In Vitro Pediculicidal Efficacy and Acute Dermal Irritation Tests of Hair Cream from the Methanolic Extract of the Stem of *Tinospora crispa* (L.) Hook. *f. et* Thoms.

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ABSTRACT

Objective. To determine the in vitro pediculicidal efficacy and acute dermal irritation in rabbits of hair cream prepared from the methanolic extract of *Tinospora crispa* (L.) Hook. *f. et* Thoms. known in the Philippines as *makabuhay*.

Methods. Several concentrations of methanolic extract, an aqueous and alcoholic extract, from *makabuhay* were tested for in vitro pediculicidal efficacy based on methods established by earlier studies. The 60% concentration of the methanolic extract, found to be the most efficacious, was made into a hair cream and was tested for in vitro pediculicidal efficacy against Kwell® Reformulated Shampoo (Permethrin 1%). The same product was evaluated for acute dermal irritation in rabbits.

Results. The 60% concentration of the methanolic extract formulated into a hair cream showed in vitro pediculicidal efficacy based on stringent criteria of 100%, 96.4%, 85.7%, 78.6%, 67.9%, 64.3% and 64.3% after 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, and 6 hours of observation, respectively, as compared with the 100% efficacy of Kwell® Reformulated Shampoo (Permethrin 1%). The hair cream passed the dermal irritation test on abraded and intact skin of rabbits.

Conclusion. The hair cream from the 60% methanolic extract of *makabuhay* was shown to possess some in vitro pediculicidal efficacy and was found to be non-irritating. This herbal pediculicide may be an alternative treatment for lice infestation.

Key Words: Tinospora crispa, makabuhay, herbal pediculicide, pediculicidal efficacy

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Based on the master's thesis entitled "Safety, Efficacy, and Stability of a Pediculicidal Hair Cream from the Methanolic Extract of the Stem of *Tinospora crispa* (L.) Hook. *f. et* Thoms."

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Introduction

Pediculosis or lice infestation is widespread, affecting people around the world regardless of socio-economic status.[1] There are as many as six million to 12 million cases worldwide each year. Most of these cases are in children between the ages of three and 10 years old, but other family members may become infested as well.[2] In the Philippines, the report of the Department of Education's Health and Nutrition Center by R.A. Ronda, which appeared in the October 8, 2008 issue of the Philippine Star, cited pediculosis as the second most common health problem among public school children next to tooth decay and dental caries.[3]

This research study was conceived in order to address the problem of pediculosis or head lice infestation prevalent among children of school age in urban poor and rural communities in the Philippines through the use of herbal pediculicide, *Tinospora crispa* (L.) Hook. *f. et* Thoms., known in the country as *makabuhay*.

In previous studies, *makabuhay* has been shown to be effective in the treatment of scabies.[4,5] The aqueous extract of the plant has been used as a pesticide in infestation of rice by insect pests.[6,7] The fresh stem juice obtained by crushing the plant part using a mortar and pestle which has also been shown to possess an in-vitro pediculicidal effect based on the percentage killing rate of 85% and 100% after 30 minutes and 45 minutes, respectively, relative to permethrin's 100% efficacy. This study showed that the *makabuhay f*resh stem juice possesses in vitro pediculicidal activity.

Drugs used to treat lice infestation are known as pediculicides and are most commonly neurotoxin-based chemical agents like pyrethrin and permethrin. The safety of these agents is questionable, with growing concerns regarding resistance to their formulations.[8] These products are also too expensive for poor Filipinos, hence the need for a safe, effective, and affordable alternative pediculicidal agent to treat lice infestation in the affected population of the Philippines.

Herbal pediculicides have been used in traditional medicine. Aside from being low cost, they are believed to be less toxic but still effective in the treatment of head lice infestation.[8] However, there is limited information about these agents. More research is needed to assess the safety

and effectiveness of these compounds before they can be fully utilized in treating lice infestation.[9]

The aim of this study was to show the safety and efficacy of a pediculicide prepared from the methanolic extract of the stem of *makabuhay* through an in vitro pediculicidal efficacy test and in-vivo dermal irritation test. This study is an essential part of a larger study to develop an effective, safe, and stable pediculicidal hair cream from *makabuhay*.

