Original Article

TIPS Trends in Phramaceutical Sciences 2015: 1(4): 243-250.

# Protective effect of methanolic extracts of *Thymus vulgaris* L. against cyclophosphamide-induced DNA damage in mouse bone marrow cells using the micronucleus test

Abbas Salmani[1], Ali Asghar Kosari[1], Aliyar Pirouzi[1], Marjan Omidi[3], Mehdi Mohsenzadeh[2*]

[1]Department of Research Center, Gerash Faculty of Medical Sciences, Shiraz University of medical Sciences, Shiraz, Iran.
[2]Cellular & Molecular Gerash Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[3]Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

## Abstract

Cyclophosphamide is a chemo-therapeutic agent used in the treatment of various cancers and autoimmune diseases. This composition has cytotoxic and clastogenic properties. The purpose of this study was to evaluate the protective effect of methanol extracts of *Thymus vulgaris* L. against DNA damage induced by cyclophosphamide in mouse bone marrow cells by the micronucleus test. The extract concentrations of 375, 750, 1500 mg/kg were injected intraperitoneally (Ip) into mice for 7 consecutive days. One hour after the last injection, cyclophosphamide 50 mg/kg Ip was injected. 24 hours after cyclophosphamide injection, the animals were killed and the samples of bone marrow were prepared and stained using the standard methods. For each sample, 1000 cells of polychromatic erythrocytes (PCE) and the same number of normochromatic erythrocyte (NCE) and the cells containing their micronucleus were counted. Cyclophosphamide increased the frequency of micronuclei polychromatic erythrocytes (MnPCE) and decreased cell proliferation (PCE/PCE+NCE). All doses of extracts significantly reduced the micronucleus frequency ratio ($P$<0.05). The cells proliferation ratio (PCE/PCE+NCE) was also increased. The best effect in reducing the micronucleus frequency was at 1500 mg/kg dosage. *Thymus* extract is able to reduce the clastogenic and cytotoxic effects of cyclophosphamide, due to its antioxidant properties, playing a protective role.

*Keywords*: Chemotherapy, Cyclophosphamide, DNA destruction, Micronucleus test, Thyme extract.

## 1. Introduction

Cancer is one of the main concerns of human communities. As a specific treatment for cancer, chemotherapy can be used with or withouth radiation therapy. It is stated that the chemotherapeutic agents can affect both normal bone marrow and malignant cells proliferation(1). Cyclophosphamide is one of the chemotherapeutic agents used for the treatment of various cancers, such as acute and chronic leukemia and malignant lymphoma. This agent can also be indicated for autoimmune diseases, in spite of its approved cytotoxicity and clastogenic properties. This compound is toxic, due to its alkalizing effect and creation of a crosslink between the two strands of DNA, breaking the DNA and protein synthesis inhibition (2). In the liver, cyclophosphamide is converted to aldophosphamide by the cytochrome P-450 enzyme. Then, aldophosphamide is converted to its active metabolites phosphoramide mustard and acrolein, spontaneously (3). Phosphoramide mustard has antineoplastic effect; while acrolein interferes with tissues antioxidant defense system, which leads to the formation of free radicals and has many toxic effects such as cell death,

*Corresponding Author*: Mehdi Mohsenzadeh, Cellular & Molecular Gerash Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
Iran.Email: mohsenzade@sums.ac.ir


Recieved:19/10/2015; Accepted:21/11//2015

apoptosis, necrosis and tumorogenesis (4). Free radicals are unstable structures that can damage DNA molecules and finally cause mutation(5).

The flavonoids and phenolic compounds from many natural plants have antioxidant and anti- clastogenic properties. Thyme with narrow leaves, frommint family (Lamiaceae), falls into this category (6). It is a perennial plant with a height up to 40 cm, which grows in dry weather and between rocks and mountains slopes in altitudes up to 1200 m. It is found in the western part of Gerash city, Fars province of Iran (7). Thyme oil has many properties like antispasmodic, carminative, antifungal, disinfectant, anthelminthic, and antirheumatic effects (8). Thyme with narrow leaves consist of various ingredients such as thymol, carvacrol, luteolin, –p-cymene, linalool, gamatripyn, camphor, cineole, burneol, beta-caryophyllene, trypeneol, pinene, luteolin, and so on (9). Many investigators have studied thyme properties, including: antibacterial (10), antifungal (11), natural food preservative (12), antioxidant (13), and analgesic (14). So far its protective effects against DNA damage hasn't been studied. Because of the anti-cancer and antioxidant properties of phenolic compounds, this herb might protect the DNA destruction induced by cyclophosphamide.

