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Full Paper

Antigenotoxicity of Depsidones Isolated from Brazilian Lichens

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Abstract: Although phenolic compounds produced by lichens have been widely investigated in antitumor assays, only a small number have been evaluated for mutagenicity and genotoxicity. This study evaluated protocetraric, hypostictic, psoromic, and salazinic acids for their potential genotoxic or antigenotoxic activity against somatic cells of *Drosophila melanogaster*. These compounds were isolated from the lichens *Parmotrema dilatatum*, *Pseudoparmelia sphaerosphora*, *Usnea jamaicensis*, and *Parmotrema cetratum*, respectively, collected from the Brazilian Cerrado biome. The compounds were evaluated at 0.75, 1.5, 3.0, and 6.0 mmol L^{-1} using the SMART test, employing standard and high-bioactivation crosses of *Drosophila melanogaster*. Doxorubicin (DXR) was the positive control. Psoromic and salazinic acids proved toxic at 6.0 mM. None of the compounds evaluated exhibited mutagenicity, but each of them significantly reduced genetic damage caused by DXR, proving antigenotoxic when tested on somatic cells of *D. melanogaster*.

Keywords: depsidones; genotoxicity; lichens; phenolic compounds; SMART assay

1. INTRODUCTION

Natural products, whether in the form of extracts, isolated substances, or synthetic and semi-synthetic compounds, have been evaluated for their utility in medicine, the food industry, and agriculture (*e.g.*, as pest control agents). Their sources are not limited to higher plants, but include mosses, fungi, algae, and lichens—all of them producing an abundance of bioactive substances. In lichens, the acetate–polymalonate route yields phenolic compounds (depsides, depsidones, quinones, anthraquinones, xanthones, dibenzofurans, usnic acids, and other products). Many of these compounds have been evaluated for their activity as antimicrobials, against a wide range of bacteria and fungi; as antitumor agents, inhibiting growth in a large panel of tumor cells; as antivirals, inhibiting replication of viruses, including HIV; and as inhibitors of enzymes such as 5-lipoxygenase, protein tyrosine phosphatase, α-glucosidase, and aldose reductase; among many other activities investigated [1-3].

Despite the marked activity exhibited by many lichen compounds, few have been evaluated for mutagenic or genotoxic properties, crucial for their safe use as drugs or in other applications. In an early investigation, Shibamoto and Wei [4] evaluated the mutagenicity of usnic, physodalic, and physodic acids. More recently, usnic, diffractaic, olivetoric, and psoromic acids have been investigated for their mutagenic and genotoxic potential [5-10].

The wing somatic mutation and recombination test (SMART) using *Drosophila melanogaster* was developed to detect loss of heterozygosity in suitable gene markers that express detectable phenotypes in wing cells. Rapid and inexpensive, the method quantifies, in an unambiguous and highly reproducible manner, the recombinogenic and mutagenic potential of chemical and physical agents [11, 12]. Two crosses—namely, standard (ST) and high-bioactivation (HB)—are typically used [13]. The ST cross, obtained from strains expressing basal levels of the metabolizing cytochrome P450 enzyme Cyp6A2, is employed to detect direct-acting genotoxins. The HB cross, obtained from strains

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expressing high levels of Cyp6A2, is used to detect indirect-acting genotoxins that exert their genotoxic activity only when metabolized [13, 14].

The present study employed the SMART assay to evaluate the genotoxic and antigenotoxic activities of protocetraric, hypostictic, psoromic, and salazinic acids.

2. MATERIAL AND METHODS

General experimental procedures

TLC was performed on pre-coated silica gel 60 GF_{254} plates (0.20 mm, Macherey-Nagel) and the spots were visualized by spraying the plates with a 10% sulfuric acid/methanol solution, followed by heating. Nuclear magnetic resonance (NMR) spectra were taken on a Bruker DPX-300 spectrometer using the solvent as an internal reference. Melting points were recorded on a Uniscience do Brasil 498 apparatus.

