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THE EFFECT OF PLUMERIA ACUMINATA AIT ON OESTROUS CYCLE AND ACUTE ORAL TOXICITY STUDY IN C3H FEMALE ALBINO MICE

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ABSTRACT

Objective: The objective of the present study is to evaluate the oral toxicity of the methanolic stem extract of *Plumeria acuminata* Ait. (a plant used by the Mising tribes of Assam, India to cure reproductive related disorders) and study its effect on the reproductive cycle of female albino mice.

Materials and Methods: The extracts were tested for their effect on the estrous cycle at two dose levels of 200 and 400mg/kg/day. The vaginal smears were then examined twice daily for two weeks and identification of the different phases of the estrous cycle was done. Acute oral toxicity study was done by administering different doses of the extract and observed individually for weight loss, mortality and any other physiological disorder. Results were then compared between the treated to that of the control group.

Result: Results have shown that none of the treated group of animals showed any symptoms associated with toxicity such as dieresis, convulsion, etc. There were also no cases of mortality observed till 72 h after the last dose. The estrous cycle was prolonged in the treated groups with decrease in the number of cycles. There was a marked increase in the diestrous phase while the mestrous phase was significantly decreased.

Conclusion: The methanolic stem extract of *P. acuminata* Ait. is none toxic and can be considered as safe to experimental animals. The disruption in the normal estrous cycle in mice caused by the extract also is an indication that the plant possesses potent anti-fertility effect.

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INTRODUCTION

The plant *Plumeria acuminata* Ait. commonly known as "Temple tree" or Champa" locally known as "Golainchi" belongs to the genus Plumeria of the Apocynaceae family. It is a deciduous plant with smooth and juicy stem with rich white latex that easily breaks. This plant is used as a medicinal plant and is native to Mexico, Colombia, Central America and Venezuela. About 2000 species belonging to 155 genera are dispersed primarily in the tropical and subtropical region of which 8 species are known to occur in India (Farooque *et al*., 2012). The primary among them are the *P. acuminata* and *P.rubra.* From ancient times this plant has been used by the different tribes and traditional healers in curing different types of ailments. The different parts of the plant like the leaves, flower, bark and oil extracted from the plant is extensively used as a remedy for diarrhea, pain, fever and as a cure for itch in many countries (Hua and Geng-Tao, 1992; Formica and Regelson, 1995; Aruoma and Cuppett, 1997). This plant has been reported to have wide range of pharmacological activities and regarded as a universal remedy in Ayurvedic medicines occupying its position as a resourceful plant having a broad spectrum of medicinal activities. Decoction of 12 - 24 gm of dried material is used to control diarrhea and dysentery (Begum *et al*., 1994). Decoction of leaf extract is applied to cracks and eruptions of the soles of the feet. Extract or infusion from leaves is used to control asthma (Pand and Mehrotra, 1960). The milky juice of the stem and leaf is used to treat skin disease such as scabies, herpes and ulcers (Raju, 2000; Prajapati *et al*., 2004). The fruit are eaten in West Indies, however, in India it has been used as an abortifacient (Bobbarala *et al*., 2009). The plant extract may also be taken as cooling agents with tea for prevention for heart stroke. The juice of the plant act as rubifecient in rheumatic pain (Tembare *et al*., 2012). The latex after mixing with coconut oil is applied

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to affected area to treat rheumatism, arthritis and skin lesion (Shinde *et al*., 2014). A recent survey conducted by the author in some upper Assam (India) Districts revealed that the stem of *Plumeria acuminata* has been used traditionally by the Mising tribes of Assam for curing different types of reproductive related disease.

The stem of the plant was grinded along with the leaf of Saura (*Streblus aspera* Lour.) and root of Kootora sak (*Amaranthus viridis* Linn.) and the juice administered to women suffering from different sorts of reproductive problems. In order to validate their claim, this plant was selected for the present study. Since all aspects relating to reproduction and pregnancy requires the normal functioning of the estrous cycle, any substances which brings about changes in the estrous cycle would indicate the anti-fertility effect of a compound. Thus, the estrous cycle may be regarded as a surrogate marker and this property has been frequently utilized in evaluating the anti-fertility effect of any substance (Long and Evans, 1922). Moreover, the estrous cycle of the female albino mice is very short which lasts for only 4–5 days. This short cycle makes it convenient to study the changes that can be produced by administering exogenous agent. Thus keeping all the facts in mind, this study was conducted with an aim to investigating the toxicity effect of methanolic stem extract of *P. acuminata* and its influence on estrous cycle in female albino mice.

