Evaluation of Possible Toxic Effects on Male Reproductive Performance and Pregnancy Outcomes in *Andrographis Paniculata* Treated Rats

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Abstract

Andrographis paniculata has been extensively used in Malay traditional medicine for promotion of well-being. However, the information of its potential toxic effects on male reproductive system are lacking. Therefore, the present study was aimed to investigate the possible effect of 50% ethanolic extract of *A. paniculata* on male reproductive performance and pregnancy outcome parameters. Fifty male rats received extract of *A. paniculata* by gavaging for 70 days at the doses of 0.5, 1, 10, 100 and 1000 mg/kg, while another 10 male rats were given distilled water. Following *A. paniculata* treatment, each male was mated with three untreated females, 3 hours daily for 14 days. Mating, pregnancy and fertility indices were measured. 50% of female rats were sacrificed on day 21 pregnancy (PO I) while another 50% of female rats were sacrificed after 21 days post-partum (PO II). Any resorptions and malformations were also recorded. Results showed that there were no significant differences observed in reproductive performance parameters. Pregnancy outcomes from PO I did not show any significant difference in maternal and fetal parameters. Meanwhile in PO II, there were no significant differences in maternal parameter. Nonetheless, the percentages of mortality rate in fetuses were significantly increased in dose dependent manner in *A. paniculata* treated groups. In conclusion, treatment of 50% ethanolic extract of *A. paniculata* in male rats had no adverse effects on reproductive performance and pregnancy outcomes. However, high mortality rate of fetuses on day 21 of post-partum may require further investigation.

KEYWORDS: *Andrographis paniculata*, toxicity, male reproductive performance, pregnancy outcomes

1. INTRODUCTION

Andrographis paniculata is a plant that belongs to the family of *Acanthaceae*. It is one of the important herbal medicines which have been used for centuries in Asia to treat various illnesses (Sattayasai *et al.*, 2010). Phytochemical investigations of *A. paniculata* revealed the presence of diterpenes, flavonoids and polyphenols (Sareer *et al.*, 2014). Extensive studies over the last decades have reported that this herbal plant is useful as an anti-inflammatory agent (Xia *et al.*, 2004), anti-microbial activity (Mishra *et al.*, 2009b), anti-thrombotic activity (Thisoda *et al.*, 2006), anti-malarial (Mishra *et al.*, 2009a), immunostimulant (Xu *et al.*, 2007), hypotensive and hypoglycaemic agent (Zhang and

Tan, 2000) and used for treatment of upper respiratory tract infection (Coon and Ernst, 2004).

In Malaysia, *A. paniculata* has been used traditionally to treat diabetes and hypertension, even though the benefits of its usage have not been justified scientifically. The potential toxic effects of *A. paniculata* for instance are still controversial (Shahid, 2011). A study by Nagalekshmi *et al*. (2011) found that the extracts of *A. paniculata* offered protection against hepatotoxicity induced by paracetamol. In the line with this, Allan *et al*. (2009) failed to show any toxicity effects of standardized *A. paniculata* extract (10% andrographolide) in male rats. In contrast, *A. paniculata* has been demonstrated to have anti-fertility and reproductive toxicity (Akbarsha and Murugaian, 2000). This suggests that *A. paniculata* might have toxicity effects that warrant further investigation.

The present study was designed to investigate the possible toxic effects of *A. paniculata* on male reproductive performance indices and pregnancy outcomes. These include comparison of the mating, pregnancy and fertility indices in *A. paniculata* treated male rats and control rats. This investigation is extremely important in order to evaluate the safety of *A. paniculata* consumption.

2. MATERIALS AND METHODS

2.1 Preparation of plant extract

The extraction of *A. paniculata* was performed using a mixture of 50% ethanol and 50% water by NOVA laboratories, Selangor, Malaysia. Standardized extract of *A. paniculata* was supplied in liquid form, dark green color. The extract was subjected to freeze drying at -50°C overnight. Later, the dried pure *A. paniculata* extract was scraped using a spatula and was pulverized into powder. The extract was then reconstituted in distilled water to give the required doses of 0.5, 1, 10, 100 and 1000 mg/kg body weight applied in this study.

