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ORIGINAL ARTICLE

Varthemia iphionoides and *Pelargonium graveolens* Extracts as a Treatment of Breast Cancer Implanted in Diabetic Mice

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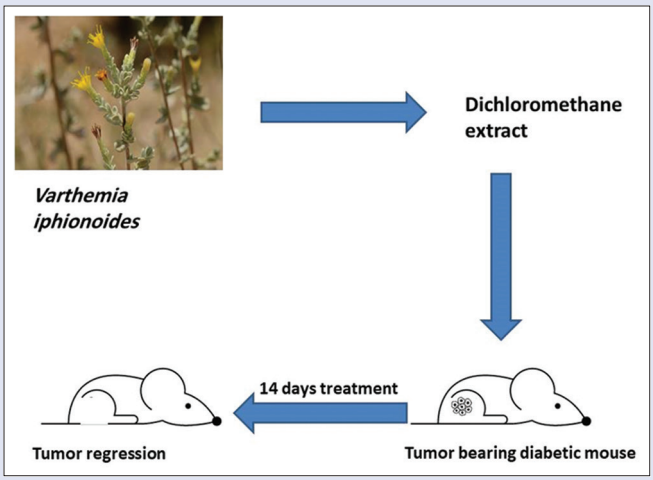
ABSTRACT

Background: The relationship between cancer and type 2 diabetes is well documented. However, studies are very limited to test new therapies for both diseases in the same biological system. This study was conducted to test the potential of two antidiabetic plants from Jordan (*Varthemia iphionoides* and *Pelargonium graveolens*) to treat breast cancer implanted in diabetic mice. **Materials and Methods:** Different solvent extracts of both plants were prepared, and the *in vitro* antiproliferative activity was tested against MCF-7, T47D, and EMT6/P breast cancer cell lines in addition to Vero normal cell lines. Normal as well as diabetic Balb/C mice were transplanted with EMT6/P cell line, and *in vivo* antitumor activity was assessed for the most potent plant extract according to the *in vitro* results. Histological examination of tumors was performed using standard hematoxylin and eosin staining protocol. Apoptosis was detected using TUNEL colorimetric assay. Vascular endothelial growth factor expression of cancer cells was detected using ELISA. Aspartate aminotransferase, alanine aminotransferase, and creatinine were measured as well as interferon-gamma, interleukin-2 (IL-2), IL-4, and IL-10. **Results:** *V. Iphionoides* dichloromethane (DCM) extract was the most potent extract and could inhibit cell growth of breast cancer cell lines (EMT6, MCF-7, and T47D). It showed high ability in targeting growth and progression of breast cancer inoculated in diabetic and non-diabetic mice. **Conclusion:** *V. iphionoids* DCM extract is a promising therapeutic option to treat breast cancer in diabetic cases. However, further studies are essential to characterize the active ingredients in this extract.

Key words: Anticancer activity, diabetes, dichloromethane extract, hypoglycemic effect, *Varthemia iphionoids*

SUMMARY

- Different solvent extracts were prepared from two plants (*Varthemia iphionoides* and *Pelargonium graveolens*) and tested to treat breast cancer in diabetic mice. *Varthemia iphionoides* dichloromethane extract showed the highest activity to treat breast cancer in diabetic mice.



Abbreviations used: VEGF: Vascular endothelial growth factor; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); JRBG: Jordan Royal Botanical Garden; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS: Fetal bovine serum, LD_{50}: Median lethal dose; STZ: Streptozotocin; rTdT: Terminal deoxynucleotidyl transferase and recombinant; PBS: Phosphate-buffered saline; Streptavidin HRP: Horseradish peroxidase-labeled streptavidin; DAB: Diaminobenzidine, AST/GOT: Aspartate aminotransferase; ALT/GPT: Alanine aminotransferase; SPSS: Statistical Package for the Social Sciences.

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INTRODUCTION

Diabetes and cancer are two of the four main noncommunicable diseases in addition to cardiovascular and respiratory diseases. Both diseases represent a global health problem, and their prevalence is in continuous rise with time.[1]

In Jordan, the reported number of cancer cases is increasing dramatically and is considered as the second cause of death after cardiovascular diseases.[2]

Diabetes has heavy economic costs, in term of the annual costs of treatment, including medications, which was estimated at about 654 million Jordanian Dinars.[3] Jordan has the ninth highest incidence of diabetes compared with neighboring countries.[4]

Evidence from large recent cohort studies showed the existence of a higher cancer incidence among patients with type 2 diabetes including endometrial, colon, and hepatic cancer.[5] To date, the potential reasons

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for this association remain unclear. Experimentally, an increase in cancer progression was observed in diabetic and/or hyperglycemic mice.[6]

Angiogenesis (blood vessel formation) is an essential step in cancer growth and metastasis; one of the most important factors in angiogenesis is vascular endothelial growth factor (VEGF) which is highly expressed in growing tumors. High levels of VEGF were detected in sera of children and adult diabetic patients, which may explain the high incidence of cancer in diabetic patients.[7,8] Patients with diabetes and cancer have a poorer prognosis compared with those without diabetes.[9] Diabetes and hyperglycemia are associated with shorter remission periods, shorter survival times, and higher mortality rates.[10]

Medicinal plants had been used widely in many diseases including cancer and diabetes.[11] Antidiabetic medicinal herbs are of special interest, especially if they have anticancer activity.[12]

In Jordan, there are around 109 plant species that are proven for their antidiabetic properties and widely used to treat diabetes.[13] The high dependence of patients on these plants may be related to the side effects associated with conventional antidiabetic drugs.[14] Although there is an increase in their consumption, many studies are still needed in order to understand the mechanism of action of these plant extracts and also to evaluate their effects and safety.[15]

Varthemia iphionoides and *Pelargonium graveolens* are two medicinal plants from Jordan used traditionally for the treatment of diabetes.[16] These plants had been tested for various biological activities including *in vitro* antiproliferative effect [Table 1]. On the other hand, many plants growing in Jordan were tested for their anticancer effects.[17,18] However, these plants were not tested to treat cancer in diabetic mice.

