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In vivo and *in vitro* Immunmodulatory Activities of *Nerium oleander* Aqueous Leaf Extract in Rabbits

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Abstract: The *in vitro* effect of *Nerium oleander* (NO) aqueous leaf extract on humoral and phagocytic activity and *in vivo* cellular immune response were studied in rabbits. Treatment of rabbits with the extract at 75 mg kg^{-1} body weight subcutaneously not only diminished the production of antibodies, but also exerted an inhibition on delayed type hypersensitivity reaction and phagocytic activity, whereas treatment of rabbits at 50 and 25 mg kg^{-1} body weight subcutaneously caused stimulation of the immune system. These results suggest the NO aqueous leaf extract exerts a marked immunomodulatory effect on the rabbits, immune system.

Key words: *In vivo*, *in virto*, immunmodulatory activities, leaf extract, rabbit

INTRODUCTION

Adelfa (*Nerium oleander*) is amember of family *Apocynaceae* (Dog bane family). It is an ornamental shrub, densely branched tree, 1-10 m tall (Galey, 1998). This plant grows outdoors in warmer regions and sometime it is grown as a house plant. It is widely cultivated in Mosul (Iraq) along roadsides, edges of woods and gardens. There are records that the plant can be used as arodenticide, insecticide and for indigestion, fever, ringworm, leprosy, venereal diseases (Galey, 1998; Bose *et al*., 1999; Rhaymah *et al*., 2006). Herbal products of the plant are increasingly used for their effects on the immune system (Chumpon *et al*., 2002 b). These products can affect the host immune system and therefore, they could be beneficial in the treatment of immune related diseases, or alternatively, they could cause inadvertent side effects. In previous reports we observed that aqueous leaf extracts of *Nerium indicum*, *Cedrela tubiflora* and *Trichilia elegans* exert inhibitory activities on both murine and human complement activation and phagocytosis mediated by mouse peritoneal exudate cells (Bhatia and Sandeep, 2004; Benencia *et al*., 1995; Benencia *et al*., 2000). In this study, we describe, in rabbits, the effects of aqueous leaf extract of *Nerium oleander* on several rabbits immune parameters closely related to humoral immunity, delayed type hypersensitivity response (cellular immunity) and phagocytic activity.

MATERIALS AND METHODS

Animals: The study included forty local breed rabbits, of both sexes, 1-2 years age, (body weight 1-1.5 kg). Animals were divided into 8 groups of 5 rabbits each.

Preparation of plant extract: *Nerium oleander* fresh green leaves were used. The specimens were collected in Mosul city (Iraq) in late spring. The plant was properly identified. Fresh plant leaves were washed with distilled water. A 500 g quantity of the plant material was cut into small pieces and blended using an electrical blender with 500 mL of 10 m *M* potassium phosphate buffer (pH 7.2). The mixture obtained was pressed through acheesecloth and the filtrate was centrifuged at 10000×g for 1 h. The supernatant fluid was separated and sterilized by filtration through nitrocellulose membrane (pore size 0.22 μm) obtaining a clear solution, which was dried by lyophilization. Sterile extract were stored at-20°C until used (Nores *et al*., 1997).

The study methods: Rabbits of all groups (1, 2, 3, 4, 5, 6) were treated s.c. with the aqueous solution of the leaf extract of *Nerium oleander* daily (-2, -1, 0, 1, 2 days). Animals of the first and second groups were treated at a dose rate of 75 mg kg^{-1} body weight. The extract was dissolved in 1 mL of Phosphate Buffered Saline (PBS). Animals of the 3rd and 4th groups were treated at a dose rate of 50 mg kg^{-1} body weight. While those of the 5th and 6th groups were treated at a dose rate of 25 mg kg^{-1} body weight. Animals of the 7th and 8th groups (control groups) were treated with equal volume of PBS.

