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RESEARCH

The potential organo-toxicity safety of Morpholine and Crinum jagus in rats

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ABSTRACT

Background: *Crinum jagus* is a medicinal plant used by traditional medicine healers and has active components as morpholine, hamayne and lycoline. Anticonvulsant and other activities of the plant exist in literature. Our previous work showed reno and hepatotoxicity.
Aim: To carry out a comparative chronic toxicity study of *Crinum jagus* bulbs and pure morpholine in rats in order to demonstrate their organo-toxicity or safety at therapeutic doses.
Methods: Animals consisted of 50 young rats of average starting-weight of 80.6grams and about 60 adult mice of average weight of 20.6grams. Crinum jagus was collected from Igbodo, Delta State of Nigeria while morpholine was got from Anuihe Herman-CHINA. The bulbs of Crinum jagus were air dried for about ten days when a constant weight was achieved, chopped and ground. 200grams each of the white paste was macerated in 2 litres each of water, ethanol and petroleum ether. The filtrates were evaporated to dryness pooled into one solid mass and further fractionated in 100ml each of petroleum ether, ethyl acetate and n-butanol. The filtrated-fractions were evaporated to semi-solid state and then pooled together before finally dried to a solid state. Acute toxicity study was carried on the fractionated extract and morpholine using Lorke's and Karber's methods respectively. High and low doses of both morpholine and fractionated extracts were chosen as one fifth and one tenth of their LD_{50} respectively (effective doses).Young albino rats of average weight of 80.6grams were divided into 5 groups (A-E) of seven females and three males per cage. 111.8mg/kg and 223.6mg/kg of fractionated extracts and 0.221mg/kg and 0.442mg/kg of morpholine were orally administered to the rats once daily for a total period of 13weeks. The last group was given 2ml of injection water to act as negative control. At the end of 13th week and beginning of the 14th week, the animals under anaesthesia were bled from the orbital sinus for hormonal assay test while the testes, ovaries, brain and hearts were excised, weighed, preserved in formalin then sent for histological study.

Results: LD_{50} of fractionated extracts of Crinum jagus was got as 1,118.03mg/kg IP in mice while that of Morpholine was 2.22mg/kg IP in mice. The reproductive hormones of all animals treated with the two separate doses of fractionated extracts or morpholine were within reference

ranges and compared with negative controls. The histology of the testes, ovaries, brains and hearts were essentially normal and compared with negative control.

Conclusion: Chronic toxicity study affords us the opportunity to demonstrate the adverse effects of standard, prospective or new drugs. Morpholine and Crinum jagus bulbs extracts at therapeutic doses are non toxicity to the reproductive organs, brain and heart of adult rats.

Key Words: Morpholine; Crinum jagus; Reproductive hormones and organs; Brain; Heart; Neuroprotection; Alzheimer's disease.

1. INTRODUCTION

1.1: *Crinum*

Crinum is the largest tropical genus of the *amaryllidaceae/lilaceae* family (Edema and Okanieme, 2002). *Crinum jagus* is a medicinal plant used in traditional medicine either singly or in a combination with *Chromoleana odorata* and *Emilia prateramisa* (both of Asteraceae family) in treatment of all forms of convulsive state.

Crinum jagus (*C.jagus*) is variable specie that occurs in tropical Africa. Its leaves may be broad in some forms, whereas they are narrower or parallel in other forms.

The plant may be found in swampy conditions, seasonal wetlands or in grassland (Savanna). Some plants are remarkably fragrant, flowered, vanilla scented while others may have little or no scent/odor. Buds are enclosed in several sheathing bracts.

It is commonly called St Christopher's lily in English *Ulede* and *Obilokpo* in local dialects (Azikiwe *et al*., 2012).

The bulbs are ground, macerated in hot palm-oil and used in treatment of all types of seizures in traditional medicine (Azikiwe *et al*., 2012).

Morpholine, Hamayne and Lycorine have earlier been isolated as active alkaloids of *C. jagus* (Edema and Okanieme, 2002; Thi-Ngoc *et al*., 2002).

Earlier work on the crude extract of Crinum jagus revealed anti mitotic activity (Nwakama et al., 2010).

