RESEARCH ARTICLE

Comparative Evaluation of Antioxidant Activity and Phytochemical Composition of Bentong Ginger Extracts and Kelulut Honey

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Abstract Bentong ginger (*Zingiber officinale* Roscoe var. Bentong) and Kelulut honey (*Heterotrigona itama*) are recognized natural antioxidants, yet their synergistic potential remains underexplored. This study evaluated the antioxidant activities of Bentong ginger, Kelulut honey, and their combinations (10% ginger with 15% or 20% honey) using DPPH and ABTS radical scavenging assays, alongside LCMS/MS QTOF profiling to identify key contributing phytochemicals. Both assays demonstrated concentration-dependent antioxidant activity, with the 10% ginger and 15% honey mixture exhibiting the strongest effect and a pronounced synergistic interaction (CI = 0.66). LCMS/MS QTOF analysis revealed diverse bioactive constituents across samples, including phenolic acids, oxygenated terpenoids, flavonoids, fatty acid derivatives, and flavin-related metabolites, supporting the enhanced radical-scavenging capacity observed in the mixtures. Overall, the findings demonstrate that combining Bentong ginger with Kelulut honey significantly augments antioxidant potency and provides a strong scientific basis for the development of natural health products or functional formulations utilizing their synergistic antioxidant properties.

Keywords: Bentong ginger, Kelulut honey, synergy, antioxidant activity, LCMS/MS QTOF analysis.

Introduction

Natural products, particularly those derived from plants, have long been explored and utilized as sources of therapeutic agents and medicinal formulations. Malaysia, with its tropical climate and fertile landscape, is rich in biodiversity and supports the cultivation of numerous medicinal plants and natural resources. Among these, ginger and honey have been widely recognized for their nutritional and medicinal properties and have been used for centuries across various cultures. Their therapeutic applications continue to evolve as new biological activities and health-promoting effects are discovered [1].

Ginger, scientifically known as *Zingiber officinale*, is a medicinal plant belonging to the Zingiberaceae family [2]. Peninsular Malaysia alone contains more than 160 species from 18 genera, contributing to the approximately 1600 species identified globally including *Zingiber officinale* Roscoe var. Bentong [3]. Ginger is extensively used both as a culinary spice, owing to its characteristic aroma and pungency, and as a traditional remedy. Numerous studies have documented its rich profile of bioactive constituents, including 6-shogaol, 6-gingerol, 10-gingerol, 12-gingerol, gingerdiones, gingerdiols, paradols, 6-dehydrogingerols, 3,5-diacetoxy-6-gingerdiol, and 5-acetoxy-6-gingerol. Among these, 6-gingerol and 6-shogaol are considered the primary contributors to ginger's antioxidant and other biological activities [4].

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Honey, a natural sweetener produced from floral nectar collected by honeybees, is another well-established traditional remedy. Beyond its conventional use as food, honey is incorporated in nutritional supplements and topical formulations. Its composition comprising carbohydrates, amino acids, vitamins, minerals, organic acids, and a wide array of bioactive compounds such as flavonoids and polyphenols varies according to floral origin and bee species [5]. These bioactive molecules contribute to honey's antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, enabling it to modulate oxidative stress and support immune function [5]. Both ginger and honey exhibit potent antioxidant and anti-inflammatory activities. Their combined use may offer enhanced protective effects through synergistic interactions, yet current research has predominantly examined their properties individually. Studies investigating their synergistic antioxidant capacity, anti-inflammatory potential, or combined bioactivities remain limited.

The present study aimed to evaluate the antioxidant activities of Bentong ginger and Kelulut honey (*Heterotrigona itama*), individually and in combination, using the DPPH and ABTS radical scavenging assays. In addition, Liquid Chromatography Tandem Mass Spectrometry Quadrupole Time-of-Flight (LC-MS/MS QTOF) was employed to identify and profile the antioxidant bioactive compounds present in each sample. These complementary analytical approaches allowed for simultaneous assessment of radical scavenging capacity and in-depth characterization of chemical constituents. By elucidating the synergistic antioxidant effects and bioactive compound profiles of Bentong ginger and Kelulut honey, this study provides new insights that may support the development of improved natural health products aimed at mitigating oxidative stress-related conditions.

Materials and Methods

Preparation of ginger extract and Kelulut honey

Bentong ginger was obtained from a local market in Bentong, Pahang, and a voucher specimen was deposited at the Herbarium of Universiti Kebangsaan Malaysia (Voucher ID: ID030/2022) [6]. Fresh rhizomes were washed, sliced into thin pieces, and dried in a hot-air oven at temperatures below 45 °C until they became completely dry and brittle. This procedure was modified from previous studies [6,10]. The dried slices were then ground into a fine powder. The dried powder was macerated in 70% (v/v) ethanol at an approximate solid-to-solvent ratio of 1:10 (w/v) for 48 hours at room temperature with occasional stirring to allow complete extraction of ethanol-soluble compounds. Ethanol was selected as the extraction solvent due to its broad solvation capacity for polar and moderately polar phytochemicals, its GRAS status, and its widespread use in comparable ginger extract studies [6]. The extract was subsequently filtered through Whatman No. 1 filter paper (11 µm pore size), and the solvent was removed using a rotary evaporator at 45 °C. The crude extract was then freeze-dried at −90 °C until all remaining moisture was eliminated, resulting in a dry Bentong ginger extract. On the other hand, Kelulut honey was harvested from a stingless bee farm located at Universiti Teknologi Malaysia, Johor. The freshly collected honey was aliquoted into 50-mL Falcon tubes to avoid repeated freeze–thaw cycles, then stored at 4 °C until further analysis. For the combination mixtures, crude Bentong ginger and Kelulut honey extracts were prepared at different concentration ratios by combining ginger extract with the honey base extract. A 10 mg/mL stock solution of Bentong ginger extract and a 250 mg/mL stock solution of Kelulut honey extract were first prepared. Two ginger–honey combination samples were then formulated using the following ratios: 10% (w/v) ginger with 15% (w/v) honey, and 10% (w/v) ginger with 20% (w/v) honey [7] The ginger ration was fixed at 10% to reflect a practically relevant and solubility-compliant level, while honey was varied at 15% and 20% to investigate the effect of increasing the honey proportion on the combined antioxidant response.

