İstanbul Ecz. Fak. Derg. / J. Fac. Pharm. Istanbul
44(1) 2014 pp.11-30

The cyto- and genotoxic potantials of the herbal mixtures frequently used in Turkey

Anıl Candaş Aygan, Buket Alpertunga, Gül Özhan*

Department of Pharmaceutical Toxicology, Faculty of Pharmacy,
Istanbul University, 34116 Istanbul, Turkey

Abstract: Despite the profound therapeutic advantages possessed by herbs, some constituents of herbs have been shown to be potentially toxic. Knowledge on their safety is inadequate although the concerns have been raised over the lack of quality controls. We aimed to determine safety of six herbal mixtures used as tea, included forty plant species, chosen on the basis of their frequency of medicinal use and commercial importance in Turkey. Their cytotoxic activities were evaluated by determining mitochondrial succinate dehydrogenase (XTT) and extracellular lactate dehydrogenase (LDH) activities on human cerviks cell line (HeLa). For their genotoxic activities, two bacterial mutation assays, Ames assay with *Salmonella typhimurium* TA98 and TA100 strains and *umu* assay with *S. thyphimurium* TA1535/pSK1002 strain, were used. The 50% inhibition concentration (IC_{50}) values of the extracts for LDH and XTT tests were 6.52-63.53 and 18.75-104.67 mg/mL, respectively. In the genotoxicity studies conducted by *umu* assay, no extracts possessed genotoxic activities at 6.25-25 mg/mL. On the contrary, Ames bacterial mutagenicity assay conducted at the same concentrations revealed that (i) some extracts were shown mutagenic activities with metabolic activation (ii) TA100 strain was more sensitive than TA98 strain to the extracts, (iii) especially, three herbal mixtures may include ingredients shown mutagenic activities both in two strains and with metabolic activation. Our findings showed that herbal teas have some significant toxic effects. Therefore, the researchers and/or national authorities should consider that the use of herbal products may be harmful to human health.

Key words: herbal mixtures, Ames assay, *umu* assay, LDH test, XTT test

***Correspondence:** gulozhan@istanbul.edu.tr

Introduction

Herbal products have been commonly used by all cultures throughout history and provided an endless source in traditional and alternative medicine. World Health Organization (WHO) reports that 70%-80% of the world population confide in herbs for primary health care (1). Especially for the past few decades, herbs have been more and more consumed by the people without any prescription (2). Despite the profound therapeutic advantages possessed by herbs, some constituents of herbs have been shown to be potentially toxic. In addition to their natural ingredients acting upon the body, their quality was influenced by many toxic contaminants originating from industries, agriculture and private households including mutagenic and carcinogenic effects with long and widespread usage (3-5). Moreover, herbal prescriptions are generally a mixture of several herbs containing several active ingredients that exhibit various pharmacological activities (6-7). Scientific studies on their safety and toxicity are inadequate although the concerns have been raised over the lack of both quality control and scientific evidence of the efficacy and safety of herbs (8-9). Their quality controls must be defined as the status of a drug, which determined its identity, purity, content, physical and biological properties, and manufacturing process. Indeed, compared with synthetic drugs, the criteria and the approach for herbal products are much more complex (10). Therefore, people should be aware of the use of herbal products and the possible complications associated with herbal products.

By the European Food Safety Authority (EFSA), protocols and guidance documents discussing the assessment of the safety of herbs for use in both foods and medicines have been recently issued by the International Life Sciences Institute, the Institute of Medicine/National Research Council, the Union of Pure and Applied Chemistry, the European Medicines Agency (EMEA). In the regulatory context, safety assessment can have bearing on whether certain products should be restricted and removed from the market, or have augmented safety information on labelling. In many instances where little toxicity information exists on a specific herbal product or its ingredients, regulatory decisions on risk mitigation activities are likely to take a cautious approach, until further information is obtained which can potentially clarify the toxicity of the product, and reduce uncertainty in the risk assessment of herbs (11-13).

Turkey is an internationally important floristic centre because of its geographic location, climate, and the presence of nearly 10.000 natural plant species (14-15). Because herbs are readily available without prescriptions in the market, their increased consumption is a crucial problem in Turkey as well as in other countries. Most of herbs have not been subjected to chemical, toxicological, pharmacological or clinical investigations and have been ignored by national health authorities. Therefore, in the present study, it was screened for cyto- and genotoxic properties of the extracts obtained from six different herbal mixtures containing a total of forty plant species and frequently used in Turkey (Table 1). To assure the extraction of both polar and non-polar compounds from plants, chloroform, methanol and water were used as extraction solvents. Their cytotoxic activities were evaluated by XTT and LDH cytotoxicity tests on HeLa cell line. For their genotoxic activities, two bacterial mutation assays, Ames assay with *Salmonella typhimurium* TA98 and TA100 strains and *umu* assay with *S. thyphimurium* TA1535/pSK1002 strain, were used. The genotoxicity assays were also conducted in the presence of a mammalian mixture of liver enzymes, the S9 microsomal fraction, to mimic in vivo activation process.

