Antioxidant, antimicrobial and immunomodulatory activities of facial serum formulation containing *Melaleuca cajuputi* essential oil

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ABSTRACT

**Aims:** The aim of the study was to evaluate the *in vitro* antioxidant, antimicrobial and immunomodulatory activities of facial serum that was formulated with *Melaleuca cajuputi* essential oil (MCEO).

**Methodology and results:** The MCEO was obtained from the leaves of *M. cajuputi* and it was added into facial serum formulations in different concentrations (1%, 2% and 4%, v/v). The antioxidant potential of the formulated facial serum was evaluated using a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The time-kill assay was used to test the antimicrobial activity kinetics of different facial serum formulations against *Cutibacterium acnes*. The immunomodulatory activities of the formulations were tested using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, cytokine assay and Griess assay. The MCEO-formulated facial serum showed significantly higher (p<0.05) antioxidant activity at 2% (v/v) and 4% (v/v) when compared to the facial serum without MCEO. Time-kill kinetic assay had shown the MCEO formulated facial serum (1%, 2% and 4% v/v) exhibited a bactericidal effect against *C. acnes* in a concentration-dependent manner after 4-8 h. MCEO formulated facial serum at concentrations of 1%, 2% and 4% (v/v) significantly enhanced (p<0.05) the viability of the macrophages after 48 h. The level of cytokines such as IFNγ, TNFα, TGFβ1, IL6, IL17 and IL23 was significantly high (p<0.05) in the treated macrophages at a concentration of 4%. Macrophages treated with MCEO-formulated facial serum at 4% concentration exhibited significantly higher nitric oxide production (p=0.018) than the untreated macrophages.

**Conclusion, significance and impact of study:** MCEO-formulated facial serum possesses antioxidant, antimicrobial and immunomodulatory activities. This study highlights MCEO as a potential natural ingredient in facial serum formulations for cosmeceutical industries.

**Keywords:** Antioxidant, antimicrobial, facial serum, immunomodulator, *Melaleuca cajuputi* essential oil

INTRODUCTION

*Cutibacterium acnes*, previously recognized as *Propionibacterium acnes* is a facultative anaerobic Gram-positive bacterium that appears as a normal microbiota on human skin (Brook and Frazier, 1991). Sebaceous gland-rich sites of the skin, such as the face and the upper trunk, are the areas where an ample amount of *C. acnes* can be found (Perry and Lambert, 2011). Even though the bacterium helps in the maintenance and support of the skin’s natural microbial balance, it is not always beneficial and can cause the pathogenesis of acne vulgaris. Acne, which is a chronic inflammatory and recurrent skin condition, is a disease that involves blockage and inflammation of the pilosebaceous unit, such as hair, hair follicle, erector pili muscle and sebaceous gland. Being recognized as one of the most prevalent diseases globally, acne vulgaris has affected more or less 10% of the world’s population (Hay et al., 2014). Acne can cause severe impacts on patients’ social and psychological well-being despite it being a non-life-threatening disease. The impacts are far more prominent for patients with severe symptoms such as the occurrence of scarring. Patients with acne often apply topical skin care products with antiseptic properties to manage their dermatologic conditions. Antimicrobial therapies are effective in alleviating the symptoms by reducing the abundance of *C. acnes* on the skin (McLaughlin et al., 2019). Various antimicrobial agents, including chlorhexidine, tricosan, alcohol and povidone-iodine, are utilised by cosmeceutical companies in their topical skin care products to treat bacterial skin infections (Hoang et al., 2021). Considering today’s rising concerns over the long-term safety effects of synthetic chemicals in...
cosmetic formulations, the consumers and cosmeceutical industries have turned towards the use of natural ingredients.

Among the potential natural medicinal plants to be investigated for its use in topical skincare products is *Melaleuca cajuputi*, locally known as ‘pokok kayu putih’ or ‘pokok gelam’. *M. cajuputi* is a medicinal plant in the Myrtaceae family. The plant has been used traditionally for the treatment of bruises, flu, gout, insect bites, rheumatism, skin infections and toothache (Shariff-Rad et al., 2017). Several studies have shown that *M. cajuputi* oil, commonly known as cajuput oil, which is extracted from leaves and flowers, possesses antioxidant properties (Al-Abd et al., 2015; Noor et al., 2020). In addition, the antibacterial activity of cajuput oil against Gram-positive and Gram-negative bacterial strains including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Salmonella enterica* have been identified in numerous studies (Steir et al., 2013; Al-Abd et al., 2016; Dahiya, 2016; Bua et al., 2020).

