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Aphrodisiac Activity of Clove Leaves (*Syzygium aromaticum* L.) Ethanol Extract and Fractions in Wistar rats

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Abstract: Erectile dysfunction (ED) and premature ejaculation are prevalent male sexual dysfunctions affecting various age groups. This study explores the potential aphrodisiac effects of Clove leaves (*Syzygium aromaticum* L.), a plant rich in secondary metabolites, including alkaloids, flavonoids, saponins, and steroids. Fractionation of the ethanol extract yielded n-hexane, ethyl acetate, and water fractions. Phytochemical screening revealed the presence of alkaloids, flavonoids, and saponins in the water fraction, while steroids were detected in the ethanol extract, ethyl acetate, and n-hexane fractions. In vivo tests on male rats demonstrated that the ethanol extract, n-hexane, and ethyl acetate fractions significantly reduced mounting latency (ML), increased mounting frequency (MF), and accelerated intromission latency (IL), indicating heightened sexual arousal and endurance. Moreover, these fractions delayed ejaculatory latency (EL) and increased ejaculation frequency (EF), akin to the positive control, X-Gra. Notably, the n-hexane fraction showed the closest efficacy to X-Gra. The presence of steroids in the active fractions suggests their role in eliciting aphrodisiac effects. Further isolation and purification of the active compound(s) may optimize therapeutic outcomes. This study underscores the potential of Clove leaf fractions as natural aphrodisiacs, warranting further investigation for clinical applications.

Introduction

The inability of the male reproductive organ to engage in sexual intercourse due to the failure to achieve penile erection for sexual satisfaction is known as erectile dysfunction (ED) (1, 2). Erectile dysfunction affects men of all age groups, particularly those aged between 40 and 70 (3, 4). Premature ejaculation is a male sexual dysfunction characterized by frequent or almost constant ejaculation approximately 1 minute before or within the vagina during penetration, and the inability to delay ejaculation in (almost) every penetration, resulting in negative consequences such as worry, anxiety, frustration, or avoidance of sexual relations (5, 6).

Various efforts have been studied to address sexual dysfunction issues, one of which involves the use of plants with aphrodisiac properties. Aphrodisiacs act as sexual stimulants and can enhance libido (7, 8). There are numerous plants that can be used as natural herbal remedies for aphrodisiac purposes in traditional Indonesian medicine, one of which is the clove plant (8, 9). The leaves of the clove plant are among the plants that contain saponins, flavonoids, tannins, sterols, and essential oils, acting as natural antioxidants (10, 11). Clove leaf oil contains phenolic components such as eugenol and eugenol acetate, as well as caryophyllene and sesquiterpenes, suggesting its potential as an aphrodisiac that enhances sexual desire (9). However, thus far, the testing of the aphrodisiac effect of Clove leaves has only extended to its extract and oil (9, 12, 13), with no comparison yet regarding the aphrodisiac effects of the polar and non-polar fractions from the ethanol extract of Clove leaves. Therefore, this study serves as a preliminary investigation to screen which fraction produces the highest aphrodisiac effect and to identify the likely compound groups responsible for such effects.

Experimental Section

Materials

Distilled water, hydrochloric acid (HCL), acetic acid, aluminum foil, Dragendorff LP, 96% ethanol, ferric chloride ($FeCl_3$), gel, hydrochloric acid (HCl), sulfuric acid (H_2SO_4), cotton, chloroform, 10% sodium hydroxide (NaOH), 10% sodium chloride (NaCl), n-hexane, carboxymethyl cellulose sodium 0.5%, cling film, clove plant raw material (*Syzygium aromaticum* L), copper acetate, and X-gra (Capsule, 150 mg, PT. Phapros Tbk., Indonesia).

Plant Preparation and Extraction

The leaf of *S. aromaticum* (clove leaf) was collected from Sibado Village, Sirenja Subdistrict, Donggala District. The selected plant material was verified by Universitas Tadulako, UPT. Sumber Daya Hayati Sulawesi (*Herbarium Celebense*), to ensure that the leaves indeed belonged to the clove plant. The clove leaves are air-dried under sunlight and subsequently powdered using a blender. A quantity of 400 g of clove leaf powder was extracted using the maceration method with 98% ethanol as the solvent in a volume of 1 L. Maceration was carried out for 3 days at room temperature (25±2°C). The liquid extract was then filtered to eliminate coarse particles/powder from the clove leaves, and it was concentrated using a rotary evaporator at a temperature of 40°C, 150 rpm, and 200 mBar. The concentrated extract was then dried at room temperature with the aid of a fan until partially dry, and its weight was measured to determine the percentage yield.

