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Isolation of Three Flavonoids from *Withania Somnifera* Leaves (*Solanaceae*) and their Antimicrobial Activities

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***Abstract*— The methanolic crude extract of *Withania somnifera* leaves (Solanaceae) were screened for Phytoconstituents, the extracts revealed the presence of flavonoids, steroids, alkaloids, saponins and tannins. A three flavonoids glycoside: 7, 3', 4'-trihydroxy flavone-3-O-rhmnosyl, Quercetin -3-O-galactosyl and 5, 7, 4`- triahydroxy-methyl-3-O-galactosyl flavonol:were isolated and its biological activity was evaluated. The structures of these flavonoids were characterized on the basis of their IR, UV, NMR and MS spectroscopic data**

***Index Terms*— *Withania somnifera*, Isolation, flavonoids glycoside, Quercetin and biological activity.**

I. INTRODUCTION

Withania somnifera, commonly known as *ashwagandha*, Indian ginseng, and winter cherry, has been an important herb in the Ayurvedic and indigenous medical systems for over 3000 years. Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent, and more recently to treat bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia [1]. Clinical trials and animal research support the use of ashwaganda for anxiety, cognitive and neurological disorders, inflammation, and Parkinson's disease. Ashwaganda's chemopreventive properties make it a potentially useful adjunct for patients undergoing radiation and chemotherapy [2]. It is grows about 30- 150 cm in height in India Pakistan, Babgla Desh, Sri Lanka and parts of northern Africa. It is also found in Congo, South Africa, Egypt, Morocco, Jordan, Pakistan and Afganistan [3]. Flavonoids can be considered as derivatives of the 2-phenylchromone parent compound composed of two phenyl rings, and a heteocyclic ring referred to as the A-, B- and C- rings [4]. Flavonoids are secondary metabolites characterized by flavan nucleus [5] and a C_6-C_3-C_6 carbon-skeleton [6]. These are group of structurally related compounds with a chromane-type skeleton having phenyl substituent in C_2-C_3 position [7]. The flavonoids belong to one of the most bioactive compounds which naturally exist in the plant kingdom. Different naturally occurring flavonoids have been described and subcategorized into flavones, flavans, flavanones, isoflavonoids, chalcones, aurones and anthocyanidines [8, 9]. These flavonoids have remarkable biological activities, including inhibitory effects on enzymes, modulatory effect on some cell types, protection against allergies, antiviral, anti-malarial, antioxidant, anti-inflammatory and anti-carcinogenic properties[8, 9]. A number of flavones, flavonols, flavanones, and isoflavones, as well as some of their methoxy, isoprenyl, and acylated derivatives, show antibacterial activity [10]. Most bacteria can be divided into two groups based on a stain developed by the Danish physician Hans Christian Gram in 1884[11]. This procedure is based on the fact that Gram-positive bacteria retain a crystal violet-iodine complex through decolorization with alcohol or acetone and Gram-negative bacteria do not. Gram-positive bacteria possess a thick peptidoglycan layer in their cell wall whereas Gram- egative bacteria possess a thin peptidoglycan layer plus a lipopolysaccharide outer membrane [12].

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II. MATERIALS AND METHODS

General Experimental Procedure

Analytical grade reagents were used for analysis of some flavonoids from *Withania somnifera*. The UV spectra recorded on a Shimadzo 1601 Spectrophotometer and UV lamp was used for localization of fluorescent spots on TLC and PC. The IR spectra were run on a Thermo Nicolet FTIR 300 as KBr disks, using Shimadzu IR-8400 Spectrophotometer. Nuclear Magnetic Resonance spectra were run on a JEOL DELTA ESP- 500 MHZ NMR Spectrophotometer. Melting points were determined on a Kofler Hot- Stage Apparatus and were uncorrected. The mass spectra were recorded by direct probe EIMS using a Shimadzu GCMS- QP5050 spectrometer with ionization induced by electron impact.

Collection of the Plant Materials

The leaves of ***Withania somnifera*** (Solanaceae) were collected in March 2010 from Erkowit (east of Sudan) and authenticated by Dr. Hassan Mustafa Hassan, Dept. of botany, University of Khartoum, and a voucher sample was deposited in the herbarium of this department.

