*European Journal of Medicinal Plants*

***21(3): 1-12, 2017; Article no.EJMP.38239***
***ISSN: 2231-0894, NLM ID: 101583475***

# Egyptian *Withania somnifera* L., Chemotype and Comparative *in vitro* Cytotoxic Activity of Extracts and Isolated Withanolides

**Rasha A. Nassra[1], Rahma Sr. Mahrous[2], Hoda M. Fathy[2*], Rasha M. Abu El-Khair[2] and Abdallah A. Omar[2]**

*[1]Department of Medical Biochemistry, Faculty of Medicine, Alexandria University, 1 El-Khartoum Square – Azarita, Alexandria, Egypt.*
*[2]Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, 1 El-Khartoum Square – Azarita, Alexandria, Egypt.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Author RSM (MSc student) contributed in collecting plant sample. Author RAN contributed to cytotoxicity studies and statistical data analysis. Authors RSM, HMF and RMAEK participated in phytochemical procedures, interpretation of the isolated compounds and in performing the docking studies and synergy analysis. Author AAO designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.*

***Article Information***

DOI: 10.9734/EJMP/2017/38239
Editor(s):
(1) Elena Maria Varoni, Dipartimento di Scienze Biomediche, Chirurgiche ed Odontoiatriche, University of Milan, Italy.
(2) Marcello Iriti, Professor, Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Daniela Hanganu, "Iuliu Hatieganu"University of Medicine and Pharmacy, Romania.
(2) Claudia Maricel Mattana, University National of San Luis, Argentina.
(3) K. Kalimuthu, Government Arts College, Bharathiar University, Coimbatore, India.
(4) Geetha Ramakrishnan, Sathyabama University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/22343

***Original Research Article***

***Received 19th November 2017***
***Accepted 13th December 2017***
***Published 18th December 2017***

## ABSTRACT

**Aims:** To investigate the chemotype of the Egyptian *Withania somnifera* and explore the cytotoxic activity of different extracts as well as the major isolated withanolides on four cell lines of common cancer variants in Egypt, both *in vitro* and using computational methods.
**Study Design:** Preparation of the different plant parts extract, determination of the chemotype of the Egyptian plant and bioactivity guided isolation of its major constituents.

**Corresponding author: E-mail: hodasherif@alexu.edu.eg, hoda.sherif@alexu.edu.eg, hodasherif@hotmail.com;*

**Place and Duration of Study:** Department of biochemistry, faculty of medicine and department of pharmacognosy, faculty of pharmacy between November 2014 and July 2016.
**Methodology:** We included four parts of the plant to be investigated on the four common cancer cell lines found in Egypt (liver, breast, lung and colon), *in vitro* MTT assay as well as docking analysis with the epidermal growth factor receptor identified as cancer molecular targets were performed, synergy analysis was carried on the most active withanolides.
**Results:** Roots extract showed selective cytotoxic activity against colon cancer cells while the 50% ethanolic extract of leaves showed selective inhibition against breast cancer cells. All investigated extracts and withanolides E, C and S showed cytotoxic activity against liver cancer cells with the highest activity manifested by the leaves extracts. Withanolide C showed anti-proliferative activity against breast cancer cells. The highest cytotoxic activity aganist HepG2 was observed for a combination of the active withanolides (E, C, and S) with $IC_{50}$ value < 0.1 µg/ml.
**Conclusion:** Egyptian *Withania somnifera* is a subtype of chemotype III with different biological activity than the Indian one. Withanolides E, C and S might be a potential lead in the development of new anti-cancer treatment modalities.

*Keywords: Withania somnifera; withanolide C; withanolide E; synergy analysis, chemotype III.*

## 1. INTRODUCTION

Plants have been a prime source of effective conventional drugs for the treatment of cancer, and while the actual compounds isolated from the plants frequently may not serve as the drugs, they provide leads for the development of potential novel agents. *Withania somnifera* (L.) Dunal family Solanaceae (W.S.) also known as Ashwagandha is a well reputed plant in India as a multi-purpose medicinal agent and is well cherished for the cytotoxic activity of its extracts and isolates. Several studies have been conducted to evaluate its effectiveness in prevention and treatment of several forms of cancer [1,2]. Aqueous extract of WS leaves has selective cancer cell growth arrest activity tested both *in vitro* and *in vivo* [3]. Roots extract exhibited potent cytotoxic effect on human malignant melanoma [4], demonstrated apoptosis in squamous cervical cell line and showed chemopreventive effects against skin cancer in Swiss albino mice [5,6].

Withanolides, the major active components of W.S. were proved to exhibit antioxidant, antitumor, as well as immunomodulatory activities [7]. Several studies proved the antitumor activity of withaferin A against different types of cancer such as breast, pancreatic, lung, colon, and cervical [8]. Withanolide E effect is through targeting apoptosis in renal cancer cells [9].

Among the most notable cancer molecular targets identified to date are the members of the epidermal growth factor receptor (EGFR)/ErbB family: among which are EGFR1 (also known as ErbB1 and HER1), and EGFR2 (also known as HER2/neu and ErbB2). The EGFR is a transmembrane receptor tyrosine kinase expressed in several types of cancer, such as breast, lung, esophagus, head and neck. EGFR and its family members are the essential contributors of a complex signaling cascade modulating growth, differentiation, adhesion, migration and survival of cancer cells. As a result of the intercalating role in progression of cancer, EGFR and its family members have appeared as interesting candidates for anti-cancer therapy [10].

Nowadays, monoclonal antibodies and small molecule inhibitors of tyrosine kinase (TKI) are emerging therapeutic strategies. Previous reports revealed that chemotherapy has the risk to affect normal cells together with cancer cells and thus therapies using compounds that selectively inhibit a target molecule (monoclonal antibodies or TKI) in a more specific subpopulation of cancer cells will have a direct effect on disease progression. Also, specific inhibition of receptor tyrosine kinases may be a coherent strategy to inhibit cancer cell function that relies on its downstream signaling pathways. That's why specific inhibitors currently under preclinical and clinical evaluation might be a better strategy [11].

