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"Brazilian ginseng" (*Pfaffia glomerata* Spreng. Pedersen, Amaranthaceae) methanolic extract: cytogenotoxicity in animal and plant assays

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ABSTRACT

Pfaffia glomerata (Spreng) Pedersen is a medicinal plant largely used as an adaptogenic herb. The species is naturally found in Africa and Americas and is the subject of pharmaceutical and commercial interest, mainly due to the accumulation of β-ecdysone in its roots. Brazil is considered the greatest supplier of *P. glomerata* in the world. Due to the morphological similarity of its roots to those of *Panax ginseng* (Korean ginseng), the species became known as the Brazilian ginseng. In this study, we performed the cytogenotoxic evaluation of *P. glomerata* root extract in rodent bone marrow micronucleus and *Allium cepa* assays. Taking into consideration the therapeutic dosage of *P. glomerata*, three methanolic extract dilutions were used in the Micronucleus assay. Each animal was treated with two consecutive doses with a 24-h interval. To assess the cytogenotoxicity of the extract, 2000 polychromatic erythrocytes per animal were analyzed. For the *Allium cepa* assay, onion seeds were exposed during 24 h to six methanolic extract dilutions. Cell cycle and chromosomal alterations were quantified. The results of the Micronucleus assay showed genotoxic effects for all treatments investigated, considering the increase in the number of micronucleated cells. In the *A. cepa* assay the methanolic extract induced alterations in the cell cycle. Aneugenic and clastogenic effects were observed. The results indicate cytogenotoxic activity of *P. glomerata* extract in animal and plant cells. Thus, the ingestion of *P. glomerata* for medicinal purposes should be done carefully. The results also lead to the possibility of investigating the potential of the species in the research of prospective compounds for cancer treatment and for isolation of biologically active substances.

1. Introduction

In spite of the multiplicity of definitions of natural product-derived drug, it is plausible to affirm that between 25% and 50% of the currently marketed drugs have their origins in natural products (Kingston, 2011).

The use of natural products may elicit harmful effects depending on their incorrect use. On the other hand, these effects can also be investigated as means of discovering and isolating new biologically active substances. Possible adverse effects caused by the use of medicinal plants or natural products are related to cell cycle disorder or genetic material instability (known as cytogenotoxic effects). Several animal and plant assays are currently available, allowing the proper evaluation of those effects. The *in vivo* rodent bone marrow micronucleus assay is widely used for genotoxicity assessment (Hwang and Kim, 2012; Rebouças et al., 2013; Alves et al., 2014).

In addition to the animal assays, different plant models have been largely used to evaluate DNA damages (Leme and Marin-Morales, 2009). In the *Allium cepa* assay, it is possible to observe cellular, chromosomal and cell cycle alterations (Leme and Marin-Morales, 2009).

Currently, stress or stress-related disorders have been considered as the major cause of several diseases (Pawar and Shivakumar, 2012). Medicinal plants, especially those classified as adaptogens, constitute an important alternative to treat different disorders (Mendes and Carlini, 2007; Pawar and Shivakumar, 2012). The best-known example of an adaptogen plant is the Korean ginseng (*Panax ginseng* C.A. Mey.).

Some species of the genus *Pfaffia* (Amaranthaceae), such as *P. glomerata*, are known as Brazilian ginseng due to the morphological similarity of its roots to those of *Panax ginseng* as well as to the comparability of their pharmacological use (Zimmer et al., 2006; Mendes, 2011). *P. glomerata* occurs naturally in Africa and the Americas, being

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widely distributed in the Brazilian territory (Gomes et al., 2010). Additionally, Brazil is considered the most important center of assessment of *P. glomerata* roots for medicinal, nutritional and cosmetic purposes (Gomes et al., 2006).

P. glomerata has been reported to be used in the treatment of rheumatism and diabetes, as well as tonic and aphrodisiac (De Oliveira, 1986; Freitas et al., 2004; Marques et al., 2004; Neto et al., 2005). Numerous medicinal applications have been documented for the species, such as gastro-protectant (Freitas et al., 2003), healing (Carneiro et al., 2010), analgesic and anti-inflammatory (Neto et al., 2005) and hypoglycemiant (Sanches et al., 2001). Until the present moment, the main components isolated from *Pfaffia* spp. roots are stigmasterol, sitosterol, allantoin, pfaffic acid and their glycosides (nortriterpenoid saponins) (Nakai et al., 1984; Nishimoto et al., 1984; Shiobara et al., 1993a, 1993b). In *P. glomerata*, different substances have been reported: triterpenoid (glomeric acid), nortriterpenoid (pfameric acid), ecdysterone, rubrosterone, oleanolic acid and glucopyranosil oleanolate (Shiobara et al., 1993a). β-ecdysone (ecdysteroid) has been found only in *P. glomerata* and, therefore, has been considered as a chemical marker for the species (Shiobara et al., 1993a; Zimmer et al., 2006). Our research group has shown in previous article that β-ecdysone is genotoxic in rodent bone marrow and *Allium cepa* assays (Neves et al., 2016).

