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Research Article

HAIR GROWTH ACTIVITY AND SAFETY TEST OF ETHOSOMAL GEL ETHYL ACETATE FRACTION OF *NOTHOPANAX* LEAVES (*NOTHOPANAX SCUTELLARIUM* MERR.)

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ABSTRACT

Objective: The aims of this study were to know the hair growth activity from the ethosomal gel of ethyl acetate fraction compared to the nonethosomal gel of ethyl acetate fraction, as well as its ability to penetrate into the skin and its safety from ethosomal gel testing. **Methods:** The hair growth activity was measured by three parameters: Hair length, hair thickness, and hair weight. **Results:** The results obtained are shown that the ethosomal gel with concentration 1% has better hair growth activity rather than the ethosomal gel with concentration 0.5% and the nonethosomal gel but it has similarity to the positive control (minoxidil gel with concentration 2%). *In vitro* penetration test using Franz diffusion cells shown that the ethosomal gel with concentration 1% has cumulative penetration of quercetin higher than nonethosomal gel, which value was 3.3175±0.02 μgcm^{-2} for the ethosomal gel with concentration 1%; and 2.7663±0.19 μgcm^{-2} for the nonethosomal gel with flux values for the ethosomal gel with concentration 1% and the nonethosomal gel, respectively, are 0.4147±0.01 μgcm^{-2}h^{-1} and 0.3458±0.02 μgcm^{-2}h^{-1}. **Conclusion:** As for the safety test using hen's egg test-chorioallantoic membrane (HET- CAM) safety test) have shown that both concentrations 0.5% and 1% of the ethosomal gel can generate mild irritation for topical application.

Keywords: *Nothopanax scutellarium*, Ethosome, Gel, Hair growth, Franz diffusion cell, Hen's egg test-chorioallantoic membrane.

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INTRODUCTION

Hair is an integral part of the body and grows almost in every part of it. Hairs have several functions, namely, to protect the head from the direct exposure to sunlight and to maintain the body temperature and for the esthetic [1]. The cycle of hair growth is comprised three stage, which is anagen (hair growing phase), catagen (hair resting phase), and telogen phase (hair falling phase). Normally, there are 40-100 hairs fallen from the head in a day. If the number of hair falling exceeds the normal number, then baldness may occur and can lead to the loss of confidence [2].

Nothopanax scutellarium Merr. is believed to have the activity of promoting hair growth [3,4]. In which is supported by several studies, such as a study conducted by Sa'diah [5] who found that ethanol 70% extract suspension with 7.5% *Nothopanax* leaves may have hair growth promoting activity with the similar conclusion yet using different forms of product, particularly 1% *Nothopanax* fraction hair tonic.

Despite the mechanism on how it may affect hair growth is still unknown, in an experiment carried out by upadhyay [6], it was found that polar compound such as flavonoid has the ability to strengthen the capillary wall of blood vessel inside hair follicle so that it will increase the blood circulation. According to Perez-Vizcanio *et al.* [7], quercetine, a flavonoid-type compound, can act as a vasodilator. Furthermore, in correlation to alopecia aerate, there was a study found that the administration of 10 μM quercetin subcutaneously may help the condition [8].

However, the difficulty of herbal product to penetrate into the skin is one of the major issues faced in using herbal ingredients. Consequently, this problem often lead to the aimed targeted effect was not achieved. Ethosomes are a lipid vesicle consist of phospholipid, alcohol (ethanol or isopropyl alcohol) with relatively high concentration and water. Compare to another form, ethosome is faster in penetrating through the skin layer and increase the transdermal flux significantly [9].

The novelty of this study, the hair growth promoting activity and safety test of *Nothopanax* leaves ethosomal gel ethyl acetate fraction which had not been performed before. Gel form was used in this experiment because of its ability to spread evenly on the skin, to moisture, and its practicality.

This study aims to evaluate the ethyl acetate fraction of *Nothopanax* leaves in ethosomal gel form in promoting hair growth as well as its penetration into the skin layer and to evaluate the safety of the product.

