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Full Length Research Article

EVALUATION OF PHYTOCHEMICAL, IN VITRO ANTIBACTERIAL AND RATE OF KILL ASSAY OF TERMINALIA AVICENNIOIDES LEAF AGAINST SOME BACTEREIA ASSOCIATED WITH DIARRHOEA

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ABSTRACT
The study was aimed to evaluate the phytochemical constituents, *in vitro* antibacterial and rate of kill assay of *Terminalia avicennioides* leaf extract against some bacteria associated with diarrhoea. The phytochemical constituents of the ethanol of *Terminalia avicennioides* leaf, aqueous, n- butanol, and ethyl acetate fractions of the leaf extract were determined using standard analytical methods. The antibacterial activities of the leaf extract and extract fractions against clinical isolates of *Salmonella* Typhimurium and *Escherichia coli* isolated from diarrhoeaic patients were determined *in vitro* by agar diffusion, dilution and time - kill methods. The result of phytochemical screening revealed the presence of carbohydrates, cardiac glycosides, saponins, flavonoides, tannins, alkaloids, phenols and triterpenes. The crude extract and extract fractions of *T. avicennioides* leaf were effective against the test bacterial isolates at varied concentration of extracts but the n-butanol fraction was more effective with Minimum inhibitory and Minimum Bactericidal Concentration of 6.25 and 12.5mg/ml. The clinical isolates of *E. coli* and *S.* Typhimurium were completely killed within 180 minutes of exposure to ethanol leaf extract and extract fractions at varied MBCs of 12.5 mg/ml and 25.0 mg/ml. The clinical isolates of *E. coli* and *S.* Typhimurium were more susceptible to n-butanol fraction within 120 minutes of exposure to the extract fraction.

Keywords: Phytochemical constituents, *in vitro* antibacterial activity, rate of kill assay, *Terminalia avicennioides* leaf

INTRODUCTION
Enteric bacteria are important pathogenic group because of their involvement in a number of diarrhoeal diseases that account for a significant number of deaths among infants and adults living in developing countries. Some strains of *Salmonella* and *Escherichia coli* are emerging as significant agents of diarrhoea worldwide and have also become endemic in many parts of developing countries including Nigeria (Ali *et al*., 2023). Traveler's diarrhoea is common in those who travel to developing countries and results from exposure to bacterial pathogens most commonly enterotoxigenic *E. coli*. Non-typhoidal *Salmonella, Campylobacter*, *Yersinia enterocolitica. E. coli* are the most common gastrointestinal pathogens. *Salmonella* related cases are often of great concern due to the organism's high microbial resistance (Musa *et al*., 2016). Exposure to infectious agents is the major risk factor for acute diarrhoea. Bacteria and viruses are often transmitted by the fecal-oral route, so hand washing and hygiene are important to prevent infection (Centres for Disease Control and Prevention, 2020). Medications such as antibiotics and drugs that contain magnesium products are also common offenders. Dietary changes can also lead to acute diarrhoea. Drug resistance in any *Enterobacteriaceae* member can result in extended hospitalization, longer illness duration and a higher risk of an invasive infection (Dairo *et al*., 2017). According to previous studies people who have been hospitalized due to gastrointestinal pathogens, have a higher risk of developing irritable bowel syndrome (Helms *et al*., 2006; Musa *et al*., 2016)
Despite the numerous number of antibiotics used for effective treatment of diarrhoea, there is a need to search for other alternatives due to the growing incidence of drug resistance among bacterial pathogens coupled with the rising costs and low therapeutic index of many synthetic drugs especially in developing countries with weak economic indices (Bulus *et al.,* 2011; Musa *et al*., 2016).

Natural plants with their wide range of phytochemicals have been proved to be effective against bacterial pathogens and are also found to possess numerous health related effects (Mann *et al.,* 2008). They are cheaper than synthetic drugs and easily accessible. Species of plants belonging to the family Combretaceae have been tested for their antimicrobial activities against some pathogenic microorganisms. Numerous African *Terminalia* species have potent antibacterial activity against a broad spectrum of medicinally important species of bacteria (Mbwambo *et al.,* 2011).The West African species *T. avicennioides*, which is widely used in Nigerian traditional medicine, has been reported to have good antimicrobial activity (Mann *et al.,* 2008; Musa *et al*., 2016).

The genus *Terminalia* belongs to the family Combretaceae. It is commonly called "Indian laurel" and locally referred to as "baushe" in Hausa, "Edo" in Igbo, and "Idiogon" in Yoruba. It comprises of approximately 200-250 species of medium to large flowering trees. *Terminalia* species are widely distributed in the savanna region of Africa and in the North -West of Nigeria, with the majority occurring in the Southern part of the continent. The ethanol leaf extract was reported to have some antibacterial effect against diarrhoeal pathogens like *Salmonella* Typhimurium and *Escherichia coli.* Traditionally, ointments of powdered leaves of *T. avicennioides* are used to treat rheumatic pains and other swollen joint conditions. Moreover, few studies have evaluated the anti diarrhoeal effect of *T. avicennioides* in Kaduna State. Such investigation demonstrated the inhibitory effect of *T. avicennioides* against *E. coli* and *S.* Typhimurium (Musa *et al*., 2016). Others include the prevalence and determinants of diarrhoea among infants in selected primary Health Care Centers in Kaduna North Local Government Area,

Nigeria and evaluation of phytochemicals and antibacterial properties of *Terminalia avicenioides* crude extract against selected bacteria from diarrhoeic patients attending some General Hospitals in Kaduna metropolis (Musa *et al*., 2016; Dairo *et al*., 2017). The present study was aimed to evaluate the phytochemical constituents, *in vitro* antibacterial and time kill assay of *Terminalia avicenioides* leaf against some bacteria associated with diarrhoea in Kaduna State

MATERIALS AND METHODS

Study Area

Yusuf Dantsoho Memorial Hospital (YDH) Tudun Wada, Gwamna Awan General Hospital (GAH) Kakuri and Barau Dikko University Teaching Hospital (BDUTH) within Kaduna metropolis

Collection and Preparation of Plant Material

Fresh leaves of *Terminalia avicennioides* plant (Guil and Perr) were collected from Karaukarau village in Zaria Local Government area of Kaduna State, Nigeria in July 2022. The plant was identified and authenticated at the Biological Sciences Department, Kaduna State University, Nigeria and assigned a voucher number, 104. The leaves were thoroughly washed in a running tap, air dried under shade for two weeks and grounded to coarse powder using a mortar and pestle.

