Research Article

Evaluation for *In-vitro* Antibacterial Activity of Selected Medicinal Plants against Food-Borne Pathogens

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ABSTRACT

Folkloric medicinal plants are commonly used all over the world and linked with different cultural believes. In most developing countries, including Ethiopia, where many people live under poor hygienic conditions; and has low access to modern health care system, the chance infection with food borne disease is undoubtedly high. As a result, peoples visit traditional healers as an alternative to the modern health care system. To this effect, five traditional medicinal plants (*Vernonia amygdalina*, *Nigella sativa*, *Ocimum sauve*, *Ruta graveolens* and *Ocimum lamifolium*) were collected for evaluation of *in vitro* antibacterial activity. Soxhlet extraction method and agar disk diffusion techniques were used to obtain the crude extracts as well as for antibacterial activity test. All the petroleum ether, chloroform and methanol crude extracts of the five medicinal plants and volatile oil were tested against standard reference strains, including *Escherichia coli* (ATCC25722), *Staphylococcus aureus* (ATCC25903), *Shigella sonnei* (ATCC259131) and *Salmonella typhimurium* (ATCC13311). The essential oil extract of *Nigella sativa* seeds showed better activity against the Gram negative bacteria *Salmonella typhimurium* at concentration of 3.7 µl. Based on our observation, it could be concluded that the crude and volatile oil extracts of the plants are candidate products for treatment of gastrointestinal pains having gone through additional investigation on pharmacokinetics and toxicity of the extracts.

Keywords: Medicinal plants, MIC, *Vernonia amygdalina*, *Nigella sativa*, *Ocimum sauve*, *Ruta graveolens*, *Ocimum lamifolium.*

INTRODUCTION

Traditional medicinal plants have indefinite therapeutic role in the world. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been extracted from natural sources, many based on their use in traditional medicine. Various medicinal plants have been used for years in daily life to treat disease all over the world. There are more than 35,000 plant species being used in various human cultures around the world for medicinal purpose[1]. At moment effective drug discovery from medicinal plants in Pharmacognosy are well practiced because of the increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microorganisms[2]. The drugs Morphine, codeine, quinine, aspirin and Taxol are examples of some well-known plant derived standardized drugs[3]. Discovery of huge amount of therapeutic drugs from folkloric medicinal plants may solve problems arising from multidrug resistance food borne pathogenic microorganisms. Foods borne illness are global problem in developing and developed countries.

Food spoilage or deterioration is predominantly caused by the growth of microorganisms on unhygienic food products and consuming it lead to illness. All persons are at risk of food borne illness. Each year in the United States, food borne illnesses infect an estimated 76 million people. More than 300,000 people are hospitalized and 5,000 people die because of the illnesses[4]. The young, old and immune compromised are more susceptible to complications.

According to Kansas Department of Health and Environment Division of Health[5]. *Bacillus cereus*, *Staphylococcus aureus*, and *Clostridium botulinum* are well-documented toxin producing food borne agents. *E. coli* O_{157}:H_7 and *Shigella spp.* also produce toxins that cause disease, which may lead to severe complications.

In Ethiopia, the majority of peoples that lives in rural area and the poor people in urban areas rely mainly on traditional medicines to meet their primary health care needs. Even if their culture and attitude contributed to their usage of traditional medicine, they have no scientifically proved know how. As a result, most of the people may get exposed to unnecessary health problem due to unfortunate custom of the traditional medicinal plants. In Ethiopia vast knowledge on the traditional uses of medicinal plants is not fully documented and most of the knowledge is conveyed from one generation to the other through words of mouth. The danger of losing valuable information is thus high considering the increasing cultural change, mobility and displacement of communities due to several factors. Moreover, traditional healers have passed on their knowledge only to the members of their own families. This practice of secretive transfer of information has been accompanied with the negligence of existing generation due to expansion of modern medicine. Traditional healers are confused with a condition, where they could hardly find their successors

