Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(2):231-236

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Phytochemical screening and antioxidant activity of methanolic fraction from the leaves of *Crescentia cujete* L. (Bignoniaceae)

Francisco Glaudson Granja Parente[1], Ana Paula de Oliveira[1], Cristiane Maria Souza de Castro Rodrigues[1], Raimundo Gonçalves de Oliveira Júnior[1], Iza Miranda Melo Paulo[1], Xirley Pereira Nunes[1], David Marrero Delange[2] and Jackson Roberto Guedes da Silva Almeida[1*]

[1]Núcleo de Estudos e Pesquisas de Plantas Medicinais, Universidade Federal do Vale do São Francisco, 56.304-205, Petrolina, Pernambuco, Brasil
[2]Centro de ProductosNaturales, Centro Nacional de Investigaciones Científicas (CNIC). Calle 198 entre 19 y 21, Atabey, Playa, Apartado Postal 6414, La Habana, Cuba

---

ABSTRACT

Phytochemical screening of the methanolic fraction from the leaves of Crescentia cujete was carried out.The total phenolic content of the fraction was determined by the Folin-Ciocalteu method. Total flavonoid content also was measured. Antioxidant activity was evaluated by using DPPH radical scavenging,β-carotene/linoleic acid bleaching and compared with ascorbic acid, BHA and BHT, as well as by ABTS free radical decolorization and compared with Trolox®. The total phenolic content was of 50.47 ± 0.08 mg of gallic acid equivalent/g while the total flavonoid content was of 47.41 ± 0.01 mg of catechin equivalent/g.The antioxidant activity of methanolic fraction by DPPH radical scavenging (measured as IC_{50})was of 34.01 ± 4.97µg/ml, and by ABTS free radical decolorization (measured as IC_{50}) was of 3.80 ± 0.20 µg/ml. The methanolic fraction exhibited good antioxidant potential by DPPH and ABTS methods while in the β-carotene method the activity was very low. The significant presence of flavonoids and tannins measured in phytochemical screening and the total phenolic and flavonoid contents can be the main source of antioxidant activity of the fraction. Further studies will be conducted to confirm the antioxidant activity of methanolic fraction through other assays.

Keywords: *Crescentia cujete*, Bignoniaceae, antioxidant activity, flavonoids.

INTRODUCTION

Bignoniaceae, the trumpet creeper or catalpa, it's a family of the mint order of flowering plants (Lamiales). It includes about 110 genera and more than 800 species of trees, shrubs, and, more often, vines, chiefly of tropical America, tropical Africa, and the Indo-Malayan region. Because of its numerous climbing vines, the family is an important part of tropical forest ecosystems. Among the important ornamental and useful members are the African tulip tree (*Spathodea campanulata*), calabash tree (*Crescentia cujete*) and many others [1].

Crescentia cujete, popularly known as the gourd tree or calabash tree, is a species of plant that is native to Central and South America [2].The fruit is the most striking feature, which is a stiff gourd, woody shell and a jelly-like pulp that are incorporated various seeds [3].Folk medicine cites the use of the fruit pulp to treat respiratory problems (asthma, for example) and also as laxative. The decoction of barks is commonly used for diarrhea and to clear wounds. The powdered leaves are used as a poultice for headaches and internally as a diuretic and in the treatment of hematomas and tumors. Fruit decoction can be used to treat bronchitis, diarrhea, stomachaches, urethritis, cold, cough and asthma [4]. The leaves and barks have shown remarkable anti-inflammatory and antibacterial activities, as well as therapeutic potential on disease processes caused by destabilization of biological membranes [5]. Naphtoquinones [6], iridoids and iridoid glycosides, aucubin, plumieride and asperuloside have already been

reported as the constituents of the leaves of this species[7]. In general, the chemical content of the plant comprises citric acid, crescentic acid, tartaric acids, tannins, β-sitosterol, stigmasterol, α- and β-amyrin, stearic acid, triacontanol, palmitic acid, flavonoids (quercetin, apigenin), 3-hydroxyoctanol glycosides and *p*-hydroxybenzoyloxy glucose [8].

