REPORT

IN VIVO ANTIOXIDANT ACTIVITY OF *ZATARIA MULTIFLORA* BOISS ESSENTIAL OIL

FARIBA SHARIFIFAR*[1], AMIN DERAKHSHANFAR[2], GHOLAMREZA DEHGHAN-NUDEH[3], NAJMA ABBASI[2], REZA ABBASI[1], REZA REZAEI GHARAEI[1], ABED KOOHPAYEH[4] AND MOHAMMAD DANESHPAJOUH[3]

[1]*Kerman University of Medical Sciences, Pharmaceutics Research Center, Department of Pharmacognosy, School of Pharmacy, Kerman, Iran*
[2]*Bahonar university, School of Veterinary, Kerman, Iran*
[3]*Kerman University of Medical Sciences, Department of Pharmaceutics, School of Pharmacy, Kerman, Iran*
[4]*Kerman Azad University, Kerman, Iran*

ABSTRACT

Zataria multiflora Boiss. (Lamiaceae) is an endemic plant to Iran with many traditional uses. We have reported previously *in vitro* antioxidant activity of the essential oil of the plant. In the present work we aimed to evaluate the antioxidant activity of the oil in rat. Antioxidant activity was measured by the test of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition and inhibition of lipid peroxidation by measuring the index of thiobarbituric acid reactive substances (TBARs). Three doses of 100, 200 and 400 µL/ kg of the essential oil of *Z. multiflora* (ZMO) was administered to animals by *i.g* routh for 10 days. The blood was collected in 11th day through direct puncture and the liver was rapidly excised. The histopathology studies of the animals was compared to animals in butylated hydroxyl toluene (BHT) group. The results showed that ZMO in all tested doses significantly was able to scavenge DPPH radical ($p<0.05$). ZMO also decreased TBARs in a dose dependent manner. No alteration in LFT enzymes or changes in histopathology of the liver was considered in ZMO treated groups. The results indicated that ZMO might be used in healthy medicine and food industry.

Keywords: *Zataria multiflora;* essential oil; lipid peroxidation; malondialdehyde

INTRODUCTION

Studies show that free radicals affect the biological systems which lead to different cell damages. Antioxidants are compounds which can protect the human from oxidative agents through different mechanisms. Interest in natural antioxidants especially those with plant origin is being increased because of common side effects of synthetic antioxidants in foods (Shahidi *et al*., 2009). Essential oils are herbal secondary metabolites which have gained interest because of their relatively low side effects (Ormancey *et al*., 2001; Sawamura, 2001). In continuing for finding the medicinal plants with antioxidant activity from Iran (Sharififar *et al*., 2010, Sharififar *et al*., 2009; Sharififar *et al*., 2007a; Yassa *et al*., 2005), the methanol extract and essential oil of *Zataria multiflora* Boiss (Avishane-shirazi) have exhibited considerable antioxidant effect (Sharififar *et al*., 2007b). This plant belongs to Lamiaceae and is native to Iran. It's essential oil has been proposed for disorders of respiratory and gastrointestinal system (Zargari, 1999). Furthermore the plant oil is a down-regulator of MDM2 gene expression which highlights the effectiveness of this oil in malignant disease (Vasiri Gohar *et al*., 2010). At the present work, we aimed to evaluate the antioxidant activity of the *Z. multiflora* oil and its toxicity for medicinal and neutraceutical purposes.

*Corresponding author: e-mail: fsharififar@kmu.ac.ir

MATERIALS AND METHODS

Plant materials

The aerial parts of *Zataria multiflora* were collected from Kerman, Iran in June 2008 and identified by Dr. Mirtajadini, Department of Botany, Bahonar University, Kerman, Iran. A voucher specimen of the plant (KF1249) is deposited at the Herbarium, School of Pharmacy, Kerman, Iran.

Chemicals

2-Thiobarbituric acid (TBA), trichloroacetic acid, butylated hydroxytoluene (BHT) and 1,1-diphenyl-2-picryl hydrazyl (DPPH) were purchased from Sigma (Deisenhofen, Germany). The other chemicals were of analytical grade.

Essential oil isolation

The plant essential oil was isolated by hydro-distillation method using Cleavenger instrument. After drying with anydrous sodium sulphate, stored in refrigerator until tested and analyzed.

