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ISOLATION OF EPICATECHIN AND ANTIMICROBIAL EVALUATION OF *INDIGOFERA WELWITSCHII* (FABACEAE)

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ABSTRACT

Proper phytochemical investigations of medicinal plants derived constituent has become of necessary owing to their versatile application in medicine. *Indigofera welwitschii* of the family *fabaceae*; is also among the species not fully investigated for its medicinal value. It is use in traditional medicine in managing skin problems. The aim is to isolate and characterize phenolic compounds from the EAF of the aerial parts of *Indigofera welwitschii* and evaluate its antimicrobial potential. The plant material upon collection was prepared and extracted with methanol. The resulting methanol extract was successively partitioned into hexane (HEF), chloroform (CHF), ethylacetate (EAF), n-Butanol (NBF) fractions and residual aqueous (AQF) fraction respectively. Silica gel chromatographic separation of EAF using gradient elution and repeated gel filtration using serphadex LH-20 was employed to isolate pure compound. A portion of the crude methanol extract (CME) and fractions was subjected to preliminary phytochemical and antimicrobial assessments. The organisms were clinical isolates of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Methicillin Resistant Staphylococcus aureus* and *Pseudomonas aeruginosa*. Agar well diffusion technique was used to determine the susceptibility of the extract (CME) and the fractions while microbroth dilution method was used to determine both the MIC, and MBC. The structure was elucidated using physico-chemical test, 1D- & 2D-NMR. (-)-Epicatechin (a flavan-3-ol) was identified. The activity against all bacterial isolates tested reveal the zones of growth inhibition range of between 12-25 mm at a concentration of 50 and 100 mg/mL. EAF and NBF have the highest activity with lowest MIC/MBC ranges of between 31.25 -62.50 mg/mL

Keywords: Antimicrobial, Epicatechin, *Indigofera welwitschii*, NMR spectroscopy, Phytochemical.

INTRODUCTION

The proper understanding of phytochemicals is essential for drug discovery and for the development of lead molecules against major disease conditions (Awuchi *et al*., 2020). Standard extraction procedures for crude drugs examination are often necessary to obtain medicinal and therapeutically desired constituents using appropriate analytical grade solvents. *Indigofera welwitschii* var. *welwitschii* also known and cited as *Microcharis welwitschii* (Ateba *et al*., 2021) belongs to the family *fabaceae* (*Leguminaceae*) which is ranked the third largest family of the blossoming plants after *Orchidnaceae* and *Asteraceae* with approximately 650 genera and 18000 different species and the second-largest family of medicinal plants, with more than 490 species used in traditional medicine (Dzoyem *et al*., 2014). The plant exists as a shrub and grows very well during raining season. It's mainly distributed in Africa around Ghana, Nigeria, Cameroun, Congo, Mozambique, Malawi and Angola (Darbyshire *et al*., 2015; Schrice, 2013). Traditional medicine revealed an existing ethno-botanical contention of using *Indigofera welwitschii* leaf powder in petroleum jelly which is claimed to be

effective treating dermatological problems. This contention was however not proven scientifically (Rahman *et al*., 2018) and over 60 *Indigofera* species which are reported in traditional medicine are not proven scientifically; thus, their uses depend on the country and the species, but similarities have been noticed (Gerometta *et al*., 2020). *Indigofera* species are widely employed in traditional medicine all around the world, against many ailments; the family is well distributed across all tropical and subtropical regions of the world (Su *et al*., 2008). Some 75% of these species are restricted to Africa and Madagascar (Ponmari *et al*., 2014). Phytochemical studies have led to the identification of over 200 compounds, notably polyphenls and terpenoids. A number of phytochemicals such as lignins, triterpenes, steroids, alkaloids, flavonoids, acylphloroglucinols, saponins, tannins, quinines, rutin, cafffeic acid, gallic acid, myricetin, galangin and quercetin have been reported from the genus (Rahman *et al*., 2018).

