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Seaweed wonders: Phytochemical, antibacterial property and burn wound healing effect of Nagkayasan (*Chaetomorpha crassa*) extract vaseline based ointment

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Abstract

This study aimed to determine the phytochemical, antibacterial property and wound healing effect of Nagkayasan (*Chaetomorpha crassa*) Vaseline based ointment in induced wound on Sprague Dawley rats. The experiment was laid using Analysis of Variance (ANOVA) in four treatments with three replicates each. In treatment 1, rat wounds were given a 0.1g of Nagkayasan antiseptic ointment. In treatment 2, rat was also given a 0.2g of Nagkayasan antiseptic ointment, in treatment 3, rat wound are left untreated. In treatment 4, rat wound were given a positive control which is the Mupirocin. Wound length among groups are gathered, properly recorded and analyzed. As to toxicity and dermal irritation evaluation of the Nagkayasan antiseptic ointment, the animals were observed for behavioural and clinical changes. The animals were found to be normal during the observation period and exhibited none of the previously mentioned changes. Results on the measured wound contraction of the wounds showed that there is significant on day 3 observation but not significant on day 6, day 9 and day 12. Likewise, the presence of seven secondary metabolites in Nagkayasan seaweed and the susceptibility of the two test pathogens *E. coli* and *S. aureus* on Nagkayasan Vaseline based ointment is a proved that the seaweed has antiseptic property.

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Introduction

Seaweeds are macrobenthic (large and attached) forms of marine macroalgae. They have a simplified, primitive structure compared to higher plants. The vegetative plant body has no roots, stems and leaves and is generally called "thallus". They vary in forms, some are branching, leaf-like or bushy type, club-like, featherlike, while some form sponge-like encrustations on hard substrates like the rocks, corals and boulders. Seaweeds are generally photosynthetic plants.

They can be found in the intertidal and subtidal zones of the sea. Generally, green and brown seaweeds can be found from intertidal to shallower portion of the subtidal zone. Red seaweeds are distributed in both zones but its corraline type of species is found in the deeper portion of the subtidal zone. On the study conducted by Baleta (2016), there were 34 species of seaweeds documented in Nangaromoan, Sta. Ana, Cagayan. Seaweeds offer a wide range of therapeutic possibilities both externally and internally, direct or indirectly, when used as foods, because they have soft tissues. Also, simply eating unprocessed dried seaweeds can yield many healing benefits. Many physical ailments in both human and even in animals can be regularly resolved with the simple addition of seaweeds to their respective diets Ganzon-Fortes, (1991).

The use of seaweeds, which abounds in the country, helps to augment the present situation of the Filipino people to be self-reliant as to the maintenance of our health especially the low- income group of the community. Seaweed derived natural products are extremely important as sources of medicinal agents. Reverting back to herbal medicine explores the efficacy and effectiveness of seaweeds to address the increasing use of antibiotic and significant resistance to the drug. In fact, seaweeds contain many bioactive and pharmacologically important compounds such as alginate, carrageen and agar as phycocolloids. This study is designed to investigate an alternative burn wound healing ointment derived from seaweed ethanolic extract of Nagkayasan (*Chaetomorpha crassa*), through several approaches including bioassays and molecular biology tools.

Likewise, the finding of antibacterial activity of the extract is hoped to have potential in producing alternative antiseptic agents from natural resources, against resistant bacteria *Escherichia coli* and *Staphylococcus aureus* to reduce the infections and fatality. Likewise, the burn wound healing effect will be determined to established its antiseptic potential.

Generally, this study aimed to determine the phytochemical, antibacterial property and wound healing effect of Nagkayasan (*Chaetomorpha crassa*) Vaseline based ointment in induced wound on Sprague Dawley rats.

