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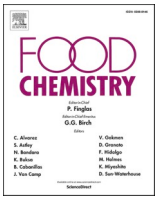

Characterization of honeys produced by sympatric species of Afrotropical stingless bees (Hymenoptera, Meliponini)

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ABSTRACT

We investigated the effect of bee species identity and harvesting methods on the chemical composition and antiradical activity of 53 honey samples, produced by six stingless bee species in western Kenya (Kakamega forest). Our results illustrate that none of the assayed parameters significantly varied between the honey samples harvested by "punching holes" (n = 25) and "squeezing" (n = 28) methods. By contrast, species identity drove significant differences in the assayed parameters. Positive correlations between the antiradical activity and the phytochemicals (phenols and flavonoids) were observed, and honeys from *Liotrigona* sp. exhibited the highest amounts of phenols (214 mg GAE/100 g), flavonoids (73.0 mg QE/100 g) and antiradical activity (76.2%). The physicochemical analyses confirm the need to establish separate stingless bee honey standards for moisture, free acidity, invertase, electrical conductivity, and HMF, as these parameters significantly diverged from the set limits for *Apis mellifera* honey.

1. Introduction

Honey is a complex mixture of mainly sugars and other substances made by honey bees and some related insects from nectar or honeydew (Machado De-Melo, Almeida-Muradian, Sancho, & Pascual-Maté, 2018). Honey produced by *Apis* bees is commercialized around the world, and has received considerable attention for its nutritional and health benefits (Rao, Krishnan, Salleh, & Gan, 2016). Yet, besides *Apis mellifera* (AM), stingless bees (SB) constitute another, lesser-known group of honey-producing bees within the family Apidae (tribe Meliponini). Unlike AM, which is found on all continents except in Antarctica and in deserts or permafrost regions of the world, SB are essentially found in tropical and subtropical regions, with about 500 species distributed across 32 genera (Grüter, 2020). These bees have distinct features like the presence of a vestigial sting, the capacity to collect nectar or honeydew from flowers of creeping and other small plants, due to their small body size. Furthermore, they have a trend of building broods in a horizontal or vertical position, and a habit to store nectar and pollen in pots rather than combs as in the case of AM (Gonzalez, Amith, & Stein, 2018; Nkoba, Raina, Muli, Mithöfer, & Mueke, 2012). Stingless bee colonies can be kept in man-made hives, just like AM - a traditional activity known as meliponiculture (Grüter, 2020; Perichon, Heard, & Schouten, 2020) - to facilitate the collection of honey, which is used by rural communities in tropical, subtropical and savanna regions of the world as folk medicine, for cultural rituals, as source of both sugars and micronutrients, or for income generation, including in a context of crop pollination (Grüter, 2020; Heard, 1999; Nkoba et al., 2012).

Stingless bee honey (SBH) is steadily gaining acceptance among consumers due to its contrasting and appealing flavor and aroma, a more fluid texture and slow crystallization, all contributing to its high commercial potential (Rao et al., 2016). Another characteristic of this honey is its resistance to form hydroxymethylfurfural (HMF) when subjected to high temperatures (Biluca et al., 2014), which is an added value for its use in pharmaceutical and food industries where the negative effects associated with excess HMF should be avoided (Shapla, Solayman, Alam, Khalil, & Gan, 2018). A more recent study revealed that SBH is the only natural product known to be enriched with a biologically active sugar (trehalulose), which has both antidiabetic and acariogenic properties (Fletcher et al., 2020). These recent findings among others suggest that current and future research into the chemical properties of SBH, offers opportunities to better characterize these under-investigated natural product (and for some, non-timber forest products), while discovering novel molecules relevant to human.

Investigations into the chemistry and bioactive compounds found in

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SBH face multiple challenges, among which the characterization of the many potential sources of variation, including the potential for species-specific compositional and functional profiles of honeys, the impact of the botanical resources exploited by the foraging bees and their variation in space across their distribution range, as well as the harvesting methods and timing (Leonhardt, Heard, & Wallace, 2014). Moreover, the sub-Saharan SB (i.e., all species occurring in the area that lies south of the Sahara in Africa) are perhaps the least known of all stingless bees to date, as their taxonomy is still being discussed (Eardley & Kwapong, 2013), and the study of their honeys is still in its infancy (Nordin, Sainik, Chowdhury, Saim, & Idrus, 2018).

In this study, we aim to contribute to filling these gaps by investigating the compositional and functional profiles of honeys collected in colonies from a range of different stingless bee species kept in man-made hives around Kakamega forest (0°09′N, 34°50′E) in western Kenya, a mid-altitude tropical rainforest, UNESCO world heritage Center, and the last remnant of the ancient Guineo-Congolian rainforest (Zimmerman, 1972). Specifically, we investigated (i) the influence of the species on the composition and antiradical activity of honeys stored by six SB species of economic importance (*Meliponula togoensis*, *M. ferruginea, M. lendliana, M. bocandei, Liotrigona* sp. and *Plebeina armata*), among communities around Kakamega forest (Nkoba et al., 2012), and (ii) the extent to which the honey harvesting methods employed by the beekeepers influence the chemical composition and antiradical activity of those honeys. Being the first multi-species survey and analyses of SBH from Kakamega forest, these findings will be an essential prerequisite to the establishment and scaling out of optimal harvesting methods, with the goal of contributing to the definition of species-specific quality standards in SBH across sub-Saharan Africa, as well as to test and document the possible human health benefits of SBH *via* the quantification of their phytochemicals and their antiradical activity.

2. Materials and methods

2.1. Study location - Kakamega forest (Kenya)

The surveys were conducted in five meliponaries located in homesteads at Chirobani (2 meliponaries), Ivihiga (1 meliponary), Kiborkok (1 meliponary) and Isiekuti (1 meliponary), nearby the indigenous forest portion of Kakamega forest near Kisumu in western Kenya (Latitude: 0° 17′ 18.00″ N; Longitude: 34° 51′ 13.19″ E). Chirobani and Ivihiga meliponaries are in the western portion of the forest, while the Kiborkok and Isiekuti meliponaries are in the most southern end of the Kakamega forest.

