Effectiveness of pineapple and papaya leaf combination for dysmenorrhea pain relief in mice (*Mus musculus*)

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Abstract

Dysmenorrhea is a common gynecological condition in women, often attributed to excessive prostaglandin production, significantly impacting daily activities. Papaya leaves, known for their medicinal properties, are a chosen herbal remedy, albeit with a bitter taste, necessitating combination with pineapple fruit. This study aimed to assess the effectiveness of a combination of papaya leaf extract and pineapple fruit as an alternative treatment for primary dysmenorrhea. In an experimental research design employing the posttest-only control group, 32 male mice were divided into seven groups, injected with acetic acid as a pain inducer, and their writhing responses were recorded for 45 minutes at 15-minute intervals. Data analysis using the ANOVA test revealed significant differences in the number of writhing responses in mice ($p<0.05$) compared to the positive control group, followed by the Duncan test. The percentage of analgesic protection was as follows: mefenamic acid (61.01%), pineapple fruit extract (62.78%), papaya leaf extract (63.39%), a combination of pineapple and papaya leaf extracts with a dose ratio of 3:1 (73.21%), 2:2 (47.32%), and 1:3 (37.78%). In conclusion, the combination of pineapple extract and papaya leaves in a dose ratio of 3:1 demonstrated the most effective pain reduction.

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Introduction

Dysmenorrhea is the term for pain and cramps in the pelvis experienced by women during menstruation.[1] The pain is caused by contractions of the uterine wall due to the high production of uterine prostaglandins (PGF2α and PGF2).[2] In general, dysmenorrhea is more common in young women of reproductive age, with the highest occurrence in adolescents, and has a prevalence variation between 60% to 90%.[3,4] According to Ju *et al.* (2014),[5] several developed countries have a fairly large prevalence of dysmenorrhea women, such as Australia with a number of dysmenorrhea women at 71.7%, while Japan has a presentation of dysmenorrhea women at 76.1%. In Indonesia, there is uncertainty regarding the prevalence of dysmenorrhea. A previous epidemiological study noted that out of 240 women in Central Jakarta aged 11-22 years, 87.5% of the respondents had primary dysmenorrhea with details of mild symptoms (20.48%), moderate symptoms (64.76%), and severe symptoms (14.76%). Although not considered a life-threatening disorder, dysmenorrhea can affect the quality of life and social relationships of women who have it.[6]

Not only is the number of incidents quite high, but dysmenorrhea is also a gynecological condition that greatly interferes with women's daily activities.[7] Severe symptoms experienced by women have been described as characteristically sharp and intermittent, felt in the suprapubic area, which worsens in the first few hours of menstruation and culminates with maximum blood flow.[8] Moreover, severe symptoms are accompanied by systemic symptoms such as nausea, vomiting, diarrhea, fatigue, fever, and insomnia. Due to these severe symptoms, some women (3-33%) require 1-3 days of complete rest every menstrual cycle, leading to absenteeism from school or work. According to World Health Organization (WHO) data in Indonesia, 15% of individuals with dysmenorrhea complain of limited activities due to the discomfort

of this gynecological condition. There is even data indicating that almost 10% of female students are absent each month due to illness, whether related to dysmenorrhea or other causes.[9]

There are several ways to relieve the symptoms of dysmenorrhea, including reducing physical activity, taking pain relievers, and using herbs.[7,8,10] The main cause of dysmenorrhea is the overproduction of pain precursors, namely prostaglandins; thus, the pain relievers and herbs consumed aim to reduce prostaglandin production.[8] Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen are commonly used to relieve pain, but they come with side effects such as headache, dizziness, dysuria, drowsiness, loss of appetite, nausea, acne, acute increase in asthma, vomiting, and gastrointestinal bleeding.[11] Consequently, many phytotherapeutic treatments have been developed to minimize side effects in addressing dysmenorrhea. Herbal medicine can serve as an alternative to NSAIDs, effectively reducing menstrual pain.[10] Research conducted by Abidah in 2017 demonstrated that papaya leaf extract reduced menstrual pain levels and prostaglandin levels in primary dysmenorrhea.[12]

