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Full Length Research Paper

Mutation preventive and antigenotoxic potential of methanol extracts of two natural lichen

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We aimed to determine the mutagenic, antimutagenic and antigenotoxic effects of *Rhizoplaca chrysoleuca* and *Rhizoplaca melanophthalma*'s methanol extracts on the known mutagens against two different organisms using mitotic index (MI) and Ames-*Salmonella* assay systems. For the MI assay the genotoxic dose of NaN_3 was defined on *Zea mays* seeds and different dose of the lichen extract used as anti-mutagen (5, 10, 20, 40 µg/plate). Observed data showed that methanol extracts prevent the cytotoxic effect of NaN_3 partially. In addition, the antimutagenic activities of the methanol extracts were investigated against 9-AA in TA1537 and NaN_3 in TA1535 strains of *Salmonella typhimurium*. Extracts show antimutagenic effect against 9-AA-induced mutation in TA1537 strain at all tested concentrations. The inhibition rates ranged from 70.73 to 85.71% (*R. chrysoleuca*, 0.5 µg/plate - 5 µg/plate). The results show that these natural compounds have an ability to reduce or prevent the effects of these mutagenic substances.

Key words: Ames, *Salmonella,* antimutagenicity, genotoxicity, mitotic index, *Rhizoplaca chrysoleuca, Rhizoplaca melanophthalma.*

INTRODUCTION

Lichens which grow on rocky coasts, soil and plant cover exist from an association with a heterotrophic mycobiont as a fungus and an autotrophic photobiont as an alga or a cyanobacterium (Jeon et al., 2009; Lange et al., 1999). These mutualistic symbionts have variously characteristic properties different from their basic component. These properties enable lichens to be used in various areas. For example, some of them are used in the perfume and dye industry and they are also used for the removal of toxic metals from different substances like water, air etc. (Bingol et al., 2009; Ugur et al., 2003). Many of them have been used as a biomonitor to measure air pollution by detecting SO_2 and heavy and radioactive metals. The role of photoactive lichen substances in photosynthesis has been examined in environmental pollution (John, 1988; Speta, 1986).

In addition to this, there are some lichen metabolites which act as a safeguard against endogenic and exogenic agents which threaten genome. For the determination of the biological activities of these metabolites, there are various studies like antimicrobial, immunostimulating, antioxidative, antiulcerogenic, analgesic/antipyretic and anti-inflammatory (Halama and Haluwin 2004; Kumar and Müller, 1999; Huneck, 2001; Ingolfsdottir, 2002; Türk et al., 2003; Tay et al., 2004; Yilmaz et al., 2004; Behera et al., 2006; Choudhary et al., 2005). It was noted that these metabolites have many biological activities including antifungal, antiviral, antiprotozoal, antiproliferative, antibiotic, antitumor,

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allergenic, plant growth inhibitory, antiherbivore, and enzyme inhibitory too (Behera et al., 2005; Ingolfsdottir, 1997; Müller, 2001; Perry et al., 1999; Yamamoto et al., 1998; Wei et al., 2008; Gulluce et al., 2006; Huneck, 1999).

In addition, many metabolites_obtained from about 60 lichen species are present in different types of antimicrobial, anticancer, antiallergen, immunogical, and expectoral drugs (Kirmizigul et al., 2007). For example; protolichesterinic acid isolated from *Cetraria islandica* L. (Ach.) inhibited the growth of malignant cell lines (Ogmundsdottir et al., 1998). On the other hand, one of them, usnic acid, isolated from *Rhizoplaca* species has been used widely in the pharmaceutical and cosmetic industries because of its high antimicrobial activity (Cansaran et al., 2006). These naturally occurring compounds also act as inhibitors of mutagenesis cytotoxicity or environmental carcinogen protectors like polysaccharide CFP-2 from lichen reduced the viability of HL-60 and K562 cells due to apoptotic pathway and telomerase activity, suggesting their possible therapeutic potential against cancer (Zeytinoglu et al., 2008; Lin et al., 2003).

