American Journal of Plant Sciences, 2015, 6, 3038-3044
Published Online December 2015 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2015.619298

Antioxidant Activity and Phytochemical Profile of *Spondias tuberosa* Arruda Leaves Extracts

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Received 15 October 2015; accepted 4 December 2015; published 7 December 2015

Abstract

***Spondias tuberosa* Arruda (umbuzeiro), a Brazilian semiarid plant, is a species of great economic, social, and ecological importance. In folk medicine, the leaves have been used in the treatment of diabetes, inflammation, stomach and uterine pains, and constipation. In this study, the antioxidant properties of ethyl acetate and methanol leaves extracts were evaluated *in vitro* using different methods: free radicals elimination by DPPH and ABTS assays, and transition metal reduction by phosphomolybdenium assay. In addition, a phytochemical study was also carried out. The methanolic leaves extracts showed the strongest antioxidant activity and the higher values for total phenolic and flavonoids. The results showed that *S. tuberosa* leaves have antioxidant activity and this seems to be related to the phenolic content.**

Keywords

Umbuzeiro, Phenolic Compounds, Flavonoids, Antioxidants

1. Introduction

The oxidative stress induced by free radicals is considered a primary factor in neurodegenerative diseases as

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Alzheimer's, Parkinson's and cardiovascular diseases as atherosclerosis [1]. The *in vitro* ability of phenolic compounds in the free radicals elimination and pro-oxidant metals reduction may explain the effectiveness in the treatment of many chronic non-transmissible diseases [2]. Natural antioxidants derived from plants are generally required to neutralize the damage caused by reactive oxygen species (ROS) to the cells [3].

Antioxidant compounds can be defined as substances that show at low concentrations the capability to inhibit or retard substrate oxidation when compared to oxidizable substrates [4]. Antioxidants such as flavonoids, tannins, coumarins, curcumanoids, xanthones, phenols, lignans and terpenoids have been found in various plant products as fruit, leaves, seeds and oils [5].

Spondias tuberosa Arruda (Anacardiaceae), known as "umbuzeiro", is a tropical plant that plays a major role in the Northeast of Brazil as an important nutritional resource [6]. It is endemic to the Caatinga zone and produces flowers and fruits during the dry season [7], when most species of plants remain in a completely deciduous state [8] [9]. In addition, the fruits represent an investment for the local population as food for humans and animals [10] [11]. This species has great ecological, social, economic, and cultural importance [12]. The "Umbu", the fruit of *S. tuberosa* provides a source of vitamins (B1, B2, B3, A, and C) and minerals (calcium, phosphorus and iron) [13] and possesses antibacterial activity [14]. In popular medicine, leaves are also used for treating several pathologies as diabetes, inflammations, uterine pain, stomach pain and constipation [15].

The aim of this study was the evaluation of the phytochemical profile and the determination of total phenolics, flavonoids, and the antioxidant activity of the ethyl acetate and methanol leaves extract of *S. tuberosa* by different methods (ABTS, DPPH and phosphomolybdenum assays).

2. Material and Methods

2.1. Plant Material

The plant was collected in Catimbau National Park, Pernambuco, Northeast of Brazil, at April 2014. The botanical identification was made by the herbarium staff of the Instituto de Agronômico de Pernambuco (IPA) and a voucher specimen was deposited in the herbarium (No. 91090).

2.2. Extract Preparation

The leaves were dried in an oven at 45˚C. The material was triturated in mill (Tecnal/Willye mill/ET-650) to obtain a powder. The extracts were obtained in a mechanical Accelerated Solvent Extractor (ASE 350 Dionex). The extracts were concentrated under a nitrogen stream in a heating block at 60˚C. 20 g of the powder were transferred to the cells of the ASE and extracted with ethyl acetate and methanol and then dried at 50˚C using a rotary evaporator.

