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Nejib Getahun Tadesse , Bruk Abraha Fitwi * , Adem Hiko Woshie

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In Vitro Antibacterial Evaluation of Leaf Extracts of Medicinal Plants in the Treatment of *Escherichia coli, Klebsiella pneumoniae,* and *Salmonella* Calve Diarrhea

Nejib Getahun[1,2], Bruk Abraha Fitwi[2]*, Adem Hiko[2]

[1] Jarso District Livestock and Fishery Office, East Hararghe Zone, Oromia Region, Ethiopia
[2] Haramaya University, College of Veterinary Medicine, P.O. Box 138, Dire Dawa, Ethiopia
* Correspondence: abraha.ashebr@gmail.com

Abstract

This study employed the disc diffusion and broth microdilution tests to evaluate the antibacterial activities of the methanolic leaf extract of five medicinal plants (*Vernonia amygdalina, Cordia africana, Calpurnia aurea, Croton macrostachyus, and Melia azedarach)* against *Salmonella, Escherichia coli,* and *Klebsiella pneumoniae*. The extracts of *Calpurnia aurea, Melia azedarach,* and *Vernonia amygdaline* showed comparable effects ($P > 0.05$) with gentamicin. The isolates and laboratory strains were most susceptible to the original concentration (1000 mg/ml) of *Vernonia amygdaline* on *Salmonella* (19.33 to 20.00 mm) and *E. coli* (21.33 mm). *Vernonia amygdaline* (1000 mg/ml) showed significantly ($P < 0.05$) superior activity on clinical isolates of *Salmonella* and *E. coli.* At 1000 mg/ml, *Melia azedarach* had significant ($P < 0.05$) superiority over other plants against *K. pneumoniae.* The minimum inhibitory concentration (MIC) ranged from 6.09 mg/ml (*E. coli*) to 48.75 mg/ml (*Salmonella*), and the minimum bactericidal concentration (MBC) value ranged from 12.18 mg/ml (*E. coli*) to 97.5 mg/ml (*Salmonella*). *Calpurnia aurea* and *Vernonia amygdalina* had all the tested secondary metabolites, except for cardiac glycosides. The yield percentage ranged from 7.9 % (*C. macrostachyus*) to 11.9 % (*Calpurnia aurea* and *Cordia africana*). These results support the potential of plant-based extracts as alternative antimicrobial agents.

Keywords: antibacterial activities; diarrheagenic bacteria; medicinal plant extract; minimum bactericidal concentration; minimum inhibitory concentration; phytochemical constituents

1. Introduction

Antibacterial treatment is utilized broadly in animal medicine to treat bacterial infections, including calf loose bowels, with the main target being coliforms and *Salmonella* [1, 2, 3). The unpredictable utilization of antibacterial is related to the development of antibacterial resistance, particularly in gram-negative bacteria [4]. Particularly, the treatment of emerging strains of bacteria causing diarrhea in calves has become difficult due to their increased resistance to available antibiotics [5, 6, 7, 8, 9]. The development of antibacterial resistance among pathogens is not only a developing concern in veterinary pharmaceuticals, but also to people [10]. Thus, sometimes, a large proportion (up to 60 %) of isolates shared between diarrheic calves and contact children exhibited multidrug resistance to commonly used medications [11].

Gram-negative bacteria in the *Enterobacteriaceae* are significant inhabitants of the animal gut and are closely related to antibiotic resistance. Concerning expanding antibacterial resistance, lowering antibiotics in food-producing animals focuses on alternative and preventive medications. From these, phytotherapy can be used as an alternative to antibiotics in gastroenteric pathogens [12, 13].

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Compared to chemically synthesized mono-target drugs, the multicomponent composition of medicinal plants offers a special advantage, such as synergistic or added substance impacts within the living being [14, 15].

For centuries, medicinal plants have been an integral part of disease prevention and treatment in both humans and animals worldwide [16, 17, 18]. Several medicinal plants are known to inhibit the growth or completely inactivate the microbes [19, 20, 21, 22]. Also, some medicinal plants are known to modulate the immune system, which may well be utilized as part of medication for infectious diseases [23]. Phytochemicals may act synergistically with antibiotics, such as blocking efflux pumps and controlling biofilms, which allows low antimicrobial usage and enhanced efficacy [12, 24, 25, 26].

In low-resource setting areas, the lack of modern veterinary services and their inaccessibility have led livestock owners to rely on traditional healers and herbalists [27]. Traditional animal healthcare practices offer low-cost alternatives when commercial drugs and veterinary services are either unavailable or too expensive [28]. In Ethiopia, plants have been used for centuries as a source of medicine to treat various ailments, and traditional medicine is a crucial part of Ethiopian culture [29], in which an estimated 90% of the livestock population is treated with traditional medicines [30]. According to the New Partnership for Africa's Development, the use of indigenous traditional medicine by the population in Ethiopia is estimated to be between 60% and 79% [31]. The far-reaching utilization of conventional pharmaceuticals in communities of Ethiopia might be credited to social adequacy, viability, physical availability, and financial reasonableness as compared to cutting-edge diarrheal pharmaceuticals [32].