Therefore investigation of the antimutagenic properties of these substances becomes a very important strategy for prevention or treatment of various diseases related to mutagenesis. Based on this information, the purpose of this study was to determine the antimutagenic and antigenotoxic properties of methanol extracts of two *Rhizoplaca* species (*R. chrysoleuca, R. melanophthalma)* against known mutagens NaN_3 and 9-AA. The colourless one called NaN_3 is the gas-forming component in many car airbag systems and also used in airplane escape chutes. The other one Aminoacridine is an antiseptic and disinfectant. Although there are limited studies, we did not come across any study about antimutagenic features of these species or their substances.

MATERIALS AND METHODS

Chemicals

Direct acting mutagens Sodium azide (NaN_3) and 9-Aminoacridine (9-AA) were obtained from Sigma-Aldrich (St. Louis, USA) and Merck (Hohenbrunn, Germany) respectively. Other solvents and pure chemicals including magnesium sulphate ($MgSO_4$), sodium ammonium phosphate ($Na_2NH_2PO_4$), D-glucose, D-biotin, sodium chloride (NaCl), L-histidine HCl, sodium phosphate-dibasic (Na_2HPO_4), crystal violet, citric acid monohydrate, potassium phosphate-dibasic (K_2HPO_4), sodium phosphate-monobasic (NaH_2PO_4) were also obtained from Difco (New Jersey, USA), Fluka (Steinheim, Germany), Merck (Darmstadt, Germany) and Sigma (St. Louis, USA).

Lichen material

R. chrysoleuca and *R. melanophthalma* lichens used in the study were collected from the entrance of Kosk Village (1853 m) in Erzurum city in Turkey. The collection and identification of species was made by Dr. Ali ASLAN from Ataturk University Education Faculty of Kazim Karabekir Department of Biology Teacher Training. Herbarium samples of the materials (*R. chrysoleuca* - KKEF-703; *R. melanophthalma* - KKEF-704) have been deposited at the herbarium of Kazim Karabekir Education Faculty, Atatürk University, Erzurum - Turkey.

Extraction method

Dried lichen samples were ground into powder and then 10 g of the material was extracted by adding 200 mL of methanol in room temperate for four hours. For the extraction, Soxhlet extractor (Isopad, Heidelberg, Germany) was used. Then the extract was filtered with Whatman filter paper (no. 1) and then methanol was vaporized by rotary evaporator (Buchi Labortechnic AG, Flawil, Switzerland).

Test organisms

Salmonella strains

For the Ames assay TA1535 (ATCC® Number: 29629) and TA1537 (ATCC® Number: 29630) strains of *Salmonella typhimurium* were used. These strains were provided by The American Type Culture Collection - Bacteria Department of Georgetown University, Washington, USA.

Zea mays seeds

Zea mays seeds were used for Mitotic Index test obtained from the Department of Field Crops, Faculty of Agriculture, Ataturk University, Erzurum, Turkey.

Ames-*Salmonella* test

The bacterial mutagenicity and antimutagenicity assays were performed as described before (Mortelmans and Zeiger, 2000). The known mutagens NaN_3 (in distilled water - 1 µg/plate) for *S. typhimurium* TA1535 and 9-AA (in methanol - 10 µg/plate) for *S. typhimurium* TA1537 were used as positive controls and DMSO 10% was used as negative control in these studies.

In the mutagenicity test performed with TA1535 and TA1537 strains of *S. typhimurium*, 100 µl of the overnight bacterial culture, 50 µl test compounds at different concentrations (0.05, 0.5, 5 µg/plate in 10% DMSO), and 500 µl phosphate buffer were added to 2 ml of the top agar containing 0.5 mM histidine/biotin. The mixture was poured onto minimal glucose plates. Histidine independent revertant colonies and viable cells were scored on plates after incubation at 37°C for 48 or 72 h.

In the antimutagenicity test performed with the same strains, 100 µl of the overnight bacterial culture, 50 µl mutagen, 50 µl test compounds at different concentrations (0.05, 0.5, 5 µg/plate in 10% DMSO), and 500 µl phosphate buffer were added to 2 ml of the top agar containing 0.5 mM histidine/biotin. The mixture was poured onto minimal glucose plates. Histidine independent revertant colonies and viable cells were scored on plates after incubation at 37°C for 48 or 72 h.

