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Full Length Research Paper

Antibacterial activity and phytochemical screening of some important medicinal plants against human diarrheal pathogens in Adama city, Ethiopia

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The methanol and chloroform plant extracts of *Withania somnifera*, *Azadirachta indica*, *Croton macrostachyus* and *Leonotis nepetifolia* showed significant antibacterial activity against 160 clinical isolates of *Salmonella* and *Shigella* sp collected from Pediatric Division of The Adama Referral Government Hospital, Adama, Ethiopia. The stool specimens were collected from children belonging to the age group of 0-10 years. Growth inhibitions were determined using standard agar dilution method. Preliminary screening of *W. somnifera*, *A. indica*, *C. macrostachyus* and *L. nepetifolia* revealed the presence of alkaloids, glycosides, saponins, phenols, tannins, flavanoids and terpenoids in the extracts. All the four plant extracts showed antimicrobial activity against the clinical isolates tested and it increased with increasing concentrations. *A. indica* extract exhibited maximum inhibition on both the diarrheal pathogens tested bacterial species at a concentration of 2 mg/ml. The antibacterial and phytochemical screening insured the importance of these plant extracts and can be speculated for use in the future.

Key words: Antibacterial activities, diarrheal pathogens, phytochemical extracts, growth inhibition.

INTRODUCTION

Infectious diseases account for the approximately one-half of all deaths in tropical countries (Sadeghian et al., 2011). Bacteria naturally develop resistance to antimicrobial drugs. In recent years, however, the overuse and misuse of antibiotics has caused a growing emergence of multidrug-resistant pathogens. The incidence of epidemics due to drug resistant micro organisms and the emergence of new diseases pose challenges in public health concerns. Historically, plants have already proved to be alternative source to the currently existing anti-infective agents. Plant derived medicines have made contributions to human health and well-being (Mathabe et al., 2006).

Plants have limitless ability to synthesize secondary metabolites such as tannins, terpenoids, alkaloids, flavo-noids, glycosides and phenols which have been found to have antimicrobial properties (Koehn and Carter, 2005). Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe and according to WHO, 80% of the world's population relies on plant-based traditional medicines for their primary healthcare needs (WHO, 2002). Medicinal plants would be the best source to obtain a variety of drugs in developing countries. There has been a revival of interest in herbal medicines, to control major diseases and the need to discover new molecular structures as lead compounds. The healing activity may be slow with the use of plant extracts but have permanent cure against various diseases.

Literature reviews divulge the fact that antimicrobial studies on the infections caused by the diarrheal pathogen in children and the use of phyto-medicines on them is lacking. The present study is an attempt to validate the antimicrobial potential of *Withania somnifera*,

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Table 1. Traditional medicinal uses of selected medicinal plants.

S/N	Botanical Name	Family	Vernacular Name	Traditional medicinal use
1	*Withania somnifera*	Solanaceae	Gizawwa	Gastrointestinal disorder, diarrhea, tumors, ulcers, abdominal distension, stomach ache, chest pains, headache, scabies and skin fungus
2	*Azadirachta indica*	Meliaceae	Niimi	Arthritis, birth control, cancer, dental care, diabetes, malaria, rheumatism, stress, ulcers, asthma, fever, cough, chicken pox, inflammations, foot fungi, ring worm, scabies, leprosy, intestinal worms, earaches, nosebleeds, and abdominal distension
3	*Croton macrostachyus*	Euphorbiaceae	Bissanna	Epilepsy, malaria, diabetes, rabies, fever, ring worm, skin rash, snakebite, venereal diseases, dysentery, gonorrhea, tape worms and diarrhea
4	*Leonotis nepetifolia*	Lamiaceae	Rasi kimirri	Treatment of cough, fever, cold, skin disease, menstruation problems, uterine, hemorrhage, headache, abdominal distension, dysentery, tuberculosis, snake bites and stings

Azadirachta indica, *Croton macrostachyus* and *Leonotis nepetifolia* methanol and chloroform plant extracts against the bacterial diarrheal pathogens such as *Shigella* and *Salmonella* isolated from the local pediatric patients of Adama city, Ethiopia and their comparison with the available and commonly treated antibiotics with a view of searching a novel therapeutics as a remedy for treating the diarrheal infections.

