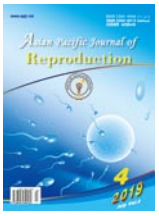

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Effect of lyophilized aqueous leaf extract of *Aquilaria subintegra* on aphrodisiac properties in mice

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ABSTRACT

Objective: To investigate the effects of *Aquilaria subintegra* leaf aqueous extracts on the aphrodisiac properties including sexual behaviour, testosterone level, percentage of pregnancy, number of offspring and male to female ratio of offspring in ICR mice.

Methods: In this experiment, each male cohabitated with one female in a polysulfone cage. 30 ICR male mice were divided into 6 groups that received normal saline (the control group), 50 mg/kg, 100 mg/kg, 200 mg/kg, 500 mg/kg, and 1 000 mg/kg body weight of *Aquilaria subintegra* leaf aqueous extracts orally for 21 days consecutively. Sexual behavior, percentage of pregnancy, number of offspring and male to female ratio of offspring in ICR mice were measured according to the established methods. Testosterone level was measured by using enzyme-linked immunosorbent assay.

Results: Mice that received *Aquilaria subintegra* leaf aqueous extracts at 50 mg/kg body weight (day 0) had significantly higher mount frequency as compared to the control group; groups treated with 100, 500, 1 000 mg/kg body weight extracts produced a greater number of offsprings when compared to the control group. All aphrodisiac parameters were similar between the treatment groups and the control group, indicating that *Aquilaria subintegra* leaf aqueous extract did not significantly alter the aphrodisiac parameters.

Conclusions: *Aquilaria subintegra* leaf aqueous extracts have no effect on the aphrodisiac properties, but could increase the breeding rate in mice.

1. Introduction

Malaysia has become the reservoir for numerous medicinal plants[1] where approximately 8 300 species are found in Peninsular Malaysia and 12 000 species are recorded in Sabah and Sarawak area[2]. Basically, the effectiveness and popularity of medicinal plants depend on the user's experiences and Malaysian folks' belief[3]. Interest in treating male infertility by using medicinal plants is rising in both developing and developed countries[4]. The World Health Organization defined infertility as a state where couples failed to get pregnant after one year of regular unprotected intercourse[5].

Researchers are now attempting to formulate drugs from plant origin to overcome infertility issues. Plant medicine or also known as phytotherapy with aphrodisiac properties provides a safer

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way to counteract with various problems associated with male infertility[6]. The phytotherapy has become more popular among the community because it is natural, cheap, has fewer side effects, easily accessible and has good therapeutic outcome[7]. There are various species of medicinal plants such as *Eurycoma longifolia*[8], *Lunasia amara*[9] and *Hibiscus sabdariffa*[10] that have been investigated by local researchers due to their capability to enhance sex drive and likewise, they can overcome infertility problem. Another potential plant for aphrodisiac and fertility purpose that is still not frequently investigated belongs to the genus of *Aquilaria* which is *Aquilaria (A.) subintegra*. This species is also known as agarwood and it belongs to the family of Thymelaeaceae.

Agarwood leaves are frequently used in folk medicine in many countries for the promotion of good health and treatment of many ailments. Many previous studies have proved scientifically the therapeutic purposes of agarwood leaves which are potentially strong to treat Alzheimer's disease[11], good as laxative agent[12], antipyretic and anti-inflammatory agents[13], antioxidant agent[14] and also anti-lipase activity[15].

The potential of agarwood leaves to treat various diseases is due to the presence of phytochemical constituents therein. The phytochemical constituents found by previous researchers in *Aquilaria* leaf extract are flavonoid glycosides[16], 2-(2- phenylethyl) chromenes[16], genkwanin 5-O- β -primeveroside and mangiferin[12], iriflophenone 2-O- α -rhamnoside and iriflophenone 3,5-C- β -diglucoside[12] and also diterpenoids[17]. Besides, the chemical constituents that consist of alkaloids, tannins, saponins, flavonoids, and terpenoids are also present in the *Aquilaria* leaf extract[18]. Traditionally, the use of agarwood as an aphrodisiac agent already claimed by several prior researchers[19]. There are some phytochemicals that possess to aphrodisiac properties which are saponins[20], alkaloids[21], as well as flavonoids[22], since these phytochemicals have androgen enhancing and antioxidant properties.

