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Phytochemical Analysis and Antioxidant Activity Assessment of *Croton linearifolius* Stem Extracts

Análise Fitoquímica e Avaliação da Atividade Antioxidante dos Extratos dos Caules de *Croton linearifolius*

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ABSTRACT

Croton linearifolius is a species with promising medicinal potential. This study investigated the antioxidant activity, total phenolic and flavonoid contents, and conducted chemical prospecting of its ethanolic stem extract and fractions. The extract was obtained by exhaustive percolation with 70% hydroethanolic solution, followed by liquid-liquid partitioning using hexane, dichloromethane, and ethyl acetate. In vitro tests were used for chemical analysis, and antioxidant activity was evaluated by DPPH and FRAP methods. The ethyl acetate fraction showed the highest antioxidant activity (80.62% in DPPH), with elevated phenolic and flavonoid levels, indicating potential for herbal medicine development.

keywords *Croton linearifolius*, antioxidant activity, phenolic compounds, flavonoids

RESUMO

Croton linearifolius é uma espécie com potencial medicinal promissor. Este estudo avaliou a atividade antioxidante, os teores de fenólicos totais e flavonoides, além da prospecção química do extrato etanólico e de suas frações obtidas dos caules. O extrato foi preparado por percolação exaustiva com solução hidroetanólica a 70%, seguido de partição líquido-líquido com hexano, diclorometano e acetato de etila. A análise química foi realizada por testes in vitro, e a atividade antioxidante pelos métodos DPPH e FRAP. A fração de acetato de etila apresentou a maior atividade antioxidante (80,62% no DPPH), indicando potencial para uso fitoterápico.

palavras-chave *Croton linearifolius*, atividade antioxidante, compostos fenólicos, flavonoides

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Introduction

Medicinal plants have long served as a valuable source of bioactive compounds, playing a fundamental role in the treatment of various pathological conditions. These compounds, commonly referred to as secondary metabolites, fulfill essential functions within the plant and also exhibit important therapeutic properties for humans, such as antioxidant, anti-inflammatory, antimicrobial, and anticancer activities. Among the most studied bioactive compounds are flavonoids, phenolic acids, terpenes, and alkaloids, which have demonstrated promising antioxidant properties. These properties are of particular interest in the context of diseases associated with oxidative stress, such as cardiovascular diseases, cancer, and neurodegenerative conditions. The therapeutic potential of these compounds has driven significant research into their use in modern medicine, offering alternative treatments and complementary therapeutic approaches to conventional drugs.

The *Croton* genus, belonging to the Euphorbiaceae family, consists of approximately 1300 species distributed globally (Webster, 1994). This genus, which includes trees and shrubs, is predominantly found in tropical and subtropical regions, with some species extending into cooler areas (Palmer & Pitman, 1972; Sharma et al., 2013). The name *Croton* is derived from the Greek word "*kroton*", meaning tick, due to the resemblance of its seeds to ticks. *Croton* species have played a key role in traditional medicine, particularly in Africa, where they are used to treat a variety of conditions, such as sexually transmitted infections, diabetes, malaria, fever, inflammation, and cancer (Iwu, 1997; Kambizi & Afolayan, 2001; Moremi et al., 2021; Oliver-Bever, 1986).

Phytochemical studies with *Croton* species have demonstrated the presence of several secondary metabolites, such as alkaloids, terpenoids (including diterpenes, triterpenes, and sesquiterpenes), flavonoids, and other phenolic compounds, which contribute to their therapeutic properties (Júnior et al., 2022). These compounds not only exhibit antioxidant and antimicrobial effects but also demonstrate significant antidiabetic, antihy- pertensive, anti-inflammatory, and anticancer activities (Kambizi & Afolayan, 2001; Moremi et al., 2021).The phytochemical diversity within the *Croton* genus makes it a promising source for the discovery of new therapeutic agents.

Brazil, home to one of the world's greatest biodiversities, is home to approximately 356 *Croton* species, with a significant number distributed across tropical ecosystems, such as the Caatinga, Atlantic Forest, and Amazon (Santos et al., 2005). Croton species in Brazil, particularly within the Caatinga biome, are notable for their diverse therapeutic applications in folk medicine. Many species are recognized for their anti-inflammatory, antimicrobial, antinociceptive, and gastroprotective activities, among others (Júnior et al., 2022). For instance, *C. linearifolius*, a species native to Brazil's semi-arid region, is traditionally used to treat conditions such as gastrointestinal disorders, pain, and inflammation (Silva et al., 2010).

The stems of *C. linearifolius* remain underexplored in phytochemical research, as most studies have focused on its leaves and roots, where compounds such as flavonoids, terpenes, and saponins have been identified (Coelho-de-Souza et al., 2013).

