STUDES ON ANTIBACTERIAL AND ANTICANCER ACTIVITY OF *NERIUM OLEANDER* EXTRACTS

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Keywords: *Nerium oleander;* antibacterial and anticancer activity; extraction; isolation; aqueous extract; alcoholic extract; hexane extract.

The active substances such as volatile oils (11.1 %) and tannins (39.2 %) from *Nerium oleander* plant (*Nerium oleander*) is reported. Some mineral elements such as sodium (107 ppm), calcium (96 ppm) and potassium (73 ppm) were found in the *Nerium oleander* seeds. The anti-bacterial activity of extracts from *Nerium oleander* showed the ability of inhibition in pathogenic bacteria *Escherichia Coli* and *Aurous Staphylococcus* for all different extracts by vary inhibition diameters for different active substances, concentrations and bacteria. The effect of *Nerium oleander* extracts (after the chemical assay) on the growth of ($L_{20}B$) cell line was studied using *in vitro* system and compared with anticancer drug cisplatin (cis-Pt) as a positive control. The cancer cells were treated with different concentrations of the extract for each of the three treatments. The cytotoxic activity was tested by inhibition rate as parameter. The results showed significant differences (P<0.05). There was strong correlation between the three treatments and the different concentrations in comparison with cisplatin.

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Introduction

Nerium oleander,[1] toxic in all its parts, is an evergreen shrub or small tree in the dogbane family *Apocynaceae*. It is the only species currently classified in the genus *Nerium*. It is most commonly known as oleander, from its superficial resemblance to the unrelated olive Olea. It is so widely cultivated that no precise region of origin has been identified, though southwest Asia has been suggested. The ancient city of Volubilis in Morocco may have taken its name from the Berber name oualilt for the flower.[2] Oleander is one of the most poisonous of commonly grown garden plants.

Figure 1. *Nerium oleander* plant

Oleander shrub, Morocco *N. oleander* is either native or naturalized to a broad area from Mauritania, Morocco, and Portugal eastward through the Mediterranean region and the Sahara (where it is only found sporadically), to the Arabian peninsula, southern Asia, and as far East as Yunnan in southern parts of China.[3] It typically occurs around dry stream beds.

Bud of a white-flowered cultivar is an ornamental gardening. Oleander grows well in warm subtropical regions, where it is extensively used as an ornamental plant in landscapes, in parks, and along roadsides. It is drought-tolerant and will tolerate occasional light frost down to 10 °C (50 °F).[4] It is commonly used in landscaping freeway medians in California, Texas and other mild-winter states in the Continental United States because it is upright in habit and easily maintained. Its toxicity renders it deer-resistant. It is tolerant of poor soils and drought. Oleander can also be grown in cooler climates in greenhouses and conservatories, or as indoor plants that can be kept outside in the summer. Oleander flowers are showy and fragrant and are grown for these reasons. Over 400 cultivars have been named, with several additional flower colours not found in wild plants having been selected, including red, purple, pink, and orange; white and a variety of pinks are the most common. Many cultivars also have double flowers. Young plants grow best in spaces where they do not have to compete with other plants for nutrients.

Nerium oleander has historically been considered a poisonous plant because some of its compounds may exhibit toxicity, especially to animals, when consumed in high amounts. Among these compounds are oleandrin and oleandrigenin, known as cardiac glycosides, which are known to have a narrow therapeutic index and can be toxic when ingested.