Despite the importance of the species, studies that evaluate the cytogenetoxic effects of *P. glomerata* extracts are incipient.

The present study reports the cytogenotoxicity evaluation of *P. glomerata* root methanolic extract in bone marrow cells of Wistar rats and meristematic cells of *Allium cepa.*

2. Material and methods

2.1. Plant material

Pfaffia glomerata (accession number 46) was kindly provided by Dr. Wagner Campos Otoni (Federal University of Viçosa-MG, Brazil). The material was cultivated at the Plant Experimental Station of Federal University of Juiz de Fora-MG (Brazil) in greenhouse with controlled conditions. Voucher material was deposited at the Leopoldo Krieger Herbarium-UFJF-Brazil (number: CES 63317).

2.2. Methanolic extract preparation and β-ecdysone content determination

Plant roots with 1 year of cultivation were collected, washed thoroughly with tap water, dried (at 45 °C), cut into small pieces, pounded and then macerated at room temperature in methanol (10%, w/v) for approximately 15 days. The solvent was renewed every 3 days. The resulting mixture was filtered and the filtrate was concentrated by methanol evaporation at 45 °C under reduced pressure using a rotary evaporator (Büchi Rotavapor® R-114TM, Switzerland). The extract was further concentrated standing it for one week in a glass recipient containing silica. Qualitative analysis of β-ecdysone and quantitation of its content was determined by the HPLC fingerprint analysis on a Shimadzu® (Tokyo, Japan) chromatography system composed by diode array SPD-MP 10A vp detector set at 248 nm, degasser DG14-14A, quaternary pumps FCV-10 ALVP, controller LC-10ATVP and automatic injector SIL-10AF. The separation was performed in an ACE C18 (150 × 4.6 mm, 5-μm particle size) column (Nova Analítica®, São Paulo, Brazil) at room temperature. The mobile phase consisted of a mixture of water and acetonitrile (84:16, v/v) at a flow rate of 1.2 mL min^{-1} in an isocratic elution, and volume of injection of 20 μL. As a reference standard, β-ecdysone (CAS 5289-74-7, Sigma®, USA — nominal content of 93%) was used. Stock solutions of reference standard and *P. glomerata* methanolic extract (final concentration = 1000 μg mL^{-1}) were diluted in a mobile phase, filtered through a 0.45-μm filter membrane (Millipore®, USA) and transferred to HPLC vials. Both solutions were assayed in duplicate, and peak areas corresponding to β-ecdysone were compared to the calibration curve allowing the amount of β-ecdysone to be quantified. The calibration curve was obtained from linear least-squares regression analysis plotting the peak area of *P. glomerata* methanolic extract *versus* the reference standard concentrations. Data acquisition and interpretation were performed using CLASS-Vp version 5.42 software.

2.3. Experimental design

Two assays were employed for cytogenotoxicity evaluation: Rodent bone marrow micronucleus and *Allium cepa* assays.

2.3.1. Rodent bone marrow micronucleus assay

The daily dose of β-ecdysone recommended for human (considered therapeutic) is 0.0823 mg/Kg (e.g. *Pfaffia glomerata*, Brazilian Ginseng Herbarium, Registry in the Ministry of Health-Brazil 118600027). Considering the correlation between the weights of humans and rats, a methanolic extract dilution containing the same amount of β-ecdysone was tested in rodents: methanolic extract dilution (MED) at 1.2798 mg/mL (treatment M1). This solution contains 0.0247 mg/mL of the β-ecdysone (1.93% of the methanolic extract, see results). Each animal with 300 g (weight) received 1 mL of this solution resulting in the exposure to the same dose used in humans (0.0823 mg/Kg) (treatment M1). The solution volume received by each animal was adjusted according to its weight (between 0.95 and 1.09 mL). Additionally, two other treatments were investigated: 5 × M1 (M2): 0.4115 mg/Kg and 10 × M1 (M3): 0.8230 mg/Kg of β-ecdysone present in Methanolic extract dilutions. The volume received by each animal was calculated in the same manner described for the M1 treatment.

