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Formulation of Spinach Leaf Extract (*Amaranthus tricolor* L.*)* Shampoo and Antifungal Activity Test Against *Pityrosporum ovale*

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ABSTRACT: Red spinach leaves contain flavonoid, tannin, alkaloid, saponin and terpenoid that have potential as antifungal compounds. This research is experimental. The purpose of this reserach is is to find out extract red spinach leaves can inhibit the growth of *Pityrosporum ovale* and to find out that red spinach leaf extract can be formulated in shampoo. Extraction was carried out using the remaceration method with 70% ethanol solvent and then tested for phytochemical content. Antifungal activity testing of red spinach leaf extract using concentrations of 15; 30; 45; 60 and 75% using the well method with PDA media incubated at 37 for 3x24 hours. The results showed the average inhibition zone activity against *Pityrosporum ovale* at a concentration of 75% of 19.17 mm statistically showed a significant difference. Formulation of shampoo preparations was carried out on red spinach leaf extract at a concentration of 15% with variations in the formula of Cocamide DEA and CMC-Na. The physical characteristics of the shampoo preparation include organoleeptic test, homogeneity, pH, viscosity, adhesion, spreadability and foam height. The results of testing the antifungal activity of shampoo preparations in formula 1 (17.5 mm); formula 2 (17.83 mm); and formula 3 (16.83 mm), based on statistical tests of antifungal shampoo preparations with a p-value (> 0.05) variations in the concentration of Cocamide DEA and CMC-Na have no significant effect on antifungal activity.

Keywords: antifungal; red spinach; *Pityrosporum ovale*; shampoo

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INTRODUCTION

Dandruff is defined as excessive shedding of the scalp, leading to sizable scales that resemble flakes, together with an oily residue (Ginting *et al*., 2021). The proliferation of Pityrosporum ovale is linked to dandruff. Pityrosporum ovale is a yeast or unicellular fungus classified within the genus Malassezia. It is regarded as the primary source of dandruff, despite being a normal component of the scalp's flora (Simanjuntak & Butar-butar, 2019).

Red spinach leaves (Amaranthus tricolor L.) are derived from the red spinach plant, recognized for its secondary metabolite chemicals. It possesses the capacity to impede the proliferation of Pityrosporum ovale. (Rahmawati *et al*., 2021). Flavonoids demonstrate antifungal properties mainly by denaturing proteins. Saponins demonstrate antifungal properties by causing the lysis of microbial cells (Nasrul & Chatri, 2024). Alkaloids have antibacterial effects via disrupting the structural integrity of microbial cell walls (Komang *et al*., 2017). Tannins act as antifungal agents by obstructing the formation of ergosterol, the primary sterol produced by fungi and an essential structural element of the fungal cell wall (Nasrul & Chatri, 2024). In addition to their antifungal properties, secondary metabolites exhibit considerable potential as antioxidants, anticancer agents, and anti-inflammatory substances (Nasrul & Chatri, 2024).

Shampoos are hair care formulations that diminish oil, dandruff, and other pollutant particles that may build on the hair shaft (Mardiyanti & Timur, 2024). Red spinach leaves have antifungal properties due to the presence of secondary metabolite chemicals that interfere with the functionality of the cytoplasmic membrane in fungal cells. The antifungal efficacy of red spinach leaf extract at a 100% concentration against Candida albicans, as determined by the disc diffusion method, resulted in an inhibition zone measuring 16.4 mm (Rahmawati & Retnaningrum, 2022). Shampoo formulations primarily consist of surfactants as active synthetic cleansing agents, preservatives to prevent microbial degradation, viscosity enhancers for optimal pourability in clear, cream, and liquid shampoos, and pH regulators to align the shampoo's pH with that of the scalp (Hia, 2019).

This research aims to explore the possibilities and advantages of red spinach leaves in formulating a herbal antifungal shampoo that demonstrates efficacy against Pityrosporum ovale, the fungus responsible for dandruff. This study may serve as a reference for the antifungal efficacy of red spinach leaves against the dandruff fungus, Pityrosporum ovale.