In the present study, in order to detect the genotoxic or carcinogenic chemicals, the micronucleus assay was used. This method was first proposed by Schmid W., and is applicable both *in vitro* and *in vivo* conditions (15). Based on the principle of micronucleus assay, in the anaphase of chromatids, the chromosome fragments became acentric due to exposure to chemicals or ionizing radiation, while the centric chromosomes move to spindle poles. After telophase, the remaining fragments deform to a one or more secondary cores that is called micronuclei. This method can be used in proliferating tissues such as liver, bone marrow cells, peripheral blood, and cultured cells, although it is preferable to use the bone marrow (16). Micronuclei in cells without nuclei (red blood cell precursors in bone marrow) are called polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE), which are easily distinguished (17). Frequency of erythrocyte micronucleus in bone marrow cells reflects the chromosomal abnormality induced by chemical agents.

## 2. Materials and Methods

### *2.1. Animals*

In this study, we examined forty-five adult male mice (Balb/c strain) aged 8-9 weeks, with an approximately weight of 35 ± 2 gr in a standard condition.

### *2.2. Plant extract*

The flowers and young leaves of thyme were collected in the spring, and their authenticity was confirmed by Jangjoo proposal (plant No. 34 contained in Table 83) (7). The aerial portions were dried at the room temperature for 4 days. The wooden particles were separated and the remaining parts were soaked (100 g of dried herb in 1 L of 75% methanol) for 72 hours.

After that, the extract was purified by Whatman filter paper No. 1. This procedure were repeated four times to clear the extract, then the filtrate was concentrated by rotary machine at 40 °C. These processes were repeated several times to obtain sufficient extract. The extract was resolved in distilled water and propylene glycol in a ratio of 4 to 1.

### *2.3. Treated Animals*

A total of 45 male mice (Balb/c strain) were equally divided into nine groups. The first group served as the control group and was fed on a basal diet without any injection. In the second group (solvent control), the solvent (water and propylene glycol in a ratio 4:1) without the extract was injected for 7 days continuously intraperitoneally (Ip) (10 ml/kg). In the third group (positive control), cyclophosphamide (50 mg/kg) was injected once (10 ml/kg) Ip. It was shown previously that this dose led to micronuclei formation (18). In the fourth group, 375 mg/kg/day of the thyme methanol extract was injected daily at 9 am for 7 consecutive days, Ip (10 ml/kg). In the fifth group, 750 mg/kg/day of the thyme methanol extract were injected daily at 9 am for 7 consecutive days, Ip (10 ml/kg). In the sixth Group, 1500 mg/kg/day of the thyme methanol extract daily was injected at 9 am for 7 consecutive days, Ip (10 ml/kg). In the seventh Group, 375 mg/kg/day of the thyme methanol extract was injected daily at 9 am, Ip (10

ml/kg) for 7 consecutive days. One hour after the last dose, cyclophosphamide (50 mg/kg) was discontinued. In the eighth Group, 750 mg/kg/day of the methanol extract was injected daily at 9 am, Ip (10 ml/kg) for 7 consecutive days. One hour after the last dose, on the 7th day cyclophosphamide (50 mg/kg) was injected, Ip. In the ninth Group, 1500 mg/kg/day of the thyme methanol extract was injected at 9 am for 7 consecutive days, Ip (10 ml/kg). One hour after the last dose on the 7th day, cyclophosphamide (50 mg/kg) was injected Ip.

### *2.4. The micronucleus test*

In all groups, 24 hours after cyclophosphamide injection Ip, the mice were killed through the cervical spinal cord transection. Both femoral bones were removed completely, then the epiphyseal portions were isolated (15). Bone marrow was gently aspirated by means of a syringe containing 1 ml of fetal bovine serum (Gibco). The suspension was collected in a test tube and centrifuged at 1500 rpm for 5 minutes. After centrifugation, the solution on top of the tube was discarded and the deposited cells were dissolved in the remainder of the serum. After that, a drop of this solution was placed on each of a set of three lamella and the smears were prepared, stained and fixed on laboratory temperature for 24 hours without chemical fixation. We used May Grunwald and Giemsa (Merck) for staining. May Grunwald staining was applied to differentiate the PCE and NCE cells and Giemsa for staining the micronuleoli. In a good preparation, a NCE and PCE appear as yellow-orange and blue-violet in color, respectively (Figure 1). We used emersion oil with light microscope with magnification of 100 times for counting the cells. 1000 cells of PCE per animal were counted; also at the same power-field, NCE and polychromatic erythrocyte containing micronucleus (MnPCE) were counted accurately. For detection of toxic effect on bone marrow cells, we counted PCE/PCE+NCE per 1000 cells of PCE. We studied two variables in this investigation: A) MnPCE cell and B) the ratio of PCE/PCE+NCE.

