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Research Article

Comparative Analysis of Bioactive Compounds in Stingless Bee Honey from Wetland and Non-Wetland Vegetation

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ABSTRACT

Stingless bee honey (SBH) produced by the stingless bee species *Heterotrigona itama* in South Kalimantan is a biologically active natural product with therapeutic potential. This study examines differences in bioactive compounds—molecules that affect living organisms—between SBH from wetland and non-wetland vegetation. The goal is to show how a terrestrial landscape influences functional properties. Using untargeted metabolomics, a technique for surveying many small molecules without preselection, via LC-HRMS (Liquid Chromatography-High-Resolution Mass Spectrometry), we identified 141 distinct metabolites (small molecules involved in metabolism), with 23 compounds present in all samples. These shared metabolites support the mitigation of obesity, wound healing, cardiovascular health, neuroprotection, and gastrointestinal function. Hierarchical cluster analysis, a statistical method for grouping data, using retention time (the time a compound takes to pass through the system), area (the size of the signal on the detector), and molecular weight, revealed distinct compositional clusters between wetland- and non-wetland-derived SBH, suggesting vegetation type shapes metabolite profiles. Wetland SBH contained 198 compounds, while non-wetland samples had 193. These findings highlight how ecological origin modulates the bioactive compounds in SBH and influences their use in nutraceuticals (foods with health benefits) and functional foods. By establishing a metabolomic baseline for SBH, this work advances understanding of its region-specific health benefits and underscores the importance of ecosystem-conscious apiculture (beekeeping) in optimizing bioactive yield.

1. Introduction

Honey is a naturally sweet substance produced by bees through enzymatic processing (the use of enzymes to chemically alter substances) of plant nectar, floral secretions, or exudates (fluids secreted by certain insects) from other insects. This product undergoes enzymatic modification, dehydration (removal of water), and maturation (development to a stable state) within hive structures, resulting in its final stable form. Chemically, honey consists mainly of sugars—primarily fructose and glucose—with organic acids, enzymes, and solid particles as secondary constituents. Its physicochemical

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characteristics (physical and chemical properties), such as color, flavor, aroma, and consistency, vary with botanical and geographical origins (Codex Alimentarius Commission 2001). Historically, honey has been vital for humans. Evidence suggests honey-producing bees evolved about 40 million years ago. Beyond its role as a sweetener, honey has been empirically validated for therapeutic applications (Nayik *et al.* 2014).

Stingless bee honey (SBH) is recognized as a functionally distinct natural product with unique bioactive properties. It is produced by a species of the *Tetragonula* spp. (National Standardization Agency of Indonesia 2018) and *Melipona* spp. (Yaacob *et al.* 2017). SBH forms as bees enzymatically modify floral nectar using species-specific salivary secretions and cephalic gland enzymes (Syafrizal *et al.* 2020). SBH stands apart from Apis honey due to greater translucency, lower viscosity, and a complex, balanced flavor. It also has significant therapeutic potential, which derives from its diverse phytochemical composition (Yaacob *et al.* 2017).

South Kalimantan is renowned for its rich biodiversity. This is exemplified by the honey produced by the stingless bee species *Heterotrigona itama* in wetland vegetation (Karnia *et al.* 2019; Satriadi *et al.* 2023). Stingless bees are a group of bees from the tribe Meliponini, characterized by their lack of functional stingers and their ability to produce honey. Wetlands, as defined by Gell *et al.* (2023), encompass swamps, brackish areas (regions where freshwater and seawater mix), peatlands (waterlogged areas with accumulated organic matter), and natural or artificial waters. These can be permanent or temporary, stagnant or flowing, and can be fresh, brackish, or salty. Wetlands also include areas of marine waters with depths less than 6 meters at low tide. Indonesia's wetlands are globally significant, covering about 10.8% (20.6 million hectares) of its terrestrial landscape (Khairiyati *et al.* 2022). South Kalimantan alone encompasses 382,272 hectares of these ecologically critical zones. These habitats sustain a diverse array of flora (plants) and fauna (animals) (Annisa *et al.* 2021).

The wetlands of South Kalimantan feature dominant vegetation, including palm trees (*Arecaceae* spp.), a family of tropical trees, and *Melaleuca cajuputi* subsp. *Cumingiana* (galam), a species of paperbark tree, is a critical floral component of this ecosystem (Syahdi *et al.* 2016; Sarah *et al.* 2022). This botanical diversity leads to heterogeneous nectar composition, meaning nectar varies considerably between plant species. Such diversity directly influences the phytochemical profile of SBH (stingless bee honey). Prior research has established that SBH's bioactive constituents—compounds with biological effects—vary significantly according to the plant species used as forage (Sousa *et al.* 2016; Silva *et al.* 2021). Meliponiculture, or the keeping of stingless bees, is feasible in wetland landscapes, particularly in peatlands (waterlogged soils rich in organic matter) and adjacent upland regions. However, spatial variation in floral resources across habitats likely drives divergent synthesis of secondary metabolites (plant-derived compounds not essential for basic metabolism) in SBH (Arifin *et al.* 2022). Key physicochemical properties, including color and flavor, are modulated by mineral content, pollen diversity, and phenolic abundance (amount of antioxidant compounds) parameters. These are intrinsically linked to honey's origin (Sulieman *et al.* 2013; Sayusti *et al.* 2025).

Previous investigations on *Heterotrigona itama* honey have mainly used targeted analytical methods. These methods have characterized phenolic acids (chemical compounds with antioxidant properties, such as catechin, chlorogenic acid, p-coumaric acid, and cinnamic acid (Majid *et al.* 2020; Shamsudin *et al.* 2022), physicochemical parameters (measurable physical and chemical properties) like moisture, pH (acidity level), and sugar profiles (the types and amounts of sugars present) (Ramlan *et al.* 2024), and antioxidant activity (the honey's ability to neutralize free radicals) (Shamsudin *et al.* 2022; Chuah *et al.* 2023). Such approaches provide only a partial chemical portrait, as they target pre-defined compound classes and may miss novel or unexpected metabolites (small molecules produced in metabolism). To date, a comprehensive untargeted metabolomics study using LC-HRMS (Liquid Chromatography-High-Resolution Mass Spectrometry, a technique for identifying a wide range of molecules simultaneously) has not been applied to *Heterotrigona itama* honey from Indonesian populations. The influence of distinct ecological habitats, especially wetland versus non-wetland vegetation, on the full metabolome (the complete set of metabolites present) of this species remains unexplored. This study addresses these gaps by using untargeted LC-HRMS metabolomics to compare complete metabolite profiles of SBH (stingless bee honey) across contrasting ecological niches in South Kalimantan. Beyond investigating systematic differences in bioactive compounds (biologically active chemical compounds) between SBH from wetland and non-wetland vegetation, this work also aims to uncover novel metabolites that targeted approaches may not reveal.

