	Isocratic elution

Characterization and Identification of isolated compounds:

- Compound R3 was identified by:

1- Thin layer chromatography (TLC)
2- High performance liquid chromatography (HPLC)
3- Ultra Violet (UV)
4- High performance thin layer chromatography (HPTLC)

- Compounds R4 &R5 was identified by:

1- High performance liquid chromatography (HPLC)
2- Ultra Violet (UV)
3-High performance thin layer chromatography (HPTLC)

3. Results and discussions

3.1 Extraction& fractionation of extracts

Before chromatographic analysis, plant components are usually separated from each other based on differences in polarity and solubility, because the crude extract contains various chemical components with different polarities. Crude plant extracts are fractionated with a series of different solvents (petroleum ether, chloroform, ethyl acetate and n-butanol). Each fraction undergoes different chromatographic techniques to further separate, purify, identify and isolate the main phytochemical components. The yield percentage (% w/w) of hexane and chloroform parts of the plant extract was determined, and the results are shown in Table (4):

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Table (4) the yield percentage (% w/w) of hexane and chloroform parts of the plant extract.

Part of plant	Fraction (f)	Weight in gram	Percentage in %w/w In the whole plant
Cold extraction of root (maceration)	Hexane f. (defatting)	1.5	0.0044
	Chloroform f.	2.7	0.007

Preliminary phytochemical investigation of different fractions: as shown in table (5)

Table (5) phytochemical investigation of different fractions

Fraction (f)	Alkaloid test	Steroid test
Hexane f. (root)	----------	+
Chloroform f. (root)	+	----------

(+) means the presence while (----):- means test not done.
The phytochemical examination results of each part confirmed the presence of steroids, alkaloids and also provided more selected information about the location of each active ingredient. Therefore, it will help further identification and isolation.

3.2 Thin layer chromatography for the hexane fraction

Using different solvent systems and standard products (β-sitosterol and stigma sterol), sprayed with vanillin-sulfuric acid reagent and heated in an oven at 105°C for 5 minutes in both the leave and root of Withania somnifera, the staining of these two compounds appeared in hexane, as shown in Figure (1a), (1b),

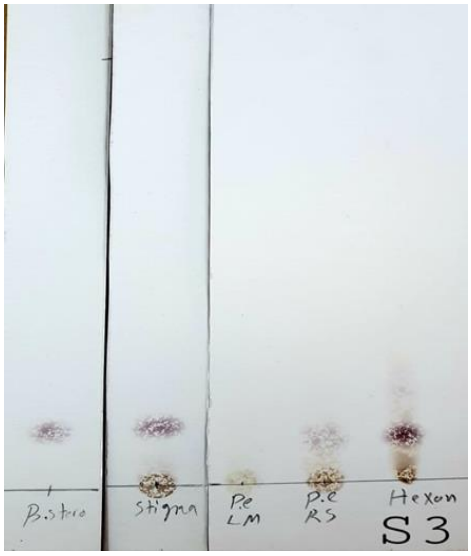

Figure (1a)

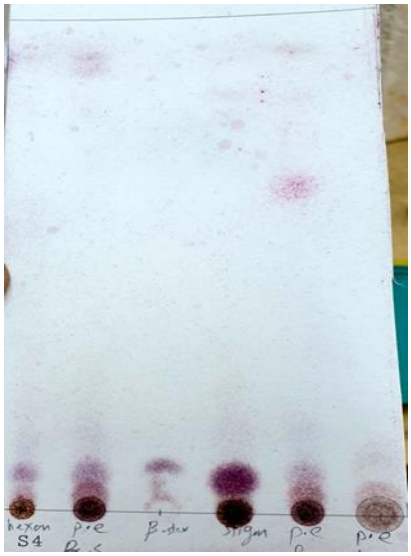

Figure (1b)

Figure (1a): Thin layer chromatography for hexane fraction with beta-sitosterol std. (B), stigmastirol std. (S), for root developed in solvent system S3: Dichloromethane: Hexane (50:50) and spraying with vanillin-sulfuric acid reagent.
Figure (1b): Thin layer chromatography for hexane fraction with beta-sitosterol std. (B), stigmastirol std. (S), for root developed in solvent system S4: Methanol: Hexane (30:70) and spraying with vanillin-sulfuric acid reagent.

-Analysis, Isolation and identification of different active constituent (secondary metabolites):

High-performance liquid chromatography (HPLC):-

1.A. High-performance liquid chromatography (HPLC) examination of the hexane fraction of root by using mobile phase was composed of 0.1% TFA (trifluoroacetic acid) aqueous solution (a) and ACN (acetonitrile) (b):As shown in figure (2a1),(2a2).

