Toxic Effect of *Nerium Oleander* Leaves Extract on Biochemical Parameters in Rabbits Serum

R. A. Salih and A. A. Alkhayyat
Department of Physiology and Pharmacology- College of Veterinary Medicine/ Baghdad University

Abstract

The study involved collection of the *Nerium oleander* leaves of two seasons (summer and winter) in 2014 which was taken from Baghdad (Amiriya). The leaves prepared by drying, grinding, the extraction which was carried by soxhlet apparatus with organic solvent (hexane). The pilot study (by using two rabbits for each dose of *N. oleander* extracts of the two seasons) was done for determining the range of doses which used. The median lethal dose (LD50) which was carried out by "up and down" method was (94.36) mg/kg B.Wt. for summer extract and (79.75) mg/kg B.Wt. for winter extract of *N. oleander* leaves. The chronic toxicity were carried out on rabbits by giving 10% of the dose that obtained by acute toxicity study and by considering different serum parameters after two weeks and four weeks of daily treatment. These parameters include serum potassium, calcium levels, serum ALT, AST and ALP levels. Biochemical study shown serum liver enzymes (ALT, AST, and ALP) was elevated in both groups (summer and winter) as compare to digoxin and control groups in different times (two weeks and four weeks), also increase serum K^+ level as hyperkalemia and decrease serum Ca^{++} level as hypocalcaemia. We concluded that the *N. oleander* leaves extract has toxic effect on liver enzymes (ALT, AST and ALP), also effect on serum calcium and potassium levels, also the winter extract more effect than that in summer extract.

Key words: *Nerium Oleander* Leaves Extract, Biochemical Parameters, Rabbits Serum
E-mail: bakir20062006@yahoo.com

التأثير السام لمستخلص اوراق نبات الدفلة على التغييرات الكيمياوية الحيوية في مصل الارانب

رنا عبد الله صالح وعلي عزيز الخياط
كلية الطب البيطري/ جامعة بغداد

الخلاصة

تضمن البحث جمع اوراق نبات الدفلة *Nerium oleander* للموسمين الصيفي والشتوي من بغداد ثم تجفيفها وطحنها بالمطحنة الكهربائية grinder، عملية الاستخلاص تمت بطريقة الاستخلاص بالهكسان باستخدام جهاز السوكسليت soxhlet apparatus. استخدمت الدراسة الاولية pilot study (وذلك بإجرائها في اثنين من ارانب التجربة لكل جرعة من مستخلص نبات الدفلة لكل موسم) لمعرفة مدى الجرع الممكن استخدامها. تم تحديد الجرعة الوسطية القاتلة LD50 بطريقة "up and down" للموسمين بالارانب، وكانت الجرعة الوسطية القاتلة LD50 94.36 ملغم/كغم من وزن الارنب للمستخلص الصيفي و79.75 ملغم/ كغم من وزن الارنب للمستخلص الشتوي. اجريت دراسة السمية المزمنة للارانب بتجريعها 10% من الجرعة الناتجة من دراسة السمية الحادة، وتم ذلك باخذ عدة معايير او اختبارات بعد اسبوعين واربعة اسابيع من التجريع اليومي. تضمنت هذه المعايير مستوى أيون البوتاسيوم والكالسيوم بالمصل، مستوى انزيمات الكبد ALT, AST and ALP بالمصل. قيمت بعض التغيرات الكيموحيوية والتي تضمنت انزيمات الكبد (ALT, AST and ALP) التي ارتفعت بكل المجاميع مقارنة بمجموعة السيطرة بكلا الفترتين، وارتفاع نسبة البوتاسيوم وقلة نسبة الكالسيوم بالمصل.نستنتج بان مستخلص اوراق الدفلة *N.*

Oleander ذو تأثير سام على انزيمات الكبد ALP and AST, ALT وكذلك تؤثر على نسبة الكالسيوم والبوتاسيوم في مصل الدم، وكان المستخلص الشتوي اكثر تأثيرا من المستخلص الصيفي.