2.2. Honey samples

The dataset consisted of 53 honey samples collected from hives maintained by beekeepers around Kakamega forest in December 2019 (Table 1). Two extraction methods were employed; “punching holes” through the honey pots to allow the honey to drip out (n = 25) and “squeezing” the honey out of the honey pots after scrapping them (n = 28). All samples were collected in the field, stored in hermetic plastic bottles, and then transported in electric cooler box (charged using car charge) at −18 °C back to the African Reference Laboratory for Bee Health at the *International Centre of Insect Physiology and Ecology (icipe)* in Nairobi (Kenya) where they remained stored at −18 °C prior to the laboratory analyses described below. All samples were handled the same, right from harvest until analyses.

Table1
Global Positioning System (GPS) coordinates of sampling sites.

Sites	Number of meliponaries	Latitude	Longitude	Elevation (meters)
Chirobani	2	N 00.22193°	E 034.92786°	1587
Isiekuti	1	N 00.15983°	E 034.85622°	1623
Kiborgok	1	N 00. 25769°	E 034.75108°	1493
Ivihiga	1	N 00. 28113°	E 034.95480°	1613

The honey samples were produced by *Liotrigona* sp. (n = 3), *Meliponula bocandei* (n = 4), *M. ferruginea* (n = 17), *M. lendliana* (n = 1), *M. togoensis* (n = 24) and *Plebeina armata* (n = 4) (Fig. 1). These species are characterized by a diverse series of morphological and ecological traits: *M. bocandei* species has a large body size (7.0 mm) and organizes its brood in clusters, while the *M. ferruginea* and *M. togoensis* specie are smaller than *M. bocandei* but larger in body size (5.1–5.9 mm) than *P. armata* (3.3–5.2 mm). On the other, *Liotrigona* sp. has a body size of between 2.1 and 4.2 mm. *M. ferruginea*, *M. togoensis* and *P. armata* organize their brood in horizontal combs. In the wild, the *M. bocandei* and *M. togoensis* nest in tree cavities while *P. armata* is an underground cavity nester and its nests are only found in termite mounds. *M. ferruginea* species nests either in cavities in trees (trunk, branch), underground or in walls of human residential houses (Ndungu et al., 2017; Nkoba et al., 2012).

2.3. Chemicals and reagents

Chemicals and solvents were of analytical or HPLC grade. HMF standard, Folin-Ciocalteu’s phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), water, methanol, potassium hydrogen phosphate, disodium hydrogen phosphate, p-nitrophenyl-α-D-glucopyranoside, tris-hydroxymethyl amino methane, proline standard, formic acid (99%), ninhydrin, ethylene glycol monomethyl ether, 2-propanol, 0.45 μm nylon filters, $NaNO_2$, $AlCl_3$, NaOH, quercetin, sodium carbonate, and gallic acid, were supplied by Sigma-Aldrich Co. (Kobian, Kenya).

2.4. Instrumentation

We used pH and conductivity meters (Jenway 3540, Essex, England), a handheld digital refractometer (Atago, Tokyo, Japan), a water activity meter (WA – 60A, Guangzhou Landtek Instruments, China), a UV/Vis spectrophotometer (Jenway 6850, Kobian, Kenya), and a high performance liquid chromatography (HPLC) (1260 series, Agilent Technologies, Santa Clara, CA, USA).

2.5. Physicochemical analyses

Physiochemical parameters were determined as per the International Honey Commission (IHC, 2009) except for the Water activity (a_w) and sugars in °Brix, which were determined as per (Yap, Chin, Yusof, & Chong, 2019) and AOAC (2005), respectively.

2.5.1. pH and free acidity

A pH and conductivity meter was used to measure the pH of 10 g of honey in 75 ml of carbon dioxide free distilled water. Free acidity was done by titrating the same sample solution with 0.1 M NaOH until a pH 8.3 was attained.

2.5.2. Moisture content

The moisture content was determined using a refractometric method. The refractive indices of honey samples were measured at room (20 to 25 °C) temperature using a handheld digital refractometer and the corresponding moisture contents (%) were recorded.

2.5.3. Water activity (a_w)

This was determined at 25 °C by means of a water activity meter as previously described (Yap et al., 2019).