Numerous works showed the distinctive antibacterial and phytochemical constituents of ethnomedicinal plants, suggesting their application in the treatment of infections, especially in dealing with drug-resistant microorganisms [33, 34, 35]. There has been an extensive use of therapeutic plants with antidiarrheal properties [36]. As of late, many of these therapeutic plants have drawn impressive attention and are being studied to logically assess their antidiarrheal activity. For instance, *Calpurnia Aurea, Croton macrostachyus,* and *Vernonia amygdaline* have been tested for their antibacterial efficacy in vitro and in vivo in different countries [37, 38,-39, 40]. Despite these reports, further research on the phytochemicals and antibacterial properties of certain community-used plants is essential in the decision-making of traditional healers. Because phytochemical profiles and antibacterial efficacy of medicinal plants can vary significantly by geographic region, elevation, and ecological conditions [41, 42]. This study was conducted to determine the in vitro antibacterial activities and phytochemical constituents of leaf extracts of *Vernonia amygdaline, Cordia africana, Calpurnia aurea, Croton macrostachyus,* and *Melia azedarach* against diarrheagenic bacteria in Eastern Hararghe, Ethiopia.

2. Methods and Materials

2.1. Study Area

Jarso area is the Eastern portion of the Hararghe, which is found at around 561 km from Addis Ababa. The Jarso area is additionally one of the 21 sites of East Hararghe. It is separated into 19 Laborer Affiliations and has a range of 487.7 km^2. The population is 187000, and the crude population density is 192 people per km^2. The bordering areas of Jarso include Harari (south), Dire Dawa (north), Kombolcha (west), and Somali (east), and Gursum (southeast). Topographically, this zone lies between 9024'59.99''N scope and 42009'60.00''E longitude. As a result, Jarso is characterized by an ideal agro climatic zones which run in height from 1050 to 3030. Jarso area animals' populations were 125768 cattle, 7685 sheep, 255780 goats, 4347 camels, 375456 poultry, 57 steeds, 5 donkeys, 8670 nectar bee hives and 5867 jackasses [43].

2.2. Study Setting

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2.2.1. Sampling of Diarrheic Calves

Animals that were included in this study were three-month-old calves that were diarrheic and came to the Jarso district veterinary clinic for administration from January 2020 up to July 2021. Fecal samples were collected using sterile test tubes containing buffered peptone water. Parallelly, rectal swabs were taken for direct bacterial culture. All the samples were transported in an icebox under cold chain to the Haramaya University College of Veterinary Medicine Microbiology Research Laboratory.

2.2.2. Experimental Design

An experimental modeling randomized in six (four treatments and two controls) blocks (groups) was arranged for different concentrations of crude extract from medicinal plants (Table 1). Such blocks were prepared for each of the study plant extracts to be tested against the three selected isolates from calf diarrhea.

Table 1. Experimental treatment for assessing the mean zone of inhibition.

Group	Treatment	Concentration (mg/mL)
i	leave extract	1000 (Original extract)
ii	leave extract	780
iii	leave extract	390
iv	leave extract	195
v	+ve control (Gentamicin 10 μg)	Not applicable
vi	-ve control (DMSO 99.2 %)	Not applicable

2.3. Isolation of Test Organism

Diarrheic calves were recruited from the same district to isolate *E. coli*, *Salmonella*, and *K. pneumoniae*. Rectal swab samples were inoculated on MacConkey (MC) agar, followed by Eosin Methylene blue (EMB) agar for *E. coli* isolation. The isolated colonies were judged based on their cultural characteristics, morphology with Gram's staining, oxidase test, and biochemical tests such as carbohydrate fermentation, indole, methyl red, H_2S production, Voges-Proskauer, urea hydrolysis, and citrate utilization according to Quinn et al. [44]. Isolation and identification of *K. pneumoniae* followed the same protocol as *E. coli*, except for the use of EMB agar. Colonies displaying mucoid, lactose-fermenting characteristics were presumptively identified as *Klebsiella*. For *Salmonella* detection, rectal swabs were conducted following ISO 6579-1:2017[45] standard, including pre-enrichment in buffered peptone water (HiMedia), followed by selective enrichment using Rappaport-Vassiliadis broth at the recommended temperature [45]. Loopfuls from both enrichments were plated on XLD agar (red colonies with black centers) and Brilliant Green Agar (red colonies and medium). Presumptive *Salmonella* colonies were sub-cultured on nutrient agar (Oxoid) and tested for catalase, oxidase, Gram's reaction, TSI reaction, lysine iron agar, citrate utilization, urease test, and SIM medium reaction. Isolates yielding a TSI profile of alkaline slant/acid butt with H_2S production, positive lysine decarboxylation, positive citrate utilization, negative urea hydrolysis, and negative indole production were confirmed as *Salmonella* spp. The standard laboratory strains of *Salmonella* Typhimurium (ATCC 13311), *K. pneumoniae* (ATCC 700603), and *E. coli* (ATCC 25922) were utilized from the Ethiopian Public Health Institute (EPHI).

2.4. Identification and Collection of the Plant Materials

Plants were collected from the Jarso area based on their wider application by traditional healers. Thus, five species of plants (Table 2) that have been broadly utilized by local healers in different zones of the Jarso area for the treatment of animal diarrheal diseases were selected in January 2020. After collecting, recognizable proof of the plants was done, and voucher numbers were given by Haramaya

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University Herbarium (HUHE). The plant leaves were washed with refined water to expel earth and soil particles and subjected to processing. Thus, they were prepared into small pieces and spread out on clean paper sheets, then allowed to dry in a shaded area at room temperature. After approximately two weeks, the dried pieces were collected carefully and ground independently using a crushing machine of an electrical process (GM1001Retsh, Germany). Finally, the fine powder was kept in labeled bottles with air-tight caps at 4 °C for future utilization.

