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Full Length Research Paper

Effect of marjoram extract treatment on the cytological and biochemical changes induced by cyclophosphamide in mice

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Marjoram (*Origanum majorana* L., Family: Lamiaceae) is a common spicy medicinal herb, used as a home remedy for the treatment of different ailments. It is well recognized with its popular name "Marjoram". It is also used worldwide in food for better flavor, both in dry form and as fresh vegetable. Marjoram is a rich source of polyphenols which are known natural antioxidants. Marjoram ethyl alcohol extract treatment was evaluated for its effect on bone marrow, micronucleated polychromatic erythrocytes and selected biochemical parameters in mice. In addition, its protective role against the side effects of cyclophosphamide (CP, an established anticancer drug) was also studied. Marjoram treatment 125 mg/kg was found to be the minimum effective dose which could induce changes in some of the parameters investigated. However, CP treatment in mice brought highly significant changes in cytological as well as biological parameters included in the present study. Marjoram was added to CP treatment in order to evaluate the effects of CP and Marjoram combined treatment in mice. Marjoram was found to protect any changes occurring in RNA, DNA, and protein contents in the liver and testes of treated mice as compared with the control. It was interesting to notice that marjoram treatment did not interfere with the desired cytotoxic activity of CP.

Key words: Marjoram (*Origanum majorana* L.), cyclophosphamide (CP), bone marrow, micronucleated polychromatic erythrocytes, mitodepression, protein and nucleic acid contents.

INTRODUTION

Origanum majoranum L. (Lamiaceae) is found all over Asia, Arabian peninsula, Africa, America and Europe (El-Ashmawy et al., 2007). It is much recognized by its common names, such as sweet marjoram and origano (Al-Howiriny et al., 2009; Christman, 2010). Marjoram was initially used by hippocrates as an antiseptic agent. It is a well-liked home remedy for chest infection, cough, sore throat, rheumatic pain, nervous disorders, cardiovascular diseases, epilepsy, insomnia, skin care, flatulence and stomach disorders (Bremness, 1994; Faleiro et al., 2005; Yazdanparast and Shahriyary, 2008). Many of the folklore medicinal claims about marjoram were confirmed in different experimental models (Busatta et al., 2008). Marjoram alcoholic, aqueous extracts, and essential oil could protect liver and kidney damage, and lead acetate injury (El-Ashmawy et al., 2005; 2007a, b; Abd El-Ghany and El-Metwally, 2010). The anti-proliferative activity of marjoram was confirmed in human lymphoblastic leukemia cell line Jurkat (Papageorgiou et al., 2008; Abdel-Massih et al., 2010). The essential oil obtained from the flowering heads of marjoram has aromatic smell and contain high percentage of polyphenols and monoterpenes which are established antioxidants (Novak et al., 2000). It is worth mentioning that marjoram volatile oil was also found to control oxidative damages induced by ethanol toxicity to brain, liver, and fertility (Aheme et al., 2007; El-Ashmawy et al., 2007a, b). Marjoram oil mixed with other substances is used in aromatherapy (Hazzit et al., 2006; Aromatherapy, 2011). Ursolic acid, carnosic acid, and carnosol isolated from marjoram possessed free radical scavenging properties (Chung et al., 2001; Vagi et al., 2005; Miron et al., 2011). Ursolic acid was found to reduce Abeta-induced oxidative injury (Heo et al., 2002). Furthermore,

it also showed inhibitory effect on acetyl cholinesterase in Alzheimer's disease (Chung et al., 2001). Origanum/Marjoram species, have been reported to show significant antimicrobial activity and its prolonged use may reduce gut bacteria (Meschino, 2005; Hazzit et al., 2006; Leeja and Thoppil, 2007). Marjoram oil was found effective against *Beneckea natriegens*, *Ervinia carotovora*, and Moraxella species. It also affected the growth of *Staphylococcus aureus* and *Aspergillus niger* (Deans and Svoboda, 1990). The emulsified oil of marjoram was reported to possess strong antiparasitic activity (Force et al., 2000). Marjoram was also found effective against food-borne pathogens and spoilage bacteria in minced meat (Dragland et al., 2003; Barbosa et al., 2009). Marjoram is also added to mouth rinse and its cures inflamed skin wounds and infections (Bremness 1994; McCarroll et al., 2008; Suntar et al., 2011). Fresh as well as dry marjoram is one of the most popular culinary herbs. It is used globally in salad, meat and vegetable dishes for flavor and as a natural antioxidant to avoid the use of synthetic antioxidants in food (Arts and Hollman, 2005; Shan et al., 2005; D'Antuono and Elementi, 2006; Yanishlieva et al., 2006; Hossain et al., 2008; Machado et al., 2010).