MATERIALS AND METHODS

Collection and preparation of plant materials

The stem of *P.acuminata* was collected from Dhemaji District of Assam (India) in January, 2015. Collection and preservation of plant specimens was followed using standard method (Jain and Rao, 1977). A voucher specimen of collected plant was organized and herbarium sheets prepared. Authentication of the herbarium sheets was done by the Department of Botany, Gauhati University, where a herbarium specimen (Accession No. -GUBH-17872/10-03-2015) of this collection was deposited. The collected stem after proper washing was pealed of its bark, cut into small pieces, shade-dried to a constant weight and grinded to a uniform powder. The extraction was prepared by soaking 100 g of the dry powdered plant materials in 500ml of methanol in a Soxhlet apparatus for about 36 hour by continuous hot percolation. The mixture then was filtered through Whatman No. 1filter paper and concentrated by using a rotary evaporator with the water bath set at 40°C. It yielded a semi-solid sticky mass (paste) concentrate of dark brown colour which was stored at 0°C. Fresh sample was prepared from this extract just before administration into the animal.

Vehicles used in the experiment

Vehicle used for administration was prepared by mixing Tween-80(1%) with normal saline at the ratio of 1:10. This was then used as a vehicle for administering different doses of the plant extract in all experimental groups.

Experimental Animals

The experiments were conducted using twelve weeks old young healthy female Swiss albino mice (C3H strain) and weighing about 23-25g. The animals were obtained from the Pasture Institute, Directorate of Health Services, Govt. of Meghalaya, Shillong (India). They were maintained under standard conditions of natural photoperiod, temperature (26 ± 3) ˚C and humidity. They were fed with standard pellet diet, vitamins, mineral supplements and water was given *ad libitum*. The animals were kept in polypropylene cages in the Department of Zoology, Cotton College Guwahati, as approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals. The experimental protocol was approved by the Institutional Ethical Committee on the Use and Care of Experimental Animals, Guwahati University, Assam, India prior to the commencement of the study.

Acute oral toxicity studies

Acute oral toxicity study was carried out as described by (Harborne, 1984). Literature surveys of conventional LD50 tests prove that, the females are somewhat more sensitive than male, even if there is little difference in sensitivity between the sexes (Lipnick *et al*., 1995; OECD, 2001). Normally female mice weighing about 22-28g were randomly selected for this experiment. The mice were divided into six groups containing three animals in each group. In order to allow for acclimatization to the laboratory conditions, the mice were kept in cages 5 days prior to dosing. Before administration of the dose, the animals were fasted for 18-20 h with water ad libitum.

Then the plant extract were administered in a constant volume over the series of doses to be tested by varying the concentration of the dosing preparation. The five different doses of 200, 700, 1000, 2000 and 4000mg/kg body weight respectively were administered to different groups of mice separately. The test substances were given by gavages using a feeding tube and administered in a single dose. After administration, food may be withheld for a further another 1-2 hours. The animals were observed individually for weight loss, mortality and any other physiological disorder after dosing at least once during the first 30 minutes, with particular attention given during the first 4 hours, periodically during the first 24 hours and daily subsequently, for a total of 3 days. One group was taken as the control group and this group received treatment with vehicle (Tween-80, 1%) only (Handa and Sharma, 1990).

Experimental design of the study

The adult normal cyclic mice exhibiting three consecutive regular estrous cycles were used in the experiment. The animals were randomly divided into three equal groups of five mice per group. To assess the effects of the extract on estrous cycle in normal female mice, the animals were treated with the different doses of the extracts for a period of 14 consecutive days. The extracts were administered orally using a feeding tube at 24h interval and administrations of the different dose of the extract are done as follows:

- **Group 1** (Vehicle control group): 1% Tween-80 solution (1%, v/v, 0.2 ml/mouse/day)
- **Group II** (Low dose group): 200mg/kg bt. Wt./mouse /day of the plant extract in 0.2ml 1% Tween-80 solution
- **Group III** (High dose group): 400 mg/kg bt. Wt./mouse /day of the plant extract in 0.2ml 1% Tween- 80 solution.

Effect on the estrous cycle

The mice or rats have short length of the estrous cycle and this makes them perfect model for fertility studies. The estrous cycle in a female mice is characterized by four different phases, diestrus, proestrus, estrus and metestrus. This cyclical pattern is completed within 4–5 days. These four stages can be microscopically identified using the vaginal smear (washing/Lavage) method as described by (Nelson *et al*., 1982: Goldman *et al*., 2007; McLean *et al*., 2012). For this the mouse was held up by the base of its tail and head. Then the polished, shortened tip of a pipette was inserted into the vaginal orifice. Care should be taken not to introduce it more than 1 mm as cervical stimulation may induce pseudopregnancy and cause injury to them (Sinha *et al*., 1978; Nelson *et al*., 1982). A drop of distilled water was gently expelled into the vagina and aspirated back into the tip and this was repeated 3 to 4 times. Then the drop of water was transferred to a slide (smear) which was allowed to dry. One drop of methanol for fixing was added to the smear for 30 sec and stained for a minimum of 30 min in freshly prepared 2% Giemsa blood stain in distilled water. Sequentially between each sampling the pipette was rinsed in a tap water, 70% ethanol, and again distilled water.

