

REVERSIBLE SPERMATOXIC EFFECT OF *Andrographis paniculata* METHANOL EXTRACT IN SPRAGUE DAWLEY RATS

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ABSTRACT

Andrographis paniculata is a herbal plant in the Acanthaceae family with potential to treat diabetes and cardiovascular and as an antifertility agent. The purpose of this study was to determine the reversible antifertility effect of methanol extract *A. paniculata* on sperm quality and spermatogenesis. A total of 28 male *Sprague Dawley* rats were assigned into two groups; control group (K1) and treated group (K2) with 14 rats in each group. The treated group was force-fed with a dose of 800 mg/kg for 14 days. On day 15, seven rats from each group were sacrificed. There was significant decrease ($p < 0.05$) in sperm count, viability and sperm motility grade in K2 compared to K1, however, the percentage of normal sperm morphology in both groups showed no difference. Histologically, the lumens of seminiferous tubule were seen less packed with sperm and the Leydig cells were regressed. The damaged Sertoli cells were seen and spermatogenesis was also inhibited in K2. The study was proceeded for another 14 days without treatment to determine the reversible effect of the extract. On day 29, the remainder seven rats from each group were sacrificed for the same parameter analysis. Data showed the sperm count, viability and the sperm motility grade in K2 increased significantly ($p < 0.05$). The testes histology showed that normal spermatogenesis, seminiferous tubule filled with sperm and normal Leydig and Sertoli cells. It appeared that the methanol extract of *A. paniculata* have an antifertility effect on sperm quality and possess spermatotoxic feature in spermatogenesis activity and the effect was reversible.

Key words: *Andrographis paniculata*, Sperm quality, Spermatogenesis, Reversible antifertility

INTRODUCTION

Rodents are of great concern due to the destruction they caused. The increasing numbers of the rodent population need to be taken seriously since they caused severe damages to the Malaysian agricultural economy as well as spreading lethal diseases to the people. There are several species of rodents identified as pests such as *Rattus argentiventer*, *Rattus rattus diardi*, *Rattus tiomanicus*, *Rattus vorvegicus* and *Rattus exulans* (Singleton & Petch, 1994). The average numbers of litters that can be produced by a female rat are from 8 to 17 per pregnancy. These numbers are estimated to be higher if it is not controlled (Lam *et al.*, 1990).

Rodents are associated to various crops such as rice and oil palms. According to Rubber Industry Smallholders' Development Authority (RISDA), these vertebrate rodents caused about 5 to 10% loss in oil palm plantation. These rodent pests could

attack throughout many stages of the oil palm tree and eat the fruit of the plant causing damage in oil palm tree (Kamaruddin, 2009). According to Ramli (2012), 77 hectares of paddy field in Alor Setar were attacked by rats that led to RM 157,000 loss per year. Rats are also a well-known disease carrier. They are a host to bacteria, parasites and even certain viruses. Direct contact to these mammals could cause lethal diseases such as leptospirosis, salmonellosis and typhoid fever (Lai *et al.*, 2013). Leptospirosis is a zoonotic disease than can cause pre-mature birth, birth defect, miscarriage and in some cases death in livestock animal (Protokol Veterinar Malaysia, 2011).

Several measures such as biological, physical and also chemical approaches have been conducted in order to control the rat population. However, these methods did not work efficiently. The chemical approach, in particular, caused a long-term effect on the ecosystem. Hence, researches have been done using herbs as contraceptive agents to reduce the population in an eco-friendly method.

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Andrographis paniculata is a family of *Acanthaceae* that grows widely in the South East Asia specifically in China and India. It has been used extensively in traditional medicine as it promotes health and treats various types of illnesses. *A. paniculata* is also known with different names such as Sambiloto, Kalmegh, Chuan-Xin-Lian, Senshinren, *Green Chiretta* and *King of Bitters* (Jarukamjorn & Nemoto, 2008). Studies reported that *A. paniculata* contains diterpenes, flavonoids and polyphenols (Sareer *et al.*, 2014). Andrographolide is one of the bioactive components in *A. paniculata* that can be found mostly at the leaves, approximately 2% (Jarukamjorn & Nemoto, 2008). *A. paniculata* shows hepatoprotective characteristic by inhibiting the elevation of γ -glutamyl transpeptidase, glutathione-S-transferase and lipid peroxidation activity in the liver through hexachlorocyclohexane inhibition (Trivedi & Rawal, 2000, Trivedi & Rawal, 2001).

