Chapter 6
Antigenotoxic Effect of Some Lichen Metabolites

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Abstract Naturally occurring compounds can have protective effects towards mutagens and carcinogens as shown by numerous studies. Several lichen species have taken quite much the attention of researchers since their extracts and compounds have been used on traditional medicine to cure different diseases such as ulcer, arthritis, tuberculosis and cancer throughout the ages. Although a wide variety of scientific investigations on the biological activities of lichen extracts and their constituent have been performed, there are quite less research on their genotoxicity/antigenotoxic activity. Up to date, most results for genotoxic/antigenotoxic activities of lichens have been obtained for lichen extracts using the Ames/*Salmonella*/microsome, the *Escherichia coli* WP2 microsome, chromosome aberration, micronucleus, sister chromatid exchange and the single-cell gel electrophoresis assays. In the present chapter, findings on the antigenotoxic/genotoxic activities and its mechanisms will be evaluated. By using the most common bacterial and nonbacterial assays, extracts of various lichen species have been shown to have promising antigenotoxic activity with quite less genotoxic activity. Lichen extracts may have a possible therapeutic potential and therefore this must be further investigated by other multiple in vitro bioassays for the development of therapeutic agents.

Abbreviations

2-AF	2-Aminofluorene
4-NPD	4-Nitrophenylenediamin
8-oxo-dG	8-Oxo-2′-deoxyguanosine, 8-hydroxy-2′-deoxyguanosine
9-AA	9-Aminoacridine
AFB1	Aflatoxin B_1
BrdU	Bromodeoxyuridine
CA	Chromosome aberration
CBS	Colloidal bismuth subcitrate
COMET	Single-cell gel electrophoresis

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HPL	Human peripheral blood lymphocytes
IMA	Imazalil
MI	Mitotic index
MMC	Mitomycin C
MMS	Methyl methanesulfonate
MN	Micronucleus
MNNG	*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
SCE	Sister chromatid exchange
SCGE	Single-cell gel electrophoresis

6.1 Introduction

Naturally occurring organic compounds from a variety of organisms including medicinal plants can act as inhibitors of genotoxicity (Ipek et al. 2003, 2005; Jayaprakasha et al. 2007; Zeytinoglu et al. 2008; Kayraldız et al. 2010; Hoshina and Marin-Morales 2014). Investigation of biological activities of natural extracts or their fractions using a series of in vitro and in vivo bioassays is very important and becoming a popular area to develop new therapeutic agents. Numerous studies on the biological potential of several classes of natural agents, dietary constituents, hormones and vitamins have shown to act as genotoxicity inhibitors as well as cytostatic or environmental carcinogen protectors (Okai et al. 1996; Scarpato et al. 1998; Ingolfsdottir et al. 2000; Mersch-Sundermann et al. 2004). Also investigation of possible genotoxicity of such agents takes the attention of researchers because of their use in folk medicine or possible application potential. The most of medicinal plants used traditionally have never been subjected to toxicological tests such as that required for modern pharmaceutical compounds. However, research has shown that quite many plants which are used in traditional medicine or other area may have genotoxic or carcinogenic properties (Santos et al. 2009; Nieminen et al. 2002). Therefore, it becomes very important to search compounds or extracts derived from plants which contain a variety of compounds for their nontoxic, antigenotoxic or genotoxic properties.

Lichen species have taken quite much the attention of researchers since their extracts and compounds have been used in traditional medicine in Europe, Asia and Northern America (Richardson 1988; Cabrera 1996; Tilford 1997). Although extracts of lichens have been subjected to many scientific investigations for their several biological activities such as immunostimulating, analgesic, antiulcerogenic, antipyretic, antimicrobial, antioxidative and antitumour (Kumar and Müller 1999; Ingolfsdottir et al. 2000; Ingolfsdottir 2002; Türk et al. 2003; Tay et al. 2004; Yılmaz et al. 2004; Halici et al. 2005; Karunaratne et al. 2005; Behera et al. 2006; Zeytinoglu et al. 2008), there are quite less research on their genotoxic/antigenotoxic activity. Scientific investigation of antigenotoxic and genotoxic properties of lichens includes in vitro and in vivo studies, mostly using their extracts. Up to date, most results for genotoxicity/antigenotoxicity of lichens

come from using the aqueous, methanol, acetone or *n*-hexane extracts. In the present chapter, findings on the antigenotoxic/genotoxic activity of lichen extract or secondary metabolites and the mechanisms will be evaluated.

6.2 Bioassays for the Antigenotoxicity/Genotoxicity of Lichens

The Organization for Economic Co-operation and Development (OECD 2012) and the European Centre for the Validation of Alternative Methods (ECVAM 2012) have largely investigated the validation of mutagenicity tests. A set of assays are recommended to determine the genotoxicity of a test agent. The methods most frequently used for the assessment of genotoxic/antigenotoxic activity of lichen extracts or its components based on bacterial short-term assays and mammalian test system are recommended by the OECD and the ECVAM. The Ames/Salmonella/microsome (Ames) and the *Escherichia coli* WP2 tryptophan reverse mutation (WP2) assays are the most common bacterial systems, and MN, CA, SCE and COMET are the most common nonbacterial systems used up to date.

