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Evaluation of in vitro Antioxidant Properties of Methanol Extract and Fractions of *Cola rostrata* (K. Schum) Leaves

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ABSTRACT

Background and Purpose: Medicinal plants are used for the treatment of diseases because of the presence of therapeutic phytochemical constituents. Antioxidants play important roles in preventing and mitigating diseases. The plant *Cola rostrata* K. Schum (Sterculiaceae) is used in traditional medicine for pain relief, diabetes, erectile dysfunction, dental hygiene, and other conditions. The objective was to evaluate the total phenolic content and in vitro antioxidant properties of *C. rostrata* leaf extract and fractions.

Methods: The dried and pulverized leaves of the plant were soaked in methanol for 72 h for extraction. Fractionation of the methanol extract was done using n-hexane, ethyl acetate and methanol in a column. The extract and fractions were screened for phytochemical constituents. The total phenolic content was determined using the Folin-Ciocalteu's assay. In vitro antioxidant properties were evaluated using the 2,2-diphenyl-1-picyhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and hydroxyl radical scavenging assay against ascorbic acid (standard).

Results: The phytochemical screening revealed the presence of alkaloids and flavonoids among others. The total phenolic content was higher in the extract (1.34 mg/g GAE) compared to the fractions. In the DPPH assay, the n-hexane fraction had lower IC_{50} (0.93 µg/mL) than the standard (3.44 µg/mL). In the FRAP assay, the ethyl acetate fraction had EC_{50} of 0.71 µg/mL which was lower than that of the standard (1.34 µg/mL). In the hydroxyl radical scavenging assay, the n-hexane had lower IC_{50} (0.37 µg/mL) than the standard (1.45µg/mL).

Conclusion: The findings suggest that the leaves of *C. rostrata* have potential antioxidant property which may be due to the high phenolic content.

INTRODUCTION

Free radicals can be stabilized or inactivated by antioxidant chemical components before they damage cells (Vishnoi et al., 2018). Antioxidants can be enzymatic or non-enzymatic in nature. Superoxide dismutase, glutathione peroxidase, and catalase are examples of enzyme-based antioxidants, whereas non-enzymatic antioxidants include ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione, carotenoids, and flavonoids. Enzymatic antioxidants may not be enough to sustain optimal cellular activities in the presence of oxidative stress, and non-enzymatic (dietary) antioxidants may be necessary (Dias *et al.*, 2020). Antioxidants either in the form of raw extracts or their chemical constituents are particularly powerful at stopping the damaging processes brought on by oxidative stress (Shah, 2015).

Consuming antioxidant-rich fruits and vegetables on a regular basis has been shown to lower the chance of developing chronic disease (Panche *et al.*, 2016). Different synthetic antioxidant substances such as ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione, carotenoids, lower reactive oxygen species. These synthetic antioxidant molecules have been linked to negative side effects (Lourenço *et al.*, 2019). For example, it has been proven that ascorbic acid is cytotoxic and can cause apoptosis. Additionally, the availability, affordability, and lability of synthetic antioxidants limit their use (Akimat *et al.*, 2021). Dietary supplements have attracted a lot of attention as potential replacements for synthetic antioxidants. In vitro antioxidant assays have been used for evaluating the antioxidant potentials of natural products (Okolo and Orisakwe, 2021). Such assays include 2,2-diphenyl-1-picyhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and hydroxyl radical scavenging assay.

Cola rostrata K. Schum (Sterculiaceae) is a perennial tree found in lowland rain forests in tropical Africa, particularly in southern Cameroon, Gabon, and southeast Nigeria (Odion *et al.*, 2019). It is a plant that is used in traditional medicine in Nigeria to treat insomnia, headaches, and diarrhea (Essien *et al.*, 2015). Previous studies have confirmed the presence of bioactive constituents of therapeutic significance (Odion *et al.*, 2013)which could combat oxidative stress-mediated pathologies. Since synthetic antioxidants have been associated with undesirable side effects, there have been searches for natural antioxidants from plants. There is paucity of information regarding the in vitro antioxidant activity of *C. rostrata* K. Schum leaves. This study assessed the in vitro antioxidant properties of the methanol extract (MEcr) and its subsequent fractions.

