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^{1}H NMR Profiling and Antioxidant Activity of Chloroform, Ethyl Acetate, and Aqueous Fractions of *Bauhinia rufescens* Stem Extract

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ABSTRACT
This study investigates the metabolite profile and antioxidant potential of aqueous, ethyl acetate, and chloroform fractions of *Bauhinia rufescens* stem extract.The stem bark was extracted using 85% methanol. ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy was employed to analyze the metabolites present in each fraction, and antioxidant activity was evaluated using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. The results revealed significant differences in metabolite composition across the fractions, with the aqueous fraction exhibiting the highest antioxidant activity. At a concentration of 1000 µg/mL, the aqueous fraction (AQ) exhibited 94.74 ± 0.58% inhibition, while the ethyl acetate (AE) fraction showed 94.33 ± 0.37%, and the chloroform (AC) fraction exhibited 81.59 ± 0.97%. The antioxidant activity decreased as the concentration reduced, with the aqueous fraction still maintaining the highest activity across other concentrations. A total of 22 metabolites were tentatively identified, including flavonoids, phenolics, and alkaloids, which are known for their bioactive properties. These findings suggest that *Bauhinia rufescens* extracts, particularly the aqueous fraction, could serve as a potential source of natural antioxidants. Further studies are needed to isolate and characterize the bioactive compounds responsible for the observed effects.

Keywords:
Bauhinia rufescens,
NMR profiling,
Antioxidant activity.

INTRODUCTION

Medicinal plant research focuses on validating traditional herbal practices, aiming to isolate bioactive compounds and standardize the crude extracts used in traditional medicine (Sofowora, 1986). Plant-based remedies are highly diverse, varying across different cultures (Farnsworth, 1976), and they are generally considered less toxic with fewer side effects compared to synthetic drugs. The identification of phytochemicals with antimicrobial properties plays a key role in the discovery of new medicines (Zhang *et al.*, 2015). Over 30% of pharmacological preparations are derived from plant compounds (Shinwari & Gilani, 2003) making the investigation of medicinal plants crucial for understanding their therapeutic efficacy and safety (Verpoorte, 2000). Common plant parts used in traditional medicine include leaves, flowers, bark, stems, and roots (Ita & Offiong, 2013). Continuous research into medicinal plants has led to the discovery of valuable therapeutic agents (Patil, 2011).

The genus *Bauhinia*, commonly known as "pata-de-vaca" (cow's paw) or "unha-de-boi" (ox's nail), belongs to the Fabaceae family, which includes approximately 18,000 species worldwide. These species are primarily found in tropical regions of Africa, Asia, and the Americas, where they are widely used in folk medicine. In Brazil, more than 60 native species of *Bauhinia* have been documented, and their leaves are often used in popular medicine, particularly for the treatment of diabetes (Pianoski *et al.*, 2020).

Bauhinia rufescens, referred to as "Matsattsagi" by the Hausa people of Nigeria, is a tiny tree with greenish-yellow to white blossoms that grows to a height of 5 to 8 meters. The plant can be found all over West and Central Africa (Babalola, 2006; Gill, 1992). The bark is used to treat smallpox and respiratory ailments, and the roots, when boiled and minced, are used to treat leprosy, syphilis, venereal disorders, diabetes, malaria, typhoid fever, and other common ailments (Abdel-razakh *et al.*, 2023).

The metabolomics approach in plants involves a comprehensive and systematic analysis of the complete

set of metabolites present within plant tissues. Nuclear magnetic resonance (NMR) spectroscopy is one of the most utilized analytical tools for applications in plant metabolomics. It provides information about the metabolites in plants. The secondary metabolites identification is made possible based on the chemical shift provided by NMR spectroscopy (Kim *et al.*, 2006).

