

Antiproliferative activities of *Artemisia herba-alba* ethanolic extract in human colon cancer cell line (HCT116)

Giulio Lupidi,¹ Massimo Bramucci,¹
Luana Quassinti,¹ Enzo Fornari,¹
Luca Avenali,¹ Hala Khalife,²
Hala Gali-Muhtasib²

¹School of Pharmacy, Drugs and Health Products, University of Camerino, Camerino, Italy; ²Department of Biology, American University of Beirut, Beirut, Lebanon

Abstract

Artemisia herba-alba (AHE) is a plant commonly used in traditional medicine for the treatment of various ailments. Here, we investigated the antioxidant and antitumor activity of the aqueous and ethanol extracts of AHE in human colon cancer HCT116 cells. The antioxidant activity was measured by DCFH assay, while antitumor effects were assessed by cell viability assays, cell cycle progression by flow cytometry, and DNA fragmentation analysis in addition to investigating the expression of key cell cycle and apoptotic proteins. While the aqueous extract had no antineoplastic effects, the ethanol extract significantly decreased HCT116 viability (IC₅₀ of 51 µg/mL at 24 h) and inhibited the production of reactive oxygen species (ROS). Treatment of HCT116 cells with the ethanol extract also caused dramatic increase in the PreG₁ population with concomitant decrease in cycling cells, provoked DNA fragmentation, significant increase in the expression levels of p53 and Bax proteins and activated pro-apoptotic caspase-3. The results obtained suggest that the ethanol extract of AHE could be used as an easily accessible source of natural antioxidants and as potential phytochemicals against colon cancer.

Introduction

Artemisia herba-alba Asso. (syn: *A. inculata* Del.) (AHE), commonly known as the desert wormwood (Arabic name: Sheeh), is a prominent perennial plant of the Irano-Turanian steppes of Spain, North Africa and the Middle East.^{1,2} It is mainly used as an aromatisant for tea and in folk medicine for the treatment of colds, coughs, intestinal disturbances, as an antidiabetic agent.^{3,4} The antileishmania activity of AHE water extract was investigated *in vitro* against *Leishmania tropica* and

Leishmania major demonstrating interesting effects.⁵ Other common uses of the plant extract include antihelminthic, antimicrobial, antispasmodic or vasorelaxant effects.⁵⁻⁷ This species is also recommended for neurological disorders as the ethanolic extract of AHE has shown activity in the GABAA-benzodiazepine receptor assay.⁸ The beneficial effects of the plant are thought to be due to its high antioxidant content, which are even greater than those of green and black tea.⁹ Monoterpene hydrocarbons and oxygenated monoterpenes, such as camphor, 1,8-cineole, *p*-cymene and davanone, are the most abundant components in AHE essential oil,¹⁰ and the presence of flavonoids and eudesmanolides as isolated classes of constituents in AHE essential oil is reported.^{11,12}

Naturally occurring polyphenolic antioxidants have been recognized as one of the most effective classes of colon cancer-preventive agents, since they reduce oxidative stress, a known contributor to carcinogenesis, with little or no systemic toxicity.¹³ The lack of detailed mechanistic data about these natural compounds has impeded bringing them to the main stream of medicines for colon cancer prevention or therapy. There is an urgent need for developing novel targeted therapies considering that current therapeutic modalities for colon cancer are associated with toxicity and side effects.¹⁴⁻¹⁶

In the present study, we investigated the antitumor activity of *Artemisia herba-alba* in HCT116 human colon cancer cells and determined its potential to cause cell cycle changes, DNA ladder formation and modulate p53, p21 and bax proteins. HCT116 is an aggressive, microsatellite unstable and growth-hormone independent colon cancer cell line which provides an excellent model system not only for basic studies, but also for evaluation of clinical treatment and management of human colon carcinoma. The ethanol extract was found to induce cell death as evidenced by the significant reduction in cell viability, enhanced DNA fragmentation, and increase in the PreG₁ population in addition to the activation of pro-apoptotic caspase-3.

Materials and Methods

Preparation of ethanolic and water extracts of *Artemisia herba-alba*

Artemisia herba-alba Asso was selected and collected from *Dabbous* store in Beirut, Lebanon. The plants were already dried when bought and had to be recommended by at least two herbalists. Voucher samples are stored at the Department of MCA of the University of Camerino, Italy (accession numbers CAME No.

Correspondence: Hala Gali-Muhtasib, Department of Biology, American University of Beirut, Lebanon.