Fractination

In this study, we used a stepwise fractionation procedure to separate compounds from a plant extract using n-hexane, ethyl acetate, and water. This procedure is based on the increasing polarity of the solvents, with n-hexane being the least polar and water being the most polar. In the fractionation process, n-hexane, comprising 10 times the volume of the extract, was introduced into a centrifuge tube. Subsequently, the tube underwent vigorous shaking for 5 minutes, followed by centrifugation at 10,000 rpm for 10 min. The resulting upper phase (n-hexane) was carefully collected, and the lower phase (extract) was then transferred to a new centrifuge tube. A similar procedure was repeated for the second fractionation, where ethanol, in a volume 5 times that of the extract, was vigorously shaken with the centrifuge tube, followed by centrifugation at 10,000 rpm for 10 min. The upper phase (ethyl acetate) was collected, and the lower phase (extract) was again transferred. For the final fractionation, water, at a volume 10 times that of the extract, was added to the centrifuge tube, shaken for 5 minutes, and then centrifuged at 10,000 rpm for 10 min. The upper phase (water) was collected, while the lower phase (extract) was discarded. This sequential process allowed for the systematic separation of compounds based on their solubilities in n-hexane, ethanol, and water, contributing to the isolation of distinct fractions for further analysis.

Phytochemical Screening

Alkaloid

The identification of alkaloids involved the utilization of Dragendorff's reagent. A few drops of Dragendorff's reagent were added to the plant extract, and the occurrence of orange-red precipitates or color changes was observed, signifying the presence of alkaloids. This reaction was visually assessed.

Flavonoid

Flavonoids were identified using a distinctive reaction with HCl and magnesium. A few drops of concentrated HCl were added to the plant extract, followed by the addition of a small piece of magnesium. The development of a pink to red coloration was observed, indicating the presence of flavonoids.

Saponin

Saponins were identified through a froth test. The plant extract was vigorously shaken, resulting in the formation of a stable foam. Additionally, HCl was added to the shaken extract, enhancing the frothing. The persistent and stable froth formation was noted, confirming the presence of saponins in the plant extract.

Tannin

Tannins were identified through a reaction with a mixture of $FeCl_3$ and NaOH 10%. The reagents were added to the plant extract, leading to the development of a blue-black or greenish-black color, indicating the presence of tannins. This color change was visually assessed.

Steroid

Steroids were identified using a mixture of acetic anhydride and sulfuric acid. The reagent was added to the plant extract, resulting in the formation of a bluish-green coloration, signifying the presence of steroids. This visual reaction is an indicative of steroid-sulfuric acid complex formation.

In Vivo Test

Animal Preparation and Grouping

The preparation of experimental animals was conducted in accordance with stringent ethical guidelines and welfare considerations, following the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research provided by the Committee on Animal Research and Ethics. Animal ethics approval (letter number: 2576/UN.28.1.30./K/2019) was obtained from Universitas Tadulako prior to the commencement of

the study. Suitable housing conditions, including adequate space, proper ventilation, and a controlled environment replicating their natural habitat, were provided for the animals.

The experimental subjects consisted of male and female white rats (Rattus norvegicus) weighing approximately 250 g, characterized by good health, cleanliness, and activity. After an acclimatization period, a total of 30 male rats and 60 female rats were distributed into 6 treatment groups and housed separately in different cages with a male-to-female ratio of 1:2. The first group (Group I) served as the normal group (administered with a 0.5% NaCMC solution), the second group (Group II) received X-Gra at 150 mg/KgBW as the positive control, the third group (Group III) was given ethanol extract at 250 mg/KgBW, the fourth group (Group IV) received n-hexane fraction at 250 mg/KgBW, the fifth group (Group V) was administered ethyl acetate fraction at 250 mg/KgBW, and the sixth group (Group VI) was treated with water fraction at 250 mg/KgBW. The rationale for determining the test sample dose is based on a previous study that used a dose of 500 mg/KgBW of a 50% ethanol extract from clove leaves (9). We reduced this to 250 mg/KgBW because our samples are fractions that are more concentrated and contain fewer matrix components compared to the extract.