Methods

Methanolic extract from the stem of *makabuhay* was tested at different concentrations for in vitro pediculicidal efficacy and acute dermal irritation in rabbits, alongside the formulation and testing of a stable finished product, a hair cream from a 60% concentration of the extract (Figure 1). Product formulation trials were done at the UP Manila College of Pharmacy, Industrial Pharmacy Department.

Choice of hair cream

Hair cream was chosen as the delivery form for the pediculicidal preparation because creams can achieve more intimate and prolonged contact with the hair and scalp, being more viscous compared with other formulations. This property is important for a pediculicidal agent, which kills through contact with lice. Hair creams also have the advantage of being easier to spread on the hair with the use of a comb. It is also easy to remove by washing with water. The hair cream formulation also enables the *makabuhay* extract to be prepared as a stable pediculicide at a maximum concentration of 60%.

In vitro pediculicidal efficacy test (Figure 2)

The test for in vitro pediculicidal efficacy was adapted from the method of Heukelbach, using adult lice and nymphs, and not eggs.[10] The lice used in the test were obtained from recruited children using procedures prepared for this purpose and approved by UP Manila–National Institute of Health Research Ethics Committee.

Nine (9) treatment groups were tested for in vitro pediculicidal efficacy consisting of seven (7) concentrations of methanolic extract from the stem of *Tinospora crispa* (10%, 20%, 30%, 40%. 50%, 60%, and 100%). The 60% aqueous extract was obtained by decoction of the coarsely powdered stem and the 60% ethanolic extract was prepared by maceration. A commercially available formulation with 10% Permethrin (Kwell®-Reformulated Shampoo, GSK), no treatment,, and the cream base (0% extract) were used as controls.

The final formulation of hair cream using 60% methanolic extract from *makabuhay* was also tested for pediculicidal efficacy as part of its quality test and stability studies.

For each of the 13 experimental groups (10 treatments including the hair cream and three control groups), a total of 28 adult lice and nymphs were tested. Lice clasping hair strands were immersed completely in the material to be tested for 20 minutes and then placed with hairs onto Whatman filter paper in Petri dishes. Pools of the products were wiped from the lice using a jeweller's forceps. The lice

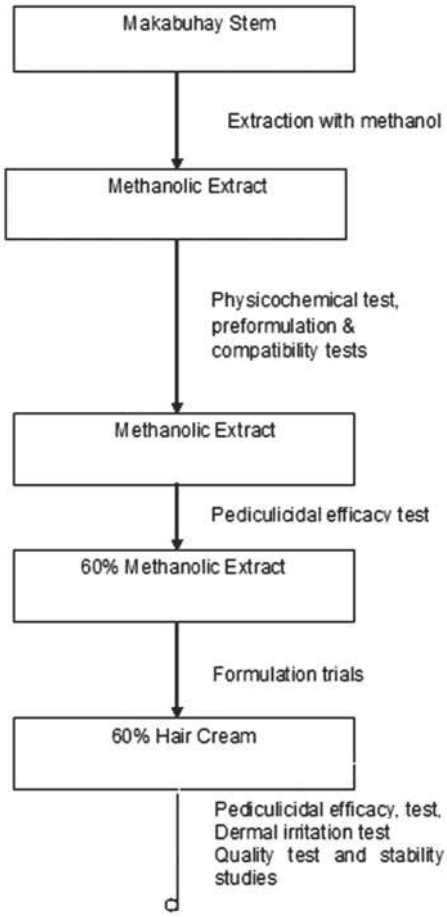

Figure 1. Safety, efficacy and stability studies of pediculocidal hair cream from *makabuhay*

in the no-treatment control group were placed directly on moistened filter paper without any treatment. They were not dipped in water or wiped with forceps, as these actions have been shown in previous studies to have no effect on lice activity levels. To prevent lice from desiccation, the filter paper was moistened with about 200 μL of tap water. To simulate treatment on an infested host, head lice were washed in tap water after 20 min and placed in a new Petri dish with unused filter paper. Seven treated lice were placed in each of the four Petri dishes lined with moistened filter paper and examined after 30 minutes, one, two, three, four, five, and six hours to see if killed (K), weakened (W) or remained active or unaffected (A) by the treatment. Lice on the filter paper were examined under a dissecting microscope and magnifying lens by a single observer in all

