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Antimutagenicity Activity of Different Fractions of *Zataria multiflora*, *Achillea wilhelmsii* and *Camellia sinensis* using Ames Test

[1]Gholamreza Dehghan-Noodeh, [2]Fariba Sharififar, [1]Mohammad Hassan Moshafi, [3]Effat Behravan, [4]Ali Dehghan-Noodeh and [2]Reza Rezaei-Gharaeei

Cancer and some other mutation-related diseases are still remained among the most difficult ones of clinical aspects. Regards to the mention issue attention is drawn to dietary anti-mutations as cancer preventative agents. The presence of anti-mutagenic agents in medicinal plants such as *Zataria multiflora*, *Achillea wilhelmsii* and *Camellia sinensis* can be considered due to their antioxidants properties. So, in the present work, different fractions of these plants were assessed for their anti-mutagenic effects using Ames method. Aerial parts of the tested plants after collection, drying and milling were extracted using petroleum ether, chloroform and 80% methanol consecutively by percolation method. The dried extracts under vacuum were examined for their anti-mutagenic effects. The minimum inhibition concentration (MIC) of the extracts was evaluated by ager dilution method. Various concentrations under their measured MIC were used for anti-mutagenic test. Each extract 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 of *Z. multiflora* exhibited anti-mutagenicity effect in the presence of TA98, while only its methanolic fraction was active against TA100. Methanolic and chloroform fractions of *C. Sinensis* showed strong anti mutagenicity against TA98 only, while the methanolic fraction of *A. wilhelmsii* showed anti-mutagenicity effect in the presence of both TA98 and TA100. Considering the presence of flavonoids in methanolic fraction and high antimutagenicity effect of this fraction, flavonoids are probably responsible for anti-mutagenic effect of the plants.

Key words: Ames test, *Zataria multiflora*, *Achillea wilhelmsii*, *Camellia sinensis*, anti mutagenic, *Salmonella tiphymurium*

[1]Pharmaceutics Research Center, Faculty of Pharmacy,
[2]Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran
[3]Department of Pharmacodynamic and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[4]Department of Life Sciences, Angila Ruskin University, Cambridge, CB1 1PT, UK

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For further information about this article or if you need reprints, please contact:

Fariba Sharififar
Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran

Tel.: +98 341 3205020
Fax: +98 341 3205003

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INTRODUCTION

Cancer is a genetic disorder in which normal cells growth control has been lost and mutation is an important factor in carcinogenesis. Surgery, radiotherapy, chemotherapy and biological therapy are the most common strategies in cancer treatment. Usually chemotherapy and biological treatment are considered component of systemic treatment (Bishop and Schiestl, 2001). Several drugs from herbal sources such as paclitaxel, vincristine, podophyllotoxin and camptothecin currently are available for treatment of cancer (Cragg *et al*., 2005; Newman *et al*., 2003). Many of mutagenic compounds have vegetable origin for instance plant alkaloid RG Isogravacridon chlorine which is a combination of potential mutagen (Mans *et al*., 2000). There are some reports for antimutagenic effect of flavonoids in the literature (Miyazawa and Hisama, 2003; Miyazawa *et al*., 2000). The other antimutagen compounds such as chlorophyll have been identified and are under more investigations (Bhattacharya, 2011). Mamyran alkaloids are also being investigated in this relation and likely to have anti-cancer impact in mammals (Paulini *et al*., 1997). The potential anticancer effects along the impact of anti mutagen studies have been done on various compounds, especially natural materials. Researchers have concluded that there is a significant correlation between the anti mutagen material and antioxidants (Ferruzzi *et al*., 2002). The reaction of the DNA is the possible mechanisms for anti mutagen compounds. There are several ways for study of anti mutagenesis of different compounds. One of the most commonly used methods is Ames test (Grover and Bala, 1993). Basically, molecular research on single-cell organisms and bacteria has more advantages in comparison to animals because these cells can allow the researcher to parse the exact genetic composition. A large number of living cells is needed in mutagenesis and anti-mutagenesis test and this is met in a bacterial system. These systems are simple, fast and cost-effective (Mortelmans and Zeiger, 2000). Microorganisms used in this study were from two strains of TA98 and TA100 of *Salmonella typhi*. All these bacterial strains have mutations in their histidine and so, the synthesis of enzymes related to biosynthesis of histidine cannot be drawn. These mutations could have their ability in synthesis of histidine back affected by chemical or environmental factors (Issazadeh *et al*., 2012). In the present work, we have studied antimutagenicity effect of different fractions of *Zataria multiflora* and *Achillea wilhelmsii*, two Iranian endemic plants and *Camellia sinensis*. *Zataria multiflora* Boiss. Belongs to Lamiaceae family and known in Persian as Avishane-shirazi. Previous studies have shown anticholinesterase, antioxidant, antibacterial, anti viral activity and effect on expression of mdm2 and atm for this plant (Arabzadeh *et al*., 2013; Gohar *et al*., 2010; Sharififar *et al*., 2011, 2012b, 2007). The plant of *Achillea wilhelmsii* K. Koch belongs to Asteraceae. Presence of a variety of phytochemicals such as volatile oils, saponins, sesquiterpenelactones and flavonoids has been reported in this plant (Fathi *et al*., 2011). This plant has anti inflammatory and immune-stimulating effects (Sharififar *et al*., 2012a, 2009). *Camellia sinensis* L. from Theaceae family contains a variety of phytochemicals such as flavonoids, catechins, alkaloids (Liao *et al*., 2001). This plant has many activities like antioxidant (Ni *et al*., 2003). The ratione behind the selection of these plants is due to their antioxidant effect which has been reported in the literature.