METHODS

Tools

Tools used in this research were autoclave (All American®), digital analytical balance (Ohaus®) incubator (Memmert®), oven (WTC binder®), ose needle (Rofa®), petri dish (Pyrex), caliper, pH meter (Mettle toledo), rion viscometer and laminar air flow (ESC0).

Materials

Research materials: red spinach leaves, Pityrosporum ovale fungal culture, PDA (E. Merck), 10% DMSO (E. Merck) and ketoconazole powder (PT.Phapros). Formula components: Na lauryl sulfate (E.Merck), cocamide DEA (technical) , CMC-Na, citric acid (technical), menthol, 70% ethanol (technical), distilled water (technical).

The Preparation of Extraction

Red spinach leaf dry powder was measured to ±1 kg and subsequently macerated with 70% ethanol for three consecutive 24-hour periods, with solvent replenishment occurring daily. The filtrate was isolated and evaporated in a water bath until a viscous extract was produced (Guntarti & Ruliyani, 2020).

Phytochemical Screening of Red Spinach Leaf Extract

Alkaloid

0.5 grams of extract was homogenized in CHCl3 and ammonia, followed by the addition of 1 mL of 2N H2SO4. The liquid was agitated, resulting in the formation of two distinct layers. Subsequently, three drops of Dragendorff's reagent were included. The formation of an orange precipitate signifies the presence of alkaloids (Usman *et al*., 2024).

Flavonoids

0.1 grams of extract was solubilized in 2 mL of ethanol. Subsequently, magnesium powder was introduced, followed by the addition of five drops of hydrochloric acid (HCl). The formation of a red color signifies the presence of flavonoids (Usman *et al*., 2024).

Saponins

0.1 grams of extract was measured, followed by the addition of 10 mL of warm water. The mixture is agitated until the froth attains a height of 1 cm. The foam signifies the existence of saponins (Usman *et al*., 2024).

Tannins

0.1 grams of extract were dissolved in 2 mL of water, followed by the addition of three drops of FeCl3. A dark blue alteration signifies the presence of tannins or a blackish-green hue (Usman *et al*., 2024).

Terpenoids

0.1 grams of extract was dissolved in 2 mL of ethanol, followed by the addition of 10 drops of glacial acetic acid and three drops of Liebermann-Burchard reagent. The presence of steroids and terpenoids was confirmed as affirmative upon the formation of a blue-green ring and a violet color ring (Yasser *et al*., 2022). The diameter of each was subsequently measured using a caliper (Yusuf *et al*., 2020). The inhibitor zone underwent analysis.

Antifungal Testing

Preparation of PDA

Approximately 39 g of Potato Dextrose Agar (PDA) was measured and placed into an Erlenmeyer flask, subsequently dissolved in 1000 mL of distilled water. The solution was heated to its boiling point to provide uniform mixing. Upon completion of the homogenization procedure, the solution was chilled to approximately 36-37°C. Measure the pH of the medium, aiming for a range of 4.5 to 5.5. If the pH has not attained the requisite acidity level, it is modified by incorporating a 10% tartaric acid solution. The Erlenmeyer flask was sealed with cotton, gauze, and brown paper to preserve sterile conditions. The media underwent sterilization via autoclaving at 121°C for 15 minutes at

a pressure of 2 atm. Following the sterilizing procedure, 20 mL of chloramphenicol was incorporated into the media under laminar airflow conditions (Azzahra *et al*., 2020)

Preparation of 2% ketoconazole Solution

Two grams of ketoconazole were measured and subsequently dissolved in 100 mL of Aquadest, followed by homogenization until a uniform solution was achieved (Yusuf *et al*., 2020).

Preparation of Test Fungal Suspensions

The test fungus was made by taking one ose of *P. ovale* colony and diluting it with sterile potato dextrose broth medium until the turbidity matched that of the standard McFarland 0.5 solution (Yanthi *et al*., 2021).

Preparation of Test Solution

The ethanol extract of red spinach leaves, at concentrations of 15%, 30%, 45%, 60%, and 75%, was solubilized in 10 mL of DMSO.

