Immunostimulatory and anti-inflammatory properties of *Silene succulenta* Forssk.

Mohamed A.M. El Gendy[a], Hemaia M. Motawe[b], Maii A.I. Maamoun[b], Radwa H. El-Akad[b], Ahmed H. Afifi[b]

[a]Department of Pharmacognosy, Drug Bioassay-Cell Culture Laboratory, [b]Department of Pharmacognosy, Pharmaceutical and Drug Industries Research Institute, National Research Centre, ElBohouth Street, (Former El Tahrir), P.O. 12622 Dokki, Giza, Egypt

Correspondence to Mohamed A.M. El Gendy, PhD, Department of Pharmacognosy, Drug Bioassay-Cell Culture Laboratory, Pharmaceutical and Drug Industries Research Institute, National Research Centre, 33 ElBohouth Street (Former El Tahrir), Dokki, PO Box 12622, Dokki, Giza, Egypt.
Tel: +20 111 473 6415, fax: +20 3337 0931;
e-mail: elgendy@ualberta.ca

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Background and objectives
Immunomodulation using medicinal plants is a useful alternative to classical conventional chemotherapy for several diseases, including cancer and autoimmune disorders. Therefore, the aim of this study was to investigate the immunomodulatory properties of *Silene succulenta* Forssk. extract that is a common herb growing in Egypt.
Materials and methods
S. succulenta was collected from Mediterranean coast region of Egypt, dried in shade, ground, and extracted using methanol. The effect of the plant extract on immune cells was tested using murine splenocytes and human peripheral blood mononuclear cells (PBMCs) proliferation colorimetric assays. A bioassay-directed fractionation was applied to identify the most active fraction and gas chromatography–mass spectrometry (GC–MS) was used to analyze it. The effect of the plant extract and its active fraction on human interleukin-2 (IL-2) was determined using enzyme-linked immunosorbent assay. Furthermore, the effect on macrophage nitric oxide production ability, macrophage viability, and HL-60-differentiated cells to neutrophil ROS production was tested.
Results and conclusion
Our results demonstrated that *S. succulenta* extract induced murine splenocytes and PBMCs proliferation in a concentration-dependent manner. Through bioassay-directed fractionation, we identified hexane subfraction as the active fraction of the extract and identified 18 compounds using GC–MS with major components of palmitic acid (33.19%) and phthalic acid, di(6-methylhept-2-yl) ester (20.22%). The extract and its active fraction induced human IL-2, whereas they inhibited macrophage nitrite-production ability, and neutrophil reactive oxygen species production level. We concluded that *S. succulenta* possesses several immunostimulatory and anti-inflammatory properties that can be utilized to treat several diseases.

Keywords:
Interleukin-2, Peripheral blood mononuclear cells proliferation, *Silene succulenta*, Splenocytes proliferation

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Introduction

Immunomodulation using medicinal plants can be considered as a useful alternative to classical conventional chemotherapy for several diseases. Immunomodulation can be used, especially when there is a need to activate host's impaired immune response in certain diseases, including cancer, or when a selective immunosuppression is required in situations like autoimmune disorders [1]. Several plants showed promising immunomodulatory properties such as *Polygala senega* L., *Viscum album* L., and *Asparagus racemosus* Willd [2,3]. In addition, other herbal plants have been considered as possible biological-response modifiers and adjunct cancer therapies such as ginseng (*Panax ginseng* C.A.Mey.), mistletoe (*Viscum album* L.), echinacea (*Echinacea purpurea* (L.) Moench, *E. angustifolia* DC., and *E. pallida* (Nutt.) Nutt.), and black cumin (*Nigella sativa* L.). Such plants induce the immune system to secrete several signals and mediators, including numerous cytokines [1,2,4].

For example, ginseng induces proliferation of lymphocytes, maturation and activation of macrophages, dentritic, and natural killer cells with elevated levels of several cytokines and mediators such as interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-12, TNF-α, INF-γ, and nitric oxide. These effects were attributed to several mechanisms including activation

of mitogen-activated protein kinase (MAPK) and nuclear factor κ-light-chain enhancer of activated B cell (NF-κB) signaling pathways, which both are under the control of toll-like receptors [5]. Another example is echinacea that possesses immunostimulatory and anti-inflammatory properties by inducing NK cells and macrophage activations and dentritic cell differentiation accompanied by elevated levels of IL-1, IL-6, IL-10, IL-12, TNF-α, INF-β, and nitric oxide. Moreover, it inhibited cyclooxygenase and 5-lipoxygenase activity. Similar to ginseng, MAPK and NF-κB are 2 main mechanisms of echinacea immunomodulatory/anti-inflamatory effects [1].

Silene succulenta Forssk. (family: Caryophyllaceae) is a common herb growing in the Northwest Mediterranean coast region of Egypt and has been widely used as a remedy for several diseases, including cancer [6]. Despite there is a report that the extract can affect macrophage functions and act as an antioxidant [7], however, a detailed immunologic investigation of the plant extract is yet to be determined. The main objective of the current study is to investigate the immunomodulatory properties of *S. succulenta* extract. The immunologic effect of the extract was tested using murine splenocytes and human peripheral blood mononuclear cells (PBMCs) proliferation assays, identifying the most active fraction using a bioassay-directed fractionation, analyzing it using gas chromatography–mass spectrometry (GC–MS), determining the level of human IL-2, testing the effect on macrophage nitric oxide production ability and viability, and finally testing the effect on ROS production of HL-60 differentiated to neutrophils.