The EGFR signaling axis has been shown to play a key role during liver regeneration following acute and chronic liver damage, as well as in cirrhosis and hepatocellular carcinoma. The treatment of hepatocellular carcinoma (HCC) cells with EGFR-specific tyrosine kinase inhibitors or neutralizing antibodies induces cell

cycle arrest and apoptosis and increases chemo sensitivity [12].

In order to gain more understanding of the structure–activity relationships observed at the EGFR, molecular docking of the most potent inhibitors withanolide E and withanolide C isolated from the Egyptian *W. somnifera* and EGFR was performed on the binding model based on the EGFR complex structure (PDB Code 1M17).

HER2, which is over-expressed in 20% to 25% of breast cancers, is the most well established therapeutic target in breast cancer [13].

Withanolides which exhibited the lowest $IC_{50}$ against breast cancer cell lines were chosen for proceeding molecular docking with EGFR2 (HER2) performed on the binding model based on the HER2 complex structure (PDB Code 3rcd complexed with TAK-285).

Three different chemotypes of W.S. have been identified and designated types I, II and III [14]. Chemotype III is characterized by the presence of nine withanolides E-M [15]. In Italy [16], Sicilian W.S. has been proved to possess all the characters of Israeli chemotype III (i.e. absence of substitution at C-4, configurations 20S and 14 α -OH, and prevalence of 17 α -OH compounds), except for an evident prevalence of withanolide J not E as Israeli chemotype Ill.

In Turkey, two chemotypes have been reported. The one from Izmir showed low content of withanolide E with an increase towards E, S and H, while those originating from Adana showed low content of withanolide H and an increase towards H, J, K, S and E [17].

Although the plant is widely distributed in the Egyptian flora, it stands as valuable resource yet to be explored as few studies were carried out on the Egyptian plant focusing mainly on its cytotoxic activity showing that its root extract can inhibit proliferation of hepatocellular and breast cancer cells [18,19].

Furthermore, the light petroleum extract of leaves showed activity against Ehrlich Ascites carcinoma [20].

The pattern of distribution of cancer in Egypt, based upon results of National Cancer Registry Program (NCRP), indicated the increased burden of liver cancer whilst breast cancer occupied the first rank among females [21]. Colorectal cancer is the third most common worldwide after lung and breast cancers [22].

The goal of this study is to investigate the chemotype of the Egyptian *Withania somnifera* and explore the cytotoxic activity of different extracts as well as the major isolated withanolides on four cell lines of common cancer variants in Egypt. In addition, the use of computational methods to explore the effects of a combination of the most active withanolides aganist liver and breast cancers using synergy analysis and performing molecular docking studies of these withanolides on EGFR and HER2 receptors.

## 2. MATERIALS AND METHODS

### 2.1 General Experimental Procedures

Melting points were determined in open glass capillaries using Stuart capillary (Stuart Scientific Stone, Staffordshire, UK) and are uncorrected. $^{1}H$ and $^{13}C$ NMR spectra were recorded on Bruker Avance III (Switzerland) (at 400 and 100 MHz, respectively) in DMSO,d6 or $CDCl_3$ solution, using TMS as the internal standard. Chemical shift values are expressed in δ (ppm). The 1D and 2D NMR experiments (COSY, HSQC and HMBC) were used to assign the structure of the isolated compounds. ESI-MS spectra were carried on Waters Xevo G2-S Q-TOF mass spectrometer (Milford, MA, USA) in both positive and negative ion modes and EI-MS spectra were carried on a Finnigan-Mat SSQ 700 at 70 ev. Coulmn chromatography (CC) was carried out using Merck silica gel 60 (0.063–0.20 mm). Preparative TLC was used for the isolation of compounds on precoated Merck silica gel 60 F254 plates

### 2.2 Plant Material and Extraction

*Withania somnifera* (L.) Dunal plant was collected from Alexandria, Egypt in November 2014. The plant was kindly identified by Professor Atef M. Ibrahim, Department of Pomology, Faculty of Agriculture, Alexandria University, Egypt. A voucher specimen (WS105) has been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Alexandria University, Egypt.

Dried roots, leaves, ripe and unripe fruits of W.S. were powdered and 10 g of each was macerated in ethanol in a 1:10 w/v ratio for 24 hrs. The dried

leaves marc was successively extracted with 50% ethanol in $H_2O$. All extracts were filtered, concentrated and were used for *in vitro* cytotoxic assay.

## 2.3 Isolation of Withanolides from leaves of W.S

The dried powdered leaves (600 g) were defatted with light petroleum, the marc was air dried then extracted with 75% ethanol. The later was filtered and the solvent was evaporated in vacuo to give a semisolid residue (40 g) which was re-dissolved in 90% ethanol then extracted successively with light petroleum and methylene chloride. The later (11.2 g) was chromatographed on a silica gel column packed in methylene chloride. Elution was carried with methylene chloride with increasing concentrations of ethyl acetate followed by methanol.

Fraction V eluted with 30% ethyl acetate in methylene chloride (2.5 g) was sequentially purified by CC on silica gel column using isocratic elution of ethyl acetate - methylene chloride (1:1) to afford 1 (25 mg) and 2 ( 40 mg). Fraction IX eluted with 5% methanol in ethyl acetate was purified using preparative TLC with mobile phase $CHCl_3$:MeOH:Acetone (9:1:0.5, v/v) to isolate 3 (20 mg) and 4 (30 mg). Compound 5 (50 mg) was isolated from fraction X eluted with 20% methanol in ethyl acetate.

## 2.4 Semi Quantitative Determination of the Relative Ratios of Withanolides

Dried powdered leaves of *W. somnifera* (0.5 g) were separately extracted with 10 ml methanol using ultrasound at room temperature for 20 min. The extract was filtered and concentrated under vacuum, then quantitatively transferred into a 10 ml volumetric flask and adjusted to the final volume with methanol, filtered through a 0.45 µm membrane filter prior to application. The methanolic extract of leaves was applied in a triplicate manner (volume 5 µl) in the form of bands on pre-coated TLC silica gel plates (10 × 6 cm) by means of Camag Linomat IV automated spray-on band applicator.