While papaya leaves have been studied for their ability to alleviate dysmenorrhea symptoms,[13] their very bitter taste necessitates the addition of a flavoring agent to maintain the essence without compromising palatability. The inclusion of organic acids from fruits, known to mask the bitter taste of papaya leaves, is a viable option.[14] Pineapple fruit, with its organic acids, can be an alternative to mitigate the bitter taste. Additionally, the bromelain enzyme content in pineapple has analgesic activity, supporting the analgesic effects of papaya leaf extract.[15] The combination formulation is expected to have a potentiating effect without causing contraindicated effects. Several studies have explored the treatment of primary dysmenorrhea using papaya leaves and pineapple fruit in the form of juice or extract, but research on the combination of pineapple fruit extract and papaya leaves has not been undertaken before. Therefore, researchers are interested in understanding the analgesic activity of a combination of papaya leaf extract and pineapple fruit as a treatment for primary dysmenorrhea. This study was conducted to determine the effectiveness and optimal combination of papaya leaf extract and pineapple fruit in treating symptoms of primary dysmenorrhea using animal models.

Materials and Methods

Research design

This study was an experimental study with a posttest-only control group design, aiming to test the effectiveness of the combination of papaya leaves and pineapple fruit as a treatment for primary dysmenorrhea.

Plant materials

The papaya leaves used in this study came from the Bekasi area, weighing approximately 5 kg. The leaves were selected based on age, ensuring they were neither too old nor too young, and were fresh, green in color, and not wilted or diseased. For this study, Bogor honey pineapple weighing approximately 2.8 kg was used. The selected pineapple was a young pineapple with a pale-yellow color and a slightly hard texture.

Preparation of extract

Papaya leaves were cleaned and cut into several pieces, then dried in an oven at 50°C for approximately 3 days. The dried papaya leaves were then mashed into a powder. The extraction method used was maceration, with a sample and solvent ratio of 1:5. Maceration was repeated three times. The obtained macerate was filtered, collected, and then evaporated with a vacuum evaporator at 40°C.[16]

Pineapple fruit was washed, cut into small pieces, and then blended with ice cubes and phosphate buffer at pH 7. The blended pineapple fruit was filtered three times. The filter results were centrifuged at 3000 rpm at 4°C for 20 minutes. The supernatant obtained was separated from the pellet and stored in the freezer. Furthermore, the centrifuged filtrate underwent the freeze-drying method at 40°C to obtain the crude extract of pineapple fruit.[17]

Determination of total flavonoids

The total flavonoid assay was conducted using the aluminium chloride-based method.[18] This quantitative method measures total flavonoids based on a standard curve equivalent to quercetin. Quercetin standards were prepared with a concentration series of 1, 5, 10, 15, and 20 mg/mL, respectively, with ethanol as the solvent. Subsequently, 0.5 mL of the standard solution was pipetted separately into a test tube, and then 0.1 mL of 10% $AlCl_3$, 1.5 mL of ethanol, 0.1 mL of sodium acetate, and 2.8 mL of water were added. The solution was shaken, allowed to stand for 30 minutes, and then measured at an absorbance of approximately 425 nm using UV-Vis spectrophotometry. Samples were prepared at a concentration of 3000 ppm, reacted, and measured according to standards. The total flavonoid concentration of the sample was determined from the calibration curve. The percentage of total flavonoids as quercetin in the extract, using the standard curve, is calculated using the following formula:[19]

$$\% = \frac{Cp \times V \times f}{W} \times 100$$

Information:
C_p = Flavonoid content of the extract
V = Volume of test solution before dilution
f = Dilution factor of the test solution
W = Weight of test material