The reactive species of oxygen and nitrogen are naturally generated by human metabolism in various physiological conditions. They play an important role in the normalfunctioning of the body, such asin phagocytosis. When the productionof thesefree radicalsis exacerbated, the human body usesits efficientantioxidant system. However, in the oxidative stress there is an imbalancebetween the production ofpro-oxidants and antioxidants, with a predominance ofpro-oxidants.Thisoverproductionof free radicals cancause numerousproblemsto the body, such as aging andcell death, cancerinduction, propagation of AIDS in HIV-positive patients and many others [9].It is necessary to search for new sources of natural antioxidants, since many of the synthetic antioxidants are having different levels of toxicity, which is of concern.

The aim of this study was to evaluate the antioxidant activity of the methanolic fraction from the leaves of *Crescentia cujete* by three different *in vitro* tests and determine the total phenolic and flavonoid contents.

EXPERIMENTAL SECTION

Plant material

The leaves of *Crescentia cujete* L. were collected in Casa Nova, State of Bahia, Brazil, in February 2013. A voucher specimen (nº 22603) was deposited in theHerbarium Vale do São Francisco (HVASF) of the Universidade Federal do Vale do São Francisco. The collected material was dried in an oven with air circulation at 45°C and then pulverized in a mill.

Extraction

The dried and powdered leaves (1429 g) were repeatedly extracted three times during 96 h with 95% EtOH at room temperature. The extractive solution was concentrated under vacuum on a rotary evaporator until complete removal of the solvent, yielding 195.0 g of crude ethanol extract (CEE-EtOH).The CEE-EtOH was suspended in a mixture of MeOH:H_2O (3:7) and extracted successively with hexane, chloroform ($CHCl_3$)and ethyl acetate (AcOEt)by vacuum liquid chromatography, in crescent order of polarity to obtain the respective fractions. The methanolic fraction (MF-MeOH) was reserved under refrigeration for conducting the tests.

Preliminary phytochemical screening

The MF-MeOH was analysed on thin layer plates of silica gel 60 F_{254} aluminum supports, applied with a micropipette and eluted in different solvent systems as previously described [10], seeking to highlight the main groups of secondary metabolism (Table 1).

Table No 1. Elution systems and revelators used to characterize the main secondary metabolites from the methanolic fraction of the leaves of *Crescentia cujete* by thin layer chromatography

Phytochemicals	Elution systems	Revelators
Alkaloids	Toluene: ethyl acetate: diethylamine (70:20:10, v/v)	Dragendorff reagent
Anthracenes derivatives	Ethyl acetate: methanol: water (100:13.5:10, v/v)	10% ethanolic KOH reagent
Coumarins	Toluene: ethyl ether (1:1 saturated with acetic acid 10%, v/v)	10% ethanolic KOH reagent
Flavonoids and tannins	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26, v/v)	NEU reagent
Lignans	Chloroform: methanol: water (70:30:4, v/v)	Vanilin phosphoric reagent
Mono and diterpenes	Toluene: ethyl acetate (93:7, v/v)	Vanilin sulfuric reagent
Naphthoquinones	Toluene: formic acid (99:1, v/v)	10% ethanolic KOH reagent
Triterpenes and steroids	Toluene: chloroform: ethanol (40:40:10, v/v)	Lieberman-Burchard reagent

Total phenolic content

Total phenolic contents were assayed using the Folin-Ciocalteu reagent, it is based on the method reported by Slinkard and Singleton [11]and only the volumes were reduced. An aliquot (40 µl) of a suitable diluted MF-MeOH fraction was added to 3.16 ml of distilled water and 200 µl of the Folin-Ciocalteu reagent, and mixed well. The mixture was shaken and allowed to stand for 6 min, before adding 600 µl of sodium carbonate solution, and shaked to mix. The solutions were left at 20 °C for 2 hours and the absorbance of each solution was determined at 765 nm against the blank and plot absorbance vs. concentration. Total phenolic content of the fraction (in triplicate) was expressed as mg gallic acid equivalents per gram (mg GAE/g) through the calibration curve with gallic acid. The calibration curve range was 50-1000 mg/l (R^2= 0.9981).

