Effect of Salvia triloba L. f. Extracts on Neoplastic Cell lines

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Abstract

The search for novel anticancer drugs continues. This work includes a preliminary study of the effect of two crude extracts of Greek or Mediterranean sage (Salvia triloba L. f.) on three malignant human cell lines and one malignant murine cell line. A boiling water extract and a methanolic extract were prepared from dried leaves of S. triloba. Yields of extraction were 9.8 and 22.4%, respectively. Tested cell lines included human larynx epidermoid carcinoma (HEp-2), human rhabdomyosarcoma (RD), human glioblastoma multiforme (AMGM5) and murine mammary adenocarcinoma (AMN3).

Both extracts exhibited time-dependent, cell specific inhibitory effects on HEp-2, RD and AMN3 malignant cell lines. AMGM5 cell line was resistant to the effects of both extract as inhibition could only be recorded after 72 hrs of exposure to the highest extracts concentrations, 625 and 1250 µg/ml. In addition, growth of HEp-2, RD and AMN3 cells under treatment with either extract was biphasic during the first 48 hrs of treatment as cells were stimulated at lower concentrations and inhibited at higher concentrations.

It seems likely that extracts of Salvia triloba may act at various therapeutic levels, especially in cancer treatment, but further research is required to evaluate the practical values of therapeutic application.

Keywords: Salvia triloba, cytotoxicity, cell line, biphasic effect.

1. Introduction

Natural products have long been a fertile source of cure for cancer, which is projected to become the major cause of death in this century (Mukherjee et al., 2001). Of these natural products, plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasoning, beverages, cosmetics, dyes and medicine. Most of this therapy involves the use of plant extracts or their active components (Craig, 1999).

Crude plant extracts from Dioscorea membranacea (Thailand) (Itharat et al., 2004), Platycodon grandiflorum A. DC. (Korea) (Lee et al., 2004), and Entanda abyssinica (Tanzania) (Kamuhawba et al., 2000) have shown significant cytotoxic effects against malignant cell lines in vitro. Mediterranean Sage (Salvia triloba L. f.) is the most popular medicinal herb in Palestine, Jordan and Lebanon (Abu-Rmaileh & Afifi, 2000; Ali-Shtayeh et al., 2000; Gali-Muhtasib & Affara, 2000). Twenty species of Salvia grow wildly in Jordan (Oran & Al-Eisawi, 1998). The leaves of the plant are boiled and used as a herbal tea for treatment of bloating, gastric disorders, abdominal pain,
oral infections, gum and tooth pains, headaches, cough, influenza and cold, feminine sterility, skin disorders, nervous conditions, asthma, rheumatism and diabetes (Oran & Al-Eisawi, 1998; Abu-Rmaileh & Afifi, 2000; Ali-Shtayeh et al., 2000; Perry et al., 2003; Salah & Jager, 2005).

The aqueous and oil extracts of sage have been shown to possess antioxidant, anti-inflammatory, anticancer and antimicrobial activities (Kamatou et al., 2007; Kamatou et al., 2008; Kamatou et al., 2010). Salvia is the largest and the most important genus of the family Lamiaceae. Plants belonging to this genus show high diversity in their secondary metabolites as well as in pharmacological effects. Several species of salvia are included in many pharmacopoeias. They are used for alimentary, pharmacological and cosmetic purposes (Kamatou et al., 2005; Perry et al., 2003).

In this study, an aqueous and a methanolic extract were prepared from the dried leaves of Salvia triloba L. f. and tested for cytotoxicity against four types of neoplastic cell lines. Both extracts showed cytotoxic effects against Hep-2, RD and AMN3 cell lines, while AMGM5 cells showed resistance to both extracts.

2. Materials and Methods

2.1. Cell Lines

Human larynx epidermoid carcinoma (HEP-2), human rhabdomyosarcoma (RD), human cerebral glioblastoma multiforme (AMGM5) and murine mammary adenocarcinoma (AMN3) cell lines were all kindly provided by the Iraqi Center for Cancer and Medical Genetics Research (ICCMGR), Baghdad, Iraq and were maintained at 37°C.

2.2. Reagents

Cell lines were grown on RPMI-1640 medium (Sigma, USA) with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin.

