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Full Length Research Paper

Detection of congenital anomalies in *Mus musculus* induced by crude leaf extracts of *Goniothalamus amuyon* (Blanco) Merr. and *Alstonia macrophylla* Wall. Ex G. Don

Annabelle A. Herrera*, Anne Monica O. Dee and Lerrie Ann Ipulan

Institute of Biology, College of Science, University of the Philippines, Diliman 1101 Quezon City, Philippines.

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A significant portion of congenital malformations in newborn infants is caused by teratogenic exposure. By gaining information on the teratogenicity of a substance, it can be avoided especially during the crucial stages of pregnancy and by knowing the antiteratogenic effects of a substance; it can be used to counteract the effects of known teratogens. The objective of this study is to evaluate the teratogenic activities or antiteratogenic capabilities of the crude extracts of two plants, *Goniothalamus amuyon* and *Alstonia macrophylla*, which are widely used by the indigenous Aetas of Morong, Bataan, Philippines for various ailments from colds to cholera. Inbred strain of ICR mice were treated with varying concentrations of the plant extract, with and without the supplementation of vitamin A, an established teratogen in excessive doses, to test for antiteratogenicity or teratogenicity of the extract. Reproductive performances and morphological malformations were assessed. From the results, mainly from the presence of a high frequency of morphological abnormalities in the extract-treated foetuses, it can be said that the two plant extracts have a potential for teratogenicity, though the dose dependency of the teratogenic ability of the extract was not established. Further studies are needed to elucidate the exact compound in the crude extract involved in the teratogenic activity and the specific mechanism of action employed.

Key words: Antiteratogenic, hypervitaminosis A, *Goniothalamus amuyon*, *Alstonia macrophylla.*

INTRODUCTION

Reproductive risks comprise a wide range of abnormalities, which possibly emanate via genetic means or via influence of environmental factors (Brent, 2004) or via an interaction of both (Monie, 1963). Quite a large percentage of reproductive problems involve congenital malformations in newborn infants.

Unknown causes including teratogens cause congenital malformations in newborns at 65 - 75% (Brent, 2004). Teratogens cause malformations by upsetting the natural developmental processes of a growing embryo (Keeler, 1984). Devising means of counteractin teratogenic effects in pregnant mothers is imperative.

*Corresponding author. E-mail: annabelle.a.herrera@gmail.com. Tel: (02)922-3585, (02) 920-5471. Fax: (02)920-5471.

The objective of this study is to evaluate any teratogenic or antiteratogenic activities of the crude extract of two different plants widely used by indigenous Aetas of Morong, Bataan, Philippines for various ailments from colds to cholera: *Goniothalamus amuyon* and *Alstonia macrophylla*. *Goniothalamus* belongs to family Annonaceae. The species *macrophyllus* is a source of medicine for various illnesses like malaria and cholera. However, the root extract can cause abortion and chemical analysis shows the presence of styryl-lactone derivatives, acetogenins and aporphine alkaloids, all of which exhibit cytotoxicity (Jantan et al., 2005).

Alstonia belongs to the family Apocynaceae with a variety of medicinal properties such as treatment for malaria, fever, pain, spasms and psychosis (Oze et al., 2007). Indole alkaloids from *A. macrophylla* extracted from the root bark exhibited cytotoxic effects, according

to the study by Keawpradub et al. (1999).

The significance of this study is to help reduce the occurrence of congenital malformations in countless victims. By gaining information on the teratogenicity of a substance, it can be avoided and by gaining knowledge on the antiteratogenic effects of a substance, it can be utilized to counteract the effects of teratogens. Diabetes, for example, causes high blood glucose levels that can result to teratogenic complications in the foetus (Pettitt et al., 2003).

In addition, with a study such as this, the discovery of medication to counteract the teratogenic activities of some inevitably required drugs such as anti-epilepsy during pregnancy is possible. Lamotrigine, an anti-epileptic drug, was observed to be teratogenic in animals (Prakash et al., 2007). Another anti-epileptic drug, gabapentin, showed teratogenicity in an experiment involving mice and thus, is also not advisable to take during pregnancy (Prakash et al., 2008). Hence, antiteratogenic treatment is a must.

