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Research Paper

Chemical and biological study of *Withania somnifera* (L.) dunal leaves growing in Upper Egypt: Beni-Suef region

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ABSTRACT

As a part of our research programme of investigating some medicinal plants cultivated in Beni-Suef Governorate (Egypt) as anticancer agents. Bioassay-guided fractionation study using brine shrimp lethality and Ehrlich Ascites carcinoma assays revealed that the PE extract (PE) of *Withania somnifera* (L.) Dunal leaves retained most cytotoxic activity among other extracts. The total phenolics, flavonoids and tannins content of the total alcoholic extract were determined. The contents of PE of the fatty acids (FA) and the unsaponifiable matter (USM) were determined using GC/MS. The results showed that total phenolic, flavonoids and tannins content were 365mg, 2.6mg and 38.9mg/1g dry weight respectively. GC/MS analysis revealed presence of 38 and 25 compounds in FA and USM, respectively. In this study, we report the isolation and identification of a new fatty alcohol: "3, 7, 11, 16 tetramethyl-heptadecane-2-en-1ol" using HR-ESI-MS, 1D and 2D NMR spectroscopic data, along with four known sterols: lupeol, stigmasterol, *β*-sitosterol and cholesterol from USM by chromatographic techniques. The cytotoxicity of the new fatty alcohol was further studied using K562 cell line and Lactate Dehydrogenase Assay (LDH). Results showed 52% inhibition of K562 cells at a concentration of 0.033µM and IC_{50} at 64.2µM. It could be concluded that *Withania somnifera* leaves could serve as an anticancer agent and an adjuvant therapy in resistance to chemotherapy.

Key words: *Withania somnifera;* GC/MS; Fatty alcohol; Cytotoxicity.

INTRODUCTION

Chemoprevention by plant-derived compounds has emerged as an accessible and promising approach to cancer control and management (Badria et al., 1994; 2007). Many phytochemicals displaying a wide array of biochemical and pharmacologic activities were recently reported to be effective in the suppression of transformation, hyperproliferation, and inflammatory processes that initiate and promote carcinogenesis, as well as angiogenesis and metastasis (Alarif et al., 2013a; b). Therefore, the current study aims to evaluate the potential anticancer activity of

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Withania somnifera (L.) Dunal (synonym*: Physalis somnifera* L.) a perennial shrub, belonging to family Solanaceae. It is also known as Ashwagandha, Indian ginseng and Winter cherry. It is widely used in different parts of the world for all age groups of patients without any side effects even during pregnancy (Gupta and Rana, 2007; Mishra et al., 2000; Sharma and Karandikar, 1986). The extracts as well as different isolated bioactive constituents of *W. somnifera* have been reported to possess adaptogenic, general tonic, cytotoxic, anti-convulsant, immunomodulatory, and antioxidant activity (Mishra et al., 2000). It was used traditionally for its neurological effects as its root was used as sedative (Bhattacharya et al., 2000). The leaf found to have regenerative properties on brain cell synapses in mice and human cell line *in vitro* due to its withanolides content (Kuboyama et al., 2005; Tohda et al., 2005). The plant is efficient in the treatment of arthritis, geriatric, behavioral and stress related problems (Gupta and Rana, 2007; Mishra et al., 2000; Dhuley, 2001; Kaur et al., 2001; Mirjalili et al., 2009). 1, 4-Dioxane and ergosterol derivatives, octacosane, stigmasterone, stigmasterol and sitostanone were reported as isolated compounds from the PE fraction of *Withania somnifera* root (Misra et al., 2012; Chatterjee et al., 2010). Palmitic, oleic, linoleic and linolenic acids were previously reported from leaf and root. Porphyrines, carotenoids, pheophytin, campesterol and *β*-sitosterol were also reported from leaf (Chatterjee et al., 2010).

MATERIALS AND METHODS

***General Experimental Procedures*:** High resolution mass spectra (HRMS) were measured using a Bruker BioApex spectrometer. 1D, 2D NMR spectra were recorded on a Varian AS 400 MHz spectrometer and UV-visible spectrophotometer on Shimadzu UV- 1650 PC. GC/MS analysis was carried out on a Thermo Fisher Scientific gas chromatography equipped with a Thermo TR-capillary column (polysiloxane column coated with 50% methyl and 50% phenyl groups) (5ms) and mass detector Trace DSQ mass spectrometer which was operated in DSQ mode. Helium was carrier gas at a flow rate of 1ml/min. The injector was operated at 200ºC and the oven temperature was programmed as follows; 50ºC for 1min. then gradually increased to 300ºC at 5min. (5°C /min). Identification of components was based on a comparison of their mass spectra with those of Wiley and NBS Libraries and those described by Adams as well as comparison of their retention indices with literature.

