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ORIGINAL ARTICLE

Comparison of Antioxidant Levels and Anti-Inflammatory Activities of Kelulut Honey Harvested at Different Month of Intervals and its Chemical Compositions

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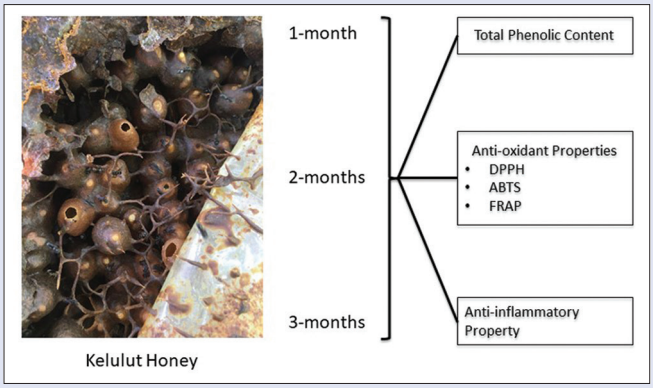
ABSTRACT

Background: Harvesting time might influence the biological activities of honey, which is crucial for honey quality. **Objectives:** This study aims to evaluate the optimum time for harvesting kelulut honey (KH) by determining its antioxidant and anti-inflammatory activities and chemical composition at different harvesting times. **Materials and Methods:** KH harvested at three different intervals (1, 2, and 3 months) was supplied by Rimbunan Hijau Bee Farm, located in Sarawak, Malaysia. The total phenolic content (TPC) and antioxidant level of KH were determined using Folin–Ciocalteu reagent and 2,2-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), and ferric-reducing ability of plasma assays, respectively. The cytotoxicity and anti-inflammatory effects of KH were evaluated on murine macrophage cells (RAW 264.7) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lipopolysaccharide (LPS)-induced nitric oxide (NO) production. The chemical composition of KH was analyzed by gas chromatography-mass spectrometry (GC-MS). **Results:** TPC and antioxidant levels of KH collected at different intervals showed no statistically significant difference ($P > 0.05$). KH at 1% concentration showed no cytotoxic activity toward RAW 264.7 cells after 24 h incubation. However, 1% KH showed no inhibition on NO production in LPS-induced RAW cells compared to the control. GC-MS revealed that hydroxymethylfurfural was present with the highest concentration in all samples. **Conclusion:** This study suggests that harvesting time does not influence the biological activities of KH.

Key words: Anti-inflammatory, antioxidant, kelulut honey, month interval, nitric oxide

SUMMARY

- The quality of honey greatly depends on the floral and geographical origin. In addition, processing, handling, and storage time also influence the composition of honey. The current study hypothesized that different harvesting times might affect the quality of the kelulut honey (KH). Based on the data obtained, harvesting time at 1, 2, and 3 months does not influence the total phenolic content and antioxidant and anti-inflammatory activities of KH.



Abbreviations used: TPC: Total phenolic content; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP: Ferric-reducing ability of plasma; NO: Nitric oxide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LPS: Lipopolysaccharide; GC-MS: Gas chromatography-mass spectrometry; KH: Kelulut honey; HMF: Hydroxymethylfurfural; Trolox: 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal bovine serum; Na_2CO_3: Sodium carbonate; CO_2: Carbon dioxide.

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INTRODUCTION

Honey is a natural product-derived food that originates from the floral nectar and processed by honeybees.[1,2] Honey comprises more than 200 bioactive compounds including polyphenols such as phenolics and flavonoids.[3] Many studies have suggested that these polyphenols are considered as major contributors to the antioxidant potential in honey.[4-6]

Antioxidant is the element that delays, avoids, or eliminates oxidative damage, which would otherwise leads to the overproduction of free radicals.[7] Studies have shown that dietary antioxidants (polyphenols) can prevent diseases associated with oxidative damage such as atherosclerosis and inflammatory joint disease[8,9] by scavenging free radicals and reducing lipid peroxidation.[10,11]

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In recent years, Kelulut honey (KH) has been the attention of researchers and botanists due to its higher amount of dietary antioxidant.[4,12] KH is a type of honey produced by *Trigona* or *Melliponine* species, a stingless type of bee.[13] It has a distinct sourish flavor and is more diluted compared to other types of honey such as Tualang honey.[14] Previous studies have shown that KH exhibits various pharmacological properties such as anti-inflammatory,[15] antimicrobial,[16] anti-proliferative,[17] and anticancer properties.[18,19]

The chemical constituents of the honey greatly depend on the floral and geographical origin of honey. In addition, the composition of honey is influenced by processing, handling, and storage time.[20,21] Many factors such as heating and prolonged storage influence the quality and the chemical composition of honey, particularly on hydroxymethylfurfural (HMF) formation and diastase enzyme deactivation.[22] However, none of the information is available on the effect of different harvesting times on the quality of honey, especially KH. If significant variations in the phenolic content, antioxidant levels, and anti-inflammatory activities exist due to the harvesting period, then KH should be ideally harvested from honeycomb only after a certain interval or at a specific time to maximize its therapeutic potentials. Perhaps, this is the first study to concern with this issue. Hence, the study was carried out to compare the antioxidant levels and anti-inflammatory activities of KH harvested at different intervals.

MATERIALS AND METHODS

Chemicals

All the solvents and reagents used in this study were of analytical grade. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), and hydrochloric acid (37%) were purchased from Calbiochem® (Darmstadt, Germany); phosphate-buffered saline, Griess reagent, ferric (III) chloride, potassium ferricyanide, sodium carbonate, and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); Folin–Ciocalteu reagent was purchased from R&M Chemicals (R&M Chemicals, Marketing, Essex, UK); gallic acid and dexamethasone were purchased from Merck (Darmstadt, Germany); Dulbecco's Modified Eagle Medium (DMEM) was purchased from HiMedia® Laboratories (Mumbai, Maharashtra, India); trypan blue was purchased from ScienCell™ (San Diego, California, USA); sodium dodecyl sulfate and dimethyl sulfoxide (DMSO) were purchased from Amresco Inc. (Ohio, USA); potassium peroxodisulfate and 2'2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Nacalai Tesque (Kyoto, Japan); methanol and fetal bovine serum (FBS) were purchased from Fisher Scientific (Loughborough, Leicestershire, UK).

