www.bti.org.in
ISSN 0974-1453
Research Article

IN-VITRO ANTIBACTERIAL ACTIVITIES OF SOME TRADITIONAL MEDICINAL PLANTS AGAINST FOOD-BORNE BACTERIAL PATHOGENS IN WOLDIA DISTRICT, ETHIOPIA

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ABSTRACT

Multi-drug resistant strains of bacteria increase the occurrence of bacterial infections that cannot be treated with conventional antimicrobial agents. Hence, the study assessed the antibacterial activity of some traditional medicinal plants against common food-borne bacterial pathogens. Five traditional medicinal plant leaves namely *Withania somnifera, Otostegia integrifolia, Calpurnia aurea, Discopodium penninervium* and *Croton macrostachyus* were selected by ethnobotanical techniques for crude extraction through soaking method. Petroleum ether, chloroform, and acetone were used as solvents for the extraction, and Agar disc diffusion method was performed for antibacterial activity tests. *Escherichia coli* (ATCC25722), *Staphylococcus aureus* (ATCC25903), *Shigella sonnei* (ATCC259131) and *Salmonella typhimurium* (ATCC13311) were the standard reference strains used to test the biological activity of the crude extracts. Data were analyzed using Microsoft Office Excel spreadsheet 2007 and Genstat 13th edition. Most of the crude extracts showed antibacterial activities in varied concentrations. The MIC values of the crude extracts were ranged between 50mg/ ml and 12.5 mg/ml. At higher concentration, most of the extracts were showed best activities. *Withania somnifera* acetone extract have been shown better pre-antibacterial activity against gram-positive bacteria. While *D.*

penninervium **chloroform extract was showed the best activity at lowest MIC value of 12.5mg/ml. The crude extracts of the five traditional medicinal plant species are candidate products for the treatment of gastrointestinal ailments caused by the tested bacterial. While, additional investigation on the structural identification of the crude extracts, toxicity tests, and other screening process were recommended for further study.**

Keywords: Antibacterial activities, ***Croton macrostachyus, Calpurnia aurea, Discopodium penninervium*****, Food borne pathogens,** ***Otostegia integrifolia, Withania somnifera***

INTRODUCTION

Nearly all cultures and civilizations from ancient times to the present day use herbal medicines, which represent a vast untapped source of medicine as antimicrobial sources to cure infections (Palombo, 2009). There are more than 35,000 plant species being used in various human cultures around the world for the medicinal purpose (Koshy *et al*., 2009). In the ways, the rapid development of multi-drug resistant strains of bacteria has increased the occurrence of bacterial infections that cannot be treated with conventional antimicrobial agents. Food-borne illnesses are a global problem and mostly it is caused by bacterial pathogens. In the United States more than 300,000 people are hospitalized and 5,000 people died from foodborne illnesses (Delaware Health and S ocial service, 2009). According to Pirbalouti *et al*. (2010) report, *Escherichia coli*, *Staphylococcus aureus, Bacillus cereus, Salmonella* and *Shigella* species are the most common foodborne bacterial pathogens, which has great human health complication. Hence, attempts were made to discover new antimicrobial agents from plants and other natural sources (Parekh and Chanda, 2007). According to Taylor (2005), there are at least 120 distinct chemical substances derived from plants that are considered important drugs and are currently in use in one or more countries in the world. Approximately 25% of the drugs used today are plant origin (Bruce, 2002). Drugs obtained from plants have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor, antibacterial and antioxidant activities (Buhler, 2000). The drugs, morphine, codeine, quinine, aspirin, and Taxol are examples of some well-known plant derived standardized drugs (Van-Wyk *et al*., 1997). Due to its long period of practices and existences, traditional medicines have become integral parts of the cultures of Ethiopian people. Ethnobotanical surveys conducted in Ethiopia illustrated the use of plants as a source of traditional medicines since time immemorial for combating different ailments. However, scientific investigations that proved the efficacy of traditional medicinal plants are limited in Ethiopia. Hence, the authors in the present have attempted to assess the traditional uses and antibacterial activities of some Ethiopian traditional medicinal plants found in namely*: Croton macrostachyus, Withania somnifera, Discopodium penninervium, Otostegia integrifolia* and *Calpurnia aurea*

against common food-borne bacterial pathogens.

