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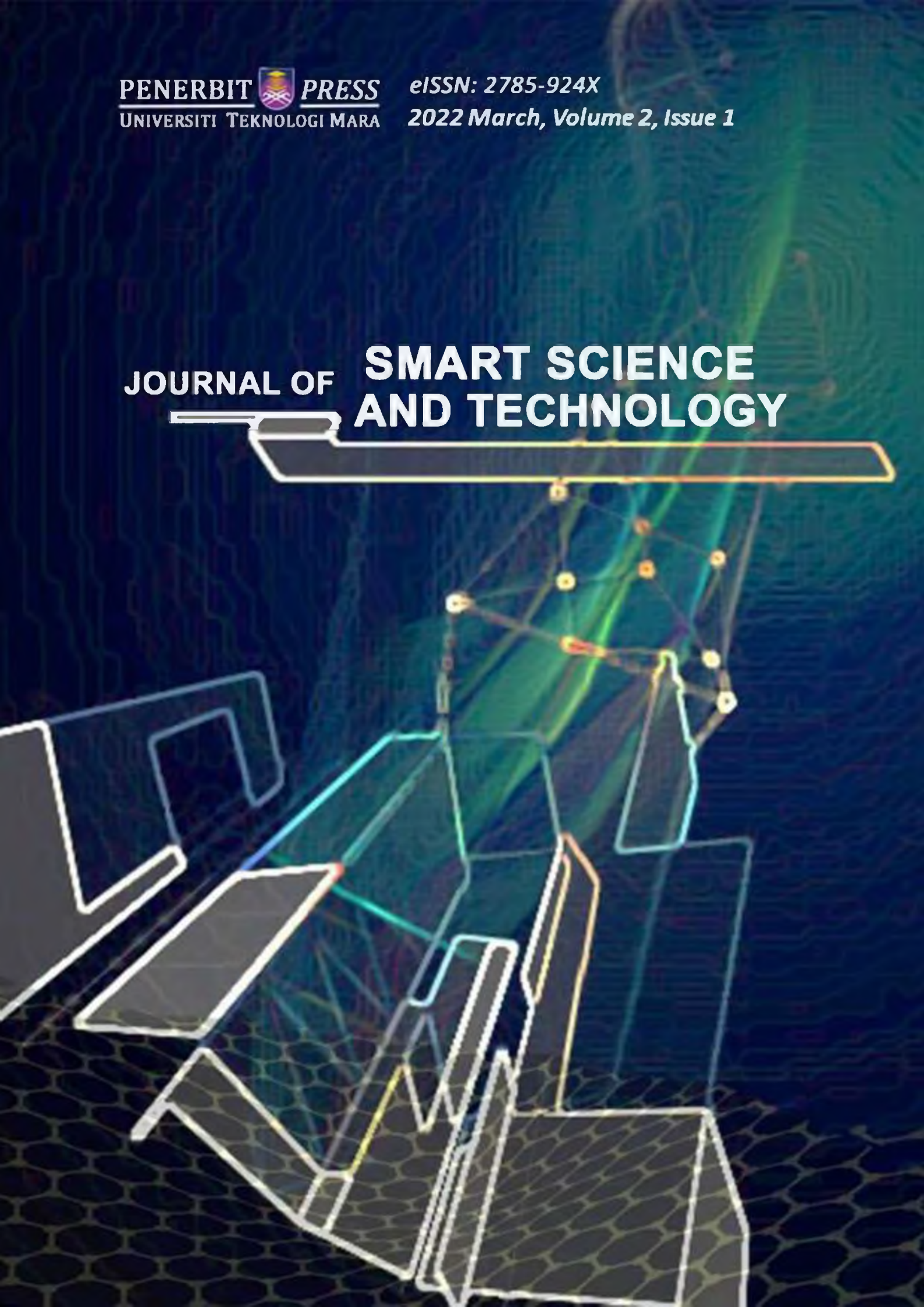

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In vitro Anti-ageing Activities of the Extracts of Low-Grade Pineapple and Lime Key from Sob Prab Cooperative Limited, Lampang, Thailand

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Abstract

In this study, the low-grade pineapple and lime key from Sob Prab Cooperative Limited, Lampang, Thailand were selected to investigate the anti-ageing activities. The pineapple (PA-J) and lime key juices (LM-J) were squeezed and then the pineapple and lime key residues were extracted by maceration in 95% (v/v) ethanol (PA-E and LM-E) and boiled in distilled water (PA-W and LM-W). The extraction yields from all extracts ranged from 14.58 to 23.01, with the presence of terpenoids as a phytochemical. For anti-ageing activities of all extracts, it was found that the LM-J extract showed the highest antioxidant activity including free radical scavenging, metal chelation and lipid peroxidation activities, whereas the PA-E extract exhibited the whitening effect including tyrosinase inhibition activity and anti-melanogenesis on B16F10 cells, and the stimulation of collagen biosynthesis on human dermal fibroblasts. In addition, all extracts did not show cytotoxicity on human dermal fibroblasts at 0.1 mg mL^{-1}. This study suggested that the PA-E and the LM extracts might be beneficial to be an active ingredient for anti-ageing cosmetics.