Skincare regimes and cosmetics are part of most people’s daily routines. Serum products are often used for facial cleansing, beauty maintenance as well as to reduce acne. In light of the growing concern towards the usage of chemical-based products, which consist of harmful ingredients and increasing antibiotic resistance due to antibiotic use in acne treatment, scientific research that seeks evidence for the efficacy of natural-based products are needed urgently (Dessiniotia and Katsambasb, 2022). The role of *M. cajuputi* essential oil (MCEO) as an antimicrobial agent against *C. acnes* and its potential as a natural ingredient to be formulated in facial serum for skincare and facial cleanser is still unclear. This study reports scientific information about the in vitro antioxidant, antimicrobial and immunomodulatory activities of different concentrations of MCEO facial serum formulation.

**MATERIALS AND METHODS**

**Plant material and extraction**

*Melaleuca cajuputi* leaves had been obtained in Pasir Puteh, Kelantan, Malaysia. Species authentication was certified by Kulliyah of Pharmacy, International Islamic University Malaysia (IIUM) (voucher number: PIIUM 0304). The MCEO was extracted from the leaves via steam distillation (Noor et al., 2020). The MCEO was stored at 4 °C before use.

**MCEO-formulated facial serum**

Facial serum (a prototype) was prepared by a local cosmetic manufacturer in Kota Bharu, Kelantan. MCEO and Tween 20 (50%, v/v) diluted in phosphate-buffered saline were mixed at a 1:1 ratio by using the mechanical homogenizer. Then, the MCEO was aseptically added to the facial serum and constantly stirred at room temperature to final concentrations of 1%, 2% and 4% (v/v). The MCEO-formulated facial serum was stored at 4 °C prior to use.

**Test microorganisms**

*Cutibacterium acnes* (ATCC-11827) were purchased from the American Type Culture Collection (ATCC, USA) and stored at -20 °C before use.

**Cell line**

Mouse macrophage cell line J774A.1 (ATCC#TIB-67) was bought from the American Type Culture Collection (ATCC, USA). Supplemented with 10% fetal bovine serum (FBS) (Caisson Labs, USA) and 1% penicillin-streptomycin (Gibco, USA), these cells were cultured in a complete medium comprising of fresh Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) media. Following that, the cells were incubated in an incubator at 37 °C with 5% carbon dioxide (CO₂).

**Inoculum preparation**

*C. acnes* was grown on nutrient agar (NA; Oxoid, Belgium) for 3 days at 37 °C under anaerobic conditions by using the Anaerogen Compact system (Oxoid, Belgium). The inoculum was freshly prepared by suspending the colonies in nutrient broth (NB; Oxoid, Belgium), followed by an adjustment to a turbidity of 0.5 McFarland standards (1.5 × 10⁶ CFU/mL) based on the optical density (OD) measurement at 620 nm.

**Determination of antioxidant activity**

The antioxidant activity of the MCEO-formulated facial serum was determined by using a DPPH radical scavenging assay based on the procedure described in the past study (Al-Abd et al., 2015). An aliquot (120 µL) of 0.25 mM DPPH solution (Sigma Aldrich, USA) in methanol (Nacalai Tesque, Japan) and 30 µL of the formulations without MCEO and with MCEO at 1%, 2% and 4% (v/v) concentrations were mixed vigorously. Then, the mixture was left in the dark for 30 min at room temperature. The absorbance was measured at 518 nm using a microplate reader (Bio-Rad, USA). DPPH in methanol was used as a blank, while the ascorbic acid (40 mg/mL) (Sigma Aldrich, USA) was utilised as a positive control. The following formula was used to calculate the percentage of antiradical activity (AA%) of the extracts (Al-Abd et al., 2015):

\[
AA\% = 100 - \left[ \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}}} \right] \times 100
\]

Where \(\text{Abs}_{\text{sample}}\), \(\text{Abs}_{\text{blank}}\) and \(\text{Abs}_{\text{control}}\) are the absorbance values of the extract, blank and control samples, respectively.
Time-kill kinetic assay

