Taylor & Francis
Taylor & Francis Group

Journal of Toxicology and Environmental Health, Part A
Current Issues

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: www.tandfonline.com/journals/uteh20

Usnic acid attenuates genomic instability in Chinese hamster ovary (CHO) cells as well as chemical-induced preneoplastic lesions in rat colon

Nayane Moreira Machado, Arthur Barcelos Ribeiro, Heloiza Diniz Nicolella, Saulo Duarte Ozelin, Lucas Henrique Domingos Da Silva, Ana Paula Prado Guissone, Francisco Rinaldi-Neto, Igor Lizo Limonti Lemos, Ricardo Andrade Furtado, Wilson Roberto Cunha, Alexandre Azenha Alves De Rezende, Mário Antônio Spanó & Denise Crispim Tavares

To cite this article: Nayane Moreira Machado, Arthur Barcelos Ribeiro, Heloiza Diniz Nicolella, Saulo Duarte Ozelin, Lucas Henrique Domingos Da Silva, Ana Paula Prado Guissone, Francisco Rinaldi-Neto, Igor Lizo Limonti Lemos, Ricardo Andrade Furtado, Wilson Roberto Cunha, Alexandre Azenha Alves De Rezende, Mário Antônio Spanó & Denise Crispim Tavares (2019) Usnic acid attenuates genomic instability in Chinese hamster ovary (CHO) cells as well as chemical-induced preneoplastic lesions in rat colon, Journal of Toxicology and Environmental Health, Part A, 82:6, 401-410, DOI: 10.1080/15287394.2019.1613274

To link to this article: https://doi.org/10.1080/15287394.2019.1613274

JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH, PART A
2019, VOL. 82, NO. 6, 401–410
https://doi.org/10.1080/15287394.2019.1613274

Taylor & Francis
Taylor & Francis Group

Usnic acid attenuates genomic instability in Chinese hamster ovary (CHO) cells as well as chemical-induced preneoplastic lesions in rat colon

Nayane Moreira Machado[a], Arthur Barcelos Ribeiro[b], Heloiza Diniz Nicolella[b], Saulo Duarte Ozelin[b], Lucas Henrique Domingos Da Silva[b], Ana Paula Prado Guissone[b], Francisco Rinaldi-Neto[b], Igor Lizo Limonti Lemos[b], Ricardo Andrade Furtado[b], Wilson Roberto Cunha[b], Alexandre Azenha Alves De Rezende[a,c], Mário Antônio Spanó[a], and Denise Crispim Tavares [b]

[a]Institute of Biotechnology, Federal University of Uberlândia, Uberlândia, MG, Brazil; [b]University of Franca, Franca, SP, Brazil; [c]Faculty of Integrated Sciences of Pontal, Federal University of Uberlândia, Ituiutaba, MG, Brazil

ABSTRACT

Usnic acid (UA) is one of the pharmacologically most important compounds produced by several lichen species. To better understand the mechanism of action (MOA) of this important substance, this study examined the genotoxicity attributed to UA and its influence on mutagens with varying MOA using the micronucleus (MN) test in Chinese hamster ovary cells (CHO). Additional experiments were conducted to investigate the effect of UA on colon carcinogenesis in Wistar rats employing the aberrant crypt focus (ACF) assay. *In vitro* studies showed a significant increase in the frequency of MN in cultures treated with the highest UA concentration tested (87.13 µM). In contrast, UA concentrations of 10.89, 21.78, or 43.56 µM produced an approximate 60% reduction in chromosomal damage induced by doxorubicin, hydrogen peroxide, and etoposide, indicating an antigenotoxic effect. In the ACF assay, male Wistar rats treated with different UA doses (3.125, 12.5, or 50 mg/kg b.w.) and with the carcinogen 1,2-dimethylhydrazine exhibited a significantly lower incidence of neoplastic lesions in the colon than animals treated only with the carcinogen. Data suggest that the MOA responsible for the chemopreventive effect of UA may be related to interaction with DNA topoisomerase II and/or the antioxidant potential of the compound.

KEYWORDS

(+)-Usnic acid; genotoxicity; antigenotoxicity; anticarcinogenic effect; chemoprevention

Introduction

Multiple and continuous exposure of DNA to environmental factors such as mutagenic or genotoxic agents, as well as to endogenous factors such as sustained oxidative stress and chronic inflammatory processes, might trigger the process of carcinogenesis (Acésio et al. 2017; Dylawerska et al. 2017; Korniluk et al. 2017; Langie et al. 2015; Saha et al. 2017; Tuttis et al. 2018). In view of the limitations and difficulties in early cancer detection, as well as the adverse effects of available treatments, studies conducted in recent decades focused on development of methods designed to prevent the onset of neoplasia (Benetou, Lagiou, and Lagiou 2015). Natural products are of particular interest as chemopreventive agents because of low toxicity and potential efficacy profiles (Gontijo et al. 2018; Greenlee 2012; Kotecha, Takami, and Espinoza 2016; Rabi and Bishayee 2009; Tuttis et al. 2018).

Among the large number of unique secondary metabolites synthesized by lichens, usnic acid (UA) is one of the pharmacologically most important compounds produced by species of the genera *Alectoria, Cladonia, Evernia, Flavocetraria, Lecanora, Ramalina*, and *Usnea* (Chen et al. 2017; Cocchietto et al. 2002; Prokopiev et al. 2017). Pure UA has been used in creams, toothpastes, mouthwashes, deodorants, and sunscreen products as an active ingredient or as a preservative (Ingolfsdottir 2002). Several lichen species contain (+) and (–) optically active forms of UA that differ in the position of the methyl group attached to carbon 12. The enantiomers of UA exhibit a broad spectrum of biological properties, including antiangiogenic, anti-inflammatory, antimicrobial, antioxidant, antiproliferative, antitumor, and cytotoxic activities against cancer cell lines (Chen et al. 2014; Fernández-Moriano et al. 2017; Galanty et al. 2017; Ingolfsdottir 2002; Koparal 2015; Mayer et al. 2005;

CONTACT Denise Crispim Tavares denisecrispim2001@yahoo.com University of Franca, Avenida Dr. Armando Salles Oliveira 201, Franca, SP 14404-600, Brazil; Mário Antônio Spanó maspano@ufu.br Institute of Biotechnology, Federal University of Uberlândia, Avenida Pará, 172 - Campus Umuarama, Uberlândia, MG 38400-902, Brazil

Nguyen et al. 2014; Ribeiro-Costa et al. 2004; Silva et al. 2017; Su et al. 2017; Suwalsky et al. 2015; Thadhani and Karunaratne 2017; Yu et al. 2016).

