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Phytochemical screening and antioxidant activity of the ethanol extracts of the bark of *Hibiscus sabdariffa*

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Abstract
Hibiscus sabdariffa, commonly known as Roselle and locally referred to as 'Chukai' in Bangladesh, is renowned for its diverse medicinal properties. Although widespread research has explored the antioxidant potential of the fruit, the bark part remains under-investigated. This study inspects the phytochemical profile and antioxidant activity of bark extracted with ethanol solvent. Phytochemical screening identified the presence of carbohydrates, alkaloids, and flavonoids. Antioxidant potential was assessed through the measurement of total antioxidant capacity, total phenolic content, total flavonoid content, and the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. The total antioxidant capacity observed was 4.235 mg/g ascorbic acid equivalent, phenolic content was 6 mg/g gallic acid equivalents, and flavonoid content was 5.896 mg/g as quercetin equivalents. In the DPPH assay, IC_{50} values for ascorbic acid and bark extract were 19.9 µg/mL and 154.87 µg/mL, respectively. Overall results reveal that the Roselle bark possesses a limited antioxidant capacity compared to ascorbic acid, although having some bioactive compounds.

Keywords: *Hibiscus sabdariffa*, phytochemical, antioxidant, medicinal plant, ascorbic acid, DPPH

Introduction

Medicinal plants are a rich source of diverse chemical compounds that exhibit a range of bioactive properties, including antioxidant, antimicrobial, anticancer, and antiviral effects [1]. Therefore, these plants are recognized as valuable reservoirs of phytochemicals with potential therapeutic applications. Among these, a major class of phytochemicals demonstrates strong antioxidant activity [2, 3]. Antioxidants protect the body from the damaging effects of free radicals, mainly the reactive oxygen species (ROS). A balance between antioxidant levels and ROS is essential for physiological homeostasis. When ROS production exceeds the antioxidant capacity, the resulting imbalance leads to oxidative stress, causing inflammation and tissue damage [4, 5]. The most effective approach is to use natural antioxidants since synthetic antioxidants can produce compounds that may lead to cancer. Moreover, natural antioxidants are abundant in all plant parts. Therefore, research for the identification of natural antioxidants from plants has rocketed [6]. Natural antioxidants, which include phenolic acids like the most important flavonoids, and carotenoids, might neutralize the harmful effects of ROS [7].

Natural substances known as flavonoids exhibit a characteristic phenolic structure and are separated into many compounds (Flavonols, flavones, flavanols, flavanones, anthocyanidins, and chalcones) [8]. Several factors influence natural products' antioxidant capability. These include extraction methods, solvents and the composition of the extract [9].

Biologically active organic compounds and secondary plant metabolites present in plants are referred to as phytochemicals. While some of these compounds are highly poisonous, others are pharmacologically active [10]. Among these, flavonoids are a prominent group of phytochemicals associated with significant health benefits. Research suggests that flavonoids may play a preventive role against chronic diseases, most importantly cancer. Other chronic conditions includes diabetes, cardiovascular diseases, alzheimer's disease, and various ocular disorders [11]. It is estimated that fruits and vegetables contain around 5,000 distinct dietary phytochemicals, many of which remain unidentified or insufficiently characterized [12].

Epidemiological and medical studies indicate that many of these compounds function as antioxidants, contributing to disease prevention [13]. Therefore, the best and most prudent use of naturally occurring materials depends on the results of phytochemical investigations, which are crucial for discovering new sources of molecules with medical relevance that are both therapeutically and industrially important [14].

Fig 1: Roselle Plant

Roselle (*Hibiscus sabdariffa*) is an annual or perennial herb most commonly found in tropical and subtropical regions. The plant is around 2-2.5m tall and belongs to the Malvaceae family. The antioxidant content of this plant can vary significantly based on environmental factors, and harvesting practices [15]. The calyces of the plant, either dried or fresh, are commonly used in making confectioneries and herbal beverages. For centuries, roselle has been recognized for its medicinal properties, assisting in treating chronic diseases [16]. Its pharmacological effects include anticancer, anti-inflammatory, antihypertensive, antihyperlipidemic, and antibacterial activities [17]. These beneficial effects are associated with the biologically active phytochemicals [18]. Roselle flower is particularly rich in flavonol polyphenols, which exist in simple and polymerized forms. Key flavonoids identified in its extracts include hibiscitrin (hibiscetin-3-glucoside), sabdaritrin and various types of gossypetin glucosides. Additionally, luteolin and quercetin was reported in the methanolic extracts derived from the flowers [19]. These phytochemicals significantly contribute to the antioxidant properties and potential health benefits of *Hibiscus sabdariffa*. Extensive research has been conducted on the leaves, fruit, and flowers of *Hibiscus sabdariffa*, highlighting their diverse phytochemical profiles and associated health benefits. However, to our knowledge, no investigation has been carried out on the phytochemical composition and antioxidant activities of the bark extract. Therefore, this study aims to fill this gap by systematically identifying the phytochemicals present in the bark and evaluating their antioxidant properties. In the study, ethanol has been used to extract the components from the bark.

