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PHENOLIC QUANTIFICATION AND ANTIOXIDANT ACTIVITY OF *ANAXAGOREA DOLICHOCARPA* AND *DUGUETIA CHRYSOCARPA* (ANNONACEAE)

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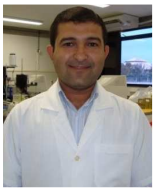

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ABSTRACT

This work presents the phenolic quantification and evaluation of *in vitro* antioxidant activity of *A. dolichocarpa* and *D. chrysocarpa* are the two species of the Annonaceae family. The total phenolics content of the plant extracts was determined by the Folin-Ciocalteu method. Antioxidant activities were evaluated by using DPPH radical scavenging and β-carotene-linoleic acid bleaching and compared with ascorbic acid, BHA and BHT used as reference compounds. The most significant total phenolic content was of 252.50 ± 7.02 mg of gallic acid equivalent/g for $CHCl_3$ extract from *A. dolichocarpa*, which presented the best antioxidant activity (IC_{50} 55.64 ± 5.24 µg/ml) for DPPH scavenging. For *D. chrysocarpa*, the more significant total phenolic content was of 213.11 ± 9.87 for AcOEt extract, with IC_{50} value of 26.97 ± 1.90 µg/ml for DPPH scavenging. BHT was the most effective antioxidant. The results obtained show that phenolic compounds contribute to the antioxidant activity of the extracts. Further studies will be conducted to isolate the chemical constituents responsible for antioxidant activity.

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KEYWORDS

Phenolic quantification, antioxidant activity, *Anaxagorea dolichocarpa*, *Duguetia chrysocarpa*, Annonaceae

INTRODUCTION

The Annonaceae is a large family comprising *ca*. 135 genera and 2500 species which are distributed mainly in tropical and subtropical regions of the world [1]. Chemical studies with species of this family have reported the isolation of terpenoids (mainly diterpenes), essential oils which the composition is predominantly of monoterpenes and sesquiterpenes, and alkaloids, especially isoquinoline alkaloids [2].

The genus *Anaxagorea* comprises approximately 30 species distributed in Central America and South America. Plants of this genus have previously yielded aporphine alkaloids, fatty acids, polyprenols, cyanogenic glucosydes, neolignans and steroid [3]. The species *Anaxagorea dolichocarpa* has a wide geographical distribution, being the neotropical species of Annonaceae most common and well distributed. In Brazil, it occurs in the states of Amapá, Amazonas, Acre, Rondônia, Goiás, Maranhão, Paraíba, Pernambuco, Bahia and Rio de Janeiro [4]. Chemical studies involving this species reported the isolation of two aporphine alkaloids anaxagoreine and asimilobine [5] as well as volatile components of the essential oil extracted from the fruits [6,7].

The genus *Duguetia* has nearly 80 known species native to tropical America [8]. In the past two decades, chemical studies of the genus have grown in number, although pharmacological and biological evaluations have not been conducted so extensively. Few chemical data are available on this genus, despite the considerable number of species. Several isoquinoline-derived alkaloids and sesquiterpene-type structures have been reported [9,10]. Aporphine alkaloids are common in Annonaceae family and some derivatives are isolated from *Duguetia* genus [11,12]. Other chemical studies, realized with species of this genus, isolated alkaloids and volatile constituents from *Duguetia flagellaris* and *Duguetia trunciflora* [13,14]; alkaloids and cinnamate derivative from *Duguetia gardneriana* [15]. There is no previous report on chemical studies of *D. chrysocarpa*.

The alkaloids are the major chemical constituents of various species of Annonaceae. Few studies report the occurrence of phenolic compounds in these species, mainly flavonoids, although they can be found in some genera and species of Annonaceae native to Brazil [16]. Studies that show the evaluation of the antioxidant activity of extracts obtained from species of this family are also few. Thus, the main goal of the present study is to determine the total phenolic content and to explore the potential *in vitro* antioxidant properties of the extracts of *A. dolichocarpa* and *D. chrysocarpa*.