This provides a more comprehensive understanding of ecological drivers shaping honey's functional properties (characteristics that contribute to its health benefits).

2. Materials and Methods

2.1. Sample Collection

This study analyzed six *Heterotrigona itama* stingless bee honey (SBH) samples collected across South Kalimantan, Indonesia. Three samples were sourced from wetland vegetation regions: Gambut (Banjar Regency), Barito Kuala Regency, and Muara Harus (Tabalong Regency). The remaining three were procured from non-wetland vegetation, including Balangan Regency, Pengaron (Banjar Regency), and Bati-Bati (Tanah Laut Regency) (Figure 1).

Honey was collected directly from managed colonies of SBH housed in meliponiculture hives at each of the six sampling locations. Honey collection was performed by opening the hive box and carefully accessing the honey storage area. Honey was aspirated directly from the honey pots using a sterile syringe or small suction pump fitted with a clean tube. Collected honey was transferred immediately into clean, sterile amber glass vials, labeled with location and sample identification codes, and stored at 4°C until further analysis. The amount collected per colony was sufficient for metabolomic analysis while leaving the majority of honey reserves intact.

2.2. Untargeted Metabolomic

The bioactive composition of SBH samples was characterized using an untargeted metabolomics workflow utilizing liquid chromatography–high-resolution mass spectrometry (LC-HRMS). Samples were dissolved in methanol (MeOH, a common polar solvent) and analyzed in both positive and negative ionization modes, following a protocol adapted from metabolomic methodologies (Windarsih *et al.* 2022). Laboratory testing was conducted at the National Research and Innovation Agency's regional laboratory in Yogyakarta, Indonesia.

2.3. Data Processing

Semi-quantitative data are tabulated and visualized through comparative Venn diagrams. In this study, analytical variables included RT (retention time), area (chromatographic peak area), and MW (molecular weight) for all detected components across samples. Inter-sample variations in RT, area, and MW were evaluated using hierarchical cluster analysis with the Ward method and Euclidean distance, as implemented in Origin 2024b. This approach enabled classification of SBH samples based on shared and distinct metabolite profiles, with dendrograms illustrating compositional similarities and clustering patterns.

3. Results

Metabolomic profiling identified 141 compounds across all SBH samples. In comparative analyses, distinct compositional variations were observed between SBH derived from wetland and non-wetland vegetation. Specifically, wetland-associated SBH exhibited a higher metabolite diversity (198 compounds) compared to non-wetland SBH (193 compounds), as detailed in Table 1. This divergence highlights the impact of ecological niche on phytochemical richness, suggesting that wetland vegetation likely contributes unique bioactive compounds to the honey matrix.

The Venn diagram in Figure 2 delineates metabolomic overlaps and distinctions among SBH samples. Comparative analysis revealed habitat-specific metabolite profiles: Wetland-associated SBH samples (SBH Wetland 01, SBH Wetland 02, and SBH Wetland 03) exhibited 17, 2, and 6 unique compounds, respectively, while non-wetland SBH samples (SBH Non-Wetland 01, SBH Non-Wetland 02, and SBH Non-Wetland 03) each contained nine exclusive metabolites. A core set of 23 metabolites was ubiquitously present across all samples, possibly indicating the presence of conserved biochemical pathways in Heterotrigona itama honey production. The names and formulas of compositional profiles are available in Table 2.

In LC-HRMS-based untargeted metabolomics, RT denotes the duration a compound requires to elute from the chromatographic column. This parameter is critical for compound identification and quantification, as reproducible RT values enhance the accuracy of metabolite annotation in complex matrices such as SBH. Hierarchical clustering analysis (Figure 3) revealed minimal RT variability between SBH Wetland

Table 1. Metabolomic profiling of bioactive compounds in *Heterotrigona itama*-derived stingless bee honey from wetland and non-wetland vegetation

Samples	Detected compounds (n(%))	
SBH Wetland 01	75	53.19
SBH Wetland 02	62	43.97
SBH Wetland 03	61	43.26
SBH Non-Wetland 01	70	49.65
SBH Non-Wetland 02	58	41.13
SBH Non-Wetland 03	65	46.10

