

Chemical Constituents, Larvicidal Effects and Radical Scavenging Activity of *Tetracera breyniana* Schlttdl. (Dilleniaceae)

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ABSTRACT

Extracts and fractions from the leaves and stems of *Tetracera breyniana* Schlttdl. were evaluated against the fourth instar *Aedes aegypti* larvae and ability to scavenge free radicals. Fractions that provided the best results were fractionated on silica gel column to afforded three flavonoids (quercetin, 7-O-methylquercetin and 7-O-methylkaempferol) and two terpenoids (β -sitosterol and betulinic acid). These compounds were identified on basis of their physical and NMR spectral data and by comparison with literature data. With exception of quercetin, all other compounds are been described for the first time in the investigated species. In the larvicidal assays, when compared to synthetic insecticide Temephos, only hexane fraction from stem was effective (LD₅₀ 72.08 μ g/mL). In the DPPH assays, EtOAc fractions from the leaves (CI₅₀ 74.15 \pm 14.73 μ g/mL) and stem (IC₅₀ 39.87 \pm 13.46 μ g/mL), of which quercetin was isolated, showed the best results when compared with the positive standards used while CHCl₃ fractions of both plant parts, of which methylated flavonols were isolated, showed only a moderate activity. Possibly these constituents are responsible in part for the radical scavenging activities observed. Subsequently, evaluation of all isolated compounds will be needed to confirm the active component.

INTRODUCTION

Dengue is a major public health problem in the world. According to World Health Organization (WHO) estimates, 0.5 million people with dengue hemorrhagic fever (DHF) require hospitalization each year and approximately 2.5% of these individuals die from DHF (WHO, 2008). Annually, there may be over 50-100 million dengue infections in tropical and subtropical areas (Renjana and Thoppil, 2013), and so these diseases have emerged as a major international health problem and a notable increase in dengue cases (77.7%) has been reported by Brazilian health authorities (Oliveira *et al.*, 2010).

In recent years, there has been a global trend toward the use of natural substances present in vegetables and other sources as antioxidant. Several of these substances may interfere with the oxidation process by reacting with free radicals (Silva *et al.*, 2010). When these species had an excessive formation induces oxidative damage to biomolecules, which eventually causes numerous diseases (Gülçin *et al.*, 2006; Edewor-Kuponiyyi, 2013).

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The family Dilleniaceae comprises 10-14 genera and ca. 500 species, with a pantropical and subtropical distribution (Kubitz, 2004; Horn, 2009). In the Neotropics are found 102 species distributed in six genera *Curatella*, *Davilla*, *Doliocarpus*, *Neodillenia*, *Pinzona*, and *Tetracera* (Bruniera and Groppo, 2010). In Brazil, 82 species of these genera are present in these genera (Fraga and Stehmann, 2010). According to Horn (2007), this family is divided into four subfamilies and the genus *Tetracera*, belonging to subfamily Delimoideae, contains about 45 species with a pantropical distribution. Species of this genus have been used in folk medicine for the treatment of various diseases and infections (Tona *et al.*, 2004; Nguyen *et al.*, 2004; Fenner *et al.*, 2010; Umar *et al.*, 2010; Lawal *et al.*, 2011) and several biological properties such as antiplasmodial (Tona *et al.*, 2004), antimycobacterial (Lowal *et al.*, 2011), anti-HIV and anti-reverse transcriptase (Kwon *et al.*, 2012), anti-hyperglycemic (Umar *et al.*, 2010), antioxidant (Kukongviriyapan *et al.*, 2003; Lock *et al.*, 2005), anti-ulcerogenic (Fenner *et al.*, 2006; Oluwole *et al.*, 2008), DNA polymerase β inhibition (Ma *et al.*, 1999), glucose-uptake activity (Lee *et al.*, 2009), and xanthine oxidase inhibition (Nguyen *et al.*, 2004) were reported.

