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Review Article

IN VITRO ANTIBACTERIAL ACTIVITIES OF METHANOL AND AQUEOUS EXTRACTS OF LEAVES OF LEPIDIUM SATIVUM, AZADIRACHTA INDICA AND MORINGA OLEIFERA AGAINST SELECTED PATHOGENIC BACTERIA

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ABSTRACT

People use medicinal plants as diet, and for treatment of infectious and noninfectious diseases and they use brief procedures like frying and cooking to do so. Medicinal plants; Moringa oleifera, Azadirachta indica, and Lepidium sativum which is believed to have active components that help to treat and manage various diseases were investigated for their antibacterial activities against Staphylococcus aureus, Salmonella Typhi, Streptococcus agalactiae and Shigella boydii. Solvent methanol and aqueous were used for extraction of crudes by means of maceration. Susceptibility testing was determined by using disc diffusion method and Minimum inhibitory concentration was determined by broth dilution method. Heat treated plant material activity against test pathogen was aimed to identify resistance capacity of plant material at different interval of time and temperature. All plant extracts under study was active against all tested pathogen after exposure to 45°C for 30 min. Many of the people in the study area were illiterate and they did not have awareness about the ways use of medicinal plants. They use the medicinal plants by cooking and frying for different purposes. The antibacterial activities of the non-heat-treated extracts of Azadirachta indica were relatively low; the results of this study show that extracts of Azadirachta indica have better residual antibacterial activities. The minimum inhibitory concentration value was ranged from 2.5-10mg/ML and determined by selecting the lowest concentration of plant extract that completely inhibited the growth of the organism in the broth medium as detected by the unaided eye.

Keywords: Medicinal plants; Antibacterial activity; Disc diffusion; Heat treatment; Salmonella Typhi

INTRODUCTION

Medicinal plants play a key role in the development and advancement of modern studies by serving as a starting point for the development of novelties in drug [1] and various modern drugs were extracted from medicinal plants through the use of plant material as indigenous cure in folklore or traditional system of medicine [2]. Plant materials will continue to play a major role in the primary health care as therapeutic remedies in many developing countries. Herbal remedies were known to treat many infectious diseases throughout the history of humankind. Thus, the discovery of medicinal plants as source of antimicrobial agents is useful in expanding the wide variety of antibiotics available [3]. The majority of these herbal plants contain substances, which are precursors for the synthesis of conventional drugs, or substances that can be used for therapeutic purposes. Herbal medicines form the basis of therapeutic use in the developing countries, but recently, there has been an increase in the use of herbal medicines in the developed world too [4]. Plants have been used traditionally for centuries and modern scientific studies have shown the existence of good correlation between the traditional or folkloric application and modern drugs against a variety of infectious diseases. Thus, the existence of such correlation strengthens the search for pharmacological active components from plants [5]. Bacterial resistance to antibiotics represents a serious problem for clinicians and the pharmaceutical industry. In the last three decades, although few antibiotics were produced, their clinical efficacy was threatened by the emergence of multi drug-resistant pathogens [6]. A great efforts are being made to reverse this trend, and one of them is the wide spread screening of medicinal plants from the traditional system of medicine hoping to get some newer, safer, and more effective agents that can be used to fight infectious

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diseases [7]. With the advancement in Science and Technology, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drugs [8].

Thus, the aim of this study was to assess invitro antibacterial activity of selected medicinal plant species based on their traditional medicinal uses for infectious diseases treatment in local community of Oromia, Eastern Ethiopia. There is a strong positive motivation for further research into the antibacterial components of Azadirachta indica, Lepidium sativum and Moringa oleifera considering the fact that plants are naturally endowed with a variety of bioactive compounds. Although some of these plants are cultivated as food and for other purposes in different parts of Ethiopia, there has been little study conducted in Ethiopia with respect to their antibacterial activities against Staphylococcus aureus, Streptococcus agalactiae, Salmonella Typhi and Shigella boydii. The general objective of this study was, therefore, to determine the antibacterial activities of methanol and aqueous leaf extracts of Garden cress (Lepidium sativum), Neem (Azadirachta indica) and Drumstick tree (Moringa oleifera) against selected food born bacterial pathogens.

