1. Introduction

Erectile dysfunction (ED) is an inability to achieve or maintain penile erection sufficiently while engaging sexual intercourse[1]. This common form of sexual dysfunction has a deep impact on the self-esteem and quality of life in men[2]. ED is expected to exceed 300 million men by the year 2025[3]. Thus, Viagra® (Sildenafil citrate), the first-in-class phosphodiesterase type 5 inhibitor, was introduced in 1998 for the treatment of ED and has been demonstrated to be effective in treating this need[3]. Before Viagra® has drawn the public attention, men have already been seeking and rising different variety of substances in order to restore their sexual activities[1-4]. It has been discovered that there are many natural resources that are hypothetically known as aphrodisiac[5]. Some examples of popular Malaysian plants currently undergoing extensive research as male aphrodisiac are Eurycoma longifolia Jack and Andrographis paniculata[6]. Beside these plants, marine resources known to have aphrodisiac values are sea cucumbers and oysters[7,8]. The other is Aplysia dactylomela (A. dactylomela), a species of local sea slug known as ‘dugu–dugu’, which is popular among the Bajaus of Semporna, Sabah. They consume A. dactylomela raw to warm up their bodies before going to the sea[9]. Although there is no significant study on the aphrodisiac property of this marine creature previously, but there has been a study on marine resources like oyster which shares the same phylum Mollusca with A. dactylomela. Furthermore, it is believed that A. dactylomela contains high level of steroids. This is...
supported by Wang and Croll who stated that steroids have been known for decades to exist in the molluscs[10]. Therefore, in this study, A. dactylomela was selected to evaluate its aphrodisiac property.

2. Materials and methods

2.1. Sample collection

A. dactylomela were obtained from the Bajaus in Semporna, Sabah, Malaysia and stored at -40 °C in a deep freezer until further used.

2.2. Animals

Male and female imprinting control region mice averaging 30 g body weight were maintained under standardized conditions with temperature 22 °C, relative humidity 60%–70% and 12 h light–dark cycle for at least 1 week before used. Food and water were given ad libitum.

2.3. Reagents preparation

Sildenafil citrate or Viagra® (5 mg/kg) was dissolved in dimethyl sulfoxide (DMSO) upon commencement of the treatment. Meanwhile, Tween 80 (10%) was prepared by dissolving 10 g of Tween 80 into 1 000 mL of distilled water. The solution was mixed thoroughly and kept in a reagent bottle until used. Preparation of normal saline solution (0.9%) was done by dissolving 9 g of NaCl into 1 000 mL of distilled water. Meanwhile, preparation of DMSO (10%) solution was done by dissolving 10 g of DMSO into 100 mL of distilled water. Each solution was mixed thoroughly and kept in a reagent bottle until used.

2.4. Sample preparation

2.4.1. Aqueous extraction

About 100 g of A. dactylomela sample was thawed from the deep freezer and dissected to obtain only the muscle part while the visceral organs being removed which yield 50 g. The muscle was then washed 2 to 3 times thoroughly under running tap water to remove all traces of dirt and debris, and then chopped into small pieces before rinsing them again with distilled water. Next the chopped sample was blended with distilled water using electrical blender with ratio of 1:4. The mixture was then first filtered using the gauze pad and followed by filtration using Whatman filter paper No. 1. The filtered extract was kept at −80 °C in a freezer for 48 h before freeze dried. A. dactylomela powder was diluted with saline prior to treatment.

2.4.2. Lipid extraction

About 120 g of A. dactylomela muscle obtained by the above procedure was chopped into small pieces before rinsing them again with distilled water. Next, the sample was extracted by method described by Folch et al.[11] with slight modification. The chopped sample was dried in an oven at 70 °C until no change in mass was observed. The dried material was first blended to produce a fine powder. The dried sample of A. dactylomela was weighed before being dissolved in solvent. The weighed dried form of A. dactylomela was then homogenized with 200 mL of solvent mixture consisting of 150 mL chloroform and 50 mL methanol (3:1, v/v). The mixture was then shaken on the orbital shaker for 3 d before filtration using Whatman filter paper No. 1. The filtrate was collected and the solvent containing extracted compound was then removed by means of a rotary evaporator at 60 °C. Finally, the extract was dried in fume cupboard giving the final yield of extract in the form of a wax–like substance which was later diluted with 10 % Tween 80 prior to treatment.