This study was conducted to test the capacity of two antidiabetic plants (*V. iphionoides* and *P. graveolens*) to treat breast cancer implanted in diabetic mice.

MATERIALS AND METHODS

Plant material

V. iphionoides (voucher number: WHT-11) was collected from wild sources (Jordan Valley), while *P. graveolens* (voucher number: WHT-12) was collected from Jordanian gardens. Plant identification was done by Jordan Royal Botanical Garden experts.

Preparation of plant extracts

Freshly harvested plant materials (aerial parts of the plant) were dried at 40°C; methanol and dichloromethane (DCM) extracts were prepared by extracting 100 g of the dried material using Soxhlet, for 8-h duration. Both solvents were used previously to prepare extracts for anticancer activity testing.[19] Water and water/methanol in a portion of 80:20 ml extractions were prepared by soaking of 100 g of dried powder for 8 h at 40°C. Percentage yield was calculated for each extract [Table 2]. Each extract was completely dried and stored at −20°C until used.

Phytochemical screening

Qualitative phytochemical screening for each extract was performed according to the standard methods described by Trease and Evans (2009).[20]

2, 2′-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) antioxidant assay

The antioxidant activity of the most potent extracts was measured using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (antioxidant kit) catalog number CS0790, Sigma-Aldrich, USA. The principle of the antioxidant assay is based on the method described by Jeong *et al.*[21] with modifications.

Table 1: The two study plants (a) *Varthemia iphionoides* and (b) *Pelargonium graveolens*, with their documented pharmacological activities

(a) *V. iphionoides*				
Part used	**Type of extract**	**Constituent**	**Activity**	**References**
Aerial	Methanolic	Crude extract	Antipseudomonal activity	[51]
Aerial	Ethyl acetate	sesquiterpene, selina-4,11 (13)-dien-3-on-12-oic acid	Antibacterial (*S. aureus, B. subtilis, M. luteus, E. coli, B. cereus,* and *S. enteritidis*)	[52]
Aerial	Acarbose aqueous extracts	Crude extract	Dual inhibition of α-amylase and α-glucosidase (significant dose-dependent)	[53]
Aerial	Hexane, ethyl acetate, chloroform, ethanol, and water extracts	Polyphenol, flavonoid	Cytotoxic activity against human myelocytic leukemia	[41]
Aerial	Ethanol extract	Flavonoids	Cytotoxic/free radical scavenger	[16]
Aerial	Ethanol	Polysaccharides, polyphenols, flavonoids, saponins.	Geroprotective activities	[54]
Shoot	Methanol/aqueous	Phenolics	Antioxidant with scavenging activity	[55]
(b) *P. graveolens*				
Part used	**Type of extract**	**Constituent**	**Activity**	**References**
Leaves	Aqueous extract	Essential oil (menthone, geraniol, linalool, caryophyllene)	Against pancreatic triacylglycerol lipase, α-amylase, and glucosidase	[56]
Flowers, leaves, stems	Supercritical carbon dioxide Steam distillation	Citronellol geraniol, terpene hydrocarbons Essential oil components	Diabetes, diarrhea, gallbladder problems, gastric ulcers, jaundice, liver problems, sterility and urinary stones	[57]
Aerial	Hydrodistilled in clevenger-type apparatus	Citronellol trans-geraniol 10-epi-γ-eudesmol, isomenthone, and linalool	Essential oil antioxidant, anticancer activities	[58]
Leaves	Hydrodistillation	Monoterpenoids, sesquiterpenoids	Hypoglycemic and antioxidant activities	[59]
Leaves	Hydrodistillation	Oxygenated monoterpenes citronellol, citronellyl formate, and menthone/isomenthone	Hypoglycemic and hypolipidemic properties	[60]
Essential oil	Essential oil	Essential oil	Immunomodulatory	[61]
Essential oil	Essential oil	Phenolics	Insecticidal activities	[62]

P. graveolens: *Pelargonium graveolens*; *S. aureus*: *Staphylococcus aureus*; *B. subtilis*: *Bacillus subtilis*; *M. luteus*: *Micrococcus luteus*; *E. coli*: *Escherichia coli*; *B. cereus*: *Bacillus cereus*; *S. enteritidis*: *Salmonella enteritidis*; *V. iphionoides*: *Varthemia iphionoides*

Table 2: Yield *Varthemia iphionoides* and *Pelargonium graveolens* extracts

Extract	Yield (g/100 g)
Varth/DCM	4.0
Varth/Water	0.5
Varth/Meth	6.0
Varth/Water/Meth	2.0
Pelar/DCM	5.0
Pelar/Water	3.0
Pelar/Meth	9.0
Pelar/Water/Meth	3.0

Varth: *V. iphionoides*; Pelar: P. graveolens; DCM: Dichloromethane; Meth: Methanol; *P. graveolens*: *Pelargonium graveolens; V. iphionoides: Varthemia iphionoides*

In vitro antiproliferative activity assay

The antiproliferative activity of each plant extract was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. EMT6/P (mouse breast), MCF-7, T47D (human breast) carcinoma cell lines and Vero (monkey kidney) normal cell line were cultured in 96-well microplates (100 µl; 1.5×10^4 cells per well) in a medium containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin, and 0.1% gentamicin. Cells were incubated for 24 h at 37°C in a 5% CO_2-enriched atmosphere. After that, EMT6, T47D, and MCF-7 cells were treated starting with 5 mg/ml of each type of plant extracts down to 39.063 µg/ml. For Vero cell line, cells were treated with 10 mg/ml down to 78.125 µg/ml of each extract. All cell lines were incubated for 48 h. Then, MTT was added to the wells according to the manufacturer's instructions (Sigma-Aldrich, Missouri, USA). Cell viability (percentage survival) was calculated compared to negative control cells (contains only tissue culture media and 0.05% methanol or DCM). The calculated IC_{50} represents the treatment concentration that showed a lethal effect on 50% of cells.

