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Enhancing the bioactive profile of lemon-based beverages through varietal honey supplementation: a multi-assay antioxidant evaluation

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ABSTRACT

Introduction: Lemon (*Citrus limon*) is one of the most widely cultivated citrus fruits in the world. Its bioactive components, citric acid plays a crucial role in energy metabolism through the citric acid cycle and demonstrates antioxidant activity by neutralizing harmful free radicals. Several studies have suggested a potential link between lemon consumption and blood pressure regulation.

Objectives: This study aimed to compare the antioxidant activity of lemon-based beverages formulated with three types of honey (Calliandra, Acacia honey, and Kelulut) using complementary antioxidant assays (CUPRAC, FRAP, and DPPH), and to explore their potential relationship with hypertension-related biomarkers such as F2-isoprostanes, ADMA, and SDMA.

Methods and Materials: Formulations included F0 (lemon juice control, 50 mL), F1 (lemon + 40 mL Calliandra honey), F2 (lemon + 40 mL Acacia honey), and F3 (lemon + 40 mL Kelulut honey). Antioxidant capacity was measured by CUPRAC (pH 7.0, 30 min at 25 ± 1 °C), FRAP (pH 3.6, 30 min at 37 °C), and DPPH (0.1 mM, 30 min at 25 ± 1 °C) assays using UV–Vis spectrophotometry. Each sample was analyzed in triplicate (n = 3). Results were expressed as ascorbic acid equivalents (AAE) and Trolox equivalents (TE).

Results: Significant differences were observed among formulations ($p < 0.0001$). The Acacia honey formulation (F2) exhibited the highest antioxidant activity across CUPRAC (3282.33 ± 1.64 µg/mL AAE; 5169.00 ± 100.22 µg/mL TE), FRAP (749.40 ± 4.06 µg/mL AAE; 288.83 ± 1.80 µg/mL TE), and DPPH (22.74 ± 1.04 µg/mL AAE; 27.71 ± 0.28 µg/mL TE) assays, which, according to Duncan's test, were significantly higher than F0, F1, and F3 ($p < 0.05$).

Conclusion: Lemon Acacia honey beverages demonstrated superior and broad-spectrum antioxidant activity compared to other formulations, likely due to the presence of flavonoids such as quercetin, kaempferol, and apigenin. These findings support the potential role of Acacia honey in modulating oxidative stress biomarkers, warranting further in vivo and clinical investigations.

KEYWORDS

Citrus Lemon, Honey types, Antioxidant Activity, CUPRAC, FRAP, DPPH assays.

INTRODUCTION

Lemon (*Citrus limon*) is one of the most widely cultivated citrus fruits in the world. It is commonly used in food, cooking, preservation, and beverages due to its distinctive flavor and beneficial properties. The growing demand for fresh lemons is largely driven by their rich content of natural phytochemicals, including citric acid, ascorbic acid, dietary fiber, essential oils, minerals, carotenoids, and flavonoids[1]. Among its bioactive components, citric acid plays a crucial role in energy metabolism through the citric acid cycle and demonstrates antioxidant activity by neutralizing harmful free radicals[2].

Several studies have suggested a potential link between lemon consumption and blood pressure regulation. In a study

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involving Turkish hypertensive patients, 40% reported consuming lemon juice as part of their alternative therapy[3]. Similarly, in healthy Japanese women, lemon intake was significantly associated with lower systolic blood pressure[4]. The underlying mechanism may involve the inhibition of NADPH oxidase, reduction of reactive oxygen species (ROS), and modulation of endothelial dysfunction markers such as asymmetric dimethylarginine (ADMA). These mechanisms are also linked to the reduction of lipid peroxidation products like F2-isoprostanes, which serve as oxidative stress biomarkers[5].

