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The Antibacterial Activity of Black Honey, Yellow Honey, and Kelulut Honey from Aceh Region and the Characterization of their Chemical Composition using GC-MS

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Honey has been used since ancient times for its taste, aroma, and numerous therapeutic properties, including antioxidant, antibacterial, anti-inflammatory, antiviral, antiparasitic, antimicrobial, and antidiabetic activities. This study aims to investigate the antibacterial properties and chemicals characterization of three different type of honeys (Black Honey, BH; Yellow Honey, YH; and Kelulut Honey, KH) from Aceh region. Kirby-Bauer disc diffusion method were performed to screen pure honey samples for antibacterial activity against wound-infecting bacteria; i.e. two Gram-positive (*Staphylococcus aureus*, and *Staphylococcus hominis*) and two Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacterial strains. Zone of inhibition were measured and compared with a positive control. The chemical characterizations of honeys were performed by using Gas Chromatography-Mass Spectroscopy (GC-MS). The Kirby-Bauer disc diffusion method showed that Black Honey (BH) exhibited the strongest antibacterial effect against *Staphylococcus aureus*; *Staphylococcus hominis*; *Escherichia coli*; and *Pseudomonas aeruginosa* with the zone of inhibitions were 18.15±0.73 mm; 17.73±0.52 mm; 17.59±0.70 mm; and 15.52±0.86 mm respectively. The GC-MS analysis showed that all honeys contains 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl compound, where the largest amount was found in Black Honey (BH) with the percentage area of 24.94%. The GC-MS analysis also exhibited that Black Honey (BH) had the highest carbohydrate content (69.54%), followed by KH (36.54%) and YH (28.40%). In conclusion, the superior antibacterial activity of Black Honey (BH) is correlated with its higher pyranone and carbohydrate compounds. The future study should evaluate the effects of Black Honey (BH) and it's formulation as wound healing dressing by in vivo on experimental animal.

Keywords: Honey; antimicrobial; Gram-positive bacteria; Gram-negative bacteria; FT-IR spectroscopy; GC-MS

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Honey is a sweet and viscous substance produced by honey bees and other types of bees. Bees produce honey from flower nectar or the secretions of other insects, such as honeydew. Typically, bees store honey in wax structures known as honeycombs, made from a mixture of wax and resin, depending on the bee species [1]. In Indonesia, various types of honey are produced by different bee species, such as *klanceng* honey, which is produced by *klanceng* bees and is beneficial for anti-inflammatory purposes due to its phenolic compounds. Another example is forest honey, produced by forest bees, the largest honey producer in the world [2, 3]. The nutritional content of forest honey includes niacin, riboflavin, pantothenic acid, calcium, magnesium, manganese, potassium, phosphorus, and zinc. Forest honey is also highly effective in alleviating diarrhoea symptoms [4].

The chemical composition of honey is highly dependent on the sources of the plants that the bees feed on [5]. However, in general, 80% of the main composition of honey is sugars such as glucose, sucrose, and fructose [6]. There are at least 22 types of sugar compounds found in honey, generally in the

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form of dextrose and laevulose. Researchers believe that sugar contained in honey is important role in the antimicrobial properties of honey. In addition to, other compounds such as phenols, flavonoids, phenols, amino acids and minerals were reported have strong correlation in bacterial inhibitory capacity of the honeys [7, 8, 9]. Chauhan reported that the potential and effectiveness of honey as an antimicrobial is highly dependent on the type or kind of honey, the botanical origin of the honey, the origin of the bees, the health of the bees, and the processing and collection methods of the bees [10]. For example, Manuka honey which comes from Leptosperm sp., has broad-spectrum antibacterial activity [11, 12].

Additionally, honey possesses physicochemical and microbiological characteristics that can be used as quality parameters. Numerous studies have been conducted to evaluate the quality of specific types of honey [13, 14, 15]. In Indonesia, the quality standards for honey are regulated by the Indonesian National Standard [16]. In this study we investigated the antibacterial properties of three honey types from the Aceh region: Black Honey (BH), Yellow Honey (YH), and Kelulut Honey (KH). We also determined their chemical composition by using Gas Chromatography-Mass Spectroscopy (GC-MS).

EXPERIMENTAL

Honey Samples

The honey samples (BH, YH, and KH) used in this study were collected from various honey gatherers in different locations, particularly from village forests. The KH honey was specifically obtained from honey farmers who cultivated bees in residential areas.

Figure 1 shows the origin of the honey samples, while **Table 1** details the sources and origins of the honey used. The samples were brought to the laboratory and stored at room temperature for further analysis.