Extraction of Withania somnifera Leaves constituents

Powdered air-dried leaves (1.0 Kg) of *Withania somnifera* were ground in a Warring Blender to a fine powder and extracted with chloroform to remove chlorophyll, resinous and waxy material. The ground material was extracted with 85% aqueous methanol and the slurry was allowed to stand at room temperature for 24 hours with occasional stirring. The solvent-containing extract was then decanted and filtered by vacuum filtration. The extraction of the ground leaves were further repeated twice with the same solvent and twice with 50% aqueous methanol [13].The four filtrates from each extraction was combined and the excess solvent was evaporated under reduced pressure at 40°C using a rotary evaporator to give crude extracts, (53g) of a solid mass and then kept into a glass container to use.

Micro-organisms collection and maintenance

The microorganisms used in the study: Bacillus subtilis, Escherichia coli, Neisseria gonorrhoeae, Pseudomonas aeruginosa and Staphylococcus aureus. were obtained from stock culture in the Department of Botany, University of Cairo, Cairo City, Egypt. The organisms were stored on agar slant in McCartney bottles and kept in the refrigerator, prior to subculture.

Preparation of standard test organisms

One ml aliquots of 24 hours broth cultures of test organisms (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*) were aseptically distributed onto nutrient agar slopes and incubated at 37 C for 24 hours. The bacterial growth was harvested and washed off with sterile normal saline, to produce a suspension containing about 10^8- 10^9 colony forming units per ml. The suspension was stored in the refrigerator at 4 C till used. Serial dilution of stock suspension, were made in sterile normal saline, and 0.02 ml volumes of the appropriate dilution were transferred onto the surface of dried nutrient agar and the drop was left to dry, and then incubated at 37 C for 24 hours [14].

Testing for antibacterial activity

To determine the activity of methanolic extract and pure component of *Withania somnifera* against the five standard organisms. The cup-plate agar was adopted with some minor modification. (2 ml) Of the standard bacteria stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45 C. (20 ml) Aliquots of incubated agar were distributed into sterile Petri dishes. The agar was left to settle and each plate was cut and agar discs were removed. Alternated cups were filled with 0.1 ml sample of each of the extract (pure component) and allowed to diffuse at room temperature for two hours. The Petri dishes were then incubated in the up right position at 37 C for 18 hours. After incubation, the diameter of the resultant growth inhibition zones were measured, and averaged.

Preliminary Phytochemical screening of the Withania somnifera

Crude Methanolic Extracts

The methanolic extracts were tested for the presence of active constituents such as flavonoids, steroids, terpenoids, alkaloids, saponins, and tannins. The following standard procedures were used:

Flavonoids

i. To about (3 ml) of the methanolic extract few drops of methanolic aluminum chloride were added. Formation of a yellowish colour indicates the presence of flavonoids [15].

ii. To about 3ml of the methanolic few drops of aqueous potassium hydroxide solution were added. A dark yellow colour is taken as a positive test for flavonoid compounds.

iii. Shinoda Test: Small pieces of Magnesium ribbon followed by few drops of concentrated hydrochloric acid were added to a small amount of methanolic extract of the plant material. Formation of a crimson red colour indicates the presence of flavones in glycosidic linkage [16].

Steroids and Terpenoids

(40 ml) aliquot of the methanolic extract was evaporated to dryness on a water bath and the cooled residue was stirred with petroleum ether to remove most of the colouring matter. The residue was extracted with (20 ml) chloroform. The chloroform solution was dehydrated over anhydrous sodium sulphate.

(5 ml) portion of the solution was mixed with (0.5 ml) acetic anhydride, followed by two drops of concentrated sulphuric acid. A green colour was observed indicating the presence of steroids and no pink-violet colour was observed indicating the absence of terpenoids [17].

Alkaloids

About (30 ml) aliquot of the methanolic extract was evaporated to dryness on a water bath. (5 ml) of 2N hydrochloric acid were added and the solution was heated with stirring in a water bath for 10 minutes. The mixture was cooled and filtered. (2 ml) of the filtrate, were added to few drops of Mayer reagent (Potassium mercuric iodide solution). A precipitate was observed indicating the presence of alkaloids[18].

Saponins

20 ml of water was added to 0.25 g of the extract in 100 ml beaker and boiled, filtered and then the filtrates used for the tests:

*Froth Test***:** 5 ml of the filtrate was diluted with 20 ml of water and shaken vigorously. A stable froth (foam) up on standing indicates the presence of saponins [19].

Tannins

To about (25 ml) of the methanolic extract was evaporated to dryness on a water bath and the residue was extracted with n-hexane and filtered. The hexane- insoluble portion was stirred with (10 ml) of hot saline solution (0.9% w/v of sodium chloride and freshly prepared distilled water).

