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Bioassay guided isolation of quercetin and β-sitosterol from *Chrozophora senegalensis* (Lam.) crude extract

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Abstract
The study was aimed at isolation of bioactive compounds from *Chrozophora senegalensis*, traditionally used for treating ailments. Plant sample was extracted with methanol using maceration and fractionated with solvents of varying polarity. Fractions were screened for antibacterial activities against clinical isolates. The most active ethylacetate fraction was evaluated for antioxidant potentials and purified on column chromatography. The antibacterial potential was significant relative to the standard with inhibition zones ranging from 10.43 - 21.53 mm against Gram-positive and Gram-negative bacteria. Antioxidant activity evaluation yielded fifty percent inhibitory concentrations (IC_{50}) ranging from 6.30 - 7.46 µg/mL while that of quercetin standard ranged from 5.01 - 8.71 µg/mL using standard procedures. The purification effort yielded quercetin and β-sitosterol. The two compounds are known to possess antioxidant and antibacterial properties. Consequently, the isolation of these phytomolecules from *Chrozophora senegalensis* crude extract partly justifies its use in traditional medicine.

Keywords: Quercetin, β-sitosterol, antibacterial, antioxidant, *Chrozophora senegalensis*

Introduction
Medicinal plants had been in use for thousands of years across cultures for therapeutic purposes and are considered as the cornerstone for modern medicine [1]. Estimates show that about 65-80% of global populace rely on medicinal plants for healthcare [2]. In recent years, interest in medicinal plants has surged, driven largely by the quest for new drugs and the desire for more natural and holistic healthcare approach. This heightened impetus for medicinal plants research is further facilitated by technological advances in separation methods, robust high throughput bioassays, innovative structure elucidation methods and the need to combat both incurable and emerging diseases with new lead compounds [3, 4]. Several metabolites of plant origin such as catechin, ellagic acid and sanguinarine with excellent pharmacological properties had been isolated and identified [5].

Chrozophora senegalensis of the Euphorbiaceae family is indigenous to the arid and semi-arid regions of Africa and mostly prevalent in countries such as Senegal, Nigeria, and Sudan. It has attracted attention due to its numerous traditional medicine applications and adaptation to harsh environmental conditions. Traditionally, the plant is used in the management and treatment of diarrhea, boils, conjunctivitis, stomachache and syphilis [6]. *Chrozophora senegalensis* is characterized by its distinctive morphological features, including a shrub-like growth form, simple leaves, and small, clustered flowers. The plant thrives on sandy soils and can withstand prolonged drought periods [7].

In vitro bioassays are crucial in the search for antimicrobial therapeutic agents. These assays are foundational in the evaluation of new antibiotics, which are urgently needed given the alarming rise in antimicrobial resistance [8]. Antimicrobial resistance has been reported as one of the top ten global public health challenges facing humanity challenges of century [9]. Similarly, Antioxidant bioassays help in identifying compounds that can mitigate oxidative stress by scavenging free radicals, chelation of metal ions, and enhancing the body's endogenous antioxidant defenses. Some commonly used antioxidant assays include the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay, and the FRAP (ferric reducing antioxidant power) assay [10]). The present study adopted FRAP, FICA and H_2O_2 assays for the evaluation of the antioxidant potentials of the crude extract fractions.

Isolation of bioactive compounds is a crucial step in natural product research, pharmacology, and biochemistry. Among the various techniques employed, column chromatography stands out for its efficiency, simplicity and affordability. Column chromatography involves the separation of compounds based on their differential interactions with the stationary phase (solid) and the mobile phase (liquid or gas). This technique has been instrumental in the isolation of pure compounds from complex mixtures [11]. In our continued effort on the phytochemical investigation of *Chrozophora senegalensis* [12, 13], we report for the first time the isolation and characterization of quercetin and β-sitosterol from *Chrozophora senegalensis* crude ethyl acetate extract.

Materials and Methods

Solvents, Reagents and Equipment

Analytical grade solvents (Loba Chiemie Company products) used include hexane, dichloromethane, ethylacetate, acetone and methanol purchased from Emmaco Surgical & Scientific Ltd Kaduna State Nigeria. Other reagents and materials include dimethyl sulfoxide, ascorbic acid, rutin, Gentamicin (Pfizer), Mueller Hinton agar and petri dishes. Silica gel 60 (70-230 mesh) and precoated aluminium thin layer plates 20 x 20 cm (silica gel 60 F_{254}) also obtained from Merck KGaA, Dermstadt, Germany purchased from Emmaco Surgical & Scientific Stores Ltd Kaduna State. NMR spectra data was obtained using AVANCE II 400 MHz Bruker in $CDCl_3$ while the IR data was processed on FT-IR Perkin Elmer spectrometer.

Sample collection

Chrozophora senegalensis was collected on November 30th, 2023 from Gwani Village Yamaltu-Deba Local Government Area of Gombe State. The plant was identified and assigned a voucher number GSUH556 by a Botanist in Department of Biological Science of Gombe State University Nigeria.

Preparation, Extraction and Fractionation

The *Chrozophora senegalensis* sample obtained was dried to a constant weight at room temperature under shade. The dried plant sample was pulverized to powder using motorized miller (blender). The powdered plant sample was kept in polythene bags under cool and dry condition until required for use.

The powdered *Chrozophora senegalensis* sample (1.5 kg) was extracted with methanol for a period of 7 days with occasional shaking using maceration method at room temperature. The extract was filtered with a muslin cloth and clarified using Whatman No.1 filter paper. The filtrate was concentrated on a rotary evaporator at 45ºC to obtain a crude extract. The crude methanol extract of *Chrozophora senegalensis* was partitioned using three different solvents; n-hexane (HEX), ethyl acetate (EtOAc) and dichloromethane (DCM). Each extraction process was repeated six times with 250 mL portion of each solvent to obtain sufficient quantity of each fraction that can be used for possible column chromatography separation. The n-Hex, EtOAc and DCM portions obtained were combined and evaporated to dryness on a rotary evaporator at 45ºC. This resulted in three different plant extract fractions. These were then subjected to antibacterial test. Fraction with the best antibacterial activity was evaluated for antioxidant potential and then subjected to column chromatography purification.

