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Toxicological Evaluation of *Aquilaria subintegra* Leaf Extract in Male ICR Mice

NUR HIDAYAT CHE MUSA, HANIZA HANIM MOHD ZAIN*, HUSNI IBRAHIM, NOR NASIBAH MOHD JAMIL AND M. T. A. MOHAMMED[1]

Department of Biology, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia
**(e-mail : haniza@fsmt.upsi.edu.my; Mobile : 0060 19512 7712)*

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ABSTRACT

The purpose of this study was to examine the toxic impact of aqueous extract of *Aquilaria subintegra* leaf (AEAS) on body weight (b. w.), relative organ weight (ROW), fatality rate, and sperm variables in male ICR mice. In acute toxicity study, the treated animals received a single dose of AEAS at 2000 mg/kg b. w., while in sub-acute toxicity study; the treated groups received 50, 100, 150, 200, 500 and 1000 mg/ kg b.w. of AEAS orally for 21 days consecutively. The control group received normal saline only. All experimental animals were survived until the end of the experiment except in the treated group that received AEAS 1000 (20%). Mild sedative effects manifested as sleepiness in treated groups receiving higher doses of AEAS during the first two to three hours after administration. The number of abnormal sperm significantly decreased in AEAS 2000 (acute) and AEAS 100, 150 and 1000 (sub-acute) compared to control. However, sperm motility significantly increased in almost all AEAS treated groups. Thus, AEAS was non-toxic to the treated mice and had therapeutic potential to improve sperm quality and enhanced breeding rate, specifically in mice.

Key words : *Aquilaria subintegra* leaf, aqueous extract, toxicity studies

INTRODUCTION

In new drug discoveries, specifically from the plant, the data obtained from toxicity studies are required to enhance the confidence in its safety for the future's initial human clinical trials (Porwal *et al.*, 2017). The utilization of medicinal plants has gained public attention as an alternative therapy to replace conventional medicine in treating various diseases because it is ineffective, abusive, and has side effects on its consumers (Kiranmai and Kiran, 2014).

In recent years, the increasing usage of plant-based drugs as the alternative way to treat various diseases demands toxicity evaluation because not all medicinal plants are safe for human consumption without undergoing any toxicological investigations (Zahi *et al.*, 2015). Scientists are currently needed to undertake toxicological studies on plant-based medications due to the increased conviction in the safety of plant-based medications for human usage. Therefore, toxicological investigations should be directed to determine the security and viability of plant medication that fits well for human utilization.

Another potential medicinal plant that has yet to be studied for its toxicity is *Aquilaria subintegra*, which belongs to the Thymelaeaceae family. *A. subintegra* is an endemic agar-wood plant to Southern Thailand and is only found in the Narathiwat Province area (Harvey-Brown, 2018). Leaf part of agar-wood plant is generally utilized in traditional medication in numerous nations to encourage better health and treat various ailments. Lately, agar-wood leaves have been commercially marketed as 'gaharu' tea (Yong, 2017).

The phytochemical constituents of *Aquilaria* leaf extract already discovered are terpenoids (Rahman *et al.*, 2016; Batubara *et al.*, 2019), saponins (Batubara *et al.*, 2019; Hashim *et al.*, 2019), glycosides (Rahman *et al.*, 2016), tannins (Rahman *et al.*, 2016; Batubara *et al.*, 2019), phenol (Hashim *et al.*, 2019), flavonoid (Duan *et al.*, 2015; Hashim *et al.*, 2019),

[1]Environment Research Center, University of Technology-Iraq, Iraq.

alkaloid (Hashim *et al.*, 2019) and steroid (Kang *et al.*, 2014; Hashim *et al.*, 2019). Various scientific research have been done on the impact of agar-wood leaf extracts on hepatoprotective (Alam *et al.*, 2016), anti-cancer (Fatmawati and Hidayat, 2016), Alzheimer's disease (Bahrani *et al.*, 2014), therapeutic laxative agent (Kakino and Hara, 2016), anti-diabetic activities (Said *et al.*, 2016), anti-arthritic activity (Rahman *et al.*, 2016), anti-lipase activity (Ibrahim *et al.*, 2018) as well as can increase the breeding rate in mice (Che Musa *et al.*, 2019b).

