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Research Article

A study on compounds with spermicidal potential in *Calophyllum inophyllum* L. seed shell towards *Mus musculus*

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Abstract
Calophyllum inophyllum L is a plant found on the coast and is known to have pharmacological effects such as anti-oxidant, anti-cancer, anti-viral and anti-inflammatory. This study aimed to determine the ability of the ethanolic extract of the sampling seed shells as a spermicide for albino mice (*Mus musculus*), determine the growth inhibition of Candida albicans, and identify its compounds. The extraction of the *C. inophyllum* L seed shell was carried out by maceration. The quality of motility and abnormal morphology of mice sperms were observed under the microscope. The fungus inhibition assay was carried out by diffusion agar, and the identification of the active compounds by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC/MS-MS). Sperm motility in ethanolic extract at the concentrations of 0.1, 0.3, and 0.5% were 10.85, 10.05, and 10.85%, respectively, and the abnormal morphology was 63.50, 65.62, and 72.67%, respectively. The partition results of the n-hexane extract showed that motility and abnormal morphology values at the concentrations of 0.1, 0.3, and 0.5% were 79.21 and 79.93%, and the abnormal morphology was 8.49, 4.30 and 0.00%, respectively. Fraction F6 at the concentration of 0.3% showed that its motility was 0.00% and abnormal morphology was 65.86%, so it did not provide inhibition against the *C. albicans* fungus. Fraction F6 contained compounds that had spermicidal potential were 2,5-Dioxo-1- pyrrolidinyl N-[(benzyloxy) carbonyl]-D-phenylalaninate, raubasine, and N-(4-{[2,4-Diamino-7,8-dyhydro-6 pteridinyl) methyl] (methyl) amino} benzoyl) L-glutamic acid or methotrexate through to cytotoxic effects.

Keywords: *Calophyllum inophyllum* L, 2,5-Dioxo-1- pyrrolidinyl N-[(benzyloxy) carbonyl]-D-phenylalaninate, Methotrexate, Raubasine, Spermicide

INTRODUCTION

The *Calophyllum inophyllum* L. has toxic properties (Emilda, 2019; Sukadana *et al*., 2009). Nyamplung seed shells have cytotoxic activity in vitro against HeLa cells with an LC50 value of 63.09 ppm (Sukadana *et al*., 2011) and the shells and seeds have cytotoxic activity against WiDr cells with an LC50 value of 42.47 ppm (Fathani (2020). *C. inophyllum* L seeds are known to contain secondary metabolite compounds tannin,

alkaloids, and saponins, which can act as spermicides (Asif, 2013; Dimas *et al*, 2023; Merillon and Ramawat, 2020) and the seed oil is known to contain flavonoids, steroids, and saponins (Safrina *et al*., 2020; Umamagheswari, 2017). In the shells and seeds of nyamplung, it is known there are polar toxic compounds such as triterpenoids, coumarins, flavonoids, saponins, and resins (Irfan and Isnatin, 2021). The toxicity and compound content of the *C. inophyllum* L plant strengthens the suspicion that this plant has the potential to be a spermicide (Donkor, 2020). The effectiveness of a spermicide can be seen from its ability to reduce the quality of spermatozoa. Parameters of good spermatozoa quality are spermatozoa that have agile motility and good morphology. (Ashfahani *et al*., 2010). The motility and morphology of spermatozoa are closely related to the fertilization process. The present study aimed to_determine the spermicidal effect of *Calophyllum inophyllum* L seed shell extract on the motility and morphology of mice spermatozoa *in vitro* and anti-fungal tests against floral *Candida albicans* (Sobel, 1999).

MATERIALS AND METHODS

Material

The material used in the research was the seed shell of the *C. inophyllum* L plant. These plants were obtained from the coastal area of Gilimanuk Negara, Jembrana-Bali. Determination of plant taxonomy was carried out at Unit Pelayanan Teknis-Lembaga Ilmu Pengetahuan Indonesia (UPT- LIPI), Plant Conservation Center "Eka Karya" Bedugul Bali Botanical Garden. The biological material used in this research was 25 male of albino mice *Mus musculus* Balb/c strain aged 2-3 months with a body weight of 20-30 g, obtained from the Faculty of Veterinary Medicine, Udayana University.