Keeping in view the common use and healing potential of marjoram, the present study was designed to explore the cytological and biochemical effects of its ethanol extract. The response of marjoram treatment on the changes induced by a well known mutagen, cyclophosphamide (CP) in mice were investigated. The results obtained are presented in this study.

EXPERIMENTAL

The aerial part of marjoram was collected from Jezan region of Saudi Arabia. The plant material was identified by the taxonomy unit of the Research Centre for Medicinal, Aromatic, and Poisonous Plants, King Saud University, Riyadh, Saudi Arabia where the herbarium specimen was kept for record purpose. The powdered plant material was extracted in a soxhlet apparatus with 95% ethanol. The solvent was evaporated at reduced pressure. Thus, the obtained semisolid marjoram extract was kept in a refrigerator till used. A fresh solution of the extract was prepared in distilled water before administration.

The study was conducted in male mice (aged 6 to 7 weeks, SWR, home bred), weighing 23 to 27 g. The animals (5 mice each) were randomly assigned to different treatment and control groups. All animals were maintained under standard conditions of humidity, temperature, and light (12 h dark, 12 h light). Mice in each group were fed with purina chow diet and had free access to water. A dose of 500 mg/kg body weight was used as the highest dose of the extract (Tanira et al., 1988; Al-Harbi et al., 1994). The freshly prepared aqueous suspension of the extract was given orally (gavage) to the animals for seven days. The experimental groups were as follows:

(1) Untreated control (distilled water);
(2) Marjoram 125 mg/kg/day;
(3) Marjoram 250 mg/kg/day;
(4) Marjoram 500 mg/kg/day;
(5) Cyclophosphamide (CP, 100 mg/kg, i.p.);
(6) Marjoram 125 mg/kg/day pretreatment (7 day) + CP 100 mg/kg, i.p.);
(7) Marjoram 250 mg/kg/day pretreatment (7 day) + CP 100 mg/kg, i.p.);
(8) Marjoram 500 mg/kg/day pretreatment (7 day) + CP 100 mg/kg, i.p.).

CP was injected (100 mg/kg body weight, group 5) 30 h before sacrifice. The animals in groups 6, 7, and 8, received CP i.p. injection simultaneously with the last dose of marjoram. The femoral cells were collected in fetal calf serum. After centrifugation, the cells were spread on slides and air-dried. Coded slides were fixed in methanol and stained with May Gruenwald solution followed by Giemsa. The polychromatic erythrocytes (PCE, 100 per mouse) were screened for micronuclei and the mitodepression was obtained by PCE/NCE (normaochromatic erythrocytes) ratio (Al-Bekairi et al., 1991; 1992).

Biochemical procedures

The levels of protein and nucleic acid in liver and testes were determined according to the following procedure: The liver and testes from the same animals were quickly excised, frozen in liquid nitrogen and stored at -20°C until they were analyzed for total protein and nucleic acids (RNA, DNA). Total protein was determined by the method of Lowry et al. (1951). The standard method described earlier was used to determine the levels of nucleic acids (Al-Harbi, 1993).

Minimum effective dose determination

A separate experiment was designed to evaluate the minimum effective dose of marjoram. The additional experimental groups of mice consisted of:

(9) Untreated control (distilled water);
(10) Marjoram 31.25 mg/kg/day;
(11) Marjoram 62.50 mg/kg/day;
(12) Marjoram 125 mg/kg/day;
(13) CP 100 mg/kg, i.p.;
(14) Marjoram 31.25 mg/kg/day pretreatment (7 days) + CP 100 mg/kg, i.p.;
(15) Marjoram 62.50 mg/kg/day pretreatment (7 days) + CP 100 mg/kg, i.p.;
(16) Marjoram 125 mg/kg/day pretreatment (7 days) + CP 100 mg/kg, i.p.

The cytological and biochemical studies were conducted according to the already described protocol.
Statistical analyses were carried out by using Student-Newman-Keuls multiple comparison test.

RESULTS AND DISCUSSION

The results of the present study on the effects of marjoram treatment in mice are presented in Tables 1 to 4. There was no increase in the incidence of micronucleated PCE in marjoram-treated mice (Groups 2, 3 and 4). There were no significant changes observed in nucleic acid (RNA, DNA) and protein contents of liver and testes in the treatment groups as compared to the control. Earlier, several herbs and spices were reported

Table 1. Effect of *O. majorana* (Marjoram ethanol extract) treatment on the frequency of micronuclei in bone marrow cells of mice.