Many studies have been reported to this herbal plant which has the potential as in anti-malarial (Misra *et al.*, 1992 & Chander *et al.*, 1995), hypotensive and hypoglycaemic (Zhang & Tan, 2000) and also anti-inflammatory (Hidalgo, 2005) activities. In another study, the anti-fertility effect of 2 g/kg dried *A. paniculata* for 6 weeks in both male and female rats was confirmed (Zoha *et al.*, 1989). Akbarsha *et al.* (1990) reported that *A. paniculata* caused arrest in spermatogenesis, decreased sperm count and motility and also morphology abnormalities. Another study reported that the administration of andrographolide from *A. paniculata* (25 mg/kg and 50 mg/kg for 48 days) has been demonstrated to have anti-fertility property and reproductive toxicity (Akbarsha & Murugaian, 2000). In female rats, *A. paniculata* aqueous extract (1 g/kg for 4, 6 and 8 weeks) caused infertility by lowering the reproductive hormone such as Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), oestrogen and progesterone (Sakilah *et al.*, 2009).

Even though rodents are more of a pest and menace, diminishing all rat population is not tolerable in order to maintain a balanced ecosystem. Thus, the present study was designed in order to develop an eco-friendly rodent pesticide through investigating the effect of methanol extract *A. paniculata* in regulating male rat fertility and its possible reversible effect in fertility.

MATERIALS AND METHODS

Preparation of plant extract

Fresh leaves of *Andrographis paniculata* (*A. paniculata*) were obtained from Ladang Puchong, Fakulti Pertanian, Universiti Putra Malaysia (UPM),

Selangor. The leaves were dried in the oven for 72 hours at 40°C and made into powder. The powdered herb was extracted using absolute methanol through a Soxhlet machine. The extract later was concentrated in a rotary evaporator (Büchi Rotovapor® R-200/205), yielding dark green liquid extract. The extract was kept refrigerated at 4°C to maintain its freshness.

Animal husbandry and treatment

A total of 28 male Sprague Dawley rats aged eight weeks (120-200 g) were obtained from the Animal House, Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor. All animals were acclimatized to the experimental condition one week prior to the experiment. The rats were fed with standard pellet diet and allowed free access to water *ad libitum*. The rats were kept in PVC cages at controlled room temperature with 12 hours light / 12-hour dark cycle.

The male rats were randomly divided into two groups with 14 rats in the control group (K1) and treated group (K2) respectively. Previous study has been made by Fatihah (2015) with two different dosages; 800 mg/kg and 1600 mg/kg respectively, both dosages showed adverse effects in the testes histology. Thus, in this study, a lower dosage at 800 mg/kg of methanol extract *A. paniculata* was used. K1 was orally administered with 1 ml of distilled water while K2 received a dose of 800 mg/kg of methanol extract *A. paniculata* respectively, once daily for 14 days. On day 15, seven rats of each group were sacrificed for sperm quality and histology of testes. To determine the reversible effect, the rats remain untreated for the next 14 days and were sacrificed on day 29. This study was approved by the Animal Ethics Committee of Faculty of Medicine, Universiti Kebangsaan Malaysia (FST/2013/MAHANEM/31-JAN./492-FEB.-2013-FEB.-2015).

Sperm counts and motility

The caudal epididymis was minced and suspended in 15 mL of Biggers – Whitten - Whittingham medium (Whittingham *et al.*, 1971) prior to incubation in 5% CO₂ incubator for 30 minutes at 37°C to allow the sperm to swim up. A total of 10 μ l sperm suspension was used for sperm count and motility. Sperm count were assessed using the 'Improved Neubauer Haemocytometer' based on the WHO manual (2010) with modification. Sperm motility grade was determined based on WHO Laboratory Manual (2010).

