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Antibacterial Activity of *Bidens pilosa* Extracts on *Escherichia coli* O157: H7 Isolated from Apparently Healthy Individuals

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Authors' contributions

This work was carried out in collaboration between both authors. Author MKO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author DEF managed the literature searches, managed the analyses of the study, cross checked and authenticated the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

This study was carried out to isolate *Escherichia coli* O157:H7 from fecal samples of children within the age range of 0-5 years from Mother and child hospital, Anchorite daycare, Womens' forum daycare and FUTA daycare all in Akure, Ondo State. It is also aimed at extracting the bioactive component of the palnt using different solvents and determining the efficacy of the plant extracts on the bacterium by agar well diffusion and evaluating their minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC). The strain showed a high frequency of single or multiple drug resistance (MDR) to the antibiotics tested. The phytochemical screening of the extracts revealed various constituents which include: flavonoid, alkaloid, phenol, saponin, terpenoid, cardiacglycoside and tannin, 70% ethanol, cold water and chloroform were employed for the plant extraction. The ethanol extract of the plant was more potent, showing higher zone of inhibition, followed by water extract and the chloroform extract showed no zone of inhibition. The

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MIC exhibited was 50 mg/mL, 70 mg/mL for ethanol and 100 mg/mL and 120 mg/mL for water, and also the MBC exhibited was 70 mg/mL, 110 mg/mL, 150 mg/mL for ethanol and 140 mg/mL, 160 mg/mL and 200 mg/mL for water. The result suggests that the preparation of the plant exhibited significant *in- vitro* antibacterial activity against the bacterium and may be employed for the routine treatment of this infection as an alternative to antibiotics.

Keywords: Bidens pilosa; Escherichia coli; phytochemicals.

1. INTRODUCTION

Toxigenic *Escherichia coli* (TEC) strains are important food-borne pathogens responsible for gastroenteritis with manifestations of crampy abdominal pains, bloody diarrhea and kidney failure especially in children [1]. The incidence of toxigenic *Escherichia coli* in human cases of diarrhea in Nigeria is on the increase Smith et al. [2].

Smith et al. [2] reported the prevalence of TEC 0157 in human stool samples to be 31.6%. The prevalence in female ranged from 0% in woman age 40-49 years to 19% in those aged 0-5yrs. In males, the prevalence ranged from 0.0% in those age 30-49 years to 11.9% in those age 0-5yrs. A significantly higher prevalence of TEC 0157 was observed in Lagos (35.0%) with greater rate of meal consumption and more eateries than in Zaria (23.7%) which had a lower rate of meal consumption and fever eateries [2]. Shiga–toxin producing *E. coli* belongs to clones of zoonotic *E. coli* of different O serogroups. The organisms evolve and acquire specific virulence factors which enable them to infect and colonize the human colon, usu*ally without invasion of the blood stream [3]. STEC once they have been ingested cause Bloody diarrhea (BD), severe colitis and Hemolytic uremic syndrome (HUS) which is associated with devastating or life threatening systemic manifestation. This hemolytic uremic syndrome (HUS) usually results from shiga toxins (Stx) produce from bacteria in the intestine that act systemically in the sensitive cells in the kidneys, brain and other organs [4,5].

Humans become infected with STEC by ingestion of contaminated food and water or by direct contact with animals, resulting in sporadic cases of disease or outbreaks, involving up to several thousand individuals [6,7]. Other sources of infection include meal, ready-to-eat sausages, raw milk, cheese, lettuce, unpasteurized apple cider and juice, cantaloupes, alfalfa sprouts, radish sprouts [8]. The epidermiology of outbreaks of STEC in humans is linked to the consumption of contaminated bovine products [9,10,11,12].