MATERIALS AND METHODS

Test organism

Inbred mice of ICR strain, four to five weeks old, were given two weeks in the laboratory as an acclimatization period to aid in adjusting to the laboratory conditions. Food and water *ad libitum* were provided as well as other requirements of the College of Science Animal Care and Use Committee. At six weeks old, the females were already used for mating.

Plant extraction

Leaves of *G. amuyon* and *A. macrophylla* obtained from Morong, Bataan, Philippines were used. These were washed, air-dried for two weeks and cut into small pieces. They were immersed in ethanol for 24 h and filtered afterwards. The filtrate was extracted at 40°C using a rotary evaporator.

Treatment of test organism

The mice were put together in a cage in a male to female ratio of either 1: 1 or 1: 2 depending on the availability of male breeders. Upon the detection of the vaginal plugs and sperm presence in the vagina, which signify the first day of gestation, the female mice were each separated, labelled and monitored.

For each plant extract, six treatments were conducted, plus the positive and negative controls. For each treatment, five mice were used. The different treatments performed per plant extract were: (1) 5 mg/ml of plant extract alone, (2) 10 mg/ml of plant extract alone, (3) 20 mg/ml of plant extract alone, (4) 5 mg/ml of plant extract with vitamin A, (5) 10 mg/ml of plant extract with vitamin A and (6) 20 mg/ml of plant extract with vitamin A.

The different concentrations of plant extract were administered via gavage feeding at 0.5 ml per day for eight days, from the eighth day to the fifteenth day of gestation. For the treatments with vitamin A, 0.02 ml (2,500 IU) of vitamin A was added to each administration of plant extract. Vitamin A supplement alone was used as the positive control while distilled water was used as the negative control.

Dissection

At the sixteenth day of gestation, pregnant mice were dissected to expose the embryos. Number of implants, number of resorptions and the number of live and dead foetuses were noted. The gestation index, percentage of dead implants, percentage of pregnant mice with resorptions and percent resorption were computed for. The fetuses were obtained and fixed overnight in 4% formaldehyde. These were used for the examinations on morphology and skeletogenesis.

Analysis of morphology

The following morphological features were observed: foetal weights, skin condition, head shape, optic and optic vesicles and forelimbs and hindlimbs and their digits. The percentage of foetuses with morphological malformations was computed for by dividing the number of foetuses with malformations by the total number of foetuses, and then multiplying the quotient by 100%.

Statistical analysis

Kruskal-Wallis nonparametric test was performed on the data with a P value less than 0.05. Further significant differences were determined by the Mann-Whitney nonparametric test.

RESULTS

Reproductive performance

For the first plant extract, *G. amuyon* (Table 1), the implantation indices of the different treatments are not significantly different. For the gestation index, the negative control, positive control and the 10 mg/ml plant extract only treatments exhibited the highest value of 100.00, while the treatment of 20 mg/ml plant extract only exhibited the lowest value of 78.57. Only the 5 mg/ml plant extract and the 20 mg/ml plant extract treatments resulted in dead implants (Figure 1). Both positive and negative controls resulted in no females with resorptions, while the 20 mg/ml treatment and all treatments of the different concentrations of plant extract plus vitamin A resulted in 100.00% females with resorptions.

In the treatments of different concentrations of plant extract only, the percentage of foetuses with morphological abnormalities increased as the concentration increased. All treatments involving vitamin A resulted in 100.00% of the foetuses with morphological abnormalities, while the negative control had 0.00% morphological abnormalities. The average weights of each of the implants per treatment did not vary significantly, except for the implants obtained from treatments involving vitamin A, which were extremely low.