Tel. +961.1.350000 - Fax: +961.1.744461.

E-mail: amro@aub.edu.lb

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0010). The dried plant material was grounded and stored in brown glass bottles until use. For preparing the ethanol or water extracts, 5 grams of the powder was dissolved either in 50 mL of absolute ethanol or in 50 mL of distilled water, respectively. Afterwards, the mixture was homogenised by Ultra-Turrax disperser and centrifuged at 12,000 × *g* for 10 min and the extract was filtered. The ethanol was evaporated with the rotor-vapor, obtaining a viscous gel which was stored at -20°C in a dark ampule. The water extract mixture was lyophilised, and the dried mixture was stored at -20°C.

Determination of total phenolics

The amount of total soluble phenolics (TPH) was determined according to the Folin-Ciocalteu method.¹⁷ Different samples of water and ethanol extracts were prepared: solutions of 3 mg/mL of water extract and 1 mg/mL of ethanol extract. The Folin Reactive was prepared by bringing 3 mL of Folin-Ciocalteu phenol reagent to a 30 mL final volume with distilled water; 75% Na₂CO₃ solution was prepared and added in the assay to activate the reaction. The titration curve was obtained using quercetin as a standard control. The absorbance was measured at 765 nm in a Varian Cary-1 spectrophotometer. The total phenolic content was determined as quercetin equivalents per mg of extract.

Measurement of intracellular ROS generation

The production of intracellular reactive oxygen species (ROS) was measured using the oxidation-sensitive fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (DCFH₂) according to published protocols.¹⁸ Briefly, cells were harvested and washed in HBSS/HEPES supplemented with 0.2% BSA

and loaded for 60 min. at 37°C, 5% CO₂ with 10 µg/mL of DCDHE. For fluorescence measurements, cells were harvested by trypsinisation and resuspended in 1 mL of HBSS/HEPES supplemented with 1% fetal bovine serum (FBS), to prevent dye leakage, transferred to a quartz cuvette, incubated at 37°C and maintained under continuous stirring. Artemisia extract was then added (25, 50 and 100 µg/mL) and the fluorescence intensity was monitored for 30 min by spectrofluorimetry. The excitation wavelength was set at 488 nm and the emission wavelength was 543 nm. The effect of the extract was also evaluated in the presence of 200 µM *tert*-butyl hydroperoxide (t-BHP).

Cell proliferation

Human colon cancer HCT116 (+/+ p53) cells were kindly provided by Dr. Carlos Galmarini (Institut National de la Sante et de la Recherche Medicale, Lyon, France). Cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated FBS (all obtained from Sigma, St. Louis, MO, USA). The cells were grown in a humidified atmosphere of 5% CO₂ in air at 37°C. The viability of HCT116 cells was determined by the standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in 96-well plates in 100 µL of growth medium (5×10³ cells/well). After 24 h, cells were exposed to different concentrations of AHE extracts (3.125 to 200 µg/mL) containing 0.5% of ethanol which was applied as control (vc) and incubated for 24 h or 48 h. Cell viability was quantified by the ability of living cells to reduce the yellow dye thiazolyl blue tetrazolium bromide (MTT) to a purple formazan product. At the end of incubation, 10 µL MTT (5 mg/mL in phosphate-buffered saline, PBS) per well was added and incubated for 4 h at 37 °C. The formazan product was then dissolved in 100 µL dimethyl sulphoxide (DMSO) after aspirating the medium and the absorbance was measured at 540 nm using a Titertek Multiscan MicroElisa (Labsystems, Helsinki, Finland). Cell viability was calculated as the percentage ratio of the sample absorbance to the reference control. IC₅₀ represents the extract concentration showing a lethal effect on 50% of the cells.

Flow cytometric analysis of DNA content

Flow cytometric analysis of DNA content was determined according to published protocols.¹⁹ HCT116 human colon cancer cells were seeded in 100 mm dishes at 1.2×10⁵ cells per well. They were then incubated and allowed to grow to 50% confluence after which they were treated with AHE extract (1.45 µg/mL of polyphenols and 2.50 µg/mL of polyphenols) for 24 h or 48 h.

