Cytogenetic and Cytotoxic study of *Micromeria myrtifolia* Extract on Animal and Human Cancer Cell Line

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Abstract

The study was designed to evaluate the cytogenetic effect of *Micromeria myrtifolia* methanolic extract and cyclophosphamide in albino male mice (*in vivo*). The cytogenetic evaluation included the metaphase index of bone marrow. Two doses 200 and 400mg/kg of extract and one doses of cyclophosphamide 15mg/kg were investigated as a positive control. Additionally the cytotoxic effect of *Micromeria myrtifolia* on two cancer cell line was carried out. The chemical detection of the flavonoids, polysaccharides and alkaloids was also carried out. The chemical detection for active compounds revealed that the methanol extract was positive for flavonoids and polysaccharides and it was negative for alkaloids. Also the result showed that *M. myrtifolia* caused a significant increase in metaphase index of mice bone marrow cells in comparison with the negative control (distilled water) and positive controls (Cyclophosphamide). The methanolic extract showed some inhibitory effect on L20B and RD cell line growth rate after 72 hr in comparison with control. From this study we conclude that the *M .myrtifolia* extracts were effective in enhancing the values of immunological parameters by increasing the metaphase index of mice bone marrow cells and the study prove that the *M .myrtifolia* extract has significant cytotoxic activity on two types of tumor cell lines.

دراسة وراثية خلوية سمية لمستخلص نبات الزوفا *Myrtifolia micromeria* في الخلايا السرطانية للانسان والحيوان

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الخلاصة

أجريت الدراسة لمعرفة التأثير الوراثي السمي للمستخلص الميثانولي لنبات الزوفا عقار السايكلوفوسفومايد في ذكور الفئران البيض، كما تضمنت هذه الدراسة معامل الاقسام الخلوي الاستوائي لخلايا نقي العظم، وقد تم استخدام جرعتان بواقع 200 و400 ملغم من المستخلص وجرعة واحدة من عقار السايكلوفوسفومايد بتركيز 15 ملغم/ كغم، فضلا عن التأثير السمي لنبات الزوفا عبر خطين من الخلايا السرطانية، كما تم اجراء الكشف الكيميائي عن مركبات الفلافينودات والسكريات المتعددة والقلويدات، وقد اظهر الكشف الكيميائي احتواء المستخلص الميثانولي على الفلافينودات والسكريات المتعددة وخلوه من القلويدات، كما أظهرت هذه الدراسة وجود زيادة معنوية في معامل الانقسام الخلوي الاستوائي لنقي العظم في الفئران مقارنة مع السيطرة السالبة (الماء المقطر) والسيطرة الموجبة (السايلكوفوسفومايد) وأظهر المستخلص الميثانولي تأثير تثبيطي في نمو خطي الخلايا السرطانية (RD) و (L20B) بعد مدة تعريض بلغت 72 ساعة مقارنة مع السيطرة ومن هذه الدراسة نستنتج بان مستخلص نبات الزوفا ذو قابلية على تعديل قيم المعاملات المناعية من خلال زيادة طور الانقسام الاستوائي في خلايا نخاع العظم للفئران المختبرية واثبتت هذه الدراسة ان نبات الزوفة ذو فعالية سمية واضحة في خطي الخلايا السرطانية.

Introduction

Herbal supplements are dietary supplements that contain herbs, either singly or in mixture. A herb also called a botanical is a plant or plant part used for its scent, flavor, and/or therapeutic properties. Products made from botanicals that are used to maintain or improve health have been called herbal supplements, botanicals, or phytomedicines (20).

Herbal remedies and alternative medicines are used throughout the world, and in the past, herb often represented the original sources of most drugs. The plant kingdom has provided an endless source of medicinal plants first used in their crude forms as herbal teas, syrups, infusions, ointments, liniments and powder (24).

Interest in a large number of traditional natural products has increased (30). It has been suggested that aqueous and ethanolic extract from plants used in allopathic medicine are potential sources of antiviral and anti tumor agents (8). Furthermore, the selection of crude plant extracts from screening programs has the potential of being more successful in its initial steps than the screening of pure compounds isolated from natural products (15).