Table 1: Ingredients of six herbal mixtures in the study.

Sample No	Ingredients	Usage
1	*Salvia triloba, Matricaria chamomilla, Tilia cordata, Thymus serpyllum, Rosa canina, Mentha piperita*	against flu, cough
2	*Malus domestica, Rosa gallica, Althae officinalis, Hibiscus sabdariffa, Tilia cordata, Caryophyllus aromaticum, Rosa canina, Matricaria chamomilla, Cinnamomum zeylanicum, Zingiber officinale*	for immunsupression, and against flu, cough
3	*Salvia triloba, Pimpinella anisum*, *Matricaria chamomilla*, *Rosmarinus officinalis, Nigella sativa, Malus domestica, Tilia cordata, Urtica diocia, Thymus serpyllum, Linum usitatissimum, Coriandrum sativum, Lavandula angustifolia, Melissa officinalis, Mentha piperita, Foeniculum vulgare*	as sedative
4	*Artemisia vulgaris*, *Aesculus hippocastanum, Nigella sativa, Anethum graveolens, Thymus serpyllum, Cymbopogon citratus, Prunus mahaleb, Glycyrrhiza glabra, Hypericum perforatum, Zingiber officinale*	for gastrointestinal disorders
5	*Pinus brutia, Nigella sativa, Anethum graveolens, Cocos nucifera, Cymbopogon citratus, Prunus mahaleb, Glycyrrhiza glabra, Crocus sativus, Citrus aurantium, Zingiber officinale*	as aphrodisiac
6	*Salvia triloba, Juniperus communis, Matricaria chamomilla, Rosmarinus officinalis, Calluna vugaris, Hibiscus sabdariffa, Urtica diocia, Thymus serpyllum, Linum usitatissimum, Melissa officinalis, Myrtus communis, Foeniculum vulgare, Crocus sativus, Thuya articulata, Cassia acutifolia, Camilla sinensis*, *Avena sativa, Cinnamomum zeylanicum*	for weight-loss

Material and methods

Materials

Ames microplate fluctuation (MPF™) 98/100, *umu*C Easy CS, Cytotox-XTT and Cytotox-LDHe kits were purchased from Xenometrix (Allschwil-Switzerland). Also, lyophilized rat liver S9 fraction was purchased from Xenometrix (Allschwil-Switzerland). Positive controls (2-nitrofluorene, 4-nitroquinoline N-oxide, 2-aminoanthracene) were obtained from Sigma (St. Louis, USA). Dimethylsulfoxide (DMSO), trypsin, triton X-100 and the other chemicals were obtained from the different companies (Biomatik, Canada; Merck, Germany; Fluka, Switzerland). Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin and and phosphate buffer saline (PBS) were purchased from Multicell-Wisent Inc. (Quebec, Canada).

Herbal extraction

Six different herbal mixtures containing forty plant species were purchased from local markets. The herbal mixtures were selected randomly based on their availability and their frequencies in traditional usage. According to declaration of company, their ingredients were confirmed by Prof. Dr. Emine Akalın in the Department of Pharmaceutical Botany, Istanbul University. Their ingredients and usage have been shown in Table 1. To assure the extraction of both polar and non-polar compounds, the herbal mixtures were extracted with chloroform, methanol and water; 2.5 g dried and mixed ground herbs were extracted with 25 mL of chloroform or methanol in a sonication bath (25ºC) for 30 min. The extracts were concentrated by rotary evaporator and dried under a gentle stream of nitrogen at 40 ^{0}C to give solid residue. The solid residues were dissolved in 1 mL DMSO, then were filtered and sterilized using 0.45 µm filters. For their decoctions with water, 2.5 g dried and mixed ground herbs were extracted with 100 mL of boiling water for 30 min. The extracts were filtered using 0.45 µm filters. The final concentration was 2500 mg/mL for the chloroform and methanol extracts, while the final concentrations of the water extract were 60 and 100 mg/mL for genotoxicity and cytotoxicity tests, respectively. In all assays, they were further diluted to produce at the concentrations of 2-50 mg/mL.

Cytotoxicity tests

Cell culture and treatment: The cervical cancer cell line (HeLa), easier to cultivate in comparison to other cell lines, were used. Cell line was provided by Department of Genetics, Istanbul University, Turkey. The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% streptomycin and penicillin at 37°C in a 5% CO_2 and 95% O_2 in a humidified cell incubator. 10^4 cells were seeded into each well of 96-well plates. After 24 h of incubating period, the culture medium was removed and the extracts were added to wells in various concentrations. The exposure concentrations were determined as 3.125-25 mg/mL for the chloroform and methanol extracts, and 6 mg/mL for water extracts. After 24 h of incubation, cytotoxicity test was performed using XTT and LDH tests according to the manufacturer's protocols.