The antifungal activity test of red spinach leaf extracts

The antifungal test was performed in a laminar airflow chamber to ensure aseptic conditions. Autoclaved PDA media is transferred in volumes of up to 10 mL into a sterile petri dish. Subsequently, the medium was infused with 1 mL of Pityrosporum ovale suspension and equilibrated by rotating the Petri dish. Subsequently, once the media has solidified, create a mark on the petri dish and form a small well using a volume pipette. Then, introduce the extract concentration along with positive and negative controls into the well. Incubate at 37°C for a minimum of 72 hours until fungal growth occurs, after which the diameters of the colonies were measured using calipers (Yusuf *et al*., 2020). The inhibitor zone underwent analysis.

Shampoo manufacturing process

Shampoo formulation derived from research (Ginting *et al*., 2021) (Table 1). CMC-Na was measured and placed in hot water for several minutes until it expanded, then agitated gently to create muchilago (mass 1). Transferred 20 mL of heated water into a beaker, thereafter added sodium lauryl sulfate, and swirled until complete dissolution occurred (mass 2). Mass 2 was incrementally incorporated into mixture 1 while stirring gently until a homogeneous consistency was achieved, followed by the addition of Cocamide DEA, which was then mixed until uniform. The combination was subsequently incorporated into the homogenized menthol solution, followed by the addition of red spinach leaf extract, and mixed until uniformly distributed. The conclusive solution was contained in a 50 mL bottle (Br. Ginting *et al*., 2021).

Table 1. Shampoo Formula

Ingredients	Formula % (b/v)			
	F0	**F1**	**F2**	**F3**
Red spinach leaf extract	0	15	15	15
Sodium lauryl sulfate	5			
Cocamide DEA	2			
CMC-Na	1,5			
Citric acid	1			
Menthol	0,12			
Aquadest	Up to 100			

Spreadability test

The shampoo was measured at 1 gram utilizing a preparation glass as a medium. The second preparation glass was positioned on the sample and subjected to a load of 50-150 grams during one minute. Subsequently, the diameter of the spread created for each load variation was quantified. The results were computed to derive the mean value. The spreadability range achieved was between 5 and 7 cm. (Meida *et al*., 2024).

Adhesion test

0.25 g of shampoo was measured and positioned between two items, thereafter subjected to pressure from a 1 kg weight for a duration of 5 minutes. A glass object was placed on the testing apparatus, and a weight of 80 grams was applied. The duration for the release of the item glass was documented, with the gel preparation's adhesion deemed acceptable if the release time exceeded 1 second (Yuniarsih *et al*., 2023).

Foam formation test

Ten milliliters of shampoo were measured in a cup and swirled three times for testing. The foam height was recorded within five minutes. The foam height was recorded. The criteria for shampoo foam range from 1.3 to 22 cm (Sambodo & Salimah, 2021).

Data Analysis

The mean diameter of the inhibition zone was analyzed quantitatively by SPSS. Data were not normally distributed and homogeneous ($P < 0.05$), so the analysis was carried out using the Mann-Whitney test and continued with the Kruskal-Wallis test at the 95% confidence level.

RESULTS AND DISCUSSION

The extract of red spinach leaves exhibited organoleptic properties characterized by a blackish-green hue, a viscous texture, and a notable aroma. Phytochemical screening of red spinach leaf extract revealed the presence of active chemicals, including alkaloids, saponins, tannins, flavonoids, and terpenoids (Table 2). Seventy percent ethanol shown efficacy as a solvent for extracting these chemicals.

Evaluation of the antifungal efficacy of Red Spinach Leaf Extract in inhibiting the growth of Pityrosporum ovale. The negative control, consisting of 10% DMSO, did not yield an inhibition zone, but the positive control with 2% ketoconazole exhibited an inhibition zone averaging 17.5 mm in width, indicating robust antifungal action. According to the research of Rahmawati & Retnaningrum (2022), a zone of inhibition measuring 5-10 mm is classified as moderate, 10-20 mm as strong, and 20 mm or beyond as extremely strong. The zone of inhibition generated by red spinach leaf extract at a concentration of 75% in this investigation exceeded that of the positive control, which was 2% ketoconazole. Rahmawati and Retnaningrum (2022) documented inhibitory zone widths of 6.1 mm and 7.9 mm at doses of 60% and 75%, respectively. The inhibitory zone in this investigation had an elevated value. The zone of inhibition against Pityrosporum ovale observed in this investigation exceeded that against Candida.