الكلمات المفتاحية: مستخلص اوراق نبات الدفلة، التغييرات الكيمياوية الحيوية، مصل الارانب

Introduction

Oleander is an evergreen shrub or small tree from 1 to 10 m tall containing gummy sticky sap in the dogbane family Apocynaceae. Oleander is an idiom for plants of the *N. oleander L*, *N. indicum*, and *Nerium odorum* species. Common names include soland, lorierbol, rosebay, and rose laurel and kaner (1). This plant grows outdoors in warmer regions, and sometime is grown as a house plant. It's widely cultivated in Mosul and Baghdad (Iraq) on roadsides, edges of woods and gardens (2, 3). Oleander contains cardiac glycosides (cardinolide). The main cardiac glycoside of oleander is oleandrin, also other glycoside is nerioside (4), and both of them can be isolated from all parts of the plant. Action of the poisons in oleander is similar to the action of the heart drug digitalis, and cardiac glycosides are a class of compounds used to treat congestive heart failure by increasing myocardial contractile force (5). Oleandrin is a cardiac glycoside derived from Nerium oleander, which has been used for many years in Russia and China for this purpose. In contrast to its use for the treatment of heart failure, preclinical and retrospective patient data suggest that cardiac glycosides (e.g., digoxin, digitoxin, ouabain, and oleandrin), may reduce the growth of various cancers including breast, lung, prostate, and leukemia (6), The important pharmacological activities are antinociceptive, anti-inflammatory, antibacterial, anticancer and CNS depressant activity (7). The usual symptoms of toxicity are severing gastroenteritis, cardiac irregularities and increased heart rate is common. Cardiac glycoside may cause hyperkalemia due to ability to inhibit the Na^+-K^+-ATPase pump (8, 9). In low doses the glycoside have a beneficial therapeutic effect on the heart by increasing the force of contraction and increasing cardiac output (10, 11).

Materials and Methods

Fresh leaves of local planted oleander were collected from Baghdad, in January and July. Then the leaves were dried at room temperature in open air and were ground by an electrical grinder. Then extracted by hexane with soxhlet apparatus, the extracts were dissolved in the propenyl glycol and administrated orally to rabbits at dose of 10% of median lethal dose(LD50) of extract was conducted to estimate the toxic potency using "up and down" method (12) LD50 = (Final test level) + (Value from table) (Difference between dose levels). The ranges of toxic doses were estimated by primary (Pilot) study for the extract which was done for determining the range of doses which used; by using two rabbits for each dose of *Nerium oleander* extract of the two seasons. Sixteen rabbits were divided equally to two groups that were given increasing doses of the extract orally (by stomach tube), the number of dead animals were recorded during 24 hrs. Fourty rabbits (local breed), 1-2 years old weighing 1-3 kg were used for the study. The animals were adapted for 2 weeks and allocated at the weighted groups. The fourty rabbits were divided into four groups, equally (10 rabbits in each group) which were used as follows: The first group was treated with distilled water as control, the second group treated with digoxin for comparison, the third group was administrated with different doses of *N. oleander* leaves extract (summer season), and the fourth group was treated with different doses of *N. oleander* leaves extract (winter season). Blood sample were collected after two and four weeks after administration from rabbit's heart directly by (5cc syringe) in a dry, clean and sterile centrifuge tubes, and then left few minutes allowed to be clotted at room temperature before circulation by centrifuge at (3000) rpm for 20 minutes to separate the clear sera which were put in eppendorf tube by

micropipette till performing the biochemical analysis (13). The procedure of liver enzymes according (14): Estimation of aspartate aminotransferase activity using automatic analyser (Mindray) ACCENT-200: Principle of the method: Optimized, modified method according to International Federation of Clinical Chemistry (IFCC), without pyridoxal phosphate.

L-aspartate + 2-oxoglutarate $\xrightarrow{AST}$ oxalacetate + L-glutamate

Oxalacetate + NADH + H^+ $\xrightarrow{MDH}$ malate +NAD^+

The rate of absorbance changing at λ= 340 nm directly proportional to aspartate aminotransferase activity.

