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Effects of the Removal of Pigment on the Antioxidant Property of *Hibiscus sabdariffa Calyx Extracted* with Methanol

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Abstract: *Hibiscus sabdariffa is a widely consumed medicinal plant known for its rich phytochemical content and antioxidant potential. This study evaluated the antioxidant properties of methanol-extracted calyx using spectrophotometric and biochemical assays. Given the global interest in natural antioxidants for therapeutic and food preservation purposes, this study provides insight into the efficacy of H. sabdariffa as a viable source. The methanolic extracts were subjected to UV-Visible spectroscopic analysis and antioxidant activity assays: DPPH radical scavenging, Ferric Reducing Antioxidant Power (FRAP), and Inhibition of Lipid Peroxidation (ILP) were conducted at the concentration (µg/ml) of 500, 250, 125 and 62.5 respectively across the three samples A, B and C (pigmented, de-pigmented and desorbed) to evaluate comparative efficiency. The UV spectrum revealed maximum absorbance at 550 nm, for sample A (3.000), sample B (0.312) and sample C (4.472)indicating the presence of phenolic compounds in varying degrees. DPPH assay showed that the desorbed sample exhibited the highest free radical scavenging activity at 76.9±0.2 (µg/ml), 67.04±0.1 (µg/ml), 48.22±0.29 (µg/ml) and 32.85±0.05 (µg/ml), while the de-pigmented sample had the lowest at 68.24±0.22 (µg/ml), 55.72±0.03 (µg/ml), 25.63±26.9 (µg/ml)and 14.02±0.7 (µg/ml). This trend is similar with FRAP assay, desorbed sample was found to be strongest (91.59±0.03 (µg/ml), 86.9±0.1 (µg/ml), 76.8±0.03 (µg/ml) and 59.92±0.09 (µg/ml) and de-pigmented sample being the weakest (80.6±0.02 (µg/ml), 75.58±0.03 (µg/ml), 60.55±0.09 (µg/ml) and 36.59±0.03 (µg/ml)). The results' trend was also similar to the ILP assay; the desorbed pigment provided a higher inhibition of lipid peroxidation (69.41±0.2 (µg/ml), 58.63±0.07 (µg/ml), 43.63±0.29 (µg/ml) and 29.07±0.44 (µg/ml)) compared to the original and de-pigmented samples. In conclusion, antioxidant activity may be correlated with pigment concentration. These findings suggest that H. sabdariffa pigment extracted with methanol possesses potent antioxidant capacity suitable for nutraceutical applications.*

Keywords: *Hibiscus pigment, methanolic extract, antioxidant activity, nutraceutical applications.*

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1.0 Introduction

Oxidative stress, resulting from an imbalance between reactive oxygen species production and the body's antioxidant defence system, is associated with cellular and molecular damage and has been implicated in the development and progression of several chronic diseases. Consequently, considerable attention has focused on natural sources of antioxidants that could be used in pharmaceutical, nutraceutical, and food applications. Medicinal plants are particularly important sources of naturally occurring antioxidant compounds, including phenolics, flavonoids, anthocyanins, tannins and other secondary metabolites. The widespread use of medicinal plants in traditional healthcare systems has therefore stimulated continued scientific investigation into their phytochemical composition and biological activities. *Hibiscus sabdariffa* L., commonly known as roselle, is one of the plants that has received considerable attention because of its nutritional, medicinal and industrial importance.

Hibiscus sabdariffa belongs to the family Malvaceae and is widely cultivated in tropical and subtropical regions(Kéllou *et al.*, 2024; Kokare*et al.*, 2025). The plant is valued particularly for its fleshy calyces, which are consumed as herbal beverages, food ingredients and traditional medicinal preparations. The calyx is also an important source of natural pigments, especially anthocyanins, which give it its characteristic red to deep-purple colour. Beyond their colouring properties, these pigments have biological activities that have attracted interest in food, pharmaceutical, and cosmetic applications. The chemical composition of *H. sabdariffa* calyx includes anthocyanins, phenolic acids, flavonoids, organic acids and other phytochemicals that may contribute individually or synergistically to its biological properties (Alarcón-Alonso *et al*., 2012; Da-Costa *et al*., 2014). These compounds have been associated with several reported biological activities of H. sabdariffa, including antioxidant, anti-inflammatory, antihypertensive, and hepatoprotective effects. Among the biological properties attributed to *H. sabdariffa*, antioxidant activity is particularly important. Studies have demonstrated that extracts of the calyx can scavenge free radicals and reduce oxidizing agents, with the observed activity generally attributed to their phenolic and flavonoid constituents as well as anthocyanin pigments. However, several factors influence the antioxidant capacity of plant extracts, including plant variety, geographical origin, extraction solvent, extraction conditions, and the relative concentration and interaction of individual phytochemicals. Methanol is frequently employed for the extraction of phenolic and flavonoid compounds because of its effectiveness in solubilizing a broad range of polar and moderately polar bioactive constituents. Consequently, researchers have widely investigated methanolic extracts of H. sabdariffa for their antioxidant properties (Odigie *et al*., 2003; Alarcón-Alonso *et al*., 2012).

Anthocyanins are of particular interest because they serve a dual function as natural pigments and biologically active compounds. Their antioxidant properties stem from their ability to donate hydrogen atoms or electrons and interact with reactive oxygen species. However, the contribution of anthocyanins to the overall antioxidant capacity of *H. sabdariffa* extracts remains a subject of

scientific interest because the calyx contains several other antioxidant constituents. Phenolic acids, flavonoids and other non-pigmented compounds may also contribute substantially to the antioxidant activity of the extract. Therefore, removing pigments provides an opportunity to investigate whether the extract's antioxidant activity is predominantly associated with its coloured constituents or whether the non-pigmented phytochemical fraction retains substantial antioxidant activity.