Furthermore, ecent studies highlight the pharmacological potential of these species, including gastroprotective and antiulcerogenic activities (Cartaxo et al., 2010).

Given the promising therapeutic activity of this species, it is essential to explore the phytochemical composition and antioxidant activity of the stem extracts. Preliminary studies have highlighted the rich diversity of secondary metabolites in *Croton* species, but there remains a significant gap in understanding the bioactive compounds present in the stems of *C. linearifolius* and their specific contribution to the therapeutic effects.

The antioxidant activity of *C. linearifolius* and other *Croton* species has garnered particular attention due to their role in neutralizing free radicals and reducing oxidative stress, a key factor in the development of chronic diseases such as cancer, cardiovascular diseases, and neurodegenerative conditions. The identification of major bioactive compounds, such as flavonoids, phenolic acids, and terpenes, and their correlation with antioxidant properties may provide valuable insights into the therapeutic potential of *C. linearifolius*.

This study aims to address the existing gap in the literature regarding the stems of *C. linearifolius*. Through detailed phytochemical analysis and evaluation of antioxidant activity via *in vitro* assays, the research will contribute to a better understanding of the bioactive substances in this species, offering new perspectives on their potential for therapeutic applications. Moreover, this study seeks to highlight the value of native plants from the Caatinga biome, supporting the sustainable use of these species for the development of new medicinal products.

Materials and methods

Plant material acquisition

Stems of *Croton linearifolius*, Figure 1, were gathered from Contendas do Sincorá National Forest in Contendas do Sincorá, Bahia State, Brazil. Exsiccates of the collected material were sent to the UEFS Herbarium for analysis, identification, and cataloging under accession number HUEFS 146620.

Figure 1 - Stems of the species *C. linearifolius*.

The research was conducted at the Laboratory of Natural Product Research (LAPRON), affiliated with the Center for Applied Chemistry Research (NUPESQ), located at the State University of Southwest Bahia (UESB) in Itapetinga, Bahia.

Experimental

Preparation of ethanolic extract

The stems of *C. linearifolius* were dried in a circulating air oven for 72 hours at a temperature of 40 °C and subsequently ground in a knife mill. After processing, the plant material was extracted by exhaustive percolation with 70% ethanol, and the obtained solution was evaporated in a rotary evaporator (Fisatom) at a temperature of 50 °C and 80 rpm to obtain the ethanolic extract (EE). The extract was stored in amber bottles and kept in the refrigerator until analysis.

Fractionation of the ethanolic extract

Considering the current emphasis on adopting “greener” methodologies in scientific research, the choice of solvents used in fractionation processes must align with environmentally sustainable principles. The use of toxic solvents, particularly organochlorine solvents, often conflicts with these guidelines. However, in this study, the solvents were selected based on their compatibility with the physicochemical properties of the target compounds, their extraction efficiency, and their role in achieving effective fractionation of the ethanolic extract (EE).

The fractionation of the ethanolic extract was carried out using a liquid-liquid partitioning technique. A total of 17 g of EE was initially dissolved in 50 mL of a 70% hydroethanolic solution to provide a hydrophilic matrix for sequential extractions with solvents of increasing polarity. Hexane, dichloromethane, and ethyl acetate were chosen to obtain the respective fractions: hexane (FH), dichloromethane (FD), and ethyl acetate (FAE). Each solvent serves a specific purpose in this methodology. Hexane was used to extract nonpolar compounds, such as lipophilic molecules, while dichloromethane, despite its toxicity, was selected for its intermediate polarity and efficiency in isolating compounds such as alkaloids and terpenoids. Ethyl acetate, a less toxic and biodegradable solvent, was employed to recover semipolar compounds.

Finally, the hydroalcoholic fraction (FHA) was obtained from the remaining solution, retaining hydrophilic compounds. While the use of dichloromethane may appear contradictory to the principles of green chemistry, its selection was justified by its ability to effectively isolate compounds essential to the objectives of this study. The use of this solvent was minimized, and efforts were undertaken to ensure proper handling and disposal in accordance with environmental safety standards.

Phytochemical prospecting

The phytochemical screening followed the methodology outlined by Matos (2009) to investigate the presence of secondary metabolites, including anthocyanins, alkaloids, catechins, coumarins, flavonoids, tannins, triterpenoids, and xanthones. The qualitative analysis was based on characteristic reactions, such as color changes, precipitation, foam formation, and pH-dependent coloration, which indicate the presence of these compounds.

