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Phytochemical constituents and *in vitro* antioxidant capacity of *Tabernaemontana catharinensis* A. DC

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ABSTRACT

Introduction: Free radicals induce numerous diseases by lipid peroxidation, protein peroxidation, and DNA damage. It has been reported that numerous plant extracts have antioxidant activities to scavenge free radicals. In this present study we determined the *in vitro* antioxidant capacity and quantified of total phenolics, flavonoids, tannins and alkaloids of *Tabernaemontana catharinensis* crude extract and fractions leaves.

Methods: The antioxidant potential was evaluated by 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging and thiobarbituric acid reactive species (TBARS) methods, total phenolics content was determined using the Folin–Ciocalteu assay, flavonoids, tannins and alkaloids were determined by spectrophotometer.

Results: Crude extract and fractions showed inhibition against TBARS, ethyl acetate was the most effective fraction (IC_{50} = 6.71 ± 0.19 μg/mL), subsequently by butanolic (26.15 ± 0.08 μg/mL), dichloromethane (43.25 ± 0.12 μg/mL) and crude extract (61.09 ± 0.05 μg/mL), respectively. Moreover, the DPPH assay, presented IC_{50} value ranged of 4.64 ± 1.25 to 27.78 ± 0.93 μg/mL. Contents of total phenols, flavonoids, tannins and alkaloids of *T. catharinensis* followed the order: ethyl acetate > butanolic > dichloromethane fractions > crude extract.

Conclusion: The present study, we found that the crude extract and fractions of *T. catharinensis* showed good antioxidant activity. Among the samples tested, the ethyl acetate fraction showed better activity than others.

1. Introduction

The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent biological activities, no side effects and economic viability.[1] The biological activities of plants may be due to the presence of a diverse group of chemical compounds including steroids, glycosides, phenolics, alkaloids, tannins, anthocyanins, flavonoids.[2]

Several authors have researched different extracts of plants in order to improve knowledge about antioxidant capacity.[3–5] Many studies have shown that natural antioxidants in medicinal plants are closely related the prevention or suppression of aging and many diseases associated with oxidative stress; cancer, cardiovascular diseases, rheumatoid arthritis, autoimmune diseases, among others.[6] These beneficial effects have been partly attributed to antioxidants, which may play important roles in inhibition of free radicals and oxidative chain-reactions within tissues and membranes.[7] Phenolic compounds have antioxidant properties because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen and hydroxyl radicals.[8]

Tabernaemontana catharinensis A. DC. (syn. *Peschiera catharinensis* A. DC. Miers) belongs to family Apocynaceae, a family consisting of 415 genera and 4555 species.[9,10] Recently, about 99 species from *Tabernaemontana* genus were reported of which 44 occur in America. *Tabernaemontana* species are well known for indole alkaloids and several pentacyclic triterpenoids with approximately 240 structurally different bases already described. These types of compounds are

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responsible for many pharmacological activities such as: anti-leishmanial; antibacterial; antitumoral; antiinflamatory; hypoglycaemic, analgesic and cardiotonic actions.[11,12] *T. catharinensis* is popularly known as 'jasmim' (jasmine), 'leiteira de dois irmãos' (milkweed), and 'casca de cobra' (snake skin) and it occurs in Argentine, Uruguay, Paraguay and Southern Brazil. In folk medicine, it is used as an antidote for snakebites, to relieve toothache, as a vermifuge to eliminate warts and, as anti-inflammatory.[13]

Considering the importance of identifying and quantifying compounds with capacity antioxidant, mainly in the crude extract because it is the common from of usage in popular medicine,[2] this study were determined the levels of phenolics, flavonoids and total tannins in crude extract and dichloromethane, ethyl acetate and n-butanol fractions of *T. catharinensis* leaves. Simultaneously, antioxidant properties were investigated using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radicals and thiobarbituric acid reactive species (TBARS) methods.