Kelulut honey samples

KH produced specifically by *Trigona itama* and *Trigona apicalis* was supplied by Rimbunan Hijau (RH) Bee Farm, Sibu, Sarawak, Malaysia. In this study, KH was collected at three different harvesting times (1, 2, and 3 months). Three batches of KH were collected for each interval. The collected KH samples were stored in a sterile airtight glass container at 4°C prior to analyses.

Determination of total phenolic content

The total phenolic content (TPC) of the KH samples was determined by using the Folin–Ciocalteu assay as described by Armania *et al.*[23] In brief, 0.5 mL of KH was reacted with 2.5 mL of 10% (v/v, in distilled water) Folin–Ciocalteu reagent and 2.0 mL of 7.5% (w/v, in Milli-Q water) sodium carbonate solution (Na_2CO_3). After incubation at 40°C for 1 h, the absorbance of the reaction mixture was measured at 765 nm using a UV-visible spectrophotometer against a reagent blank. Gallic acid at various concentrations (3.9–1000 μg/mL) was used as the standard for the construction of calibration curve. The TPC of KH was expressed in microgram gallic acid equivalent per gram of KH (μg GAE/g KH). The KH and standard were prepared in triplicates of two independent experiments.

Determination of antioxidant properties

2,2-Diphenyl-2-picrylhydrazyl hydrate scavenging activity

The DPPH radical scavenging activity of KH was measured according to the protocol described earlier.[23] In brief, 50 μL of KH and 195 μL of 0.1 mM DPPH solution in methanol were mixed in a 96-well microplate and incubated in the dark at room temperature for 1 h. Subsequently, the absorbance was measured at 540 nm using a microplate reader against a blank solvent. Trolox at various concentrations (3.9–1000 μg/mL) was used as the standard for the construction of calibration curve. The percentage of DPPH radical scavenging activity was calculated and expressed in microgram of Trolox equivalent antioxidant capacity per gram of KH (μg TEAC/g KH). The KH and standard were prepared in triplicates of two independent experiments.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

The ABTS radical scavenging activity of KH was measured according to the protocol described earlier[23] with slight modifications. Prior to measurement, ABTS+ solution was prepared by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate (ratio of 1:1, v/v in Milli-Q water). After incubation for 16 h in the dark at room temperature, the ABTS+ solution was diluted to an absorbance of 0.700 ± 0.005 at 734 nm using a microplate reader against Milli-Q water as a blank. To measure the ABTS radical scavenging activity, 200 μL of the adjusted ABTS+ solution was added to 20 μL of KH. The mixture was incubated for 6 min in the dark at room temperature. Finally, the absorbance was immediately measured at 734 nm using a microplate reader against a blank solvent. Trolox at various concentrations (3.9–1000 μg/mL) was used as the standard for the construction of calibration curve. The percentage of ABTS radical scavenging activity was calculated using the following equation and expressed in microgram of Trolox equivalent antioxidant capacity per gram of KH (μg TEAC/g KH). The KH and standard were prepared in triplicates of two independent experiments. ABTS radical scavenging activity was calculated by following the formula in DPPH assay.

Ferric-reducing ability of plasma assay

The ferric-reducing ability of plasma (FRAP) assay described by Armania *et al.*[23] was modified to be performed in a 96-well plate. In brief, 50 μL of KH was mixed with 1 M hydrochloric acid (75 μL), 0.1% (w/v) potassium ferricyanide solution (75 μL), 1% (w/v) sodium dodecyl sulfate solution (25 μL), and 0.2% (w/v) ferric (III) chloride (25 μL). After incubation for 20 min at 50°C, the absorbance of the reaction mixture was measured at 750 nm using a microplate reader (E16 OneTech Medical Equipment Co., Ltd., Guangzhou, China) against a reagent blank. Trolox at various concentrations (3.9–1000 μg/mL) was used as the standard for the construction of calibration curve. The FRAP of KH was expressed in microgram of Trolox equivalent antioxidant capacity per gram of KH (μg TEAC/g KH). The KH and standard were prepared in triplicates of two independent experiments.

Cell culture

Murine RAW 264.7 macrophage cell line was purchased from American Type Culture Collection (ATCC®) (ATCC accession no. TIB-71)

(Manassas, VA, USA). The cells were cultured in DMEM supplemented with 5% FBS and incubated at 37°C in a humidified chamber with 5% carbon dioxide (CO_2). The cells used for each experiment were of less than 18 passage numbers.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The MTT assay of the KH samples in RAW 264.7 cells was performed as described by Ng *et al.*[24] with slight modifications to determine the cytotoxicity. Concisely, the cells (2×10^4 cells per well) were seeded in a 96-well plate. After 24 h of incubation period, the cells were treated with various concentrations (0.5%–3%; v/v) of KH. Untreated cells were included as control. After 24 h incubation, 30 µL of the MTT solution (2 mg/mL) was added into each well, and the plate was incubated at 37°C for 4 h in a dark condition. Subsequently, the supernatant was removed and then, 100 µL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 540 nm using a microplate reader. The percentage of cell viability was calculated using the following equation, $[(A_{control} - A_{sample}) \times 100 / A_{control}]$ and the highest concentration of KH that was non-toxic (at least 90% of cell viability) to RAW 264.7 cell was further selected for the anti-inflammatory assay using LPS-induced RAW 264.7 cells. The MTT assay for KH was performed in triplicates of three independent experiments.