MATERIALS AND METHODS

Materials

The sample was obtained from BALITTRO and then was determined in Indonesian Institute of Sciences (LIPI). Phospholipon 90G from GMBH Lipoid, Germany, carbopol from PT. Surya Dermato, Indonesia, Veet ®, Other reagents were purchased from Sigma-Aldrich, Singapore. New Zealand rabbits weight 2500-3000 g were obtained from Livestock Research Center, Bogor, Indonesia and Sprague-Dawley mouse age 2-3 months, weight ±150 g were obtained from Bogor Agricultural Center, Indonesia.

Extraction and fractionation of *Nothopanax* leaves

Dried *Nothopanax* leaves powder was macerated in chloroform (48 hrs) and then ethyl acetate (72 hrs) with 1:7 ratio, respectively. Macerate then was evaporated with rotary vacuum evaporator until the thick extract was obtained which then was dispersed in warm aquadest and sonicated for 5 minutes. The fractionation of the extract was performed using n-hexane. After the separation between water fraction and n-hexane fraction had occurred, the water fraction was re-fractioned using ethyl acetate and was then evaporated with rotary vacuum evaporator at temperature 40-50°C until the thick extract was obtained.

Preparation of *Nothopanax* leaves fraction ethosome

Nothopanax leaves fraction ethosome was prepared using composition (Table 1) that had been formulated and utilizing modified thin film hydration method [10].

Formulation and preparation of ethosomal gel ethyl acetate fraction of *Nothopanax* leaves

According to a study conducted by Rifkia [11], the ethyl acetate fraction with concentration 0.5% and 1% have stronger hair growth activity. Hence, in this experiment, those concentrations were used (Table 2).

Hair growth activity test

Hair growth activity test was performed in a completely randomized design based on the modified method by Tanaka *et al*. [12]. The back of male New Zealand rabbit weight 2500-3000 was served as the tested site. Six equal sizes of rectangles (2.2 cm × 2.5 cm) which act as the tested site were marked using a marker. Six rectangles were treated with six different samples, consisting of normal control, negative control, positive control (2% minoxidil gel), 0.5% *Nothopanax* leaves fraction ethosome, 1% *Nothopanax* leaves fraction ethosome, nonethosome gel.

Hen's egg test-chorio allantoic membrane (HET-CAM) safety test

There are three testing groups consist of negative control group (0.9% NaCl), positive control group (1% sodium lauryl phosphate), and *Nothopanax* leaves fraction ethosomal gel (0.5% and 1%); three eggs were used in each group. Fertile egg <7 days old from leghorn chicken weighing between 50 and 60 g were selected and incubated at 37.5°C for 24 hrs. On the 8th day of incubation, the eggs were observed under the light. The broken eggs were taken out; the egg's air cavity was marked and cut. The inner membrane of the egg was then moisturized with 0.9% NaCl and slowly peeled with tweezers. Eggs then were treated with the sample according to their group. The reaction occurred for 300 seconds was observed. In the second unit, the reaction and the time of the occurrence were noted, calculated, Cazedey *et al*. 2009 [13] and categorized (Table 3).

Penetration test using Franz diffusion cell

In vitro penetration test

The skin of Sprague-Dawley mouse age 2-3 months was served as the membrane used in the test. After the mouse being put down with ether, the mouse's hairs were shaved and the skin was sliced 0.6±0.1 mm thick. The penetration test was carried out using Franz diffusion cell with 1.77 cm^2 of diffusion area and 15 mL of compartment volume.

To the skin, 1 g sample was applied. In minutes 10, 30, 60, 90, 120, 180, 240, 300, 360, 420, and 480, using the syringe, 1 mL sample was taken out and replaced with the mixture of phosphate solution pH 7.4 and tween 80 with the same volume. The concentration of the sample was then determined using high-performance liquid chromatography (HPLC). The cumulative amount of quercetine penetrated for each diffusion area was calculated using the formula [14-16,19].

RESULTS AND DISCUSSION

Extraction and fractionation of *Nothopanax* leaves

The *Nothopanax* leaves were extracted using maceration. This method was chosen because it is believed that high temperature may reduce the total flavonoid concentration by 15-78%. From the process, 32.79 g ethyl acetate thick fraction was obtained with characteristics as described in Table 3. The concentration of ethyl acetate extract and ethyl acetate fraction were determined using HPLC using quercetin as the standards, resulting 1.64% for the extract and 5.83% for the fraction. Hence, showing that fractionation procedure may increase flavonoid concentration.