Specifically, this study aimed:

1. To determine the secondary metabolites, present in the ethanolic extract of Nagkayasan;
2. To determine the physico-pharmaceutical characteristics of the Nagkayasan Vaseline base ointment;
3. To determine the inhibitory effect of the Nagkayasan ethanolic vaseline base ointment against *Escherichia coli* and *Staphylococcus aureus*;
4. To determine the burn wound healing effect of the different dosage of Nagkayasan vaseline base ointment by measuring the wound lenght contraction in burn wound of rats;
5. To compare the effect of the wound healing effect of the Nagkayasan vaseline base ointment and the standard drug Mupirocin (Bactroban) in burn wound of rats; and
6. To determine if there is a significant difference on the wound length contraction of burn wound in Sprague Dawley rat when treated with different dosage of Nagkayasan.

Materials and methods

Materials

Graduated cylinders, Beakers, evaporating dish, Erlenmeyer's flasks, Volumetric flasks, Porcelain Buchner funnel, Glass funnel, Glass rod, Graduated pipette, Spatula, Test tubes, Test tube holders, Test tube rack, Test tube brush, Petri dish, Forceps, inoculating loop, Cork borer, Reagent sprayer, Filter paper, Water bath, Auto clave, Oven, Incubator, Mettle balance, Tracing paper, Medicine dropper, Nescafe glass jar, rat cage, were the materials used

in the study. The reagents that was used in the study are as follows: 80% ethyl alcohol, , 2M HCl, NaCl powder, Dilute HCl, Anhydrous sodium carbonate, Felhings solution, NaOH, Mercuric iodide, Potassium iodide, Iodine, Vanillin powder, Antimony (|||) chloride, Bismuth (|||) nitrate, Ferric chloride, 10% HCl, 10% NaCl, Magnesium ribbon, Acetone, Acetic anhydride, Concentrated H_2SO_4, Acetic acid, Chloroform, n-Butanol, Ethyl acetate, Potassium ferricyanide, Napthanol, Nutrient agar, eucalyptus oil and vaseline gel.

Research Design of the Study

The experimental research design was an actual laboratory set-up using Complete Randomized Design (CRD). Four phases were included in the pursuit of the study.

Phase 1. This phase includes the collection and extraction of the seaweed material and phytochemical screening;

Phase 2. This phase covers the formulation and physico-pharmaceutical evaluation of the seaweed ointment.

Phase 3. This phase covers the antibacterial assay against *E. coli* and *S. aureus.*

Phase 4. The burn wound healing effect of Nagkayasan ointment on Sprague dawley rat.

Treatments

For the antibacterial assay there were four treatments and replicated three times as follows:

T1- Vaseline gel

T2- Eucalyptus oil

T3- Nagkayasan ethanolic extract

T4- Nagkayasan antiseptic ointment

For the burn wound healing effect there were four treatments and replicated three times as follows:

T1- 0.1g of Nagkayasan antiseptic ointment

T2- 0.2g Nagkayasan antiseptic ointment

T3- negative control (untreated)

T4- Positive control (Mupirocin)

Procedure

Collection and preparation of the seaweed material

Nagkayasan and other seaweeds are abundant in the intertidal zones of Sta, Ana, Cagayan. On the study conducted by Abaleta (2016), Nagkayasan (*Chaetomorpha crassa)* is one of the most abundant species present in Nangaromoan, Sta. Ana, Cagayan. The Nagkayasan seaweed was gathered at the shore of Nangaromoan, Sta Ana, Cagayan as washed-up seaweed. They were washed thoroughly and air-dried on wire trays for two days.

Preparation of the seaweed extract

Two hundred grams of the prepared seaweed sample was placed in an Erlenmeyer flask. Two hundred millimeter of 80% ethyl alcohol was added. The flask was stoppered tightly and left in a room temperature with constant shaking. After 48 hours, the material was blended and filtered through cheesecloth and finally filtered through a Buchner funnel lined with a filter paper. The flask and the seaweed material were rinsed with fresh portions of the ethanol solution. The seaweed residue was discarded.