Extraction of Plant Material

Eight hundred grams (800g) of powdered leaf was dissolved in one (1) litre of 70% ethanol and left for 72hours with constant shaking. The filtrate collected in a crucible was concentrated in a water bath for two days at 40^0C to obtain dry crude extracts.

Fractionation of Crude Extract of *T. avicennioides* Leaf

Using separation funnel method (Musa *et al*., 2016), 120 g of the crude extract was dissolved gradually in 1.5 liters of sterile distilled water in a separating funnel. Two hundred and fifty milliliters (250 ml) of ethyl acetate was added to 250 ml of the extract solution (1:1v/v) the funnel was then shaken vigorously and allowed to stand for 15 minutes to allow for the separation of organic solvent from the mixture. The ethyl acetate was removed living the debris. The process was repeated twice to ensure complete separation. The same procedure was performed sequentially using n-butanol and ethyl acetate solvents. An aqueous layer (residue) which remained in the funnel was removed as the Residual Aqueous Fraction (RAF). Each fraction was collected in a beaker, labelled and evaporated to dryness at 40 ^{0}C using a rotary evaporator. Each extract fraction was tightly covered in a clean container and kept in a refrigerator maintained at 4 ^{0}C for further use.

Phytochemical Screening of Crude Extracts and Extract Fractions of *Terminalia avicennioides* Leaf

The phytochemical screening of crude extracts and extract Fractions of *Terminalia avicennioides* Leaf was conducted in accordance with the methods of Evans (2009) and Tiwari *et al.* (2011) to ascertain the presence or absence of carbohydrates, cardiac glycosides, saponins, flavonoids, tannins, alkaloids anthraquinones, phenols, steroids, and triterpenes in the leaf extract and extract fractions of *Terminalia avicennioides*

Detection of Carbohydrates

To 5ml of distilled water, 0.2 g of an extract was dissolved and filtered. The filtrate was used to test for the presence of carbohydrates as follows:

Molisch's Test**:** Filtrate was treated with drops of alcoholic a-naphthol solution in a test tube. The formation or non-formation of a violet ring at a junction indicated the presence or absence of carbohydrates.

Benedicts Tests**:** Filtrate was treated with Benedict's reagent and heated gently. Formation or non-formation of orange red precipitate indicated the presence or absence of reducing sugars.

Fehling's Test**:** Filtrate was hydrolyzed with diluted HCl, neutralized with alkali and heated with Fehling's A and B solutions. Formation or non-formation of red precipitate indicated the presence or absence of reducing sugars.

Detection of Cardiac glycosides

Keller-Kiliani Test: Five milliliters (5 ml) of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was mixed with 1ml of concentrated sulphuric acid. Formation or non-formation of brown ring at the interface, characteristic of cardenolides, indicated the presence or absence of a deoxysugar. A violet ring (may or may not appear below the brown ring, while in the acetic acid layer, a greenish ring may or may not form gradually), indicated the presence or absence of cardiac glycosides.

Detection of Saponins

Froth Test: To 1 g of extract 20 ml of distilled water was added and shaken in a graduated cylinder for 15 minutes. Formation or non-formation of 1cm layer of foam indicated the presence or absence of saponins.
Foam Test**:** Zero point five gram (0.5 g) of extract was shaken with 2 ml of water. Persistent production or non-production of foam for 10 minutes indicated the presence or absence of saponins.

Detection of Flavonoids

Alkaline Reagent Test**:** To 2 ml of water 0.2 g of extract was dissolved and treated with few drops of sodium hydroxide solution formation of intense yellow colour, which became colourless on addition of dilute acid or non-formation of yellow colour indicated the presence or absence of flavonoids.
Lead Acetate Test**:** To 2 ml of water 0.2 g of extract was dissolved and treated with 3 drops of lead acetate solution. Formation or non-formation of yellow colour precipitate indicated the presence or absence of flavonoids

Detection of Tannins

To 2 ml of water 0.2 g of extract was dissolved. About 1 ml of 1 % gelatin solution containing sodium chloride was added. Formation or non-formation of a white precipitate indicated the presence or absence of tannins
Ferric Chloride Test**:** To 2 ml of water 0.2 g of extract was dissolved and 1 % ferric chloride solution was added. Formation or non-formation of blue, green or brownish green colour indicated the presence or absence of tannins

Detection of alkaloids

Mayer's Test**:** Filtrates were treated with Mayer's reagent (Potassium mercuric Iodide). Formation or non formation of a

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yellow colour precipitate indicated the presence or absence of alkaloids.

Wagner's Test**:** Filtrates were treated with Wagner's reagent (iodine in potassium iodide). Formation or non-formation of a brown/reddish precipitate indicated the presence or absence of alkaloids.

Dragendoff's Test**:** Filtrates were treated with dragendroff's reagent (solution of potassium bismuth iodide). Formation or non-formation of a red precipitate indicated the presence or absence of alkaloids. Harger's Tests**:** Filtrates were treated with Herger's reagent (Saturated picric acid solution). Formation or non-formation of a yellow colour precipitate indicated the presence or absence of alkaloids.

Detection of Anthraquinones:
Five grams (5 g) of each extract was stirred with 10 ml of aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of benzene. The benzene layer was then separated and 10% ammonia solution was added to half of its volume. Formation or non-formation of a pink red or violet colouration in the ammonia phase (lower layer) indicated the presence or absence of anthraquinone derivatives in the extract.

Detection of Phenols
Ferric Chloride Test: To 2 ml of water 0.2 g of extract was dissolved and treated with 3 drops of ferric chloride solution. Formation or non formation of bluish black colour indicated the presence or absence of phenols.

Detection of Phytosterols and Triterpenes
Salkowski's Test: To 2 ml of water 0.2 g of extract was dissolved and treated with chloroform and filtered. The filtrate was treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance or non appearance of golden yellow colour indicated the presence or absence of triterpenes.
Libermann Burchard's Test: To 2 ml of water 0.2 g of extract was dissolved and treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added. Formation or non-formation of brown ring at the junction indicated the presence or absence of phytosterols.