They were then harvested by trypsin release and washed twice with PBS. The pellet was resuspended in an ice-cold solution of 70% ethanol in PBS (v/v). After 2 h of fixation, cells were washed twice with PBS. Fixed cells were stained by suspension in a solution of propidium iodide (100 µg/mL final concentration; Molecular probes, Eugene, OR) and 1% RNase A (Sigma Chemical Co, St. Louis, MO). Distribution of cell cycle phases with different DNA contents was determined using a FACScan flow cytometer (Becton- Dickinson, San Jose, CA). Cells that were less intensely stained than G₁ cells (sub G₁ cells) in flow cytometric histograms were considered apoptotic. Analysis of cell cycle distribution and the percentage of cells in the G₁, S and G₂/M phases of the cell cycle were determined using Cell Quest.

DNA gel electrophoresis

Conventional agarose gel electrophoresis was done as described previously with some modifications.²⁰ Briefly, HCT116 (3×10⁶ cells) were exposed to AHE ethanolic extract at a final concentration of 50 µg/mL. After 24 h of incubation, cells were washed with PBS, trypsinised, and centrifuged at 500 × g for 5 min. The pellet was suspended in 400 µL of ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.2% Triton X-100) for 15 min. The samples were then centrifuged at 12,000 × g for 15 min. The supernatant was collected, proteinase K (1 mg/mL) was added, and then incubated for 2 h at 56°C. RNase was added to the supernatant to a final concentration of 0.5 mg/mL, and the resulting suspension was incubated for 2 h at 56°C. To each tube, 0.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed and centrifuged at 12,000 × g for 2 min to separate the DNA containing upper aqueous phase. To the resulting aqueous phase, two volumes of ice-cold absolute ethanol and 1/10th volume of 5 M sodium acetate were added and incubated overnight at -20°C to precipitate DNA. DNA was pelleted by centrifuging at 12,000 × g for 10 min at 40°C; the supernatant was aspirated and the pellet was washed with 1 mL of 70% ethanol. After repeating the above centrifugation step and removing the last traces of the supernatant fraction, the pellet was allowed to dry at room temperature and resuspended in 10 µL of TBE buffer (90 mM Tris, 90 mM borate, and 10 mM EDTA, pH 8.0) and 2.5 µL of sample buffer (30% glycerol, 0.25% bromophenol blue). DNA samples were resolved on 1 % agarose gel containing 0.1 µg/mL ethidium bromide using TBE as the running buffer for 150 min at 40 V. The gel was then visualised under a UV transilluminator.

Caspase-3 activity

Activation of caspase-3 was measured by using colorimetric Caspase-3 Assay Kit (Sigma, St. Louis, MO, USA). Cells incubated

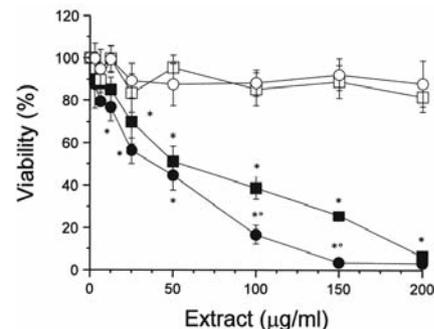


Figure 1. *Artemisia herba-alba* ethanolic extract reduces the viability of HCT116 cells. Cells were grown in microtiter plates and exposed to ethanolic or water extract (3.125 to 200 µg/mL). Cell viability was assessed after 24 h (■, □) and 48 h (●, ○) for ethanolic (■, ●) and water (□, ○) extracts by MTT assay. Comparisons between treated and control cells were evaluated using t-test. The level of significance was set at 0.05 (*P<0.05 vs control, °P<0.05 vs 24h).

