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Full Length Research Paper

Effect of extracts from field and *in vitro* plants of *Petiveria alliacea* L. on plasmidial DNA

Soares B. O.[1], Oliveira M. B. N.[2], Mansur E.[1], Dantas F.J.S.[2], De Mattos J. C. P.[2], Caldeira-de-Araujo A.[2] and Gagliardi R. F.[1]*

[1]Núcleo de Biotecnologia Vegetal – Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier 524, PHLC, Sala 602, 20550-013 Maracanã, Rio de Janeiro, Brazil.
[2]Laboratório de Radio e Fotobiologia, Universidade do Estado Rio de Janeiro, PAPC, Avenida Vinte e Oito de Setembro 87, Fundos - 4°andar, 20551-030 Vila Isabel, Rio de Janeiro, RJ. Brazil.

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***Petiveria alliacea* L. is a native herbaceous species from the Amazon region traditionally used in folk medicine for its various pharmacological activities. This study aimed to perform a comparative assessment of genotoxic and antigenotoxic potential of field- and *in vitro*-grown plants. Specimens from different populations from Rio de Janeiro State were used as donor plants for *in vitro* culture on Murashige and Skoog (MS) medium. Aqueous extracts from field and *in vitro* plants were evaluated by analyzing their effect on the integrity of pUC 9.1 plasmid. DNA genotoxic potential was assessed after treatment of plasmid DNA with increasing concentrations of aqueous extracts (1.0, 5.0 to 10.0 mg/ml) from the populations studied and the *in vitro* plants originated from them. The electrophoretic analysis showed changes in the structural conformation of plasmid DNA, indicating the occurrence of single- and double-strand breaks caused by the extracts, demonstrating the genotoxic potential at higher concentrations. The antigenotoxic potential was assessed based on plasmid protection induced by the extracts after DNA treatment with stannous chloride. Thus, data also pointed to a antigenotoxic effect of extracts against oxidative damage. In addition, the intensity of the effect of extracts on plasmid DNA varied according to origin of plants.**

Key words: Antigenotoxicity, bioactivity, DNA topology, genotoxicity, micropropagation.

INTRODUCTION

Petiveria alliacea L. (Phytolaccaceae) is a plant endemic to the Amazon Forest. In Brazil, it is popularly known as “guiné” or “tipi.” The leaves are widely used in folk medicine and African-Brazilian cults (Ponte et al., 1996; Azevedo and Silva, 2006; Gomes et al., 2008). Research on the biological effects of this species has aroused great interest regarding its therapeutic and prophylactic use. Various pharmacological uses have been demonstrated, such as analgesics (Di Stasi, 1988), insect repellent (Johnson et al., 1997),

*Corresponding author. E-mail: gagliard@uerj.br.

(Ponte et al., 1996), acaricide (Johnson et al., 1997), antibacterial (Szczepan et al., 1972; Williams et al., 2003), antifungal (Benevides et al., 2001), and treatment for bovine viral diarrhea virus (BVDV) (Ruffa et al., 2002). Many of these effects may result from a cytotoxic mechanism also described for this species as the inhibition of antioxidant status *in vitro* and *in vivo*, featuring pro-oxidant effects in a concentration-dependent manner (Andrade et al., 2012). On the other hand, components of the extracts have shown a well-defined antioxidant activity (Delle-Monache et al., 1996; Okada et al., 2008), as well as immunomodulatory (Quadros et al., 1999) and neuropharmacological activities (Cifuentes et al., 2001). Research in the last two decades focused on polysulfides with antineoplastic action produced in this species (Mata-Greenwood et al., 2001; Rosner et al., 2001; Ruffa et al., 2002; Williams et al., 2003; Williams et al., 2007; Webster et al., 2008).

Paradoxically, both beneficial and harmful effects are common to many plant extracts by the large amount of substances produced from secondary metabolism. Previous work on *P. alliacea* revealed the presence of triterpenoids, saponins, polyphenols, coumarins, benzaldehyde, benzoic acid, flavonoids, fredelinol, pinitol and allantoin, varying their concentrations in the root, stems and leaves (De Sousa, 1990; Kubec et al., 2002, 2003; Delle-Monache and Suarez, 1992; Delle-Monache et al., 1996). Either alone or together, these substances can cause different biological effects in humans.

Despite the importance of these plants and their pharmacological activities, few studies have reported on the specific action of crude extracts on DNA coupled with an assessment of genotoxic potential. Such data are crucial since toxicity is a key determinant in the use of phytochemicals for medicinal purposes. Despite the risks, demand is increasing for plants with medicinal properties, as described earlier, causing a simultaneous increase in extraction and erosion in natural plant populations possibly containing the genotypes of greatest interest. However, the use of tissue culture methods can produce bioactive substances by providing for the multiplication of specific genotypes, but without the influence of environmental factors. Among these techniques, micropropagation allows for the multiplication (Borgaud et al., 2001; Tripathi and Tripathi, 2003; Lima et al., 2010) of genetically uniform and healthy plants in a short time. In addition, the controlled culture environment allows the manipulation of environmental factors to minimize or modify their effects (Vanisree et al., 2004). This technique develops plant stocks for commercial and industrial use without depleting natural resources and also facilitates genetic improvement, management, germplasm exchange and conservation (Rout and Das, 2000). Baseline genotoxicity can be used to monitor the quality of plants produced in culture for pharmacological purposes and can be quantified through the assessment of conformational changes in supercoiled plasmid DNA structure (De Mattos et al., 2000). Therefore, this study aimed to analyze both the genotoxic and antigenotoxic potential of extracts from different populations of field and *in vitro* plants of *P. alliacea* based on the effect of the extracts on the DNA molecule.

MATERIALS AND METHODS

Plant

Samples were obtained from spontaneously growing populations in different regions of Rio de Janeiro State. These plants were potted and kept in a greenhouse with 50% shade for identification and preparation of extracts. The species were identified by Dr. Alexandre G. Christo (Rio de Janeiro Pontifical Catholic University), and voucher specimens were kept at the Herbarium of Rio de Janeiro State University (HRJ 11.131 - 11.711 - 11.710 - 11.618, respectively). Samples were tagged according to area codes: (i) MG (22° 64' 32.18"S and 43° 12' 22.26"W, elev. 13 m); (ii) MH (22° 51' 24.46"S and 43° 22'13.75"W, elev. 17 m); (iii) NT (22° 53' 55.95"S and 43° 05' 09.37"W, elev. 54 m); and (iv) VI (22° 54' 57.57"S and 43° 14' 18.54"W, elev. 22 m). Botanical characterization of these samples was previously reported (Soares et al., 2013) and the plants were classified as *P. alliacea* L. based on the morphological characteristics, as well as the herbaceous habit and garlic odor. However, plants collected from MH, VI, and NT were identified as belonging to the variety *P. alliacea alliacea*. Plants collected from MG were identified as var. *P. alliacea tetrandra* (B.A. Gomes) Hauman (Marchioretto, 2010). Harvested plants were maintained in the greenhouse of the Plant Biotechnology Center at Rio de Janeiro State University. The seeds produced by these samples were used to initiate *in vitro* cultures.

Culture growth conditions

The *in vitro* cultures were incubated in growth chambers at 30 ± 2°C and 16 h photoperiod under an irradiance of 46 µmol $m^{-2}s^{-1}$ supplied by cool white fluorescent lamps.

In vitro germination

Seeds from different populations were used to establish primary cultures *in vitro*. Aiming to decontaminate the seeds were washed three times with detergent in tap water, immersed in 70% ethanol for 5 minutes and then in 1% NaOCl solution for 5 minutes. In addition, incubation was performed with 1% Benlate® and 1% Agrimicina® for 15 min. Afterwards, the seeds were inoculated on MS medium (Murashige and Skoog, 1962) without growth regulators (MS0) supplemented with 0.04 g/L Benlate® and 300 mg/L Agrimicina®, and incubated in flasks for one week under culture conditions, as described earlier. After this period, all flasks containing microorganismal contaminates were discarded, and the microorganism-free seeds were transferred to MS medium and maintained under the same growth conditions.

Micropropagation

Shoot apices and nodal segments to the third position from the top down (0.5 cm long) were excised from *in vitro*-grown plants derived from seed germination and cultured on MS medium

solidified with 0.7% agar plus 30 g/L sucrose. The pH was adjusted to 5.8 before autoclaving for 15 min at 121°C. The shoots were transferred to MS medium supplemented with Indole-3-acetic acid (IAA) 0.6 µM for rooting. These cultures were incubated in growth chambers at the described conditions. Primary regenerants in the form of whole plants were subcultured six to eight weeks by inoculation of shoot apices on growth regulator-free MS medium. The *in vitro* plants were used as donors of leaf explants for preparation of the extracts.

Extract preparation

After leaves were dried at 45°C for 48 h, the aqueous extract was prepared by boiling 10% wt/wt of the dried powdered plant leaves in sterile distilled water for 10 min. The aqueous extract was then left to cool at room temperature overnight.

Extraction of plasmid DNA

Cultures of *Escherichia coli* DH5αF'IQ were used as host for plasmid pUC 9.1. Plasmid DNA was extracted using the Invisorb® Spin Plasmid Mini Two plasmid extraction kit. The quantification of DNA was performed by absorbance at 260 nm.

DNA topology analysis

In order to evaluate possible genotoxic activity, aliquots of plasmid DNA (100 ng) were incubated with aqueous extracts (1.0, 5.0 to 10.0 mg/ml) at room temperature for 60 min. In order to assay the possible antigenotoxic potential of plant extracts, 1.11 mM stannous chloride ($SnCl_2$) solution was mixed with the extracts, added to the plasmid suspension and incubated at the same conditions described earlier. Stannous chloride was used as a positive control because it is a reactive oxygen species generator and can induce DNA strand breaks. After incubation, 10 µl of each sample were subjected to electrophoresis in a 0.8% normal melting point agarose gel (100 V for 30 min in 1× TAE buffer, pH 8.0). The gel was stained with ethidium bromide (0.5 mg/ml), visualized under a transilluminator system and digitalized with a Cannon XYZ system. Each assay was repeated at least three times, and the DNA bands from the best result were quantified (%) through Image J software, ver. 1.46 U.

Statistical analysis of DNA strand breaks

The data collected from light densitometer scanning gave us the null events percentage [no breaks = p (0; µ)] for each of the extracts tested. In this way, using the Poisson distribution, it is possible to obtain the mean value of breaks for each of the concentrations from the percentage of DNA supercoiled forms, as follows: $\mu = -\ln p(0; \mu)$ (Remington and Schor, 1985).

RESULTS

Micropropagation

The samples of *P. alliacea* collected in the field (Figure 1A, C, E and G) were introduced into the culture through *in vitro* germination of their seed, constituting different lineages *in vitro* (MG, NT, MH and VI) which were maintained by monthly subcultures (Figure 1B, D, F and H). From these primary cultures, more subcultures were performed monthly using shoot apices and nodal segments as explants which showed regeneration frequencies of 90 to 100% independent of position. Growth initiation occurred from apical or axillary buds after 6 to 7 days.

Genotoxic potential

The plasmid treated with *P. alliacea* aqueous extracts from field samples (Figure 2A, C, E and G; lanes 3, 4 and 5) and *in vitro* plants (Figure 2B, D, F and H; lanes 3, 4 and 5) caused single- (Figure 2A, C, E and G; lane 3) and double-strand breaks (Figure 2B and F; lanes 3, 4 and 5). In the *in vitro* samples from MG and VI, double-strand breaks were observed in lane 5. This treatment led to the conversion from Form I (supercoiled intact DNA) to Form III (open circle). This effect was dependent on both the concentration of the extract and the origin of the plants. Thus, different profiles were observed between NT field and *in vitro* samples, where apparently *in vitro* extracts of all plants were able to cause DNA breaks at different concentrations (Figure 2A and B; lanes 3, 4 and 5), as shown by quantitative analysis of corresponding bands on the gel and the decrease in Form I (supercoiled plasmid DNA), along with the presence of broken DNA strands, including Form II (circular) and/or Form III (open circle), as shown graphically in Figure 2A and B. MG field and *in vitro* samples showed a similar electrophoretic profile, as demonstrated through quantitative analysis showing that field extracts produced both single- (Form II) (Figure 2C, lanes 3 and 4) and double-strand breaks (Form III) (Figure 2C, lane 5), while low concentrations of *in vitro* extracts produced only DNA changes as indicated by Form III (Figure 2D, lanes 3 and 4). DNA changes as indicated by Form II appear just at the highest concentration of the extract (Figure 2D, lane 5). MH field samples only showed significant formation of double- and single-strand breaks at the highest tested concentration (Figure 2E, lane 5). On the other hand, all concentrations of *in vitro* samples exhibited double- and single-strand break formations, starting from the lowest concentration (Figure 2F, lanes 3, 4 and 5). VI field samples showed single-strand breaks at all tested concentrations, while no double-strand breaks could be detected (Figure 2E, lanes 3, 4 and 5). VI *in vitro* samples also showed single-strand breaks at the lowest concentration (Figure H, lane 3), while concentrations shown in lanes 4 and 5 showed both kinds of lesions (Figure H, lanes 4 and 5).