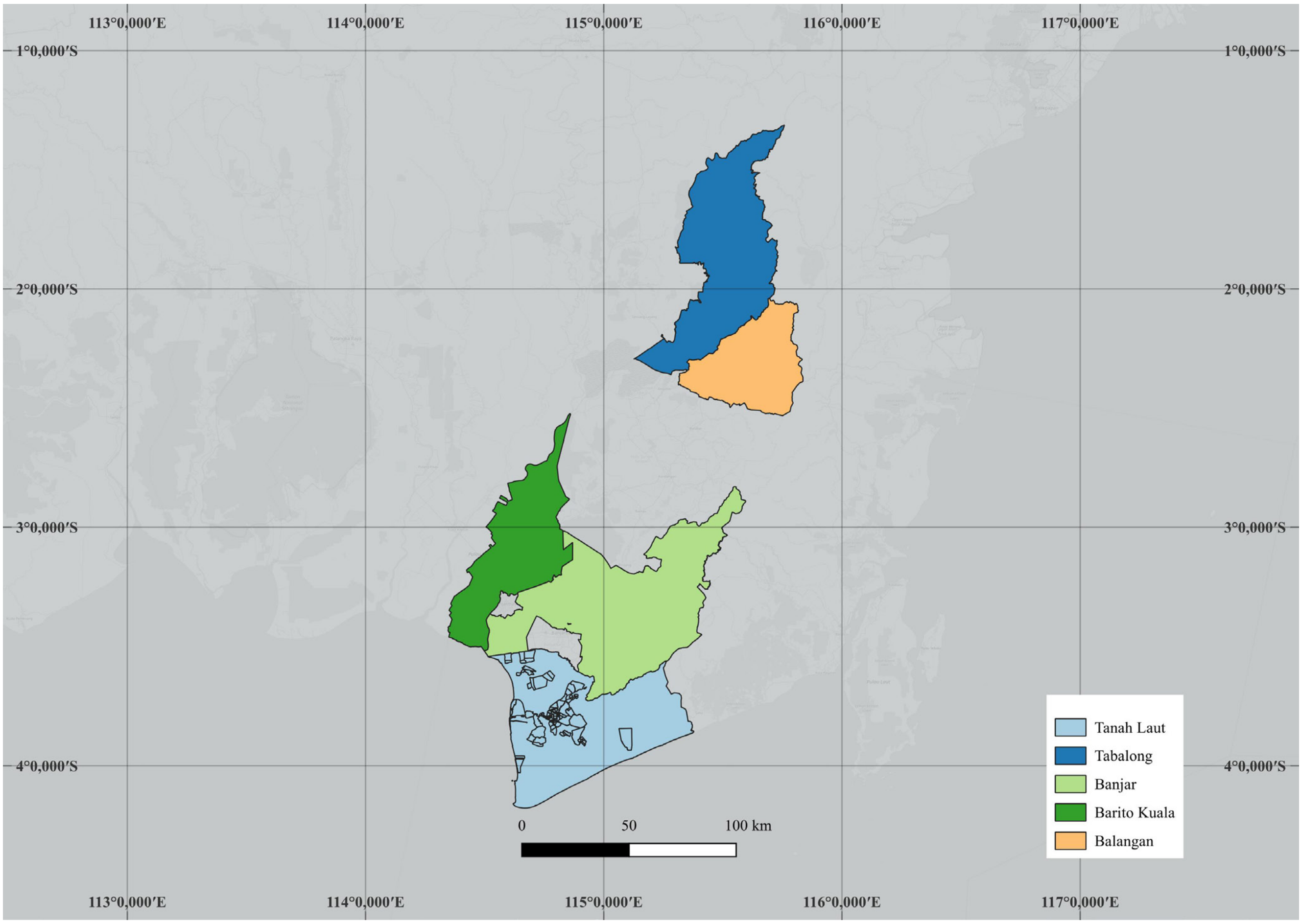

Figure 1. The sampling location

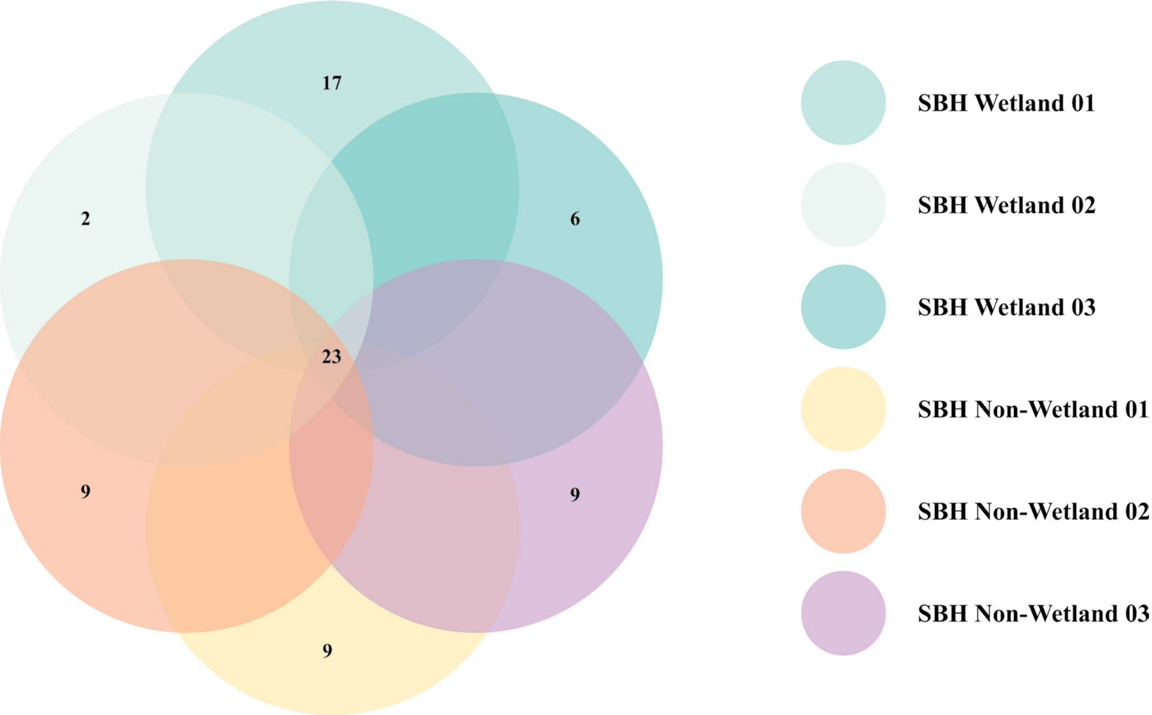

Figure 2. Venn diagram depicting metabolomic overlap among *Heterotrigona itama*-derived stingless bee honey samples from wetland and non-wetland vegetation

Table 2. Common bioactive compounds in all *Heterotrigona itama* stingless bee honey samples

Name	Formula
(+/-)-Muscone	$C_{16}H_{30}O$
1-(14-methylhexadecanoyl) pyrrolidine	$C_{21}H_{41}NO$
1-Stearoylglycerol	$C_{21}H_{42}O_4$
2,2,6,6-Tetramethyl-1-piperidinol (TEMPO)	$C_9H_{19}NO$
2-Amino-1,3,4-octadecanetriol	$C_{18}H_{39}NO_3$
2-Deoxyhexopyranose	$C_6H_{12}O_5$
5-Hydroxymethyl-2-furaldehyde	$C_6H_6O_3$
Bis(4-ethylbenzylidene) sorbitol	$C_{24}H_{30}O_6$
Bis(methylbenzylidene) sorbitol	$C_{22}H_{26}O_6$
C14-Dihydroceramide	$C_{32}H_{65}NO_3$
C16-Dihydroceramide	$C_{34}H_{69}NO_3$
Capsi-amide	$C_{17}H_{35}NO$
Cinnamic acid	$C_9H_8O_2$
Citric acid	$C_6H_8O_7$
D (+)-Phenyllactic acid	$C_9H_{10}O_3$
Furfural	$C_5H_4O_2$
L-Phenylalanine	$C_9H_{11}NO_2$
L-Pyroglutamic acid	$C_5H_7NO_3$
L-α-PALMITIN	$C_{19}H_{38}O_4$
MFCD00801046	$C_{18}H_{34}O$
Stearamide	$C_{18}H_{37}NO$
Tridemorph	$C_{19}H_{39}NO$
α,α-Trehalose	$C_{12}H_{22}O_{11}$

01 and SBH Non-Wetland 03, suggesting similar RT profiles.