1.1 *Bidens pilosa* Plant

Bidens pilosa is an erect, perennial herb widely distributed across temperate and tropical regions. *B. pilosa* is either glabrous or hairy, with green opposite leaves that are serrate, lobed, or dissected. It has white or yellow flowers, and long narrow ribbed black achenes (seeds). It grows to an average height of 60 cm and a maximum of 150 cm in favorable environments. *B. pilosa* prefers full sun and moderately dry soil. However, it can grow in arid and barren land from low to high elevations. With the advantage of being fast- growing, in the 1970s, the Food and Agricultural Organization actively promoted the cultivation of *B. pilosa* in Africa [13]. *B. pilosa* propagates via seeds. A single plant can produce 3000–6000 seeds. Dry mature seeds from *B. pilosa* can be germinated in 3 to 4 days in moist soil or after being soaking in water. Seeds are viable for at least 3 years [14]. Minimal agricultural techniques are required for *B. pilosa* cultivation. Due to its invasive tendencies, *B. pilosa* is generally considered to be a weed [15] and is thought to have originated in South America and subsequently spread all over the world. *Bidens* species and their varieties bear vernacular names based on their characteristics. For example, *Bidens* species are known by such names as Spanish needles, beggar's ticks, devil's needles, cobbler's pegs, broom stick, pitchforks, and farmers' friends in English and some other languages because of their sticky achenes and are sometimes known as “xianfeng cao” (“all bountiful grass”) in Chinese because of their prosperous growth.

1.2 Traditional Uses

The plant can be used as an herb and as an ingredient in teas or herbal medicines. Its shoots and leaves, dried or fresh, are utilized in sauces and teas [16,17]. All parts of *B. pilosa* plant, the whole plant, the aerial parts (leaves, flowers, seeds, and stems), and/ or the roots, fresh or

dried, are used as ingredients in folk medicines. It is frequently prepared as a dry powder, decoction, maceration or tincture [18]. Generally, this plant is applied as dry powder or tincture when used externally, and as a powder, maceration, or decoction when used internally, *B. pilosa* either as a whole plant or different parts, has been reported to be useful in the treatment of more than 40 disorders such as inflammation, immunological disorders, digestive disorders, infectious diseases, cancers, metabolic syndrome, wounds, and many others [19,20].

2. MATERIALS AND METHODS

2.1 Study Population

Ethical clearance and permit was given by Mother and Child Hospital, "Akure Ondo" State, Nigeria. The study population consisted of one hundred and fifty eight apparently healthy children consisting of fifty- six males and ninety-eight females with their age range between 0-5 years. Sterile sample bottles were used to collect the feces, which were collected at Mother and child hospital Akure, Anchorites school, Womens forum daycare centre and Federal University of Technology Daycare centre, Ondo State and immediately transported to the Microbiology Postgraduate Research Laboratory, Federal University of Technology Akure for processing.

2.2 Methods of Isolation

The samples were inoculated by pour plate method onto freshly prepared eosin methylene blue (EMB) agar plates and incubated at 37°C for 24 hours. Presumptive identification of *E. coli* was based on the characteristic green metallic sheen on the EMB agar plates, between three to five representative colonies were streaked again on the plates containing eosin methylene blue agar. These plates were then incubated aerobically at 37°C for 24 hours Cheesborough [21]. After incubation, the cultural and morphological characteristic of distinct, well isolated colonies were studied. These included the shape, size, elevation, edges, opacity, surface and color representative were picked per plate and biochemically confirmed using methyl red, indole, catalase, simon citrate and sugar such as: mannitol, glucose, sucrose, lactose to check for acid and gas production, stock cultures of pure isolates were labelled and stored accordingly at 4°C for further use.

2.3 Detection of Hemorrhagic *Escherichia coli*

The culture media used for the detection of the bacterium was Sorbitol MacConkey agar, which was prepared according to the manufacture specification. The colonies were picked from the slants with an inoculating loop and streaked on sorbitol macConkey agar plates. These plates were then incubated aerobically at 37°C for 24 hours Vernozy-Rosand and Roze [22]. After incubation, the colonies which are pale brown/ colorless indicate the strain for O157 and this was further confirmed using the slide agglutination test with O157 antiserum.

2.4 Processing and Extraction of *Bidens pilosa*

Fresh leaves were air dried for four weeks until fully crispy, and later leaves were pounded using clean mortar and pestle, then pulverized into fine powder by blending in a high-speed blender. They were separately kept in an airtight container to avoid the absorption of moisture. 200 g of the powdered sample were soaked in 1500 millimeter of 70% ethanol, chloroform and water respectively as solvents to extract the bioactive compounds. Each container was labeled appropriately and left for 72 hours (3 days). After this period, it was sieved using muslin cloth and then filtered using no 1 Whatmann filter paper. The filtrates were vaporized to dryness using rotary evaporator. The ethanolic, chloroform and water (solid) extract was preserved in a sterile bottle at 4°C ready for use.

