

## Properties of macerated herbal oil

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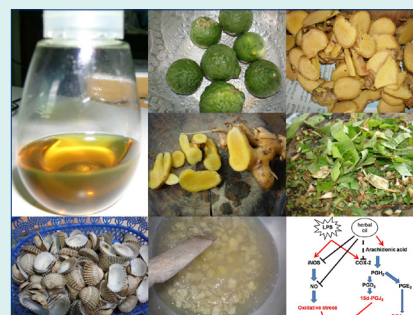
### Abstract

**Introduction:** The addition of herbs into hot sesame oil could increase the oil-pulling efficiency of sesame oil. The aim of present study was to modify the proportion of herbs and sesame oil with the addition of other ingredients including menthol, camphor, and borneol and improve the medicinal properties and the scent of the oil.

**Methods:** Macerated herbal oil was prepared by heat extraction of five species of herbs (*Zingiber cassumunar*, *Zingiber zerumbet*, *Plantago major* Linn, *Citrus hystrix*, and *Amomum biflorum*) with hot sesame oil. The study was performed to evaluate the anti-oxidant, anti-inflammatory, and anti-bacterial properties of this macerated herbal oil.

**Results:** Macerated herbal oil was evaluated for antioxidant activity using DPPH and ABTS assays. It was shown that at dilution 1:2 in DMSO, the macerated herbal oil had DPPH and ABTS radical scavenging activities equal to 63% and 22%, respectively. Macerated herbal oil dilution 1:8 in DMSO demonstrated ferric reducing capacity equivalent to ascorbic acid (0.208 μM) and had reducing power equivalent to *butylated hydroxytoluene* (BHT) 7.41 μg/mL. MTT assay was performed using immortalized human mesenchymal stem cells (HMSCs) as a cell culture model. The result indicated that the cytotoxic concentration of the macerated herbal oil was  $\geq 2.5$  μL/mL in complete DMEM. Anti-inflammatory effects were evaluated using the nitrite assay and RT-PCR. It was found that the macerated herbal oil could inhibit nitrite accumulation in culture media. Change in the expression of COX-2, Nrf2, and NF-κB in RT-PCR confirmed the anti-inflammatory activity of the macerated herbal oil.

**Conclusion:** It could be concluded that the macerated herbal oil could inhibit nitrite accumulation in culture media, which might be the inhibitory effect of the macerated herbal oil on COX-2 or Nrf2, the downstream modulator of the COX-2 pathway. Further intensive studies are needed for the optimization before bringing this macerated herbal oil into clinical application.



### Introduction

Macerated herbal oil has been used as a remedy for joint, muscle, and skin inflammation in India and Southeast Asia since the ancient times and even in the present time. It is well known that sesame oil has anti-inflammatory properties and has always been used as an alternative medicine in many parts of Asia.<sup>1</sup> Currently, there are many scientific studies trying to prove that sesame oil has therapeutic effects, including: prevention of oxidative stress and lipid peroxidation in liver,<sup>2,3</sup> restoration of kidney function,<sup>4</sup> healing of intestinal and cardiac

systems,<sup>5,6</sup> relieving pneumonia and bronchitis in rats,<sup>7</sup> and osteoprotection.<sup>8</sup> Sesame oil contains important ingredients such as sesamol,<sup>9-12</sup> linoleic acid, and oleic acid,<sup>13</sup> which might play important roles in rendering such therapeutic effects.

Sparingly water soluble ingredients in herbs are insoluble in water but can be dissolved in solvents like methanol, ethanol, or acetone. According to the polarity of phytoconstituents, the solvents for extraction are optional. Hexane is a solvent that can extract most of the fatty acids, lipids, waxes, steroids, triterpenoids, steroidal

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alkaloids and non-polar components. Methanol is suitable for the extraction of components like saponins, alkaloids, glycosides, carotenoids, flavonoids, and phenolics. Hot oil maceration is the method for extracting the phytoconstituents because maceration is a simple and effective method for extracting oil soluble components from herbal material. Sesame oil has medicinal properties of its own that can be utilized as an adjunct to the healing properties of the macerated herbs. Then Sesame oil was selected in this study. Moreover, the use of sesame oil did not need the hexane or methanol elimination process. For example, sabinene and terpinen-4-ol in *Zingiber cassumunar*<sup>14,15</sup> and zerumbone in *Zingiber zerumbet*<sup>16</sup> are water insoluble, so sesame oil can be employed to extract these substances from herbs. Moreover, the combination of sesame oil and herbal ingredients might provide benefits in terms of preserving the quality of active ingredients and preventing the lipid oxidation.

In the present study, sesame oil was heated with five species of herbs. These herbs have been widely studied and are readily available in local markets. The selected species of herbs were the ones traditionally used, including *Z. cassumunar*, *Z. zerumbet*, *Plantago major* Linn, *Citrus hystrix*, and *Amomum biflorum*. The active ingredients in the herbs have specific characteristics as follows:

*Zingiber cassumunar* Roxb.: This species has been widely researched and it was shown that terpinen-4-ol was the active compound which caused leukemic cells apoptosis.<sup>14</sup> It also has anti-inflammatory and anti-cancer properties.<sup>17-21</sup>

*Zingiber zerumbet* (L.) Smith: *Z. zerumbet* has many research results corroborating its medicinal properties. Its active ingredient zerumbone has antitumor,<sup>22</sup> antinociceptive,<sup>23</sup> anti-inflammatory, and antimicrobial properties.<sup>24,25</sup> Moreover, the extract of this plant was proven to be nontoxic to cells, without any genetic mutation.<sup>26</sup>

*Plantago major* Linn: *P. major* is recognized in the family Plantaginaceae. A variety of researches have demonstrated the antiviral, antileukemic, and wound-healing effects of this plant,<sup>27,28</sup> together with its antioxidant and anticancer activities.<sup>29,30</sup> The polysaccharide extract from the seeds of this plant have been used as a matrix for controlled release of drugs (drug-releasing matrix).<sup>31</sup>

*Citrus hystrix* DC: *C. hystrix* is a common tropical herb in the family Rutaceae. The leaves and peels of *C. hystrix* have antimicrobial and antioxidant properties,<sup>32,33</sup> because this plant contains many phenolic compounds.<sup>34</sup> The essential oils from the leaves and peels of *C. hystrix* demonstrate anti-bacterial effects on pathogens in the respiratory tract.<sup>35</sup>

*Amomum biflorum* Jack: *A. biflorum* is in the family Zingiberaceae. This plant has been proven to have high contents of total phenolic compounds with antioxidant and anti-tyrosinase properties.<sup>36</sup>

To improve the properties of macerated herbal oil, the proportion of herbs and sesame oil are needed to be modified. In the present study the amount of herbs and

sesame oil were modified and other ingredients such as menthol, camphor, and borneol were added to improve the scent of the oil. The bioactivity of this traditional medicine was tested with human mesenchymal stem cells (HMSCs) because HMSCs exhibited changes in the expression level of inflammatory mediator gene under various conditions.<sup>37,38</sup> Before bringing this alternative medicine into clinical application as a topical drug for local inflammation or infection, the quality of the macerated herbal oil should be proved and improved for better therapeutic outcome.

