

Antioxidant, α -amylase and α -glucosidase inhibitory activities of Ethyl gallate Isolated from the stem bark of *Acacia nilotica* (L) Delile

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Abstract

Acacia nilotica (L). Dilile is a plant used traditionally against cancer, antidiarrheal, antimicrobial, antiinflammation, antifungal and antidiabetic activities have all been documented. Despite the reported traditional uses of this plant in the management of diabetes mellitus there is no report on the chemical constituents responsible for this activity. The dried pulverized stem bark was extracted with 95% ethanol and the crude extract was partitioned with water, ethyl acetate. The ethyl acetate soluble fraction (C4) was subjected to column chromatography to afford ethyl gallate (C1) and a chromatographic fraction (C3) respectively. The chemical structure of ethyl gallate was established on the basis of NMR and compared with literature. C1, C3 and C4 were evaluated for antioxidant property using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) as well as α -amylase and α -glucosidase inhibitory activities. The result revealed that the ethyl acetate soluble fraction, ethyl gallate and the chromatography fraction exhibited dose dependent DPPH radical scavenging activity with ethyl gallate showing the highest activity with IC_{50} of 6.64 ± 1.00 μ g/ml comparable to ascorbic acid with IC_{50} of 4.94 ± 0.37 μ g/ml respectively. The chromatographic fraction (C3) showed the highest activity against ABTS, while both the ethyl acetate fraction and ethyl gallate exhibited ferric reducing property. Ethyl gallate however showed weak inhibitory activity against α -amylase and α -glucosidase in comparison to the standard drug acarbose. Ethyl gallate isolated from *Acacia nilotica* exhibited strong antioxidant activity which may justify the potentials of this plant as source of antioxidant in the management of diabetes mellites.

Key words: *Acacia nilotica*, antioxidant, ethyl gallate, α - amylase, α -glucosidase

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Introduction

Reactive oxygen/nitrogen species have been implicated in the pathogenesis of diseases such as cancer, cardiovascular diseases, diabetes, neurodegenerative disorder among others as a result of generation of free radicals. These radicals are scavenged by compounds capable of donating a hydrogen atom or mediate their activity via activation of antioxidant enzymes (Donne *et al.*, 2006; Oboh, 2006). Attention has now shifted to natural substances usually medicinal plants as source of antioxidants found to aid in the alleviation of diseases by obstructing the oxidation of macromolecules (Silva *et al.*, 2004). Diabetes mellitus is a metabolic disorder characterized by hyperglycemia as a result of defects in insulin secretion or insensitivity (American Diabetes Association, 2012). The International diabetic federation estimated that more than 537 million adults worldwide have diabetes as at 2024 and this is expected to rise to 643 million people by 2030, and the contributing factors include urbanization, aging population, decreasing physical activity and rise in overweight and obesity rate. According to a report by the International Diabetes of Nigeria, as at 2024, there is reported 3% prevalence of diabetes in adults in Nigeria, which translates to about 3 million cases of diabetes in Nigeria. Despite the availability of antidiabetic drugs, the rising cost of production of these drugs has led to an astronomical increase in the cost of management of diabetes to avoid complication, hence the need to screen our ethnomedicinal plants used in the management of diabetes as a lead discovery for antidiabetic drugs. *Acacia nilotica* (Del) is a medicinal plant of the family Fabaceae with over 1,400 species in the genus *Acacia* (Seigler, 2003). It is widely distributed in the tropical and sub-tropical regions of the world including Nigeria, Mozambique, Senegal and Ghana (Pedley, 1986). The plant has diverse ethnomedicinal uses which include; the leaves are used to treat diarrhea and Inflammation in Northern Nigeria (Agunu *et al.*, 2005), the leaves, bark and pods are used in Ayurvedic medicine practice against cancer, fever and menstrual problem (Kalaivani and Martin, 2010), the leaves, pods as well as the stem bark have been reported as a traditional antidiabetic remedy in Pakistan, Egypt and Nigeria (Saha, 2018). The antidiabetic potentials of the aqueous extract of the leaves have been reported (Abubakar Gidado *et al.*, 2018), while Abdul Rauf *et al.* (2024) reported the α -glucosidase and α -amylase inhibitory activities of the crude methanol extract of the stem bark of *Acacia nilotica*. We have previously reported the inhibitory activity of the ethyl acetate soluble fraction of this plant against a panel of disease related protein kinases (Ahmadu *et al.*, 2024). Numerous bioactive principles such as gallic acid, naringenin, kaempferol and catechin derivatives have been isolated from this plant (Kalaivani *et al.*, 2011). The isolation of two new Peltogynoids from the chloroform extract as well as ethyl gallate and catechin from the ethyl acetate soluble fraction

