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# *In Vitro* and Antioxidant Evaluation of a Nanoemulsion Formulation Containing Kojic Monooleate for Hyperpigmentation Treatment

**Nur Farzana Jaslina[1, 2], Siti Efliza Ashari[1, 2, 3]*, Rosfarizan Mohamad[4], Nur Hana Faujan[1, 2, 3]**
[1]Integrated Chemical BioPhysics Research, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[3]Centre of Foundation Studies for Agricultural Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[4]Department of Industrial Bioprocess, Faculty of Biomolecular and Science Biomolecular, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
*Correspondence author (e-mail: ctefliza@upm.edu.my)

Running Head: Kojic monooleate (KMO) was incorporated into a formulation as the sole active ingredient, and the formulation was analysed for cytotoxicity using EpiDermTM tissue culture and melanin and tyrosinase inhibition using B16-F1 melanoma cells to confirm its capability to treat hyperpigmentation.

Cell damage caused by exposure to UV radiation, as an external stress-inducing factor, may contribute to skin hyperpigmentation. Kojic monooleate (KMO) was used as an active in a nanoemulsion formulation for hyperpigmentation treatment. The objective of this study was to analyse the cytotoxicity and efficacy of a nanoemulsion formulation containing KMO. The formulation was prepared using high and low energy emulsification techniques and analysed for cytotoxicity and melanin and tyrosinase inhibition. Then, it was investigated for total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition, and elastase inhibition. The formulation inhibited tyrosinase and melanin by 74.14% and 36.80%, respectively, at 1 mg/mL concentration. It also contained a substantial amount of phenolic and flavonoid compounds with the values of 8.14 ± 0.03 mg/g eq. to gallic acid and 1.55 ± 0.03 mg/g eq. to rutin, respectively. The formulation also inhibited DPPH free radicals by 12.99% at 100 µg/mL. It also possessed the capability to prevent photodamage, as it inhibited elastase by 27.91% at 1000 µg/mL. The KMO nanoemulsion was also found to be non-irritant. In short, the KMO nanoemulsion formulation could provide good effects if applied to the skin frequently and safe for daily applications.

**Key words:** Formulation; nanoemulsions; skin pigmentation inhibitor; cytotoxicity assessment; antioxidants

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Production and distribution of melanin affect the colouration of skin, hair, and eyes [1]. Hyperpigmentation and melasma are the results of abnormal production of melanin throughout the skin [2-4]. Melanin is synthesised via the process of melanogenesis in melanoma and melanocytes inside the epidermal layer of human skin [4-6]. Tyrosinase is a key enzyme in the biosynthesis of melanin, hence making it the key target to inhibit the synthesis of melanin [1, 7-8].

During melanogenesis, tyrosinase catalyses the conversion of L-tyrosine to 3,4-dihydroxyphenyl L-alanine (L-DOPA), followed by the oxidation of L-DOPA to dopaquinone. Finally, dopaquinone polymerises to form melanin [9-10]. Melanin produced is transferred to neighbouring keratinocytes, which is then distributed via various routes to the surface of the skin [11-12]. Uncontrollable tyrosinase will result in overproduction of melanin, hence causing hyperpigmentation [13].

Prolonged exposure to UV radiation triggers the production of reactive oxygen species (ROS), which are chemically unstable, thus leads to tissue damage, photoaging, sunburn, wrinkles, photosensitivity, and DNA damage [14-15]. In human skin, melanin plays a critical role in protection against solar ultraviolet (UV) radiation, preventing mutations in cellular DNA and various environmental oxidative

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stresses [16-18]. Apart from that, formulations rich in antioxidants are often used to help protect the skin as they can scavenge free radicals by contributing an additional oxygen molecule to free radicals [19-20]. Antioxidants are any substances that can inhibit oxidation by specifically quenching free radicals or by chelation of redox metals. Therefore, antioxidants are very useful in cosmetic formulations [20].

Phenolics are natural antioxidants with a high ability to scavenge ROS, such as hydrogen peroxide, superoxide, and peroxynitrite radicals. According to a recent theory [21], plants use phenolic compounds as a defence mechanism to prevent molecular damage by counteracting ROS. Higher total phenolic content indicates higher antioxidant activity [21-22]. Flavonoids are secondary metabolites from plants that exhibit antioxidant, anti-inflammatory, and anti-bacteria activities [23-24]. The compounds contain high phenolic antioxidants such as kojic acid, vitamin C and niacinamide, often used in cosmetic products as a defense against free radicals [25].

Kojic acid ((5-hydroxymethyl)-1, 4-pyrone) is a potent tyrosinase inhibitor produced by fungal species such as Aspergillus, Acetobacter, and Penicilium [23, 26-27]. A derivative of kojic acid, kojic monooleate (KMO) improves the storage instability, oil-solubility, and toxicity of kojic acid [4, 26-27]. KMO is also found to be a better tyrosinase inhibitor compared to kojic acid and exhibits strong antioxidant activity [4].

