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Evaluation of the Safety of Three Phenolic Compounds from *Dipteryx alata* Vogel with Antiophidian Potential

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Abstract

Phenolic compounds from *Dipteryx alata* Vogel were assayed against the *in vitro* neurotoxic effect induced by *Bothrops jararacussu* (Bjssu) venom. Mutagenicity was assessed by the Ames test using *Salmonella typhimurium* strains TA98, TA97a, TA100, and TA102, in experiments with and without metabolic activation. Anti-bothropic activity was obtained by using mouse phrenic nerve-diaphragm (PND) preparation and myographic technique. Control experiments with physiological Tyrode solution were used for keeping the PND preparations alive (n = 4). Concentrations of phenolic compounds were as follow: protocatechuic and vanillic acids (200 µg/mL, n = 4), vanillin (50 µg/mL, n = 4). These compounds were used alone or pre-incubated with the venom (40 µg/mL), 30 min prior the addition to the organ bath (n = 4). Phenolic compounds significantly inhibited the neuromuscular blockade of Bjssu in the following order of potency: vanillic acid > protocatechuic = vanillin. Vanillic acid added 10 min after the Bjssu venom was also able to avoid the venom-blockade evolution. The mutagenicity assay indicated that all phytochemicals were unable to increase the number of revertants, demonstrating the absence of mutagenic activity. This study

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demonstrated both the safety and therapeutical potential of the three phenolic compounds as novel complementary anti-bothropic agents.

Keywords

Ames Test, Baru, *Bothrops jararacussu* Venom, Vanillic Acid, Vanillin

1. Introduction

Natural phenolic compounds have an aromatic ring bearing one or more hydroxyl or etherified substituents, being known due the ability to complex proteins by hydrogen bonding. Among them, compounds such as protocatechuic (1, PCA) and vanillic (2, VA) acids, both universal among the angiosperms [1]; and the aldehyde vanillin (3, VN) have closely related structures (**Figure 1**), which justify the similarity in their biological activity [2].

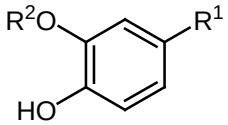
Dipteryx alata Vogel (Leguminosae), a native plant from the Brazilian savannah and popularly known as *baru* [3], contains 18 compounds already identified and among them the three phenolic derivatives (PCA, VA, and VN) of biomedical relevance [4].

The biological activity of these compounds has been characterized, and revealed their potential as antioxidants [5], scavengers of active oxygen species and electrophiles [6], blockers of nitration [7], and metal chelators [8]. Despite of their environmental relevance considering the endangered situation of the Brazilian Cerrado biome, a preliminary survey for biological activities justifies the bio-prospection, due to the potential of *baru* as a source for medicinal use, nutritional food, pharmaceutical, and cosmetic compounds. The controlled bio-prospection could allow the valorization of Cerrado's plants, and their sustainable use, contributing to the environment protection.

One of the medicinal interests on *baru* compounds is their use as anti-ophidian medicine. *Bothrops* snakebites, including the *Bothrops jararacussu* snake, are the most relevant snake accidents in Brazil, not only because the number of accidents, but also by the severity of symptoms, which includes high level of pain, inflammation, hemorrhage and myonecrosis. The attributed clinical signs result from proteases/phospholipases/thrombin-like enzymes and peptides present in the venom [9] [10]. Despite of the systemic antigen-antibody action of the antiserum, the local manifestations of *Bothrops* envenomation are only partially avoided [11]. Thus, strategies to minimize the effects at the bite local would corroborate to avoid unwanted sequels, such as a limb amputation.

Nanotechnology, an innovation of the pharmaceutical sciences, can contribute to the development of a supplementary medicine in order to improve serum therapy [12]. Nevertheless, before this step is achieved, the safety assessment is a crucial protocol.

In this study, PCA, VA, and VN from *Dipteryx alata* were assayed in a pre-incubation model of a mouse phrenic nerve-diaphragm (PND) preparation, used to measure the *in vitro* neuromuscular activity of *B. jararacussu* venom [13]. The mutagenic activity of these compounds were assessed by the *Salmonella* microsome assay (Ames test), using *S. typhimurium* test strains TA98, TA97a (to detect frameshift mutations), TA100 (to detect base-pair-substitution mutations) and TA102 (normally used to detect mutagens that cause oxidative damage and base-pair-substitution mutations), in the presence or absence of *in vitro* metabolizing systems [14]-[16]. Results of genetic toxicological tests, combined with an adequate pharmacology profile, have been used to



1: $R^1 = COOH$, $R^2 = H$
2: $R^1 = COOH$, $R^2 = CH_3$
3: $R^1 = CHO$, $R^2 = CH_3$

Figure 1. The structures of tested antibothropic phenolics. 1: protocatechuic acid (PCA), 2: vanillic acid (VA), 3: vanillin (VN).

approve clinical trials of novel drug candidates [17].