2.3.2. Allium cepa assay

Six methanolic extract dilutions (MEDs) (8, 16, 32, 64, 128 and 256 mg/mL, called M4 to M9 to distinguish from M1 to M3, were investigated.

2.3.3. Rodent bone marrow micronucleus assay

Twelve-week old male Wistar rats (*Rattus norvegicus Berkenhout, 1769*) were used in the experiment. Animals weighing 285–327 g were obtained from the vivarium of the Federal University of Juiz de Fora (UFJF). The animals were kept in cages, in a climate-controlled environment (23 ± 2 °C) with a 12-h light/dark cycle and 40–60% of relative humidity. Food (Nuvital, Colombo, PR, BR) and water were available *ad libitum*. The Wistar rats were randomly divided into five experimental groups. A group of five animals was used to evaluate each treatment (M1, M2, and M3). The remaining two groups were used as negative and positive controls (distilled water and MMS — 80 mg/kg, respectively).

The animals were submitted to the treatments for two consecutive days with a 24 h interval. The animals were euthanized by total exsanguination under anesthesia (intraperitoneal injection of 5% xylazine — 10 mg/Kg and 2% ketamine — 90 mg/Kg solution) 24 h after the final dose. This study was carried out in strict accordance with the internationally accepted principles for laboratory animal use and care (US Guidelines, NHI publication #85-23, revised in 1985). All procedures with animals were approved by Ethics Committee on Animal Experimentation of Federal University of Juiz de Fora, Minas Gerais, Brazil (protocol number: 61/2013).

For micronuclei analysis, the bone marrow from one femur of each animal was flushed out using saline solution and centrifuging for 5 min (1500 rpm). The supernatant was discarded and smears were made on clean slides. The slides were fixed with ethanol and stained with Giemsa (Gollapudi and Kamra, 1979). In order to evaluate the genotoxic effects of *P. glomerata* methanolic extract, 2000 polychromatic erythrocytes (PCE) per animal were analyzed and the micronucleated cells were counted. Possible cytotoxic effects were investigated by the PCE:NCE (polychromatic: normochromatic erythrocytes) ratio in 1000 erythrocytes per animal. The slides were coded and blindly scored

using a light microscope at 1000× magnification. The mean number of micronucleated polychromatic erythrocytes (MnPCE) in individual rats was used as the experimental unit. The standard deviation was calculated based on the differences among animals within the same group.

2.3.4. Allium cepa assay

Seeds of *A. cepa* cv. Baia Periforme (Feltrin) were pre-exposed to distilled water for root emergence and later submitted to extract treatments during 24 h (M4 to M9).

Distilled water and 4×10^{-4}MMMS (Metil Methanesulfonate, Sigma®, USA) were used as negative and positive controls, respectively.

After exposure, nine root tips (ranging from 1.0 to 1.5 cm) were collected from each treatment, washed in distilled water and fixed in fresh cold ethanol:acetic acid (3:1) solution. The treatments were arranged in a completely random design with three repetitions (three meristems were analyzed per repetition). The slides were prepared by the squashing technique. Briefly, the roots were hydrolyzed in HCl 5N for 20 min; the meristematic regions were covered with coverslips and carefully squashed into a drop of 45% acetic acid solution. The coverslips were removed with liquid nitrogen and the slides were stained with 5% Giemsa (Merck®, Germany). For mitotic indices analysis, six fields of each slide were randomly chosen and analyzed under a stereomicroscope (40× objective). At least 300 cells were analyzed in each field, totaling 1800 cells per slide. With the purpose of estimating the amount of chromosomal abnormalities, all dividing cells of each slide were evaluated. The following abnormalities were quantified: chromosome adherence, c-metaphase, later segregation, multipolarity, chromosome losses, fragments, bridges and micronuclei.

2.4. Statistical analysis

The data are shown as mean ± standard deviation (SD). Statistical evaluation of the data was performed by one-way analysis of variance (ANOVA) followed by the Dunnett's test ($p < 0.05$).

3. Results

3.1. β-ecdysone quantification

The retention time of the β-ecdysone standard and the compound in the methanolic extract sample was the same (1.23 min) and the peak areas of the chromatogram were 4,105,133 mA and 369,559 mA respectively for these samples (Fig. 1). Considering the purity of the β-ecdysone standard (93%) (CAS 5289-74-7, Sigma®, USA) it was possible to calculate the β-ecdysone content in the methanolic extract of *P. glomerata* (1.93%). Considering this value, each methanolic extract ilutions (MEDs) here investigated had the following amounts of β-ecdysone: M1 (0.0247 mg/mL), M2 (0.1235 mg/mL), M3 (0.2470 mg/mL), M4 (0.1544 mg/mL), M5 (0.3088 mg/mL), M6 (0.6176 mg/mL), M7 (1.2352 mg/mL), M8 (2.4704 mg/mL), M9 (4.9408 mg/mL).

