

Research Article

UPLC-ESI-MS/MS Profile and Antioxidant, Cytotoxic, Antidiabetic, and Antiobesity Activities of the Aqueous Extracts of Three Different *Hibiscus* Species

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Received 8 February 2020; Revised 20 May 2020; Accepted 3 June 2020; Published 25 June 2020

Academic Editor: Casimiro Mantell

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The aqueous extracts of *Hibiscus calyphyllus* (HcA), *Hibiscus micranthus* (HmA), and *Hibiscus deflersii* (HdA) growing in Saudi Arabia did not receive enough attention in phytochemical and biological studies. This inspired the authors to investigate the phytochemicals of these extracts for the first time using UPLC-ESI-MS/MS in negative and positive ionization modes. The analysis afforded the tentative identification of 103 compounds including phenolic compounds, flavonoids, and anthocyanins. Moreover, *in vitro* evaluations of their cytotoxic, antioxidant, antidiabetic, and antiobesity activities were carried out. The results showed that aqueous extract of *Hibiscus calyphyllus* had the highest activity as an antioxidant agent ($SC_{50} = 111 \pm 1.5 \mu\text{g/mL}$) compared with ascorbic acid ($SC_{50} = 14.2 \pm 0.5 \mu\text{g/mL}$). MTT assay was used to evaluate cytotoxic activity compared to cisplatin. *Hibiscus deflersii* showed the most potent cytotoxic effect against A-549 (human lung carcinoma) with $IC_{50} = 50 \pm 5.1 \mu\text{g/mL}$, and *Hibiscus micranthus* showed a close effect with $IC_{50} = 60.4 \pm 1.7 \mu\text{g/mL}$. *Hibiscus micranthus* showed the most potent effect on HCT-116 (human colon carcinoma) with $IC_{50} = 56 \pm 1.9 \mu\text{g/mL}$ compared with cisplatin ($IC_{50} = 7.53 \pm 3.8 \mu\text{g/mL}$). HcA and HdA extracts showed weak cytotoxic activity against A-549 and HCT-116 cell lines compared to the other extracts. Eventually, *Hibiscus deflersii* showed astonishing antidiabetic ($IC_{50} = 56 \pm 1.9 \mu\text{g/mL}$) and antiobesity ($IC_{50} = 95.45 \pm 1.9 \mu\text{g/mL}$) activities using *in vitro* α -amylase inhibitory assay (compared with acarbose ($IC_{50} = 34.71 \pm 0.7 \mu\text{g/mL}$)) and pancreatic lipase inhibitory assay (compared with orlistat ($IC_{50} = 23.8 \pm 0.7 \mu\text{g/mL}$)), respectively. In conclusion, these findings are regarded as the first vision of the phytochemical constituents and biological activities of different *Hibiscus* aqueous extracts. *Hibiscus deflersii* aqueous extract might be a hopeful origin of functional constituents with anticancer (on A-549 cell line), antidiabetic, and antiobesity activities. It might be a natural alternative remedy and nutritional policy for diabetes and obesity treatment without negative side effects. Isolation of the bioactive phytochemicals from the aqueous extracts of aerial parts of *Hibiscus calyphyllus*, *Hibiscus micranthus*, and *Hibiscus deflersii* and estimation of their biological effects are recommended in further studies.

1. Introduction

Hibiscus (Malvaceae) consists of approximately 200 species widely distributed in tropical and subtropical regions of the world [1]. *Hibiscus* is a genus of herbs, shrubs, and trees [2]. Phytochemical investigation of *Hibiscus* has been reported to contain many classes of secondary metabolites including anthocyanins, flavonoids, steroids, terpenoids, alkaloids, quinones, and sesquiterpene [2].

Many *Hibiscus* species are valued as ornamental plants and are cultivated in gardens [2]. Fruits of some species are used as food; a soft drink is provided from flowers of some species (*H. sabdariffa* L.) and also used in food industry, for example, in cakes, wines, syrups, jellies, puddings, and cold or hot beverages and as a colorant for herbal teas [1]. Since ancient times, *Hibiscus* has been used in traditional folk medicine for different disorders.

Various pharmacological effects have also been shown for *Hibiscus* and its components such as antihypertensive, antiatherosclerotic, antioxidant, antihypercholesterolemic, hypolipidemic, antinociceptive, anti-inflammatory, antipyretic, analgesic, antifungal, antibacterial, antifertility, antidiabetic, anticancer, antimutagenic, chemopreventive, anthelmintic, and anticonvulsant activities [1, 3, 4].

Hibiscus deflersii (HdA), which is native to Ethiopia and grown as ornamental plant worldwide, is 1 m high erect perennial or annual leafy untidy shrub of bright green narrow dentate leaves surrounding bright crimson-red flowers. Many interesting pharmacological activities of HdA had been reported; its leaves extract is used to treat cardiac disorders and diabetes. However, its flower infusion and extract are used as demulcent and emollient, while its decoction is used for the treatment of bronchial catarrh [5].

Hibiscus micranthus (HmA), which is commonly distributed from south to western part of Saudi Arabia, is a 45 cm shrub with heavy leaves, white flowers, and short pedicels with very distinctive capsules of pea-size fruits and is distributed vastly in Saudi Arabia, India, tropical Africa, and Ceylon. Its flowers and fruits exhibited antidiabetic and laxative activities when used orally, and when applied topically it is used as antidandruff agent. The plant also showed anti-inflammatory, antipyretic, antitumor, antimicrobial, and antiviral activities. Literature detects a wide range of phytochemicals in HmA as flavonoids, phenolic acids, fatty alcohols, fatty acids, β -sitosterol, and alkanes [5].

Hibiscus calyphyllus (HcA) is 1 m high leafy shrub characterized by bright yellow flower with dark red center surrounded by simple wide serrate leaves. It is commonly found in Jazan, south-western region of Saudi Arabia. The ethyl acetate fraction of this plant showed potent antioxidant activity [5].

Hibiscus aerial parts are considered as food crops consumed as hot or cold beverages (aqueous extract). Numerous scientific papers have been published discussing the chemical contents of different fractions of HcA, HdA, and HmA, which showed their high biological effectiveness, having antioxidant, antidiabetic, antiobesity, and cytotoxic activities. For our knowledge, the previous literature on *Hibiscus* species suffers from insufficiency of detailed information on the phytoconstituents of aqueous extracts of Saudi HdA, HcA, and HmA and their biological activities. Therefore, the aims of the present research were (i) to perform direct analysis of aqueous extracts, which relies on UPLC coupled with ESI-MS/MS detection, (ii) to detect the antioxidant, antidiabetic, antiobesity, and anticancer activities of aqueous extracts of HdA, HcA, and HmA; and (iii) to anticipate the components responsible for antioxidant, antidiabetic, antiobesity, and anticancer activities.

2. Materials and Methods

2.1. Plant Material. Aerial parts of three different species of genus *Hibiscus* were collected from As-Sarawat mountains, Jabal As-Sahla', and Aseer province, in Saudi Arabia (2,556 m height above sea level), during March 2009. Taxonomical authentication of plant samples was performed by

Prof. Dr. Mohamed Yousef from the Pharmacognosy Department, College of Pharmacy of King Saud University, and voucher specimens (*H. calyphyllus* no. HA-234, *H. deflersii* no. HA-567, and *H. micranthus* no. HA-16240) were kept at the herbarium of the department.

2.2. Preparation of Extracts. Air-dried powder aerial parts of selected plant samples (600 g) were individually extracted with distilled water at 100°C with continuously shaking for 3 hrs. The marc of each plant material was extracted thrice under similar conditions by repeating the above-mentioned procedure. The aqueous extracts were then filtered by centrifugation, and the filtrates were pooled. The obtained filtrates were freed from the solvent by freeze-drying to get dark brown solid masses. The weight of resulted residues was 34.02, 26.25, and 36.41 g for *H. calyphyllus* (HcA), *H. deflersii* (HdA), and *H. micranthus* (HmA), respectively.

2.3. Chemicals and Reagents. Sigma Aldrich (Hamburg, Germany) provided all the chemical and reagents used throughout the experiments, including HPLC grade methanol ($\geq 99.9\%$), acetonitrile, water for ESI-MS analyses, reagent grade formic acid ($\geq 95\%$), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (freshly dissolved in methanol at a concentration of 0.004%), ascorbic acid (99%), Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, dinitrosalicylic acid (DNS, colour reagent), *p*-nitrophenyl butyrate (NPB, $\geq 98\%$), and potassium phosphate buffer (pH 6.0). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cisplatin, DMSO, porcine pancreatic lipase and α -amylase enzyme, acarbose ($\geq 95\%$), and orlistat ($\geq 98\%$) were used as control. Human lung carcinoma (A-549) and human colon carcinoma (HCT-116) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

2.4. UPLC-ESI-MS/MS. Ultra-performance liquid chromatography with electrospray ionization quadrupole-linear ion trap-tandem mass spectrometry analysis, performed on ESI-MS positive and negative ion acquisition mode, was carried out on a XEVO TQD triple quadrupole instrument. Method in a multiple-reaction monitoring (MRM) mode was employed for the quantitative determination of phytochemicals. The crude *Hibiscus* extracts were analyzed by UPLC, in order to obtain chromatographic profiles of the more polar portions of the extracts, which contain phenolic and flavonoid compounds. The samples were dissolved in HPLC grade methanol, filtered through 0.2 μ m membrane disc filter, and resulting solution concentrations were in the range of 0.2 to 0.5 mg/mL, depending on each crude extract.