Antigenotoxic potential

The antigenotoxic potential of the extracts was evaluated based on their capacity to block or reduce the occurrence

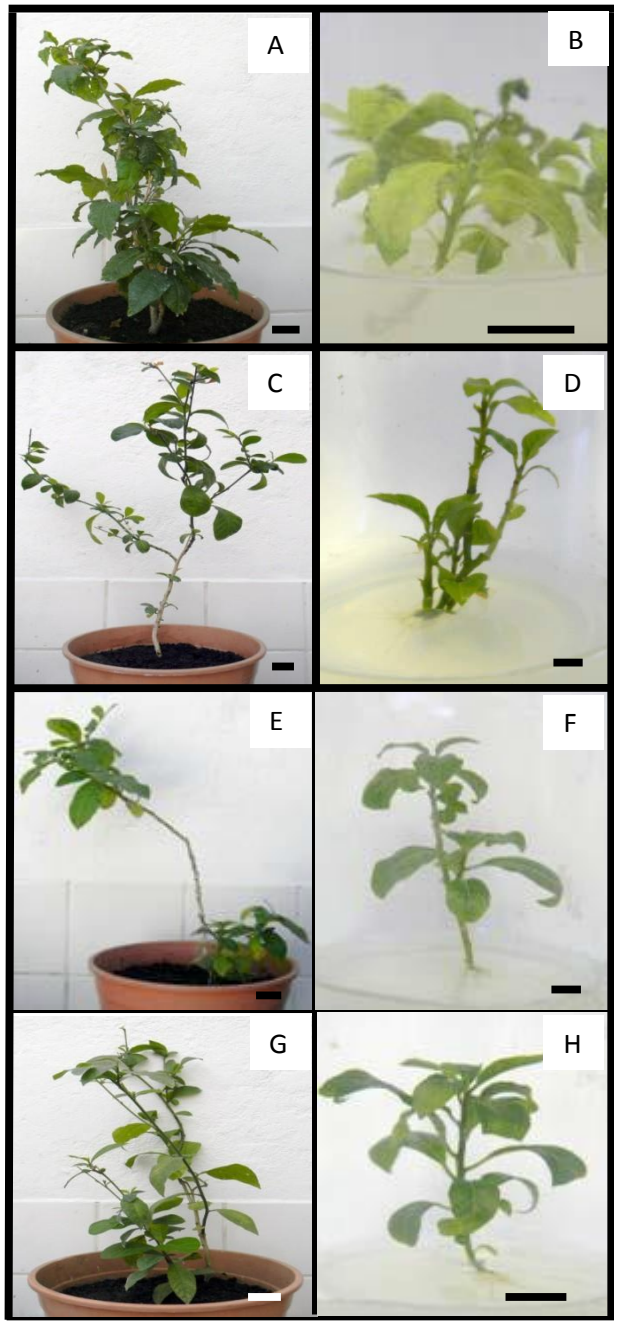

Figure 1. *Petiveria alliacea* L. from field samples: A) MG field; B) MG *in vitro*; C) NT field; D) NT *in vitro*; E) MH field; F) MH *in vitro*; G) VI field; H) VI *in vitro*. Bar = 1 cm

of DNA strand breaks when plasmid DNA molecules were challenged with $SnCl_2$. This experimental protocol is also based on plasmid DNA mobility through agarose gel submitted to an electrical field. The plasmid DNA (pUC 9.1) treated with $SnCl_2$ led to the DNA conversion from Form I to Forms II and III (De Mattos et al., 2000), a pattern that was used as positive control for this type of injury (Figure 2, lane 2). As verified by comparing lanes 6 and 8 in Figure 2B, a protective effect was observed in the presence of NT extracts from *in vitro* plants derived from this population. This effect was also detected in MH field samples (Figure 2E, lanes 7 and 8) and VI field samples (Figure 2G, Lanes 6 and 7). However, in the MG *in vitro* samples, this effect either did not appear (Figure 2D) or was inconclusive (Figure 2F, lanes 6, 7 and 8). The genotoxic/antigenotoxic effects of extracts were measured by densitometry (Figure 2) and further analyzed through Poisson distribution (Figure 3).

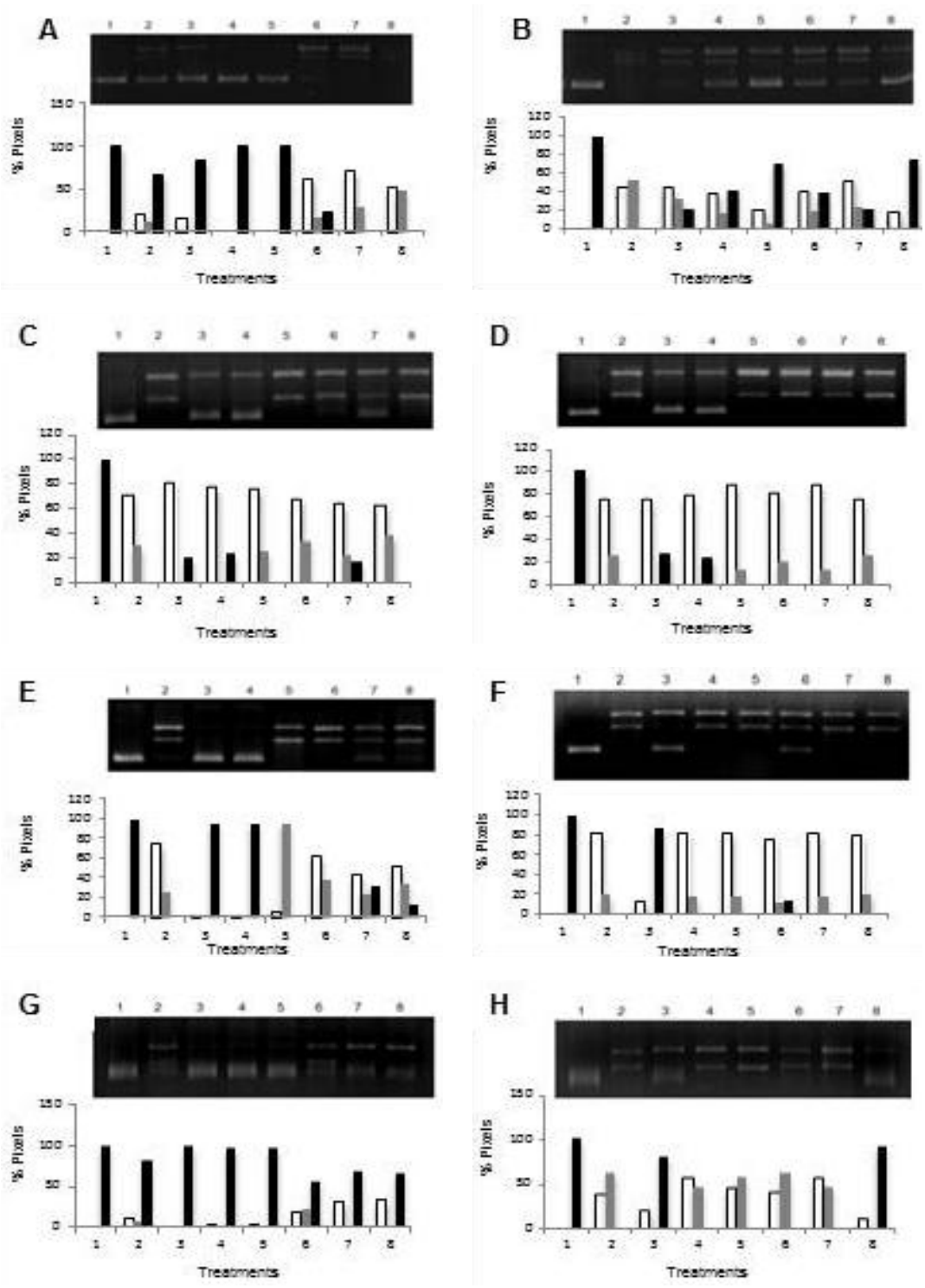

Figure 2. Qualitative and semi-quantitative evaluation of the effects of *P. alliacea* aqueous extract on plasmid pUC 9.1 DNA. Bands corresponding to aliquots of the suspension of plasmid (100 ng) treated with extracts (0-1.0-5.0-10 mg/mL). Lanes 1 and 2 respectively show negative and positive controls. Lanes 3, 4 and 5 correspond to the genotoxic potential of the aqueous extract, while lanes 6, 7 and 8 show the antigenotoxic effect of the extract against stannous chloride: A) NT field plant; B) NT i*n vitro* plant; C) MG field plant; D) MG *in vitro* plant; E) MH field plant; F) MH i*n vitro* plant; G) VI field plant; H) VI *in vitro* plant. Graphical bars represent DNA densitometric measurements by Image J. Bars correspond to lanes: 1 – pUC 9.1; 2 – p UC 9.1 + 1.11 mM Sn Cl_2; 3 – p UC 9.1 + 1 mg/ml extract; 4 – pUC 9.1 + 5 mg/ml extract; 5 – pUC 9.1 + 10mg/L extract; 6 – pUC 9.1 + 1 mg/ml extract + Sn CL2; 7 - pUC 9.1 + 5 mg/ml extract + $SnCl_2$, 8 - pUC 9.1 +10 mg/ml extract + $SnCl_2$.

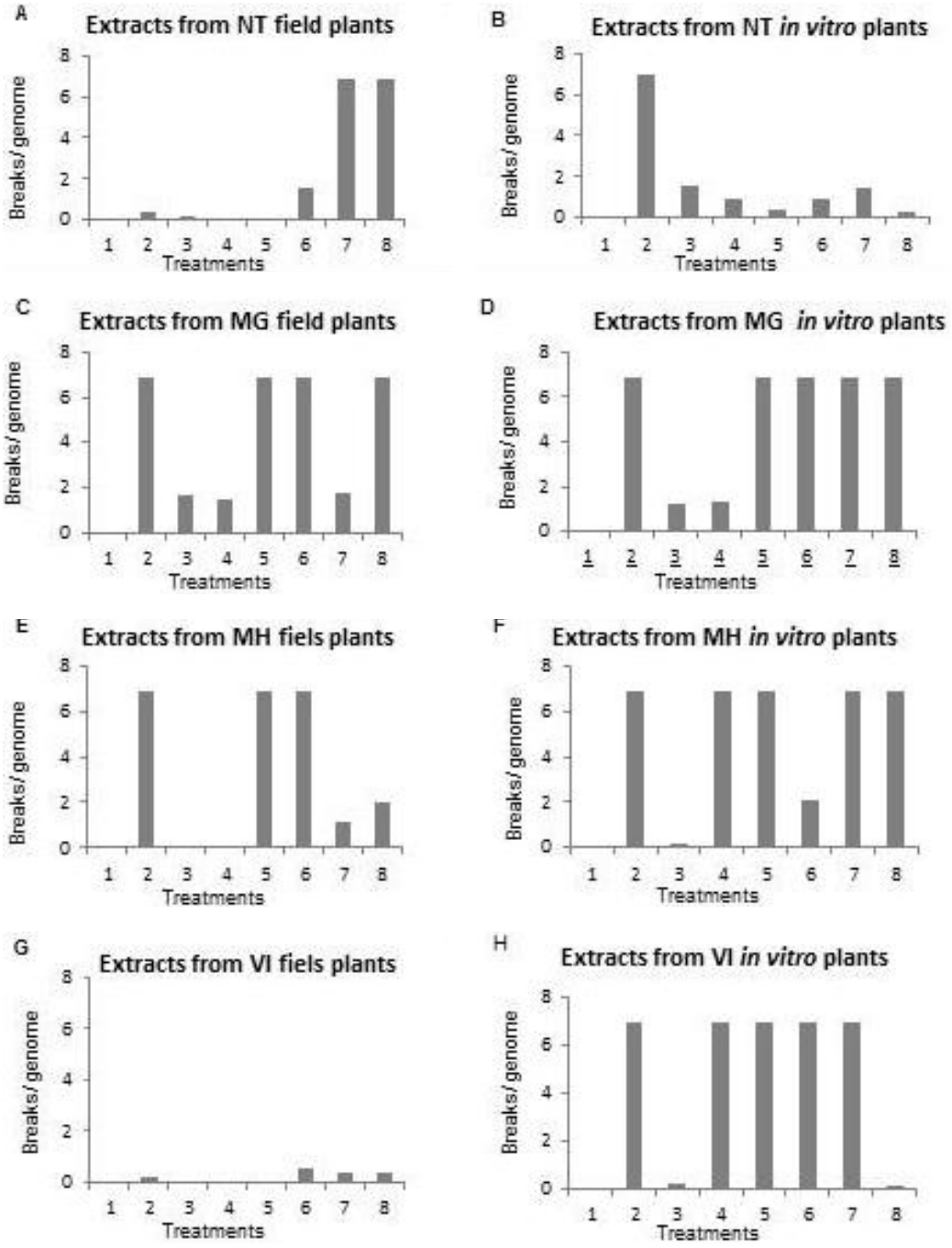

Figure 3. Number of single-strand breaks/genome in plasmid DNA treated with extracts. Bands corresponding to aliquots of the suspension of plasmid (100 ng) treated with extracts (0 to 1.0 to 5.0 to 10 mg/ml). Columns 1 and 2, respectively show negative and positive controls. Columns 3, 4 and 5 correspond to the genotoxic potential of the aqueous extract, while lanes 6, 7 and 8 show the antigenotoxic effect of the extract against stannous chloride. Analysis of densitometric data performed by Image J gave the null events percentage, that is, no breaks, for each of the treatments tested. Using Poisson distribution, we have obtained the mean value of breaks for each of the concentrations, as follows: $\mu = -\ln p(0; \mu)$. Number of single-strand breaks/genome represents the means of three isolated experiments.

The data presented as the DNA-single-strand breaks (DNA-SSB) per kilobase of plasmidal DNA (mean value) is according to the genotoxic/antigenotoxic potential of the extracts. (Figure 3A, B, C, D, E, F, G, H).