The treatment of 20 mg/ml plant extract with vitamin A did not yield any actual implants due to incidences of aborted pregnancies or resorptions exhibited by the uteri. Since the exact number of implantations could not be determined, most indices could not be computed for and the data obtained from the mentioned treatment was not

Table 1. Effects of the plant extract of *G. amuyon* on the reproductive performance and organogenesis of ICR mice.

	dH_2O	5	10	20	5 VA	10 VA	20 VA	VA
Implantation index	10.00	10.50	11.00	14.00	10.00	11.50	NI	13.00
Gestation index	100.00	90.48	100.00	78.57	0.00	0.00	NI	100.00
% dead implants	0.00	9.52	0.00	21.43	100.00	100.00	NI	0.00
% female with resorptions	0.00	80.00	40.00	100.00	100.00	100.00	100.00	0.00
% fetuses with morphological abnormalities	0.00	76.19	93.94	92.86	100.00	100.00	NI	100.00
Average implant weight	0.47	0.35	0.49	0.51	0.03	0.04	NI	0.47

dH_2O – distilled water as negative control, 5 – 5 mg/ml plant extract only, 10 – 10 mg/ml plant extract only, 20 – 20 mg/ml plant extract only, 5 VA – 5 mg/ml plant extract with 0.02 ml vitamin A, 10 VA – 10 mg/ml plant extract with 0.02 ml vitamin A, 20 VA – 20 mg/ml plant extract with 0.02 ml vitamin A, VA – 0.02 ml vitamin A as positive control, NI – no implants countable. Implantation index = Total number of implants/total number of pregnant mice. Gestation index = (Total number of live implants/total number of implants) (100%). % Dead implants = 100 – gestation index. % Females with resorptions = (Total number of females exhibiting signs of resorptions/total number of pregnant mice) (100%). % Foetuses with morphological abnormalities = (Total number of foetuses with morphological abnormalities/total number of foetuses) (100%).

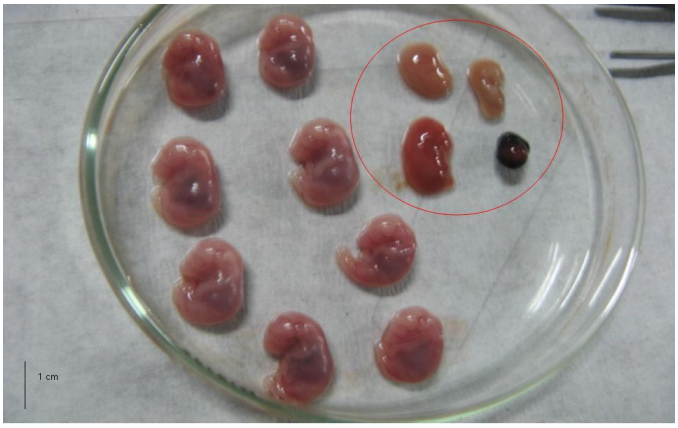

Figure 1. Implants from a day 16 pregnant ICR mouse treated with 20 mg/ml *G. amuyon* plant extract only.

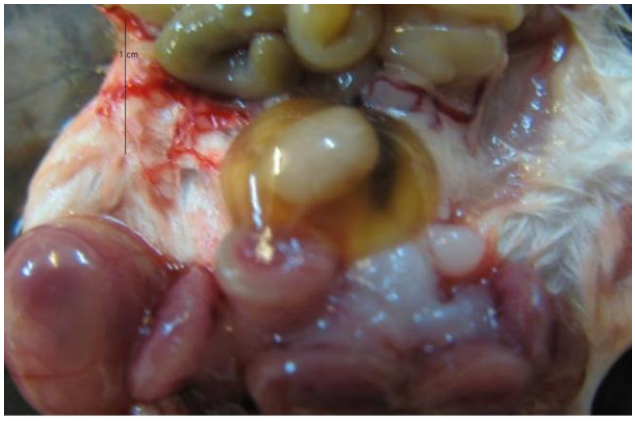

Figure 2. Resorption.

included in the statistical analysis.

For the other plant extract, *A. macrophylla* (Table 2), the implantation index exhibited no deducible trend. The gestation index was rather high for all treatments which yielded implants except for the 5 mg/ml plant extract with vitamin A treatment. Among the treatments which yielded implants, only 20 mg/ml plant extract and 5 mg/ml plant extract plus vitamin A resulted in dead implants (Figures 2 - 3). Both positive and negative controls resulted in no females with resorptions, while the 20 mg/ml treatment and all treatments of the different concentrations of plant extract plus vitamin A resulted in 100.00% females with resorptions.