Mobile phase: dichloromethane: toluene: methanol: acetone 15: 2: 1: 1 which was used in our earlier publication for quantitative estimation of withanolide S [23] was used for development. Visualization with (methanol/ sulphuric acid) spray reagent followed by heating at 120℃ for 10 min. Photos of the TLC plate after visualization were taken using Nikon camera under UV light at 366 nm, images were sharpened and resized then densitometric analysis was carried using Sorbfil® videodensitometer software. Relative ratios were calculated by the software using area under the peaks.

## 2.5 Cell Viability Assay

### 2.5.1 Cell culture

Human hepatocellular carcinoma cell line (HepG2), human non-small cell lung cancer cell line (A540), human breast cancer cell line (MCF7) and colon cancer cell line ($CaCo_2$) were preserved and obtained frozen in liquid nitrogen (-180℃) from Vacsera, Giza, Egypt.

Cells were cultivated in T25 culture flasks (CELLSTAR®, Greiner Bio-One) in high glucose Dulbecco's Modified Eagle Medium (DMEM) with 2 mM L-glutamine (Lonza, Switzerland), supplemented with 10% fetal bovine serum FBS (Seralab, UK), and 100 U/ml penicillin / 2 mg/ml streptomycin (Invitrogen Corporation, Grand Island, NY) at 37℃ in a 5% $CO_2$, 95% humidified air incubator.

Exponentially growing cells were trypsinized (Trypsin-EDTA, Lonza, Switzerland) and resuspended in antibiotic-containing medium (100 units penicillin G and 0.1 mg of streptomycin/ml). Cells showing 70-90% confluency were harvested. Afterwards, dilutions were made to give the appropriate cell densities 1 x 106 cells/ml for inoculation onto 96-wellmicrotiter plates (final cell number/well was equal to 1×105 cells in 100 µl culture media) and incubated for 24 hours before addition of the test extracts in concentrations ranging from1000-1.95 µg/ml in triplicate and incubated for 48 hrs before applying the MTT assay.

Serial dilutions of test extracts were prepared by dissolving in Dimethyl sulfoxide (DMSO) followed by dilution with DMEM to yield the final DMSO concentration in the assay well as 0.2%. Cisplatin (ALEXIS Biochemical, Lausen Switzerland), was used as a positive control.

The cell viability was calculated with respect to DMSO control.

### 2.5.2 MTT cytotoxicity assay

Anti-proliferative activity against the four cell lines was estimated by the 3-[4,5-dimethyl-2-thiazolyl]-

2,5-diphenyl- 2H-tetrazolium bromide (MTT) assay [24], which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells giving the purple-colored precipitate of formazan which was then dissolved in 150 µl of DMSO by proper mixing. The color absorbance of each well was recorded at 575 nm in a Bio-Rad microplate reader, using DMSO as a blank. Then, the half maximal growth inhibitory concentration ($IC_{50}$) value of the test extracts was calculated.

The relative cell viability was expressed as the mean percentage of viable cells compared to control non-treated cells and the $IC_{50}$ of the studied extracts were calculated by the trend line equation.

Viability % = Average absorbance (OD) test / Average Absorbance (OD) control x100

## 2.6 Statistical Analysis

All results are presented as means of triplicate experiments. All experiments were conducted in triplicate and values expressed as mean ± standard deviation, correlations were carried out using the correlation and regression in the EXCEL program.

## 2.7 Synergy Analysis

The combination of the most active withanolides were evaluated using Compusyn® software and expressed as the combination index (CI) which is based on the Median-Effect Principle derived from enzyme kinetic models as developed by Chou and Talalay [25]. This method quantifies synergistic, additive or antagonistic effects at different doses. The fraction affected (Fa) at each concentration was calculated (i.e., a Fa of 0.9 equals 10% of viable cells). Fa indicates the fraction of cell death induced by drug treatment.

## 2.8 Molecular Docking

The X-ray crystallographic structure of EGFR complexed with 4-anilinoquinazoline inhibitor erlotinib (PDB Code: 1M17) and the X-ray crystallographic structure of HER2 complexed with TAK (PDB Code: 3RCD) were obtained from the RCSB Protein Data Bank.

The three dimensional structure of withanolides was derived by Avogadro software. The accurate docking was performed using the docking tool iGEMDOCK v2.0. Based on the binding energy in kcal/mol. Numbers of runs taken are 70 and max interactions were 2000 with population size 200 and with an energy threshold of 100 also at each step least 'min' torsions/translations/ rotations are tested and the one giving lowest energy is chosen. The hydrophobic preference and electrostatic preference were set to 1.00. The binding site of the target was identified at a distance 8 Å.

## 3. RESULTS AND DISCUSSION

The $^{1}H$ and $^{13}C$-NMR of all the five isolated compounds (Figs. 1, table 1and 2) showed the characteristics for withanolide structure where signals for α, β unsaturated δ-lactone moiety were observed at approximately (δ C 150, 122 and 166) and were assigned for C (24), C (25) and C (26) respectively. In addition to five methyl singlets and olefinic signals assigned for H-2 and H-3.

