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PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF FRACTIONS OF *SIDA ACUTA* AGAINST SOME REFERENCE ISOLATES OF BACTERIA

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ABSTRACT
The use of medicinal plants could be an excellent source of drugs to overcome the problem of antibiotic resistance. This study was undertaken to determine the phytochemical analysis and antibacterial activities of Ethanol leaf extract fractions of *Sida acuta* against *Escherichia coli* (ATCC 43888), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (*ATCC* 9027) and *Bacillus subtilis* (ATCC 6633). The phytochemical constituents of the fractions of the crude extract were determined using standard methods. Furthermore, the antibacterial activity of the leaf fractions against the reference isolates of bacteria were determined using agar well diffusion and broth dilution methods, at varied concentrations of the extract and using ciprofloxacin antibiotic as control. Preliminary phytochemical screening showed the presence of alkaloids, saponins, glycosides, tannins, flavonoids, cardiac glycosides, terpenes and phenols. The N-butanol and aqueous fractions of the leaf of *S. acuta* demonstrated significant antibacterial activities at ($P \leq 0.05$) against the isolates of bacteria, while chloroform and ethyl acetate do not. The n-butanol fraction however, exhibited the highest activity against all bacterial isolates with MICs and MBCs of 37.5 mg/mL and 75.0 mg/mL respectively. Therefore, the observed antibacterial activities revealed that *Sida acuta* could be used for the treatment of infections caused by the test bacterial isolates.

Keywords: Phytochemical Screening, Antibacterial activity, *Sida acuta,* Fractions.

INTRODUCTION
Medicinal plants are distributed worldwide, but they are most abundant in the tropical countries (Calixto, 2020). It is estimated that plant materials have provided the models for 50 % chemotherapeutic drugs (Murray, 2021). Medicinal plants have been used by mankind for their nutritional and therapeutic values since the beginning of human civilization. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of these agents in traditional medicine (Lamidi *et al*., 2019).

Many pharmacological industries have produced a number of new antibiotics in the last three decades, but resistance to these drugs by microorganisms is constantly increasing. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized frequently as therapeutic agents (El- Said *et al*., 2018).Therefore, immediate action is required to combat the problem, by encouraging researchers to develop new drugs; more so of herbal origin as synthetic drugs are known to cause side effects (Iwu, 2019).

Sida acuta commonly known as “Stubborn grass’’, “Isekotu’’ in Yoruba, “Udo’’ in Igbo and “Kalkashin kwado’’ in Hausa is one of those plants currently used by indigenous people for the management of some health problems (Murray, 2021). It belongs to the family *malvaceae*, species *Sida acuta*. It is found on most soil types, except seasonally flooded days or soils derived from limestone. All parts of this tree, including leaves, barks, roots and flowers are used for medicinal purposes (Onajobi, 2018). Medicinal plants such as *Sida acuta* has been asserted to provide various culinary and medicinal properties. These medicinal properties exert bacteriostatic and bactericidal effects on some bacteria. These effects have been attributed to compounds like peptides, alkaloids, essential oil and flavonoids which are some of the major components in these plants (Okigbo and Igwe, 2017).
The plant is used for various medicinal purposes. In Nigeria, whole plant and leaves of *Sida acuta* is used for the treatment of malaria, ulcer, fever, gonorrhea, abortion, breast cancer, poisoning, inflammation, feed for livestock, stops bleeding, treatment of sores, wounds among others (Kayode *et al*., 2019).

MATERIALS AND METHODS

Collection, Authentication and Preparation of Plant Material
Fresh leaves, of *Sida acuta* plant were collected within kawo Kaduna North Local Government area of Kaduna State. The plant was identified and authenticated by a taxonomist at the Department of Biological Science, Kaduna State University and assigned voucher number (2214) which was deposited at the herbarium section of the Department. The leaves of *Sida acuta* were washed thoroughly under running water and dried under room temperature at 28 ˚C for 14 days. It was grounded into coarse powder using mortar and pestle and stored in separate air tight bottles.