2. Materials and methods

2.1. Chemicals, apparatus and general procedures

All chemicals and solvents were of the analytical grade obtained from Sigma Chemical Co. (St. Louis, MO, USA). The absorbance for phenolics, flavonoids, tannin, DPPH and TBARS assay were performed in a Shimadzu-UV-1201 (Shimadzu, Kyoto, Japan) spectrophotometer.

2.2. Plant collection and extractions

Leaves of *T. catharinensis* were collected in Bossoroca (Rio Grande do Sul State of Brazil) in September of 2009 (coordinates 28°65'93″ S and 55°01'27″ W). A dried voucher specimen is preserved in the herbarium of the Department of Biology at Federal University of Santa Maria by register number SMBD 12355. The leaves were dried at room temperature and powdered in a knife mill. The powder of leaves (1580.02 g) was macerated at room temperature with 70% ethanol for a week with daily shake-up. After filtration, the extract was evaporated under reduced pressure to remove the ethanol, was then suspended in water and partitioned successively with dichloromethane, ethyl acetate and n-butanol. The yield of the extract and fractions was calculated by the formula:

$$\text{Yield}(\%) = (\text{Mass of the extract/mass of material}) \times 100.$$

2.3. Determination of total phenolics contents

The determination of total phenolic content was performed by the Folin–Ciocalteu method with slightly modifications.[14] The samples were read at 730 nm in spectrophotometer. The total phenolics content was expressed in milligrams equivalents of gallic acid (GAE) per gram of each fraction. The equation obtained for the calibration curve of gallic acid in the range of 0.005–0.030 mg/mL was $Y = 11.969 - 0.0454$ ($r = 0.9984$).

2.4. Determination of total flavonoids contents

The determination of flavonoids was performed as described by Boligon et al.[8] The absorbance was determined by spectrophotometer at 420 nm. Ethanol was used as a blank. The equation obtained for the calibration curve of quercetin in the range of 0.012–0.200 mg/mL was $Y = 4.4087 - 0.0140$ ($r = 0.9952$). The content of flavonoids was established as quercetin mg/g dry extract. The experiments were conducted in triplicate.

2.5. Determination of total tannins contents

The tannins content was performed using the method described by Morrison et al,[15] with some modifications. Samples in concentrations of 0.25 mg/mL, 5 mL of solution A (1 g vanillin in 100 mL of methanol) and solution B (8 mL HCl in 100 mL of methanol) were used to experiment. The samples were read at 500 nm in spectrophotometer. The total tannins content was expressed in milligrams equivalents of catechin per gram of each fraction. The equation obtained for the calibration curve of catechin in the range of 0.001–0.025 mg/mL was $Y = 0.00015x + 0.005$ ($r = 0.9979$).

2.6. Radical-scavenging activity – DPPH assay

The radical-scavenging activity of *T. catarinensis* crude extract and fractions was quantified in the presence of DPPH$^{\bullet}$ stable radical, according to a slightly modified method.[16] Spectrophotometric analysis was used to measure the free radical-scavenging capacity (RSC) and to determine the scavenging concentration or inhibitory concentration (IC_{50}). The DPPH quenching ability was expressed as IC_{50} (the extract concentration (μg/mL) required to inhibit 50% of the DPPH in the assay medium).

The crude extract and fractions were tested at six different ethanol dilutions at 7.81, 15.62, 31.25, 62.50, 125 and 250 μg/mL. Each sample was mixed with 1.0 mL of DPPH 0.3 mM in ethanol solution. After 30 min, the absorption was measured at 518 nm. A solution of DPPH (1 mL, 0.3 mM) in ethanol (2.5 mL) was used as a negative control and ascorbic acid in the same concentrations used for the fractions and the crude extract provided the positive control. The test was performed in triplicate and the calculation of the antioxidant activity followed the equation:

$$\%\,\text{Inibition} = 100 - \left[\left(\left(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}\right)\Big/\text{Abs}_{\text{control}}\right) \times 100\right]$$

Where: $\text{Abs}_{\text{sample}}$ is absorbance of each fraction; $\text{Abs}_{\text{blank}}$ is absorbance of fractions without adding the DPPH; $\text{Abs}_{\text{control}}$ is absorbance the solution of ethanol in DPPH.