2.5. Phytochemical screening

The entire phytochemical screening test was done according to the procedures of Raman[12].

2.5.1. Alkaloids testing (Dragendoff’s test)

A small amount of both aqueous and lipid extracts were diluted with distilled water and methanol, respectively. Each diluted extract was then mixed with 1 mL of 1 mol/L HCl in a test tube followed by adding 2 to 5 drops of Dragendoff’s reagent. The yellow to orange precipitate indicates a positive result.

2.5.2. Terpenoids and steroids testing (Liebermann–Burchard test)

A total of 2 mL of each diluted extract was transferred into a spot test plate and allowed to dry before mixing properly with 3 to 5 drops of acetic anhydride. Then, 1 to 2 drops of concentrated sulphuric acid was added (from the wall of spot test plate to let the acid to mix slowly). The color changes were observed, blue color indicates a positive result for terpenoids whereas purple color indicates positive for steroids.

2.5.3. Flavonoids testing

Two milliliters of each diluted extracts was mixed with three pieces of magnesium coils into different test tubes, followed by 0.5 mL of HCl and the solutions were allowed to mix and settle for 10 min. The color changes were observed, a red color indicating a positive result.

2.5.4. Saponins testing

Distilled water was added to the diluted samples until about
three fourth of the test tube’s height. Then, the solutions were vigorously shaken for a few minutes and allowed to stand for 15–20 min. The detection of the saponins content was classified as no froth (negative), froth less than 1.0 cm (weakly positive), froth 1.2 cm high (positive) and froth greater than 2.0 cm (strongly positive).

2.6. Mounting frequency

Assessment of mating was measured based on methods by Subramoniam et al.[13] with slight modification. Sixteen imprinting control region male mice were divided into 8 groups of 2 mice per group. Three treated groups received 50, 100 and 200 mg/kg body weight aqueous extract of A. dactylomela, respectively, while another 3 treated groups received lipid extract of A. dactylomela: 50, 100 and 200 mg/kg body weight, respectively. The positive control group received Sildenafil citrate (5 mg/kg), while the negative control group received normal saline. All doses were administered (i.p.) in a volume of 10 mL/kg body weight.

Mice were observed for 3 h and their behaviors were scored blindly as described[14]. Male mice were placed individually in a clear cage. After 1 h of administration of the extracts, a female mouse was introduced into the cage. The animals remain paired for 3 h. The number of mounts was recorded during 15 min observation period at the start of each hour. All experiments were performed from 09:00 to 14:00 h on sunny days.

2.7. Statistical analysis

In this study, recorded values were expressed as mean±SEM. Statistical significant was determined using Tukey’s test and independent t-test. Values with $P<0.05$ were considered significant.

3. Results

3.1. Phytochemical screening

Phytochemical screening of A. dactylomela showed presence of major metabolites of alkaloids, flavonoids and saponins for aqueous extract and steroids abundantly present in lipid extract (Table 1).

Table 1 Phytochemical screening test of A. dactylomela.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Alkaloid</th>
<th>Terpenoids</th>
<th>Steroid</th>
<th>Flavonoids</th>
<th>Saponins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>c</td>
</tr>
<tr>
<td>Lipid</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>+</td>
<td>b</td>
</tr>
</tbody>
</table>

+: Absence; +: Weakly presence; ++: Mildly presence; +++: Strongly presence; b: Froth less than 1 cm (weakly positive); c: Froth 1.2 cm high (positive).

3.2. Effects of A. dactylomela extracts on mounting behavior

Mounting frequency for the effect of different doses of aqueous and lipid extracts of A. dactylomela are shown in Table 2. The mean for aqueous and lipid extracts of A. dactylomela were 0.00±0.00 and 10.22±5.56, respectively.