2. MATERIALS AND METHODS

2.1. Plant material

The stem barks of *Anaxagorea dolichocarpa* Sprague & Sandwith were collected in the city of Santa Rita, State of Paraíba, Brazil, in February 2006. A voucher specimen was deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), of the Federal University of Paraiba, with the code Agra & Góes 5543.

The fruits of *Duguetia chrysocarpa* Maas were collected in Santa Rita, State of Paraiba, Brazil, in January 2004. A voucher specimen (Agra 5538) was deposited at the

Herbarium Prof. Lauro Pires Xavier (JPB), of the Federal University of Paraiba.

2.2. Extraction

The stem barks of *A. dolichocarpa* (2000 g) as well as the fruits of *D. chrysocarpa* (2000 g), dried and pulverized, were subjected to maceration with 95% EtOH for 72 hours. The EtOH solution was concentrated under vacuum yielding 64 and 107 g of crude ethanolic extract of *A. dolichocarpa* (Ad-EtOH) and *D. chrysocarpa* (Dc-EtOH), respectively. The extracts were suspended in MeOH:H_2O (3:7) and partitioned with hexane, chloroform ($CHCl_3$) and ethyl acetate (AcOEt) in crescent order of polarity to obtain the respective extracts.

2.3. Preliminary phytochemical screening

Preliminary phytochemical analysis of the ethanol extracts was carried. The presence of alkaloids was tested with Dragendorff's and Mayer's reagents, flavonoids with HCl and Mg powder, phenols with ferric chloride and steroids and terpenoids by Liebermann-Burchard reaction [17].

2.4. Total phenolic content

Total phenolic contents were assayed using the Folin-Ciocalteu reagent, it is based on the method reported by Slinkard and Singleton [18], only the volumes have been adjusted. An aliquot (40 µl) of a suitable diluted extracts were 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 = 0.9985). All samples were performed in triplicates.

2.5. Antioxidant activity

2.5.1. Chemicals

Folin-Ciocalteu reagent, β-carotene, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Fluka. Ascorbic acid was purchased from Dinâmica, Brazil. Linoleic acid and gallic acid were obtained from Vetec, Brazil. Recordings were made in a UV-VIS Spectrometer QUIMIS, Brazil. All reagents and solvents were of analytical grade.

2.5.2. DPPH Free Radical Scavenging Assay

The free radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazil (DPPH) assay [19, 20]. Sample stock solutions (1.0 mg/ml) of the 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.

2.5.3. 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 [21]. 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 ^{o}C 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 ^{o}C 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 = $[1 - (A_0 – A_t) / (A_0^0 – A_t^0)] \times 100$; 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.

2.6. Statistical analysis

All determinations were conducted in triplicates and the data are expressed as mean ± SD. The IC_{50} values were calculated by linear regression using by GraphPad Prism® 5.0 program. Values were considered significantly different at $p < 0.05$.

3. RESULTS AND DISCUSSION

Plants have been the basis of traditional medicines throughout the world for thousands of years and continue to provide new remedies to humankind; a great deal of effort has therefore focused on using available experimental techniques to identify natural antioxidants from plants [22]. Phenolic compounds are among the major classes of antioxidants compounds. The phenolic compounds of plants fall into several categories such as simple phenolics, phenolic acids (derivatives of cinnamic and benzoic acids), coumarins, flavonoids, stilbenes, tannins and lignans. Nitrogen compounds such as alkaloids also present antioxidant activity [23].

Preliminary analysis demonstrated that ethanolic extracts of *A. dolichocarpa* and *D. chrysocarpa* contain phenols, alkaloids, steroids and terpenoids. However, the crude extracts were found to be negative for the presence of flavonoids.

The extracts of two species were evaluated in terms of their total phenols content (TP) and the antioxidant properties were evaluated using the 2,2-diphenyl-1-picrylhidrazyl (DPPH) radical scavenging and β-carotene/linoleic acid bleaching assays (Table 1). The extracts were evaluated by comparing with ascorbic acid, BHA and BHT, which are well-known commercial antioxidants.

