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# Antimicrobial and Antiproliferative Activities of Extracts and Compounds Isolated from Varthemia (*Varthemia iphionoides* Bloiss)

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## ABSTRACT

The present study was designed to evaluate the antimicrobial activity and antiproliferation activity against human leukemia (HL-60) cells *in vitro* for hexane, ethyl acetate, ethanol and aqueous extracts of aerial parts of *Varthemia iphionoides*. The ethyl acetate extract showed a pronounced antibacterial activity against four bacterial and three candidal species. Hexane and ethanol extracts showed a pronounced antiproliferation effect on human leukemia (HL-60) cells ($P < 0.05$), with a 89.0 and 62.3 inhibition percentage, respectively at 200 µg/mL. Fractionation of ethyl acetate and ethanol extracts and further purification by columns chromatography afforded sesquiterpene, selina-4, 11(13)-dien-3-on-12-oic acid (**1**) from ethyl acetate extract and three 3-*O*-methylated flavones; 5,7,4'-trihydroxy-3,6-dimethoxy-flavone (**2**), 5,7,4'-trihydroxy-3,5'-dimethoxyflavone (**3**) and 5,4'-dihydroxy-3,7,5'-trimethoxy-flavone (**4**) from ethanol extract. Compound (**1**) showed a pronounced antibacterial activity against studied microorganisms except *Listeria monocytogenes* and showed almost no inhibitory effect on the proliferation of HL-60 cells. Compound (**4**) exhibited the highest anticandidal activity and great antiproliferative activity against leukemia (HL-60) cells with inhibition percentage of 66.7 at 200 µg/mL. Compounds **2** and **3** inhibited completely the proliferation of human leukemia (HL-60) cells at 200 µg/mL and showed low antimicrobial activities.

**Keywords:** antibacterial, flavones, HL-60 cells, sesquiterpene

**Abbreviations: ATCC**, American type culture collection; **CDCl3**, deuterated chloroform; **CFU**; colony-forming unit; **FBS**, fetal bovine serum; **FT/IR**, fourier transform infrared spectrometer; **HL**, human leukemia; **IR**; infrared, **KBr**, potassium bromide; **MeOH**, methanol; **MTT**, 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide; **NMR**, nuclear magnetic resonance; **PSG**, penicillin-streptomycin-glutamine; **UV**, ultraviolet

## INTRODUCTION

Plants produce a high diversity of secondary metabolites for defense and survival in the ecosystem. Secondary metabolites have been shown to exhibit numerous biological activities that promote positive health effects, which justified research on traditional medicine focused on the characterization and isolation of the biologically active compounds from these plants (Newman *et al*. 2003; Kilani *et al*. 2008). Furthermore, plant secondary metabolites and their derivatives may be used safely and effectively against drug-resistant microbial pathogens and they could play an important role in the treatment of many diseases (Newman *et al*. 2000, 2003; Butler 2004).

Wilkins and Board (1989) reported that more than 1,340 plants are known to be potential sources of antimicrobial compounds but few active compounds were isolated and identified (Saxena and Vyas 1986; Thomann and Baeurmann 1993; Elgayyar *et al*. 2001).

Many diseases such as atherosclerosis, Parkinson, Alzheimer and cancer are mainly linked to oxidative stress due to free radicals formation in the body (Addis and Warner 1991; Halliwell 1994; Rice-Evans *et al*. 1997; Lu and Foo 2000; Emerit *et al*. 2004). Phenolic antioxidants, which are widely distributed in the plant kingdom and present in considerable amounts in fruits, vegetables, spices, medicinal herbs and beverages reported to have protective effect against damages in organs caused by free radicals and to have beneficial health effect against many human diseases such as diabetes, cancers, and coronary heart diseases (Middleton and Kandaswami 1993; Broadhurst *et al*. 2000). These protective effects have been mostly ascribed to their scavenging, metal chelating and antioxidant activities (Kameoka *et al*. 1999; Al-Dabbas *et al*. 2006). Thus, phenolic compounds in plants may be linked to the lower incidence and lower mortality rates of cancer (Rejiya *et al*. 2009).

Varthemia (*Varthemia iphionoides*) is a perennial, bushy plant, 20-50 cm long, with a woody base and many basal, unbranched stems, hairy to sticky, aromatic. Leaves oblong, simple, entire, sub-sessile, densely hairy, and grayish. Heads 2-5 mm in diameter, florets yellow-orange surrounded by oblong involucres (Afifi *et al*. 1991; Al-Dabbas *et al*. 2005).

