Mansoura Journal of Biology Vol. 38(2) December, 2013.

DETERMINATION OF LD_{50} AND EVALUATION OF SUB TOXICITY OF OTTELIONE A IN MICE

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Received: (12/ 8/ 2012)

ABSTRACT

On the basis of acute inter peritoneal (ip) toxicity studies, 370mg/kg body weight induce 50% death in mice, $1/10^{th}$ of the dose was selected to investigate the safety profile of ottelione A in terms of its oxidative stress. Acute toxicity (LD_{50}) test was performed with ottelione A in mice by ip injection. The sub acute toxicity studies were performed in mice after 15 days of OTTE administration, while alfa-fetoprotein and oxidative stress were monitored. The LD_{50} value of 370 mg/kg was obtained. The sub acute toxicity study evaluated insignificant changes in body weight and survivals of both OTTE-treated and control mice. The results also showed insignificant change in lipid peroxidation, glutathione and catalase levels in the liver of OTTE-treated mice. Meanwhile, these results were associated with significant increase in the activity of SOD in the liver of OTTE-treated mice as compared with the control. In conclusion, these results indicate that ottelione A is safe at the tested dose. This results point to the potential use of ottelione A in case characterized by oxidative stress.

Key words: LD_{50}, antioxidants, oxidative stress, Ottelione A

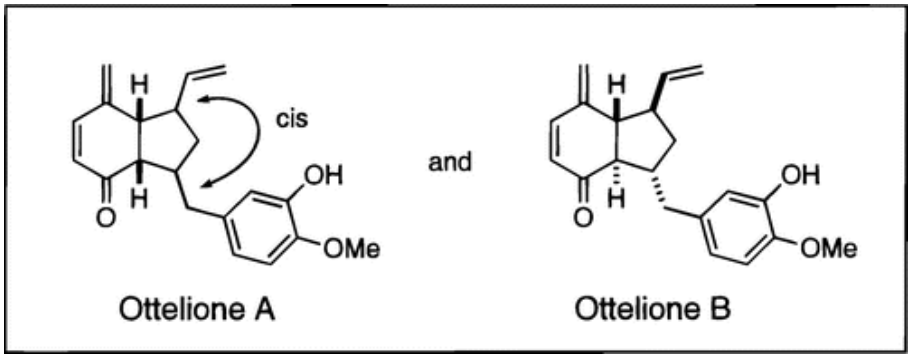
INTRODUCTION

Interest in natural plants to afford treatments for pain or curatives for a variety of diseases or recreational custom reaches back to the

earliest time of history. Ottelia alismoides (duck lettuce) is a freshwater herb. Its native variety is the tropical and warmer areas of Africa, Asia and Australia **(Cook & Urmi-Kong, 1984).** Ottelia alismoides grows partially submerged with rosetted, broadly cordate, floating leaves on long petioles. Its distribution in Egypt is rare, but it blooms annually in rice fields and irrigation channels during the summer months **(Ayyad, et al., 1998).** In the present study, O. alismoides was collected from the irrigation channels of the Nile Delta in Egypt in June 2009 and August 2010.

The leaves of Ottelia alismoides are used ethno medically across Africa and south Asia without scientific basis or safety concerns. Determination of its toxicological profile will provide supportive scientific evidence in favor of its continuous usage. Demand is progressively increased not only in developing countries but also in the industrialized nations **(Srivastera, et al., 1996)**. World Health Organization (WHO) estimates that approximately 80% of the developing world's population meets their Primary Healthcare needs through traditional medicine **(Farnsworth, et al., 1985).** About 25% of prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material **(Abere, et al., 2010).**

Following extraction and purification of the extract, two similar chromatographically and spectroscopically compounds known as ottelione A and B were isolated. Ottelione A is the main active component and showed quite remarkable biological activity. In Vitro, Ottelia alismoides crude extract (OTTE) showed to be an efficient inhibitor of tubulin polymerization and can disassemble preformed microtubules **(Combeau, et al., 2000).** Ottelione A was isolated from Ottelia alismoides collected in the Nile Delta, Egypt by the Hoye group **(Ayyad, et al., 1998)** and showed antitubercular activity and remarkable in vitro cytotoxicity against various cancer cell lines **(Li, et al., 1995).** Ottelione B was a stereoisomer of ottelione A and showed inert biological activity.