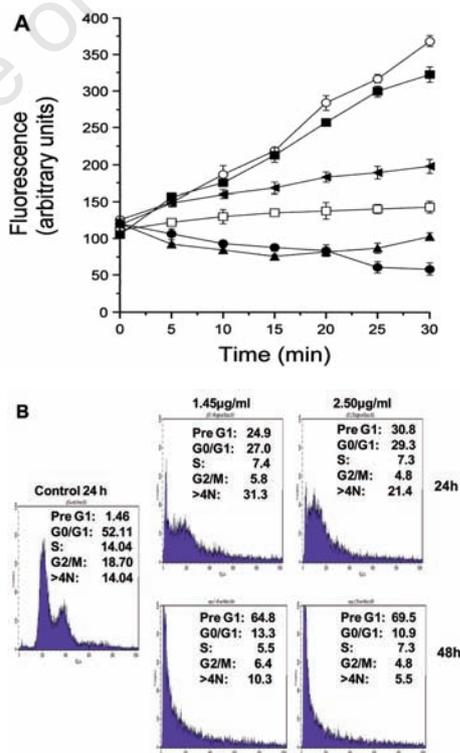


Figure 2. *Artemisia herba-alba* ethanolic extract increases the PreG1 population and enhances the generation of reactive oxygen species. (A) Cells, loaded with the fluorescent dye DCDHE, were treated with 100 µg/mL (p) of ethanolic extract or with vehicle (□). The scavenger activity of the ethanolic extract at 25 µg/mL (■), 50 µg/mL (t), and 100 µg/mL (●) was also evaluated in the presence of 200 µM *tert*-butyl hydroperoxide (t-BHP) (○). (B) Cells were treated with 1.45 µg/mL or 2.5 µg/mL of ethanol extract for 24 h and 48 h and cell cycle profiles were analyzed by flow cytometry after DNA staining with propidium iodide. In A-C, values represent the mean (± SD) of three independent experiments.

with 50 or 100 $\mu\text{g/mL}$ of AHE extract for different times (1, 2, 4, 12 and 24 h.) were harvested, washed with PBS and the pellets were resuspended in lysis buffer (50 μL) for 20 min. Lysates were centrifuged at $20,000 \times g$ for 10 min at 40°C , and supernatants were collected and added to 96-well plates. Final reaction buffer (100 μL) and caspase-3 colorimetric substrate (Ac-DEVDpNA 10 μL) were then added to each well. Plates were incubated at 37°C for 2 h and optical density was measured at 405 nm with a Titertek Multiscan MicroElisa (Labsystems, Helsinki).

Western blot analysis

HCT116 human colon cancer cells were treated at 50% confluence with the AHE ethanol extract for 24 h. At the end of the incubation period, 30 mg of cytoplasmic protein extracts were prepared and resolved on 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (1% non fat dry milk/1% Tween 20 in PBS) for 1 h at room temperature, and then incubated with appropriate monoclonal primary antibodies (human reactive p53 from Santa Cruz Biotechnology, p21 from Biosource, and Bax from BD Biosciences). The blot was then incubated with appropriate secondary horseradish peroxidase-conjugated antibody and detected by chemiluminescence and autoradiography using X-ray film. GAPDH was used as loading control.

Statistical analysis

Results are expressed as means \pm standard deviation (SD). Statistical analysis was performed using SPSS Student Version 11.0 Software Package. Comparisons between different treatments were evaluated using a one-tailed Student's t-test. The level of significance was set at 0.05.

Results

Since ancient times, plants have been used to treat various diseases, and their uses have been reported in the ancient literature of indigenous systems of medicine.²¹ The search for new anti-cancer drugs is one of the most prominent areas of natural products research, and in recent years there is considerable interest in identifying novel botanicals that selectively induce growth arrest of cancer cells without affecting normal cells. Several natural compounds have been found to exhibit chemopreventive activities both *in vitro* and *in vivo*. These compounds are able to induce apoptosis in a number of experimental models of carcinogenesis,^{21,22} and to prevent the development of colon cancer both *in vitro* and *in vivo*.²³⁻²⁷

Artemisia herba-alba Asso is a plant that is used extensively in folk medicine in the Mediterranean region.^{1,2,28-33} Despite reported biological, pharmacological, and toxicological activities of *Artemisia herba-alba*, there has been no published work on the effects of extracts or components of this plant on cancer cells. In this study we investigated, for the first time, the antioxidant and anti-colon-cancer activities of AHE Asso. The effects of aqueous and ethanolic extracts of AHE on HCT116 human colon cancer cell viability was determined using MTT assay. Cells were exposed to various concentrations of AHE extracts for 24 h and 48 h, and only the ethanolic extract significantly ($P < 0.05$) reduced the viability of HCT116 cells in a dose- and time-dependent manner (Figure 1). The inhibitory activity was 83% after 48 h in the presence of 100 $\mu\text{g/mL}$ of ethanol extract. The IC_{50} values were 50.92 $\mu\text{g/mL}$ (44.53-58.23 $\mu\text{g/mL}$, 95% confidence interval) and 32.73 $\mu\text{g/mL}$ (27.73-38.65 $\mu\text{g/mL}$, 95% confidence interval) for 24 h and 48 h of incubation, respectively. The aqueous extract

showed no effect on colon cancer cell viability (Figure 1), therefore, all later experiments were done with the ethanol extract.