Antibacterial Activity Assay

Petri dishes were filled with 20 mL of sterilized molten Mueller-Hinton agar. Each petri dish was inoculated with 10 µL of a bacterial suspension containing 10^6 cfu/mL. Filter paper discs 6 mm in diameter were briefly soaked in 500 µg/mL solution of the different fractions of the plant crude extract and placed on the inoculated solidified agar. Gentamicin discs at 10 µg/mL served as positive controls; while 10% DMSO discs served as negative controls. The plates were incubated at 37ºC for 18-24 hrs. Diameters of bacterial growth inhibition zones were measured in millimeters using a transparent meter rule. Each test was performed in triplicate, and the average inhibition zone diameter was calculated and expressed as Mean ± S.D. [13].

Minimum Inhibitory Concentration (MIC) using Broth Dilution Method

To determine the MIC of the plant extract, broth dilution method was used with slight modifications [14]. Nine serial, two-fold dilutions of plant extracts were prepared and ranged from 500 - 3.91 µg/mL. Test tubes 10 and 11 served as negative and positive controls respectively. For each bacterial species, three sets of nine test tubes were used. Each test tube contained 2 mL of culture medium, 2 mL of plant extract solution in serial dilution and 10 µL of bacterial suspension. Tubes 10 and 11 also received 2 mL of culture medium. To test tube 10 was added 10 µL of bacterial suspension (negative control) and to test tube 11 was added 10 µL of bacterial suspension and 10 µg/mL of gentamicin (positive control). The tubes were incubated at 37ºC for 24 hrs and observed for bacterial growth. The lowest concentration of the extract that inhibited bacterial growth was recorded as the MIC.

Minimal Bactericidal Concentration (MBC)

The MBC is the lowest concentration of the extract that killed 99.9% of the bacteria after 18-24 hrs of incubation at 37 °C. To determine the MBC, the contents of the test tubes without turbidity or visible bacterial growth in the MIC test were transferred to sterile Mueller-Hinton agar plates and incubated at 37 °C for 18-24 hrs. The MBC is the concentration of the extract where no bacterial colonies grew on the agar plate after 18-24 hrs of incubation. Each experiment was performed in triplicate [14].

Antioxidant Activity Evaluation

The ferric reducing antioxidant power and ferrous ion chelating activity were evaluated using previously described method [13]. Additionally, the hydrogen peroxide assay was used to assess the antioxidant activity of the crude extract fractions in accordance with the method reported by Dibala *et al.* [15].

Column Chromatography

The column chromatography purification was performed according to the procedure reported by Kwaji *et al.* [16] with slight modification. *Chrozophora senegalensis* crude extract (8 g) was pre-adsorbed onto silica gel 60 and then loaded to a column already packed with silica gel 60 (70-230 mesh) using the wet slurry procedure. Gradient elution was performed with hexane/ethyl acetate and ethyl acetate/methanol at 5% volume change in polarity of the eluting solvents; (100.00), (95.05), (90.10), (85.15), (80.20), (75.25), (70.30) etc. to yield several fractions of 100 mL each. A total of 153 fractions

were collected. Fractions were concentrated and combined based on their TLC profile. Fractions 58-50 contained a single spot and was coded IB-1. Similarly fractions 109-112 contained a single spot and was coded IB-3. Fractions containing single spot are considered pure and were subsequently recrystallized from acetone to give pure compounds.

Spectroscopic Analysis of Isolates

Characterizations of isolates were performed using nuclear magnetic resonance spectroscopy, NMR (^{1}H and ^{13}C) and FT-IR spectra data and literature comparison.

Results and Discussions

Antibacterial Activities

The n-Hex, DCM and EtOAc fractions of *Chrozophora senegalensis* crude extract were tested on clinical bacteria isolates and the Ethyl acetate fraction displayed significant to moderate activity against all tested bacteria (Table 1). Furthermore the evaluation of MIC and MBC carried out on the Ethyl acetate fraction revealed MIC values of 5.21 - 31.25 µg/mL and MBC values of 15.63 - 83.33 µg/mL which were considered as significant for all the bacterial pathogens. For the bacterial pathogens with the ratio of MBC/MIC < 4, the effect of the extract was considered bactericidal but if MBC/MIC > 4, the effect is non-bactericidal (Table 2) based on the classification reported by Yohana *et al.* [14]. Consequently, the effect of the ethylacetate extract was bactericidal to *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumoniae* and nonbactericidal to *Enterococcus faecalis.*

Table 1: *C. senegalensis* Extract Fractions Inhibition Zones (mm).at 120 mg/mL

Bacterial	Hexane (mm)	Dichloromethane (mm)	Ethyl acetate (mm)	Gentamicin (10 µg/ml)
E. coli	ND	13.86±1.31	21.53±1.86	26.33±1.58
B. cereus	ND	ND	18.62±1.76	20.00±0.00
S. typhi	ND	07.41±1.07	11.63±1.71	20.13±1.63
P. vulgaris	8.65±1.43	07.62±1.02	10.43±1.22	20.00±1.00
E. faecalis	9.12±1.17	ND	10.49±1.32	9.55±1.31
S. aureus	ND	ND	ND	17.67±1.15
P. aeruginosa	ND	ND	ND	18.17±1.76
K, pneumonia	8.83±1.79	14.91±1.83	20.68±1.76	13.52±1.22

Keys: *P. aeruginosa = Pseudomonas aeruginosa; B. cereus= Bacillus cereus;*
S. typhi= Salmonella typhi; P. vulgaris= Proteus vulgaris;
E. faecalis= Enterococcus faecalis; S. aureus = Staphylococcus aureus;
E, coli = Escherichia coli; K. pneumonia = Klebsiella pneumonia

Table 2: MIC and MBC of Ethyl acetate fraction.