Table 2. Medicinal Plants Employed in the Jarso district for treatment of Calf Diarrhea.

Scientific name	Local name	Family name	Parts used	Voucher No
Calpurnia aurea	Ceekaa	Polygonaceae	Leaf	HUHE0000009429
Melia azedarach	Kiniinaa	Meliaceae	Leaf	HUHE00000010959
Cordia africana	Waddeeysa	Boraginaceae	Leaf	HUHE0000002154
Croton macrostachyus	Makkanniisa	Fabaceae	Leaf	HUHE0000006858
Vernonia amygdaline	Eebichaa	Asteraceae	Leaf	HUHE0000005110

HUHE = Haramaya University Herbarium.

2.5. Crude Extract Preparation

Plant materials were extracted using 99.8% methanol (Ranchem industry, Turkey) by maceration. Thus, 100 g of each powder was mixed with 500 mL of methanol in a flask. The flasks were placed out on an orbital shaker (GFL 3006, Germany) with 80 rpm at room temperature for 72 hours. The extracts were then filtered through Whatman No. 1 filter paper (Unused Delhi, India). The extracts were concentrated under reduced pressure at 37 °C employing a Buchi rotating evaporator (RE-5299 Henan, China). The distinctive extracts were measured and recorded (**Eq. 1**) before being transferred into labeled vials and stored at 4°C. The sterility of extracts was confirmed by plating on Mueller-Hinton agar (Becton Dickinson and Company, Cockeysville, MD, USA) [46]. The percentage yield of the crude extracts was determined using the following formula:

$$Percentage\ yield = \frac{x1 - x2}{y} x\ 100\ (\mathbf{Eq.1})$$

Where x1 = Weight of container with crude extract, x2 = Empty container, and y = the weight of the soaked powder [47].

2.6. Phytochemical Screening

Unrefined plant extracts were screened for various phytochemical constituents to establish a link between these secondary metabolites and antibacterial activity. Tests were conducted for alkaloids, flavonoids, glycosides, phenolic compounds, saponins, sterols, and tannins using standard methods [48, 49] (**Supplementary 1**).

2.7. Antibacterial Activity Assay of Crude Extracts

2.7.1. Inoculum Standardization

Standard and clinical isolates were inoculated onto a nutrient agar plate medium and incubated for 24 hours at 37 °C. The overnight-grown colonies were used to prepare test suspensions and adjusted to the 0.5 McFarland turbidity standard (~1×10^8 CFU/ml) according to Clinical and Laboratory Standards Institute [50].

2.7.2. Agar Disc Diffusion

The disc diffusion technique, as described elsewhere [46, 50] was used to test the zone of inhibited growth. Whatman No. 1 filter paper was used to create 6 mm diameter discs, which were sterilized at 180 °C for 30 minutes. Three concentrations of crude extracts (780, 390, and 195 mg/ml)

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were prepared using Dimethyl Sulfoxide (DMSO, 99.2%) as solvent. The fourth concentration was 1000 mg/ml (100%) of crude extract (original extracts). Each sterile disc receives 20 µl of each extract and is left to dry for 30 minutes in room temperature. This was done with a subsequent addition of 10 µl at a time, followed by repeated application after being dried. A bacterial culture suspension (1.5 × 10^8 CFU/ml) was added to Petri plates of Mueller-Hinton Agar (MHA) medium. The crude extract impregnated discs were then placed on the surface of MHA in triplicate. Negative control discs (DMSO impregnated) and positive control (Gentamicin 10 µg/ml) were used for comparison. After allowing all the plates to stand at room temperature for 30 to 60 minutes, they were incubated at 37 °C for 18-24 hours. The resulting zones of growth inhibition around the discs were measured in millimeters (mm) using a ruler, followed by calculating the mean zones of inhibition.

2.7.3. Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined by the tube dilution method [51]. The extracts were diluted to decreasing concentrations (390 mg/mL to 0.76 mg/mL) in serial twofold dilutions using the nutrient broth in tubes. Both positive (bacterial suspension only) and negative control (diluted plant extract) were included. Then, 1 ml of bacterial suspension was transferred to the treatment test tubes. After 24 hrs of incubation at 37°C, the tubes were checked for any evidence of growth (turbidity). The tube with the lowest concentration of the extract with no turbidity (matching the negative control tube) was judged as MIC. All tests were conducted in triplicate, and mean values were recorded for analysis.

2.7.4. Minimum Bactericidal Concentration of Crude Extracts

Test tubes with no turbidity from the MIC test were aseptically sub-cultured to antibacterial-free nutrient agar plates, using a sterile wire loop and incubated at 37ºC for 24h. Then observed for evidence of bacterial colonies. The minimum bactericidal concentration (MBC) was judged as the lowest concentration at which no bacterial colony was observed on the agar plates. All tests were conducted in triplicate, and mean values were recorded for analysis.