Organoleptic Properties of Honey

The organoleptic properties of the BH, YH, and KH honey samples, including color, aroma, and taste, were analyzed.

Figure 1. MAP of Aceh Province and the origin of honeys.

Table 1. Description of the honey samples and their origins.

No.	Kinds of Honeys	The Origin of Honeys			
		Regency/City	District	Village	Source Honey
1.	BH	Bener Meriah Regency	Bandar	Pondok Baru	Village forest
2.	YH	Bener Meriah Regency	Timang Gajah	Damaran Baru	Village forest
3.	KH	Banda Aceh City	Syiah Kuala	Darussalam	Honey farmer

Antibacterial Activity

The Kirby-Bauer disk diffusion method was employed to determine the antibacterial activity. Nineteen grams of Mueller Hinton Agar (MHA) medium was added to 500 mL of sterile distilled water and then heated to boiling using magnetic stirring. The media was sterilized in an autoclave at 121°C for 15 minutes. The media (25 mL) was poured into a Petri dish and allowed to solidify. A suspension of bacteria (Gram-negative, *Escherichia coli* and *Pseudomonas aeruginosa*; Gram-positive, *Staphylococcus aureus* and *Staphylococcus hominis*) were applied to the solidified MHA media using a cotton swab. The paper disk, previously soaked in a positive control solution (Gentamycin 30 µg and Vancomycin 25 µg were used as positive control for *Staphylococcus aureus* and *Staphylococcus hominis* respectively; Ciprofloxacin 30 µg and Erythromycin 25 µg were used as positive control for *Escherichia coli* and *Pseudomonas aeruginosa* respectively), was placed on the media. The zone of inhibition formed after incubating the media at 37°C for 24 hours was measured to assess the antibacterial activity of the samples [17].

FT-IR Analysis

The samples were analyzed for functional group content using Fourier-Transform Infrared Spectroscopy (FT-IR spectroscopy). The FT-IR spectrum analysis was performed at wave numbers ranging from 400-4000 cm^{-1}.

GC-MS Analysis

Sample preparation: A small amount of each honey sample was used directly for GC-MS analysis. GC-MS analysis: The GC-MS analysis was conducted using the TG-SQC system qualification column (Thermo Scientific™ TRACE 1310 GC) in tandem with the Thermo Scientific™ ISQ LT Single Quadrupole Mass Spectrometer. The autosampler used was the Thermo Scientific™ TriPlus™ RSH Autosampler with an HP-5MS column and TG-WAXMS with a length of 15 m, a diameter of 0.25 mm, and a film thickness of 0.25 µm. Spectroscopic detection was performed by the Single Quadrupole Mass Spectrometer. The maximum temperature was set at 330/350°C. The relative quantity of the chemical compounds present in each sample was expressed as retention time (min.) and percentage based on the peak area (% area) produced in the chromatogram were analyzed.

RESULTS AND DISCUSSION

Organoleptic Properties of Honey

The organoleptic properties of the honey, including color, aroma, and taste, were evaluated. The results are presented in **Table 2** below. Table 2 shows that BH and KH have black and brown colors with a faint aroma and a bitter-sweet or slightly bitter taste. Meanwhile, YH is yellow with a strong aroma and a sweet taste. Honey is a natural substance with a sweet taste, produced by bees from nectar or plant sap. This nectar is collected, processed, and combined with specific compounds by bees, then stored in hexagonal combs. Honey is highly nutritious, containing important compounds such as saccharides, amino acids, vitamins, enzymes, organic acids, phenols, aromatic substances, pigments, essential oils, vitamins, and minerals [18, 19, 20].

Antibacterial Activity

The antibacterial activity of the three types of honey was tested against four bacterial strains: *S. aureus*, *S. hominis* (Gram-positive strains), *P. aeruginosa*, and *E. coli* (Gram-negative strains). The results showed that BH exhibited the strongest antibacterial activity against all four bacterial strains, with inhibition zones ranging from 15.52 mm to 18.15 mm, indicating very strong activity. In contrast, KH demonstrated the strongest activity against *S. aureus*, with an inhibition zone of 17.73 mm. Interestingly, YH showed no antibacterial activity against any tested bacteria (Figure 3 and Table 3), which may be attributed to its lower carbohydrate content than BH and KH. The frailty antibacterial activity of YH is expected due to high content of 5-Hydroxymethylfurfural (5-HMF). Robert and Shapla et al, reported that HMF is produced by the degradation of sugars through the nonenzymatic Maillard reaction (Figure 2) [21, 22]. Sugar plays a very important role in the anti-microbial activity of honey, resulting in reduced antibacterial activity [21].

