Cancer chemopreventive effect of dietary Zataria multiflora essential oils

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Abstract: Zataria multiflora Boiss., with the common name Avishan-e-Shirazi, is native to Iran. This herb has been found to possess varied pharmacological properties. In the present study, for the first time, colon chemopreventive effects of Z. multiflora essential oils (0.01% and 0.1% in the diet) in rats treated with 1,2-dimethylhydrazine (DMH) were demonstrated. For this purpose, the oxidative stress/antioxidant parameters (lipid peroxidation, glutathione, superoxide dismutase, catalase, and ferric reducing ability of plasma) concomitant with xenobiotic metabolizing enzymes (CYP450 and GST) were considered. Moreover, the colonic β-catenin protein was examined in colon tissues followed by histopathological analysis. The results showed that the dietary intervention of Z. multiflora oils in tumor-bearing rats induced with DMH caused significant modulatory effects on DMH-metabolizing enzymes, but with lack of oxidative stress/antioxidant status. In parallel, the elevated protein β-catenin induced by DMH decreased significantly in treatment groups. However, the decreased tumor formations in histopathological biopsies in treated groups further confirmed these results. Thus, with reference to histopathological and biochemical data, it can be safely concluded that inhibition of colon premalignant lesions induced by DMH was mediated by the interference of Z. multiflora oils through the modulatory effect of DMH-metabolizing enzymes in association with β-catenin and no impact of antioxidant/oxidative stress state.

Key words: Zataria multiflora, essential oils, colon tumorigenesis, oxidative stress, xenobiotic metabolizing enzymes

1. Introduction

Zataria multiflora Boiss. (Lamiaceae), with the common name Avishan-e-Shirazi, is native to central and southern parts of Iran (Amin et al., 1991). This herb is not only used as food flavoring, but is also diversely utilized in traditional medicine for its antiseptic, analgesic, antispasmodic, and antiinflammatory properties (Moazzafarian, 1996; Hosseinzadeh et al., 2000; Ramezani et al., 2005). The cytotoxicity and antibacterial effects of Zataria multiflora essential oils (Amin et al., 2010; Malekinejad et al., 2012) have also been reported. In addition, the radioprotective effect of Zataria multiflora extract against genotoxicity induced by γ-irradiation in human blood lymphocytes was noted (Hosseinimehr et al., 2011). Hosseinimehr et al. also reported the chemoprotective effects of Zataria multiflora extract against genotoxicity induced by cyclophosphamide in mice bone marrow cells. It can thus be soundly suggested that the chemopreventive activity of Zataria multiflora essential oils may have intense application potential, especially in the colon cancer treatment.

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Colorectal cancer is one of the most common cancers in the United States (James et al., 2002; Stone et al., 2004). Annually, around one million new cases of colorectal cancer are diagnosed and half a million mortalities are reported worldwide (Stone et al., 2004). However, according to the Iranian Annual National Cancer Registration Report, colorectal cancer is respectively the third and fifth most common cancer form in women and men (Ministry of Health, Islamic Republic of Iran, 2007). Effectually, the incidence of colorectal cancer has increased during the past 25 years (Mosavi-Jarrahi et al., 2005).

Considering the effect of lifestyle on induction of colon tumors via different environmental carcinogen (Choudhary and Hansen, 1998; Doyle et al., 2007; Harriss et al., 2009), 1,2-dimethylhydrazine (DMH) was detected as a potent colon specific carcinogen. Hepatic DMH metabolism by cytochrome p450 (CYP450) results in the production of active intermediates azoxymethane and methylazoxymethanol in the liver, which are further transported into the colon (Perše and Cerar, 2005; Dadkhah et al., 2011). Methylazoxymethanol decomposition leads
to methylidazonium ion formation and methylate cellular components such as DNA in colonic epithelial cells resulting in β-catenin gene mutation (Reynoso-Camacho et al., 2011). In the nucleus, β-catenin complexes with TCF/LEF family members, which functioning as transcriptional activators (Fuchs et al., 2004). In normal epithelial cells, cytosolic β-catenin interacts with APC, Axin, glycogen synthase kinase-3β, and other proteins, leading to phosphorylation of Ser and Thr in the N-terminal region of β-catenin, followed by ubiquitination and proteasomal degradation (Fuchs et al., 2004; Katoh, 2005; Wang et al., 2006). Mutation in β-catenin or APC prevents the phosphorylation and consequently β-catenin proteasomal degradation, thus leading to β-catenin/TCF/LEF complexes’ accumulation in the nucleus and activation of downstream target oncogenes such as c-myc, c-jun, and cyclin D1, ultimately giving rise to colon cancer (Perše and Cerar, 2005; Wang et al., 2006; Sirnes et al., 2014).

