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Research Article

Tentative Identification of Compounds, Antioxidant, and Antimicrobial Activity of the Edible Part of *Benincasa hispida* L. Fruit (Cucurbitaceae)

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Abstract

The edible part of *Benicasa hispida* (Thunb.) Cogn. fruit is traditionally used in Southeast Sulawesi to treat high blood pressure, typhoid fever, and body cooling. The present study evaluated the chemical compounds present in the 80% ethanol of the edible part of the plant using phytochemical screening and an LC-MS analysis, antioxidant activity based on assays on total phenolics content (TPC), total flavonoids content (TFC), and DPPH, and antimicrobial activity towards *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Phytochemical screening revealed the presence of tannins, flavonoids, terpenoids, steroids, and saponins in the extract. As many as eighteen compounds (**1-18**) were tentatively identified in the extract, including sugars, a simple phenolic, a tricarboxylic acid, a peptide, flavonoids, quinic acid derivatives, phytosterols, triterpenoids, and saponins. The extract exhibited remarkable antioxidant activity with an SC_{50} value of 23.4 µg/mL, although its TPC (1.1±0.1 mg GAE/g extract) and TFC (1.0±0.1 mg QE/g extract) values were considered in low amounts. The extract was found inactive to inhibit the microbial growths of all tested microbes. However, raffinose (**3**) present in the extract might be beneficial as a prebiotic to promote a healthy human gut. The study concludes that the 80% ethanol extract of the edible part of *B. hispida* fruit could be used to develop natural antioxidant agents and nutraceuticals.

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INTRODUCTION

Benincasa hispida (Thunb.) Cogn. is a herbaceous climber that belongs to the Cucurbitaceae family. It is one of the Cucurbitaceae crops known as wax gourd due to its white wax-covered fruit. The fruit's edible part (mesocarp) is white, spongy, and succulent. This part is the main part of the plant used by natives in making food[1]. In some countries like Malaysia, India, and China, the plant is traditionally used to treat coronary diseases, gastrointestinal tract problems, urinary tract and kidney stones problems, metabolism ailments, constipation, fever, diabetes, and obesity[2,3]. In Southeast Sulawesi, the plant is known by different vernacular names by locals, such as *konduru* (Bombana, Konawe, Baubau, Muna, Kolaka), *kundur* (Kendari), and *sudeng* (Bugis, Kolaka). Traditionally, the edible part of *B. hispida* fruit is processed as soup or juice and given orally to treat high blood pressure[4,5], typhoid fever[6], and for cooling the body. For daily food consumption, this part is processed as vegetable soups like *sayur bening* (clear soup) and *sayur santan* (coconut soup). Hence, based on medicinal uses and functional nutrition, the fruit of *B. hispida* is easy to find in local markets.

Despite its traditional uses, studies on chemical compounds and biological activities of *B. hispida* from Southeast Sulawesi are still a handful, which only one report on antibacterial activity[7]. Hence, the current status of *B. hispida* emerges for further investigation. The present study evaluated the chemical compounds of the 80% ethanol extract of edible part of *B. hispida*

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fruit using phytochemical screenings and an LC-MS analysis, antioxidant activity based on assays on total phenolics content (TPC), total flavonoids content (TFC), and DPPH, as well as antimicrobial activity towards *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*.

MATERIALS AND METHODS

Materials

Chemicals

Ethanol (cat. no: 1.00983), HPLC grade of acetonitrile (cat. no: 1.00030), Folin-Ciocalteu (FC) reagent (cat. no: 1.09001), ascorbic acid (cat. no: 1.00468), dimethyl sulfoxide (DMSO, cat. no: 1.02952), sodium bicarbonate (cat. no: 1.06239), aluminum sheet thin layer chromatography (TLC) plate (silica gel 60 GF254, 0.25 mm, 20 × 20 mm, cat. no: 1.05554), Mueller-Hinton agar (MHA, cat. no: 1.05437), and potato dextrose agar (PDA, cat. no: 1.10130) were obtained from Merck (Darmstadt, Germany). Gallic acid (cat. no: G7384) dan quercetin (cat. no: G4951) were obtained from Sigma-Aldrich (St. Louis, US). Meanwhile, aluminum chloride hexahydrate (cat. no: 898) was purchased from Loba Chemie (Mumbai, India), and 2,2-diphenyl-1-picrylhydrazyl (DPPH, cat. no: MB263) was purchased from HiMedia (Mumbai, India). Deionized water (Waterone) was purchased from OneMed (Jakarta, Indonesia). Chloramphenicol and ketoconazole were purchased from Kimia Farma (Jakarta, Indonesia).

Microbial cultures

Microbial cultures used were *S. typhi* ATCC 14028, *E. coli* ATCC 35218, *S. aureus* ATCC 25923, and *C. albicans* ATCC 10231, which represent Gram-negative bacteria, Gram-positive bacteria, and fungi, respectively.

Plant sample

Fruits of *B. hispida* were freshly collected from local markets in Kolaka regency, Southeast Sulawesi, Indonesia, in February 2022. The sample was taxonomically authenticated using a literature1, and its voucher specimen (number BH001) was deposited at the Laboratorium Terpadu USN Kolaka. The morphology of the fruit is displayed in **Figure 1**.

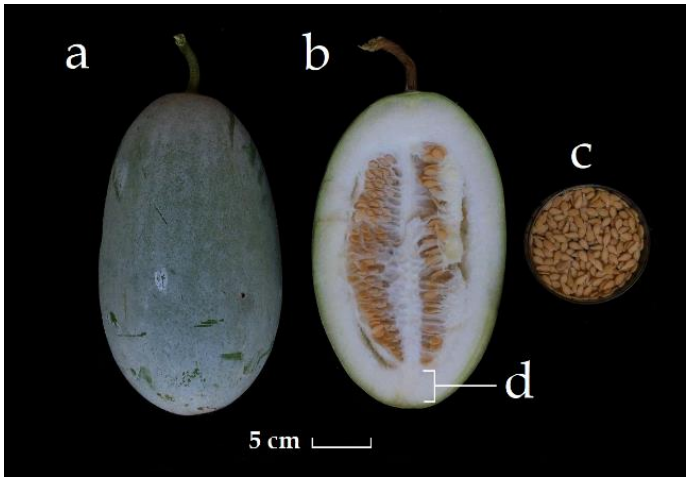


Figure 1. Fruits of *B. hispida* (**a**), cross-sectional of the fruit (**b**), seeds (**c**), and the edible part (**d**).