Extraction of Plant Material
Plant chemicals were extracted by cold maceration method as described by Ogbeba *et al*. (2017). About three hundred (300g) of leaves, of *Sida acuta* were suspended into 2000mL of 70 % Ethanol in conical flask. The mixture was stirred vigorously with a sterile glass rod, and kept in tightly sealed vessels at room temperature. The mixture was left for 72 h with constant shaking. The mixture was filtered off with sterile filter paper (Whatman no 1 filter paper) into a clean conical flask. Filtrate was transferred into the sample holder of the rotary evaporator at a room temperature of 28 ˚C. The semi- solid extract produced was kept under a ceiling fan to dry.

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The standard extract obtained was weighed and stored in a refrigerator at 4 ˚C until required for use. The percentage yield of the ethanol crude extracts of leaves of *Sida acuta* was calculated using the formular Percentage Yield of Plant Extract (%) $= \frac{W_2 - W_1}{W_0} \times 100$

Where W_1 = Weight of the container in grams
W_2 = Weight of container + Extract
W_0 = Weight of powdered leaf, root or bark

Fractionation of Ethanol Leaf Extract of *Sida acuta*

The ethanol leaf extract was fractionated using different grades of organic solvents of increasing polarities (Chloroform, Ethyl acetate and N- butanol) in order to separate the active components present. The test was carried out using partitioning method in accordance with the method described by Akindele *et al*. (2019) with little modification.

A quantity of leaf extract (30 g) was suspended in 200 mL of distilled water then poured in separating funnel followed by addition of 400 mL chloroform solution. Layer between two different solvents appeared in separation funnel which was sealed (Tene *et al*., 2016). Funnel was shaken vigorously for 25 min and pressure was released at regular intervals. After 25 min two layers were separated, containing fractions of chloroform and aqueous in the funnel. The chloroform fraction was separated from the aqueous and it was collected in a sterile beaker. 400 mL of ethyl acetate was also poured in the funnel, two different solvents appeared and were separated. The ethyl acetate fraction was collected in a beaker and lastly, 400 mL of n – butanol solution was poured in the funnel, two layers were separated containing n- butanol and aqueous fraction. Each fraction was collected in a separate container. Procedure was repeated twice and the obtained fractions were placed in rotary evaporator at 100 rpm. The soluble fractions were weighed, labeled and stored in refrigerator at 4 ˚C until required for use.

Phytochemical Screening of the Soluble Fractions of *Sida acuta*

The phytochemical screening was carried out according to the method described by Ogbeba *et al*. (2017).All soluble fractions of *Sida acuta* were screened for the presence of Alkaloids, Flavonoids, Saponins, Phenol, Tannins, Steroids, Anthroquinone, Terpenoids, Phlobatannins and Cardiac glycosides.

Test for Alkaloids (Mayers test):

Alkaloid solution produced white yellowish precipitate when few drops of Mayer's reagents were added to 0.5 g of the extract.

Test for Flavonoids (Sodium hydroxide test):

About 0.5 g of the extract were dissolved in 2 mL of 10% aqueous NaOH solution and filtered, a change in color from yellow to colourless on addition of dilute hydrochloric acid indicates the presence or absence of flavonoids.

Test for Saponins (Frothing test):

About 0.5 g of the ethanol extract was mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. Positive result is indicated by presence or absence of honey comb appearance.

Test for Phenols (Ferric chloride test):

One ml of extract was added to few drops of neutral 5 % ferric chloride solution in test tubes. A dark green color indicates the presence or absence of phenolic compounds.

Test for Tannins (Ferric Chloride test):

One ml of water and 1-2 drops of 0.1 % ferric chloride solution was added to 1 mL of ethanol extract of *S. acuta* and the blue color observed indicates the presence or absence of gallic tannins and the green black color indicates the presence or absence of catecholic tannins.

