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Phytochemical Analysis, Antibacterial and Antioxidant Activities of Leaf Extracts of *Strychnos Innocua* Del.

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ABSTRACT

Strychnos innocua is a shrub, or small tree which grows up to 10 m tall. It belongs to the family Loganiaceae and is often straight-stemmed. The root decoction is taken as a remedy for gonorrhoea, and the fresh roots extracts are used to treat snakebite. The aim of the study was to use established methods to assess the phytochemical content, antibacterial, and antioxidant properties of hexane, chloroform, ethyl acetate, and methanol extracts of *Strychnos innocua* leaves. All of the extracts contained flavonoids, alkaloids, tannins, terpenoids, and saponins. The ethyl acetate extract yielded flavonoids and tannins (3.4 percent and 13.8 percent, respectively). In comparison to the other extracts, the methanol extract had the highest levels of phenolic (10.44%) and saponins (4.2%). The methanol extract has the highest alkaloid concentration (6.0%). With a mean zone of inhibition of 11 to 18 mm, the extracts were effective against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhi*, and *Escherichia coli*. The minimal inhibitory and bactericidal concentrations were 3.125 and 12.50 g/mL, respectively. At 250 g/mL, the antioxidant activity of hexane, chloroform, ethyl acetate, and methanol extracts were 59.2, 56.1, 88.8, and 91.3%, respectively. When compared to ascorbic acid, the ethyl acetate extract exhibits a reductive potential of 0.91 nm (1.3 nm). Finally, the extracts of *S. innocua* contained a variety of phytochemicals, showed significant antibacterial and antioxidant activities

Keywords: Antimicrobial activity, Antioxidant, Phytochemical analysis, Secondary metabolites, *Strychnos innocua*, 2, 2-diphenyl-1-picryl hydrazyl radical.

Introduction

Strychnos innocua is a deciduous shrub that grows to a height of 2 to 18 meters. Snakebite is treated with its root extract, which is also used as an aphrodisiac and to treat gonorrhoea. The bark and twigs are infusions that help babies grow and give birth. Its fruit pulp is also used as eardrops and to treat diarrhea.[1] The seeds have emetic effects. The vitamins, carotenoids, and flavonoids found in fruits and vegetables are responsible for their antioxidant activity. [2] Antioxidants protect cells from the harmful effects of reactive oxygen species (ROS), such as superoxide anion (O_2^{2-}) and peroxyl ($ROO^{\cdot}$) reactive radicals produced by normal human metabolism.[3] Polyphenols (such as catechins) have high redox capabilities, neutralizing free radicals and decomposing peroxide.[4] Both wild and domesticated plants contain bioactive phytochemicals. Flavonoids, phenolics, tannins, and saponins are among them.[5] These compounds are in the spotlight because of their medicinal role in the treatment of a variety of chronic diseases, including cancer, cardiovascular disease, and gastrointestinal disease, as well as anti-ageing, anticancer, and protective effects for neurodegenerative, diabetes mellitus, and obesity.[6-7] Antioxidants are compounds that inhibit the majority of oxidation reactions that begin with the generation of free radicals. Antioxidants trap free radicals, delaying or preventing damage to live organisms' cells and tissues.

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Reducing agents are another name for antioxidants.[8] Antioxidants are important because they can reduce oxidative stress, which is one of the factors that can harm biological molecules.[9] They're used in food preservation, cosmetics, and the prevention of gasoline and rubber degradation, among other things. Carotenoids, flavonoids, lutein, and polyphenols are antioxidant chemicals found in plants and fruits.[10] Microbial drug resistance is currently of great interest in the scientific community.[11-12] Microbial strains that have developed multidrug resistance (MDR) pose a serious threat to global public health. The use of herbal items as a new lead in the creation of improved medications against microbial strains is becoming increasingly popular.[13]
Despite the fact that this plant is widely utilized in ethnomedicine, there is little or no available literature on the on its antibacterial, antioxidant, or phytochemical properties As a result, the goal of this work is to use established methodologies to assess the phytochemical content, antibacterial, and antioxidant activity of *Strychnos innocua* leaf extracts.