The inhibitory zone observed in the region exposed to red spinach extract is attributable to the bioactive substances it contains, including alkaloids, flavonoids, saponins, tannins, and terpenoids. Alkaloids contribute to antifungal activity by obstructing the cellular respiration system and protein synthesis, ultimately leading to fungal cell death. Flavonoids impede nutrient absorption in fungal cells, hence hindering their growth and ultimately leading to their demise.

Table 2. Phytochemical Screening Results of Red Spinach Leaf Extracts

Compound	Reagent	Reference	Result	
Alkaloid	Dragendroff	Orange red precipitate (Usman *et al*., 2024)	Red precipitation	Positive
Flavonoid	Mg powder + concentrated HCl	Red, yellow or orange color (Usman *et al.,* 2024)	Red color	Positive
Saponin	Distilled water	Foam Usman *et al.,* 2024)	Formation of foam	Positive
Tannin	$FeCl_3$	Blackish green or dark blue Usman *et al.,* 2024)	Blackish green	Positive
Terpenoid	Liebermann Burchard	Ring brown or violet (Niuwa *et al.,* 2021)	Brown ring	Positive

Table 3. Antifungal Test Results of Red Spinach Leaf Extract

Treatment	Mean ±SD
Ketokonazole 20% Control (+)	17,5±0,5[a]
DMSO 10% Control (-)	0
RSLE 15%	15,43±0,21[b]
RSLE 30%	16,10±0,53[b]
RSLE 45%	16,50±0,50[b]
RSLE 60%	18,50±0,50[b]
RSLE 75%	19,17±0,58[c]

Note: A significant difference (p<0,05) with Mann Whitney test
RSLE = Red Spinach Leaf Extract
a = There a significant difference (p<0,05) between RSLE 75%
b = There is no a significant difference between RSLE 15%, 30%, 45%, 60%
c = There is a significant difference (p<0,05) between Control (+), RSLE 15, 30%, 45% and 60%

Saponins function as antifungals by building complexes with membrane sterols, resulting in fungal cell death, whereas tannin compounds hinder cellular respiration and protein biosynthesis, leading to the demise of fungal cells (Rahmawati & Retnaningrum, 2022). Terpenoids can damage the fungal plasma membrane, leading to ion homeostasis abnormalities in bacterial cells (Attamimi & Yuda, 2022). This thorough analysis elucidates the mechanisms by which alkaloids disrupt fungal respiration and protein synthesis, resulting in cellular mortality. Flavonoids limit fungal development by impairing nutrient absorption and destroying cellular components. The research highlights the increasing interest in plant-based antifungals owing to escalating medication resistance (Esmaeili *et al*. 2025).

Evaluation of Red Spinach Leaf Extract Loaded Shampoo

Organoleptic test result

Organoleptic results in formula 0 with a thick form, clear white color and odorless. The shampoo preparation formulation has a thick shape, dark green color and cherry blossom odor. This is consistent with research conducted by Puspitasary et al. (2019) that found that increasing the concentration of Na-CMC did not affect the color and odor of the resulting product. The study also showed that adding 6-10% Cocomide DEA did not affect the color of the shampoo product, but did increase the odor. This indicates that the combination of CMC-Na (2-4%) and Cocomide DEA (1.5-2.5%) did not affect the color and aroma of the product (Eryaputri et al., 2023)