To enhance both palatability and nutritional value, lemon is increasingly being formulated into functional beverages with natural sweeteners such as honey[6]. In addition to being a healthier sweetener, honey provides numerous bioactive compounds. It has been shown to improve nitric oxide (NO) bioavailability and vascular function, particularly through components like ferulic acid and flavonoids[7]. Honey exhibits various biological activities, including antioxidant, antihypertensive, anti-inflammatory, and antimicrobial effects. The composition of honey varies depending on floral origin, bee species, geography, and seasonality[8].

In this study, three honey varieties Calliandra, Acacia, and Kelulut were selected based on their distinct botanical and entomological origins. Calliandra honey, produced by wild *Apis dorsata* bees from Calliandra flower nectar, is rich in flavonoids and has demonstrated antihypertensive effects[7]. Acacia honey comes from the nectar of acacia leaf axils by mellifera bees in the acacia forests of Dumai, Riau. Meanwhile, Kelulut honey, obtained from stingless bees (*Trigona* spp.), contains high levels of phenolic acids and has been shown to possess strong antioxidant and antimicrobial activity[9].

The combination of lemon and various honey types offers a promising approach to reduce oxidative stress and hypertension. While honey has been shown to improve lipid profiles, inflammatory markers, and blood pressure[10], comparative studies on the antioxidant performance of lemon-honey beverages and their relationship with oxidative stress biomarkers remain limited.

Antioxidant activity can be assessed using several methods, including CUPRAC, FRAP, and DPPH assays. Each method offers different insights depending on the chemical nature of antioxidants involved. CUPRAC assesses electron-donating capacity at physiological pH, FRAP measures reducing power in acidic pH, and DPPH reflects hydrogen atom transfer[11]. These complementary assays provide a comprehensive evaluation of antioxidant potential, especially when comparing samples with diverse bioactive content.

This study aimed to compare the antioxidant activity of lemon-based beverages formulated with three different types of honey Calliandra, Kelulut, and Honeydew using CUPRAC, FRAP, and DPPH assays, and to explore their potential association with oxidative stress biomarkers such as F2-isoprostanes, ADMA, and SDMA linked to hypertension.

MATERIALS AND METHODS

Preparation for Lemon and Honey Extract

This study used ripe *Citrus x limon* (L.) Osbeck lemons and three types of honey: Calliandra (*Apis mellifera*), Acacia Honey (*Apis mellifera*) and Kelulut (*Trigona spp.*). The lemon variety was selected for its genetic resilience, productivity, and suitability for product development. Fresh lemons (~15 kg per batch) were sourced from farmers in Ciampea, Bogor, while honey (totaling 3,660.6 mL) was obtained from local markets and beekeepers.

The production process began with washing the lemons, cutting them into approximately 5 × 5 cm pieces, and extracting the juice using a manual fruit press machine (Heavy Duty Manual Juice Extractor). This yielded around 11.25 L of fresh lemon juice, equivalent to 50 mL per serving. The juice was then mixed with one of the three honey varieties in equal proportions (40 mL or 57 g per serving). The final mixture was pasteurized at 90°C for 18 seconds.

The ratio of lemon juice to honey (50:40 mL; 1.25:1) was chosen within the general formulation range reported in functional beverage studies (1:1–1:3) to balance palatability and functional potential[4,12].

Table 1. The formulations of lemon and honey

Ingredients	Type of Honey		
	F1*	F2*	F3*
Lemon	50	50	50
Honey	40	40	40
Total	90	90	90

F1 = Calliandra, F2 = Acacia Honey, F3 = Kelulut.

Antioxidant activities measurement (Cupric Ion Reducing Antioxidant Capacity (CUPRAC), Ferric reducing antioxidant power (FRAP), and DPPH Free radical scavenging assay)

The antioxidant activity of the lemon-honey beverage was evaluated using three established methods: Cupric Ion Reducing Antioxidant Capacity (CUPRAC), Ferric Reducing Antioxidant Power (FRAP), and DPPH free radical scavenging assay.