Animal ethical approval

All experiments related to the use of experimental animals in this study received an Ethical Clearance statement approval from the Research Ethics Commission of the Faculty of Medicine, Udayana University, Denpasar, Bali, Indonesia.

Methods

The dried powder of the *C. inophyllum* L seeds shell was macerated using 96% ethanol solvent (Kartika, 2018). The concentrated ethanol extract was suspended in a solvent mixture of ethanol and water (7:3), and then the ethanol was evaporated to obtain a water extract. The water extract obtained was then partitioned successively with n-hexane and chloroform to obtain n-hexane, chloroform, and water extracts, each was evaporated to obtain concentrated extracts of n-hexane, chloroform, and water. The third extracts were tested for motility and morphology on mice (*M. musculus*) spermatozoa.

The spermicide test was done by taking cauda epididymis secretions by sacrificing mice by neck discollation and then dissecting them. The cauda epididymis was taken and placed into a petri dish containing 0.9% physiological NaCl. The cauda epididymis was separated from the testis by cutting the proximal part into a watch glass containing 1 mL of physiological NaCl, then the proximal part of the cauda was cut slightly with scissors, then the cauda was gently pressed until the epididymal fluid came out and was suspended in 0.9% NaCl, then stirred until homogeneous. The spermatozoa suspension was then mixed with Nyamplung seed shell extract in a 1:1 ratio. The mixture was stirred until homogeneous, and one drop was placed on a clean glass object. The observations were made on the quality of spermatozoa, including motility and morphology.

Determination of motility

Analysis of spermatozoa motility was carried out by dripping 1 drop of semen on a glass slide. The droplets were of the same size for each examination. Then, covered with a glass cover for spermatozoa motility in a visual field and an assessment of spermatozoa motility (Suhadi and Arsyad, 1983) was done:

a. Spermatozoa with good motility, namely fast movement, straightforwardness, agility, and activeness (%).
b. Spermatozoa with poor motility i.e. any movement other than spermatozoa with good motility (%)
c. Non-motile spermatozoa (%).

Analysis of the speed of spermatozoa movement was measured by calculating the time (seconds) required to travel 1 box of the microhemocytometer by motile and straight spermatozoa. The value of the movement speed was measured in units of µm/second (Hartamto, 1985).

Determination of morphology

The spermatozoa morphology was evaluated by staining Eosin nigrosine by dripping the suspension at the end of the object glass. The extract was added in a ratio of 1:1, and then Eosin nigrosine dye was added. The number of drops was taken, to be the same size, then sand. Then smear preparations were made and then aired until dry. The preparations were then observed und be er a microscope with a magnification of 40× to count the number of damaged spermatozoa. Morphology can be calculated using the following formula:

$$\%\ \text{Morphology abnormal cell} = B/(B+A) \times 100\% \qquad \text{Eq.1}$$

information
A: Number of normal spermatozo
B: Number of abnormal spermatozoa

Spermicidal tests on motility and morphology were carried out on column fractions and isolates in the same way.

***Candida albicans* Inhibitory test**

One to two oses (inoculating loop) of *Candida albicans* were taken and applied to the surface of the SDA, then incubated at room temperature for 1-2 days. The fungal suspension was added with NaCl until a certain turbidity according to the 0.5 Mc Farland standard (108 CFU/ml). Then a sterile cotton swab was dipped into the fungal suspension, pressed on the tube wall until the cotton was not too wet, and applied to the surface of the SDA. A well of a diameter of 6 mm was made and incubated at 37°C for 1-2 days and then measured the diameter of the inhibition zone (Wulan and Rian, 2023).