Previously Leandro et al. (2013) observed that UA significantly reduced the frequencies of micronuclei (MN) and DNA damage induced by the mutagen methyl methanesulfonate (MMS) both *in vitro* and *in vivo* test systems. The present study aimed to contribute to the knowledge regarding the genotoxicity induced by UA and its influence on mutagens with different mechanisms of action (MOA) using an MN frequency test in Chinese hamster ovary cells (CHO). Additional experiments were conducted to investigate the effect of UA on colon carcinogenesis in Wistar rats.

Material and methods

Chemicals

The UA (Figure 1) used in this study was kindly provided by Wilson Roberto Cunha, PhD, from the Natural Products Research Group of the University of Franca, Franca, São Paulo, Brazil. Additional information on the isolation and purification of UA may be found in Leandro et al. (2013). For the experiments, UA was dissolved in dimethylsulfoxide (DMSO, 0.02%; CAS 67-68-5, Sigma-Aldrich). Doxorubicin (DXR; Eurofarma Laboratórios S/A, Ribeirão Preto, São Paulo, Brazil; 0.92 µM) and hydrogen peroxide (H_2O_2; CAS 7722-84-1, Sigma-Aldrich; 100 µM) were dissolved in sterile distilled water immediately before use, and etoposide (VP-16; CAS 33419-42-0, Sigma-Aldrich; 1.7 µM) was dissolved in 0.5% DMSO. The colon carcinogen 1,2-dimethylhydrazine dihydrochloride (DMH; CAS 306-37-6, Sigma-Aldrich) was dissolved immediately before use in 1 mM ethylenediaminetetraacetic acid (EDTA; CAS 60-00-4, Synth®, Diadema, São Paulo, Brazil).

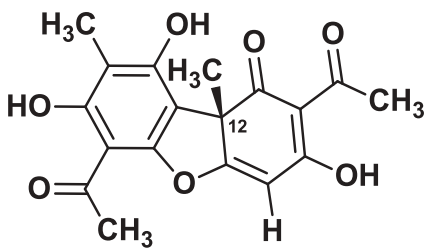

Figure 1. Chemical structure of (+)-usnic acid.

In vitro test system

Cells and culture conditions

The CHO cell line was maintained as a monolayer in plastic culture flasks (25 cm^2) with HAM-F10 (Sigma-Aldrich) and DMEM (Sigma-Aldrich) (1:1) culture medium supplemented with 10% fetal bovine serum (FBS, Nutricell, Campinas, São Paulo, Brazil), antibiotics (0.01 mg/ml streptomycin and 0.005 mg/ml penicillin, Sigma-Aldrich) and 2.32 mg/ml HEPES (Sigma-Aldrich), and incubated at 37°C in a BOD-type incubator. The cell cycle time was 12 hr under these conditions, and the experiments were conducted using CHO cells after the 4th passage. During this period, cells adapt to the culture conditions and actively proliferate with an exponential increase in cell density. This cell population is considered the most viable at this time, and it is therefore recommended to assess cellular function at this stage.

Assessment of genotoxicity and antigenotoxicity

Concentrations of UA of 21.78, 43.56, or 87.13 µM were used for genotoxicity assessment based upon the non-genotoxic concentrations determined in a previous study (Leandro et al. 2013). For antigenotoxicity assessment, non-cytotoxic or genotoxic concentrations (10.89, 21.78, or 43.56 µM) were combined with three mutagens with differing MOA: DXR, H_2O_2, or VP-16. Negative (no treatment), solvent (DMSO, 0.02%), and positive controls (mutagens) included. The protocol was performed in triplicate on three different days to ensure reproducibility.

Cytokinesis-block micronucleus (CBNM) assay

The CHO cells were seeded into a culture flask containing 5 ml HAM-F10/DMEM medium supplemented with 10% FBS and incubated for 25 hr. Subsequently, cells were treated with UA for 3 hr (OECD 487, 2016). After treatment, cells were washed twice with PBS and fresh serum-supplemented medium containing 3 µg/ml cytochalasin B (Sigma-Aldrich) added. The cells were incubated for an additional 17 hr. At harvest time, the cells were rinsed with 5 ml PBS, trypsinized with 0.025% trypsin–EDTA, and centrifuged for 5 min at 850 *g*. The pellet was hypotonized in 1% sodium citrate at 37°C and homogenized. This cell suspension was centrifuged under the same conditions. The

pellet was resuspended in methanol:acetic acid (3:1) and again homogenized. Fixed cells were then transferred to slides, stained with 3% Giemsa, and analyzed under a light microscope.

The criteria established by Fenech (2000) were used for the analysis of MN and binucleate cells. For analysis, 1,000 cells were counted per culture, for a total of 3,000 binucleate cells per treatment. Cytotoxicity of the treatment was evaluated by calculating the nuclear division index (NDI), determined by the analysis of 500 cells per culture, for a total of 1,500 cells per treatment group. Cells with well-preserved cytoplasm containing 1 to 4 nuclei were counted and the NDI was calculated using the following equation according to Eastmond and Tucker (1989):

$$NDI = \frac{[M1 + 2(M\,2) + 3(M\,3) + 4(M\,4)]}{N} \quad (1)$$

where *M1–M4* are the number of cells with 1, 2, 3, and 4 nuclei, respectively, and *N* is the total number of viable cells.

In vivo test system

Animals

Male Wistar rats (*Rattus norvegicus*) weighing approximately 120 g were provided by the Faculty of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. The animals were kept in plastic boxes in an experimental room under controlled conditions of temperature (22 ± 2°C) and humidity (50 ± 10%) under a 12-hr light-dark cycle, with free access to regular rat chow and water. The study was conducted in accordance with internationally accepted *Principles for the Use and Care of Laboratory Animals* (8th edition, National Research Council – 2011) and the experimental protocol was approved by the Ethics Committee on Animal Care of the University of Franca (No. 1574270916).

Experimental design

Doses of UA of 3.125, 12.5, or 50 mg/kg b.w. were utilized. The doses were selected based upon a previous study in which UA produced antigenotoxic effects in bone marrow cells and hepatocytes of Swiss mice (Leandro et al. 2013). The animals received 4 subcutaneous (s.c.) injections of DMH (40 mg/kg b.w.; 0.5 ml) twice a week (2nd and 5th day) for 2 weeks (weeks 2 and 3) and euthanized at the end of the 5th week, as described by Senedese et al. (2013). After one week acclimation, animals were divided into 8 treatment groups: negative control (EDTA, 0.37 µg/L; 0.5 ml, s.c.); Tween 80 (CAS 9005-64-5, Synth®; 5%); UA (50 mg/kg b.w.; 1 ml); positive control (DMH, 160 mg/kg b.w.); Tween 80 (5%) and DMH, and UA (3.125, 12.5, or 50 mg/kg b.w.; 1 ml) plus DMH. UA and Tween 80 were administered to rats by gavage 5 times a week for 2 weeks (weeks 2 and 3) during DMH treatment (Figure 2). Body weight and water consumption were measured three times a week throughout the experimental period. Each treatment group consisted of 5 animals.