2. Material and Methods

2.1. Plant Material and Extraction

The barks of an adult *Dipteryx alata* Vogel tree were collected in Pedro Afonso (Tocantins, Brazil), and identified by Institute of Agronomy of Campinas. The voucher specimen was deposited (IAC 50629) at the herbarium of Institute of Agronomy of Campinas. The *D. alata* barks (1.269 kg) were dried at 37˚C over 48 h and then powdered, ground in a mill, macerated (200 g, during 5 days) in 2 L of 70% ethanol, being the suspension percolated (under protection against light) at 20 drops/min, resulting in a 20% (m/v) hydroalcoholic extract. Then, the extract was concentrated under reduced pressure and lyophilized, providing a residue of 170 g, reaching 85% of efficiency [18].

2.2. Isolation

Part of the above described residue (50 g) was dissolved in a 80:20 MeOH:H_2O mixture, and partitioned successively with the corresponding solvents to give hexane (1.5 g), dichloromethane (CH_2Cl_2, 18 g), ethyl acetate (EtOAc, 3.7 g) and methanol (MeOH residue, 21 g) fractions. The CH_2Cl_2 fraction was submitted to a silica-gel flash column chromatography and eluted with hexane-EtOAc (9:1 to EtOAc) to give 12 subfractions. These subfractions were further successively flash-chromatographed in silica gel and purified by Sephadex LH-20 column chromatography, eluted with hexane-CH_2Cl_2-MeOH-H_2O (2:2:1) to yield 18 compounds, among them the phenolic derivatives protocatechuic acid (PCA, 1), vanillic acid (VA, 2) and vanillin (VN, 3) [4].

2.3. Compounds Solubilization

In order to use the phenolic derivatives in the pharmacological assays (see below), they were previously solubilized as follows: PCA (compound 1) in 30 µL of dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA); VA and VN (compounds 2 and 3, respectively) in 15 µL of polyethylene glycol (PEG 400). The concentration of the solubilizing agents did not cause changes on basal response of the neuromuscular preparations, according to Cintra-Francischinelli *et al*. [19].

2.4. Pharmacological Assays

2.4.1. Crude Snake Venom

Bothrops jararacussu venom (Bjssu) was collected from two adult specimens kept in the "Serpentário do Centro de Estudos da Natureza"—*Center for Nature Studies Snake Pit*-CEN. The venom was lyophilized and certified by Professor Dr. José Carlos Cogo from University of Vale do Paraiba, Univap, SP, Brazil.

2.4.2. Animals

Male Swiss white mice (26 - 32 g) were supplied by Anilab (Animais de Laboratório, Paulínia, SP, Brazil). The animals were housed at 25˚C ± 3˚C on a 12 h light/dark cycle and they had access to food and water *ad libitum*. This study (protocol number A013/CEUA/2011) was approved by the institutional Committee for Ethics in Research of University of Vale do Paraiba, and the experiments were performed following the guidelines of the Brazilian College for Animal Experimentation.

2.4.3. Mouse Phrenic Nerve-Diaphragm Muscle (PND) Preparation

The phrenic nerve-diaphragm [20] was obtained from mice previously anesthetized with halothane (Cristália, Brazil) and killed by exsanguination. The diaphragm was removed and mounted under a tension of 5 g/cm in a 5 mL organ bath containing aerated Tyrode solution (control) with the following composition (mM): NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 0.49; NaH_2PO_4 0.42; $NaHCO_3$ 11.9; and glucose 11.1. After equilibration with 95% O_2/5% CO_2 (v/v), the pH of this solution was 7.0. The PND preparations were indirectly stimulated with supramaximal stimuli (4× threshold, 0.06 Hz, 0.2 ms) delivered from an electrical stimulator (model ESF-15D, Ribeirão Preto, Brazil) directly to the nerve by bipolar electrodes. Isometric twitch tension was recorded with a force displacement transducer (cat. 7003, Ugo Basile, Italy) coupled to a 2-Channel Recorder Gemini physio-

graph device (cat. 7070, Ugo Basile) via a Basic Preamplifier (cat. 7080, Ugo Basile). The PND myographic recording was performed according to Ferraz *et al*. [21]. PND was allowed to stabilize for at least 20 min before the experiments.

