

Phytochemical and Antioxidant Properties of *Manilkara zapota* (L.) P Royen Fruit Extracts and its Formulation for Cosmeceutical Application

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ABSTRACT

Manilkara zapota (L.) P fruit extracts were investigated for its bioactive compounds and their potential of natural antioxidant properties for cosmeceutical application. In vitro antioxidant activity of hexane, ethyl acetate, and ethanol extracts were evaluated by 1,1-diphenyl-picrylhydrazyl (DPPH) radical scavenging activity, β -carotene bleaching activity assays (BCB) and oxygen radical absorbance capacity (ORAC) methods. Total phenolic (TPC), total flavonoid (TFC) and total tannin (TT) of the extracts were determined. Antioxidant activity by DPPH scavenging activity has a good correlation with phenolic and flavonoid content $R^2=0.9905$ and $R^2=0.9924$. Whereas, no correlation was shown between ORAC and TPC values ($R^2<0.1$). Liquid chromatography mass spectrometry (LCMS) was used to analyze the amount of targeted phenolic and fatty acids in EtPE, EaPE and ethyl acetate seed extract (EaSE). EaPE and EtPE extracts demonstrated strong antioxidant activity than HSE ($3.09 \pm 0.08 \mu\text{g/mL}$, $2.57 \pm 0.18 \mu\text{g/mL}$ and $7.54 \pm 0.17 \mu\text{g/mL}$ ($p<0.05$), respectively). However, EtPE extract was chosen to be formulated and studied as ethanol is much cheaper than ethyl acetate as solvent for extraction. The formulation exhibited non-toxic property and thus safe to be explored in cosmeceutical application. .

Keywords: Antioxidant activity, Phytochemical, *Manilkara zapota*, Cytotoxicity

INTRODUCTION

Skin is the largest and the most important organ as protective barrier from oxidative injury caused by free radical. Free radical such as reactive oxygen species (ROS) is very harmful which are produced by condition of sun exposure, air pollution, ionization radiation and smoking. ROS are proficient in oxidizing lipids, proteins and DNA foremost to serious skin disorder including high pigmentation, premature ageing and skin cancer. While, the sign of ageing that normally found such as wrinkles, sagging and dryness skin. There are several approaches reported in protecting against the degenerative effects of ROS and among of them is through high consumption of antioxidants [1].

Plant based antioxidants are more interest than synthetic antioxidants due to their relatively nontoxic and wide acceptance by consumers [2]. The potent antioxidants such as vitamin C and Vitamin E can be found naturally in fruit and vegetables (apple, banana, broccoli, garlic, orange juice, green tea, olive oil, palm oil, and sun flower oil). Fruits and vegetables are rich in phytochemicals with medicinal properties such as antioxidants [3], anti-inflammatory [4], anti-cancer and anti-microbial [5]. The entire part of fruits (pulp, peel and seed) is rich in bioactive compounds, such as phenolic constituents, carotenoids, vitamins and dietary fiber [6].

Polyphenolic constituents are secondary metabolites and easily destructed with environment stress that responsible many cure and incurable diseases. Polyphenolic constituents can be classified into water soluble (phenolic acid, flavonoids and quinones) and water in-soluble compounds (condensed tannin and lignin). Some restriction of polyphenolic compounds are i) fast release, ii) low solubility, iii) low permeation, iv) low bioavailability and v) easy destruction against environment stress [7]. However, they are very effective in preventing radical chain reactions, delaying or inhibiting oxidation process and retarding lipid peroxidation process affected by reactive oxygen species (ROS) [8]. Therefore, the aim of this research has been focused toward exploring natural antioxidants from plant sources.

Manilkara zapota (L.) P Royen fruit can be a good source of nutrient, containing bioactive polyphenolic compounds [9-11]. *M. zapota* also known as the sapodilla belongs to Sapotaceae family and is one of the major crops in India, Southern Mexico, Caribbean and Central America as well as in South East Asia. *M. zapota* fruit is very popular in Malaysia, Thailand, Singapore, Cambodia and Indonesia with a pleasant sweet flavor is usually used in the beverage industry. The tree is evergreen which grows in wide range of climatic conditions and all tropical lands and in some areas grown in plantation. *M. zapota* shows medicinal properties such as antioxidant activity [9], anti-inflammatory [12], anti-bacterial [5] and anti-tumor [13]. It is mostly used in the beverage industry. However, details on natural antioxidant analysis of *M. zapota* fruit extract for cosmeceutical application are limited. No research on the cytotoxicity study of the *M. zapota* extract in cosmeceutical formulation was ever reported.

Therefore, in this work, the polyphenolic, fatty acid and volatile compounds of *M. zapota* fruit extracts in different solvent were studied. The total phenolic content (TPC), total tannin content (TT) and total flavonoid (TFC) were evaluated. The *M. zapota* in terms of antioxidant properties was formulated in nanoemulsion system and the cytotoxicity was evaluated.

MATERIALS AND METHODS

Chemicals

Hexane, ethyl acetate, ethanol, Folin-Ciocalteu reagent, gallic acid, 1,1-diphenyl-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), linoleic acid, rutin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 2,2-Azobis (2-methyl propionamide) dihydrochloride (AAPH), fluorescein sodium salt, β -carotene and alpha tocopherol were obtained from Sigma-Aldrich (Germany). Sodium carbonate (Na_2CO_3), polyvinylpyrrolidone (PVPP), aluminum chloride (AlCl_3), acetonitrile and Tween 80 were purchased from Merck (Darmstadt, Germany). All solvents and reagents were of analytical or high performance liquid chromatography (HPLC) grade.

Plant samples

M. zapota fruits were obtained from TESCO supermarket in Selangor, Malaysia. The fruits were kept at room temperature, cleaned and washed. The seeds were collected and separated. The peel was removed and the pulp was sliced into small and thin portions. The pulp, peel and seeds were dried under sun shade. Then, they were ground separately using a blender. The grounded pulp, peel and seed were soaked separately in hexane at room temperature for 24 h and were replaced with new hexane for three days. These procedures were repeated by using ethyl acetate and ethanol as solvents successively.