Plant collection and extract preparation

Parmotrema dilatatum (Vain.) Hale (Parmeliaceae), *Parmotrema cetratum* (Ach.) Hale (Parmeliaceae) and *Pseudoparmelia sphaerospora* (Nyl.) Hale (Parmeliaceae) were collected near Piraputanga village, in Aquidauana county, Mato Grosso do Sul state, Brazil (20°27′21.2″S, 55°29′00.9″W; alt. approx. 200 m). *Usnea jamaicensis* Ach. (Parmeliaceae) was obtained from decor stores. Species identification was carried out by Prof. Mariana Fleig, of the Universidade Federal do Rio Grande do Sul, Prof. Marcelo P. Marcelli, of the Instituto de Botânica de São Paulo, and Philippe Clerc, of the Herbarium of Geneva, Switzerland. Voucher specimens were deposited at the Campo Grande Herbarium of the Universidade Federal de Mato Grosso do Sul (CGMS 49840 for *P. dilatatum*, CGMS 37950 for *P. cetratum*, CGMS 49837 for *P. sphaerospora*, CGMS 49838 for *U. jamaicensis*).

Thalli of *P. dilatatum*, *P. cetratum, P. sphaerospora*, and *U. jamaicensis* were separately powdered and extracted with chloroform (2×), followed by acetone (3×), at room temperature, and subsequently concentrated *in vacuo*. The concentrated acetone extracts were then treated with a small volume of acetone in an ice bath and centrifuged. This procedure was repeated until a purified compound was obtained from each lichen. Protocetraric acid was obtained from *P. dilatatum*, hypostictic acid from *P. sphaerospora*, psoromic acid from *U. jamaicensis*, and salazinic acid from *P. cetratum*. The structures of these compounds were confirmed by NMR spectra (Figures S1-S9, Supplementary Material) and were concordant with the literature [15-17].

Genotoxic activity: somatic mutation and recombination test (SMART)

The SMART assay with *D. melanogaster* was performed according to the methodology described by Fernandes et al. [18]. Three strains were used for cross breeding: (1) the "multiple wing hairs" ("mwh") strain, of *mwh/mwh* genetic constitution; (2) the "flare-3" strain, of $flr^3/In(3LR)TM3\ rip^p sep\ l(3)89Aabx^{34e}$ and Bd^S genetic constitution; and (3) the "ORR; flare-3" strain, of $ORR/ORR;\ flr^3/In(3LR)TM3,\ rip^p sep\ l(3)89Aabx^{34e}$ and Bd^S genetic constitution. This last strain inherits chromosomes 1 and 2 from the Oregon R (R) line (which is DDT resistant), carrying genes responsible for a high level of metabolizing enzymes of P(CYP)6 A2–type cytochrome [13]. Two crossings were performed between these strains: (*a*) the standard (ST) cross, from "mwh" males and "flare-3" virgin females [19], and (*b*) the HB cross, from "mwh" males and "ORR; flare-3" virgin females [13].

Eggs from both crossings were collected over 8 h in culture flasks containing a solid agar-agar base (4% w/v) covered with a layer of biological yeast supplemented with sugar. Groups of third-instar (72 ± 4 h) larvae were transferred to glass vials containing alternative medium (1.5 g of instant mashed potato flakes, Yoki, Brazil) and assayed following two protocols: (1) for genotoxicity evaluation, each compound was separately tested at concentrations of 0.75, 1.5, 3.0, and 6.0 mmol L^{-1}; (2) for antigenotoxicity evaluation, the same concentrations were employed in association with 2.0 mmol L^{-1} doxorubicin (DXR). For both protocols, DXR (2.0 mmol L^{-1}) and solvent (Milli-Q water, 1% Tween-80, and 3% ethanol) were used as the positive and negative controls, respectively.

Emerging adults carrying one of two genotypes—namely, marker trans-heterozygous (MH; $mwh\ +/+flr^3$) or balancer-heterozygous (BH; $mwh+/+\ TM3,\ Bd^S$)—were collected and fixed in 70% ethanol. The wings were mounted on slides in Faure's solution (30 g of gum arabic, 50 g of chloral hydrate, 20 mL of glycerol, and 50 mL of water) and examined for the

occurrence of mutant spots using an optical microscope at 400× magnification.

The chi-squared test was employed to interpret the toxicity assay. Results were considered statistically significant when $p < 0.05$.

Statistical analysis

For each treatment, the frequencies of each type of spot (single small, single large, or twin) and the total frequency of spots per fly, for each treatment, were compared in pairs (negative control *versus* compounds; DXR alone *versus* compounds + DXR), in accordance with the multiple-decision procedure proposed by Frei and Würgler [20], allowing four possible diagnoses: positive, negative, inconclusive, or weakly positive. The relative frequencies of each group were compared using Kastenbaum and Bowman's conditional binomial test [21] at a significance level of 5%. However, since false positive results can occur, all final weakly positive results were analyzed with the non-parametric U-test [22].