2.4.4. Experimental Protocols

Control PND preparations (n = 4) were submitted to Tyrode nutritive solution in order to maintain them. Other PND preparations were submitted to the following phenolic derivatives concentrations, which were based in previous studies [21]: PCA and VA (200 µg/mL, n = 4), VN (50 µg/mL, n = 4) and *B. jararacussu* venom 40 µg/mL (n = 4). New PND preparations were also pre-incubated with the same concentrations of the phenolic derivatives, during 30 min prior to addition into the organ bath. This assay was carried out in order to verify the ability of the phenolic compounds to neutralize the *in vitro* neurotoxic effect of the Bjssu crude venom (n = 4).

2.5. *In Vitro* Mutagenicity Assay

Mutagenic activity was tested by the *Salmonella*/microsome assay, using the *S. typhimurium* tester strains TA98, TA100, TA102 and TA97a [22], which were kindly provided by B. N. Ames (Berkeley, CA, USA), with and without metabolization by the preincubation method [15]. The strains from frozen cultures were grown overnight for 12 - 14 h, in Oxoid Nutrient Broth No. 2. The S9 fraction, prepared from livers of Sprague-Dawley rats treated with the polychlorinated biphenyl mixture Aroclor 1254 (500 mg/kg), was purchased from Molecular Toxicology Inc. (Boone, NC, USA) and freshly prepared before each test. The metabolic activation system consisted of 4% of S9 fraction, 1% of 0.4 M $MgCl_2$, 1% of 1.65 M KCl, 0.5% of 1 M D-glucose-6-phosphate disodium, 4% of 0.1 M NADP, 50% of 0.2 M phosphate buffer, and 39.5% sterile distilled water [15]. The phenolic compounds of *D. alata* extract were dissolved in DMSO in order to obtain the nontoxic concentrations. The tested concentrations were selected based on a preliminary toxicity test. In all subsequent assays, the upper limit of the dose range tested was either the highest nontoxic dose or the lowest toxic dose determined in this preliminary assay. Toxicity was apparent either as a reduction in the number of histidine revertants (His+), or as an alteration in the auxotrophic background (*i.e.*, background lawn). The concentrations varied from 0.78 to 6.25 mg/plate for PCA, 0.39 to 3.13 mg/plate for VA and 0.1 to 0.78 mg/plate for VN.

All concentrations of the phenolic compounds to be tested were previously added to 0.5 mL of 0.2 M sodium phosphate buffer (pH 7.4), or to 0.5 mL de 4% S9 mixture, with 0.1 mL of bacterial culture and then incubated at 37˚C for 20 min. Next, 2 mL of top agar (0.6% agar, histidine and biotin 0.5 mM each, and 0.5% NaCl) was added, and the mixture was poured on to a plate containing minimal glucose agar (1.5% Bacto-Difco agar and 2% glucose in Vogel-Bonner medium E). The plates were incubated at 37˚C for 48 h and the His(+) revertant colonies were counted manually. All experiments were carried out in triplicate. The standard mutagens used as positive controls in experiments without S9 mix were 4-nitro-*O*-phenylenediamine (10 µg/plate) for TA98 and TA97a, sodium azide (1.25 µg/plate) for TA100 and mitomycin (0.5 µg/plate) for TA102. 2-anthramine (1.25 µg/plate) was used with TA98, TA97a and TA100 and 2-aminofluorene (1.25 µg/plate) with TA102 in the experiments with metabolic activation. DMSO (solvent) was used as a negative control (50 µL/plate).

The mutagenic index (MI) was calculated for each concentration tested, and considered as the average number of revertants per plate obtained by the test compound divided by the average number of revertants per plate in the negative (solvent) control. A sample was considered mutagenic when a dose-response relationship was detected and a two-fold increase in the number of mutants (MI ≥ 2) was observed with at least one concentration [23].

2.6. Statistical Analysis

Each experimental protocol from the pharmacological assays was repeated at least four times and the results are shown as mean ± SEM. The number of experiments (n) is indicated in the legend of each figure. Student's *t*-test was used for statistical comparison of the data and the confidence level was set as 5% (alpha = 0.05). The results of the mutagenicity tests were analyzed with the Salanal statistical software package (US Environmental Protection Agency, Monitoring Systems Laboratory, Las Vegas, NV, version 1.0, from Research Triangle Institute, RTP, North Carolina, USA), adopting the Bernstein *et al*. [24] model. The data (revertants/plate) were assessed by analysis of variance (ANOVA), followed by linear regression.