Group	Treatment and dose (mg/kg body weight)	Polychromatic erythrocytes screened	Micronucleated polychromatic erythrocytes %	Normo-chromatic erythrocytes screened	PCE/NCE ratio Mean ± SEM
1	Control (distilled water)	5285	0.37 ± 0.06	5100	1.04 ± 0.06
2	*O. majorana* (125)	5070	0.28 ± 0.07	5390	0.95 ± 0.07
3	*O. majorana* (250)	5355	0.31 ± 0.15	5278	1.02 ± 0.07
4	*O. majorana* (500)	5130	0.30 ± 0.05	5563	0.93 ± 0.05
5	Cyclophosphamide (CP, 100) i.p.	5427	4.87 ± 0.23**	6290	0.81 ± 0.05*
6	*O. majorana* (125) + CP (100) i.p.	5063	3.60 ± 0.28*	6515	0.78 ± 0.08*
7	*O. majorana* (250) + CP (100) i.p.	4989	2.70 ± 0.30**	6044	0.83 ± 0.06*
8	*O. majorana* (500) + CP (100) i.p.	5072	3.45 ± 0.25**	6807	0.75 ± 0.06*

*P < 0.05; **P < 0.001 (Student-Newman-Keuls multiple comparisons test). Five animals were used in each group. Treatment groups 2, 3, 4 and 5 were compared to group 1. Combined treatment groups 6, 7, and 8 were compared with group 5.

Table 2. The minimum effective dose of *O. majorana* (Marjoram EtOH extract) required to affect the frequency of micronuclei induced in bone marrow cells of mice by (CP) Cyclophosphamide treatment.

Group	Treatment and dose (mg/kg body weight)	Polychromatic erythrocytes screened	Micronucleated polychromatic erythrocytes %	Normo-chromatic erythrocytes screened	PCE/NCE ratio Mean ± SEM
9	Control (distilled water)	5195	0.32 ± 0.03	4627	1.12 ± 0.05
10	*O. majorana* (31.25)	5103	0.28 ± 0.03	5226	0.98 ± 0.07
11	*O. majorana* (62.50)	5769	0.27 ± 0.05	5309	1.09 ± 0.07
12	*O. majorana* (125)	5355	0.22 ± 0.03	4933	1.09 ± 0.08
13	Cyclophosphamide (CP, 100) i.p.	5458	4.67 ± 0.25**	7762	0.70 ± 0.04**
14	*O. majorana* (31.25) + CP (100) i.p.	5817	4.52 ± 0.38	8285	0.71 ± 0.08
15	*O. majorana* (62.50) + CP (100) i.p.	5900	3.95 ± 0.46	9578	0.66 ± 0.09
16	*O. majorana* (125) + CP (100) i.p.	5035	2.52 ± 0.30*	8507	0.59 ± 0.07

*P < 0.05, **P < 0.001 (Student-Newman-Keuls multiple comparisons test). Five animals were used in each group. Groups 10, 11, 12 and 13 were statistically compared with group 9. Groups 14, 15 and 16 were statistically compared with group 13.

to possess a wide range of biological activities leading to defend tissues against O2-induced damage. Marjoram species also offered a clear cut protection against H_2O_2–induced DNA damage showing its antioxidant potential (Shan et al., 2005; Yanishlieva et al., 2006; Aheme et al., 2007). In another recent study, a related species *Orthophytum compactum* showed high antioxidant potential with an IC(50) = 2 ± 0.1 mg/L, and its ethanol extract (56 mg/L) showed some activity against human breast cancer cells MCF7 (Babili et al., 2011). During the present experiment, marjoram treatment did not caused mitodepression as compared to the control. These results demonstrated that marjoram treatment is devoid of any cytotoxic or mutagenic effects in the given dose range (Tables 1 and 2). The results of current study are fully in agreement with the

Table 3. The effect of *O. majorana* (Marjoram ethanol extract) and combined treatment with CP and marjoram extract on protein and nucleic acid contents of liver in mice. (Mean ± S.E.M).