and their activity against protein kinase have been reported (Ahmadu, 2010; 2017). Despite the reported hypoglycemic activity of the various plant parts of *Acacia nilotica*, there is no report on the α -amylase, α -glucosidase inhibitory and Antioxidant activities of chemical constituents from the stem bark of *Acacia nilotica*. In this present work, we report herein the Isolation of ethyl gallate from the ethyl acetate soluble part of the ethanolic extract and the investigation of the antioxidant as well as α -amylase and α -glucosidase inhibitory activities.

Materials and Methods

Plant Collection

The stem bark of *Acacia nilotica* was collected in Zaria, Kaduna State, in July, 2024 and was authenticated by US Gallah of the Herbarium Unit, Department of Biological Sciences, Kaduna State University, Kaduna, where a voucher specimen was deposited.

Extraction and Isolation

The powdered stem bark (300 g) was extracted with 2x2.5L of 95% ethanol at room temperature for 7 days, and the combined ethanolic extract was concentrated using a rotary evaporator to obtain a dark brown mass (35 g). A portion of the crude ethanol extract (30 g) was suspended in 300 ml of water and successively partitioned with 1.5 L each of ethyl acetate and n-butanol to afford their soluble fractions, which weighed 10.1 and 3.2 g, respectively. A portion of the ethyl acetate fraction (9.7 g) was packed in a column (4 cm x 20 cm) and eluted gradually starting with dichloromethane, followed by stepwise addition of methanol as follows: 99:1, 98:2, 97:3, 96:4, 95:5, 90:10, 80:20, 70:30, 60:40; 50:50; 30:70; 10:90, and finally methanol (100%). Aliquots (50 mL) were collected, and the progress of separation was checked on thin-layer chromatography (TLC) using solvent system I: dichloromethane: ethyl acetate (2:3), and solvent systems II and III: ethyl acetate; dichloromethane: methanol and water (15:8:4:1; 6:4:4:1), respectively. Elution with 10% methanol in dichloromethane afforded compound I (35 mg).

Antioxidant Studies

The DPPH assay was carried out according to the method of Brand-Williams *et al.* (1995), with some modifications as previously discussed (Ahmadu *et al.*, 2025) briefly, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (8 mg) was dissolved in methanol (100 mL) to give a solution concentration of 80 μ g/mL. To determine the scavenging activity, 100 μ L of DPPH reagent was mixed with 100 μ L of sample in a 96-well microplate and incubated at room temperature for

30 min. After incubation, the absorbance was measured at 514 nm using a microplate reader, and 100% methanol was used as a control. DPPH scavenging activity was estimated using the following formula:

$$\% \text{ DPPH scavenging} = (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100.$$

Where A_{blank} represents the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} represents the absorbance of the test sample/standard.

The 2,2-azabinos (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) assay was performed according to the method described by Arnao *et al.* (2020), with some modifications. 7mM 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 2.45 mM potassium persulphate were mixed and dissolved in double distilled water. The solution was then diluted with distilled water at a 1:9 v/v ratio. A 190 μL volume of reagent was pipetted into a microtiter well, followed by the addition of 10 μL of sample/standard (ascorbic acid). The absorbance was measured at $\lambda=735$ nm. A reagent blank reading was taken, and after the addition of the sample, the absorbance reading was taken after 6 min of initial mixing. Antioxidant activity was estimated using the following formula:

$$\% \text{ ABTS scavenging} = (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100$$

For the FRAP assay, solutions were prepared as described by Benzie and Strain (1996). The FRAP working solution was prepared by mixing 10 volumes of acetate buffer (300 mM, pH 3.6) with 1 volume of 2,4,6-tripyridyl-S-triazine (TPTZ) (40 mM dissolved with 40 mM HCl) and 1-volume of ferric chloride (20 mM in water). The microplate FRAP assay was performed as previously described, with minor modifications. Sample solutions (20 μL) were added directly to the 96-well microplate, followed by 280 μL of working FRAP solution. The mixtures were shaken, incubated at 37°C in the dark for 30 min, and the absorbance was measured at 593 nm using a microplate reader. The values were estimated in μM ferrous (Fe II) equivalents.