The plate incorporation method was used to assess the results of mutagenicity and antimutagenicity assays (Maron and Ames, 1983).

For the mutagenicity assays, the mutagenic index was calculated for each concentration, which is the average number of revertants per plate divided by the average number of revertants per plate with the negative (solvent) control. A sample was considered mutagenic

Table 1. The mutagenicity assay results of methanol extracts on *S. tyhimurium* TA1535 and TA1537 strains.

Test ıtems	Concentration (µg/plate)	Number of revertants			
		S. typhimurium TA1535		*S. typhimurium* TA1537	
		Mean ± S.E.	Mutat. %	Mean ± S.E.	Mutat. %
NaN_3**	1	515.50 ± 02.90ö		504.00 ± 02.86ö	
9-AA**	40				
DMSO*** (µl/plate)	100	27.75 ± 00.95		36.50 ± 01.94	
R. chrysoleuca	0.05	22.25 ± 00.48ö	-	28.25 ± 01.11	-
	0.5	23.50 ± 00.87ö	-	31.00 ± 00.71	-
	5	21.75 ± 01.03ö	-	27.75 ± 00.63	-
R. melanophytalma	0.05	25.00 ± 01.08	-	27.25 ± 00.75	-
	0.5	22.75 ± 01.11	-	32.00 ± 01.29	-
	5	24.50 ± 00.87	-	31.00 ± 01.22	-

*p < 0,05. **NaN_3 and 9-AA were used as positive controls for S. *typhimurium* TA1535 and TA1537 strains, respectively. ***DMSO % 10 (Dimethyl sulfoxide) was used as negative control.

when they were observed a dose-response relationship and a two-fold increase in the number of mutants with at least one concentration was observed (Vargas et al., 1993; Varella et al., 2004; Evandri et al., 2005; Santos et al., 2008).

For the antimutagenicity assays, the inhibition of mutagenicity was calculated by using the following equation (M: number of revertants/plate induced by mutagen alone, S_0: number of spontaneous revertants, S_1: number of revertants/plate induced by the extract plus the mutagen):

$$\% \text{ Inhibition} = [(M-S_1)/(M-S_0)]\times 100$$

25 to 40% inhibition was defined as moderate antimutagenicity; 40% or more inhibition as strong antimutagenicity; and 25% or less inhibition as no antimutagenicity (Ikken et al., 1999; Negi et al., 2003; Evandri et al., 2005).

Mitotic index test

Equal size of *Zea mays* seeds were chosen and surface sterilized with 5% (w/v) NaOCl for 6 min. After sterilization, seeds were washed with double-distilled water and dried using sterile filter paper. Fifteen seeds were germinated in 15 cm diameter Petri dishes on two layers of sterile Whatman No. 1 filter paper. 400 µl of the NaN_3 was found as mutagen or genotoxic dose (no germination). This dose was added to each petri solution expected to control. Four different doses of the lichen extract were selected from dose determine test and used as antigenotoxic (5, 10, 20, 40 µg/plate).

The lichen extract and the mutagen were mixed with 7 ml distilled water and added to each plate. Only double distilled water was used for the control group. Two replicates were made for each concentration. The petri dishes were allowed to germinate at 23°C in the incubator (Binder, Tuttlingen, Germany) for 72 h. The root tips of germinated seeds were fixed in acetic acid-alcohol (1:3) for 24 h and then they were transferred in 70% alcohol and stored at +4°C in the fridge. For mitotic preparation, root tips were removed from the alcohol and washed with tap water (at least twice) and hydrolyzed in water bath with 1 N HCl, at 60°C for 15 min.

Then these were dyed with Feulgen reactive for 4h. Finally the last parts of root tips which had dyed very densely were cut and their crushed preparates in 45% acetic acid were made. MI was calculated as the ratio of the number of dividing cells to the total number of cells.