XTT test; The principle of test is based on the formation of water-soluble orange formazan with the reduction of yellow tetrazolium salt by the mitochondrial succinate dehydrogenase, which belongs to the mitochondrial respiratory chain and is only active in viable cells, in the presence of an electron coupling reagent (16). As to test protocol, the exposed cells were washed with PBS and 200 µL/well of fresh culture medium were added. XTT-I (2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide inner salt) and XTT-II (buffer) solution were mixed at 1:100 ratio. Then, 50 µL of this mixture was added to all wells. The plate was incubated for 3 h at 37°C, 5% CO_2. After 3 h, the content of the well was mixed by pipetting up and down. Then, optical density (OD) in every well was read at 480 nm with a reference wavelength at 680 nm.

LDH test; Lactate dehydrogenase is stable cytoplasmic enzyme, present in all cells and rapidly released into the cell culture supernatant upon membrane damage or cell lysis. Lactate dehydrogenase reduces pyruvate to lactate by oxidizing reduced nicotinamide adenine dinucleotide (NADH) to oxide form (NAD^+). Spectrophotometrical measurement of NADH consumption is the principle LDH test (17). As to the test protocol, 20 µL of the supernatant were transferred from each well to a new 96-well plate. Then, the reaction was started by adding 200 µL LDH-II (NADH) and 40 µL LDH-III (Pyruvate) to every well. The absorbance of NADH was read kinetically at 340 nm for 25 min. LDH I was reconstitution solution.

In both cytotoxicity tests, negative (untreated, culture medium) and solvent (1% DMSO) controls were used. In LDH test, triton X-100 (1%) was used as positive control because of giving maximum activity. For each extract, four concentrations were tested in triplicates and each test was repeated twice.

Evaluation of the results; 50% inhibition concentration (IC_{50}) was used for cytotoxic activity. IC_{50} value was expressed as the concentration of sample caused an inhibition of 50% in enzyme activities in cells. For calculation, the absorbance values of samples were compared with the absorbance values of solvent/positive controls after all absorbance values were corrected by subtracting the absorbance of blank.

In XTT test, IC_{50} was calculated according to the following formula as the percentages of solvent control; *% inhibition = 100 - (corrected mean* OD_{sample} *x 100 / corrected mean* $OD_{\text{solvent control}}$*)*

In LDH test, the mean ΔOD/min for each well was firstly determined. Secondly, extracellular lactate dehydrogenase activity was calculated as unit (nmol NADH consumed/min/mL) by using the following formula: *NADH consumption = (ΔOD/min x 0.260 x 1000) / (6.2 x 20)* (0.260 mL was reaction volume in mL; 1000 µL was to convert for calculation the result in mL; 6.2 was mM extinction coefficient of NADH at 340 nm; 20 µL was volume taken for the assay).

Genotoxicity tests

Bacterial strains and culture medium; The strains used for the Ames assay were *S. typhimurium* TA98 and TA100 detected base-pair substitution mutations and frame shift mutations, respectively. In *umu* assay, *S. typhimurium* TA1535/pSK1002 strain was used for the different kinds of genotoxic lesions. All strains were obtained from kits (Ames MPF™ 98/100, *umu*C Easy CS). Strains were preserved frozen in 15% glycerol at -80°C.

Metabolic activation system (S9 mix); Lyophilized rat liver S9 fraction induced by Aroclor 1254 was used as 30% percent in assays. 30% of S9 mix was prepared immediately before the use by combining the reagent mix in a sterile tube. For Ames assay, S9 mix content was 0.083 mL 1.00 M KCl, 0.080 mL 0.25 M $MgCl_2.6H_2O$, 0.063 mL 0.20 M glucose-6-phosphate, 0.250 mL 0.04 M NADP, 1.270 mL 0.20 M NaH_2PO_4 buffer and

0.750 mL S9 fraction. For *umu* assay, S9 mix content was 0.070 mL 1.00 M KCl, 0.067 mL 0.25 M $MgCl_2.6H_2O$, 0.053 mL 0.20 M glucose-6-phosphate, 0.211 mL 0.04 M NADP and 0.098 mL S9 fraction (18).

Ames assay; Amino acid-dependent *S. typhimurium* strains cannot grow to form colonies in the absence of an external histidine source. Colony growth is resumed if a reversion of the mutation occurs, allowing the production of histidine to be resumed. Spontaneous reversions occur with each of the strains; mutagenic compounds cause an increase in the number of revertant colonies relative to the background level. The Ames MPF™ assay kit is a liquid microplate modification of the traditional Ames assay. In that assay, catabolic activity of revertant cells decreases the pH of solution resulting in colour change from purple to yellow and the results are evaluated by counting positive and negative wells (19-20).