Bacteria	MIC(µg/ml)	MBC(µg/ml)	MBC/MIC
Escherichia coli	5.21 ± 1.83.	15.63 ± 0.00	3
Salmonella typhi	7.81 ± 0.00	26.04 ± 7.37	3
Enterococcus faecalis	6.51 ± 1.83	31.35 ± 0.00	5
Klebsiella pneumoniae	31.25 ± 0.00	83.33 ± 29.46	3

Antioxidant activity

Ferric Reducing Antioxidant Power

The % FRAP of the fractions revealed that ethyl acetate fraction exhibited good reducing ability of 67.79% at 50 µg/mL relative to Rutin with 80.59%. The IC_{50} value was found to be 7.46 µg/mL (Fig. 1) for EtOAc fraction and 5.37 µg/mL for standard rutin (Fig. 2). This showed a strong antioxidant potential for EtOAc crude extract fraction relative to the standard antioxidant. The observed strong antioxidant activity indicates the EtOAc crude extract fraction ability to neutralize free radicals and ultimately reduce oxidative stress in biological systems as reported by Oliveira-Silva *et al.* [17].

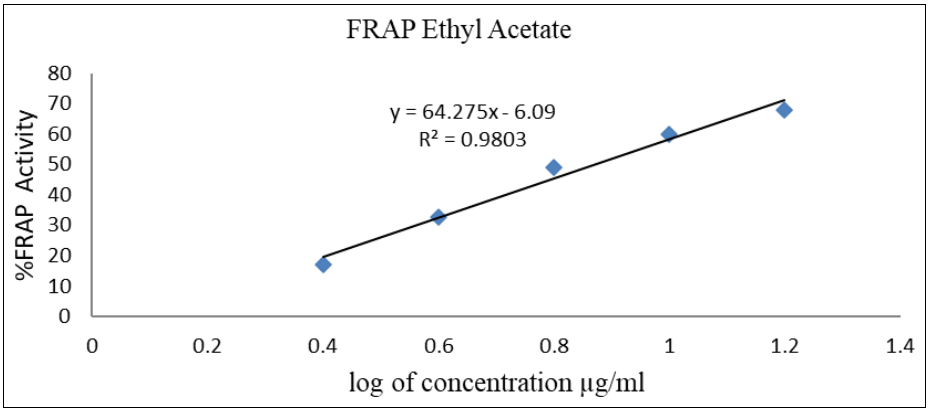

Fig 1: % FRAP Ethylacetate fraction of *C. senegalensis* (IC_{50} = 7.46 µg/mL)

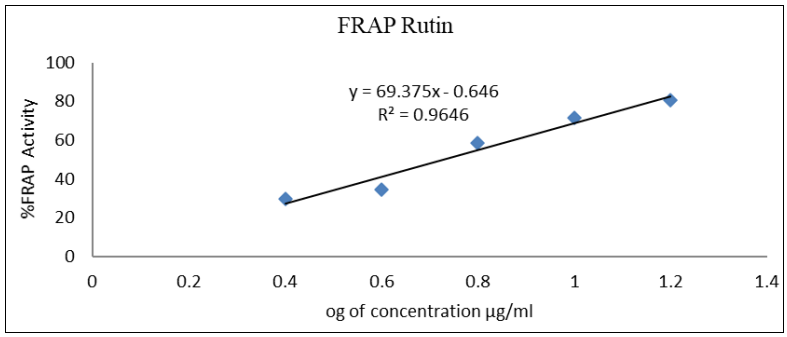

Fig 2: % FRAP of the Rutin Standard (IC_{50} = 5.37 µg/mL).

Ferrous Ion Chelating Activity

The % ferrous ion chelating activity of the fractions revealed that the EtOAc fraction exhibited high FICA value of 94.65% at 50 µg/mL relative to the standard rutin (96.62%). This gave a corresponding IC_{50} value of 4.07 µg/mL for EtOAc fraction (Fig. 3) and 8.71 µg/mL for the standard EDTA (Fig.4). This is consistent with the findings of Oliveira-Silva *et al.* [17].

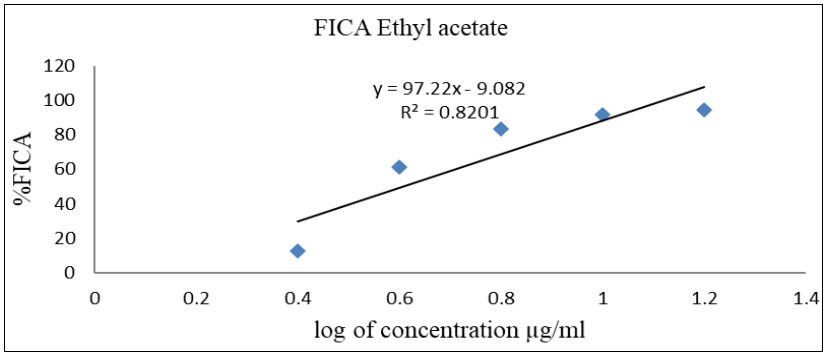

Fig 3: FICA assay of *Chrozophora senegalensis* (IC_{50} = 4.07 µg/mL)

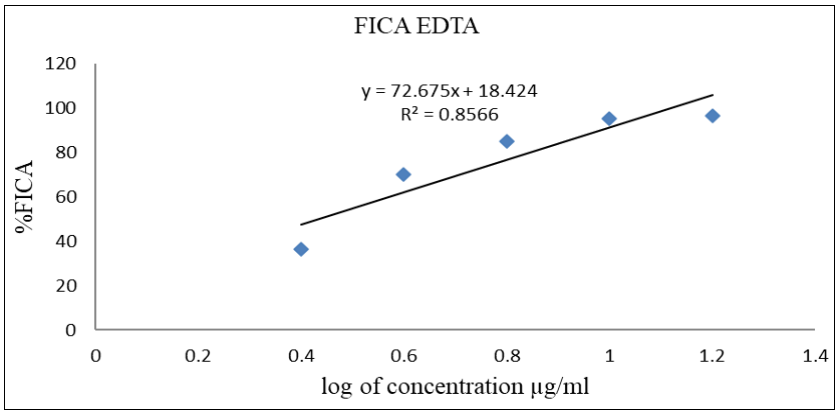

Fig 4: Rutin FICA assay of *Chrozophora senegalensis* (IC_{50} = 8.71 µg/mL).