Procedure:

Using automatic analyser (mindray) ACCENT-200: kinetic method

1-Reagent and 2-Reagent were ready to use.

For reagent blank deionized water was recommended.

Estimation of alanine aminotransferase activity using automatic analyser (Mindray) ACCENT-200:

Principle of the method:

Optimized, modified method according to International Federation of Clinical Chemistry (IFCC), without pyridoxal phosphate.

L-alanine + 2-oxoglutarate $\xrightarrow{ALT}$ pyruvate + L-glutamate

Pyruvate + NADH+H^+ $\xrightarrow{LDH}$ Lactate + NAD^+

The rate of absorbance changing at λ=340 nm is directly proportional to alanine aminotransferase activity.

Procedure:

Using automatic analyser (mindray) ACCENT-200: kinetic method

1-Reagent and 2-Reagent were ready to use.

For reagent blank deionized water was recommended.

Estimation of alkaline phosphatase activity using automatic analyser (Mindray) ACCENT-200:

Principle of the method:

Kinetic method recommended by International Federation of Clinical Chemistry (IFCC).

2-amino-2-methyl-1-propanol + p-nitrophenylophossphate+H_2 $\xrightarrow{AP}$ 4-nitrophenol + 2-amino-2-methly-1-propanol phosphate

The rate of 4-nitrophenol formation is directly proportional to the ALP activity.

Procedure:

Using automatic analyzer (mindray) ACCENT-200: kinetic method

1-Reagent and 2-Reagent are ready to use.

For reagent blank deionized water was recommended.

Serum potassium is measured by the use of a flame photometer or ion-selective electrode. The procedure is rapid, simple, and reproducible. The DxC800 system uses indirect (or diluted) I.S.E. (ion selective electrode) methodology to measure calcium concentration in serum, a calcium ion selective electrode measures un-bound free calcium ions in solution, the system determines calcium concentration by measuring calcium ion activity in solution, when the sample buffer mixture contacts the electrode, calcium ions complex with the ionophore at the electrode surface, changes in potential develop at the electrode surface as the reaction occurs, these changes in potential are referenced to a sodium reference electrode, the reference signal is used in calculating the analyze concentrations based on the Nernst equation (15, 16).

- **Statistical analysis:** Data were analyzed statistically using the Microsoft Program (SPSS). Statistical analysis of data was performed on the basis of Two-Way Analysis of Variance (ANOVA) using a significant level of ($P<0.05$). Specific group differences were determined using least significant differences (LSD).

Result and Discussion

- **Median Lethal Dose (LD50):** The LD50 results of two seasons in rabbits were shown in tables (1, 2):

Table (1) LD50 of *N. oleander* leaves extract on rabbits in summer

Doses mg/kg	Concentration (mg/ml)	Dead and lived Rabbits
62.5	12.5	O
75	15.0	O
87.5	17.5	O
100	20.0	O
112.5	22.5	X
100	20.0	X
87.5	17.5	X
75	15.0	O

-*O for lived rabbits and X for dead rabbits.

(OOOOXXXO)

LD50= Xf + Kd

= 75 + (+1.549) ×12.5

= 75 + (19.3625)

= 94.3625 mg/kg

Table (2) LD50 of *N. oleander* leaves extract on rabbits in winter

Doses (mg/kg)	Concentration (mg/ml)	Dead and lived Rabbits
37.5	7.5	O
50	10.0	O
62.5	12.5	O
75	15.0	O
87.5	17.5	X
75	15.0	X
62.5	12.5	O
75	15.0	O

-*O for lived rabbits and X for dead rabbits.