Plant Material: Fresh leaves of *Withania somnifera* (L.) Dunal were collected from Beni-Suef, Egypt in January, 2010, identified by Dr. Mohamed El-Gibali, Senior Botanist, Department of Plant Taxonomy, Faculty of Science, Cairo University. A voucher specimen (BUPD 27) is deposited in Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University.

Reagents: Silica gel (60-120 mesh, Merck) was used for column chromatography (CC). Precoated aluminum sheets of silica gel 60 F_{254}, 0.25 mm (Merck, Darmstadt, Germany) were used for TLC and for monitoring the fractions from CC. All solvents used were of analytical grade. *p*-anisaldehyde and 1% vanillin sulfuric were used as spraying reagents for detection of steroids and triterpenes.

Preparation of extracts: Dried leaves (2kg) were exhaustively extracted with 70% ethanol. The collected extract was dried and dissolved in the least amount of distilled water and successively fractionated using a separating funnel and solvents of increasing polarity: PE, methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol and water (H_2O).

Preparation of FA and USM from PE extractive: PE extractive of *Withania somnifera* leaves was saponified according to a published procedure (Finar, 1973).

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This was performed by refluxing 2g of the extractive with 20ml of alcoholic potassium hydroxide (10 % w/v) and 8ml benzene**.** The solvent was distilled off to dryness and the residue was dissolved in 40ml water. The resulted solution was then extracted with several portions of diethyl ether (5×50ml) till complete extraction of USM (0.6g). The aqueous alkaline mother liquors was acidified and extracted with diethyl ether to obtain FA (0.7g). About 0.5g of the fatty acid mixture was separately methylated by dissolving in 50ml methanol containing 2.5ml sulfuric acid for preparation of fatty acid methyl ester (FAME) which was then subjected to GC/MS analysis (Finar, 1973).

Extraction and isolation of components from USM: USM (0.6 g) was subjected to a silica gel CC (40 g), (50 x 3.8 cm). Stepwise gradient elution was carried out using *n*-hexane and EtOAC. Fractions of 10 ml each were collected and concentrated. Similar fractions monitored by TLC using *n*- hexane/EtOAc (7:3 v/v) and concentrated to dryness to afford 19 fractions. Fraction 11 was found as one spot by TLC examination and designated compound **1** (34 mg). Fraction 12 "38.5 mg" was subjected to a silica gel CC (15 g), (25 x 1cm). Stepwise gradient elution was carried out using *n*-hexane and acetone. Fractions of 10 ml each were collected and concentrated. Similar fractions were monitored by TLC using *n*-hexane/acetone (7:3 v/v) and concentrated to dryness. Sub-fraction 8 gives compound **2** (4mg), sub-fraction 9 gives compound **3** (7mg), sub-fraction 10 gives compound **4** (17 mg) and sub fraction 11 gives compound **5** (5mg). These isolated compounds were identified by ^{1}H- and ^{13}C-NMR and by co- TLC against authentic.

Total phenolics, flavonoids and tannins determination: Total phenolic contents (TPC) of *Withania somnifera* leaves was determined by Folin – Ciocalteu method. It was expressed as mg of Gallic acid equivalent (GAE)/1g dry weight. Total flavonoid contents (TFC) of *Withania somnifera* leaves was determined by Aluminum chloride method and the flavonoids content was expressed as mg Rutin equivalent (RE) /1g dry weight. Total Tannin Content **(**TTC) was determined by casein precipitation method. After the residual phenolic content was determined the filtrate using the Folin-Ciocalteu method, the difference between the two steps represents the TTC (El Zalabani et al., 2012; Silva et al., 2011).

The total phenolics, flavonoids and tannins content were determined for the first time in *Withania somnifera* (L.) Dunal leaves growing in Egypt. Results showed that the contents were 365mg, 2.573mg and 38.9mg /1g dry weight, respectively.