Sperm viability and morphology

For sperm viability, a moving sperm is counted as viable and non-moving sperm considered dead. The sperm was analysed using light microscope at

400X magnification. For the assessment of the sperm morphology, 10 μ L of sperm suspension was smeared on a clean glass slide. The slide was allowed to dry overnight and stained using Giemsa staining. A total of 200 sperm were observed under phase contrast microscope at 400X magnification. The criteria of Wyrobek and Bruce (1975) were employed for the evaluation of sperm morphology.

Testes histology

Testes were fixed in a Bouin's Solution for overnight, washed with 0.9% NaCl, dehydrated through graded concentration of ethanol, embedded in paraffin wax, sectioned at 5 μ m thickness and stained using Mallory staining. Testicular spermatogenesis was observed under light microscope.

Reversible studies

Reversible studies were conducted by discontinuing the treatment of methanol extract *A. paniculata* for the next 14 days. The rats were sacrificed and the same parameters were analysed following withdrawal of the 14 days' treatment.

Statistical Analysis

Minitab version 16 was used in this study for statistical analysis. The data presented as mean \pm standard error of means (SEM) and statistical significance was tested by oneway ANOVA test. The value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Sperm count, motility, viability and morphology

Data showed that there was statistically significant depression in the caudal epididymal sperm count; motility and viability after 14 days administration of methanol extract *A. paniculata* (Table 1). Sperm count in the treated group (K2) with the total mean of $(11.4 \pm 0.17) \times 10^6$, in comparison to control group (K1), $(17.9 \pm 0.16) \times 10^6$. Based on the result attained, the percentage of

viability in K2 (67.24 ± 11.20 %) is lower compared to K1 (94.3 ± 0.87 %). Meanwhile, the sperm motility grade was reduced from the progressive movement in K1 to non-progressive movement in K2. However, no statistically significant differences occurred among the control and treated group in the sperm morphology.

Testes histology

In the control group, normal features with successive stage of spermatogenesis and normal interstitial space were observed (Figure 1). The lumen of seminiferous tubule was filled with sperm. Testicular tissue sections in the treated group indicated that the methanol extract of *A. paniculata* caused changes where the effect caused the decreasing number of Sertoli cells, spermatogonia cells and Leydig cells. Furthermore, the increase of interstitial space in the matrix also appeared in the treated group (Figure 2).

Reversible studies

The treatment of the extract was discontinued after 14 days treatment and the same parameters; sperm count, motility, viability and morphology and testes histological studies were obtained. All the parameters were found to be recovered. Sperm count, motility and viability of the treated group were significantly increased in comparison to the control group while there was no significant difference in the sperm morphology of each group. The sperm count of K2 increased significantly with the mean of $(30.7 \pm 0.09) \times 10^6$ in comparison to K1, $(20.1 \pm 0.08) \times 10^6$. The sperm viability in K2 also improved significantly with the percentage of 95.96 ± 0.94 % in comparison to K1, 94.04 ± 0.96 % (Table 2). The sperm motility of K2 was in normal range with progressive movement congruent to K1. Histologically, the number of Sertoli cells regained, regeneration of Leydig cells, a complete development in spermatogonia cells were found in the treated group (Figure 3). In addition, the interstitial space in the matrix of the treated group was also found decreased.

Table 1. The effect of methanol extract of *A. paniculata* on sperm quality after 14 days of treatment. Values are mean \pm SEM

Group	Sperm Count ($\times 10^6$)	Sperm Motility	Sperm Viability %	Sperm Morphology % (Normal)
Control (K1)	17.87 ± 0.16	Progressive	94.30 ± 0.87	90.36 ± 3.62
Treated (K2)	$11.35 \pm 0.17^*$	Non-progressive	$67.24 \pm 11.20^*$	90.43 ± 2.03

*Significantly different compared to control ($p < 0.05$).