Unfortunately, in the utilization of high-efficiency drugs, serious side effects cannot be ruled out. As a result, more attention has been paid to natural alternatives with fewer side effects. Hence, this study was specifically conducted to evaluate, for the first time, the colon chemopreventive activity of Iranian Zataria multiflora essential oils in rat model colon carcinogenesis induced by DMH. For this purpose, the antioxidant/oxidative parameters and major DMH-metabolizing enzymes were considered followed by estimation of β-catenin protein levels in treated groups.

2. Materials and methods

2.1. Preparation of Zataria multiflora essential oils
Essential oils were extracted from Zataria multiflora Boiss. (Lamiaceae) aerial parts using a Clevenger-type apparatus (Fatemi et al., 2012). The extraction was carried out for 2 h and the resultant oil was stored in dark glass bottles in a freezer (−20 °C) until further use.

2.2. Induction of colon tumor in rats
Young male Wistar rats (100 ± 20 g) purchased from the Pasteur Institute of Iran were maintained at 25 ± 2 °C with a 12-h light/12-h dark cycle. Animal studies were approved by the Medical Ethics Committee of Tarbiat Modares University. This ethics committee was based on the World Medical Association Declaration of Helsinki (adopted by the 18th World Medical Assembly, Helsinki, Finland, in June 1964).

DMH was dissolved in 1 mM EDTA just before use and the pH was adjusted to 6.5 with 1 mM NaOH to ensure the stability of the chemical. The rats were randomly assigned to 4 groups (8 rats/group). The rats in group 1 received 0.5 mL of EDTA, the vehicle of the DMH, subcutaneously (s.c.) once a week for 18 weeks and served as the DMH group. Groups 3 and 4 were given DMH injections (30 mg/kg b.w.) and diets containing 0.01% and 0.1% of Zataria multiflora essential oil respectively, and they were considered as treated groups. The diet containing the essential oil was simultaneously initiated with DMH treatment and continued until experiment termination (180 days). At the end of the experiment, the animals were anesthetized and blood was collected by heart puncture. Animals were then sacrificed; liver tissues were removed and processed for biochemical assays.

2.3. Colon tumor enumeration
At the termination of the experiment (180 days), the animals were sacrificed and their colons were removed. For this purpose, the animals were cut open along the longitudinal axis from the cecum to the anus and flushed with isotonic saline. The colons were divided into 3 sections and designated as section a – proximal colon, section b – middle colon, and section c – distal colon. The respective incidences, inhibitions, numbers, positions, and sizes of tumors were recorded and the colons were fixed in 10% neutral buffered formalin (Sigma) and embedded in paraffin. Later, the tissue sections (6 mm) were stained with hematoxylin and eosin (H&E) for histological observation. Finally, the colon tumors were classified according to morphology, extent of invasion, and differentiation.

2.4. Preparation of tissue homogenate and plasma
At the end of experimentation (180 days), the heparinized blood samples were collected by heart puncture and centrifuged at 3000 × g for 10 min to obtain plasma. Liver and colon samples were immediately transferred to ice-cold containers and homogenized (20% w/v) in the appropriate buffer using a homogenizer (Heidolph Diax 600).

