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RESEARCH ARTICLE

Efficacy of Acute Oral Supplementation of *Aquilaria malaccensis* Leaves Aqueous Extract on Adult Female Sprague Dawley Rat Growth Performance

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Abstract *Aquilaria malaccensis* belongs to the Thymelaceae family and is frequently encountered in select states of Peninsular Malaysia, notably Terengganu, Kelantan, Pahang, and Johor. Its favorable pharmacological and nutritional attributes have attracted the attention of experts in the pharmaceutical and food industries. They are currently investigating its potential as an organic substitute herb for the formulation of diverse medicinal commodities. In spite of its growing utilization as a supplementary component, it is crucial to acknowledge that improper or excessive consumption of *Aquilaria malaccensis* leaf extract might pose a risk of oral toxicity. To evaluate this aspect, an acute study was carried out to investigate both the immediate and delayed toxic repercussions of aqueous extract from *Aquilaria malaccensis* leaves on rats during a 14-day span.The study involved twenty-four female Sprague Dawley rats, divided into four groups: Control (C); 1 ml of distilled water, Treatment 1 (T1); 1 g of *Aquilaria malaccensis* per kg of body weight, Treatment 2 (T2); 2 g per kg of body weight, and Treatment 3 (T3); 3 g per kg of body weight. The data were analyzed using appropriate statistical methods; one-way analysis of variance (ANOVA) for parametric data and the Chi-Square test for non-parametric data.The results indicated that both T2 and T3 led to a significant increase in the mean weight of the organ (i.e.,ovary) compared to the control group. However, no significant differences were observed among the treatment groups with regard to weekly food intake (WFI), feed conversion ratio (FCR), and body weight gain (BWG) throughout the 14-day acute oral toxicity assessment. In conclusion, this preliminary study involving female rats suggests that doses of *Aquilaria malaccensis* up to 3 g/kg of body weight do not result in immediate (within 3-4 hours) or delayed toxic effects over a 14-day period, as evidenced by behavioral and physical, and growth parameter assessments (weekly food intake (WFI), feed conversion ratio (FCR), and body weight gain (BWG). The study indicates that exposing the animals to *Aquilaria malaccensis* aqueous extract at doses of 1 g, 2 g, and 3 g/kg of body weight does not adversely affect their overall condition. No instances of mortality or severe clinical effects were observed in any of the female rats during this acute oral toxicity study.

Keywords: *Aquilaria malaccensis,* Growth Performance, Leaves Extract, Oral Toxicity, Phytochemicals.

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Introduction

Traditional herbal remedies have gained popularity within communities as an alternative to modern pharmaceuticals, largely due to the perceived health advantages attributed to their pharmacological constituents. The Food and Agriculture Organization (FAO) states that approximately 80% of the population turns to natural herbal resources to support their overall fitness and well-being [1]. Examples of such plants include Ginseng (*Panax ginseng*), Gingko *(Gingko biloba*), Chamomile *(Matricaria chamomilla)*, Tongkat Ali *(Eurycoma longifolia*), and Kacip Fatimah *(Labisia pumila).* These herbs have been utilized since ancient times owing to their associated benefits [2, 3].

Among the Aquilaria species, *Aquilaria malaccensis* stands out as the predominant source of Gaharu in Malaysia, surpassing other Aquilaria varieties in popularity [4]. Large-scale cultivation of *Aquilaria malaccensis* has been noted among farmers in Malaysia [5]. Apart from *Aquilaria malaccensis*, several other Aquilaria species thrive in the Malaysian rainforests, including *Aquilaria microcarpa, Aquilaria hirta, Aquilaria beccariana,* and *Aquilaria rostrata* [6, 7]. *Aquilaria malaccensis*, in particular, holds significant promise in pharmaceutical advancements. Its pharmacological attributes, encompassing anti-cancer effects on human colon cancer cells, alleviation of discomfort, stomach-warming properties to prevent vomiting, and asthma relief, position it as the most esteemed among the Aquilaria species [8, 9, 10, 11, 12]. This botanical gem has also found its place in traditional East Asian medicine due to its manifold benefits.