2.8. Data Analysis

Data obtained from the test were entered into a Microsoft Excel 2010 spreadsheet, cleaned, and analyzed using Statistical Package for Social Sciences (SPSS version 20, Armonk, NY: IBM Corp). Zone of inhibition, MIC, and MBC were presented using descriptive statistics such as mean and standard error of mean. The difference in the mean value between treatment variables was carried out using one-way ANOVA, followed by Tukey's Post Hoc Multiple Comparison test. The significance level was set at $P < 0.05$.

3. Results

3.1. Inhibition of Microbial Growth

The current plant extracts have a growth inhibitory effect with a mean zone of inhibition ranging from 10.0 to 21.33 mm (Table 3). The highest record (21.33 mm) was noted with the *Vernonia amygdaline* (1000 mg/ml) against *E. coli* and *Cordia africana* (1000 mg/ml) against *Salmonella*. Likewise, *Calpurnia aurea* at 100% concentration shows 20.00 mm inhibition against *Klebsiella pneumonia.* Also, *Vernonia amygdaline* at 100% concentration shows 19.33 to 20.00 mm inhibition against *Salmonella.* The highest activity of *Croton macrostachyus* extract (1000 mg/ml) was recorded at ~16.00 mm against *E. coli* (clinical isolate) and *Salmonella* (standard strain). Regarding *Melia azedarach* extract, the highest activity was against *Salmonella,* with 19.67 mm inhibition. Generally, for *Calpurnia aurea, Cordia africana,* and *Vernonia amygdaline*, the highest concentration (original extract) exhibited a significantly higher ($P < 0.05$) inhibition zone than the respective lowest concentration (195 mg/mL). Among the plants, *Vernonia amygdaline* (1000 mg/ml) showed significantly ($P < 0.05$) superior activity on clinical

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isolates of *Salmonella* and *E. coli*, while the highest concentration of *Calpurnia aurea* showed a significantly higher ($P < 0.05$) inhibition against the standard strain of *K. pneumoniae*. *Melia azedarach* and *Calpurnia aurea* on *K. pneumoniae*, *Vernonia amygdaline* on *Salmonella* and *E. coli*, showed similar inhibitory activity ($P > 0.05$) compared with Gentamicin, while the negative control (DMSO) showed no inhibition in all test pathogens. The study also showed susceptibility variations between the clinical isolates and standard strains (Table 3).

Table 3. The zone (mean ± SEM) of bacterial growth inhibition (mm) using different concentrations of crude plant extracts compared with standard antibacterial drugs.

Plants	Concentrations (mg/ml)	*K. pneumonia*		*Salmonella*		*E. coli*	
		Clinical	Standard	Clinical	Standard	Clinical	Standard
Calpurnia aurea	1000	15.67±0.3^{b}	20.00±0.6^{a,b}	15.00±0.0^{f}	17.67±1.2^{b,f}	17.0 ±0.3	15.33±0.3
	780	13.00±0.0^{c}	16.00±2.5^{c}	14.33±0.3^{cz}	15.67±0.3^{c}	14.5±0.3	15.33±0.3
	390	13.00±0.6^{d}	13.00±0.6^{d}	14.00±0.6^{d}	14.00±0.6^{d}	14.5±0.5^{d}	15.00±0.6^{d}
	195	11.00±0.0^{e}	11.67±0.3^{e}	13.00±0.0^{e}	12.67±0.3^{e}	13.0±0.3^{e}	13.33±0.3^{e}
Cordia africana	1000	15.00±0.6^{b}	12.00±0.0	13.00±0.3^{f,g}	21.33±0.9^{b}	15.5±0.3	14.00±0.6
	780	12.33±0.9^{c}	13.00±0.6^{c}	14.33±0.3^{d}	13.33±0.3^{c}	14.67±0.3	13.67±0.7
	390	12.33±1.2^{d}	12.00±0.6^{d}	14.00±0.3^{cz}	14.00±0.6^{d}	14.3±0.9^{d}	13.00±0.0^{d}
	195	11.00±0.0^{e}	11.00±0.0	12.00±0.3	12.33±0.3^{e}	13.67±0.3^{e}	12.00±0.0^{e}
Croton macrostachyus	1000	14.33±0.3	13.00±0.6	15.00±0.0^{f}	15.00±0.0^{f}	16.67±0.3	15.00±0.0
	780	12.00±0.0^{c}	12.67±0.3^{c}	14.00±0.6^{cz}	15.33±0.3^{c}	14.67±0.3	14.67±0.3
	390	11.67±0.3^{d}	12.00±0.6^{d}	15.33±0.3^{d}	15.00±0.6^{d}	15.00±0.6^{d}	14.00±0.0^{d}
	195	10.00±0.0^{e}	11.00±0.6^{e}	14.00±0.0^{e}	13.00±0.0^{e}	14.00±0.0^{e}	12.00±0.0^{e}
Melia azedarach	1000	17.00±0.0^{ab}	15.33±0.3	14.33±0.3^{f,g}	19.67±0.3^{b}	15.67±0.3	15.33±0.3
	780	13.00±0.0^{c}	13.00±0.0^{c}	13.33±0.3^{z}	15.00±0.0^{c}	15.00±0.0	14.00±0.0
	390	12.00±0.0^{d}	11.00±0.6^{d}	14.00±0.0^{d}	14.00±0.0^{d}	15.00±0.0^{d}	13.00±0.0^{d}
	195	10.33±0.3^{e}	10.00±0.0^{e}	12.00±0.0	12.00±0.0^{e}	13.00±0.0^{e}	12.00±0.0^{e}
Vernonia amygdaline	1000	15.33±0.3^{b}	15.33±0.7	19.33±0.3^{ab}	20.00±1.2^{b}	21.33±0.9^{a,b}	21.0±1.2^{a,b}
	780	13.00±0.0^{c}	12.00±0.3^{c}	15.00±0.0^{c}	14.67±0.3^{c}	19.00±2.0^{a,c}	19.00±1.5^{c}
	390	12.00±0.0^{d}	12.00±0.0^{d}	12.67±0.3	14.00±0.6^{d}	15.00±0.0^{d}	14.00±0.6^{d}
	195	10.00±0.0^{e}	10.00±0.0^{e}	12.00±0.0	11.00±0.0^{e}	13.00±0.0^{e}	13.00±0.0^{e}
Gentamycin	**10µ**	**18.00±0.5^{a}**	**19.67±0.9^{a}**	**19.00±0.3^{a}**	**29.67±0.7^{a}**	**22.33±1.06^{a}**	**23.67±0.78^{a}**