Phytochemical component identification

To identify tannins, 5.0 mL of distilled water was added to 2.0 mL of the hydroethanolic solution in a test tube, followed by filtration and the addition of 1-2 drops of 10% ferric chloride solution. Blue coloration suggests the presence of hydrolysable tannins, while green coloration indicates condensed tannins.

To identify flavonoids, metallic magnesium fragments and drops of 5% sodium hydroxide solution were added to 2.0 mL of the hydroethanolic solution in another test tube. The observation of varied coloration suggests the presence of different flavonoid structures.

To identify alkaloids, 2.0 mL of the hydroethanolic solution was mixed with 2.0 mL of 0.1 M hydrochloric acid solution and heated for 10 minutes. After cooling and filtration, a few drops of Mayer, Dragendorff, and Wagner recognition reagents were added. The formation of slight turbidity or precipitate indicates the possible presence of alkaloids.

To identify saponins, 2 mL of the hydroethanolic solution were mixed with 5.0 mL of boiling water in a test tube. After cooling and vigorous agitation followed by 20 minutes of rest, the presence of saponins was classified by foam formation.

Evaluation of antioxidant activity

The assessment of antioxidant activity of the extracts obtained from the stems of *C. linearifolius* was carried out using two different methods: DPPH free radical scavenging and ferric ion reducing power (FRAP).

DPPH free radical scavenging method

The quantitative evaluation of antioxidant activity was conducted following the methodology described by Rufino et al. (2007). Initially, the reaction kinetics of the ethanolic extract were performed by spectrophotometric readings of the samples reacting with DPPH every five minutes, observing the reduction in sample absorbance until stabilization. The time determined by this test was used in the experiments with the obtained samples.

For the tests, five dilutions of each sample were prepared: EE, FH, FD (2.0; 4.0; 6.0; 8.0; 10.0 mg.mL^{-1}), and for FAE, lower concentrations were used (0.5; 1.0; 1.5; 2.0; 2.5 mg.mL^{-1}), as well as positive controls, rutin (0.05; 0.1; 0.15; 0.20; 0.25 mg.mL^{-1}) and quercetin (0.025; 0.05; 0.075; 0.10; 0.125 mg.mL^{-1}). Then, 0.1 mL of each sample dilution was transferred to test tubes in triplicate.

In a dark environment, 3.9 mL of 0.06 Mmol.L^{-1} methanolic DPPH solution was added to the tubes and homogenized in a vortex. After 40 minutes (time determined by the reaction kinetics of the ethanolic extract conducted previously), readings were taken using a Shimadzu UV-Vis 1800 spectrophotometer at a wavelength of 515 nm. The blank used for the readings consisted solely of methanol.

From the absorbance readings of the samples, the Effective Concentration 50 (EC50) was calculated, which corresponds to the amount of sample required to reduce the initial concentration of DPPH free radical by 50%, and the final value was expressed in μg.mL^{-1}, along with the percentage of inhibition, calculated using the equation (1):

$$\% = \left(\frac{\text{ABS DPPH} - \text{ABS Sample}}{\text{ABS DPPH}} \right) \times 100, \tag{1}$$

where ABS DPPH is the absorbance reading of the pure DPPH solution and ABS Sample is the recorded the ability of the samples and standards to scavenge the DPPH free radical, with those producing a higher percentage of inhibition being the most efficient.

The antioxidant activity of the samples was also expressed by the antioxidant activity index (AAI) proposed by Scherer and Godoy (2009). This index facilitates comparing the antioxidant strength of different extracts and is calculated according to equation (2):

$$AAI = \frac{\text{Initial concentration of DPPH } (\mu\text{g.mL}^{-1})}{\text{EC50 } (\mu\text{g.mL}^{-1})}. \quad (2)$$

The concentration of DPPH used in the assay corresponded to 24 μg.mL^{-1} for a solution of 0.06 Mmol.L^{-1}. Antioxidant activity is considered poor when AAI < 0.5, moderate when AAI is between 0.5 and 1.0, strong when AAI is between 1.0 and 2.0, and very strong when AAI > 2.0.

Method of ferric ion reducing power

The evaluation of the antioxidant activity of the ethanolic extract (EE) and the hexane (FH), dichloromethane (FD), and ethyl acetate (FAE) fractions by the FRAP method was carried out following the methodology described by Rufino et al. (2006), with modifications. The FRAP reagent was prepared by mixing 25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL of a 10 mM TPTZ solution, and 2.5 mL of a 20 Mmol.L^{-1} ferric chloride aqueous solution, to be used immediately after preparation.