## Materials and methods

### Macerated herbal oil preparation and total phenolic content

All the herbs were washed and sliced. A volume of 1500 mL of cold pressed sesame oil (purchased from a local manufacturer) was heated with 300 g of crushed cockle shells on a charcoal stove for 10 minutes. The quantities including 400 g of *Zingiber cassumunar*, 200 g of *Z. zerumbet*, 50 g of *P. major* Linn, 200 g of *C. hystrix* peels, 50 g of *C. hystrix* leaves, and 100 g of *A. biflorum* were added into the heated sesame oil. All the components were slowly stirred for 1.5 hours. Finally, 150 g of menthol, 50 g of camphor, and 50 g of borneol were added to improve the scent of the oil. After 1.5 hours of heat treatment, the oil was filtered through a delicate sieve and left for cooling down. The herb residual was separated using Whatman No. 1. Clear yellow oil with the soft aroma of the herbs was aliquoted in closed containers before storing at 4°C until use. Main components of macerated herbal oil are shown in Table 1.

Total phenolic assay was performed by the following method. Briefly, the macerated herbal oil was diluted with dimethyl sulfoxide (DMSO) to the dilutions of 1:2, 1:4, 1:8, 1:16, and 1:32. Thereafter, 50 µL of each dilution was mixed with 2.5 mL Folin Ciocalteu reagent, and 2.0 mL Na<sub>2</sub>CO<sub>3</sub> was added and mixed before incubating at 45°C for 15 minutes. A blue color was observed, and the absorbance was measured at a wavelength of 764 nm. The mixture of

**Table 1.** Main components of macerated herbal oil

Components	Weight percent (wt%)
Sesame oil	47
Crushed cockle shells	10
<i>Zingiber cassumunar</i> Roxb.	13
<i>Zingiber zerumbet</i>	7
<i>Plantago major</i> Linn.	2
Peels of <i>Citrus hystrix</i>	7
Leaves of <i>Citrus hystrix</i>	2
<i>Amomum biflorum</i>	3
Menthol	5
Camphor	2
Borneol	2
Total	100

2.5 mL Folin Ciocalteu reagent and 2.0 mL  $\text{Na}_2\text{CO}_3$  was used as blank for the zero setting. A standard calibration curve was prepared using different concentrations of gallic acid in methanol. Three replicate assays were performed for each dilution, and the total phenolic concentrations were calculated from the standard curve and reported as mg/mL equivalent to standard gallic acid.

#### **DPPH radical scavenging and ABTS assay**

DPPH radical scavenging and ABTS assay were performed. The macerated herbal oil was diluted with DMSO to the dilutions of 1:2, 1:4, 1:8, and 1:16. Then 50  $\mu\text{L}$  of each dilution was mixed with 2,950  $\mu\text{L}$  of 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent and allowed to react in the dark at room temperature for 15 minutes. The absorbance was measured at a wavelength of 515 nm using distilled water to set zero. Standard trolox was used as the positive control. DMSO solution of volume 2,950  $\mu\text{L}$  plus 50  $\mu\text{L}$  of each dilution was used as a sample blank. DPPH solution of volume 2,950  $\mu\text{L}$  plus 50  $\mu\text{L}$  DMSO was used as a control for calculation. Three replicate assays were performed for each dilution, and the %DPPH scavenging activity was calculated by equation (Eq. 1):

$$\% \text{Inhibition} = \frac{\text{Absorbance control} - (\text{Absorbance sample} - \text{Absorbance sample blank})}{\text{Absorbance control}} \times 100 \quad (\text{Eq. 1})$$

For the ABTS assay, 10  $\mu\text{L}$  of each dilution was mixed with 990  $\mu\text{L}$  of working 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) reagent (14 mmol/L ABTS reagent plus 5 mmol/L potassium persulfate solution in the ratio 1:1). The mixture was allowed to stand for 1 minute before measuring at 734 nm using distilled water to set zero. Standard trolox was used as the positive control. Working ABTS reagent should have absorbance around  $0.700 \pm 0.02$  (0.68–0.72) at 734 nm. Three replicate assays were performed for each dilution and the inhibition percentage was calculated by Eq. 2:

$$\% \text{Inhibition} = \frac{\text{Absorbance working reagent} - \text{Absorbance sample}}{\text{Absorbance working reagent}} \times 100 \quad (\text{Eq. 2})$$

#### **Ferric reducing capacity and reducing power**

The macerated herbal oil was diluted with DMSO to the dilutions of 1:8, 1:16, 1:32, 1:64, and 1:128. Then 60  $\mu\text{L}$  of each dilution was mixed with 1,800  $\mu\text{L}$  of the cocktail solution. After that, 180  $\mu\text{L}$  of deionized water was added to the reaction tube. The reaction was mixed and the absorbance was detected at 593 nm as a baseline. The reaction mixture was incubated again at 37°C for 4 minutes, following which the absorbance of the mixture was measured again at 593 nm within 4 minutes. Three replicate assays were performed for each dilution. Various concentrations of ascorbic acid were used to generate the standard curve. The ferric reducing capacity was calculated from the standard curve and reported as ferric reducing capacity equivalent to ascorbic acid.

For reducing power, 2.5  $\mu\text{L}$  of each dilution was mixed with 2.5 mL PBS. Then 2.5 mL of 1% potassium

ferricyanide ( $\text{K}_3\text{Fe}[\text{CN}]_6$ ) was added to the mixture and incubated at 50°C for 20 minutes. After the reaction cooled down, 2.5 mL of 10% trichloroacetic acid was added and mixed. The reaction was centrifuged at 3000 rpm for 10 minutes. A volume of 2.5 mL of the supernatant was separated and mixed with 2.5 mL of deionized water. A volume of 0.5 mL of 0.1% ferric chloride was added to the reaction and allowed to stand for 10 minutes. The absorbance was measured at 700 nm. Three replicate assays were performed for the statistical analysis. Various concentrations of *butylated hydroxytoluene* (BHT) were used to generate a standard curve. The reducing power was calculated from the standard curve and reported as reducing power equivalent to BHT.