In India, *Crinum jagus* is used in treatment of snake bites (Ode and Asuzu, 2006; Gomes *et al*., 2010).

Crinum jagus bulb has also recently been shown to possess anti-asthmatic activity (Sonibare and Gbile, 2010). In another recent work antioxidant and antihaemorrhagic activities of *C.jagus* were documented (Ode *et al*., 2010).

The bulbs of Crinum jagus is also used in treatment of chronic cough, malaria, sores and possess antibacterial activity (Osakwe *et al*., 2011).

Broad spectrum anticonvulsant activity of Crinum jagus has been demonstrated in experimental animals (Azikiwe *et al*., 2012).

The crude methanolic extract and the purified fractions of the bulb of Crinum jagus exhibited anti-mycobacterial activity which is an indication of promising potential of this plant for the development of anti-tuberculosis agent (Akintola *et al*., 2013).

In a 14week comparative chronic toxicity study, Brambaifa *et al*., 2014 found that low dose of morpholine and both high and low doses of *C. jagus* had no significant changes in the renal functions tested parameters and their histological patterns were also normal compared to control. But, the high dose of morpholine however exhibited increases in urea, creatinine, sodium, potassium, bicarbonate and chloride with the histological pattern showing necrotizing effects.

1.2: Morpholine

Out of the three main active component of *C. jagus* morpholine appears to be the only specific components. Other active components of *C. jagus* hamayne and lycorine are not only found in other plants (Berkov *et al*., 2007), they are found in most other species of *Crinum* (Houghton *et al*., 2004). Morpholine extract from *Crinum jagus* is liquid at room temperature and above but as crystalline solid at temperatures below 14-15^{0}C. It has a strong smell of ammonia, slimy and colourless (Edema and Okanieimen, 2002).

Its other names are diethylenimide oxide and 1,4- oxazinane tetrahydro-1,4 oxazine (Klaus et al., 2003). The Chinese originally obtained morpholine from some plants but, many companies today rely on the synthetic form. Dilutions usually, 0.1-5% of morpholine are used in treatment of seizures and other ailments in Chinese traditional medicine (Emily-Fans, 2009).

Morpholine has a chemical structural formula of C4H9ON. Synthetic morpholine is soluble in water and most other solvents. It has a boiling point of 128.4, melting point of 4.6 and igniting (firing point of 310). It has the same relative density with water, has strong smell of ammonia and interlaced soft sweet smell typical of amines (Klaus *et al*., 2003). It can be synthesized in the Laboratory by neutralizing ethanolamine with sulfuric acid (Klaus *et al*., 2003).

Morpholine is used as a chemical emulsifier in waxing of fruits and as structural building block in the preparation of certain antibiotic like linezolide (Klaus *et al.*, 2003). But, morpholine is classified as a toxic chemical (Klaus *et al*., 2003).

Morpholines have been shown to block T-receptors of calcium ion channels (Ku *et al*., 2006). Morpholines also have been shown to possess antienzymes activity against some parasites including plasmodium falciparum as well as inhibition of cyclooxygenase (Khan *et al*., 2005).

Methoxy and hydroxyl morpholine derivatives of phencyclidine have recently been shown to exhibit analgesic/pain perception activity in rats (Ahmadi *et al*., 2011).

It has a potency of a selective antiseizure activity against PTZ-induced seizure that is superior over valproic acid, a standard drug against absence seizure in man (Azikiwe *et al*., 2013)

A dose dependent renotoxicity of morpholine but not Crinum jagus has been demonstrated. The present chronic toxicity study therefore aims at finding out if the reproductive organs are speared thus paving ways for non effect on fertility. Histology of heart and brain shall also be undertaken for a guide as no readily assessable biochemical parameter exists for the duo.