Protein level test

Measurement of the protein content of pineapple extract was conducted based on the Bradford test method (1976). Bovine serum albumin (BSA) was used as a standard with concentrations of 0, 0.0625, 0.125, 0.25, and 0.5 mg/mL, each with a volume of 10 mL. To perform the test, 25 µL of each BSA concentration was pipetted into a new test tube, and 1.25 mL of Bradford reagent was added. The solution was homogenized with a vortex and then incubated for 5 minutes. After incubation, the absorbance of the solution was measured at a wavelength of 595 nm. The concentration and absorbance data of the standards were expressed as x and y, respectively, and connected to form the graph of the linear regression equation.

For the measurement of the protein content of pineapple extract, 25 µL of the extract was pipetted into a test tube, and 1.25 mL of Bradford reagent was added. The solution was homogenized with a vortex and then incubated for 5 minutes. After incubation, the absorbance of the solution was measured at a wavelength of 595 nm, and this process was repeated three times. The absorbance data (y) of the sample were entered into the standard regression equation to determine the protein content of the pineapple extract.

Preparation of mice

The experimental subjects consisted of healthy male mice (*Mus musculus*) aged 2-3 months, weighing between 20-30 grams. The sample size, determined using the Federer formula with a 20% dropout addition, totaled 28 mice. Before treatment, the mice underwent a 2-week to 1-month adaptation period, during which they were provided ad libitum access to water and CP 552 type pellet feed for approximately 8-12 hours daily. The mice were housed in stainless steel cages measuring 30 x 20 x 20 cm, featuring a cage bottom covered by 1 cm thick husks replaced every three days. The cages were situated in a well-ventilated room with indirect sunlight, and cleanliness was maintained by cleaning drums and feeding areas at least three times a week. To ensure randomization, the samples were divided into 7 groups using the simple random sampling method, where each mouse was assigned a number and then randomly selected by the researchers.

Analgesic assay (*in vivo* test)

The test for the analgesic effect of the extract is conducted following the procedure outlined in reference.[16] The analgesic power test was conducted on male mice (*M. musculus*) using the chemical excitatory method with a 1% acetic acid solution as the pain inducer. The papaya leaf and pineapple fruit extract were prepared as a stock solution of 20 mL, then administered to mice in a quantity of 1 mL. After a 30-minute interval following the extract administration, mice in each treatment group were injected with 1% acetic acid, also in a volume of 1 mL, using an aqueous solvent. Mice subjected to the acetic acid-inducing solution exhibited pain endurance by wriggling on the abdomen and retracting their legs. The observed and calculated amount of writhing was monitored over a 45-minute period at 15-minute intervals.

Determination of analgesic protection

The evaluation of analgesic activity using the acetic acid induction method involves assessing the percent protection and percent effectiveness of the analgesic. The percentage protection against acetic acid is determined by comparing the number of stretches in each group to the control and calculating the percentage reduction in stretching. The formula for calculating analgesic protection is as follows:[20]

$$\text{Analgesic protection } (\%) = \frac{Nc - Nt}{Nc} \times 100\%$$

Here, Nc represents the number of stretches in the negative control, and Nt represents the number of stretches in the test animals from each treatment group. The percent analgesic can be determined from the difference in the amount of stretching, Nc - Nt.

Experimental design

The combination dose was prepared at 500 mg/kg body weight. Considering the average weight of mice is 20 g, the dose variation was 10 mg per 20 g body weight. Oral administration to mice is restricted to a maximum volume of 1 mL. Therefore, a stock of 20 mL is created, containing 200 mg of extract, ensuring that each mL contains 10 mg of extract. The mice were divided into seven groups, each with four repetitions, based on the treatment, as outlined in the following Table 1.

Statistical analysis

SPSS 2.5 software was used to analyze data on mice's wriggling results. All data are presented in the form of an average value and standard deviation. The data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's test. Data with a P value <0.05 are considered significant.