Methods

Sample preparation

A sample of the fruit was washed using tap water and drained. The edible part (**Figure 1d**) was separated from the skin and seeds by cutting. This part (26.514 kg) was dried at an optimized temperature of 50°C for three days using an oven (Maksindo, Indonesia). The dried edible part was coarsely powdered using a dry blender and yielded coarse powder (0.822 kg) with a percentage yield of 3.1% and a water content of 96.9%. The coarse powder was then kept in a zip lock pouch until use.

Sample extraction

The coarse powder of the edible part of *B. hispida* fruit (0.274 kg) was macerated using 80% ethanol as the solvent. Maceration of the sample was executed for 3 × 48 hours at room temperature. The 80% ethanol extract was obtained after manual filtration, and the solvent was evaporated using a vacuum rotary evaporator (Biobase, China). Then, the extract was weighed and stored in a refrigerator at 4°C until use.

Phytochemical screening

Phytochemical screenings were performed according to a previous work[8] to detect the presence of alkaloids, tannins, flavonoids, terpenoids, steroids, and saponins in the 80% ethanol extract of the edible part of *B. hispida* fruit. The qualitative observation was also made based on results on color intensities for alkaloids (reddish orange), tannins (brownish green/dark blue), flavonoids (red), terpenoids (reddish brown), steroids (fluorescence greenish-yellow), and the formation of foam for saponins with the scale of high (+++), fair (++), and low (+) of their presence in the extract.

LC-MS/MS analysis

Chemical compounds in the 80% ethanol extract were evaluated using a UPLC aligned with an MS analyzer (Waters Xevo Tandem Quadrupole (TQD) Mass Spectrometer, Waters, Ireland) according to our previous method[9] with some adaptations. Separation of the compounds was performed using a reverse phase UPLC BEH C18 column (2.1 × 50 mm, particles 1.7 µm) and gradiently eluted with water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The elution was performed for 20 minutes with a flow rate of 0.4 mL/minute (**Table I**). The extract was dissolved in ethanol (90%) and filtered using a Millex membrane filter of 0.22 µm. The injection volume was 5 µL. For mass analysis, a positive mode electrospray ionization (ESI) was performed using the following parameters: mass range of m/z 50 to m/z 1200, cone voltage 30 V, capillary voltage 3.0 kV, and source temperature 500°C. The m/z values of separated peaks and their fragmentations were analyzed using online mass databases (PubChem, ChemSpider, CheBI, KEGG, mzCloud, and Mass Bank). The identification of tentative compounds as natural products was cross-checked using the Dictionary of Natural Products and LOTUS (Natural Products Online).

Table I. Gradient elution

Time	%A (water-0.1% formic acid)	%A (acetonitrile-0.1% formic acid)
0 minute	95	5
15 minutes	0	100
5 minutes	95	5

Total phenolics content (TPC) and total flavonoid content (TFC) assays

Both TPC and TFC values in the 80% ethanol extract were spectrophotometrically evaluated using Folin-Ciocalteu and aluminum chloride methods, respectively, according to previous reports[9,10], with some modifications. The 80% ethanol extract was diluted in ethanol (96%), and the final concentration in the reaction mixture was 1000 µg/mL. For TPC, 20 µL of diluted extract in a 96-well microplate was added with 100 µL of Folin-Ciocalteu (10%, v/v, in water) and incubated for 5 minutes at room temperature. Then, the mixture was added with 80 µL of sodium bicarbonate (7.5%, w/v, in water) and re-incubated for 30 minutes at room temperature in the dark. The absorbance of the reaction mixture was read at 765 nm using a microplate reader (Spectrostar Nano, BMG Labtech, Germany). Meanwhile, for TFC, the reaction mixture was 100 µL of diluted extract and µL of aluminum chloride (2%, w/v, in water). The mixture was incubated for 15 minutes, and its absorbance was read at 435 nm using a microplate reader. Both TPC and TFC experiments were repeated three times. Concentrations of phenolics and flavonoids in the diluted extract were calculated using equations from their respective linear standard curves, that is, a gallic acid standard curve (absorbance = (0.083 × concentration of gallic acid in µg/mL) + 0.2006, $r^2 = 0.990$) for TPC and a quercetin standard curve (absorbance = (0.0459 × concentration of quercetin in µg/mL) + 0.0189, $r^2 = 0.999$) for TFC. Both curves were established by plotting the absorbance versus concentrations ranging from 1 to 31.2 µg/mL. Then, TPC (mg GAE/g of extract) and TFC (mg QE/g of extract) values were calculated using the **Equation 1**, where C was the concentration of gallic acid or quercetin plotted using the linear standard curves, FV was the final volume used for dilution, d was the dilution factor, and W was the weight of 80% ethanol extract.

$$mgGAE\ or\ mg\ QE: \frac{(C \times FV \times d)}{W} \qquad \textbf{[1]}$$

DPPH assay

The radical scavenging activity of the 80% ethanol extract was evaluated using qualitative and quantitative DPPH assays according to the previous methods[9,10] with slight modifications. For qualitative analysis, five serial solutions of the extract in 96% ethanol were spotted at 20 µL on the TLC plate, then dipping the plate into a DPPH solution (0.4 mM in 96% ethanol) for 10 seconds. After drying with an air dryer, the plate was incubated for 30 minutes in the dark at room temperature. The white zone formed around the spot against the purple DPPH on the plate, indicating radical scavenging activity. The final sample concentrations in the plate ranged from 12.5 to 200 µg/spot. Ascorbic acid was used as the positive control of the assay. For quantitative analysis, the reaction mixture consisted of 100 µL of extract solution (0.8 to 100 µg/mL, in 96% ethanol) and 100 µL of DPPH solution (40 µg/mL, in 96% ethanol) was incubated for 15 minutes in the dark at room temperature. Then, the reaction mixture absorbance was read at 515 nm. The percentage of radical scavenging activity (%RSA) was calculated using the **Equation 2**. The absorbance of the sample was corrected using the sample blank containing sample and ethanol. The 50% radical scavenging concentration (SC_{50}) values of the extract and positive controls (ascorbic acid, gallic acid, and quercetin) were obtained from a nonlinear regression (curve fit) using GraphPad Prism 5.

$$\%RSA: \frac{(Absorbance\ of\ DPPH - Absorbance\ of\ sample)}{Absorbance\ of\ DPPH} \times 100\% \qquad \textbf{[2]}$$

Antimicrobial assay

The antimicrobial activity of the 80% ethanol extract was evaluated using an agar well diffusion method according to the previous studies[11,12]. The extract solution was made in DMSO-normal saline (1-2%). The activity was first screened at a high concentration of 10,000 µg/well. Chloramphenicol (20 µg/well) and ketoconazole (0.5 µg/well) were used as the positive controls of the assay for bacteria and fungi, respectively. The surface of solidified media MHA or PDA in a Petri dish was inoculated with 1 mL of microbial culture (0.5 McFarland). Wells for extract, positive control, and solvent control were prepared by aseptically punching the inoculated media using yellow tips and discarding the tips. After that, samples were added to the wells accordingly (40 µL/well), and the Petri dish was incubated at 37 °C for 24 hours for bacteria and 48 hours for fungi. The observation of a clear zone around the well was done two times at 18 and 24 hours for bacteria and 24 and 48 hours for fungi. The diameter of the clear zone as the inhibition zone indicated the antimicrobial activity.

