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PHOTOPROTECTIVE ACTIVITY ETHANOLIC EXTRACTS AND CREAM FORMULATION OF CAMELLIA SINENSIS AND AZADIRACHTA INDICA

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ABSTRACT

Ultraviolet (UV) rays can cause many diseases such as erythema and skin cancer when exposed in excess. Thus, it is important to protect oneself from UV rays by application of sunscreen. However, several issues were found regarding sunscreen effectiveness in previous studies causing researchers to study on plants as alternative sunscreen. *Camellia sinensis* (Tea) and *Azadirachtaindica* (Neem) have shown potential for its individual photo-protective activity in previous study but no literature have been found for both plants working synergistically. Therefore, this research aims to evaluate the photo-protective activity of *C. sinensis* and *A. indica* cream formulations with ratio of 1:1. Both plants' leaves underwent soxhlet extraction separately and concentrated using rotary evaporator. The concentrated extracts were tested for their individual and combination of both plants antioxidant and *in-vitro* sun protective factor (SPF) activity using spectrophotometer. Oil-in-Water (O/W) formulations were made from the extract in tea to neem with ratio of 1:0, 0:1 and 1:1 and its SPF activity were tested. The SPF activity of the neem cream showed the highest SPF value of 1.32±0.15, followed by combination of tea neem cream (1:1) with SPF value of 1.00±0.21 and tea cream with SPF value of 0.93±0.00. With the significance of 0.143 ($p<0.05$), this research concluded that there is no significant synergistic photo-protective activity between *C. sinensis* and *A. indica* cream formulations.

KEYWORDS: *Camellia sinensis, Azadirachtaindica,* Photo protective, SPF and O/W emulsion.

INTRODUCTION

Ultraviolet (UV) radiation can cause harm to human if prolong exposure occurs. Such damages include sunburn and skin cancer. World Health Organization states that between 2 to 3 million non-melanoma skin cancers and 132 000 melanoma skin cancer occurs yearly and these numbers are expected to increase due to depletion of ozone layer occur each year. Hence, it is important to protect oneself from UV by regular application of sunscreen.

A study done by Bakos *et al.*, 2002 showed that when the use of sunscreens was compared to no use at all, it appeared to show a progressive protection as the SPF increased. However there are several issues have been brought up regarding the usage of sunscreen in previous studies such as most sunscreen have moderate efficacy, some of the ingredients in a sunscreen such as amino benzoic can cause photo sensitivity reaction and their chemo-preventive activity are poorly understood (Yusuf *et al., 2007)*. Therefore, researchers began to study on plant as an alternative photo-protection agent as most of the plants contain phenol such as phenolic acid, flavonoids and high molecular weight poly phenols which can act as UV blockers (Svobodova *et al.,* 2003). Both *Camellia sinensis* and *Azadirachtaindica* have shown potential for its individual photo-protective activity (Kaur & Saraf, 2011; Gupta, 2013). However, no previous literatures have shown them working synergistically. Therefore, this study is done to evaluate their photo-protective activity when combine in a formulated emulsion.

METHODOLOGY

Plant Collection

Both plant's leave were collected in Selangor, Malaysia whereby the tea leaves (SK2426/14) were collected from Bukit Cheeding BOH Estate, Banting while the neem leaves (SK 2427/14) were collected from housing area in seksyen 13, Shah Alam. Both plant's leave were authetified by Institute of Bioscience, Universiti Putra Malaysia.

Preparation of Plant Extract

500g of plant's leave were cleaned, made to coarse powder and packed in the Soxhlet apparatus for hot extraction process. Extraction was performed with ethanol (90%) at

temperature of 50°C to 60°C. The extract then was concentrated by recovering the solvent through rotary evaporator (Kaur and Saraf, 2011).

Antioxidant Activity Determination

The antioxidant activity of extract was determined using reducing power estimation method. 5mg of extract and ascorbic acid were dissolved separately in 1.0mL of deionised water with 2.5mL of phosphate buffer (0.2M, pH 6.6) and 2.5mL of 1% potassium ferricyanide (10 mg/ml).The mixtures were incubated at 50°C for 20 minutes and left to cool at room temperature. Then, it was mixed with 2.5 mL of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 minutes. Finally, 2.5 ml of supernatant solution was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% of ferric chloride. The end mixture then was diluted up to 10ml with distilled water. It was allowed to stand for 10 minutes. Absorbance was measured at 700 nm in a visible spectrophotometer against blank, which are the mixture of all reagents in the same quantity as added while preparing samples. It was then compared with standard (ascorbic acid). Increase absorbance of the reaction mixture indicates increase antioxidant activity via reducing power with reference to equal amount of ascorbic acid (Kaur and Saraf, 2011).