In vitro α -glucosidase inhibition assay

α -Glucosidase inhibitory activity was assessed using a standard method (Nair *et al.*, 2013) with slight modifications. Briefly, 60 μL of the sample solution and 50 μL of 0.1 M phosphate buffer (pH 6.8) containing α -glucosidase solution (0.2 unit/ml) were incubated in 96 well plates at 37°C for 20 min. After pre-incubation, 50 μL of 5 mM p-nitrophenyl- α -D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37°C for another 20 min. The reaction was stopped by adding 160 μL of 0.2 M Na_2CO_3 to each well, and absorbance readings (A) were recorded at 405 nm using a microplate reader and compared to a control that had 60 μL of buffer solution instead of the compound.

Alpha Amylase assay

The assay system comprised the following components in a total volume of 260 μL : 40 μL of PBS (0.02M, pH 6.9), 100 μL of glucose oxidase reagent, 40 μL of each soluble starch (2 g/L), inhibitor solution, and enzyme solution (2 units/mL). Briefly, the enzyme solution was mixed with the plant extract and pre-incubated on a hot plate for 10 min at 37°C. The reaction was initiated by pipetting the starch solution into the wells and incubating for another 15 min. Finally, 100 μL of glucose oxidase reagent was added, and the absorbance was measured at 505 nm after 15 min. As a negative control, 40 μL of phosphate-buffered saline was used instead of the sample, and the absorbance was measured in parallel with the samples. For each sample concentration, a sample blank was added in parallel. For kinetic studies, immediately after the addition of starch, the GOD reagent was added, and the readings were taken at 1 min intervals for 45 min at 505 nm. The results are expressed in terms of the IC_{50} value.

Statistical analysis

All experiments were performed in triplicate. Data are presented as \pm SEM. Graphs were plotted using GraphPad Prism version 8.0. Significant differences between samples were calculated by comparing means using one-way ANOVA. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using Microsoft Excel 2016 (Microsoft Inc.). USA.

Results

Compound 1 was isolated as an off-white solid (35 mg). The UV spectrum showed maximum absorption at 225 and 326 nm, consistent with phenolic compounds. The proton NMR spectrum revealed signals for aliphatic methyl and oxygenated methylene protons at $\delta=1.34$ and 4.27 ppm, respectively, consistent with methyl and methylene protons, as well as an aromatic proton at $\delta=7.04$ ppm, consistent with ethyl gallate. The ^{13}C -NMR spectrum confirmed the compound to be ethyl gallate from the identification of the carbonyl carbon signal at $\delta=168\text{ppm}$. Compound 1 was identified as ethyl gallate as previously reported (Ahmadu *et al.*, 2017)

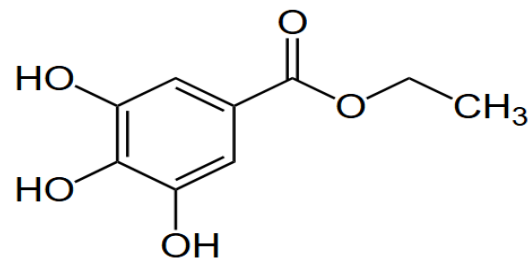


Figure 1: Ethyl gallate

UV (Methanol): 225, 326 nm

NMR (^1H , CD_3OD): $\delta(\text{ppm})$: 1.34 (t) H-1), 3.32, 4.27 (q, H-5), 7.04 (s);

NMR (^{13}C , CD_3OD): $\delta(\text{ppm})$: 14.1; 62; 110.0; 140; 147; 168.

In vitro Antioxidant Activity

DPPH Scavenging Activity: DPPH is a stable free radical that accepts electrons or hydrogen atoms to become a stable diamagnetic molecule. Antioxidants transfer electrons or hydrogen atoms to DPPH, neutralizing its free radical character to form 1–1 diphenyl–2–picryl hydrazine. This causes the DPPH to change its color from purple to yellow, and the degree of change in color, measured at 517 nm, reflects the antioxidant potential of the compound or extract (Sochor et al., 2010). In this study, the antioxidant activity of the ethyl acetate soluble fraction (C4), isolated compound; ethyl gallate (C1), column fraction (C3), and the standard (ascorbic acid) increased in a concentration-dependent manner from 10 $\mu\text{g/mL}$ and almost plateaued at 20 $\mu\text{g/mL}$, as shown in Figure 1. At 10 $\mu\text{g/mL}$, C3 and C1 exhibited a greater percentage of inhibition than ascorbic acid. C1 had an IC_{50} of 6.64 ± 1.00 in the DPPH assay, which is comparable to the IC_{50} of ascorbic acid (4.94 ± 0.37).