Data analysis

Data from TPC, TFC, DPPH, and antimicrobial assays were obtained from three repeated experiments (n = 3) and presented as mean ± standard deviation (SD). SC_{50} values were determined using GraphPad Prism 5 (GraphPad Inc., California, US).

RESULTS AND DISCUSSION

Yield of extraction and phytochemical screening

The maceration of the edible part of *B. hispida* fruit using 80% ethanol as the solvent yielded a reddish-brown extract with a percentage yield of 17.3% (47.49 g). Phytochemical screenings of this extract showed the occurrence of tannins, flavonoids, terpenoids, steroids, and saponins (**Table II**). Meanwhile, alkaloids were not detected, possibly due to either their absence or low amounts in the extract since previous studies have reported the presence of pyrazine derivatives and volatile nitrogen-containing compounds in *B. hispida* fruit[13]. Additionally, considering the polarity of the solvent used, these compounds might not be extracted during extraction. On the contrary, terpenoids were qualitatively present in high amounts by observing an intense reddish-brown layer during the experiment, suggesting the presence of polar terpenoids in the extract.

Table II. Phytochemical groups detected in the 80% ethanol extract of edible part of *B. hispida* fruit

Phytochemical group	Detection	Qualitative observation
Alkaloid	Absent	-
Tannin	Present	+
Flavonoid	Present	+
Terpenoid	Present	+++
Steroid	Present	+
Saponin	Present	+

Tentative identification of compounds

The LC-MS/MS analysis of the 80% ethanol extract of the edible part of *B. hispida* fruit yielded variable peaks of compounds in the total ion mass chromatogram starting from the retention time (T_R) of 0.52 to 16.20 minutes (**Figure 2**). Some major peaks are also observed based on their peak height, such as at the retention time of 0.52, 9.52, 9.71, and 14.19 minutes. The analysis of molecular ion and fragmentation patterns of the separated peaks resulted in the tentative identification of 18 compounds, including sugars (**1, 3**), a simple phenolic (**2**), a tricarboxylic acid (**4**), a peptide (**5**), flavonoids (**6, 7, 8**), quinic acid derivatives (**9, 10**), phytosterols (**11, 12, 13**), triterpenoids (**14, 18**), and saponins (**15, 16, 17**). These compounds are listed in **Table III**, and their structures are displayed in **Figure 3**.

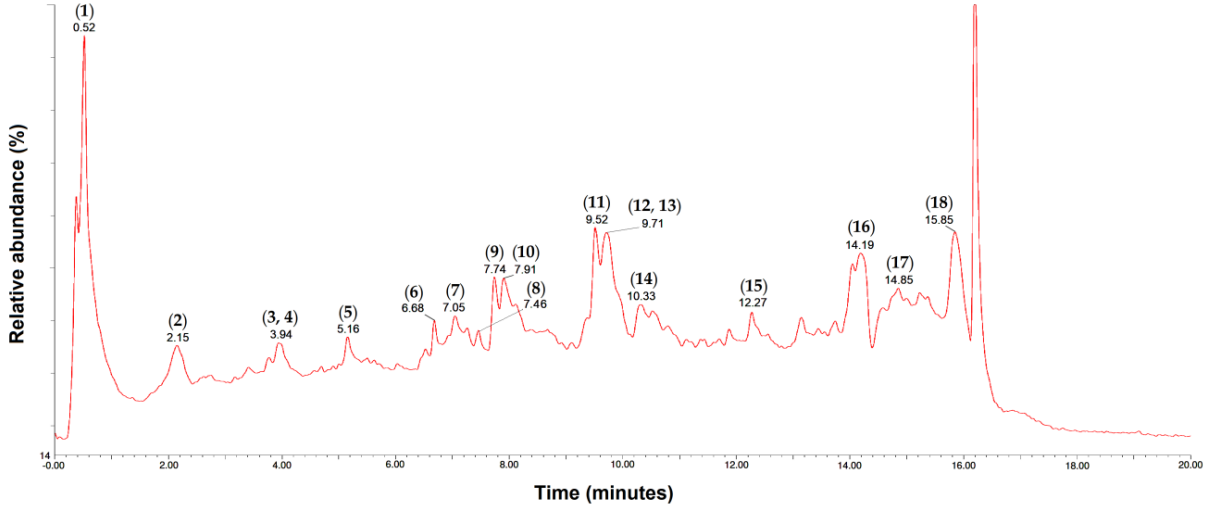


Figure 2. Total ion chromatogram (TIC) of compounds in the 80% ethanol extract of edible part of *B. hispida* fruit.

Table III. Tentative compounds identified in the 80% ethanol extract of edible part of *B. hispida* fruit