Method of Cream Preparation

In this study, oil in water (O/W) creams or emulsion was prepared by using 3 groups of extracts in tea:neem ratio of 1:0, 0:1 and 1:1. To prepare base; aqueous phase that consist of potassium hydroxide (1%w/w) was dissolved in deionized water (85%w/w), followed by addition of glycerin (5%w/w), and tea extract (2%w/w). The resulting mixture was heated up to 80°C. At the same time, oil phase consisting of stearic acid (4%w/w), coconut oil (5%w/w) and petroleum jelly (1% w/w) were added and heated at 80°C. After that, oil phase was added to the aqueous phase drop by drop at 80°C with continuous stirring for 20 min to 25 min. Then, the mixture was homogenized at 8000 rpm till uniform emulsion was obtained. The emulsion was poured into wide mouth container and stored at different temperature for cream stability test (Kale *et al.*, 2012).

SPF Determination of Ethanolic Extracts and Creams

The *in vitro* determination of 3 different groups of extract and cream (1:0, 0:1, 1:1) were done by method described by Mansur *et al.*, 1986. For extract sample preparation, 100 mg of extract was dissolved in 10 ml of ethanol, which will give out 10 mg/ml of extract (Shekar *et al.*, 2012). For cream sample preparation, 0.5 g of cream was dispersed in 100 ml of distilled

water and homogenized by ultrasonication for 5 min. The obtained dispersion was filtered with a filter paper and the first 10 ml was discarded. Then 2 ml of filtered solution was adjusted to 50 ml using distilled water (Smaoui *et al.*, 2013). The absorbance of the extracts were determined from 290 nm to 320 nm at every 5 nm interval, using ethanol as blank for extract and distilled water for cream. (Shekar *et al*., 2012).

$SPF_{spectrophotometric} = CF \times \sum_{290}^{230} EE(\lambda) \times 1(\lambda) \times Abs(\lambda)$

Where CF (correction factor) is 10, EE (λ) is erythmogenic effect of radiation with wavelength λ, Abs is Spectrophotometric absorbance values at wavelength λ. The values of EE(λ)×1(λ) are constant and can be refer to Table 1. The obtained absorbance values are multiplied with EE(λ)×1(λ) and then their summation is taken and multiplied with correction factor to obtain the SPF values (Mansur *et al*., 1986).

Cream Stability Test

Stability tests were performed on samples kept at 2°C ± 0.1°C (in cold room), 27°C ± 0.1°C (in room temperature), and 40°C ± 0.1°C (in oven) for 28 days with observations done at various intervals. The cream characteristics, colour, liquefaction, phase separation, centrifugation test and pH of creams were analysed during the observation (Smaoui *et al.*, 2013).

Statistical Analysis

All the measurement and test were done in triplicate and the values were expressed in Mean±Standard error. One-way ANOVA analysis was done on result for SPF determination of cream only; while Tukey correction was used to determine significant differences for the comparison. Differences were considered statistically significant if $p<0.05$.

RESULTS

Table 1 Constant value for SPF Spectrophotometric formula.

Wavelength (λ nm)	EE x I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
Total	1

Antioxidant Activity Determination of Extracts

Based on figure 1, tea extract showed the highest antioxidant activity by 65.4%±1.24, followed by tea neem (1:1) extract with 63.5%±1.30 and neem extract with 31.1% ± 0.78.

SPF Determination of Extracts

Based on figure 2, tea extract showed the highest SPF value of 12.01 ± 0.89 followed by tea neem (1:1) extract with SPF value of 7.59 ± 0.29. Neem extract showed the lowest SPF value with 5.24 ± 0.65.

