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Antimutagenic Activity of The Methanol Extract of *Helichrysum plicatum* ssp. *plicatum*

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The present study was conducted to evaluate the antimutagenic activity of methanol extract of *Helichrysum plicatum* ssp. *plicatum* plants. Antimutagenic activity of the extract was estimated by employing AMES- *Salmonella*/microsome assay by using *Salmonella typhimurium* TA1535 and TA1538 strains against direct acting mutagens sodium azide (NaN_3), 4-nitro-1-quinoline oxide and the S9-dependent mutagen 2-amino fluorene (2AF). In this study, standard plate incorporation method was preferred to determine antimutagenic activity. In the absence of S9 metabolic activation all three doses of plant extract showed statistically significant ($p < 0.05$) antimutagenic activity on TA1535 strain, but no on the TA1538 strain. In the presence of S9 microsomal fraction, plant extract exerted moderate antimutagenic activity against the 2AF mutagen and reduced mutant colonies in the TA1535 and TA1538 strains. The present results indicate that *H. plicatum* ssp. *plicatum* extract has antimutagenic activity.

Key Words: *Helichrysum plicatum* ssp. *plicatum*, Methanol extract, Antimutagenicity, AMES *Salmonella*/microsome test system, S9-Metabolic activation system.

INTRODUCTION

Plants have commonly been used in medicine both as a traditional remedy and industrial products[1]. The evidences which were obtained from epidemiological, *in vivo* and *in vitro* experimental studies showed that plant metabolites might have preventive effects against various diseases including cancer. These studies have increased antimutagenic and antigenotoxic investigations[2]. It is known that antimutagens or anticarcinogens play active role to struggle the factors connected with cancer etiology[3]. In addition to this research, the chemical compounds which

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have antimutagenic and anticarcinogenic properties, have become obligatory because of increasing mutation ratio and cancer risk in human being[4]. Also, to research molecular mechanism of tumor genesis and be able to offer biomedical preventive benefits to tumor formation make these studies inevitable[1].

To determine the antimutagenic activities of plant metabolites with *in vivo* studies by using experiment animals are not preferred beginning test systems because of their high cost and long time requirement[5]. So researchers have developed many test systems for anticarcinogenity studies which are cheap and require short time[6]. AMES-*Salmonelle*/microsome test system is one of the important shorting time test systems that are commonly used for determining antimutagen/anticarcinogens or on the contrary mutagen/carcinogens[1].

The genus *Helichrysum* belongs to the tribe Inuleae in the Asteraceae family and is known for its aromatic and therapeutic properties. It is a large family of about 600 species worldwide[7-9]. This genus is represented in Turkish flora by 18 taxa, of which 9 are endemic[10]. The plants of this genus are notable for their involucres, made up of a large number of bracts located in several rows and which are yellow and shiny[11,12]. Some members of genus *Helichrysum* have been well characterized with respect to their secondary metabolites, largely dominated by alkaloids, flavonoids, phloroglucinols and tannins[13,14] with antibacterial and antioxidant properties. However, the antimutagenic effect of any *Helichrysum* species growing in Anatolia has not been studied so far. The objective of this study is to evaluate antimutagenic activity of *Helichrysum plicatum* ssp. *plicatum*.

EXPERIMENTAL

Mutagens, direct acting mutagens sodium azide (NaN_3), 4-nitro-1-quinoline-oxide (4NQO) and the S9-dependent mutagen 2-aminofluorene (2AF), were obtained from Sigma-Aldrich Co (St. Louis, MO, USA). All solvents and pure chemicals, dextrose, L-histidine, D-biotin, L-histidin.HCl, sodium phosphate (monobasic, $NaH_2PO_4 \cdot H_2O$), crystal violet, potassium phosphate (dibasic, anhydrous, K_2HPO_4), sodium ammonium phosphate, magnesium sulfate ($MgSO_4.H_2O$), sodium chloride, citric acid monohydrate ($Na_2NH_2PO_4.4H_2O$), sodium phosphate (dibasic, $Na_2HPO_4.H_2O$), nicotinamide adenine dinucleotide phosphate (NADP) were also obtained by Sigma, Merck and Fluka.