#### **MTT assay and nitrite assay**

MTT assay was performed following the protocol manual of assay.<sup>39</sup> Immortalized HMSCs (passage 5) (*Health Science Research Resources Bank*, Osaka, Japan) were cultured at a seeding density of  $1 \times 10^5$  cells/well into a 6-well plate. Cells were allowed to adhere for 24 hours in complete Dulbecco's Modified Eagle Medium (DMEM) at 37°C and 5%  $\text{CO}_2$ . The macerated herbal oil was diluted in DMSO to the dilutions of 1:4, 1:8, 1:16, 1:32, and 1:64. Then 100  $\mu\text{L}$  of each dilution was added to 10 mL of complete DMEM. Thus, the final concentrations of the macerated herbal oil in 1 mL DMEM were 2.5  $\mu\text{L}$ , 1.25  $\mu\text{L}$ , 0.625  $\mu\text{L}$ , 0.313  $\mu\text{L}$ , and 0.156  $\mu\text{L}$ , respectively. After the cells were allowed to settle for 24 hours, the cell viability was determined using the MTT assay. Briefly, the entire DMEM was discarded and replaced with macerated herbal oil supplemented DMEM (3 mL/well). The cell culture was maintained at a temperature of 37°C in a 5%  $\text{CO}_2$  incubator for 48 h. The macerated herbal oil supplemented DMEM was discarded and replaced with 1 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (0.5 mg MTT in 1 mL complete DMEM). The reaction was maintained at 37°C in 5%  $\text{CO}_2$  for 2 hours. The MTT supplemented DMEM was discarded and 1 mL of DMSO was added. The absorbance of the formazan product was measured at 570 nm and the background was corrected at 630 nm. The percentage of the cell viability was compared to that of control which was cultured in DMEM plus DMSO (100  $\mu\text{L}$  DMSO in 10 mL DMEM). Three replicate assays were performed for the statistical analysis.

For the nitrite assay, the HMSCs (passage 5) were cultivated in a 6-well plate at a seeding density of  $1 \times 10^5$  cells/well using phenol red-free DMEM containing 10% FBS. After 24 hours, the cells were incubated with bacterial lipopolysaccharide (LPS; 2  $\mu\text{g}/\text{mL}$ ), macerated herbal oil (0.2  $\mu\text{L}/\text{mL}$ ), and LPS (2  $\mu\text{g}/\text{mL}$ ) plus macerated herbal oil (0.2  $\mu\text{L}/\text{mL}$ ) or DMSO for 24 hours, and then the culture media was subjected to the nitrite assay. Briefly, 800  $\mu\text{L}$  of the culture medium was mixed with 800  $\mu\text{L}$  of the Griess reagent and left at room temperature for 10 minutes. The absorbance was measured at 550 nm using water to set zero. A standard curve was plotted using serial concentrations of sodium nitrite. Three replicate assays

were performed for the statistical analysis.

### Relative quantitative real-time PCR

For the anti-inflammatory assay (pre-treatment), cells were incubated for 2 hours with the macerated herbal oil (0.2 µg/mL) before being treated with bacterial LPS (2 µg/mL), or DMSO (100 µL DMSO in 10 mL DMEM) for 24 hours. For the anti-inflammatory assay (co-treatment), cell culture was performed as described in the nitrite assay. In the anti-inflammatory assay (post-treatment), cells were incubated for 2 hours with bacterial LPS (2 µg/mL) before being treated with the macerated herbal oil (0.2 µg/mL), or DMSO (100 µL DMSO in 10 mL DMEM) for 24 hours. For the investigation of the redox gene expression upon long-term exposure, the HMSCs (passage 5) were seeded with complete DMEM containing 10% FBS into a 6-well plate at a seeding density of  $1 \times 10^5$  cells/well. After 24 hours, the cells were incubated with the macerated herbal oil (0.1 µL/mL) or DMEM + DMSO for 12 days.

After the culture period, the total RNA was extracted from the HMSCs using NucleoSpin® RNA II, according to the protocol provided by the manufacturer. A volume of 10 µL total RNA was treated again with 1 µL rDNase (Macherey-Nagel) and incubated at 37°C for 10 minutes. A 40 µL cDNA reaction volume was prepared with the use of 5× iScript Reverse Transcription Supermix (Bio-Rad), as described by the manufacturer's protocol. Reverse transcription was performed in Eppendorf Mastercycler® using the following program: priming at 25°C for 5 minutes, reverse transcription at 42°C for 30 minutes, and enzyme inactivation at 85°C for 5 minutes. cDNA was stored at -20°C until real-time PCR was performed. No-RT control, which contained all the reaction components except for the reverse transcriptase, was used to detect the contamination of the genomic DNA. The primer pairs for real-time PCR are shown in Table 2.

The cDNA was diluted 1:10 and RT-PCR was performed with a SYBR Green RT-PCR mastermix (Toyobo) in LightCycler480 (Roche). The 10-µL reaction was composed of 5 µL of SYBR Green RT-PCR master mix, 1 µL of cDNA template, 3.5 µL of distilled water, and 0.25 µM of target-specific primer. The polymerase chain reaction protocol consisted of 95°C pre-incubation for 30 seconds, followed by 45 cycles of 95°C for 5 seconds, 55°C for 10 seconds, and 74°C for 15 s. After the real-time PCR, a melting curve analysis was performed to demonstrate the

specificity of the PCR product as a single peak. A control, which contained all the reaction components except for the template, and no-RT control were included in all the experiments. Three replicate assays were performed for the statistical analysis. The relative quantification for the gene expression was normalized to GAPDH using Pfaffl method (Light Cycler®480 software release 1.5.0 SP4).

