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Phytochemical screening, antioxidant and antibacterial activity of extracts from the flowers of *Neoglaziovia variegata* (Bromeliaceae)

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ABSTRACT

The phytochemical screening, antioxidant and antibacterial activities of extracts from the flowers of Neoglaziovia variegata were investigated. It was also evaluated the total phenolic and flavonoid contents by the Folin-Ciocalteu and aluminum chloride methods, respectively. Antioxidant activities of the extracts were evaluated by using of 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging and β-carotene-linoleic acid bleaching and compared with ascorbic acid, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) used as reference compounds. The antibacterial effect was evaluated by the method of microdilution. Preliminary analysis demonstrated that the extracts were found to be positive for the presence of anthracene derivatives, flavonoids, tannins, mono and diterpenes. The most significant total phenolic content was of 249.63 ± 2.79 mg of gallic acid equivalent/g for chloroform extract ($CHCl_3$), and 242.59 ± 12.64 for ethyl acetate extract (AcOEt). The total flavonoids content was of 483.31 ± 50.65 mg of catechin equivalent/g for $CHCl_3$ extract. The ethanolic extract presented the best antioxidant activity (IC_{50} 5.97 ± 0.22 μg/ml) for DPPH scavenging. BHA was the most effective antioxidant. Antioxidant activities and total phenolic contents were found to be highly correlated. The ethanolic extract showed activity against most of the microorganisms tested, especially Bacillus cereus, Enterococcus faecalis and Staphylococcus aureus.

Keywords: *Neoglaziovia variegata*, Bromeliaceae, antioxidant activity, antibacterial activity, medicinal plants.

INTRODUCTION

The Bromeliaceae, one of the largest botanical families of the New World, is distributed extensively in tropical America [1]. This family comprises 58 genera and 3172 species [2]. Considering the large number of species of the Bromeliaceae family few have been studied chemically so far. Despite this, there is a considerable amount of identified compounds, which mostly belong to the class of triterpenoids and flavonoids [3].

Neoglaziovia variegata belongs to the family Bromeliaceae, subfamily Bromelioideae, and is popularly known in Brazil as "caroá". This species can be commonly found in the Brazilian Caatinga vegetation (dry woodland characteristic of semi-arid regions of Northeastern Brazil) [4]. This species is native to the lower stratum of the

Brazilian Caatinga. It has striped leaves, flowers protected by bracts with bright coloration and fruits as juicy berries. This species constitutes one of the most used raw material for use in craftsmanship in the semi-arid region of Brazil, generating jobs and income for many families. Its leaves are used in fiber extraction which is used for manufacturing string, hats, purses, rugs, hammocks, fishing nets and fabrics. The "caroá" plant, however, has been collected directly in the Caatinga in an extrativism manner, without any systematization of cultivation, having practically disappeared in some regions [5]. Previous studies realized by our group showed the antioxidant activity and acute toxicity [6] as well as the antinociceptive effect of the ethanolic extract from the leaves of *N. variegata* [7].

In our continuing search of the Brazilian Caatinga medicinal plants to combine biodiversity conservation with drug discovery, the aim of this study was to investigate the chemical composition, antioxidant and antibacterial activities of extracts from the flowers of *N. variegata*. Recently, a qualitative characterization by HPLC-DAD of the main phenolic compounds from ethanol extract of *Hymenaea martiana* and the evaluation of its antioxidant activity was carried out [8].

EXPERIMENTAL SECTION

Plant material

The flowers of *Neoglaziovia variegata* (Arruda) Mez were collected in the city of Petrolina, State of Pernambuco, Brazil, in January of 2011. The samples were identified by a botanist from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD). A voucher specimen (6441) was deposited at the Herbarium Vale do São Francisco (HVASF) of the Federal University of San Francisco Valley.

Extraction

The dried and powdered flowers (1174 g) were repeatedly extracted three times during 72 h with 95% EtOH at room temperature. The extractive solution was concentrated under vacuum yielding after distillation of solvent, 44 g of crude ethanol extract (Nv-EtOH). The Nv-EtOH was suspended in a mixture of H_2O:MeOH (7:3) and extracted successively with hexane, $CHCl_3$ and AcOEt in crescent order of polarity to obtain the respective extracts [9], which yielded 8, 4 and 7 g, respectively.