Oxidative stress, driven by the overproduction of reactive oxygen and nitrogen species (ROS and RNS), plays a key role in the development of chronic diseases such as cancer, diabetes, and cardiovascular diseases (Arika *et al.*, 2019). Free radicals, which are unstable molecules with unpaired electrons, cause cellular damage, leading to these conditions if not properly neutralized by antioxidants (Halliwell, 2011). As part of their regular metabolic activities, plants are known to produce a variety of bioactive phytochemicals. The natural antioxidant qualities of such phytochemicals, which are present in both food and medicinal plants, have been extensively researched. Bioactive substances like phenolics, flavonoids, and tannins are abundant in many plant parts, such as leaves, stems, bark, seeds, and roots. These substances can have the ability to serve as antioxidants and counteract excess free radicals (Adebo & Medina-Meza, 2020; Atanasov *et al.*, 2015). According to epidemiological and clinical research, several of these phytochemicals have antioxidant properties that provide protection and disease prevention by either deactivating lipid free radicals or stopping hydroperoxides from breaking down into free radicals (Maisuthisakul *et al.*, 2007; Unuofin *et al.*, 2018). To date, various phytochemical and antioxidant properties of *Bauhinia rufescens* have been reported (Aliyu *et al.*, 2009; Hamidu *et al.*, 2009). However, to the best of my knowledge, the metabolite profile and the impact of solvent polarity on the antioxidant activity across different fractions of this plant have not been documented. Therefore, the primary objective of this research is to profile the metabolites in the Chloroform, Ethyl Acetate, and Aqueous Fractions of *Bauhinia rufescens* methanol extracts and evaluate their antioxidant activity.

MATERIALS AND METHODS

Collection and preparation of plant materials

The plant sample was authenticated at the Herbarium of Ahmadu Bello University, Zaria, Nigeria, and reference specimens were stored for future use under the code ABU0900230., fresh stem bark of the *Bauhinia rufescens*were collected from the Hadejia-Nguru Wetland in Jigawa State, Nigeria, in June 2021. It was thoroughly washed with deionized water to remove surface impurities. The plant material was then air-dried for three weeks. The dried, plant material was ground into powder using a mortar and pestle. The powdered, air-dried plant bark was subsequently soaked in 85% methanol and filtered through Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure at 40°C until completely dry. The concentrated 85% methanol extract was then fractionated using a modified Kupchan partitioning method (Van Wagenen *et al.*, 1993) yielding n-hexane, chloroform, ethyl acetate, and aqueous fractions. The solvents were evaporated to give the following fractions: n-hexane, chloroform, ethyl acetate, and aqueous.

Preparation of Samples for NMR Analysis

All ^{1}H NMR spectra were acquired on a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., Palo Alto, California, USA), operating at a frequency of 499.887 MHz at room temperature (25°C). A pre-saturation (PRESAT) pulse sequence was employed to reduce water (H_2O) interference, with 64 scans conducted. For the ^{1}H NMR analysis, 1 mL of NMR-grade methanol was mixed with 15 mg of each chloroform, ethyl acetate, and aqueous fractions of *Bauhinia rufescens* stem extracts. The mixture was vortexed for 10 minutes, sonicated for 10 minutes, and centrifuged for 10 minutes. Subsequently, 0.6 mL of the supernatant was transferred into 5 mm NMR tubes for analysis.

Antioxidant Activity

The antioxidant activity of the chloroform, ethyl acetate, and aqueous fractions of the stem extract of *Bauhinia rufescens*, as well as the standard, was measured based on the radical scavenging effect using the stable 2,2-diphenylpicrylhydrazyl (DPPH) free radical assay, as modified by (Braca *et al.*, 2002). Working solutions of the extracts were prepared in methanol, and ascorbic acid was used as the standard at a concentration of 0.01 g/mL. A 0.004% DPPH solution was prepared in methanol. To measure the radical scavenging activity, 2 mL of the sample solution was mixed with 2 mL of the DPPH solution, along with the standard solution separately. The solution mixtures were kept in the dark for 30 minutes, after which the optical activity was measured at 517 nm using a spectrophotometer. Methanol (2 mL) mixed with DPPH solution (0.004%, 2 mL) served as the blank. The optical density was recorded, and the percentage inhibition was calculated using the following equation:

$$AA\% = \frac{Ab - Aa}{Ab} \times 100 \quad (1)$$

where

AA = % Antioxidant activity

A_b = Absorbance of blank

Aa = Absorbance of the test sample.

RESULTS AND DISCUSSION

Table 1: Mean and standard deviation of antioxidant activity of the fractions *Bauhinia rufescens*stem extract

S/N	CONC (µg/ml)	AQ	AE	AC	AA
1	1000	94.74±0.58	94.33±0.373	81.59±0.97	87.976±0.59
2	500	93.08 ±0.65	92.92±0.515	80.578±0.841	87.033±0.86
3	250	92.589 ±0.723	91.958 ±0.743	76.671±0.82	84.95 ±0.69
4	125	91.259±1.340	87.571±1.66	74.63±0.82	81.31±0.69
5	62.5	86.57 ±4.07	83.934 ±0.502	71.820±0.505	76.48 ±1.95
6	31.25	78.35±0.769	79.97±0.796	66.127±2.04	63.13 ±1.81
7	15.25	64.88±0.721	68.54±2.91	56.15±2.02	56.40 ± 1.07
8	7.81	59.59±0.954	55.812±1.32	54.87±87	52.47 ±1.26