CUPRAC Assays

The antioxidant capacity using the CUPRAC method was determined by reacting the sample or standard solution with the

CUPRAC reagent (a mixture of 10^{-3} M $CuCl_2$, 7.5×10^{-3} M neocuproine in ethanol, and 1 M ammonium acetate buffer, pH 7.0). The reaction mixture consisted of 1,000 µL of the test solution and 3,000 µL of the CUPRAC reagent. The mixture was incubated for 30 minutes at room temperature (25 ± 1°C) under light-protected conditions, and the absorbance was measured at 450 nm using a UV-Vis spectrophotometer.

FRAP Assays

The FRAP method was performed based on the reduction of Fe^{3+} to Fe^{2+}, forming an intense blue Fe^{2+}-TPTZ complex. The FRAP reagent consisted of 2,500 µL sodium acetate buffer solution (pH 3.6), 250 µL TPTZ solution (10 mM in ethanol), and 250 µL $FeCl_3 \cdot 6H_2O$ solution (20 mM in 5 M HCl). A 10 µL sample was added to the reagent mixture and incubated at 37°C for 30 minutes under light-protected conditions. Absorbance was measured at 595 nm using a UV-Vis spectrophotometer. Vitamin C and Trolox were used as standards at concentrations ranging from 5–40 µg/mL.

Assay DPPH

Antioxidant activity by the DPPH method was performed using 0.1 mM DPPH solution in ethanol (pH ~7.0). The samples (F0–F3) were diluted in ethanol to a final concentration of 5% v/v. A total of 1,000 µL of the diluted sample was mixed with 2,000 µL of the DPPH solution, vortexed, and incubated at room temperature (25 ± 1°C) for 30 minutes under light-protected conditions. Absorbance was measured at 515 nm using a UV-Vis spectrophotometer. Vitamin C (0–10 µg/mL) and Trolox (0–20 µg/mL) were used as positive controls. Antioxidant capacity was expressed as percentage inhibition and IC_{50} values. The percentage of antioxidant activity (AA%) was calculated using the following equation:

$$AA\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Experimental conditions and replication

All measurements (CUPRAC, FRAP, and DPPH) were performed in triplicate for each formulation. Analyses were conducted simultaneously in a single batch, since the purpose of this study was to compare antioxidant activity among formulations rather than to validate inter-day variability. Experimental conditions were kept constant under controlled laboratory settings, including pH (according to each assay), temperature (25 ± 1°C for CUPRAC and DPPH; 37°C for FRAP), reaction time (30 minutes), and protection from light during incubation.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Prior to statistical testing, data were assessed for normality and homogeneity of variances. Statistical analysis was performed using one-way analysis of variance (ANOVA) in IBM SPSS Statistics. Post-hoc comparisons were conducted using Duncan's multiple range test to determine significant differences among the means. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Antioxidant activity of lemon honey beverage

The CUPRAC (Cupric Reducing Antioxidant Capacity) assay evaluates the ability of antioxidant compounds to reduce Cu^{2+} to Cu^{+} ions. In this system, the Cu^{2+}-neocuproine complex ($Cu(Nc)_2^{2+}$) is reduced to a yellow-colored $Cu(Nc)_2^{+}$ complex, measured at 450 nm. CUPRAC is sensitive to polyphenols, flavonoids, and vitamins typically found in lemon and honey. One advantage of CUPRAC compared to DPPH is its stability and lower susceptibility to interference from non-antioxidant compounds[13,14].

As shown in Table 2, the antioxidant capacity significantly differed among the four formulations (F0–F3), with $p < 0.0001$ across all assays.