With respect to the former field ,and over the last two decades, an expanding body of evidence from epidemiological and laboratory studies has demonstrated that some edible plants as a whole, or their identified ingredients, have substantial protective effects on human mutagenesis and/or carcinogenesis (18). In this regard, a progress was made to under stand the biochemical mechanisms of dietary and medicinal anti-mutagens and anti-carcinogens, and the investigators have broaden the horizons to cover various aspects of chemoprevention by edible photochemical or their mixtures (29).

The immune system is further related target of the medicinal plant research. In this context, it is interesting to note that it has been recognized for several decades that nutrition and health are closely interrelated, and much research has focused on the nutrition effect on the immune system and its proper functioning (16). More recently, the effect of nutrition on chronic degenerative disease has become an area of intense study, bringing about a shift in the concept of optimal nutrition away from merely preventing diseases stemming from nutrient deficiencies to reducing the risk of chronic diseases (13). One group of nutrients thought to play a vital role in such disease prevention is antioxidants. Evidence has been accumulating over the past few years that many plant constituents not previously thought of

as separate nutrients, for example the phenolic compounds, can act as powerful antioxidants and immune modulators (26).

Micromeria spp. (Labiatae) are perennial herbs or chamaephytes. The extract of these plants has been reported to have some medicinal value, for example, the leaves have been reported to possess anti-inflammatory and antimicrobial effects and are also used against some other human ailments (inflamed eyes, wounds, skin infections, stomachache, chest pain, colds, fevers, and others (21).

These plants are Known to be a rich source of essential oil contents (mono and sesquiterpenes especially thymol, carvacrol), and flavanoids, to which, the medicinal effects of *Micromeria* have been ascribed (28).

This study aimed to extract the active compounds from *Micromeria myrtifolia* and tested the cytogenetic and cytotoxicity effect of the plant.

Materials and methods

Laboratory Animals:

Albino male mice (*Musmusculus*) were used to carry out the investigations of the present study. They were obtained from Biotechnology Research Center (Al-Nahrain University). Their age range was 8-9 weeks, and their weight was 23-27 grams at the beginning of experiments. They were caged in the animal house of the supplier, in which the temperature was 23-26°C, and a light: dark periods of 10:14 hours/day.

The animals had free excess to diet (standard pellets) and drinking water during all experiments.

Plant Extraction:

The plant powder was extracted with methanol solvents,50 grams of the powder were extracted in the solvent at 45°C for three hours using the soxhlet apparatus. The resulted extract was concentrated by rotary evaporated at 45°C, and the dry deposit was obtained, the methanol deposit extract was dissolved in sterile distilled water to prepare the doses (25).

Experimental Design:

Two doses of *Micromeria myrtifolia* were used to assess the cytogenetic effects of extract, and their modulating effects of the drug cyclophosphomide in albino male mice. Two stages were used in the evaluation.

First Stage:

In this stage, the cytogenetic effects on mitotic index of *Micromeria myrtifolia* and cyclophosphamide were investigated. The animals were divided into three groups:

1.GroupI: treated with distilled water (negative controls = 4 animals).

2.GroupII: treated with cyclophosphamide at a dose of 15 mg/kg (positive controls = 4 animals).

3.Group III: treated with two doses of the *Micromeria myrtifolia* plant extract (200, 400 mg/kg) (8 animals).

The tested materials were given orally as a single dose 0.1 ml per a day for 7 days. Then the mice were sacrificed in day 8 for laboratory assessments. The total numbers of mice in this stage were 16 animals.

Second Stage:

Cell line study of the plant *Micromeria myrtifolia* was carried out in Biotechnology Research Center/Al-Nahrain University . In this study, the preliminary screening on cytotoxic activity of *Micromeria myrtifolia* was carried out .

The screening involved the investigation of cytotoxicity of methanolic extract of *Micromeria myrtifolia*, then the extract were evaporated until complete dryness. The screening of cytotoxicity was carried out on tumor cell lien L20B and RD.