MATERIALS AND METHODS

Chemicals: Two strains of TA98 and TA100 of *Salmonella* were obtained from University Shahed of Medical Sciences, Tehran, Iran. All the used chemicals were from analytical grade.

Plant materials: Plant samples were dried after gathering from habitat. Aerial part of the plants were milled and passed through the sieve. An amount of 500 g of plant was extracted with different solvents in increasing polarity order consecutively by percolation method (petroleum ether, chloroform and methanol 80%, respectively). The extracts were concentrated in vacuum and dried in oven at 40°C. Dried extracts kept at -20°C until experiments.

Antimutagenicity assay: At the first, a minimum glucose agar (MGA) medium was prepared. Broth cultures of each strain of bacteria developed 12 hours before the test. A volume of 1 mL histidine-biotin solution was added to each 10 mL agar solution. Agar was kept at 40-45°C. A volume of 0.1 mL of the bacterial strains was cultured and the contents of a sterile vial of 2 mL of agar poured on the MGA plates and left overnight. A uniform agar layer made on a rotating shaker. Plates were incubated at 37°C and the number of returned colonies was counted 48 h after incubation (Mortelmans and Zeiger, 2000). The overnight medium prepared by using a mother plate of each bacterial strain. A proper colony was chosen from mother plate and transferred into the nutrient broth medium by a sterile loop. Tubes were placed in a shaker incubator (210 rpm) at 37°C for 12 h to have an ideal microbial growth rate. Plant extracts were diluted based on MIC concentrations (3.125, 6.25, 12.5 and 25 mg mL^{-1}). Agar culture medium

was enriched by adding 10 mg mL^{-1} sterile histidine-biotin solution after autoclaving. A volume of 0.5 mL of each concentration of plant extract, 0.1 mL of overnight culture of bacterial strains, 0.2 mL histidine-biotin solution and 0.1 mL of mutagen solution (sodium azide) and specific dilution (1.5 μg mL^{-1}) transferred to the tubes with a pipette in sterile conditions. Finally 2 mL of agar medium transferred to a vial at 45°C and mixed immediately and poured on MGA plate smoothly. Solvent of plant extracts was used as negative control. The plates were incubated at 37° C for 48 h and the number of revertant colonies counted. The flat grass field of the plates by using a microscope verified the correct test conditions. Bacterial strains tested for a specific mutation, had special characteristics and in the case of returning the mutation to the bacteria because of any reason, they lost their obtained properties. The number of spontaneous returned of each bacteria strain was approximately acceptable but outside of that range was considered abnormal due to contaminated materials and equipment, mutagenic agents of the previous experiments, higher concentrations of histidine and environmental factors such as light, etc. (Mortelmans and Zeiger, 2000). The normal levels of spontaneously returned each bacterial strain, was calculated as the following: (120-200) for TA100 and (30-50) for TA98.

Evaluation of the number of spontaneously returned strains was not essential before making anti-mutation test, since negative control plates had no testing samples and only bacterial strains existed in them by Mortelmans and Zeiger (2000).

The number of colonies corresponding to each dilution of test substance and positive and negative controls were noted. Anti mutagenicity ratio calculated by dividing revertant colonies average in positive control on revertant colonies average in desired dilution.

$$\text{Mutation frequency ratio} = \frac{(C_m - C_n)}{C_v}$$

where, C_m is the number of colonies of mutants, C_n is the number of spontaneous colonies for negative control and C_v is the number of colonies of viable cells. The ratio equal or greater than 2 is considered as anti antimutagenic agent (Ren *et al*., 2006).

