ORIGINAL ARTICLE

Ethyl Acetate Fraction of *Solanum nigrum* L.: Cytotoxicity, Induction of Apoptosis, Cell Cycle in Breast Cancer Cells, and Gas Chromatography-Mass Spectrometry Analysis

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Abstract

Objective: The objective of this study was to evaluate the cytotoxic, apoptotic, and cell cycle effect of four fractions obtained from the whole aerial part of *Solanum nigrum*, against three cancer cell lines (MCF-7, A2780, and HT29) and one normal cell line (MRC-5). **Materials and Methods:** MTT assay was used to assess the cytotoxicity of the four fractions. Then, the most active fraction (ethyl acetate) was further examined by annexin V propidium iodide (PI)/FITC apoptosis assay, cell cycle analysis in MCF7 cells followed by gas chromatography-mass spectrometry (GC-MS) analysis. **Results:** The highest growth inhibition activity was obtained by the ethyl acetate fraction (IC_{50} 10–12 µg/ml) against the three cell lines and that fraction spared the normal cell line MRC5 with IC_{50} of 31.78 µg/ml. Annexin V PI/FITC revealed a dose-dependent induction of apoptosis in MCF7 cells when treated the ethyl acetate fraction; which also caused a non-dose dependent S phase arrest. The GC-MS analysis of the ethyl acetate fraction showed fatty acids and esters of fatty acids as the significant components. **Conclusion:** The ethyl acetate fraction from *S. nigrum* strongly inhibited the growth of breast, ovarian, and colon cancer cell lines, and it was three folds selective against the normal fibroblasts. It also caused the induction of early apoptosis and S phase cell cycle block at 10 µg/mL. Future bioassay-guided fractionation of the ethyl acetate fraction could reveal the active compounds, which can be more investigated in further studies.

Key words: Apoptosis, cell cycle, cytotoxicity, gas chromatography-mass spectrometry, *Solanum nigrum*

INTRODUCTION

Cancer is one of the main causes of death around the globe, and the number of cases is estimated to be 21 million by 2030.[1-3] In 2018, 1,735,350 cancer cases and 609,640 cancer deaths took place in the United States.[4] There are several therapeutic approaches for cancer treatment, including chemotherapy and inhibition of negative immune regulation; however, high cost and chemotherapy-associated complications, make it unfavorable choice in some cases. Moreover, strategies to prevent the onset of cancer are still in its infancy.[5] Thus, the discovery and development of novel

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anticancer drug(s) remain one of the most significant thrust areas in modern biological research.[2]

Moreover, medicinal plants serve as nature's gift to humans to help them pursue better health by their bioactive compounds, which have been used in traditional practices since ancient times. Several medicinal plant species inhibit the progression and development of cancer.[6-9] More than 60% of the clinically approved anticancer drugs are derivatives of medicinal plant.[10-12] Interestingly, herbal medicines are still considered as the primary source of treatment in underdeveloped countries.[13] There is enormous scientific interest in the identification of drug molecules or use herbal formulations against cancer because they are considered to be less toxic to normal cells and more tolerable.[2] Vincristine, vinblastine, docetaxel, and paclitaxel are some of the most important natural anticancer.[14] However, these drugs also have some limitations.[6]

Sudan has over 3000 documented species of flowering plants belonging to 170 families and 1280 genera. *Solanum nigrum* L. (Solanaceae) is locally known in Sudan as "Enab el Deibor Elmugad El Aswad." It is a small rounded plant with berry fruits, which is edible in different areas of Sudan and used in traditional medicine for the treatment of various diseases, including tumors. Worldwide, scientists investigated the different parts of *S. nigrum*, as it demonstrated potential antioxidant, hepatoprotective, antitumor, cytostatic, anticonvulsant, antiulcerogenic, and anti-inflammatory effects.[15-23] Moreover, many of the local traditional healers in Sudan claim the use of *S. nigrum* to treat cancer. Thus, it triggered interest in our research group to study this plant. Previously, whole *S. nigrum* extracts were investigated for anticancer potential on PC-3 human prostate cancer cells and Hela cervical cancer cells, revealing a high percentage of growth inhibition against both cell lines.[24] Therefore, this study aims to investigate the cytotoxic activity of *S. nigrum* fractions against other cancer cell lines representing vital tissues: Human breast cells MCF-7, human ovarian cells A2780, and human colon cells HT29; and to determine their selectivity against the normal non-transformed fibroblast MRC5 using MTT assay. In addition, selected fractions were investigated by gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

All chemicals, reagents, and diagnostic kits were purchased from Sigma-Aldrich (Saudi Arabia) unless otherwise specified.