Materials and methods

Chemicals

Roswell Park Memorial Institute (RPMI) 1640 medium, histopaque 1077, concanavalin A (Con A), phytohemagglutinin (PHA-M), LPS from *E. coli* O127:B8, Griess reagent, all-trans retinoic acid (RA), and [3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazoliumbromide] (MTT) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Colorless RPMI media, penicillin–streptomycin, trypsin/EDTA solution, L-glutamine, fetal bovine serum (FBS), and human IL-2 ELISA kit (Catalog number: EH2IL2) were obtained from Invitrogen (Carlsbad, California, USA). Nitroblue tetrazolium (NBT) was obtained from Loba Chemie, India, miglumine indomethacinate (Liometacen ampouls) was from El-Nile Co. for Pharmaceuticals (Egypt).

Plant collection, extraction, and fractionation

S. succulenta herb was collected in Spring 2020 from Halazine valley, Marsa Matrouh, Northwest Mediterranean coast region of Egypt, GPS coordinates: Latitude: 31.3529, Longitude: 27.23725. The plant was identified by an expert of plant taxonomy, Dr. Ashraf Soliman, Faculty of Science, Cairo University, and a voucher specimen was kept in the herbarium of our department with a given number B-267.

The plant was dried in solar oven with temperature below 40°C in shade, and ground using gutter mill. Hundred grams of the powdered plant was extracted by maceration using 450 ml methanol, concentrated under reduced pressure using Rotavapour (Buchi-R114, Flawil, Switzerland), freeze-dried, and finally stored in our laboratory in −20°C freezers (25% w/w).

Dried crude extract was suspended in water and was successively and exhaustively fractionated, by liquid–liquid partitioning, with ethyl acetate, then by distilled water to provide nonpolar and polar fractions, respectively. Thereafter, a further fractionation of the ethyl acetate fraction was carried out between hexane and methanol. Organic fractions were separated and dried under reduced pressure using rotavapour, whereas the aqueous fraction was freeze-dried. All fractions were kept in a freezer until tested biologically [8].

Gas chromatograph–mass spectrometry

GC–MS analysis was carried out for the active hexane subfraction of *S. succulenta* extract using a Shimadzu GC–MS-2010 Plus (Koyoto, Japan) equipped with Rxi-1MS-fused bonded column (30 m×0.25-mm i.d.×0.25-μm film thickness) (Restek, USA) equipped with a split–splitless injector. The temperature of the column started with 50°C for 3 min (isothermal), programmed to reach 300°C at a rate of 5°C/min, and kept constant at 300°C for 10 min (isothermal). Injector temperature was set at 280°C and helium carrier gas flow rate was kept at 1.37 ml/min. Mass spectra were recorded applying the following condition: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 220°C. The sample was diluted to 1% v/v and was injected with split mode (split ratio 1 : 15). Identification of each compound is based on its mass spectrometric data and retention indices in comparison with those of the National Institute of Standards and Technology (NIST) Mass Spectral Library (December 2011) and literature.

Cell culture

Raw 264.7 is a murine monocyte/macrophage cell line and HL-60 is a human acute promyeloblastic leukemia cell line, and both cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI media, supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in 75-cm^2 tissue culture flasks at 37°C in a humidified CO_2 incubator. Cells were subcultured every 3–5 days using cell scrapper or centrifugation for Raw 264.7 cells and HL-60 cells that grow as suspended cells, respectively.

Animals

Six male Swiss Albino mice weighing 20–22 g were obtained from the Theodor Bilharz Institute (Giza, Egypt) and used for the preparation of murine splenocytes. A conditioned atmosphere of 22±2°C was maintained and kept on a standard pellet diet and water *ad libitum*. Animal procedures were approved and in accordance with the regulations of the National Research Centre Medical Research Ethics Committee, Egypt, final approval number 19-296.

Human volunteers

Three human volunteers were employed for blood donation. The volunteers were selected between 45 and 55 years old with a good health profile. All procedures for collecting blood samples were approved by the National Research Centre Medical Research Ethics Committee, Egypt, and according to the regulations of Declaration of Helsinki–ethical principles for medical research, registration number 19-296.