While turning to natural herbal sources as an alternative to conventional medications is a practice gaining traction, it is important to recognize the potential for herbal toxicity [13]. The introduction of herbal supplements can result in toxicity for consumers when not prepared correctly, used excessively without proper indication, or consumed over extended periods [13, 14, 15]. A further factor contributing to herbal toxicity is the general perception among the public that all natural products are inherently safe for consumption [16,17]. Additionally, insufficient familiarity with specific herbs and supplementary products among consumers can also lead to toxic effects following their use [15, 18, 19].

The acute oral toxicity study aimed to investigate the consequences of a single administration within a 24-hour window and the potential for delayed mortality over a 14-day period [20]. This study focused on short-term effects, specifically examining immediate safety, efficacy, and physiological responses by observing how a test substance quickly reveals side effects following oral dosing [20]. The manifestations of toxic effects were typically evident within minutes, hours, or days (up to approximately 2 weeks) [21]. The dose of the test material that could result in the death of 50% of a tested animal group signifies an unsafe level for consumption [22, 23].

To date, there has been a limited number of studies conducted to ascertain the immediate toxicological effects of *Aquilaria malaccensis* specifically in female animals. Prior research in this area, has primarily focused on male subjects [30]. Hence, the key aim of this study is to uncover how supplementing *Aquilaria malaccensis* affects various parameters, such as Physical and Behavioural Assessment (PB), Weekly Feed Intake (WFI), Body Weight Gain (BWG), Feed Conversion Ratio (FCR), and Relative Organ Weight (ROW) after the administration of *Aquilaria malaccensis* to female rats.The selected dosages (1 g, 2 g, and 3 g/kg) correspond to those used in previous pertinent studies and adhere to established norms for studying acute toxicity. This consistency provides a reference point for understanding results and simplifies the analysis of dose-dependent responses.

Materials and Methods

Extract Preparation

Aquilaria malaccensis leaves were gathered from the Merchang Karas Forest Reserve situated in Merchang, Terengganu, Malaysia. The location was chosen due to the availability of *Aquilaria malaccensis* leaves samples. During sampling, *Aquilaria malaccensis* trees were identified according to Identification Manual of Aquilaria Species [27]. The leaves were weighed, then thoroughly cleansed with clear water. Subsequently, the leaves were evenly laid out on parchment paper and subjected to a period of air-drying at ambient temperature spanning three successive days, until their weight reached a consistent value [28].Dried leaves were then pulverized using a Waring blender (Waring Commercial, USA) and weighed before macerated for 24 hours in distilled water at a sample-to-solvent ratio of 1:10 [28, 29]. The maceration process of leaves took place for 24 hours before sample was put into ultrasonic extractor (Wisd, Korea) for 15 minutes to speed up the extraction rate of active pharmaceutical ingredients. Samples were then centrifuged under 5000 rpm for 4 minutes [28] and filtered using Whatman paper No. 4. The filtered supernatants were concentrated using a rotary vacuum evaporator (Heidolph, Germany) at 40ºC-50ºC until dark-brown crude dry extracts were obtained. Subsequently, these dry crude extracts were subjected to measurement of weight and stored at a temperature of 4℃ to prevent any potential chemical degradation. During dose preparation, distilled water was used to facilitate the dry crude extract administration to animals using oral gavage according to dose calculated [26, 30].

Phytochemical Screening

The analysis of secondary metabolites through qualitative phytochemical screening was undertaken to ascertain the presence of various compounds, including tannins, saponins, glycosides, flavonoids,

terpenoids, steroids, and alkaloids, within the aqueous extract of *Aquilaria malaccensis* leaves. The following tests has been conducted to detect the presence of these phytochemicals.

Table 1. The phytochemical and test that has been conducted during phytochemical screening

Phytochemical content	Test conducted
a) Tannins	Ferric Chloride Test
b) Saponins	Froth Test
c) Glycosides	Glycosides Test
d) Flavonoids	Conc. H_2SO_4 Test
e) Steroids	Steroid test
f) Terpenoids	Salkowski test
g) Alkaloids	Dragendoff's Test

Experimental Animals

24 sexually matured adult Sprague Dawley female rats were used in this study. The decision to opt for female rats was rooted in their elevated sensitivity to toxicity as compared to male rats [24, 25, 26]. Healthy adult female rats aged between 8-9 weeks with average starting weight between 180-200 g were allocated randomly in separated individual cages with a steel cover consisting of absorbent bedding with sufficient amount of commercial pellets (Gold Coin Feedmills, Malaysia), set up in a temperature-controlled environment at 22 ± 3°C, 50-60% humidity, and 12-hour light (0800-2000) / 12-hour dark cycle (2000-0800). A week before the trials, the rats were acclimatised to the laboratory condition for 7 days. Organisation for Economic Co-operation and Development (OECD) Test guideline 423 was referred to and slight changes were made to match to laboratory conditions [26]. This trial has been conducted in facilities approved by Centralized Laboratory Management Centre (CLMC), Universiti Sultan Zainal Abidin (UniSZA), Besut Campus, Terengganu, Malaysia.