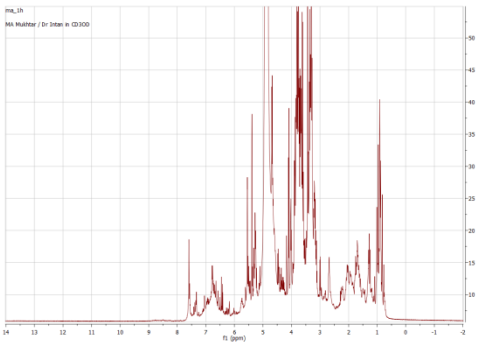

Fig 1:[1]HNMR spectrum of aqueous fraction

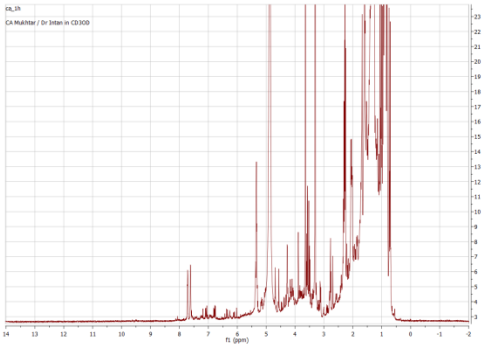

Fig 2:[1]HNMR spectrum of Ethylacetae fraction

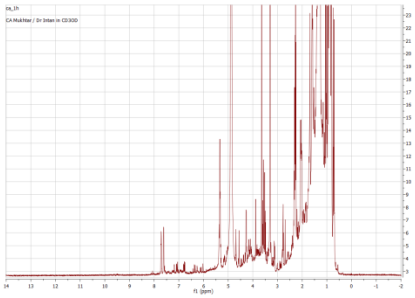

Fig 3: [1]HNMR spectrum of Chloroform fraction

Table 2: Putative metabolites identified from the fractions of *Bauhinia rufescens* stem extract

*	Putative metabolite	Chemical shift	AQ	AE	AC	Reference
1	Sucrose	5.37d 4.22d	√	-	-	(Sousa *et al.*, 2012)
2	Glucose and fructose	3.63 – 3.80m	√	√	-	(Ade-Ajayi *et al.*, 2011)(More et al., 2022)
3	Kaemferol	6.18d, 6.38d, 8.06d	√	-	-	(Satake *et al.*, 2007)
4	Quercetin 3 -O-β-glucuronide	6.17d,8.47d,7.29dd	√	-	-	(Satake *et al.*, 2007)
5	Epicatechin	7.04,6.93m,4.95m	√	√	-	HMDB
6	Luteolin	6.20s,6.44d,6.5s,6.89t	√	√	-	Pubchem
7	Choline	3.2s	√	√	-	(Pantami *et al.*, 2020)
8	Fumaric acids	6.56s	√	-	-	((Bakiri *et al.*, 2017)
9	Astilbin	5.17d 4.67d 3.79d	-	√	√	((Tlhapi *et al.*, 2021)

10	Apegenin	3.05s	-	√	-	(Pantami*et al.*, 2020)
11	Stilbene	1.66s 1.77s 3.37s 7.41d 5.19m	-	√	-	(Satake *et al.*, 2007)
13	Frieldelin	1.97m 1.77m 1.29s	-	√	√	(Sousa *et al.*, 2012)
14	Lupeol	1.36m 1.02s 0.98s 0.77s	-	-	√	(Tlhapi *et al.*, 2021)
15	Oleic oil	0.86t 1.24m 1.26m	-	√	√	(Pantami *et al.*, 2020)
16	Lutein	1.00s	-	-	√	(Pantami*et al.*, 2020)
17	Quinic acids	1.6m 1.8m	-	√	√	pubchem

Key: AQ: Aqueous fraction, AE: Ethyl acetate fraction AC: Chloroform fraction and AA Ascorbic acids, √ = Present, - = Absents