Experimental animals

Animal care and use were conducted according to standard ethical guidelines, and all of the experimental protocols were approved by the Research and Ethical Committee at the Faculty of

Pharmacy-Applied Science University (approval number: 2016-PHA-9).

Ninety female Balb/c mice (3–6 weeks old, 23–25 g body weight) were used in this study. Separate cages with wooden shaving were used to keep mice. The environmental parameters in the animal room were as follows: 50%–60% humidity and 25°C temperature with continuous ventilation.

Selection of the plant extract for *in vivo* study

Based on the *in vitro* results, the most potent extract against the *in vitro* EMT6 cell line was *V. iphionoides* DCM extract; therefore, it was selected for the *in vivo* study.

Acute toxicity study (median lethal dose screening)

In order to select the dose ranges for actual median lethal dose (LD_{50}) for the most potent plant extract (*V. iphionoides* DCM extract), a pilot study was conducted on small group of animals; plant extract was dissolved in phosphate-buffered saline (PBS) containing 5% Tween 80 as cosolvent, and an emulsion was formed. For screening of the LD_{50}, five groups of animals ($n = 6$ for each group) were prepared and treated with *V. iphionoides* DCM plant extract in a range between 500 and 750 mg/kg (Akhila *et al*. 2007, OECD Guidelines' Procedure 420 2001).[22]

Screening chronic toxicity study of *Varthemia iphionoides* dichloromethane extract

In order to select and assess the safety of the dose of the plant extract which will be given to the animals for chronic use. Six groups of Balb/C mice ($N = 3$) for each group were selected (age of 6 weeks and average weight of 20–23 g) to be tested, which injected with 100, 150, 200, 250, and 300 mg/kg of the *V. iphionoides* DCM extract for 2 weeks. These values were selected based on the results of acute toxicity test. We took concentration around the therapeutic dose, and mice were treated for 14 days which is the duration of our treatment procedure. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and serum creatinine were measured before and after the study, which were all comparable to the normal control mice values.

Diabetes induction

Diabetes was induced by intraperitoneal injection of a freshly prepared streptozotocin (STZ) solution (40 mg/kg) prepared in phosphate buffer (0.1 M, pH 4.5) to overnight-fasted mice every day during a week. During this time, animals were allowed to eat normally. STZ-treated animals were considered as diabetics when their fasting blood glucose levels were above 150 mg/dL.[23,24] Only diabetic mice were selected for the study groups.

Measuring blood glucose during plant therapy

Blood samples were collected every day by tail vein sampling technique using 25-gauge needles. One hundred microliters were collected from each mouse, and serum levels of glucose were measured using a glucose detection kit. Procedure and instructions were conducted in accordance with kit with catalog no. QRXQC16W (Arcomex, Jordan). For reference, blood glucose was also measured using a blood glucose meter (ACCU-CHEK Performa, Mannheim, Germany).

Tumor inoculation

The mouse mammary tumor cells (EMT6/P) were harvested by trypsinization, centrifuged, washed, and resuspended in Minimal Essential Medium (MEM) medium at a density of 1×10^6/100 µl. Cell viability was assessed using trypan blue exclusion method. Mice (6 weeks old, 20–25 g weight) were injected subcutaneously in the abdominal area using a 23-gauge needle syringe with 1×10^6 cells suspended in 100 µl PBS.

Antitumor activity testing of *Varthemia iphionoides* dichloromethane extract

Tumor-bearing mice were placed in six groups ($n = 10$ for each group) so that the average tumor volumes for all groups are closely matched.

- Group A1: Control, diabetic mice, with implanted cancer, were treated with vehicle
- Group A2: Diabetic mice, with implanted cancer, were treated with *V. iphionoides* DCM extract
- Group A3: Diabetic mice, with implanted cancer, were treated with metformin (80 mg/kg)
- Group B1: Control mice with implanted cancer, not diabetic, were treated with vehicle
- Group B2: Mice with implanted cancer, not diabetic, were treated with *V. iphionoides* DCM extract
- Group B3: Mice with implanted cancer, not diabetic, were treated with metformin (80 mg/kg)
- Group C1: Control mice without implanted cancer, diabetic, were treated with vehicle
- Group C2: Mice without implanted cancer, diabetic, were treated with *V. iphionoides* DCM extract.

Treatments began 14 days following tumor cell inoculation.

Mice were monitored during the 2-week treatment period, and the tumor size was measured every 2 days using the equation: length × width 2×0.5.[25] After the last dose, tumor-bearing mice in all groups were

sacrificed, and their tumors were dissected and stored in 10% salined formalin or further testing.

Histological examination of tumor sections

Formalin-fixed specimens were gradually dehydrated and embedded to prepared paraffin blocks. Sections of 5 μm thickness were prepared using microtome; standard hematoxylin and eosin (H and E) procedure was used to stain different sections. Light microscope equipped with computer-controlled digital camera was used to visualize images on the slides.

Apoptosis detection in tumor sections

Degree of apoptosis induced by each treatment was detected using the DeadEnd Colorimetric TUNEL System. The DeadEnd Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3`-OH DNA ends using the terminal deoxynucleotidyl transferase and recombinant enzyme. Horseradish peroxidase-labeled streptavidin is then bound to these biotinylated nucleotides which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine. Using this procedure, apoptotic nuclei are stained dark brown. Detailed and step-by-step procedure was done in accordance with the DeadEnd Colorimetric TUNEL System G7362 (Promega, USA).