who could scientifically prove and document the significance of traditional knowledge on medicinal plants for coming generation[6]. In addition of the above problems most of the Ethiopian research emphasized on the check-list form of the selected medicinal plants rather than evaluating efficacy of the potential plant extracts and phytochemical compounds. Considering the aforementioned issue, five medicinal plants (*Ruta graveolens*, *Vernonia amygdalina*, *Nigella sativa*, *Ocimum lamifolium* and *Ocimum Sauve*) which have been used as folk medicine for treatment of abdominal diseases, were selected for evaluation of antibacterial activity of their crude and essential oil extracts against *Escherichia coli* (ATCC25722), *Staphylococcus aureus* (ATCC25903), *Shigella sonei* (ATCC259131) and *Salmonella typhimurium* (ATCC13311). The finding of this study is, therefore, helpful to create a bridge for aforementioned knowledge gap by evaluating antimicrobial active of the crude extracts and essential oil of some of the commonly used medicinal plants against common food borne bacterial pathogens.

MATERIALS AND METHODS

Sampling Site and Study Area

The sampling sites were Serbo town and Jimma town, located 330 and 353 km southwest of Addis Ababa, respectively. Evaluation of the antimicrobial activities of the plant extracts and phytochemical constituency was carried out in Microbiology Laboratory, Department of Biology, College of Natural Sciences, Jimma University. Moreover, plant material extraction of the candidate traditional medicinal plants was conducted in Chemistry Laboratory, College of Natural Science, Jimma University.

Collection and Identification of Plant Materials

Plants or plant parts used in folklore medicine were collected from different areas of Jimma Zone particularly from Serbo town's wild places. But plants selected from Jimma town were purchased from market. Based on the ethnomedicinal survey information obtained from local inhabitants, five plants were found most important for the treatment of stomach-ache in the study area. These plants are 'Anchebi' (*Ocimum sauve*), 'Tena Adam' (*Ruta graveolens*), 'Damma kessie' (*Ocimum lamiifolium*), 'Tikur Azmuid' (*Nigella sativa*) and 'Yegrawa Qitel' (*Vernonia amygdalina*). Each specimen was labeled, numbered, annotated with the date of collection, the locality and their medicinal uses and their approximate dosages of administration were recorded. The specimens were identified at Jimma University by experienced plant taxonomist, Voucher specimens were deposited at Jimma university herbarium.

Extraction of the Study Plant Materials

The seeds of *Nigella sativa* and leaves of *Vernonia amygdalina, Ocimum sauve, Ruta graveolens* and *Ocimium lamifolium* were extracted by Soxhlet apparatus in gradient extraction by using solvents of different polarity. Petroleum ether, chloroform and methanol have been used for extraction. According to modified Samidurai and Saravanakumar[7] method the clean shade dried leaves and seeds of the study plants were crushed into small pieces by mortar and pestle and twenty five gram of the powdered plant material was placed in the extraction thimble. The plant material was extracted successively in 500ml of petroleum ether, chloroform and methanol. After extraction, the solvent mixture evaporated by using rotary evaporator at 40 °C reduced temperature to get concentrated extracts. The concentric extract was placed in to desiccators for more than 15 days for removing the remaining solvent mixture and to obtained the dried crude extract.

Extraction of Essential oil using Hydro Distillation Technique

Extraction of essential oil was conducted according to modified Abed[8] method. Briefly, clean dried seeds of *Nigella sativa* (100gm) and fresh leave of *Ruta greaveolens* (100gm), *Ocmium lamifolium* (60gm), *Vernonia amygladyia* (100gm) and *Ocmium Sauve* (100gm) were placed into a distillating pot; 500 ml of water was added and the mixture was subjected to conventional steam distillation using the Clevenger apparatus for 3-6 hours. The oil was then separated and the remaining moisture content was absorbed by adding anhydrous sodium sulphate. The oil obtained was placed in glass vial sealed with parafilm and kept in refrigerator protected from direct light.

