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Original Article | Artículo Original

Phytochemical study and antioxidant capacity of three fractions from the stem of *Caesalpinia bahamensis* Lam.

[Estudio fitoquímico y actividad antioxidante de tres fracciones del tallo de *Caesalpinia bahamensis* Lam.]

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Abstract

Context: *Caesalpinia bahamensis* Lam. is a medicinal plant used by the Cuban population to treat renal and hepatic diseases. However, this species lacks scientific studies that support its biological applications.

Aims: To evaluate the chemical composition and the antioxidant capacity of fractions obtained from the stem of *Caesalpinia bahamensis* Lam.

Methods: A continuous extraction of the stem was made by maceration using a battery of solvents of increasing polarity: chloroform, ethyl acetate and methanol. All fractions were analyzed by TLC and phytochemical screening. The compounds of the chloroform fraction were identified by GC/MS, while the ethyl acetate and methanol fractions were characterized by UV-Vis spectroscopy. The antioxidant capacity was evaluated by the DPPH and FRAP assays.

Results: Ten compounds were identified by GC/MS of the chloroform fraction, associated with fatty acids, terpenoids and phytosterols. The major compounds of this fraction were octacosanol, monopalmitin and palmitic acid. The presence of flavonoids in the ethyl acetate and methanol fractions was demonstrated by phytochemical screening, TLC and UV spectroscopy. The three fractions showed antioxidant capacity in the DPPH assay, with the methanol fraction (IC_{50}=11.1 µg/mL) being the most active. The ethyl acetate fraction (equivalent to 100.7 µmol ascorbic acid) and the methanol fraction (equivalent to 37.3 µmol ascorbic acid) showed antioxidant capacity in the FRAP assay at concentrations of 125 µg/mL and 1000 µg/mL, respectively.

Conclusions: The fractions evaluated showed antioxidant capacity in the DPPH and FRAP assays, possibly associated with the presence of phenols and flavonoids.

Keywords: antioxidant activity; *Caesalpinia bahamensis*; fatty acids; flavonoids; GC/MS.

Resumen

Contexto: *Caesalpinia bahamensis* Lam. es una planta medicinal usada por la población cubana para el tratamiento de enfermedades hepáticas y renales, sin embargo, existen pocos estudios científicos que avalen sus propiedades biológicas.

Objetivos: Evaluar la composición química y la capacidad antioxidante de fracciones obtenidas del tallo de *Caesalpinia bahamensis* Lam.

Métodos: Se realizó una extracción continua del tallo de la planta por maceración usando una batería de disolventes en polaridad creciente: cloroformo, acetato de etilo y metanol. A todas las fracciones se les realizó un tamizaje fitoquímico y CCD. Los compuestos de la fracción clorofórmica fueron identificados por CG/EM, mientras que las fracciones acetato de etilo y metanol fueron caracterizadas mediante espectroscopía UV. La capacidad antioxidante se evaluó mediante los ensayos DPPH y FRAP.

Resultados: Se identificaron diez compuestos de la fracción clorofórmica mediante CG/EM, asociados con estructuras de ácidos grasos, terpenoides y fitoesteroles. Los compuestos mayoritarios de esta fracción fueron: octacosanol, monopalmitina y ácido palmítico. La presencia de flavonoides en las fracciones acetato de etilo y metanol fue demostrada por tamizaje fitoquímico, CCD y espectroscopía UV. Las tres fracciones mostraron capacidad antioxidante por DPPH, siendo la fracción metanólica (IC_{50}=11.1 µg/mL) la más activa. Por otra parte, las fracciones acetato de etilo (100.7 µM equivalentes de ácido ascórbico) y metanol (37.3 µM equivalentes de ácido ascórbico) mostraron capacidad antioxidante en el ensayo de FRAP a concentraciones de 125 µg/mL y 1000 µg/mL, respectivamente.

Conclusiones: Las fracciones evaluadas mostraron capacidad antioxidante en los ensayos DPPH y FRAP, posiblemente asociada a la presencia de flavonoides y fenoles.

Palabras Clave: actividad antioxidante; ácidos grasos; *Caesalpinia bahamensis*; flavonoides; GC/MS.

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INTRODUCTION

Reactive oxygen species (ROS) are formed as a consequence of the metabolism of aerobic organisms (Choudhury et al., 2017). It is known that an overproduction of these could be the cause of chronic diseases such as cancer, cardiovascular and neurodegenerative diseases (Huang, 2018). In normal health conditions, the body has control mechanisms to prevent the damage caused by these substances (Tadhani et al., 2007). However, when the balance between oxidants and antioxidants breaks down, so-called oxidative stress is generated (Gutteridge and Halliwell, 2018).