The aqueous extracts of *Varthemia iphionoides* are commonly used in local Jordanian folk-medicine for treatment of gastrointestinal disorders (Afifi *et al*. 1991), the treatment of patients with diabetes mellitus (Afifi *et al*. 1997), have been found to have an antispasmodic effect on the smooth muscles of rabbits (Afifi *et al*. 1990). Extracts possessed antioxidant (Al-Dabbas *et al*. 2006a) and antimicrobial activity (Afifi *et al*. 1991; Al-Dabbas *et al*. 2005).

The aim of this study was to evaluate the antimicrobial and HL-60 cells antitumor activities of extracts and isolated compounds from *V. iphionoides* aerial parts.

## MATERIALS AND METHODS

### Chemicals

Silica gel 60 (0.063-0.200 mm, Merck, Germany) was used in silica gel column chromatography; antimicrobial susceptibility test

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and tetracycline discs were purchased from Oxoid (Oxoid Ltd., Hampshire, UK). Other chemicals were of the highest analytical grade purchased from Sigma-Aldrich Chemical Industries (St. Louis, MI, USA).

## General experimental procedures

NMR spectra were measured at 500 and 125 MHz at 27°C in $CDCl_3$ on a JEOL FX-500 spectrometer. Optical rotation was measured at 22°C using a JASCO P-1030 spectropolarimeter. IR (KBr) and UV (MeOH) were recorded on JASCO FT/IR 5300 and Hitachi U-3310 spectrophotometers.

## Plant material

Complete mature herbs of *V. iphionoides* (stem, leaves and flowers) were collected from the Yajouz area, 15 km east of Amman, Jordan in April 2008. The botanical identification of collected material was done by Professor H. Takruri of the Department of Nutrition and Food Technology, Faculty of Agriculture, University of Jordan. A voucher specimen (vi-17-002) was deposited in the Faculty of Pharmacy, University of Jordan, Amman, Jordan

## Preparation of extracts

Air-dried and powdered aerial parts (200 g) were extracted with *n*-hexane, ethyl acetate and ethanol successively using a Soxhlet apparatus for 5 h for each solvent. The extracts were dried under reduced pressure to yield 3.1% (w/w) of hexane extract, 3.6% of ethyl acetate extract and 2.4% of ethanol extract. The water extract was prepared as follows: the ground aerial parts of the plant (300 g) were extracted with boiling distilled water (1500 mL) for 10 min with continuous stirring, and then left for 24 h at room temperature. The filtrate was evaporated to dryness under vacuum at 40°C on the rotary evaporator to give an 18.5% w/w yield. All extracts were kept in a refrigerator at 4°C

## Microorganisms

Five bacterial strains and four fungal strains were used in this study: *Listeria monocytogenes* (ATCC 7644), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25925), *Bacillus cereus* (ATCC 14579), *Salmonella typhimurium* (ATCC 14028), *Candida albicans*, *Candida glabrata* and *Candida tropicalis* (clinical isolates). Bacteria were cultured in nutrient broth (Oxoid) at 37°C for 12 h and were maintained on nutrient agar slants (Oxoid) at 4°C. Cultures of yeast were grown in malt broth (Oxoid) at 28°C for 2 days and were maintained at 4°C on potato dextrose agar (Difco, USA) plates. Bacterial strains were obtained from stock cultures (Microbiologics, Inc., USA) of the Department of Biological Science of the Jordan University of Science and Technology, Irbid, while Candida clinical strains were obtained from the Department of Pathology-Microbiology, University of Jordan, Amman, Jordan.

## Antimicrobial activity assay

A preliminary disk diffusion test was used to investigate the antimicrobial activities of the extracts and isolated compounds from *V. iphionoides*. The bacteria or fungi strains were grown in liquid medium for 20 h to yield a final concentration of $10^6$-$10^7$ CFU/mL. Next, aliquots of 20 µl of each bacterial suspension was mixed with 3.5 mL of nutrient soft agar and seeded into the hard nutrient agar previously poured into Petri dish (plastic, 94 × 16 mm, Greiner Bio-One GmbH, Austria) (Al-dabbas *et al*. 2005). For *Candida* isolates culture, 0.2 mL of each yeast suspension was also spread onto potato dextrose agar plates. A sterile paper disk (Oxoid), 6 mm in diameter, containing 1000 µg of different extracts or of the isolated compounds was placed onto the center of the nutrient agar or potato dextrose agar plates and incubated for 24 h at 37°C for bacteria and for 48 h at 28°C for *Candida* isolates. The inhibition of bacterial or yeast growth was evaluated by measuring the diameter of the transparent inhibition zone around each disc. The average of three measurements was taken. Control discs were loaded with the same solvents and dried using the same method as the treated discs. Tetracycline (50 µg/disc) and the antifungal miconazole nitrate (40 µg/disc, DentFem-Chemie, Germany) were used as standard antibiotic.

