Structural Elucidation and Antibacterial Activity Studies of Leaf Extracts of *Withania somnifera*

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Abstract: Withania somnifera (W. somnifera), *a small, woody shrub in the Solanaceae family, has been studied using a phytochemical test, antibacterial activity and partial characterizations. Air-dried and powdered leaves of the plant were extracted with maceration over an electrical shaker using the solvent chloroform and methanol. After crude extracts of the plant were concentrated, the diffusion antibacterial susceptibility test was carried out on 25, 50, 75, and 100 mg/mL of chloroform and methanol crude extract. The bacteria used were* S. aureus, S. pneumonia, E. coli, *and* S. typhi. *Each antibacterial activity test was carried out three times. The most active crude extract of the plant was subjected to a phytochemical test and fractionation with column chromatography. Chloroform and methanol extract of the plant inhibits all cultures of four bacteria. Both chloroform and methanol extract of* W. Somnifera *inhibits both gram-positive and negative bacterium with a comparable inhibition zone with the standard antibiotics, amoxicillin, gentamicin, and cefoxitine. In addition, it gives a maximum inhibition zone than that of amoxicillin, starting from 25 to 100 mg/mL. Methanol extract of* W. somnifera *contains phenolic, alkaloids, flavonoids, tannins, and phytosteroids. Partial characterization of pure fractions by using ^{1}H-NMR, ^{13}C-NMR, Dept-135 NMR, and IR spectroscopy, the compound WS-1 affords withaferin A. Withaferine A shows antibacterial activity with an inhibition zone of 11, 10.5, 11, and 9 mm against the bacterium* S. aureus, S. pneumoniae, E. coli, *and* S. typhi *respectively.*

Keywords: *antibacterial test; inhibition zone; partial characterization; withaferin A*

INTRODUCTION

Traditional medicine is health practices or approaches incorporating plant, animal, and mineral-based medicines [1]. Reports show that global acceptance and use of herbal medicines and related products continue to increase exponentially [2]. The widespread use of traditional medicine could be attributed to cultural acceptability, perceived efficacy against certain diseases, physical accessibility, and affordability compared to modern medicine. All this necessitates investigating the status of medicinal plant resources and the knowledge base associated with them [3-4].

Phytochemical progress has been aided enormously by developing rapid and accurate methods of screening plants for particular chemicals, and the emphasis is inevitably on chromatographic techniques. The continuous chemical technology improved isolation, structural characterization, and further synthesis of the pharmacologically active plant secondary metabolites [5].

Withania somnifera (*W. somnifera*) is a small, woody shrub in the *Solanaceae* family. It is an erect, evergreen shrub, 30–150 cm high [6]. *W. somnifera* has many applications, such as treating inflammation and fevers and protecting against infection or illness [7]. This plant extract is a potent immune-stimulator, antioxidant and anticarcinogenic [8]. *W. somnifera* is a popular herb used in traditional home medicine and remedies that have been practiced for thousands of years. Although trusted for its wide health benefits, the active principles of *W. somnifera* effects have not been understood to a large extent. Only recently, few studies on cell and animal models have demonstrated mechanisms of its anti-inflammatory, anti-cancer, anti-diabetic, anti-stress, antioxidant, neuroprotective, and immuno-modulatory potentials [9].

Ethanol extracts of *W. somnifera* demonstrated the highest antibacterial activity, followed by the aqueous extract. Acetone extract showed less antibacterial activity against most test bacterial pathogens but the highest activity against *S. aureus*. The activity of the extracts was comparable to that of standard antibiotic Gentamicin [10].

Previous phytochemical studies on *W. somnifera* comprised various chemical constituents such as withanolides, withanine, withananine, somniferinine, somniferiene, tropanol, scopoletin, and several sitoindosides are reported from aerial parts, roots, and berries of *W. somnifera* [11-12].