Keywords

Thai fruits; Cosmetics; Community; Wastes; Anti-ageing; Antioxidant

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1 Introduction

In the ageing process, dermal fibroblasts are incapable to produce collagen and elastin fibers properly, leading to a decrease in the collagen level, and stimulating the collagen to cross-link form, resulting in loss of skin elasticity[1]. Tyrosinase is a key regulating enzyme related to melanogenesis on melanocytes. Melanin has a variety of biological functions such as pigmentation of eyes, hairs, skin and also sun protection. However, the overproduction of melanin can cause signs of ageing such as darker skin, an increase in the number of dark

spots, and melasma[2]. Radical oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) and superoxide radical ($O_2^{\bullet-}$), which were stimulated by sunlight (UVA and UVB), can cause directly severe oxidative damage to the skin, resulting in reduced collagen biosynthesis as well as up-regulation of tyrosinase activity[3,4].

Presently, plant extracts have received much attention as an alternative source of ingredients for cosmetic products. Phytochemical constituents in plants such as flavonoids, phenolic compounds, saponins, tannins, xanthones, and alkaloids of several plants have justified their potential use for antioxidants, anti-aging, and anti-melanogenesis[5,6]. Several studies demonstrate the collagen biosynthesis and anti-melanogenesis of many natural plant extracts. Chutoprapat et al.[7] reported that the hulls of bambara groundnut (*Vigna subterranea*) extracted by maceration in 95% (v/v) ethanol shows the highest in the anti-melanogenesis on murine melanoma (B16F10) cells which was superior to that of kojic acid about 1.6-fold. The leave extracts from Star grass (*Hypoxis aurea* Lour.) demonstrate the collagen biosynthesis on dermal fibroblasts, tyrosinase inhibition on B16F10 cells, and anti-oxidant activities[8]. In addition, the ethanolic extracts of *Lespedeza cuneata* G. Don can be applied to a potential agent for the treatment of skin disorders by keeping skin tissue maintenance and regulating melanogenesis via inhibition of collagenase and elastase, and also inhibition of tyrosinase expression and activity[9].

Smooth Cayenne pineapple (*Ananus comosus* L.); and lime key (*Citrus aurantifolia* (Christm.) Swingle) are the major agricultural fruits from the community crop of the agriculturist members of Sob Prab Cooperative Limited, Lampang, Thailand. However, a number of the fruits are of low-grade quality each year. The value-added of these low-grade fruits such as new product development should be promoted by the Cooperative in supporting the members to increase the income and reduce the cost for disposing of the fruit wastes. The primary knowledge of these fruits before product development including biological and pharmaceutical activities should be investigated. Previous reports found that lime key juice and peel extract showed antioxidant and anti-microbial activities[10,11]. Lin et al.[12] reported that the essential oils from the lime key (*C. aurantifolia*) (LEO) exhibits potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging activity, and are effective as inhibitors of lipid peroxidation, which leads to reduced LDL (low-density lipoprotein) to counteract hyperlipidemia. The pineapple extracts demonstrate many biological activities including antioxidant, ani-tyrosinase and anti-melanogenesis[13,14]. Tacorin is a crude protein extracted from *A. comosus* stem which exhibits promise wound healing therapeutic agent via increasing the expression of transforming growth factor β (TGF-β) and reducing the expression of matrix metalloproteinase 2 (MMP-2)[15]. Vrianty et al.[13] found that the extract of *A. comosus* core (PEC) by 70% ethanol extraction exhibits antioxidant activity (IC_{50} of 87.46 μg mL^{-1}) and tyrosinase enzyme inhibition (IC_{50} of 62.27 μg mL^{-1}). Subsequently, Arshad et al.[14] found that 2% of methanolic extract from *A. comosus* on the cream products, significantly reduces the skin irritation or erythema, melanin pigment and sebum contents *in vivo*.

However, the study on anti-ageing activities including anti-melanogenesis and collagen biosynthesis of the pineapple and lime key is not found. This study aimed to investigate the phytochemical constituents and the *in vitro* anti-ageing potential efficiency and cytotoxicity in order to evaluate its potential application in cosmetic and cosmeceutical applications.

2 Method

2.1 Preparation and Extraction

The pineapple (PA) and lime key (LM) in this study were obtained from Sob Prab Cooperative Limited, Lampang, Thailand during January-March, 2019. The fruits

were washed with tap water, air dried, and cut into small pieces. The PA and LM fruits were squeezed by a juice extractor (J), the peel residues were dried at 60°C, and extracted by maceration in 95% ethanol with shaking for 2 days (E) and boiling in distilled water for 2 hours (W). After that, the extracts were filtered through a filter paper, evaporated by a rotary evaporator, and dried by a freeze dryer. Finally, the dried extracts were kept in an amber bottle at 4°C before being used. The extraction yields were calculated on the dry weight basis.

2.2 Phytochemical Screening

All extracts were screened for the phytochemical constituents, including glycoside, steroids, flavonoids, saponin, carotenoids, terpenoids, and coumarin by the previously described processes[16]. The qualitative results are expressed as (+) for the presence, and (−) for the absence of phytochemicals.

2.3 Antioxidant Activities

2.3.1 Free radical scavenging activity

The DPPH free radical scavenging activity of the extracts was assayed as described previously[7]. Briefly, 100 µL of the extracts and/or L-ascorbic acid (Sigma-Aldrich, USA) (positive control) at the various concentrations and 100 µL of DPPH (Sigma-Aldrich, USA) solution (0.2 mg mL^{-1} in ethanol) were added into each well of a 96-well microplate, and then incubated at room temperature for 30 minutes in dark room. The absorbances were measured at 570 nm by a microplate reader.

