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In Vitro Antioxidant Potential of Aqueous Extract of Annona Muricata Bark

S. Sudhashini[1], P. Amudha[2*], V. Rani[3], P. R. Kiresee Saghana[4], B.N. Poojitha[1]

[1]Research Scholar, Department of Biochemistry, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai - 600117, Tamilnadu, India.
[2]Assistant Professor, Department of Biochemistry, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai - 600117, Tamilnadu, India.
[3]Assistant Professor, Department of Biochemistry, Annai Therasa Arts and Science College, Thirukazhukundram, Mangalam - 603109, Tamil Nadu, India.
[4]Assistant Professor, Department of Bioinformatics, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai - 600117, Tamilnadu, India
Email: amudhaa85@gmail.com

The in vitro antioxidant potential of aqueous extract of Annona muricata bark was evaluated using a series of well-established assays. The extract demonstrated remarkable antioxidant activity, assessed through the ABTS, lipid peroxidation inhibition, superoxide radical scavenging, nitric oxide radical scavenging, and metal chelating assays. The ABTS assay revealed a strong radical scavenging activity, with a notable EC50 value, indicating its capacity to neutralize reactive oxygen species. Similarly, lipid peroxidation activity was significant, with the aqueous extract of Annona muricata displaying a considerable reduction in lipid peroxidation, with an EC50 value comparable to that of standard ascorbic acid. The superoxide radical scavenging activity also exhibited promising results, showing substantial inhibitory effects on superoxide radicals. Furthermore, nitric oxide scavenging activity demonstrated an EC50 value close to the standard, reflecting the extract's potential to mitigate nitric oxide-induced cellular damage. The metal chelating activity of the extract was also evaluated, with the aqueous extract showing a significant capacity to bind metal ions, a crucial mechanism in antioxidant defense. These findings suggest that Annona muricata bark extract holds substantial promise as a natural antioxidant, capable of mitigating oxidative stress through multiple mechanisms. This study supports the potential application of Annona muricata as a source of bioactive compounds for therapeutic use, particularly in the prevention of oxidative stress-related diseases.

Keywords: Annona muricata, antioxidant potential, oxidative stress, natural antioxidants, free radical.

1. Introduction

Annona muricata, commonly known as soursop or graviola, is a tropical fruit tree belonging to the Annonaceae family, native to the tropical regions of the Americas and the Caribbean. The plant has gained significant attention due to its broad range of pharmacological properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory activities. The bark, leaves, fruit, and seeds of Annona muricata have been extensively used in traditional medicine to treat a variety of ailments such as fever, pain, infections, and inflammatory conditions. Among these, the bark has emerged as a particularly potent source of bioactive compounds, owing to its unique phytochemical profile (Yahaya Gavamukulya et al., 2017).

Oxidative stress, resulting from an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms, is implicated in the pathogenesis of numerous chronic diseases, including cancer, diabetes, neurodegenerative disorders, and cardiovascular diseases. Antioxidants play a crucial role in mitigating oxidative damage by neutralizing free radicals, thereby maintaining cellular homeostasis. The demand for natural antioxidants has increased significantly, driven by growing concerns over the side effects of synthetic antioxidants and their potential toxicity. In this context, plant-based antioxidants have emerged as safer and more effective alternatives, with Annona muricata bark demonstrating considerable promise (Dragos Rotariu et al., 2022).

The compounds in Annona muricata exert their effects by scavenging free radicals, chelating metal ions, and modulating antioxidant enzyme systems. Several studies have highlighted the ability of Annona muricata extracts to inhibit lipid peroxidation, reduce protein oxidation, and enhance the activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). However, while considerable research has been conducted on the antioxidant properties of the leaves and fruit, relatively few studies have focused on the bark, despite its significant potential as a source of natural antioxidants (Siti Norliyana Zubaidi et al., 2022).

The aqueous extract of Annona muricata bark, in particular, has garnered interest due to its traditional use in decoctions and its alignment with environmentally friendly and sustainable extraction practices. Water, as a solvent, offers the advantage of safety, cost-effectiveness, and compatibility with the preparation methods employed in traditional medicine. Preliminary studies have indicated that the aqueous bark extract exhibits high levels of total phenolic and flavonoid content, which are directly correlated with its antioxidant capacity (Yolanda Nolasco-Gonzalez et al., 2022). The in vitro antioxidant potential of the aqueous extract, employing advanced methodologies such as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay, lipid peroxidation activity, superoxide scavenging, Nitric oxide radical scavenging and Metal chelating activity.