For each compound, inhibition percentages were calculated from the control-corrected frequency of clones per 10^5 cells (FC) and the frequency of mutation (FM), as follows:

FC = {(DXR alone) – [(DXR alone) – (compound + DXR)]/(DXR alone)} × 100 [23];

FM = (FC in BH individuals) / (FC in MH individuals).

The recombination frequency (FR) was calculated as FR = 1 – FM.

3. RESULTS AND DISCUSSION

The SMART assay was performed to evaluate the genotoxic activities of protocetraric, hypostictic, psoromic, and salazinic acids (Figure 1) on the offspring of ST and HB crosses of *D. melanogaster* chronically treated with one of these compounds at 0.75, 1.5, 3.0, and 6.0 mmol L^{-1}.

None of the compounds proved genotoxic, with frequencies of clone formation per cell division ranging from 0.41×10^{-5} to 2.15×10^{-5} for the ST cross and from 0.72×10^{-5} to 3.07×10^{-5} for the HB cross, therefore not differing significantly from negative controls (1.6×10^{-5} for ST and 2.25×10^{-5} for HB crosses) (Figure 2). At the highest concentration, however, only psoromic and salazinic acids proved toxic, significantly reducing survival rates in treated animals ($p \leq 0.05$), compared with the negative control, which yielded negative or inconclusive results at this concentration.

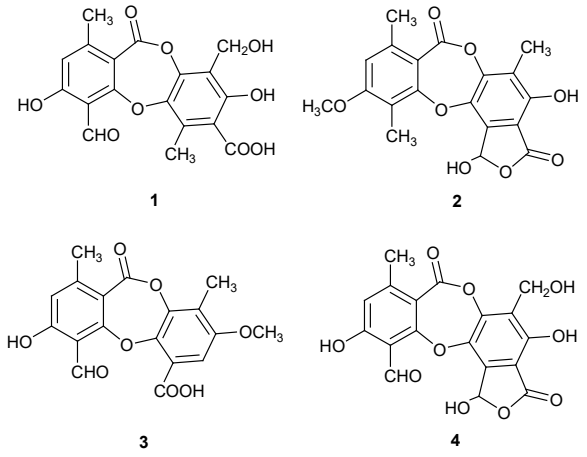

Figure 1. Structures of protocetraric (**1**), hypostictic (**2**), psoromic (**3**), and salazinic (**4**) acids.

Similar genotoxicity levels for ST and HB crosses indicate that the enzyme system involved in cellular detoxification via cytochrome P450 does not interfere with the genotoxic effect of compounds on somatic cells of *D. melanogaster* [24].

The compounds were evaluated not only for their ability to prevent or induce damage in genetic material when employed per se, but also for their ability to prevent DNA damage when administered in association with DXR—an antineoplastic anthracycline antibiotic that damages DNA by interacting with cytosine and guanine, leading to formation of DNA adducts, which may cause sister chromatid exchanges, chromosome aberrations, and interaction with topoisomerase II, preventing religation of double strands, with permanent DNA damage and subsequent non-homologous recombination events [25]. In addition, DXR generates radicals and oxidative stress, facilitating lipid peroxidation and ultimately inflicting oxidative damage to DNA [26].

Figure 3 shows the frequencies of clone formation in the progeny of ST and HB crosses treated with non-toxic 0.75-3.0 mmol L^{-1} concentrations of one of the acids in association with 0.2 mmol L^{-1} DXR (Tables S1-S8, Supplementary Material). Again, a consistent pattern was observed for all four acids, with 72-100% (mostly >80%) inhibition of mutation events caused by DXR in

descendants of ST and HB crosses. Hypostictic acid inhibited mutagenic events by 100% both in the ST cross, when employed at 3.0 mmol L^{-1}, and the HB cross, when used at 1.5 mmol L^{-1} and higher concentrations. Despite their antimutagenic activity, none of the compounds evaluated had significant influence on DXR-induced recombination (Tables S1-S8, Supplementary Material). As revealed in previous studies using the SMART assay, the principal mutational contribution of DXR was related to its ability to induce recombination.