MATERIALS AND METHODS

Collection and processing of plant material

Fresh leaves of *Cola rostrata* were harvested from a forest in Nsukka, Enugu State, Nigeria, where they naturally flourish. The plant was identified by a taxonomist attached to the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State, Nigeria where a voucher specimen (InterCEDD 104) was deposited. The leaves were washed in tap water to remove dust and were shade-dried for two weeks. The dried leaves were then ground using a miller, weighed, packaged in an airtight container with clear labels, and kept at room temperature prior to extraction.

Preparation of methanol extract

Maceration was carried out by placing 528 g of finely ground leaves in 4.5 L of analytical-grade methanol for 72 h based on a modified method (Ekalu and Habila, 2020). The mixture was periodically stirred and was filtered twice; first with muslin cloth and later with Whatman filter papers # 1. The filtrate was concentrated using a rotary evaporator at 50℃ and then dried over a hot air oven at 35℃. Prior to usage, the concentrated extract (MEcr) was weighed, labeled, and kept in a refrigerator at 4℃.

Fractionation of extract

A 20 g of MEcr was fixed in 200 g of silica gel with methanol to form slurry. The slurry was poured into long glass column. The column was eluted with n-hexane (500 mL, 3 times), ethyl acetate (500 mL, 3 times) and finally with 1000 mL of methanol (Ostberg-Potthoff *et al.*, 2019).

Qualitative phytochemical screening

Standard qualitative phytochemical tests were done on the crude methanol extract and the various fractions (Harborne, 1998). The presence or absence of a specific phytochemical group was determined by visual examination of color or fronting.

Determination of total phenolic contents

The Folin-Ciocalteu technique adopted from Do *et al.* (2014), was used to measure the total phenolic content of the extract and fractions with some changes. In triplicates, 1 mL of the extract was combined with 2 mL of the Folin-Ciocalteu reagent. The mixture was heated for 30 min at 40℃ with 20 s of shaking and read at 765 nm absorbance. Gallic acid was used as a standard for plotting the calibration curve. The total phenolic contents of the extract and fractions were calculated as mg of gallic acid equivalents (GAE) per gram (g).

DPPH free radical scavenges assay

The is based on the assay method described by (Nithya and Madhavi, 2017) and it involves the use of 1,1-dipheny-2-picrylhydrazyl (DPPH) radical scavenging assay. Briefly, the methanol extract and fractions were made in five different concentrations (0.001, 0.01, 0.1, 1, and 10 g/mL). Different concentrations of ascorbic acid were made in the same concentrations as well. A quantity (0.5 mL) of 0.3 mM DPPH in methanol was added to a clean test tube after 1 mL of each sample under study had been added. After shaking, the mixture was allowed to stand at room temperature in the dark for 15 min. As a starting point, blank solutions containing 2.5 mL of the

solution of extract or fraction under study and 1 mL of methanol were utilized. Ascorbic acid at the same concentrations as the examined extract and fractions were employed as the positive control, whereas the negative control contained 2.5 mL of DPPH solution and 1 mL of methanol. The absorbance was read at 517 nm using a spectrophotometer after incubation in the dark. Three replicates of the experiments were run. The equation below was used to calculate the DPPH radical scavenging activity (Yeo and Shahidi, 2019).

$$\% \text{ Radical scavenging activity} = \frac{A_c - A_s}{A_c} \times 100$$

Where A_s is the absorbance of the sample, and A_c is the absorbance of the control. The half maximal inhibitory concentration (IC_{50}) of the extract and each fraction was computed from a plot of percentage DPPH free radical inhibition versus the extract concentration.

Ferric reducing power (FRAP) assay

The assay carried out based on the method described by Guchu *et al.* (2020) with some modifications. In summary, 2 mL of phosphate buffer (0.2 M, pH 6.6), 2 mL of potassium ferricyanide, and 5 different concentrations of extract and fractions (25, 30, 100, 200, and 400 g/ml) and ascorbic acid were combined. Each mixture was incubated for 20 min at 50℃. Following the addition of 2 mL of 10% trichloroacetic acid (TCA), the mixture was centrifuged at 1000 rpm for 10 min. Of the supernatant, 2 mL was aspirated and combined with 1 mL of ferric chloride (0.1 %) and 2 mL of distilled water. A UV-Vis spectrophotometer was then used to measure and record the absorbances spectrophotometrically at 700 nm in triplicates. The graph of absorbance at 700 nm against extract/fraction concentrations was used to identify the concentrations of the extract or fraction that produced an absorbance value of 0.5 and was taken as the median effective concentration (EC_{50}).