Test Microorganisms and Microbial Culture

Three Gram negative bacteria; (*Escherichia coli ATCC 25722*, *Salmonella typhimurieum ATCC 13311* and *Shigella sonnei ATCC259131)* and one *Gram* positive bacteria (*Staphylococcus aureus ATCC 25903)* known to cause food borne bacterial infection were used to evaluate the antimicrobial activity of crude extracts and essential oil of medicinal plants. The test microorganisms were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) clinical bacteriology laboratory, Addis Ababa. The Bacterial strains were reactivated by sub culturing in nutrient broth at 37°C and maintained on nutrient agar slant at 4°C for further activity test.

Antibacterial Activity Test via Agar Disk Diffusion Assay

Tambekar[9] Agar disk diffusion method was used to evaluate the antibacterial activities of medicinal plant extracts. The 24 hours plate cultures of 0.5 Mc Farland 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 methanol crude extracts were dissolved in tween-20 solvent. The stock solutions were prepared at amount of 100mg/ml for each solvent extracts. The blotting paper discs (6 mm diameter) were soaked in various dilute solvent extracts, and dried for 5 minutes to avoid flow of extracts in the test media. Antibacterial

activity of potential plant extract against bacterial pathogens by disc diffusion technique were identified after incubation for 24 hr. at 37°C, by measured the zone of inhibition of growth in mm.

Tween-20 solvent was used as negative control.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration of the crude extracts and essential oils were determined as described by Komuraiah[10].

To obtain the minimum inhibitory concentrations; the extracts that exhibited considerable activity were diluted with nutrient broth in a series of six test concentrations 20mg/ml, 6.66 mg/ml, 2.22mg/ml, 0.74mg/ml, 0.24mg/ml and 0.08 mg/ml.

For volatile oils the concentrations were 100µl, 33.33 µl, 11.11 µl, 3.70 µl, 1.23 µl, and 0.41µl. Then 0.1ml (100µl) of McFarland standard inoculums (1-2 × 10^8 cfu/ml) was added to each test tube. Control tubes were maintained simultaneously.

The tubes were incubated aerobically at 37°C for 24 h. The lowest concentration of extract that produced no visible bacterial growth (no turbidity) when compared with control tube was regarded as Minimum Inhibitory Concentration (MIC).

Phyto-chemical Analysis

The most common Phyto-chemicals (secondary metabolites) such as alkaloids, glycosides, flavonoids, tannins, saponins and phenolics present in powdered forms of the study five medicinal plants were analyzed following methods described in literature[11-13].

Test for Tannins

Half gram of the powdered plant materials were boiled in 10 ml of distilled water in a 100 ml beaker sized and then filtered; few drops of 0.1% ferric chloride ($FeCl_3$) were added.

Formation of brownish green or a blue-black coloration indicates the presence of tannins.

Test for Alkaloids

From about 0.5 g of powdered plant materials boiled in 10 ml of prepared acid alcohol and filtered, about 5 ml of the filtrate was taken and 2 ml of dilute ammonia added.

Then 5 ml of chloroform was also added and shaken gently. The chloroform layer was extracted with 10 ml of acetic acid. Formation of a cream with Mayer's reagent confirms the presence of alkaloids.

Test for Saponins

To 0.5 g of powdered plant materials in a test tube, 5 ml of distilled water was added and the mixture was vigorously shaken. Formation of a froth Persistent for 30 min confirms the presence of saponins.

Test for Flavonoids

To a portion of an aqueous filtrate of the powdered plant materials about 5 ml of dilute ammonia solution was added. Concentrated sulphuric acid (1 ml) addition and yellow colorations that disappeared on standing indicated the presence of flavonoids.

Test for Cardiac Glycosides

To 2 ml alcoholic filtrate plant materials, 1 ml glacial acetic acid and 1-2 drops of $FeCl_3$ was added and 1 ml of concentrated H_2SO_4 followed.

Appearance of a violet ring below the brown ring confirms positive reaction for cardiac glycosides.