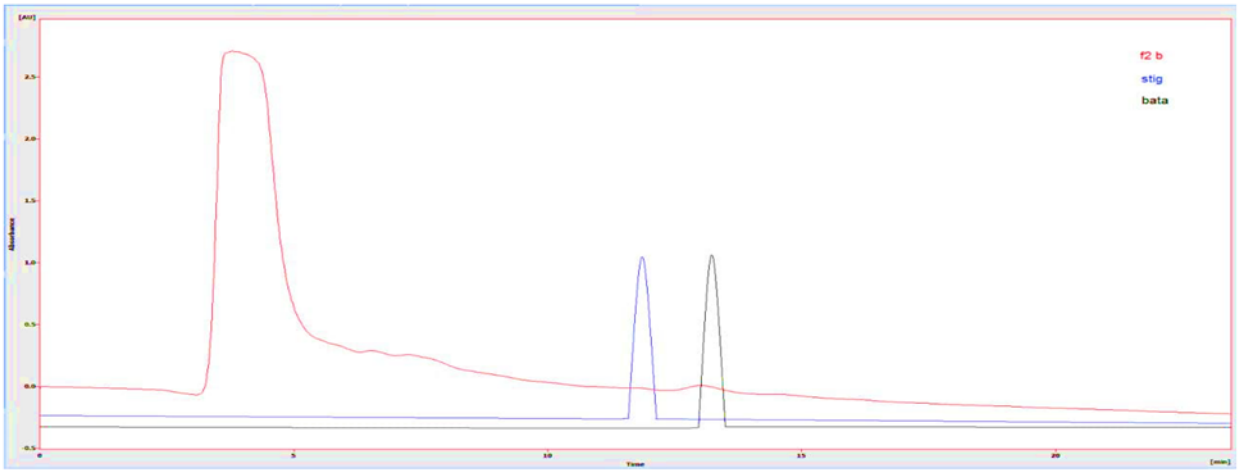

Figure (2a1): High-performance liquid chromatography (HPLC) examination of the hexane fraction of root by using ACN (acetonitrile) (b) as a mobile phase, with beta-sitosterol and stigmasterol standards.

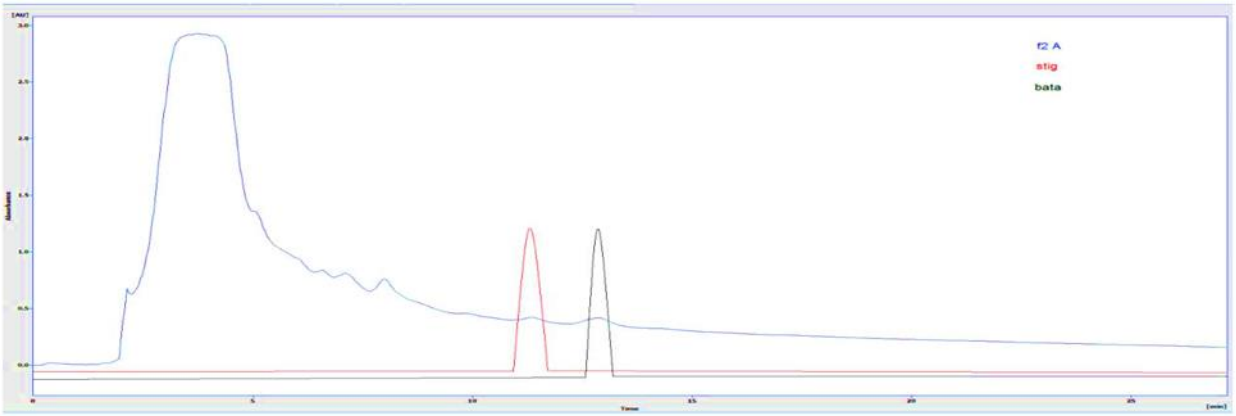

Figure (2a2): High-performance liquid chromatography (HPLC) examination of the hexane fraction of root by using0.1% TFA (trifluoroacetic acid) aqueous solution (a) as a mobile phase, with beta-sitosterol and stigmasterol standards.

- As shown in figures above there was matching in beaks of steroid standards (beta-sitosterol and stigmasterol) with the beaks of Hexane fraction.so this gave indication of presence of these active constituents.

1. B. Compounds (R4, R5) separated (isolated) and identified by using Hplc chromatography with using Acetonitrile: DW: acetic acid (60: 25: 5) as mobile phase.as shown in figure (2b1), (2b2).

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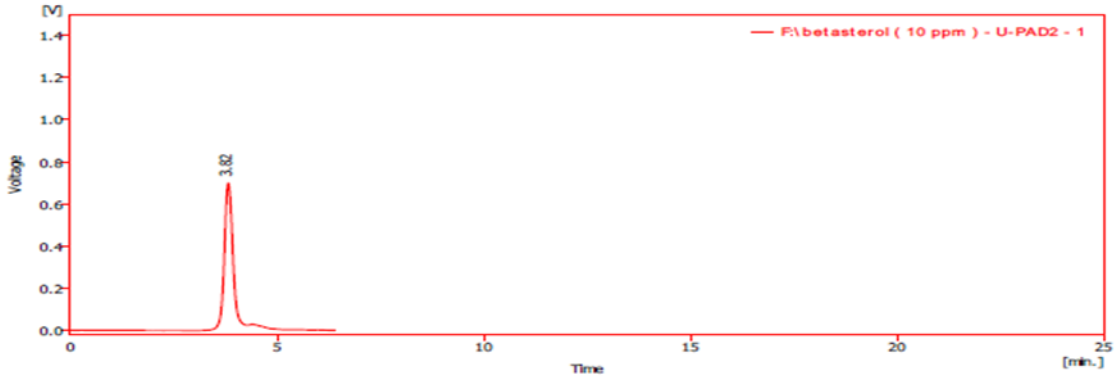
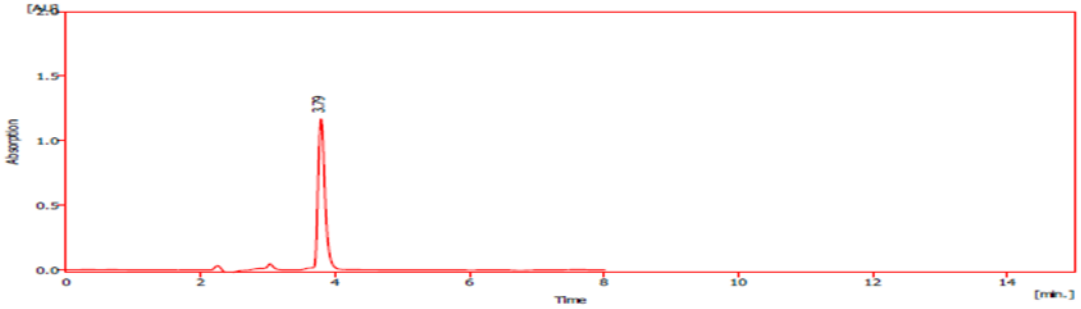