RESULTS AND DISCUSSION

The results of maceration of 1.2 kg of dried powder of *C. inophyllum* L seed shell (water content 8.06 ± 0.83%) with 96% ethanol for 12 × 24 hours produced 20.08 g of the concentrated extract with a dark brown-black color or a yield of 1.67%. Spermicide testing for motility in ethanol extracts and partitioned extracts were 10.85, 10.05, and 10.95% at a concentration of 0.1; 0.3; and 0.5%, respectively, while the extract n-hexane had a very high spermicidal effect with a motility value were 8.49; 4.30; and 0.00% at the same concentration. The normal spermatozoa motility category is > 50%, and if the spermatozoa are < 50%, the sperm motility is known in the subfertile category (Peter *et al*., 2021).

Spermicide testing for morphological abnormalities in ethanol extracts and partition extracts at a concentration of 0.1, 0.3, and 0.5%, respectively, showed that the morphological value of cell damage was 63.50, 65.62, and 72.67%, while the partition results showed that n-hexane had a very high spermicidal effect with a morphological abnormality value of 77.84, 79.21 and 79.93. The sperm morphology of 0.5% n-hexane extract shows an abnormal shape with a morphology value of 79.93%, as shown in Fig. 1 and normal sperm morphology is shown in Fig. 2.

The observation on spermatozoa motility and morphology parameters showed a significant decrease in all extract treatments compared to control and placebo. In the control and placebo, it was found that the spermatozoa had a high level of motile ability, namely 100% alive with progressive movement and no visible morphological damage to certain parts of the spermatozoa. The motility of the partitioned extract showed that n-hexane at a concentration of 0.5% had the highest spermicidal effect with a motility value of 0.00%. Normal spermatozoa motility was > 50% and if spermatozoa motility was < 50%, the sperm motility was categorized as subfertile sperm (Peter *et al*., 2021).

According to Toelihere (2010), the classification of spermatozoa abnormalities has several categories namely the primary category includes a head that is too large (macrocephalic), a head that is too small (microcephalic), a short head that is wide, flat, elongated and piriform, bent, enlarged or connected abaxially at the base of the head, and the tail circular, broken, or split. Secondary abnormalities were characterized by a broken spermatozoa tail, a head without a tail, and a folded middle part. This showed that the *C. inophyllum* L seed shell had active sub-

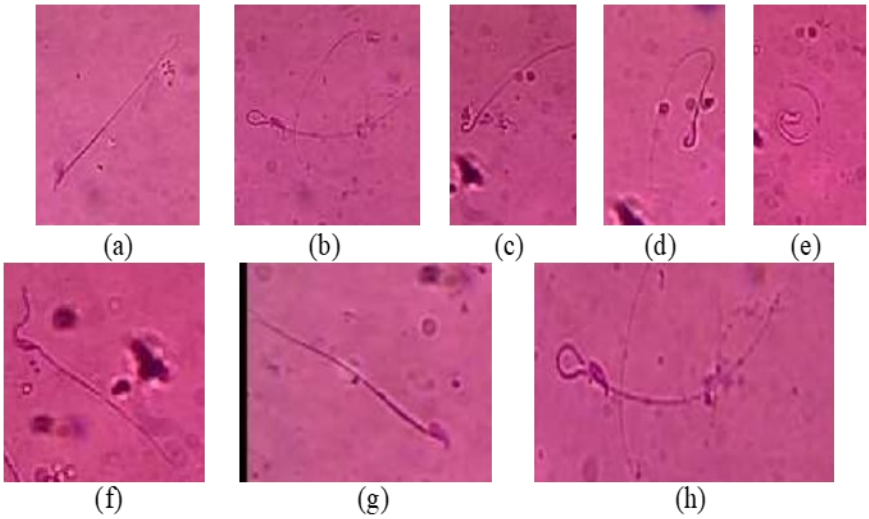


Fig. 1. *(a), Sperm with a folded tail (b), Sperm with a folded neck (c), Sperm with a bent neck (d), Sperm with an abixal neck and a bent tail (e), Sperm with a circular body and a rounded tail (f), Sperm with a bent neck (g), Sperm tail bent (h), Sperm neck bent abixally*