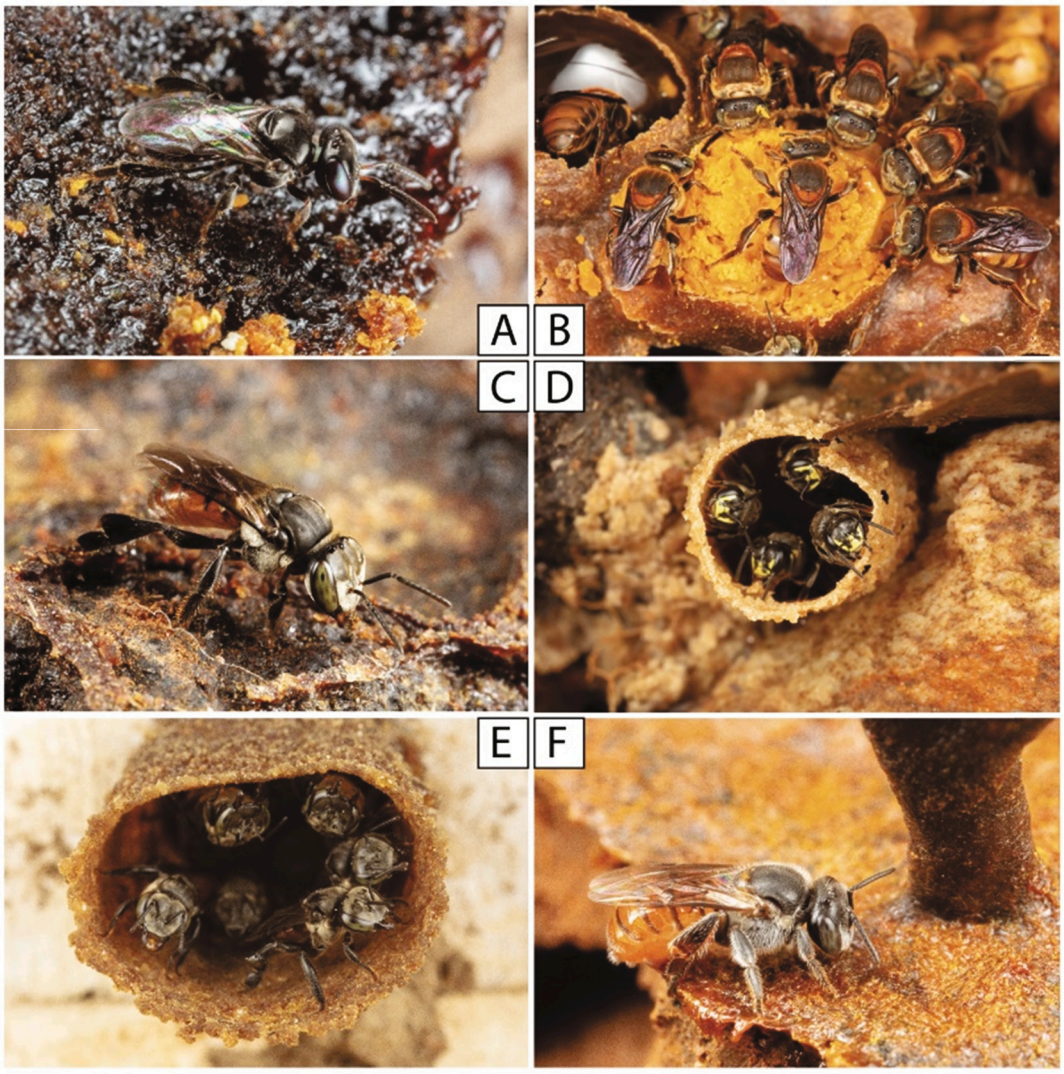

Fig. 1. Sympatric Afrotropical species investigated in this study. A. *Liotrigona* sp. worker at nest entrance; B. Workers of *Meliponula (Meliponula) bocandei* inside their nest; C. Worker of *Meliponula (Axestotrigona) ferruginea* inside its nest; D. Workers of *Meliponula* (*Meliplebeia*) *lendliana* at nest entrance; E. Workers of *Meliponula (Axestotrigona) togoensis* at nest entrance; F. *Plebeina armata* worker inside a its nest hosted in an underground termite colony. All photographs by NJ Vereecken.

2.5.4. Electrical conductivity

Electrical conductivity was measured using a pH and conductivity meter for a 20% (w/v) solution of honey suspended in distilled water. The meter was calibrated with conductivity solution before taking the readings of honey solutions and conductance was given in mS/cm.

$S_H = K^*G$

where;

S_H = electrical conductivity of the honey solution in mS/cm
K = cell constant in cm^{-1} (0.93)
G = conductance in mS

2.5.5. Invertase activity

This was determined spectrophotometrically. Five grams of the sample were dissolved in 25 ml buffer solution (11.66 g of potassium hydrogen phosphate, and 2.56 g of disodium hydrogen phosphate in 1 L of water), to make honey solution. Then, five ml of the substrate solution (6.03 g of p-nitrophenyl-α-D- glucopyranoside in 1 L of buffer solution) was transferred into two separate test tubes and incubated in a water bath for 5 min at 40 °C. After incubation, 0.5 ml of the honey solution was added to one test tube (sample) and 0.5 ml of reaction terminating solution (363.4 g of tris- hydroxymethyl amino methane in 1 l of water) to the other test tube (blank). The test tubes were further incubated for 20 min after which 0.5 ml of reaction terminating solution was added to the sample and 0.5 ml of honey solution to the blank. The absorbance of the sample against the blank was measured after 15 min at 400 nm using a UV/Vis spectrophotometer.

Invertase in invertase number (IN) = 21.64 * A_{400} nm
Where: 21.64 is the slope

2.5.6. Proline content

The spectrophotometric method was used as follows; 0.5 ml of the honey solution (5% w/v) was placed in one tube, 0.5 ml of water (blank) into a second tube and 0.5 ml of proline standard solution into two other tubes. To these tubes, 1 ml of formic acid (99%) and 1 ml of ninhydrin solution (3% w/v in ethylene glycol monomethyl ether) was added. The tubes were capped and stirred for 15 min and then transferred into a boiling water bath (100 °C) for 15 min. After 15 min, the tubes were moved to a water bath at 70 °C for 10 min. Finally, 5 ml of the propanol-water solution (1:1) was added to each tube, capped immediately then left to cool before the absorbances were measured 45 min later at 510 nm. Proline content was calculated as follows;

Proline content(mg/kg) = Es/Ea*E1/E2*80

where;

Es = Absorbance of the sample solution
Ea = Absorbance of the proline standard solution (average of two readings),
E1 = mg proline taken for the proline solution preparation
E2 = Weight of honey in g.
80 = Dilution factor

2.5.7. Hydroxymethylfurfural (HMF)

The HMF was determined using high performance liquid chromatography (HPLC) equipped with a diode array detector (DAD, G1315D), a binary pump and an auto sampler, all from Agilent. Separation was on an Agilent C18, 4.6 × 100 mm, 3.5 μm column. Briefly, honey samples (10 g) were diluted to 50 ml with distilled water, filtered using a 0.45 μm nylon filter and injected (10 μl) into an HPLC system. The HPLC method included an isocratic mobile phase of 85% water and 15% methanol with a flow rate of 1.0 ml/min at 30 °C. The detection wavelength was 285 nm. HMF concentration in the samples was calculated by comparing the corresponding peak areas of the samples and HMF standard (1 – 10 ppm) curve after correcting for the dilution of the samples. HMF was expressed in mg/kg honey.

2.5.8. Sugars (^{o}Brix)

The total sugars (soluble solids) were determined according to AOAC (2005), by recording a reading of the honey sample in a handheld digital refractometer.

2.6. Phytochemical contents analyses

2.6.1. Total flavonoids content (TFC)

They were determined using the method of Dowd as in published procedures (Mokaya, Bargul, Irungu, & Lattorff, 2020). Briefly, 1 ml extract of the sample (1 g of honey in 4 ml of water) was mixed with 4 ml of distilled water, then 300 μl of 5% $NaNO_2$ was added and mixed. After 5 min, 300 μl of 10% $AlCl_3$ was added and left for 1 min before adding 2 ml of 1 M NaOH and 2.4 ml of distilled water. The absorbance was measured against the blank (the mixture minus the sample) at 510 nm. Quercetin (Q) was used to generate a calibration curve (20 – 200 μg/ml), and TFC were expressed as mg Q equivalent (E)/100 g honey.