Thus, the presence of numerous phytochemical constituents by previous researchers specifically saponins, alkaloids and flavonoids, as well as the subjective opinion given by local communities, further becomes the basis of the aphrodisiac investigation. Therefore, this study aimed to investigate the effects of the aqueous extract of a species of agarwood - *A. subintegra* on sexual behaviour, testosterone level, percentage of pregnancy, number of offspring and male to female ratio of offspring in Institute of Cancer Research (ICR) mice.

2. Materials and methods

2.1. Plant materials

A. subintegra with herbarium number NHCM004 was collected from one of the agarwood plantations in Tanjong Malim, Perak, Malaysia in November 2015. Species were identified by Associate Prof. Dr. Fatimah Mohamed from Biology Department, Universiti Pendidikan Sultan Idris and deposited at the Herbarium of Universiti Pendidikan Sultan Idris.

2.2. Plant sample extraction

Two kilograms fresh leaves of *A. subintegra* were collected from the agarwood plantation in Tanjong Malim, Perak, Malaysia. The leaves were washed, air dried, and ground using an electrical grinder to form a fine powder. 800 g of the powder was soaked in 8 L of distilled water for 24 h at room temperature with occasional stirring. The filtrate obtained was oven dried at 55 ℃ for 48 h followed by freeze-drying for 72 h[14]. The brown crude extract obtained was stored at -20 ℃ prior to further use.

2.3. Experimental design

The experiment was performed on healthy male ICR mice of twelve to fourteen weeks old and body weight of (34 ± 6) g. Repeated oral dose administration was carried out in this experiment. In this study, 30 ICR male mice were divided into 6 groups, with 5 mice in each group. The first group was the control group, which received 10 mL/kg body weight of normal saline orally. The other five groups received a suspension of five different doses of *A. subintegra* leaf aqueous extracts at the doses of 50, 100, 200, 500 and 1 000 mg/kg body weight respectively, and they were treated with *A. subintegra* crude extract using plastic syringes attached to ball-tipped stainless steel feeding needle daily for 21 days consecutively. The 21 days of experimental period was chosen due to the gestation period of mice that takes about 21-23 days. This condition was therefore essential to count offspring birth at the end of the experiment. All oral administrations were administered daily at the same point of time between 08:00 am and 09:00 am. The administration volume was 10 mL/kg body weight of the animal[23]. The amount of the crude extract was calculated based on the body weight of the animal and dissolved in distilled water before administered directly to the mice. This calculation based on the formula provided by a previous study[24] as below:

$$\text{Dosage (mg)} = \text{Body weight of animal (g)} \div 1\,000\ \text{(g)} \times \text{dose of extract (mg)}$$

Before the administration of *A. subintegra* crude extract, the animals were fasted overnight. During the period of the experiment, food and water were given *ad libitum*.

2.4. Parameters in assessing plant with aphrodisiac potential

2.4.1. Sexual behaviour test

The sexual behaviour observation was done on day 0, 7, 14 and 21. Day 0 was considered as a starting point of the experiment because all treated mice received their first six different doses at Day 0. Immediately after administration of the extract, the male mice were individually placed in their cages. 15 min later, a non-estrous female

was introduced into the cage. The observation was done on 0, 7, 14 and 21 days after being orally treated with the agarwood aqueous extract. The experiment was conducted at 09:00 a.m until 2:00 p.m in the same animal laboratory and under the light of the same intensity. The non-estrous female mice were introduced into the cages of male animals with 1 male to 1 female ratio[25]. The occurrence of events and phases of mating were recorded using closed-circuit television video camera for about 2 h[26]. The behavioral observations were carried out by taking into account the following parameters described by a previous study[27]. Mounting behaviour was determined and characterized by the parameters: A) Mount frequency – The average number of the mount by a male mouse without intromission during 120 min observation; B) Mount latency – The lag time in minutes from the introduction of female in the cage to the first mount. And intromission behaviour was evaluated according to these parameters: A) Intromission frequency – The average number of intromission during 120 min observation: B) Intromission latency – The time in minutes for the first intromission after the introduction of female in the cage.

2.4.2. Testosterone estimation

After 21 days of treatment with *A. subintegra* leaf aqueous extracts, blood samples about 0.8-1.0 mL were drawn from the mice' hearts by cardiac puncture method. The blood samples were collected in test tubes while the mice were put under mild ether anesthesia in the morning of day 22. And the samples were kept at room temperature for 1 h. After coagulation, the tubes were placed in the centrifuge at 3 000 rpm for 15 min to collect the plasma prior to testosterone determination[28]. Sera was pipetted by micro pipettes and transferred into new label tubes, sealed with parafilm and stored to freeze at -20 ℃ before being used in the measurement of testosterone using enzyme-linked immuno sorbent assay. Serum testosterone concentration of the experimental animals was assayed by using the procedure outlined in the manufacturer's instruction manual (Testosterone ELISA Kit ab108666).