Preparation of *Nothopanax* leaves fraction ethosome

The ethosome which was obtained using thin film hydration technique came out in a dark green suspension and had a particular *Nothopanax* leaves odor. From the characterisation performed (Table 4), it was found that ultrasonication process can reduce the particle size (Table 5); particles in the suspension were evenly distributed which was shown by polydispersity index; and the suspension that was produced was stable as the zeta potential values was more positive than +30 mV and There was a significant variation (more negative than −30 mV) [17]. There was a significant variation ($p < 0.05$) in week III between both the ethosomal gel 0.5 % and ethosome gel 1 % groups and the normal control and the negatives control groups (Table 6). There was an increase in the hair shaft thickness in week IV (Table 7). There was no lysis reaction and coagulation found in all four group (Table 8). According to the irritation score generated (Table 9).

Formulation and preparation of ethosomal gel ethyl acetate fraction of *Nothopanax* leaves

The nonethosome gel and ethosome gel 1% produced a quite similar green color finished product with a particular odor of *Nothopanax* leaves, whereas the ethosome gel 0.5% has a lighter green appearance. This variation is believed caused by the different percentage of ethosome in each gel product. Under a microscope, all of the produced gel products look homogenous (Fig. 2).

As for pH, the ethosomal gel produced have pH ranging from 5.51 for ethosomal gel 0.5%, 5.62 for ethosomal gel 1% and 6.31 for nonethosomal gel.

Table 1: *Nothopanax* leaves fraction ethosome ingredients

Materials	Concentration (%)
Nothopanax leaves ethyl acetate fraction	2
Soy phosphatidilcholine	2
Ethanol	20
Propylene glycol	5
Phosphate buffer pH 5	Ad 100

Table 2: Ethosomal gel ethyl acetate fraction of *Nothopanax* leaves formulation

Materials	Concentration (%b/b)		
	F1	F2	F3 (non ethosomal gel)
Nothopanax leaves fraction ethosome	25	50	-
Nothopanax leaves ethyl acetate fraction	-	-	1
Propylene glycol	15	15	15
Carbomer	0.5	0.5	0.5
TEA	0.2	0.2	0.2
Sodium metabisulfite	0.1	0.1	0.1
Methyl paraben	0.14	0.14	0.14
Propyl paraben	0.02	0.02	0.02
Demineralised water	Ad 100	Ad 100	Ad 100

TEA: Triethanolamine

Table 3: HET-CAM irritation category

Score	Irritation category
0-0.9	Nonirritant
1-4.9	Low irritant
5-8.9 or 5-9.9	Mild irritant
9-21 or 10-21	High irritant

Cazedey, *et al.* 2009

Table 4: *Nothopanax* leaves ethyl acetate fraction characteristics

Characteristics	Results
Physical form	Semi-solid
Color	Dark green
Odor	Mangkokan leaves odor
Taste	Astringenous
pH	6.34
Water content	4.74%
Drying shrinkage	5.26%
Total ash content	0.95%

Table 5: *Nothopanax* leaves fraction ethosome characteristic

Characteristics	Results
Particle size	776.2 nm±1.22 (before ultrasonication) 110.5 nm±1.78 (after sonication)
Polydispersity index (PDI)	0.393±0.04
Zeta potential	−31.3±0.1 mV
Ethosome vesicle morphology	Spherical and no particle aggregation appear
Entrapment efficiency	50.32%

The result of the measurement of particle size distribution shows that particle size underwent an increase to 240.5±3.16 nm with potential-determining ion 0.443±0.05 and zeta potential zeta −72.9±0.76 mV (Fig. 4). This increase may be resulted from the use of homogenizer when mixing the ethosome with gel base in which making the ethosome vesicle to aggregate and increase the vesicle size.

In stability test, whether in low temperature (4°±2°C), room temperature (27°±2°C), or high temperature (40°±2°C), the ethosomal gel 0.5%, ethosome gel 1% and nonethosome gel did not demonstrate any change in color and odor until week 12. For pH, there was a decline in each formula, yet the pH still ranging in the skin pH from 4.5 to 6.5. In cycling test, it was found that there was no change in odor and color of ethosomal gel 0.5%, ethosomal gel 1% and nonethosomal gel, and there was no syneresis took place after 12 days so that it can be said that both of the products were stable.