2.6.2. Total phenols content (TPC)

They were quantified following the Folin–Ciocalteu method as described in published protocols (Mokaya et al., 2020). One gram of honey was diluted with 20 ml of distilled water. To 1 ml of the honey solution in a test tube, 5 ml of 0.2 N Folin–Ciocalteu reagent was added. After 5 min, 4 ml of 75 g/l sodium carbonate was added before the mixture was incubated at room temperature (20 to 25 °C) for 2 h. The absorbance was read at 760 nm against a water blank. Gallic acid (GA) standard was used to yield a calibration curve (0 – 250 μg/ml). The TPC was expressed as mg of GAE/100 g honey.

2.7. Analysis of radical scavenging activity (RSA)

2.7.1. DPPH radical scavenging activity

The spectrophotometric method as previously reported was used with minor modifications (Mokaya et al., 2020). To 0.75 ml methanolic honey solution (50 mg/ml), 1.5 ml of 20 mg/l DPPH solution (2 mg of DPPH in 100 ml of methanol) was added and the mixture was incubated for 15 min at room temperature in the dark. The absorbance was measured at 517 nm against a blank sample (0.75 ml honey solution with 1.5 ml methanol). The control sample consisted of 0.75 ml methanol mixed with 1.5 ml DPPH solution, with methanol as its blank. The antiradical activity was expressed as a percentage inhibition, relative to the control sample.

Radical scavenging activity (RSA) expressed as % inhibition = [(control absorbance − sample absorbance)/control absorbance] * 100%

2.8. Statistical analyses

2.8.1. Analysis of honey similarity – Impact of species and harvesting methods.

All the assayed parameters were done in triplicate. We used the vegan package (version 2.0–5) in RStudio (version 1.1.456) (RStudio Team, 2016) for R (R Core Team, 2018) to perform a multivariate analysis of similarities (ANOSIM) using the average Bray-Curtis distances among samples to test statistically whether there was a significant difference in honey composition between species, and the harvesting methods. Non-linear multidimensional scaling (NMDS) plot was used to highlight the effects induced by the bee species and harvesting methods on honey composition and its antiradical activity. The NMDS was computed using the function "metaMDS" of the "vegan" package in RStudio (version 1.1.456) (RStudio Team, 2016) for R (R Core Team, 2018). Box plots were done using R v3.6.2 (R Core Team, 2018), the factoextra (v1.0.6, Kassambara & Mundt, 2019) and ggplot2 v3.2.1 (Wickham, Chang, & Wickham, 2016) packages, to show variation of phytochemicals between the species irrespective of the harvesting methods. Kruskal-Wallis test was used to check for species-specific patterns in each assayed parameter, as nine of the twelve parameters failed the Shapiro's test for normality ($p > 0.05$).

2.8.2. Pairwise comparisons of honey parameters

The pairwise comparisons of stingless bee honey parameters described above were visualized with the *ggpairs* function in the *GGally* package (version version 1.4.0.) (Schloerke et al., 2018) in RStudio (version 1.1.456) (RStudio Team, 2016) for R (R Core Team, 2018); a loess regression was fitted to the observed data with 95% confidence band intervals around the fit. The scatter plot produced also allows the computation of Pearson's correlation coefficient for each pair of variables, both irrespective of the species and harvesting methods (i.e., "punching holes" vs. "squeezing").

3. Results and discussion

3.1. Harvesting methods and bee species in relation to honey properties

The ANOSIM analyses showed no significant impact of the harvesting methods on the stingless bee honey composition and antiradical activity (R-stat = 0.0069, p = 0.3223), but a significant effect of the target species on the same properties (R-stat = 0.274, $p < 0.001$). These similarities and differences are summarized in the NMDS plot (Fig. 2). This agreed with the Kruskal-Wallis test results for species-specific patterns in each of the assayed parameter (Table 1 and Fig. 2), which showed that 66.7% of the parameters varied significantly among the studied species. Stress value for the two- dimensional plot was equal to 0.174 and non-metric goodness-of-fit measured with the Shepard's diagram was $r^2 = 0.970$, allowing a safe interpretation of the data. Convex hulls comprising samples associated to each honey harvesting method were overlapping to a considerable extent, indicating no impact of the harvesting method on the honey properties (Fig. 2a), a result consistent with the ANOSIM analysis above. By contrast, as shown in Fig. 2b of the NMDS analyses, a series of samples associated to different stingless bee species appeared more clearly separated into discrete clusters. This is particularly the case for samples associated to bees in the genera *Liotrigona* and *Plebeina* which appeared to cluster further outside the otherwise overlapping convex hulls comprising samples scattered over a large area of the phenotypic space, and associated to the remaining four

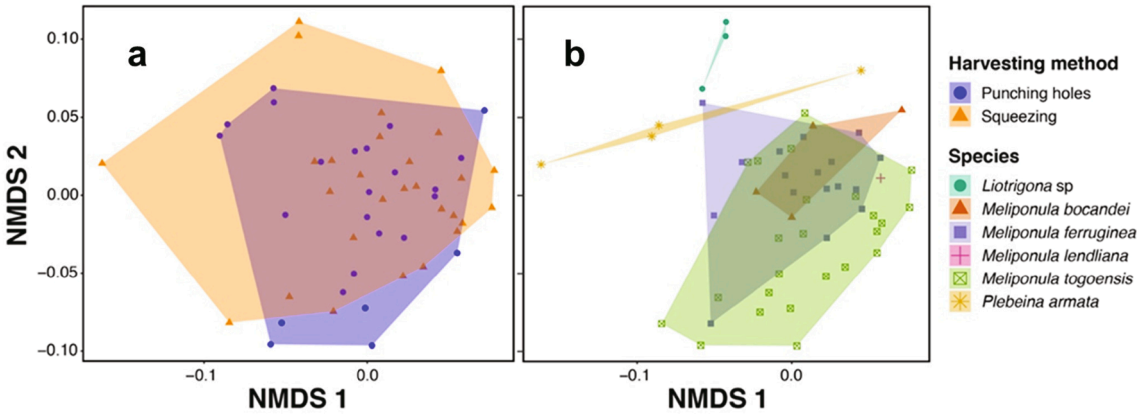

Fig. 2. Non-linear Multidimensional Scaling (NMDS) ordination plots showing the differentiation of honey samples grouped into two convex hulls comprising samples associated to different harvesting methods (*a*), and into six convex hulls comprising samples associated to different Afrotropical stingless bee species (*b*).

species in the genus *Meliponula* (Fig. 2b).