MATERIALS AND METHODS

Materials

Glass wares

Measuring cylinders of various sizes (Pyrex England), Column of different sizes and dimensions, Beakers of different sizes (Pyrex England), Separating funnel, volumetric flasks (Pyrex England), thin layer chromatographic plates silica 60F256, Silica gel of mesh size 60-120 µm (Qualikems), Sephadex LH-20 and Capillary tubes

Equipment

Electronic weighing balance (MetlersPr 63 Switzerland), NMR spectrometer (Agilent technologies 400 MHz) (Multi User laboratory, Ahmadu Bello University, Zaria) among others.

Reagents

The solvent used are of analytical grade. They include Methanol, Chloroform, N-hexane N- butanol, Ethylacetate, Acetone and Dichloromethane

Experimental organisms

Staphylococcus aureus, Bacillus subtilus, Methicillin resistant Staphylococcus aureus, *Escherichia coli* and *Pseudomonas aeruginosa* (Microbial strain bank of the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto State, Nigeria).

Standard drugs

Tinidazole/ciprofloxacin disc (50/5µg) and Terbinafine citrate disc (32µg) Oxoid limited UK

Methods

Collection of the plant material

The aerial parts of the plant material (*Indigofera welwitschii*) was collected in July, 2019 from Batagarawa local government area in Katsina State, Nigeria. It was identified via taxonomic means at the Herbarium unit of the department of Botany, Ahmadu Bello University Zaria, Nigeria by a taxonomist, Mallam Sanusi Namadi (voucher number ABU01702). The aerial part of the plant was air dried under shade, grounded to powder, labeled and preserved.

Extraction of plant material

The aerial parts of *Indigofera welwitschii* (2.2 kg) was extracted continuously in methanol (10 L) using maceration method for 72 hours. The solvent was removed and the filtered extract was allowed to dry yielding a green residue subsequently referred to as the crude methanol extract (CME).

Partitioning of the crude methanolic extract (CME)

The crude methanol extract (120 g) was suspended in distilled water and filtered; the water-soluble portion was successively partitioned using n-hexane (1500 mL), chloroform (1000 mL), ethylacetate (2500 mL) and n-Butanol (1500 mL) to obtain n-hexane fraction (HEF), chloroform (CHF), ethyl-acetate (EAF), n-butanol (NBF) and the residual aqueous (AQF) fractions, respectively.

Preliminary phytochemical screening

The crude extract and the fractions were subjected to preliminary phytochemical screening using standard procedures to identify the presence of various chemical constituents like alkaloids, flavonoids, and tannins etc.

Column chromatographic separations of Ethylacetate fraction (EAF)

10 g of dry ethylacetate fraction was measured and dissolved in small amount of methanol. It was then mixed and adsorbed completely in small quantity of Silica gel powder (60 – 120 mesh size), dried, triturated and then loaded on top of the column. The column was packed using wet slurry method with Silica gel powder (60 – 120 mesh size) being the stationary phase while the sample was applied using dry loading method (Cannell, 1998). Silica gel gravity column chromatography was carried out by gradient elution method in the silica gel packed column (5×100 cm) using different solvent combinations starting with n-hexane (100 %), hexane: ethylacetate (9:1) to hexane: ethylacetate (1:1) followed by ethylacetate (100 %), ethylacetate: methanol (9:1) up to 100 % methanol. The column fractions of 50 mL each were collected and monitored by TLC sprayed with sulphuric acid reagent and visualizing under ultra violet light (254 and 366 nm). This gradient elution using the various solvent systems yielded One hundred and ten collections of smaller fractions were made. These fractions were pooled together based on similarities on their TLC profiles. This yielded twenty sub fractions labeled A_1-A_{20}**.** Co TLC of these fractions was further conducted and similar fractions were pooled together to yield six major sub fractions of varying quantities labeled B_1-B_6. Fractions B_1, B_2 and B_3 were then subjected to further purifications to obtain pure compounds

The gel filtration was performed using Sephadex LH-20 (Sigma) where the Sephadex was suspended in methanol and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set while the sample was dissolved in small volume of the eluent and applied to the top of the column.