The assay was conducted according to manufacturer's protocol. Semi-solid strains (*S. typhimurium* TA98 and TA100) in vials were thawed for 5 min. 200 µL growth medium was added to each of the vials to obtain homogenous suspensions of strains. 25 µl of the every suspension was added to a mixture of 10 mL growth medium and 10 µL ampicillin (50 mg/mL) in 50 mL culture tubes. Negative control was devoid of strains. The culture tubes were loosely capped, to allow aeration, and incubated in a shaker at 37°C, 250 rpm for 14-16 h. The overnight grown cultures were diluted 10 times with growth medium and the absorbance was measured at 600 nm. The assay was continued if the absorbance for the overnight grown culture and negative control were measured approximately 0.25 and 0.005, respectively. If not, the assay was stopped because the strain was not proper for the assay and the possibility of contamination was high. 1 mL of the overnight grown cultures was added to 3 mL growth medium in 50 mL culture tubes and reincubated in the environmental shaker at 37°C, 250 rpm for 90 min. The absorbance of the reincubated culture was measured at 600 nm. The assay was continued if the absorbance of reincubated culture was measured approximately 1.5-1.9.

Mutagenic potentials of the extracts were assessed in absence and presence of S9 mix in sterile medium. The final concentration of S9 mix in the assay was 4.5% v/v. Positive controls were 2-nitrofluorene (2 µg/mL) and 4-nitroquinoline N-oxide (0.1 µg/mL) without metabolic activation

and 2-aminoanthracene (5 µg/mL) with S9 mix. DMSO was used as the negative control.

Reincubated culture was diluted in exposure medium and exposed to the extracts in 24-well plates for 90 min at 37°C in the presence or absence of S9 mix. The exposure concentrations were determined as 6.25, 12.5, 25 and 50 mg/mL for the chloroform and methanol extracts, and 2 and 4 mg/mL for water extracts.

At the end of 90 min, 2.8 mL of indicator medium was added to each well of the 24-well plates. This mixture from each well was distributed into 48 wells of a 384-well micro-titer plate (50 µL per well) and was incubated at 37°C in a dry incubator for 48 h. Medium contains a pH indicator dye which changes from purple to yellow on bacterial growth. After 48 h incubation at 37°C, the plates were scored by optical determination for yellow wells.

Evaluation of the results: The number of positive (yellow) wells out of 48 wells in triplicate were counted and compared with the negative control. The criteria used to evaluate the Ames results were the fold increase in number of positive wells over the solvent control baseline, and the dose dependency. The fold increase of revertants relative to the solvent control was determined by dividing the mean number of positive wells at each dose by the solvent control baseline. The solvent control baseline was defined as the mean number of positive wells in the solvent control plus 1 standard deviation (SD). All solvent controls from an experiment with identical conditions (same day, same bacterial culture, solvent and incubation conditions) were combined. An increase of >2-fold relative to the baseline was classified as positive for that dose. Positive responses of >2-fold relative to the baseline at more than one dose with a dose-response led to the test sample being classified as positive. A test sample was classified as negative where no response >2 times the baseline and no dose-response was observed. To evaluate dose-response, student's t-test (1-sided, unpaired) was used. *P* value <0.05 was considered to be statistically significant. Each experiment was repeated at least twice.

umu assay: S. typhimurium TA1535 strain includes pSK1002 plasmid. If genotoxic lesions are produced by the samples, the *umu*C gene is induced as part of the bacterial SOS response in repair pathway. The plasmid pSK1002 contains the *umu*C gene fused to the *lac*Z reporter gene. The

induction of *lac*Z is measured by the conversion of colourless ONPG substrate (o-nitrophenyl-β-D-galactopyranoside) to the yellow product o-nitrophenol by the *lac*Z-encoded β-galactosidase (21).

The assay was conducted according to the manufacturer's protocol. Semisolid *S. typhimurium* strain TA1535/pSK1002 was used in vial. TG medium (200 µL) was added to the vial to obtain homogenous suspensions of strain. 10 µL of ampicillin (50 mg/mL) was added to 10 mL TG medium (¼TGA medium) in 50 mL culture tubes. 50 µL of *Salmonella* suspension was mixed with 10 mL TGA medium. Negative control was devoid of bacteria. The culture tubes were loosely capped, to allow aeration, and incubated in a shaker at 37°C, 250 rpm for 14-16 h. The overnight grown cultures were diluted 10 times with TG medium and the absorbance was measured at 600 nm.

Like Ames assay, mutagenic potentials of the extracts were assessed in absence and presence of S9 mix in *umu* assay. The final concentration of S9 mix in the assay was 0.75% v/v. Positive controls were 4-nitroquinoline N-oxide (0.463 µg/mL) without metabolic activation and 2-aminoanthracene (1.85 µg/mL) with S9 mix. DMSO was used as the negative control.