pH test result

The required pH is 5.0-9.0 (Sambodo & Salimah, 2021). The pH of shampoo preparations to inhibit the fungus *Pityrosporum ovale* must be in the physiological pH range of the scalp, which is not irritating and remains effective in fighting the fungus, in the pH results in three shampoo preparations containing red spinach leaf extract, the one that is more fulfilling is formula one with a pH of 5.85. Research conducted by Prayadna et al. (2017) showed that increasing the concentration of Cocomide DEA to 2.3 and 5% increased the pH of shampoo by 6.8, 7.2, and 10.95, respectively. Cocomide DEA is alkaline. Another study conducted by Puspitasary et al. (2019) also showed that increasing CMC-Na to 3, 4,5 and 6% also increased the pH value. These data indicate that increasing Cocomide DEA and CMC-Na causes an increase in pH towards alkaline.

Viscosity test result

The viscosity results on the preparation of red spinach leaf shampoo meet the viscosity requirements of shampoo preparations, namely 400-4000 cPs. The higher the concentration of CMC-Na and Cocamide DEA in the preparation, the higher the viscosity of the preparation. The higher the viscosity result of the preparation, the shampoo preparation can stick to the scalp longer. Research conducted by Eryani et al. (2023) stated that the higher the concentration of CMC_Na, then it can increase viscosity of preparation higher. Research conducted by Eryaputri et al. (2023) stated that a 6% concentration of Cocomide DEA resulted in a shampoo viscosity of 1970 cPs to 2730 cPs at a 10% concentration. This indicates that increasing the consistency of Cocomide DEA and CMC-Na increases the shampoo viscosity.

Spreadability test result

Shampoo containing red spinach leaf extract showed the results of the spreadability test that are in accordance with the criteria, which are in the range of 5-7 cm (Meida *et al*., 2024). The results are influenced by the different concentrations of thickeners in the formulation, where a decrease in viscosity contributes to an increase in the spreadability value. Based on the results of the spreadability test in Table 4 between shampoo F0 (without red spinach leaf extract) and F1 (15% red spinach leaf extract), the addition of red spinach leaf extract decreases the spreadability of the shampoo, but increasing the concentration of Cocomide DEA and CMC-Na decreases the spreadability. The value of the adhesion test has a relationship with the spreadability, where the smaller the spreadability, the longer the time needed to adhere, and conversely, the greater the spreadability of the ointment, the faster the time needed for the ointment to adhere, due to the thick consistency (Arinata et al., 2025). The results of the adhesion test in Table 4 show that shampoo F3 with 4% Cocomide DEA and 2.5% CMC-Na with the greatest adhesion should produce a shampoo preparation with the smallest spreadability. The data is in accordance with this study that F3 with the greatest adhesion has the smallest spreadability value, namely (5.61 ± 0.15 cm). The adhesion value is smaller than shampoo

with formula F0 (6.27±0.20 cm), F1 (6.11±0.25 cm), and F2 (6.86±0.52 cm). Increasing the concentration of Cocomide DEA and CMC-Na decreases the spreadability of the shampoo preparation. When viewed from F0 and F1, the addition of red spinach leaf extract decreases the spreadability of the shampoo preparation.

Adhesiveness test result

In this adhesion test, it less than one second, thus F0, F1, F2 and F3 met the requirements of adhesion of shampoo. Based on the results of the adhesion test, adhesiveness of preparation increase directly proporsional with increasing concentration of CMC. The increase in viscosity in the preparation is due to the increase in CMC-Na. The CMC-Na excipient has the characteristic of producing a thick, gel-like substance. This viscosity increases with the increase in the concentration of CMC-Na. Research conducted by Eryani et al. (2023) showed that shampoo with CMC-Na 3, 4, and 5% produced adhesive strengths of 8.32±1.52, 14±1.73, and 20.6±2.08 seconds, respectively. Research conducted by Eryaputri et al. (2023) showed that increasing the concentration of Cocomide 6-10% resulted in an increase in viscosity. The increase in viscosity is directly proportional to the increase in adhesive strength. These data indicate that F3 should have the greatest adhesive strength because it has the largest concentration of Cocomide DEA and CMC-Na compared to other formulas. The increase in viscosity is a function directly proportional to adhesive strength. The greater the viscosity of the preparation, the greater the adhesive power produced, which means the longer the adhesion time of the preparation. The results of this study show that F3 shampoo, which has the highest concentration of Cocomide DEA and CMC-Na, has the greatest adhesive power (4±0.58) compared to formulas F0 (1±0.12 seconds), F1 (1±0.12 seconds), and F2 (2±0.52 seconds).

