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Metabolomic Profiling and *In Silico* Analysis of Acacia Honey–Lemon Bioactives: Potential DDAH-1 Interactions

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ABSTRACT

The metabolite composition of Acacia honey–lemon formulations and the potential interactions of their bioactive compounds with dimethylarginine dimethylaminohydrolase 1 (DDAH-1), an enzyme involved in asymmetric dimethylarginine (ADMA) metabolism, remain insufficiently characterized. This study aims to characterize the bioactive profile of an Acacia honey–lemon formulation using an integrated analytical and computational approach. Metabolite profiling was performed using LC–MS/MS, followed by HPLC-based quantification of hesperidin to support its selection as a literature-supported and HPLC-guided docking ligand. Selected compounds were subsequently evaluated by molecular docking against DDAH-1. LC–MS/MS analysis putatively annotated 93 metabolites within retention times of 0.94–31.74 minutes, including flavonoids, phenolic compounds, organic acids, vitamins, and alkaloids. HPLC analysis showed significant differences in hesperidin concentrations among formulations ($p<0.001$), with the Acacia honey–lemon formulation showing the highest measured concentration (1054.98 ± 31.08 mg/100 mL). Molecular docking predicted hesperidin as the ligand with the strongest interaction with DDAH-1, with a binding affinity of −11.7 kcal/mol. The Acacia honey–lemon formulation exhibited a diverse putatively annotated metabolite profile and the highest HPLC-measured hesperidin concentration among the formulations tested. Hesperidin showed the strongest predicted binding affinity for DDAH-1 among the ligands evaluated.

Keywords: acacia honey; DDAH-1; lemon; metabolite profiling; molecular docking

INTRODUCTION

Bees are an important livestock commodity that produce high-value functional products, particularly honey. Honey is a natural supersaturated solution produced from floral nectar through collection, enzymatic modification, and storage by honey bees (Khan *et al*., 2018). Honey consists mainly of fructose and glucose (~80%), along with water, amino acids, enzymes, vitamins, minerals, polyphenols, and flavonoids (Din *et al*., 2025). These constituents contribute to its antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory activities (Bouali *et al*., 2022).

Among monofloral honeys, acacia honey is widely produced in managed beekeeping systems and is derived from Acacia flowers, including Acacia mangium and Robinia pseudoacacia (Mărgăoan *et al*., 2021). The interaction between bees, forage plants, and the cultivation environment influences the bioactive composition of acacia honey, including bee-derived proteins, enzymes, and peptides (Ustadi *et al*., 2017).

Acacia honey was selected a priori as the primary honey variety in the present study based on previous findings demonstrating its consistently favorable antioxidant performance when combined with lemon. Jayadi *et al*. (2025) reported that the Acacia honey–lemon formulation exhibited favorable antioxidant capacity across the CUPRAC, FRAP, and DPPH assays compared with formulations prepared using other honey varieties. These findings provided the scientific basis for selecting the Acacia honey–lemon formulation for subsequent LC–MS/MS-based metabolite profiling and molecular docking. Proteins and enzymes in honey, such as those involved in hydrogen peroxide formation and antimicrobial activity, contribute significantly to its biological properties. The biological characteristics of Acacia honey as a product of managed bees may therefore confer specific molecular activities that warrant further mechanistic investigation.

Honey is frequently combined with fruits and other natural ingredients to develop functional food and beverage products. Lemon (*Citrus limon*) is particularly relevant because it provides an acidic matrix and contains phenolic compounds, flavonoids, carotenoids, limonoids, vitamin C, and minerals (Shakour *et al*., 2020). Previous studies on honey–lemon formulations have primarily focused on physicochemical characteristics, antioxidant capacity, sensory properties, and microbiological quality (Jayadi *et al*., 2025).

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Although previous studies have examined the physicochemical, sensory, antioxidant, microbiological, and storage properties of honey–lemon products, their metabolite composition and molecular interactions remain insufficiently characterized. In particular, limited information is available on the putative metabolite profile of Acacia honey–lemon formulations, hesperidin levels, and interactions of their bioactive compounds with targets related to endothelial function. Therefore, this study integrated LC–MS/MS-based putative metabolite profiling, HPLC-based hesperidin quantification, and molecular docking with dimethylarginine dimethylaminohydrolase-1 (DDAH-1), a protein involved in asymmetric dimethylarginine metabolism, nitric oxide bioavailability, and endothelial function. Molecular docking was used to predict ligand–protein interactions but not to establish DDAH-1 activation or inhibition. This study aims to characterize the putative metabolite profile of an Acacia honey–lemon formulation, quantify hesperidin in lemon-only and honey–lemon formulations, and evaluate the predicted interactions of hesperidin and other selected bioactive compounds with DDAH-1, providing a hypothesis-generating basis for their potential relevance to endothelial function.

MATERIALS AND METHODS

This study employed a sequential analytical framework to evaluate the bioactive potential of honey–lemon formulations. The analytical stages included: (1) LC–MS/MS-based metabolite profiling of the Acacia honey–lemon formulation; (2) putative annotation of secondary metabolites from the LC–MS/MS data; (3) HPLC-based quantitative determination of hesperidin across the honey–lemon formulations to provide complementary analytical support for its selection as a citrus-related marker and principal docking ligand; and (4) in-silico evaluation of hesperidin and other selected ligands against dimethylarginine dimethylaminohydrolase-1 (DDAH-1) as a vascular-related molecular target.

