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Effect of ethanol and aqueous extract of *Moringa oleifera* on bacteria isolated from wound infection

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Abstract

The aqueous and ethanol extracts of *Moringa oleifera* used for the treatment of infectious disease were tested for their active against gram positive and gram-negative bacteria isolated from burns infection culture using the broth dilution and disc diffusion method. Results of this study revealed the presence of phytochemical which were active against gram positive and negative bacteria. Ethanol extracts of plant showed highest activity other than aqueous extract. The minimum inhibitory concentration (MIC) of the aqueous extracts on the test organism was 25- 100 mg\ml, while that of the ethanol extract ranged between 25 -50 mg\ml on the test organisms, the minimum bacterial concentration (MBC) ranging between 25-100 mg\ml for ethanol extract, and 25-200 mg\ml for aqueous extracts. The highest activity at 45 ^{o}C was demonstrated by the ethanol extracts of *S. rosmarinus against Staphylococcus aureus* and *Klebsiella spp*. In this study plant extracts against gram negative bacteria showed activity in acidic pH only in contrast of gram positive bacteria which were constant in plant extract. *M. oleifera* contained essential elements at higher levels. The results of this study suggest the possibility of using the ethanolic extracts of plant in treating diseases caused by the test organisms, especially when prepared at acidic pH.

Keywords: Plant extract; *Moringa oleifera*; Antimicrobial activity; Bacteria

1. Introduction

Plants play a vital role in maintaining human health and contribute towards improvement of human life. They are important components of medicines, cosmetics, dyes, beverages etc. [1] Although hundreds of plant species have been tested for antimicrobial Properties [2].

There are many cases of infection by drug resistant bacteria whereas few drugs are available effective for the treatment of such patients. Thus, it is urgently necessary to discover or develop new drugs that are effective on such drug resistant bacteria. We have been trying to discover novel compounds, such as antimicrobial compounds and inhibitors of drug resistance systems in bacteria, [3] that are effective against multidrug-resistant bacteria. Though *Moringa oleifera* L.is known as one of the herbs that has antimicrobial activity, there are few papers that have showed its antibacterial activity, as well as has shown anti-fungal, anti-viral properties that make it a useful weapon in combating many illnesses [4]. *M.oleifera* is cultivated in several countries mainly to obtain dried leaves to be used as raw material in medicine, perfumery and food industry [5]. Oleander comprises one of the largest genera of flowering plants in the world [6]. A large number of preservative compounds have been introduced on to the market but many of them have not gained acceptance because of chemical toxicity, Lowe efficacy, high cost, or corrosiveness [7].Certain wood preservatives have been banned or limited for some applications such as chromated copper arsenate in some European countries, the United States, and Japan [8] Since some natural extractives contain tannin or have toxic effects against biotic agents, they could be preferred for protection of wood or wood based objects against destroying organisms [9].

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Ability of natural plant extracts to protect wood against degrading fungi and insects have been one possible approach. Oleander is one of the most poisonous plants and contains numerous toxic compounds. Many of Oleander's relatives have similar leaves and contain toxic compounds. The entire plant including the milky white sap is toxic and any part can cause an adverse reaction. Oleander is also known to hold its toxicity even after drying [10]. The objective is developing new wood preservatives [11and 8]. The goal of this investigation is to discover plant products that inhibit micro-organisms, especially that cases wound infection.

2. Material and methods

2.1. Collection of plant samples

The medicinal plants used for the experiment was identified according to various literatures, and including other pertinent taxonomic literature. The part of the plant used is the leaves. Collected plants were washed thoroughly and chopped into small pieces shade dried and grinded into powdered form. Clean and dry separating funnel was taken.

2.2. Test microorganisms

Bacterial species Shigella dysenteriae; Aeromonas hydrophila; Escherichia coli; Enterobacter spp; Klebsiella spp; Pseudomonas aeruginosa and *Staphylococcus aureus* were all obtained from the microbiology laboratory in Mustansiryah University.