2.4.3. Percentage of pregnancy, number, and male to female ratio of offspring

Each male cohabitated with one female in a cage and used for sexual behaviour and orientation activity parameters in this fertility test. The females were observed for the pregnancy rate and the number of offspring were recorded[29]. The sex's detection was done at four to five weeks after the birth of the offspring. Previous studies had already claimed that the birth of offspring which had a favour to male offspring was closely related to the enhancement of sexual behaviour of the treated animal in aphrodisiac properties investigation[30,31]. It was important to measure male to female ratio of offspring in this study.

2.5. Statistical analysis

Data analysis was carried out by using IBM-SPSS statistics software program (Version 20.0). The results were expressed as mean ± standard deviation (mean ± SD). Statistical analyses were performed using one-way analysis of variance and followed by Tukey's test for parametric multiple comparisons between the control and the treatment groups.

2.6. Ethics

This study was done under proper research ethics and care that was approved by Universiti Pendidikan Sultan Idris Research Committee (approval No. P20142002610).

3. Results

3.1. Sexual behaviour

The effects of the various dose of *A. subintegra* leaf aqueous extracts on sexual behaviour were presented in Table 1. Among all the physical indices of sexual behaviour monitored in male mice, only *A. subintegra* leaf aqueous extracts at 50 mg/kg body weight resulted in significantly higher (P<0.05) mount frequency on day 0 as compared to the control group. Conversely, the other treatment groups showed significantly lower (P<0.05) mount frequency on day 0, day 7, day 14 and day 21 except *A. subintegra* leaf aqueous extracts at 100 (day 14) and 200 (day 0; day14) mg/kg body weight as compared to the control group. However, intromission frequency, intromission latency and mount latency for the treatment groups remained similar to the control group.

3.2. Testosterone level estimation, number, and male to female ratio of offspring and percentage of pregnancy

Table 2 showed the effects of *A. subintegra* leaf aqueous extracts on the number of offspring, male to female ratio of offspring, percentage of pregnancy and testosterone level. There was significantly higher (P<0.05) number of offspring in *A. subintegra* leaf aqueous extracts at 100, 500 and 1 000 mg/kg body weight as compared to the control group. The percentage of pregnancy for the treatment groups remained similar to the control group with 100%. But, the *A. subintegra* leaf aqueous extract at all doses showed no significant changes in testosterone concentration and in male to the female ratio when compared with the control group.

Table 1. Effect of aqueous leaf extracts of *Aquilaria subintegra* (AEAS) on sexual behaviour parameters in male ICR mice.