Discussion

The antioxidant potential of the fractions was assessed using the DPPH assay, with results presented in Table 1. The findings revealed concentration-dependent radical scavenging effects for all fractions. The aqueous and ethyl acetate fractions exhibited significant antioxidant activity, comparable to that of ascorbic acid, with scavenging rates exceeding 90% at concentrations of 1000 µg/ml and 500 µg/ml. In contrast, the chloroform fraction displayed relatively lower antioxidant activity, achieving a maximum scavenging rate of 81.59% at 1000 µg/ml. The differences in antioxidant activity and metabolite composition among the aqueous, ethyl acetate, and chloroform fractions of *Bauhinia rufescens* stem extracts can be attributed to the distinct solubility profiles of compounds in each solvent. Such solvent-dependent variations in activity have been previously reported by (Barchan *et al.*, 2014). The superior antioxidant activity of the aqueous and ethyl acetate fractions is likely due to the presence of flavonoids such as quercetin 3-O-glucuronide, kaempferol, luteolin, and apigenin, which are widely recognized for their free radical scavenging activities. As Luteolin, kaempferol, apigenin, and quercetin are common flavonol glycosides, known for their anti-inflammatory and antioxidant activities (Tian et al., 2021). Kaempferol in particular, exhibits potent antioxidant activity and shows promise for anticarcinogenic, anticancer, and neuroprotective effects (Bangar *et al.*, 2023) and also Excellent antioxidant properties of catechins have also been reported (Grzesik *et al.*, 2018). Therefore, it can be suggested that these flavonoids may play a significant role in the observed antioxidant activities.

The ethyl acetate fraction (Table 2) on the other hand, contains bioactive substances including choline and stilbene. Xia *et al.*, (2022) reported stilbene's antioxidant qualities, while Mehta *et al.*, (2009) noted the health benefits of choline. The ethyl acetate fraction's high activity may likely be result of these bioactive substances. In comparison, the chloroform fraction, containing lipophilic compounds such as lupeol, oleic oil, and friedelin, that showed moderate antioxidant potential. Friedelin has demonstrated marked antioxidant and liver protective effects (Sunil *et al.*, 2013). While lupeol, although a potent anti-inflammatory compound, has relatively low antioxidant activity at low concentrations (Alghamdi *et al.*, 2022). Moreover, another study reported that apigenin, at higher doses, exhibit significant antioxidant activity and a potential role in restoring corticosterone balance (Alghamdi *et al.*, 2022).

The differences in metabolite composition across fractions can be attributed to the polarity of the solvents used during fractionation, which selectively solubilize compounds based on their polarity. Aqueous extraction favors the isolation of hydrophilic compounds such as flavonoids and catechins due to their high solubility in water, resulting from their polar nature. Ethyl acetate, being moderately polar, is capable of extracting both hydrophilic and lipophilic compounds, including stilbenes and choline. In contrast, chloroform, a non-polar solvent, predominantly extracts lipophilic compounds like lupeol and friedelin. These variations in solvent polarity and the affinity of secondary metabolites for specific solvents explain the presence of certain metabolites in specific fractions while others are absent.

This finding aligns with the results of Wakeel *et al.*, (2019) who reported that solvent polarity significantly affects metabolite solubility, with polar solvents effectively dissolving flavonoids. The absence of sugars, catechins, and quercetin in the chloroform fraction can be explained by their polar nature, which limits their solubility in non-polar solvents like chloroform. This observation is consistent with the findings ofAlavi *et al.*, *(*2014) , who reported the limited solubility of polar compounds in non-polar solvents.

The solubility of sugars in the aqueous fraction can be attributed to their polar characteristics. This finding corroborates the work of Van Putten *et al.*, (2014) who demonstrated the solubility of sugars in water-methanol mixtures. Similarly, the presence of catechin in the ethyl acetate fraction can be justified by the findings of Arotiba *et al.*, (2009), who reported the extraction of catechins in ethyl acetate. This is due to the moderate polarity of ethyl acetate, which makes it suitable for dissolving compounds like catechin that are neither excessively hydrophilic nor hydrophobic. Finally, the solubility of lupeol, lutein, and oleic acid in chloroform can be explained by their hydrophobic characteristics, which align well with chloroform's non-polar nature. This highlights the effectiveness of solvent polarity in determining the selective extraction of metabolites during fractionation.

These findings highlight the rich bioactive composition of aqueous, ethyl acetate and chloroform fractions of *Bauhinia rufescens*, making them promising candidates for therapeutic applications.

CONCLUSION

The present study highlights the antioxidant potential and unique metabolite profiles of *Bauhinia rufescens* fractions, with the aqueous fraction displaying the most promising results in the DPPH assay. The use of NMR profiling has enabled the identification of key metabolites, including flavonoids and phenolics, that contribute to the antioxidant activity observed. These findings support the potential of *Bauhinia rufescens* as a valuable source of natural antioxidants for pharmaceutical applications. Future work should focus on the isolation and structural elucidation of the bioactive compounds, as well as exploring their mechanisms of action in biological systems.

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