Hair growth activity test

In this research, the hair length data was processed statistically with SPSS 20 using Shapiro-Wilk test for normal distribution test and levene test for homogeneity test. In week I and II, due to the possibility of rabbit hair still in the telogen phase or the adaptability of the rabbit, the data obtained were not distributed normally; hence, the procedure continued with Kruskal-Wallis test. In week III and week IV, the data obtained were distributed normally and homogeneously, and the ANOVA test was carried out as well as the *post hoc* LSD afterward.

There was a significant variation ($p<0.05$) in week III between both the ethosomal gel 0.5% and ethosome gel 1% groups and the normal control and negative control groups, between nonethosomal gel and both normal control and negative control, and between positive control and both normal control and negative control, whereas between normal control and negative control were shown insignificant variation ($p>0.05$) which most likely mean that there was no indication of hair growth activity.

In week IV, there was a significant variation ($p<0.05$) between ethosomal gel 1% and ethosomal gel 0.5%, nonethosomal gel group as well as normal control and negative control groups which may demonstrate that ethosomal gel had the best hair growth promoting activity but not as good as positive control (Fig. 3). However, the insignificant variation ($p>0.05$) still occurred between normal control and negative control.

There was an increase in the hair shaft thickness in week IV. From Fig. 4, it was found that the largest size of hair shaft was demonstrated by ethosomal gel 1% followed by nonethosomal gel, ethosomal gel 0.5%, normal control, negative control and positive control. The increase in the hair thickness was possibly caused by the vasodilatory effect from the flavonoid content in the *Nothopanax* leaves, which consequently increasing the nutritional input to the hair follicle.

According to the result, there was no significant variation between control negative and control normal ($p>0.05$). As for positive control, ethosomal gel 0.5%, ethosomal gel 1%, and nonethosomal gel, there was a significant variation ($p<0.05$) demonstrated with normal

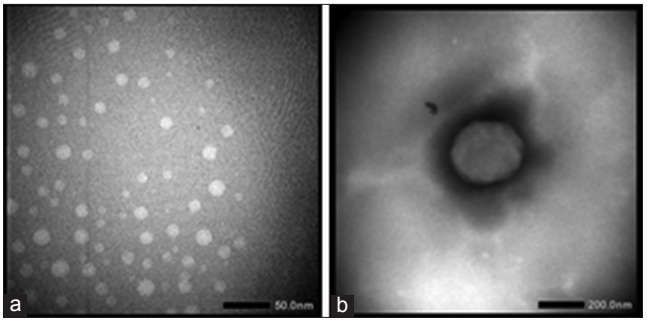

Fig. 1: Vesicle morphology observation using transmission electron microscope. (a) ×20.000 magnification, (b) ×80.000 magnification

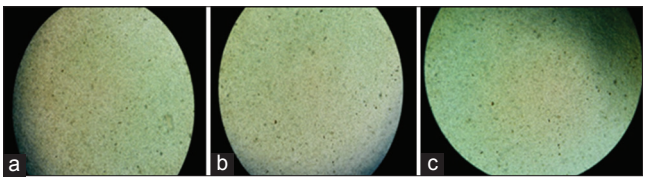

Fig. 2: Homogeneity test of ethosomal gel with ×10 magnification. (a) Ethosomal gel 0.5%, (b) ethosomal gel 1%, (c) nonethosome gel