TGA medium and S9 mix were added to each wells of plate. Then, the extracts were added to wells at 6.25, 12.5 and 25 mg/mL concentrations for all extracts. The plates were incubated at 37°C, 120-150 rpm for 2 h. During the 2 h, a second plate was prepared with TG medium with freshly added ampicillin to all wells (for 1 plate: 28 µL ampicillin stock (50 mg/mL) to 28 mL TG medium). After 2 h, 30 µL of the contents of the first plate was transferred to the second plate. The second plate was read to obtain OD_{600}. Then, the second plate was incubated 37°C, 120-150 rpm for 2 h. During the 2 h, a third plate was prepared with 150 µL B-buffer/ONPG mixture (for 1 plate: 15 mL of B buffer, 40.5 mL 2-mercaptoethanol, 1 mL ONPG solution) and pre-warmed to 28°C. At the end of the 2 h incubation, the second plate was mixed and read the OD_{600}. Then, 30 mL of each wells of the second plate was transferred to the third plate. The third plate was incubated at 28°C, 120-150 rpm for 30 min. After 30 min, 120 mL of stop reagent was added to each well. The plate was mixed and read the OD_{420}.

Evaluation of the results: For each sample, the Growth factor (G), the β-galactosidase activity (relative units) (U) and the Induction ratio (IR)

were calculated as follows:

$G=(OD_{600,S} - OD_{600,B}) / OD_{600,N} - OD_{600,B})$
(OD_{600}-absorbance at 600 nm; S-sample; B-blank; N-negative control)

$U=(OD_{420,S} - OD_{420,B}) / OD_{600,S} - OD_{600,B})$
(OD_{420}-absorbance at 420 nm; OD_{600}-absorbance at 600 nm; S-sample; B-blank)

$IR=(OD_{420,S} - OD_{420,B}) / [(OD_{420,N} - OD_{420,B}) \times G]$
(OD_{420}-absorbance at 420 nm; S-sample; B-blank; N-negative control)

The whole test is considered valid if the positive controls reach IR ≥1.5. The average OD_{600} of the negative controls of the second plate should increase by IR ≥1.5 during the 2 hr incubation (growth control). A sample is considered genotoxic if IR ≥1.5 and G ≥0.5. To decide if sample is mutagenic, we also evaluated dose-response for all extracts. So, a test sample was classified as negative where no response IR ≥1.5 and no dose-response were observed. To evaluate dose-response, student's t-test (1-sided, unpaired) was used. *P* value <0.05 was considered to be statistically significant. The results were determined with *umu*C Easy CS Excel Programme. Each experiment was repeated at least twice.

Results

It was determined that all extracts were cytotoxic to HeLa cells by using XTT and LDH tests. The IC_{50} values for LDH and XTT tests were 6.52-63.53 and 18.75-104.67 mg/mL, respectively. Sample 5 was the most cytotoxic herbal mixture for three extracts according to data obtained from both of tests (Table 2). As it can be seen in the results, the extracts were disturbed both on membrane permeability and mitochondrial activity of cell.

Table 2: Cytotoxic activity observed by using XTT and LDH tests in HeLa cell line exposed to the extracts of the selected six herbal mixtures.

Sample No	Extraction	IC_{50} (mg/mL)	
		LDH	XTT
1	Methanol	63.53	84.73
	Chloroform	24.41	27.41
	Water	20.96	33.14
2	Methanol	21.67	23.53
	Chloroform	22.78	21.33
	Water	48.43	104.67
3	Methanol	59.45	35.94
	Chloroform	59.45	26.86
	Water	6.52	83.71
4	Methanol	19.12	25.12
	Chloroform	23.64	21.33
	Water	9.54	23.47
5	Methanol	11.12	18.75
	Chloroform	18.52	19.80
	Water	18.59	53.53
6	Methanol	36.91	21.90
	Chloroform	19.86	19.48
	Water	60.64	55.92

To evaluate of the genotoxic potentials of the extracts, Ames and *umu* assays were carried out. The studied concentrations were determined on the basis of the maximum permissible concentrations of the test conditions. We took into consideration that herbs are usually used several times per day as tea and %1-5 (v/w) proportions are suggested for intake on-time.

Table 3 shows the mutagenic activity of the extracts of the herbal mixtures in the Ames test with and without microsomal activation on the TA98 and TA100 strains.

The chloroform extracts were mutagenic mostly in the presence of metabolic activation. Sample 1, 2 and 4 were no mutagenic, sample 3 was mutagenic only to TA100 at the highest concentration (2.79 fold) with S9. Sample 5 and 6 were mutagenic to TA100 at the studied concentrations (≥2.05 fold) with S9. The mutagenic activities of sample 5 and 6 to TA100 were observed concentration-dependently. Besides, sample 5 was mutagenic at 12.5 and 50.0 mg/mL (≥2.41 fold) with/without S9 and at 6.25 and 12.5 mg/mL (2.95 fold) with S9 to TA98 (Table 3).