KMO was encapsulated in a nanoemulsion formulation as an active compound for hyperpigmentation treatment. Its characterisation and stability study have been reported in a previous study [28]. The main objective of this study was to analyse the efficacy and irritancy of KMO nanoemulsion formulation for cosmeceutical application. Skin diseases involve physical appearance, hence they often affect the emotional state of consumers, which eventually results in diminished personal satisfaction. Therefore, a treatment to reduce melanin production with less side effects was studied.

## MATERIALS AND METHODS

### 1. Materials

Castor oil (CO), N- Succinyl-Ala-Ala-Ala-p nitroanilide (SANA), and elastase type IV from porcine pancreas were purchased from Sigma, USA. Lemon essential oil (LO) (Argentine origin) and carboxylic acid (Trolox) were purchased from Aldrich, USA. Tris (hydroxymethyl) aminomethane was purchased from Sigma-Aldrich, USA. DPPH free radicals were purchased from Calbiochem, Germany. Sodium chloride, potassium chloride, anhydrous potassium dihydrogen orthophosphate, and anhydrous disodium hydrogen orthophosphate were purchased from Bio Basic Canada Inc. B16-F1 melanoma cell line (ATCC CRL-6323) was purchased from American Type Culture Collection. Reconstructed epidermal model EpiDermTM, EPI-100-NMM-SIT/ assay medium, MTT diluent, SDS solution, and nylon mesh (EPI-MESH) were purchased from MatTek, USA. All chemicals used were of analytical, food, or cosmetic grade classes.

### 2. Instrumentation

Sonication was performed using an ultrasonic bath sonicator (Power Sonic 405, Hwashin Technology Co., Seoul, Korea). Nanoemulsion formulations were prepared using both high and low energy emulsification techniques using a high shear homogenizer (T25 digital; IKA-Werk, GmbH & Co. KG, Staufen im Breisgau, Germany) and an overhead stirrer (RW20 digital; IKA-Werk, GmbH & Co. KG, Staufen im Breisgau, Germany), respectively. For total phenolic content, total flavonoid content, DPPH inhibition activity, and elastase inhibition activity, absorbance measurements were performed using a UV-visible spectrophotometer (Shimadzu model UV-1650).

### 3. Preparation of Nanoemulsion Formulations Containing Kojic Monooleate

Nanoemulsion formulations were prepared using both high and low energy emulsification techniques as described by a previous study [28], and the droplet size of the formulations was determined to ensure the formulations obtained were identical to the previous study. In brief, the oil and aqueous phase were prepared separately. For the oil phase, KMO (10.0% w/w) was added to castor oil (3.37% w/w) in a beaker. For the aqueous phase, tween 80 (3.19% w/w) and xanthan gum (0.70% w/w) were added to deionised water (81.68% w/w). Both phases were stirred continuously at 30°C before being subjected to sonication for 20 min using a bath sonicator (Power Sonic 405, Hwashin Technology Co., Seoul, Korea). After sonication, the aqueous phase was homogenised, while the oil phase was added dropwise into the aqueous phase during homogenisation process that took place for 15 min at 6,000 rpm using a high shear homogeniser (T25 digital; IKA-Werk, GmbH & Co. KG, Staufen im Breisgau, Germany). Then, the mixture was further homogenised for 3 h at 250 rpm using a low shear homogeniser (RW20 digital; IKA-Werk). Lastly, a mixture of lemon oil and 0.7% (w/w) of potassium sorbate was added into the mixture and further homogenised for 15 min at 250 rpm. An identical formulation without KMO was prepared as a control formulation.

### 4. Melanin Inhibition Assay

#### 4.1. Sample Preparation

The KMO formulation was diluted using distilled water at different concentrations, ranging from 0.01 mg/mL to 5 mg/mL. The samples were vortexed and filtered using 0.2 µm syringe filters.

### 4.2. Cell Viability Assay

B16-F1 melanoma cells were seeded into 24-well plates and cultured until they reached 90% confluence. Then, the cultured cells were treated with the KMO formulation at various concentrations (0.01 to 5 mg/mL), 0.1 mg/mL zinc sulphate (positive control), and 0.1 mg/mL kojic acid (negative control). 0.1 mg/mL kojic acid was chosen as a negative control since kojic acid is known for its toxicity effect on cells. After 72 h incubation, MTT reagent (3-(4, 5-Dimethylthiazol-2-YI)-2, 5-Diphenyltetrazolium bromide) was added into each well and incubated again for 3 h at 37°C. Then, the cells were centrifuged and dissolved in dimethyl sulfoxide (DMSO) before measuring the absorbance using a spectrophotometer at 690 nm. Relative cell viability was expressed as a percentage of a sample of treated cells to control cells (Equation 1).

$$\% \text{ Cell viability} = \frac{A_{sample}}{A_{control}} \times 100 \quad \text{(Eq. 1)}$$

Where $A_{sample}$ = Absorbance of sample

And $A_{control}$ = Absorbance of control

### 5. Melanin Assay

B16-F1 melanoma cells were seeded into 24-well plates and cultured until they reached 90% confluence, as described by a previous study [29]. Then, the cultured cells were treated with the KMO formulation at various concentrations (0.01 to 1 mg/mL) and kojic acid (0.1 mg/mL). After 72 h incubation, the absorbance of extracellular melanin was measured using a spectrophotometer at 690 nm. A synthetic melanin was used as a standard. All experiments were performed in triplicate and melanin inhibition was calculated using Equation 2.