Collection of Clinical Bacterial Isolates
A total of 154 Clinical isolates of *E. coli* and 31 clinical isolates of *S.* Typhimurium isolated from diarrhoeaic patients were obtained from Yusuf Dantsoho Memorial Hospital (YDH) Tudun Wada, Gwamna Awan General Hospital (GAH) Kakuri and Barau Dikko University Teaching Hospital (BDUTH) Kaduna. Of the 154 isolates of *E. coli*, 67 (43.5 %) were collected from YDH, 45 (29.2 %) from GAH and 42 (27.2 %) from BDUTH. Of the 31 isolates of *S.* Typhimurium collected, 15 (48.4 %) were from YDH, 5 (16 %) from GAH and 11 (35.5 %) from BDUTH respectively. Typed strains of *E .coli* and *S.* Typhimurium (*E .coli* ATCC 25922 and *S.* Typhimurium ATCC 14028) were sourced from the National Veterinary Research Institute (NVRI), Vom, Jos, Plateau state. All isolates were inoculated in nutrient agar slants, labelled, placed in ice-packs and transported to the laboratory of the Department of Microbiology, Kaduna State University. Clinical Isolates were confirmed using standard microbiological methods (Cheasbrought, 2010) and then stored in a refrigerator operating at 4 ^{0}C, till required

Antibacterial Susceptibility Test

Preparation of Extract Concentration
One gram (1g) each of ethanol crude extracts, aqueous, n-butanol and ethyl acetate fractionsof leaf of *T. avicennioides* were weighed and added to 10 ml each of 10 % dimethyl sulfoxide (DMSO) to obtain 100 mg/ml stock solutions of each extract. Using two-fold serial dilution, concentrations of 50 mg/ml, 25 mg/ml and 12.5 mg/ml were prepared from each stock solution. The different concentrations were labeled and kept in bijou bottles for subsequent use (Srinivasan *et al.,* 2009).

Preparation of Turbidity Standard and Standardization of bacterial inoculum.
McFarland standard was prepared by diluting 1 ml of concentrated sulphuric acid with 99 ml of sterile distilled water (1% v/v). Another 1 % (w/v) solution of barium chloride was also prepared by dissolving 0.5 g of dehydrated barium chloride in 50 ml distilled water. Zero point six milliliters (0.6 ml) of barium chloride solution was mixed with 99.4 ml of H_2SO_4 solution to yield 1% v/v barium sulphate suspension. The turbid solution (McFarland Scale No 1) was used as a reference to adjust the turbidity of the bacterial suspension. A colony of cells from an overnight growth culture of a test bacterium was added to 2 ml of sterile physiological saline as suspension medium using a sterile wire loop. The bacterial suspension was compared to 0.5 McFarland standards (1.5 x 10^8 CFU/ ml) under a white background with contrasting black lines (Cheesbrough, 2010).

Antibacterial Activities of Ethanol Crude Extracts and Extract Fractions of *T. avicenniodes* against Clinical Isolates of *E. coli* and *S.* Typhimurium
Agar well diffusion method, described by Srinivasan *et al*. (2007) was used to determine the antibacterial activity of the various test concentration of the extracts of *Terminalia avicennioides* against clinical and reference isolates of *E. coli* and *S.* Typhimurium. Using a micropipette about 100 µl of standardized inoculum of a bacterial suspension was inoculated into Mueller Hinton agar plates (in triplicates) and spread evenly over the entire surface of the plates using a sterile swab stick. The plates were left for 10 minutes before wells were dug in the agar using 8 mm sterile cork borer. One hundred microliter (100 µl) volume of the various concentrations of extracts (100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml) were each filled in to the wells. Additional wells were filled with dimethylsulfuroxide to serve as negative controls. The plates were left for 10 minutes at room temperature for diffusion of extracts into the agar to take place and then incubated at 37^{0}C for 24 hours. The cultures were examined for zones of growth and the diameter of each zone was measured in millimeters. The means were calculated to the nearest whole number.

Determination of Minimum Inhibitory Concentration (MIC)
The MICs of crude extracts against the clinical and reference strains of *E. coli* and *S.* Typhimurium were determined using broth dilution method described by Andrews (2001). One milliliter (1 ml) of extract concentration was added to a test tube containing 9 ml of Mueller Hinton broth. One hundred micro liters (100 µl) each of a standardized inoculum of a test bacterium was added to mixtures of different concentrations of extracts with Mueller Hinton broth.

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The test tubes were incubated at 37^0 C for 24 hours. The growth of bacteria in the broth were examined which were indicated by the turbidity of the broth. However, the lowest concentration of the extract which inhibited the growth of a test organism was recorded as the minimum inhibitory concentration (MIC). Negative controls were set up as follows; Mueller Hinton broth only and Mueller Hinton broth with extract. While positive control comprised of Mueller Hinton broth and the test organism

Determination of Minimum Bactericidal Concentration (MBC)
The Minimum Bactericidal Concentration (MBC) was determined from the MIC tube that showed no growth. An inoculum from the tube was sub cultured on to nutrient agar plate and incubated at 37 ^{0}C for 24 hours. The lowest concentration of extract that yielded no growth was the Minimum Bactericidal Concentration (MBC). The negative controls were nutrient agar only and nutrient agar with extracts only (Andrews, 2001).

Determination of the Rate of Kill
Assay for the rate of kill of a clinical isolate of each test bacterium and the control isolates by ethanol crude extracts of *T. avicenioides* leaf and the active fractions were evaluated in accordance with the method described by Odenholt *et al.* (2001). One milliliter (1 ml) from each standardized culture suspension of test bacteria was added to 9 ml of selected MBC of extract in a sterile test tube such that the final test suspension contained approximately 10^6 cfu/ml of the test bacteria. The test suspensions were kept in a water bath and maintained at 37^0C. One milliliter (1 ml) of a test suspension was withdrawn at predetermined intervals of 30, 60, 120, 180 and 240 minutes. Tenfold serial dilutions were carried out with sterile normal saline containing 3% tween 80. Zero point one milliliter (0.1 ml) of each dilution was aseptically plated out in duplicates on nutrient medium using, pour plate method for viable counts. Control plates were also prepared and contained the bacterial cells without the extracts. All plates were later incubated at 37 ^{0}C for 24 hours. The emergent bacterial colonies were counted and compared with bacterial counts from control cultures. Colony counts were plotted against time intervals on a semi log graph paper to obtain the rate of kill standard curve for each selected concentration of the extracts. The same experiment was carried out by substituting the extract with metronidazole at varied concentrations of 100 mg/ml and 50 mg/ml to serve as another control.

RESULTS
The yield of ethanol crude extract of *Terminalia avicenniodes* leaf after extraction is 18.8 %. The yields of fractions of the crude extract after fractionation of the crude extract are 28.1%, 13.2% and 5.7% respectively. The results of phytochemical screening of ethanol leaf extract and extract fractions of *T. avicennioides* revealed the presence of alkaloids, flavonoides, tannins, saponins, phenols, triterpenes, cardiac glycosides and steroids (Table 1). Steroids were only found in ethyl acetate fraction. Alkaloid was absent in ethyl acetate fraction but present in aqueous and n-butanol fractions.

