Research Article

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

Hanan M. Al-Yousef 1, Wafaa H. B. Hassan, 2 Sahar Abdelaziz, 2 Musarat Amina 1, Rasha Adel, 2 and May A. El-Sayed 2

1 Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
2 Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

Correspondence should be addressed to Hanan M. Al-Yousef; halyousef@ksu.edu.sa

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

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* species suffers from insufficiency of detailed information on the phytoconstituents of aqueous extracts of *HdA, HcA, and HmA*; and (iii) to anticipate the components responsible for antioxidant, antiinflammatory, antipyretic, and anticancer activities. Literature detects a wide range of phytochemicals in HmA as flavonoids, phenolic acids, fatty alcohols, fatty acids, sitosterol, and alkanes [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 antiadipogenic 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, 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, antiadipogenic, 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, antiadipogenic, antiobesity, and anticancer activities of aqueous extracts of HdA, HcA, and HmA; and (iii) to anticipate the components responsible for antioxidant, antiadipogenic, 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 (≥99.9%), acetonitrile, water for ESI-MS analyses, reagent grade formic acid (≥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, dinitrosalicyclic acid (DNS, colour reagent), p-nitrophenyl butyrate (NPB, ≥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 (≥95%), and orlistat (≥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 quadruple 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 μm 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 μm–2.1 × 50 mm; 50 mm × 1.2 mm inner diameter; 1.7 μm particle size) at 0.2 m/mL 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%; 2–5 min, linear gradient from 10 to 30%; 5–15 min, linear gradient from 30% to 70%; 15–22 min, linear gradient from 70% to 90%; and 22–25 min, isocratic elution at 90%; 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–QqQ–LIT–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 (Rt) and mass spectrum with reported data and library search (such as FooDB (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 μg/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 λmax of 517 nm against DPPH radical without antioxidant (control) and the reference compound ascorbic acid (5, 10, 20, 40, 80, 160, and 320 μg/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:

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

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 (μg/mL) to determine scavenging capacity (SC50), 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 μg/mL gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO2 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 μg/mL to 500 μg/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% CO2 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:

\[
\text{cell viability} = \left(1 - \frac{\text{ODt}}{\text{ODc}}\right) \times 100\%,
\]

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

The relation between surviving cells and each extract concentration (1–500 μg/mL) was plotted to get the survival curve of each tumor cell line after treatment with the tested extract. The 50% inhibitory concentration (IC50), 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 ± 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 µg/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 – As/Ac) × 100, where Ac is the absorbance of control and As is the absorbance of tested extracts. Acarbose was used as a control [11]. The IC₅₀ 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 IC₅₀ 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 ± 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 µg/mL) was prepared in 0.1 mM potassium phosphate buffer (pH 6.0). Samples with different concentrations (7.81–1000 µg/mL) were preincubated with 100 µg/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 multiple-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 – As/Ac) × 100, where As is the absorbance in the presence of test substance and Ac is the absorbance of control. The IC₅₀ value was defined as the concentration of pancreatic lipase inhibitor required to inhibit 50% of its activity under the assay conditions. Estimation of IC₅₀ 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 ± SD.