Nitric oxide assay

To determine the anti-inflammatory activity of the tested KH, nitric oxide (NO) production assay was performed as described previously by Kim *et al.*[25] with slight modifications. In brief, RAW 264.7 cells (1×10^5 cells per well) were pretreated with the selected concentration of the tested KH (1%, v/v) for 24 h. Subsequently, the culture supernatant was aspirated. To confirm that the selected concentration of the tested KH did not induce NO production in the RAW 264.7 cells prior to being further stimulated with LPS, the NO production in the culture supernatant was quantified by Griess reagent. Then, the cells were stimulated with 10 µg/mL LPS (150 µL, diluted in culture media) for 24 h. Dexamethasone (0.1 µM) was included as a positive control. The inhibitory effect of KH on NO production was measured by Griess reagent. After stimulation with LPS, 100 µL aliquots of the cell culture supernatant were transferred to a new 96-well microplate. An equal volume of Griess reagent was added into each well. After incubation for 10 min at room temperature, the absorbance was measured at 540 nm using a microplate reader.

Gas chromatography-mass spectrometry analysis

KH samples were analyzed by gas chromatography equipped with mass spectrometry (GC-MS-QP2010 Plus-Shimadzu). The GC specifications were as follows: column oven temperature: 50°C, injector temperature: 280°C (split mode with the ratio being adjusted to 20:1, injection volume = 0.1 µL), flow control mode: linear velocity (51.3 cm/s), and column flow: 2.00 mL/min. The flow rate of the helium carrier gas was set to 1 mL/min. The total run time was 60 min. Mass spectra were obtained at the m/e of 40–700 and electron ionization at 70 eV. The chromatograms of the KH samples were identified by comparing their mass spectra with NIST08 library data and the GC retention time against known standards.

Statistical analysis

The data were expressed as mean ± standard error of mean. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, Version 22) (International Business Machines Corp., New York, USA), and $P < 0.05$ was considered statistically significant. The results were analyzed by using one-way analysis of variance and followed by Tukey or Dunnett's test to identify the significant difference between the tested KH.

RESULTS

Total phenolic content of kelulut honey

To quantify the TPC in the KH harvested at different intervals (1, 2, and 3 months), Folin–Ciocalteu assay was performed. In the present study, three batches of KH harvested at different intervals were collected. Table 1 illustrates the TPC of KH harvested at different intervals. The comparison of TPC levels was performed either by batches [Table 1] or combination of the three batches [Table 1] of KH harvested at different intervals. As shown in Table 1, the TPC of KH harvested at different intervals varied from 569.97 ± 31.63 to 800.50 ± 43.23 (batch A), 615.20 ± 40.63 to 729.47 ± 47.06 (batch B), and 617.20 ± 28.02 to 674.95 ± 22.38 (batch C) µg GAE/g KH. There was a statistically significant difference ($P < 0.05$) in the TPC of KH harvested at different intervals in batch A but not for other batches, where the KH harvested after 3 months of maturation exhibited the highest value [Table 1]. From the combination of the three batches [Table 1], the TPC of KH harvested at different intervals varied from 630.69 ± 18.38 to 717.12 ± 29.95 µg GAE/g KH in the following order: 1 month >2 months >3 months. However, the TPC levels showed no statistically significant difference ($P > 0.05$) among the intervals.

Antioxidant levels (2,2-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, and ferric-reducing ability of plasma) of kelulut honey

Table 2 illustrates the antioxidant levels of the KH harvested at different intervals (1, 2, and 3 months), as determined by DPPH, ABTS, and FRAP assays. Comparison of antioxidant levels was performed either by batches [Table 2] or combination of the three batches [Table 2] of the KH harvested at different intervals.

For batch A, the antioxidant levels of KH harvested at different intervals varied from 117.64 ± 3.83 to 169.73 ± 2.20 (DPPH), 141.03 ± 9.83 to

Table 1: Total phenolic content of kelulut honey harvested in different month intervals (1, 2, and 3 months)

a		
Batch	**Harvesting time (month interval)**	**TPC (µg GAE/g KH)**
A	1	800.50±43.23[a]
	2	569.97±31.63[b]
	3	659.69±27.52[b]
B	1	704.04±38.61[a]
	2	729.47±47.06[a]
	3	615.20±40.63[a]
C	1	646.81±58.12[a]
	2	674.95±22.38[a]
	3	617.20±28.02[a]
b		
Harvesting time (month interval)		**TPC (µg GAE/g KH)**
1		717.12±29.95[a]
2		658.13±24.94[a]
3		630.69±18.38[a]

Comparison of TPC was performed by (a) batches and (b) combination of the three batches of KH harvested at different month intervals. Data are expressed as mean±SEM of three replicates. Data with different superscripts (*,#) in the same batch (a) or column (b) are considered significant. Results were analyzed by one-way ANOVA and followed by Tukey test to identify the significant difference between the tested KH. TPC is expressed as microgram of gallic acid equivalent per gram of KH (µg GAE/g KH). TPC: Total phenolic content; SEM: Standard error of mean; ANOVA: Analysis of variance; KH: Kelulut honey; GAE: Gallic acid equivalents