6.2.1 Bacterial Short-Term Assays

The Ames and the WP2 assays are short-term bacterial reverse mutation assays specifically designed to detect a wide range of chemicals or other agents which can produce genetic damage. The Ames employs several histidine-dependent *Salmonella* strains, each carrying different mutations in various genes in the histidine operon, pointing different mutagen acting mechanisms. The recommended combinations of *S. typhimurium* strains by OECD in the Ames test are given in Table 6.1 (reviewed by Mortelmans and Zeiger 2000). When the *Salmonella* strains carrying

Table 6.1 Genotype of the most commonly used *Salmonella* tester strains

Strains/allele	Mutation type	DNA target	Reversion event
TA97/hisD6610	Deletion	-C-C-C-C-C-C-	Frameshifts
TA98 TA1538/*hisD3052*	Deletion	-C-G-C-G-C-G-C-G-	Frameshifts
TA100 TA1535/*hisG46*	Deletion	-G-G-G-	Base pair substitution
TA102 TA104/hisG428	Wild type Deletion	TAA (ochre)	Transition/transversion
TA1537/hisC3076	Deletion	+1 frameshift (near -C-C-C-run of Cs)	Frameshifts

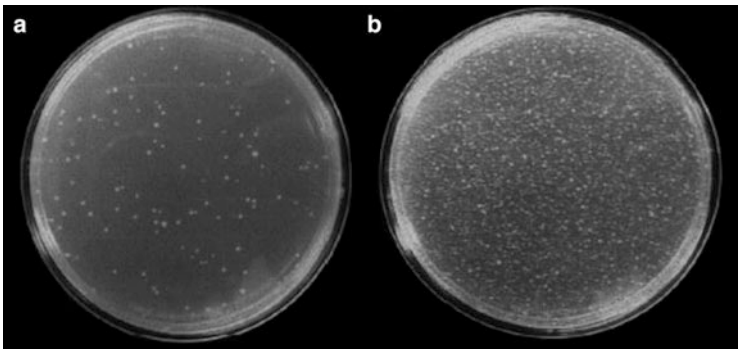

Fig. 6.1 Ames test plates of TA100 strain of *Salmonella typhimurium*. (**a**) Control: spontaneous revertants; (**b**) a mutagenic dose response to sodium azide (from Mortelmans and Zeiger 2000)

mutations in *his* gene are grown on a minimal media agar plate with a trace of histidine, only those bacteria that revert to histidine independence are able to form colonies (Fig. 6.1). When a mutagen is added to the plate, the number of revertant colonies per plate is increased (Maron and Ames 1983; Mortelmans and Zeiger 2000). Ames assay provides a very sensitive study of potentially mutagenic pathways for the metabolism of compounds in both the absence and the presence of a rat liver microsomal system (S9 mix).

Base pair substitution (A:T to G:C or G:C to A:T) and frameshift mutations (deletions) in *S. typhimurium* strains are represented to identify both types of mutation caused by a test compound. Therefore, differences in the activity of a test compound acting in these strains may yield some insight into how the compounds interact with the DNA of bacteria. Additionally, some genetic markers have been developed to make the strains more sensitive to certain types of mutagens.

The WP2 assay detects trp(−) to trp(+) reversion at a site blocking a step in the biosynthesis of tryptophan prior to the formation of anthranilic acid. The different auxotrophic WP2 strains all carry the same A:T base pair at the critical mutation site within the *trpE* gene. The most widely used *E. coli* WP2 strains, each carrying the *trpE* mutation, areWP2 (wild type for DNA repair), WP2 (pKM101), WP2 *uvrA*, WP2 *uvrA* (pKM101) and WP2 (pKM101) (Mortelmans and Riccio 2000). The assay is currently used by many researchers in conjunction with the Ames assay for screening chemicals for their mutagenicity. The Ames assay procedures are the same as for WP2 assay with the exception that limited histidine instead of limited tryptophan is used. International guidelines have been established for performing these mutagenicity assays. These assays are used worldwide as an initial screen to determine the mutagenic/antimutagenic potential of new chemicals, drugs or natural product from plants or animals.

Conversely, the antimutagenicity of a compound against a selected positive mutagen can be investigated when the two chemicals are co-administered to the bacteria in both test systems. Using known mutagenic compounds as “positive controls”, it is possible to study whether tested components can reduce DNA damage.

6.2.2 Nonbacterial Short-Term Assays

At present, several antigenotoxicity/genotoxicity assays which include the chromosome aberration (CA), micronucleus (MN), somatic mutation and recombination test (SMART), sister chromatid exchange (SCE) and the single-cell gel electrophoresis (SCGE) or COMET assays are available, and they are recommended to be used as a set for investigations.