3. Results and Discussion

3.1. UPLC-ESI-MS/MS. Identification of the chemical composition of the aqueous extract of the Hda, Hm, 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 (Rₜ) were identified depending on their MS² 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-H₂O]⁺ 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 MS² 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-glycosides 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 vicenin (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 MS² spectrum that confirm the mono-C-hexose-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
| Comp. no. | Compound name                        | Rt (min) | [M−H]− (m/z) | [M+H]+ (m/z) | MS2 fragments (m/z) | Hda | HcA | HmA | References |
|----------|-------------------------------------|----------|--------------|--------------|---------------------|-----|-----|-----|------------|
| 1        | L-ascorbic acid                     | 0.30     | 177          | 133, 129, 127, 113, 103, 101, 57 | √   | √   | —   | 1           |
| 2        | Oleuropein                           | 0.76     | 459          | 409, 529, 545, 377, 341, 307, 257, 215, 179 | √   | √   | —   | 2           |
| 3        | Succinic acid*                      | 0.86     | 119          | 101          | —                   | √   | —   | —   | —          |
| 4        | Tyrosol isomer                      | 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   | —   | 3           |
| 6        | Hydroxyctisic acid derivative       | 1.36     | 593          | 209          | —                   | √   | —   | —   | —          |
| 7        | Butein                              | 1.43     | 273          | 163, 143, 137 | √   | —   | —   | 4           |
| 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   | —   | 5           |
| 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-    | 6.31     | 607          | 475 (M−H−132)−, 299 (M−H−132−176)−, 179 | √   | —   | 11  | —          |
| pentoside |                      |          |              |              | —                   | √   | —   | 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-             | 7.02     | 563          | 545 (M−H−18)−, 443 (M−H−120)−, 431 (M−H−132)−, 353 (M−H−132−90)−, 341 (M−H−120−90)−, 311 (M−H−132−120)− | √   | —   | 13  | —          |
| pentoside isomer |          |          |              |              | —                   | √   | —   | 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       | Apin (apigenin-7-apiosylglucoside)  | 7.29     | 563          | 443, 413, 269 (M−H−132−162)− | √   | —   | 5, 18       |
| 23       | Apin 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, 333, 339 | √   | 13  | —   | —          |
| 31       | Luteolin derivative                 | 7.98     | 737          | 285          | —                   | 18, 21 |
| 32       | Schaftoside (apigenin 6-C-glucoside 8-C-riboside) | 7.99 | 565 | 415(M+H−150)−, 409, 391, 379, 361, 349, 337, 325, 307, 295, 273 | √   | 1, 22 |