Test for Steroids (Sulphuric acid test):

Five drops of concentrated H_2SO_4 were added to 0.5 g of the extract. The colour change from violet to green or blue confirms the presence or absence of steroids in sample.

Test for Terpenoids (Salkowski test):

About 0.5 g of the extract was diluted with 5 mL of distilled water, it was mixed in 2 mL of chloroform, and 3 ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration at the interface showed positive results for the presence or absence of terpenoids.

Test for Cardiac Glycosides (Keller-Killani test):

About 0.5 g of the extract was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This mixture was under layed with 1 mL of concentrated sulphuric acid. A brown ring at the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout the thin layer.

Test for Glycosides (Ferric Chloride test):

About 0.5 g of the extract was diluted with 5 mL of distilled water in a test tube, three drops of ferric chloride solution were added, presence or absence of green to black precipitate indicates the hydrolysis of glycoside.

Test for Phlobatannins: (hydrochloric acid test):

when 1 mL of extract was boiled with 1 mL of 1 % aqueous hydrochloric acid, deposition of a red precipitate indicates a positive or negative result.

Collection of Test Bacteria

The Reference isolates of bacteria used in this study include *Staphylococcus aureus* (ATCC 6538)*, Escherichia coli* (ATCC 43888)*, Bacillus subtilis* (ATCC 6633) and *Pseudomonas aeruginosa* (ATCC 9027)*.* The isolates were collected from National Vetenary Research Institute (NVRI), VOM, Jos, Plateau State. All isolates were collected in nutrient agar slants, labeled, placed in a cold box and transported to the post graduate laboratory of the department of microbiology Kaduna State University. Isolates were incubated at 37 °C for 24 h.

Reconfirmation of Bacteria

The Reference isolates were reconfirmed using standard bacteriological methods and conventional Biochemical tests. An inoculum from an overnight growth culture of the test bacteria were streaked on freshly prepared plates using appropriate media for

each bacterium. The isolates were sub cultured on Mannitol Salt Agar (MSA) for *S. aureus*, Nutrient Agar (NA) for *P. aeruginosa, centrimide* agar for *B. subtilis* and Eosin-Methylene Blue Agar (EMB) for *E. coli.* The media for each isolate was prepared according to the manufacturer's instruction. Colonies showing different pigmentations were Gram stained and sub-cultured on each appropriate media. Pure cultures were further analyzed for indole, catalase, coagulase, methyl-red, Voges Proskauer, motility, oxidase and citrate utilization. All the confirmed isolates were kept on agar slants and stored in a refrigerator at 4 ℃ until required (Cheesbrough, 2018).

Preparation of Extract and Antibiotic Concentrations

Three grams (3 g) each of the chloroform, Aqueous, N- butanol and Ethyl acetate leaf fractions of *Sida acuta* were weighed and 10mL each of 10 % dimethyl sulfoxide (DMSO) were added to obtain 300 mg/mL stock solutions of each extract. Using two- fold serial dilution, concentrations of 150 mg/mL, 75 mg/mL and 37.5 mg/mL were prepared from each stock solution. Similarly, one gram (1g) of Ciprofloxacin was weighed and dissolved in 10mL of 10 % DMSO to obtain 100 mg/ml of the antibiotic concentration .The different concentrations, were labeled and kept in bijou bottles for subsequent use (Shrivastava *et al*., 2018)**.**

Standardization of Bacterial inoculums

The turbid solution (McFarland Scale No. 1) was used as a reference to adjust the turbidity of the bacterial suspension. Some quantity of test bacteria from overnight growth culture was added to 2mL of sterile physiological saline as suspension medium. The bacterial suspension was compared to 0.5 McFarland standards (1.5×10^8 CFU mL) under a white background with contrasting black lines (Cheesbrough, 2018).