RESULTS

Isolation and Purification of Compound AM2 (Epicatechin)

Gel filtration chromatography (Sephadex LH-20) of B_2

Repeated gel filtration chromatography of fraction **B_2** obtained from the pooling of the silica gel column chromatography of the ethylacetate fraction of the crude methanolic extract of *Indigofera welwitschii* gave 3 major fractions coded B_2A_1, B_2A_2, B_2A_3 (Table 1) respectively.

Gel filtration Chromatography (Sephadex LH-20) of C3

The fraction **C3**, obtained from the repeated gel filtration chromatography of fraction **B_2A_2**; sub fraction of **B2** of the silica gel column of ethylacetate fraction of the crude methanolic extract of *Indigofera welwitschii* (Table 1) was subjected to further repeated gel filtration chromatography which led to the isolation of a yellow or a yellowish-brown substance in collections 3-9 **(D2)** coded AM6 (Plate I).

Table 1: Gel filtration Chromatography (Sephadex LH-20) of B_2

S/N	No of Eluates (B_2)	Code
1	1 – 6	B_2A_1
2	7 – 19	**B_2A_2**
3	20 – 24	B_2A_3
	B_2A_2	
1	1 – 2	C1
2	3 – 7	**C2(AM2)**
3	8 – 18	**C3**
	C3	
1	1-2	D1
2	3-9	**D2(AM6)**
3	10-12	D3

Plate I: TLC Chromatogram of C3(Gel filtration)

Thin layer chromatographic analysis of AM6

Compound AM6 gave a single homogenous spot on TLC using two solvent systems viz; Chloroform: Methanol 3:1 and Chloroform: Ethylacetate 2:8. The isolated compound appear as a yellowish brown amorphous solid which is partially soluble ethylacetate and completely soluble in acetone and methanol and positive to ferric chloride and shinoda test

Plate II: TLC chromatogram of Compound AM6 using Chloroform: Methanol 3:1

Table 2: R_f value of AM6

Solvent System	No of Spots	R_f Value	Solubility
CHL: ME 3:1	1	0.74	Acetone, Methanol
EA:CHL:ME 15:8:4	1	0.68	Acetone, Methanol

The isolated compound has a sharp melting point range of 166 to 170 ^{0}C which indicates its purity and it tested positive to ferric chloride reagent suggesting the presence of phenolic nucleus (Silva *et al*., 1998). The ^{1}H-NMR and ^{13}C-NMR spectrum of the isolated compound exhibited chemical shift values typical of flavonoids (Hye *et al*., 2009; Jung *et al*., 2012; Nasir *et al*., 2015). The presence of an AX system (1, 2, 3, 5-tetrasubstituted benzene ring A) was discerned from the protons at δ_H 5.92 (1H, d, *J*= 4.0 Hz, H-8) and δ_H 5.82 (1H, d, *J*=2.6 Hz, H-6) while an ABX system (1, 3, 4-trisubstituted benzene ring B) was depicted via the protons at δ_H 6.82 (1H, d, *J*= 4.0 Hz, H-2$^{'}$), δ_H 6.71 (1H, dd, *J*=1.6, 9.48 Hz, H-6$^{'}$) and δ_H 6.69 (1H, d, *J*= 8.0, H-5$^{'}$). The spin-spin coupling confirmed that carbon C-4 consists of two protons consistent with the flavan skeleton while the presence of an aliphatic ring was clearly discerned from the proton chemical shift values observed at δ_H 4.52 (1H, s, H-2) and δ_H 3.92 (1H, m, H-3) representing an oxymethine and a carbinol proton respectively, typical of a saturated