Effect of *nerium oleander* aqueous leaf extract on the humoral immunity: Animals of the first, third and fifth groups were used in this experiment. The animals of these groups were immunized with 2×10^8 of a sheep erythrocytes (SRBC) in 0.1 mL of PBS suspension administered intraperitoneally on day 0. Five days after immunization, blood was collected. The blood sample was incubated for 1 h at 37°C, centrifuged and the sera

were pooled. The sera were incubated for 30 min at 56°C in order to inactivate the complement and stored at -20°C until use (Huson and Hay, 1980). Titration of rabbit haemagglutinating antibodies was used by a micro technique employing 96 wells microplates (David *et al*., 2003). Briefly, each well of the plate received 25 μL of serial two fold dilutions of sera in PBS and each well received an additional 25 μL volume of 1 % (v/v) SRBC suspension in PBS. After incubating the mixtures for 2 h at room temperature the haemagglutinating capacity of the sera was read. Titers of sera were determined as the reciprocal of the maximal dilution presenting positive haemagglutination.

Effect of *nerium oleander* aqueous leaf extract on the cellular immunity: The effect of *Nerium oleander* aqueous leaf extract on Delayed-Type Hypersensitivity (DTH) response in rabbits was done in the 2nd, 4th and 6th groups. Animals of these groups were sensitized by intraperitoneal injection of a suspension containing 1×10^6 SRBC in 0.2 mL of PBS (day 0). Seven days later (day + 7), the flank skin of each rabbit was carefully freed of hair, using hair clipper. A circular area was marked and wiped with 70% ethanol. An aliquot of 1×10^8 SRBC suspensded in 50 μL of PBS was inoculated intradermaly using tuberculin syringes into the right side of the flank region of each animal in the center of the marked circular area for elicitation of the DTH reaction. The left side of the flank of the each animals was inoculated with 50 μL of PBS as a control. Observation was made within 24 h (+8 day) to monitor the degree of swelling and redness of the skin. Mean diameter and skin fold thickness were measured by a caliper. The difference between the means of right and left side skin thickness gave a degree of swelling which was used for group comparisons (Schultz, 1982).

Effect of *nerium oleander* aqueous leaf extract on the phagocytic activity: The phagocytic activity of the treated and control animals was tested by using the Nitroblue Tetrazolium (NBT) reduction assay (Courreges *et al*., 1994). Briefly, 1.5 mL of 0.2 % NBT dye was mixed with the same amount of the peritoneal fluid in Hank's Balanced Salts Solution (HBSS) for each animal in silicon-coated test tubes to prevent adhesion of phagocytic cells. The mixture was incubated at 37°C for 30 min and washed twice with PBS. Thin smears were made onto clean glass slides, stained with 10 % Giemsa and examined under oil immersion lens (×100). Cells taking blackish color as a result of formazan reduction (positive cells) (Metacalf *et al*., 1986) and those remaining intact were counted up to 100 cells. Calculation was based on the:

$$\text{Phagocytic Index (PI)} = \frac{\text{Number of (NBT) positive cells}}{\text{Total number of phagocytic cells}} \times 100$$

Statistics: The statistical significance of the data was determined by Student s t-test. A P value less than 0.05 was taken as significant (Petrie and Watson, 1999).

RESULTS

Effect of *Nerium oleander* aqueous leaf extract on the humoral immunity: The effects of different doses of the extract on the production of the haemagglutinating antibodies in rabbits are shown in Table 1. Significant differences between treated and control animals were observed in the first, third and fifth groups. The treatment of the ainmals at a dose rate 75 mg kg^{-1} body weight of the extract significantly inhibited ($p<0.05$) the levels of the haemagglutinating antibodies as compared to the control group, whereas treatment at the dose rates of 50 and 25 mg kg^{-1} body weight significantly stimulated the levels of the haemagglutinating antibodies as compared to the control group.

The effect of *Nerium oleander* aqueous leaf extract on Delayed-Type Hypersensitivity (DTH) response in rabbit: As shown in Table 2, administration of the *Nerium*

Table 1: Effect of *Nerium oleander* aqueous leaf extract on on the production of the haemagglutinating antibodies in rabbits

Treatment groups	HAU (mean±SE)
1st (75 mg kg^{-1} body weight)	30.2±2.7 *
3rd (50 mg kg^{-1} body weight)	120.0±16.0*
5th (25 mg kg^{-1} body weight)	149±11.3*
Control	80.0±12.1*

HAU:is considered the reciprocal of highest dilution of serum with evident agglutination, * Significant at $p<0.05$

Table 2: The effect of *Nerium oleander* aqueous leaf extract on delayed-type hypersensitivity (DTH) response in rabbit