Since the generation of reactive oxygen species (ROS) is an important mode of action of many anticancer agents, we explored whether cell death induced by AHE extract is due to its pro-oxidant effects. ROS generation was measured using the fluorescent dye DCF. Cell treatment with ethanolic extract (100 $\mu\text{g/mL}$) decreased the fluorescent signal progressively to almost 50% of the control value after 15 min of incubation, suggesting a strong antioxidant activity (Figure 2A). Protection against exogenous oxidative stress was further evidenced by incubating cells in the presence of 200 μM t-BHP. The inhibition of ROS production was dose-dependent increasing with the amount of the ethanol extract.

To further determine and confirm whether or not the observed reduction in cell viability by the AHE extract was related to induction of apoptosis, the subdiploid fraction (characteristic of apoptosis) was measured by PI staining

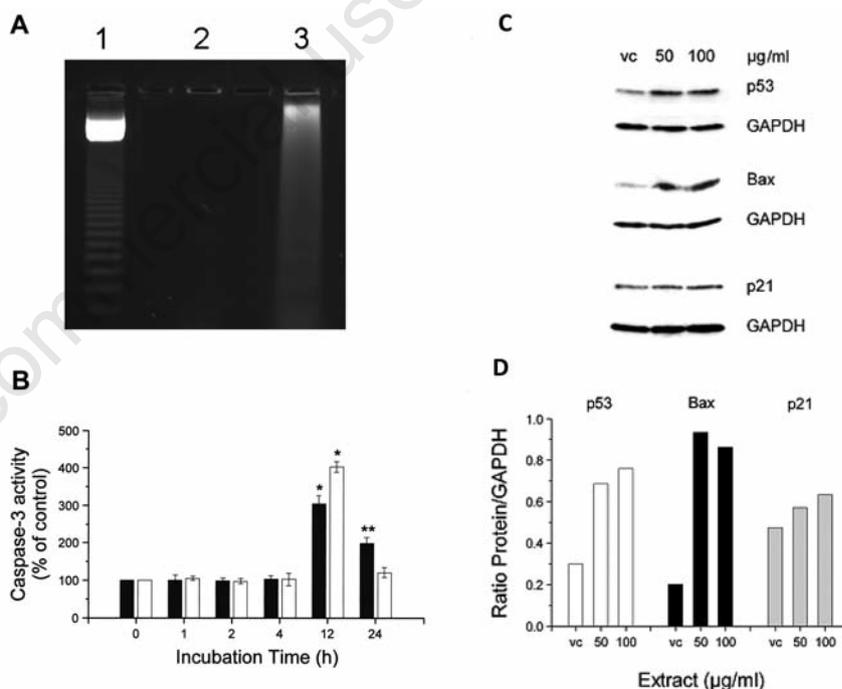


Figure 3. *Artemisia herba-alba* ethanolic extract causes DNA fragmentation, activates caspase-3 and increases p53 and Bax proteins. A) HCT116 cells were incubated with vehicle (lane 2) or with 50 $\mu\text{g/mL}$ of AHE ethanolic extract for 24 h (lane 3) and genomic DNA prepared as described in Materials and Methods. Lane 1 represents 123-bp DNA size marker. A representative of three independent experiments is shown; B) Caspase-3 activation was determined in the cytosolic fraction of HCT116 cells using colorimetric assay, after treatment with 50 (■) or 100 (□) $\mu\text{g/mL}$ of AHE for 1, 2, 4, 12 and 24 h. Data represent the mean (\pm S.E.) from three independent experiments versus control. (**) $P < 0.001$, (*) $P < 0.05$, *vs* control; C) For western blotting, cells were treated with vehicle (vc) or with the indicated concentrations of ethanolic extract for 24 h and whole cell lysates were prepared. Blots were incubated with mouse anti-p53, anti-Bax, and anti-p21 antibodies and then incubated with anti-mouse secondary horseradish peroxidase-conjugated antibody. Bands were detected by luminescence and autoradiography. GAPDH was included as a loading control; D) Bands were quantified using image capture and analysis system (Kodak MI, Rochester, NY, USA).

of DNA content with flow cytometry. We investigated the mode of action of the extract on cellular parameters, including cell cycle distribution and apoptosis induction using extract concentrations that range below and above the IC₅₀. Treatment with 1.45 µg/mL and 2.50 µg/mL of the AHE ethanol extract for 24 h or 48 h caused dramatic increases in the pre-G₁ population (a hallmark of apoptosis and necrosis) with respect to control cells in addition to a significant decrease in the proportion of cells in G₀/G₁, S and G₂/M phases (Figure 2B).

