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IDENTIFICATION AND QUANTIFICATION OF PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY IN COWPEAS OF BRS XIQUEXIQUE CULTIVAR[1]

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ABSTRACT - Cowpea (*Vigna unguiculata* [L.] Walp.) is one of the most important legumes produced in tropical and subtropical regions throughout the world, especially in the developing countries in Africa, Latin America, and Asia. It is the main source of protein, calories, dietary fiber, minerals, and vitamins for a large segment of the world population. Cowpea is also a potential functional food with a range of bioactive compounds, including phenolic compounds. This legume is grown mainly in the North and Northeast regions of Brazil, but is also consumed in other regions, and is thus important for the farmers who depend on this crop for income. This study identified and quantified phenolic compounds in the cowpea cultivar BRS Xiquexique. Such quantification reveals the functional characteristics of cowpeas, mainly as a source of antioxidants, which will be essential to add value to this food and to expand its forms of consumption. The extracts were analyzed using an HPLC model LC-20AT, equipped with a manual injector. For the HPLC analysis, standard solutions were prepared with pure phenolic acids such as gallic acid, quercetin, caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid, catechin, and epicatechin. The major phenolic compounds identified were catechin (2.07± 0.329 mg 100 g^{-1}), epicatechin (0.48 ± 0.130 mg 100 g^{-1}), gallic acid (67.19 ± 6.200 mg 100 g^{-1}), ferulic acid (32.07 ± 0.753 mg 100 g^{-1}), and chlorogenic acid (3.08 ± 0.489 mg 100 g^{-1}). We observed that the BRS Xiquexique cultivar contains functional phenolic compounds, especially gallic acid and ferulic acid, demonstrating the antioxidant potential of cowpea.

Keywords: *Vigna unguiculata*. Bioactive. Chemical composition.

IDENTIFICAÇÃO E QUANTIFICAÇÃO DE COMPOSTOS FENÓLICOS E DA ATIVIDADE ANTIOXIDANTE NO FEIJÃO-CAUPI EM GRÃOS DA CULTIVAR BRS XIQUEXIQUE

RESUMO - O feijão-caupi (*Vigna unguiculata* [L.] Walp.) é uma das mais importantes leguminosas produzidas em regiões tropicais e subtropicais do mundo, principalmente nos países em desenvolvimento da África, América Latina e Ásia. Para um grande segmento da população mundial é a principal fonte de proteínas, calorias, fibras alimentares, minerais e vitaminas. Possui ainda compostos bioativos, destacando-se os compostos fenólicos. É produzido no Norte e Nordeste, consumido também em outras regiões do país e apresenta grande importância para os agricultores que sobrevivem desta cultura. O objetivo do presente estudo foi identificar e quantificar os compostos fenólicos no feijão-caupi, cultivar BRS Xiquexique. Tal determinação permitirá conhecer características funcionais do feijão-caupi, como fonte de antioxidantes, o que será de primordial importância para agregar valor funcional e nutricional a este alimento e ampliar as formas de consumo. Os extratos foram analisados por CLAE em equipamento modelo LC-20AT, com injetor manual. Para as análises foram preparadas soluções padrões com os ácidos fenólicos puros, tais como: ácido gálico, quercetina, ácido cafeico, ácido clorogênico, ácido ferúlico, ácido p-cumárico, catequina e epicatequina. Foram identificados catequina (2,07 ± 0,329 mg 100 g^{-1}), epicatequina (0,48 ± 0,130 mg 100 g^{-1}), ácido gálico (67,19 ± 6,200 mg 100 g^{-1}), ácido ferúlico (32,07 ± 0,753 mg 100 g^{-1}) e ácido clorogênico (3,08 ± 0,489 mg 100 g^{-1}) em concentrações importantes. Concluiu-se que a cultivar BRS Xiquexique contêm compostos funcionais, destacando-se o ácido gálico e o ácido ferúlico com maiores teores, comprovando o potencial antioxidante do feijão-caupi.

Palavras-chave: *Vigna unguiculata*. Bioativos. Composição química.

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INTRODUCTION

Studies of antioxidants in foods are currently widespread because of their action in delaying or inhibiting oxidative damage to cells. Given the magnitude of antioxidants preventing diseases and promoting health, it is of utmost importance to identify these compounds for use.