The percentage of growth inhibition was calculated according to (22), and according to following equation:

$$\text{Growth inhibition } \% = \frac{Control - Treatmentcell}{Control} \times 100$$

Laboratory Methods:

Mitotic index:

The metaphase index was assessed on somatic cells obtained from the bone marrow of experimental animal mice, according to a pre-established method (4), which was based on the following steps:

1. The animal was injected intraperitoneally with 0.25 ml of colchicin solution with concentration of 1mg/ml, and after two hours, the animal was sacrificed by cervical-dislocation.
2. The animal was dissected, and femur bone was removed and transferred to two Petri dishes containing 5 ml of PBS.

3. The femur bone was cleaned from muscles and other tissues, and both ends were cut. Then, the bone marrow was obtained with PBS 5 ml using disposable insulin syringe, and collected in a test tube.
4. The cell suspension of tube was gently pipette, and centrifuged 2000 rpm for 5 minutes.
5. After discarding the supernatant, the cell deposit was suspended in 10 ml of a warm 37°C, hypotonic KCl (0.075M), and incubated for 30 minutes in a water bath 37°C, with shaking every 5 minutes.
6. The tube was centrifuged 2000 rpm for 5 minutes, and the supernatant was discarded.
7. The cell deposit was slowly suspended in 5 ml of cooled fixative 4ºC, and incubated for 30 minutes at 4°C.
8. Step 7 was repeated, and the cell deposit was gently suspended in 1-2 ml of cooled fixative, to prepare a single cell suspension.
9. Few drops 4-5 of the fixed cell suspension were dropped vertically from a height of about 3 feeton cleaned slides to give chance for nuclei and chromosomes to spread well.
10. The slides were air-dried, stained with Giemsa stain for 15 minutes, rinsed with distilled water, and left to dry at room temperature.
11. The slides were examined under oil immersion lens 100X, and at least 1000 cells (divided and non-divided cells) were scored. Then, the percentage of metaphase cells (metaphase index) was calculated according to the following equation:

$$\text{Metaphase index (\%)} = \left(\frac{\text{Number of Metaphase Cells}}{\text{Total Count}} \right) \text{x } 100$$

Cell line procedure:

This was applied according to the method adopted by (1).

Results and Discussion

Metaphase index:

In the present study, only cells at metaphase were scored in samples of bone marrow and there for the metaphase index was based on the percentage of these cells. A treatment with Cyclophosphamide caused a significant reduction in the metaphase index 2.44% as compared to the negative control 2.76 %. In contrast the two doses of extract were associated with a significant increased index 6.2% and 9.6% respectively as compared with both negative and positive control (Table, 1).

Plant extract preparation is effectively and extensively used for their medicinal properties, and they have become increasingly popular worldwide (5).

Interest in a large number of traditional natural products has increased (32; 30). It has been suggested that aqueous and ethanolic extract from plants used in allopathic medicine are potential sources of antiviral and anti-tumor agents (8). Furthermore, the selection of crude plant extracts from screening programs has the potential of being more successful in its initial steps than the screening of pure compounds isolated from natural products (15).

One of the current strategies for drug discovery involved the study of plant materials based on the ethnobotanical usage. The search for anticancer drugs, use of a plant or plant materials for the treatment of certain cancer-related disease can provide a guide for further studies, this includes, cancer treatment, immune disorders, infectious diseases, parasitic diseases and viral diseases (9).

The results indicate that Cyclophosphamide with its dose resulted in the reduction of MI in mouse bone marrow cells. This may be related to the proteins required for mitosis which were not produced at the same quantities, or the code was not reached the cell to induce it to proliferate, or the drug may cause the death of bone marrow cells (31) or due to defect occurred in the mitotic spindle composition during cell division (27).

The plant extract were significantly effective in increasing the metaphase index of bone marrow cells, but the extent effect was dose dependent. The lymphocytes are originated in the bone marrow through the lymphoid lineage progenerater, which is the outcome hemopoitic stem cell proliferation.

The latter outcome was investigated in term of bone marrow metaphase index, which was significantly increased in mice treated

with the dose of plant extract, and therefore, their absolute peripheral count was excepted to be increased. *M. myrtifolia* extract contain polysaccharides, and in this regard Biringanine and co-workers 2004 (6) suggested that several plant extracts of the family extract have some therapeutically activities that could be dependent on the extracts content of polysaccharides, and also it has been recently demonstrated that polysaccharides isolated from fungal spp. (*Ganoderma lucidum*) accelerated the recovery of bone marrow cells and total leucocyte count in immunosuppressed mice (34).

