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Phytochemical screening, *in vitro* antioxidant, photoprotective and hemolytic activities of ethyl acetate extracts of the fruits and branches from *Spondias tuberosa* (umbu)

Triagem fitoquímica, atividades antioxidante, fotoprotetora e hemolítica *in vitro* dos extratos de acetato de etila dos frutos e ramos de *Spondias tuberosa* (umbu)

Cribado fitoquímico, actividades antioxidante, fotoprotectora y hemolítica *in vitro* de extractos de acetato de etilo de frutos y ramas de *Spondias tuberosa* (umbu)

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Amanda Dias de Araújo
ORCID: https://orcid.org/0000-0002-5899-9617
Universidade Federal de Pernambuco, Brazil
E-mail: amandabiologa1@gmail.com
Fernanda Granja da Silva Oliveira
ORCID: https://orcid.org/0000-0002-6924-2740
Universidade Federal do Vale do São Francisco, Brazil
E-mail: fernanda.gso@hotmail.com
Francinete Francis Lacerda
ORCID: https://orcid.org/0000-0002-7130-0778
Instituto Agronômico de Pernambuco, Brazil
E-mail: cavalcanti.fran@hotmail.com
Carlos Eduardo Sales da Silva
ORCID: https://orcid.org/0000-0002-9037-0827
Universidade Federal de Pernambuco, Brazil
E-mail: carlossalles10@yahoo.com.br
Bruno Souza dos Santos
ORCID: https://orcid.org/0000-0003-4644-0047
Universidade Federal de Pernambuco, Brazil
E-mail: souza.farmacia@hotmail.com
Clovis Macêdo Bezerra Filho
ORCID: https://orcid.org/0000-0003-0826-7976
Universidade Federal de Pernambuco, Brazil
E-mail: clovisfilho@gmail.com
Bárbara de Azevedo Ramos
ORCID: https://orcid.org/0000-0002-1166-8183
Universidade Federal de Pernambuco, Brazil
E-mail: barbara.a.ramos@hotmail.com
Wolfgang Harand
ORCID: https://orcid.org/0000-0001-8745-7190
Instituto Nacional do Semiárido, Brazil
E-mail: wolfgang.harand@insa.gov.br
Nicácio Henrique da Silva
ORCID: https://orcid.org/0000-0002-8562-3850
Universidade Federal de Pernambuco, Brazil
E-mail: nicacio_hsilva@hotmail.com
Patrícia Maria Guedes Paiva
ORCID: https://orcid.org/0000-0003-3467-708X
Universidade Federal de Pernambuco, Brazil
E-mail: ppaiva63@yahoo.com.br
Jackson Roberto Guedes da Silva Almeida
ORCID: https://orcid.org/0000-0002-0867-1357
Universidade Federal do Vale do São Francisco, Brazil
E-mail: jackson.guedes@univasf.edu.br
Márcia Vanusa da Silva
ORCID: https://orcid.org/0000-0002-2221-5059
Universidade Federal de Pernambuco, Brazil
E-mail: marciavanusa@yahoo.com.br
Maria Tereza dos Santos Correia
ORCID: https://orcid.org/0000-0003-4920-9975
Universidade Federal de Pernambuco, Brazil
E-mail: mtscorreia@gmail.com

Abstract
Spondias tuberosa is a medicinal plant used to treat inflammatory conditions and digestive disorders. The phytochemical screening, antioxidant, photoprotective and hemoytic activities of ethyl acetate extracts from the fruits and branches were investigated. Antioxidant activities were evaluated using DPPH, ABTS and phosphomolybdenum assays. The photoprotective and hemolytic activities was evaluated by the spectrophotometric method. Identification of compounds classes from the ethyl acetate branches extract was performed by HPLC. TLC analysis demonstrated presence of flavonoids, cinnamic derivatives, triterpenes and steroids in both extracts, and proanthocyanidins and leucoanthocyanidins only in branches, and it was identified by HPLC gallic acid, chlorogenic acid, caffeic acid, ferulic acid in the ethyl acetate extract of branches. Ethyl acetate branches extracts showed the best results: total phenolic compounds (83.88 ± 0.2 mg GAE/g), flavonoids (11.24 ± 2.0 mg QE/g), DPPH (88.80 ± 0.4 %), ABTS (68.92 ± 0.7%), phosphomolybdenum (27.94 ± 0.26%) and sun protection factor (SPF) with 15.50 ± 0.41. In addition, both extracts did not present hemolytic activity. Therefore, the branches extracts can be used in the production of photoprotective application with phytocosmetics. This work is the first report of photoprotective activity of *Spondias tuberosa* (umbu).
Keywords: *Spondias tuberosa*; Umbu; Photoprotection; Hemolytic activity; Antioxidants.