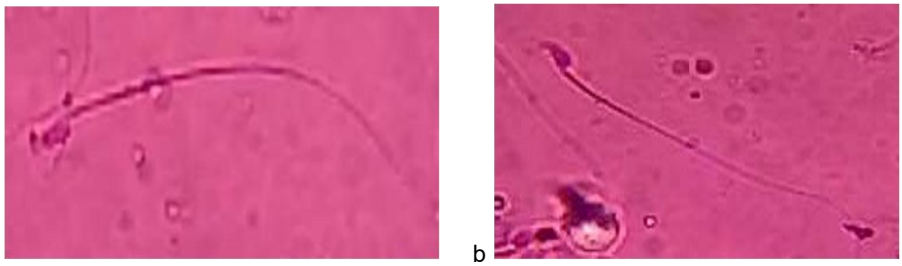


Fig. 2. *(a) and (b) Normal spermatozoa*

stance significantly reduces spermatozoa's quality, especially for motility and morphology parameters. This decrease in the quality of spermatozoa is thought to be caused by the presence of active substances in the *C. inophyllum* L seed shell, which are cytotoxic or have a spermicidal effect on spermatozoa.

The results of separation using column chromatography of the n-hexane extract produced 26 eluates, which were then analyzed using TLC to group eluates with the same stain and separation pattern. The mobile phase used in the Fraction combining process was the best eluent n-hexane-chloroform (9:1). The results of combining TLC obtained 15 fractions. Due to consideration of the weight of the fractions obtained, fractions F2, F4, F9, and F10 were not subjected to further research stages, so only 11 fractions were tested for motility and morphological abnormalities at a concentration of 0.3%. Based on 11 fractions tested on spermatozoa, only fraction F6 had a 0% percentage of motility and 67.85% damage to spermatozoa morphology. The identification results using LC-MS/MS on F6 obtained a chromatogram with 14 peaks at different retention times where only 12 compounds were successfully identified based on the database approach, presented in Fig. 3 and Table 1.

Of the 12 compounds that were identified based on the database approach, the compounds that have the potential to act as spermicides were: 2,5-Dioxo-1-pyrrolidinyl N-[(benzyloxy) carbonyl]-yD-phenylalanine has the potential to be spermicidal through cytotoxic effects and hormonal effects thereby disrupting hormonal balance. Raubasine has the potential to be a spermicide because of its toxic properties, which damage spermatozoa cells in the tail, thereby reducing spermatozoa motility. N-(4-{[2,4-Diamino-7,8-dyhydro-6pteridinyl) methyl] (methyl) amino} benzoyl)-L-glutamic