Collection and identification of plant specimens

Plant specimens were collected from Al-Gazira State, Sudan from April to June 2012 by Mr. Suraj Soluman identified by Dr. Haider Abedelgadir, a plant taxonomist, the herbarium of the Medicinal and Aromatic Plants Research Institute (MAPRI), National Research Center, Khartoum-Sudan, as *S. nigrum* L. A voucher specimen (No. MAP/2012/10) was deposited in MAPRI herbarium. The leaves and stems were separately ground to powder using a mortar and pestle.[24]

Preparation of extracts and fractions

One hundred gram of the powdered leaves was macerated successively in chloroform, 80% methanol and kept for 5 days at room temperature with occasional shaking. Each mixture was then filtered, and the filtrate was evaporated to dryness in an evaporating dish on a steam bath at a temperature of 70°C. The process was repeated 4 times with intervals of 5 days. Then, methanolic extract of *S. nigrum* whole aerial plant extract (100 g in 500 ml methanol, yield 23.6%) was prepared using cold extraction, which was further fractionated by dissolving 50 g in 250 mL of distilled water, which was shaken before adding 100 mL of ethyl acetate. The ethyl acetate layers were combined and evaporated under reduced pressure using rotary evaporator (Buchi, Switzerland). The aqueous layer was shaken again with 100 mL of n-butanol. The butanol layers were further combined and evaporated under reduced pressure. The aqueous layer was lyophilized using freeze-drier (Tribal, USA), then stored at −4°C. The extraction process resulted in four fractions: Chloroform, ethyl acetate, n-butanol, and aqueous.

Cytotoxicity assay

The cytotoxicity of the four *S. nigrum* fractions was evaluated by MTT assay against MCF7, A2780, HT29, and MRC5 cells. Each of the three cell lines and the normal fibroblasts was cultured in 96-well (3×10^3/well) and incubated at 37°C overnight (CO_2 5%, humidity 100%). Final concentrations of fractions were 0, 6.25, 12.5, 25, 50, and 100 µg/mL in media (Dimethyl sulfoxide [DMSO] 0.1%). A positive control (5-fluorouracil: 5-FU) was used at the same final concentrations. Each concentration was tested in triplicates, and the experiment was repeated twice. The plates were incubated for 72 h, followed by addition of MTT and incubation for 3 h; then, the supernatant was aspirated and 100 µL of DMSO added to each well. Absorbance read on the multi-plate reader (Bio Rad PR4100, USA) at A_{550} fraction concentration causing 50% inhibition (IC_{50}) compared to control cell growth (100%) was determined. GraphPad Prism version 5.00 was used for analysis.[25]

Annexin V propidium iodide (PI)/FITC apoptosis assay

The most active fraction (ethyl acetate) was subjected to apoptosis induction determination using annexin V PI/FITC assay. MCF7 cells were cultured in 6 well plates (1×10^5 cells/well) for overnight at 37°C, followed by treatment with the ethyl acetate fraction (final concentrations: 0, 5, 10, and 20 µg/mL). After 24 h, the supernatant was collected in tubes and kept on ice, and remaining cells

were trypsinized, incubated at 37°C and pooled with the supernatant. Cells were centrifuged at 350 g for 5 min and washed with phosphate-buffered saline (PBS), and the pellet was re-suspended in binding buffer (100 µL) and annexin V PI/FITC (10 µL). The tubes were incubated at room temperature in the dark for 30 min. Binding buffer (400 µL) and 10 µL PI were added. Samples were analyzed by flow cytometry (Beckman Coulter FC500, USA) within 1 h. Viable cells were differentiated from early and late apoptotic/necrotic cells by annexin V (X-axis) and PI staining (Y-axis).[26]