However, the "punching holes" method is encouraged as it results in clean honey, thus minimizing subsequent processing steps, i.e., it eliminates the need for filtration. Furthermore, this method allows for a faster repair of the honey pots by the bees compared to scrapping and squeezing pots, which destroys the pots completely. Any method (harvesting or processing) that negatively affects the honey chemical composition, particularly the physicochemical properties, lowers the quality of that honey (Hempattarasuwan, Settachaimongkon, & Duangmal, 2019).

We hypothesize that the species-specific honey profiles illustrated by the clustering of samples according to the bee species are largely associated to the fact that different co-occurring stingless bee species might exploit slightly different ecological niches and host plants for the collection of honeydew and nectar (Shamsudin et al., 2019). Therefore, characterizing the foraging patterns of African stingless bees as part of their ecological and climatic niche, and the identification of their host plants from mixed-species pollen loads, will undoubtedly be of significance for future research.

Further, our results agree with the findings of a previous study that illustrated how ten different stingless bee species in Brazil, produce honey of varying composition (Biluca, Braghini, Gonzaga, Costa, & Fett, 2016). Similar results were also reported by Espinoza-Toledo and colleagues, where they observed clustering of honey samples according to the species identity (*Melipona solani, M. beecheii,* and *Scaptotrigona mexicana*), in the same region (Espinoza Toledo et al., 2018).

3.2. Physicochemical properties

The results for physicochemical properties are presented in Table 2 as the mean ± standard deviation.

3.2.1. Water activity (a_w) and moisture content

Water activity depict the available water for microbial growth in foods, and 0.6 is the minimum a_w for the osmophilic yeast to thrive and cause unwanted fermentation. The a_w ranged from 0.70 to 0.77, where the highest values were from *M. lendliana* and the lowest values were from *M. bocandei* and *M. ferruginea* samples. These results corroborated with those reported for *Kelulut* honey (0.79) (Yap et al., 2019), and *Trigona carbonaria* honey (0.74) (Oddo et al., 2008). Despite its high a_w content, SBH is relatively resistant to fermentation due to its low pH and high free acidity, which deters the growth of microbes.

High moisture could favor unwanted honey fermentation during storage caused by osmophilic yeast, thus a useful quality control criterion (Nordin et al., 2018). Samples from all the species had higher moisture values than the recommended limit (≤20%) for AMH (Codex Alimentarius Commission, 2001). The values varied between 26.1 and 35.9%. Of the studied species, *M. lendliana* sample reached the highest while *M. ferriginea* samples had the least amount of moisture. A similar range of values have been cited for stingless bee honey in the past studies, e.g., a range of 23.1 – 43.5% was recorded for honey samples from ten species of Brazil (Biluca et al., 2016), and 25.0 – 47.0% for Thailand samples (Chuttong, Chanbang, Sringarm, & Burgett, 2016). Also, a range of 25.1 – 35.0 % was recorded for Ethiopian *Meliponula*

Table 2
Influence of stingless bee species on the physicochemical properties of stingless bee honey (mean ± SD).

Species	n	Water activity (a_w)	Moisture (%)	pH	Free acidity (meq/kg)	Electrical conductivity (mS/cm)	Proline (mg/kg)	Invertase activity (IN)	HMF (mg/kg)	Sugars (% Brix)
***Liotrigona* sp.**	**3**	0.73 ± 0.02	29.1 ± 1.4	4.0 ± 0.1	270 ± 38	1.6 ± 0.3	771 ± 142	8.9 ± 1.1	11.6 ± 18.0	71.0 ± 1.4
Meliponula bocandei	**4**	0.70 ± 0.02	27.6 ± 6.3	4.4 ± 0.5	48 ± 24	0.6 ± 0.1	457 ± 125	2.8 ± 2.3	11.2 ± 11.7	72.4 ± 6.3
Meliponula ferriginea	**17**	0.70 ± 0.03	26.1 ± 2.6	4.9 ± 0.4	38 ± 26	1.0 ± 0.2	443 ± 99	3.7 ± 1.9	10.6 ± 10.4	73.9 ± 2.6
Meliponula lendliana	**1**	0.77	35.9	4.5	52	1.2	491	1.1	13.4	64.1
Meliponula togoensis	**24**	0.74 ± 0.03	31.2 ± 3.2	5.0 ± 0.5	30 ± 14	1.1 ± 0.1	379 ± 188	4.5 ± 4.0	15.8 ± 10.0	68.8 ± 3.2
Plebeina armata	**4**	0.71 ± 0.03	27.2 ± 2.7	4.0 ± 0.3	141 ± 63	0.6 ± 0.1	415 ± 204	4.6 ± 2.9	55.2 ± 45.4	72.9 ± 2.7
Chi-squared		18.5	21.9	20.8	20.6	25.5	10.6	9.22	7.25	21.9
df		5	5	5	5	5	5	5	5	5
p-value		≤0.05	<0.001	<0.001	<0.001	<0.001	>0.05	>0.05	>0.05	<0.001

The analyses were done following Kruskal-Wallis test for $p \leq 0.05$. n = number of samples, df = degree of freedom, and IN = invertase number, SD = standard deviation.

beccarii honey samples (Gela, Hora, Kebebe, & Gebresilassie, 2021). The two parameters (a_w and moisture) had a strong positive correlation ($r^2 = 0.703$, Fig. 3). Environmental factors during harvesting are among the key determinants of honey's moisture content. The high moisture content in SBH may therefore be due to the nature of their habitat i.e., the humid tropical and subtropical regions (Nordin et al., 2018).