Table 2: Antioxidant levels of kelulut honey harvested in different month intervals (1, 2, and 3 months)

a				
Batch	**Harvesting time (month interval)**	**DPPH (µg TEAC/g KH)**	**ABTS (µg TEAC/g KH)**	**FRAP (µg TEAC/g KH)**
A	1	167.21±2.97[a]	194.30±7.57[a]	512.93±24.60[a]
	2	117.64±3.83[b]	141.03±9.83[b]	361.99±10.51[b]
	3	169.73±2.20[a]	218.82±16.92[a]	401.47±17.70[b]
B	1	98.22±4.61[a]	178.28±4.86[a]	457.95±14.63[a]
	2	171.76±2.83[b]	236.92±12.22[b]	471.75±8.10[a]
	3	182.08±4.47[b]	119.85±3.69c	479.26±33.58[a]
C	1	157.96±3.56[a]	161.61±11.42[a]	426.18±8.22[a,b]
	2	121.63±2.50[b]	203.15±7.66[b]	479.26±33.58[b]
	3	141.57±3.20[c]	175.78±10.86[a,b]	382.49±9.36[a]
b				
Harvesting time (month interval)		**DPPH (µg TEAC/g KH)**	**ABTS (µg TEAC/g KH)**	**FRAP (µg TEAC/g KH)**
1		141.13±7.69[a]	178.06±5.59[a]	465.69±12.75[a]
2		137.01±6.21[a]	193.70±11.07[a]	437.66±17.22[a]
3		164.46±4.51[b]	171.48±11.73[a]	421.07±15.91[a]

Comparison of antioxidant levels was performed by (a) batches and (b) combination of three batches of KH harvested at different month intervals. Data are expressed as mean±SEM of three replicates. Data with different superscripts ($^{*, \#, +}$) in the same batch (a) or column (b) are considered significant. Results were analyzed by one-way ANOVA and followed by Tukey tests. Antioxidant levels of KH is expressed as microgram of trolox equivalent antioxidant capacity per gram of KH (µg TEAC/g KH). SEM: Standard error of mean; ANOVA: Analysis of variance; KH: Kelulut honey; DPPH: 2,2-diphenyl-2-picrylhydrazyl hydrate; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP: Ferric-reducing ability of plasma; Trolox: 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; TEAC: Trolox equivalent antioxidant capacity

218.82 ± 16.92 (ABTS), and 361.99 ± 10.51 to 512.93 ± 24.60 (FRAP) µg TEAE/g KH [Table 2]. Similar results were obtained from the DPPH and ABTS assays, where the KH harvested after 3 months of maturation exhibited the highest antioxidant levels. In contrast, the KH harvested after 1 month of maturation exhibited the highest antioxidant levels when measured using the FRAP assay. However, statistical analysis revealed no statistically significant difference ($P > 0.05$) between the antioxidant levels of the KH harvested after 1 and 3 months of maturation based on the DPPH and ABTS assays.

For batch B, the antioxidant levels of KH harvested at different intervals varied from 98.22 ± 4.61 to 182.08 ± 4.47 (DPPH), 119.85 ± 3.69 to 236.92 ± 12.22 (ABTS), and 457.95 ± 14.63 to 479.26 ± 33.58 (FRAP) µg TEAE/g KH [Table 2]. Here, the antioxidant levels of the KH harvested after 3 months of maturation exhibited the highest value as determined by the DPPH and FRAP assays. In the ABTS assay, the KH harvested after 2 months of maturation showed the highest antioxidant levels ($P < 0.05$) compared to that of other months. However, statistical analysis revealed no statistically significant difference ($P > 0.05$) between the antioxidant levels of the KH harvested after 2 and 3 months of maturation based on the DPPH and FRAP assays.

For batch C, the antioxidant levels of the KH harvested at different intervals varied from 121.63 ± 2.50 to 157.96 ± 3.56 (DPPH), 161.61 ± 11.42 to 203.15 ± 7.66 (ABTS), and 382.49 ± 9.36 to 479.26 ± 33.58 (FRAP) µg TEAE/g KH [Table 2]. As depicted in Table 2, the antioxidant levels of the KH harvested after 2 months of maturation exhibited the highest value as determined by the ABTS and FRAP assays. In the DPPH assay, the KH harvested after 1 month of maturation showed the highest antioxidant levels ($P < 0.05$) compared to that of other months. However, statistical analysis revealed no statistically significant difference ($P > 0.05$) between the antioxidant levels of the KH harvested after 1 and 2 months of maturation based on the FRAP assay.

From the combination of the three batches [Table 2], the antioxidant levels of KH harvested at different month intervals varied from 137.01 ± 6.21 to 164.46 ± 4.51 (DPPH), 171.48 ± 11.73 to 193.70 ± 11.07 (ABTS), and 421.07 ± 15.91 to 479.26 ± 33.58 (FRAP) µg TEAE/g KH. Overall, the antioxidant levels showed no statistically significant difference ($P > 0.05$) among the intervals when measured by the ABTS and FRAP assays. In the present study, similar antioxidant results were obtained from the ABTS and DPPH assays. However, the antioxidant levels determined by the FRAP assay were higher twofold compared to the other assays used in this study.

Cell viability of RAW 264.7 cells treated with kelulut honey

Prior to the anti-inflammatory assay, the ideal concentration of KH that does not cause toxicity in RAW 264.7 cells was determined. MTT assay was performed to evaluate the cytotoxic activity of KH harvested at different harvesting intervals on RAW 264.7 cells. The viability of RAW 264.7 cells after treatment with various concentrations (0.5%–3%) of KH was determined. In the present study, the concentration of KH that resulted in cell viability higher than 90% was considered non-toxic to the RAW 264.7 cells. Figure 1a-c presents the percentages of cell viability of RAW 264.7 cells after treatment with various concentrations of KH harvested at different intervals for all the three batches, after 24 h of incubation. The results show that cell viability of RAW 264.7 cells statistically significantly decreased ($P < 0.05$) in a concentration-dependent manner after being treated with different concentrations of KH harvested at different intervals. As shown in Figure 1a-c, the percentages of cell viability of the RAW 264.7 cells after treatment with 1% of KH harvested at different intervals were more than 92%. This indicates that KH at 1% of concentration has no cytotoxicity toward the RAW 264.7 cells after 24 h of incubation. Hence, this concentration was further selected for the anti-inflammatory assay.