Since ancient times, man has used plants for medicinal and nutritional purposes (Rodríguez et al., 2015) and recent studies have shown that the consumption of fresh fruits, vegetables and teas has been related to the prevention of cancer and cardiovascular diseases (Tadhani et al., 2007). It is suggested that this tendency could be linked to the presence of phenolic compounds and flavonoids in plants, metabolites well studied for their antioxidant properties (Estrela et al., 2017).

The antioxidant and antitumoral activity *in vitro* of the genus *Caesalpinia* has been demonstrated for several of its species, such as *C. sappan* (Saenjum et al. 2010), *C. bonduc* (Ogunlana et al., 2015), *C. pulcherrima* (Hsu et al., 2012), *C. pluviosa* (Zanin et al., 2015) and C. *decapetala* (Wei et al., 2013). On the other hand, *C. bahamensis* is a medicinal plant that has been used for the treatment of kidney and liver diseases and vulgarly known as "brasilete" (Roig, 2012). Recently, the cytotoxic effect of the dichloromethane extract of the bark of this species against SK-Mel-28 (human melanoma), MDA-MB-231 (human mammary adenocarcinoma) and 5637 (human bladder carcinoma) cells was demonstrated (Setzer et al. 2015). However, the antioxidant effect of the plant has not been demonstrated yet and knowledge about its chemical composition it is scarce.

In this sense, this study contributes to the knowledge of the species *Caesalpinia bahamensis* Lam. (*Leguminosae*). Phytochemical analysis of three fractions obtained from the stem of the plant is carried out, and the antioxidant effect of the aforementioned fractions in the DPPH and FRAP assays is demonstrated for the first time.

MATERIAL AND METHODS

Chemicals

All substances were purchased from Thermo-Fisher Scientific (United Kingdom) unless otherwise stated.

Plant material

Stems of *C. bahamensis* were collected in March 2017 at Cañada Arroyón, Artemisa, Cuba (22°46'45.7"N 83°04'18.6"W). The material was identified in the National Botanical Garden of Cuba, where a voucher specimen (No. 85369) was deposited. The materiel was dried in an oven (AI-SET-DNE 600, Shanghai, China) at 40°C during seven days and milled (Manesti, Italy) until the size of the particles was less than 2 mm.

Fractionation

To obtain the fractions, a continuous extraction of the stem of the plant was made by maceration using a battery of solvents of increasing polarity: chloroform, ethyl acetate and methanol. The extraction was carried out for seven days for each solvent at room temperature and in the absence of light, using a ratio of 1 g of drug per 10 mL of solvent.

Characterization of fractions

Phytochemical screening

The phytochemical screening was done through the assays of Sudan (fats and oils), Dragendorff (alkaloids), Mayer (alkaloids), Wagner (alkaloids), Baljet (lactones and coumarins), Liebermann-Burchard (triterpenes and steroids), Fehling (reducing sugars), foaming (saponins), ferric trichloride (polyphenols), ninhydrin (amino acids), Bornträger (anthraquinones) and Shinoda (flavonoids). The results were analyzed by color changes

of the extracts by applying the mentioned reagents (Miranda and Cuéllar, 2000).

TLC profile

TLC was carried out on plastic plates covered with silica gel F254 (Merck). The chloroform fraction was developed with *n*-hexane/ethyl acetate (7:3) as the mobile phase, while the ethyl acetate and methanol fractions were developed with chloroform/methanol (9:1). Sulphuric acid 5 % in ethanol was used as revelator.

UV profile

The ethyl acetate and methanol fractions were analyzed by UV spectroscopy on a spectrophotometer (Lambda 35, Perkin Elmer, Singapore). Previous to analysis, the samples were diluted in 10 mL of the extraction solvent. Spectra were recorded in scan mode (200-700 nm).

Gas Chromatography/Mass Spectrometry

The chloroform fraction was analyzed by GC/MS on a Trace 2000 instrument (TRACE 2000 GC, Interscience Thermo Quest, Belgium). Prior to analysis, 300 µL of the test sample was derivatized by adding 100 µL of the N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% of trimethylsilyl chloride (BSTFA + 1% TMCS) reagent and 100 µL of chloroform. The mix was stirred and maintained at 30°C for 30 min. An HP-5Ms (30 m, 0.25 mm ID x 1.0 µm) column (Hichrom Limites, UK) was used. The inlet and detector temperatures were 280°C and 250°C, respectively. Helium gas was used as the mobile phase. The identification of compounds was done using the NIST 2000 data base.

