Research Article

Evaluation of *Rhinacanthus nasutus* for Utilizing as Sebum-controlled Facial Toner

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ABSTRACT

Rhinacanthus nasutus has been officially registered as traditional herbal medicine since 2011 as a tincture form of medication. It has properties in treating skin diseases caused by fungus, ringworm, and athlete's foot. The major bioactive compounds in *R. nasutus* are rhinacanthins. For cosmetics, *R. nasutus* extract (RNE) has been formulated as shampoos, soap bars, and liquid soaps. However, facial toner with RNE has not yet been found. Therefore, an objective of this study is to develop a sebum-controlled facial toner containing RNE. The total tannin content was studied and compared using Folin-Ciocalteu colorimetric assay. The astringent test was studied on hemoglobin precipitation. RNE showed astringent activity at AC_{50} by 1.06 ± 0.08 mg/mL while tannic acid as standard showed astringent activity at AC_{50} by 0.03 ± 0.00 mg/mL. Cytotoxic effect against human fibroblasts was evaluated with sulforhodamine B assay, and the result showed that RNE was safe in a concentration range between 0.001 to 1.0 mg/mL. In the development of toner, RNE was selected at concentration level of 0.20 %w/w, based on cell survival and astringent activity. At this concentration, it had astringent activity at 58.04% cell survival rate at 73.04%. Stability of formulated toners were evaluated by 6 heating-cooling cycles. The results showed that all formulas had an appropriate stability profile. The result from this study suggested that in order to formulate an efficient sebum-controlled toner containing RNE, other plant extracts with high astringent potency should be further combined. Further studies on *R. nasutus* herb such as anti-collagenase and anti-tyrosinase activities should be considered to confirm the potential of using RNE as cosmetic products.

Keywords: *Rhinacanthus nasutus*, Tannin, Sebum-controlled, Oily skin, Cosmetics

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Introduction

Rhinacanthus nasutus (RN) is an herb in the Acanthaceae family. It is commonly known as white crane flower, snake jasmine or 'Thong pun chung' in Thai. RN is an herb commonly found in South and Southeast Asia. The leaves and/or roots of RN are traditionally used to prepare balm for the treatment of skin infection [1]. Other than this benefit, it can be topically used as a treatment for many skin diseases, including skin rashes, psoriasis, eczema, and inflammatory skin disorders. Based on this reason, RN should have benefits for acne and inflammatory skin conditions.

RN was reported to contain many biological activities, which were anti-bacterial, anti-fungal, antioxidant and anti-inflammation effect [2]. There are many phytochemicals in RN leaves. Presence of flavonoids, alkaloids, anthraquinones, polyphenols, and phytosterols saponins including tannin were reported with the ethanolic extract of the leaves [3]. Tannin is considered as one among important phytochemicals in plants. It is produced in higher plants as a response to stressors. It is a water-soluble phenolic compound known for its various biological activities, including antioxidant and anti-inflammatory activities. The structure of tannin, which contains phenolic rings and hydroxyl groups, strongly relates to its activities including antioxidant and protein-binding activities [4]. In the food industry, tannin is considered as an anti-nutrient factor, in which it binds to the proteins and leads to the poor absorption of protein from food. Plants with high tannin content usually have astringency taste, which occurs from the precipitation of protein in taste buds [5]. However, for cosmetics, tannin is considered as an interesting ingredient. Other than its antioxidant and anti-inflammatory activity, tannin can bind to the protein on the skin pore and lead to the reduction of sebum secretion and pore size [6]. Based on this reason, the plants with high tannin content are used for health promotion and disease prevention. Examples of the plants with high tannin are tea, chestnut tree, some legume seeds, pine bark, emblica and *Terminalia spp.* While naphthoquinone class, rhinacanthins, were reported as a major and important phytochemical in RN which accounted for many biological activities of the plant, however, its cosmetic benefits were not yet reported.