2.5. Biochemical assays: lipid peroxidation
A weighed portion of liver was homogenized in phosphate buffer (100 mM, pH 7.0) and used to measure the concentration of thiobarbituric acid reacting substances (TBARS) as an indicator of lipid peroxidation. Lipid peroxides are unstable and decompose to form a complex series of compounds including malondialdehyde (MDA). The concentration of TBARS was measured spectrophotometrically according to the instructions of the kit purchased from Enzo Life Sciences, Inc. (UK). The MDA assay kit is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA that yields a stable chromophore with maximal absorbance at 586 nm. Finally, the level of lipid peroxidation was obtained by MDA standard curve.

2.5.1. Glutathione estimation
Glutathione (GSH) was estimated in liver homogenate based on the protocol of the purchased kit from BioVision,
In this assay, a unique buffer eliminates protein thiol interference and stabilizes GSH. A chromogenic reagent (o-phthalaldehyde) then reacts with GSH, generating a fluorescent compound that can be measured by spectrofluorometer. The concentration of GSH is calculated by a standard curve.

2.5.2. Determination of superoxide dismutase and catalase enzyme activities
The activities of superoxide dismutase (SOD) and catalase (CAT) were estimated in liver homogenate using commercial kits (BioVision, Inc.) and following the instructions given by the company. The SOD assay kit utilizes WST-1, which produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method.

The CAT assay kit provides a highly sensitive and simple assay for measuring CAT activity in biological samples. In the assay, CAT first reacts with \( \text{H}_2\text{O}_2 \) to produce water and oxygen. The unconverted \( \text{H}_2\text{O}_2 \) reacts with an OxiRed probe to produce a product, which can be measured at 570 nm. CAT activity is reversely proportional to the signal.

2.5.3. Glutathione S-transferase activity
Liver cytosolic glutathione S-transferase (GST) activity was measured spectrophotometrically using CDNB as a substrate with reference to kit instructions from BioVision. The GST assay kit is based upon the GST-catalyzed reaction between GSH and the GST substrate, CDNB (1-chloro-2,4-dinitrobenzene). The product of this reaction produces dinitrophenyl thioether, which can be detected by spectrophotometer at 340 nm.

2.5.4. CYP450 activity
CYP450 activity was studied in liver homogenate according to the procedure described in the kit purchased from Enzo Life Sciences. The CYP450 assay kit is based upon the transmission of electrons between NADPH and CYP450 substrate, resulting in demethylation of the substrate and formation of formaldehyde. The formaldehyde reacts with formaldehyde detection reagent, producing a compound with fluorescence activity. The formaldehyde formation is calculated by a standard curve.

2.5.5. Ferric reducing ability of plasma assay
Ferric reducing ability of plasma (FRAP) assay was performed using TPTZ reagent as described by Benzie and Strain (1996). FRAP level was calculated by plotting a standard curve of absorbance against the \( \mu \text{mol/L} \) concentration of Fe(II) standard solution.

2.5.6. Measurement of \( \beta \)-catenin at protein levels
\( \beta \)-Catenin levels in colonic preparations were measured quantitatively using a commercially available kit (Roche Diagnostic GmbH, Germany). The assay was performed according to the manufacturer's instructions. The \( \beta \)-catenin ELISA kit uses 2 antibodies that immobilize \( \beta \)-catenin on a microtiter plate. The HRP enzyme conjugated with the second antibody then generates a product that can be detected by spectrophotometer at 450 nm.

2.6. Statistical analysis
Data are presented as mean ± standard error of mean (SEM). The results were subjected to one-way ANOVA followed by Tukey's honestly significant difference test using SPSS 19.0. Significance was defined at \( P < 0.05 \).

3. Results

3.1. Tumor characterizations and stages in tumor-bearing rats treated with Zataria multiflora essential oils
At the termination of the experimental period (180 days), total number of tumors in all groups (except the control group) was 106 tumors with 100% incidence in DMH-treated rats (Table 1 and 2). Collectively in the treated groups, 4 tumors were allocated to section a, 30 tumors to section b, and 93 tumors to section c. The average numbers and sizes of tumors by colon length significantly decreased in the treatment groups (0.01% and 0.1% essential oil) in comparison to the DMH-treated rats (\( P < 0.05 \)), although with some differences seen in sections a, b, and c (Table 1).