In Ethiopia, *W. somnifera* is used for the treatment of hepatic [13] and blackleg [14]. The plant is also efficient in treating snake bites [15]. The bark and leaves of the plant are used to treat the extended flow of menstruation/menometrorrhagia. The root and leaf part of the plant treats gallstone [16]. Therefore, the aim of this study was extraction, isolation, antibacterial activity study, and structural elucidation of chemical constituents from *W. somnifera* by using ^{1}H-NMR, ^{13}C-NMR, DEPT-135, and IR spectroscopic techniques.

■ EXPERIMENTAL SECTION

Study Area, Sample Collection, and Preparation

This study was conducted in North Wollo Zone Woldia town, about 525 km North of Addis Ababa. Taxonomic identification and giving the scientific name of the plant were made by botanical science experts in the Woldia University Biology department. The plant leaves were first carefully collected and washed with water to remove dirt and dust particles. Then it was allowed to dry in air at room temperature. The dried plant materials were then crushed into powder and ready for extraction.

Instrumentation

IR spectrum was recorded with KBr pellets using Perk-Elmer BX Infrared Spectrometer in the range of 4000–400 cm^{-1}. ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 spectra were recorded on Bruker advanced 400 MHz spectrometers with TMS as the internal standard. Analytical TLC was conducted on 0.2 mm thick aluminum-coated TLC plates. Detection was done by using 254 nm and 365 nm UV light. Column chromatography was carried out using silica gel 60 (Merck).

Procedure

Method of extraction

Three hundred and fifty grams of the powdered plant material was extracted with hexane and then by chloroform. The marc was then extracted with methanol for 72 h. Both chloroform and methanol extract were then filtered with Whatman filter paper and evaporated using a Rotary Evaporator. Then different concentrations (25, 50, 75, and 100 mg/mL) of chloroform and methanol crude extracts were prepared using serial dilution for an antibacterial susceptibility test.

Disc diffusion antibacterial susceptibility test

All Petri dishes were washed and autoclaved at 121 °C for 15 min and the hot Petri dishes were allowed to cool under the refrigerator (Laminar air flow) before any test. All bacteria strains brought from the Ethiopian health and research institute were sub-cultured on nutrient broth and incubated for 48 h. Then, the cultured bacteria were transferred to nutrient agar in slant form, incubated for 24 h, and preserved in a refrigerator until the plant extract was ready for the test. From the preserved bacteria, one or two colonies of the bacteria were taken with the help of a loop and immersed

in 4–6 mL screw-capped vials, which contain sterilized nutrient broth, and incubated for 24 h, totally called test suspension. The test suspension turbidity was compared with the standard 0.5 McFarland solution by shaking both test suspension and McFarland solution differently on the electrical shaker and checking the turbidity difference until the turbidity became the same. After confirming the required turbidity of the test suspension against McFarland 0.5 turbidity, the test suspension was brushed on Muller Hinton agar three times by using a different and sterile (none toxic) swab after dipping the swab into the standardized suspension of bacteria and excess fluid was expressed by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab was streaked in three directions and continuously brushed over the Muller Hinton Agar or by rotating the plate for complete coverage of the agar surface.

After inoculation or injection of test suspension on the Muller Hinton Agar, the inoculated plates were allowed to stand for 3–5 min but no longer than 15 min. The readily prepared sterile paper discs were loaded with different concentrations of the plant extract at; 25, 50, 75, and 100 mg/mL separately. The above concentrations were made with serial dilution from methanol and chloroform extract of *W. somnifera*. Unlike methanol extract, the chloroform extract was not dissolved in distilled water; hence chloroform was used to prepare the above concentrations for chloroform extract of the plants. Chloroform by itself has antimicrobial activity. So, to remove or decrease the efficacy of the chloroform, the chloroform was removed by exposing the readily loaded disc to the atmosphere for 4 min. Hence, the chloroform was evaporated and removed since the chloroform was volatile, and to increase the diffusion rate of the extract, and sterile distilled water was sprayed on readily loaded discs. In addition, two free paper discs were loaded on the medium as a negative control in which both discs were immersed in the chloroform, and one was dried for 4 min, whereas the other was not. Finally, the diffuse paper discs were placed suitably on the medium and then transferred to an incubator at 37 °C for 24 h. Similarly, the standard antibiotic Cefoxitin for the gram-negative bacterium (*E. coli* and *S. typhi*), amoxicillin, and gentamicin for the gram-positive bacterium (*S. aureus* and *S. pneumonia*) were placed and used as a positive control. The antibacterial activity was recorded by measuring the width of the clear inhibition zones around the discs using a zone reader (mm) called digital Caliber [17].