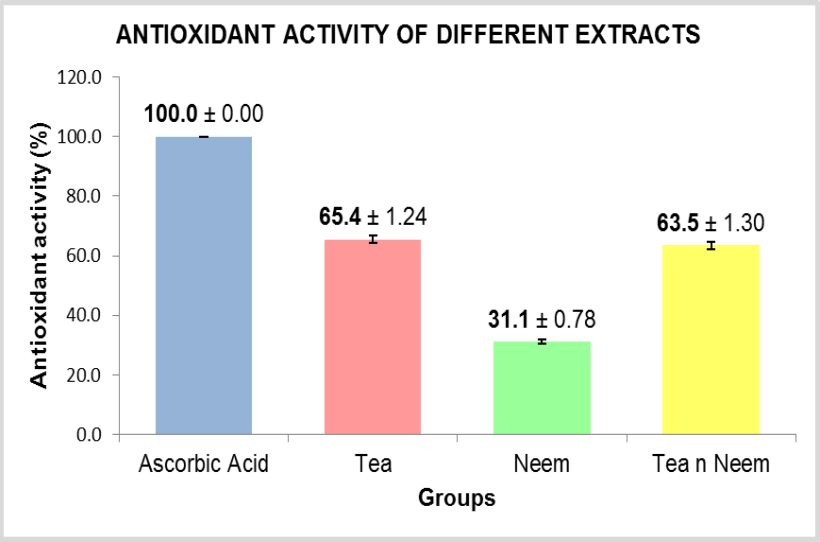

Figure 1 Antioxidant activities of extracts when compared with ascorbic acid as the standard.

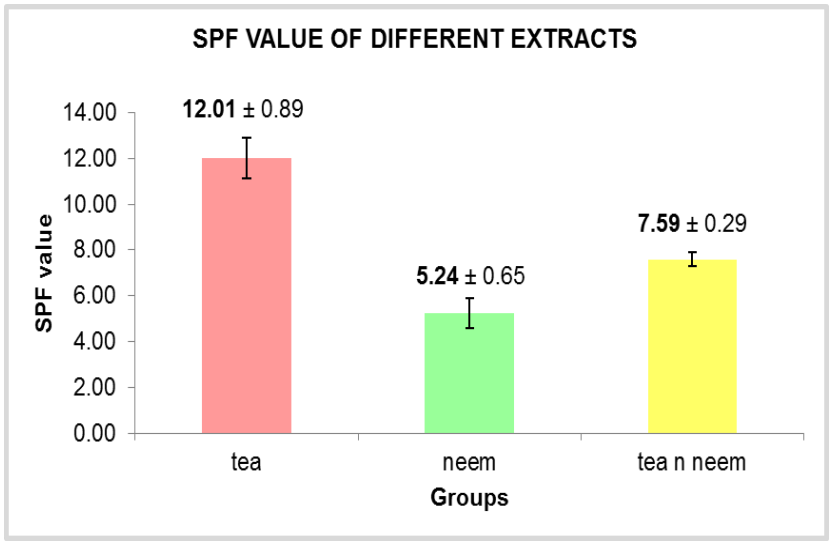

Figure 2 The *in vitro* SPF value of extracts by using method proposed by Mansur *et al.*

Table 2 Cream stability test on tea and neem cream with observation done at fresh, 24 hours, 14 days, 21 days and 28 days

Days		Fresh		24 Hours		14 Days		21 days		28 Days	
Cream		T	N	T	N	T	N	T	N	T	N
Characteristic	4°C	S	S	S	S	S	S	S	S	S	S
	27°C	S	S	S	S	S	M	S	M	S	M
	40°C	S	S	S	S	S	O	S	O	S	O
Colour	4°C	B	G	B	G	B	G	B	G	B	G
	27°C	B	G	B	G	SD	SD	SD	SD	SD	SD
	40°C	B	G	B	G	MD	MD	MD	MD	MD	MD
Liquefaction	4°C	-	-	-	-	-	-	-	-	-	-
	27°C	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	+	-	+	+	+
Phase separation	4°C	-	-	-	-	-	-	-	-	-	-
	27°C	-	-	-	-	-	-	-	-	-	-
	40°C	-	-	-	-	-	+	-	+	-	+
Centrifugation test	4°C	-	-	-	-	-	-	-	-	-	-
	27°C	-	-	-	-	-	-	-	+	-	+
	40°C	-	-	-	-	-	+	-	+	-	+

T = Tea, N =Neem, S = Smooth, M = Mould, O = Oily, B = Beige, G = Green, SD = Slight darker, MD = More darker, - = Absent, + = Present

Table 3 Cream stability test on tea neem (1:1) cream with observation done at fresh, 24 hours, 14 days, 21 days and 28 days