A time-kill assay was performed against the C. acnes using MCEO-formulated facial serum at concentrations of 1%, 2% and 4% (v/v). C. acnes was grown on nutrient agar in a Petri dish for 3 days at 37 °C under anaerobic conditions by using the Anaerogen Compact system (Talib and Saleh, 2015). Individual colony of C. acnes was isolated and suspended in sterile normal saline. The density of the bacterial culture was adjusted to a 0.5 McFarland standard. This suspension was diluted 1:100 in sterile nutrient broth. Following that, the MCEO-formulated facial serum was added, and the test tubes were incubated anaerobically for 24 h at 37 °C. Five µL of the inoculated broth were aseptically inoculated onto nutrient agar plates at time intervals of 0, 2, 4, 8, 12 and 24 h. The agar plates were incubated in an anaerobic system at 37 °C for 48 h. The untreated C. acnes were used as growth control while C. acnes treated with vancomycin (Sigma Aldrich, USA) (2 µg/mL) were utilized as a positive control. The procedure to determine the colony-forming unit (CFU) of the C. acnes was performed in triplicate. A graph of log CFU/mL was plotted against time.

MTT assay

A total of 2 × 10⁵ macrophages (J774A.1) per well were incubated in three 96-well plates in a CO₂ incubator for 24 h. The medium (DMEM) was discarded, and the macrophages were treated with 100 µL of medium containing formulated facial serum with MCEO (1%, 2% and 4%, v/v) and without MCEO (0%). The untreated wells were used as negative controls. The macrophages treated with 5 µg/mL lipopolysaccharide (LPS) were used as positive control. The plates were incubated for 24 h, 48 h and 72 h in a CO₂ incubator at 37 °C. Once the medium was discarded, 10 µL of MTT (Nacalai Tesque, Japan) solution (5 mg/mL) and 90 µL of medium were added into each well. Then the plates were incubated for 4 h in a CO₂ incubator at 37 °C. 50 µL of stop solution (DMSO; Sigma Aldrich, USA) was added, and the plates were incubated for 30 min. The optical densities (OD) were measured at 570 nm by using a microplate reader (Bio-Rad, USA). The percentage of viability was calculated using the following formula (Lin and Lin, 2020):

\[
\text{Viability} = \frac{\text{OD}_{\text{treated cells}}}{\text{OD}_{\text{untreated cells}}} \times 100
\]

Cytokine assay

For the detection of mouse T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17) related cytokines, cytokine assay was performed using a multi-analyte ELISArray kit (Qiagen, USA) according to the manufacturer’s instructions with cell culture supernatants as samples. The cells were seeded with 5 × 10⁴ cells/mL in a 96-well plate and incubated overnight at 37 °C before being treated with MCEO-formulated facial serum at a concentration of 4% (v/v). After 48 h of incubation, the cell culture supernatants were collected and analyzed based on the manufacturer’s instructions (according to the layout of the catalogued multi-analyte ELISArray kit). Through a microplate reader (Bio-Rad, USA), the absorbance was measured at 450 nm within 30 min of stopping the reaction.

Griess reagent assay

The nitric oxide production by macrophages treated with four different concentrations of MCEO-formulated facial serum (0-4%, v/v) was determined using the Griess Reagent System (Promega, USA) based on the manufacturer’s instruction. The macrophages (1 × 10⁵ cells/well) were incubated in a 96-well plate in a CO₂ incubator at 37 °C for 24 h. Then the medium (DMEM) was discarded and the macrophages were treated with 100 µL of MCEO-formulated facial serum. After 72 h, the culture supernatant was collected for nitric oxide analysis. 50 µL of samples were added into a 96-well plate, and 50 µL of sulphanilamide solution was added to it, followed by incubation at room temperature for 10 min in the dark. Finally, 50 µL of NED (N-(1-Naphthyl) ethylenediamine) solution was added and incubated for 10 min at room temperature in the dark. Through a microplate reader (Bio-Rad, USA), the absorbance was measured at 540 nm. The concentration of nitric oxide was calculated by generating a standard curve using 50 µL of nitrite standard at different concentrations. The untreated macrophages served as a negative control and the experiment was performed in triplicate.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software version 8. Results are expressed as the mean of three readings ± standard error (SE). For the analysis of the DPPH radical scavenging activity and macrophage viability, the statistical significance (p<0.05) of the differences between means was assessed by an analysis of variance (ANOVA) and post-hoc test (Dunnett’s test). The statistical significance (p<0.05) of the differences between the means of cytokines level was assessed using a paired sample t-test. The statistical significance (p<0.05) of the differences between means of the nitric oxide concentration was assessed by an analysis of variance (ANOVA) and post-hoc test (Tukey’s test).