Anti-inflammatory activity of kelulut honey on lipopolysaccharide-induced nitric oxide production in RAW 264.7 cells

NO production assay was performed to evaluate the anti-inflammatory activity of KH harvested at different intervals on RAW 264.7 cells. The cells were pretreated with 1% of KH harvested at different intervals for 24 h prior to being induced with LPS. NO levels in the LPS-induced RAW 264.7 cells after pretreatment with KH were determined by Griess reagent and compared with the control cells. Figure 2 presents the NO levels of LPS-induced RAW 264.7 cells after being pretreated with 1% of the KH harvested at different intervals for all the three batches. To evaluate the

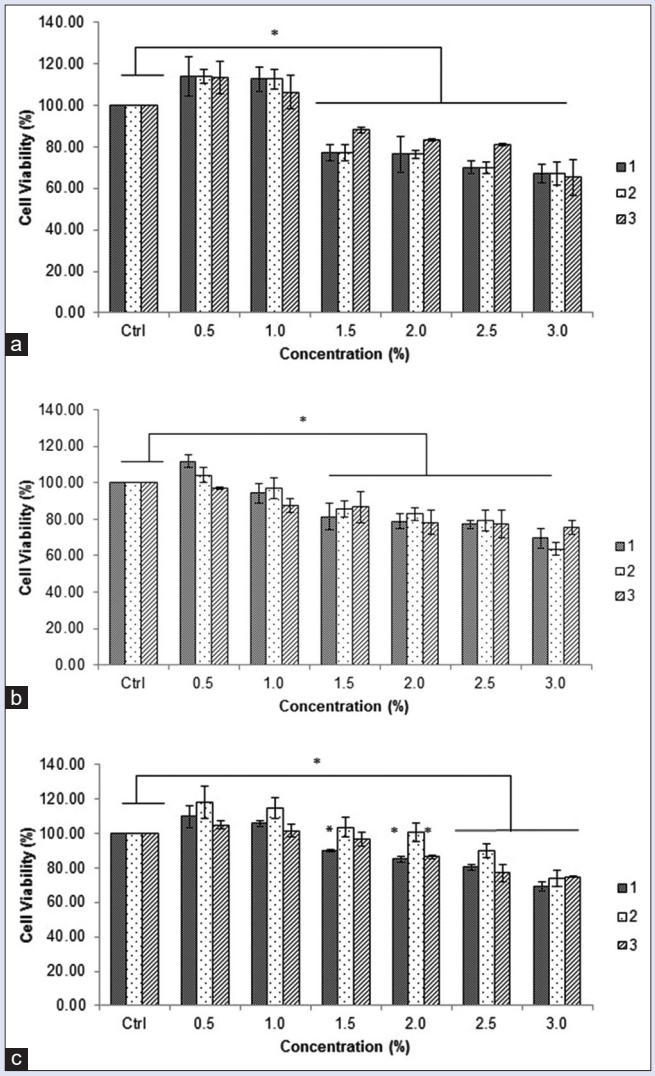

Figure 1: Percentages of cell viability of RAW 264.7 cells after treated with kelulut honey harvested in different month intervals (1, 2, and 3 months). Three batches of samples were collected for each month interval (a-c). Comparison of percentages of cell viability was performed by (a-c) three batches of kelulut honey. Data are expressed as mean ± standard error of mean of three replicates. Results were analyzed by one-way analysis of variance and followed by Dunnett's test. The means marked with * are significantly different with $P < 0.05$ as compared to untreated cells (Ctrl)

validity of the inflammatory model used in this study, dexamethasone was used as a positive control. NO production was statistically significantly reduced ($P < 0.05$) in the cells treated with dexamethasone compared to the control cells (LPS-induced RAW 264.7 cells) [Figure 2]. However, NO production was not inhibited after pretreatment with 1% of KH harvested at different intervals for all the three batches. Overall, the selected concentration (1% KH) showed no inhibition of NO production in the LPS-induced RAW 264.7 cells among all the KH harvested at different intervals compared to the control cells (LPS-induced RAW 264.7 cells) [Figure 2].

Chemical composition of kelulut honey analyzed by gas chromatography-mass spectrometry

A GC-MS analysis was carried out on all the samples of KH collected in different batches and intervals [Table 3]. The common chemical compounds

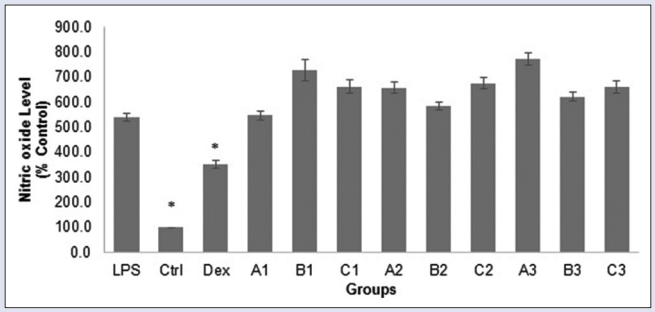

Figure 2: Effect of kelulut honey (at concentration of 1%) harvested at different month intervals (1, 2, and 3 months), on LPS-induced nitric oxide production in RAW 264.7 cells. Comparison of nitric oxide levels was performed by (a-c) batches of kelulut honey harvested at different month intervals. Data are expressed as mean ± standard error of mean of three replicates ($n = 3$). Results were analyzed by one-way analysis of variance followed by Dunnett's tests. *Significantly different with $P < 0.05$ as compared to control cells (LPS only). Alphabet indicated for batch while numeric indicated for month(s). Ctrl: Control without LPS; Dex: Dexamethasone; LPS: Lipopolysaccharide

present in all the samples included HMF, d-allose, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, and 4H-pyran-4-one, 3,5-dihydroxy-2-methyl. Among the chemical compounds, HMF presented with the highest concentration. The multichemical composition present in the KH may contribute to its medicinal activity, including the antioxidant and anti-inflammatory properties.