### *2.5. Statistical analysis*

We used one way analysis of variance (ANOWA) and POs-Hoc tests for showing a significant difference between the groups and to indicate in which groups at 0.05 (95% CI), the differences are significant.

## 3. Results

### *3.1. Clastogenic and cytotoxic effects*

There are significant differences between cyclophosphamide (positive control) and the control group ($p<0.05$). Data showed that cyclophosphamide increased the frequency of micronucleus and decreased cell proliferation (Table 1). This study didn't show any significant differences between methanol extract and the control group on MnPCE frequency and cell proliferation ratio (PCE/PCE+NCE) ($p<0.05$). Therefore, in this research the selected doses of thyme extract didn't have any clastogenic and cytotoxic effects (Table 1).

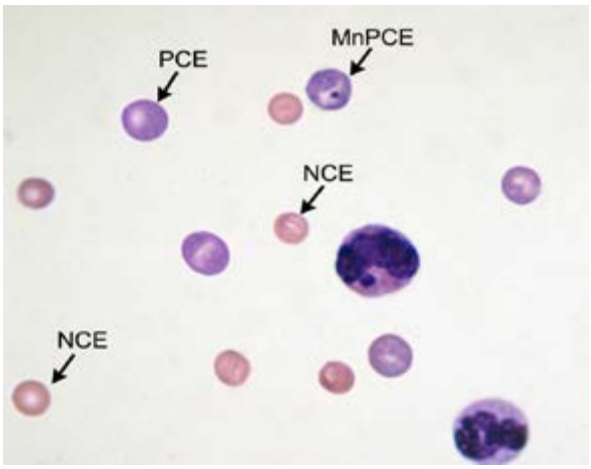


Figure 1. Bone marrow cells: normochromatic erythrocytes (NCE) in yellow-orange, polychromatic erythrocytes (PCE) in blue-violet, and larger cells containing micronucleus polychromatic erythrocytes (MnPCE).

Table 1. Results of the effect of solvents, cyclophosphamide, and diverse concentrations of methanol extract of thyme on the frequency of micronucleus polychromatic erythrocytes (MnPCE) and the cell proliferation ratio (PCE/PCE+NCE).

| Group | Treatment | MnPCE/1000PCE±SD | PCE/PCE+NCE ±SD |
|---|---|---|---|
| 1 | Control | 5.25±1.10 | 0.47±0.03 |
| 2 | Solvent (distilled water + propylene glycol) | 6.00±1.29 | 0.48±0.02 |
| 3 | Cyclophosphamide (50 mg/kg/bw) | 97.50±10.94 | 0.30±0.03 |
| 4 | Thyme methanol extract (375mg/kg/day) | 6.25±1.25 | 0.49±0.04 |
| 5 | Thyme methanol extract (750 mg/kg/day) | 6.50±2.32 | 0.46±0.01 |
| 6 | Thyme methanol extract (1500 mg/kg/day) | 6.00±1.95 | 0.48±0.08 |
| 7 | Cyclophosphamide+thyme methanol extract (375mg/kg/day) | 47.50±6.35 | 0.41±0.06 |
| 8 | Cyclophosphamide+thyme methanol extract (750 mg/kg/day) | 36.00±5.47 | 0.38±0.07 |
| 9 | Cyclophosphamide+thyme methanol extract (1500 mg/kg/day) | 33.50±5.00 | 0.42±0.08 |

PCE=polychromatic erythrocytes, NCE=normochromatic erythrocytes.

As can be seen in Table 1, for every dosage of extracts (375, 750, 1500 mg/kg/day), the frequency of MnPCE cells was reduced compared with the group who received only cyclophosphamide ($p$<0.05). With increasing dosages of extracts from 375 to 750 mg/kg/day, there was a statistically significant reduction in MnPCE frequency ($p$<0.05). Although this property was seen with an increased concentration from 750 to 1500 mg /kg /day, it was not statistically significant ($p$<0.05). There was a little reduction of micronucleoli with a dosage more than 750 mg/kg/day of thyme extract.