To investigate whether AHE triggers DNA fragmentation, genomic DNA was isolated from cells incubated for 24 h with 50 µg/mL of ethanolic extract and analyzed on 1% agarose-gel electrophoresis. Figure 3A shows the presence of typical DNA laddering, suggesting the activation of apoptotic processes by the ethanol extract. To elucidate the apoptogenic effects of AHE, we evaluated the extract effects on caspase-3. Caspase-3 is a well-known executor enzyme in the apoptotic pathway. Treatment of HCT116 cells with IC₅₀ concentrations of the ethanol extract markedly enhanced caspase-3 activity with respect to the control (Figure 3B). Caspase-3 significantly increased in the cells after 12 h of incubation with AHE, and no higher expression levels of caspase-3 were observed at 24 h. Since caspase activation was observed, the effects of the AHE extract on the regulation of apoptosis-related genes such as Bax, p53, and p21 were investigated. We conducted Western blot analyses to determine the levels of Bax, p53 and p21 proteins and as expected, the levels of Bax protein in cells incubated with AHE ethanolic extract increased significantly (Figure 3C). Bax levels increased by 4.6 and 4.3 fold with respect to control after 24 h of incubation with 50 and 100 µg/mL of ethanol extract, respectively (Figure 3D). Similarly, the expression of p53 protein was increased (2.3 and 2.5 fold with respect to control) and moderate increase in p21 protein expression was observed in response to treatment with the ethanol extract. The pro-apoptotic protein Bax can indirectly activate caspase-3 through activation of caspase-9. The increase in Bax expression, an index of apoptotic cell death, suggests the involvement of the mitochondrial pathway in AHE-induced apoptosis.

In conclusion, this study shows that exposure of HCT116 human colon cancer cells to *Artemisia herba-alba* ethanol extract decreases cell viability, causes DNA damage, and induces apoptosis via the activation of caspase-3 and increase in Bax and p53 proteins. Further biochemical and molecular studies of the ethanol extract should be carried out in different types of cancer cell lines and in animal models of colon cancer to establish its therapeutic efficacy. As reported, sesquiterpene lactones, which are among the prominent natural products

found in *Artemisia* species, could be the promising candidates for the observed effects.³⁴ Further investigations to identify and characterize the efficacious phytotherapeutic bioactive compounds of *Artemisia herba-alba* are currently in progress, however, our data support the role of AHE ethanol extract against colon cancer.