2.3.2 Metal chelating activity

The metal chelating activity of the extracts was assayed by the Ferrous ion chelating (FIC) method as described previously[7]. Briefly, 50 µL of the extracts and/or Ethylenediaminetetraacetic acid (EDTA) (Lobal Chemie, India) (positive control) at the various concentrations, 50 µL of 1 mg mL^{-1} iron (II) chloride ($FeCl_2$), and 50 µL of 1 mg mL^{-1} of ferrozine in 1% hydrochloric acid (HCl) (TCI, USA), were mixed into each well of a 96-well microplate. After that, the mixture was incubated at room temperature for 60 minutes in the dark room. The absorbance of the mixture was measured at 570 nm by a microplate reader.

2.3.3 Lipid peroxidation inhibition

The lipid peroxidation activity of the extracts was assayed by the Ferric thiocyanate (FTC) method as described previously[7]. Briefly, 50 µL of the extracts and/or α-tocopherol (Sigma-Aldrich, USA) (positive control) at various concentrations, 50 µL of linoleic acid in 50% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA), and 50 µL of 5 mM ammonium thiocyanate (NH_4SCN) (Sigma-Aldrich, USA) were added into each well of a 96-well microplate. Then, the mixture was added 50 µL of 5 mM NH_4SCN (Sigma-Aldrich, USA) and 50 µL of 2 mM $FeCl_2$, and incubated at 37°C for 60 minutes. The absorbances were measured at 450 nm by a microplate reader.

The inhibition percentages (%) of the DPPH, FIC and FCT methods were calculated as follows:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

where A_0 was the absorbance of the control and A_1 was the absorbance of the treatments.

The concentrations providing 50% scavenging (SC_{50} mg mL^{-1}), 50% chelation (MC_{50} mg mL^{-1}), and 50% peroxidation (LC_{50} mg mL^{-1}) were calculated from the graph plotted between % inhibition and the extract concentrations.

2.4 Tyrosinase Inhibition Activity

The tyrosinase inhibition activity of the extracts was modified dopachrome method as described previously[7]. Briefly, 50 µL of extracts and/or kojic acid as a positive control (Sigma-Aldrich, USA) at various concentrations, 50 µL of 0.1 mg mL^{-1} L-tyrosine, 50 µL of 200 U mL^{-1} mushroom tyrosinase (Sigma-Aldrich, USA) in 0.1 mM phosphate buffer (pH 6.8), and 50 µL of 0.1 mM phosphate buffer were added into each well of a 96-well microplate and then

incubated at 37°C for 60 minutes. The absorbances were measured at 570 nm by a microplate reader. The tyrosinase inhibition percentages (%) were calculated following Equation 1. The concentrations providing 50% inhibition (IC_{50} mg mL^{-1}) were calculated from the graph plotted between % tyrosinase inhibition activity and the extract concentrations.

2.5 In vitro Anti-ageing Activities by Cell Cultures

The murine melanomas (B16F10) and human dermal fibroblasts were used for the investigation of anti-melanogenesis and collagen biosynthesis. These cells were obtained from American Type Culture Collection (ATCC), Virginia, USA. The cells were maintained under the standard conditions at 37°C under a 5% CO_2 atmosphere in the Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU mL^{-1} of penicillin and 100 mg mL^{-1} of streptomycin.

2.5.1 Anti-melanogenesis activity on B16F10 murine melanomas

Anti-melanogenesis on B16F10 murine melanomas was examined as described previously[7]. Briefly, the B16F10 cells (2.5×10^5 cells) were seeded into 6-well plates and incubated at 37°C in a CO_2 incubator for 24 hours. After that, the extracts and/or kojic acid at the suitable concentration were co-treated with 10 µM alpha-Melanocyte stimulating hormone (α-MSH) (Sigma-Aldrich, USA) and incubated at the same condition for 72 hours. After incubation, the medium was removed, and then the cells were washed with 1X Phosphate buffer saline (PBS), dissolved in 500 µL of 10% (w/v) NaOH, and further incubated at 60°C for 1 hour. The absorbance was measured at 450 nm by a microplate reader. The percentages of the anti-melanogenesis were calculated as follows:

$$\% \text{ Anti-melanogenesis} = 100 - [(M_t/M_c) \times 100] \quad (2)$$

where, M_t was the melanin content of the samples, and M_c was the melanin content of the control.

2.5.2 Collagen biosynthesis stimulation on human skin fibroblasts

The collagen biosynthesis was performed according to the previously described processes[7]. Briefly, the human skin fibroblasts cells (5×10^5 cells) were seeded into 6-well plates and incubated at 37°C in a CO_2 incubator for 24 hours. After that, the extracts and/or ascorbic acid at the suitable concentration were added and incubated at the same condition for 24 hours. Then, the cells were dyed 1 mL of 0.1% (w/v) Sirius red solution in saturated picric acid for 1 hr. The dye was removed, and the cells were washed with 1 mL of 10 mM HCl for 5 times. The cells were dissolved with 0.1 M NaOH. The absorbances were measured at 540 nm by a microplate reader. The percentage of the collagen content was calculated as follows:

$$\%\text{Collagen biosynthesis} = (C_t/C_c) \times 100 \quad (3)$$

where, C_t was the collagen content of the samples-treated, and C_c was the collagen content of the control.

