CORRELATION BETWEEN TPC AND TFC WITH ANTIOXIDANT ACTIVITY OF *Piper sarmentosum* EXTRACT AND ITS FORMULATION FOR COSMETIC PURPOSES

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Abstract: This study investigates the characteristics of an antioxidant cream made from the methanol extract of *Piper sarmentosum* leaves, which is locally known as the wild betel or *pokok kadok* in Malay. The secondary metabolites of the leaves were subjected to phytochemical tests to detect the presence of natural compounds. Antioxidant activity was described by its total phenolic content (TPC) and total flavonoid content (TFC), which was assessed by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. A phase diagram was constructed to find a possible region to formulate an antioxidant cream. In phytochemical screening, the methanolic extract showed positive presence of alkaloids, flavonoids, steroids, terpenoids and tannins. In quantitative analysis of antioxidative components, besides having significantly higher TFC content compared with quercetin ($P<0.0001$), the extract of *P. sarmentosum* leaves also displayed high phytochemical content and was proven to be an efficient free radical scavenger and reducing agent compared with ascorbic acid ($p=0.0121$). It was observed that the phytochemical compounds in the leaf extract like alkaloids, terpenoids, flavonoids and tannins were the major contributors of antioxidant activity. The leaf extract was also a suitable ingredient to produce a cream with good spreadability, homogeneity, consistency, appearance and pH.

Keywords: *Piper sarmentosum*, antioxidant, total phenolic content, total flavonoid content, Phase diagram

Introduction

Throughout history, herbs and natural therapies have been used to improve health and they have become important ingredients in medicine and cosmetics. Some terrestrial plants, marine organisms, animals, microbes and fermentation products contain active components that may have therapeutic effects. Others have high content of anti-oxidants that have potential to enhance health and wellbeing, besides maintaining a youthful look.

Nowadays, cosmetic products are manufactured with more natural ingredients, especially those used to reduce scars. They usually come in creams, which are effective and easy to apply. Those products are a result of applying colloidal stability through microemulsion. To ensure a long shelf life, paraben is used as a preservative in cosmetic products because of its antifungal and antibacterial activity. However, there are drawbacks in using paraben in cosmetics, and many users are becoming aware of its adverse effects. Studies have found it to be carcinogenic, besides being disruptive to the endocrine system. It may also cause developmental and reproductive toxicity, and induce allergies. On the other hand, natural ingredients are more preferable as it allows the use of alternative solvents and renewable products in manufacturing, which are safer to human health (Chemat *et al.*, 2012). Thus, this study tries to formulate a cream containing *P. sarmentosum* leaf extract using the microemulsion of non-ionic surfactants (Tween 80 and Span 65).

Piper sarmentosum or the wild betel (locally known as *pokok Kadok* in Malay), is a

common herb in Southeast Asia. It mostly used for culinary purposes, but it may also be good for keeping diabetes under control. It contains different classes of antioxidative compounds like alkaloids, steroids, phenylpropanoids, C-benzylated dihydroxyflavone and phenylpropanoyl amides (Shahzad, 2017). Therefore, this study intends to explore the use of *P. sarmentosum* as a new cosmetic and skin healing product that is high in antioxidant activity.

Materials and Methods

Preparation of Methanol Leaf Extract

Alkaloid detection

Mayer's test was used to detect the presence of alkaloids, with a small amount of leaf extract added to diluted HCl and mixed with Mayer's reagent. Presence of alkaloids was determined with the formation of yellow precipitates (Tiwari *et al.,* 2011).

Flavonoid detection

The alkaline reagent test was used to detect flavonoids. Two to three drops of NaOH solution were added into the leaf extract as described by Tiwari *et al.* (2011). The presence of flavonoids was confirmed when the solution turned intense yellow and become colorless again with the addition of a diluted acid.

Steroid detection

The Liebermann-Burchard test was used to detect steroids using the protocols described by Singh *et al.* (2012). Briefly, 0.5 ml of the extract was mixed with 2.0 ml of acetic anhydride. The mixture was then cooled in a bowl of ice. About 1.0 ml of concentrated H_2SO_4 was added and the appearance of a blue green ring would confirm the positive presence of steroids (Singh *et al.,* 2012).