Resumo
Spondias tuberosa é uma planta medicinal usada para tratar doenças inflamatórias e distúrbios digestivos. Foram investigadas as atividades fitoquímica, antioxidante, fotoprotetora e hemótica dos extratos de acetato de etila dos frutos e ramos. As atividades antioxidantes foram avaliadas pelos ensaios de DPPH, ABTS e fosfomolibdênio. As atividades fotoprotetora e hemolítica foram avaliadas pelo método espectrofotométrico. A identificação das classes de compostos do extrato dos ramos de acetato de etila foi realizada por HPLC. A análise por TLC demonstrou a presença de flavonóides, derivados cinâmicos, triterpenos e esteróides em ambos os extratos, e proantocianidinas e leucoantocianidinas apenas em ramos, e foi identificada por HPLC ácido gálico, ácido clorogênico, ácido caféico, ácido ferúlico no extrato de acetato de etila dos ramos. Extratos de ramos de acetato de etila apresentaram os melhores resultados: compostos fenólicos totais (83,88 ± 0,2 mg GAE/g), flavonóides (11,24 ± 2,0 mg QE/g), DPPH (88,80 ± 0,4%), ABTS (68,92 ± 0,7%), fosfomolibdênio (27,94 ± 0,26%) e fator de proteção solar (FPS) com 15,50 ± 0,41. Além disso, ambos os extratos não apresentaram atividade hemolítica. Portanto, os extratos dos ramos podem ser utilizados na produção de aplicações fotoprotetoras com fitocosméticos. Este trabalho é o primeiro relato da atividade fotoprotetora de *Spondias tuberosa* (umbu).
Palavras-chave: *Spondias tuberosa*; Umbu; Fotoproteção; Atividade hemolítica; Antioxidantes.

Resumen
Spondias tuberosa es una planta medicinal que se usa para tratar afecciones inflamatorias y trastornos digestivos. Se investigó el cribado fitoquímico, antioxidante, fotoprotector y hemoítico de extractos de acetato de etilo de frutos y ramas. Las actividades antioxidantes se evaluaron mediante ensayos de DPPH, ABTS y fosfomolibdeno. La actividad fotoprotectora y hemolítica se evaluó mediante el método espectrofotométrico. La identificación de clases de compuestos a partir del extracto de ramas de acetato de etilo se realizó mediante HPLC. El análisis de TLC demostró presencia de flavonoides, derivados cinámicos, triterpenos y esteroides en ambos extractos y proantocianidinas y leucoantocianidinas solo en ramas, y se identificó por HPLC ácido gálico, ácido clorogénico, ácido cafeico, ácido ferúlico en el extracto de acetato de etilo de ramas. Los extractos de ramas de acetato de etilo mostraron los mejores resultados: compuestos fenólicos totales (83,88 ± 0,2 mg GAE/g), flavonoides (11,24 ± 2,0 mg QE/g), DPPH (88,80 ± 0,4%), ABTS (68,92 ± 0,7%), fosfomolibdeno (27,94 ± 0,26%) y factor de protección solar (SPF) con 15,50 ± 0,41. Además, ambos extractos no presentaron actividad hemolítica. Por tanto, los extractos de ramas se pueden utilizar en la producción de aplicaciones fotoprotectoras con fitocosméticos. Este trabajo es el primer reporte de actividad fotoprotectora de *Spondias tuberosa* (umbu).
Palabras clave: *Spondias tuberosa*; Umbu; Fotoprotección; Actividad hemolítica; Antioxidantes.

1. Introduction

Spondias tuberosa (Anacardiaceae) is a endemic and native Caatinga specie. The plant species is popularly known as "umbuzeiro" (Omena et al., 2012). Several plant parts are largely used in folk medicine (branches, stem bark, fruits, roots, resin and leaves) in the treatment of various pathologies: inflammation, infections, venereal diseases, digestive disorders, diarrhea and also antiemetic and tonic activity (Siqueira et al., 2016)

Oxidative stress responses caused by ultraviolet radiation (UV) can induce a several harmful effects on the skin, such as induction of immunosuppression, skin carcinogenesis and premature photoaging (Vilela et al., 2011). Plants are known for

produce various antioxidants against molecular damage caused by ROS. Phenolics compounds are the main antioxidants obtained from plants (Neto et al., 2016), encompassing the flavonoids.