(OOOOXXOO)

LD50= XF + Kd

= 75 + 0.38 × 12.5

= 75 + (4.75)

= 79.75 mg/kg

The present study targeted to determine the LD50 in rabbits administrated orally with *Nerium oleander* hexane leaf extract. The LD50 was carried by "up and down" method. The estimated dose which killed half of rabbits was 94.3625 mg/kg in summer and 79.75 mg/kg in winter, this indicated that concentration of toxic substances in hexane extract (non polar) in winter is more toxic than that in summer due to accumulation of lipid in *N. oleander* leave plant increased in cold season because of decline plant growth in this period (17). After the oral administration of the *N. oleander* leaves extract for the rabbits for both seasons (summer and winter) at two and four weeks, the result as following:

- **Serum Calcium and potassium levels:** Serum Ca^{++} levels decreased but not significantly in two weeks among all groups, while in four weeks after treatment showed winter group decreased significantly as compared to control group, while digoxin and summer groups showed non significant decrease, the winter extract group were more toxic than the other two and this may be due to the presence of different active ingredient which may be more powerful than pure digoxin. Furthermore differences in time of exposure (2 weeks and 4 weeks) illustrates non significant differences in control, digoxin and summer groups, but significant decrease in winter group, this result are showed in (table 3).

Table (3) Serum calcium levels (mEq/L) in rabbits after administration of winter extract, summer extract and digoxin in two times

Group / Time	Control	Digoxin	Summer	Winter
After 2weeks	18.36±0.46 Aa	17.26±0.46 Aa	17.10±0.42 Aa	17.38±1.88 Aa
After 4 weeks	17.06±0.49 Aa	16.48±0.17 Aa	16.74±0.36 Aa	11.00±0.15 Bb

-LSD= 1.81
-Different capital letters refer to significant differences between different groups at (P<0.05).
-Different small letters refer to significant differences between different times at (P<0.05).

Serum K^+ levels after two weeks showed non significant increase between digoxin and control group, but significant increase between extract groups (summer and winter) as compared to control and digoxin, while there was no significant increase between summer and winter groups. The same results was obtained with the samples after four weeks of treatment. Increased levels of serum K^+ according to time of exposure (2 weeks and 4 weeks) within same group showed no significant increase between control group as well as in digoxin group, or in summer group, but there was significant increase differences in winter group, this may be explained on the basis of cardiac glycoside content of this sample (table 4). Also as a result to oleander inhibition of Na^+-K^+ pump which cause hyperkalemia (18).

Table (4) Serum potassium levels (mEq/L) in rabbits after administration of winter extract, summer extract and digoxin in two times

Group / Time	Control	Digoxin	Summer	Winter
After 2weeks	4.56±0.13 Ba	5.56±0.20 Ba	6.36±0.33 Aa	6.64±0.07 Ab
After 4 weeks	4.90±0.24 Ba	5.82±0.13 Ba	7.20±0.24 Aa	7.80±1.43 Aa

-LSD=1.04
-Different capital letters refer to significant differences between different groups at (P<0.05).
-Different small letters refer to significant differences between different times at (P<0.05).

The increased intracellular sodium inhibits sodium-dependent calcium transport out of the cytoplasm, resulting in increased intracellular calcium, and thus increased inotropy. In overdose, digoxin and oleander inhibition of the sodium-potassium ATPase frequently results in hyperkalemia (18, 19, 20).

- **Serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP):** The ALT which is found in the hepatocytes is considered as the more specific test to liver function (21). The serum levels of ALT after two weeks of treatment showed non significant increase between digoxin, summer, winter groups as compared to control group, or between each other, this may be due to that affected liver tissue was not enough to cause released of ALT to blood stream. Also, after four weeks there were non significant increase between digoxin and control group, but significant increase between the two seasons extract groups as compared to control and digoxin and this may be attributed to that the extract may contain different active ingredients that effect and give this result, while there was no significant increase differences between winter and summer group. Considering time of exposure (two weeks and four weeks) there was no significant increase in control group, or in digoxin group, but significant increase in summer group and in winter group (table 5) due to liver damage, and some time elevation in other than liver such as heart (22).