The UPLC system was a mass spectrometer, Waters Corporation, Milford, USA. The reverse-phase separations were performed (ACQUITY UPLC BEH C18 Column, 1.7 μ m–2.1 $\times$ 50 mm; 50 mm $\times$ 1.2 mm inner diameter; 1.7 μ m particle size) at 0.2 mL/min flow rate. A previously reported gradient program was applied for the analysis [6]. The mobile phase comprised acidified water containing 0.1%

formic acid (A) and acidified methanol containing 0.1% formic acid (B). The employed elution conditions were as follows: 0–2 min, isocratic elution at 10% B; 2–5 min, linear gradient from 10 to 30% B; 5–15 min, linear gradient from 30% to 70% B; 15–22 min, linear gradient from 70% to 90% B; and 22–25 min, isocratic elution at 90% B; finally, washing and reconditioning of column were done. Electrospray ionization (ESI) was performed in both negative and positive ion modes to obtain more data. The parameters for analysis were set using negative ion mode as follows: source temperature 150°C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440°C, cone gas flow 50 L/h, and desolvation gas flow 900 L/hr. Mass spectra were detected in the ESI between m/z 100 and 1000 atomic mass units. Chemical constituents were identified by their ESI-QqLIT-MS/MS spectra and fragmentation patterns. The peaks and spectra were processed using the MassLynx 4.1 software and tentatively identified by comparing their retention time (R_t) and mass spectrum with reported data and library search (such as FoodDB (<http://www.Foodb.ca>)).

2.5. Antioxidant Activity. The antioxidant activity of extracts was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University by the DPPH free radical scavenging assay in triplicate, and average values were considered.

2.5.1. DPPH Radical Scavenging Activity [7]. Freshly prepared (0.1 mM) solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and different tested extracts prepared at 5, 10, 20, 40, 80, 160, and 320 $\mu\text{g/mL}$ in methanol were vigorously mixed and allowed to stand for 30 min at room temperature in the dark [8]. The absorbance values of the resulting solution were recorded with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201) at λ_{max} of 517 nm against DPPH radical without antioxidant (control) and the reference compound ascorbic acid (5, 10, 20, 40, 80, 160, and 320 $\mu\text{g/mL}$). All the determinations were performed in three replicates and averaged. The percentage inhibition of the DPPH radical was calculated according to the following formula:

$$\% \text{ DPPH radical - scavenging} = \left[\left\{ \frac{(AC - AS)}{AC} \right\} \times 100 \right], \quad (1)$$

where AC is the absorbance of the control solution and AS is the absorbance of the sample in DPPH solution.

The percentage of DPPH radical scavenging was plotted against each extract concentration and ascorbic acid ($\mu\text{g/mL}$) to determine scavenging capacity (SC_{50}), which is the concentration required to scavenge DPPH by 50% (i.e., concentration giving 50% reduction in the absorbance of a DPPH solution from its initial absorbance).

2.6. Evaluation of Cytotoxicity. HdA, HmA, and HcA were tested for their cytotoxic activity against human lung carcinoma (A-549) and human colon carcinoma (HCT-116)

cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) against DMSO and cisplatin as negative and positive controls, respectively. These mammalian cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were propagated on Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer, and 50 $\mu\text{g/mL}$ gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week.

For antitumor assay, the tumor cell lines were suspended in medium at concentration of 5×10^4 cell/well in Corning® 96-well tissue culture plates and then incubated for 24 hrs. The tested extracts were then added to 96-well plates (six replicates) to achieve eight concentrations for each extract ranging from 1 $\mu\text{g/mL}$ to 500 $\mu\text{g/mL}$. Six vehicle controls with media or 0.5% DMSO were run for each 96-well plate as a control. After incubation for 24 h, the numbers of viable cells were determined by the MTT test. Briefly, the media were removed from the 96-well plates and replaced with 100 μL of fresh culture DMEM without phenol red; then, 10 μL of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of phosphate buffered saline (PBS)) was added to each well including the untreated controls. The 96-well plates were then incubated at 37°C and 5% CO_2 for 4 hrs. An 85 μL aliquot of the media was removed from the wells, and 50 μL of DMSO was added to each well, mixed thoroughly with the pipette, and incubated at 37°C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (Sunrise, Tecan, Inc., USA) to determine the number of viable cells, and the percentage of viability was calculated:

$$\text{cell viability \%} = \left[1 - \left(\frac{\text{OD}_t}{\text{OD}_c} \right) \right] \times 100\%, \quad (2)$$

where OD_t is the mean optical density of wells treated with the tested sample and OD_c is the mean optical density of untreated cells.

The relation between surviving cells and each extract concentration (1–500 $\mu\text{g/mL}$) was plotted to get the survival curve of each tumor cell line after treatment with the tested extract. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose-response curve (log extract concentration on x -axis vs. percentage viability from untreated cells on y -axis) for each concentration through nonlinear regression analysis (dose-response inhibition, log inhibitor vs. normalized response-variable slope) using GraphPad Prism 5 software (GraphPad Software, San Diego, California) [9, 10]. All experiments were repeated at least three times. Results are reported as means $\pm$ SD.

2.7. In Vitro Antidiabetic Assay

2.7.1. α -Amylase Inhibition Method. In α -amylase inhibition method, the enzyme solution was prepared by dissolving α -amylase in 20 mM phosphate buffer (6.9) at a

concentration of 0.5 mg/mL. One mL of the extract of various concentrations (7.81–1000 $\mu\text{g/mL}$) and 1 mL of enzyme solution were mixed together and incubated at 25°C for 10 min. After incubation, 1 mL of starch (0.5%) solution was added to the mixture and further incubated at 25°C for 10 min. The reaction was then stopped by adding 2 mL of dinitrosalicylic acid (DNS, colour reagent), heating the reaction mixture in a boiling water bath (5 min). After cooling, the absorbance was measured calorimetrically at 565 nm. The inhibition percentage was calculated using the following formula: % inhibition = $(1 - A_s/A_c) \times 100$, where A_c is the absorbance of control and A_s is the absorbance of tested extracts. Acarbose was used as a control [11]. The IC_{50} value was defined as the concentration of α -amylase inhibitor needed to inhibit 50% of its activity under the assay conditions.

Nonlinear regression analysis using GraphPad Prism 5 software (GraphPad Software, San Diego, California) was conducted to calculate IC_{50} from graphic plots of the dose-response curve for each applied concentration. Each experiment was performed in triplicate, and all values are represented as means $\pm$ SD.

2.8. In Vitro Antiobesity Using Pancreatic Lipase Inhibitory Assay. The lipase inhibition activity of plant extract was determined by the method in [12]. In this assay, the porcine pancreatic lipase activity was measured using *p*-nitrophenyl butyrate (NPB) as a substrate. Lipase solution (100 $\mu\text{g/mL}$) was prepared in a 0.1 mM potassium phosphate buffer (pH 6.0). Samples with different concentrations (7.81–1000 $\mu\text{g/mL}$) were preincubated with 100 $\mu\text{g/mL}$ of lipase for 10 min at 37°C. The reaction was then started by adding 0.1 mL NPB substrate. After incubation at 37°C for 15 min, *p*-nitrophenol amount released in the reaction was measured using multiplate reader. Orlistat was used with the same concentrations as a control. The results were expressed as percentage inhibition, which was calculated using the following formula: inhibitory activity (%) = $(1 - A_s/A_c) \times 100$, where A_s is the absorbance in the presence of test substance and A_c is the absorbance of control. The IC_{50} value was defined as the concentration of pancreatic lipase inhibitor required to inhibit 50% of its activity under the assay conditions. Estimation of IC_{50} was done from dose-response curve graphic plots for each concentration by nonlinear regression analysis using GraphPad Prism 5 software. Each experiment was performed in triplicate, and all values are represented as means $\pm$ SD of triplicates.

3. Results and Discussion

3.1. UPLC-ESI-MS/MS. Identification of the chemical composition of the aqueous extract of the HdA, HmA, and HcA was carried out by UPLC-ESI-MS/MS in negative and positive ion modes. Totally, 103 secondary metabolites arranged according to retention time (R_t) were identified depending on their MS^2 information given by the precursor ion's mass, their fragments, known fragmentation patterns for the given classes of compounds, and neutral mass loss, as

well as comparison with the available literature and searching in an online database [13] as shown in Table 1. Figure 1 shows the base peak chromatograms of the three aqueous extracts.

3.1.1. Phenolic Compounds. Phenolic acid derivatives are mostly glycosides; their fragmentation stage started with the cleavage of the glycosidic linkage to provide the m/z of the phenolic acid and the corresponding neutral mass loss of sugar molecules (–162 Da), and then neutral mass losses of hydroxyl (–18 Da), methyl (–15 Da), or carboxylic (–44 Da) groups were helpful in identification of the specific phenolic acid. Methyl gallate (72) [17] and its derivative (85) [39] and syringic acid derivative (76) [33] were identified. Compound 33 and its isomer (89) were tentatively identified as 4-hydroxybenzoic acid while compound 55 and its isomer (59) were tentatively identified as 3-hydroxybenzoic acid [13].

Tyrosol (4) and its isomers (26, 67, and 94) were characterized by two fragments: m/z 77, corresponding to the aromatic ring; m/z 93, corresponding to the phenol group, respectively [15]. Tyrosol precursor ion at m/z 121 does not refer to the $[M + H]^+$ ion, but to the $[M + H - H_2O]^+$ according to [15]; this may be due to in-source fragmentation, even under mild ionization conditions.