Antioxidant assays

Free radical scavenging capacity

The used method was a modification to that described by Tabart (2009) The assay is based on the reduction of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•). Ten concentrations of the extract were prepared for the analysis; 500 µL of each solution was mixed with 1500 µL of DPPH reagent (0.075 mg/mL). A mix of 250 µL dimethyl sulfoxide (DMSO) with 250 µL of distilled water was used as blank for the methanol fraction, while for the ethyl acetate and chloroform fractions 500 µL of distilled water was used as a blank. The reaction was left in the dark for 30 min and, subsequently, UV absorbance was measured at 517 nm. Each determination was performed in triplicate. Trolox was used as reference compound. The percent of inhibition was calculated using the following formula:

% inhibition of the DPPH= [(Abs control - Abs sample)/Abs control] x 100

Where: Abs control: Abs of blank +DPPH and Abs sample: Abs of fraction +DPPH

The mean effective concentration (IC_{50}) was determined with the help of the Graphprism 5.0 statistical program.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done according to Benzie and Strain (1996) with some modifications. The FRAP reagent was prepared *in situ* by mixing 0.1 mol/L of sodium acetate buffer (pH 3.6), 10 mmol/L of TPTZ (2, 4, 6-tris(2-pyridyl)-s-triazine) and 20 mmol/L of ferric chloride (10: 1: 1, v: v: v) and then warmed at 37 °C before using. The fractions were prepared at the concentration of 2 mg/mL, and four successive dilutions of the samples to be tested were prepared. Test samples (30 µL) and water (90 µL) were allowed to react with 900 µL of the FRAP solution for 30 min in the dark. Readings of the colored product (ferrous tripyridyltriazine complex) were then done at 593 nm. The blank consisted of 120 µL of water and 900 µL of reagent. The results were expressed as µmol equivalent of ascorbic acid, according to the standard curve of ascorbic acid (100-1000 µmol/L).

Statistical analysis

Values were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed with SPSS 18.0. For multiple comparisons, one-way ANOVA was used followed by

Dunnett post-hoc test. Values of $p<0.05$ were considered statistically significant.

RESULTS

Characterization of fractions

Phytochemical screening

Phytochemical screening of the chloroform fraction allowed to detect only the presence of oils and fats. A slight pink coloration in the Liebermann-Burchard assay suggested the presence of triterpenes and steroids at low concentrations.

In the ethyl acetate fraction, lactones, coumarins, triterpenes, steroids, reducing sugars, phenolic compounds, quinones, and flavonoids were evidenced. In the methanolic fraction the same components as those found in the ethyl acetate fraction were observed, but the color changes were stronger, suggesting a higher concentration of metabolites. In addition, this extract showed the presence of saponins. The results of the Dragendorff, Mayer and Wagner assays were negative, indicating the absence of alkaloids in the fractions obtained from the stem of *C. bahamensis*.

TLC profile

Table 1 shows the retention factor and the observed color after revelation of the three fractions. When revealing the chromatogram of the chloroform fraction with sulphuric acid (H_2SO_4) and heat, around six spots with purple to reddish-brown color were observed, indicating the presence of triterpenoids and phytosterols. In the ethyl acetate fraction, around three spots were observed with yellow, orange and light purple color, indicative for the presence of flavonoids and triterpenoids. The spots at Rf of 0.58 and 0.69 observed in ethyl acetate fraction were observed in methanol fraction too.

UV profile

The ethyl acetate and methanol fraction were analyzed by UV spectroscopy. Fig. 1 shows the UV spectrum of the fractions. In both cases, two characteristic bands of flavonoids were observed, the first one at 285 nm and the second one at 445 nm.

Table 1. Retention factor and observed color in Thin Layer Chromatography (TLC) of the fractions of *C. bahamensis*

Spot	Fractions					
	Chloroform		Ethyl-acetate		Methanol	
	Rf	Color	Rf	Color	Rf	Color
1	0.07	red-brown	0.46	light purple	-	-
2	0.22	red-brown	0.58	yellow	0.58	yellow
3	0.29	red-brown	0.69	orange	0.69	orange
4	0.35	purple	-	-	-	-
5	0.51	purple	-	-	-	-
6	0.85	red-brown	-	-	-	-

The color that appears is the one that is observed after revealing the plate with sulphuric acid (H2SO4).