Flavonoids absorb electromagnetic radiation in the ultraviolet range (UV) and visible and thus have a defensive role in plants against to UV radiation from sunlight (Cooper-Driver, 2001). The flavonoids eliminate the radicals induced by UV radiation, moreover, might provide protective effect against UV radiation by as strong screens UV absorbing (Silva Almeida & Oliveira-Júnior et al., 2013). The UV radiation spectrum is subdivided into three bands, according to the wavelength can be classified: UVA, UVB and UVC. The UVA radiation present the longest wavelengths (320-400 nm), and it is characterized as inducer of oxidative processes in the skin (Popim et al., 2008).

For these reasons, the use of sunscreen products has been stimulated for the prevention of severe skin diseases. Nowadays, one of the trends of cosmetic market is the search for products from natural resources (Oliveira-Júnior et al., 2013). Several plant extracts and oils have been used in cosmetic products such as sunscreen because of the photoprotective action (Deuschle et al., 2015). In this context, the aim of this work was to evaluate the antioxidant, photoprotective and hemolytic potential of ethyl acetate extracts of the fruits and branches of the *S. tuberosa*.

2. Methodology

The present work is an applied, experimental and quantitative research, involving several stages described below.

Plant material

Fruits without stones and branches were collected in Catimbau National Park in Pernambuco, in the Northeast of Brazil, in April 2015 (collection authorization: nº 26743-3 ICMBio). The plant material were identified and authenticated at the Herbarium of the Agronomic Institute of Pernambuco (IPA), with the voucher number 91090.

Extraction

The plant material (fruits and branches) were dried at 45˚C and were processedin mill (Willye® mill/ET-650) to form the powder. The mechanical Accelerated Solvent Extraction (ASE 350 Dionex®) was used to obtain the extracts. It's was used 20g of fruits and the branches powder were placed to the cells of the Accelerated Solvent Extractor, concentrated under a nitrogen stream in a heating block at 60˚C and extracted with ethyl acetate, right after were transferred for a rotary evaporator and dried at 50˚C.

Determination of Total Phenolics content

Total Phenols was determined by Folin-Ciocalteu method (Li et al., 2008). The 200 µL of diluted sample were mixed to 1000 mL of Folin-Ciocalteu reagent (1:10) passing 4 minutes was added 0.8 mL of saturated sodium carbonate solution (75 g/L). After 120 minutes at room temperature, protected from light, absorbance was measured at 765 nm. The Gallic acid (50 - 500 mg/L) was used for calibration of standard curve. Results were expressed as milligram of gallic acid equivalent (mg GAE)/g of dry weight of extract the plant.

Quantification of the Flavonoids

Flavonoid contents were measured by aluminum chloride colorimetric method (Woisky and Salatino, 1998). Five hundred microliters of diluted sample were added to 500 µL of Aluminum chloride (Prepared in 2% methanol). After 30 min protected from light at room temperature, the absorbance was measured at 420 nm. The quercetin was used as standard (curve: 5-35 mg/mL) and the results were expressed in mgQE (milligram quercetin equivalent)/g dry weight of extract the plant.

Phytochemical Analysis

The phytochemical screening was performed by TLC (thin layer chromatography) (Harborne et al., 1998; Roberts et al., 1957) were investigated: flavonoids, saponins, cinnamic derivatives, phenylpropanoids, triterpenoids, steroids, mono- and sesquiterpenes, alkaloids, coumarins, proanthocyanidins and leucoanthocyanidins, and quinones, were utilized systems and revealers adequate for each classes of compounds.

HPLC Analysis

The High performance liquid chromatography carried in HPLC-DAD Agilent® 1200 using a C18 column C18 Zorbax® SB (250 x 4,6 mm, 5 µm) at 30 ± 0.8°C. HPLC analysis were carried out using a mobile phase consisting 0.3% acetic acid (A) and acetonitrile (B) was applied for a total time of 20 min. Gradient: 0 min: 92 (A) % and 8 % (B); 15 min: 65% (A) and 35 % (B); 17 – 20 min: 92% (A) and 8 % (B). The flow rate constant: 2.4 mL / min. and the detection carried out at 256 and 360 nm to acquire UV spectra.