The specific objectives of the present study were:

- To determine antibacterial activities of non-heat-treated crude extracts of plants.
- To investigate the effect of heat on crude extracts of the plant materials.
- To determine the MIC of the non-heat-treated crude extracts of plant materials.

MATERIALS AND METHODS

Preparation of Extracts

The plants were identified and authenticated properly at the Herbarium of Haramaya University. The plant leaves were separated, washed and cleaned thoroughly with tap water and then with distilled water and air-dried in shade at botanical laboratory for several weeks. Plant extracts were prepared in accordance with the methods described in [9] with minor modifications. About 40 g of the powder was separately soaked in 200 mL of methanol and aqueous, in a 400 mL stoppered reagent bottle and the mixture were shaken for 72 hrs using an electrical shaker. The resulting mixture was first filtered with cheesecloth, then with Whatman No1 filter paper. The filtrates were then separately concentrated in vacuum using Rotary Evaporator at 37oC- 40oC. The methanol extracts were transferred carefully to labeled vials and allowed to permit evaporation of residual solvents at room temperature for 3-4 days while the aqueous extracts were dried using Lyophilizer alpha 1-2 LD plus-Martin Christ to prepare crude powder. Then the dried extracts were stored in sterile bottles and kept in refrigerator until further use. Heat treatment of plant crude extracts was done using the method described by [10] with some modifications. Dried extracts of plant materials were kept under 45, 50, and 55^{o}C for both 30 and 60 min. Then the samples were cooled at room temperature and stored in a refrigerator at 4^{o}C until further use. The disc diffusion method [11] was used to evaluate the antibacterial activity.

Preparation of micro-organism culture

The test bacterial pathogens, i.e. Staphylococcus aureus ATCC25923, Streptococcus agalactiae ATCC 12338, Salmonella Typhi ATCC13311 and Shigella boydii ATCC9202, were obtained from Ethiopian Public Health Institution (EPHI), Addis Ababa, Ethiopia. All the bacterial strains were grown and maintained on nutrient agar slants [9]. Fresh bacterial cultures were prepared by sub-culturing stock bacterial cultures into freshly prepared nutrient agar and incubating at 37oC for 24 hours. The colonies formed were picked up with a sterile inoculating loop and transferred into sterile saline solution and the turbidity was adjusted to the 0.5 McFarland's standard solution [11, 12].

Preparation of Stock Solution and Serial Dilution

Solutions of 200 mg/mL were prepared by reconstituting 1 g of each of the dried crude powder in 5 mL of respective solvent (i.e. methanol and aqueous). From this stock solution, 150 mg/mL, 125 mg/mL and 100 mg/mL working solutions were prepared and used for the susceptibility testing. Another stock solution of crude extract 20 mg/mL was prepared by reconstituting 0.1 g of each of the dried crude powder in 5 mL of methanol and aqueous solution. Five sterile test tubes were arranged on a test tube rack and 1 mL of sterile solvent solution was dispensed into them. From the stock solution, 1 mL of extract was transferred into the first test tube and then successive two-fold serial dilutions of the extracts were carried out. The resultant concentrations in the test tubes were 10, 5, 2.5, 1.25, 0.625 mg/mL [12]. They were used, along with the stock, for determination of minimum inhibitory concentration.

Determination of the Antimicrobial Activities of the Crude Extracts

Mueller Hinton agar was prepared in the plates as the media for the test microorganisms. Sterile filter paper discs (Whatman No1, 6 mm) [11] and the discs were sterilized. Each sterile disc was impregnated individually with 3 mg of 150 mg/ml, 125 mg/ml and 100 mg/ml concentration using a micropipette. Then discs impregnated with concentrations of non-heat treated and heat-treated plant extracts were placed on the spread plated Muller Hinton agar surface using sterile forceps. Disc impregnated with Amoxicillin (5 µg/mL) was used as positive control while a disc soaked with pure solvent (methanol and aqueous) was used as a negative control. The Petri dishes were then incubated at 37°C for 24 hrs. After incubation, the diameters of the zone of inhibitions were measured by using transparent ruler and the mean values of three readings were recorded.