To determine the antioxidant activity of the extract and fractions using the FRAP method, a concentration of 2000 μg.mL^{-1} of each sample (ethanolic extract and fractions) was used, except for the ethyl acetate fraction, which was used at a lower concentration of 500 μg.mL^{-1}. Under dark conditions, 90 μL aliquots of each sample were transferred to test tubes (in triplicate). Then, 270 μL of deionized water and 2.7 mL of the FRAP reagent were added. The samples were vortexed and maintained in a water bath at 37 °C for 30 minutes. After this time, absorbance readings were taken at 595 nm, using the FRAP reagent as a blank to calibrate the spectrophotometer.

The standard curve was prepared with ferrous sulfate solutions at concentrations of 500, 1000, 1500, and 2000 μM. The same procedure used for the samples was applied to the ferrous sulfate standard curve concentrations. Subsequently, absorbance readings were plotted on the Y-axis, and the ferrous sulfate concentrations (in μM) were plotted on the X-axis, yielding the linear regression equation (3):

$$y = 0.000667x - 0.043500, \quad (3)$$

with a correlation coefficient of $R^2 = 0.998182$. The average absorbance values of the samples were substituted into the previously obtained equation to determine the amount of ferrous ions (Fe^{2+}) present in the sample, equivalent to the concentration in μM of $FeSO_4 \cdot 7H_2O$. The resulting value was then converted to mg of $FeSO_4 \cdot 7H_2O$ per gram of extract, and the final result was expressed as mg of Fe^{2+} per gram of extract.

The use of only four points in the standard curve for quantification by the FRAP method was adopted based on the robustness of the methodology and the high correlation coefficient obtained ($R^2 = 0.998182$). This coefficient demonstrates a strong linearity between the selected points, ensuring the reliability of the standard curve for determining the concentrations of ferrous sulfate (Fe^{2+}) in the evaluated extracts and fractions. The selection of the points (500, 1000, 1500, and 2000 μM) was based on the concentration range most relevant for the analysis, adequately covering the necessary interval for the calculations and ensuring the accuracy of the results.

Additionally, this approach was aligned with the experimental conditions described by Rufino et al. (2006), with modifications that did not compromise the validity of the method.

Determination of total phenolic content

The determination of total phenolic content in the ethanolic extract (EE) and fractions was conducted following the methodology described by Sousa et al., (2007), using visible region spectroscopy with the Folin-Ciocalteu method, with modifications. For the experiment, a concentration of 2000 μg.mL^{-1} was used for EE, FH, FD, and a concentration of 500 μg.mL^{-1} was used for FAE, with methanol as the solvent for the preparation of all solutions.

In 10 mL volumetric flasks, 500 μL aliquots of each sample were mixed with 500 μL of the Folin-Ciocalteu reagent and 6 mL of deionized water for 60 seconds. Then, 2 mL of 15% aqueous solution of sodium carbonate

(Na_2CO_3) were added to the mixture, and the solutions were mixed for another 30 seconds. Finally, the solutions were made up to 10 mL with deionized water. After 2 hours, absorbance readings were taken at 750 nm using a spectrophotometer, with methanol and all reagents except the samples as blanks. All samples were prepared in triplicate, and the procedure was conducted in the dark.

The total phenolic content was determined by interpolating the absorbances of the samples into a linear regression equation obtained from the standard curve constructed with gallic acid standards (40, 80, 120, 160, and 200 μg.mL^{-1}), and the results were expressed in mg equivalent of gallic acid (EGA) per gram of extract. The linear regression equation is given by equation (4):

$$y = 0.006177x - 0.074867, \tag{4}$$

with a correlation coefficient $R^2 = 0.999344$.

Determination of total flavonoids content

The concentration of total flavonoids was determined by a colorimetric assay using aluminum chloride as a chromophoric agent, according to the methodology described by Santos and Blatt 1998, with modifications. For the assay, a concentration of 1000 μg.mL^{-1} was used for EE, FH, FD, and a concentration of 250 μg.mL^{-1} was used for FAE, with methanol as the solvent.

In 5 mL volumetric flasks, 1 mL aliquots of each sample were mixed with 500 μL of a 10% methanolic solution of aluminum chloride, and the volume was completed with a 5% methanolic solution of acetic acid. After 30 minutes, absorbance readings were taken at 425 nm using a spectrophotometer, with methanol and all reagents except the samples as blanks. All samples were prepared in triplicate, and the procedure was conducted in the dark.

To determine the flavonoid content, a standard curve of quercetin was constructed using concentrations of 5.0, 10.0, 15.0, 20.0, and 25.0 μg.mL^{-1}, with the absorbances of the samples interpolated into the linear regression equation. The total flavonoid content was expressed as mg equivalent of quercetin (EQ) per gram of extract. The linear regression equation obtained by equation (5):

$$y = 0.017527x + 0.015100, \tag{5}$$

with a correlation coefficient $R^2 = 0.998599$.