MATERIALS AND METHODS

Collection of plant materials

Four different fresh plants namely *W. somnifera*, *A. indica*, *C. macrostachyus* and *L. nepetifolia* were collected from the Hetosa Worida, Arsi zone of Ethiopia and transported to the Adama Science and Technology University, Adama. The plants were authenticated and identified by the National Herbarium, Addis Ababa University. The plants were thoroughly washed in tap water and air-dried in shade before the extractions were made. The profile of the above mentioned medicinal plants are listed in Table 1.

Solvent extractions

The dried whole plant material of *W. somnifera*, *A. indica*, *C. macrostachyus* and *L. nepetifolia* were powdered with the warring blender and 100 g of the shade-dried powder was filled and extracted successively with methanol and chloroform separately using shaker at 70 rpm for 48 h. The solvent extracts were concentrated using a Rota-vapor (Buchi R-205 Switzerland) at 40°C under reduced pressure and preserved at 5°C in airtight glass container throughout the study.

Collection of clinical pathogens

The clinical bacterial isolates of Salmonella (n = 80) and Shigella sp. (n = 80) were obtained from the Diagnostic Microbiology Laboratory, Adama Referral Government Hospital, Adama, Oromia Region, Ethiopia for a period of 3 months starting from February to April 2014. These isolates were sub-cultured on Salmonella-Shigella agar (SS agar) media and MacConkey agar media and were incubated at 37°C for 48 h and standard biochemical tests were carried out for identification of the genus. After identification, the cultures were suspended in saline for further assaying with plant extracts.

Isolation and identification of bacterial isolates

The diarrheal pathogens (*Salmonella* and *Shigella* sp.) were isolated and identified based on the following tests: Gram staining, Nitrate reduction test, Urease test, Indole test, Methyl red test, Voges – proskauer test, Citrate, Catalase test, Triple sugar iron test and Mannitol Motility tests according to Brock et al. (2009). The pure and isolated microbes were used for antimicrobial assay.

Standard strain and antibiotics

Standard strain of *Escherichia coli (*ATCC 29522) was also kindly provided by the hospital authorities for comparative studies. Concurrently, chloramphenicol, tetracycline and norfloxacin were used as positive controls and these were procured from the diagnostic microbiology laboratory, Adama Referral Government Hospital, Adama, Ethiopia.

Evaluation of antimicrobial activity

The antimicrobial effects of the plant extracts on the clinical isolates were evaluated using standard agar dilution method (CLSI 2007). To 15 ml of Mueller-Hinton agar medium containing either the plant extract or the antibiotics were added and distributed to each Petri dish. Concentrations ranging from 0.025 to 4 mg/ml and 0.001

to 0.06 mg/ml were used for plant extracts and antibiotics; respectively. Then the isolates were inoculated onto the agar surface before incubation. Triplicate experiments were conducted for each plant extract and suitable positive and negative controls were included with their respective solvents. *E. coli* ATCC29522 were used as a positive control for comparative purpose.

Data analysis

The results are statistical ANOVA based on the three replicates done for each of the four crude extracts of the medicinal plants. Percent inhibitions of each treatment are averaged and compared with that of the negative control:

$$\text{Growth inhibition (\%)} = \frac{DT - DC \times 100}{DC}$$

where: DT - average diameter of bacterial colony with extract treatments; and DC - average diameter of bacterial colony in control treatments.

Phytochemical analysis

Alkaloids

About 5 g of the sample was taken in 250 ml of 20% acetic acid in ethanol and kept for 4 h. This was filtered and the extract was concentrated using the water bath until the volume reduced to one-fourth of the original volume. Then concentrated NH4OH was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitation was collected by filtration and weighed (Obadoni and Ochuko, 2001).

Glycosides

About 5 ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlaid with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout the thin layer.

Saponins

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion (Trease and Evans, 2000).

Phenols

The fat free sample was boiled with 50 ml of ether for 15 mins. 5 ml of extract was pipetted into a 50 ml flask, and then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrate amyl alcohol were also added. The sample was made up to the mark and left to react for 30 min. The absorbance of solution was recorded using a spectrophotometer at 505 nm (Obadoni and Ochuko, 2001).

