

Spermicide of Papaya Seed Oil and Compounds

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ABSTRACT

This study aimed to determine the spermicidal activity of papaya seed oil (*Carica papaya* L) from local Bali varieties against Wistar rat spermatozoa by evaluating sperm quality and the correlation between the percentage of motility and viability spermatozoa to identify its active compounds. Papaya seed oil was obtained by extracting papaya seeds using n-hexane solvent. The study was designed as follows; group of spermatozoa Wistar rat were treated with NaCl 0.9% (control, P₀), with Sodium carboxy methyl cellulose (NaCMC) 0.5% (P₁), with n-hexane extract 0.1%(b/v) (P₂), with n-hexane extract 0.3%(b/v) (P₃), and with n-hexane extract 0.5%(b/v) (P₄). Column chromatography was used to separate the n-hexane extract into two fractions, A and B. The spermicide test for fractions A and B followed the same design as the test for n-hexane extract but until the P₇ group. The result showed that fraction B exhibited higher spermicidal activity, with 23.3% motility and 33.8% of viability sperm at 100 ppm. The identification of fraction B by LCMS showed at least 12 mixture compounds were venoterpine, vitamin K5, 5-phenyl-2-pyridinamine, harmalan, styrylquinoline, 1,2,3,4-tetrahydro-1-benzoyl-2-methyl-4-phenylamino-quinoline, calcitroic acid, siderol, N-benzyl-linoleamide, (3β)-3-(diisopropylamino)androst-5-en-17-one, pipereicosalidine, and 25-azacholesterol. Spermicidal activity may be a synergistic effect of several compounds that have a cytotoxic activity such as styrylquinoline, 1,2,3,4-tetrahydro-1-benzoyl-2-methyl-4-phenylamino-quinoline, siderol, and pipereicosalidine.

1. Introduction

Indonesia is the fourth most populous country globally with a population exceeding 270 million (WordBank 2019). The potential of human resources makes Indonesia a country with potent economic power that can grow very large if it can be managed properly. However, the rapid and expansive population growth poses significant challenges and needs careful attention to allocating and managing natural resources, education, health, and large employment management. Population growth in a particular area is endogenous, depending upon both natural increase and migration decisions. The natural increase, resulting from fertility and mortality dynamics further contributes to population growth (Schultz 2008).

In order to prevent the emergence of new and significant problems, for example food and natural

resource crises, it is important to control the current population growth. Pregnancy and birth control offer viable solutions to address this population growth. Contraception is one way to prevent pregnancy, by inhibiting the process of ovulation, fertilization, and implantation. Consequently, it is one of the most important tools available to individuals, both women and men to achieve their desired family size (United Nations 1994).

However, male participation in contraception remains considerably much lower than women (male = 6.34%, female = 93.66%). More contraception is aimed at women, while it is still very limited in men. Unfortunately, female contraceptives work based on hormones, specifically estrogen and progesterone, have resulted in adverse health effects, contributing to various degenerative diseases and malignancies. Given Indonesia's abundant natural resources, including potential in pregnancy and birth, exploring contraception methods for men becomes an optimal strategy. Using natural materials is one of the ways

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to explore new male plants with the second-largest drug potential in the world.

Spermicidal methods, including vaginal barrier methods is one modern method of contraception (Bradley *et al.* 2012). These methods function by altering the integrity of the sperm plasma membrane and the vaginal pH, creating a hostile environment for spermatozoa (Colin and Krishna 2023). Contraceptive aids such as jelly, foam tablets, sponges, or condoms often contains nonoxynol-9 (or N-9) compounds. N-9 is a chemical detergent that damages sperm plasma membranes and exhibits spermicidal activity. However, it does not protect against sexually transmitted diseases (STDs) or HIV (Human Immunodeficiency Virus) due to its detergent properties. Its frequent use even increases the risk of HIV acquisition. This outcome is believed because of the N-9-induced mucosal inflammation is crucial for accurately assessing the safety of potential vaginal microbicides and contraceptives (Irina *et al.* 2011; Cates and Harwood 2011). Hence, it is very important to find natural alternative materials that are suitable, safe, non-detergent, and that have the potential as a spermicide to replace N-9 compounds.