Oily skin is the one among several cosmetic concerns in teenagers and young adults. Oily skin is a condition that may induce a negative effect on a client's lifestyle such as hard to apply makeup and may have a negative effect on self-esteem [7]. Oily skin is also related to a higher chance of acne. There are two major cosmetic active ingredients to manage oily skin, which are sebum-controlling agents and sebum absorbing agents [8]. Tannin is an interesting sebum-controlling agent utilized in cosmetic products. Example of a famous plant extract containing tannin is witch hazel (*Hamamelis virginiana*) is commonly used in many toners. However, this plant extract needs to be imported from other countries.

Based on the aforementioned, this study was aimed to evaluate cosmetic utilization potential of RN leaves extract by evaluation of total tannin content, *in vitro* astringent efficacy, and fibroblast toxicity. After that, RN leaves extract was prepared in a form of solution and evaluated for its irritation and allergy and sebum-controlled efficacy in human volunteers.

Materials and Methods

Plant extract and chemicals

RN extract (RNE) was purchased from Krung Thep Chemi, Bangkok Thailand (voucher number SKP 0011814, identified by Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand was reported from the supplier). It was reported to be extracted from the leaves and stalks with ethanol, then mixed with 49.90% w/w propylene glycol as a vehicle and 0.10% w/w sodium benzoate as a preservative.

The chemicals and reagents used in this experiment were purchased from local distributors in Thailand. The ingredients for formulating and preparing placebo toner and toner containing RNE were purchased from local distributors in Thailand in cosmetic grade.

Ferric chloride ($FeCl_3$), Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), swine hemoglobin (Sigma-Aldrich, Inc., USA), tannic acid (Tokyo Chemical Industry, Japan) and 95% ethanol (Avantor Performance Materials, Inc., Malaysia) were in analytical grade.

Deionized water was prepared with Merck Millipore system (Merck KGaA, Germany)

Evaluation of total tannin content

Total tannin content was evaluated according to the previous method described by Kumar and Chaiyasut [9]. Tannic acid was used as a standard. The result was expressed in a term of mg tannic acid equivalent (TE)/ g extract.

Evaluation of in vitro astringent efficacy

In vitro astringent efficacy was evaluated by the method described by Kumar and Chaiyasut (2017), with slight modification. One ml of 1 mg/mL of swine hemoglobin in DI water was mixed with 1000 µL of the 1 mg/mL solution of various plant extracts in DI water by using a vortex mixer. The mixture was further centrifuged at 1000 x g, 25°C for 10 min. A supernatant was collected and measured for the absorbance at 407 nm with a UV/visible spectrophotometer. Tannic acid was used as a positive control and DI water was used as control. Astringent activity was calculated by the following equation,

$$\text{Astringent activity (\%)} = \frac{\text{Actrl-Asample}}{\text{Actrl}} \times 100$$

When, Actrl was the absorbance of the control, Asample was the absorbance of the sample. The results were expressed in a term of AC_{50} which was the concentration of the sample that had 50% astringent activity.

Fibroblast cytotoxicity assay

Cell culture

Adult human primary dermal fibroblast (HDFa, PCS-201-012) was purchased from American Type Culture Collection (ATCC). It was cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a 5% humidified incubator.

Cytotoxicity evaluation

Cytotoxic effect against HDFa was evaluated with sulforhodamine B (SRB) assay, according to the method described by Vichai and Kirtikara [10]. Sodium lauryl sulfate (SLS) was used as a positive control group, DI water was used in a control group. Various concentrations of SLS and RNE, in the range between 0.001 - 10 mg/mL were studied.

Preparation of toner containing RNE

The formula compositions of placebo toner and RNE toner were shown in Table 1. For the preparation technique, RNE was mixed with PEG-40 hydrogenated castor oil before the addition of butylene glycol. After that, another ingredient was added to the mixture with moderate agitation. Then, deionized water was added. Finally, the mixture was mixed until homogenous liquid was obtained. The color of both formulas was adjusted to the same level to prevent volunteers from knowing which one is the RNE toner to reduce bias from the clinical study.