Removing natural pigments from plant extracts is relevant to their potential industrial application. Although the intense colour of *H. sabdariffa* extracts is desirable in some food and beverage applications, it may be undesirable in products where colour, colour stability, or visual appearance is an important consideration. Pigment removal could therefore facilitate incorporating H. sabdariffa bioactive compounds into products where a colourless or less intensely coloured extract is preferred, including certain beverages, pharmaceutical formulations, nutraceutical preparations, and cosmetic products. However, removing pigments may simultaneously remove compounds that contribute significantly to antioxidant activity. Therefore, it is important to determine whether pigment removal reduces, retains, or alters the extract's antioxidant properties.

Previous investigations have extensively documented the antioxidant activity and phytochemical composition of *H. sabdariffa*, but comparatively less attention has been given to determining the specific contribution of its pigments to the overall antioxidant activity of methanolic calyx extracts. Most studies have evaluated the antioxidant capacity of crude or whole extracts, thereby measuring the combined effects of pigmented and non-pigmented constituents. Consequently, information remains limited on how antioxidant activity changes after pigment removal and whether the non-pigmented fraction retains appreciable antioxidant potential. This represents an important knowledge gap, particularly for developing pigment-reduced *H. sabdariffa* extracts for applications where colour is undesirable.

A comparative assessment of pigmented, de-pigmented and desorbed fractions can provide useful information about the relationship between pigment content and antioxidant activity. Complementary antioxidant assays are particularly important because different assays measure different aspects of antioxidant behaviour. The DPPH radical-scavenging assay provides an indication of the ability of an extract to neutralize a stable free radical, whereas the ferric reducing antioxidant power (FRAP) assay measures reducing capacity. Inhibition of lipid peroxidation (ILP), on the other hand, indicates the extract's ability to prevent oxidative degradation of lipids. Employing these complementary assays can therefore provide a more comprehensive assessment of changes in antioxidant activity associated with pigment removal.

This study's significance lies in its potential to clarify whether pigment removal from *H. sabdariffa* calyx extracts substantially affects their antioxidant properties. Showing that antioxidant activity is retained after pigment removal would support the development of less-coloured H. sabdariffa extracts for applications where intense pigmentation is undesirable. Conversely, a substantial reduction in antioxidant activity after pigment removal would provide evidence that pigmented constituents contribute significantly to the extract's antioxidant capacity. Such information is relevant to the rational development and utilization of *H. sabdariffa* extracts in nutraceutical, food, pharmaceutical and other bioactive-product applications (Adusei, 2020)

Therefore, this study aimed to evaluate the effect of pigment removal on the antioxidant

properties of methanolic extracts of *Hibiscus sabdariffa* calyx by comparing pigmented, de-pigmented and desorbed samples. The antioxidant properties of the samples were assessed using DPPH radical-scavenging activity, ferric reducing antioxidant power (FRAP), and inhibition of lipid peroxidation (ILP). The study was designed to determine whether pigment removal alters the antioxidant capacity of the extract and to provide evidence on the contribution of pigmented constituents to the overall antioxidant activity of *H. sabdariffa* calyx.

2.0 Materials and Methods

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2.0.1 Sample Collection and Preparation

Hibiscus sabdariffa L., popularly known as "zobo" in Nigeria, was locally obtained from a farm in Tungan Mallan, Paikoro Local Government Area, Niger State, Nigeria. A taxonomist in the Department of Biological Sciences, Ibrahim Badamasi Babangida University, Lapai, Nigeria, authenticated the plant material and deposited it in the university herbarium with voucher number IBBU210511.

The calyces were air-dried at ambient temperature for the specified period and then ground into a powder using a mortar and pestle. The sample preparation procedure described by Adisa *et al*. (2024) was adopted with minor modifications.

2.0.2 Preparation of Methanolic Extract and Pigment Removal

One hundred grams (100 g) of the powdered H. sabdariffa calyx was extracted with 1,000 mL of methanol in a beaker. The mixture was allowed to stand for 24 h, then decanted and filtered to obtain the methanolic extract. The extract was divided into two portions designated A and B.

Portion A was retained as the original, pigmented methanolic extract. Portion B was subjected to de-pigmentation by treatment with 25 g of charcoal as a local adsorbent. The mixture was warmed for approximately 5 min, then allowed to cool. The charcoal was separated from the extract by decantation followed by filtration to obtain the depigmented extract (B).

The pigments adsorbed onto the charcoal were subsequently desorbed using 180 mL of methanol. The charcoal–methanol mixture was warmed for approximately 5 min and filtered to obtain the desorbed pigment extract (C). Thus, the three fractions investigated were designated as follows: A, original pigmented extract; B, depigmented extract; and C, desorbed pigment extract.

The three extracts were concentrated using a simple distillation apparatus at 55–60 °C. The concentrated extracts were subsequently used for the antioxidant and phytochemical analyses.

2.0.3 UV–Visible Spectrophotometric Analysis

The UV–Visible absorption spectra of samples A, B and C were recorded using a UV–Visible spectrophotometer (Model 54G-Tecno-Pova). The spectra were used to compare the absorbance characteristics of the original, depigmented, and desorbed extracts and to assess changes associated with pigment removal and recovery.