Murine splenocytes proliferation assay

Swiss Albino male mice (n=6) were sacrificed using cervical dislocation technique, spleens were aseptically removed, and cell suspension from each mouse was prepared. Increasing concentrations of *S. succulenta* extract were incubated with splenocyte suspension in RPMI media with 10% heat-inactivated FBS from each mouse in 96-well microplates (2.5×10^5 cells/well) at 37°C and 5% CO_2 atmosphere. Con A (5 mcg/ml) was used as a positive control as it is an activator of T-lymphocyte proliferation. After 72 h of incubation, 40 µl of 8 mM MTT solution was added to each well for 4 h. Thereafter, the plates were centrifuged using plate rotor at 3000 rpm for 20 min and media were decanted, 100 µl of isopropanol was added to each well, and incubated in a humidified CO_2 incubator at 37°C for 1 h to dissolve the formazan crystals. The absorbance was measured using a Biochrom Anthos Zenyth 200-multiwell microplate reader (Bio-chrom Ltd, USA) at 570 nm and a reference wavelength of 690 nm. Splenocytes proliferation index (PI) was calculated according to the formula: ((Reading of treatment-Reading of DMSO)/Reading of DMSO) × 100 [9].

Human PBMCs proliferation assay

Blood samples (15 ml per volunteer) from three healthy donors were collected in heparin-treated 50-ml Falcon centrifuge tubes. Thereafter, samples were diluted by adding the same volume of sterile PBS in the tubes and mixed gently. Density-gradient Histopaque 1077 solution (20 ml) was carefully pipetted in another 50-ml sterile Falcon tube. Equal volume of diluted blood sample from each donor was gently overlaid onto the density-gradient reagent. Tubes were centrifuged at 400g for 30 min at room temperature, which separate the PBMCs from other blood components [10]. PHA-M (10 mcg/ml) was used as a positive control as it stimulates T-lymphocyte proliferation. All other procedures are similar to murine splenocytes proliferation assay [11,12].

Determination of human interleukin-2 (IL-2) level

PBMCs were isolated from three male human volunteers and distributed in 24-well cell culture plates with a density of 10^6 cell/well in colorless RPMI 1640 media, containing 10% heat-inactivated FBS. PBMCs were incubated with increasing concentration of *S. succulenta* extract or PHA-M (10 mcg/ml) as a positive control for 24 h at 37°C in a humidified CO_2 incubator. Thereafter, media with cells were collected in 1.5-ml centrifuge tubes, centrifuged at 400g for 5 min, the supernatant was isolated, and the concentration of IL-2 was determined using human IL-2 ELISA kit (EH2IL2, Invitrogen, USA) [13].

Nitric oxide assay

RAW 264.7 cells were seeded in 24-well plates at a density of 5×10^5 cells/well and incubated for 24 h at 37°C and 5% CO_2. Thereafter, media of each well were aspirated and fresh FBS-free RPMI media were replaced. Different concentrations of plant extracts were incubated with the cells for 1 h before stimulation with 1 mcg/ml of LPS for 24 h. The presence of nitrite was determined in cell culture media using Griess reagent. Briefly, 50 mcl of cell culture medium with an equal volume of Griess reagent in a new 96-well plate was incubated at room temperature for 10 min. Then, the absorbance was measured at 540 nm using Biochrom Anthos Zenyth 200-multiwell microplate reader (Bio-chrom

Ltd, USA). The amount of nitrite in the media was calculated from sodium nitrite ($NaNO_2$) standard curve [14].

Macrophage-viability assay

After reading nitric oxide assay, media were decanted, and the viability of Raw 264.7 cells was determined by incubation with MTT salt as mentioned under murine splenocytes proliferation assay [7].

ROS production of HL-60-differentiated neutrophils

All-trans-retinoic acid (RA) was used for generating neutrophils from HL-60 cells. RA from stock solution was added to a final concentration of 1 μM and incubated for 1 week. Cell density was rigorously kept at 1.5×10^5 cells/ml as the percentage of differentiated cells depended on cell density. *S. succulenta* extract/fractions were incubated with HL-60 differentiated to neutrophils (250,000 cells/1.5-ml tube) for 1 h before incubation with LPS (1 mcg/ml) as a known activator of neutrophils for another hour. Cell activation and generated reactive oxygen species was assessed using nitroblue tetrazolium solution (NBT) 0.2% w/v. Isopropanol (100 μl) was added to each tube for 20 min and the color intensity was measured at 570/690 nm [15,16].

Statistical analysis

All results are expressed as mean±SEM, and statistical analysis of each treatment group was determined using one-way ANOVA followed by Student–Newman–Keuls post hoc test. SigmaStat 3.5 program for Windows, Systat Software Inc. (San Jose, California, USA) was used to carry out statistic operations of all experiments.