DPPH Scavenging Assay

The antioxidant activity of Bentong ginger, Kelulut honey, and their mixtures was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay in a 96-well microtiter plate [7]. A 0.04 mg/mL DPPH solution was freshly prepared by dissolving 0.4 mg of DPPH powder in 10 mL of dimethyl sulfoxide (DMSO).

Crude Bentong ginger extract was dissolved to obtain a 10 mg/mL stock solution, while crude Kelulut honey extract was prepared at 250 mg/mL. Two-fold dilution of 100 µL of sample stock solutions resulted in final concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, 0.0391, 0.0195 mg/mL for Bentong ginger sample and 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.9063, 1.9531, 0.9766, and 0.4882 mg/mL for Kelulut honey sample [6]. The concentrations of 10% Bentong ginger and 15% and 20% of Kelulut honey were prepared before being mixed. 1 mL of 10% Bentong ginger was mixed with 1 mL of 15% Kelulut honey and 1 mL of 20% Kelulut honey. The two- fold dilution of 100 µL of both mixed sample stock solutions yielded 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.9063, and 0.4882 mg/mL for the first

ratio and 300, 150, 75, 37.5, 9.375, 4.6875, 2.3438, 1.1719, and 0.586 mg/mL for the second ratio. Before two-fold dilution, 100 µL of 70% ethanol solution was added to each well of the 96-well microtiter plate, except for the sample wells.

Next, 100 µL of 0.04 mg/mL DPPH solution was added to each well except blank wells and all solutions was mixed well. The mixture of the solutions was incubated in the dark room at room temperature for 30 minutes. After 30 minutes of incubation, each sample's absorbance was measured using a microplate reader at a wavelength of 517 nm. The control well contained 100 µL of 70% ethanol mixed with 100 µL of DPPH solution. The blank well contained 100 µL of sample mixed with 100 µL of 70% ethanol. Each well in the microtiter plate had a final volume of 200 µL. The assay was performed in triplicate, and percentage inhibition was calculated using Equation 1. Results were reported as mean inhibition percentages (n = 3).

$$Percentage\ of\ inhibition\ (\%) = \left(\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100\%$$

Equation 1

The IC_{50} value for each sample was determined by plotting the percentage of DPPH radical inhibition against the corresponding sample concentrations. IC50represents the concentration of extract required to reduce the initial DPPH absorbance by 50%, indicating the antioxidant strength of the sample.

ABTS assay

The assay was performed according to the method described by previous study with some modifications that involved the use of a 96-well microtiter plate [8]. A stock ABTS solution (7 mM) was prepared by dissolving ABTS powder in distilled water. Separately, a 2.45 mM potassium persulfate ($K_2S_2O_8$) solution was prepared. Equal volumes of the two solutions were mixed to generate the ABTS radical cation ($ABTS^{\bullet+}$). The mixture was kept at room temperature in the dark for 12–16 hours to allow complete formation of the $ABTS^{\bullet+}$ radical. After incubation, the $ABTS^{\bullet+}$ solution was diluted with 70% ethanol to obtain an absorbance of 0.70 ± 0.005 at 734 nm, which served as the working solution.

Bentong ginger extract, Kelulut honey, and their mixtures (10% ginger with 15% or 20% honey) were prepared at the same stock concentrations used for the DPPH assay, and two-fold serial dilutions of 100 µL were made to obtain final concentrations identical to those used in the DPPH experiment. For the assay, 100 µL of each diluted sample was mixed with 100 µL of the $ABTS^{\bullet+}$ working solution in the wells of the microtiter plate. The plate was then incubated at room temperature for 30 minutes in the dark to prevent photodegradation of the radical solution. Following incubation, the absorbance of each mixture was recorded at 734 nm using a microplate reader. All samples were assayed in triplicate, and the percentage of inhibition was calculated using the same equation applied in the DPPH assay. The IC50values, representing the concentration required to scavenge 50% of the $ABTS^{\bullet+}$ radicals, were determined by plotting the percentage inhibition against the corresponding sample concentrations.

Data analysis

All experimental data were analyzed using two-way analysis of variance (ANOVA) to compare the antioxidant activities among Bentong ginger, Kelulut honey, and their mixture samples. Differences between groups were considered statistically significant at $p < 0.05$. Results were expressed as mean values, and statistical significance was accepted only when the calculated *p*-values were below the 0.05 threshold [9].

Sample preparation for LCMS/MS QTOF Analysis

For LC-MS/MS QTOF analysis, Bentong ginger, Kelulut honey, and their combination samples were prepared at concentrations of 10 mg/mL, 250 mg/mL, and 300 mg/mL, respectively. Bentong ginger and Kelulut honey were weighed according to the desired concentrations and dissolved in 900 µL of cold 80% ethanol prepared with distilled water. The samples were vortexed thoroughly and then chilled at −20 °C for 30 minutes to enhance clarity prior to centrifugation at 14,000 rpm for 10 minutes. Following centrifugation, the clear supernatant was collected and further diluted using 80% ethanol to obtain a final concentration below 1 mg/mL, as required for LC-MS/MS QTOF analysis to minimize ion suppression and detector overload, ensuring optimal sensitivity and accurate compound identification. All solvents used for sample preparation were LC-MS compatible.

To prevent clogging of the LC-MS/MS QTOF column, each sample was passed through a 0.45 µm or 0.22 µm PTFE syringe filter into an LC-MS vial to remove undissolved solids and particulates. The vials were clearly labeled with sample identification, solvent composition, and final concentration before submission to the University Laboratory Management Centre, Universiti Teknologi Malaysia, for analysis.