ring C (Jung *et al*., 2012). Two hydrogen atoms with chemical shift values at δ_H 2.48 (1H, *dd*, 2.0, 8.0 Hz, H-4a) and δ_H 2.83 (1H, dd, *J*= 4.0, 16.0 Hz, H-4b) assignable to C-4 is characteristic of 3-flavan-type flavonoid (Hye *et al*., 2009; Jung *et al*., 2012; Nasir *et al*., 2015).The *ortho* coupled protons with resonances at δ_H 6.82 (1H, d, *J*= 4.0 Hz, H-2'), δ_H 6.71 (1H, dd, *J*=1.6, 9.48 Hz, H-6') and δ_H 6.69 (1H, d, *J*= 8.0, H-5') form the first aromatic ring while the second aromatic ring exhibited a proton spin system comprised of *meta*-coupled protons with resonances observed at δ_H 5.82 (1H, d, *J* = 2.6 Hz) and δ_H 5.92 (1H, d, *J* = 3.5 Hz) which were assigned to H-6 and H-8 protons placed on carbons C-6 (δ_C 94.83) and C-8 (δ_C 94.45) respectively based on COSY correlation. The chemical shift value for H-2 (δ_H 4.52) which appeared as a broad singlet is an indication that the compound is an epicatechin rather than catechin. The ^{13}C-NMR indicated the presence of 15 carbon atoms. The compound exhibited seven aromatic methine carbon peaks at *δc* 98.19 (C-6), 94.45 (C-8), 99.85 (C-10), 128.9 (C-1'), 118.74 (C-2'), 114.65 (C-5') and 128.32 (C-6'), five quaternary oxygenated carbon atoms at δ_C156.94 (C-5), 152.55 (C-7), 157.24 (C-9), 145.19 (C-4') and 144.71 (C-3') and the three aliphatic carbons at *δc* 82.69 (C-2), δ_C 67.28 (C-3) and the methylene carbon at δ_C 29.05 (C-4). The basic structure of this compound was therefore deduced as 3, 5, 7, 3', 4'–pentahydroxyflavanol, commonly known as (-) – epicatechin. The presence of a methylene carbon at *δc* 29.05 (C-4) (Antonelli *et al*., 2007) and the absence of a downfield signal at around *δc* 82.69 (C-2) confirms it further to be an epicatechin rather than catechin (De mello *et al*., 1996; Petereit, 2002). However, the results of the 2D-NMR (HSQC, ^{1}H-^{1}H COSY and HMBC) spectroscopy of the compound were used to assign the carbon atoms to their respective protons and established the connectivity between the various protons and carbons linkages within the molecule; The HSQC experiment in particular, established the attachment of various protons to their respective carbons while the ^{1}H-^{1}H COSY established the correlations between the protons at δ_H 3.9 (H-3) δ_H 2.47 (H-4a), δ_H 2.83 (H-4b) and δ_H 2.47 (H-4a) and δ_H 2.83 (H-4b) which confirmed the assignment of ring C while the cross peaks correlations observed between δ_H 6.7 (H-2') and δ_H 7.41 (H-6') further substantiate the assignment of ring B. The correct assignment of the protons, carbons and their linkages in the molecule was confirmed through the cross peaks detected on the HMBC spectroscopy as summarized on Table (3) which fully establishes the structure of the compound as an epicatechin. Furthermore, the HMBC spectrum showed correlations of H-5' proton with carbons C-1', C-3' and C-4'. Thus, the ABD proton spin system was placed on ring B. Ring C on the other hand displayed a saturated system with two germinal protons resonating at δ_H 2.83 (1H 4_a, dd, *J* = 4.0, 16.0 Hz) and δ_H 2.48 (1H 4_b, dd, *J* = 4.5, 8.0 Hz) and they were placed at C-4 (δ_C 26.70). The large coupling constant confirms the stereochemical configuration of it to be Trans at C-2 and C-3 Figure (1). Comparison with a reference NMR data (Orisakeye and Olugbade, 2014; yusuf *et al*., 2019) (Table 4) showed a good match for the flavanoidal compound; (2R, 3 R)-3,4-dihydro-2-(3,4-dihydroxyphenyl)-2H-chromene-3,5,7 triol (Epicatechin)