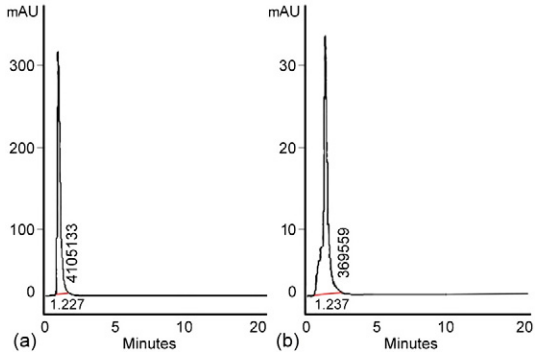

Fig. 1. HPLC profile for β-ecdysone quantification. (a) β-ecdysone (Sigma®). (b) Methanolic extract.

3.2. Rodent bone marrow micronucleus assay

The PCE/NCE ratio and the percentage of micronucleated polychromatic erythrocytes (MnPCE) are displayed in Table 1. No significant difference was observed in the PCE/NCE ratios. On the other hand, upon comparison to the negative control, a significant increase of micronuclei induction in bone marrow cells of rats was observed after treatments with all *P. glomerata* extract dilutions (MEDs) (Table 1). Even at the lowest MED (M1 — 0.0823 mg/kg) evaluated, significant effects were observed. The micronucleus percentage of this treatment was 2.78× higher than that one observed for the negative control (Table 1). A representative image of the micronuclei is shown in Fig. 3k.

3.3. Allium cepa Assay

Reductions in the mitotic index were observed for treatments higher than or equal to M5 (16 mg/mL) (Fig. 2a). The above-described exposure to *P. glomerata* MEDs also changed the proportions of mitotic phases (Fig. 2b–e). Higher concentrations (M6—32 mg/mL to M9—256 mg/mL) caused an increase of the prophase index (Fig. 2b). Additionally, comparing to the negative control, the M5 treatment (16 mg/mL) significantly increased the percentage of cells in metaphase and telophase (Fig. 2c and 2e).

MEDs-induced chromosome and cytological alterations (Table 2). It was possible to observe adherent and c-metaphases, later segregation and multipolarity segregation in anaphases, chromosomal losses, fragments and anaphase-telophase bridges.

The total percentage of alterations seemed to be a suitable criterion of the observed effects. The value of this parameter increased in comparison to the negative control and significant effects were noted for all treatments (M4—8 mg/mL to M8—128 mg/mL), except for M9 (256 mg/mL) (Table 2). Considering all MEDs in which it was possible to observe significant effects, the increase in the number of chromosomal alterations, comparing to the negative control, varied according to the type of chromosome abnormalities. The increase was of 3.8 times for chromosome adherence; 3.3 times for c-metaphase; 6.7 times for later segregation; 2.2 times for multipolar anaphase/telophase; 2.8 times for chromosomal losses and 2.4 times for chromosome bridges (Table 2).

Some treatments gave more alterations than the positive control: M6 (32 mg/mL) and M7 (64 mg/mL) increased the number of cells with chromosome adherence; M9 (256 mg/mL) induced an increase in c-metaphase cells, while M6 (32 mg/mL), M7 (64 mg/mL) and M8 (128 mg/mL) increased the number of chromosome bridges (Table 2).

Table 1
PCE/NCE ratio in bone marrow of Wistar rats and micronucleus (MnPCE) frequencies after exposure to *Pfaffia glomerata* methanolic extract by gavage.

Treatments	PCE/NCE ± S.D. (1000 erythrocytes)	MnPCE ± S.D. (2000 PCE)
NC	1.82 ± 0.68	0.59 ± 0.18[b]
PC	1.30 ± 0.29	1.32 ± 0.27[a]
M1	1.16 ± 0.38	1.64 ± 0.45[a]
M2	1.31 ± 0.15	1.83 ± 0.53[a,b]
M3	1.07 ± 0.18	2.05 ± 0.12[a,b]

PCE — polychromatic erythrocyte; NCE — normochromatic erythrocyte; MnPCE — micronucleated polycromatic erytrocyte; NC — Negative control = distilled water; PC — Positive control = 80 mg kg^{-1} day^{-1} of methyl methanesulfonate (MMS); M1 = Methanolic extract at concentration 4.2642 mg mL^{-1} (contains 0.0823 mg kg^{-1} of β-ecdysone); M2 = Methanolic extract at concentration 21.3212 mg mL^{-1} (contains 0.4115 mg kg^{-1} of β-ecdysone); M3 = Methanolic extract at concentration 42.6424 mg mL^{-1} (contains 0.823 mg kg^{-1} of β-ecdysone). The statistically different means comparing to the negative and positive controls are marked with ([a]) and ([b]), respectively (Dunnett's test, $p < 0.05$).