Group	Treatment and dose (mg/kg body weight)	DNA (µg/100 mg)	RNA (µg/100 mg)	Protein (mg/100 mg)
1	Control (distilled water)	217. 15 ± 7.78	630 ± 22	15.35 ± 0.33
2	*O. majorana* (125)	209.67 ± 5.50	625 ± 19	15.27 ± 0.29
3	*O. majorana* (250)	216.33 ± 5.83	600 ± 17	15.32 ± 0.21
4	*O. majorana* (500)	224.42 ± 6.65	590 ± 22	15.25 ± 0.23
5	Cyclophosphamide (CP, 100) i.p.	160 .18 ± 6.22*	465 ± 18***	13.51 ± 0.20***
6	*O. majorana* (125) + CP (100) i.p.	179.46 ± 6.77	550 ± 24	14.76 ± 0.35
7	*O. majorana* (250) + CP (100) i.p.	200. 52 ± 9.48	530 ± 20*	15.22 ± 0.19
8	*O. majorana* (500) + CP (100) i.p.	193.75 ± 7.83	595 ± 17**	15.28 ± 0.14

*P < 0.05; **P < 0.001 (Student-Newman-Keuls multiple comparisons test). Five animals were used in each group. Treatment groups 2, 3, 4 and 5 were compared to group 1. Combined treatment groups 6, 7 and 8 were compared with group 5.

Table 4. The effect of marjoram (*O. majorana* ethanol extract) and combined CP and Marjoram extract, on protein and nucleic acid contents of testes in mice (Mean ± S.E.M.).

Group	Treatment and dose (mg/kg body weight)	DNA (µg/100 mg)	RNA (µg/100 mg)	Protein (mg/100 mg)
1	Control (distilled water)	410. 5 ± 14.0	325.5 ± 16.0	10.2 ± 0.23
2	*O. majorana* (125)	400.6 ± 13.5	320.4 ± 19.5	10.2 ± 0.15
3	*O. majorana* (250)	403.5 ± 10.0	310.6 ± 17.4	9.9 ± 0.12
4	*O. majorana* (500)	395.4 ± 6.5	307.5 ± 11.0	10.0 ± 0.13
5	Cyclophosphamide (CP, 100) i.p.	383.5 ± 9.0	305.0 ± 14.0	9.6 ± 0.16
6	*O. majorana* (125) + CP (100) i.p.	385.6 ± 6.7	310.0 ± 15.5	9.7 ± 0.30
7	*O. majorana* (250) + CP (100) i.p.	376. 5 ± 10.5	315.5 ± 20.0	10.0 ± 0.19
8	*O. majorana* (500) + CP (100) i.p.	380. 0 ± 9.5	320.5 ± 17.0	9.8 ± 0.24

P> 0.05 (Student-Newman-Keuls multiple comparisons test). Five animals were used in each group. Treatment groups 2, 3, 4 and 5 were compared to group 1. Combined treatment groups 6, 7 and 8 were compared with group 5.

earlier reports where marjoram treatment was establish to possess free radical scavenging and a dose dependent anti-proliferative effect on human leukemic cell line due to the presence of various active chemical constituents (Shan et al., 2005; Yanishlieva et al., 2006; Abdel-Massih et al., 2010; Mossa and Nawwar, 2011).

CP is employed widely alone or in combination with other drugs for the treatment of neoplastic illness (Hamsa and Kuttan, 2011). CP toxicity is mainly due to its active metabolites acrolein and phosphoramide mustard (Bhatia et al., 2006). In the present study, CP-treatment (Group 5) caused a significant ($P < 0.05$) mitodepression and highly significant ($P < 0.001$) increase in the incidence of micronucleated PCE as compared to the controls. In the CP-treated animals, a significant reduction in protein and nucleic acid contents of liver were observed as compared to the control. However, the effect was less profound in testes protein and nucleic acid contents as compared to the control group mice. The use of CP as adjuvant chemotherapy for better safety and tolerance of docetaxel was successful in cases of breast cancer (Coleman, 2003; Watanabe et al., 2010). All the observed changes in the current study were in agreement with earlier findings, which added further support to the established cytotoxic and mutagenic properties of CP (Al-Bekairi et al., 1992; Al-Harbi et al., 1994). CP treatment is also recognized to induce secondary malignancy and other toxic effects, therefore, all parameters were supposed to be observed carefully to avoid the complications caused by prolonged CP-treatment (Jankovic and Radosavljevic, 2007). Cyclophosphamide is also found to cause transitional cell carcinoma of bladder in children (DeLair et al., 2005). In addition, the preventive protocols for handling antineoplastic drugs should also remain effective (Travis et al., 1995; Moretti et al., 2011).