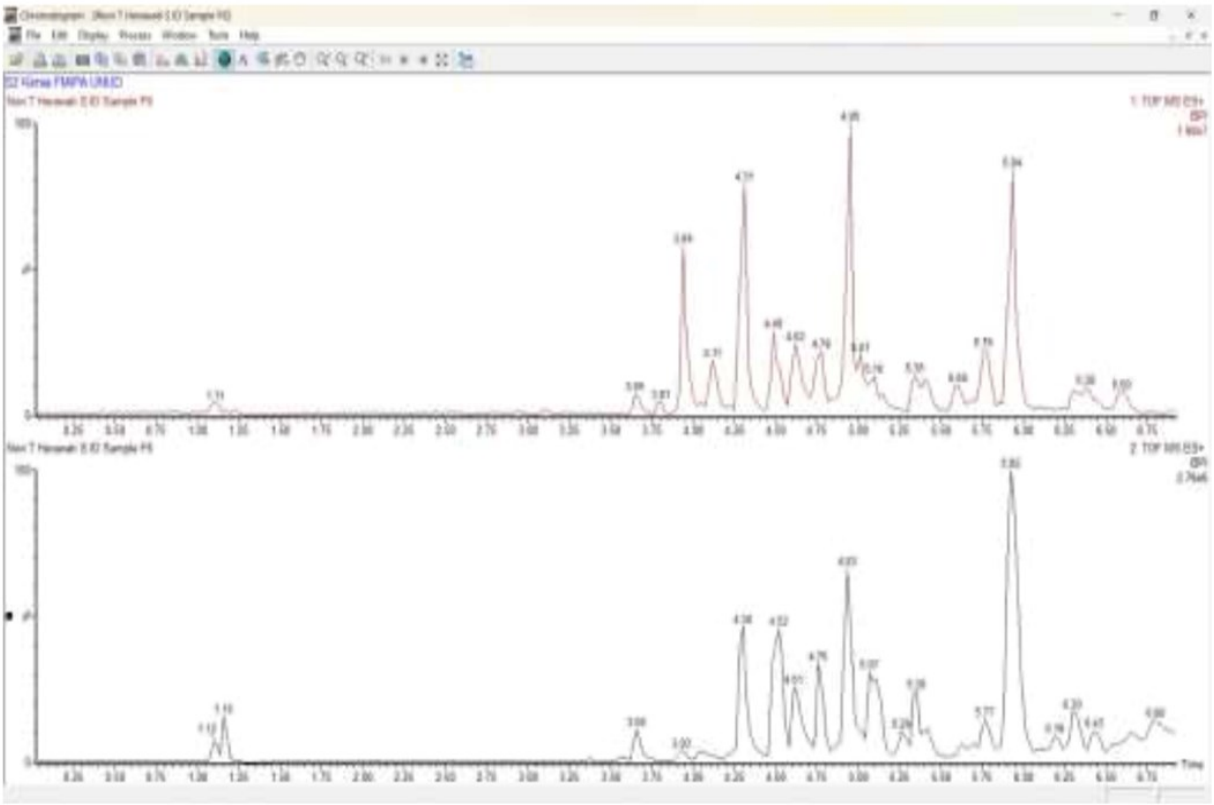


Fig. 3. *Chromatograph of F6 fraction with Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS)*

Table 1. Compound identification results for F6 using Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS)

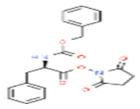
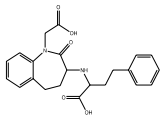
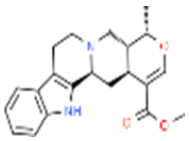
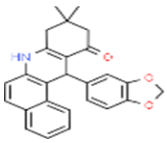
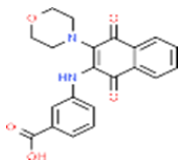
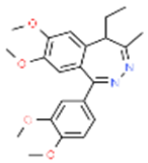
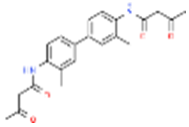
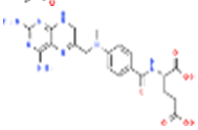
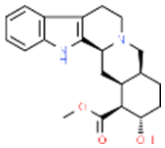
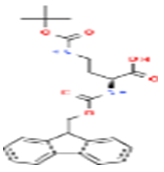
No.	Retention Time	Ion $M+H^+$ (m/z)	Chemical Formula	Alleged Compound	Structure
1.	3.65	397.1401	$C_{21}H_{21}N_2O_6$	Z-D-PHE-OSU-2,5-Dioxo-1-pyrrolidinyl N-[(benzyloxy)carbonyl]-D-phenylalaninenate	
2.	3.81	397.1401	$C_{18}H_{12}N_{12}$	Unidentified	
3.	3.93	397.1759	$C_{22}H_{25}N_2O_5$	Benazeprilat	
4.	4.31	353.1854	$C_{21}H_{25}N_2O_3$	Raubasine	
5.	4.48	398.1792	$C_{26}H_{24}NO_3$	12-(1,3Benzodioxo 1-5-yl)-9,9-dimethyl-8,9,10,12-tetrahydrobenzo[a] acridin-11(7H)-one	
6.	4.62	397.1294	$C_{21}H_{19}N_2O_5$	3-[[3-(4-morpholinyl)-1,4-dioxo-2-naphthalenyl]amino]benzoic acid	
7.	4.77	383.1968	$C_{22}H_{27}N_2O_4$	Tofisopam	
8.	4.95	569.3123		Unidentified	
9.	5.06	381.1809	$C_{22}H_{25}N_2O_4$	Naphtanilide G	
10.	5.40	457.1970	$C_{20}H_{25}N_8O_5$	(Methotrexate) N-(4-{[2,4-Diamino-7,8-dihydro-6-pteridinyl)methyl](methyl) amino}benzoyl)-L-glutamic acid	
11.	5.76	355.2018	$C_{21}H_{27}N_2O_3$	Rauwolscine	
12.	5.93	441.2023	$C_{24}H_{29}N_2O6$	FMOC-DAB(BOC)-OH	
13.	6.32	429.1896		Unidentified	
14.	6.40	945.5433		Unidentified	