As shown in Table 2, both Zataria multiflora essential oil treatments could inhibit the formation of tumors. The tumor incidences in Zataria multiflora essential oil-treated groups (0.01% and 0.1% in the diet) were reduced to 87.5% and 75%, respectively. However, the tumor inhibition rates were 43.3% and 56.6%, respectively, in the treated groups. Although in all the experimental groups, the tumor incidence in the colon tissues was allocated to sections in the order of \( c > b > a \), the highest inhibition rate was observed in section a.

The effects of dietary Zataria multiflora essential oils on the total tumor numbers in DMH-treated rats based on the tumor classifications for proximal, middle, distal, and total colons are shown in Table 3. Cumulatively in the colon length, after 6 months of treatment, 11 tumors were assigned to tubular adenoma, 23 tumors to carcinoma in situ, and 72 tumors to invasive adenocarcinoma in all groups except the negative control group. In the treated groups, 3 and 8 tubular adenoma tumors were identified in sections a and b, respectively. The 23 tumors categorized as carcinoma in situ were distributed as 1, 8, and 14 tumors in sections a, b, and c, respectively. In addition, 14 and 58 tumors were recognized as invasive adenocarcinoma in sections b and c, respectively. It is important to mention here that invasive adenocarcinoma, especially in section c, had a higher rate of tumor incidence in the colon of treated rats. Furthermore, the invasive adenocarcinoma tumors, which numbered 45 in DMH-treated rats, were reduced to 18 and 9 in essential oil-treated groups, respectively.
Table 1. Effect of dietary *Zataria multiflora* essential oils (EOs) on the number and size of the tumors in proximal, middle, distal, and total colons of the DMH-treated rats.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>No. rats examined</th>
<th>No. rats with tumor</th>
<th>Total no. tumors</th>
<th>No. tumors/rat</th>
<th>Tumor size (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>a    b    c    T</td>
<td>a    b    c    T</td>
<td>a    b    c    T</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>0</td>
<td>0    0    0    0</td>
<td>0    0    0    0</td>
<td>0    0    0    0</td>
</tr>
<tr>
<td>DMH</td>
<td>8</td>
<td>8</td>
<td>4    15   34   53</td>
<td>0.5 ± 0.3′</td>
<td>1.9 ± 0.7′</td>
</tr>
<tr>
<td>DMH + 0.01% EOs</td>
<td>8</td>
<td>7</td>
<td>0    9    42   30</td>
<td>0 ± 0″</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>DMH + 0.1% EOs</td>
<td>8</td>
<td>6</td>
<td>0    6    17   23</td>
<td>0 ± 0″</td>
<td>0.7 ± 0.4</td>
</tr>
</tbody>
</table>

The rats in group 1 received 0.5 mL of EDTA, the vehicle of the DMH, s.c. once a week for 18 weeks and were considered as the control group. The rats in group 2 received 0.5 mL of DMH dissolved in EDTA (30 mg/kg b.w.) by injection (s.c.) once a week for 18 weeks and served as the DMH group. Groups 3 and 4 were given DMH injections (30 mg/kg b.w.) and diets containing 0.01% and 0.1% of *Zataria multiflora* EOs respectively for 6 months and were considered as treated groups. The colons were divided into 3 sections (a: proximal colon, b: middle colon, c: distal colon, T: total length of the colon). Values are mean ± SEM obtained from 8 animals in each group. *: P < 0.05 is considered significantly different from control group within each parameter. **: P < 0.05 is considered significantly different from DMH-treated group within each parameter.
3.2. Histopathological examinations in DMH-induced colon tumor in rats treated with Zataria multiflora essential oils