2.2 Animal husbandry and treatment

A total of 60 adult male rats and 180 adult female rats of Sprague Dawley were obtained from Animal Research and Service Centre, Universiti Sains Malaysia (USM), Kubang Kerian, Kelantan, Malaysia. The rats weighing between 180-200 g (aged 8-10 weeks) were housed in PVC cages and maintained under controlled environmental condition at temperature of 22 ± 25°C with a 12 h light / 12 h dark cycle. All animals had free access to tap water and were fed with a standard pellet diet. All rats were allowed to acclimatize for one week prior to the experiment. The male rats were previously mated until they become sexually experienced to attest fertility (Dasuki *et al.*, 2011).

The male rats were randomly divided into six groups (I-VI) of 10 each. Group I were orally administered with 0.4 ml of distilled water (vehicle) whilst groups II, III, IV, V and VI received 0.5, 1, 10, 100 and 1000 mg/kg of *A. paniculata* extract in 0.4 ml of

vehicle respectively, once daily for 70 days (OECD, 2001). All experimental procedures on rats were conducted in accordance to USM Guide For The Care and Use of Laboratory Animals and approved by Animal Ethics Committee USM/PPSP/050(1).

2.3 Male reproductive performance

Following *A. paniculata* treatment, each male rat was separated into a single cage and was allocated with three untreated female rats (1:3 ratio), 3 hours daily for a duration of 14 days. During this period, at least two estrous cycles of female rats should have elapsed while exposed to the males (Kuriyama and Chahoud, 2004). Vaginal smears from the female rats were collected daily after 3 hours test time. The day of sperm detection in the vaginal smear was considered as day 0 of pregnancy. Mating (number of males that make any of the three females sperm positive within 14 days/total number of males involved in mating x 100), pregnancy (number of pregnant females/number of sperm positive females x 100) and fertility indices (number of days elapsed until the male rats had first fertilized its female partner) were measured according to the standard procedures (Wan *et al.*, 2013).

2.4 Pregnancy outcome

On day 21 of pregnancy (PO I), 50% of the females per group were sacrificed by diethyl ether inhalation. The peritoneal cavity was opened to reveal the ovaries and uteri away. The ovaries were removed and the number of corpora lutea was counted. The uterine horn was sectioned longitudinally and pre-implantations loss [(number of corpora lutea – number of implantations)/number of corpora lutea) X 100], resorption [(number of implantations – number of live fetuses)/number of implantations X 100], live birth index (number of live offspring/number of offspring delivered) and mortality rate (number of dead fetuses/number of offspring delivered X 100) were recorded. All the remaining pregnant females were allowed to give birth to their offspring. The day of birth was designed as postnatal day 0. At postnatal day 21 (PO II), all dams were sacrificed and subjected to postmortem examination. Viability index (number of live offspring at lactation day 4/number of live offspring delivered) and lactation index (number of live offspring at day 21/number of live offspring born X 100) were calculated accordingly. All the fetuses were blotted dry and then their body weight and sex ratio (number of male offspring/female offspring) were determined. The fetuses were observed for obvious external malformations immediately and preserved in 10% formaldehyde (Tian *et al.*, 2011).

2.5 Statistical analysis

One-way ANOVA was performed on a normal distribution data, followed by Bonferroni post-hoc test in case of overall significant effects. If the data did not assume a normal distribution, Kruskal Wallis test was performed followed by Mann Whitney U test for comparison between groups. Proportion was analyzed using Chi square test. The level of significant difference was defined as $p<0.05$. The statistical analyses were performed using SPSS software (version 20.0).

3. RESULTS

3.1 Male reproductive performance

The data concerning on the male reproductive performance parameters such as mating, pregnancy and fertility indices are shown in Table 1. The results revealed that there were no significant changes in mating, pregnancy and fertility indices when *A. paniculata* treated male rats were mated with healthy fertile females.

Table 1: Effects of *A. paniculata* on mating, pregnancy and fertility indices.