Papaya (*Carica papaya* L.) belonging to the family Caricaceae is commonly known as papaya in English, Papita in Hindi, and Erandakarkati in Sanskrit. The different parts of papaya such as fruit, fruit juice, root, leaves, bark, latex, and seed contain various chemical constituents and pharmacological properties (Tarun and Yash Prashar 2015). Among these parts, the seeds are particularly interesting to study. It can be used as a potential source of oil (papaya oil), which is rich in beneficial triacylglycerols, oleic acid (about 90%), linoleic acid (about 5%), ω -6 essential fatty acid (Puangsri *et al.* 2005; Malacrida *et al.* 2011; Samaram *et al.* 2013; Samaram *et al.* 2014; Labarca *et al.* 2015; Samaram *et al.* 2015). The seeds also contain carpaine, caricin, glucotropacolin, glucosinolates, benzyl isothiocyanate, and an enzyme myrosin. In addition, papaya seeds have pharmacological properties such as carminative, anti-fertility agents in males (complete loss of fertility attributing to decline in sperm motility and alteration in their morphology), counter-irritant, as pasta in the treatment of ringworm and psoriasis also antibacterial (Kartikar and Basu 1998; Kusemiju *et al.* 2002; Vidya 2005; Tarun and Yash Prashar 2015).

Papaya seeds possess remarkable antibacterial properties and effective against infections caused by *E. coli*, *Salmonella*, and *Staphylococcus*. Additionally, papaya seeds may protect the kidneys against toxin-induced kidney failure. The seeds can eliminate intestinal parasites, and help detoxify the liver. Additionally, they can be used as a skin irritant to help reduce fever. Papaya seeds are recognized as a remedy for piles and typhoid and possess anti-helminthic and anti-amoebic properties. Interestingly, dried papaya seeds resemble peppercorns and can be used similarly. Grinding a few seeds over a meal, particularly protein-rich ones, provides an easy way to add extra enzymes to your diet and improve your digestive health (Arvind *et al.* 2013).

In the context of male contraception, papaya oil seed can be used as a spermicide. Papaya oil can be obtained by extracting papaya seeds using n-hexane solvent, followed by evaporation of the solvent. Honeydew variety papaya has been identified to have spermicidal activity (Lohiya *et al.* 2000). Verma and Chinoy (2002) stated that papaya seed extract could influence the contractions of the epididymal cauda tubules, resulting in infertility (Lohiya *et al.* 2002). However, the infertility activity, particularly the *in vitro* spermicidal properties of the local Balinese papaya variety known as Gedang, has yet to be previously investigated. Therefore, we want to explore and evaluate the spermicidal properties of n-hexane concentrated extracts from young Balinese papaya seeds on the quality of Wistar rat spermatozoa. The study aims to examine the sperm immobilization effect of the n-hexane extract of *Carica papaya* seeds on rat spermatozoa *in vitro*, with the ultimate goal of exploring its potential as a vaginal spermicidal contraceptive.

2. Materials and Methods

2.1. Materials

The papaya seeds used in this study are young white seeds from the local Bali papaya (gedang) obtained from Bali's Badung area and its surroundings. The chemicals used were ethanol (pa), n-hexane (pa), Sodium carboxy methyl cellulose (NaCMC), ethyl acetate (pa), potassium bromide (KBr) concentrated sulfuric acid, acetic acid, silica gel GF452, silica gel 60, physiological NaCl 0.9%, eosin nigrosin citrate, 90% alcohol, and distilled

water. While the equipment was a set of glass tools, rotary evaporator Buchi R 114 and vacuum Buchi B 169, a chromatography set, TLC, Camber, 254 nm, and 366 nm UV lamps, microscope binocular (Olympus, Japan), micropipette, micro tube (Eppendorf), object glass, cover glass, and LC-MS/MS (Waters ACQUITY UPLC®H-Class System).

2.2. Methods

Papaya seeds were dipped in hot ethanol and then drained to dry. About 1 kg of dried seeds was crushed until to powder; then, it was extracted with ethanol. A rotary vacuum evaporator evaporated solvent ethanol to obtain a viscous extract. This extract was partitioned into n-hexane and evaporated to produce a viscous oil seed papaya. Following, n-hexane extract was tested on healthy sperm to determine the average percentage of motility and viability of spermatozoa Wistar rats before and after treatment. Spermicide potency is based on spermatozoa quality, and the correlation between the percentage of motility and viability with the design study is as follows:

P_0 = group of spermatozoa wistar rat with NaCl 0.9%(control)

P_1 = group of spermatozoa wistar rat with NaCMC 0.5%

P_2 = group of spermatozoa wistar rat with n-hexane extract 0.1%(b/v)

P_3 = group of spermatozoa wistar rat with n-hexane extract 0.3%(b/v)

P_4 = group of spermatozoa wistar rat with n-hexane extract 0.5%(b/v)

Following, the n-hexane extract was separated by column chromatography (silica gel 60, n-hexane: ethyl acetate (1:1)) to obtain 2 fractions (fractions A and B). Both fractions were tested spermatozoa quality to determine the average percentage of motility and viability of spermatozoa Wistar rat at various concentrations with design study as followed:

