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Atividade Antioxidante e caracterização do óleo essencial das raízes de Piper marginatum Jacq.

Antioxidant activity and characterization of the essential oil from the roots of Piper marginatum Jacq.

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Abstract

Piper marginatum Jacq (Piperaceae) is known as "caapeba cheirosa", is a plant native to Central and South America, which is used in folk medicine to treat stomach problems. Because of its use in folk medicine and common cultivation in home gardens on the outskirts of Porto Velho - RO, this study aimed to extract, identify and quantify the essential oil of fresh roots, as well as its antioxidant activity. The extraction of essential oil from the roots was carried out by hydrodistillation in Clevenger apparatus modified. The analysis of the components of essential oil was by GC/MS and 25 allowed the identification of chemical components, most of which sesquiterpenes: (E) -anetol, (Z) -anetol, safrole, germacrene-D-germacrene B and bicyclogermacrene, which showed higher levels compared to other constituents, and the dominant classes of the essential oils were monoterpenes, sesquiterpenes and phenylpropanoid. In the antioxidant activity test were determined effective concentrations (EC50) and Antioxidant Activity (% AA). The following CE50 and %AA values were found: Ginkgo biloba (used as reference) 46,96 mg.L-1 and 75.26 mg.L-1 for the essential oil from the roots of P. marginatum. The methodology used for the antioxidant activity was adequate, and the essential oil showed significant antioxidant activity.

Keywords: *phenylpropanoids; sesquiterpenes; monoterpenes; Piper; Piperaceae.*

Resumo

Piper marginatum Jacq (Piperaceae) é conhecida como caapeba cheirosa, é uma planta nativa da América Central e do Sul, a qual é utilizada na medicina popular para o tratamento de problemas gástricos. Devido ao seu uso na medicina popular e cultivo comum nos quintais da periferia de Porto Velho – RO, este trabalho objetivou extrair, identificar e quantificar o óleo essencial da raízes frescas, assim como sua atividade antioxidante. A extração de óleo essencial das raízes foi realizada por hidrodestilação em aparelho de Clevenger modificado. A análise dos componentes do óleo essencial foi através da CG/EM e permitiu identificar 25 componentes químicos, sendo a maioria sesquiterpenos dentre os quais: (E)-anetol, (Z)-anetol, safrol, germacreno-D, germacreno-B e biciclogermacreno, que apresentaram teores superiores em relação aos demais constituintes, e as classes predominantes dos óleos essenciais foram: monoterpenos, sesquiterpenos e fenilpropanóides. No teste de atividade antioxidante foram determinados a concentrações efetivas (CE50) e Atividade Antioxidante (%AA). Os seguintes valores de CE50e %AA foram encontrados: Ginkgo biloba (usado como referência) 46,96 mg.L-1 e 75.26 mg.L-1 para o óleo essencial das raízes de P. marginatum. A metodologia utilizada para a atividade antioxidante mostrou-se adequada, e o óleo essencial apresentaram atividade antioxidante expressiva.

Palavras-chave: *fenilpropanóides; sesquiterpenos; monoterpenos; Piper; Piperaceae.*
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Introduction

The Piperaceae family belongs to the Piperales order and it is one of the most primitive families of Angiosperms. It is a predominantly tropical family, which currently comprises four genera (Piper, Peperomia, Sarchorhachis, and Ottonia); the Piper and Peperomia are the most representative genera with approximately 2,000 to 1,700 species, respectively (MABBERLEY, 1997; SOUZA, 2005). The Piper genus has about 2,000 identified species (WANKE et al., 2007), which are easily found in both hemispheres in tropical and subtropical regions.