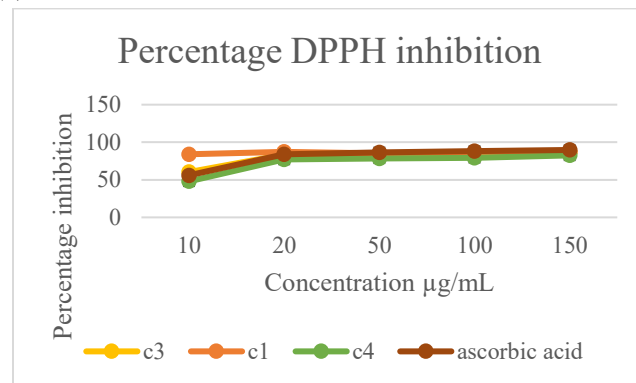


Figure 2: Percentage of DPPH Inhibition of C1, C3, C4 and ascorbic acid

Table 1: IC_{50} ($\mu\text{g/mL}$) of C1, C3, C4 and ascorbic acid

	DPPH	ABTS
C1(Ethyl gallate)	6.64 ± 1.00	6.56 ± 3.80
C3	9.04 ± 1.70	2.76 ± 1.11
C4	6.84 ± 0.07	40.38 ± 0.56
Ascorbic acid	4.94 ± 0.37	56.19 ± 3.95

values are given as mean \pm S.E.M (n = 3)

ABTS Assay

The result of the ABTS assay which corroborated that of DPPH radical scavenging activity is shown in figure 3. C1 had the best ABTS activity when compared with the ethyl

acetate soluble fraction and ascorbic acid. The ABTS activity of the column fraction (C3) is also greater than the ethyl acetate soluble fraction (C4).

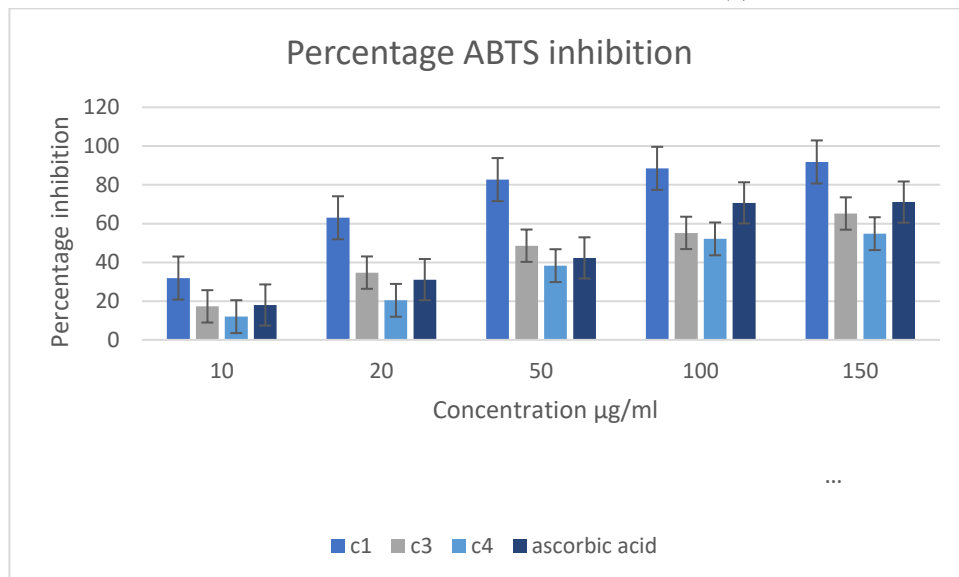


Figure 3: Percentage (%) ABTS activity of C1, C3, C4, and ascorbic acid. Values are given as mean \pm S.E.M.

Antioxidant Power of ethyl acetate, chromatographic fractions, and ethyl gallate.

