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Original Research Article

Antioxidant Activity of *Pandanus amaryllifolius* Leaf and Root Extract and its Application in Topical Emulsion

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Abstract

***Purpose**: To develop Thai pandan (Pandanus amaryllifolius) as an antioxidant ingredient for topical emulsion.*
***Methods**: Dried leaf and root of P. amaryllifolius (Pandanceae) were extracted separately by maceration with either ethanol or propylene glycol. Antioxidant capacity was investigated by 2,2-diphenyl-1-picry hydrazyl radical (DPPH) and linoleic acid peroxidation method. The total phenolic content was measured by Folin-Ciocalteau assay. Oil-in-water topical emulsions containing the individual extracts were prepared and tested for stability.*
***Results**: Propylene glycol extract exhibited higher DPPH activity and total phenolic content than the ethanol extract while the DPPH activity of the leaf extract was higher than that of the root. The 50 % inhibition concentration (IC_{50}) value of leaf and root extracts was 0.810 and 2.340 mg/ml, respectively. Although the antioxidant activity of the crude extracts was lower than that of standard vitamin C and butylated hydroxytoluene (BHT), the ethanol/propylene glycol solution extract (ethanol was reduced to 50 % extract volume) showed higher inhibition (90.1 %) of linoleic acid peroxidation than 200 ppm of vitamin C (57.1 %) and BHT (71.1 %). An oil-in-water emulsion containing 3 % of the ethanol and propylene glycol extract showed creamy texture with medium viscosity and demonstrated good stability under accelerated aging test.*
***Conclusion**: The results indicate a potential for the development of P. amaryllifolius leaf extract as an antioxidant ingredient in topically applied formulations.*

***Keywords:** Pandanus amaryllifolius, Extraction, Antioxidant, Emulsion, Stability.*

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INTRODUCTION

The leaf of *Pandanus amaryllifolius* Roxb., commonly known as pandan, is often used to give a refreshing, fragrant flavor to south-east Asian dishes [1]. Besides its culinary value, pandan leaves are used in the perfume industry and also medicinally as a diuretic, cardio-tonic and anti-diabetic [2]. Furthermore, the leaves are used to refresh the body, reduce fever, and relieve indigestion [3], and are reported to contain various alkaloids [4] and unglycosylated pandamin protein which exhibits antiviral activity against human viruses, herpes simplex virus type-1 and influenza virus [5]. The leaves also contain quercetin [6], carotenoids, tocopherols, tocotrienols and essential oils [7]. The major compound responsible for the unique pleasant aroma of *P. amaryllifolius* is 2-acetyl-1-pyrroline. Additional 30 aroma components have been found of which

the main ones are hexanal, 2-hexenal, 3-methyl pyridine, 2-penten-1-ol, nonanal, benzaldehyde and linalool [8]. It has been further reported that the ethanol extract of the leaves cultivated in Malaysia exhibited excellent heat-stable antioxidant property [9]. Also, it has been reported that the decoction of *P. amaryllifolius* root and rhizome has been traditionally used in treating diabetic patients. The compound 4-hydroxybenzoic acid has been identified and it showed a hypoglycemic effect in normal rats after the oral administration of 5 mg/kg [10].

Oil-in-water emulsion is commonly formulated for topical pharmaceuticals and cosmetics. It is thought that inclusion of antioxidants in the products can offer better protection and possibly correct the damage caused by free radicals. Natural antioxidants, such as polyphenols in edible herbs, are believed to be safer than synthetic antioxidants. Recently, there has been an increase in the use of polyphenolic compounds in cosmetics [11,12]. This trend indicates a need for the study of similar compounds when incorporated in finished products.

Pandan has long been used in food but there have been few studies of its topical pharmaceutical and cosmetic applications. Thus, in this work, an attempt has been made to formulate a topical oil-in-water emulsion containing Thai pandan extract as an antioxidant ingredient and to evaluate its total phenolic content and antioxidant activity.

EXPERIMENTAL

Plant materials and reagents

P. amaryllifolius was collected in August 2009 from Chiangrai province, Thailand and identified by Dr. Tipsuda Tangtragoon (Department of Biology, Maejo University, ChiangMai, Thailand). A voucher specimen (no. QSBG 56488) has been kept at Queen Sirikit Botanic Garden Herbarium, Chiangmai, Thailand for future reference.