### Nitric oxide scavenging assay

Sodium nitroprusside generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions, can be measured by using the Greiss reagent. The macerated herbal oil was used as a scavenger of nitric oxide, which led to a decrease in the nitric oxide level. A volume of 2 mL of sodium nitroprusside (10 mM) was mixed with 0.5 mL of PBS and 0.5 mL of the macerated herbal oil dilutions of 1:256 and 1:512, and then incubated at 25°C for 150 minutes. After that, 0.5 mL of the previous reaction was mixed with 1 mL of 1% sulfanilamide and incubated at room temperature for 5 minutes. Thereafter, 1 mL of 0.1% naphthyl ethylenediamine dihydrochloride was added and incubated at 25°C for 150 minutes. The absorbance was read at 537 nm using water to set zero. The reaction of the same reagents with 0.5 mL DMSO was used as a control. The %inhibition was calculated by Eq. 3:

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

### Anti-bacterial activity

This experiment was performed in order to determine the lowest concentration of an antimicrobial agent that can kill bacteria in a given time. Briefly, the macerated herbal oil was diluted 10-fold in brain-heart infusion (BHI) broth ( $10^0$ – $10^{-7}$ ) and *Streptococcus mutans* (DMST 187777) was diluted in brain-heart infusion (BHI) broth to a concentration of 0.5 McFarland standard using an OD measurement at 600 nm. Thereafter, 100 µL of each 10-fold macerated herbal oil dilution and 100 µL of the bacterial suspension were added into each well of a 96-well tissue culture plate. As a negative control, an equivalent volume of BHI was added to the bacterial cultures instead of the 10-fold macerated herbal oil dilution. As a positive control, 0.12% chlorhexidine was added to the bacterial cultures instead of the 10-fold macerated herbal oil dilution. For the minimum bactericidal concentration measurement,

**Table 2.** Human target genes for relative quantitative PCR

Target genes	Primer sequences (5'→3')
Cyclooxygenase-2 (COX-2)	F: CCCTGGGTGTCAAAGGTAA R: GCCCTCGCTTATGATCTGTC
Nuclear factor erythroid-derived 2-related factor 2 (Nrf2)	F: AGTGGATCTGCCAACTACTC R: CATCTACAACGGGAATGTCTG
NF-kappa B (NF-κB)	F: ATGGCTTCTATGAGGCTGAG R: GTTGTGTTGGTCTGGATGC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: AAGGGCTCATGCCACAGTC R: GGATGACCTTGCCACAG

10  $\mu$ L aliquots from a 96-well tissue culture plate that were previously treated with macerated herbal oil were spread on the BHI agar plates and incubated at 37°C for 24 hours under anaerobic conditions.

### Statistical analysis

The student's *t* test and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test were used to determine the significant difference between the two samples from the three independent assays. The data are presented as mean  $\pm$  standard deviation (SD). The statistical significance was set at  $P < 0.05$ .

## Results

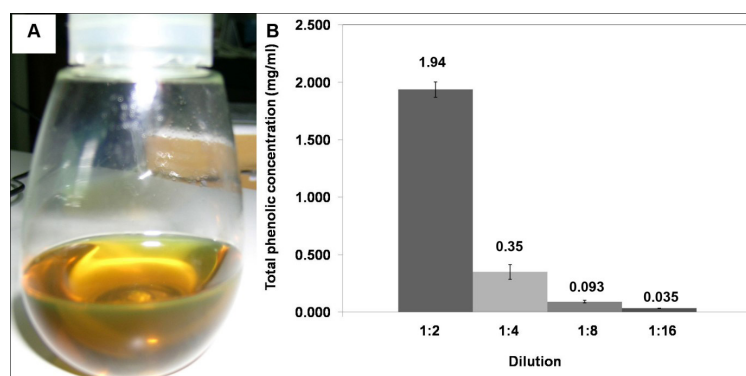
### Macerated herbal oil preparation

After 1.5 hours of heat treatment, the oil was filtered through a delicate *sieve*. The oil was then left for cooling down and the other residual was separated using Whatman No. 1. Clear yellow herb-scented oil was obtained, as shown in Fig. 1A, which was stored at 4°C until use. Macerated herbal oil was tested for the total phenolic content using the Folin Ciocalteu method. The result revealed that the macerated herbal oil dilution 1:2 had a total phenolic concentration equivalent to the concentration of gallic acid, 1.94 mg/mL. The concentration of the phenolic

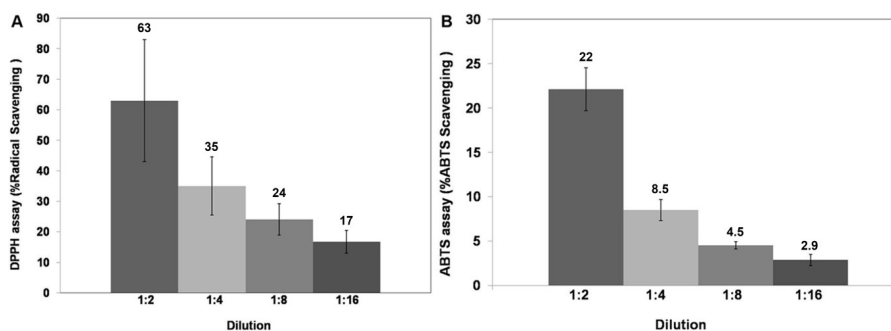
compound decreased unproportional to the dilution (Fig. 1B). This result indicated that phenolic compounds were present in the prepared macerated herbal oil.

### DPPH radical scavenging assay and ABTS radical scavenging assay

The antioxidant activity of the macerated herbal oil was tested using the DPPH assay and the ABTS assay. DPPH is a stable free radical with purple color. When the number of DPPH free radicals reduces, a yellow color is generated, and the reduction in their number results in the decrease of absorbance at 515 nm. The macerated herbal oil was diluted in DMSO to various dilutions. After the DPPH radicals had reacted with the macerated herbal oil, the absorbance was measured and compared to the absorbance of the DPPH reagent. The antioxidant activity of the macerated herbal oil was reported as %radical scavenging, as presented in Fig. 2A. The antioxidant activity of the macerated herbal oil was confirmed with the ABTS assay. ABTS reacted with potassium persulfate to generate ABTS free radicals which are blue in color. The reduction in the number of the ABTS radicals with several dilutions of the macerated herbal oil caused a decrease in the blue color, which could be measured at 734 nm. The %ABTS radical scavenging activity of the macerated herbal oil was calculated and is as presented in Fig. 2B.



**Fig. 1. (A) Macerated herbal oil:** clear yellow herb aroma oil was obtained in this experiment. **(B) Total phenolic concentration:** the macerated herbal oil was diluted in DMSO to dilutions 1:2, 1:4, 1:8, and 1:16 which had total phenolic concentrations equivalent to gallic acid 1.94 mg/mL, 0.35 mg/mL, 0.093 mg/mL, and 0.035 mg/mL, respectively.