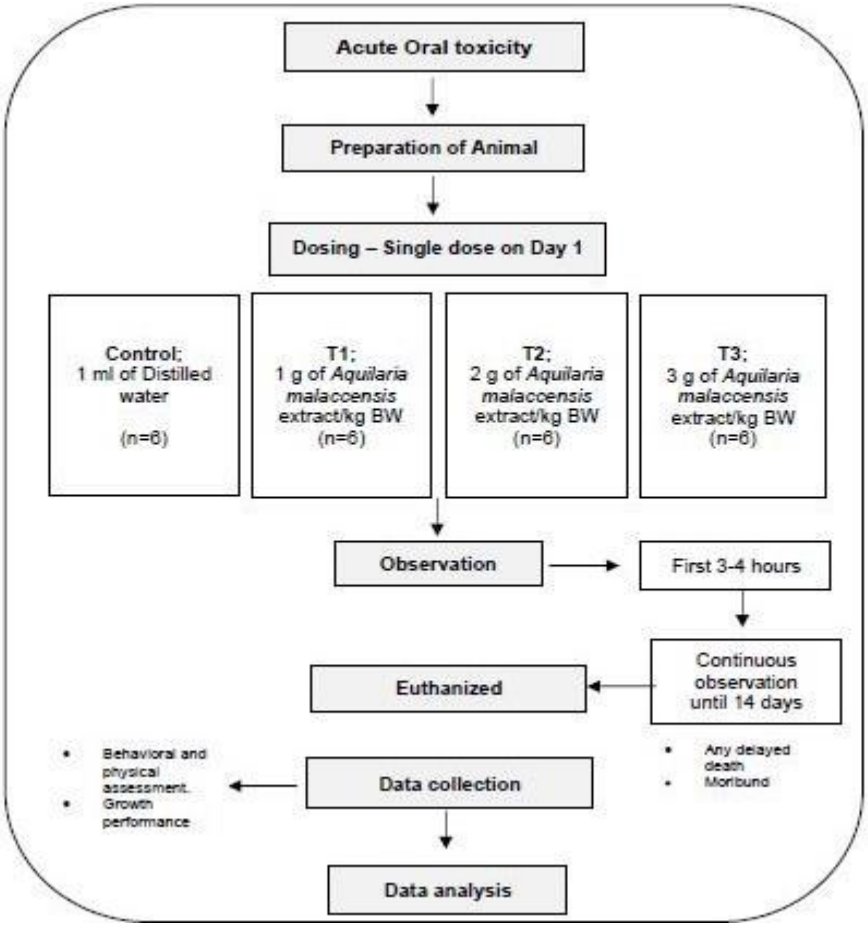


Figure 1. Flowchart illustrates the experimental design of acute oral toxicity study of *A. malaccensis*

Acute Oral Supplementation of Aquilaria malaccensis Leaves Extract

This acute oral toxicity study was designed to assess the effects of a single administration within 24 hours, critically in the first 3-4 hours) and possible delayed death within the period of 14 days [26].Twenty-four (n=24; n=6 per group) female Sprague Dawley rats with average body weight of 180-200 g were assigned randomly into four treatment groups.; consisting of Control (C: administered with 1 ml of distilled water), Treatment 1 (T1: 1 g of *Aquilaria malaccensis* /kg body weight), Treatment 2 (T2: 2 g of *Aquilaria malaccensis* /kg body weight), and Treatment 3 (T3: 3 g of *Aquilaria malaccensis* /kg body weight), respectively. The chosen doses (1 g, 2 g, and 3 g/kg) were based on what has been used in previous studies (mostly in male animal models) and are in line with established standards for studying how substances can be immediately harmful. This consistency makes it easier to compare results and understand how the effects change with different amounts [26,30].