Table 1: Metabolites identified in the aqueous extracts of *Hibiscus deflersii* (HdA), *H. micranthus* (HmA), and *H. calyphlla* (HcA) using UPLC–ESI–MS in negative and positive ionization modes.
| Comp. no. | Compound name | Rt (min) | [M−H]− (m/z) | [M+H]+ (m/z) | MS² fragments (m/z) | HdA | HcA | HmA | References |
|----------|---------------|----------|---------------|--------------|---------------------|-----|-----|-----|------------|
| 33       | 4-Hydroxybenzoic acid | 7.99     | 139           | 121, 111, 105, 97, 93, 79 | √ | | | | 1 |
| 34       | Peonidin-3-(p-coumaroyl-glucoside) | 8.27     | 609           | 301(M+H-146-162) | √ | | | | 23 |
| 35       | Kaempferol-3-(p-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           | 565, 545(M−H-18), 473(M−H-90)’, 443(M−H-132)’, 353(M−H-120-90)’, 341(M−H-132-120)’ | √ | | | | 13 |
| 37       | Kaempferol-3-O-glucoside | 8.41     | 447           | 285 | | | | | 6, 8 |
| 38       | Gamma-eudesmol rhamnoside derivative | 8.47     | 577           | 439, 397, 379, 367, 349, 321, 293, 249, 197, 127 | | | | | 1 |
| 39       | L-ascorbic acid isomer | 8.55     | 177           | 133, 129, 127, 113, 103, 101, 57 | √ | | | | 1 |
| 40       | Peonidin-3-(p-coumaryl-glucoside) isomer | 8.69     | 609           | 301(M+H-146-162) | √ | | | | 23 |
| 41       | Vicenin isomer | 8.89     | 593           | 473(M−H-120)’, 395, 383(M−H-120-90)’, 338, 327, 298 | | | | | 15, 25 |
| 42       | Apin 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-132-120)’ | √ | | | | 6, 13 |
| 44       | Unknown | 9.04     | 319           | 239, 204, 195, 97 | | | | | — |
| 45       | Kaempferol-3-(p-coumaryl-glucoside) isomer | 9.12     | 593           | 447(M−H-146)’, 327, 285(M−H-146-162) | | | | | 24 |
| 46       | Peonidin-3-(p-coumaryl-glucoside) isomer | 9.37     | 609           | 609, 579, 463(M+H-146)’, 301(peonidin)(M+H-146-162)’ | | | | | 23 |
| 47       | Methyl apigenin-C-rhamnoside-O-glucoside* | 9.38     | 593           | 327(M+H-162-104)’, 297(M+H-162-134)’ | | | | | — |
| 48       | Diosmetin-7-O-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-352feruloylglucuronide)’ | | | | | 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-O-dihexoside | 11.38    | 593           | 269(M+H-162-162) | | | | | 9 |
| Comp. no. | Compound name                                      | $R_t$ (min) | $[M-H]^-$ (m/z) | $[M+H]^+$ (m/z) | MS² 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-rhamnoglucoside 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 glucorone*          | 12.46       | 797             | 445 (M–H–176–176)$^-$, 299 (M–H–176–176–146)$^-$, 237, 205 | ✓   | —   |
| 69       | Apin 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–CO₂)$^-$, 195, 171 | ✓ ✓ | —   |
| 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       | Apin isomer                                       | 21.89       | 565             | 433 (M + H-132)$^+$, 413, 271 (M + H-162–132)$^+$ | ✓ ✓ | 5   |
| 85       | Methyl gallate                                    | 23.06       | 325             | 183 (M – H–142)$^-$ | ✓ ✓ | 29  |
| 86       | Gallocaechin 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; Rₜ: retention time; Rut.: rutinoside; Glc.: glucose; HdA: Hibiscus deltoefers; HcA: H. calyphyllus; HmA: H. micranthus.
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 a 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 (Rt 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 (Rt 7.25 min) with [M + H]− ion peak at m/z 593 was tentatively identified as kaempferol-3-O-rutinoside/luteolin-7-O-rutinoside.
Apigenin-7-O-apiosylglucoside (apin) (22) and its iso-
mer (69) were tentatively identified from the MS profile of peak at Rt 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 apin 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 MS2 base peak fragment ion at m/z 435 [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 MS2 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 (Rt 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 (Rt 9.55 min) was identified as luteolin hexoside ([M+H]+ ion at m/z 449 and MS2 ion at m/z 287 [M+H−Glucose]−). The precursor ion of compound 48 was detected at m/z 607 [M−H]− and its characteristic MS2 fragment ion at m/z 299 [M−H−2×Glucose]−, 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 MS2 fragment ion at m/z 283 [M−H−rutin]−, 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 (Rt 10.68 min) as it produced a [M+H]+ ion at m/z 579 with MS2 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 MS2 ions at m/z 269 [M−H−162−162]− was tentatively identified as apigenin-1-O-dihexoside [21]. Compound 63 [M−H]− ion at m/z 623 was tentatively identified as isorhamnetin-3-O-rutinoside. The fragmentation patterns showed MS2 fragments at m/z 315 [M−H−308]− (isorhamnetin), corresponding to loss of rutinoside (−308 Da) moiety [37]. Compound 68 with [M−H]− at m/z 797 was tentatively identified as Diosmetin rhamnoglucoside feruloyl glucuronide as it showed MS2 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 MS2 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 MS2 fragmentation pattern with the previously reported data [21]. Compound 31 with [M+H]+ at m/z 737 and MS2 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 MS2 fragment ion at m/z 285, was identified tentatively as methyl apigenin derivative. Galloclatechins derivative (86) [33] and epicatechin derivative (97) [18] were recognized by comparing their MS2 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 MS2 fragment ions at m/z 301 [M+H−176−176]−. 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 MS2 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 MS2 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 MS2 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 MS2 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 MS² fragment ions at m/z 303 [M + H -176-86]+. These were indicative of a delphinidin with glucuronic acid (176 Da) and malonyl (86 Da) moieties. This compound was suggested to be delphinidin malonyl glucuronic acid.

Compound 74 showed a molecular ion peak [M + H]+ at m/z 799 and MS² 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 MS² 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 coumaryl-glucoside moieties (−308 Da) [43]. This compound was proposed to be delphinidin-3-(p-coumaryl-glucoside) derivative [35].