Biological study

Cell line and culture**:** Human chronic leukemia K562 cells were obtained from American Type Culture Collection, Rockville MD, USA. The MRC5 cells was purchased from VACSERA (Cairo, Egypt) and cultured in RPMI (Roswell Park Memorial Institute; Buffalo, New York, USA) medium supplemented with 100µg/ml penicillin-streptomycin, 2.5µg/ml fungizone, 10% heat-activated foetal calf serum, and 2 mM glutamine. Cells were allowed to grow at 37^{o}C in a humidified atmosphere of 5% CO_2 and 95% air to form a monolayer. At 60-70% confluence cells were subcultured; first they were washed with phosphate-buffered saline (PBS), then trypsinized with 3ml of 0.25% trypsin in 0.03% EDTA, then washed with fresh medium and seeded at 1x10^{4} cells/well in a 96-well microplate. The reagent kit for the assay of LDH was purchased from Biorex Diagnostics (Antrim, UK). Methotrexate, 5-flourourasil (5-FU), and Cisplatin were kindly offered by the Oncology Center, Mansoura University, Mansoura 35516 Egypt. Ehrlich Ascites female mice were purchased from national cancer institute at Cairo, Egypt. Brine shrimp eggs were obtained from Antoun- aquarium, Cairo, Egypt.

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Ehrlich ascites in vitro assay: Different concentrations of the tested samples were prepared (100, 50 and 25µg/ml DMSO). Ascites fluid from the peritoneal cavity of the donor animal (contains Ehrlich cells) was aseptically aspirated. The cells were grown partly floating and partly attached in a suspension culture RPMI 1640 medium, supplemented with 10% foetal bovine serum (FBS). They were maintained at 37ºC in a humidified atmosphere with 5% CO_2 for 2h. The viability of the cells used in control experiments (without the drug) exceeded 95% as determined by the microscopical examination using a hemocytometer and using trypan blue stain (stains only the dead cells) using 5-FU as control (Fujita et. al.,1988; Badria et al., 2007).

***Microwell cytotoxicity assay using Artemia salina (Brine shrimp)*:** A solution of sea water was prepared by dissolving 32.5g salt in IL distilled water. One mg of brine shrimp, *Artemia salina* (Leach), eggs was added in a hatching chamber (22x32cm) and kept under an inflorescent bulb for 48 h for the eggs to hatch into shrimp larvae (nauplii). Fifty milligrams of (tested extracts/fractions) or 1mg of pure sample were dissolved in 5ml of 70% methanol to prepare stock solution for both pure compounds and extract/fractions and several concentrations of each were tested in triplicates. Ten larvae (nauplii) of *Artemia salina* were transferred into each vial and the volume completed into 5ml with sea salt solution immediately after adding the nauplii, 24 h later, DMSO was used as control. The number of surviving shrimp at each concentration was counted, recorded and LD_{50} was calculated using ED50V10 Excel software (Meyer et al., 1982; Solis et al., 1993).

MTT assay on MRC5 cell line: Cytotoxicity was assessed by MTT assay according to the manufacturer´s recommendations (Roche Diagnostics GmbH, Mannheim, Germany). Experiments by three replicates, and data represented as the mean±standard deviation (SD). This assay relies on the ability of viable cells to reduce a yellow tetrazolium salt (MTT; Sigma) metabolically to a purple Formosan product. This reaction takes place when mitochondrial reductase enzymes are active. Cells were grown in 96-well plates (1×10^4/200µl/well). After incubation with the reagents, the medium was removed and the cells were treated with 20µl of MTT (5mg/ml) for 3h at 37˚C. Subsequently, 100µl of DMSO were added and the solubilized Formazan product was spectrophotometrically quantified by the aid of a microtiter plate reader, Power Wave XS (Bio Tek, Winooski, VT, USA), at 570nm (Hansen et. al.,1989).

% Viability = (Mean absorbance of sample/ Mean absorbance of control) × 100

IC_{50} was calculated using ED50V10 Excel software.

***Anti-leukemic cell cultures and cell growth inhibition assay*:** K562 cell line was grown in suspension culture at 37ºC in RPMI-1640 medium supplemented with 10% non-dialysed FBS, 2 mM L-glutamine, 100 units/ml of penicillin and 10µg/ml of streptomycin. For the cell growth inhibition assay, K562 cells were set up at $1x10^5$ cells /well in Costar 24-well plates. Cells were allowed to grow undisturbed for 24h before addition of "drugs". After 48h incubation with drugs at 37ºC, viable cell were counted using the trypan blue exclusion method and Taxol was used as standard (Ma et al., 2010).