Hydrogen Peroxide Assay

The $\%H_2O_2$ activity for the EtOAc fraction was 81.52% at 50µg/mL relative to that of standard rutin with 92.61% at the same concentration. The calculated IC_{50} value was 6.31µg/mL (Fig. 5) for EtOAc fraction and 5.01 µg/mL for the standard ascorbic acid (Fig. 6). These findings are consistent with previous studies that reported the strong antioxidant activity of *Chrozophora senegalensis*, often attributing this property to its high content of flavonoids, phenolics, and terpenoids [13, 18].

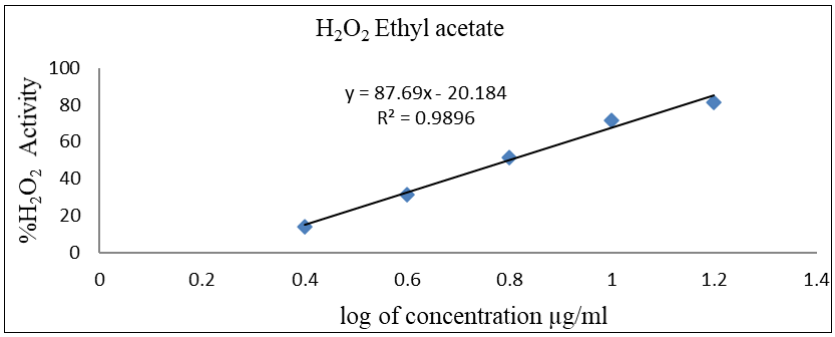

Fig 5: H_2O_2 assay of *Chrozophora senegalensis* (IC_{50} = 6.31 µg/mL).

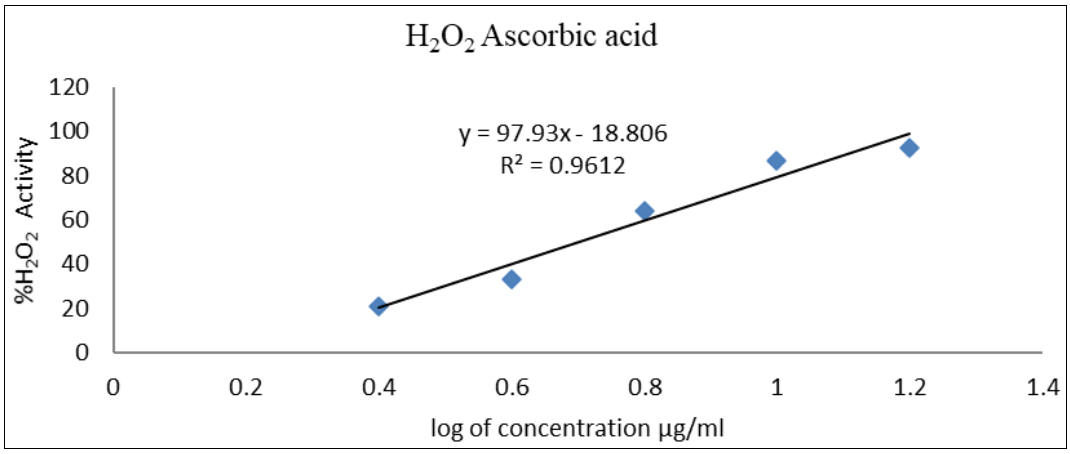

Fig 6: Ascorbic acid Hydrogen peroxide assay (IC_{50} = 5.01 µg/mL).

The result of H_2O_2 assay showed a linear increase in hydrogen peroxide activity over the concentration range of 3.125 - 50 µg/mL for EtOAc fraction and ascorbic acid. The EtOAc fraction showed potent hydrogen peroxide scavenging activity.

Characterization of Isolates

FT-IR Spectrum of Isolate IB-1

The isolated compound was obtained as white crystals (IB 1).

Based on the IR spectrum (Fig. 7), OH stretching was seen at 3354.58 cm^{-1}, the C=O vibration of an aryl ketone was seen at 1617.46 cm^{-1}. The decreased frequency is due to the resonance effect of the aromatic ring. An aromatic C=C vibration was observed at 1508.84 cm^{-1}, while an OH bending phenolic vibration was observed at 1334.28 cm^{-1}. The C-O vibration was seen at 1052.95 cm^{-1}. The assignment of the frequencies is consistent with literature [19].

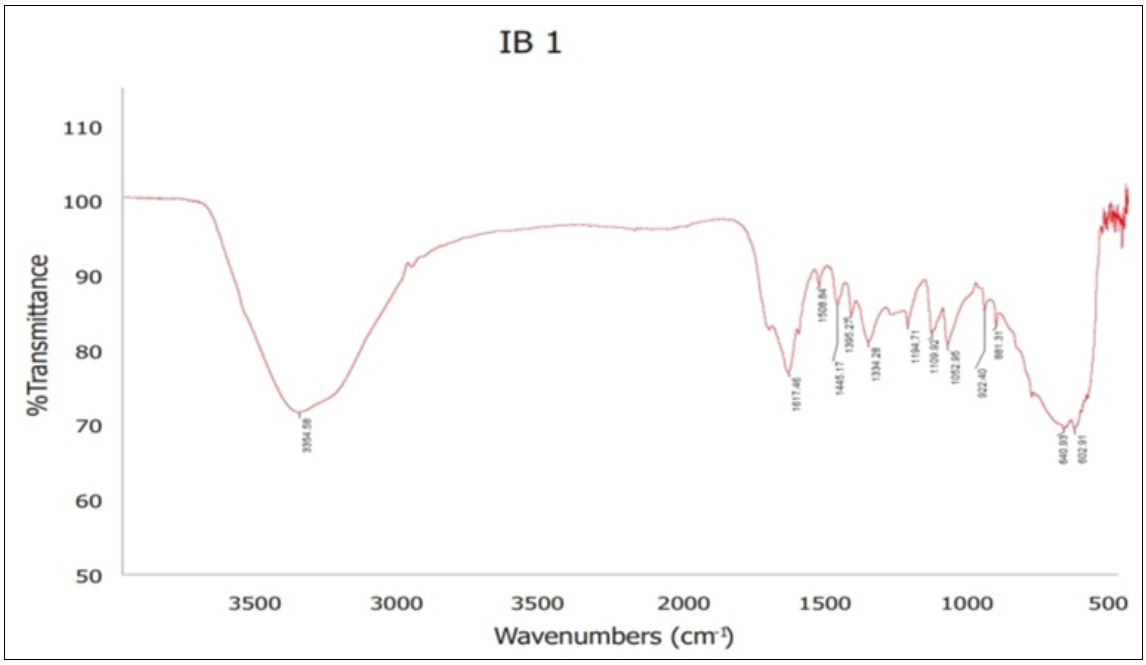

Fig. 7: IR Spectrum of the isolated compound (IB 1)