Phytochemical test and fractionation

After the crude extract's antibacterial efficacy was confirmed, identifying the secondary metabolites was studied through the phytochemical test. Phytochemical analysis for major constituents of the plant extract was done using standard qualitative methods as described by various authors [18-20].

Detection of alkaloids. Extracts were dissolved individually in dilute hydrochloric acid (HCl) and filtered. The filtrates were used to test for the presence of alkaloids as follows; (1) Mayer's test; filtrates were treated with Mayer's reagent (potassium mercuric iodide). The formation of yellow-colored precipitate indicated the presence of alkaloids. (2) Dragendorff's test; Filtrates were treated with Dragendorff's reagent (solution of potassium bismuth iodide). The formation of orange-yellow precipitate indicated the presence of alkaloids.

Detection of saponins. Foam test; a small amount of extract was shaken with little quantity of water. If foam produced persists for 1 min, it indicates the presence of saponins.

Detection of phytosterols. Extracts were treated with chloroform and filtered. The filtrates were treated with a few drops of concentrated sulfuric acid (H_2SO_4), shaken, and allowed to stand. The appearance of golden yellow color indicated the presence of triterpenoids.

Detection of phenols. A Ferric chloride ($FeCl_3$) test was used to detect phenols. Extracts were treated with a few drops of ferric chloride solution. The formation of bluish black color indicated the presence of phenols.

Detection of flavonoids. To the alcoholic solution of extracts, a few fragments of magnesium ribbon and concentrated HCl was added. The appearance of a magenta color after a few mins indicates the presence of flavonoids.

Detection of terpenoids. Terpenoids were detected by copper acetate test. Extracts were dissolved in water

and treated with a few drops of copper acetate solution. The formation of an emerald green color indicated the presence of terpenoids.

Test for tannins. The presence of tannins was tested by diluting 1 mL of the active solvent extract with distilled water and then adding a few drops of 5% ferric chloride ($FeCl_3$) solution. Blue-black/dark precipitate indicates the presence of tannins.

After the phytochemical test, compounds were isolated by column chromatography over silica, and the purity of each fraction was checked by thin layer chromatography (TLC).

Fractionation with column chromatography (CC)

Initially, the slurry was prepared by mixing silica gel with hexane. The column was then packed with the slurry and allowed to stand until a clear or bubble-free pack of columns was obtained. The crude extract is then mixed with hexane till the mixture becomes very viscous or slurry. This adsorbed sample was then applied to the top of column chromatography.

As described below in Table 1, elution was done with an appropriate solvent ratio (hexane/chloroform, chloroform/ethyl acetate, ethyl acetate/methanol) by varying the polarity starting from a less polar solvent and progressing to a highly polar solvent to get fractions.

The purity of each fraction was monitored by using analytical TLC plates. Antibacterial susceptibility tests for the selected fractions followed the same procedure for the crude extract. Finally, the pure fractions which showed antibacterial activity were subjected to structural elucidation.

RESULTS AND DISCUSSION

Extraction Yield

The crude extract yield and percent yield obtained from 72 h chloroform and methanol solvent extraction of air dried and coarsely crushed leaves of *W. somnifera* were 98 g (28%) and 112 g (32%), respectively. The color of chloroform and methanol extract *W. somnifera* leaves was dark green.