Total flavonoid content

Total flavonoid content was determined by using a colorimetric method previously described [12]. Briefly, 0.30 ml of the MF-MeOH, or (+)-catechin standard solution were mixed with 1.50 ml of distilled water in a test tube followed by addition of 90 µl with distilled water and mixed well. The absorbance was measured immediately against the blank at 510 nm using a spectrophotometer (QUIMIS, Brazil) in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results were expressed as mg of catechin equivalents per gram of extracts (mg CE/g) through the calibration curve with catechin. The calibration curve range was 50-1000 mg/l (R^2 = 0.9930).

DPPH free radical scavenging assay

The free radical scavenging activity was measured using the 2,2-diphenyl-1-pycrilhydrazil (DPPH) as say previously described [13].Sample stock solutions (1.0 mg/ml) of extracts were diluted to final concentrations of 243, 81, 27, 9, 3 and 1 µg/ml, in ethanol. One ml of a 50 µg/ml DPPH ethanol solution was added to 2.5 ml of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity (AA) using the following formula: AA% = [(absorbance of the control – absorbance of the sample)/ absorbance of the control] x 100. Ethanol (1.0 ml) plus plant fraction solution (2.5 ml) were used as a blank. DPPH solution (1.0 ml) plus ethanol (2.5 ml) was used as a negative control. The positive controls [ascorbic acid, BHA (butylated hidroxyanisole)and BHT (butylated hidroxytoluene)] were those using the standard solutions. Assays were carried out in triplicate. The IC_{50} values were calculated by linear regression using by GraphPad Prism 6.0 program.

β-Carotene bleaching test

The β-carotene bleaching method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion[13].The rate of β-carotene bleaching can be slowed down in the presence of antioxidants. β-carotene (2 mg) was dissolved in 10 ml chloroform and to 2 ml of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40 ^oC and 100 ml of distilled water were added, then the emulsion was vigorously shaken during two minutes. Reference compounds (ascorbic acid, BHA and BHT) and sample fraction were prepared in ethanol. The emulsion (3.0 ml) was added to a tube containing 0.12 ml of solutions 1 mg/ml of reference compounds and sample fraction. The absorbance was immediately measured at 470 nm and the test emulsion was incubated in a water bath at 50 ^oC for 120 min, when the absorbance was measured again. Ascorbic acid, BHA and BHT were used as positive control. In the negative control, the extracts were substituted with an equal volume of ethanol. The antioxidant activity (%) was evaluated in terms of the bleaching of the β-carotene using the following formula: % Antioxidant activity = [1 - (A_0 – A_t) / (A_0^0 – A_t^0)] x 100; where A_0 is the initial absorbance and A_t is the final absorbance measured for the test sample, A_0^0 is the initial absorbance and A_t^0 is the final absorbance measured for the negative control (blank). The results are expressed as percentage of antioxidant activity (% AA). Tests were carried out in triplicate.