Table 3: IR Spectrum data of the isolated compound (IB 1)

S/No	Frequency (cm^{-1})	Type of Vibration
1	3354.58	O-H intermolecular
2	1617.46	C=O keto group
3	1508.84	C=C aromatic
4	1334.28	O-H phenolic
5	1052.95	C-O

Proton (^1H) NMR of IB-1

The ^1H NMR spectrum of IB-1 revealed characteristic chemical shifts at δ_H 6.27 (1H, d), 6.44(1H, d), 6.74(1H, dd), 7.40 (1H, dd), 7.69 (1H, dd), 9.47 (1H, s), 9.49 (1H, s) and 12.47 (1H, s) ppm (Fig. 8, Table 4). The signals between δ 6.27 and 7.7 ppm represent aromatic protons, at C-6, C-8, C-2', C-5' and C-6'; δ_H 9.47 ppm represents the phenolic protons of rings A and B and the highly deshielded signal at12.47 ppm indicates that of the hydroxyl proton at C-5 partially bonded to the ketone functional group of ring C. These chemical shifts are consistent with those found in the structure of quercetin [20, 21].

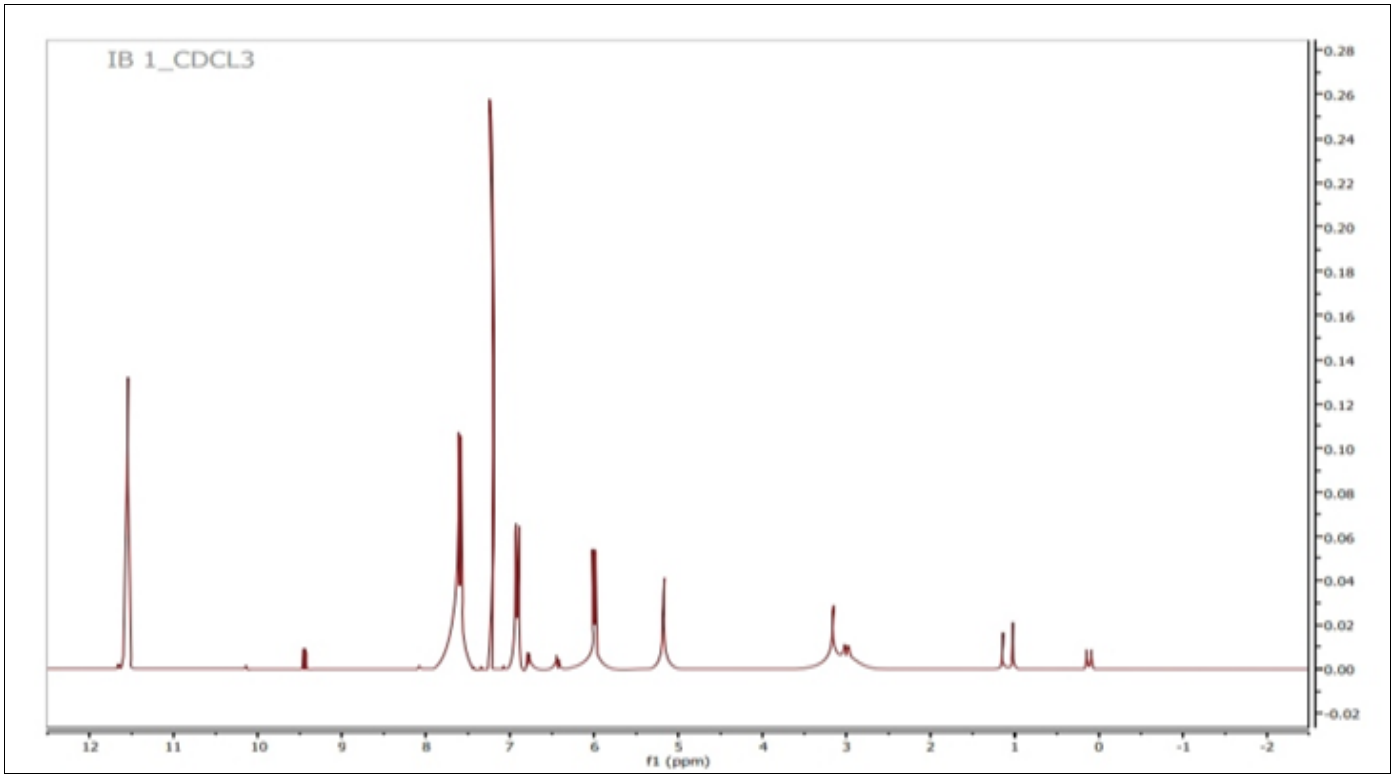

Fig 8: H^1 NMR Spectrum of the isolated compound (IB 1)

Carbon-13 (^{13}C) NMR of IB-1

The ^{13}C NMR spectrum (Fig. 9) further supports the identification of IB-1 as quercetin, with notable signals at δ 96.3(C-8), 101.7 (C-6), 105.6(C-10), 115.3(C-2'), 115.9 (C-5'), 122.5(C-1'), 128.5(C-6'), 137.1(C-3), 145.2(C-3'), 145.7(C-4'), 146.2(C-2), 157.4 (C-9), 161.0 (C-5), 163.3(C-7), and 176.8(C-4) ppm (Table 4). These assignments agree with previously reported ^{13}C spectrum data for quercetin (Fig.10). The characteristic carbonyl carbon chemical shift of flavone structure at δ_C 176.8 ppm was observed. This is consistent with literature reports [20, 21].