DISCUSSION

This study was designed to compare the antioxidant levels and anti-inflammatory activities of KH harvested at different intervals (1, 2, and 3 months). The results indicate that harvesting time (1, 2, and 3 months) does not influence the TPC, antioxidant levels, and anti-inflammatory activities of KH.

Plant polyphenols and flavonoids such as phenolic acid, gallic acid, tannic acid, and ellagitannin have been reported to be effective as singlet oxygen scavengers, reducing agents, and hydrogen atom donators.[26] Many studies have suggested that polyphenols and flavonoid compounds are major contributors to the antioxidant potential in honey.[4-6] Therefore, prior to the evaluation of the antioxidant levels, the TPC was quantified. Quantification by Folin–Ciocalteu assay revealed that the amount of TPC varied in the following order: 1 month >2 months >3 months [Table 1]. However, there was no significant difference in the TPC levels of KH harvested at different intervals (1, 2, and 3 months). This result suggests that the maturation time of KH in beehive does not influence the TPC. However, the result of this study is not in agreement with that of Moniruzzaman *et al.*[27] The study revealed that harvesting time does influence the TPC of Malaysian Acacia honey collected at different months during a 2-year time span (September 2010 and December 2012), where the honey collected at the beginning of the year showed the highest phenolic content. The differences could be due to the shorter study period for the current study, and thus might not have been affected by the two monsoon wind seasons, the southwest monsoon from late May to September and the northeast monsoon from October to March.

To evaluate the antioxidant levels in KH, three different assays were used in this study, namely, DPPH, ABTS, and FRAP assays. As shown in Table 2, the antioxidant levels of the KH harvested at different intervals varied. However, the antioxidant levels showed no statistically significant difference ($P > 0.05$) among the intervals based on ABTS and FRAP assays. This indicates that the maturation time of KH in beehive does not

Table 3: Chemical composition identified in the nine samples of kelulut honey by using gas chromatography-mass spectrometry analysis

Number	Compound	Peak area (%)	RT
	A1		
1	Acetic acid, methyl ester (CAS) Methyl acetate	0.44	2.62
2	2-Propanone, 1-hydroxy- (CAS) Acetol	0.48	2.89
3	Propanoic acid, 2-oxo- (CAS) Pyruvic acid	0.78	2.99
4	Propanoic acid	0.45	3.12
5	2-Propyn-1-ol (CAS) Propargyl alcohol	0.88	3.59
6	1-Hydroxy-2-butanone	0.21	3.85
7	Propanoic acid, 2-hydroxy-, ethyl ester, (S)-	0.84	3.94
8	Propane, 2-nitro-	0.41	4.13
9	Propanoic acid, 2-oxo-, methyl ester (CAS) Methyl pyruvate	0.55	4.28
10	2-Furancarboxaldehyde (CAS) Furfural	5.50	4.89
11	1.2-diacetylhydrazine	0.99	5.21
12	Furfural	0.65	5.71
13	FURFURYL ALCOHOL	1.59	6.17
14	2-Propanone, 1-(acetyloxy)- (CAS) Acetol acetate	0.59	6.68
15	Furan<2-acetyl->	1.58	7.64
16	Ethanone, 1-(2-furanyl)-	0.78	7.90
17	2 (5H)-Furanone	0.60	8.17
18	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	0.61	8.36
19	2-Cyclopenten-1-one, 2-hydroxy-	1.47	8.54
20	2 (5H)-Furanone, 5-methyl-	0.49	8.83
21	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	1.68	9.65
22	Corylon	0.48	10.93
23	Phenylacetaldehyde	1.18	11.07
24	Methyl 2-furoate	1.41	11.53
25	2,3-Dihydro-5-hydroxy-6-methyl-4H-pyran-4-one	0.70	11.63
26	2,5-DIMETHYL-4-HYDROXY-3 (2H)-FURANONE	1.24	11.79
27	4H-Pyran-4-one, 3-hydroxy-2-methyl- (CAS) Maltol	0.92	12.39
28	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	4.00	13.11
29	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	1.81	14.20
30	5-Formyl-2-furfurylmethanoate	1.71	14.53
31	HMF	25.56	15.28
32	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	1.06	15.48
	A2		
1	Oxirane, 2,3-dimethyl- (CAS) 2,3-Epoxybutane	0.24	2.52
2	2-Propanone, 1-hydroxy- (CAS) Acetol	0.36	3.43
3	FURFURAL	0.83	4.94
4	2-Cyclopenten-1-one, 2-hydroxy-	0.95	8.33
5	(S)-2-Hydroxypropanoic acid	1.05	8.50
6	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	0.60	9.55
7	Phenylacetaldehyde	0.28	11.06
8	2,3-Dihydro-5-hydroxy-6-methyl-4H-pyran-4-one	0.61	11.99
9	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-	8.06	13.36
10	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.41	13.98
11	HMF	37.47	15.15
12	1,2,3-Propanetriol, monoacetate	2.35	15.27
13	D-Allose	17.10	19.57
	A3		
1	Oxirane, 2,3-dimethyl-, cis-	0.24	2.54
2	FURFURAL	0.75	4.85
3	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	0.43	9.55
4	Phenylacetaldehyde	0.25	11.19
5	2,5-Dimethyl-4-hydroxy-3 (2H)-furanone	0.33	11.85
6	Furyl hydroxymethyl ketone	0.47	11.93
7	2,3-Dihydro-5-hydroxy-6-methyl-4H-pyran-4-one	0.56	11.99
8	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	9.14	13.38
9	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.38	13.96
10	HMF	38.33	15.18
11	1,2,3-Propanetriol, monoacetate	2.84	15.29
12	D-Allose	19.17	19.57
	B1		
1	Formic acid, ethenyl ester	0.31	2.52
2	.BETA.-IONONE EPOXIDE	0.12	2.67

Contd...

Table 3: Contd...