2.3. Phytochemical Analysis

The phytochemical screening of the plant extracts was performed by thin layer chromatography (TLC) according to Harborne [16] and Roberts *et al.* [17]. Aliquots of ten microliters of the extracts of the extracts were applied on silica gel chromatography plates, using elution systems and suitable developers to investigate the presence of saponins, flavonoids, cinnamic derivatives, phenylpropanoids, triterpenoids, steroids, mono- and sesquiterpenes, alkaloids, proanthocyanidins and leucoanthocyanidins, coumarins, and quinones.

2.4. Determination of Total Phenolic Content

Total phenolic content was determined by the Folin-Ciocalteu method to Li *et al.* [18] Two hundred microliters of diluted sample were added to 1 mL of 1:10 diluted Folin-Ciocalteu reagent. After 4 min, 800 mL of saturated sodium carbonate solution (75 g/L) was added. After 2 h of incubation at room temperature, protected from light, the absorbance at 765 nm was measured in triplicate. Gallic acid (0 - 500 mg/L) was used for calibration of standard curve. The results were expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of plant extract.

2.5. Determination of Flavonoids

The determination of flavonoids follows the methodology proposed by Woisky and Salatino [19] to 0.5 mL of

diluted samples, was added 0.5 mL of 2% $AlCl_3$ (w/v) solution prepared in methanol. After 30 minutes of incubation at room temperature, protected from light, the absorbance at 420 nm was measured in triplicate. The results were expressed as milligram quercetin equivalent (mg QE)/g dry weight of plant extract.

2.6. Antioxidant Activity Using 2,2-Azino-Bis-(3 Ethylbenzothiazoline)-6-Sulfonic Acid (ABTS)

According to Silva *et al.* [20], the ABTS assay is based on the generation of chromophore cationic radical obtained from the oxidation of ABTS by potassium persulfate. The oxidation reaction was prepared with 7 mM ABTS stock solution plus 140 mM potassium persulfate (final concentration) and the mixture was left in the dark at room temperature (23˚C - 25˚C) for 12 - 16 h (time required for radical formation) before its use. The ABTS + solution was diluted in ethanol to an absorbance of 0.7 (±0.02) units at 734 nm. The effect of extract amount on the antioxidant activity was carried out using aliquots of 30 µL, and mixing with 3 mL diluted ABTS + solution. The absorbances at 734 nm were measured at different time intervals (6, 15, 30, 45, 60 and 120 min). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a reference standard. The values of oxidative inhibition percentage were calculated and plotted as a function of the reference antioxidant concentration (Trolox) and expressed as Trolox equivalent antioxidant capacity (TEAC, µM). All determinations were carried out in triplicate.

2.7. DPPH Radical Scavenging Assay

The DPPH free radical scavenging activity of the extracts was performed according to Brand-Williams *et al.* [21] with some modifications. A methanolic DPPH stock solution (200 µM) was further diluted in methanol to obtain a UV-VIS absorbance between 0.6 - 0.7 at 517 nm, obtaining the DPPH working solution. Different concentrations of the extracts (40 µL) were mixed with DPPH solution (250 µL) and after 30 min incubation in darkness the absorbances were read at the same wavelength mentioned above. The measurements were triplicated and their scavenging activities were calculated based on the percentage of DPPH scavenged.

2.8. Total Antioxidant Capacity by Phosphomolybdenum Assay

According to Pietro *et al.* [22], the total antioxidant capacity (% TAC) was evaluated by phosphomolybdenum assay. An aliquot of 0.1 mL of sample solution (100 µg/mL) was combined with 1 mL of reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 90˚C for 90 min. Afterward, the absorbance was measured at 695 nm against a blank (1 mL of reagent and 0.1 mL of solvent). Total antioxidant activity was expressed in relation to ascorbica-cid.

2.9. Statistical Analysis

Pearson correlation analysis was performed using a Statistical (Statistical Statsoft, Tulsa) software; *P*-values < 0.05 were considered significant.

3. Results and Discussion

The phytochemical screening was performed to identify the classes of chemical compounds present in the extracts. Other studies have already demonstrated the antioxidant activity of sterols, terpenoids, oils, flavonoids, alkaloids and other phenolic compounds [23] [24].