Maximum area profiles demonstrated comparable compound abundances between SBH Wetland 01 and SBH Non-Wetland 02 (Figure 4). Cinnamic acid displayed the highest relative abundance in SBH Wetland 02, surpassing levels observed in SBH Wetland 03, SBH Non-Wetland 01, and other tested samples.

Molecular weight (MW) determination in SBH enables precise identification of bioactive compounds, such as flavonoids and antioxidants, which underpin its health-promoting properties. Additionally, MW analysis supports the differentiation of SBH botanical origins by resolving distinct chemical profiles, a critical factor in authenticity assessment. As illustrated in Figure 5, MW distributions exhibited notable similarity across samples: SBH Wetland 03 closely aligned with SBH Non-Wetland 01, SBH Non-Wetland 02 with SBH Non-Wetland 03, and SBH Wetland 01 with SBH Wetland 02. There is no apparent difference in MW among the samples.

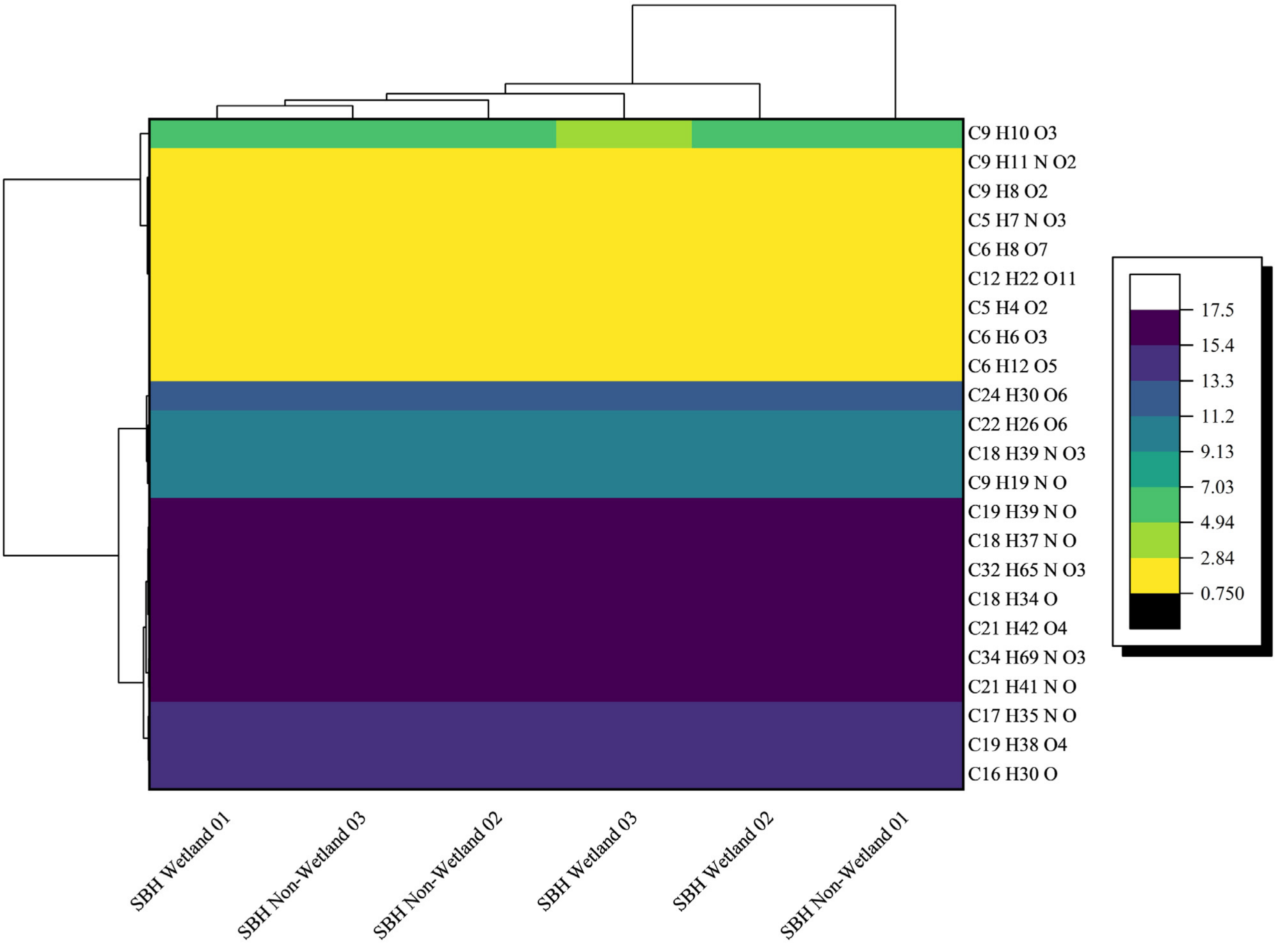

Figure 3. Hierarchical clustering analysis heatmap with dendrogram of retention time profiles