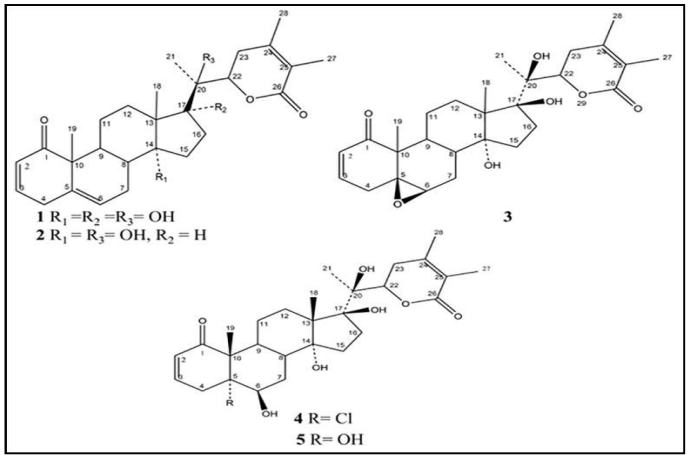


**Fig. 1. Chemical structures of the isolated withanolides 1-5**

**Table 1.$^{13}$C-NMR spectral data for compounds (1-5)**

| Carbon | 1[a] | 2[a] | 3[b] | 4[b] | 5[b] |
|---|---|---|---|---|---|
| | δ C | δ C | δ C | δ C | δ C |
| 1 | 204.4 | 204.3 | 203.4 | 201.6 | 204 |
| 2 | 127.8 | 127.9 | 128.9 | 127.9 | 128.2 |
| 3 | 145.3 | 145.27 | 146.4 | 143.15 | 142.7 |
| 4 | 33.3 | 33.48 | 32.65 | 37.7 | 35.6 |
| 5 | 135.4 | 135.2 | 62.03 | 82.4 | 76.8 |
| 6 | 124 | 124.9 | 63.2 | 73.83 | 73.9 |
| 7 | 25.04 | 25.3 | 26.35 | 29.21 | 29.4 |
| 8 | 36.02 | 35.16 | 33.9 | 34.16 | 33.6 |
| 9 | 32.1 | 36.34 | 37.07 | 34.83 | 34.1 |
| 10 | 50.3 | 50.88 | 48.23 | 52.9 | 51.8 |
| 11 | 21.9 | 22.13 | 23.1 | 22.6 | 22.7 |
| 12 | 26.7 | 32.58 | 34.72 | 34.81 | 32.4 |
| 13 | 51.02 | 47.44 | 54.2 | 54.56 | 54.7 |
| 14 | 88.4 | 85.03 | 81.82 | 83.3 | 83.1 |
| 15 | 32.9 | 32.01 | 30.5 | 31.03 | 31.2 |
| 16 | 30.6 | 20.64 | 36.24 | 36.25 | 36.3 |
| 17 | 89.8 | 49.35 | 87.6 | 87.85 | 88 |
| 18 | 18.9 | 17.4 | 20.75 | 21.31 | 21.4 |
| 19 | 18.5 | 18.95 | 14.9 | 16.47 | 15.6 |
| 20 | 76.7 | 75.33 | 78 | 78.5 | 78.6 |
| 21 | 19.5 | 21.33 | 19.45 | 19.7 | 19.9 |
| 22 | 80.4 | 81.34 | 81.35 | 81.5 | 81.9 |
| 23 | 32 | 31.8 | 32.46 | 32.37 | 34.9 |
| 24 | 150.3 | 149.02 | 151.35 | 151.26 | 151.4 |
| 25 | 121.06 | 122.03 | 120.54 | 120.6 | 120.7 |
| 26 | 165.4 | 166.14 | 166.45 | 166.48 | 166.6 |
| 27 | 12.4 | 12.47 | 12.6 | 12.6 | 12.6 |
| 28 | 20.6 | 20.52 | 20.75 | 20.76 | 20.8 |

*a) In $CDCl_3$ , b) in DMSO,d6*

**Compound 1:** ESIMS spectrum showed a prominent peak at m/z 493.26 $[M +Na]^+$ indicating a molecular formula $C_{28}H_{38}O_6$, $^1$H and $^{13}$C-NMR spectra showed additional olefinic signals at δ H 5.63 (H-6), δ 135.4 (C-5) and δ 124 (C-6) indicating an unsaturation in ring B between (C-5) and (C-6), as suggested by HSQC and HMBC correlations confirming its identity as withanolide J [26].

**Compound (2):** ESIMS spectrum showed a prominent peak at m/z 477.7 $[M +Na]^+$ indicating a molecular formula $C_{28}H_{38}O_5$. Spectroscopic data of (2) was almost identical to that of (1) except for the absence of α -OH group at (C-17) as it appeared at δ 49.35 along with (H-17) at δ H 2.19 confirming its identity as withanolide G [26].

**Compound (3):** The molecular formula $C_{28}H_{38}O_7$ was determined from ESIMS spectrum showing a prominent peak at m/z 509.25 $[M +Na]^+$. The presence of 5 β, 6 β –epoxy group was concluded from $^{13}$C signals from oxygenated quaternary and oxymethine carbons at δ 62.03 (C-5) and δ 63.2 (C-6) as well as the H signal at δ 3.2 (H-6) establishing its identity as withanolide E [27-31].

**Withanolide S (5):** Its isolation and characterization was reported in our previous study [32].

**Compound (4):** NMR spectra was closely related to those of withanolide S (5), except for (C-5) signal deshielded to δ 82.4 in (4) suggesting the presence of C-5 chloro group rather than hydroxyl which was confirmed by the appearance of the molecular ion peak at m/z 545.23 $[M +Na]^+$ for a molecular formula of $C_{28}H_{40}O_7Cl$ in the ESIMS spectrometry along with ions containing $Cl^-$ isotope at m/z 547.23. In the EIMS spectrum, two small informative peaks were observed at m/z 432 and 414 representing the elimination of 3 and 4 molecules of water and hydrochloric acid, respectively. Assignments of all functional groups were achieved by 2D-NMR spectra confirming it is withanolide C [27].