2.6 Cytotoxicity Test

All extracts were tested for cytotoxicity on human skin fibroblasts by the MTT assay as described previously[7]. The human skin fibroblasts (1×10^4 cells) were seeded into a 96-well plate, and incubated at 37°C under a 5% CO_2 atmosphere for 24 hours. After that, the cells were treated with the extracts at various concentrations and incubated at the same condition for 24 hours. Then, the medium was removed, and the cells were added 100 µL of 0.5 mg mL^{-1} MTT solution, and further incubated for 4 hours. After incubation, the blue-violet crystals were dissolved with 100 µL of DMSO, and gently mixed for 15 minutes. The absorbances were measured at 550 nm by a microplate reader. The percentage of cell viability was calculated by comparison

to 100% viability of untreated cells (control) as the following:

$$\% \text{ Cell viability} = [A_S/A_C] \times 100 \quad (4)$$

where, A_s is absorbance at 550 nm of treated cells and A_C is absorbance at 550 nm of untreated cells.

2.7 Statistical Analysis

All determinations were performed in triplicate (n = 3). Data were presented as mean ± standard deviation (S.D.). Statistical differences between the control and treated groups were tested by ANOVA with the Tukey test.

3 Results

3.1 Extraction Yields and Phytochemical Constituents of the Extracts from Pineapple and Lime Key

The pineapple (PA-J) and Lime key juices (LM-J) were squeezed and freeze dried, and then, pineapple and lime key residues were extracted by maceration in ethanol (PA-E and LM-E) and boiled in distilled water (PA-W and LM-W). The extraction yields of all pineapple and lime key extracts ranged from 14.58% to 23.01%. The most phytochemicals found in those extracts were terpenoids and coumarin (Table 1).

3.2 Anti-oxidant Activities of the Extracts from Pineapple and Lime Key

Table 2 demonstrated that all extracts exhibited the three antioxidant activities including free radical scavenging activity (SC_{50}), metal chelation (MC_{50}), and lipid peroxidation inhibition (LC_{50}). The highest DPPH radical scavenging activity was found in the Lime key juice (LM-J) with the SC_{50} of 0.034 ± 0.006 mg mL^{-1}, which were superior to L-ascorbic acid (SC_{50} of 0.046 ± 0.005 mg mL^{-1}) ($p < 0.05$). Subsequently, the extracts from the lime key juice (LM-J) with the MC_{50} of 0.432 ± 0.044 mg mL^{-1}, and the lime key peel residues by boiling in distilled water (LM-W) with the MC_{50} of 0.459 ± 0.038 mg mL^{-1} showed the highest metal chelating activity, whereas, EDTA as a positive control gave MC_{50} of 0.025 ± 0.002 mg mL^{-1}, while the pineapple peel residues extracted by boiling in distilled water (PA-W) with the LC_{50} of 0.267 ± 0.030 mg mL^{-1} gave the highest lipid peroxidation inhibition, which was comparable to α-tocopherol (LC_{50} of 0.202 ± 0.011 mg mL^{-1}) ($p < 0.05$).

Table 1. The extraction yields and phytochemical constituents of the extracts from pineapple and lime key.

Samples	% Yields	Phytochemical constituents						
		Glycosides	Steroids	Flavonoids	Saponins	Carotenoids	Terpenoids	Coumarins
LM-J	23.01	−	−	−	−	−	+	+
LM-W	14.83	+	+	−	−	−	+	+
LM-E	14.58	−	−	+	+	−	+	+
PA-J	17.12	+	+	−	−	−	+	−
PA-W	15.18	+	−	+	−	−	+	+
PA-E	15.46	−	−	+	+	+	+	+

Note: The qualitative results are expressed as (+) for the presence and (−) for the absence of phytochemicals. PA and LM are pineapple and lime key respectively. J is the extraction by a squeezer. E is the extraction by maceration in 95% ethanol with shaking. W is the extraction by boiling in distilled water.

Table 2. Antioxidant activities of the extracts from pineapple and lime key.

Samples	Antioxidant activities		
	Free radical scavenging activity [SC_{50} (mg mL^{-1})]	Metal chelation [MC_{50} (mg mL^{-1})]	Lipid peroxidation inhibition [LC_{50} (mg mL^{-1})]
LM-J	0.034 ± 0.006^{a}	0.432 ± 0.044^{B}	0.405 ± 0.017^{iii}
LM-W	0.056 ± 0.001^{c}	0.459 ± 0.038^{B}	0.352 ± 0.085^{ii}
LM-E	0.079 ± 0.003^{e}	2.074 ± 0.280^{D}	0.404 ± 0.016^{iii}
PA-J	0.055 ± 0.015^{c}	1.450 ± 0.310^{C}	0.338 ± 0.026^{ii}
PA-W	0.063 ± 0.006^{d}	1.312 ± 0.095^{C}	0.267 ± 0.030^{i}
PA-E	0.228 ± 0.005^{f}	2.111 ± 0.101^{D}	0.439 ± 0.050^{iii}
L-ascorbic acid	0.046 ± 0.005^{b}	N.D.	N.D.
EDTA	N.D.	0.025 ± 0.002^{A}	N.D.
α-tocopherol	N.D.	N.D.	0.202 ± 0.011^{i}

Note: The data are expressed as mean ± SD and the different superscript letters ($^{a-f}$ for SC_{50}, $^{A-D}$ for MC_{50}, and $^{i-iii}$ for LC_{50}) in the column indicate significant differences by Tukey test at $p < 0.05$. PA and LM are pineapple and lime key respectively. J is the extraction by a squeezer. E is the extraction by maceration in 95% ethanol with shaking. W is the extraction by boiling in distilled water.