One day before the trials, the rats underwent an overnight fasting regimen prior to dosing to ensure the standardization of exposure conditions [30]. This fasting protocol aids in guaranteeing that the primary determinant of the study's outcomes remains the test substance itself, without potential interactions stemming from recently ingested substances. The Organisation for Economic Co-operation and Development (OECD) Test guidelines 423 was referred [26]. The trials were conducted in facilities approved by Centralized Laboratory Management Centre (CLMC), Universiti Sultan Zainal Abidin (UniSZA), Besut Campus, Malaysia and all methods applied in this study were approved by UniSZA Animal and Plant Research Ethics Committee (UAPREC) with reference numbers: UAPREC/04/044 and UAPREC/04/045.

Physical and Behavioural Assessment (PB)

Following the oral administration of the test substances using oral gavage, a thorough examination was conducted during a crucial 4-hour window immediately after dosing. This observation aimed to monitor the immediate reactions to *Aquilaria malaccensis* extract, with each individual female rat being closely observed [26, 30]. Subsequently, a daily continuous monitoring was carried out over a span of 14 days, focusing on identifying signs like moribund conditions or delayed mortality. Various parameters such as the condition of fur, eyes, mucous membranes, and the movement of the animals were attentively recorded [30, 31].

Weekly Feed Intake (WFI)

Daily evaluations were performed to quantify and document feed consumption. At a consistent designated time each day, the residual feed was subjected to weighing. To maintain uniformity, a predetermined quantity of pre-measured feed (30 grams) was consistently provided to the rats daily via the feeding tray. The feed leftover including the spillage at the bottom of the cage was weighed and measured. Weekly feed intake was calculated on (Day 7 and Day 14) [34, 35].

$$\textit{Feed Intake} = \textit{Preweighed Feed (30 g/day)} - \textit{Feed Left Over (R} \quad (1)$$

Feed Conversion Ratio (FCR)

To monitor the growth performance of the rats supplemented with *Aquilaria malaccensis* aqueous extract within 14 days of treatment, the Feed Conversion Ratio (FCR) was computed by using this formula [36]

$$\textit{FCR} = \textit{Feed Intake by Animal (g)} / \textit{Animal Weight Gain (g)} \quad (2)$$

Relative Organs Weight (ROW)

On the 15th day, the animals underwent euthanasia procedures. The rats were anesthetized and subjected to carefully conducted sacrifice methods, following appropriate handling procedures to minimize any potential sources of stress. 5 ml of Diethyl Ether, acting as an anaesthetic agent, was introduced onto a cotton gauze pad and placed within a euthanasia glass container [37, 38]. The individual animals were then introduced into the container one at a time, allowing them to inhale the agent until achieving a state of unconsciousness. Subsequent to attaining unconsciousness, dissection procedures were carried out [39]. The retrieval of organs was carried out with precision, utilizing forceps and a scalpel. The weights of organs such as the liver, spleen, heart, adrenal gland, kidney, ovary, and uterus were meticulously documented. The organ weight was determined using the Organ-To-Body Weight Ratio (%) [40]:

$$\textit{ROW= (Organ weight (g)) / (body weight of the animal on sacrifice day (g)) x100} \quad (3)$$

Body Weight Gain (BWG)

Prior to introducing the freshly pre-weighed feed, the animals were taken out of their cages and their weights were measured. The weekly increase in body weight for the animals was determined at each week interval, specifically on Day 7 and Day 14. The calculation of body weight gain (BWG) followed this formula [41].

$$BWG = (final\ body\ weight - initial\ body\ weight) / (Initial\ Body\ Weight) \times 100 \quad (4)$$

Statistical Analysis

Analyzing daily data for an extended period can lead to a substantial amount of data, which might be challenging to interpret and present. Thus, weekly intervals were used as it can make the sets of data and interpretation more manageable. Parametric data such as relative organs weights' (ROW) was analysed using Analysis of Variance, one-way ANOVA (SPSS, Version 24.0), this was followed by the application of Tukey's post-hoc test to assess pairwise differences. Between-within repeated ANOVA was used to test the main effects of the treatment and time and the interaction effects between the two factors on body weight gain (BWG), weekly feed intake (WFI), and feed conversion ratio (FCR). Data were expressed in Mean ± Standard Error of Mean (S.E.M) and the *p*-value. The value of $p<0.05$ was considered significant. Whereas, the non-parametric data (physical and behavioural assessment) were analysed by using Chi-Square Test.