The increasing demand for means that favor a healthy life has driven the research of new substances that satisfy these needs. Consumption of vegetables has been associated with a healthy diet. In addition to their nutritional potential, these foods contain different phytochemicals, many of which perform biological functions, especially those capable of antioxidant action (LIMA et al., 2004).

Antioxidants are substances that can delay or inhibit oxidative damage, preventing the initiation or propagation of oxidation chain reactions, thereby preventing diseases caused by free radicals in the body (SILVA; ROCHA; CANNIATTI-BRAZACA, 2009). These radicals are unstable and highly reactive molecules, produced naturally via metabolic processes or biological dysfunction, which are combated by antioxidants, such as phenolic compounds and flavonoids (BARREIROS; DAVIS, 2006), which are produced in the body or obtained through the diet.

Several sources of natural antioxidants exist, and some are widely found throughout the plant kingdom. Phenolic compounds originate from secondary metabolism of plants, being essential during their growth and reproduction. In addition, they are also formed under stress conditions such as infections, injuries, and UV radiation. (ANGELO; JORGE, 2007).

The cowpea (Vigna unguiculata [L.] Walp.) is an important legume produced in largely in Africa, Latin America, and Asia. It is a rich source of proteins, calories, dietary fiber, minerals, and vitamins for a large number of people globally (PHILLIPS et al., 2003; CARVALHO et al., 2012). Although it is primarily produced in limited regions in Brazil, it is consumed throughout the country, and is therefore important for farmers. Identification of antioxidant compounds in cowpea will reveal its functional characteristics, which will be of paramount importance to add value to this crop for increasing its consumption.

MATERIAL AND METHODS

Samples

The BRS Xiquexique cowpea cultivar was provided from the Brazilian Agricultural Research Company (Embrapa Meio Norte), Teresina - PI, Brazil, which is 72 meters high, 5 ° 5 ' South Latitude and 42 ° 48' of West Longitude.

Preparation of gallic acid standard curve

For preparation of the stock solution, 0.125 g of gallic acid (vacuum oven dried) was diluted in a volumetric flask with deionized water. One milliliter (50 ppm), 2 mL (100 ppm), 3 mL (150 ppm), 5 mL (250 ppm), 10 mL (500 ppm) and blank (0 ppm) of the mixture were further diluted in with deionized water such that the final volume was 100 mL. Then, 100 µL of each solution was pipetted into a 10 mL volumetric flask, 0.5 mL of Folin-Ciocalteu reagent was added, and the solution was stirred. After 5 minutes, 1.5 mL of 20% sodium carbonate solution was added, shaken, and the volume was made up to 10 mL with deionized water. After incubating for 2 hours at 24 °C, protected from light with aluminum foil, absorbance values at 765 nm were measured in a 10 mm cuvette. Data are presented as a plot graph (mg L^{-1}) of gallic acid versus absorbance.

Extraction of phenolic compounds for analysis

The extracts were obtained according to the methodology of Rufino et al. (2010). To obtain the acetonic extract (A), 0.2 g of the sample was weighed and crushed in a mortar after drying in an oven at 50 °C. Then, 8 mL of 80% acetone was added to the sample, treated with ultrasound for 1 hour, followed by centrifugation of the homogenate at 4,000 rpm for 15 minutes. The supernatant was collected, and the volume was made up to 10 mL with deionized water.

Determination of total phenolics

To determine the total phenolics, 2 mL of deionized water was added to a 10 mL volumetric flask, followed by addition of 100 µL of the extract and 0.5 ml of the Folin-Ciocalteu reagent. The solution was stirred, and incubated for 5 minutes. Then, 1.5 mL of 20% sodium carbonate was added, the mixture was shaken, and was diluted with deionized water to a final volume of 10 mL. After incubating for 2 hours at room temperature and protecting from light with aluminum foil, the absorbance at 765 nm was measured in a 10 mm cuvette according to the methodology of Singleton and Rossi (1965).

Determination of total flavonoids

For the determination of total flavonoids, the method described by Kim, Jeong and Lee (2003) and modified by Blasa et al. (2006) was employed. One milliliter of the extract was mixed with 0.3 mL of 5 % m/v sodium nitrite ($NaNO_2$). After 5 minutes, 0.3 mL of 10 % m/v aluminum chloride ($AlCl_3$) was added. After incubation for 6 minutes, 2 mL of

1 M sodium hydroxide (NaOH) were added, and absorbances of the samples were measured at 425 nm in a spectrophotometer (BEL 1102, Monza, Milan, Italy). Different concentrations of quercetin (0 to 100 mg L^{-1}) were used for obtaining a standard curve, and the results were expressed in milligrams equivalent to quercetin (mg EQ 100 g^{-1} sample).