2.1 Determination of DPPH Radical-Scavenging Activity

The radical-scavenging activity of the extracts was determined using the method described by Siddartha *et al*. (2022), with appropriate modifications. A 0.004% (w/v) solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was prepared in methanol. One millilitre (1 mL) of the DPPH solution was mixed with 1 mL of each extract at different concentrations. Gallic acid was used as the reference antioxidant.

The extract solutions were prepared by dissolving 0.05 g of the dried extract in 50 mL of methanol. We prepared the test solutions at concentrations of 62.5, 125, 250, and 500 µg/mL. The reaction mixtures were thoroughly mixed and incubated at 25 °C for 30 min in the dark. We measured absorbance at 517 nm using a spectrophotometer. All measurements were performed in triplicate.

The percentage DPPH radical-scavenging activity was calculated using Equation 1:

$$\% \, inhibition = \frac{Ablank - Asample}{Ablank} \times 100 \quad (1)$$

where: A_{blank} is the absorbance of DPPH radical + methanol; A_{sample} is the absorbance of DPPH radical + sample extract or standard.

2.2 Determination of Ferric Reducing Antioxidant Power

The reducing capacity of the extracts was determined using the ferric-reducing method described by Ryszard and Ronald (2019). Different concentrations of the extracts and gallic acid standard (12.5–100 µg/mL) were prepared in 1 mL of distilled water. Each prepared solution was mixed with 3.0 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide.

The mixtures were incubated at 50 °C for 20 min. Subsequently, 2.5 mL of 10% trichloroacetic acid was added, and the mixtures were centrifuged at 3,000 rpm for 10 min. A 2.5 mL aliquot of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3$). The absorbance was measured at 700 nm against an appropriate reagent blank using a spectrophotometer.

2.3 Determination of Percentage Inhibition of Lipid Peroxidation

The inhibition of lipid peroxidation by the extracts was determined using a modified thiobarbituric acid reactive substances (TBARS) method described by Geetha *et al*. (2013). Mix egg homogenate (0.5 mL, 10% v/v) with 0.1 mL of the test extract or gallic acid standard (10 mg/mL), then make up to 1 mL with distilled water. Then, add 0.05 mL of $FeSO_4$ solution and incubate for 30 min.

After incubation, add 1.5 mL acetic acid and 1.5 mL thiobarbituric acid prepared in sodium dodecyl sulfate. Vortex the mixture thoroughly, then heat at 95 °C for 60 min. After cooling to room temperature, 5 mL of butan-1-ol was added, and the mixture was centrifuged at 12,000 rpm for 10 min. The absorbance of the upper organic layer was measured at 532 nm.

The percentage inhibition of lipid peroxidation was calculated using equation 2:

$$\%Inhobitionoflipidperoxidation = \left(I - \frac{E}{C}\right) \times \frac{100}{1} \quad (2)$$

where (C) is the absorbance of the fully oxidized control and (E) is the absorbance measured in the presence of the test extract.

2.4 Qualitative Phytochemical Screening

Qualitative phytochemical screening was carried out on the extracts using standard chemical tests to determine the presence of selected classes of secondary metabolites.

2.4.1 Test for Tannins

The test, as described by Sahira and Catherine (2015). Two millilitres (2 mL) of the sample was mixed with 2 mL of distilled water in a test tube, followed by the addition of five drops of lead acetate solution. The mixture was shaken thoroughly, and the formation of a coloured precipitate was recorded as a positive indication for tannins.

2.4.2 Test for Flavonoids

(a) Sodium Hydroxide Test

Two millilitres (2 mL) of the sample was placed in a test tube, and 10 mL of sodium hydroxide solution was added. The development of yellow coloration was recorded as a positive indication for the presence of flavonoids (Evans *et al*., 2002).

(b) Ammonia Solution Test

Each sample (0.2 g) was dissolved in 3 mL of distilled water, shaken thoroughly and filtered. Subsequently, 3 mL of 25% ammonia solution was added to the filtrate, and the test tube was exposed to air. The development of a gradually appearing green coloration was recorded as a positive indication for flavonoids (Sahira & Catherine, 2015).

2.4.3 Test for Glycosides

The test for glycosides was carried out as described by Sahira and Catherine (2015). Each sample (0.2 g) was dissolved in 2 mL of chloroform in a test tube. Carefully add concentrated sulfuric acid (H_2SO_4) to form a separate lower layer. The formation of a

reddish-brown ring at the interface was recorded as a positive reaction.

2.4.4 Test for Saponins

Saponins were detected using the frothing test described by Ambarkar*et al*. (2011). Each sample (0.5 g) was shaken vigorously with 10 mL of distilled water in a test tube. The mixture was warmed in a hot-water bath for approximately 5 min, then shaken vigorously. Persistent frothing was recorded as evidence of saponin presence.

3.0 Results and Discussion

3.1 UV-Visible Analysis

he UV–Visible absorption spectra of the methanolic extracts obtained before and after pigment removal are presented in **Figure 1**. The figure compares the absorption characteristics of the original extract (Sample A), de-pigmented extract (Sample B), and desorbed pigment extract (Sample C). The comparison provides evidence of changes in the concentration and spectral characteristics of the coloured constituents following adsorption and subsequent desorption.

Fig. 1 shows that Sample A exhibited the strongest absorption in the visible region, with the highest absorbance occurring approximately within the 500–550 nm region. This strong visible absorption is consistent with anthocyanin pigments, which produce the characteristic red colouration of *H. sabdariffa* calyces. Anthocyanins have conjugated chromophoric structures that enable strong absorption in the visible region (Maciel*et al*., 2022). The relatively high absorbance of Sample A therefore reflects the substantial pigment content retained in the original methanolic extract.