The genus Piper is mainlydistributed in the tropical and subtropical region of the world and has been extensively investigated as the source of new natural products with potential antifungal, antitumoral, antioxidant,antiplasmodial, and tripanocidal properties (Lago et al., 2009). The geographical distributions of species of the Piper genus, in the American continent, occur in Central America, Antilles, and South America. In Brazil, these species occur in the states of Amazonas, Acre, Amapá, Pará, Piauí, Ceará, Pernambuco, Bahia, Rio de Janeiro, Minas Gerais, Paraná, Santa Catarina, São Paulo, Distrito Federal, and Mato Grosso (GUIMARÃES e GIORDANO, 2004; NAVICKIENE et al., 2000).

Several species from Amazon have been already studied, such as P. belte, P. nigrum, P. amapaense, P. duckei, P. bartlingianum, P arboreum. Piper species are large producers of essential oils (MAIA et al., 2001), the oils of Piper in the Amazon have showed terpenoid and phenylpropanoid compounds as major constituents, always with the predominance of one over the other (SILVA et al., 2011; ANDRADE et al., 2011). The biological activity of Piper species is very diverse and also widely used in folk medicine to treat many diseases (VIEIRA, 1992; DI STASI e HIRUMA-LIMA, 2002; LORENZI e MATOS, 2002).

Piper marginatum Jacq. is popularly known as “caapeba cheirosa” or “malvarisco” (in Brazil), its roots are used as an infusion to treat pain in general, fever, gasses, gonorrhea, liver diseases, and as an antidote against snake bite. Its fruits are used as a substitute for black pepper, using them as a condiment (ALMEIDA, 2008), its roots are also used as tea, plaster, bath and compress to treat headache, flu, furuncles, insect bites, swelling, inflammation in the legs, and to relieve pain, as well as to disinfect and heal wounds (PEREIRA et al., 2007), the decoction of leaves of P. marginatum has been used by against liver and vesicle diseases, and as tonic with carminative and antispasmodic action (Maia et al., 2001).

Given the regional importance of P. marginatum, this study aimed to extract, identify and analyze the antioxidant activity of the essential oil obtained from its roots by capturing the free radical 2,2-diphenyl-1-picryl-hidrazila (DPPH), in samples collected in Porto Velho - RO.

Materials and methods

Plant Material Collection

The plant material was collected in the outskirt of Porto Velho, the state capital of Rondônia (8° 46’49.41” S, 63° 53’07.06” W), in January/2010. The species identification was carried out in the Rondoniense Herbarium, located at the Federal University of Rondônia – UNIR, *Campus* of Porto Velho, where its voucher specimen was deposited (No. 905). The plant material was collected in the morning, packed and transported to the Research Laboratory for Chemistry of Natural Products, at the Federal University of Rondônia, where the essential oil was extracted from the plant roots.

Extraction of the Essential Oils

The fresh plant material (1.0 kg of roots) were ground and subjected to oil extraction in a modified Clevenger apparatus, obtaining 1.4 mL of essential oil from the roots (EORPM); subsequently, the oil was treated with sodium sulfate anhydride (Synth) to be dehydrated, and then it was kept in an amber tube, properly sealed, and storage in a refrigerator at 10°C until the gas chromatography analysis.

Analysis of the Essential Oils

The essential oils were analyzed by using an equipment from ThermoElectron (TRACE GC ULTRA – DSQ model) equipped with a capillary column (30 m x 0.25 mm) dimethylpolysiloxane DB-5 (J & W) (25 m x 0.20 mm, 0.20 μm), using Helium as a carrier gas (flow rate of 1.0 $mL.min^{-1}$), injector temperature (Split model) of 250ºC, flame ionization detector (FID) at 270°C, column temperature of 35–180ºC/3°C/min and 180–250°C/10ºC.min^{-1}, and injected volume of 0.02 μL pure oil. The mass spectra were: electron impact at 70 eV and scanning of the masses in the *Full Scan* mode 43–650. The chemical constituents of the essential oils were identified through the spectrum studies from the database of the spectra library “NIST” (National Institute of Standards and Technology) and complemented by comparison with the device library, literature data and Kovats retention indices (IK) (ADAMS, 1995). These analyses were performed in the Biogeochemistry Laboratory of the Federal University of Rondônia.