**Fig. 2. DPPH and ABTS assays:** the macerated herbal oil was diluted in DMSO to dilutions of 1:2, 1:4, 1:8, and 1:16, and the antioxidant activity was evaluated using the DPPH assay (A) and the ABTS assay (B). The results were presented as %radical scavenging (%inhibition).

### Ferric reducing capacity and reducing power

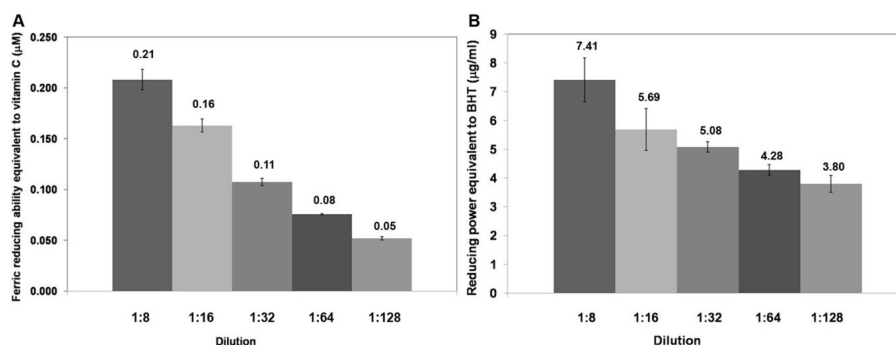
The ferric reducing capacity relies on the principle that the antioxidants can transfer electrons to the  $\text{Fe}^{3+}$  ions to become  $\text{Fe}^{2+}$  ions which have blue color. Absorbance can be measured at a wavelength of 593 nm.  $\text{Fe}^{2+}$  ions were formed after the reaction with the various concentrations of the macerated herbal oil, and the absorbance was compared to the standard curve. The antioxidant activity of the macerated herbal oil was reported as ferric reducing capacity equivalent to ascorbic acid (Fig. 3A).

The experiment was confirmed with reducing power which relies on a similar principle. Ferric ions ( $\text{Fe}^{3+}$ ) in the  $\text{FeSO}_4$  solution possess the ability to obtain electrons from other substances in order to form  $\text{Fe}^{2+}$ . Various concentrations of the macerated herbal oil were made to react with the  $\text{FeSO}_4$  solution, and then the absorbance was measured at 700 nm, which was proportional to the concentration of the ferrous ion ( $\text{Fe}^{2+}$ ). The increase in the absorbance indicated the considerable potential of the macerated herbal oil as a reducing agent. The absorbance was compared to the ability of BHT which was used to

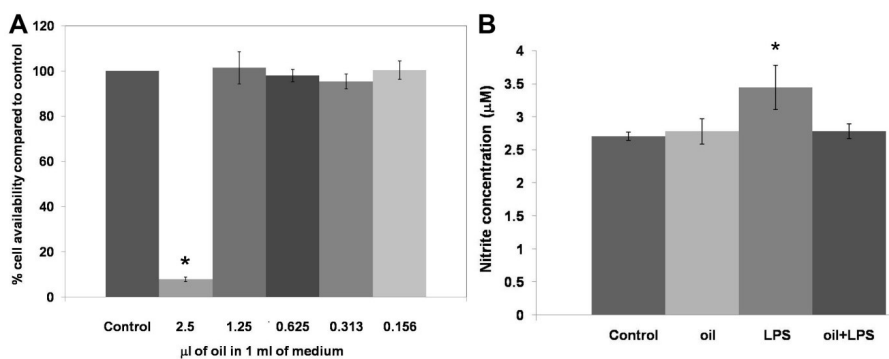
generate the standard curve. The result was reported as reducing power equivalent to BHT (Fig. 3B).

### MTT assay and nitrite accumulation assay

Various concentrations of the macerated herbal oil were prepared using 100  $\mu\text{L}$  of the macerated herbal oil dilutions at 1:4, 1:8, 1:16, 1:32, and 1:64 and were added to 10 mL of complete DMEM. Thus, the final concentrations of the macerated herbal oil in 1 mL DMEM were 2.5  $\mu\text{L}$ , 1.25  $\mu\text{L}$ , 0.625  $\mu\text{L}$ , 0.313  $\mu\text{L}$ , and 0.156  $\mu\text{L}$ , respectively. The MTT assay indicated that only the 2.5  $\mu\text{L}/\text{mL}$  concentration showed cytotoxic effect on the HMSCs (Fig. 4A). Taking into consideration the result of the MTT assay, the macerated herbal oil at the concentration of 0.2  $\mu\text{L}/\text{mL}$  (100  $\mu\text{L}$  of macerated herbal oil, dilution 1:50, in 10 mL of complete DMEM) was used for the nitrite assay. The accumulation of nitrite is an indicator of NO synthesis, which was measured in the culture medium using the Griess reagent. The HMSCs were seeded into a 6-well plate at a density of  $1 \times 10^5$  cells/well and treated with the macerated herbal oil with or without LPS (2  $\mu\text{g}/$



**Fig. 3 (A) Ferric reducing capacity:** the macerated herbal oil was diluted in DMSO to dilutions 1:4, 1:8, 1:16, and 1:32 which had ferric reducing capacities equivalent to ascorbic acid 0.351  $\mu\text{M}$ , 0.093  $\mu\text{M}$ , 0.035  $\mu\text{M}$ , and 0.006  $\mu\text{M}$ , respectively. **(B) Reducing power:** the macerated herbal oil was diluted in DMSO to dilutions 1:4, 1:8, 1:16, and 1:32 which had reducing powers equivalent to BHT 0.351  $\mu\text{g}/\text{mL}$ , 0.093  $\mu\text{g}/\text{mL}$ , 0.035  $\mu\text{g}/\text{mL}$ , and 0.006  $\mu\text{g}/\text{mL}$ , respectively.