Number	Compound	Peak area (%)	RT
	B1		
3	2-Propenoic acid, methyl ester	0.08	3.05
4	2-Propanone, 1-hydroxy- (CAS) Acetol	0.25	3.38
5	ETHYL LACTATE	0.11	3.83
6	Furfural	0.44	4.87
7	2-Furanmethanol	0.10	5.63
8	(S)-2-Hydroxypropanoic acid	1.30	8.05
9	2-Cyclopenten-1-one, 2-hydroxy-	0.31	8.27
10	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	0.29	9.53
11	Phenylacetaldehyde	0.18	11.07
12	2,5-Dimethyl-4-hydroxy-3 (2H)-furanone	0.28	11.78
13	Furyl hydroxymethyl ketone	0.39	11.98
14	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	7.78	13.31
15	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.30	13.92
16	5-Formyl-2-furfurylmethanoate	0.66	14.47
17	HMF	32.43	15.08
18	1,2,3-Propanetriol, monoacetate	3.71	15.24
19	5-Acetoxymethyl-2-furaldehyde	1.33	15.66
20	D-Allose	19.51	19.47
	B2		
1	Carbamic acid, monoammonium salt (CAS) Ammonium carbamate	0.18	2.57
2	2-Propanone, 1-hydroxy- (CAS) Acetol	0.25	3.83
3	Furfural	0.99	4.89
4	Muramic acid	3.31	8.28
5	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-on	0.25	9.54
6	Phenylacetaldehyde	0.19	11.07
7	2,5-Dimethyl-4-hydroxy-3 (2H)-furanone	0.25	11.80
8	Furyl hydroxymethyl ketone	0.32	11.93
9	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	7.42	13.33
10	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.29	13.92
11	5-Formyl-2-furfurylmethanoate	0.60	14.47
12	HMF	34.23	15.08
13	5-Acetoxymethyl-2-furaldehyde	0.64	15.67
14	D-Allose	18.38	19.47
	B3		
1	Propanoic acid, 2-oxo- (CAS) Pyruvic acid	0.32	2.52
2	2-Propanone, 1-hydroxy- (CAS) Acetol	0.30	3.50
3	FURFURAL	0.50	4.94
4	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	0.31	9.56
5	Phenylacetaldehyde	0.34	11.07
6	2,5-Dimethyl-4-hydroxy-3 (2H)-furanone	0.29	11.79
7	Furyl hydroxymethyl ketone	0.49	11.93
8	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	9.09	13.34
9	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.36	13.93
10	5-Formyl-2-furfurylmethanoate	0.65	14.47
11	HMF	35.81	15.11
12	1,2,3-Propanetriol, monoacetate	2.69	15.22
13	D-Allose	21.54	19.54
	C1		
1	Propanedioic acid (CAS) Malonic acid	0.58	2.53
2	Methylglyoxal	0.40	2.68
3	ETHYL LACTATE	0.52	3.80
4	Methylglyoxal	0.25	3.95
5	2,3-Butanediol	0.20	4.14
6	Furfural	0.37	5.08
7	2-Furanmethanol	0.14	5.84
8	2-Cyclopenten-1-one, 2-hydroxy-	0.43	8.37
9	Propanoic acid, 2-hydroxy-, methyl ester, (.+/-.)-	0.38	8.50
10	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	0.36	9.59
11	Phenylacetaldehyde	0.33	11.07
12	Furyl hydroxymethyl ketone	0.56	11.95
13	2,3-Dihydro-5-hydroxy-6-methyl-4H-pyran-4-one	0.67	12.01

Contd...

Table 3: Contd...

Number	Compound	Peak area (%)	RT
	C1		
14	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	11.23	13.40
15	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.28	13.98
16	HMF	39.73	15.14
17	1,2,3-Propanetriol, monoacetate	2.67	15.26
18	D-Allose	17.98	19.58
	C2		
1	Oxirane, 2,3-dimethyl-, cis-	0.25	2.54
2	2-Propanone, 1-hydroxy- (CAS) Acetol	0.12	3.43
3	2-Butanone, 3-hydroxy-	0.27	3.81
4	Furfural	0.37	4.93
5	2-Furanmethanol	0.10	5.69
6	2-Heptanol	2.59	8.31
7	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	0.26	9.55
8	Phenylacetaldehyde	0.17	11.07
9	2,5-Dimethyl-4-hydroxy-3 (2H)-furanone	0.25	11.82
10	Furyl hydroxymethyl ketone	0.32	11.94
11	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	7.55	13.36
12	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.25	13.94
13	HMF	33.88	15.11
	C3		
1	Oxirane, 2,3-dimethyl-	0.21	2.57
2	Propanoic acid, 2-hydroxy-, ethyl ester, (S)-	0.16	3.82
3	FURFURAL	0.15	1.97
4	2,4-Dihydroxy-2,5-dimethyl-3 (2H)-furan-3-one	0.22	9.56
5	Phenylacetaldehyde	0.16	11.07
6	2,5-Dimfethyl-4-hydroxy-3 (2H)-furanone	0.21	11.82
7	Furyl hydroxymethyl ketone	0.39	11.94
8	2,3-Dihydro-5-hydroxy-6-methyl-4H-pyran-4-one	0.42	12.00
9	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	7.78	13.37
10	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.27	13.95
11	5-Formyl-2-furfurylmethanoate	0.64	14.48
12	HMF	34.10	15.11
13	D-Allose	20.61	19.52

Three batches of samples were collected for each month interval (A1-A3, B1-B3, and C1-C3). Alphabet indicated for batch while numeric indicated for month(s). HMF: Hydroxymethylfurfural; RT: Retention time

influence the antioxidant levels. However, the previous study conducted by Moniruzzaman *et al*.[27] revealed that harvesting time does influence the antioxidant levels of Malaysian Acacia honey collected at different months during a 2-year time span (September 2010 and December 2012), where the honey collected at the beginning of the year showed the highest antioxidant levels as determined by DPPH assay. The study suggested that phenolic compounds are the contributing factor to the antioxidant capacity of Malaysian Acacia honey.