All treatments except for the negative control resulted in 100.00% of the foetuses with morphological abnormalities. Average implant weights showed no significant difference except for the 5 mg/ml plant extract plus vitamin A treatment, which was rather low.

The treatment of 10 mg/ml plant with vitamin A extract and 20 mg/ml plant extract with vitamin A did not yield any actual implants, and thus most indices could not be computed for. The data obtained from the mentioned treatments therefore, were not included in the statistical analysis.

Morphological abnormalities

As a basis for comparison, the foetuses from the negative control treatment were observed for gross morphology (Figure 4) and features which differed from the negative control were considered abnormal. The head was generally round with an elongated snout. The skin was smooth and porous. The eyes and ears were prominent. The digits on the forelimbs and hindlimbs were separate from one another.

Morphological abnormalities were observed in the following parts: the head, skin, eyes, ears and digits of the limbs. Malformations in the head shape and in the limbs were most common. Head shape was angular in a number of live foetuses from all the treatments except for the negative control (Figure 5B). Reduced elongation in the snout was also observed in fetuses (Figure 5C). Wrinkling of the skin was abnormal (Figure 6) as opposed

Table 2. Effects of the plant extract of *A. macrophylla* on the reproductive performance and organogenesis of ICR mice.

	dH_2O	5	10	20	5 VA	10 VA	20 VA	VA
Implantation index	10.00	4.00	7.50	11.00	9.00	NI	NI	13.00
Gestation index	100.00	100.00	100.00	90.91	22.22	NI	NI	100.00
% dead implants	0.00	0.00	0.00	9.09	77.78	NI	NI	0.00
% female with resorptions	0.00	80.00	60.00	100.00	100.00	100.00	100.00	0.00
% fetuses with morphological abnormalities	0.00	100.00	100.00	100.00	100.00	NI	NI	100.00
Average implant weight	0.47	0.45	0.46	0.42	0.17	NI	NI	0.47

dH_2O – distilled water as negative control, 5 – 5 mg/ml plant extract only, 10 – 10 mg/ml plant extract only, 20 – 20 mg/ml plant extract only. 5 VA – 5 mg/ml plant extract with 0.02 ml vitamin A, 10 VA – 10 mg/ml plant extract with 0.02 ml vitamin A, 20 VA – 20 mg/ml plant extract with 0.02 ml vitamin A, VA – 0.02 ml vitamin A as positive control, NI – no implants countable. Implantation index = Total number of implants/total number of pregnant mice. Gestation index = (Total number of live implants/total number of implants) (100%). % Dead implants = 100 – gestation Index. % Females with resorptions = (Total number of females exhibiting signs of resorptions/total number of pregnant mice) (100%). % Foetuses with morphological abnormalities = (Total number of foetuses with morphological abnormalities/total number of foetuses) (100%).

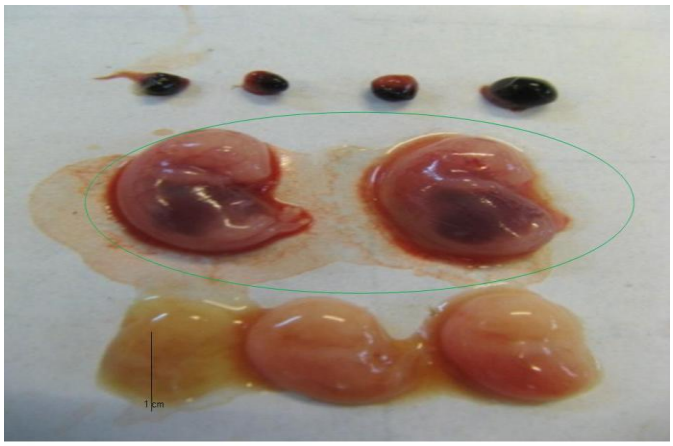

Figure 3. Implants from a day 16 pregnant ICR mouse treated with 5 mg/ml *A. macrophylla* plant extract with vitamin A.