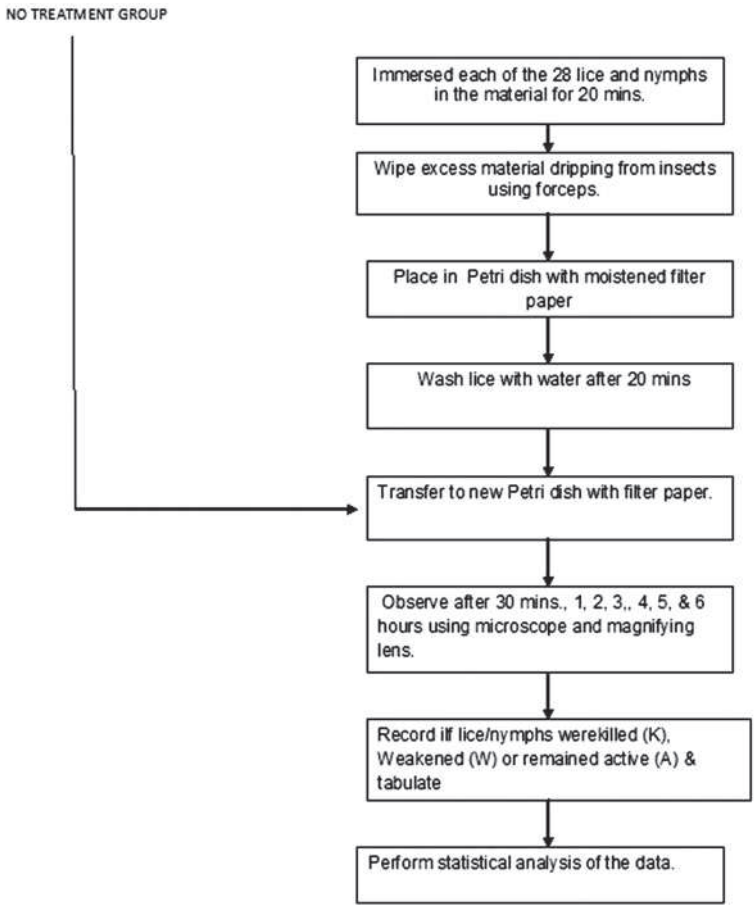

Figure 2. Pediculocidal efficacy test

cases to prevent inter-observer variability. The lice in each Petri dish were closely monitored and the observations (K,W, A) were tabulated.

There were two criteria used in the pediculicidal efficacy tests. First was the *stringent criteria,* wherein death of the adult lice and nymphs were recorded only when there is absolutely no movement observed (K). The second was the *unstringent criteria,* when adult lice or nymphs are considered "killed"(more properly neutralized) even if there are still signs of movement in the insects, such as movement of claws and legs in place and/or peristaltic movement of the gut observed under the microscope (W+K).

Statistical treatment of data

The data obtained was analyzed using Stata Version 8 (STATA Corporation) to compare differences in counts of the experimental groups using Two Way Repeated Measures ANOVA followed by Scheffe's Multiple Comparison Test.[11] A potential positive effect exists when there is at least one concentration of the methanolic extract with a significant difference in effect as compared with the effect of the negative control groups (no treatment and cream base), and, conversely, if the most effective concentration of the extract and the finished product prepared from the extract have no significant difference in effect with that of the commercially available product (Kwell® Reformulated). Multiple Comparison Tests (MCT) were applied after ANOVA to identify treatment pairs which indicate possible significant differences for each time period of observation.