Determination of vascular endothelial growth factor in EMT6/P cells

EMT6/P cells were dispensed into three separated tissue culture flasks at an optimized concentration of 1,500,000 cells/10 ml of complete tissue culture medium. After 24 h, the media in each flask were completely removed, and the attached cells were treated with 150 mg/ml of *V. iphionoides* DCM extract. Cells were incubated for 48 h; after that, the media of each flask were transferred into sterile tubes, and the attached cells were harvested by employing trypsinization technique and washed using PBS for 2–3 min. After washing, cells were transferred to the sterile tubes and centrifuged at 1500 rpm and 4°C for 10 min and resuspended in 1 ml of ×1 cell lysis buffer. This was repeated three times for maximum cell lysis. The supernatant was transferred to a new tube and diluted 5-folds with ×1 sample diluent buffer for further analysis. Then 100 μl of each supernatant of different treatments were pipette in duplicates into 96-well plate precoated anti-VEGF antibody and incubated for 2.5 h. Later on, the wells were washed 4 times with ×1 wash solution, and a 100 μl of ×1 prepared biotinylated detection antibody was added to each well and incubated for 1 h at room temperature with gentle shaking. Later on, the wells were washed again 4 times with ×1 wash solution. After washing, a 100 μl of Horseradish peroxidase-conjugated streptavidin is pipetted to the wells and incubated for 45 min, at room temperature with gentle shaking. The wells are again washed as before, and a 100 μl of TMB substrate solution is added and incubated for 30 min, in a dark place at room temperature with gentle shaking. Later, 50 μl of stopping solution is added to each well, and the intensity of the color was measured at 450 nm immediately. The sample values are then read off the standard curve.

Assessment of possible liver and kidney toxicity in mice

Investigation in the possibility of developing liver toxicity toward the use of the plant extract was established through measuring AST and ALT liver enzymes using AST/GOT kit and ALT/GPT kit, respectively. Procedure and instructions were conducted in accordance with kits with catalog no. M11531i-20 and M11533i-20 (Biosystems, Spain) for measuring AST and ALT, respectively. In order to investigate whether the use of *V. iphionoides* DCM extract will result in potential nephrotoxicity, serum levels of creatinine were measured using creatinine detection kit. Procedure and instructions were conducted in accordance with kit with catalog no. C130613 (Arcomex, Jordan).

Assessment for immune system function by determination of interferon-gamma, interleukin-2, interleukin-10, and interleukin-4 levels in serum sample

Serum levels of interferon-gamma (IFN-γ), interleukin-2 (IL-2), IL-10, and IL-4 were measured for representative samples of mice from all study groups using quantitative ELISA kits. This is a 4.5-h solid-phase ELISA designed to measure mouse IFN-γ, IL-2, IL-10, and IL-4 serum levels. Principle of the assay is the same for all cytokines, by employing the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for each cytokine has been precoated onto 96-well microplate. Detailed and step-by-step procedure was done in accordance to catalog no. MIF00 and M4000B (R and D, USA)

Statistical analysis

All data generated during the course of the research were analyzed statistically by one-way ANOVA test to determine statistical significance (for change in tumor size and blood sugar between tested *in vivo* groups). The level of significance for the differences between means within each tested *in vivo* group was computed by Tukey's honestly significant difference. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 20. $P < 0.05$ was considered statistically significant. IC_{50} (the concentration at which there is a 50% cell death in comparison to negative control cells) was calculated using nonlinear regression in the Statistical Package for the Social Sciences (SPSS) version 20 (IBM, Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Antiproliferative activity of *Varthemia iphionoides and Pelargonium graveolens* extracts

For the two study plants, the anticancer activity of eight plant extracts (water, water/methanol 8:2, methanol, and DCM) was evaluated against three cancer cell lines (EMT6/P, MCF-7, and T47D) and Vero normal cells.

Most of the extracts displayed cell growth inhibition against the subjected breast cancer cell lines (EMT6/P, MCF-7, and T47D) in a dose-dependent manner. For *V. iphionoides* extracts, DCM extract had the lowest IC_{50} value against EMT6/P cell line (IC_{50} = 117 μg/ml) compared with T47D (IC_{50} = 160 μg/ml) and MCF (IC_{50} = 270 μg/ml) [Table 3]. A similar response was observed for methanolic extract, and the highest activity was against EMT6/P cell line followed by T47D and MCF cell lines with IC_{50} values of 14, 210, and 330 μg/ml, respectively. Water extract had shown lower IC_{50} values against the three cancer cell lines (IC_{50} >1000 μg/ml). Furthermore, water methanolic extract had shown lower IC_{50} value than that of water extract against EMT6/P cell line followed by T47D and MCF cell lines (IC_{50} values = 145, 210, and 330 μg/ml), respectively [Table 3].

The results of this part showed that the most potent extract with the lowest IC_{50} value was found to be *V. iphionoides* DCM extract. This may indicate that the nonpolar active principles are responsible for most of the antiproliferative activities in this plant. Previous studies reported a pronounced cytotoxic effect of *V. iphionoides* on human cancer cell lines such as leukemia (HL-60) using hexane, chloroform, and ethanol extracts.[26] Other studies showed the effect of crude extracts of this plant on other human cancer cell lines.[27]

P. graveolens water extract activity was much more noticed than that of *V. iphionoides* water extract, with the lowest IC_{50} value against EMT6/P cell line followed by MCF-7 and T47D cell lines (IC_{50} values = 410, 590, and 610 μg/ml, respectively). For water/methanolic extract, activity was much more noticed with T47D cells followed by MCF-7 and EMT6/P cell lines (IC_{50} values of 590, 700, and 860 μg/ml, respectively) [Table 3].

Unlike *V. iphionoides* DCM extract, *P. graveolens* DCM extracts had shown lower activity against EMT6/P (IC_{50} = 450 μg/ml) cell line, MCF-7 cell line (650 μg/ml) and T47D (IC_{50} = 700 μg/ml). *P. graveolens* methanolic extract had shown lower activities than those of *V. iphionoides* methanolic extracts with higher IC_{50} values against EMT6/P cell line followed by MCF-7 and T47D cell lines (IC_{50} value of >1000, 950, and 790 μg/ml, respectively) [Table 3].

Previous studies on *P. graveolens* reported high anticancer activity of this plant's different extracts against many human cancer cell lines,[27-29] which is in agreement with our results.

The eight extracts had shown low activity against Vero normal cells as indicated by high IC_{50} values (IC_{50} >10 000 mg/ml), and this may indicate the safety of these extracts against normal cells.