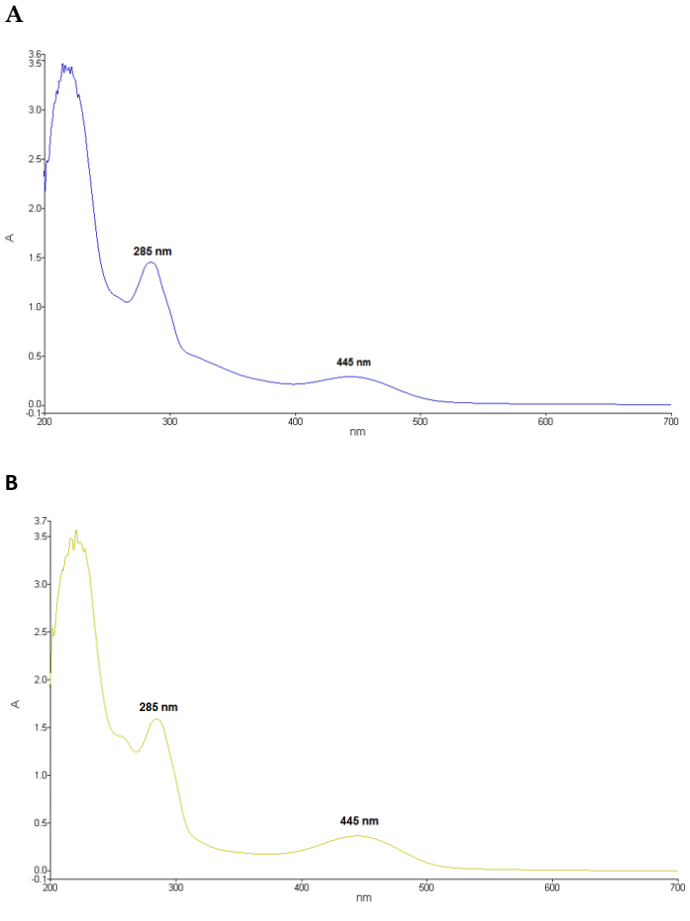

Figure 1. UV profile of ethyl acetate **(A)** and methanol **(B)** fractions obtained from the stem of *Caesalpinia bahamensis* Lam.

GC/MS analysis

Ten compounds were identified from the chloroform fraction by gas chromatography coupled to mass spectrometry (GC/MS). The presence of fatty acids was detected, and smaller amounts if terpenoids and phytosterols. The major compound was octacosanol, a high molecular weight alcohol (Table 2) (Fig. 2). The compounds were compared with the database NIST 11 and Wiley 275 of the equipment according the mass fragments obtained.

Table 2. Identified compounds of the chloroform fraction of the stem of *Caesalpinia bahamensis* Lam.

RT	Compounds	RA
7.44	t-Muurolol	1.56
7.61	α-Bisabolol	1.25
10.81	Nerolidol	0.89
10.94	Palmitic acid TMS	10.64
12.81	Linoleic acid TMS	5.27
12.87	Oleic acid TMS	4.56
13.16	Stearic acid TMS	2.71
21.52	Octacosanol TMS	26.53
23.05	β-Sitosterol	2.05
24.62	Monooeloylglycerol	6.41

Relative abundance (%) (RA): Percentage of one compound in relation to all identified compounds. Retention time (RT): Time of apparition of each compound in the chromatogram.

Antioxidant capacity

Free radical scavenger capacity

The free radical scavenger capacity of the fractions was evaluated by the DPPH assay (Fig. 3, Table 4). This assay offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological samples (Kedare and Singh, 2011). The results are expressed as IC_{50}, that is, the quantity of fraction needed to scavenger 50% of the free radicals (Bécquer et al., 2018). The three fractions showed free radical scavenger capacity; however, the methanol fraction has the best effect in this assay with an IC_{50} of 11.1 µg/mL (Fig. 3B, Table 4).

This method requires hydrophilic conditions, and for this reason, the chloroform fraction was not evaluated. The results were expressed as µM equivalent of ascorbic acid, the standard used for the analysis.

FRAP assay

The capacity the of methanol and ethyl acetate fractions to reduce Fe^{3+} to Fe^{2+} *in vitro* was measured by the FRAP assay. This method requires hydrophilic conditions, and for this reason, the chloroform fraction was not evaluated. The results were expressed as µM equivalent of ascorbic acid, the standard used for the analysis. The results displayed in Table 3 show the ability of both extracts to reduce Fe^{3+} to Fe^{2+}. In this case, the best results were obtained for the ethyl acetate fraction at a concentration of 0.125 mg/mL.