Sample Source and Formulation

Three honey–lemon beverage formulations were prepared using different honey types: Calliandra honey (LM1), Acacia honey (LM2), and Itama honey (LM3). Acacia honey was obtained from a beekeeping farm in Dumai, Riau Province, Indonesia. Calliandra honey was obtained from Jepara, Central Java, Indonesia, while Itama honey (*Trigona* sp.) was obtained from Kalimantan, Indonesia. Fresh lemons (*Citrus limon*) were sourced from Ciampea Farm, Bogor, and harvested six months after flowering. Each formulation was prepared by mixing honey and freshly squeezed lemon juice at a ratio of 50:40 mL (56:44, v/v). The mixtures were homogenized and pasteurized at 92–94 °C for 17 seconds and then stored under refrigerated conditions prior to analysis (Kim *et al*., 2015). Acacia honey–lemon (LM2) was selected as the primary formulation for LC–MS/MS-based metabolite profiling based on previous evidence demonstrating its consistently favorable antioxidant performance (Jayadi *et al*., 2025). This selection was made independently of the comparative hesperidin quantification conducted in the present study. HPLC-based determination of hesperidin was performed for the lemon-only control and all three honey–lemon formulations (L0, LM1, LM2, and LM3) to evaluate formulation-associated differences in hesperidin concentration. LM1 and LM3 were not subjected to LC–MS/MS-based metabolite profiling or molecular docking. However, because honey-only, matrix-spiked recovery, and targeted LC–MS/MS confirmation controls were not included, the observed differences in hesperidin concentration were interpreted as formulation-associated analytical differences rather than direct evidence that Acacia honey specifically enhanced or preserved hesperidin stability.

Preparation of Extracts

For LC–MS/MS-based putative metabolite profiling, only the Acacia honey–lemon formulation was analyzed**.** The Acacia honey–lemon sample was processed using a conventional extraction (CE) approach to obtain a supernatant enriched with bioactive compounds. Briefly, the sample was homogenized in 80% aqueous methanol at a ratio of 1:2 (w/v) for 30 seconds to facilitate matrix disruption and enhance the release of soluble metabolites. The resulting homogenate was subsequently subjected to orbital shaking at 200 rpm for 120 minutes at room temperature (20 ± 2°C). Following extraction, the mixture was centrifuged at 6000 × g for 15 minutes at 20 °C, and the clear supernatant was carefully recovered for subsequent analyses, in accordance with a previously described protocol (Giovagnoli-Vicuña *et al*., 2022).

LC–MS/MS-Based Metabolite Profiling and Putative Metabolite Annotation

Metabolite profiling was performed using a Vanquish UHPLC system coupled to a Q Exactive Plus Orbitrap high-resolution mass spectrometer (Thermo Scientific). Samples were filtered through a 0.2 μm nylon membrane to remove particulate matter and diluted with methanol at a ratio of 1:10 before instrumental analysis. Chromatographic separation was performed using an Accucore C18 column (100 × 2.1 mm, 1.5 μm; Thermo Scientific).

Solvent A consisted of water containing 0.1% formic acid, while solvent B consisted of acetonitrile containing 0.1% formic acid. The mobile phase was delivered at a flow rate of 0.2 mL/min. Gradient elution was performed as follows: 0–1 min, 5% B; 1–25 min, 5%–95% B; 25–28 min, 95% B; and 28–33 min, 5% B for column re-equilibration. The column temperature was maintained at 30 °C, and the injection volume was 2 μL.

Mass spectrometric detection was performed using electrospray ionization in both positive and negative modes over an m/z range of 100–1500. The heated electrospray ionization source was operated with sheath gas at 15 arbitrary units, auxiliary gas at 5 arbitrary

units, and sweep gas at 0 arbitrary units. The spray voltage was set at 4.2 kV in positive ion mode and 3.8 kV in negative ion mode. The capillary temperature and heater temperature were set at 320 °C and 200 °C, respectively, and the S-lens RF level was fixed at 50.

Metabolite annotation was performed by matching accurate mass measurements and available MS/MS fragmentation data with entries in the ChemSpider and mzCloud databases. Annotated features were further evaluated based on retention time, molecular formula, calculated mass, mass error, database matching results, peak area, and comparison with the analytical blank. Annotation confidence was assigned according to the Metabolomics Standards Initiative guidelines. Because authentic reference standards were not used for LC–MS/MS confirmation, no compound was assigned as MSI Level 1, and the detected compounds were reported as putatively annotated metabolites rather than definitively identified compounds.

Quantitative Determination of Hesperidin

To provide complementary analytical support for selecting hesperidin as the principal docking ligand, HPLC-based quantitative determination was performed on the lemon-only control and the three honey–lemon formulations. Hesperidin concentrations were evaluated to compare formulation-associated differences and assess the relevance of hesperidin for subsequent molecular docking analysis. A stock solution of hesperidin (1000 ppm) was prepared by dissolving 5.0 mg of hesperidin in methanol and diluting it to 5.0 mL in a volumetric flask. The solution was vortexed, sonicated for 30 minutes, and filtered through a 0.45 µm nylon membrane filter. Working standard solutions were prepared by diluting the stock solution with methanol–water (1:1, v/v) to obtain concentrations of 1.25, 2.5, 5.0, and 10 ppm.

All samples, including the lemon-only control and honey–lemon formulations, were analyzed using the same sample preparation procedure for HPLC-based hesperidin quantification. Briefly, 500 µL of the sample was diluted with methanol–water (1:1, v/v) to a final volume of 10.0 mL. A further dilution was performed by transferring 100 µL of the solution into 10.0 mL methanol–water (1:1, v/v), resulting in a 2000-fold dilution. Each formulation was independently prepared in three separate batches (n = 3). Each batch underwent separate sample preparation, was sonicated for 30 minutes, filtered through a 0.45 µm nylon membrane filter, and analyzed separately by HPLC. The mobile phase consisted of methanol–water–acetic acid (40:58:2, v/v/v) and was prepared by mixing 200 mL HPLC-grade methanol, 290 mL water for injection, and 10 mL HPLC-grade acetic acid, followed by sonication for 30 minutes. HPLC analysis was performed with an injection volume of 20 µL, a flow rate of 1 mL min^{-1}, and a column temperature of 45 °C. Detection was conducted at 288 nm using a C8 column, following the method of Kanaze *et al*. (2004). The final concentration was expressed as mg/L and converted to mg/100 mL. In addition to the three honey–lemon formulations, a lemon-only sample (L0) was analyzed using the same HPLC procedure as a formulation control. This control was included to provide a comparative reference for hesperidin derived from the lemon matrix and support interpretation of hesperidin concentrations measured in the honey–lemon formulations. Hesperidin concentrations from three independent replicates (n = 3) were analyzed using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT), with significance set at $p<0.05$.