Chemical investigations carried out with active extracts of different *Tetracera* species have reported mainly the isolation of flavonoids (Gurni *et al.*, 1981; Gurni and Kubitzki, 1981; Na *et al.*, 2001; Lee *et al.*, 2009) and terpenoids (Dan and Dan, 1980; Ma *et al.*, 1999; Na *et al.*, 2001; Subramanyam *et al.*, 2009) and studies have showed that some of these compounds possess important biological activities (Ma *et al.*, 1999; Subramanyam *et al.*, 2009; Nguyen and Nguyen, 2013). *T. breyniana* Schlttdl., known as “cipó-de-fogo”, is a creeper whose leaf decoction is used in Brazil against lymphatic problems (Souza, 2000). From the leaves of this species, besides proanthocyanidin some common flavonols (azaleatin, kaempferol, 5-methylkaempferol, and quercetin), glycoside (quercetin 3-O-galactopyranoside) and sulphates (kaempferol 3-sulphate and quercetin 3-sulphate) have been isolated (Gurni and Kubitzki, 1981). This work was undertaken in order to determine the phytochemicals, larvicidal effects and free radical scavenging activity of the extracts from the leaves and stem of this species. Among isolated compounds, with exception of quercetin, this work report for the first time the presence of 7-O-methylquercetin and 7-O-methylkaempferol, and terpenoids (betulinic acid and β -sitosterol) as well as evaluation of larvicidal and radical scavenging activities in the investigated species.

MATERIAL AND METHODS

Plant Material

Leaves and stem of *T. breyniana* were collected in the Área de Proteção Ambiental de Santa Rita, Marechal Deodoro, AL, Brazil, in May 2008 and identified by Rosângela P. de Lyra

Lemos of the Instituto do Meio Ambiente do Estado de Alagoas (IMA-AL), where a voucher specimen (MAC-23840) was deposited.

Extraction and Isolation

The air-dried leaves (350 g) and stems (820 g) were extracted with 90% EtOH at room temperature. The EtOH extracts (leaves: 36.1 g; stems: 17.9 g) were suspended in MeOH-H₂O (3:2) and extracted with hexane, CHCl₃ and EtOAc. Subsequently, both extracts and fractions from partition were evaluated against fourth instar of *A. aegypti* larvae and as free radical scavengers (DPPH). Fractions with promising results in the assays were further fractionated. Hexane (4.6 g), CHCl₃ (4.4 g) and EtOAc (2.2 g) fractions from leaves after successive chromatographic fractionations over silica gel with hexane-EtOAc and Sephadex LH-20 with MeOH afforded, respectively, β -sitosterol [**1** (10 mg; Macari *et al.*, 1990)], 7-O-methylquercetin [**2** (10 mg; mp 286.2-289.7°C (Kim, 2005)] and quercetin [**3** (11 mg; Kim, 2005)]. CHCl₃ fraction from stems (2.2 g) was chromatographed on silica gel column with hexane-EtOAc to provide, after recrystallizations with MeOH, betulinic acid [**4** (20 mg; mp 285.3-287.2°C; Mahato and Kundu, 1994)] and 7-O-methylkaempferol [**5**; 10 mg] (Pizzolatti *et al.*, 2003; Silva *et al.*, 2009).

The structures of the compounds (**1-5**) (Fig. 1) were identified by their physical (mp) and by interpretation of their spectral data (¹H and ¹³C NMR, including DEPT, HSQC and HMBC experiments) and by the comparison with those reported in literature.