Materials and Methods

Plant materials
Strychnos innocua leaves were obtained in May 2021, from Maigana village in Sabon Gari Local Government Area, Zaria, Kaduna State. The plant was discovered in the Herbarium of Ahmadu Bello University's (ABU) Zaria's Department of Botany, Faculty of Life Sciences. The Department's Herbarium has received a voucher specimen (Voucher Specimen Number 4100).

Extraction procedure
S. innocua leaves (500 g) were air-dried and ground into a fine powder. The powder was then extracted exhaustively and sequentially with 1000 mL each of hexane, chloroform, ethyl acetate, and methanol

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using a Soxhlet extractor. A rotary evaporator was used to concentrate each of the extracts.[14]

Phytochemical screening of extracts
Standard laboratory protocols were used to conduct the quantitative and qualitative phytochemical screening. [15-20]

Quantitative determination of phytochemical constituents
Determination of total phenolic compound (TPC)
A standard method as described by Cascant *et al.*,[21] was used to determine the total phenolic content of the hexane, ethyl acetate, and methanolic extracts. The extracts were dissolved with distilled water to a known concentration of 0.0 µg to 600 µg of tannic acid/mL. 250 µl of diluted extract or tannic acid solution was mixed with 1 mL of distilled water in a test tube followed by the addition of 250 µL of Folin-Ciocalteu reagent. The samples were agitated for 5 minutes at 27 ˚C to ensure full mixing with the Folin-Ciocalteu reagent. The final volume was built up to 6.0 mL with distilled water after 2.5 mL of 7 percent sodium carbonate aqueous solution was added. After incubating the samples for 90 minutes, the absorbance of the resulting blue colour solution was measured using a spectrophotometer at 760 nm. The experiment was carried out in triplicates.

Determination of alkaloids
Dried powdered leaves of *S. innocua* (5.0 g) were weighed into a 250 mL beaker, and 200 mL of 10% acetic acid in ethanol were added. The mixture was covered and allowed to extract. It was then filtered and concentrated to one-quarter of its original volume. Drop wise additions of concentrated ammonium hydroxide solution to the extract were made until the precipitation was complete. The solution was allowed to settle, and the precipitate was collected and washed with weak ammonium hydroxide before being filtered, dried, then weighed to determine the percentage alkaloid. [22]

Determination of saponins
Each of the plant extracts (15 g) was put in a conical flask with 100 mL of aqueous ethanol (20%). The extracts were cooked for 4 hours at 55 °C in a hot water bath with constant stirring. The mixture was filtered, and the residue was extracted again using 200 mL of 20% ethanol and 200 mL of water. Over a water bath at 90°C, the mixed extracts were reduced to 40 mL. The concentrate was transferred to a 250 mL separating funnel, which was then filled with 20 mL diethyl ether and violently agitated. The aqueous layer was saved, while the ether layer was thrown away. This purification procedure was repeated with the addition of 60 mL of n-butanol. 10 mL of 5% aqueous sodium chloride was used to wash the mixed butanol extracts twice. In a water bath, the residual solution was heated. The samples were dried in the oven to a consistent weight after evaporation, and the percentage saponins was estimated. [23]

Determination of flavonoids
A total of 5.0 g of plant material was weighed and extracted many times at room temperature with 100 mL of 80 percent aqueous methanol. Whattman filter paper No 41 was used to filter the entire solution. Over a water bath, the filtrate was evaporated to dryness and weighed to a consistent weight. The flavonoids percentage was then determined. [24]

Determination of total tannin content
A modified approach of Lamkeng *et al.*,[25] was used to determine the total tannin content. 0.5 mL Folin-Denis reagent, 1 mL Na_2CO_3 (0.5 percent W/V) solution, and distilled water up to 5 mL were added to 0.1 mL of 1 mg/mL each extract. Within 30 minutes of reaction against blank, the absorbance was measured at 755 nm. The equivalent of tannin acid was used to express the total tannin in the extract.[25]