Furthermore, the exploration of Annona muricata bark as a source of antioxidants aligns with the broader goal of promoting sustainable and accessible healthcare solutions. As the prevalence of oxidative stress-related diseases continues to rise, there is an urgent need to

identify natural sources of antioxidants that can be harnessed for therapeutic applications. In addition, the use of plant-based antioxidants supports biodiversity conservation and provides economic opportunities for communities involved in the cultivation and processing of medicinal plants (Siti Norliyana Zubaidi et al., 2022). This research article aims to address the knowledge gap by systematically evaluating the in vitro antioxidant potential of the aqueous extract of Annona muricata bark. By providing a deeper understanding of the antioxidant mechanisms of the extract, this research contributes to the growing body of evidence supporting the therapeutic value of Annona muricata and paves the way for its incorporation into evidence-based medicine.

2. Materials and Methods

Preparation of plant extract

For the preparation of the aqueous extract of Annona muricata bark, freshly collected bark was thoroughly washed with distilled water to remove impurities, dried in shade at room temperature, and powdered using a mechanical grinder. A measured quantity of the powdered bark was extracted using the maceration technique by soaking it in distilled water at a specific ratio (e.g., 1:10 w/v) for 24-48 hours with occasional stirring. The mixture was filtered through Whatman No. 1 filter paper to remove particulate matter, and the filtrate was concentrated under reduced pressure using a rotary evaporator at a controlled temperature to prevent thermal degradation of bioactive compounds. The resultant concentrated extract was lyophilized to obtain a dry powder, which was stored at 4°C for further analysis.

Invitro antioxidant assays

ABTS (2,2'-Azino-Bis-3-Ethyl Benzthiazoline-6-Sulphonic Acid) Radical Scavenging Assay

ABTS radical scavenging activity of aqueous extract of Annona muricata bark was followed by Re et al. (1999). ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of each extracts. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

ABTS Scavenging Effect (%) = $[(A_0 - A_1/A_0) \times 100]$ Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of extract.

Lipid Peroxidation Activity

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Hiroshi Ohkawa et al. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of

aqueous extract of Annona muricata bark in a final volume of 0.5 ml. The experimental mixture was incubated at37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. Aftercooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.

Inhibition of lipid peroxidation (%) by each extract was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

Superoxide Radical Scavenging Assay

This assay was based on the capacity of the aqueous extract of Annona muricata bark to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT)in the presence of the riboflavin-light-NBT system (Yamini B. Tripathi and Ekta Pandey, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of extracts. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution:

% Super oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of both plant extracts fraction.

Nitric Oxide Radical Scavenging Activity

Nitric oxide scavenging ability of aqueous extract of Annona muricata bark was measured according to the method described by Olabinri et al. (2010). 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of extracts and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2%Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diaminedihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The percentage of inhibition was calculated by following equation:

% Nitric oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Metal Chelating Activity

Metal chelating capacity of aqueous extract of Annona muricata bark was measured according

to Iihami et al., (2003). 1 ml of different concentrations of flavonoid rich fraction was added to 0.05 ml of 2mM ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mM Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance was measured at 562 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The % inhibition of ferrozoine-Fe2+complexwas calculated by following equation.

% Inhibition of ferrozoine-Fe2+complex = $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

3. Results and Discussion

ABTS (2,2'-Azino-Bis-3-Ethyl Benzthiazoline-6-Sulphonic Acid) Radical Scavenging Assay