Compound 78 was tentatively identified as malvidin derivative as it showed a protonated allylconone peak at m/z 331 corresponding to malvidin [43]. Compound 99 produced a [M + H]+ ion at m/z 871 with MS² 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 MS² fragment ions at m/z 295 [M - H -298]+; 277 [M - H -298-H₂O]+, 251 [M - H -298-CO₂]+, 195, and 171 were detected suggesting the presence of hydroxy-ocytadecenoic 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 MS² ion at m/z 133 [M + H -44]+ [13]. Moreover, compound 5 was suggested to be sucrose (MS¹ at m/z 341 [M - H]−, MS² at m/z 179 [M - H -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 (MS² at m/z 377 [M - H -glc]+) and 307 [M - H -glc -C₂H₄O]+) [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 MS² 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 MS² intense fragment ion at m/z 101 [M + H -18]+, attributable 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 (MS¹ at m/z 577 [M - H]−, MS² 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 HcA, 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 μg/mL in methanol were used. The DPPH scavenging percentage of different extracts as well as ascorbic acid and SC₅₀ 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 μg/mL and low SC₅₀ values (111 ± 1.5 μg/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 HcA and HmA displayed moderate antioxidant activities with SC₅₀ = 137.6 ± 0.3 and 135 ± 0.5 μg/mL, respectively, with ascorbic acid SC₅₀ = 14.2 ± 0.5 μg/mL as standard.

During this work, many major anthocyanins were detected in LC-MS analysis of HcA (such as peonidin di-rhamnoside, peonidin derivative, and peonidin-3-(p-
Correlation between radical scavenging ability, SC$_{50}$ 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 SC$_{50}$ 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 IC$_{50}$ values as follows: IC$_{50}$ $\leq$ 20 $\mu$g/mL highly active, IC$_{50}$ 21–200 $\mu$g/mL moderately active, IC$_{50}$ 201–500 $\mu$g/mL weakly active, and IC$_{50}$ $>$ 501 $\mu$g/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 IC$_{50}$ of 113 $\pm$ 3.4 $\mu$g/mL when compared to cisplatin, 7.53 $\pm$ 3.8 $\mu$g/mL. The higher activity of HdA (IC$_{50}$ = 50 $\pm$ 5.1 $\mu$g/mL) as a strong antioxidant may be attributed to the presence of major compounds such as butein flavonoid [58] andpeonidindipentosideanthocyanin according to Mahadevan et al. [44, 49, 59]. HdA is more cytotoxic to A-549 cells (IC$_{50}$ = 50 $\pm$ 5.1 $\mu$g/mL) than HmA (IC$_{50}$ = 60.4 $\pm$ 1.7 $\mu$g/mL) although both of them have nearly similar common major compounds, oleuropein and peonidin dipentoside.

As indicated by IC$_{50}$ values, the cytotoxicity of tested samples against HCT-116 cell is arranged as follows: HmA $>$ HcA $>$ HdA. A close cytotoxic effect on HCT-116 cell line was shown by HdA and HcA (IC$_{50}$ = 96 $\pm$ 3.2 and 92.9 $\pm$ 4.1 $\mu$g/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...
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 proantocyanidin, 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. tiliacus 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
pancreatic lipase inhibitory assay are shown in Figure 7 and Table 3. HdA exhibited higher inhibitory activity than the HmA and HcA with IC50 of 95.45 ± 1.9, 107.7 ± 1.5, and >1000 μg/mL, respectively, comparable with orlistat (IC50 = 23.8 ± 0.7 μg/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 antioesity activity. Da-Costa-Rocha et al. [48] stated that Hibiscus extract (or tea) may help in weight loss as antioesity 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 antioesity mechanisms, including 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 antioesity (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 phytochemicals 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)