Tannins

About 500 mg of the sample was weighed into 100 ml plastic bottle, after which 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipetted out into a tube and mixed with 3 ml of 0.1M FeCl3 in 0.1N HCl and 0.008 M potassium ferrocynide. The absorbance was measured with spectrophotometer at 120 nm wavelength, within 10 min. A blank sample without plant extract was prepared and absorbance was recorded at the same wavelength. A standard was prepared using tannic acid to get 100 ppm after which the absorbance was measured (Van-Burden and Robinson, 1981).

Flavonoids

About 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper no.42 (125 mm). The filter was later transferred to a crucible and evaporated to dryness over a water bath and weighted (Boham and Kocipai, 1994).

RESULTS AND DISCUSSION

Results from the preliminary screening of *W. somnifera*, *A. indica*, *C. macrostachyus* and *L. nepetifolia* are presented in Table 2 and it reveals the presence of alkaloids, glycosides, saponins, phenols, tannins, flavanoids and terpenoids in the plant extracts. Presence of tannins in *W. somnifera*, *A. indica* and *C. macrostachyus* shows the ability of these plants to play the role as antidiarrheal and antihaemorrhagic agent. Our results are comparable with the results obtained by Abalaka et al. (2012) and Qamaruddin et al. (2012). Saponins are present in the extracts of *W. somnifera*, *C. macrostachyus* and *L. nepetifolia*; they are harmless when taken orally and they have beneficial properties of lowering cholesterol levels in the body (El-Mahmood et al., 2010).

Infectious diseases of bacterial origin, such as

Table 2. Phytochemical contents of the medicinal plants.

Phytochemical content	*Withania somnifera*	*Azadirachta indica*	*Croton macrostachyus*	*Leonotis nepetifolia*
Alkaloids	+	-	+	+
Glycosides	+	+	-	+
Saponins	+	-	+	+
Phenols	-	-	-	-
Tannins	+	+	+	-
Flavanoids	+	-	-	+
Terpenoids	-	+	+	-

+, indicates presence of compounds in the plant extracts; -, indicates absence of compounds in the plant extracts.

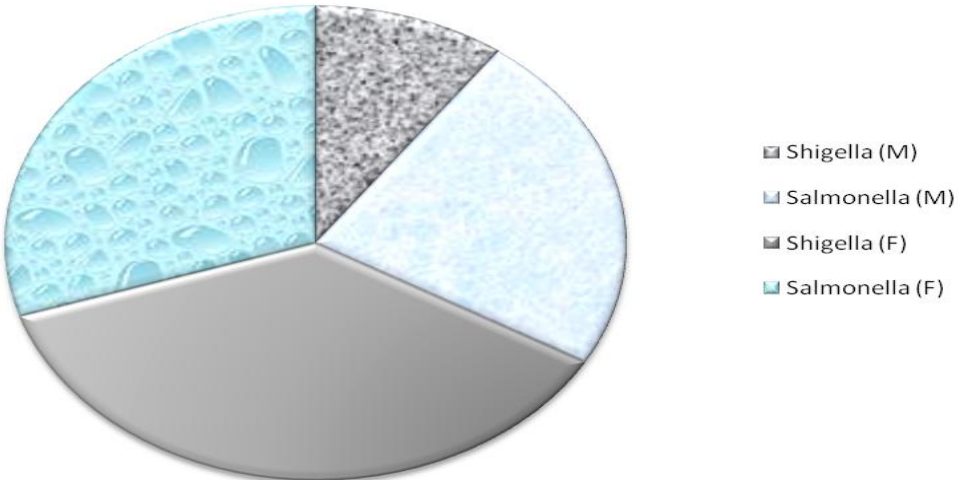

Figure 1. Prevalence of *Shigella* and *Salmonella* infections among children of both sex. Shigella (M) = % Shigella infection in male children; Salmonella (M) = % Salmonella infection in male children; Shigella (F) = % Shigella infection in female children; Salmonella (F) = % Salmonella infection in female children.

Salmonella Spp., *Shigella Spp*., etc., represent the major cause of morbidity and/or mortality in developing countries like Ethiopia. The prevalence of infection is much high in female children when compared to the male children included in this study (Figure 1). With the emergence of HIV, the task of this micro-flora has even become worse as they facilitate the infection rate by the virus or by significantly reducing the onset time of AIDS (Otshudi et al., 2000). Nowadays, there are very few, if any, antibiotics to which these micro-organisms have not developed resistance (Syed et al., 2012). The situation is further intricate by the lack of patient conformity to antibiotic regimen and by the inflated costs of the antibiotics.