2.3. Plant Material

Fresh Salvia triloba L. f. plant was obtained from local market in Jordan. Representative specimens were taken to the Hashemite University Herbarium, the Hashemite University, Zarqa, Jordan, where they were identified as Salvia triloba L. f. of the family Labiatae (Lamiaceae). Plant leaves were separated and placed in the shade inside a well-ventilated room. Dried leaves were ground to a fine powder using a coffee grinder. No sieving was applied and the powder was put in air-tight polyethylene bags and placed in the freezer at -20°C till use.

2.4. Preparation of Crude Extracts

Aqueous extract was prepared by adding boiling distilled water to 50 gm of plant material. The mixture was left to cool down at room temperature. The methanolic extract was prepared using absolute methanol. Both extracts were filtered through Whatman No. 1 filter paper and dried at 37°C. Dried powder was kept in screw-cap bottles at -20°C till use. Serial dilutions of each extract in RPMI-1640 medium were used for treating the cell lines with final concentrations of 1250 µg/ml, 625 µg/ml, 312.5 µg/ml, 156.25 µg/ml, 78.125 µg/ml and 39 µg/ml.

2.5. Cytotoxicity Assay

The protocol adopted for cytotoxicity assay using MTT (Sigma, USA) was that of Betancur-Galvis et al. (2002). The cells were plated in 96-well flat-bottomed plate. After adhesion, extract dilutions were added to the appropriate wells and the plates were incubated for 24, 48 or 72 hrs at 37°C, 5-10% CO2 in a humidified environment. Untreated cells were used as controls. The supernatants were removed from the wells of the microtitration plate at the end of each exposure period. Then 28 µl of MTT (2 mg/ml) solution in phosphate buffered saline (PBS) was added to each of the wells in the microtitration plate. The plate was covered with self-adhesive film and incubated for 1.5 hrs. at 37°C. At the end of this incubation period 130 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The plates were placed on a shaker for 15 min and the absorbency was read at 492 nm (OD492) using a multi-well spectrophotometer (Organon Teknika, Austria).

3. Results

3.1. Effect on HEP-2 Cell Line:

The aqueous extract showed a time-dependent effect on viability of HEP-2 cells. Viability decreased with time reaching its lowest after 72 hrs of treatment with all concentrations used (Fig. 1.a). The lowest percentage of cell viability resulted from treatment with 1250 µg/ml for 72 hrs and reached 34.55%. The 50% growth inhibitory concentration (GI50) was 1054.6872 µg/ml after 72 hrs of treatment. The highest three concentrations of the methanolic extract, 312.5, 625, and 1250 µg/ml, showed both time- and concentration-dependent effects (Fig. 1.b). Cell viability reached as low as 34.9, 28, and 18.7% after treatment with 1250 µg/ml for 24, 48, and 72 hrs, respectively. In addition, treatment with 625, 312.5 and with 39 µg/ml for 72 hrs caused significant reduction in cell viability, which reached 31, 55.1, and 57.9%, respectively. GI50 was 1095.5 and 377.625 µg/ml following 48 and 72 hrs of treatment, respectively.

3.2. Effect on RD Cell Line

Cell viability reached its lowest percentage after 72 hrs of exposure to all concentration of the aqueous extract (Fig. 1.c). Viability decreased to as low as 9.36, 22.1, 54.7, 57.8, and 54.66% at 1250, 625, 312.5, 156.25, 78.125, and 39 µg/ml, respectively. Under treatment with the aqueous extract, growth of RD cells was found to be biphasic (nonmonotonic) (Fig. 1.c). The aqueous extract stimulated cell growth over a range of 39-625 µg/ml. In contrast to its growth-promoting effects at lower concentrations, the aqueous extract inhibited cell growth at the highest concentration, 1250 µg/ml. However, this biphasic effect diminished with time of exposure and all concentrations showed inhibitory effects on RD cells during the 72-hour exposure period. This resulted in 50% least inhibitory concentration (LC50) of 63.9219 µg/ml, while GI50 was 580.375 µg/ml after 48 hrs and 342.75 µg/ml after 72 hrs of exposure.

The methanolic extract stimulated growth of RD cells during the first two days of incubation over a range of 39-312.5 µg/ml. In contrast to this growth-stimulating effect
at lower concentrations, the methanolic extract inhibited cell growth at the highest two concentrations, 1250 and 625 µg/ml. However, the time-dependent effect seemed to prevail over the growth-stimulatory effect after 72 hrs of exposure, resulting in inhibition of cell growth (Fig. 1.d). LC50 was 48.8282 µg/ml. After 48 and 72 hrs of exposure, GI50 was 592.125 and 590.2813 µg/ml, respectively.