Antibacterial Activity

The antibacterial activity was conducted on four bacterial species (*S. aureus*, *S. pneumonia*, *S. typhi*, and *E. coli*) over four concentrations of *W. somnifera* crude extract. Chloroform crude extract inhibited each of the four bacteria (Table 2). *S. pneumonia* was the most sensitive bacterium to the chloroform extracts of *W. somnifera* with a zone of inhibition of 18 mm, and the least sensitive bacterium to the same extract was *S. typhi*

Table 1. Solvent ratio used for column chromatography

Fraction	Solvent	Ratio	Fraction	Solvent	Ratio
1	Hexane/$CHCl_3$	10:0	17	$CHCl_3$/EtOAc	4:6
2	Hexane/$CHCl_3$	9:1	18	$CHCl_3$/EtOAc	3:7
3	Hexane/$CHCl_3$	8:2	19	$CHCl_3$/EtOAc	2:8
4	Hexane/$CHCl_3$	7:3	20	$CHCl_3$/EtOAc	1:9
5	Hexane/$CHCl_3$	6:4	21	EtOAc/MeOH	10:0
6	Hexane/$CHCl_3$	5:5	22	EtOAc/MeOH	9:1
7	Hexane/$CHCl_{3r}$	4:6	23	EtOAc/MeOH	8:2
8	Hexane/$CHCl_3$	3:7	24	EtOAc/MeOH	7:3
9	Hexane/$CHCl_3$	2:8	25	EtOAc/MeOH	6:4
10	Hexane/$CHCl_3$	1:9	26	EtOAc/MeOH	5:5
11	$CHCl_3$/EtOAc	10:0	27	EtOAc/MeOH	4:6
12	$CHCl_3$/EtOAc	9:1	28	EtOAc/MeOH	3:7
13	$CHCl_3$/EtOAc	8:2	29	EtOAc/MeOH	2:8
14	$CHCl_3$/EtOAc	7:3	30	EtOAc/MeOH	1:9
15	$CHCl_3$/EtOAc	6:4	31	EtOAc/MeOH	0:10
16	$CHCl_3$/EtOAc	5:5			

Table 2. *W. somnifera* chloroform extract antibacterial inhibition zone against *the four bacteria*

Parameters		*W. somnifera* chloroform extract				Positive control		Negative control	
Concentration (mg/mL)		25	50	75	100	AM, 30 mg	CN, 30 mg	Im	nI
Inhibition zone (mm) mean ± SD	*S. aureus*	12	12	11.6	13	10.48	18.57	8.4	-
	S. pneumonia	12	15	17	18	10.48	18.57	8	-
		25	50	75	100	cN, 30 mg			
	E. coli	10.6	10.3	15.3	15.6	24		8.5	-
	S. typhi	7.6	7.6	7.6	6.6	24		7	-

Im = paper disc immersed in chloroform, nI = immersed and then dried, SD = standard deviation, AM = amoxicillin, CN = gentamicin, cN = cefoxitin mg = milligram

with the zone of inhibition of 6.6 mm at the same 100 mg/mL concentration. *S. pneumonia*, *E. coli*, *and S. aureus* with an inhibition zone of 18, 15.6, and 13 mm, respectively, show more inhibition zone than standard antibiotics amoxicillin at 100 mg/mL of crude extract.

Methanol crude extract inhibited every four bacteria (Table 3). *E. coli* was the most sensitive bacterium to the methanol extracts of *W. somnifera,* with a zone of inhibition of 23 mm. The least sensitive bacterium to the same extract was *S. aureus,* with a zone of inhibition of 13 mm at the same 100 mg/mL concentration. All bacteria show more inhibition zone than standard antibiotics amoxicillin at 100 mg/mL concentration of crude methanol extract. In general, comparing methanol crude extract of *W. somnifera* with the standard Gram-positive antibiotics, the crude extract at 100 mg/mL was more active than amoxicillin and comparably active with gentamicin. Similarly, it was comparably active to gram-negative antibiotic cefoxitin.

As the antibacterial susceptibility test result indicated, both chloroform and methanol crude extract of *W. somnifera* inhibited all four bacteria. This indicates the plant *W. somnifera* has good antibacterial activity.

Phytochemical Test Result

The phytochemical constituent of the methanol extract of the leaves was investigated, and the obtained secondary metabolites are shown in Table 4, in which the "+" sign indicates the presence and the "–" sign indicates the absence of the respective natural product.