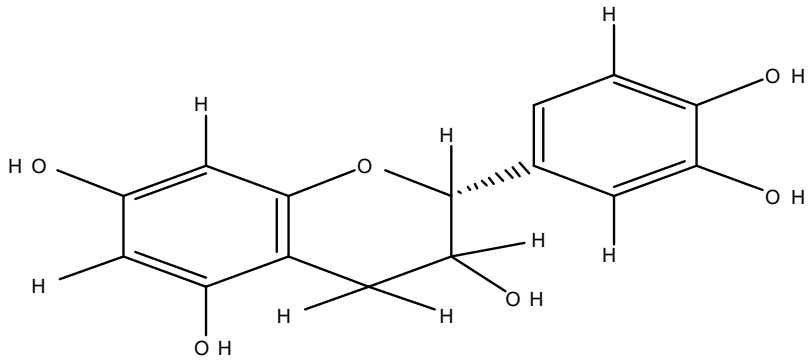

Figure1: Structure of epicatechin

Table 3:1D and 2D NMR spectral data summary for the isolated compound

Position	***δ*** ^{13}***C***	***δ*** ^{1}***H***	***J* Values**	**DEPT**	**COSY**	**HMBC**
2	81.36	4.52	1H (*brs*)	-CH	3.9 ≠ 4.52	C1', C5', C6'
3	67.28	3.92	1H m	-CH	(H-4a, H-	C10',
4	26.70	2.83	1H *dd* 4.0, 16.0	-CH_2	4b)	C9, C10, C3, C2
		2.48	1H *dd* 4.0, 8.9		2.83 ≠ 2.48 H-4a	
5	155.92			-C	H-4b	
6	94.83	5.82	1H *d* 2.6	-CH		C5, C6, C10
7	156.54			-C	5.82 ≠ 5.92	
8	94.45	5.92	1H *d* 3.2	-CH		C7, C8, C9
9	156.93			-C		
10	99.15			-CH		
1^1	130.68			-C		
2^1	113.63	6.82	1H *d* 3.5	-CH		C4', C6', C2
3^1	145.81			-C	6.70 ≠ 6.45	
4^1	144.78			-C	6.71 ≠ 6.82	
5^1	114.64	6.69	1H *d* 8.0	-C		C3', C5', C2
6^1	118.32	6.71	1H *dd* 1.6, 9.48, 16.8	-CH		C1', C2', C3'

Table 4: Comparison of ^{1}H and ^{13}C-NMR data of isolated compound with reported literature

Position	δ_H (*J* in Hz) Orisakeye and Olugbade	δ ^{13}C	δ_H (*J* in Hz)	δ ^{13}C
2	4.87(*brs*)	78.80	4.52 1H (*brs*)	81.36
3	4.20(*m*) 2.72 (*dd*, 3.3, 16.7)	66.33	3.92 1H (*m*)	67.28
4	2.85 (*dd*, 4.5, 16.7)	28.32	2.831H *dd* 4.0, 16	26.70
			2.48 1H *dd* 4.0, 8.9	
	6.02(*d*, 2.2)			
5	5.91(*d*, 2.2)	156.9		155.92
6		95.10	5.82 1H *d* 2.6	94.83
7		156.58		156.54
8	7.05(*d*, 1.7)	95.01	5.92 1H *d* 3.2	94.45
9		156.99		156.93
10	6.78(*d*, 8.1)	99.10		99.15
1^{1}	6.84(*dd*, 1.7, 8.1)	131.6		130.68
2^{1}		114.6	6.82 1H *d* 3.5	113.63
3^{1}		144.89		145.81
4^{1}		144.61		144.78
5^{1}		114.81	6.69 1H *d* 8.0	114.64
6^{1}		118.76	6.71 1H *dd* 1.6, 9.48	118.32