T_R (min)	ES(+)	Ion type	MS/MS; m/z (% base peak)	Tentative compound name	Structure number	Formula	MW
0.52	381.69	$[M+K]^+$	291, 148, 118, 104 (100)	Sucrose	**1**	$C_{12}H_{22}O_{11}$	342.29
2.15	171.44	$[M+H]^+$	171 (100)	Gallic acid	**2**	$C_7H_6O_5$	170.11
3.94	622.10	$[M+4H_2O+2Na]^+$	522 (20), 504	Raffinose	**3**	$C_{18}H_{32}O_{16}$	504.43
3.94	229.54	$[M+2H_2O]^+$	193 (60)	Citric acid	**4**	$C_6H_8O_7$	192.12
5.16	532.05	$[M+H]^+$	309 (40), 225	3-*S*-glutathione-dihydrosinapic acid	**5**	$C_{21}H_{29}N_3O_{11}S$	531.53
6.68	291.71	$[M+H]^+$	261 (100), 167	Catechin	**6**	$C_{15}H_{14}O_6$	290.26
7.05	478.99	$[M+2H]^+$	381, 327, 311 (100), 301, 229, 208, 167	Chrysoeriol-7-*O*-glucuronide	**7**	$C_{22}H_{20}O_{12}$	476.38
7.46	480.99	$[M+H]^+$	301 (100), 229, 208, 167	Myricetin-3-O-glucoside	**8**	$C_{21}H_{20}O_{13}$	480.37
7.74	339.67	$[M+H]^+$	323 (100) , 241, 218, 167	3-*p*-coumaroylquinic acid	**9**	$C_{16}H_{18}O_8$	338.30
7.91	427.79	$[M+H]^+$	411, 339, 323 (100), 241, 218, 167	4-hydroxybutyl-chlorogenate	**10**	$C_{20}H_{26}O_{10}$	426.41
9.52	409.91	$[M+H]^+$	343, 327 (100), 313, 273, 241, 213	Stigmasta-4,22*E*,25-trien-3-one	**11**	$C_{29}H_{44}O$	408.65
9.71	410.95	$[M]^+$	343, 327 (100), 313, 273, 241, 213	5-dehydroavenasterol	**12**	$C_{29}H_{46}O$	410.67
				Stigmasta-7,22*E*,25-trien-3β-ol	**13**	$C_{29}H_{46}O$	410.67
10.33	443.79	$[M+H]^+$	427 (40), 327, 233, 208	Cucurbita-5,23-diene-3β,25-diol	**14**	$C_{30}H_{50}O_2$	442.72
12.27	576.76	$[M]^+$	469, 377, 337, 279 (10), 208, 152	Daucosterol	**15**	$C_{35}H_{60}O_6$	576.84
14.19	1023.76	$[M+H+Na]^+$	815, 798, 697, 664, 614, 566, 464, 436 (20), 338, 327, 284, 217, 152	Oleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1→4)-(1→3)-*a*-L-rhamnopyranosyl-(1→2)-*a*-L-arabinopyranoside	**16**	$C_{51}H_{82}O_{19}$	999.18
14.85	1059.78	$[M+H_2O]^+$	815, 798, 686, 664, 588, 566, 464, 436, 381, 181, 152	3β-O-acetyloleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1→4)-(1→3)-*a*-L-rhamnopyranosyl-(1→2)-*a*-L-arabinopyranoside	**17**	$C_{53}H_{84}O_{20}$	1041.22
15.85	571.10	$[M+H]^+$	554 (100), 532, 441, 338, 152	25-O-acetyl-16,20-dihydroxy-3-methoxy-cucurbita-5(10),6,23-triene-3,11,22-trione	**18**	$C_{33}H_{46}O_8$	570.71

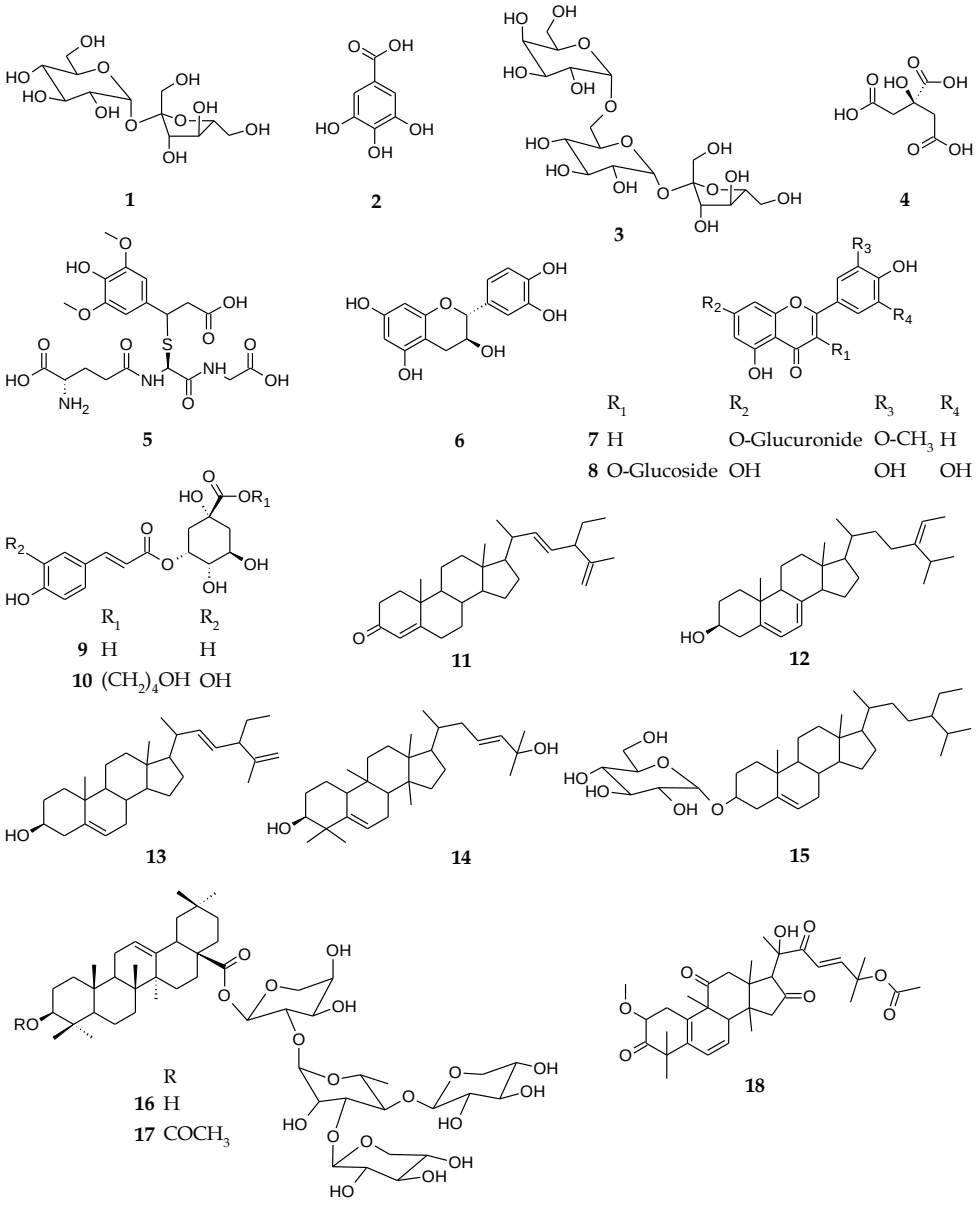


Figure 3. Structure of tentative compounds (**1**-**18**) in the 80% ethanol extract of edible part of *B. hispida* fruit.