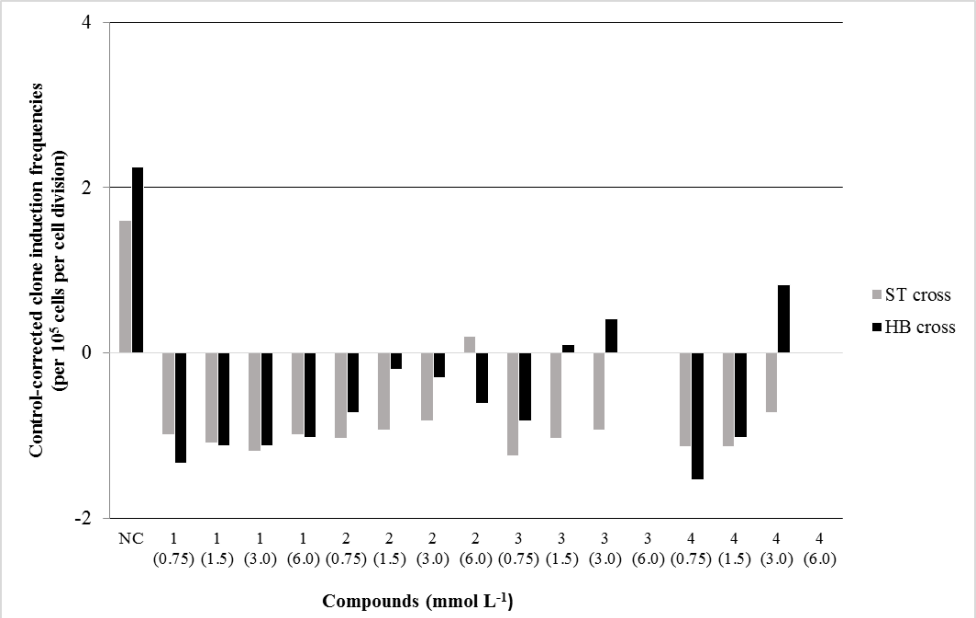

Figure 2. Control-corrected clone induction frequencies for compounds in the SMART test (NC: negative control).

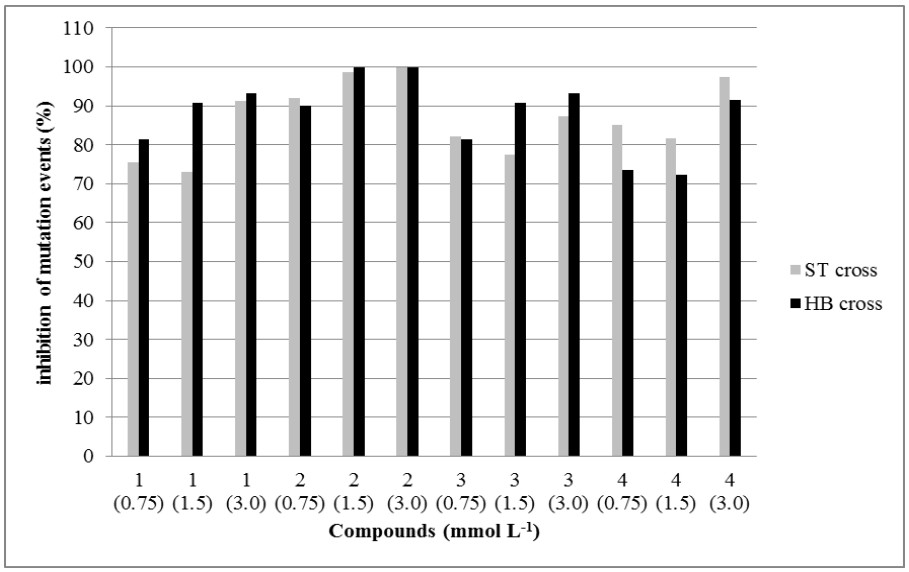

Figure 3. Inhibition of mutation events by compounds tested in association with 0.2 mmol L^{-1} DXR.

DNA changes caused by chemical compounds can trigger a complex carcinogenesis process. In normal cells carrying mutations in malignant genes, loss of heterozygosity by mitotic recombination may unchain a neoplastic mechanism. Loss of a functional copy of a heterozygous tumor suppressor gene represents an important step during neoplastic transformation [27]. Furthermore, mutations that inactivate tumor suppressor genes or alter expression of oncogenes may cause malignant transformation [28]. The compounds evaluated exhibited noteworthy biological activities and elucidating their mutagenic

profiles paves the way for their future use as protective agents against mutagenic events.

4. CONCLUSION

Protocetraric, hypostictic, psoromic, and salazinic acids isolated from Brazilian lichens exhibited antigenotoxic activity when tested on *D. melanogaster* cells, significantly reducing genetic damage caused by DXR. The antibiotic and antitumor activities of these compounds lend them for use in pharmaceutical applications, considering the proven safety of these substances (absence of DNA damage).

5. ACKNOWLEDMENTS

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