It is worth mentioning that in the present study, marjoram-treatment significantly reduced ($P < 0.05$) the intensified increase in micronucleated PCE caused by CP-treatment (Groups 6, 7 and 8) without affecting its cytotoxicity. CP- treatment is known to induce drastic biochemical changes, however, marjoram treatment inhibited the biochemical changes induced by CP-treatment in mice (Tables 3 and 4). During current investigation, it was concluded that treatment with

marjoram lower doses (62.50 and 31.25 mg/kg body weight) could not induce any significant biochemical changes. Thus, confirming 125 mg/kg body weight dose level to be the minimum effective dose which could produce statistically significant biochemical changes. Such findings also added support to the observed antimutagenic properties of marjoram treatment occurring at a dose level (125 mg/kg).

The findings of the present study, were further supported by the results of earlier experiments where treatment with different extracts of Marjoram/Origanum species exerted antimalarial, antioxidant, and protective activities (Babili et al., 2011). Such properties of marjoram might be attributed to the presence of terpenes, polyphenols, phenolic glycosides, phenolic derivatives, flavonoids, tannins, sitosterol and essential oil. In additions, the findings of the current study were further substantiated by earlier reports where different chemical compounds present in marjoram were shown to possess different biological activities including antioxidant potential and free radical scavenging properties. (Assaf et al., 1987; Nakatani, 2000; Novak et al., 2000; Heo et al., 2002; Kelly, 2004; Hazzit et al., 2006; Ahmed et al., 2009; Misharina et al., 2009; Hossain et al., 2010a, b).

Generally, the high antioxidant capacity of marjoram and other Origano species were found depending on their total phenol contents. It was followed by thyme extracts. The amount of phenolic acids, flavones, flavanones and flavonols were found to play a vital role in the antioxidant capacity of marjoram extracts (Miron et al., 2011). Such activities might be held responsible for the protective role observed by marjoram treatment (Abdel-Massih et al., 2010; Babili et al., 2011). The findings of the present experiment, are fully in agreement with earlier reports demonstrating that treatment with marjoram alcoholic, aqueous extracts, and essential oil could protect liver and kidney damage and genotoxicity persuaded by lead acetate treatment (El-Ashmawy et al., 2005; 2007a, b; Abd El-Ghany and El-Metwally, 2010).

The possibility of interference of some of marjoram phyto-constituents with CP metabolism cannot be ruled out (Dragano et al., 2010). Earlier reports also suggested that some microsomal enzymes in hepatic cells are known to alter CP metabolism under the influence of certain drug products (Sladek, 1972; Akah and Onuogu, 1993; Al-Bekairi et al., 1992; Al-Harbi et al., 1994; Yazdanparast and Shahriyary, 2008). Different anti-cancer drugs including CP, are known to induce secondary carcinoma as a part of their side effects (McCarroll et al., 2008). Combination chemotherapy and use of antioxidants have been considered to reduce the frequency of relapse, and adverse effects of anti-cancer drugs (Travis et al., 1995; Colman, 2003; Maihail et al., 2011). Among urinary bladder cancer survivors, CP-treatment increased the occurrence of subsequent neoplasms in the urinary bladder of cancer survivors (Travis et al., 1995; Cohen et al., 1998; Bermejo et al., 2009). Various plant extracts such as *Ipomoea obscura*, *Capsicum frutescens*, *Juglans regia*, *Nigella sativa*, *Trigonella foenum-graecum*, and *Withania somnifera* could ameliorate CP-induced toxicities by amending antioxidant activity and levels of certain enzymes (Bhatia et al., 2006a, b; Winters, 2006; Alenzi et al., 2010; Oboh and Ogunruku, 2010; Oboh et al., 2011; Hamsa and Kuttan, 2011). Therefore, there is need to find some natural drugs which can reduce the adverse effects of anticancer drugs without altering their cytotoxicity.

During the present study, the effect of marjoram on the incidence of micronucleated PCE induced by CP-treatment was not dose dependent and no significant increase in activity occurred after 250 mg/kg body weight dose level. These finding are fully in agreement with the earlier reported response of several other herbal drugs which could not demonstrate a dose-dependent elevation of anticlastogenic potential (Renner, 1986; Yanishlieva et al., 2006). The results of the present study supported the safe use of marjoram in spices due to its antioxidant potential, and this natural drug warrant further investigations.

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Conclusion

Marjoram was found to possess antimutagenic, antioxidant potential and low toxicity. There is need of further studies on this naturally occurring medicinal plant.

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