Leaves and root were used for extraction. All chemicals and reagents for activity study were of A.R. grade. Solvents for extraction (95 % ethanol and propylene glycol) and other ingredients used in emulsion preparation were of cosmetic grade. All spectrophotometric data were acquired using UV-Vis Spectrophotometer (Libra 522, Biochroms).

Preparation of extracts

Solution extract: *P. amaryllifolius* leaf and root samples were cut into small pieces and dried in hot-air oven (Memment UNE/UFE) at 40 ^{o}C for 48 h. The material (10 g of leaf or 20 g of root) was immersed in solvent (250 ml, ethanol or propylene glycol or ethanol/propylene glycol blends of 4:1 and 1:1 volume ratio) and macerated under different conditions, i.e. at ambient temperature (28-32 ^{o}C) for 1 and 3 days and at 50 ^{o}C for 8 h. The extract solution was filtered using a filter paper (Whatman No.1) and kept at 4 ^{o}C. Ethanol and propylene glycol were selected as solvents for extraction because they are normally used as ingredient in topically applied formulations. The plant materials (10 g of leaf or 20 g of root) were also extracted with ethanol (250 ml) at 50 °C for 8 h under sonication-assisted conditions (Ultrasonic 690D, Crest) and the solvent removed using a rotary evaporator at reduced pressure to obtain crude extracts.

DPPH radical-scavenging assay

The scavenging activity of the extracts against DPPH radicals was evaluated according to the method Que *et al* [13] with some modifications. A solution of DPPH in absolute ethanol (0.1 mM, 3 ml) was added to the extract (1 ml, as for the solution extract). The reaction was allowed to continue at ambient temperature for 30 min in the dark and then the absorbance (Abs) was measured at 517 nm. Scavenging activity (%SA) on DPPH radicals was calculated as in Eq 1. The data are expressed as mean ± SD (n = 3).

$$\%SA = \{Abs_{control} - (Abs_{sample} - Abs_{blank})/Abs_{control}\} \times 100 \quad \ldots\ldots(1)$$

Linoleic acid emulsion–thiocyanate method

The antioxidant activity of the extracts was also evaluated using the thiocyanate method [i] with some modifications. Vitamin C and butylated hydroxytoluene (BHT, 200 ppm) were used as positive controls. The reaction mixture (linoleic acid emulsion) consisted of 0.28 g of linoleic acid, 0.28 g of Tween 20 and 50 ml of phosphate buffer (0.2M, pH 7.0). The emulsion (2.5 ml) was mixed with 0.5 ml of the test sample and 2.5 ml of phosphate buffer, and incubated at 40 ^{o}C for 96 h. The mixture prepared without test sample served as control. Aliquots (0.1 ml) were taken from the incubation mixture at intervals of 24 h, mixed with 5.0 ml of 75 % ethanol, 0.1 ml of 30 % ammonium thiocyanate and 0.1 ml ferrous chloride (20mM in 3.5 % HCl), and allowed to stand at room temperature for 3 min. The absorbance of the mixture was measured spectrophotometrically at 500 nm. The inhibition of lipid peroxidation in linoleic acid emulsion was calculated at 72 h as in Eq 2.

Inhibition (%) = 100 - {(As/Ac)100} …… (2)
where Ac is the absorbance of the control reaction which contains only linoleic acid emulsion and sodium phosphate buffer, and As is the absorbance of the test sample or standard [15]. All measurements were made in triplicate.

Determination of total phenolic content

The total phenolic content of the extracts was determined by Folin-Ciocalteau total phenolic assay [16]. Gallic acid was used as a standard and a range of concentrations (50, 100, 250, 500, 750 and 1000 mg/L) was used to create a standard curve. Deionized water (1.58 ml) and Folin-Ciocalteau reagent (100 µl) was added to the extract sample, the standard, or blank (20 µl). The reaction mixture was incubated at room temperature for 5 min. Sodium carbonate solution (300 μl, 10% w/v) was then mixed with it and incubated for 90 min. The absorbance of the mixture was measured spectrophotometrically at 765 nm. The total phenolic content was calculated from a standard curve and expressed as gallic acid equivalent (GAE) mg/100 g plant sample. Determinations were made in triplicate.