Table 1: Phytochemical Constituents of Ethanol Leaf Extract and Extract Fractions of *T. avicennioides*

Constituents	Leaf Extract	Leaf Fractions		
	Ethanol	Aqueous	N – butanol	Ethyl acetate
Carbohydrates	+	+	+	+
Anthraquinones	-	-	-	-
Steriods	-	-	-	+
Triterpenes	+	+	+	+
Cardiac glycosides	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+
Flavonoides	+	+	+	+
Alkaloids	+	+	+	-
Phenols	+	+	+	+

Confirmation of Clinical Isolates of Bacteria
Results from the screening of clinical isolates of *S.* Typhimurium and *E. coli* obtained from the three hospitals show that, among the 31 (100 %) isolates of *S.* Typhimurium screened, 26 (83 %) were confirmed on the basis of their cultural and biochemical characteritics. Of the 21 confirmed isolates, 9 were from YDH, 2 from GAH and 10 from BDTH (Table 2). Among the 154 isolates of *E. coli.* screened, 63 (40.9 %) exhibited cultural and biochemical characteristics typical of *E. coli.* Of the 63 confirmed isolates, 29 were from YDH, 11 from GAH and 23 from BDTH respectively (Table 3).

Table 2: Proportions of Clinical isolates of *S.* Typhimurium Confirmed

Hospitals	No of Isolates recieved (%)	No of Isolates Confirmed (%)
YDH	15(48.4)	9 (42.9)
GAH	5(16)	2(9.5)
BDTH	11(35.5)	10(47.6)
TOTAL	31(100)	21(67.7)

KEY: YDH – Yusuf Dantsoho General Hospital, GAH – Gwamna Awan General Hospital, and BDTH – Barau Dikko Teaching Hospital, Kaduna

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Table 3: Proportions of Clinical isolates of *E. coli* Confirmed

Hospitals	No of Isolates received (%)	No of Isolates Confirmed (%)
YDH	67(43.5)	29 (46)
GAH	45(29.2)	11(17.5)
BDTH	42(27.2)	23 (36.5)
TOTAL	154	63(40.9)

KEY: YDH – Yusuf Dantsoho General Hospital, GAH – Gwamna Awan General Hospital, and BDTH – Barau Dikko Teaching Hospital, Kaduna

Antibacterial Activities of Ethanol Extract of *T. avicennioides* Leaf and Metronidazole against Clinical Isolates of *S.* Typhimurium and *E. coli.*

The responses of *S.* Typhimurium and *E. coli* isolates to ethanol leaf extract of *T. avicennioides* are presented in Table 4. All bacterial isolates were susceptible to the extract at concentrations of 12.5 mg/ml with the exception of the reference isolates. High inhibitory effect was observed on all the test isolates as the concentration increases from 12.5 - 100 mg/ml with mean zones of inhibition that ranged between 8.0 mm - 22.08±1.75 mm.

Table 4: Susceptibility of *S.* Typhimurium and *E. coli* Isolates to Ethanol Leaf Extract of *T. avicennioides.*

Bacterial Isolates Tested		Mean Zone of Inhibition (mm)			
		12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
S .yphimurium	Reference Isolates	8.0±0.00	15.00±0.00	18.00±0.00	20.00±0.00
	Clinical Isolates	8.08±6.08	13.15±5.71	18.40±0.00	21.50±0.00
E. coli	Reference Isolates	8.0±0.00	15.50±0.71	18.00±0.00	20.00±0.00
	Clinical Isolates	8.68±2.54	13.64±4.90	18.90±2.08	22.08±1.75

The MIC and MBC of the leaf extract against the test clinical isolates of *S.* Typhimurium and *E. coli* were each found to be 12.5mg/ml and 25.0mg/ml. Higher MIC (25mg/ml) and MBC (50mg/ml) were exhibited against all the reference isolates tested (Table 5).

Table 5: Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of Ethanol Extracts of *T. avicennioides* against *S.* Typhimurium.

Isolate Codes of Bacteria	MIC (mg/ml)	MBC(mg/ml)
YS5	12.5	25.0
YS8	12.5	25.0
GS1	12.5	25.0
GS2	12.5	25.0
BS4	12.5	25.0
BS7	12.5	25.0
S. Typhimurium ATCC 14028 (Control)	25. 0	50.0

Key: Y = Yusuf Dantsoho, G = Gwamna Awan, B = Barau Dikko, S = *S.* Typhimurium

Table 6: Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of Ethanol Extracts of *T.avicennioides* against *E. coli.*

Isolate Codes of Bacteria	MIC (mg/ml)	MBC(mg/ml)
YE20	12.5	25.0
YE19	12.5	25.0
GE9	12.5	25.0
GE11	12.5	25.0
BE14	12.5	25.0
BE10	12.5	25.0
E. coli ATCC 25922 (Control)	25.0	50.0

Key: Y = Yusuf Dantsoho, G = Gwamna Awan, B = Barau Dikko, S = *S.*Typhimurium

The result of antibacterial activities of different concentrations of fractions of *T. avicennioides* leaf and 50 µg metronidazole against the clinical and reference isolates of *S.* Typhimurium is presented in Table 7. All bacteria were susceptible to the extract fractions with

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mean zones of inhibition that ranged between 8.00.0±0.0 - 26.57±0.59 mm. The n-butanol fraction demonstrated higher inhibitory effect on the clinical isolates of *S.* Typhimurium (26.57±0.59) compared to the reference strain (22.67±0.58). Similar pattern of activities were also demonstrated by the leaf fractions against the clinical isolates of *E. coli* with mean zones of inhibition that ranged between 9.67±100 - 21.95±0.29. However, the ethyl acetate fraction showed more inhibitory activity (25.33±0.58) on the reference isolates of *E. coli* compared to other fractions (Table 8). All activities of the leaf fractions were found to be concentration dependent and comparable with the antibiotics used. Values in means with standard deviations followed by different letters in superscripts across rows are significantly different at P < 0.05.

Table 7: Antibacterial Activities of *T. avicennioides* Leaf Fractions and Metronidazole gainst Clinical Isolates of *S.* Typhimuriumat Different Concentrations.