Antioxidant activity test

For this evaluation, a solution of 100 μg.mL-1 of DPPH was prepared in methanol. The test solutions of EORPM were prepared in methanol at the following concentrations: 10, 50, 100, 150 and 250 μg.mL-1. The same procedure was performed for the standard solution, Ginkgo biloba (EGb 761). Then, 1 mL of DPPH solution was added to 2.5 mL of the samples, which stayed at rest for 20 min, protected from light. The blank solution was prepared with 1 mL of the solution under test with 2.5 mL of methanol, and the control consisted of 1 mL of DPPH solution and 2.5 mL of methanol. The absorbance readings were performed in a UV–Vis spectrophotometer (Shimadzu UV 1601) at 517 nm. The tests were performed in triplicate; from the means of the data we calculated the percentage of antioxidant activity (%AA) (Equation 1), and the effective concentration (EC50), which consists of the amount of antioxidant required to decrease the initial concentration of DPPH to 50%; such concentration was determined by linear regression of the values (MENSOR et al., 2001).

The DPPH scavenging activity of the ethanolic extract, eluates, and Ginkgo biloba were expressed as percentage, according to Equation 1:

$$\%AA = 100 - \left\{ \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control}} \right) \times 100 \right\}$$

The antioxidant activity tests were performed in the Phytochemistry Laboratory of the Federal University of Rondônia.

2.4 Statistical analysis

The databases were generated through the GraphPad Prism 6.0 software, performing the determination of the EC50 from a linear regression, correlating the inhibition percentage as a function of the percentage of the tested concentrations, assuming an interval of 95% ($p<0.05$); the correlation among the means were evaluated by the Tukey test, or ANOVA - one way, also assuming an interval of 95% ($p<0.05$).

3 Results and Discussion

The relationship of the chemical constituents of essential oils extracted from the roots of P. marginatum, their relative amounts, and their retention indices (IK), in total, 25 volatile components were identified, comprising approximately 95% of the total composition of the oils, are shown in Table 1:

The yield of essential oils obtained from P. marginatum roots was 0.20%; the sesquiterpenoids were the most highly represented class, as many as hydrocarbons an oxygenated compounds: d-elemene (3.44%), b-elemene (3,63%), germacrene D (8.83%), bicyclogermacrene (9.40%) and germacrene B (8.07%), but all constituents, belonging to the phenylpropanoids, predominated: (Z)-Anethol (8.01%), its isomer (E)-Anethol (10.10%) and safrol (5.78%).

Plant species does not necessarily have a predominant class of secondary metabolites, however, the EORPM analysis showed the presence of monoterpenes (oxygenated and hydrocarbon), sesquiterpenes (oxygenated and hydrocarbon) and phenylpropanoids (Table 1).

The presence of sesquiterpenes compounds in Piper oils has been previously reported (PARMAR et al., 1997; ANDRADE et al, 2008; ANDRADE et al., 2009; ANDRADE et al., 2011; SILVA, 2014). The chemical profile observed for this species is different from those reported for other parts of the plant (MACHADO et al., 1994; SANTOS et al., 2001; PINO et al., 2004; MESQUITA et al., 2005; POTZERNHEIM et al., 2006). The composition of these essential oils may be attributed to edaphoclimatic factors, compared to studies on other Piper species (MESQUITA et al., 2005; MORAIS et al., 2007; NAVICKIENE et al., 2006), in which different compositions and concentrations were described, according to the collection period and regions of such species.

The metabolic plasticity is one of the alternatives that plants use when they are face to the different environmental conditions in the same place (LARCHER, 2000). Dill (2009), carrying out a literature review on the substances of the essential oils of Brazilian Piperaceae, found that in more tropical climates these plants have a composition of essential oils with a higher incidence of sesquiterpenes and phenylpropanoids. Same results were obtained in our study, in which the sesquiterpenes represented the highest amount of the EORPM components. However, the chemical composition of essential oils analyzed showed qualitative and quantitative variation when compared to other studies carried out for the same species (ANDRADE et al., 2005; FACUNDO et al., 2007; ANDRADE et al., 2008; ANDRADE et al., 2011; SILVA et al., 2011; MORALES et al., 2013; SILVA et al., 2014), which can be assigned by the influence of local environmental conditions.