**Table 2 .$^{1}$H-NMR spectral data for compounds (1-5)**

| Position | 1[a] | 2[a] | 3[b] | 4[b] | 5[b] |
|---|---|---|---|---|---|
| | δ H(*J* in Hz) | δ H(*J* in Hz) | δ H(*J* in Hz) | δ H(*J* in Hz) | δ H(*J* in Hz) |
| 2 | 5.9, dd, (12, 4) | 5.91, dd, (12,4) | 5.95, dd, (12) | 5.81, d, (12) | 5.6,dd, (10,2) |
| 3 | 6.77, ddd, (12, 8, 4) | 6.8, ddd, (12,8,4) | 6.99, ddd, (12,8) | 6.73, dd, (12, 4) | 6.5,ddd, (10,5,2) |
| 4 | 2.86, dd, (24, 4) | 2.87, dd, (20,4) | 1.35 | 2.455, m, (20) | 1.90,dd,(19.1,4.6) |
| | 3.3, d (24) | 3.315, d, (20) | 1.67 | 3.495, d, (20) | 3.07 ,d, (19.1) |
| 6 | 5.625, d, (4) | 5.66, d, (4) | 3.2, br s | 3.84, s | 3.47,dd, (8, 4) |
| 7 | 1.85, m | 1.875, d, (4) | 1.83, m | 1.38, m, (12) | 1.25, m |
| | 2.1, m | 2.10, s | 2.08, s | 2.23, q, (12) | 1.95,m |
| 8 | 1.87, s | 1.88, s | 1.83, s | 2.09, t, (23, 8) | 2.34,m |
| 9 | 2.63, s | 2.015, d, (4) | 2.68, s | 2.51, s | 1.98,td, (12, 5) |
| 11 | 1.66, m | 1.66, d, (12) | 1.06, s | 1.34, s | 1.28,d, (14) |
| | 2.35, s | 2.24, d, (12) | 1.52, d, (8) | 2.23, q, (12, 10.7) | 2.12,dd,(14, 4.6) |
| 12 | 1.48, m, (12) | 1.835, d, 2H | 2.45, m | 2.405, d, (4) | 1.38,m, (12) |
| | 2.32, s, (12) | | 2.30, s | 2.51, s | 1.57,td, (12) |
| 15 | 1.82, s | 1.52, m, (12) | 1.16, br s | 1.17, m, (10.7) | 1.12, s |
| | 2.60, s | 1.61, m, (12) | 2.08, m | 2.23, q, (12, 10.7) | 2.15, m |
| 16 | 1.60, m, (12) | 1.63, m | 2.20, s | 1.54, m | 1.49,br d, (6) |
| | 2.22, d, (4) | 2.05, m | 2.35, s | 2.405, m, (4) | 2.37,br d, (3) |
| 17 | - | 2.19, s | - | - | - |
| 18 | 1.09, s | 1.08, s | 0.98, s | 1.03, s | 0.99,s |
| 19 | 1.27, s | 1.29, s | 1.07, s | 1.30, s | 1.12,s |
| 21 | 1.34, s | 1.32, s | 1.24, s | 1.26, s | 1.20,s |
| 22 | 4.63, dd, (12, 4) | 4.235, dd, (20, 4) | 4.63, dd, (12,8,4) | 4.682,dd,(12, 4) | 4.6,dd , (13, 3 ) |
| 23 | 2.38, m, (16) | 2.21, s | 1.52, d, (8) | 1.41, m,(10.7) | 2.37,m |
| | 2.70, d, (16) | 2.39, m | 1.4, m | 1.58, m,(10.7) | 2.37,m |
| 27 | 1.89, s | 1.91, s | 1.76, s | 1.77, s | 1.73,s |
| 28 | 1.96, s | 1.97, s | 1.90, s | 1.90, s | 1.86,s |

*a) In $CDCl_3$ , b) in DMSO,d6*

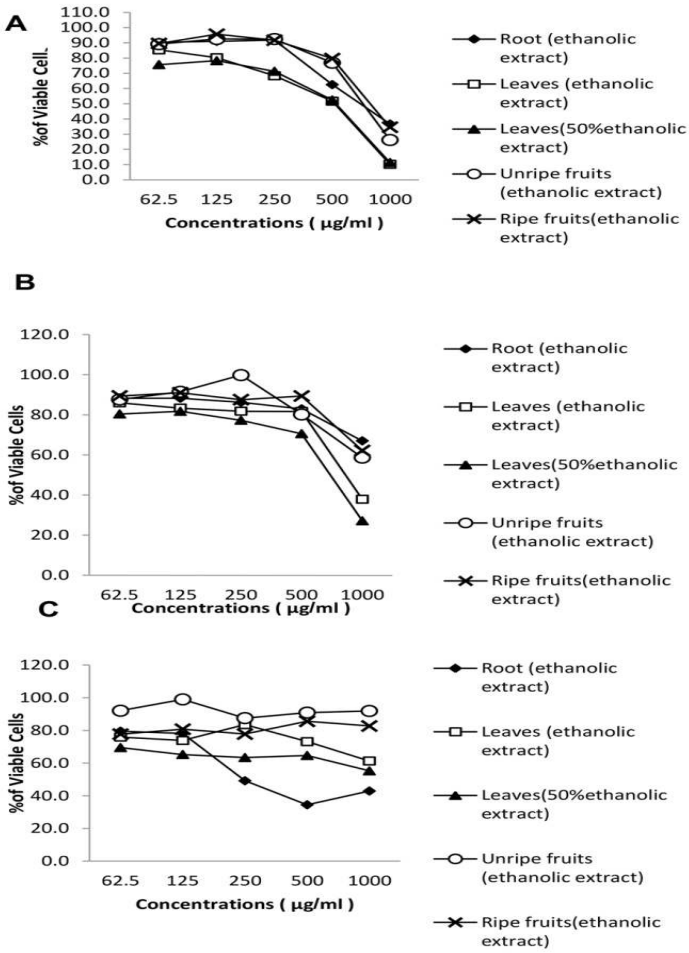


**Fig. 2. % Cell viability in liver (A), breast (B) colon (C) cancer cell lines. Data expressed as mean ± SD**

## 3.1 Determination of the chemotype of Egyptian *W. somnifera*

Semi quantitative determination of the relative ratios of the five major withanolide namely withanolides; J (1), G (2), E (3), C (4) and S (5) using sorbfil® videodensitometer software indicated their presence in a relative ratio of 19, 20, 16, 11 and 34% with an obvious high abundance of withanolide S indicating that it is an unreported subtype of chemotype III.