Toxicity studies of animals administered oleander extract concluded that rodents and birds were observed to be relatively insensitive to oleander cardiac glycosides. [5] Other mammals, however, such as dogs and humans, are relatively sensitive to the effects of cardiac glycosides and the clinical manifestations of "glycoside intoxication".[6]

However, despite the common "poisonous" designation of this plant, very few toxic events in humans have been reported. According to the Toxic Exposure Surveillance System (TESS) in 2002 there were 847 human exposures to oleander reported to poison centers in the United States.[7]

Despite this exposure level, from 1985 through 2005, only three deaths were reported. One cited death was apparently due to the ingestion of oleander leaves by a diabetic man. [8] His blood indicated a total blood concentration of cardiac glycosides of approximately 20 μg L^{-1} which is well above the reported fatal level. Another study reported on the death of a woman who self-administered "an undefined oleander extract" both orally and rectally and her oleandrin tissue levels were 10 to 39 μg g^{-1} which were in the high range of reported levels at autopsy.[9] And, finally, one study reported the death of a woman who ingested oleander 'tea'.[10] Few other details were provided.

In contrast to consumption of these undefined oleander derived materials, there is no toxicity or deaths reported from topical administration or contact with Nerium oleander or specific products derived from them. In reviewing oleander toxicity Lanford and Boor[11] concluded that, except for children who might be at greater risk, "the human mortality associated with oleander ingestion is generally very low, even in cases of moderate intentional consumption (suicide attempts)".[11]

Toxicity studies that have been conducted in dogs and rodents administered oleander extracts by intramuscular (IM) injection indicated that on an equivalent weight basis, doses of an oleander extract with glycosides ten times in excess of those likely to be administered therapeutically to humans are still safe and without any "severe toxicity observed".[12]

Experimentals

(Cisplatin) (10 mg in 20 ml solution) was provided by Ebew (Austria).

Nerium oleander was obtained from the local market. It was grounded and kept at a laboratory temperature until used, The aqueous extract was prepared by placing 40 g of *Nerium oleander* powder in the conical flask containing 200 mL of distilled water and stirred with magnetic blender for 30 minutes and then centrifuged for 15 minutes. The solution was kept in an electric furnace at 35 °C until we get the extract and from it we prepared the solutions with 5, 10, 15, 20 and 25 % concentration.

Alcohol extract was obtained by puting 50 g of *Nerium oleander* powder in a extraction unit (Soxhlet) and 350 ml of 80 % ethanol was added and heated to 40 °C and then the extract was extracted by using vacuum rotary evaporator over a period of 12 h. also at 35 °C[13] after that by the same way aqueous extract solutions prepared.[14]

The percentage of oil in the seeds, based on the Association of Official Analytical Chemists (AOAC) method[15] was estimated from the extract extracted with hexane. The volatile oils were extracted from the ether extract from which the solvent was separated.

Tannins were isolated from *Nerium oleander* by adding 75 ml of distilled water to 0.5 g of *Nerium oleander* powder The mixture put in boiled water bath for 30 minutes, then the mixture run in centrifuge at 200 cycle\minutes for a period of 20 minutes .The solution transfer to flask 100 ml and complete the volume to the mark with distilled water then added to the mixture 20 ml of 4 % lead acetate with shaking then continued and filtered. The sludge dried at 70 °C in electric furnace.[16]

Ash content was estimated by taking 2 g of *Nerium oleander* powder and burning it in an oven at 550 °C, The ash so obtained was collected and weighed.

The percentage of moisture was estimated from 2 g of *Nerium oleander* powder which was heated to 60 °C for a period of 24 hours in an electric furnace. The heated material after cooled and weighed to estimate the loss in weight.

The pH of the solution, obtained from blending 10 grams of *Nerium oleander* powder with 100 ml of distilled water and stirring the mixture using a magnetic stirrer for 10 minutes, was measured using a pH -Meter.

The presence of semi-alkaloids, carbohydrates, saponin, flavonoids, lipids, proteins, and tannins were also detected.[17,18] The results are given in Table 1.

Study the activity against pathogenic bacteria

Agar-well diffusion method, suggested by Kirby Baauer,[19] was used to measure the sensitivity of Escherichia Coli and Staphylococcus aurous bacteria (isolated and diagnosed in culture laboratory in children's hospital in Ramadi). The Mueller Hinton agar was also used to test the sensitivity of bacteria towards the extract from *Nerium oleander*. The dishes were placed in incubator at 37 °C for 24 hours and inhibition diameter was measured (Inhibition Zone)[19] in each hole by ruler.