MATERIALS AND METHODS

Identification and Preparation of plants for extraction

Ethnobotanical surveys were performed from February-May, 2013 to identify the most five preferred traditional medicinal plants, which are used for the treatment of human gastrointestinal ailments in Gubalafto District, Northern Ethiopia. Accordingly, data were collected from a total of 60 indigenous knowledgeable respondents, their age between 20-84 years and the five most preferred traditional medicinal plants, namely: *Croton macrostachyus, Withania somnifera, Discopodium penninervium, Otostegia integrifolia* and *Calpurnia aurea* were identified based on the total sum of informant use reports for in-vitro antibacterial activity tests (Table 1). Specimens of each medicinal plant species and their leaves for extraction were collected from different wild areas of Woldia District with the help and guidance of interviewed informants. The scientific names of each medicinal plant species were identified at the National Herbarium (ETH), Addis Ababa University by using taxonomic keys and descriptions given in the relevant volumes of Flora of Ethiopia and Eritrea books (Hedberg and Edwards, 1989; Edwards *et al.,* 1995; Hedberg *et al.,* 2006). Finally, the accuracy of identifications was confirmed by a senior plant taxonomist in Addis Ababa University and the voucher specimens with labels were deposited at ETH. The collected leaves of traditional medicinal plant species from the wild were disinfected, air-dried and grounded into a fine powder for extractions. The crude extractions were conducted in a chemistry laboratory, Wollo University and evaluations of the antimicrobial activities of the plant extracts were carried out in Dessie Regional Health Research Center from July-October, 2013.

Ethical considerations

Informants have informed the objectives of the research during ethnomedicinal surveys in support of collaboration letters obtained from Woldia University Research and Development Office and Woldia Town Administration Office. Finally, research data were collected from the informants after they gave us oral consent.

Extraction process

Forty grams of dried clean leaves of *Croton macrostachyus, Withania somnifera, Discopodium penninervium, Otostegia integrifolia, and Calpurnia aurea* were soaked in conical flasks containing 400 ml petroleum ether solvent. All dried leaves were soaked for 72 hours with shaking of the extracts at the intermediate time. After 3 days, the extracts were filtered by using a Voucher separatory funnels, and the collected extracts were further separated by rotary evaporator at $40^{o}C$ reduced the temperature. Finally, the crude extracts were placed in desiccators containing CaCl2. The dried extracts were stored in the refrigerator for further uses. The second and third gradient extraction procedures were performed with the same steps by using chloroform and acetone solvents.

Culture Media Used

Muller-Hinton Agar (Oxoid, UK), Nutrient Broth (Himedia, India) and Nutrient Agar (Science Company, UK) were used during the study. Muller Hinton Agar was used for the antimicrobial tests and Nutrient Broth was used for determination of minimum inhibitory concentration. Moreover, Nutrient Agar was used for routine stock cultures and subcultures.

Test microorganisms and microbial culture

Three Gram-negative bacteria namely: *Escherichia coli* (ATCC 25722), S*almonella typhimurium* (ATCC 13311) and S*higella sonnei* (ATCC259131) and one Gram-positive bacterium, it's name *Staphylococcus aureus* (ATCC 25903), those causes foodborne bacterial infections, were used to evaluate the antimicrobial activity of crude extracts of the traditional medicinal plants. The microorganism strains for the tests were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) Clinical Bacteriology Laboratory in Addis Ababa. The bacterial strains were reactivated by sub-culturing in nutrient broth at 37°C and maintained on the nutrient agar slants at 4°C for further activity.

