



## Original Research Article

## Antimicrobial Activities of Aqueous and Ethanolic Extracts from *Nerium oleander* Used in the treatment of burns infections isolates

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### ABSTRACT

The aqueous and ethanol extracts of *Nerium oleander* 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 °C was demonstrated by the ethanol extracts of *Nerium oleander* 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. *Nerium oleander* 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.

**Keyword:** Plant extract; *Nerium oleander*; Antimicrobial activity; Bacteria

### 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 *Nerium oleander* 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]. *Nerium oleander* 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, Low efficacy, high cost, or corrosiveness [7]. Certain wood preservatives have been banned or limited for some applications such as chromated copper arsenate (CCA) 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]. Ability of natural plant extracts to protect wood against degrading fungi and insects have been one possible approach for Oleander is one of the most poisonous plants and contains numerous toxic compounds. The most significant of these toxins are oleandrin and neriine, which are cardiac glycosides. They are present in all parts of the plant, but are most concentrated in the sap. Many of Oleander's relatives have similar leaves and contain toxic compounds. It is thought that Oleander may contain many other unknown or un-researched compounds that may have dangerous effects. Oleander bark contains rosagenin, which is known for its strychnine-like effects. 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 developing new wood preservatives [11, 8]. The goal of this investigation was to discover plant products that inhibit micro-organisms, especially that cases wound infection.

## **MATERIALS AND METHODS**

### **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.

### **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 Al –Mustansiriyah University.

### **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 45°C. Bacterial suspension was added into MHA to give a final concentration of  $10^7$  bacteria/ml and plated out.

### **Phytochemical screening**

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

### **Antibacterial activity**

The plate-hole diffusion assay as described by [14] was used to determine the growth inhibition of from wound infection were obtained. The tests were carried out by using a stock concentration of 500mg/ml prepared by dissolving 1g of the ethanol extract and aquatic extract into 2ml of distilled water. Nutrient agar was prepared and 25ml each was poured into sterile petri dish. This was allowed to solidify and dry. Using a sterile cork-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 100 $\mu$ l at varying concentrations of 500mg/ml, 400mg/ml and 300mg/ml respectively. This was done in triplicate and the plates were incubated at 37°C for 18hours. The antibacterial activities were observed and measured using a transparent meter rule and recorded if the zone of inhibition was 10mm [15].

#### **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 37°C, 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.

#### **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

bacteria by the plant extract. The isolated bacteria 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 37°C for 18 hours. The lowest concentration of the extract which showed no bacterial growth was noted and recorded as the MBC.

#### **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 °C, 70 °C, 100 °C and 121 °C 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].

#### **Determination of essential elements**

Three gram of dried plant were taken and mixed with 8ml of concentrated H<sub>2</sub>SO<sub>4</sub> (98%) and 2ml of HClO<sub>3</sub> (60%) in conical flask for 24 hours which covered by watch glass. Then left this mixture for 6 hours at the sand bath at 80 °C, 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].

## **RESULT AND DISCUSSION**

The result of the Phytochemical screening for Nerium oleander showed the same results are presented in Table 1. This reveals moderate concentration of alkaloids, coumarines , cardiacglycosides, ratenges, phenols, flavonoids, saponins, tannins,essential 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 *Nerium oleander*.

**Table 1: Phytochemical screening of Ethanol, Hot water and Cold water extract of *Nerium oleander***

Constituents	Ethanol extract	Hot water extract	Cold water extract
Alkaloids	+	+	+
Phenols	+	+	+
Cardiac glycosides	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Terpenes	+	+	+
Steroids	-	-	-
Tanins	+	+	+
Ratenges	+	+	+
Coumarines	+	+	+
Essensial oil	+	+	+

Regression analysis of the relationship between size of inhibition zone (mm) and 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 *Nerium oleander* 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 *Nerium oleander*, 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 *Nerium oleander*, 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)							
	Con.	P.a.	E.spp	K.spp	A.h.	S.d.	E.c.	S.a.
Ethanol Extract of <i>Nerium oleander</i>	500	23	-	9	-	-	28	28
	400	20	-	8	-	-	23	22
	300	18	-	8	-	-	20	18
Hot aqueous Extract of <i>Nerium oleander</i>	500	18	-	7	-	-	26	10
	400	18	-	-	-	-	23	9
	300	16	-	-	-	-	19	7
Cold aqueous Extract of <i>Nerium oleander</i>	500	18	-	8	-	-	18	18
	400	18	-	7	-	-	13	13
	300	15	-	-	-	-	11	9
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 *Nerium oleander* 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.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				+	+	+	+	+	+	+	+	
E.spp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
K.spp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
A.h.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
S.d.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
E.c.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			+	+		+	+	+	
S.a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	+	-	-	+		-	+	-	