In vivo dermal irritation test (Figure 3)

The in vivo dermal irritation test using the 60% hair cream, the concentration of the finished product selected for this study, was performed on the abraded and unabraded/intact skin of healthy rabbits following the procedure of the Organization of Economic Cooperation and Development (OECD) for the testing of chemicals.[12]

Two groups with three healthy rabbits per group were acclimatized to laboratory conditions for five days before testing. The rabbits were placed in individual cages at room temperature (27±3°C) and a relative humidity of 30% to 40% RH. A sufficient supply of food and water ad libitum was given to the animals. The fur of the dorsal area of each rabbit was shaved 24 hours before the test. A dose of 0.5 g of the cream was applied to the test site, approximately 6 square

cm in area. The adjacent area of untreated skin on each animal served as negative control for the test. The cream was spread on a gauze patch that was then applied to the skin. The area was covered with gauze and held in place with non-irritating tape. The patch was removed 24 hours after exposure.

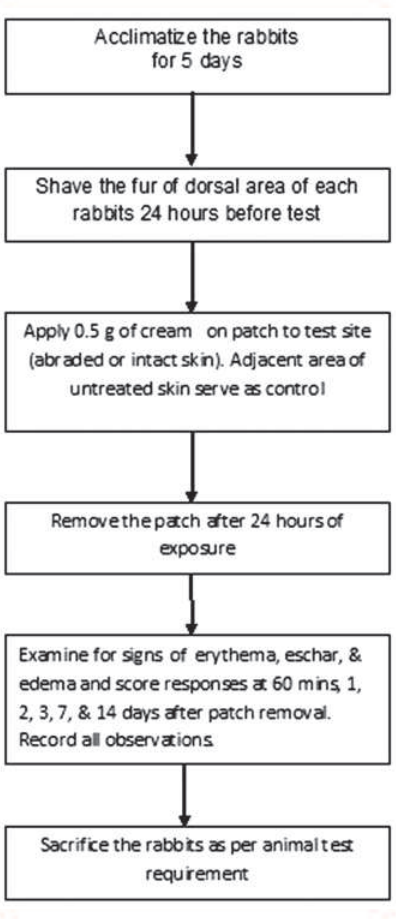

Figure 3. Acute dermal irritation test in rabbits

The duration of observation was 14 days after application. The animals were examined for signs of erythema, eschar, and edema and the responses scored at 60 minutes, 24, 48, and 72 hours, and 14 days after patch removal. Dermal irritation was scored and recorded according to the grades. Further observations were done as necessary to establish reversibility. In addition to the observation of irritation, any serious lesions and other toxic effects were noted. The rabbits were sacrificed after the test as per animal test regulation.

Results

The result of the in vitro pediculicidal efficacy tests showed that the 60% methanolic extract was the most efficacious concentration of *makabuhay* as compared to the lower methanolic concentrations of the extract, the 60% aqueous and the 60% ethanolic concentrations of *makabuhay*. The statistical test confirmed the significant difference in effect of the 60% concentrations of the methanolic extract relative to the negative controls (cream base and no treatment) and the other concentrations of the extract tested. However, Kwell® Reformulated Shampoo was found to be more efficacious than the 60% methanolic extract concentration with pediculicidal efficacy of 100% from 30 minutes up to the end of six hours of observation.

Figures 4 and 5 present the computed percentages of lice killed based on the less stringent (W+K/28*100) and stringent (K/28*100) criteria, respectively, for all experimental treatments at all time periods of observation. From the graph, it can be seen that the 60% methanolic extract concentration of *makabuhay* was 100% efficacious under the less stringent criteria (Figure 4) but with efficacy beginning with 100% (after 30 minutes of observation), decreasing to 96.4% (after 1 to 3 hours), 92.8% (after 4 hours) and 85.7% at the end of five and six hours under the stringent criteria (Figure 5). Kwell® Reformulated Shampoo exhibited a consistent 100% efficacy under both criteria. The 60% ethanolic extract of *makabuhay* also demonstrated good pediculicidal efficacy, though less than the methanolic extract.

The 60% methanolic extract was formulated into a hair cream and tested for quality. Table 1 shows the quality test results of the hair cream prepared from the 60% methanolic extract.