The essential oils from the leaves, stems and inflorescences of P. marginatum had already some of their biological activities described in some studies, such as the one conducted by Neves et al. (2008), who described the acaricide activity of these essential oils, and found the most effective action in the inflorescence oil, which had the Patchoulou as its major component (23.38%).

Santana (2009), studying the essential oil from the leaves of P. marginatum, obtained an oil with 83.2% of phenylpropanoids and observed that this oil showed a high cytotoxic activity against Aedes aegypti.

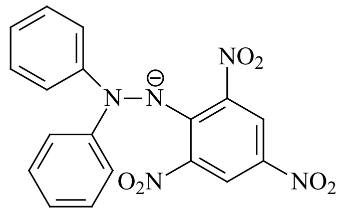
Reigada (2009) described the fungitoxic activity of P. marginatum against the fungus Cladosporium cladosporioide and Cladosporium sphaerospermum, in isolated and fixed constituents, and found that the compounds with the highest activity were the flavones sakuranetin and 4’-O-methyl-nageranetina.

Many cytotoxicity assays using essential oils have been performed with carcinogen cells (SILVA et al., 2008; PITA, 2010) since both the essential oils and their isolated constituents have shown suppressive activity

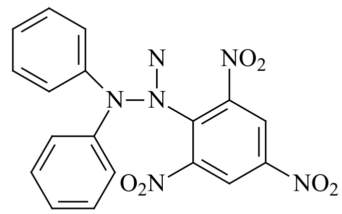
Table 1- Chemical composition (%) of the essential oils obtained from the leaves, inflorescences, stems and roots of P. marginatum Jacq.

Components	IK	EORPM	Chemical Classification
α-Pinene	939	0.90	monoterpene hydrocarbon
Canphene	954	1.90	monoterpene hydrocarbon
(Z)-Anethole	1253	8.01	phenylpropanoid
(E)- Anethole	1285	10.10	phenylpropanoid
Safrole	1287	5.78	phenylpropanoid
d-Elemene	1338	3.44	oxygenated monoterpene
a-Copaene	1377	1.13	oxygenated monoterpene
b-Bourbonene	1388	2.22	oxygenated monoterpene
b-Cubebene	1389	0.67	oxygenated monoterpene
b-Elemene	1391	3.63	oxygenated monoterpene
b-Caryophyllene	1409	5.88	sesquiterpene hydrocarbon
b-Curjunene	1434	1.73	sesquiterpene hydrocarbon
a-Humulene	1455	1.49	sesquiterpene hydrocarbon
Aromadendrene	1460	1.72	sesquiterpene hydrocarbon
Germacrene D	1485	8.83	sesquiterpene hydrocarbon
b-Selinene	1490	4.23	sesquiterpene hydrocarbon
Bicyclogermacrene	1499	9.40	sesquiterpene hydrocarbon
Germacrene A	1509	1.41	sesquiterpene hydrocarbon
δ-Cadinene	1523	1.36	sesquiterpene hydrocarbon
Germacrene B	1561	8.07	sesquiterpene hydrocarbon
Caryophyllene oxide	1583	1.44	oxygenated sesquiterpene
Globulol	1585	0.46	oxygenated sesquiterpene
Viridiflorol	1593	6.54	sesquiterpene hydrocarbon
Himachalol	1650	1.50	oxygenated sesquiterpene
α-Cadinol	1654	0.64	oxygenated sesquiterpene

Wherein: EORPM = essential oil from the roots.