Preparation of topical oil-in-water emulsion containing the extract

The oil phase consisted of cyclopentasiloxane (4 %), jojoba oil (5 %), dimethicone (2 %), shea butter (2.5 %), jojoba ester (3.5 %), Span-80 (1.7 %), Tween-80 (1.3 %), Sepigel-305 (2.5 %) and propylparaben (0.15 %) while the aqueous phase was comprised of propylene glycol (3 %), methylparaben (0.15 %) and deionized water. The oil phase and water phase were mixed and homogenized (Ultra-Turrax® T25 basic, IKA) at 65 – 70 °C for 5 min. The resulting emulsion was cooled to 40 °C and the extract was added and mixed by using homomixer until homogeneous. Ethanol and ethanol/propylene glycol (1:1) leaf extracts were used as active ingredient and their concentrations in the emulsion varied from 1 to 5 % w/w.

The viscosity of the developed products was measured with a Viscometer (RV#4, 4 rpm, Brookfield, USA). The color of products was measured with a Chromameter (Konica, Minolta). Stability prediction of emulsion product is usually performed by accelerated aging test at different storage conditions [17]. The products are generally tested for gravitational stability under centrifugation (6000 rpm, 30 min) [18] and those with no phase separation will then subjected to accelerated aging test. The product containing 3 % extract was divided into 4 samples in a well-closed 120 ml glass bottle and kept in different conditions, i.e. at 4 °C, ambient temperature (28-32 °C) , 45 °C and heating-cooling cycle (4 °C, 24 h; 45 °C, 24 h). The samples were monitored every week for 1 month.

Statistical analysis

Each experimental data point represents the mean from three independent experiments. The deviation from the mean at the 95% significance level was used to determine the differences in biological activity. The 50% inhibition concentration (IC_{50}) values were calculated from linear regression analysis.

RESULTS

***P. amaryllifolius* extracts**

The solution extracts of leaf were dark green with a unique, pleasant pandan odor while the root extracts were odorless with a light yellow color. Their pH was 6.0 ± 0.1.
The crude extract yield of the root (14.1 %) was higher than that of the leaf (9.2 %).

DPPH radical-scavenging

Extraction at 50 ^{o}C for 8 h produced extract that had higher DPPH radical-scavenging activity (96.37 ± 4.50 %) than those obtained at ambient temperature (28-32 ^{o}C)- Day 1, 64.29 ± 2.41 %; Day 3, 74.65 ± 2.62 %). The DPPH activity of the leaf extract obtained at 50 ^{o}C obtained using various solvents and solvent mixtures are shown in Table 1. It can be seen that DPPH activity was highest for propylene glycol extract followed by ethanol/propylene glycol extract (1:1 and 4:1 solvent ratio), and ethanol extract, in that order. The DPPH activity of the propylene glycol leaf extract (94.56 ± 3.35 %) was three times higher than that of the root extract (29.55 ± 1.21 %).

The data obtained also showed that sonication-assisted conditions increased the antioxidant activity of the extracts approximately two times, which indicates that sonic energy is of great help in extraction.

The 50 % inhibition concentration (IC_{50}) was graphically obtained to determine the least extract concentration that can inhibit 50 % of free radicals [19]. The results showed that the IC_{50} values of vitamin C, BHT, as well as *P. amaryllifolius* leaf and root extracts were 0.012 ± 0.001, 0.290 ± 0.007, 0.810 ± 0.009 and 2.340 ± 0.040 mg/ml, respectively.

Table 1: DPPH radical–scavenging activity of *P. amaryllifolius* leaf extracted at 50 °C for 4 h under sonication-assisted conditions.

Solvent	% DPPH activity	
	1st extraction*	2nd extraction*
Ethanol	86.36±2.56	69.61±1.08
Ethanol/Propylene glycol (4:1)	93.06±3.04	79.41±2.39
Ethanol/Propylene glycol (1:1)	93.32±1.89	90.37±3.46
Propylene glycol	94.56±3.35	92.06±3.17

** 1st extraction is maceration of leaf sample (10 g) in 250 ml solvent; 2nd extraction is maceration of the leaf sample from the first extraction (collected after solvent was removed) in fresh 250 ml solvent*

Linoleic acid emulsion system–thiocyanate inhibition

The inhibitions of the reference standards (vitamin C and BHT) and those of the extracts at various concentrations are graphically shown in Fig 1. Increase in absorbance indicates less inhibition in lipid peroxidation.