The presence of sugars (**1, 3**) in the 80% ethanol extract of the edible part of *B. hispida* fruit validated its nutritional components. The LC-MS/MS spectrum (**Figure 2**) showed a major peak at a retention time of 0.52 minutes, having a sodium adduct ion at m/z 381.69 $[M+Na]^+$. Fragment ions of this peak at m/z 118 and 104 indicated the pattern of disaccharides[14], for which sucrose (**1**) is tentatively assigned for the peak. A recent study has reported sucrose (**1**) as a component for the taste and nutrition of *B. hispida* fruit, along with glucose and fructose. This study shows that sucrose (**1**) composition decreased with fruit development while glucose and fructose increased[15]. In line with our present study, the fruit sample used was unripened at the age of approximately four months. Hence sucrose (**1**) is detected as a major compound in the extract, while glucose and fructose are undetectable. Another sugar was found at the retention time of 3.94 minutes and had an adduct ion of $[M+4H_2O+2Na]^+$ at m/z 622.10, followed by fragment ions at m/z 522 $[M+H_2O]^+$, m/z 504 $[M]^+$ and m/z 143 as the base peak. This pattern is typical of trisaccharides[14], which based on previous studies[16,17], raffinose (**3**) is tentatively identified. The family of Cucurbitaceae has been reported to produce oligosaccharides, mainly raffinose (**3**) and stachyose. Both of them are galactosyl-sucrose which plays a role as transport sugars in plants of this family[17]. Related to biological activity, polysaccharides from *B. hispida* fruit showed an antiglycation effect and antioxidant activity, which benefit for reducing glucose levels in diabetic patients[18]. Hence, more studies on the antidiabetic activity of *B. hispida* fruit are a noble challenge.

In addition to sugars, organic acids contribute to the taste and nutrition of the *B. hispida* fruit such as citric acid (**4**) and malic acid[15]. The peak of citric acid (**4**) was observed at the retention time of 3.94 minutes, overlapping with raffinose (**3**) and having a base peak of m/z 193 for a protonated molecular ion $[M+H]^+$. The citric acid (**4**) and malic acid are organic acids accumulated in the Cucurbitaceae family during fruit development. Their contents are higher in the young fruits stage and gradually decrease during maturity. The antimicrobial activity of this compound (**4**) has been reported[19].

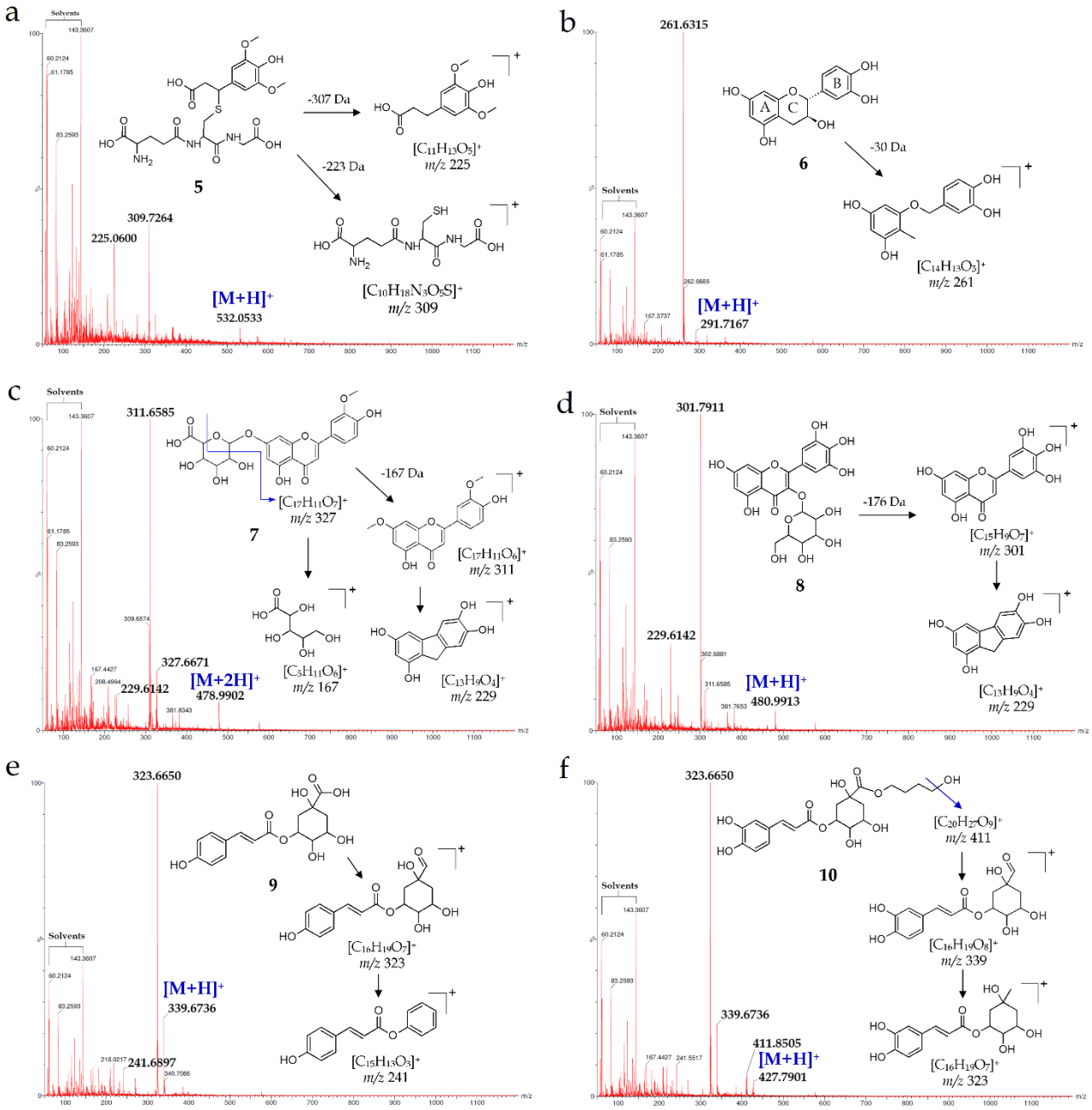
As part of the nutrition components in the *B. hispida* fruit, a peptide was also detected at a retention time of 5.16 minutes with a protonated molecular ion at m/z 532.05 $[M+H]^+$. Two fragment ions were observed at m/z 309 for a protonated glutathione and m/z 225 for a protonated sinapic acid (**Figure 4a**). The fragmentation is similar to those reported in the literature[20]. Hence, this compound was tentatively assigned as 3-S-glutathione-dihydrosinapic acid (**5**), and its presence as a natural product is first reported in the present study. The glutathione side in compound (**5**) might be a potential for antioxidant activity. Apart from the finding, a common peptide found in the Cucurbitaceae family is known as citrulline and has also been reported in the fruit of *B. hispida*[15]. However, this peptide was not detected in this study's 80% ethanol extract.