After identification of the secondary metabolite constituent of the crude extract of *W. somnifera*, the methanol extract of the plant leaves was subjected to fractionation with column chromatography.

From column chromatography 31 (F1-F31), fractions were collected. From these fractions, three fractions were grouped as fraction 1 (f^1(F13-F15 or WS-1) was used for further study since they give relatively the same Rf (retention factor) value. In addition, fraction 2 (f^2(F16) shows one spot on the TLC test. These two fractions are then individually concentrated using a rotary evaporator, and all are found to be yellowish oily. Then, they were subjected to the antibacterial test, without concentration preparation due to a shortage of load, simply to check whether they had an antibacterial activity or not. Hence, the first fraction showed better antibacterial activity against the four bacteria species than the second

Table 3. *W. somnifera* methanol extract antibacterial inhibition zone result against *the four bacteria*

Parameters		*W. somnifera* methanol extract				Positive control	
Concentration (mg/mL)		25	50	75	100	AM, 30 mg	CN, 30 mg
Inhibition zone (mm) mean ± SD	*S. aureus*	8	11	6	13	10.48	18.57
	S. pneumonia	11	11	16	14	10.48	18.57
		25	50	75	100	cN, 30 mg	
	E. coli	17	18	19	23	24	
	S. typhi	14	16	17	19	24	

Im = paper disc immersed in chloroform, nI = immersed and then dried, SD = standard deviation, AM = amoxicillin, CN = gentamicin, cN = cefoxitin mg = milligram

Table 4. Phytochemical screening for different crude extracts of *W. somnifera*

No. of sample	Natural products	Phytochemical tests	Methanol
1	Alkaloid	Dragendorff test	+
		Mayer reagent test	+
2	Phenolics	Ferric Chloride test	+
3	Tannins	Ferric Chloride test	+
4	Flavonoids	Shinoda test	+
5	Terpenoids	Noller's test	-
6	Saponins	Foam test	-
7	Phytosteroids	Salkowski test	+

Table 5. Fraction antibacterial susceptibility test result

Inhibition zone (mm)	Fraction 1 (f^1)	Fraction 2 (f^2)
S. aureus	11	-
S. *pneumonia*	10.5	-
E. coli	11	8
S. *typhi*	9	7

fraction, which was only active against *E. coli* and *S. typhi* (Table 5).

Antibacterial activities of the two fractions show a comparable less active than the crude extracts. This might be due to the loss of the synergetic effect. In addition, the antibacterial activity of fraction two (2) was less active than the standard antibiotics, but fraction one (1) shows relatively comparable activity with amoxicillin.

Partial Characterization of WS-1

Characterization of the compound (WS-1) was done using spectroscopic techniques. The IR (KBr) spectrum of the compound (WS-1) exhibited a characteristic absorption board band at 3436 cm^{-1}, which shows that the compound has a hydroxyl functional group. The C-H stretching was observed at 3011 cm^{-1}. This peak confirms the presence of the olefin group. The methyl C-H stretching in this compound is confirmed by the presence of a sharp peak at 2926 cm^{-1}. A sharp peak at 1740 cm^{-1} is due to a carbonyl group of the ester functional group. The presence of the ketone functional group was confirmed by a sharp peak around 1700 cm^{-1}. A weak band at 1655 cm^{-1} showed the presence of alkene C=C stretch. The peak at 1453 cm^{-1} indicates the presence of α, β-unsaturated groups in the compound. The presence of absorption bands between 1300–1000 cm^{-1} illustrated C-O stretches of the ester functional groups. The disappearance of a peak at 720 cm^{-1} confirms that the structure lacks a long chain. Therefore, the IR spectrum depicts the presence of hydroxyl functionality, ketone group, an ester group, and a methyl group attached to a quaternary carbon in the compound isolated (Fig. 1).

The ^{1}H-NMR spectrum of WS-1 featured chemical shift values at δ 0.9–0.83, 0.96, 1.2, and 1.6 due to the tertiary methyl protons (Table 6). A peak from δ 5.42–5.30 was assigned to vinylic protons at H-2 and H-3, respectively. A peak at δ 2.31 was due to H-4. An overlapped broad peak at δ 2.0 is due to the hydroxyl proton attached at C-4 and C-28. The C-22 methine proton appeared at δ 4.3. Another peak at 4.15 is due to the methine proton at C-4 (Fig. 2).