Evaluation of toxicity of Z. multiflora essential oil (ZMO)
The toxicity studies of the *Z. multiflora* essential oil was done on the basis of pilot experiment.

Acute oral toxicity
Healthy adult male Wistar (200-250g) were purchased from Razi institute, Tehran, Iran. They were maintained on normal diet in individual metabolic cages with access to food and water in standard conditions (in a room temperature 25 ± 2 °C with 12-h light, 12-h darkness). The study protocol complied with current ethical regulations and related rules of animal research by the Medical Ethics Committee of Kerman University of Medical Sciences and all animals used in the experiment received humane care. 32 rats (randomly divided in 4 groups) received four different doses of ZMO (200, 400, 800 and 1600 µL/kg, i.g). The animals received food and water after administration of a single dose of ZMO and the mortality of animals was observed closely (Riley, 1960).

Sub-acute oral toxicity
For sub acute studies, 24 rats were randomly divided in 3 groups. ZMO was used at doses, 200, 400 and 800 µL/kg once a day, for 10 days by i.g. Control rats received equal volume of solvent/day orally for 10 day with access to food and water. The weight of animals and the ratio of organ weight/body weight of each animal was measured (Riley, 1960).

Treatment groups
Based on the results from toxicity studies, 40 rat were randomly distributed into 5 group of 8 animals. An equal volume of the essential oil or drugs with the following content administered daily to test groups. Animals from group 1-3 received various doses of 100, 200 and 400µL/kg/day essential oil of *Z. multiflora* (ZMO) by intra gastric intubation (i.g) for 10 days, positive control (group 4) and the control group (group 5) received BHT 20mg/kg and equal volume of the solvent (DMSO-water: 2-3), respectively.

The sampling of Blood
At the 11th day of the experiment (end of the test day), blood was prepared by cardiac puncture and the liver was excised. After blood coagulation, the serum was obtained after centrifugation at 2500 rpm for 15 min. The biochemical markers including aspartate amino-transaminase (AST), alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) in the serum of different treated rats were determined at the end of 10 days.

Antioxidant experiments
DPPH assay
For evaluating the radical scavenging activity of the animal serum, DPPH assay test was used in triplicate as described by Burits and Bucar, 2000. After adding 30 µL of serum to 3 mL of a DPPH solution (0.004%), sonicated for 2 minutes in 25^{o}C and incubated for 30 minutes at room temperature. The absorbance was read at 517nm. The percent of DPPH inhibition (I %) was determined as next formula: $I\% = (A_{blank}-A_{sample}/A_{blank}) \times 100$.

Estimation of Lipid Peroxidation
The most common method for measuring the products of lipid peroxidation is TBA test which quantitatively detects malondialdehyde (MDA). Lipid peroxidation in serum was estimated by the method described by Chaturvedi, 2008. 0.1 ml of serum was mixed with 2 ml of a stock TBA reagent which contains trichloroacetic acid (TCA) 15 % w/v 0.25 M HCl and thiobarbituric acid 0.37 % w/v 0.25 M HCl. The mixture was incubated about 10 min in boiling water bath. After cooling, added to 2 mL of 1M NaOH which prepared freshly. The absorbance was read at 535 nm and the results were showed as mM/dL serum.

Histopathological Studies
Histopathological studies of the livers of animals were carried out to determine the toxic or protective effects of the essential oil on liver in comparison to BHT. For assessing the early acute hepatic damage, the biomarker enzymes of AST, ALT and ALP were determined. Immediately after the removal of liver, were fixed in formaldehyde buffer (10%).

STATISTICAL ANALYSIS

The experiments were done in triplicate. Data were expressed as mean ± S.D. The mean of groups was compared by one- way ANOVA test and comparison of means was done by post hoc of Tukey. The differences were considered significant when $p < 0.05$ and $p<0.001$.

RESULTS

The hydro-distillation of the aerial parts of *Z. multiflara* gave a pale yellow-colored essential oil (2.8% v/w). The result of the oil analysis by GC–MS has been reported previously. Those findings exhibited that thymol is the major compound (37.59%) of the oil. The other main components identified as carvacrol (33.65%) and *p*-cymene (7.72%) (Sharififar *et al*., 2007b).