The increasing incidence of diarrheal pathogens namely Shigellosis and Salmonellosis were high in children below two years of age and there is a clear correlation between the increase in age and decrease in the incidence of infection (Figure 2). Our results very well agree with the studies conducted by Otshudi et al. (2000) and Bussman et al. (2011). The spread of drug-resistant microorganisms is a big threat to successful therapy of microbial diseases especially in children (Lou et al., 2010). Therefore, there is an urgent need to search new compounds characterized by diverse chemical structures and mechanisms of action with no side-effects or allergy.

Results of antimicrobial activity of the extracts in the agar dilution method are summarized in Table 3. As expected, the methanolic extracts of *A. indica* showed maximum inhibition of both *Salmonella* (70%) and *Shigella* (80%) isolates at a concentration of 2 mg/ml which is almost comparable with tetracycline. All the four plant extracts did exhibit antibacterial activity at higher concentrations, which could be attributed to our testing with the crude extracts. From the antibacterial screening of the crude extracts of the four plants under study on selected clinical isolates, the methanolic and chloroform extracts up to a concentration of 1 mg/ml were not able to

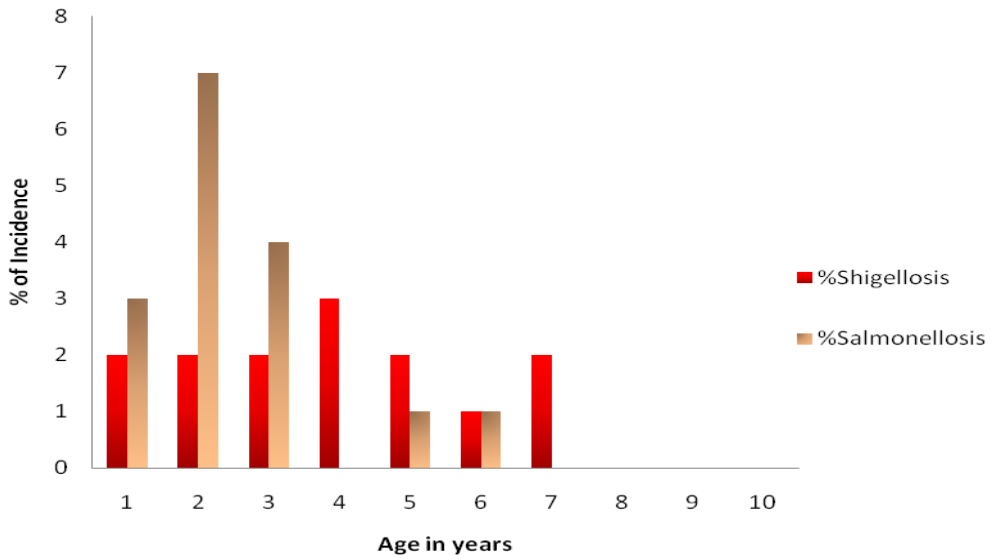

Figure 2. Incidence of *Shigellosis* and *Salmonellosis* in children with different age groups.

Table 3. Antibacterial activity of plant extracts and antibiotics against the clinical isolates (*Salmonella* and *Shigella*) and standard *E. coli* ATCC 25922.

Antibiotics/Plant extract	Growth inhibition %			
	Concentration (mg/ml)	*Salmonella*	*Shigella*	*E. coli* ATCC 25922 (positive control)
Chloramphenicol	0.005	8	0	0
	0.01	14	8	100
	0.02	43	14	100
	0.04	58	22	100
	0.08	86	32	100
	0.15	100	44	100
	0.30	100	62	100
	0.60	100	100	100
Tetracycline	0.006	0	0	100
	0.012	0	0	100
	0.25	3	0	100
	0.50	18	12	100
	0.10	46	38	100
	0.20	100	100	100
Norfloxacin	0.0001	0	14	100
	0.0002	71	21	100
	0.0004	100	100	100
Withania somnifera (ME)	1	0	0	0
	2	32	40	100
	4	96	100	100
Withania somnifera (ChE)	1	0	0	0
	2	16	20	100
	4	45	54	100

Table 3 Contd.

