Research Paper

# Free radical scavenging activity of *Pfaffia glomerata* (Spreng.) Pederson (Amaranthaceae)

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## ABSTRACT

**Objective:** To evaluate the free radical scavenging and cytotoxic activities of the butanolic (BuOH) extract, methanolic (MeOH) extract and 20-hydroxyecdysone extracted from the roots of *Pfaffia glomerata*.

**Materials and methods:** *Pfaffia glomerata* roots were collected, powdered and extracted with methanol by maceration at room temperature. The extract was concentrated under vacuum, yielding a residue, followed by a butanol extraction. The 20-hydroxyecdysone (EC) was obtained by chromatographic separation of the BuOH fraction. An amount of 10 mg of each dry extract and EC was dissolved in 0.1% dimethyl sulphoxide–phosphate-buffered-saline solution (DMSO–PBS) and screened for their capabilities on scavenging thiobarbiturate reactive substances (TBARS). The antioxidant activity of each extract was determined *in vitro* by measuring malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in erythrocyte ghosts treated with ferric-ascorbate. The investigation has also included the cytotoxicity measurement by Trypan blue exclusion test and tetrazolium reduction assay in mice peritoneal macrophages.

**Results:** The free radical scavenging activity of EC was higher than that present in the BuOH fraction. The MeOH extract showed a remarkable pro-oxidant activity. The EC-free radical reaction–inhibition was almost twice of that of the control α-Tocopherol (αT). The Trypan blue exclusion assay confirmed toxicity of the MeOH extract, whose lethality surpassed 80% of the treated macrophages after 1 h of 0.01 mg exposure per $10^6$ cells.

**Conclusions:** The present study shows the antioxidant effect of the Brazilian Ginseng. The scavenging effect was evidenced for EC as well the BuOH fraction. The MeOH extract showed cytotoxicity on mice peritoneal macrophages. Such toxicity is probably due to ginsenosides present in this latter fraction and warrants further toxicological evaluation of the Brazilian Ginseng roots.

KEY WORDS: Antioxidant, bio-protective; Brazilian Ginseng; cytotoxicity; free radical scavenger.

## Introduction

Like other medicinal plants, there are difficulties in the botanical identification of the genus *Pfaffia*, in which the popularly known Ginseng is included. *Pfaffia glomerata* (Brazilian Ginseng) is currently commercialized as *Pfaffia paniculata* (unpublished data). Oral administration of powdered roots of *P. paniculata* inhibits the growth of allogenic cancer cells in mice.[1],[2] *Pfaffia glomerata* roots are used as a tonic and aphrodisiac[3] as well as in the treatment of diabetes[4] and inflammatory diseases.[5],[6] Being one of the most popular herbs in Brazil, many users claim that there is no cytotoxicity attributed to *P. glomerata*, which might have an antioxidant property.[7] Its medicinal activity is attributed to the glomeric acid, a triterpenoid, and pfameric acid, a nortriterpenoid together with ecdysterone, rubrosterone, oleanolic acid and beta-glucopyranosyl oleanolate that were isolated from the roots of *P. glomerata*.[8] Nevertheless, there exists a controversy that members of this genus might cause oxidative stress because of their chemical properties or due to their

metabolites.[3],[9],[10] This concern is related to some of its constituents, which, if present in the crude extracts, may have a potential toxicity.[3],[9] Although extracts from the roots of *P. glomerata* seem to have a central nervous system depressant activity,[11] a protective effect against induced neurotoxicity has been related to some of its constituents.[12] The aqueous extract protects the gastric mucosa and inhibits gastric acid secretion in rats.[13] However, the presence of certain plant constituents may be responsible for the diverse array of cellular insults[10],[14] that could produce effects ranging from altered physiology to cell death.[3],[9],[10] The occurrence of these substances might reinforce the contraindication of the Brazilian Ginseng for those suffering from allergy, depression, drug addiction, hormonal imbalance, pregnancy, or cardiovascular diseases.[3],[9],[10],[15]–[17],[19] In the present study, we examined the free radical scavenging properties and the antioxidant activity of 20-hydroxy-ecdysterone (Fig. 1) as well as of the methanolic (MeOH) and butanolic (BuOH) extracts from the roots of *P. glomerata*. The cellular viability of normal mice macrophages exposed to constituents of the plant was also evaluated.