Table 2. Organoleptic properties of Black honey (BH), Yellow honey (YH), and Kelulut honey (KH).

No.	Type of Honey	Color	Aroma	Taste
1.	BH	Black	Slightly fragrant	Bittersweet
2.	YH	Yellow	Fragrant	Sweet
3.	KH	Brown	Slightly fragrant	Sweet, slightly bitter

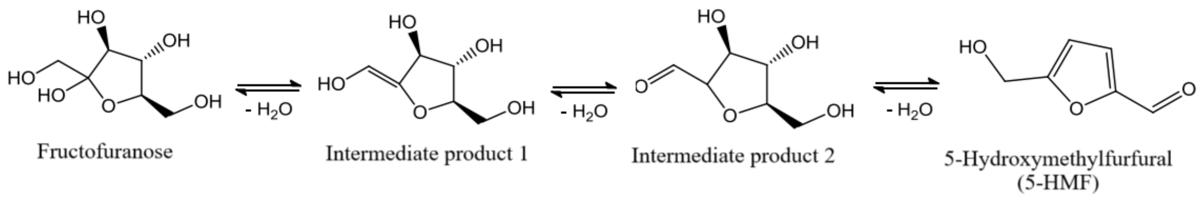

Figure 2. Formation of HMF in honey [22].

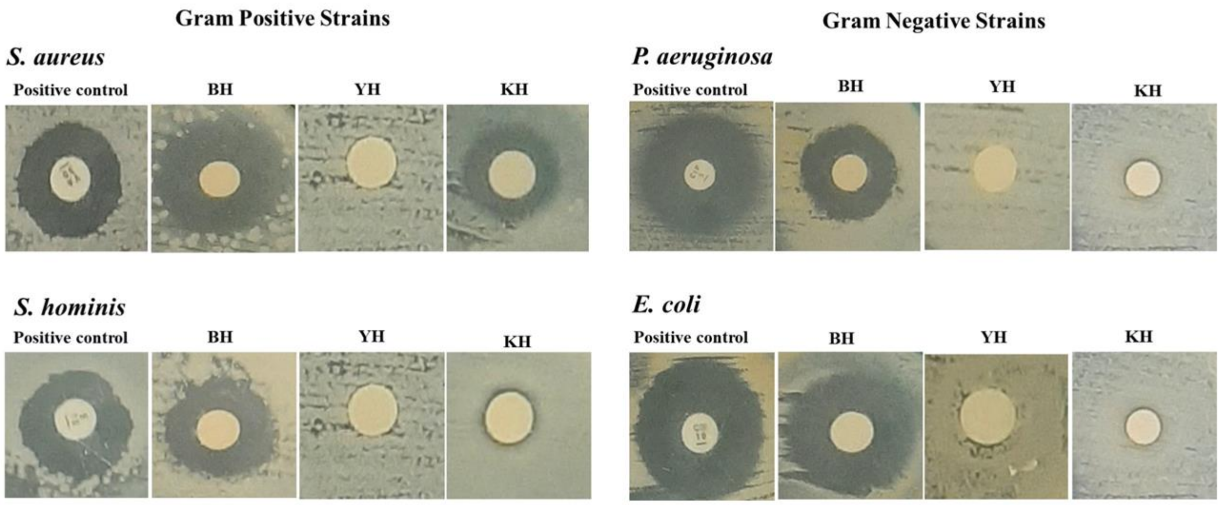

Figure 3. The antibacterial activity of three different types of honeys; Black honey (BH), Yellow honey (YH), and Kelulut honey (KH) against Gram-positive and Gram-negative strains.

Bogdanov et al. and Alvarez-Suarez et al. reported that carbohydrates are the primary contributors to the antimicrobial activity of honey, alongside other minor contributors such as phenolic compounds [23, 24]. Furthermore, several studies have shown that honey's antioxidant and antimicrobial activities are not only strongly correlated with sugar content and phenolic compounds but also influenced by the color of the honey. Dark-colored honey has been reported to contain high levels of phenolic compounds and sugars, which consequently enhances its antioxidant capacity [25]. This finding is consistent with our results, where Black honey (BH) exhibited higher carbohydrate content and antimicrobial activity than YH and KH. The antimicrobial activity of honey largely depends on the synergistic effects of the compounds present, the acidity level, peroxide content, and the osmotic pressure exerted by the honey itself [26].