Ferric reduction antioxidant power (FRAP) is another measure of the antioxidant potential of a given sample. This involves the reduction of Fe^{3+} to Fe^{2+} by donating an electron, which is an important mechanism of phenolic antioxidant action (Benzie, 1996). In the present assay, the FRAP activity was estimated from the standard curve of the absorbance against the concentration of ascorbic acid with the following regression equation: $y = 0.0009x + 0.172$ ($R^2 = 0.9581$). The chromatography fraction (C3) had the highest

activity of 55 mg AAEg^{-1} (Figure4), while the ethyl acetate fraction and ethyl gallate (C1 and C4) produced the same FRAP value (44 mgAAEg^{-1}).

There were no significant differences in the DPPH and ABTS assays. However, there was a statistically significant difference ($P < 0.05$) in the FRAP assay. Pairwise comparisons were performed between the groups using the t-test, and Bonferroni correction was performed. Statistical significance was obtained between C1 and C4 in the FRAP assay.

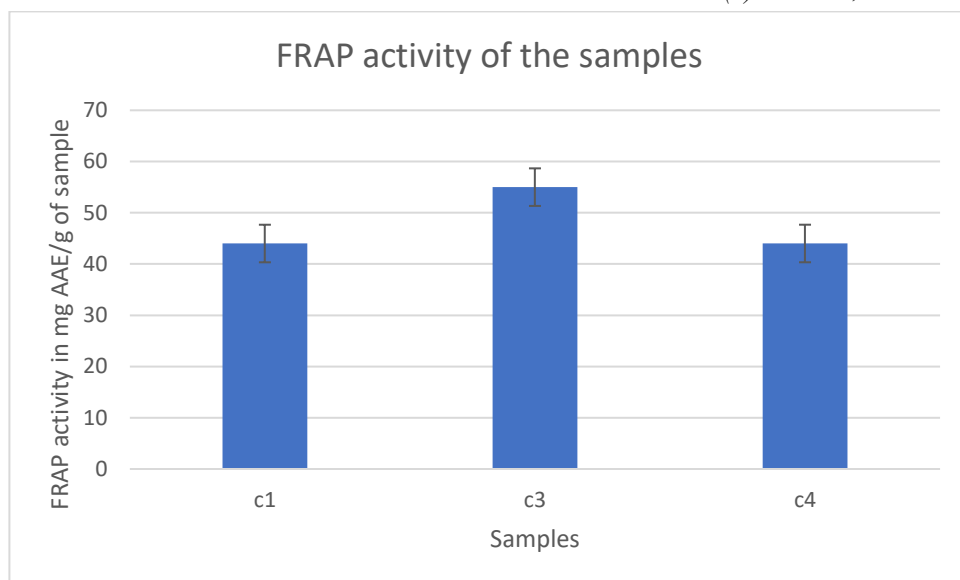


Figure 4: FRAP activities of C1, C3, and C4. Values are expressed as mean \pm S.E.M.

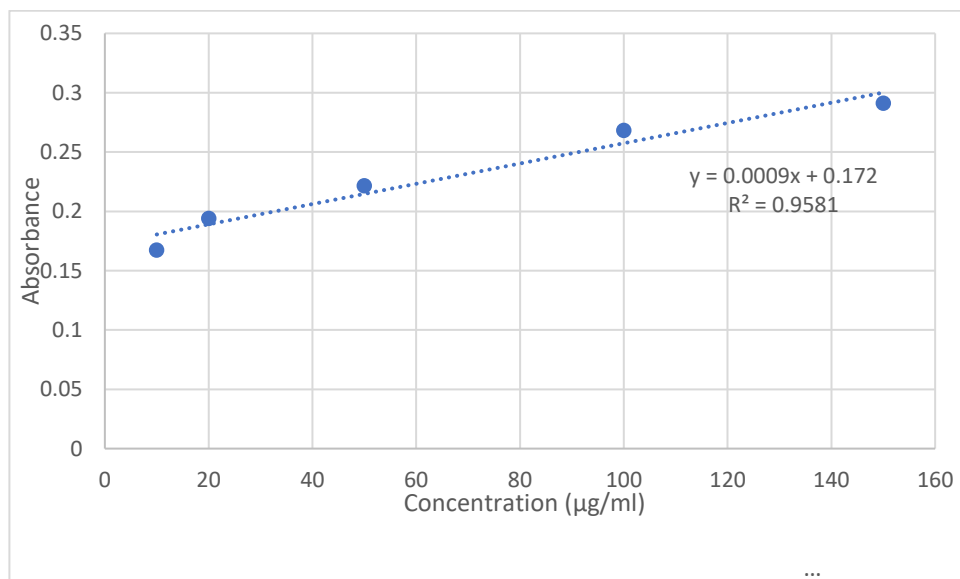


Figure 5. Calibration curve of ascorbic acid (standard) for FRAP determination

α – amylase and α – glucosidase Activity

C1 (ethyl gallate) was tested for both α -amylase and α -glucosidase activities using acarbose as the standard. The results of the α -amylase assay (Figure 6) showed a dose-dependent inhibition of the enzyme *in vitro*. C1 exhibited comparable inhibition to the standard drug acarbose, although the standard drug exhibited greater inhibition of