References

[1] C. S. Kılıç, S. Aslan, and M. Kartal MandCoskun, “Fatty acid composition of Hibiscus trionum L. (Malvaceae),” Journal of Natural Products, vol. 5, pp. 65–69, 2011.
[2] N. Vasudeva and S. K. Sharma, “Biologically active compounds from the Genus Hibiscus,” Pharmaceutical Biology, vol. 46, no. 3, pp. 145–153, 2008.
[3] S. Venkatesh, J. Thilagavathi, and D. Shyam sundar, “Antidiabetic activity of flowers of Hibiscus rosasinensis,” Fitoterapia, vol. 79, no. 2, pp. 79–81, 2008.
[4] L. B. Vinh, N. T. M. Nguyet, C. D. Thanh et al., “Chemical constituents of Vietnamese mangrove Hibiscus tiliacus with antioxidant and alpha-glucosidase inhibitory activity,” Natural Product Research, pp. 1–6, 2019.
[5] N. A. Siddiqui, H. M. Al-Yousef, T. A. Alhowiriny et al., “Concurrent analysis of bioactive triterpenes oleanolic acid and β-amyrin in antioxidant active fractions of Hibiscus calyphyllus, Hibiscus deflersii and Hibiscus micranthus grown in Saudi Arabia by applying validated HPTLC method,” Saudi Pharmaceutical Journal, vol. 26, no. 2, pp. 266–273, 2018.
[6] W. H. B. Hassan, S. Abdelaziz, and H. M. Yousef, “Chemical composition and biological activities of the aqueous fraction of Parkinsonia aculeata L. growing in Saudi Arabia,” Arabian Journal of Chemistry, vol. 12, no. 3, pp. 377–387, 2019.
[7] G. C. Yen and P. D. Duh, “Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species,” Journal of Agricultural and Food Chemistry, vol. 42, no. 3, pp. 629–632, 1994.
[8] I. Gülçin, Ö. Kürus, M. Oktay, and M. E. Büyükkoçkuroğlu, “Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (Urtica dioica L.),” Journal of Ethnopharmacology, vol. 90, no. 2-3, pp. 205–215, 2004.
[9] S. M. Gomha, T. A. Salah, and A. O. Abdelhamid, “Synthesis, characterization, and pharmacological evaluation of some novel thiadiazoles and thiazoles incorporating pyrazole moiety as anticancer agents,” Monatshefte für Chemie - Chemical Monthly, vol. 146, no. 1, pp. 149–158, 2015.
[10] T. Mosmann, “Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays,” Journal of Immunological Methods, vol. 65, no. 1-2, pp. 55–63, 1983.
[11] M. B. Narkhede, P. V. Ajjimire, A. E. Wagh, M. Mohan, and A. T. Shivashanmugam, “In vitro antiobesity activity of Caesalpinia digyna (R.) methanol root extract,” Asian Journal of Plant Science and Research, vol. 1, no. 2, pp. 101–106, 2011.
[12] Y. S. Kim, Y. M. Lee, H. Kim, J. Kim, D. S. Jang, and J. H. Kim, “Anti-obesity effect of Morus bombycis root extract: anti-li-pase activity and lipolytic effect,” Journal of Ethnopharmacology, vol. 130, no. 3, pp. 621–624, 2010.
[13] FooDB, “The Metabolomics Innovation Centre,” 2019, https://foodb.ca/.
[14] H. Zemmouri, S. Ammar, A. Boumendjel, M. Messarah, and A. El Feki, “Chemical composition and antioxidant activity of Borago officinalis L. leaf extract growing in Algeria,” Arabian Journal of Chemistry, vol. 12, no. 8, pp. 1954–1963, 2019.
[15] M. Lambert, E. Meudec, A. Verbaere et al., “A high-throughput UHPLC-QqQ-MS method for polyphenol profiling in rosé wines,” Molecules, vol. 20, no. 5, pp. 7890–7914, 2015.
[16] S. Mocco, Metabolomics Technologies Applied to the Identification of Compounds in Plants: A Liquid Chromatography-Mass Spectrometry-Nuclear Magnetic Resonance Perspective over the Tomato Fruit, Ph.D. thesis, Wageningen University, Wageningen, The Netherlands, 2007.
[17] I. M. Shi, M. S. Ali-Shtayeh, R. M. Jamous, and D. Arráez-Román, “HPLC-DAD-ESI-MS/MS screening of bioactive components from Rhus coriaria L. (Sumac) fruits,” Food Chemistry, vol. 166, pp. 179–191, 2015.
high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS),” Food Chemistry, vol. 169, pp. 336–343, 2015.

[19] E. Emile Nicoué, "Identification des anthocyanes de deux espèces de bleuets sauvages du Québec "Vaccinium angus-

tifolium" et "Vaccinium myrtilloides" et évaluation de leur potentiel antioxidant," Université Laval Quebec, Canada,

Doctoral dissertation, 2010.

[20] Q. Chang and Y. S. Wong, "Identification of flavonoids in hamketaub beans (Vigna sinensis) by high-performance liquid

chromatography–electrospray mass spectrometry (LC-ESI/MS),” Journal of Agricultural and Food Chemistry, vol. 52,

no. 22, pp. 6694–6699, 2004.