According to literatures, the antigenotoxic/genotoxic potential of lichens has been evaluated, commonly MN, SCE, CA, COMET, 8-oxo-2-deoxyguanosine (8-oxo-dG) in mammalian cell and MI in plant cell assays. The purpose of the MN test is to examine the structural and numerical chromosomal damage which formed small membrane-bound DNA fragments or micronuclei in the cytoplasm of interphase cells caused by a tested agent or by clastogens and aneugens. Micronuclei can be formed by chromosome fragments lacking a centromere or whole chromosomes which are unable to migrate during cell division. The MN test can be conducted in the presence or in the absence of cytochalasin B, which is used to block cell division and generate binucleated cells (Fig. 6.2a). The cytokinesis-block micronucleus assay is a sensitive, comprehensive and simple methodology for measuring DNA damage, cytostasis and cytotoxicity which can be scored easily in a variety of systems, in vitro and in vivo (Fenech 2007; Kirsch-Volders et al. 2011). The assay is being applied successfully for biomonitoring of in vivo genotoxin exposure, for in vitro genotoxicity testing and in diverse research fields such as nutrigenomics and pharmacogenomics.

SCE assay is another short-term test and useful for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome in mammalian and also non-mammalian cells. Various cytomolecular protocols have been used to perform the SCE assay (Bakkali et al. 2008). SCEs result from the interchange of DNA replication products and involve DNA breakage and reunion (Wilson and Thompson 2007). Detection of SCEs requires the differential staining of sister chromatids, which can be achieved generally by the incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles (Fig. 6.2b). After labelling, treatment of cells with a spindle inhibitor such as colchicine is required to accumulate cells in a metaphase-like stage of mitosis (Perry and Evans 1975; Ipek et al. 2003).

The short-term in vitro mammalian cell chromosome aberration (CA) test measures the frequency of asymmetrical structural chromosome aberrations after exposure to test chemicals or mutagens. The in vitro chromosomal aberration test may employ cultures of established cell lines or primary cell cultures. Procedures involve the stimulation of generally human peripheral blood lymphocytes (HPL) by cyclophosphamide, to divide in whole blood cultures. Cells in metaphase are analysed for the presence of chromosomal aberrations (Fig. 6.2c) (Clare 2012).

The COMET assay is used to detect the DNA strand breaks in eukaryotic cells and named due to the shape of DNA distribution seen which bears resemblance to a celestial comet. This well-established, highly sensitive, rapid and simple

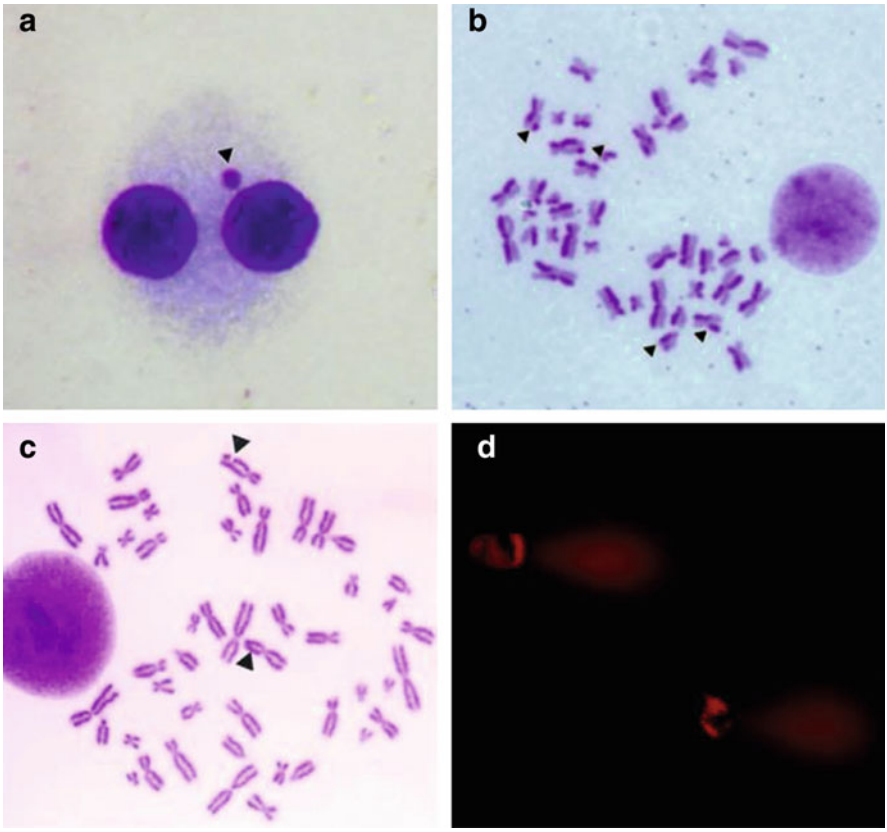

Fig. 6.2 Photomicrographs for some genotoxicity assays. (**a**) A mitogen-stimulated cytokinesis-block lymphocyte containing one MN; Giemsa staining of BrdU-incorporated chromosomes in human lymphocytes for SCE (**b**), *arrowheads* show chromosome breaks and sister union; and for CA (**c**) sister chromatids stained at different density (photograph kindly provided by Dr. B. Ayaz Tuylu). (**d**) COMET tails of chromosomes visualised by an epifluorescence microscope (photograph kindly provided by Dr. A. T. Koparal)