In contrast, Sample B showed markedly reduced absorbance across the visible region, with reported values ranging from approximately 0.022 to 0.398. The substantial reduction relative to Sample A indicates effective removal of a large proportion of the coloured constituents during charcoal treatment. This observation is consistent with reports that adsorbent materials such as activated charcoal can remove coloured compounds from plant extracts through adsorption processes (Rizkiyah*et al*., 2022). The residual absorbance in Sample B suggests that de-pigmentation was incomplete and that some chromophoric compounds remained in the extract. These may include residual anthocyanins or other phenolic and flavonoid constituents capable of absorbing in the UV–Visible region..

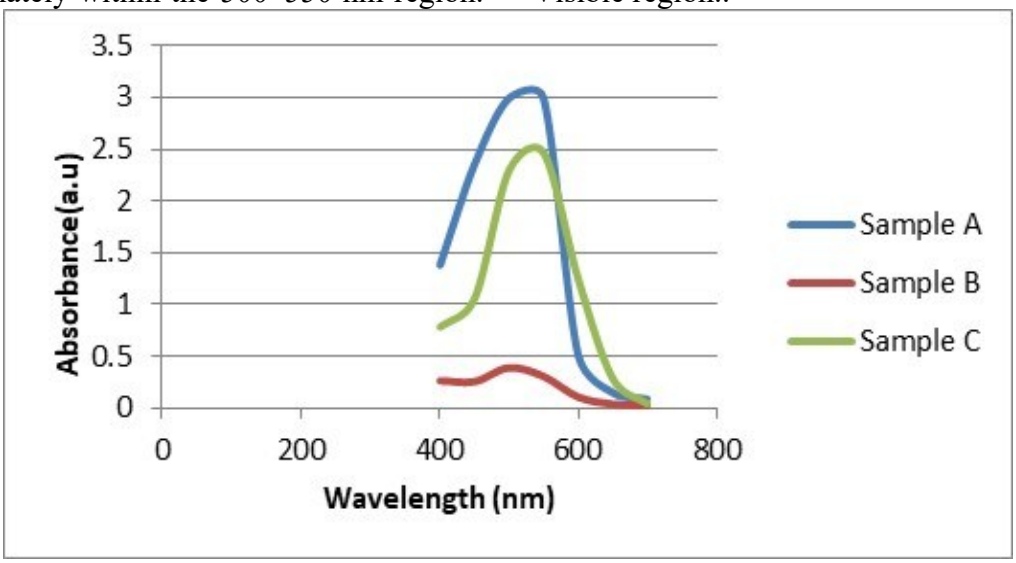


Fig. 1: UV-Visible Absorption spectrum

Key: Sample A: Original extract, Sample B: Depigmented extract, Sample C: Desorbed pigment ofthe*H. sabdariffa* calyx

Sample C, representing the desorbed fraction, exhibited considerably greater absorbance than Sample B, with absorption features extending into the visible region and a pronounced response around 500–550 nm. The higher absorbance of Sample C compared with Sample B indicates that a proportion of the compounds adsorbed onto the charcoal was recovered during methanolic desorption. The absorption maxima in the 500–550 nm region are consistent with the recovery of anthocyanin-rich coloured constituents. However, Sample C's spectral profile differed from Sample A's, suggesting that the desorption process did not fully restore the original pigment composition. Differences in solvent interaction, adsorption strength and pigment stability may influence the efficiency of pigment recovery (Airouyuwa*et al.*, 2024).

The reduction and alteration of the spectral profile following de-pigmentation and desorption may also indicate some structural modification or degradation of pigment molecules during processing. Anthocyanins are sensitive to environmental conditions, and their stability can be influenced by pH, temperature, solvent composition and processing conditions (Xue *et al*., 2024). The absorption observed in the 400–450 nm region across the samples may also reflect contributions from other phenolic or flavonoid constituents and possible pigment transformation products. However, UV–Visible spectroscopy alone cannot identify individual compounds; confirmation requires chromatographic or spectrometric techniques.

The spectral behaviourin Fig. 1 generally aligns with previous reports of strong absorption by *H. sabdariffa* anthocyanins in the visible region. Delphinidin-3-sambubioside and cyanidin-3-sambubioside are among the major anthocyanins reported in roselle calyces and contribute substantially to their characteristic colour(Larasati et al., 2024). The relatively low absorbance of Sample B therefore provides spectroscopic evidence for effective de-pigmentation, whereas the increased absorbance of Sample C demonstrates partial recovery of coloured constituents after desorption. These changes are important in interpreting the antioxidant results presented in subsequent sections.

3.2 Qualitative Phytochemical Screening

Table 1 presents the qualitative phytochemical composition of the original, depigmented, and desorbed methanolic extracts. The table compares the presence or absence of selected secondary metabolites in Samples A, B, and C after pigment removal and desorption.

Table 1 shows that tannins were detected in Samples A and C but not in Sample B. Flavonoids were detected in Sample A but were absent from Samples B and C based on the tests used. Saponins were not detected in any of the three samples, whereas glycosides were detected in Samples A and B but not in Sample C. The results therefore demonstrate qualitative differences in the phytochemical profiles of the three fractions following pigment adsorption and desorption.