Table 2 The effects of different doses and different extracts of A. dactylomela on mounting frequency of male mice.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Dose (mg/kg)</th>
<th>Mounting frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>2 h</td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Negative</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>Aqueous</td>
<td>50</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>–</td>
</tr>
<tr>
<td>Lipid</td>
<td>50</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5</td>
</tr>
</tbody>
</table>

+: No mounting frequency observed; Positive control: Sildenafil citrate; Negative control: Normal saline; Values were of three observations.

A Tukey’s post-hoc test illustrated in Tables 3 and 4 revealed that the mounting frequency was significantly increased in lipid 50 mg/kg (10.667±1.434, $P<0.01$) compared to the positive (10.00±1.53) and negative (2.00±1.16) control groups. There were no statistically significant differences between the other groups of treatments.

Table 3 Mounting frequency analysis of male mice in treated groups with positive control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mounting frequency</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs Aqueous A</td>
<td>10.000±1.434</td>
<td>&gt;0.001*</td>
</tr>
<tr>
<td>Control vs Aqueous B</td>
<td>10.000±1.434</td>
<td>&gt;0.001*</td>
</tr>
<tr>
<td>Control vs Aqueous C</td>
<td>10.000±1.434</td>
<td>&gt;0.001*</td>
</tr>
<tr>
<td>Control vs Lipid A</td>
<td>10.667±1.434</td>
<td>&gt;0.001*</td>
</tr>
<tr>
<td>Control vs Lipid B</td>
<td>5.000±1.434</td>
<td>0.048*</td>
</tr>
<tr>
<td>Control vs Lipid C</td>
<td>5.667±1.434</td>
<td>0.020*</td>
</tr>
</tbody>
</table>

A: 50 mg/kg; B: 100 mg/kg; C: 200 mg/kg; Each value represented mean±SEM, $n=3$; *$P<0.05$ (Tukey’s test).

Table 4 Mounting frequency analysis of male mice in treated groups with negative control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mounting frequency</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs Aqueous A</td>
<td>2.000±1.434</td>
<td>0.847</td>
</tr>
<tr>
<td>Control vs Aqueous B</td>
<td>2.000±1.434</td>
<td>0.847</td>
</tr>
<tr>
<td>Control vs Aqueous C</td>
<td>2.000±1.434</td>
<td>0.847</td>
</tr>
<tr>
<td>Control vs Lipid A</td>
<td>18.667±1.434</td>
<td>&gt;0.001*</td>
</tr>
<tr>
<td>Control vs Lipid B</td>
<td>3.000±1.434</td>
<td>0.458</td>
</tr>
<tr>
<td>Control vs Lipid C</td>
<td>2.333±1.434</td>
<td>0.729</td>
</tr>
</tbody>
</table>

A: 50 mg/kg; B: 100 mg/kg; C: 200 mg/kg; Each value represented mean±SEM, $n=3$; *$P<0.05$ (Tukey’s test).
4. Discussion

4.1. Phytochemical screening

Phytochemical screening can help to reveal the chemical constituents of A. dactylomela extract and the one that predominates over the others. It may also be used to search for bioactive component for further testing. The present study is parallel with Matsumoto et al., and Wang and Croll that showed steroids have been identified in various types of mollusks including octopus, slug, sea hare, land snail, soft–shell clam and oyster[10,15]. Earliest studies indicated that steroids involved in development of sexual organs[7,10,16]. Furthermore, it is also responsible for sexual maturation which involved testosterone hormone. A study showed that the slug, Arion ater has a capability to synthesize testosterone from steroid[16]. Steroids play a critical role in sexual development and are synthesized in steroidogenic tissues from a common precursor, the cholesterol[10]. This reflected the reason steroid was only found in lipid extract of A. dactylomela.

4.2. Mounting frequency

The mean differences of aqueous and lipid extracts of A. dactylomela indicated that the lipid extract was more effective as it possessed higher mean±SEM in mounting frequency compared to aqueous extract.