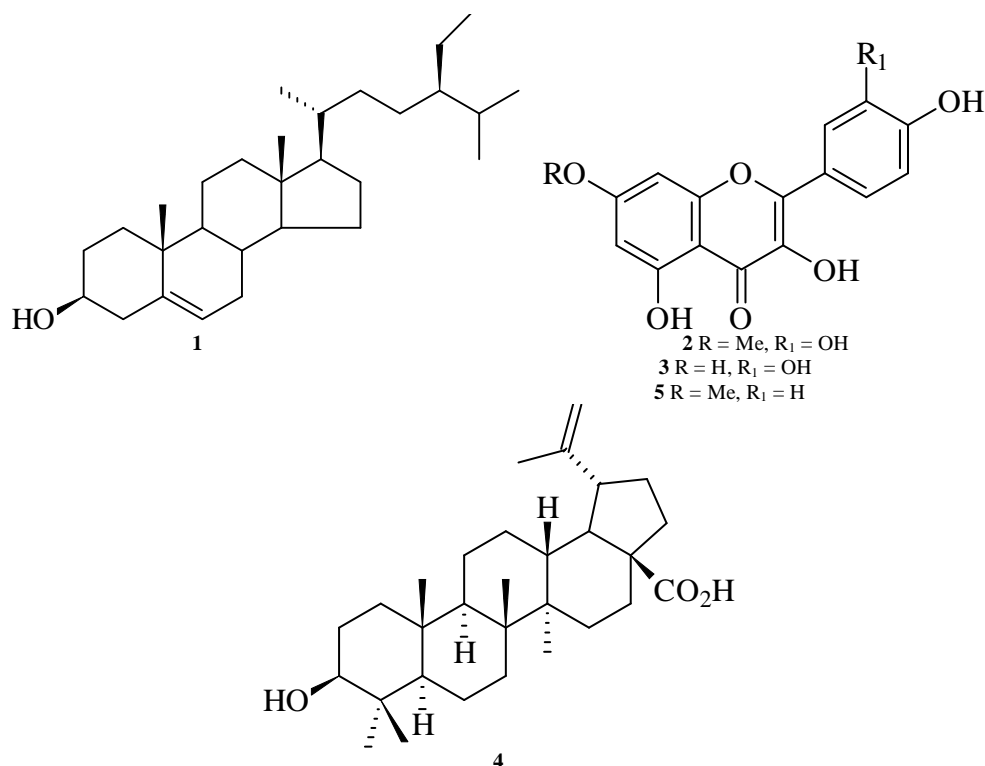


Fig. 1: Isolated compounds from *T. breyniana*.

Larvicidal assays

Fourth instar of *A. aegypti* larvae, aged four to six days, were collected from a mosquito colony maintained at insectaria of the Instituto de Química e Biotecnologia da Universidade Federal de Alagoas. The mosquitoes were kept at $27.1 \pm 4^\circ\text{C}$ and $69.9 \pm 7.8\%$ relative humidity and photoperiod of approximately 12 h. The hatching of larvae occurred in distilled water, and the feeding of adult insects was made with anhydrous glucose solution to 10% in cotton balls changed daily. The blood supply for the females was performed using the species pigeons *Columbia livia*. Larvicidal assays were performed following the recommendations of the WHO (1981) with some modifications (Oliveira *et al.*, 2010). Initially all samples were screened at 250 $\mu\text{g/mL}$. Based on the percent mortality values (mortality $\geq 75\%$), LD₅₀ values in micrograms per milliliter and their 95% fiducial limits of upper and lower confidence limits of the samples were determined.

Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating LD₅₀, and other statistics at 95% fiducial limits of upper and lower confidence limits were calculated by using Probit analysis of Finney (1971).

Free radical scavenging activity (DPPH)

These assays for all samples were conducted in triplicate, according to the methods described by Silva *et al.* (2010). Samples and standards were measured at 515 nm, using at least five different concentrations (12.5 to 100 $\mu\text{g/mL}$), against a blank (MeOH HPLC grade). In a cuvette, 0.1 mL of the samples was mixed with 0.9 mL DPPH• dissolved in MeOH (100 $\mu\text{mol/L}$) and the absorbance was monitored for 1 h.

The percentage inhibition was calculated using the equation, (%) = $[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{samples}}) / \text{Abs}_{\text{control}}] \times 100$, and IC₅₀ values were estimated by a nonlinear regression algorithm using Origin™ version 7.0 (Micronal, Northampton, MA, USA). Data were analyzed by ANOVA followed by Tukey test ($p < 0.05$). Ascorbic acid and BHT were used as controls.

RESULTS AND DISCUSSION

In the preliminary larvicidal assays, among the extracts tested only one of them showed 100% mortality against fourth instar *A. aegypti* larvae. All other no caused mortality ($\leq 25\%$) after 48 h exposure. As shown in Table 1, when compared to synthetic insecticide Temephos (LD₅₀ 8.7 $\mu\text{g/mL}$), only hexane fraction from stem (LD₅₀ 72.08 $\mu\text{g/mL}$; 95% CL 60.92-82.47) was effective. All others were inactive (LD₅₀ $> 280 \mu\text{g/mL}$; results not shown in Table 1) (Oliveira *et al.*, 2010). The isolation and purification of this extract and evaluation of these compounds will be needed to identify the active component.