Detection of Terpenoids

Terpenoids were detected using the Salkowski Test. A total of 5.0 ml extract was mixed with 2.0 ml chloroform. Concentrated H_2SO_4 was added carefully to form a single layer in the organic solution. Formation of a reddish-brown layer at the interface would indicate the presence of terpenoids (Mir & Sawhney, 2013).

Detection of Tannins

Tannins were detected using the bromine water test. A total of 1 ml extract was added with equal amount of bromine water. A reddish-brown precipitate or greenish black formation would prove the presence of tannins in the leaf extract (Singh *et al.,* 2012).

Determination of Total Phenolic Content

TPC was determined using the Folin-Ciocalteu reagent as described by Velioglu *et al*. (1998). The methanolic leaf extract was diluted three times with methanol and mixed with 2.25 ml of Folin-Ciocalteu reagent in triplicates before being left in the dark at room temperature for five minutes. A total of 2.25 ml of 60 g/L sodium carbonate solution was added and the mixtures were allowed to sit in room temperature for another 90 minutes before the absorbance was measured using a spectrophotometer at 725 nm wavelength. Gallic acid was used as a standard to compare with the extract.

Determination of Total Flavonoid Content

TFC was studied using the calorimetric method according to Dewanto *et al*. (2002). A total of 0.5 ml of methanolic extract was dissolved with 2.25 ml distilled water in triplicate test tubes. Then, 5 % NaNO2 (0.15 ml) solution was added to the mixtures. Approximately after six minutes, 0.3 ml of 10 % $AlCl_3$.6H2O solution was added and the solutions were left in room temperature for five more minutes. Then, an aliquot of 1M NaOH (1.0 ml) was added. The absorbance was measured at a wavelength of 510 nm. Quarcetin was used to compare flavonoid content with the leaf extract.

DPPH Free Radical Scavenging Assay

The free radical scavenging activity of the leaf extract was measured with 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay (Magalhaes *et al*., 2006). An aliquot of samples with various concentration diluted in 80% methanol were prepared in triplicates with 3.0 ml of 0.5M DPPH in absolute ethanol, which was then allowed to stand at ambient temperature for 30 minutes in the dark. The mixtures' absorbance was then measured using a spectrophotometer at a wavelength of 520 nm. The percentage of free radical scavenging was calculated using Equation 1 (Eq 1).

$$\text{Free radical scavenging activity (\%)} = \frac{\text{Ac-As}}{\text{Ac}} \times 100 \quad \text{(Eq 1)}$$

whereas is the absorbance of the sample and Ac is absorbance of negative control at 520 nm wavelength.

Statistical analysis

All results were presented in mean ± standard error (SEM). The IBM SPSS version 20.0 software (IBM Corp, Armonk, NY, USA) was used to analyse the data and statistical tests performed included one-way ANOVA ($P<0.05$), unpaired t test ($p<0.05$) and Pearson correlation test.

Construction of phase diagram

The titration method was used to construct the ternary phase diagram. A total of 0.5 g Tween 80 and Span 65 mixture was dissolved in nine vials of constant heptane at ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1, respectively. A very small amount of deionized water was titrated into each vial and the mixtures were vortexed until they became homogenised with each titration of water. This was followed by centrifugation at 3500 rpm for 10 minutes. The samples were observed for phase separation or changes.

For the phase diagram, the solutions that were observed as clear and isotropic were classified as one phase micro-emulsion, while solutions that were cloudy in one phase were classified as one phase macro-emulsion. The process was repeated with the addition of butanol to broaden the phase region.

Cream Formulation

All ingredients were measured according to reference formulation in Table 1. Oil phase and aqueous phase were separated in beakers and heated in a water bath at 70°C for 10-15 minutes. The oil phase was poured into the aqueous phase and stirred thoroughly until an emulsion was formed. A homogenizer was used to homogenize the emulsion into a fine, smooth texture. The cream was allowed to cool down before being divided in two air-tight vials. The *P. sarmentosum* leaf extract was added into one vial and stirred until it mixed evenly while the other vial without the leaf extract was used as a comparison control.