Toxicity studies and LD_{50} determination
The acute toxicity studies indicated the non-toxicity of ZMO at dose of 1600 µL/kg. No significant body weight changes were considered. In acute and sub acute toxicity studies, no abnormality was seen at macroscopic examination of post mortem of the animals (data have not shown). There was no abnormal weight gain in animals through the study. The ratio of organ weight/ body weight was not significantly different from control animals (table 1). The changes in biochemical and hematological parameters of animals orally treated with various doses of

ZMO were not significant from control. On the basis of obtained results, the lethal dose of ZMO was found to be more than 1600 µL/kg.

DPPH radical-scavenging activity assay

The percent of inhibition of DPPH radical of ZMO in different doses in comparison to control group are shown in Figure 1. The ZMO exhibited strong scavenging activity at all doses in a dose dependent manner which is significantly different from the control group especially at the doses of 200 and 400 µL/kg (p<0.05).

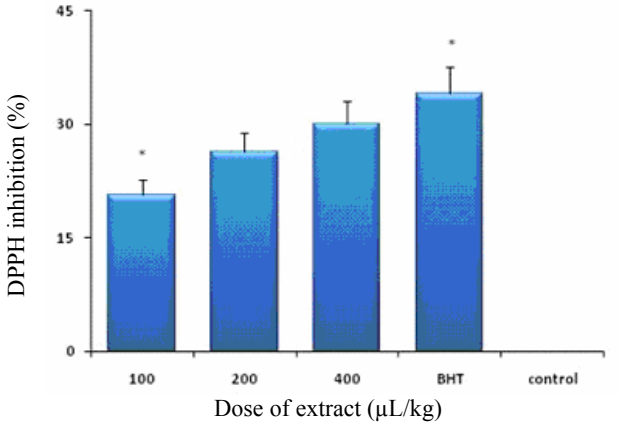

Fig. 1: Antioxidant activity doses of *Z. multiflora* (ZMO) in DPPH assay. The percent of inhibition of DPPH radical at different doses of ZMO have shown in comparison to BHT.

*P<0.05 compared with control group.

Inhibition of lipid peroxidation

ZMO administration in normal rat significantly reduced serum TBARs level ($p<0.05$) in all tested groups in a dose dependent manner (table 2). This effect is comparable with BHT. BHT has reduced TBARs levels 28.5% in comparison to control whereas ZMO has shown 10.1%, 24.4% and 32.8% reduction at doses of 100, 200 and 400 µL/kg respectively. An increasing at dose from 100-400 µL/ kg caused a 24.6% decrease in the level of serum TBARs.

The results of histopathology

Microscopic findings in BHT group revealed mild hyperemia, biliary hyperplasia, capillarization, hepato cellular degeneration and necrosis, and hydropic degeneration (Figure 2a & b). No histopathologic lesions were seen in the other groups. There was no sign of inflammation, fatty change or necrosis in ZMO treated and control animals.

DISCUSSION

Oxidative factors can induce damages in the human body which promote different disease. Our previous studies exhibited that ZMO has antioxidant activity *in vitro*. In the present work, ZMO in all doses exhibited considerable inhibitory effect on DPPH in a dose dependent manner which showed no significant difference with BHT at doses of 200 and 400µL/kg ($p<0.05$). In TBARs experiment, ZMO exhibited more activity than BHT at the dose of 400µL/kg (32.8 % in comparison to 28.5% inhibition respectively) (Table 1). The low level of TBARs in ZMO treated rats indicates that ZMO can potentially inhibit the lipid peroxidation. The peroxidation of lipids leads to generation of MDA which can cause cell damages (Yoshikawa *et al*., 1997). MDA level is a main marker of endogenous lipid peroxidation, so compounds with capacity of inhibition of lipid peroxidation, can prevent from pathologic conditions. Thymol and carvacrol have been reported recently to be major constituents in ZMO (Sharififar *et al*., 2007b), so its antioxidant activity could be due to the proton-donating effects of these oil components. Phenolic compounds by