Standardization of Inoculum

The 0.5 McFarland turbidity standard was prepared by adding 0.5 ml of a 1.175% ((W/V)) barium chloride dihydrate ($BaCl_2 \bullet 2H_20$) solution to 99.5 ml of 1% (v/v) sulfuric acid (H_2SO_4). This mix was considered to be equivalent to a cell density of 1 to 2 $x10^8$cfu/ml (Doughari, 2006). The turbidity standard is matched with a bacterial suspension used to prepare the inoculum. McFarland turbidity standard tubes were sealed with Parafilm, to prevent evaporation. Barium sulfate turbidity was compared with identical tubes containing inoculums 0.85 % NaCl saline solution.

Antibacterial activity tests

According to (Tambekar *et al*., 2008), the agar disk diffusion method was used to evaluate the antibacterial activities of the medicinal plant species leaf extracts. The 24 hours plate cultures of 0.5 McFarland standard (1 to 2 x 10^8 CFU mL−1) bacterial suspensions were uniformly spread on Mueller-Hinton Agar plate (Oxoid) to form lawn cultures. The petroleum ether, chloroform, and acetone crude extracts were dissolved in tween-20 solvent. The stock solutions were prepared in the amount of 100mg/ml for each solvent extract. The blotting paper discs (6 mm diameter) were soaked in various dilute solvent extracts and dried for 5 minutes to avoid the flow of extracts in the test media. Antibacterial activity of potential plant extracts against bacterial pathogens by disc diffusion techniques were identified after incubation for 24 hours at 37°C and the respective zones of inhibition produced by the extracts were measured and recorded. Standard antibiotic disc (Tetracycline 30 µg) and Gentamycin (10 µg) were used as a positive control and tween-20 solvent taken as a negative control.

Determination of Minimum Inhibitory Concentration (MIC*)*

For the identification of MIC values, the extracts that exhibited a considerable activity were diluted with nutrient broth in a series of four test concentrations (50mg/ml, 25 mg/ml, 12.5mg/ml and 6.25mg/ml) as described by Koshy *et al*. (2009).Then 0.1mL (100µL) of standard inoculum (1 – 2 × 10^8cfu/ml) was added to each test tube. Control tubes without extracts were maintained simultaneously. The tubes were incubated aerobically at 37°C for 24 hours. The lowest concentration of extract that produced no visible bacterial growth (no turbidity) was considered as Minimum Inhibitory Concentration (MIC).

Data analysis

The overall data obtained from the study were analyzed by Genstat 13th edition. Means and standard deviation of the triplicate tests of the antibacterial activities of the crude plant extracts were calculated by descriptive statistics. Two way ANOVA was used to test the significant variation among extracts, bacterial strains and bacterial strains and extracts using least significant difference (LCD) values. Statistical differences were considered at $P \leq 0.05$.

RESULTS AND DISCUSSIONS

Taxonomy and Traditional Uses of Extracted Traditional Medicinal Plants

The extracted five medicinal plant species were found to be under five genera and four families, of which the family, Solanaceae consisted of two species, which is the highest, whereas the others consisted of one each (Table 1). Besides the treatment of gastrointestinal ailments, the extracted traditional medicinal plants have been used for other health care remedy preparations in the study area (Table 1). In addition, these traditional medicinal plant species were also recorded as traditional medicines by other researchers in Ethiopia (Yineger and Yewhalaw, 2007; Tambekar et al., 2008; Flatie *et al.*, 2009; Lulekal *et al.*, 2013; Agize *et al.*, 2013).

Table 1.The taxonomy and traditional uses of studied medicinal plant species in Woldia District.