2.7. In vitro Fe(II)-induced lipid peroxidation in rat brains

Male Wistar rats weighing 270–320 g and with age from 3 to 3.5 months, from our own breeding colony were kept in cages of 3 or 4 animals each, with continuous access to food and water in a room with controlled temperature (22 ± 3 °C) and on a 12-h light/dark cycle with lights on at 7:00 a.m. The animals were maintained and in accordance to the guidelines of the Brazilian Association for Laboratory Animal Science (COBEA).

Rats were decapitated under mild ether anesthesia, and the encephalic tissue was rapidly dissected and placed on ice. Tissues were immediately homogenized in cold 10 mM Tris–HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at 4000 g to yield a pellet that was discarded and a low-speed supernatant one (S1) was used for the TBARS assay.[14] After centrifugation, an aliquot of 100 μl of S1 was incubated for 1 h at 37 °C with pro-oxidant agent (10 μM of $FeSO_4$) in presence or absence of plant extracts and then used for TBARS determination. The concentration ranges of each tested extract were 0.1, 5, 10, 25, 50, 100 and 200 μg/mL. TBARS production was determined as described by Puntel et al[17]

2.8. Statistical analysis

Data from the TBARS assay were analyzed statistically by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests when appropriated using the statistical software SPSS

Table 1
Yield of crude extract and fractions in percentage (%), contents of total phenols, flavonoids and tannins of *T. catharinensis*.

Crude extract and fractions	Yield (%)	Phenols[a] ± S.E.[d] (mg/g)	Flavonoids[b] ± S.E.[d] (mg/g)	Tannin[c] ± S.E.[d] (mg/g)
		Leaves		
Butanolic	8.5	413.52 ± 0.34 a	106.01 ± 0.01 a	10.23 ± 0.10 a
Ethyl acetate	4.1	562.78 ± 0.08 b	120.08 ± 0.17 b	10.03 ± 0.22 a
Dichloromethane	9.7	189.04 ± 1.09 c	80.19 ± 0.64 c	5.61 ± 0.49 b
Crude extract	10.3	135.57 ± 0.28 d	78.06 ± 0.25 c	3.78 ± 1.14 b

Averages followed by different letters in each column differ by Tukey test at $p < 0.001$.
[a] Phenols: expressed as gallic acid equivalents (GAE).
[b] Flavonoids: expressed as quercetin (mg/g fraction).
[c] Tannin: expressed as catechin (mg/g fraction).
[d] S.E.: standard error.

10.0 for Windows. TBARS graphic was constructed using the Slide Write 4.032 Bit Edition program. Statistical *p* values were calculated to quantify levels of significance for each treatment type. A significant *p* value ($p < 0.001$ when appropriate) means that there exists significant difference between the two sets of data being analyzed. One-way ANOVA followed by Tukey test were performed in the total phenolics, flavonoids, tannins and DPPH assays.

3. Results and discussion

The yields of crude extract (CE), and dichloromethane (DCM), ethyl acetate (EA) and *n*-butanol (BU) fractions obtained of the leaves are given in Table 1. The highest yield was obtained with CE (10.3%), followed by DCM (9.7%), BU (8.5%) and EA (4.1%) fractions, respectively.