The colons of the control rats (group 1) showed normal Lieberkühn glands with normal mucosal and submucosal layers and typical colonic architecture with no signs of apparent abnormality (Figure 1a). There were no microscopically observable changes, including tumors, in the colonic morphology of this group. There were also no histological evidences of neoplasia or toxicity. Histopathological study revealed not only the presence of colon tumors in the experimental group that received DMH (group 2), but also histological features of invasive adenocarcinoma. There were also dysplasia and abnormal structures in the Lieberkühn glands. In this group, neoplastic cells invaded the muscular layers and formed gland-like structures accompanying cystic dilation (Figure 1b). In DMH-treated rats receiving 0.01% Zataria multiflora essential oil (group 3), tubular adenocarcinoma with cystic dilation of the glands developed. Tumor cells invaded the muscular layers of the intestine (arrow) (Figure 1c). A tubular adenoma (arrow) with no evidence of metastasis in the lamina propria and submucosa was reported in experimental group 4, which received DMH + 0.1% Zataria multiflora essential oil. Cystic dilation of the tumor glands was clearly observed (arrow head) (Figure 1d).

3.3. The effects of Zataria multiflora essential oil on hepatic oxidative injury parameters in colon cancer induced by DMH

Table 4 shows the effects of Zataria multiflora essential oil (0.01% and 0.1% in the diet) on antioxidant/oxidative injury parameters in treated rats. DMH treatment showed considerable increase in GSH level (P < 0.05).
The experimental rats treated with both doses of *Zataria multiflora* essential oils showed significant decreases in GSH levels as compared to DMH-treated rats (P < 0.05) (Table 4).

The hepatic TBARS levels (as an indicator of the lipid peroxidation) in all the experimental groups had nonsignificant differences (P > 0.05). Although the activities of hepatic antioxidant enzymes SOD and CAT in the DMH group were considerably lower than in the control group (P < 0.05), no differences were found in the groups treated with essential oils as compared to the DMH group (P > 0.05) (Table 4).

**Table 4. Effect of dietary *Zataria multiflora* essential oils (EOs) on hepatic and plasma antioxidant/oxidative parameters.** Values are mean ± SEM obtained from 8 animals in each group. *: P < 0.05 is considered significantly different from control group within each parameter. **: P < 0.05 is considered significantly different from DMH-treated group within each parameter.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH (ng/mL, liver)</th>
<th>Lipid peroxidation (µmol/g, liver)</th>
<th>SOD activity (U/mL, liver)</th>
<th>CAT activity (mU/mL, liver)</th>
<th>FRAP (mmol/L, plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>264.67 ± 31.5</td>
<td>230.91 ± 13.5</td>
<td>2.61 ± 0.27</td>
<td>4.88 ± 0.49</td>
<td>428.12 ± 35.7</td>
</tr>
<tr>
<td>DMH</td>
<td>350.3 ± 16*</td>
<td>245.46 ± 12.2</td>
<td>0.77 ± 0.07*</td>
<td>3.13 ± 0.42*</td>
<td>908.62 ± 66.9*</td>
</tr>
<tr>
<td>DMH + 0.01% EOs</td>
<td>260.17 ± 18.2**</td>
<td>238.31 ± 13.8</td>
<td>1.24 ± 0.38</td>
<td>2.87 ± 0.47</td>
<td>736.65 ± 117.5</td>
</tr>
<tr>
<td>DMH + 0.1% EOs</td>
<td>261.3 ± 14.6**</td>
<td>242.36 ± 15.9</td>
<td>1.49 ± 0.46</td>
<td>3.36 ± 0.36</td>
<td>859.62 ± 59.15</td>
</tr>
</tbody>
</table>
3.4. The effects of Zataria multiflora essential oil on FRAP in colon cancer induced by DMH

Table 4 shows the oral administration effects of Zataria multiflora essential oil on the FRAP level of the control group and the experimental rats treated with DMH. In comparison to the control group, the FRAP level was significantly higher in the DMH group (P < 0.05), whereas administration of Zataria multiflora essential oil had no effects on the FRAP value (P > 0.05).

3.5. The effects of Zataria multiflora essential oil on GST and CYP450 activities (hepatic detoxification enzymes)

The CYP450 and GST activities in the liver of experimental rats injected with DMH (DMH group) increased considerably in relation to the control group (P < 0.05) (Figures 2 and 3). The oral administration of Zataria multiflora essential oil in treatment groups significantly decreased the hepatic CYP450 and GST activity in comparison to the DMH group (P < 0.05) (Figures 2 and 3).