The ^{13}C spectrum of a compound isolated from *W. sominifera* leaves (WS-1) (Table 6) also strengthens the fact that the chemical shift region around δ 203 is due to the presence of a ketone carbon, while the peak at δ 173.10 is for the ester carbon. The peaks from δ 120–140

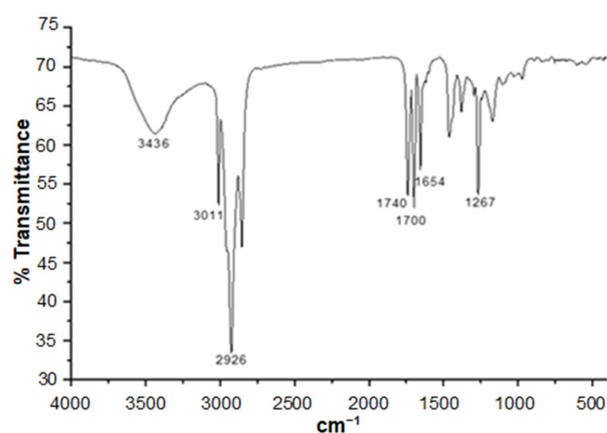

Fig 1. IR spectrum of WS-1

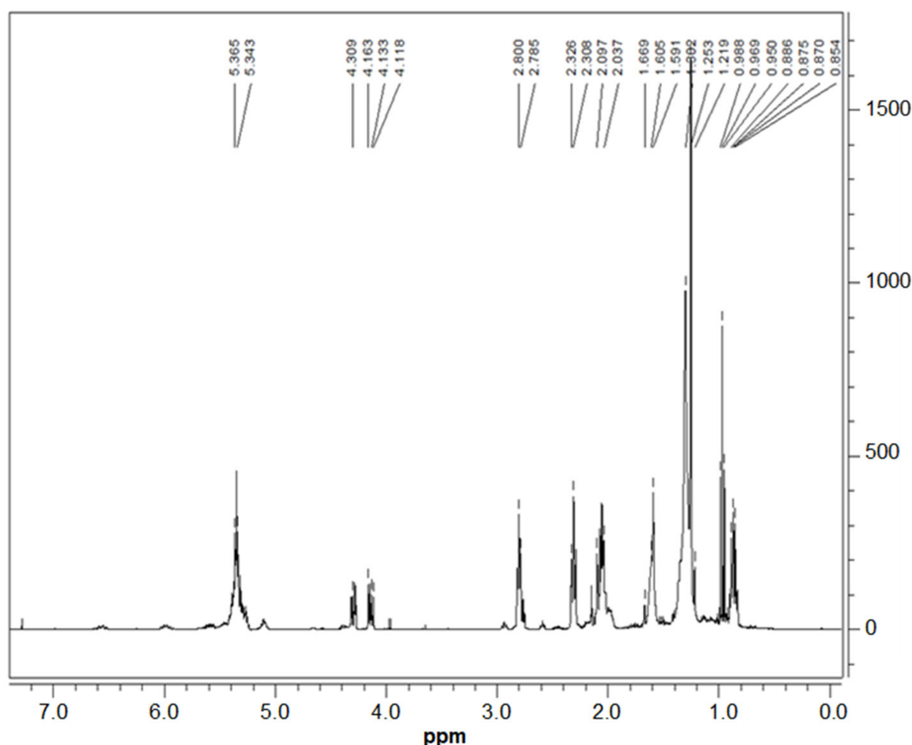

Fig 2. ^{1}H-NMR spectrum of WS-1

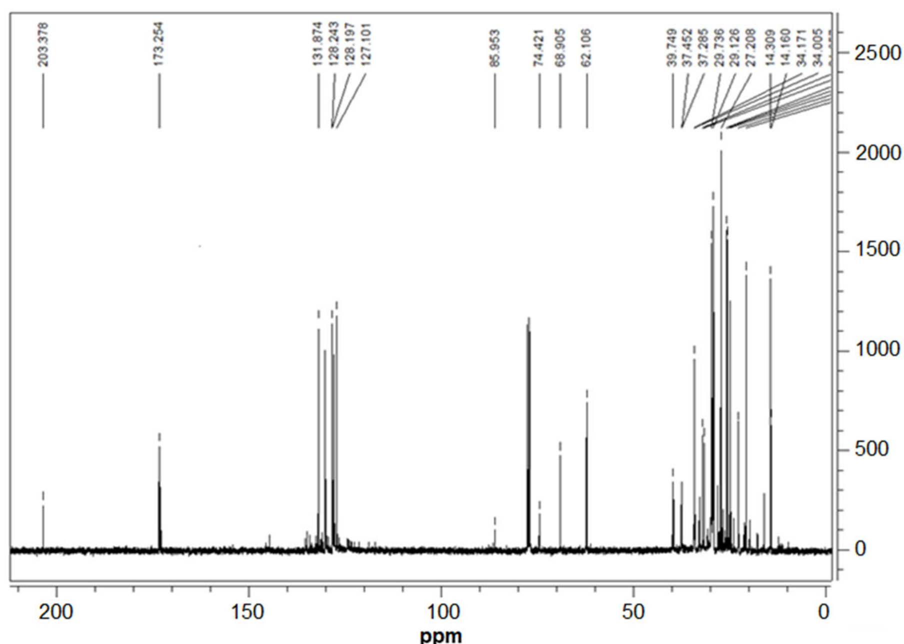

Fig 3. ^{13}C-NMR spectrum of WS-1

are for the olefinic carbons, from δ 90–60 are for the carbons connected with electron-withdrawing