## Materials and methods

*Plant*

*Pfaffia glomerata* roots were collected in January 2000 in Icaraíma/Paraná State, Brazil, and identified by a botanist (W. M. Kranz, personal communication). A voucher specimen (No. 18695) was deposited at the Herbarium of the Universidade Estadual de Londrina (FUEL), Brazil.

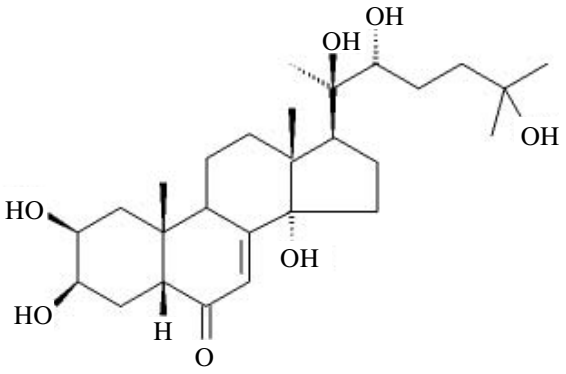
*Preparation of extract*

Powdered roots (500 g) were extracted with methanol by maceration at room temperature. The solvent was removed by distillation under vacuum to yield a 55 g solid residue (MeOH extract). The crude residue was dissolved in MeOH/$H_2O$ (9:1) and extracted five times using 200 ml *n*-butanol. The *n*-butanol was removed and concentrated to dryness giving rise to a solid residue (BuOH fraction). The BuOH fraction (3 g) was eluted with $H_2O$, MeOH and ethyl-acetate (AcOEt) in an Amberlite XAD-2 resin generating 12 fractions. Fractions 5 and 6 were eluted with methanol in a Sephadex LH-20 chromatography column. It gave 50 mg of purified 20-hydroxyecdysone (EC). The collected fractions were evaporated to dryness and analyzed by silica gel TLC and visualized by UV (254 and 366 nm) and iodine vapor.

*Screening*

In this assay, a malondialdehyde (MDA) or a 4-hydroxynonenal (4-HNE) 10 mM standard was used to construct standard curves (from 2 to 20 nmol/ml) against which unknown samples were plotted.[19]–[22] The TBARS was expressed in terms of MDA or 4-HNE concentrations in nmol/ml. All the samples were run in duplicate. The lipid peroxidation was carried out in erythrocyte ghosts by the action of a ferric-ascorbate induction. The TBARS were detected by the thiobarbituric acid method.[21] The fractions and extracts, used as dry material, were solubilized in 1 ml 10% dimethyl sulphoxide (w/v) and subsequently diluted to 1:10 (v/v) with phosphate buffered saline (0.1 M PBS, pH 7.2). The screening was carried out by oxidizing a sample material at 37°C for different intervals (30, 60, 90, 120 and 180 min) and measuring the MDA and the 4-HNE content 3 min after and

**Figure 1.** Structural formula of 20-hydroxyecdysone




60 min before the compound exposure.[7],[8],[20],[22] Samples were incubated and compared with a distilled water control in order to calculate the percentage of inhibition of malonyldialdehyde (MDA)[20],[21] and MDA plus 4-HNE.[20] Appropriate vehicle control constituted of 1% dimethyl sulphoxide subsequently diluted 1:10 (v/v) with PBS. α-Tocopherol (αT) 2000 IU was used as a positive control.

*Malonyldialdehyde (MDA) determination*

In the MDA assay, 0.65 ml of 10.3 mM *N*-methyl-2-phenyl-indole in acetonitrile was added to 0.2 ml of the previously stimulated systems (1.6 mg/ml erythrocytes ghosts, 2 mM deoxyribose, 100 mM $FeCl_3$, 100 mM ascorbate, 20 mM $KH_2PO_4$–KOH buffer, pH 7.4) either in the presence or in the absence of 0.5 ml extracts and (or) αT. After vortexing for 3–4 s and adding 0.15 ml of HCl 37%, samples were mixed well, closed with a tight stopper, and incubated at 45 °C for 60 min. The samples were then allowed to cool on ice, centrifuged, and the absorbance measured spectrophotometrically at 586 nm.