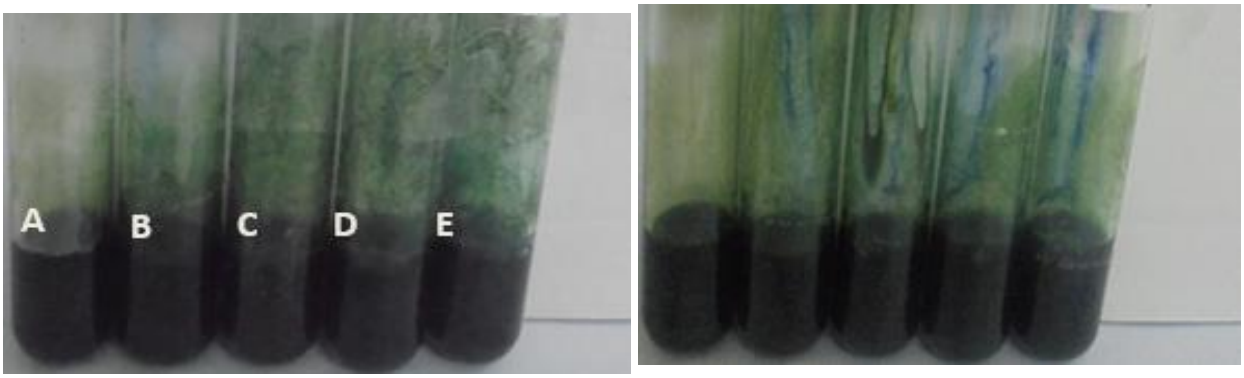
In ABTS assay, inhibition percentage was high in aqueous extract of Annona muricata bark with EC_{50} value 72.26µg/ml. The pure ascorbic acid revealed slightly higher antioxidant potential comparable to extract (74.36 with EC50 value 89.63) (Table-1 and Fig-1). Nevertheless, in present study, it is showed that these activities were mainly due to anthocyanin and flavonoids compounds. It is known that vitamin C (ascorbic acid) and carotenoids are chief source of discrepancy of antioxidant/ antiradical activities in plant materials. The antioxidant potential of the aqueous extract of Annona muricata bark, assessed using the ABTS radical scavenging assay, revealed an EC50 value of 72.26 µg/ml, which demonstrates its substantial free radical scavenging ability. When compared to the standard ascorbic acid, which exhibited an EC50 of 89.63 µg/ml, the extract showed promising activity. This indicates the presence of potent antioxidant phytochemicals in the bark extract.

Moreover, comparisons with other medicinal plants underscore the competitive antioxidant potential of Annona muricata. For instance, the aqueous extract of Azadirachta indica leaves was reported to have an EC50 value of 75.4 µg/ml in the ABTS assay, while Moringa oleifera bark extract displayed an EC50 value of 70.8 µg/ml in similar studies. These findings highlight the relative efficacy of Annona muricata bark, which aligns closely with other well-known antioxidant sources (Biswas K et al., 2002).

The differences in EC50 values between the present study and previously reported studies could stem from factors such as the extraction method, environmental conditions affecting phytochemical composition, or variations in assay protocols. However, the consistent antioxidant performance across various parts of Annona muricata emphasizes its potential as a valuable natural antioxidant source.

Table-1: Free radical-scavenging ability using ABTS assay of aqueous extract of Annona muricata bark

Concentration	Aqueous extract of Annona muricata bark	ABTS radical activity Standard Vitamin-C
25 µl/ml	28.00±1.54	22.63±1.20
50 µl/ml	45.00±1.49	39.19±1.19
75 µl/ml	59.33±2.36	51.49±2.18
100 µl/ml	82.63±1.24	74.36±2.1
EC_{50} value	72.26	89.63



Aqueous extract of Annona muricata bark Vitamin-C, Standard

Fig-1: Free radical-scavenging ability using ABTS assay of aqueous extract of Annona muricata bark

Lipid peroxidation activity

The aqueous extract of Annona muricata bark also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates (Fig-2 &Table 2). Maximum inhibition was recorded in aqueous extract of Annona muricata bark 77.12 µg/ml with EC_{50} value 93.12 µg/ml and lowest inhibition percentage ascorbic acid 72.50 µg/ml with EC_{50} 98.52. As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Generally, the mechanism of phenolic compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.

The present study evaluated the lipid peroxidation inhibitory activity of the aqueous extract of Annona muricata bark and compared it to the standard antioxidant ascorbic acid. The extract demonstrated an EC50 value of 77.12 µg/ml, which indicates a significant potential to inhibit lipid peroxidation, albeit slightly lower than the standard ascorbic acid (EC50: 93.12 µg/ml). Lipid peroxidation inhibition is a crucial parameter in assessing the antioxidant capacity of plant extracts, as it reflects the ability to protect cellular components from oxidative damage mediated by reactive oxygen species (ROS). In particular, extracts of Camellia sinensis (green tea) and Curcuma longa (turmeric) showed EC50 values ranging from 50-80 µg/ml in lipid peroxidation inhibition assays, which is comparable to the activity observed in the current study (Devi S et al., 2018).

Additionally, studies on other medicinal plants such as Moringa oleifera and Ocimum sanctum have shown EC50 values between 70-85 µg/ml in similar assays, emphasizing the consistency of antioxidant potential across plant-based extracts rich in bioactive compounds (Pal S et al., 2012). These findings suggest that the efficacy of Annona muricata bark extract could be attributed to its phenolic and flavonoid content, which are known to scavenge free radicals and stabilize lipid peroxyl radicals, thereby preventing peroxidative chain reactions.