Table 1: Modified formulation and ingredients of cream emulsion

	%	**Weight (g)**
Cetyl alcohol	10.00	3.00
Olive oil	20.24	6.07
Tween 80	23.76	7.13
H_2O	43.00	12.90
Glycerine	1.00	0.33
P. sarmentosum leaf extract	2.00	0.60
Total	100.00	30.00

Physical properties of cream and evaluation

The two prepared formulation with and without plant extract were examined and evaluated for their physical properties which is based on their texture, homogeneity and pH.

Organoleptic characteristics: Each formulation was tested for color texture, physical appearance and homogeneity. These characteristics were evaluated by visual

observation. Homogeneity and texture were tested by pressing a sufficient amount of each formulated cream sample between the thumb and index finger. The consistency of the cream and presence of coarse particles in it were used to determine the texture and homogeneity of the cream sample. Immediate feels on the skin such as stiffness, grittiness, and greasiness were also tested.

Determination of pH: 1g from each of the cream sample was measured and mixed in 25 ml of deionized water to obtain liquid form in order to fill it in the digital pH meter. The digital the pH meter used is Laquatwin pH meter, USA. Before conducting the measurements, the pH meter was calibrated with pH 7 buffer solution followed by pH 4 first. The measurements were made 3 times to calculate the mean.

Spreadability (S): A sufficient amount of cream sample from each formulation were applied and compressed to uniform thickness between two glass slides. The glass slides then were loaded with 100 g weight for 7 minutes and was observed. The time taken for the two glass slides to separate was recorder to measure the stability. The less the time taken for the glass slide to separate, the greater the spread ability of the cream. The spread ability value was then calculated using Equation 2 (Eq 2).

$$S = (m \cdot l)/t \qquad \text{(Eq 2)}$$

where S is the spreadability, m is the weight applied on the upper glass slide per gram, l is the length of the upper glass slide moved on the lower plates of the glass slide per cm, and t is the time taken for the glass slide to separate per second.

The spreadability test was also conducted by applying the cream samples directly onto the skin at the back of the palm and observed whether the spread was even and good.

Results and Discussion

The plant sample contained all five secondary metabolites which was beneficial in treatment of wounds. Some of them were advantageous and important as antioxidants, which would promote wound healing. For example, flavonoids are major antioxidants that fight free radicals that could damage the skin. The results of the phytochemical screening are tabulated in Table 2.

Table 2: Summary of phytochemical screening results *P. sarmentosum* extract

No.	Natural compound test	Result	Conclusion
1	Alkaloids	Formation of yellow color precipitate	Presence of alkaloids
2	Flavonoids	Intense yellow color to colorless	Flavonoids present
3	Steroids	Formation of blue- green ring	Presence of steroids
4	Terpenoids	Reddish-brown coloration interface	Terpenoids present
5	Tannins	Reddish-brown precipitate	Presence of tannins

Total Phenolic Content

Phenolic compounds are all popular in many plants and herbs as they act as antioxidant and free radical scavengers. A study is carried out to determine TPC in the leaves of *Piper sarmentosum*. The plant extract was compared with standard gallic acid in this study to quantify the compound present in sample, and the light absorbance was measured using a UV-Vis spectrophotometer with wavelength of 725 nm. The values for TPC are shown in Figure 1. From the figure, it is obvious that TPC in gallic acid was higher than in *P. sarmentosum*.

For this study, unpaired t test was used to compare the TPC between *P. sarmentosum* and standard gallic acid. The study showed that there was a statistically high significant difference ($p<0.01$) in TPC between *P. sarmentosum* and

gallic acid (Figure 1), which showed a mean ± Standard Error Mean (SEM) of 0.4209 ± 0.1105and 1.000 ± 0.0, respectively. The 95% confidence interval was 0.2724 to 0.8858.