3.1.2. Flavone C-Glycosides. In negative ionization mode, the presence of $[M - H - 90]^-$ and $[M - H - 120]^-$ confirmed that the compounds are mono-C-hexosylated flavonoids. The sugar on position 8 can be detected by investigation of MS^2 spectrum (i.e., the absence of the fragment peak at m/z $[M - H - 18]^-$) as in compound 27, which was identified as orientin (luteolin-8-C-glucoside) [25, 27, 31, 32], and compound 98, which was tentatively identified as kaempferol-8-C-glucoside.

The substitution of the two C-glucosides in positions 6 and 8 in compound 20 and its isomer (41) can be confirmed by the characteristic fragments at m/z 383 corresponding to $[M - H - 120 - 90]^-$ and m/z 353 corresponding to $[M - H - 120 - 120]^-$ in MS/MS spectrum. The compound was identified as vicerin (apigenin 6,8-di-C-glucoside) [26, 27]. Luteolin C-hexoside-C-pentoside (30) and its isomer (50) with $[M - H]^-$ at m/z 579 showed ion fragments at m/z 489 $[M - H - 90]^-$, m/z 459 $[M - H - 120]^-$, m/z 429 $[M - H - 150]^-$, m/z 369 $[M - H - 120 - 90]^-$, and m/z 339 $[M - H - 120 - 120]^-$ [25].

Compound 8 and its isomers (19, 36, 43) exhibited characteristic fragments at m/z 443 corresponding to $[M - H - 120]^-$, m/z 431 corresponding to $[M - H - 132]^-$, m/z 353 corresponding to $[M - H - 120 - 90]^-$, and m/z 341 corresponding to $[M - H - 132 - 90]^-$ in MS^2 spectrum that confirm the mono-C-hexoside-C-pentoside substitution in positions 6 and 8. The compound was identified as apigenin C-hexoside-C-pentoside [18, 25, 40].

Schaftoside (apigenin-6-C-glucoside-8-C-riboside) (32) and its isomer (51) showed a pseudomolecular ion peak $[M + H]^+$ at m/z 565, and the typical fragmentation pathway of C-glycosylated flavonoids resulted in the formation of ions at m/z 475 $[M + H - 90]^+$, corresponding to the loss of an

TABLE 1: Metabolites identified in the aqueous extracts of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA) using UPLC–ESI–MS in negative and positive ionization modes.

Comp. no.	Compound name	R _t (min)	[M – H] [–] (m/z)	[M + H] ⁺ (m/z)	MS ² fragments (m/z)	HdA	HcA	HmA	References
1	L-ascorbic acid	0.30		177	133, 129, 127, 113, 103, 101, 57	✓	✓	✓	1
2	Oleuropein	0.82	539		377, 341, 307, 215, 179	✓		✓	2
3	Succinic acid*	0.86		119	101	✓			—
4	Tyrosol	0.93		121	103, 97, 93, 89, 79, 77, 73, 65, 45	✓			3
5	Sucrose	0.95	341		179 (M – H-162) [–] , 161, 131, 119, 117, 113, 103, 101, 89, 87, 71, 59			✓	4
6	Hydroxycitric acid derivative	1.36		593	209		✓		1
7	Butein	1.43		273	163, 143, 137	✓			5
8	Apigenin C-hexoside-C-pentoside	1.53	563		545 (M – H-18) [–] , 443 (M – H-120) [–] , 431 (M – H-132) [–] , 353 (M – H-120-90) [–] , 341 (M – H-132-90) [–] , 311 (M – H-132-120) [–]		✓		6
9	L-ascorbic acid isomer	1.81		177	133, 129, 127, 113, 103, 101, 57			✓	1
10	N-feruloyltyramine	3.32		314	235, 181, 177, 145, 121, 103, 93, 45			✓	1
11	Cyanidin 3-O-galactoside	3.57		449	287, 137		✓		7, 8
12	L-ascorbic acid isomer	4.52		177	133, 129, 127, 113, 103, 101, 57	✓			1
13	Patuletin (6-methoxyquercetin)	4.97		333	318, 301, 169, 155	✓			9
14	Succinic acid isomer	6.05	117		99 (M – H-18) [–]	✓			10
15	Diosmetin-7-O-glucuronide-3'-O-pentoside	6.31	607		475 (M – H-132) [–] , 299 (M – H-132-176) [–] , 179			✓	11
16	L-ascorbic acid isomer	6.32		177	133, 129, 127, 113, 103, 101, 57		✓		1
17	Succinic acid isomer	6.49	117		117, 99			✓	10
18	Cyanidin 3-O-sambubioside	6.76	579		339, 285		✓		12
19	Apigenin C-hexoside-C-pentoside isomer	7.02	563		545 (M – H-18) [–] , 443 (M – H-120) [–] , 431 (M – H-132) [–] , 341 (M – H-132-90) [–] , 311 (M – H-132-120) [–]	✓			13
20	Vicenin (apigenin 6,8-di-C-glucoside)	7.10	593		473 (M – H-120) [–] , 383 (M – H-120-90) [–] , 285	✓			14, 15
21	Kaempferol-3-O-rutinoside/luteolin-7-O-rutinoside	7.25	593		285 (M – H-Rut.) [–]	✓			16/17
22	Apiin (apigenin-7-apiosylglucoside)	7.29	563		443, 413, 269 (M – H-132-162) [–]		✓		5, 18
23	Apiin isomer	7.31		565	433 (M + H-132) ⁺ , 413, 271(M + H-162-132) ⁺	✓			5
24	Cyanidin 3-O-glucoside	7.37		494	287 (M + H-162) ⁺ , 137	✓			3
25	Diosmetin C-glucoside C-pentoside*	7.37	593		413 (M – H-90-90) [–] , 383 (M – H-90-120) [–]		✓		—
26	Tyrosol isomer	7.46		121	93, 77		✓		3
27	Orientin (luteolin 8-C-glucoside)	7.46	447		357 (M – H-90) [–] , 327 (M – H-120) [–] , 297 (M – H-150) [–] , 285(M – H-162) [–]	✓			13, 15, 19, 20
28	Delphinidin malonyl glucuronide*	7.59		565	303 (M + H-176-86) ⁺		✓		—
29	Methyl apigenin derivative*	7.81		799	285	✓			—
30	Luteolin C-hexoside-C-pentoside	7.82	579		489 (M – H-90) [–] , 459 (M – H-120) [–] , 429 (M – H-150) [–] , 411, 399, 369 (M – H-120-90) [–] , 365, 353, 339 (M – H-120-120) [–] , 299			✓	13
31	Luteolin derivative Schaftoside (apigenin 6-C-glucoside 8-C-ribose)	7.98	737		285		✓		18, 21
32		7.99		565	529, 511, 475, 457, 445, 427, 415(M + H-150) ⁺ , 409, 391, 379, 361, 349, 337, 325, 307, 295, 273			✓	1, 22

TABLE 1: Continued.

Comp. no.	Compound name	R _t (min)	[M – H] [–] (m/z)	[M + H] ⁺ (m/z)	MS ² fragments (m/z)	HdA	HcA	HmA	References
33	4-Hydroxybenzoic acid	7.99		139	121, 111, 105, 97, 93, 79		√		1
34	Peonidin-3-(<i>p</i> -coumaroyl-glucoside)	8.27		609	301(M + H-146-162) ⁺		√		23
35	Kaempferol-3-(<i>p</i> -coumaryl-glucoside)	8.27	593		593, 447(M – H-146) [–] , 327, 285(M – H-146-162) [–]			√	24
36	Apigenin C-hexoside-C-pentoside isomer	8.32	563		545 (M – H-18) [–] , 473 (M – H-90) [–] , 443 (M – H-120) [–] , 431 (M – H-132) [–] , 353 (M – H-120-90) [–] , 341 (M – H-132-90) [–] , 311 (M – H-132-120) [–]	√		√	13
37	Kaempferol-3- <i>O</i> -glucoside	8.41	447		285		√		6, 8
38	Gamma-eudesmol rhamnoside derivative	8.47	577		439, 397, 379, 367, 349, 322, 293, 249, 197, 127		√		1
39	L-ascorbic acid isomer	8.55		177	133, 129, 127, 113, 103, 101, 57	√			1
40	Peonidin-3-(<i>p</i> -coumaroyl-glucoside) isomer	8.69		609	301(M + H-146-162) ⁺	√	√		23
41	Vicenin isomer	8.89	593		473 (M – H-120) [–] , 395, 383 (M-120-90) [–] , 338, 327, 298			√	15, 25
42	Apiin isomer	8.98		565	433 (M + H-132) ⁺ , 413, 271(M + H-162-132) ⁺	√		√	5
43	Apigenin C-hexoside-C-pentoside isomer	8.98	563		545 (M – H-18) [–] , 443 (M – H-120) [–] , 431 (M – H-132) [–] , 353 (M – H-120-90) [–] , 341 (M – H-132-90) [–] , 311 (M – H-132-120) [–]	√			6, 13
44	Unknown	9.04	319		239, 204, 195, 97			√	—
45	Kaempferol-3-(<i>p</i> -coumaryl-glucoside) isomer	9.12	593		447 (M – H-146) [–] , 327, 285(M – H-146-162) [–]	√			24
46	Peonidin-3-(<i>p</i> -coumaroyl-glucoside) isomer	9.37		609	609, 579, 463 (M + H-146) ⁺ , 301 (peonidin) (M + H-146-162) ⁺	√	√	√	23
47	Methyl apigenin-C-rhamnoside- <i>O</i> -glucoside*	9.38		593	327 (M + H-162-104) ⁺ , 297 (M + H-162-134) ⁺ , 285		√		—
48	Diosmetin-7- <i>O</i> -rutinoside	9.50	607		607, 577, 461 (M – H-146) [–] , 299 (M – H-146-162) [–]	√		√	26
49	Luteolin hexoside	9.55		449	287 (M + H-162) ⁺	√			5
50	Luteolin C-hexoside-C-pentoside isomer	9.63	579		369 (M – H-120-90) [–] , 339 (M – H-120-120) [–] , 322, 281, 259, 124		√		13
51	Schaftoside isomer	9.94		565	529, 511, 475, 457, 445, 427, 415(M + H-150) ⁺ , 409, 391, 379, 361, 349, 337, 325, 307, 295, 273		√		1, 22
52	Succinic acid isomer	10.01	117		99		√		10
53	L-ascorbic acid isomer	10.36		177	133, 129, 127, 113, 103, 101, 57		√		1
54	Peonidin dirhamnoside*	10.37		593	301 (M + H-146-146) ⁺	√			—
55	3-Hydroxybenzoic acid	10.49		139	105, 97, 93, 79		√		1
56	Acacetin-rhamnoglucoside	10.61	591		283 (M – H-Rut.) [–] , 103, 58	√		√	18
57	Kaempferol dimethyl ether dipentoside*	10.68		579	579, 315 (M + H-132-132) ⁺			√	—
58	Peonidin glucoside feruloyl glucuronide	10.82		815	463 (M + H-352 feruloyl glucuronide) ⁺	√			7
59	3-Hydroxybenzoic acid isomer	10.94		139	105, 97, 93, 79		√		1
60	N-feruloyltyramine isomer	10.96		314	235, 218, 181, 177, 145, 121, 103, 93, 45			√	1
61	Unknown	11.07	877		877, 813, 783, 557		√		—
62	Apigenin- <i>O</i> -dihexoside	11.38	593		269 (M – H-162-162) [–]		√		9