Table 1
Shows total phenolics (TP) and anti-oxidant activity of extracts from Anaxagorea dolichocarpa and Duguetia chrysocarpa and standards ascorbic acid, BHA and BHT.

Groups	TP (mg GAE/g)	DPPH (IC_{50}, µg/ml)	β-carotene bleaching (% AA)
Anaxagorea dolichocarpa			
EtOH	57.13 ± 4.16	142.20 ± 59.46	8.40 ± 6.22
Hexane	5.40 ± 4.23	742.00 ± 280.40	1.63 ± 0.41
$CHCl_3$	252.50 ± 7.02	55.64 ± 5.24	25.61 ± 2.93
AcOEt	245.10 ± 8.33	380.30 ± 174.90	2.71 ± 3.64
Duguetia chrysocarpa			
EtOH	191.80 ± 8.00	79.04 ± 35.80	10.98 ± 1.07
Hexane	50.47 ± 3.06	166.40 ± 65.47	11.92 ± 8.77
$CHCl_3$	117.10 ± 4.62	162.30 ± 33.84	2.57 ± 1.54
AcOEt	213.11 ± 9.87	26.97 ± 1.90	10.71 ± 8.78
Standards			
Ascorbic acid	---	3.91 ± 0.33	13.14 ± 3.84
BHA	---	2.69 ± 0.73	80.93 ± 3.45
BHT	---	0.70 ± 0.24	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 total phenolics content of the plant extracts was determined by the Folin-Ciocalteu method. This method for total phenol is useful in order to know the efficiency of extraction of phenolic in solvents. The most significant total phenolic content was of 252.50 ± 7.02 mg of gallic acid equivalent/g for $CHCl_3$ extract from *A. dolichocarpa*. For *D. chrysocarpa*, the more significant total phenolic content was of 213.11 ± 9.87 for AcOEt extract. Thus, the total phenolics may play a role in the antioxidant activity.

The scavenging activity on DPPH free radical is a common method to evaluate the antioxidative activity of plant extracts. DPPH is a stable, organic free radical extensively used to evaluate scavenging activity of antioxidants because it is sensitive enough to detect active ingredients at low concentrations. In the DPPH assay, an antioxidant scavenges the free radicals [24]. When an antioxidant is mixed with any concentration of the free radical forming sample such as DPPH, it reduces the free radical formation which is detected by decrease in the absorbance of DPPH [25]. DPPH has a purple color which is reduced to yellow-colored diphenylpicrylhydrazine. As shown in Table 1, extracts from *A. dolichocarpa* and *D. chrysocarpa* showed different antioxidant activities. The $CHCl_3$ extract from *A. dolichocarpa* had relatively strong DPPH radical scavenging activity (IC_{50} 55.64 ± 5.24 µg/ml), exhibiting high antioxidant capacity when compared to other extracts of this plant. The AcOEt extract of *D. chrysocarpa* showed the lowest IC_{50} value (26.97 ± 1.90 µg/ml), being considered the most effective antioxidant

among the extracts. The results indicate that there was a correlation between the total phenolic content and the antioxidant activity of the extracts. BHT was the most effective antioxidant (IC_{50} 0.70 ± 0.24 µg/ml). Ascorbic acid and BHA also showed an excellent scavenging activity, with values of IC_{50} of 3.91 ± 0.33 and 2.69 ± 0.73 µg/ml, respectively.

The phytochemical screening revealed the presence of alkaloids in the extracts. Recently, phytochemical investigation realized by our group led to isolation of three azaphenanthrene alkaloids from the species *Anaxagorea dolichocarpa* (data not showed). The phenolic compounds are under investigation.