The values are Mean ± S.E.M (n=3), mean values with different superscript letters differ significantly at $P < 0.05$, ^{a} comparison is between different concentrations of extracts to gentamicin, ^{b,f,g} comparison was made among plant extracts only for 1000 mg/ml, ^{c,z} comparison was made among plant extracts only for 780 mg/ml, ^{d} comparison was made among plant extracts only for 390 mg/ml, ^{e} comparison was made among plant extracts only for 195 mg/ml, the negative control has shown no antibacterial activity.

3.2. Minimum Inhibitory Concentration

The MIC values of plant extracts ranged from 6.09 mg/mL to 48.75 mg/mL (Table 4). Except for *Melia azedarach*, all the crude extracts had considerable antibacterial activity, with the lowest MIC value of 6.09 mg/mL against *E. coli* and 12.18 mg/mL against *K. pneumoniae.* The MIC value of *Melia azedarach* was higher for *K. pneumoniae* (24.38 mg/ml) and *E. coli* (12.18 mg/ml) than for other plant extracts. With *Salmonella* (clinical isolate), *Melia azedarach* showed better activity with a MIC value of 24.38 mg/ml than other plants (48.75 mg/ml).

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Table 4. The MIC (in mg/ml) of plant extracts against the tested bacteria.

Plants	*K. pneumonia*		*Salmonella*		*E. coli*	
	Clinical	Standard	Clinical	Standard	Clinical	Standard
Vernonia Amygdaline	12.18±0.00	12.18±0.00	48.75±0.00	24.38±0.00	6.09±0.00	6.09±0.00
Calpurnia Aurea	12.18±0.00	12.18±0.00	48.75±0.0	48.75±0.0	6.09±0.0	6.09±0.0
Cordia Africana	12.18±0.00	12.18±0.00	48.75±0.00	48.75±0.00	6.09±0.00	6.09±0.00
Croton Macrostachyus	12.18±0.00	12.18±0.00	48.75±0.00	48.75±0.00	6.09±0.00	6.09±0.00
Melia Azedarach	24.38±0.00	24.38±0.00	24.38±0.00	24.38±0.00	12.18±0.00	12.18±0.00
The values are Mean ± S.E.M (n=3).						

3.3. Minimum Bactericidal Concentration

The MBC of the extract of the study plant was in the range of 12.18 mg/mL to 97.5 mg/mL (Table 5). The lowest MBC concentration (12.18 mg/ml) was observed by *Calpurnia Aurea, Cordia africana*, and *Vernonia amygdaline* against *E. coli.* Overall, the same MBC values were obtained against standard and clinical isolates (Table 5).

Table 5. The MBC (in mg/ml) of plant extracts against the tested bacteria.

Plants	*K. pneumonia*		*Salmonella*		*E. coli*	
	Clinical	Standard	Clinical	Standard	Clinical	Standard
Vernonia amygdaline	24.38±0.00	24.38±0.00	48.75±0.00	48.75±0.00	12.18±0.00	12.18±0.00
Calpurnia aurea	24.38±0.00	24.38±0.00	97.5±0.00	97.5±0.00	12.18±0.00	12.18±0.00
Cordia africana	24.38±0.00	24.38±0.00	97.5±0.00	97.5±0.00	12.18±0.00	---
Croton macrostachyus	24.38±0.00	24.38±0.00	97.5±0.00	97.5±0.00	24.38±0.00	24.38±0.00
Melia azedarach	48.75±0.00	48.75±0.00	48.75±0.00	48.75±0.00	24.38±0.00	24.38±0.00
The values are Mean ± S.E.M (n=3), --- = no activity.						

3.4. Phytochemical constituents and characteristics of the crude extracts

The crude extract of plants showed varied reactions for the presence of alkaloids, saponins, tannins, and phenols. But all were negative for the cardiac glycosides, while flavonoids, terpenoids, and steroids were found in all extracts (Table 6). All the crude extracts from the leaves were green, but with varied yields (Table 7). Thus, *Calpurnia aurea* and *Cordia africana* leaves yielded the highest, while *Croton macrostachyus* yielded the lowest.