Mutagenic activities of methanol extracts were seen only in the presence of metabolic activation. The mutagenic activities were shown at 50.0 mg/mL (≤2.27 fold) in sample 1, at higher than 25.0 mg/mL (≤2.05 fold) in sample 2 to both strains. Sample 3 had no mutagenic activities. Sample 4 was mutagenic to TA98 as concentration-dependent (≥2.14 fold) and to TA100 (≤2.97 fold). Sample 5 was mutagenic at only 25.0 mg/mL (≥4.21 fold) and 12.5 mg/mL (≥3.02 fold) for TA98 and TA100, respectively. Sample 6 was mutagenic to both strains as concentration-dependent (≥2.14 fold) (Table 3).

As to their water extracts; no mutagenic activity was observed to TA98, only sample 2 was mutagen at 4 mg/ml in the presence of metabolic activation (3.48 fold). To TA100 strain, sample 1 and 2 were mutagenic independent metabolic activation (≥2.18 fold), sample 3, 4 and 5 were mutagenic with S9 (≥3.31 fold), sample 6 was no mutagenic (Table 3).

Table 3: Mutagenic activity observed by using the Ames MPF™ 98/100 assay in bacterial strains TA98 and TA100 exposed to the extracts of the selected six herbal mixtures without or with metabolic activation (S9).

Sample No	Extraction	Concentration (mg/mL)	Revertants fold increase (over baseline)			
			TA98		TA100	
			S9-	S9+	S9-	S9+
DMSO			1.92	2.03	2.25	2.08
Positive Controls			16.28	15.90	18.04	20.52
1	Chloroform	6.25	0.45	0.30	0.87	0.82
		12.5	0.74	0.74	0.87	0.62
		25.0	1.04	0.89	0.35	0.62
		50.0	1.19	0.89	1.22	0.82
	Methanol	6.25	0.83	0.57	1.37	1.64
		12.5	0.83	0.57	1.37	1.54
		25.0	1.00	0.70	1.26	1.75
		50.0	1.67	**2.27**	1.13	**2.36**
	Water	2.0	0.94	0.85	1.01	1.45
		4.0	1.00	0.54	**2.52***	**5.76***
2	Chloroform	6.25	0.27	0.89	1.57	1.17
		12.5	0.30	0.74	0.17	0.35
		25.0	0.27	0.74	0.17	1.17
		50.0	1.07	0.59	0.35	1.57
	Methanol	6.25	0.59	0.89	1.26	1.40
		12.5	0.45	1.46	1.05	1.62
		25.0	0.54	**2.23**	1.34	**2.05**
		50.0	0.89	1.78	1.34	**2.09**
	Water	2.0	0.96	0.87	1.02	1.34
		4.0	1.06	**3.48***	**2.18***	**3.90***
3	Chloroform	6.25	0.31	0.53	0.21	1.13
		12.5	0.41	0.80	0.30	1.59
		25.0	0.41	1.34	0.41	1.74
		50.0	1.22	1.54	0.56	**2.79***
	Methanol	6.25	0.21	0.45	0.51	0.05
		12.5	0.54	0.83	0.57	1.03
		25.0	0.54	0.76	0.72	0.91
		50.0	0.21	0.84	0.84	1.51
	Water	2.0	0.65	1.12	0.99	1.87
		4.0	0.47	1.61	1.49	**7.15***
4	Chloroform	6.25	0.54	0.30	0.51	1.13
		12.5	0.70	0.22	0.50	1.34
		25.0	0.71	0.38	0.70	0.92
		50.0	0.54	0.68	0.72	1.43
	Methanol	6.25	0.35	**2.41***	0.30	**2.97**
		12.5	0.47	**2.41***	0.45	**2.12**
		25.0	1.06	**3.22***	0.60	1.09
		50.0	1.74	**8.84***	0.60	0.45
	Water	2.0	1.01	0.67	0.98	1.56
		4.0	0.71	0.80	1.03	**5.23***
5	Chloroform	6.25	1.67	1.88	0.08	**2.38**
		12.5	**2.67**	**3.22**	0.28	**2.56***
		25.0	1.05	1.61	0.17	**3.66***
		50.0	**4.19***	**2.41**	0.28	**3.11***
	Methanol	6.25	0.67	1.33	0.17	1.73
		12.5	1.67	1.50	0.35	**3.02**
		25.0	1.67	**4.21**	0.52	1.67
		50.0	0.67	0.50	0.35	0.80
	Water	2.0	0.34	0.56	1.23	1.34
		4.0	0.22	0.54	1.95	**3.31***
6	Chloroform	6.25	1.33	**2.95**	0.31	**2.05***
		12.5	1.33	**2.95***	0.47	**2.12***
		25.0	0.33	1.33	0.48	**2.44***
		50.0	0.33	1.61	0.60	**2.93***
	Methanol	6.25	0.35	1.34	0.40	**2.70***
		12.5	1.05	**2.14***	0.89	**3.47***
		25.0	0.7	**5.09***	0.45	**4.75***
		50.0	0.71	**4.82***	0.30	**6.39***
	Water	2.0	0.98	1.12	0.98	1.34
		4.0	1.37	1.31	0.82	1.54

* t test *P* value (unpaired 1-sided) < 0,05. Bold: Fold increase over baseline ≥ 2.