Biochemical analysis

Blood samples were collected by cardiac puncture under anesthesia (sodium pentobarbital, 45 mg/kg b.w., i.p., 0.3 ml). Serum alanine aminotransferase

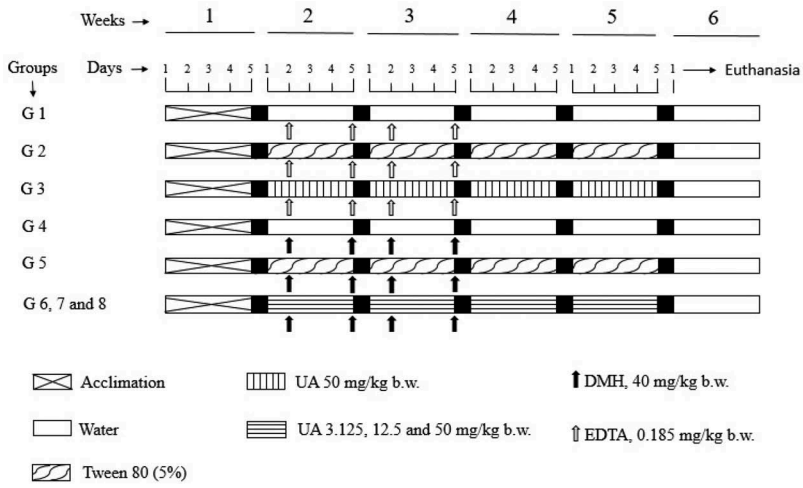

Figure 2. Schematic representation of the experimental design.

(ALT, Modified Glutamate Pyruvate Transaminase Kit, IFCC – REF: D98624; DIALAB) and aspartate aminotransferase (AST, Modified Glutamate Oxaloacetate Transaminase Kit, IFCC – REF: D98616; DIALAB) activities were measured to assess hepatotoxicity. Renal function was evaluated by the measurement of creatinine (Enzymatic Creatinine Assay Kit, PAP – REF: D95704; DIALAB) and urea (Urea UV Auto Urease Kit, GLDH – REF: D95595; DIALAB). For this purpose, approximately 400 µl blood was collected from the heart of euthanized animals and samples centrifuged for 15 min at 2500 g for separation of plasma. The samples were analyzed in an automated Mindray BS 200® analyzer based on the principle of absorption.

Analysis of aberrant crypt foci (ACF)

The procedures for ACF analysis in rat colons were performed according to Bird (1987). The animals were anesthetized with 1 ml sodium pentobarbital (45 mg/kg b.w., i.p.) and euthanized by exsanguination. After laparotomy, colons were excised, flushed with 0.9% saline, cut open along the longitudinal axis, and fixed in 10% phosphate-buffered formalin (pH 6.9–7.1) for 24 hr. Immediately prior to analysis, the colon was stained with 0.02% methylene blue for 5 min, mounted on microscope slides with the mucosal side facing upward, and observed under a light microscope at 100x magnification. Fifty sequential fields of the distal colon were screened for ACF, which were characterized by elongated, slit-shaped lumens surrounded by thickened epithelium that stained more intense than the surrounding normal crypts. The number of ACF and crypt multiplicity (number of crypts per focus) was recorded. Each colon specimen was examined by at least three observers in a double-blind manner.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) for completely randomized experiments, calculating F statistics and respective p values. In cases in which $P < .05$, treatment means were compared using Tukey's test and the minimum significant difference was calculated for $\alpha = 0.05$. Statistical analysis was performed using the GraphPad Prism 5 program.

Results

In vitro test system

The frequencies of MN in CHO cells treated with varying UA concentrations (21.78, 43.56, or 87.13 µM) and their respective controls are presented in Table 1. No significant differences in MN frequency induction were observed between cultures treated with 21.78 or 43.56 µM UA compared to negative control. In contrast, the highest concentration tested (87.13 µM) significantly increased chromosome damage compared to negative control.

Figure 3 illustrates the frequency of MN in CHO cells treated with UA combined with different mutagens. Cell cultures incubated with UA plus mutagen (DXR, H_2O_2, or VP-16) exhibited a significant reduction in MN frequency compared to respective mutagen alone, except for the treatment with 43.56 µM UA combined with DXR. In cultures incubated with UA combined with different mutagens, the gradual rise in UA concentration did not markedly modify DNA damage mediated by each mutagen, demonstrating an absence of a concentration–response relationship.

In vivo test system

Table 2 shows the mean and standard deviation of initial and final body weight, weight gain, and water consumption of Wistar rats under varying experimental conditions in the ACF assay. Rats exposed to DMH alone or combined with UA displayed reduced weight gain compared to controls (negative, Tween 80, and UA). No marked

Table 1. Micronucleus (MN) frequency and nuclear division index (NDI) in CHO cultures treated with (+)-usnic acid and the respective controls.

Treatments (µM)	MN frequency	NDI
Control	6.33 ± 1.53	1.66 ± 0.04
DMSO	7.67 ± 2.31	1.65 ± 0.04
UA 21.78	8.33 ± 3.79	1.58 ± 0.03
UA 43.56	8.00 ± 3.00	1.58 ± 0.03
UA 87.13	15.00 ± 3.00[a]	1.62 ± 0.01
DXR	22.67 ± 1.15[a]	1.68 ± 0.06

DXR – doxorubicin (0.92 µM); DMSO – dimethylsulfoxide (0.02%). For determination of MN frequency, 3,000 binucleated cells were scored per treatment (1,000 cells/treatment/repetition). The NDI was calculated by analyzing 1,500 cells per treatment group (500 cells/treatment/repetition). The values are the mean ± standard deviation.
[a]Significantly different from the negative control group ($P < 0.05$).

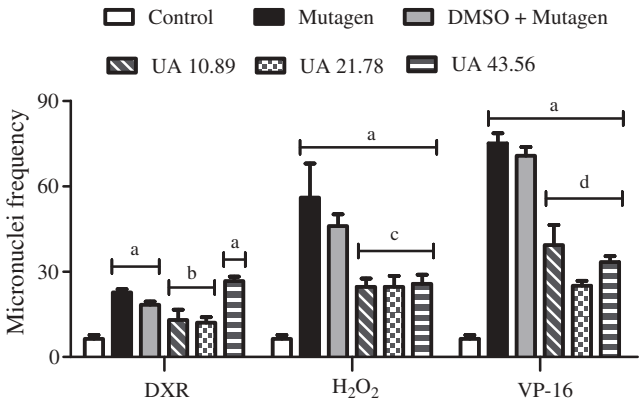

Figure 3. Frequencies of micronuclei in CHO cultures treated with different concentrations of (+)-usnic acid combined with different mutagens and their respective controls. UA – (+)-Usnic acid; DMSO – dimethylsulfoxide (0.02%); DXR – doxorubicin (0.92 μM); H_2O_2 – hydrogen peroxide (100 μM); VP-16 – etoposide (1.7 μM). A total of 3,000 binucleated cells were scored per treatment. The values are the mean ± standard deviation.