Days		Fresh	24 Hours	14 Days	21 Days	28 Days
Cream		TN	TN	TN	TN	TN
Characteristic	4°C	S	S	S	S	S
	27°C	S	S	S	S	S
	40°C	S	S	S	S	S
Colour	4°C	GB	GB	GB	GB	GB
	27°C	GB	GB	SD	SD	SD
	40°C	GB	GB	MD	MD	MD
Liquefaction	4°C	-	-	-	-	-
	27°C	-	-	-	-	-
	40°C	-	-	-	+	+
Phase separation	4°C	-	-	-	-	-
	27°C	-	-	-	-	-
	40°C	-	-	-	-	+
Centrifugation test	4°C	-	-	-	-	-
	27°C	-	-	-	-	-
	40°C	-	-	-	+	+

TN = Tea neem cream, S = Smooth, GB = Greenish beige, SD = Slight darker, MD = More darker, - = Absent, + = Present

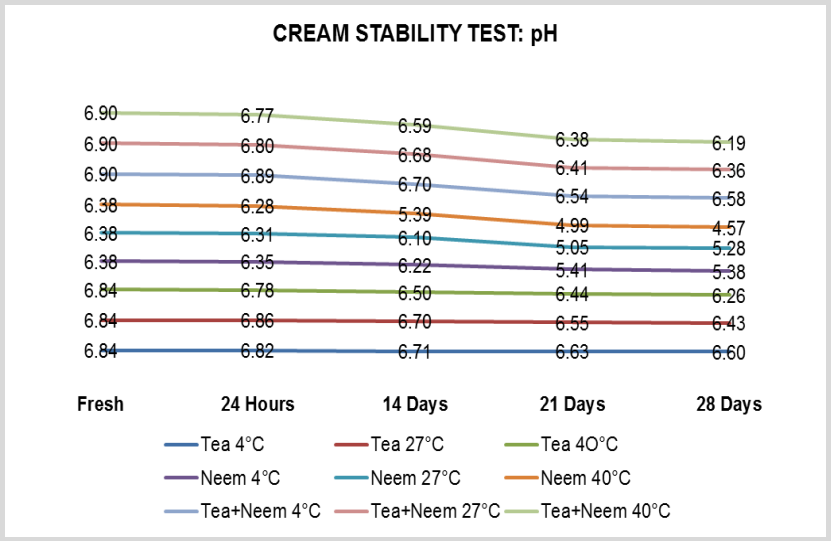

Figure 3 The pH value of creams stored at different condition which have been observed at fresh, 24 hours, 14 days, 21 days, and 28 days

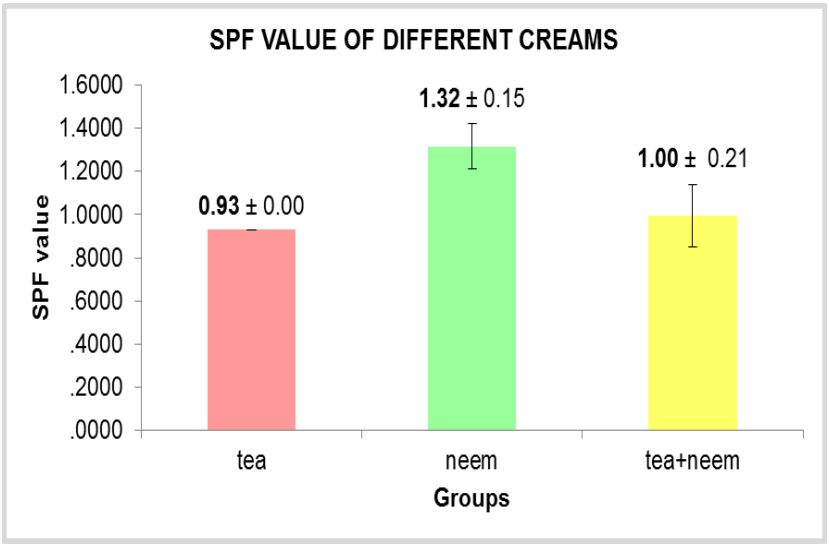

Figure 4 The SPF value of creams by using method proposed by Mansur *et al.*

Cream Stability Test

Based on table 2, table 3 and Figure 3, all the creams were assessed in 6 different aspects which are characteristics, colour, liquefaction, phase separation, centrifugation test and pH. All fresh creams showed smooth characteristic which is similar to the usual cream texture available in market. However, the characteristic for some creams were changed during 14th day observation. Neem cream stored at 27°C showed some mould development while neem cream stored at 40°C was found to be oily.

For colour aspect, fresh cream of tea showed beige colour, neem showed green colour and tea neem (1:1) showed greenish beige colour. All creams stored at 4°C able to maintain the respective colour throughout the 28 days. However, the creams stored at 27°C began to change to a slightly darker colour than the original cream colour and all cream stored at 40°C changed to darker colour during 14th day observation until the 28th day observation.