genotoxicity test is based on the lysing of cells embedded in agarose on a microscope slide to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Then electrophoresis at high pH results in structures resembling comets, observed by epifluorescence microscopy (Fig. 6.2d). The intensity of the comet tail relative to the head reflects the number of DNA breaks (Singh et al. 1988; Collins 2004; Speit et al. 2009). Depending on experimental conditions, the migrating DNA reflects the amount of single- or double-strand breaks, alkali-labile sites, including incomplete excision repair sites, but also DNA–DNA and DNA–protein cross-links (Santos et al. 2009; Verschaeve et al. 2010). A broad spectrum of DNA damage can then be detected either by visual classification of comet morphologies or from morphological parameters obtained by image analysis.

8-Oxo-2′-deoxyguanosine (8-oxo-dG) is a frequently used biomarker of oxidative DNA damage caused by free radicals and other reactive species constantly generated in vivo. Later, 8-oxo-dG is removed from DNA by the base excision repair pathway and subsequently transported into body fluids such as saliva, urine and plasma. Such oxidative damage to DNA is probably the contributor of the age-related development of diseases such as cancer. Agents that decrease oxidative DNA damage should thus decrease the risk of cancer development. Thus, the measurement of 8-oxo-dG is the commonest method of assessing DNA damage (Halliwell 2000; Türkez et al. 2012a). An assay for the measurement of 8-oxo-dG has been developed by using a monoclonal antibody specific to 8-oxo-dG (N45.1), and an ELISA (The enzyme-linked immunosorbent assay) has been well established (Toyokuni et al. 1997).

The mitotic index (MI) as a parameter for the evaluation of cytotoxic agents is the ratio of the number of cells, in a cell population, undergoing mitosis to the number of cells not undergoing mitosis. Mutagens can be detected cytologically by cellular inhibition, disruption in metaphase, induction of chromosomal aberrations and chromosomal fragmentation and disorganisation of the mitotic spindle and consequently of all subsequent dependent mitotic phases. MI is used as an indicator of adequate cell proliferation which can be measured by various plant test systems. Cytotoxicity tests, using plant test systems in vivo, such as *Allium cepa* and *Zea mays*, are validated by several researchers, who jointly performed with other organisms testing for genotoxicity (Agar et al. 2010; Gökbayrak and Sivas 2011; Aslan et al. 2012b).

6.3 Antigenotoxic/Genotoxic Potential of Lichen Extracts

Several researches have been performed on the antigenotoxicity/genotoxicity of lichens in just about the last 10 years. The studies up to date are summarised in two separate tables according to the activity assays. In Table 6.2, lichen species tested for only their antigenotoxicity or both genotoxicity and antigenotoxicity were listed. The lichen species which were tested for only their genotoxicity were listed in Table 6.3.

As indicated in Table 6.3, the earliest research for the genotoxicity of lichens has been performed using Ames mutagenicity assay for the secondary metabolites of *Hypogymnia enteromorpha* (Ach.) Nyl. by Shibamoto and Wei (1984). Then, the first report describing the therapeutic potential of lichens against drug genotoxicity was from Geyikoglu et al. (2007) (Table 6.2). Aqueous extracts of four common lichen species collected from Giresun Province in Turkey, *Dermatocarpon intestiniforme*, *Pseudevernia furfuracea*, *Parmelia pulla*, *Ramalina capitata* and *Rhizoplaca melanophthalma*, were tested for their genotoxic and antigenotoxic potentials. *Dermatocarpon intestiniforme*, *Pseudevernia furfuracea*, *Parmelia pulla* and *Ramalina capitata* were found to be antigenotoxic at 5–10 μg/ml concentration against colloidal bismuth subcitrate (CBS)-induced SCE and MN formation in human peripheral lymphocytes (HPL) in vitro. However, one other species *Rhizoplaca melanophthalma* was not antigenotoxic. The order of