LCMS/MS QTOF Analysis

The LC-MS/MS QTOF chemical profiling analysis was performed using a method modified from previous study [10] (Table 1). An Agilent 1290 Infinity LC system coupled to an Agilent 6520 Accurate-Mass Q-TOF mass spectrometer equipped with a dual electrospray ionization (ESI) source (Agilent Technologies Inc., USA) was used for compound detection. Samples were injected onto an Eclipse XDB-C18 Narrow-Bore column (150 mm × 2.1 mm, 3.5 µm), with the mobile phases consisting of 0.1% formic acid in deionized water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). A gradient elution was applied at a flow rate of 0.5 mL/min, and the column temperature was maintained at 25 °C throughout the 25-minute run time. The LC-MS system operated in MS/MS QTOF mode, detecting only positively charged ions. A capillary voltage of 3500 V was applied to enhance ion production in the ESI source, while fragmentor and skimmer voltages facilitated ion transmission through the vacuum interface and protected ions from atmospheric pressure. Appropriate gas temperature settings were used to promote efficient solvent evaporation during ionization. The mass-to-charge (m/z) scan range was sufficiently broad to enable detection of both small metabolites and larger bioactive compounds. Adequate acquisition time was set to ensure sufficient spectral collection per compound, thereby improving signal quality and accurate mass determination. Compound identification was based on accurate mass matching against the PCDL and METLIN databases. These annotations represent putative identifications with Level 2 confidence [11]. Additionally, database matches corresponding to synthetic pharmaceuticals, industrial dyes, and non-natural compounds were excluded to minimize false-positive annotation. Only phytochemicals consistent with plant and honey metabolomes were retained for discussion.

Table 1. LC-MS/MS QTOF Operating Parameters

	Parameters	Values
	Modes	MS/MS QTOF
	Ion polarity	Positive
	Vcap	3500 V
	Fragmentor Voltage	125 V
	Skimmer	65 V
	Drying Gas	10 L/min
	Gas temperature	300 °C
	Mass range (m/z)	100 (min) 3200 (Max)
	Nebulizer	45 psig
	Reference ion use	119.03632 966.000725
	Acquisition rate (spectra/s)	1.03
	Acquisition time (ms/spectrum)	973
	Transient/spectrum	9632

Combination Index (CI) Analysis

The Combination Index (CI) at 50% inhibition (ED50) was calculated using the simplified Chou–Talalay equation [12, 13]:

$$CI = \frac{D_1}{(Dx)_1} + \frac{D_2}{(Dx)_2}$$

where D_1 and D_2 represent the doses of ginger and honey in the mixture that produce 50% inhibition, and $(Dx)_1$ and $(Dx)_2$ correspond to the IC_{50} values of the individual samples. This approach provides an approximation of interaction at ED50 but does not replace full median-effect modeling.

Results and Discussion

DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate the free radical scavenging activity of Bentong ginger, Kelulut honey, and their combination. DPPH is a stable free radical with a characteristic deep purple color, which becomes pale yellow or colorless upon reduction by antioxidant compounds capable of donating hydrogen atoms [8]. In this study, the color change of the DPPH solution indicated the extent of radical neutralization by antioxidants present in the samples. As the concentration of Bentong ginger and Kelulut honey increased, the degree of DPPH reduction also increased, resulting in a gradual fading of the purple color. This demonstrated the concentration-dependent antioxidant activity of both samples.

Figure 1 illustrates the percentage inhibition of DPPH radicals by Bentong ginger at different concentrations, while Figure 2 presents the inhibition profiles of gallic acid, Kelulut honey, and the two mixture ratios of Bentong ginger and Kelulut honey. Across all samples, the free radical scavenging activity increased progressively with concentration, although minor fluctuations were observed at certain concentration points. These fluctuations are common in natural extracts and may be attributed to variations in solubility, sample matrix effects, or the presence of multiple interacting compounds within the crude extracts. Overall, the inhibition trends clearly show that both Bentong ginger and Kelulut honey possess notable antioxidant capacity, and their combination further enhances DPPH radical scavenging activity, supporting the potential synergistic effects between the two natural products.