EA and BU fractions exhibited a high content of phenolics (562.78 ± 0.08 and 413.52 ± 0.34 mg/g GAE, respectively) when compared to the DCM fraction (189.04 ± 1.09 mg/g GAE) and CE (135.57 ± 0.28 mg/g GAE). For the determination of flavonoids contents, the EA fraction exhibited the highest value (120.08 ± 0.17 mg/g quercetin), BU expressed the second highest value (106.01 ± 0.01 mg/g quercetin). On the other hand, CE showed the lowest flavonoids content (78.06 ± 0.25 mg/g quercetin). The quantification of tannin and alkaloids followed the order: BU > EA > DCM > CE (Table 1). When comparing the results obtained with phenolics, flavonoids, tannins and alkaloids contents assays, we may observe a relation between dosages for fractions. Pereira et al[18] working with *Ilex paraguariensis* found phenolics contents ranging from 277.10 to 96.82 mg/g, in our study we found higher values. Considering total phenolic contents, the results obtained for *T. catharinensis* in our study were very close to those obtained by Janovik et al[6] (*Cariniana domestica* – EA = 510.00; BU = 486.22; CE = 480.03 and DCM = 214.32 mg/g) using similar methodology. In addition, *Scutia buxifolia* showed a variation of the 323.47 to 141.09 mg/g; 145.72 to 100.37 mg/g and 176.70 to 66.67 mg/g for phenolics, flavonoids and tannin contents, respectively.[14]

In this study, *T. catharinensis* leaves exhibited very good antioxidant capacity (Fig. 1; Table 2). The DPPH assay is used as a tool to the *in vitro* evaluation of extracts and fractions and its results can indicate the presence of antioxidant compounds in plant extracts. EA was the most active fraction, the IC_{50} (the amount of extract of the plant tested necessary to decrease the concentration of initial DPPH absorbance by 50%) value obtained was 4.64 ± 1.25 μg/mL. BU showed slightly greater IC_{50} value (5.88 ± 0.19 μg/mL) than EA, being also very active, and the DCM IC_{50} was 14.49 ± 0.39 μg/mL. The lowest antioxidant capacity (IC_{50} = 27.78 ± 0.93 μg/mL) was observed for CE (Table 2). EA and BU antioxidant activities were found to be higher than the well known antioxidant ascorbic acid, commonly used as a standard (IC_{50} = 15.98 ± 1.30 μg/mL). This fact could be explained on the basis of the similarity between compounds with high antioxidant capacity extracted by these organic solvents. Many other studies have demonstrated that butanolic and ethyl acetate fractions are good sources of antioxidant compounds.[5,6,14]

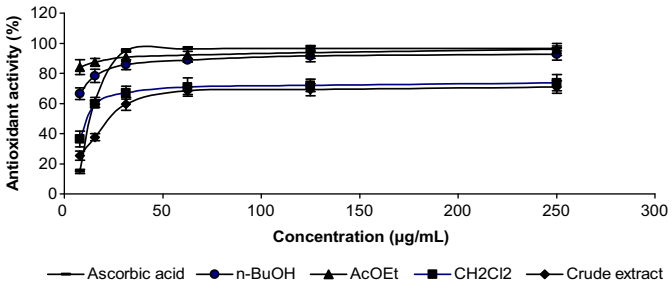

Fig. 1. Antioxidant activities of crude extract and dichloromethane, ethyl acetate and butanolic fractions from the leaves of *T. catharinensis*.

It is important to compare data obtained on *T. catharinensis* with other plants under the same assay conditions: the IC_{50} values of EA and BU from the stimulating beverage popularly drunk every day in South Brazil, *I. paraguariensis* (mate tea), were 13.26 and 27.22 μg/mL, respectively.[19] If compared with green tea (*Camellia sinensis*), the results obtained with *T. catharinensis* were 2-fold higher than those obtained for this plant, IC_{50} = 11.8 μg/mL.[11] Comparing the phenolics and flavonoids contents and IC_{50} TBARS obtained for leaves of the *T. catharinensis*, there were statistically significant differences ($p < 0.01$) between the fractions, however for tannin and IC_{50} DPPH values showed no statistical difference for BU and EA (Tables 1 and 2). Several authors have described a positive correlation between phenolics content and antioxidant capacity[8,14] using similar assay systems.