$$\% \text{ Melanin Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad \text{(Eq. 2)}$$

Where A sample = Absorbance of sample

And A control = Absorbance of control

### 6. Tyrosinase Inhibition Assay

Tyrosinase inhibition activity using L-Dopa as the substrate was spectrophotometrically determined as described by a previous study [30], with minor modifications. The KMO formulation (1 g) was mixed with 50% methanol (10 mL) and sonicated for 30 min in an ultrasonic bath, then centrifuged at 4,000 rpm for 30 min. The sample was then diluted by a dilution factor of 3.33 to reach a final concentration of 30.03%. The prepared sample was mixed with L-Dopa (2 mM) and potassium phosphate buffer (0.05 M, pH 6.5). The mixture was incubated for 5 min at room temperature for reaction to take place. Then, tyrosinase was added to the mixture and the absorbance of dopachrome was measured using microplate reader at 492 nm. Tyrosinase inhibition activity was calculated using Equation 3.

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

Where A sample = Absorbance of sample

And A control = Absorbance of control

### 7. Skin Irritation Test

Skin irritation assay using EpiDermTM tissue culture was performed as described by a previous study [31]. First, EpiDermTM tissue was transferred into a six-well plate filled with the assay medium (0.9 mL) and incubated for 60 ± 5 min at 37°C, 5% CO2 and 95% relative humidity. After 60 min of incubation, the insert from the upper wells was transferred into the lower wells of the 6-well plate which contained new assay medium (0.9 mL) and incubated again for 18 ± 3 h.

The KMO formulation (30 µL) was applied to three single EpiDermTM tissues. The cultures were incubated for 35 ± 1 min at 37°C, 5% CO2, and 95% relative humidity. Then, each EpiDermTM tissue was removed from the incubator, separated from the assay material, and washed with phosphate-buffered saline. Then, the EpiDermTM tissues were transferred into wells containing the assay medium and incubated for 24 ± 2 h.

After incubation, the assay medium was renewed and the EpiDermTM tissues were incubated for 18 h. After 18 h incubation, the EpiDermTM tissues were transferred into six-well plates containing MTT medium (300 µL, 1 mg/mL) and incubated for 3 h. Then, blue formazan salt formed by cellular mitochondria was extracted using isopropanol (2 mL). Extraction solution (200 µL) was transferred into 96-well microtiter plates for absorbance measurement at 570 nm. Isopropanol (200 µL) was used as a blank. Phosphate buffer saline was used as the negative control and 5% SDS solution was used as the positive control. Relative cell viability for each tissue was calculated as a percentage (%) relative to the mean of the negative control tissue viability.

### 8. Total Phenolic Content (TPC)

The phenolic content of the KMO formulation was determined by measuring a blue reaction as the phosphor-wolframate-phosphomolybdate complex is reduced by phenolics by using the Folin–Ciocalteu method with a slight modification [32]. First, the KMO formulation (100 µL) was added to distilled water (1 mL). Then, Folin–Ciocalteu reagent (0.5 mL) was added and the mixture was vortex. The mixture was incubated for 3 min at room temperature, before addition of Na2CO3 (1 mL, 7.5% diluted with distilled water) to the mixture. The mixture was vortex again,

before heated in an incubator at 95°C for 1 min. After incubation, the mixture was allowed to cool at room temperature. The absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Shimadzu model UV-1650) and expressed as gallic acid equivalents (GAE) per mL of formulation. A formulation without KMO acted as the control formulation. All measurements were performed in triplicate.

**9. Total Flavonoid Content (TFC)**

The determination of TFC of the KMO formulation was measured by using the aluminium chloride colorimetric method [33]. The KMO formulation (0.5 mL at 100 µg/mL concentration) was mixed with aluminium chloride (0.1 mL, 10%), potassium acetate (0.1 mL, 1 M), and distilled water (4.3 mL). Then, the mixture was incubated at room temperature for 30 min before measuring the absorbance at 415 nm using a UV-Vis spectrophotometer (Shimadzu model UV-1650). Rutin at different concentrations (0.01, 0.02, 0.03, 0.04, and 0.05 mg/mL) was used to construct the calibration curve. All measurements were performed in triplicate.