DISCUSSION

The methods based on the activation of preformed meristems (shoot tips and axillary buds), which retain the potential to recover true-to-type plants, are desirable for many biotechnological purposes. Thus, micropropagation of various pathogen-free plant species, including many medicinal plants, has been reported (Tripathi and Tripathi, 2003). *In vitro* cultures have been considered an important tool for both mass production of phytochemicals and pharmacological studies (Borgaud et al., 2001). In this way, previous reports have already described the production of plants by amplifying nodal segments on *P. alliacea* (Castellar et al., 2011). In this study, *in vitro* plants were derived from four different regions of Rio de Janeiro State, Brazil, and they were established after germination of their seeds in order to maintain intraspecific diversity, thus generating the four representative samples of the populations studied. Evaluation of electrophoretic mobility has been used to study the oxidant/antioxidant properties of different natural products (Reiniger et al., 1999; Biso et al., 2010; Hamedt et al., 2013). This approach could be used to monitor the quality of plants produced by culture for pharmacological purposes. Thus, it is possible to follow the occurrence of different types of injury (single- or double-strand breaks) based on the intensity and position of the bands produced by electrophoresis. Accordingly, the band corresponding to supercoiled DNA (Form I) becomes less intense when DNA strand breaks occur, generating the characteristic bands corresponding to Forms II (open circle) and/or Form III (linear) (De Mattos et al., 2004).

Some studies have reported on the pro-oxidant effects of this species in different experimental models (Salim, 2011; Andrade et al., 2012). Using a sea urchin model, a moderate genotoxic activity, based on cytogenetic changes, was reported (Hoyos et al., 1992), but to our knowledge, this is the first study on the effect of extracts of *P. alliacea* on the DNA molecule. Based on the evaluation of genotoxic potential, the results obtained in the present study demonstrate that aqueous extracts from *P. alliacea* have components that induce lesions in the DNA molecule. This is also the first study to evaluate the direct effects of extracts of *P. alliacea* on the DNA molecule. It is well known that many biological effects are caused by chemical substances produced in the plant metabolism, which under natural conditions are subject to climate changes, pathogens and predators, justifying the high chemical diversity produced through evolution. In artificial culture, plants are also subjected to various stress conditions, as represented by the artificial environment of culture, a known inducer of specific adaptations (Smulders and Klerk, 2010). These responses are often reflected by increased synthesis of certain substances, or even the synthesis of new substances not detected in field plants (Tripathi and Tripathi, 2003; Vanisree et al., 2004).

In this work, genotoxic effects were stronger with extracts from *in vitro* plants than those obtained from field samples. This is an interesting result since we know that the synthesis of secondary metabolites is highly induced under both natural and artificial conditions. Thus, it is possible that stress conditions in artificial cultures could be more intense than those in natural conditions. Thus, metabolites induced in the culture cannot be formed in the field due to the silencing of some genes. Moreover, the possibility of specific responses cannot be ruled out. The present results demonstrate that aqueous extracts have components capable of inducing breaks and/or alkali-labile injuries in the DNA molecule. Moreover, lesions can lead to cell inactivation, as well as mutagenic potential (Friedberg et al., 2006), justifying the need for further such studies in other experimental models, such as bacteria and eukaryotic cells. In addition, some studies suggest that various extracts or compounds of this species exhibit antioxidant or pro-oxidant activity (Carlini, 2003; Andrade et al., 2012). In this work, the antigenotoxic effect was influenced by plant origin, suggesting, in turn, the influence of some genetic variation. This antioxidant activity could also result from the different flavonoids already reported in this species (Delle-Monache et al., 1996; Okada et al., 2008).

Indeed, many studies are currently focused on the antioxidant activity of medicinal plants (Kumar et al., 2010). Most active antioxidant compounds are phenols, flavonoids, isoflavones, alpha-tocopherol and anthocyanins, and a direct relationship between antioxidant activity and phenolic compounds in plant extracts has been reported by many researchers (Gollucke-Boiago et al., 2008; Chirinos et al., 2008; Sharififar et al. 2009; Du et al. 2009; Conforti et al., 2009). The ability of different plant extracts to protect supercoiled plasmid DNA against the deleterious effects of hydroxyl radicals generated during metabolism was evaluated by a test that detects changes in the structural conformation of plasmids. This test has been widely used, with modifications to evaluate the antigenotoxicity of several plant species (De Mattos et al., 2000; Lee et al., 2002; Kumar et al., 2010). Specifically, the addition of stannous chloride in the reac-tion mixture results in the formation of hydroxyl radicals (OH•) that react with plasmid DNA, thus producing single- and double-strand breaks (Forms II and III). As such, it is possible to follow the occurrence and type of injury (single- or double-strand break) by the intensity and position of the bands produced in the agarose gel. Accordingly, the band on the supercoiled DNA becomes less intense when the DNA is broken by OH radicals, and the characteristic bands of this type of injury are more intense (De Mattos et al., 2004).

In assessing the potential antigenotoxicity of extract samples used in this work, the change in band position obtained in relation to DNA damaged by stannous chloride showed a protective effect of the extract in

agreement with the results obtained for other species (De Mattos et al., 2000, 2004). The antioxidant and antineoplastic activity of *P. alliacea* could result from the presence of different substances, including the flavonoids already reported in the species (Delle-Monache and Suarez, 1992; Delle-Monache et al., 1996; Okada et al., 2008; Soares et al., 2013). Characterization of the arrays and the possibility of applying different methods of eliciting and monitoring the material produced in culture can guarantee the safe *in vitro* production of this material, making possible the therapeutic application of herbal and natural remedies.

Conclusion

The assessment of the genotoxic potential in *P. alliacea* demonstrated the occurrence of changes in plasmid topology influenced by the concentration of extracts and genotype of the plant; however, genotoxic effects were stronger with extracts from *in vitro* plants than those obtained from field samples, possibly because of the differences between *in vivo* and *in vitro* environmental factors. On the other hand, the assessment of potential antigenotoxicity of aqueous field plant extracts and *in vitro* cultures demonstrated a protective effect against injuries caused by $SnCl_2$.

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Conflict of Interest

Authors have not declare any conflict of interest.

REFERENCES

Andrade TM, Melo AS, Dias RGC, Varela ELP, Oliveira FR, Vieira JLF, Andrade MA, Baetas AC, Monteiro MC, Maia CSF (2012). Potential behavioral and pro-oxidant effects of *Petiveria alliacea* L. extract in adult rats. J. Ethnopharmacol. 143:604-610.

Azevedo SKS, Silva IM (2006). Medical and religious plants in conventional and open-air markets of Rio de Janeiro State, Brazil. Acta Bot. Bras. 20:185-194.

Benevides PJC, Young MCM, Giesbrecht AM, Roque, NF, Bolzani VDS (2001). Antifungal polysulphides from *Petiveria alliacea* L. Phytochemistry 57:743-747.

Biso FI, Rodrigues CM, Rinaldo D, Reis MB, Bernardi CC, De Mattos JCP, Caldeira- de-Araújo A, Vilegas W, Cólus IMS, Varanda EA (2010). Assessment of DNA damage induced by extracts, fractions and isolated compounds of *Davilla nitida* and *Davilla elliptica* (Dilleniaceae). Mutat. Res. Genet.Toxicol. Environ. Mutagen. 702:92-99.

Borgaud F, Gravot A, Milesi S, Gontier E (2001). Production of plant secondary metabolites: a historical perspective. Plant Sci. 161:839-851.

Carlini EA (2003). Plants and the central nervous system. Pharmacol. Biochem. Behav. 75:501-512.

Castellar A, Gagliardi RF, Mansur E (2011). *In vitro* propagation and establishment of callus and cell suspension cultures of *Petiveria alliacea* L., a valuable medicinal plant. J. Med. Plants Res. 5:1113-1120.

Chirinos R, Campos D, Warnier M, Pedreschi R, Rees J, Larondelle Y (2008). Antioxidant properties of mashua (*Tropaeolum tuberosum*) phenolic extracts against oxidative damage using biological *in vitro* assays. Food Chem. 111:98-105.

Cifuentes CM, Serranillos MPG, Iglesias I, Fresno AMV (2001). Neuropharmacological profile of ethnomedicinal plants of Guatemala. J. Ethnopharmacol. 76:223-228.

Conforti F, Sosa S, Marrelli M, Menichini F, Statti GA, Uzunov D, Tubaro A, Menichini F (2009). The protective ability of Mediterranean dietary plants against the oxidative damage: The role of radical oxygen species in inflammation and the polyphenol, flavonoid and sterol contents. Food Chem. 112:587-594.

De Mattos JCP, Dantas FJS, Bezerra RJAC, Bernardo-Filho M, Cabral-Neto JB, Lage CAS, Leitão AC, Caldeira-de-Araujo A (2000). Damage induced by stannous chloride in plasmid DNA. Toxicol. Let. 116:159-163.

De Mattos JCP, Dantas FJS, Caldeira-de-Araujo A, Moraes MO (2004). Agarose gel electrophoresis in the classroom. Biochem. Mol. Biol. Educ. 4:254-257.

De Sousa JR, Demuner AJ, Pinheiro JA, Bretmair E, Cassels BK (1990). Dibenzyl Trisulphyde and Trans-N-methyl-4-methoxyproline from *Petiveria alliacea*. Phytochemistry 29:3653-3655.

Delle-Monache F, Menichini F, Suarez LEC (1996). Substances from *Petiveria alliacea*: II further flavanonoides and triterpenes. Gaz Chim. Ital. 126:275-278.

Delle-Monache F, Suarez LEC (1992). 6-c-formil and 6-c-hydroxymethyl flavanones from *Petiveria alliacea*. Phytochemistry 31:2481-2482.

Di Stasi LC, Costa M, Mendacolli LJ, Kirizawa M, Gomes C, Trolin GJ (1988). Screening in mice of some medicinal plants used for analgesic purposes in the state of São Paulo. J. Ethnopharmacol. 2:205-208.

Du M, Li M, Ma F, Liang D (2009). Antioxidant capacity and the relationship with polyphenol and vitamin C in Actinidia fruits. Food Chem. 113:557-562.

Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, Ellenberger T (2006). DNA Repair and Mutagenesis Washington, DC, ASM Press. pp. 1,118.

Gollucke-Boiago AP, Souza CJ, Queiroz TD (2008). (+)-Catechin and (-)-epicatechin levels of concentrated and ready to drink through storage. Int. J. Food Sci. Technol. 43:1855-1859.

Gomes PB, Noronha EC, Melo CT, Bezerra JN, Neto MA, Lino CS, Vasconcelos SM, Viana GS, Sousa FC (2008). Central effects of isolated fractions from the root of *Petiveria alliacea* L. (tipi) in mice. J. Ethnopharmacol. 120:209-214.

Hamedt AL, Ortiz IC, Huertas PAG, Saenz J, Caldeira-de-Araujo A, De Mattos JCP, Rodriguez-Gasquez MA, Chavez OT (2013). Cytotoxic, mutagenic and genotoxic evaluation of crude extracts and fractions from *Piper jericoense* with trypanocidal action. Acta Trop. 131:92-97.

Hoyos LS, Au WW, Heo MY, Morris DL, Legator MS (1992). Evaluation of the genotoxic effects of a folk medicine *Petiveria alliacea* (anamu). Mut. Res. 280:29-34.

Johnson L, Williams LAD, Roberts EV (1997). An insecticidal and acaricidal polysulfide metabolite from the roots of *Petiveria alliacea*. Pest Sci. 50:228-232.

Kubec R, Kim S, Musah RA (2002). S-Substitued cysteine derivatives and thiosulfinate formation in *Petiveria alliacea*-Part II. Phytochemistry 61:675-680.

Kubec R, Kim S, Musah RA (2003). The lachrymatory principle of *Petiveria alliacea*. Phytochemistry 63:37-40.

Kumar A, Kaur R, Arora S (2010). Free radical scavenging potential of some Indian medicinal plants. J. Med. Plants Res. 4:2034-2042.

Lee J, Kim H, Kim J, Jang Y (2002). Antioxidant property of an ethanol extract of the stem of *Opuntia ficus-indica* var. Saboten. J. Agric.