2.5 Phytochemical Screening of the Plant Extracts

The phytochemical analysis was carried out according to the method of Dawang and Datup [23] to determine the qualitative and quantitative phytochemicals present in the extracts as follows:

2.6 Qualitative Determination of Phytochemical Components of Plant Extracts

2.6.1 Test for alkaloid

Exactly 0.5 g of the extract was stirred in 5 mL of 1% aqueous HCl on a steam water bath, 1 mL of the filtrate was treated with a few drops of

Dragendorf reagent, blue black turbidity was taken as positive for alkaloid.

2.6.2 Test for saponin

Frothing properties of saponin in aqueous solution was used as screening test. Exactly 0.5 g of extract was shaken with distilled water in a test tube frothing which persist on warming was taken as positive for saponin while its absence signifies negative result.

2.6.3 Test for tannin

Exactly 0.5 g of the extract was stirred in 100 mL of distilled water, filtered and ferric chloride reagent was added to the filtrate a blue black green or blue green precipitate was taken as positive for the presence of tannin.

2.6.4 Test for flavonoid

Exactly 0.5 g of the extract was stirred in 20 mL of dilute ammonia solution a yellow colouration was observed, the disappearance of the yellow colour after the addition of 1 ml conc. H_2SO_4 indicate the presence of flavonoid.

2.6.5 Test for terpenoids

Exactly 0.5 g of the extract was mixed with 20 mL of chloroform and filtered 3ml of conc. H_2SO_4 was added to the filtrate to form a layer. A reddish brown color at the interface was observed which indicate the presence of terpeniod.

2.6.6 Test for cardiac glycosides

The followings were carried out to test for cardiac glycosides:

Legal's test: The extract was dissolve in pyridine and a few drops of 2% sodium nitroprusside with few drops of 20% NaOH were added. A deep red coloration which faded to a brownish yellow indicates the presence of cardenolides.

Lieberman's test: A 20 mL of acetic anhydride was added to 0.5 g of the extract and filter, 2 mL of concentrated H_2SO_4 was added to the filtrate. There was a color change from violet to blue or green which indicate the presence of steroids nucleus (i.e. aglycone portion of the cardiac glycoside).

Salkowski's test: 0.5 g of the extract was mixed with 20 mL of chloroform and filtered 3 mL of conc. H_2SO_4 was added to the filtrate to form a layer. A reddish brown color at the interface was observed which indicate the presence of steroidal ring.

Keller-Killiani's test: About 0.5 g of the extract was dissolve in 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was then under layered with 1 ml of conc. H_2SO_4, a brown obtain at the interface indicate the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring while in the acetic acid layer; a green ring may form just above the brown ring and gradually spread throughout this layer.

2.7 Quantitative Determination of the Phytochemical Components present in the Plants' Extracts

2.7.1 Tannin

Exactly 0.5 g of finely ground sample was weighed into a 50 mL sample bottle. 10 mL of 70% aqueous acetone was added and properly covered. The bottle were put in an ice bath shaker and shaken for 2 hours at 30ºC. Each solution was then centrifuge and the supernatant store in ice. 0.2 mL of each solution was pipetted into the test tube and 0.8 mL of distilled water was added. Standard tannin acid solutions were prepared from a 0.5 mg/mL of the stock and the solution made up to 1 mL with distilled water. 0.5 mL of Folinciocateau reagent was added to both sample and standard followed by 2.5 mL of 20% Na_2CO_3 the solution were then vortexed and allow to incubate for 40minutes at room temperature, its absorbance was read at 725nm against a reagent blank concentration of the same solution from a standard tannic acid curve was prepared Makkar and Goodchild [24].