ABTS free radical decolorization assay

The ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] free radical decolorization assay is reported as a method for determination of antioxidant activity applicable to both lipophilic and hydrophilic antioxidants [14]. Twelve hours before the beginning of the test, 5.0 ml of an aqueous solution of ABTS (7.0 mM) and 88 µl of an aqueous solution of sodium persulphate (140 mM), both stored in amber bottles, were mixed to promote the radical generation, which remained stable for sixteen hours after completion of mixing. After twelve hours, alcoholic solutions of MF-MeOH and standard (Trolox®) were prepared at a concentration of 0.1 mg/ml. Then, the ABTS radical ($ABTS^+$) was diluted in ethanol to an absorbance of 0.700 ± 0.05 at 734 nm. To obtain different concentrations of the standard and sample, mixtures were prepared in cuvettes (1 cm optical path), varying the ratio between sample volume and the volume of the solvent used (96% ethanol). The ABTS radical volume remained fixed (2.700 ml). Table 2 shows the final concentrations at the end of such mixtures. For reading the absorbance, each concentration was carried out in triplicate. The blank of each one was obtained through the replacement of the fixed volume of ABTS radical by the same volume of ethanol 96%. A negative control was prepared (also in triplicate) by mixing 2.7 ml of the ABTS radical and 0.30 ml of 96% ethanol. All cuvettes were subjected to mixing for six minutes by ultrasonification, and then the absorbance was determined at 734 nm and converted into percentage antioxidant activity (AA) using the following formula: AA% = [(absorbance of the control - absorbance of the sample)/ absorbance of the control] x 100. The IC_{50} values were calculated by linear regression using by GraphPad Prism 6.0 program.

Table No 2. Values of ratio between the volume of solvent (96% ethanol) and the MF-MeOH, and their concentrations in µg/ml

Initial Sample Concentration (mg/ml)	Final Sample Concentration (µg/ml)	Sample Volume (µl)	Ethanol Volume (µl)	ABTS Radical Volume (µl)
0.1	0.5	15	285	2700
0.1	1.0	30	270	2700
0.1	1.5	45	255	2700
0.1	2.0	60	240	2700
0.1	2.5	75	225	2700
0.1	3.0	90	210	2700

Statistical analysis

All determinations were conducted in triplicates and the data are expressed as mean ± SD. Values were considered significantly different at $p< 0.05$.

RESULTS AND DISCUSSION

Preliminary analysis (Table 3) demonstrated that MF-MeOH was positive for the presence of anthracene derivatives, flavonoids and tannins, lignans, mono and diterpenes, triterpenes and steroids, whereas for the presence of alkaloids, coumarins and naphtoquinones, the fraction was negative.This data shows the significant presence of phenolic compounds, such as anthracene derivatives, lignans and mainly flavonoids and tannins. Phenolics can be defined as aromatic hidroxylated compounds, having one or more aromatic rings with one or more hydroxyl groups, presenting a large structural diversity which is divided into different subclasses, such as flavonoids, phenolic acids, including hydroxybenzoic acids and hydroxycinnamic acids, tannins, oxidized polyphenols, stilbenes and lignans [15]. Besides the protective actions in biological systems, like anti-inflammatory and antitumor properties [16], these phenolic compounds exhibit antioxidant activity and can be classified as free radical inhibitors, peroxide decomposers, metal inactivators or oxygen scavengers [17,18].

DPPH free radical scavenging assay is widely used to evaluate the free radical scavenging of plant extracts since it is a simple, sensitive, rapid and reproducible method [19].The chemical structure of DPPH has an unpaired electron, which is delocalized throughout the molecule (giving a violet coloration to the compound). For this reason, this structure is considered a stable free radical. DPPH free radical scavenging assay is based on the antioxidant potential of a substance to scavenge the DPPH radical, reducing it to hydrazine. When a donor of hydrogen atoms is added to a DPPH solution, a change in the coloration from violet to yellow occurs, which can be measured by UV-VIS spectroscopy at 518 nm [13].The analysis result is performed by the IC_{50}, which is considered the effective concentration to achieve 50% of antioxidant activity. According to the values shown in table No. 4, the solutions that had higher antioxidant capacity were the BHA (IC_{50} = 2.74 ± 1.07 µg/ml) and ascorbic acid (IC_{50} = 2.69 ± 0.26 µg/ml), an expected result, since these substances are already described in the literature as excellent antioxidants. BHT presented an IC_{50} (12.73 ± 1.16 µg/ml) slightly more similar to MF-MeOH (IC_{50} = 34.01 ± 4.97 µg/ml). MF-MeOH, despite having a much larger IC_{50} compared to standards, showed a relative efficiency in scavenge the DPPH radical, which emphasizes the need to carry out further evaluation tests of antioxidant activity, particularly by the significant presence of phenolic compounds (Total phenolic content = 50.47 ± 0.08 mg GAE/g) and flavonoids (Total flavonoid content = 47.41 ± 0.01 mg CE/g).