Lactate dehydrogenase (LDH) inhibitory assay: MRC5 cells were treated with the isolated compounds at concentrations from 0–250µM. All chemicals were dissolved in RPMI medium and filtered through a membrane filter (0.2µm) before cell treatment. LDH activities were measured at 1, 24, 48, and 72h. Cytotoxicity was evaluated through monitoring the release of LDH into the medium. The supernatant (50µl) were drawn off from each cell culture well and assayed for LDH activity by

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measuring the absorbance at 340nm (Decker and Lohmann, 1988; Legrand et al., 1992; Hilf et al., 1976).

Statistical analysis: Data were presented as mean values ± SD. Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by posthoc Tukey's test (Statistica, StatSoft, USA). Value of $P<0.05$ was assumed as statistically significant.

RESULTS

The total 70% ethanol extract of *Withania somnifera* showed cytototoxicity against MRC5 and K562 cell lines with IC_{50}=71.7µg/ml and inhibition of 67.5% at a concentration of (10µg/ml) respectively Compared toTaxol (97.7%)

Results of Ehrlich Ascites *in vitro* assay on successive fractions, compared to the control 5-FU (IC_{50}= 2.82 ± 0.07 µg/ml), showed that PE and H_2O fractions showed the best activity (IC_{50}= 25 µg/ml for both) followed by CH_2Cl_2 and EtOAc with IC_{50}= 50 and 100 µg/ml respectively. *n*-Butanol fraction was totally inactive. Brine shrimp toxicity assay showed that PE was the most active fraction (IC_{50}= 40.9 µg/ml). Herein, USM and FAME of this fraction was chemically analyzed using GC/MS (Tables **1** and **2**).

Table- 1: GC/MS analysis of unsaponifiable matter (USM) of the petroleum ether extractive of *Withania somnifera* (L.) Dunal Leaf.

	R_t	Compound	Molecular weight	Formula	Base beak	Peak area %
1	5.39	1-Hexanol, 2-ethyl	130	$C_8H_{18}O$	57.1	0.26
2	14.62	n-Tetradecane	198	$C_{14}H_{30}$	57.1	2.26
3	16.13	2,4,6-Trimethyloctane	156	$C_{11}H_{24}$	43	0.58
4	19.29	Hexadecane "n-Cetane"	226	$C_{16}H_{34}$	57.1	2.00
5	20.37	3,6-Dimethylundecane"	184	$C_{13}H_{28}$	57.1	0.80
6	21.47	n-Nonadecane	268	$C_{19}H_{40}$	57.1	4.94
7	24.45	Hexahydrofarnesyl acetone "2-Pentadecanone, 6,10,14-trimethyl"	268	$C_{18}H_{36}O$	43.1	0.61
8	24. 67	2-Methylhexadecan-1-ol	256	$C_{17}H_{36}O$	43.1	0.33
9	25.51	Heneicosane	296	$C_{21}H_{44}$	57.1	4.15
10	29.55	à-N-Normethadol	297	$C_{20}H_{27}NO$	58.1	24.34*
11	29.95	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	268	$C_{16}H_{28}O_3$	84.1	0.78
12	30.93	Eicosane	282	$C_{20}H_{42}$	57.1	2.59
13	35.72	2-Methyloctadecane	268	$C_{19}H_{40}$	57.1	1.51
14	36. 48	"Diisooctyl phthalate"	390	$C_{24}H_{38}O_4$	149	4.28
15	41.37	n-Tetracosane	338	$C_{24}H_{50}$	57.1	11.27*
16	43.45	n-Heptacosane	380	$C_{27}H_{56}$,	57.1	1.34
17	44.42	4-Methylcholesta-8,24-dien-3-ol	398	$C_{28}H_{46}O$	55.1	1.27
18	45.26	Campesterol "Ergost-5-en-3-ol, (3á,24R)"	400	$C_{28}H_{48}O$	43.1	8.72*
19	45.65	Stigmasterol "Stigmasta-5,22-dien-3-ol, (3á,22E)-"	412	$C_{29}H_{48}O$	55.1	2.15
20	45.89	Sulfurous pentadecyl acetate	376	$C_{21}H_{44}O_3S$	57.1	1.19
21	46.34	n-Octadecyl chloride	288	$C_{18}H_{37}Cl$	57.1	3.77
22	46.61	Stigmasta-5,24(28)-dien-3-ol, (3á,24Z)-	412	$C_{29}H_{48}O$	314	12.27*
23	47.16	Cholestan-3-ol, 2-methylene-, (3á,5à)-	400	$C_{28}H_{48}O$	69.1	0.74
24	47.59	"Corynan-17-ol, 18,19-didehydro-10-methoxy"	326	$C_{20}H_{26}N_2O_2$	44	2.58
25	48.13	1-Heptatriacotanol	536	$C_{37}H_{76}O$	43.1	1.16
% total identified compounds					**96.38**	
% total identified sterols					**20.31**	
% total identified hydrocarbons					**70.07**	

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Table- 2: GC/MS analysis of fatty acid methyl ester (FAME) portions of the petroleum ether extractive of *Withania somnifera* Leaf.