The ratio of cell proliferation (PCE/PCE+NCE) was significantly different between the cyclophosphamide group and every other groups ($p$<0.05). The results indicated that the different doses of methanol extract of thyme do not have any clastogenic and cytotoxic effects.

## 4. Discussion

In this study, in order to reduce the cytotoxicity induced by cyclophosphamide, the methanol extracts of thyme with narrow leaves were used. The extracts were dissolved in water and propylene glycol in a ratio 4 to 1. To detect the clastogenicity, the solvent alone was injected to the mice and then their bone marrow smears were assessed. The observations didn′t show any changes in MnPCE and PCE/PCE+NCE parameters compared to the control group (Table 1 and Figure 1).

In the methanol extract groups (1500, 750, 375 mg/kg), the MnPCE and PCE/PCE+NCE

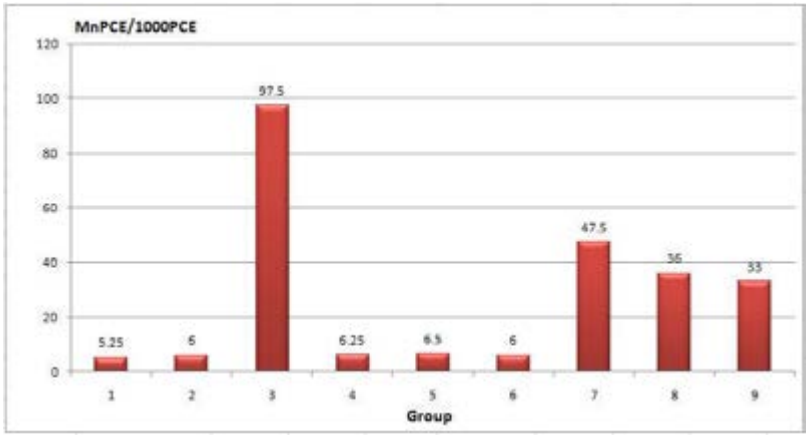


Figure 2. Frequency changes of MnPCE in the control group, solvent, cyclophosphamide, and varying concentrations of methanol extract of thyme.

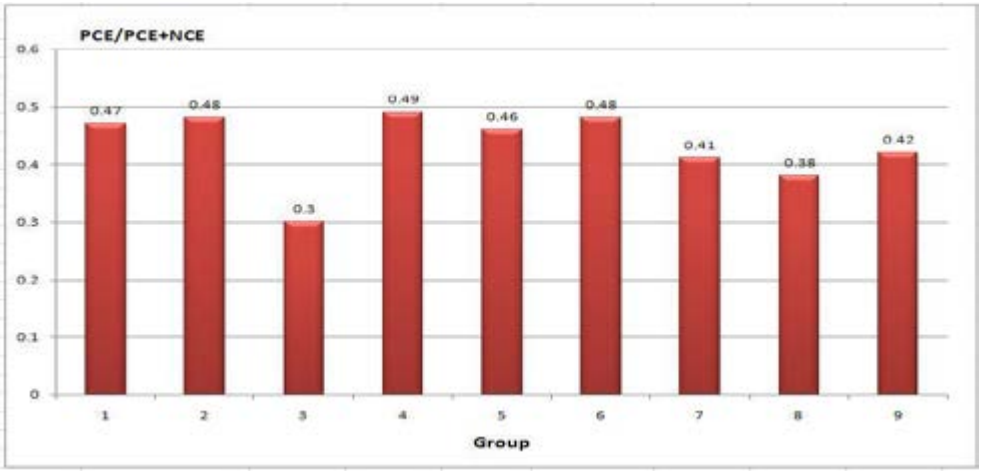


Figure 3. Variation of cell proliferation (PCE/PCE+NCE ) in the control group, solvent, cyclophosphamide, and diverse concentrations of methanol extract of thyme.

were not changed; that means thyme extract alone didn′t have any cytotoxic or clastogenic effects (Table 1). Our results indicated that the methanol extract of thyme reduced clastogenic and cytotoxic effects of cyclophosphamide in mice bone marrow cells. This was assessed by detection of MnPCE in bone marrow cells, 24 hours after the last injection.

