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Evaluation of mutagenic, recombinogenic and carcinogenic potential of (+)-usnic acid in somatic cells of *Drosophila melanogaster*

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ABSTRACT

The main of this study was to evaluate the mutagenic and carcinogenic potential of (+) − usnic acid (UA), using Somatic Mutation and Recombination Test (SMART) and the test for detecting epithelial tumor clones (*wts*) in *Drosophila melanogaster*. Larvae from 72 ± 4 h from *Drosophila* were fed with UA (5.0, 10.0 or 20.0 mM); urethane (10.0 mM) (positive control); and solvent (Milli-Q water, 1% Tween-80 and 3% ethanol) (negative control). ST cross produced increase in total mutant spots in the individuals treated with 5.0, 10.0 or 20.0 mM of UA. HB cross produced spot frequencies in the concentration of 5.0 mM that were higher than the frequency for the same concentration in the ST cross. In the highest concentrations the result was negative, which means that the difference observed can be attributed, in part, to the high levels of P450, suggesting that increasing the metabolic capacity maximized the toxic effect of these doses. In the evaluation of carcinogenesis using the wts test, the results obtained for the same concentrations of UA show a positive result for the presence of tumors when compared to the negative control. We conclude that UA has recombinogenic, mutagenic and carcinogenic effects on somatic cells in *D. melanogaster*.

1. Introduction

Lichen is an association of a heterotrophic organism (mycobiont) and an autotrophic organism (photobiont) (Martins et al., 2014), capable of producing a variety of substances with phenolic characteristics, called lichenic substances (Fernández-Moriano et al., 2015). These substances, also known as lichenic acids, are secondary metabolites, and include depsides, depsidones, benzyl esters, dibenzofurans, usnic acids, xanthones, anthraquinones and derivates of pulvinic acid (Johnson et al., 2011).

Usnic acid (UA), in addition to be considered one of the most important biologically active metabolites of lichens, has also received the most study (Ingólfsdóttir, 2002). It is characterized as a yellow pigment, of low molecular weight (344.32 g), insoluble in water and glycerol, and partially soluble in ethanol and easily soluble in ether, acetone, chloroform and ethyl acetate (Kristmundsdóttir et al., 2002). It is widely distributed in the genera Alectoria, Cladonia, Usnea, Lecanora, Ramalina and Evernia, and can be found in two enantiomeric forms: (−) - usnic acid and (+) - usnic acid (Leandro et al., 2013).

A wide range of biological activities have been reported for UA, including anti-microbial, antiviral, anti-proliferative, anti-inflammatory, antitumoral, anti-mitotic, anti-fungal and analgesic actions (Lauterwein et al., 1995; Perry et al., 1999; Kristmundsdóttir et al., 2002; Vijayakumar et al., 2000; Mayer et al., 2005; Campanella et al., 2002).

Low doses of (+)-usnic acid showed high cytotoxic activity against cancerous cells (Koparal et al., 2006). UA showed activity against the wild-type p53 breast cancer cell line MCF7 as well as the non-functional p53 breast cancer cell line MDA-MB-231 and the

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lung cancer cell line H1299 (null for p53) (Mayer et al., 2005). It has also been shown that UA has an apoptotic effect in human cancer lineages, significantly reducing cellular viability and inducing apoptosis in ovarian carcinoma, cervical adenocarcinoma, breast adenocarcinoma, colon adenocarcinoma, promielocitic leukemia and T-cell leukemia (Bačkorová et al., 2011; Bézivin et al., 2004; Burlando et al., 2009; Einarsdóttir et al., 2010; Ren et al., 2009).

However, in vitro studies have revealed that UA induces damage to cell membranes, and inhibits mitochondrial function of hepatocytes, leading to an increase in the production of reactive oxygen species, and inducing oxidative stress (Han et al., 2004; Pramyothin et al., 2004). And, as shown by Yokouchi et al. (2015), UA also induces myocardial toxicity in rats from mitochondrial dysfunction and oxidative stress.

There are limited data on the genotoxic/mutagenic/carcinogenic properties of UA. Previous studies have shown that UA induced DNA damage in V79 cells in vitro assessed by the comet assay (Leandro et al., 2013). Nevertheless, UA was not genotoxic/mutagenic in human lymphocytes in vitro, when assessed by cytokinesis-blocked micronucleus (CBMN) assay, chromosomal aberrations (CA) and micronucleus (MN) tests (Koparal et al., 2006; Polat et al., 2016) neither in V79 cells in vitro, using MN test (Leandro et al., 2013) and in Swiss mice in vivo, using MN and comet assays (Leandro et al., 2013). Furthermore, UA exerted a protective effect against the chromosomal and genome damage induced by methyl methanesulfonate (MMS) in V79 cells in vitro and in Swiss mice in vivo (Leandro et al., 2013). Thus, due to conflicting results observed in the literature, it can be concluded that genotoxicity/mutagenicity/carcinogenicity of UA needs further studies.

There are no records of research with UA using *Drosophila melanogaster* as a test organism. According to Akmoutsou et al. (2011), Drosophila is considered a model organism for genetic studies in eukaryotes, as well as in the evaluation of toxic, mutagenic and carcinogenic effects.