Acute toxicity is the toxicity produced by a pharmaceutical when it is administered in one or more doses during a period not exceeding 24 hours. Acute toxicity of a drug can be determined by the calculation of LD_{50}, i.e., the dose that will kill 50% of animals of a particular species **(Miller & Tainter, 1944 and Dietrich, 1983)** .Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use. The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans.

The aim of the present work was to determine the LD_{50} of Ottelione A and to investigate the safety profile in terms of its effect on the redox state of liver of mice.

MATERIALS AND METHODS

Chemicals

All reagents were of the highest purity available. All chemicals were purchased from Sigma-Aldrich Company Ltd. (Gillingham, United Kingdom).

Experimental animals

Three months old male BALB/c mice were maintained at the animal facility of the department of Zoology at the faculty of Science, Mansoura University on a 12 h light/12 h dark cycle under controlled conditions of temperature (20 ± 4°C) and relative humidity (55 ± 10%).

Mice had *ad libitum* access to a standard diet and pure water. All procedures for animal experimentation used were approved by the Institutional Animal Ethics Committee.

Preparation of plant extract

Whole plant of Ottelia alismoides (~500 gm), collected in June2009 and August 2010, were air dried at room temperature and stored at ~-20°C until extracted according to method of the Hoye group **(Ayyad, et al., 1998).** Plant material was shredded by hand into pieces of ~10 cm2 and soaked with petroleum ether for several days each time (2 x 3 L). The solvent was decanted, and the combined organic extracts were concentrated under vacuum to give a dark green residue (~10 g).

Acute toxicity study

"Estimation of the LD50 and percentage of mortality"

An approximate LD50 can be initially determined as a pilot study by a so called 'staircase method' using a small number of animals and increasing the doses of the drug **(Dietrich, 1983).** Male Albino mice of 5 animals per group and weighing 25±3 g were inter peritoneal injected graded doses (200-800 mg/kg body weight) of OTTE (Table 1). After injection of the OTTE the mice were observed for toxic effects after 24 h treatment. The toxicological effects were observed in terms of mortality and expressed as LD50. The animals were observed for first 2 hours and then at 6th and 24th hour for any toxic symptoms. After 24 hours, the number of died mice was counted in each group. The probit analysis was used to compute the lethality percentage of OTTE by aid of NCSS2007 software. 1/10 of the LD_{50} dose has been considered for further studies.

Sub acute toxicity study

In another experiment, mice were randomly divided into two groups comprising eight animals in each group. The first group did not receive any treatment and served as a control group. The Ottelione A (OTTE) group received 37mg OTTE/kg bw by interperitoneal injection on 1^{st} and 3^{rd} day of 15 days experimental period. This dose represents $1/10^{th}$ of the LD_{50} dose.

At the end of the experimental period, all mice were sacrificed on the next day after an overnight fast by decapitation. Blood was collected and sera were separated by centrifugation at 3000 rpm for 10 min. The

liver were isolated, weighed, cooled and homogenized 10-fold volume of ice-cold Phosphate buffer saline (50 mM, pH 7.0) for biochemical determinations. Lipid peroxidation was estimated by measuring the formation of the thiobarbituric acid reactive substances (TBARS) **(Ohkawa, et al., 1982).** The glutathione (GSH) content was determined spectro photometrically at 412 nm using 5,5′-dithiobis-2-nitrobenzoic acid (Beutler, 1982). The activity of superoxide dismutase (SOD) was determined using nitroblue tetrazolium and phenazine methosulphate **(Nishikimi, et al., 1972).** Catalase (CAT) activity was assayed as suggested by Bock et al. (1980), based on the rate of H_2O_2 degradation by the action of catalase contained in the examined samples. For SOD and CAT the homogenates were sonicated for 2 min. alfa-fetoprotein (AFP) level was estimated using colorimetric assay kit (Biovision, USA) **(Uotila, et al., 1981).** Statistical analysis of the results was performed using ANOVA followed by Newman–Keul's post hoc test.