Helichrysum plicatum ssp. *plicatum* D.C. plants were collected at flowering stage between June and August 2006 from rocky mountain areas in the vicinity of Askale-Kop (Erzurum, Turkey.) The taxonomic identification of plant materials was confirmed by a senior plant taxonomist, Meryem Sengul, in the Department of Biology, Atatürk University, Erzurum, Turkey. Collected plant materials were dried in shadow and ground in a grinder with a 2 mm diameter mesh. A voucher specimen has been deposited at the Herbarium of the Department of Biology, Atatürk University, Erzurum, Turkey (ATA HERB No: 9562).

Preparation of the methanol extract: The dried and powdered leaves (500 g) were extracted with 1 L of methanol using a Soxhlet extractor (ISOPAD, Heidelberg, Germany) for 72 h at a temperature not exceeding the boiling point of the solvent[15]. The extract was filtered using Whatmann filter paper (no. 1) and then concentrated *in vacuo* at 40 °C using a rotary evaporator (Buchi Labortechnic AG, Flawil, Switzerland) yielding a waxy material 20.04 % v/w. The extract was then lyophilized and kept in the dark at 4 °C until tested.

S9-Based metabolic activation system: Male Sprague Dawley rat liver post-mitochondrial supernatant (S9 fraction) induced by Aroclor 1254, was purchased from Moltox (Molecular Toxicology, Boone, NC, USA). The metabolic activation system (S9 mixture) was prepared from 10 % of a rat liver homogenate (S9 microsomal fraction) using NADPH Regency's (Glucose-6-phosphate, MgCl/KCl in 0.1 M phosphate buffer, pH 7.4) and NADP, the mixture was kept on ice during testing.

Bacterial strains: *S. typhimurium* strains TA1535 (ATCC® Number: 29629) and TA1538 (ATCC® Number: 29631) were provided by The American Type Culture Collection-Georgetown University, Bacteria Department, Washington, U.S.A For all assays, fresh bacterial cultures were prepared from frozen permanent cultures and incubated and shaken overnight at 37 ºC to a concentration of approximately 1-2 × 10^9 CFU/mL.

Antimutagenicity assay: Antimutagenicity assay was performed according to the method by Mortelmans and Zeiger[6] which is modified procedure reported by Maron and Ames[16]. Suitable concentrations of known mutagens for *S. typhimurium* strains, with and without S9 mixture, were chosen from the linear part of a concentration-response curve: 4-nitroquinoline-1-oxide (4NQO in DMSO 0.2 µg/plate) for TA1538 without S9, sodium azide (NaN_3 in distilled water 1 µg/plate) for TA1535 without S9; 2-aminoflourene (2AF in DMSO 600 µg/plate) for all strains with S9. 100 µL of the overnight bacterial culture, 50 µL mutagens, 50 µL test compounds at different concentrations (0.05, 0.5, 5 µg/plate in 10 % DMSO) and 500 µL of S9 mixture or phosphate buffer were added to 2 mL of the top agar containing 0.5 mM histidine/biotine. The mixture was poured onto minimal glucose plates. Histidine independent reverting colonies and viable cells were scored on plates after incubation at 37 ºC for 48 or 72 h. The experiments were repeated at least twice and each concentration was determined in triplicate. Positive controls were 2-aminofluorene with S9 for TA1535 and TA1538 and 4NQO without S9 for TA1538, NaN_3 without S9 for TA1535. The negative control was the solvent control 10 % DMSO. After incubation revertant colonies were counted and the percentage inhibition assay calculated: [1-T/M] × 100, where T is the revertants per plate in the presence of mutagen and plant extract and M is the revert ants per plate in the control (10 % DMSO, no plant extract). A non-antimutagenic effect was considered to give a value smaller than 25 %, a moderate effect a value between 25-40 % and strong antimutagenicity a value greater than 40 %[17].

Statistical analysis: The results are presented as the average and standard error of three experiments with triplicate plates/dose experiment. The data were further analyzed for statistical significance using analysis of variance (ANOVA) and the difference among means was compared by high-range statistical domain using Tukey's test. A level of probability < 0.05 was taken as indicating statistical significance.

RESULTS AND DISCUSSION

The possible antimutagenic potential of *H. plicatum* ssp. *plicatum* was examined against 4NQO, NaN_3 and 2AF in *S. typhimurium* strains TA1535-TA1538 with and without S9 microsomal fraction using standard plate incorporation assay. In order to assess antimutagenic effects of *H. plicatum* ssp. *plicatum* induction or suppression of revertant colonies was examined in *S. typhimurium* strains TA1535-TA1538. In the assay, the plant methanol extract were done at the doses 5, 0.5 and 0.05 µg/plate, the results are shown in Tables 1 and 2. All the values are expressed as mean ± standard error. The data were analyzed for statistical significance using analysis of variance (one way ANOVA), followed by Tukey's method. p values less than or equal to 0.05 were considered to indicate statistical significance.