Table 2. Summary of Antioxidant Activity Parameters of Lemon-Honey Beverages

Parameter	F0	F1	F2	F3	Significance (p-value)
CUPRAC-AAE (µg/mL)	1346.67 ± 11.59^{a}	1465.00 ± 23.43^{b}	3282.33 ± 1.64^{d}	3029.67 ± 35.02^{c}	<0.0001
CUPRAC-TE (µg/mL)	2014.33 ± 22.37^{a}	2243.67 ± 45.39^{b}	5169.00 ± 100.22^{d}	4680.00 ± 67.55^{c}	<0.0001
FRAP-AAE (µg/mL)	306.40 ± 2.89^{b}	291.10 ± 3.16^{a}	749.40 ± 4.06^{d}	409.40 ± 3.60^{c}	<0.0001
FRAP-TE (µg/mL)	88.17 ± 1.29^{b}	81.20 ± 1.45^{a}	288.83 ± 1.80^{d}	134.80 ± 1.60^{c}	<0.0001
DPPH-AAE (µg/mL)	10.21 ± 1.22^{a}	12.58 ± 1.06^{b}	22.74 ± 1.04^{d}	18.90 ± 0.26^{c}	<0.0001
DPPH-TE (µg/mL)	6.53 ± 1.53^{a}	13.30 ± 0.54^{b}	27.71 ± 0.28^{d}	23.06 ± 8.66^{c}	<0.0001

Means in the same row with different superscript letters are significantly different (Duncan's test, $p < 0.05$).

The results of the CUPRAC assay showed that all honey-containing formulations (F1–F3) exhibited significantly higher antioxidant activity than the control (F0). The F2 formulation (Acacia honey) yielded the highest activity (3282.33 ± 1.64 µg/mL AAE; 5169.00 ± 100.22 µg/mL TE), which, according to Duncan's test, was significantly different from F0, F1, and F3 ($p < 0.05$). This strong performance is likely due to the presence of active flavonoids and phenolic compounds in Acacia honey, such as quercetin and apigenin, which efficiently interact with the CUPRAC redox system[14].

The FRAP assay showed a consistent trend, with F2 (Acacia honey) again demonstrating the strongest ferric reducing capacity (749.40 ± 4.06 µg/mL AAE; 288.83 ± 1.80 µg/mL TE). According to Duncan's test, this was significantly higher than all other formulations ($p < 0.05$). The synergistic interaction between lemon vitamin C and Acacia flavonoids such as kaempferol and apigenin likely explains this enhancement[15].

The DPPH assay also revealed that F2 (Acacia honey) exhibited the strongest radical scavenging activity (22.74 ± 1.04 µg/mL AAE; 27.71 ± 0.28 µg/mL TE), significantly higher than F0, F1, and F3 ($p < 0.05$, Duncan's test). This indicates that Acacia honey possesses superior hydrogen-donating ability, consistent with its high phenolic content, including quercetin, apigenin, and phenolic acids[16]. Unlike CUPRAC and FRAP, which rely on electron transfer, DPPH emphasizes hydrogen atom transfer[17], suggesting that Acacia honey demonstrates broad antioxidant activity across multiple mechanisms.

Overall, Duncan's test confirmed that all formulations were statistically different from one another across CUPRAC, FRAP, and DPPH assays. Acacia honey (F2) consistently ranked highest in antioxidant capacity, followed by Kelulut (F3) and Calliandra (F1), while lemon alone (F0) had the lowest activity.

Differences in antioxidant capacity among test methods are influenced not only by the composition of bioactive compounds but also by environmental factors such as pH, temperature, and possible synergistic or antagonistic interactions[18]. The CUPRAC method operates at a neutral pH (~7), a relatively stable condition that mimics the physiological environment of the human body[19], allowing compounds such as flavonoids and vitamin C to remain active. In contrast, the acidic environment of the FRAP assay (pH 3.6) can promote metal ion release but may destabilize sensitive compounds. The strong performance of F2 in both assays suggests that its antioxidants (e.g., quercetin, kaempferol, apigenin) are stable and active across different conditions[20].