Parameters	Control (n=10)	*A. paniculata* extract					p value
		0.5 mg/kg (n=10)	1 mg/kg (n=10)	10 mg/kg (n=10)	100 mg/kg (n=10)	1000 mg/kg (n=10)	
[z] Mating index (%)	6/10 (60)	7/10 (70)	7/10 (70)	10/10 (100)	9/10 (90)	10/10 (100)	p>0.05
[z] Pregnancy index (%)	10/15 (67)	10/16 (63)	12/18 (67)	14/23 (61)	19/24 (79)	18/24 (75)	p>0.05
[y] Fertility index (Day)	2.00±1.50	2.00±3.00	3.00±2.00	5.50±6.00	3.00±4.00	4.50±7.00	p>0.05

[y]= Kruskal Wallis. Values are median ± inter quartile range. [z] = Chi square. *P<0.05 was considered significant. n= number of male rats.

3.2 Pregnancy outcomes

The data were presented in two ways, 50% of female rats were sacrificed on day 21 of pregnancy (PO I) and another 50% were sacrificed after 21 days post-partum (PO II).

3.2.1 Dams sacrificed on Day 21 of pregnancy

Table 2 shows that the number of sperm positive females and pregnant dams did not differ from control group at any dose levels. Nonetheless, there was a significant decreased in dams' body weight gain mated with male rats that received *A. paniculata* 1000 mg/kg compared to control group and *A. paniculata* 1 mgkg. There was statistically difference among 1 mg/kg from 0.5, 10 and 1000 mg/kg *A. paniculata* groups in the pre-implantation loss parameter. No statistically significant differences among control and *A. paniculata* treated groups were found with regard to gravid uterine weight, adjusted maternal weight, number of corpora lutea, number of implantations, implantation/litter and number of resorptions.

Table 3 shows that administration of *A. paniculata* extract to male rats at any doses had no significant effect on fetal parameters such as the number of fetuses, percentage of live fetuses, viable fetuses/litter, mortality rate, fetus weight/litter and sex ratio. However, the fetus' weight in 10 mg/kg of *A. paniculata* treated group was significantly increased as compared to control group. There were no observable malformation of fetuses from all *A. paniculata* treated and control groups with the exception of one fetus that showed

micrognathia (jaw malformation) from the dam caged with 1 mg/kg *A. paniculata* treated male rat.

Table 2: Pregnancy outcomes from dams mated with control and *A. paniculata* treated groups on day 21 pregnancy.

Parameters	Control (n=10)	*A. paniculata* extract 0.5 mg/kg (n=10)	1 mg/kg (n=10)	10 mg/kg (n=10)	100 mg/kg (n=10)	1000 mg/kg (n=10)	p value
[z] Sperm positive females (%)	6(40)	6(50)	8(54)	12(80)	9(60)	11(74)	p>0.05
[z] Pregnant dams (%)	4(67)	3(50)	5(63)	6(50)	4(45)	7(64)	p>0.05
[z] Dam body weight gain (%)	55	50	56^^	46	50	33*^^	p<0.05
[Y] Gravid uterine weight (g)	53.10 ± 30.44	46.48 ± 29.88^	69.99 ± 13.10^#√	57.06 ± 12.68#	71.80 ± 16.67√ξ	41.70 ± 42.30 ξ	p>0.05
[x] Adjusted maternal body weight (g)	271.04 ± 3.47	260.64 ± 4.04	247.57 ± 6.70	260.34 ± 8.51	247.17 ± 4.50	245.97 ± 10.16	p>0.05
[Y] Total Corpora Lutea	47	33	58	71	47	82	p>0.05
[Y] Total Implantation	42	29	58	67	45	60	p>0.05
[x] Implantation/litter	10.50 ± 1.50	9.67 ± 1.76	11.60 ± 0.51	11.17 ± 0.54	11.25 ± 0.85	8.57 ± 1.00	p>0.05
[y] Pre-Implantation loss (%)	3.85 ± 31.92	10.00 ± 29.22^	0^#ξ	7.42 ± 10.79#	3.85 ± 8.17	11.11 ± 36.75 ξ	p<0.05
[z] Resorptions total (%)	6/42 (14)	13/29 (34)	6/58 (10)	8/67 (12)	4/45 (9)	18/60 (30)	p>0.05

[x] = One-ways ANOVA. Values are mean ± SEM. [y] = Kruskal Wallis. Values are median ± inter quartile range. [z] = Chi square. *p<0.05 was considered significant. ^, ^^, √, ξ and # = Significantly different between *A. paniculata* treated groups. n= number of male rats.

Table 3: Pregnancy outcomes from dams mated with control and *A. paniculata* treated groups, and fetal parameters were evaluated at the caesarian section performed on day 21 of pregnancy.