Cell cycle analysis

Cell cycle analysis was performed using MCF7 cells, which were cultured in 6 well plates (1×10^5 cells/well in 2 mL medium), before treatment with the ethyl acetate fraction (0, 5, 10, and 20 µg/mL; 24 h). Cells were washed with cold PBS and trypsinized. The collected cells were centrifuged at 350 g for 5 min. Pellets were washed in cold PBS, centrifuged and fixed overnight in 70% ice-cold ethanol. Centrifuged cells were re-suspended in cold PBS with the addition of ribonuclease A (15 min), followed by PI (2 µL/mL). Samples were held on ice, and analyzed by flow cytometry. Data analysis of DNA contents (PI bound to DNA) of 20,000 events was carried out.[27]

GC-MS analysis

The ethyl acetate fraction analyzed by GC-MS (Shimadzu, Japan). The analysis was performed on Shimadzu GC-9AM with a flame ionization detector equipped with SE-54 capillary (internal diameter = 0.25 mm and length = 30 m). Mass spectra were recorded on Finnigan Trace DSQ (Thermo Electron Corporation) at an ionization voltage of 70 eV equipped with a DB-5 capillary column (internal diameter = 0.25 mm, film thickness = 0.25µm, and length = 30m).

RESULTS

Cytotoxicity assay

The result of MTT cytotoxicity assay for the *S. nigrum* four fractions revealed that the ethyl acetate fraction exhibited the best inhibition against the three cell lines ($IC_{50:}$ 11.20, 12.33, and 10.48 µg/mL against MCF7, A2780, and HT29, respectively); and it was 3 times less toxic against the normal cell line MRC5 with IC_{50} 31.78 µg/mL [Table 1]. The activity of ethyl acetate fraction against MCF7 was twice better compared to the activity of 5-FU against the same cell line. Thus, the activity of ethyl acetate fraction will be further investigated against MCF7 cells.

Annexin V PI/FITC apoptosis assay

The flow cytometry analysis for MCF7 cells, using annexin-V PI/FITC, treated with different concentrations of the ethyl acetate fraction for 24 h; revealed a dose-dependent induction of early apoptosis in MCF7 cells (C4: 18.9%, 30.8%, and 36.3%; for 0, 5, 10, and 20 µg/mL, respectively, Figure 1), compared to the control cells (C4: 10.6%). Moreover, the percentages of late apoptotic MCF7 cells (C2) were 18.6%, 22.2%, and 15.4% (for 5, 10, and 20 µg/mL, respectively), as compared to the percentages of late apoptotic cells detected in the non-treated MCF7 cells (control: 8.9%).

Cell cycle analysis

The cell cycle analysis was performed to detect DNA content in MCF7 cells treated with different concentrations of ethyl acetate fraction for 24 h using the PI The ethyl acetate fraction at 10 µg/mL increased the number of MCF7 cells in the S phase compared to control, but that increase was not dose-dependent [Figure 2].

GC-MS

GC-MS analysis showed that fatty acids and esters of fatty acids were detected as significant components in the ethyl acetate fraction [Table 2], while ketone and alkene were essential components in the n-butanol fraction [Table 3].

DISCUSSION

Four fractions were obtained in this study from *S. nigrum* whole plant methanolic extract. The ethyl acetate fraction exhibited high cytotoxic activity (IC_{50} 10–12 µg/ml) against the three cell lines. This finding agrees with previous studies which reported that polysaccharides, steroidal glycosides,

Table 1: Cytotoxicity of ***Solanum nigrum*** fractions against three cell lines and one normal fibroblast (MTT 72 h, IC_{50}±SD µg/ml, *n*=3)

Fraction	MCF7	A2780	HT29	MRC5
Chloroform	14.64±2.44	20.77±7.33	41.29±9.32	14.91±2.44
Ethyl acetate	11.20±1.43	12.33±4.02	10.48±0.84	31.78±4.06
N-butanol	20.05±5.87	32.58±8.51	29.67±4.22	15.08±2.49
Aqueous	19.06±3.56	32.37±4.76	24.85±5.89	25.75±3.00
5-FU	19.21±0.56	4.80±0.36	2.72±0.90	2.70±0.20