TABLE 1: Continued.

Comp. no.	Compound name	R _t (min)	[M – H] [–] (m/z)	[M + H] ⁺ (m/z)	MS ² fragments (m/z)	HdA	HcA	HmA	References
63	Isorhamnetin-3-O-rutinoside	11.59	623		315 (M – H-Rut.) [–] , 300, 271	✓			27
64	Peonidin dirhamnoside isomer*	11.69		593	447 (M + H-146) ⁺ , 301 (M + H-146-146) ⁺	✓	✓		—
65	Acacetin-rhamnoglucoiside isomer	11.69	591		283 (M – H-Rut.) [–]	✓		✓	18
66	Succinic acid isomer	11.78	117		99		✓		10
67	Tyrosol isomer	12.05		121	93, 77		✓		3
68	Diosmetin rhamnoside feruloyl glucuronide*	12.46	797		445 (M – H-176-176) [–] , 299 (M – H-176-176-146) [–] , 237, 205	✓			—
69	Apiin isomer	12.87	563		269	✓			18
70	Succinic acid isomer	12.90	117		99		✓		10
71	Peonidin dipentoside*	13.13		565	565, 301(M + H-132-132) ⁺ , 336 265, 195, 135, 91, 45	✓			—
72	Methyl gallate	13.25		185	168, 124		✓		5
73	Succinic acid isomer	13.65	117		99	✓			10
74	Delphinidin-3-arabinoside derivative	15.40		799	435, 303 (M + H-364-132) ⁺	✓			7, 23
75	Hydroxy-octadecadienoic acid derivative	15.57	593		295 (M – H-298) [–] , 277 (M – H-298-18) [–] , 251(M – H-298-18-CO ₂) [–] , 195, 171	✓			28
76	Syringic acid derivative	15.83	377		197		✓		21
77	22-Dehydrocholesterol	16.00		393	393, 273, 173, 171, 130, 125			✓	1
78	Malvidin derivative*	16.72		565	331, 147	✓			—
79	Kaempferide derivative isomer	17.40	623		299 (M – H-2 Glc.) [–] , 163	✓			29
80	Unknown	17.42		274	274, 256 (M + H-18) ⁺ , 210 (M + H-18-46) ⁺ , 111, 105, 102, 88, 71		✓	✓	—
81	Unknown	17.54		399	267, 253, 227	✓			—
82	L-ascorbic acid isomer	17.82		177	133, 129, 127, 113, 103, 101, 57		✓		1
83	Succinic acid isomer	21.49	117		117, 99		✓		10
84	Apiin isomer	21.89		565	433 (M + H-132) ⁺ , 413, 271 (M + H-162-132) ⁺			✓	5
85	Methyl gallate derivative	23.06	325		183 (M – H-142) [–]	✓			29
86	Gallocatechin derivative	24.48	561		305	✓			21
87	Succinic acid isomer	24.96	117		99		✓		10
88	Unknown	25.01		515	515, 353, 331, 313, 239			✓	—
89	4-Hydroxybenzoic acid isomer	25.28		139	121, 111, 105, 97, 93, 79	✓	✓		1
90	Succinic acid isomer	26.11	117		117, 101, 99	✓	✓		10
91	Unknown	26.66		805	615, 606, 598, 413, 391, 279, 167, 149, 113	✓			—
92	Luteolin-7-glucuronide-3'4'-pentoside	26.77	593		461 (M – H-132) [–] , 285 (M – H-132-176) [–] , 169			✓	11
93	Peonidin dipentoside isomer*	27.45		565	547, 301 (M + H-132-132) ⁺ , 259, 219, 133, 113, 85, 45	✓		✓	—
94	Tyrosol isomer	27.55		121	93, 77		✓		3
95	L-ascorbic acid isomer	27.61		177	133, 129, 127, 113, 103, 101, 57			✓	1
96	Delphinidin-3-(p-coumaroyl-glucoside) derivative	28.36		799	611, 303 (M + H-188-308) ⁺		✓		23
97	Epicatechin derivative	28.91		678	289		✓		6
98	Kaempferol-8C-glucoside*	29.40		449	329 (M + H-120) ⁺ , 299 (M + H-150) ⁺		✓		—
99	Malvidin 3-O-glucoside derivative	29.83		871	493, 331	✓			8
100	Unknown	30.00		871	593, 552, 369, 260, 105			✓	—
101	L-ascorbic acid isomer	30.41		177	133, 129, 127, 113, 103, 101, 57	✓	✓		1
102	Peonidin derivative*	30.65		799	648 (M + H-galloyl) ⁺ , 301 (peonidin)		✓		—
103	Succinic acid isomer	31.52	117		117, 99	✓			10

1: [13]; 2: [14]; 3: [15]; 4: [16]; 5: [17]; 6: [18]; 7: [19]; 8: [20]; 9: [21]; 10: [22]; 11: [23]; 12: [24]; 13: [25]; 14: [26]; 15: [27]; 16: [28]; 17: [29]; 18: [30]; 19: [31]; 20: [32]; 21: [33]; 22: [34]; 23: [35]; 24: [28]; 25: [26]; 26: [36]; 27: [37]; 28: [38]; 29: [39]. *Tentative identified; R_t: retention time; Rut.: rutinose; Glc.: glucose; HdA: *Hibiscus deflersii*; HcA: *H. calyphyllus*; HmA: *H. micranthus*.

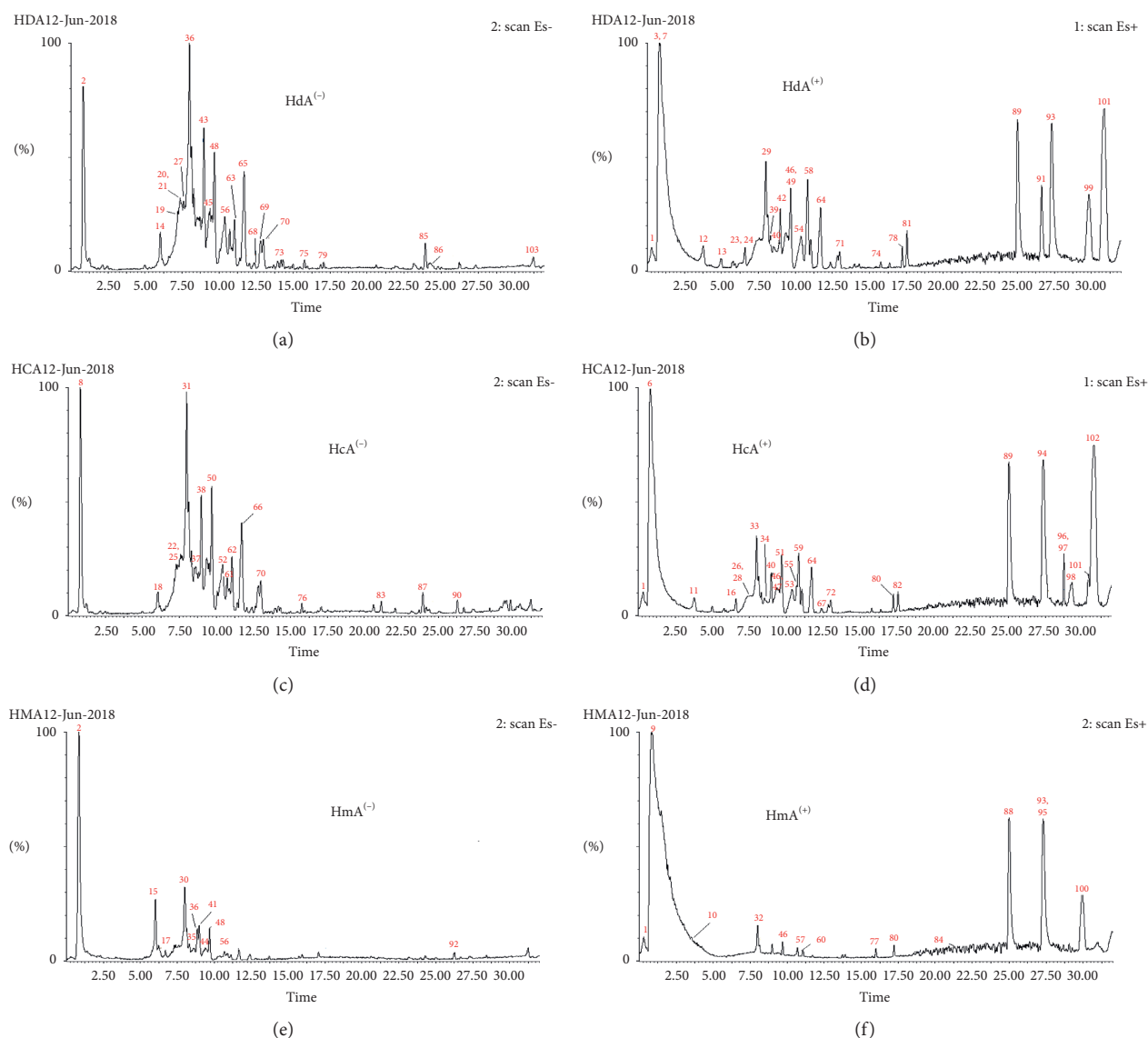


FIGURE 1: UPLS-ESI-MS chromatograms of aqueous extracts of aerial parts of *H. deflersii* (HdA), *H. calyphyllus* (HcA), and *H. micranthus* (HmA) in negative (–) and positive (+) ionization modes.