## Determination of phenolic compounds

The amount of phenolic compounds present in the extracts was determined by Folin-Ciocalteau reagent (Duh and Yen 1997). Catechol was used as the standard for a calibration curve.

## Antiproliferative activity

Human leukemia (HL-60) cell lines are a valid model and widely used to determine general antitumoral compounds (Hou *et al*. 2001; Rabah *et al*. 2004; Li *et al*. 2009). Human leukemia (HL-60) cells were obtained from the Cancer Cell Repository (Tohoku University, Japan). The cell lines were grown and maintained in a humidified incubator at 37°C and in 5% $CO_2$ atmosphere in RPMI 1640 medium (Nissui, Japan) containing 100 g/L FBS (Fetal Bovine Serum) and 10 g/L PSG (penicillin-streptomycin-glutamine) (BioWhittaker Co., Walkersville, MD).

The MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide] colorimetric assay, based on the method of Hou *et al.* (2003), is based on the reduction of MTT by mitochondrial dehydrogenase to a purple formazan product. It was used to assess the antiproliferative action of *V. iphionoides* extracts and isolated flavonoids in human leukemia HL-60 cells. The cells were suspended at a density of $2 \times 10^4$ cells/mL in RPMI 1640 medium containing 100 g/L FBS and 10 g/L PSG, then 100 µl of each was plated into each well of 96-well microtiter plates and incubated for 48 h. Different *V. iphionoides* extracts and isolated compounds added to the wells with final concentrations of 25, 50, 100 and 200 µg/mL and incubated at 37°C, 5% $CO_2$ for 48 h. Two controls were used; one contained medium and cells with sterilized water and the other contained extracts and medium without cells to check the effect of each extract color. 10 µl (0.5 mg/mL) of MTT solution was then added to each well and incubated for another 4 h. The resulting MTT-formazan product was dissolved by the addition of 100 µl of 0.04 N HCl-isopropanol solution to each well, mixing by micropipette then measuring the absorbance at 595 nm using a microplate reader (Bio-Rad, Model 550, USA).

## Isolation and identification of phenolic compounds

The ethyl acetate crude extract (3 g) was chromatographed on a silica gel (Wakogel C-300, Wako Pure Chemical Industries, Japan) column. Final purification of the antibacterial compound was carried out by recrystalization from ethyl acetate/hexane (1:1) yielding 50 mg of sesquiterpene, selina-4, 11(13)-dien-3-on-12-oic acid) (**1**) pure compound (Al-dabbas *et al.* 2005).

The crude *Varthemia* ethanol extract was fractionated with a mixture of chloroform: methanol: water (9: 1: 0.1, v/v) using normal open column chromatography packed with 300 g of silica gel 60 (0.063-0.200 mm, Merk, Germany) and 28 fractions (200 mL each) were collected (Al-dabbas *et al.* 2006). The flavonoids were isolated and re-crystallized from a mixture of chloroform and methanol (1:1) to afford pure compounds, 5,7,4'-trihydroxy-3,6-dimethoxyflavone (**2**), 5,7,4'-trihydroxy-3,5'-dimethoxyflavone (**3**) and 5,4'-dihydroxy-3,7,5'-trimethoxyflavone (**4**). The chemical identity of the isolated and purified 3-methoxyflavonols was confirmed by UV, IR, MS, $^1$H-NMR and $^{13}$C-NMR spectra (Al-dabbas *et al*. 2006).

## Statistical analysis

A completely randomized block design was used in designing the experiment. Analysis of variance and Duncan's multiple range test were carried out using statistical analysis systems (SAS 1996). Differences were considered to be statistically significant at $P \leq 0.05$.

**Table 1** Screening for antimicrobial activities[a] of *Varthemia iphionoides* extracts and compounds at a concentration of 1.0 mg/disc.