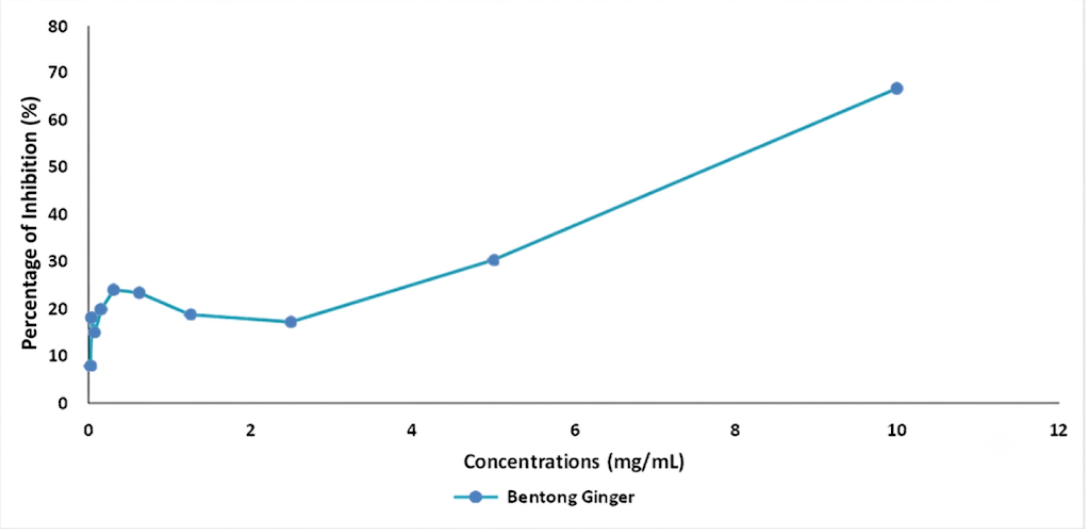

Figure 1. Percentage of DPPH free radical scavenging activity of Bentong ginger

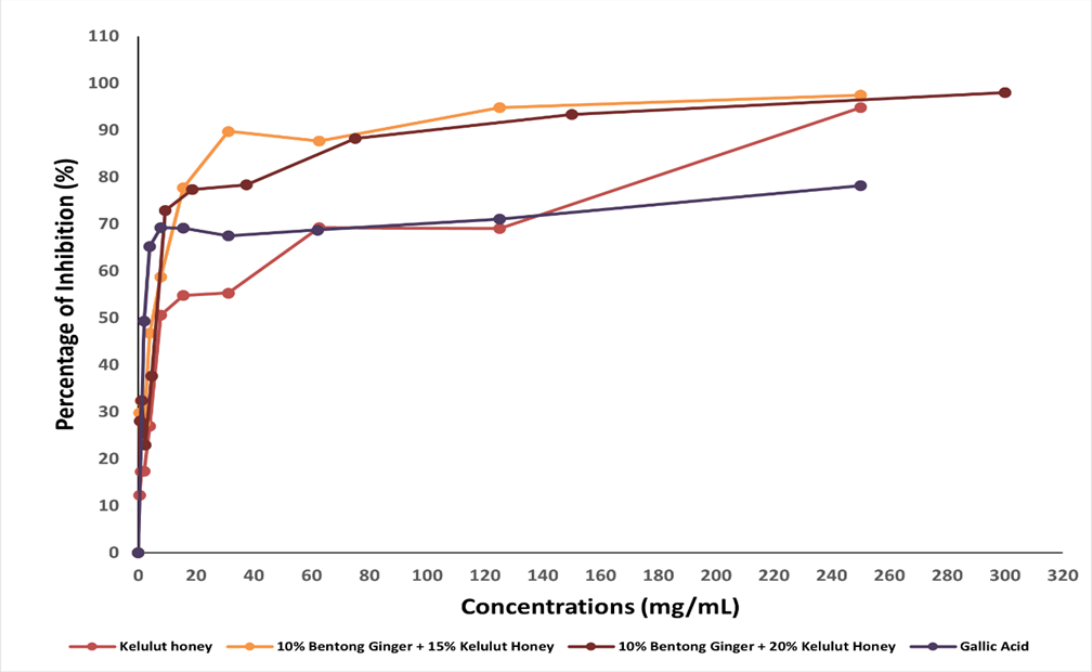

Figure 2. Percentage of DPPH free radical scavenging activity at different concentrations of Kelulut honey, and the combination of Bentong ginger with Kelulut honey

The IC_{50} value represents the concentration of a sample required to inhibit 50% of DPPH free radicals, where lower IC_{50} values indicate stronger antioxidant activity. The IC_{50} values for all samples were calculated from the percentage inhibition data presented in Figures 1 and 2 and are summarized in Table 2. Gallic acid, which is widely recognized for its strong radical-scavenging capacity, was used as the positive control for comparison with all samples. As shown in Table 2 and Figure 3, gallic acid demonstrated the highest antioxidant activity among all tested samples, yielding the lowest IC_{50} value of 0.99 mg/mL. Kelulut honey exhibited a stronger antioxidant effect than Bentong ginger, with IC_{50} values of 7.0 mg/mL and 7.7 mg/mL, respectively, indicating that both samples have comparable but moderate antioxidant capacities. The antioxidant activity of the ginger–honey mixtures varied depending on the concentration ratio. The combination of 10% Bentong ginger with 15% Kelulut honey showed the greatest enhancement in antioxidant activity, with an IC_{50} value of 4.8 mg/mL, while the 10% Bentong ginger with 20% Kelulut honey mixture demonstrated a slightly weaker effect, with an IC_{50} of 6.2 mg/mL. Although the IC_{50} values differed significantly, the inhibition curves for both mixtures exhibited similar upward trends, with percentage inhibition increasing steadily alongside concentration. This suggests that both mixtures benefit from combined antioxidant contributions, though the degree of synergy may vary with the ratio of ginger to honey. These results indicate that the mixture of Bentong ginger and Kelulut honey, particularly at the 10%:15% ratio exhibits enhanced antioxidant potential compared to the individual samples, supporting the presence of synergistic interactions between the two natural products. It should be noted that the antioxidant properties of Kelulut honey are inherently variable across batches, as they are influenced by seasonal changes in floral sources, geographical origin, harvesting method, and storage conditions [5]. In the present study, honey from a single harvest at a controlled apiary was used, which minimizes within-study variability but limits direct extrapolation to honey from other batches or origins.

The combination of Bentong ginger and Kelulut honey demonstrated higher antioxidant activity compared to the individual samples, indicating a possible synergistic enhancement of free radical scavenging capacity. Although both ginger and honey independently exhibited notable antioxidant properties, their combination resulted in a significant increase in activity. Based on the percentage inhibition values presented in the public data repository (https://data.mendeley.com/datasets/s2tbwfr8yd/1), Bentong ginger inhibited 66.79 ± 28.94% of DPPH radicals at its highest tested concentration (10 mg/mL), while Kelulut honey achieved 94.86 ± 7.98% inhibition at 20 mg/mL. The comparatively lower inhibition value for Bentong ginger is attributed to the lower concentration used in the assay, as the amount of sample and the solvent volume directly influence the final concentration and therefore the observed antioxidant capacity. Despite these concentration differences, previous findings reported that Bentong ginger possesses inherently stronger antioxidant activity than Kelulut honey due

to its high levels of gingerols and shogaols bioactive phenolic compounds known for their potent free radical-scavenging abilities [4]. The enhanced antioxidant effect observed in the ginger–honey mixtures therefore suggests that the phenolic compounds of ginger may interact synergistically with the diverse flavonoids and polyphenols present in Kelulut honey, resulting in improved radical-neutralizing capacity beyond what either sample achieves alone.

Additionally, IC_{50} values between 10–50 mg/mL indicate strong antioxidant activity, 50–100 mg/mL indicate intermediate activity, and values above 100 mg/mL reflect weak activity. In this study, all IC_{50} values obtained for gallic acid, Bentong ginger, Kelulut honey, and their combinations fell below 10 mg/mL, confirming that all samples possess strong antioxidant activity [14]. Based on the IC_{50} values derived from the DPPH free radical scavenging assay (Table 2), the antioxidant activity followed the order: Gallic acid > 10% Bentong ginger with 15% Kelulut honey > 10% Bentong ginger with 20% Kelulut honey > Kelulut honey > Bentong ginger. This ranking reinforces the observation that the mixture containing 10% Bentong ginger and 15% Kelulut honey exhibited the most substantial enhancement among the tested combinations, outperforming the individual samples. The results further support the presence of synergistic interactions in the mixture, wherein the combined phytochemicals of ginger and honey yield greater antioxidant potential than when either natural product is tested alone.