RESULTS

The results of the acute inter peritoneal toxicity tests of ottelion A (OTTE) is shown in Figure 1 and Tables 1& 2, respectively. Treatment with OTTE did not cause mortality for up to 100 mg/kg body weight but increasing mortality was recorded with increasing doses of 200-800 mg/kg body weight. However, at 800 mg/kg body weight 90% mortality was recorded. Death in each case was preceded by peri-oral tremor followed by decreased locomotor activity. The mortality percent of mice were positively correlated with the logarithm of the injected doses of OTTE (Table 2). As shown in Table 2 and Fig 1, the LD50 value of OTTE when injected ip was found to be 370.127 mg/kg body weight.

Chronic treatment with 37mg/kg/day for 15 days did not cause change in the pattern of weight gain and survivals in the OTTE-treated mice when compared to the control values (Fig 2&3 respectively).

The results indicated no change in alfa fetoprotein level in serum of OTTE treated mice compared with the control animals (Table 3).

Treatment with $1/10^{th}$ of LD_{50} dose did not induce oxidative stress as indicated by insignificant change in lipid peroxidation and glutathione

concentrations as well as catalase activity in liver (Table 3). On the other hand, the treatment with OTTE significantly increased the activity of superoxide dismutase in liver as compared with the control mice.

Table (1): The full design to estimate lethality percentiles (LD_{50}) of Ottelione A and the statistical probit analysis of ottelione A after 24 hrs of inter peritoneum injection with graded doses in adult male mice.

Group (n=5)	Doses	Actual %	Probit %	Number responding	Expected count	Differences	Calculated Chi-square
1	**200**	**20.00**	**13.40**	**1.00**	**0.67**	**0.33**	**0.19**
2	**300**	**20.00**	**35.27**	**1.00**	**1.76**	**-0.76**	**0.51**
3	**500**	**80.00**	**70.58**	**4.00**	**3.53**	**0.47**	**0.21**
4	**800**	**90.20**	**91.73**	**4.51**	**4.59**	**-0.08**	**0.02**

Table (2): The lethality percentiles of ottelione A in adult male mice at 24 hrs following intra peritoneal injection with graded doses.

Percentil	Probit	Log(Dose)	Dose
1%	2.6737	2.0069	101.5900
5%	3.3551	2.1713	148.3680
10%	3.7184	2.2590	181.5637
20%	4.1584	2.3652	231.8532
25%	4.3255	2.4056	254.4209
30%	4.4756	2.4418	276.5536
40%	4.7467	2.5072	321.5158
50%	5.0000	2.5684	370.1277
60%	5.2533	2.6295	426.0896
70%	5.5244	2.6949	495.3634
75%	5.6745	2.7312	538.4561
80%	5.8416	2.7715	590.8675
90%	6.2816	2.8777	754.5260
95%	6.6449	2.9654	923.3426
99%	7.3263	3.1299	1348.5041

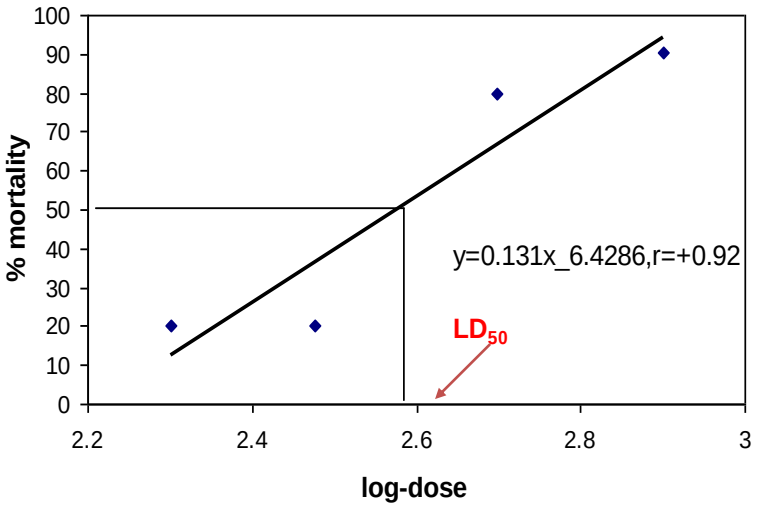


Fig. (1): Relationship between the mortality percentage of mice and logarithm of administrated doses of ottelione A.