Table 1 Formula composition of placebo toner and toner containing *Rhinacanthus nasutus* extract (RNE toner).

Ingredient	Amount (%w/w)	
	Placebo toner	**RNE toner**
Deionized water	95.25	95.05
Butylene glycol	3.00	3.00
Phenoxyethanol (and) chlorophenesin (and) glycerin (Microcare® PHC)	0.80	0.80
Water soluble perfume	0.50	0.50
PEG-12 dimethicone	0.25	0.25
PEG-40 hydrogenated castor oil	0.10	0.10
Disodium EDTA	0.10	0.10
Rhinacanthus nasutus extract	-	0.20
Coloring agent	qs	qs

Note: qs is abbreviated from 'quantum sufficit' which is Latin language, means as needed. In this case, the coloring agent was added into the formulations to creating similar color for both formulas for achieving a single blinded process in the clinical trial.

Stability evaluation of toner containing RNE

Stability of RNE toner was evaluated with heating-cooling cycles. Tested formula was kept in a 45°C hot air oven for 24 h before keeping it in a 4 °C refrigerator for another 24 h. This was counted as 1 cycle. Six consecutive cycles were used. Changes in physical appearance, color and pH were observed. Changes in the color were measured with a handheld portable spectrophotometer (CM-700d, Konica Minolta, NJ, USA). The color parameters were expressed in a term of International Commission on Illumination (CIE) Lab color space [11].

The changes in color (ΔE) were calculated with the following equation.

$$\Delta E = \sqrt{(L1^*-L2^*)^2+(a1^*-a2^*)^2+(b1^*-b2^*)^2}$$

Clinical trial

Ethical consideration

The protocol was developed according to the ethical guidelines described in Declaration of Helsinki, Belmont report and CIOMS guideline. The protocol was reviewed and approved by University of Phayao Human Ethic Committee with the certification number UP-HEC 1.3/010/66.

Recruitment of volunteers

Inclusion criteria were healthy volunteers who have oily skin, concerned about enlarged pore size, not currently on other clinical trials and no allergy history for the composition in the formulation and RNE.

Exclusion criteria were presence of any type of skin diseases or abnormalities and persons who are currently taking antihistamine or corticosteroid medications.

Withdrawal criteria were volunteers who were willing to withdraw from the study, any adverse effects from tested formulations were observed during the trial and volunteers who cannot come to the follow-up visit.

Total of 12 volunteers were recruited in this study.

Evaluation of irritation potential of formulated RNE toner

Safety profiles of the test products were evaluated with an epicutaneous closed patch test [12]. All procedures were described to all volunteers and the informed consent statements were signed prior to the test. Twenty microliters of each sample, including 1 %w/w sodium lauryl sulfate solution as positive control, deionized water as negative control, placebo toner, and RNE toner was placed on the back of the volunteers with Finn® chamber (Smart practice, USA) and kept in position for 48 h. After the time was reached, the patch was removed and the skin reaction was assessed at 30 min, 24 h and 48 h after patch removal. The skin reaction was graded for erythema and edema as follows: 0 = no reaction, 0.5 = very slight reaction, 1.0 = slight reaction, 2.0 = obvious reaction, and 3.0 = important reaction. The mean irritation index (MII) of each sample was then calculated according to the following equation:

$$MII = \frac{\sum IS24}{n}$$

When, □IS24 was the summation of irritation and edema scores at 24 h reading and n was the number of subjects included in this study.

The degree of irritation was categorized based on the MII value as non-irritation (MII<0.2), slight irritation (0.2<MII<0.5), moderate irritation (0.5<MII<1), and strong irritation (MII>1).