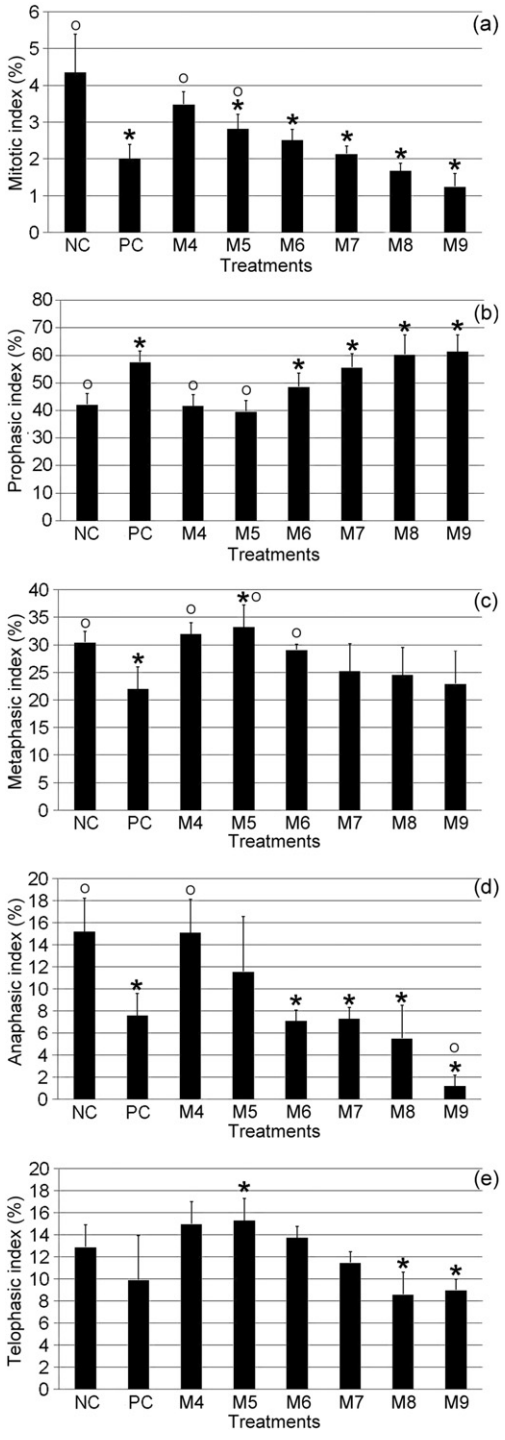

Fig. 2. Cell cycle analysis after the exposure to six methanolic extract dilutions (M4 — 8 mg/mL; M5 — 16 mg/mL; M6 — 32 mg/mL; M7 — 64 mg/mL; M8 — 128 mg/mL; M9 — 256 mg/mL). NC = Negative control (distilled water). PC = Positive control (MMS 4×10^{-4} M). (a) Mitotic index; (b) Prophase index; (c) Metaphase index; (d) Anaphase index; (e) Telophase index. Significant differences comparing to the negative and positive controls are marked with asterisks (*) and circles (o), respectively (Dunnett's test, $p < 0.05$).

Considering the micronuclei analysis as an extra variable, all treatments investigated induced significant increases in the percentage of this alteration (Table 2).

Representative images of the abnormalities observed are shown in Fig. 3.

4. Discussion

Medicinal plants, especially those with adaptogenic properties, are widely used in folk medicine. Furthermore, the World Health Organization (WHO) estimates that in some areas of Asia and Africa, 80% of the population use herbal medicines as a primary health care (Aggarwall and Mali, 2015). Despite the importance of plants as adaptogens and their several applications with therapeutic purposes, there is little information about the possible adverse effects of these plants.

In this study, the cytogenotoxic effect of an important Brazilian medicinal plant (*P. glomerata*, Brazilian Ginseng) was investigated. In popular medicine, *P. glomerata* is mainly used as tea, prepared from the decoction of the roots. It can also be employed through the ingestion of capsules that are commercially available. Commonly, the concentration of β-ecdysone in these capsules is close to 2.88 mg (e.g. *Pfaffia glomerata*, Brazilian Ginseng Herbarium, Registry in the Ministry of Health-Brazil 118600027).