3.2.2. pH and free acidity

All the samples were acidic in nature, having pH values between 4.0 and 5.0. The lowest values (most acidic) were found in *Liotrigona* sp. and *P. armata* samples, while the highest values (less acidic) were found in *M. togoensis* samples. Stingless bee honeys from Brazil had a pH range of 3.3 – 6.6, and 3.1 – 3.9 (Biluca et al., 2016; Sousa et al., 2016), and Thailand SBH samples were found to have a pH range of 3.1– 5.3 (Chuttong et al., 2016). A study on *Scaptotrigona pectoralis* honey (Moguel, Sosa-Moguel, Pino, Bolivar-Moreno, & Cuevas-Glory, 2019), also recorded a low pH value (3.6). The low pH of honey is of significance as it inhibits the growth of microbes, hence maintaining the stability, and the shelf life of honey (Lage et al., 2012).

Free acidity varied between 30 and 270 meq/kg, with *Liotrigona* sp. and *P. armata* exhibiting extraordinarily high free acidity values of 270 and 141 meq/kg, respectively. Samples from three of the studied species had values within the recommended limit for AMH (≤50 meq/kg) (Codex Alimentarius Commission, 2001). Our results were in agreement with what was observed by Nordin and coworkers (Nordin et al., 2018), where 59.6% out of the 472 stingless bee honey samples, had their free acidity values within the set standard for AMH. Previous studies have reported a similar range of values for SBH (Biluca et al., 2016; Moguel et al., 2019). Honey acidity derives from the organic acids, particularly the gluconic acid, which vary among samples based on floral composition, the bee species, and the rate of fermentation of sugars to alcohol, and further oxidation to carboxylic acids (Lage et al., 2012; Sousa et al., 2016). A strong negative correlation ($r^2 = -0.605$, Fig. 3) between the pH and free acidity was recorded, implying that the lower the pH, the higher the free acidity, and vice vasa. Just like the low pH, the high free acidity in honey is crucial as it deters microbial development (Lage et al., 2012).

3.2.3. Electrical conductivity

Honey's conductivity is due to the presence of minerals, proteins, organic acids, and other organic compounds (Nordin et al., 2018). There was variability among the studied species as the values ranged from 0.6 to 1.6 mS/cm. The lowest values were found in *P. armata* and *M. bocandei* samples, while the highest values were recorded in *Liotrigona* sp samples. Consistent with these values are those found by (Biluca et al., 2016) for Brazilian SBH samples (0.15 – 1.34 mS/cm) and (Chuttong et al., 2016) for Thailand SBH samples (0.32 – 3.10 mS/cm). Contrary, the samples from *M. subnida* and *M. scutellaris*, which showed comparatively low values that ranged from 0.30 to 0.67 mS/cm (Sousa et al., 2016). It is used alongside other parameters like palynological assay to determine honey floral origin. Strong positive correlations were recorded between the electrical conductivity and the phytochemicals, which are organic compounds ($r^2 = 0.590$ with TFC, and $r^2 = 0.592$ with TPC, respectively). When compared to the set limit for AMH (≤0.8 mS/cm), four of the studied species had high values (Commission, 2001).

3.2.4. Proline

Liotrigona sp. honeys had higher proline (771 mg/kg) than other species while *M. togoensis* honeys had the least amount (379 mg/kg). A study on Ethiopian *Meliponula beccarii* honey samples reported a comparable proline content mean of 214.5 mg/kg (Gela et al., 2021). However, a study by Sousa et al. (2016) found low proline values. Proline is one of the major free amino acids found in a abundance in honey, and is assayed as an indicator of honey maturity and to check for adulteration (Sousa et al., 2016). Since proline is also related to the floral source and the amount of pollen present in the honey it could be

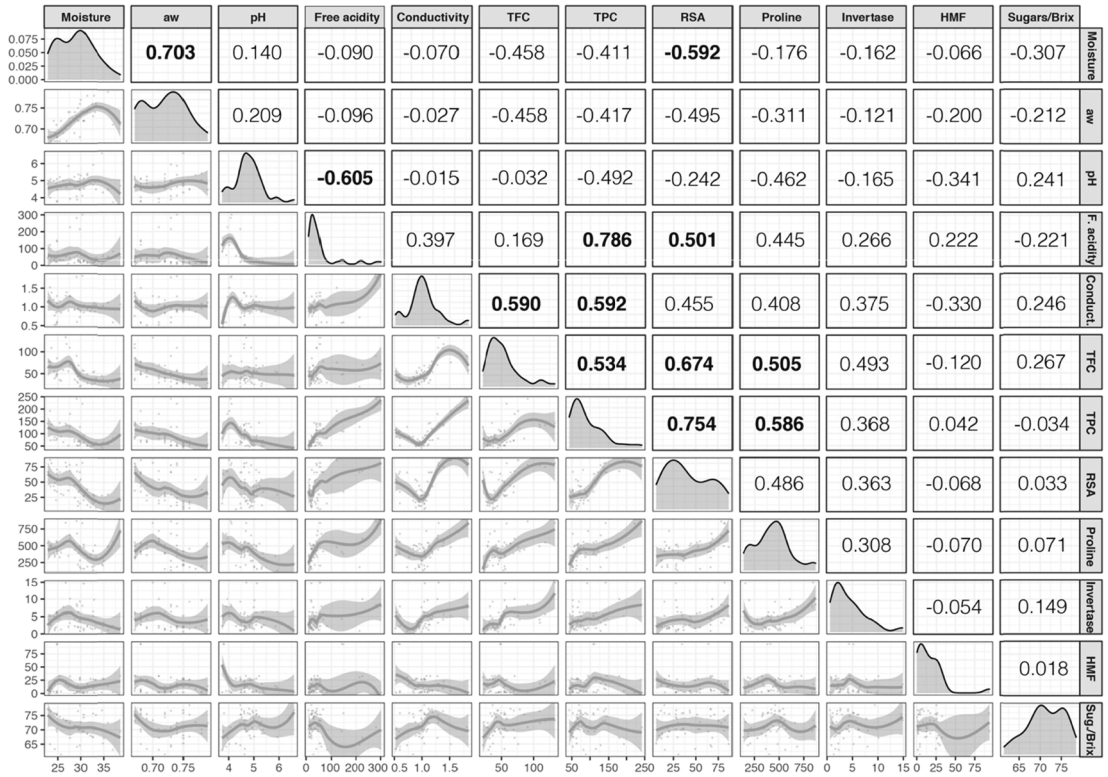

Fig. 3. Scatter plot matrix illustrating the pairwise relationships between stingless bee honey parameters. The scatter plot also shows Pearson's correlation coefficient for each pair of variables; the diagonal of the scatter matrix represents the density distribution of the data, irrespective of species or harvesting method. The results indicate that most variables are weakly correlated (values in bold indicate Pearson's correlation coefficient >±0.5).