the α -amylase enzyme at all tested doses. The α -glucosidase inhibitory activity of the isolated compound (ethyl gallate) and acarbose (standard drug) are illustrated in Figure 6. The ethyl gallate was seen not to exhibit much activity against α -glucosidase enzyme compared with the standard drug. The IC_{50} values of ethyl gallate and acarbose against the two enzymes were calculated (Table 2).

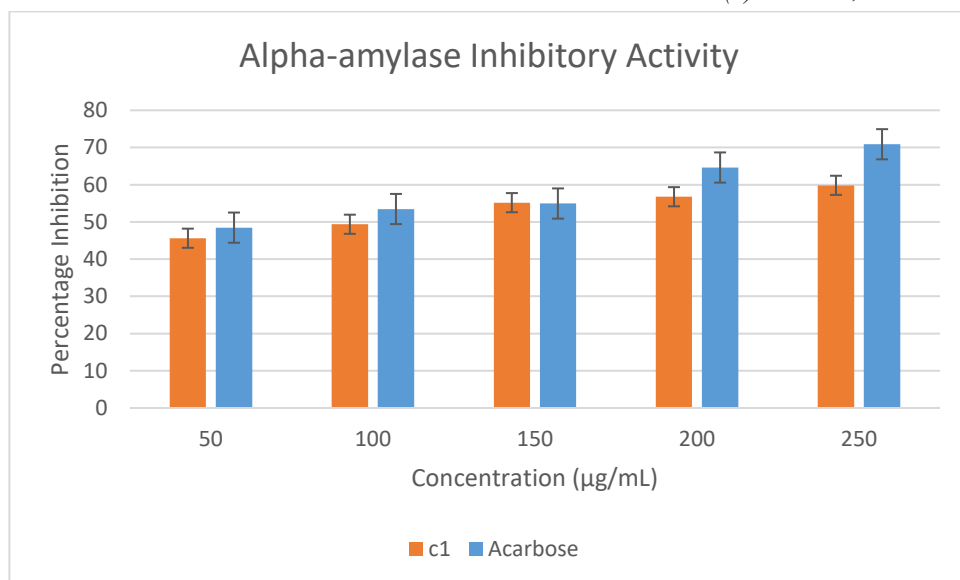


Figure 6. Percent (%) α -amylase inhibitory activity of c1 and the standard (acarbose)

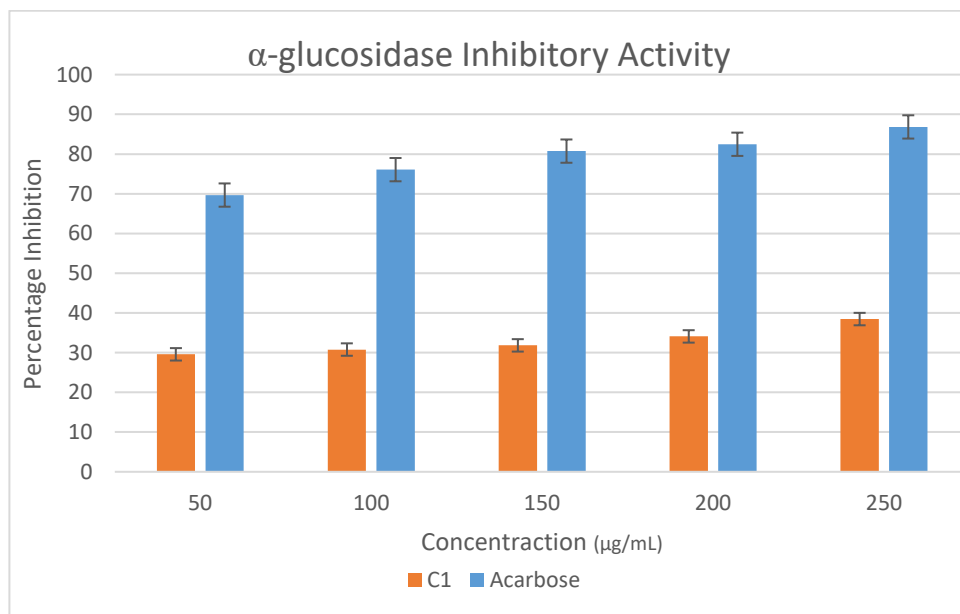


Figure 7. Percent (%) α -glucosidase inhibitory activity of ethyl gallate (C1) and the standard (acarbose)