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was carried out based on the model described by (Rajkumar *et al.*, 2010) with minor modification. A quantity (2.4 mL) of phosphate buffer (pH 7.8) was added to test tubes to create the reaction mixture. With the exception of the controls, the same test tubes received 90 mL of 1 mM phenanthroline, 150 mL of 0.1 mM hydrogen peroxide, 60 mL of 1 mM iron (III) chloride, 1.5 mL of the extract or fractions, and ascorbic acid (the standard) at various concentrations (25, 30, 100, 200, and 400 g/mL), before being incubated for 5 min at room. Radical scavenging activity was determined based on the rise in absorbance at 560 nm using the formula:

$$\% \text{ Radical scavenging activity} = \frac{Ab_c - Ab_s}{Ab_c} \times 100$$

Where Abc is the absorbance for control and Abs is absorbance for the sample

Statistical analysis

Data obtained were analyzed using Statistical Package for the Social Sciences (SPSS version 23.0). Quantitative data represent mean ± standard deviation. Data were subjected to statistical analysis using one way ANOVA plus *Tukey post hoc* test. $P<0.05$ was considered statistically significant.

RESULTS

Percentage yield

Table 1 shows the yield of the methanol extract (47 g) and the fractions of NHF, EAF, and MEF with values 5.1 g, 9.2 g, and 7.4 g respectively.

Table 1: Yield of extract and fractions of *Cola rostrata* leaves

Samples	Weight of Ground Leaves (g)	Weight of Extract (g)	Yield (g)	Percentage Yield (%w/w)
MEcr	528	-	47	8.9
NHF	-	20	5.1	25.5
EAF	-	20	9.2	46
MEF	-	20	7.4	37

MEcr = methanol extract; EAF = ethyl acetate fraction; NHF = n-hexane fraction; MEF = methanol fraction.

Qualitative phytochemical screening

Methanol extract and fractions of *C. rostrata* leaves show the presence of alkaloids, tannins, saponins, flavonoids, terpenoids, cardiac glycosides, reducing sugars, steroids, fats and oils (Table 2).

Table 2: **Phytochemical constituents of extract and fractions of *Cola rostrata* leaves**

Phytoconstituents	MEcr	NHF	EAF	MEF
Alkaloids	+	+	+	-
Tannins	+	-	+	+
Saponins	+	-	+	+
Flavonoids	+	-	+	+
Terpenoids	+	+	+	+
Cardiac glycosides	+	-	+	+
Reducing sugars	+	-	-	+
Steroids	+	+	-	-
Fat and Oils	+	+	-	-

(+) = Present; (-) = Absent; MEcr = methanol extract; EAF = ethyl acetate fraction; NHF = n-hexane fraction; MEF = methanol fraction.

Total phenolic content

Table 3 shows the total phenolic contents (mg/g GAE) in various fractions of *C. rostrata*. The total phenolic content at 1000 µg/mL ranged from 0.02 mg/g GAE in n-hexane fraction to 0.11 mg/g GAE in methanol fraction. At 500 µg/mL, the total phenolic content ranged from 0.11 in methanol fraction to 1.34 mg/g GAE in the methanol extract. At 250 µg/mL, the total phenolic content was highest (0.51 mg/g GAE) in methanol fraction and lowest in n-hexane fraction.

Table 3: Total phenolic content (mg/g GAE) extract and fractions of *Cola rostrata*

Conc. (µg/mL)	MEcr (mg/g GAE)	NHF (mg/g GAE)	EAF (mg/g GAE)	MEF (mg/g GAE)
250	0.48	0.28	0.35	0.51
500	1.34	0.46	0.27	0.11
1000	0.06	0.02	0.04	0.11

MEcr = methanol extract; EAF = ethyl acetate fraction; NHF = n-hexane fraction; MEF = methanol fraction.

A notable *in vitro* concentration-dependent DPPH radical scavenging activity was also observed in the methanol extract and fractions of *C. rostrata*, and ascorbic acid (Table 4). The percentage inhibition for ascorbic acid (standard) was higher than those of ethyl acetate fraction, n-hexane fraction, methanol fraction and methanol extract. The IC_{50} values were highest in the extract, followed by ethyl acetate fraction, ascorbic acid, methanol fraction and n-hexane fraction in that order.