SD: Standard deviation

Table 2: Gas chromatography-mass spectrometry analysis of ethyl acetate fraction

Peak no.	R. time	Area %	Molecular weight	Formula	Name
1.	19.959	1.76	148	$C_{10}H_{12}O$	Benzaldehyde, 4-(1-methyl-ethyl)
2.	33.349	168	170	$C_{10}H_{18}O_2$	6-Hydroxymethyl-1,4,4trimethyl-bicyclo (3,1,0) hexane-2-ol
3.	34.161	167	380	$C_{27}H_{56}$	Heptacosane
4.	35.819	9.35	278	$C_{16}H_{22}O_4$	1,2-Benzenedicarboxylic acid, bis (2-methyl propyl) ester
5.	37.757	30.60	282	$C_{18}H_{34}O_2$	Oleic acid
6.	38.194	1.04	508	$C_{34}H_{68}O_2$	Heptadecanoic acid, heptadecyl ester
7.	40.598	3.18	296	$C_{20}H_{40}O$	Trans-phytol
8.	41.225	19.61	210	$C_{14}H_{26}O$	7-Tetradecanal, (Z)
9.	41.586	7.12	282	$C_{18}H_{34}O_2$	Oleic acid
10.	42.316	2.16	508	$C_{34}H_{68}O_2$	Heptadecanoic acid, heptadecyl ester
11.	42.927	0.75	252	$H_{17}H_{32}O$	8-Hexadecanal, 14-methyl-,(Z)
12.	43.230	1.25	340	$C_{23}H_{32}O_2$	Phenol, 2,2'-methylenebis {6-(1,1-dimethylethyl)-4-methyl-
13.	44.976	1.81	530	$C_{34}H_{58}O_4$	1,2-Benzenedicarboxylic acid, ditridecyl ester
14	46.234	16.00	300	$C_{18}H_{36}O$	Hexadecanoic acid, 2-hydroxyethyl ester
15	48.157	2.01	283	$C_{18}H_{37}NO$	N, N-Dimethyldecanamide

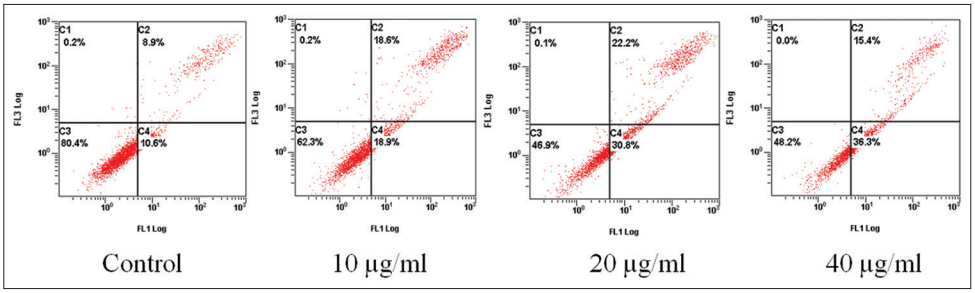

Figure 1: Histograms showing different phases of staining MCF7 cells with annexin V PI/FITC treated with ethyl acetate fraction (24 h). X-axis: Annexin V, Y-axis: PI C1: (necrosis-death, PI+/annexin V–); C2: (Late apoptosis, PI+/annexin V+); C3: (living cells, PI-/annexin V–); C4: (early apoptosis, PI-/annexin V+).*n* = 2, experiment 2×

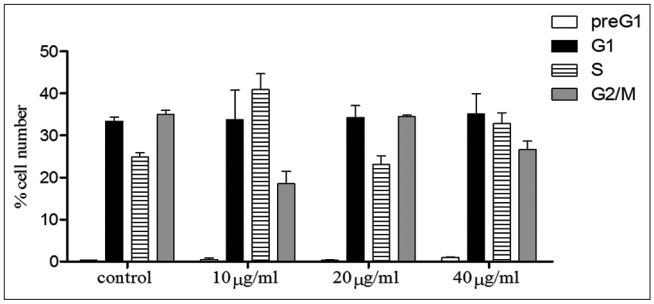

Figure 2: Effect of ethyl acetate fraction after 24 h treatment in MCF7 cell cycle distribution. Data shown are mean % ± standard deviation. (*n* = 2). The experiment repeated 2×

and glycoalkaloids, which were isolated from *S. nigrum*, showed high activity against cancer cells.[28-30]