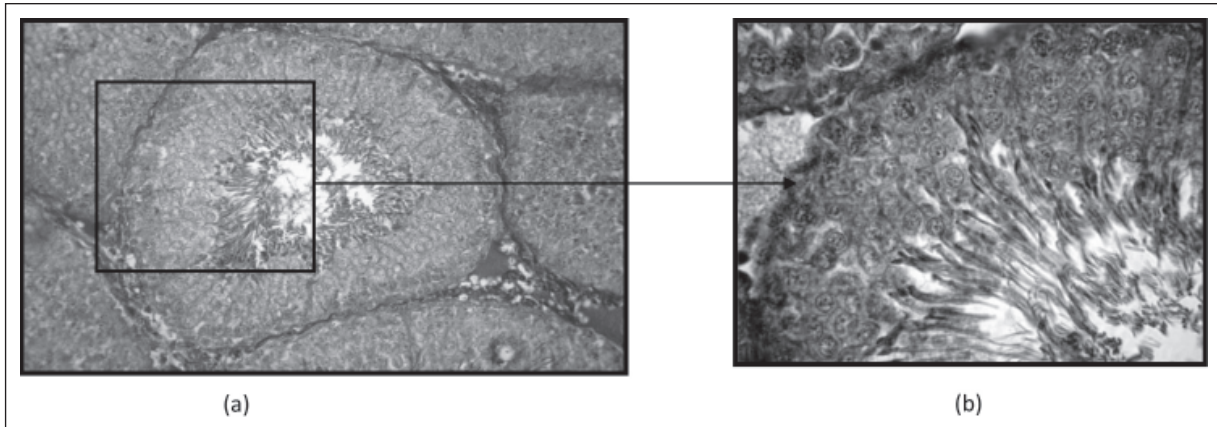


Fig. 1. Photomicrograph of rat testis cross-section in control group (K1) showing normal features with successive stage of spermatogenesis and normal interstitial space. Lumens of seminiferous tubule were filled with spermatozoa. Magnification (a) $\times 400$ (b) $\times 1000$.

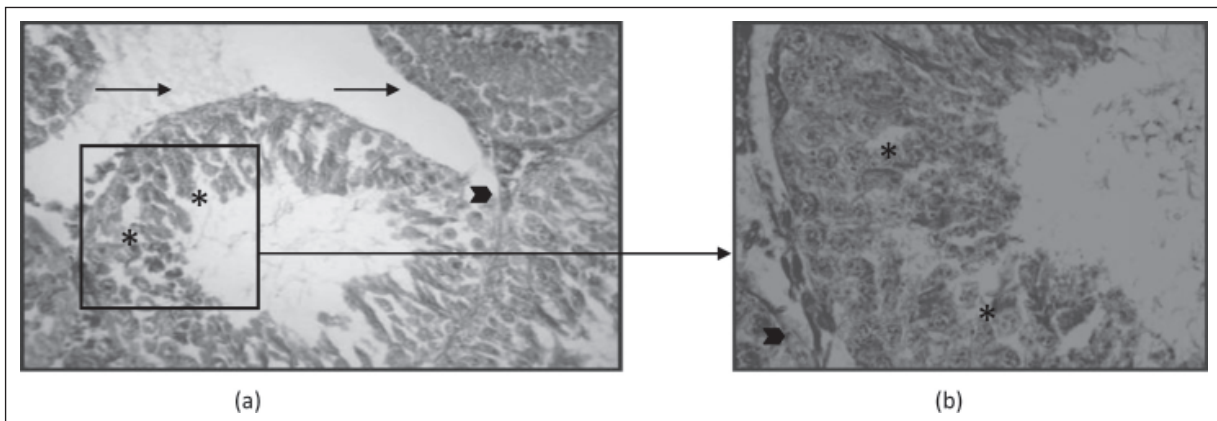


Fig. 2. Photomicrograph of rat testis cross-section in treated group (K2) after 14 days of treatment showing degeneration of spermatogenic elements with appearance of vacuole (asterix). Loosening interstitial space (arrows). Seminiferous tubule showing the shrinkage of Leydig cells (arrow head). Magnification (a) $\times 400$ (b) $\times 1000$.