*Malonyldialdehyde plus 4-Hydroxynonenal (MDA + 4-HNE) determination*

The MDA + 4-HNE were determined spectrophotometrically at 586 nm and expressed as micromolar using a calorimetric assay for lipid peroxidation.[20] Briefly, 0.65 ml of 10.3 mM *N*-methyl-2-phenyl-indole in acetonitrile was added to 1 ml of previously incubated solutions containing 0.2 ml of erythrocytes ghosts, 5 mM $FeSO_4$, 500 mM ascorbate, 2 mM octanoic acid in 0.1 M Tris–HCl, pH 7.4 and 0.5 ml of each sample, as described before. After vortexing for 3–4 s 0.15 ml of 15.4 M methanesulfonic acid was added and the samples were mixed well and incubated at 44 °C for 40 min. The samples were then allowed to cool on ice, centrifuged, and the absorbance measured spectrophotometrically at 586 nm.

*Erythrocyte ghosts (EG)*

Albino mice red blood cells (AMRBC) pellet was washed for leukocyte separation using 20 ml of TRIS buffer, pH 7.4 (6.05 g Tris, 6.42 g NaCl, 420 ml 0.1 M HCl, 580 ml de-ionized water) and centrifuged at 1600 g for 10 min.[9],[10] The superior phase was discarded and the procedure was repeated twice. An equal volume of TRIS buffer was added to final pellet and incubated for a minimum of 4 h at 4°C. Lysis of erythrocytes was performed on ice with precooled conditions. A 15 ml resealing solution (301 mg $MgSO_4$, 372 mg KCl in

500 ml of sterile water) was added to 0.5 ml of the cell suspension. Immediately, 1 ml of a stabilizing solution (53.7 g KCl, 10.5 g NaCl in 400 ml of deionized water) was added and the suspension was kept on ice for 5 min and then at 37°C for 30 min. The ghosts were centrifuged for 10 min at 3000 g and kept under refrigeration for further use.

*Cytotoxicity measurement* [16]

Cytotoxicity was measured by the Trypan blue exclusion assay.[15] Different concentrations of the EC, BuOH fraction and MeOH extract (0.1, 1, 10 and 100 $\mu$g/ml) were added to $10^6$ peritoneal macrophages maintained at culture conditions (37±1°C, 5% $CO_2$) for 1 h. Cells were washed and incubated again in the absence of the extract fractions for 24 h. An equal volume of the colorant Trypan blue (6.2 mM, NaCl 0.8 M) was then added and gently mixed. After 2 min, cells were counted using a hemacytometer. The percentage of dye-exclusion was also carried out immediately after the first cell washing. All the assays were performed five times and compared with a control system composed of cells added to vehicle instead of EC, BuOH fraction, or MeOH extract. The mitochondrial activity of the recently dead cells, which do not reduce significant amounts of tetrazolium salt (MTT), was also measured. The cells were cultured in a 96-well flat-bottom plate at the concentration of $10^6$ macrophages per milliliter and after 12 h of preconditioning, the cells were treated with various concentrations of EC, BuOH fraction, or MeOH extract for 24 h. Thereafter, culture medium was aspirated and 100 $\mu$l of 1 mg/ml MTT-PBS (w/v) was added to the cultures and further incubated for 4 h at 37 °C. The formazan crystals made due to dye reduction by viable cells were dissolved using acidified isopropanol (0.1 N HCl). The mitochondrial activity was estimated by measuring the optical density (OD) of color produced by MTT dye reduction at 570 nm.[15],[16] The level of blue color development in the control wells was designated as 100% viability and all further comparisons were based on that reference level. Blank values, indicating the absorbance of MTT and vehicle (1:1) were subtracted from all samples.

*Statistical analysis*

The results are expressed as mean±SD and mean±SEM for cytotoxicity and for the free radical scavenging activity assays respectively. The data were analyzed by a one-way analysis of variance (ANOVA). Pair-wise multiple comparisons were performed using the Student–Newman–Keuls (SNK) multiple comparison test to detect significant difference ($P$ <0.05) between the values that had more than two groups. For comparison of data between two groups, the Student's *'t'* test was carried out to detect any significant difference ($P$ <0.05). Correlations were found by Pearson's correlation coefficient in bivariate correlations. The statistical analysis was carried out using the Instat software (GraphPad, San Diego, CA, USA).