Food Chem. 50:6490-6496.
Lima SS, Moreira DL, Affonso VR, Henriques AB, Sato A, Esquibel MA, Leitão GG, Lage CLS (2010). Micro propagation of *Polygonum acre* Kunth var. *aquatile* (Mart.) Meisn and seasonal variation of tannins in acclimatized plants. J. Med. Plants Res. 4:573-578.
Marchioretto MS (2010). Phytolaccaceae in: List of species of Flora of Brazil. Botanical Garden of Rio de Janeiro.
Mata-Greenwood E, Ito A, Westenburg H, Cui BL, Mehta RG, Kinghorn AD, Pezzuto JN (2001). Discovery of novel inducers of cellular differentiation using HL-60 promyelocytic cells. Anticancer Res. 21:1763-1770.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
Okada Y, Tanaka K, Satoc E, Okajima H (2008). Antioxidant activity of the new thiosulfinate derivative, S-benzyl phenylmethanethiosulfinate, from *Petiveria alliacea* L. Organ. Biomol. Chem. 6:1097-1102.
Ponte JJ, Franco A, Silveira-Filho J (1996). Preliminary investigation on the nematocide potentiality of Guine's Plants (*Petiveria alliacea*). Phytopathol. Venezuela 9:14-15.
Quadros MR, Souza-Brito ARM, Queiroz MLS (1999). *Petiveria alliacea* L. extracts protects mice against *Listeria monocytogenes* infection-effects on bone marrow progenitor cells. Immunopharmacol. Immunotoxycol. 21:109-124.
Reiniger IW, Silva CRS, Felzenszwalb I, De Mattos JCP, Oliveira JF, Dantas FJS, Bezerra RJAC, Caldeira-de-Araujo A, Bernardo-Filho M (1999) Boldine action against the stannous chloride effect. J. Ethnopharmacol. 68:345-348.
Remington RD, Schor MA (1985). Some important distributions. In: Johnson RA, Wichern DW (Eds.), Statistics with Applications to the Biological and Health Sciences. Prentice-Hall, Inc., Englewood Cliffs, New Jersey. P 415.
Rosner H, Williams LAD, Jung A, Kraus W (2001). Disassembly of microtubules and inhibition of neurite out growth, neuroblastoma cell proliferation, and MAP kinase tyrosine dephosphorylation by dibenzyl trisulphide. Biochim. Bioph. Acta 1540:166-177.
Rout GR, Das SSP (2000). *In vitro* manipulation and propagation of medicinal plants. Biotechnol. Adv. 18:91-120.
Ruffa MJ, Ferraro G, Wagner ML, Calcagno ML, Campos RH, Cavallaro L (2002). Cytotoxic effect of Argentine medicinal plant extracts on human hepatocellular carcinoma cell line. J. Ethnopharmacol. 79:335-339.
Salim S (2011). Oxidative stress in anxiety: implications for pharmacotherapy. Am. J. Integr. Med. 1:11-21.
Sharififar F, Dehghn-Nudeh G, Mirtajaldini M. (2009). Major flavonoids with antioxidant activity from *Teucrium polium* L. Food Chem. 112:885-888.
Smulders MJM, Klerk GJ (2010). Epigenetics in plant tissue culture. Plant Growth Regul. 63:137-146.
Soares BO, Fernandes DC, Cantelmo L, Rocha LP, Pettinelli JA, Christo AG, Coelho MGP, Gagliardi RF (2013). Botanical characterization of *Petiveria alliacea* L. from Rio de Janeiro, Brazil: Systematic and functional implications. Plant Biosys. 147:411-417.
Szczepan CV, Zgorzela P, Hoyer GA (1972). Isolation, structure elucidation, and synthesis of an antimicrobial substance from *Petiveria alliacea* L. Drug Res. 22:1975-1976.
Tripathi L, Tripathi JN (2003). Role of biotechnology in medicinal plants. Trop. J. Pharm. Res. 2:243-253.
Vanisree M, Lee CY, Lo SF, Nalawade SM, Lin CY, Tsay HS (2004). Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Bot. Bull. Acad. Sin. 45:1-22.
Webster SA, Mitchell SA, Gallimore WA, Williams LAD, Ahmad MH (2008). Biosynthesis of Dibenzyl Trisulfide (DTS) from somatic embryos and rhizogenous/embryogenic callus derived from Guinea hen weed (*Petiveria alliacea* L.) leaf explants. *In Vitro* Cell. Dev. Biol. Plant 44:112-118.
Williams LAD, Rosner H, Levy HG, Barton EN (2007). A critical review of the therapeutic potential of dibenzyl trisulphide isolated from *Petiveria alliacea L* (guinea hen weed, anamu). West Indian Med. J. 56(1):17-21.
Williams LAD, Vasquez E, Klaiber I, Kraus W, Rosner H (2003). A sulfonic anhydride derivative from dybenzil trisulphide with agro-chemical activities. Chemosphere 51:701-706.

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Anatomy, histochemistry and chemical characterization of the essential oil of the gum tree *Vochysia pyramidalis* Mart

Gessiane Silva Cabral Guimarães[1], Fabiula Gomes de Morais Ribeiro[1*], Flávia Dionísio Pereira[1], Sebastião Carvalho Vasconcelos Filho[1], Fabiano Guimarães Silva[1] and Adriane da Silveira Gomes[2]

[1]Federal Instituto of Education, Science and Technology Goiás - Campus Rio Verde - Rod. Goiás Km 01 South, Rural Zone, Rio Verde - GO, Brazil.
[2]Federal Institute of Education, Science and Technology Goiás - Iporá Campus - West Avenue s/n, Output Piranhas-Iporá / GO, Brazil.

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The Gomeira (*Vochysia pyramidalis* Mart.), belonging to the family Vochysiaceae, is a plant species from the Brazilian cerrado used in folk medicine because of its reputedly anti-inflammatory, analgesic and anti-bacterial properties. However, scientific data concerning this species are scarce. Therefore, the aim of the present study was to conduct an anatomical and histochemical assessment of this plant species and characterize the chemical composition of its essential oil. For the anatomical evaluation, the leaves, stems and petals were fixed and subjected to common plant anatomy techniques. For the histochemical tests, fresh samples of the stems, leaves and petals were sectioned using a microtome and stained with different dyes to identify the chemical components. The essential oil was obtained from fresh flowers and both dry and fresh leaves using hydrodistillation and was characterized by gas chromatography coupled with mass spectrometry (GC/MS). The anatomical analysis showed several xeromorphic characteristics, which suggest that the mucilaginous channeland idioblasts are the only secretory structures present in this species. The histochemical tests revealed the presence of secondary metabolites belonging to two different chemical classes: the phenolic compounds and terpenes. The chemical composition of the essential oil was analyzed by gas chromatography–mass spectrometry (GC/MS), and hexadecanol; pentacosane and farnesol were identified as its main components.

Key words: Brazilian cerrado in Goiás state, secretory structures, farnesol, secondary metabolites, terpenes.

INTRODUCTION

The interest in renewable sources has increased in recent years as part of a general effort to promote a new sustainable economy and replace traditional processes (Piazza and Foglia, 2001). A successful outcome depends on many factors and may be achieved with the use of, for example, plant species from the Brazilian cerrado,

cerrado, which are promising but remain largely understudied. One-half of the native species are estimated to possess a type of medicinal property, but few have been adequately studied (Rodrigues and Carvalho, 2001; Gusmão et al., 2006; Souza and Felfili, 2006). Ninety-two secondary metabolites have been previously described for Vochysiaceae, and pharmacological properties have been attributed to several species of this family (Hess and Delle, 1999; Guarin Neto, 2006; Gomes et al., 2009; Jesus et al., 2009; Carnevale et al., 2011). The reported chemical components include triterpenoids, steroids and polyphenols, such as flavonoids and ellagic acid derivatives (Carnevale et al., 2011). Gomeira or Gomeira-de-macaco ("monkey gum-producing tree") are the common Portuguese names of *Vochysia pyramidalis*, which is named for its production of a gum that is eaten by monkeys. This tree species has great ornamental potential and is commonly used in urban forestry, particularly in parks and gardens, because of its stature and attractive flowers. *V. pyramidalis* has also been used for recovering degraded areas because of its rapid growth and attraction to the local fauna. Regionally, the wood of *V. pyramidalis* is used in linings and posts (Silva et al., 2009).

However, there are few studies quantitatively or qualitatively measuring the chemical composition of the cellular components of *V. pyramidalis*. The capacity of many plant species to synthesize secondary metabolites is associated with their anatomical features. Therefore, previous knowledge becomes essential because it locates the plant's secretory structures and, in certain cases, identifies the moment of full functioning of specific glands (Akaisue and Oliveira, 1987; Ming, 1994; Thadeo et al., 2009). Histochemical studies allow for the detection of secondary metabolites, which can later be quantified based on the observed intensity (Santos et al., 2009).

Therefore, anatomical and histochemical characterization are essential steps toward the identification of potentially interesting plant species in addition to locating secretion sites and/or the accumulation of biologically active products (Thadeo et al., 2009). Moreover, the chemical characterization of plant essential oils contributes to the discovery of biologically active substances of interest for the pharmaceutical, food and cosmetic industries (Silva-Santos et al., 2008). Therefore, the aim of the present study was to perform anatomical and histochemical assessments of leaves, stems and petals and to characterize the chemical components of the essential oils extracted from the leaves and flowers of *V. pyramidalis*.

MATERIALS AND METHODS

Plant material

The specimen of the studied species was identified by Gustavo Hiroaki Shimizue and deposited at the herbarium of Campinas State University (Universidade Estadual de Campinas - UNICAMP) under number 159466. The *V. pyramidalis* plant material used in the experiments was collected at the municipality of Rio Verde, Goiás, Brazil (17° 49' 464" S to 51° 02' 105" W, altitude 733 m) (Figure 1). Two collections were performed, one during Spring in October 2011 and the other during Autumn in March 2012. In the first collection (October 2011), stems, leaves and flowers were collected for anatomical and histochemical analyses, and flowers were collected for essential oil extraction. In the second collection (March 2012), leaves were collected for essential oil extraction. All collections were performed at 8 a.m.

Assay I

Anatomical analysis

For the anatomical analysis, the leaves, stems and petals of *V. pyramidalis* were collected, fixed in FAA_{50} for 24 h (Johansen, 1940) and preserved in 70% alcohol. Subsequently, the plant material was enclosed in plastic resin according to the manufacturer's instructions (Leica Historesin). Cross and longitudinal sections, approximately 5 to 7 µm in thickness, were obtained using a manual rotary microtome (Logen). For the structural analysis, the cross-sections were stained with Toluidine Blue following O'Brien and McCully (1981). The following structures were observed: the central midrib and mesophyll (leaf), xylem (stem) and parenchyma and epidermis (petals). The observations were performed with a microscope (OLYMPUS BX 61) coupled to a photographic camera (OLYMPUS DP73). For the study of the foliar surface, 0.25-cm ² samples of the leaves' middle third were subjected to epidermis dissociation with Jeffrey solution (10% nitric acid and 10% chromic acid) (Johansen, 1940), stained with 1% fuchsin and mounted in Canadian balsam for observation of the stomata type. Three slides, each containing one cross-section of each analyzed structure, were prepared for each anatomical analysis.

Assay II

Histochemical analysis

The histochemical tests were performed using fresh material from the stems, leaves and flowers. The cross-sections were treated with the following reagents: vanillin-hydrochloric acid for the tannins (Mace and Howell, 1974); Wagner reagent for the alkaloids (Furr and Mahlberg, 1981); acid phloroglucinol for the lignin (Johansen, 1940); Sudan IV for the lipids (Johansen, 1940); potassium dichromate for the phenolic compounds (Gabe, 1968); Nadi reagent for the terpenes (essential oils and resin acids) (David and Carde, 1964); Xylidine Ponceau (XP) for the total proteins (O'Brien and McCully, 1981); Schiff reagent (periodic acid-Schiff reagent - PAS) for the total carbohydrate (Maia, 1979); hematoxylin for aluminum

*Corresponding author. E-mail: fabianoifgoiano@gmail.com.

Figure 1. The flowering of *V. pyramidalis* Mart: The adult plant in its natural area of occurrence, in the Cerrado of the municipality of Rio Verde - Goiás (A); gray trunk, rough(B); and yellow inflorescence (C).
Source: Gessiane Silva Cabral Guimarães (2012).

(Al) (Polle et al., 1978) and hydrochloric acid for calcium oxalate identification (Chamberlain, 1932). Photomicrographs were obtained using a microscope (OLYMPUS BX61) coupled to a photographic camera (OLYMPUS DP73).

Assay III

Essential oil isolation

The essential oil was isolated from three types of samples: fresh flowers, fresh leaves and dry leaves. The leaves were dried in a convection oven (TE-394/2) at 45°C until a constant mass was obtained. A sample of 200 g of plant material was used in 2 L of distilled water and subjected to hydrodistillation for 2 h by a Clevenger apparatus. The collected hydrolate was extracted with dichloromethane in a ratio of ¼ of the total hydrolate volume divided by 3. Anhydrous sodium sulfate was then added for 30 minutes as a desiccant and was filtrated and evaporated under a fume hood. Following the evaporation of the solvent, the essential oil was transferred to an amber glass jar and stored at 4 ± 3°C until analysis. The plant oil content was calculated using the relationship between the mass of the extracted essential oil and plant dry mass used in the distillation.

Essential oil analysis

The chemical analyses were performed at the Chemistry Department of Lavras Federal University (Universidade Federal de Lavras - UFLA) using a gas chromatography device coupled to a quadrupole mass spectrometer (GC/MS) (Shimadzu QP5050A Kyoto, Japan) under the following operational conditions: a fused silica capillary column, model DB-5 (30-m long with an internal diameter of 0.25 mm and 0.25 µm thick film) (Shimadzu, Japan) using helium at a flow of 1 ml min^{-1} as the carrier gas and temperature-programmed heating (60°C ramped at 3°C min^{-1} up to 240°C followed by a ramp of 10°C min^{-1} up to 270°C with an isotherm of 7 and 70 min of total running time). The ionization energy of the detector was established at 70 eV, and the injection volume was 1 ml of sample diluted in dichloromethane (ultra-residue analysis; Baker, EUA) with a split ratio of 1:20. The temperatures of the injector and detector were maintained at 220 and 240°C, respectively. The analysis was performed in scanning mode at a speed of 2 scans s^{-1} and a mass range of 45 to 500 m/z.

RESULTS

Assay I

Anatomical analysis

The leaves were observed to be hypostomatic with paracytic stomata (Figure 2A and B).The epidermis consisted of one to three cell layers (Figure 2C, E and F). The parenchyma was differentiated into photosynthetic and fundamental tissue. The chlorenchyma was dorsoventral and consisted of palisade and spongy parenchyma. The palisade parenchyma was located directly below the adaxial surface of the epidermis and consisted of 1 to 2 cell layers. The palisade parenchyma cells were elongated and of equal length and became

smaller as they approached the main vein. The spongy parenchyma cells, when in the paradermal sections of the leaf surface, were isodiametric and connected to one or more palisade parenchyma cells. The spongy parenchyma cells could be characterized by a different shape than the remaining spongy cells and were connected to several cells of the palisade parenchyma (Figure 2C). The leaf vein on the adaxial surface was slightly convex and had a collateral vascular bundle that was enveloped by a bundle sheath and several fundamental parenchyma layers. A single duct was observed in the center of the main vein. Directly below the epidermis, on the adaxial and abaxial surface, two to three layers of collenchyma cells were observed (Figure 2C and D). The stem showed secondary xylem with numerous solitary vessels and a smaller number of multiple vessels with radial arrangements and simple perforation plates. The vessels were lined with axial parenchyma cells, rays, numerous fibers and mucilaginous channel (Figure 2G and H). The petals of *V. pyramidalis* showed a simple epidermis on both surfaces and a homogeneous parenchyma with collateral vascular bundles (Figure 2I). Stomata and small papillae were also observed on the petals (Figure 2J).