Olawode et al., reported that sugar is the main component of honey, where glucose and fructose are the most abundant types of sugar in honey [27]. In addition, more than 200 other additional compounds were also detected in honey such as enzymes, pigments, phenolic compounds, amino acids, proteins, lipids, vitamins and minerals [28]. Meanwhile, Santos and González stated that those thought to be responsible for the aroma, taste and microbial activity of honey are sugars, amino acids, and several other volatile compounds such as alcohol and phenylacetic esters [29, 30]. The mechanism of action of sugar as an antimicrobial is by increasing the osmotic pressure in bacterial cells, causing water to flow out of bacterial cells through osmosis. As a result, cells shrink due to dehydration and cannot survive in hypertonic sugar solutions [31, 32].

Other researchers have also noted that the antimicrobial activity of honey is greatly influenced by its sugar content, particularly the compound Methylglyoxal. Phenolic compounds and complex carbohydrates also play a role in the antimicrobial activity of honey [33]. For example, Maduka honey has been reported to exhibit activity against several Gram-positive and Gram-negative bacteria [34]. The acidity of honey also inhibits microbial growth and stimulates bactericidal action [35].

Table 3. The diameter of inhibition zones of three different types of honeys: Black honey (BH), Yellow honey (YH), and Kelulut honey (KH) against Gram-positive and Gram-negative bacteria.

No.	Microorganism		Type of honey	Diameter of inhibition zone ± SD (mm)
1.	***Gram-positive***	***S.aureus***	Black honey (BH)	18.15±0.73
			Yellow honey (YH)	8.44±0.14
			Kelulut honey (KH)	17.73±0.52
			Gentamycin (Positive control)	21.18±0.26
		S.hominis	Black honey (BH)	17.47±0.70
			Yellow honey (YH)	0.00±0.00
			Kelulut honey (KH)	15.13±0.25
			Vancomycin (Positive control)	15.13±0.25
2.	***Gram-positive***	***E.coli***	Black honey (BH)	17.59±0.02
			Yellow honey (YH)	0.00±0.00
			Kelulut honey (KH)	7.23±0.30
			Ciprofloxacin (Positive control)	26.00±0.05
		P.aeruginosa	Black honey (BH)	15.52±0.86
			Yellow honey (YH)	0.00±0.00
			Kelulut honey (KH)	7.50±0.015
			Erythromycin (Positive control)	16.42±1.57

FT-IR Analysis

Several types of honey samples were analysed using FT-IR spectroscopy. The FT-IR analysis was conducted over a 4000-400 cm-1 wavenumber range. The FT-IR spectrum of each honey extract sample was correlated with the vibrations of functional groups. The FT-IR spectra of the three types of honey (Black Honey [BH], Yellow Honey [YH], and Kelulut Honey [KH]) are shown in **Figure 4**.

The analysis results indicate that the three honey samples exhibit similar FT-IR spectral patterns with slight differences in spectral intensity. The FT-IR analysis further reveals that the spectra of BH, YH, and KH generally consist of five specific regions: Region 1 (3500-2800 cm^{-1}), Region 2 (1750-1550 cm^{-1}), Region 3 (1550-1150 cm^{-1}), Region 4 (1150-950 cm^{-1}), and Region 5 (950-600 cm^{-1}). Each region is distinguished based on the absorption of specific functional groups. The absorption of functional groups in each of the three honey samples is presented in **Table 4**.