Result Table (Uncal - F:\betasterol (10 ppm) - U-PAD2 - 1)

	Retan. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.820	1901.641	275.303	100.0	100.0	0.13	
	Total	1901.641	275.303	100.0	100.0		

Beta-sitosterol std

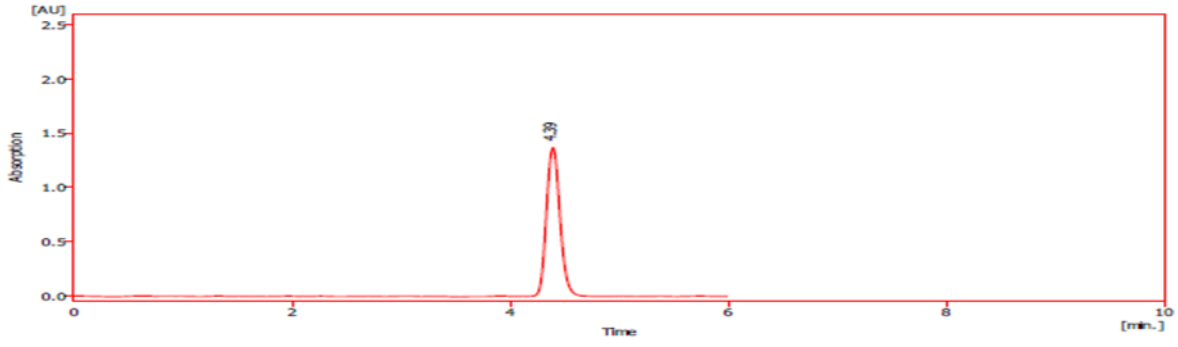


Result Table

	Retan. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.793	891.769	312.163	100.0	100.0	0.05	
	Total	891.769	312.163	100.0	100.0		

Isolated compound R_4

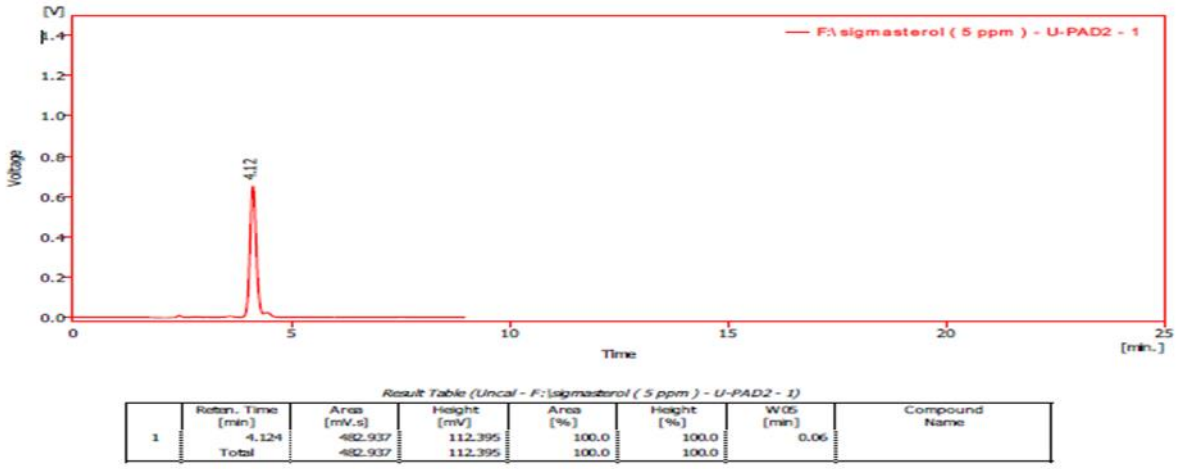
Figure (2b1): Identification of the isolated compound by HPLC using Acetonitrile: distilled water: acetic acid (60:25:5) as a mobile phase /UV- 280 nm.



Result Table

	Retan. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	4.393	11809.874	1369.636	100.0	100.0	0.14	
	Total	11809.874	1369.636	100.0	100.0		

Compound (R_5)



Stigmasterol std.

Figure (2b2): Identification of the isolated compound by HPLC using Acetonitrile: distilled water: acetic acid (60:25:5) as a mobile phase /UV- 280 nm.