Despite significant advances in the techniques used for mosquitoes control during recent decades, it continues to pose serious public health problems and many infectious diseases for humans and animals such as malaria, dengue fever, yellow fever,

filariasis, and others can be transmitted by vectors. *A. aegypti* acts as a vector for the arboviruses responsible for yellow fever and also for dengue and dengue fevers (Garcez *et al.*, 2009). Since there is no vaccine currently available for dengue and DHF prevention, the eradication of the mosquito adults and larvae, which is primarily relied on applications of synthetic insecticides or larvicides, is the most commonly adopted strategy for reducing morbidity (WHO, 2008). According to the literature, the organophosphate temephos is one of the most employed commercial insecticides for the control of this vector (Macoris *et al.*, 2007; Garcez *et al.*, 2009; Oliveira *et al.*, 2010). However, these chemicals can affect non target population and may increase mosquito resistance to insecticides. So, plants may be an alternative source of mosquito control agents because they constitute a rich source of bioactive chemicals.

In this study, free radical scavenging activity of extract and fractions from *T. breyniana*, as well as of the standards used (ascorbic acid and BHT) were evaluated by DPPH. As shown in Table 1, among the extracts tested, with exception of hexane fractions from the leaves and stems, all other samples tested showed significant antioxidant activity (IC₅₀ $< 350.78 \pm 2.71 \mu\text{g/mL}$) and the best results were obtained with the EtOAc fractions from the leaves ($74.15 \pm 14.73 \mu\text{g/mL}$) and stems ($39.87 \pm 13.46 \mu\text{g/mL}$), with IC₅₀ values comparable to standards used, of which quercetin (**3**) was isolated. Comparatively, CHCl₃ fractions of both parts of the plant showed only a moderate activity (Table 1). These fractions, less hydroxylated flavonoids (7-O-methylquercetin and 7-O-methylkaempferol) were isolated. Subsequently, evaluation of all isolated compounds will be needed to confirm the active component.

Table 1: Effects of extracts and fractions against forth instar *A. aegypti* larvae and DPPH.

Plant part	Extracts and fractions	<i>A. aegypti</i> LD ₅₀ ($\mu\text{g/mL}$) ^a	DPPH IC ₅₀ \pm DP ($\mu\text{g/mL}$)
Leaves	Crude EtOH		195.23 \pm 1.30
	Hexane		NT
	CHCl ₃		155.93 \pm 3.87
	EtOAc		74.15 \pm 14.73
	MeOH-H ₂ O		123.45 \pm 2.22
Stem	Crude EtOH		350.78 \pm 2.71
	Hexane	72.08 (95% CL 60.92-82.47)	2911.31 \pm 1.20
	CHCl ₃		192.33 \pm 1.58
	EtOAc		39.87 \pm 13.46
	MeOH-H ₂ O		106.77 \pm 4.38
	Ascorbic acid		37.37 \pm 3.18
	BHT		97.86 \pm 2.52
	Formulate Temephos	8.7 (95% CL 7.0-10.2)	

CL = confidence limits (lower and upper confidence limits). ^a Mean value of three replicates; LD₅₀ lethal dose that kills 50% of the exposed larvae; NT = not tested.

Many studies have shown that antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods and in biological systems (Gülçin *et al.*, 2006) and antioxidant capacity is widely

used as a parameter to characterize food or medicinal plants and their bioactive components (Silva *et al.*, 2010). The hydroxyl radical which is a very reactive species that is formed in biological systems has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Edewor-Kuponiya, 2013). According Silva *et al.* (2010), anti-radical activities of plant extracts are often associated with the presence of polyphenol compounds, like flavonoids, which have an important role in stabilizing lipid oxidation.

CONCLUSION

There are no reports on larvicidal and radical scavenging activities to species studied. The results of this study showed that hexane fraction from stem of *T. breyniana* possess potential for control of mosquito *A. aegypti* and open the possibility of further investigations on the efficacy of the larvicidal properties of plant product extracts. This study also showed that some extracts were effectively able to inhibit the formation of free radicals. Among the extracts tested, the best radical scavenging activities was found mainly by the most polar extracts and in some case, this activity was comparable with that of BHT or ascorbic acid. Although there is the possibility that the tannins and others pigments takes a role in scavenging activity, previous studies showed that the effects of some extracts seem to be correlated with the presence of phenolic compounds such as flavonoids. Then, among compounds isolated, quercetin, 7-*O*-methylkaempferol, 7-*O*-methylquercetin may mediate the radical scavenging activity observed in the extracts from leaves and stem of *T. breyniana*.

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