**10. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay**

This method is based on the scavenging activity by antioxidants towards DPPH radicals. When a DPPH radical is mixed with a substance that possesses antioxidant activity, the reduced form of the radical is produced, causing decolouration of deep purple colour [34]. The KMO formulation (5 µg) was mixed with PBS (1 mL, pH 7.4) and methanolic DPPH (1 mL, 100 µM) solution. After 30 min reaction, the absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Shimadzu model UV-1650). A formulation without KMO was used as a control formulation. For control, the sample was replaced with methanol. For blank, the sample and DPPH were replaced with methanol. Ascorbic acid at different concentrations (0.02 g/mL to 0.10 g/mL) was used to prepare the calibration curve. All experiments were performed in triplicate. The percentage of DPPH radical scavenged was calculated using Equation 4.

$$\% \text{ DPPH Inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100 \quad \text{(Eq. 4)}$$

Where A sample = Absorbance of sample

And A control = Absorbance of control

**11. Assessment of Elastase Inhibition**

Elastase inhibition of the KMO formulation was measured using a method described by a previous study [35], with minor modifications. Porcine pancreatic elastase (PPE, Sigma, Type IV) was used as the enzyme, while N-Succ-(Ala) 3-nitroanilide (SANA) used as the substrate to monitor the release of p-nitroanilin. In brief, Tris-HCl buffer (700 µL, 0.2 M, pH 8.0), elastase (50 µL, 2.5 U/mL), and the KMO formulation (200 µL) at different concentrations in Tris-HCl buffer were mixed and preincubated at 25°C for 20 min. After pre-incubation, the substrate (50 µL, 0.8 mM) was added to each mixture and the mixtures were incubated again for 20 min at 25°C. Then, the absorbance was measured at 293 nm using a UV spectrophotometer (Shimadzu model UV-1650). In control, Tris-HCl buffer was used in place of the formulation. Relative inhibition of the KMO formulation on the elastase activity was calculated using Equation 5:

$$\% \text{ Elastase Inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100 \quad \text{(Eq. 5)}$$

Where A sample = Absorbance of sample

And A control = Absorbance of control

## RESULTS AND DISCUSSION

**1. KMO Nanoemulsion Formulation**

The characterisation of the KMO formulation is tabulated in Table 1. The formulation was oil-in-water (O/W) nanoemulsion with droplet size of 110.01 nm and elastic rheology properties. The formulation was non-toxic as it had $IC_{50}$ > 100 µg/ mL and was stable at various conditions (4°C, 25°C, and 45°C for 90 days).

**2. Melanin Inhibition Assay**

Figure 1 shows the effect of different concentrations (0.01 – 5.00 mg/mL) of the KMO formulation on B16-F1 melanoma cell viability. The KMO formulation was not toxic to B16-F1 melanoma cells at the concentration range of 0.01 mg/mL to a maximum of 1 mg/mL. However, at concentrations higher than 1 mg/mL, the KMO formulation caused cell viability to reduce drastically by 90%. This shows that the KMO formulation was toxic to B16-F1 melanoma cells at concentrations higher than 1 mg/mL. Thus, concentrations higher than 1 mg/mL cannot be used to study the effect of a sample on melanin, as at those concentrations B16-F1 melanoma cells cannot survive, therefore disrupting the production of melanin. Therefore, the concentration range of 0.05 – 1.00 mg/mL of the KMO formulation was used to study the effect of the KMO formulation on melanin. Kojic acid was used as control, and 0.1 mg/mL was the selected concentration as a concentration higher than this causes reduction of cell viability. Zinc sulphate (0.1 mg/mL) was used as a positive control, as it reduces cell viability greatly even at lower concentrations.

**Table 1**. Characterisation of KMO formulation

| Characteristics | KMO formulation |
|---|---|
| Type of emulsion | O/ W nanoemulsion |
| Rheology measurement | Elastic (shear thinning and pseudo-plastic properties) |
| Droplet size | 110.01 nm |
| pH | 6.28 |
| PDI analysis | 0.25 ± 0.01 |
| Zeta potential | - 43.60 ± 0.23 mV |
| Conductivity | 1492.00 ± 0.03 µScm$^{-1}$ |
| Stability | 4°C, 25°C and 45°C for 90 days √<br>Centrifugal force √<br>Freeze-thaw cycles √ |
| MTT assay | $IC_{50}$ > 100 µg/ mL |

Abbreviations: KMO, kojic monooleate

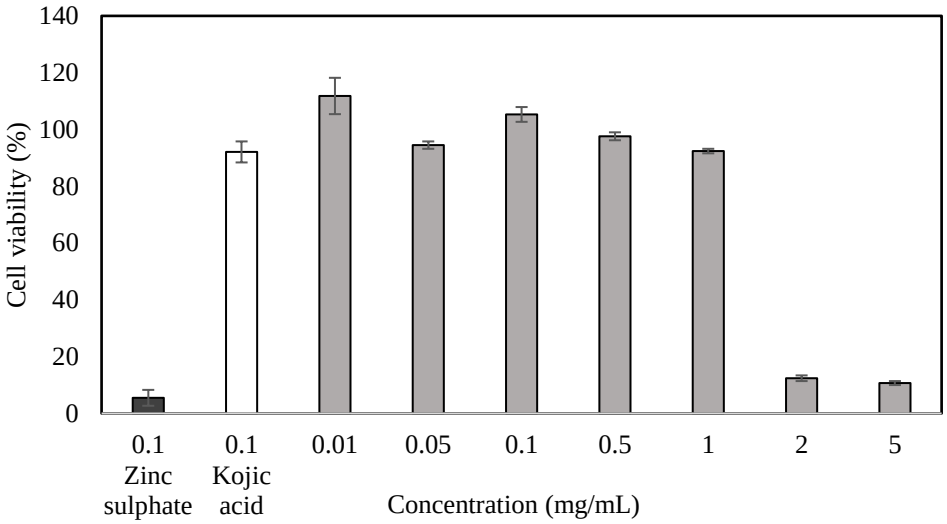


**Figure 1**. Effect of KMO formulation at various concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, 5 mg/mL), 0.1 mg/mL of kojic acid (negative control) and 0.1 mg/mL of zinc sulphate (positive control) on cell viability.