-Quantitative analysis by calibration curve to determine the total concentration of isolated plant components, (R4, R5).

Quantitative analysis of the standard by calibration curve to determine the total concentration of isolated plant components, (R4, R5), as shown in figure (2b3)

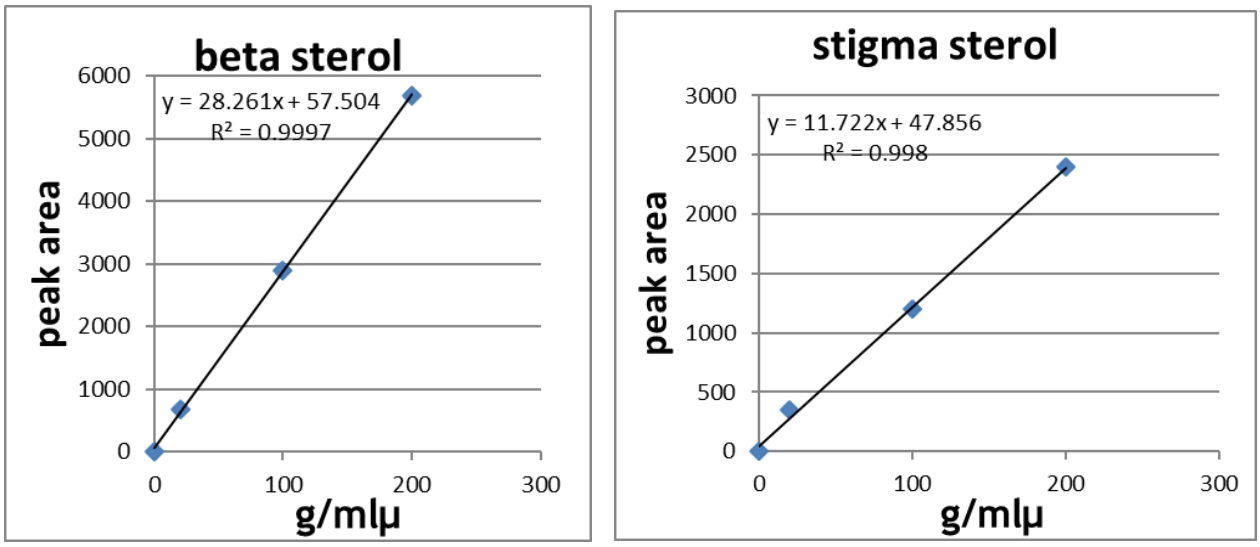


Figure (2b3) concentration of phyto-constituents in hexane fraction.

By using the standard linear equation and the area under the curve to calculate the concentration of secondary metabolite in hexane fraction of root .as shown in table (6):

Table (6) the concentration of secondary metabolite in hexane fraction of root.

f2a peak area	beta sitosterol	stigma sterol	Weight of sample solved
	1030.67	2233.97	110mg\5ml

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µg/ml	32.70	185.70	
concentration µg/mg extract	1.49	8.44	
f2 b peak area	224.38	2131.14	73mg\5ml
µg/ml	3.75	176.67	
concentration µg/mg extract	0.26	12.10	

2. A. Analysis of compound from chloroform fraction with mobile phase contains 1% aq. acetic acid solution (Solvent A) and acetonitrile (Solvent B).as shown in figure (2c1) with caffeine standard.

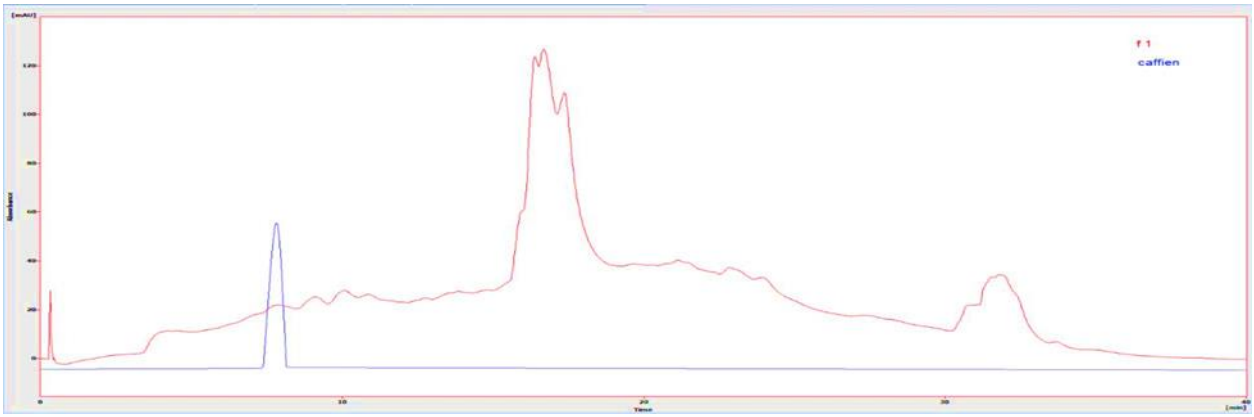
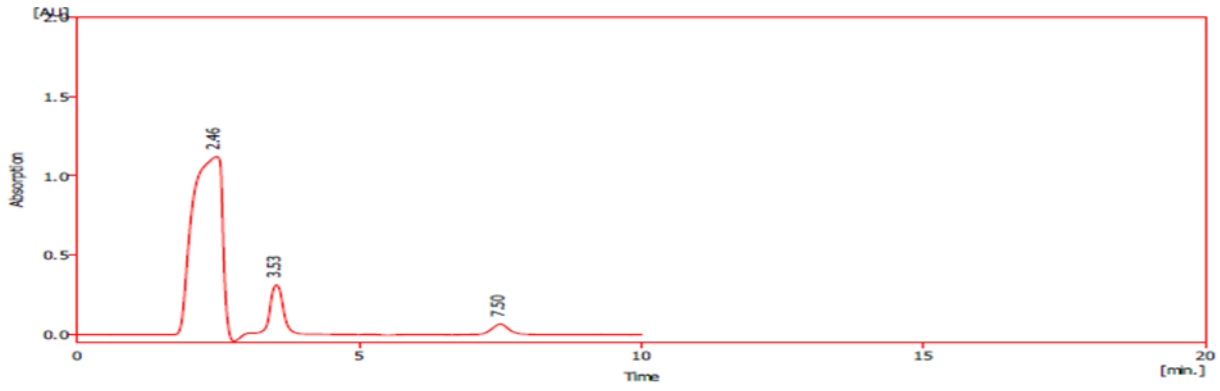