**Fig. 4 (A) Cell viability:** various concentrations of the macerated herbal oil (2.5  $\mu\text{L}/\text{mL}$ , 1.25  $\mu\text{L}/\text{mL}$ , 0.625  $\mu\text{L}/\text{mL}$ , 0.313  $\mu\text{L}/\text{mL}$ , and 0.156  $\mu\text{L}/\text{mL}$ ) were incubated with HMSCs ( $1 \times 10^5$  cells/well) for 48 h. The cell viability was determined using the MTT assay and the %viability was compared to the control group (cells cultured in DMEM + DMSO). Only the 2.5  $\mu\text{L}/\text{mL}$  concentration showed cytotoxic effect on the HMSCs (\* $P < 0.05$ ). **(B) Nitrite assay:** the macerated herbal oil concentration 0.2  $\mu\text{L}/\text{mL}$  was used in this study. Three groups of the cell cultures were designed to evaluate the NO production. The cells ( $1 \times 10^5$  cells/well) were incubated with oil, LPS, and oil + LPS for 24 h. The nitrite assay was performed with the Griess reagent. The nitrite concentrations in the culture medium were compared to the control group (cells cultured in DMEM + DMSO). The cells treated with LPS (2  $\mu\text{g}/\text{mL}$ ) showed increased nitrite accumulation in the culture medium and nitrite accumulation was decreased in the presence of the macerated herbal oil (\* $P < 0.05$ , ANOVA, Tukey post hoc).

mL). It was demonstrated that LPS alone increased the concentration of nitrite in the culture medium. When LPS and the macerated herbal oil were presented together, the level of nitrite was not different from that of control. This result indicates that the macerated herbal oil had attenuated nitrite accumulation in the culture medium, which implies a reduction in the NO synthesis (Fig. 4B). The result obtained upon treating the HMSCs with the macerated herbal oil alone was not different from that of control.

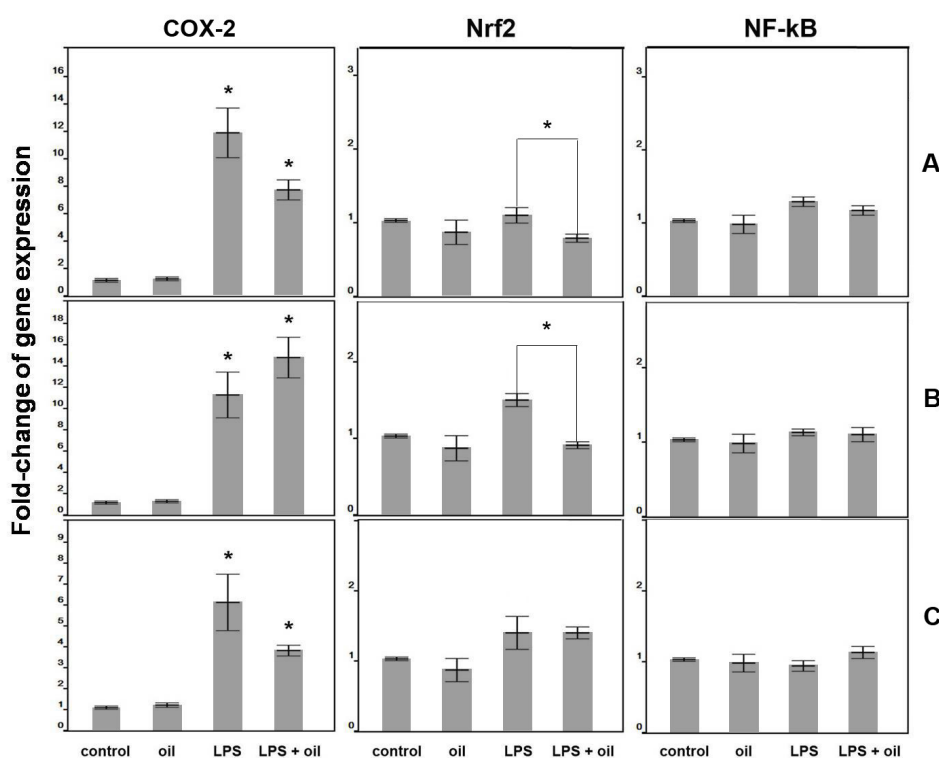
### Gene expression

The results of pre-treatment are shown in Fig. 5A. Exposure of the cells to LPS for 24 hours caused a 12.3-fold up-regulation of the *cox-2* gene, while exposure to the macerated herbal oil for 2 h followed by 24 hours of LPS treatment caused a 7.8-fold up-regulation of the *cox-2* gene. The difference between these two groups was statistically significant ( $P < 0.05$ ), which shows that

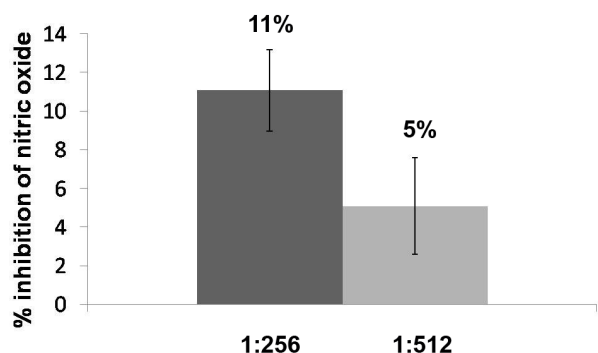
pre-treatment of macerated herbal oil (0.2  $\mu\text{L/mL}$ ) could reduce *cox-2* gene expression when it is applied before the treatment of LPS. No significant change in the *Nrf2* and *NF- $\kappa$ B* expressions were observed at the 24-hour culture period compared to the control group.

From the result of the co-treatment, as presented in Fig. 5B, it is evident that the LPS treatment and the LPS + oil treatment significantly induced the *cox-2* gene expression, which indicates that the macerated herbal oil (0.2  $\mu\text{L/mL}$ ) could not inhibit the LPS-induced *cox-2* gene expression in the HMSCs when they were presented together. However, the LPS + oil treatment resulted in the attenuation of the LPS-induced *Nrf2* gene expression. No significant change in the *NF- $\kappa$ B* expression was observed at the 24-hour culture period compared to the control group.