3.3. Effect on AMGM5 Cell Line

Fig. 1.e demonstrates that significant reduction in growth of AMGM5 cells of the aqueous extract was at the highest two concentrations after the longest incubation period: After 72 hrs of treatment, the concentrations 1250 and 625 µg/ml inhibited cell growth by 31.8 and 44%, respectively. Noteworthy, however, is that viability did not go beneath the 50% barrier under any concentration or incubation period. As a result, GI50 was >1250 µg/ml.

Also, only the highest two concentrations of the methanolic extract, 1250 and 625 µg/ml, caused significant inhibition of growth of AMGM5 cells after 72 hrs (Fig. 1.d). However, growth percentages of AMGM5 cells even under those two concentrations were still higher than 50%. GI50 was >1250 µg/ml.

3.4. Effect on AMN3 Cell Line

The aqueous extract had a time-dependent effect on viability of AMN3 cells. Cell viability decreased with incubation time and significant reduction in viability was recorded on day two of exposure (Fig 1.g). The concentrations 312.5, 625 and 1250 µg/ml caused significant reduction in cell viability after 48 hrs of incubation with AMN3 cells as cell viability reached 39, 31, and 36%, respectively. Therefore, percentages of inhibition were 61, 69, and 64%, respectively. Aqueous extract concentrations 39, 78.125, 625 and 1250 µg/ml caused significant reduction in viability after 72 hrs of exposure by lowering cell viability to 49.3, 46.57, 19.76 and 20.88%, respectively. This reflected percentages of inhibition of 50.7, 40, 80.24 and 79.12%, respectively. The aqueous extract had a biphasic effect on growth of AMN3 cells: After 24 hrs of incubation, lower concentrations significantly increased percentage of growth, while the highest concentration, 1250 µg/ml, inhibited cell growth giving 34% inhibition. Extended treatments with the different concentrations of the aqueous extract for 48 and 72 hrs diminished the biphasic effect of the extract, and the time-dependent inhibitory effect was more profound. As a result, the lower concentrations had insignificant effect on cell growth after 48 hrs of treatment, while the higher concentrations (>156.25 µg/ml) showed significant inhibitory effects. After 72 hrs of treatment, most concentrations significantly inhibited cell growth. LC50 of the aqueous extract was 42.6133 µg/ml. GI50 of the extract was 578.125 µg/ml after 48 hrs and became as low as 289.0625 µg/ml after 72 hrs of treatment.

Incubation of AMN3 cells for 24 hrs with the three lowest concentrations of the methanolic extract: 39, 78.125 and 156.25 µg/ml caused significant concentration-dependent increases in cell proliferation thus reaching 120, 150, and 167%, respectively (Fig. 1.h). However, this stimulatory effect became insignificant at the concentration 312.5 µg/ml and even inhibitory at the highest two concentrations 625 and 1250 µg/ml as cell viability went down to 39 and 40%, respectively. The second and third days of treatment with lower concentrations: 39, 78.125, 156.25 and 312.5 µg/ml had no significant effect on cell viability. However, the highest two concentrations, 625 and 1250 µg/ml, significantly decreased cell viability during the second and third day of treatment. On the second day of treatment with 625 and 1250 µg/ml, cell viability was 35 and 34% and reflecting percentages of inhibition was 65 and 66%, respectively. On the last day of treatment, viability was 54 and 17.3%, respectively, representing 46 and 82.7% inhibition. A look at Fig. 1.h explains these figures since a clear biphasic (nonmonotonic) effect is evident. The methanolic extract of S. triloba had a growth-stimulating effect over the concentrations 39-312.5 µg/ml. In contrast, the higher concentrations (>312.5 µg/ml) had inhibitory activity on the growth of AMN3 cells. GI50 was 542.7813 and 541.6875 µg/ml after 48 and 72 hrs of treatment, respectively.

4. Discussion

4.1. Effect on Human Larynx Epidermoid Carcinoma (Hep-2) Cell Line:

Plants contain several different families of natural products among which are compounds with weak estrogenic or antiestrogenic activity towards mammals. These compounds, termed phytoestrogens, include certain isoflavonoids, flavonoids, stilbens, and lignans (Dixon, 2004). Guo et al. (2004) studied the effect of daidzein, one of the most common phytoestrogens, on human nonhormone-dependent cervical cancer cells, HeLa in vitro. At most concentrations ad incubation times, cancer cells were arrested at G0/G1 phase and a time-dependent manner was found. The suppressive effects of daidzein were by means of alteration in cell cycle, apoptosis, and inhibition of telomerase activity.