P_0 = group of spermatozoa Wistar rat with NaCl 0.9%(control)

P_1 = group of spermatozoa Wistar rat with NaCMC 0.5%

P_2 = group of spermatozoa Wistar rat with fraction A 100 ppm

P_3 = group of spermatozoa Wistar rat with fraction A 300 ppm

P_4 = group of spermatozoa Wistar rat with fraction A 500 ppm

P_5 = group of spermatozoa Wistar rat with fraction B 100 ppm

P_6 = group of spermatozoa Wistar rat with fraction B 300 ppm

P_7 = group of spermatozoa Wistar rat with fraction B 500 ppm

The most active fraction after purification was examined by phytochemical identification and LCMS analysis.

2.3. Motility of Spermatozoa

Motility is seen from the percentage of spermatozoa that move forward progressively; it was determined by the formula (Angela *et al.* 2010; Herlina *et al.* 2018):

$$\% \text{ Motility} = \frac{b - a}{b} \times 100$$

a = number of progressive spermatozoa

b = the number of spermatozoa counted (100)

2.4. Viability of Spermatozoa

Spermatozoa viability is the ability of spermatozoa to survive after being diluted and is one of the important factors in determining the quality of spermatozoa, usually, it counts to living spermatozoa were taken as those not absorbing eosin red (transparent), with the formula (Angela *et al.* 2010; Herlina *et al.* 2018):

$$\% \text{ Viability} = \frac{b - a}{b} \times 100$$

a = live spermatozoa

b = number of spermatozoa assessed (100)

3. Results

Spermicide potency is based on spermatozoa quality and the correlation between the percentage of motility and viability. The n-hexane extract was tested for spermatozoa quality to determine the percentage of motility and viability of spermatozoa Wistar rats, as shown in Figure 1.

Figure 1 showed that n-hexane extract of young papaya seeds had an average percentage of motility and viability of sperm Wistar rats toward

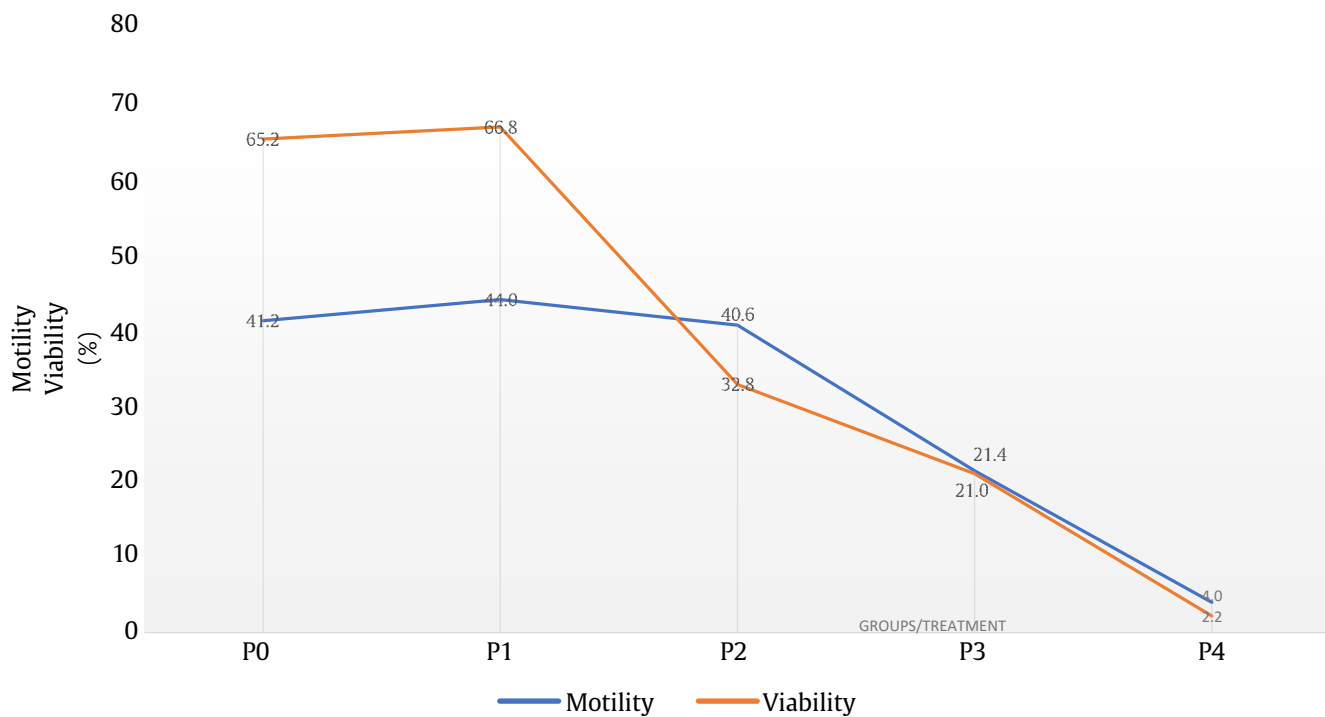


Figure 1. The mean percentage motility and viability in a group with n-hexane extract