Extraction of samples

The extraction procedure was carried out according to method of Jahan *et al.* [8] with some modifications. The process was repeated 3 times continuously for 3 days (for each solvent) at sample to solvent ratio (hexane, ethyl acetate and ethanol) of 1:10 (w/v). The supernatant was filtered through Whatman filter paper No. 1 and concentrated by a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) at 40°C and stored at -20°C for further analysis. The percentage yield was expressed as a mass of the obtained extract relative to the mass of the dried matter of the initial sample used, as described from equation 1.

$$\text{Extraction yield(\%)} = \frac{\text{Extract(g)}}{\text{Dried Extract(g)}} \times 100 \quad (1)$$

Phytochemical analysis

Determination of total phenolic content (TPC)

Total phenolic compounds were determined using Folin-Ciocalteu method [14]. This method is created on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue solution. *M. zapota* fruit extracts (1 mg) were taken in a test tube and dissolved in methanol (1 mg/mL). Different concentrations (0.05, 0.10, 0.15, 0.25, 0.50, 1.0 mg/mL) of gallic acid as standards were prepared using a serial dilution technique. *M. zapota* fruit extract (20 μL) was taken in a separate test tube and mixed with the Folin-Ciocalteu reagent (100 μL) and deionized water (1.58 mL). The samples were mixed well and kept in a dark place for 30 min. Then, 7.5% of sodium carbonate (300 μL) was added to the sample and mixed. Again the test tubes were kept in the dark to complete the reaction for 2 h. The absorbance was measured by using a UV spectrophotometer (UV-1601 Shimadzu spectrophotometer, Japan) at 765

nm. The determination of total phenols in different solvents was carried out in triplicate and the average was then calculated. The phenolic content were expressed as mg gallic acid equivalent to 100 g of dry weight.

Determination of total tannin (TT)

Tannin content in each sample was determined using insoluble polyvinyl-polyrrolidone (PVPP), which binds tannins. Briefly, 1 mg of extract dissolved in methanol (1 mg/ml), in which the total phenolics were determined, was mixed with 100 mg PVPP, vortexed and kept for 15 min at 4°C and centrifuged for 10 min at 3000 rpm. In the clear supernatant the non-tannin phenolics were determined the same way as the total phenolics. Every concentration of non-tannin (20 µL) was taken in a separate test tube and mixed with the Folin-Ciocalteu reagent (100 µL) and deionized water (1.58 mL). The samples were mixed well and kept in a dark place for 30 min. Then 7.5% of sodium carbonate (300 µL) was added to the sample test tube and shaken. The test tubes were then kept in the dark place to complete the reaction for 2 h. The absorbance was measured by using a UV-Vis spectrophotometer at a fixed wavelength 765 nm. Tannin content was calculated as the difference between total and non-tannin phenolic content as described in equation 2.

$$\text{Total tannin} = \text{total phenolic} - \text{nontannin phenolic} \quad (2)$$

Determination of total flavonoids (TFC)

Colorimetric method with minor modification was used to determine the total flavonoid contents in *M. zapota* fruit extract with different solvents as described by Shofian et al. [15]. The crude extract (0.5 mg) was taken and placed in a test tube. Then 0.1 mL of aluminum chloride solution (10%), 0.1 mL of potassium acetate (1 mol/L) and followed by 4.3 mL distilled water were added to the same test tube. The mixture was shaken using vortex mixer and the samples were incubated at room temperature for 30 min. The absorbance of the samples was measured at fixed wavelength of 415 nm by using a UV-Vis spectrophotometer. Rutin standard was used to prepare the calibration curve. The determinations of total flavonoids in different crude extracts were carried out in triplicate and the average was calculated.

Evaluation of antioxidant activity

DPPH scavenging assay

M. zapota fruit extracts from pulp, peel and seed were evaluated for their antioxidant activity using the 2, 2-diphenylpicrylhydrazyl (DPPH•) scavenging method reported by Rice-Evans [16] with minor modification. The assay is based on the discoloration of DPPH free radical after phenols addition and measuring their ability to transfer H atoms/electrons to radicals. Methanol solutions of each extract were prepared at different concentrations (1, 2, 3, 4, 5, 10 mg/mL) to a final volume of 2.5 mL. Each test tube was added with 1.0 mL of 0.004% (w/v) methanol solution of DPPH. The solutions were shaken vigorously. After 30 min of reaction at room temperature (25 ± 2°C) the absorbance was measured at 517 nm by spectrophotometer (UV-1601 Shimadzu spectrophotometer, Japan). This method was carried out in triplicate. All the samples were kept and protected from light. The radical scavenging activity of the tested crude extract samples was estimated as an inhibition percentage which was calculated using the following equation 3:

$$\% \text{Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (3)$$

where A_{control} represents the absorbance of the blank control and A_{sample} represents the absorbance of the extracts.

β-carotene bleaching assay

The antioxidant activity of the extracts was evaluated using β-carotene bleaching assay according to the modified method by Karamian [14]. A stock solution of β-carotene mixture was prepared using 2 mg of β-carotene in 10 mL chloroform (0.2 mg/mL). The mixture (2 mL) was pipetted into a round bottom flask. Then, the chloroform was removed by rotary evaporator at 40°C. Five clean test tubes with 0.2 ml of extract each were prepared at different concentrations (100, 50, 25, 5 and 1 ppm) and then 4 mg of linoleic acid followed by 400 mg of Tween 80 and 100 mL were transferred to the test tubes. An emulsion was formed and they were placed in water bath at 50°C for 2 h. During the period, absorbance of each samples were measured at 470 nm at (t=0, 60 and 120 min). The percentage of β-carotene bleaching activity was calculated as described in the following equation 4:

$$\% \text{Inhibition} = \frac{\beta\text{-carotene After 2h}}{\text{Initial } \beta\text{-carotene}} \times 100 \quad (4)$$

Oxygen radical absorbance capacity (ORAC)

The ORAC procedure used an automated plate reader (KC4, Bio Tek, USA) with 96-well plates [17]. Analyses were

conducted in phosphate buffer pH 7.4 at 37°C. Proxy radical was generated using 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) which was freshly prepared for each run. Fluorescein was used as the substrate. Fluorescence conditions were as follows: excitation at 485 nm and emission at 520 nm. The standard curve was linear between 0 and 50 mM Trolox. Results are expressed as mmol Trolox equivalent (TE)/kg of the dried sample.