## 3.2 Cytotoxicity Activity

### 3.2.1 Effect of extracts of different parts of W.S

All extracts showed different degrees of activity against liver cancer where the 50% ethanolic extract of leaves showed the highest activity with an $IC_{50}$ 497.2 µg/ml followed by its 95% ethanolic extract with an $IC_{50}$ 505.2 µg/ml. The $IC_{50}$ of extracts of unripe fruits and roots were 745.1 and 786 µg/ml respectively. Roots extract is the sole one that showed selective cytotoxic activity against colon cancer cell lines with $IC_{50}$ 567.4 µg/ml. The 50% ethanolic extract of leaves showed selective inhibition against breast cancer with an $IC_{50}$ 699.1µg/ml.

None of the extracts showed any cytotoxic activity, in the studied concentration ranges, against lung cancer cell line. Besides, ripe fruits extract didn't show any significant activity against any of the tested cell lines.

### 3.2.2 Cytotoxic activity of withanolides

Withanolides E (3) and C (4) showed potent cytotoxic activity with $IC_{50}$ values lower than 1

µg/ml. while fraction IX showed the highest cytotoxic activity with $IC_{50}$ value< 0.1 (Table 3).

### 3.2.3 Synergy analysis

As shown in (Fig. 3), among 4 combination data points all are on the synergy side (CI<1) especially at Fa 0.9, 0.95, 0.97 which are important for cytotoxic activity. At Fa 0.9 value of CI is 0.27049 indicating strong synergy between withanolides E, C and S. At Fa 0.95, CI is 0.44369 indicating synergy and at Fa 0.97 its value is 0.78896 indicating moderate synergy.

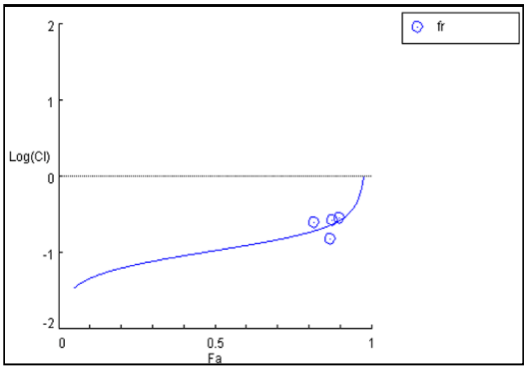


**Fig. 3. Combined cytotoxic effect of Withanolides E, C and S on Hep-G2 presented with Fa-log (CI) plot**

**(fr) Fraction IX, fraction with three withanolides (80%withanolide E ,10% of both withanolide C and withanolide S)*

### 3.2.4 Molecular docking: (Fig. 4)

*3.2.4.1 EGFR1*

There are five binding regions in EGFR1, including adenine region, hydrophobic region I and II, phosphate binding region, and sugar pocket. Region II includes hydrophobic residues such as Leu694, Leu768, Pro770, Phe771, and Leu820. Moreover, there are two acidic residues (Glu738 and Asp831) located on the helix-αC and a phosphate binding region along the sugar pocket whereas the basic residue Lys721 located near the phosphate binding region, formed the salt-bridge to Glu738 in the helix-αC [33].

Withanolide C showed hydrogen bonds with Cys773, Thr 830 and Asp831 and Van der Waals forces with Gly772, Lys721, Val702, Cys773, Asp776, Leu820, Thr830 and Asp831.

While withanolide E showed hydrogen bonds with Asp831 and Met769 and Van der Waals forces with Leu694, Val702, Thr 766, Gly772, Leu 820, Lys721, Met769, Pro770 and Thr830.

*3.2.4.2 EGFR2*

Amino acids that are critical for HER2-ligand interaction include Lys724, Lys736, and Cys805. Binding at the key amino acids results in blocking of the ATP binding site entrance and may result in inhibition of HER2 activity [34]. Withanolide S showed binding affinity -101.9.3, whilst withanolide C showed -100.4.

Withanolide S showed hydrogen bond with Met801 and Thr862, and strong Van der Waals forces with Ala751, Val734, Lys753, Leu785, Leu796, Phe864, Leu852, Thr862 and Asp863.

Whilst withanolide C showed hydrogen bonds with Lys753, Arg849, Asn850 and Asp863 and strong Van der Waal bonds with Leu726, Phe731, Val734, Arg849, Asn850, Asp863 and Phe1004.

Budhiraja etal [7], stated that the presence of 1-ketone-2 ene function in ring A and a 5,6-epoxy functionality in ring B in the withanolide structure are essential to elicit the cytotoxic activity. A typical feature in withanolide E (3) which leads to an increase in its potency against liver cancer.

**Table 3. *In vitro* cytotoxicity of withanolides on Hep-G2& MCF7**

| Compound/ Fraction N$^{o}$ | Antitumor activity | | $IC_{50}$(µg/ml) | |
|---|---|---|---|---|
| | Hep-G2 | MCF7 | Hep-G2 | MCF7 |
| 1 | - | - | 1057.1 | 3472.2 |
| 2 | - | - | 1112.7 | 1875.8 |
| 3 | ++++ | - | < 1 | 3073 |
| 4 | ++++ | ++ | < 1 | 250 |
| 5 | + | - | 971.93 | 1291 |
| Fraction IX | ++++ | + | < 0.1 | 580 |
| Cisplatin[a] | + | + | 519 | 696.4 |

*Antitumor scoring $IC_{50}$: (-) : > 1000 µg/ml, (+):(500 -1000 µg/ml), (++):(100 -500 µg/ml) (+++) : <10 µg/ml , (++++) : < 1 µg/ml [a]Positive control*