Result and Discussion

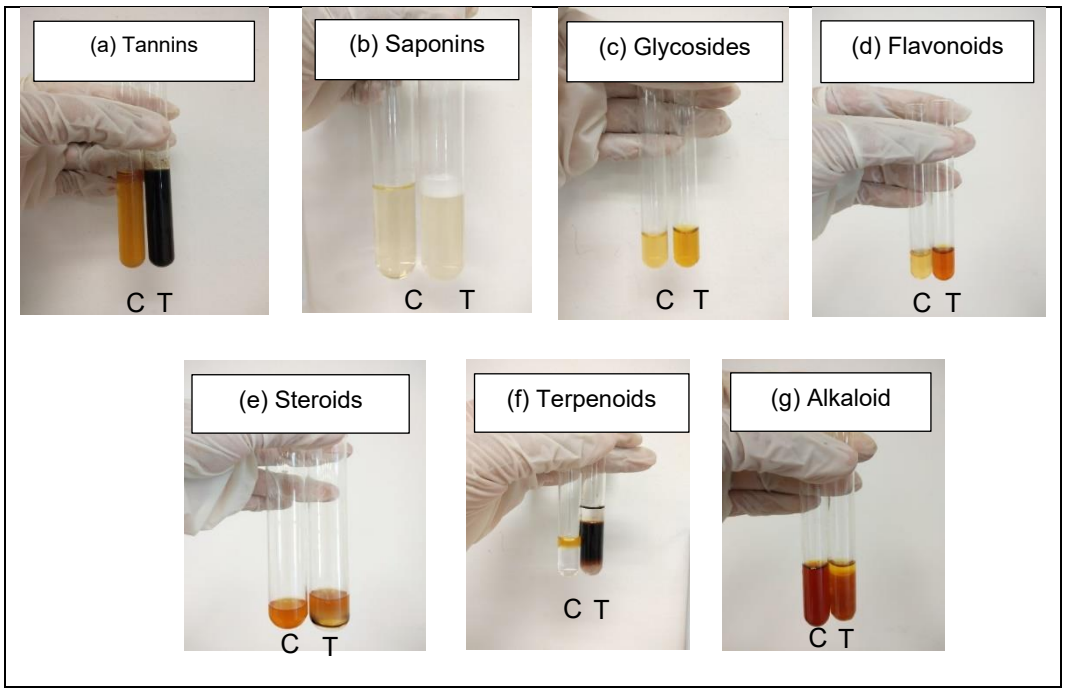
Phytochemical Content of the Leaves

The result of these phytochemical screening showed the presence of phytochemicals such as tannins, saponins, glycosides, flavonoids, steroids, terpenoids, and alkaloids *in Aquilaria malaccensis* leaves (Table 2, Figure 2).

Table 2. The presence of phytochemical content in *Aquilaria malaccensis* leaves

Phytochemical	Observation	Absence or presence
Tannins (Ferric Chloride Test)	Greenish-black precipitate indicates the presence of tannins.	+++
Saponins (Froth Test)	1 cm thick foam indicates the presence of saponins.	+++
Glycosides (Glycosides Test)	Formation of a yellow colour indicates the presence of glycosides.	+++
Flavonoids (Conc. H_2SO_4 Test)	Rapid red colour indicates the presence of alkaloid	+++
Steroids (Steroid test)	Yellow with green fluorescence indicates the presence of steroid.	+++
Terpenoids (Salkowski test)	Reddish brown formation indicates the presence of terpenoids.	+++
Alkaloids (Dragendoff's Test)	Red-brown precipitate indicates the presence of the alkaloids.	+++

(+++) indicates the presence of phytochemicals in tests conducted.



Note: (C) = Control, (T) = Tested with reagent

Figure 2. The figure illustrates the observation from the screening process

Acute Oral Supplementation Efficacy on Growth Performance

Physical and Behavioral Assessment (PB)

After the administration of the Aquilaria malaccensis extract, all treatment groups did not show toxicity-related side effects. There were no lethal effects, mortality and morbidity caused by the administration of higher dose of *Aquilaria malaccensis* aqueous extract observed. No abnormality was observed in fur, mucous membrane, or eye colour in all treatment groups; (C: control, T1: 1 g/kg, T2: 2 g/kg and T3:3 g/kg) within 14 days period of observation.