The detection of flavonoids in Sample A is particularly relevant to the antioxidant activity of the original extract. Flavonoids contain hydroxyl groups that can participate in hydrogen- and electron-transfer reactions, thereby contributing to free-radical scavenging and reducing activity. The presence of flavonoids in the original extract is therefore consistent with the antioxidant responses subsequently observed in the DPPH, ferric-reducing and lipid-peroxidation assays. Previous studies have similarly identified flavonoids and other polyphenolic constituents as important contributors to the biological activities of *H. sabdariffa* extracts (Owoade *et al.*, 2016; Chen *et al.*, 2013).

Table 1: Phytochemical results

Phytochemical	Test	Sample A	Sample B	Sample C

Tannins	Lead Acetate	+	-	+
Tannins	Ammonia	+	-	-
Flavonoids	Hydroxide	+	-	-
Saponins	Frothing	-	-	-
Glycosides	Salkowski's	+	+	-

The absence of detectable flavonoids in Sample B suggests that either these compounds were removed during charcoal treatment, their concentration fell below the qualitative test's detection limit, or their chemical reactivity changed during processing. Because qualitative phytochemical tests provide presence/absence information rather than quantitative concentrations, the results cannot establish the extent of flavonoid removal. Nevertheless, the finding is noteworthy because Sample B also demonstrated the lowest antioxidant activity across the three antioxidant assays.

Tannins were detected in Sample A and recovered in Sample C but were not detected in Sample B. This pattern suggests that some tannin-containing constituents may have been preferentially associated with the adsorbed fraction and subsequently recovered during desorption. Similarly, glycosides were detected in Samples A and B but not in Sample C, suggesting differences in the distribution or recovery of these constituents following treatment. The absence of saponins in all three samples indicates that these compounds were either absent or below the detection limit of the test employed.

Overall, the phytochemical results in Table 1 provide qualitative evidence that charcoal treatment altered the detectable phytochemical composition of the extract. However, the results should be interpreted together with the antioxidant assays rather than used alone to assign antioxidant activity to individual compound classes. Quantitative determination of total phenolic, total flavonoid and anthocyanin contents, together with chromatographic analysis, would be required to establish the specific compounds responsible for the observed changes.

3.3 Antioxidant Activity

3.3.1 DPPH Radical-Scavenging Activity

Table 2 presents the DPPH radical-scavenging activities of Samples A, B, and C at concentrations of 62.5, 125, 250, and 500 µg/mL, and Figure 2 illustrates the concentration-dependent comparison. These results were used to evaluate the ability of the three extract fractions to neutralize the DPPH radical.

As shown in Table 2, DPPH radical-scavenging activity increased progressively with increasing extract concentration in all three samples. Sample A increased from 31.06 ± 0.10 at 62.5 µg/mL to 74.95 ± 0.22 at 500 µg/mL. Sample B increased from 14.02 ± 0.70 to 68.24 ± 0.22 over the same concentration range, whereas Sample C increased from 32.85 ± 0.05 to 76.90 ± 0.20. Fig. 2 also clearly illustrates the concentration-dependent response.

The increasing DPPH-scavenging activity with concentration indicates that higher extract concentrations provide more antioxidant constituents to interact with the DPPH radical. This behaviour is consistent with the concentration-dependent antioxidant response commonly observed in plant extracts containing phenolic and flavonoid compounds.

Among the three samples, Sample C exhibited the highest DPPH-scavenging activity at all tested concentrations. At 500 µg/mL, Sample C recorded 76.90 ± 0.20, compared with 74.95 ± 0.22 for Sample A and 68.24 ± 0.22 for Sample B. A similar pattern occurred at 62.5, 125 and 250 µg/mL, where Sample C

consistently produced the highest values. Sample B, in contrast, recorded the lowest activity at every concentration.

The relatively low DPPH activity of Sample B corresponds with its low absorbance in the UV–Visible analysis and its reduced qualitative phytochemical profile. In particular, the absence of detectable flavonoids in Sample B may partly explain its lower radical-scavenging response. Nevertheless, Sample B's retained activity indicates that pigment removal did not completely eliminate the extract's antioxidant constituents. Other non-pigmented phenolic compounds or residual pigments may therefore have contributed to the activity.

The high DPPH activity of Sample C indicates that the desorption process recovered constituents with substantial radical-scavenging capacity. The result may be associated with the recovery of pigmented constituents and other antioxidant compounds that became associated with the charcoal during de-pigmentation. Anthocyanins in *H. sabdariffa*, including delphinidin- and cyanidin-derived compounds, have been reported to possess radical-scavenging properties and may contribute to the antioxidant activity of roselle extracts.

Table 2:Antioxidant Test Results of DPPH Concentration (µg/ml)

Concentration	62.50	125	250	500
Sample A	31.06±0.1	41.59±0.11	62.22±0.22	74.95±0.22
Sample B	14.02±0.7	25.63±26.9	55.72±0.03	68.24±0.22
Sample C	32.85±0.05	48.22±0.29	67.04±0.1	76.9±0.2