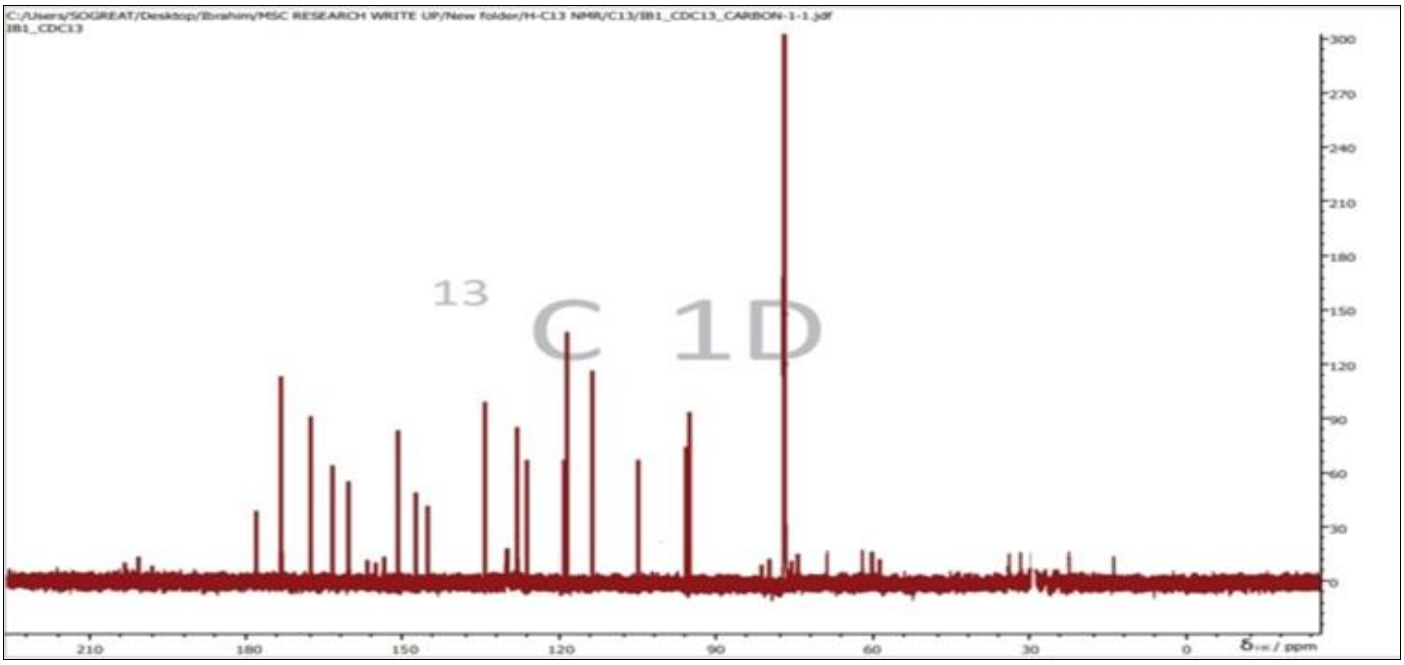

Fig 9: ^{13}C NMR Spectrum of the isolated compound (IB 1)

Table 4: (δ) ^{13}C- and ^{1}H-NMR for IB 1 and Literature data [21, 22]

S/No.	Experimental Values (ppm)		Literature values (ppm)	
C-atom	δH	δC	δH	δC
2		146.2		147.26
3	9.56	137.1	9.59	136.18
4		176.8		176.29
5	12.47	161.0	12.47	161.18

6	6.27	101.7	6.19	98.64
7	10.75	163.3	10.75	164.34
8	6.44	96.3	6.40	93.80
9		157.4		156.94
10		105.6		103.47
1’		128.5		122.41
2’	7.69	115.3	7.68	115.52
3’	9.32	145.7	9.35	145.51
4’	9.32	146.2	9.35	148.16
5’	6.74	115.9	6.88-6.90	116.06
6’	7.40	122.5	7.53-7.55	120.43

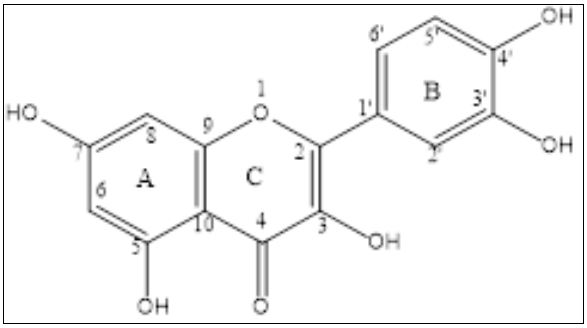

Fig 10: Quercetin

FT-IR Spectrum Result of Isolate IB-3

For IB-3, the IR Spectrum showed broad absorption peak at 3359.63 cm^{-1} indicating the presence of -OH group. The peak at 2914.86 cm^{-1} indicates the presence of aliphatic C-H stretching. The absorption peak at 1646.87 cm^{-1} indicates unsaturation of C=C absorption peak and peak at 1293 cm^{-1} indicates the presence of C-O stretching vibration. The absorption band at 1540.43 cm^{-1} indicated the bending vibration of OH and absorption band at 1472.39-1462.29 cm^{-1} is due to C-H bending of geminal dimethyl group. The absorption peak at 936 cm^{-1} is due to C=C-H group. The above frequencies are diagnostic of β-sitosterol [23].