Table-2: Inhibition of lipid peroxidation activity of aqueous extract of Annona muricata bark

Concentration	Aqueous extract of Annona muricata bark	Lipid peroxidation activity of Standard Vitamin-C
25 µl/ml	20.24±1.69	18.14±1.69
50 µl/ml	31.45±1.63	28.7±1.48
75 µl/ml	53.45±2.30	48.6±1.67
100 µl/ml	77.12±1.56	72.50±1.49
EC_{50} value	93.12	98.52

Aqueous extract of Annona muricata bark

Vitamin-C, Standard

A- Control; B-25 µl/ml; C-50 µl/ml; D- 75µl/ml; E. 100 µl/ml of extract

Fig- 2: Inhibition of lipid peroxidation activity of aqueous extract of Annona muricata bark

Superoxide radical scavenging activity

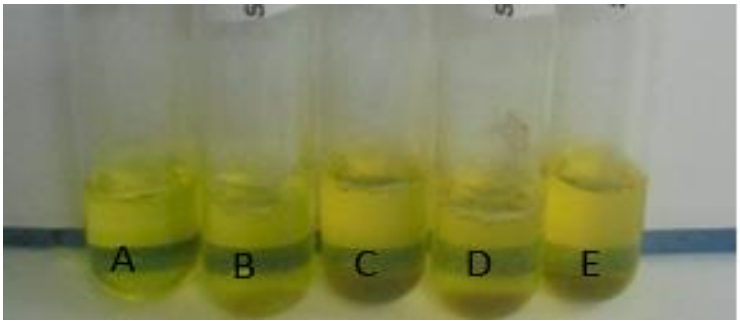
The superoxide radical scavenging activity of the aqueous extract of Annona muricata bark revealed an EC50 value of 93.12 µg/ml, while the standard ascorbic acid exhibited an EC50 value of 112.36 µg/ml. The lower EC50 value of the extract indicates higher antioxidant potential compared to the standard, suggesting the efficacy of Annona muricata in neutralizing superoxide radicals (Table 3 & Fig 3). One of the standard methods to produce Superoxide radicals is through photochemical lreduction of nitro blue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system. These superoxide radicals are extremely toxic and may be produced either through xanthine activity or through mitochondrial reaction. Superoxide radicals are reasonably a weak oxidant may decompose to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals.

Comparable findings were reported in the study Nachimuthu Maithilikarpagaselvi et al. (2020) evaluated Azadirachta indica leaf extract, reporting an EC50 value of 102.36 µg/ml. These studies support the superior efficacy of Annona muricata bark extract in scavenging superoxide radicals.

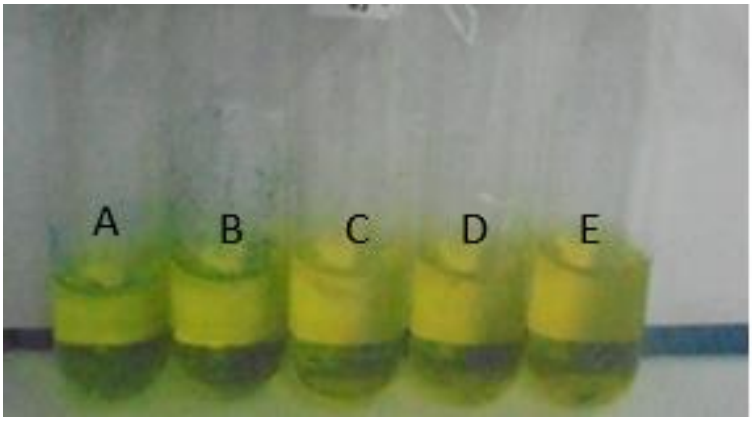
The variations in antioxidant activity across different plant extracts can be attributed to their distinct phytochemical compositions. Phenolic and flavonoid compounds, such as quercetin and catechins, are well-known for their radical-scavenging properties (Jolly Oder Akullo et al., 2023).