Preparation of standard solutions of isolated substances from *Nerium oleander*

A series of different extracts solutions have been prepared for concentrations of (5 %, 10 %, 15 %, 20 % and 25 %) mg mL^{-1}.

Study of cytotoxic effect on cancer cell line

One type of cancer cell lines ($L_{20}B$) were used to study the impact of the extracts from *Nerium oleander* on the growth of cells in laboratory.

All solutions are prepared at the same center and culturing tissues were studied in vitro under optimum conditions by the same center. The growth media used in tissue culture technique was MEM (Minimum Essential Media) was provided by Fetal Calf Serum 10 % to form a confluent monolyer, then subculture to discard the previous growth medium and the cell washed with sterilized phosphate buffer solution (PBS) by autoclave at 121 ^{o}C for 15 min and addition 2-3 min and moving the culture flask kindness. The trypsin-versene solution to discard and cells incubated at 37 ^{o}C until the cell separation from ground flask, added new growth media and redistribution of cells at the microtiter and incubated at 37 ^{o}C.[20]

Statistical Analysis

Data were analyzed by analysis of variance ANOVA. Investigation of differences between cis-platin and the relation with other groups by using the statistical program (SPSS) within significant level (P<0.05).[21]

Result and discussion

The results of the phytochemical (screening of plant materials) studies of the *Nerium oleander* are presented in Table 1.

Table 1. Results of the statements chemical substances effective in *Nerium oleander*

Active Compounds	Reagents	Indicators	Results
Alkaloids	Draggendorff	orange	+
Tannins	Ferric chloride, Lead acetate	greenish blue soln. gelatinous ppt.	+
Intense tannins	Lead acetate	light brown ppt.	+
Flavonoids	aq. NH_3	yellow soln.	+
Amino acids	Alnhidran	purple	+
Phenols	potassium ferrocyanide	greensh – blue ppt.	-
Resins	hydrochloric acid	turbid	+
Terpenoids	Salkowski	dark red	-
Saponins	mercury chloride	white ppt.	+
Carbo-hydrates	α-naphthol	purple	+
Loco antho-cyanidine	hydrochloric acid	red ppt.	+
Steroids	The same of terpenoids reagent after one day	bluish solution	+
Glycosides	Benedict	red ppt.	+

(+) and (-) indicate the positive and negative tests, respectively.

Figure 1 and Table 2 show the percentage of the active contents, tannins and volatile oils isolated from *Nerium oleander*.

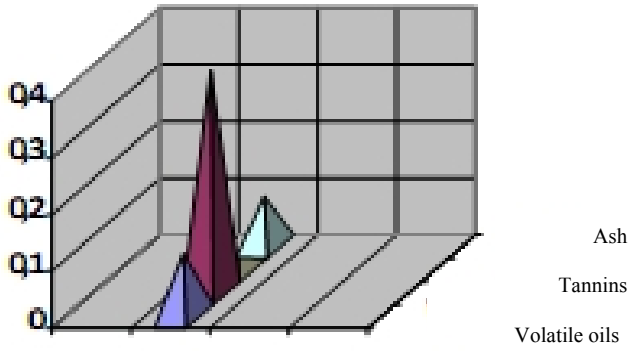

Figure 2. The percentage amounts of extracted materials

The percentage depends on several factors, including climatic conditions and different seasons of the year as the high temperature leads to the loss of more water, and that the decline reduces the loss of water from the plant.[16]

Total carbohydrates=100-(% moisture+% crude protein + % crude fat + % ash content)

The sum of percentages of proteins and carbohydrates is about 91.1 %, which is consistent with the calculated ratios published in previous paper.[22]