Table 6.2 Lichen species tested for only their antigenotoxicity and genotoxicity

Species/extract	Against	Assay	Cell types	Genotoxic	Anti-genotoxic	References
Cetraria aculeatea/ aqueous	4-NPD 2-AF NaAz	Ames	TA98 TA100	No	Yes	Zeytinoglu et al. (2008)
	MMC	MN	HPL	No	No	
Cetraria islandica/ methanol	AFB1	SCE MN	HPL	NP	Yes	Kotan et al. (2011)
	9-AA NaN_3	Ames	TA1535 TA1537	No	Yes	Aslan et al. (2012b)
		MI	*Zea mays*			
Cladonia foliacea/ methanol		Ames	TA1535 TA1537	No	Yes	Anar et al. (2013)
		WP2	*E. coli*			
	AFB1	SCE	HPL	NP	Yes	
Cladonia rangiformis/ methanol	AFB1	SCE MN	HPL	No	Yes	Kotan et al. (2013)
Dermotocarpon intestiniforme/ aqueous	CBS	SCE MN	HPL	No	Yes	Geyikoglu et al. (2007)
	$CdCl_2$	MN	HPL	No	Yes	Guner et al. (2012)
	$HgCl_2$	SCE MN	HPL	No	Yes	Türkez and Dirican (2012)
	Imazalil	CA MN	HPL	No	Yes	Türkez et al. (2012b)
Evernia prunastri/ methanol	NNNG	Ames	TA1535 TA1537	NP	Yes	Alpsoy et al. (2013)
	Acridin	WP2	*E. coli*			
	AFB1	SCE	HPL			
Lecanora muralis/ methanol	AFB1	SCE MN	HPL	NP	Yes	Alpsoy et al. (2011)
Parmelia pulla/ aqueous	CBS	SCE MN	HPL	No	Yes	Geyikoglu et al. (2007)
Peltigera rufescens (Weis) Humb./aqueous	Imazalil	CA MN	HPL	No	Yes	Türkez et al. (2012b)
Peltigera canica/ methanol	9-AA	Ames	TA1535 TA1537	No	Yes	Gormez et al. (2013)
		WP2	*E. coli*			
Pseudevernia furfuracea/ aqueous	CBS	SCE MN	HPL	No	Yes	Geyikoglu et al. (2007)
Pseudevernia furfuracea/methanol, acetone, hexane	AFB1	SCE MN	HPL	No	Yes	Türkez et al. (2010)

(continued)

Table 6.2 (continued)

Species/extract	Against	Assay	Cell types	Genotoxic	Anti-genotoxic	References
Pseudevernia furfuracea/ methanol	9-AA NaN3	Ames	TA1535 TA1537	NP	Yes	Aslan et al. (2012b)
		MI	*Zea mays*			
Ramalina capitata/aqueous	CBS	SCE MN	HPL	No	Yes	Geyikoglu et al. (2007)
Rhizoplaca melanophthalma/ aqueous	CBS	SCE MN	HPL	No	No	Geyikoglu et al. (2007)
	NaN(3)	MI	*Zea mays*	NP	Yes	Agar et al. (2010)
	9-AA	Ames	TA1537			
Rhizoplaca chrysoleuca/ methanol	AFB1	SCE MN	HPL	NP	Yes	Alpsoy et al. (2011)
	NaN(3)	MI	*Zea mays*	NP	Yes	Agar et al. (2010)
	9-AA	Ames	TA1537			
Usnea longissima/ methanol	AFB1	SCE, MN	HPL	NP	Yes	(Agar et al. 2011)
Umbilicaria vellea/methanol	AFB1	SCE, MN	HPL	NP	Yes	Aslan et al. (2012a)
Xanthoria elegans/aqueous	MMC	CA, MN SCE 8-oxo-dG	HPL	No	Yes	Aydin and Türkez (2011b), Türkez et al. (2012a)
Xanthoparmelia somloensis/ methanol	AFB1	SCE MN	HPL	NP	Yes	Aslan et al. (2012a)
Secondary metabolite						
Usnic acid	MMS	COMET	V79 cells	Yes	Yes	Leandro et al. (2013)
		MN		No		

CA Chromosome aberration, *MN* Micronucleus, *NP* Not performed, *HPL* Human peripheral blood lymphocytes, *CBS* Colloidal bismuth subcitrate, *MNNG N*-methyl-*N*′-nitro-*N*-nitrosoguanidine

antigenotoxicity efficacy against CBS was *Pseudevernia furfuracea*, *Dermatocarpon intestiniforme*, *Ramalina capitata* and *Parmelia pulla*. On the other hand, all lichen extracts tested were not genotoxic alone (Table 6.2).

After this work, fresh aqueous extract of *Cetraria aculeata* (Schreb.) Fr. which is one of the common species in Turkey was studied for its genotoxic/antigenotoxic activities in both Ames and mammalian cell systems (Zeytinoglu et al. 2008). The extract (at 0.1–500 μg/ml) exhibited strong antigenotoxic activity against three known mutagenic agents, 4-nitrophenylenediamin (4-NPD), 2-aminofluorene (2-AF) and sodium azide (NaN_3) in TA98 and TA100 strains of *Salmonella typhimurium* in the presence and absence of metabolic activation, without any

