ACUTE TOXICITY OF LEAVES ETHANOLIC EXTRACT OF *NERIUM OLEANDER* (ALDEFLA) IN LOCAL IRAQI RABBITS (*LEPUS CUNICULUS*)

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**Abstract**

This study was conducted to evaluate the acute toxicity of the one of worldwide botanical *Nerium oleander*. First of all, the leaves of plant had collected from *Nerium oleander* tree which grew up in Alneel distinct, Babil province, Iraq during August 2019. Leaves powder have been extracted by cold method of extraction by soaking in 95% ethanol. Median lethal dose (*LD*$_{50}$) of plant extract was performed in seven local healthy male Iraqi rabbits (*Lepus cuniculus*), up and down method have been used for deriving *LD*$_{50}$ after oral administration. Animals were observed during 24 hr of extract administration for any signs of toxicity in all subjected animals, while necropsy finding and histopathology for several internal organs like lung, stomach and heart have been studied in mortal animals only. The results showed, the yield extract of plant is 9.02%, while the *LD*$_{50}$ of leaves ethanolic extract is 232.23 mg/Kg.BW orally after 24 hours. Various clinical signs of toxicity were observed, briefly, salivation, dyspenea, piloeraction, hind limbs extension, depression, convolution, gasping etc. Necropsy finding included emphysema, pulmonary congestion and bronchopneumonia, mild endocarditis, fibril layer on pericardial infiltrate and inflammatory cells filaments, in stomach specifically, in glandular region there were severe erosion, lysis of mucosa extend to submucosa, most glands in submucosa show severe necrosis. Various histopathological changes were observed such as pulmonary congestion, thickening of alveolar walls, dilated pulmonary blood vessels, severe degeneration of bronchial epithelial mucosa and necrosis, also there were pleuritis and pulmonary emphysema. Heart showed, infiltration and extension of inflammatory cells to myocardium and acute pericarditis, thickened inflammatory layer replaced the flattened epicardium .whereas in stomach there were severe atrophic necrosis and sloughing of mucosal epithelium, the sub mucosal glands severely degenerated and necrotic.In conclusion: The ethanolic cold extraction is one of the useful method for obtaining significant yield extract of *N. oleander* leaves. The *N. oleander* leaves ethanolic extract is considered severely toxic in rabbits after oral administration.

**Key words:** *Nerium*, *oleander*, lethal dose, ethanolic extract, rabbit, histopathology.

**Introduction**

*Nerium oleander* known by local Iraqi name Aldefla, is a toxic plant of the *Apocynaceae* family to humans, animals and insects (Khordadmehr and Nazifi, 2018). It is a small tree evergreen with a wide distribution in world. Its flowers grow in clusters in incurable branches and show various colors including yellow, pink, red, peach and white. All parts of the plant can be very toxic to insects, several of animals and humans(Farkhondeh et al., 2020; Ghurghure et al., 2019). Children are very susceptible to the *N. oleander* toxicity. Unintentional ingestion of plant in children and use of this plant for suicide are two main lead to *N. oleander* toxic in the world. The important clinical finding consists of vomiting, nausea, diarrhea, abdominal pain, arrhythmias and hyperkalemia. The significant toxic impact of *Nerium oleander* poisoning is cardiotoxicity (ventricular arrhythmia, bradycardia, tachycardia (Farkhondeh et al., 2020). *Nerium Oleander* poisoning is not uncommon in domestic animals and man and there have been some cases of dangerous toxicosis in children and adults. *Nerium oleander* is a plant that is often grown in gardens and public areas. Various Pharmacological effects of *Nerium oleander* including antinociceptive, anti-inflammatory and anticancer activity were reported (Salih 2017). The extracts of many parts of *Nerium oleander* Leaves are used as antidiabetic remedy in the traditional medicinal systems of many parts of the world (Dey et al., 2019). *Nerium oleander* is used to produce cardiotonic medicine, this plant used as an anti-cancer
medicine and alternative medicine for many causes (Azzalini et al., 2019), but the potential toxic effects of all parts of the shrub either fresh or dried on animal and human body were documented (Serra, 2016). It contains numerous toxic compounds. The major toxic components found are the cardiac glycosides oleandrin and nerin (Abdou, Basha and Khalil, 2019).

Aldefla is toxic when consumed, the cause of toxicity is mostly due to the presence of cardiac glycoside particularly oleandrin, firstly it has a digitalis action. Many attempts are made to use it as cardiac tonic but not be successful due to narrow therapeutic index. Other toxic constituents of this plant is cardinolides and niriine which are glycosides, second the bark of the plant have rosagenin which has a strychnine action, three the signs of toxicity and the causes of death is the similar for other cardiac glycosides as digoxin (Li et al., 2020).