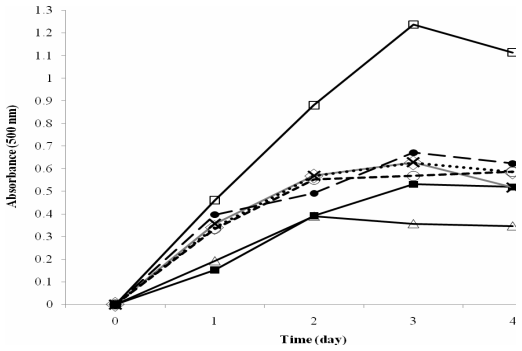

Fig 1: Ferric thiocyanate antioxidant activity of vitamin C and BHT, *P. amaryllifolius* root and leaf crude extracts: Control (□),Vitamin C 200 ppm (■), BHT 200 ppm (∆), *P. amaryllifolius* root 400 ppm (×), *P. amaryllifolius* root 200 ppm (●), *P. amaryllifolius* leaf 400 ppm (○), and *P. amaryllifolius* leaf 200 ppm (◊).

The absorbance of vitamin C, BHT and the extracts is lower than that of the control signifying the materials' ability to slow down peroxidation of linoleic acid. The inhibition of lipid peroxidation by the extracts was 49.3 ± 1.0 and 45.2 ± 1.2 % for leaf and root extracts while those of vitamin C (57.1 ± 0.9 %) and BHT (71.1 ± 1.0 %) were higher at the same concentration (200 ppm). When the concentration of the extracts was increased, inhibition increased by approximately 10 %.

The peroxidation inhibition of concentrated extract solutions, considered potentially suitable for the formulation of topical emulsion, is shown in Fig 2. The inhibition of the concentrated solution extract (ethanol/propylene glycol blend at 1:1 and ethanol was completely removed) was 90.1 ± 1.2 %, and is higher than that of the reference standards - vitamin C (57.1 ± 0.9 %) and BHT (71.1 ± 1.0 %).

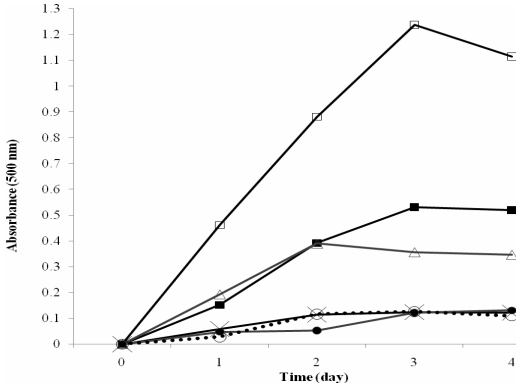

Fig 2: Ferric thiocyanate antioxidant activity of vitamin C and BHT (200ppm each), and concentrated extract solution of *P. amaryllifolius* leaf and root: Control (□), Vitamin C 200 ppm (■), BHT 200 ppm (∆), *P. amaryllifolius* leaf ethanol extract (solvent was removed until the extract volume remained at 20%) (×), *P. amaryllifolius* leaf ethanol/propylene glycol 1:1 extract (ethanol was completely revoved) (●), and *P. amaryllifolius* root ethanol extract (solvent was removed until the extract volume remained at 20%) (○).

Total phenolic content

The total phenolic content of the ethanol leaf extract (319.2 ± 15.9 mg GAE/100 g plant sample) was much higher than that of the root extract (28.8 ±1.1 mg GAE/100 g plant sample). Furthermore, sonication-assisted extraction resulted in approximately 2 to 4 times higher phenolic content. In addition, propylene glycol extract had higher total phenolic content (360.8 ± 10.8 mg GAE/100 g plant sample) than ethanol extract (319.2 ± 15.9 mg GAE/100 g plant sample).

Topical extract emulsion

The ethanol leaf extract (the extract was concentrated by removing solvent until the extract volume remained at 20% of original volume) , labeled ET20) and the ethanol/ propylene glycol (1:1) extract (the extract was concentrated by completely removing ethanol and the volume remained at 50 % of original volume), labeled EP50) were used to prepare separate emulsions. The results indicated that the products containing 1 and 3 % extract had medium viscosity with creamy texture and no phase separation observed under centrifugation.

The emulsions were creamy with light sweet, pleasant odor. ET20 emulsion was greener and less viscous than EP50 emulsion (Table 2).

