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Aphrodisiac properties of *Aquilaria malaccensis* leaves aqueous extract in ICR mice

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ABSTRACT: The study was conducted to investigate the effects of *Aquilaria malaccensis* leaves aqueous extracts on the aphrodisiac properties, which included; sexual behaviour, orientation activity, and testosterone level in ICR mice. Thirty healthy and sexually experienced male and thirty non-estrous female mice were used. In this experiment, each male was cohabitated with one female in a polysulfone cage. The mice were divided into 6 groups that received normal saline (control group), 50 mg/kg, 100 mg/kg, 200 mg/kg, 500 mg/kg, and 1000 mg/kg body weight of *A. malaccensis* leaves aqueous extract orally for 21 days consecutively. Results showed that all aphrodisiac parameters investigated in this study were similar between the treatment groups to the control group. However, two treated groups that received 100 mg/kg (day 14; day 21) and 200 mg/kg (day 0; day 21) resulted in significantly higher in mount frequency as compared to the control group. Overall, the results revealed that *A. malaccensis* leaves aqueous extract did not significantly alter the aphrodisiac parameters. Thus, this study validated that *A. malaccensis* leaves aqueous extracts lack of aphrodisiac properties in mice.

KEYWORDS: *Aquilaria malaccensis*; aphrodisiac; sexual behaviour; orientation activity; testosterone.

1. INTRODUCTION

Sexual dysfunction can be defined as the failure to accomplish a normal sexual intercourse comprising premature ejaculation, retrograded, retarded ejaculation, erectile dysfunction, arousal problems (reduced libido), compulsive sexual behaviour and orgasmic disorder [1,2]. It is common among men of any age, ethnicities, and cultural backgrounds. The most widely recognized issue in male sexual dysfunction is erectile dysfunction. Erectile dysfunction is described as the steady failure to accomplish an erection sufficient for the purpose of satisfactory sexual intercourse, or the inability to ejaculate, or both [3]. In some cases, the terms erectile dysfunction, infertility problems and sterility issues are used to describe the similar thing since it seems not easy to clearly distinguish using traditional ethnobotanical data [4].

Some major causes that might be contribute to this infertility problem also consist of psychological disturbances, i.e. performance anxiety, strained relationship, depression, stress, guilt and fear of sexual failure; neurological disorders such as Parkinson's disease, Alzhemier's disease, spinal cord or nerve injury; deficiencies in sex hormones; side effects associated with chronic use of drugs like anti-hypertensives, central agents, psychiatric medications, antiulcer, antidepressants, anti-androgens and lifestyle related problems, i.e. chronic alcohol abuse, drug abuse and cigarette smoking [5].

In developing countries like Malaysia, the inability to afford modern medical therapy for infertility problem has forced patients to seek for traditional plant medicine. Traditional plant medicine or also known as phytotherapy with aphrodisiac properties provides a safer way to counteract with various problems associated with male infertility [6]. The phytotherapy has become more popular among the community because it is being natural, cheap, having the fewer side effect, easy to access and good therapeutic outcome [7].

Some plant species have been investigated for their use as sex stimulants and fertility enhancing agent in traditional medicines including *Eurycoma longifolia* [8, 9], *Ficus deltoidea* [10], *Nigella sativa* [11], *Lunasia amara* [12], *Gynura procumbens* [13], and *Chlorophytum borivilianum* [14]. Another potential plant for aphrodisiac and

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fertility purpose that still not investigate belongs to the genus of *Aquilaria* which is *Aquilaria malaccensis*. This species also known as agarwood and it is belonging to the family of Thymelaeaceae. Five main species of agarwood remained well distributed in Malaysia including *A. malaccencis*, *A. hirta*, *A. beccariana*, *A. rostrata* and *A. microcarpa* [15].

Agarwood leaves are frequently used in folk medicine in many countries for the promotion of good health and treatment of many ailments. A lot of studies have been done by previous researchers from various countries to verify this issues scientifically. Many previous studies have been performed on the effect of different species of the agarwood leaves extracts as the treatment of anticancer [16], Alzheimer's disease [17], therapeutic laxative agent [18, 19], antipyretic and anti-inflammatory [20], antimicrobial [21, 22], and anti-hyperglycemic activity [23].