The results of post-treatment are as shown in Fig. 5C. Exposure to LPS for 2 hours followed by 24 hours of normal culture caused a 6.2-fold up-regulation of the *cox-2* gene, while exposure to LPS for 2 hours followed



**Fig. 5. (A) Gene expression of pre-treatment:** the macerated herbal oil concentration 0.2  $\mu\text{L/mL}$  was used in this study. The cells ( $1 \times 10^5$  cells/well) were treated using three different ways: (oil) cells were treated with oil for 24 h, (LPS) cells were treated with LPS for 24 h, and (LPS + oil) cells were exposed to oil for 2 h before treating with LPS for 24 h. Total RNA extraction and RT-PCR were performed to investigate the gene expression compared to control (cells cultured in DMEM + DMSO). The LPS and LPS + oil treated groups showed significant up-regulation of the *cox-2* gene compared to the control group ( $*P < 0.05$ ). The LPS + oil treated group significantly lowered the *cox-2* gene expression when compared to the LPS group ( $*P < 0.05$ , ANOVA, Tukey post hoc). LPS + oil treated groups showed significant down-regulation of the *Nrf2* gene compared to LPS group ( $*P < 0.05$ , ANOVA, Tukey post hoc). **(B) Gene expression of co-treatment:** the cells were incubated with the oil, LPS, and oil + LPS for 24 h. Both LPS and LPS + oil treated groups showed significant up-regulation of the *cox-2* gene compared to the control group ( $*P < 0.05$ , ANOVA). It was only the LPS-treated group that showed significant up-regulation of the *Nrf2* gene which was decreased in the presence of oil ( $*P < 0.05$ , ANOVA, Tukey post hoc). **(C) Gene expression of post-treatment:** the cells were treated using three different ways: (oil) cells were treated with oil for 24 h, (LPS) cells were treated with LPS for 2 h before being cultured in DMEM + DMSO for 24 h, and (LPS + oil) cells were exposed to LPS for 2 h before treating with oil for 24 hours. The LPS and LPS + oil treated groups showed significant up-regulation of the *cox-2* gene compared to the control group ( $*P < 0.05$ , ANOVA, Tukey post hoc). The LPS + oil treated group significantly lowered the *cox-2* gene expression when compared to the LPS group ( $*P < 0.05$ , ANOVA, Tukey post hoc).



**Fig. 6. Nitric oxide scavenging activity:** the nitric oxide scavenging activity of the macerated herbal oil was reflected as the %inhibition of nitric oxide. The macerated herbal oil dilutions 1:265 and 1:512 had %inhibition of nitric oxide of 11% and 5%, respectively.

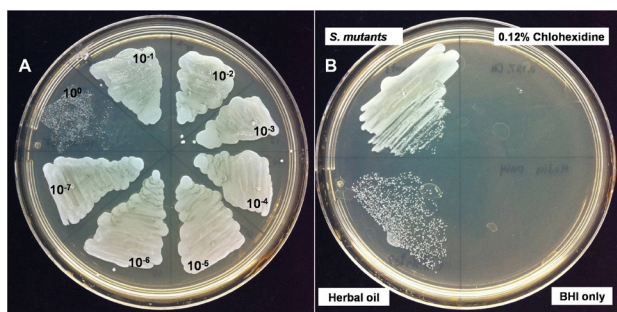
by 24 hours of macerated herbal oil treatment caused a 3.8-fold up-regulation of the *cox-2* gene. The difference between these two groups was statistically significant ( $P < 0.05$ ), which shows that post-treatment of macerated herbal oil (0.2  $\mu\text{L}/\text{mL}$ ) could reduce *cox-2* gene expression when it is applied after the treatment of LPS. However, it was observed that the macerated herbal oil could not inhibit the LPS-induced *Nrf2* gene expression in the post-treatment study. No significant change in the NF- $\kappa$ B expression was observed at the 24-hour culture period compared to the control group.

#### Nitric oxide scavenging assay

The macerated herbal oil showed NO scavenging activity up to the dilutions of 1: 512. The result, which is illustrated in Fig. 6, indicated that the herbal dilution 1: 256 had NO scavenging activity at 11%. The NO scavenging activity decreased proportionally to the dilution of the macerated herbal oil, demonstrating the activity to be at 5% at the dilution of 1:512.

#### Anti-bacterial activity

Macerated herbal oil was diluted 10-fold in BHI broth and



**Fig. 7 Anti-bacterial activity:** (A) when 100  $\mu\text{L}$  of each 10-fold macerated herbal oil dilution was added to 100  $\mu\text{L}$  bacterial suspension, only the  $10^0$  dilution showed antimicrobial activity. (B) The macerated herbal oil at dilution  $10^0$  was seen to inhibit bacterial growth when compared to the negative control (*S. mutants*). The macerated herbal oil at dilution  $10^0$  was less effective when compared to the positive control (0.12% Chlorhexidine). BHI was used as the blank control.

100  $\mu\text{L}$  of each dilution was added to 100  $\mu\text{L}$  of bacterial suspension. As shown in Fig. 7, the macerated herbal oil presented antimicrobial activity against *S. mutans*. However, the antimicrobial activity was not very effective because only a  $10^0$  dilution is capable of inhibiting bacterial growth.

#### Discussion

The total phenolic contents and the antioxidant properties of macerated herbal oil were confirmed using different methods. The study was continued to find out how the addition of menthol, camphor, and borneol into this macerated herbal oil affected cells in the culture system. HMSCs were used because this macerated herbal oil was applied to relieve inflammation of mesenchymal tissues such as bones and muscles. Results from the MTT assay showed no cytotoxicity when the culture media contained oil concentration  $< 2.5 \mu\text{L}/\text{mL}$  (the result of Coomassie blue R staining was demonstrated in the supporting data). Nitrite assay was employed for the investigation of the anti-inflammatory effects; nitrite assay is a test based on the principle that inflammation raises the activity of the inducible nitric oxide synthase (iNOS). The increased nitric oxide synthase activity is evaluated by monitoring the change in the nitrite level in the cultured media. LPS (0.2  $\mu\text{g}/\text{mL}$ ) was used in this experiment to stimulate human mesenchymal stem cell inflammation. Obviously, the concentration of nitrite in the LPS-treated group was higher than that of the control group, but when the macerated herbal oil was presented with LPS (oil + LPS), it was found that the concentration of nitrite in the cultured medium was not different from that of the control group. The cells cultured with the macerated herbal oil alone (media + oil) showed no difference from the control group, thus indicating that the nitrite level was increased in the cultured media because of LPS and that it could be decreased by co-treatment with the macerated herbal oil. The study went on further to investigate gene expressions in the cultured cells. In the co-treatment (Fig. 5B), the cells cultured in the medium that contained both the macerated herbal oil and LPS expressed significantly higher levels of *cox-2* gene when compared to control. However, there was no significant difference between the LPS-treated group and the oil + LPS treated group. The macerated herbal oil could not prevent the up-regulation of the *cox-2* gene in the LPS-induced condition. Linoleic acid is an important ingredient in sesame oil, and it can be converted into arachidonic acid in mammalian cells.<sup>40</sup> COX-2 is an inducible enzyme abundant in the cells at the sites of inflammation, and it catalyzes the conversion of arachidonic acid to prostaglandin.<sup>41</sup> Thus, when the macerated herbal oil and LPS were presented together, the combination could not inhibit the *cox-2* expression. The result indicates that the macerated herbal oil prevented the up-regulation of the *Nrf2* gene in the LPS-induced condition. Recent research work has shown that *Nrf2* has protective effect against oxidative stress. The study conducted by Itoh et al indicated that *Nrf2* regulates