Parameters	Control (n=10)	*A. paniculata* extract 0.5 mg/kg (n=10)	1 mg/kg (n=10)	10 mg/kg (n=10)	100 mg/kg (n=10)	1000 mg/kg (n=10)	p value
Number of dams	4	3	5	6	4	7	-
[y] Total number of fetuses	36	21	52	59	41	42	p>0.05
[z] Live fetuses (%)	35 (97.2)	19 (90.5)	52 (100)	59 (100)	41 (100)	42 (100)	p>0.05
[x] Viable Fetuses/Litter	8.75 ± 1.44	5.33 ± 1.20	10.40 ± 0.68	9.83 ± 0.75	10.25 ± 0.75	6.00 ± 1.45	p>0.05
[z] Mortality rate (%)	1/36 (2.8)	2/21 (9.5)	0	0	0	0	p>0.05
[y] Fetus weight (g)	4.67 ± 1.49	4.04 ± 0.81	4.79 ± 0.50	4.69 ± 1.18	5.03 ± 0.32*	4.54 ± 0.55	p<0.05
[y] Fetus weight/ Litter	4.75 ± 2.31	3.83 ± 2.95	4.76 ± 0.47	4.81 ± 1.58	5.01 ± 0.10	4.37 ± 0.54	p>0.05
[z] Sex ratio (M%/F%)	18/18 (50:50)	8/13 (38:62)	20/32 (38:62)	39/20 (66:34)	21/20 (51:49)	25/17 (60:40)	p>0.05

[x] = One-ways ANOVA. Values are mean ± SEM. [y] = Kruskal Wallis. Values are median ± inter quartile range. [z] = Chi square. *P<0.05 was considered significant. ^ = Significantly different among *A. paniculata* treated groups. n= number of male rats.

3.2.2 Dams sacrificed on Day 21 post-partum

Table 4 shows that the number of sperm positive was significantly increased in female rats impregnated by males administered with *A. paniculata* extract at the dose of 100 mg/kg. Treatment of *A. paniculata* at any dose levels had no significant effect on the percentage of pregnant dams, maternal weight gain, uterine weight and adjusted maternal weight. The other parameters evaluated such as total corpura lutea, total implantation, implantation/litter, pre-implantation loss and resorptions total also did not suggest modifications.

As can be seen in Table 5, *A. paniculata* extract did not produce any adverse effect on the number of newborn, live birth index, mortality rate, viable pups/litter, pups growth rate, sex ratio and viability index within the doses range tested. Although the pups from 10 mg/kg *A. paniculata* group showed significant reduction in body weight at birth but the result of pup weight/litter ratio did not show any significant difference when compared to control group. With regard to lactation index, 1 mg/kg *A. paniculata* group showed a significant reduction as compared to control group. On the other hand, mortality rate was significantly increased in three *A. paniculata* extract treated groups (1, 100, 1000 mg/kg). Finally, there were no significant differences observed in pups' body weight at postnatal day 21.

Table 4: Pregnancy outcomes from dams mated with control and *A. paniculata* treated groups on day 21 post-partum.

Parameters	Control (n=10)	*A. paniculata extract* 0.5 mg/kg (n=10)	1 mg/kg (n=10)	10 mg/kg (n=10)	100 mg/kg (n=10)	1000 mg/kg (n=10)	p value
Females involved	15	12	15	15	15	15	-
[y] Sperm positive females (%)	9 (60)	10 (84)	10 (67)^	11 (74)$^{\xi}$	15 (100)*^$^{\xi}$	13 (87)	p<0.05
[y] Pregnant dams (%)	6 (67)	7 (70)	7 (70)	8 (73)	15 (100)	11 (85)	p>0.05
[y] Maternal weight gain (%)	4.44	3.87	-3.08	8.12	-2.36	1.38	p>0.05
[x] Uterine weight (g)	0.31 ± 0.05	0.36 ± 0.04	0.39 ± 0.08	0.43 ± 0.06	0.36 ± 0.03	0.39 ± 0.04	p>0.05
[x] Adjusted maternal weight (g)	257.77±15.30	260.49±10.34	230.75±17.68	257.57 ±10.25	241.84±10.41	245.34±8.01	p>0.05
[x] Total corpura lutea	97	119	127	125	244	169	p>0.05
[x] Total implantation	70	76	82	95	163	102	p>0.05
[x] Implantation/Litter	11.67 ± 0.61	11.14 ± 0.86	11.71 ± 0.71	11.88 ± 0.72	10.47 ± 0.70	9.27 ± 0.71	p>0.05
[x] Pre-implantation loss (%)	26.00 ± 6.78	33.93 ± 5.12	30.25 ± 8.47	22.50 ± 3.51	35.59 ± 4.23	38.53 ± 4.94	p>0.05
[y] Resorptions total (%)	11/70 (16)	20/76 (26)	20/82 (24)	16/95 (17)	39/163 (24)	17/102 (17)	p>0.05