Table 3. Ferric reducing antioxidant potential (FRAP) values of different fractions of *Caesalpinia bahamensis* Lam in terms of ascorbic acid equivalents (AAEq).

Fraction	Concentration (mg/mL)	FRAP (µmol/mL ascorbic acid equivalents)
Methanol	1	37.3 ± 9.6[b]
	0.5	NE
	0.25	NE
	0.125	NE
Ethyl acetate	1.0	382.3 ± 13.5[a]
	0.5	337.3 ± 12.5[a]
	0.25	239.0 ± 11.6[a]
	0.125	100.7 ± 14.4[b]

Data is expressed as mean ± SEM. Different letters represent statistical differences (ANOVA, Dunnet post-hoc test; $p<0.05$). NE: Not effect.

DISCUSSION

Cancer, cardiovascular and neurodegenerative diseases and diabetes mellitus are among the leading causes of death worldwide, so their prevention and treatment are part of the objectives of health institutions and the scientific community in general (Heron, 2010).

Table 4. Concentration of different fractions of *Caesalpinia bahamensis* at DPPH radical scavenging activity 50% (IC_{50}).

Fraction	DPPH IC_{50} [µg/mL]
Methanol	11.1 ± 0.7[a]
Ethyl acetate	23.6 ± 1.2[a]
Chloroform	154.1 ± 5.3[b]

Data is expressed as mean ± SEM. Different letters represent statistical differences (ANOVA, Dunnet post-hoc test; $p<0.05$).

Recent studies have linked the prevention of these diseases with the consumption of antioxidants in the diet, and many researchers have focused their efforts on finding new sources of antioxidants, with medicinal and food plants being one of the most studied resources in this field (Adegbola et al., 2017; Fareed et al., 2017; Franco and Martínez, 2017; Ravi et al., 2018). In this sense, flavonoids, phenolic acids, carotenoids, and tocopherols have been the most studied natural antioxidants and have been related to the capacity to scavenge free radicals and to reduce Fe^{3+} to Fe^{2+} (Brewer, 2011). The presence of poly-unsaturated fatty acids has also been related to the activity of scavenging free radicals (Brewer, 2011).

On the other hand, *Caesalpinia bahamensis* is a medicinal plant on which there are few references in the literature. So far, its diuretic effect in Wistar rats (Felipe et al., 2011) and its antimicrobial (Abreu et al., 2017) and antitumor (Setzer et al., 2015) activity *in vitro* has been reported. Also, seventy-four compounds were identified by GC/MS in a non-polar fraction of the methanolic extract (Felipe et al. 2017).

In this study, the methanol and ethyl acetate fractions showed the best antioxidant activity in the DPPH and FRAP assays, which could be due to the strong presence of flavonoids and phenolic compounds detected by phytochemical screening, thin layer chromatography, and UV spectroscopy. The chloroform fraction also showed an antioxidant effect in the DPPH assay, which in this case can be related to the presence of polyunsaturated fatty acids such as oleic, palmitic and linoleic acid identified by GC/MS.