The mixture was cooled and filtered and the volume adjusted to (10 ml) with more saline solution. (5 ml) of this solution was treated with few drops of ferric chloride solution, a blue colour was formed indicating the presence of tannins [20].

Isolation of flavonoids

The dry extract was loaded on a polyamide 6S column chromatography (80 x 3 cm). The column was eluted with water, and then water -Methanol mixtures of decreasing polarity and 7 fractions were collected. The major fractions obtained were combined into three fractions after chromatographic analysis. Fraction A (5 g) was fractionated by **PC**, using Whatman No 3 sheets and (n-butanol: acetic acid: water) upper layer as solvent system followed by Sephadex LH-20 column eluted with methanol (100%) and methanol/ water (9: 1), to afford two pure compounds W_1 (29 mg) and compound W_2 (36mg). Fraction **B** (3 g) was subjected to column chromatography on cellulose and n-BuOH saturated with water as an eluted solvent to give two major sub fractions, then each of them was separately fractionated on a Sephadex LH-20 to yield pure compound W_3 (27 mg) and compound W_4 (21 mg) .

III. RESULTS AND DISCUSSION

The phytochemical screening of *Withania somnifera* constituents in methanolic extracts revealed the presence of alkaloids, Steroids, saponins and phenolic compounds (flavonoids, and tannins). The weight and percentage yield of pure compounds extracted from *Withania somnifera* are shown in **Table 1**.

Table 1

Weight and percentage yield of some compounds extracted from *W. somnifera* leaves

components	weight	Percentage yield
Compound W_1 (mg)	29	0.58
Compound W_1 (mg)	36	0.72
Compound W_1 (mg)	27	o.77

The Rf values and colour reactions of pure compounds are shown in **Table 2**.

Table 2

The Rf values and colour reactions of pure compounds

Comp.	*Rf values x 100*				***Colour reactions***		
No	**H_2O**	**HoAc 15%**	**BAW**	**TBA**	**UV**	**UV +NH_3**	**$AlCl_3$**
W_1	**41**	**47**	**49**	**60**	**Deep purple**	**Yellow**	**Yellow**
W_2	**38**	**43**	**59**	**46**	**Deep purple**	**Yellow**	**Yellow**
W_3	**34**	**62**	**67**	**19**	**Fluorescent Light blue**	**Fluorescent y green**	**Yellow**

The IR spectroscopic analysis of pure compounds extracts of *Withania somnifera* leaves gave the following characteristic absorption peaks as shown in **Table 3**.

Table 3

IR Spectroscopic data Region (Wavenumber (cm-1)

components	W_1	W_2	W_3
C-H aromatic bending	631, 779	799	597, 818
C-O aromatic stretching	1178	1173	1176
C= C aromatic stretching	1608	1442	1653
C=O stretching	1655	1654	1710
C-H, alkanes stretching	2923	2925	2927
O-H (stretch)	3399	3399	3381

The Pure compounds dissolved in methanol, were subjected to UV spectrophotometric investigation in 4 ml capacity quartz cells using Shimadzu UV recording spectrometer Model UV 240 in **table 4** [21].

Table 4
UV Spectrum data of pure compounds extracted from *W. somnifera*

Comp. No	MeOH	NaOMe	$AlCl_3$	$AlCl_3$/ HCl	NaOAc	NaOAc/ H_3BO_3
W_1	267, 300 sh, 350	275, 326 sh, 402	270, 303sh, 350, 400	274, 303sh, 355, 386	274, 303sh, 379	266, 297sh, 359
W_2	257, 300sh 360	272, 327 sh, 409	273, 295 sh, 425	268, 300sh, 362, 400	275, 310 sh, 380	260,300sh, 375
W_3	250, 296 sh, 350	240, 260 sh, 380	270, 10sh, 340sh, 445	260, 350 sh, 420	260, 380, 430	255, 300sh, 390

Table 5
The antibacterial activity of crude and pure compounds extracted from *Withania somnifera*

Sample		Inhibition zone diameter (mm / mg sample)				
		B acil subtilis **(G^+)**	***Escherichi coli*** (G^-)	***Neisseria gonorrhoeae*** **(G^-)**	***Pseudomonas aeruginosa*** **(G^-)**	***S taphylococcus aureus*** **(G^+)**
Control: DMSO		0.0	0.0	0.0	0.0	0.0
Standard	**Tetracycline Antibacterial a**	27	31	33	29	26
Crude extract		9	13	9	13	13
W_1		12	11	12	12	11
W_2		9	9	8	9	9
W_3		13	14	15	10	13

The methanolic extract of the leaves of *W. somnifera* was fractionated by polyamide column chromatography to give several fractions, which were further chromatogramphed on Sepadex LH 20 and cellulose to afford three compounds w_1, w_2 and w_3. The structures of these flavonoids were characterized on the basis of the IR show in table 1. The NaOMe, $AlCl_3$, $AlCl_3$/ HCl, and fused NaOAc/ H_3BO_3 reagents were separately added to the methanolic solution of the investigated material and UV measurements were then recorded in (table 4) to determine the position of hydroxyl groups.