Table 2: Properties of emulsion cream containing 3 % *P. amaryllifolius* leaf extract

Property	ET20 emulsion	EP50 emulsion
Color	Green	Tinted green
L*, a*, b*	L* = 86.67±0.30 a* = -7.09±0.07 b* = 20.78±0.08	L* = 92.88±0.20 a* = -3.05± 0.03 b* = 6.12±0.19
pH	6.11	6.26
Viscosity	35,000 cps	36,600 cps

L is the lightness of the color (L*= 0 yields black and L*= 100 indicates diffuse white); a* indicates red and green hue where negative values indicate green while positive values indicate red; b* is yellow and blue color where negative values indicate blue and positive values indicate yellow*

Stability of emulsion product

At the end of the accelerated stability test, the color of the product slightly changed to a lighter green colour and the characteristic pandan odor was less intense. Additionally, pH was practically unchanged except for at 45 °C where it decreased from 6.11 to 5.70 in ET20 emulsion and from 6.26 to 5.90 in EP50 emulsion.

Product viscosity

Initially, the product had a creamy texture with medium viscosity. After accelerated-aging test, as Fig 3 shows, the viscosity of the emulsion was largely unchanged after storage at ambient temperature. But the viscosity of ET20 product stored at 45 °C and subjected to heating-cooling cycle, decreased (20 - 50 %) while that stored at 4 °C showed a 30 % increase over time. EP50 product viscosity at 4 and 45 °C increased from 10 - 30 %. Furthermore, decrease in viscosity (44 %) was obtained when EP50 product was subjected to heating - cooling cycle. Texture change was noticeable when emulsion viscosity varied by 30 % or more.

Product color

The results were recorded as coordinates of CIELAB or L*, a*, b* values in Table 3 with the color difference (∆) of each parameter also shown. L* represent the lightness of the color (L*= 0 yields black and L*= 100 indicates diffuse white), a* indicates red and green hue (negative values indicate green while positive values indicate red) and b* positions between yellow and blue (negative values indicate blue and positive values indicate yellow. Color difference in L* value ($\Delta L^* = L^*_{wt} - L^*_{w0}$) showed a decline for all conditions except ambient temperature. The greatest change (8% decreased) was found for the emulsion product containing ET20 when subjected to heating - cooling cycle, and this indicates the lightness is decreased. ∆a* ($\Delta a^* = a^*_{wt} - a^*_{w0}$) increased over time indicating that the green hue decreased. ∆b* ($\Delta b^* = b^*_{wt} - b^*_{w0}$) decreased which indicates the blue hue was dominant and the yellow hue is minimal. The total color difference (∆E*) between initial value (W0) and values in week 4 was also calculated as in Eq 3 [20].

$$\Delta E^* = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2} \quad \ldots\ldots\ldots\ldots\ldots\ldots(3)$$

The result, (∆E*) which incorporates changes in the three components (L*, a*, b*) was highest when the product was subjected to heating - cooling treatment; this was followed by treatment at 45 °C, ambient temperature and 4 ˚C in that order. The product containing ET20 showed the highest ∆E* value. The greatest total color difference after 1 month storage occurred with the heating - cooling treatment (7.6). Thus overall, the color of the emulsion remained stable as ∆E* was < 10 [21] and only slightly changed to lighter green compared with the initial color.

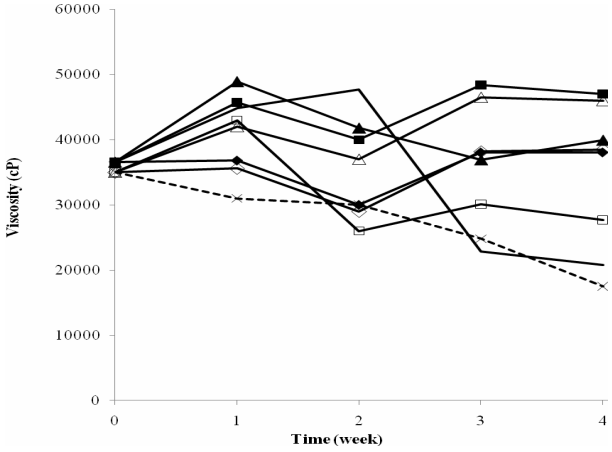

Fig 3: Viscosity of emulsion containing 3 % *P. amaryllifolius* leaf extract following storage at various conditions: Ambient Temp. ET20 (◊), 45 °C ET20 (□), 4 °C ET20 (∆), Heating-cooling ET20 (×), Ambient Temp. EP50 (♦), 45 °C EP50 (■), 4 °C EP50 (▲), heating-cooling, ET20 (—). ***Note:*** ET20 contains ethanol extract while EP50 contains ethanol/propylene glycol extract.

