ORIGINAL ARTICLE

Antimutagenic activity of major fractions of *Zataria multiflora* Boiss by Ames method

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Zataria multiflora is a medicinal plant that has been interested in antimutagenicity effect because of its high antioxidant activity and richness of flavonoids. Antimutagenicity effect of total extract of the plant has been reported previously. Aerial parts of *Z. multiflora* were extracted by petroleum ether, chloroform and 80% methanol by liquid-liquid extraction method consequently. The fractions were concentrated in vacuum and dried at 40°C in oven. The genotype of two standard strains of *Salmonella typhimurium* (TA98, TA100) was confirmed by the evaluation of two important factors of histidine requirement and the presence of R factor. The minimum inhibition concentration (MIC) of the fractions against these two strains was determined by agar dilution method. From each fraction, various concentrations less than MIC were studied for anti-mutagenic test. The sample along with bacterial strain and mutagen agent were incubated at 37°C for 48 h. The number of revertant colonies was counted and compared with control plates. Our results showed that all fractions especially petroleum ether and chloroform ones maintain the number of colonies in the standard range in control plates and prevent from the growth of many strains of bacteria and increase of revertant colonies enhancement in a concentration-dependent manner. This effect was prominent against TA100 starin. Methanolic fraction exhibited anti-mutagen activity just in the highest used concentration in the presence of TA98.

Key words: *Ames test, anti-mutagenicity, fraction, phenylpropanoids, Salmonella typhimurium, Zataria multiflora*

INTRODUCTION

The available anticancer drugs have restricted uses and different side effects, so the need for drugs with different mechanisms and fewer side effects, is felt. Research for newer and especially herbal drugs has always attracted the attention of researchers.[1] The Ames test is one of the initial tests used in preliminary screening of compounds with antimutagenicity effect because of its simplicity, quickness, and convenience.[2] This test uses several strains of *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis (histidine operon), so they cannot synthesis some enzymes needed for histidine biosynthesis. A reversion back of these mutants to a prototrophic state, influenced by different chemical or environmental factors, can be studied in order to achieve mutagen and anti-mutagen compounds.[3] Several studies have pointed out that many mutagenic and carcinogenic damages are caused by free radicals.[4] So it is expected compounds with antioxidant effect would be a good candidate for finding new antimutagenic agents.

Zataria multiflora Boiss known as Avishane-Shirazi belongs to Lamiaceae family that has antiviral, antibacterial and antioxidant effects.[5] Our recently research has shown that the methanolic extract of *Z. multiflora* has antioxidant and antimutagenicity effects,[6] and because of a close relationship between antioxidant and antimutagenicity effects, the aim of this study was to evaluate the antimutagenicity effect of major fractions of methanolic extract of *Z. multiflora* by Ames method.

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MATERIALS AND METHODS

Zataria multiflora was gathered from Koohpayeh in June 2012, Kerman Province, Iran and scientifically identified in department of Pharmacognosy, Kerman Faculty of Pharmacy and a voucher specimen was deposited in Herbarium Center (KF 1249). Aerial parts of the plant were dried in shade.

Fractionation of the plant extract

An amount of 500 g of plant materials was extracted with methanol 80% to give total extract. A part of this extract was fractioned with petroleum ether by liquid-liquid extraction method for 72 h. The extract was collected each 24 h and replaced with fresh solvent. Final extracts were mixed and dried under vacuum to give a viscos extract. The extraction was continued consecutively with chloroform and 80% methanol.

Antimicrobial effect and cytotoxicity of samples

At first, minimum inhibition concentration (MIC) of the fractions against two strains of *S. typhimurium* (TA98, TA100) was evaluated by agar dilution method. From each fraction, various concentrations less than MIC were studied for anti-mutagenicity test. A volume of 20 ml of Mueller-Hinton agar medium containing different dilutions of each fraction of *Z. multiflora* (50, 25, 12.5, 6.25, 3.12, mg/ml) was transferred to a plate and bacterial cultures was done on each plate. A plate without extract was considered as positive control to ensure the growth of microbes.