As shown in Table 1 and Figure 1, the clastogenic effects of cyclophosphamide was shown with an increasing frequency of MnPCE, from 5.25 to 97.50 in the control and cyclophosphamide groups, respectively ($p$<0.05). Moreover, the cytotoxic effects of cyclophosphamide has been shown (Figure 3) with decrease of the ratio of PCE/PCE+NCE from 0.47 in the control group to 0.30 in the cyclophosphamide group, which was statistically significant ($p$<0.05).

Cyclophosphamide is an alkylating agent that produces free radicals. These unstable molecules alkylate the guanine base in n-7 location of the DNA arm, leading to cross linkage between DNA arms and crushing the DNA molecules, causing mutation (19). One of the most important methods against mutation is to trap these free radicals (20). Gamal-Eldeen *et al*. showed that algae Sargassum dentifolium have the ability to collect the OH radicals and active oxygens, so has a protective effect against cyclophosphamide-induced abnormalities (21). Zhang *et al.* worked on ginsenoside, a triterpene of ginseng plant, which reduces the deleterious effects of cyclophosphamide on DNA, apoptosis of bone marrow cells, and peripheral blood lymphocytes (22). Vilar *et al.* reported anticlastogenic effects of Ginkgo biloba extract against cyclophosphamide and mitomycin C on the bone marrow of mice and suggested that Ginkgo biloba have both direct and indirect anticlastogenic capacities (23).

In the present research, thyme extract had excellent antioxidant properties. The important components of *Thymus vulgaris* extract with narrow leaves are thymol, carvacrol, linalool, and luteolin (9). Thymol is a monocyclic-phenolic compounds with antioxidant and anti-cancer properties, which protects the DNA (24). Carvacrol ia an aromatic compound with anti-cancer, antioxidant, and neuroprotective activities (25). Besides, the anticlastogenic effect of luteolin is very strong against carcinogenic substance Trp-p-2 (26). Linalool, an alcoholic monoterpene has also anti-tumor effect (27). Therefore, the protective properties of thyme extract against cyclophosphamide-induced clastogenic effect could be due to brooming of free radicals.

The methanol extracts of thyme with decreasing the frequency of MnPCE and increasing PCE/PCE+NCE had good effects against the cyclophosphamide induced toxicity. As shown in Table 1, the 375 mg/kg injection dose of thyme extract was able to decrease MnPCE frequency from 97.5 to 47.5 and increase the cell proliferation ratio (PCE/PCE+NCE) from 0.30 to 0.49 in the control and cyclophosphamide groups, respectively, which were statistically significant ($p$<0.05).

Although a higher abundance of MnPCE were seen with increasing the dosage to 750 mg/

kg ($p$<0.05), this was not true with the dosage of 1500 mg/kg ($p$<0.05). This means the more effective dose of thyme extract was 750 mg/kg.

## 5. Conclusion

In general, the results of this study were as follows: 1- Thyme extract alonedidn't show any clastogenic and cytotoxic effects in mice bone marrow cells. 2- Thyme methanol extract can protect the mice bone marrow cells against cyclophosphamide induced cytotoxicity. 3- The most effective dose of thyme extract is 750 mg /kg.

## Acknowledgements

The authors appreciated all consultants of the research center of Gerash Medical College.

## Conflict of Interest

None declared.