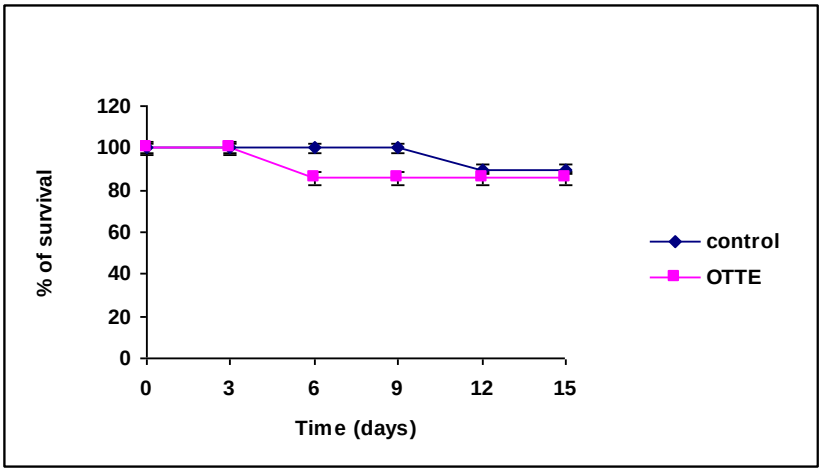


Fig. (2): Body weight change of control and Ottelione A (OTTE) treated mice along 15 days.

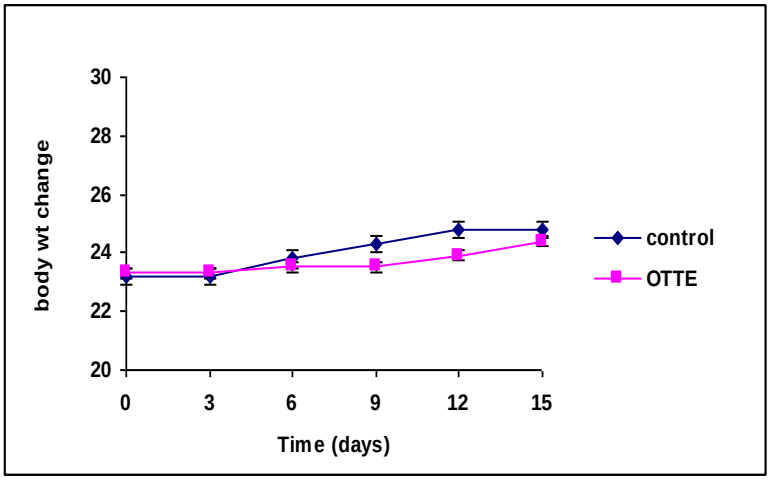


Fig. (3): Percentage of survival of control and ottelione A (OTTE) treated mice along 15 days

Table (3): Concentration of alfa-fetoprotein in serum, hepatic lipid peroxidation product (TBARS), glutathione (GSH) contents as well as superoxide dismutase (SOD) and catalase (CAT) activities in adult male mice injected ip with ottelione A compared with control.

	AFP (ng/ml)	TBARS (µg/g)	GSH (mg/g)	SOD (Unit/g)	CAT (µM H_2O_2/Sec/g)
Control group Mean ± SE	7.74 ± 0.55	133.20 ± 7.41	0.24 ± 0.003	75.05 ± 3.40	39.12 ± 0.91
OTTE group Mean ± SE	6.66 ± 0.11	134.74 ± 4.03	0.26 ± 0.004	112.01 ± 7.51[a]	36.02 ± 0.91

n=10

[a] significance at ($p \leq 0.05$) level

DISCUSSION

Natural products provide a wealth to medicine and are considered great sources of new drugs, and attempts to characterize their bio-safety have gained momentum in many pharmaceutical and food processing applications **(Cowan, 1999).** Plants such as fruits, vegetables and medicinal herbs contain a wide variety of free radical-scavenging molecules, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and some other endogenous metabolites that are rich in antioxidant activity **(Velioglu, et al., 1998, Zheng & Wang, 2001; Cai, et al., 2003 and Samarth, 2008).**

Because drugs that show specific inhibitory activity for cancer cell growth are usually cytotoxic, we evaluated the cytotoxicity of OTTE in mice by evaluating the LD_{50} after ip injection with OTTE in mice. The calculated concentration OTTE which causes a 50% morality was 370 mg/kg. In the acute toxicity study, the OTTE did not produce any mortality up to the 100mg/Kg dose level. There were no significant changes in the behavior in relation to the posture, diarrhea, mood and motor activity. The animals did not convulse nor exhibited writhing. The acute lethality, LD_{50}, of OTTE indicated a relatively high safety profile. The increase in body weight after drug administration usually indicate absence of toxicity as decrease in body weight is an index of toxic effect of a compound **(Kiran, et al., 2012 and Okoye, et al., 2012).** The present study is in agreement with this finding and showed insignificant change in both body weight and survivals of animals after the treated period with $1/10^{th}$ of the LD_{50} of ottelione A.