The total volume of the filtrate was concentrated to about 200ml in vacuo using reflux methodology using a hot bath to evaporate the alcohol. A flame test was also done to ensure that no alcohol remained in the extract. The concentrate was then stored in a tightly stoppered container and kept in the refrigerator until use.

Phytochemical Screening

A 100ml of Nagkayasan ethanolic extract was submitted at the Natural Product Research and Innovation Center at CSU Andrews Campus for phytochemical screening. The Nagkayasan ethanolic extract of the seaweed was tested for the presence of the following algal constituents: alkaloids, glycosides, tannins, saponins, flavanoids, coumarins and tripenoids.

Ointment Production by Fusion Method Adopted from Rao kodati et al., (2011)

In the preparation of 50% Nagkayasan aseptic ointment the investigators used 100ml of Nagkayasan ethanolic extract, 100 grams of Vaseline gel base, and

5ml of Eucalyptus oil as scent. Each ingredient was mixed and heated gently with stirring then cooled. One hundred (100ml) of Nagkayasan ethanolic extract was then added slowly to the above melted ingredients and stirred thoroughly until the mass cooled down and a homogeneous product was formed. The ointment was then packed in a wide mouth container.

Physico-pharmaceutical Evaluation of Ointment adopted Kumari, S. et al., (2017)

pH measurement adopted from Rajeev Malviya et al., (2016)

According to Hamedelnei E. (2015) the standard pH for all ointment was between five to seven (5-7). The pH of the formulation was determined using a digital pH meter. One gram of ointment was dissolved in 25ml of distilled water and the electrode was then dipped in to cream formulation for 30 min until constant reading was obtained. And constant reading was noted.

Homogeneity

According to Hamedelnei E. (2015) the ointment was tested for homogeneity by visual inspection. Homogeneity and texture were tested by pressing a small quantity of the formulated cream on the thumb and index finger. It was tested for their appearance with no lump. Other characteristics such as odor, smoothness, and grittiness were determined by visual inspection.

Antimicrobial Assay

The antibacterial assay of the samples was done by DOST Regional Standard Laboratory. The Nagkayasan ointment, Nagkayasan ethanolic extract, the base ingredients Vaseline base and eucalyptus oil was tested for their antibacterial activity using the paper disc diffusion technique. In this study, *S. aureu* and *E. coli* was obtained from the UPLB-BIOTECH through DOST -R2 was used as the test organisms.

Data Gathering

After 24 hours of incubation, the diameter of zones of inhibition was measured in millimeters using the Vernier caliper with the aid of magnifier.

Clear and well defined zones of inhibitions around the discs was observed.

Data Analysis

For six (6) mm diameter of disc, the diameter of zones of inhibition was observed and corresponding influences: <10 mm maybe expressed as inactive, 10-13 mm, partially active, 14-19 mm active, >19 mm very active (Guevara, 2005).

Analysis of Variance was used to determine the significant difference on the mean of the zone of inhibition of the leaves extract. Least Significant Difference was further used to compare the means of the zone of inhibition.

Discarding Microorganisms

After measuring the zones of inhibition, the Petri dishes used will be soaked in Lysol solution and will be autoclaved for 30 minutes at 15psi to eradicate the bacteria and to avoid contamination.

Burn Wound Healing Procedures

Creation of Burn Wound

All the surgical interventions were carried out under sterile conditions under general anesthesia. The predetermined area for wound infliction at the back of the animal was prepared for surgery by removing hairs with surgical blade. The animals were anaesthetized and placed on the operation table in its natural position.

Burn wound model. A cylindrical metal rod was heated over the open flame until flamed red and pressed to the shaved and disinfected surface for 6 s on selected dorsal area of animal under light anesthesia (lidocaine). Animals were placed in their respective cages.

Application of extract

The Nagkayasan ointment was applied using a medical swab. Using individual swab for 0.1g and 0.2 g Nagkayasan ointment and 0.2 of Mupirocin® was applied e for 12 days in the burn wound of Sprague Dawley rats.