Determination of antioxidant activity

- *ABTS*

Firstly, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical was prepared. For this preparation, 0.0384 g of the ABTS reagent was weighed and dissolved in 10 ml of deionized water. Subsequently, the potassium persulfate solution (2.45 mM) was prepared by dissolving 0.0331 g of potassium persulfate in 25 mL of deionized water. Then, 5 mL of the ABTS solution and 5 mL of the persulfate solution were mixed, homogenized, and transferred to an amber flask, and kept protected from light for 16 hours at room temperature.

After activation of the $ABTS^{+}$ radical, an aliquot of 1 mL of the formed radical was diluted in 50 mL of ethanol. Absorbance measurements were performed in 10 mm cuvettes at 734 nm, until the absorbance stabilized at 0.700 (for absorbance value of less than 0.700, more radical was added; for higher values, more ethanol was added). After adjustment, an aliquot of 2.9 mL of the solution was transferred to a cuvette. Immediately, 60 µL of the sample extract were added, and absorbance was again measured after 7 minutes. The results were expressed in µM TEAC (Trolox equivalent antioxidant capacity) per 100 g of sample. For this, a standard Trolox curve (standard antioxidant) was prepared, and the absorbance values of the extracts were plotted against this curve to obtain the results in µM TEAC/100 g, according to the method of Re et al. (1999).

- *DPPH*

DPPH (0.0394 g) was dissolved in 10 mL of methanol. Then, the solution was diluted to 1:100 using 80% v/v methanol solution to adjust the initial absorption to 0.800. An aliquot of 2.9 mL of this adjusted solution was pipetted into a 10 mm cuvette. The initial absorbance was measured, 100 µL of the extract was added, and the cuvette was incubated for 30 minutes in the dark. The results were compared to the standard antioxidant, Trolox, and were expressed in µM TEAC/100 g of sample, according to method given by Kim et al. (2006).

Determination of anthocyanins

The total anthocyanin content was determined using the pH difference method (GIUSTI; WROLSTAD, 2001). To a 0.2 mL aliquot of diluted sample, 1.8 mL of potassium chloride solution (pH 1.0) was added, homogenized, and stored for 10 minutes in the absence of light, and an equivalent procedure was performed in a solution of sodium acetate (pH 4.5). The absorbance was measured using a spectrophotometer (BEL 1102, Monza, Milan, Italy) at the maximum wavelength of each sample and at 700 nm in buffer solutions of pH 1.0 and pH 4.5. The blank used was distilled water. The absorbance was measured at the maximum absorption wavelength (420 nm) and at 700 nm.

Absorbance was calculated as follows:

$$A = (A_{máx.vis} - A_{700nm})_{pH\ 1.0} - (A_{máx.vis} - A_{700nm})_{pH\ 4.5}$$

The concentration of monomeric pigments can be calculated from this absorbance and expressed as cyanidin-3-glycoside (MW = 449.2, and ε = 26,900).

Monomeric anthocyanins (mg/100g) = (A × MW × DF × 100)/(ε × 1).

A = Absorbance; MW = molecular weight; DF = dilution factor; ε = molar absorptivity.

Identification and quantification of phenolics compounds using HPLC

The extracts were analyzed using high performance liquid chromatography (HPLC), according to Pereira et al. (2004) and Tiberti et al. (2007). Analyses of the phenolic compounds were carried out using a Shimadzu chromatograph, model LC-20AT, equipped with manual injector SIL-20AC, CBM-20ª controller and SPD-M20A diode array detector. The column used was a C18 stationary phase, Shim-Pack VP-ODS-2 (25 cm x 0.5 cm, with a 5 µm particle, Shimadzu).

Trifluoroacetic acid (0.1% v/v) was used as the mobile phase in deionized water as eluent A, and acetonitrile was used as eluent B. Prior to the analysis, the system was equilibrated with a mixture of 9.5:0.5 eluents A and B, respectively. The gradient was as follows: 5% B (10 minutes), 5-100% B (40 minutes), 100% B (5 minutes), and 5% B (5 minutes). The temperature was maintained at 35 °C, and the eluent flow was maintained at 1 $mL.min^{-1}$ during the analysis. Detection was performed from 190 nm to 400 nm.