	R_t	Compound	Molecular weight	Formula	Base beak	Peak area %
1	4.01	10-Undecenoic acid, methyl ester	198	$C_{12}H_{22}O_2$	55.1	0.01
2	4.37	1-Hexadecanol, 2-methyl-	256	$C_{17}H_{36}O$	43.1	0.05
3	4.58	Nitro-L-arginine	219	$C_6H_{13}N_5O_4$	44	0.02
4	4.86	Methyl 5-(2-undecylcyclopropyl) pentanoate	310	$C_{20}H_{38}O_2$	41.1	0.35
5	5.30	Methyl tetradecanoate	242	$C_{15}H_{30}O_2$	74	1.59
6	5.78	Undecanedioic acid, dimethyl ester	244	$C_{13}H_{24}O_4$	55.1	0.04
7	6.58	Tetradecanoic acid, 12-methyl-, methyl ester	256	$C_{16}H_{32}O_2$	74.1	0.09
8	6.84	Dodecanedioic acid, dimethyl ester	258	$C_{14}H_{26}O_4$	55.1	0.33
9	7.15	Pentadecanoic acid, methyl ester	256	$C_{16}H_{32}O_2$	74.1	0.83
10	7.50	2-Pentadecanone, 6,10,14-trimethyl- "Hexahydrofarnesyl acetone"	268	$C_{18}H_{36}O$	43.1	0.06
11	7.98	"Octadecanoic acid, 9,10-epoxy-, cis-"	298	$C_{18}H_{34}O_3$	41.1	0.07
12	9.27	N-Acetylglycine	117	$C_4H_7NO_3$	74	22.94*
13	10.03	2-Hexadecenoic acid, methyl ester, (E)	268	$C_{17}H_{32}O_2$	87.1	2.86
14	10.38	"Lauric acid, ethyl ester"	228	$C_{14}H_{28}O_2$	88.1	1.11
15	10.93	"Margaric acid methyl ester"	284	$C_{18}H_{36}O_2$	74.1	0.80
16	11.28	Cyclopropanedodecanoic acid, 2-octyl-methyl ester	366	$C_{24}H_{46}O_2$	44	0.07
17	11.59	16-Octadecenoic acid, methyl ester	296	$C_{19}H_{36}O_2$	41.1	0.03
18	12.50	3-Tetradecen-5-yne, (E)-	192	$C_{14}H_{24}$	79.1	39.44*
19	12.88	Ergoline-8-methanol, 10-methoxy-1,6-dimethyl-, (8á)	300	$C_{18}H_{24}N_2O_2$	270	7.01*
20	13.14	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- Linolenic acid, methyl ester	292	$C_{19}H_{32}O_2$	95.1	7.41*
21	13.48	9,12-Octadecadienoic acid, methyl ester, (E,E)- "Linolelaidic acid, methyl ester"	294	$C_{19}H_{34}O_2$	67.1	0.51
22	14.24	9-Octadecenoic acid (Z)-, methyl ester "Oleic acid, methyl ester"	296	$C_{19}H_{36}O_2$	55.1	0.12
23	16.18	Eicosanoic acid, methyl ester "Methyl arachisate"	326	$C_{21}H_{42}O_2$	67.1	3.48
24	16.51	4,8,12,16-Tetramethylheptadecan-4-olide"5-Methyl-5-(4,8,12-trimethyltridecyl) dihydro-2(3H)-furanone"	324	$C_{21}H_{40}O_2$	99.1	1.02
25	16.85	Methyl 4-methoxy-4,8,12,16-tetramethylheptadecanoate	370	$C_{23}H_{46}O_3$	145.1	0.29
26	17.18	Eicosanoic acid, ethyl ester	340	$C_{22}H_{44}O_2$	88.1	0.14
27	17.72	Heneicosanoic acid, methyl ester	340	$C_{22}H_{44}O_2$	74.1	2.25
28	18.00	11,13-Dihydroxy-tetradec-5-ynoic acid, methyl ester	270	$C_{15}H_{26}O_4$	43.1	0.45
29	18.69	Undecanoic acid, 11-bromo-methyl ester	278	$C_{12}H_{23}BrO_2$	74.1	0.13
30	19.29	Docosanoic acid, methyl ester "Behenic acid, methyl ester"	354	$C_{23}H_{46}O_2$,	74.1	2.67
31	20.20	Docosanoic acid, ethyl ester	368	$C_{24}H_{48}O_2$	88.1	0.15
32	20.73	Tricosanoic acid, methyl ester"Methyl tricosanoate"	368	$C_{24}H_{48}O_2$	74.1	0.59
33	21.12	2-Bromooctadecanal	346	$C_{18}H_{35}BrO$	43.1	0.02
34	25.27	9-Hexadecenoic acid	254	$C_{16}H_{30}O_2$	105.6	0.02
35	25.65	Cyclopropanedodecanoic acid,2-octyl-,methyl ester	366	$C_{24}H_{46}O_2$	43.1	0.06
36	26.55	Agaricic acid" 1,2,3-Nonadecanetricarboxylic acid, 2-hydroxy-"	416	$C_{22}H_{40}O7$	44	0.01
37	29.78	Triacontanoic acid, methyl ester "Methyl melissate"	466	$C_{31}H_{62}O_2$	74.1	0.49
38	31.35	Folic Acid	441	$C_{19}H_{19}N_7O_6$	44	0.05
% total identified compounds					**97.56**	