Test for Phenolics

To 2 ml of alcoholic or aqueous plant filtrate, 1 ml of 1% ferric chloride solution was added. After a minute appearance of blue or green color indicated presence of phenols.

Data Analysis

The overall data obtained from the study were analyzed by Statistical Analysis System soft ware version 9.2 (SAS 9.2).

During analysis; means and standard deviation of the replicates test of the antibacterial activities of both the crude plant extracts and essential oils were calculated. Statistical differences was considered at P-value less than 0.05 ($P<0.05$).

RESULTS AND DISCUSSION

Sociodemographic Characteristics of the Study Population

A total of 150 interviewee participated in provision of information pertaining to the use of medicinal plants and all the participants were aged ≥ 21 years.

Of the total interviewees, 51 (34%) were males and the rest 99 (66 %) were females with male to female ratio of almost 2:1. Of the respondents, 146 (97.33 %) were non-herbalist and the remaining 4 (2.66 %) were local herbalist.

Results of analysis of the gathered information showed that the people have been using traditional medicinal plants for treatment of stomach ache.

Based on the respondents opinion, the degree of importance of the major traditional medicinal plants were prioritized and the ranking was as follows:

Ruta graveolens (local name; 'Tena Adam') 45 (30 %), *Nigella sativa* ('Tikuir Azmuid') 26 (17.33 %), *Ocimum sauve*, ('Anchebi') 16 (10.66%), *Ocimum lamifolium* ('Damma Kessie') 13 (8.66%) and *Vernonia amygdalina* ('Girawa') 9 (6%). The profile of traditional medicinal plants used in this study is shown below (Table1).

Table 1: Profile of the five Medicinal plants commonly used in the Study area

S. No	Name of Medicinal Plants	Local Name	Family	Plant Parts Used	Traditional Uses	Place of Collection
1.	*Ruta graveolens*	Tena Adam	Rutaceae	Leaves	Stomach ache, Flavouring	Jimma town
2.	*Nigella sativa*	Tikuir Azmuid	Ranunculaceae	Seeds	Stomach ache, as flavor	Jimma town
3.	*Vernonia amygdalina*	Girawa	Astraceae	Leaves	Stomach ache	Serbo kebele
4.	*Ocimum sauve*	Anchebi	Lamiaceae	Leaves	Stomach ache	Serbo kebele
5.	*Ocimum lamifolium*	Damakessie	Lamiaceae	Leaves	Stomach ache, treatment	Serbo kebele

Table 2: Soxhlet method extracted antibacterial activities (at 100mg/ml Conc.)

Name of Extracts	*Bacterial Strains*			
	Staphylococcus aureus (ATCC25903)	*Salmonella typhimurium (ATCC13311)*	*Shigella sonnei (ATCC 259131)*	*Escherichia coli (ATCC 25722)*
NSPE	10.5 ± 0.7^{c}	10.5 ± 0.7^{c}	10 ± 0^{c}	11.5 ± 0.7^{c}
NSCE	16 ± 1.4^{b}	NA	15.5 ± 0^{b}	NA
NSME	9.5 ± 0.7^{c}	7.5 ± 0.7^{c}	NA	9.5 ± 0.7^{c}
OLPE	NA	NA	NA	NA
OLCE	9.5 ± 3.53^{c}	NA	NA	NA
OLME	NA	NA	NA	17.5 ± 3.53^{b}
OSPE	15.5 ± 0.7^{b}	NA	NA	NA
OSCE	22 ± 1.4^{a}	NA	NA	10.5 ± 0.7^{c}
OSME	9.5 ± 0.7^{c}	NA	NA	NA
RGPE	12.5 ± 3.5^{c}	NA	NA	NA
RGCE	9.5 ± 0.7^{c}	NA	NA	NA
RGME	NA	NA	9.5 ± 0.7^{c}	NA
VAPE	22.5 ± 3.5^{a}	NA	NA	NA
VACE	19 ± 1.4^{a}	NA	NA	NA
VAME	20.5 ± 0.7^{a}	NA	NA	NA