% MI = Total number of dividing cells / Total counted cells x 100

The results are expressed as means ± standard error. All statistical analyses were performed using SPSS 17.0 computer program (SPSS Inc.). The data were compared with ANOVA test using a significant level of $p < 0.01$ (Agar et al., 2005; Kiran and Sahin, 2005).

RESULTS

Mutagenic and antimutagenic effects of the lichen extracts on *S. Typhimurium* strains

In order to assess the mutagenic and antimutagenic effects of *Rhizoplaca* species, induction or suppression of revertant colonies were examined in *S. typhimurium* strains. The results of mutagenicity assay of Lichen extracts are presented in Table 1. In the bacterial mutagenicity tests, mutagenic activity of *R. chrysoleuca* and *R. melanophytalma* extracts were not seen on any strain (Table 1). The obtained data showed that both of the extracts did not show antimutagenic activity on NaN_3 applied TA1535, but all concentrations of these extracts showed significant antimutagenic activity on TA1537 except 5 µg/plate of *R. melanophytalma*. The inhibition rates of these extracts were between 70.73% (*R. chrysoleuca* - 0.5 µg/plate) to 85.71% (*R. chrysoleuca* - 5 µg/plate). The antimutagenic activities of methanol extracts were tested at three different concentrations

Table 2. The antimutagenicity assay results of methanol extracts on *S. tyhimurium* TA1535 and TA1537 strains.

Test items	Concentration (µg/plate)	Number of revertants			
		***S. typhimurium* TA1535**		***S. typhimurium*TA1535**	
		Mean ± S.E.	**Mean ± S.E.**	**Mean ± S.E.**	**Mean ± S.E.**
NaN_3**	1	515.50 ± 02.90ö		504.00 ± 02.86ö	
9-AA**	40				
DMSO*** (µl/plate)	100	27.75 ± 00.95		36.50 ± 01.94	
R. chrysoleuca	0.05	519.00 ± 03.16	-	130.25 ± 03.09	74.16*
	0.5	524.50 ± 04.57	-	147.50 ± 07.62	70.73*
	5	517.50 ± 01.55ö	-	72.00 ± 01.47	85.71*
R. melanophytalma	0.05	525.50 ± 02.40	-	102.00 ± 03.76	79.76*
	0.5	547.25 ± 07.74	-	96.50 ± 03.48	80.85*
	5	518.50 ± 02.66	-	410.50 ± 08.27	18.55

* $p < 0.05$. **NaN_3 and 9-AA were used as positive controls for S. *typhimurium* TA1535 and TA1537 strains, respectively. ***DMSO %10 (Dimethyl sulfoxide) was used as negative control.

Table 3. The antimutagenicity assay and MI results of methanol extracts on *Zea mays* root tips.

Test items	Concentration (µg/plate)	*Zea mays* Mitotic index ± S.E
NaN_3**	800	No Germination
Negative Control***	-	20.16 ± 0.79
R. chrysoleuca	5	11.61 ± 0.88*
	10	15.64 ± 0.47*
	20	15.82 ± 0.45*
	40	16.99 ± 0.65*
R. melanophytalma	5	13.22 ± 1.41*
	10	15.20 ± 0.92*
	20	15.47 ± 1.19*
	40	16.51 ± 1.13*

*p < 0,05.
**NaN_3 were used as positive controls.
***Distilled water was used as negative control.

(0.05, 0.5, 5 µg/plate).The results were evaluated by using standard plate incorporation method and summarized in Table 2.

Antigenotoxic effects of the lichen extracts on *Zea mays* root tips

Mutagenic dose of the NaN_3 established as 800 µg/plate. Dose levels above 800 µg/plate also show cytotoxic effect (no germination). However doses of lichen extracts above 40 µg have low applicability. But four tested doses of lichen extracts lower than the threshold dose did not have any effect compared with the control. So the antigenotoxic properties of the *R. chrysoleuca* and *R. melanophthalma* extracts were tested against mutagenic agents NaN_3 on *Zea mays* root tips. Germination was observed in all Petri. The lichen extracts exhibited anti-genotoxic activity against NaN_3 induced mutagenicity, in a slightly dose-dependent manner. The results are also presented in Table 3.