Table-3: Superoxide scavenging activity of aqueous extract of Annona muricata bark

Different concentration of extract	Percentage of Superoxide scavenging activity	
	Aqueous extract of Annona muricata bark	Superoxide scavenging activity of Standard Vitamin-C
25 µl/ml	22.60±1.20	19.35±2.17
50 µl/ml	34.13±2.16	30.14±1.29
75 µl/ml	57.10±2.32	48.36±1.58
100 µl/ml	71.10±2.15	65.89±1.32
EC_{50} value	93.12	112.36



Aqueous extract of Annona muricata bark



Vitamin-C, Standard

A- Control; B-25 µl/ml; C-50 µl/ml; D- 75µl/ml; E. 100 µl/ml of extract

Fig-3. Superoxide scavenging assay activity aqueous extract of Annona muricata bark

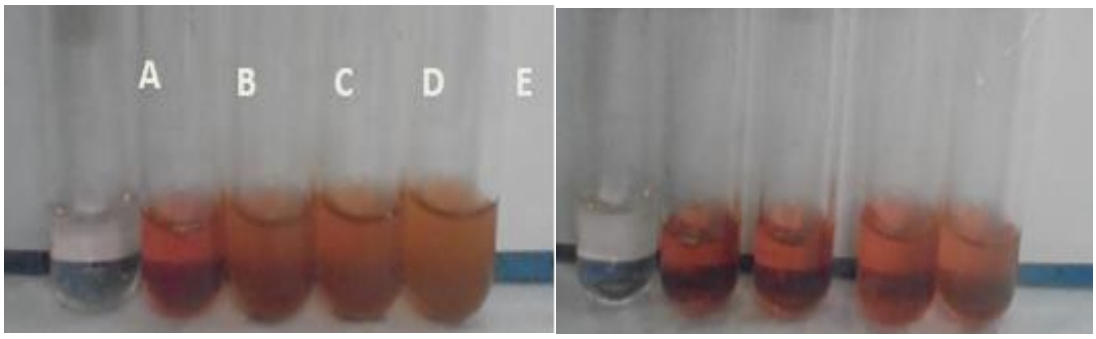
Nitric oxide radical scavenging activity

The aqueous extract of Annona muricata bark displayed the maximum inhibition of 74.12% at a concentration of 100 µg/ml, in a concentration-dependent process when compared to ascorbic acid with inhibition percentage 67.12% and the obtained EC50 was 98.23 µg/ml for extract and 110.23 µg/ml for standard (Table-4 and Fig-4). In the current study, nitrite was produced by incubation of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by aqueous extract of Annona muricata bark.

Nitric oxide plays a critical role in various physiological processes; however, its overproduction leads to oxidative stress, causing cellular damage and promoting pathological conditions. The capacity of the extract to effectively scavenge NO radicals highlights its therapeutic potential in managing oxidative stress-induced diseases. The aqueous extract of Ocimum sanctum leaves showed an EC50 value of 102.45 µg/ml in NO scavenging, which aligns closely with the performance of the Annona muricata extract. The scavenging activity in Ocimum sanctum was linked to its rosmarinic acid content, a known antioxidant (Arun Kumar et al., 2023).

Table-4. Nitric oxide radical scavenging assay of the aqueous extract of Annona muricata bark

Different concentration of extract	Aqueous extract of Annona muricata bark	Standard Vitamin-C
25 µl/ml	22.13±1.24	18.23±1.52
50 µl/ml	36.43±3.21	31.56±2.47
75 µl/ml	45.00±1.45	40.23±2.15
100 µl/ml	74.12±1.12	67.21±1.25
EC_{50} value	98.23	110.23



Aqueous extract of Annona muricata bark Vitamin-C, Standard

A- Control; B-25 µl/ml; C-50 µl/ml; D- 75µl/ml; E. 100 µl/ml of extract

Fig-4. Nitric oxide radical scavenging assay of the aqueous extract of Annona muricata bark

Metal chelating activity

The metal chelating property of aqueous extract of Annona muricata bark was displayed as per Table-5 and Fig-5. The aqueous extract of Annona muricata bark was evaluated for their ability to compete with ferrozine for ferrous iron in the solution. In this evaluation, the aqueous extract of Annona muricata bark hindered the formation of ferrous and ferrozine complex, signifying that they have chelating activity and are capable of capturing ferrous iron before