3. Results and Discussion

The deforestation process and associated factors have been studied. Both science and technology have been used to protect human health and environment, and to promote innovative green-business practices [25]. Plants with medicinal properties take important role in the sustainability concept. This concept creates and maintains the condition in which human beings and nature can coexist in a productive harmony, allowing social, economic and other requirements of the present and future generations [26].

The Brazilian biome known as *Cerrado* has been extensively threatened in the last decades. Many species of plants could disappear even before their medicinal properties could be studied [27]. *D. alata* is a very appreciated specimen by the Cerrado population due to its great value for wood-industry, to recover deforested areas, and specially as a food source [28]. In addition, its medicinal properties as antiophidian agent was previously recognized [4] [21] [29].

The antiophidian properties of three natural phenolic compounds PCA (1), VA (2), and VN (3) found in *D. alata* [4], whose structures are shown in **Figure 1**, is showed here for the first time in the literature.

Vanillic acid is an oxidized form of VN and exhibits more free radical scavenging activity than VN [30]. VA has antioxidant, antimicrobial and anti-mutagenic activities and can exhibit a chemopreventive effect in experimentally induced carcinogenesis in rats [31]-[34].

Moreover, VA can scavenge free radical species, having cardioprotective properties, and it could repress fibrogenesis and inflammation in the chronically injured liver [35]-[37]. VN is used as a flavoring agent in food and cosmetics, having well-studied antimicrobial [38] [39], anti-mutagenic, antioxidant, and anti-carcinogenic activities [39]-[41].

Vanillic acid and PCA are commonly derivatives of hydroxybenzoic acid or benzoic acid. According to Anter *et al*. [42], PCA did not exhibit any genotoxic effect. However, it has an antigenotoxic property against the hydrogen-peroxide effects, exhibiting tumoricidal activity, and apoptosis-induction in HL-60 leukemic cells.

Figure 2 shows the pharmacological effect of the phenolic compounds. VN exhibited bigger potency (around 4×) than VA and PCA, since only 50 µg/mL vanillin was used in comparison to the 200 µg/mL of VA and PCA. VN also exhibited a facilitatory effect measured by increased twitches amplitude, at least during 40 min ($p < 0.05$ when compared to the control group).

Probably the facilitatory effect of VN was associated to its reactive electrophilic character. The ideal phytochemical substance for further neutralization assays with Bjssu could be VA, since it showed the better profile, having no significant difference with control (Tyrode solution). It is important to observe that VN and PCA

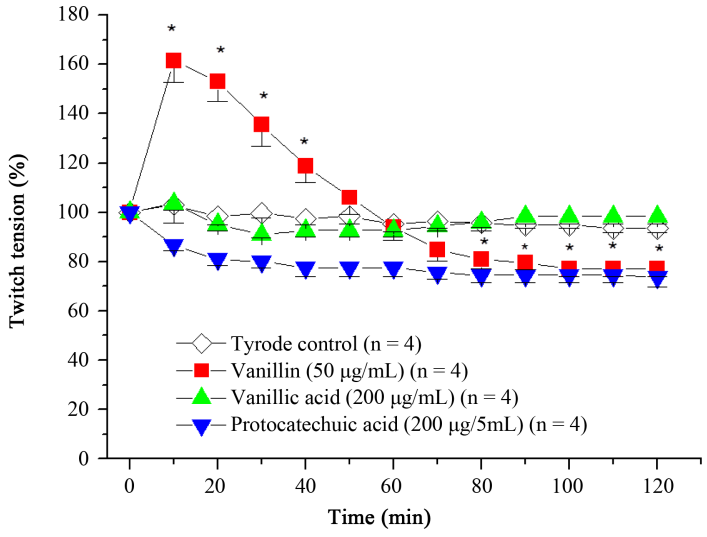


Figure 2. Pharmacological activity evaluation (mouse phrenic nerve-diaphragm preparation, indirect stimuli). The phenolic compounds profile at the selected concentrations and number of experiments (n) are shown in the figure. Each point represents the mean ± SEM. *= $p < 0.05$ in comparison with the Bjssu venom.

showed significant differences when compared to the control group from 80 min to 120 min.

Figure 3 shows the *in vitro* preincubation with each phytochemical prior the addition of Bjssu venom and the effect of the crude Bjssu venom alone. The *in vitro* irreversible neuromuscular blockade of *B. jararacussu* venom (Bjssu) is well-known [13].