Determination of MIC

Minimum inhibitory concentration was evaluated for extracts of non-heat-treated plant material. The MIC of each extract was determined by using concentrations (10, 5, 2.5, 1.25 and 0.625 mg/mL) of the plant extracts by using the broth dilution method [13]. 4 mL of the NB was pipetted into each of 6 test tubes. 0.1 mL of the prepared successive two-fold serial dilutions of the crude extract concentrations were mixed with the nutrient broth. Thereafter, 0.1 ml of the standardized inoculum of test pathogens was dispensed into each of the test tubes containing the suspension of nutrient broth and the crude extract. Then, all test tubes were properly corked and incubated at 37°C for 24 hrs. The tube with lowest concentration of the extract showing no growth after incubation was taken as the MIC [14].

Percentage Residual Antibacterial Activity

The percentage residual antibacterial activity (%RAA) was calculated using the following formula:

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Where, % RAA = Percentage residual antibacterial activity,

ZIAHT = Mean zone of inhibition after heat treatment and

ZIBHT = Mean Zone of inhibition before heat treatment.

RESULTS AND DISCUSSION

Antibacterial Activity of Untreated Crude Extracts

The results bring out that all plant extracts were effective in inhibiting the growth of test pathogens with varying inhibitory effects as depicted in table 1. For both solvent extract M. oleifera was the most effective extract retarding growth of Stp. aureus and Sal.Typhi at all concentrations while aqueous extracts of M. oleifera had no inhibitory activity against Str.agalactiae at 125 mg/mL concentration. In contrast methanol extracts of M. oleifera shows high zone of inhibition at 150 mg/mL against Stp. aureus, similar result was observed in the study of [15] who investigated antibacterial activity of the methanol extract of M. oleifera using the well diffusion technique and reported that the most significant activity of this plant's extract was seen against Stp. aureus. According to present study preparing an extract with methanol was shown to provide a better antibacterial activity as in the results obtained by [16]. However, the inactivity of a plant extract does not indicate the absence of bioactive constituents, nor that the plant is inactive. According to [17], active compounds may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed. The present study also implies that the activities was dose dependent and influence of solvent on extraction of metabolites required for antibacterial activity, thus the availability of crude is depending on type of solvent used for extraction and concentration of the plant material. Beside this, some factors such as rotary evaporator used during extraction of crude also trigger influence on the activities of crudes based on boiling point of solvent used for extraction. For instance, the temperature used for methanol and water is different (i.e. the temperature used during Rotary evaporator is 45oC for both solvents; because of water require high temperature than methanol, it require long duration of time than methanol). This implies that activities of water extracted plant material has reduced due to the fact that time used during rotary evaporator has influence on crude extracts when compared to low boiling point solvents. Hence, the aqueous extracted crude extract has lower zone of inhibition than methanol crude extracts.