Our results demonstrated the cytogenotoxic effect of methanolic extract of *P. glomerata.*

In the *Allium cepa* assay, **s**ignificant reductions in the mitotic index (MI) were observed for some treatments. Mitotic rates significantly lower than the negative control may indicate an action on the growth and development of the organism exposed to the agent tested (Leme and Marin-Morales, 2009). This effect was observed when the methanolic extract was used in concentrations equal or higher than M5 (16 mg/mL).

In addition to the mitotic index, the phase indices were also analyzed. For some treatments (M6 to M9), a significant increase in the percentage of cells in prophase was observed. This effect may represent a blockage in the checkpoint between prophase and metaphase, namely *Chfr* (Scolnick and Halazonetis, 2000). This effect has been previously observed when a chemical agent (noctodasol) induced the microtubules depolymerization (Scolnick and Halazonetis, 2000).

The presence of numerous cellular and chromosomal abnormalities here observed corroborate with the hypothesis of the interference on the mitotic spindle assembly and stopping in the *Chfr* checkpoint, previously described by Scolnick and Halazonetis (2000). Moreover, c-metaphase, chromosome loss, multipolarity and later segregation are alterations typically associated with the malfunction of the mitotic spindle (Leme and Marin-Morales, 2009). All of these alterations were observed after the exposure to the extract.

The exposure to *P. glomerata* extract also induced a significant increase of bridges in anaphase and telophase cells. Chromosomal rearrangements or breaks in the chromosome end can induce the formation of bridges (Singh, 2003).

The adherent chromosomes, another common cytogenotoxic effect, are derived from a toxic effect on the chromatin organization, more precisely on the proteinaceous matrix of chromatin, and are very often an irreversible effect which leads to cell death (Fiskesjö and Levan, 1993; Fernandes et al., 2009).

The induction of chromosomal adherence was a strong effect observed in the results. The predominance of this alteration in comparison to other chromosomal changes was previously reported by Silva et al. (2011) when they evaluated the cytotoxic effect of the essential oils of two species of *Heterothalamus*.

Using rodent bone marrow micronucleus assay, no statistical differences in the PCE/NCE ratios were observed in any treatment when compared to the negative control, indicating no cytotoxic effects. However, an increase in the percentage of micronucleated polychromatic erythrocytes (MnPCE) was observed for all treatments investigated. The MnPCEs are widely used as biomarker to assess genotoxicity in bone marrow of rats (Fromowitz et al., 2012) and several studies have demonstrated the genotoxic activity of natural products by the analysis of this endpoint such as, extract of *Rubus imperialis* (Alves et al., 2014), extract of *Himatanthus articulates* (Rebouças et al., 2013) and essential oil of *Zanthoxylum piperitum* (Hwang and Kim, 2012). Similarly,

Table 2
Cell and chromosome alterations observed on meristematic cells of *Allium cepa* after 24 h exposure to *P. glomerata* methanolic extract.

Treatments	%Total	Adherence	C-metaphase	Later segregation	Multipolarity	Chromosomal loss	Fragments	Bridges	Micronuclei
NC (dH_2O)	9.32^{b}	8.36^{b}	2.31	1.23^{b}	2.94^{b}	2.32^{b}	0.21	4.12	0.32
PC (MMS 4×10^{-4} M)	28.34^{a}	29.55^{a}	4.55	10.34^{a}	6.76^{a}	6.12^{a}	0.68	5.41	0.30
M4 (8 mg mL^{-1})	17.11^{ab}	23.68^{a}	2.37	6.78^{ab}	4.11	7.60^{a}	0	6.21	0.81^{ab}
M5 (16 mg mL^{-1})	18.78^{ab}	26.89^{a}	6.72^{a}	8.75^{a}	3.45^{b}	6.42^{a}	0.32	6.33^{a}	0.72^{ab}
M6 (32 mg mL^{-1})	21.34^{ab}	36.27^{ab}	5.76^{a}	9.87^{a}	5.26^{a}	5.79^{a}	0.28	9.52^{ab}	0.85^{ab}
M7 (64 mg mL^{-1})	19.34^{ab}	40.65^{ab}	6.25^{a}	9.75^{a}	6.23^{a}	5.28^{a}	0	14.21^{ab}	0.79^{ab}
M8 (128 mg mL^{-1})	27.89^{a}	32.86^{a}	7.51^{a}	10.11^{a}	7.80^{a}	6.65^{a}	0	13.26^{ab}	0.90^{ab}
M9 (256 mg mL^{-1})	14.32^{b}	31.37^{a}	12.75^{ab}	4.56^{ab}	2.31^{b}	7.90^{a}	0	7.14^{a}	0.66^{ab}