2.7.2 Saponin

The spectrophotometric method of Brunner [25] was used for Saponin determination. 2 g of the finely grinded sample will be weighed into a 250 mL beaker and 100 mL of Isobutyl alcohol or (But-2-ol) will be added. Shaker will be used to shake the mixture for 5 hours to ensure uniform mixing. The mixture will now be filter with No 1 Whatman filter paper into 100 mL beaker containing 20 mL of 40% saturated solution of magnesium carbonate ($MgCO_3$). The mixture

obtain again will be filter though No 1 Whatman filter paper to obtain a clean colorless solution. 1 mL of the colorless solution will be taken into 50 mL volumetric flask using pipette, 2 mL of 5% iron (iii) chloride ($FeCl_3$) solution will be added and made up to the mark with distill water. It would be allow standing for 30 min for the color to develop. The absorbance is read against the blank at 380 nm.

2.7.3 Flavonoid

The total flavonoid content of the extract was determined using a colourimeter assay developed by Chang et al. [26]. 0.2 mL of the extract was added to 0.3 mL of 5% $NaNO_3$ at zero time. After 5 min, 0.6 mL of 10% $AlCl_3$ was added and after 6 min, 2 mL of 1M NaOH was added to the mixture followed by the addition of 2.1 mL of distilled water. Absorbance was read at 510 nm against the reagent blank and flavonoid content was expressed as mg rutin equivalent.

2.8 Cardiac Glycosides

The procedure described by Sofowora [27] was used 10 mL the extract pipetted into a 250 mL conical flask. 50 mL chloroform was added and shaken on vortex mixer for 1 hour. The mixture was filtered into 100 mL conical flask. 10 mL of pyridine and 2 mL of 29% of sodium nitroprusside were added and shaken thoroughly for 10 min. 3 mL of 20% NaOH was added to develop a brownish yellow colour. Glycosides standard (Digitoxin). A concentration which range from 0 – 50 mg/mL were prepared from stock solution the abs was read at 510 nm.

2.9 Terpenoid

The procedure described by Sofowora [27] was used 1 g of finely grounded sample was weighed into a 50 mL conical flask 20 mL of chloroform: methanol 2:1 was added the mixture was shaken thoroughly and allowed to stand for 15 min at room temp. The suspension was centrifuge at 3000rpm the supernatant was discarded and the precipitate was re-washed with 20 mL chloroform: methanol 2:1 and then re-centrifuge again the precipitate was dissolve in 40 mL of 10% Sodium dodecyl sulfate solution. 1mL of 0.01M ferric chloride was added and allowed to stand for 30 min before taken the absorbance at 510 nm. The STD Terpenoid (alpaterpineol) concentration ranging from 0-5 mg/ml from the stock solution.

2.10 *In vitro* Assay

2.10.1 Reconstitution of the ethanolic, chloroform and water extracts

The Solomon-Wisdom et al. [28] method was used. The crude extract of ethanolic, chloroform and water was reconstituted with 30% dimthylsulphoxide (DMSO) and concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 mg/mL were prepared and filtered with a sterile milipore membrane filter.

2.10.2 Bacterium tested

A total of 11 *Escherichia coli* O157:H7 strains were isolated and screened at the Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria were employed for this study. The stockcultures were maintained at 4°C Nutrient agar.

2.10.3 Preparation of standard inocula for *in vitro* assay

The method described by Chukwuka et al. [29] was employed in preparing the standard inoculums of the clinical isolates for *in vitro* assay. Overnight colonies were transferred to a tube of sterile saline. The bacterial suspension was compared to the 0.5McFarland standards against a sheet of white paper on which black lines were drawn. The bacterial suspension was adjusted to the proper density as the 0.5 McFarland by adding sterile saline or more bacterial growth. Then bacterial suspension was diluted to obtain 10^6cfu/mL.