Compound W_1 absorbs at λ_{max} (MeOH) 267, 350 nm due to the benzoyl and cinnamoyl chromophores of the flavonol moiety [22]. NaOMe exhibited a bathochromic shift of +52 nm indicating a 4`- OH. NaOAc gave about +7 nm indicating the 7-OH, also the shift of boric acid spectrum indicated a catechols moiety **[21]**. ^{1}H-NMR (500 MHz, DMSO-d_6) figure (1) shows δ (ppm): δ 3.0 – 3.5 (m, 3H) assigned for a methoxyl function; δ 4.2 – 5 (m, 6H) accounting for sugar protons; δ 6.04 (s, 1H) and δ 6.25 (d, 1H), accounting for C_6- and C_8- H's respectively. The resonances at δ 6.84 and 7.98 ppm were assigned for the aromatic H's of B- ring. The froth test [23] revealed the glycosidic nature of compound (W_1), after completed acid hydrolysis and subsequent paper chromatography the sugar was identify as galactose.

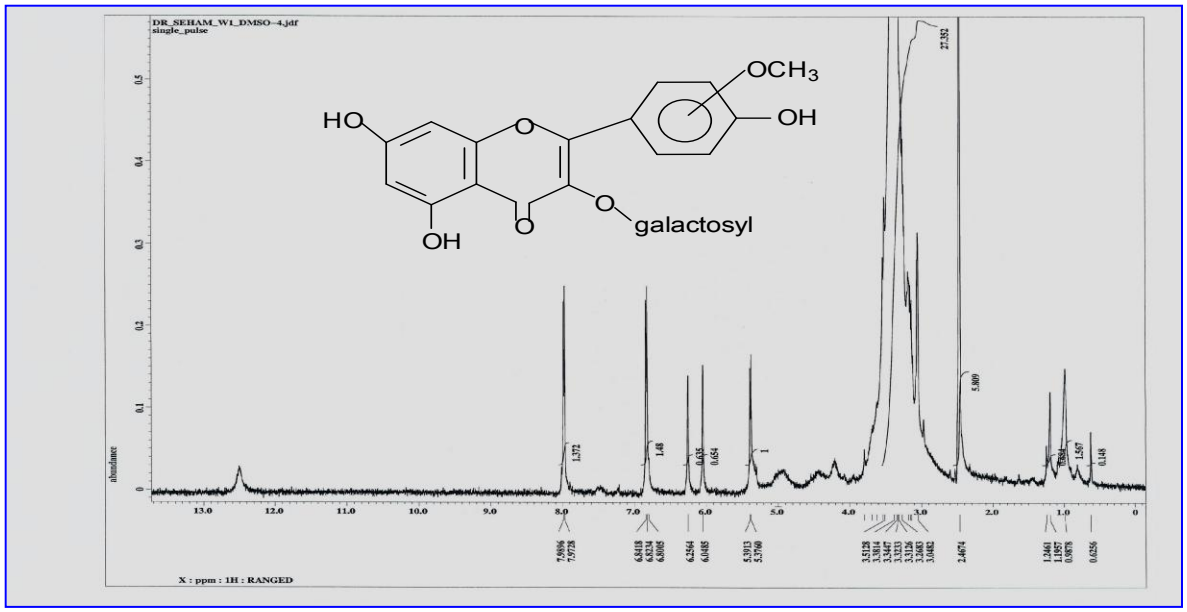

Figure (1) ^{1}H NMR spectrum data of compound $W_{1.}$

Compound W_2 absorbs at λ_{max} (MeOH) 267, 350 nm, the NaOMe spectrum exhibited a bathochromic shift of + 49 nm (**table 4**) indicated the presence of 4`-OH and absence of 3-OH. The results of UV shift and ^{1}H-NMR elucidate the structure feature of compound w_2 in (**figure: 2**).