Method of ferric ion reducing power

The evaluation of the antioxidant activity of the ethanolic extract (EE) and the hexane (FH), dichloromethane (FD), and ethyl acetate (FAE) fractions by the FRAP method was conducted following the methodology described by Rufino et al. (2006), with modifications. The FRAP reagent is prepared by mixing 25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL of a 10 mM TPTZ solution, and 2.5 mL of a 20 mM aqueous solution of ferric chloride, to be used immediately after preparation.

To determine the antioxidant activity of the extract and fractions using the FRAP method, a concentration of 2000 μg.mL^{-1} of each sample (ethanolic extract and fractions) was used, except for the ethyl acetate fraction, which was used at a lower concentration of 500 μg.mL^{-1}. In the dark, 90 μL aliquots of each sample were transferred to test tubes (in triplicate). Subsequently, 270 μL of deionized water and 2.7 mL of the FRAP reagent were added. The samples were vortexed and maintained in a water bath at 37 °C for 30 minutes. After this time, absorbance readings were taken at a wavelength of 595 nm, using the FRAP reagent as a blank to calibrate the spectrophotometer.

The standard curve was prepared with ferrous sulfate solutions at concentrations of 500, 1000, 1500, and 2000 μM. The same procedure used for the samples was performed with the concentrations of the ferrous sulfate standard curve. Subsequently, absorbance readings were plotted on the Y-axis and ferrous sulfate concentrations (in μM) on the X-axis, obtaining the linear regression equation given by equation (6):

$$y = 0.000667x - 0.043500, \tag{6}$$

with a correlation coefficient $R^2 = 0.998182$. The mean absorbance values of the samples were substituted into the previously obtained equation to determine the amount of ferrous ions (Fe^{2+}) present in the sample,

equivalent to the concentration in µM of $FeSO_4.7H_2O$. Subsequently, the found value was converted to mg of $FeSO_4.7H_2O$ per gram of extract, and the final result was presented in mg of Fe^{2+} per gram of extract.

Determination of total phenolic content

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In 10 mL volumetric flasks, 500 μL aliquots of each sample were mixed with 500 μL of the Folin-Ciocalteu reagent and 6 mL of deionized water for 60 seconds. Then, 2 mL of 15% aqueous solution of sodium carbonate (Na_2CO_3) were added to the mixture, and the solutions were mixed for another 30 seconds. Finally, the solutions were made up to 10 mL with deionized water. After 2 hours, absorbance readings were taken at 750 nm using a spectrophotometer, with methanol and all reagents except the samples as blanks. All samples were prepared in triplicate, and the procedure was conducted in the dark.

The total phenolic content was determined by interpolating the absorbances of the samples into the linear regression equation obtained from the standard curve constructed with gallic acid standards (40, 80, 120, 160, and 200 μg.mL^{-1}. The results were expressed in mg equivalent of gallic acid (EGA) per gram of extract. The linear regression equation obtained by the equation (7):

$$y = 0.006177x - 0.074867, \tag{7}$$

with a correlation coefficient $R^2 = 0.999344$.

Determination of total flavonoids content

The concentration of total flavonoids was determined by a colorimetric assay using aluminum chloride as a chromophoric agent, according to the methodology described by Santos and Blatt (1998), with modifications. For the assay, a concentration of 1000 μg.mL^{-1} was used for EE, FH, and FD, and a concentration of 250 μg.mL^{-1} was used for FAE, with methanol as the solvent.

In 5 mL volumetric flasks, 1 mL aliquots of each sample were mixed with 500 μL of 10% methanolic solution of aluminum chloride, and the volume was completed with 5% methanolic solution of acetic acid. After 30 minutes, absorbance readings were taken at 425 nm using a spectrophotometer, with methanol and all reagents except the samples as blanks. All samples were prepared in triplicate, and the procedure was conducted in the dark.

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$$y = 0.017527x + 0.015100, \tag{8}$$

with a correlation coefficient $R^2 = 0.998599$.

Results e discussion

Yield of fractions obtained from crude extract

From 17 g of the crude extract, 1.83 g of the hexane fraction (FH), 2.2 g of the dichloromethane fraction (FD), and 0.83 g of the ethyl acetate fraction (FAE) were obtained through a liquid-liquid partitioning process. Mass and percentage yields for the extracts are presented in Table 1. The extraction yields of the fractions indicate that the constituents present in the plant stems are predominantly polar, as the highest yield was obtained for the hydroalcoholic fraction, where the most polar compounds are found, especially glycosylated derivatives.