Figure 2 shows melanin inhibition (%) by different concentrations (0.05 – 1.00 mg/mL) of the KMO formulation and kojic acid as a positive control. Kojic acid inhibited melanin by 72.1% at 0.1 mg/mL. The KMO formulation possessed lower melanin inhibition with percentage of inhibition of 38% at 1 mg/mL. To compare the KMO formulation and kojic acid, 1 mg/mL of the KMO formulation was equivalent to 0.1 mg/mL of kojic acid, as 1 mg/mL of the KMO formulation contained only 10% (w/w) of KMO. However, this finding suggests that kojic acid possesses stronger inhibition towards melanin compared to KMO. A previous study had reported inhibition by 25% towards melanin using 62.5 µg/mL of KMO as the study sample [4]. Another research has reported that kojic acid was effective in reducing melanin content equivalent to hydroquinone, however the side effects of kojic acid included redness, stinging, and exfoliation [36]. This work suggested that kojic acid has better inhibition towards melanin compared to KMO. Melanin overproduction results in skin hyperpigmentation [37], hence a KMO nanoemulsion formulation that was able to inhibit melanin with less side effects was studied.

## 3. Tyrosinase Inhibition Assay

Figure 3 shows the standard curve for tyrosinase inhibition using kojic acid at different concentrations (1.75 × 10-4, 3.50 × 10-4, 7.50 × 10-4, 1.50 × 10-3, and 0.03 % v/v), with $y = 11.197x + 39.241$ and $r^2 = 0.963$. Tyrosinase was often used as the key target for the screening of new inhibitors since tyrosinase plays a major role in melanin biosynthesis [38].

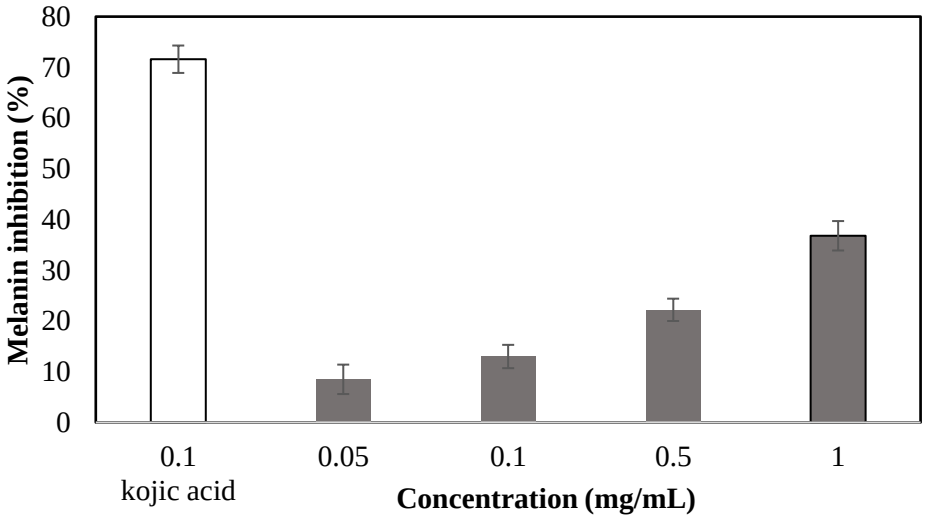


**Figure 2**. Melanin inhibition (%) of KMO formulation at various concentrations (0.01, 0.05, 0.1, 0.5, 1 mg/mL) and 0.1 mg/mL of kojic acid (positive control).

Figure 4 shows tyrosinase inhibition using L-DOPA as substrate by the KMO formulation and the control formulation. The KMO formulation showed substantial tyrosinase inhibition (74.14%), while the control formulation possessed 41.86% inhibition. Good inhibition on tyrosinase by the control formulation might be due to the presence of *Citrus limon* (lemon) essential oil. A previous research has reported that *Citrus limon* essential oil showed significant inhibition of L-DOPA by mushroom tyrosinase (45 – 50% inhibition at 167 µg/mL concentration) [39]. This shows that the addition of KMO itself contributed to approximately 32.28% inhibition towards tyrosinase. In comparison, kojic acid inhibited tyrosinase by 91.14% at 0.003% (v/v). These data suggest that kojic acid possesses significantly better tyrosinase inhibition activity compared to KMO, however its toxicity and side effects are major drawbacks for consumers. A previous study has reported 62.5 µg/mL of KMO exhibited 40% cellular tyrosinase inhibition acivity and approximately 80% of mushroom tyrosinase inhibition activity [4]. This suggests that the findings in this research are in line with the previous research, as 0.3 mL/mL of the KMO formulation (10% KMO; 0.03 mL/mL) showed 32.28% inhibition towards tyrosinase, while 0.0625 mg/mL of KMO showed 40% inhibition towards tyrosinase. To compare with a plant-based formulation, a formulation containing 1% ellagic acid and three plant extracts (Folium Eucalypti extract, Folium Juglandis extract, and Cortex Castanea extract) inhibited tyrosinase only by 66.53% [40].