Solvents	Conc.	Agents	Zone of Inhibition (ZI) in mm			
			Stp. aureus	*Str. agalactiae*	*Sal.*Typhi	*Sh. boydii*
	150mg/mL	*M.oleifera*	13.3 ± 1.0^{Aa}	11.0 ± 1.0^{Ab}	13.2 ± 1.97^{Aa}	8.8 ± 4.0^{Bc}
		A.indica	11.3 ± 6.07^{Ba}	9.67 ± 5.58^{Aa}	11.3 ± 1.53^{Ba}	8.2 ± 5.0^{Bb}
Methanol	125mg/mL	*L.sativum*	11.67 ± 1.00^{Ba}	10.3 ± 1.155^{Ab}	12.3 ± 5.8^{Ca}	9.2 ± 5.7^{Ab}
		M.oleifera	11.3 ± 2.65^{Aa}	8.3 ± 5.9^{Ab}	11.0 ± 1.73^{Aa}	7.0 ± 0.0^{Ac}
		A.indica	9.4 ± 2.52^{Ba}	8.0 ± 1.0^{Ab}	10.0 ± 1.00^{Ba}	6.7 ± 1.15^{Ac}
		L.sativum	10.4 ± 3.1^{Ca}	8.3 ± 1.53^{Ab}	10.67 ± 5.8^{Aa}	7.67 ± 5.8^{Bb}
Aqueous	150mg/mL	*M.oleifera*	10.6 ± 2.89^{Aa}	8.4 ± 5.8^{Ab}	10.0 ± 1.73^{Aa}	8.0 ± 1.0^{Ac}
		A.indica	8.8 ± 1.53^{Ba}	8.7 ± 1.155^{Aa}	8.4 ± 4.51^{Ba}	7.6 ± 1.53^{Ab}
		L.sativum	9.3 ± 1.53^{Ca}	9.6 ± 3.5^{Ba}	9.0 ± 1.0^{Ca}	8.8 ± 1.53^{Ba}
	125mg/mL	*M.oleifera*	9.0 ± 1.0^{Aa}	0	8.2 ± 2.52^{Ab}	6.9 ± 1.2^{Ac}
		A.indica	7.3 ± 6.43^{Ba}	7.3 ± 0.577^{Aa}	7.0 ± 1.0^{Ba}	6.6 ± 5.3^{Ab}
		L.sativum	7.7 ± 1.155^{Ba}	7.6 ± 6.67^{Ab}	7.6 ± 6.61^{Ca}	7.5 ± 2.0^{Ba}
Control	Solvents (N.C)	3 mg disc	0	0	0	0
	Amoxicillin (P.C)	5 µg disc	30.0 ± 3.61^{a}	29.3 ± 3.5^{a}	26.6 ± 1.53^{b}	25.67 ± 2.1^{c}

Antibacterial Activities of Heat-Treated Crude Extracts

The results showed that antibacterial activities were reduced with heat treatment from temperatures of 45-55oC for both 30 and 60 minutes. Relatively higher retention of antibacterial activity was observed for 30 minutes of heat treatment. Previous researchers had also reported that heat treatment (including pasteurization and sterilization) could lead to decomposition of active phytochemicals [18]. The heat-treated antibacterial activities of crude extracts were observed in table 2. The activities of all crude extracts were reduced to zero by heat treatment at a temperature of 60°C for both 30 and 60 min. The extracts of all tested plant species (M. oleifera, A. indica and L. sativum) retained their antibacterial activities after 30 min of heat treatment at 45°C against all test pathogen.People use medicinal plants like moringa oliefera, leaf for consumption by cooking; thus by using the same procedure for cooking cabbage. In case of Lepidium sativum they use the seeds for treatment of many stomach and intestinal diseases by frying on frying pan and leaves by cooking even if they need to treat any types of diseases either infectious or noninfectious diseases. In case of Azadirachta indica leaf first they ground into small peace and then they boil it very well. They do this for the sake of reducing the bad smell of the leaves because the leaves of neem have bad smell during grinding. This is due to having no further concern with the nature of plant material and lack of awareness regarding to usage of medicinal plants. It is important to explore the nature of active constituents of plant crude extracts, including their stability to heat treatment. For example, previous studies carried out for some medicinal plants such as garlic showed that the antibacterial activity was retained even at higher temperature [19].

The present study showed that the extract sustained the antibacterial activity at higher temperature. Nonetheless, there was a considerable disappear in the antibacterial activity of plant crude extracts at higher temperature (55°C), i.e. showed no antibacterial activities. The data also showed that some of the plant crude extracts were more sensitive to heat treatment than others as from the result. This was indicated by methanol extracts of A. indica, which lost its antibacterial activities against Sh. boydii after just 30 minutes of exposure to 50°C. Similarly, the methanol extract of A. indica and L.sativum lost its antibacterial activity against Sh. boydii after 30 minutes of exposure to 50°C, while the aqueous extracts of A. indica and M.oleifera lost its activities against Sh. boydii after 60 minutes of exposure to 45°C and that of aqueous extracts A. indica lost its activities against all test pathogens after 60 minutes of exposure to 50°C. Zone of inhibition of positive control was the same for both table one and two against all test pathogen.