Hesperidin was selected as the principal docking ligand based on its well-established occurrence in citrus and complementary HPLC quantification, rather than definitive identification in the untargeted LC–MS/MS dataset, because targeted LC–MS/MS confirmation using an authentic hesperidin standard was not performed.

In Silico Analysis of DDAH-1

Protein–protein interaction and pathway analysis. Candidate ligands for the *in silico* analysis included compounds putatively annotated from the LC–MS/MS dataset and hesperidin, which was included separately based on literature evidence and complementary HPLC findings. Additional phytochemical information was retrieved from Dr. Duke's Phytochemical and Ethnobotanical Databases using Acacia honey as the primary query. Chemical structures of the selected ligands were obtained in SMILES format from the PubChem database for subsequent *in silico* analyses.

Protein–protein interaction (PPI) analysis was conducted to investigate the involvement of dimethylarginine dimethylaminohydrolase-1 (DDAH-1) in hypertension-related biological pathways. Figure 1 shows the DDAH-1 structure before and after preparation. Interaction networks were generated using the STRING database (https://string-db.org/), followed by KEGG-based pathway enrichment analysis (https://www.kegg.jp/) to identify signaling pathways associated with vascular regulation and blood pressure control.

Drug-likeness evaluation (Lipinski's Rule of Five). Lipinski's Rule of Five evaluated the drug-likeness and potential membrane permeability of the selected bioactive compounds. Assessment was performed based on standard parameters, including molecular weight below 500 g/mol, logP values less than 5, no more than five hydrogen bond donors (HBD), no more than ten hydrogen bond acceptors (HBA), and molar refractivity

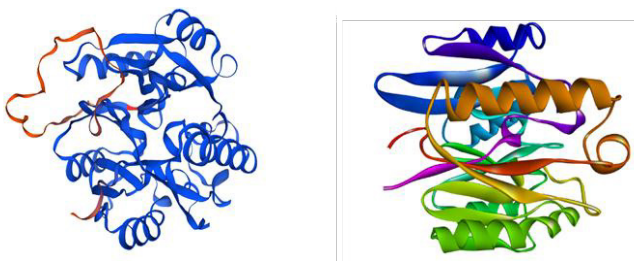


Figure 1. Human DDAH-1 structure (*Homo sapiens*) obtained from the Protein Data Bank (PDB ID: 3P8E, chain A): (a) before preparation and (b) after preparation.

ranging from 40 to 130. Compounds meeting at least four of these five criteria were considered to exhibit favorable drug-like characteristics.

Ligand and receptor preparation, molecular docking, and visualization. Structural preparation and energy minimization were performed using PyRx integrated with AutoDock Vina to generate energetically stable ligand conformations. Binding affinity values obtained from PyRx served as indicators of ligand–receptor binding strength (Astria *et al*., 2023).

The crystal structure of human DDAH-1 from *Homo sapiens* was obtained from the Protein Data Bank using PDB ID 3P8E, with a crystallographic resolution of 2.49 Å. Chain A was selected as the receptor model for molecular docking analysis. The receptor was prepared by removing crystallographic water molecules, non-essential heteroatoms, ions, and the co-crystallized ligand LN7. Polar hydrogens and charges were added before conversion into PDBQT format for docking analysis. The co-crystallized ligand LN7 was used as a reference ligand/positive control to provide a comparative docking benchmark.

Molecular docking was performed using PyRx 0.8 integrated with AutoDock Vina. The grid box was centered on the DDAH-1 active-site region, with center coordinates x = 18.9298, y = −0.6836, and z = −0.7606, and grid dimensions of 49.71 Å × 49.51 Å × 46.96 Å. The exhaustiveness value was set to 8, and nine docking poses were generated for each ligand.

As an additional validation step, the co-crystallized ligand LN7 from DDAH-1 (PDB ID: 3P8E) was redocked using PyRx/AutoDock Vina. The best redocked LN7 pose showed a binding affinity of −6.0 kcal/mol and an RMSD of 1.668 Å relative to the crystallographic LN7 pose, indicating that the docking protocol reproduced the native binding orientation within the commonly accepted threshold of 2.0 Å.

RESULTS

Profile of Bioactive Compounds in Acacia Honey–Based Formulation with Lemon

UPLC–MS/MS analysis putatively annotated 93 metabolites within retention times of 0.94–31.74 minutes based on accurate mass measurements, available MS/MS spectral information, and database matching. The annotated metabolites represented several compound classes, including flavonoids, phenolic compounds, organic acids, vitamins, alkaloids, and sugars. Among these, citric acid, malic acid, pyroglutamic acid, and glutarylcarnitine were detected at retention times between 1 and 3 min, while the selected representative metabolites summarized in Table 1 showed retention times ranging from 1.1 to 6.6 minutes. Table 1 also presents their names, molecular formulas, retention times, molecular weights, and compound classes, while Figure 2 shows the overall UPLC–MS/MS chromatographic profile.

Table 1 shows that the compound with the highest molecular weight was 1.5-anhydro-1-(2,4,6-trihydroxyphenyl) hexitol ($C1_2H_{16}O_8$; MW 288.08), detected at a retention time of 1.07 minutes. The presence of flavonoids, vitamin-related metabolites, phenolic metabolites, aromatic carboxylic acids, and alkaloid-related metabolites indicates that the Acacia honey–lemon formulation contains diverse putatively annotated metabolite classes.