Many herbs have a long history of use and of claimed health benefits. However, herbal supplements and botanicals have potent pharmacologic activity and, consequently, contribute to potential adverse effects and drug interactions (20).

Table (1): Metaphase index of bone marrow cells (mean ± standard error) of albino male mice treated with *Micromeria myrtifolia* concentrated filtrate, distilled water (negative controls) and cyclophosphamide drug (positive control).

Groups		Dose (mg/kg)	Mean ± Standard Error %	Treatment Efficiency (%)	Statistical Evaluation
Positive Control (Cyclophosphamide Drug)		15	2.44±0.43	-18.44	A
Negative Control (Distilled Water)		0.00	2.76±0.21		A
M.myrtifolia extract	First dose	200	6.2±0.3	147.03	B
	Second dose	400	9.6±0.30	34.69	C

Different letters in the same column: significant difference ($P \leq 0.05$) between means.

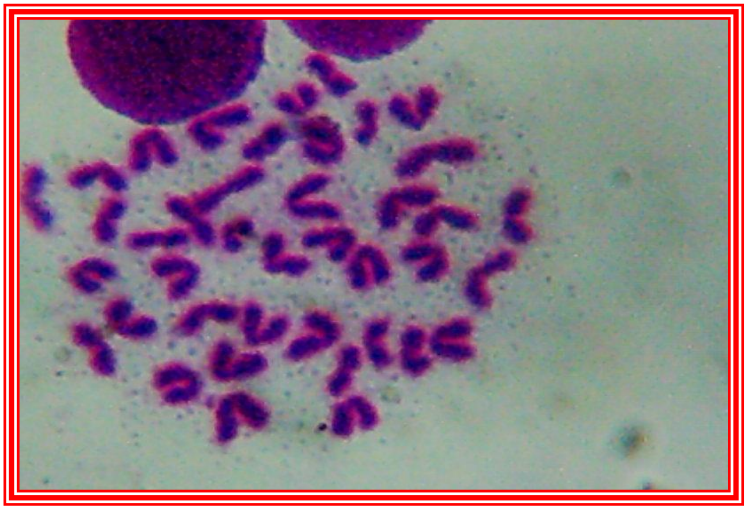


Figure (1): Cell in metaphase stage taken from mice treated with extract showing normal chromosome**.**

Cytotoxicity of Plants Extracts on Tumor Cells:

The results of plant extract on their effect on both cell lines shows there are also a significant differences ($P<0.05$) between means of cell viability of each L20B and RD cultures treated with *Micromeria myrtifolia* extract(Table 2).*Micromeria myrtifolia* cause a significant decrease ($P<0.05$) in cell viability of L20B and RD cell line for all concentrations to reach maximum significant decrease at concentration 1000µg/ml in comparison with the negative control(Figure 2 ; Figure 3).

However, natural products provide an inexhaustible source of anticancer drugs in terms of both variety and mechanism of action (23).

The use of herbal supplements by cancer patients in the preoperative period is prevalent and consistent with the substantial increase in the use of alternative medical therapies by cancer patients (14). Anywhere from 25% to 85% of cancer patients are seeking alternative and complementary nutritiona4l therapies for prevention or during cancer treatment. The use of these therapies is highest among patients with breast cancer 80% to 85% (19), prostate cancer 27% to 43% (17), and head and neck cancer 25% (10). In a study of 820 cancer patients receiving chemotherapy or radiation therapy, 29.1% reported using complementary integrative nutritional therapies that were not prescribed by their physician (14).