2.3. Culture medium and inoculum

The stock cultures of microorganisms used in this study were maintained on Plate Count Agar slants at +4°C. Cell suspensions were prepared by inoculation of each bacterium into 10 ml of Nutrient broth. Incubation was performed at 37°C for 24 h. On the next day Mueller-Hinton Agar (MHA) was prepared and cooled to -5 °C. Bacterial suspension was added into MHA to give a final concentration of 10^7 bacteria/ml and plated out.

2.4. Phytochemical screening

The plant extracts was screened for phytochemical constituents using standard procedures of analysis [12 and 13].

2.5. Antibacterial activity

The plate-hole diffusion assay as described by Ogundipe et al.,[14] was used to determine the growth inhibition of bacteria by the plant extract. The isolated bacteria from wound infection were obtained. The tests were carried out by using a stock concentration of 500 mg/ml prepared by dissolving 1g of the ethanol extract and aquatic extract into 2ml of distilled water. Nutrient agar was prepared and 25 ml each was poured into sterile petri dish. This was allowed to solidify and dry. Using a sterile cock-borer of 9mm diameter three equi-distant holes per plate were made in the set agar and were inoculated with 0.5ml over night suspension of the bacteria. Thereafter, the wells (holes) were filled with the extract solution volume 100µl at varying concentrations of 500 mg/ml, 400mg/ml and 300 mg/ml respectively. This was done in triplicate and the plates were incubated at 37 ºC for 18hours. The antibacterial activities were observed and measured using a transparent meter rule and recorded if the zone of inhibition was ‹10mm [15].

2.6. Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration where no visible turbidity is observed in the test tube (bacteriostatic concentration). The Reuben et al.,[16] was employed. In this method, the broth dilution technique was utilized where the plant extract was prepared to the highest concentration of 500mg/ml (stock concentration) in sterile distilled water and serially diluted (two-fold) to a working concentration ranging from 0.780 mg/ml to 200mg/ml using nutrient broth and later inoculated with 0.2ml suspension of the test organisms. After 18 hours of incubation at 37ºC, the test tubes were observed for turbidity. The least concentration where no turbidity was observed was determined and noted as the minimum inhibitory concentration (MIC) value.

2.7. Minimum Bacterial Concentration (MBC)

The MBC is defined as the lowest concentration where no bacterial growth is observed (bactericidal concentration). This was determined from the broth dilution resulting from the MIC tubes by sub culturing to antimicrobial free agar as described by Usman et al. [17].In this technique, the contents of the test tubes resulting from MIC was streaked using a sterile wire loop on agar plate free of bacteria and incubated at 37ºC for 18 hours. The lowest concentration of the extract which showed no bacterial growth was noted and recorded as the MBC.

2.8. The effect of heat and pH on medicinal plant extract

The samples of plant extract (one vial of 100 ml) were provided to determine the effect of heat on it, test samples were heated 45 ºC, 70 ºC, 100 ºC and 121 ºC for 15 min. [18]. To determine the effect of pH, extracts were treated at pH ranges of 3 to 8 using 1 N HCl and 1 N NaOH solutions respectively in series of test tubes for 1 h and then tested for antibacterial activity [19].

2.9. Determination of essential elements

Three gram of dried plant were taken and mixed with 8ml of concentrated H2SO4 (98%) and 2ml of HClO3 (60%) in conical flask for 24 hours which covered by watch class. Then left this mixture for 6 hours at the sand bath at 80C° , until the digestion material converted to white powder. Then add 8ml of deionized water to this powder and the trace elements were determined by flame atomic absorption spectrophotometer [20].

3. Results and discussion

Table 1 Phytochemical screening of Ethanol, Hot water and Cold water **e**xtract of *M. oleifera*

Number	Constituents	Ethanol extract	Hot water extract	Cold water extract
1	**Alkaloids**			
	i.Dragendorff's test ii.Meyer's test	+	+	+
2	Phenols			
		+	+	+
3	**Cardiac glycosides**			
	Killer-killanis test	+	+	+
4	**Flavonoids**			
	i. Shinoda's test ii. $FeCl_3$ test	+	+	+
5	**Saponins**			
	Frothing test	+	+	+
6	**Terpenes**			
	Salkowski test	+	+	+
7	**Steroids**			
	Libarman-Burchard's test	-	-	-
8	**Tanins**			
	i.$FeCl_3$ test ii.Lead acetate test	+	+	+
9	**Ratenges**			
		+	+	+
10	**Coumarines**			
		+	+	+
11	**Essensial oil**			
		+	+	+