Analysis of *M. zapota* fruit extracts bioactive compounds by liquid chromatography-mass spectrometry (LC-MS)

Waters ACQUITY UPLC systems with a quaternary pump, refrigerated autosampler, column heater, Waters Single Quadrupole Detector (SQD) were used for all LC-MS analyses. Chromatographic separations were performed on ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm × 1.7 μm) at 35°C. The mobile phase composed of solution A (Water (LCMS Grade)+0.1% Formic acid) and solution B (Methanol (LCMS Grade)+0.1% Formic acid). The gradient program was started with 75% A and 25% B for 8.5 min and then increased to 100% B; held for 11.5 min and reinitialized to 75% A and 25% B in 5 min at both ionization modes. A flow rate of 0.2 ml/min was used and 1 μL of sample were injected. Samples were filtered through a 0.22 μm Milipore filter (Milipore, Bedford, MA) prior to UPLC injection. The mass raw data was analyzed by Masslynx MS Software version 4.1. Automated quantification with MassLynx targeted quantitative analysis was performed by QuanLynx (Waters Technologies). The results are expressed as mg/100 g of *M. zapota* fruit extract.

Analysis of volatile compounds by gas chromatography mass spectroscopy (Gcms)

The volatile compounds present in EtPE, EaPE and EaSE was identified by GCMS-QP5050A Shimadzu system with a splitless injector (250°C), at a split ratio of 20.0 using DB-5MS column (ZEBRON ZB-FFAP 30 m × 0.25 mm I.D × 0.25 μm of fil thickness). Helium was used as a carrier gas at the rate of 1 mL/min, 1 μL of sample was injected keeping ion source temperature 260°C and interface temperature at 260°C. The column temperature was kept at 100°C for 1 min after injection and then increased at the rate of 10°C min to 275°C which was held for 20 min.

Formulation of EtPE extract

EtPE was selected to be formulated in nanoemulsion system. Nanoemulsion was prepared by high energy emulsification (high shear homogenizer) followed by low energy emulsification method (overhead stirrer). EtPE extract was dissolved in palm kernel oil esters (PKOEs) and surfactant (Span 80: Tween 80) as the oil phase. The oil phase was slowly titrated into the aqueous phase and constantly homogenized at 6500 rpm for 20 min using a high shear homogenizer (PT3100, Kinematica AG, Switzerland). The formulation formed was cooled down to room temperature while stirring at 350 rpm for 30 min using an overhead stirrer (RW20 digital, IKA-Werke, Germany). The formulation was further characterized on the cytotoxicity study.

MTT cell viability assay

The toxicity of the EtPE formulation were investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT assay. Cells with the concentration of 1×10^5 cells in 100 μL were seeded onto 96-well culture plates and incubated overnight at 37°C under 5% CO₂ atmosphere for cell attachment. 20 μL of sample diluted in Dulbecco's modified Eagle's medium (DMEM) was added to each well, which yielded the final concentrations of 0.01-1000 μg/mL with a tenfold serial dilution. The same procedure was utilized for to blank. Dimethyl sulfoxide (DMSO) concentration was maintained at 0.1%. Fresh medium 20 μL was added into the control wells to make the final volume of 200 μL and medium alone (without cell and sample) was used as blank. After 3 h of incubation, 20 μL of MTT solution (5 mg/mL in Phosphate-buffered saline (PBS)) was added into each well and incubated further for 4 h. The medium was then aspirated and the insoluble formazan salt in each well was dissolved with 100 μL of 0.1% DMSO. The plates were swirled for 10 min. All the MTT assays were performed in triplicate. The absorbance of the formazan solution was measured by using a microplate reader (Thermo Scientific, USA) at a wavelength of 570 nm. The cell viability (%) related to control wells with cell culture medium was calculated as the following equation 5.

$$\text{Cell viability(\%)} = \frac{\text{Absorbance Sample}}{\text{Absorbance Control}} \times 100 \quad (5)$$

Statistical analysis

Statistical analyses were performed by one-way ANOVA with $P < 0.05$. Values are expressed as mean ± SD of three replicates.

RESULTS

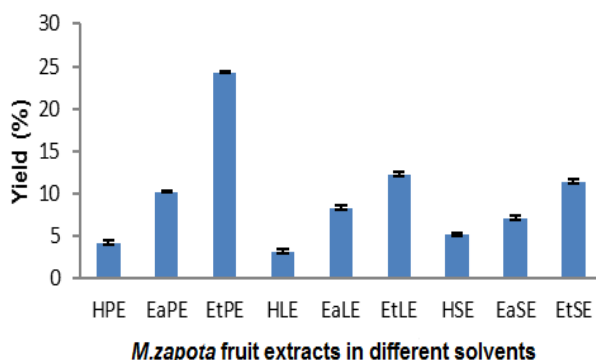
Extraction of *M. zapota* fruit in different solvents (Hexane, Ethyl Acetate and Ethanol)

Figure 1 shows the percentage yield of *M. zapota* fruit extracts in different solvents (hexane, ethyl acetate and ethanol). At similar temperature and time condition, the total yield (%) of hexane, ethyl acetate and ethanol extracts were found to be 12.61%, 25.67% and 47.95 %, respectively. Ethanol exhibited the high yield due to high solubility of bioactive compounds of *M. zapota* fruit in high polarity of solvent. The bioactive compounds such as phenolic acid, flavonoid and tannin. The percentage yield of extracts increased as the polarity of the solvent system was increased. The values were 4.21 ± 0.22 , 10.21 ± 0.12 and 24.27 ± 0.10 (%) for *M. zapota* pulp extract using hexane (HPE), ethyl acetate (EaPE) and ethanol (EtPE), respectively. Similar trend was observed in peel (3.20 ± 0.23 , 8.32 ± 0.25 , 12.28 ± 0.22 (%)) and seed extracts (5.20 ± 0.24 , 7.14 ± 0.27 , 11.4 ± 0.28 (%)). Polar solvents are frequently used for recovering polyphenols from fruit and vegetables. Previous study reported by Hatami *et al.* [18] showed that different polarity of solvents, exhibited different of percentage yield. Other than that, the percentage yield was also reported to be related to the temperature, pressure and time [19]. Therefore, in order to extract high yield of plant extract, there are several factors to be considered.