An increase in cell viability was recorded for Hep-2 on the first and second days of exposure to the aqueous and the methanolic extracts. However, the effect was lower on the second and became inhibitory on the third day of exposure to result in the reduction of cell viability by the end of the 72nd hour of exposure. Li and his colleagues (2002) reported that ursoolic acid (UA) and oleanolic acid (OA), which are triterpene acids distributed widely in plants all over the world, had an inhibitory effect on human colon carcinoma cell line HCT15. Proliferation assays showed that proliferation of UA- and OA-treated cells slightly increased at 24 hrs and significantly decreased at 48 hrs and 60 hrs. Cell cycle analysis showed that treated cells gradually accumulated in G0/G1 phase, with a concomitant decrease of cell population in S period and no detectable apoptotic fraction. Therefore, it was concluded that UA and OA had significant anti-tumor activity with the possible mechanism of action of inhibiting tumor cell proliferation through cell-cycle arrest.

In his work on the effect of black tea polyphenols and terpenoids and green tea terpenoids, Sa’eed (2004) recorded a slight increase in density of Hep-2 cells at low concentrations on the third day of exposure, while higher concentrations decreased cell density. LeBail et al. (1998) showed that flavonoids at low concentrations significantly
Figure 1. Effect of S. triloba extracts on viability of cell lines: (a) effects of the aqueous extract on HEp-2, (b) effects of the methanolic extract on HEp-2; (c) effect of the aqueous extract on RD, (d) effect of the methanolic extract on RD; (e) effect of the aqueous extract on AMGM5, (f) effect of the methanolic extract on AMGM5; (g) effect of the aqueous extract on AMN3, (h) effect of the methanolic extract on AMN3.
enhanced the proliferation of human breast cancer cells MCF-7. The response was dose-dependent. In contrast, they reduced MCF-7 cell proliferation at high concentrations.

4.2. Effect on Rhabdomyosarcoma (RD) Cell Line:

Both the aqueous and methanolic extracts of *S. triloba* had biphasic (nonmonotonic) effects on Rhabdomyosarcoma (RD) cells *in vitro* (i.e. hormesis). Compared with inhibition of HEP-2 cell, RD inhibition by both the aqueous and methanolic extracts of *S. triloba* was higher. This reflected the sensitivity of RD cells compared with HEP-2 cells. Al Hilli (2004) showed in his study on the effect of crude extracts of *Cyperus rotundus* L. that RD cells were more sensitive to each of the hexane, aqueous, and ethanolic extracts compared with HEP-2 cells. Highest inhibition was recorded after 72 hrs of treatment with any of the extracts. Exposure of HEP-2 and RD cells to green and black tea terpenoids and polyphenols also revealed that RD cells were more sensitive than HEP-2 cells (Sa’eed, 2004). Among HEP-2, RD-228 and Vero cells tested for long-term survival studies, RD-228 cell line was more sensitive to the total alkaloid fraction of the methanolic extracts of *Solanium pseudocapsicum* (Vijayan et al., 2004).

4.3. Effect on AMGM5 Cell Line:

AMGM5 cells showed little sensitivity to the aqueous and methanolic extracts of *S. triloba* compared with HEP-2 and RD cells (GI₅₀ >1250 µg/ml). Only higher concentrations (625-1250 µg/ml) were able to exhibit inhibitory effects towards AMGM5 cells after 72 hrs of incubation, while lower concentrations caused insignificant or significant stimulatory effects.

Glioblastoma is usually rapidly fatal (Stupp et al., 2005). Glioblastomas are the most common and aggressive type of malignant glioma. They are characterized by rapidly dividing cells, invasion into normal brain, and a high degree of vascularity. At present, there is no effective treatment for glioblastoma (Ouafik et al., 2002). Tamoxifen has been shown to have cytotoxic activity against glioma cells at high doses. Tamoxifen caused dose-dependent growth inhibition in human glioma cell lines U87MG, U137MG and U138MG. However, a slight increase in cell growth was recorded at low concentrations of tamoxifen (≤10 µM) (Hui et al., 2004).

The current standard of care for newly diagnosed glioblastoma is surgical resection to the extent feasible, followed by adjuvant radiotherapy (Stupp et al., 2005). Oil extract of *Salvia triloba* and higher concentration may play role in inhibition of this neoplastic cell line.