Table 2. The antibacterial activities of heat-treated leaf extracts against pathogenic bacteria

Solvents	T°(°C)	Antibacterial agent	Time (min)	Mean Zone of Inhibition (ZI) in mm			
				Stp. aureus	*Str. agalactiae*	*Sal.*Typhi	*Sh. boydii*
Methanol	45	*M. oleifera*	30	$10.3 \pm .322^{Aa}$	$8.8 \pm .625^{Ab}$	$9.0 \pm .851^{Ab}$	$8.3 \pm .58^{Ac}$
		A. indica		$7.4 \pm .361^{Ba}$	$8.0 \pm .681^{Bb}$	$8.2 \pm .436^{Bb}$	$7.1 \pm .153^{Ba}$
		L. sativum		$9.6 \pm .56^{Aa}$	$8.30 \pm .61^{Bb}$	$9.3 \pm .67^{Aa}$	$8.3 \pm .58^{Ab}$
		M. oleifera	60	$8.0 \pm .00^{Aa}$	$8.37 \pm .551^{Aa}$	$8.57 \pm .513^{Ab}$	7.3 ± 1.13^{Ac}
		A. indica		$7.10 \pm .66^{Ba}$	$7.4 \pm .451^{Ba}$	$7.47 \pm .379^{Ba}$	$6.5 \pm .569^{Bb}$
		L. sativum		$8.2 \pm .681^{Aa}$	$7.6 \pm .31^{Bb}$	$7.6 \pm .057^{Bb}$	$7.3 \pm .70^{Ab}$
	50	*M. oleifera*	30	9.3 ± 1.15^{Aa}	$7.67 \pm .153^{Ab}$	$8.2 \pm .153^{Ac}$	7.2 ± 1.04^{Ab}
		A. indica		$6.53 \pm .116^{Bb}$	$7.3 \pm .252^{Aa}$	$7.3 \pm .21^{Ba}$	0
		L. sativum		$7.6 \pm .70^{Ca}$	$7.3 \pm .322^{Aa}$	$8.3 \pm .20^{Ab}$	$6.5 \pm .473^{Bc}$
		M. oleifera	60	$7.6 \pm .557^{Aa}$	$7.1 \pm .557^{Ab}$	$7.57 \pm .404^{Aa}$	$6.7 \pm .265^{c}$
		A. indica		0	$6.34 \pm .322^{Bb}$	$6.60 \pm .50^{Ba}$	0
		L. sativum		$6.8 \pm .20^{Ba}$	$6.50 \pm .10^{Ba}$	$7.5 \pm .551^{Ab}$	0
Aqueous	45	*M. oleifera*	30	8.7 ± 1.30^{Aa}	$8.4 \pm .46^{Aa}$	$8.3 \pm .40^{Aa}$	$7.2 \pm .51^{Ab}$
		A. indica		$7.1 \pm .51^{Ba}$	$7.53 \pm .50^{Bb}$	$7.63 \pm .12^{Bb}$	$6.7 \pm .44^{Bc}$
		L. sativum		$7.5 \pm .44^{Ba}$	$8.3 \pm .58^{Ab}$	$8.2 \pm .551^{Ab}$	$7.5 \pm .473^{Aa}$
		M. oleifera	60	$7.5 \pm .681^{Aa}$	$7.8 \pm .15^{Aa}$	$7.7 \pm .30^{Aa}$	0
		A. indica		$6.9 \pm .85^{Bb}$	$7.1 \pm .21^{Bb}$	$6.8 \pm .346^{Bb}$	0
		L. sativum		$7.1 \pm .153^{Ba}$	$6.80 \pm .10^{Ba}$	$6.6 \pm .10^{Bb}$	$6.6 \pm .551^{b}$
	50	*M. oleifera*	30	$8.5 \pm .21^{Aa}$	$7.53 \pm .551^{Ab}$	$7.3 \pm .70^{Ab}$	$6.6 \pm .361^{c}$
		A. indica		0	$6.47 \pm .42^{Ba}$	$6.60 \pm .35^{Ba}$	0
		L. sativum		$6.8 \pm .20^{Ba}$	$7.0 \pm .252^{Ca}$	$7.3 \pm .436^{Ab}$	0
		M. oleifera	60	$7.1 \pm .153^{a}$	$6.67 \pm .58^{b}$	$6.8 \pm .985^{Ab}$	0
		A. indica		0	0	0	0
		L. sativum		0	0	$6.47 \pm .31^{A}$	0