Antioxidant activity

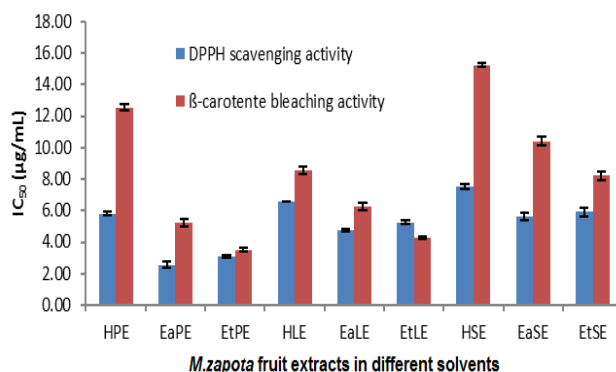
Figure 2 shows antioxidant activity of *M. zapota* fruit extracts in different solvents by DPPH scavenging activity and β -carotene bleaching assay (BCB). Results indicated that all the extracts potentially scavenged the free radicals. They were expressed as the half maximal (50%) inhibitory concentration (IC₅₀). The lowest IC₅₀ represent the highest antioxidant activity. Vitamin C, gallic acid and trolox (Tro) were used as the positive controls.

The ability of *M. zapota* extracts to inhibit the discoloration of DPPH from purple to yellow was spectrophotometrically measured. The results showed that the EaPE and EtPE exhibited relatively high antioxidant activity with low IC₅₀



HPE: Hexane Pulp Extract; EaPE: Ethyl Acetate Pulp Extract; EtPE: Ethanol Pulp Extract; HLE: Hexane Peel Extract; EaLE: Ethyl Acetate Peel Extract; EtLE: Ethanol Peel Extract; HSE: Hexane Seed Extract; EaSE: Ethyl Acetate Seed Extract; EtSE: Ethanol Seed Extract

Figure 1: Percentage yield of *M. zapota* fruit extract in different solvents (n-Hexane, ethyl acetate and ethanol)



HPE: Hexane Pulp Extract; EaPE: Ethyl Acetate Pulp Extract; EtPE: Ethanol Pulp Extract; HLE: Hexane Peel Extract; EaLE: Ethyl Acetate Peel Extract; EtLE: Ethanol Peel Extract; HSE: Hexane Seed Extract; EaSE: Ethyl Acetate Seed Extract; EtSE: Ethanol Seed Extract

Antioxidant activity represented in IC₅₀ values and the error bars represent standard deviations (n=3)

Figure 2: Antioxidant activity of *M. zapota* fruit extracts in different solvents

values of $2.57 \pm 0.18 \mu\text{g/mL}$ and $3.09 \pm 0.08 \mu\text{g/mL}$, respectively. The lowest antioxidant activity was found in HSE ($7.54 \pm 0.17 \mu\text{g/mL}$). This could be due to less phenolic compound in HSE extract. The antioxidant ability using β -carotene bleaching was measured and the results obtained were varied, ranged from 3.52 ± 0.12 to $15.24 \pm 0.14 \mu\text{g/mL}$. EtPE exhibited the highest antioxidant activity (IC_{50} : $3.52 \pm 0.12 \mu\text{g/mL}$) through BCB assay, whilst HSE showed the least antioxidant activity (IC_{50} : $15.24 \pm 0.14 \mu\text{g/mL}$) in the bleaching of β -carotene.

Antioxidant activity (DPPH scavenging activity) has a good correlation with phenolic and flavonoid content $R^2=0.9905$ and $R^2=0.9924$ (The data not shown). These results were in good agreement with those obtained by Bedawey *et al.* [20] who found highly positive relationship existed between total phenolics and antioxidant activity in many plant species. EtPE showed the highest amount of TPC, TFC and also exhibited high antioxidant activity using DPPH scavenging activity and BCB assay. The harmful oxidation caused by free radicals in the human body can be blocked by phenoxide ions that are delocalized in these antioxidants, which scavenge the free radicals and detoxify the organism. In addition, phenolic compounds, which are effective proton donors, are responsible in stabilizing lipid oxidation and antioxidant activity.

The pulp section of *M. zapota* fruit showed high antioxidant activity by DPPH and β -carotene bleaching assays compared to peel and seed extract. However, all *M. zapota* extracts could be classified as very active antioxidant ($\text{IC}_{50}<50 \mu\text{g/mL}$). Muhtadi *et al.* [21] reported that, IC_{50} could be classified to 4 categories where IC_{50} of less than $50 \mu\text{g/mL}$, was listed as very active, $50\text{-}100 \mu\text{g/mL}$ was listed as active, $100\text{-}200 \mu\text{g/mL}$ was listed as quite active and more than $200 \mu\text{g/mL}$ which was listed as inactive as an antioxidant.

In this study, a variation in antioxidant capacity ranging from 49.72 to 3210.49 mmol TE/kg extract was observed. Ethyl acetate seed extract (EaSE) showed the highest ORAC values (3210.49 mmol TE/kg extract); whilst hexane peels extract (HLE) showed the least of ORAC values (49.72 mmol TE/kg extract). The highest total amount of ORAC values was found in *M. zapota* seed extract which 5329.30 mmol TE/kg extract followed by pulp (1953.30 mmol TE/kg extract) and peel (1479.8 mmol TE/kg extract)

Isabelle *et al.* [22] reported that *M. zapota* fruit without the peel and seeds showed high ORAC activity as compared to the others fruits such as palm, strawberry and banana. However, to date there is no report on the antioxidant activity by ORAC on *M. zapota* seed extract. The antioxidant ability using ORAC of *M. zapota* fruit extract is plotted against TPC (Figure 3). The finding showed that, there was no correlation between ORAC and TPC of *M. zapota* fruit extracts ($R^2<0.1$). Isabelle *et al.* [22] reported that the antioxidant capacity by ORAC of vegetables extract correlated well to TPC, however for the ORAC of fruit extract, no correlation was observed with TPC.

The ORAC method is relevant to an *in vivo* conditions as it is using a biologically relevant free radical source (peroxyl radical) which is free radical commonly found in human biology system. This biochemical assay measures the antioxidative degeneration of fluorescein. Antioxidants are able to protect fluorescein after exposure to 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) radicals.