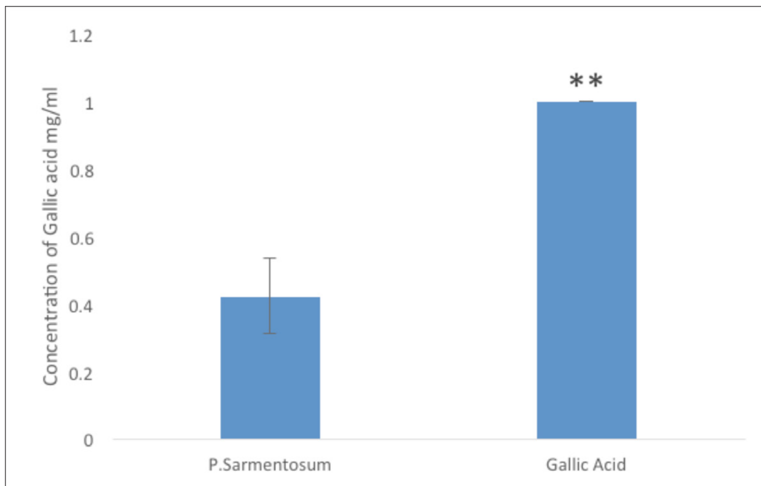

Figure 1: Total phenolic content of *Piper sarmentosum* and gallic acid. ** indicates significant difference (p<0.01). Error bars represent standard deviation

Total Flavonoid Content

The study shows that the Total Flavonoid Content (TFC) found was determined in comparison with standard quercetin. It was proven that the TFC content in *P. sarmentosum* was significantly higher than quercetin. The plant extract was compared with standard gallic acid to quantify the compound present in the sample. The values for TFC are shown in Figure 2. From the figure, it was obvious that TFC in *P. sarmentosum* was higher than in quercetin. For this study, unpaired t test was used to compare the TFC between *P. sarmentosum* and quercetin, and a very significant difference (p<0.0001) was observed0. The mean concentration of TFC in the leaf extract and quercetin was 36.75 ± 0.1146 and 1.000 ± 0.0, respectively. The 95% confidence interval was 36.07 to -35.44.

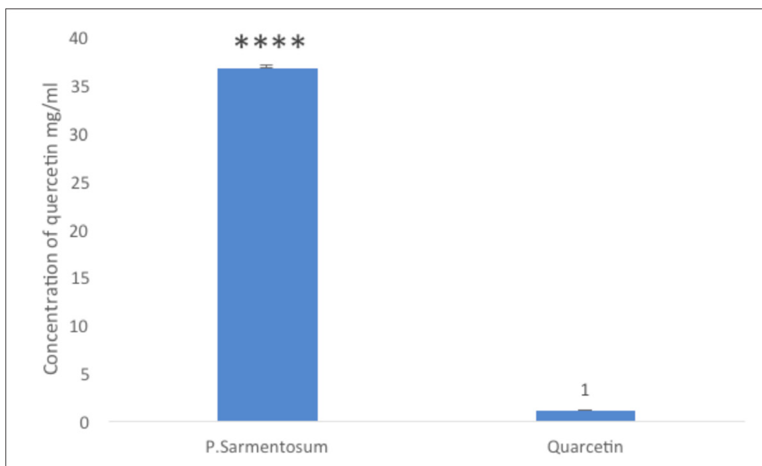

Figure 2: Total flavonoid content of *Piper sarmentosum* and quercetin. ****indicates significant difference (p<0.0001). Error bars represent standard deviation

DPPH Scavenging Assay

The antioxidant activity of the leaf extract was determined through DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay with ascorbic acid as a positive control. A negative control was also performed to calculate the percentage of scavenging effect. The leaf extract displayed excellent scavenging effect on DPPH radicals, which was in the range of above 1,500 % compared to ascorbic acid, which was below the range of 250 % at concentration of 3 mg/ml (Figure 3). Unpaired t test showed a significant difference between the antioxidant activity of *P. sarmentosum* extract and ascorbic acid (P<0.05), which had a mean scavenging percentage of 1,518 ± 296.8 and 226.2 ± 0.4496, respectively. The 95 % confidence interval was -2116 to -468.0. The F test also found a significant difference between the variances (*p*<0.0001).