groups or treatment. Based on MANOVA analysis, tests of between-subjects effects showed that treatment effect on both dependent variables, the percentage of motility and viability is significant. A further test to see the difference mean between treatments (group) as significantly at the level 0.05 using multiple comparisons Bonferroni test. The percentage of motility groups P_0 , P_1 , and P_2 , different significantly (Sig. <0.05) against P_3 and P_4 , but P_0 was not significantly different (Sig. >0.05) toward P_1 and P_2 groups. Because P_0 vs P_1 and P_0 vs P_2 were not significantly different (Sig. >0.05), this means that the movement of spermatozoa cells (motility) in the group of Wistar rats given 0.9% NaCl solution was not different from that given 0.5% NaCMC and treated with 0.1% n-hexane extract of papaya seeds. However, spermatozoa cells that were given 0.3% and 0.5% n-hexane extract gave an average effect on the motility of 21.4% and 4.0%, respectively, which means that the dilution of 0.3% n-hexane extract of papaya seeds was only able to reduce cell motility about half more (51.9%) than the control (P_0), while the dilution of 0.5% n-hexane extract was able to reduce cell motility 97.08% against the control P_0 . For the percentage viability, the P_0 group was not significantly different from P_1 (Sig. >0.05), which means that both control and the effect of the 0.5%

NaCMC dilution were the same, so as a comparison, only the control group P_0 was used. Groups P_2 , P_3 , and P_4 difference significantly concerning P_0 (Sig.<0.05), as did P_2 vs P_3 , P_2 vs P_4 , and P_3 vs P_4 were significantly different (Sig. <0.05). Analysis of Pearson Correlation between Dependent Variables (% Motility and Viability) is 0.806 with a significance of 0.000 which indicated that there was a strong and significant positive correlation between the two variables, especially in the P_3 and P_4 groups. This showed that the spermatozoa cells of Wistar rats given n-hexane extract at a concentration of 0.3% and 0.5% affect cell viability and motility so that they had potential as spermicide agents.

Separation of 2 g of n-hexane concentrated extract by column chromatography (silica gel 60, n-hexane: ethyl acetate (1: 1)) resulted in 60 eluates which were then fractionated with TLC to produce 2 fractions A as much as 0.2580 g and B of 0.1715 g. The spermicide potency of both fractions was determined based on the correlation between the percentage of motility and the viability of the fractions at various concentrations groups as shown in Figure 2.

The results of the Bonferroni test between P_0 and P_1 on the motility and viability variables were not significantly different (Sig. >0.05), meaning that one of the two controls could be used as a comparison.