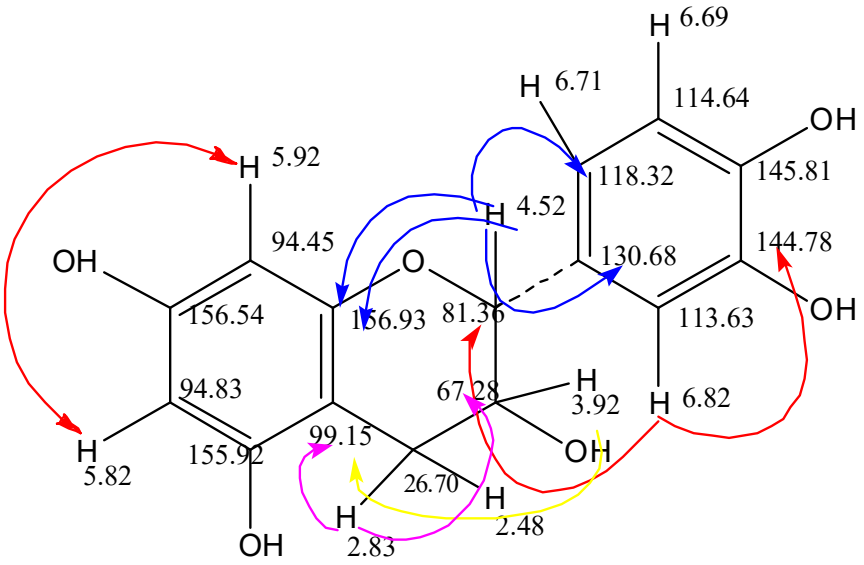

Figure 2: Some Major HMBC Correlations of Epicatechin

Antibacterial Evaluation

Antibacterial zone of inhibition

The antibacterial zone of inhibition expressed as mean and standard deviation was measured using a meter rule in millimeter (mm) and obtained in triplicate. The test organisms, the crude methanol extract and the various fractions were coded to ease their expression in the tables. Highest zone of inhibition against the test organisms was exhibited by ethylacetate (EAF) and n-butanol (NBF) fractions at a concentration of 50 mg/mL and 100 mg/mL; as demonstrated in table 5 and 6 respectively .

Table 5: Antibacterial zone of inhibition of crude methanol extract and the fractions (n-Hexane, Chloroform, Ethyl acetate and n-Butanol) at 100 mg/mL

	Mean Zone of Inhibition (mm)						
Test organisms	**CME**	**HEF**	**CHF**	**EAF**	**NBF**	**CPT (50/5 µg)**	**DMSO (10 %v/v)**
SA	16.33±0.58	15.33±0.58	16.00±0.00	22.33±0.58	20.33±0.58	32.00	00.00 ± 0.00
BS	12.33±0.58	16.33±0.58	14.33±0.58	25.33±0.58	23.33±0.58	32.00	00.00 ± 0.00
PA	12.33±0.58	12.33±0.58	17.33±0.58	24.33±0.58	20.33±0.58	32.00	00.00 ± 0.00
EC	13.33±0.58	12.33±0.58	12.33±0.58	20.33±0.58	20.33±0.58	32.00	00.00 ± 0.00
MRSA	8.60±0.50	0.00±0.00	0.00±0.00	22.33±0.58	24.00±0.58	32.00	00.00 ± 0.00

MIC/MBC of crude methanol extract and the fractions (n-Hexane, Chloroform, Ethyl acetate and n-Butanol)

Ethylacetate (EAF) fraction recorded the highest activity with lowest minimum inhibitory concentration and minimum bactericidal concentrations (MIC/MBC) while crude methanol extract (CME) showed activity with minimum bactericidal concentrations (MBC) as demonstrated in table 7