In order to obtain a more detailed understanding of the toxicity and the mutagenic and carcinogenic profile of the substance, the somatic mutation and recombination test - SMART and the test for detection of epithelial tumor clones (*wts*) were used, both with *D. melanogaster*. Potential for mutation, recombination and carcinogenesis, as well as toxicity of exposure to different concentrations were determined.

2. Material and methods

2.1. (+) − Usnic acid

The UA (Fig. 1) used in this study was kindly provided by Denise Crispim Tavares PhD, of the Natural Products Research Group of the University of Franca (UNIFRAN), Franca, SP, Brazil. Additional information about the isolation and purification of UA can be found in Leandro et al. (2013).

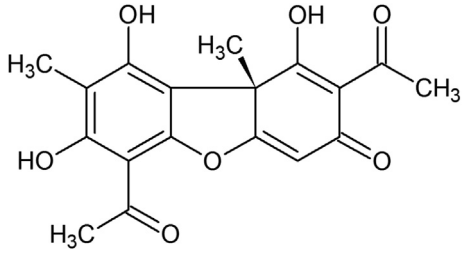

Fig. 1. Structural formula of (+)- usnic acid.

2.2. Somatic mutation and recombination test (SMART) in somatic cells of D. melanogaster

2.2.1. Lineages of D. melanogaster

Three mutant lineages of *D. melanogaster*, used for the SMART, were supplied by Dr. Ulrich Graf, of the Swiss Federal Institute of Technology in Zurich (ETHZ), Schwerzenbach, Switzerland. The stocks of these lineages are kept in tubes containing culture medium prepared with water, yeast (*Saccharomyces cerevisae*), agar, banana and nipagin, inside a B.O.D. incubator at a temperature of approximately 25 °C and relative humidity of 60% and photoperiod of 12 h of light and 12 h of darkness.

The three lineages used: *mwh*, *flr*^{3} and *ORR* possess the genetic markers *multiple wing hairs* (*mwh*, 3–0.3) and *flare-3* (*flr*^{3}, 3–38.8) (Graf et al., 1989). In the lineage resistant to *DDT*, *Oregon R* (R), the gene *RI* at position 65.0 on chromosome-2, is responsible for high levels of cytochrome P450 expression, typical of this lineage. Chromosomes 1 and 2 of lineage *flr*^{3} were therefore substituted with chromosomes 1 and 2 of the lineage *Oregon R* (R) (Frölich and Würgler, 1989).

2.2.2. Crosses

Three *D. melanogaster* strains with the genetic markers multiple wing hairs (*mwh*, 3–0.3) and flare-3 (*flr*^{3}, 3–38.9) were used: (1) multiple wing hairs (*mwh/mwh*); (2) flare-3 (*flr*^{3}*/In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx34*^{e} *and Bd*^{S}); and (3) ORR; flare-3 (*ORR/ORR; flr*^{3}*/In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx34*^{e} *and Bd*^{S}). The ORR; flare-3 strain has chromosomes 1 and 2 from a DDT-resistant Oregon R(R) line, which are responsible for a high constitutive level of cytochrome P450 enzymes (Graf and van Schaik, 1992). Two types of crosses were conducted for the experiment: Standard Cross (ST) using virgin females flare-3 crossed with males multiple wing hairs (Graf et al., 1984, 1989); High Bioactivation (HB) Cross using virgin females *ORR; flr*^{3} crossed with males multiple wing hairs (Graf and van Schaik, 1992).

These crosses produced two types of progeny: transheterozygous individuals for the marker genes (MH), and heterozygous individuals for the chromosome TM3 (BH) (Graf et al., 1984). The BH individuals are different phenotypically from the MH individuals due to the presence of serrations on the edges of their wings, characteristics conferred by the marker *Bd*^{S} (Graf et al., 1984).

2.2.3. Treatment

After the lineages were crossed, the couples were transferred to tubes containing hatching medium, where they remained for 8 h. After 72, ±4 h from the start of the hatching period, the third-stage larvae were collected, washed with water and collected with the aid of a fine mesh sieve.

The larvae collected were placed in glass tubes containing 1.5 g instantaneous mashed potato (Yoki Alimentos, São Bernardo do Campo, SP, Brazil) with 5 mL of different concentrations of UA (5.0; 10.0 or 20.0 mM). The UA was dissolved in a mixture of 1% Tween-80 and 3% ethanol in ultrapure water, which was used as a negative control, while urethane was used for the positive control (10.0 mM). The concentrations were chosen based on the survival rates of a dose-response test. Where the larvae were counted before distribution and collected in previously identified tubes. After hatching they were stored in 70% ethanol for later analysis.

After removing the wings of the flies under a stereoscopic microscope, the slides of the treated adult wings were mounted using Faure's solution [gum arabic (30 g), glycerol (20 mL), water (50 mL) and chloral hydrate (50 g)] and analyzed under an optical microscope at a magnification of 400 to identify spots of mutant hairs that could be classified as simple (*mwh* or *flr*^{3}) or twin (*mwh* and