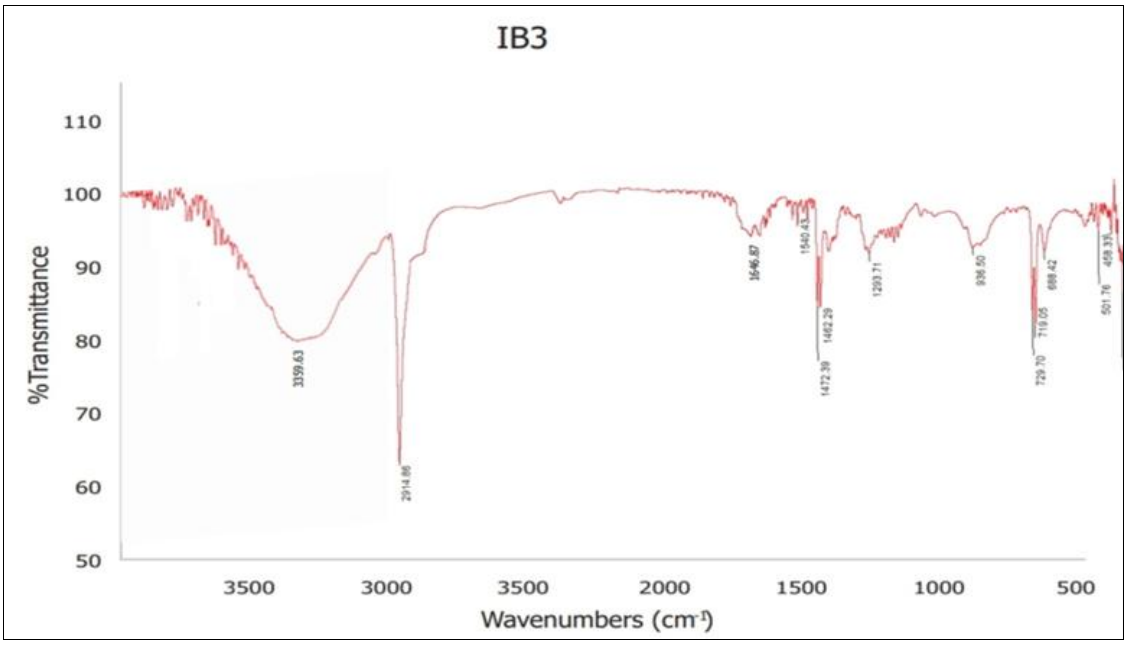

Fig 11: IR Spectrum of the isolated compound (IB 3)

Table 5: IR Spectrum data of the isolated compound (IB-3)

Bands (cm^{-1})	Interpretation
3359.63	Stretching vibration of OH
2914.86	Stretching vibration of CH alkane (symmetric and asymmetric)
1646.87	Stretching vibration of C=C
1540.43	Bending vibration of OH
1472.39-1462.29	Bending vibration of isopropyl chain
1293.71	Bending vibration of C-O of 2° alcohol

Proton (^{1}H) NMR of IB-3

The ^{1}H NMR spectrum for IB-3 exhibits key signals that match the typical profile of β-sitosterol. The signals include δ 5.29 (dd, J = 7.2 Hz, 1H), which corresponds to the olefinic proton at C5-6; a characteristic signal in sterols δ 3.97 (tt, J = 10.1, 5.1 Hz, 1H), representing the hydroxyl proton at C-3, another signature of sterols. δ 1.03 (s, 3H) and δ 0.69 (d, J = 9.1 Hz, 3H), corresponds to methyl groups at C-19 and C-18, respectively and are characteristic of β-sitosterol, as reported in prior studies [24,23].

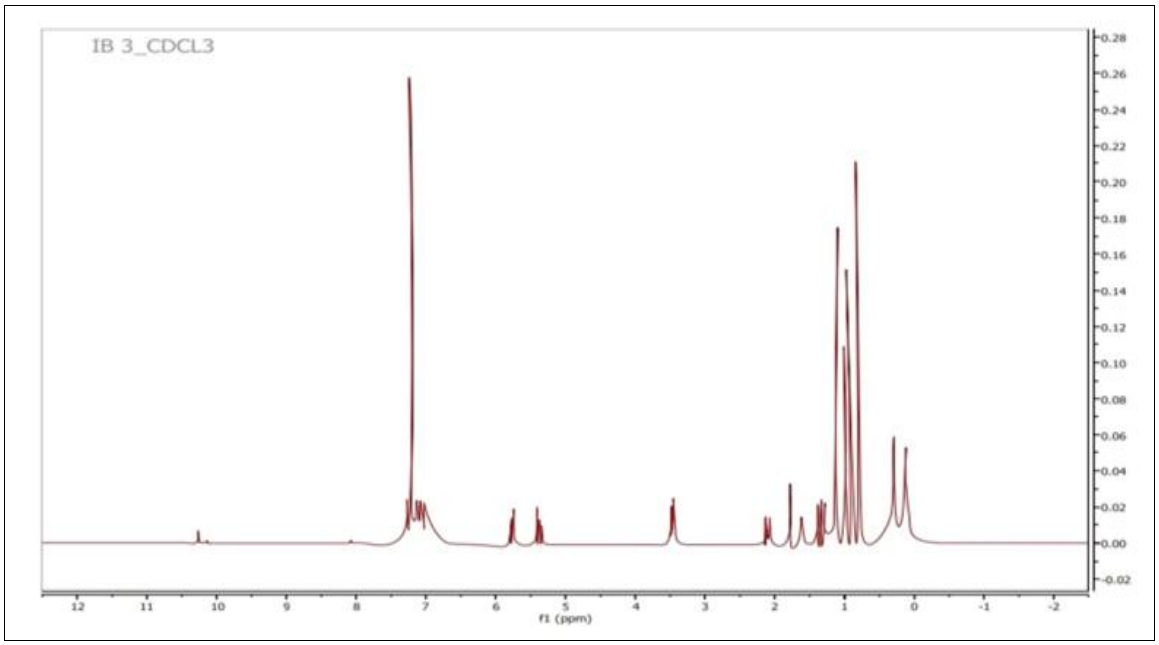

Fig 12: ^{13}C-NMR Spectrum of the isolated compound (IB 3)

^{13}C NMR of IB-3

The ^{13}C NMR data for IB-3 (Fig. 13; Table 6) provides further confirmation of the β-sitosterol molecule. The spectral signals includes δ 11.4, 11.8, 18.7, 19.3 and 19.4, all correspond to six methyl carbons, δ_C 31.9-32.0, δ 36.3, and δ 56.5-56.8, which are typical of carbon atoms found in a steroid structure. The chemical shifts δ_C 122.0 and δ_C 140.8 represent the carbons of the double bond in the ring, typically found in β-sitosterol at C-5 and C-6, confirming the sterol's unsaturated site [24, 25]. The combined NMR data match well with those reported for β-sitosterol, making the identification of IB-3 as β-sitosterol reliable (Fig. 14). Studies on β-sitosterol had consistently shown similar chemical shifts in both ^{1}H and ^{13}C NMR spectra, thus confirming this structure identification [23, 25].