[a]Significantly different from the negative control group ($P< .05$).[b]Significantly different from the DXR group ($P< .05$).[c]Significantly different from the H_2O_2 group ($P< .05$).[d]Significantly different from the VP-16 group ($P< .05$).

differences were observed between groups for the other variables.

Analysis of the biochemical parameters demonstrated significantly higher serum ALT levels in animals treated with UA (12.5 or 50 mg/kg b.w.) plus DMH compared to those treated only with DMH. No significant differences were found for liver (AST and ALT) or renal (creatinine and urea) function parameters when the treatment was compared with negative control. These findings indicate an apparent absence of hepatotoxic and nephrotoxic effects following different treatment regimes (Figure 4).

Aberrant crypt foci were noted only in colons of DMH-treated rats (Figure 5) but not in negative control or groups receiving UA or Tween 80 alone (data not shown). The numbers of ACF and aberrant crypts (AC) were significantly lower in groups administered different UA doses (3.125, 12.5 or 50 mg/kg b.w.) during DMH treatment compared to rats injected only with DMH. Animals treated with Tween 80 plus DMH did not differ significantly from DMH-treated rats. The number of AC and AC/ACF ratio obtained for all groups administered DMH revealed a higher frequency of foci with one crypt (data not shown). A gradual elevation in UA dose did not produce a proportional reduction in DMH-induced ACF, demonstrating an absence of a dose–response relationship.

Discussion

The results of the MN frequency test in CHO cells demonstrated genotoxicity attributed to UA at the highest concentration (87.13 μM). In contrast, previously Leandro et al. (2013) found no marked genotoxic effect of UA in V79 cells in the MN frequency test at any of the concentrations tested (43.56, 87.13, 174.24, or 348.48 μM). O'Donovan (1990) and Erexson, Periago, and Spicer (2001) reported that CHO cells are more sensitive to MN induction than V79 cells and that both cell lines vary significantly in their ability to metabolize genotoxins.

Prokopiev et al. (2019) used the comet assay to examine the genotoxic potential of (+)- and (−)-UA (1, 10, 50, or 100 mg/kg b.w.) in liver and kidney cells of CD-1 mice after 1, 3, 6, 12, and 24 hr treatment. A genotoxic effect attributed to UA was only observed 1 hr after oral administration at doses of 50 or 100 mg/kg b.w. In contrast, Leandro et al. (2013) found no marked UA-induced genotoxic effect in mouse bone marrow cells (MN test) or hepatocytes (comet assay) at doses of 25–200 mg/kg. The discrepancies in UA-

Table 2. Mean (±SD) initial weight, final weight, weight gain and water consumption of Wistar rats treated with different (+)-concentrations of usnic acid (UA) over the 4 weeks of analysis. DMH – 1,2-dimethylhydrazine (160 mg/kg b.w.); Tween 80 (5%). The values are the mean ± standard deviation.

Treatments (mg/kg b.w.)	Initial weight (g)	Final weight (g)	Weight gain (g)	Water consumption (mL/animal/day)
Control	165.2 ± 5.12	428.0 ± 21.89	262.8 ± 21.65	59.32 ± 11.06
Tween 80	138.6 ± 11.78	349.6 ± 36.84	211.0 ± 32.46	50.78 ± 7.80
UA 50	126.3 ± 7.37	334.6 ± 39.55	208.3 ± 32.35	69.17 ± 17.84
DMH	155.8 ± 19.48	316.0 ± 29.60	160.2 ± 41.01[a]	54.93 ± 14.65
Tween 80 + DMH	145.8 ± 5.97	320.8 ± 33.79	175.0 ± 31.98[a]	53.94 ± 15.25
UA 3.125 + DMH	151.0 ± 14.61	326.2 ± 54.51	175.2 ± 44.04[a]	56.02 ± 15.40
UA 12.5 + DMH	146.0 ± 10.46	322.6 ± 33.46	176.6 ± 27.79[a]	55.10 ± 10.10
UA 50 + DMH	136.6 ± 5.59	265.2 ± 47.88	128.6 ± 44.94[a]	51.62 ± 14.60

[a]Significantly different from the negative control group ($P< 0.05$).

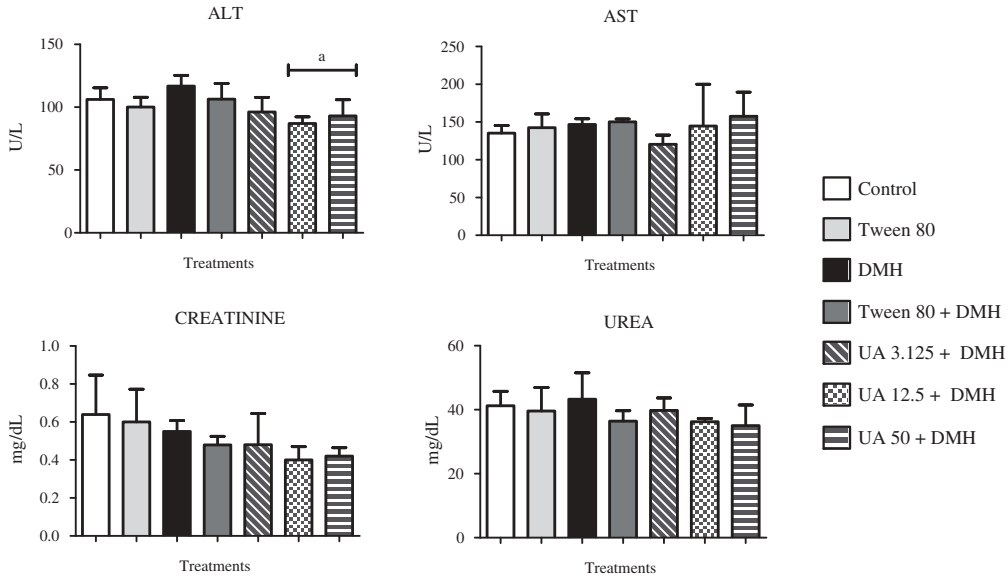

Figure 4. Serum levels of (a) aspartate aminotransferase (AST); (b) alanine aminotransferase (ALT); (c) creatinine, and (d) urea in Wistar rats treated with (+)-usnic acid and DMH. UA – (+)-Usnic acid; DMH – 1,2-dimethylhydrazine (160 mg/kg b.w.); Tween 80 (5%).