Table 1. Results of quality test of the 60% hair cream

Test Parameter	Actual Result
Appearance	Smooth moist cream
Color	Brownish black
Odor	Mint
pH	4 to 5
Particle size	16.75 μm (coarse emulsion)
Spreadability	6.0 mm
TLC Profile	Two (2) Distinct purple spots;1st (0.412), 2nd(0.575)
HPLC (for berberine)	0.89%
% Methanol (Gas Chrom.)	0.13%
Pediculicidal Efficacy	>80%Killed first 2 hours to 64.3% after 6 hours
Microbial limit	Passed

It can also be seen from figure 5 that the in vitro pediculicidal efficacies of the hair cream based on the stringent criteria were: 100%, 96.4%, 85.7%, 78.6%, 67.9%, 64.3% and 64.3% at 30 mins, one, two, three, four, five, and six hours of observation, respectively. There was a decrease in efficacy of the hair cream as compared with the extract, probably due to insufficient mixing of extract and cream additives.

The same batch of the 60% hair cream was subjected to dermal irritation test on the abraded and intact skin of healthy rabbits. A sample of the 60% hair cream was

Experimental Groups	30 mins	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours
Cream Base	50	53.6	64.2	82.1	78.6	78.6	64.3
10% Meth. Extract	71.4	50	17.9	7.1	10.7	17.9	17.9
20% Meth. Extract	3.6	10.7	3.6	0	3.6	7.1	3.6
30% Meth. Extract	82.1	75	67.8	57.1	53.6	53.6	50
40% Meth. Extract	100	100	82.1	60.7	53.6	75	71.4
50% Meth. Extract	100	100	92.8	85.7	82.1	78.5	75
60% Meth. Extract	100	100	100	100	100	100	100
60% Aq. Extract	7.1	0	0	0	0	0	0
60% Ethan. Extract	100	100	100	100	100	92.9	85.7
100% Meth. Extract	100	100	100	100	100	100	100
No Treatment	3.6	7.1	0	0	7.1	7.1	3.6
Kwell®	100	100	100	100	100	100	100
60% Hair Cream-Meth	100	100	96.4	89.3	85.7	78.6	78.6

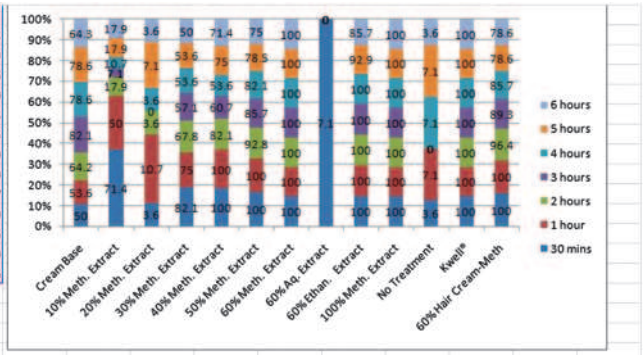

Figure 4. Percentage of lice killed using less stringent (unstringent) criteria

	30 mins.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours
Cream Base	0	0	0	0	25	39.2	21.4
10% Meth. Extract	21.4	10.7	0	3.6	3.6	3.6	3.6
20% Meth. Extract	3.6	0	0	0	0	0	3.6
30% Meth. Extract	82.1	67.9	53.6	46.4	35.7	32.1	32.1
40% Meth. Extract	92.9	92.9	57.1	32.1	21.4	21.4	21.4
50% Meth. Extract	100	96.4	78.6	67.8	35.7	28.6	28.6
60% Meth. Extract	100	96.4	96.4	96.4	92.8	85.7	85.7
60% Aq. Extract	0	0	0	0	0	0	0
60% Ethan. Extract	100	100	100	96.4	85.7	78.6	78.6
100% Pure Extract	100	100	100	100	100	100	100
No Treatment	0	0	0	0	0	0	0
Kwell®	100	100	100	100	100	100	100
60%Hair Cream-Meth.	100	96.4	85.7	78.6	67.9	64.3	64.3