Statistical analysis: The results are expressed as mean±SD of three separate measurements. Significant differences between tested groups were compared by ANOVA and differences with $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

One useful approach to search for new anti-cancer drugs, is the study of natural compounds that mostly belong to the new structural classes. Study the species that traditionally have been used in cancer treatment is useful as a research model to find new anticancer agents. Previous study show that medicinal plants containing flavonoids could be a good candidate for anti-cancer (Chahar *et al*., 2011). From the geneticists view, live non-mammalian systems such as bacteria, yeasts and so on have many advantages. These systems are simple economical, fast and allow a detailed analysis of their genetic composition (Vujosevic and Blagojevic, 2004). Since total extract of the plant has composed of a variety of different substances (including alkaloids, flavonoids, saponins, etc.) with different structures and solubility and due to different effects of these substances on each other, so we intended to fractionate tested extracts to near to separate active fractions. So, different fractions of the tested plants prepared by different solvent systems in increasing polarity order like petroleum ether, chloroform and methanol 80% with percolation method. Most of secondary metabolites of medicinal plants are heat-sensitive, so for avoiding from destroying the bioactive in medicinal plants by heating, we have used percolation method for extraction. Before testing the anti mutagenicity of drug or plant extract on a certain strain of *S. tiphy* MIC should be determined. Agar dilution method was used to determine the MIC and concentrations lower than MIC was used for Ames test. A field of grass in all the measured samples and counting the number of revertant colonies recorded the results to show the anti mutagenicity of samples. This indicates no contamination of the samples at all stages and therefore no negative effect on the results of the Ames test. According to the results, plates containing microbial extract, the mutagenic substances and tested extracts were tangible and had substantial change in the number of the revertant colonies in comparison to positive control; so, herbal extracts have anti-mutagenic effect. The obtained results of antimutagenesis test showed that petroleum ether and methanolic fractions of *Z. multiflora* extract were able to control the number of revertant plate and kept within the standard range. These fractions prevent from the growth of bacterial strains as well as sharp increase in number revertant (Table 1, 2). Chloroform fractions of this plant decreased the number of revertant colonies in the presence of AT98 only. This fraction of *Z. multiflora* significantly reduced the number of revertant colonies (Table 3) and a significant increase in antimutagenicity ratio in the presence of TA98 in comparison to positive

Table 1: Number of revertant colonies of *S. typhi* in the presence of petroleum ether fractions of three medicinal plants in different concentrations

		No. of revertant colonies					
		Concentration (mg mL^{-1})					
Bacterium		25	12.5	6.25	3.125	Control-	Control+
S. (TA98)	A	-	830±98.5	706±64.3	953±128.5	84.3±8.2	1750±50
	B	-	-	843±60.2	966±175.6	71±17.5	1536±77.63
	C	-	-	923±92.9	876±92.9	53±12.6	1523±248.26
S. (TA100)	A	1330±121.2	2150±229.12	2583±425.2	3233±416.3	151±10.4	2900±50
	B	-	-	1766±175.5	2566±125.8	176±15.2	3053±150
	C	-	-	2083±76.37	1833±125.8	158±23.6	3200±229.1

A: *Zataria multiflora*, B: *Achillea wilhelmsii*, C: *Camellia sinensis*. Each experiment was repeated three times and the results were reported as mean±SEM

Table 2: Number of revertant colonies of *S. typhi* in the presence of methanolic fractions of three medicinal plants in different concentrations

		No. of revertant colonies					
		Concentration (mg mL^{-1})					
Bacterium		25	12.5	6.25	3.125	Control-	Control+
S. (TA98)	A	293±90.18	410±65.6	520±75.5	683±76.4	62±16.6	1573±94.5
	B	-	700±132.3	666±152.8	1066±152.75	71±15.3	1406±51.3
	C	-	550±217.9	516±104.0	743±92.9	54±12.0	1410±127.7
S. (TA100)	A	-	1016±125.9	1020±163.7	976±125.0	143±20.3	3150±180.3
	B	-	1083±125.9	1483±104.0	1316±175.5	165±18.1	3183±292.9
	C	-	-	1666±208.2	2266±378.6	149±28.3	3050±217.99

A: *Zataria multiflora*, B: *Achillea wilhelmsii*, C: *Camellia sinensis*. Each experiment was repeated three times and the results were reported as mean±SEM

Table 3: Number of revertant colonies of *S. typhi* in the presence of chloroform fractions of three medicinal plants in different concentrations

		No. of revertant colonies					
		Concentration (mg mL^{-1})					
Bacterium		25	12.5	6.25	3.125	Control-	Control+
S. (TA98)	A	416±104.1	586±80.8	690±36.1	856±40.4	61±12.6	1540±120
	B	-	1520±111.4	1750±180.3	1833±76.4	71±7.6	1603±204.04
	C	333±125.8	400±100	490±65.6	466±61.1	53±7.6	1360±115.32
S. *t* (TA100)	A	2133±208.2	2000±264.6	2506±110.2	2860±150.9	246±30.6	3066±152.75
	B	-	1950±132.3	2133±208.2	2833±125.8	273±58.6	3126±161.65
	C	_	_	2800±100.0	3116±401.0	273±25.7	2803±126.62