Groups	Mount frequency	Mount latency (min)	Intromission frequency	Intromission latency (min)
Control				
Day 0	21.20 ± 1.17	15.78 ± 1.72	1.20 ± 1.68	13.50 ± 1.60
Day 7	17.60 ± 1.14	2.43 ± 1.44	0.00 ± 0.00	6.05 ± 0.00
Day 14	17.20 ± 1.70	3.23 ± 1.80	0.00 ± 0.00	0.00 ± 0.00
Day 21	22.40 ± 1.91	1.18 ± 1.59	0.00 ± 0.00	0.00 ± 0.00
AEAS 50				
Day 0	39.50 ± 1.70*	6.16 ± 1.10	2.25 ± 1.50	42.35 ± 0.00
Day 7	6.00 ± 1.83*	2.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 14	4.00 ± 1.83*	7.98 ± 0.72	0.00 ± 0.00	0.00 ± 0.00
Day 21	11.25 ± 1.63*	1.09 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
AEAS 100				
Day 0	10.00 ± 1.83*	7.31 ± 1.81	0.60 ± 1.34	43.52 ± 0.00
Day 7	6.25 ± 1.06*	2.80 ± 1.22	0.00 ± 0.00	0.00 ± 0.00
Day 14	10.25 ± 1.40	2.38 ± 1.78	0.00 ± 0.00	0.00 ± 0.00
Day 21	13.00 ± 1.83*	10.05 ± 1.36	0.00 ± 0.00	0.00 ± 0.00
AEAS 200				
Day 0	28.20 ± 1.70	8.77 ± 1.01	0.20 ± 0.45	119.36 ± 0.00
Day 7	5.80 ± 1.79*	33.19 ± 1.40	0.00 ± 0.00	0.00 ± 0.00
Day 14	14.80 ± 1.49	8.77 ± 1.66	0.00 ± 0.00	0.00 ± 0.00
Day 21	5.80 ± 1.35*	10.64 ± 1.52	0.00 ± 0.00	0.00 ± 0.00
AEAS 500				
Day 0	10.20 ± 1.32*	22.69 ± 1.36	2.60 ± 1.81	3.50 ± 0.00
Day 7	5.20 ± 1.39*	22.13 ± 1.99	3.00 ± 1.12	14.41 ± 0.00
Day 14	8.80 ± 1.59*	4.23 ± 1.73	0.00 ± 0.00	0.00 ± 0.00
Day 21	6.20 ± 1.56*	2.24 ± 1.16	0.00 ± 0.00	0.00 ± 0.00
AEAS 1 000				
Day 0	8.75 ± 1.22*	45.84 ± 1.45	0.00 ± 0.00	0.00 ± 0.00
Day 7	0.00 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 14	0.00 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day 21	7.50 ± 1.70*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as mean±SD; *: compared with the control group at $P<0.05$ on the same day of the observation period; $n=5$ in each group. The control group received 10 mL/kg body weight of normal saline orally. The other five groups (AEAS 50, AEAS 100, AEAS 200, AEAS 500 and AEAS 1 000 groups) received *Aquilaria subintegra* leaf aqueous extracts at the doses of 50, 100, 200, 500 and 1 000 mg/kg body weight, respectively. The data of mount latency (2.07±0.00; 1.09±0.00) and intromission latency (6.05±0.00; 42.35±0.00; 43.52±0.00; 119.36±0.00; 3.50±0.00; 14.41±0.00) obtained are due to that the only one treated mouse performed these sexual behaviour parameters during the observation period. Besides, the other mice in the same group became inactive and sleepy throughout 2 h of observation.

Table 2. Effects of aqueous leaf extracts of *Aquilaria subintegra* (AEAS) on the number of offspring, male to female ratio of offspring, percentage of pregnancy and testosterone level in mice.

Groups	Control	AEAS 50	AEAS 100	AEAS 200	AEAS 500	AEAS 1 000
Number of offspring	9.20 ± 1.17	9.80 ± 1.27	14.00 ± 0.71*	12.20 ± 1.92	13.60 ± 1.70*	13.60 ± 1.14*
Male/female ratio of offspring	0.76 ± 0.18	0.70 ± 0.17	0.76 ± 0.13	0.80 ± 0.14	0.72 ± 0.20	0.85 ± 0.10
Percentage of pregnancy (%)	100	100	100	100	100	100
Testosterone level (ng/mL)	2.98 ± 1.25	1.58 ± 0.24	4.97 ± 1.90	1.93 ± 0.33	1.66 ± 0.24	3.20 ± 1.80

Data are expressed as mean±SD; *: compared with the control group at $P<0.05$; $n=5$ in each group.

4. Discussion

In the present study, the sexual behaviour parameters observed were mount frequency, intromission frequency, mount latency and intromission latency. Despite the fact that ejaculation frequency was not performed in this study, both mount and intromission frequencies were already sufficient, which are valuable indices of vigour, libido, and potency[32]. Besides, it was exceptionally hard even for a skilled observer to distinguish between intromission and ejaculation in observation of sexual behaviour[33].

The plant-derived drugs are of more importance and they give large contributions to human health, especially for the reproductive purpose[34]. Generally, mount and intromission latencies were used as the indicators of sexual motivation[35]. However, mount latency and intromission latency are inversely proportional to sexual motivation. In this way, the decrease in the mount and intromission latencies was the positive result for the extract which might imply stimulation of sexual motivation and arousability. It may also be the sign of boosted sexual appetitive behaviour in experimental animals which further supports the sexual improvement effect of the plant extract[35].