In the present UPLC–QToF–MS analysis, the compound (2S)-3-phenyl-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydro-2 furanyl] methyl}amino) propanoic acid was detected at a retention time of 2.18 minutes, while Hex-2-ulose was detected at retention times of 1.08 and 2.39 minutes. Although the untargeted LC–MS/MS analysis provided a broad putative metabolomic profile of the Acacia honey–lemon formulation, hesperidin was not assigned as a definitively confirmed metabolite from this dataset.

Table 1. Active compound profile in lemon honey beverages

Metabolite name	Molecular weight (G/Mol)	Retention time	Ionization mode	Molecule formula	Ontology
1,5-Anhydro-1-(2,4,6-trihydroxyphenyl)hexitol	288.0844	1.072	[M+H]+1	$C_{12}H_{16}O_8$	Flavonoid
Dehydroascorbic acid	174.0158	1.115	[M-H]-1	$C_6H_6O_6$	Vitamin derivative
Pyrogallol	126.0318	1.08	[M+H]+1	$C_6H_6O_3$	Phenol
Benzoquinone	108.02	2.578	[M+H]+1	$C_6H_4O_2$	Quinone
Stachydrine	143.0946	1.077	[M+H]+1	$C_7H_{13}NO_2$	Proline derivative
1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid	230.1054	6.686	[M+H]+1	$C_{13}H_{14}N_2O_2$	Tetrahydro-β-carboline (THβC) derivatives
8-Methyl-8-azabicyclo[3.2.1]oct-3-yl (3S)-1,2-dithiolane-3-carboxylate	273.0845	1.393	[M+H]+1	$C1_2H_{19}NO_2S_2$	Alkaloid tropane
Lotustralin	261.1211	1.103	[M+H]+1	$C_{11}H_{19}NO_6$	Cyanogenic glycoside
2-C-methylerythritol 4-phosphate	216.0397	1.195	[M-H]-1	$C_5H_{13}O_7P$	Sugar phosphate (MEP pathway)
2-Furoic acid	112.0151	1.104	[M-H]-1	$C_5H_4O_3$	Heteroaromatic carboxylic acid
(2S)-3-Phenyl-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]methyl}amino) propanoic acid	327.131	2.18	[M+H]+1	$C_{15}H_{21}NO_7$	Flavonoid glikosida

Hesperidin Quantification in Honey–Lemon Beverage Formulations

Following LC–MS/MS-based putative metabolite profiling, HPLC quantification showed significant differences in hesperidin concentrations among the honey–lemon formulations ($p<0.001$), with LM2 (Acacia honey–lemon) exhibiting the highest measured level and providing complementary analytical support for its selection as the principal docking ligand (Table 2). The hesperidin standard curve showed a linear response over the concentration range of 1.25–10 ppm, with the regression equation $y = 0.0831x - 0.0024$, where y represents the chromatographic peak area and x represents hesperidin concentration in the diluted sample. The LM2 formulation (Acacia honey + lemon) exhibited the highest calculated hesperidin concentration, at 10,549.7 ± 310.75 µg/mL, equivalent to 1054.98 ± 31.08 mg/100 mL.

For the Acacia honey–lemon formulation, the chromatographic peak areas of 0.42180, 0.43893, and 0.44710 corresponded to diluted sample concentrations of 5.1047, 5.3108, and 5.4091 mg/L, respectively. After correction using the total dilution factor of 2000, these values were equivalent to 10,209.39, 10,621.66, and 10,818.29 mg/L, corresponding to 1020.94, 1062.17, and 1081.83 mg/100 mL.

In Silico Interaction of Bioactive Compounds from Acacia Honey and Lemon with DDAH-1

The initial step of the *in silico* study involved ligand preparation, in which five of the ten putatively annotated metabolites in the Acacia lemon–honey sample—hesperidin, dehydroascorbic acid, pyrogallol, citric acid, and 2-furoic acid—were selected for molecular docking against DDAH-1 (Tables 1 and 3). The selected ligands were prepared using PyRx based on AutoDock Vina, and energy-minimized to obtain stable conformations. The remaining five compounds—benzoquinone, stachydrine, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, 8-methyl-8-azabicyclo[3.2.1]oct-3-yl (3S)-1,2-dithiolane-3-carboxylate, and lotustralin—were excluded because of high chemical reactivity, structural complexity potentially affecting docking reliability, limited relevance to DDAH-1-mediated cardiovascular mechanisms, and, for some compounds, potential toxicity or receptor specificity primarily related to neural targets. Thus, the five selected ligands