Qualitative analysis of phytochemicals

The extracts were evaluated on thin layer plates of silica gel 60 F_{254} aluminum supports, applied with a micropipette and eluted in different solvent systems as described by Wagner and Bladt [10, 11], 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 extracts of flowers of *Neoglaziovia variegata* by thin layer chromatography.

Phytochemicals	Elution systems	Revelators
Alkaloids	Toluene: ethyl acetate: diethylamine (70:20:10, v/v)	Dragendorff reagent
Anthracene 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)	Vanillin phosphoric reagent
Mono and diterpenes	Toluene: ethyl acetate (93:7, v/v)	Vanillin 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 [12], and only the volumes have been reduced [13]. An aliquot (40 µl) of a suitable diluted EtOH, hexane, $CHCl_3$ and AcOEt extracts was added to 3.16 ml of distilled water and 200 µl of the Folin–Ciocalteu reagent, and mix well. The mixture was shaken and allowed to stand for 6 min, before adding 600 µl of sodium carbonate solution, and shake 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 contents of the extracts (three replicates per treatment) were 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 = 0.9938). All samples were performed in triplicates.

Determination of Total Flavonoid Content

Total flavonoid content was determined by using a colorimetric method described previously [14]. Briefly, 0.30 ml of the EtOH, hexane, $CHCl_3$ and AcOEt extracts, or (+)-catechin standard solution were mixed with 1.50 ml of

distilled water in a test tube followed by addition of 90 µl of a 5% $NaNO_2$ solution. After 6 min, 180 µl of a 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for another 5 min before 0.6 ml of 1 M NaOH was added. The mixture was brought to 330 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 (R^2 = 0.9948). The calibration curve range was 50-1000 mg/l.

DPPH Free Radical Scavenging Assay

The free radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazil (DPPH) assay [15]. 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 extracts solutions (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 and BHT) 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 5.0 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 [16]. 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 was added, then the emulsion was vigorously shaken during two minutes. Reference compounds (ascorbic acid, BHA and BHT) and sample extracts 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 extracts. 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.

Microorganisms

The reference bacterial strains used in this study were obtained from National Institute of Quality Control in Health (INCQS/FIOCRUZ - Brazil). The microorganisms used were: *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonela enterica* (ATCC 10708), *Serratia marcescens* (ATCC 13880), *Shigella flexneri* (ATCC 12022) and *Staphylococcus aureus* (ATCC 25923).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The antibacterial effect was evaluated by the method of microdilution [17] as recommended by The National Committee for Clinical Laboratory Standards [18]. Initially a stock solution of 25 mg/ml of extracts was prepared using an aqueous solution of 20% DMSO (v/v). It was transferred 200 µl of this dilution to the microplate containing 200 µl of Müller-Hinton broth. Then, serial dilutions were performed resulting in concentrations of 6250, 3120, 1560, 1250, 780, 390, 190 and 90 µg/ml. The inoculum containing 5 x 10^5 CFU ml^{-1} (0.5 in McFarland scale) was added to each well. It was reserved wells in microplate for sterility control of the broth, the bacterial growth and the action of antimicrobial reference (Gentamicin). For gentamicin was used an initial concentration of 1.6 mg/ml, which was diluted to concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125 µg/ml. The microplates were incubated under conditions of aerobically for 18-24 h at 37 ° C when 10 µl of 2,3,5-triphenyl-tetrazolium (CTT) 2% were added to each well to detect the color change of the CTT (colorless) to red, reflecting the bacterial metabolism active. The MIC was defined as the lowest concentration of the extracts that visibly inhibited the bacterial growth. To determine the MBC, aliquots of 10 µl were withdrawn from each well containing the extracts and transferred to Petri dishes containing agar Müller-Hinton. The plates were incubated for 24 h at 37 °C. The appearance of bacterial colony for a given concentration indicates that does not was able to kill 99.9% or more bacterial inoculum used. Assays were performed in triplicate. The density of the extracts was employed to convert µl/ml in mg/ml. The latter being used to express the MIC and MBC.