In vivo study

V. iphionoides DCM extract was the most potent extract (IC_{50} = 117 μg/ml) against the mouse breast cancer cell line (EMT6/P) *in vitro*. Accordingly, it was selected to treat breast cancer cell line inoculated in mice in the *in vivo* study.

The pilot LD_{50} study revealed that the highest nonlethal concentration was 650 mg/kg and the lowest lethal concentration was 750 mg/kg. The LD_{50} value of *V. iphionoides* DCM extract was calculated according to the method of Akhila and Alwar.[21] The LD_{50} value of the *V. iphionoides* DCM (aerial parts) extract was 750 mg/kg. For the *in vivo* study, we used a concentration of 300 mg/kg as a therapeutic dose. This concentration was determined based on the results of chronic toxicity study.

Treatment of tumor-bearing mice with *V. iphionoides* DCM extract could attenuate cancer progression, and this was noticed by the percentage of change in tumor size when compared with untreated mice.

Significant increase in tumor was noticed in all control groups having both cancer and diabetes. The increase in tumor size was much less in *V. iphionoides* DCM extract-treated groups. About one-fourth of animals were totally cured in the treated group compared with no cured animals at all in the control group. Dramatic tumor size reduction was noticed with non-diabetic cancer-bearing mice treated with the plant extract with a percentage of cured animals (70%) compared with untreated control groups and all mice could survive [Table 4]. These observed results suggest a strong effect of this plant extract on tumor progression when used to treat cancer and a positive but less effect on tumor progression when used to treat cancer in diabetic mice.

Several studies had verified that patients with diabetes and cancer have a poorer prognosis compared with those without diabetes. Diabetes and hyperglycemia are associated with shorter remission periods, shorter survival times, and higher mortality rates.[9,10,30]

The effect of *V. iphionoides* DCM extract on blood sugar levels was tested in all treated groups [Table 3]. There was a significant reduction in blood sugar values among the diabetic mice groups, with and without cancer. None of diabetic mice with cancer had been totally cured. In the control group, 25% of tumor-bearing diabetic mice treated with the plant extract were totally cured; this could be related also to the hypoglycemic effect of this extract [Table 5]. Hypoglycemic effect of *V. iphionoides* could be one of the factors that participate in tumor regression observed in this study. These results are consistent with Previous studies that showed the reduction of plasma glucose levels in tumor-bearing animals may be responsible, directly or indirectly, for the significantly prolonged survival compared to normal-fed controls.[31,32]

Phytochemical screening of *V. iphionoides* DCM extract indicated the presence of phytosterols, in addition to terpenoids [Table 6]. It is known that DCM is specially used for the selective extraction of terpenoid.[33] The presence of these two phytochemicals may be related to the strong

Table 3: The half maximum inhibitory concentration values of *Varthemia iphionoides* and *Pelargonium graveolens* different extracts

Plant extract	IC_{50} (μg/ml)			
	EMT6 cell line	MCF cell line	T47D cell line	Vero normal cells
Varth/DCM	120±5.0	270±10.0	160±5.3	>10,000
Varth/Water	998.8±2.8	2000±20.0	1050±50.0	>10,000
Varth/Meth	145±5.0	330±10.0	210±5.0	>10,000
Varth/Water/Meth	520±8.7	720±13.0	900±10.0	>10,000
Pelar/DCM	450±5.0	650±10.0	700±10.0	>10,000
Pelar/Water	410±20.0	590±5.0	610±10.0	>10,000
Pelar/Meth	998.28±57.0	950±10.0	790±10.0	>10,000
Pelar/Water/Meth	860±10.0	700±10.2	590±10.0	>10,000

IC_{50}: Half maximum inhibitory concentration; DCM: Dichloromethane; Meth: Methanol; Varth: *V. iphionoides*; Pelar: *P. graveolens*; *P. graveolens*: *Pelargonium graveolens*; *V. iphionoides*: *Varthemia iphionoides*

Table 4: Effect of different treatments on tumor size and mortality

Group/treatment	Initial tumor size (mm^3)	Final tumor size (mm^3)	Change in tumor size %	Cured mice %	Percentage death
A1/vehicle	475.77	2611	+449±2.698	0	40
A2/plant extract 300 mg/kg	311.1	1060.78	+240±2.462*	25	11
A3/metformin (80 mg/kg)	397.02	1624	+309.80±2.556*	50	11
B1/vehicle	445.67	1783.44	+300.16±2.544	20	11
B2/plant extract	299.43	182.19	−39.1±1.032*	70	0
B3/metformin (80 mg/kg)	382.4	405.03	+6.00±1.747*	50	0

Data were expressed as mean±SEM. For statistical difference measurement, values of each treatment were compared with the negative control; *P<0.05. Group A1 was used as a negative control for Groups A2 and A3. Group B1 was used as a negative control for Groups B2 and B3. A1: Group with cancer and diabetes treated with vehicle; A2: Group with cancer and diabetes treated with *V. iphionoides* DCM extract (300 mg/kg); A3: Group with cancer and diabetes treated with metformin (80 mg/kg); B1: Control group with cancer not diabetic treated with vehicle; B2: Group with cancer not diabetic treated with *V. iphionoides* DCM extract (300 mg/kg); B3: Group with cancer not diabetic treated with metformin (80 mg/kg); SEM: Standard error of mean; DCM: Dichloromethane; *V. iphionoides: Varthemia iphionoides*

anticancer properties of this plant extract. Terpenoids are a large group of natural compounds, which had proved opportunities in cancer therapy.[34,35] Phytosterols which are plant sterols structurally similar to cholesterol[36,37] also enable programmed antitumor responses.[38] Remarkable inhibitory actions on lung, stomach, ovarian, and breast cancers were reported for phytosterols via multiple mechanisms of action.[39,40]

Furthermore, in our attempt to correlate the anticancer activity of *V. iphionoides* DCM extract to its phytochemical composition, antioxidant test (ABTS) was performed. An antioxidant value for the extract suggested an alternative mechanism for the observed anticancer and hypoglycemic effects. The highest antioxidant activity was observed in *V. iphionoides* water extract. This result could be explained by the presence of phenolic compounds in this extract.[41] Phenolics were not detected in V. *iphionoides* DCM extract as indicated by phytochemical screening tests.