The result of the Phytochemical screening for *M.oleifera* showed the same results are presented in Table 1. This reveals moderate concentration of alkaloids, coumarines , cardiacglycosides, ratenges, phenols, flavonoids, saponins, tannins,essensial oil and terpenes some of which chemical compounds have been associated to antibacterial activities and thus have curative properties against pathogens [21] except steroids no of plant extracts contain it. Phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds are secondary metabolites of plant that serve as defense mechanisms against predation by many microorganisms [22]. This may therefore explain the demonstration of antimicrobial activity of *M.oleifera.*

Regression analysis of the relationship between size of inhibition zone (mm) and plant crude extract concentration (Log value) showed that there was a significant correlation between concentrations of tested plant extracts and the mean inhibition zone of pathogenic isolates. The in vitro antibacterial activities are shown in Table 2. As is shown, a wide spectrum activity against some of bacterial strains studied. Amongst the Gram-positive and Gram-negative bacteria, Gram positive bacteria S. aureus were inhibited by plant extract. Indifference ethanol extract of *M.oleifera* was more effective compare two aqueous extract for the same plant ,.All Gram negative bacteria i.e. E. spp ,S. dysenteriae, A . hydrophila were found to be resistant to all of the extracts of *M.oleifera*, Exceptionally *K .spp, E . coli* and *P. aeruginosa* showed zone of inhibition . Were as all gram negative bacteria i.e. *S. Dysenteriae, A . hydrophila, K . spp , E . coli and P . aeruginosa* gave antibacterial activity as zone of inhibition around the extract of *M.oleifera*, but only E . spp was resistant to all of the extract preparation. The demonstration of antibacterial activity against both gram positive and gram negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds **[**23**]**.Several workers have reported that many plants possess antimicrobial properties including the parts which include; flower, bark, stem, leaf, etc. It has been shown that when solvents like ethanol, hexane and methanol are used to extract plant, most of them are able to exhibit inhibitory effect on both gram positive and gram negative bacteria [24].

Out of the two solvents used for extraction, the ethanol extracts showed the highest activity against the test organisms, followed by the aqueous extracts (hot & cold). Different solvents have been reported to have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent [22]. Ethanol extracts in this study might have had higher solubility for more phytoconstituents, consequently the highest antibacterial activity. The demonstration of antimicrobial activity by water extracts provides the scientific basis for the use of these plants in the traditional treatment of diseases, since most traditional medicine men use water as their solvent in which the decoctions are prepared.

Table 2 Antibacterial Activity of Plant Extracts against Test Organisms

Extract/concentration Mg/ml	**Zone of inhibition (mm)**							
	Co.	***P.a.***	***E.spp***	***K.spp***	***A.h.***	***S.d.***	***E.c.***	***S.a.***
Ethanol Extract of *M.oleifera*	500	22	-	9	-	-	26	28
	400	20	-	8	-	-	21	23
	300	19	-	8	-	-	20	18
Hot aqueous Extract of *M.oleifera*	500	16	-	6	-	-	25	11
	400	16	-	-	-	-	23	9
	300	14	-	-	-	-	18	7
Cold aqueous Extract of *M.oleifera*	500	16	-	8	-	-	18	18
	400	16	-	7	-	-	12	12
	300	15	-	-	-	-	11	8
Control(water)	-	-	-	-	-	-	-	-
Control(Ethanol)	-	-	-	-	-	-	-	-

The minimum inhibitory concentration MIC and minimum bactericidal concentration MBC results are shown in Tables 3, 4,. These tables reveal that the ranges of activity for both MIC and MBC are 0.780 to 200mg/ml. The highest MIC and MBC values is an indication that either the plant extracts are less effective on some bacteria or that the organism has

the potential of developing antibiotic resistance, while the low MIC and MBC values for other bacteria is an indication of the efficacy of the plant extracts.