Results and discussion

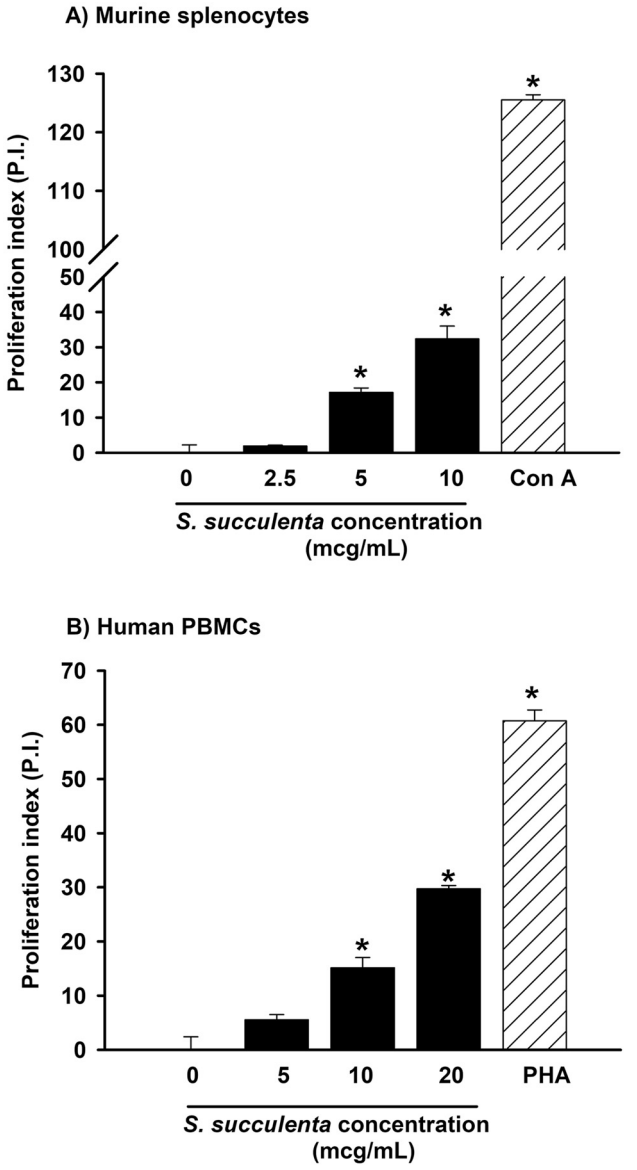
Effect of *S. succulenta* methanolic extract on murine splenocytes proliferation

To investigate the immunomodulatory effects of *S. succulenta* herb methanolic crude extract, several experiments were adopted. First, we tested the effect of *S. succulenta* methanolic crude extract on murine splenocytes proliferation. Our results showed that *S. succulenta* methanolic crude extract induced murine splenocytes proliferation in a concentration-dependent manner with (2±0.2)%, (17±1.3)%, and (32±3.6)% increase in PI with 2.5, 5, and 10 mcg/ml, respectively (Fig. 1a). The effect was statistically significant at 5 and 10 mcg/ml. Con A was used as a positive control that induced splenocytes PI by (125 ±0.9)% (Fig. 1a). It was mentioned previously that murine splenocytes consist mainly of lymphocytes and macrophages [17]. Our finding may suggest an effect of the plant extract on lymphocytes and/or macrophages.

Effect of *Silene succulenta* methanolic extract on PBMCs proliferation

To confirm the obtained results on murine immunologic cells, it was necessary to use human immunologic cells from healthy volunteers. Similar results were obtained when increasing concentrations of *S. succulenta* methanolic crude extract were incubated with PBMCs obtained from three human volunteers. *S. succulenta* methanolic crude extract significantly induced PBMCs proliferation with (5±0.94)%, (15 ±1.9)%, and (30±0.63)% increase in P.I. with 5, 10,

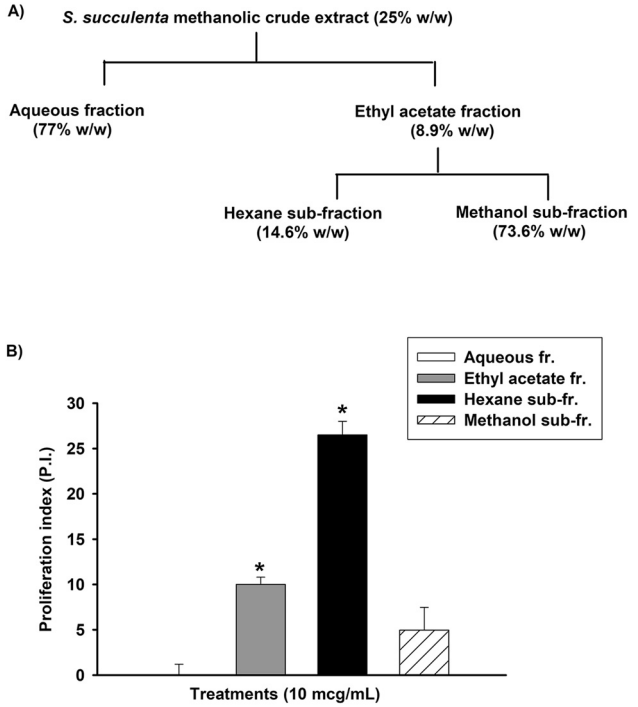
Figure 1



Effect of *Silene succulenta* extract on murine splenocytes proliferation (a) and human peripheral blood mononuclear cells (PBMCs) proliferation (b). Data were expressed as average proliferation index (P.I.), ±SEM (n=3). *P value less than 0.05 compared with the same treatment of DMSO-treated group.

and 20 mcg/ml, respectively (Fig. 2b). The effect was significantly different at higher concentrations, namely, 10 and 20 mcg/ml. Higher concentrations were used with human PBMCs due to the observed reduced sensitivity of human immune cells compared with murine splenocytes. PBMCs composed mainly of lymphocytes (70–90%), monocytes (10–20%), and minute amount of dendritic cells (1–2%) [13]. Both murine splenocytes and PBMCs proliferation models were widely used to test the immunomodulatory effects of several compounds and herbal extracts [9,17–19].