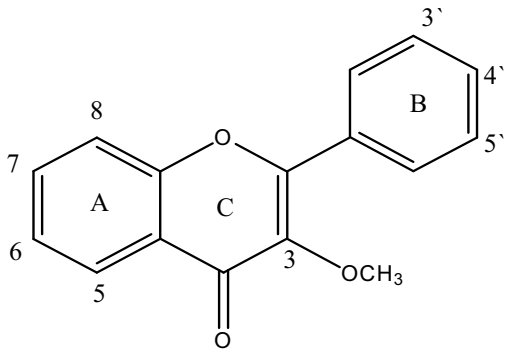
| Microorganism | Water extract | Ethanol extract | Ethyl acetate extract | Hexane extract | Comp 1 | Comp 2 | Comp 3 | Comp 4 | Tetracycline standard[c] | Miconazol nitrate[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| *Listeria monocytogenes* | –[b] | – | 3.5 | – | 2.0 | – | – | – | 2.0 | – |
| *Escherichia coli* | – | – | 3.0 | – | 11.5 | 4.0 | 3.5 | 6.0 | 24.0 | – |
| *Staphylococcus aureus* | – | – | 5.5 | – | 7.5 | 6.0 | 3.5 | 5.5 | 25.0 | 10.0 |
| *Bacillus cereus* | – | – | 5.0 | – | 9.0 | 5.5 | 2.0 | 2.0 | 23.0 | 4.0 |
| *Salmonella typhimurium* | – | – | 7.0 | – | 10.0 | 2.0 | 2.5 | 3.5 | 24.5 | – |
| *Candida albicans* | 2.0 | 3.0 | 5.0 | 2.5 | 8.0 | 3.5 | 5.0 | 13.0 | – | 20.0 |
| *Candida tropicalis* | 3.0 | 3.5 | 4.0 | 2.0 | 6.5 | 3.0 | 7.0 | 12.0 | – | 14.0 |
| *Candida glabrata* | 3.5 | 3.0 | 5.5 | 3.0 | 7.0 | 2.0 | 5.5 | 9.0 | – | 17.0 |

[a] Diameter in mm of the zone of inhibition (n = 3). Solvents of the extracts and of the antibiotic produced no inhibition.
[b] No inhibition zone.
[c] (25 µg/disc )of tetracycline hydrochloride was used as antibacterial standard.
[d] (40 µg/disc ) of miconazol nitrate antifungal standard.

## RESULTS AND DISCUSSION

### Antimicrobial effect of extracts and isolated compounds

The antimicrobial effects of the four extracts of the aerial parts of *V. iphionoides* are shown in **Table 1**. The ethyl acetate extract from the aerial parts of *V. iphionoides* was the most effective against the microbial species used in this study. Of the isolated compounds (**Figs. 1, 2**), the sesquiterpenes, selina-4, 11(13)-dien-3-on-12-oic acid (**1**) from the ethyl acetate extract was the most active compound against tested bacteria and showed good activity against tested yeast: *E. coli*, *S. aureus*, *B. cereus*, *S. typhimurium*, *C. albicans*, *C. tropicalis* and *C. glabrata* with inhibition zones of 11.5, 7.5, 9.0, 10.0, 8.0, 6.5 and 7.0 mm, respectively. The inhibition of *C. albicans*, *C. tropicalis* and *C. glabrata* by compound **4** was greater than that of tested bacteria with inhibition zones of 13.0, 12.0 and 9.0 mm, respectively and was slightly effective against *E. coli* and *S. aureus* with inhibition zones of 6.0 and 5.5 mm, respectively. Compound **3** exhibited anticandidal activity, especially *C. tropicalis*, more than against bacteria. The inhibition of *E. coli*, *S. aureus* and *B. cereus* by compound **2** was greater than that of *S. typhimurium* and *Candida* spp. None of the extracts or of the isolated compounds or standard antibiotic was able to inhibit *L. monocytogenes*.




| Compound | $R_5$ | $R_6$ | $R_7$ | $R_4$‘ | $R_5$` |
|---|---|---|---|---|---|
| **2** | OH | $OCH_3$ | OH | OH | H |
| **3** | OH | H | OH | OH | $OCH_3$ |
| **4** | OH | H | OH | OH | OH |

**Fig. 1 Flavones isolated from *Varthemia iphionoides*.**

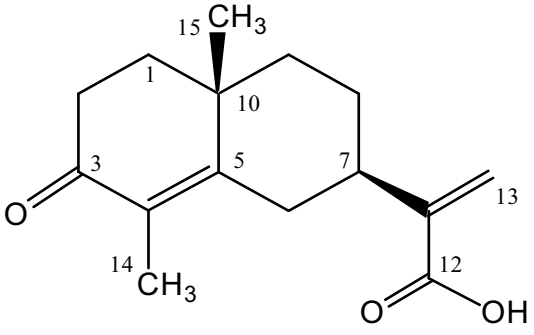



**Fig. 2 Structure of selina-4, 11(13)-dien-on-12-oic acid (1).**

**Table 2** The contents of phenolic compounds (catechol equivalent) of *Varthemia iphionoides* extracts.

| Extract | Total phenolic compounds (mg/g) extract[a] |
|---|---|
| Water | 50.23 ± 1.84 b |
| Ethanol | 72.37 ± 1.63 a |
| Ethyl acetate | 40.43 ± 5.30 c |
| Hexane | 6.18 ± 1.06 d |

[a] Values are means ± standard deviation of two different experiments in each three measurements were made. Means in the column followed by different letters are significantly different ($P < 0.05$).