Antibacterial activity
Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, and *Salmonella typhi* were all tested against the extracts. For bacterial screening, the agar dissemination technique was utilized. [26] The media was cleaned for 15 minutes at 121°C after being cultured overnight. The disinfected media was added to each test bacteria (0.1 mL). The germs were evenly distributed throughout the media. At the focal point of the infected medium, a hole of diameter (5 mm) was drilled, and the extract (0.1 mL) was poured into the holes. The medium were hatched for 24 hours at 37 °C and the zone of microorganism inhibition was observed.

Determination of minimum inhibitory concentration of the extracts
The soup was heated in test tubes for 8 minutes at 122 degrees Celsius, then chilled. At 37°C, the bacteria solution was cultured for 6 hours. The bacteria had the same turbidity as McFarland (1.5 x 108 cfu/mL). The extracts were 20 g/ mL, 10 g/ mL, 5 g/ mL, 2.5 g/ mL, and 1.25 g/mL in concentration. The test organisms were introduced to these concentrations in exactly 0.1 mL increments and cultured at 37 °C for 24 hours. The minimal inhibitory concentration (MIC) was determined in a test tube with no turbidity. [27] A typical antibacterial agent, ciprofloxacin, was utilized as a positive control.

Determination of minimum bactericidal concentration of the extracts
The media were distributed into sterile plates once they had been prepared and sterilised. The media were chilled to 37 degrees Celsius. The contents of the MIC-containing test tubes were then transferred to the medium and incubated at 37°C for 24 hours.

Determination of DPPH radical-scavenging activity of S. innocua leaf extracts
Baliyan et *al.*, (2022)[28] proposed a method for determining the scavenging activity of 2, 2- diphenyl-1-picryl hydrazyl radical. In methanol, extracts with concentrations of 15 g/mL, 30 g/mL, 60 g/mL, 120 g/mL, and 240 g/mL were produced. A positive standard of vitamin C (ascorbic acid) was utilized. 0.3 mM DPPH radical was added to the various amounts of each chemical (1 mL). The following formula was used to compute the percentage (percent) of radical – scavenging activity:

$$\text{Radical scavenging activity} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The total antioxidant capacity
Total antioxidant capacity (TAC) was determined using the phosphomolybdenum technique.[28] The extract was combined with sodium phosphate (28 mM), sulphuric acid (0.6 M), and ammonium molybdate (0.3 mL) (4 mM). The mixture was then incubated at 95 °C for 90 minutes after cooling. A UV-VIS spectrophotometer was used to measure absorbance (695 nm).

Ferric-Ion-Reducing antioxidant property
Standard procedures were used to determine the extracts' decreasing antioxidant power. [29] In test tubes, 2.5 mL of each extract (250, 200, 150, 100 and 50 g/mL), 1 percent potassium ferric cyanide (2.5 mL), and phosphate buffer solution (2.5 mL, 0.2 M, pH = 6.6) were combined for 20 minutes at 50°C. After that, 2.5 mL of 10% trichloroacetic acid was added to the mixture and thoroughly shaken. The mixture was diluted with 2.5 mL distilled water and 0.5 mL 0.1 percent $FeCl_3$ for 10 minutes. At 700 nm, the absorbance was measured.

Statistical analysis
All data were presented as the mean ±standard deviation of three different determinations. P-values ($P \geq 0.05$) were used to show the level of significant difference.

Results and Discussion

Hexane and ethyl acetate extracts both contained terpenes (Table 1). The ethyl acetate and methanol extracts contained tannins, saponins, and flavonoids. The methanol extract contained carbohydrates and cardiac glycosides, however the flavonoids concentration was less than 11 mg/g.