* Major compounds R_t: retention time

N-Normethadol (24.34%), Stigmasta-5, 24(28)-dien-3-ol (12.27%), n-Tetracosane (11.27 %) and Campesterol (8.72 %) were the major identified compounds in USM and 3-Tetradecen-5-yne (39.44%), N-acetylglycine (22.94%), linolenic acid methyl ester (7.41%) and ergoline-8-methanol, 1, 6-dimethyl-10-methoxy (7.01%) were the majors in FAME.

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Compound 1 yellow, oily residue (34 mg). It gives violet color with 10% p-anisaldehyde/H_2SO_4 reagent and positive reaction with Dragendorff's reagent. The molecular formula was assigned as $C_{21}H_{42}O$ on the basis of negative mode HR-ESI–MS: m/z = 327.27636 $[M+NH_4-H]^-$ (Calculated for $C_{21}H_{45}ON$ 327.28890), ^{1}H- NMR, ^{13}C-NMR and 2-DNMR (Table **3**) (Figures **1- 7**).

Table-3: NMR data of Compound 1 [3,7,11,16 tetramethyl-heptadecane-2-en-1ol] (400MH$_Z$ in pyridine).

Position	δ C	Multiplicity	δ H	HMBC
1	59.73	CH_2.OH	4.47(*d*, *J* = 8 Hz, 2H)	C2& C3
2	127.04	CH	5.76(*t*, 1H)	C4 &C21
3	136.55	Q		
4	40.96	CH_2	2.05 (*t*, 2H)	C2, C3, C5 &C21
5	26.31	CH_2		
6	37.75	CH_2		
7	33.78	CH		
8	38.34	CH_2		
9	25.90	CH_2		
10	38.46	CH_2		
11	33.86	CH		
12	38.51	CH_2	1.14 (*d*, *J* = 1.1 Hz, 2H)	C10&C13
13	25.59	CH_2		
14	30.82	CH_2	1.43(*m*, 2H),	C16
15	40.38	CH_2	1.13 (*d*, *J* = 1.1 Hz, 2H)	
16	29.00	CH	1.31(*d*, *J* = 4 Hz 1H)	
17	23.65	CH_3	0.90 (*s*, 3H)	
18	23.55	CH_3	0.90 (*s*, 3H)	C15, C16 & C17
19	20.68	CH_3	0.89(*s*, 3H)	C11&C12
20	20.73	CH_3	0.89(*s*, 3H)	
21	17.01	CH_3	1.68 (*s*, 3H)	C2,C3&C4