%Total = total percentage of alterations. NC = Negative control (distilled water); PC = postitive control (MMS — Metil Methanesulfonate). The statistically different means comparing to the negative and positive controls are marked with (^{a}) and (^{b}), respectively (Dunnett's test, $p < 0.05$).

micronucleated cells revealed the genotoxic effect in the *Allium cepa* assay. During the toxicity analysis, the micronuclei evaluation can also help to distinguish between a clastogenic and an aneugenic effect (Butenandt and Karlson, 1954). In general, the loss of chromosomal fragments results in the formation of smaller micronuclei than those formed when an entire chromosome is lost. In the present study, we observed different micronuclei sizes, showing the possible occurrence of both processes (clastogenicity and aneugenicity) caused by *Pfaffia* extract.

Treatments that represent concentrations close to the therapeutic dosage of extracts were evaluated. The treatment M1 (methanolic extract dilution) that had the β-ecdysone therapeutic dosage demonstrated a cytogenotoxic action. Increase in the percentage of micronucleated polychromatic erythrocytes (MnPCE) was also observed in this treatment. It is important to emphasize that animals received the same concentration of β-ecdysone present in one capsule of commercial products of *P.glomerata*. In *Allium cepa* assay, one of the treatments (M8) that represents a human therapeutic dosage also showed cytogenotoxic effects and constitutes additional evidence that *P. glomerata* usage should be prudently evaluated.

One of the main components frequently reported in *Pfaffia* extracts is the β-ecdysone, an ecdysteroid produced by insects and plants (Dinan,

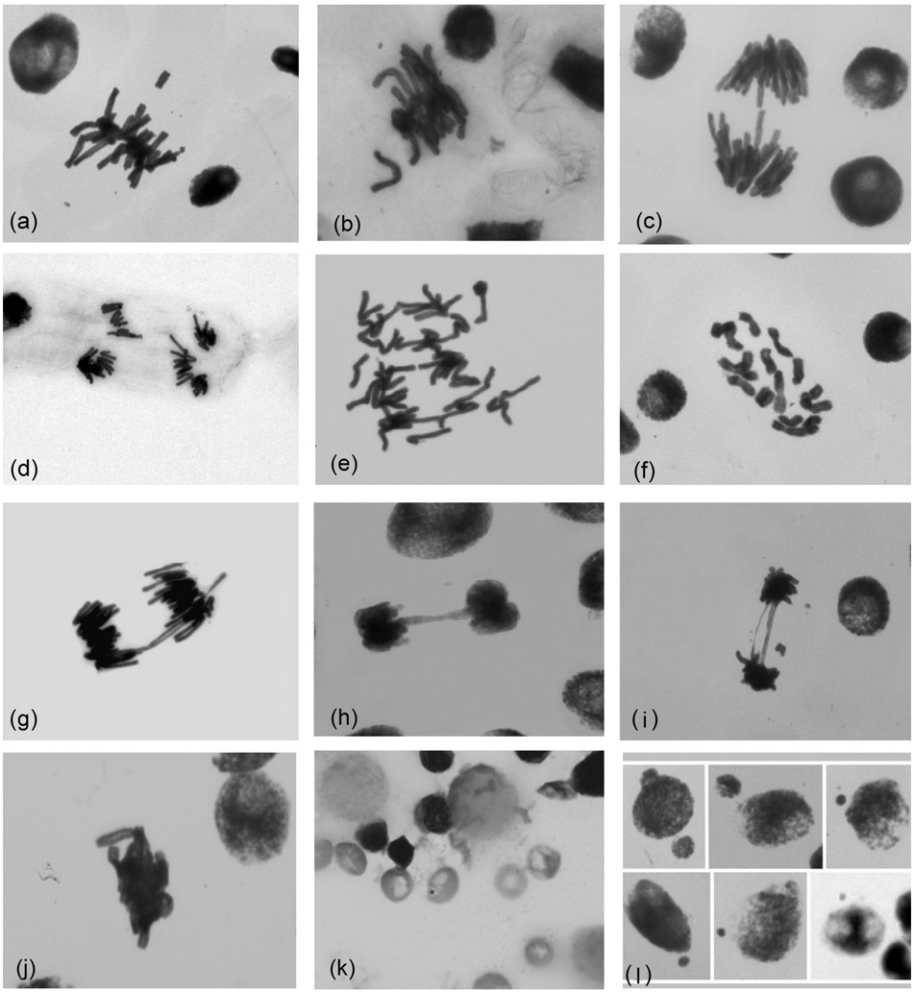

Fig. 3. Chromosome/cellular abnormalities. (a) Chromosomal fragments; (b) Chromosome loss; (c) Later segregation; (d) and (e) Multipolarity; (f) c-metaphase; (g) Anaphase bridge; (h) Telophase bridge; (i) Bridges and fragment in anaphase; (j) Chromosomal adherence; (k) Micronuclei in bone marrow cell; (l) Micronuclei of different sizes in *Allium cepa* cells.