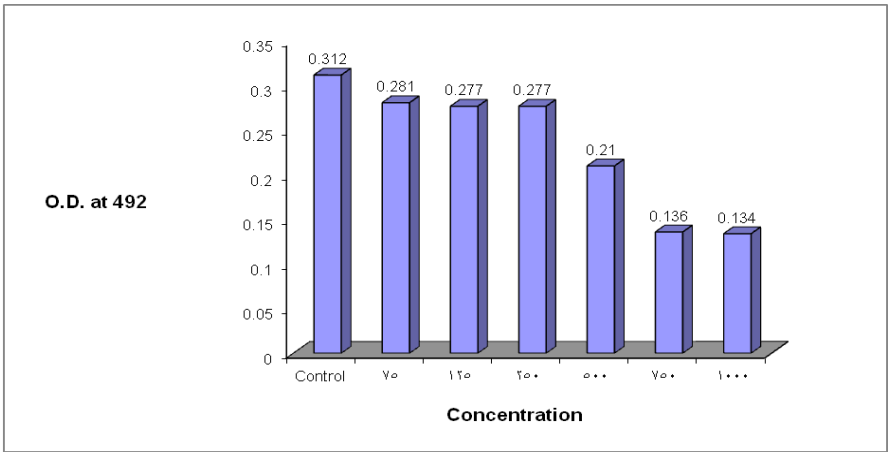
Such findings can be considered important, especially if we consider that methanol extract of members of Lamiaceae family showed anti- carcinogenic effects, *in vivo* and *in vitro* and the dose was effective in this regard (3; 11). Equally important, carcinogenesis is normally preceded by mutation induced by different agents, especially those that have oxidant effects(33; 2).

The terpens are a class of essential oil terpens that have many biological and pharmaceutical activities, which can be useful to treat human disease; for example, volatile terpens as monoterpens sesquiterpens are known to have several pharmacological activities including antibacterial, antifungal, antispasmodic (7), also they can be used as potentiators of anti tumor agents which can increase bioavailability of an orally administered hydrophopic pharmaceutical compound by inhibition of cytochrome p450 and /or decreasing of p-glycoprotein drug transport (12).

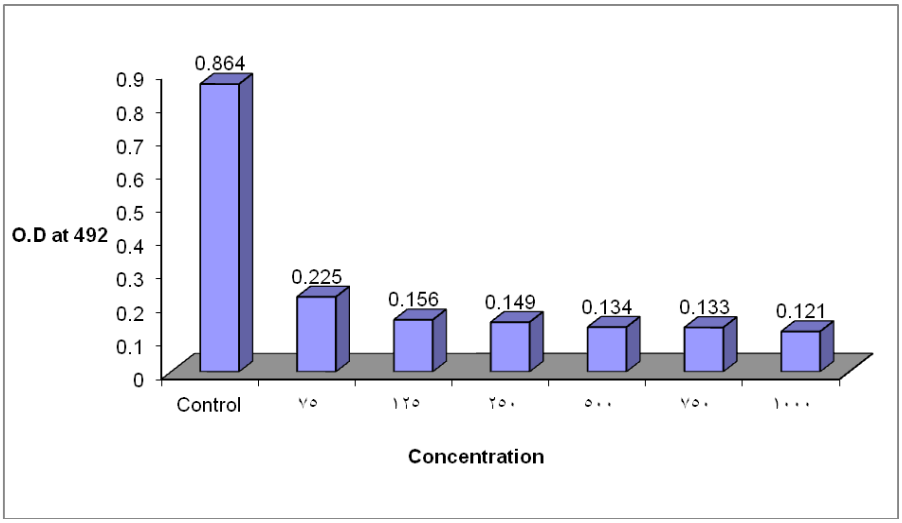
Table(2): Cytotoxic effect of *Micromeria myrtifolia* extract on the growth of cancer cell line(L20B,RD).

Concentration µg/ml	**Cell viability (Absorbance) % (m)**	
	L20B	**RD**
	M.myrtifolia	***M.myrtifolia***
Negative control	**A 0.312**	**A 0.864**
75	**B 0.281**	**B 0.225**
125	**B 0.277**	**C 0.156**
250	**B 0.277**	**C 0.149**
500	**C 0.210**	**D 0.134**
750	**D 0.136**	**D 0.133**
1000	**D 0.134**	**E 0.121**

Differences A,B,C,D,E are significant ($p<0.05$) to compression column.



Figure(2): Growth inhibition percentage of *Micromeria myrtifolia*on L20B after 72hr.



Figure(3): Growth inhibition percentage of *Micromeria myrtifolia*on RD after 72hr.

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