Table 4 shows the genotoxicity results of the extracts by *umu* assay. The chloroform extracts of sample 2, 3 and 6 were not mutagenic. Sample 4 and 5 were mutagenic at 25.0 mg/mL (IR≥1.556 with S9 and at IR≥1.500 without S9, respectively). Sample 1 induced *umu* gene expression concentration-dependently with S9 (IR≥1.559). The methanol extracts of sample 2 and 3 were mutagenic at 25.0 mg/mL (IR≥1.589), and sample 4 was mutagenic at 12,5 mg/mL (IR≥1.712) with S9. The water extracts of sample 3, 4 and 5 were not mutagenic. Sample 1, 2 and 6 were mutagenic at 25 mg/mL with/without S9 (IR≥1.670). As indicated in evaluation of the results in *umu* assay, only chloroform extract of sample 1 was significantly shown weak mutagenic activities in the presence of metabolic activation (P <0.05).

Discussion

Herbs used in traditional medicine are generally assumed safe due to their long-term use and they are "natural". This concept is largely hazardous and the uncontrolled use of the herbal products by general population claim for studies on their side effects and toxicity (22). However, there is a lack of controlled studies on the plants and the risks of their consumption are unknown. Indeed, there are usually limited data obtained from *in vitro*, *in vivo* and human, which are used as the basis for risk characterization and the establishment of safe intakes (11).

The fractionation of complex mixtures and the chemical identification of their components are almost impossible and not reasonable. Furthermore, the mixture's genotoxic and cytotoxic effect potentials are possibly different from the sum of the effects of the components (7). To predict the genotoxic effects of the consumption of the complex mixtures on human health, Ames and *umu* assays can be useful tools in the quantification of the genotoxic activity of the complex mixtures and the different responses of the several *Salmonella* strains can help in the identification of the classes of genotoxic compounds present in the samples (21, 23-24).

However, a positive result does not necessary indicate the substance as a carcinogen. It confirms that the substance is not mutagenic to the particular bacterial strain used and for the genetic endpoint tested. For carcinogenicity a 2 year carcinogenicity test would have to be performed by testing the effect of the mutagenic sample in mice and rats (male and female) (25). Generally studies on herbal products are related to their antimutagenic/anticarcinogenic potentials (26-30).

Table 4: Mutagenic activity observed by *umu*C Easy CS assay in TA1535/pSK1002 exposed to the extracts of the selected six herbal mixtures without or with metabolic activation (S9).

Sample No	Extraction	Concentration (mg/mL)	Growth factor (G)		Induction factor (IR)	
			S9-	S9+	S9-	S9+
DMSO			0.967	1.002	1.000	1.000
Positive control			1.005	0.994	6.692	3.600
1	Methanol	6.25	1.180	0.913	0.906	0.863
		12.5	1.235	0.858	0.868	1.368
		25.0	1.22	1.090	0.834	0.964
	Chloroform	6.25	1.248	0.901	0.941	**1.559**
		12.5	1.340	0.781	0.774	**1.571**
		25.0	1.162	0.988	0.744	**2.006**
	Water	6.25	1.139	1.034	1.011	1.199
		12.5	1.107	1.053	1.181	1.422
		25.0	1.074	0.955	**1.670**	**2.142**
2	Methanol	6.25	1.118	1.027	0.935	1.281
		12.5	1.094	1.109	1.006	1.376
		25.0	1.129	1.360	1.081	**1.679**
	Chloroform	6.25	1.161	0.800	1.007	1.061
		12.5	1.090	0.789	1.247	1.003
		25.0	0.417	0.712	1.165	0.979
	Water	6.25	1.581	2.068	0.943	0.876
		12.5	1.503	2.404	1.076	1.186
		25.0	2.094	3.215	**1.772**	**1.728**
3	Methanol	6.25	1.049	1.036	0.817	1.449
		12.5	1.101	1.085	0.911	1.490
		25.0	1.125	1.166	1.174	**1.589**
	Chloroform	6.25	1.140	1.029	0.800	1.130
		12.5	1.120	1.056	0.904	1.361
		25.0	1.030	1.013	1.009	1.324
	Water	6.25	1.095	0.979	0.842	1.141
		12.5	1.155	0.995	1.121	1.352
		25.0	1.680	0.980	1.184	1.498
4	Methanol	6.25	0.903	1.085	0.734	1.133
		12.5	1.136	1.136	1.125	**1.712**
		25.0	1.017	1.125	1.007	1.239
	Chloroform	6.25	1.065	1.041	0.846	1.043
		12.5	1.091	1.069	0.862	1.203
		25.0	1.178	1.117	0.918	**1.556**
	Water	6.25	1.105	1.076	0.930	1.123
		12.5	1.121	1.092	1.105	1.147
		25.0	1.093	1.148	1.260	1.244
5	Methanol	6.25	0.980	1.139	0.853	0.842
		12.5	1.020	1.193	0.948	0.902
		25.0	1.024	1.351	1.049	0.799
	Chloroform	6.25	0.960	1.082	0.830	0.988
		12.5	0.938	1.110	1.179	1.008
		25.0	0.884	1.081	**1.500**	1.159
	Water	6.25	0.987	1.011	0.863	1.040
		12.5	1.043	1.06	0.953	1.166
		25.0	1.059	1.049	1.068	1.466
6	Methanol	6.25	1.018	1.108	1.043	1.015
		12.5	1.060	1.166	1.171	1.183
		25.0	1.008	1.325	1.172	1.192
	Chloroform	6.25	0.973	0.965	1.030	1.030
		12.5	1.092	1.092	1.174	1.024
		25.0	1.096	1.096	1.107	0.972
	Water	6.25	0.996	1.028	1.104	1.192
		12.5	1.059	1.110	1.415	1.439
		25.0	1.153	1.217	**1.774**	**1.798**