Assay II

Histochemical analysis

The histochemical tests were negative for tannins and alkaloids (Table 1). The phloroglucinol reagent detected lignin in the cell wall of the vessel elements and fibers in the leaves and stems. Sudan IV stained the lipids in the cuticle of all studied organs and numerous droplets of oil in the homogenous parenchyma of the petals. The potassium dichromate test identified non-lignin phenolic compounds in all organs in the epidermis and palisade parenchyma regions, a portion of cells (idioblasts) in the midrib of the leaves, fundamental parenchyma of the stem cortex and pith and parenchyma of the petals. The stained phenolic compounds were not tannins because the results of the vanillin-hydrochloric acid test were negative. On the leaves and stems, the Nadi reagents revealed the presence of terpenes in the isolated cells of the fundamental parenchyma (idioblasts), and were widely distributed in the homogeneous parenchyma of petals. The total proteins were stained by XP in the leaf and stem, and storage proteins were not detected. In the parenchyma of padding cells and mucilaginous channel, which, including idioblasts, are the only secretory structures of the plant, the presence of total hydrocarbons was detected by staining using PAS. Hematoxylin staining showed the presence of Al in epidermal cells of both the adaxial and abaxial surfaces of the leaf. The calcium oxalate crystals were of the druse type and were observed in leaves, stems and petals. The crystals were soluble in hydrochloric acid, and no effervescence was observed.

Assay III

Essential oil isolation and analysis

The obtained essential oils showed a faint yellow color with a strong odor. The oil yields were 0.002% from fresh flowers and dry leaves, 0.0018% from fresh leaves and a 0.0019% mean yield for *V. pyramidalis*. Twenty-six compounds were identified by GC/MS during the chemical analysis: 13 sesquiterpene hydrocarbons, 6 oxygen-containing sesquiterpenes, 4 oxygen-containing monoterpenes, 2 alcohols and 1 long-chain hydrocarbon, as shown in Table 2. Altogether, 77.05% of the compounds from the essential oil of dry leaves, 86.99% from fresh leaves and 87.85% from fresh flowers were identified. The sesquiterpene hydrocarbons fraction was predominant in essential oils from dry leaves (20.40%), fresh leaves (8.14%) and fresh flowers (11.16%). The components of the oxygen-containing fraction in the oil of fresh flowers were similar to those from fresh leaves (10.54 and 10.40%, respectively) but lower than in dry leaves (17.76%). Low concentrations of monoterpenes were detected, 6.18% in dry leaves, 7.13% in fresh leaves and 6.60% in fresh flowers. Other compounds (alcohols and hydrocarbons) were also identified, totaling 32.71% in dry leaves, 61.32% in fresh leaves and 59.55% in fresh flowers (Table 2). Sixteen compounds were observed in the 3 samples (aromadendrene, bicyclogermacrene, δ-cadinene, α-copaene, cyclosativene, caryophyllene oxide, cis-cis-farnesol, lanceol, (E)-nerolidol, geraniol, cis-limonene oxide, linalool, α-terpineol, heptadecanol, hexadecanol and pentacosane). The relative abundance of several compounds identified in the fresh leaves decreased when they were dried (lanceol, geraniol, linalool, α-terpineol, hexadecanol and pentacosane) and others were no longer detected (β-bourbonene, germacrene B, germacrene D, viridiflorene, spathulenol and viridiflorol). However, the concentration of aromadendrene, bicyclogermacrene, δ-cadinene, α-copaene, cyclosativene, caryophyllene oxide, farnesol, (E)-nerolidol, cis-limonene oxide and heptadecanol increased after the leaves were dried. Allo-aromadendrene and α-bulnesene were identified only in the oils from dry leaves, whereas β-bisabolene and β-selinene were detected only in the oils from flowers. The main compounds from the essential oils of flowers and fresh leaves were hexadecanol (25.84 to 27.90%) and pentacosane (25.90 to 27.89%). In dry leaves, the main compounds were pentacosane (19.34%) and farnesol (9.91%) (Table 2).

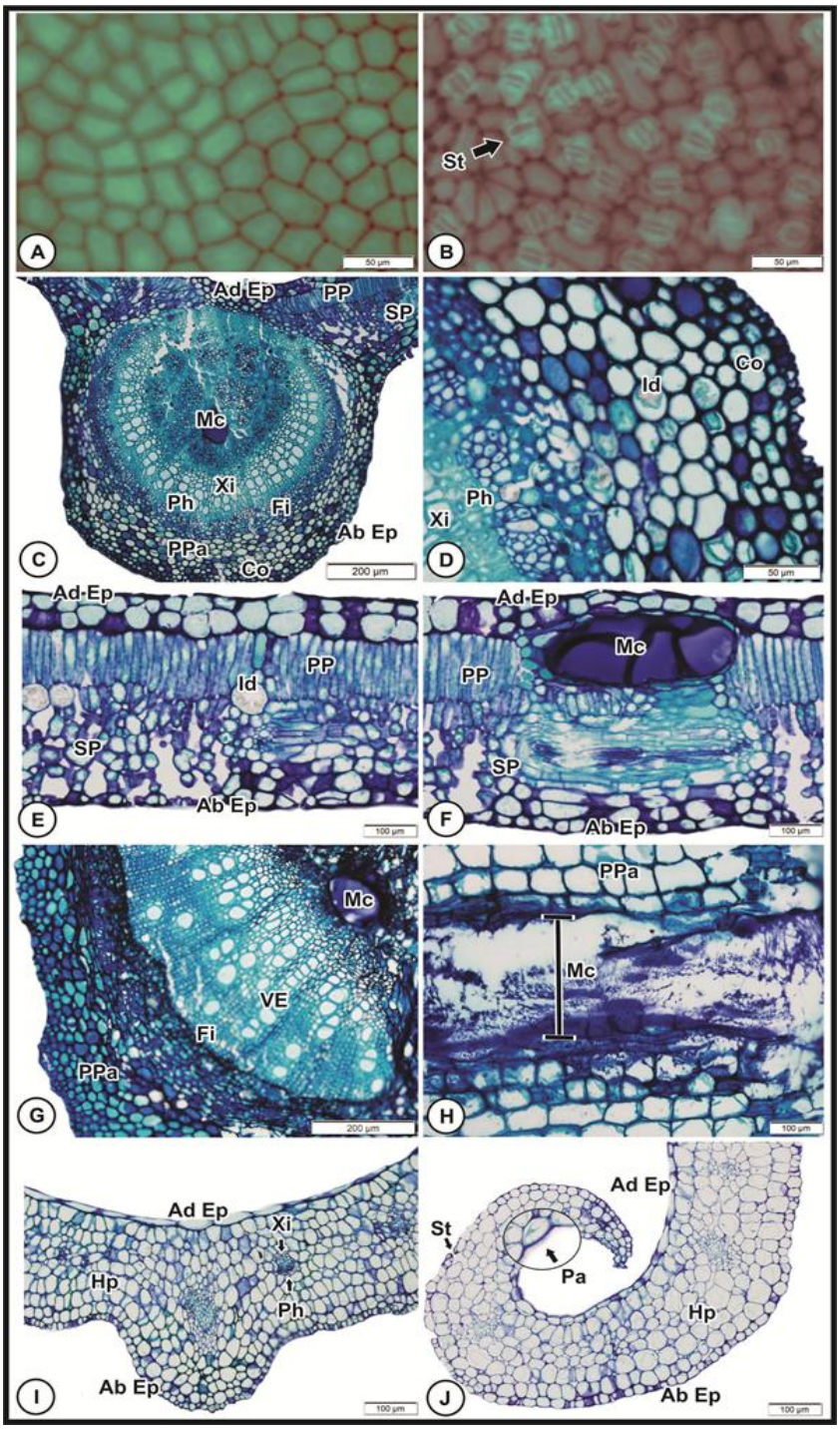

Figure 2. *V. pyramidalis* epidermis dissociation: A - The abaxial surface showing stomata (arrow) and B - The adaxial surface showing the absence of stomata. The cross-sections stained with Toluidine Blue: C - Main vein (leaf); D - CollenchymaandParenchyma of padding(leaf); E and F – Mesophyll (leaf); G - Xylem (stem); H-Longitudinal section of themucilaginouschannel(stem); I and J - Homogeneous parenchyma (petal); Ep Ad - Adaxial epidermis; Ep Ab - Abaxial epidermis; PP - Palisade parenchyma; Id- Idioblasts; SP- Spongy parenchyma; Mc – Mucilaginous channel; Fl - Phloem; Xi - Xylem; PPa - Parenchyma of padding; Fi - Fibers; Co - Collenchyma;VE - Vessel elements; - Hp - Homogeneous parenchyma; St – Stomata; Pa - Featuredinpapillae.

Table 1. The histochemistry of the leaves, stem and petals of *V. pyramidalis* Mart. for the detection of the main classes of metabolites (Rio Verde – GO, 2012).

Test	Coloration	Target compound	Plant organ used		
			Leaf	Stem	Petal
Vanillin-hydrochloric acid	Red	Tannins	-	-	-
Wagner	Red	Alkaloids	-	-	-
Phloroglucinol	Reddish	Lignin	+	+	-
Sudan IV	Orange/Reddish	Lipids	+	+	+
Potassium dichromate	Brown	Phenolic compounds	+	+	+
Nadi	Blue	Terpenes	+	+	+
Xylidine Ponceau	Red	Total proteins	+	+	NP
Schiff's reagent	Purple magenta	Total hydrocarbons	+	+	NP
Hematoxylin	Turquoise blue to violet	Aluminum	+	-	NP
Hydrochloricacid	Crystal dissolution without effervescence	Calcium oxalate	+	+	+

Abbreviations: + (positive reaction; - negative reaction; NP not performed).

DISCUSSION

Assay I

Anatomy

The anatomical study of *V. pyramidalis* allowed the identification of several common characteristics of species of the Vochysiaceae family. Barbosa (1999) observed that the stomata of *V. thyrsoidea* Pohl, *V. sessilifolia* Warm., *V. rufa* Mart. and *V. magnifica* Warm. were restricted to the abaxial surface of leaves and that the epidermis was simple on the leaf surfaces of both *V. magnifica* and *V. tucanorum*. The author further observed a larger number of xeromorphic characteristics in *V. thyrsoidea* than in the other species studied, with *V. thyrsoidea* presenting a multilayered adaxial epidermis and representing the only species with a simple, or at times bilayered, hypodermis. The leaf anatomy of *V. thyrsoidea* was dependent on the typical environmental conditions of rupes trian fields. *V. rufa* and *V. Cinnamomea* demonstrated epidermal cells with larger anticlinal than periclinal cell walls, similar to some epidermal cells observed in *V. pyramidalis* (the remaining cells had smaller anticlinal than periclinal cell walls). Similarly to *V. pyramidalis,* the presence of a single mucilage canal was observed in *V. magnifica, V. rufa* and *V. thyrsoidea,* and idioblasts were observed in *V. sessilifolia*, *V. rufa* and *V. magnifica*.

In the majority of species examined, the spongy parenchyma was formed by eight cell layers. In *V. pyramidalis*, five to eight cell layers were observed, where as only five layers were observed in *V. cinnamomea*. *V. thyrsoidea* demonstrated the largest number of cell layers, ranging from twelve to thirteen. As observed for *V. pyramidalis*, the palisade parenchy main the majority of Vochysiacea species was generally formed by two cell layers. However, some species, such as *V. thyrsoidea*, demonstrated three to four cell layers. In accordance with the present study, Paula et al. (2000) performed a cross-section analysis of the secondary xylem of *V. pyramidalis* and identified solitary vessels, simple perforation plates, a secretory channel in the axial parenchyma and abundant fibers. The identical characteristics were observed in *V. tucanorum*, although the fibers were less numerous than in *V. pyramidalis*. Xeromorphic features are present in species of the family Vochysiaceae, which suggests physiological and anatomical adaptations, thus resulting in adaptive success to arid environments with high radiation rates and nutrient-poor soils (Fahn and Cutler, 1992). The leaf is the plant organ that shows the greatest structural variation in response to climatic changes (Dickison, 2000). Such features, which include a dorsiventral mesophyll, developed vascular tissue associated with fibers and the presence of stomata confined to the abaxial leaf surface, were evident in the leaf blades and mucilage cells of *V. pyramidalis.*

Assay II

Histochemistry

According to Mayworm et al. (2011), Vochysiaceae species show a high oleaginous potential. The histochemical tests revealed that the oils were mainly present in the homogeneous parenchyma of *V. pyramidalis* petals. Several species of the family Vochysiaceae possess idioblasts containing crystals, either in the spongy parenchyma or at the transition with the palisade parenchyma (Barbosa, 1999). The druse crystals observed for *V. pyramidalis* were composed of calcium

Table 2. The percentage of compounds identified in the essential oil of V. pyramidalis Mart. isolated from different samples. Rio Verde - GO, 2012.