Table 6. Phytochemical compositions of the crude extract of leaves of plants.

Secondary metabolite tested	Tested plants				
	Calpurnia aurea	*Cordia africana*	*Croton macrostachyus*	*Melia azedarach*	*Vernonia amygdaline*
Flavonoids	+	+	+	+	+
Alkaloids	+	_	+	+	+
Saponins	+	_-	+	_-	+
Tannins	+	+	_-	_-	+
Steroid	+	+	+	+	+
Terpenoids	+	+	+	+	+
Phenols	+	_-	-_	_-	+
Cardiac Glycoides	-_	_-	_-	_-	_-
- Absence, + Presence					

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Table 7. Plant parts and yielded extract using 99.8% concentrated methanol.

Plant names	Part used	Amount macerated(g)	Yield (g)	Yield (%)	Color of extract
Calpurnia aurea	Leaf	100	19.05	11.9	Green
Cordia africana	Leaf	100	18.89	11.9	Green
Croton macrostachyus	Leaf	100	16.54	7.9	Green
Melia azedarach	Leaf	100	18.15	9	Green
Vernonia amygdaline	Leaf	100	20.79	10.9	Green

4. Discussion

To address the shortage of new antibacterial options and the increasing prevalence of antibiotic resistance, plants may offer a promising solution. Evaluating the antibacterial effects of herbal medicines for treating bacterial infections, such as those causing neonatal calf diarrhea, is crucial for addressing animal health issues [52]. The present study evaluated crude plant extracts for their antibacterial activity against *K. pneumoniae, Salmonella,* and *E. coli* isolates obtained from cases of diarrhea in calves.

The finding on *Vernonia amygdaline* against clinical isolates of *E. coli* (21.33 mm), *Salmonella* Typhimurium (19.33 mm), and *K. pneumoniae* (15.33 mm), is agreeable with the values reported in multiple geographic and experimental contexts. Thus, Nmema et al. [53] reported a range from 13-20 mm for *K. pneumoniae* and 13-18 mm for *S.* Typhi at various concentrations of ethanol extract using agar well diffusion. Moreover, Sa'idu et al. [54] showed the inhibitory activity of different concentrations of the plant against *K. pneumoniae* (7-14 mm) using agar well diffusion of methanolic extract. Similarly, Ugwu et al. [55] reported better activity of the plant for *E. coli* (16 mm) than *Klebsiella* spp (13 mm) and *Salmonella* spp (12 mm). Moreover, the observed similarity between the clinical isolate and laboratory strain is in line with the findings of Ugwu et al. [55] on *E. coli* strains, which showed no difference in zone of inhibition across the test concentration of the plant. Using ethanolic extract, 7-14.5 mm for *E. coli*, and 7.5 – 11.5 mm for *K. pneumoniae* [56]. Generally, organisms become more sensitive to the plant extract as the concentration increases [57].

Similarly, Adetunde et al. [58] reported that ethanolic and aqueous extracts of the plant have broad antimicrobial activity (Gram-positive, Gram-negative, yeast), but the ethanol extract showed inhibitory activity against *E. coli* (19 mm) compared with *Salmonella*, and only the aqueous extract showed activity against *S.* Typhi (19 mm). However, contrary to our finding, Ugochi et al. [59] showed better activity of the plant against *K. pneumoniae* (16.4 mm) than *E. coli* (14.10 mm) and *Salmonella* (5.4 mm). Likewise, Jarmai et al. [60] reported a lack of activity of the methanolic extract of the plant against *E. coli*, while better activity against *K. pneumoniae* (18 mm) and a moderate value on *Salmonella* Typhi (9 mm). Using a methanolic extract, 18.0- and 16.0-mm inhibition were recorded for K. *pneumoniae* and *E. coli*, respectively, using 4 mg/ml of concentration on agar well diffusion test [61]. As low as 11 mm was recorded for clinical isolates of *Salmonella* using ethanol extract [62]. Moreover, as low as 7 mm for *E. coli* and 4 mm for *K. pneumoniae* was reported using aqueous extract [63]. Using agar well diffusion test, *V. amygdalina* leaves from Ethiopia have shown antimicrobial activity against the laboratory standard strains, 19.2 mm for *E. coli* and but no activity for *S.* Typhi [64].

The observed *V. amygdaline* MIC value for *K. pneumoniae* (12.18 mg/ml), *E. coli* (6.09 mg/ml), and *Salmonella* (24.38-48.75 mg/ml), which is similar to the value of 6.25 mg/ml for *E. coli* and 25 mg/ml for *Salmonella* reported elsewhere [55], 50 mg/ml for *Salmonella* [62], but it was higher than value of 0.78 for *E. coli* [64], 5 mg/ml for *K. pneumoniae* [54], 6.25 mg/ml for *Klebsiella* [55]. On the other hand, the current finding was lower than the values of 25 mg/ml for *E. coli* and *K. pneumoniae* using ethanol extracts [56], value of 50 mg/ml for *E. coli* and 25 mg/ml for *K. pneumoniae* [63], 50 mg/ml for *K. pneumoniae* [60]. The current MBC value for *E. coli* (12.18 mg/ml), *K. pneumoniae* (24.38 mg/ml), and *Salmonella* (48.75 mg/ml) is similar with the MBC value of 25 mg/ml for *K. pneumoniae*, but lower than the value for *E. coli* (25 mg/ml) [63], 50 mg/ml for *K. pneumoniae* [60], and 100 mg/ml for *Salmonella* [62]. The disparities could be due to different methods of extraction, such as using aqueous extract