Fractions	Concentration (Mg/ml)	MZI±SD *S.*Typhimurium (C)	MZI±SD *S.*Typhimurium (R)
Aqueous	100	21.91±0.4280^{a}	20.67±0.58^{a}
	50	15.09±0.42^{b}	15.67 0.58^{a}
	25	8.00±0.00^{c}	8.00±0.00^{c}
	12.5	8.00±0..00^{c}	11.0±0.00^{b}
n-butanol	100	26.57±0.59^{a}	22.67±0.58^{a}
	50	20.04±1.46^{b}	17.00±1.00^{a}
	25	15.30±0.56^{c}	16.67±2.08^{a}
	12.5	8.00±0.00^{d}	13.00±1.00^{bc}
Ethyl acetate	100	24.09±1.51^{a}	22.00±1.00^{a}
	50	22.52±1.24^{a}	19.67±1.53^{a}
	25	17.43±3.41^{b}	19.33±0.58^{a}
	12.5	14.43±4.22^{c}	11.33±1.53^{b}
Metronidazole	50µg	27.00±0.00	25.00±0.00

Values are means ± SD, n=3. Means ±SD followed by different letters in superscripts across rows are significantly different at P < 0.05,
Key: SD=Standard Deviation, C = Clinical isolate, R = Reference strain, MZI=Mean diameter of zone of inhibition.

Table 8: Antibacterial Activities of *T. avicennioides* Leaf Fractions and Metronidazole against Clinical Isolates of *E. coli* at Different Concentrations.

Fractions	Concntration (Mg/ml)	MZI±SD *E. coli* (C)	MZI ±SD *E. coli* (R)
Aqueous	100	20.19±0.67^{a}	22.67±0.58^{a}
	50	17.28±1.06^{a}	18.33 0.58^{a}
	25	14.75±1.48^{b}	11.44±5.43^{b}
	12.5	11.32±2.73^{b}	0.00±0.00^{c}
n-butanol	100	21.95±0.29^{a}	24.67±0.58^{a}
	50	20.46±0.66^{b}	21.67±0.58^{a}
	25	17.37±0.72^{c}	12.67±0.58^{b}
	12.5	12.54±4.11^{c}	13.33±0.58^{b}
Ethyl acetate	100	19.67±0.95^{a}	25.33±0.58^{a}
	50	16.49±1.38^{a}	20.67±1.53^{b}
	25	9.67±100^{b}	16.67±0.58^{b}
	12.5	10.96±3.12^{c}	12.67±0.58^{c}
Metronidazole	50µg	26.0±0.00	27.0±0.00

Values are means ± SD, n=3. Means ±SD followed by different letters in superscripts across rows are significantly different at P < 0.05,
Keys: SD=Standard Deviation, C = Clinical isolate, R = Reference strain, MZI=Mean diameter of zone of inhibition.

The result of MIC and MBC of aqueous (AQ), n-butanol (BL) and ethyl acetate (EA) fractions of *T. avicennioides* leaf extracts against clinical and reference isolates of *S.* Typhimurium and *E. coli* is presented in Table 9. The BL fraction had the lowest MIC and MBC of 6.25 mg/ml and 12.5 mg/ml against all the test isolates of bacteria. The EA fraction showed MIC range of 6.25 - 12.5 mg/ml and MBC range of 12.5 - 25 mg/ml. The fraction with the highest MIC and MBC was the aqueous fraction which ranged between 12.5 and 25 mg/ml - 25 and 50mg/ml respectively.

Table 9: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Active Fractions of *T. avicennioides* Leaf Extract against *E. coli* and *S.* Typhimurium.

Bacteria	Leaf Fractions of *T. avicennioides*					
	AQ(mg/ml)		BL(mg/ml)		EA(mg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
E.coli Cl	12.5	25.0	6.25	12.5	6.25	12.5
E.coli Rl	12.5	25.0	6.25	12.5	12.5	25.5
*S.*TyphimuriumCl	12.5	25.0	6.25	12.5	12.5	25.5
*S.*Typhimurium Rl	25.0	50.0	6.25	12.5	6.25	12.5

Key: CL = Clinical Isolate, Rl = Reference Isolate, AQ =Aqueous fraction, BL= n butanol fraction, EA=Ethyl acetate fraction

The profile rate of kill of ethanol crude extracts of *T. avicennioides* leaf and its active fractions at different Minimum Bactericidal Concentrations (MBC) are presented as follows;
Figure 1 shows the result of bactericidal effect of ethanol crude

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extract of *T. avicennioides* leaf (I), aqueous (II), n-butanol (III) and ethyl acetate fractions (IV) against clinical and reference isolates of *S.* Typhimurium (C and R) at MBC of 12.5 mg/ml and 25.0 mg/ml. The densities of C and R isolates of *S.* Typhimurium were reduced to 4.0 $\log_{10}$ and 4.1 $\log_{10}$ within 30 minutes of exposure to crude extract of *T. avicennioides* when compared to the control culture. The populations of C and R isolates of *S.* Typhimurium were further reduced to 3.6 $\log_{10}$ and 3.7 $\log_{10}$ within 60 minutes. When the contact time was increased to 120 minutes, the C and R isolates of *S.* Typhimurium were reduced to 2.5 $\log_{10}$ and 2.0 $\log_{10}$. All bacteria were completely wiped out when the contact time was increased to 180 minutes. A similar trend of bactericidal effect was observed when bacteria were treated with aqueous fractions at MBC of 25mg/ml against C isolates and 50 mg/ml against R isolates at contact times of 30 and 60 minutes. But within 120 minutes, all the isolates of *S.* Typhimurium were reduced to 2.3 $\log_{10}$. The n-butanol fraction also demonstrated high bactericidal effect against C and R isolates of *S.* Typhimurium at MBCs of 12.5 mg/ml. The densities of C and R isolates of *S.* Typhimurium were reduced to 4.0 $\log_{10}$ within 30 minutes of exposure. Within 60 minutes, the populations of C and R isolates of *S.* Typhimurium were reduced to 3.6 $\log_{10}$ and 3.5 $\log_{10}$. At contact time of 120 minutes, the populations of C and R isolates were completely wiped out. Similar reduction in populations of bacteria were also observed when the C and R isolates of *S.* Typhimurium were treated with 25 mg/ml and 12.5 mg/ml of ethyl acetate fractions within 30 and 60 minutes of contact time. When the time was increased to 120 minutes the populations of C and R isolates were reduced to 2.5 $\log_{10}$ and 2.3 $\log_{10}$ respectively.