Table 2. IC_{50} value of Ginger Bentong, Kelulut Honey and combination of Bentong ginger with Kelulut honey for free radical scavenging activity

Type of samples	IC_{50} Values (mg/mL)
Gallic Acid (Control)	0.99
Bentong Ginger	7.7
Kelulut Honey	7.0
10% Bentong Ginger: 15% Kelulut Honey	4.8
10% Bentong Ginger: 20% Kelulut Honey	6.2

ABTS Radical Cation Decolorization Assay

The antioxidant capacity of Bentong ginger, Kelulut honey, and their mixtures was also evaluated using the ABTS radical cation decolorization assay. The ABTS reagent, formally known as 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), forms a stable blue-green radical cation ($ABTS^{\bullet+}$) that is commonly used to assess the antioxidant activity of biological samples. Antioxidants present in the Bentong ginger and Kelulut honey samples reduced the $ABTS^{\bullet+}$ radicals by donating hydrogen atoms, converting the radical cation into a colorless neutral form [9]. As a result, the extent of decolorization observed in the reaction mixture directly reflected the antioxidant strength of each sample. Higher levels of decolorization corresponded to greater radical-scavenging activity, demonstrating that the antioxidant capacity measured by the ABTS assay increased proportionally with the concentration of Bentong ginger, Kelulut honey, and their respective mixtures.

Figure 3 presents the percentage inhibition curve for Bentong ginger, while Figure 4 illustrates the inhibition profiles for gallic acid, Kelulut honey, and the two ginger–honey mixtures at various concentration ratios. All plot curves demonstrated a consistent increase in ABTS radical scavenging activity with rising sample concentration, indicating that the antioxidants present in Bentong ginger and Kelulut honey effectively reduced the ABTS radical cation. This concentration-dependent response confirms the significant antioxidant capacity of both natural products. Based on the IC_{50} values shown in Table 3, Bentong ginger exhibited the lowest IC_{50} value of 0.02 mg/mL, indicating the strongest antioxidant activity among all tested samples. This was followed by gallic acid, which showed an IC_{50} of 0.4 mg/mL. The low IC_{50} value of Bentong ginger suggests that only a small amount of the extract was required to neutralize 50% of the ABTS radicals, reflecting its high phenolic and antioxidant content. In contrast, Kelulut honey produced the highest IC_{50} value (2.1 mg/mL), indicating comparatively lower but still substantial antioxidant activity. The combined samples demonstrated notable enhancements in antioxidant capacity. The 10% Bentong ginger with 20% Kelulut honey mixture showed an IC_{50} of 0.7 mg/mL, while the 10% Bentong ginger with 15% Kelulut honey mixture produced a slightly higher IC_{50} of 0.8 mg/mL. These results indicate that the presence of abundant ginger bioactive compounds, such as gingerols and shogaols, likely contributed to increased radical scavenging when combined with the diverse polyphenols found in Kelulut honey. Although Bentong ginger displayed the strongest ABTS radical scavenging activity individually, its performance in the DPPH assay was slightly lower than that of Kelulut honey, reflecting differences in the sensitivity of each assay to specific antioxidant compounds. Some variability in absorbance readings may be attributed to the natural color and turbidity of the extracts. Bentong ginger produced a cloudy solution, which may have affected the accuracy of absorbance measurements even at the high detection wavelength of 734 nm. Despite this, the

percentage inhibition values across all samples, were consistently above 50% and increased steadily with concentration, supporting the strong antioxidant potential of both Bentong ginger and Kelulut honey (https://data.mendeley.com/datasets/v4vb656j2r/1).