EA, BU and DCM fractions of *T. catharinensis* significantly inhibited iron-induced TBARS production in brain preparations (for all fractions *p* values were 0.001; Fig. 2). The inhibitory potency was in the following order: EA (IC_{50} = 6.71 ± 0.19 μg/mL) > BU (26.15 ± 0.08 μg/mL) > DCM (43.25 ± 0.12 μg/mL) > CE (61.09 ± 0.05 μg/mL) (Table 2). The brain is particularly susceptible to free radical damage because of its high consumption of oxygen and its relatively low concentration of antioxidants enzymes and free radicals scavengers. Several studies have focused in the use of natural therapeutic antioxidant compounds that can afford

Table 2
IC_{50} (μg/mL) values of the DPPH and TBARS assay for crude extract and fractions of *T. catharinensis*.

T. catharinensis	IC_{50} DPPH ± S.E. (μg/mL)	IC_{50} TBARS ± S.E. (μg/mL)
Butanolic	5.88 ± 0.19 a	26.15 ± 0.08 a
Ethyl acetate	4.64 ± 1.25 a	6.71 ± 0.19 b
Dichloromethane	14.49 ± 0.39 b	43.25 ± 0.12 c
Crude extract	27.78 ± 0.93 c	61.09 ± 0.05 a
Ascorbic acid	15.98 ± 1.30 d	–

IC_{50}: concentration required to inhibit 50% of the activity. S.E.: standard error; averages followed by different letters in each column differ by Tukey test at $p < 0.001$.

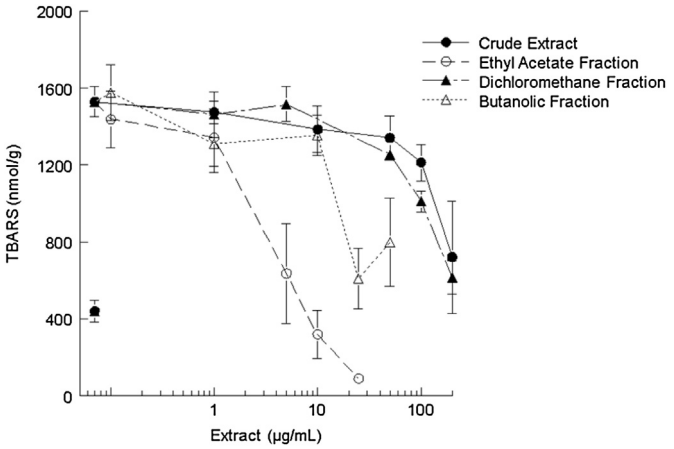

Fig. 2. Effects of different concentrations of crude extract, ethyl acetate dichloromethane, and butanolic fractions from the leaves of *Tabernaemontana catharinensis* on Fe(II) (10 µM)-induced TBARS production in brain homogenates. Data show means ± SEM values average from 3 to 4 independent experiments performed in duplicate.

protection in a variety of *in vitro* and *in vivo* models of human pathologies, including neurotoxicity models.[17]

Boligon et al[14] and Zadra et al[20] working with medicinal plants, such as, *S. buxifolia*, *Nasturtium officinale* and *Solanum guaraniticum* found effects on lipid peroxidation *in vitro*, these species showed the great antioxidant activity that is directly related to the presence of phenolics compounds and flavonoids.

4. Conclusion

The results clearly indicated that CE and fractions of *T. catharinensis* showed good antioxidant activity. Among all the samples analyzed, EA fraction showed better antioxidant activity by the DPPH and TBARS assays, which can be attributed to its high content of total phenolics and flavonoids. Taken together, our results indicate that this plant has antioxidant potential and can be a promising source of natural antioxidants. However, more detailed *in vivo* studies are required to establish the safety and bioavailability of *T. catharinensis*.

Conflicts of interest

All authors have none to declare.

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