Table 6: Antibacterial zone of inhibition of crude methanol extract and the fractions (n-Hexane, Chloroform, Ethyl acetate and n-Butanol) at 50 mg/mL

	Diameter zones of inhibition (Mean ± SD) (mm) 50 mg/mL						
Bacteria Isolates	**CME**	**HEF**	**CHF**	**EAF**	**NBF**	**CPT (50/5 µg)**	**DMSO (10 %v/v)**
SA	14.33 ± 0.58	10.33 ± 0.58	12.00 ± 0.00	21.33 ± 0.58	18.33 ± 0.58	32.00	00.00 ± 0.00
BS	11.33 ± 0.58	10.33 ± 0.58	12.33 ± 0.58	20.33 ± 0.58	20.33 ± 0.58	32.00	00.00 ± 0.00
PA	11.33 ± 0.58	8.33 ± 0.58	14.33 ± 0.58	22.33 ± 0.58	17.33 ± 0.58	32.00	00.00 ± 0.00
EC	11.33 ± 0.58	7.33 ± 0.58	10.33 ± 0.58	17.33 ± 0.58	16.33 ± 0.58	32.00	00.00 ± 0.00
MRSA	6.03 ± 0.50	0.00±0.00	0.00±0.00	18.33 ± 0.58	18.59 ± 0.58	32.00	00.00 ± 0.00

Data are expressed as Mean± S.D. Key: **SA**= *Staphylococcus aureus*; **PA**= *Pseudomonas aeruginosa*; **EC**= *Escherichia coli*; **BS**= *Bacillus subtilis*; **CPT**: Ciprofloxacin/Tinidazole; **DMSO** = Dimethyl sulphoxide **MRSA**= Methicillin resistant *Staphylococcus aureus;* **MIC**: Minimum Inhibitory Concentration; **MBC**: Minimum Bactericidal Concentration; **CHF**= Chloroform, **CME** = Crude methanol Extract, **EAF** = Ethyl acetate Fraction, **NBF** = n butanol Fraction, **HEF**= Hexane fraction

Table 7: MIC/MBC of Crude Methanol Extract and the Fractions (n-Hexane, Chloroform, Ethyl acetate and n-Butanol)

	Concentration (mg/mL) MIC/MBC				
Bacteria Isolates	**CME**	**HEF**	**CHF**	**EAF**	**NBF**
SA	125.00/250.00	125.00/125.00	62.50/62.50	31.25/31.25	62.50/125.00
BS	125.00/250.00	125.00/125.00	31.25/62.50	31.25/31.25	62.50/125.00
PA	125.00/250.00	125.00/125.00	31.25/62.50	31.25/31.25	62.50/125.00
EC	125.00/250.00	125.00/125.00	31.25/62.50	31.25/31.25	62.50/125.00
MRSA	125.00/250.00	250.00/250.00	125.00/250.00	31.25/31.25	125.00/250.00

Key: **SA**= *Staphylococcus aureus*; **PA**= *Pseudomonas aeruginosa*; **EC**= *Escherichia coli*; **BS**= *Bacillus subtilis*; **TCF**= Tinidazole/Ciprofloxacin; **DMSO**= Dimethyl sulphoxide **MRSA**= Methicillin resistant *Staphylococcus aureus;* **MIC**: Minimum Inhibitory Concentration; **MBC**: Minimum Bactericidal Concentration; **CHF**= Chloroform, **CME** = Crude methanol Extract, **EAF** = Ethyl acetate Fraction, **NBF** = n butanol Fraction, **HEF**= Hexane fraction

CONCLUSION

The plant in general has shown possession of weak antibacterial activity and thus it is a weak antibacterial agent. The isolated and characterized compound of epicatechin from the ethylacetate fraction of aerial part of *Indigofera welwitschii* is to the best of my literature search is the first report of isolation of this compound from the plant.

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