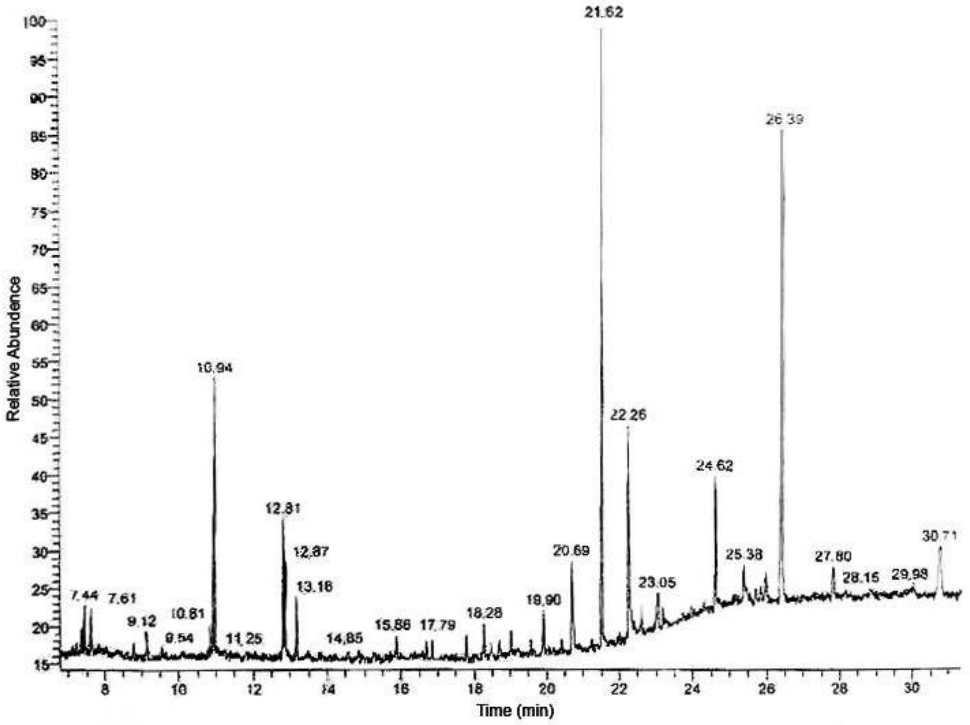

Figure 2. Chromatogram GC-MS of the chloroform fraction of *Caesalpinia bahamensis* Lam.

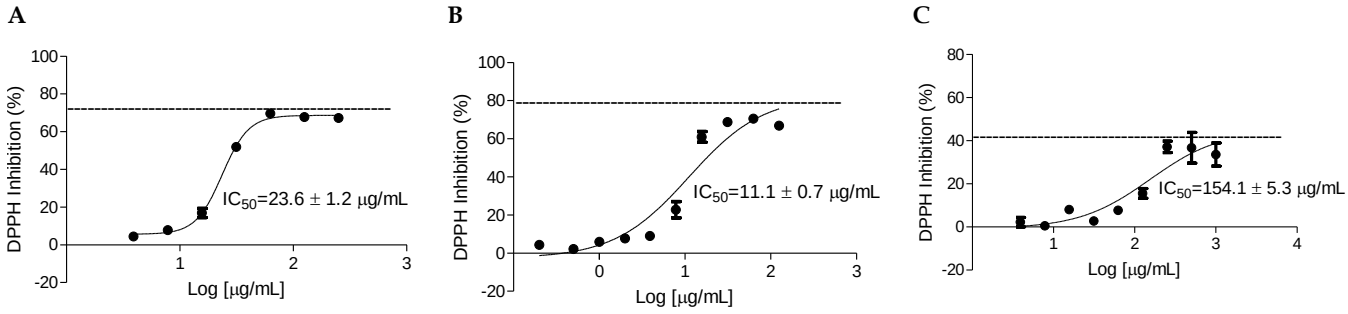

Figure 3. Concentration-response curves of the DPPH assay.

In each chart appear represented % inhibition of DPPH *vs.* log of the concentration (µg/mL) of the fraction) and the corresponding inhibitory concentration 50% (IC_{50}). **A:** ethyl acetate fraction; **B:** methanol fraction; and **C:** chloroform fraction.

The compounds identified in the chloroform fraction of the stem of *Caesalpinia bahamensis* correspond to those found in a petroleum ether fraction of the methanolic extract of this species (Felipe et al., 2017). On the other hand, Dominicis et al. (1995) have reported the presence of flavonoids and the absence of alkaloids in the species through phytochemical screening techniques. Here, the presence of these compounds is confirmed by TLC and UV spectroscopy. Finally, for the first time, the antioxidant effect of this species is demonstrated because in the genus it has been widely studied; however, more in-depth studies are needed to corroborate the antioxidant activity of this plant.

CONCLUSIONS

A phytochemical study of three fractions from the stem of *Caesalpinia bahamensis* Lam. was performed where were identified ten compounds in the chloroform fraction by GC/MS. The fractions showed antioxidant activity by DPPH and FRAP assays. The three fractions studied showed free radical scavenger capacity; however, the methanol and ethyl acetate fractions showed of the best effects, possibly associated at presence of flavonoids and phenols in the fractions analyzed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Felipe A	Hernández I	Gutiérrez Y	Scull R	Carmenate LM	Pieters L	Rodeiro I	Delgado R
Concepts or ideas	x	x				x	x	x
Design	x	x				x	x	x
Definition of intellectual content	x							
Literature search	x		x		x			
Experimental studies	x		x	x	x			
Data acquisition	x	x		x	x		x	
Data analysis	x	x	x				x	x
Statistical analysis		x					x	
Manuscript preparation	x							
Manuscript editing			x			x	x	x
Manuscript review	x	x	x	x	x	x	x	x

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