Scientific names	Local names (in Amharic)	Family	No. of reports by informants	Disease treated	Parts Used	Method of preparations	Voucher specimen Number
Withania somnifera (L.) Dunal in DC.	Giziewa	Solanaceae	30	Gastrointestinal	Leaves	Crush, squeeze then drink	GC048
				Impotency	roots	Peel, chew and absorb the juice	
				Evil spirit	All parts	Fumigate the house	
Otostegia integrifolia Benth.	Tinjut	Lamiaceae	25	Stomach ache	leaves	Rub, squeeze then drink	GC141
				To kill flea	Stem and leaves	Fumigate the reservoir	
*Calpurnia aurea (*Ait.) Benth.	Digita	Fabaceae	23	Diarrhea	Leaves	Rub, squeeze then drink	GC020
Discopodium penninervium Hochst.	Segeleteji ni	Solanaceae	21	Stomach ache and diarrhea	Leaves	Rub and squeeze then drink with water	
				Evil spirit	leaves	Fumigate the house/immerse in water then spray the home	
Croton macrostachyus Del.	Mekanisa	Euphorbiaceae	18	Gastrointestinal problems	Leaves	Rub, squeeze then drink with water	GC130
				Fibril illness	leaves	Boiled and fumigate all the body	

Extracted Yields

The crude extract amounts of the traditional medicinal plants greatly vary according to the plant species and solvent type used for the extraction (Table 2). Chloroform extract of *O. integrifolia* extract yield was the highest (11.5 %) followed by *D. penninervium* Petroleum ether extract (8.5%). The highest yields of extracted plant species could reflect the compatibility nature of chemical components of plants and solvents used. In addition, the variation found in the amounts of the same plant species crude extracts could be the different solubility of chemical compounds with respect to different solvents (Mofor *et al.*, 2013).

Table 2. Extraction yields of medicinal plant species from 40-gram sample

Name of Medicinal Plant species	Solvents Used For Extractions	Yields (%)
D. penninervium	Petroleum ether	8.5
	Chloroform	2.75
	Acetone	4.25
C. aurea	Petroleum ether	6
	Chloroform	5.25
	Acetone	5
W. somnifera	Petroleum ether	5.25
	Chloroform	4.5
	Acetone	1.25
O. integrifolia	Petroleum ether	5
	Chloroform	11.5
	Acetone	6
C. macrostachyus	Petroleum ether	2
	Chloroform	3.5
	Acetone	3.75

Antibacterial activities of crude extracts

There were significant vibrations in the antibacterial activities of 15 extracts from the bacterial strains (Table 3). *Calpurnia aurea* and *W. somnifera* acetone extracts showed better activities against the test strain *S. aureus* with a mean zone of inhibition values of 15.6±4 and 20±1 mm respectively. The finding showed that acetone extract of *W. somnifera* has broad spectrum against both gram-positive and gram-negative bacteria except *S. sonnei*. The finding matched with the earlier conclusion made by (Pandey et al., 2011). The finding also agreed with the previous study investigated by Al-Ani *et al*. (2013) that *W. somnifera* acetone extracts were active against *S. typhimurium* and *E. coli.* In addition, other studies confirmed that different extracts of *W. somnifera* were found active against antifungal and antibacterial pathogens (Satish and Mahesh, 2008; Senthil and Vinoth, 2011; Singariya *et al*., 2012; Subbaiah and Savithramma, 2013). Methanol extracts of *C. aurea* also showed an activity against *S. typhimurium, Shigella* spp., *E. coli* and *S. aureus* (Umer *et al*., 2013) and *E. coli* and *S. aureus* (Adedapo *et al*., 2008).

The current study revealed that petroleum ether and chloroform extracts of *C. macrostachyus* showed antibacterial activities for S. *aureus* and *S. sonnei* but Acetone extracts only active for *S. typhimurium.* Related to the current finding, the methanol extracts *C. macrostachyus* showed antioxidant and antifungal activities (Mofor *et al*., 2013). In Ethiopia, the plant, *C. macrostachyus* root, fruit and leaf extracts were also active against antimalarial activity (Mekonnen, 2014 and Mohammed *et al*., 2014).