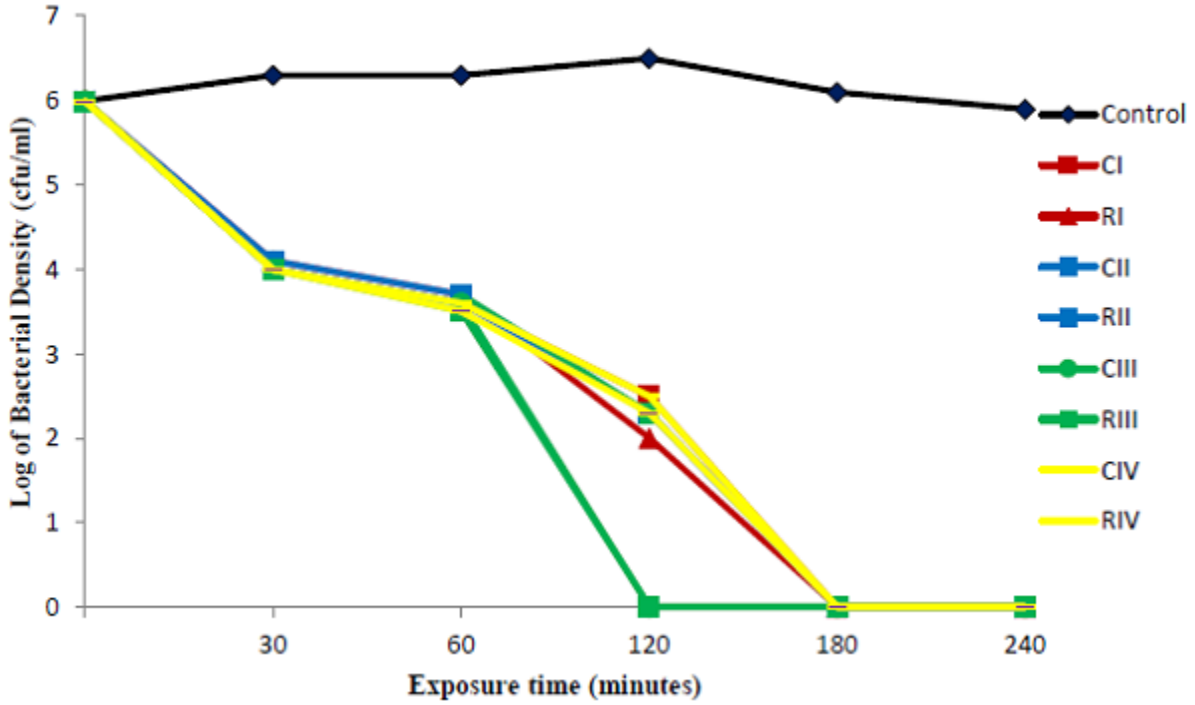

Figure 1: Profile rate of kill of clinical and reference islates (C and R) of *S.* Typhimurium by ethanol leaf extract (I), aqueous fraction (II), n-butanol faction (III) and ethyl acetate fraction (IV).

In Figure 2, the C and R isolates were reduced to 4.0 $\log_{10}$ and 3.9 $\log_{10}$ within 30 minutes of contact with 100 mg/ml of antibiotic. Within 60 minutes, they were reduced to 3.7 $\log_{10}$ and 2.8 $\log_{10}$ and were completely wiped out when the contact time was increased to 120 minutes. Similarly within 30 minutes of contact with 50 mg/ml of antibiotics, the C and R isolates of *S.* Typhimurium were both reduced to 3.9 $\log_{10}$.. Within 60 minutes, they were reduced to 3.2 $\log_{10}$ and 3.0 $\log_{10}$ and were completely wiped out when the contact time was increased to 120 minutes.

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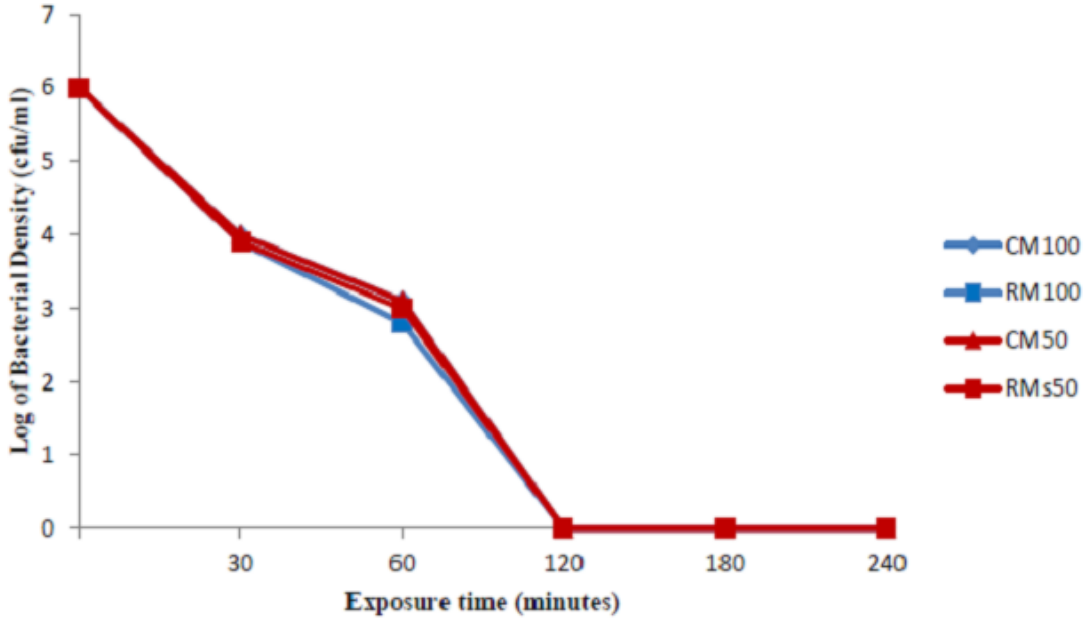

Figure 2: Profile rate of kill of clinical and reference isolates (C and R) of *S.* Typhimurium by metronidazole (M) at varied concentrations of 100 mg/ml and 50 mg/ml

Within 30 minutes of exposure to 25 mg/ml MBC of ethanol crude extracts, the densities of both C and R strains of *E. coli* were also reduced to 4.1 $\log_{10}$. When the time interval was increased to 60 minutes, the populations were further reduced to 3.8 $\log_{10}$ and 2.3 $\log_{10}$ at 120 minutes. They were wiped out within 180 minutes. The aqueous fraction at 25 mg/ml MBC reduced the populations of *E. coli* cells to 4.1 $\log_{10}$ within 30 minutes. Within 60 minutes, the C and R isolates of *E. coli* were reduced to 3.8 $\log_{10}$ and 3.9 $\log_{10}$ and within 120 minutes, they were reduced to 2.3 $\log_{10}$ and 2.6 $\log_{10}$ respectively. Within 180 minutes, the populations of all bacteria were completely wiped out when compared to populations of bacteria in the control culture (Figure 3).