[a]Significantly different from the DMH group ($P < .05$).

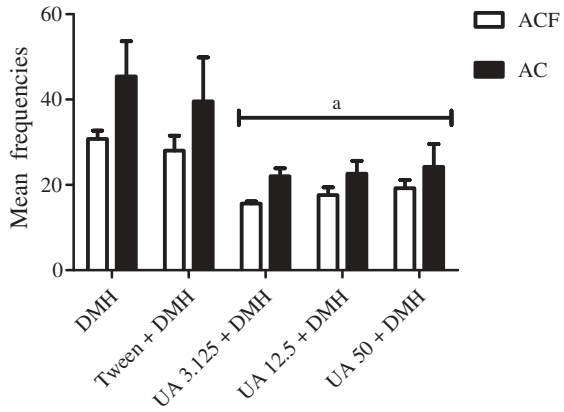

Figure 5. Frequencies of aberrant crypt foci (ACF) and aberrant crypts (AC) observed in distal colons of Wistar rats treated with different (+)-usnic acid doses and DMH. UA – (+)-Usnic acid; DMH – 1,2-dimethylhydrazine (160 mg/kg b.w.); Tween 80 (5%). Fifty consecutive fields were analyzed per animal, corresponding to 250 fields per treatment group. The values are the mean ± standard deviation.

[a]Significantly different from the DMH group ($P < .05$).

mediated genotoxicity may be related to differences in the experimental conditions. In the study of Leandro et al. (2013), DNA damage was determined 24 hr after administration of UA with no genotoxicity detected. On the other hand, Prokopiev et al. (2019) found UA-initiated genotoxic effects in liver after 1 and 3 hr and in kidneys after 1 hr, but no significant response was noted after longer periods. These findings indicate the occurrence of a rapid induction and repair of DNA damage. In addition, UA induced no marked genotoxic effect in the MN frequency test using Swiss mouse bone marrow (Leandro et al. 2013). Negative results in appropriate *in vivo* assays are generally considered sufficient to demonstrate an absence of a significant genotoxic risk (ICH 2012).

In an attempt to contribute to the understanding of MOA underlying the antigenotoxic effect previously reported by Leandro et al. (2013), the influence of UA was investigated on genotoxicity induced by mutagens with differing MOA such as DXR, H_2O_2, and VP-16. DXR, an anthracycline antibiotic, is a key chemotherapeutic drug employed for cancer treatment, although its use is limited by chronic and acute adverse effects (Quiles et al. 2002). Anthracyclines such as DXR are DNA topoisomerase II inhibitors. This enzyme is involved in fundamental biological processes, including DNA replication, transcription, DNA repair, and chromatin remodeling. DXR binds to DNA topoisomerase II and stabilizes an intermediate reaction in which the DNA strands are cut and covalently linked to tyrosine residues of DNA topoisomerase II, creating a ternary DXR–DNA–DNA topoisomerase II complex that alters DNA structure and impedes its synthesis (Minotti et al. 2004). Further, the quinone present in the molecular structure of DXR may be

oxidized to a semiquinone radical. Semiquinone radicals react rapidly with oxygen to generate superoxide (O_2^-) and H_2O_2 that are converted to highly reactive hydroxyl radicals, inducing DNA damage (Finn, Findley, and Kemp 2011; Injac and Strukelj 2008; Venkatesh et al. 2007).

Our data demonstrated that UA reduced DXR-induced genotoxicity. The MOA underlying the protective effect of UA against DXR may be related to modulation of DNA topoisomerase II activity and/or the antioxidant properties of this compound.

In an effort to contribute to the understanding of UA-mediated MOA involved in the observed antigenotoxicity, the influence of UA on genotoxicity induced by H_2O_2 and VP-16 was also examined. H_2O_2 is an oxidizing agent that initiates oxidative DNA damage through production of a hydroxyl radical (OH^-), which generates multiple DNA modifications, including base damage, sugar damage, and DNA-protein crosslinks (Živković et al. 2017). VP-16 is a potent anticancer agent that inhibits DNA topoisomerase II, leading to the production of DNA breaks. Usnic acid was also able to decrease chromosomal damage induced by H_2O_2 and VP-16, indicating a protective effect against genotoxicity initiated by the three mutagens DXR, H_2O_2, and VP-16.

The precise molecular MOA of lichen-specific metabolites are almost unknown. However, several investigators demonstrated the binding of UA to DNA and its antioxidant potential (Bolton, Dunlap, and Dietz 2018; Hasinoff et al. 1996; Jacob et al. 2013; Plsíkova et al. 2014; Smith et al. 2014). Plsíkova et al. (2014) examined the interactions between lichen metabolites (parietin, atranorin, UA, and gyrophoric acid) and calf thymus DNA using molecular biophysics and biochemical methods. The tested lichen metabolites were identified as DNA topoisomerase II catalytic inhibitors that reduce clastogenicity of DNA topoisomerase II poisons such as DXR and VP-16 (Hasinoff et al. 1996).

According to Leandro et al. (2013), the antioxidant activity of UA may explain its protective effect against MMS-induced genotoxicity *in vitro* and *in vivo*. MMS is a well-known DNA-damaging alkylating agent that forms DNA mono-adducts and crosslinks, resulting in DNA breakage or base substitution mutations (Jenkins et al. 2005). Alkylating agents were also noted to produce rapid depletion of glutathione S-transferase in mammalian cells, generating oxidative stress (van de Water, Zoeteweij, and Nagelkerke 1996). Suwalsky et al. (2015) demonstrated that UA molecules interact with the lipid bilayers of cell membranes in human erythrocytes acting as an antioxidant by blocking access of oxidants to cell membranes.

An oxidizing environment may trigger the intracellular expression of genes with protective function, but some molecules also exert an influence on biomolecules that participate directly or indirectly in the expression of genes related to the antioxidant response. An important cellular defense mechanism that attenuates the adverse effects of reactive oxygen species (ROS) is nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates expression of various enzymes that protect against oxidative stress (Forman, Davies, and Ursini 2014; Sosa et al. 2013). In this context, Chen et al. (2017) observed that UA significantly increased protein levels of Nrf2, promoted Nrf2 translocation to the nucleus, up-regulated antioxidant response element (ARE) activity, and induced the expression of Nrf2-regulated targets, including glutathione reductase, glutathione S-transferase and NAD(P)H quinone oxidoreductase-1 (NQO1), in a human hepatoma cell line (HepG2).