Table 3 Minimum Inhibitory Concentration (MIC) values for Bacterial Isolates Against *M.oleifera* extracts

Bacteria-a Isolates	**Extract concentration (mg/ml)**																										
	0.780			**1.560**			**3.125**			**6.25**			**12.5**			**25**			**50**			**100**			**200**		
	E	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**
P.a.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	I	I	+	+	+	+	+	+	+	+	+
E.spp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K.spp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A.h.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S.d.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.c.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	I	+	+	I	+	+	+
S.a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	+	-	-	+	I	-	+	+	-

Key: *S.d = S. dysentriae; S.a = S. aureus; A.h.= A . hydrophila; E.c.= E. coli ; E.spp. = Enterobacter spp: K.spp = Klebsiella spp: P.a.=P . aeruginosa*

- = Resistance (growth of bacteria); + = Concentrations show no turbidity (inhibition of bacterial growth); I = least concentration showing no turbidity (MIC); E=Eethanol extract; H= Hot aqueous extract; C= Cold aqueous extract

Table 4 Minimum Bacterial Concentration (MBC) Values for Bacterial Isolates against *M.oleifera* extracts

Bacteri-a Isolates	**Extract concentration (mg/ml)**																										
	0.780			**1.560**			**3.125**			**6.25**			**12.5**			**25**			**50**			**100**			**200**		
	E	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**	**E**	**H**	**C**
P.a.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B	B	B	+	+	+	+	+	+	+	+	+
E.spp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K.spp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A.h.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S.d.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.c.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B	B	-	+	+	B
S.a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B	-	-	+	-	-	+	B	-	+	+	-

S.d = S. dysentriae; S.a = S. aureus; A.h.= A . hydrophila; E.c.= E. coli ; E.spp. = Enterobacter spp: K.spp = Klebsiella spp: P.a.=P . aeruginosa

- = Resistance (growth of bacteria); + = Concentrations show no turbidity (inhibition of bacterial growth); B= = Minimum Bactericidal (MBC); E=Ethanol extract; H= Hot aqueous extract ; **C=** Cold aqueous extract

Result of the effect of temperature on the plant extracts showed that various temperature ranges of 45, 70, 100 and 121^{o}C had various effect on the antimicrobial activity of the extracts (Fig 4,5and 6), in methanol extracts of *M.oleifera* the 45 ^{o}C was the effective temperature (diameter of zone of inhibition 25 mm).,while A. hydrophila had the constant activity in different used temperatures .As can clearly be seen by this figures, the rest bacteria did not response to these temperatures in ethanol, both aqueous extract (No zone of inhibition).

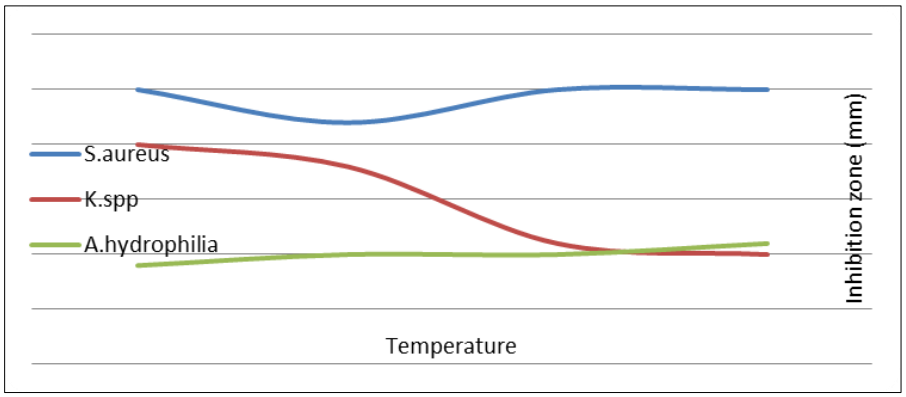

Figure 4 Effects of temperature on antimicrobial activity of ethanol extract *M. oleifera*