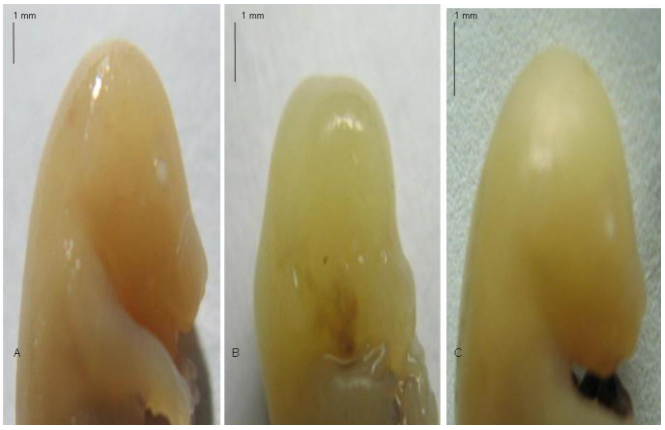

Figure 5. Different head shapes of the foetuses.

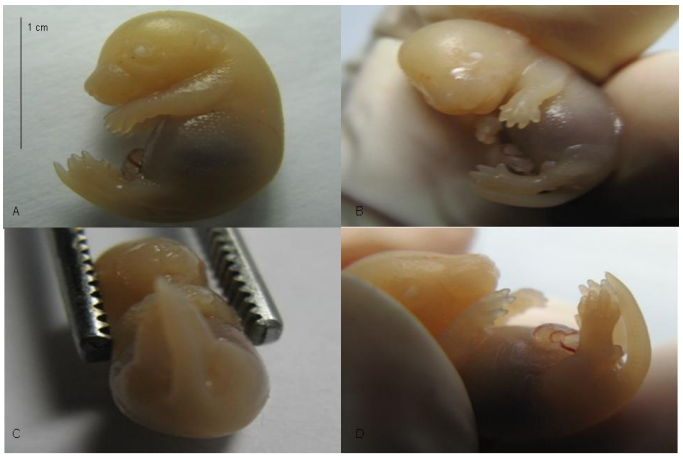

Figure 4 (A - D). Features of a normal foetus from a day 16 negative control pregnant ICR mouse.

Figure 6. Excessive wrinkling of skin in a foetus.

to the smooth skin of the negative control. Eyes and ears

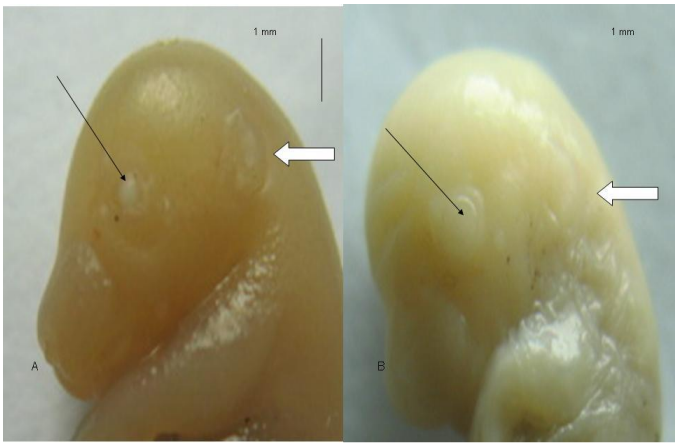

Figure 7 . Differences in the extent of eye and ear development.

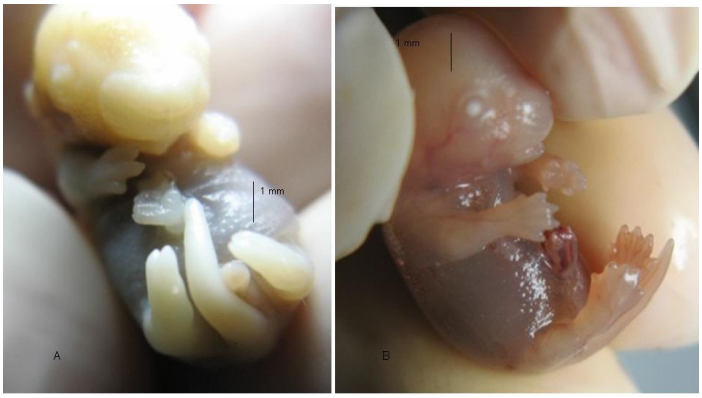

Figure 8. Abnormalities in the development of digits.

were present in all foetuses but these were under-developed in some of the foetuses considered as dead implants (Figure 7). Abnormalities in the digits of both forelimbs and hindlimbs occurred mostly by either failure of digits to separate fully or by the lack of some digits (Figure 8).