## Results

The scavenging assays presented a statistically significant interaction between the factors of treatment and time, based on the generation of MDA ($P$ = 0.004) and MDA + 4-HNE ($P$ < 0.001). Significant differences on the free radical contents were established by considering the time and proportional antioxidant capability of each sample (Fig. 2). There was no significant effect due to the vehicle control on the generation of any species of thiobarbituric-acid-reactive-substances (TBARS), MDA or HNE. We observed that the control, constituted of red blood cell ghosts added with $Fe^{2+}$-ascorbate incubated for 60 min at 37 °C ($control_{60\ min}$), augmented between 1.06 and 4.73 times its content of TBARS after 3 min incubation ($control_{3\ min}$). The addition of 2000 IU αT reduced the generation of MDA and MDA + HNE by 4.26 and 2.08 times, respectively. We detected a significant decrease in MDA and HNE content, after the addition of BuOH fraction and of the purified EC. The addition of the MeOH extract enhanced MDA and the MDA + HNE by 4.69 and 1.65 times, respectively. In this case, the association of 2000 IU of αT was not effective in decreasing TBARS concentration significantly. The BuOH fraction doubled the elimination of the initial MDA production, independent of being added to αT. Similar result was observed with the EC treatment, where the MDA was reduced by 1.1 times. The highest scavenging activity was observed in the system in which the 4-HNE was measured. The BuOH fraction presented an additive effect with αT, reducing 5.76 times the free radical contents in the system. The TBARS were reduced 4.2 and 3.62 times by the treatments with EC and BuOH fraction, respectively. The antioxidant activities of the systems added with the BuOH fraction and with αT could similarly maintain the antioxidant protection for 1 h. The system treated with EC conserved its scavenging capability for, at least, 2 h (data not shown). We detected 76% of cellular death on the Trypan blue exclusion assay, when mice macrophages were exposed to the concentration of 10 $\mu$g per $10^6$cells MeOH extract per milliliter for 1 h. After 24 h incubation cell lethality was 80%. The treated cells had the typical apoptotic appearance (data not shown) and caused a concentration-dependent inhibition of MTT reduction (Table 1). The difference between control and treated cells was statistically significant at 10 μg MeOH extract per $10^6$ macrophages ($P$ < 0.01). Cells treated with the MeOH extract showed mitochondrial activity, which was reduced to 14% when compared to either the BuOH fraction or purified EC. No toxicity observed following the treatments with EC or BuOH (Table 1).

## Discussion

In the present study, we showed that apart from 20-ecdysone (EC) and the BuOH fraction of *P. glomerata*, the MeOH