H and E staining procedure is one of the principle techniques in histology, and it is widely used in medical diagnosis. Applying H and E stains to tumor sections of approximately similar volume from different study groups revealed the ability of *V. iphionoides* DCM extract to induce necrosis in wide regions in tumors implanted in mice [Figure 1]. Our results are consistent with previous findings where DCM extract displayed a significant cytotoxic activity against HeLa cell line in culture and aponecrotic cell death confirmed using H and E morphological staining.[42]

Table 5: Effect of different treatments on blood sugar

Group/treatment	Percentage change in blood sugar
A1/vehicle	+33±0.016
A2/plant extract	−19±0.074*
A3/metformin (80 mg/kg)	−28±0.055*
C1/plant extract	−2.02±0.002
C2/vehicle	1.30±0.001*

$$\text{Percentage change in blood sugar} = \frac{\text{Final blood glucose value - initial blood sugar value}}{\text{Initial blood glucose value}} \times 100$$

Data were expressed as mean±SEM. For statistical difference measurement, values of each treatment were compared with the negative control; *P<0.05. Group A1 was used as a negative control for Groups A2 and A3. Group C1 was used as a negative control for C2. A1: Group with cancer and diabetes treated with vehicle; A2: Group with cancer and diabetes treated with *V. iphionoides* DCM extract (300 mg/kg); A3: Group with cancer and diabetes treated with metformin (80 mg/kg); C1: Diabetic group as control; C2: Diabetic group treated with plant extract; SEM: Standard error of mean; DCM: Dichloromethane; *V. iphionoides: Varthemia iphionoides*

Another targeted pathway in cancer treatment mechanism responsible for the observed anticancer activity is an induction of apoptosis (the process of programmed cell death). In cancer, this process is not working due to the upregulation of antiapoptotic genes and downregulation of apoptotic genes, so cells will continue in division and proliferation.[43] *V. iphionoides* DCM extracts were able to induce apoptosis in tumor-bearing mice [Figure 2]. Even no previous works had dealed with *V. iphionoides* extracts to detect

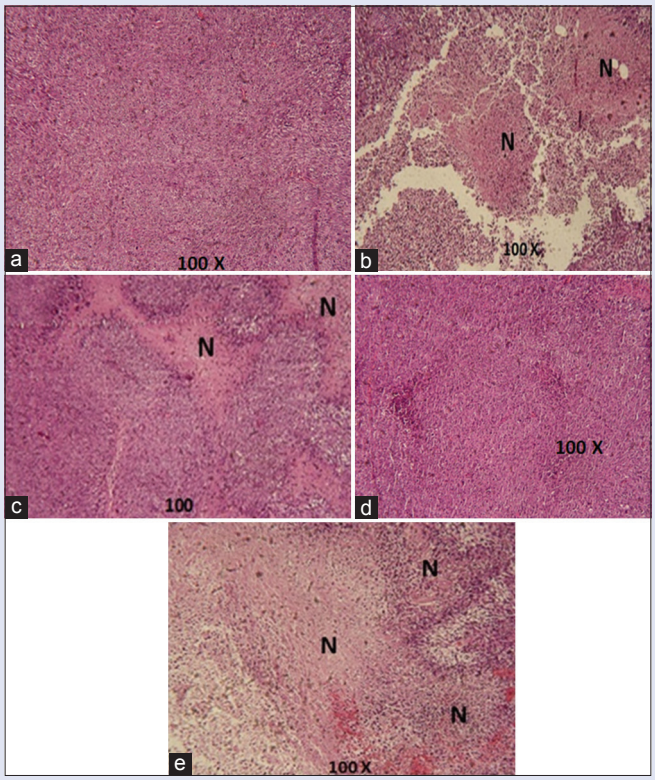

Figure 1: Hematoxylin and eosin staining of tumors. Control group with cancer and diabetes treated with vehicle (a), group with cancer and diabetes treated with plant extract (b), group with cancer and diabetes treated with metformin (80 mg/kg) (c), control group with cancer not diabetic treated with vehicle (d), group with cancer not diabetic treated with *Varthemia iphionoides* dichloromethane extract (300 mg/kg) (e). N: Necrotic area

Table 6: Preliminary phytochemical screening of different solvent extracts of (a) *Varthemia iphionoides* and (b) *Pelargonium graveolens*

(a) *Varthemia iphionoides* extracts							
V. iphionoides	**Phytochemical test result**						
	Flavonoids	**Terpenoids**	**Tannins**	**Saponins**	**Phenols**	**Alkaloids**	**Phytosterol**
Water	+	+	−	+	+	−	/
Water/Meth	+	+	−	+	+	−	/
Meth	+	+	−	−	+	−	/
DCM	−	+	−	−	−	−	+
(b) *Pelargonium graveolens* extracts							
P. graveolens	**Phytochemical test result**						
	Flavonoids	**Terpenoids**	**Tannins**	**Saponins**	**Phenols**	**Alkaloids**	**Phytosterol**
Water	+	+	−	−	+	−	/
Water/Meth	+	+	−	−	+	−	/
Meth	+	+	−	−	+	−	/
DCM	−	+	−	−	−	−	+

+: Present; −: Absent;/: Not tested; DCM: Dichloromethane; Meth: Methanol; *P. graveolens*: *Pelargonium graveolens*; *V. iphionoides*: *Varthemia iphionoides*

apoptosis in tumor cells; many studies had shown induction of apoptosis for other plants' DCM extract. Results of these studies indicate an effective inhibition of growth and apoptosis induction in cancer treated with DCM fractions.[44] Other data suggest the potential application of DCM extracts to treat breast cancer by apoptosis induction.[45]