Bothrops jararacussu venom has two basic phospholipase A_2 homologues, namely bothropstoxin-I (BthTX-I, a Lys49-PLA2) [43] and bothropstoxin-II (BthTX-II, an Asp49-PLA2) [44] [45]. BthTX-I is considered the main myotoxin from the venom since it is able to reproduce *in vitro* the neurotoxicity and the myonecrosis of the crude venom [43], being this characteristic the main reason of the interest in the myotoxin. BthTX-I has a presynaptic nature at 0.35 µM, which is not sufficient to cause muscle fiber depolarization [46]. The Asp49 to Lys49 substitution in the catalytic center (only in the calcium-binding loop) explains the lack of enzymatic action in BthTX-I, due to the loss of ability to bind Ca^{2+} [47].

Chemically, the mechanism of interaction between the snake venom and the plant includes hydrogen-bonds, electrostatic bonds, Vand der Waals forces, hydrophobic bonds, formation of inactive acid-base complexes protein precipitation and covalent bonds [48]-[52]. The tested phenolic compounds protected the PND preparation against the neurotoxic effect of the venom in the following order: VA > PCA = VN.

Acid-base complexation does not explain PCA activity, since PCA did not show the same ability in neutralizing the venom neuromuscular blockade as VA, and the phenolic groups probably have an important role. The chemical difference between VA and PCA is the methylation of the meta-hydroxyl group. This methylation did facilitate the interaction between the *para*-hydroxyl groups with venom's constituents, making VA a better venom-inhibitor than PCA. PCA has both hydroxyl groups bonded intramolecularly. Interestingly, VA was isolated from the active fraction 7 of *D. alata* against Bjssu [29], showing the importance of biomonitoring studies.

Vanillic acid was also evaluated after 10 min of Bjssu venom action (**Figure 4**), in a post-venom model. Even in this condition, VA was able to counteract the venom myotoxic activity, significantly protecting ($^{*}p < 0.05$) the tissue against the venom damage.

This post-venom model has been commonly used to observe the plant extract potency, in a better mimic model of the ophidian accident than the preincubation model. Hydroalcoholic extracts of leaves from *Casearia gossypiosperma* [53] and *Vellozia flavicans* [54] were validated using the same post-venom model. In all cases, the initial damage induced by the crude Bjssu venom was irreversible, but the damage progression was controlled, conferring an anti-bothropic property to those plants.

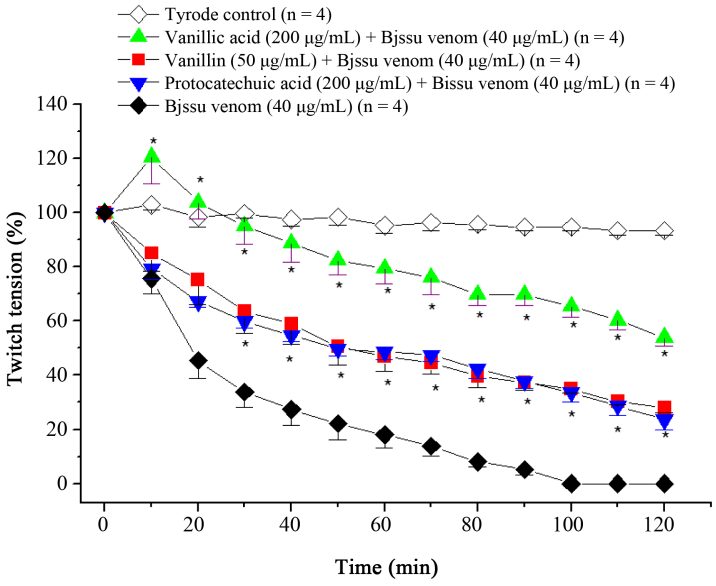


Figure 3. Pharmacological activity evaluation (mouse phrenic nerve-diaphragm preparation, indirect stimuli). Each phenolic compound was preincubated prior Bjssu addition. The concentrations and the number of experiments (n) are shown in the figure. Each point represents the mean ± SEM. *= $p < 0.05$ in comparison with the Bjssu venom.