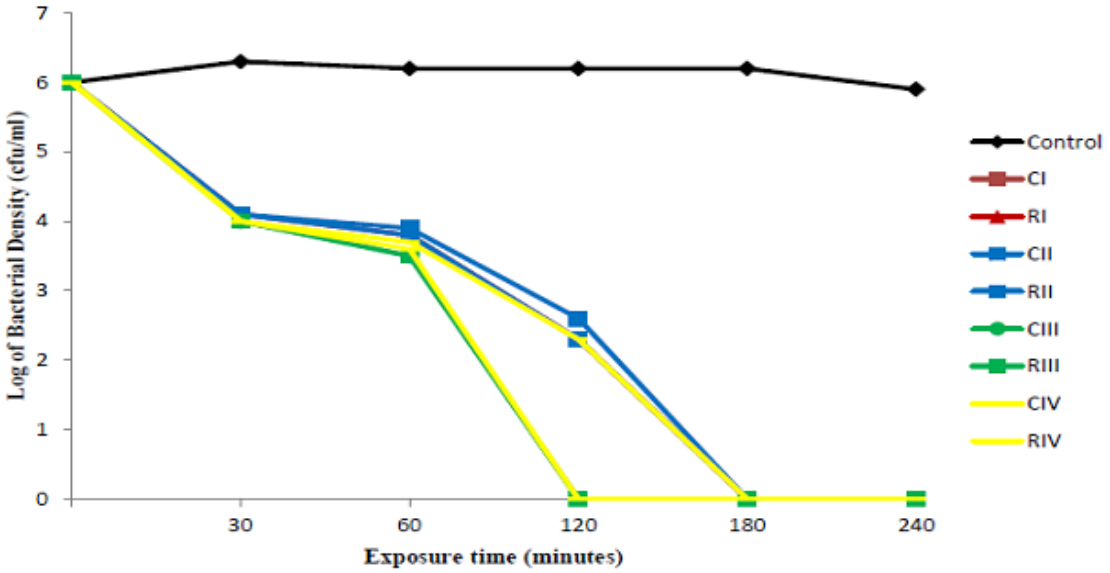

Figure 3: Profile rate of kill of clinical and reference isolates (C and R) of *E. coli* by ethanol leaf extract (I), aqueous fraction (II), n-butanol faction (III) and ethyl acetate fraction (IV)

The n-butanol fraction also showed high bactericidal activity against the *E.coli* isolates at a concentration of 12.5 mg/ml of MBC. Within 30 minutes, the populations of C and R isolates of *E. coli* were both reduced to 4.0 $\log_{10}$. They were further reduced to 3.5 $\log_{10}$ within 60 minutes. All cells were completely wiped out when the contact time was increased to 120 minutes compared to the populations of bacteria in other plates. The Ethyl acetate fraction also exhibited appreciable bactericidal activity at MBC of 12.5

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mg/ml and 25 mg/ml against C and R strains of *E. coli*. Within 30 minutes exposure to fraction, both C and R isolates were reduced to 4.0 log_{10}. Within 60 minutes of exposure, they were reduced to 3.7 log_{10} and 3.6 log_{10}. Within 120 minutes, the populations of *E. coli* R isolates were completely wiped out. The population of *E. coli* clinical isolate was reduced to 2.3 log_{10} and was later wiped out within 180 minutes respectively.

Figure 4 shows the result of the standard antibiotic metronidazole used against C and R isolates of *E. coli*. The C and R isolates were reduced to 4.0 log_{10} and 3.9 log_{10} within 30 minutes of contact with 100 mg/ml of antibiotics. Within 60 minutes, they were reduced to 3.1 log_{10} and 2.8 log_{10} and were completely wiped out when the contact time was increased to 120 minutes. Similarly, the C and R isolates of *E. coli* were reduced to 4.0 log $_{10}$ and 3.9 log_{10} with 50 mg/ml of antibiotics and were further reduced to 3.1 log_{10} and 3.0 log_{10} within 60 minutes and later wiped out when the contact time was increased to 120 minutes.

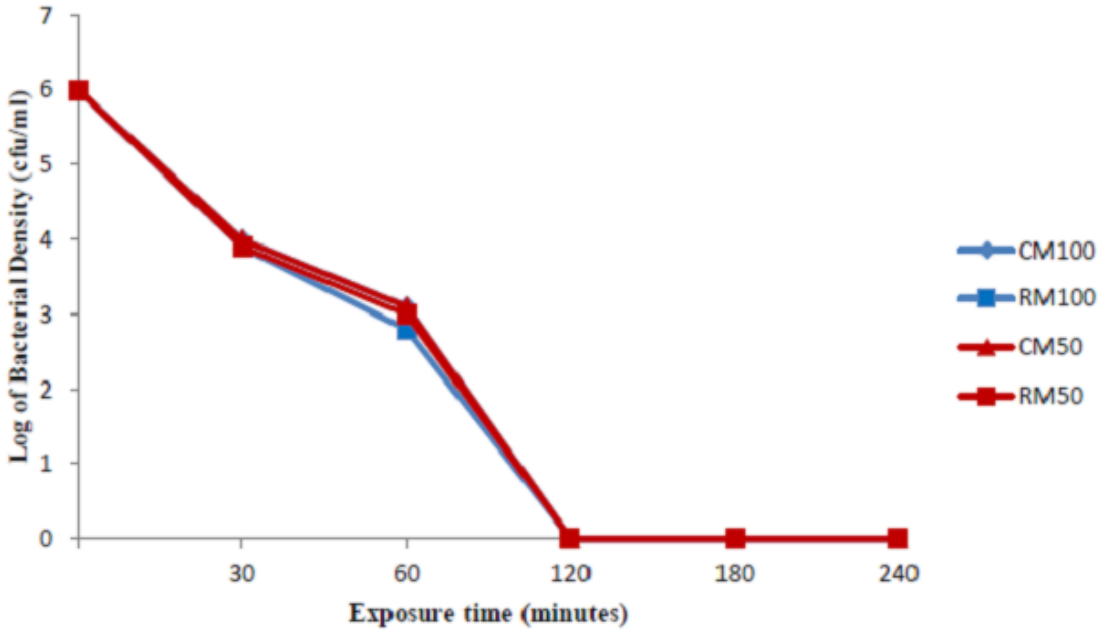

Figure 4: Profile rate of kill of clinical and reference isolates (C and R) of *E.coli* by metronidazole (M) at varied concentrations of 100 mg/ml and 50 mg/ml