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Table 1: Qualitative phytochemical screening of extracts of *Strychnos innocua*

Phytochemical	Hex	Chl	EtAc	Meth
Terpenes	+	+	+	+
Carbohydrate	-	-	+	+
Cardiac glycoside	-	-	+	+
Saponins	-	-	-	+
Flavonoids	-	-	+	+
Alkaloids	_	+	+	+
Anthraquinones	-	-	-	-

KEY: - = Negative, + = Positive, Hex = Hexane extract, Chl = Chloroform extract, EtAc = Ethyl acetate extract, Meth = Methanol extract

In comparison to the methanol extract (10.4 mg/g), flavonoid concentration was lower in the hexane (6 mg/g) and ethyl acetate (3.4 mg/g) fractions. The methanol fraction has the highest polyphenolic concentration (10.44 mg/g), followed by the ethyl acetate extract (10.3 mg/g), and the hexane extract (1.98 mg/g). Tannin content was found to be highest in the ethyl acetate extract (13.8 mg/g), followed by the methanol fraction (2.8 mg/g), and lowest in the hexane extract (0.48 mg/g). The methanol fraction has the most saponin (4.2 mg/g), followed by ethyl acetate (2.0 mg/g) and hexane extract (0.6 mg/g). The hexane extract (2.4 mg/g) contained the lowest concentration of alkaloids, whereas the methanol extract (6.0 mg/g) had the most (Table 2). The result of the antimicrobial activity of the extracts is shown in Table 3. The hexane extract was sensitive to all the test bacteria, except *Staphylococcus aureus, Bacillus subtilis* and *Staphylococcus aureus, Bacillus subtilis* with inhibition zone ranging from 11 to 18 mm. The chloroform extract was active against *Bacillus subtilis, Klebsiella pneumonia, Escherichia coli* and *Salmonella typhi,* with a zone of inhibition each ranging from 16 to 18 mm. The ethyl acetate extract was active against all the bacteria, except *Pseudomonas aeruginosa.*

Table 2: Quantitative phytochemical screening of extracts of *Strychnos innocua*

Phytochemical (mg/g)	Hex	EtAc (mg/g)	Meth (mg/g)
Alkaloids	2.4	3.2	6.0
Flavonoids	0.6	3.4	10.4
Saponins	0.6	2.0	4.2
Tannins	0.48	13.8	2.8
Phenolics	1.98	10.3	10.44

KEY = Hex = Hexane extract, EtAc = Ethyl acetate extract, Meth = Methanol extract

Table 3: The zone of inhibition of extracts of *S. innocua*

Bacteria	Bacteria concentration	Zone of Inhibition (mm)				
		Hex	Chl	EtAc	Meth	Cipro
Staphylococcus aureus	100	-	-	18	-	40
	50	-	-	16	-	
	25	-	-	14	-	
	12.5	-	-	12	-	
Bacillus subtilis	100	-	18	18	16	33
	50	-	16	16	14	
	25	-	14	14	12	
	12.5	-	12	12	11	
Escherichia coli	100	16	16	16	18	39
	50	14	14	14	16	
	25	12	13	12	14	
	12.5	11	12	11	12	
Pseudomonas aeruginosa	100	-	-	-	16	42
	50	-	-	-	15	
	25	-	-	-	14	
	12.5	-	-	-	12	
Klebsiella pneumonia	100	18	16	16	-	40
	50	16	14	14	-	
	25	14	12	12	-	
	12.5	12	11	11	-	
Salmonella typhi	100	18	18	18	-	36
	50	16	16	16	-	
	25	14	14	14	-	
	12.5	12	12	12	-	

KEY: Hex = Hexane extract, Chl = Chloroform extract, EtAc = Ethyl acetate extract, Meth = Methanol extract, Cipro = Ciprofloxacin

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The inhibition zone ranged from 11 to 18 mm. The methanol extract had a zone of inhibition of 16, 18 and 18 mm against *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* respectively. The zones of inhibition of the extracts were lower than that of ciprofloxacin, a standard antibacterial agent. The MIC and MBC values ranged from 3.125 to 12.50 µg/mL (Table 4).