Table 2. Percentage of active combatants in *Nerium oleander*

Active combatants	Percentage, %
Volatile oils	11.1
Tannins	39.2
Moisture	2.1
Ash	8.8
PH	6.0

Nerium oleander contains sodium is 107 ppm, calcium and potassium are 96 and 73 ppm, respectively, all are important functional and metabolic metals for human body.[21]

Table 3 shows the anti-bacterial activity of *Nerium oleander* extracts towards pathogenic *Escherichia Coli* and *Aurous Staphylococcus* bacteria. The water extracts showed higher activity at 25 mg mL^{-1}, the inhibition diameter was 22 mm for *Aurous Staphylococcus* and 19 mm for *Escherichia Coli.*

The inhibition action of alcohol extract is due to the presence of flavonoids, tannins, and some of phenolic compounds which have a biological influence on many bacteria races due to the presence of hydroxyl groups which have the ability to form hydrogen bonds with hydroxyl group in these compounds and water molecules in bacterial cell. Since the water is 90 % of weight and therefore it will disables dynamic actions in bacterial cell.[23] The phenolic compounds have the ability to coagulate the bacterial cell proteins and destroy enzymes involved in the manufacture of necessary amino acids to increase cell division.[24]

Table 3. Effect of aqueous extract of *Nerium oleander* in different concentrations on growth of pathogenetic bacterial races

Conc. in mg mL^{-1}	Inhibition diameter, mm	
	Staphylococcus aurous	*Escherichia Coli*
25	22	19
20	17	13
10	14	11
10	11	9
5	9	8

Table 4. Inhibition effect of extract and cis-Pt on cancer line ($L_{20}B$) with different concentrations after 72 h exposure time.

Treatment conc. µg mL^{-1}	Inhibition rates, %		
	cis-Pt	Aqueous extract	Alcohol extract
31.25	26.55	28.98	27.10
62.5	29.95	30.88	30.09
125	45.10	46.65	45.99
250	71.97	72.76	71.45

Study of Cytotoxic Effect

Cancer cell lines were used to study the effect of *Nerium oleander* extracts on the growth of cells in laboratory to know the efficiency of extracts as anti-tumors. cancer cell line type mice transformed cell line ($L_{20}B$) used with different concentrations comparable with anticancer drug cisplatin as a positive control after 72 h exposure time.

We calculate the proportion of cell numbers under the optimal conditions for growth in the absence of extracts, so that the output is the control group. Extracts were added to study its effects on cell growth in selected lines.

Extracts were divided into three groups, first one included a hot water extract, second was hot alcoholic extract and the third had cis-Pt. The result were statistically analyzed by ANOVA. The results demonstrated the impact of compounds on cell number ratio when using cell line ($L_{20}B$). It is clear that hot alcoholic extract have the greatest influence on the proportion of growth cell number and the effect was significant (P<0.05). This result is compared with published literature.[25] The effect of aqueous extract was also significant (P<0.05) but the percentage of inhibition - as in the Figure 3 was less than alcoholic extract (Table 4, Figure 3).

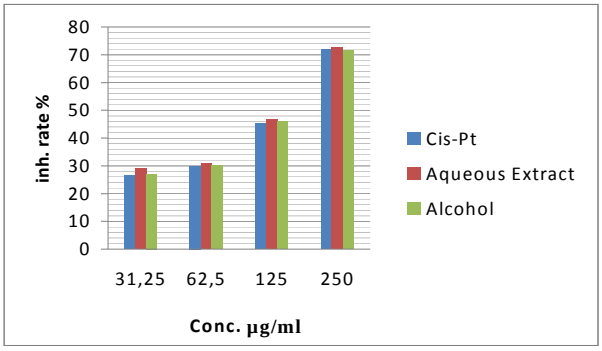

Figure 3. The comparison of inhibition rates between three treatments with cis-Pt drug in cell line $L_{20}B$

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Received: 09.01.2014.
Accepted: 01.02.2014.