Antibacterial Activities of Fractions of *Sida acuta* Leaf Extract Against Reference Isolates of Bacteria

The antibacterial activity of each soluble fraction (aqueous (AQ), Chloroform(CL), n-butanol (BL), and ethylacetate (EA)) against the reference isolates of *E. coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* were evaluated using agar well diffusion method of sensitivity as described by Bauer *et al*.(2020). About 100μl of standardized inoculum of each bacteria was inoculated on Mueller Hinton agar plates (in triplicates) and spread evenly with sterile swab stick. Wells of 9 mm diameter size was cut with a sterile cork borer and each was filled with 100μl of crude extract fractions at varied concentrations of 300, 150, 75 and 37.5 mg/mL respectively. The plates were left at room temperature for 10 min and then incubated for 24 h at 37 ˚C. The diameter of zone of inhibition was measured. A solution constituting the diluent, DMSO was used as a negative control. The activities of the leaf extract fractions were compared with a standard antibiotic, ciprofloxacin.

Determination of Minimum Inhibitory Concentrations (MIC) of Soluble Fractions of Ethanol Leaf Extract of *Sida acuta*

The MICs of crude extract against the reference isolates of *E. coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* were determined using broth dilution method. The following concentrations of extracts: 300 mg/mL, 150 mg/mL, 75 mg/mL and 37.5 mg/mL were prepared by ten –fold serial dilutions.

One ml of extract concentration was added to a test tube containing 9 mL of Mueller Hinton broth. About 100 µl each of a standardized inoculum of the test bacterium was added to mixtures of different concentrations of extracts with Mueller Hinton broth. The test tubes were incubated at 37 ˚C for 24 h. The growth of bacteria in the broth examined were indicated by the turbidity of the broth. However, the lowest concentration of extract which inhibited the growth of a test bacteria was recorded as the Minimum Inhibitory Concentration (MIC) (Andrew, 2019). Negative controls were set up as follows Mueller Hinton broth only and Mueller Hinton broth with extract. While positive control comprised of Mueller broth with typed strain and Mueller Hinton broth with antibiotics.

Determination of Minimum Bactericidal Concentrations (MBC) of Soluble Fractions of Ethanol Leaf Extract of *Sida acuta*

The positive MIC tubes were sub-cultured on nutrient agar plates with proper labels followed by incubation at 37 °C for 24 h. They were examined for growth of bacteria. The tube with minimum concentration of extract in which the growth was completely stopped was noted as the minimum bactericidal concentration. The negative controls were nutrient agar only and nutrient agar with extracts only (Andrew, 2019).

RESULTS

Percentage Weights of Fractions of Ethanol leaf extract of *S. acuta*

The percentage weights of fractions of ethanol leaf extract of *S. acuta* are presented in Table 1. The fraction with the highest percentage weight was n-butanol (28.92 %), followed by Aqueous (20.38 %), chloroform (20.09 %) and then the ethyl acetate fraction (4.15 %).

Phytochemical constituents of Fractions of Ethanol leaf extract of *S. acuta*

The results of phytochemical screening of fractions of ethanol leaf extract of *S. acuta* as presented in Table 2, indicated that the phytocompounds screened for which include Alkaloids, flavonoids, phenols, tannins, steroids, anthroquinone, terpenoids, phlobatannins and cardiac glycosides were present in all the fractions with the exception of saponins in n- butanol, alkaloids and terpenoids were absent in aqueous, while steroids were absent in ethyl acetate fractions. However, chloroform had fewer compounds.

Antibacterial Activities of *S. acuta* Leaf Fractions and Ciprofloxacin Against Isolates of *E.coli, S.aureus*, *B. subtilis* and *P. aeruginosa*

The antibacterial activities of *Sida acuta* leaf fractions and ciprofloxacin again st *E .coli* ATCC (43888), *P. aeruginosa* ATCC (9027), *S. aureus* ATCC (6538) and *B. subtilis* reference isolates were presented in Table 3. Two of the four partially purified fractions (aqueous and n-butanol) exhibited appreciable antibacterial activities against the test bacterial isolates at concentrations of 300, 150, 75 and 37.5 mg/mL of extract fractions. The n-butanol fraction exhibited higher activity against all the isolates with mean zones of inhibition ranging from 10.25 ± 0.35 to 23.50 ± 0.00 mg/mL. Followed by the aqueous fraction with mean zones of inhibition ranging from 9.75 ± 0.35 to 20.75 ± 0.35 mg/mL. However, chloroform and ethyl acetate fractions showed no activity against all the bacterial isolates.