2.10.4 Antibacterial assay of *Bidens pilosa* extracts on test bacterium

To determine the sensitivity of the clinical bacteria to the extracts of leaves, method of NCCL [30] was adopted. 0.1 mL of the bacterial suspension was drawn out with the aid of a sterile pipette and was aseptically introduced into sterile petri dishes. Sterilized Mueller Hinton Agar (MHA, Difco, USA) that had been cooled to about 45°C was aseptically poured into the petri dishes containing 0.1 mL of the bacterial isolates; each petri dish was gently swirled in a clockwise direction in order to ensure that the bacterium is homogeneously distributed with MHA. The plates were then allowed to stand for 40 minutes for the inoculated bacteria to be established in the medium. After 40 min, four wells each for the clinical isolates were aseptically bored on each

agar plate using a sterile cork borer (10 mm) at allowance of 30 mm between opposite wells and the edges of the petri dishes. 0.1 mL of each reconstituted extracts was then introduced into each well in the plates using sterile pipette. Two out of the four wells bored was used for negative and positive control. For negative control, 0.1mL of the reconstituting agent (30% DMSO) was used while for the positive control 0.1mL of ofloxacin (16 mg/mL) was used. The plates were incubated at 37°C for 24 hours. The resulting zones of inhibition were measured using a transparent metre rule. The experiment was done in triplicates and the average reading was taken to be the zone of inhibition of the test bacterium [30].

2.10.5 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of the extract was determined using the broth dilution method reported by Cheesbrough [21]. In this method, different concentrations of the crude extracts were prepared at (400, 200, 100, 50, 25 and 12.5 mg/mL) for first screening and MBC (200, 180, 160, 140, 120 and 100 mg/ml), MIC (150, 130, 110, 90, 70 and 50 mg/mL) for second screening. Mueller-Hinton Broth (MHA) was prepared and 5mL was drawn out with sterile pipette into test tubes, then 0.1 mL of inoculum of the bacterial isolates (1 x 10^6cell/mL) was inoculated into each test tube and mixed thoroughly. With the aid of sterile pipette, one millimeter of the different concentrations of ethanolic and water extracts was withdrawn into each test tube containing the broth culture of each of the isolates. The test tubes were then incubated at 37°C for 24 hrs. Growth in each tube was checked for by using a spectrophotometer (Beckman model 35). Growth inhibition was indicated by low turbidity while growth was indicated by high turbidity. The concentration at which there was no/less growth as indicated by clear broth is taken as the minimum inhibitory concentration. The MBC was determined by taking a loopful from each tube that showed no growth during MIC determination and streaked onto extract free agar plates, incubated at 37°C for 24 hours. The least concentration at which no growth was observed was noted as the MBC [21].

2.11 Statistical Analysis

Analysis of data was done using one way analysis of variance (ANOVA) and Means were compared by Duncan's Multiple Range Test at 95% confidence level using SPSS 16.0 version. Difference was considered significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

The ethanolic extracts of *Bidens pilosa* showed higher activity in inhibiting the test organisms than the water and chloroform extracts, this may further confirm the affirmation that more active compounds are released by ethanol extraction than water. While the chloroform extracts showed no inhibition, the extracts were tested at 50, 75, 100, 125, 150, 200, 250, 300, 350 and 400 mg/mL concentration against eleven test organisms out of which two of the extracts (ethanol and water) recorded significant antibacterial activity against all the test organisms only at concentration of 400 mg/mL, though antibacterial activity was not observed in chloroform extracts of the plants which may be as a result of the solvent not been able to extract the plants' biologically active ingredient there by making the extracts less potent enough for antibacterial activities in this studies. This result corroborates the work of Doss et al. [31] where antibacterial activity was not observed in petroleum ether and chloroform extracts against all the pathogens used in the work. The significant antibacterial activity of *Bidens pilosa* indicates the presence of potent biologically active constituents such as tannins, flavonoids, alkaloids and saponins. The presence of these potent biologically active constituents though in varying percentages might be responsible for the antibacterial activity of *B. pilosa* observed in this present study. Flavonoids have been found to be an effective antibacterial substance probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls and this might have contributed to the antibacterial activity of the plant. Tannins and alkaloids antimicrobial action may be attributed to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins. Cowan [32] has reported reviews on the antimicrobial properties of tannins which reports that tannins can be toxic to filamentous fungi, yeasts and bacteria. All these properties of the biologically active constituents might have conferred the antibacterial activities of *Bidens pilosa*. All the test organisms were highly susceptible to the commercial antimicrobial agents (Ofloxacin) used at 16 mg/mL for positive control and DMSO showed no inhibitory effects on the test organisms as negative control.