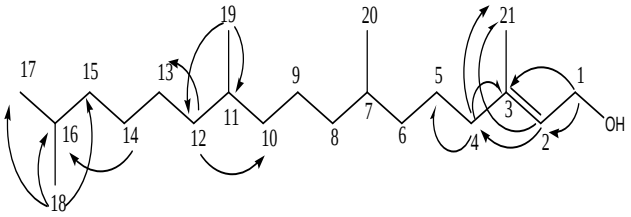

Fig. **1**: Compound **1**

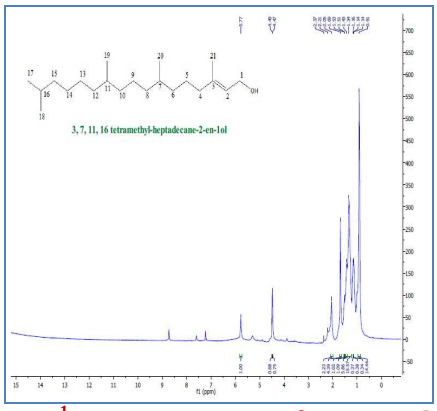

Fig.2: ^{1}H-NMR spectrum of compound 1

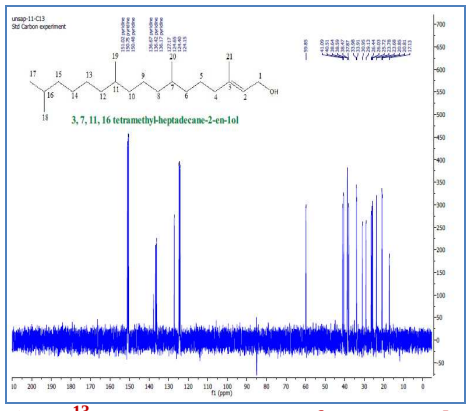

Fig.3: ^{13}C-NMR spectrum of compound 1

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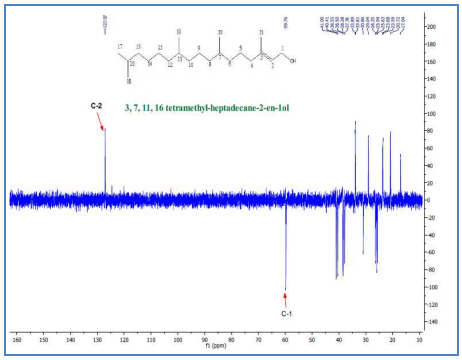

Fig.4: DEPT spectrum of compound 1

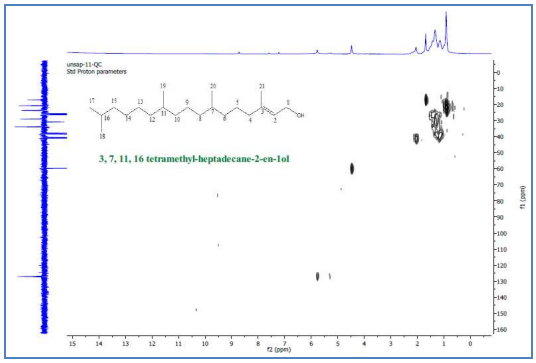

Fig.5: HMQC spectrum of compound 1

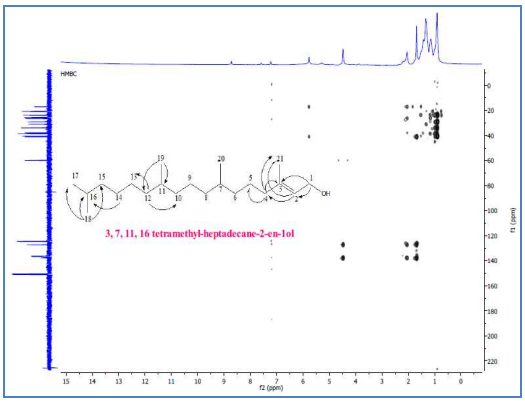

Fig.6: HMBC spectrum of compound 1

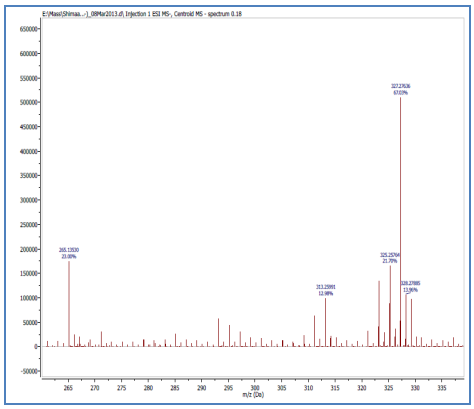

Fig.7: mass [M-H] spectrum of compound 1

Compound **1** was identified as **3, 7, 11, 16 tetramethyl-heptadecane-2-en-1ol**. This is the first report for this compound to be isolated from a natural source. This compound was tested against K562 cell line at a concentration of 0.33µM and results showed 52.5% inhibition. A mechanistic study was carried out by testing the compound as LDH inhibitor. It showed IC_{50} = 64.2 µM compared to 5-FU, Cisplatin and Methotrexate (**Table 4**).