The phytochemical constituents found by previous researchers in *Aquilaria* leaves extract are flavonoid glycosides [24], 2-(2-phenylethyl)chromenes [25], lignans [26] and diterpenoids [27]. Besides, the chemical constituents that consist of alkaloids, tannins, saponins, flavonoids, and terpenoids also are available in the *Aquilaria* leaves extract [28, 16]. Traditionally, the utilization of agarwood as aphrodisiac agent already claimed by several previous studies [29-31]. There are some phytochemicals that possess to aphrodisiac properties which are saponins [3, 32], alkaloids [33] as well as flavonoids [34, 35] since these phytochemicals have androgen enhancing and antioxidant properties. Thus, the presence of various phytochemical constituents by previous researchers specifically saponins, alkaloids, and flavonoids as well as the subjective opinion that provided by local communities additionally become the backbone for the aphrodisiac investigation.

However, no studies have been reported till date on agarwood leaves extracts that may act as aphrodisiac agent in men scientifically although it has been used traditionally for years specifically in Malaysia. This study was therefore designed to investigate the effects of the aqueous extract of a species of agarwood; *A. malaccensis* on the sexual behaviour, orientation activity and testosterone estimation of ICR male mice to validate the use of the plant, mentioned as an aphrodisiac in the folklore medicine.

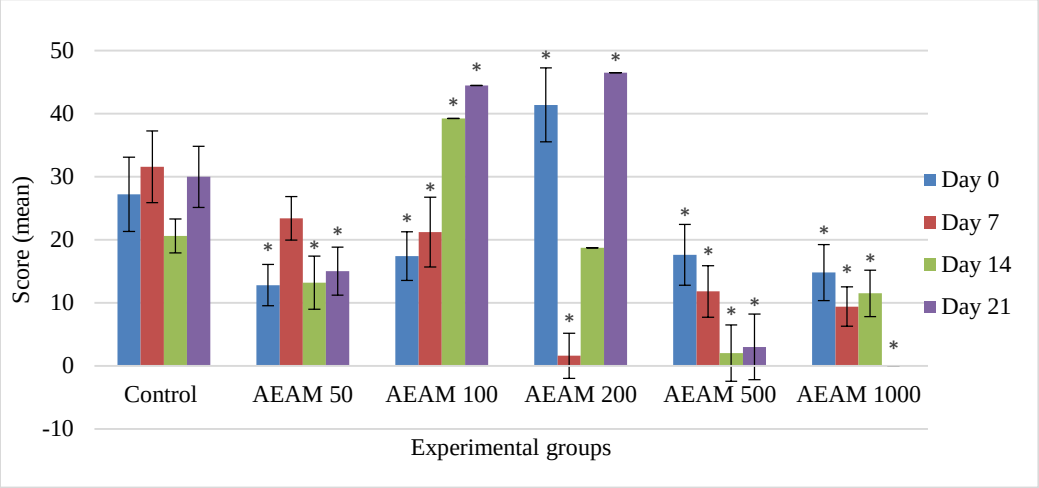
2. RESULTS AND DISCUSSION

The study was conducted to investigate the effects of *Aquilaria malaccensis* leaves aqueous extracts (AEAM) on the aphrodisiac properties, which included; sexual behaviour, orientation activity and testosterone level in ICR mice.

2.1. Effect on sexual behaviour

In the present study, the sexual behaviour parameters observed were mount frequency (MF), intromission frequency (IF), mount latency (ML) and intromission latency (IL). 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 [36]. Besides, it was exceptionally hard even for a skilled observer to distinguish between intromission and ejaculation in observation of sexual behaviour [37]. The effects of the various dose of crude extracts on sexual behaviour are summarized in Table 2. Among all the physical indices of sexual behaviour monitored in male mice, AEAM 100 (day 14; day 21) and AEAM 200 (day 0; day21) resulted in significantly higher in mount frequency as compared to the control group. Contrariwise, the other treatment groups showed significantly lower ($p<0.05$) in mount frequency for all observation days except AEAM 200 (day 14) as compared to the control group (Figure 1).