[21] R. O. Bakr, S. A. E. H. Mohamed, and N. Ayoub, "Phenolic

profile of Centaurea aegyptiaca L. growing in Egypt and its
cytotoxic and antiviral activities,” African Journal of Traditional, Complementary and Alternative Medicines, vol. 13,
no. 6, pp. 135–143, 2016.

[22] O. Al Kadhi, A. Melchini, R. Mithen, and S. Saha, “Develop-
oment of a LC-MS/MS method for the simultaneous detec-
tion of tricarboxylic acid cycle intermediates in a range of
biological matrices,” Journal of Analytical Methods in Chemistry, vol. 2017, Article ID 5391832, 12 pages, 2017.

[23] F. Ferreres, C. Grosso, A. Gil-Izquierdo, P. Valentão, and

C. Azevedo, “HPLC-DAD-ESI/MSn analysis of phenolic compounds for quality control of Grindelia robusta Nutt. and
bioactivities,” Journal of Pharmaceutical and Biomedical Analysis, vol. 94, pp. 163–172, 2014.

[24] A. Sinela, N. Rawat, C. Mertz, N. Achir, and M. Fulcrand

HandDornier, “Anthocyanins degradation during storage of Hibiscus sabdariffa extract and evolution of its degradation
products,” Food Chemistry, vol. 214, pp. 234–241, 2017.

[25] S. Ammar, M. Del Mar Contreras, O. Belguith-Hadrich,

A. Segura-Carretero, and M. Bouaziz, “Assessment of the
distribution of phenolic compounds and contribution to the
antioxidant activity in Tunisian fig leaves, fruits, skins and
pulps using mass spectrometry-based analysis,” Food and
Function, vol. 6, no. 12, pp. 3663–3677, 2015.

[26] D. Barreca, E. Bellocco, C. Caristi, and U. Leuzzi, “Flavonoid
profile and radical-scavenging activity of Mediterranean sweet
lemon (Citrus limetta) juice,” Food Chemistry, vol. 129,
no. 2, pp. 417–422, 2011.

[27] R. M. IbrahimLin, A. M. El-Halawany, D. O. Saleh et al.,
“HPLC-DAD-MS/MS profiling of phenolics from Securigera
securidaca flowers and its anti-hyperglycemic and anti-
hyperlipidemic activities,” Revista Brasileira de Farmacognosia,
vol. 25, no. 2, pp. 134–141, 2015.

[28] I. C. Rodríguez-Medina, R. Beltrán-Debón, V. M. Molina
et al., “Direct characterization of aqueous extract of Hibiscus
sabdariffa using HPLC with diode array detection coupled to
ESI and ion trap MS,” Journal of Separation Science, vol. 32,
no. 20, pp. 3441–3448, 2009.

[29] M. Simirgiotis, J. Benites, and C. Areche, “Antioxidant ca-
pacities and analysis of phenolic compounds in three endemic
nolana species by HPLC-PDA-ESI-MS,” Molecules, vol. 20,
no. 6, pp. 11490–11507, 2015.

[30] U. Shin, “Negative atmospheric pressure chemical ionisation
low-energy collision activation mass spectrometry for the characterisation of flavonoids in extracts of fresh herbs,”
Journal of Chromatography A, vol. 902, no. 2, pp. 369–379,
2000.

[31] V. Spinola, J Pinto, and P. C. Castilho, “Identification and quantification of phenolic compounds of selected fruits from
Madeira Island by HPLC-DAD-ESI-MSn and screening for
their antioxidant activity,” Food Chemistry, vol. 173, pp. 14–
30, 2015.

[32] C. A. Ledesma-Escobar, F. Priego-Capote, and M. D. Luque
De Castro, “Characterization of lemon (Citrus limon) polar
extract by liquid chromatography-tandem mass spectrometry
in high resolution mode,” Journal of Mass Spectrometry,
vol. 50, no. 11, pp. 1196–1205, 2015.

[33] M. B. Hossain, D. K. Rai, N. P. Brunton, A. B. Martin-Diana,
and B. R. Catherine, “Characterization of phenolic compo-
sition in lamiaceae species by LC-ESI-MS/MS,” Journal of
Agricultural and Food Chemistry, vol. 58, no. 19, pp. 10576–
10581, 2010.