arabinose unit (riboside), and at m/z 445 $[M + H - 120]^+$, corresponding to the loss of a glucose unit. Further fragmentations of the sugar moieties were observed to generate $[M + H - 186]^+$ ions at m/z 379, $[M + H - 240]^+$ ions (forming the base peaks) at m/z 325, and $[M + H - 270]^+$ ions at m/z 295 [34].

Diosmetin C-glucoside C-pentoside (25) showed a pseudomolecular ion peak $[M - H]^-$ at m/z 593. The typical fragmentation pathway of C-glycosylated flavonoids resulted in the formation of ions at m/z 413 $[M - H - 90 - 90]^-$, corresponding to the loss of an pentoside moiety, and at m/z 383 $[M - H - 120 - 90]^-$, corresponding to the loss of a glucose moiety.

3.1.3. Flavonoid O,C-Glycosides. Flavonoid O,C-glycosides are distinguished by the lack of the aglycone ion. Only the precursor ion $[M - H]^-$ is detected in addition to the ions

resulting from the interglycosidic linkage cleavage including the key fragmentation ions at $[M - H - 120]^-$ and $[M - H - 120 - 162]^-$ or $[M - H - 90 - 146]^-$ with or without $[M - H - 18]^-$. On the basis of these rules, compound 47 (R_t 9.38 min) with a $[M + H]^+$ ion at m/z 593 was tentatively identified as methyl apigenin-C-rhamnoside-O-glucoside as it showed MS² fragments at m/z 327 $[M + H - 162 - 104]^+$, corresponding to loss of O-glucoside moiety (–162 Da), m/z 297 $[M + H - 162 - 134]^+$, corresponding to additional loss of C-rhamnosyl moiety (–134 Da), and m/z 285 (methyl apigenin).

3.1.4. Flavonoid O-Glycosides. Fragmentation pattern of flavonoid O-glycosides is characterized by the loss of the sugar moiety [41, 42], and as a result a deprotonated aglycone ion is yielded in MS². Compound 21 (R_t 7.25 min) with $[M - H]^-$ ion peak at m/z 593 was tentatively identified as kaempferol-3-O-rutinoside/luteolin-7-O-rutinoside

based on the ESI mass data and the 308 Da neutral loss leaving fragment ion at m/z 285 corresponding to kaempferol/luteolin aglycones [29, 37]. Diosmetin-7-*O*-glucuronide-3'-*O*-pentoside (15) (R_t 6.31 min) produced a $[M - H]^-$ ion at m/z 607 with MS^2 fragments at m/z 475 $[M - H - 132]^-$, corresponding to loss of pentosyl moiety, and 299 $[M - H - 132 - 176]^-$, corresponding to loss of additional glucuronyl moiety [23].

Apigenin-7-*O*-apiosylglucoside (apiin) (22) and its isomer (69) were tentatively identified from the MS profile of peak at R_t of 7.29 and 12.87 min with $[M - H]^-$ at m/z 563 and MS/MS base peak fragment ion at m/z 269, which gave the loss of 294 Da (162 + 132 Da) (apiosylglucoside moiety) [17, 30]. Furthermore, other three apiin isomers (23, 42, and 84) were tentatively identified from the MS profile of peaks at retention time of 7.31, 8.98, and 21.89 min with $[M + H]^+$ at m/z 565 and MS^2 base peak fragment ion at m/z 433 $[M + H - 132]^+$, corresponding to apiosyl moiety loss, and at m/z 271, which indicated the loss of 294 Da (162 + 132 Da) (apiosylglucoside moiety) [17]. Compounds 35 and 45 with $[M - H]^-$ ion at m/z 593 were tentatively identified as kaempferol-3-*O*-(*p*-coumaryl glucoside). The fragmentation pattern showed MS^2 fragments at m/z 447 $[M - H - 146]^-$, corresponding to loss of rhamnose (−146 Da) moiety, and at m/z 285 $[M - H - 146 - 162]^-$, corresponding to additional loss of glucose moiety (−162 Da). They both gave the product ion at m/z 285 corresponding to the kaempferol aglycone [28]. Compound 37 (R_t 8.41 min) with $[M - H]^-$ at m/z 447 was tentatively identified as kaempferol-3-*O*-glucoside from ESI $^-$ mass data and the neutral loss of 162 Da for glucosyl moiety [18, 20]. By the same manner, compound 49 (R_t 9.55 min) was identified as luteolin hexoside ($[M + H]^+$ ion at m/z 449 and MS^2 ion at m/z 287 $[M + H - \text{glc.}]^+$). The precursor ion of compound 48 was detected at m/z 607 $[M - H]^-$ and its characteristic MS^2 fragment ion at m/z 299 $[M - H - \text{rut.}]^-$, related to deprotonated diosmetin, and consequently it was tentatively identified as diosmetin-7-*O*-rutinoside [36]. Additionally the precursor ion of compounds 56 and 65 was detected at m/z 591 $[M - H]^-$ and its characteristic MS^2 fragment ion at m/z 283 $[M - H - \text{rut.}]^-$, related to deprotonated acacetin, and consequently it was tentatively identified as acacetin-rhamnoglucoside and its positional isomers [30]. Compound 57 was tentatively identified as kaempferol dimethyl ether dipentoside (R_t 10.68 min) as it produced a $[M + H]^+$ ion at m/z 579 with MS^2 fragments at m/z 315 $[M + H - 132 - 132]^+$, corresponding to loss of dipentosyl moieties. Compound 62 with a $[M - H]^-$ at m/z 593 and MS^2 ions at m/z 269 $[M - H - 162 - 162]^-$ was tentatively identified as apigenin-*O*-dihexoside [21]. Compound 63 $[M - H]^-$ ion at m/z 623 was tentatively identified as isorhamnetin-3-*O*-rutinoside. The fragmentation patterns showed MS^2 fragments at m/z 315 $[M - H - 308]^-$ (isorhamnetin), corresponding to loss of rutinose (−308 Da) moiety [37]. Compound 68 with $[M - H]^-$ at m/z 797 was tentatively identified as diosmetin rhamnoside feruloyl glucuronide as it showed MS^2 fragments at m/z 445 $[M - H - 176 - 176]^-$, corresponding to loss of feruloyl (−176 Da) and glucuronide (−176 Da) moieties, and 299 $[M - H - 176 - 176 - 146]^-$, corresponding to additional loss of

rhamnose moiety (−146 Da). According to [39], kaempferide derivative (79) was tentatively identified by its molecular ion $[M - H]^-$ at m/z 623 and fragmentation pattern containing specific ion at m/z 299 $[M - H - 162 - 162]^-$, corresponding to loss of two glucose moieties, and ion at m/z 163. According to [23], the diglycosylated flavonoid (92) was detected as it possessed a pseudomolecular ion $[M - H]^-$ at m/z 593 with MS^2 fragment ions at m/z 461 $[M - H - 132]^-$, corresponding to pentosyl moiety loss, as well as other less abundant ion at m/z 285 $[M - H - 132 - 176]^-$ resulting from the loss of glucuronyl moiety (−176 Da). This compound was identified as luteolin-7-glucuronide-3'/4'-pentoside.

3.1.5. Flavonoid Aglycones. Patuletin (6-methoxy quercetin) (13) was identified by comparing its MS^2 fragmentation pattern with the previously reported data [21]. Compound 31 with $[M - H]^-$ ion at m/z 737 and MS^2 fragment ion at m/z 285 was determined to be luteolin derivative [30, 33], while compound 29, which gave a $[M + H]^+$ ion at m/z 799 and MS^2 fragment ion at m/z 285, was identified tentatively as methyl apigenin derivative. Galocatechin derivative (86) [33] and epicatechin derivative (97) [18] were recognized by comparing their MS^2 fragmentation pattern with the previously published data.

3.1.6. Anthocyanins. A total of 9 anthocyanin derivatives have been detected in *Hibiscus*. Compounds 34, 40, 46, 54, 58, 64, 71, 93, and 102 were tentatively identified as peonidin derivatives. Thus, mass data of compound 34 and its isomers (40 and 46) were proposed to be peonidin-3-(*p*-coumaroyl-glucoside) as they showed a molecular ion peak $[M + H]^+$ at m/z 609 and MS^2 fragment ions at m/z 301 $[M + H - 162 - 146]^+$. These were indicative of a peonidin with glucose (162 Da) and coumaroyl (146 Da) moieties [35].