Total phenolics, tannin content and total flavonoid

Table 1 shows the summary of the phytochemical analysis of TPC, TFC and TT of *M. zapota* fruit extract at different parts (pulp, peel and seeds). The TPC obtained range from 1.34 ± 0.07 to $23.01 \pm 0.09 \text{ mg GAE/g}$. Ethanol pulp extract (EtPE) showed the highest TPC which was $23.01 \pm 0.09 \text{ mg GAE/g}$ whereas the lowest content was measured in hexane seed extract (HSE) ($1.34 \pm 0.07 \text{ mg GAE/g}$) ($p<0.05$). The TFC of *M. zapota* extracts varied from 52.45 to 179.14 mg Rutin/g. The highest TFC was detected in EtPE ($179.14 \pm 0.06 \text{ mg Rutin/g dry weight}$) and the lowest was in HSE ($52.45 \text{ mg Rutin/g dry weight}$). While TT was varied from 0.55 to 21.12 mg GAE/g. Ethanol peel extract (EtLE) obtained the highest TT ($21.12 \pm 0.63 \text{ mg GAE/g}$) and HSE had the lowest tannin content ($0.55 \pm 0.06 \text{ mg GAE/g}$) ($p<0.05$). TPC, TFC and TT of *M. zapota* extracts were high in ethanol as the solvent system followed by ethyl acetate and hexane. Thenmozhi and Rajan [23] reported that, major polyphenols compounds can be detected in the ethanol extract.

The standard curve of gallic acid for determination of total phenolic content was drawn and the regression equation was: TPC of *M. zapota* extracts exhibited good correlation to its TFC. Ethanol extract had the highest in TPC and TFC followed by ethyl acetate and hexane extracts, respectively. Ethanol has been proven as an effective solvent to extract phenolic compounds. Other than that, ethanol is safe for human consumption [22]. The results obtained showed that different part of fruits gave different amount of phenolic content. *M. zapota* pulp extract showed the highest phenolic content compared to the peel and seed extract.

Flavonoids are secondary metabolites with high therapeutic potential, including cardioprotective [24], anti-inflammatory, antimicrobial [25], and antitumor [26]. In this study, Rutin was used as standard compound in the

detection of flavonoid content. The regression equation for total flavonoid determination was: Similar to TPC, ethanol extract exhibited the highest amount of flavonoid compounds followed by ethyl acetate and hexane. Flavonoids have the capability as radical scavenger due to molecular structure of flavonoids which contained hydroxyls group that could donate an electron (H^+) to radicals such as hydroxyl ($HO\cdot$), superoxide ($O_2\cdot^-$) and peroxy ($ROO\cdot$) and thus neutralizing them [26].

Tannin, commonly referred to tannic acid is water soluble polyphenolic compounds which are present in plants. The regression equation for total tannin determination was: The highest tannin content of *M. zapota* extract was detected in ethanol, followed by ethyl acetate and hexane. Tannin serves as natural defense mechanism against microbial infections and can be used as antimicrobial properties [27]. Murshid *et al.* [28] reported that, tannin was also responsible for antioxidant activity.

Analysis of phenolic and fatty acid compounds by liquid chromatography mass spectroscopy (LCMS)

Among of *M. zapota* fruit extracts, ethanol pulp extract (EtPE) showed the highest TFC (phenolic content) and TFC (flavonoid content) while ethanol peel extract (EtLE) exhibited the highest TT (Tannin content), respectively. Moreover, EtPE, EaPE and EaSE were the most potent in scavenging free radical. Several phenolic and fatty acid compounds were identified in these extracts, using Liquid chromatography mass spectroscopy (LCMS). LCMS serves as a highly screening and confirmatory tool, which is generally a crucial technique for analyzing phenolic components in plant samples [29].

Table 2 shows the phenolic and fatty acid compounds present in EtPE, EaPE and EaSE along with their retention time which are detected within 10 min. Phenolic are antioxidant compounds which act as agents to neutralize harmful free radicals (ROS). The phenolic compounds can be classified into flavonoid (quercetin) and phenolic derivatives (hydrobenzoic and hydroxynamic acid) such as gallic acid, protocatechuic acid, vanillic acid, ferulic acid. All of the bioactive compounds contain of aromatic ring and hydroxyl group. The hydroxyl groups were responsible to antioxidant, anti-inflammatory and antimicrobial properties.

Among the 18 tested phenolic and fatty acid compounds, protocatechuic acid (PCA) (1) was the most abundant in EtPE extract (114.80 ± 10.23 mg/100 g), whilst least in EaPE (12.11 ± 0.01 mg/100 g) and EaSE (9.10 ± 1.02 mg/100 g). Gallic acid (2) as the first compound appeared at 1.34 min was found to be 44.24 ± 1.40 mg/100 g in EtPE, 12.11

Table 1: Summary of the phytochemical analysis of TPC, TFC and TT of *M. zapota* fruit extract at different parts (pulp, peel and seeds)

Parts of <i>M. zapota</i>	Solvents	Total phenols (mg GAE/g DW)	Total Flavonoid (mg Rutin /g DW)	Tannin Content (mg GAE/g DW)
EtPE	Ethanol	23.01 ± 0.09	179.14 ± 0.06	20.98 ± 0.16
EtLE		22.60 ± 0.05	160.32 ± 0.03	21.12 ± 0.63
EtSE		11.03 ± 0.13	97.23 ± 0.11	10.47 ± 0.12
EaPE	Ethyl acetate	8.33 ± 0.06	85.24 ± 0.02	6.87 ± 0.14
EaLE		12.33 ± 0.08	110.28 ± 0.01	8.80 ± 0.07
EaSE		11.31 ± 0.04	116.06 ± 0.03	8.91 ± 0.11
HPE	Hexane	1.72 ± 0.05	68.52 ± 0.01	1.01 ± 0.12
HLE		8.82 ± 0.37	88.28 ± 0.02	7.48 ± 0.40
HSE		1.34 ± 0.07	52.45 ± 0.03	0.55 ± 0.06

GAE: Gallic Acid Equivalent; DW: Dried Weight; EtPE: Ethanol Pulp Extract; EtLE: Ethanol Peel Extract; EtSE: Ethanol Seed Extract; EaPE: Ethyl Acetate Pulp Extract; EaLE: Ethyl Acetate Peel Extract; EaSE: Ethyl Acetate Seed Extract; HPE: Hexane Pulp Extract; HLE: Hexane Peel Extract; HSE: Hexane Seed Extract