In the present study, the antioxidant levels determined by FRAP assay were higher twofold compared to those of the DPPH and ABTS assays. A previous study revealed that the antioxidant levels determined by FRAP assay increased as polarity of the solvent increased.[28] Since in the present study KH and Trolox (standard) were diluted in methanol, it is believed that methanol (a high polarity solvent) influenced the antioxidant levels in the FRAP assay. Similar antioxidant results were obtained from the ABTS and DPPH assays because both methods used a strongly colored stable radical compound.[29]

The DPPH and ABTS assays are spectrophotometric techniques based on quenching of stable colored radicals (ABTS• + or DPPH) and show the radical scavenging ability of antioxidants..[30,31] In the DPPH assay, the reduction of purple DPPH radicals resulted in the formation of yellow nonradical form of DPPH.[32] Meanwhile, in the ABTS assay, the reduction of blue-green ABTS radicals resulted in the formation of a colorless stable form of ABTS.[31] FRAP assay is the simplest and rapid method to evaluate the antioxidant levels of compounds based on the reduction of potassium ferricyanide.[23] In this assay, the color changes to dark blue as ferric ion is reduced to ferrous ion.[33] In contrast to DPPH and ABTS assays, FRAP assay does not require the use of any exclusive chemicals and it is also a highly reproducible method.[34]

Inflammatory diseases such as asthma, autoimmune disease, and inflammatory bowel disease occur due to the imbalance of natural antioxidants. This eventually leads to free radical productions from different biological and environmental sources.[35] Compounds that display antioxidant capacity in honey such as phenolic acids and flavonoids have received attention among researchers due to their role in the prevention of inflammatory diseases associated with oxidative stress such as asthma, autoimmune disease, and inflammatory bowel disease.[8,9] The phenolic compounds capable to stabilize cell membranes from being destroyed by free radicals, therefore preventing cell inflammation.[11] Hence, phenolic compound functionally serves as a free radical scavenger and eventually reduces lipid peroxidation. Ahmadi-Motamayel *et al*.[36] reported that high concentration of honey can cause toxicity to the cells due to the high content of sugar in the honey. This will eventually cause the cells to become hypotonic and lead to cell shrinkage and cell death.[37] Thus, prior to the anti-inflammatory assay, the ideal concentration of KH that does not cause toxicity effect in RAW 264.7 cells was determined by MTT assay.

Based on the MTT assay, the cell viability of RAW 264.7 cells statistically significantly decreased ($P < 0.05$) in a concentration-dependent manner after treatment for 24 h with different concentrations of KH harvested at different intervals [Figure 1a-c]. As shown in Figure 1, the percentages of cell viability of RAW 264.7 cells after being treated with 1% of the KH harvested at different intervals were more than 92%. A percentage of cell viability higher than 90% is considered non-toxic to the RAW 264.7 cells.[38] This indicates that 1% KH has no cytotoxic activity toward RAW 264.7 cells after 24 h. Hence, this concentration was selected for subsequent anti-inflammatory assays.

To evaluate the anti-inflammatory activities of KH, the inhibition of NO production was quantified in LPS-induced RAW 264.7 cells. The selected concentration (1% KH) did not inhibit NO production in LPS-induced RAW 264.7 cells for all KH harvested at different intervals compared to the control cells (LPS-induced RAW 264.7 cells) [Figure 2]. This result indicates that the selected concentration of KH does not show anti-inflammatory activities in the LPS-induced RAW 264.7 cells. However, surprisingly, NO production in LPS-induced RAW 264.7 cells significantly increased after being treated with KH harvested at different intervals compared to the control cells (LPS-induced RAW 264.7 cells) [Figure 2]. Quantification of NO production after pretreatment with 1% KH showed no significant production of NO in RAW 264.7 cells prior to being induced with LPS, compared to the control cells (unpublished data). Thus, this indicates that the pretreatment of RAW 264.7 cells with KH does not stimulate NO production in the cells. Therefore, it is postulated that the pretreatment of RAW 264.7 cells with a suitable concentration of KH (in this case, 1%) is probably able to enhance the growth of RAW 264.7 cells. Previous studies revealed that Manuka honey promotes tissue growth and proliferation of fibroblasts and epithelial cells by stimulating the release of cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α).[39,40] In this study, NO production was significantly reduced in the cells treated with dexamethasone compared to the control cells (LPS-induced RAW 264.7 cells), indicating that the anti-inflammatory model used in this study is valid. Dexamethasone is a corticosteroid drug commonly used to treat inflammation.[41] It exerts its functions by binding to the intracellular receptor (glucocorticoid receptor) and ligand-inducible transcription factor, which belong to the nuclear receptor superfamily.[42] In the present study, LPS increased the release of NO levels in the RAW 264.7 cells fourfold compared to the untreated cells [Figure 2]. A previous study reported that NO level increased two- to fourfold in LPS-induced RAW 264.7 cells compared to untreated cells.[43] LPS is the most powerful activator of macrophages, which stimulates the production of pro-inflammatory cytokines such as TNF-α.[44] Although the current study showed that KH did not significantly reduce NO production induced by LPS in RAW 264.7 cells, it does not mean that KH does not exhibit anti-inflammatory property. KH may suppress inflammation via other mechanisms, and thus further studies are needed to unveil the underlying mechanism of anti-inflammation by KH.

CONCLUSION

This study suggests that harvesting time (1, 2, and 3 months) does not influence the TPC, antioxidant, and anti-inflammatory activities of KH. In addition, KH at 1% failed to suppress NO production induced by LPS. However, further investigation by increasing the concentration of KH or another anti-inflammatory assay is needed to confirm the biological activity of KH.

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Conflicts of interest

There are no conflicts of interest.

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