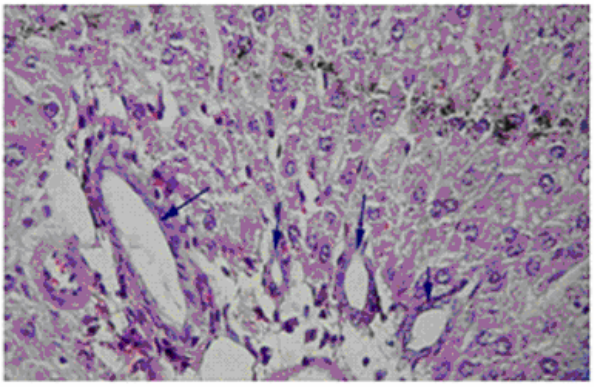

Fig. 2(a): Microscopic findings in BHT treatment group, biliary hyper-plasia. 4 bile ducts are seen in a portal area (arrows). (400, H&E

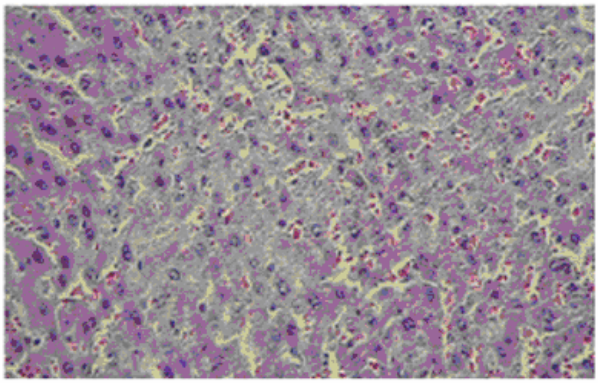

Fig. 2(b): Microscopic findings in BHT treatment group, Mild hyperemia and capillarization of the liver. (200, H&E

Table 1: The effects of oral administration of ZMO (100, 200 and 400µl/kg) on the organ weight /body weight ratio of rats (g/g)

Group	Liver × 10−3	Spleen × 10−3	Kidney × 10−3
ZMO group (100µl/kg)	17.5 ± 1.38	1.8 ± 0.4	3.1 ± 0.5
ZMO group (200µl/kg)	16.9 ± 2.6	1.6 ± 0.1	3.6 ± 0.4
ZMO group (400µl/kg)	14.9 ± 0.5	1.1 ± 0.09	3.2 ± 0.1
BHT	16.2 ± 3.9	1.7 ± 0.06	3.2 ± 0.03
Control	16.7 ± 1.5	1.6 ± 0.4	3.6 ± 0.03

Values are expressed as mean S.D. (n=6).

Table 2: The Effect of ZMO on animal serum of TBARS, AST, ALT, ALK, urea and craetinine in comparison to control

Group	TBARS (nmol/dl)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Urea (mg/dL)	Creatinine (mg/dL)
ZMO group (100µl/kg)	101.9 ± 14.3*	320.5 ± 87.4	131.5 ± 13.3	511.25 ± 77.0	40.35 ± 4.40	0.65 ± 0.06
ZMO group (200µl/kg)	85.7 ± 13.4**	275 ± 41.4	140.5 ± 24.8	397.25 ± 55.8	45.5 ± 1.73	0.624 ± 0.09
ZMO group (400µl/kg)	76.2 ± 10.1***	331.41 ± 54.4	149.32 ± 4.2	452.63 ± 52.6	49.55 ± 1.33	0.418 ± 0.02
BHT	81.0 ± 7.6***	208.2 ± 18.3	115.8 ± 13.3	468.8 ± 67.2	41.2 ± 2.38	0.64 ± 0.05
Control	113.4± 21.5	263.25 ±21.92	140.5 ± 36.0	391.5 ± 88.31	52.0 ± 12.35	0.575 ± 0.09

inhibiting the chain reaction of oxidation can retard or stop the oxidation of other molecules (Sepici-Dince *et al*., 2007). More importantly, our data demonstrated that even at the highest dose, ZMO showed no toxicity on liver in tested doses. Histopathology studies indicated that no lesion were seen in ZMO treated animals, whereas mild hyperemia, biliary hyperplasia, capillarization, hepato cellular degeneration and necrosis, and degeneration have been occurred in the animals treated with BHT (Figure 2a & b). For finding the mechanism of antioxidant effect of ZMO, further studies are needed to separate and identify the biologically active component(s). The results of the recent work show that ZMO at all tested doses significantly inhibited lipid peroxidation. It is for the first time that *in vivo* antioxidant activity has been reported for ZMO. Our results indicate that ZMO could be considered for medicinal purposes and its biological properties especially antioxidant activity may be an important support for its rational use.

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