Ferric reducing antioxidant power (FRAP)

Table 5 shows the concentration-dependent increase in absorbance values at a wavelength of 700 nm. The ethyl acetate fraction displayed higher absorbance values than those found for the extract and fractions at all concentrations. Compared to those derived from the extract and fractions, ascorbic acid showed a greater absorption. The EC_{50} value for the ethyl acetate fraction was lower than those of the extract, fractions as well as the reference ascorbic acid. The highest EC_{50} value was by the extract, which was followed by n-hexane, methanol fraction, standard ascorbic acid, and ethyl acetate in that order

DPPH free radical scavenging activity

Table 4: In vitro percentage DPPH percentage inhibition activity

Concentration (µg/mL)	Methanol Extract (% inhibition)	n-Hexane Fraction (% inhibition)	Ethyl Acetate Fraction (% inhibition)	Methanol Fraction (% inhibition)	Ascorbic Acid (% inhibition)
0.01	73.63	62.43	73.68	65.24	74.31
0.1	78.33	78.55	83.75	75.29	83.20
1	73.68	82.76	85.63	82.57	88.92
10	86.46	88.25	91.18	86.82	91.80
IC_{50} (µg/mL)	5.82 ±0.32	0.93 ± 0.05	3.67 ± 0.22	1.32 ± 0.15	3.44 ± 0.28

n= 3

Table 5: In Vitro ferric reducing antioxidant power (FRAP)

Concentration (µg/mL)	Methanol Extract (Absorbance)	n-Hexane Fraction (Absorbance)	Ethyl Acetate Fraction (Absorbance)	Methanol Fraction (Absorbance)	Ascorbic acid (Absorbance)
25	0.050 ± 0.006	0.067 ± 0.009	0.100 ± 0.010	0.077 ± 0.007	0.137 ± 0.009
30	0.093 ± 0.009	0.177 ± 0.003	0.193 ± 0.009	0.147 ± 0.015	0.303 ± 0.013
100	0.153 ± 0.090	0.270 ± 0.006	0.310 ± 0.010	0.260 ± 0.006	0.383 ± 0.003
200	0.250 ± 0.017	0.397 ± 0.012	0.437 ± 0.009	0.373 ± 0.012	0.517 ± 0.023
400	0.410 ± 0.208	0.483 ± 0.009	0.560 ± 0.011	0.490 ± 0.060	0.653 ± 0.015
EC_{50} values (µg/mL)	2.67 ± 0.15***	1.91 ± 0.10**	0.71 ± 0.04	1.75 ± 0.11	1.34 ± 0.08

Values are Mean ± SEM (standard error of mean). n=3;$^{**}P<0.01$;$^{***}P<0.001$ compared to ascorbic acid.

Table 6: In vitro percentage OH• scavenging activity

Concentrations (µg/mL)	Methanol Extract (% inhibition)	n-Hexane Fraction (% inhibition)	Ethyl Acetate Fraction (% inhibition)	Methanol Fraction (% inhibition)	Ascorbic acid (% inhibition)
25	0.00	0.00	2.5	0.00	33.33
30	15.46	50.90	67.59	52.60	65.83
100	55.19	73.29	78.25	69.63	76.83
200	70.70	79.35	83.50	76.57	81.36
400	75.67	82.44	86.03	82.17	85.36
IC_{50}(µg/mL)	$2.28 \pm 0.13^{***}$	0.37 ± 0.01	$2.26 \pm 0.12^{***}$	0.38 ± 0.02	1.45 ± 0.09

(-) = no inhibition. n=3 $^{***}P<0.001$ versus standard.

DISCUSSION

Oxidative stress develops when the body produces more reactive oxygen/nitrogen species (ROS/RNS) and its antioxidant defenses are less effective (Surai *et al.*, 2019). For aerobic organisms and healthy cells, the production of reactive ROS and RNS is inevitable and happens at a controlled rate (Valavanidis *et al.*, 2013). As a result of the substantial rise in ROS/RNS production under oxidative stress, membrane lipids, proteins, and nucleic acids are pathologically altered (Lü *et al.*, 2010). Ageing and a number of pathogenic processes, such as atherosclerosis, cancer, ischemia reperfusion injury, and neurodegenerative illnesses, are linked to the oxidative degradation of these biomolecules (Thanan *et al.*, 2014). Humans have complex antioxidant systems that work to prevent the harmful effects of oxidative stress in order to preserve homeostasis in the redox system and safeguard the body against ROS and RNS (Surai *et al.*, 2019). Antioxidant defense mechanisms in the body are both endogenous and exogenous (Bouayed and Bohn, 2010). Carotene, L-ascorbic acid (vitamin C), and α-tocopherol and tocotrienols (vitamin E) are exogenous sources of antioxidants that come from the foods (Bouayed and Bohn, 2010). The usage of synthetic antioxidants to combat oxidative stress has been linked to harmful health effects (Lourenço *et al.*, 2019; Liu and Mabury, 2020). Natural antioxidants from plants are becoming more popular as a replacement for synthetic antioxidants due to their supposed safety, accessibility, and affordability (Jain *et al.*, 2019).