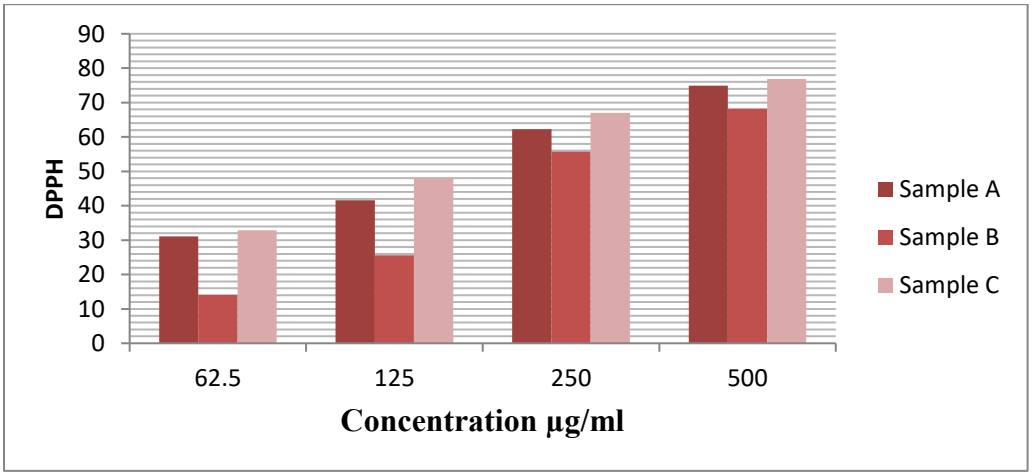


Figure 2:Column chart showing the concentration-dependent DPPH radical scavenging activity of samples A, B and C

The relative performance of Samples A, B and C in Table 2 and Figure 2 therefore suggests an association between pigment removal and changes in antioxidant activity. However, the results do not by themselves establish that anthocyanins were solely responsible for the higher activity of Sample C. The desorbed fraction may contain both pigments and co-extracted phytochemicals. Consequently, the observed antioxidant differences should be interpreted as changes associated with the de-pigmentation/desorption process rather than as direct proof of the antioxidant activity of a specific pigment.

3.3.2 Ferric Reducing Antioxidant Power

Table 3 presents the ferric-reducing activities of Samples A, B, and C at different concentrations, and Fig. 3 illustrates their concentration-dependent responses. The assay indicates the reducing capacity of antioxidant constituents in the extracts.

As shown in Table 3, all three samples exhibited increasing reducing activity as the extract concentration increased. Sample A increased from 51.59 ± 0.07 at 62.5 µg/mL to 88.62 ± 0.02 at 500 µg/mL. Sample B increased from 36.59 ± 0.03 to 80.60 ± 0.02, while Sample C increased from 59.92 ± 0.09 to 91.59 ± 0.03 over the same concentration range. The same concentration-dependent pattern is evident in Fig. 3.

Sample C recorded the highest reducing activity at every concentration tested. At 500 µg/mL, Sample C showed an activity of 91.59 ± 0.03, compared with 88.62 ± 0.02 for Sample A and 80.60 ± 0.02 for Sample B. At the lowest concentration of 62.5 µg/mL, Sample C also exhibited the highest value (59.92 ± 0.09), followed by Sample A (51.59 ± 0.07) and Sample B (36.59 ± 0.03).

The lower reducing capacity of Sample B indicates that de-pigmentation was associated with reduced electron-donating capacity of the extract. This observation is consistent with the DPPH results, where Sample B also exhibited the lowest activity. The agreement between the two assays strengthens evidence that charcoal treatment altered the extract's antioxidant composition.

Sample A displayed intermediate reducing activity, indicating that the original extract contained substantial reducing constituents. Sample C exceeded Sample A, suggesting that the desorbed fraction contained a relatively high concentration of compounds capable of reducing ferric ions under the assay conditions. This may reflect enrichment of certain compounds during adsorption and subsequent desorption, although the present data do not establish the precise mechanism responsible for this increase.

The results presented in Table 3 and Fig. 3 further demonstrated that pigment removal does not necessarily result in complete loss of antioxidant activity. Rather, de-pigmentation reduced the measured reducing capacity, while subsequent desorption produced a fraction with greater reducing activity than both the de-pigmented and original extracts. This observation suggests that compounds associated with the charcoal contributed significantly to the measured antioxidant response.

The present results should nevertheless be interpreted cautiously because ferric-reducing assays measure overall reducing capacity and cannot distinguish between anthocyanins, phenolic acids, flavonoids, organic acids or other reducing compounds. Quantitative chemical profiling would therefore be required to establish which compounds accounted for the differences among Samples A, B and C.

Table 3: Result of FRAP Antioxidant Test (µg/ml)

Concentration	62.50	125	250	500
Sample A	51.59±0.07	72.33±0.1	80.7±0.03	88.62 ±0.02
Sample B	36.59±0.03	60.55±0.09	75.58±0.03	80.6±0.02
Sample C	59.92±0.09	76.8±0.03	86.9±0.1	91.59±0.03