Table 1 - Yield of fractions after partitioning of the EE.

Fractions	Mass Yield (g)	Yield (%)
Hexane (FH)	1.83	10.8
Dichloromethane (FD)	2.2	12.9
Ethyl Acetate (FAE)	0.83	4.9
Hydroalcoholic (FHA)	10.6	60.6
Total	15.46	90.0

Phytochemical prospecting

Through the in vitro phytochemical prospecting of the ethanolic extract from the stems of *C. linearifolius*, it was possible to identify the presence of classes of secondary metabolites such as flavonoids, condensed tannins, saponins, and alkaloids, constituents that have a wide variety of biological activities, such as antimicrobial, antioxidant, and antitumor properties.

Plants rich in tannins are used in folk medicine for the treatment of various diseases, such as arterial hypertension, stomach problems, urinary system issues, and general inflammatory processes. Flavonoids hold pharmacological importance as they exhibit anticarcinogenic, anti-inflammatory, antiallergic, and antibacterial properties. The complexation of saponins with cholesterol, a remarkable property attributed to saponins, has led to a significant number of studies aimed at evaluating the use of saponins in the diet to reduce serum cholesterol levels (Price et al., 1987). Janeway (2006) linked the action of saponins on the immune system to the healing process, as these substances can modify membrane permeability, facilitating the passage of immune cells to the site of injury. Alkaloids are a structurally diverse class of secondary metabolites characterized by a wide range of biological activities, such as anticholinergic, emetic, antimalarial, antihypertensive, hypnoanalgesic, amoebicidal, antiviral, myorelaxant, anesthetic, antitumor, antitussive, and cholinergic, among others. Prior knowledge of the classes of chemical components found in plants is of great importance because once the presence of certain chemical groups is detected, phytochemical and biological studies are directed accordingly.

Evaluation of antioxidant activity - DPPH free radical scavenging method

The antioxidant activity of the extracts and fractions was evaluated using the DPPH free radical scavenging method. The results are presented in Table 2.

Table 2 - Antioxidant activity results using the DPPH method.

Samples	Inhibition (%) $\pm$ SD	CE50 (μg.mL^{-1})	IAA
EE	74.85 $\pm$ 0.36	148.17	0.16
FH	18.18 $\pm$ 0.24	-	-
FD	67.17 $\pm$ 0.87	324.75	0.074
FAE	80.62 $\pm$ 3.77	24.50	1.0
Rutin	59.87 $\pm$ 1.55	4.95	4.84
Quercetin	90.59 $\pm$ 0.63	1.54	15.5

SD (standard deviation); - (Not calculated)

Table 2 shows the antioxidant activity results using the DPPH method. The evaluation of the antioxidant activity results allows us to conclude that the ethyl acetate fraction (FAE) exhibits the highest antioxidant potential, as evidenced by its superior inhibition of the DPPH free radical, with 80.62%. This result indicates that the compounds present in FAE are highly efficient in neutralizing free radicals. In contrast, the hexane fraction (FH) demonstrated the lowest antioxidant activity, with only 18.18% inhibition, which is consistent with its lower ability to interact with DPPH radicals.

The antioxidant activity observed in the extracts and fractions can be attributed to the presence of phenolic compounds, which are known for their reducing properties due to the hydroxyl groups in their structures. These groups allow the phenolic compounds to donate hydrogen atoms or electrons to free radicals, including the DPPH radical used in this experiment. As these compounds are typically polar, they tend to dissolve more easily in polar solvents. This explains why the ethyl acetate fraction, a more polar solvent compared to

hexane and dichloromethane, exhibited better antioxidant performance. The higher polarity of ethyl acetate allows it to extract a higher proportion of phenolic compounds, enhancing its antioxidant capacity.

On the other hand, the hexane and dichloromethane fractions, which are less polar, showed lower efficiency in neutralizing free radicals. Hexane, being a nonpolar solvent, primarily extracts nonpolar compounds, such as lipids, which are less effective at donating electrons or hydrogen atoms to free radicals. As expected, the dichloromethane fraction, also less polar, showed intermediate antioxidant activity (67.17%), likely due to a mixture of polar and nonpolar compounds, which may have limited its ability to donate electrons or hydrogen radicals to the DPPH radical.

The ethanolic extract (EE) exhibited moderate antioxidant activity (74.85% inhibition), lower than that of FAE but higher than the FH and FD fractions. This can be attributed to the fact that the ethanolic extract contains a diverse range of compounds, both polar and nonpolar, resulting in interactions among them, which may impair the ideal antioxidant activity of certain reducing molecules. Compounds with more polar characteristics are less likely to interact with nonpolar substances, which may reduce the overall antioxidant effectiveness of the ethanolic extract compared to FAE, which is more selective in its composition.