Table (5) Serum ALT (IU/L) levels in rabbits after administration of winter extract, summer extract and digoxin in two times

Group / Time	Control	Digoxin	Summer	Winter
After 2weeks	64.60±6.85	65.00±2.97	65.60±18.77 b	65.80±8.41 b
After 4 weeks	76.60±1.29 B	78.20±0.67 B	111.40±8.84 Aa	127.60±25.91 Aa

-LSD=28.42
-Different capital letters refer to significant differences between different groups at (P<0.05).
-Different small letters refer to significant differences between different times at (P<0.05).

The AST present in hepatocytes, red blood cells and in cardiac and skeletal muscles (23). The serum level of AST after two weeks of treatment showed significant increase between digoxin and control group. Also, there was significant increase between two extract groups as compared to control group may be due to effect on liver tissue and heart in this treated groups, while no significant increase between summer group and digoxin group, also significant increase between winter group as compared to digoxin group this may be attribute to different active ingredients found in the extract which give this effect, while there was no significant increase differences between both seasons extract. After four weeks no statistical increase between digoxin and control groups, but significant increase alteration between summer and winter groups. Furthermore significant increase between summer and winter groups as compare to digoxin group was found, this may also referred to active ingredients content present in extract while digoxin is only a pure substance, also there was significant increase differences between winter and summer groups this may be due to more amount of oleandrin found in winter extract. Considering times of exposure between two weeks and four weeks there no statistical increase within control and within digoxin groups, but significant increase differences were seen in summer group, and winter group this may be referred to liver and heart damage (table 6).

Table (6) Serum AST (IU/L) levels in rabbits after administration of winter extract, summer extract and digoxin in two times

Group / Time	Control	Digoxin	Summer	Winter
After 2weeks	53.60±4.86 Ca	65.20±6.47 Ba	68.60±11.14 ABb	87.00±14.81 Ab
After 4 weeks	63.00±4.01 Ca	81.20±8.81 Ca	104.40±10.82 Ba	189.00±17.45 Aa

-LSD=20.71
-Different capital letters refer to significant differences between different groups at (P<0.05).
-Different small letters refer to significant differences between different times at (P<0.05).

The ALP is an intrahepatic enzyme found in lining cells of the biliary ducts, bone and placenta (24). The level of serum ALP after two weeks of treatment showed significant increase between digoxin group and control group due to effect on liver tissue, but non significant increase between summer and winter groups as compared with control. However accomplished was statistical increase altering between digoxin and summer groups may be due to effect of digoxin are more on the vital organ, while no significant increase between winter group and digoxin group, or between summer group and winter group. Furthermore after four weeks showed only significant increase within digoxin group as compared to control group due to its toxic effect. Considering time of exposure (two and four weeks) there was no significant increase differences within all groups (table 7).

Table (7) Serum ALP (IU/L) levels in rabbits after administration of winter extract, summer extract and digoxin in two times

Group / Time	Control	Digoxin	Summer	Winter
After 2weeks	69.60±11.65 B	116.00±19.82 A	74.20±14.88 B	91.60±16.17 AB
After 4 weeks	56.20±7.24 B	95.00±24.11 A	81.00±16.29 AB	73.60±9.92 AB

-LSD=30.77

-Different capital letters refer to significant differences between different groups at (P<0.05).

The levels of ALT, AST and ALP in the blood normally are low, those enzymes are considered as biomarkers of hepatic affection and elevated of these enzymes due to a major permeability or cell rupture (21, 25, 26). LD50 evaluated to rabbits by (2) who using subcutaneous injection with *N. oleander* watery leaf extract, and (27) who utilized daily dosage of aqueous extract *N. oleander* leaves and flower orally to evaluation symptoms outcome, both of them found statistical elevate to aspartate and alanine aminotransferase exertion, serum sodium- potassium, and depress blood cholinesterase action within erythrocytes and plasma, which may resemble to certain degree the present study concerning (ALT, AST and ALP) enzymes. Also (27) showed that these abnormal changes that seen in both hematological and biochemical parameters were due to the damage that occur in most organs of the mice, including heart, kidney lung, spleen and liver. In fact lesions were noticed in these all organs after the killing of the mice, liver and spleen enlargement were also observed. Diarrhea was also recorded in the treated animals when compared with untreated ones. However, histopathological study has been made by some local and national researchers in which they mention that oleander cause significant tissue destruction in many organs (28) in goat and in rat (29). However, organ destruction is due to the toxic content of oleander glycoside which was shown to act directly on the heart, causing cardiac arrhythmias and heart block (30), and on gastrointestinal tract causing enteritis, abdominal pain and diarrhea (31, 32). Conclusions*: Nerium oleander* leaves extract have toxic effect, which affected on levels of calcium, potassium and some liver enzymes (ALT, AST and ALP) in rabbits serum, also the winter extract have more toxic effect than that in summer extract.