Considering the antigenotoxic effect of UA against DNA damage mediated by the three different mutagens, UA action on colon carcinogenesis in rats was also investigated. The results revealed a chemoprotective effect of UA against preneoplastic lesions induced by DMH, indicating that UA may prevent DNA damage and formation of preneoplastic lesions in the early stage of colon cancer.

The carcinogen DMH, when metabolized, releases azomethane, azoxymethane, methylazoxymethanol, ethane, carbon dioxide, and methyldiazonium, which methylate DNA, RNA, and proteins (Choudhary and Hansen 1998; Swenberg et al. 1979). These metabolites are responsible for the methylation of DNA bases in various organs, including proliferating epithelial cells (Chang 1984). Ghadi et al. (2012) found that DMH also induced carcinogenesis through increased OH^- production, which, in the presence of metal ions, may contribute to lipid peroxidation. Thus, in agreement with *in vitro* results, the observed protective effect of UA counteracting colon carcinogenesis may be related, at least in part, to an antioxidant activity.

Odabasoglu et al. (2006) and Halici et al. (2005) determined the gastroprotective effects of UA on indomethacin-induced gastric ulcers in rats. Data showed a reduction in gastric lesions and increased levels of antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, and reduced glutathione, suggesting that the gastroprotective effects of UA might be attributed to an action on diminished oxidative damage. Similarly, Su et al. (2014) observed a protective effect of UA on lipopolysaccharide-induced acute lung injury in mice, which might be related to the suppression of excessive inflammatory responses and oxidative stress in lung tissue.

The lack of a significant dose–response effect of UA observed in the present study might be due to erratic absorption across the cell membrane and consequent poor bioavailability of the substance in the cell. In addition, dose–response assessment is complicated by the fact that many chemopreventive agents exert simultaneous protection at different levels (Knasmüller et al. 2002). The lack of a dose–response effect of UA might therefore be attributed to activation of different MOA depending upon the dose tested.

Conclusions

In conclusion, at the highest concentration tested UA exerted an antigenotoxic effect against damage induced by DXR, H_2O_2, and VP-16 in CHO cells under the present experimental conditions. This lichen metabolite also showed protective activity against colon carcinogenesis in rats. Data suggest that UA may be utilized as a potential chemoprotective agent and that this effect may be attributed to an action on DNA topoisomerase II and/or to antioxidant potential of this compound. Further studies are necessary to elucidate the MOA involved in the effects of UA on genomic instability.

Author Contributions

All authors participated in the design of the study, interpretation and analysis of the data, and revision of the manuscript. WR Cunha was responsible for the isolation of the chemical substance. MA Spanó and DC Tavares coordinated and supervised the study.

Conflict of interest

The authors declare no conflicts of interest.

Funding

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior: [Grant Number 001]; Fundação de Amparo ã Pesquisa do Estado de São Paulo: [Grant Number 307817/2015-6]; Conselho Nacional de Desenvolvimento Científico e Tecnológico: [Grant Number 307817/2015-6].

ORCID

Denise Crispim Tavares http://orcid.org/0000-0003-4646-5914

References

Acésio, N. O., G. S. Carrijo, T. H. Batista, J. L. Damasceno, M. B. Côrrea, M. G. Tozatti, and D. C. Tavares. 2017. Assessment of the antioxidant, cytotoxic, and genotoxic potential of the *Annona muricata* leaves and their influence on genomic stability. *J. Toxicol. Environ. Health A* 80:1290–300. doi:10.1080/15287394.2017.1377653.

Benetou, V., A. Lagiou, and P. Lagiou. 2015. Chemoprevention of cancer: Current evidence and future prospects. *F1000Research* 4:1–10. doi:10.12688/f1000research.5779.5.

Bird, R. P. 1987. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: Preliminary findings. *Cancer Lett.* 37:147–51.

Bolton, J. L., T. L. Dunlap, and B. M. Dietz. 2018. Formation and biological targets of botanical *o*-quinones. *Food Chem. Toxicol.* 120:700–07. doi:10.1016/j.fct.2018.07.050.

Chang, W. W. 1984. Histogenesis of colon cancer in experimental animals. *Scand. J. Gastroenterol. Suppl.* 104:27–43.

Chen, S., V. N. Dobrovolsky, F. Liu, Y. Wu, Z. Zhang, N. Mei, and L. Guo. 2014. The role of autophagy in usnic acid-induced toxicity in hepatic cells. *Toxicol. Sc.* 142:33–44. doi:10.1093/toxsci/kfu154.

Chen, S., Z. Zhang, T. Qing, Z. Ren, D. Yu, L. Couch, and L. Guo. 2017. Activation of the Nrf2 signaling pathway in usnic acid-induced toxicity in HepG2 cells. *Arch. Toxicol.* 91:1293–307. doi:10.1007/s00204-016-1775-y.

Choudhary, G., and H. Hansen. 1998. Human health perspective of environmental exposure to hydrazines: A review. *Chemosphere* 37:801–43.

Cocchietto, M., N. Skert, P. Nimis, and G. Sava. 2002. A review on usnic acid, an interesting natural compound. *Naturwissenschaften* 8:137–46. doi:10.1007/s00114-002-0305-3.

Dylawerska, A., W. Barczak, A. Wegner, W. Golusinski, and W. M. Suchorska. 2017. Association of DNA repair genes polymorphisms and mutations with increased risk of head

and neck cancer: A review. *J. Med. Oncol.* 34:197–204. doi:10.1007/s12032-017-1057-4.

Eastmond, D. A., and J. D. Tucker. 1989. Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. *Environ. Mol. Mutagen.* 13:34–43.

Erexson, G. L., M. V. Periago, and C. S. Spicer. 2001. Differential sensitivity of Chinese hamster V79 and Chinese hamster ovary (CHO) cells in the *in vitro* micronucleus screening assay. *Mutat. Res.* 495:75–80. doi:10.1016/S1383-5718(01)00199-1.

Fenech, M. 2000. The in vitro micronucleus technique. *Mutat. Res.* 455:81–95. doi:10.1016/S0027-5107(00)00065-8.

Fernández-Moriano, C., P. K. Divakar, A. Crespo, and M. P. Gómez-Serranillos. 2017. Protective effects of lichen metabolites evernic and usnic acids against redox impairment-mediated cytotoxicity in central nervous system-like cells. *Food Chem. Toxicol.* 105:262–77. doi:10.1016/j.fct.2017.04.030.

Finn, N. A., H. W. Findley, and M. L. Kemp. 2011. A switching mechanism in doxorubicin bioactivation can be exploited to control doxorubicin toxicity. *PLoS Comput. Biol.* 7:e1002151. doi:10.1371/journal.pcbi.1002244.