When compared to other studies, the results of this study are in line with the findings of previous research on the antioxidant activity of *Croton* species. For example, a study by Santos-Alves (2017) demonstrated that the antioxidant activity of the extract from *C. cordiifolius* bark ranged from 4.54% to 44.63%, with the EC50 value above 1000 μg.mL^{-1}, indicating a significantly lower antioxidant potential compared to the ethanolic extract in this study. Additionally, Costa et al. (20172) evaluated the antioxidant potential of extracts from *C. argyrophyllus* and observed that the ethyl acetate fraction showed an inhibition percentage of 95.06 $\pm$ 0.14% with an EC50 value of 4.27 $\pm$ 0.016 μg.mL^{-1}, values higher than those found in this study but still consistent with the higher antioxidant potential of the ethyl acetate fraction.

Furthermore, a study by Brito (2014) demonstrated that the ethyl acetate fractions from *C. linearifolius* showed high DPPH radical inhibition, with values of 89.90% and 83.47%. These results are very similar to those found in this study for the ethyl acetate fraction, confirming the high antioxidant efficiency of this fraction.

Method of ferric ion reducing antioxidant power

The antioxidant activity was also evaluated using the FRAP method, and the results are shown in Table 3.

Table 3 - Antioxidant activity results using the FRAP method.

Samples	**mg Fe^{2+}. g^{-1} extract**
EE	4.21
FH	2.27
FD	3.66
FAE	16.55
Rutin	82.60
Quercetin	254.74

Based on the FRAP test results, significant differences were observed among the evaluated fractions and extracts regarding their ability to reduce Fe^{3+} to Fe^{2+}, which is indicative of antioxidant activity. The ethyl acetate fraction (FAE) showed the highest reduction capacity, with 16.55 mg Fe^{2+}.g^{-1} of extract, highlighting its high antioxidant activity compared to the other fractions and the ethanolic extract (EE), which exhibited 4.21 mg Fe^{2+}.g^{-1} of extract.

These results support the efficiency of the FAE in neutralizing reactive oxygen and metal species, similar to what has been observed in other studies with *Croton* species, where fractions containing phenolic compounds, such as flavonoids, demonstrated strong antioxidant activity. The behavior observed in the FRAP test can be explained by the presence of flavonoids and other phenolic compounds in the ethyl acetate fractions, which are known for their ability to donate electrons and chelate metals, thus increasing the reduction capacity of metal ions.

FAE, being richer in phenolic compounds, showed higher antioxidant capacity, reflecting its efficiency in reducing Fe^{3+}. This is consistent with the literature, which highlights flavonoids as key compounds for

antioxidant activity due to their hydroxyl groups that facilitate the donation of electrons or hydrogen atoms to free radicals (Nardi et al., 2003; Rocha et al., 2021).

In contrast, the hexane fraction (FH) showed the lowest antioxidant capacity in the FRAP test, with 2.27 mg $Fe^{2+}.g^{-1}$ of extract, which can be attributed to the predominant presence of non-polar compounds, such as lipids, which have a reduced ability to donate electrons or hydrogen atoms, as evidenced by other studies indicating that lipophilic compounds generally show lower antioxidant activity compared to phenolic compounds (Rocha et al., 2021).

Additionally, the dichloromethane fraction (FD) showed an intermediate reduction activity, with 3.66 mg $Fe^{2+}.g^{-1}$ of extract, which can be explained by the presence of a mixture of polar and non-polar compounds, similar to what was observed in the study by Lopes et al. (2004), which also noted antioxidant activity in *Croton* fractions, but with varying intensities depending on the polarity of the compounds present.

When compared with the positive standards rutin and quercetin, Table 3, which exhibited exceptional antioxidant activities, with values of 82.60 and 254.74 mg $Fe^{2+}.g^{-1}$ extract, respectively, the *Croton* extracts and fractions exhibited more moderate activities, but still considerable, especially the ethyl acetate fraction, which approached the values observed for the standards. This reflects the importance of phenolic compounds, such as flavonoids, in antioxidant activity (Rocha et al., 2021).

Determination of total phenolics and flavonoids

Based on the results of the total phenolics and flavonoids quantification in the *Croton* samples, presented in Table 4, we can draw an interesting connection between the flavonoids identified in species of the *Croton* genus and their observed antioxidant activity, highlighting the diversity of bioactive compounds present within this genus. Phytochemical studies conducted on various *Croton* species reveal the presence of a wide range of flavonoids, which are particularly relevant to the antioxidant potential of these plants. The relationship between the flavonoids found in the ethanolic extract and ethyl acetate fraction (FAE) and those described in other *Croton* species helps to better understand the antioxidant properties observed.