So far, no study was carried out to examine the toxicity effect of *A. subintegra* leaves aqueous crude extract (AEAS). Thus, the current study is required to provide empirical evidence for its alleged folklore use, as there are no scientific data on the systemic toxic effects of single and repeated doses of AEAS in the literature. Several parameters, including mice body weight, fatality rate, relative organ weight and sperm variables were assessed.

MATERIALS AND METHODS

All methodologies used in this investigation were carried out in accordance with appropriate research ethics, as endorsed by the research committee of Universiti Pendidikan Sultan Idris.

A. subintegra fresh leaf was acquired from agar-wood grower in Tanjong Malim, Perak, Malaysia. The species has been recognized and stored at Universiti Pendidikan Sultan Idris' Herbarium with the voucher number : NHCM004. Leaf samples were washed thoroughly, air-dried and crushed into a coarse powder using an electrical grinder. 800 g of the coarse powder sample was immersed in 8 l of distilled water for 24 h at room temperature, with periodical stirring. Then, the filtrate obtained was oven-dried for 48 h at 55°C before being freeze-dried for 72 h. The brown crude sample obtained was kept in the freezer at -20°C prior to further use.

The experiment was conducted on 12 to 14 week old healthy male ICR mice weighing 34±6 g. For acute and sub-acute toxicity tests, mice were randomly split into two groups and seven groups, each with five animals. The animals were housed in polypropylene cages under standard animal housing conditions. Food and drink, on an *ad libitum* basis, were given throughout the experimental period. Five days before the experiment started, the animals were allowed to acclimate (Ghan *et al.*, 2016). The plant extract was administered via oral gavage technique in both the toxicity tests. About 12 h (overnight), no food was given to the male mice prior to the extract administration.

The total of 10 healthy male ICR mice were split into two groups (n = 5) which were treated group and control group. The treated group received 2000 mg/kg body weight (Veeresh *et al.*, 2017) of AEAS, and the control group was fed the equivalent volume of normal saline (NaCl). After administering AEAS and NaCl, the mice's behaviour was constantly watched using a CCTV video camera for the first 4 h and then once daily for the next 14 days. The experimental animals' body weight was measured on day 0, 7 and 14. Besides, abnormal behaviour and fatality were observed among experimental animals (Dahham *et al.*, 2016). On day 15, animals were anesthetized with anesthetic ether and sacrificed in the morning.

Oral repetitive dose was done based on the Organization of Economic Co-operation and Development (OECD) Guideline No. 407. In this experiment, 35 mature male mice were grouped into seven. The administration volume of plant extract (AEAS) and NaCl was 10 ml/kg b. w. of the experimental animal (Ghan *et al.*, 2016). The extract dose was calculated according to the formula provided by Erhirhie *et al.* (2014). The treated mice received 50, 100, 150, 200, 500 and 1000 mg/kg b. w. of AEAS orally for 21 days consecutively, while the control group received normal saline. The animals were observed daily for any irregular signs, including urination, an anomaly in eyes, skin, and fur, breathing difficulty, mucus secretion, diarrhea, micturition and drowsy symptoms. The number of fatalities was also recorded (Zahi *et al.*, 2015). The mice's body weight was determined on days 0, 7, 14 and 21.

The animals were sacrificed in the morning of day 15 and day 22 for acute toxicity and sub-acute toxicity study, respectively. Liver, kidney, and both side of testis and epididymis were gently excised and weighed separately. Each animal's relative organ weight was then calculated using the previous studies' formula (Zahi *et al.*, 2015).

After being sacrificed, all experimental animals were subjected to sperm analysis, including sperm motility, morphology and count. Each animal's epididymis was dissected and put in a petri dish with 5 ml of pre-heated (37°C) normal saline solution. The epididymis was sliced and immersed in 5 ml of pre-heated NaCl (0.9%). The suspension of sperm obtained previously was added with 1% eosin Y in a 1 : 1 ratio. Approximately 10 µl of the stained sperm suspension was pipetted into the chamber and left to settle for 5 min before counting sperm. Sperm motility evaluation was done under an inverted microscope at 20x magnification as quickly as possible after a mouse was dissected. The sperm suspension was examined within 5 min after their isolation from the epididymis. 10 µl of the sperm suspension prepared previously was pipetted into the counting chamber and classified as either motile or non-motile sperm. The total number of sperm was tallied after the non-motile sperm was counted. Sperm motility was measured as the proportion of motile sperm to the actual sum of sperm counted. The data obtained were expressed in percentage.