Some phenolics, including flavonoids also detected in the 80% ethanol extract of the edible part of *B. hispida* fruit. Gallic acid (**2**) was assigned to a peak at the retention time of 2.15 minutes, having a protonated molecular ion $[M+H]^+$ at m/z 171.44[21]. Furthermore, flavonoids (**6, 7, 8**) in the 80% ethanol extract were consecutively observed at the retention time of 5.16, 6.68, and 7.05 minutes, having molecular ions at m/z 291.71, m/z 478.99, and m/z 480.99, respectively. The molecular ion at m/z 291.71 was a protonated ion $[M+H]^+$, which is the base peak at m/z 261, showing a prominent fragment of catechin (**6**) after a loss of its hydroxyl group (3-OH) attached to the ring C of the structure and fission of that ring (**Figure 4b**). On the other hand, the molecular ion at m/z 478.99 was predicted as chrysoeriol-7-O-glucuronide (**7**) based on its base peak at m/z 311 after the elimination of a glucuronide group (167 Da). The presence of this group in the structure was further confirmed by a fragment ion at m/z 167 in the spectrum (**Figure 4c**). Furthermore, the molecular ion at m/z 480.99 had a base peak at m/z 301, indicating the fragment ion of myricetin after the elimination of the O-glucoside group (179 Da), for which myricetin-3-O-glucoside (**8**) is tentatively assigned for this peak (**Figure 4d**). Both compounds **7** and **8** had a fragment ion at m/z 229, which is predicted as a structural arrangement of flavone and flavonol skeletons, especially those that have 5, 7, 3', and 4'-hydroxy substitution, such as apigenin, myricetin, and quercetin. Previous studies have reported the occurrence of gallic acid (**2**) in the Cucurbitaceae family[22] and *B. hispida* from Malaysia[23]. Similarly, catechin (**6**), along with gallic acid (**2**), is also present in the *B. hispida* fresh juices from Romanian[24] and has been isolated from the dried flesh of *B. hispida* from Hainan, China[25]. As far as our search, compounds **7** and **8** have yet to be reported from *B. hispida*. However, various flavones and flavonols, as well as their glycosides, have recently been reported from the pericarp of *B. hispida* fruit also from China[26], which supported the findings of the present study.

In addition to flavonoids, two quinic acid derivatives (**9** and **10**) peaks were consecutively observed at the retention time of 7.74 and 7.91 minutes, respectively. The molecular ion of the first peak was at m/z 339.67, with a base peak at m/z 323 after a loss of a hydroxyl group. Further fragment ions of this peak were at m/z 241, 218, and 167, showing a pattern of 3-p-coumaroylquinic acid (**9**) (**Figure 4e**). This compound has recently been reported from the seed of *Cucumis sativus*[27]. Compound **10** was predicted to have a skeleton as in compound **9** since some fragmentation patterns are similar. The LC-MS/MS spectrum showed additional fragment ions at m/z 427 as a protonated molecular ion $[M+H]^+$ and m/z 411 for a loss of one hydroxyl group. The ion at m/z 411 has been reported for butyl chlorogenate[28], which further fragment ion at m/z 339 indicated an elimination of a butoxy side ($C_4H_8O^+$, 72 Da) from the main skeleton of chlorogenate (**Figure 4f**). Hence, the compound was tentatively identified as 4-hydroxybutyl-chlorogenate (**10**). The occurrence of chlorogenic acid has been reported in the Cucurbitaceae family[22]. However, to the best of our knowledge, the occurrence of 4-hydroxybutyl-chlorogenate (**10**) in the family has yet to be reported.

Two cucurbitanes (**14**, **18**) were observed in the LC-MS/MS spectrum at the retention time of 10.33 and 15.85 minutes, respectively. Compound **14** had a protonated molecular ion $[M+H]^+$ at m/z 443.79 with fragment ions at m/z 427 for a loss of methyl group attached to the side chain and m/z 327 for successive cleavage of that chain (**Figure 4g**). Further fragmentation of this compound (**Table II**) tentatively deduced it as cucurbita-5,23-diene-3β,25-diol (**14**), which has been reported from the seeds of *Sicana odorifera* (Cucurbitaceae)[29]. Compound **18** was suggested to have a protonated molecular ion $[M+H]^+$ at m/z 571.10 with a base peak at m/z 554, indicating a loss of a methyl group from the 3-methoxy group in ring A of the proposed structure. The fragment ion at m/z 441 also suggested the compound to have a cucurbita-5(10),6,23-

triene-3,25-diol 3β-form skeleton[30]. Hence, the compound was tentatively deduced as 25-O-acetyl-16,20-dihydroxy-3-methoxy-cucurbita-5(10),6,23-triene-3,11,12-trione (**18**). The plausible MS/MS fragmentation of compound **18** is displayed in **Figure 4h**.