(1)
DPPH
cor: violeta



(2)
Hidrazina
cor: amarelo pálido

Figure 1 - Radical (1) and non-radical forms (2) of DPPH
Reference: Adapted from ALVES et al., 2010

against diverse types of cancer, including, cancer of colon, gastric, breast, lung tumors and leukemias (DE ANGELES, 2001).

The antioxidant activity of EORPM was evaluated by capturing the DPPH free radical (2,2-diphenyl-1-picryl -hydrazyl), which is characterized as a stable free radical due to the delocalization of the unpaired electron throughout the molecule. This delocalization confers to this molecule a violet color, characterized by an absorption band of ethanol at about 520 hm (ALVES et al., 2010). This test is based on the measurement of the antioxidant capacity that a given extract or substance has to scavenge the DPPH, reducing it to hydrazine, which has a pale yellow color of (Figure 1), reducing the absorbance up to 515 hm (RUFINO et al., 2007) .

The addition of the Piper oils to the DPPH solution caused a colorreduction in its optical density at 517 nm.. The values for the antioxidant activity were expressed as effective concentration values of 50% (EC50), which is the antioxidant activity required to reduce to 50% the initial concentration of DPPH. As a positive control it was used the standardized extract of Ginkgo biloba (EGb 761), and as a negative control it was used the methanol; these results were obtained by tabulating the data. The results were calculated using the equation of the line (Graph 1), with an EC50 equal to 75.26 mg.L-1 for the EORPM, and mg.L-1 for the Ginkgo biloba.

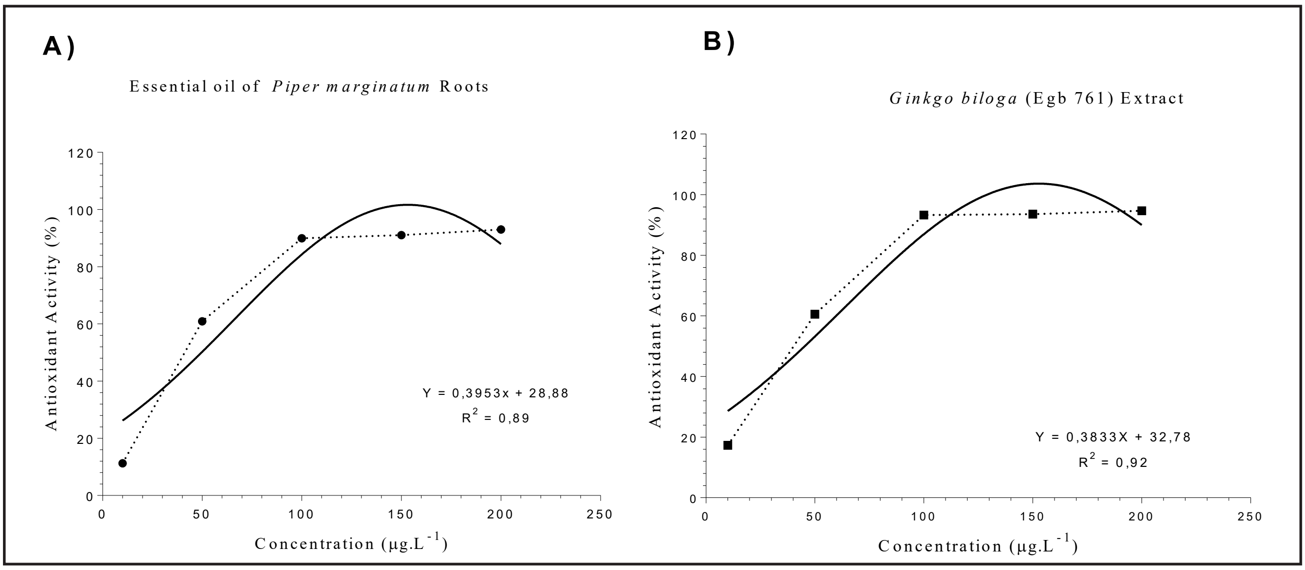
These results are shown both in Graph 1 and in Table 2, in which we may verify the significant difference among all tested concentrations. Many plants and herbs considered as medicinal have had their antioxidant activity studied, and many secondary metabolites have shown biological activity, among these activities, the antioxidant is prominent, however, this activity directly dependent on the concentration and class (flavonoids, tannins, terpenes, alkaloids, etc.) of these secondary metabolites (MACARI et al., 2004).