Ethical considerations

All mice used were obtained from the laboratory of the Department of Pharmacology, Faculty of Medicine, University of North Sumatra in Indonesia. The mice were housed in stainless-steel cages measuring 30 x 20 cm and were fed CP 552 type pellets during the treatment period. All ethical procedures used in this study adhere to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NRC 1996) and have been approved by the ethics committee of the Pharmacology Department of FK USU with No. 0859/KEPH-FMIPA/2021.

Results

Quantitative phytochemical of papaya leaf and pineapple extract

Based on Table 2, the results of the total flavonoid test showed that papaya leaf extract with a concentration of 3,000 µg/mL had a flavonoid content of 12.034 mg EK/g extract or 1.2034%. The protein content test of pineapple extract was carried out using the Bradford method, where the protein content was obtained from the linear regression equation of the bovine serum albumin (BSA) cal-

Table 1. Single extract dose and combination administration in mice groups.

Group	Treatment	Pineapple fruit extract (dose mg/kg bw)	Papaya leaf extract (dose mg/kg bw)
1	Positive control mefenamic acid 0.13 ml/20 g bw (k^+)	-	-
2	Negative control na-cmc 0.5% 20 ml (k^-)	-	-
3	Pineapple fruit extract	500	-
4	Papaya leaf extract	-	500
5	Combination of pineapple fruit extract and papaya leaves in a 3:1 dosage ratio		375125
6	Combination of pineapple fruit extract and papaya leaves in a 2:2 dosage ratio		250250
7	Combination of pineapple fruit extract and papaya leaves in a 1:3 dosage ratio		125375

BW, Body weight.

ibration curve. The results of the protein content test showed that pineapple fruit extract contained a relatively high amount of protein.

Effectiveness of combination papaya leaf and pineapple extract in mice

Data on the number of mice writhing are presented in Figure 1. Stretching symptoms that arise are observed for 45 minutes with an interval of 15 minutes. The writhing response is in the form of the legs and stomach being pulled back due to the pain caused by acetic acid induction. Mice injected with 50% Na-CMC had the highest number of stretches. This indicates that the 50% Na-CMC solution has no effect on relieving pain because it has no active substance and does not cause bias. Of all the sample extracts tested, both single extracts and a combination of extracts from papaya leaves and pineapple fruit were able to reduce the amount of stretching. The amount of writhing that decreased with time intervals indicated that the combination of extracts was able to relieve pain caused by acetic acid induction. Based on the ANOVA analysis at the 5% test level, the number of ticks in the mice showed significantly different results ($p < 0.05$) compared to the positive control group, so Duncan's further test was carried out.

Of the 7 test groups, it can be seen that the combination of pineapple and papaya leaf extracts with a ratio of 3:1 is the combination that produces the least wriggling of mice with an average of 22.5 writhing and with a decrease in the amount of writhing as the test time increases (Table 3). These results were better when compared with the positive control mefenamic acid. From Duncan's test, it can be concluded that the mice injected with the negative control had the highest number of stretches. Mefenamic acid injection as a positive control showed analgesic protection of 61.01%. Meanwhile, each single extract of papaya leaf and pineapple fruit produced analgesic protection of 63.39% and 62.78%, respectively. The highest analgesic protection was produced by the test sample that resulted in the least amount of writhing, namely the combination of extracts of pineapple and papaya leaf in a ratio of 3:1, with an analgesic protection of 73.21%.