Contrary results were obtained from the present study because *A. subintegra* leaf aqueous extracts were lacking in aphrodisiac properties. Furthermore, the administration of these extracts in higher doses caused a sedative effect that eventually leads to the reduction in sexual behaviour and orientation activity towards female and towards male self. This finding was supported by the earlier study because they found that the administration of agarwood leaf aqueous extract also resulted in the reduction of motor activities in treated mice due to the sedative effect as compared to the control group[34]. Additionally, the animals treated with agarwood extracts in the present study also did not indicate enough attraction to the females but rather, they appeared to be tired or sleepy and were not ready to move towards the females. This is in agreement with the findings of other researchers[36,37] who observed sexual behaviour parameters with *Aquilaria malaccensis* leaf, *Bulbine natalensis* stem, and *Massularia acuminata* root in male rodents, respectively. This sedative effect also resulted in the reduction of mount frequency and intromission frequency in the treated mice that received *A. subintegra* leaf aqueous extracts and it obviously appeared after 15 min to 30 min during the observation period.

In the investigation of aphrodisiac properties produced by *A. subintegra* leaf aqueous extracts, some parameters such as testosterone estimation, percentage of pregnancy, number of offspring and male to female ratio of offspring were also evaluated. Basically, the increment of testosterone levels stimulates sexual behaviour in humans[38]. The increase in testosterone serum levels in the blood sample of experimental animals is considered as solid proof for the plant extract possessing aphrodisiac enhancing properties, but a decreased testosterone level might be considered as the result of the lack of aphrodisiac properties[39].

The present study discovered that the oral administration of *A. subintegra* leaf aqueous extracts for 21 consecutive days had not significantly altered the level of testosterone in blood serum as compared to the controls. This finding was supported by earlier study while working on the aqueous extract of *Garcinia kola* seeds[40]. They noticed that the insignificant difference was recorded in reproductive hormones; especially testosterone in their study is an indication that gonadotropin releasing-hormone-luteinizing hormone signaling was not affected. Thus, they found that there were no enhancements recorded in sexual behaviour and orientation activity (towards female and towards male self) parameters in treated animals. According to the present study, 100 mg/kg body weight of *A. subintegra* leaf aqueous extracts found to be the best dose to increase the testosterone level in the mice. However, this positive result is not sufficiently capable to improve sexual behaviour scoring in the treated group due to the sleepiness symptom found during the observation period.

All the females which were fertilized by the males treated with *A. subintegra* leave aqueous extracts as well as fertilize by the control males became pregnant. This result was consistent with the finding from the previous study while working on *Argyreia nervosa* towards male mice[30]. As indicated by previous studies[29,38], they claimed that the increase in pregnancy rate in the treated groups with *Pedalium murex* might be due to the healthy viable sperm and boost of sexual desire of the male animals. Besides, they found that all the offspring of treated groups were normal and healthier, demonstrating the safety of the plant-based drug and the lack of any teratogenic potential. The offspring of control, as well as agarwood treated animals, were born in normal condition and this finding suggested that the agarwood extracts may be lacking in teratogenic effect. This finding is parallel with the previous studies working on stem bark, leaves and seeds of *Ficus platyphylla* in promoting fertility effects in female rats[41].

However, in our study only *A. subintegra* leaf aqueous extracts at 100, 500 and 1 000 mg/kg body weight demonstrated the significant increment in the number of offspring recorded in comparison with the control group. The increase in the number of offspring is also probably due to the healthy production of sperm cells in the treated groups that received *A. subintegra* leaf aqueous extracts rather than in the control groups. The possible explanation for this finding is probably due to the presence of some phytochemical constituents and minerals in the agarwood leaves such as flavonoids[42], phenols[43], selenium[44], zinc[44], and also copper[45] that can act as antioxidants which are vital in enhancing healthy sperm production. The presence of antioxidant properties in agarwood leaves is already stated in previous studies[14]. Thus, the healthy condition of sperm cells was one of the important factors to ensure successful fertilization process that leads to the enhancement of the number of offspring production in treated mice.

In conclusion, the results of the present study showed that the male mice treated with *A. subintegra* leave aqueous extracts in the presence of non-estrous female did not increase their sexual performance for 2 h during the observation period at day 0, 7, 14 and 21. The prolonged treatment with *A. subintegra* leaf aqueous extracts caused an inconsistent decrease in sexual behaviour recorded in treated animals due to noticeable sleepiness symptoms, especially in the treated groups that received higher doses of *A. subintegra* leaf aqueous extracts. Otherwise, the result obtained for the number of offspring in treated groups showed that *A. subintegra* leaf aqueous extracts at 100, 500 and 1 000 mg/kg is capable of increasing the breeding rate in mice by producing more healthy offspring in a short time.

Conflict of interest statement

All the authors declare that there is no conflict of interest.

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