Materials and Methods

Collection of the bark

The roselle bark was collected from the Chandpur district of Bangladesh during the fruit maturation stage. The plant was subsequently identified taxonomically at Botany Department of Jahangirnagar University, Bangladesh.

Extraction process

Bark parts were chopped into pieces and dried under indirect sunlight for five days to ensure complete drying, the dried bark pieces were kept in an oven for 72 hours set at a temperature of 60 °C. The dried bark was then ground into a coarse powder using an attrition-type grinder. Total 120g powder was obtained from the process. Powdered bark was extracted with ethanol using a Soxhlet extractor at room temperature. The extraction process continued until the solvent flowing from the powdered sample became completely colourless, indicating that the extraction was complete. Subsequently, a rotary evaporator was employed to evaporate the extracts under vacuum until they reached a dry, concentrated form.

Chemicals and reagents

The following reagents and chemicals were utilized in this study: Molisch's reagent, Mayer's reagent, Hager's reagent, Wagner's reagent, Dragendroff's reagent, Folin-Ciocalteu reagent (FCR), sodium hydroxide, sulfuric acid, chloroform, ferric chloride, aluminium Chloride, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, quercetin, and gallic acid. To ensure the reliability and reproducibility of results, all the chemicals and reagents were chosen analytical grade commercially and kept in the appropriate storage condition.

Sample preparation

The sample solution was prepared by suitably treating small volumes of decolorized, dried extracts. The stock solution was kept in the fridge at 4 ^{0}C. All experiments were done within one week after the completion of the extraction procedure. The extract was diluted with appropriate solvents when required. Freshly prepared samples were used for phytochemical tests.

Phytochemical Screening

Various qualitative phytochemical tests such as test for Carbohydrate, Glycoside, Glucoside, Saponin, Steroid, Tannin, Flavonoid, and Alkaloid were performed according to the following methods [20-21].

Carbohydrate Test (Molisch's Test)

A small quantity of the extract solution was placed in a test tube, followed by the addition of two drops of Molisch's reagent, which is a solution of α-naphthol in ethanol. Subsequently, 1 mL of concentrated sulfuric acid was carefully added by allowing it to flow down the side of the test tube. The presence of carbohydrates was indicated by the formation of a distinct purple ring at the interface between the extract solution and Molisch's reagent-sulfuric acid mixture.

Small amount of the extract solution was dissolved in 1 mL of water in a test tube. Two drops of sodium hydroxide solution were then added. Yellow colour in the reaction indicated the presence of glycosides in the extract.

Glycosides Test (Fehling solution test)

In a test tube, a small quantity of the extract solution was taken and combined with several drops of diluted sulfuric acid. The mixture was heated until boiling. Afterwards, a few drops of 0.1 M sodium hydroxide (NaOH) were added to the mixture. Subsequently, both components of Fehling's solution (A and B) were introduced, and the mixture was boiled once more. The development of a brick-red precipitate serves as an indication of a positive glucoside test.

Saponin Test

2 mL of the extract was mixed with 20 mL of deionized water in a test tube. The test tube was subjected to violent shaking for two minutes. The foam that was seen suggested that

saponins were present.

Steroid Test
A small volume of the extract solution was added to a test tube, followed by the addition of 2 mL of chloroform. 1 mL concentrated sulfuric acid (H_2SO_4) was then carefully introduced along the side of the test tube. The presence of steroids was indicated by the chloroform layer changing to a red coloration.

Tannin Test
A small volume of the extract solution was added to a test tube containing 10 mL of distilled water. Subsequently, a few drops of a 5% ferric chloride ($FeCl_3$) solution were introduced to the mixture. The appearance of a greenish-black precipitate confirmed the presence of tannins.