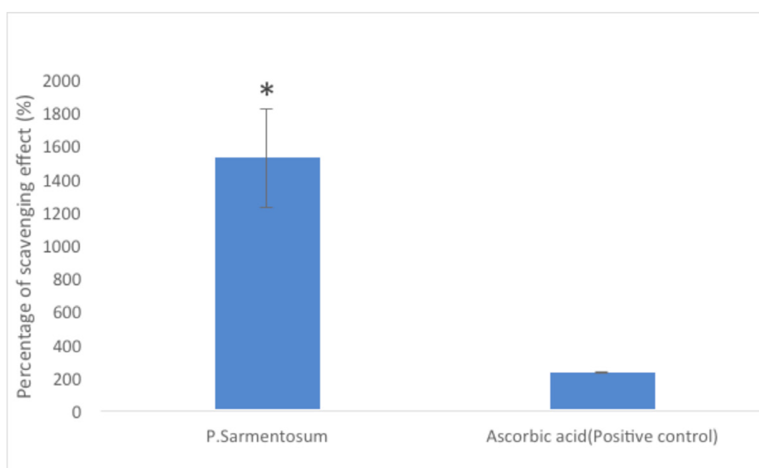

Figure 3: The scavenging activity of *Piper sarmentosum* and ascorbic acid assayed by DPPH free-radical scavenging method. * indicates significant difference (*p*<0.05). Error bars represent standard deviation

The antioxidant in the sample would turn from purple to yellow as it was oxidized by DPPH. The percentage of scavenging effect was very high probably because the total antioxidant content in the sample was lower than those in the absorbance control. Apart from that, the DPPH coloration might also be too concentrated or had been slightly damaged due to light exposure.

Correlation

An analysis of correlation was used to study phytochemical contents and antioxidant activity of *P. sarmentosum*. Correlations between TPC, TFC and antioxidant activities were analyzed using Pearson correlation as stated in Table 3. From the table, the DPPH assay had positive correlation with TPC and TFC (r=0.9343 and r=1, respectively). It was proven that phytochemicals such as TPC and TFC present in *P. sarmentosum* basically contributed to the total antioxidant activity that it exhibited. Both showed a correlation in the assay, therefore, TPC and TFC might be major contributors of the antioxidant activity in the plant extract.

Table 3: Pearson correlation analysis (r values) of TPC, TFC and antioxidant activity

TPC	0.9343**
TFC	1****

**Correlations were significant (p<0.01)

****Correlations were significant (p<0.0001)

Construction of Phase Diagrams

A ternary phase diagram was constructed to find the suitable region for formulating a cream

containing a natural ingredient. In order to do so, a ternary phase diagram consisting of water and non-ionic emulsifiers was constructed. Along the process, the phase behavior of the system was observed and the results were presented in the diagram. An oil phase used in this study was heptane, whereas the non-ionic surfactants were Tween 80 (Polyoxyethylene 20 Sorbitan Monooleate) and Span 65 (Sorbitan Tristearate), which is shown in Figure 4.

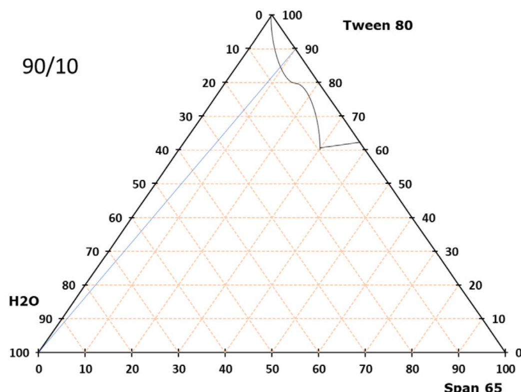

Figure 4: Ternary phase diagram of Water-Tween 80-Span 65 system with heptane as an oil phase

Tween 80 (HLB = 15) and Span 65 (HLB = 2.1) were chosen since they were readily available in the laboratory and their colloidal structures had been characterized by many groups as well as other researches. Figure 4 shows the area of the one-phase isotropic region for water/Span65/Tween 80 with heptane 50 % as a constant oil phase. It was observed that both Tween 80 and Span 65 were completely miscible with each other. A maximum water solubility of seven weight per cent of water was observed at Tween 80 to Span 65 weight ratio of 90:10. The isotropic solution region was observed to be very small on the ternary phase diagram. So, another phase diagram with the addition of butanol (Figure 5) was drawn to obtain a larger isotropic region.