Ames test

Overnight growth culture was prepared using mother plates of each bacterial strain. A single colony from the mother plate was inoculated to nutrient broth medium and was grown for 12 h with shaking at 37°C (210 rpm) up to the optimal microbial growth. A volume of 0.5 ml of each plant fraction (in different dilutions less than MIC), 0.1 ml of overnight bacterial culture, 0.2 ml of histidine-biotin solution (0.5 mmol of each) and 0.1 ml of sodium azide (1.5 μg/ml) as the mutagen, were transferred into test tubes and mixed thoroughly. 2 ml of this prepared semisolid agar was immediately spread on the layer of minimal glucose agar plates in the petri dish. After complete solidification, dishes were put in incubator. After 48 h of incubation at 37°C, the number of prototrophic revertant colonies was counted in each plate.[7] This experiment was triplicated with each dilution of the extract and each strain of bacteria. A plate containing surface agar, bacterial strain and the certain mutagen was considered as positive control. The solvent with surface agar and bacterial strain was used as a negative control.

Ratio of antimutagenicity

The ratio of antimutagenicity was expressed as the ratio of the average number of revertant colonies in the positive control to that in a certain concentration of plant fraction. Antimutagenicit potential was assumed if the ratio was 2.0 or higher. The percentage of inhibition was calculated as follows:[8]

$$I\% = ([A - B]/A) \times 100$$

Where A and B are the number of revertant colonies in each plate, in the presence of positive control and mutagen or/and sample. The number of colonies in the negative control should be subtracted from the numerator and denominator. The inhibition of 25–40% is considered medium antimutagenicity effect and over 40% and under 25% are considered as positive and negative antimutagenicity effect respectively.

Statistical analysis

Statistical analysis of data was performed using SPSS software (IBM corporation) and differences with $P < 0.05$ was considered significant.

RESULTS

Minimum inhibition concentration of different fractions of *Zataria multiflora*

The results of MIC determination of different fractions of *Z. multiflora* have given in Table 1. The least MIC was due to petroleum ether fraction against both *S. typhimurium* TA98 and TA100.

Antimutagenicity properties of fractions

Evaluation of the number of revertant colonies of *S. typhimurium* (TA98, TA100) was done in the presence of petroleum ether, chloroform and methanolic fractions of *Z. multiflora* in different concentrations. Each experiment was repeated in triplicate and results were reported as mean ± standard error of the mean [Figures 1-3].

These results show that petroleum ether fraction of *Z. multiflora* reduced the number of revertant colonies of both TA98 and TA100 strains in a concentration-dependent manner at concentrations of 0.195, 0.195 and 0.0975 mg/ml. Antimutagenicity effect was prominent against TA100 strain at all tested concentrations (with over 40% inhibition and ratio of antimutagenicity over 2.0), whereas average of antimutagenicity effect against TA98 strain (I% between 25 and 40) was considered just at 0.195 mg/ml. This test was negative in the other concentrations [Figure 1 and Table 2].

Table 1: Determination of MIC of total extract and different fractions of *Zataria multiflora* Boiss against two strains of *Salmonella typhimurium* (TA98, TA100)

Fractions (mg/ml)	Bacterial strains	
	Salmonella typhimurium TA98	*Salmonella typhimurium* TA100
Petroleum ether	0.781	0.39
Chloroform	1.56	3.125
Methanol	50	25

MIC: Minimum inhibition concentration

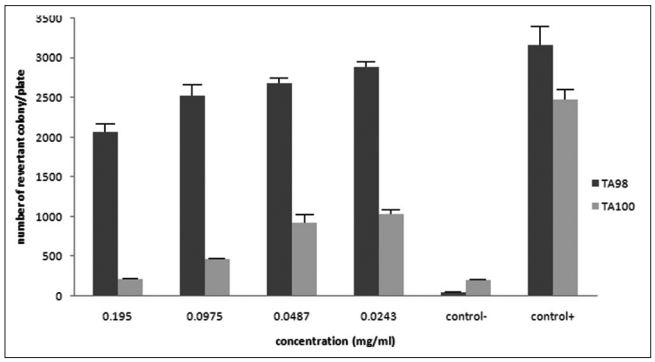

Figure 1: Effect of petroleum ether fraction of *Zataria multiflora* on the number of *Salmonella typhimurium* (TA98, TA100) revertant colonies. Results are reported as mean ± standard error of the mean