Petroleum ether extract of *O. integrifolia* showed the highest activities against the gram-negative bacteria, *S. typhimurium*, *S. sonnei* and *E. coli* with a mean zone of inhibition values of 12.3±0.5, 6±2.3 and 16.3±3.5 mm respectively. According to Tadesse *et al*. (2001), study on the composition, antimicrobial and antioxidant activity of *O. integrifolia* showed the extracted oils were also active against bacterial pathogens. However, activities against the gram-negative bacteria were null for *C. aurea* acetone extract and minimal for *W. somnifera* acetone extract. Likewise, *D. penninervium* petroleum ether extract was not showed activities against both the gram-positive and gram-negative bacterial strains (Table 3). The average mean difference between extracts, bacterial strains and bacterial strains and extracts greater than the LCD values showed that a significant difference in antibacterial activities between and among the five medicinal plant species (Table 3).

Generally, all the studied medicinal plant species have not shown equivalent activities. There might be several factors such as season of collection, habitat, ways of the collection, drying and storage place of medicinal plant parts, type of bacterial strain, the safety of extracts and materials used for culturing purpose, which could affect or reduce the antimicrobial activities of traditional medicinal plants (Rao, 1995). In addition, this study substantiated the use of informant consensus results (informant use reports) from ethnomedicinal plants study for antibacterial bioassay screening.

Table 3. Antibacterial activities of extracted medicinal plant species (at concentration 100 mg/ml)

Key: NA; Extract has no activity. Values are mean inhibition zone (mm) ± S.D of the triplicates. Least significant differences of mean (5% level) value between extracts is 0.997; between bacterial strains is 0.515 and between bacterial strains and extracts type is 1.995.

Extracted traditional medicinal plant species	Solvents used for extractions	Mean zone of inhibition on bacterial strains			
		***S. aureus* (ATCC25903)**	***S. typhimurium* (ATCC13311)**	***S. sonnei* (ATCC259131)**	***E. coli* (ATCC25722)**
D. penninervium	Petroleum ether	NA	NA	NA	NA
D. penninervium	Chloroform	12.3±0.57	NA	NA	NA
D. penninervium	Acetone	9.6±2.5	NA	NA	NA
C. aurea	Petroleum ether	13±5	NA	6±0	NA
C. aurea	Chloroform	NA	6.3±0.57	NA	7.6±0.57
C. aurea	Acetone	15.6±4	NA	NA	NA
C. macrostachyus	Petroleum ether	7.3±1.5	NA	6.3±0.5	NA
C. macrostachyus	Chloroform	6±0	NA	10.6±1.15	NA
C. macrostachyus	Acetone	NA	8.3±3.2	NA	NA
O. integrifolia	Petroleum ether	9.3±1.15	12.3±0.57	6±2.3	16.3±3.5
O. integrifolia	Chloroform	NA	NA	6.6±0.57	NA
O. integrifolia	Acetone	10.3±1.5	NA	NA	NA
W. somnifera	Petroleum ether	NA	NA	7.3±0.57	NA
W. somnifera	Chloroform	11.6±1.5	NA	NA	NA
W. somnifera	Acetone	20±1	10.3±0.57	NA	6.3±0.57

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration is the lowest concentration of plant extracts at which the extracts inhibit the test strains. From the pre-antibacterial activity tests, 23 extracts that showed better inhibition zones were taken for the evaluation of minimum inhibitory concentrations. Consequently, a series of different concentrations were prepared at 50mg/ml, 25mg/ml,12.5mg/ml and 6.25mg/ml (Table 4).

From the evaluated traditional medicinal plant extracts, DCE had the best MIC against the test strain *S. aureus*; with 12.5mg/ml value. A related study conducted in Nigeria showed that the ethanol extracted leaves of *Vernonia amygdalina* had MIC value of 100mg/ml against *S. aureus* (Ibrahim *et. al.*, 2011). But, the current results showed that DCE can inhibit the aforementioned gram-positive bacteria with a low concentration of 12.5mg/ml value. The WCE, WAE and OAE showed MIC values of 25mg/ml against the gram-positive *S. aureus.* The finding is matched to a study conducted by Ogundare (2011). Most of the extracts were active at MIC values of 50mg/ml against different strains (Table 4).