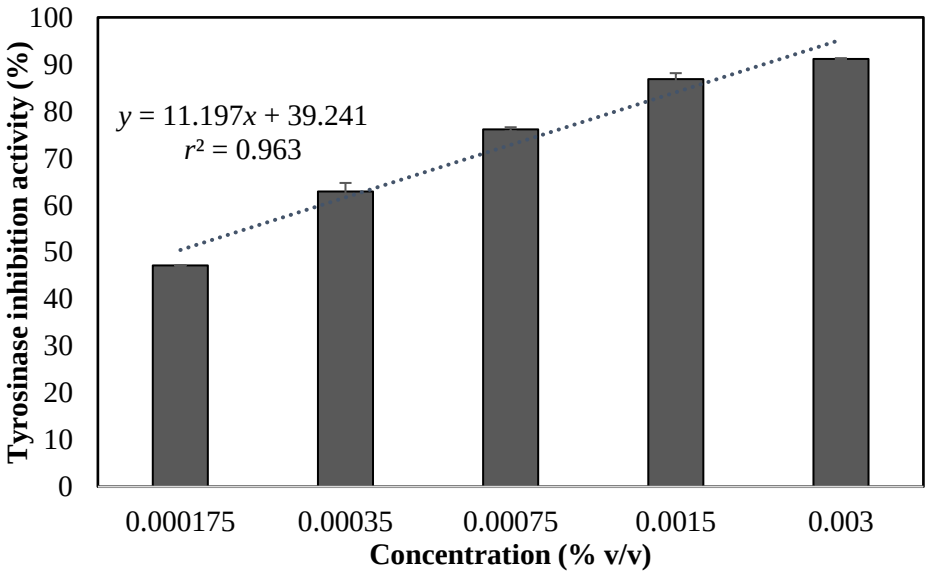


**Figure 3**. Tyrosinase inhibition activity of kojic acid at different concentrations (0.000175, 0.00035, 0.00075, 0.0015, and 0.003 % v/v).