TABLE-1
ANTIMUTAGENICITY OF PLANT EXTRACTS OF *Helichrysum plicatum* ssp. *plicatum* TO *Salmonella typhimurium* (TA1538, TA1535) WITH METABOLIC ACTIVATION (S9)

Test item	Conc. (µg/plate)	Number of revertant colonies			
		Salmonella typhimurium TA1535		*Salmonella typhimurium* TA1538	
		Mean ± SE	Inhibition (%)	Mean ± SE	Inhibition (%)
Negative control, DMSO		5.33 ± 1.52		12.66 ± 2.52	
Positive control, 2AF	600	405.00 ± 14.18		122.66 ± 6.66	
Helychrysum pilicatum ssp. *pilicatum*	5.00	207.00 ± 25.24*	49	124.00 ± 6.11	-
	0.50	209.33 ± 8.41*	48	124.33 ± 5.23	-
	0.05	233.33 ± 16.89*	42	126.00 ± 6.11	-

*p < 0.05, DMSO = Dimethyl sulfoxide was used as negative control; 2AF = 2-Aminoflourene, was used as positive control.

When assayed with S9-based metabolic activation, every three doses of the plant extract caused statistically significant ($p < 0.05$) antimutagenic activity in the only single strains. The inhibition effect has been observed, respectively 49-48-42 % on the TA 1535; whereas no inhibition effect observed on the TA1538 strain (Table-1).

In the absence of S9 microsomal fraction, plant extract exerted moderate anti-mutagenic activity against the 4NQO and NaN_3 mutagens and reduced mutant colonies in theTA1535 and TA1538 strains. *H. plicatum* ssp. *plicatum* induced inhibition bound to concentration-dependent: 5 µg/plate induced moderate antimutagenic activity both TA1535 and TA1538, reducing histidine-prototrophic reverting colonies by 36-22 % whereas lower concentrations (0.05 and 0.5 µg/plate) not reduced histidine-independent revertant colonies (Table-2).

TABLE-2
ANTIMUTAGENICITY OF PLANT EXTRACTS OF *Helichrysum plicatum* ssp. *plicatum* TO *Salmonella typhimurium* (TA1538, TA1535) WITHOUT METABOLIC ACTIVATION (S9)

Test item	Conc. (µg/plate)	Number of revertant colonies			
		Salmonella typhimurium TA1535		*Salmonella typhimurium* TA1538	
		Mean ± SE	Inhibition (%)	Mean ± SE	Inhibition (%)
Negative control, DMSO	0.2	34.67 ± 4.22		10.25 ± 1.31	
Positive control, 4NQO NaN_3	1.0	255.00 ± 11.81		196.50 ± 10.01	
Helychrysum pilicatum ssp. *pilicatum*	5.00	178.33 ± 9.53*	36	148.33 ± 4.70*	22
	0.50	292.00 ± 8.32	–	184.33 ± 3.18	–
	0.05	282.33 ± 9.28	–	200.00 ± 6.43	–

*p < 0.05, DMSO = Dimethyl sulfoxide was used as negative control; 4NQO = 4-Nitroquinoline-1-oxide and NaN_3 = Sodium azide, were used as positive control.

In the presence of S9 mix, the observed antimutagenic activity for *H. plicatum* ssp. *plicatum* 2AF mixture may be related to a blockade of the cytochrome-dependent system P450. The results demonstrate that the antimutagens exist in the fractions may interact with some enzyme system of microsomal cytochrome P450 in the liver homogenates which are necessary for activation of promutagens. With the studies of some researchers on antimutagenic, antitumoral and anticarcinogenic activities of plant extracts revealed that the biochemical components of plant do not act *via* single mechanism. They interfere with the metabolic activation of promutagens, act as blocking agents, form adducts with ultimate metabolite and scavenge free radicals[18].

At the study conducted on the different species of this plant by Reid *et al.*[19] it was determined that there is no antimutagenic effect of this plant. On the contrary, it was observed that this plant has mutagenic effect 5 µg/plate and 0.5 µg/plate doses on the *S. typhimurium* TA98-TA100. It is thought that this diversity is raised from the differences of chemical composition that may change as related to species and ecological different. The antimutagenic activities of extracts result from not only the capacity that can effect the enzymatic activation process but also having the characteristics that can collect the free radicals.