Measuring wound healing Activity

Wound healing activity was determined based on the procedure of Sharma et.al. (2011). The wound healing activity can be measured by assessing two parameters such as percentage (%) of wound contraction and comparison of wound mean area among the controls. Wound area was traced using a transparent ruler and was scaled in a paper. Percent wound contraction was calculated using the following formula:

Percentage of wound contraction

$$= \frac{\text{Initial wound size-specific size}}{\text{Initial wound size}} \times 100$$

Statistical Analysis

Analysis of Variance was used to determine the significant difference on the mean of the different treatments per day of observation (3days, 6 days, 9 days and 12 days). Least Significant Difference was further used to compare the means of the zone of inhibition.

Results and discussions

This chapter shows the result of the analysis made in the study and interpretation of the data gathered on the phytochemical screening, physico-pharmaceutical characteristics, antibacterial assay and wound healing effect of the Nagkayasan ointment.

Phytochemical Screening

Table 1. Phytochemicals present in Nagkayasan (*Chaetomorpha crassa*).

Phytochemicals	Ethanol Extraction
Alkaloids	+
Coumarin	+
Flavonoids	+
Glycosides	+
Tannins	+
Terpenoids	+
Saponins	+

Legend: + (present), - (not present)

Table 2 showed the phytochemical constituents of Nagkasayan ethanol extraction. It showed that there were seven bioactive components present in the ethanolic extract of Nagkayasan which consist of alkaloids, coumarin, flavonoids, glycosides, tannins, terpenoids and saponins. The presence of numerous secondary metabolites of the seaweed contributed on its nomination for the development of natural product such as antiseptic ointment. The presence especially of tannins alkaloids, saponins and flavonoids are good indicator that the seaweed extract has antibacterial potential as established in many studies.

Physico-pharmaceutical Evaluation

Table 2. Physico-pharmaceutical evaluation of the Nagkayasan antiseptic ointment.

	Standard	Observation
pH	5-7	Slightly basic (6.95)
Color	Yellow gold	Pale gold
Odor	-	minty
Smoothness	Smooth	Smooth
Grittiness	Free from grittiness	Non-gritty
Homogeneity and appearance with no lumps	Homogenous	Homogenous with no lumps

Table 2 showed the physico-pharmaceutical evaluation of ointment as to the following: pH was measured 6.95 which is slightly acidic but remains within the standard pH for ointment, odor was described by using the olfactory sense which smelled minty because of the eucalyptus as added scent in the ointment, grittiness was based on the study of Shelke, Y.U., *et al.* (2015) which stated that the ointment should be sooth and free from grittiness. The ointment was homogenous, and it appeared with no lumps as compared to the study of Hamedelniel, E. (2015). The results showed that the gathered data were similar to the standard data of observation.

Table 3. Zone of inhibition of the Nagkayasan antiseptic ointment and individual base ingredients against *Escherichia coli (*mm*).*

Treatment	R1	R2	R3	Total	Mean	Qualitative Description
T1- Vaseline Base Gel	6	6	6	18	6.00	Inactive
T2-Eucalyptus oil	11	9	9	29	9.67	Partially active
T3-Nagkayasan Ethanolic Extract	26	25	20	71	23.67	Very Active
T4-Nagkayasan Antiseptic Ointment	25	29	27	81	27.00	Very Active

According to Dr. Vijendra Nalamothu (2015) the importance of conducting the physico-pharmaceutical evaluation of ointment is that in the formulation components, a simple change in properties, such as pH, viscosity, the relative amounts of oil, water, surfactants, stabilizers, droplet size, ionic nature, or the method of preparation, can often influence skin absorption and efficacy. Table 3 showed that *E. coli* is susceptible to both T3 Nagkayasan ethanolic extract and T4 Nagkayasan antiseptic ointment with very active effect of 23.67 mm and27 mm zones of inhibition respectively. On the other hand, Vaseline base gel was found to be inactive while the eucalyptus oil shows partially active effect on *E. coli* with zone of inhibition of 9.67mm. The result indicates the antibacterial property of the Nagkayasan extract against *E. coli*.