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Determination of Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of Fractions of *S. acuta* Leaf Extract on Test Bacterial Isolates.

Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of the active fractions of *S. acuta* leaf against the test bacterial isolates were determined. The MICs and MBCs of aqueous (AQ) and n-butanol (BL) fractions of *S. acuta* leaf extract on reference isolates of *E. coli, S. aureus*, *P. aeruginosa* and *B. subtilis* were presented in Table 4. The BL demonstrated the highest activity with the lowest MIC and MBC of 37.50 and 75.00 mg/mL against all the reference isolates. This was followed by Aqueous (AQ) fraction with MIC and MBC range of 37.5 to 75.00 and 75 to 150 mg/mL respectively. Therefore, BL fraction could be preferred solvent for extraction of compounds from the leaf of *S. acuta* plant.

Table 1: Percentage weights of Soluble Fractions of *Sida acuta* Leaf Ethanol per 30g of Extract

Leaf fractions	Weight(g)	Percentage weight (%)
Aqueous	5.707	20.38
Chloroform	5.626	20.09
Ethyl acetate	4.150	14.82
N-butanol	8.097	28.92

Table 2: Phytochemical Screening of *S.acuta* Leaf Ethanol Fractions

		S. acuta Leaf fractions			
Constituents	**Method**	**N-butanol**	**Chloroform**	**Aqueous**	**Ethyl acetate**
Alkaloids	Mayers	+	-	+	-
Flavonoids	NaoH	+	-	+	-
Saponins	Frothing test	-	-	+	+
Terpenoids	Ferric chloride	+	-	-	+
Tannins	Ferric chloride	+	+	+	+
Steroids	Sulphunic acid	+	+	+	-
Terpenes	Ferric chloride	+	-	+	+
Cardiac glycosides	Keller kellani	+	+	+	+
Glycosides	Ferric chloride	+	+	+	+
Phlobatannis	HCL	+	+	+	+

KEY + = Detected - = Not detected

Table 3: Antibacterial Activities of Ethanol Leaf Fractions of *Sida acuta* and Ciprofloxacin against Reference Isolates of Bacteria at Different Concentrations

Fractions	Conc. mg/Ml	MIZ±SD			
		E. coli	*P. aeruginosa*	*S. aureus*	*B. subtilis*
Aqueous	300	20.75±0.35^{a}	19.50±0.00^{ab}	16.50±1.41^{b}	19.75±3.89^{b}
	150	20.50±1.41^{a}	12.50±0.00^{b}	14.25±1.06^{b}	14.75±2.47^{c}
	75	10.75±0.35^{c}	10.75±0.35^{c}	12.25±0.35^{c}	11.25±0.35^{cd}
	37.5	9.75±0.35^{c}	0.00±0.00^{d}	11.75±0.35^{c}	0.00±0.00^{d}
N-butanol	300	21.50±0.71^{a}	20.75±0.35^{a}	23.50±0.00^{a}	18.50±3.54^{b}
	150	14.50±1.41^{b}	15.75±0.35^{b}	20.75±0.35^{a}	16.75±1.06^{b}
	75	11.75±1.06^{c}	13.00±0.71^{b}	15.50±0.00^{b}	14.50±4.95^{c}
	37.5	10.25±0.35^{c}	10.75±0.06^{c}	12.75±1.06^{c}	12.00±0.71^{cd}
Ethyl acetate	300	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	150	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	75	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	37.5	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
Chloroform	300	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	150	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	75	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	37.5	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
Ciprofloxacin	100	26.00±0.00^{a}	25.00±0.00^{a}	27.00±0.00^{a}	35.00±0.00^{a}