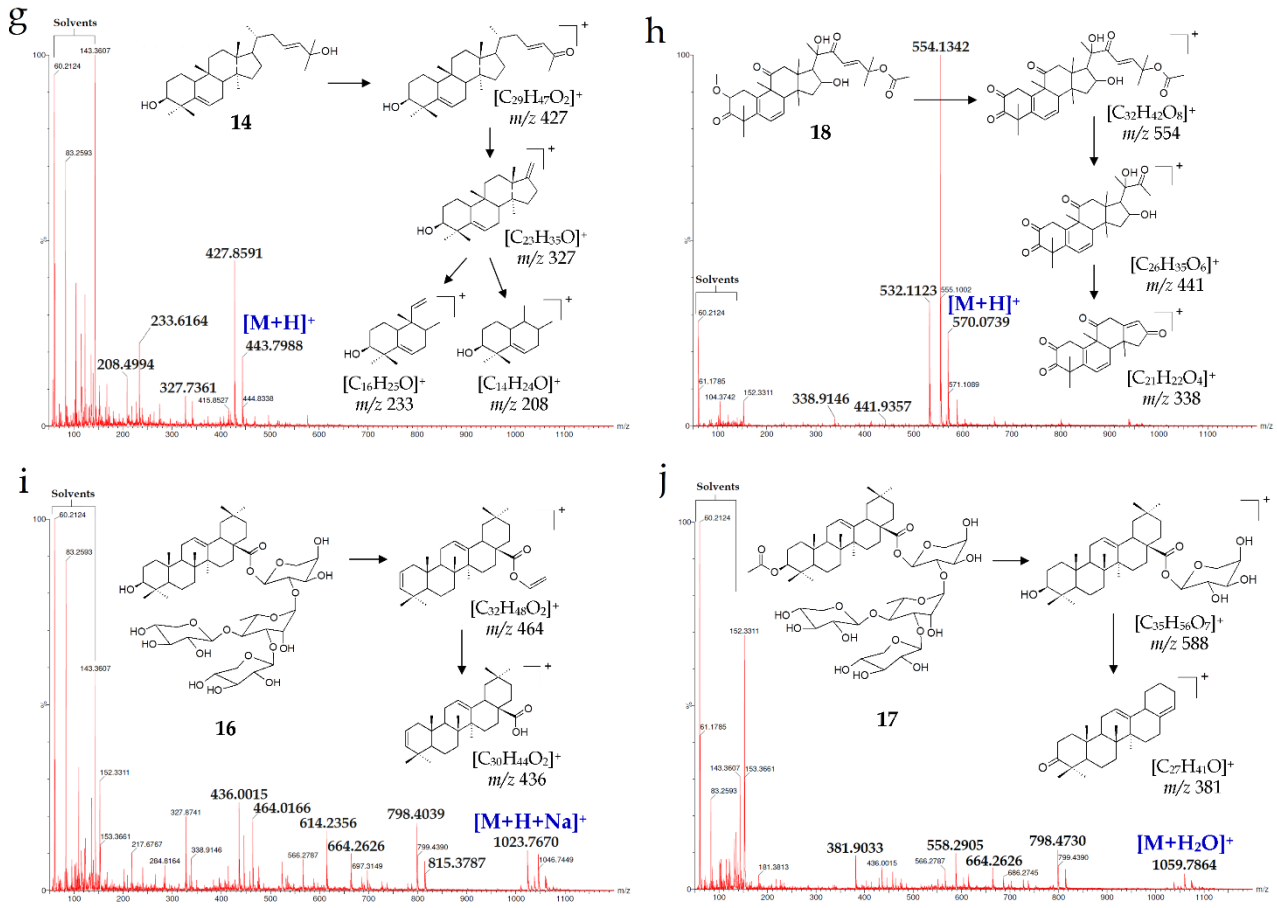


Figure 4. The plausible MS/MS fragmentation of compounds **5-10**, **14**, and **16- 18**.

Two peaks of oleananes with sugar moieties (**16**, **17**) were observed in the LC-MS/MS spectrum (**Figure 2**). The first peak appeared at the retention time of 14.19 minutes and suggested having a protonated and a sodium adduct ion $[M+H+Na]^+$ at m/z 1023.76. The fragment ions at m/z 815, 798, and 697 indicated the elimination of two xylopyranoses (2 x $C_5H_9O_5$, 298 Da) from the compound, followed by fragment ions at m/z 664, 614, and 464 for losing rhamnosyl and arabinopyranosyl sides from the oleanane skeleton. In addition, the fragment ion at m/z 436 indicated the oleanolic acid after losing its hydroxyl functionality at C-3 (**Figure 4i**). The compound was then tentatively deduced as oleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1→4)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**16**), which has recently reported from the 70% ethanol extract of the sun-dried fruits of *B. hispida*[31]. Another peak was observed at the retention of 14.85 minutes and suggested to have a molecular ion with water adduct $[M+H_2O]^+$ at m/z 1059.78. Some fragmentations (m/z 798, 664, and 436) of this molecular ion were similar to those in compound **16**, while fragment ion at m/z 588 indicated the eliminations of three sugar moieties (xylopyranosyl-xylopyranosyl-rhamnosyl; $C_{16}H_{27}O_{12}$, 411 Da) and an acetyl group (43 Da) (**Figure 4j**). Hence, compound **17** was suggested as a derivative of compound **16**, for which 3β-O-acetyloleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1→4)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**17**) was tentatively assigned for the peak. Further isolation and more spectroscopic measurements are promising works to reveal the exact structure of this compound.

Total phenolic content, total flavonoid content, and antioxidant activity

Phenolics and flavonoids have been correlated to antioxidant activity in plants and foods[32]. The 80% ethanol extract of the edible part of *B. hispida* fruit contained low amounts of phenolics and flavonoids (**Table IV**). Despite low amounts of phenolics and flavonoids, the extract showed promising antioxidant activity towards DPPH radicals assayed qualitatively on a TLC plate (**Figure 5a**) and quantitatively using spectrophotometric measurement (**Figure 5b**) with an SC_{50} value of 23.4 µg/mL. Studies have shown that good antioxidant activities do not always define by the high amount of phenolics and flavonoids[9,10] but might be affected by the interactions among them either to promote synergistic effects or to result in

antagonistic effects[33]. Three compounds (**2**, **6**, **8**) identified in the extract have been reported to exhibit potent antioxidant activities[34-36]. Meanwhile, according to our search, the antioxidant activity of compound **7** (a flavonoid) has not yet been reported. The interactions of these compounds might promote synergistic effects, which resulted in the DPPH radical scavenging activity in the extract. The activity was compared to ascorbic acid, gallic acid, and quercetin as the positive controls. The activity of the edible part of the fruit was also considered more potent when compared with the seed[37]. Hence, this edible part could be used as a good source of natural antioxidants for nutraceutical and pharmaceutical developments.