Where: NSPE, Nigella Sativa Petroleum Ether Extract; NSCE, Nigella Sativa Chloroform Extract; NSME, Nigella Sativa Methanol Extract; OLPE, Ocimium lamifolium petroleum Ether Extract; OLCE, Ocimium lamifolium Chloroform Extract; OLME, Ocimium lamifolium Methanol Extract; OSPE, Ocimium sauve petroleum Ether Extract; OSCE, Ocimium sauve Chloroform Extract; OSME, Ocimium sauve Methanol Extract; RGPE, Ruta graveolens petroleum Ether Extract; RGCE, Ruta graveolens Chloroform Extract; RGME, Ruta graveolens Methanol Extract; VAPE, Vernonia amygdalina Petroleum Ether Extract; VACE, Vernonia amygdalina Chloroform Extract; VAME, Vernonia amygdalina Methanol Extract, NA; Not have Activities. Values are mean inhibition zone (mm) ± S.D of the triplicates and the superscripted similar letters show absence of statistical significance (P<0.05) among the values. Statistically significance considered at p < 0.05.

Antibacterial Activities of Essential Oil Extract

Table 3: Antibacterial activities of essential oil extract (at 150µl conc.)

Name of essential oil	Bacterial strains			
	Staphylococcus aureus (ATCC25903)	*Salmonella typhimurium (ATCC13311)*	*Shigella sonnei (ATCC259131)*	*Escherichia coli* (ATCC25722)
NS-O	10 ± 0^{c}	25 ± 5.6^{a}	9.5 ± 0.7^{c}	12.5 ± 0.7^{c}
RG-O	9 ± 0^{c}	10.5 ± 0.7^{c}	NA	NA

Where, NS-O = Nigella sativa essential oil; RG-O= Ruta graveolens essential oil; NA= No activity. Values are mean inhibition zone (mm) ± S.D of the triplicates and similar letters show absence of statistical significance (P<0.05) among the values. Statistically significance considered at p < 0.05.

Table 4: Phytochemical constituent of the Selected Medicinal Plants

Tests for	Name of Medicinal Plants				
	Vernonia amygdalia	*Nigella sativa*	*Ocimum sauve*	*Ruta geraveolens*	*Ocimum lamifolium*
Alkaloids	-	±	-	+	-
Tannins	+	+	-	+	+
Saponins	+	+	+	+	+
Flavonoids	+	+	-	+	-
Cardiac Glycosides	+	-	+	-	-
Phenols	+	+	+	+	+

Where - Indicate absence; + Indicate presence; ± Indicate slight presence/absence

Antibacterial Activity of Crude Extracts

The study plants were extracted via soxhlet method accordingly, mean antibacterial activity values of the triplicate plant extracts (at concentration of 100mg/ml) were recorded as given above (Table 2). Petroleum ether extract of *Vernonia amygdalina* and chloroform extract of *Ocimum sauve* had better activities against *Staphylococcus aureus* with mean value 22.5 ± 3.5 and 22 ± 1.4 respectively. Methanolic extract of *Ocimum lamifolium* had better activity against the gram negative bacteria (*Escherichia coli*) with mean value 17.5 ± 3.53. In contrast; the petroleum ether extract of *Ocimum lamifolium* did not showed any activities against all the tested strains as shown above (Table2).

Phytochemical Screening of Selected Medicinal Plants

All the evaluated medicinal plants have saponins and Phenols, but differ in their constituents of Alkaloids, tannins, flavonids and Cardiac Glycosides (Table 4). The presence of Alkaloids in *Nigella sativa* is doubtful as there were in consistence between experiments.

All the crude extracts of five medicinal plants (*Ruta graveolens, Nigella sativa, Ocimum sauve, Vernonia amygdalina and Ocimum lamifolium*) obtained from soxhlet method of extraction were evaluated for antibacterial activity. Accordingly, petroleum ether extract of *Vernonia amygdalina* showed better activity against Staphylococcus *aureus* with zone of inhibition 22.5mm diameter (Table 2). Other study also showed that the ethanolic extracts of *Vernonia amygdalina* had active against the gram positive *Staphylococcus aureus*[14].