Percentage Residual Antibacterial Activity

In this study, the percentage residual antibacterial activities were calculated only for result having the highest inhibition zone (i.e. for 150 mg/mL and after heat treatment at 45oC for 30 minutes). In

this study, the untreated plant extracts showed a better antibacterial activity than the heat-treated ones. This is used to look thoroughly in order to understand which plant is sensitive to the heat and which is resistant by comparing the percentage calculated from the result obtained before and after heat treatment. As can be seen from the table 3, the percentage residual antibacterial activities of crude extracts ranged from 6.7–35% for both solvents. The lowest values of the percentage indicate that the inhibition zone of the crude extract of heat treated and non-heat-treated plant material was more or less similar and vice versa. Although the antibacterial activities of the non-heat-treated extracts of A. indica were relatively low, the results of this study show that extracts of A. indica have better residual antibacterial activities than the other two plants extract against all test pathogens except Sal. Typhi. This implies that this plant has sensitive to heat as compared to the remaining two test plants.

Table 3 Percentages mean residual antibacterial activity.

Solvents	Antibacterial agent	Zone of inhibition (ZI) in mm			
		Stp.aureus	*Str.agalactiae*	*Sal.*Typhi	*Sh.boydii*
Methanol	*M.oleifera*	23	20	32	7
	A. indica	35	22	28	13
	L. sativum	18	14	24	10
Aqueous	*M. oleifera*	18	12	17	6.7
	A. indica	19.4	14	9.2	13.5
	L.sativum	19.5	8	9	15

Determination of (MIC)

In the present study, the MIC of the extracts ranged from 1.25 mg/ml -10 mg/mL. The MIC was determined by selecting the lowest concentration of plant extract that completely inhibited the growth of the organism in the broth medium as detected by the unaided eye. The results of the MIC determination for the different plant extracts against the test pathogens are shown in table 4. According to [20], the broth dilution method requires preparing various dilutions of the compound under test in a suitable solvent and, as a result, the extracts had to be dried and re-dissolved. Hence, aqueous extracts have lower MIC as compared to methanol extracts because the plant material may contain the non-polar parts of molecules that frequently do not dissolve the intermediate polar or non-polar components of the dried extract. Using water mixable solvents such as methanol was used as optional solvent to enhance the extraction of non-water soluble material, because methanol has potential to dissolve both polarities. The higher MIC indicates that the plant extracts had weaker activities of killing or inhibiting the test pathogens and vice versa. The strongest activity was exhibited against the methanol extracts of m.oliefera against Stp. Aureus and Sal.Typhi, and methanol extracts of L. sativum against Sal.Typhi.

Table 4. The MIC of leaf extracts against four pathogenic bacteria

Source of crude Extract	Solvent Used	MIC of the methanol and aqueous leaf extracts (mg/mL) against selected bacterial pathogens			
		Stp. aureus	*Str. agalactiae*	*Sal.*Typhi	*Sh. boydii*
M. oleifera	MeOH	1.25	2.5	1.25	2.5
	Aqs.	5.0	5.0	5.0	5.0
A. indica	MeOH	5.0	5.0	2.5	5.0
	Aqs.	10.0	NCI	5.0	NCI
L. sativum	MeOH	2.5	5.0	1.25	5.0
	Aqs.	5.0	NCI	2.5	NCI

MIC= minimum inhibitory concentration, MeOH= Methanol, Aqs. =aqueous, Stp. = Staphylococcus, Str. = Streptococcus, Sal. = Salmonella, Sh. = Shigella, NCI= none of the concentrations was inhibitory

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