The antioxidant activity by the β-carotene bleaching test is determined in a multiphase system in which water and lipids coexists in the presence of an emulsifier. In this procedure, the β-carotene acts as a target molecule for the free radicals formed by oxidation of linoleic acid. This attack promotes rapid degradation (bleaching) of β-carotene, which can be delayed by the presence of a free radical scavenger. The antioxidants will compete with the β-carotene by the alkylperoxyl radicals released by oxidizing environment [20].According to the results shown in table No. 4, MF-MeOH had a percentage of antioxidant activity (AA% = 5.76 ± 2.23) much lower than the positive control: BHA (AA% = 43.12 ± 10.02), BHT (AA% = 40.70 ± 7.96) and ascorbic acid (AA% = 1.94 ± 1.79). This low percentage of inhibition may indicate that MF-MeOH has mostly hydrophilic antioxidants, which are usually not effective against lipophilic free radicals. To reinforce this assumption, ascorbic acid (which has a highly hydrophilic structure) showed a significantly lower activity when compared with BHA and BHT.

The ABTS free radical decolorization as say has an important advantage over the methods of determining antioxidant activity, which is the ability to determine the activity of hydrophilic and lipophilic antioxidants [14].The ABTS radical is generated by the oxidation reaction between potassium persulphate with the aqueous solution of ABTS. The antioxidant potential is analyzed based on the bleaching (from dark green to light green) of ABTS radical solution, which is monitored by UV-VIS spectroscopy at 734 nm [21].Through the values shown in table No. 4 it is possible to verify that the antioxidant activity of MF-MeOH (IC_{50} = 3.80 ± 0.20 µg/ml) was very significant, approaching to the result obtained for the standard Trolox® (IC_{50} = 1.03 ± 0.03 µg/ml). This result suggests a mutual

contribution of hydrophilic and lipophilic antioxidants, since the method is capable of measuring the activity of both types. However, being the methanol fraction, the majority contribution probably came from the water-soluble antioxidants present is this fraction.

Table No 3. Preliminary chemical characterization of MF-MeOH from the leaves of *C. cujete*

Phytochemicals	Result
Alkaloids	-
Anthracene derivatives	+
Coumarins	-
Flavonoids and tannins	+++
Lignans	+
Mono and diterpenes	+
Naphtoquinones	-
Triterpenes and steroids	+

(-)not detected;(+) low presence; (++) moderate presence; (+++) strong presence.

Table No 4. Total phenolic (TP), total flavonoids (TF) and antioxidant activity of MF-MeOH from the leaves of *C.cujete*

	TP (mg GAE/g)	TF (mg CE/g)	DPPH (IC_{50}, µg/ml)	β-carotene bleaching (%AA)	ABTS (IC_{50}, µg/ml)
MF-MeOH	50.47 ± 0.08	47.41 ± 0.01	34.01 ± 4.97	5.76 ± 2.23	3.80 ± 0.20
Ascorbic acid	---	---	2.69 ± 0.26	1.94 ± 1.79	---
BHA	---	---	2.74 ± 1.07	43.12 ± 10.02	---
BHT	---	---	12.74 ± 1.16	40.70 ± 7.96	---
Trolox®	--------	--------	--------	--------	1.03 ± 0.03

The IC_{50} values were obtained by interpolation from linear regression analysis with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. Values are given as mean ±SD (n=3).