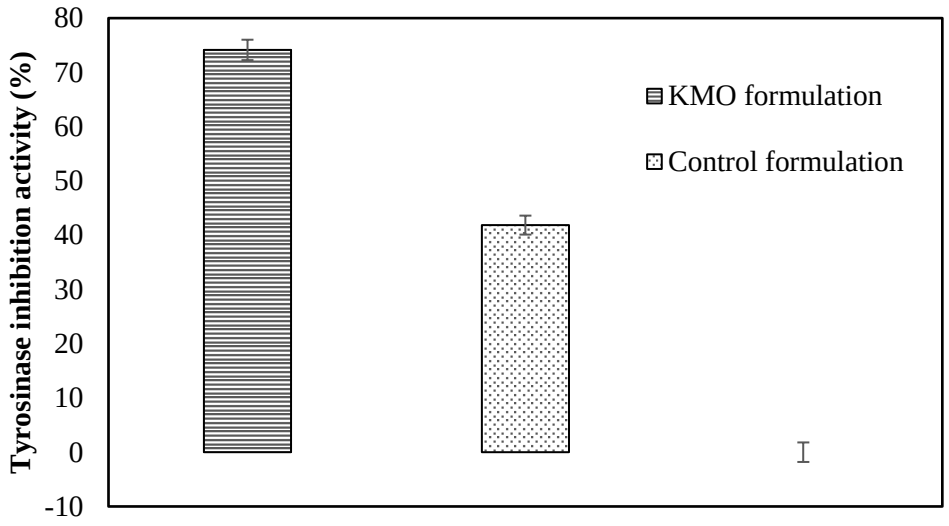


**Figure 4**. Tyrosinase inhibition activity of KMO formulation and control formulation at 30.03% (v/v) concentration.

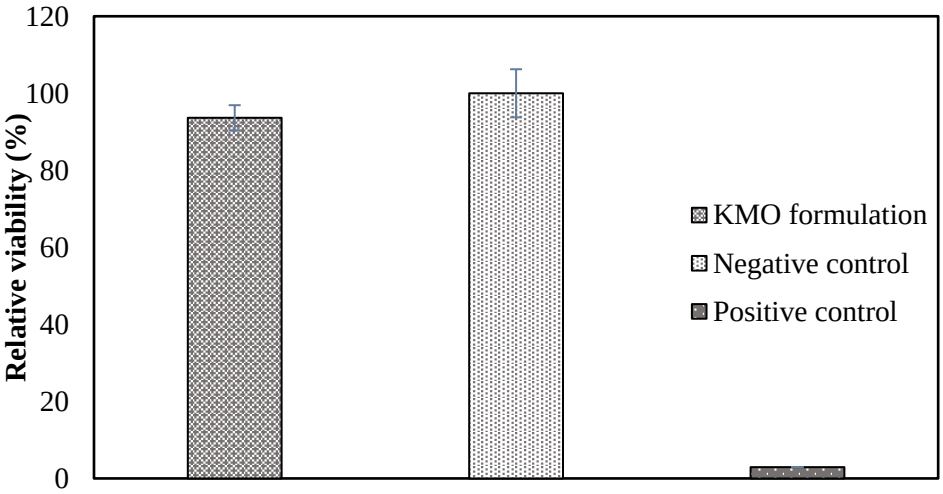


**Figure 5**. Skin irritation assay of KMO formulation and control formulations at 30.03% (v/v) concentration using EpiDerm™ tissue culture.

## 4. Skin Irritation Test

Figure 5 shows skin irritation test on EpiDerm™ tissue culture by the KMO formulation, phosphate buffered saline (negative control), and 5% SDS solution (positive control). Mean tissue viability lower than 50% is predicted to be irritant on human skin, while mean tissue viability higher than 50% is non-irritant and safe to human skin. The KMO formulation showed 93.60% of mean viability, which means the KMO formulation was non-irritant and predicted to be safe *in vivo*.

## 5. Total Phenolic Content (TPC)

TPC of the KMO formulation is given in Table 2. The KMO formulation (100 µL/mL) showed a high phenolic content of 8.14 ± 0.03 mg of phenolic compounds per g of sample of gallic acid equivalent (GAE) (the standard curve equation: $y = 3.68x + 0.03$, $r^2 = 0.98$). This shows that KMO contributed high phenolic content in the formulation. TPC is directly proportional to antioxidant activity [41]. Phenolic compounds possess antimicrobial and anti-aging properties besides antioxidant activity [42]. This finding suggests that the KMO formulation possessed higher antioxidant activity, as well as antimicrobial and anti-aging properties. A previous research reported that 4 well-known commercialised brands in India contained a polyphenol content ranging from 0.825 to 5 mg/g equivalent to gallic acid at 100 µL /mL concentration [42].

**Table 2**. TPC of KMO formulation and control formulation.

| Sample | mg/g eq. to gallic acid |
|---|---|
| KMO formulation | 8.14 ± 0.03 |
| Control formulation | 2.45± 0.13 |

**Table 3**. TFC of KMO formulation and control formulation.

| Sample | µg/g eq. to rutin |
|---|---|
| KMO formulation | 1547.0 ± 0.0298 |
| Control formulation | 28.5 ± 0.0005 |

## 6. Total Flavonoid Content (TFC)

Table 3 shows TFC of the KMO formulation and the control formulation with the amounts of 1547.0 ± 0.03 and 28.5 ± 0.01 µg/g equivalent to rutin at 100 µg /mL, respectively. This shows that KMO contains high flavonoid content related to antioxidant activity. Flavonoid compounds possess antioxidant activity and anti-radiation properties, which contribute to the protection of the skin from UV rays [43]. Recent studies showed that 100 µg/mL of cream formulations containing extracts of *Musa accuminata* (L.), *Psidium gujava* (L.), and *Pyrus communis* (L.) have 53.22 µg/g total flavonoid content equivalent to quarcetin [15].

Flavonoids are a class of phenolic compounds. Therefore, flavonoid content will be lower than phenolic content, and this supports the result obtained for both TFC and TPC of the KMO formulation and the control formulation, as the KMO formulation and the control formulation had phenolic content higher than total flavonoid content [24]. Correlations between antioxidant activity, DPPH inhibition and TFC would be lower than those of TPC, as the aluminium chloride method is specific to detect flavones and flavonols only. Thus, TFC measured might differ from the actual flavonoid content present [44].

## 7. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

Inhibition of DPPH (%) is shown in Figure 6. At 100 µg/mL concentration, the KMO formulation and the control formulation showed 12.99% and 11.69% inhibition towards DPPH free radicals, respectively. Meanwhile, a previous research has also revealed that KMO inhibits DPPH by 59.01% at 1000 µg/mL [4]. Hence, it is safe to say that the KMO formulation possessed high antioxidant content and high capability to scavenge free radicals. To compare with another formulation, at 100 µg/mL, a formulation containing 4% extract of lyophilised blueberry showed only 8% DPPH inhibition [45].