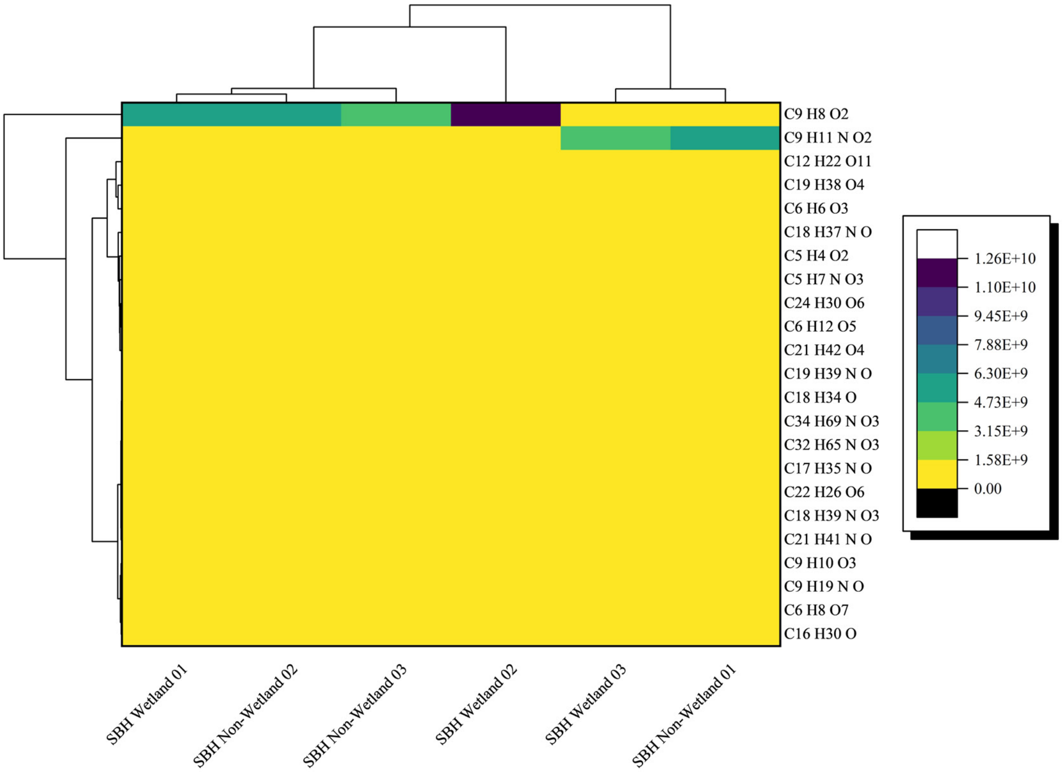

Figure 4. Hierarchical clustering analysis heatmap with dendrogram of area profiles

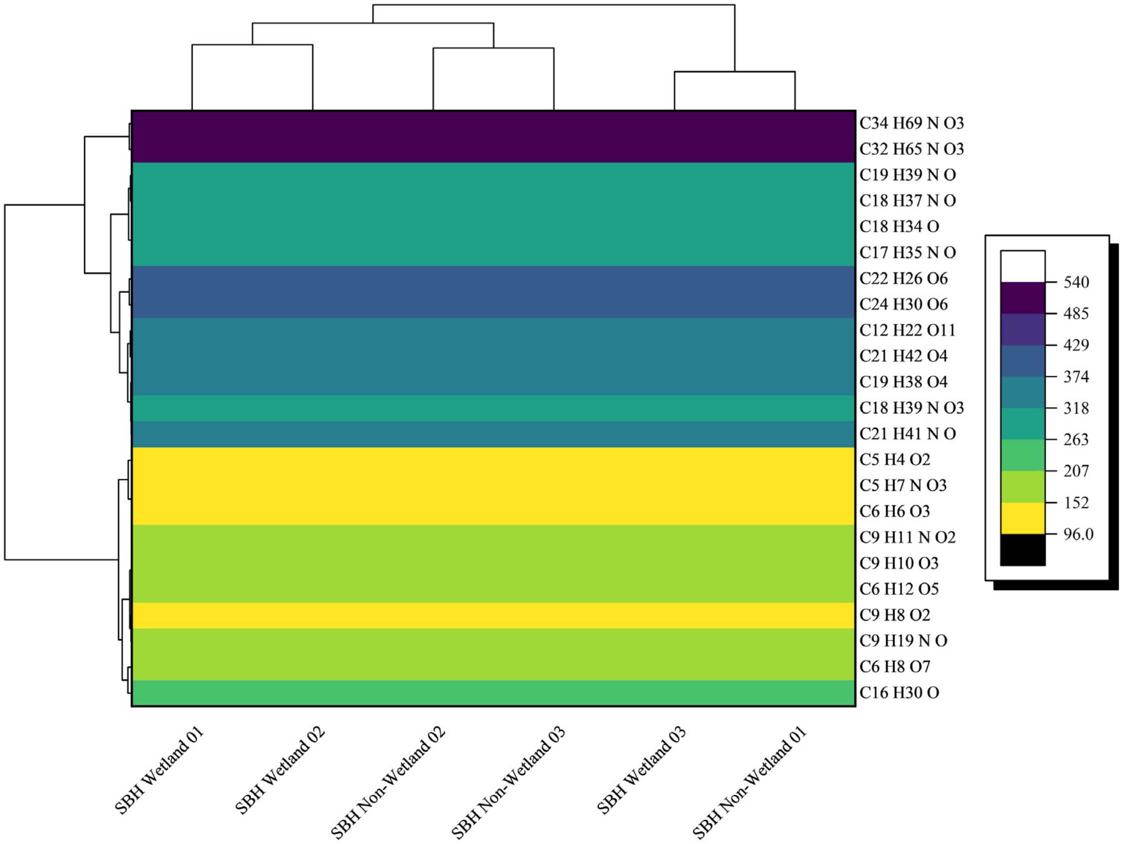

Figure 5. Hierarchical clustering analysis heatmap with dendrogram of molecular weight profiles

4. Discussion

Several identified compounds have been shown to exhibit documented therapeutic properties. These include muscone, 2-amino-1,3,4-octadecanetriol, 5-hydroxymethyl-2-furaldehyde, bis(4-ethylbenzylidene)sorbitol, cinnamic acid, citric acid, d(+)-phenyllactic acid, l-phenylalanine, and α,α-trehalose. These pharmacologically active metabolites collectively underpin SBH's nutraceutical applications.

4.1. Muscone

Muscone was consistently detected across all analyzed SBH samples. Limited research exists on muscone's occurrence in apicultural products, and no prior studies have documented its presence in honey matrices. Naturally biosynthesized in the abdominal gland secretions of male musk deer (Wang *et al.* 2020). Recent preclinical evidence highlights the therapeutic potential of muscone. Oral administration of muscone in rat models subjected to chronic restraint stress significantly alleviated depressive-like behaviors and inflammation, supporting its potential efficacy as an antidepressant agent (Liu *et al.* 2022).

There are two plausible mechanisms that may explain how muscone formed in SBH. First, stingless bees possess complex exocrine gland systems, including the Dufour's gland, Nasanov gland, mandibular glands, and salivary or cephalic glands, which collectively produce a diverse array of volatile and non-volatile metabolites. These glandular secretions contribute enzymes, antimicrobial peptides, and secondary metabolites to the honey matrix during the enzymatic processing of nectar. Macrocyclic ketones structurally related to muscone have been identified in the cuticular and glandular secretions of various hymenopteran species (Bergström 2008), suggesting that muscone in SBH may originate from glandular contributions during honey processing.