At 50 g/mL, the extracts' free-radical scavenging activity ranged from 27.4 to 42.3 percent. The activity ranged from 31.3 to 55.1 percent at 100 g/mL. The radical-scavenging activity ranged between 34.6 and 73.9 percent at 150 g/mL. The extracts' radical-scavenging activity was determined to be between 40.5 and 89.8 g/mL at 200 g/mL. The methanol extract has the highest antioxidant activity (91.3%), followed by the ethyl acetate extract (88.8%). (Figure 1). The inhibitory concentration (IC_{50}) of the standard, ascorbic acid, was 14.0 g/mL in the DPPH assay, 877 g/mL in the hexane extract, 505 g/mL in the chloroform extract, and 105 g/mL in the ethyl acetate extract, and 86 g/mL in the methanol extract.

At 50 g/mL, total antioxidant activity ranged between 29 and 30 nm; at 100 g/mL, total antioxidant activity ranged between 31.3 and 55.1 nm; and at 150 g/mL, total antioxidant activity ranged between 34.6 and 73.9 nm. The absorbance of the extracts at 200 g/mL was found to be between 40.5 and 89.8 nm, however at 250 g/mL, the absorbance was found to be between 59.2 and 91.3 nm. At 50, 100, 10, 200, and 250 g/mL, ascorbic acid had absorbance values of 27, 61, 114, 179, and 237 nm, respectively (Figure 2).

The absorbance of hexane, chloroform, ethyl acetate, and methanol extracts ranged between 0.040 and 0.006 nm at 50 g/mL in the ferric ion-reducing power experiment. At 100 g/mL, the absorbance ranged from 0.015 to 0.091 nm, whereas at 150 g/mL, the absorbance ranged from 0.056 to 0.129 nm. At 200 g/mL, the extract's absorbance ranged from 0.114 to 0.517. The absorbance ranged between 0.157 and 0.905 nm when the concentration was 250 g/mL. At 50, 100, 150, 200, and 250 g/mL, ascorbic acid had absorbances of 0.396, 0.606, 0.982, 1.653, and 1.954 nm, respectively (Figure 3).

The phytochemicals found in *Strychnos innocua* extracts were identical to those found in the *Senna mimosoides* and *Cissus quadrangularis*. [30-31] Methanol and ethyl acetate solvents exhibit good extractability of polar chemicals such as alkaloids, flavonoids, tannins, and saponin, according to Wang *et al..*, (2012).[32] In these solvents, phytochemicals are extremely soluble. Chemical compounds' bioavailability and dissolving capability against diverse solvents are critical in the extraction process.[33] Tannins are one of the main active compounds in wine, fruits, and beer that act as antioxidants.[34] They suppress HIV replication selectively and have antiviral, antibacterial, and anticancer properties.[35] *Strychnos innocua* leaves contain a significant amount of flavonoids, according to the findings of this study. Flavonoids also have biological properties, such as the ability to protect against bacteria and viruses.[36]

The extracts of the leaves contained a significant amount of saponin. Alkaloids are chemical molecules with a wide range of pharmacological properties, including anticancer, antibacterial, and antihyperglycemic properties.[37] Cocaine, caffeine, and nicotine, for example, are used as psychotropics and stimulants. Plants produce steroids, which are known to have antibacterial and insect repellent characteristics.[38] The average diameter of the zone of inhibition was higher than 18 mm, and the MIC was less than 100 µg/mL, indicating a powerful antibacterial action.[39-41] This is in agreement for the antimicrobial activity reported for extracts of *E. globulus*, *A. indica* and *G. glabra* extracts.[42] *In vitro* studies have demonstrated that secondary metabolites in plants have strong antibacterial action[43-46]. As a result, it's possible that leaf extracts are broad-spectrum antibacterial agents, and that their activity is linked to the existence of secondary metabolites.