RESULTS

DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging assay of MCEO formulated facial serum showed antioxidant activity in a concentration-dependent manner which ranging from 23.09% to 73.55% (Figure 1). Formulation with 1% (v/v) MCEO demonstrated no significant difference in antioxidant activity as compared to facial serum without MCEO. However, at 2% (v/v) and
Figure 1: DPPH radical scavenging assay of MCEO formulated facial serum at different concentrations (*p<0.05).

4% (v/v), the facial serum displayed significantly high antioxidant activity.

Antibacterial activity against *C. acnes*

After 8 h of treatment, 1% (v/v) and 2% (v/v) MCEO-formulated facial serum exhibited bactericidal effects, reducing the starting log CFU/mL (5.477) by greater than 3 log$_{10}$ CFU/mL after 4 h of treatment. 4% (v/v), the facial serum displayed significantly high antioxidant activity.

Viability of macrophages J774A.1

The facial serum formulated with MCEO improved the viability of macrophage cells. The viability of macrophages was significantly enhanced compared to the untreated macrophages at concentrations of 2% (v/v) and 4% (v/v) (Figure 3). Meanwhile, the viability of macrophages treated with facial serum without MCEO showed no significant differences with untreated macrophages at 24, 48 and 72 h.

Cytokine level

Studies on the effect of cytokine level following treatment with the 4% (v/v) MCEO-formulated facial serum had shown that the interleukin (IL)-6, IL17A, IL23, Interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα) and transforming growth factor-beta (TGF-β1) were significantly higher in treated macrophages (Figure 4). Other cytokines including IL2, IL4, IL5, IL10, IL12 and IL13, however had shown no significant differences between treated and untreated macrophages.

Nitric oxide production

The results of the nitric oxide assay are shown in Figure 5. The macrophages treated with 4% (v/v) MCEO-formulated facial serum demonstrated significantly (p=0.018) higher nitric oxide production as compared to the untreated macrophages. There was no significant difference between the nitric oxide production of treated macrophages (0%, 1% and 2%, v/v) as compared to the untreated macrophages. In addition, nitric oxide production was significantly higher in macrophages treated with 4% (v/v) MCEO-formulated facial serum as compared to the facial serum without MCEO (p=0.031).

Figure 2: Time-kill curves of *C. acnes* treated with MCEO formulated facial serum at different concentrations.
Figure 3: Viability of macrophages (J774A.1) treated with different concentrations of MCEO formulated facial serum after 24, 48 and 72 h (*p<0.05).

Figure 4: Th1- Th2- Th17-related cytokines analysis of macrophages after 48 h treatment with 4% MCEO formulated facial serum (*p<0.05).

DISCUSSION
Rising concerns about the long-term safety effects of synthetic antimicrobial chemicals in cosmetic formulations have shifted the cosmeceutical industries toward the utilization of natural ingredients (Thiyagarasaiyar et al., 2020; Morais et al., 2021). The antimicrobial agent from natural plants offers a promising potential to enhance the effectiveness of topical skin care products by inhibiting the growth of C. acnes (Sinha et al., 2014; Orchard and van Vuuren, 2017; Rybczyńska-Tkaczyk et al., 2023). In this study, we investigated the antioxidant, antimicrobial and immunomodulatory activity of a facial serum that was formulated with natural essential oil from local M. cajuputi leaves. The radical scavenging activity of the MCEO- serum formulation was increased in a dose-dependent manner. A similar finding was reported previously in which high antioxidant activity was highlighted in M. cajuputi solvent extracts (Batubara et al., 2009; Al-Abd et al., 2015). The high scavenging activity exhibited by this plant extracts is believed to be attributed to the phenolic contents of the sample, flavonoid and the presence of hydroxycinnamic acids such as caffeic acid phenyl ester.
Figure 5: The nitric oxide production by macrophages treated with MCEO serum formulation at 0%, 1%, 2% and 4% (v/v) concentrations (*p<0.05).