In this test, liquefaction can be seen only on creams stored at 40°C. Neem cream stored at 40°C showed the earliest presence of liquefaction which was during 14th day of observation. The neem cream showed slight liquefaction on 14th day of observation, increase in liquefaction on 21st day of observation and no further increase for 28th day of observation. Tea neem (1:1) cream showed slight liquefaction on 21st day of observation and no increase of liquefaction can be seen during 28th day observation. Tea cream showed slight liquefaction only on the 28th day of observation.

All creams showed absence of phase separation throughout the 28 days of observation except for neem and tea neem (1:1) cream stored at 40°C. Phase separation can be seen on 14th day of observation until 28th day of observation for neem cream stored at 40°C. Tea neem (1:1) cream stored at 40°C showed presence of phase separation only on the 28th day of observation.

Centrifugation test were done on all creams and only some creams showed phase separation after being centrifuged. Neem cream stored at 40°C showed phase separation after centrifuged during 14th day observation until 28th day observation. However, for neem cream stored at 27°C and tea neem (1:1) cream stored at 40°C showed phase separation after centrifuged during 21st day observation until 28th day observation.

All the fresh cream showed pH in the range of 6.0 to 7.0. However, a progressive decrease can be seen throughout the 28 days observation but still maintained in the pH range of 6.0 to 4.5.

SPF Determination of Creams

Based on figure 4, the SPF values of neem cream showed the highest with SPF value of 1.32 ± 0.15 followed by tea neem cream with SPF value of 1.00± 0.21 and tea cream came in last with SPF value of 0.93 ± 0.00. The result for this test were analysed for one way ANOVA ($p>0.05$) where the significance obtained from this study is 0.143.

DISCUSSIONS

Antioxidant Activity Determination of Extracts

Antioxidant activities of the extracts are determined using ferric reducing power estimation method whereby the presence of extract's antioxidants would reduce fe^{3+} into fe^{2+} which suggests that the extract is an electron donor and could neutralize free radicals (Zhu *et al.*, 2001).

In this study, tea extract showed the highest antioxidant activity which may be due to high presence of phenol content especially polyphenols such as (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin-3-gallate (EGCG) (Katiyar and Elmets, 2001). The combination of tea and neem (1:1) extract showed no sign of synergistic antioxidant activity. This may occurred due to presence of compound in neem extract that may cause deleterious effect on tea extract which makes the antioxidant activity level to be lower than tea extract but higher than neem extract. Previous findings had shown neem to have good antioxidant activity (Sithisarn *et al.*, 2005). However when compared to the other 2 group of extracts in this study, it has the lowest antioxidant activity.

SPF Determination of Extracts

In-vitro SPF determinations of extracts were done by taking the absorbance readings of each extracts and applied it into Mansur mathematical equation. This method is one of the common methods done for *in-vitro* determination of SPF of a compound in many studies (Gupta, 2013; Kaur & Saraf, 2011; Oliveira-Junior *et al.*, 2013; Shekar *et al*., 2012).

Tea extract showed the highest SPF value in this study which may be due to the fact that tea has the highest antioxidant activity as determined earlier. Neem has the lowest SPF value which was in coherent with the antioxidant activity result earlier where neem has the lowest antioxidant activity when compared to the other extracts group. The combination of tea and neem extracts also does not show any synergistic photo-protective activity. This occurrence may be due to compound present in neem which may decrease the amount of antioxidant of tea thus reducing the overall SPF value. From this test results, the study showed that the antioxidant activity of a plant does relate to its SPF value where the higher the antioxidant activity the higher the SPF value.

Cream Stability Test

Emulsions are thermodynamically unstable (Kontogiorgos *et al.*, 2004). Therefore, cream stability test are test that is use to observe how emulsion or cream are affected to the different storage conditions which then gives the idea on the overall its storage stability. This is very crucial for preliminary study of a new cream formulation.

All creams maintain their smooth characteristic except for neem cream stored in 27°C and 40°C. Neem cream stored at 27°C showed sign of mould development which may be due to improper sterilization method throughout the process of making and storing the emulsion. On the other hand, neem cream stored at 40°C became oily signify that the phases already began to separate. This occurrence was due to improper homogenization process during emulsion production (Abdurrahman &Rosli, 2006). All the other creams managed to maintain their smooth characteristic which showed that they undergo a proper process during emulsion development.