Evaluation of sebum-controlled efficacy of RNE toner

This study was a single blinded, split-face, placebo control trial. Volunteers were asked to apply placebo toner on the left side of their face and RNE toner on the right side of their face. They were asked to apply the tested products twice a day, in the morning and in the evening with a cotton pad. Sebum contents

of the face were measured at three areas for each side of the face, which were forehead, cheek, and chin. The measurements were carried at baseline, 7, 14, 21 and 28 days after application of the tested product with Sebum Collector together with the DermaLab Combo (Cortex Technology, Denmark) device. Analysis was carried with Sebum Module analysis software provided by the device. Percent change in sebum content of each volunteer was calculated by using this following equation.

$$\% \text{ Change in sebum content} = \frac{(S_{before} - S_{after})}{S_{before}} \times 100$$

When, S before was sebum content at baseline and S after was sebum content at specific time (day 14 or day 28, after treatment).

Statistics

All of the data were reported in the term of mean ± SD of triplicate experiment. Significance of differences was evaluated by using Student's t-test (paired t-test by comparison between before treatment and after treatment) at the confidence level 95% by using SPSS version 17.0 (IBM Analytics, USA). Values of $p<0.05$ indicated statistically different.

Results and Discussion

In this study, RNE was a dark brownish-green colored viscous liquid with green, herb characteristic odor. Total tannin content of RNE was 43.52 ± 2.65 mg TE/g extract, evaluated by Folin-Ciocalteu colorimetric assay. This assay was carried based on the electron transfers from polyphenols, including tannin, to the phosphomolybdic/phosphotungstic acid complex of the reagent in an alkaline medium. This complex normally has yellow color from Mo (VI), when electron transfer happens, the transition state of molybdenum will be changed into Mo(V) which has blue color. The changes in the color can be measured with a visible spectrophotometer. Higher intensity of the blue color indicates higher content of tannin. When comparing the tannin content in RNE and other common astringency plant from the study of Kumar and Chaiyasut [9], it was found that RNE had comparable amount of tannin (5.46 ± 0.32 mg TE/g sample) to the 3 vegetable extracts cultivated in Northern Thailand of which hog plum had the highest amount of tannin at 8.69 ± 0.68 mg TE/g sample, white popinac had 7.49 ± 0.36 mg TE/g sample, and phak phai had 6.32 ± 0.16 mg TE/g sample.

For the evaluation of astringent activity, RNE showed astringent activity at AC_{50} of 1.06 ± 0.08 mg/mL while tannic acid showed astringent activity at AC_{50} of 0.03 ± 0.00 mg/mL. This suggested that RNE was less potent than tannic acid, since RNE contained other phytochemicals which may disturb the astringency process, and tannic acid is a pure phytochemical. Based on previous work of Son et al. (2013) which studied the tannin content and astringent activity of green apple rind extract, and the results showed that it had higher astringent activity (101.7 ± 5.3%) from tannin content of green apple rind extract of 30.48 mg TA/mg of dry matter [6]. Ditthawutthikul et al. reported that *Ficus fistulosa* extract contained 35.9 ± 0.0 μg of tannic acid equivalent per gram of extract, and its astringent activity was 87.45 ± 1.76% [13].

These may imply that other phytochemicals in the crude extract might play an important role for astringent activity.

RNE showed no cytotoxic effect against HDFa in the concentration range between 0.001 – 1.0 mg/mL, with the cell survival rate of 78.37 – 94.00%. However, in the higher concentration at 10 mg/mL, RNE was toxic against HDFa, with the cell survival rate of 7.60 ± 0.24%. Positive control, SLS was toxic to HDFa with a survival rate at concentration level 0.1 and 1.0 mg/mL of 10.84 ± 1.63% and 8.41 ± 0.60%, respectively.

Next, the toner containing RNE, and placebo toner were prepared, with the technique described above. Both toners had the same color, since the coloring agent was added to prevent the volunteers from knowing which one is the RNE toner or placebo, to reduce bias during the clinical trial.

The changes in pH and color value, measured in CIE Lab scale before and after accelerated stability study with heating-cooling cycles were shown in Table 2. After six consecutive heating-cooling cycles, both toners showed neither sedimentation nor color changes. The ΔE from placebo toner was 0.58 and RNE toner was 0.33. The previous report of Mokrycki and Tatol [11], suggested that when ΔE less than 1 the differences in color was unnoticeable with naked human eye. Based on this reason, formulated toners were physically stable. Further studies on chemical compositions and chemical stability may be conducted.