To validate the method, the following parameters were used: limits of detection (LD) and quantification (LQ), reproducibility of areas under the peaks of phenolic compounds through analyses of ten replicates of the patterns, and the recovery (AOAC, 2005). The limit of detection was calculated as the lowest concentration providing a chromatographic signal that was three times higher than the noise signal. The limit of quantification was calculated as the minimal concentration capable of providing a chromatographic signal five times greater than the noise signal.

For the analyses, standard solutions were prepared using pure phenolic acids, such as gallic acid, quercetin, caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid, catechin, and epicatechin. One milligram of each standard acid was diluted in mobile phase (0.1% Milli-Q water of ATF) to make up to 10 mL volume, then the samples were filtered and injected into the chromatograph. Two milliliters of sample were collected and placed in glass tubes, where they were evaporated in Centrivap for 3 hours at 40 °C. After evaporation, the compounds were resuspended in the mobile phase (1 mL), the material was filtered, and then injected into the chromatograph. The analyses were done in quadruplicate, 60 minutes for each sample. The results were expressed in mg per 100 g of dry matter.

Data analyses

A database was developed. The determinations were performed in triplicate or quadruplicate (determinations by HPLC), and the data obtained were presented as means and standard deviation.

RESULTS AND DISCUSSION

Total phenolic, flavonoid, and total anthocyanin concentrations

Table 1 shows the content of total phenolic compounds, flavonoids, and anthocyanins obtained from the cowpea cultivar. Data are means of three replicates in acetone extracts, expressed in dry basis.

The cultivar BRS Xiquexique presented 199.05 ± 1.98 mg equivalent of gallic acid-GAE 100 g^{-1}. Based on a study by Marathe et al. (2011), which classified legumes such as common bean, cowpea, chickpeas, soybeans, and peas into three different groups according to their phenolic compound content, the raw cowpea cultivar analyzed in the present work can be classified under moderate phenolic content (> 100 and < 200 mg GAE 100 g^{-1}).

Table 1. Total phenolics, flavonoids, and total anthocyanins from cowpea cv BRS Xiquexique.

Bioactive Compound	**(GAE 100 g^{-1})***
Total phenolics	199.05 ± 1.98
Flavonoids	67.96 ± 0.54
Anthocyanins	Nd**

*Gallic acid (GAE) equivalents. **Nd – not detected. Means of three repetitions ± SD.

Giami (2005) analyzed four strains of cowpea, and obtained levels ranging from 99 to 196 mg 100 g^{-1}, which are in agreement with those obtained in the present study. In a study by Zia-Ul-Haq et al. (2013), higher levels of phenolic compounds (1,190 - 1,620 mg GAE 100 g^{-1}) were observed in the flours of four cultivars of raw cowpea consumed in Pakistan.

Several factors may interfere with the content of phenolic compounds in legumes, such as genetic and environmental factors, as well as factors inherent in the extraction conditions of these compounds, such as the type of solvent used. Thus, this may justify the differences observed in the content of these compounds, as compared to those observed in other studies.

The cultivar BRS Xiquexique presented high flavonoid content (67.96 mg 100 g^{-1}). Behling et al. (2004) reported that flavonoid content in foods consumed daily as 44 mg in cereals, 79 mg in potatoes, 45 mg in grains and nuts and 162 mg in vegetables and herbs. The cultivars of cowpea presented high levels of these compounds. Compared with Brazilian fruits, the cowpea cultivars evaluated were higher than the levels observed by Barreto, Benassib and Mercadante (2009) for jackfruit (22.3 ± 0.2 mg EQ 100 g^{-1}), for nectarine (23.7 ± 1.2 mg EQ 100 g^{-1}) and for star fruit (42.6 ± 2.3 mg EQ 100 g^{-1}).

Wang et al. (2008) analyzed 40 selected legumes, including cowpea, and verified high levels of total flavonoids for cowpea (44.19 mg 100 g^{-1}) and (25.29 mg 100 g^{-1}). Cowpea contains high levels of flavonoids, myricetin, and quercetin, and low levels of genistein, kaempferol, and daidzein.