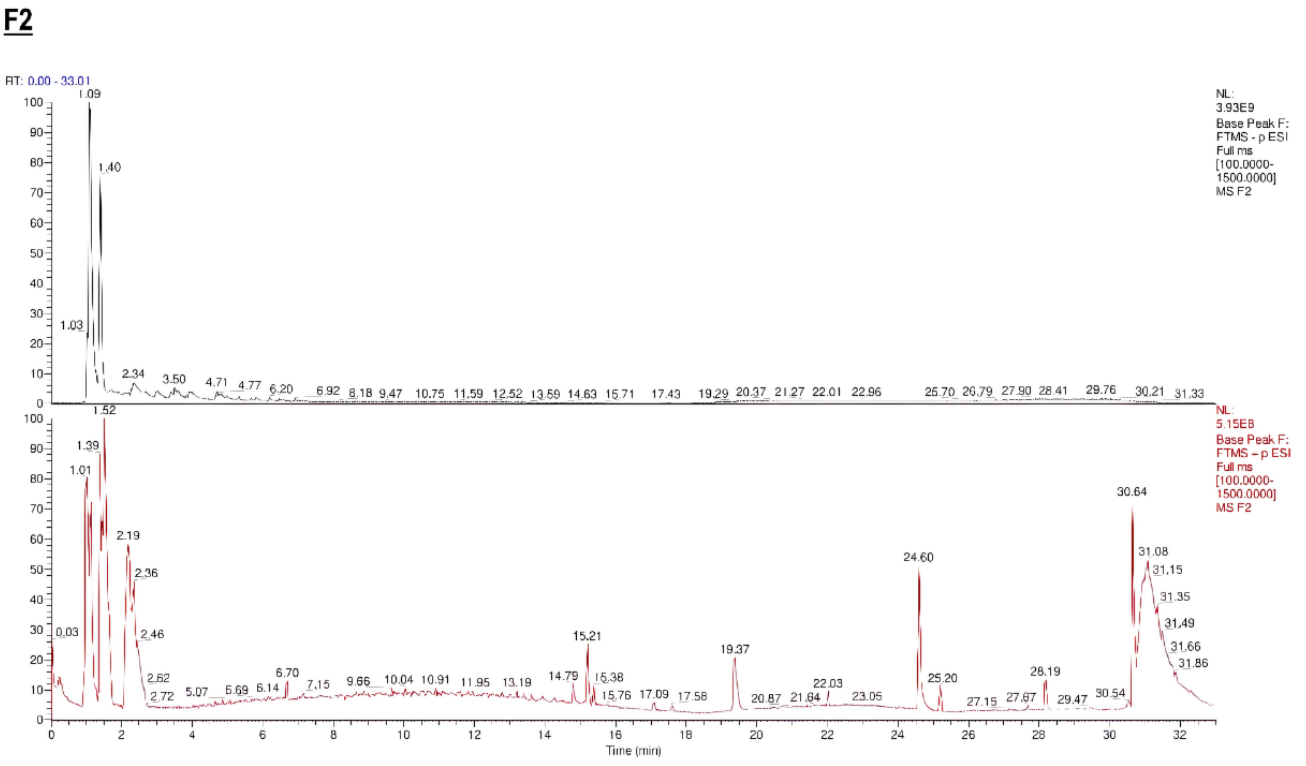


Figure 2. Chromatogram of UPLC-MS/MS results for honey lemon supernatant

Table 2. Hesperidin content in different honey–lemon beverage formulations

Variable (µg/mL)	Formula				P-value
	L0	LM1	LM2	LM3	
Hesperidin	9703.97 ± 496.3[b]	7184.35 ± 57.96[a]	10549.7 ± 310.75[c]	9107.90 ± 198.53[b]	<0.001*

Note: L0= lemon-only control LM1 = Calliandra honey + lemon; LM2 = Acacia honey + lemon; LM3 = Itama honey + lemon. [abc] Values within the same row sharing identical superscript letters indicate no significant difference among treatments ($p>0.05$), whereas different superscript letters indicate statistically significant differences based on one-way ANOVA followed by Duncan's Multiple Range Test (DMRT). *$p<0.05$ indicates statistical significance.

Table 3. Ligand preparation based on Lipinski's rule of five

Ligand	Molecular characteristics				
	Weight molecule (MW) < 500	Lipophilicity (LogP < 5)	Hydrogen bond acceptors (<10)	Hydrogen bond donors (≤5)	Molar refractivity (40–130 cm³/mol)
Hesperidin	×	√	×	×	×
Pyrogallol	√	√	√	√	√
Citric acid	√	√	√	√	√
Dehydroascorbic acid	√	√	√	√	√
2 Furoid acid	√	√	√	√	√

represented major metabolite classes, including flavonoids, vitamin derivatives, phenolics, and organic acids, allowing the docking analysis to focus on structurally and functionally representative compounds with potential relevance to oxidative stress and endothelial function-related pathways.

Based on Lipinski rule predictions (Table 3), four of the five tested ligands, pyrogallol, citric acid, dehydroascorbic acid, and 2-furoic acid, fulfilled all Lipinski criteria. These compounds exhibited favorable molecular weight, lipophilicity, and hydrogen-bonding capacity, suggesting good theoretical oral bioavailability and sufficient permeability across lipid membranes to reach systemic circulation. In contrast, hesperidin violated three Lipinski parameters, including molecular weight greater than 500 Da, excessive hydrogen bond acceptors (>10), and molar refractivity below the optimal range (40–130 cm³/mol). These characteristics indicate relatively high polarity and limited membrane permeability.

The molecular docking results demonstrated that hesperidin exhibited the lowest binding energy (−11.7 kcal/mol), indicating the strongest predicted interaction with the DDAH-1 receptor among the tested ligands (Table 4). The reference ligand LN7 showed a predicted binding affinity of −6.0 kcal/mol and was used as a comparative docking benchmark. Table 4 shows that hesperidin formed multiple predicted hydrogen-bonding, electrostatic, and hydrophobic interactions with DDAH-1 residues.

The substantially lower binding energy observed for hesperidin suggests that this compound may form a more stable complex with the Dimethylarginine dimethylaminohydrolase-1 active site compared with the other tested ligands. Hesperidin interacted with DDAH-1 residues, including GLU101, LEU301, LYS198, VAL105, VAL106, GLU108, and ALA31, through conventional hydrogen bonds, carbon–hydrogen bonds, electrostatic interactions, and hydrophobic interactions (Figure 3).

In contrast, pyrogallol, citric acid, dehydroascorbic acid, and 2-furoic acid exhibited higher predicted binding energy values ranging from −6.0 to −5.0 kcal/mol. Pyrogallol interacted with VAL106, GLU101, ALA104, VAL105, and VAL154. Citric acid interacted with GLU101, SER299, PHE156, LYS198, and LEU301, whereas dehydroascorbic acid interacted with GLY151, VAL106, PHE156, and LYS198. The 2-furoic acid ligand interacted with ARG121, ASP102, CYS297, PHE99, and HIS196. Overall, these findings indicate that hesperidin exhibited the strongest predicted interaction with the DDAH-1 active-site region among the tested ligands. However, this result should be interpreted as a computational prediction of ligand–protein interaction and not as evidence that hesperidin modulates the DDAH–ADMA–NO pathway or vascular regulation.