Treatment groups	Skin fold thickness (mm) on day 0	Skin fold thickness (mm) after 24 h
2nd (75 mg kg^{-1} body weight)	3.1±1.7	1.5±2.1*
4th (50 mg kg^{-1} body weight)	2.3±0.3	3.3±0.2*
6th (25 mg kg^{-1} body weight)	3.2±0.01	4.8±0.03*
Control	2.1±0.7	2.2±0.3

* Significant at $p<0.05$, Values are mean±SE

Table 3: Effect of *Nerium oleander* aqueous leaf extract on the phagocytic activity

Groups	Phagocytic Index (mean±SE)
1st and 2nd (75 mg kg^{-1} body weight)	31.0±0.4*
3rd and 4th (50 mg kg^{-1} body weight)	73.0±0.2*
5th and 6th (25 mg kg^{-1} body weight)	89.0±0.2*
Control	66.0±0.01

* Significant at $p<0.05$

oleander aqueous leaf extract at a dose rate of 75 mg kg^{-1} body weight (second group) caused a significant reduction of the skin swelling (thickness). When the extract was given at the dose rate of 50 and 25 mg kg^{-1} body weight, it caused a significant increase in the skin swelling (swelling and redness at the site of injection) as compared to pre immunization record and the control group.

Effect of *Nerium oleander* aqueous leaf extract on the phagocytic activity: Similar to what recorded with humoral and cellular immunity, as animals of the first and second groups (treated at the dose rate of 75 mg kg^{-1} body weight) produced a significant reduction in the percentage of nitroblue tetrazolium positive cells in comparison to control groups. In the third, forth, fifth and sixth groups, there was a significant increase in the percentage of nitroblue tetrazolium positive cells in comparison to control groups (Table 3).

DISCUSSION

In present study we demonstrated that the aqueous leaf extract of *Nerium oleander*, exerted a marked immunomodulatory effect on the rabbits immune system. The *in vitro* immunomodulatory effect of *Nerium indicum* leaves extract was reported earlier (Bhatia and Sandeep, 2004). The inhibitory and the stimulatory effect of the extract on the production of the haemagglutinating antibodies in the rabbits against SRBC may be attributed to the decrease and increase in the number of the antibody producing cells, respectively (Tizard, 1987). When we studied, the effect of the extract on DTH, we found that the extract interfered with the sensitization of T cells (Tizard, 1987; Valentine and Lawrence, 1971) (at the a dose rate of 75 mg kg^{-1} body weight), while DTH reactions observed on the skin of the experimental animals treated with the extract at the dose rate of 50 and 25 mg kg^{-1} body weight 24 h post inoculation expressed the existence of cellular mediated immunity response. The cutaneous reaction is attributed to liberation of lymphokines, skin reactive factor and monocytes chemotactic factor from sensitized T-cell (Valentine and Lawrence, 1971). Thickening and the reddening of skin in immunized animals are attributed to vasodilatation that causes increase capillary permeability and local influx of mononuclear cells at the site of inoculation (Valentine and Lawrence, 1971; Rose *et al*., 1986). In this study, we also recorded a significant decrease in the percentage of NBT positive cells in comparison to control groups. Animals of the other treated groups (50 and 25 mg kg^{-1} body) produced a significant increase in the the percentage of NBT positive cells in comparison to control groups. Phagocytosis is a vital biological process in elimination of a foreign agent from the body, revealing a non specific cell mediated immune reaction (Roitt *et al*., 1993). The increase in the the percentage of NBT positive cells, indicates an increased in phagocytic activity, which enhances stimulation of macrophages (Burrelll, 1979). Several reports have shown that some plants and herbs such as *Azadirachta indica* (Vander *et al*., 1987), ginger and tea were consistently immunosuppressive agents, whereas *dong quai*, milk thistle and St. john, s wort were consistently immunostimulatory *in vitro* (Chumpon *et al*., 2002a, b). Our results are the first report of an extract obtained from NO capable of inhibiting or stimulating antibody production and causing DTH.

CONCLUSION

Taking together all the previously mentioned facts, we can conclude that the NO extract has interesting immunomodulating properties. At present we are developing phytochemical studies in order to establish the chemical nature of the principles responsible for the activity reported in this research.

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