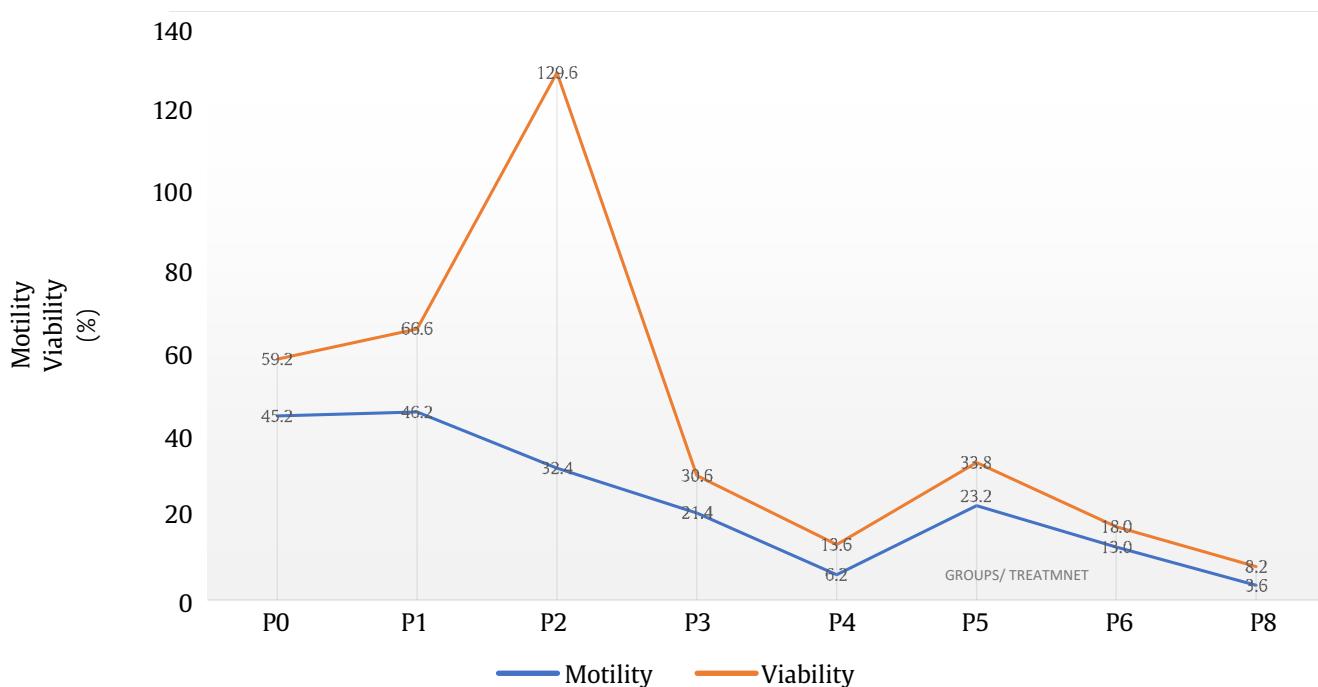


Figure 2. The mean of percentage motility and viability in a group of Column Chromatography Fractions

Furthermore, differences between groups for motility variables were as follows; the groups P_2 vs P_0 , P_3 vs P_0 , P_4 vs P_0 , P_5 vs P_0 , P_6 vs P_0 , P_7 vs P_0 , P_2 vs P_3 , P_2 vs P_4 , P_2 vs P_5 , P_2 vs P_6 , P_2 vs P_7 , P_3 vs P_4 , P_3 vs P_6 , P_3 vs P_7 , P_4 vs P_5 , P_4 vs P_6 , P_5 vs P_6 , P_5 vs P_7 , and P_6 vs P_7 were significantly different (Sig. <0.05) except for the P_3 vs P_5 and P_4 vs P_7 groups which was not significantly different (Sig. >0.05). The difference between groups for viability variables was as follows; the groups P_2 vs P_0 , P_3 vs P_0 , P_4 vs P_0 , P_5 vs P_0 , P_6 vs P_0 , P_7 vs P_0 , P_2 vs P_3 , P_2 vs P_4 , P_2 vs P_5 , P_2 vs P_6 , P_2 vs P_7 , P_3 vs P_4 , P_3 vs P_6 , P_3 vs P_7 , P_4 vs P_5 , P_5 vs P_6 , and P_5 vs P_7 , were significantly different (Sig. <0.05) except for the P_3 vs P_5 , P_4 vs P_6 , and P_4 vs P_7 groups which was not significantly different (Sig. >0.05). Based on statistical analysis, it could be concluded that fraction A (concentration 300 ppm) with fraction B (concentration 100 ppm) and fraction A (concentration 500 ppm) with fraction B (concentration 500 ppm) had the same effect on the percentage of motility and viability as or not significantly different, while fraction A (concentration 500 ppm) and fraction B (concentration 300 ppm) only had the same effect on the percentage of viability. The result from Pearson Correlation between Dependent Variables (% Motility and Viability) was 0.965 with a significance of <0.001 which indicated that there was a strong and significant positive correlation between the two variables, especially in the P3 and P5 groups.

In other words, fraction A (concentration of 500 ppm) and fraction B (concentration of 100 ppm) both affected motility and viability so that they were potential as spermicide materials, but fraction B at a concentration of 100 ppm had the most potential as a spermicide.

The results of the analysis by LCMS showed that the fraction B has any peaks with Retention time (Rt) as shown in Figure 3.