DISCUSSION

Profile of Bioactive Compounds in Acacia Honey–Based Formulation with Lemon

The UPLC–QToF-MS/MS analysis revealed a diverse putative metabolite profile in the Acacia honey–lemon beverage, which may partly reflect its botanical origin because the chemical composition of monofloral honey is strongly influenced by its predominant nectar source (Mădaş *et al*., 2019). The putatively annotated metabolites represented several chemical classes, including phenolic and flavonoid-related compounds, vitamins, alkaloids, and organic acids. These compound classes have previously been associated with antioxidant, antimicrobial, and anti-inflammatory activities (National Center for Biotechnology Information, 2025) and align with previous reports on lemon-derived materials and honey (Bouali *et al*., 2022), as well as the recognized biological relevance of natural phenolics, flavonoids, and alkaloids (Chidananda *et al*., 2025). Some putatively annotated compounds, particularly tetrahydro-β-carboline derivatives and 2-furoic acid, may also be associated with processing-related reactions, suggesting that both botanical origin and processing may contribute to the observed chemical profile (National Center for Biotechnology Information, 2025).

Phenolic and flavonoid-related constituents are particularly relevant to endothelial function because of their reported roles in attenuating oxidative stress and preserving nitric oxide (NO) bioavailability. Oxidative stress may also increase asymmetric dimethylarginine (ADMA) by impairing dimethylarginine dimethylaminohydrolase (DDAH) activity, thereby affecting the ADMA–DDAH–eNOS pathway (Pietro *et al*., 2020). However, the LC–MS/MS findings represent chemical annotation rather than evidence of direct biological activity and do not demonstrate activation or inhibition of DDAH-1. Therefore, their relevance to endothelial function remains hypothesis-generating. Hesperidin was not explicitly putatively annotated by LC–MS/MS and was instead quantified separately by HPLC; previous RP-HPLC studies reported retention times of approximately 4.89–5.32 minutes depending on chromatographic conditions (Chauhan *et al*., 2025).

Table 4. Interaction of complex molecules hesperidin, pyrogallol, citric acid, dehydroascorbic acid, 2 furoic acid with DDAH 1

Compounds	Interaction characteristics			Binding energy (kcal/mol)
	Point interaction	Chemistry bond	Type	
Hesperidin	N:UNK0:H -A:LEU301:O	Hydrogen bond	Conventional hydrogen bond	–11.7
	N:UNK0:H -A:GLU101:OE1	Hydrogen bond	Conventional hydrogen bond	
	A:VAL106:HN -N:UNK0:O	Hydrogen bond	Conventional hydrogen bond	
	A:LYS198:HZ2 -N:UNK0:O	Hydrogen bond	Conventional hydrogen bond	
	A:LYS198:HZ3 -N:UNK0:O	Hydrogen bond	Conventional hydrogen bond	
	N:UNK0:H10 -N:UNK0:O	Hydrogen bond	Carbon hydrogen bond	
	N:UNK0:H28 -A:GLU101:OE1	Hydrogen bond	Carbon hydrogen bond	
	N:UNK0:H33 -A:GLU101:OE1	Hydrogen bond	Carbon hydrogen bond	
	A:VAL105:HA -N:UNK0:O	Hydrogen bond	Carbon hydrogen bond	
	A:VAL105:HA -N:UNK0:O	Hydrogen bond	Carbon hydrogen bond	
	A:GLU108:OE2 -N:UNK0	Electrostatic	Pi-Anion	
	N:UNK0:C -A:ALA31	Hydrophobic	Alkyl	
	N:UNK0:C -A:LEU301	Hydrophobic	Alkyl	
Pyrogallol	N:UNK1:H -A:VAL106:O	Hydrogen bond	Conventional hydrogen bond	–5.7
	N:UNK1:H -A:GLU101:O	Hydrogen bond	Conventional hydrogen bond	
	N:UNK1:H -A:ALA104:O	Hydrogen bond	Conventional hydrogen bond	
	A:GLU101:OE1 -N:UNK1	Electrostatic	Pi-Anion	
	A:VAL105:HG12 -N:UNK1	Hydrophobic	Pi-Sigma	
	N:UNK1 -A:VAL154	Hydrophobic	Pi-Alkyl	
Citrit acid	N:UNK1:H -A:GLU101:OE1	Hydrogen bond	Conventional hydrogen bond	–6
	N:UNK1:H -A:GLU101:OE1	Hydrogen bond	Conventional hydrogen bond	
	N:UNK1:H -A:SER299:O	Hydrogen bond	Conventional hydrogen bond	
	A:PHE156:HN -N:UNK1:O	Hydrogen bond	Conventional hydrogen bond	
	A:LYS198:HZ2 -N:UNK1:O	Hydrogen bond	Conventional hydrogen bond	
	A:LEU301:HN -N:UNK1:O	Hydrogen bond	Conventional hydrogen bond	
Dehydroascorbic acid	N:UNK1:H -A:GLY151:O	Hydrogen bond	Conventional hydrogen bond	–5.7
	A:VAL106:HN -N:UNK1:O	Hydrogen bond	Conventional hydrogen bond	
	A:PHE156:HN -N:UNK1:O	Hydrogen bond	Conventional hydrogen bond	
	A:LYS198:HZ2 -N:UNK1:O	Hydrogen bond	Conventional hydrogen bond	
2 furoic acid	A:ARG121:NH1 -N:UNK1	Electrostatic	Pi-Cation	–5
	A:ASP102:OD2 -N:UNK1	Electrostatic	Pi-Anion	
	A:CYS297:SG -N:UNK1	Other	Pi-Sulfur	
	N:UNK1 -A:PHE99	Hydrophobic	Pi-Pi T-shaped	
	A:HIS196:CE1 -N:UNK1:O	Hydrogen bond	Carbon hydrogen bond	

Because authentic reference standards were not used for LC–MS/MS confirmation, the detected compounds were reported as putatively annotated rather than definitively identified, as accurate mass matching, database comparison, and available MS/MS spectra are insufficient for MSI Level 1 identification. Accordingly, hesperidin docking should be interpreted as an HPLC-guided and literature-supported *in silico* exploration rather than an extension of LC–MS/MS-confirmed hesperidin identification.