2. METHODOLOGY

2.1: Plant extraction:-

Extraction was divided into two stages. The first stage involved crude extraction in polar (water), semi polar (ethanol) and non polar (petroleum ether) solvents. The second stage involved further extraction (fractionation) of the pooled crude extract got from the first stage. The bulbs of Crinum jagus were air dried for about ten days when a constant weight was achieved, chopped and ground. 200grams each of the white paste was macerated in 2 litres each of water, ethanol and petroleum ether. The filtrates were evaporated to dryness pooled into one solid and further fractionated in 100ml each of petroleum ether, ethyl acetate and n-butanol. The filtrated-fractions were evaporated to semi-solid state and then pooled together before finally dried to a solid state. The fractionated was refrigerated until required.

2.2: Phytochemistry

Method of Phytochemistry was based on the method of Trease and Evans (1987) and as applied by Akah *et al*., 2003; Azikiwe *et al*., 2009**.** Phytochemistry was carried out to test for presence of alkaloids, flavonoids, tannins, terpenoids, glycosides, reducing sugars, fats/oils, resins, proteins, acidic substances and carbohydrates.

2.3: Acute Toxicity (LD_{50}) study) on fractionated extract

Lorke's 1983 involved the use of 13 animals and divided into two stages. At the first phase, a total of 9 male mice of average weight of 20.5grams were used (Akah et al., 2003; Akhila *et al*., 2007)

The animals were divided into 3 groups (A-C) of 3 animals per group. The fractionated extracts of *Crinum jagus* re-dissolved in water and administered intraperitoneally to the mice at doses of 10mg/kg, 100mg/kg and 1000mg/kg. All animals had unrestricted access to water and animal feed and were then observed in their cages for 24hours. The animals were observed for possible signs of toxicity (anorexia, drowsiness, apnoea, immobility, twitches and irritation) and, or death. All dead animals were immediately removed from the cage as soon as possible once death was observed, counted and recorded.

The second stage followed the end of the first stage and 4 new mice were selected and placed 1per cage. They were separately given 2500mg/kg, 5000mg/kg, 7500mg/kg and 10,000mg/kg and were further observed for the next 24hours.

LD_{50} was then calculated as the geometric mean of the lowest dose that killed an animal (2500) and the highest dose that did not kill any animal (1000). Geometric mean was calculated as the average of the square roots of the lowest dose that killed an animal and the highest dose that did not kill any animal. With the LD_{50} obtained, the presumable effective doses were taken thus one-fifth of LD_{50} as high dose (HD) and one-tenth as low dose (LD).

2.4: Acute Toxicity (LD_{50}) study) on Morpholine

All the animals at the first stage of Lorke's died hence Karber's method. An initial pilot study was carried out using a total of 4 mice of average weight of 20.5g. The animals were divided into four cages (A-D) with an animal per cage and given 10mg/kg, 1000mg/kg, 5000mg/kg and 10,000mg/kg of morpholine intraperoteneally, respectively. All animals were then observed for 24 hours for any sign of acute toxicity.

All animals that received morpholine died thus necessitating another pilot study in which 1mg/kg, 4mg/kg, 6mg/kg and 8mg/kg of morpholine was administered intraperitoneally, respectively, to another set of four mice. All animals were again observed for 24 hours.

The main study commenced subsequently, with 48 mice of average weight of 20.5g. The mice were divided into 8 groups (A-H) of 6 mice per group. Animals in group G received 1ml/kg of water for injection intraperitoneally, and thus served as negative control.

Morpholine was administered in doses of 0.5mg/kg, 1.0mg/kg, 1.5mg/kg, 2.0mg/kg, 2.5mg/kg, 3.0mg/kg and 4.0mg/kg, respectively to mice in groups A to G.

All animals had unrestricted access to drinking water and animal feed throughout the 24-hour duration of the experiment.

All animals were observed for signs of toxicity as described earlier. Dead animals were counted and recorded against the dosage group.