Table 4. Zone of inhibition of the Nagkayasan antiseptic ointment and individual base ingredients against *Staphylococcus aureus (*mm*)*.

Treatment	R1	R2	R3	Total	Mean	Qualitative Description
T1- Vaseline Base	6	6	6	18	6.00	Inactive
T2-Eucalyptus oil	10	11	9	10	10.00	Partially active
T3-Nagkayasan Ethanolic Extract	28	27	24	79	26.33	Very Active
T4-Nagkayasan Antiseptic Ointment	32	29	34	95	31.67	Very Active

Table 4 showed that T4 Nagkayasan antiseptic ointment has the highest zone of inhibition with 31.67 mm followed by T3 Nagkayasan ethanolic extract. Both T3 and T4 have a very active effect on *S. aureus*. The Vaseline base showed inactive effect while the eucalyptus oil has a zone of inhibition of 10 mm which is partially active against *S. aureus*. Eucalyptus oil is found to have antibacterial and wound healing effect (Xu *et al.*, 2015).

The presence of seven secondary metabolites in Nagkayasan seaweed and the susceptibility of the two test pathogens *E. coli* and *S. aureus* is a proved that the seaweed ointment has antiseptic property. Based from the Table 5 above, the adjusted p-value of both *E. coli and S. aureus* are less than the alpha (0.05000), therefore, it is concluded that the difference between the treatments are statistically significant.

Wound Healing Effect

For the acute dermal toxicity study, the rats were given an increasing dose of 0.1g , 0.2g, 0.5g and 1.0g and were observed for behavioral and clinical changes for a period 144 hours.

Table 5. Analysis of Variance at p<0.05000 for the zone of inhibition of the two test pathogen.

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	P
E. coli	280.4000	3	70.1000	31.33333	10	3.133333	22.3723	0.000056
S. aureus	540.0000	3	135.0000	2.00000	10	0.200000	675.0000	0.000000

Analysis of Variance (Marked differences are significant at p< 0.05000)

Table 6. Dermal toxicity test of Nagkayasan ointment on Sprague Dawley rats.

No. of rats	Dosage (g)	Observed dermal irritation and inflammation (hours)							Remarks
		1	24	48	72	96	120	144	
1	0.1	0	0	0	0	0	0	0	Normal (no changes observed)
1	0.2	0	0	0	0	0	0	0	Normal (no changes observed)
1	0.5	0	0	0	0	0	0	0	Normal (no changes observed)
1	1.0	0	0	0	0	0	0	0	Normal (no changes observed)

Table 6 showed the skin reaction and dermal irritation of test animals. The increasing dosage of 0.1g to 1.0g shows no signs of observable changes both on the behaviour and skin appearance of the rats. This indicates that the Nagkayasan Vaseline based ointment is not toxic to the test animals.

Burn Wound Healing Effect in Sprague Dawley rats

The wound healing activity was studied using four treatments as follows: T1 (0.1g Nagkayasan oinment), T2 (0.2g Nagkayasan ointment), T3 (untreated) T4 (Mupirocin). The mean wound length on day 3 was (2.30mm) (1.60mm) (1.93mm) (1.70mm); on day 6 was (1.77mm) (1.47mm) (1.83mm) (1.50mm), on day 9 (1.60mm) (1.27mm) (1.47mm) (1.47mm), and the mean wound length closure of burn wound model on day 12 was (1.00mm) (0.87mm) (1.10mm) (1.03mm) respectively.

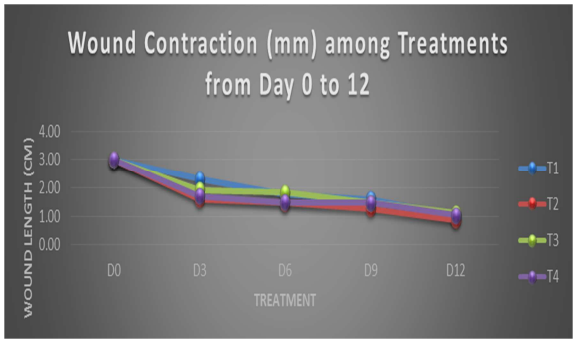

Fig. 1. Wound healing activity of the four treatments in terms of wound length.