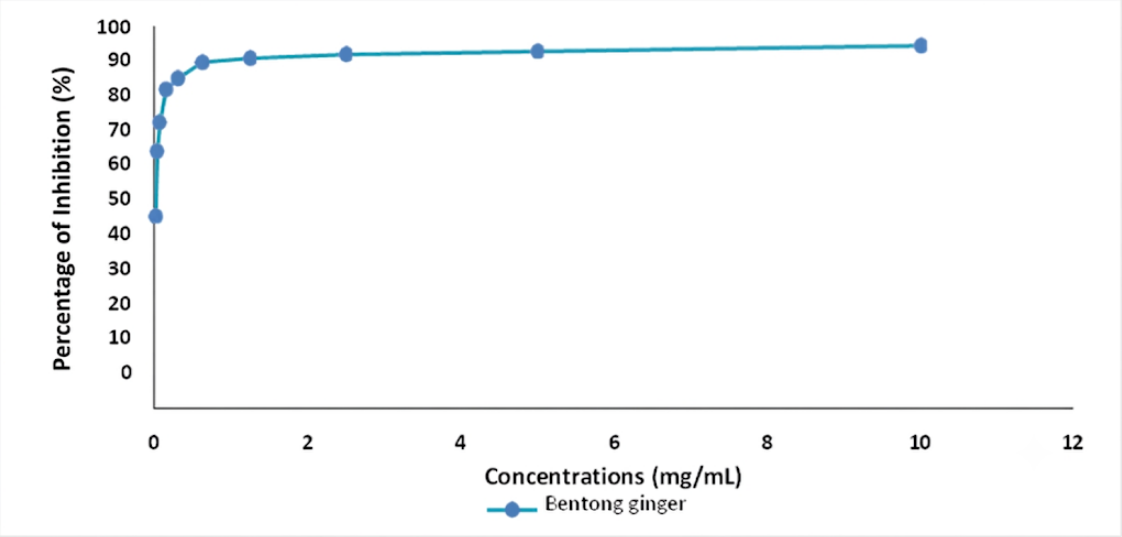

Figure 3. Percentage ABTS radical cation scavenging activity of Bentong ginger

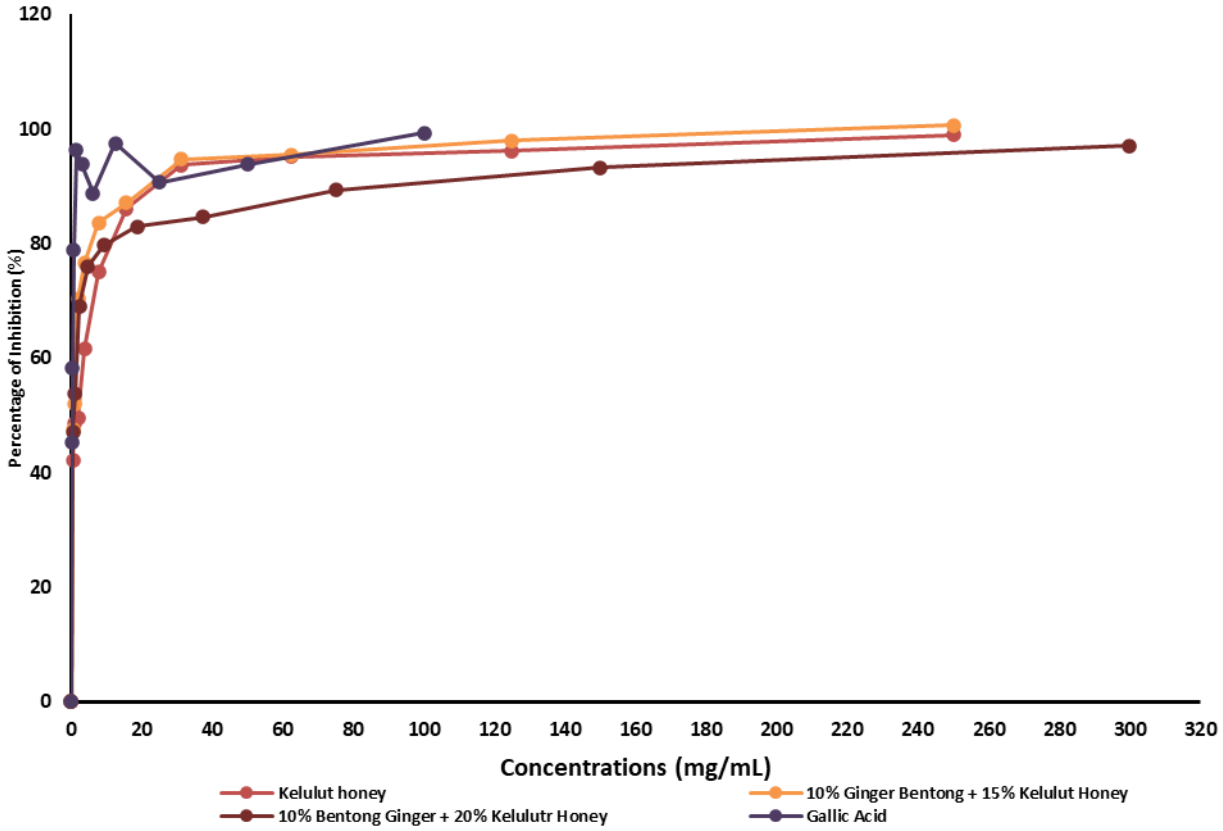

Figure 4. Percentage of ABTS radical cation scavenging activity of different concentration of Kelulut honey,and the combinations of Bentong ginger with Kelulut honey.

Table 3. IC_{50} value of Ginger Bentong, Kelulut Honey and combination of Bentong ginger with Kelulut honey for radical cation scavenging

activity.

Sample(s)	IC_{50} Values (mg/mL)
Gallic Acid	0.4
Bentong Ginger	0.02
Kelulut Honey	2.1
10% Bentong Ginger: 15% Kelulut	0.8
10% Bentong Ginger: 20% Kelulut	0.7

Putative Identification of Antioxidant Bioactive Compounds by LCMS/MS QTOF

LCMS/MS QTOF Profiling of Antioxidant Compounds in Bentong Ginger

LCMS/MS QTOF analysis of Bentong ginger extract resulted in the putative annotation of several phytochemical constituents with confidence scores exceeding 70%, as presented in Table 4. Compound annotation was performed based on accurate mass matching and database comparison; therefore, the identifications represent putative assignments (Level 2 confidence) [15]. The annotated compounds were primarily classified into sesquiterpenoids, phenolic derivatives, and flavin-related compounds, which are consistent with the known phytochemical composition of ginger rhizomes.

Table 4. The putatively annotated compounds and their classifications in Bentong ginger with confidence score of more than 70%

Antioxidant compounds (Retention time)	Confidence score	Mass	Compound classification
7(14)-Bisabolene-2,3,10,11-tetrol (12.736, 13.062, 13.419 and 15.582)	98.87	272.1984	Sesquiterpenoid
Threo-Syringoylglycerol (15.826)	93.64	244.0953	Phenolic
2,6-Dihydroxybenzoic acid (13.238)	86.09	154.0270	Phenolic
Methyl 2,4,6-trihydroxybenzoate (18.358 and 19.014)	85.52	184.0367	Phenolic
5-Carboxyvanillic acid (19.014)	76.47	212.0312	Phenolic
Lumichrome (15.834)	70.98	242.0794	Flavins

Among the identified constituents, 7(14)-Bisabolene-2,3,10,11-tetrol was detected at multiple retention times with a high confidence score (12.736, 13.062, 13.419 and 15.582 min; confidence score 98.87), suggesting the presence of oxygenated sesquiterpenoid derivatives. Sesquiterpenes are well-documented major components of ginger and are responsible for its characteristic aroma and various biological activities [1]. The presence of oxygenated functional groups in such terpenoid derivatives may enhance redox properties and contribute to antioxidant potential. In addition to terpenoids, several phenolic compounds were putatively annotated, including threo-syringoylglycerol (15.826 min; 93.64), 2,6-dihydroxybenzoic acid (13.238 min; 86.09), methyl 2,4,6-trihydroxybenzoate 18.358 and 19.014 min; 85.52), and 5-carboxyvanillic acid (19.014 min; 76.47). Phenolic derivatives are widely recognized for their radical scavenging activity due to their ability to donate hydrogen atoms or electrons and stabilize reactive species through resonance structures [16]. The detection of hydroxy- and methoxy-substituted aromatic compounds in the extract provides a plausible chemical explanation for the antioxidant activity observed in the DPPH and ABTS assays.

Threo-syringoylglycerol, a phenylpropanoid-related compound, may arise from lignin-associated metabolic pathways and contains structural features associated with antioxidant behavior [17]. Similarly, benzoic acid derivatives such as 2,6-dihydroxybenzoic acid and 5-carboxyvanillic acid are common plant

secondary metabolites that possess redox-active functional group [18,19]. Methyl 2,4,6-trihydroxybenzoate, an esterified phenolic derivative, may influence polarity and stability while retaining antioxidant capacity [20]. In addition, lumichrome, classified as a flavin-related compound, was detected with moderate confidence (15.834 min; 70.98). Lumichrome is a known riboflavin degradation product that can occur naturally in plant systems and may exhibit redox activity, potentially contributing to the overall antioxidant profile of the extract [21]. The LCMS findings indicate that Bentong ginger contains structurally diverse phytochemicals, particularly phenolic and terpenoid derivatives, which are consistent with its reported antioxidant properties.