However, mount and intromission latencies for the treatment groups remained similar to the control group. The dose-dependent increase in the time spent for the first mount and intromission being recorded among the treated groups compared to the control group. Nil values were recorded on intromission frequency and intromission latency in all groups because the mice being passive and sleepy starting from day 7 until the end of observation period (day 21) especially the treated mice groups that received the crude extract. Generally, mount and intromission latencies were used as indicators of sexual motivation [38]. However, they also claimed that ML and IL 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 [38, 39].



Note, *the mean difference is significant at $p < 0.05$

Figure 1. Various doses effect of *A. malaccensis* aqueous leaves extract in mean scoring of mount frequency towards mice.

Contrary results were obtained from the present study because AEAM was lack of aphrodisiac properties. Furthermore, the administration of AEAM in high dose caused sedative effect in terms of reducing sexual behaviour and orientation activity towards female and towards self. Additionally, the treated animals with AEAM also did not indicate enough attraction to the females but rather appeared to be tired or sleepy and were not ready to move towards the females. This agreed with the findings of [1] and [40] who observed sexual behaviour parameters with 100 mg/ kg body weight of *Bulbine natalensis* stem and 200 mg/ kg body weight of *Massularia acuminata* root respectively in male rats. This condition also resulted in reducing of MF and IF in the treated animals and this sedative effect obviously appeared after 30 minutes of observation period. Though, mount and intromission latencies for the treatment groups showed insignificantly different as compared to control group. Moreover, observation at each experimental period in this investigation found that the sexual function in treated mice was not enhanced, especially in a few parameters of sexual behaviour such as IF and IL which had no value (Table 2). A similar finding was reported on *Garcinia kola* seeds in male Wistar rats [41].

The higher values of mount and intromission latencies observed in AEAM treated mice is one of the main indicator of an increase in the hesitation time of the male mice towards the female mice [1, 39]. This situation was demonstrated when the vast majority of the treated mice were not attracted to their opposite gender. Besides, the dissimilarity in the values of MF and IF were also being recorded in treated animals throughout the experimental period. This situation was supported by the previous study [39], they suggested that every number of mount recorded in treated animals was not determined the success of intromission (the phase that recognized when the copulatory organ enters the vagina during a mount).

2.2. Effect on orientation activity

Orientation activity parameters were observed from the polysulfone cage side when the extract treated male mice were introduced to the female mice. The effects of the various dose of AEAM on orientation behaviour are summarized in Table 3. The mean orientation score towards environment (rearing) showed significantly higher ($p<0.05$) in AEAM 200 on day 7 as compared to the control group. The orientation activity towards environment (climbing) revealed significantly higher ($p<0.05$) in AEAM 50 (day 0), AEAM 100 (day 0), AEAM 200 (day 0) and AEAM 500 (day 21) than the control group. In contrast, AEAM 50, AEAM 100 and AEAM 200 on day 14 was found to have significantly lower ($p<0.05$) in climbing than the control group.

Table 2. Effect of *Aquilaria malaccensis* leaves aqueous extracts on mean scoring of sexual behaviour in male mice

Group (am)	Mount frequency (mean ± SD)	Mount latency (minutes) (mean ± SD)	Intromission frequency (mean ± SD)	Intromission latency (minutes) (mean ± SD)
Control				
Day 0	27.20±5.89	2.09±1.81	3.60±5.68	19.95±13.30
Day 7	31.60±5.68	1.74±1.41	0.20±0.45	6.05±0.00
Day 14	20.60±2.70	4.67±4.44	0.00±0.00	0.00±0.00
Day 21	30.00±4.85	1.10±0.82	0.00±0.00	0.00±0.00
AEAM 50				
Day 0	12.80±3.27*	4.04±4.76	0.00±0.00	0.00±0.00
Day 7	23.40±3.44	7.01±10.73	0.20±0.45	67.21±0.00
Day 14	13.20±4.21*	19.65±15.54	0.00±0.00	0.00±0.00
Day 21	15.00±3.81*	9.33±15.18	0.40±0.89	48.23±0.00
AEAM 100				
Day 0	17.40±3.85*	5.36±6.22	0.00±0.00	0.00±0.00
Day 7	21.20±5.54*	1.10±0.78	0.00±0.00	0.00±0.00
Day 14	39.25±0.00*	1.93±2.42	1.20±2.68	60.54±0.00
Day 21	44.50±0.00*	0.94±0.97	0.20±0.45	0.00±0.00
AEAM 200				
Day 0	41.40±5.86*	1.68±2.16	0.80±1.10	5.24±0.00
Day 7	1.60±3.58*	25.19±0.00	0.00±0.00	0.00±0.00
Day 14	18.75±0.00	2.11±1.74	0.75±0.96	57.74±68.55
Day 21	46.50±0.00*	1.65±1.12	0.50±1.00	98.20±0.00
AEAM 500				
Day 0	17.60±4.83*	3.70±4.34	4.80±2.17	26.23±0.00
Day 7	11.80±4.09*	1.36±0.00	0.00±0.00	0.00±0.00
Day 14	2.00±4.47*	14.09±0.00	0.00±0.00	0.00±0.00
Day 21	3.00±5.20*	1.54±1.25	0.00±0.00	0.00±0.00
AEAM 1000				
Day 0	14.80±4.44*	3.11±2.57	0.00±0.00	0.00±0.00
Day 7	9.40±3.13*	24.26±34.99	0.00±0.00	0.00±0.00
Day 14	11.50±3.70*	5.37±6.15	0.00±0.00	0.00±0.00
Day 21	0.00±0.00*	0.00±0.00	0.00±0.00	0.00±0.00