Discussion

Free radicals, which are natural byproducts of cellular metabolism, have been implicated in the pathogenesis of several diseases, including diabetes, inflammation, cancer, and cardiovascular disorders (Costa *et al*, 2017; Chaudhury *et al*, 2023). Polyphenolic compounds, widely distributed in plant species, have been extensively documented for their antioxidant and free radical scavenging activities, primarily through mechanisms involving electron donation (Khalid *et al*, 1998).

In the present study, the antioxidant potentials of isolated ethyl gallate, along with column and ethyl acetate-soluble fractions, were assessed using DPPH, ABTS, and FRAP assays, with ascorbic acid serving as the standard reference compound. In the DPPH assay, ethyl gallate (C1) exhibited an IC_{50} value of 6.64 ± 1.00 $\mu\text{g/mL}$, closely comparable to that of ascorbic acid (4.94 ± 0.37 $\mu\text{g/mL}$). Interestingly, in the ABTS assay, ethyl gallate demonstrated significantly greater activity, with an IC_{50} of 6.56 ± 3.80 $\mu\text{g/mL}$,

substantially lower than that of ascorbic acid (56.19 ± 3.95 $\mu\text{g/mL}$), suggesting a superior radical scavenging capacity in this system. Among the fractions tested, the column fraction (C3) showed the highest antioxidant activity in the ABTS assay, with an IC_{50} value of 2.76 ± 1.11 $\mu\text{g/mL}$, the column fraction (C3) also showed the highest reducing power in the FRAP assay with 55 mgAAEg^{-1} these findings are consistent with previously reported data on polyphenolics (Asif, 2015). Overall, the results highlight the potent antioxidant capacity of ethyl gallate. Polyphenols have been known to possess antioxidant activity thereby offering protection against oxidative stress; thus, intake of these polyphenols have been associated with reduction in reactive oxygen induced diseases (Foyzun *et al*, 2022). A large number of polyphenols have been reported in the leaves and pods of *Acacia nilotica* (Sadiq *et al*, 2017) and these has corroborated the earlier reports on the antioxidant ability of natural polyphenolics which may be attributed to their effective electron-donating ability, in some cases exceeding that of ascorbic acid (Baba *et al*, 2024; Boulmouk *et al*, 2021). Among the therapeutic applications of *A. nilotica* is in the management of diabetes (Ahmad *et al*, 2008). Khalaf *et al* (2023) have reported that the ethyl acetate soluble fraction of the stem bark of the hydro alcohol stem bark extract of *Acacia nilotica* improved lipid profile and insulin sensitivity as well as ameliorates oxidative stress in high fatty diet. One of the therapeutic approaches involved in the management of diabetes is reduction of glucose uptake from the gastrointestinal tract (GIT) by the inhibition of α -amylase and α -glucosidase (Fajirah *et al*, 2018). Examples of drugs in this category include acarbose, miglitol and voglibose. Plant extracts are known to possess α -amylase and α -glucosidase inhibitory activity (Nair *et al*, 2013). Ethyl gallate showed weak inhibitory activity against α -amylase and α -glucosidase enzymes in comparison to standard drug acarbose, this indicate that the antioxidant activity of ethyl gallate may be responsible for the hypoglycemic property of *Acacia nilotica*, since high blood level of glucose in diabetic patient can lead to the generation of free radicals and reactive oxygen species which in turn leads to damage of cellular macromolecules and progression of diabetes, hence antioxidant therapy is one of the strategies in the management of diabetes.

Conclusion

The ethyl acetate soluble fraction, the chromatography sub fraction as well as ethyl gallate have been shown to exhibit antioxidant activity and weak α -glucosidase and α -amylase inhibitory activities thus justifying the ethnomedicinal importance of *Acacia nilotica* in the management of diabetes mellitus.

Conflicts of Interest

None declared

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