3.3 Tyrosinase Inhibition Activity of the Extracts from Pineapple and Lime Key

In Figure 1, the results found that the lime key juice (LM-J), the pineapple juice (PA-J), and the pineapple peel residues extracted by boiling in distilled water (PA-W) extracts with the 0.05 ± 0.03 mg mL^{-1}, 0.06 ± 0.01 mg mL^{-1}, and 0.02 ± 0.02 mg mL^{-1} respectively, exhibited the highest tyrosinase inhibition activity which were more potent than kojic acid (IC_{50} of 0.02 ± 0.01 mg mL^{-1}) ($p < 0.05$).

3.4 In vitro Anti-ageing Activities of the Extracts from Pineapple and Lime Key

In vitro anti-ageing activities of the extracts from pineapple and lime key including anti-melanogenesis on B16F10 cells and collagen biosynthesis stimulation on human dermal were investigated. The proper concentration of the pineapple and lime key extracts, and the standards (kojic acid and L-ascorbic acid) was 0.1 mg mL^{-1} which exhibited no cytotoxicity on both cells (data did not show).

All extracts showed anti-melanogenesis on B16F10 cells after being treated for 72 hours. The lime key juice (LM-J), the lime key peel residues extracted by boiling in distilled water (LM-W), and the pineapple peel residues extracted by maceration with ethanol (PA-E) extracts inhibited the highest melanin production of $24.98 \pm 1.09\%$, $26.25 \pm 1.28\%$, and $27.38 \pm 1.14\%$ respectively whereas kojic acid as a positive control gave $34.64 \pm 1.43\%$ as shown in Figure 2.

The collagen stimulating effect of the extracts from pineapple and lime key were investigated by Picro Sirius Red Stain. Figure 3 exhibited the collagen biosynthesis stimulation on human dermal fibroblasts of all extracts after treated for 24 hours. The results found that all extracts could stimulate collagen biosynthesis except the pineapple peel residues extracted by boiling in distilled water (PA-W) extract. The pineapple peel residues extracted by maceration in ethanol (PA-E) especially demonstrated the highest collagen biosynthesis on human dermal fibroblast cells ($9.00 \pm 1.21\%$), which was lower than that of L-ascorbic acid ($12.00 \pm 1.81\%$).

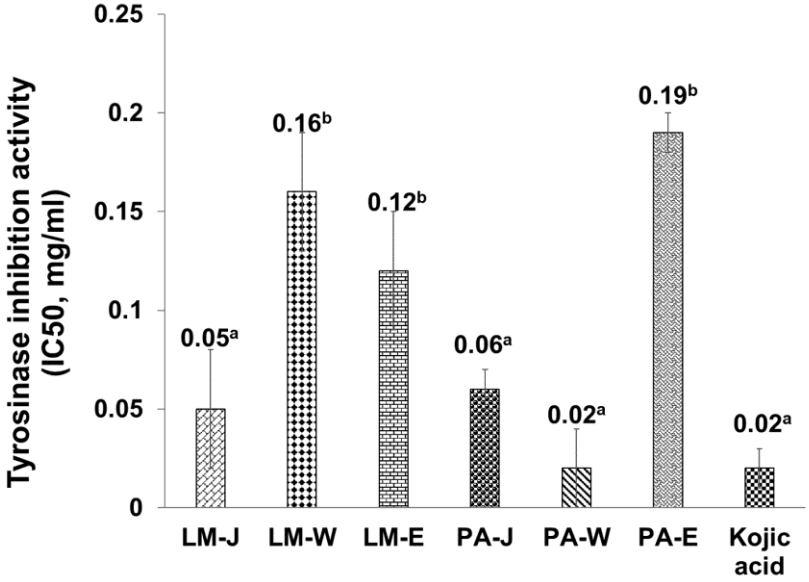

Figure 1. Tyrosinase inhibition activity of the extracts from pineapple and lime key. Superscript asterisks ($^{a-b}$) in the column indicate significant differences by the Tukey test at $p < 0.05$. PA and LM are pineapple and lime key respectively. J is the extraction by a squeezer. E is the extraction by maceration in 95% ethanol with shaking. W is the extraction by boiling in distilled water.