Anthocyanins were not detected in the cowpea cultivar BRS Xiquexique. Previous reports in literature that aimed at identifying and quantifying anthocyanins, flavonoids, and condensed tannins in cowpea samples were performed in cultivars with dark seed coat, as these bioactive compounds are concentrated in this grain region (HA et al., 2010; OJWANG; DYKES; AWIKA, 2012; OJWANG et al., 2013). Our study confirms the statements of Akond et al. (2011) on the direct relationship between the bean tegument color and the anthocyanin levels, as the cultivar BRS Xiquexique presents a light-colored tegument and no detectable anthocyanin.

Similar results were reported by Huber (2012) on evaluation of common beans of the cultivars BRS9435-comet (brown), Xamego (black), and the G-2358 (white) lineage. Highest tannin content was observed in the Xamego crude cultivar (11.21 mg g^{-1} catechin), but the presence of such compounds in the G-2358 strain was not detected. No condensed

tannins were detected in cooked black and brown beans in a study by Ranilla, Genovese and Lajolo (2009), suggesting that formation of insoluble complexes between proteins and tannins and between carbohydrates and tannins in whole grains inhibited extraction of the compounds by the solvent, and therefore, the non-detection thereof in the method using the vanillin reagent. Analyzing six distinct genotypes of cowpea, Ojwang et al. (2013) did not identify these compounds using the HCl-vanillin test in white or green tegument grains. They suggested genetic control over the accumulation of tannins or proanthocyanidins.

Antioxidant activity

The antioxidant activity in the cowpea cultivar analyzed using the DPPH and ABTS free radical capture method is shown in Table 2.

Xu and Chang (2012) analyzed the health promotion effects related to the antioxidant activity of 13 legumes consumed in the United States, including peas, lentils, soybeans, chickpeas, cowpea beans, and common beans, showed using the DPPH method that the antioxidant activities of yellow soybean and black beans were 107 μmol TEAC 100 g^{-1} and 1940 μmol TEAC 100 g^{-1}, respectively. The values obtained in the present research using the DPPH method were higher than those reported for yellow peas (358 μmol TEAC 100 g^{-1}), chickpeas (294 μmol TEAC 100 g^{-1}), green peas (277 μmol TEAC 100 g^{-1}), and yellow soybean (107 μmol TEAC 100 g^{-1}), and lower than those for cowpeas (707 μmol TEAC 100 g^{-1}).

Table 2. Antioxidant activities of the cowpea cultivar BRS Xiquexique determined using DPPH and ABTS methods.

Method	(μmol TEAC* g^{-1})
DPPH	575.4 ± 2.98
ABTS	608.5 ± 2.09

*Trolox equivalent antioxidant capacity (TEAC).
Means of three tests ± standard deviation (SD).

Results for antioxidant capacity and phenolic contents higher than those obtained in the present study were verified by Deng et al. (2013), who analyzed cowpea marketed in China. They observed antioxidant activity between 1727 and 2312 μmol TEAC 100 g^{-1} using the ABTS method between 1357–1924 μmol Fe (II) 100 g^{-1} using the ferric reducing antioxidant power (FRAP) method, and total phenolic content of 717–939 mg GAE 100 g^{-1}. Marathe et al. (2011) also found different results than those observed in the present study for cowpeas with brown and red teguments. They reported antioxidant activities higher than 12.0 μmol TEAC g^{-1} using the ABTS method and higher than 400 μmol TEAC g^{-1} using the DPPH method. These high contents of phenolic compounds reported in this work can be explained by color of the tegument, which influences the antioxidant capacity. Therefore, the observed antioxidant values were lower in the present research because the beans showed lighter tegument colors (white and green).

However, antioxidant activities observed in the present study were higher than those reported by Oboh (2006), who evaluated the ability of raw cowpea samples (two of white and three of brown tegument) to sequester the DPPH free radical, obtaining percentages of free radical inhibition in the range of 5.5–29.9%. In the present study, higher percentages of DPPH radical inhibition were observed, ranging from 40–50% for the cultivars.

It is worth noting that the in vitro antioxidant activity measured in the present study suggests bioactivity, but does not determine it. Moreover, antioxidant activity is related to the phenolic content, to the configuration of hydroxyl groups, and to the conjugated double bounds.

Identification and quantification of phenolic compounds

The HPLC chromatograms of phenolic compounds identified in the BRS Xiquexique cultivar and the standards of epicatechin, gallic acid, chlorogenic acid, and ferulic acid are presented in Figures 1 and 2.