For the colour aspect, fresh cream of tea showed beige colour, neem showed green colour and tea neem (1:1) showed greenish beige colour. Only creams stored at 4°C were able to maintain their original colour while the other creams stored at 27°C and 40°C turned to be slightly and more darker than the original fresh colour. These events may be due to the separation of oil phase promoted at higher temperature where the oil phase begins to move to the upper part of emulsion and causes colour changes (Smaoui *et al.*, 2013). However, due to the low temperature of 4°C, the creams which were stored in this condition are stable and do not undergo oily phase separation throughout the 28 day of observation.

The flow properties of emulsion do relate with the emulsion's viscosity (Nasirideen *et al.*, 1998). Increase in liquefaction are results from decrease in viscosity which occurs due to emulsion separation and starting from the emulsion preparation, the temperature and time processes begin can contribute to its separation (Herbert *et al.*, 1988). Presence of liquefaction in all creams stored at 40°C showed that the creams lose its viscosity due to hydrolysis which occurred at higher temperature. Other creams that did not showed any sign of liquefaction were proved to be stable.

Presence of phase separation can be seen in neem and tea neem cream stored at 40°C which may be due to an event called creaming. Creaming can occur due to separation of emulsion droplets as a cream layer separates from the continuous phase via gravitational force (Derick,

2000). Some factors contribute to creaming include large size of droplet, low droplet concentration and reduce viscosity (Mc Clements, 2005). Tea cream phase remain stable throughout the 28 days of observation proving that it is stable.

The instability of an emulsion may only come after several months or years of storage and not during the 28 days or cream stability test. Therefore, centrifugation test are done in order to speed up the potential destabilization process. In this study, neem and tea neem cream stored at 40°C showed presence of phase separation after centrifuge. However, these creams already showed presence of phase separation even before the centrifugation test which means that they were not stable even before the testing. On the other hand, neem cream stored at 27°C showed presence of phase separation only after centrifugation test. This occurrence means that the cream may become unstable after the 28 days of cream stability test. Other creams showed no sign of phase separation even after centrifugation test which means that all those creams were very stable. This stability can be achieved by proper homogenization speed during emulsion formulation that might have prevented the breakage of the formulations during testing (Abdurahman and Rosli, 2006).

The pH range for human skin are from 4.5 to 6.0 and all new formulation of emulsion must be in this range in order to be approved for the market (Matousek *et al*., 2003). In this study, the fresh cream showed pH which is higher than 6.0 and this may result in possible irritation when topical application is done. However, along with the 28 days of observation, all the creams showed a steady decrease of pH. Changes in pH can be an indicator that there are occurrences of chemical reactions that can give an idea on the quality of the final product. All the creams stored at 40°C showed the highest pH difference from the fresh cream pH when compared to the creams that are stored at 4°C and 27°C. This may be due to destabilization caused by hydrolysis which was promoted at higher temperature (Smaoui *et al*., 2013). Overall, after the stability test ends, all the creams managed to maintain their pH within the suggested range.

SPF Determination of Creams

The result for this test showed opposite pattern from the determination of antioxidant activity and SPF value of the extract whereby it showed neem cream to have the highest SPF value followed by tea neem cream and tea cream. This may occur due to interaction of chemicals employed in the formulations which can affect the SPF value (Mishra *et al.*, 2012). Thus, it causes the SPF value of neem cream to be increase and tea cream to be decrease when

compared to original extract. Other than that, the SPF value for those creams are much lesser than the extract's SPF value which may be due to only 2% of the extract were taken for this formulation and unwanted interaction occurred between extracts and chemical employed in the formulation (Mishra *et al.*, 2012).

One-way ANOVA analysis showed increased significance of 0.143 than the expected 0.05. This showed no significant synergistic activity in combined tea and neem ethanolic extract cream with ratio of 1:1.

CONCLUSIONS

Antioxidant activity of plant may not be directly proportional to the SPF value of that plant. However, many plant that showing high SPF value does have high antioxidant activity. In this study, tea extract does show the highest value for both antioxidant activity and SPF value.

The addition of other compounds and some procedures during the formulation development process may affect the extract activity and the cream stability. After formulation development, this study showed that neem cream had the highest SPF value which is totally opposite from the extract's SPF value. However, due to improper homogenization, neem cream showed the least stability. On the other hand, tea cream proved to be the most stable with the least SPF value.

In conclusion, the study showed that there is no significant synergistic photo protective activity between *C. sinensis* and *A. indica* cream formulations.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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