S/No	Compound	Classification	Dry leaf (%)	Fresh leaf (%)	Fresh flower (%)	KI
1	Allo-aromadendrene	SH	0.18	0.00	0.00	1439
2	Aromadendrene	SH	1.94	0.87	0.54	1458
3	Bicyclogermacrene	SH	2.56	1.59	1.71	1500
4	β-Bisabolene	SH	0.00	0.00	3.56	1505
5	β-Bourbonene	SH	0.00	1.94	2.35	1387
6	α-Bulnesene	SH	5.89	0.00	0.00	1509
7	δ-Cadinene	SH	0.86	0.10	0.15	1522
8	α-Copaene	SH	4.99	1.00	2.02	1368
9	Cyclosativene	SH	3.98	0.59	0.37	1368
10	Germacrene B	SH	0.00	0.80	0.03	1559
11	Germacrene D	SH	0.00	0.79	0.23	1483
12	β-Selinene	SH	0.00	0.00	0.11	1485
13	Viridiflorene	SH	0.00	0.46	0.09	1494
	Total	-	20.40	8.14	11.16	-
14	Caryophyllene oxide	OS	1.95	0.17	0.90	1582
15	Cis-cis-Farnesol	OS	9.91	4.60	3.26	1715
16	Lanceol	OS	0.30	0.57	0.12	1759
17	(E)-Nerolidol	OS	5.60	1.89	1.73	1561
18	Spathulenol	OS	0.00	2.69	3.74	1575
19	Viridiflorol	OS	0.00	0.48	0.79	1589
	Total	-	17.76	10.40	10.54	-
20	Geraniol	OM	1.03	1.98	2.09	1249
21	cis-Limonene oxide	OM	1.00	0.30	0.04	1137
22	Linalool	OM	2.48	2.98	2.05	1097
23	α-Terpineol	OM	1.67	1.87	2.42	1187
	Total	-	6.18	7.13	6.60	-
24	Heptadecanol	AL	5.98	5.53	7.81	1698
25	Hexadecanol	AL	7.39	27.90	25.84	1869
26	Pentacosano	HYD	19.34	27.89	25.90	2489
	Total	-	32.71	61.32	59.55	-
	Total identified (%)	-	77.05	86.99	87.85	-

Sesquiterpene hydrocarbon (SH); Oxygen-containing sesquiterpene (OS); Oxygen-containing monoterpene (OM); Alcohol (AL); Hydrocarbon (HYD); KI –Kovat Index obtained for the compounds.

oxalate, which is the typical composition of plant cell crystals (Fahn, 1990). The presence of phenolic compounds and terpenes are closely related to the wide occurrence of idioblasts in leaves, stems and petals. The idioblasts may contain mucilaginous substances, such as oils and several types of crystals, differing mainly from the chemical compounds stored in the other parenchymal cells (Apezzato-da-Glória and Carmello-Guerreiro, 2006). According to Castro et al. (2004), phenolic compounds possess anti-ulcerogenic properties and wound-healing and antiseptic actions. Regarding terpenes, the study by Bortalanza et al. (2002) in *V. divergens* Pohl identified the efficiency of tormentic acid, a pentacyclic triterpene, against neuropathy and persistent pain because of inflammatory processes. The presence of this class of secondary compounds may be a characteristic common to the genus. The Al detection test showed similar results to those obtained by Andrade et al. (2011), in which the cuticle cell walls and cells of the adaxial and abaxial epidermis of *V. pyramidalis* were strongly stained by hematoxylin. The location of Al in the tissues was detected by the purple color typical of Al stained with

hematoxylin in Vochysiaceae species (*Qualea grandiflora, Callisthene principais* and *V. pyramidalis*). In the literature, *V. pyramidalis* is considered an Al accumulating species (Silva Júnior and Pereira, 2009). According to Haridasan (1982), numerous native bushes and trees of the Brazilian cerrado accumulate Al in their leaves. This element is toxic for many plants; however, numerous species, particularly those native to the cerrado, have developed different detoxification mechanisms. The presence of this element in *V. pyramidalis* cell walls may be explained by the fact that Al ions can compete with, and prevail over, calcium ions (Ca^{+2}) for pectin binding sites (Kinraide, 1998).

Assay III

The isolation and analysis of essential oil

According to Silva-Santos et al. (2008), terpenic compounds may show relevant biological actions, such as antibiotic, analgesic and anti-inflammatory properties, and with potential applications in such areas as dermatology, cardiology, gastroenterology, neurology or oncology. The presence of terpenes in the leaves, stems and petals of *V. pyramidalis* was shown using histochemical testing and confirmed by CG/MS analysis. GC/MS characterized the composition of essential oil as mainly a mix of volatile mono- and sesquiterpenes. The results showed that the chemical composition of the samples differed both qualitatively and quantitatively. Greater differences were observed between dry and fresh samples in terms of the abundance of their components. This difference may be explained by the presence of volatile compounds, temperature or enzymatic degradation (Moure et al., 2001; Lima et al., 2003). However, several components are more abundant in samples of fresh material, namely the main compounds, with the exception of farnesol, for which the increase in its percentage may be a consequence of the drying process that can favor its extraction (Park el al., 2001). Farnesol is an important chemical compound that is widely used by the cosmetic industry as a bacteriostatic agent in soaps, deodorants and skin care products. Farnesol has the potential to help treat dermal infections by *Staphylococcus aureus* previously treated with beta-lactams. Farnesol is characterized by its bacteriostatic action against *S. aureus* (Akiyama et al., 2002) and *Candida albicans* (Sato et al., 2004). *V. pyramidalis* shows secondary compounds commonly observed in its family. However, further studies are required to identify the factors that influence the chemical composition and yield of essential oils and evaluate the potential of their chemical components using phytochemical and pharmacological analyses, to use *V. pyramidalis* as a medicinal plant or to use the plant in such areas as the cosmetic and food industries.

Conclusion

Several xeromorphic characteristics were observed on the leaf blade of *V. pyramidalis.* The anatomical studies suggest that mucilaginous channel and idioblasts are the only secretory structures present in this plant species. The histochemical tests revealed the presence of 2 classes of secondary metabolites: phenolic compounds and terpenes. The presence of these metabolites is related to the wide distribution of idioblasts in the leaves, stem and petals. Twenty-six compounds were identified through GC/MS, representing a mean of 83.96% of the total chemical compounds in essential oil with the highest abundance of hexadecanol, pentacosane and farnesol.

Conflicts of Interest

All authors report no conflict of interest.

REFERENCES

Akaisue G, Oliveira F (1987). Farmacobotânica, vol. 1. Pharmakon: São Paulo.

Akiyama H, Oono T, Huh WK, Yamasaki O, Ogawa S, Katsuyama M, Ichikawa H, Iwatsuki K (2002). Actions of farnesol and xylitol against Staphylococcus aureus. Chemotherapy 48(3):122-128.

Andrade LRM, Barros LMG, Echevarria GF, Amaral LV, Cotta MG, Rossatto DR, Haridasan M, Franco AC (2011). Al-hyperaccumulator Vochysiaceae from the Brazilian Cerrado store aluminum in their chloroplasts without apparent damage. Environ. Exp. Bot. 70:37-42.

Apezzato-Da-Glória B, Carmello-Guerreiro SM (2006). Anatomia vegetal, 2nd ed. Viçosa: UFV.

Barbosa AR (1999). As espécies do gênero Vochysia Aubl. (Vochysiaceae) ocorrentes no Estado de São Paulo. Dissertação de Mestrado em Biologia Vegetal. Universidade Estadual de Campinas: Campinas.

Bortalanza LB, Ferreira J, Hess SC, Delle Monache F, Yunes RA, Calixto JB (2002). Anti-allodynic action of the tormentic acid, a triterpene isolated from plant, against neuropathic and inflammatory persistent pain in mice. Eur. J. Pharmacol. 453:203-208.

Carnevale NFC, Pilon AC, Silva DHS, Bolzani VS, Gamboa IC (2011). Vochysiaceae: secondary metabolites, ethnopharmacology and pharmacological potential. Phytochem. Rev. 10(3):413-429.

Castro HG, Ferreira FA, Silva DJH, Mosquim PR (2004). Contribuição ao estudo das plantas medicinais-Metabólitos secundários, 2nd ed. Viçosa.

Chamberlain CJ (1932). Methods in plant histology, 5th ed. The University of Chicago Press: Chicago.

David R, Carde JP (1964). Coloration différentielle des inclusions lipidiques et terpéniques des pseudophylles du Pin maritime au moyen du réactif nadi. C. R. Acad. Sci. 258:1338-1340.

Dickison WC (2000). Integrative plant anatomy. San Diego: Harcourt Publishing House/Academic Press.

Fahn A (1990). Plant anatomy, 4th ed. Pergamon Press: Oxford.

Fahn A, Cutler D (1992). Xerophytes. Gebruder Borntraeger: Berlin.

Furr M, Mahlberg PG (1981). Histochemical analyses of lacticifers and glandular trichomes in *Cannabis sativa*. J. Nat. Prod. 44:153-159.

Gabe M (1968).Techniques histologiques. Masson e Cie: Paris.

Gomes RC, Bonarum F, Darin DD, Seito LN, Di Stasi LC, Dokkedal AL, Vilegas W, Brito ARMS, Hiruma-Lima CA (2009). Antioxidante action of methanolic extract and buthanolic fraction of *Vochysia tucanorum*

Mart. in the gastroprotection. J. Ethnopharmacol. 121:466-471.
Guarin Neto GO (2006). O saber tradicional Pantaneiro: as plantas medicinais e a educação ambiental. Rev. Eletrôn. Mestrado Educ. Ambiental 17:71-79.
Gusmão E, Vieira FA, Fonseca Júnior EM (2006). Biometria de frutos e endocarpos de Murici (*Byrsonima verbascifolia* Rich. ex A. Juss.). Cerne 12(1):84-91.
Haridasan M (1982). Aluminum accumulation by some Cerrado native species in central Brazil. Plant Soil 65:265-273.
Hess SC, Delle Monache F (1999). Divergioic acid, a triterpene from *Vochysia divergens*. J. Braz. Chem. Soc. 10(2):104-106.
Jesus NZT, Lima JCS, Silva RM, Espinosa MM, Martins DTO (2009). Levantamento etnobotânico de plantas popularmente utilizadas Como antiúlceras e antiinflamatórias pela comunidade de Pirizal. Brasil: Nossa Senhora do Livramento-MT. Rev. Bras. Farmacogn. 19(1):130-139.
Johansen DA (1940). Plant microtechnique.New York: McGraw Hill Book Company.
Kinraide TB (1998). Three mechanisms for the calcium alleviation of mineral toxicities. Plant Physiol. 118:513-520.
Lima HRP, Kaplan MAC, Cruz AVM (2003). Influencia dos fatores abióticos na produção e variabilidade de terpenóides em plantas. Floresta Ambiente 10(2):71-77.
Mace ME, Howell CR (1974). Histochemistry and identification of condensed tannin precursor in roots of cotton seedlings. Can. J. Bot. 52(11):2423-2426.
Maia V (1979). Técnica histológica. Atheneu: São Paulo.
Mayworm MAS, Buckeridge MS, Marquez UML, Salatino A (2011). Nutritional reserves of Vochysiaceae seeds: chemical diversity and potential economic uses. An. Acad. Bras. Cienc. 83(2):523-531.
Ming LC (1994). Estudo e pesquisa de plantas medicinais na Agronomia. Hortic. Bras. 12:9-3.
Moure A, Jose MC, Daniel F, Domínguez JM, Jorge S, Herminia D, Núñez MJ, Parajó JC (2001). Natural antioxidants from residual sources. Food Chem. 72(2):145-171.
O'Brien TP, McCully ME (1981). The study of plant structure: principles andselected methods. Melbourne: Termarcarphi.
Park KJ, Yado MKM, Brod FPR (2001). Estudo de secagem de pêra bartllet (*Pyrus* sp.) em fatias. Braz. J. Food Technol. 21(3):288-292.
Paula JE, Silva Júnior FG, Silva APP (2000). Caracterização anatômica de madeiras nativas de matas ciliares do centro-oeste brasileiro. Sci. Forestalis 58:73-89.
Piazza GJ, Foglia TA (2001). Repeseed oil for oleochemical usage. Eur. J. Lipid Sci. Technol. 103(7):450-454.
Polle E, Konzac CF, Kittrick JA (1978). Visual detection of aluminium tolerance levels in wheat by hematoxylim staining of seedling roots. Crop Sci. 18:823-827.
Rodrigues V, Carvalho DA (2001). Levantamento etnobotânico de plantas medicinais no domínio Cerrado na região do Alto Rio Grande-Minas Gerais. Ciên. Agrotechnol. 25(1):102-123.
Santos MCA, Freitas SP, Aroucha EMM, Santos ALA (2009). Anatomia e histoquímica de folhas e raízes de vinca (*Catharanthus roseus* (L.) G Don). Rev. Biol. Ciências Terra 9:24-30.
Sato T, Watanabe T, Mikami T, Matsumoto T (2004). Farnesol, a morphogenetic autoregulatory substance in the dimorphic fungus *Candida albicans*, inhibits hyphae growth through suppression of a mmitogen-activated protein kinase cascade. Biol. Pharm. Bull. 27:751-752.
Silva Júnior MCS, Pereira BAS (2009). 100 Árvores do Cerrado Matas de Galeria. Rede de sementes do Cerrado: Brasília.
Silva-Santos A, Antunes MAS, Bizzo HR, D'Avila LA, Souza-Santos LC, Souza RC (2008). Analysis of uses of essential oils and terpenics/ terpenoids compounds by pharmaceutical industry through USPTO granted patents. Rev. Bras. Plant Med. 10(1):8-15.
Souza CD, Felfili VM (2006). Uso de plantas medicinais na região de Alto Paraíso de Goiás, GO, Brasil Acta. Bot. Bras. 20(1):135-142.
Thadeo M, Meira RMSA, Azevedo AA, Araújo JM (2009). Anatomia e histoquímica das estruturas secretoras da folha de Casearia decandra Jacq. (Salicaceae). Rev. Brasil Bot. 32:329-338.

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Full Length Research Paper

Antibacterial activity of tannins from *Psidium guineense* Sw. (Myrtaceae)

Cinthia Gracielly Rodrigues[1], Perácio Rafael Bueno Ferreira[2]*, Cintia Sorandra Oliveira Mendes[1], Ronaldo Reis Júnior[1], Henrique Maia Valério[I], Igor Viana Brandi[3] and Dario Alves de Oliveira[1]

[1]Bioprospecting and Genetic Resources Laboratory, State University of Montes Claros, Brazil.
[2]Plant Tissue Culture Laboratory, Federal University of Viçosa, Brazil.
[3]Institute of Agrarian Sciences, Federal University of Minas Gerais, Brazil.