* The mean difference is significant at $p<0.05$ level
n=5 in each group

Besides, the orientation towards female in all treated groups showed the reduction in sniffing as compared to the control group except AEAM 50 (day 0). The orientation towards female (licking) also caused significantly lower ($p<0.05$) for their mean of scoring in AEAM 50 (day7; day 14), AEAM 500 and AEAM 1000 as compared to the control group. However, highly significant ($p<0.05$) of licking also being recorded for AEAM 50 and AEAM 100 on day 0 as compared to the control. The orientation towards self (genital grooming) was found to be gradually decreased along the addition of experimental period in all treated groups. But, among them AEAM 200 (day 7), AEAM 500 (day 7 and day 21) and AEAM 1000 (day 7 and day 21) showed the significantly lower ($p<0.05$) in genital grooming when compared to the control group.

During observation period, the male mice in all groups, upon introduction, reacted with immediate advances toward the females and displayed sexual interest towards female mice such as chasing, genital sniffing and licking which eventually ended into mounting and intromission. However, the prolong treatment with AEAM caused inconsistently decrease in orientation activity especially towards female and towards self that recorded in treated animals. In contrast, orientation activity which was exploration depicted to be higher in treated groups than control group specifically in AEAM 500 and 1000. This result is parallel with the finding from the previous study [41]. They reported that there were no sexual enhancing properties in the aqueous extract of Garcinia kola seeds in male Wistar rats.

Table 3. Effect of *A. malaccensis* leaves aqueous extracts on mean scoring of orientation activities in male mice.