4. Discussion

Our result identified that Fraction B was the most potential spermicide. The results of the phytochemical test showed that the B fraction was positive for triterpenoid compounds, with Liberman-Burchard reagent producing red purple color. Many plants known for their spermicidal effects contain saponin triterpenoids, flavonoids, and phenol compounds (Farnsworth and Waller 1982). The decrease in motility and viability in Wistar rat spermatozoa is caused by substances contained in the n-hexane extract of young papaya seeds. Among these substances, triterpenoids exhibit anti-fertility activity, which can reduce spermatozoa count and even eliminate them. Saponins and triterpenoids could affect the cell membrane, causing it to shrink which resulted in a decrease

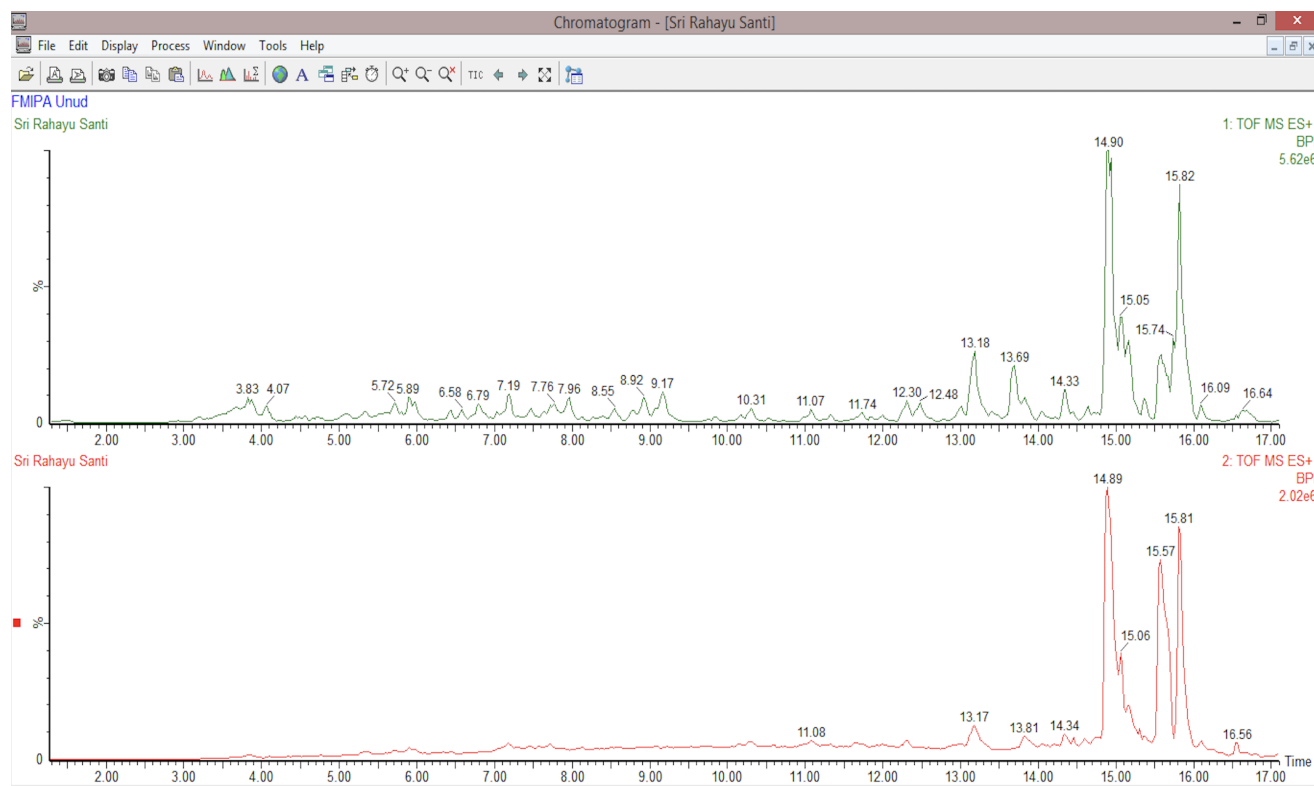


Figure 3. Chromatograph of the fraction B

in the integrity of the cell membrane (Miltaine *et al.* 2001). Another possibility was, the decrease in spermatozoa motility and viability could be caused by the disruption of spermatozoa permeability, disrupting the transportation of food (nutrients) for the spermatozoa. Membrane permeability was closely related to nutrient transport which played an important role in cell metabolism. With the disruption of the permeability of the spermatozoa membrane, the nutritional needs will be disrupted and the spermatozoa cells will die. (Pathak *et al.* 2000). This follows the opinion of (Jayendran *et al.* 1984) who stated that the permeability of spermatozoa is closely related to the motility and viability of spermatozoa.

Based on chromatograph Liquid Chromatography (LC) in Figure 3. showed that the fraction B contains at least 12 compounds with structure molecular as shown in Table 1.