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

+ = Concentrations show no turbidity (inhibition of bacterial growth)

l = least concentration showing no turbidity (MIC)

E = Ethanol extract

H = Hot aqueous extract

C = Cold aqueous extract

**Table 4: Minimum Bacterial Concentration (MBC) Values for Bacterial Isolates against *Nerium oleander* extracts**

Bacteria 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. dysenteriae*; *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°C had various effect on the antimicrobial activity of the extracts (Fig 1, 2 and 3), in methanol extracts of *Nerium oleander* the 45 °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 these figures, the rest bacteria did not response to these temperatures in ethanol, both aqueous extract (No zone of inhibition).

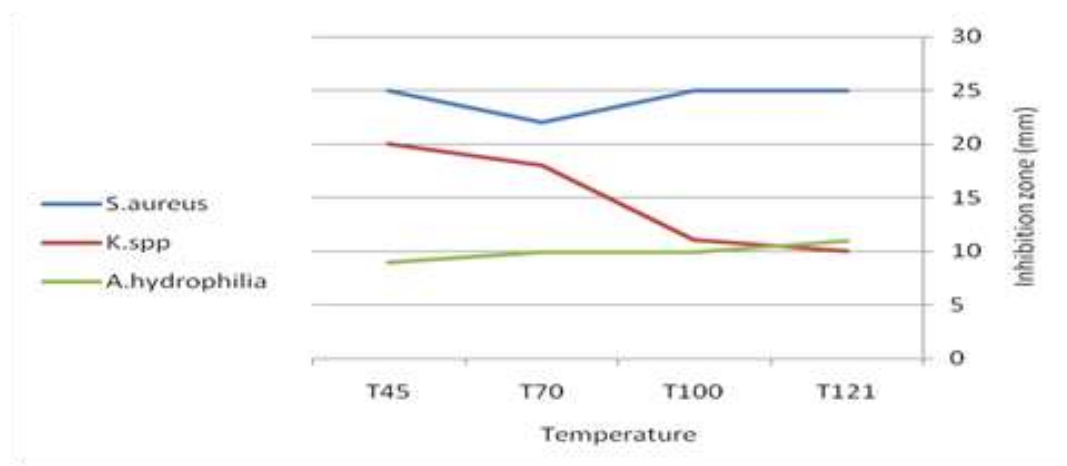


Fig 1. Effects of temperature on antimicrobial activity of ethanol extract *Nerium oleander*

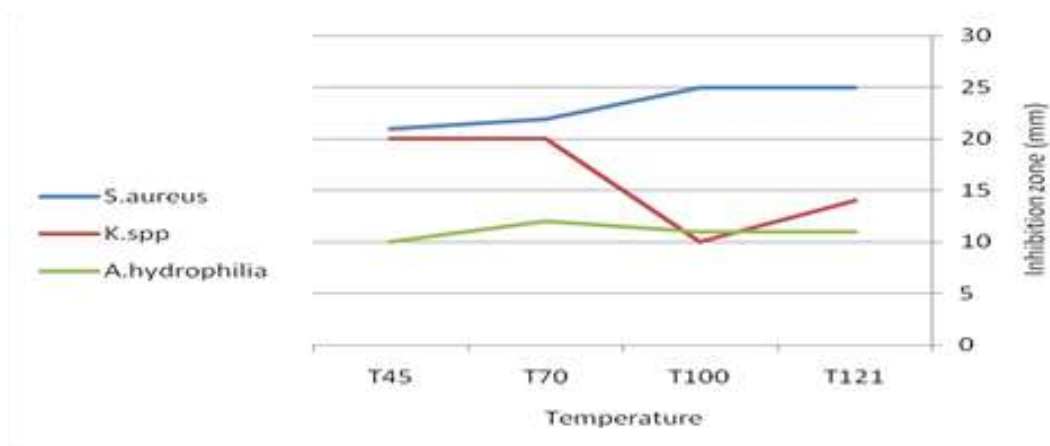


Fig 2. Effect of temperature on antimicrobial activity of hot aqueous extract *Nerium oleander*