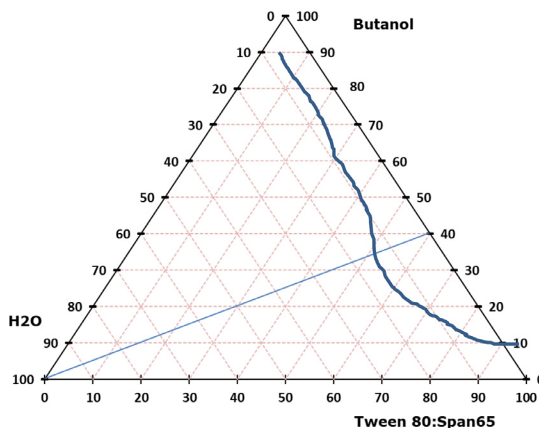

Figure 5: Ternary phase diagram of Water-Butanol-Tween 80 to Span 65 system with heptane as an oil phase

The 90:10 ratio (Tween 80 to Span 65) was then used as the mixed non-ionic surfactant apex and titrated with a third component, which was butanol. The figure showed a similar pattern of the isotropic region to that of the water/Tween 80/Span 65 system, but with an increased amount of the maximum water solubility of 20 % by weight. The maximum water solubility occurred at the butanol/Tween 80 to Span 65 (90:10) weight ratio of 40:60. This suggested that a higher amount of mixed surfactants were required to achieve the maximum water solubility and to form the colloidal structures. However, the region was still not suitable to formulate a cream due to its unstable phase and narrow region. Thus, a formulation derived from a ternary phase diagram constructed by Doreen (2008) with slight modification of essential oils was adapted to formulate two formulations of cream samples for this study.

Cream Formulation and Characterization

Organoleptic characteristics

Table 4 shows the results of the organoleptic characteristics for both cream samples with and without *P. sarmentosum* leaf extract. From the results, the mean pH of the cream with the extract was 4.28 and 4.70 for the cream without (Table 5). The pH of the creams was suitable to apply on skin as the pH of human skin was in the range of 4 to 6.

Table 4: The organoleptic characteristics of cream samples with and without *P. sarmentosum* leaf extract

No.	Organoleptic properties	With plant extract	Without plant extract
1	Physical appearance	Opaque	Opaque
2	Colour	Green	White
3	Texture	Viscous	Viscous
4	Homogeneity	Present	Present
5	Immediate skin feeling	Greasiness, moisturizing, spreadable	Greasiness, moisturizing, spreadable

Table 5: The pH readings in both of cream samples

No.	Tests	With plant extract	Without plant extract
1	First test	4.42	4.89
2	Second test	4.19	4.79
3	Third test	4.24	4.42
4	**Mean**	**4.28**	**4.70**

Spreadability

The spreadability of both cream samples were calculated using Equation 2. Spreadability is an ability of a cream or gel to be evenly spread on the skin. From the calculation, the spreadability mean of cream sample with and without plant extract was 26.94 g cm/s and 25.85 g cm/s, respectively, as shown in Tables 6 and 7. The result of spreadability denoted the extent of area to which the prepared formulations readily spread on application to skin, while homogeneity ensured that no lumps were formed (Pattanayak *et al.,* 2011).

Table 6: Spreadability of cream sample with plants extract

First test	$S = \frac{100.2.5}{12} = 20.83g\ cm/s$
Second test	$S = \frac{100.3.5}{10} = 35.00g\ cm/s$
Third test	$S = \frac{100.3.0}{12} = 25.00g\ cm/s$
Mean	26.94 g cm/s

Table 7: Spread ability of cream sample without plants extract

First test	$S = \frac{100.2.5}{13} = 19.23g\ cm/s$
Second test	$S = \frac{100.3.0}{12} = 25.00g\ cm/s$
Third test	$S = \frac{100.3.0}{9} = 33.33g\ cm/s$
Mean	25.85 g cm/s

The sample creams were tested directly at the back of the palm. When it was applied and rubbed on the skin, the creams exhibited decreasing viscosity. The more the creams were rubbed onto the skin, the easier they spread.

Conclusion

There is a strong positive correlation between TPC and TFC with free radical scavenging activity of the *P. Sarmentosum* leaf extract, thereby showing it was a good source of antioxidants and may have the potential to become a product that can promote wound healing. A previous study had found that the plant extract was biocompatible and non-toxic towards human liver tissue. Plus, the plant extract had also been studied for its potential in reducing blood sugar levels. The medicinal potential of *P. Sarmensotum* should be explored further, especially in experiments to see how efficient it promotes wound healing and prevent the formation of scars.

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