Table 2. The effect of methanol extract *A. paniculata* on sperm quality after discontinuation of treatment for 14 days

Group	Sperm Count ($\times 10^6$)	Sperm Motility	Sperm Viability %	Sperm Morphology % (Normal)
Control (K1)	20.07 \pm 0.08	Progressive	94.04 \pm 0.96	85.64 \pm 6.41
Treated (K2)	30.69 \pm 0.09*	Progressive	95.96 \pm 0.94*	82.71 \pm 10.79

*Significantly different compared to control group ($p < 0.05$).

DISCUSSION

Daily oral administration of 800 mg/kg body weight methanol extract of *A. paniculata* for 14 days caused inhibitory effect towards sperm quality and spermatogenesis. A significant decrease in sperm count, motility and viability of the treated rats suggested that methanol extract *A. paniculata* has

a spermatotoxic effect. The *A. paniculata* and its bioactive components, andrographolide were reported to affect spermatogenesis by preventing cytokinesis of dividing sperm cell lines by the presence of abnormal Sertoli cells (Akbarsha & Manivanan, 1993). The previous study reported that andrographolide produced similar results of low sperm count and motility when administered orally

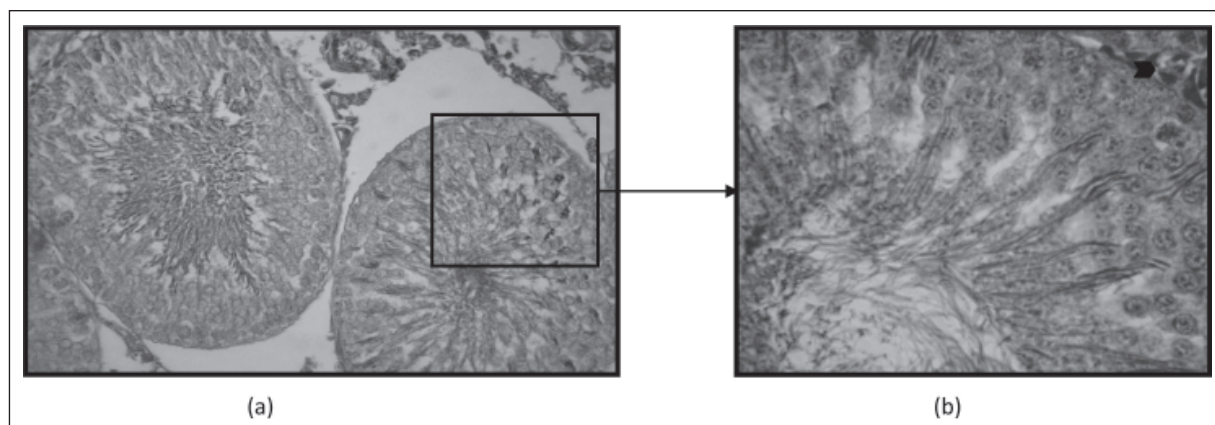


Fig. 3. Photomicrograph of rat testis cross-section in treated group (K2) after treatment been discontinued for 14 days showing the recovery effect of spermatogenic elements. Lumen of seminiferous tubule were filled with the sperm cells. (a) $\times 400$ (b) $\times 1000$.

to Wistar albino male rats (Akbarsha & Murugaian, 2000).

Testes histology of the treated group showed that the methanol extract *A. paniculata* caused changes in spermatogenesis. The affected tubules showed loosening of germinal epithelium and mixing of spermatids of different stages of spermatogenesis. The regression of Leydig cells also contribute to the inhibition of spermatogenesis (Figure 2). The reduction of testosterone hormone could lead to low sperm production in the testes. Leydig cells are important in producing testosterone. The regression of these cells will interrupt the secretion of testosterone and finally inhibit the development of the sperm. As reported in the previous study, Fatihah & Mahanem (2015), testosterone level decreased significantly in the methanol extract *A. paniculata* (800 mg/kg and 1600 mg/kg respectively for 14 days) treated rats compared to control group. The presence of abnormal Sertoli cells ceased the sperm development due to nutrient insufficiency. This was strengthened by Akbarsha *et al* (1990), who reported that *A. paniculata* stops spermatogenesis by inhibiting cytokinesis in spermatogonia cells and damaging the Sertoli cells. Janarthanan (1990) reported that andrographolide has antifertility effect by increasing the cholesterol, phosphatase acid and phosphatase alkaline activity.