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 demonstrated that EtOH and hexane extracts were positive for the presence of anthracene derivatives, flavonoids and tannins, mono and diterpenes. The hexane extract also showed positive reaction for the presence of triterpenes and steroids. The $CHCl_3$ extract was positive for the presence of anthracene derivatives, coumarins, flavonoids and tannins, mono and diterpenes and naphtoquinones. The ethyl acetate extract was positive for the presence of coumarins, flavonoids and tannins, lignans and naphtoquinones. All extracts were negative for the presence of alkaloids. The presence of compounds in the extracts ranged to low presence (+) to strong presence (+++). Some classes of secondary metabolites were not detected in extracts.

As expected, phenols were found in extracts of flowers. Among the phenolic compounds, flavonoids and tannins occurred more frequently. These results are in agreement with the major classes of secondary metabolites found in the family Bromeliaceae [3]. These results are presented in Table 2.

Table No 2. Phytochemical characterization of extracts from the flowers of *Neoglaziovia variegata*.

Phytochemicals	EtOH	Hexane	$CHCl_3$	AcOEt
Alkaloids	-	-	-	-
Anthracene derivatives	++	++	+	-
Coumarins	-	-	++	++
Flavonoids and tannins	++	++	++	+++
Lignans	-	-	-	++
Mono and diterpenes	+	+++	++	-
Naphthoquinones	-	-	+	+
Triterpenes and steroids	-	++	-	-

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

Chemistry of natural products is a research field with endless potential, and is especially important in countries possessing great biodiversity. Reactive oxygen species (ROS) including oxygen-centered radicals and some non-radical derivatives of oxygen cause oxidative stress to cells [19]. In recent years there is an intensive increase in researches objecting the evaluation of the antioxidant activity of extracts and other materials from natural sources, since antioxidant compounds could be applied to treat and prevent cancer and cardiovascular diseases as well as to the aging process [20].

Table 3 summarizes the results from the quantitative determination of phenolic and flavonoids as well as the effect of extracts from *Neoglaziovia variegata*, ascorbic acid, BHA and BHT on the DPPH free radical scavenging and β-carotene-linoleic acid bleaching test.

The content of phenolic compounds in the studied extracts expressed in equivalent of gallic acid/g of extract (GAEq/g) varied between 27.85 ± 8.34 and 249.63 ± 2.79 mg/g for hexane and $CHCl_3$ extracts, respectively. The highest amounts were found in the $CHCl_3$ and AcOEt extracts (249.63 ± 2.79 and 242.59 ± 12.64, respectively). The level of flavonoids, expressed in catechin equivalents (CEq) in mg/g of plant extract, varied from 187.80 ± 9.28 to 483.31 ± 50.65 mg/g. The highest amounts were found for the $CHCl_3$ extract.

Assays based on the scavenging of DPPH has been widely used to measure the antioxidant activity of different phenolic compounds and the results obtained are, in most cases, in agreement with those derived by lipid peroxidation assays in oils [21, 22]. DPPH is one of a few stable available organic nitrogen radicals and has a UV-vis absorption maximum at 515-518 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of color [23]. The data showed that the EtOH and AcOEt extracts exhibited excellent free radical scavenging activity. The EtOH extract showed better antioxidant activity than BHT using by DPPH method, with a value of IC_{50} of 5.97 ± 0.22 μg/ml. BHA was the most effective antioxidant, with a value of IC_{50} of 3.50 ± 3.17 μg/ml. In some cases the extracts with strong antiradical activity are abundant in flavonoids or phenolic compounds.

The antioxidant activity of extracts was also evaluated by the β-carotene/linoleate bleaching method. This 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. β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The rate of the β-carotene bleaching can be slowed down in the presence of antioxidants [24]. This

method is one of the antioxidant assays suitable for plant extracts. The addition of extracts, ascorbic acid, BHA and BHT prevented the bleaching of β-carotene to different degrees. All extracts had lower antioxidant activity than BHT and BHA.