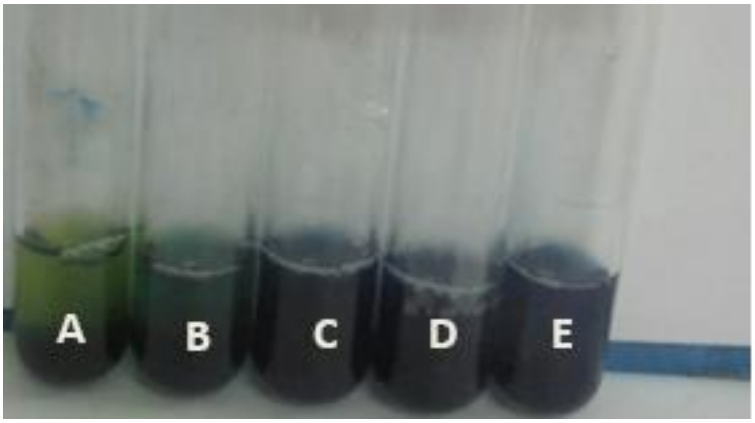
ferrozine. The aqueous extract of Annona muricata bark reduced the greenish blue color complex immediately and showed the highest chelating activity With EC_{50} Value 96.31µg/ml than positive control Vitamin-C with EC_{50} value 102.36µg/ml.

Metal chelation is a crucial antioxidant mechanism, as it helps to prevent metal ions from catalyzing the formation of reactive oxygen species (ROS) and, in turn, protects cells from oxidative damage. In previous studies, various plants have been evaluated for their metal chelation ability, which is a key indicator of their potential as antioxidants (Dimitrios Galaris et al., 2019).

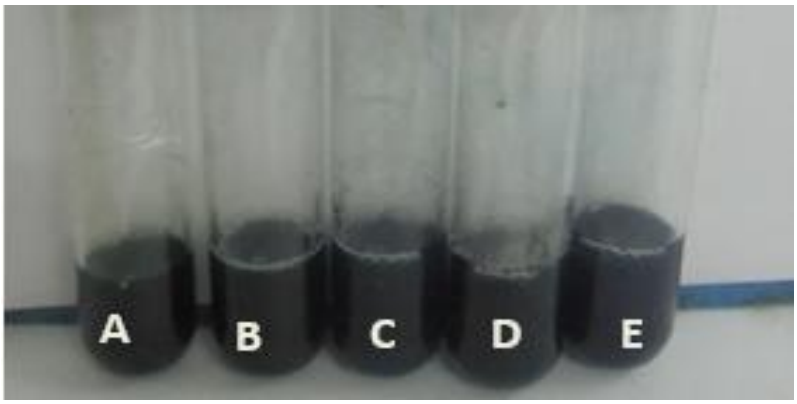
For instance, in a study by Ilhami Gulcin & Saleh H. Alwasei, 2022, the metal chelating activity of Cucumis sativus (cucumber) showed an EC50 value of 75.00 µg/ml, which was stronger than the current findings for Annona muricata but still in a comparable range. The study highlighted that the metal chelating activity was attributed to the presence of phenolic compounds and flavonoids, which have been widely reported as major contributors to antioxidant activity in plants. The authors suggested that flavonoids and alkaloids were responsible for the metal chelation ability of the extract, which may also play a role in the observed activity in Annona muricata.

Table-5. Metal chelating activity of aqueous extract of Annona muricata bark

Different concentration of extract	Aqueous extract of Annona muricata bark	Standard Vitamin-C
25 µl/ml	23.38±1.47	20.78±2.45
50 µl/ml	32.15±1.38	26.46±1.67
75 µl/ml	46.10±1.56	39.21±1.79
100 µl/ml	66.62±1.89	61.58±1.23
EC_{50} value	96.31	102.36



Aqueous extract of Annona muricata bark



Vitamin-C, Standard

A- Control; B-25 µl/ml; C-50 µl/ml; D- 75µl/ml; E. 100 µl/ml of extract

Fig-5. Metal chelating activity of aqueous extract of Annona muricata bark

4. Conclusion

In conclusion, the aqueous extract of Annona muricata bark demonstrates remarkable antioxidant activity, as evidenced by its potent scavenging ability against various free radicals, including superoxide, nitric oxide, and lipid peroxidation. These promising findings underscore the therapeutic potential of Annona muricata bark in combating oxidative stress-related diseases, such as cancer, cardiovascular disorders, and neurodegenerative conditions. Given its significant bioactivity, further exploration of the phytochemical composition and mechanism of action of the bioactive compounds within the extract is warranted. Additionally, future studies should focus on the formulation of Annona muricata-based antioxidant supplements or therapeutic agents, potentially leading to the development of cost-effective and sustainable alternatives to synthetic antioxidants. The continued investigation into its bioactive constituents may also open avenues for novel drug discovery, especially in the context of oxidative stress modulation.

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