Table 2 Changes in pH and color of placebo toner and toner containing *Rhinacanthus nasutus* extract (RNE toner) after stability study with 6 heating-cooling cycles (HC).

Evaluated parameters	Placebo toner		RNE toner	
	Before HC	After HC	Before HC	After HC
pH	5.11 ± 0.01	5.15 ± 0.01	5.53 ± 0.01	5.52 ± 0.01
CIELab color scale				
L*	59.33 ± 0.14	59.77 ± 0.02	59.20 ± 0.58	59.51 ± 0.23
a*	0.24 ± 0.01	0.19 ± 0.01	0.06 ± 0.08	0.02 ± 0.02
b*	3.70 ± 0.01	4.07 ± 0.03	3.72 ± 0.37	3.62 ± 0.02
ΔE	-	0.58	-	0.33

Before the test products were given to the volunteers, safety of the products was evaluated with epicutaneous closed patch test. The MII scores of tested compounds and products were described in Table 3. From Table 3, 1% SLS, which is a positive control, showed an MII score of 0.67 which indicated moderate irritation. SLS is a known skin irritant, and it was used as a positive control. While DI water, a negative control, was non-irritating to skin with a MII score of 0.00. Both placebo toner and RNE toner were also shown non-irritating to the skin, with MII score of 0.00 and 0.00, respectively.

Table 3 Mean irritation index (MII) score of tested compounds and formulations.

Tested compounds/formulations	MII score
1% sodium lauryl sulfate solution	0.67
Deionized water	0.00
Placebo toner	0.00
Toner containing *Rhinacanthus nasutus* extract	0.00

The volunteers were then asked to apply formulated toners, with the placebo toner on the left side of the face and RNE toner on another side. Since the study was single blinded and the color of both toners were adjusted to the same intensity, the volunteers were not known which side of the face used the active RNE toner. This was for reducing the bias and placebo effect that may happen during the study, since volunteers might think that the RNE toner is better than placebo and lead to false positive results.

During the study period, facial sebum levels were measured at three areas, which were forehead, cheek, and chin. The measurement of sebum was done with the absorbent technique with the sebum collector strip which absorbed the sebum from the area, so the measurement area must be changed. The results suggested that both toners significantly reduced the sebum content on the skin from day 7 after application ($p<0.05$) (Figure 1 - 3). For the area that was treated with RNE toner, at day 14 after application, the % change of sebum content, from baseline of the forehead area was -53.07%, cheek area was -50.24%, and chin area was -43.98%. At day 28 after application, the % change of sebum content from baseline of the forehead area was -48.70%, cheek area was -61.05%, and chin area was -32.88%. The % change of sebum reduction in those three facial areas seemed promising. However, the sebum-controlling efficacy of RNE toner was not significant different when compared with placebo toner.