Group	Activity score (mean ± SD)					
	Towards environment			Towards female		Towards self
	Exploration	Rearing	Climbing	Sniffing	Licking	Genital grooming
Control						
Day 0	4.60 ± 1.67	6.40 ± 4.16	17.00±5.10	12.00 ± 4.40	11.00±1.87	5.20±2.17
Day 7	2.60 ± 0.89	6.40 ± 5.59	16.40±4.34	9.80 ± 5.81	13.80±5.63	4.80±1.64
Day 14	2.60 ± 0.89	6.20 ± 4.55	24.40±3.21	5.20 ± 1.92	13.00±4.85	3.20±0.84
Day 21	1.80 ± 0.45	5.20 ± 3.42	10.80±5.63	2.80 ± 0.84	7.20 ± 3.03	5.20±1.30
AEAM 50						
Day 0	5.00 ± 2.65	6.40±3.29	26.80±4.66*	27.80±5.50*	19.4±4.39*	5.40±1.14
Day 7	1.40 ± 0.55	2.00±1.87	8.20±4.21	3.40 ± 1.52	5.40±4.51*	3.40±1.34
Day 14	1.60 ± 0.89	5.40 ± 3.21	13.80±3.70*	2.40 ± 1.95	3.60±2.19*	3.20±1.64
Day 21	2.20 ± 1.10	5.60 ± 3.29	12.20±3.35	2.00 ± 1.00	3.60 ± 0.89	3.20±2.28
AEAM 100						
Day 0	5.00±1.22	10.20±3.77	32.40±3.21*	6.40±5.73	10.20±3.27	3.80±1.48
Day 7	1.20±0.84	2.20±1.48	11.00±5.83	3.00±1.00	7.00±3.08	3.20±1.10
Day 14	1.60±0.55	5.00±1.87	15.00±5.79*	2.80±1.10	8.00±4.42	2.80±1.92
Day 21	2.00±0.71	2.80±2.28	12.8±2.86	2.60±1.52	6.60±3.78	3.60±2.88
AEAM 200						
Day 0	3.60±0.89	7.40±5.32	27.40±4.93*	15.00±4.64	18.80±2.95*	3.60±1.14
Day 7	3.80±5.26	14.80±4.38*	26.6±4.67	5.20±5.36	6.80±3.03	0.02±0.45*
Day 14	1.20±0.84	6.00±5.16	8.25±1.50 *	4.40±3.97	7.60±3.58	3.20±3.83
Day 21	2.00±1.22	11.25±2.87	17.75±3.77	3.60±1.95	7.40±3.97	3.80±2.86
AEAM 500						
Day 0	1.80±0.84	1.60±0.55	12.40±5.18	3.80±2.39	4.60±3.51*	4.20±3.77
Day 7	3.40±1.95	8.60±4.45	18.20±4.38	1.60±2.61*	3.60±1.34*	1.80±2.05*
Day 14	4.00±1.58	6.40±4.22	22.80±5.26	1.80±1.30	3.40±3.21*	1.40±0.55
Day 21	2.20±1.30	5.40±4.39	19.00±4.18*	1.40±0.55	2.20±1.92	1.00±0.71*
AEAM 1000						
Day 0	2.40±1.14	1.20±0.84	11.80±3.90	2.20±1.10*	4.40±2.88*	2.00±0.71
Day 7	2.80±2.17	5.20±4.76	16.80±7.40	1.40±1.95*	2.40±3.36*	0.60±0.89*
Day 14	2.75±0.96	5.00±2.94	23.00±4.83	1.75±0.96	2.50±3.32*	1.50±1.00
Day 21	2.50±1.29	6.25±4.43	14.75±3.59	0.50±0.58	0.75±1.50*	0.50±1.00*

* The mean difference is significant at $p<0.05$ level (n=5 in each group)

2.3. Effect on testosterone level

The effect of the oral administration of AEAM on testosterone level is presented in Table 4. The extract showed no significant changes ($p<0.05$) in testosterone concentration at all doses administered when compared with the control. However, AEAM 200 showed slight increase and the other treated groups showed slight decrease in testosterone level as compared to the control. In general, elevated testosterone level also boosts the sexual behaviour in humans [42, 43]. Therefore, an increase in testicular and serum free testosterone concentration will confirm aphrodisiac potential inherent in the plant extract. Commonly, high level of testosterone in the male reproductive system is closely related to the increase of Luteinizing hormone (LH) concentration and the presence of secondary phytochemical which capable to mimic the function of LH to stimulate interstitial cells in testosterone production [44].

Table 4. Effect of aqueous leaves extract of *A. malaccensis* on testosterone level in male mice.

AM	Treatment Groups (mean ± SD)					
	Control	AEAM 50	AEAM 100	AEAM 200	AEAM 500	AEAM 1000
Testosterone concentration (ng/ml)	3.08±1.15	3.07±2.06	2.91±1.00	3.12±0.96	2.41±1.28	2.16 ± 0.89

* The mean difference is significant at $p<0.05$ level (n=5 in each group)

Contrary result was obtained from the present study because there was no significant changes recorded in testosterone concentration at all doses administered when compared with control (Figure 2). Clinical information on testosterone likewise proposes that a slight increment in the levels of the hormone in adult males results in a moderate but significant increase in sexual desire and libido [45, 36]. Surprisingly, in the present study the slight increment in testosterone level in AEAM 200 was not associated with the changes in sexual behaviour and orientation activity (towards self and towards females) because the scoring for these parameters were remained similar with the other treated groups. Thus, the sedative effect was found to be the main factor to reduce the score recorded in sexual behaviour and orientation activity (towards self and towards females) in experimental animals.