3.6. The effects of Zataria multiflora essential oil on colonic β-catenin protein level in colon cancer induced by DMH

As shown in Figure 4, the levels of β-catenin in colonic tissues of DMH-treated rats were significantly (P < 0.05) increased. Nevertheless, the colonic β-catenin level that increased due to DMH administration decreased in comparison to the control value (P < 0.05) in groups treated with Zataria multiflora essential oil (0.01% or 0.1% in the diet).

4. Discussion

The essential oil extracted from Z. multiflora cultivated in Shiraz, Iran, exhibited in vitro antioxidant activity with major compounds such as thymol (61.8%), carvacrol (10.5%), p-cymene (7.5%), and γ-terpinene (4.4%) (Fatemi et al., 2012). However, in the present study, we effectively, for the first time, demonstrated the chemopreventive activity of Iranian Z. multiflora against DMH-induced colon carcinogenesis. The mechanism(s) of this process could partly be due to the amendment of the xenobiotic metabolizing enzymes concomitant with decreased β-catenin protein level with no antioxidant/oxidative stress states. Our previous findings also validated the colon chemopreventive activities of caraway essential oils and seed powder as well as Nigella sativa seed powder in DMH-induced colon tumors through the modulatory effect of the DMH-metabolizing enzymes and β-catenin protein level (Dadkhah et al., 2011, 2014a, 2014b; Allameh et al., 2013).

As far as detoxification of DMH, popularly known as an environmental carcinogen, is concerned, DMH primarily undergoes metabolism in the liver by CYP450, resulting in the production of electrophilic diazonium ions that elicit oxidative stress (Fiala et al., 1977, 1987; Rijnkels and Alink, 1998) manifesting its action in the colon tissue and thus leading to colon cancer. In this study, the increased CYP450 activity after DMH treatments confirmed these reports, indicating the possible induction of CYP450 due to DMH metabolism in phase I of xenobiotic metabolism (Figure 2). Suppression of CYP450 by the seeds (Figure 2) reduced the DMH reactive metabolite formation, which in turn led to lower DMH carcinogenic effect by inhibiting the methylating DNA, RNA, or protein of colonic epithelial cells (Choudhary et al., 1998).
The enhanced level of β-catenin after significant decrease of DMH treatment due to Z. multiflora essential oil treatments (Figure 4) led to diminished DMH carcinogenesis, thus in turn confirming the essential oil's chemoprevention effects. In the present experiment, the rats treated with Z. multiflora essential oils could adapt to the enzyme interference with DMH metabolism (CYP450 and GST), leading to β-catenin depression (Figures 2–4). Moreover, β-catenin protein did not accumulate in the cytoplasm, resulting in low levels of β-catenin/TCF/LEF complexes in the nucleus and decreased activation of downstream target oncoproteins such as c-myc, c-jun, and cyclin D1. This eventually led to decreased tumor formation, resulting in weak progression of colon carcinogenesis (Fuchs et al., 2004; Katoh, 2005; Perše and Cerar, 2005; Wang et al., 2006; Sirnes et al., 2014).

The present results are in concurrence with the studies of Sadik and Shaker (2013), who reported that standardized pomegranate extract minimized all the aberrant alterations in the studied Wnt genes in colonic tissues of the DMH+pomegranate group in relation to the DMH-induced colon cancer group. Silibinin supplementation to DMH-treated rats restored the GSH-dependent enzyme levels but decreased the levels of β-catenin, PCNA, argyrophilic nuclear organizer regions, and cyclin D1 (Sangeetha et al., 2012).

In conclusion, this study indicated that dietary Z. multiflora essential oils possess colon chemopreventive properties through modulatory DMH-metabolizing enzyme activities, i.e. CYP450 and GST, concomitant with decreased levels of β-catenin protein. Contrastingly, the antioxidant/oxidative stress parameters were not involved in prevention mechanism(s) of the essential oils.

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References