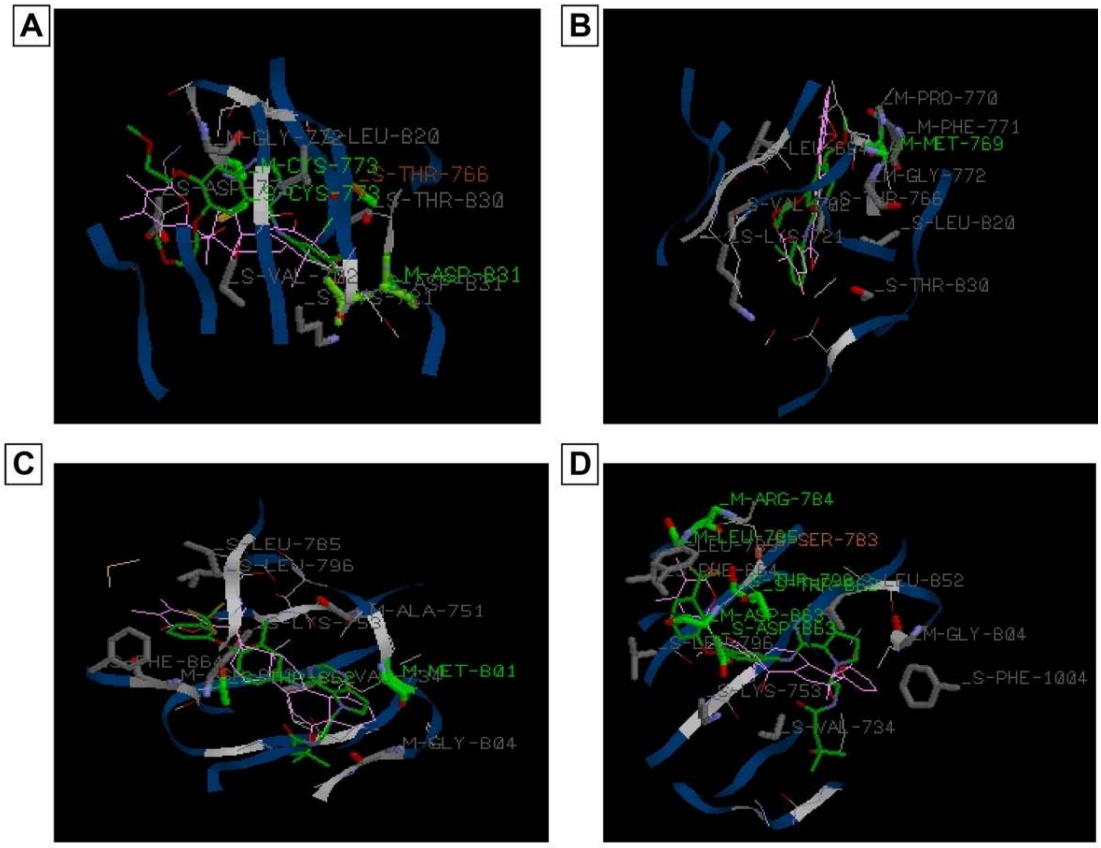


**Fig. 4. Predicted binding model of withanolide C (a) and E (b) to EGFR1 and binding models of withanolide C (c) and S (d) to EGFR2. The EGFR1 and EGFR2 were shown as a ribbon drawing. The side chains of the active site residues and withanolide C, E and withanolide S are represented as stick model**

No loss of activity was observed when the 5,6-epoxy functionality was replaced by a 5α-chloro, 6 β-hydroxyl system in withanolide C (4). Furthermore, the presence of Cl atom increased the effect against breast cancer as expressed by the $IC_{50}$ values.

Withanolides J and G (1 and 2) showed weaker activity against the cell lines tested and this may be attributed to the presence of unsaturation at C5 and C6 as shown by Machin and Veleiro [35]. As for withanolide S (5), the decrease in activity in comparison with withanolide E and C against liver cancer may correlates with the presence of a 5α-hydroxyl group.

Molecular docking results, along with the biological assay data, suggest that withanolide C possesses higher cytotoxic activity than withanolide E.

## 4. CONCLUSION

In conclusion, the present study confirmed that the Egyptian plant is a subtype of chemotype III. Leaf extract demonstrated the highest activity against liver cancer. Further experiments are needed to elucidate the exact mechanisms involved in such effect.

Withanolides E and C were most effective in inhibiting the growth of liver cancer cells and the use of withanolides E, C and S in a combination augmented significantly the anti-proliferative potency than when used separately, most probably due to a synergistic mechanism but further studies on other combinations ratios are recommended to determine the optimum dose. Thus, it can be postulated that withanolides might be a potential lead in the development of new anti-cancer treatment modalities.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## ACKNOWLEDGEMENTS