groups, and the others from δ 12–40 are for the aliphatic group. The ^{13}C-NMR spectrum showed the presence of twenty-eight carbons (Fig. 3).

The DEPT-135 spectrum showed 21 peaks corresponding to the methyl, methylene and methine carbons. The suggested compound (WS-1) has four methyl groups at δ 14.11, 14.31, 16.02, and 20.56. There are seven methylene groups at δ 22.72, 24.83, 25.61, 29.01,

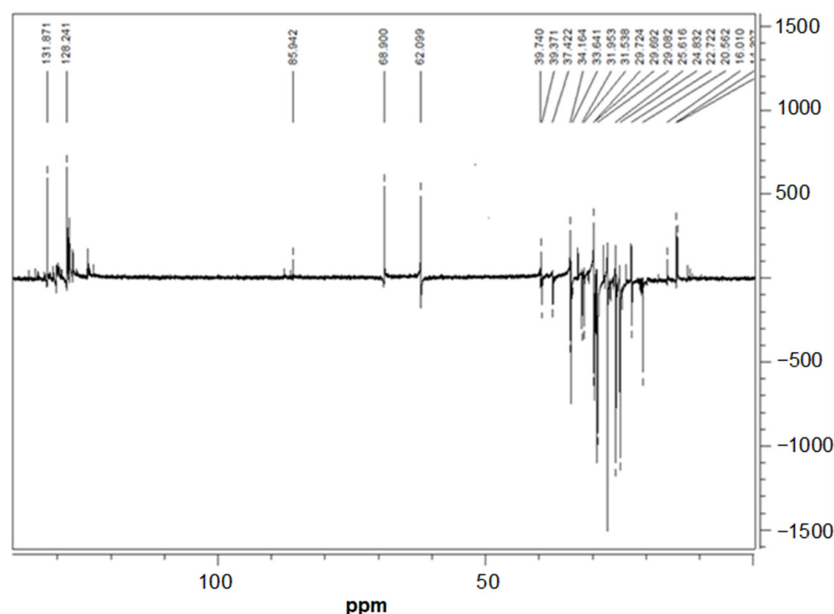

Fig 4. DEPT-135 spectrum of WS-1

Table 6. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral data for WS-1 and comparison with reported compound