Forman, H. J., K. J. Davies, and F. Ursini. 2014. How do nutritional antioxidants really work: Nucleophilic tone and para-hormesis versus free radical scavenging in vivo. *Free Radic. Biol. Med.* 66:24–35. doi:10.1016/j.freeradbiomed.2013.05.045.

Galanty, A., P. Koczurkiewicz, D. Wnuk, M. Paw, E. Karnas, I. Podolak, and M. Michalik. 2017. Usnic acid and atranorin exert selective cytostatic and anti-invasive effects on human prostate and melanoma cancer cells. *Toxicol. In Vitro* 40:161–69. doi:10.1016/j.tiv.2017.01.008.

Ghadi, F. E., A. Malhotra, A. R. Ghara, and D. K. Dhawan. 2012. Selenium as a modulator of membrane stability parameters and surface changes during the initiation phase of 1,2-dimethylhydrazine induced colorectal carcinogenesis. *Mol. Cell. Biochem.* 369:119–26. doi:10.1007/s11010-012-1374-z.

Gontijo, D. C., M. A. N. Diaz, G. C. Brandão, P. C. Gontijo, A. B. D. Oliveira, L. G. Fietto, and J. P. V. Leite. 2018. Phytochemical characterization and antioxidant, antibacterial and antimutagenic activities of aqueous extract from leaves of *Alchornea glandulosa*. *J. Toxicol. Environ. Health A* 81:805–18. doi:10.1080/15287394.2018.1492479.

Greenlee, H. 2012. Natural products for cancer prevention. *Semin. Oncol. Nurs.* 28:29–44. doi:10.1016/j.soncn.2011.11.004.

Halici, M., F. Odabasoglu, H. Suleyman, A. Cakir, A. Aslan, and Y. Bayir. 2005. Effects of water extract of *Usnea longissima* on antioxidant enzyme activity and mucosal damage caused by indomethacin in rats. *Phytomedicine* 12:656–62. doi:10.1016/j.phymed.2004.06.021.

Hasinoff, B. B., J. C. Yalowich, Y. Ling, and J. L. Buss. 1996. The effect of dexrazoxane (ICRF-187) on doxorubicin-and daunorubicin-mediated growth inhibition of Chinese hamster ovary cells. *Anticancer Drugs* 7:558–67.

ICH. 2012. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. ICH - S2(R1) Genotoxicity testing and data interpretation for pharmaceuticals intended for human use. https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm.

Ingolfsdottir, K. 2002. Usnic acid. *Phytochemistry* 61:729–36.

Injac, R., and B. Strukelj. 2008. Recent advances in protection against doxorubicin-induced toxicity. *Technol. Cancer Res. Treat.* 7:497–516. doi:10.1177/153303460800700611.

Jacob, D. A., E. G. Gibson, S. L. Mercer, and J. E. Deweese. 2013. Etoposide catechol is an oxidizable topoisomerase II poison. *Chem. Res. Toxicol.* 26:1156–58. doi:10.1021/tx400205n.

Jenkins, G. J. S., S. H. Doak, G. E. Johnson, E. Quick, E. M. Waters, and J. M. Parry. 2005. Do dose response thresholds exist for genotoxic alkylating agents? *Mutagenesis* 20:389–98. doi:10.1093/mutage/gei054.

Knasmüller, S., H. Steinkellner, B. J. Majer, E. C. Nobis, G. Scharf, and F. Kassie. 2002. Search for dietary antimutagens and anticarcinogens: Methodological aspects and extrapolation problems. *Food Chem. Toxicol.* 40:1051–62.

Koparal, A. T. 2015. Anti-angiogenic and antiproliferative properties of the lichen substances (-)-usnic acid and vulpinic acid. *Z. Naturforsch. C* 70:159–64. doi:10.1515/znc-2014-4178.

Korniluk, A., O. Koper, H. Kemona, and V. Dymicka-Piekarska. 2017. From inflammation to cancer. *Ir J Med Sci* 186:57–62. doi:10.1007/s11845-016-1464-0.

Kotecha, R., A. Takami, and J. L. Espinoza. 2016. Dietary phytochemicals and cancer chemoprevention: A review of the clinical evidence. *Oncotarget* 7:52517–29. doi:10.18632/oncotarget.9593.

Langie, S. A., G. Koppen, D. Desaulniers, F. Al-Mulla, R. Al-Temaimi, A. Amedei, and A. K. Charles. 2015. Causes of genome instability: The effect of low dose chemical exposures in modern society. *Carcinogenesis* 36:61–88. doi:10.1093/carcin/bgv031.

Leandro, L. F., C. C. Munari, V. L. F. L. Sato, J. M. Alves, P. F. Oliveira, D. F. P. Mastrocola, and W. R. Cunha. 2013. Assessment of the genotoxicity and antigenotoxicity of (+)-usnic acid in V79 cells and Swiss mice by the micronucleus and comet assays. *Mutat. Res.* 753:101–06. doi:10.1016/j.mrgentox.2013.03.006.

Mayer, M., M. A. O'Neill, K. E. Murray, N. S. Santos-Magalhães, A. M. A. Carneiro-Leão, A. M. Thompson, and V. C. Appleyard. 2005. Usnic acid: A non-genotoxic compound with anti-cancer properties. *Anticancer Drugs* 16:805–09.

Minotti, G., P. Menna, E. Salvatorelli, G. Cairo, and L. Gianni. 2004. Anthracyclines: Molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* 56:185–229. doi:10.1124/pr.56.2.6.

National Research Council. 2011. *Guide for the care and use of laboratory animals*. 8th ed. The National Academies Collection: Reports funded by National Institutes of Health. doi:10.17226/12910.

Nguyen, T. T., S. Yoon, Y. Yang, H. B. Lee, S. Oh, M. H. Jeong, and K. Y. Lee. 2014. Lichen secondary metabolites in *Flavocetraria cucullata* exhibit anti-cancer effects on human cancer cells through the induction of apoptosis and suppression of

tumorigenic potentials. *PLoS ONE* 9:e111575. doi:10.1371/journal.pone.0111575.

O'Donovan, M. R. 1990. 1,8-dinitropyrene: Comparative mutagenicity in Chinese hamster V79 and CHO cells. *Mutagenesis* 5:275–78.

Odabasoglu, F., A. Cakir, H. Suleyman, A. Aslan, Y. Bayir, M. Halici, and C. Kazaz. 2006. Gastroprotective and antioxidant effects of usnic acid on indomethacin-induced gastric ulcer in rats. *J. Ethnopharmacol.* 103:59–65. doi:10.1016/j.jep.2005.06.043.

OECD (2016), *Test no. 487: In Vitro mammalian cell micronucleus test*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. doi:10.1787/9789264264861-en.