The interval mean of the number dead in each group of animals was used as well as the difference between doses for the same interval. The product of interval mean and dose difference was obtained. The sum of the product was divided by the number of animals in a group and the resulting quotient was subtracted from the least lethal dose to all animals in a group in order to obtain LD_{50} value.

$$LD_{50} = \text{The apparent least dose lethal to all in a group} - \frac{\sum (a \times b)}{N}$$

where *N* is the number of animals in each group, **a** is the dose difference and **b** is the mean mortality (Akhila *et al*., 2007; Deora *et al*., 2010, Azikiwe *et al*., 2014)

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2.5: Chronic toxicity study

Fifty young rats of average starting weight of 80.6grams were divided into 5 cages (A-E) of 7 females and 3 males per cage. They were fed rats/mice pellets and had non-restricted access to drinking water. They were allowed one week acclimatization before commencement of experiment. 111.8 and 223.6mg/kg of fractionated extract of Crinum jagus were orally administered to A and B respectively while groups C and D received 0.22 and 0.44mg/kg of morpholine also respectively. Group E was given 2ml/kg to act as negative control. These doses were given daily for 13 weeks and weekly weights were taken and recorded. At the end of the 13th week and beginning of the 14th week the animals were anaesthesized with chloroform while blood samples were collected from the orbital sinus into khan tubes (Parasuraman *et al*., 2010).

The animals under anaesthesia were then bled from the orbital sinus for hormonal assay test while the testes, ovaries, brain and hearts were excised, weighed, preserved in formalin then sent for histological study.

2.6. Data presentation and Statistical analysis

Data were presented as mean±Standard deviation of organ weight and number of bleeding spots while comparative histological architecture was presented as micrograph. Statistical analysis was done with SPSS version 16.0 using one-way ANOVA and students t-test for comparative statistics. P≥0.05 was adjudged non significant.

3. RESULTS

Results on solvent extraction, fractionations and photochemistry are as reported in Azikiwe *et al*., 2012. LD_{50} of Crinum jagus was got as 1,118.03mg/kg IP in mice while that of Morpholine was got as 2.218mg/kg IP in mice (Tables 1 and 2).

Table 1a

Stage one LD_{50} study. (LORKE'S METHOD).

Doses of Extract or Morpholine	NO of Animals	Death-Morpholine	Death- EXTRACT
10mg/kg	3	3	NIL
100mg/kg	3	3	NIL
1000mg/kg	3	3	NIL

**No animal died at the first stage thus requiring further study to possibly get to the closest acute lethal dose.*

Table 1b

Stage TWO LD_{50} study. (LORKE'S METHOD)

Doses of Extract	NO of Animals	Death
2500mg/kg	1	1
5000mg/kg	1	1
7500mg/kg	1	1
10,000mg/kg	1	1

**At the second stage all animals died thus LD_{50} was taken as the geometry mean of the product of the square root of 1000 and 2500 to give a final value of 1118.033mg/kg (IP) in mice.*

Table 2

Morpholine Acute Toxicity (Karber's Method)

Doses mg/kg	No.Dead	Dose difference (a)	Mean Mortality (b)	a X b
0.5	Nil	Nil	0	0
1.0	Nil	0.5	0	0
1.5	Nil	0.5	0	0
2.0	4	0.5	2	1

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2.5	4	0.5	4	2.0
3.0	5	0.5	4.5	2.25
4.0	6	1.0	5.5	5.5

Results on Hormonal Study:-

The hormonal assay biochemical results were all within the reference ranges and comparable with non treated animals (Table 3).

Table 3

Effects of test-substance on Hormonal Assay in rats (Mean ± SD)

Doses mg/kg or ml/kg	FSH(mlIU/ml)	LH(mlIU/l)	OESTRO (pg/ml)	PROGEST (ng/ml)	TESTO (ng/ml)
PF111.8	30.18.±2.6	11.03.±0.56	520.7.±236.9	23.91.±3.9	7.00.±2.0
PF223.6	30.00.±1.9	10.88.±0.01	519.7.±214.4	24.20.±5.0	6.91.±2.0
Morpholine.0.221	30.10.±2.9	11.01.±0.56	520.7.±230.5	23.52.±4.3	7.02.±3.0
Morpholine.0.442	29.79.±3.4	11.33.±0.26	521.8.±216.2	24.23.±3.5	7.12±1.9
Water.2	30.17.±2.2	11.03.±0.56	520.7.±236.9	24.21.±5.9	7.00.±2.8

Shows that all tested hormones were not significantly different from control group.