Figure (2c1): Hplc of chloroform fraction with caffeine standard.

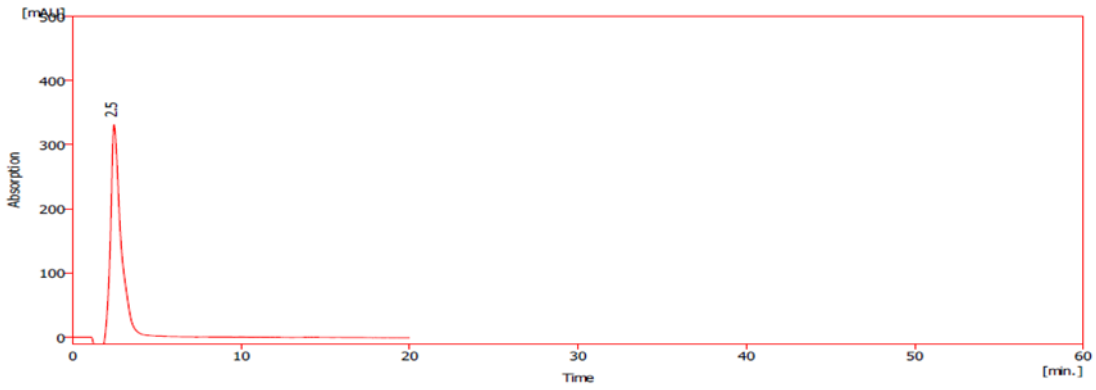
2. B. High-performance liquid chromatography (HPLC) examination, separation and identification of the chloroform fraction of root and isolated compound R3with mobile phase Methanol: D.W: formic acid (70:25:5) and caffeine standard.as shown in figure (2c2).



Result Table

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.457	41727.393	1147.854	87.0	75.5	0.63	
2	3.533	4818.577	305.554	10.0	20.1	0.24	
3	7.503	1423.239	66.421	3.0	4.4	0.32	
	Total	47969.209	1519.830	100.0	100.0		

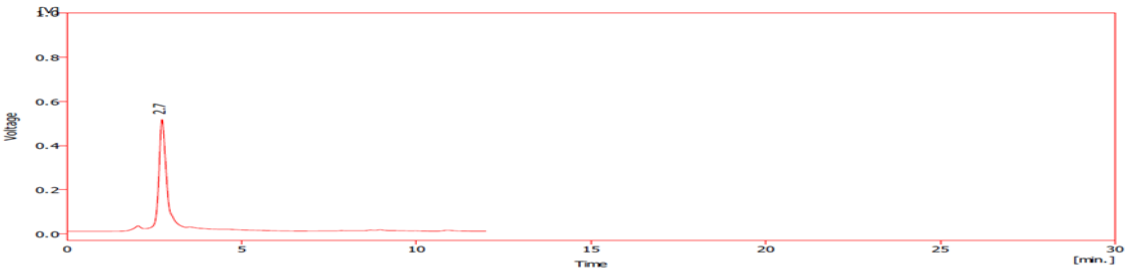
Chloroform fraction



Result Table

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.453	1351.823	97.421	100.0	100.0	0.25	
	Total	1351.823	97.421	100.0	100.0		

Caffeine standard



Result Table

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.720	3624.443	364.533	100.0	100.0	0.17	
	Total	3624.443	364.533	100.0	100.0		

Compound R_3

Fig (2c2) Identification of the isolated compound (R3) by HPLC with mobile phase Methanol: D.W: formic acid (70:25:5) and caffeine standard.