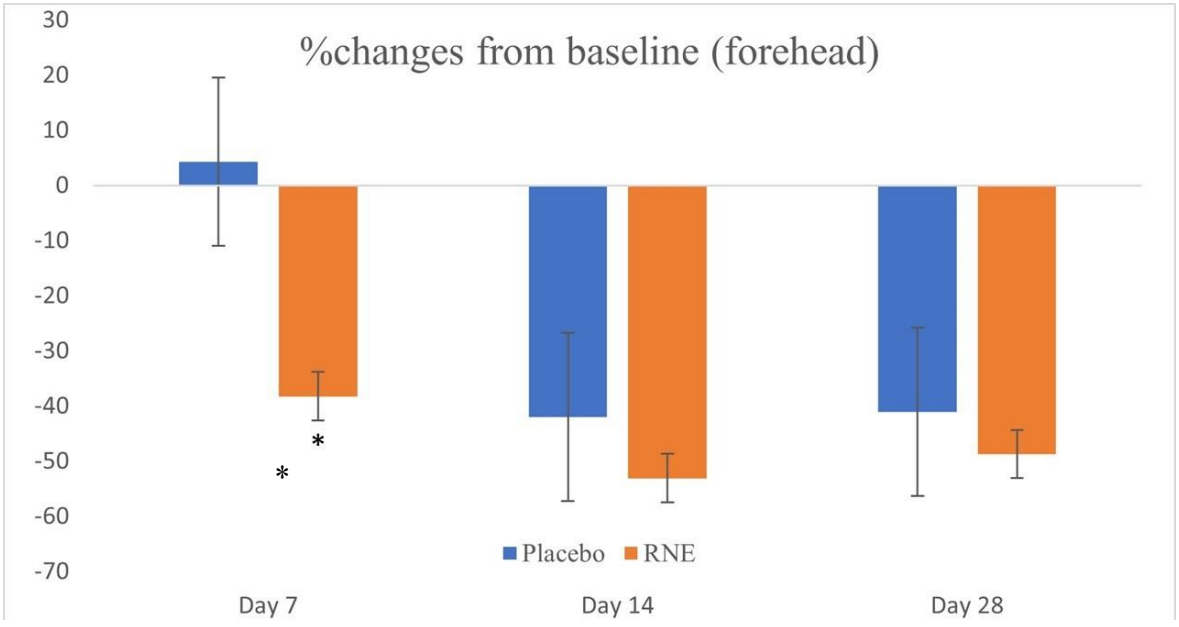

Figure 1 % Changes in sebum content from baseline at forehead area.

Note: * indicated significant differences between placebo toner and toner containing *Rhinacanthus nasutus* extract ($p<0.05$)

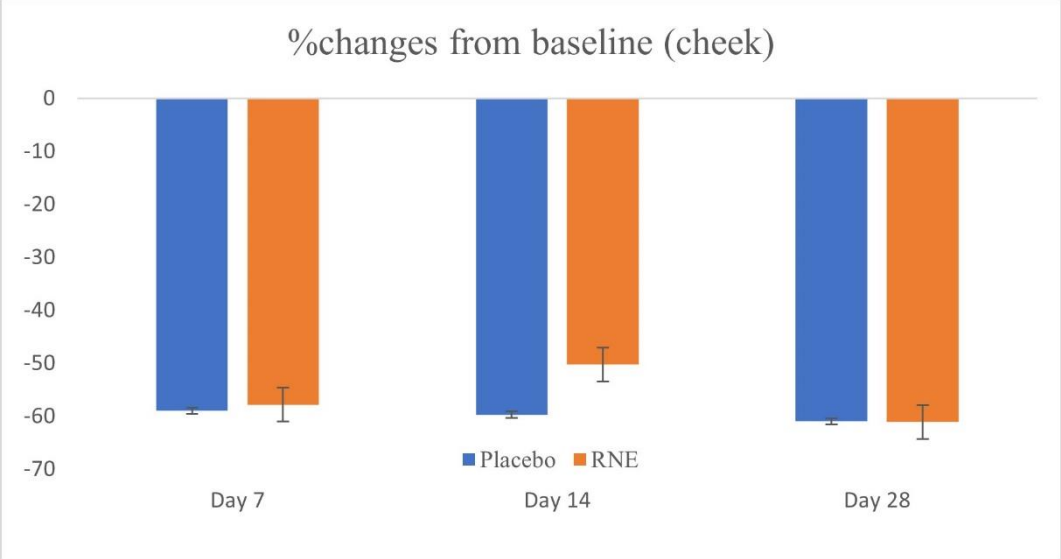

Figure 2 % Changes in sebum content from baseline at cheek area.