In the case of reversible study, the rats from K2 showed that the sperm quality was restored after the treatment ceased. The total sperm count, motility and viability of the group increased significantly and in fact higher than control group particularly in sperm count. Based on the testes histology, it showed that discontinuing consumption of the methanol extract of *A. paniculata* regained the normal order of spermatogenesis process. Normal Leydig cells and Sertoli cells appeared in the

reversible group. It is believed that this resulted from the positive feedback in the rats' body system. Methanol extract of *A. paniculata* given for the first 14 days were metabolised and excreted within the second 14 days of this experiment, which leads to the normal development of sperm and Leydig cell. These results are congruent to a study reported by Akowuah *et al* (2008). The research showed that the andrographolide excreted through urine after 24 hours of oral administration was less than 2%. This evidence that the diterpenoid was absorbed and metabolized in the body. In the study by Akowuah *et al* (2009), it was reported that the andrographolide in plasma was reduced after three hours of consumption. It was also reported that after six hours of consumption, the level of andrographolide in the serum was reduced significantly in comparison to the first hour of administration (Fatihah, 2015; Fatihah & Mahanem, 2015). Hence, this supports the current study where the effect of andrographolide can be regulated and reversible.

In contrast to previous studies (Akbarsha & Manivanan, 1993; Akbarsha & Murugaian, 2000), Mkrtychyan *et al* (2005) reported that the consumption of Kan Jang mixture with *A. paniculata* (60 mL for 13 days) showed no significant negative effect on male semen quality and fertility. Another study also reported that the consumption of andrograpolid a major component in *A. paniculata* (50 mg/kg for 2, 4, 6, and 8 weeks) resulted in improved fertility and has the aphrodisiac-like effect that was comparable to sildenafil (Sattayasai *et al.*, 2010). Additionally, Dasuki *et al* (2015) reported that the consumption of ethanol extract of *A. paniculata* (0.5 mg/kg to 100 mg/kg for 70 days) showed no adverse effects on the reproductive performance and pregnancy outcomes.

Even though the mechanism in which *A. paniculata* affected male fertility are still debatable, some factors at least contribute to the result need to be considered such as type of solvent used in herbs extraction and type of extract. Different types of solvents used for extraction lead to different outcomes (Babu *et al.*, 2009). Solvent used in the herb extraction was based on the polarity (Barwick, 1997). According to Kumoro *et al* (2009), employing polar organic solvents such as methanol, ethanol and water which contain hydroxyl group obtained high extract yields of *A. paniculata* in comparison to the non-polar organic solvent. This is due to andrographolide has a strong polarity since the hydroxyl and the carbonyl groups are attached in its ring (Kumoro *et al.*, 2009). However, it is reported that adding water to methanol or ethanol would reduce the content of andrographolide in the extract and hydrolyse into deoxyandrographolide. Hence, this could be one of the many factors that contribute to the unclear result.

The isolation of a single bioactive component in the herb extract caused different effects compared to the crude form extracts. Ng (2006) reported that saponin in the crude is favoured in pharmacological activities such as antithrombotic, anti-atherosclerotic, fibrinolytic, antioxidant and cardioprotective effects while individual saponin such as propanaxatriol and propanaxadiol help to improve liver inflammation and the apoptosis in atherosclerotic animal, while gensenoside Re (G-re) contributes in the immunological adjuvant activity (Sun *et al.*, 2006).

Although there are many conflicting issues regarding the antifertility effect of *A. paniculata*, the results of the present study suggest that methanol extract of *A. paniculata* reduced sperm quality and spermatogenesis and discontinuing the consumption will reverse the antifertility effect. Overall, this result supports the potential of methanol extract *A. paniculata* in regulating male fertility and can be benefited as eco-friendly rodent pesticide.

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