Treatment and Observation

The extracts, fractions, and X-Gra were suspended in 0.5% NaCMC. Male rats in groups 2-6 were administered the respective suspension solutions as described in the previous section. The administration of these suspension solutions continued for 14 days, after which the male rats were placed together with female rats in a single cage for each group. Prior to cohabitation, the male rats were acclimated to their cages for 5 minutes under red light (75W). Two days earlier, the female rats were given estradiol valerate in accordance with the study conducted by Brawer et al (1978). Their interactions were observed and recorded based on parameters described in the previous study, including mounting latency (ML), intromission latency (IL), ejaculatory latency (EL), mounting frequency (MF), intromission frequency (IF), and ejaculation frequency (EF).

Statistical Analysis

The ML, MF, IL, IF, EL, and EF values from each group were tested for normality and homogeneity to determine the appropriate post-hoc test. Subsequently, One-Way ANOVA and Tukey tests were employed to analyze whether there were significant differences among groups with normal and homogenous data. Meanwhile, the Kruskal-Wallis test was applied to groups with data that were not normal or homogenous. This statistical analysis was conducted using RStudio (Version 4.2.1, RStudio Inc, Boston, USA).

Result and Discussion

Extract and Fractions Yield

The obtained dried extract amounted to 98 g, with a yield percentage of 24.5%. After fractionation, the n-hexane, ethyl acetate, and water fractions were obtained in quantities of 8.35 g, 6.28 g, and 1.77 g, respectively, with yield percentages of 8.5%, 6.4%, and 1.8%. The decrease in percentage yield of the last fraction is a common occurrence with increased levels of fractionation. This phenomenon is attributed to the significant removal of compounds during the initial fractionation process, particularly as the content has already been extensively sieved. The phytochemical contents within the extract and fractions are elaborated in the following section.

Phytochemicals in Extract and Fractions

Table 1 shows that the ethanol extract indicates the presence of all types of secondary metabolites analyzed except for tannins. Meanwhile, the water fraction shows detection for alkaloids, flavonoids, and saponins. The solubility of alkaloids in water is due to their ionic form, making them more soluble in water than in organic solvents (14). Flavonoids, typically polar in nature, are also detected in the water fraction (15). Saponins were present in water but not in ethyl acetate and n-hexane. Tannin was not detected in the extract and all fractions, while steroids were only detected in the extract, ethyl acetate, and n-hexane fractions.

Table 1. Secondary metabolites identified in clove leaf ethanol extract and its fractions.

No.	Phytochemicals	Results			
		Ethanol	Water	Ethyl Acetate	N-Hexane
1.	Alkaloid	+	+	+	-
2.	Flavonoid	+	+	-	-
3.	Saponin	+	+	-	-
4.	Tanin	-	-	-	-
5.	Steroid	+	-	+	+

Aphrosidiacs Activity

The results of normality and homogeneity tests showed that MF, IF, and IL data were normally and homogeneously distributed ($p>0.05$). Therefore, One Way Anova and Tukey-test were used for statistical analysis of these parameters. ML, EF, and EL did not follow normal and homogenous distributions ($p<0.05$), so the Kruskal-Wallis test was applied.

Regarding ML, which measures the time from pairing male and female rats to the first mounting within 30 minutes, Group II exhibited the lowest average ML value, followed by Group IV, V, III, VI, and I

(refer to Figure 1). No significant difference was observed between Group II and the fraction groups (Group IV and V) except for Group VI. Group II and IV also showed a significant difference compared to Group III. All treated groups were significantly different from Group I, except for Group VI. This indicates that animals administered with X-Gra, ethanol extract, n-hexane fraction, and ethyl acetate fraction from Clove leaves were capable of accelerating the increase in the test animals' arousal, as indicated by a faster ML compared to the negative control and water fraction. It also suggests that the steroid group is responsible for this effect, as this group is present in the ethanol extract, n-hexane fraction, and ethyl acetate fraction, whereas it is absent in the water fraction. The use of steroids is known to increase sexual desire and they naturally occur in our bodies to regulate sexual desire (16, 17).

MF measures the frequency of male rats mounting female rats within 30 min. Once again, Group II displayed the highest average MF value, followed by Group VI and V. While these three groups did not show significant differences among themselves, they were significant when compared to Group I, III, and VI. This suggests that Groups II, VI, and V tend to restore the test animals' desire to resume mounting compared to the negative control group, ethanol extract group, and water fraction group (18). It is also evident here that the ethanol extract containing steroids has a relatively lower effect compared to the n-hexane and ethyl acetate fractions. This might be attributed to a higher concentration of steroids in the latter two fractions, resulting in a larger amount of steroids being administered at the same dose compared to the ethanol extract.