useful for the characterization of the botanical sources of honey. As per the Codex Alimentarius Commission (2001), the proline limit for AMH should be ≥ 180 mg/kg, and all the studied stingless bees honey samples met this limit.

3.2.5. Invertase activity

The lowest values were observed in *M. lendliana* (1.1 IN), while the largest values were found in *Liotrigona* sp honeys (8.9 IN). Three of the studied species had values above the minimum limit (≥4 IN) for AMH (Codex Alimentarius Commission, 2001). Previous studies have reported comparable values for invertase activity in SBH, e.g., in Malaysian SBH, the range was between 0.27 and 4.94 IN (Julika et al., 2020), and an average of 22.0 IN for *S. pectoralis* honey (Moguel et al., 2019).

Invertase is used as indicator of honey freshness or prolonged storage, due to its high sensitivity to heat and tendence to deteriorate over time. The detected low enzyme activity for some of the studied samples, which were freshly obtained from the honey pots, may be regarded as a natural feature for SBH, rather than an index of scarce freshness or prolonged storage. Therefore, the use of enzyme activity as an indicator of freshness, as is commonly used for AMH, may not be applicable for SBH (Nordin et al., 2018).

3.2.6. Hydroxymethylfurfural (HMF)

HMF is an important honey quality parameter, widely used as an indicator of honey freshness and adulteration from external sources (Sousa et al., 2016). HMF is generally absent in freshly harvested honey, but it tends to surge over time. It is formed by the reaction of sugars, particularly fructose, with the acids. The range for HMF was from 10.6 to 55.2 mg/kg and the highest values were measured in the samples of *P. armata* while the lowest values were in *M. ferruginea* samples. Most of the studied species (5), showed HMF values within the range established by the Codex Alimentarius Commission (2001) of ≤ 40 mg/kg for AMH. A study by Holanda et al. (2012) revealed that HMF of *M. fasciculata* honey varied between 5.44 and 70.79 mg/kg. Equally, a range of 28.0 – 58.3 mg/kg was reported for Brazilian SBH (Nascimento et al., 2015). Normally, under proper storage conditions, and low temperatures it forms slowly, but when honey is exposed to high temperatures, poor storage conditions, or addition of boiled sucrose, the HMF rises (Shapla et al., 2018).

3.2.7. Sugars (°Brix)

The sugars, especially reducing sugars, influence the energy value of honey, thus they are an immediate source of energy for bees (Sousa et al., 2016). The sugars ranged from 64.1 (*M. lendliana* sample) to 73.9°Brix (*M. ferruginea* samples). A similar range (55.2–76.1°Brix) was recorded by (Biluca et al., 2016) for SBH samples from Brazil. Compared to AMH, the values for SBH are low due to their high water content and low sugars. For instance, Nordin and colleagues (Nordin et al., 2018)