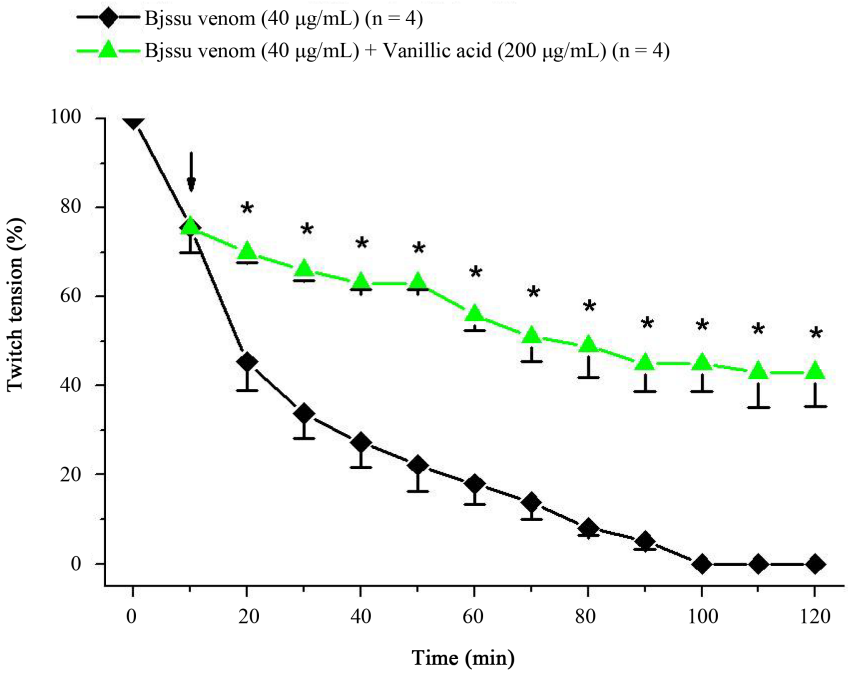


Figure 4. Pharmacological activity evaluation (mouse phrenic nerve-diaphragm preparation, indirect stimuli) of Vanillic acid in a post-venom model. The concentrations and the number of experiments (n) are shown in the figure. Each point represents the mean ± SEM. * = $p < 0.05$ in comparison with the venom. Arrow: time of Vanillic acid addition.

The balance between the therapeutic and toxicological effects of a compound is a very important measure of its usefulness as a drug. Therefore, the determination of the potential mutagenic effect of any drug under development is mandatory [55]. The Ames assay, which is recommended for testing the mutagenicity of chemical compounds with potential pharmacological application [56] was used in the present study.

In previous studies, Esteves-Pedro *et al*. [18] showed that the *D. alata* Vogel extract had no mutagenic effect by Ames test on the strains tested, in either the presence or absence of metabolic activation. To complement the preliminary results [18] and considering the promising results obtained in the present study, the mutagenic activity of the isolated compounds of *D. alata* Vogel extract was also assessed (**Tables 1-3**). These Tables list the mean number of revertants/plate (M), the standard deviation (SD) and the mutagenic index (MI) after the treatments with VA, PCA and VN respectively, observed in *S. typhimurium* strains TA98, TA100, TA102 and TA97a in the presence (+S9) and absence (−S9) of metabolic activation.

The mutagenicity assays show that none of the phenolic compounds induced any increase in the number of revertant colonies compared to the negative control group, indicating the absence of any mutagenic activity. The absence of mutagenicity against *S. typhimurium* bacterial strains in the Ames assay of these compounds is a positive step towards determining its safe use in medicine. Considering the biological properties of these compounds, a lack of mutagenic effect in the bacterial systems tested is highly relevant.

In addition, the genotoxic and anti-genotoxic effects of VA were determinated on mitomycin C-induced DNA damage in human blood lymphocyte cultures *in vitro* by the cytokinesis-block micronucleus test and the alkaline comet assay. The results showed that VA could prevent oxidative damage to DNA and chromosomes when used at appropriate low doses [57]. VA also induced an inhibitory effect on the mutagenicity of 3-(5-nitro-2-furyl) acrylic acid (5NFAA) and sodium azide [58]. Stagos *et al*. [59] evaluated the mutagenicity of the PCA; and the results showed no mutagenic effect and no significant effect on bleomycin-induced mutagenicity. According to Shaughnessy *et al*. [60], VN is a dietary antimutagen that reduces the spontaneous mutant frequency in *S. typhimurium* strain TA104 (*hisG*428, *rfa*, *uvrB*, pKM101) by 50%, when added to assay plates.

Taken together our results, which are also corroborated with data from literature, these phytochemicals are not mutagenic, and they act as antimutagens according to other studies [39] [41] [42] [57] [59]. These results should stimulate new research in order to provide medicines using these safe molecules and nanotechnology to treat

Table 1. Mutagenic activity expressed as the mean and standard deviation of the number of revertants/plate and the mutagenic index (in brackets), for the strains TA98, TA100, TA102, and TA97 of *S. typhimurium* after treatment with phytochemical 4-hydroxy-3-methoxybenzoic (Vanillic acid) isolated from *D. alata* Vogel, with (+S9) and without (−S9) metabolic activation.