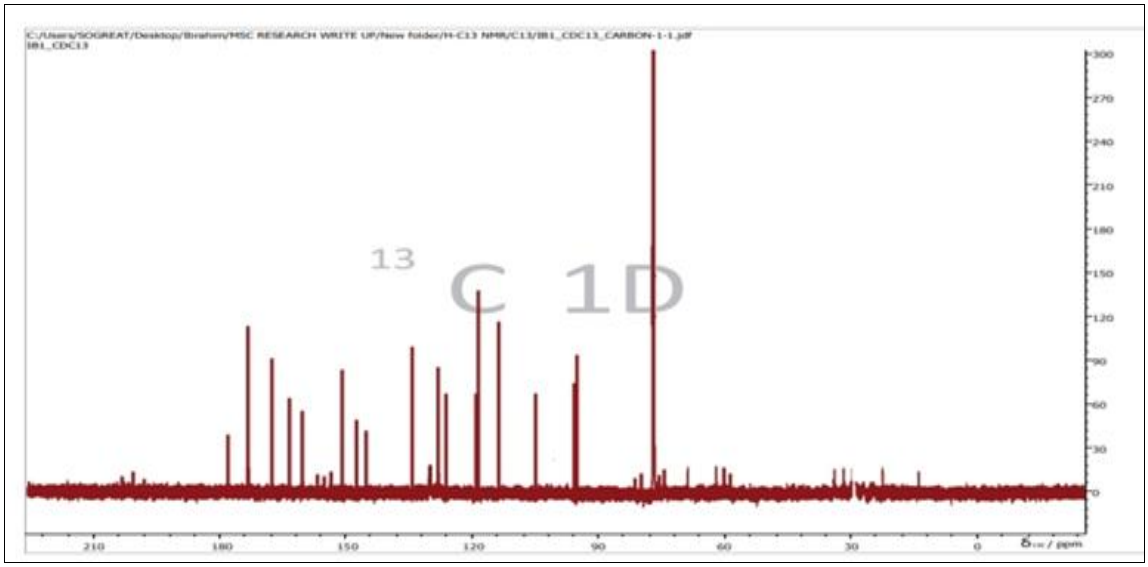

Fig 13: ^{13}C NMR Spectrum of the isolated compound (IB 3)

Table 6: δ_H- & δ_C for IB-3 &Literature [23, 26]

IB 3			Literature Data	
Carbon atom	**δH**	**δC**	**δH**	**δC**
1		36.7		37.3
2		31.6		31.9
3	3.57	71.2	3.52	71.9
4	2.38	42.3	2.27	42.4
5		140.8	-	140.8
6	5.29	122.0	5.35	121.8
7		31.9		31.7
8		31.9		32.0
9	0.92	50.2	0.92	50.2
10		36.3	-	36.6

11		21.2		21.2
12	2.00	39.9	1.99	39.8
13		43.90	-	42.3
14		56.8		56.8
15		25.9		24.4
16		28.1		28.3
17		56.5		56.1
18	0.69	11.8	0.69	12.3
19		19.3		19.5
20		34.3		36.2
21	0.82	24.3	0.79	26.2
22		45.7		46.1
23	1.03	24.5	1.14	26.1
24	0.69	12.0	0.91	12.3
25	1.85	29.2	1.63	29.2
26		19.4		19.9
27	0.80	18.7	0.79	19.1
28	1.25	22.9	1.24	23.1
29	0.84	11.4	0.83	12.0

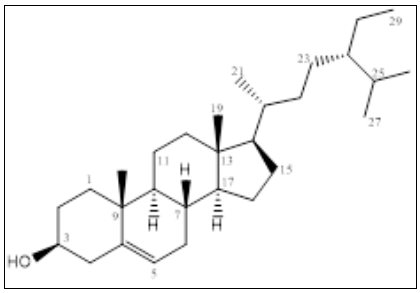

Fig 14: β-Sitosterol

Conclusion

The observed antibacterial and antioxidant activities and the successful isolation and characterization of bioactive compounds from *Chrozophora senegalensis* ethylacetate crude extract partly confirm its medicinal potentials. The two compounds isolated (Quercetin and β-sitosterol) may be partly responsible for the observed bioactivities and hence could justify the use of plant in traditional medicine 'for the treatment of some ailments and diseases. Although the isolation of quercetin derivatives had been reported from *Chrozophora senegalensis* but this is first time of Quercetin aglycone and β-sitosterol isolation from *Chrozophora senegalensis* to the best of our knowledge. This indicates that the quercetin and its derivatives coexists in the plant in addition to other bioactive compounds such as β-sitosterol. The presence of these phytochemical compounds partly justifies the use of *Chrozophora senegalensis* in traditional medicine.

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Conflict of Interest

Authors declare that conflict of interest does not exist.

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Appendices

The following Tables provides sample raw data for each antioxidant method used for assessment of antioxidant activity.

Table 5: FRAP of Ethyl Acetate fraction of *C. senegalensis*

Concentration (µg/ml)	Rutin (%) % Inhibition	Ethyl Acetate (%) % Inhibition
3.125	29.59	17.21
6.25	34.50	32.63
12.5	58.34	49.00
25	71.25	60.02
50	80.59	67.79

Table 6: Ferrous Ion Chelating Activity (FICA) of *C. senegalensis*

Concentration (µg/ml)	EDTA % Inhibition	*Ethyl Acetate (%)* %Inhibition
3.125	36.34	12.68
6.25	70.14	61.13
12.5	84.79	83.38
25	94.93	91.63
50	96.62	94.65

Table 7: Hydrogen peroxide Scavenging Activity of fraction of *C. senegalensis*

Concentration (µg/mL)	Rutin (%) % Inhibition	*Ethyl Acetate* (%) % Inhibition
3.125	21.23	13.85
6.25	33.38	31.38
12.5	64.00	51.69
25	86.46	71.38
50	92.62	81.54