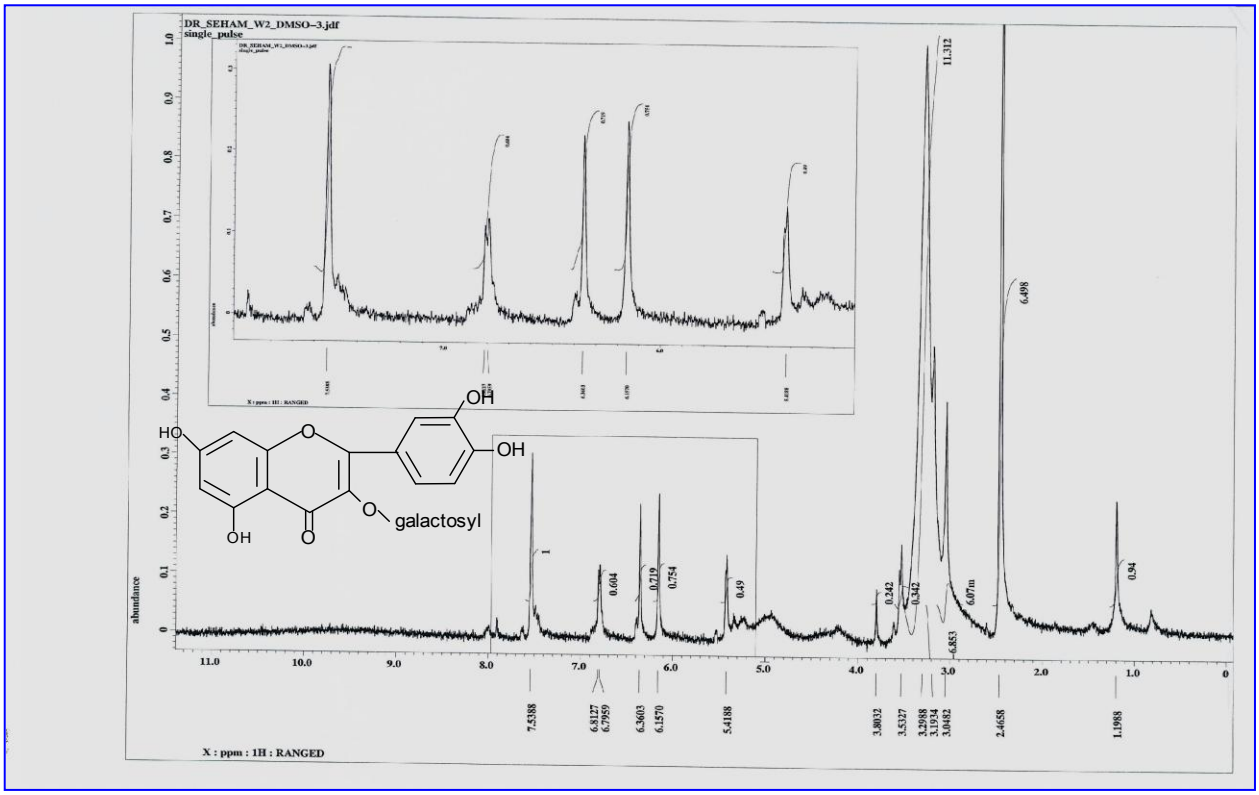

Figure (2) ^{1}H NMR spectrum data of compound W_2

The UV spectral data of compound W3 in MeOH characterized a flavone with three hydroxyl groups. The addition of NaOMe gave a bathochromic shift of 30 nm (**table 4**) indicated of 4`-OH. Also 7-OH and catechol moiety in ring B were characterized by shift reagents of NaOAc and NaOAc/ H_3BO_3 respectively. The ^{1}H-NMR spectrum of W_3 (**Figure: 3**) showed: δ 3.2- 3.9 (m) assigned for sugar protons. Furthermore, there was only one anomeric proton signal of sugar moiety

attached to a flavonoid skeleton (doublet at δ 5.4) proving the presence of rhamnosyl moiety (with a methyl group at 1.1 ppm). The resonances at δ 6.15 and 6.7(d, 2H) were assigned for C_6- and C_8- H's also signals at δ 6.92 (s, 1H), 7.03 (s, 1H) and 7.41 (d, 1H) are typical for a flavone B-ring with 3`- and 4`-OH functions. The resonance at δ 8 ppm is characteristic of C_5-H.