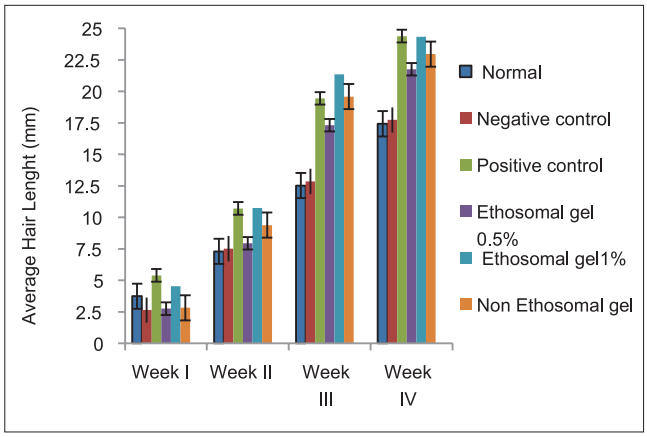

Fig. 3: Average hair length week I - IV

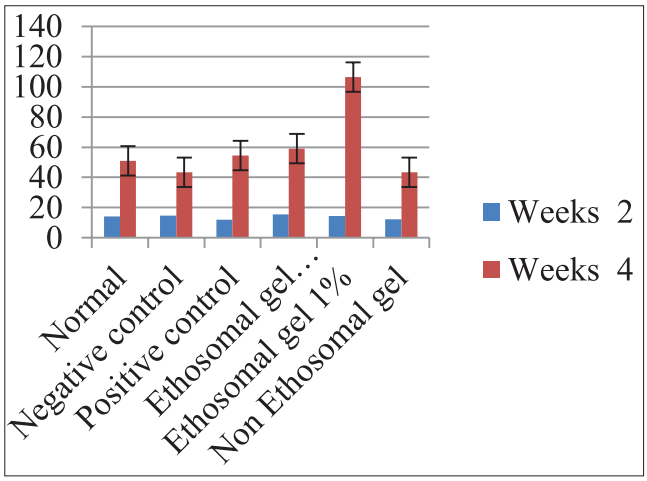

Fig. 4: Average hair thickness week II and week IV

Table 6: Average hair length per week

Treatment	Average hair length (mm)±SD			
	Week I	Week II	Week III	Week IV
Normal control	3.75±5.12	7.30±3.79	12.53±2.96	17.43±2.7
Negative control	2.65±5.3	7.52±3.38	12.86±3.4	17.74±3.01
Positive control	5.40±7.28	10.71±4.42	19.45±4.96*#	24.39±4.54*#
Ethosomalgel 0,5%	2.76±5.52	7.95±3.78	17.32±4.8*#	22.51±2.29*#
Ethosomalgel 1%	4.54±5.74	10.76±5.14	21.36±4.53*#	24.33±4.05*#
Non ethosome	2.83±5.65	9.39±3.89	19.59±4.8*#	22.96±3.88*#

(*) Significant variation (p<0.05) toward normal control, (#) significant variation (p<0.05) toward negative control

Table 7: Average hair weight week IV

Treatment	Average hair weight (mg)±SD
Normal control	27.08±13.7
Negative control	27.1±12.6
Positive control	59.48±6.3*#
Ethosomal gel 0,5%	49.2±13.3*#
Ethosomal gel 1%	49.63±15.7*#
Non Ethosomal gel	44.63±7.9*#

(*) Significant variation (p<0.05) toward normal control, (#) significant variation (p<0.05) toward negative control, SD: Standard deviation

Table 8: Average time of CAM reaction (second)

Test group	Average time of CAM reaction (second)±SD		
	Haemorrhage	Lysis	Coagulation
Negative control	-	-	-
Positive control	33.33±8.7	-	-
Ethosomal gel 0.5%	188±53.7	-	-
Ethosomal gel 1%	185±15.1	-	-

CAM: Chorio allantoic membrane, SD: Standard deviation

Table 9: Irritant score after the application of tested product

Test group	Irritant score			
	CAM 1	CAM 2	CAM 3	Mean±SD
Negative control	0	0	0	0
Positive control	7.11	4.58	4.30	5.33±1.6
Ethosomal gel 0,5%	0.85	2.45	2.35	1.88±0.9
Ethosomal gel 1%	2.01	1.65	2.13	1.93±0.3

CAM: Chorio allantoic membrane, SD: Standard deviation

control and negative control. For ethosomal gel 0.5%, ethosomal gel 1%, and nonethosomal gel, there was no significant variation with positive control. Therefore, it may be concluded that the hair growth activity regarding weight of ethosomal gel 0.5%, ethosomal gel 1% and nonethosomal gel were equal with positive control.

According to the results of three parameters tested, ethosome gel 0.5%, ethosome gel 1%, and nonethosome gel have the hair growth activity similar to control positive. Ethosome gel 0.5% and 1% have better activity compare to nonethosome gel, which indicating that the ethosome gel can increase the skin penetration of a drug. However, the best result was shown by ethosome gel 1%.