Figure 2



A flowchart of the process of fractionation of *S. succulenta* extract (a) and a bioassay-directed fractionation using PBMCs proliferation assay (b). Data were expressed as average P.I., ±SEM (n=3). **P* value less than 0.05 compared with the same treatment of DMSO-treated PBMCs.

Bioassay-directed fractionation and GC–MS analysis

As indicated in Fig. 2, the ethyl acetate fraction significantly induced human PBMCs proliferation activity more than the aqueous fraction. Moreover, further fractionation of ethyl acetate showed that hexane subfraction induced PBMCs proliferation by (26.5±1.5)%, whereas the methanol subfraction induced it by (5±2.5)% (Fig. 2a and b). Therefore, we chose hexane subfraction for GC–MS analysis and for further experiments.

The GC–MS profiling of the n-hexane fraction of *S. succulenta* herb revealed the identification of 18 compounds as presented in Table 1. Palmitic acid was the major identified compound and represents 33.19% of the total fraction followed by phthalic acid, di(6-methyl-hept-2-yl) ester (20.22%). Other fatty acids/esters, including myristic acid (1.04%), stearic acid (0.94%), and palmitic acid methyl ester (2.97%), have been also identified (Table 1). Moreover, aromatic compounds, saturated and unsaturated aliphatic hydrocarbons, fatty alcohol, fatty alcohol ester, and phytosterols were presented in the n-hexane subfraction (Table 1). In agreement with our hypothesis, it was mentioned that palmitic acid possesses several immunologic effects through

Table 1 Compounds identified by GC–MS in the n-hexane subfraction of *Silene succulenta*

Peak	Rt	Compound	Molecular formula	RI_{exp}	RI_{lit}	Content (%)	Identific-ation
1	5.350	p-Xylene	C_8H_{10}	850	850.5	0.94	MS, RI
2	7.993	Benzene, 1-ethyl-3-methyl	C_9H_{12}	944	944	0.63	MS, RI
3	7.993	Benzene, 1-ethyl-2-methyl	C_9H_{12}	975	968	1.43	MS, RI
4	9.723	Decane	$C_{10}H_{22}$	999	1000	0.64	MS, RI
5	12.915	Undecane	$C_{11}H_{24}$	1098	1100	2.63	MS, RI
6	16.015	Undecane, 2,4-dimethyl	$C_{13}H_{28}$	1198	1200	1.95	MS, RI
7	26.532	1-Hexadecene	$C_{16}H_{32}$	1585	1589	0.73	MS, RI
8	30.076	Tetradecanoic acid (myristic acid)	$C_{14}H_{28}O_2$	1737	1740	1.04	MS, RI
9	31.126	Octadecene	$C_{18}H_{36}$	1784	1793	0.90	MS, RI
10	33.624	Hexadecanoic acid, methyl ester (palmitic acid methyl ester)	$C_{17}H_{34}O_2$	1901	1909	2.97	MS, RI
11	34.514	Hexadecanoic acid (palmitic acid)	$C_{16}H_{32}O_2$	1945	1946	33.19	MS, RI
12	37.364	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-acetate	$C_{22}H_{42}O_2$	2090	2114	0.93	MS, RI
13	37.739	(Z,Z) 9,12-Octadecadien-1-ol	$C_{18}H_{34}O$	2097	2069	3.62	MS, RI
14	38.201	Octadecanoic acid (stearic acid)	$C_{18}H_{36}O_2$	2161	2134	0.94	MS, RI
15	41.338	4,8,12,16-Tetramethyl-heptadecan-4-olide	$C_{21}H_{40}O_2$	2308	2364	1.46	MS, RI
16	44.431	Phthalic acid, di(6-methyl-hept-2-yl) ester	$C_{24}H_{38}O_4$	2493	2475	20.22	MS, RI
17	55.628	Campesterol	$C_{28}H_{48}O$	3275	3305	2.61	MS, RI
18	62.176	24-methylenecycloartenol	$C_{31}H_{52}O$	3753		8.26	MS, RI

RI_{exp}, retention index, determined experimentally on an RXI-1MS column; RI_{lit}, published retention indices.

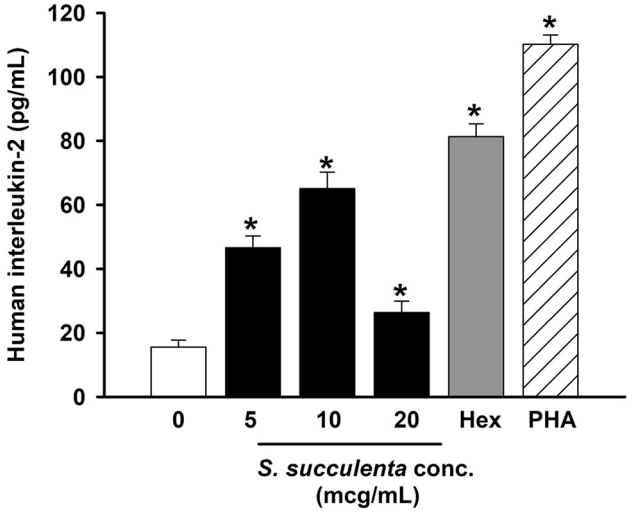
activation of dendritic cells (DCs) that can stimulate T-cell responses and regulate their differentiation [20].