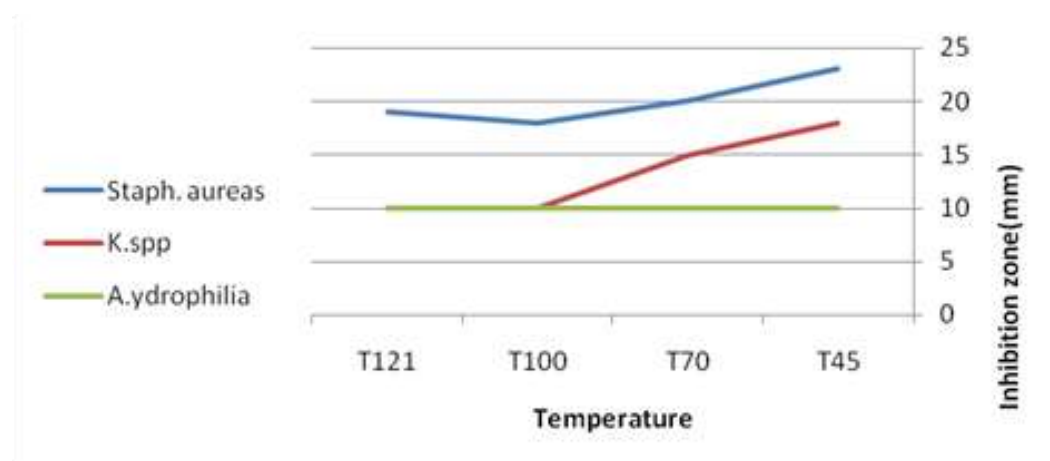


Fig 3. Effect of temperature on antimicrobial activity of Cold aqueous extract *Nerium oleander*

The activity slightly increased at acidic pH (3 to 5). While at alkaline pH the activity of the plant extracts reduced except for *A. hydrophilia* in plant extracts (Fig 4, 5 and 6). The antibacterial activity of the extracts

slightly increased at acidic pH. Increase in activity of phytoconstituents 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 *P.aeruginosa*.

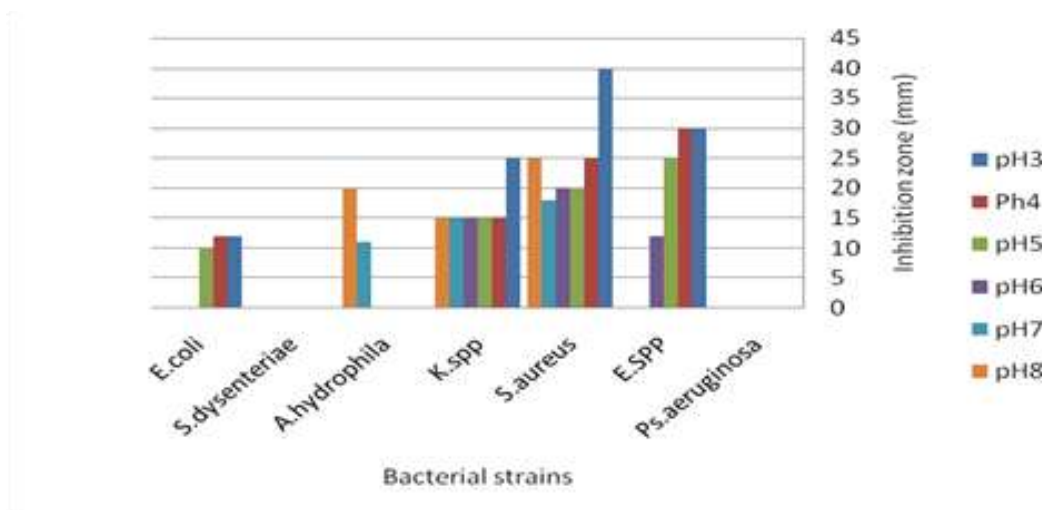


Fig 4. Effects of pH on antimicrobial activity of ethanol extract *Nerium oleander*