Moreover, the results showed insignificant difference in the levels of serum alfa fetoprotein between the OTTE-treated mice and control groups (Table 3). This is an indication of no biochemical changes related to carcinogenicity and suggest potential use for antitumor effects.

Reactive oxygen species (ROS) and other free radicals are considered the main cause of organ injury and systemic dysfunction. Plant extracts are known to affect many functions of the vital organs, especially the liver. Hepatic toxicity was one of the most significant effects was likely responsible for the early deaths observed after

treatment with toxic extracts. The present studies showed that the hepatic redox state did not affected following ip administration of OTTE to mice. This evidence is clearly appears by insignificant change in lipid peroxidation and glutathione levels in liver. Lipid peroxidation, an autocatalytic free radical chain propagating reaction, is known to be associated with pathological conditions of cells. Malondialdehyde (MDA), the end product of lipid peroxidation, was reported to be higher in stressed tissues than in non-stressed or pathological organs.

Oxidative stress is the specific cellular stress where the ideal physiological ratio of oxidants to reductants is altered in favor of oxidants, creating species such as oxygen radicals **(Cadenas & Davies, 2000).** Our findings show that OTTE, given ip, up-regulated SOD activity, which is a principal antioxidant in liver. Under normal conditions, reactive oxygen species (ROS) are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione peroxidase. The free radical scavenging enzyme, SOD is present in all oxygen metabolizing cells and its function is to provide a defense against the potentially damaging reactivity of superoxide radical.

The present finding may play a role in the prevention of oxidative damage in liver and might help in cases where over production of superoxide radicals may rise such as ischemia /reperfusion. The free radical hypothesis supported the fact that the antioxidants effectively inhibit the tumor. Since OTTE up-regulated SOD activity in liver it is possible to use OTTE extract to inhibit the growth and proliferation of tumor. OTTE showed to be an efficient inhibitor of tubulin polymerization and can disassemble preformed microtubules and attenuate tumor proliferation **(Combeau et al., 2000).**

CONCLUSION

In conclusion, these results indicated that the ottelione A is safe at the dose tested, and calls for caution in use at higher doses in treatment in cases characterized by free radicals production.

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تقدير الجرعة المميتة وتقييم سمية الاوتيليون (أ) في الفئران

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علي ضوء الدراسات التي تجري لاختبار السمية الحادة للمواد الطبيعية تم التوصل إلي أن ٣٧٠ مجم/ كجم من مركب ottelione A يتسبب في وفاة ٥٠ % من فئران التجارب وقد تم اختيار١٠/١ من هذه الجرعة لاختبار فاعلية استخدام هذ المركب بأمان كمضاد للأكسدة. أجري اختبار السمية لمادة ottelione A من خلال الحقن في الغشاء البريتونى لفئران التجارب ثم أجريت بعض الاختبارات بعد مرور ١٥ يوم من معالجه الفئران بالجرعة المحددة ومنها تحديد نسبة (AFP) والجهد التأكسدي. وقد دلت النتائج علي عدم حدوث تغير في معدل وزن الفئران أو الوفاة في كلا من المجموعة الضابطة والمجموعة المعالجة بمركب ottelione A . أيضا اظهرت النتائج عدم حدوث تغيير في مستوي كلا من الإجهاد التأكسدي للدهون (MDA) في الكبد وبعض الإنزيمات المضادة للأكسدة (GSH & CAT) في الكبد في المجموعة المعالجة ولكن أظهرت النتائج زيادة في نشاط إنزيم (SOD) في الكبد في المجموعة المعالجة مقارنة بالمجموعة الضابطة. ومما تقدم يمكن القول أن هذه النتائج أظهرت إمكانية استخدام الجرعة المحددة من مركب ottelione A بأمان وتوصي الدراسة باستخدام هذا المركب في الحالات المصاحبة بإنتاج بعض الشقوق الحرة.