Discussion

This study assessed the analgesic potential of a papaya or *Carica papaya* leaves extract (CPE) and pineapple or *Ananas comosus* extract (ACE) combination for alleviating primary dysmenorrhea pain, a condition characterized by lower abdominal pain before and during menstruation without pelvic damage.[7,21] The pain's onset involves complex pathways, including hormonal changes, anti-inflammatory responses, and immune system activity. Prostaglandin overproduction, particularly prostaglandins F2α and E2, is considered a primary cause of dysmenorrhea,[2,22] linked to hormonal changes such as decreased progesterone levels due to factors like stress and diet.[23,24] The prolonged presence of arachidonic acid, coupled with menstruation-related intracellular damage, contributes to excessive prostaglandin production.[2,25] This overproduction can result in uterine hypercontraction, vasoconstriction, ischemia, hypoxia, and inflammatory responses, releasing pro-inflammatory molecules like cytokines, bradykinins, and prostaglandins.[22,26] These molecules activate receptors on nociceptor neurons, increasing sensitivity and triggering pain perception. Additionally, prostaglandins act as sensitizing agents, lowering the nociceptive activation threshold and potentially leading to hyperalgesia, allodynia, and increased pain perception.[27,28]

The combination of CPE and ACE, administered in mice using the acetic-acid writhing test, demonstrated significant analgesic activity by reducing writhing and increasing percent protection. This aligns with prior research, which established the analgesic properties of CPE and ACE.[29] CPE acts as a peripheral analgesic, blocking pain transmission to the peripheral or central nervous system. ACE exhibits antinociceptive activity, reducing pain in the acetic-acid writhing test model.[30] Other studies also mention that pineapple juice and papaya leaves can reduce menstrual pain levels and prostaglandin levels in individuals with dysmenorrhea.[12,31] The combined CPE and ACE in this study effectively reduced pain in acetic acid-induced mouse models, attributed to flavonoids. CPE's flavonoids inhibit prostaglandin production by blocking COX-1, COX-2, and 5-lipoxygenase active sites, and suppressing COX-2

Table 2. Flavonoids and protein levels in papaya leaf and pineapple extracts.

Phytochemistry extract	Qualitative	Quantitative
Flavonoids	+	12.034 mg EK/g extract
Proteins	+	0.275 mg/mL

Table 3. Treatment group writhing time (45 minutes).

Group	The amount of stretching				Average ± SD
	1	2	3	4	
Negative control (Na-CMC)	98	88	82	68	84±12.54
Positive control (Mefenamic acid)	39	30	34	28	32.75±4.85^{abc}
Pineapple extract	30	31	30	34	31.25±1.89^{ab}
Papaya leaf extract	38	32	29	24	30.75±5.85^{ab}
Combination of pineapple extract and papaya leaf 3:1	30	26	22	12	22.5±7.72a
Combination of pineapple extract and papaya leaf 2:2	39	38	31	69	44.25±16.88^{bc}
A combination of pineapple and papaya extracts 1:3	55	54	53	47	52.25±3.59^{c}

*a, b, c, d Different letters indicate a significant difference between samples at the 5% level of significance (Duncan's test). *SD, Standard deviation.

gene expression.[32,33]

ACE is known to contain the enzyme bromelain, which can serve as an alternative to NSAIDs in the treatment of pain due to its analgesic and anti-inflammatory activity.[34] Numerous studies have highlighted the anti-inflammatory and analgesic properties of bromelain, elucidating various mechanisms and pathways. Among these, the most prominent is bromelain's ability to reduce bradykinin levels, a pain mediator.[35] The reduction in bradykinin levels occurs through the inhibition of bradykinin synthesis at the inflammation site, achieved by depleting plasma kallikrein and inhibiting plasma exudation with bromelain.[36] In addition to its impact on the bradykinin pathway, bromelain has been reported to induce a decrease in prostaglandin E2 concentrations by inhibiting COX-2 and reducing glutamate concentrations, a neurotransmitter involved in pain perception.[37,38]