The determination of plant material toxicity was based on external indicators exhibited by animals. These indicators encompassed the display of symptoms like ruffled fur, signaling stress or illness, as well as unusual ocular and nasal discharge, and abnormal movements such as hyperactivity or numbness within the specified observation period. Additionally, a threshold for toxicity was set at a level that either resulted in a 50% mortality rate within a group of test animals or triggered specific observable signs [34, 42, 43, 47].Healthy rats, on the other hand, typically exhibited distinct behaviors indicative of well-being, including active movement within their cages and the presence of bright red eyes, as a consequence of proper grooming practices [44, 45]. These signs of vitality were consistently demonstrated by rats across all treatment groups.

Weekly Feed Intake (WFI)

The findings from this acute study showed no significant differences ($p>0.05$) in weekly feed intake in all treatment groups as presented in Table 3.

Table 3. The effect of *Aquilaria malaccensis* aqueous extract supplementation on Weekly Feed Intake (WFI)

Weekly Feed Intake	Control	Treatment 1 g/kg BW	Treatment 2 g/kg BW	Treatment 3 g/kg BW
Week 1	66.833 ± 2.286	67.167 ± 1.905	68.167 ± 2.509	66.667 ± 3.242
Week 2	79.500 ± 3.394	78.667 ± 2.716	80.500 ± 0.671	79.833 ± 2.613

The data are presented as Mean ± Standard Error Mean (SEM).

In the realm of toxicological investigations, any modification in physiological parameters, such as a decline in the weekly feed intake of animals, typically signifies potential toxicity [30, 48]. Derived from the acquired outcomes, it can be discerned that the single dose of *Aquilaria malaccensis* aqueous leaf extract administered at doses of 1, 2, and 3/kg did not exert any discernible influence on the feed intake patterns of the animals throughout the entire 14-day treatment period across all treatment groups.

Feed Conversion Ratio (FCR)

No statistically significant distinctions were identified ($p>0.05$) in the Feed Conversion Ratio (FCR) among the animal groups subjected to different treatments during the first and second weeks of the 14-day administration period for the higher doses of *Aquilaria malaccensis* aqueous extract (1 g, 2 g, and 3 g/kg of body weight), when contrasted with the control group. This observation is detailed in Table 4.

Table 4. The effect of *Aquilaria malaccensis* aqueous extract supplementation on Feed Conversion Ratio (FCR)

Feed Conversion Ratio	Control	Treatment 1 g/kg BW	Treatment 2 g/kg BW	Treatment 3 g/kg BW
Week 1	4.338 ± 0.400	3.735 ± 0.215	3.749 ± 0.309	4.146± 0.530
Week 2	4.310 ± 0.505	4.355 ± 0.300	4.097 ± 0.100	3.992 ± 0.278

The data are presented as mean ± standard error mean (SEM).

The insignificant changes in FCR among the group could indicate that the rats' metabolic processes and nutrient utilization were relatively stable across the treatment conditions [67]. This was relatable with the feed intake of the animal, where there is none of the animal has shown the abnormal pattern or reduction in feed intake. The absence of significant changes in FCR may also suggest that the treatments did not adversely affect the rats' digestive processes or overall health, as reflected in their feed conversion efficiency [67]. The insignificant of FCR values among the groups also has suggested that these treatments did not disrupt the animals' ability to convert feed into body weight [67]. While there was no significant changes were observed in FCR in this study, there might be other aspects of the rats' physiological responses that were affected by the treatments, warranting further investigation.

Body Weight Gain (BWG)

The results indicate that there were no statistically significant differences ($p>0.05$) observed in the increase of body weight within all treatment groups when compared to the control group during both Week 1 and Week 2. These findings are presented in Table 5.

Table 5. The effect of *Aquilaria malaccensis* aqueous extract supplementation on Body weight gain (BWG).