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[63] and ethanol extract [56, 62]. The presence of phytochemicals such as tannins, flavonoids, alkaloids, saponins, phenols and terpenoids is in agreement with previous reports [61, 57, 54, 60, 55]. Similarly, Ahmad et al. [62] reported that cardiac glycosides are not found in the plant using ethanol extraction. Several compounds from the investigated classes of phytochemicals were reported for their antibacterial activities [65, 57]. The yield of *Vernonia amygdaline* of leaves extract (10.9%) is higher than the percentage reported (7.16%) of Noumedem *et al.* [66].

The present study underscored the better inhibition zones exhibited by *Calpurnia aurea,* 15.67-20.00 mm for *K. pneumoniae*, 15.0-17.67 mm for *Salmonella* Typhimurium, and 15.33-17.0 mm for *E. coli*. These results significantly surpass the inhibition zones reported by Umer et al. [38], who found only 10 mm, 11 mm, and 14 mm, respectively, for these bacterial species. It is similar to the 15.63 mm documented by Demeke *et al.* [67] and 11.47-15.67 mm by Mulatu [68] against *E. coli* using ethanol extract. Moreover, the current finding is within the range 15 to 19 mm reported by Wasihun et al. [40] using ethanol extract. This suggests a potent efficacy, potentially influenced by factors such as the extraction method and agroecology. Additionally, *Calpurnia aurea* demonstrated strong effectiveness against *Salmonella* Typhimurium, aligning with Abise [69], who reported an inhibition zone of 17.66 mm, while higher than 11.5 to 13.23 mm by Mulatu [68] using ethanol extract for the same isolates. Using agar well diffusion test, *C. aurea* leaves from Ethiopia have shown antimicrobial activity against the laboratory standard strains, 11.0 mm for *E. coli* and 10.3 mm for *S.* Typhi [64]. Nevertheless, these findings remain lower than the inhibition zones observed for the antibiotic gentamicin, which measured 19.00 to 29.67 mm for *Salmonella*. In this study, the MIC values of 12.18 mg/ml (*K. pneumoniae),* 48.75 mg/ml (*Salmonella),* and 6.09 mg/ml (*E. coli)* for *Calpurnia aurea* differ from Abise [69], who reported an MIC value of 1.25 mg/ml for *E. coli.* Likewise, 12.5 for *S.* Typhi and *E. coli* was reported by Assefa et al. [64]. Extracts with strong antibacterial activity typically had low MIC values, while those with weaker activity had higher MIC values.

This study's phytochemical analysis of *Calpurnia aurea* leaf extracts revealed the presence of alkaloids, flavonoids, saponins, phenols, tannins, steroids, and terpenoids. Umer et al. [38] and Mulatu [68] also identified these compounds but did not report steroids. The bioactivity of the extracts varied depending on the extraction solvents, geographical source, harvest time, storage conditions, and drying methods [70]. Antibacterial properties are attributed to bioactive compounds like tannins, which may inactivate enzymes and affect microbial adhesion [71]. Flavonoids and saponins also demonstrate antibacterial activity by interacting with protein structures [72]. The extraction yield in this study was 11.9%, slightly higher than 9.1 % (ethanol extract) by Mulatu [68], and 10.02 % by Wasihun et al. [40], suggesting that the secondary metabolites in *Calpurnia aurea* leaves are predominantly polar. Variations in extraction techniques and plant parts may account for differences in yields. Moreover, a higher percentage (14.64 %) was reported from Ethiopia [64].

The 15 mm zone of inhibition exhibited by *Cordia africana* extract against clinical isolates of *E. coli* and *K. pneumoniae* is lower than the report of Alhadi *et al.* [73] from Sudan, which showed 18-19 mm zone against *E. coli*. The MIC of *E. coli* (6.09 mg/ml) is according to the findings of Alhadi *et al.* [73], who reported 6.25 mg/ml for the methanol extract of leaves. While lower values were reported, 0.128 mg/ml for *S.* Typhimurium and 512 mg/ml for *E. coli* standard strains using methanolic bark extract from Nigeria [74]. These suggest the superior activity of the plant against some pathogens, which is also evidenced by the current observation of the highest inhibition zone of inhibition (21.33 mm) against the standard strain of *S.* Typhimurium as compared with the other plants. The phytochemical analysis of Methanolic root bark extract of *Cordia africana* revealed the presence of tannin, flavonoids, terpenoids, and saponins, and the absence of alkaloids in line with the report from Sudan by Alhadi *et al.* [73]. As compared with the current result on the yield (11.9%), Alhadi *et al.* [73] recorded a higher extraction percentage yield (15.75%) from methanol extraction of *Cordia africana* leaves.