ABTS Assay

The ABTS ((2,2-Azino-Bis-(3 Ethylbenzothiazoline)-6-Sulfonic Acid; Sigma-Aldrich) free radical scavenging activity of the extracts was performed (Uchôa et al., 2015). The was prepared an 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 for radical formation. The ABTS+ solution was diluted in ethanol to an absorbance of 0.7 (±0.02) at 734 nm. Antioxidant activity from extract was carried out using aliquots of 30 µL the extracts (1mg/ml) mixing with 3 mL diluted ABTS+ solution. The samples were measured at different time intervals (6, 15, 30, 45, 60 and 120 min) at 734 nm. The percentage oxidation inhibition were calculated and plotted as a function of the reference standard antioxidant concentration (Trolox -(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich, St. Louis, MO, USA) and expressed in Trolox equivalent antioxidant capacity (TEAC, µM).

DPPH assay

Antioxidant activity using DPPH method (Brand-Williams et al. 1995) was performed with some modifications. The was prepared the solution of DPPH 200 µM in methanol to obtain an absorbance of 0.6 - 0.7 at 517 nm. Concentrations of the extracts (1000 µg/mL – 31.25 µg/mL) were mixed with DPPH solution, after 30 min incubation in darkness, were read the absorbance (517 nm). Gallic acid was used as standard. The methanol was used as control. The antioxidant activities were calculated based on the percentage of DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich) radical scavenging using the formula:

$$\%\,\mathbf{DPPH} = \frac{(\mathbf{Ac}-\mathbf{As})}{\mathbf{Ac}}\ x\ 100$$

Ac: Control absorbance.

As: Sample absorbance.

Phosphomolybdenum Assay

Total antioxidant activity (% TAA), was evaluated by phosphomolybdenum method (Prieto et al., 1999). Was used of 0.1 mL of extract solution (100 µg/mL) mixed with 1 mL of reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were carried the boiling water bath at 90˚C for 90 min. Afterward, the absorbance was measured at 695 nm against a control: 1 mL of reagent and 0.1 mL of solvent. The %TAA was expressed in relation to ascorbic acid and calculated by the following formula:

$$\% \, \mathbf{TAA} = \frac{(\mathbf{As} - \mathbf{Ac})}{\mathbf{Aaa} - \mathbf{Ac}} \, x \, 100$$

Ac: absorbance of the control.

As: absorbance of extract.

Aaa: absorbance of ascorbic acid.

Hemolytic Assay

Erythrocytes from citrated blood were isolated by centrifugation (1500 rpm for 10 min at temperature: 4°C). Afterward removal of plasma and buffy coat, the erythrocytes were washed with phosphate-buffered saline (PBS; pH 7.4) three times and was resuspended using the same buffer and a 1% erythrocyte suspension was prepared. The tubes received: 1.1 mL of erythrocyte suspension and 0.4 mL of extract at various concentrations (31.25–1000 µg/mL). The solvent (saline solution 0.9%) was used with negative control and the positive control received 0.4 mL of Triton X-100. Afterward 60-min incubation at room temperature, cells were centrifuged and the supernatant was used to measure the absorbance (540 nm) of the released hemoglobin (De-Qiang et al., 2011). The results was calculated from triplicate assays. The relative hemolytic activity was expressed in relation to Triton X-100 and calculated by the formula:

$$\mathbf{Relative\ hemolytic\ activity}\ (\%) = \frac{(\mathbf{As} - \mathbf{Ab})\boldsymbol{x}\ \mathbf{100}}{\mathbf{Ac} - \mathbf{Ab}}$$

Ab: absorbance of the negative control.

As: absorbance in the assay with extract.

Ac: absorbance in the assay with Triton X-100. Hemolysis concentration was calculated.

FPS Determination: Sun Protection Factor *in vitro*

The photoprotective activity was evaluated by spectrophotometric method of diluted solutions (Mansur et al., 1986). The extracts were previously dried on oven at 40 ºC for 60 minutes. Dilutions were prepared in the concentrations (5, 25, 50 and 100 $mg.L^{-1}$). Scans from 260 to 400 nm with intervals of 5 nm were realized. A spectrophotometer was used, with quartz cuvettes with 1 cm optical path for the acquisition of the spectra. Calculations of the Sun Protection Factor (SPF) were made considering the intervals λ determined.

$$\mathbf{SPF} \ = \mathbf{FC} \, \mathbf{x} \, \Sigma_{320}^{290} \, \boldsymbol{x} \, \boldsymbol{EE}(\boldsymbol{\lambda}) \boldsymbol{x} \, (\boldsymbol{abs} \, (\boldsymbol{\lambda})$$

Where FC = Correction factor; = sum of the absorption from 290 to 320 nm, EE = eritemogenic effect; abs = absorption. The values of EE used to the SPF calculation were the same found in the literature.