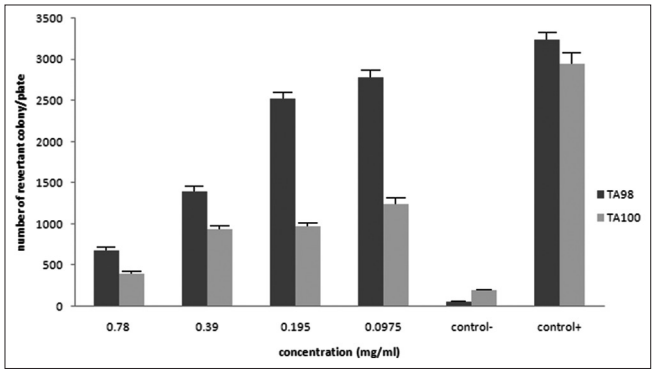

Figure 2: Effect of chloroform fraction of *Zataria multiflora* on the number of *Salmonella typhimurium* (TA98, TA100) revertant colonies. Results are reported as mean ± standard error of the mean (*t*-test analysis)

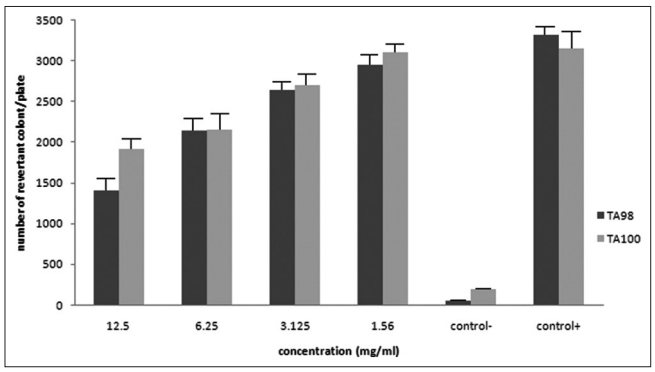

Figure 3: Effect of methanolic fraction of *Zataria multiflora* on the number of *Salmonella typhimurium* (TA98, TA100) revertant colonies. Results are reported as mean ± standard error of the mean

Chloroform fraction of *Z. multiflora* also reduced the number of revertant colonies of *S. typhimurium* [Figure 2], which reaches the highest reduction against both TA98 and TA100 strains at 0.78 mg/ml. A strong antimutagenicity effect (inhibition over 40%) was observed in the TA100 strain at all concentrations (the ratio of antimutagenicity was over 2.0). A strong antimutagenicity against TA98 strain and antimutagenicity ration more than 2 was observed in 0.78 and 0.039 mg/ml [Figure 2 and Table 3].

As shown in Figure 3, methanolic fraction of *Z. multiflora* reduced the number of revertant colonies of *S. typhimurium* that reaches maximum effect against both TA98 and TA100 strains at 12.5 mg/ml. The strong inhibition (over 40%) and high antimutagenicity ratio (2) against TA98 was observed at 12.5 mg/ml. The other concentrations were inactive against TA100 [Figure 3 and Table 4].

DISCUSSION

One of the useful methods to search for finding the new anticancer drugs is studying the mechanisms of natural compounds, most of which belong to different chemical structures and have distinct mechanisms of action. In Iranian traditional medical, some of the plants such as green tea, thyme and yarrow have been suggested for cancer treatment. Study of such species could be used as a logical ration behind looking for new anticancer drugs. In the other word, several studies have shown anti-cancer effect of some of the flavonoids and plants containing them and such plants could be a good candidate for anticancer studies.[9]

For the detection of mutagenic and antimutagenic substances, several systems have been developed and applied. One of these is living and nonmamalian systems such as bacteria, yeast that have many advantages from geneticists's sight of view. Ames test is used because of convenience, efficiency and being ideal method due to the salient properties of these microorganisms, such as fast proliferation, small size, and little space radius.[10]

As regards, plant total extracts have an array of secondary metabolites with diverse biological activities, different molecular structures and solubility. In previous research, antimutagenic effect of total extract of *Z. multiflora* has been established, and it was expected that fractionation of this plant would be suit to achieve active compounds of the plant. Initially, fractionation of total extract was performed using petroleum ether, chloroform and 80% methanol by liquid-liquid extraction. Preliminary analysis showed the highest antimutagenicity effect of methanolic extract of *Z. multiflora*. So in the present work, major fractions of methanolic extract of *Z. multiflora* have been evaluated for antimutagenicity effect.

At the first, MIC of samples was determined by agar dilution method. Various concentrations less than MIC were used in Ames test. After implementation of the Ames test and confirmation of green background, the number of revertant colonies in each plate, was recorded. Review on any of the tested strains indicates that the number of spontaneous revertant colonies (negative control) were in the normal range and the number of mutated colonies (positive control) increased to appropriate levels [Figures 1-3]. No contamination was induced through the experiments, so there was no negative effect resulted in the Ames test.