Bold: Fold increase over baseline ≥ 2.

In Turkey, consumers can buy herbal products without a prescription from the market without proper scientific evaluation, and without any mandatory safety and toxicological studies. Because of that, the present study was conducted to investigate the cytotoxic and genotoxic effects of some herbal mixtures often used in our country. There are genotoxicity data available individually for these herbs. Among them, *Melissa officinalis* was genotoxic, mutagenic and might have carcinogenic potential. The presence of phenolic compounds, especially caffeic acid derivatives, might be correlated to its genotoxic activity. The spectrum of mutations revealed a great trend for base substitutions, mainly in guanines and adenines (22). An ethanolic extract of *Melissa officinalis* was also endowed with antigenotoxic/antimutagenic properties, and its use in pre-treatment could diminish the induction of DNA damage by an alkylant agent (31). In one study, the mutagenicity of *Coriandrum sativum* (hot water, methanol and hexane) was studied in TA98 and TA100 strains by the Ames assay. The extracts were not mutagenic in either of the strains, with/without S9 metabolic activation (32). Contrary to that, Mahmoud et al. (33) reported its mutagenic activities in the Ames assay with TA98 and TA100 strains. When Ebeed et al. (34) investigated the potential antimutagenic and cancer chemoprevention effects of the hot water crude extract of *Foeniculum vulgare* seeds in mice and *Drosophila*, they observed that the extract might have slight genotoxic effects on mice rather than *Drosophila*. Hydroalcoholic extracts of *Foeniculum vulgare* fruits, *Matricaria chamomilla* flowers, *Mentha piperita* were genotoxic (35). Some studies reported licorice extracts, glycyrrhizin and glycyrrhetic acid obtained from *Glycyrrhiza* sp. were negative in TA98 and TA100 strains (36-37). Only Martinez et al. (38) found licorice extract was mutagenic in TA100 strain, but not in TA98. It was reported that the oral LD_{50} for saffron (*Crocus sativa*) in mice was 20.7 g/kg administered as a decoction (39). A number of previous studies suggest that saffron possesses anticarcinogenic activities and has no cytotoxic effect on non-malignant cells (40-41), while exhibiting cytotoxic inhibitory activity against different animal and human malignant cells (41-43). Similarly, *Cymbopogon citratus, Zingiber officinale, Hypericum perforatum* and *Matricaria chamomilla* had cyotogenotoxic potential (35, 44-45).

Generally, herbal prescriptions are generally a mixture of several herbs containing several active ingredients that exhibit various activities in a

single preparation. Because of this, results obtained from the present study should be beneficial data.

In conclusion, under our assay conditions, we observed that some of the herbal mixtures used as herbal teas for the treatment of diseases and disorders such as flu, cough, migraine, nervous tension, ingestion problems, various types of spasms, liver and gall bladder disorders showed cytotoxic and mutagenic potencies (especially with metabolic activation). Investigation on the active constituents of these herbs may provide useful comparative information in the future even if identification of all compounds present in herbs is a difficult task. Also, it will be beneficial to evaluate the carcinogenicity of these plants in order to assess the risks for human health. Standardization of methods and quality control data on safety and efficacy are required for understanding of the use of herbal products. Contrary to popular belief that "natural are safe", herbs can cause significant toxic effects, drug interaction and even morbidity or mortality. We believed that the article is not a bias against herbal products. The purpose of this article is to raise the awareness of the researchers and/or national authorities regarding the use of herbal products without hazard to human health.

Acknowledgement

This work was supported by the Research Fund of Istanbul University (18434/30115).

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