Effect of *Silene succulenta* methanolic extract and its active hexane subfraction on human IL-2 level

The next step was to test whether the extract and its active fraction can induce the level of IL-2 as a known immune modulator released mainly by the helper T lymphocytes [21]. Indeed, the extract significantly induced the level of human IL-2 when compared with DMSO-treated cells with 2-, 3.2-, and 0.7-fold increase with 5, 10, and 20 mcg/ml of the extract, respectively (Fig. 3). Most importantly, the hexane-active subfraction (10 mcg/ml) significantly induced IL-2 level by five-fold when compared with vehicle-treated control (Fig. 3). PHA-M was used as a positive control that showed six-fold increase of human IL-2 at 10 mcg/ml (Fig. 3). Palmitic acid (PA) has been identified to be the major component of the active-hexane subfraction. As PA can stimulate T-cell response [20], it may justify the elevated level of IL-2 by the extract and its active-hexane subfraction.

IL-2 is a biological-response modifier that can improve the body's natural response to disease [22]. IL-2 stimulates growth and differentiation of T-cell response, it induces $CD8^+$ T cells and NK-mediated cytotoxic effector function, proliferation of and immunoglobulin (Ig) production by activated B-lymphocytes, and enhancement of antigen presentation by monocytes. It can be used in immunotherapy to treat cancer. It enhances the ability of the immune system to kill tumor cells and may interfere with blood flow to the tumor. Aldesleukin (Proleukin, ATC code L03AC01) is a recombinant IL-2 used in treating malignant melanoma and renal-cell carcinoma [22]. These findings confirm the immunostimulatory effects of the extract and may provide one of its modes of action as a promising immunostimulatory agent.

Figure 3

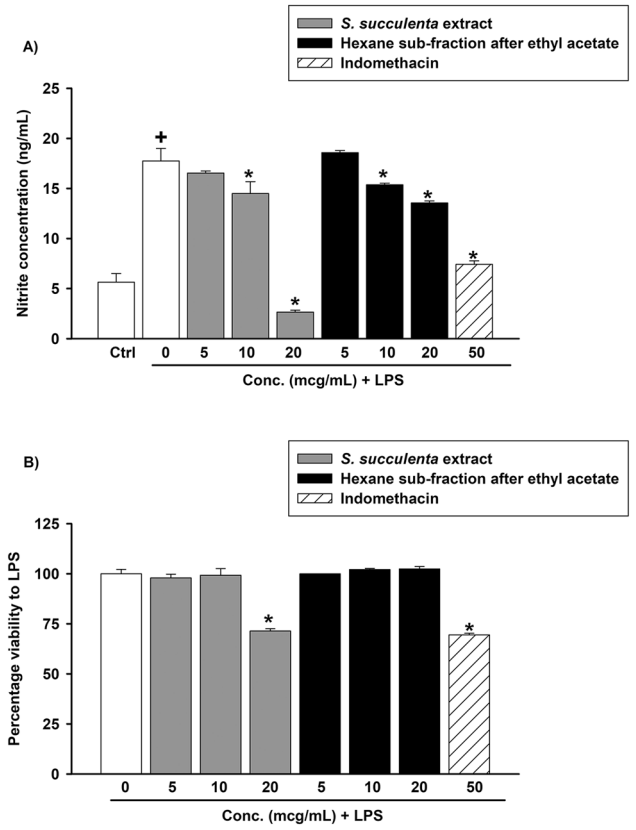


Effect of *Silene succulenta* extract and hexane subfraction (10 mcg/ml) on human IL-2 level. Data were expressed as average±SEM (n=3). *P value less than 0.05 compared with the same treatment of DMSO-treated peripheral blood mononuclear cells.

Effect of *Silene succulenta* methanolic extract and its active-hexane subfraction on Raw 264.7 cells

As the plant extract affected murine splenocytes and human PBMCs, and both of them consist mainly of lymphocytes and macrophages/monocytes, therefore, it was important to test the effect of the plant extract on macrophages exemplified by nitric oxide production (detected as amount of nitrite) and viability assays. Figure 4a depicts that LPS significantly induced the nitrite level by 3.15-fold, whereas pretreatment with the plant extract reduced that level in a concentration-dependent manner. The plant extract reduced LPS-mediated nitrite level by (6.8±0.2)%, (18.3±1.2)%, and