Body Weight Gain (g)	Control	Treatment 1 g/kg BW	Treatment 2 g/kg BW	Treatment 3 g/kg BW
Week 1	15.313 ± 1.364	16.023 ± 1.878	18.737 ± 2.121	19.943 ± 1.274
Week 2	15.670 ± 1.263	16.573 ± 1.823	19.323 ± 2.202	20.753 ± 1.058

The data are presented as mean ± standard error mean (SEM).

No decrease in body weight was noted across all subjects. These findings distinctly reveal that the progression in body weight of the subjects remained unaffected by the dose administered over the course of the 14-day observation period. In a previous study addressing acute toxicity, it was posited that a dosage level of a plant extract could be categorized as toxic if a reduction of 10% or more in body weight was observed among the treated subjects [58]. The outcomes derived from this present study offer compelling evidence that the *Aquilaria malaccensis extract* did not elicit substantial modification in body weight of the animals across all groups.

Relative Organ Weight (ROW)

Upon the culmination of the 14-day observation period, significant difference in the mean weights of all organs were not identified ($p>0.05$), with one exception. Notably, the weight of the ovaries displayed a significant increase ($p<0.05$) within the T2 and T3 groups when juxtaposed with the control group, as depicted in Table 6.

Table 6. The effect of *Aquilaria malaccensis* aqueous extract supplementation on Relative Organ Weight (ROW)

Organ	*n*	Control	Treatment 1 g/kg BW	Treatment 2 g/kg BW	Treatment 3 g/kg BW
Liver	*6*	4.15 ± 0.074	4.03 ± 0.077	3.99 ± 0.054	3.98 ± 0.066
Lung	*6*	0.58 ± 0.025	0.61 ± 0.018	0.64 ± 0.086	0.65 ± 0.025
Heart	*6*	0.35 ± 0.007	0.35 ± 0.006	0.36 ± 0.011	0.36 ± 0.012
Spleen	*6*	0.23 ± 0.010	0.24 ± 0.016	0.24 ± 0.009	0.25 ± 0.010
Kidney	*6*	0.37 ± 0.008	0.37 ± 0.004	0.38 ± 0.008	0.40 ± 0.013
Adrenal	*6*	0.02 ± 0.002	0.02 ± 0.002	0.02 ± 0.002	0.02 ± 0.002
Ovary	*6*	**0.02 ± 0.003^{a}**	**0.02 ± 0.002^{ab}**	**0.03 ± 0.002^{b}**	**0.03 ± 0.002^{b}**
Uterus	*6*	0.21 ± 0.024	0.19 ± 0.008	0.19 ± 0.013	0.18 ± 0.014

The data are presented as mean ± standard error mean (SEM).

Organ weights serve as crucial indicators of toxic effects within the realm of toxicological investigations [59]. The significant elevation in ovarian weight observed in the T2 and T3 groups, when contrasted with the control group, is plausibly attributed to the potential interference of alkaloid and steroid constituents present in the leaves. Alkaloids and steroids, as pertinent phytochemicals essential to reproduction might exert an influence on female reproductive hormones, thereby contributing to the observed alteration [50],

The inclusion of tannins in the equation further warrants consideration. The presence of tannins could potentially evoke toxicity and prompt inflammation in specific organs [51]. The ingestion of minute quantities of tannin compounds may confer health benefits due to their recognized anti-carcinogenic and antioxidant properties [54]. However, at elevated dosages, tannin compounds have been documented to yield toxic effects in animals [51].

Additionally, the amplified protein content in the proximate analysis of *Aquilaria malaccensis* leaves, as outlined in prior research, could underlie the observed increment in ovarian weight. The augmented protein content could indeed underpin various aspects of female ovulatory processes, as supported by previous studies [28, 63, 64, 66]. Notably, protein plays a pivotal role in ovum generation and fertility by instigating follicular formation within the ovary, thereby culminating in ovarian weight augmentation [65].

Conclusion

To summarize, the oral supplementation of *Aquilaria malaccensis* in this 14-day acute study did not result in any noticeable signs of toxicity or mortality among the female rats. Moreover, the growth-related parameters such as weekly feed intake (WFI), feed conversion ratio (FCR), and body weight gain (BWG) remained unchanged even with the administration of higher extract doses. Collectively, these findings suggest that the maximum dosage of *Aquilaria malaccensis* aqueous extract, particularly at 3 g/kg, can be considered safe for consumption within a limited timeframe of 14 days.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgment

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