The sperm suspension staining procedure was done using Eosin Y (1%) at 1 to 5 ratio (1 : 5). 10 µl stained was added in 50 µl of sperm suspension. Then, 10 µl of stained sperm sample was pipetted into the chamber and allowed to air dry before being examined under the microscope. The criteria for sperm abnormalities were determined based on the findings of earlier studies (Takeda *et al.*, 2016). Abnormalities recorded were no hook, pinhead, bent head, coiled tail, bent tail and hairpin loop. An inverted microscope with a magnification of 40x was used to examine 200 randomly selected sperm from each mouse. The data obtained were measured in the percentage of the abnormal characteristic.

The data were presented as mean±SEm. Analysis data were done using one-way ANOVA supplemented by Tukey's test for multiple parametric comparison. The data obtained were considered significantly different at $P<0.05$ when the treatment groups were compared with the control group.

RESULTS AND DISCUSSION

The use of plant medicine as an alternative approach to treating various diseases has been growing recently, but their side effects and toxicity are yet unknown. Toxicology studies must be conducted to guarantee the security and efficacy of medicinal plants for human usage. Acute and sub-acute toxicity were both assessed using AEAS in this study. Based on acute toxicity study**,** there was no abnormal signs and fatality recorded in AEAS 2000. The plant extract dose level was detrimental if it caused the treated animals to lose 10% or more of their original body weight on the first day of the experiment (Wang *et al.*, 2019). Therefore, the results showed that the mice given AEAS 2000 were in good health because their body weight dropped by only 5.09%, which was less than 10% of their original body weight. Besides, no significant changes were recorded in the relative organ weight of the treated mice

Table 1. Relative organ weights of mice treated with AEAS 2000 for 14 days

Experimental groups	Relative organ weight (Mean±SEm)			
	Testis	Epididymis	Kidney	Liver
Control	0.60±0.03	0.36±0.04	1.49±0.13	4.90±0.53
AEAS 2000	0.55±0.03	0.37±0.01	1.29±0.12	4.61±0.49

Five mice in each group. *Significantly different at $P<0.05$.

Table 2. Effect of acute toxicity study on sperm variables in mice

Experimental groups	Sperm variables (Mean±SEm)		
	Sperm abnormality (%)	Sperm count (10^6/ml)	Sperm motility (%)
Control	43.78±0.57	1.01±0.15	49.22±1.18
AEAS 2000	33.84±1.32*	1.05±0.10	63.23±1.95*

Five mice in each group. *Significantly different at $P<0.05$.

organs (testis, epididymis, kidney and live) with AEAS 2000 compared to control (Table 1). However, a single dose of AEAS 2000 caused significant differences ($P<0.05$) in sperm morphology (abnormality) and motility ($P<0.05$) as compared to the control group (Table 2).

Based on the sub-acute toxicity results, it was astonishing to notice that the mice receiving higher doses of AEAS became inactive and spent their time more on sleeping. The sedative effect produced by agar-wood was mentioned by earlier researchers worldwide (Bahrani *et al.*, 2014; Che Musa *et al.*, 2019b). A similar finding was obtained by Che Musa *et al.* (2019a) in toxicological evaluation of *Aquilaria malacensis* leaves aqueous extract. They concluded that agar-wood leaves aqueous extract was safe to be used in the *in vivo* study.

Fig. 1 illustrates the experimented animal's body weight changes in grams that had received AEAS and NaCl (control) for 21 consecutive days. Day 0 was considered as a starting point for body weight changes in experimented animals. The body weight increment in all experimental groups was recorded on day 14 and 21. There was a slight decrease and increase in treated groups' body weight on day 7 and day 14. However, a gradual increase in body weight was noticeable on the final day of the experimental period.

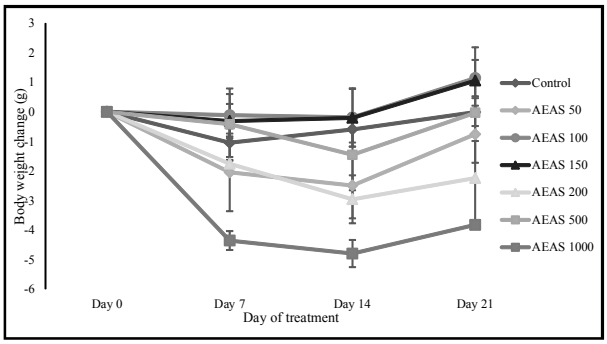

Fig. 1. The changes of experimental animal's body weight.