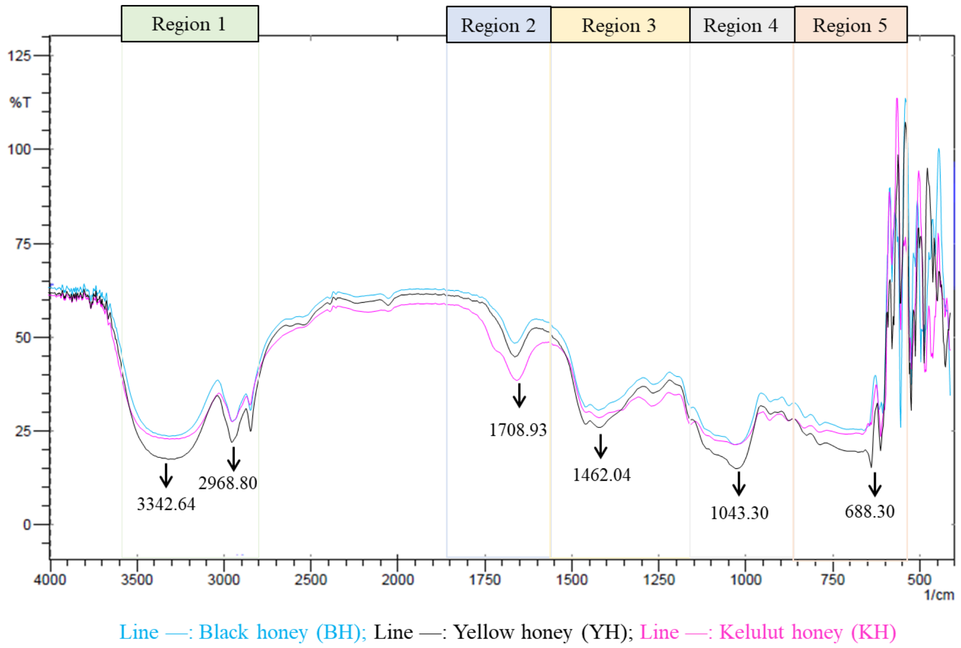

Figure 4. The FT-IR spectrum of three different types of honey: Black Honey (BH), Yellow Honey (YH), and Kelulut Honey (KH).

Table 4. FT-IR spectrum of five specific regions from three different types of honey: Black Honey (BH), Yellow Honey (YH), and Kelulut Honey (KH).

Region spectrum	Wavenumber	Functional groups
Region 1	3500-2800 cm^{-1}	-O-H stretching (carboxylic acids) -N-H stretching (free amino acids) -C-H stretching (carbohydrates)
Region 2	1750-1550 cm^{-1}	-O-H bending (water) -C=O stretching (carbohydrates)
Region 3	1500- 1150cm^{-1}	-C-O stretching (carbohydrates) -C=O stretching (ketones) -C-H stretching (carbohydrates)
Region 4	1150-950 cm^{-1}	-C-O stretching (carbohydrates) -C-C stretching (carbohydrates) -C-C stretching ring vibration (mainly from carbohydrates)
Region 5	950-600 cm^{-1}	-C-H bending (mainly from carbohydrates) -Ring vibration (mainly from carbohydrates) -Anomeric region of carbohydrates