Treatments		Number of revertants (M ± SD)/plate and (MI)							
		TA 98		TA 100		TA 102		TA 97a	
	mg/plate	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Vanillic acid	0.0[a]	28 ± 2	24 ± 2	104 ± 15	95 ± 6	271 ± 18	461 ± 21	125 ± 15	96 ± 10
	0.39	28 ± 5 (1.0)	21 ± 3 (0.9)	113 ± 11 (1.1)	95 ± 11 (1.0)	241 ± 25 (0.9)	518 ± 13 (1.1)	98 ± 13 (0.8)	98 ± 5 (1.0)
	0.78	41 ± 2 (1.5)	18 ± 3 (0.8)	115 ± 15 (1.1)	88 ± 14 (0.9)	237 ± 13 (0.9)	522 ± 16 (1.1)	108 ± 10 (0.9)	108 ± 2 (1.1)
	1.56	31 ± 5 (1.1)	21 ± 2 (0.9)	91 ± 7 (0.9)	89 ± 7 (0.9)	268 ± 10 (1.0)	500 ± 20 (1.1)	113 ± 9 (0.9)	103 ± 13 (1.1)
	2.34	27 ± 2 (1.0)	20 ± 1 (0.8)	96 ± 11 (0.9)	84 ± 7 (0.9)	315 ± 8 (1.2)	487 ± 15 (1.1)	96 ± 3 (0.8)	103 ± 18 (1.1)
	3.13	27 ± 5 (1.0)	21 ± 4 (0.9)	99 ± 8 (0.9)	92 ± 12 (1.0)	293 ± 19 (1.1)	471 ± 13 (1.0)	84 ± 6 (0.7)	97 ± 5 (1.0)
	Ctrol+	2064 ± 87[b]	1213 ± 33[e]	1252 ± 124[c]	1870 ± 69[e]	1173 ± 47[d]	1822 ± 102[f]	1968 ± 77[b]	1850 ± 67[e]

M ± SD = mean and standard deviation; MI = mutagenicity index; [a]Negative control: dimethylsulfoxide (DMSO-50 µL/plate); Ctrol+ = Positive control-[b]4-nitro-*o*-phenylenediamine (NOPD-10.0 µg/plate-TA98, TA97a); [c]sodium azide (1.25 µg/ plate-TA100); [d]mitomycin (0.5 µg/plate-TA102), in the absence of S9 and [e]2-anthramine (1.25 µg/plate-TA 97a, TA98, TA100); [f]2-aminofluorene (10.0 µg/plate-TA102), in the presence of S9.

Table 2. Mutagenic activity expressed as the mean and standard deviation of the number of revertants/plate and the mutagenic index (in brackets), for the strains TA98, TA100, TA102, and TA97 of *S. typhimurium* after treatment with phytochemical 3,4-dihydroxybenzoic acid (Protocatechuic acid) isolated from *D. alata* Vogel, with (+S9) and without (−S9) metabolic activation.

Treatments		Number of revertants (M ± SD)/plate and (MI)							
		TA 98		TA 100		TA 102		TA 97a	
	mg/plate	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Protocatechuic acid	0.0[a]	28 ± 2	24 ± 2	104 ± 15	95 ± 6	271 ± 18	461 ± 21	125 ± 15	96 ± 10
	0.78	30 ± 7 (1.1)	24 ± 6 (1.0)	113 ± 17 (1.1)	94 ± 11 (1.0)	273 ± 6 (1.0)	502 ± 34 (1.1)	116 ± 10 (0.9)	105 ± 16 (1.1)
	1.56	26 ± 2 (0.9)	22 ± 5 (0.9)	104 ± 3 (1.0)	100 ± 9 (1.0)	261 ± 18 (1.0)	475 ± 51 (1.0)	121 ± 24 (1.0)	101 ± 4 (1.1)
	3.13	24 ± 2 (0.9)	19 ± 4 (0.8)	100 ± 9 (1.0)	96 ± 6 (1.0)	257 ± 6 (0.9)	492 ± 37 (1.1)	124 ± 12 (1.0)	120 ± 2 (1.3)
	4.69	25 ± 2 (0.9)	21 ± 2 (0.9)	94 ± 19 (0.9)	97 ± 7 (1.0)	299 ± 29 (1.1)	498 ± 7 (1.1)	124 ± 4 (1.0)	113 ± 26 (1.2)
	6.25	32 ± 9 (1.1)	17 ± 3 (0.7)	114 ± 10 (1.3)	103 ± 7 (1.1)	372 ± 10 (1.4)	490 ± 21 (1.1)	107 ± 6 (0.9)	120 ± 18 (1.3)
	Ctrol+	2064 ± 87[b]	1213 ± 33[e]	1252 ± 124[c]	1870 ± 69[e]	1173 ± 47[d]	1822 ± 102[f]	1968 ± 77[b]	1850 ± 67[e]