The present study revealed that the lipid extract of A. dactylomela could enhance male sexual behavior in normal mice for all doses (50, 100 and 200 mg/kg) administered. Similarly, previous study concluded that alcohol extract was used and found to be active in relation to sexual behaviour[7,13,14,17].

Mounting frequency of male mice administered with lipid extract at 50 and 100 mg/kg were found to be higher compared to extract at 200 mg/kg over the 3 hour observation. This might be due to failure to produce greater response as the dose increased which is known as maximal efficacy[18]. Thus, at concentration of 200 mg/kg of lipid extract, maximal efficacy has been reached. In relation to time, it was illustrated that the effect of the lipid extract of A. dactylomela diminished over time. During the 2nd and 3rd hour of treatment, the bioactive compound seems to have undergone metabolic degradation[19].

In this experiment, it was observed that the aqueous extract of A. dactylomela did not produce any mounting behavior. The observed mice were very passive at higher doses, 100 and 200 mg/kg within the observation period. This result deviated with the dose–response principles, where larger doses would produce larger effects. However, the observations obtained are probably due to the effect of particular drug which enhanced the responses at lower dose and showed no observable effect at higher dose. At higher dose, it would impair the response[7].

Mounting frequency was broadly used in the studies of aphrodisiac[1,13,14,17,20], thus it was taken as a primary parameter in this study. Huge difference between aqueous and lipid extract on the mean of mounting frequency suggested that the active compound inside the extract was responsible for the mounting frequency in all doses administered. Lipid extract will cross the blood brain barrier causing an increase in several anterior pituitary hormones concentration at the limbic system which stimulates the production of testosterone[21,22].

Libido and mounting frequency are enhanced by elevated testosterone levels[14,23], and the response will decrease when optimum concentration of hormone is reached. These negative feedback principles were due to the testosterone concentration in the blood increasing to its optimum and inhibiting the release of gonadotrophin–releasing hormone by the hypothalamus that resulted in less gonadotrophin–releasing hormone being secreted into the blood that flow to the anterior pituitary. Therefore, anterior pituitary would reduce luteinizing hormone concentration in the systemic blood and cause leydig cells to secrete low testosterone level[24].

Statistical analysis from Tukey’s test showed there was no significant increase in mounting frequency after administration of aqueous extract 50, 100, 200 mg/kg and lipid extract 100, 200 mg/kg compared to both positive and negative control groups. Reasons brought to this insignificant result was because of other internal and external factors such as physiological condition[25], sounds, diet, lighting, and female pheromone which is a chemical substance secreted externally by some animals (especially insects) that influences the physiology or behavior of other animals of the same species[7].

Contrarily, there was a statistically significant difference between the lipid 50 mg/kg compared to the positive and negative groups. Based on this evidence, it showed that the administration of lipid extract of A. dactylomela at 50 mg/kg concentration possessed substantial value in aphrodisiac property more than the positive control, Sildenafil citrate. This suggested that the active constituent in the lipid extract of A. dactylomela at this concentration is more effective than Sildenafil citrate on mounting frequency test. The standard Sildenafil citrate drug was used only as a reference for quantitative comparison and not for the mechanisms of the reaction.

This finding provides preliminary scientific evidence that A. dactylomela extract was pharmacologically tested possesses an aphrodisiac value. Lipid extract of A. dactylomela showed significant evidence compared to
aqueous extract in mounting frequency of male mice at doses of 50, 100 and 200 mg/kg. As the incidence of male sexual dysfunction is increasing, which need more and rapid search of the natural products with aphrodisiac potentials, this A. dactylomela could be treated as one of the alternative medications from natural means to mend sexual activity in men. Align with the detection of steroid in lipid extract through phytochemical screening test, the lipid extract especially at concentration of 50 mg/kg showed significant difference \( P<0.05 \) with both positive and negative controls in mounting frequency test. It was found that the extract significantly increased mounting frequency and indicated an improvement in sexual behaviour of the treated animals. In contrast, as it is no detection of steroid in aqueous extract, there was no mounting behaviour been observed in aqueous extract of A. dactylomela.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**Acknowledgements**

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**References**