Venoterpine was an antineoplastic agent, a substance that inhibited or prevented the proliferation of neoplasms (Royal Society of Chemistry 2022), vitamin K5 could reduce transfusion-transmitted septic reactions from contaminated plasma products (Fei *et al.* 2017),

5-Phenyl-2-pyridinamine had mutagenic activities (Jiao *et al.* 2018), harmalan were psychoactive substances with differing pharmacological profiles (Hans *et al.* 1985). Styrylquinoline behaved as integrase binding inhibitor HIV-1 which was a key enzyme in the replication cycle of the retrovirus since it catalyzes the integration of the reverse transcribed viral DNA into the chromosomal DNA (Jean-François and Didier 2010).1,2,3,4-tetrahydro-1-benzoyl-2-methyl-4-phenylamino-quinoline, generally the compounds had quinoline ring has been found to possess cytotoxicity, antimalarial, antibacterial, antifungal, anthelmintic, cardiotoxic, anticonvulsant, anti-inflammatory, and analgesic activity (Akranth *et al.* 2013). Calcitroic Acid (CTA) was a VDR agonist and mediates biological effects similar to 1,25(OH)₂D₃, which have shown antirachitic and anti-inflammatory activity (Yu *et al.* 2021). Siderol has been cytotoxic activity or antiproliferative (Ekaterina-Michaela *et al.* 2020), N-benzyl linoleamide had the potential to inhibit soluble epoxide hydrolase (sEH) (Nalin *et al.* 2020), pipericosalidine was known as cytotoxic and anti-inflammatory activity (Ziyan Guo *et al.* 2019), and 25-azacholesterol inhibited the sterol

Table. 1 Data analysis compounds in the fraction B

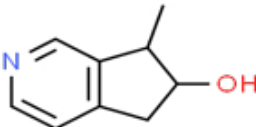
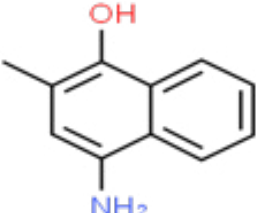
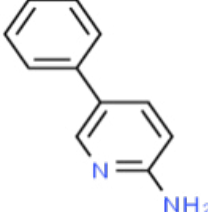
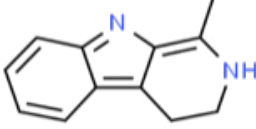
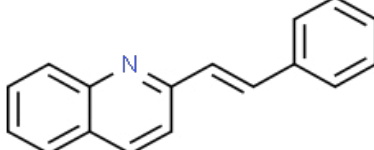
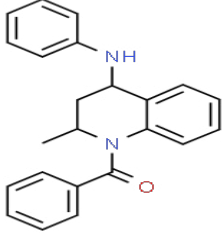
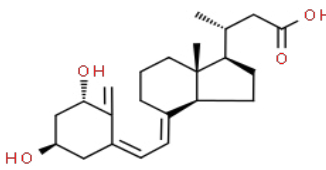
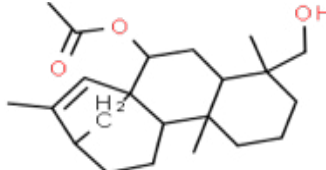
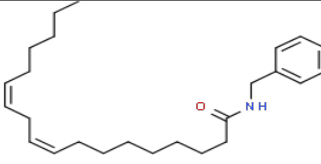
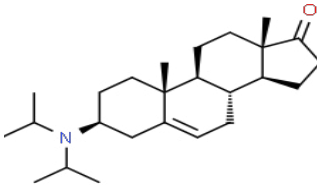
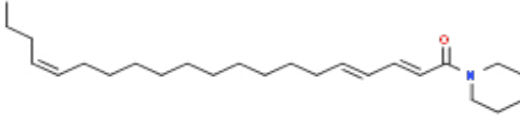
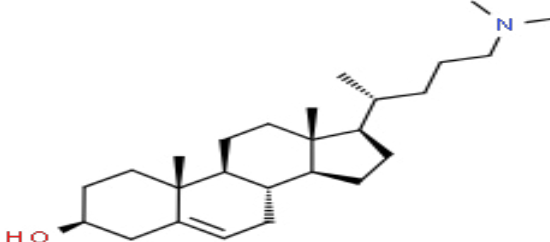
Retention time	Ion M ⁺ m/z	Chemical formula	% Ifit	Alleged compound	Structure
3.826	150.0919	C ₉ H ₁₁ NO	96.25	Venoterpine	
5.718	174.0919	C ₁₁ H ₁₁ NO	86.75	Vitamin K5	
5.893	171.0922	C ₁₁ H ₁₀ N ₂	78.47	5-Phenyl-2-pyridinamine	
7.166	185.1079	C ₁₂ H ₁₂ N ₂	97.38	Harmalan	
7.735	232.1126	C ₁₆ H ₁₃ N	99.62	Styrylquinoline	
11.074	343.1810	C ₂₃ H ₂₂ N ₂ O	99.89	1,2,3,4-tetrahydro-1-benzoyl-2-methyl-4-phenylamino-Quinoline	
12.305	375.2535	C ₂₃ H ₃₄ O ₄	59.16	Calcitroic acid	
13.184	347.2586	C ₂₂ H ₃₄ O ₃	92.10	Siderol	