The toxic effects of plant or their active alkaloids lead to infiltration of cells with hemorrhage and severe negative variations in the lung, induce lesions and infiltration of inflammatory cells into the portal places with scattered necrosis of hepatocytes in the liver, heart toxicity of the plant in the cardiac were included, induced multiple degrees of hemorrhage, myocardial degeneration and necrosis. It also lead to arrhythmia, sinus bradycardia and in ECG records prolonged P-R interval. The toxic effects of N. oleander are typically related to its inhibitory effects on the Na-K ATPase pump in the cellular membrane (Karthik et al., 2020).

Due to wildly distribution of this plant which is may contribute into various toxicosis in animals which may grazing and in human regarding un-intentional ingestion. We are planned to assessment its acute toxicity which is considered the important tool for assessment the risk of any chemical, biological and botanicals.

**Materials and Methods**

Leaves of plant Nerium oleander are collected from Babil province, A.L. Neel at 2019 August (Fig. 1). The plant is 3 meters in long with dark-green leathery lanceolate leaves and has white flowers. It is dried at room temperature with opened windows and door for one week, then grind by using electrical grinder and kept in the tightly stopped glass bottle and kept in the refrigerator under 4°C till used. The powder of Nerium oleander leaves is extracted by ethanol (Chem. Lab NV, Belgium). Fifty g of plant dried powder have been soaked into 500 ml of 95% ethanol with continuous stirring for two hours by using magnetic stirrer machine and kept in conical flask in ice bath for 24 hours in refrigerator, then filtered by using piece of gauze, after that the filtrate filtered one more by using apparatus of Buchner through 0.4 millipore filter under negative pressure (27). The yield extract was calculated by applying the following equation:

\[
\text{Yield extract} = \frac{\text{weight of extract (gm)}}{\text{weight of N.oleander crude powder (gm)}} \times 100
\]

(Banso and Adeyemo, 2006).

• **Animals:** A total of seven male local Iraqi rabbits (Lepus cuniculus) purchased from the animal house of college of veterinary medicine, university of Baghdad, their weight range is 1000-1700 gm and age 6-12 month, the animals raised in the animal house of college of veterinary medicine, university of Baghdad for four weeks to acclimatization under suitable condition of temperature 25±1°C and dark/light cycle 12/12 hours, the animals fed ad labium with standard pellet, green grass and water in air-conditioned room.

**Assessment of Median Lethal Dose (LD<sub>50</sub>)**

The median lethal dose of leaves ethanolic extract of N. oleander is assessed by up and down method (Dixon, 1965) briefly, N. oleander leaves extract was given orally by gastric gavag to the animals at range of

**Fig. 1:** Nerium Oleander (Aldefla) from AL Neel district, Babel, Iraq.

**Fig. 2:** Ehanolic extract of N. oleander leaves extract.
doses between 150-240 mg/kg BW and the difference in doses was 30 mg/kg BW. LD$_{50}$ was calculated after 24 hours observation of dead and live animal by applying the following equation:

\[
LD_{50} = x_f + kd
\]

\[
x_f = \text{last dose administrated,} \\
k = \text{constant,} \quad d = \text{difference between dose}
\]

• **Pathology:** Each dead rabbit has been dissected as soon as for necropsy finding and microscopic lesion of several organs like heart, lung and stomach. The animal is examined grossly after 10 minute from death. The organs preserved in 10% formalin buffer for fixation before embedding the tissues in paraffin block, 3-10 micron thickness of histological sections were prepared by microtome. All histological sections have been stained by hematoxylin and eosin stain (H&E) (AL-Naqeeb and Yousif, 2011).

**Results**

Extraction yield of *N. oleander* leaves ethanolic extract is dark green in color, amorphous crystals (Fig. 2), it is yield about 54.12 g from 600 g crude powder which is representing 9.02%.

The oral median lethal dose (LD$_{50}$) of *N. oleander* ethanolic extract that found by up and down method is 232.23 in local male Iraqi rabbits (*Lepus cuniculus*) for 24 hr (Table 1).

\[
LD_{50} = x_f + kd
\]

\[
LD_{50} = 210 + (0.741 \times 30)
\]

\[
LD_{50} = 232.23 \text{ mg/kg BW orally in rabbit after 24 hours.}
\]

\[
Kd = 0.741
\]

• **Clinical findings:** The morbid rabbits showed various signs of poisoning like depression, pawing the ground, anorexia, piloeraction, dyspnea, slightly watery salivation, extended of hind limb while the fore limb is unaffected, tachypnea, froth in the mouth. Table 2, shows the most important symptoms with the timing of their emergence and disappearance within 24 hours after the dose with the leaves ethanolic extract of *N. oleander*.