Table IV. TPC, TPC, and DPPH radical scavenging activity (values as mean ± SD, n = 3)

Sample	TPC (mg GAE/g of extract)	TFC (mg QE/g of extract)	% RSA (at 100 µg/mL)	SC_{50} (µg/mL)
80% ethanol extract	1.1 ± 0.1	1.0 ± 0.1	92.5 ± 0.7	23.4
Ascorbic acid	-	-	99.6 ± 2.2	2.8
Gallic acid	-	-	97.1 ± 0.9	3.8
Quercetin	-	-	99.5 ± 2.4	9.5

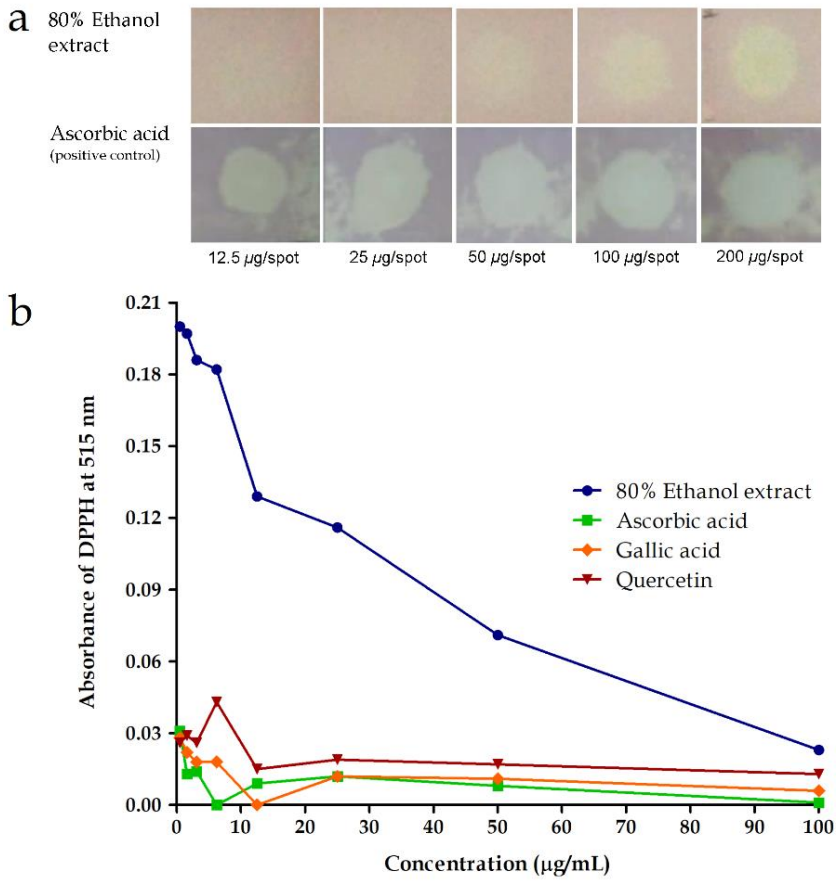


Figure 5. Qualitative (**a**) and quantitative (**b**) DPPH radical scavenging activity of 80% ethanol extract of the edible part of *B. hispida* fruit.

Antimicrobial activity

Some studies have shown the antimicrobial activity of the *B. hispida* fruit and its seeds toward bacteria[37,38]. From these studies, it is concluded that *B. hispida* displayed antibacterial activity at high doses. However, in the present study, a high dose (10,000 µg/well) of the 80% ethanol extract of the edible part of *B. hispida* fruit was found not to inhibit all the tested microbes, including the fungus *C. albicans* (**Table V**). Meanwhile, chloramphenicol and ketoconazole, as the positive controls, were consistently active in inhibiting the growths of the tested bacteria and fungus, respectively. Previous studies reported the antibacterial activity of *B. hispida* fruit and seed using water and 95% ethanol as solvents in the extraction[37,38]. The choice of solvents might affect the extraction of active compounds from the sample. In addition, the sugars (**1, 3**) might promote microbial growth as they act as nutrients. Thus, their presence might hinder the growth inhibition activity by active compounds in the extract. However, in the human gut, these sugars, especially raffinose (**3**), can act as a prebiotic that

promotes the activity and growth of good gut bacteria and reduce the abundance of pathogenic bacteria[39]. It might explain the traditional use of *B. hispida* fruit soup to treat typhoid fever. However, concerning the previous studies, further research is still needed to explore the antimicrobial compounds in *B. hispida*. For example, gallic acid (**2**), citric acid (**4**), and catechin (**6**) have been reported for antimicrobial activity[18,40]. However, considering the low amounts of phenolics and flavonoids in the extract, their activity might not be detectable.

Table V. Antimicrobial activity (values as mean ± SD, n = 3)

Sample	Concentration (µg/well)	Inhibition zone (mm)							
		S. typhi		*E. coli*		*S. aureus*		*C. albicans*	
		18 h	24 h	18 h	24 h	18 h	24 h	24 h	48 h
80% ethanol extract	10.000	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Chloramphenicol	20	19.77 ± 2.6	18.79 ± 2.7	24.99 ± 0.9	25.50 ± 1.4	18.97 ± 1.5	19.06 ± 1.5	-	-
Ketoconazole	0.5	-	-	-	-	-	-	22.75 ± 1.4	19.54 ± 0.9

CONCLUSION

The edible part of *B. hispida* fruit contains tannins, flavonoids, terpenoids, steroids, and saponins. Among them, eighteen compounds were tentatively identified, including sugars (**1, 3**), a simple phenolic (**2**), a tricarboxylic acid (**4**), a peptide (**5**), flavonoids (**6, 7, 8**), quinic acid derivatives (**9, 10**), phytosterols (**11, 12, 13**), triterpenoids (**14, 18**), and saponins (**15, 16, 17**). Despite low contents of phenolics and flavonoids, 80% ethanol extract of this part exhibited potent DPPH radical scavenging activity. The extract did not show antimicrobial activity towards all tested microbes. However, raffinose (**3**) as a potent prebiotic for human gut bacteria might explain the traditional use of *B. hispida* fruit in relieving typhoid fever. Hence, the study concluded that the edible part of *B. hispida* fruit could be used in the development of natural antioxidant agents and nutraceuticals.

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AUTHORS' CONTRIBUTION

Carla Wulandari Sabandar: the project leader responsible for research design, research management, results validation, manuscript writing and editing. **Harni Sartika Kamaruddin**: project team member who contributed ideas for research design and provided technical assistance for antimicrobial assay. **Reskiya Nur Insani**: a candidate for the Barchelor's degree of Pharmacy who conducted the experimental works, data collection, and data analysis. **Rana Triana Amin**: a fresh undergraduate student who helped the experimental work on antimicrobial assay and data collection as well as analysis. **Zulkifli**: a middler undergraduate student who helped in antioxidant assay and data analysis. **Tien**: a research collaborator who provided facilities for spectrophotometric measurements.

DATA AVAILABILITY

The supporting data of the article are accessible from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

The authors have no conflict of interests to disclose. All authors are fully responsible for the content and writing of this article.

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