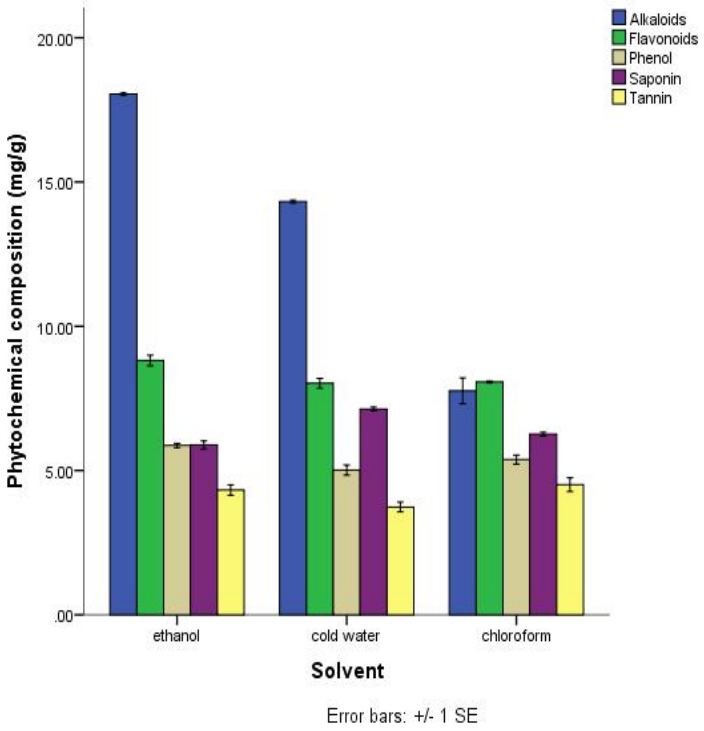

Fig. 1. Quantitative phytochemical analysis of *B. pilosa* extracts

The antibiotics sensitivity of the test organisms, when compared with the inhibitory activities of the extracts indicated that a good percentage of the plant extracts which showed antibacterial activity competed well with standard antimicrobial agents used. Also most of these phytochemical compounds have been known to exert antibacterial activity on bacteria. According to Fowler et al. [33], constituents such as flavonoids, alkaloids and tannins exhibit antibacterial activity due to their biofilm and efflux inhibitory activity.

Table 1. Results of the qualitative phytochemicals in plant extracts

Phytochemical	BE	BCW	BC
Saponin	+	+	-
Tannin	+	+	+
Flavonoid	+	+	+
Alkaloid	+	+	+
Terpenoid	+	+	-
Phenol	+	+	+
Cardiacglycosides	+	+	+

Keys: +=Positive; -=Negative; BE=Bidens pilosa ethanol extract; BCW= Bidens pilosa coldwater extract and BC= Bidens pilosa chloroform extract

MIC values were lower than MBC values, suggesting that the extracts were bacteriostatic at lower concentration but bactericidal at higher concentration. Therefore, low MIC and MBC values obtained indicated that, the ethanol extract is effective as an antibacterial. This is consistent with the report of Mushore and Matuvhunye [34], Usman and Osuji [35] who reported the same trend. From the result, the active extracts of *Bidens pilosa* has shown that the ethanol extract had a higher antibacterial activity than that of water extract. The ethanol extract had a lower MIC and MBC at 50/70 mg/mL and 70/110/150 mg/mL respectively, yet for water extract; the MIC and MBC were observed to be 100/120 mg/mL and 140/160/200 mg/mL respectively. These results showed that the ethanol extract was more powerful against the test bacterium than water extract. All the results on the MIC and MBC of the ethanol and water extracts were consistent with the initial results showing the antibacterial activity (inhibition zone) against the test bacterium. As with inhibition zone, ethanol extract was observed to have a lower MIC and MBC than water extract against the test bacterium. This was expected because the bigger the inhibition zones the lower the MIC and MBC will be and also the lower the polarity, the higher the activity.