Compounds 71 and 93 produced a $[M + H]^+$ ion at m/z 565 with MS^2 fragments at m/z 301 $[M + H - 132 - 132]^+$. These were indicative of a peonidin with two pentoside moieties (264 Da) and were tentatively identified as peonidin dipentoside, while compounds 54 and 64 produced $[M + H]^+$ ion at m/z 593 with MS^2 fragments at m/z 301 $[M + H - 146 - 146]^+$ (peonidin). These were indicative of peonidin with two rhamnoside moieties (292 Da) and were tentatively identified as peonidin dirhamnoside. Compound 58 was tentatively identified as peonidin glucoside feruloyl glucuronide as it produced a $[M + H]^+$ ion at m/z 815 with MS^2 fragment ions at m/z 463 $[M + H - 176 - 176]^+$, corresponding to the loss of a feruloyl (−176 Da) and glucuronide (−176 Da) moieties, and at m/z 301 $[M + H - 176 - 176 - 162]^+$ (peonidin), corresponding to the loss of a glucose unit. Compound 102 was tentatively identified as peonidin derivative as it showed a $[M + H]^+$ ion at m/z 799 with MS^2 fragment ions at m/z 648 $[M + H - 151]^+$, corresponding to the loss of a galloyl (−151 Da) moiety, and at m/z 301 (peonidin).

The mass spectra of compounds 11 and 24 showed their protonated aglycon ions $[M + H]^+$ to be m/z 287, corresponding to cyanidin. These protonated aglycon ions were all formed by loss of a sugar moiety with 162 units from their $[M + H]^+$, indicating that they are anthocyanidin

monoglucosides or monogalactosides. This suggests the presence of cyanidin 3-*O*-galactoside (11) and cyanidin 3-*O*-glucoside (24). The m/z values of compound 18 detected at m/z 579 in the negative ion mode are similar to those of cyanidin 3-*O*-sambubioside [24].

Compounds 28, 74, and 96 were tentatively identified as delphinidin derivatives. Compound 28 showed a molecular ion peak $[M + H]^+$ at m/z 565 and MS^2 fragment ions at m/z 303 $[M + H - 176 - 86]^+$. These were indicative of a delphinidin with glucuronide (176 Da) and malonyl (86 Da) moieties. This compound was suggested to be delphinidin malonyl glucuronide.

Compound 74 showed a molecular ion peak $[M + H]^+$ at m/z 799 and MS^2 fragment ions at m/z 435 $[M + H - 364]^+$ (delphinidin-3-arabinoside) and m/z 303 $[M + H - 364 - 132]^+$, corresponding to loss of arabinose moiety. These were indicative of a delphinidin-3-arabinoside derivative [19, 35]. Compound 96 showed a molecular ion peak $[M + H]^+$ at m/z 799 and MS^2 fragment ions at m/z 611 $[M + H - 188]^+$ and at m/z 303 $[M + H - 188 - 308]^+$. These were indicative of a delphinidin with coumaroyl-glucoside moieties (−308 Da) [43]. This compound was proposed to be delphinidin-3-(*p*-coumaroyl-glucoside) derivative [35].

Compound 78 was tentatively identified as malvidin derivative as it showed a protonated aglycone ion peak at m/z 331 corresponding to malvidin [43]. Compound 99 produced a $[M + H]^+$ ion at m/z 871 with MS^2 fragments at m/z 493, corresponding to malvidin-3-*O*-glucoside [20], and at m/z 331 $[M + H - 378 - 162]^+$, corresponding to malvidin [43], with one glucose moiety (162 Da), and was identified as malvidin 3-*O*-glucoside derivative [20].

3.1.7. Fatty Acid Derivatives. For compound 75, a pseudomolecular ion peak $[M - H]^-$ at m/z 593 was observed with MS^2 fragment ions at m/z 295 $[M - H - 298]^-$; 277 $[M - H - 298 - H_2O]^-$, 251 $[M - H - 298 - CO_2]^-$, 195, and 171 were detected suggesting the presence of hydroxy-octadecadienoic acids derivative [38].

3.1.8. Miscellaneous Compounds. For L-ascorbic acid (1) and its isomers (9, 12, 16, 39, 53, 82, 95, and 101), a protonated pseudomolecular ion was observed at m/z 177 and MS^2 ion at m/z 133 $[M + H - 44]^+$ [13]. Moreover, compound 5 was suggested to be sucrose (MS^1 at m/z 341 $[M - H]^-$, MS^2 at m/z 179 $[M - H - \text{glc.}]^-$, 161, 131) [16]. It was previously reported that L-ascorbic acid and sucrose were identified in *Hibiscus* species as *H. sabdariffa* contains higher amount of ascorbic acid compared to orange and mango [44–46].

Oleuropein (2) showed a deprotonated pseudomolecular ion at m/z 539 (MS^2 at m/z 377 $[M - H - \text{glc.}]^-$ and 307 $[M - H - \text{glc.} - C_4H_6O]^-$) [14]. It was previously reported that oleuropein was identified in *Hibiscus* [47]. Succinic acid (14) and its isomers (17, 52, 66, 70, 73, 83, 87, 90, and 103) were recognized by comparing their MS^2 fragmentation pattern with the reported data [22]. They showed a deprotonated molecular ion at m/z 117 and an intense fragment at m/z 99, attributed to the loss of water molecule [22], while compound 3 showed a protonated molecular ion at m/z 119 with

MS^2 intense fragment ion at m/z 101 $[M + H - 18]^+$, attributed to the loss of water molecule (−18 Da); thus, it was tentatively identified as succinic acid.

Hydroxycitric acid derivative (6) and butein chalcone (7) were identified by comparison with published data [13, 17], respectively. Hydroxycitric acid is the principal organic acid found in the calyces of *Hibiscus* according to [48]. Moreover, compound 38 was identified as a sesquiterpenoid derivative, gamma-eudesmol rhamnoside derivative (MS^1 at m/z 577 $[M - H]^-$, MS^2 at m/z 439 $[M - H - 138]^-$, corresponding to gamma-eudesmol, 293 $[M - H - 138 - 146]^-$, corresponding to loss of rhamnose moiety (−146 Da)) [13]. N-feruloyltyramine (10), its isomer (60), and 22-dehydrocholesterol (77) were recognized by comparing their MS/MS fragmentation pattern with the reported data [13].

3.1.9. Unidentified Compounds. Finally, seven compounds (44, 61, 80, 81, 88, 91, and 100) with the pseudomolecular ions $[M - H]^-$ at m/z 319 and 877 and $[M + H]^+$ at m/z 274, 274, 399, 515, 805, and 871, respectively, could not be identified. Furthermore, of the 103 compounds identified, 7 compounds are unidentified; 46 compounds in HdA, 42 compounds in HcA, and 25 compounds in HmA have been reported in the present study (Table 1). In conclusion, combination of accurate mass measurement and LC ability to separate isomeric compounds can be considered a powerful tool in the identification of polyphenol diversity in three species of the *Hibiscus* genus even in the absence of standards, but the stereochemical differentiation between the large number of isomers that were found in our species, for example, isomers of luteolin C-hexoside-C-pentoside, apigenin C-hexoside-C-pentoside, and cyanidin rutinoside, was not possible with our methodology.

3.2. Antioxidant Activity. It is well known that plant phenols and flavonoids in general are highly effective free radical scavengers and antioxidants. Thus, they are used for the prevention and cure of various disorders which are mainly associated with free radicals. Series of concentrations ranged from 5 to 320 $\mu\text{g/mL}$ in methanol were used. The DPPH scavenging percentage of different extracts as well as ascorbic acid and SC_{50} values (the concentration required to scavenge DPPH by 50%) are shown in Figures 2 and 3, respectively. HcA exhibited the highest antioxidant activity as indicated by its high DPPH scavenging percentage (65%) at 320 $\mu\text{g/mL}$ and low SC_{50} values ($111 \pm 1.5 \mu\text{g/mL}$). Its activity can be attributed to its contents of polyphenolic compounds such as phenolic acids, flavonoids, and anthocyanins (i.e., apigenin C-hexoside-C-pentoside, luteolin C-hexoside-C-pentoside, luteolin derivative, 4-hydroxybenzoic acid, tyrosol and peonidin derivative, and succinic acid). Unfortunately, both HdA and HmA displayed moderate antioxidant activities with $SC_{50} = 137.6 \pm 0.3$ and $135 \pm 0.5 \mu\text{g/mL}$, respectively, with ascorbic acid $SC_{50} = 14.2 \pm 0.5 \mu\text{g/mL}$ as standard.

During this work, many major anthocyanins were detected in LC-MS analysis of HcA (such as peonidin dirhamnoside, peonidin derivative, and peonidin-3-(*p*-

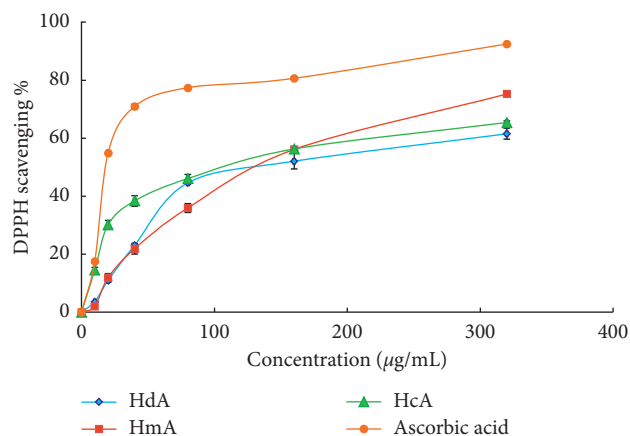


FIGURE 2: DPPH scavenging capacity of aqueous extracts of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA).