References

1. Ozmaie, S.; Akbaril, G.; Asgharil, A.; Sakhal, M. & Mortazavi, P. (2013). Experimental Oleander (*Nerium oleander*) Poisoning in Sheep: Serum biochemical changes and pathological study. Ann. Biol. Res., 4 (1): 194-198.
2. Al-Farwachi, M. I.; Rhaymah, M. S. & Al-Badarani, B. A. (2008). Acute toxicity of *Nerium oleander* aqueous leaf extract in rabbits. Iraqi J. Vet. Sci., 22 (1): 1-4.
3. Salih, R. A. (2008). Study of acute toxicity of different extracts of Oleander (*Nerium Oleander*) Leaves in Mice. A Thesis of M. Sc.
4. Wasfi, I. A.; Zorob, O.; Al-Katheeri, N. A. & Al-Awadhi, A. M. (2008). A fatal Case of Oleandrin Poisoning. Forensic Sci. Int., 179 (2-3):31-36.
5. Kjeldsen, K.; Norgaard, A. & Gheorghiade, M. (2002). Myocardial Na-K-ATPase: The Molecular Bases for the hemodynamic effect of Digoxin Therapy in Congestive Heart Failur. Cardiovascular Research, 55(4):710-713.
6. Frese, S.; Frese-Schaper, M.; Andres, A. C.; Miescher, D.; Zumkehr, B. & Schmid, R. A. (2006). Cardiac glycosides initiate Apo2L/TRAIL-induced apoptosis in non-small cell lung cancer cells by up-regulation of death receptors 4 and 5". Cancer Res., 66 (11): 5867-5874.
7. Gupta, V. & Mittal, P. (2010). Phytochemical and pharmacological potential of *Nerium Oleander*. Int. J. Pharm. Sci. Res., 1(3):21-27.
8. Xie, Z. & Askari, A. (2002). Na^+-K^+-ATPase as a signal transducer. Eur. J. Biochem., 269:2434-2439.
9. Schoner, W. & Scheiner, B. G. (2007). Endogenous and exogenous cardiac glycosides: Their roles in Hypertension, Salt Metabolism, and Cell Growth. Am. J. Physiol. Cell Physiol., 293:509-536.