Table- 4: IC_{50} [µM] of Compound 1 on MRC5 by LDH Assay, after 72h.

Compound	IC_{50} [µM][a]	Positive control	IC_{50} [µM][a]
		Methotrexate	0.087 ± 0.01
Compound **1**	64.2± 0.335	Cisplatin	61 ± 0.035
		5-FU	2.82 ± 0.07

[a]Data shown are the mean ± SD of three experiments. The means were significantly different across the samples.

Four compounds (**2**, **3**, **4** and **5**) have been also isolated from USM and their structures were confirmed by means of co-TLC with authentics and comparing their ^{1}H-NMR spectral data with those published as lupeol (Compound **2**) (Jamal, et al., 2008), stigmasterol (Compound **3**) (Jamal et al., 2008; Habib et al., 2007), *β*-sitosterol (Compound **4**) **(**Ghazala and Shameel, 2005; Muhit et al., 2010) and cholesterol (compound **5**) (Sabry, 2012). Lupeol and cholesterol were isolated for the first time from *Withania somnifera* (L.) Dunal leaves.

DISCUSSION

In a trial to correlates the bioactivity of *Withania somnifera* (L.) Dunal leaves growing in Egypt with its chemical contents, the antitumor activity of the total 70% EtOH

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extract, successive fractions and isolated compounds were evaluated. The chemical pattern of the total ethanol extract was evaluated by estimation of the total phenolics, flavonoids and tannins content. The results revealed that the major contents were the phenolics and tannins which could explain the activity of the water fraction. The total flavonoids content were found as minor in this plant. It was reported that flavonoids and flavonoid glycosides were isolated from EtOAc and *n*-butanol fractions respectively (Jayasinha et al., 1999).

This result is in agreement with the obtained data which showed weak activity with the EtOAc fraction while the *n*-butanol fraction was completely inactive. Based on the results of biological screening; PE fraction was chosen for further phytochemical study. Herein, it was chemically investigated for its FA and USM contents using GC/MS analysis. The FA portion contains about 40% saturated and 58 % unsaturated FA where it's major unsaturated FA was linolenic acid methyl ester (7.41%).

Generally essential fatty acids play a positive role as anticancer but contradictory some fatty acids may promote the tumor growth (Phoon et al., 2001, Razanamahefa et al., 2000). Exposure of tumor cells to polyunsaturated fatty acids (PUFA) induces apoptosis in these cells by augmenting free radical generation and lipid peroxidation. They also potentiate the cytotoxicity of anti-cancer drugs (Das, 2011). The GC/MS of the USM showed that it contains 20.31% steroids. The majors were stigmasta-5, 24(28)-dien-3-ol (12.27%) and campesterol. Some of the steroids and steroidal lactones isolated from *Withania somnifera* (L.) Dunal proved to be active as anticancer agents against several cell lines (Jayaprakasam et al., 2003; Singh et al., 2011). USM was further proceeded for isolation its bioactive constituents. Five compounds were isolated from the PE fraction and identified. Compound **1** was new and identified as a fatty alcohol. Its structure was elucidated using 1 and 2D NMR and HR-MS spectroscopic techniques, as presented in results. The anticancer activity of this compound was studied. It showed moderate activity against K562 cell line. A mechanistic study was carried out using LDH inhibitory assay and the compound showed comparable results to the standard Cisplatin. It was reported that lupeol (Compound **2**), was tested for its cytotoxic activity against different cancer cell lines (Breast, Cervix, Colon, Haematopoietic and Lymphoid tissue, Lung, Ovary and Skin cancer (Agarwal, 2012). The other three isolated compounds (compounds **3**, **4** and **5**) were sterols in nature. Stigmasterol (Compound **3**) was tested for its cytotoxic activity against Haematopoietic and Lymphoid tissue cancer (Agarwal, 2012). It was reported that *β*-sitosterol (Compound **4**) was also tested against several types of cancer cell lines to assess its cytotoxic activity against (Brain, Breast, Intestine, Lung, Nasopharyngeal, Ovary and Prostate) (Agarwal, 2012).

CONCLUSION

It could be concluded from the presented data that the isolated compounds from *Withania somnifera* (L.) Dunal leaves could serve as anticancer, complementary therapy and/or chemo-sensitizing agent against cancer resistant to chemotherapy.

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