The antibacterial activity of ethyl acetate extract and compound **1** from our previous study (Al-dabbas *et al.* 2005) also showed similar results on different bacterial strains. Al-Afifi *et al.* (1991) also studied the antimicrobial activity of the aqueous crude extract and four isolated compounds from *V. iphionoides* at a rather lower concentration (300 µg/disc) and found that 3',3'-di-methylquercetin was active against the fungi *Fusarium solani* and *Candida tropicalis*, while kumatakenin and jaceidine were more active against *F. solani*, *Aspergillus parasiticus* and *C. tropicalis*.

### Total phenolic compounds content

The total phenolic contents in *V. iphionoides* as catechol equivalents were greatest in the ethanol extract (**Table 2**), followed by aqueous extract, ethyl acetate and hexane extracts in decreasing order. Phenolic content was lowest in the hexane extract, perhaps due to the limited solubility of hexane-extracted material in water, the solvent used in this experiment.

The results suggest that the antimicrobial activity of ethyl extract depends not only on the presence of phenolic compounds but also on the presence of other secondary metabolites or to the synergistic effect of secondary metabolites in the extracts (Gordana *et al.* 2007).

### Antiproliferation activity of extracts and isolated compounds

The hexane and ethanol extracts of *V. iphionoides* exhibited significant ($P < 0.05$) inhibitory effects on the proliferation of leukemia HL-60 cells *in vitro* by the MTT assay (**Table 3**), with 89% suppression with the hexane extract and 62.3% with the ethanol extract at 200 µg/mL. The aqueous and ethyl acetate extracts showed limited activity. Compounds **2** and **3** completely inhibited leukemia HL-60 cells at 200 µg/mL. Compound **4** showed good antiproliferation activity (66.7%) while Compound **1** showed poor antiproliferation activity (7.0%) at the same concentration.

**Table 3** Percentage inhibition of human leukemia cells (HL-60) of different extracts and isolated compounds from *Varthemia iphionoides* at a concentration of 200 mg/L.

| Extract | % Inhibition of leukemia cells[a] |
|---|---|
| Water | 5.4 ± 4.2 e |
| Ethanol | 62.3 ± 6.5 c |
| Ethyl acetate | 15.3 ± 3.3 d |
| Hexane | 89.0 ± 4.50 b |
| 1 | 7.0 ± 2.8 e |
| 2 | 100.0 ± 0.0 a |
| 3 | 100.0 ± 0.0 a |
| 4 | 66.7 ± 2.1 c |

[a] Values are means ± standard deviation of two different experiments in each three measurements were made. Means in the column followed by different letters are significantly different ($P < 0.05$)

The pronounced inhibitory effect of *V. iphionoides* extracts against human leukemia (HL-60) cells *in vitro* is possibly due to the synergistic antiproliferative effects of flavones, because plant flavonoids are known to inhibit several biochemical events associated with cellular growth (Middleton and Kandaswami 1993; Kanadaswami *et al.* 2005) and its extracts may induce antitumor activity. Phenolic compounds within plant extracts are correlated with antitumor and antioxidant activities and they can act as free radical scavengers and terminate the radical chain reactions that may occur in fat (Sato *et al.* 1996). The differences in the phenolic compound structure and/or different functional groups substitution on the backbone structure will surely influence the free radicals scavenging and antitumor activities. Therefore, the ability of *Varthemia* extracts to retard the formation of free radicals is very possibly related to the flavone content of these extracts and their ability to donate protons to free radicals (Kanadaswami *et al.* 2005).

## CONCLUSIONS

Results of the present work showed that the ethyl acetate extract was superior to other extracts of this plant in its antimicrobial activity and contain substantial amounts of biologically active ingredients other than phenolic compounds like the isolated sesquiterpene (**1**) as measured by the degree of inhibition of studied microorganisms. Ethanol and hexane extracts were the most effective as antiproliferative extracts against human HL-60 cells and the isolated flavonols from ethanol extracts showed strong antiproliferation activity suggesting that *V. iphionoides* is a promising plant.

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