When determining total phenolic content, the Folin-Ciocalteau method is a quick and popular assay. Because of the high concentration of phenol in themethanol extract and fractions, it is possible that *C. rostrata* has the ability to scavenge free radicals. The ability of plant extracts with polyphenol components to absorb free radicals and behave as electron or hydrogen donors explains their antioxidant action (Medini *et al.*, 2014). It is inappropriate to base the determination of antioxidant activity on just one antioxidant experimental paradigm (Spiegel *et al.*, 2020). For evaluating antioxidant activity in practice, a variety of in vitro evaluation approaches are taken into account (Subedi *et al.*, 2014). In DPPH radical scavenging assay, substances are regarded as antioxidants if they can scavenge free radicals in vitro and affect color changes. As previously shown by Odion *et al.* (2013) in root bark extracts of *C. rostrata*, which is comparable to the present investigation, the DPPH scavenging ability of our examined plant displayed a concentration-dependent relationship. Based on our study, theIC_{50} of 0.93 g/ml of n-hexane fraction shows stronger radical scavenging activity than that of ascorbic acid. The ferric ion (Fe^{3+}) can be reduced to ferrous ion (Fe^{2+}) using the FRAP method (Sadananda *et al.*, 2014). The results of this investigation showed that at wavelengths of 700 nm, absorbance values increased concentration-dependently. The ethyl acetate fraction displayed absorbance values that were noticeably greater than those of other fractions and crude at every tested concentration. In addition, the relationship between EC_{50} values and antioxidant efficiency is inverse. As a result, ethyl acetate fraction had stronger ferric reducing antioxidant capacity than ascorbic acid and other fractions, with an EC_{50} of 0.71 g/ml. Furthermore, the antioxidant properties of the extract and fractions were evaluated using well established criteria (Boligon *et al.*, 2014) an they all appear to be extremely potent with EC_{50} values less than 50 g/ml. The extract and fractions also showed a concentration-dependent increase in hydroxyl scavenging activity (Agbor *et al.*, 2014). According to Fidrianny *et al.* (2015), the half maximum (IC_{50}) values for the crude and fraction were less than 50ug/ml, making them extremely potent. Numerous studies have demonstrated that medicinal plants contain active components that are responsible for their antioxidant action (Subedi *et al.*, 2014). This bioactivity is believed to be as a result of

various antioxidant-rich phytochemicals found in medicinal plants.

The examined plant extracts contained flavonoids, phenols, and tannins, among other antioxidant phytochemicals, according to qualitative phytochemical profiling. Notable phytochemicals such as flavonoids and phenols in the leaves may have contributed to the antioxidant efficacy. The reductive or oxidative activities of these phytochemicals enable the neutralization of free radicals and their counteracting effects, are assumed to be the source of their antioxidant capacity (Boligon *et al.*, 2014). Many of these secondary metabolites have powerful reductive properties, which are thought to be responsible for the decreased prevalence of oxidative stress-related illnesses that increase morbidity and mortality (Cosme *et al.*, 2020). One of the phenolic class of compounds found in plants is flavonoid. They have a number of pharmacological effects, such as anti-inflammatory and anti-tumor properties, and can function as antioxidants to protect cells from the harmful effects of free radicals (Cosme *et al.*, 2020). Antioxidant and reactive species neutralizing power of flavonoids depends on their structure, where their hydroxyl atoms are located, and other characteristics (Nithya and Madhavi, 2017). These compounds show strong scavenging effects of harmful radicals that are linked to several diseases.

CONCLUSION

The methanol leaf extract and fractions of *Cola rostrata* exhibited antioxidant capacity. As a result of this preliminary study, *C. rostrata* leaves appear to be a valuable herbal medicine that should be explored further.

CONFLICT OF INTEREST

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AUTHORS CONTRIBUTIONS

FKA: conceptualization, methodology and writing; KOO: conceptualization, writing-review, editing and data curation; JAI: project administration, conceptualization and investigation; COU: formal analysis, data curation and writing; ANO: methodology, investigation and writing.

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