Foam formation test result

Foam height testing was carried out to assess the ability of surfactants to produce foam, with a literature reference that shows the range of foam height on the same ranges from 1.3 to 22 mm. The higher the resulting foam can help the preparation in inhibiting fungi because the preparation can distribute its active ingredients evenly. Shampoo preparation without extract (F0) and shampoo with 15% red spinach leaf extract (F1) with the same concentration of Cocomide DEA (2%) and CMC-Na (1.5%), F0 produced a foam height that was not much different (6.34±0.60) with F1 (6.63±0.67). Variations in the concentration of Cocomide DEA (2-4%) and CMC-Na (1.5-2.5%) produced a foam height that was not much different, which means that variations in the concentration of Cocomide DEA and CMC-Na did not affect the foam height. This is different from the research conducted by Eryaputri et al. (2023) which showed that increasing Cocomide DEA resulted in a high increase in foam. Evaluation of of Red Spinach Leaf Extract Loaded Shampoo can be seen in Table 4.

Antifungal Activity Test Red Spinach Leaf Extract Shampoo Preparations

The results of the antifungal activity test of red spinach leaf extract loaded shampoo have a zone of inhibition with a strong category based on Rahmawati & Retnaningrum, (2022) that the zone inhibition of 5-10 mm falls into the moderate category, 10-20 mm into the strong category, and =20 mm / > into the very strong category gainst *Pityrosporum ovale.* Based on statistical analysis the results of the *Kruskal-Wallis* statistical test obtained an Asymp. Sig value > 0.05, indicating no significant differences among the test groups for the antibacterial inhibition zone variable, the results of *Mann Whitney* test formula 0 indicates significant differences ($p<0.05$). Antifungal result of red spinach leaf extract loaded shampoo can be seen in Table 5.

Table 4. Evaluation of Red Spinach Leaf Extract Loaded Shampoo

Parameters (n=3)	Formula			
	F0	F1	F2	F3
pH	5.11±0,01	5.85±0,02	5.93±0,00	6±0,01
Viscosity (cPs)	2200±0,00	2300±0,34	2700±0,42	2900±0,5
Spreadibility test (cm)	6,27±0.20	6,11±0.25	6,86±0.52	5.61±0.15
Adhesiveness Test (second)	1±0.12	1±0.12	2±0.52	4±0.58
Foam height (cm)	6.34±0.60	6.63±0.67	6.5±0.78	6.1±0.55

Note
F0 : Shampoo containing CMC 1,5%
F1 : Shampoo containing RSLE 15% + CMC 1,5%
F2 : Shampoo containing RSLE 15% + CMC 2%
F3 : Shampoo containing RSLE 15% + CMC 2,5%

Table 5. Antifungal Result of Red Spinach Leaf Extract loaded Shampoo

Treatment	Zone of inhibition (mm)			Mean±SD
	R1	R2	R3	
Ketokonzole 20% Control (+)	17,5	15	16	16,17±1,26
F0*	0	0	0	0±0
F1	18,5	16,0	16,0	17,5±1,32
F2	18,5	17,5	17,5	17,83±0,71
F3	16,0	16,5	18	16,83±1,04

Note: * indicates significant differences ($p<0.05$) with the *Mann Whitney* test

CONCLUSIONS

Red spinach leaf extract has antifungal activity against the growth of *Pityrosporum ovale.* The shampoo containing red spinach leaf extract has antifungal activity against *Pityrosporum ovale* fungi with an effective extract concentration of 15%. The evaluation results of the shampoo preparation meet the standard characteristics of shampoo preparations.

AUTHOR CONTRIBUTION

Marcia, Margareta, Nanda: literature search; experimental studies; data analysis; manuscript preparation.

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