Second, muscone may originate from botanical sources via the foraging activity of *Heterotrigona itama*. The wetland vegetation of South Kalimantan, including *Melaleuca cajuputi* and various *Arecaceae* species, is chemically complex, and some plant species produce macrocyclic terpene derivatives that may be incorporated into nectar or pollen and subsequently transferred to the honey matrix. The terpenoid biosynthetic pathway, particularly the mevalonate pathway operating in certain plants and microorganisms, can produce cyclopentanone and cyclopentadecanone derivatives as secondary metabolites (Hsieh *et al.* 2021; Isah *et al.* 2022).

4.2. 2-Amino-1,3,4-Octadecanetriol

The organic compound 2-amino-1,3,4-octadecanetriol demonstrates antibacterial activity. Phytosphingosines are long-chain amino alcohols with 18 carbon atoms, typically found in plants, and possess antibacterial properties. Glenz *et al.* (2022) reported that phytosphingosine, at a concentration of 15.9 µg/mL, exhibited 95% eradication efficacy against *Pseudomonas syringae pv. tomato, Agrobacterium tumefaciens*, and *Rhizobium radiobacter*. 2-Amino-1,3,4-octadecanetriol is the systematic name for phytosphingosine, a long-chain sphingoid base that constitutes a core structural unit of phytosphingolipids, a major class of sphingolipids found in yeast, fungi, plants, and invertebrates (Pruett *et al.* 2008). Phytosphingosine in SBH most likely originates from plant sphingolipids (e.g., glycosylceramide), which are rich in phytosphingosine, carried in nectar and pollen, then undergo intensive fermentation and enzymatic hydrolysis during honey maturation by bee glycosidase and hive microbes (Wright *et al.* 2003; Seo *et al.* 2021; Steinberger *et al.* 2021; Rosińska *et al.* 2025). The botanical profile of nectar and pollen, which is strongly influenced by local flora, also shapes the composition of these metabolites (Leponiemi *et al.* 2023; Chege *et al.* 2025).

A mechanistically plausible secondary endogenous source is de novo sphingolipid biosynthesis in bee tissue, given that this pathway is highly conserved in eukaryotes. The pathway begins with the condensation of L-serine and palmitoyl-CoA by serine palmitoyltransferase to form 3-ketosphinganine, which is then reduced to sphinganine and N-acylated to dihydroseramide (Wigger *et al.* 2019; Green *et al.* 2021; Jamjoum *et al.* 2024; Mahawar and Wattenberg 2025). In other organisms, hydroxylation at the C-4 position by the enzyme sphinganine C-4-hydroxylase yields phytosphingosine, suggesting that a similar pathway may operate in insects (Mashima *et al.* 2020; Smith and Merrill 2002; Merrill 2025). The consistent detection of C14- and C16-dihydroceramide in all of our honey samples is consistent with active de novo sphingolipid metabolism, which indirectly supports the contribution of endogenous sources to this class of compounds in *Heterotrigona itama* honey (Merrill 2002; Siddique *et al.* 2015; Wigger *et al.* 2019; Lachkar *et al.* 2021).

4.3. 5-Hydroxymethyl-2-Furaldehyde

5-Hydroxymethyl-2-furfural (HMF), a well-established marker of food quality and freshness (Polovková and

Šimko 2017), exhibits potential therapeutic properties. Preclinical studies demonstrate that intraperitoneal administration of HMF significantly enhances survival in mouse models of permanent forebrain ischemia. Ya *et al.* (2012) attribute this effect to HMF's antioxidant capacity.

5-Hydroxymethylfurfural is the main product of two reactions, namely the acid-catalyzed degradation of hexose and the decomposition of 3-deoxy-α-D-glucose in the Maillard reaction. 5-Hydroxymethylfurfural is formed from hexose, including oligo/polysaccharides that are hydrolyzed into hexose, in an acidic environment, during heating or at lower temperatures with low pH (Shapla *et al.* 2018). Mechanistic studies on rapeseed honey show that free acids in honey catalyze fructose and glucose to form HMF (Yang *et al.* 2019). Stingless bee honey generally has a pH between 3.2 and 3.8 and high free acidity (Zawawi *et al.* 2022; Begna *et al.* 2024; Zheng *et al.* 2025). Fructose has been reported to have an HMF formation rate 31.2 times higher than glucose, in line with the explanation that ketohexoses (fructose) are more selective in producing HMF than aldohexoses (glucose) (Shapla *et al.* 2018). Meanwhile, HMF formation through the Maillard reaction requires basic organic compounds such as amino acids. Previous studies have shown that the amino acid content in SBH is relatively low, so the Maillard reaction may be less dominant in the mechanism of HMF formation in SBH compared to acid-catalyzed hexose degradation reactions (Sousa *et al.* 2016; Andrade-Velásquez *et al.* 2023).

4.4. Bis(4-Ethylbenzylidene) Sorbitol

Bis(4-ethylbenzylidene) sorbitol exhibits antibacterial activity, as evidenced by its identification in *Nigella sativa* (black cumin) extract, where it demonstrated efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) (Anidya *et al.* 2023). No previous studies have directly reported the presence of bis(4-ethylbenzylidene) sorbitol in honey. However, it is known that sorbitol is a polyol commonly produced from carbohydrate biomass and readily participates in further chemical transformations (Zhang *et al.* 2016). The phenylpropanoid pathway yields various aromatic acids and alcohols that can be converted into substituted benzyl aldehydes via microbial/plant enzymatic transformations (Ramanjaneyulu *et al.* 2015; Qiu *et al.* 2024). In an acidic environment, polyols such as sorbitol can undergo condensation with aromatic aldehydes, and chemical studies demonstrate strong sorbitol–aromatic interactions under acid-catalyzed conditions (Zhang *et al.* 2016). Bis(4-ethylbenzylidene) sorbitol in SBH most likely forms through non-enzymatic condensation between sorbitol (of sugar origin) and 4-ethylbenzaldehyde (of phenylpropanoid origin), facilitated by the honey's acidic nature and phenolic composition.