For those considered as dead implants (Figures 1 - 3), mere general body shape and gross morphology were severely altered, some of which could not be discerned anymore such as those obtained from mice treated with 5 and 10 mg/ml of *G. amuyon* plant extract only (Figure 9).

DISCUSSION

In both plant extracts, the yield of implants from the mice of only plant extract treatments was greater than that of the yield of implants from the mice of plant extract with vitamin A treatments. Mice under the treatments of 20 mg/ml *G. amuyon* extract with vitamin A, 10 mg/ml *A. macrophylla* extract with vitamin A, and 20 mg/ml *A. macrophylla* extract with vitamin A yielded no countable implants at all. For this reason, the mentioned treatments were not used in most of the calculations and analytical procedures. These findings contribute to the evidence that the plant extracts are most probably not antiteratogenic.

The implantation index is the total number of implants over the number of pregnant females and determines whether the variation in treatments affects the capacity of the treated female mice to accommodate a certain number of implants. In the *G. amuyon* treatments at different concentrations of plant extract, the implantation indices computed did not vary significantly with each other and with the controls. In the *A. macrophylla* set up, however, the presence of large deviations in the indices can be noted, although this cannot conclude that the implantation index varies with the concentrations of treatments given since the number of pregnant mice from which implants were obtained are very few, for instance, only one under the 5 mg/ml extract only treatment, which exhibited the greatest deviation among the index values. No sound conclusion of the effects of treatment on the implantation index can thus be inferred from that data.

Gestation index is simply the number of live foetuses over the total number of implants obtained expressed in percentage form, while the percentage of dead implants is simply the opposite of this, calculated by deducting the gestation index from 100. In a way, this determines the mortality rate of the foetuses. Both the gestation indices of the negative and positive controls exhibited a 100.00% gestation index, along with a few of the other treatments. With the established teratogenicity of the positive control, it was still able to result in a 100.00% gestation index. Treatments which resulted in a gestation index of less than 100.00% are thus potentially even more teratogenic than the positive control, such as 20 mg/ml of *G. amuyon* extract treatment and the plant extracts with vitamin A treatments, resulting in no greater than 25.00% gestation index.

Even though the treatments of 5 mg/ml of *A. macrophylla* extract only, 20 mg/ml of *A. macrophylla* extract only and 5 mg/ml of *A. macrophylla* extract with vitamin A yielded implants. Statistical analysis could not be performed on the indices involving numbers of implantations from the obtained data of the mentioned treatments. In these treatments, only one pregnant mouse replicate per treatment was able to exhibit implantations whose exact numbers could be determined. This is due to the observation that, although signs of pregnancy were noted, most of the uteri were already highly damaged, possibly due to the toxicity of the plant extract. More importantly, these observations indicate that there are no antiteratogenic properties being exhibited by these plant extracts.

Resorption is described as a manifestation of an aborted pregnancy or implant (Figure 1). In relation to the last two indices mentioned, the implants obtained from the study which were not considered as live foetuses