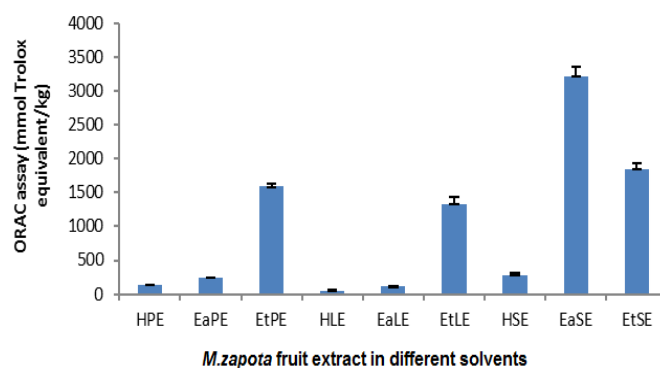


Figure 3: Antioxidant activity of *M. zapota* fruit extract in different solvent by oxygen radical absorbance capacity (ORAC)

± 0.01 mg/100 g and 23.24 ± 1.08 mg/100 g in EaPE and EaSE, respectively. Quercetin (3) the last compound observed at 9.03 min found to be 23.10 ± 2.40 mg/100 g and ferullic acid (4) (22.80 ± 1.20 mg/100 g) in EtPE. Figure 4 shows some of phenolic and fatty acid compound in *M. zapota* extracts.

The fatty acid profile of EtPE, EaPE and EaSE extracts contain of linoleic acid (polyunsaturated), stearic acid, oleic acid (monounsaturated), palmitic acid and myristic acid. Long chain polyunsaturated fatty acid (LC-PUFAs) linoleic acid (5) was abundant in EaSE (95.32 ± 1.20 mg/100 g), EaPE (68.11 ± 1.80 mg/100 g) and EtPE (38.35 ± 0.24 mg/100 g). The highest oleic acid (6) was found in EaSE (70.34 ± 0.21 mg/100 g), EaPE (59.41 ± 0.20 mg/100 g) and EtPE (23.68 ± 0.52 mg/100 g). Linoleic acid is an omega-6 fatty acid, required for proper skin function. Increasing levels of essential fatty acid (EFAs; omega-3 and omega-6) can increase cell membrane fluidity, enhance barrier function and repair, decrease trans-epidermal water loss, moisturize, improve cell immunity, and acts as an anti-inflammatory agent [30]. Oleic acid is good for percutaneous absorption enhancer and it is widely used in transdermal delivery of drug [31].

Table 2: Shows the phenolic and fatty acid compounds present in EtPE, EaPE and EaSE along with their retention time which are detected within 10 min

Standards references	Retention time (min)	Sample extracts (mg/100 g extract)		
		Ethanol pulp extract (EtPE)	Ethyl acetate pulp extract (EaPE)	Ethyl acetate seed extract (EaSE)
Gallic acid	1.34	44.24 ± 1.40	38.14 ± 1.12	23.24 ± 1.08
Protocatachuic acid	2.08	114.80 ± 10.23	12.11 ± 0.01	9.10 ± 1.02
Resorcinol	2.11	15.70 ± 4.90	ND	4.21 ± 0.05
4-hydroxybenzoic acid	3.25	1.56 ± 0.52	0.18 ± 0.14	0.54 ± 1.20
Vanillic acid	3.68	3.70 ± 0.15	1.24 ± 1.26	0.24 ± 1.30
Caffeic acid	3.75	2.80 ± 1.40	ND	1.36 ± 0.05
Syringic acid	3.98	0.38 ± 0.06	ND	ND
Epicatechin	5.22	10.20 ± 0.40	2.24 ± 0.28	3.15 ± 0.21
Coumaric acid	5.66	1.70 ± 0.76	ND	ND
Ferullic acid	6.58	22.80 ± 1.20	5.21 ± 0.21	ND
Myricitrin	8.06	1.69 ± 0.76	0.08 ± 0.12	0.04 ± 0.02
Quercetin	9.03	23.10 ± 2.40	05.24 ± 0.04	3.18 ± 0.25
Kaempferol	5.24	ND	ND	12.12 ± 0.20
Linoleic acid	2.68	38.35 ± 0.24	68.11 ± 1.80	95.32 ± 1.20
Stearic acid	4.14	17.42 ± 0.11	21.25 ± 1.30	27.24 ± 0.80
Oleic acid	3.24	23.68 ± 0.52	59.41 ± 0.20	70.34 ± 0.21
Palmitic acid	3.02	10.32 ± 0.18	12.21 ± 1.10	14.04 ± 1.30
Myristic acid	2.11	3.21 ± 0.16	4.14 ± 1.30	8.26 ± 1.20

ND: Not Detected

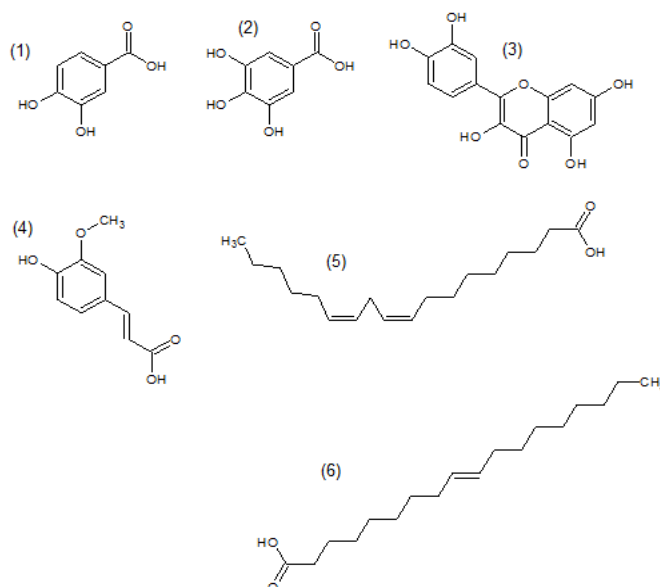


Figure 4: Structure of major phenolic and fatty acid compounds identified in EtPE, EaPE and EtSE

In cosmetic and pharmaceutical applications, EFAs are used to enhance the absorption of bioactive and could also be used as carrier oils [32]. EFAs are important in human diet because they cannot be produced by the body and must be obtained from food. EFAs reach of 74% in epidermis skin layer and decreasing with age and causing roughness and sensitivity skin. Therefore, the used of EFAs in cosmetics could increase the EFAs in the skin layer. Long chain omega-3 or -6 polyunsaturated fatty acids (PUFAs) can lower the production of reactive oxygen species (ROS), thus decreasing the risk of various diseases and improves. The effect of omega-3 long chain PUFAs on ROS is stronger than saturates, monounsaturates and polyunsaturates of the omega-6 series.