- Ultra Violet (UV)

Identification of the isolated compound (R3) by (UV):

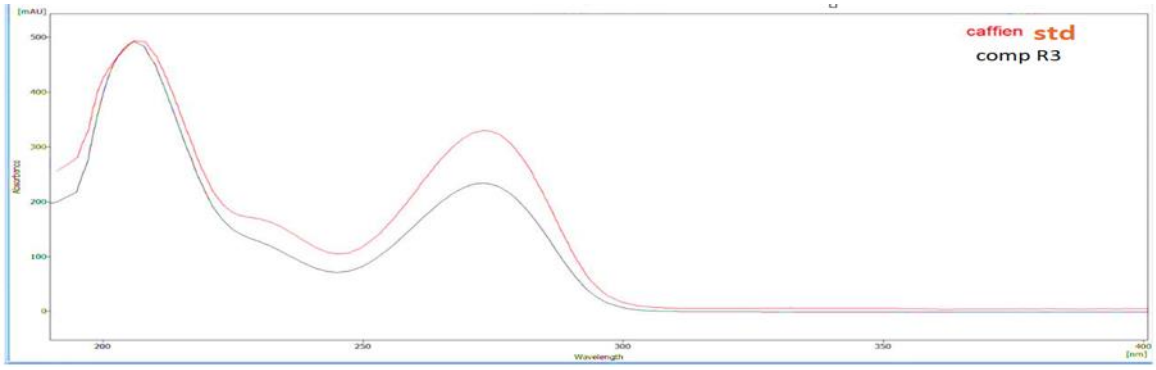


Figure (3a) UV analysis of compound R3 and caffeine standard.

Identification of the isolated compound (R4), (R5) by (UV):

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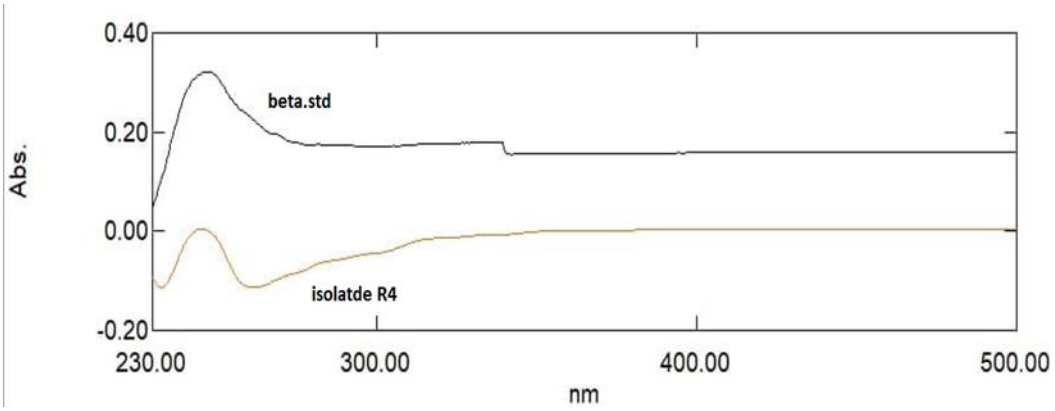
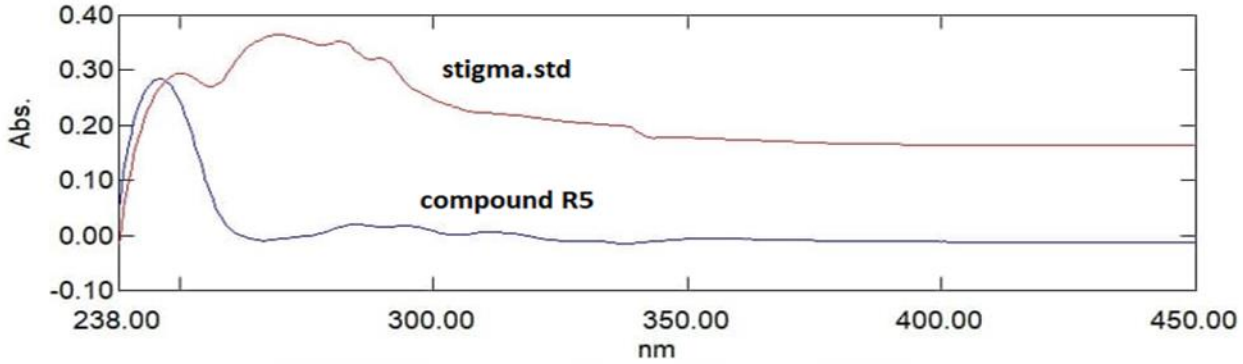


UV of isolated compound R4 with beta-sitosterol standard.

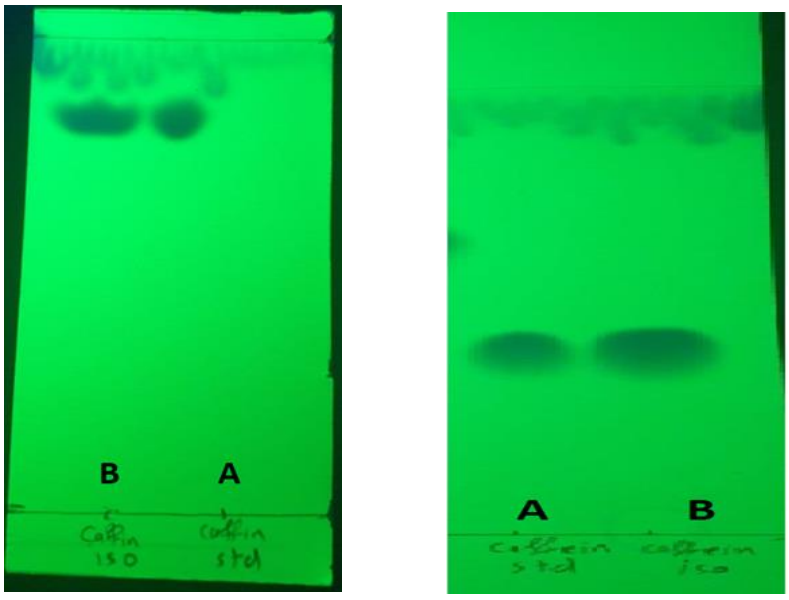


UV of isolated compound R5 with stigma sterol standard.