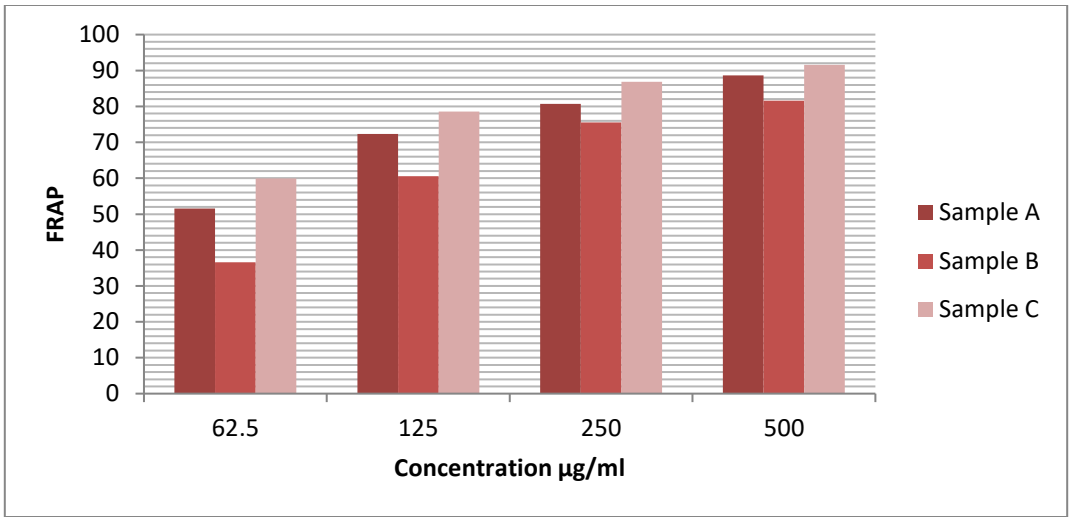


Fig. 3: Column chart showing the concentration-dependent antioxidant power of samples A, B and C

3.3.3 Inhibition of Lipid Peroxidation

The inhibition of lipid peroxidation by Samples A, B and C at concentrations ranging from 62.5 to 500 µg/mL is presented in Table 4, while the corresponding concentration-dependent responses are illustrated in Fig. 4. This assay provides a complementary assessment of antioxidant activity by determining the ability of the extracts to inhibit oxidative degradation of lipids.

Table 4 shows that inhibition of lipid peroxidation increased with increasing extract concentration in all samples. Sample A increased from 27.41 ± 0.10 at 62.5 µg/mL to 66.32 ± 0.03 at 500 µg/mL. Sample B increased from 9.98 ± 0.64 to 59.01 ± 0.33, whereas Sample C increased from 29.07 ± 0.44 to 69.41 ± 0.20. Fig. 4 clearly demonstrates the concentration-dependent pattern.

Sample C exhibited the highest inhibition of lipid peroxidation at all tested concentrations. At 500 µg/mL, its inhibition value was 69.41 ± 0.20, compared with 66.32 ± 0.03 for Sample A and 59.01 ± 0.33 for Sample B. At 62.5 µg/mL, Sample C also showed the highest activity (29.07 ± 0.44), followed by Sample A (27.41 ± 0.10) and Sample B (9.98 ± 0.64).

The comparatively low activity of Sample B is consistent with the results obtained from the DPPH and ferric-reducing assays. The reduction in lipid-peroxidation inhibition after pigment removal suggests that some compounds removed during charcoal treatment contributed to the extract's ability to protect lipids against oxidative damage. However, the retained activity of Sample B indicates that de-pigmentation did not completely eliminate the antioxidant constituents capable of inhibiting lipid oxidation.

The high activity of Sample C may be associated with the recovery of antioxidant constituents from the adsorbent during methanolic desorption. Because the desorbed fraction showed the highest activity in all three assays, the results suggest that constituents concentrated in, or recovered from, the adsorbed fraction contributed substantially to the antioxidant response. Anthocyanins and other phenolic compounds in H. sabdariffa have been reported to have antioxidant properties and may participate in inhibiting oxidative reactions.

The pattern observed in Table 4 and Fig. 4 is therefore consistent with the DPPH and ferric-reducing results: Sample C demonstrated the highest antioxidant activity, Sample A showed intermediate activity, and Sample B showed the lowest activity. The agreement among the three assays is important because they evaluate different aspects of antioxidant behaviour. DPPH primarily reflects radical-scavenging capacity, the ferric-reducing assay reflects reducing capacity, and the lipid-peroxidation assay evaluates protection against oxidative degradation of lipids.

Table 4: Antioxidant test result of inhibition of lipid peroxidation concentration (µg/ml)

Concentration	62.50	125	250	500
Sample A	27.41±0.1	36.99±0.12	54.57±0.22	66.32±0.03
Sample B	9.98±0.64	21.04±26.9	47.07±0.03	59.01±0.33
Sample C	29.07±0.44	43.63±0.29	58.63±0.07	69.41±0.2

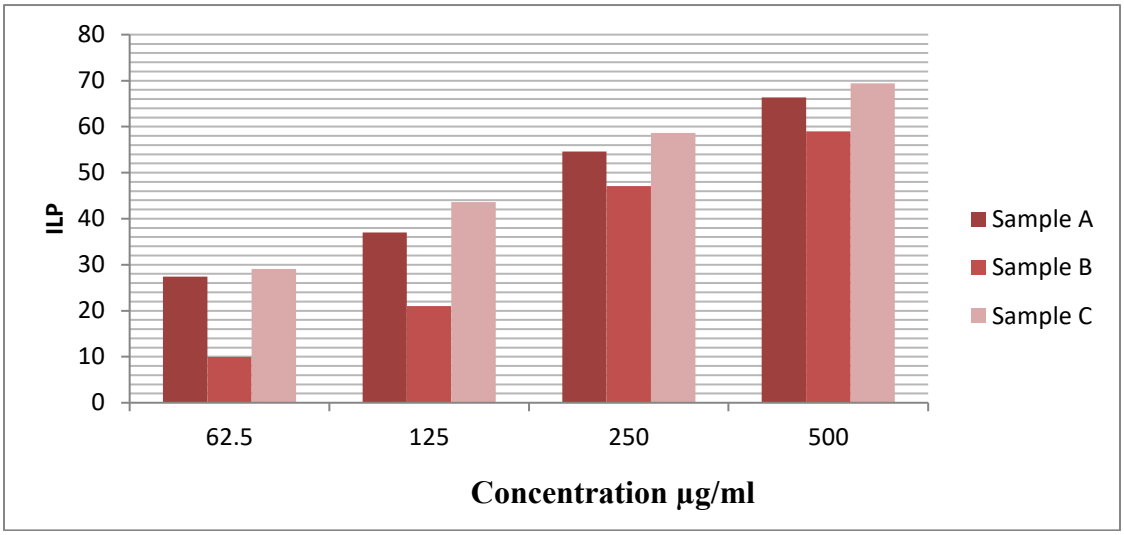


Fig.4:Column chart showing the concentration-dependent inhibition of lipid peroxidation of samples A, B and C.