The complete acid hydrolysis of compound W_3 and subsequent UV studies of the resultant aglycone using the shift reagent $AlCl_3$ indicated a 3-hydroxyl and this is precisely the cite of glycosylation. The aglycone of compound W3 seems to be trihydroxy flavone.

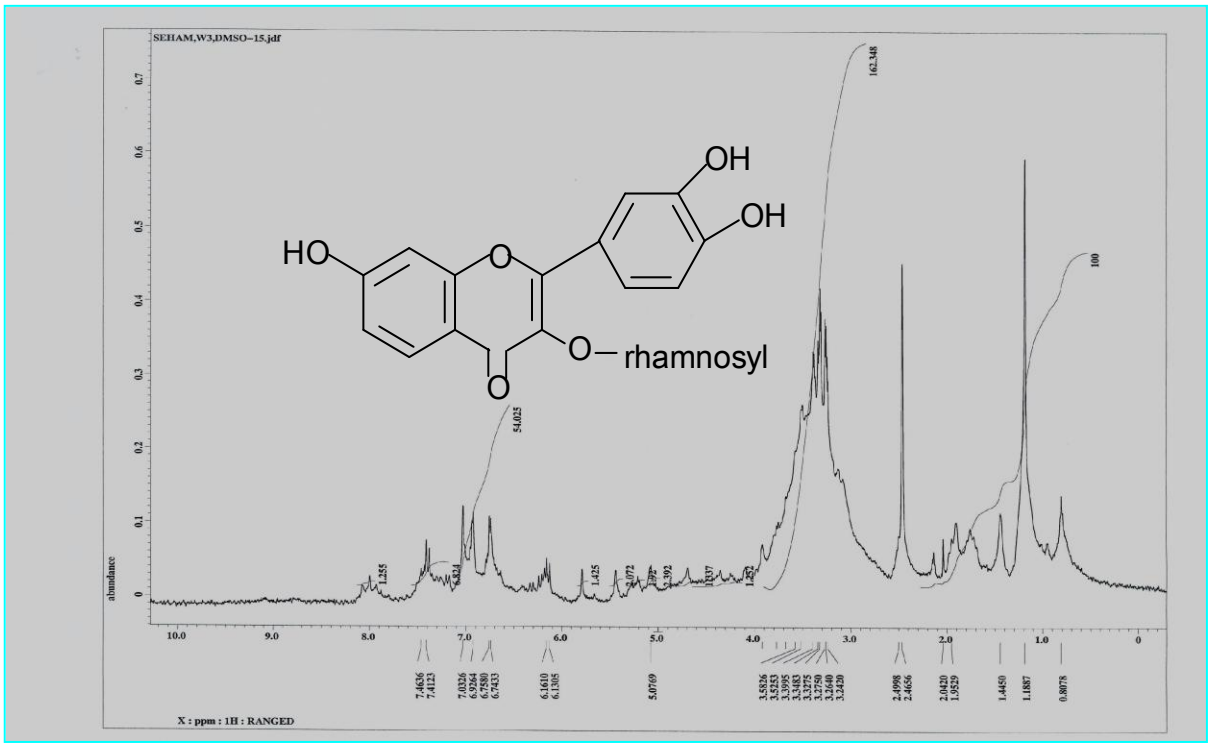

Figure (3) ^{1}H NMR spectrum data of compound W_3.

The antibacterial activity results of crude extract and pure compounds of *Withania somnifera* against five human pathogens was carried out in **table 5**. The cup-plate agar diffusion methods were adopted with some minor modifications. The test organisms were: *Bacillus subtilis, Escherichia coli, Neisseria gonorrhoeae, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The crude extract of *Withania somnifera* showed moderate inhibition against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* and weak inhibition against *Neisseria gonorrhoeae* and *Bacillus subtilis.* Compound W_1 exhibited a moderately inhibition against all five organisms but less than their crude extract. Compound W_2 showed weak inhibition against all organisms. Compound W_3 exhibited high inhibition against all organisms accept *Pseudomonas aeruginosa* where it is low activity.

IV. CONCLUSION

A detailed phytochemical study of *Withania somnifera* leaves were carried out. From the methanolic extract of *Withania somnifera* a three flavonoids were isolated and purified using different chromatographic techniques. These compounds (W_1, W_2 and W_3) were identified via spectroscopic tools: IR, UV, ^{1}H NMR, and MS spectroscopy. The crude extract and pure compounds exhibited moderate activity against the human pathogens: *Bacillus subtilis, Escherichia coli, Neisseria gonorrhoeae, Pseudomonas aeruginosa* and *Staphylococcus aureus.*

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