A: *Zataria multiflora*, B: *Achillea wilhelmsii*, C: *Camellia sinensis*. Each experiment was repeated three times and the results were reported as mean±SEM

Table 4: Results of antimutagenicity ratio of different fractions of three tested medicinal plants analyzed by the Ames test (on *S.*, TA100) in different concentrations

	Antimutagenicity ratio								
	Zataria multiflora			*Achillea wilhelmsii*			*Camellia sinensis* q2		
Concentration (mg mL^{-1})	A	B	C	A	B	C	A	B	C
25	2.1	1.4	-	-	-	-	-	-	-
12.5	1.3	1.5	3.1	-	1.6	2.9	-	-	-
6.25	1.1	1.2	3.1	1.7	1.4	2.1	1.5	1	1.8
3.125	1	1.1	3.2	1.2	1.1	2.4	1.7	1	1.3

A: Petroleum ether fraction, B: Chloroform fraction, C: Methanolic

Table 5: Results of antimutagenicity ratio of different fractions of three tested medicinal plants analyzed by the Ames test (on *S. typhimuirum*, TA98) in different concentrations

	Antimutagenicity ratio								
	Zataria multiflora			*Achillea wilhelmsii*			*Camellia sinensis*		
Concentration (mg mL^{-1})	A	B	C	A	B	C	A	B	C
25	-	3.6	5.3	-	-	-	-	4.08	-
12.5	2.1	2.6	3.8	-	1.05	2	-	3.4	2.5
6.25	2.4	2.2	3.02	1.8	1	2.1	1.6	2.7	2.7
3.125	1.8	1.8	2.3	1.6	1	1.3	1.7	2.9	1.9

A: Petroleum ether fraction, B: Chloroform fraction, C: Methanolic fraction

control ($p<0.05$) (Table 5), while the methanolic fraction showed these effects only in the presence of TA100 (Table 4). A variety of flavonoids, terpenoids and phenolic acids are present in *Z. multiflora* (Sharififar *et al*., 2011), therefore due to antimutagenicity of flavonoids and phenolic compounds, the antimutagenicity of the plant might be attributed to them. These results also show that petroleum ether and methanolic fractions of *A. wilhelmsii* kept the number of revertant plate within the standard range and prevented largely from both growth of bacterial strains and sharp increase in the number of revertant colony, however chloroform fraction could not inhibit mutagenesis (Table 1-3). Methanolic extract of *A. wilhelmsii* caused a strong decrease in the number of revertant colonies with a value ratio of antimutagenesis near 2 (Table 4-5). Phytochemical studies have reported the presence of falvonoids, essential oils, saponins and tannins in this species. Petroleum ether and methanolic fractions of *C. sinensis* kept the number of revertant plate within the standard range and prevented from the growth of bacterial strains and the sharp increase in the number of revertant largely. The number of revertant colonies was reduced in a concentration manner. Chloroform fraction of *C. sinensis* exhibited antimutagenesis effect only in the presence of TA_{98} (Table 2). The presence of phenolic compounds, catechins, tannins and alksloids has been reported in this plant (Liao *et al*., 2001; Ni *et al*., 2003). All fractions of this plant could significantly reduce the number of revertant plates at concentrations of 3.125, 6.25 and 25 mg mL^{-1}, respectively in the presence of TA_{98} and at 6.25, 12.5 and 25 mg mL^{-1} in presence of TA_{100} ($p<0.05$) (Table 1-3). Generally, the mechanism behind the antimutagenesis effect of different compounds might be due to the following: detoxification of active components, presence of nucleophilic group, nitrosation inhibition, direct inhibition of carcinogen compounds, modifying the metabolites leading to production of mutagenic and carcinogenic substances, electrophilic substance blockage, collection of active oxygen, reduction of point mutation and protection of nuclear enzyme. There are some researches indicating that flavonoids could inhibit nitrosation (Diying *et al*., 2007). Presence of a carbonyl group in C4 core of flavonoid is necessary for antimutagenic properties. The position of hydroxyl groups is also important and determining. Considering the solubility of most of flavonoids in methanol, the antimutagenesis of this fraction of the plants are mostly might be pertained to them.

CONCLUSION

Considering the antioxidant and antimutagenesis effects of methanolic fractions of the tested palnts, it seems that probably antimutagenic effect of flavonoids might be strongly related to antioxidant effect of this fraction. It also drawn that amongst the different fractions of three tested plants, methanolic extract of *Z. multiflora* exhibited the greatest antimutagenesis effect and would be a good candidate for further studies.

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