The result obtained for the analysis of EORPM antioxidant activity may be explained by the fact that the essential oils are generally composed of terpenes, which are considered as natural antioxidants, according to Grassmann et al. (2002). This group of secondary metabolites is widely distributed in the Piper genus of the Legal Amazon (DILL, 2009; MAIA e ANDRADE, 2009; ANDRADE et al., 2009), and in other regions of Brazil, as shown in the study performed by Potzernheim et al. (2006) who obtained relevant results collecting Piperaceae in the mountainous region of Brasília, and verified that the P. dilatatum (81%), P. hispidum (84.5%) and P. arboreum sbsp. arboreum (68.7%) have predominant terpene compounds.

Usually, the oils rich in sesquiterpene hydrocarbons exhibit antioxidant properties. A good example is the oil of Eupatorium polystachyum, dominated by β -caryophyllene (15.4%), germacrene D (9.4%), and bicyclogermacrene (19.2%), which showed significant antioxidant activity by DPPH assay (SOUZA et al., 2007). Furthermore, the sesquiterpenes β-caryophyllene (13.2%), β -selinene (10.6%), and α-selinene (9.7%) were also found in the extract of Alpinia galanga, which showed antioxidant activity by the β -carotene/linoleic acid assay, with an inhibition of 70% (MAYACHIEW e DEVAHASTIN, 2008).

Santos et al. (2011), analyzing the essential oil from the leaves of P. marginatum in the in vitro growth of F. oxysporum colonies, using the concentration of 10 μL, observed that after 92 hours of bioassay, there was an average growth diameter of 22.5 mm, whereas, for the control with no use of essential oil, the average diameter was 69.9 mm, thus demonstrating that the essential oil from the leaves of P. marginatum has an inhibitory effect on the in vitro growth of F. oxysporum.

Therefore, the data obtained in this study may evidence that the plant studied herein has anti-inflammatory and/or antioxidant activity, since the essential oils may mitigate the damage effects caused by the oxidative stress, by capturing the hydroxyl radicals (SOUZA et al., 2007). Moreover, the essential oils showed phenolic compounds and sesquiterpenes known for their ability to scavenge free radicals such as the superoxide anion (LARSON, 1988).



Graph 1 - Antioxidant Activity: A) of EORPM; B) of Ginkgo biloba by the DPPH method.

Table 2: Antioxidant activity means (%) of the EORPM. These results are expressed as mean ± standard error of the mean (S.E.M.); means followed by * in the same column are significantly different at 5% by Tukey test with regards to the control (Ginkgo biloba).

Sample	Concetration (µg.mL-1)				
	200	150	100	50	10
EORPM	93.04 ± 0.04*	91.07 ± 0.12*	89.93 ± 0.38*	60.88 ± 0.32*	11.28 ± 0.51*
GK	94.10 ± 0.35*	93.73 ± 0.63*	93.92 ± 0.42*	75.46 ± 1.20*	17.16 ± 1.42*

Wherein: EORPM = essential oil from the roots, GK = *Ginkgo biloba*.

Conclusion

The chemical constituents of the extracted essential oils were mainly the phenylpropanoids, sesquiterpenes, and monoterpenes. The evaluation of the antioxidant activity showed no significant difference regarding to the Gingko biloba, but the essential oil chemotype obtained in this study gives evidence that the studied plant has a possible biological activity (anti-inflammatory and/or antioxidant), however, further studies (in vitro and in vivo) should be carried out to supplement the information presented in this study.

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