Flavonoids Test
A small volume (1 mL) of the extract solution was placed in a test tube, followed by the addition of a few drops of concentrated hydrochloric acid. The immediate development of a red color confirmed a positive result for the presence of flavonoids.

Alkaloid Test
1 mL extract solution was neutralized with 1-2 drops of diluted sulfuric acid and then divided into four separate test tubes. To each tube, three drops of the respective reagents were added: Mayer's reagent to the first tube, Hager's reagent to the second, Wagner's reagent to the third, and Dragendroff's reagent to the fourth. The formation of a white cream precipitate in the first tube, a yellow crystalline precipitate in the second tube, a brownish-black precipitate in the third tube, and an orange or orange-red precipitate in the fourth tube indicated the presence of alkaloids in the solution.

***In-vitro* assessment of antioxidant activities**
The antioxidant activity of *Hibiscus sabdariffa* bark was evaluated through the determination of total phenolic content, flavonoid content, total antioxidant capacity, and free radical scavenging ability using the following methodologies:

Determination of Total Phenolic Content [22]
The total phenolic content was evaluated using the Folin–Ciocalteu Reagent (FCR) assay. To each test tube, 5 mL of a 10-fold diluted FCR solution was added to 1 mL of the plant extract. Subsequently, 4 mL of sodium carbonate solution was introduced into each tube. For the standard solution, the reaction was allowed to proceed at 25 °C for 30 minutes, while the extracts were incubated for one hour under the same conditions to ensure complete reaction. The absorbance of the resulting solutions was measured at 765 nm using a UV spectrophotometer, with eathanol serving as the control. The total phenolic content in the ethanolic extracts was expressed as gallic acid equivalents (GAE) and calculated using the following formula:

$$C = (c \times V)/m$$

Here,
C = total content of phenolic compounds in GAE (mg/g) of plant extract)
c = concentration of gallic acid determined from the calibration curve (mg/mL)
V = extract volume (mL)
m = weight of the pure plant ethanolic extract

Determination of Flavonoids Content [23-24]
To measure flavonoid content, the aluminium chloride was used. In a test tube, 1 mL of the extract was mixed with 3 mL of ethanol. Then 0.2 mL of a 10% aluminum chloride solution and 0.2 mL of a 1 M potassium acetate solution were added. The mixture was then diluted with 5.6 mL of distilled water. The reaction was allowed to proceed for 30 minutes at room temperature. After the incubation, the absorbance was recorded at 415 nm against a blank sample using a UV-visible spectrophotometer. The total flavonoid content was then calculated in quercetin equivalents (mg QE/g extract) based on a quercetin standard curve.

$$C = (c \times V)/m$$

Here,
C = total flavonoid content in quercetin (mg/g) of plant extract
c = Quercetin concentration determined from the calibration curve (mg/mL)
V = extract volume (mL)
m = weight of the pure ethanolic extract

Determination of Total Antioxidant Capacity [25]
The phosphomolybdenum assay was employed for this purpose. The experiment is based on the reduction of Mo(VI) to Mo(V) by antioxidant compounds, forming a quantifiable green complex. The procedure involved preparing a reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. In a test tube, 3 mL of freshly prepared reagent solution was mixed with 0.3 mL (2-3 drops) of the sample extract. The reaction was then incubated at 95 °C for 90 minutes. After cooling down to room temperature, the absorbance of the resulting solution was measured at 695 nm using a spectrophotometer, with a blank prepared under identical conditions but without the sample extract. The total antioxidant capacity, expressed in mg of ascorbic acid equivalents per g of plant extract, was calculated using the formula:

$$A=c\times VmA$$

Where,
A =antioxidant capacity in mg/g
c = concentration of ascorbic acid (mg/mL) obtained from a standard calibration curve
V = volume of the extract (mL)
m = mass of the pure extract (g)

Free Radical Scavenging Capacity [26-27]
DPPH (2,2-diphenyl-1-picrylhydrazyl) method was employed to determine free radical scavenging capacity. Chemically, the delocalization of the electron of DPPH gives a deep violet colour in solution. When DPPH is mixed with a substrate (AH), able to donate a hydrogen atom, DPPH gets reduced with the loss of this violet colour. The procedure involves adding 0.2 mL of plant extract solutions to test tubes, followed by 2 mL of a 0.5 mM DPPH solution in each tube. The mixtures were then incubated at room temperature for 30 minutes to allow the reaction to proceed. After incubation, absorbance was measured at 517 nm using a UV spectrophotometer, with the results compared to a blank solution containing only ethanol.