4.5. Cinnamic Acid

The phenolic compound, ubiquitous in honey matrices (Iftikhar *et al.* 2022), was detected in all samples of the analyzed SBH. While this study did not explicitly quantify antioxidant activity, cinnamic acid, a prominent phenolic acid, was consistently identified with high abundance across samples. Emerging evidence highlights its therapeutic versatility: Naghavi *et al.* (2021) demonstrated that topical application of cinnamic acid at concentrations below 1% accelerates dermal wound healing without adverse effects. Furthermore, supplementation with cinnamic acid in high-fat diet-induced obese rat models significantly improves obesity-related symptoms, including epididymal fat gain, insulin resistance, glucose intolerance, and dyslipidemia, without causing liver or kidney toxicity (Lee *et al.* 2022).

Cinnamic acid is the first product of the phenylpropanoid pathway, formed from L-phenylalanine via the catalysis of phenylalanine ammonia-lyase, which catalyzes the non-oxidative deamination of L- phenylalanine to trans-cinnamic acid and ammonium ion (Hyun *et al.* 2011; Vargas-Tah and Gosset 2015; Kukil and Lindberg 2022). This reaction is a commitment step that transfers carbon from primary metabolism to the secondary phenylpropanoid pathway (Hyun *et al.* 2011; Vargas-Tah and Gosset 2015). Cinnamic acid is then hydroxylated by cinnamate-4-hydroxylase to form p-coumaric acid and is subsequently modified (methylation, esterification, etc.) to yield p-coumaric, caffeic, ferulic, and sinapic acids (Vargas-Tah and Gosset 2015; Zeng *et al.* 2020; Kukil and Lindberg 2022).

Phenylpropanoids, including cinnamic acid and its derivatives, are widespread secondary metabolites in vascular plants; lignans, flavonoids, coumarins, and cinnamaldehyde all originate from the cinnamate skeleton (Vargas-Tah and Gosset 2015; Gao *et al.* 2023). Studies on various plants confirm that (E)-cinnamic acid is a key intermediate of L- phenylalanine in photosynthetic tissues and aroma-storing organs (Gonda *et al.* 2018; Zeng *et al.* 2020; Gao *et al.* 2023). This supports the assertion that phenylpropanoid-rich oil-producing plants, such as Melaleuca, are a logical source of cinnamic acid in nectar and subsequently in honey, although the species *Melaleuca cajuputi* has not yet been specifically studied.

4.6. Citric Acid

Citric acid plays a crucial role in mitigating oxidative damage within cells, thereby potentially enhancing immune function and safeguarding vital organs such as the liver and brain (Singh *et al.* 2022). Its anti-inflammatory efficacy is evidenced by the downregulation of pro-inflammatory cytokines in experimental models (Hara and Watanabe 2015). Additionally, citric acid modulates glucose metabolism by enhancing insulin sensitivity, offering therapeutic promise for hyperlipidemic and diabetic conditions (Yadikar *et al.* 2022). The compound also enhances the bioavailability of essential minerals, such as iron, which is crucial in combating anemia (Singh *et al.* 2022). Furthermore, citric acid synergistically enhances the effectiveness of various antimicrobial agents, making it potent against a broad spectrum of bacterial species, including antibiotic-resistant strains (Lee *et al.* 2002).

Citric acid is the primary intermediate in the citric acid cycle, which oxidizes acetyl-coenzyme A to produce energy in nearly all aerobic organisms (Choi *et al.* 2021; Guo *et al.* 2022; Arnold and Finley 2023). This cycle consists of a series of interconnected tricarboxylic acids (Guo *et al.* 2022; Arnold and Finley 2023). The citric acid cycle is also the primary energy pathway in eukaryotic cells, including bee glandular tissues; intermediates such as citrate can be exported from the mitochondria to the cytosol and the extracellular environment (Choi *et al.* 2021; Guo *et al.* 2022). Metabolomic studies of honeybees show that bees produce and secrete organic acids from the citric acid cycle into the intestinal lumen, which are then utilized by gut bacteria; citrate concentrations in the intestine increase significantly solely from the metabolism of sugar-fed bees, without any contribution from pollen (Quinn *et al.* 2024).

4.7. D(+)-Phenyllactic Acid

Phenyllactic acid demonstrated selective antimicrobial activity in in vitro studies using a vaginitis rat model, exhibiting toxicity against *Gardnerella vaginalis* and *Candida albicans*, primary pathogens associated with vaginal infections, while causing minimal adverse effects on commensal *Lactobacillus* spp. Furthermore, phenyllactic acid downregulated mast cell activation (Kwon *et al.* 2024). D-3-phenyllactic acid inhibitors were found to inhibit a variety of gram-positive bacteria commonly found in humans and foodstuffs, such as *Staphylococcus aureus* and *Enterococcus faecalis*, as well as gram-negative bacteria from humans, including *Providencia stuartii* and *Klebsiella oxytoca*. Mechanistic insights from scanning electron microscopy revealed that D-3-phenyllactic acid disrupts the integrity of *Listeria monocytogenes* cells, inducing morphological alterations, including loss of cell rigidity, cytoplasmic swelling, and eventual membrane lysis (Dieuleveux *et al.* 1998).

D(+)-3-phenyllactic acid is a naturally occurring phenolic organic acid commonly found in honey and various fermented products, and is widely recognized as a characteristic metabolite produced by lactic acid bacteria (Luo *et al.* 2020; Cheng *et al.* 2022). In lactic acid bacteria, phenyllactic acid is synthesized via the aromatic amino acid catabolic pathway, in which L-phenylalanine is first transaminated by aromatic aminotransferase to phenylpyruvic acid, which is then reduced by lactate dehydrogenase to 3-phenyllactic acid. The D-lactate dehydrogenase isoenzyme produces D-phenyllactic acid, while L-lactate dehydrogenase produces L-phenyllactic acid (Cheng *et al.* 2022; Wenger *et al.* 2023).

The L-phenylalanine to phenylpyruvic acid to phenyllactic acid pathway has been confirmed in several food-grade lactic acid bacteria species, including *Lactiplantibacillus plantarum, Lacticaseibacillus rhamnosus, Leuconostoc* spp., and *Pediococcus acidilactici*, where the addition of phenylalanine significantly increases phenyllactic acid production (ss 2016; Cheng *et al.* 2022; Zhang *et al.* 2022; Lunavath *et al.* 2023; Wenger *et al.* 2023; Kassym *et al.* 2024). In the context of SBH, lactic acid bacteria inhabiting propolis-coated honey pots provide a semi-closed fermentative environment highly conducive to this pathway, with L-phenylalanine detected in the honey matrix serving as the primary substrate for phenyllactic acid biosynthesis. D-phenyllactic acid is known to exhibit stronger antimicrobial activity than L-phenyllactic acid, with membrane-damaging effects and the ability to inhibit biofilm formation in various bacteria and fungi (ss 2016; Cheng *et al.* 2022; Zhang *et al.* 2022; Lunavath *et al.* 2023; Wenger *et al.* 2023; Kassym *et al.* 2024). Therefore, the consistent presence of D-phenyllactic acid across all SBH samples supports its role as a key component of the SBH's natural antimicrobial defense system.