Figure 4

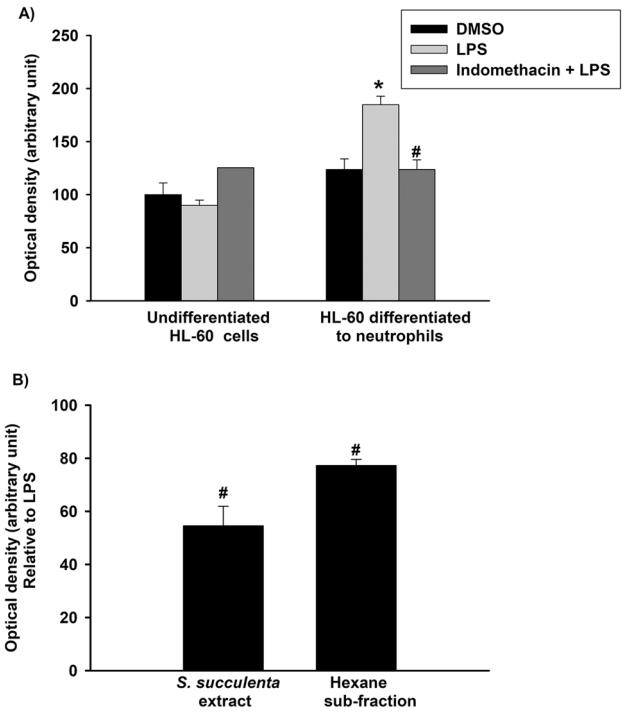


Effect of *Silene succulenta* extract on murine Raw 264.7 macrophage nitrite production (a), and cell viability (b). Griess reagent was used to determine the nitrite level. (b) After the 24-h incubation, viability was determined using MTT salt. Data were expressed as average±SEM (n=3). +P value less than 0.05 compared with control, *P value less than 0.05 compared with LPS.

(85±0.2)% with 5, 10, and 20 mcg/ml, respectively. The effect was statistically significant at 10 and 20 mcg/ml (Fig. 4a). Moreover, the obtained effect was not due to cytotoxicity toward macrophages as the extract showed only (29±1.15)% cytotoxicity at 20 mcg/ml (Fig. 4b).

To a lower degree, the hexane-active subfraction showed (0±0.21)%, (13.4±0.15)%, and (23.6±0.19)% reduction in LPS-mediated nitrite level with 5, 10, and 20 mcg/ml, respectively (Fig. 4a). Interestingly, the hexane-active subfraction did not show any cytotoxicity to macrophages (Fig. 4b). Indomethacin (50 mcg/ml) was used as a known anti-inflammatory and a positive control, which showed (58±0.34)% reduction of nitrite level that was accompanied with (31±0.8)% cytotoxicity (Fig. 4a and b). Our finding may suggest a modulatory effect on macrophage function with little or no effect on its viability. In agreement with our results, it was mentioned previously that *S. succulenta* can affect nitrite production in Raw 264.7 cells [7].

Figure 5



Differentiation of HL-60 to neutrophils (a) and effect of *Silene succulenta* extract and its active-hexane subfraction on LPS-mediated neutrophil ROS production (b). HL-60 was differentiated using all-trans-retinoic acid (RA, 1 μM) for 7 days and then treated with Silene succulenta extract or its active subfraction for 1 h before incubation with LPS (1 mcg/ml) for another 1 h before incubation with LPS (1 mcg/ml) for another 1 h. Thereafter, NBT was incubated for 0.5 h, the resultant blue formazan compound inside the cells was dissolved using isopropanol, and the absorbance was measured at 570/690 nm. Data were expressed as average±SEM (n=3). *P value less than 0.05 compared with control, #P value less than 0.05 compared with LPS.

Effect of *Silene succulenta* methanolic extract and its active-hexane subfraction on neutrophil function

Human neutrophils represent 60% of leukocytes and they are phagocytic cells that release several mediators in response to inflammatory and other immunological reactions [23]. NBT test measures ROS generated by leukocytes. NBT becomes reduced by free oxygen radicals forming a blue-black compound, formazan. The reaction can be monitored spectrophotometrically and it reflects the ROS-generating activity in the cytoplasm of the cells. Moreover, the results of NBT staining strongly correlate with ROS levels assessed by chemiluminescence [24].

As indicated in Fig. 5, differentiated neutrophils are stimulated by LPS treatment more than the nondifferentiated HL-60 cells that showed nearly no effect of LPS stimulation. Moreover, microscopic examination was carried out using Wright–Giemsa-stained cells confirming the structure of neutrophils. Pretreatment with *S. succulenta* extract or its active-hexane subfraction significantly reduced the ROS level generated by LPS treatment confirming the anti-inflammatory properties of the extract (Fig. 5).

Conclusion

S. succulenta extract possesses several immunomodulatory effects. It acts as immunostimulant extract by inducing proliferation of murine splenocytes and human PBMCs, and inducing human IL-2 levels. Furthermore, it possesses anti-inflammatory effects by inhibiting LPS-mediated macrophage nitrite-production level and LPS-mediated ROS in neutrophils.

Acknowledgments

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Conflicts of interest

There are no conflicts of interests.

References

1 Catanzaro M, Corsini E, Rosini M, Racchi M, Lanni C. Immunomodulators inspired by nature: a review on curcumin and echinacea. Molecules 2018; 23:2778.