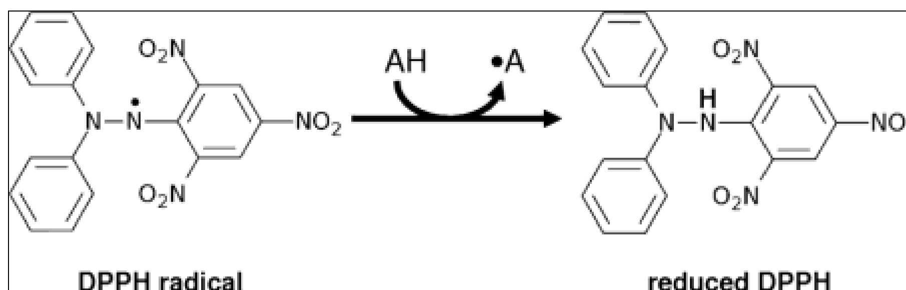

Fig 2: DPPH reaction

The following equation was used to determine the percentage (%) of inhibitory activity.

(A0 – A1)/A0} X 100

A0 is the control absorbance reading, and A1 is the extract or standard absorbance reading. The IC_{50} was then determined by plotting the percentage of inhibitions against concentration on the graph. The IC_{50} value was determined by plotting the percentage of inhibition against concentration on a graph.

Results

Phytochemical Screening

Phytochemical investigation or screening is an evaluation process for the detection of plant constituents through chemical analysis. In this experiment, the presence of various reported compounds was identified by using various standard qualitative tests as well as the presence of other constituents in *Hibiscus sabdariffa bark* extract. The results are summarized in Table 1.

Table 1: Phytochemical screening of *Hibiscus sabdariffa* bark extract

Metabolites	(+) =Positive reaction (-) =No reaction
Carbohydrates	+
Glycosides	-
Glucoside	-
Saponins	-
Steroids	-
Tannins	-
Flavonoids	+
Alkaloids	+

Total Phenol Content: The total phenolic content of the ethanolic extract of the bark of *Hibiscus sabdariffa* was calculated using the standard curve of gallic acid (y=0.009581x + 0.1079, R^2=0.9842, Figure 1). Measurements were done in triplicates. The total phenol contents was found 6.0925 mg/g, gallic acid equivalent.

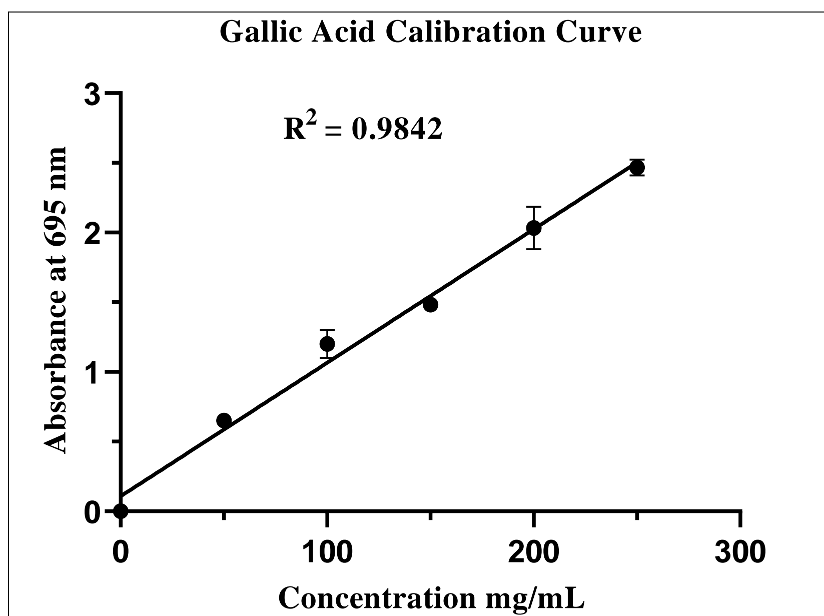

Fig 3: Linear Regression Analysis of Gallic Acid Standard

Flavonoid Content

The total flavonoid content was determined using a standard curve of quercetin, described by the equation y=0.0092x−0.0256y = 0.0092x - 0.0256y=0.0092x−0.0256 with an R^2 value of 0.9898 (Figure 2), and was expressed as quercetin equivalents (QE) per gram of extract. All measurements were performed in triplicate. The flavonoid content in the ethanolic extract of *Hibiscus sabdariffa* bark was found to be 5.896 mg/g in quercetin equivalents.