4.8. L-Phenylalanine

Fitzgerald *et al.* (2020) demonstrated that oral administration of concentrated phenylalanine reduces energy intake and attenuates postprandial blood glucose levels in healthy male subjects. Further preclinical studies corroborate its metabolic benefits. Acute L-phenylalanine supplementation suppresses short-term food intake, whereas chronic administration significantly decreases both caloric consumption and body weight in diet-induced obesity rat models (Alamshah *et al.* 2017). Beyond its role in metabolic regulation, L-phenylalanine exhibits cardiovascular therapeutic potential, as evidenced by its capacity to ameliorate vascular dysfunction in hypertensive rodents (Heikal *et al.* 2018).

L-Phenylalanine is an essential aromatic amino acid that cannot be synthesized de novo by mammals and must therefore be obtained from plant-based sources in the diet (Hyun *et al.* 2011; Pascual *et al.* 2016). This amino acid serves a dual function as a building block of proteins and as the primary precursor of the phenylpropanoid pathway, with phenylalanine ammonia-lyase catalyzing the non-oxidative deamination of L-phenylalanine to trans-cinnamic acid (Hyun *et al.* 2011; Tohge *et al.* 2013; Pascual *et al.* 2016; Cotinguiba *et al.* 2022).

Plant nectar and pollen contain proteins and free amino acids, including L-phenylalanine, which are collected by *Heterotrigona itama* workers and incorporated into the developing honey matrix. Pollen is known as a rich source of protein, approximately 7–40% protein, and provides a large reserve of essential amino acid precursors (Pascual *et al.* 2016). During honey processing in the hive, secretions from the bees' head glands, particularly the hypopharyngeal glands, supply various enzymes, including proteases that degrade the proteins in nectar, pollen, and royal jelly into peptide fragments and free amino acids (Rossano *et al.* 2012; Hu *et al.* 2019). Thus, the concentration of L-phenylalanine detected in SBH reflects the combined contribution of plant biosynthesis, the amino acid content of nectar and pollen, and the proteolytic activity of bees during honey maturation.

4.9. α,α-Trehalose

Oral trehalose supplementation (3.3 g/day) for 12 weeks significantly reduced C-reactive protein (CRP) levels, a biomarker of systemic inflammation, which correlated with improved quality of life and ameliorated neuropsychiatric symptoms, including depression and chronic stress (Hashemian *et al.* 2023). Trehalose also demonstrates therapeutic efficacy in rare metabolic disorders. Intravenous trehalose infusion in patients with Niemann-Pick disease induced systemic metabolic modulation (Khoshakhlagh *et al.* 2023).

α,α-Trehalose is a non-reducing disaccharide composed of two glucose molecules linked by an α,α-1,1-glycosidic bond (Tang *et al.* 2018; Chen *et al.* 2022; Sevriev *et al.* 2024). In insects, including bees, trehalose is synthesized primarily in the fat body via the trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase pathway, in which trehalose -6-phosphate synthase catalyzes the formation of trehalose-6-phosphate from uridine diphosphate glucose and glucose-6-phosphate, which is then dephosphorylated by trehalose-6-phosphate phosphatase to yield free trehalose (Shukla *et al.* 2015; Tang *et al.* 2018; Yang *et al.* 2023; Chaudhari *et al.* 2025). The resulting trehalose is released into the hemolymph and is generally present at higher concentrations than glucose, providing a rapid energy reserve for flight muscles, supporting chitin synthesis, and protecting cells from heat, drought, and oxidative stress (Shukla *et al.* 2015; Yoshida *et al.* 2016; Tang *et al.* 2018; Yang *et al.* 2023).

Untargeted metabolomics has garnered increasing attention in the study of SBH due to its capacity to resolve the complex chemical diversity underlying this natural product's unique bioactivity and health-promoting attributes. By employing HRMS-based metabolomics, researchers achieve comprehensive profiling of SBH's metabolites without prior knowledge of their presence, enabling the detection and quantification of known and novel compounds. The chemical composition of SBH is inherently heterogeneous, shaped by ecological variables (e.g., botanical origin, geographical location, and bee species), which complicates standardization but enriches its value as a regionally distinct commodity. This variability is pivotal for regions pursuing geographical indication, as metabolomics identifies species and location-specific metabolic signatures that authenticate provenance and deter commercial fraud (Chuah *et al.* 2023).

A critical application of untargeted metabolomics is in quality assurance, specifically addressing the issue of adulteration. A pervasive challenge in apicultural industries is detecting synthetic additives or degradation products that compromise the physicochemical integrity of SBH. Concurrently, this study elucidates the bioactive metabolites responsible for SBH's therapeutic properties, including antioxidant, anti-inflammatory, and antimicrobial activities.

While untargeted metabolomics holds substantial analytical potential for SBH research, it faces challenges due to the complexity of honey's chemical composition. Accurate metabolite annotation requires advanced analytical instruments and access to comprehensive spectral libraries, which are currently limited for understudied matrices, such as SBH. Methodological inconsistencies in sample preparation, inter-instrument variability in sensitivity, and disparities in data processing algorithms further introduce analytical variability, complicating cross-study comparability. To address these limitations, future research must prioritize standardizing metabolomic protocols. Curating SBH-specific metabolite libraries enriched with high-resolution mass spectral data and biosynthetic pathway annotations will facilitate robust compound annotation and quantification. Such advancements are critical to unlocking SBH's full phytochemical potential and translating its health-promoting properties into validated nutraceutical applications.

Untargeted metabolomics is a powerful analytical tool for resolving the phytochemical complexity of SBH. This methodology is critical for quality assurance protocols, safeguarding against adulteration, and elucidating bioactive metabolites responsible for SBH's health-promoting properties. As analytical frameworks and spectral databases evolve, this approach will deepen the understanding of SBH's unique biochemical signatures, thereby advancing its translational applications in ethnomedicine and pharmaceutical innovation.

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