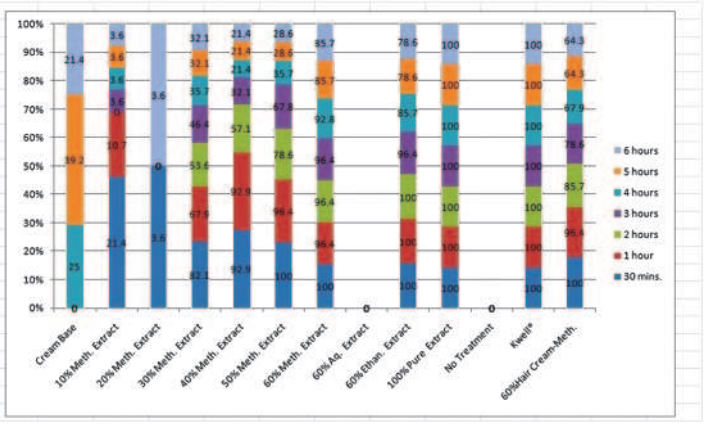

Figure 5. Percentage of lice killed using stringent criteria

submitted for testing for dermal irritation in healthy rabbits at the Industrial Technology Development Institute (ITDI) of the Department of Science and Technology (DOST). The result of the tests in both abraded and unabraded/intact skin of healthy rabbits showed that the product is not a skin irritant in either skin condition. There was no erythema, eschar, or edema formation observed during the entire period of observation. No adverse reactions were noted at the end of 14 days.

Discussion

Makabuhay is one of the most popular Philippine medicinal plants because of its many uses. Quisumbing reported the following traditional uses of the drug: the aqueous extract is used in the treatment of stomach trouble, indigestion and diarrhea and as tonic; a preparation mixed with coconut oil is effective for rheumatism and flatulence in children; the decoction of the stem is used as a wash for tropical ulcers, itches, ordinary and cancerous wounds.[13] A lotion consisting of two parts extract and one part refined vegetable oil has been reported as effective in the treatment of scabies.[4] An in vitro efficacy study of the plant juice showed its potential pediculididal activity.[14]

This study was on the pediculicidal efficacy of the methanolic extract of the stem of *makabuhay* prepared into a hair cream. It differs from the previous study by Reyes et al. in that it involved tests for pediculicidal efficacy of different types of extract and a hair cream prepared from the methanolic extact at a longer observation period of 6 hours. The longer observation period proved to be significant in fully assessing pediculicidal efficacy. Both studies confirm the pediculicidal activity of the juice and extracts from the stem of *makabuhay*.

The pediculicidal efficacy tests of the different concentrations of the methanolic extract–cream base mixtures, 60% aqueous and 60% ethanolic extracts showed that the 60% methanolic extract concentration was the most efficacious. Thus, a 60% hair cream was prepared from the

methanolic extract of the stem of *makabuhay* mixed with compatible excipients. It proved to be efficacious as a pediculicidal preparation, though not as efficacious as the prototype drug, Kwell® Reformulated Shampoo (Permethrin). As seen in the study and as reported in the literature, lice are capable of "sham death". This means that lice and nymphs may be inactivated by the pediculicidal agent for some time during the period of observation only to recover afterwards. This explains why the reported mortality rate of *makabuhay* hair cream changes from 100% after 30 minutes of observation to 85.7% after two hours of observation and 64.3% after six hours. Thus, Heukelbach et al. prescribed observation periods of ≥ six hours and pediculicides resulting in not less than 80% mortality rates to be registered as drugs.[10] The 60% *makabuhay* hair cream killed 86% of the lice and nymphs after two hours of observation but only 64% remained inactive after six hours; the others had recovered. This result is consistent with the research findings of Heukelbach and his associates assessing the in vitro efficacy of several commercial pediculicides, only two out of seven pediculicides (Tea TreeGel® from *Melaleuca alternifolia* and Quellada® Permethrin) met the criteria satisfactorily.[10]

Statistical analysis of the data collected from the tests for pediculicidal efficacy confirmed findings of the efficacy of the 60% methanolic extract-cream base mixture and the 60% hair cream. The results of Two Way Repeated Measure ANOVA combined with Scheffe's Multiple Comparison Test provided an objective basis in interpreting the claim of pediculicidal efficacy of *makabuhay*. The efficacy of the herbal pediculicide was also found to be time–dependent, being inversely proportional with time. This means that the longer the time of observation, which was from 30 minutes to six hours, the lower the recorded percentage efficacy of the 60% hair cream from the methanolic extract of *makabuhay*. This result is consistent with the ability of lice to exhibit "sham death."