References

1. Al-Qura'n S. Ethnopharmacological survey of wild medicinal plants in Showbak, Jordan J Ethnopharmacol 2009;123:45-50.
2. Saleh MA, Belal MH, el-Baroty G. Fungicidal activity of *Artemisia herba-alba* Asso (Asteraceae). J Environ Sci Health B 2006;41:237-44.
3. Al-Shamaony L, Al-Khazraji SM, Twajj HA. Hypoglycemic effect of *Artemisia herba-alba*. II. Effect of a valuable extract on some blood parameters in diabetic animals. J Ethnopharmacol 1994;43:167-71.
4. Boriky D, Berrada M, Talbi M, Keravis GF. Eudesmanolides from *Artemisia herba-alba*. Phytochemistry 1996;3:309-11.
5. Hatimi S, Boudouma M, Bichichi M et al. In vitro evaluation of antileishmania activity of *Artemisia herba-alba* Asso. Bull Soc Pathol Exot 2001;94:29-31.
6. Yashphe J, Segal R, Breuer A, Erdreich-Naftali G. Antibacterial activity of *Artemisia herba-alba*. J Pharm Sci 1979;68:924-5.
7. Skiker M, Mekhfi H, Aziz M et al. *Artemisia herba-alba* Asso relaxes the rat aorta through activation of NO/cGMP pathway and K(ATP) channels. J Smooth Muscle Res 2010;46:165-74.
8. Salah SM, Jager AK. Two flavonoids from *Artemisia herba-alba* Asso with in vitro GABA_A-benzodiazepine receptor activity. J Ethnopharmacol 2005;99:145-6.
9. Abid ZB, Feki M, Hedhili A, Hamdaoui MH. *Artemisia herba-alba* Asso (Asteraceae) has equivalent effects to green and black tea decoctions on antioxidant processes and some metabolic parameters in rats. Ann Nutr Meta 2007;51:216-22.
10. Abou El-Hamd M, El-Sayed M, Hegazy M et al. Chemical constituents and biological activities of *Artemisia herba-alba*. Rec Nat Prod 2010;4:1-25.
11. Haouari M, Ferchichi A. Essential oil composition of *Artemisia herba-alba* from Southern Tunisia Molecules 2009;14:1585-94.
12. Hudaib M, Aburjai T. Composition of the essential oil from *Artemisia herba-alba* grown in Jordan. J Essen Oil Res 2006;18:301-4.
13. Tseng TH, Lee YJ. Evaluation of natural and synthetic compounds from East Asiatic folk medicinal plants on the mediation of cancer. Anticancer Agents Med Chem 2006;6:347-65.
14. Baer-Dubowska W. Cancer chemopreventive agents-drugs for the 21st century? Acta Pol Pharm 2006;63:369-73.
15. Boursi B, Arber N. Current and future clinical strategies in colon cancer prevention and the emerging role of chemoprevention. Curr Pharm Des 2007;13:2274-82.
16. Imai M, Kikuchi H, Denda T et al. Cytotoxic effects of flavonoids against a human colon cancer derived cell line, COLO 201: a potential natural anti-cancer substance. Cancer Lett 2009;276:74-80.
17. Katsube T, Tabata H, Ohta Y et al. Screening for antioxidant activity in edible plant products: comparison of Low-Density Lipoprotein Oxidation Assay, DPPH, radical scavenging assay and Folin-ciocalteu assay. J Agric Food Chem 2004;58:2391-6.
18. Le Bel C, Ischiropoulos H, Bondy SC. Evaluation of the probe 2,7-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992;5:227-31.
19. Riccardi R, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nature Protocols 2006;1:1458-61.
20. Herrmann M, Lorenz HM, Voll R et al. A rapid and simple method for the isolation of apoptotic DNA fragments. Nuc Acid Res 1994;22:5506-7.
21. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768-80.
22. Coseri S. Natural products and their analogues as efficient anticancer drugs. Mini Rev Med Chem 2009;9:560-71.
23. Kyle JA, Sharp L, Little J et al. Dietary flavonoid intake and colorectal cancer: a case-control study. Br J Nutr 2010;103:429-36.
24. Link A, Balaguer F, Goel A. Cancer chemoprevention by dietary polyphenols: Promising role for epigenetics. Biochem Pharmacol 2010;80:1771-92.
25. Landis-Piwowar KR, Milacic V, Dou QP. Relationship between the methylation status of dietary flavonoids and their growth-inhibitory and apoptosis-inducing activities in human cancer cells. J Cell Biochem 2008;105:514-23.
26. Braun L, Cohen M. Herbs and Natural Supplements: An Evidence-based Guide, 2nd ed. Elsevier, Harcourt Australia; 2007.
27. Blijham GH. Chemotherapy of colorectal cancer. Anticancer Drugs 1991;2:233-45.
28. Bailey C, Danin A. Bedouin plant utilization in Sinai and Negev. Econ Bot 1981;35:145-62.
29. Jouad H, Haloui M, Rhouioui H et al. Ethnobotanical survey of medicinal plants used for the treatment of diabetes, cardiac

- and renal diseases in the North centre region of Morocco (Fez–Boulemane). *J Ethnopharmacol* 2001;77:175-82.
30. Segal R, Feuerstein I, Danin A. Chemotypes of *Artemisia herba-alba* in Israel based on their sesquiterpene lactone and essential oil constitution. *Phytochemistry* 1987;15:411-6.
31. Ziyat A, Legssyer A, Mekhfi H et al. Phytotherapy of hypertension and diabetes in oriental Morocco. *J Ethnopharmacol* 1997;58:45-54.
32. Tahraoui A, El-Hilaly, Israili ZH, Lyoussi B. Ethnopharmacological survey of plants traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province). *J Ethnopharmacol* 2007;110:105-17.
33. Zeggwagh N-A, Farid O, Michel JB et al. Cardiovascular effect of *Artemisia herba-alba* aqueous extract in spontaneously hypertensive rats. *Methods Find Exp Clin Pharmacol* 2008;30:375-81.
34. Ghantous A, Gali-Muhtasib H, Vuorela H et al. What made sesquiterpene lactones reach cancer clinical trials? *Drug Discov Today* 2010;15:668-78.

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