Table. 1 Continued

Retention time	Ion M ⁺ m/z	Chemical formula	% Ifit	Alleged compound	Structure
14.351	370.3110	C ₂₅ H ₃₉ NO	93.31	N-Benzyl-Linoleamide	
14.878	372.3266	C ₂₅ H ₄₁ NO	99.42	(3β)-3-(Diisopropylamino) androst-5-en-17-one	
15.820	374.3423	C ₂₅ H ₄₃ NO	99.97	Pipereicosalidine	
16.088	388.3579	C ₂₆ H ₄₅ NO	82.90	25-Azacholesterol	

transmethylase enzyme (Haughan *et al.* 1995). Of the several compounds whose activity has been identified in the fraction B (concentration of 100 ppm), most of these compounds have a cytotoxic activity that has the potential to damage spermatozoa cells so that the quality of spermatozoa decreases. The synergism of the cytotoxic properties of these compounds was proven to be able to reduce the quality of rat spermatozoa by destroying the head of the sperm cell so that the cell movement could not be straight and agile or the sperm cells died. (Figure 4B) displayed the image of unhealthy sperm cells, indicating the n-hexane extract's detrimental effects. Upon exposure to the extract for 30 seconds, the sperm cell heads exhibited damaged and appeared unclear after being stained with eosin-nigrosin. Dead sperm cells were observed with compromised cell membranes, allowing eosin-nigrosin entered the cell causing osmosis pressure

of extract. These findings suggested that, the extract possesses anti-fertility activity, reducing the quality and even killing spermatozoa to have spermicidal activity.

In conclusion, based on statistical analysis, the quality of sperm is determined by its percentage of motility and viability. There were no significant differences observed between combinations of Fraction A (300 ppm) and fraction B (100 ppm), as well as fraction A (500 ppm) and fraction B (500 ppm). Both had similar effects on the percentage of motility and viability. It was observed that even at a lower concentration of 100 ppm, fraction B significantly impacted the percentage of sperm motility and viability. It indicated that fraction B was particularly active as a spermicide, with a percentage of 23.3% motility and 33.8% of viability. The potency of fraction B as a spermicide, could be attributed to the synergistic effect of its cytotoxic compounds.

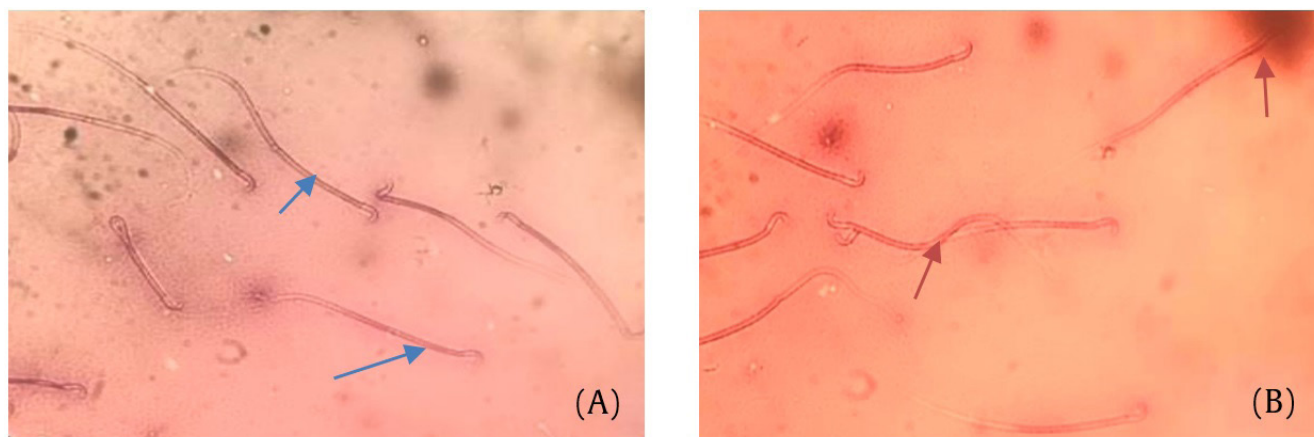


Figure 4. (A) Normal shape of Wistar rat sperm cells before being given n-hexane extract (→), and (B) dead sperm cells after dripping papaya seed n-hexane extract shown red cell of eosin-negrosin (→)

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