[x] = One-way ANOVA. Values are mean ± SEM. [y] = Chi square. *P<0.05 was considered significant. ^ and [ξ] = Significantly different among *A. paniculata* treated groups. n= number of male rats.

Table 5: New born parameters from dams mated with *A. paniculata* treated male rats.

Parameters	Control (n=10)	*A. paniculata extract* 0.5 mg/kg (n=10)	1 mg/kg (n=10)	10 mg/kg (n=10)	100 mg/kg (n=10)	1000 mg/kg (n=10)	p value
[x] Total newborn	59	60	62	79	120	86	p>0.05
Postnatal Day 1							
[x] Live birth index (%)	100	97	97	96	96	100	p>0.05
[y] Mortality rate (%)	0	2/60 (3)	2/62 (3)	3/79 (4)	5/120 (4)	0	p>0.05
[x] Viable pups/Litter	9.83 ± 0.60	8.57±1.25	8.86±1.26	9.88±1.08	8.00±0.84	7.82±0.77	p>0.05
[x] Birth weight	6.52±0.06	6.85±0.07	6.12±0.09	5.58±0.12*****	6.41±0.09	6.23±0.06	p<0.05
[x] Pups weight/ Litter	6.53 ± 0.13	6.92 ± 0.18	6.05 ± 0.23	5.64 ± 0.32	6.38 ± 0.25	6.28 ± 0.13	p>0.05
[y] Pups Growth rate (%)	474.31±14.92	493.75±9.74	477.42±28.32	527.94±16.23	463.49±11.20	524.99±14.03	p>0.05
[y] Sex Ratio (M%/F%)	30/29 (51:49)	30/30 (50:50)	35/27 (56:44)	46/33 (58:42)	58/62 (48:52)	40/46 (47:53)	p>0.05
[y] Viability index (%)	58/59 (98)	58/58 (100)	44/60 (73)	73/76 (96)	102/115 (89)	73/86 (85)	p>0.05
Postnatal Day 21							
[y] Lactation index (%)	58/59 (98)	58/58 (100)	31/60 (52)*	73/76 (96)	92/115(80)	65/86 (76)	p<0.05
[y] Mortality rate (%)	1/59 (2)	0	31/62 (50)***	6/79 (8)	28/120 (23)**	21/86 (24)***	p<0.01 p<0.001
[x] Pups body weight (g)	37.12±0.87	40.59±0.66	36.06±1.58	35.08 ± 0.59	37.57±0.81	39.15±0.86	p>0.05

[x] = One-way ANOVA. Values are mean ± SEM. [y] = Chi square. *P<0.05 was considered significant **p<0.01 and***p<0.001.
n= number of male rats.

4. DISCUSSION

The evaluation of reproductive performance and pregnancy outcomes provide measures of the functional consequences of reproductive injury (Manson and Kang, 1994). Oral administration of *A. paniculata* at different dose levels ranging from 0.5 of up to 1000 mg/kg body weight did not affect the male reproductive performance in any deleterious ways. Though there was no significant effect in mating index observed in our study, treatment with *A. paniculata* 10 and 1000 mg/kg exhibited 100% positive response while the control group showed only 60%. Similar trends are observed in a study by Mkrtchyan *et al*. (2005), where standardized extract of *A. paniculata* (fixed combination Kan Jang) showed no significant negative effects on the male fertility but rather it exhibited a positive trend in fertility indices. Our finding is further supported with a previous research by Renu (2005). The author indicated that treatment with 95% *A. paniculata* ethanolic extract for four week at 10 and 1000 mg/kg showed an increase of 90% and 80% in libido index respectively.