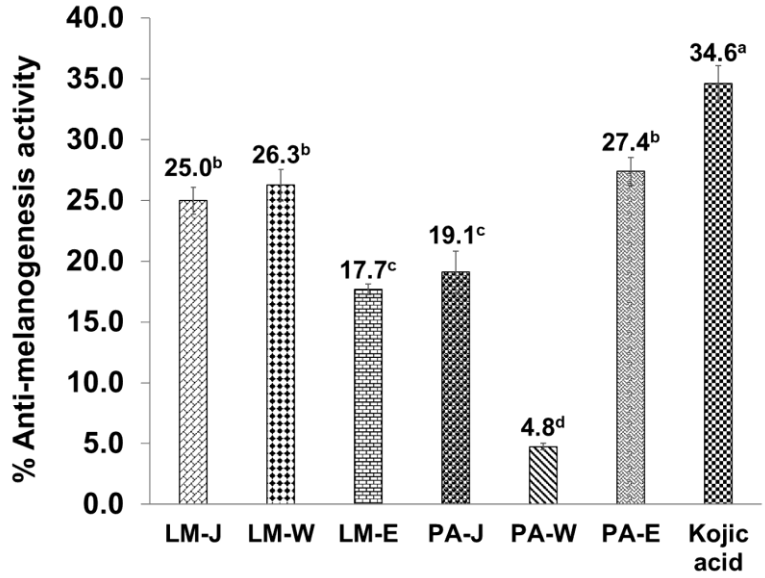

Figure 2. Anti-melanogenesis on B16F10 cells of the extracts from pineapple and lime key at concentration of 0.1 mg mL^{-1}. Superscript asterisks ($^{a-d}$) in the column indicate significant differences by the Tukey test at $p < 0.05$. PA and LM are pineapple and lime key respectively. J is the extraction by a squeezer. E is the extraction by maceration in 95% ethanol with shaking. W is the extraction by boiling in distilled water.

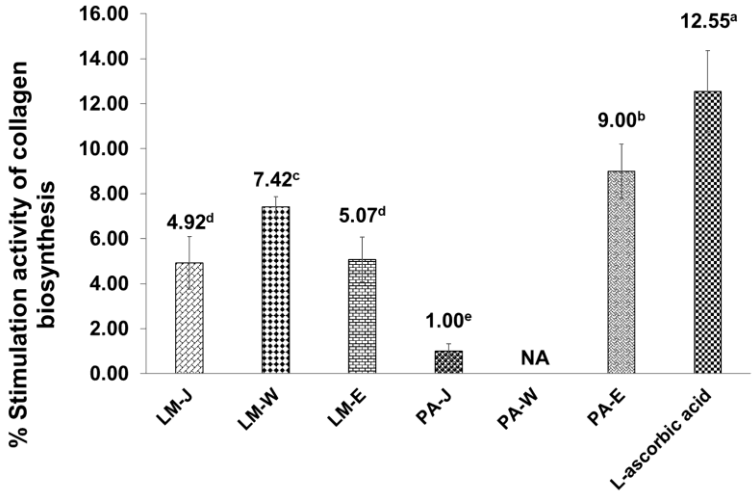

Figure 3. Collagen biosynthesis stimulation on human skin fibroblasts of the extracts from pineapple and lime key at concentration of 0.1 mg mL^{-1}. The data are expressed as mean ± SD and different superscript asterisks ($^{a-e}$) in the column indicate significant differences by Tukey test at $p < 0.05$. PA and LM are pineapple and lime key respectively. J is the extraction by a squeezer. E is the extraction by maceration in 95% ethanol with shaking. W is the extraction by boiling in distilled water.

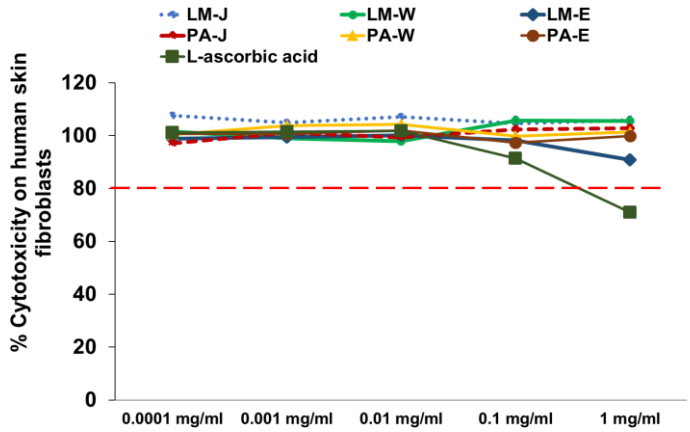

Figure 4. Cytotoxicity on human dermal fibroblasts of the extracts from pineapple and lime key. PA and LM are pineapple and lime key respectively. J is the extraction by a squeezer. E is the extraction by maceration in 95% ethanol with shaking. W is the extraction by boiling in distilled water.

3.5 Cytotoxicity on Human Dermal Fibroblasts of the Extracts from Pineapple and Lime Key

The cytotoxicity on human dermal fibroblats of the extracts from pineapple and lime keys was investigated for safety evaluation. The cell viability of the extracts was compared to the control (untreated). The results showed that all pineapple and lime key extracts at the concentration of 1.0 mg mL^{-1} and below did not show cytotoxicity on human dermal fibroblast cells after treated for 24 hours, since the cell viability was higher than 80%. The results met the criteria of Nemati et al.[17].

4 Discussion

Pineapple (*A. comosus*) and lime key (*C. aurantifolia*) are fruit crops which have great economic importance in Asia and South American cuisines, and they can be consumed fresh or cooked[18,19]. Previously, Ali et al.[20] reported that lemon juice has

several important chemical components, including citric acid, and Vitamin C, and it contains high concentrations of polyphenols, flavonoids, coumarins, and γ-terpinene, which lend to their antioxidant. Likewise, the pineapple extracts were reported to contain phytochemical constituents such as vitamin A and C, flavonoids, saponins, steroids, and triterpenoids, as well as showing antioxidant, antimicrobial, anti-diabetes, and anti-inflammatory activities[13,21]. In this study, terpenoids are found to contain the most phytochemical constituent in the extracts of pineapple and lime key that are the low-grade fruits from the community crop from Sob Prab Cooperative Limited, Lampang, Thailand. In addition, the ethanolic extraction demonstrates the presence of flavonoids, saponins and carotenoids whereas glycosides are found in the extracts by boiling in distilled water. The differences in the extraction process, solvent, temperature, and nature of plants may affect the extraction yields and their phytochemicals[22,23]. The phytochemical constituents found in the pineapple and lime key extracts might be the response for the anti-ageing activities.