Hesperidin Analysis from Acacia Honey and Lemon

Following LC–MS/MS-based putative metabolite profiling, HPLC-based quantification was conducted to determine hesperidin concentrations across the honey–lemon formulations and provide complementary analytical support for its selection as a principal docking ligand. The HPLC results showed that LM2 (Acacia honey–lemon) had the highest measured hesperidin concentration among the tested formulations. This finding provided formulation-level context for the subsequent molecular docking analysis.

The higher measured hesperidin concentration in LM2 provides quantitative context for the docking analysis but should not be interpreted as direct validation of the predicted molecular interaction or as evidence that Acacia honey enhances, preserves, or stabilizes hesperidin. HPLC quantification and molecular docking provide complementary but distinct evidence by measuring compound concentration and predicting ligand–protein interactions, respectively. Hesperidin, a flavanone glycoside widely distributed in citrus fruits, including lemon (Imperatrice *et al*., 2022), was therefore selected as a literature-supported and HPLC-guided principal docking ligand and has been reported to exhibit antioxidant activity (Hajimahmoodi *et al*., 2014). Hesperidin may coexist with honey-derived phenolic compounds such as caffeic acid, quercetin, and pinocembrin; however, any potential synergistic effects require validation using controlled experimental designs.

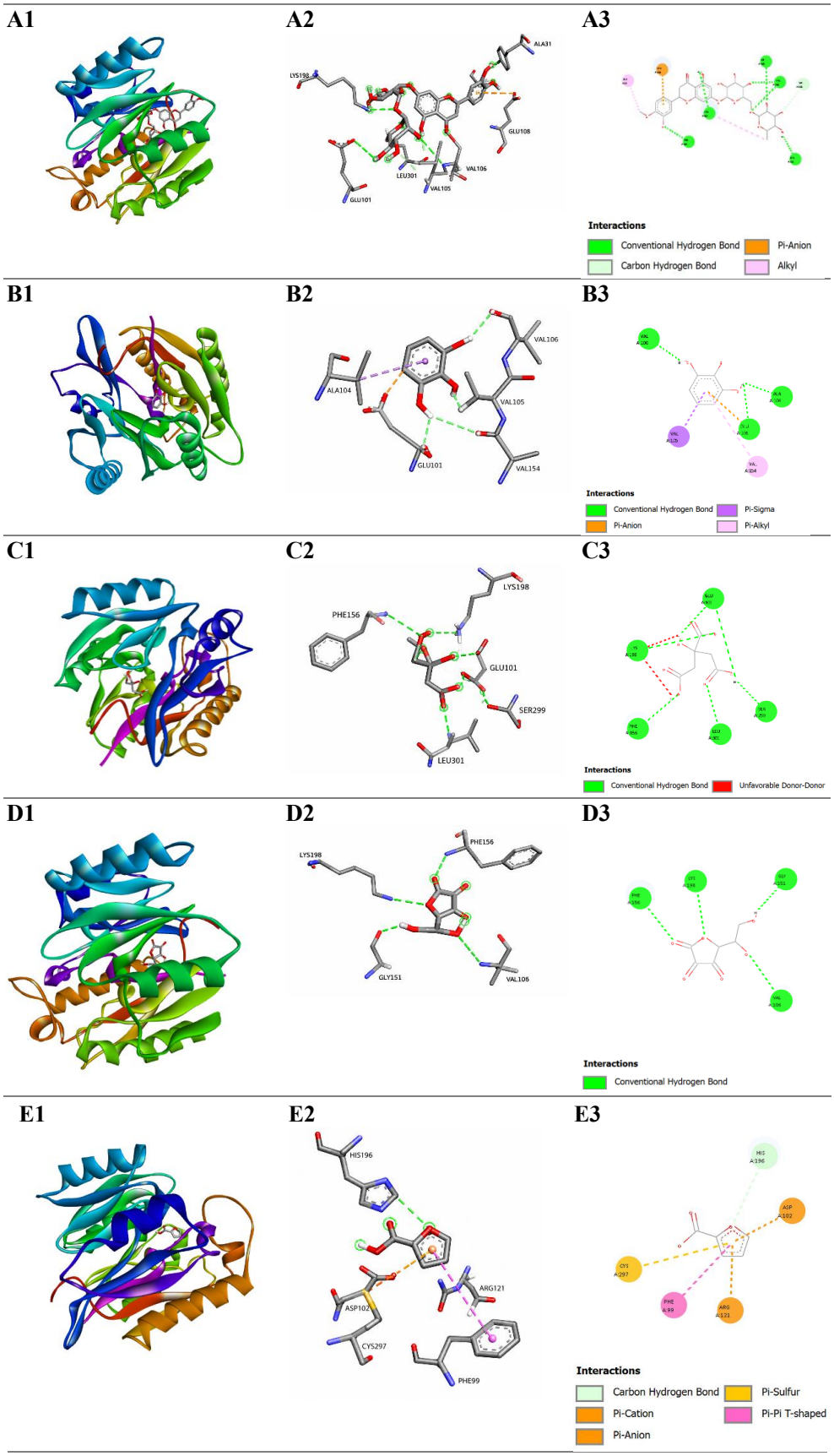


Figure 3. Molecular docking of the DDAH-1 protein receptor with (A) hesperidin, (B) pyrogallol, (C) citric acid, (D) dehydroascorbic acid, and (E) 2-furoic acid. Panels 1 and 2 show the three-dimensional (3D) visualization of the ligand–receptor complexes, while panel 3 presents the two-dimensional (2D) interaction diagrams.