M ± SD = mean and standard deviation; MI = mutagenicity index; [a]Negative control: dimethylsulfoxide (DMSO-50 µL/plate); Ctrol+ = Positive control-[b]4-nitro-*o*-phenylenediamine (NOPD-10.0 µg/plate-TA98, TA97a); [c]sodium azide (1.25 µg/ plate-TA100); [d]mitomycin (0.5 µg/plate-TA102), in the absence of S9 and [e]2-anthramine (1.25 µg/plate-TA 97a, TA98, TA100); [f]2-aminofluorene (10.0 µg/plate-TA102), in the presence of S9.

several pathological conditions, such as snakebite envenoming.

4. Conclusion

Phenolic compounds from *D. alata* significantly protected the neuromuscular preparation against the irreversible neuromuscular blockade-induced by *B. jararacussu* venom, at different levels: VA > PCA = VN, by unclear mechanisms. VA significantly inhibited the venom-blockade evolution in a post-venom model. Moreover, the results indicated the absence of any mutagenic activity by Ames test; it is important to guarantee its safe use in humans.

Table 3. Mutagenic activity expressed as the mean and standard deviation of the number of revertants/plate and the mutagenic index (in brackets), for the strains TA98, TA100, TA102, and TA97 of *S. typhimurium* after treatment with phytochemical 4-hydroxy-3-metoxibenzaldehído (Vanillin) isolated from *D. alata* Vogel, with (+S9) and without (−S9) metabolic activation.

Treatments		Number of revertants (M ± SD)/plate and (MI)							
		TA 98		TA 100		TA 102		TA 97a	
	mg/plate	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Vanillin	0.0[a]	20 ± 2	30 ± 2	115 ± 7	121 ± 9	313 ± 24	411 ± 17	151 ± 8	143 ± 8
	0.10	20 ± 3 (1.0)	33 ± 3 (1.1)	103 ± 8 (0.9)	142 ± 9 (1.2)	375 ± 15 (1.2)	453 ± 27 (1.1)	171 ± 11 (1.1)	192 ± 6 (1.3)
	0.20	17 ± 1 (0.9)	35 ± 3 (1.2)	111 ± 11 (1.0)	148 ± 11 (1.2)	430 ± 19 (1.4)	496 ± 13 (1.2)	205 ± 22 (1.4)	167 ± 12 (1.2)
	0.39	19 ± 3 (0.9)	30 ± 4 (1.0)	110 ± 2 (1.0)	143 ± 10 (1.2)	422 ± 43 (1.4)	503 ± 14 (1.2)	186 ± 12 (1.2)	163 ± 7 (1.1)
	0.59	16 ± 2 (0.8)	37 ± 3 (1.2)	108 ± 6 (0.9)	144 ± 13 (1.2)	367 ± 26 (1.2)	489 ± 27 (1.2)	167 ± 17 (1.1)	167 ± 5 (1.2)
	0.78	20 ± 2 (1.0)	34 ± 6 (1.1)	108 ± 10 (0.9)	116 ± 3 (1.0)	325 ± 41 (1.0)	445 ± 21 (1.1)	173 ± 7 (1.1)	170 ± 13 (1.2)
	Ctrol+	1319 ± 41[b]	1696 ± 41[e]	1708 ± 27[c]	1480 ± 52[e]	1220 ± 24[d]	1825 ± 55[f]	1875 ± 62[b]	1623 ± 48[e]

M ± SD = mean and standard deviation; MI = mutagenicity index; [a]Negative control: dimethylsulfoxide (DMSO-50 µL/plate); Ctrol+ = Positive control-[b]4-nitro-*o*-phenylenediamine (NOPD-10.0 µg/plate-TA98, TA97a); [c]sodium azide (1.25 µg/ plate-TA100); [d]mitomycin (0.5 µg/plate-TA102), in the absence of S9 and [e]2-anthramine (1.25 µg/plate-TA 97a, TA98, TA100); [f]2-aminofluorene (10.0 µg/plate-TA102), in the presence of S9.

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