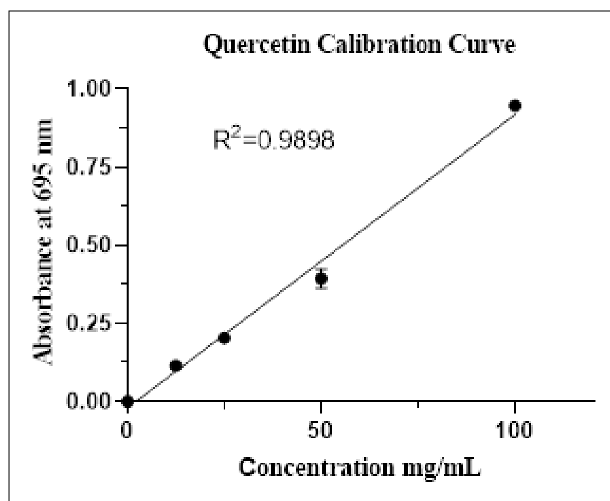

Fig 4: Linear Regression Analysis of Quercetin Standard

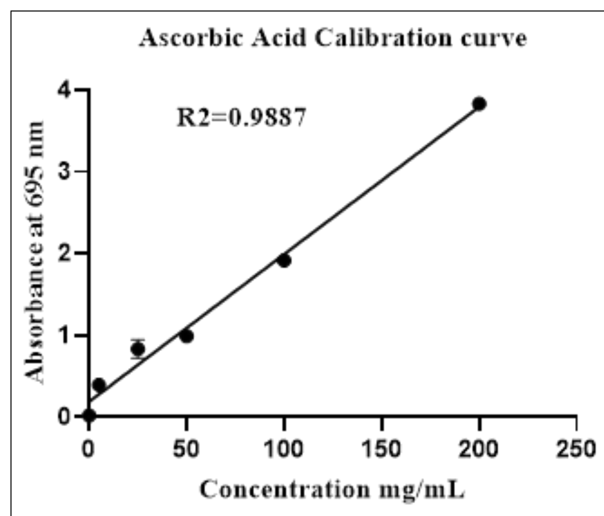

Fig 5: Linear Regression Analysis of Ascorbic Acid Standard

Total Antioxidant Capacity

The standard curve of ascorbic acid was utilized to measure the total antioxidant capacity. (y= 0.01804x + 0.1889, R^2=0.9887, Figure 3). To establish the curve, measurements were done in triplicate. Total antioxidant activity was found 4.235 mg/g, equivalent of ascorbic acid.

DPPH Scavenging Activity

The ability of antioxidants to scavenge free radicals has been extensively assessed using DPPH. Plotting the percent (%) of inhibition versus log concentration allowed for the calculation of the IC_{50} value from the graph. The curve of percent (%) inhibition against log concentration is illustrated in Figure 4.

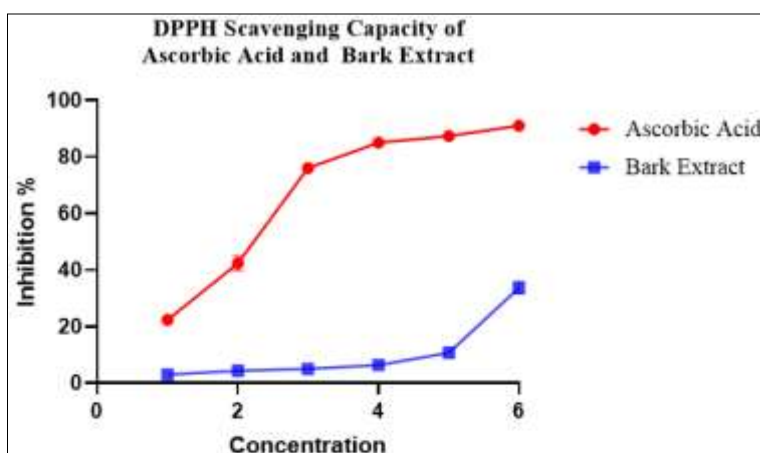

Fig 5: DPPH radical scavenging activity of Ascorbic acid (Standard) vs *Hibiscus sabdariffa* bark extract.

DPPH scavenging activity was calculated by determining the IC_{50} value of the standard and bark extracts. The IC_{50} value of the standard ascorbic acid was 19.9 µg/mL. However, the IC_{50} value of the bark extract was found 154.87µg/mL.