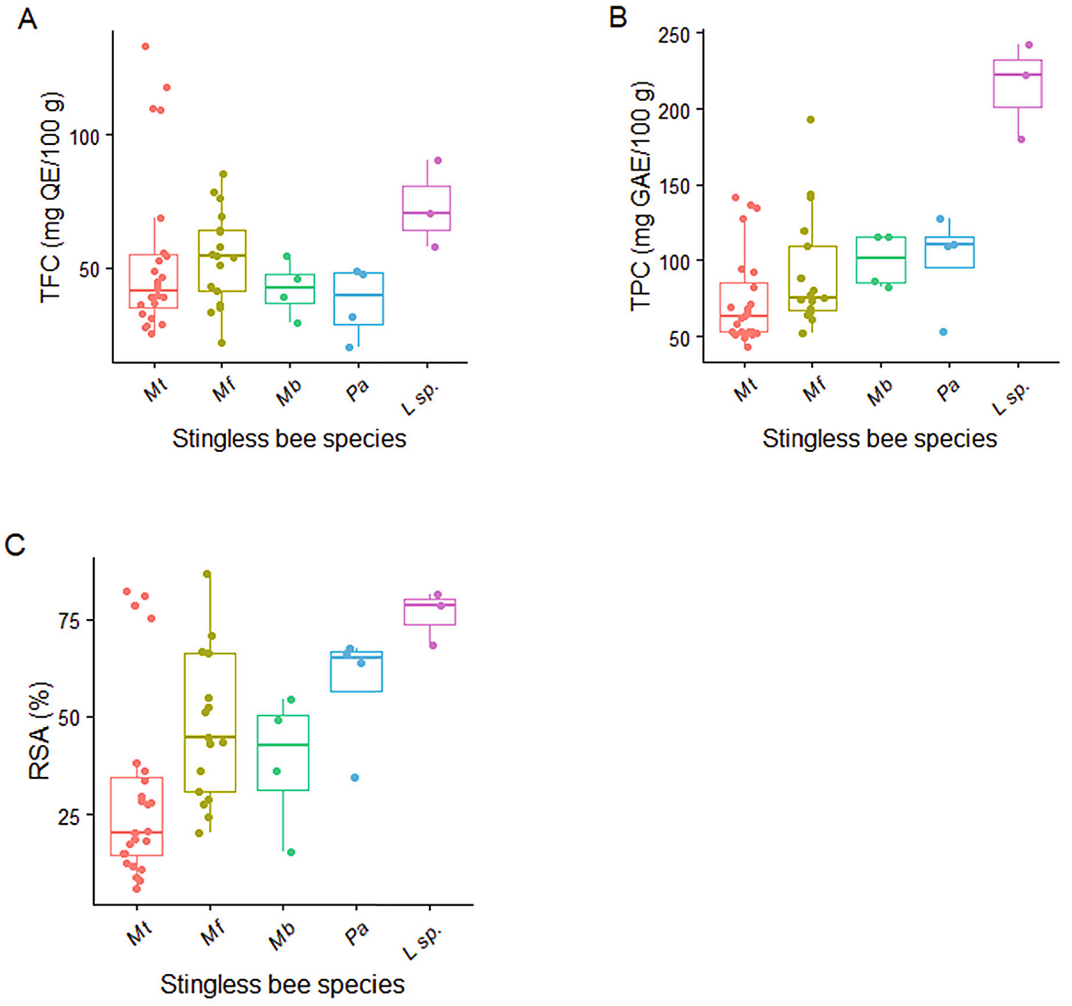

Fig. 4. Box plots explaining variation in phytochemical contents (A and B), and antiradical activity (C). *L_sp* = *Liotrigona* sp., *Pa* = *Plebeina armata*, *Mb* = *Meliponula bocandei*, *Mf* = *Meliponula ferriginea*, and *Mt* = *Meliponula togoensis*. *M. lendliana* was not included, because it had a single data point (one sample). Significant variations across species were observed for TPC (Chi-squared = 15.3, df = 5, $p \leq 0.05$), and RSA (Chi-squared = 16.0, df = 5, $p \leq 0.05$), but not for TFC (Chi-squared = 9.21, df = 5, $p > 0.05$).

noticed a range of 64.5 to 75.8°Brix in SBH compared to a range of 78.77 to 316.92°Brix in AMH, in the papers they reviewed. More sugars in honey results in a high osmotic pressure, which discourages development of microbes, thus favoring longer shelf life of honey.

The physicochemical data support the need to establish separate quality standards for SBH, to avoid unfair rejection of otherwise good honey, agreeing with other researchers who have voiced similar concerns (Chuttong et al., 2016; Nordin et al., 2018).

3.3. Phytochemicals

These results are shown in Fig. 4. The TPC ranged from 57 to 214 mg GAE/100 g, with *M. lendliana* sample recording the least amount while *Liotrigona* sp. honeys recording the highest amount (Fig. 4, A). Previous studies on SBH recorded comparable values, e.g., scales of 10.3 – 98.0 mg GAE/100 g (Biluca et al., 2016) and 31.5 – 126.6 mg GAE/100 g (Sousa et al., 2016) were recorded for honeys from different stingless bee species. Similarly, a study on AMH samples from Kenya found a comparable range of values (Mokaya et al., 2020).

The TFC ranged from 28.7 to 73.0 mg QE/100 g (Fig. 1, B). Honeys from *Liotrigona* sp. showed the highest TFC, and samples from *M. lendliana* demonstrated the lowest TFC. The TFC values for the current study are higher than those previously cited for this type of honey (Sousa et al., 2016), and this could be due the differences in floral composition in different geographic regions and the different producing species. Compared to AMH from Kenya, a significant difference ($p < 0.0001$) was observed (Mokaya et al., 2020), with SBH having the highest values. The presence of these compounds (phytochemicals) in honey is an indication of its good quality (Ranneh et al., 2018), as they have been associated with most of the biofunctional properties of honey such as immune-stimulation, antimicrobial, anticancer, anti-inflammatory, and antioxidant (Cianciosi et al., 2018). This was true even in the current study, as evidenced by the strong positive correlations between the antiradical activity (RSA) vs phenols ($r^2 = 0.754$) and RSA vs flavonoids ($r^2 = 0.674$), as shown in Fig. 3. It is important to note that not only the quantity, but also the quality of the phytochemicals is responsible for the abovementioned biofunctional properties.

3.4. Radical scavenging activity (RSA)

The *in-vitro* radical scavenging activity ranged from 30.0 to 76.2% (Fig. 1, C), which was comparable to the findings reported for Malaysian SBH (90% for 40 mg/ml honey concentration) by Ranneh et al. (2018). Honeys from *Liotrigona* sp. exhibited the highest scavenging activity, with all its samples exhibiting >50% scavenging ability (Fig. S1), while *M. togoensis* samples had the least activity. A study by Sousa et al. (2016) recorded a low range of 11.2 to 46.9%, despite having used a higher concentration (100 mg/ml) than the one used in the present study (50 mg/ml). The antiradical activity was greatly influenced by the phenols and flavonoids (Fig. 3). Free radicals have been found to cause damage to biomolecules like DNA, RNA, proteins, and cell membranes, which eventually may lead to the development of diseases including cardiovascular dysfunctions and cancer (Cianciosi et al., 2018). Therefore, exogenous intake of antiradical molecules through the diet is vital to help counteract the damaging effects of free radicals (Cianciosi et al., 2018).

4. Conclusions

This study demonstrated that the bee species identity was a significant driver of the compositional profiles of SBH, with significant impacts on the physicochemical profile, phytochemical contents, and antiradical activity. On the other hand, there were no significant differences in honey composition between samples obtained through "punching holes" and "squeezing" harvesting methods. However, we support the use of "punching holes" as opposed to "squeezing", because with the latter method, solid debris are introduced in honey, and the pots are destroyed leaving the bees with a difficult task of rebuilding new pots.

Our results further showed that most of the studied samples were rich in phytochemicals (phenols and flavonoids) and exhibited significant radical scavenging activities, especially, *Liotrigona* sp. samples. We as well noted that moisture, free acidity, invertase, electrical conductivity, and HMF, in some of the studied SBH samples, failed to comply with the set standards for AMH. Therefore, we concur with those who have proposed the need to establish separate standards for SBH. We are confident that more coordinated research is required in this field with large-scale, structured surveys comparing stingless bee honeys to *Apis mellifera* honeys in sub-Saharan Africa and beyond.

CRediT authorship contribution statement

Hosea O. Mokaya: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Kiatoko Nkoba:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition. **Robert M. Ndunda:** Investigation, Formal analysis, Writing - review & editing. **Nicolas J. Vereecken:** Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130597.

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