Ajila *et al.* [33] reported that, PCA (1) exhibited antioxidant, anti-ageing and anti-cancer activities. GA (2) naturally found in fruits has several biological activities including antioxidant, antityrosinase, antimicrobial, anti-inflammatory and anticancer activities. However GA has poor water solubility (11.5 mg/mL) [34]. Quercetin (3) is a flavonoid compound and it is naturally present in vegetables, fruits, non-alcoholic drinks and medicinal plants. Many beneficial effects of quercetin have been described, including anti-inflammatory and anti-allergic effects as well as having potential to diminish or prevent some types of cancer [35]. In addition, ferulic acid (4) is a hydroxynamic acid, with strong antioxidant and potent antimicrobial property.

Analysis of volatile compounds by gas chromatography mass spectroscopy (GCMS)

Figure 5 shows the chromatogram of EtPE (a), EaPE (b) and EaSE (c). Several peaks of volatile compounds were observed. GCMS is one of the greatest techniques to detect the constituents of volatile substance, long chain, branched chain hydrocarbons, alcohols acids and esters. The identification of the volatile compounds was confirmed based on the peak area, retention time, molecular weight and molecular formula.

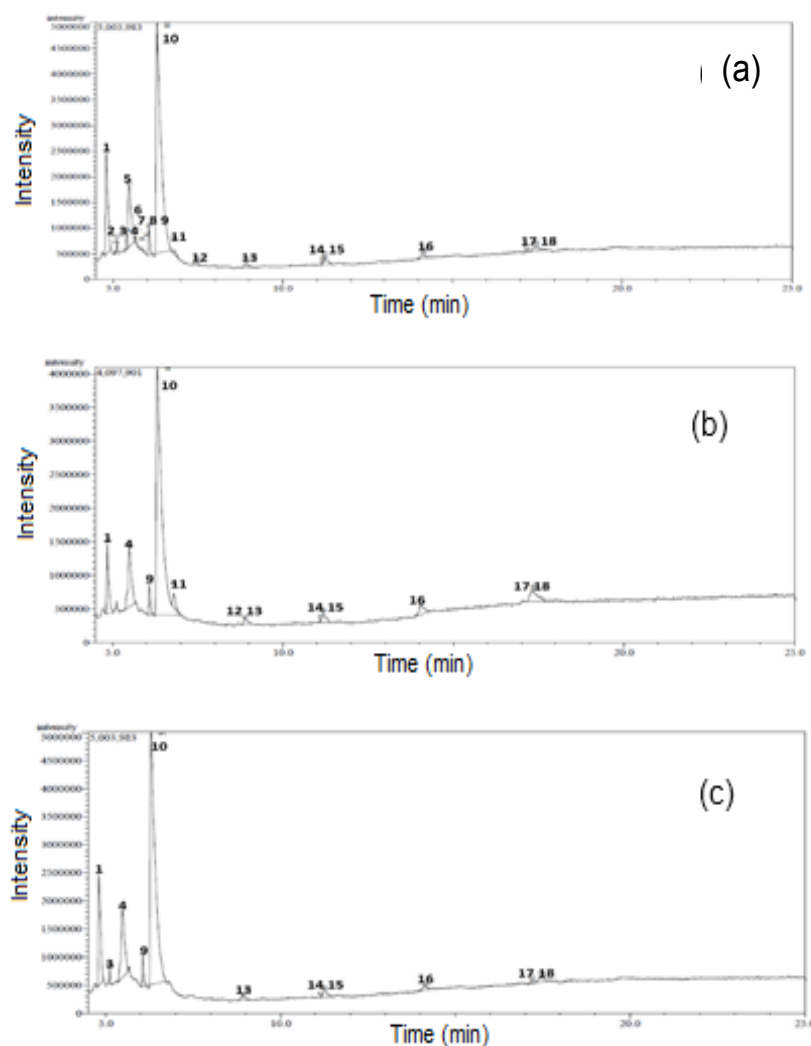


Figure 5: GC-MS chromatogram of EtPE (a), EaPE (b) and EaSE (c) fruit extract of *M. zapota*

Table 3: Shows the percentage of volatile compounds in EtPE, EaPE and EaSE extracts along with retention time, molecular formula and molecular weight

No.	Retention time (min)	Compounds	EtPE Peak Area%	EaPE Peak Area%	EaSE Peak Area%	Molecular formula	Molecular weight (g/mol)
1	4.801	5-Methyl-2(3H)-furanone	12.93	7.54	13.39	C ₅ H ₆ O ₂	98
2	5.075	Methyl beta-D-Ribopyranoside	0.13	ND	ND	C ₆ H ₁₂ O ₅	164
3	5.111	Dodecanol	0.82	ND	0.84	C ₁₂ H ₂₆ O	186
4	5.425	Glyceraldehyde	1.35	10.52	13.17	C ₃ H ₆ O ₃	90
5	5.471	Glycolic acid	12.72	ND	ND	C ₂ H ₄ O ₃	76
6	5.668	1,3-cyclohexanedione	0.35	ND	ND	C ₆ H ₈ O ₂	112
7	5.754	methyl diethanolamine	0.10	ND	ND	C ₇ H ₁₃ NO ₂	119
8	5.854	1-hexadecanol	0.34	ND	ND	C ₁₆ H ₃₄ O	242
9	6.060	Formic acid	2.33	2.58	2.41	C ₁ H ₂ O ₂	86
10	6.298	1,2,3-Propanetriol	62.46	67.11	64.67	C ₃ H ₈ O ₃	92
11	6.796	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	0.63	2.05	ND	C ₆ H ₈ O ₄	144
12	8.877	Capric acid methyl ester	ND	1.14	ND	C ₁₁ H ₂₂ O ₂	186
13	8.923	Palmitic acid methyl ester	0.85	1.12	0.88	C ₁₇ H ₃₄ O ₂	270
14	11.169	Tetradecanoic acid	0.15	0.31	0.15	C ₁₄ H ₂₈ O ₂	228
15	11.237	8-Octadecenoic acid, methyl ester	1.70	2.16	1.76	C ₁₉ H ₃₆ O ₂	296
16	14.157	Palmitic acid	0.95	2.45	0.98	C ₂₀ H ₄₀ O ₂	312
17	17.198	Stearic acid	0.31	0.21	0.32	C ₁₈ H ₃₆ O ₂	284
18	17.465	Oleic acid	1.37	4.13	1.42	C ₁₈ H ₃₄ O ₂	282