2 Singh N, Tailang M, Mehta SC. A review on herbal plants as immunomodulators. Int J Pharma Sci Res 2016; 7:3602–3610.

3 Ganju L, Karan D, Chanda S, Srivastava KK, Sawhney RC, Selvamurthy W. Immunomodulatory effects of agents of plant origin. Biomed Pharmacother 2003; 57: 296–300.

4 Salem ML. Immunomodulatory and therapeutic properties of the Nigella sativa L. seed. Int Immunopharmacol 2005; 5:1749–1770.

5 Kim JH, Kim DH, Jo S, Cho MJ, Cho YR, Lee YJ, Byun S. Immunomodulatory functional foods and their molecular mechanisms. Exp Mol Med 2022; 54:1–11.

6 Badawy SA, Hassan AR, Abu El Wafa SA, Mohammad AI. UPLC-MS/MS profiling and antitumor activity of *Silene succulenta* Forssk growing in Egypt. Azhar Int J Pharm Med Sci 2021; 1:58–62.

7 Ghonime M, Emara M, Shawky R, Soliman H, El-Domany R, Abdelaziz A. Immunomodulation of RAW 264.7 murine macrophage functions and antioxidant activities of 11 plant extracts. Immunol Invest 2015; 44:237–252.

8 Afifi AH, El-Desoky AH, Kato H, Mangindaan REP, de Voogd NJ, Ammar NM, *et al.* Carteritins A and B, cyclic heptapeptides from the marine sponge Stylissa carteri. Tetrahedron Lett 2016; 57:1285–1288.

9 Courreges MC, Benencia F, Coulombie FC, Coto CE. In vitro and in vivo activities of Melia azedarach L. aqueous leaf extracts on murine lymphocytes. Phytomedicine 1998; 5:47–53.

10 Kizhakeyil A, Ong ST, Fazil M, Chalasani MLS, Prasannan P, Verma NK. Isolation of human peripheral blood T-lymphocytes. Methods Mol Biol 2019; 1930:11–17.

11 Ocklind G. Stimulation of human lymphocytes by phytohemagglutinin (PHA) in a new ultra-microtest plate. Immunobiology 1986; 171:339–344.

12 Ozaki M, Iwanami A, Nagoshi N, Kohyama J, Itakura G, Iwai H, *et al.* Evaluation of the immunogenicity of human iPS cell-derived neural stem/progenitor cells in vitro. Stem Cell Res 2017; 19:128–138.

13 Kleiveland CR. Peripheral blood mononuclear cells. In: Verhoeckx K, Cotter P, López-Expósito I, Kleiveland C, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H, editors. The impact of food bioactives on health: in vitro and ex vivo models. Cham (CH): Springer; 2015. 161–167.

14 Maksimovic Z, Malencic D, Kovacevic N. Polyphenol contents and antioxidant activity of Maydis stigma extracts. Bioresour Technol 2005; 96:873–877.

15 Moghanloo E, Ghorbani E, Beikverdi MS, Badameh P, Rezaei S, Piroozmand A, *et al.* The netosis formation of HL-60 cell differentiated to neutrophil-like cells by LPS. J Human Environ Health Promotion 2018; 4:138–143.

16 Breitman TR, Selonick SE, Colins SJ. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci 1980; 77:2936–2940.

17 Hwang SA, Dasgupta A, Actor JK. Cytokine production by non-adherent mouse splenocyte cultures to Echinaceae extracts. Clin Chim Acta 2004; 343:161–166.

18 Yang EJ, Song KS. The ameliorative effects of capsidiol isolated from elicited Capsicum annuum on mouse splenocyte immune responses and neuroinflammation. Phytother Res 2021; 35:1597–1608.

19 Bouamama S, Merzouk H, Latrech H, Charif N, Bouamama A. Royal jelly alleviates the detrimental effects of aging on immune functions by enhancing the in vitro cellular proliferation, cytokines, and nitric oxide release in aged human PBMCS. J Food Biochem 2021; 45: e13619.

20 Nicholas DA, Zhang K, Hung C, Glasgow S, Aruni AW, Unternaehrer J, *et al.* Palmitic acid is a toll-like receptor 4 ligand that induces human dendritic cell secretion of IL-1β. PloS One 2017; 12: e0176793.

21 Floros T, Tarhini AA. Anticancer cytokines: biology and clinical effects of interferon-alpha2, interleukin (IL)-2, IL-15, IL-21, and IL-12. Semin Oncol 2015; 42:539–548.

22 Baigent G, Barngrover D. Recombinant interleukin-2 (aldesleukin) for oncology and HIV disease and recombinant protein treatment (Fabrazyme) for Fabry's disease (No.14 in a series of articles to promote a better understanding of the use of genetic engineering). J Biotechnol 2002; 95:277–283.

23 Ear T, McDonald PT. Cytokine generation, promoter activation, and oxidant-independent NF-κB activation in a transfectable human neutrophilic cellular model. BMC Immunol 2008; 9:1–17.

24 Esfandiari N, Sharma RK, Saleh RA, Thomas AJJr, Agarwal A. Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. J Androl 2003; 24:862–870.