The analgesic activity of the ACE and CPE combination was assessed using an intraperitoneal acetic acid-induced writhing model, a common method to evaluate peripheral analgesic activity.[39] Acetic acid injection induces visceral pain by releasing endogenous pro-inflammatory mediators, including prostaglandins, serotonin, and bradykinin, stimulating peripheral nociceptive neurons and causing pain and stretching responses.[26,30,40] The local inflammatory response involves the release of arachidonic acid via COX-1 and COX-2, impacting the biosynthesis of prostaglandins E2 and F2 (PGE2, PGF2).[41] Nociception from acetic acid injection is linked to cytokine release from mast cells and resident macrophages, including interleukin (IL-1β, IL-8) and tumor necrosis factor-alpha (TNF-α).[42] The tested compounds' analgesic activity was determined by reduced total nociceptive score and writhing frequency, along with an increased protection percentage, manifesting as back arching, abdominal muscle contraction, and hind limb stretching.[43] The ACE and CPE combination significantly exhibited analgesic activity, suggesting potential intervention in the prostaglandin pathway. Further research, including testing prostaglandin levels and evaluating the combination's impact on pro-inflammatory mediators like IL-1β, IL-8, TNF-α, and COX-2 expression, is warranted to understand its mechanisms in primary dysmenorrhea intervention.[44,45]

While the acetic acid injection stretch model is recognized for simplicity and sensitivity in assessing analgesic activity, it may yield false-positive results due to its lack of selectivity. Therefore, additional testing methods, such as the formalin test, which mimics acute and chronic pain through neurogenic and central nociceptive mechanisms, should be employed to confirm the analgesic activity of the ACE and CPE combination.[46] Furthermore, an oxytocin-induced writhing test is recommended to assess pain inhibition of uterine hypercontraction in cases of primary dysmenorrhea.[26] In conclusion, the analgesic effects of the ACE and CPE combination were demonstrated in the acetic acid-induced writhing model, suggesting a potential intervention in the prostaglandin pathway. However, further research, including the assessment of prostaglandin levels and the impact on pro-inflammatory mediators, is needed to fully understand the mechanisms involved in the combination's role in primary dysmenorrhea. Additionally, employing more selective testing methods will enhance the validity of the findings.

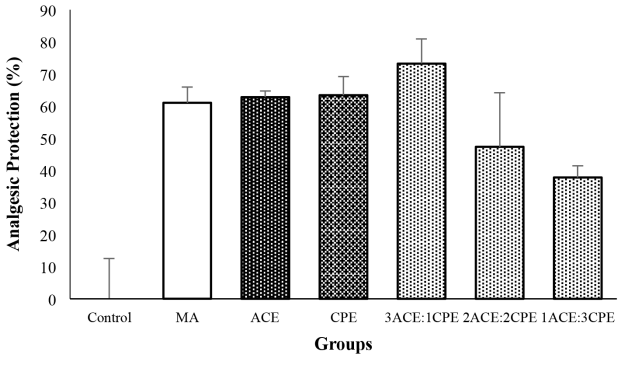

Figure 1. Graph of the percentage of analgesic protection for each test group. (MA: Mefenamic Acid; ACE: *Ananas comosus* extract; CPE: *Carica papaya* leaves extract; 3ACE:1CPE: Combination of *Ananas comasus* (pineapple) extract and papaya leaf 3:1 etc).

Conclusions

Administration of papaya leaf extract and pineapple fruit, either as a single dose or in various combinations, exhibits analgesic activity in mice induced with acetic acid. Among the five extract groups, the combination of pineapple extract and papaya leaves in a 3:1 ratio demonstrates the most effective pain relief. This combination proves to be comparable to mefenamic acid, the positive control. Further research is warranted, such as geliat tests with formalin and oxytocin, along with assessments of prostaglandin levels, IL-1β, IL-8, TNF-α, and COX-2 expression levels. These investigations aim to elucidate the mechanisms underlying the impact of the combined extract of pineapple fruit and papaya leaves on primary dysmenorrhea pain. Additionally, this study should extend its scope to include research on adolescents with primary dysmenorrhea, subjecting them to acute, chronic, and sub-chronic toxicity tests involving the combination of pineapple fruit extract and papaya leaves.

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