A comparative analysis of the ethanolic extract of *Duguetia chrysocarpa* with a standard discretamine by thin layer chromatography revealed that this alkaloid can be present in the extract. The antioxidant activity presented by the ethanol extract may be related to the presence of discretamine. This alkaloid is a representative constituent of many species of Annonaceae and the *Duguetia* genus. The antioxidant activity of discretamine isolated from *Xylopia langsdorffiana* was also reported in the literature [2]. It appears that discretamine have a strong hydrogen-donating capacity and can efficiently scavenge DPPH radicals. The presence of phenolic hydroxyls in this compound appeared essential for scavenger properties.

Other method used for evaluation of antioxidant activity of the extracts was the β-carotene/linoleic acid system. In the β-carotene bleaching assay, linoleic acid releases a free radical with hydrogen atom, causes the β-carotene to lose its chromophore and orange color. The antioxidants extracted from plants can prevent this process by neutralizing the free radical [24]. In this model, the extracts showed weak to moderate antioxidant activity, and the most active extract was the $CHCl_3$ extract from *A. dolichocarpa* with percentage of antioxidant activity of 25.61 ± 2.93, moderate activity. BHA was as effective as BHT, and much more effective than ascorbic acid. There was no significant difference between BHT and BHA.

4. CONCLUSION

In summary, the present study demonstrates for the first time that *A. dolichocarpa* and *D. chrysocarpa* contain phenolic compounds which can serve as natural sources of antioxidants and that the extracts shown to possess antioxidant activity using *in vitro* models. Further research will be conducted to reach the substance responsible for antioxidant activity of extracts.

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REFERENCES

1. L. W. Chatrou, H. Rainer, P.J.M. Maas. Annonaceae (Soursop Family). In: N. Smith, S.A Mori, A. Henderson, D.W. Stevenson, S.V. Heald (eds.), *Flowering Plants of Neotropics*; New York BotanicalGarden, New York, USA, 2004; pp. 18-20.
2. Silva, M.S., Tavares, J.F., Queiroga, K.F., Agra, M.F., Barbosa-Filho, J.M., Almeida, J.R.G.S., Silva, S.A.S. Alkaloids and other