The preliminary phytochemical analysis for the *S. tuberosa* leaves (**Table 1**) revealed the presence of high levels of flavonoids, triterpenes. The phytochemical profile results showed that the plant extract has molecules with high technological potential for the development of new drugs with application in the treatment and prevention of various diseases.

In the determination of total phenolics and flavonoids, the results showed that the methanol solvent was better than ethyl acetate to extract phenolic compounds (**Table 2**) which may be explained by its good polarity and solubility for phenolic compounds extracted from plants [25] [26].

The lower polarity solvent, the ethyl acetate, showed much lower capacity for extracting phenolic compounds,

Table 1. Phytochemical profile of the methanolic and ethyl acetate extracts of the *S. tuberosa* leaves.

Secondary metabolites	Extract			
	Standards	Elution system	Ethyl acetate	Methanol
Flavonoids	Quercetin and rutin	A	+++	+++
Cinnamic derivatives	Chlorogenic acid	A	+	+
Triterpenes and steroids	*β*-sitosterol	B	+++	+++
Mono and sesquiterpenes	Thymol	C	−	−
Alkaloids	Pilocarpine	A	−	−
Coumarins	Coumarin	D	−	−
Condensed proanthocyanidins and leucoanthocyanidins	Catechin	A	−	−

A—AcOEt-HCOOH-AcOH-H_2O (100:11:11:27 v/v); B—Toluene:AcOEt (90:10 v/v); C—Toluene:AcOEt (97:03 v/v); D—$CHCl_3$-MeOH (98:2 v/v). + = Presence and − = Absence; + = low, ++ = intermediate, +++ = high.

Table 2. Total phenolic and flavonoids compounds quantification from *S. tuberosa* leaves extracts. Media ± SD (n = 3).

Sample	Total phenolic content (mgGAE/g)	Flavonoid content (mgQE/g)
Ethyl acetate extracts	75.69 ± 0.73	10.24 ± 0.66
Methanolic extracts	100.07 ± 0.02	15.74 ± 0.04

GAE: gallic acid equivalent. QE: quercetin equivalent.

compared with the more polar solvent. Numerous studies have shown a strong relationship between total phenolic content and antioxidant activity in fruits, vegetables and medicinal plants [27]. Phenolic compounds have been reported to have multiple biological effects including antioxidant activity. In addition, they can act in the free radical elimination or prevent its formation [28].

It has been reported that most of the antioxidant activity may be associated with phytochemicals such as flavonoids, isoflavones, anthocyanins, flavones, catechins and other phenolic compounds [29].

There are several methods to determine the antioxidant capacity of phytochemical compounds. The methods used in this study to determine the antioxidant capacity of the ethyl acetate and methanolic extract were ABTS, DPPH and phosphomolybdenum assay. Antioxidants act in many of biological responses as inflammation and immunity, they function as signaling mechanisms for redox regulation. Even at minimal levels of oxidative stress, they are strongly detected and then the protective antioxidant mechanism is put into action, which is essential for maintaining the structural integrity of proteins. Recently, particular attention has been made on the antioxidant properties of plants derived from dietary food constituents [29].

The ABTS assay results were shown in **Figure 1**. All extracts (1 mg/ml) of *S. tuberosa* had antioxidant activity as a function of the time according to the Pearson correlation coefficient ($r = 0.958$, $p < 0.05$), which means that the beneficial antioxidant effect of the extracts improves significantly over time.

The methanol leaves extract had the highest antioxidant capacity with oxidation inhibition rate of 70.25% ± 0.49% after 120 min equivalent to TEAC of 1489.99 ± 12.02 µM of Trolox, while the ethyl acetate extract showed 58.724% ± 0.93% inhibition with TEAC of 1211.11 ± 22.68 µM of Trolox after 120 min.

The assay of the DPPH radical elimination expressed as a percentage of the radical reduction was presented in **Table 3**. The radical elimination activity was detected only in the methanolic leaves extract (68.12% ± 2.67%). The percentage of radical reduction for the ethyl acetate extract cannot be calculated due to the formation of precipitates in the sample in contact with the DPPH radical. According to the Pearson correlation coefficient, there is a positive correlation between the methanol leaves extract concentration and the free radicals elimination ($r = 0.967$, $p < 0.05$).