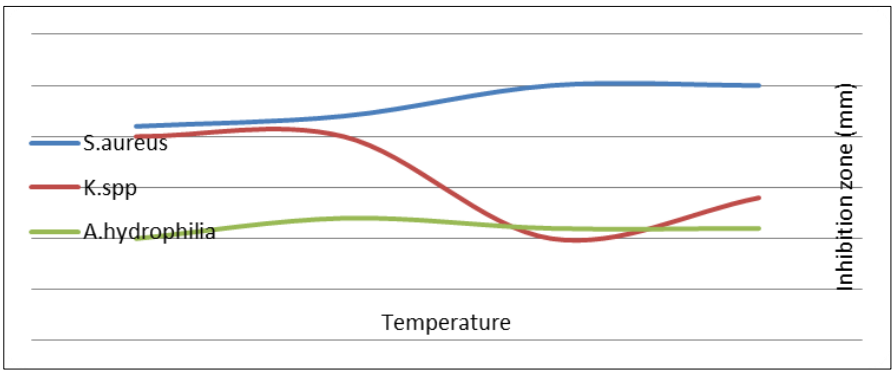

Figure 5 Effect of temperature on antimicrobial activity of Hot aqueous extract *M.oleifera*

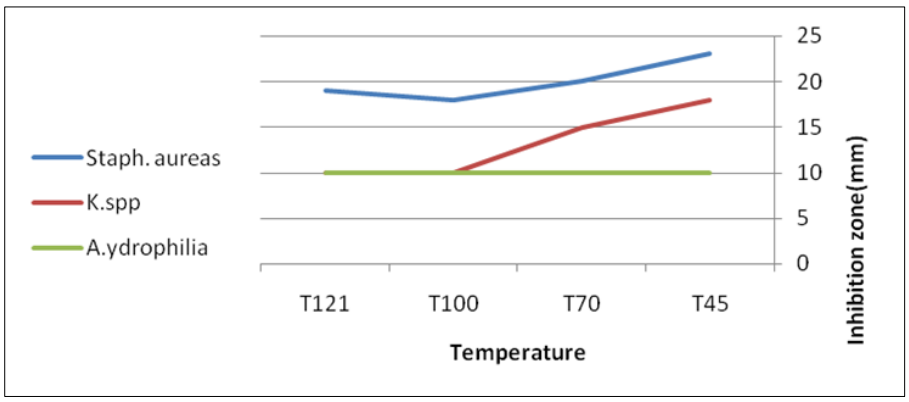

Figure 6 Effect of temperature on antimicrobial activity of Cold aqueous extract *M.oleifera*

The activity slightly increased at acidic pH (3 to 5), while at alkaline pH the activity of the plant extracts reduced except for A. hydrophila in plant extracts (Fig 7,8and 9). The antibacterial activity of the extracts slightly increased at acidic pH. Increase in activity of phyotoconstituents in the presence of acidic medium has earlier been reported [25]. The local application of these plants involves the addition of high doses of potash which is a strong basic salt, and for the fact that the activity of the extracts reduced at alkaline pH in this study, it may explain why the plant concoction is taken for longer period of time before any curative effect is noticed. In this study noticed that gram positive bacteria S. aureus gave constant result in plant extracts. While plant extracts against gram negative bacteria especially *E.coli* and E.spp showed activity in acidic pH only, and these activity was stable in plant extracts application. As well as lactose fermented bacteria K.spp inhibited in different pH, but with low inhibition zone, similar to PS.aeruginosa .