Statistical analysis

The determinations were performed in triplicates. The data obtained were expressed as mean ± SD. The concentration needed for 50% of hemolysis was calculated by linear regression analysis.

3. Results and Discussion

The phytochemical analysis demonstrated that both extracts were positive for the presence of flavonoids, cinnamic derivatives, triterpenes and steroids and positive for proanthocyanidins and leucoanthocyanidins only to extract the branches (Table 1). The main substances found in the extracts studied possess a great antioxidant potential (Simic et al., 1994; Ramalho et al., 2006).

Table 1. Phytochemical profile of the ethyl acetate fruits and branches extracts of the *S. tuberosa*.

Secondary metabolites	Standards	Elution system	Extract	
			Fruits	Branches
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-H2O (100:11:11:27 v/v); B: Toluene:AcOEt (90:10 v/v); C: Toluene:AcOEt (97:03 v/v); D: CHCl3-MeOH (98:2 v/v). (+) = Presence and (-) = Absence; (+) = low, (++) = intermediate, (+++) = high. Source: Authors (2020).

The Table 2 demonstrates the results from the quantitative determination of phenolic and flavonoids and effect of extracts in radical scavenging the DPPH, ABTS and phosphomolybdenum.

Table 2. Total phenolic content (TPC), total flavonoid (TF) DPPH, ABTS and TAA of extracts from *Spondias tuberosa*. Mean ± SD (n = 3).

Sample	TPC (mgGAE/g)	TF (mg QE/g)	DPPH %	ABTS %	TAA %
Fruits	43.67 ± 1.3	6.58 ± 0.2	2.01 ± 1.6	55.14 ± 3.0	21.26 ± 1.3
Branches	83.88 ± 0.2	11.24 ± 2.0	88.80 ± 0.4	68.92 0.7	27.94 ± 0.26

Values are given as mean ± SD (n=3). Source: Authors (2020).

In agreement Table 2, significant contents of phenolic compounds and flavonoids were found. These are major classes of secondary metabolites found in the genus *Spondias* (Silva et al., 2014).

Flavonoids are important bioactive compounds from plants biosynthesized from the phenylpropanoids way (Machado et al., 2008). This group of secondary metabolites can be highlighted with a high therapeutic potential, such as antioxidant, anti-inflammatory and inhibiting unregulated cell proliferation (Machado et al., 2008), demonstrating the importance of this class of substance found in the extracts of *S. tuberosa*.

The hemolytic assay showed that extracts did not exhibit hemolytic activity since the extract of the fruits and branches have maximum degrees of hemolysis of 3.127% and 0.043%, respectively, at a concentration of 1000 µg/mL. Hemolysis refers to the lysis or rupture of red blood cell membranes allowing the release of hemoglobin into plasma. The absence of hemolytic activity in human erythrocytes from processed extracts suggest that in the analyzed concentrations they did not have the lysis capacity of the red blood cells.

The SPF values were calculated. The fruits extract showed FPS: 0.83 ± 0.11, values obtained were not significant, according to the National Health Surveillance Agency (ANVISA, 2012), which indicates 6.0 for the minimum value for SPF for sunscreen products. On the other hand, the SPF values obtained with the branches extract was 15.50 ± 0.41, showed values above the indicated in the National Health Surveillance Agency (ANVISA, 2012), demonstrating the biotechnological potential of this species.

These results are related to the content of total phenolic compounds and antioxidant activity. Other studies have shown that plants that absorb in the ultraviolet region have in their composition metabolites such as flavonoids, tannins, anthraquinones, alkaloids and polyphenols (Violante et al., 2009). The ethyl acetate extract of the branches posses in its composition great amounts of phenolic compounds. According to Costa et al., 2015 the presence of the metabolite indicates a potential absorption of UV radiation.