VEGF is a potent signal protein that stimulates angiogenesis. Upregulation of VEGF is well defined in many tumor types, and blocking or inhibition of this pathway is an attractive target in cancer prevention and therapy.[46] Blocking angiogenesis deprives tumors of oxygen and nutrients which result in the reduction of tumor proliferation and expansion. Angiogenesis inhibition has a role in the observed antiproliferative effect of *V. iphionoides* DCM; in this study, the inhibition of VEGF expression was most obvious in cells treated with *V. iphionoides* DCM extract as indicated by the value of VEGF level of (90 pg/ml), compared with untreated EMT6/P cells' VEGF level (330 pg/ml) [Table 7]. No previous studies had tested *V. iphionoides* extracts for its ability to suppress VEGF expression, but some experiments showed that the inhibition of cancer in mice was significant in the DCM extract-treated mice, and the antitumor effect may be associated with the downregulation of the expression of VEGF.[47]

Changes in the immune system due to the exposure to *V. iphionoides* DCM extract were also explored through measuring levels of IFN-γ, IL-2, IL-4, and IL-10 [Table 8]. It was noticed that there was increasing in the production of IL-2 and IFN-γ, which are key cytokines in Th1 antitumor immune response in all bearing tumor groups. Such results can be explained by the ability of *V. iphionoides* DCM extract to stimulate the immune system. Levels of IL-4 did not elevate significantly in any plant extract-treated cancer group, and this suggests that *V. iphionoides* DCM extract stimulates Th1 and not Th2 immune response. In normal cases, there is a balanced ratio of Th1/Th2 cytokines. Increased concentrations of Th2 cytokines were observed in patients having different tumor types,[48] and this was noticed with cancer-bearing mice.

Table 7: The effect of *Varthemia iphionoides* dichloromethane extract (150 µg/ml) on vascular endothelial growth factor expression level, compared with vascular endothelial growth factor values obtained from negative control (nontreated cancer cells) and mouse normal control

Treatment	VEGF concentration (pg/ml)
Mouse normal control	80±7.071
VDCM extract	90±14.142*
Doxorubicin	160±7.071*
Negative control	330±21.213

*P<0.05. Data were expressed as mean±SEM. For statistical difference measurement, values of each treatment were compared with the negative control. SEM: Standard error of mean; VEGF: Vascular endothelial growth factor

IL-10 levels in all groups were high compared with normal mice (P < 0.05) but much less than that of tumor-bearing mice and tumor-bearing diabetic mice. IL-10 which may suppress the expression of Th1 cytokines also can enhance B-cell survival, proliferation, and antibody production.[49] Elevation in IL-10 level indicates activation of the Treg.[50] This could conclude that immune modulation may be resulted from plant extract which enhanced the

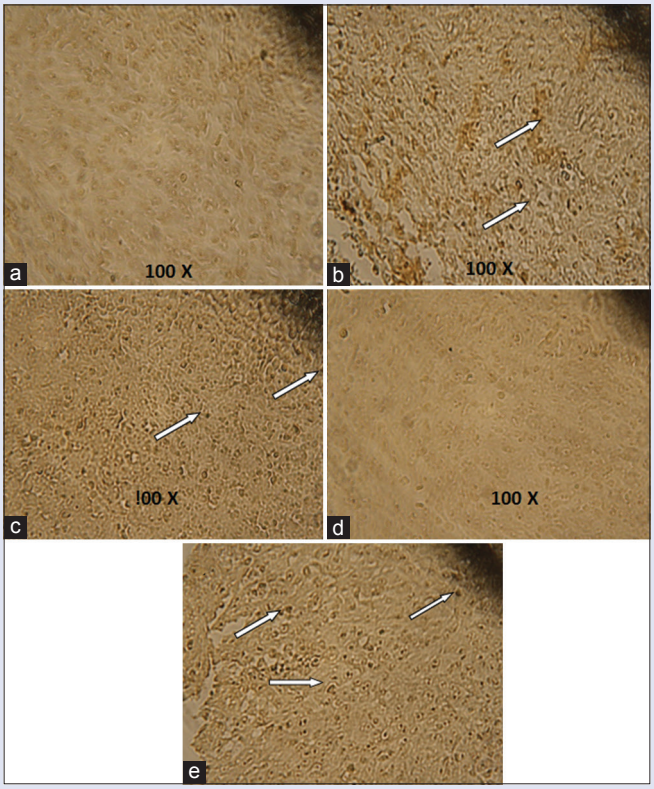

Figure 2: Colorimetric TUNEL assay for detection of apoptosis in tumor sections. Control group with cancer and diabetes treated with vehicle (a), with cancer and diabetes treated with *Varthemia iphionoides* dichloromethane (300 mg/kg) (b), with cancer and diabetes treated with metformin (80 mg/kg) (c), control with cancer not diabetic treated vehicle (d), with cancer not diabetic treated with *Varthemia iphionoides* DCM extract (300 mg/kg) (e). Arrow: Toward nucleus of dead cells

Table 8: The effect of different treatments on interferon-γ, interleukin-2, interleukin-4, and interleukin-10

Treatment	IL-10	IL-4	IL-2	IFN-γ
A1/nontreated	155.0±7.071#	130.0±1.414#	100.0±14.142#	126.0±8.485#
A2/extract	122.0±3.535*,#	95.0±7.071*,#	212.5±17.677*,#	105.0±7.071*,#
A3/metformin	55.0±7.071*	122.5±3.535*	180.0±14.142*,#	107.5±17.677*,#
B1 nontreated	31.0±2.828#	320.0±28.284#	74.0±5.656	160.0±14.142#
B2 VDCM extract	45.0±7.071*,#	237.5±53.033#	115.0±7.071*,#	395.0±134.350*,#
B3 metformin	68.5±3.535*,#	306.0±8.485#	245.0±63.639*,#	114.5±26.162*,#
C1/vehicle	34.5±7.778#	190.0±56.568#	74.0±5.656	80.0±14.142
C2/VDCM extract	62.5±3.535*,#	107.5±24.748*,#	85.0±7.071*,#	97.5±24.748*
Normal control	18.5±0.707	116.5±4.949	64.5±6.363	58.2±16.617