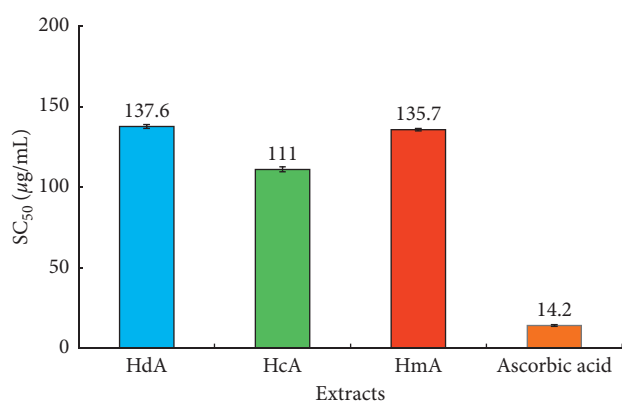


FIGURE 3: SC₅₀ of antioxidant activity of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA).

coumaroyl-glucoside)). According to [49], anthocyanin's high antioxidant activity was evidenced and is related to its structure, including the type, number, and position of the substituents in the flavylium cation. 3'-Hydroxyl group in cyanidin forms a catechol structure in the B ring, stabilizes the semiquinone radical, and forms a stable quinone that inhibits free radicals, such as DPPH, while 3',5'-dihydroxyl group of delphinidin forms a pyrogallol structure in the B ring that has more delocalized electrons to stabilize the free radical generated in the medium. Ethyl acetate fraction of HcA, in a previous study, showed more significant antioxidant activity (SC₅₀ = 17.6 ± 1.8 µg/mL) than the other extracts of HdA and HmA [5]. Many potential pathways for phenolic compounds to act as antioxidants were listed such as inhibiting free radical formation, peroxide decomposition, oxygen radical absorbance, free radical scavenging, suppression of singlet oxygen, increasing the levels of endogenous defenses, chelating of metal ions, and enzymatic inhibition [50, 51]. There is a direct relation between the total polyphenolic content and the antioxidant activity [52] as polyphenolic compounds such as phenolic acids, flavonoids, and anthocyanins may be responsible for the antioxidant activity on a large proportion [51].

Correlation between radical scavenging ability, SC₅₀ values, and the identified phenolic acids in LC-MS analysis is considerable as extracts with higher flavonoids and/or phenolics contents showed higher antioxidant activity and lower SC₅₀ value. Salem et al. [53] stated in a previous study that flowers and leaves of *H. rosa-sinensis*, *H. sabdariffa* calyx extract, and *H. platanifolius* leaves extract possessed antioxidant activity that may be attributed to anthocyanins, flavonoids, and ascorbic acid content.

3.3. Cytotoxicity Assay. About 70% of death, in low- and middle-income countries, is caused by cancer [54]. For new effective anticancer drug discovery, screening of the cytotoxic activity of the plant extracts and natural products is necessary [55]. Edible plants are excellent resources of anticancer agents [56].

In our study, *in vitro* cytotoxic activity of the applied samples against tested cell lines using MTT assay and cisplatin as a positive standard showed a decrease in cell viability in dose-dependent manner as illustrated in Figure 4 and Table 2. Evaluation was based on IC₅₀ values as follows: IC₅₀ ≤ 20 µg/mL highly active, IC₅₀ 21–200 µg/mL moderately active, IC₅₀ 201–500 µg/mL weakly active, and IC₅₀ > 501 µg/mL inactive, which is in a good accordance with the American National Cancer Institute protocol [57].

In case of A-549 cell line, the cytotoxicity of the applied extracts was arranged as follows: HdA > HmA > HcA. Unfortunately, HcA exhibited the weakest cytotoxic activity against A-549 cell line with IC₅₀ of 113 ± 3.4 µg/mL when compared to cisplatin, 7.53 ± 3.8 µg/mL. The higher activity of HdA (IC₅₀ = 50 ± 5.1 µg/mL) as a strong antioxidant may be attributed to the presence of major compounds such as butein flavonoid [58] and peonidin dipentoside anthocyanin according to Mahadevan et al. [44, 49, 59]. HdA is more cytotoxic to A-549 cells (IC₅₀ = 50 ± 5.1 µg/mL) than HmA (IC₅₀ = 60.4 ± 1.7 µg/mL) although both of them have nearly similar common major compounds, oleuropein and peonidin dipentoside.

As indicated by IC₅₀ values, the cytotoxicity of tested samples against HCT-116 cell is arranged as follow: HmA > HcA > HdA. A close cytotoxic effect on HCT-116 cell line was shown by HdA and HcA (IC₅₀ = 96 ± 3.2 and 92.9 ± 4.1 µg/mL, respectively). The cytotoxic activity of HmA may be attributed to N-feruloyltyramine as it was reported as a cytotoxic agent in a previous study [60], and this cytotoxic activity may be enhanced by the presence of ascorbic acid according to [61, 62].

Our results are in agreement with those reported for the cytotoxicity of flavonoids, phenolic acids, and terpenes content which are major constituents identified in HmA in that study [63–66]. In a previous study, different extracts (ethyl acetate, chloroform, petroleum ether) of HcA, HdA, and HmA showed strong anticancer property against human hepatocellular carcinoma (HepG2) and human breast carcinoma (MCF-7) cell lines [63]. Leaves, calyx, and stem extracts of other species of *Hibiscus* (such as *H. sabdariffa*, *H. rosa-sinensis*, *H. micranthus*, *H. vitifolius*, and *H. syriacus*) have shown promising cytotoxic activity against many

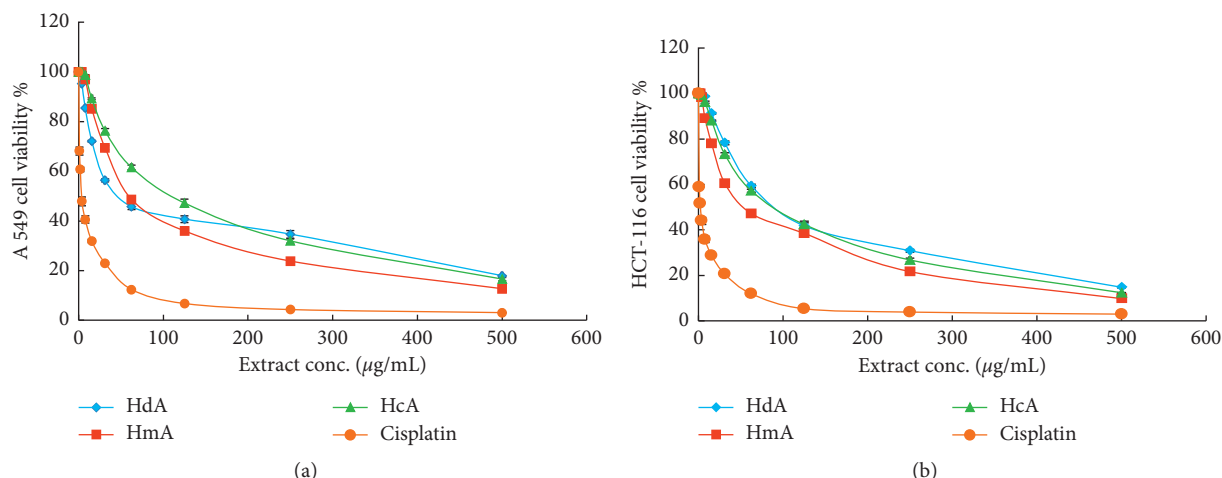


FIGURE 4: *In vitro* cytotoxic activity of aqueous extracts of tested *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA) against A-549 (a) and HCT-116 (b) cell lines.

TABLE 2: IC₅₀ of tested aqueous extracts of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA) against A-549 and HTC-116 cell lines.

Tested extracts	IC ₅₀ (μg/mL)	
	A-549 cell line	HCT-116 cell line
HdA	50 ± 5.1	96 ± 3.2
HcA	113 ± 3.4	92.9 ± 2.8
HmA	60.4 ± 1.7	56 ± 1.9
Cisplatin	7.53 ± 3.8	2.43 ± 4.1

These are the means of three determinations. The data are presented as μg/mL.

cancer cells such as breast, lung, and human leukemia cells (HL-60) and liver cancer cell lines, and they showed potent cytotoxic activity against human lung cancer cell line (A-549) that may be attributed to the presence of flavonoids, tannins, triterpenes, phenols, steroids [67, 68], polyphenolic compounds, such as protocatechuic acid, anthocyanins such as delphinidin-3-sambubioside, and myristic acid and uncarinic acid A [45, 48, 52].

3.4. Antidiabetic Assay. Diabetes mellitus (DM) is a persistent disorder that is incurable due to the deficiency of insulin that affects 10% of the population. It is expected to extend the number of diabetic individuals to 230 million in 2025. There are many side effects for drugs currently used in DM treatment, so herbal medicines are highly recommended for the treatment of diabetes instead of other synthetic drugs [3]. Since ancient times, DM has been treated orally using folklore medicine with several medicinal plants or their extracts [65].