Figure (3b) UV analysis of compounds R4, R5 and beta-sitosterol & stigma sterol standards.

-Thin layer chromatography (TLC) for identification of Compound R3:-

This Identification method done with tow solvent systems (S7), (S8) to confirm the identity of compound (R_3). With using caffeine standard.as shone in figure (4).



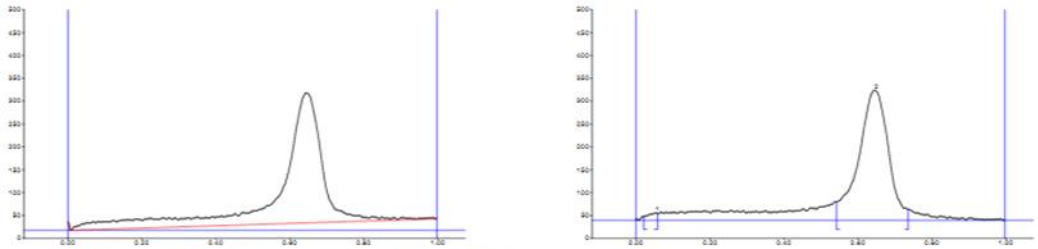
S7 S8

Fig (4) TLC analysis for the isolated compound R3 (B) from chloroform fraction of the root and caffeine

std (A) and S7: acetone: D.W: ammonia (90:7:3), S8:chloroform: ethyl acetate: formic acid (60:40:10) as amobile phase,UV (254 nm).

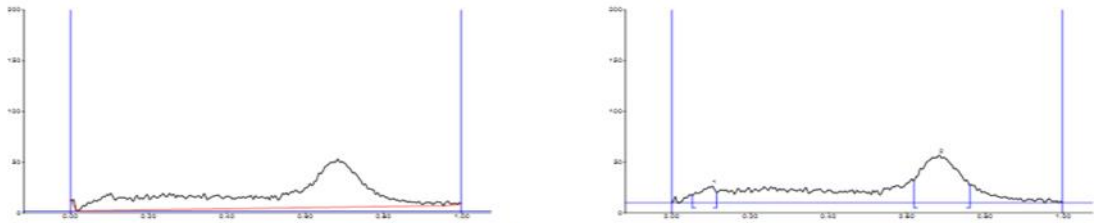
As shown in fig (4) the compound R3 and the caffeine standard correspond the same Rf value and same colore under uv.

-High performance thin layer chromatography (HPTLC)
HPTLC for identification of compoundsR4, R5: as shown in figure (5)



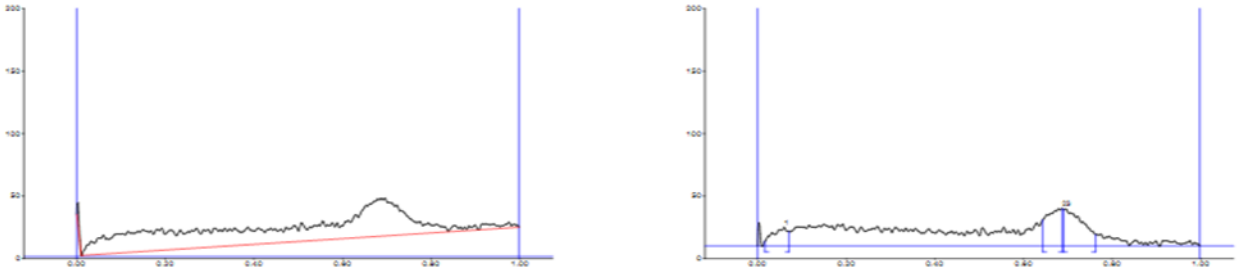
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	8.4	0.05	16.2	5.38	0.06	14.4	324.8	1.82	unknown *
2	0.54	40.1	0.64	284.4	94.62	0.74	23.7	17557.6	98.18	unknown *

Hptlc of beta-sitosterol standard



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	7.7	0.10	16.6	26.00	0.11	10.1	519.8	13.20	unknown *
2	0.62	21.7	0.68	47.1	74.00	0.76	17.4	3418.6	86.80	unknown *

Hptlc of compound (R_4)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	4.0	0.06	15.2	20.04	0.07	11.6	404.7	17.86	unknown *
2	0.64	21.2	0.69	30.4	40.09	0.69	30.1	826.1	36.46	unknown *
3	0.69	29.2	0.70	30.2	39.87	0.76	9.4	1034.9	45.68	unknown *