4.4. Effect on AMN3 Cell Line:

Growth of AMN3 cells due to treatment with the aqueous and methanolic extracts of *S. triloba* was biphasic (nonmonotonic). However, the highest concentration, 1250 µg/ml, of both extracts showed time- and dose-dependent inhibitory effects. AMN3 cells were more sensitive to the aqueous extract than to the methanolic extract (LC₅₀ of the aqueous extract was 42.6133 µg/ml and GI₅₀ of the aqueous extract was 289.0625 µg/ml after 72 hrs, while GI₅₀ of the methanolic extract was 542.6875 µg/ml after 72 hrs of treatment). In addition, AMN3 cells were more sensitive to crude extracts of *S. triloba* than HEP-2, RD, and AMGM5 cells.

Induction in absorbance of AMN3 cells was reported after 24 hrs of exposure to low concentrations of hexane extract of *Cyperus rotundus* L. (Al Hilli, 2004), and due to exposure to low concentrations of black tea polyphenols for 72 hrs (Sa’eed, 2004). AMN3 cells were more sensitive to the aqueous extract of *C. rotundus* L than to the hexane or ethanolic extracts (Al Hilli, 2004). In addition, AMN3 cells were more sensitive than HEP-2 and RD cells to the crude extracts of *C. rotundus* L. (Al Hilli, 2004) and green tea polyphenols and terpenoids (Sa’eed, 2004).

Phytoestrogens are a chemically diverse group of compounds made by plants. Although estrogens stimulate the growth of many breast tumors, there is a negative correlation between the incidence of breast cancer and the phytoestrogens-rich diet of certain Asian populations. To begin resolve this paradox, the estrogenic properties of genistein and quercetin, two flavonoid phytoestrogens particularly abundant in soybeans were analyzed. At minimal effective concentrations, both flavonoids stimulated the proliferation of MCF-7 (estrogen-dependent) and MCF-7SH (estrogen-independent) cell lines of human breast cancer. At high concentrations, such as those reached with a soy-rich diet, genistein and quercetin were strong cytotoxic agents that even killed estrogen receptor-independent HeLa cells. Therefore, it was concluded that the mode of action of phytoestrogens and the balance between being risk or chemopreventive factors for breast cancer might depend on the dietary load.

Tamir et al. (2000) studied the estrogenic properties of glabridin, the major isoflavon in licorice (*Glycyrrhiza glabra* L.) root. The effect of increasing concentrations of glabridin on the growth of three breast tumor cells (T-47D, MCF-7, and MDA-MB-468) was biphasic. Glabridin showed an estrogen receptor-dependent, growth-promoting effect at low concentrations (10 nM-10μM) and estrogen receptor-independent anti proliferative activity at concentrations of >15μM.

Studies of the effect of dietary phytoestrogen biochanin A on proliferation of MCF-7 cells also showed that biochanin A exhibited biphasic regulation on MCF-7 cells. At concentration of less than 10 µg/ml, cells responded to biochanin A by increasing cell growth and de novo DNA synthesis. The addition of biochanin A at concentrations of greater than 30 µg/ml significantly inhibited cell growth and DNA synthesis in a dose-dependent fashion resulting in an IC₅₀ value of 40 µg/ml (Hsu et al., 1999).

Sukardiman and his colleagues (2000) found that the flavonoid pinostrobin inhibited DNA topoisomerase I activity resulting in the cleavage of DNA and thus cytotoxicity towards cell culture of human mammary carcinoma. Panaro et al. (1999) observed that flavone acetic acid (FAA), a synthetic flavonoid, mediated G₂/M cell cycle arrest in NMU (mammary carcinoma cells of the rat). Morphological cytogenetic analysis demonstrated a colcemid-like effect of FAA on cytokinesis by causing accumulation of condensed C-metaphases, which are caused by colcemid and named accordingly.

Conolly and Lutz (2004) tried to promote a scientific discussion of the concept termed "hormesis" due to the fact...
that for reactions of a complex biological system to a toxicant, nonmonotonic (biphasic) dose-effect relationships can be observed showing a decrease at low dose followed by an increase at high dose, or vice versa. Calabrese and Baldwin (1998) suggested that chemical hormesis was a reproducible and relatively common biological phenomenon.

5. Conclusion:

_Salvia triloba_ has cytotoxic effect against some cancer cell lines. It seems likely that these plants may act at various therapeutic levels, but further studies are required to evaluate the practical values of therapeutic application.

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