Table 6.3 Lichen species tested for only their genotoxicity

Species/extract/secondary metabolite	Assay	Cell types	Genotoxic	References
Aspicilia calcerea/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011a)
Bryoria capillaris/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011b)
Cetraria chlorophylla/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011a)
Hypogymnia physodes/ methanol	CA, MN	HPL	Yes	Ari et al. (2012)
Hypogymnia physodes/aqueous	CA, MN	HPL	No	Türkez et al. (2012c)
Peltigera rufescens/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011b)
Physcia aipolia/aqueous	CA, MN	HPL	No	Aydin and Türkez (2011a)
Ramalina polymorpha/aqueous	CA, MN	HPL	No	Türkez et al. (2012c)
Usnea florida/aqueous	CA, MN	HPL	No	
Secondary metabolite				
Physodic acid (from *Hypogymnia enteromorph*)	Ames	TA100	No	Shibamoto and Wei (1984)
Physodalic acid (from *Hypogymnia enteromorph*)			Yes	
Usnic acid	MNPCEs	Mouse PCEs	Yes	Al-Bekairi et al. (1991)
	MN	HPL	No	Koparal et al. (2006)
	CA, MN	HPL	No	Polat et al. (2013)

CA Chromosome aberration, *MN* Micronucleus, *HPL* Human peripheral blood lymphocytes, *PCEs* Polychromatic erythrocytes, *MNPCEs* Micronucleated PCEs

mutagenic activity (Table 6.1). Preincubation of bacteria with the extract prevented the mutagenic activity of 4-NPD in the higher range in both strains grown without metabolic activation than those grown with metabolic activation. It was suggested that the antigenotoxic potential of the extract was higher in the absence of metabolic system and in inhibiting frameshift mutations. Results indicate a direct and specific activation of the extracts. However, in a further investigation, the extract of *Cetraria aculeata* (Schreb.) Fr. does not have antigenotoxic activity against mitomycin C (MMC) in terms of MN formation in HPL. The extract was not also genotoxic alone in the mammalian system. According to the overall results, the extract of *C. aculeata* is significantly antigenotoxic in the bacterial system, whereas it is not capable of inhibiting MN formation in MMC-induced human peripheral blood cells, and that is pointing at different effects in two bioassay systems.

Recently, more investigations have been performed with an aqueous extract of *Dermatocarpon intestiniforme* in cultured HPL (Table 6.2). The extract at 25 and 50 ppm concentration conferred protection against cadmium chloride ($CdCl_2$)

(30 ppm)-induced MN formation despite its non-genotoxicity in the cells (Guner et al. 2012). It was also revealed that the SCE and MN rates induced by mercury chloride ($HgCl_2$) were alleviated in the cells treated with 50 μg/ml of the extract (Türkez and Dirican 2012). The extract was also antigenotoxic against imazalil (IMA)-induced CA and MN formation in cultured HPL. The lymphocytes were treated in vitro with varying concentrations of the lichen extract (25, 50 and 100 μg/ml) and tested in combination with imazalil (336 μg/ml). The extract alone was not genotoxic, and when combined with IMA treatment, it reduced the frequency of CAs and the rate of MNs (Türkez et al. 2012b). According to the overall results of MN, CA and SCE assays performed, the extract of *Dermatocarpon intestiniforme* is quite antigenotoxic against different types of clastogens or aneugens which cause the structural and numerical chromosomal damage.

One other aqueous extract of lichen species Peltigera rufescens and *Xanthoria elegans* (25, 50 and 100 μg/ml) has been assessed by four genotoxicity end points including CA, MN, SCE and 8-oxo-dG assays in HPL (Türkez et al. 2012a, d). Imazalil- and MMC-induced frequencies of four genotoxic indices were diminished by the extract, indicating its inhibitory effect on oxidative DNA damage of reactive agents beside the structural and numerical chromosomal damages. The extract and its secondary metabolites may have a potential to decrease the risk of cancer development.

The antimutagenic and antigenotoxic effects of methanol extracts of *Rhizoplaca chrysoleuca* and *Rhizoplaca melanophthalma* against known mutagens have been evaluated in two different organisms as a plant and bacteria using different assays (Agar et al. 2010). Extracts (5–40 μg/plate) prevented NaN_3-induced mitotic index partially in *Zea mays* seeds. Furthermore, they were antimutagenic against 9-aminoacridine (9-AA)-induced mutation in TA1537 strain at all tested concentrations (0.5–5 μg/plate) in Ames test. The inhibition rates ranged from 70.73 to 85.71 %.

Several investigators have been focused on the possible antigenotoxic potential of lichens against a well-known mutagen aflatoxin B_1 (AFB1). Türkez et al. (2010) reported the antigenotoxic activity of another lichen species *Pseudevernia furfuracea* using its three diverse extracts as methanol, acetone and *n*-hexane. All the lichen extracts did not induce a significant number of SCEs and MN in cytokinesis-blocked HPL. Moreover, their results indicated that AFB1-induced SCEs were inhibited by the application of 50 μM methanol or acetone extracts. The positive effect of methanol, acetone and ether extracts in decreasing the incidence of MN in comparison with an unprotected level was attained when cultures were treated simultaneously with AFB1 and the extracts. Agar et al. (2011) reported that methanol extracts obtained from *Usnea longissima* suppress the mutagenic effects of AFB1 in HPL examined by the SCE and MN tests. Kotan et al. (2011, 2013) also found that AFB1-induced genotoxicity has been suppressed by the methanol extract of another lichen species *Cetraria islandica* and *Cladonia rangiformis*. The results showed that the frequencies of SCE and MN level decreased when 5 and 10 mg/ml concentrations of the extract were added to AFB1-treated cultures. The methanol extracts of *Rhizoplaca chrysoleuca* and

Lecanora muralis, 5 and 10 μg/ml (Alpsoy et al. 2011), and *Umbilicaria vellea* and *Xanthoparmelia somloensis* (Aslan et al. 2012a) were antigenotoxic against AFB1-induced SCE and MN formation in HPL in vitro.