Smears were taken daily between 8-10 am in the morning and 5-7pm in the evening. The effect of extract on the estrous cycle was monitored for 14 days (Gasco *et al*., 2005). With the help of a microscope at 10 X, the examination of the different stages of estrous cycle was done and classified as to the different stage of the cycle according to criteria modified from those of Thung *et al*. (1956) and Byers *et al*. (2012). Four stages were distinguished: The mice is said to be in the diestrus (DE) phase if the leucocytes constitutes the majority of the cells in the smear; proestrus (PE) phase is represented by the presence of a large number of nucleated cells; oestrus (E) phase is marked by the presence of large numbers of cornified epithelial cells in the smear; and the presence of scattered squamous epithelial cells and plenty of neutrophils in the smear indicates the metestrus (ME) phase (Goldman *et al*., 2007). The proportions of the different types of cells were taken into account while determining the different phases of the estrous cycle (Long and Evans, 1922).

Statistical analysis

All the observations are expressed as Mean ± SEM. The tests for significance were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett Multiple Comparison Test. The significance level was considered at $P<0.05$.

RESULTS

Effect of the plant extract on acute oral toxicity study

The result on the effect of methanolic stem extract of *P. acuminata* on oral acute toxicity study is presented in Table-1. After the administration of the plant extract, behavioural pattern of the animals were observed for first 6 h, followed by 14 h in the animals in both control (1% Tween-80) and extract-treated groups. Results have shown that toxic symptoms or mortality was not observed in any of the treated group of animals. The extract fed mice were as healthy as those that were given normal saline with Tween-80. The animals did not exhibit significant changes in behaviour, water consumption, impairment in food intake and other postural abnormalities. Other symptoms associated with toxicity such as dieresis, convulsion, etc., were also not observed after administration of the extract from the first dose till 72 h after the last dose.

Effect of the plant extract on estrous cycle in mice

Administration of methanolic stem extract of **P. acuminata** orally at the two dose of 200 and 400 mg/kg body weight/day induced dose dependent effects on the normal cyclicity of adult female mice. The vaginal smear when examined in the animals treated with 200 mg/kg body weight/day of the extract, the duration of metestrus decreased by 1.47 fold while the duration of diestrus phase was prolonged by 1.14 fold with appearance of large number of leucocytic cells. However, the duration of proestrus and estrus phase was decreased though this decrease was non-significant. On the other hand, at a higher dose i.e. treatment with 400 mg of the extract induced a 1.19 fold increase in the length of diestrous phase while the metaestrus phase was found to decrease by 1.56 fold. Moreover, in this fourteen day treatment period the number of cycles showed a decline indicating an enhancement in the length of a complete estrous cycle. The total length of one complete cycle was increased to 7-8 days.

DISCUSSION

To establish the efficacy and safety of plant derived products and drugs for human use, carrying out toxicological assessment in experimental animals is very essential to predict toxicity and to determine the 'safe' doses of the extract. In the present study, the metanolic stem extract of *P. acuminata* Ait. showed no sign of acute toxicity when administered orally up to a dose level of 2000mg/kg bw. The animals showed no stereotypical symptoms connected with toxicity, such as convulsion, diarrhea or increased dieresis.

Table 1. Acute oral toxicity study. Table shows no major changes in the animals including behavioural changes or mortality in the methanolic extract fed animals up to the dose of 4000mg/kg bw

Group	Dosage mg/kg BW	Observation
Control	Tween-80	All the animals showed no stereotypical symptoms associated with toxicity, such as diarrhea, convulsion, increased diuresis or mortality.
Methanolic stem extract of *Plumeria acuminata* Ait	200 700 1000 2000 4000	

Table 2. Effect of *P. acuminata* Ait. on the different phases of the estrous cycle in the adult female mice

Numbers of days/hours(mean ±SEM) spent in each phase					
Animal Group	Proestrus	Estrus	Metestrus	Diestrus	No. of E. Cycles
I.Vehicle control	1.30±0.11	1.80±0.22	2.50±0.22	8.30±0.34	2.33±0.12
II. 200mg/Kg	1.2±0.12	1.70±0.20	1.70±0.12*	9.50±0.35*	1.82±0.09*
III.400mg/kg	1.1±0.10	1.40±0.19	1.60±0.10*	9.90±0.24**	1.63±0.06**

Value for each group is expressed as Mean±SEM, n=5. Here *p<0.05, ** p<0.01 and no asterisk denotes non-significant.