DISCUSSION

P. amaryllifolius leaf extracted in propylene glycol had higher DPPH radical-scavenging activity than that extracted in ethanol which may indicate that antioxidant active components in *P. amaryllifolius* are more soluble in a more polar propylene glycol solvent [22]. The propylene

Table 3: Color variation of emulsion products under various storage conditions

Colour Difference	Emulsion containing ET20											
	ΔL*				Δa*				Δb*			
	AT[a]	45˚C	4˚C	HC[b]	AT[a]	45˚C	4˚C	HC[b]	AT[a]	45˚C	4˚C	HC[b]
W0/W1	0.38	-2.49	-0.98	-4.08	0.73	0.77	0.09	0.48	-0.86	1.62	0.38	1.55
W0/W2	0.66	-4.08	-1.40	-4.51	1.30	1.17	0.28	0.90	-2.00	1.02	0.59	1.03
W0/W3	0.34	-3.80	-1.00	-5.42	1.51	1.64	0.52	1.37	-2.05	-0.15	0.44	0.45
W0/W4	0.94	-5.28	-1.41	-7.44	1.84	1.82	0.75	1.26	-3.21	-1.14	-0.23	0.48
Colour Difference	Emulsion containing EP50											
	ΔL*				Δa*				Δb*			
	AT[a]	45˚C	4˚C	HC[b]	AT[a]	45˚C	4˚C	HC[b]	AT[a].	45˚C	4˚C	HC[b]
W0/W1	1.00	-1.05	-1.21	-1.07	0.17	0.24	-0.01	0.18	-0.42	0.10	-0.28	0.09
W0/W2	0.61	-1.62	-1.61	-2.18	0.40	0.37	0.16	0.28	-0.11	0.33	0.25	-0.01
W0/W3	0.31	-2.03	-2.23	-5.64	0.79	0.70	0.35	0.39	-0.37	0.07	-0.19	-0.15
W0/W4	0.68	-1.88	-1.50	-5.77	0.61	0.42	0.06	0.18	-0.44	-0.09	-0.09	-0.64

[a] *AT = ambient temperature (28-32 °C)* , [b] *HC= heating-cooling cycle*
ΔL is the color difference between initial (W0) and subsequent week (Wt), (L^*_{wt} - L^*_{w0}); Δa* is the color difference between initial (W0) and subsequent weeks (Wt), (a^*_{wt} - a^*_{w0}); Δb* is the color difference between initial (W0) and subsequent week (Wt), (b^*_{wt} - b^*_{w0})*

glycol extract also showed higher content phenolic compounds and this can be linked to its higher DPPH activity [23].

Incorporation of the extract in a heterogeneous emulsion system resulted in a product with antioxidant characteristics. Topical emulsion systems generally consist of multiple phases in which lipid and water coexists with some emulsifiers [24]. However, it should be noted that the concentration of the extract in the linoleic acid emulsion system used was relatively high (approx. 9 %), and therefore further investigation in bulk oil-in-water emulsion system containing various levels of extract is required.

Propylene glycol extract may not be the suitable choice for topical application due to the fact that a high content in a formulation may result in an oily feeling. Hence, a concentrated mixture of ethanol/propylene glycol (1:1) extract was tested.

Rheological properties of emulsions are important not only for physical characterization but are also parameters indicating system quality. Thus, stability test was performed to ensure that the products meet the intended physical, chemical and performance characteristics when they stored under various conditions. It has been noted that tolerance of stability after 1 - 2 heating/cooling cycles is considered as: stable if the change in viscosity is < 10 %; acceptable, if the viscosity is higher than 10 % but not more than 20 %; and unstable if > 20 % [25]. The viscosity results obtained reveal that the developed products were stable. Moreover, the results are very useful for selecting optimal storage condition of products. Furthermore, the colour stability results obtained also indicate that the color of the products, which is mainly derived from the extract, was basically stable. The findings of this study are useful as they may aid in extending the use of *P. amaryllifolius* leaf in current traditional practice in foods [**Error! Bookmark not defined.**] to industrially produced topical products.

CONCLUSION

P. amaryllifolius extracted in propylene glycol solvent exhibited higher DPPH radical scavenging activity and total phenolic content than that extracted in ethanol, while the leaf demonstrated higher antioxidant activity than the root. The oil-in-water topical emulsion containing 3 % leaf extract possessed good stability under accelerated aging conditions. Thus pandan leaf extract is a potential suitable natural antioxidant, which can serve as an alternative to synthetic antioxidants used in topical emulsion. However, further work on the stability and efficacy of the extract in bulk emulsions is required in this regard.

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