In the present study, *in vitro* α-amylase inhibitory activity of the applied samples, evaluated using different doses (7.81–1000 μg/mL), showed significant inhibition of carbohydrate hydrolyzing enzymes (α-amylase) in dose-dependent manner as illustrated in Figures 5 and 6. HdA exhibited higher α-amylase inhibitory activity (78.85 ± 1.8%,

IC₅₀ = 56.22 ± 1.9 μg/mL) than that of HcA (68.0 ± 0.4%, IC₅₀ = 103.9 ± 1.5 μg/mL) and HmA (63.58 ± 1.9%, IC₅₀ = 149.07 ± 2.1 μg/mL) against acarbose standard with IC₅₀ = 34.71 ± 0.7 μg/mL. The higher activity of HdA (IC₅₀ = 56.22 ± 1.9 μg/mL) may be attributed to the presence of oleuropein. Jemai et al. [69] previously reported that oleuropein prevents some metabolic diseases related to oxidative stress such as diabetes, hypercholesterolemia associated with diabetes, and cardiovascular complications which are very predominant in diabetics, due to its hypoglycemic activity as it enhances peripheral glucose uptake or insulin release and stimulates the synthesis of liver glycogen through its antioxidant power.

In agreement with our results, other studies have reported that aerial parts of HdA were used as potential antidiabetics due to the presence of flavonoids [70]. The flowers and fruits of HmA were found effective in diabetes [71]. The reported hypoglycemic activity of methanol leaf extract of *H. sabdariffa* and *H. rosa-sinensis* and flowers extract of *H. vitifolius* and *H. tiliaceus* may refer to the presence of flavonoids, phenols, tannins, alkaloids, and saponins [48, 65, 72].

3.5. Antiobesity Activity. Overweight and obesity are chronic disorders that are considered as a growing issue influencing both adults and children. Obesity is defined as irregular or excessive fat accumulation caused by the imbalance between energy intake and expenditure. The vast majority of metabolic disorders such as cardiovascular disease, dyslipidemia, hypertension, and diabetes may be due to obesity or overweight [45, 73]. The inhibition of the digestion and absorption of dietary fats is a promising remedy for obesity. Natural products are preferable to obesity drugs such as orlistat which have many side effects (i.e., development of cardiovascular problems, restlessness, sleeping disorder, and stomach pain) [73].

Results of the antiobesity activity of the three *Hibiscus* species aqueous extracts grown in Saudi Arabia using *in vitro*

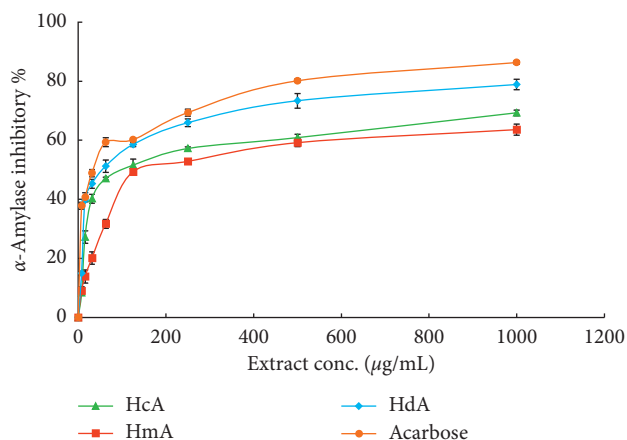


FIGURE 5: *In vitro* α -amylase inhibitory activity of aqueous extracts of tested *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA).

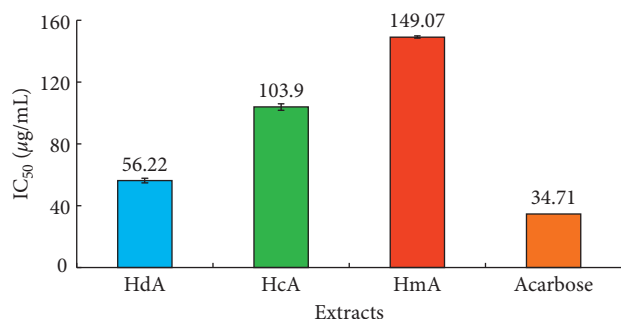


FIGURE 6: IC_{50} of α -amylase inhibitory activity of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA) aqueous extracts and standard acarbose.

pancreatic lipase inhibitory assay are shown in Figure 7 and Table 3. HdA exhibited higher inhibitory activity than the HmA and HcA with IC_{50} of 95.45 ± 1.9 , 107.7 ± 1.5 , and $>1000 \mu\text{g/mL}$, respectively, comparable with orlistat ($IC_{50} = 23.8 \pm 0.7 \mu\text{g/mL}$) as standard. Lipase inhibitory activity of HdA may be attributed to the presence of anthocyanins (peonidin-3-(*p*-coumaroyl-glucoside), peonidin dirhamnoside, peonidin glucoside feruloyl glucuronide, peonidin dipentoside, malvidin derivative, and malvidin-3-O-glucoside derivative) and organic acids (such as succinic, ascorbic, and 4-hydroxybenzoic acid). It was reported that polyphenol compounds such as anthocyanins [74] and organic acids [75] are responsible for the antiobesity activity. Da-Costa-Rocha et al. [48] stated that *Hibiscus* extract (or tea) may help in weight loss as antiobesity agent due to its effects on fat absorption-excretion, inhibition of the activity of α -amylase, starch absorption, and blocking sugars. Moreover, aqueous extract of *Hibiscus* species showed a powerful inhibition of triglyceride accumulation as whole extract was more active than isolated polyphenols.

In a previous study, aqueous extract of *H. sabdariffa* (with anthocyanins being major compounds) exhibited many potential antiobesity mechanisms, including

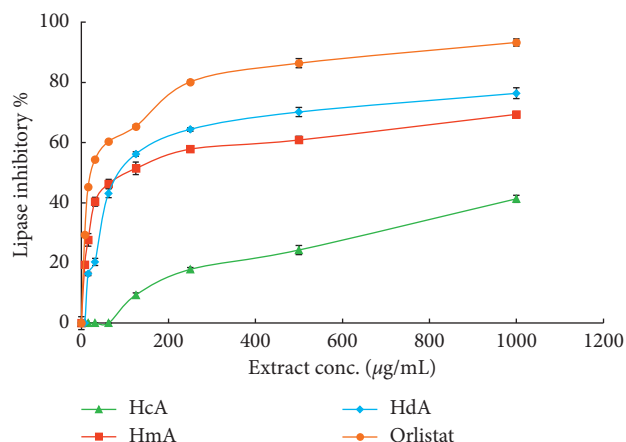


FIGURE 7: *In vitro* lipase inhibitory activity of aqueous extracts of tested *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA) in comparison with orlistat standard.

TABLE 3: IC_{50} of tested aqueous extracts of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA) against pancreatic lipase enzyme.

	IC_{50} ($\mu\text{g/mL}$)
Tested extracts	
HdA	95.45 ± 1.9
HcA	>1000
HmA	107.7 ± 1.5
Orlistat standard	23.8 ± 0.7

These are the means of three determinations. The data are presented as $\mu\text{g/mL}$.

antihyperglycemic activity, reduction in plasma cholesterol level, inhibition of gastric and pancreatic lipase enzymes, thermogenesis stimulation, inhibition of lipid droplet accumulation in fat cells, and fatty acid synthase inhibition [76]. Drinking a cup of *Hibiscus* tea after meals can reduce the absorption of dietary carbohydrates and assist in weight loss [45].

4. Conclusion

Phenolic compounds, flavonoids, and anthocyanins were identified in three different *Hibiscus* species using UPLC-ESI-MS/MS analysis. HcA showed the highest *in vitro* antioxidant activity compared with other tested extracts, and this activity can be attributed to its contents of polyphenolic compounds such as apigenin C-hexoside-C-pentoside, luteolin C-hexoside-C-pentoside, luteolin derivative, 4-hydroxybenzoic acid, and tyrosol and peonidin derivative in addition to presence of anthocyanin contents such as peonidin dirhamnoside, peonidin derivative, and peonidin-3-(*p*-coumaroyl-glucoside). HdA showed the most potent effect on human lung carcinoma (A-549) cell line, which may be attributed to the presence of major compounds such as butein flavonoid and peonidin dirhamnoside and peonidin dipentoside anthocyanins, with the highest activities as antidiabetic (due to oleuropein presence) and as antiobesity (may be attributed to the presence of major anthocyanins

such as peonidin-3-(*p*-coumaroyl-glucoside), peonidin dirhamnoside, and peonidin glucoside feruloyl glucuronide in addition to organic acids (such as succinic, ascorbic, and 4-hydroxybenzoic acid)). The results recommend that HdA need further studies for the possible use as anticancer, antidiabetic, and antiobesity agent as it might be a natural alternative remedy and nutritional policy for diabetes and obesity treatment without negative side effects. Isolation of the bioactive phytochemical from the HcA, HmA, and HdA and estimation of their biological effects are recommended in further studies.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors made considerable contributions to the manuscript. HA, ME, MA, RA, and SA designed the study. ME, WH, HA, SA, MA, and RA performed the experiments. ME, SA, HA, and WH interpreted the results. ME, HA, and SA wrote the manuscript. All authors revised the manuscript and approved it for publication.

Acknowledgments

The authors thank Prof. Dr. Mohamed Yousef, Pharmacognosy Department, College of Pharmacy, King Saud University (KSU), for the plant identification. This research was carried out with personal funds from the authors.

Supplementary Materials

Table S1: metabolites identified in the aqueous extract of three different *Hibiscus* species: *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA), using UPLC-ESI-MS in negative and positive ionization modes. Table S2: IC₅₀ of tested aqueous *Hibiscus* extracts of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA) against A-549 and HTC-116 cell lines. The data are presented as µg/mL. Table S3: IC₅₀ of tested aqueous *Hibiscus* extracts of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphyllus* (HcA) against pancreatic lipase enzyme. The data are presented as µg/mL. (Supplementary Materials)

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