Table 4 - Results of quantification tests for total phenolics and flavonoids

Samples	**Total Phenolics** (mg of GAE / g extract)	**Total Flavonoids** (mg of EQ / g extract)
EE	58.43	15.67
FH	30.37	11.94
FD	59.21	21.64
FAE	105.04	73.62
FHA	22.00	-

Flavonoids such as quercetin, kaempferol, and taxmarixetin are commonly identified in various *Croton* species, including *C. steenkampianus*, *C. sylvaticus*, and *C. zambesicus* (Aderogba, Ndhlala, Rengasamy, & Van Staden, 2013; Aderogba, Ndhlala, & Van Staden, 2013; Prozesky, 2004). These compounds play a crucial role in the antioxidant properties of the plants due to their ability to donate electrons and hydrogens to free radicals, as well as their metal-chelating capacity, which is directly reflected in the higher antioxidant activity of the ethanolic and ethyl acetate fractions observed in the DPPH and FRAP assays.

For example, quercetin has been isolated from several *Croton* species, including *C. zambesicus* and *C. sylvaticus*, and is one of the most well-known flavonoids for its potent antioxidant properties (Aderogba et al., 2011; Ndhlala et al., 2013). Similarly, the *Croton* samples analyzed in this study showed significant concentrations of total flavonoids, with the ethyl acetate fraction (FAE) being particularly rich in flavonoids such as quercetin, which can be correlated with its superior antioxidant activity.

The presence of flavonoids in high concentrations in the ethanolic and ethyl acetate fractions suggests that these fractions may contain compounds similar to those isolated from other *Croton* species, such as vitexin and isovitexin, which have also been associated with antioxidant activities (Aderogba et al., 2011; Mohamed et al., 2009). Furthermore, the fractions with higher concentrations of phenolic compounds and flavonoids, such as FAE, also exhibited superior performance in antioxidant assays, suggesting that the antioxidant properties of *Croton* are closely linked to the presence of these bioactive compounds.

For example, the identification of flavonoids such as vitexin, found in *C. zambesicus* (Mohamed et al., 2009), and quercetin, present in several *Croton* species (Prozesky, 2004), confirms the importance of these compounds for the antioxidant activity observed in the analyzed *Croton* samples. Previous phytochemical studies, such as those by Aderogba et al. (2011), identified flavonoid glycosides such as vitexin and aglycones like quercetin in various parts of *Croton* plants. These flavonoids possess potent antioxidant properties, which are consistent with the results observed in the present study, where the fractions with higher total flavonoid concentrations, such as FAE, exhibited greater capacity to neutralize free radicals.

Similarly, other *Croton* species, such as *C. gratissimus* and *C. sylvaticus*, also contain flavonoids that play significant roles in protecting against oxidative stress, as evidenced by the discovery of kaempferol and taxmarixetin in earlier studies (Aderogba, Ndhlala, & Van Staden, 2013). These connections between the flavonoids identified in the analyzed fractions and the flavonoid compounds described in other *Croton* species reinforce the idea that flavonoids are key compounds for the antioxidant properties of plants within this genus.

Flavonoids, especially those found in the ethanolic and ethyl acetate fractions, such as quercetin and vitexin, play a central role in the antioxidant activities observed in the *Croton* samples. This provides a basis for future investigations into their therapeutic potential and applications in natural products aimed at protecting against diseases related to oxidative stress.

Conclusions

This study allowed for the qualitative evaluation of the chemical constituents present in the ethanolic extract obtained from the stems of *Croton* linearifolius. Through phytochemical screening, the presence of classes of secondary metabolites such as flavonoids, condensed tannins, saponins, and alkaloids was detected, constituents that exhibit a wide variety of biological activities. Quantitative tests confirmed that the species under study has antioxidant potential and high concentrations of total phenolics and flavonoids, predominantly found in the ethyl acetate fraction. This underscores the importance of isolating and identifying the constituents present in the species under study to elucidate its structure and chemical composition.

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Author contributions

C. T. S. de Franca participated in investigation, methodology, data curation, writing – original draft. M. M. da S. Passos participated in data curation, formal analysis, visualization, validation, writing – review & editing. M. A. R. Costa participated in writing – original draft, writing – review & editing, conceptualization, validation. E. L. Oliveira participated in methodology, formal analysis, visualization, writing – review & editing. S. A. Gualberto participated in conceptualization, supervision, validation, writing – review & editing.

Conflicts of interest

No potential conflict interest was reported by the authors.

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