According to these results, it was concluded that plant extracts from *Neoglaziovia variegata* have potent antioxidant activity, achieved by scavenging abilities observed against DPPH. The existing data give new information for the antioxidant potential and polyphenolic content of plant species that have not been traditionally used as medicinal plant.

Table No 3. Total phenolics (TP), total flavonoids (TF) and antioxidant activity of extracts from the flowers of *Neoglaziovia variegata*.

Extract	TP (mg GAEq/g)	TF (mg CEq/g)	DPPH (IC_{50}, µg/ml)	β-carotene (% AA)
EtOH	223.70 ± 10.56	308.31 ± 12.51	5.97 ± 0.22	0.81 ± 0.70
Hexane	27.85 ± 8.34	187.80 ± 9.28	391.20 ± 43.10	11.54 ± 1.75
$CHCl_3$	249.63 ± 2.79	483.31 ± 50.65	53,94 ± 2.74	14.57 ± 3.86
AcOEt	242.59 ± 12.64	212.19 ± 35.21	13.43 ± 0.49	5.06 ± 0.93
Ascorbic acid	---	---	4.72 ± 2.67	7.50 ± 2.12
BHA	---	---	3.50 ± 3.17	80.93 ± 3.45
BHT	---	---	17.87 ± 2.98	86.77 ± 1.14

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).

The discovery of new antibiotics is important due to the increasing incidence of multiple resistances of pathogenic microorganisms to drugs that are currently in clinical use [25, 26]. The results for evaluation of the antibacterial activity for extracts of *N. variegata* are shown in Table 4, and are expressed as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The antibacterial activity was evaluated against eight reference bacteria. The ethanolic extract showed activity against most of the microorganisms tested, especially for strains Gram-positive *Bacillus cereus*, *Enterococcus faecalis* and *Staphylococcus aureus*. Moreover, excellent results have been identified in Gram-negative strains such as *Escherichia coli*, *Salmonela choleraesuis, Serratia marcescens*, *Shigella flexneri*, but mainly *Klebsiella pneumoniae*, a multidrug-resistant bacteria, producing carbapenemase [27]. This is the first study that shows that extracts of *N. variegata* have antibacterial activity.

Table No 4. Antibacterial activity of extracts from the flowers of *Neoglaziovia variegata*.

Microorganisms	MIC (µg/ml)					MBC (µg/ml)				
	EtOH	Hexane	$CHCl_3$	AcOEt	GEN	EtOH	Hexane	$CHCl_3$	AcOEt	GEN
Bacillus cereus	*	1560	3120	3120	0.4	*	1560	3120	3120	0.4
Enterococcus faecalis	*	3120	12500	12500	0.4	*	3120	12500	12500	0.4
Escherichia coli	390	1560	3120	3120	*	3120	3120	3120	3120	0.4
Klebsiella pneumoniae	390	780	1560	1560	0.05	3120	1560	6250	12500	0.05
Salmonela choleraesuis	390	1560	1560	1560	0.05	3120	6250	3120	6250	0.05
Serratia marcescens	390	190	1560	1560	*	1560	6250	6250	6250	0.025
Shigella flexneri	390	780	1560	1560	*	1560	6250	12500	12500	0.025
Staphylococcus aureus	*	780	1560	1560	0.025	3120	6250	12500	12500	0.025

MIC: minimal inhibitory concentration. MBC: minimum bactericidal concentration. (*) absence of bacterial increase at all concentrations tested (n=3). EtOH= ethanolic extract; Hexane= hexanic extract; $CHCl_3$= chloroform extract; AcOEt= ethyl acetate extract; GEN= gentamicin.

CONCLUSION

This study showed that the ethanol extract of *N. variegata* and the ethyl acetate extract obtained by partition contain substantial amount of phenolics which are responsible for its marked antioxidant activity as assayed through *in vitro* models. *N. variegata* could be a good source of antioxidant phenolics. It was demonstrated that the extracts contains high content of phenolic compounds and flavonoids. The antioxidant activity presented by the extracts is related to the presence of these compounds. The flavonoids present in the extracts could be responsible by antibacterial activity presented in this study.

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