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The aim of this study was to quantify and evaluate the antibacterial activity of tannins extracted from leaves of *Psidium guineense*, obtained using two different isolation methods, against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The first extraction method was used to isolate condensed tannins (Method A) and the second to isolate mixtures rich in esters of gallic acid and glucose (Method B). Both extraction methods yielded high concentrations of tannins, with 312 and 257 mg/g of dry material obtained using Methods A and B, respectively. These compounds formed halos of growth inhibition in *S. aureus* and *P. aeruginosa* cultures. Tannins isolated by method B gave rise to larger inhibition halos than those isolated by method A. Analysis of the antibacterial activity of tannins isolated by method B revealed a minimum inhibitory and bactericidal concentration of 256 µg/ml for *S. aureus* and 512 µg/ml for *P. aeruginosa*. These results demonstrate that *P. guineense* is a promising source of pharmacologically active tannin molecules, and further studies are therefore necessary to determine the toxicity of the plant and ensure its safe use for animal and human health.

Key words: *Psidium guineense*, tannins, isolation, antibacterial activity, *Staphylococcus aureus, Pseudomonas aeruginosa*.

INTRODUCTION

Psidium guineense, commonly known as *araçá*, is a shrub of the family Myrtaceae with a height of 2 to 2.5 m and is native to and widely dispersed in tropical America (González et al., 2005). The roots of *P. guineense* have been traditionally used in Brazil as antidiarrheals and diuretics, and the bark is used in tanneries due to its high tannin content (Rodrigues and Carvalho, 2001). A study by González et al. (2005) demonstrated the antimicrobial

*Corresponding author. E-mail: peracio.bueno@gmail.com. Tel: +55 (31)38992930.

potential of the pulp of *P. guineense* fruit and Fernandes et al. (2012) realized a growth inhibitory effect of *P. guineense* extract on antimicrobial agents against methicillin-resistant *Staphylococcus aureus* strains due for it combination with beta-lactams, carbapenems and fluoroquinolones in antibiotic, corroborating the hypothesis that *P. guineense* extract have synergistic effect in antimicrobial activity.

Some species of the family Myrtaceae are promising targets for exploration through chemical and pharmacological studies. Studies of *Psidium guajava* and *Eugenia uniflora* have demonstrated that extracts from the leaves of these plants show diverse pharmacological activities, such as anti-inflammatory, antimicrobial, antimalarial, hypoglycemic, antispasmodic and antioxidant effects (Schapoval et al., 1994; Lee et al., 2000; Gutiérrez et al., 2008). Multiple classes of chemicals with important pharmacological activities have already been isolated from plants belonging to this family (Meckes et al., 1996; Begum et al., 2002; Salib and Michael, 2004).

The ability of tannins to form complexes with macromolecules is the basis both for their ecological properties in controlling insects, fungi and bacteria and for their pharmacological activities (Santos and Mello, 1999). Plants rich in tannins are employed in traditional medicine to treat a number of diseases (Haslam, 1996; De Bruyne et al., 1999). Extensive analyses have revealed diverse biological activities of this class of substances (Matsuo et al., 1994; Haslam, 1996; Lee et al., 2000), including their ability to inhibit the growth of microorganisms (Scalbert, 1991; Akiyama et al., 2001; Ferreira et al., 2012).

Considering the inhibitory effect of tannins on microorganisms, the notable presence of these compounds in the leaves of *P. guineense* and the paucity of studies concerning this species, this study aimed to quantify and evaluate the antibacterial activity of tannins extracted from leaves of *P. guineense*.

MATERIALS AND METHODS

Collection of the botanical material and preparation of the extract

Leaves of *P. guineense* were collected from 10 randomly chosen wild plants in (Savannah Brazilian area) northern area of the state of Minas Gerais, Brazil (16°51'00"S, 43°41'49"W). A sample of the botanical material was deposited in the herbarium of the State University of Montes Claros (n° 606). The extraction method was adapted from Salminen et al. (1999). Briefly, 20 g of dry and pulverized leaves were suspended in 100 ml of 70% acetone with 0.1% ascorbic acid and agitated for one hour. After centrifugation at 10,000 g for 10 min, the pellet was re-extracted four times with the same solvent. The acetone from the combined extracts was then evaporated in a rotary evaporator under a vacuum at 30°C, and the extract was subsequently filtrated using filter paper Whatman's No. 1 and concentrated in a forced air convection oven at 30°C until the volume was reduced as much as possible. A sample of the resulting raw extract was used for evaluation of the minimum inhibitory concentration. The remaining portion was diluted with an equal volume of 95% ethanol and centrifuged at 10,000 g in order to remove the pellet.

Isolation of tannins

The crude extract was then subjected to one of two methods of tannin isolation, designated here as A and B. Method A was proposed by Asquith and Butler (1985) for the isolation of fractions rich in tannins. The hydrophilic fraction resulting from the extraction process, which was dissolved in 95% ethanol, was applied to a Sephadex LH-20 column (3 × 30 cm) equilibrated with 95% ethanol. The column was washed with 95% ethanol at a flow rate of 1 ml/min. Fractions of 10 ml were collected and read with a spectrophotometer at a wavelength of 280 nm until their absorbances reached approximately zero. The ethanol used in the washes was discarded after calculating the yield of the combined fractions. The column was then eluted with 50% aqueous acetone at a flow rate of 1 ml/min until the Sephadex returned to its characteristic color (white) and the elute became less intense in color. Fractions of 4 ml were collected, and their absorbances at 435 nm were determined. The fractions were analyzed for the presence of tannins using the gelatin precipitation test (Strumeyer and Malin, 1975). Tannin-positive fractions were combined, and the acetone was completely removed from the combined fractions by evaporation under reduced pressure at 30°C. The aqueous sample was extracted three times with an equal volume of ethyl acetate, and the organic phase (upper) was discarded. After the third extraction, traces of ethyl acetate remaining in the aqueous fractions were removed in a forced air convection oven at 30°C. The aqueous sample was then lyophilized and subjected to antibacterial activity testing, and the radial diffusion in agarose gel method was used for total tannin quantification.

Method B was proposed by Hagerman and Klucher (1986) to isolate tannins from mixtures rich in esters of gallic acid and glucose. The aqueous sample resulting from the extraction process and dissolved in 95% ethanol was applied to a Sephadex LH-20 column (40 ×3 cm) equilibrated with 95% ethanol. The Sephadex gel was washed with 95% ethanol at a flow rate of 0.5 ml/min, and 10 ml fractions were collected until their absorbance readings at 280 nm reached approximately zero. The ethanol used in the washing was discarded after calculating the yield of the combined fractions. The tannins were then eluted from the gel with 50% acetone and 0.001 M ascorbic acid. Fractions of 4 ml were collected, and their absorbances at 435 nm were determined. The fractions were analyzed for the presence of tannins using the gelatin precipitation test (Strumeyer and Malin, 1975), and the tannin-positive fractions were combined. Acetone was completely removed from the combined fractions by evaporation under reduced pressure at 30°C. The aqueous sample was lyophilized and subjected to antibacterial activity analysis using the disc diffusion method, and the quantification of total tannins was measured using radial diffusion in agarose gels.

Quantification of total tannins

The concentration of total tannins present in the lyophilized samples was determined by radial diffusion in agarose gels (Hagerman, 1987). Five milligrams of the sample was dissolved in 100 µl of 50% methanol. Perforations were made in a previously prepared gel with the aid of a cylinder 2.8 mm in diameter. Twenty microliters of lyophilized sample dissolved in methanol was

introduced into each perforation. After 96 h of incubation at 30°C, the diameters of the rings were measured in millimeters. The quantity of precipitated tannins was determined using a calibration curve for tannic acid (5 to 15 mg/ml). The data are presented as the area of the tannin-protein precipitate in cm^2 per g of sample dry mass.

Antibacterial activity assay

The tests for bacterial growth inhibition were carried out with *S. aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853). Standard strains seeded in Brain Heart Infusion (BHI; Biobrás®) agar with mineral oil (Ideal®) were cultured in Müller-Hinton agar (Biobrás®) and incubated for 24 h in an oven at 35°C. Bacterial suspensions in 0.98% NaCl with turbidity equivalent to McFarland Scale 0.5 (1×10^8 cells/ml) were prepared from recent cultures. The suspensions of the tested microorganisms were seeded in Petri dishes with Müeller-Hinton agar surface with the aid of a sterile swab. The disk diffusion test was based on the National Committee for Clinical Laboratory Standards (NCCLS M2-A8, 2003).

Lyophilized tannin samples obtained using both isolation methods were dissolved in dimethyl sulfoxide [DMSO (70 mg/ml)] and applied to sterile Blank discs 6 mm in diameter (Cecon). These discs were then deposited on the agar, and the dishes were incubated at 35°C for 24 h. After incubation, the halo of inhibition formed around the discs was measured in millimeters. Commercial discs with 30 µg chloramphenicol (Laborclin®) were used as a positive control for *S. aureus,* and discs with 30 µg tetracycline (Laborclin®) were used for *P. aeruginosa*. Discs infused with 10 µl DMSO were used as a negative control.

The broth microdilution test, based on the NCCLS standard M7-A6 (2003), was used to determine the minimum inhibitory concentration (MIC) of the isolated samples and raw extract. Concentrations of isolates and raw extract from 0.5 to 1024 µg/ml were tested. The inoculated microdilution dishes were incubated at 35°C for 24 h in microplates. Resazurin, an indicator of microbial growth, was used to aid the analysis. The minimum inhibitory tannin concentration was defined as the lowest concentration that resulted in no microbial growth, as indicated by the blue color of the resazurin. Then, the tannin dilutions that showed no microbial growth were divided into smaller increments to more precisely define the minimum bactericidal concentration (MBC). The antibacterial activity assay was performed in 5 repetitions.

Statistical analysis

The data were analyzed with the R statistical system version 2.8.0 using generalized linear models. The appropriate model was estimated from the elimination of non-significant variables tested from the full model, which is known as the backward method (Crawley, 2005).

RESULTS

The chromatograms as shown in Figure 1 track absorbances of the ethanolic wash fractions at 280 nm and the fractions eluted with acetone at 430 nm using a spectrophotometer for methods A (Asquith and Butler, 1985) and B (Hagerman and Klucher, 1986). The gel of the Sephadex LH-20 adsorbs tannins in alcohol and releases them in aqueous acetone. The chromatograms show that during elution with 95% ethanol, a discernable absorption region was observed up to approximately 400 min for method A (Figure 1a) and up to nearly 600 min for method B (Figure 1b). The other substances remained firmly adhered to the top of the column until they were eluted with 50% acetone, forming a single peak in both methods. The single peak eluted with aqueous acetone began to register after 700 min with method A (Figure 1a) and after 1500 min with method B (Figure 1b). For all the fractions eluted with 50% acetone (which correspond to the final peak), formation of a precipitate was observed in the gelatin precipitation test.

Regarding the yield of the fractions eluted from the column, there was no difference between the two isolation methods. The fractions eluted with ethanol had a yield of 43.68±3.8%, and those eluted with acetone showed a yield of 22.05±1.77%. Thus, approximately 66% of the total material applied to the column was recovered.

The equation of the tannin standard curve generated by radial diffusion in agarose gels (Figure 2) was $y= 4.4924x + 0.3902$, $R^2= 0.9974$. The quantity of total tannins present in the sample obtained from isolation method A (312 ± 8 mg/g of dry material) was significantly greater than that from method B (257±8 mg/g of dry material) (ANOVA, $P<0.01$).

The results of the antibacterial disk diffusion assay are as shown in Figure 3 and are expressed as the diameter of the inhibition halo. The compounds isolated by methods A and B formed growth inhibition halos for both the tested bacterial strains. Despite showing lower tannin content, the sample originating from isolation method B presented a larger inhibition halo (18.2±0.10 mm) compared to method A (15.8±0.12 mm), against *P. aeruginosa*. The ethyl acetate used for the washing fractions in method A may have removed phenolic compounds, such as flavonoids, simple phenols and even polyphenols, which would act synergistically with tannins in the aqueous phase. Because the sample obtained by method B showed a larger inhibition halo, further analyses of its MIC and bactericidal level were performed.

The minimum bactericidal and inhibitory concentrations of the raw extract and the tannin samples isolated by method B are as shown in Table 1. MBC was equal to the inhibitory concentration for both strains. These results demonstrate that this tannin isolation method effectively isolated compounds with antibacterial activity, because the isolated sample presented an MBC sixteen times smaller than the raw extract (personal data). According to the classification proposed by Aligianis et al. (2001), MIC values of ≤0.5 mg/ml are considered strongly inhibitory in the case of vegetable matter.