In this study, *Croton macrostachyus* showed a maximum inhibition zone of 16.67 mm and 15.00 mm against clinical and standard *E. coli* isolates, respectively. In their study, Sendeku *et al.* [75] reported a higher value of inhibition against standard (25.00 mm) and clinical isolates (20.00 mm) of

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the same organism using methanol as a solvent. However, far lower values were reported by Jackie *et al.* [76] on *E. coli* (9.0 mm) and *Salmonella* Typhimurium (2.3 mm). The possible reason for the discrepancy between the present finding and other studies could be the difference in the concentration of methanol [77] and plant parts used, where 80% methanol and steam bark were used in the other studies, whereas 99.8% was used in the present study. The current MIC values of *Croton macrostachyus* extracts against the test pathogens (Table 4) are not in line with a previous report on methanol extracts of leaves and roots of *Croton macrostachyus* on *E. coli* with MIC value of 250 mg/ml [78]. The phytochemicals reported in the present study, such as saponins, flavonoids, terpenoids, steroids, and alkaloids, were also reported previously by Alemu *et al.* [79]. The 7.9% extract yield of *Croton Machrostachyus* is slightly higher than the report of Kiristos *et al.* [80], on methanolic stem bark extract (5%). Plant parts (leaf, bark, or root) and their source can influence the quality of an extract [81, 82].

Melia azedarach exhibited the highest inhibition at a concentration of 100% against the standard strain of *Salmonella* (19.67 mm), followed by clinical isolates *K. pneumoniae* (17.00 mm), *E. coli* (15.67 mm), and *Salmonella* (15.33 mm). The current findings, 14.0 mm (390 mg/ml) and 13.0 mm (195 mg/ml) on *E. coli*, are consistent with the reported range of 12.0 - 16.7 mm (300 mg/ml) and 10.0-13.0 mm (150 mg/ml) from Nigeria [83], but far higher than the reported value of 5.64 mm at 700 mg/ml against *E. coli* with ethanol extracts elsewhere [84]. The current value for *K. pneumoniae* (10.33 mm at 195 mg/ml) is lower than the reported value of 12.67 mm at 200 mg/ml for *Klebsiella* by Al-Khafaji et al. [85], but with different plant parts and extraction method. However, the same authors reported a lower value (7.33 mm) than the current finding (13.00) against *E. coli* at similar concentrations. The MIC of *Melia azedarach* against *E. coli* (12.18 mg/ml) is far lower than the reported value of 1400 mg/ml against *E. coli*, but with ethanolic extract [84]. *Melia azedarach* was found to be negative for various phytochemicals (Table 6), which is not in line with the report showing the presence of tannins, saponins, and phenolics [86]. Moreover, the authors reported a 6.72% extraction yield of *Melia azedarach* methanol leaves extracted, which is lower than the current finding (9%). On the other hand, a similar finding (9.3% yield) from ethanolic extract was reported elsewhere [84].

In the present study, clinical isolates revealed that *E. coli* exhibited the highest susceptibility to leaf extracts from the five tested plants, followed by *Salmonella* and K. pneumoniae (Table 3). This variation in susceptibility may be attributed to differences in capsule structure and composition among the bacterial species. Notably, the capsule thickness in *K. pneumoniae* has been reported to thickest. According to Amak et al. [87] and Schembri et al. [88], the inner layer of the capsule consists of densely packed fiber bundles arranged perpendicularly to the outer membrane. The more robust and complex capsule of *K. pneumoniae* may contribute to its reduced permeability and resistance to antimicrobial agents, including plant-derived extracts [89, 90). Plant secondary metabolites affect the bacterial cells in different ways such as cell wall disruption [91], inhibit biofilm formation and microbial DNA synthesis [92], inhibit energy synthesis, and bacterial toxins production [93, 94]. Secondary metabolites also have antidiarrheal activities through different mechanisms, such as inhibition of histamine release by saponins [95], inhibition of autacoids and prostaglandins release by terpenoids [96, 97], and astringent action of phenols [95]. The antidiarrheal effect coupled with antibacterial activity may be useful in the management of diarrheal states, especially in conditions where modern antidiarrheal drugs and antibiotics are not available.

5. Conclusion

Ethiopian plants have long been the source of important products with therapeutic value. The agar disc diffusion assay revealed that *Vernonia amygdaline, Cordiana africana, Calpurnia aurea, Croton macrostachyus, Melia azedarach* demonstrated good antibacterial activities against *Salmonella, E. coli,* and *K. pneumoniae* at different concentrations. The significant antibacterial activity against clinical isolates, with *E. coli* showing the highest susceptibility, followed by *Salmonella* and *K. pneumoniae*, justifies their traditional use for the treatment of calf diarrhea infections. Notably, the MBC values in the current study were lower than some previously reported for *V. amygdalina*, which further

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supports the potential efficacy. The lower antibacterial concentrations of *Vernonia amygdaline, Cordiana africana, and Calpurnia aurea* showed for *E. coli* and *K. pneumoniae* reinforce the potential of these drugs in treating coliform-associated diarrhea. The inhibitory activity of *Vernonia amygdaline, Melia azedarach*, and *Calpurnia aurea* was comparable to gentamicin. These results support the potential of plant-based extracts as alternative or complementary antimicrobial agents, especially in the face of rising antibiotic resistance and limited availability of commercial drugs. Further phytochemical analysis and in vivo studies are recommended to identify active constituents and assess therapeutic applicability and the synergistic or antagonistic effects of active components. For more diversified medicinal uses conservation priority should be given to medicinal plants.

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