![Fig. 3: Lung of rabbit after administration of *Nerium oleander* leaves by ethanolic extract at dose 240 mg/kg Bw.](image)

![Fig. 4: Heart of rabbit after administration of *Nerium oleander* leaves by ethanolic extract at dose 240 mg/kg Bw.](image)

![Fig. 5: Stomach of rabbit after administration of *Nerium oleander* leaves by ethanolic extract at dose 240 mg/kg Bw.](image)

![Fig. 6: Section of lung shows: mild interstitial pneumonia (In), pulmonary emphysema (E) & congestion (C). H&E stain. 40x](image)
for different doses.

- **Necropsy Finding**: The necropsy finding of dead animals showed, there were severe emphysema, pulmonary congestion and bronchopneumonia (Fig. 3), while in heart we are observed was clots, fibrinous exudate, mild endocarditis, fibril layer on pericardial infiltrate and inflammatory cells filaments, (Fig. 4) and, in stomach specifically, in glandular region there were severe erosion, lysis of mucosa extend to submucosa most glands in submucosa show severe necrosis (homogenous and structure less) (Fig. 5).

**Histopathological finding**

The histopathological finding of organs rabbit after administration of ethanolic extract of *Nerium Oleander* leaves at dose 240 mg/kg Bw. orally lungs in rabbit which have been observed are pulmonary congestion, thickening of alveolar walls, dilated pulmonary blood vessels filled with blood and few inflammatory cells, severe degeneration of bronchial epithelial mucosa and necrosis, also there were pleuritis and pulmonary emphysema (Fig. 6). Heart showed, infiltration and extension of inflammatory cells to myocardium and acute pericarditis, thickened inflammatory layer replaced the flattened epicardium (Fig. 7). Whereas the histopathological finding in stomach included severe atrophic necrosis of mucosal epithelium, complete sloughing in other section, the sub mucosal glands severely degenerated and necrotic of stomach) and showed the submucosal glands severely degenerated and necrotic (Fig. 8).

### Discussion

Acute toxicity defines the adverse effects of a substance that outcome either from a single exposure or from many exposures in a short period of time (typically less than 24 hours). To be pronounced as acute toxicity, the adverse effects should happen within 14 days of the administration of the substance, also refer to poisonous state (Hatif, Kafi and Alkhayyat, 2010). *Nerium Oleander* leaves decoction give to male rat injected intramuscularly in both hind limbs in two groups, group one injected 5 ml/kg and group two injected 10 ml/kg. The results refer to both doses can induce acute phase condition (Abbasi et al., 2017; Klaassen and toxicology, 1986).

Median lethal dose (LD<sub>50</sub>) is the dose essential to kill half the members of a examined population after a specified test duration. It is a tool to measure the acute toxicity of a chemical active ingredient. Also use to detect the toxicity of plant, to ass therapeutic dose of plant from through test LD<sub>50</sub> (Saliem, 2010). The estimated LD<sub>50</sub> of ethanolic extract of *Nerium Oleander* leaves in current study is 232.23 mg /kg.Bw orally in rabbit could be scheduled in the grade 4 severely toxic agent according to classification dependent by (Hayes and Loomis, 1996). The clinical signs that have been observed on exposed rabbit included depression, anorexia, piloeraction, dyspenia, slightly watery salivation, extended of hind limb while the fore limb is unaffected and tachypeniea. These clinical signs are in agreement with those observed after one hour in mice exposed ethanolic extract of *Nerium oleander* leaves at of 520 mg/kg body weight.

<table>
<thead>
<tr>
<th>Initial dose (mg/kg .BW)</th>
<th>Different between doses (mg/kg .BW)</th>
<th>Last dose (mg/kg .BW)</th>
<th>Outcomes*</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; orally (mg/kg .BW after 24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>30</td>
<td>210</td>
<td>OOOXOXO</td>
<td>232.23</td>
</tr>
</tbody>
</table>

* X= Dead animal; O= alive animal

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**Table 1**: The outcome of median lethal dose (LD50) of orally administration of ethanolic extract of *Nerium oleander* leaves in Local Iraqi rabbit (*Lepus cuniculus*) calculated by up and down method.

**Fig. 7**: Section of heart shows: mild endocarditis with clot (c), fibrin exudate and inflammatory cells (red arrow). H&E stain. 40x.