Table 2: Inhibitory effect and antimutagenicity ratio of petroleum ether fraction of *Zataria multiflora* against two strains of *Salmonella typhimurium* (TA98, TA100)

Concentration (mg/ml)	Bacterial strains			
	Salmonella typhimurium TA98		*Salmonella typhimurium* TA100	
	Inhibition (%)	Ratio of antimutagenicity	Inhibition (%)	Ratio of antimutagenicity
0.195	34.9	1.5	91.9	12.4
0.0975	20.4	1.2	81.7	5.4
0.0487	15.2	1.1	63.1	2.7
0.0243	8.9	1	58.6	2.4

Table 3: Inhibitory effect and antimutagenicity ratio of chloroform fraction of *Zataria multiflora* against two strains of *Salmonella typhimurium* (TA98, TA100)

Concentration (mg/ml)	Bacterial strains			
	Salmonella typhimurium TA98		*Salmonella typhimurium* TA100	
	Inhibition (%)	Ratio of antimutagenicity	Inhibition (%)	Ratio of antimutagenicity
0.78	79.2	4.8	86.8	7.6
0.390	56.9	2.3	68.2	3.8
0.195	22	1.2	67.2	3
0.0975	13.9	1.1	57.9	2.3

Table 4: Inhibitory effect and antimutagenicity ratio of methanol fraction of *Zataria multiflora* against two strains of *Salmonella typhimurium* (TA98, TA100)

Concentration (mg/ml)	Bacterial strains			
	Salmonella typhimurium TA98		*Salmonella typhimurium* TA100	
	Inhibition (%)	Ratio of antimutagenicity	Inhibition (%)	Ratio of antimutagenicity
12.5	57.8	2.3	39	1.6
6.25	35.6	1.5	31.7	1.4
3.125	20.3	1.2	14.1	1.1
1.56	11.2	1.1	1.2	1

The obtained results showed that different fractions of *Z. multiflora* caused significant changes in the number of revertant colonies in comparison to positive control. According to the theory of Prof. Ames, mutagenicity or antimutagenicity is proportional to the concentration of desired substance.[2] In this study, petroleum ether fraction of the plant kept the number of revertant colonies of *S. typhimurium* in low range and prevented the high growth of bacterial strains (especially TA100) and inhibited a sharp increase in the number of revertant colonies. With increasing the concentration of this fraction, the number of revertant colonies has dropped. Chloroform fraction exhibited lower antimutagenicity effect that was prominent against the TA100 strain. Among the different concentrations, methanol fraction was active against TA98 just in the highest concentration. In all fractions, a relationship between concentration and activity was seen, so that with increasing the concentration, the number of the revertant colonies has been decreases [Figures 1-3]. A frame-shift and base-pair type of mutation has been identified by TA98 and TA100 strains respectively.[11] Petroleum ether and chloroform fractions of *Z. multiflora* have shown the greatest effect against TA100 strain, so it can be concluded that the active antimutagenic substances in these two fractions exert their effect by preventing from base-pair type of mutation. Contrary, methanolic fraction was active against TA98 at the highest concentration that is due to preventing from frame-shift type of mutation. Essential oil of *Z. multiflora* mainly contains carvacrol, thymol that belong to phenyl propanoids, limonene and paracymene that are from monoterpenes. In addition, compounds such as rosmarinic acid, oleanolic acid and luteolin have been reported in this plant.[12] It is probable that a part of essential oil can be extracted through petroleum ether fraction. Terpenoids and phenolic compounds associated with chlorophyll pigments are found in petroleum ether too. Antimutagenic effects of terpenoids and phenyl propanoids has been confirmed and might be responsible for antimutagenic effect of petroleum ether fraction.[4,12] Antioxidant effect of methanolic extract and essential oil of *Z. multiflora* has been reported. According to these activities of the plant and emphasise on its antimutagenic effects of in the present study, it can be concluded that:

- Theory and mechanism of anticancer, antioxidant and cytotoxic effects are associated with antimutagenic effects of the plant
- Antimutagenic effects in the Ames test regarding the type of its mechanism are dependent on the microbial

strain. It is possible that a strain would be sensitive to antimutagenic effects of a lead substance than the other

- Mutagens that are used in the Ames test may react with the antimutagen substance and lead to the prevention of the occurrence of antimutagenic effect, while the shift mutagens with different mechanisms show these effects.

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