constituents from *Xylopia langsdorffiana* (Annonaceae). *Quím. Nova*, 32 (6): 1566-1570, (2009).
3. Díaz, A.M.P. Neolignans from *Anaxagorea clavata*. *Phytochemistry*, 44 (2): 345-346, (1997).
4. Pontes, A.F., Barbosa, M.R.V., Maas, P.J.M. Flora Paraibana: Annonaceae Juss. *Acta Bot. Bras.*, 18 (2): 281-293, (2004).
5. Hocquemiller, R., Rasamizafy, S., Moretti, C., Jacquemin, H., Cavé, A. Anaxagoreine, a new alkaloid isolated from two species of the genus *Anaxagorea*. *Planta Med*, 41: 48-50, (1981).
6. Fournier, G., Hadjiakhoondi, A., Charles, B., Leboeuf, M., Cavé, A. Volatile components of *Anaxagorea dolichocarpa* fruit. *Biochem. Syst. Ecol.*, 22 (6): 605-608, (1994).
7. Andrade, E.H.A., Oliveira, J., Zoghbi, M.G. Volatiles of *Anaxagorea dolichocarpa* Spreng. & Sandw. and *Annona densicoma* Mart. growing wild in the state of Pará, Brazil. *Flavour Frag. J.*, 22: 158-160, (2007).
8. Muhammad, I., Dunbar, D. C., Takamatsu, S., Walker, L. A., Clark, A. M. J. Antimalarial, cytotoxic, and antifungal alkaloids from *Duguetia hadrantha*. *J. Nat. Prod.*, 64: 559-562, (2001).
9. Carollo, C. A., Siqueira, J. M., Garcez, W. S., Diniz, R., Fernandes, N. G. *N*-Nitrosoanonaine and *N*-nitrosoxylopine, aporphine alkaloids from *Duguetia furfuracea*. *J. Nat. Prod.*, 69: 1222-1224, (2006).
10. Carollo, C. A., Hellmann, A. R., Siqueira, J. M. Sesquiterpenoids from the essential oil from leaves of *Duguetia furfuracea* (Annonaceae). *Biochem. Syst. Ecol.*, 33: 647–649, (2005).
11. Leboeuf, M., Cavé, A., Bhaumik,P. K., Mukherjee, B., Mukherjee, R. The phytochemistry of the Annonaceae. *Phytochemistry*, 21: 2783-2813, (1982).
12. Silva, D. B., Tulli, E. C. O., Militão, G. C. G., Costa-Lotufo, L. V., Pessoa, C., Moraes, M. O., Albuquerque, S., Siqueira, J. M. The antitumoral, trypanocidal and antileishmanial activities of extract and alkaloids isolated from *Duguetia furfuracea*. *Phytomedicine*, 16: 1059-1063, (2009).
13. Fechine, I. M., Navarro, V. R., Cunha, E. V. L., Silva, M. S., Maia, J. G. S., Barbosa-Filho, J. M. Alkaloids and volatile constituents from *Duguetia flagellaris*. *Biochem. Syst. Ecol.*, 30: 267-269, (2002).
14. Fechine, I. M., Lima, M. A., Navarro, V. R., Cunha, E. V. L., Silva, M. S., Barbosa-Filho, J. M., Maia, J. G. S. Alcalóides de *Duguetia trunciflora* Maas (Annonaceae). *Rev. Bras. Farmacogn.*, 12: 17-19, (2002).
15. Almeida, J. R. G. S., Lúcio, A. S. S. C., Barbosa-Filho, J. M., Agra, M. F., Silva, M. S., Cunha, E. V. L., Uchoa, D. E. A., Braz-Filho, R. Alkaloids and a new cinnamate derivative from *Duguetia gardneriana*. *Biochem. Syst. Ecol.*, 35: 456-458, (2007).
16. Santos, D. Y. A. C., Salatino, M. L. F. Foliar flavonoids of Annonaceae from Brazil: taxonomic significance. *Phytochemistry*, 55: 567-573, (2000).
17. F. J. A. Matos. *Introdução à fitoquímica experimental*, 2ª ed., Fortaleza, Edições UFC, 1997.
18. Slinkard, K., Singleton, V. L. Total phenol analysis: automation and comparison with manual methods. *Am. J. Enol. Viticult.*, 28: 49-55, (1977).
19. Mensor, L. L., Menezes, F. S., Leitão, G. G., Reis, A. S., Santos, T. C., Coube, C. S., Leitão, S. G. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytother. Res.*, 15: 127-130, (2001).
20. Falcão, D. Q., Costa, E. R., Alviano, D. S., Alviano, C. S., Kuster, R. M., Menezes, F. S. Atividade antioxidante e antimicrobiana de *Calceolaria chelidonioides* Humb. Bonpl.

& Kunth. *Braz. J. Pharmacogn.*, 16 (1): 73-76, (2006).
21. Wannes, W. A., Mhamdi, B., Sriti, J., Jemia, M. B., Ouchikh, O., Hamdaoui, G., Kchouk, M. E., Marzouk, B. Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. *italica* L.) leaf, stem and flower. *Food Chem. Toxicol.*, 48 (5): 1362-1370, (2010).
22. Krishnaiah, D., Sarbatly, R., Nithyanandam, R. A review of the antioxidant potential of medicinal plant species. *Food Bioprod. Process.*, 89 (3): 217-233, (2011).
23. Velioglu, Y. S., Mazza, G., Gao, L., Oomah, B. D. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J. Agric. Food Chem.*, 46 (10): 4113-4117, (1998).
24. Chen, Y., Huang, B., He, J., Han, L., Zhan, Y., Wang, Y. *In vitro* and *in vivo* antioxidant effects of the ethanolic extract of *Swertia chirayita*. *J. Ethnopharmacol.*, 136 (2): 309-315, (2011).
25. Elayaraja, A., Vijayalakshmi, M., Devalarao, G. *In vitro* free radical scavenging activity of various root and rhizome extracts of *Acorus calamus* Linn. *International Journal of Pharma and Bio Sciences*, 1 (4): 301-304, (2010).