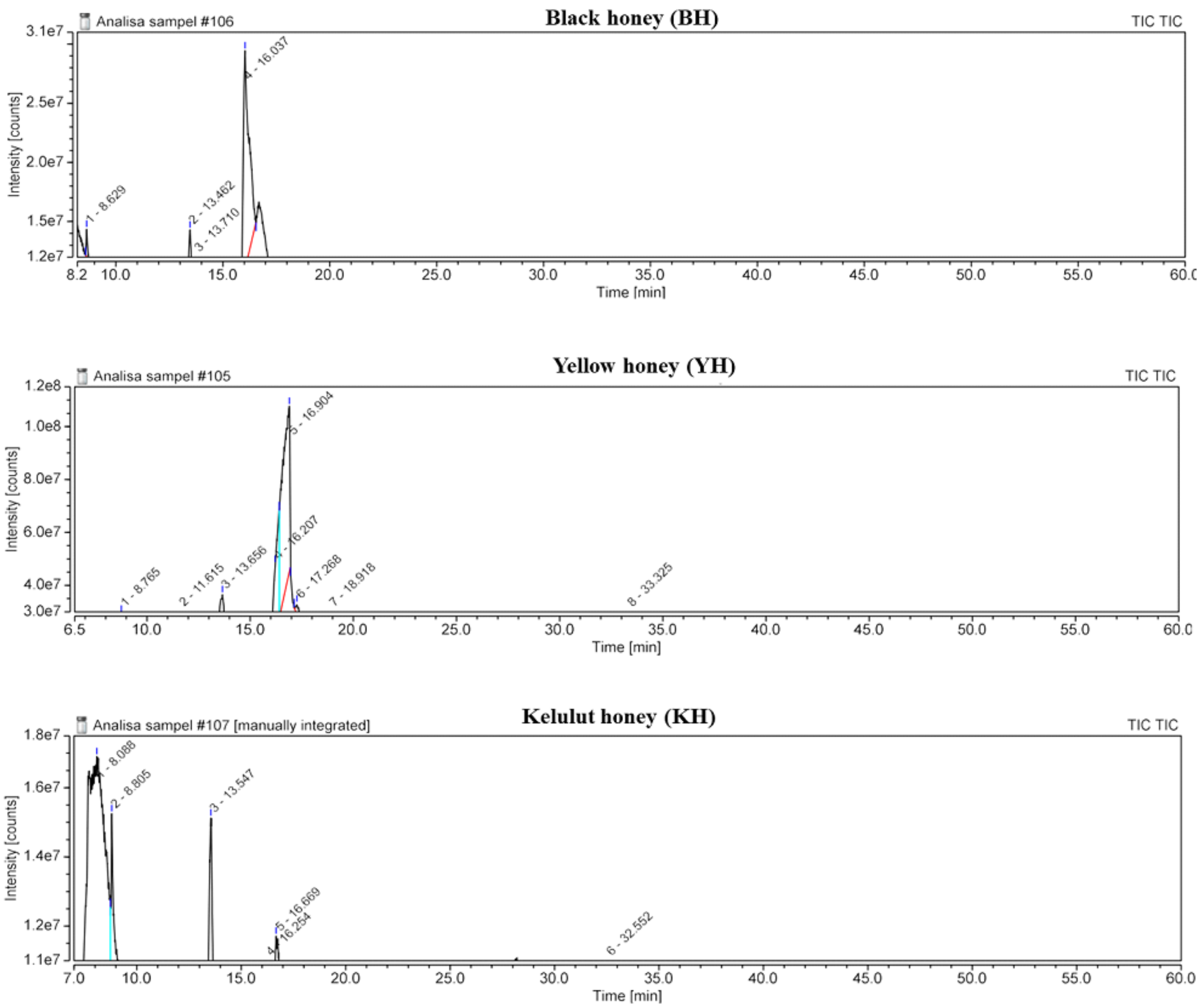

Figure 5. The GC-MS spectrum of three different types of honey: Black Honey (BH), Yellow Honey (YH), and Kelulut Honey (KH).

The analysis also shows that the absorption in Region 1 at 3342.64 cm^{-1} corresponds to the -OH group derived from water molecules. Meanwhile, the absorption at 2968.80 cm^{-1} is attributed to C-H stretching and -C-N, likely originating from carboxylic acids and free amino acids [36].

The absorption in Region 2, particularly at 1708.93 cm^{-1}, is associated with -C=O stretching from ketone groups in fructose and aldehyde (-CH=O) from glucose present in honey [37, 38]. The specific absorptions in Regions 3, 4, and 5, ranging from 1550 cm^{-1} to 600 cm^{-1}, are characteristic of the fingerprint region, corresponding to the absorption of -C-O, -C-C, and -C-H groups, which are derived from various carbohydrates such as sucrose, glucose, and fructose [39, 40, 41]. The most noteworthy absorption occurs in Region 3 at 1462.04 cm^{-1}, suspected to originate from the vibrations of -O-CH and -C-C-H groups, likely stemming from carbohydrate structures. The absorption in Region 4, within the wavenumber range of 1150-950 cm^{-1}, corresponds to the C-O group in C-OH within carbohydrates. Lastly, the absorption in Region 5, spanning 950-600 cm^{-1}, is characteristic of -C-H and -C-C group absorptions derived from carbohydrate compounds [42].

GC-MS Analysis

GC-MS analysis was performed on three types of honey (BH, YH, and KH). The GC-MS analysis results for these honeys are shown in **Figure 5**.

The analysis revealed that BH contains the highest number of compounds, followed by YH and KH, with 4, 8, and 6 compounds, respectively. The GC-MS results also indicated that all three types of honey contain the compound 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, with varying percent areas. BH exhibited the highest percent area, followed by KH and YH, with values of 24.95%, 8.11%, and 2.70%, respectively. This compound, known as pyranone ($C_6H_8O_4$), is a six-membered ring compound. The GC-MS analysis also revealed two main components in the honey samples used in this study: carbohydrates and other compounds. The chemical structures of these two components are illustrated in **Figure 6**. Honey is highly nutritious and rich in amino acids, vitamins, minerals, and other essential organic compounds. Honey is also a significant source of carbohydrates, as it is a sweet, concentrated solution [43, 44]. The chemical composition of honey is greatly influenced by its origin, geographical location, and environmental conditions [45, 46].

Table 5. The chemical compositions of three different types of honey: Black Honey (BH), Yellow Honey (YH), and Kelulut Honey (KH).