Results of histopathological analysis:-

The organs included the brain, heart, ovary and testis. These tested organs remained essentially normal for all doses as compared with control. Results are presented as typical cases to reduce unnecessary repetition of the same details (Figs. 1-4).

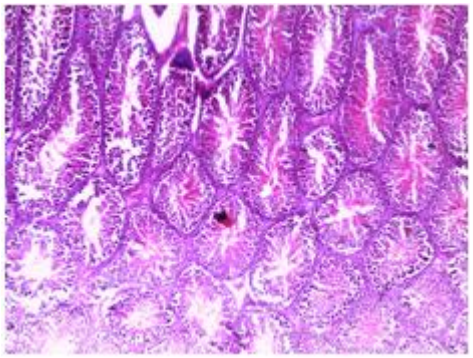

Figure 1

Histology of rat's testis after 13-14 weeks chronic dosing with either Crinum jagus extract or pure morpholine. The testis was histological normal compared with control.

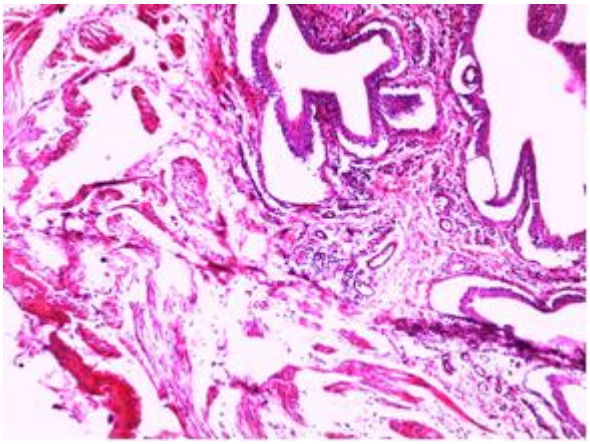

Figure 2
Histology of rat's ovary after 13-14 weeks chronic dosing with either Crinum jagus extract or pure morpholine. The testis was histological normal compared with control.

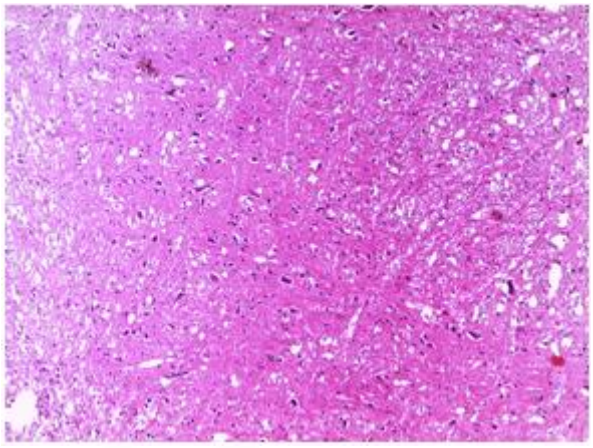

Figure 3
Histology of rat's brain after 13-14 weeks chronic dosing with either Crinum jagus extract or pure morpholine. The testis was histological normal compared with control.

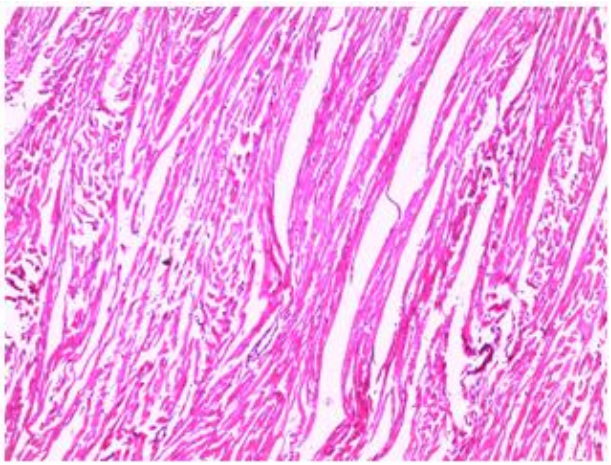

Figure 4
Histology of rat's heart after 13-14 weeks chronic dosing with either Crinum jagus extract or pure morpholine. The testis was histological normal compared with control.