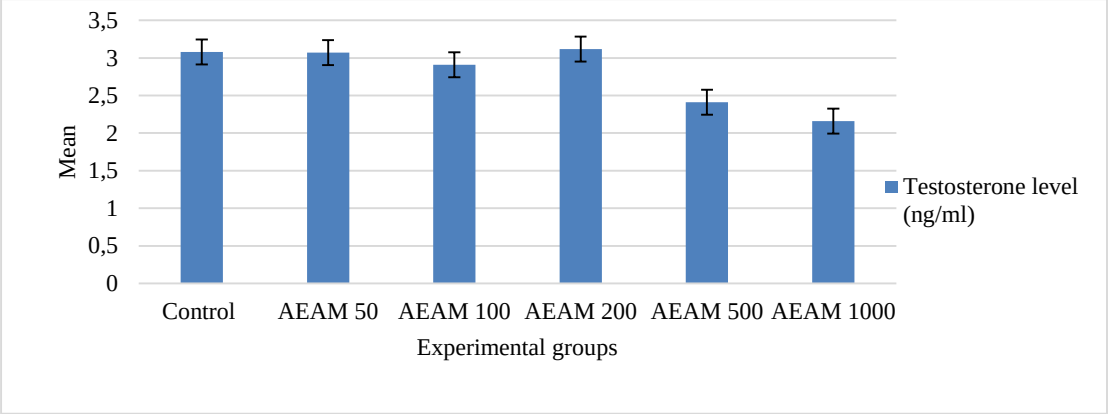


Figure 2. Various doses effect of *A. malaccensis* aqueous leaves extract in testosterone level estimation towards mice

3. CONCLUSION

The results of the present study show that the treated male mice with an aqueous extract of *A. malaccensis* leaves (AEAM) in the presence of non-oestrous female did not increase their sexual performance for two hours observation period at day 0, 7, 14 and 21. The prolong treatment with AEAM caused inconsistently decrease in sexual behaviour and orientation activity (towards female and towards self) parameters that recorded in treated animals especially in AEAM 1000. This study has thus discovered that the aqueous leaves extract of *A. malaccensis* possess lack of aphrodisiac properties towards mice.

4. MATERIALS AND METHODS

All methods that applied in this study was done under proper research ethics and care that was approved by Universiti Pendidikan Sultan Idris research committee.

4.1. Plant sample extraction

The fresh leaves of *A. malaccensis* were collected from Agarwood Al-Hilmi plantation in Behrang, Perak, Malaysia. The plant specimen was collected in November 2015, identified and deposited at the Herbarium of Universiti Pendidikan Sultan Idris. The plant specimen was assigned a voucher numbers NHCM001. In plant sample extraction the leaves were washed, air dried, and ground using electrical grinder to form the fine powder. 800 g of the powder were macerated in 8L of distilled water for 24 h at room temperature with occasional stirring. The mixtures were filtered using cloth filter at room temperature [46] and the filtrate obtained were oven dried at 55°C for 48 hours [47], followed by freeze drying for 72 h [48]. The brown crude extract obtained was stored at -20°C prior to further use.

4.2. Experimental design

Repeated oral dose administration was carried out in this experiment. In this test, 30 adult male mice were used. 30 male mice were divided into 6 groups of 5 mice each group and treated with crude extract using plastic syringes attached to ball-tipped stainless steel feeding needle daily for 21 days consecutively as the following in Table 1. The administration volume was 10 mL/kg b.w of the animal [49]. The amount of crude extract was calculated based on the body weight of the animal and dissolved in distilled water before administered directly to the mice [50]. Before the administration of crude extract, the animals were fasted overnight. During the experiment period, food and water were given *ad libitum*.

Table 1. The different doses of *A. malaccensis* aqueous leaves extract (AEAM)

Group	Treatment
Control	Mice received 10 ml/kg body weight of normal saline (n=5)
AEAM 50	Mice received 50 mg/kg body weight /day crude extract (n=5)
AEAM 100	Mice received 100 mg/kg body weight /day crude extract (n=5)
AEAM 200	Mice received 200 mg/kg body weight / day crude extract (n=5)
AEAM 500	Mice received 500 mg/kg body weight / day crude extract (n=5)
AEAM 1000	Mice received 1000 mg/kg body weight / day crude extract (n=5)