The heightened production of ROS due to UV radiation results in oxidative damage to macromolecules, proteins and lipids (Sander et al., 2010), activating or inactivating enzymes and changing signaling cascades. An important point to prevent skin damage induced by UV radiation is the application of compounds with antioxidant activity. Among these are compounds include those derived from plant extracts. The combination of polyphenols in sunscreens and in products for skin care could provide an effective strategy to reduce these harmful effects and thus help prevent skin cancer and photoaging due to exposure to sunlight (Nichols et al., 2010; Heo et al., 2001).

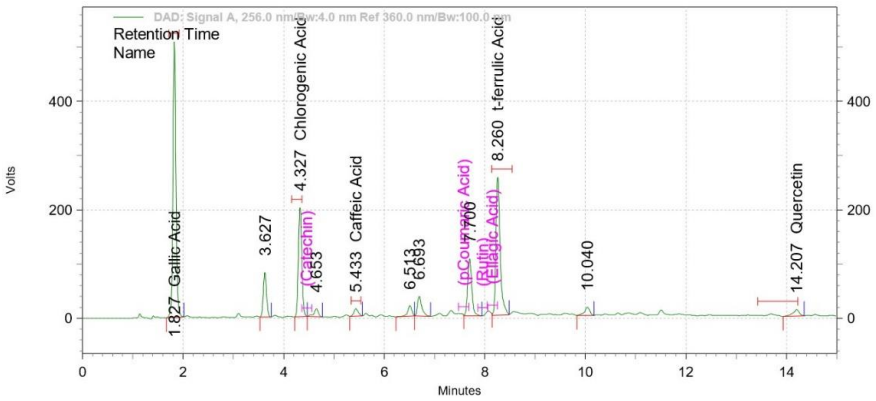
Antioxidants act by different mechanisms sunscreens, providing protection to the skin through the cellular response modulation, and combination of these would provide better sunscreen effect to the skin (Murray et al., 2008). Ethyl acetate branches extract showed better results for this reason, HPLC was used to identify compounds classes. The quantification of the phenolic compounds from the chromatographic profile (fingerprint) obtained by HPLC-DAD is shown in the table and figure below (Table 3 and Figure 1).

Table 3. Major phenolic compounds identified in ethyl acetate extract of *S. tuberosa* by HPLC.

Compounds	Retention time (min)	Content (µg/mg)
Gallic acid	1.81	19.4
Chlorogenic acid	4.24	106.2
Caffeic acid	5.44	2.36
Ferulic acid-t	8.36	6.88
Ellagic acid	8.42	1.5

Source: Authors (2020).

Figure 1. HPLC analysis in branches extracts using a mobile phase consisting 0.3% acetic acid (A) and acetonitrile (B) applied for a total running time of 20 min. Gradient: 0 min: 92 (A) % and 8 % (B); 15 min: 65% (A) and 35 % (B); 17 – 20 min: 92% (A) and 8 % (B), which considerably improved the separation between the peaks.



Source: Authors (2020).

The HPLC analysis the demonstrates important bioactive substances present in the ethyl acetate extract of branches of the *S. tuberosa*. Mainly constituted by phenolic compounds, the extract demonstrated important results for the antioxidant and photoprotective evaluation. Several searches have shown that polyphenolic compounds extracted are effective antioxidants *in vitro* (Kähkönen et al., 1999; Rice-Evans et al., 1997).

Chlorogenic acid, the major compound among the phenolics analyzed in this study, showed anti-diabetic and anti-lipidemic effects (Ong et al., 2013; Meng et al., 2013), corroborating the traditional use of this plant. Showed that chlorogenic acid decreased the formation of ROS and increased glutathione levels in hepatoma cells (Granado-Serrano et al., 2007). Murine keratinocytes when exposed to UVB radiation and treated with chlorogenic acid present reduction of AP-1 and NF-kB and MAPK phosphorylation (JNK, p38, ERK, MKK4) (Feng et al., 2005).

4. Conclusion

The results indicates that *S. tuberosa* possess important phenolic compounds which can serve as antioxidants and photoprotective agents. Phenolic compounds present in extracts could be responsible by photoprotective effect presented in this study, mainly for the ethyl acetate branches extract that showed better results compared to ethyl acetate fruits extracts,

moreover showed no hemolytic activity. These data indicate the great biochemical and biotechnological potential of *S. tuberosa* and can aggregate chemical and medicinal value to a Caatinga native species. Further investigation of this species will be realized to reach the substance responsible for antioxidant and photoprotective activities from extracts with possible future development of pharmaceuticals and cosmetics products.

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