DISCUSSION

Although lichens produce an arsenal of unique biochemical compounds for an adaptation in marginal habitats, up to present there are limited investigations about their bioactivity such as their antimutagenic effects (Zeytinoglu et al., 2004).

The present study focused on assessing the effects of methanol extracts of *R. chrysoleuca and R. melanophytalma* against NaN_3 and 9-AA induced damage in different organisms. Applied mutagens were NaN_3 for TA1535 and *Z. mays* seeds, 9-AA for TA1537. 9-AA did not show any significant effect on *Zea mays*. According to data we observed, none of the extracts have toxic activity on *Zea mays* seeds or on *Salmonella* strains. Just in *Zea mays* seed the dose above 400 µl caused a stress on seeds briefly. But in the bacterial strains, there was no observation of any mutagenicity of performed extracts. In the bacterial assay system with *S. typhimurium* TA1535 and TA1537 strains and MI test with *Zea mays*, the antimutagenic activity of the lichen extracts against mutagens NaN_3 and 9-AA was observed.

When NaN_3 was applied to *Zea mays* seeds with lichen extracts, the division ratio came closer to the control

value depending on the increase of lichen extracts. However extracts did not show antimutagenic effect against TA1535, whereas NaN_3 is a mutagen and one of the most powerful mutagen in crop plants (Al-Qurainy and Khan 2009). In addition it is known to be highly mutagenic in several organisms, including micro-organisms and animals (Rines 1985; Veleminsky and Angilis, 1987; Owais and Kleinhofs, 1988). The mutagenicity is mediated through the production of an organic metabolite of azide compound (Owais and Kleinhofs, 1988). This metabolite enters into the nucleus, interacts with the DNA and creates point mutation in the genome. Previous studies clearly showed that the mutagenicity of this mutagen is mediated through the production of an organic metabolite of azide called L-azidoalanine (Owais and Kleinhofs, 1988; Kleinhofs et al., 1978). Presumably this promutagen NaN_3 must be metabolized by plant cells to the mutagenic agent. The production of this metabolite was found to be dependent on the enzyme O-acetylserine sulfhydrylase. This enzyme catalyses the condensation of azide with O-acetylserine to produce azidoalanine (Kredich 1971; La Velli and Mongolg, 1987). Azide anions are a potent inhibitor of the proton pump (Kleinhofs and Owais, 1978) and alter the mitochondrial membrane potential. These effects may hamper ATP biosynthesis resulting in decreased of ATP (molecules) which may slow the germination rate or reduce germination (percentage). So in *Zea mays* seedling, lichen extracts presumably bind to azide ions or blocking O-acetylserine sulfhydrylase enzyme and prevent the creation of L-azidoalanine. But in mutant TA 1535 strain of *Salmonella* the same activity can not obtained. This is clearly showed that the mechanism of NaN_3 which effect DNA is different in tested organisms.

On the other hand, the genotoxicity of acridines has been studied in phage, bacteria, fungi, insects, plants, cultured mammalian cells, and mammals (Ferguson and Denny, 1991). These mutagens known as intercalator agents, bind to DNA noncovalently by intercalation. Through intercalation, they induce frameshift mutations at hotspots in which a single base, especially guanine, is repeated (Neidle and Abraham, 1984; Ferguson and Denny, 1990). Present study suggested that both extracts obtained from *R. chrysoleuca and R. melanophytalma* have antimutagenic activity in TA1537 strain at all tested concentrations (0.05, 0.5, 5 µg/plate). These phytochemicals can decrease the rate of mutation by inactivating the mutagens or by inhibiting the metabolic activation of promutagens.

Conclusion

In summary, any chemical which is able to cause damage to the DNA is a reliable plant and bacterial assays, and should be considered as having the potential of damaging the DNA of other organisms in the environment. Nevertheless, if a compound has an ability to decrease chemical induced damage in any tested organisms, then this property may apply to damage in other organisms too. Therefore antimutagenic assays of these lichen species should be further performed for determination of their pharmaceutical and ecological properties.

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