Table 2. Diameter of the zone of inhibition of the F6 fraction against *Candida albicans*

No.	Control or Fraction	Repetition N	Diameter of the zone of inhibition (cm)
		1	0
1.	Control	2	0
	(ethanol)	3	0
		4	0
		1	0
2.	F6	2	0
		3	0
		4	0

N is the number of replications of the control or fraction

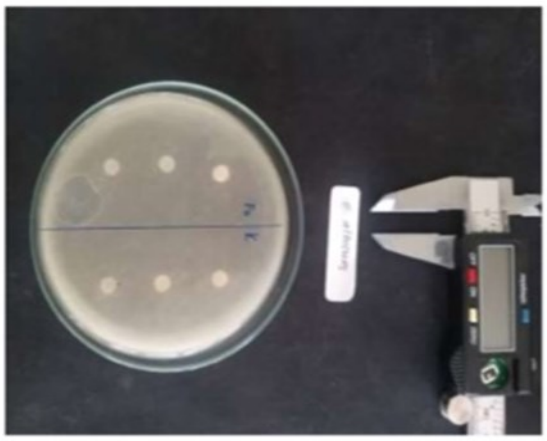


Fig. 4. *Inhibition zone of F6 against Candida albicans*

acid or methotrexate has the potential to be a spermicide because of its antineoplastic properties, namely being able to divide cells by destroying DNA so that apoptosis occurs, preventing the development and spread of cells (antiproliferative) is embryotoxic which can induce the number, motility, and viability of the sperm.

Fraction 6 (F6) with the compounds contained in it at a concentration of 0.3% did not affect the growth of *C. albicans* (Table 2 and Fig. 4), which is a fungus that is needed as a normal flora in the vagina and indicates a healthy condition of the intimate organs (Rebecca *et al*., 2024; Brooks *et al*., 2008).

Conclusion

The present study concluded that n-hexane extract of *C. inophyllum* L seed shell was the most toxic, with a spermicidal effect on the motility and morphology of mice spermatozoa at a concentration of 0.1, 0.3, and 0.5%, respectively, were 8.49, 4.30, and 0.00%. The morphology of damage from the neck to the tail of spermatozoa cells was 77.84, 79.21, and 79.93%. Fraction F6 of n-hexane extract was the most active, with a motility percentage of 0.00% and morphology of 65.86% Fraction F6 of the n-hexane extract of the *C. inophyllum* seed shell did not provide inhibition against the *C. albicans* fungus. Fraction F6 contained compounds that had spermicidal potential were: 2,5-Dioxo-1- pyrrolidinyl N-[(benzyloxy) carbonyl]-D-phenylalaninate, raubasine, and N-(4-{[2,4-Diamino-7,8-dyhydro-6pteridinyl) methyl] (methyl) amino} benzoyl)-L-glutamic acid or methotrexate. The compound 2,5-Dioxo-1-pyrrolidinyl N-[(benzyloxy) carbonyl]-D-phenylalaninate is a group of alkaloids thought to have spermicidal properties that can cause spermatozoa abnormalities through cytotoxic effects and hormonal effects, thereby disrupting hormonal balance. The raubasine compound can interfere with the activity of the ATP-ase enzyme in the middle part of the spermatozoa tail cell membrane so that the homeostasis of sodium and potassium ions will be disrupted, disrupting cell membrane permeability and nutrient transport required for cell metabolism. Methotrexate can affect the quality of spermatozoa cells because it has antiproliferative properties that can damage DNA at the beginning of apoptosis and prevent the development and spread of cells, which will affect normal cells, suppress growth and be embryotoxic.

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Conflict of interest

The authors declare that they have no conflict of interest.

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