Peak No.	RT (min.)	Compound	Relative area (%)
Black Honey (BH)			
1.	8.63	DL-Arabinose	2.03
2.	13.46	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	24.94
3.	13.71	N-Acetyl-d-galactosamine	6.97
4.	16.04	6-Acetyl-β-d-mannose	60.54
		Total	100.00
Yellow Honey (YH)			
1.	8.77	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	2.70
2.	11.62	DL-Arabinose	1.42
3.	13.66	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	16.24
4.	16.21	6-Acetyl-β-d-mannose	19.16
5.	16.90	5-Hydroxymethylfurfural	52.66
6.	17.27	Melezitose	1.89
7.	18.92	Maltose	4.30
8.	33.32	Melbiose	1.63
		Total	100.00
Kelulut Honey (KH)			
1.	8.09	Dihydroxyacetone	55.34
2.	8.81	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	8.11
3.	13.55	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	18.01
4.	16.25	6-Acetyl-β-d-mannose	5.67
5.	16.67	2-Deoxy-D-galactose	11.57
6.	32.55	D-Mannose	1.29
		Total	100.00

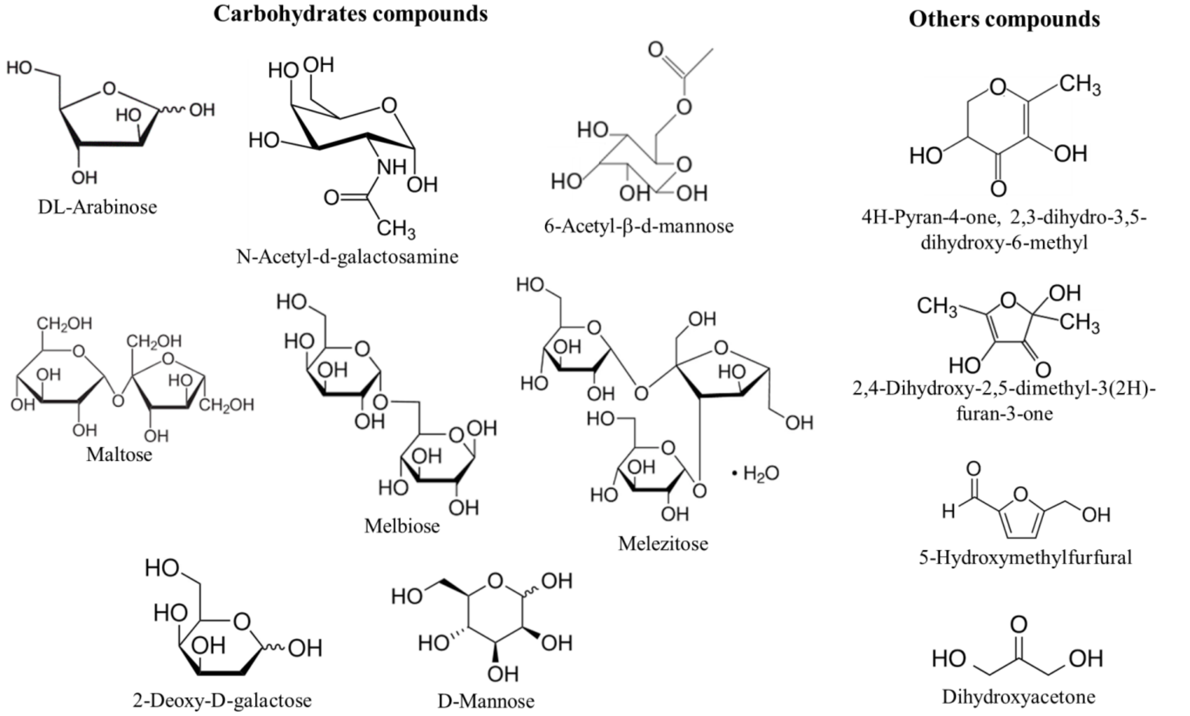

Figure 6. The carbohydrates and other compounds of three different types of honey: Black honey (BH), Yellow honey (YH), and Kelulut honey (KH), analyzed by GC-MS.

The carbohydrate content in honey is complex, with 70% being monosaccharides (primarily glucose and fructose) and 10-15% being disaccharides. The analysis also showed that YH contains melezitose (a trisaccharide) with a percent area of 1.89%. This finding is consistent with research conducted by De La Fuente, which reported that blossom honey contains a very small amount of melezitose.

In contrast, honeydew honey contains a relatively high amount of this compound [47, 48]. According to Kang and Yoo, the sugar composition in honey significantly affects its physicochemical properties, such as hygroscopicity, viscosity, and crystallization. The sugar content in honey is also greatly influenced by the type of honey, the source of nectar, the origin of the honey, and the environmental conditions (climatic conditions) [49, 50].