Fig. 1 showed the reduction of wound area of different treatments over a period of 12 days. The fig. also shows the wound length in millimetre (mm) of the test animals for 12 days. Wound contraction was noted on all the treatments, however T2 (0.2g Nagkayasan Ointment) shows the highest wound healing effect registering 0.87mm wound length after 12 days followed by T1 (0.1g Nagkayasan Ointment) and T4 (Mupirocin) with 1.0mm and 1.03mm respectively. T3 (untreated) registered the lowest wound length at 1.10mm. The data shows that the higher the dose of the Nagkayasan ointment the higher the wound healing effect, application of the ointment help to increased wound contraction. However, local and systematic factors such as infection oxygenation, stress, hormone, ischemia, age and nutrition could possibly delay wound healing process still considered.

The Nagkayasan antiseptic ointment, showing clinically fast rate of wound contraction maybe due to the presence of flavonoid, tannins, saponins, alkaloids, and glycosides which are mediators of inflammation and possess significant antimicrobial properties. It suggests that the Nagkayasan Vaseline base ointment has promoted wound healing activity. The burn wound model showed wound healing in both 0.1g and 0.2g of Nagkayasan ointment which was comparable with the standard drug.

Table 7. Analysis of Variance of the Nagkayasan antiseptic ointment, negative control, positive control on their effect to the burn-wound model on Sprague-Dawley rats.

Days of observation	Analysis of Variance p- value
Day 3	Significant 0.034382115
Day 6	Non-significant 0.155148101
Day 9	Non-significant 0.106189459
Day12	Non-significant 0.772368287

Table 5 showed that only in day three that the four treatments were significantly different on their wound healing effect. On the other hand, day 6, day 9 and day 12 are not significantly different. It implies that Nagkayasan ointment are more effective on the onset of wound healing process.

Conclusions

In light of the foregoing results and discussions, the following conclusions are in order:

1. The Nagkayasan ethanolic extract contains secondary metabolites such as alkaloids, glycosides, tannins, saponins, flavanoids, coumarins and tripenoids which has pharmaceutical importance.
2. The Nagkayasan vaseline base ointment passed the standard values for the physico-pharmaceutical evaluation with the following characteristics: The Nagkayasan Vaseline based ointment is pale yellow in color, eucalyptus in odor, non-gritty, smooth and no lumps texture. Also, the pH is 6.95 which are within the standard of an ointment for skin application.

3. The Nagkayasan Vaseline base ointment and Nagkayasan ethanolic extract are both susceptible to the test pathogens *E. coli* and *S. aureus* with very active effect.
4. That there is a significant difference on the reaction of the pathogens to the different treatments of Nagkayasan and ingredient base.
5. The increasing dosage application of 0.1g to 1.0g of Nagkayasan vaseline base ointment shows no signs of observable changes both on the behaviour and skin appearance of the rats.
6. Nagkayasan Vaseline base ointment has promoted wound healing activity. The burn wound model showed wound healing in both 0.1g and 0.2g of Nagkayasan ointment which was comparable with the standard drug.

Recommendations

In the light of the forgoing conclusions the following recommendations are hereby recommended.

1. The investigators highly recommend the use of Nagkayasan seaweed for burn wound healing and antiseptic purposes.
2. The investigators recommend that the Nagkayasan antiseptic ointment should undergo human skin reaction test for commercialization, despite the fact that all the ingredients are safe for human utilization.
3. The investigators recommend using other microbes to include pathogenic fungi in the antimicrobial test.
4. The investigators recommend also recommend the formulation of the different concentration of Nagkayasan extract in the ointment product.

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