Mechanism of this antimutagenic activity may be due to its antioxidant activity suggested by previous reports[12,14]. The antimutagenic activity also might be related to on its ability to change membrane lipids and permeability of ion channels as suggested by others[18,20,21].

According to these results, the activity degree of the components can be examined by the chemical components of the plant extract and adding functional group to the isolated agent matter. The antimutagenic activities of extracts result from not only the capacity that can affect the enzymatic activation process but also having the characteristics that can collect the free radicals. The protective mechanism of various compounds that have structural differences each other can be multi-factorial.

Because of this reason, the mechanism of plant originated natural compounds which have antimutagenic effect on human being, can be demonstrated with *in vivo* studies. As related to these results with the detailed studies of plant originated compounds, it can be possible to change them into more useful products for human being.

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REFERENCES

1. T. Roncada, V.E.P. Vicentini and M.S. Mantovani, *Toxicol. In Vitro*, **18**, 617 (2004).
2. F.I. Abdulaev, L. Riveron-Negrete, H. Caballero-Orgeta, J.M. Hernandez, I. Perez-Lopez, R. Pereda-Miranda and J.J. Espinosa-Aguirre, *Toxicol. In Vitro*, **17**, 731 (2003).
3. V. Karekar, S. Joshi and S.L. Shinde, *Mutat. Res-Gen Tox. En.*, **468**, 183 (2000).
4. P.E. Hartman and D.M. Shankel, *Environ. Mol. Mutagen.*, **15**, 145 (1990).
5. E. Ipek, E. Searching Carvacrol's Mutagenic and Antimutagenic Activity with AMES and SCE. Master Thesis, Enstitu of Natural and Applied Science, Anadolu University, Eskisehir, Turkey (2003).
6. K. Mortelmans and E. Zeiger, *Mutat. Res.*, **455**, 29 (2000).
7. O. Hilliard, Flora of Southern Africa, Part 7 Inuleae, Fascicle 2 Gnaphaliinae. Government Printer, Pretoria, South Africa (1983).
8. P.G. Wilson, *Nuytsia*, **8**, 447 (1992c).
9. T. Mesfin Tadesse & Reilly, in eds.: D.J.H. Hind, C. Jeffrey and G.V. Pope, 17. A Contribution to Studies on *Helichrysum* (Compositae- Gnaphalieae)-A Revision of the Species of North-East tropical Africa. In: Advances in Compositae Systematics, Royal Botanic Gardens, Kew, pp. 379-450 (1995).
10. P.H. Davis, Flora of Turkey and the East Aegean Islands, University Press: Edinburg, Vol. 5 (1982).
11. L. Peyron and M. Roubaud, *Soap Perfum. Cosmet.*, **43**, 726 (1970).
12. M. Aslan, D.D. Orhan, N. Orhan, E. Sezik and E. Yesilada, *J. Ethnopharmacol.*, **109**, 54 (2007).
13. T.G. Dekker, T.G. Fourie, F.D. Snyckers and C.F. Vandersckyf, *S. Afr. J. Chem-S-Afr. T.*, **36**, 114 (1983).
14. B. Tepe, M. Sokmen, H.A. Akpulat and A. Sokmen, *Food Chem.*, **92**, 89 (2005).
15. A. Sokmen, B.M. Jones and M. Erturk, *J. Ethnopharmacol.*, **67**, 79 (1999).
16. D.M. Maron and B.N. Ames, *Mutat. Res.*, **113**, 173 (1983).
17. P.S. Negi, G.K. Jayaprakasha and B.S. Jena, *Food Chem.*, **80**, 393 (2003).
18. I.E. Ipek, H. Zeytinoglu, S. Okay, B.A. Tuylu, M. Kurkcuoglu and K.H.C. Baser, *Food Chem.*, **93**, 551 (2005).
19. K.A. Reid, J. Maes, A. Maes, J. van Staden, N. De Kimpe, D.A. Mulholland and L. Verschaeve, *J. Ethnopharmacol.*, **106**, 44 (2006).
20. A. Ultee, M.H.J. Bennik and R. Moezelaar, *Appl. Environ. Microbiol.*, **68**, 1561 (2002).
21. R. Aeschbach, J. Loliger, B.C. Scott, A. Murcia, J. Butler, B. Halliwell and O.I Aruoma, *Food Chem. Toxicol.*, **32**, 31 (1994).

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