HET-CAM safety test [18]

There was no lysis reaction and coagulation found in all four groups. According to the irritation score generated, negative control was categorized as non-irritant, positive control as mild irritant, ethosome gel 0.5% and ethosome gel 1% as low irritant. The irritant properties of ethosomal gel is possibly caused by propylene glycol content in the gel.

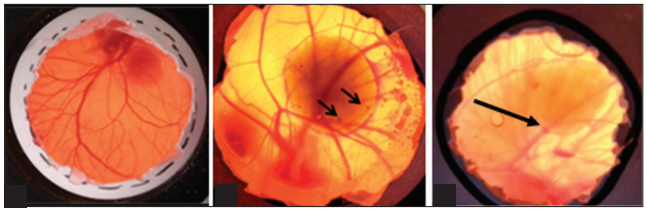

Fig. 5: Chorio allantoic membrane (CAM) reaction. (Description: (a) Normal CAM, (b) ethosomal gel 0.5% CAM; (c) ethosomalgel 1% CAM)

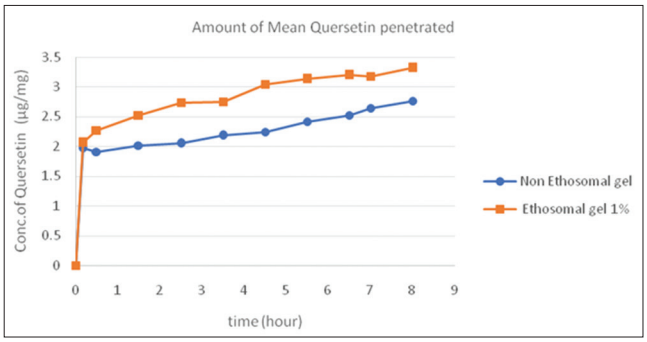

Fig. 6: Cumulative amount of quercetine penetrated for ethosomal gel 1% and nonethosomal gel

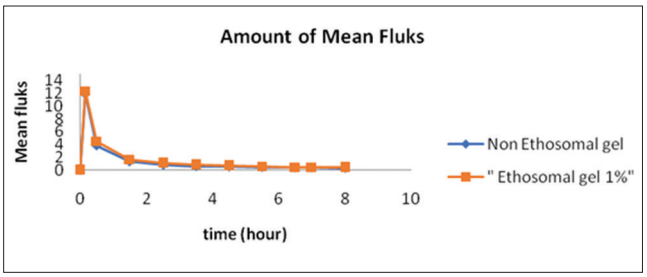

Fig. 7: Ethosomal gel 1% and nonethosomal gel flux

The result of the measurement of particle size distribution shows that particle underwent an increase 240,5±3.16. Cumulative the penetration test was performed, it was found that the amount of the quercetine penetrated is 3.3175±0.02 µgcm^{-2} for ethosome gel 1% and 2.7663±0.19 µgcm^{-2} for nonethosome gel, whereas for ethosome gel 0.5%; the result was too low and could not be detected during HPLC analysis (Fig. 6). This is most likely due to the high concentration of ethanol in ethosome which served as the penetration enhancer that may lead to the change of stratum corneum permeability. With the distraction of lipid bilayer in stratum corneum, ethosome may easily penetrate into the skin. The low amount of the quercetine penetrated may be resulted from the presence of lipid in the membrane that may disrupt the active compound penetrating optimally.

From Fig. 6 it was that at the beginning of the release of active substance, the steady state had not been reached yet as there was still a variation in the concentration gradient. The concentration gradient is an active substance thrust force in crossing the membrane by passive diffusion. After the steady state was achieved, flux (Fig. 7) is not affected any longer by concentration leading to the flatter curve.

CONCLUSION

Ethyl acetate fraction (1%) ethosomalgel has better activity of hair growth promotion was compared to that of 0.5% ethyl acetate fraction ethosomal gel, nonethosomal gel, negative control or normal control, but not better than positive control. Ethyl acetate fraction ethosomal gel has the cumulative amount of quercetine penetrated and flux higher than nonethosome gel in *in vitro* penetration test. 0.5% ethyl acetate fraction ethosomal geland 1% ethyl acetate fraction ethosomal gelmay be mildly irritating if used topically.

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