Several natural plants which have been used in traditional medicine have been screened for phytochemicals, bioactive compounds, and pharmacological properties including anti-ageing activity to find out new promising natural ingredients for cosmetic applications. Anti-ageing activities including antioxidant, anti-melanogenesis, tyrosinase inhibition activity and collagen biosynthesis of natural plants have been worldwide reported. Radical oxygen species (ROS) are responsible for the age-associated damage at the cellular and tissue levels[24]. In this study, the lime key juice (LM-J) extract shows the greatest antioxidant activities since it demonstrates the highest inhibition of the three mechanisms, including free radical scavenging, metal chelation, and lipid peroxidation. This might be due to the levels of terpenoids and coumarins found in the LM extract, as well as the extraction process by squeezer and freeze dryer do not destroy the heat-labile substances. Similarly, Oikeh et al.[10] showed that the lime key juice concentrate, its content alkaloids, phenols, flavonoids, steroids, terpenoids, and saponins, especially reducing sugar and cardiac glycosides leading to antioxidant activities of 6.25 $\pm$ 0.06% on inhibitions of the DPPH-radical at 1 mg mL^{-1} lime key juice and 173.25 $\pm$ 0.25 μmol L^{-1} Fe^{2+} g^{-1} of the extract on FRAP. Moreover, Rungpanit and Pirak[25] reported that the ethanolic extracts of tangerine and lime key peel at ratio 1:1 for 90 minutes, were the potential rich source of phenolic compound, and natural antioxidant with the DPPH-radical scavenging, ABTS and FRAP method of 82.75 $\pm$ 0.06%, 3.14 $\pm$ 0.02 μgGAE mL^{-1} and 0.73 $\pm$ 0.01 μg mL^{-1} Fe^{2+} respectively.

Senol et al.[26] reported that the extracts of *Citrus species* from Turkey demonstrate the metal-chelating capacity at 500 μg mL^{-1}, and also the leaf and peel extracts of *C. aurantium* had effective metal chelating activity, H donor ability, reducing power ability, superoxide anion radical, nitric oxide radical and hydroxyl radical scavenging activities[27].

It is well known that tyrosinase plays a critical role in the melanogenic pathway since it can promote the production of melanin by assessing the hydroxylation of L-tyrosine to L-DOPA (monophenolase) and the oxidation of L-DOPA to DOPA-quinone (diphenolase)[28]. Therefore, the effect on anti-tyrosinase activities might become the most prominent approach for reduced melanin production on the anti-melanogenesis process. Several plants that have a tyrosinase inhibition and anti-melanogenesis properties have been used for cosmetic purpose, particularly in skin whitening[8,29,30].

The pineapple and lime key extracts, especially the lime key juice (LM-J) extracts can strongly inhibit the anti-melanogenesis on B16F10 cells as well as the mushroom tyrosinase activity, which are superior to kojic acid, a tyrosinase inhibitor. These results are close to the previous report showing the tyrosinase inhibitor in the peel-off mask of lime key peel ethanolic extract results obtained about 18.86%[22]. Additionally, the 70% ethanolic extract of pineapple core and peel have demonstrated tyrosinase

inhibition potential with IC_{50} values of 62.27 and 56.93 ± 5.46 μg mL^{-1} respectively[13,20]. There are many reports demonstrating that terpenoids, coumarins, and flavonoids can affect tyrosinase inhibition activity and anti-melanogenesis.

Liu-Smith et al.[31] described that Flavonoids such as genistein, apigenin, Kaempferol exhibit melanogenic or anti-melanogenic effects are mainly via α-MSH-stimulated microphthalmia-associated transcription factor (MITF) and/or the melanogenesis enzymes tyrosinase, DCT2 or TYRP-1.

The auraptene, which is a geranyloxy coumarin found in the Ferula species, has the anti-melanogenic activity through direct tyrosinase inhibition and could modulate the expression of major melanogenesis-related genes including tyrosinase, tyrosinase-related protein 1, and dopachrome tautomerase in the murine melanoma cell line[32].

Kamauchi et al.[33] also reported that coumarin derivatives by chemical synthesis showed tyrosinase inhibition and anti-melanogenesis activity on B16 melanoma cells. During ageing, the collagen biosynthesis in the skin is reduced and higher collagenases level, leading to skin wrinkling and loss of elasticity[23]. Many plant extracts can stimulate the collagen biosynthesis on skin cells, which can be used for reducing wrinkles and increasing tissue repairs. The ethanolic extracts of Star grass and the aqueous extract of straw mushroom (*Volvariella volvacea*) exhibited stimulation of collagen biosynthesis on human skin fibroblasts, which was superior to vitamin C[8,34]. This may be another important mechanism to evaluate the possibility in using plant extracts as an ingredient in anti-ageing cosmetic and food supplements.