KEY: Means with same letter(s) in a column are not significantly different according to Duncan Multiple Range Test (DMRT) at P ≤ 0.05 level of significant. Values are means ± standard deviations, MZI = Mean diameter Zone of Inhibition

Table 4: Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of Active Fractions of *S. acuta* Leaf Extract on Reference Isolates of Bacteria

BACTERIA	MICs and MBCs of Active Fractions (mg/ml)			
	Aqueous		n- butanol	
	MIC	MBC	MIC	MBC
E. coli (RI)	75.00	150.0	37.50	75.00
P.aeruginosa (RI)	75.00	150.0	37.50	75.00
S. aureus (RI)	37.50	75.00	37.50	75.00
B. subtilis (RI)	75.00	150.0	37.50	75.00

KEY: RI= Reference Isolate

DISCUSSION

Preliminary screening of compounds present in ethanol leaf fractions revealed the presence of many secondary metabolites. Phytocompounds are known to be biologically active and can aid the antibacterial activities of *S. acuta* through different mechanisms. Alkaloids for example, being one of the compounds present in *S. acuta* is one of the largest groups of phytochemicals in plants, with amazing effects in humans, leading to the development of a powerful pain killer medication (Musa *et al.,* 2020).One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms which have been widely studied for their potential use in the elimination and reduction of human cancer cells (Akinpelu, 2019). It is evidence from the current research that phenols, tannins, terpenoids,

flavonoids and cardiac glycosides are active against a wide range of antimicrobial activity (Musa *et al*., 2016). Similar compounds were observed by (Akinlandeswari *et al*., 2015 and Musa *et al*., 2016).Therefore, these constituents present could be attributed to the reason why *S. acuta* is used for treatment of many bacterial infections.

The lack of activities demostrated by the ethylacetate and chloroform fraction, could be due to the polarity of the solvent. The probable active compounds such as Alkaloids and Flavonoids present in N- butanol and Aqueous fractions which are polar in nature, could lead to improved antibacterial activities compared to the ethyl acetate and chloroform fractions, that are non-polar. The fractionation process might have concentrated the active components in the n-butanol and aqueous fractions of the leaf extracts of *S. acuta*. However, the differences in polarities of the different organic solvents as well as the solubilities of compounds in the solvents must have led to improved antibacterial activities. This result is in conformity with the findings of (Akinlandeswari *et al*., 2020) who reported appreciable antibacterial activity of *S. acuta* leaf extract on both gram positive and gram negative bacteria.

In the present study, the susceptibilities of test bacteria to the active fractions of *S. acuta* leaf were also found to be concentration dependent meaning the higher the concentration the higher the activity which were similar to the previous findings of Fine boy *et al*., 2019 .Certain compounds present in the extract fractions could be responsible for the observed antibacterial activities. The differences in the MICs and MBCs of fractions on the test bacterial isolates could be due to selective antibacterial activities of these fractions and also due to strain differences or perhaps due to genetic contents of bacterial isolates used in the study. However, Musa *et al*., (2021) reported lower MIC and MBC (12.5, 25 and 6.25, 12.5) for *E. coli* and *S. aureus* respectively on *Mitracarpus scaber*. The effectiveness of N- butanol of the leaf extract against all the isolates of bacteria tested compared to other fractions further shows that the active components present in the crude leaf extracts could be more enhanced in n-butanol fraction due to its high polarity and the compound solubility in n-butanol solvent. Similar research was conducted by (Musa *et al*., 2016) who reported n-butanol fraction of *T. avicennioides* having the highest activity with lower MIC of 6.25mg/ml and MBC of 12.5 mg/ml against all the test bacterial isolates of *E.coli* and *S.typhymurium*

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