A limitation of this study is that targeted LC–MS/MS confirmation of hesperidin using an authentic standard was not performed. Therefore, hesperidin was not claimed as a definitively confirmed compound in the untargeted LC–MS/MS dataset. The rationale for selecting hesperidin as the principal docking ligand was based on its well-documented occurrence in lemon and other citrus matrices, its reported biological relevance to oxidative stress and endothelial function, and the complementary HPLC-based quantification conducted before the molecular docking analysis. In addition, honey-only, matrix-spiked recovery, and stability controls were not included. Consequently, the observed differences in hesperidin concentration among formulations should not be interpreted as direct evidence of hesperidin stabilization by Acacia honey.

In Silico Interaction of Bioactive Compounds from Acacia Honey and Lemon with DDAH-1

Molecular docking was used to predict interactions between selected bioactive compounds and DDAH-1, consistent with previous in silico studies showing that honey-derived phenolic compounds can interact with specific protein targets (Shamsol *et al.* 2023). The ligands were selected based on their biochemical relevance, structural characteristics, molecular polarity, and reported relationships with oxidative stress and endothelial function. Hesperidin was prioritized as a literature-supported and HPLC-guided ligand because it is a major lemon flavonoid associated with antioxidant, anti-inflammatory, endothelial-function, and NO-related effects (Rizza *et al*., 2011). Dehydroascorbic acid, pyrogallol, 2-furoic acid, and citric acid were included as representative vitamin-related, phenolic/aromatic, and organic-acid compounds with physicochemical characteristics relevant to exploring their predicted interactions with DDAH-1.

Lipinski's rule of five evaluates drug-likeness based on molecular weight, hydrogen-bond donors and acceptors, and log P (Benet *et al*., 2016). Pyrogallol, citric acid, dehydroascorbic acid, and 2-furoic acid fulfilled the evaluated criteria, whereas hesperidin showed less favorable physicochemical characteristics, although intestinal deglycosylation may improve its absorption properties (Nemeth *et al*., 2003). Log P further reflects the balance between solubility and lipophilicity (Broccatelli *et al*., 2018). In molecular docking, a more negative binding energy indicates a stronger predicted ligand–protein interaction (Rifai *et al*., 2020), but both Lipinski and docking results remain theoretical predictions rather than direct evidence of oral bioavailability or biological effectiveness.

The present study did not measure ADMA concentration, DDAH-1 enzymatic activity, nitric oxide production, oxidative stress markers, endothelial function, or blood pressure-related outcomes. Therefore, the docking findings should be interpreted only as predicted molecular interactions and not as evidence of DDAH–ADMA–NO pathway regulation.

As shown in Figure 4, hesperidin is a flavanone glycoside composed of a hesperetin aglycone linked

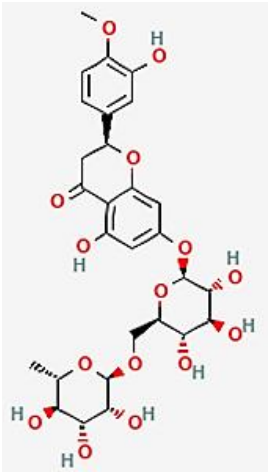


Figure 4. Chemical structure of hesperidin (Wdowiak *et al*., 2022)

at the C7 position to a rutinoside moiety consisting of rhamnose and glucose (Wdowiak *et al*., 2022). Its multiple hydroxyl and oxygen-containing functional groups provide potential hydrogen-bond donor and acceptor sites, while its aromatic rings may facilitate π-based interactions with amino acid residues. In the present study, these structural characteristics align with the multiple predicted interactions between hesperidin and DDAH-1 residues, including LEU301, GLU101, VAL106, LYS198, VAL105, GLU108, and ALA31. Nevertheless, these structural features and docking interactions indicate only predicted molecular compatibility and do not establish whether hesperidin activates or inhibits DDAH-1.

Detailed ligand–DDAH-1 interactions are summarized in Table 4, with hesperidin showing more diverse predicted interactions than the other ligands. Redocked LN7 showed a binding affinity of −6.0 kcal/mol and an RMSD of 1.668 Å, supporting docking setup acceptability, while 2-furoic acid showed the weakest predicted affinity and served as a low-affinity internal comparison ligand.

CONCLUSION

This study characterized the bioactive profile of an Acacia honey–lemon formulation using LC–MS/MS, HPLC, and molecular docking. LC–MS/MS putatively annotated flavonoids, phenolic compounds, vitamins, organic acids, and alkaloids; HPLC showed the highest measured hesperidin concentration in the Acacia honey–lemon formulation; and docking predicted hesperidin as the ligand with the strongest DDAH-1 interaction. These findings remain preliminary and hypothesis-generating because targeted LC–MS/MS confirmation, honey-only controls, matrix-matched recovery and stability testing, and functional DDAH-1 assays were not performed. The results may support the characterization and value addition of honey as a bee-derived livestock product and provide a basis for future animal biology studies. Future studies should include targeted LC–MS/MS confirmation using authentic hesperidin standards with retention-time matching, precursor-ion confirmation, and diagnostic product-ion

confirmation, along with replicate-based validation, matrix-matched calibration, spike-recovery and stability testing, and assessments of DDAH-1 activity, ADMA, nitric oxide, oxidative stress, and endothelial function.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used an AI tool for language translation and grammatical editing. After using this tool, the author(s) carefully reviewed and revised the content as necessary and took full responsibility for the accuracy and integrity of the final manuscript.

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