EtPE: Ethanol Pulp Extract; EaPE: Ethyl Acetate Pulp Extract; EaSE: Ethyl Acetate Seed Extract of *M. zapota*; ND: Not Detected

The highest peak was found to be 1, 2, 3-Propanetriol or glycerol (glycerin). Glycerol is suitable to be used in food, pharmaceutical and cosmetic industry. Vegetable based glycerol has moisturizing effect and widely used in skin care products. Crude glycerol can be applied in the number of processes such as fermentation, oxidation, esterification and transesterification, biomass liquefaction, oligomerization and polymerization, carboxylation, glycerolysis, hydrogenolysis, dehydration or dihydroxylation.

Table 3 shows the percentage of volatile compounds in EtPE, EaPE and EaSE extracts along with retention time, molecular formula and molecular weight. They were 5-methyl-2(3H)-furanone (12.93%), methyl beta-D-ribopyranoside (0.13%), dodecanol (0.82%), glyceraldehyde (1.35%), glycolic acid (12.72%), 1,3-cyclohexanedione (0.35%), methyl diethanolamine (0.1%), 1-hexadecanol (0.34%), formic acid (2.33%), 1,2,3-propanetriol (62.46%), palmitic acid methyl ester (0.85%), tetradecanoic acid (0.15%), 8-octadecenoic acid, methyl ester (1.70%), palmitic acid (0.95%), stearic acid (0.31%) and oleic acid (1.37%) in EtPE extract.

While 5-methyl-2(3H)-furanone (7.54%), glyceraldehyde (10.52%), formic acid (2.58%), 1,2,3-propanetriol (67.11%), 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (2.05%), palmitic acid methyl ester (1.12%), tetradecanoic acid (0.31%), 8-octadecenoic acid, methyl ester (2.16%), palmitic acid (2.45%), stearic acid (0.21%) and oleic acid (4.13%) in EaPE, followed by 5-methyl-2(3H)-furanone (13.39%), dodecanol (0.84%), glyceraldehyde (13.17%), formic acid (2.41%), 1,2,3-propanetriol (64.67%), palmitic acid methyl ester (0.88%), tetradecanoic acid (0.15%), 8-octadecenoic acid, methyl ester (1.76%), palmitic acid (0.98%), stearic acid (0.32%) and oleic acid (1.42%) in EaSE.

The EtPE, EaPE and EaSE can be used as new active compounds having antioxidant properties for topical application. The uses of *M. zapota* fruit extract in cosmeceutical products are still limited and a new source to be explored. The natural antioxidant of *M. zapota* extract could replace the synthetic compounds such as propyl gallate (PG), tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) that are normally used in the cosmetic industry. Other than that, the uses of monoethanolamine, diethanolamine sodium laureth sulfate, triethanolamine, as synthetic antioxidant have shown adverse reactions such as allergic contact dermatitis, irritant contact dermatitis, phototoxic and photo-allergic reactions to consumer skin [36].

Formulation study containing EtPE extract

In the formulation study, extract of EtPE of *M. zapota* was selected to be used as an active compound. Ethanol is non-toxic solvent that is safe and can be used on human skin. Ethanol peel extract (EtLE) and ethanol seed extract (EtSE) were not chosen in formulation study due to the least in antioxidant activity of the extracts. Therefore, EtPE

nanoemulsion was formulated and was further evaluated on the accelerated stability with respect to freeze/thaw cycle, centrifugation force at different temperature. EtPE nanoemulsions successfully withstood freeze/thaw cycle with no alteration in particle size. There were no sign of precipitation after centrifugation and no phase separation was observed at different temperature. Freeze/thaw cycles can provide a rough assessment on the physical stability of different formulations at different storage conditions. Centrifugation can accelerate the rate of creaming or sedimentation which demonstrates the breakdown of an emulsion system due to gravitational force.

In order to further confirm the safety of nanoemulsion, cytotoxicity study was evaluated. Toxicity of the formulation was tested on fibroblast cell line (3T3). Concentration-dependent cytotoxicity of the formulation was tested by MTT colorimetric assay to evaluate the cellular response of the 3T3 cells. The MTT assay indicated that relative viability decreased as the concentration of samples increases to more than 50% at highest concentration of 1000 µg/mL. This is a good indication on the safety of the EtPE extract in formulation since IC₅₀ higher than 1000 µg/mL is considered non-toxic [37]. The survival of cell was found to be higher due to the presence of oil in the formulation. The cells were able to survive without showing any sign of toxicity which proved that the formulation containing EtPE extract are non-toxic and safe to be used as active ingredient in cosmeceutical application.

CONCLUSION

Ethanol pulp extract (EtPE), ethyl acetate pulp extract (EaPE) and ethyl acetate seed extract (EaSE) of *M. zapota* exhibited the highest antioxidant activity among all the *M. zapota* extracts. Higher content of phenolic and flavonoid in EtPE than other extracts have contributed to the stronger antioxidant activity. In addition, antioxidant activity of extract is also affected by higher level of PUFA and volatile compounds. Phenolic, flavonoid and tannin are very soluble in the high polarity solvents. Ethanol extract had the highest in TPC, TFC and TT followed by ethyl acetate and hexane extracts, respectively. Therefore, EtPE was selected to be formulated and then the stability and cytotoxicity of the formulation were evaluated. EtPE formulation proved that the EtPE extract is safe and non-toxic and can be used as a new source of active compound in cosmeceutical application against skin ageing.

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