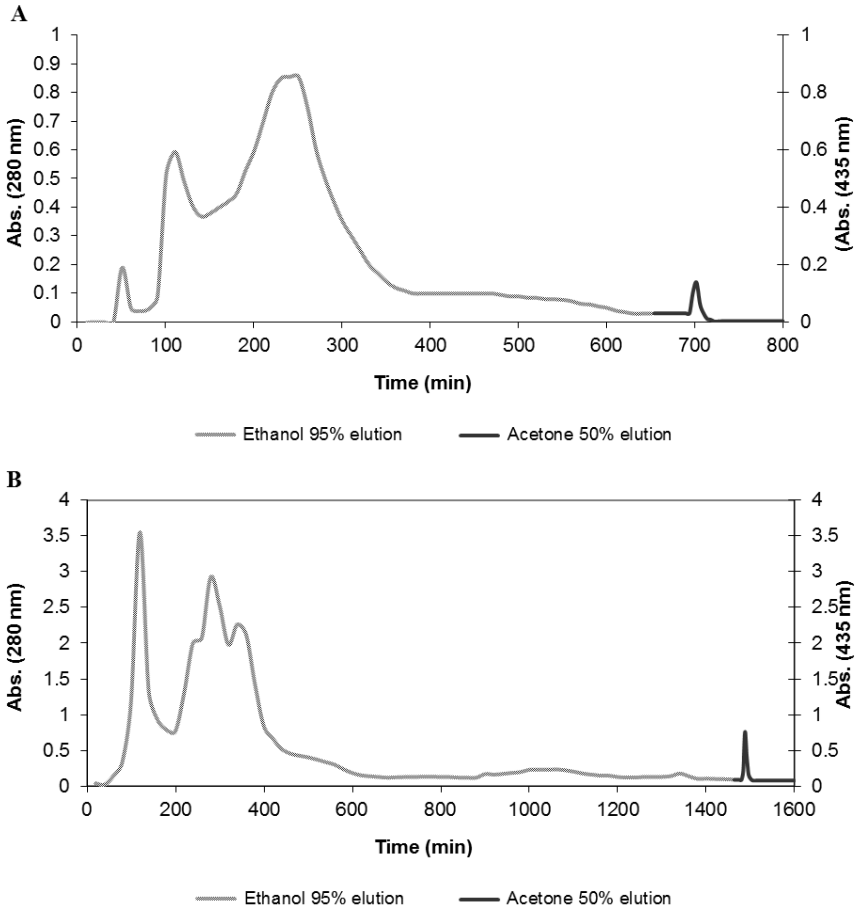

Figure 1. Tracking of the fractionation of phenolic compounds through their adsorption in Sephadex LH-20 and their elution with 95% ethanol (at 280 nm) followed by 50% acetone (at 435 nm). (A) Method A; (B) Method B. Abs: Absorption.

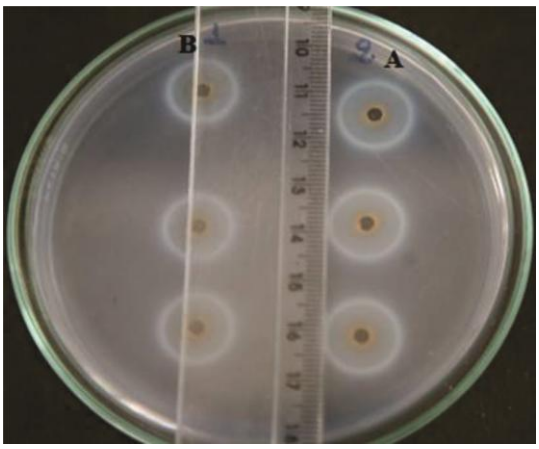

Figure 2. Halos of tannin-protein precipitate formed in the quantification of total tannins by radial diffusion in agarose gels from samples obtained using isolation methods A (right) and B (left) from *P. guineense* leaves.

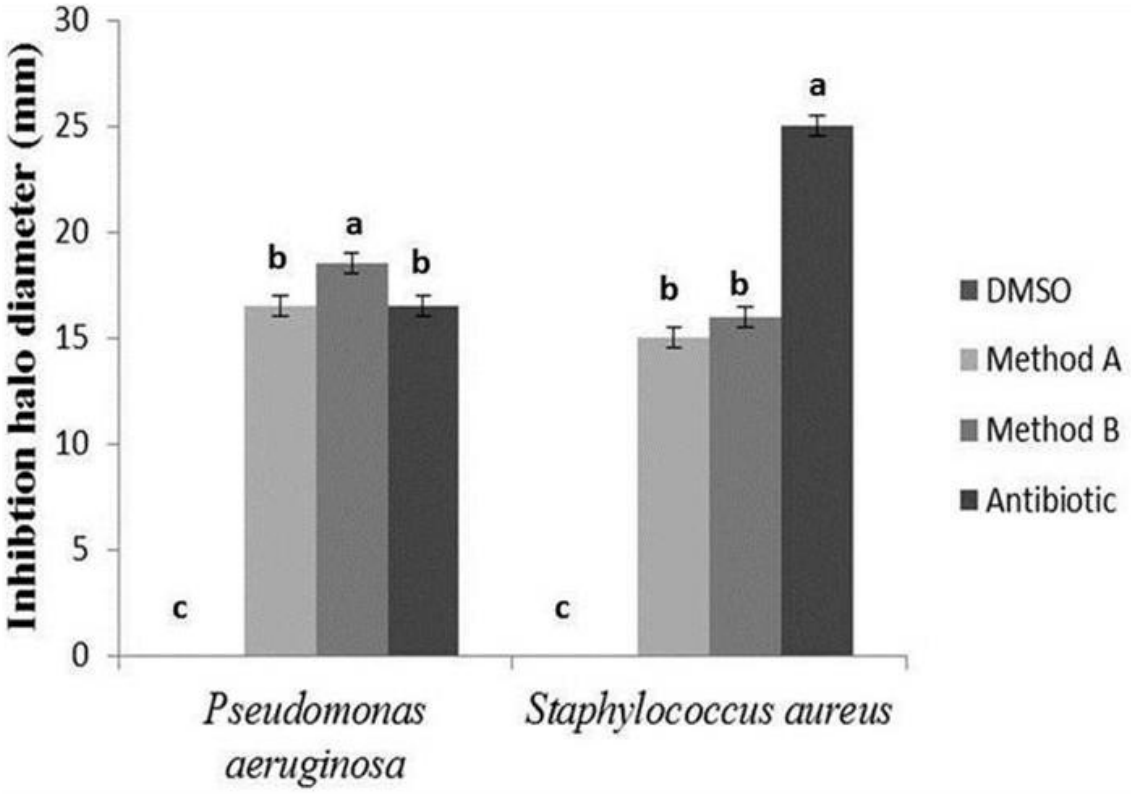

Figure 3. Measurements of the diameter of the bacterial growth inhibition halo induced by the samples obtained using the two isolation methods and by the positive (tetracycline, chloramphenicol) and negative (DMSO) controls. *Values are the means ± Standard deviations of three replicated experiments. Means followed by the same letter do not differ statistically among themselves.

Table 1. Antibacterial activity of raw tannin extract isolated from leaves of *P. guineense*.

Micro-organism	Origin	MIC and MBC (mg/ml)*	
		Raw extract	Isolated tannins
S. aureus	ATCC 25923	4.01	0.256
P. aeruginosa	ATCC 27853	8.20	0.512

*CIM and CBM showed the same values for trials with raw extract and with isolated tannins.

DISCUSSION

The percentages obtained by Wang and Lee (1996) for extracting tannins from *Areca catechu* fruits were 47 and 30% for the ethanol and acetone fractions, respectively, resulting in a total recovery of approximately 77%. The tannin content of the samples from both methods was higher than that obtained by Wang and Lee (1996) from *Areca catechu* fruits (160 mg/g of dry material).

The inhibitory effects of different classes of tannins against various microorganisms have been widely studied. According to Haslam (1996), tannins act on micro-organisms by binding to proteins and adhesins, inhibiting bacterial enzymes, rupturing the cell membrane and scavenging microbial substrates. Kumar and Vaithiyanathan (1990) suggested that tannins directly inhibit microbial function in the rumen by complexing with bacterial cells or indirectly by reducing the availability of nitrogen and sulfur for microbial protein synthesis. Scalbert (1991) listed 33 studies documenting the antimicrobial properties of tannins. Sakanaka et al. (1996) reported the role of polyphenols in inhibiting growth and cellular adherence of

the oral bacteria *Porphyromonas gingivalis*, which is responsible for the majority of acute periodontitis cases. De Bruyne et al. (1999) evaluated the antimicrobial activity of a series of proanthocyanidin dimers and verified that the minimum inhibitory concentration was >100 µg/ml for *Escherichia coli, P. aeruginosa, Salmonella paratyphi, Enterobacter cloacae, Mycobacterium fortuitum, S. aureus* and *Candida albicans*. Djipa et al. (2000) correlated the antibacterial activity demonstrated by *Syzygium jambos* (Myrtaceae) to its high tannin content, and Akiyama et al. (2001) iden-tified tannic acid as a possible adjuvant to antibiotics in the treatment of *S. aureus* infections. Panizzi et al. (2002) observed that fractions of tannins and other phenols ex-tracted from *Rubus ulmifolius* showed high antimicrobial activity. González et al. (2005) proved the antibacterial potential of the raw extract from the peel and pulp of *P. guineense* fruit, and they demonstrated that fractions isolated from this extract using petroleum ether, dichloromethane, ethyl acetate and water also had anti-microbial effects, which were attributed to the presence of tannins, flavonoids and terpenes in the fruits of this species.

Thus, *P. guineense* represents a promising source of molecules with pharmacological and antibacterial activities, and it contains high tannin content in its leaves, which exhibit a diverse set of biological activities, as extensive studies have shown.

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Conflict of Interest

Authors have not declare any conflict of interest.

REFERENCES

Akiyama H, Fujii K, Yamasaki O, Oono T, Iwatsuki K (2001). Antibacterial action of several tannin against *Staphylococcus aureus*. J. Antimicrob. Chemoth. 4(48):487-491.

Aligianis N, Kalpoutzakis E, Mitaku S, Chinou IB (2001).Composition and antimicrobial activity of the essential oil of two *Origanum* species. J. Agric. Food Chem. 9(49):4168-4170.

Asquith TN, Butler LG (1985). Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. J. Chem. Ecol. 11(11):1535-1544.

Begum S, Hassan SI, Siddiqui BS, Shaheen F, Ghayur MN, Gilani AH (2002). Triterpenoids from the leaves of *Psidium guajava*. Phytochemistry 4(61):399-403.

Crawley MJ (2005). Statistical: An introduction using R. Jhon Wiley e Sons: New York,USA. P 761.

De Bruyne T, Pieters L, Deelstra H, Vlietinck AJ (1999). Condensed vegetable tannins: biodiversity in structure and biological activities. Biochem. Syst. Ecol. 4(27):445-459.

Djipa CD, Delmée M, Quetin-Leclercq J (2000). Antimicrobial activity of bark extracts of *Syzygium jambos* (L.) Alston (Myrtaceae). J. Ethnopharmacol. 1-2(71):307-313.

Fernandes TG, Mesquita ARC, Randau KP, Franchitti AA, Ximenes EA (2012). In Vitro Synergistic Effect of *Psidium guineense* (Swartz) in combination with Antimicrobial Agents against Methicillin-Resistant *Staphylococcus aureus* Strains. Scientific World J. 2012:158237.

Ferreira PRB, Mendes CSO, Rodrigues CG, Rocha JCM, Royo VA, Valerio HM, Oliveira DA (2012) Antibacterial activity tannin-rich fraction from leaves of *Anacardium humile.* Ciên. Rural 10(42):1861-1864.

González AMN, González MBR, Pinto NLS. 2005. Phytochemical study and antibacterial activity of *Psidium guineense* Sw (choba) against *Streptococcus mutans*, causal agent of dental caries. Rev. Cubana Med. Plant 10:3-4

Gutiérrez RMP, Mitchell S, Solis RV (2008). *Psidium guajava*: A review of its traditional uses, phitochemistry and pharmacology. J. Ethnopharmacol. 17(117):1-27.

Hagerman AE (1987). Radial diffusion method for determination tannin in plant extracts. J. Chem. Ecol. 3(13):437-449.

Hagerman AE, Klucher KM (1986). Tannin-protein interation. In Plant Flavonoids in biology and Medicine: biochemical, pharmacological, and structure-activity relationships, Cody V, Middleton E, Harborne J (eds). Alan R. Liss: New York. pp. 67-76.

Haslam E (1996). Natural polyphenols (vegetable tannins) as drugs and medicines: possible modes of action. J. Nat. Prod. 2(59):205-215.

Kumar R, Vaithiyanathan S. (1990). Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. Anim. Feed Sci. Technol. 1-2(30):21-38.

Lee MH, Chiou JF, Yen KY, Yang LL (2000). EBV DNA polymerase inhibition of tannins from *Eugenia uniflora.* Cancer Lett. 2(154):131-136.

Matsuo T, Hanamure N, Shimoi K, Nakamura Y, Tomita I (1994). Identification of (+)- galoocatechin as a bio-antimutagenic compound in *Psidium guajava* leaves. Phytochemistry 4(36):1027-1029.

Meckes M, Calzada F, Tortoriello J, Gonzalez JL, Martinez M (1996). Terpenoids isolated from *Psidium guajava* hexane extract with depressant activity on central nervous system. Phytother. Res. 7(10):600-603.

NCCLS (2003). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 6th edn. Approved Standard, M7-A6. National Committee for Clinical Laboratory Standards: Wayne, PA.

NCCLS (2003). *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 8th edn. Approved standard, M2-A8. National Committee for Clinical Laboratory Standards: Wayne, PA.

Salib JY, Michael HN (2004). Cytotoxic phenylethanol glycosides from *Psidium guaijava* seeds. Phytochemistry 14(65):2091-2093.

Salminen JP, Ossipov V, Loponen J, Haukioja E, Pihlaja K (1999). Characterisation of hydrolysable tannins from leaves of *Betula pubescens* by high-performance liquid chromatography-mass spectrometry. J. Chromatogr. A 2(864):283-291.

Santos SC, Mello JCP (1999). Taninos. In *Farmacognosia: da planta ao medicamento*, Simões CMO, Schenkel EP, Gosmann G, Mello JCP, Mentz LA, Petrovick PR (eds). Universidade Federal do Rio Grande do Sul: Porto Alegre. pp. 614-656.

Scalbert A (1991). Antimicrobial properties of tannins. Phytochemistry 12(30):3875-3883.

Schapoval EES, Silveira SM, Miranda ML, Alice CB, Henriques AT (1994). Evaluation of some pharmacological activities of *Eugenia uniflora* L. J. Ethnopharmacol. 3(44):137-142.

Strumeyer DH, Malin MJ (1975). Condensed tannins in grain sorghum: isolation, fractionation and characterization. J. Agric. Food Chem. 5(23):909-914.

Wang CK, Lee WH (1996). Separation, Characteristics, and Biological Activities of Phenolics in Areca Fruit. J. Agric. Food Chem. 8(44):2014-2019.

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