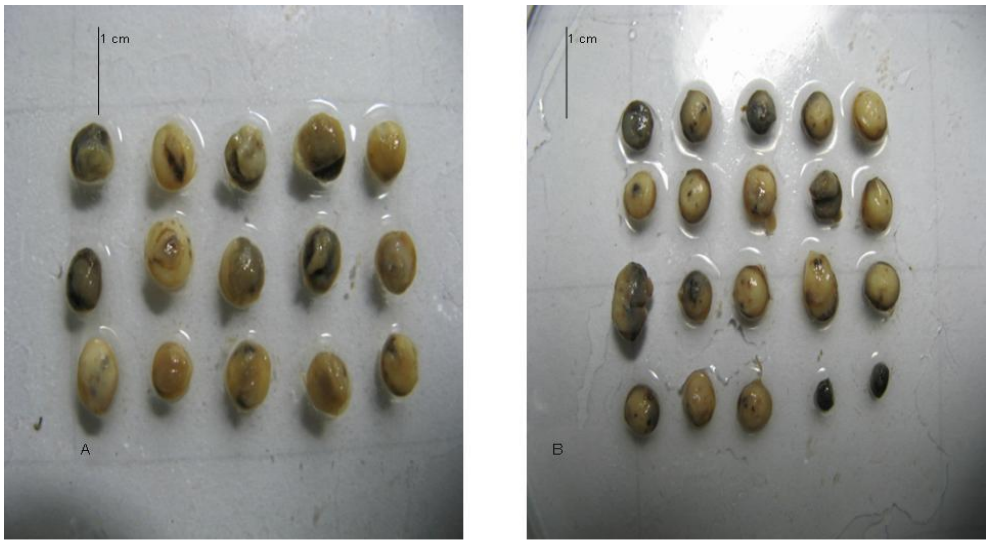

Figure 9. Dead implants.

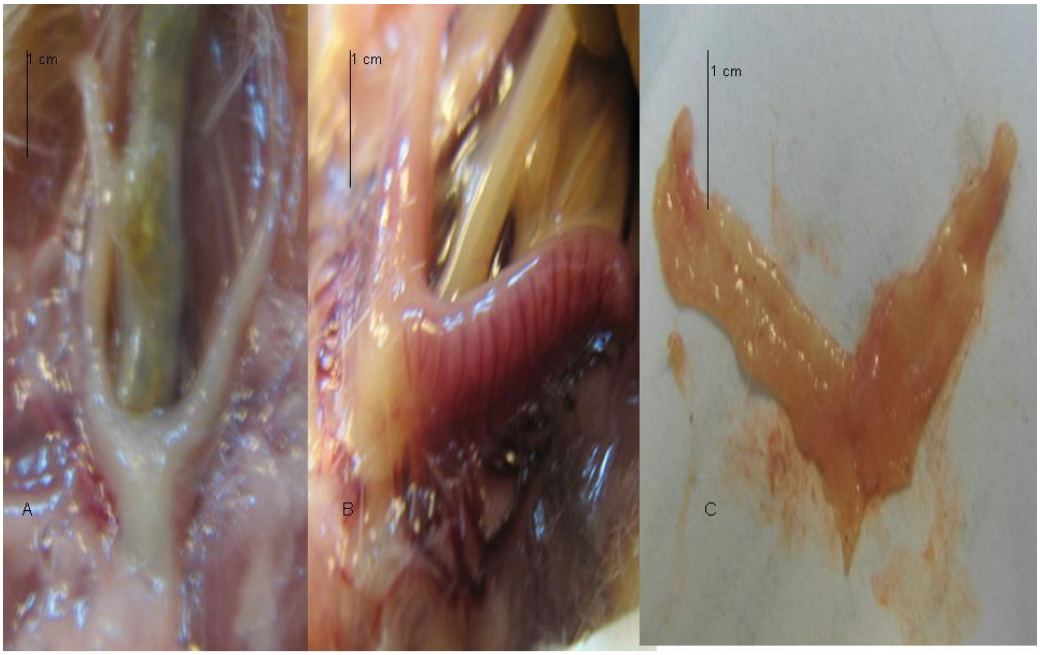

Figure 10. Uteri from dissected ICR mice: (A) uterus from a not pregnant untreated ICR mouse, (B) highly vascularized uterus from a 10 mg/ml A. macrophylla extract treated pregnant ICR mouse and (C) thick walls seen in uterus in B, cut open

under gestation index, in other words, the dead implants, were instead considered as resorptions or aborted implants. The presence of resorption was also characterized by the thick uterine walls and high degree of vascularization (Figure 10 B).

The percentage of female mice with resorptions implies the degree of susceptibility to teratogenicity possessed by the mice in response to the treatments. Thus, presence of resorptions is one effect of teratogenic activity. In both plants, the percentages of female mice with resorptions which were computed for each treatment were no lower than 40.00% for all the treatments except the controls and thus, giving implications on the potential teratogenicity of the plant extracts.