Table 2. The minimum inhibitory concentrations (mg/ml) of water and ethanolic extract of *B. pilosa* on *E. coli* O157:H7

Isolates	Water	Ethanol	Chloroform
1	100	50	0
2	120	70	0
3	100	50	0
4	100	50	0
5	100	70	0
6	100	50	0
7	120	50	0
8	100	50	0
9	100	70	0
10	100	50	0
11	100	70	0

Table 3. The minimum bactericidal concentrations (mg/mL) of water and ethanolic extract of *Bidens pilosa* on *E. coli* O157:H7

Isolates	Water	Ethanol	Chloroform
1	160	110	0
2	200	150	0
3	140	110	0
4	160	70	0
5	200	150	0
6	160	110	0
7	160	110	0
8	200	150	0
9	200	110	0
10	140	70	0
11	160	110	0

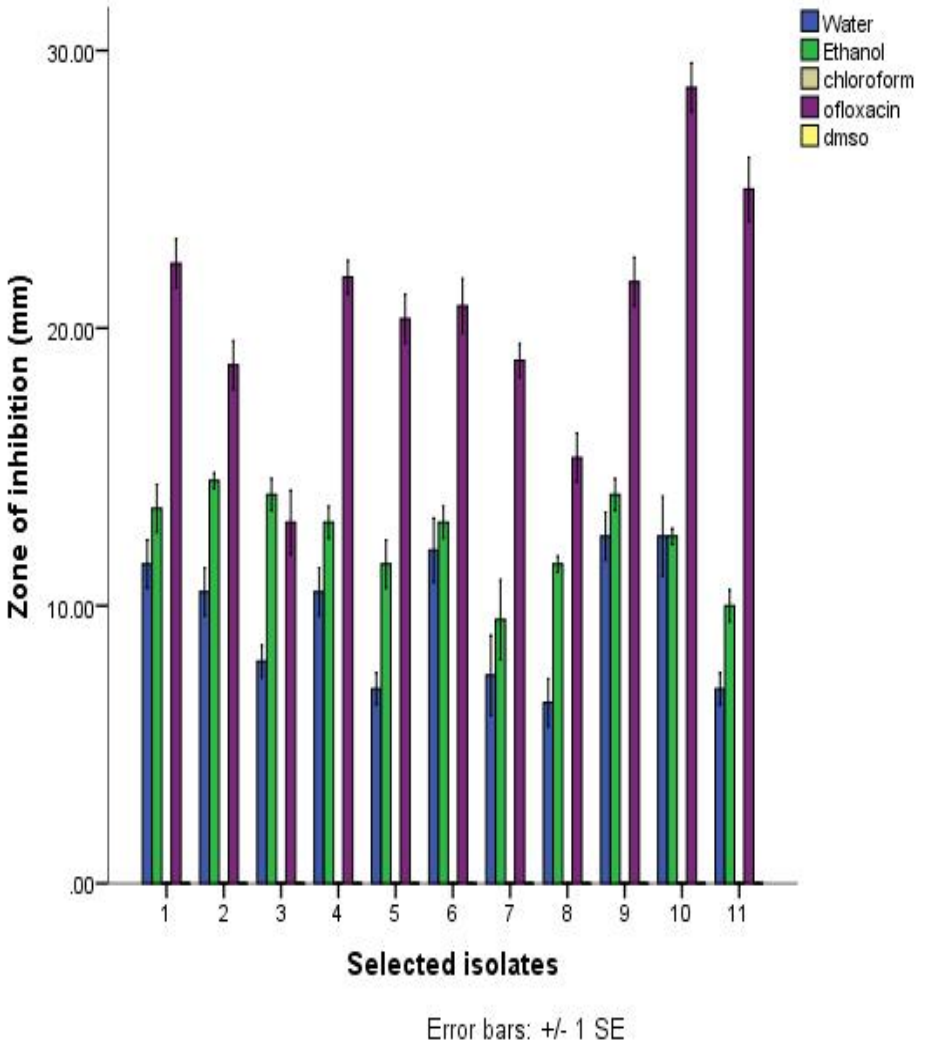

Fig. 2. Mean diameter of zones of inhibition (mm) of *B. pilosa* on test bacterium at 400 mg/mL

4. CONCLUSION

The result obtained in this research has shown that *Bidens pilosa* has some antibacterial property against *E. coli* O157:H7 and effective against infection caused by this bacterium.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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