The methanol extract of *Evernia prunastri* (Huds.) Willd. was a strong antimutagenic on TA1537 and WP2 strains of *E. coli* with 37.70 % and 69.70 % inhibition rates against *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and acridine-induced mutagenicity, respectively. Co-treatments of HPL with the extract and AFB1 decreased the frequencies of SCE (Alpsoy et al. 2013).

The genotoxic and antigenotoxic effects of methanol extract of *Cladonia foliacea* (Huds.) Willd. were studied using WP2, Ames (TA1535 and TA1537) and SCE test systems by Anar et al. (2013). According to their results, 5 μM concentration of AFB1 changed the frequencies of SCE. When 5 and 10 μg/ml concentrations of extract were added to AFB1, the frequencies of SCE were decreased. On the other hand, the extract was not mutagenic in Ames (*Salmonella typhimurium* TA1535, TA1537) and *Escherichia coli* WP2 test systems, while it has antimutagenic activity.

Pseudevernia furfuracea and *Cetraria islandica* were tested using their methanol extracts for both their genotoxic and antigenotoxic activities. The extracts of two species were not mutagenic in Ames and *Zea mays* mitotic index test systems. Furthermore, some extracts showed significant antimutagenic activity against 9-AA in Ames test. Inhibition rates for 9-AA mutagenicity ranged from 25.51 % (*Pseudevernia furfuracea*, 0.05 μg/plate) to 66.14 % (*Cetraria islandica*, 0.05 μg/plate). In addition, all of the extracts were significantly antimutagenic against NaN_3, increasing the MI values of *Zea mays* (Aslan et al. 2012b). Gormez et al. (2013) showed that the methanol extract of *Peltigera canina* posses an antigenotoxic potential in Ames and WP2 tests.

Another eight lichen species collected from Erzurum and Artvin provinces in Turkey, *Aspicilia calcarea*, *Bryoria capillaris*, *Cetraria chlorophylla*, *Hypnogymnia physodes*, *Peltigera rufescens*, *Physcia aipolia*, *Ramalina polymorpha* and *Usnea florida*, have been tested for only their genotoxicity of the water extracts in cultured HPL as given in Table 6.3 (Aydin and Türkez 2011a, b; Türkez et al. 2012c). All tested lichen extracts up to 500 or 1,000 mg/l concentration had no genotoxic effects on the cell by the application of CA and MN assays, however exhibiting antioxidant properties. The methanol extract of *Hypogymnia physodes* (L.) Nyl. was studied for its genotoxicity using CA and MN tests in HPL culture. Relatively higher concentrations are required for its genotoxic activity (Ari et al. 2012).

6.4 Antigenotoxic/Genotoxic Potential of Lichen Secondary Metabolites

Lichen secondary metabolites exert various biological actions such as antitumour, antimicrobial, anti-inflammatory, apoptotic and cytotoxic activities (Ingolfsdottir et al. 1997; Vijayakumar et al. 2000; Huneck 2001; Tay et al. 2004; Yılmaz

et al. 2004; Mayer et al. 2005; Einarsdottir et al. 2010; Mitrovic et al. 2011; Molnar and Farkas 2010). Usnic acid is one of the most abundant lichen secondary metabolites studied for its biological activities as given above. It has been used widely in the pharmaceutical and cosmetic industry, due to its high antimicrobial activity (Ingolfsdottir 2002). Furthermore, usnic acid exhibited antiproliferative effect on human leukaemia cell (K562) and endometrial carcinoma (Ishikawa, HEC-50) cells (Carderelli et al. 1997; Kristmundsdottir et al. 2002).

A few findings present about the genotoxic/antigenotoxic activities of lichen secondary metabolites (Karunaratne et al. 2005). The earliest genotoxicity reports for the secondary metabolites of lichens come from Shibamoto and Wei (1984). They have tested usnic acid, physodic acid and physodalic acids isolated from *Hypogymnia enteromorph* (Ach.) Nyl. for their mutagenicity in the Ames assay (Table 6.3). Among them only physodalic acid exhibited significant mutagenicity against *Salmonella typhimurium* strain TA 100 with or without S9 mix in both plate-incorporation and preincubation assays. (+) -Usnic acid and (−) usnic acid isolated from *Ramalina farinacea* and *Cladonia foliacea*, respectively, have been found to be non-genotoxic due to the absence of MN induction in HPL (Koparal et al. 2006).