From the *G. amuyon* data, the average weights of each of the implants per treatment did not vary significantly. The implants obtained from treatments involving vitamin A exhibited extremely low weights due to the in ability to

obtain any live foetuses from them. The aborted implants were drastically lower in weight as compared to the live foetuses. From the *A. macrophylla* data, the average implant weights also did not vary significantly. In 5 mg/ml plant extract plus vitamin A treatment, the average implant weight was rather low as compared to the rest of the treatments, also due to the tremendous drop in the gestation index.

The presence of morphological malformations also indicates teratogenic effects. Determination of morphological abnormalities involved comparison with the negative controls. In the positive control, malformation of the limbs, particularly digit development was present in all of the foetuses. Additionally, malformations in the head shape and in the limbs were most common among the remaining treatments, suggesting possibly the targets of teratogenic activity of the plant extracts. However, incidence of other types of malformations may also indicate minor side effects of the teratogenic plant extracts.

The percentage of the foetuses exhibiting morphological abnormalities was 0.00% in only the negative control, signifying no signs of teratogenicity exhibited. The percentage of the foetuses exhibiting morphological abnormalities in the positive control was 100.00%, which implies high extent of teratogenicity. From the *G. amuyon* data, all foetuses from treatments involving vitamin A exhibited morphological abnormalities and thus signify teratogenicity. However, the treatments of the plant extract also gave high percentages of foetuses with malformations, also signifying teratogenic effects. From the *A. macrophylla* data, all foetuses from all treatments exhibited morphological abnormalities, also signifying teratogenic manifestations. It can be concluded in terms of morphological features that the two plant extracts possess teratogenic properties.

According to previous studies, large number of the species of *Goniothalamus* were being utilized as a traditional means of nonspontaneous abortion (Wiart, 2007), while species of *Alstonia* were claimed to have caused teratogenic effects (Arulmozhi et al., 2007). The difference that lies in this particular research is that the effects of these extracts are also studied in combination with the effects of an already established teratogen, as the plant extracts in query may possibly reduce or further aggravate the teratogenic activity of this teratogen. Additionally, Bienengräber and Fanghänel (1997) also observed that timing and dosage can change the activity of certain compounds. Herrera (2007) found out that teratogenic *Annona squamosa*, a member of the Family Annonaceae, along with *Goniothalamus*, showed signs of antiteratogenicity when used at low concentration but, from the results of this study, this was not the case in *G. amuyon* and *A. macrophylla*.

With the observation that *G. amuyon* and *A. macrophylla* are teratogenic, it is obvious that the six principles of teratology were observed that caused disruption in normal embryogenesis. Supplementing the results in this study are the findings of teratogenicity of *G. amuyon roots* (Wiart, 2007) due to the array of cytotoxic acetogenins, styryl-lactones and various alkaloids (Wu et al., 1991; Zhang et al., 1999; Hisham et al., 2003; Wattanapiromsakul et al., 2005), which are anti-cancer but also potentially teratogenic. The cytotoxicity of acetogenins was said to employ inhibitory activities on mitochondrial enzymes (Wiart, 2007) and thus, even normal and healthy cells may be affected by the cytotoxicity. Keawpradub et al. (1999), Gupta et al. (2002), Baliga et al. (2004), Jagetia and Baliga (2004), Arulmozhi et al. (2007), Oze et al. (2007) and Shetty et al. (2007) found anti-fertility and anti-cancer effects of the chemical components of *Alstonia* species. It has anti-oxidant properties not capable of fighting the effects of the excess vitamin A teratogen.

CONCLUSION AND RECOMMENDATIONS

It can be concluded that both plants extracts, *G. amuyon* and *A. macrophylla*, exhibit teratogenic effects on mice foetus. This is based mainly on the results of the percentage of pregnant mice which exhibited resorptions and by the results of the percentage of foetuses exhibiting morphological abnormalities. Additionally, the dose dependency of the teratogenic activities of these plant extracts cannot be completely determined. On the other hand, these plants do not possess antiteratogenic properties to counteract the teratogenicity of vitamin A.

For future studies, a more specific extraction by the use of separation procedures, such as chromatography techniques, may be performed to be able to isolate and identify the specific compound involved in the teratogenic activity of the crude plant extract. Mechanisms of action of the compound should also be analyzed.

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