Authors are grateful to Dr. Armelle Vallat, Institute of Chemistry, University of Neuchâtel, Switzerland for carrying out the mass spectral analysis of the withanolides.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Nema R, Khare S, Jain P, Pradhan A. Anticancer activity of *Withania somnifera* (Leaves) flavonoids compound. Int J Pharm Sci Rev Res. 2013;19(1):103.
2. Yadav B, Bajaj A, Saxena M, Saxena AK. In vitro anticancer activity of the root, stem and leaves of *Withania Somnifera* against various human cancer cell lines. Indian J Pharm Sci. 2010;72(5):659–63.
3. Wadhwa R, Singh R, Gao R, Shah N, Widodo N, Nakamoto T, et al. Water extract of ashwagandha leaves has anticancer activity: Identification of an active component and its mechanism of action. PLoS ONE. 2013;8(10).
4. Halder B, Singh S, Thakur SS. *Withania somnifera* root extract has potent cytotoxic effect against human malignant melanoma cells. PLoS ONE. 2015;10(9).
5. Jha AK, Jha M, Kaur J. Ethanolic extracts of *Ocimum sanctum, Azadirachta indica* and *Withania somnifera* cause apoptosis in SiHa cells. Res J Pharm Biol Chem Sci. 2012;3(2):557–62.
6. Prakash J, Gupta SK, Dinda AK. *Withania somnifera* root extract prevents DMBA-induced squamous cell carcinoma of skin in swiss albino mice. Nutr Cancer. 2002;42(1):91–7.
7. Budhiraja RD, Krishan P, Sudhir S. Biological activity of withanolides. J Sci Ind Res. 2000;59(November):904–9.
8. Vanden Berghe W, Sabbe L, Kaileh M, Haegeman G, Heyninck K. Molecular insight in the multifunctional activities of Withaferin A. Biochem Pharmacol. 2012;84(10):1282–91.
9. Henrich CJ, Brooks AD, Erickson KL, Thomas CL, Bokesch HR, Tewary P, et al. Withanolide E sensitizes renal carcinoma cells to TRAIL-induced apoptosis by increasing cFLIP degradation. Cell Death Dis. 2015;6(2):e1666.
10. Grandis JR, Sok JC, Malfettone A, Rubini V, Siciliano M, Benedetto A Di, et al. Signaling through the epidermal growth factor receptor during the development of malignancy. Pharmacol Ther. 2004; 102(1):37–46.
11. Seshacharyulu P, Ponnusamy MP, Haridas D, Jain M, Ganti AK, Batra SK. Targeting the EGFR signaling pathway in cancer therapy. Biochemistry. 2012; 16(1):15–31.
12. Komposch K, Sibilia M. EGFR signaling in liver diseases. Int J Mol Sci. 2016;17.
13. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Treat. 2012;136(2):331–45.
14. Abraham A, Glotter E, Kirson I, Lavie D. A chemotaxonomic study of *Wlthania* (L.) dun. Phytochemistry. 1968;7(6):957–62.
15. Glotter E, Kirson I, Abraham A, Lavie D. Constituents of *Withania somnifera* Dun-XIII. The withanolides of chemotype III. Tetrahedron. 1973;29(10):1353–64.
16. Vitali G, Conte L, Nicoletti M. Withanolide composition and in vitro culture of Italian *Withania somnifera*. Planta Med. 1996; 62(3):287–8.
17. Özgüven M, Sener B. Drug yield and withanolide contents of *Withania somnifera* (L.) dunal cultivated in Turkey. In: International Symposium on Medicinal and Aromatic Plants, XXIII IHC. 1990;219–28.
18. Ahmed WA, Mohamed A, Nasser EA, Doaa E. Potential toxicity of egyptian ashwagandha: Significance for their therapeutic bioactivity and anticancer properties. Int J Sci Res. 2015;4(2):2170–6.
19. Ahmed WA, M OH, Elhefny MA, Elnasr A, El Syed N. The first molecular insights to the effect of ashwagandha and propolis on cancer cells: Comparative study. In: International Conference on Advances in Agricultural, Biological and Environmental sciences. London(UK). 2015;136–41.

20. Abdelgawad S, Ma G, Hetta M, Ross S, Badria F. Chemical and biological study of *Withania somnifera* (L.) dunal leaves growing in Upper Egypt: Beni-Suef region. J Nat Prod. 2015;8.
21. Ibrahim AS, Khaled HM, Mikhail NN, Baraka H, Kamel H. Cancer incidence in egypt: results of the national population-based cancer registry program. J Cancer Epidemiol. 2014;2014.
DOI: 10.1155/2014/437971
22. Gado A, Ebeid B, Abdelmohsen A, Axon A. Colorectal cancer in Egypt is commoner in young people: Is this cause for alarm? Alexandria J Med. 2014;50(30):197–201.
23. Mahrous RS, Fathy HM, Abu EL-Khair RM, Omar AA. Validated thin-layer chromatographic method for the identification and monitoring of the effect of the extraction method on the yield and phytochemical constituents of Egyptian *Withania somnifera* leaves. J Sep Sci. 2017:*(in press).*
DOI: 10.1002/jssc.201700764
24. Horiuchi N, Nakagawa K, Sasaki Y, Minato K, Fujiwara Y, Nezu K, et al. In vitro antitumor activity of mitomycin C derivative (RM-49) and new anticancer antibiotics (FK973) against lung cancer cell lines determined by tetrazolium dye (MTT) assay. Cancer Chemother Pharmacol. 1988;22(3):246–50.
25. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984; 22(C):27–55.
26. Gottlied HE, Kirson I. 13C NMR spectroscopy of the withanolides and other highly oxygenated C28 steroids. Org Magn Reson. 1981;16(1):20–5.
27. Bessalle R, Lavie D. Withanolide C, A chlorinated withanolide from *Withania somnifera*. Phytochemistry. 1992; 31(10):3648–51.
28. Frolow F, Ray AB, Sahai M, Glotter E, Gottlieb HE, Kirson I. Withaperuvin and 4 deoxyphysalolactone, two new ergostane-type steroids from Physalis peruviana (Solanaceae). J Chem Soc Perkin Trans 1. 1981;1029–32.
29. Glotter E, Abraham A, Gunzberg G, Kirson I. Naturally occurring steroidal lactones with a 17α-oriented side chain. Structure of withanolide E and related compounds. J Chem Soc, Perkin Trans 1. 1977;(4):341–464.
30. Kirson I, Abraham A, Sethi PD, Subramanian SS, Glotter E. 4[beta]-Hydroxywithanolide E, a new natural steroid with a 17[alpha]-oriented side-chain. Phytochemistry. 1976;15(2):340–2.
31. Lavie D, Kirson I, Glotter E, Rabinovich D, Shakked Z. Crystal and molecular structure of withanolide E, a new natural steroidal lactone with a 17α-side-chain. J Chem Soc, Chem Commun. 1972;877–8.
32. Mahrous RS, Ghareeb D, Fathy HM, Abu EL-Khair RM, Omar AA. The protective effect of Egyptian *Withania somnifera* against alzeheimer's. Med Aromat Plants. 2017;6(285).
33. Liu B, Bernard B, Wu JH. Impact of EGFR point mutations on the sensitivity to gefitinib: Insights from comparative structural analyses and molecular dynamics simulations. Proteins Struct Funct Genet. 2006;65(2):331–46.
34. Yang SC, Chang S Sen, Chen CYC. Identifying HER2 inhibitors from natural products database. PLoS ONE. 2011;6(12).
35. Machin RP, Veleiro AS, Nicotra VE, Oberti JC, J MP. Antiproliferative activity of withanolides against human breast cancer cell lines. J Nat Prod. 2010;73(5):966–8.




*Peer-review history:*
*The peer review history for this paper can be accessed here:*
*http://sciencedomain.org/review-history/22343*