IL measures the time from pairing male and female rats to the first intromission, while IF measures the number of intromissions performed by male rats (19). In terms of the IL parameter, Group II has the lowest value, followed by Group V, IV, and III, and these four groups are not significantly different. However, they are all significantly different when compared to Group I and VI. This indicates that these four groups significantly stimulate the acceleration of intromission. Meanwhile, in the IF parameter, Group II exhibits the highest and significantly different value compared to all other groups. This implies that the group administered with X-Gra can prolong the sexual endurance of the test animals. In addition to Group II, Group IV and V also show high and significant IF values compared to Group I and VI.

EL measures the time from the first intromission to ejaculation, while EF denotes the number of ejaculations. Regarding the EL parameter, Group II, IV, and V have the highest and significantly different values compared to Group I and III. This confirms that these three groups can enhance sexual endurance and delay ejaculation. Furthermore, based on EF values, Group II and IV can increase ejaculation frequency in some rats with an average increase of 1.5 times. From all these test parameters, it is evident that X-Gra, as a positive control, and the n-hexane fraction can stimulate the sexual desire of the test animals, prolong the duration of intromission, and increase the frequency of ejaculation.

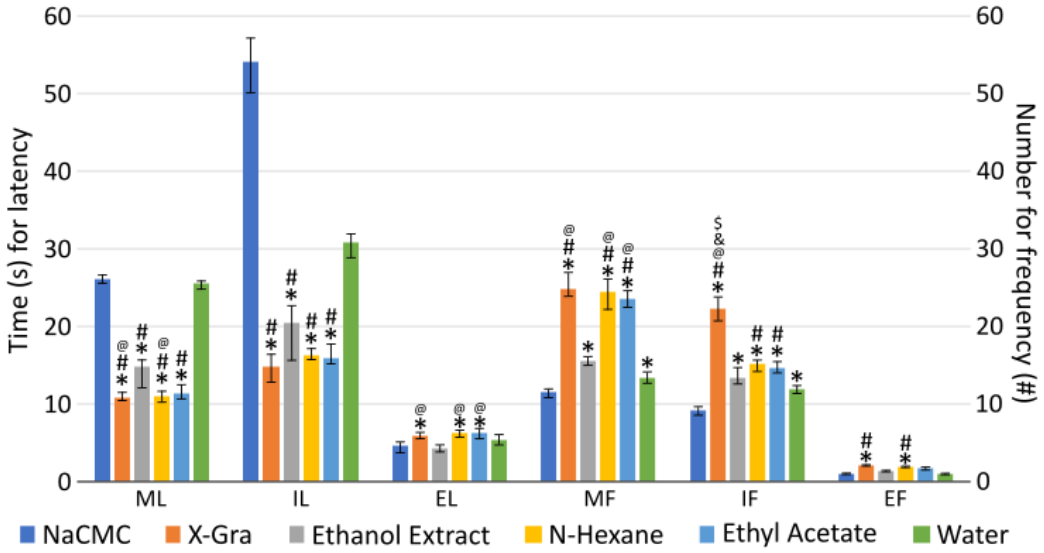

Figure 1. Interaction values of male and female rats after 14 days treatment. Note: ML = mounting latency, IL = intromission latency, EL = ejaculatory latency, MF = mounting frequency, IF = intromission frequency, and EF = ejaculatory frequency. (*, $p<0.05$) shows a significant difference to group normal, (#, $p<0.05$) shows significant difference to group positive control (X-Gra), (@, $p<0.05$) shows significant difference to ethanol extract group, (&, $p<0.05$) shows significant difference to N-hexane group, and ($, $p<0.05$) shows significant difference to ethyl acetate group.

Conclusion

Based on the results obtained, the ethanol extract of clove leaves contains alkaloids, flavonoids, saponins, and steroids. Among its three fractions, only n-hexane and ethyl acetate contain steroids. The ethanol extract, n-hexane fraction, and ethyl acetate fraction from clove leaves exhibit a high aphrodisiac effect, nearly equivalent to the effect produced by X-Gra, with the n-hexane fraction being the closest. It is essential to note that the n-hexane fraction is still in a mixed matrix form, resulting in a lower effect compared to the positive control. Based on the metabolite content, it is likely that the steroid compound is responsible for the aphrodisiac effect produced. Isolating the responsible compound with optimal purity allows for the generation of a more optimal effect.

Declarations

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Conflict of Interest

The authors declare no conflicting interest.

Data Availability

The unpublished data is available upon request to the corresponding author.

Ethics Statement

Animal ethics approval (letter number: 2576/UN.28.1.30./K/2019) was obtained from Universitas Tadulako.

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Not applicable.

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