Recently, the genotoxic and antigenotoxic potentials of (+) usnic acid against methyl methanesulfonate (MMS)-induced chromosomal and genome damage have been evaluated in mammalian cells in vitro and in vivo (Leandro et al. 2013). Usnic acid alone induced DNA damage at concentrations of 60 and 120 g/ml determined by the COMET assay. However, it has not induced MN formation in V79 cells at the concentrations tested, and not any genotoxic effects were observed in vivo. The combined administration of usnic acid and MMS significantly reduced the frequencies of MN and DNA damage in vitro and in vivo when compared to treatment with MMS alone (Table 6.2). Polat et al. (2013) also showed the nonmutagenicity of usnic acid by two assays as CA and MN. Mice were treated orally with aqueous suspensions of (+) usnic acid in a single dose of either 100 or 200 mg/kg. The slight increase in the micronucleated polychromatic erythrocytes (MNPCEs) without affecting DNA synthesis was reported, and an effect of usnic acid on spindle apparatus was suggested (Al-Bekairi et al. 1991) (Table 6.3).

Usnic acid triggered the oxidative stress and disruption of the normal metabolic processes of breast cancer cell line MCF7 and lung cancer cell line H1299 (null for p53); however, it was not involved in DNA damage. It was suggested that the property of usnic acid as a non-genotoxic anticancer agent that works in a p53-independent manner makes it a potential candidate for novel cancer therapy (Mayer et al. 2005).

6.5 Conclusion

The methods most frequently used for the assessment of genotoxic and antigenotoxic activities of lichen extracts and products in vitro and in vivo are described above. These methods are not meant to be comprehensive of all existing

methods, but more must be in consideration for further investigation of the genotoxicity for their safety assessment or antigenotoxicity of especially secondary metabolite alone or in combination for their synergistic activities. Positive results of an in vitro/in vivo test indicate that the tested substance is genotoxic or antigenotoxic, and negative results indicate that the test substance is not genotoxic under the conditions of the assay performed. Genotoxicity and antigenotoxicity of lichens have appeared to be evaluated using several types of assays by detecting direct or indirect base substitution and frameshift mutagenicity (Ames and WP2), clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to dysfunction of mitotic apparatus) (MN), numerical and structural DNA damage (CA) and DNA strand breaks (COMET).

Accumulating data from the short-term in vitro and in vivo studies showed that lichen extracts could possess antigenotoxic effects. There are a small number of results for extracts which do not have antigenotoxic effects. Generally used tests for this purpose were common bacterial tests as Ames and WP2 and human lymphocytes tests as MN and SCE. However, there is a gap in the data about the lichen genotoxicity/antigenotoxicity since some group studied only mutagenicity, others antigenotoxicity without genotoxicity. Most findings are extremely promising that lichens may have therapeutic potential at least for cancer because of their antigenotoxic activities without genotoxic activity. The extracts of nine species of lichens out of 16 species tested, *C. aculeata*, *C. islandica*, *C. foliacea*, *D. intestiniforme*, *P. pulla*, *P. canica*, *P. furfuracea*, *R. capitata* and *X. elegans*, have antigenotoxic activities, but they are not genotoxic (Table 6.1). The extracts of seven species as *C. islandica*, *E. prunastri*, *L. muralis*, *R. chrysoleuca*, *U. longissima*, *U. vellea* and *X. somloensis* are antigenotoxic, but not tested for their genotoxic activities. On the other side, the extracts of *C. aculeata* and *R. melanophthalma* are neither genotoxic nor antigenotoxic for the human peripheral blood lymphocytes. The extracts of other six lichen species tested are not also genotoxic except for *H. physodes* (Table 6.2).

There are minor evidences about the genotoxic and antigenotoxic activities of the secondary metabolites of lichens. Interestingly, usnic acid shows variation in its effects since it is either genotoxic or antigenotoxic according to the results of COMET assay, but not genotoxic according to MN assay; however, it is genotoxic in vivo. Although physodic acid is nonmutagenic, physodalic acid is mutagenic in the same assay system.

Also variation in the effective doses of the extract on different cells or test systems suggests the necessity of more in vitro and in vivo antigenotoxicity studies to know the exact potential of the extract, and then it may find an application for treatments. Further investigation to complete the gap and more data for other lichen species will be so useful for their possible therapeutic application.

The mechanisms of antigenotoxic action of all these lichen extracts are not completely known but appear to be due to antioxidative potentials of their secondary metabolites as described in Chap. 1. Because, most of the extracts have been investigated for their antigenotoxicity and antioxidant activities, also indicated quite strong antioxidative activity (Türkez et al. 2010; Aydin and Türkez 2011a, b; Kotan et al. 2011; Polat et al. 2013). The chemopreventive potential of

several lichen extracts or secondary metabolites against DNA damage induced by known compounds such as AFB1, MMS and CBS, strongly indicates that lichens can be a resource of new therapeutics.

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