(Mishra et al., 2012; Loganayaki et al., 2013). The antioxidant activity of MCEO in cosmeceutical products will be beneficial for anti-aging and preventing oxidative damage to the skin cells and tissues caused by free radicals (Addor, 2017).

The bactericidal effects of MCEO formulations were observed at 1%, 2% and 4% (v/v) concentrations. The bactericidal effect is defined as an agent that is capable of reducing more than 3-fold of log_{10} colony forming unit (CFU/mL) or inhibiting 99.9% of bacterial growth from the initial inoculum (Aung et al., 2016). In this study, the minimum concentration of MCEO in the formulation that was capable of preventing *C. acnes* growth was 1% (v/v). The growth of *C. acnes* was exponentially dropped after four hours of treatment. The killing rate of the formulation was progressively increased at higher concentrations to reflect the concentration-dependent killing effect. Different concentrations might induce different killing efficacy over the course of treatment. Antimicrobial agents at higher concentrations would alter various biological responses in the bacteria to induce effective and rapid cell death (Bernier and Surette, 2013). The killing rate of the MCEO formulation was significantly higher at 4% (v/v) compared to the untreated macrophages and macrophages treated with formulation without the addition of MCEO. The findings indicate that the formulation appears to have no cytotoxic effects in vitro. To our knowledge, this is the first study that investigates the effect of MCEO on macrophage viability. Our findings suggest that MCEO may improve host immunological responses against bacterial infections by stimulating macrophage growth.

Cytokines are the chemical signaling molecules responsible for most of the biological effects in the immune system, including inflammatory responses. TNFα is a cytokine that is involved in the acute phase of inflammation by the recruitment of neutrophils and the activation of arachidonic acid metabolism (Naseri et al., 2018). IFNγ is a TH1 cytokine that is predominant in innate immunity against intracellular bacteria and protozoa. The IFNγ induces a high level of pro-inflammatory cytokines by stimulating the macrophage activation to further promote inflammatory responses and enhance macrophage phagocytic ability to eliminate the invading intracellular pathogen (Mosser and Edwards, 2008; Raetz et al., 2013). In this study, we observed significantly higher anti- and pro-inflammatory cytokine expressions following treatment with 4% (v/v) MCEO facial serum formulation. This result explains the higher level of TNFα expressions as the IFNγ might be responsible for stimulating the macrophage to express TNFα. A higher level of TNFs suggests that MCEO may stimulate inflammation, which is normally beneficial for the host to fight against any invading intracellular pathogens. However, a prolonged inflammatory process has been reported to cause tissue damage (Abdulkhaleq et al., 2018). Besides, the 4% (v/v) MCEO-serum formulation enhanced the IL6, IL17 and IL23 expressions of the treated macrophages. IL6 and IL17 are...
proinflammatory cytokines involved in the generation and propagation of inflammation (Tanaka et al., 2014). IL23 works in conjunction with IL6 to promote the development of Th17 cells (Duvallet et al., 2011). As mediators of cellular immunity, Th17 has a crucial part in activating other immune cells, such as B cells and cytotoxic T cells, as well as in the regulation of immune responses (Tesmer et al., 2008).

Nitric oxide (NO) is a signaling molecule that plays an important role as the biological mediator and regulator of inflammation (Zhai et al., 2009). This study showed that macrophages treated with serum formulation containing 4% (v/v) MCEO produced significantly higher levels of NO than macrophages treated with lower concentrations (1-2%). It suggests that a high concentration of MCEO might induce an inflammatory response and may cause tissue destruction (Botta et al., 2008). High NO levels might be attributed to the stimulation of inducible NO synthase (iNOS) expression by TNFα (Naseri et al., 2018). An improved understanding of MCEO's immunomodulatory activity will assist in making the decision regarding its application as a value-added ingredient in cosmeceutical formulations.

CONCLUSION

This study demonstrated the promising antioxidant, antibacterial and immunomodulatory potential of MCEO constituents in facial serum formulations. MCEO could enhance the antioxidant and immunomodulatory activities and, therefore, potentiate the bactericidal effects of the formulated facial serum against C. acnes growth. The findings also suggest that MCEO has potential applications as an antimicrobial agent in topical skincare products at minimum effective concentrations since a higher concentration of MCEO might stimulate unwanted inflammatory responses. Further research, however, is necessary to examine its mechanisms for the observed bioactivities, in vivo efficacy and toxicity.

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