LCMS/MS QTOF Profiling of Antioxidant Compounds in Kelulut Honey

LCMS/MS QTOF analysis of Kelulut honey resulted in the putative annotation of several compounds with confidence scores exceeding 70%, as summarized in Table 5. The annotated features were detected at distinct retention times ranging from 15.826 to 23.668 minutes and were assigned based on accurate mass matching against spectral databases.

Table 5. The putatively annotated compounds and their classifications in Kelulut honey with Confidence Score of more than 70%

Antioxidant compounds (Retention time)	Confidence score	Mass	Compound classification
Stearamide (18.279)	99.10	283.2879	Fatty acid amide
Palmitoyl-EA (15.826, 17.588)	90.25	299.2837	Fatty acid amide
Fukiic acid (19.167)	88.14	272.0522	Phenylpropanoic acid
2-Hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinol (23.668)	86.96	562.4371	Polyprenylayted hydroquinone (phenol)
Methyl 2,4,6-trihydroxybenzoate (18.588, 19.164)	85.65	184.0367	Phenolic
Herniarin (17.766)	83.26	237.1719	Coumarin (phenolic)
Naringenin 7,4'-dimethyl ether (18.419)	80.69	300.0987	Flavonoids

The detected compounds were primarily classified into fatty acid amides, phenolic derivatives, flavonoids, coumarins, and quinone-related structures, which are consistent with recent metabolomic investigations of Kelulut honey. Stearamide (18.279 min; confidence score 99.10) and palmitoyl-ethanolamide (15.826 and 17.588 min; confidence score 90.25) were reported in honey metabolomes, potentially from floral nectar and bee secretions, adding to chemical diversity though not primary phenolics [10]. Several phenolic-related compounds were also putatively annotated. Fukiic acid (19.167 min; 88.14) and methyl 2,4,6-trihydroxybenzoate (18.588 and 19.164 min; 85.65) represent phenylpropanoic and hydroxybenzoic acid derivatives, respectively. Phenolic acids are well established contributors to honey antioxidant capacity through hydrogen atom transfer and electron donation mechanisms [16]. The detection of hydroxy-substituted aromatic structures provides a plausible explanation for the radical scavenging activity observed in the DPPH and ABTS assays.

The presence of 2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinol (23.668 min; 86.96), classified as a polyprenylated hydroquinone derivative, suggests the occurrence of quinone-related compounds. Quinone and hydroquinone structures are redox-active molecules capable of participating in electron transfer reactions, which may contribute to antioxidant behavior in complex matrices. Herniarin (17.766 min; 83.26%), a coumarin derivative, and naringenin 7,4'-dimethyl ether (18.419 min; 80.69), a flavonoid derivative, further support the presence of plant-derived phenolic constituents in Kelulut honey. Flavonoids and coumarins have been widely reported in Kelulut honey and are recognized as key contributors to antioxidant properties [22].

Overall, the LCMS profiling indicates that Kelulut honey contains structurally diverse bioactive compounds, particularly phenolic and flavonoid derivatives, alongside lipid-related metabolites. These findings are consistent with recent metabolomic studies highlighting the complex phytochemical composition of Kelulut honey and its associated antioxidant potential.

LCMS/MS QTOF Profiling of Antioxidant Compounds in 10% Bentong Ginger with 15% Kelulut

LCMS/MS QTOF analysis of the 10% Bentong ginger and 15% Kelulut honey mixture resulted in the putative annotation of several compounds with confidence scores above 70%, as presented in Table 6. The detected features were observed at retention times ranging from 8.876 to 21.364 minutes. The annotated compounds were classified mainly into benzamides, fatty acids, fatty acid amides, terpenoid esters, and flavin-related derivatives.

Table 6. The putatively annotated compounds and their classifications in 10% Bentong ginger with 15% Kelulut honey with confidence score of more than 70%

Antioxidant compounds (Retention time)	Confidence score	Mass	Compound classification
N-[2-(4-Hydroxyphenyl) ethyl] benzamide (8.876)	96.24	241.1095	Benzamides
7,10-Hexadecadienoic acid (19.793)	93.67	252.2083	Fatty acid
Oleoyl Ethanolamide (21.364)	83.43	325.2986	Fatty acid amide
Linalyl propionate (11.815)	82.95	210.1610	Acyclic
Lumichrome (13.666)	79.68	242.0798	Flavin

N-[2-(4-hydroxyphenyl)ethyl] benzamide was detected at a retention time of 8.876 minutes with a confidence score of 96.24% and classified as a benzamide derivative. Structurally, this compound contains a hydroxy-substituted aromatic ring, which may contribute to antioxidant behavior through hydrogen atom donation and electron transfer mechanisms [23]. Additionally, 7,10-Hexadecadienoic acid (19.793 min; 93.67%) was identified as a polyunsaturated fatty acid. Fatty acids are commonly reported in both plant extracts and honey and may originate from floral lipids or bee metabolic processes [10]. Besides, oleoyl ethanolamide (21.364 min; 83.43%), classified as a fatty acid amide, further supports the presence of lipid-derived metabolites in the mixture. Fatty acid ethanolamides have been detected in natural matrices and may arise from lipid metabolism or enzymatic modification processes. Linalyl propionate (11.815 min; 82.95%), identified as an acyclic terpenoid ester, likely originates from floral volatile components and is consistent with the terpenoid profile associated with ginger and nectar-derived compounds [10]. Terpenoid esters may indirectly contribute to antioxidant potential through complementary redox interactions within complex phytochemical systems. Lumichrome (13.666 min; 79.68%), classified as a flavin-related compound, represents a riboflavin-derived metabolite that may arise from honey. As noted by previous studies on Western Australian honeys, the lack of hydroxyl substitution on the lumichrome benzene ring precludes the hydrogen-atom transfer necessary for activity in DPPH-based assays [21].

In summary, the LCMS profiling of the 10:15 mixture indicates the presence of structurally diverse compounds, including phenolic-related aromatic derivatives, unsaturated fatty acids, fatty acid amides, terpenoid esters, and flavin-related metabolites. The coexistence of these chemically distinct classes suggests a complementary phytochemical composition that may underlie the enhanced antioxidant activity observed in the radical scavenging assays. Nevertheless, as the compound identifications are putative and based solely on accurate mass matching, further confirmation using authentic standards and targeted MS/MS analysis would be required for definitive structural validation and quantitative assessment.