It was observed positive results in the ABTS and DPPH test sample, indicating that the extracts had comparable activities in both assays. However, only the methanolic leaves extract showed antioxidant activity in both assays (ABTS and DPPH).

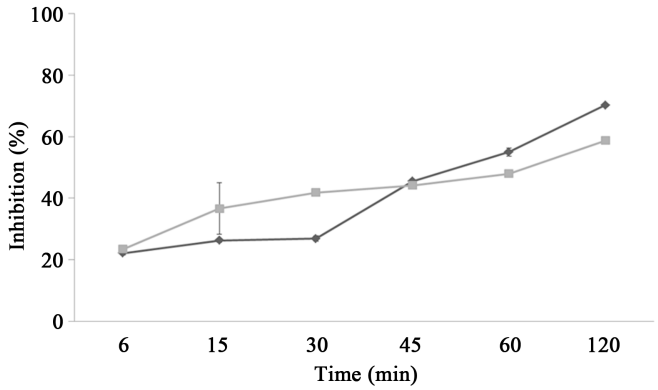

Figure 1. Effect of the incubation time on antioxidant activity of *S. tuberosa* leaves extracts in ABTS assay. Ethyl acetate ( ), Methanol extract ( ).

Table 3. Antioxidant activity of methanolic extract of *S. tuberosa* leaves in different concentrations. Gallic acid was used as standard. Mean ± SD (n = 3).

Extract Concentration (µg/mL)	DPPH RSA%
1000	68.12 ± 2.67
500	49.66 ± 1.66
250	35.29 ± 1.17
125	18.41 ± 1.40
62.5	18.03 ± 0.79
31.25	9.47 ± 0.36

RSA %: Percentage of DPPH radical reduction activity after 30 min.

Regarding the applicability of each assay reported by our results, DPPH is a free radical that is obtained directly without preparation (ready to dissolve), while the ABTS is a cation (ABTS • +), which should be generated by enzymatic activity (peroxidase and myoglobin) or chemical (manganese dioxide and potassium persulfate) reactions [30] [31].

The total antioxidant activity (CAT) assay performed by phosphomolybdenum, which molybdenum ion reduction capability of *S. tuberosa* extracts indicating that both extracts (methanolic and ethyl acetate) leaves were antioxidants. However, differences were observed in the antioxidant activity between these two types of extracts. The methanolic extract showed better activity (31.02% ± 0.01 TAC) than the ethyl acetate extract (22.58% ± 0.03% TAC) using ascorbic acid as standard. Likewise the other methods, the methanol was the most effective solvent for extracting the antioxidant with potential secondary metabolites from *S. tuberosa* leaves in comparison with ethyl acetate.

According to the results, we note that there was a significant difference between the results of phosphomolybdenum and ABTS/DPPH assays, which can be explained by the fact that the hydrogen transfer electrons from antioxidant varies with its chemical structure [32]. In addition, non-phenolic compounds such as tocopherols and ascorbic acid may also act as reducer, thus cannot be observed a positive relationship between phenolic content and phosphomolybdenum reduction activity [33]. Other compounds, such as carotenoids, which were not measured in this study, can be present in the extract and could contribute to antioxidant activity in the samples.

4. Conclusion

These results showed that leaves extracts of *S. tuberosa* possess antioxidant activity in all methods analyzed. The use of methanol was an efficient method of extraction of secondary metabolites with antioxidant activity compared to the use of ethyl acetate. The antioxidant properties of the secondary metabolites of the leaves ex-

tract of this plant may represent a potential source of components that could improve the health, being applied as functional foods or incorporated biomolecules into pharmaceutical or nutraceutical preparations.

Acknowledgements

The authors acknowledge the support given by the laboratório de produtos Naturais and Laboratório de Biologia Molecular, Departamento de Bioquímica of the Universidade Federal de Pernambuco (UFPE), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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