Carbon number	^{1}H-NMR Chemical shift [δ] value of WS-1	^{1}H-NMR Chemical shift [δ] value of reported compound	^{13}C-NMR Chemical shift [δ] value of WS-1	^{13}C-NMR Chemical shift [δ] value of reported compound	DEPT-135 Multiplicity
1	--	--	203.37	204.2	---
2	5.3	5.89	128.24	127.5	-=CH-
3	5.4	6.57	131.87	148.6	=CH-
4	4.16	4.9	68.9	67.3	-CH-
5	--	--	62.11	81.3	Quaternary C
6	2.8	3.16	62.01	52.7	-CH-
7	2.13	2.15	29.74	38.5	$-CH_2-$
8	1.28	1.66	29.69	36.6	-CH-
9	2.03	1.39	34.17	47.0	-CH-
10	--	--	37.28	59.2	Quaternary C
11	1.67	1.81	22.72	28.5	$-CH_2-$
12	1.63	1.95	25.61	40.3	$-CH_2-$
13	--	--	34.11	44.3	Quaternary C
14	1.3	1.18	37.45	56.3	-CH-
15	1.68	1.70	24.83	25.2	$-CH_2-$
16	1.65	1.38	31.95	24.2	$-CH_2-$
17	1.35	1.23	39.74	53.1	-CH-
18	0.96	0.75	14.11	12.3	$-CH_3$
19	1.2	1.25	16.02	10.3	$-CH_3$
20	2.3	1.94	31.54	40.3	-CH-
21	0.83-0.9	0.99	14.31	13.6	$-CH_3$
22	4.13	4.44	85.95	80.1	-CH-
23	2.8	2.52	29.01	30.8	$-CH_2-$
24	--	--	127.1	157.8	Quaternary C
25	--	--	128.12	126.4	Quaternary C
26	--	--	173.25	168.5	--
27	4.3	4.34	39.37	56.5	$-CH_2-$
28	1.6	2.09	20.56	20.2	$-CH_3$

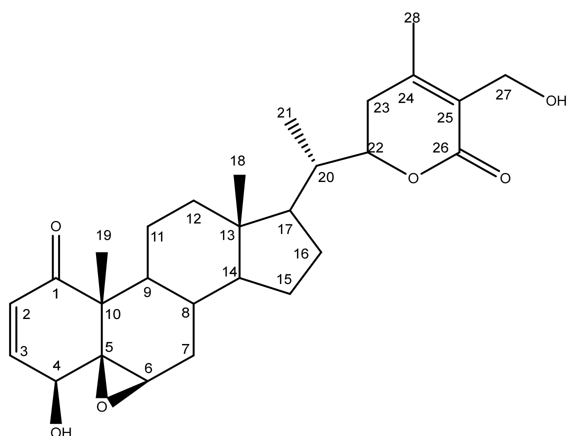

Fig 5. Proposed chemical structure of WS-1

29.74, 31.95, and 39.37. There are 10 methine groups, 2 in the olefinic region at δ 128.24 and 131.87 and 5 in the aliphatic region at δ 29.69, 31.54, 34.17, 37.45 and 39.74, respectively. The rest methine groups are found at δ 62.01, 68.9, and 85.95. So, the remaining seven carbons must be quaternary carbons. Therefore, the compound has seven quaternary carbon atoms at δ 34.11, 34.17, 62.11, 127.1, 128.12, 173.10, and 203.37 (Fig. 4 and Table 6).

Based on the spectra data from ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 and the information obtained from literature, the compound is a withaferine A [21-23] (Fig. 5).

■ CONCLUSION

Methanol and chloroform extract of *W. somnifera* shows relatively good antibacterial activity against *E. coli*, *S. aureus*, *S. pneumonia*, and *S. typhi*. Methanol leaf extract of *W. somnifera* contains secondary metabolites, and the isolation of this compound with chromatographic techniques affords withaferine A. Furthermore, the structure of the compound withanolide was determined using spectral data from IR, ^{1}H-NMR ^{13}C-NMR, Dept-135, and data from reported compounds.

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