## 5. References

1. Flora SD, Izzotti A, D'Agostini F, Balansky RM, Noonan D, Albini A. Multiple points of intervention in the prevention of cancer and other mutation-related diseases. *Mutat. Res*. 2001;480:9-22.
2. Hales BF, Barton TS, Robaire B. Impact of paternal exposure to chemotherapy on offspring in the rat. *J. Natl. Cancer Inst*. 2005;34:28-31.
3. Qiu J, Hales BF, Robaire B. Damage to rat spermatozoal DNA after chronic cyclophosphamide exposure. *Biol Reprod.* 1995;53:1465-73.
4. Kern JC, Kehrer JP. Acrolein-induced cell death: a caspase-influenced decision between apoptosis and oncosis/necrosis. *Chem. Biol. Interact*. 2002;139:79-95.
5. Bergamini CM, Gambetti S, Dondi A, Cervellati C. Oxygen, reactive oxygen species and tissue damage. *Curr. Pharm. Des*. 2004;10:1611-26.
6. Duthie GG, Duthie SJ, Kyle JA. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. *Nutr Res Rev.* 2000;13:79-106.
7. Jangjoo M. Collected, Naming Scientific and of Phytochemical Plants of the Fars Region Gerash: Tehran University of Medical Sciences; 1373.
8. Zargari A. Medicinal plants. Vol. 3, Tehran Univ.; 1997. p. 590.
9. García-Risco MR, Vicente G, Reglero G, Fornari T. Fractionation of thyme (*Thymus vulgaris* L.) by supercritical fluid extraction and chromatography. *J Supercrit Fluids.* 2011;55:949-54.
10. Rota MC, Herrera A, Martínez RM, Sotomayor JA, Jordán MJ. Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. *Food Control*. 2008;19:681-7.
11. Kumar A, Shukla R, Singh P, Prasad CS, Dubey NK. Assessment of *Thymus vulgaris* L. essential oil as a safe botanical preservative against post harvest fungal infestation of food commodities. *Innov Food Sci Emerg Technol.* 2008;9:575-80.
12. Nguefack J, Dongmo J, Dakole C, Leth V, Vismer H, Torp J, et al. Food preservative potential of essential oils and fractions from *Cymbopogon citratus*, *Ocimum gratissimum* and *Thymus vulgaris* against mycotoxigenic fungi. *Int. J. Food Microbiol.* 2009;131:151-6.
13. El-Nekeety AA, Mohamed SR, Hathout AS, Hassan NS, Aly SE, Abdel-Wahhab MA. Antioxidant properties of *Thymus vulgaris* oil against aflatoxin-induce oxidative stress in male rats. *Toxicon.* 2011;57:984-91.
14. Taherian AA, Babaei M, Vafaei AA, Jarrahi M, Jadidi M, Sadeghi H. Antinociceptive effects of hydroalcoholic extract of *Thymus vulgaris*. *Pak J Pharm Sci.* 2009;22:83-9.
15. Schmid W. The micronucleus test. *Mutation Research.* 1975;31:9-15.
16. Müller W-U, Streffer C. Micronucleus assays. Advances in mutagenesis research: Springer; 1994:1-134.
17. Fenech M. The *in vitro* micronucleus technique. *Mutat Res.* 2000;455:81-95.
18. Serpeloni JM, dos Reis MB, Rodrigues J, dos Santos LC, Vilegas W, Varanda EA, *et al. In vivo* assessment of DNA damage and protective effects of extracts from *Miconia* species using the comet assay and micronucleus test. *Mutagenesis.* 2008;23:501-7.
19. Aguilar-Mahecha A, Hales BF, Robaire B. Chronic cyclophosphamide treatment alters the expression of stress response genes in rat male germ cells. *Biol Reprod.* 2002;66:1024-32.
20. Tiwari AK. Imbalance in antioxidant

defence and human diseases: Multiple approach of natural antioxidants therapy. *Curr. Sci.* 2001;81:1179-87.

21. Gamal-Eldeen AM, Abo-Zeid MA, Ahmed EF. Anti-genotoxic effect of the Sargassum dentifolium extracts: Prevention of chromosomal aberrations, micronuclei, and DNA fragmentation. *Exp. Toxicol. Pathol.* 2013;65:27-34.

22. Zhang QH, Wu CF, Yang JY, Mu YH, Chen XX, Zhao YQ. Reduction of cyclophosphamide-induced DNA damage and apoptosis effects of ginsenoside Rb1 on mouse bone marrow cells and peripheral blood leukocytes. *Environ. Toxicol. Pharmacol.* 2009;27:384-9.

23. Vilar J, Leite K, Chen LC. Antimutagenicity protection of *Ginkgo biloba* extract (Egb 761) against mitomycin C and cyclophosphamide in mouse bone marrow. *Genet. Mol. Res.* 2009;8:328-33.

24. Slamenova D, Horvathova E, Sramkova M, Marsalkova L. DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured *in vitro*. *Neoplasma.* 2007;54:108-12.

25. Mastelic J, Jerkovic I, Blažević I, Poljak-Blaži M, Borović S, Ivančić-Baće I, *et al.* Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. *J Agric Food Chem.* 2008;56:3989-96.

26. Samejima K, Kanazawa K, Ashida H, Danno G-i. Luteolin: a strong antimutagen against dietary carcinogen, Trp-P-2, in peppermint, sage, and thyme. *J Agric Food Chem.* 1995;43:410-4.

27. Ravizza R, Gariboldi MB, Molteni R, Monti E. Linalool, a plant-derived monoterpene alcohol, reverses doxorubicin resistance in human breast adenocarcinoma cells. *Oncology reports.* 2008;20:625-30.