3.4 Comparative Interpretation of the Antioxidant Results

The three antioxidant assays show a consistent overall pattern across the extract fractions. As shown in Tables 2–4 and Figs. 2–4, Sample C generally exhibited the highest antioxidant activity, Sample A showed intermediate activity, and Sample B recorded the lowest activity. Furthermore, antioxidant activity increased with increasing extract concentration in all three assays.

The consistency of this pattern provides evidence that the de-pigmentation treatment substantially altered the antioxidant properties of the *H. sabdariffa* calyx extract. The lower antioxidant activity of Sample B, together with its markedly reduced UV–Visible absorbance and altered qualitative phytochemical profile, suggests that charcoal treatment removed or reduced constituents that contributed to antioxidant activity. Conversely, the relatively high antioxidant activity of Sample C indicates that methanolic desorption recovered a considerable proportion of antioxidant-active constituents associated with the adsorbed fraction.

The results also indicate that the antioxidant activity of *H. sabdariffa* calyx cannot be attributed exclusively to its pigments. Sample A retained strong antioxidant activity, while Sample B retained measurable activity despite substantial pigment removal. This indicates that non-pigmented constituents also contribute to the extract's antioxidant properties. At the same time, the consistently lower activity of Sample B than Samples A and C suggests that pigment-associated constituents may contribute significantly to overall antioxidant capacity.

The higher antioxidant activity of Sample C than Sample A is particularly noteworthy. This observation may indicate preferential adsorption and subsequent concentration of antioxidant-active constituents on the charcoal surface followed by their recovery during methanolic desorption. It may also reflect differences in the relative composition of the fractions rather than simply an increase in pigment concentration. Therefore, the present findings support an association between pigment removal and antioxidant activity but do not establish a direct quantitative relationship between anthocyanin concentration and antioxidant capacity.

Taken together, the UV–Visible results in Fig. 1, phytochemical findings in Table 1, and antioxidant results presented in Tables 2–4 and Figs. 2–4 demonstrate that pigment removal significantly changes the chemical and antioxidant characteristics of *H. sabdariffa* calyx methanolic extracts. The de-pigmented fraction exhibited reduced antioxidant activity, whereas the desorbed fraction showed the highest activity across the three assays. These findings indicate that pigment-associated constituents contribute to the antioxidant properties of the calyx extract, while non-pigmented constituents also contribute significantly.

However, further quantitative analysis of total phenolic content, total flavonoid content and individual anthocyanins would be necessary to establish the chemical basis of the differences observed among Samples A, B and C. Such analyses would provide stronger evidence on whether the observed antioxidant changes are directly associated with pigment concentration or with broader changes in phytochemical composition caused by adsorption and desorption.

4.0 Conclusion

This study evaluated the effect of pigment removal on the antioxidant properties of methanolic extracts of *Hibiscus sabdariffa* calyx by comparing the original extract (Sample A), de-pigmented extract (Sample B), and desorbed pigment extract (Sample C). The UV–Visible spectral analysis showed substantial differences among the three samples: Sample A showed strong absorption in the visible region associated with the characteristic pigments of H. sabdariffa; Sample B showed markedly reduced absorbance after de-pigmentation; and Sample C showed partial recovery of the visible-absorbing constituents after desorption.

Qualitative phytochemical screening further showed differences in the detectable phytochemical composition of the three fractions. In particular, flavonoids were detected in the original extract but not in the de-pigmented and desorbed samples, while tannins and glycosides showed different distributions among the fractions. These changes indicate that charcoal treatment and subsequent desorption altered the methanolic extract composition.

The DPPH, ferric-reducing and lipid-peroxidation assays consistently showed concentration-dependent antioxidant activity. Across the three assays, Sample C generally exhibited the highest antioxidant activity, followed by Sample A, whereas Sample B demonstrated the lowest activity. The reduced activity of the de-pigmented extract indicates that the removal of pigment-associated constituents was accompanied by a reduction in antioxidant capacity. However, the

retention of measurable antioxidant activity in Sample B also demonstrates that non-pigmented constituents contribute to the overall antioxidant properties of *H. sabdariffa* calyx.

Overall, the findings show that pigment removal alters the antioxidant properties of H. sabdariffa calyx methanolic extracts and suggest that pigment-associated constituents contribute significantly to their antioxidant activity. The strong antioxidant response in the desorbed fraction further indicates that antioxidant-active constituents can be recovered after adsorption. These findings provide useful evidence for developing pigment-reduced or pigment-recovered H. sabdariffa extracts for food, nutraceutical, and related applications. However, quantitative determination of total phenolic, flavonoid and anthocyanin contents, together with chromatographic characterization of individual compounds, is recommended to establish the specific chemical constituents responsible for the differences in antioxidant activity among the three fractions.

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Conflict of interests

The authors declare no conflict of interest.

Authors' Contributions

Adisa, M. J. conceptualised the study and supervised the research. Olanipekun, B. E. contributed to sample preparation and experimental procedures. Sulaiman, K. contributed to antioxidant analyses and data

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