LCMS/MS QTOF Profiling of Antioxidant Compounds in 10% Bentong Ginger with 20% Kelulut

LCMS/MS QTOF analysis of the 10% Bentong ginger and 20% Kelulut honey mixture resulted in the putative annotation of several compounds with confidence scores exceeding 70%, as presented in Table 7. The annotated features were detected at retention times ranging from 8.749 to 12.530 minutes.

Table 7. The putatively annotated compounds and their classifications in 10% Bentong ginger with 20% Kelulut honey with confidence score of more than 70%

Antioxidant compounds (Retention time)	Confidence score	Mass	Compound classification
N-[2-(4-Hydroxyphenyl) ethyl] benzamide (8.749)	91.53	241.1094	Benzamides
7(14)-Bisabolene-2,3,10,11-tetrol (10.007, 10.238, 10.498, and 12.530)	84.49	272.1978	Sesquiterpenoid
Linalyl propionate (11.559)	83.65	210.1611	Acyclic monoterpenoids

The detected compounds were similar with the mixture of 10% ginger and 15% honey. The phytochemical profiles of both mixtures are anchored by the presence of N-[2-(4-Hydroxyphenyl) ethyl] benzamide, which serves as the highest-scoring marker in both the 15% honey formulation (8.876 min; 96.24 confidence) and the 20% honey formulation (8.749 min; 91.53 confidence). Similarly, the acyclic monoterpenoid linalyl propionate was identified across both samples, appearing at 11.815 min with an 82.95 score in the 15% mixture, and at 11.559 min with an 83.65 score in the 20% mixture.

Significant contrasts emerge in the diversity of specialized metabolites identified. The 15% honey mixture (Table 6) uniquely displays a broader range of lipid and flavin derivatives, including the fatty acid 7,10-Hexadecadienoic acid (19.793 min; 93.67 score), the fatty acid amide oleoyl ethanolamide (21.364 min; 83.43 score), and the flavin lumichrome (13.666 min; 79.68 score). While lumichrome is identified here, literature suggests it lacks direct radical scavenging activity in DPPH assays because it lacks the necessary hydroxyl groups on its benzene ring to participate in primary antioxidant reactions. Conversely, the 20% honey mixture (Table 7) shows a more specialized terpenoid profile with the addition of the sesquiterpenoid 7(14)-Bisabolene-2,3,10,11-tetrol, which was detected across multiple retention times (10.007, 10.238, 10.498, and 12.530 min) with a confidence score of 84.49. Unlike lumichrome, which lacks radical scavenging activity due to the absence of hydroxyl groups in its benzene ring, this bisabolene derivative contains four hydroxyl groups that facilitate antioxidant activity through hydrogen atom donation. These hydroxyl groups allow the molecule to neutralize free radicals, thereby contributing to the mixture's overall antioxidant potential [24]. Additionally, as an acyclic monoterpenoid, linalyl propionate (11.559 min; 83.65 score) may also contribute to the antioxidantive activities [25].

Compared to the 10:15 mixture, the 10:20 formulation exhibited a narrower range of annotated compounds above the confidence threshold. Nevertheless, the coexistence of phenolic-related aromatic derivatives and oxygenated terpenoids in the 10:20 mixture provides a plausible chemical basis for its antioxidant activity. Overall, the LCMS findings support the hypothesis that the antioxidant activity of the ginger–honey formulations arise from structurally diverse phytochemicals derived from both botanical and bee-mediated sources. However, as the compound identifications are putative, further confirmation using authentic standards and MS/MS fragmentation analysis would be necessary for definitive structural validation.

Combination Index Analysis

The combination index (CI) was calculated based on the Chou–Talalay method using IC_{50} values obtained from the DPPH assay since this assay exhibited increase antioxidant capacities compared to ABTS assays. The analysis conducted revealed that 10% ginger:15% honey mixture exhibited a CI value of 0.66, indicating strong synergistic interaction (CI < 0.7), whereas the 10% ginger:20% honey mixture showed a CI value of 0.86, corresponding to moderate synergy [13]. These findings demonstrate that the 10:15 formulation provides a more optimal interactive antioxidant effect. The observed synergy may be attributed to complementary redox mechanisms between phenolic derivatives and oxygenated terpenoids identified through LCMS analysis.

From a translational perspective, ginger-based synergistic antioxidant compositions have previously been patented, most notably a United States invention combining ginger with other herbal extracts such as honeysuckle and sophora, for topical antioxidant skin-care applications (US Patent No. US8043637B2). While the present formulation is similarly directed towards topical application, it differs fundamentally from such prior art in that it pairs Bentong ginger with Kelulut honey, a combination not

covered by existing ginger–herb antioxidant patents. The optimized 10:15 ginger-to-honey ratio identified here therefore offers a scientifically standardized compositional basis for the development of topical antioxidant formulations, representing a meaningful advancement over currently available ginger- and honey-based preparations.

Conclusions

This study demonstrates that Bentong ginger and Kelulut honey possess strong intrinsic antioxidant activities, and their combination, particularly the 10% ginger and 15% honey formulation, produces a markedly enhanced and synergistic effect. The DPPH and ABTS assays confirmed superior radical-scavenging performance for this ratio, supported by LCMS/MS QTOF profiling that revealed a diverse array of complementary phytochemicals, including phenolic acids, terpenoids, flavonoids, fatty acid derivatives, and flavin-related compounds. Combination index analysis confirmed synergistic antioxidant interactions at both ginger–honey ratios tested, with the 10:15 formulation exhibiting strong synergy (CI = 0.66) and the 10:20 formulation demonstrating moderate synergy (CI = 0.86). Overall, the findings highlight the potential of ginger–honey formulations as natural, effective antioxidant sources and support future development of functional foods or nutraceutical products based on these synergistic bioactive combinations. Nevertheless, it should be noted that this study assessed antioxidant activity at a single timepoint, and the long-term stability of the formulations under real storage conditions remains to be investigated in future studies.

Conflicts of Interest

The best antioxidant composition of Bentong ginger and kelulut honey has been filed for patent under patent filing number PI2025008556.

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