Potential Link between Antioxidant Activity and the Reduction of Blood Pressure and Oxidative Biomarkers

Oxidative stress and endothelila dysfunction mechanisms in the development of hypertension, primarily driven by excess reactive oxygen species (ROS) from sources such as NADPH oxidase and mitochondria. These ROS reduce nitric oxide (NO) bioavailability, impair endothelial function, and promote eNOS uncoupling, which further produces ROS instead of NO—creating a cycle of vascular damage and inflammation[21]. Endothelial dysfunction is also a well-known predictor of cardiovascular events.

Key biomarkers such as asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are used to assess endothelial dysfunction and oxidative stress. ADMA directly inhibits eNOS, while SDMA interferes with arginine transport, both reducing NO availability. F2-isoprostanes also serve as stable indicators of lipid peroxidation and oxidative stress[22].

Natural products like Calliandra and Acacia honey are rich in phenolic compounds and flavonoids with strong antioxidant potential. Supplementation with Calliandra honey has been shown to enhance antioxidant enzyme activity (SOD, CAT, GPx) and lower malondialdehyde (MDA) levels in hypertensive rats while stingless bee honey has demonstrated protective effects against oxidative DNA damage and iNOS expression[23].

Lemon also provides vitamin C and flavonoid glycosides such as hesperidin and eriocitrin, which exhibit antihypertensive properties. In a study using hypertensive rats, lemon flavonoids significantly reduced systolic blood pressure by inhibiting angiotensin-converting enzyme (ACE), with hesperidin showing greater efficacy than epigallocatechin gallate[24].

Thus, combining tropical honey and lemon may offer complementary effects by reducing oxidative stress and blood pressure through antioxidant enhancement, anti-inflammatory activity, and ACE pathway inhibition. This synergy may help modulate biomarkers like ADMA and F2-isoprostanes, although clinical validation remains necessary.

In this study, antioxidant activity was evaluated using CUPRAC and FRAP rather than DPPH, due to their electron transfer mechanisms, which more accurately reflect physiological redox states[25]. CUPRAC operates at neutral pH (~7), simulating plasma conditions, while FRAP measures metal ion reduction[26]. In contrast, DPPH based on hydrogen atom transfer is sensitive to lipophilic antioxidants and less applicable to clinical oxidative biomarkers[27]. Interestingly, the F2 formulation (lemon + Acacia honey) consistently showed the highest antioxidant activity across CUPRAC, FRAP, and DPPH assays. This suggests that Acacia honey possesses broad-spectrum antioxidant potential, being effective in both electron transfer (CUPRAC and FRAP) and hydrogen atom transfer (DPPH) mechanisms.

Such robust in vitro activity strengthens the rationale for its potential biological relevance, particularly in protecting endothelial function and reducing oxidative stress. The synergistic interaction between lemon flavonoids (e.g., hesperidin,

eriocitrin) and Acacia honey polyphenols (e.g., quercetin, apigenin) may help modulate key biomarkers such as ADMA, SDMA, and F2-isoprostanes, thereby supporting future investigations into its role in blood pressure regulation and cardiovascular protection.

CONCLUSION

This study compared the antioxidant activity of lemon-based beverages containing Calliandra, Acacia honey, and Kelulut honey using CUPRAC, FRAP, and DPPH assays, and explored their potential relationship with oxidative stress biomarkers linked to hypertension, such as F2-isoprostanes, ADMA, and SDMA.

Among the formulations, Acacia honey (F2) consistently exhibited the highest antioxidant capacity across CUPRAC, FRAP, and DPPH assays, indicating strong electron-donating and hydrogen-donating potential, likely due to stable flavonoids such as quercetin, kaempferol, and apigenin. The superior redox activity of F2 supports its potential role in reducing biomarkers like F2-isoprostanes and ADMA, both of which are closely associated with vascular oxidative stress and hypertension.

Thus, the Acacia honey formulation (F2) represents a promising candidate for functional beverage development aimed at mitigating oxidative stress and supporting blood pressure management, highlighting the need for further in vivo and clinical validation.

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