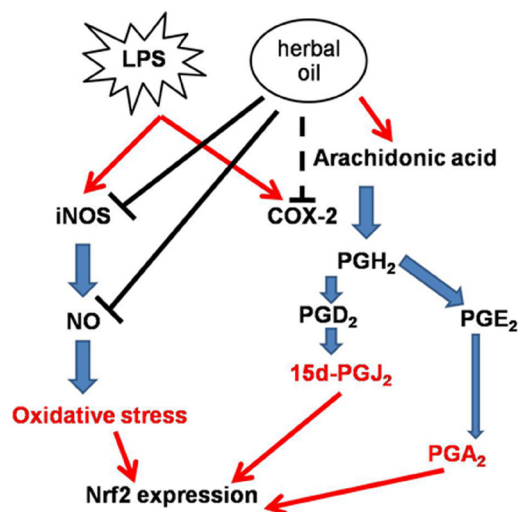


the inflammation process downstream of 15d-PGJ<sub>2</sub> by recruitment of inflammatory cells and by regulation of the gene expression within those cells.<sup>41</sup> Previous study by Wang et al showed that polyunsaturated fatty acids induce Nrf2 signaling pathway and that *Nrf2* plays a role in the suppression of LPS-induced inflammation.<sup>42</sup> Thus it is possible that polyunsaturated fatty acids in the herbal oil could be able to do so. Pre- and post-treatment with the macerated herbal oil (0.2 μL/mL) could reduce the *cox-2* gene expression when it was applied before or after the treatment of LPS. However, it was observed that the reduction in the *cox-2* gene expression in the pre- and post-treatment groups could not reach the baseline because the up-regulation of the *cox-2* gene in the pre- and post-treatment groups were significantly higher than that of the control group. Thus, evidently, post-treatment with the macerated herbal oil could not inhibit the LPS-induced *Nrf2* gene expression. However, the *Nrf2* gene expression in the case of pre-treatment with the macerated herbal oil could not be interpreted because the treatment with LPS alone did not show significant up-regulation of the *Nrf2* gene (it could be noticed that the treatment with LPS alone significantly induced *Nrf2* gene expression except that of the pre-treatment study). Thus, it could be postulated that the macerated herbal oil might play its anti-inflammatory role at the *cox-2* or *Nrf2* level, depending on the duration of the application. Furthermore, this study showed that the macerated herbal oil contained NO scavenging activity; NO is one of the mediators which regulates *Nrf2* activity.<sup>43</sup> It could be postulated that the NO level decreased because of two reasons: Firstly, the macerated herbal oil attenuated iNOS activity and, secondly, the macerated herbal oil acted as the NO scavenger. These reasons explain the attenuation of the LPS-induced *Nrf2* expression. The postulated mechanism has been presented in Fig. 8. No significant change in the *NF-κB* expression was found.

Various kinds of macerated herbal oil have been used in dental clinics with the aim of maintaining oral hygiene.<sup>44</sup> In the present study, the macerated herbal oil showed bactericidal activity but its efficiency as an antimicrobial agent is in need of improvement. The important components which might contribute to antimicrobial property and anti-inflammatory property were shown in the supporting data. Moreover, it was possible that the addition of menthol and borneol probably contributed to the antibacterial activity of the macerated herbal oil.<sup>45</sup> Because of the cytotoxic effect occurred in HMSCs when menthol, camphor, and borneol were added to the macerated herbal oil (Fig. 4A) so this macerated herbal oil should be applied for external use only. From this study, the use of macerated herbal oil could be applied as an alternative medical product.

## Conclusion

The macerated herbal oil dilution 1:2 contained total phenolic concentration equivalent to gallic acid 1.94 mg/mL. The antioxidant activity of the macerated herbal oil was evaluated and confirmed with various antioxidant



**Fig. 8 Postulated mechanism:** LPS-induced inflammatory responses by increasing expressions of iNOS and *cox-2*. iNOS and *cox-2* produced other inflammatory mediators which induce *Nrf2* expression. When macerated herbal oil and LPS were presented together in co-treatment, linoleic acid in the macerated herbal oil was used as a precursor of arachidonic acid which might accelerate LPS-induced *cox-2* expression. However, the macerated herbal oil could inhibit iNOS expression together with its NO scavenging activity leading to a reduction in the NO level, which resulted in the down-regulation of the *Nrf2* gene expression. Nevertheless, the macerated herbal oil might be able to inhibit LPS-induced *cox-2* expression when it is applied before or after the LPS. In the pre-treatment study, the HMSCs were exposed to the macerated herbal oil only 2 h before replacing with LPS, so it is possible that the oil might have employed its protective effects on the cells before the addition of LPS, which led to the reduction in the *cox-2* gene expression. In the post-treatment study, after the 2-h treatment with LPS, the HMSCs were exposed to the macerated herbal oil for 24 h. When the macerated herbal oil was added, it could express the anti-inflammatory effect by bringing about the reduction in the *cox-2* gene expression.

assays, namely, DPPH, ABTS, ferric reducing capacity, and reducing power. The MTT assay using HMSCs as the culture model pointed out that the cytotoxic concentration of the macerated herbal oil was  $\geq 2.5$  μL/mL. The anti-inflammatory effects were evaluated using nitrite assay and RT-PCR. It was found that the macerated herbal oil could inhibit nitrite accumulation in culture media. The results from RT-PCR demonstrated the inhibitory effect of the macerated herbal oil on *cox-2* or *Nrf2*, the downstream modulator of the *cox-2* pathway. Although the macerated herbal oil demonstrated bactericidal activity, the quality of the same needs to be improved.

## Ethical approval

There is none to be declared.

## Competing interests

No competing interests to be disclosed.

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## Research Highlights

### What is current knowledge?

✓ This article proved that the macerated herbal oil contains medicinal properties especially antioxidant and anti-inflammatory effects on HMSCs.

### What is new here?

✓ The mechanism of anti-inflammatory effect of macerated herbal oil on HMSCs was demonstrated.

✓ The bactericidal activity of the macerated herbal oil was observed in this study.

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