Plsíkova, J., J. Stepankova, J. Kasparkova, V. Brabec, M. Backor, and M. Kozurkova. 2014. Lichen secondary metabolites as DNA-interacting agents. *Toxicol. In Vitro* 28:182–86. doi:10.1016/j.tiv.2013.11.003.

Prokopiev, I. A., E. V. Filippov, G. V. Filippova, and N. P. Gladkina. 2017. Genotoxicity of usnic-acid enantiomers in vitro in human peripheral-blood lymphocytes. *Cell Tissue Biol* 11:141–46. doi:10.1134/S1990519X17020031.

Prokopiev, I. A., G. Filippov, E. Filippova, I. Voronov, I. Sleptsov, and A. Zhanataev. 2019. Genotoxicity of (+)- and (-)-usnic acid in mice. *Mutat. Res.* 839:36–39. doi:10.1016/j.mrgentox.2019.01.010.

Quiles, J. L., J. R. Huertas, M. Battino, J. Mataix, and M. C. Ramírez-Tortosa. 2002. Antioxidant nutrients and adriamycin toxicity. *Toxicology* 180:79–95.

Rabi, T., and A. Bishayee. 2009. Terpenoids and breast cancer chemoprevention. *Breast Cancer Res. Treat.* 115:223–39. doi:10.1007/s10549-008-0118-y.

Ribeiro-Costa, R. M., A. J. Alves, N. P. Santos, S. C. Nascimento, E. C. Gonçalves, N. H. Silva, and N. S. Santos-Magalhães. 2004. *In vitro* and *in vivo* properties of usnic acid encapsulated into PLGA-microspheres. *J Microencapsul* 21:371–84. doi:10.1080/02652040410001673919.

Saha, S. K., S. B. Lee, J. Won, H. Y. Choi, K. Kim, G. M. Yang, and S. G. Cho. 2017. Correlation between oxidative stress, nutrition, and cancer initiation. *Int J Mol Sci* 18:1544. doi:10.3390/ijms18071544.

Senedese, J. M., J. M. Alves, I. M. de Souza Lima, E. A. P. de Andrade, R. A. Furtado, J. K. Bastos, and D. C. Tavares. 2013. Chemopreventive effect of *Copaifera langsdorffii* leaves hydroalcoholic extract on 1,2-dimethylhydrazine-induced DNA damage and preneoplastic lesions in rat colon. *BMC Complement. Altern. Med.* 13:3–11. doi:10.1186/1472-6882-13-3.

Silva, C. R., K. S. N. Marinho, T. D. S. Silva, D. K. S. Ferreira, G. M. Aguiar, M. C. B. Martins, and N. H. Silva. 2017. Teratogenic effect of usnic acid from *Cladonia substellata* Vainio during organogenesis. *Biomed. Res. Int.* 2017:1–7.

Smith, N. A., J. A. W. Byl, S. L. Mercer, J. E. Deweese, and N. Osheroff. 2014. Etoposide quinone is a covalent poison of human topoisomerase IIβ. *Biochemistry* 53:3229–36. doi:10.1021/bi500421q.

Sosa, V., T. Moliné, R. Somoza, R. Paciucci, H. Kondoh, and M. E. LLeonart. 2013. Oxidative stress and cancer: An overview. *Ageing Res. Rev.* 12:376–90. doi:10.1016/j.arr.2012.10.004.

Su, Z. Q., Y. H. Liu, H. Z. Guo, C. Y. Sun, J. H. Xie, Y. C. Li, and H. M. Chen. 2017. Effect-enhancing and toxicity-reducing activity of usnic acid in ascitic tumor-bearing mice treated with bleomycin. *Int. Immunopharmacol.* 46:146–55. doi:10.1016/j.intimp.2017.03.004.

Su, Z. Q., Z. Z. Mo, J. B. Liao, X. X. Feng, Y. Z. Liang, X. Zhang, and X. P. Lai. 2014. Usnic acid protects LPS-induced acute lung injury in mice through attenuating inflammatory responses and oxidative stress. *Int. Immunopharmacol.* 22:371–78. doi:10.1016/j.intimp.2014.06.043.

Suwalsky, M., M. Jemiola-Rzeminska, C. Astudillo, M. J. Gallardo, J. P. Staforelli, F. Villena, and K. Strzalka. 2015. An in vitro study on the antioxidant capacity of usnic acid on human erythrocytes and molecular models of its membrane. *Biochim. Biophys. Acta* 1848:2829–38. doi:10.1016/j.bbamem.2015.08.017.

Swenberg, J. A., H. K. Cooper, J. Bücheler, and P. Kleihues. 1979. 1,2-dimethylhydrazine-induced methylation of DNA bases in various rat organs and the effect of pretreatment with disulfiram. *Cancer Res.* 39:465–67.

Thadhani, V. M., and V. Karunaratne. 2017. Potential of lichen compounds as antidiabetic agents with antioxidative properties: A review. *Oxid. Med. Cell Longev.* 2017:1–10. Article ID 2079697. doi:10.1155/2017/2079697.

Tuttis, K., D. L. M. G. Da Costa, H. L. Nunes, A. F. L. Specian, J. M. Serpeloni, L. C. D. Santos, and I. M. S. Cólus. 2018. *Pouteria ramiflora* (Mart.) Radlk. extract: Flavonoids quantification and chemopreventive effect on HepG2 cells. *J. Toxicol. Environ. Health A* 81:792–804. doi:10.1080/15287394.2018.1491911.

van de Water, B., J. P. Zoeteweij, and J. F. Nagelkerke. 1996. Alkylation-induced oxidative cell injury of renal proximal tubular cells: Involvement of glutathione redox-cycle inhibition. *Arch. Biochem. Biophys.* 327:71–80. doi:10.1006/abbi.1996.0094.

Venkatesh, P., B. Shantala, G. C. Jagetia, K. K. Rao, and M. S. Baliga. 2007. Modulation of doxorubicin-induced genotoxicity by *Aegle marmelos* in mouse bone marrow: A micronucleus study. *Integr. Cancer Ther.* 6:42–53. doi:10.1177/1534735406298302.

Yu, X., Q. Guo, G. Su, A. Yang, Z. Hu, C. Qu, and X. Chai. 2016. Usnic acid derivatives with cytotoxic and antifungal activities from the lichen *Usnea longissima*. *J. Nat. Prod.* 79:1373–80. doi:10.1021/acs.jnatprod.6b00109.

Živković, L., S. Borozan, A. Čabarkapa, D. Topalović, U. Ciptasari, V. Bajić, and B. Spremo-Potparević. 2017. Antigenotoxic properties of *Agaricus blazei* against hydrogen peroxide in human peripheral blood cells. *Oxid. Med. Cell Longev.* 2017:1–9. Article ID 8759764. doi:10.1155/2017/8759764.