Table 4: Minimum inhibitory concentration and minimum bactericidal concentration of extracts of *Strychnos innocua* leaves

Organisms	MIC (µg/mL)				MBC (µg/mL)			
	Hex	Chl		Meth	Hex	Chl	EtAc	Meth
Staphylococcus aureus	12.5	12.5	6.25	12.5	12	12.5	6.25	12.5
Bacillus subtilis	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
Escherichia coli	3.125	6.25	3.15	3.125	3.125	6.25	3.125	
Pseudomonas aeruginosa	3.125	12.5	6.5	6.25	6.25	12.5	6.25	
Klebsiella pneumonia	12.3	3.125	3.125	12.5	12	3.125	3.125	
Salmonella typhi	12.5	6.25	6.25	12.5	12.5	6.25	6.25	

Key: Hex = Hexane extract, Chl = Chloroform extract, EtAc = Ethyl acetate, Meth = Methanol extract, AA = Ascorbic acid

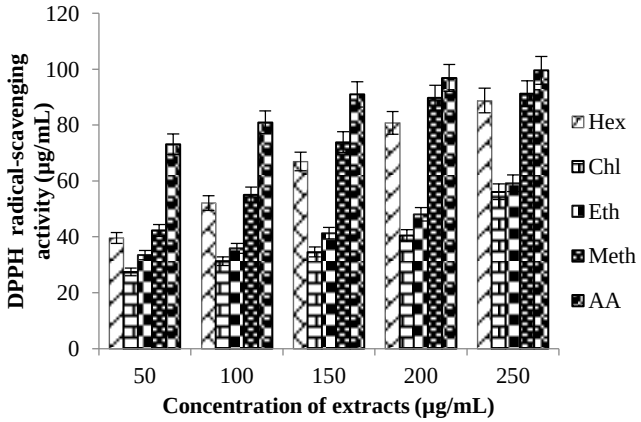

Figure 1: DPPH radical-scavenging activity of *Strychnos innocua* leaf extracts.
KEY = Hex = Hexane extract, Chl = Chloroform extract, EtAc = Ethyl acetate extract, Meth = Methanol extract, AA = Ascorbic acid a-d = Means with error bars having different superscripts are significantly different P < 0.05

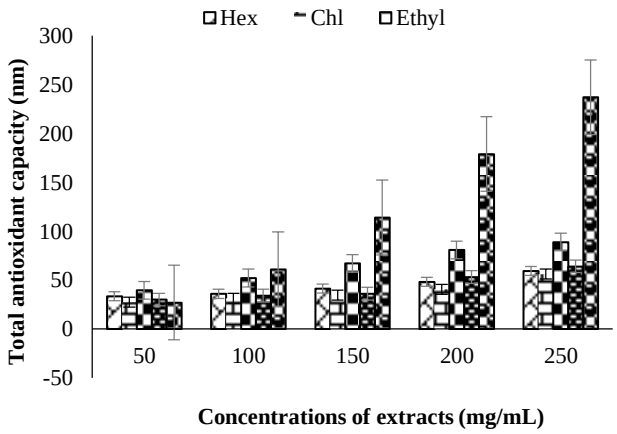

Figure 2: Total antioxidant activity of *Strychnos innocua* leaf extracts.
KEY = Hex = Hexane extract, Chl = Chloroform extract, EtAc = Ethyl acetate extract, Meth = Methanol extract, AA = Ascorbic acid

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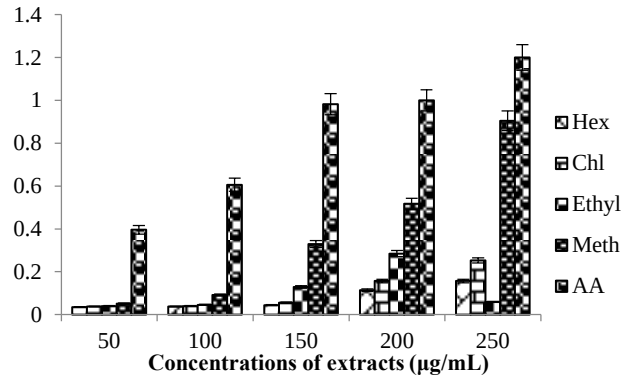