The best plant extracts that showed antibacterial activities were considered for MIC determination. Based on the issue, the MIC values crude extracts of VAPE, VACE, OSCE and RGPE were 6.66 mg/ml against *Staphylococcus aureus*. The mentioned extracts possessed different antibacterial activity as reported by[14-16]. Whereas MIC values of VAME and OLCE were 20 mg/ml against *Staphylococcus aureus*. The NSME and OSCE were MIC values 20 mg/ml and 6.66mg/ml against *Escherichia coli* respectively. None of the selected crude extracts showed activities against *Shigella sonnei* and *Salmonella typhimurium* within test concentration range.

The activities of essential oils extracted from folkloric medicinal plants were evaluated on different standard strains. The triplicate measured zone of inhibition was calculated and presented in the form of mean ± standard deviation as showed in the above (Table 3).

The essential oil of *Nigella sativa* has the highest activity against *Salmonella typhimurium* with inhibition zone diameter of 25±5.6; it has the least effect, if any, on *Shigella sonnei.* Likewise, essential oil of *Ruta graveolens* has no activity on both *Shigella sonnei and Escherichia coli.* The degree of antibacterial property depends up on several factors such as age of the plant, duration of storage, temperature; preparation of media could also indirectly affect the activities of extracts[17].

Moreover, Extracted essential oils that showed inhibition in the pre-activity test were selected for evaluation of minimum inhibitory concentration. Accordingly essential oil *Nigella sativa* showed no any activity against *Staphylococcus aureus* and *Shigella sonnei* within the evaluated concentration ranges (0.41-100 µl). However, the MIC values for *Salmonella typhimurium and Escherichia coli were* 3.70µl and 33.33 µl, respectively. Likewise, essential oil of *Ruta graveolens* has no any visible activities against the three test strains but *Staphylococcus aureus* with MIC value of 33.33 µl.

The *Nigella sativa* seeds essential oil showed MIC values 3.7 µl against *Salmonella typhimurium* and 33.33 µl against *Escherichia coli.* Similar finding was reported by[18]. Also at MIC value of 33.33 µl the Essential oil of *Ruta graveolens* did shown an activity against *Staphylococcus aureus*. The finding was also confirmed by[19].

The presences of different Secondary metabolites have contributed to antimicrobial properties observed. Our finding confirmed all the study plants contain secondary metabolites such as Saponins, Phenols and Tannins except in *Ocimum sauve.*

Hence, presence of these metabolites considerably important for antibacterial activities through different mechanisms. For instance, Shimada[7] reported that Tannin has been found to form irreversible complexes with proline rich protein resulting in the inhibition of bacterial protein synthesis.

Also the phenolic compounds are thought to be toxic to micro organisms, inhibiting the enzymes which are essential for the growth of microorganisms[20]. Others like alkaloids, saponins, flavonoids and cardiac glycosides were found to have *in vitro* antimicrobial properties[21].

Generally Phytochemicals present in medicinal plants are used for treating intestinal disorders such as diarrhea and dysentery[22]. Therefore, in our finding presence of different phytochemicals supports the practice of traditional healers for treatments of abdominal ache.

CONCLUSION

The study showed that all the selected medicinal plants have been showed different antimicrobial properties. The variation may arises from solvents used for extraction, the concentration used, time of plant collection, method of extraction and type of *in vitro* tests used. As the factors in consider from all, *Nigella sativa* seeds oil showed better antibacterial activities at least (3.7µl) concentration against *Salmonella typhimurium*. Not only the essential oils the crude extracts of VAPE, VACE, OSCE and RGPE showed better activities against *Staphylococcus aureus* up to 6.66mg/ml. The OSCE also showed promising activity against both gram-positive and gram-negative bacteria at 6.66mg/ml.

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