DISCUSSION

The use of *T, avicenioiodes* in the treatment of different kinds of ailments, among many tribes in Nigeria, could be attributed to the presence of secondary metabolites detected in the extract of *T, avicenioiodes* leaf hence, supporting its traditional use (Man *et al*., 2008; Musa *et al*., 2016). Phytocompounds are known to be biologically active and can aid the antibacterial activities of *T. avicenniodes* through different mechanisms. Reports have shown that several compounds belonging to the investigated classes of metabolites showed antibacterial activities (Musa *et al.,* 2016; Hassan *et al*., 2022). Alkaloids for example, being one of the compounds present in *T. avicenniodes* 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 (Kengni *et al.,* 2013). Reports have also indicated that alkaloids possess analgesic properties in addition to anti-inflammatory properties which were reported for most alkaloids derived from medicinal plants (Misonge *et al*., 2015). Tannins and flavonoids are widespread and have low toxicity compared to other active plant compounds (Bushra *et al*., 2009). They possess antioxidant, astringency, bitterness and colour properties (Mamta *et al*., 2013). Saponins protect plants against attack by potential pathogens. They have also been reported to possess analgesic and anti-inflammatory activities (Jaker *et al*., 2013). Triterpenes detected in the extracts of *T. avicenniodes* have been reported to possess analgestic, anti-inflammatory, anti-cancer, anti-malaria, anti-viral, anti-bacterial activities and inhibition of cholesterol synthesis (Abdelmohsen *et al*., 2014). Cardiac glycosides have direct action on heart thereby helping to support its strength and rate of contraction when it is failing (Abdelmohsen *et al*., 2014) Carbohydrates are the main components of cell wall and protoplasm and have been widely recognized to play important roles in diverse biological processes, including viral and bacterial infections, cell growth and proliferation, cell-cell communication, as well as immune response (Majaw and Moringthem, 2009).

The susceptibility of bacterial isolates to ethanol crude extract could be attributed to the type of solvent used for extraction. It has been reported that the nature and composition of some biologically active components are enhanced in the presence of ethanol due to the stronger extraction capacity of ethanol as well as solubility of the compounds in the solvent which may be responsible for the antibacterial activity (Tiwari *et al.,* 2011). Additionaly, there is high amount of polyphenols in ethanol that helps in penatration and degradation of cell walls and seeds efficiently compared to other solvents (Musa *et al*., 2016; Hassan *et al*., 2022)

The differences in susceptibilities of *E. coli* and *S.* Typhimurium isolates to different concentrations of crude extracts of *T. avicennioidies* could be attributed to strain or species differences (Hassan *et al*., 2022) The low MICs and MBCs of all the extracts against the test bacterial isolates, shows that the plant is bactericidal in action. This conforms with the findings of Man *et al*. (2008) who evaluated the antimicrobial activity of crude extracts of *T. avicennioides* against respiratory tract pathogens and the findings of Musa *et al*. (2016) as well as Mann and Kuta (2014) who evaluated the antibacterial activities of selected medicinal plants and crude extracts of *T. avicennioides* against humans and fish pathogenic bacteria.

The high activity demonstrated by n-butanol fraction compared to other fractions also shows that the active components present in the crude extract could be more enhanced in n -butanol fractions probably due to the polarity of the solvent and the solubility of compounds in the solvent. Similar findings were reported by Suleiman *et al.* (2013) and Musa *et al.* (2016) who conducted similar studies on *T. avicennoides* plant. The low MICs and MBCs of the leaf fractions against the isolates of bacteria is an indication that the fractions of *T. avicennioides* could be more suitable in treating diseases associated with *E. coli* and *S.* Typhimurium and the active ingredients present in the extract fractions could be responsible for the observed antibacterial activities. The differences in the MIC and MBC of fractions on the test bacterial isolates could be due to strain differences or genetic contents of bacterial isolates used in the study.

The significant rapid reduction in the viable cell counts of bacterial isolates incubated with different MBCs of the leaf extract and fractions of the extract at different time intervals justified the effectiveness of *T. avicennioides* extracts on *S.* Typhimurium and *E. coli* clinical isolates. The high bactericidal effect of n-butanol fraction further justified its efficacy and suggestive use as an alternative to metronidazole in the treatment of diarrhoeal diseases caused by the investigated bacterial isolates. The present findings on the rate of kill assay is similar to the findings reported by Akinpelu *et al*. (2009) where they use the stem bark extract of *Afzelia africana* to kill about 95.8% of *E. coli* cells within 105 minutes. A similar study was also conducted by Ukwubile *et al*. (2013), who evaluated the bactericidal effects of *Bridelia ferruginea* and *T. avicennioides* at ½ x MIC and 2x MIC within 8 hours against *Staphylococcus aureus* and Methicilin Resistant *Staphylococcus aureus* (MRSA). The observed differences between the activities of other fractions of *T. avicennioide* when compared with the standard antibiotic metronidazole against *S.* Typhimurium and *E. coli* isolates could be due to the mixtures of compounds present in the various fractions compared to the pure compounds contained in the standard antibiotic metronidazole, that solely contain 100% of pure compound as active ingredients (Ukwubile *et al*., 2013) However, the partially purified n-butanol fraction of the leaf extract may contain less amount of these mixtures, judged by the observed bactericidal effects (Akinpelu *et al*., 2009; Ukwubile *et al*., 2013). Moreover, the current result on the rate of kill assay provided basis for evaluation of the level of toxicity of plant extract and isolation and purification of promising chemical constituents present in *T. avicennioides* through activity guided bioassay, which could be an interesting tool in both animal and human studies and in drug development.

Conclusion

The phytochemical constituents detected in the leaf extract of *T. avicenioides* are carbohydrates, alkaloids, flavonoids, tannins, saponins, phenols, triterpenes, cardiac glycosides and steroids. The ethanol crude extract of *T. avicenioides* leaf, the aqueous, n-butanol and ethyl acetate fractions of the leaf were highly effective against the clinical isolates of *S.* Typhimurium and *E. coli.* The activity of each extract was found to be concentration dependent. The n - butanol fraction demonstrated more activity with low MIC and MBC of 6. 25 and 12.5 mg/ml against the test isolates of bacteria compared to other fractions. The growth inhibition and efficacy of ethanol crude extract of *T. avicennioides* leaf and the fractions was also dependent on time and concentration. There was a rapid reduction in the viable cell counts of bacterial isolates when incubated with different minimum inhibitory concentrations of the leaf extract and fractions of the extract within 120-180 minutes. All bacteria were completely killed within 120 minutes of exposure to n-butanol fraction at 25mg/ml minimum inhibitory concentration. N-butanol fraction of *T. avicennioides* leaf could be used to treat diarrhoeal diseases caused by *S.* Typhimurium and *E. coli.*

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Competing Interest: Authors have declared that no competing interests exist among them.

Authors Contributions

Musa, F.M designed the entire aspect of the work. Muhammad-Idris Z.K. wrote the first draft of the manuscript with literature search. Wartu, J.R. wrote the second draft of the manuscript. All authors read and approved the final manuscript.

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