Furthermore, this report may be the first to report the effect of the pineapple and lime key extracts on the stimulation of collagen biosynthesis on human skin fibroblasts. It is revealed that the pineapple peel residues extracted by maceration in ethanol (PA-E) extract can stimulate collagen biosynthesis more than the other extracts. This might be due to the phytochemicals such as terpenoids, coumarins, flavonoids, saponins especially carotenoids responsible for the highest activity on the extracts. Asiatic acid and asiaticoside are the most active of the triterpenes from *Centella asiatica* which can stimulate collagen synthesis at low doses[35]. Darawsha et al.[36] reported that carotenoids, polyphenols, and estradiol could protect dermal fibroblasts from oxidative stress-induced damage through a reduction in ROS levels. It also suggests that a natural carotenoid-rich extract can prevent the ageing-related collagen I degradation in the dermis and improve the extracellular matrix[37]. Additionally, the pineapple has the ability to stimulate wound healing via increased expression of TGF-β and reduced expression of MMP-2 to prevent the degradation of collagen[15,18].

The different cytotoxicity of the extracts depends on a specific plant extract and the cell types which affect the different responses[38]. All extracts from pineapple and lime key have no cytotoxicity on human dermal fibroblasts leading to indicate no toxicity for skin application, which would be beneficial to be developed as cosmetics and cosmeceuticals.

The Pearson's correlations of the anti-ageing activities of the extracts from pineapple and lime key are shown in Table 3, and the correlation coefficient values (R) are described as weak ($R = \pm 0.00$ to ± 0.49), moderate ($R = \pm 0.50$ to ± 0.79) or strong ($R = \pm 0.80$ to ± 1.00)[39]. Our study revealed that the Pearson's correlations of the *in vitro* anti-ageing activity of the pineapple and lime key extracts between the collagen biosynthesis (CM) and anti-melanogenesis (AM) with the R of $+0.835$ were significantly classified as high positive correlation ($p < 0.05$), which means if the collagen biosynthesis stimulation of the extracts is increasing, the anti-melanogenesis would be dramatically increased as well. On the other hand, the R of the lipid peroxidation (LC) between the metal chelation (MC); and tyrosinase inhibition (IC) were classified to be having moderate positive relationships.

Although, the correlations of most anti-ageing activities of the extracts were a

positive relationship, the negative strong relationships could have appeared especially between the AM and the IC with the *R* of −0.873. Generally, the cellular tyrosinase is a key enzyme of melanogenesis. This study however indicates that the mushroom tyrosinase inhibition activity did not relate to the *in vitro* anti-melanogenesis on B16F10 cells. It has been reported that the water extract of *P. atlantica* subsp. mutica is significantly against mushroom tyrosinase activity but cannot inhibit cellular tyrosinase activity. The butanol extract shows mushroom tyrosinase inhibition but not for anti-melanogenesis[40,41].

Table 3. The Pearson's Correlation (R^2) of the anti-ageing activities of the extracts from pineapple and lime key.

Anti-ageing activities	The Pearson's Correlation (R^2)					
	SC	MC	LC	IC	AM	CB
SC	-	0.497	0.478	0.114	0.241	−0.044
MC	0.497	-	0.727	0.174	0.140	−0.074
LC	0.478	0.727	-	0.789	−0.503	−0.583
IC	0.114	0.174	0.789	-	−0.873*	−0.795
AM	0.241	0.140	−0.503	−0.873*	-	0.835*
CB	−0.044	−0.074	−0.583	−0.795	0.835*	-

Note**:** *Correlation is significant at the 0.05 level (2-tailed). Free radical scavenging activity (SC), Metal chelating activity (MC), Lipid peroxidation inhibition (LC), Tyrosinase inhibition activity (IC) are calculated from $1/SC_{50}$ (mL mg^{-1}), $1/MC_{50}$ (mL mg^{-1}), $1/LC_{50}$ (mL mg^{-1}) and $1/IC_{50}$ (mL mg^{-1}), respectively. Anti-melanogenesis activity (AM) and Collagen biosynthesis stimulation (CB) are the % collagen biosynthesis stimulation and the % anti-melanogenesis activity.

Moreover, the suppression of melanogenesis on cell culture might not be only from the inhibition of cellular tyrosinase, the other mechanisms via down-regulation of MITF, and tyrosinase-related protein-1 & 2 (TRP 1 & 2) might respond for *in vitro* anti-melanogenesis. On the other hand, the correlations between the anti-ageing activities and the pineapple and lime key extracts on this research works have small sample sizes (n = 6) which affect the inference and prediction of relationships. However, there is a very common situation in day-to-day data analysis. Therefore, the increasing of sample size would be better for clearer explanation of the relationships between biological activity including the anti-ageing activities and natural extracts[42].

5 Conclusion

The low-grade pineapples and lime keys are the main problems after cultivation for many crops in Thailand including the community crop from Sob Prab Cooperative Limited, Lampang Province. The pharmaceutical and biological activities of these two fruits would be beneficial to promote the value-added and alternative utilization such as product development. Overall, our results highlight that the pineapple peel residues extracted by maceration in ethanol (PA-E) extracts and the Lime key juice (LM-J) extract demonstrate the excellent antioxidant and tyrosinase inhibition activities as well as the *in vitro* anti-ageing activities on cell-based assays including anti-melanogenesis and collagen biosynthesis stimulation. This suggested that the PA-E and LM-J extracts have a promise to be further developed as anti-ageing agents in cosmetic, cosmeceutical, and food supplement applications.

Conflict of Interest

No conflict of interest.

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