In this study, several types of sugars were identified in the various honey samples tested, with BH containing three types of sugars, YH containing five, and KH containing three. The percentage of sugars (carbohydrates) in the three types of honey is shown in **Figure 7**. The GC-MS analysis revealed that BH has the highest carbohydrate percentage, at 69.54%, followed by KH and YH, at 36.54% and 28.40%, respectively. Bentabol Manzanares et al. reported that the total carbohydrate content in blossom honey and honeydew honey is 73.89% and 78.45%, respectively. The total carbohydrate content found in this study is lower than that reported by Manzanares et al. [51]. Interestingly, BH contains N-Acetyl-D-galactosamine (GalNAc) with a relatively high percent area (6.97%), while YH and KH did not detect any amino sugars. GalNAc is an amino sugar derived from galactose. Nazia et al. noted that 11 aminoglycoside residues were detected in honey based on analyses using MIP-SPE and chromatography with tandem mass spectrometry [52].

The GC-MS analysis also showed that, in addition to carbohydrate compounds, there is another group of compounds (other compounds) detected in the three types of honey used, with YH having the highest percentage of other compounds at 71.60%, followed by KH and BH at 63.45% and 30.46%, respectively. The analysis also revealed that the compound pyranone (4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl) was detected in all types of honey, with varying percentages, with BH having the highest percentage at 24.94%. Interestingly, dihydroxyacetone was detected in KH honey with a very high percentage of 55.34%. Auguistine reported that fourteen chemical constituents have been identified by GC-MS in Cameroon honey, with the major chemical constituents were 3, 5-Dihydroxy-6-methyl-2, 3-dihydro-4H-pyran-4-one (8.91%); 2, 4-Dimethyl-1-pentanol (9.23%); 2-Butoxyethyl acetate (11.11%); and 2-Furancarboxaldehyde, 5-hydroxymethyl (36.02%). These substances are recognized to have some biological activities, including anti-infammatory, antifungal, antioxidant properties, and makeup wound healing power activity [53].

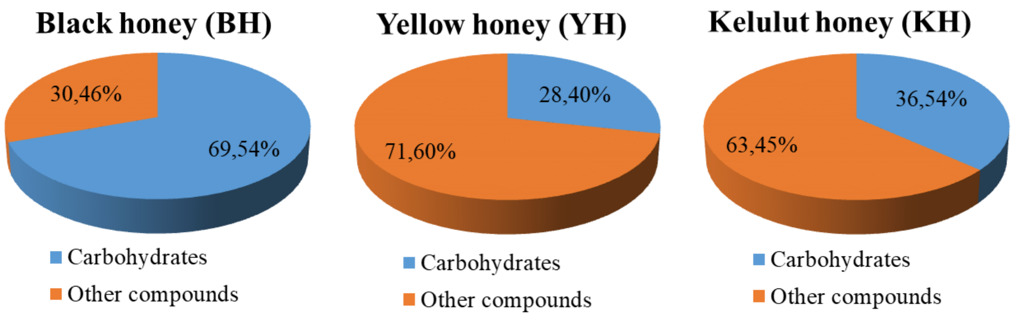

Figure 7. The percentage of carbohydrate compounds and other compounds in three different types of honey: Black honey (BH), Yellow honey (YH), and Kelulut honey (KH) analyzed by GC-MS.

Other compounds, such as furanone (2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one), were also detected in YH and KH honey with relatively high percentages of 16.24% and 18.01%, respectively. Alissandrakis et al. reported that phenylacetaldehyde (32.90%) and 3-hydroxy-4-phenyl-2-butanone (14.70%) are the main components in thyme honey [54]. Other research has identified flavonoids such as pinobanksin, pinocembrin, and chrysin as the main components in Manuka honey. Other compounds such as luteolin, quercetin, 8-methoxykaempferol, isorhamnetin, kaempferol, and galangin were also identified in Manuka honey [55].

CONCLUSION

The antimicrobial activity showed that Black Honey (BH) exhibited the strongest antibacterial effect compared with Kelulut Honey (KH) and Yellow Honey (YH) against wound-infecting bacteria; i.e. two Gram-positive (*Staphylococcus aureus*, and *Staphylococcus hominis*) and two Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacterial strains with the zone of inhibitions in the range between of 15.52±0.86 mm and 18.15±0.73 mm. The GC-MS analysis showed that Black Honey (BH) had a largest amount of pyranone and carbohydrate compounds with the percentage area of 24.94% and 69.54% respectively. The superior antibacterial activity of Black Honey (BH) is correlated with its higher pyranone and carbohydrate compounds.

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