Figure 3: Ferric ion-reducing power of *Strychnos innocua* leaf extracts.
KEY = Hex = Hexane extract, Chl = Chloroform extract, EtAc = Ethyl acetate extract, Meth = Methanol extract, AA = Ascorbic acid

Plant secondary metabolites suppress germs by establishing hydrogen bonds with nucleic acid bases, preventing bacteria from synthesizing DNA and RNA. As a result, leaf extracts may have the same mode of action against the selected species.[47] The scavenging activity of the leaf extracts was proportionate to the rise in extract concentration, indicating that the extract has increasing reducing ability.[48-49] Similar scenario was also reported for the antioxidant activities of *Aconitum chasmanthum*[50]. *S. innocua* leaves have been shown to exhibit radical-quenching activity by converting DDPH to hydrazine. The DPPH is a persistent radical that decolorizes a solution by reacting with antioxidant substances.[51]The findings of this investigation demonstrated that the methanol extract of *S. innocua* leaves may have reducing properties, indicating that it has a high percentage antioxidant activity (91.3% at 250 µg/mL). Because it is easy and repeatable, DPPH radicals are most typically utilized in the spectrophotometric approach of determining antioxidant capacity[52]. The extracts were found to have high antioxidant activity and the ability to stop lipid oxidation, making them useful in the treatment of metabolic illnesses. The transition from the Mo^{+6} to the green phosphate/Mo^{+5} complex is linked to total antioxidant capacity. At the highest concentrations of 250 mg/mL (Fig. 2), total antioxidant capacity (88.8 mg/g) was detected, which corresponds favourably with the value of the reference standard for ascorbic acid.[52]
This total antioxidant capacity is higher tha**N** that recorded for edible plants commonly found in East Asia and the Middle East by Kukharenko et al., (2020).[53] In general, the presence of compounds with reducing characteristics is linked to the presence of chemicals that operate by terminating free-radical chain reactions by donating hydrogen atoms.[54] The creation of the Fe^{2+} compound enhanced the absorbance of the extracts, as did the concentration, as seen in the reference antioxidant (Figure 3). In all of the tests, the extracts of *S. innocua* leaves had a significant antioxidant activity. The ability of bioactive substances to lose electrons was linked to their antioxidant properties.[55] The ability of bioactive substances to lose electrons was linked to their antioxidant properties**.**[55] The fact that the ethyl acetate extract has a higher absorbance than ascorbic acid suggests that it may operate as an oxygen scavenger.[56] When compared to ascorbic acid (1.95 nm), the methanol extract of *S. innocua* has a moderate reductive potential of 0.90 nm at 250 µg/mL, indicating that it is a good reducing agent. **T**his is similar to that reported for crude extracts and essential oils of *Syzygium cumini* leaves by Mohamed *et al.*, (2013).[56] The fact that the ethyl acetate extract has a higher absorbance than ascorbic acid suggests that it may operate as an oxygen scavenger.[57] When compared to ascorbic acid (1.95 nm), the methanol extract of *S. innocua* has a moderate reductive potential of 0.90 nm at 250 µg/mL, indicating that it is a good reducing agent. [57]

Conclusion

In conclusion, the extracts of *S. innocua* leaves contain a variety of plant secondary metabolites in appreciable quantities and possessed significant antimicrobial and antioxidant activities. This could explain the use of this plant in folklore medicine in the treatment of metabolic and infectious diseases. There has been no report on the detailed scientific and pharmacological activities of this plant before now. Further studies are ongoing to isolate pure and active compounds from the extracts.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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