10. Joubert, J. P. J. (1989). Cardiac Glycosides In: Toxicants of Plant Origin .Volume 2: Cheeke P. R., Ed. Boca Rafon, F.L.:CRC Press, PP. 61-96.
11. Cheeke, P. R. (1998). Natural Toxicants in Feeds, Forages and Potsonons Plants, Edition 2 .Danville: Interstate Publishers, PP. 390-409.
12. Dixon, W. J. (1965). The "Up and Down" method for small samples. Am. Stati. Assoc. J., 60: 967-978.
13. Rhaymah, M. S.; Al-Farwachi, M. I. & Al-Badrani, B. A. (2011). Chronic Toxicity of *Nerium Oleander* Aqueous Leaf Extract in Rabbits. Al-Anbar J. Vet. Sci., 4:88-93.
14. Ahmed, R. M. (2002). Assessments of liver enzymes (AST, ALT and ALP) activity in Sudanese patients with type 2 diabetes mellitus. Sudan University of Science and Technology College of Graduate Studies.
15. Synchron, B. C. (2007). Clinical Systems Chemistry Information Manual.
16. Rastegar, A. (1990). Clinical methods: The history, physical and laboratory examinations.3rd Edition. Chapter 195. Serum potassium.
17. Meletiou-Chrisyou, M. S.; Banilas, G. P. & Bardis, C. (2011). Plant biomonitoring: Impact of urban environment on seasonal dynamics of storage substances and chlorophylls of oleander. Global NEST Journal, 13(4): 395-404.
18. Kassop, D.; Donovan, M. S.; Cohee, B. M.; Mabe, D. L.; Wedam, E. F. & Atwood, J. E. (2014). An unusual case of cardiac glycoside toxicity. Int. J. Cardiol., 170: 434-444.
19. Hack, J. B. & Lewin, N. A. (2002). Cardiac Glycosides. In: Goldfrank, L. R.; Flomenbaum, N. E.; Lewin, N. A. Goldfrank's Toxicologic Emergencies, 7th ed., New York: McGraw-Hill Companies, Inc., PP. 724-740.
20. Levine, M.; Nikkanen, H. & Pallin, D. J. (2011). The effects of intravenous calcium in patients with digoxin toxicity. J. Emerg. Med., 40 (1): 41-46.
21. Al-Hadithy, H. A. H.; Badawi, N. M. & Mahmood, M. M. (2013). Estimation of serum liver enzymes activities in Awassi sheep. The Iraqi J. Vet. Med., 37(1): 115-120.
22. Kasper, D.; Braunwald, E.; Fauci, A.; Hauser, S.; Longo, D. & Jameson, J. (2005). Harrison's principles of internal medicine. 16th ed. New York: McGraw-Hill.
23. Evans, G. O. (2009). Animal Clinical Chemistry: A Practical Handbook for Toxicologists and Biomedical. 2nd Ed., Published by CRC Press, An Imprint of Taylor and Francis Group. Boca Raton London, New York, Chapter 2 General Enzymology, PP. 17-36.
24. Copeland, R. A. (2000). Enzymes. In: A Practical Introduction to Structure, Mechanism and Data Analysis. Second edition. A John Wiley and Sons, INC., Publication New York/ Chichester/ Weinheim/ Brisbane/ Singapore/ Toronto.
25. Nwafor, P. A.; Ekpo, M.; Hadezi, T. W.; Okokon, J. & Bassey, A. L. (2006). Acute toxicity potential of methanolic extract of smilax kranssiana leaves in rats. Int. J. Pharmacol., 2(4): 463-466.
26. McPherson, R. A. & Pincus, M. R. (2007). Henry's Clinical Diagnosis and Management by Laboratory Methods. 21st ed. Saunders Elsevier, 1405.
27. Altaee, M. F. (2011). In vivo toxicity study of *Nerium oleander* leaves and flowers aqueous extracts in mice. (Cytogenetic, biochemical and hematological study). Baghdad Sci. J., 8(1): 366-372.
28. Aslani, M. R.; Movassaghi, A. R.; Janati-Pirouz, H. & Karazma, M. (2007). Experimental oleander (*Nerium oleander*) poisoning in goats: a clinical and pathological study. Iranian J. Vet. Res., 8(1): 58-63.
29. Yahaya, M. A.; Al-Farhan, A. H. & Adam, S. E. (2000). Preliminary toxicity study on the individual and combined effects of Citrullus colocynthis and Nerium oleander in rats. Fitoterapia, 71(4): 385-391.
30. Siemens, L. M.; Galey, F. D. & Johnson, B. (1995). The clinical, cardiac and pathophysiological effect of oleander toxicity in horse. J. Vet. Inter. Med., 9:217-222.
31. Oryan, A.; Maham, A. & Rezakani, M. (1996). Morphological studies on experimental oleander poisoning in cattle. Zentral. Vet. Med., 43:625-634.
32. Radostitis, O. M.; Gay, C. C.; Blood, D. C. & Hinch, K. W. (2000). Veterinary Medicine: textbook of disease of cattle, sheep, pig and horse. 9th ed., Philadelphia, WB Sanders Company.