Data were expressed as mean±SEM. For statistical difference measurement, values of each treatment were compared with the negative control, *P<0.05; #P<0.05 compared with normal control. Group A1 was used as a negative control for Groups A2 and A3. Group B1 was used as a negative control for Groups B2 and B3. Group C1 was used as a negative control for C2. All groups were compared with the normal control. A1: Group with cancer and diabetes treated with vehicle; A2: Group with cancer and diabetes treated with *V. iphionoides* DCM extract (300 mg/kg); A3: Group with cancer and diabetes treated with metformin (80 mg/kg); B1: Control group with cancer not diabetic treated with vehicle; B2: Group with cancer not diabetic treated with *V. iphionoides* DCM extract (300 mg/kg); B3: Group with cancer not diabetic treated with metformin (80 mg/kg); C1: Diabetic group as control; C2: Diabetic group treated with plant extract; IL: Interleukin; SEM: Standard error of mean; DCM: Dichloromethane; *V. iphionoides: Varthemia iphionoides*

Th1 anticancer immune response and reduced the inhibitory effect of IL-10.

In order to evaluate whether the administration of *V. iphionoides* DCM extract might result in hepatic and/or nephrotoxicity, serum levels of creatinine, AST, and ALT were measured for all treatments. High levels of AST and ALT were detected in all treatments. However, the increase in the level of AST and ALT was much more with groups having diabetes or cancer or both diseases [Table 9]. This elevation may be justified by the presence of the disease itself. In *V. iphionoides* DCM extract-treated groups, creatinine levels were closer to the normal control values [Table 10].

Table 9: Aspartate aminotransferase and alanine aminotransferase levels for different treatments

Treatment	AST (IU/L)	ALT (IU/L)
A1/nontreated	91.71±1.212$^{\#}$	51.29±1.619$^{\#}$
A2/VDCM extract	61.26±1.506$^{*,\#}$	42.32±1.644$^{*,\#}$
A3	48.80±0.141$^{*,\#}$	27.39±0.427$^{*,\#}$
B1/nontreated	93.89±2.750$^{\#}$	31.18±0.579$^{\#}$
B2/VDCM extract	60.06±0.749$^{*,\#}$	37.86±1.513$^{*,\#}$
B3/metformin	71.15±0.813$^{*,\#}$	16.11±0.629*
C1/VDCM extract	46.70±0.494$^{\#}$	18.7±0.494$^{\#}$
C2/nontreated	62.65±2.584$^{*,\#}$	20.75±0.530$^{*,\#}$
Normal control	56.63±1.859	16.83±0.728

Data were expressed as mean±SEM. For statistical difference measurement, values of each treatment were compared with the negative control; **P*<0.05. All groups were compared with the normal control; #P<0.05. Group A1 was used as a negative control for Groups A2 and A3. Group B1 was used as a negative control for Groups B2 and B3. Group C1 was used as a negative control for C2. All groups were compared with the normal control. A1: Group with cancer and diabetes treated with vehicle; A2: Group with cancer and diabetes treated with *V. iphionoides* DCM extract (300 mg/kg); A3: Group with cancer and diabetes treated with metformin (80 mg/kg); B1: Control group with cancer not diabetic treated with vehicle; B2: Group with cancer not diabetic treated with *V. iphionoides* DCM extract (300 mg/kg); B3: Group with cancer not diabetic treated with metformin (80 mg/kg); C1: Diabetic group as control; C2: Diabetic group treated with plant extract; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; SEM: Standard error of mean; DCM: Dichloromethane; *V. iphionoides*: *Varthemia iphionoides*

Table 10: Serum creatinine levels for different treatments among groups compared with normal control group with cancer and diabetes treated with vehicle

Treatment	Creatinine (μmol/L)
A1/nontreated	0.550±0.014$^{\#}$
A2/VDCM extract	0.465±0.049$^{\#}$
A3/metformin	0.820±0.028$^{*,\#}$
B1/nontreated	0.610±0.021$^{\#}$
B2/VDCM extract	0.485±0.021$^{*,\#}$
B3/metformin	0.475±0.021*
C1/VDCM extract	0.630±0.042$^{\#}$
C2/nontreated	0.535±0.120$^{\#}$
Normal control	0.440±0.042

Data were expressed as mean±SEM. For statistical difference measurement, values of each treatment were compared with the negative control, **P*<0.05. Group A1 was used as a negative control for Groups A2 and A3. Group B1 was used as a negative control for Groups B2 and B3. Group C1 was used as a negative control for C2. All groups were compared with the normal control, $^{\#}$*P*<0.05. A1: Group with cancer and diabetes treated with vehicle; A2: Group with cancer and diabetes treated with *V. iphionoides* DCM extract (300 mg/kg); A3: Group with cancer and diabetes treated with metformin (80 mg/kg); B1: Control group with cancer not diabetic treated with vehicle; B2: Group with cancer not diabetic treated with *V. iphionoides* DCM extract (300 mg/kg); B3: Group with cancer not diabetic treated with metformin (80 mg/kg); C1: Diabetic group as control; C2: Diabetic group treated with plant extract; SEM: Standard error of mean; DCM: Dichloromethane; *V. iphionoides*: *Varthemia iphionoides*

CONCLUSION

V. iphionoids DCM extract represents a newly proposed mechanism to target cancer and diabetes when present in the same animal. It inhibits cell growth of breast cancer cell lines (EMT6, MCF-7, and T47D) and was safe on normal cells. Furthermore, it inhibits tumor growth and progression of breast cancer inoculated in mice. Antitumor activity of this plant extract is mediated through induction of apoptosis, lowering blood glucose, inhibition of VEGF expression, and stimulation of immune system. However, further experimental studies are required to explore safety and to determine its mechanism of action. Furthermore, further phytochemical analysis is required in order to determine the specific chemical profile, mainly for terpenoids and phytosterols contents, which may be responsible for the observed effects.

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Conflicts of interest

There are no conflicts of interest.

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