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4. DISCUSSION

Some drugs and chemicals become active only after some biotransformation thus their active metabolites may now cause harm to the kidney on its way out of the body. Every substance also has some degree of toxicity depending on the dosage, route of administration and duration. Our present study could not demonstrate toxicity to the reproductive system, brain and heart at chronic administration of both high and low doses of morpholine and fractionated (PF) extracts of Crinum jagus. It may be stated that chronic administration of Crinum jagus and morpholine in the epileptics could spear these above organs of theirs.

According to Health-Canada, a governmental agency for food and drugs, morpholine is neither carcinogenic nor teratogenic and causes no toxicity on its own but, forms complexes with nitrates to form N-nitrosmorpholine that is carcinogenic.

Man is commonly exposed to morpholine through preserved fruits but at doses of 4.2ng/kg body weight per day, no harm is done but, whether sufficient nitrosmorpholine may eventually be formed on chronic ingestion remains a big question to be answered (Health Canada, 2002). This agency administered 0.48mg/kg body weight per day to rats and found no significant toxic effects.

Chronic inhalation studies however exhibited toxicity in rats exposed to 250 and above parts per minute for 6hours and in 5 days per week as reported by different authors (CDC-2003).

Most known antiepileptics are not only toxic they exhibit a paradoxical seizure activity on chronic administration, especially in myoclonic seizure of Dravet syndrome (White, 2010).

Amaryllidaceae alkaloids exhibit a wide range of physiological effects, of which the acetylcholinesterase (AChE) inhibitory activity is the most relevant with the alkaloidal extract from Crinum. jagus presenting the highest neuroprotective activity in both pre- and post-treatments against a glutamate excitotoxic stimulus (Calderón *et al*., 2010; Cortes et al., 2015).

The bulbs of Crinum jagus and Crinum glaucum are used in traditional medicine in southern Nigeria for memory loss and other mental symptoms associated with ageing and alkaloidal extracts of bulbs from each species showed inhibition of acetylcholinesterase, an activity exploited therapeutically to raise the depressed levels of acetylcholine in the brain associated with Alzheimer's disease (Houghton *et al*., 2004). In particular, galanthamine represents the first prescription drug emanating from the Amaryllidaceae after its approval by the FDA in 2001 for the treatment of Alzheimer's disease.

Donepezil, galanthamine, and tacrine are therapeutic acetylcholinesterase (AChE) inhibitors used for the treatment of Alzheimer's disease. Glutamate induces neurotoxicity via necrotic neuronal death and mediated through nicotinic acetylcholine receptors (nAChRs) (Takatori, 2006). This author also showed that donepezil and galanthamine protect cortical neurons against acute glutamate treatment-induced neurotoxicity at steps before, and that tacrine protects at steps after, nitric oxide radical formation. On the other hand, the neuroprotective effects of donepezil and galanthamine, but not of tacrine, against neurotoxicity induced by moderate glutamate treatment were mediated through the phosphatidylinositol 3-kinase-Akt pathway (Takatori, 2006).

The lycorine series of alkaloids have also garnered widespread interest as cytotoxic agents and were amongst the earliest of the Amaryllidaceae constituents to exhibit such activity. The anticancer agent is related to pancratistatin due to the potency, selectivity, low toxicity and high tolerability typifying targets of this series of alkaloids (Nair and van-Staden., 2014). Lycorine was the only alkaloid that displayed moderate topoisomerase-I inhibitory activity compared to other alkaloids from Crinum genera (Niño *et al*., 2007).

From the foregoing and our present study, one dire say that the neuroprotective activity of Crinum jagus and one its major active constituents, morpholine must have accounted for the normal histology pattern of the brain. Glutamate is neurotoxic substance as well as involved in the pathogenesis of epilepsy. Activity against glutamate induced neurotoxicity could therefore be likened to the killing of two birds with just one stone.

5. CONCLUSION

Chronic toxicity study affords us the opportunity to demonstrate the adverse effects of standard, prospective or new drugs. Morpholine and Crinum jagus bulbs extracts at therapeutic doses are non toxicity to the reproductive organs, brain and heart of adult rats.

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