4.3. Parameter in assessing plant with aphrodisiac activity

4.3.1. Sexual behaviour test

Healthy and sexually experienced male albino mice that showing brisk sexual activity were selected for the study. The methods that implemented in this study was approved by Universiti Pendidikan Sultan Idris research ethics committee. The experimental animals were divided into six groups of five animals each and kept singly in separate polysulfone cages during the experiment as mention in the experimental design above. First group represents the control group, which received 10 ml/kg of normal saline orally. The other five groups were received suspension of five different doses of water extract of *A. malaccensis* at the doses of 50,100, 200, 500 and 1000 mg/kg body weight respectively. All oral administrations were done daily at the same point time of between 08:00 am and 09:30 am for 21 days. The observation was done on 0, 7, 14 and 21 days after orally treated with different doses of agarwood aqueous extract. The experiment was conducted at 09:00 a.m until 2:00 p.m in the same laboratory and under the light of the same intensity. The non-estrous female mice were introduced into the polysulfone cages of male animals with 1 female to 1 male ratio. During observation period, any jerking movement of the mating area was avoided to enable the mice to chase each other; and cleaning of the mating area was performed after each trial, since the urine trails left by one mouse might alter the sexual behavior of the next mouse [51]. The occurrence of events and phases of mating were recorded using CCTV video camera about 2 hours [52].

The behavioural observations were carried out by taking into account the following parameters that described by [53] and [54].

Mounting Behaviour – It was determined and characterized by following parameters.

(A) Mount frequency – The average number of mount by a male mice without intromission during 2 hours observation

(B) Mount latency- The lag time in minutes from the introduction of female in the cage to first mount.

Intromission Behaviour – It was evaluated according to these following aspects.

(A) Intromission frequency – The average number of Intromission during 2 hours observation

(B) Intromission latency- The time in minutes for first intromission after introduction of female in the cage.

4.3.2. Orientation activity test

The same mice in sexual behaviour were used in this test. The test was done in 30 minutes early of the sexual behaviour observation method [55]. The orientation activity was carried out about three weeks of treatment and analyzed in three segments [56, 57].
Orientation behaviour of male mice were determined using following method of scoring:
Orientation towards female – (1 for every sniffing and 2 for every licking)
Orientation towards self – (1 for non-genital grooming and 2 for genital grooming)
Orientation towards environment – (1 for exploration, 2 for *rearing and 3* for climbing)
The cumulative score at 0, 7, 14 and 21 days of the treatment of experimentation were recorded using CCTV video camera.

4.3.3. Testosterone estimation

After 21 days of treatment with plant extract, blood samples were drawn from the mice' hearts by cardiac puncture method. Blood samples were collected in test tubes while the mice are put under mild ether anesthesia in the morning of day 22. Test tubes that contain blood samples were gathered and left at room temperature for 1 hour. After coagulation, the tubes were placed into the centrifuge at 3000 rpm for 15 min to collect the plasma prior to testosterone determination [58]. Sera was pipetted by micro pipettes and transferred into new, label tubes, sealed with parafilm and stored to freeze overnight at -20°C before being used in measurement of testosterone using ELISA.

4.3.3.1. Blood collection method

In general, blood withdrawal by cardiac puncture is considered terminal procedures of the study to collect a single, good quality and large volume of blood and must be performed only after ensuring that the animal is under surgical anesthesia [59]. Blood collection method was described by [60]. In this procedure 1 ml syringe and a 20 gauge x 1.5" needle were used. The procedure starts by palpating heart of the mice and inserting 5mm needle from the center of the thorax towards the animal's chin, 5-10 mm deep and the syringe was held 25-30 degrees away from the chest. The needle was withdrawn after sufficient amount of blood able to be collected and blood was transferred into the test tube. Lastly, the secondary method of euthanasia was performed to ensure that the animal is deceased.

4.3.3.2. ELISA method

Serum testosterone concentration of the experimental animals was assayed using the procedure outlined in the manufacturer's instruction manual (Testosterone ELISA Kit ab108666).

4.4. Statistical analysis

The results were expressed as mean ± SD. Statistical analyses were performed using one-way ANOVA and followed by Tukey's test for parametric multiple comparisons between the control and the treatment groups. The values were considered significantly different when the p value was less than 0.05 ($p < 0.05$).

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