Discussion

The ethanolic extract of *Hibiscus sabdariffa* barks contained some important phytocompounds, according to the study's outcomes. The screening of phytochemicals demonstrated that the sample was positive for carbohydrates, flavonoids and alkaloids. It has been demonstrated that hydrocarbon molecules have antibacterial effects [28, 29]. These phytocompounds' isolation and purification could one day be helpful in the development of innovative medications for the treatment of ailment [30].

The reducing capacity of the sample was measured using the Folin–Ciocalteu Reagent (FCR) in order to figure out the total phenolic compounds. Phenolic compounds are widely recognized for their antioxidant properties, playing a vital role in neutralizing free radicals and protecting against oxidative stress. The phenolic concentration obtained in this study indicates that *Hibiscus sabdariffa* bark is a meaningful source of phenolic compounds. Further research is required to isolate and characterize these phenolic compounds to better understand their individual contributions to the extract's overall biological activity. Overall, the phenolic content of the *Hibiscus sabdariffa* bark extract offers potential for health applications, especially in areas related to oxidative stress and inflammation.

The plant's flavonoid components indicate that it has medicinal benefits [28, 31, 32]. Flavonoid molecules with functional groups have antioxidant activities and prevent lipid peroxidation and the angiotensin I-converting enzyme [33, 34]. According to this study, the ethanolic extract of *Hibiscus sabdariffa* bark has 5.896 mg/g of flavonoids, which are equivalents to quercetin. The amount we detected suggests that this plant could be an important source of these beneficial compounds. Interestingly, most research on *Hibiscus sabdariffa* has focused on its calyces or leaves, which are also rich in flavonoids. Even so, our findings indicate that the bark hasn't gotten nearly as much attention despite having considerable flavonoid content. This makes the bark potentially useful for pharmacological applications as an extra or substitute source. The potential of the bark extract in

medication development, particularly for oxidative stress-related disorders, might be investigated further. In the future, identifying the particular flavonoids found in the bark and evaluating each one's potential health advantages may help identify more medicinal applications for this plant.

The total antioxidant capacity of the bark extract was found to be 4.235 mg/g, measured in ascorbic acid equivalents. This result indicates that the extract has a moderate ability to neutralize free radicals, which play a key role in oxidative stress. Despite the lower antioxidant potency, the presence of bioactive compounds like flavonoids and phenols still makes this bark extract worth exploring, particularly for other therapeutic properties, such as anti-inflammatory or antimicrobial activities. Further research could help isolate and identify the compounds contributing to this antioxidant capacity.

When DPPH free radical reacts with hydrogen donors, it is converted to the equivalent hydrazine, which is then used to determine antioxidants' free radical scavenging capacity. Stable free radicals can be formed by DPPH in methanol or aqueous solutions. Greater activity in scavenging free radicals is shown by lower absorbance [35]. When an electron or hydrogen radical from an antioxidant component was accepted, the molecule's absorbance at 517 nm disappeared, transforming it into a stable diamagnetic molecule[36]. The experimental data indicates that the ethanolic extract of *Hibiscus sabdariffa* bark has a moderate capacity to scavenge free radicals. Figures 4 shows that the percentage of DPPH scavenging by the extract is lower compared to ascorbic acid. The IC_{50} value, which measures the concentration needed to inhibit 50% of free radicals, reveals a moderate difference between the extract and the standard. Ascorbic acid, a potent antioxidant, has a low IC_{50} of 19.9 µg/ml, whereas the bark extract has a much higher IC_{50} of 154.87 µg/mL**,** indicating it requires a greater amount to achieve the same antioxidant effect. The results suggests that *Hibiscus sabdariffa* bark extract possesses a low amount of antioxidant capacity and is not a potential candidate for natural antioxidant.

Conclusion

Our study revealed that ethanolic bark extract of *Hibiscus sabdariffa* contains flavonoids and phenols, both known for their antioxidant properties. However, despite these bioactive compounds, the bark extract's overall antioxidant potential was found low, as shown by its total antioxidant capacity and a higher IC_{50} value in the DPPH assay. Therefore, Rosella bark is not a good candidate for antioxidant research. However, it could be recommended to investigate the bark part in different solvents to find out all the active constituents. This outcome of the study could be important in future efforts to design nutraceuticals and pharmaceuticals.

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Conflict of interests

The authors declare no conflict of interest in this study.

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