One of the hallmarks of apoptosis is the externalization of phospholipid phosphatidylserine (PS) by translocation from the inner to the outer layer of the plasma membrane for recognition of phagocytes during the early stage of apoptosis. Hence, PS can serve as a specific target for the detection of early apoptotic cells. Besides, polyphenolic rich extracts of *S. nigrum* inhibited cancer cell growth through the induction of apoptosis, and suppression of angiogenesis, down the expression of CD31 and AKT/mTOR pathways. The induction of apoptosis was confirmed in this study by flow cytometric annexin V PI/FITC assay; as the MCF7 cells treated with the ethyl acetate fraction exhibited a dose-dependent induction of apoptosis.[31-33]

Solamargine (steroidal alkaloid glycoside), which was previously isolated from *S. nigrum*, caused cell cycle arrest at G2/M, upregulated the expression of the caspase-3 protein, and induced apoptosis in human hepatoma SMMC-7721 and HepG2 cells.[28] While in this study, treatment of MCF7 cells with the ethyl acetate fraction caused non-specific cell cycle arrest, as it increased the number of cells in the S phase at

Table 3: Gas chromatography-mass spectrometry analysis of n-butanol fraction

Peak no.	R. time	Area %	Molecular weight	Formula	Name
1.	6.788	3.06	114	$C_7H_{14}O$	Butanone
2.	6.792	0.46	158	$C_8H_{14}O_3$	Isobutyric anhydride
3.	7.785	2.64	116	$C_7H_{16}O$	Di-n-propylecarbinol
4.	7.958	26.39	130	$C_8H_{18}O$	1-butoxybutane
5.	7.785	2.64	116	$C_7H_{16}O$	Din-propylcarbinol
6.	8.282	7.81	144	$C_8H_{16}O_2$	Isobutyl isobutyrate
7.	8.650	3.02	100	$C_6H_{12}O$	3-Hydroxy-1-hexene
8.	9.391	4.30	128	$C_8H_{16}O$	3-Methyl-4-heptanone
9.	9.547	2.00	144	$C_8H_{16}O_2$	Isobutyl isobutyrate
10.	9.798	9.00	140	$C_{10}H_{20}$	4-Ethyl-3-octene
11.	10.736	3.77	198	$C_{14}H_{30}$	Hexane, 3,3,4,4,-tetraethyl
12.	12.972	17.88	140	$C_{10}H_{20}$	4-Ethyl-3-octene
13.	13.911	7.03	140	$C_{10}H_{20}$	4-Ethyl-3-octene
14.	8.282	7.81	327	$C_{21}H_{28}O_4$	2,3-dimethoxy-4-(3-phenyl-1-propynyl) -4-(butyldimethylsilyl) oxy-2-cyclobutene-1-one

10 and 40 µg/mL, compared to untreated cells. Barton-burke demonstrated that the non-specific cell cycle increase caused by the ethyl acetate fraction represented the killing of cancer cells in either resting or dividing phases.[34]

CONCLUSION

S. nigrum ethyl acetate fraction strongly inhibited the growth of breast, ovarian, and colon cancer cell lines and spared the normal cells. In addition, it induced dose-dependent early apoptosis and non-dose-dependent S phase block. However, extensive research is needed to isolate pure compounds from *S. nigrum* and to test their mechanisms of action.

CONFLICTS OF INTEREST

The authors declare that they have no competing interest

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AUTHORS' CONTRIBUTIONS

Ehssan H. O. Moglad: Plant extraction, cytotoxic activities and wrote the draft; Muddathir S. Alhassan: GC-MS analysis; Eshtiyag A. Abdalkareem interpreted the result; Ashraf N. Abdalla: Annexin V PI/FITC apoptosis assay, cell cycle assay; Masaki Kuse: GC-MS analysis and revised the final paper.

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