In PO I study, the female rats that cohabited with 1000 mg/kg *A. paniculata* treated male rats showed a significant decrease in maternal weight gain. The maternal deaths as well as the decrease in weight gain during pregnancy are indications that there

was maternal toxicity (Mello *et al.*, 2005). These maternally toxic doses of any substance also proved to be embryofeto-toxic as revealed by three outcomes evaluated: embryolethality, prenatal growth retardation and fetal malformations (Wang *et al.*, 2012). It is possible that reduction in maternal weight gain is not related to the treatment since *A. paniculata* extract were given to the males, not females. Unhealthy conditions, diseases or early pregnancy may also be the reason for the lowering of the percentage body weight gain in female rats during the pregnancy period (Patrick and Marie, 1998). No other maternal parameters showed significant different. As for fetal parameters, only 100 mg/kg *A. paniculata* treated group showed a significant increase in fetal weight as compared to control. However, fetus weight/litter parameter did not show any significant effect. Thus, it seems that *A. paniculata* did not produce any sign of toxicity in both maternal and fetal parameters based on the pregnancy outcomes observations at day 21 of pregnancy.

As for PO II, there was a significant increase in the number of sperm positive in females mated with *A. paniculata* male treated rats. This situation may arise due to higher mating index among the *A. paniculata* treated groups in reproductive performance study. Previous study by Sattayasai *et al*. (2010) also revealed that treatment of *A. paniculata* for four weeks was able to improve sexual motivation and performance, as well as serum testosterone in mice. The other maternal parameters did not show any modifications. Similarly, ingestion of *A. paniculata* had no effect on general fetal parameters at day 21 of post-partum. However, in post natal day 21 there was a significant increase in mortality rate of fetuses among *A. paniculata* treated groups. Signs of maternal well-being are usually observed in developmental toxicity studies, i.e. decrease in maternal weight gain, decreased in food and water consumption, appearance of clinical signs and organ toxicity and mortality (Elbetieha *et al.*, 2001). According to this study, the decreased in maternal weight gain observed in three dose levels of *A. paniculata* (1, 100 & 1000 mg/kg) correspond to the increase in mortality rate of the fetuses (Chahoud *et al.*, 1999). Thus it is likely that the adverse effect in mortality rate does not necessarily reflect to the treatment of *A. paniculata* in male rats.

The most common teratogenic effects attributed to toxic substances are spontaneous abortion, congenital malformations, intrauterine growth retardation, mental retardation, carcinogenesis and mutagenesis (Eluwa *et al.*, 2010). In mice and rats, even malformations as severe as neural tube defects, fused or missing ribs, and fused or scrambled sternebrae could be caused by maternal or paternal toxicity (Mello *et al.*, 2005). Gross external examination of fetuses revealed no obvious abnormality or malformation in all fetuses delivered from dams mated to *A. paniculata* treated paternal groups except for one fetus from 1 mg/kg *A. paniculata* group which showed micrognathia. Abnormalities or malformation of fetuses may also be caused by maternal or paternal genetic factors, environmental factors and other factors related to diet. The cause of approximately 40% of malformation is unknown and 12-25% of congenital defects are purely genetic defects (Gardner *et al.*, 2014). Teratogenicity is a very difficult condition to relate the cause and effects relationships. Only controlled epidemiological studies can be expected to detect a relationship between environmental factors such as drug exposure and pregnancy outcomes (Zomerdijk *et al.*, 2014). These studies must be

performed by unbiased researchers who are able to statistically analyze the population and correct for confounding factors (Speight and Holford, 1997). Animal studies cannot be true predictors of teratogenicity due to wide inter- and intra- species variations in the pharmacokinetic of the drugs concerned including placental transfer (Jagetia and Baliga, 2003). Thus, the single observation of micrognathia in our study could be a single case which might be a natural incidence in the rat population of our laboratory.

5. CONCLUSION

Administration of 50% ethanolic extract of *A. paniculata* in male rats for 70 days showed no observable pattern of toxicity on male reproductive performance and pregnancy outcome parameters. The plant seems to be safe to male rats however higher mortality rate in fetuses at day 21 post-partum may require detailed investigation in order to confirm its safety.

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