**Fig. 8**: Section of stomach shows: (E) erosion and, (n) severe degeneration and necrosis of gastric glands (Arrows). H&E stain. 40x.
Table 2: Signs of poisoning and mortality outcome that developed according to different doses of *N. Oleander* leaves Ethanolic Extract in Local Male Iraqi Rabbits (*Lepus cuniculus*) according to Up and Down method.

<table>
<thead>
<tr>
<th>Dose mg/kg BW</th>
<th>Clinical signs during 24 hrs</th>
<th>Time of signs appeared</th>
<th>Results (O, X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 mg</td>
<td>depression, anorexia, piloeraction, dyspeniea, slightly watery salivation, extended of hind limb while the fore limb is unaffected, tachypeniea.</td>
<td>After five hours from administration</td>
<td>O</td>
</tr>
<tr>
<td>180 mg</td>
<td>depression, anorexia, piloeraction, dyspeniea, slightly watery salivation, extended of hind limb while the fore limb is unaffected, tachypeniea.</td>
<td>After three hours from administration</td>
<td>O</td>
</tr>
<tr>
<td>210 mg</td>
<td>depression, anorexia, piloeraction, dyspeniea, slightly watery salivation, dragging of hind limb while the fore limb is unaffected, tachypeniea.</td>
<td>After three hours from administration</td>
<td>O</td>
</tr>
<tr>
<td>240 mg</td>
<td>sever convulsion, gasping, slightly watery salivation, piloeraction, extruded of eye ball, head down, incoordination, circling around itself.</td>
<td>After 15 minute from administration</td>
<td>X</td>
</tr>
<tr>
<td>210 mg</td>
<td>depression, anorexia, piloeraction, dyspeniea, slightly watery salivation, dragging of hind limb while the fore limb is unaffected, tachypeniea.</td>
<td>After two hours from administration</td>
<td>O</td>
</tr>
<tr>
<td>210 mg</td>
<td>depression, anorexia, piloeraction, dyspeniea, slightly watery salivation, dragging of hind limb while the fore limb is unaffected, tachypeniea.</td>
<td>After two hours from administration</td>
<td>O</td>
</tr>
</tbody>
</table>

was administered orally, the signs of toxicosis have appeared, abdominal pain, frequent urination, weakness, diarrhea, depression, convulsive movement and death (Saliem, 2010). While clinical signs of aqueous extract of *Nerium oleander* in female goat that appeared after 1 hr from receiving the extract were abdominal pain, convulsion, ruminatalotony, depressin, convulsion and death (Aslani *et al*., 2007). The clinical signs that observed on rabbits of our study were not fully agree with a study conducted in dogs have been administrated orally 0.25g.kg^-1 of fresh ground leaves of *Nerium oleander*, when several dogs died after the ingestion, but all showed signs of poisoning such as vomiting, diarrhea, nausea, apathy, conjunctiva congestion, dehydration, abdominal pain, tremors, diarrhea, loss of appetite and tenesmus. Loss of appetite was prominent in 30% of the dogs even after the treatment. The first obtainable clinical sign was vomiting, within 27- 75 minutes of administration of ground green leaves. Second presented sign was loss of appetite, after the intoxication and after the treatment, with 40% of the animals having these signs for as long as 12 hours after giving (Camplesi *et al*., 2017). The clinical signs appeared in sheep after 30 minute from take dried *Nerium Oleander* leaves were decrease heart rate (cardiac pauses), tachyarrthemia, mild to moderate tympany, abdominal pain, polyuria and pollakiuria (Aslani *et al*., 2004).