The pure (100%) methanolic extract was found to be as efficacious as the prototype drug. However, it must be remembered that prepared dosage forms have their advantages over crude extracts such as ready availability and convenience of administration. The 60% pediculicidal hair cream is better to use in this respect. The hair cream from the *makabuhay* extract may be considered a natural alternative to permethrin-based pediculicides, particularly in cases of drug allergy and drug resistance.

The result of the dermal irritation test of the 60% hair cream showed that the product is not a skin irritant to both abraded and intact skin of rabbits. Since rabbits have more sensitive skin than humans to irritant substances,[15] it may be said that this test is a good indicator that the product can be applied to the skin and scalp without danger of acute irritation.

One more safety concern remains, in that the product uses methanol as the solvent of extraction. Methanol is classified as a Class 2 solvent by the International Conference on Harmonization (ICH).[16] Class 2 solvents are those which must be limited in herbal preparations due to their potential adverse effects. Thus, the methanol in the *makabuhay* extract must be determined and limited to ensure safety of the product. The presence of methanol in the extract and the 60% hair cream was measured by gas chromatography and were found to be 2.12% in the extract and 0.13% in the hair cream respectively. The reduction of methanol in the extract was accomplished by drying the extract obtained using the rotary flask evaporator immediately after continuous extraction. During processing, the methanol in the extract was further removed by heating the extract at 70ºC to 80ºC (above the boiling point of methanol at 64.7 ºC) on a water bath and continuing the heating during the mixing of the extract with the excipients at 70ºC to 80ºC to form the cream. With this procedure, reduction of the methanol in the final product can be ensured to a safe level.

The ICH guidelines provide options for establishing limits to Class 2 solvents in terms of permitted daily exposure (PDE) given the dose of the drug and the concentration of the solvent in the drug. Based on these guidelines, the exposure of the patient to the use of the hair cream given 10 g to 20 g topical use daily was computed. The computed daily exposure was between 13 mg to 26 mg respectively (assuming 0.13% methanol content in the hair cream). These values were below the permitted daily exposure (PDE) of 30 mg per day for methanol .[16]

Conclusion and Recommendation

In vitro pediculicidal efficacy of the methanolic extract and the hair cream were tested based on the method of Heukelbach. The most efficacious concentration of the extract was identified to be the 60% concentration of the methanolic extract–cream base mixture. The 60% concentration of the methanolic extract formulated into a hair cream showed in vitro pediculicidal efficacy based on stringent criteria of 100%, 96.4%, 85.7%, 78.6%, 67.9%, 64.3% and 64.3% after 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, and 6 hours of observation, respectively. The 60% *makabuhay* hair cream apparently killed 86% of the lice and nymphs after two hours of observation, but the percentage declined to 64.3% after six hours, below Heukelbach's prescribed 80% efficacy. This observation can be attributed to the ability of lice for stasis or "sham death," a period of inactivity upon exposure to the pediculicidal agent after which they may recover. The 60% hair cream prepared from the extract passed the acute dermal irritation test. This means the product was not a skin irritant to both abraded and unabraded or intact skin.

The following are recommendations for future research:

1. Perform fractionated bioassay for pediculicidal activity using different solvents, particularly including ethanol, which resulted in good pediculicidal results alongside methanol. This method may identify the solvent fractions possessing more potent pediculicidal efficacy at lower concentrations as compared with the crude methanolic extract. The active principles of the fractions may later be isolated and purified and used to prepare the appropriate dosage form for a safer, more effective and stable herbal pediculicide of *makabuhay.*
2. Continue safety tests and conduct clinical studies using the hair cream from the methanolic extract of *makabuhay* as a pediculicide.

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Erratum to Vios S, Chua A, Guerrero M, et al. SigN-PQ Neuropathic Pain Questionnaire Development and Validation in English and Filipino Languages. Acta Medica Philippina. 2010; 44(3):10-17.

In Appendix B, Item 6 - "Is the pain stabbing in character?" *"Para bang sinasaksak ang sakit?"* should have been omitted.