Hptlc of Stigmasterol standard

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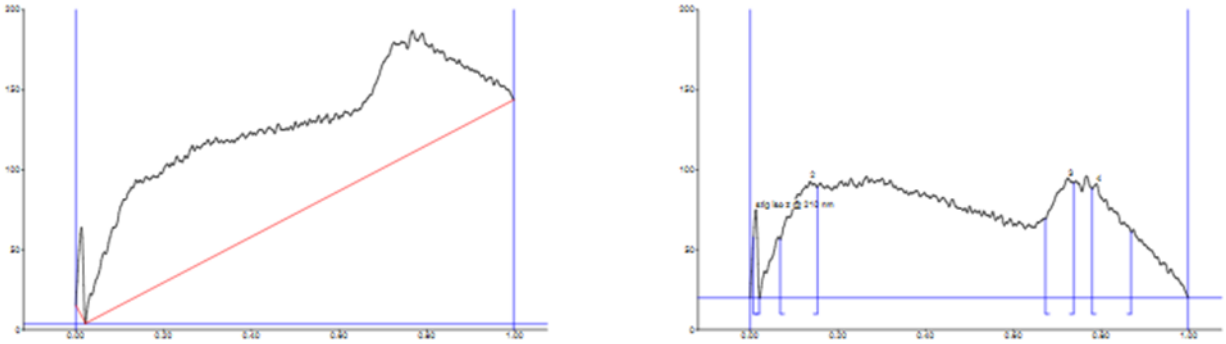
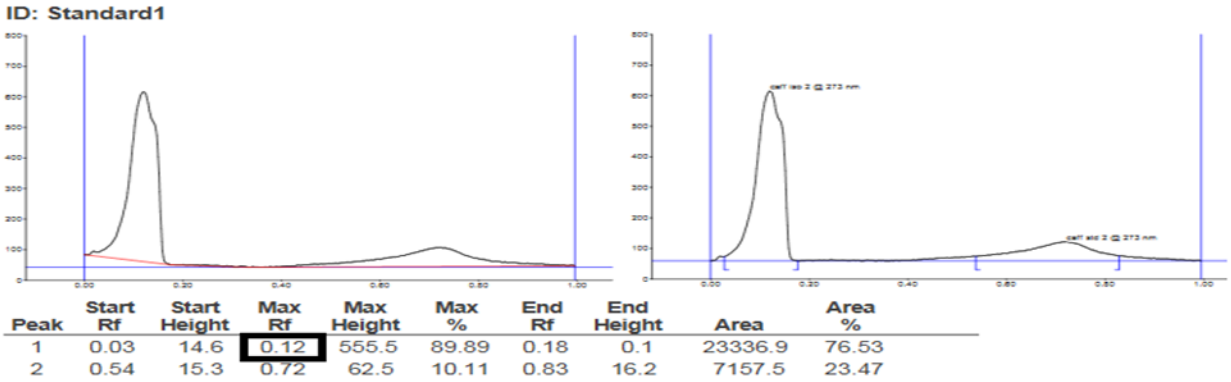


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.01	38.0	0.01	55.2	20.10	0.02	0.1	357.5	3.52
2	0.07	36.8	0.14	73.1	26.60	0.15	69.2	3513.0	34.60
3	0.67	49.4	0.73	75.0	27.29	0.74	72.0	2861.7	28.19

Hptlc of Compound (R_5)

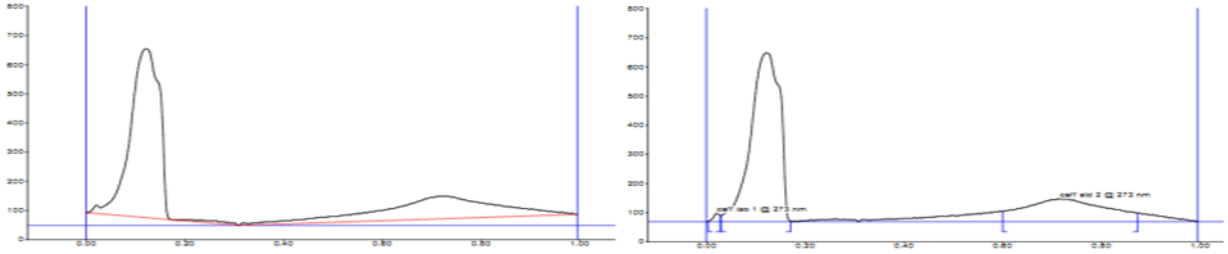
Figure (5) HPTLC for identification of compoundsR4, R5 with phytosterols standards.

-HPTLC for identification of compound R3: as shown in figure (6)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	14.6	0.12	555.5	89.89	0.18	0.1	23336.9	76.53
2	0.54	15.3	0.72	62.5	10.11	0.83	16.2	7157.5	23.47

Caffeine standard.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.00	0.3	0.02	28.2	4.12	0.03	20.5	280.3	0.76
2	0.03	20.6	0.12	578.4	84.52	0.17	1.3	26487.4	71.93
3	0.60	34.7	0.72	77.7	11.36	0.88	29.1	10056.7	27.31

Compound R3

Figure (6) HPTLC of caffeine standard and compound R3.

We may deduce from the aforementioned findings that the isolated compound (R3) is caffaine, compound (R4) is beta-sitosterol, and compound (R5) is stigma sterol.

4. Conclusion

Three metabolites were obtained from the plant extract: beta-sitosterol and stigmasterol were separated from the hexane fraction of the root of *Withania somnifera*, and caffaine was isolated from the chloroform fraction.

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