The finding was most likely attributable to the stress condition created by the force-feed technique of NaCl and AEAS that were done daily for 21 days. Mice were susceptible to stress and difficult to cope and adapt to unfamiliar routines (Beery and Kaufer, 2015). This study was also supported by previous research on stress-induced experiment towards Balb/c mice (Xu *et al.*, 2018). They found out that the unfamiliar daily routine of mice, such as the stress-exerted treatment on them, caused a reduction in their body weight.

There was no significant difference in the relative organ weight of testis, epididymis, kidney and liver in AEAS compared to the control group (Table 3). The organs weights of liver and kidney were relatively sensitive signs in toxicity studies (Sanyal *et al.*, 2016). Based on the toxicity finding, there was no detrimental effect on internal organs studied, including liver, kidney, testis and epididymis in the AEAS treated groups compared to the control group. Thus, all the treated mice's organs were probably healthy because the treated mice showed no detectable side effects throughout the experimental period.

The administration of AEAS for 21 days caused a decrease in sperm abnormality percentage of treated groups as compared to the control (Table 4). No hook, bent head, pinhead, coiled tail, bent tail, and hairpin loop were among the six forms of sperm abnormalities found in all experimental groups, as shown in Fig. 2. Similar sperm abnormalities were discovered in a previous study while working with *A. malaccensis* leaves aqueous extract (Che Musa *et al.*, 2019a). The percentage mean of abnormal sperm for AEAS 100, 150 and 1000

Table 3. Relative organ weights of mice that treated with AEAS for 21 days

Experimental groups	Relative organ weight (Mean±SEm)			
	Testis	Epididymis	Kidney	Liver
Control	0.65±0.06	0.41±0.06	1.37±0.13	3.88±0.42
AEAS 50	0.65±0.05	0.37±0.02	1.32±0.11	4.15±0.35
AEAS 100	0.59±0.04	0.36±0.06	1.30±0.09	3.99±0.30
AEAS 150	0.63±0.05	0.37±0.06	1.23±0.17	3.84±0.28
AEAS 200	0.66±0.05	0.37±0.05	1.24±0.06	4.02±0.32
AEAS 500	0.63±0.05	0.34±0.06	1.17±0.10	3.62±0.17
AEAS 1000	0.68±0.11	0.39±0.06	1.25±0.14	4.02±0.26

Five mice in each group. *Significantly different at $P<0.05$.