[34] R. Colombo, J. H. Yariwake, and M. McCullagh, “Study of C-
and O-glycosylflavonones in sugar cane extracts using liquid
chromatography–exact mass measurement mass spectrome-
try,” Journal of the Brazilian Chemical Society, vol. 19, no. 3,
pp. 483–490, 2008.

[35] R. Stein-Chisholm, J. Beaulieu, and C. Grimm, “LC-MS/MS
and UPLC-UV evaluation of anthocyanins and anthocyan-
dins during rabbiteye blueberry juice processing,” Beverages,
vol. 3, no. 4, p. 56, 2017.

[36] A. Brito, A. Gattuso, J. Ramirez, C. Areche, B. Sepúlveda, and
M. Simirgiotis, “HPLC-UV-MS profiles of phenolic com-
pounds and antioxidant activity of fruits from three citrus
species consumed in northern Chile,” Molecules, vol. 19,
no. 11, pp. 17400–17421, 2014.

[37] M. P. Rodríguez-Rivera, E. Lugo-Cervantes, and
P. Winterhalter, “Metabolite profiling of polyphenols in peels of Citrus limetta Risso by combination of preparative high-
speed countercurrent chromatography and LC-ESI-MS/MS,”
Food Chemistry, vol. 158, pp. 139–152, 2014.

[38] M. A. Farag, S. T. Sakna, N. M. El-Fiky, M. M. Shabana, and
L. A. Wessjohann, “Phytochemical, antioxidant and antidi-
abetic evaluation of eight Bauhinia L. species from Egypt
using UHPLC-PDA-qTOF-MS and chemometrics,” Phyto-
chemistry, vol. 119, pp. 41–50, 2015.

[39] A. A. Chernonosov, E. A. Karpova, and E. M. Lyakh,
“Identification of phenolic compounds in Myrciaria bracteata
leaves by high-performance liquid chromatography with a
diode array detector and liquid chromatography with tandem
mass spectrometry,” Revista Brasileira de Farmacognosia,
vol. 27, no. 5, pp. 576–579, 2017.

[40] W. Y. Sayadi, J. S. Jin, Y. A. Cho et al., “Determination of polyphenols in three Capsicum annuum L. (bell pepper)
varieties using high-performance liquid chromatography-
tandem mass spectrometry: their contribution to overall
antioxidant and anticancer activity,” Journal of Separation
Science, vol. 34, no. 21, pp. 2967–2974, 2011.

[41] K. Ablajan, Z. Abliz, X.-Y. Shang, J.-M. He, and R.-P. Zhang,
“Structural characterization of flavonol 3,7-di-O-glycosides
and determination of the glycosylation position by using
negative ion electrospray ionization tandem mass spec-
trometry,” Journal of Mass Spectrometry, vol. 41, no. 3,
pp. 352–360, 2006.

[42] R. Wang, M. Ye, H. Guo, K. Bi, and D.-a. Guo, “Liquid
chromatography/electrospray ionization mass spectrometry
for the characterization of twenty-three flavonoids in the
extract of Dalbergia odorifera,” Rapid Communications in
Mass Spectrometry, vol. 19, no. 11, pp. 1557–1565, 2005.

[43] A. Andrade, R. Heydari, Z. Talebpour et al., “Study of new
extraction methods for separation of anthocyanins from red
grape skins: analysis by HPLC and LC-MS/MS,” Journal of
Liquid Chromatography & Related Technologies, vol. 31,
no. 17, pp. 2686–2703, 2008.
[74] T.-W. Huang, C.-L. Chang, and E.-S. Kao, “Effect of Hibiscus sabdariffa extract on high fat diet-induced obesity and liver damage in hamsters,” *Food & Nutrition Research*, vol. 59, no. 1, Article ID 29018, 2015.

[75] D. Abdalla, I. F. Peréz-Ramírez, J. Pérez-Jiménez, and G. M. R. Nava, “Comparison of the bioactive potential of roselle (*Hibiscus sabdariffa* L.) calyx and its by-product: phenolic characterization by UPLC-QTOF MS and their anti-obesity effect in vivo,” *Food Research International*, vol. 126, Article ID 108589, 2019.

[76] J. W. Yun, “Possible anti-obesity therapeutics from nature - a review,” *Phytochemistry*, vol. 71, no. 14-15, pp. 1625–1641, 2010.