Azadirachta indica (ME)	0.025	0	0	0
	0.05	35	42	0
	1.0	65	75	55
	2.0	70	85	100
Azadirachta indica (ChE)	0.025	0	0	0
	0.05	18	20	0
	1.0	23	35	40
	2.0	45	52	100
Croton macrostachyus (ME)	0.025	15	0	0
	0.05	29	0	0
	1.0	42	35	38
	2.0	82	65	100
Croton macrostachyus (ChE)	0.025	0	0	0
	0.05	9	0	0
	1.0	21	16	62
	2.0	44	39	100
Leonotis nepetifolia (ME)	0.025	0	0	0
	0.05	10	0	0
	1.0	38	35	57
	2.0	72	60	100
Leonotis nepetifolia (ChE)	0.025	0	0	0
	0.05	19	6	0
	1.0	36	21	45
	2.0	88	56	100

All the values are mean ± standard error of mean of three determinations: ME = methanolic extract, ChE = chloroform extract.

inhibit bacterial growth appreciably suggesting the probable variations between the organic solvents used that result in different extract profiles (both in quality and quantity). The discernible possibility may be because the active ingredients of the plants were at lower concentrations to effect growth inhibitions (El-Mahmood et al., 2010). Overall, Salmonella strains were more susceptible to the methanolic extracts of the medicinal plants as compared to Shigella strains. However, chloroform extracts of *L. nepetifolia* did show promising inhibiting activity (88%).

It is believed that plants which are rich in a wide variety of secondary metabolites, belonging to chemical classes such as tannins, terpenoids, alkaloids, and polyphenols are generally superior in their anti-microbial activities (Cowan, 1999). The mechanism of action of tannins is based on their ability to bind proteins thereby inhibiting cell protein synthesis. Flavonoids are a diverse group of plant secondary metabolites, almost universally present in higher plants in relatively high concentrations (Abd et al., 2013). They have a wide range of biological activities that curtail largely from their ability to bind to proteins. Earlier studies have also shown that quinolizidine alkaloids, lectins, non-protein amino acids and tannins are the major components of *C. macrostachys* (Bussman et al., 2011). The antibacterial activity of this plant extract against clinical isolates of *Shigella and Salmonella* in the current study might be due to the presence of alkaloids which is in agreement with the results of previous study (Mathabe et al., 2006).

The results of this study allow us to infer that both Shigella and Salmonella causing diarrhea in children in long applications of the present day antibiotics might turn out to be mostly drug resistant (Martin-Betello, 1980; Obadoni et al., 2001). Multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune supervision and

allergic reactions (Mahesh and Satish, 2008). This probably explains the use of these plants by the indigenous people against a number of infections since generations.

As of now, little work has been done on the antimicrobial activity and plausible medicinal applications of the phytochemical compounds and hence extensive investigations are needed such as *in vivo* studies on this plant necessary to determine toxicity of the active constituents, their side effects, pharmacokinetics properties to exploit the bioactive principles,for therapeutic utility in treating the childhood diarrheal infections (Otshudi et al., 2000; Qamaruddin et al., 2010). The antibacterial activities can be enhanced if the active components are purified and adequate dosage determined for proper administration. At last, the need of the hour is the development of an effective phytocompound into an exploitable herbal product, which is devoid of side effects and drug resistance problem (WHO, 2002).

Botanical antibacterial strategies may be related to particular phytochemicals and to plant extracts characterized by the inhibitory activity of bacterial efflux pump, quorum sensing or other resistance development against the present drugs (Cowman, 1999; Koehn and Carter, 2005; Lou et al., 2010). Different plant-derived extracts and essential compounds demonstrated antibacterial activity, interfering in particular with the proteins synthesis, enzymes inhibition, production of cell wall complexes, formation of disulfide bridges and intercalation with cell wall and/or DNA, among others. Resurgence of interest and increasing consumer demand for effective and safe natural products requires programs and studies to select active antimicrobial molecules. The present study corroborates with the study of many investigators in this field.

The traditional use of plants as medicines in several countries and the recent recommendations to use natural products as an important source of antimicrobial molecules provides the basis for these studies.

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