Changes in body weight were not noticed. Deaths or mortality due to toxicity during the 72 h period at the doses tested were also not found. This study on toxicity was in strong consistent with earlier published work on different crude extract from medicinal plants that were evaluated by many researchers. The leaves of *P. acuminata* were found to be non toxic up to the dose level of 2000mg/kg bw over a period of 24 hrs (Gupta *et al*., 2006). Male mice fed daily with the methanolic leaf extract of *P. acuminata* at the dose of 300, 600 and 1,200 mg/kg body weight constantly for 270 days showed no signs of abnormalities in the test groups as compared to the controls. Other parameters like liver, kidney functions hematology and blood chemical values in treated groups were found to be normal in comparison with the control group (Gomathi *et al*., 2012). Previous literature on the plant extracts belonging to some plants of the same genus have also reported them as nontoxic at different dose level which further supports the results of the present study. Hence, conclusions can be drawn that the plant extract under investigation can be considered as safe to conduct experiments on mice. On the basis of this study, the dose pattern for conducting the experiment on mice can also be predetermined with a low dose of 200mg/kg and a high dose of 400mg/kg body weight.

The female estrous cycle in animals is the time between periods of sexual receptivity. In mice and rats, this cycle is completed in 4/5 day intervals and reoccurs after that (Maligalig, 2001). It comprises the recurring of several different physiological and morphological changes in the vagina, uterus and ovaries that are brought about by reproductive hormones which in turn, is regulated by the secretion of gonadotropins hormones (FSH and LH) and hypothalamic releasing factor from the pituitary (Lerne, 1969). The reproductive functions like normal estrus cycle, implantation, ovulation and subsequent sustenance of pregnancy etc., all dependent upon the coordinated action of estrogen and progesterone. The level of estrogen and progesterone reaches its peak during proestrus and decline to a low level at the end of estrus. During metaestrous, this level intermediates and in diestrus phase on the other hand circulating progesterone levels are low while the estrogen levels are at their intermediate levels (Biswal, 2014). Therefore, a perfect balance is highly essential in their levels for maintaining fertility. Any interruption in their levels will result in unbalanced ovarian functions that will have an effect the estrus cycle and thereby fertility.

The results of the present study on the effect of *P. acuminata* on estrous cycle of female mice showed that the extract caused a significant dose dependent alteration in the duration of the different phases of the estrous cycle. The normal periodicity of the cycle as seen in the control group was replaced by a vague representation where the diestrus phase were prolonged along with non-significant shortened duration of the proestrus and estrus phase. Moreover, the numbers of cycles were also reduced. Since the only matting phase in the animals is the estrus phase, its non alteration in duration at both the doses and the prolongation in the diestrous phase, as manifested from our study could possibly suggest some anti-fertility effect of the plant extract under investigation. Thus, the decrease in the number of the estrous cycle as evident from this study corresponds to a decrease in the time during which ovulation occurs (reduction in the rate of ovulation) resulting in decreased fertility (Nayaka *et al*., 2014). Prolongation of the diestrous phase suggests that the extract might have interfered with the hormonal synthesis that might have brought about changes in the various phases of the estrus cycle and the number of cycles. The persistence diestrus phase indicates that the extract could possibly interferes with the actions of estrogen, progesterone, FSH and LH that are responsible for the control of the estrus cycle in female animals through its action on the pituitary-gonadal axis. Similar findings were also reported by different workers working on different plant extract like *Mimosa pudica* root extract (Ganguly *et al*., 2007), *Carica papaya* seeds (Raji *et al*., 2005), *Hibiscus rosasinensis* flowers (Kholkute *et al*., 1976) etc. A number of phyto-chemical constituents present in plants such as alkaloids, flavonoids, saponins, glycosides and phenolic compounds are structurally or chemically related to natural hormones. These compounds have been documented for their effect on the reproduction of animal such as causing disruption in the estrous cycle in experimental animals (Fairley *et al*., 1972; Ifeanyi *et al*., 2011; Verma *et al*., 2016; Soni *et al*., 2016).

Conclusion

The present study on methanolic stem extract of *Plumeria acuminata* on female mice indicates that the extract is none toxic to experimental animals and can be considered as safe. Also evidence from the study indicates that the extract caused disruption in the normal estrous cycle in mice which is an indication that the extract might have interfered with the normal hormone level of the animal which will have a bearing on the fertility of the animal. Further work on the mechanism(s) of its action on fertility in mice is underway.

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