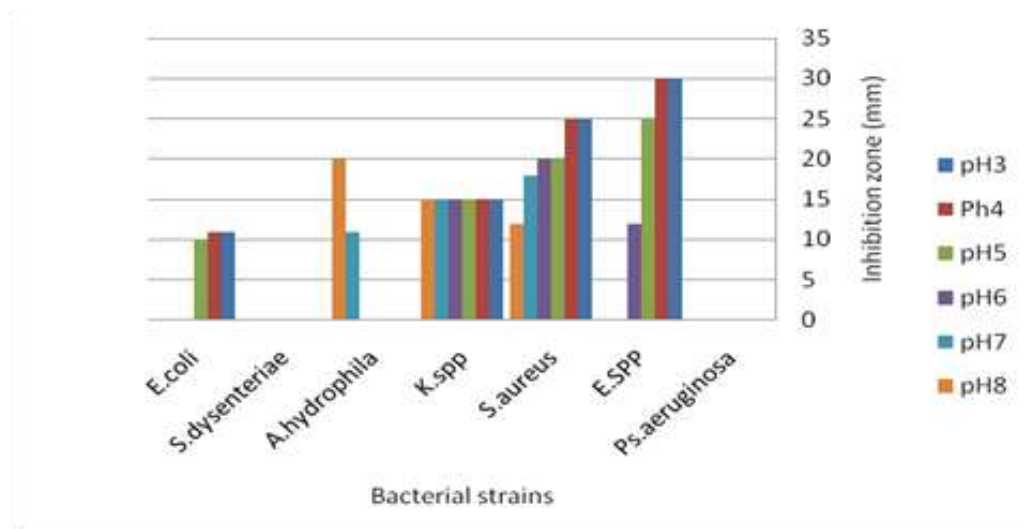
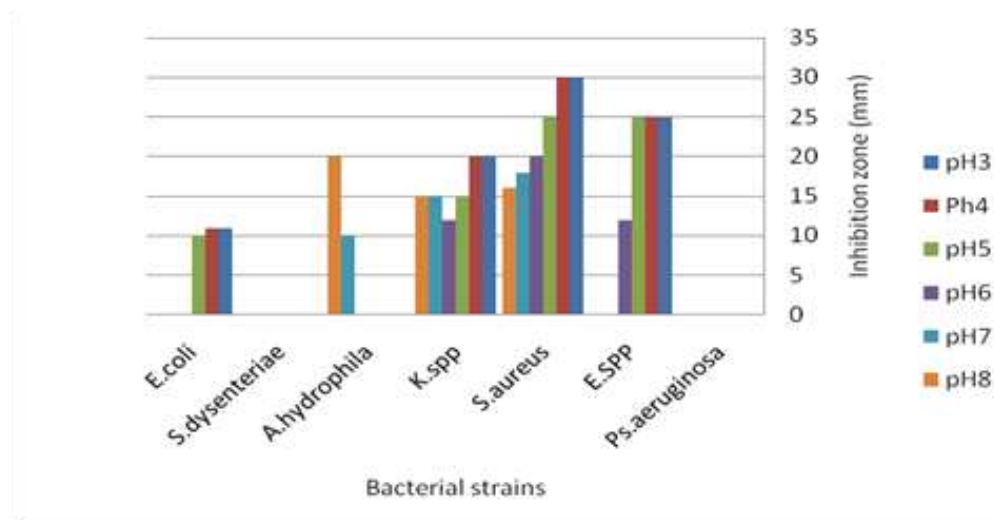


Fig 5. Effect of pH on antimicrobial activity of hot aqueous extract *Nerium oleander*





**Fig 6. Effect of pH on antimicrobial activity of Cold aqueous extract *Nerium oleander***

Ten elements, Ca, Co, Cu, Mn, Fe, K, Na, P, Zn and Pb, were determined in *Nerium oleander* and Table 5. *Nerium oleander* 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.

#### CONCLUSION

Our results indicate that some plant extracts have inhibitory effect for growth of microorganism include the bacteria under studies due to presence of some active compound in these plants which act as natural antibiotics that can use it instead of antibiotics after experiments have performed on it.

**Table 5: Essential elements concentration of *Nerium oleander* .**

Elements	Concentration	<i>Nerium oleander</i>
Pb	ppm	0.6
Na	ppm	594
K	%	1.2
Ca	%	0.92
Fe	ppm	700
Zn	ppm	63.6
P	%	0.35
Mn	ppm	5.7
Co	ppm	2.5
Cu	ppm	6.5

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