Table 4. A minimum inhibitory concentration of different medicinal plant extracts against the test strains

Bacterial strains	Name of Extracts	MIC in mg/ ml
E. coli	WAE	25
	OPE & CCE	50
S. typhimurium	WAE, OPE, CCE, & CRAE	50
S. sonnei	WPE, CRPE, OPE, OCE, CPE, CRCE, & CRPE	50
S. aureus	CPE, CRCE, DAE CRPE & OPE	50
	OAE, WCE, & WAE	25
	DCE	12.5

Keywords: DPE (*Discopodium penninervium* Petroleum Ether Extract); DCE (*Discopodium penninervium* Chloroform Extract); DAE (*Discopodium penninervium* Acetone Extract); CPE (*Calpurnia aurea* Petroleum Ether Extract); CCE (*Calpurnia aurea* Chloroform Extract); CAE (*Calpurnia aurea* Acetone Extract); WPE (*Withania somnifera* Petroleum Ether Extract); WCE (*Withania somnifera* Chloroform Extract); WAE (*Withania somnifera* Acetone Extract); OPE (*Otostegia integrifolia* Petroleum Ether Extract); OCE (*Otostegia integrifolia* Chloroform Extract); OAE (*Otostegia integrifolia* Acetone Extract); CRPE (*Croton macrostachyus* Petroleum Ether Extract); CRCE (*Croton macrostachyus* Chloroform Extract); CRAE (*Croton macrostachyus* Acetone Extract).

In classifying the efficiency of plant extracts against gram-positive and gram-negative bacteria, the much greater number could be expected to be active against gram-positive than gram-negative bacteria (Panthi and Chaudhary, 2006). A possible explanation for the case may lie in the significant differences between the outer layers of gram-negative and gram-positive bacteria. Gram-negative bacteria possess an outer membrane and a unique periplasmic space not found in gram-positive bacteria (Shan *et al.*, 2007).The resistance of gram-negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules (Shan *et al.*, 2007). The same author (Shan *et al.*, 2007) also associated the reason with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside.

CONCLUSIONS

The activities of all extracted traditional medicinal plant species against the test strains confirmed the potentials of indigenous knowledgeable persons in the treatment of ailments. This also highly supported the roles of traditional medicinal plant species in the scientific investigations like in the discovery of new drugs. The antibacterial properties of the crude extracts of all the selected medicinal plants were dependent on concentrations. The different solvents in the extraction increased activity variations within the tested strain in the same plant part. The crude extracts of WAE, DCE, and OPE could be used to isolate bioactive natural products that may serve as

basic sources for the development of new antimicrobial compounds. Further work is needed to identify and characterize the pure chemical compounds of tested traditional medicinal plants, which is responsible antimicrobial properties.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors had a significant intellectual contribution towards the design of the study, data collection and analysis and write-up of the manuscript. Getnet Chekole conducted data collection in the field, identified the plants, laboratory work, analyzed the data, wrote the draft manuscript and followed it up through revisions up to submission and after. Baye Wodajo took part in field data collection, laboratory work and checked its final version. Mulugeta Mulat did laboratory work, edited the draft manuscript and checked its final version.

ACKNOWLEDGMENTS

We thank Woldia University for the research grant and the people of Woldia District for their participation during ethnobotanical data collection. We also express our gratitude to Woldia District Administrative and Information Affairs Offices, Wollo University Chemistry Department, Dessie Regional Health Research Laboratory Center, Ethiopian Health and Nutrition Research Institute, and Addis Ababa University Taxonomists for their information and consent letter, apparatus permission for extraction, support during biological activities test, contribution of standard bacterial strains and in the identification plants, respectively.

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