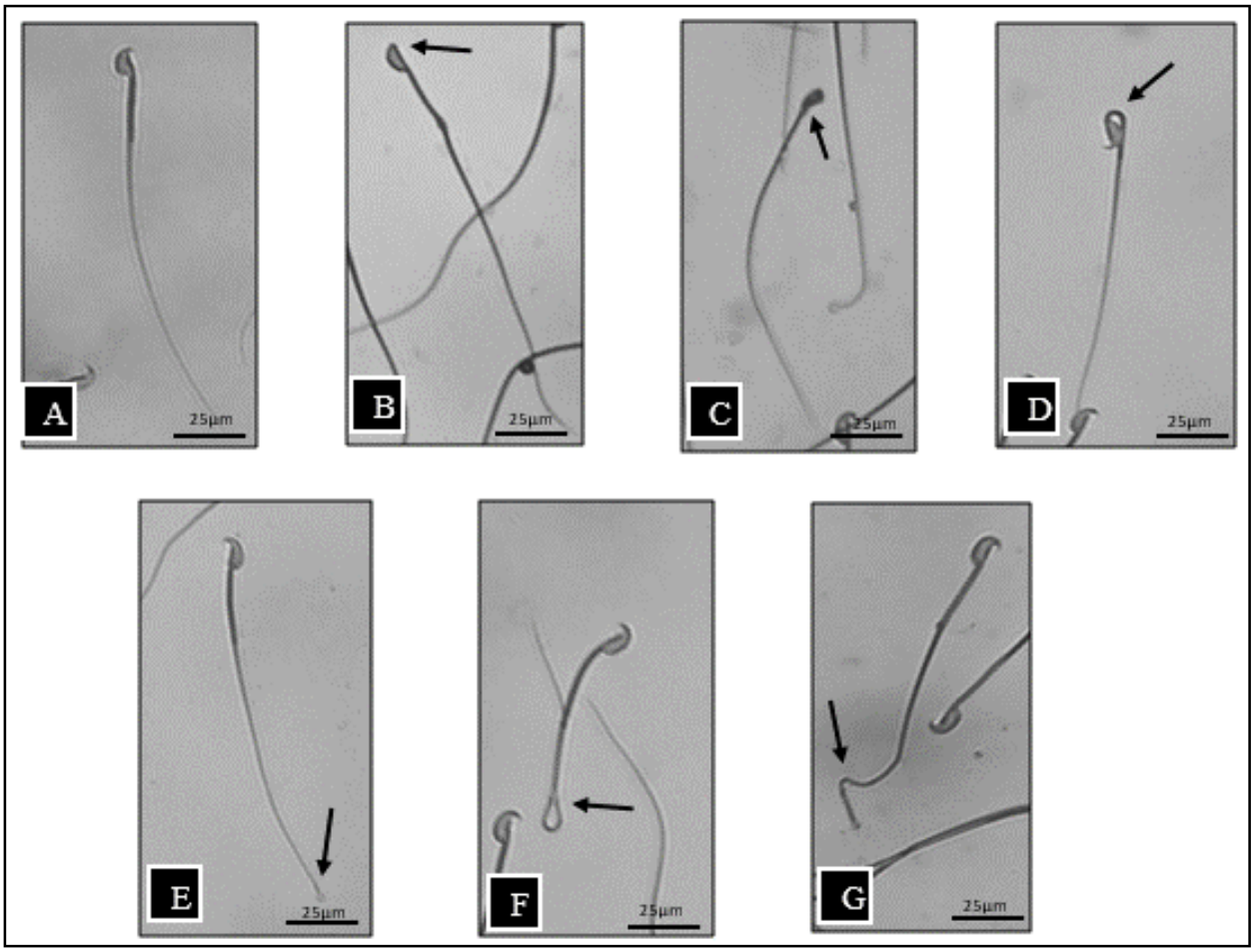

Fig. 2. Seven sperm morphological criteria of male ICR mice observed using an inverted microscope at 40x magnification, (A) Normal sperm, (B) No hook, (C) Pinhead, (D) Bent head, (E) Coiled tail, (F) Hairpin loop and (G) Bent tail.

Table 4. Effect of subacute toxicity study on sperm variables of mice that were treated with AEAS for 21 days

Experimental groups	Sperm variables (Mean±SEm)		
	Sperm abnormality (%)	Sperm count (10^6/ml)	Sperm motility (%)
Control	37.08±1.51	0.93±0.15	45.26±1.35
AEAS 50	34.73±0.67	1.24±0.24	62.93±1.21*
AEAS 100	27.05±0.83*	1.60±0.07	69.30±1.25*
AEAS 150	27.05±0.73*	1.63±0.27	69.55±2.08*
AEAS 200	34.43±0.30	1.72±0.06	70.41±0.82*
AEAS 500	36.33±0.43	1.70±0.23	70.22±1.49*
AEAS 1000	27.29±0.75*	0.92±0.07	68.05±0.29*

Five mice in each group. *Significantly different at P<0.05.

were significantly lower (P<0.05) than the control group (Table 4). This was due to the presence of numerous phytochemical constituents, particularly flavonoids, which aided in sperm production by promoting the growth and development of mature sperm cells (Shahraki *et al.*, 2015).

The presence of antioxidants for this plant leaf was not investigated in this study, but it was confirmed from previous studies (Begum, 2016) that agar-wood leaf extraction had naturally occurring antioxidant properties. It was also possible that the agar-wood leaf extract used in this study reduced abnormalities in sperm

cell morphology via the same mechanism. Moreover, AEAS 50, 100, 150, 200, 500 and 1000 showed a significant difference in sperm motility percentage compared to the control group (Table 4). These findings suggested that AEAS with antioxidant properties aided in the prevention of sperm cellular damage induced by reactive oxygen species (ROS), resulting in enhanced sperm motility and decreased sperm abnormalities in AEAS-treated animals.

CONCLUSION

This study indicated that AEAS was relatively safe for consumption with a mild sedative effect. The findings also proposed that AEAS improved sperm quality in mice and could be used as an alternative treatment in the future, particularly to cure the infertility problem. Further investigation on the usage of *A. subintegra* leaves aqueous extract is required to provide more scientific information specifically for its therapeutic purposes.

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