As shown in Table 3, gallic acid showed the highest phenolic compound, followed by ferulic acid. Epicatechin and chlorogenic acid were identified at lower concentrations. In a study by Nderitu et al. (2013), ferulic acid was identified in cowpea, a result consistent with that observed in this research. It can be inferred that the high content of ferulic acid in higher content can be expected in a legume.

According to Yao et al. (2013) and Zhang et al. (2013), cowpea has an anti-glycemic and anti-inflammatory effect, potentially useful in reducing the complications in diabetic people. Its antioxidant action due to its bioactive compounds is also an important feature of its functionality.

The present study showed that because of the presence of bioactive compounds in BR Xiquexique of cowpea, this cultivar can be used as a source of compounds like gallic acid and ferulic acid for the control of chronic non-communicable diseases, such as diabetes and obesity.

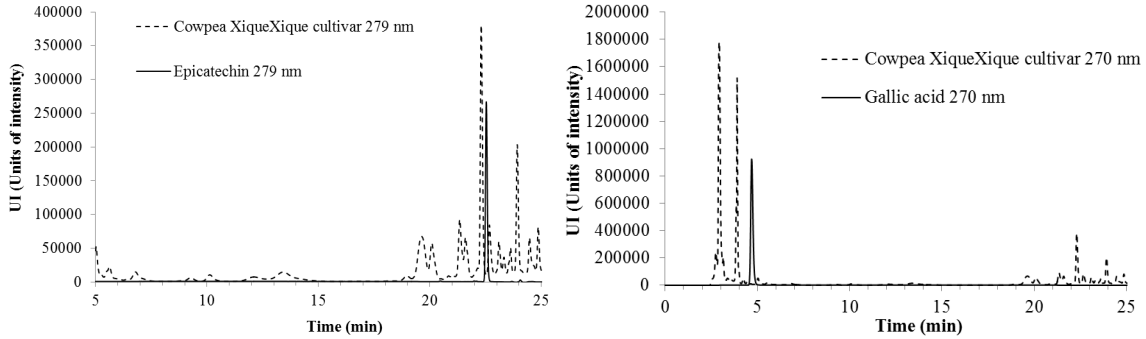

Figure 1. HPLC chromatograms of cowpea BRS cultivar Xiquexique extracts and the standards, epicatechin (measured at 279 nm) and gallic acid (measured at 270 nm).

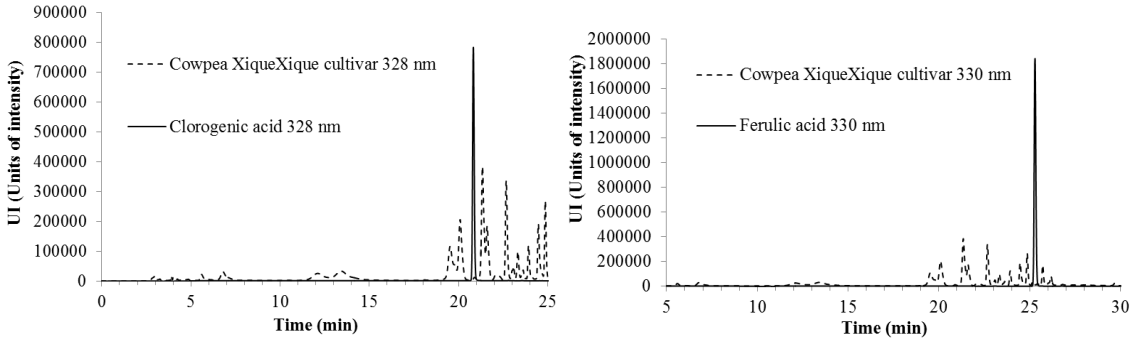

Figure 2. HPLC chromatograms of cowpea BRS cultivar Xiquexique extracts and the standards, chlorogenic acid (measured at 328 nm) and ferulic acid (measured at 330 nm).

Table 3. Identification and quantification of the phenolics compounds detected in the extracts of cowpea cultivar BRS Xiquexique.

Phenolic compound	**(mg 100 g^{-1})**
epicatechin	0.48±0.130
gallic acid	67.19±6.200
ferulic acid	32.07±0.753
chlorogenic acid	3.08±0.489

Means of four tests ± standard deviation (SD).

CONCLUSION

It was concluded from the results that the cultivar BRS Xiquexique contains bioactive compounds, containing gallic acid and ferulic acid at high levels, proving the antioxidant and functional potential of cowpea.

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