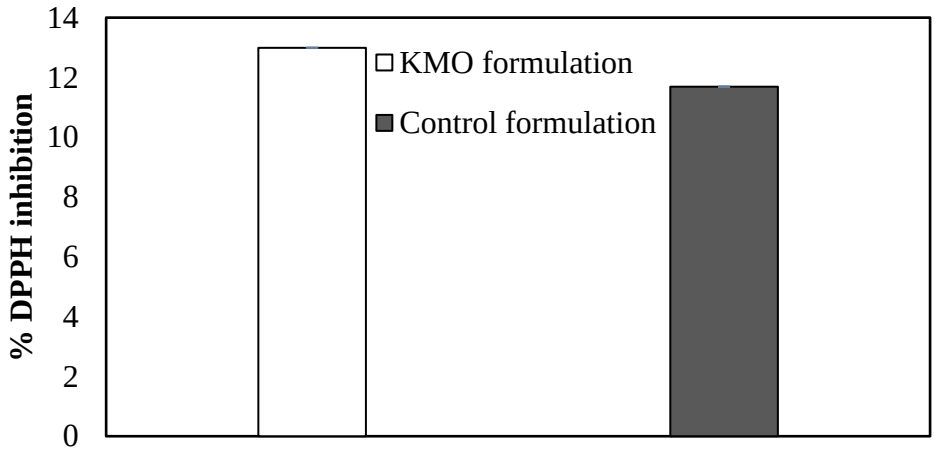


**Figure 6**. DPPH inhibition (%) of KMO formulation and control formulation at 100 µg/mL concentration.

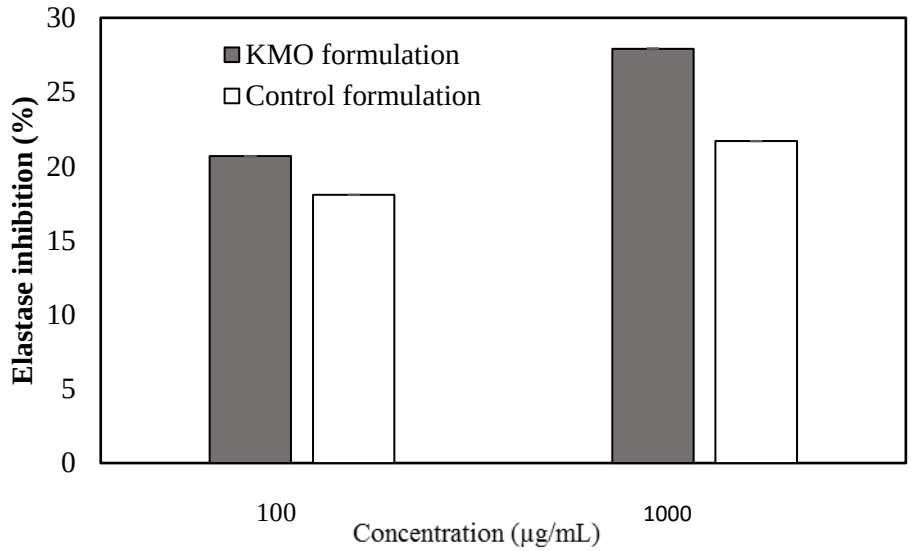


**Figure 7**. Elastase inhibition (%) of KMO formulation and control formulation at 100 µg/mL and 1000 µg/mL concentration.

Phenolics and flavonoids are able to reduce DPPH radicals due to their hydrogen donating ability [46]. The results obtained in this study show that DPPH radical scavenging activities of the KMO formulation and the control formulation might be contributed by their hydrogen donating ability. The phenolic and flavonoid contents of the control formulation might be contributed by *Citrus limon* (lemon) essential oil, as clearly stated by a previous study that lemon essential oil exhibits strong antioxidant activities [47]. The ability of the formulations to scavenge free radicals would contribute to the protection of the skin from side effects of UV rays, such as hyperpigmentation and skin aging [42].

**8. Assessment of Elastase Inhibition**

Figure 7 shows elastase inhibition of the KMO formulation and the control formulation at 100 and 1000 µg/mL. The KMO formulation inhibited elastase by 20.68% and 27.91% at 100 and 1000 µg/mL, respectively. While the control formulation inhibited elastase by 18.07% and 21.69%, respectively. The inhibitory effect of the formulations could be correlated to total polyphenol and flavonoid contents in KMO [48]. These results support the capability of the KMO formulation to scavenge free radicals (in antioxidant part), hence the ability to inhibit elastase and contribute to the prevention of skin aging apart for its main function to treat hyperpigmentation. The previous researcher mentioned that phenolic compounds, such as flavonoids and tannins, are the primary cause for the inhibitory effect on elastase and hyaluronidase activity [49]. To compare with another study, a cream containing polyherbal phytophospholipid complex for skin aging treatment inhibited elastase by 23% at 1000 µg/mL [20].

## CONCLUSION

The KMO formulation exhibited inhibition towards both tyrosinase and melanin. It also contained phenolics and flavonoids. It exhibited antioxidant activity, as confirmed by DPPH radical inhibition assay. The formulation also showed good elastase inhibition. It was also confirmed to be non-irritant when tested using EpiDerm™ tissue culture. In short, these findings suggest that the KMO formulation could provide good effects if applied to the skin frequently in terms of reducing hyperpigmentation and wrinkles.

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## AUTHOR CONTRIBUTION STATEMENT

Nur Farzana Jaslina performed the experiments, analysed the data, and wrote the article. Siti Efliza Ashari conceived and designed the experiments. Rosfarizan Mohamad and Nur Hana Faujan participated in data analyses. The authors declare that they have no conflict of interest in their authorship or publication of this contribution.

## DISCLOSURE STATEMENT

There is no conflict of interest

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