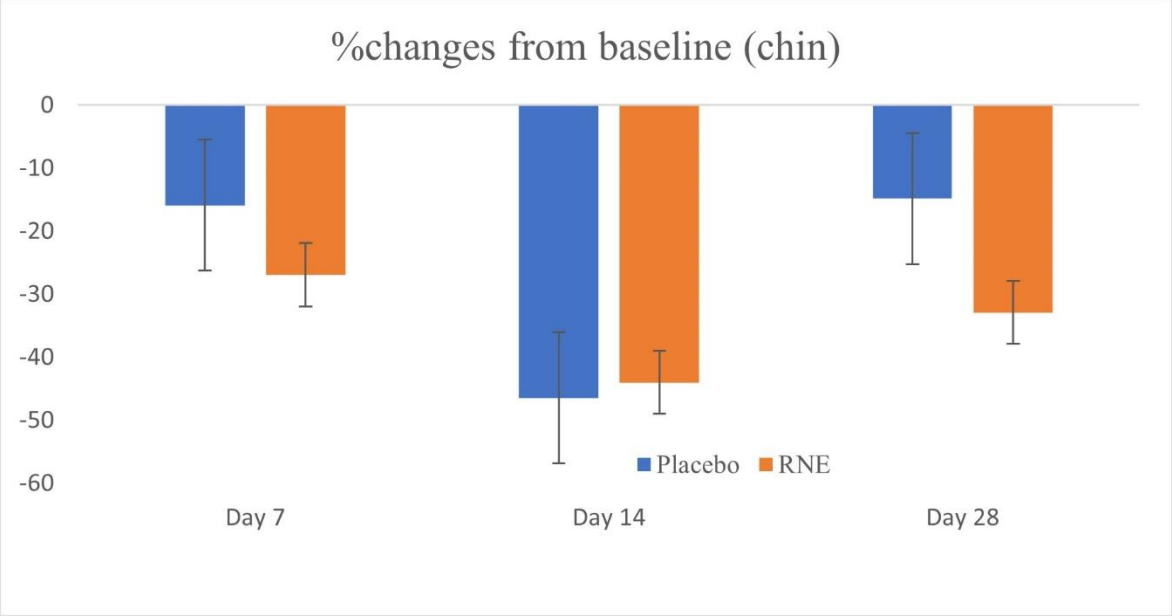

Figure 3 % Changes in sebum content from baseline at chin area.

The results suggested that RNE alone may not be effective as a sebum-control toner and other plant extracts or active ingredients with astringent potential should be used together for a synergistic effect. From the study of Juan Wei et al. [14] showed that the formulated sebum control essence which combined 3 different active ingredients (*Cleome gynandra* leaves extract, *Epilobium fleischeri* extract, and *Saccharomyces*/ zinc ferment) showed positive sebum controlled efficacy not only in a reduction of an excessive amount of sebum secretion (21.8% within 8h and 30.05% after 28 days) but also the formulated essence could attenuate other skin problems which are induced by an excessive amount of sebum secretion such as enlarged pores, blackheads and milia in long-term use.

Conclusion

RNE is a well-known plant used in the treatment of various skin diseases, including skin infection and inflammation. In this study, we demonstrated that RNE contained tannin with a total tannin content of RNE was 43.52 ± 2.65 mg TE/g extract, evaluated by Folin-Ciocalteu colorimetric assay. RNE also exhibited good in vitro astringent activity. However, RNE had less potent astringent activity than standard tannic acid with AC_{50} at 1.06 ± 0.08 mg/mL, while the standard tannic acid had AC_{50} at 0.03 ± 0.00 mg/mL. The safety of RNE was further evaluated by HDFa cytotoxicity. It was found that RNE was safe under the concentration range between 0.001 - 1.0 mg/mL, with the cell survival rate of 78.37 – 94.00%. Toner containing RNE was successfully formulated. It showed good physical stability and safety in human volunteers, with an MII score of 0.00. However, RNE toner was not effective in sebum controlling since the reduction of sebum is not different from placebo. Further studies on other in vitro activities, such as, anti-inflammatory activity and chemical stabilities of the formulation should be considered. The effects of RNE on other skin parameters, such as pigmentation and wrinkles might be further studied in the volunteers to confirm the potential of using RNE as cosmetic products.

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