The estimated LD$_{50}$ of ethanolic extract of *Nerium oleander* leaves in current study is (232.23 mg /kg.Bw orally in rabbit is more than the LD$_{50}$ s/c injected aqueous leaf extract *Nerium oleander* in rabbit which was 157.37 mg / kg B. wt, but fall into the same grade of toxicity (Al-Badrani, Rhaymah and Al-Farwachi, 2008). While the median lethal dose of leaves *Nerium oleander* ethanolic extract is 520 mg/kg body weight were giving orally to male mice (Saliem, 2010). Whereas several studies found the orally LD$_{50}$ in mice of hexane leaf extracts of *N oleander* has red, pink and white flowers was 325, 300 and 350mg/kg respectively (6). The oral LD$_{50}$ of aqueous extract of *Nerium oleander* female goat is 110mg/kg BW (12). The LD$_{50}$ that has been found in rabbits in current study is disagree with the LD$_{50}$ of *N. oleander* leaves collected from Amiriya/Baghdad-IRAQ in summer and winter, which were extracted by hexane and derived by “up and down” method were 94.36, 79.75 mg/kg BW respectively (Salih and Alkhayyat, 2016), on the other hand the oral LD$_{50}$ of dried *Nerium Oleander* leaves was 110 mg /kg.BW in sheep which possessed clinical signs of poisoning appeared too early after 30 minutes of administration (Aslani *et al*., 2004), whereas the oral LD$_{50}$ of *N. oleander* leaves after a simple extraction from biological samples, for example, in cattle is 50 mg/ kg.BW. The leaf fragments found in the rumen contents were small and not simply isolatable from the rest of the contents. Though, there were leaves in the forage that were clearly distinct by the direct examination. Oleander poisoning due to active components contained in the plant are cardiac glycosides belonging to the cardenolides that are very toxic to many species, from human to insects (Rubini *et al*., 2019).
The toxicity of *Nerium oleander* due to the present of the highest concentrations of cardiac glycosides are in the seeds and roots of the common pink *Nerium oleander*, while the highest component of oleandrin are found in the leaves of the plant. Furthermore, *N. oleander* with red to pink flowers has higher concentrations of cardiac glycosides than *Nerium oleanders* with white flowers (Khordadmehr and Nazifi, 2018). So the thought of decrease in LD$_{50}$ rate resulted from administration *N. oleander* leave ethanolic extract to rabbits in current study comparable to that found in different literature may be due less abundant of oleanrin leaves of plant with white flower which has been used. *Nerium oleander* Leaves have high amount of cardiac glycoside and flavonoids compounds from methanolic extract of flower (Vu, Bui and Nguyen, 2016). Oleander toxicity is a common problem in several parts of the world. Oleander poisoning due to oleandrin (the chief cardiac glycoside of oleander) and nerin, which lead damage by inhibiting the plasmalemmal Na’/K’ ATPase (Abdou, Basha and Khalil, 2019). Oleander contains cardiac glycosides called cardinolide. Cardiac glycoside may cause hyperkalemia because of ability to inhibit the Na+K+-ATPase pump, leading to increased intracellular sodium and calcium and decreased intracellular potassium (Salih, 2017). The concentration of oleandrin in *N. oleander* tissues is about 0.08% (Salama et al., 2019). *Nerium oleander* is cardiotoxic plant and Its toxicity is due to the content of cardioactive glycosides, specially oleandrin, found throughout the plant (Botelho et al., 2018). The ethanol extracts of *Nerium oleander* leaves which have the highest amount of entirephenolics and flavonoids, appeared the highest antioxidant and cause toxicity of plant (Einiali et al., 2018). Oleander poisoning due to active components contained in the plant are cardiac glycosides belonging to the cardenolides that are very toxic to many species, from human to insects (Rubini et al., 2019).

Extraction yield is mass of extract or mass of dry matter is used as indicator of the extraction condition and it is the amount of component of interest obtained from extractions output. The effect of extraction environments on yield. There are several steps to get the phytochemicals from plant, for example milling, grinding, homogenization and extraction. The extraction method is the important step to minimize interfering from compounds that may co-extract with the board compounds. It is supposed that bioactive compounds from plant foods may have health beneficial effects and reduce the risk of diseases (Dhanani et al., 2017). The yield of *Nerium oleander* leaves extraction has been extracted by 95% ethanol is 9.02%. This is closely with ethanol extract of *Nerium oleander* leaves was (8.46%) extracted with absolute ethanol 100 ml for 24 h. at room temperature, were collected in late May 2014 from University of Sistan, Baluchestan southeast of Iran (Einiali et al., 2018) and the our result better than this studies by the non-polar solvents via petroleum ether (2.87%) was less as compared to the yield obtained from the polar solvents by ethanol and water 5.7% and 6.8% respectively. The fraction yield obtained from the successive extraction using the solvents petroleum ether, ethanol and aqueous extract obtained by maceration, from root of Nerium Oleander (Kawalekar et al., 2012), while disagree with the yield of extract 26%, 18% by Soxhlet method by the two hydro-methanoic and hydro-ethanolic extracts respectively of *Nerium oleander* leaves 20g of fresh leaves extract by ether petroleum and then dry for 10 minutes at room temperature, after that solute is placed in Soxhlet (Malika, Badiaa and Farida, 2016). The other studies showed extraction yields for every kilogram of leaves *Nerium oleander* by ethanolic extract was 1.03% (de Melo et al., 2020).

**Conclusion**

The ethanolic cold extraction is one of the useful method for obtaining significant yield extract of *N. Oleander* leaves. The *N. Oleander* leaves ethanolic extract is considered severely toxic in rabbits after oral administration.

**Conflict of interest**

The authors declare that they have no conflict of interest

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