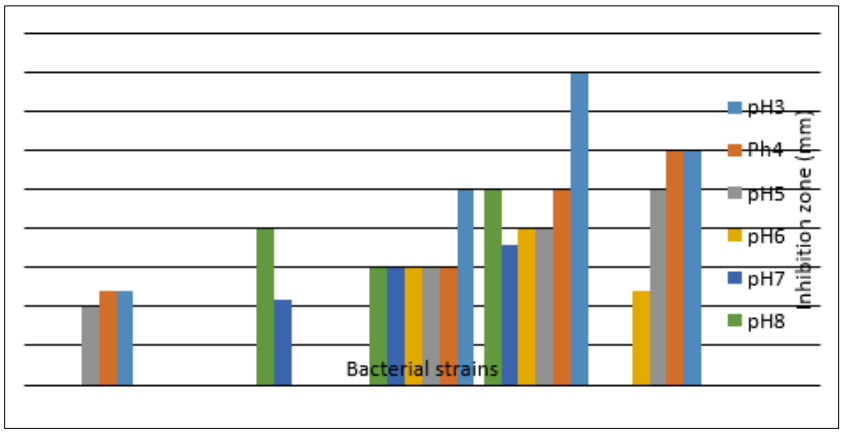

Figure 7 Effects of pH on antimicrobial activity of ethanol extract *M.oleifera*

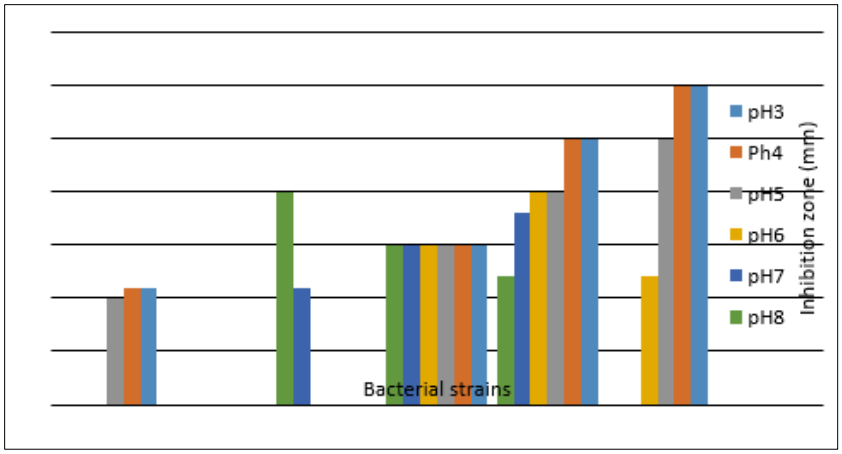

Figure 8 Effect of pH on antimicrobial activity of hot aqueous extract *M.oleifera*

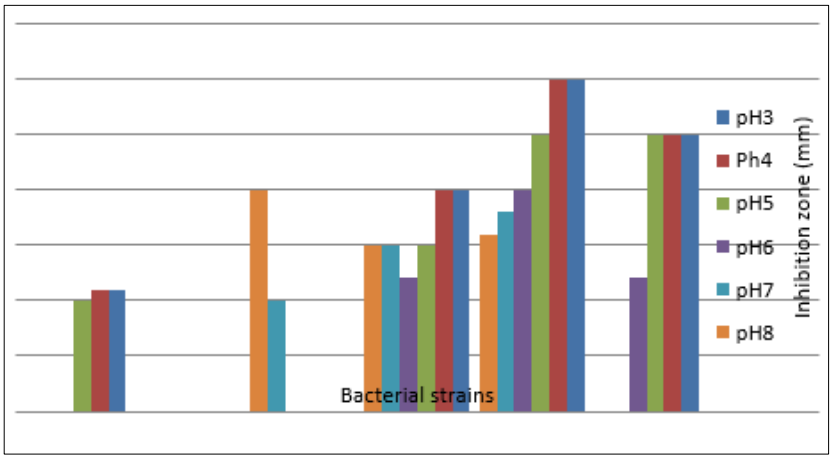

Figure 9 Effect of pH on antimicrobial activity of Cold aqueous extract *M.oleifera*

Ten elements, Ca, Co, Cu, Mn, Fe, K, Na, P, Zn and Pb, were determined in *M.oleifera* and Table 7. *M.oleifera* contained essential elements (Mn, Fe, K,Na,P and Pb,) at higher levels.. Therefore, it may not produce any health risks for human consumption, if other sources of toxic metal contaminated food are not taken at the same time.

Table 7 Essential elements concentration of *M. oleifera*

Elements	Concentration	*S. rosmarinus*
Pb	ppm	0.6
Na	ppm	582
K	%	1.1
Ca	%	0.82
Fe	ppm	720
Zn	ppm	61.4
P	%	0.45
Mn	ppm	7.1
Co	ppm	3.5
Cu	ppm	6.4

4. Conclusion

Results of this study demonstrated by the aid of *M.oleifera* extracts revealed that this plant has antimicrobial activity against test organisms and this may be suggest the use of this extract in treatment of infectious diseases.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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