CONCLUSION

This study showed that the MF-MeOH obtained by partition contains a significant amount of phenolic compounds, which are the possible responsible for antioxidant activity measured by some*in vitro* models. Once the tested sample is a methanol fraction, the substances contained therein are mainly water-soluble, which restricts the antioxidant potential checked for the presence of hydrophilic antioxidants, being more expressive by DPPH and ABTS methods. MF-MeOH of *Crescentia cujete* could be a good source of substances (mainly phenolics) that can minimize the effects caused by free radicals, requiring, however, more tests, both *in vitro* and *in vivo* to confirm this property.

Acknowledgements
The authors are grateful to Brazilian agency CNPq for financial support.

REFERENCES

[1] "Bignoniaceae." Encyclopaedia Britannica. Britannica Academic. Encyclopædia Britannica Inc.Web. 28 Oct. **2015**. <http://academic-eb-britannica.ez21.periodicos.capes.gov.br/EBchecked/topic/65081/Bignoniaceae>.
[2] G Wang; W Yin; ZY Zhou; KL Hsieh; JK Liu,*J. Asian Nat Prod Res*, **2010**, 12(9), 770-775.
[3] JE Espitia-Baena; HR Duran-Sandoval; J Fandino-Franky; F Díaz-Castillo; HA Gómez-Estrada,*Rev. Cubana Plant. Med*, **2011**, 16(4), 337-346.
[4] JF Morton,*Econ Bot*, **1968**, 22(3), 273-280.
[5] MS Parvin; N Das; N Jahan; MA Akhter; L Nahar; ME Islam,*BMC Res. Notes*, **2015**, 8(1), 1-7.
[6] CE Hetzel; AA Gunatilaka; TE Glass; DG Kingston; G Hoffman; RK Johnson,*J. Nat. Prod*, **1993**, 56(9), 1500-1505.
[7] K Agarwal; SP Popli,*Fitoterapia*, **1992**, 63(5), 476.
[8] N Das; ME Islam; N Jahan; MS Islam; A Khan; MR Islam; MS Parvin,*BMC Complement. Altern.Med*., **2014**, 14(1), 45.
[9] SML Vasconcelos; MOF Goulart; JBF Moura; V Manfredini; MS Benfato; LT Kubota,*Quim. Nova*, **2007**, 30(5), 1323-1338.
[10] H Wagner; S Bladt. Plant Drug Analysis: A Thin Layer Chromatography Atlas, 2nd Edition, Springer, New York, **1996**.
[11] K Slinkard; VL Singleton,*Am. J. Enol. Vitic.*,**1977**, 28(1), 49-55.
[12] V Dewanto; X Wu; RH Liu,*J. Agric. Food Chem.*, **2002**, 50(10), 3010-3014.
[13] CQ Alves; JM David; JP David; MV Bahia; RM Aguiar,*Quim. Nova*,**2010**, 33(10), 2202-2210.
[14] EM Kuskoski; AG Asuero; AM Troncoso; J Mancini-Filho; R Fett,*Ciênc. Tecnol. Aliment.*,**2005**, 25(4), 726-732.

[15] J Cote; S Caillet; G Doyon; JF Sylvain; M Lacroix,*Crit. Rev. Food Sci. Nutr*. **2010**, 50(7), 666-679.
[16] NG Puttaraju; SU Venkateshaiah; SM Dharmesh; SMN Urs; R Somasundaram,*J. Agric. Food Chem*., **2006**, 54(26), 9764-9772.
[17] JD Dziezak,*Food Technol*, **1986**, 40(9), 94-102.
[18] K Yagi,*Agric. Biol. Chem*., **1970**, 45(9), 594-598.
[19] P Mayachiew; S Devahastin,*Food Sci. Tech.*, **2008**, 41(7), 1153-1159.
[20] P Terpinc; H Abramovic,*Food Chem*, **2010**, 121(2), 366-371.
[21] Y Chen; B Huang; J He; L Han; Y Zhan; Y Wang,*J. Ethnopharmacol*, **2011**, 136(2), 309-315.