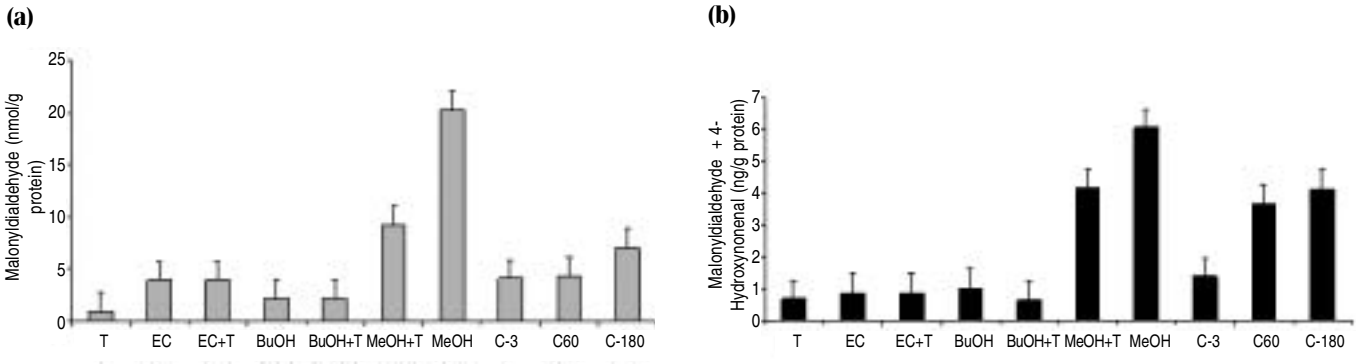
**Table 1**

**Cytotoxicity upon mouse peritoneal macrophages exposed to different extracts of *P. glomerata* roots**

| *System* | *mg per $10^6$ cells* | *Lethality *(%) After 1 h* | *After 24 h* | *Mitochondrial activity (%)* |
|---|---|---|---|---|
| EC | 10 | 0.9±0.03$^{\dagger}$ | 4.2±0.3$^{\S}$ | 100$^{\dagger\dagger}$ |
| BuOH | 10 | 1.1±0.82$^{\dagger}$ | 5.5±0.5$^{\S}$ | 100$^{\dagger\dagger}$ |
| MeOH | 0.01 | 76±1.03$^{\ddagger}$ | 83.0±1.22$^{\P}$ | 14 |
| Vehicle * | 0.0 | 0.7±0.09$^{\dagger}$ | 5.0±1.1$^{\S}$ | 100$^{\dagger\dagger}$ |

Values are mean±SD. * Vehicle-control constituted of 1% dimethyl sulphoxide subsequently diluted 1 : 10 (v/v) with PBS. $P$ < 0.01 in comparison to Control, MeOH, BuOH and EC (One-way ANOVA). $^{\dagger,\ddagger,\S,\P,\dagger\dagger}$ Groups that share a superscript were not significantly different.

**Figure 2a and 2b.** Scavenging activity of *P. glomerata* against malonyldialdehyde (MDA) (A) and malonyldialdehyde plus 4-hydroxinonenal (MDA+4-HNE) (B) generated in red blood cells ghost with $Fe^{2+}$-ascorbate free radicals generation system after 60 min at 37°C following the addition of 2000 UI/ml αT, or of 10 mg/ml ecdysone (EC), EC plus 2000 UI/ml αT (EC+T), butanolic fraction (BuOH), BuOH plus 2000 UI/ml αT (BuOH+T), methanolic extract (MeOH), MeOH plus 2000 UI/ml αT (MeOH+T). C-3, C-60 and C-180 represent the MDA and MDA+4-HNE generated in the vehicle-control system 3 min before and 60 and 180 min after incubation in the absence of other substances. Values are expressed as mean±SEM. $P$ <0.05.




extract did not demonstrate any antioxidant activity *in vitro*. At a concentration of 10 μg/$10^6$ peritoneal macrophages, MeOH extract induced a strong cytotoxic effect. It seems that MeOH extract induced the generation of free radicals with significant morphological scores of all cells treated, rather than an expected protection against any kind of TBARS in particular. Although its constituents were not individually identified, this should not affect the conclusion of the experiment because all the systems were treated the same, except for the exposure to EC, BuOH fraction or MeOH extract. The importance of the concentration of 10 $\mu$g/$10^6$ peritoneal macrophages MeOH extract for living organisms is indeed uncertain. We were unable to retrieve any information on the concentration of toxic constituents in the Brazilian Ginseng from the medical literature. The few toxicological studies of Ginseng constituents[5],[9],[10],[20] suggest that ginsenosides could decrease the antioxidant activity. Further investigations are necessary to evaluate the chemical constitution and pro-oxidant activity of the constituents in the MeOH extracts of *P. glomerata*. The relative content of polar substances, such as hydroxylated radicals, to whose presence the TBARS generation might be involved, is largely variable among different commercially available Ginseng products.[24] These variations result in different pharmacologic properties, and sometimes even antagonistic. Hence, it is not surprising to find significant variation in cytotoxicity among EC, the BuOH fraction and the MeOH extract. Since the Brazilian Ginseng might contain many types of ginsenosides, future studies should evaluate the contribution of various ginsenosides towards toxicity. The absence of mitochondrial function (MTT assay) observed on macrophages treated with the MeOH fraction, reinforced its capability of generating defects in apoptosis signaling pathways. Our findings show that Brazilian Ginseng content may also possess cytotoxic property contraindicating its popularity as a crude-powder,[3],[9],[10] which may pose concerns on the safety of *P. glomerata* for treating oxidative stress. Although *in vitro* toxicity may not reflect the circumstances in humans further investigation and monitoring of the adverse effects of Ginseng are warranted. Before more information becomes available, it may be considered that the use of Brazilian Ginseng should be undertaken with caution. Information may also be collected regarding the possible adverse effects in those who had consumed the Brazilian Ginseng.

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