2001; Dinan et al., 2001; Lafont and Dinan, 2003; Nowickyj et al., 2008; Kapur et al., 2010) that exhibit several pharmacological and medicinal effects in mammals (Lafont and Dinan, 2003; Dinan and Lafont, 2006; Festucci-Buselli et al., 2008; Gorelick-Feldman et al., 2008). The compound may be successfully extracted using methanol (Rates and Gosmann, 2002; Flores et al., 2009). However, in spite of its pharmacological importance, studies which evaluated possible harmful effects of β-ecdysone or other ecdysteroids are rare. Cytogenotoxic effects of pure β-ecdysone (Sigma®) have been recently described by Neves et al. (2016) in a study that showed adverse effects of this substance in rodent bone marrow micronuclei and *Allium cepa* assays. β-ecdysone also induces increase in the frequency of clastogenic and aneugenic changes (adherence, later segregation, multipolarity, chromosome loss and bridges), thus confirming the results of exposure to the extract.

Additionally, several adverse effects have been described after abusive or prolonged use of high doses of ginseng extracts. Together, they are known as "Ginseng Abuse Syndrome" and the main symptoms are hypertension, nervousness, insomnia and diarrhea (Vigo et al., 2003).

Here, we also observed that *P. glomerata* methanolic extract is potentially genotoxic both to rat bone marrow and *A. cepa* cells. These results corroborate to the cytogenotoxic potential of pure β-cdysone (Sigma®), which was previously reported by our research group in the same cell types (Neves et al., 2016).

The triterpenenoid saponins are another important chemical constituent of *Pffafia* species (Vigo et al., 2003; Sparg et al., 2004) and cannot be discarded in the interpretation of the results. The saponins already known to be found in the *Pfaffia* species, called pfaffosides (A, B, C, D, E and F) were isolated from *Pfaffia paniculata*, another medicinal species of the genus (Nakai et al., 1984; Nishimoto et al., 1984). The presence of saponins has been demonstrated in *P. glomerata* (Sparg et al., 2004), although the complete identification has not been reported so far. Many biological activities were attributed to different saponins isolated from plants including haemolytic properties, antimicrobial, insecticidal, and molluscicidal activities (Lee et al., 1999). Additionally, several saponins showed cytotoxic and antitumor activity (by cell cycle arrest and apoptosis induction) (Liu et al., 2000; Matsuzaki et al., 2003; Yun, 2003; Man et al., 2010), including some isolated from *Panax ginseng* (Liu et al., 2000; Matsuzaki et al., 2003; Man et al., 2010). Thus, some of the effects reported for *P. glomerata* in this study could be also due to the presence of saponins. Unfortunately, the lack of availability of saponins isolated from *P. glomerata* hinders the specific evaluation of this component. Our results open a new possibility to investigate the antitumor activity of *Pfaffia glomerata*. This biological activity has been already reported for another *Pfaffia* species, *P. paniculata* (Matsuzaki et al., 2003; Silva et al., 2005; Matsuzaki et al., 2006; Nagamine et al., 2009; Silva et al., 2010), reinforcing the possibility of observing the same effect in *P. glomerata*.

5. Conclusion

To summarize, our results indicate cytogenotoxic activity of the methanolic extract of *Pfaffia glomerata*. The cytogenotoxicity was mainly observed by aneugenic alterations, interference on the mitotic spindle operation, and opens the possibility of investigating the use of *P. glomerata* for cancer treatment.

It is important to highlight that this is the first *in vivo* genotoxic assessment for *P. glomerata* methanolic extract in bone marrow of Wistar rats. The results obtained using the two different cell types (rodent and *A. cepa*) strongly suggest the rational use of the species. Our data also reinforce the importance of the assessment of the cytogenotoxic potential of roots, fruits and other parts of plants, preferentially with different study model systems, in order to help to protect human health. Meanwhile, our research group has been working on the chemical and cytogenotoxic characterization of *P. glomerata* aqueous extract to better understand its effects.

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