Phytochemical investigation of unused parts of *Hibiscus sabdariffa*

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Abstract: Twelve compounds have been isolated from the unused parts of *Hibiscus sabdariffa*. The isolated compounds were identified as oleic acid (**1**), *β*-sitosterol (**2**), lupeol (**3**), oleanolic acid (**4**), betulinic acid (**5**), 5α, 8α-Epidioxyergosta-6,22-dien-3β-ol (**6**), 5'-Methoxy Propacin (**7**) Aquillochin (**8**), *β*-sitosterol glucoside (**9**), 5,8-dihydroxy dodeca-5,7-dienedioic acid (**10**), gallic acid (**11**) and kaempferol 3-*O*-(6-*O*-trans-*p*-coumaroyl)-*β*-D-glucopyranoside (trans tiliroside) (**12**). The chemical identity of these compounds was elucidated based on spectroscopic data (NMR, UV, MS and IR spectra). This is the first report to indicate isolation of these compounds from *H. Sabdariffa* (except *β*-sitosterol). Compounds **7**, **8**, **11** and **12** displayed a remarkable antioxidant activity compared to ascorbic acid.
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1. Introduction

Medicinal plants constituents are known to be localized in one or more of plant organs e.g. leaves, flowers, bark……ect. [1], while significant amounts remained untapped due to lack of proper management. For example *Hibiscus sabdariffa* in which the calyx of the flower is the main part being used to obtain the active constituents. The unused portion of the medicinal plants represents a serious environmental hazard due to lack of the proper disposal. Some being used as animal feed or dumped in the fields and water ways.

Previous phytochemical studies on *Hibiscus* genus have reported the presence of steroid, triterpene, flavanoid, coumarinolignan, naphthoquinones and naphtopyranes [2-6]. However anthocyanin, flavanoids, hydroxy cinnamic acids were found to be the main active constituents of *H. sabdariffa* isolated from calyx and epicalyx extracts [7-9].

H. sabdariffa extracts have demonstrated to have a broad range of therapeutic effects [10] such as hepatoprotective [11], antioxidant [12-13], anti-obesity [14], anticholesterol [15], anticancer [16], inhibition of the contractility of rat bladder and uterus [17], antibacterial [18], and antihypertensive [19].

This research was conducted to isolate the chemical constituents of the unused parts of *H. sabdariffa* and evaluate its potential use in pharmacy and medicine.

2. Results and discussion

Twelve compounds have been isolated from the aerial parts (except calyx and epicalyx) of *H. sabdariffa*. Compounds **1**, **2**, **3**, **4**, **5**, **9**, **11** and **12** (Figure 1) were identified by comparative study to those cited in the literature [20-23]. These compounds are oleic acid (**1**), *β*-sitosterol (**2**), lupeol (**3**), oleanolic acid (**4**), betulinic acid (**5**), *β*-sitosterol glucoside (**9**), gallic acid (**11**) and kaempferol 3-*O*-(6-*O*-trans-*p*-coumaroyl)-*β*-D-glucopyranoside (trans tiliroside) (**12**).

Compound (**6**) was isolated as rosette crystals. APT spectrum revealed the presence of 28 carbon atoms which suggested that compound (**6**) is a steroidal skeleton of the cholestane type [24]. It showed four methine carbon signals due to two disubstituted double bonds at δ_C 135.5 (C_6), 135.3 (C_{22}), 132.3 (C_{23})and 130.9 (C_7). ^{1}H-NMR spectrum showed two proton signals at δ_H 6.23 (H_6, *d*, *J* = 8.2), and 6.49 (H_7, *d*, *J* = 8.2) indicating isolated double bond. Although there are several possible positions e.g. C_4, C_5 and C_{22}, for a double bond in the framework of steroids, the chemical shift and *cis* coupling of these protons were indicative of the presence of C_6 double bond with 5,8-epidioxy functionality [25]. The second disubstituted double bond was assigned to the side chain double bond at C_{22}. This is evidenced by the downfield proton signals at δ_H 5.14 (1H, *dd*, *J* = 15.1, 8.2), 5.24 (1H, *dd*, *J* = 15.3, 7.4) which were assigned for the *trans* H_{23} and H_{22}, respectively. Based on these findings, the structure of **6** was defined as 5,8-epidioxysteroid possessing two double bonds at C_6 and C_{22}. The carbon signal at 66.6 ppm was assigned to C_3 as indicated from its proton multiplet at δ_H 3.94.

The structure of compound (**6**) was confirmed by comparison with reported data [26-27] as 5α, 8α-Epidioxyergosta-6,22-dien-3β-ol. This is

the first report of the isolation of this compound from family malvaceae.

Compound (**7**) was isolated as white amorphous powder. The presence of an oxygenated coumaryl skeleton was suggested from the UV maxima at 321 and 223 nm [28]. This was supported by the presence of two proton doublets at δ_H 6.22 and 7.81 (J = 9.6 Hz) assigned to H_3 and H_4 of the α-pyrone ring system. APT spectrum (Table 1) revealed the presence of 9-carbon signals besides the coumarin chromophore. ^{1}H-NMR spectrum (Table 1) suggested the presence of a tri-substituted symmetric aryl moiety as revealed from a proton singlet at δ_H 6.69 (2H). The presence of three aromatic methoxyl groups was confirmed from ^{1}H-NMR, APT spectrum and HMBC correlations (Table 1).

APT spectrum showed two oxygenated methine carbons at δ_C 81.2 ($C_{7'}$) and 74.0 ($C_{8'}$) and a methyl at δ_C 16.6 ($C_{9'}$) indicating a propane moiety. This was confirmed from the presence of a proton doublet at δ_H 4.65, J = 8.2, a proton multiplet at δ_H 4.29 and a methyl doublet at 1.16, J = 6.4. Furthermore, ^{1}H-^{1}H COSY correlated the proton signal at δ_H 4.65 ($H_{7'}$) with the proton signal at δ_H 4.29 ($H_{8'}$) and the latter with the proton signal at δ_H 1.16 ($3H_{9'}$). Taken together, these data indicated the presence of a phenyl propanoid moiety. HMBC correlations (Table 1) between the proton signal at δ_H 4.65 ($H_{7'}$) with carbon signal at δc 105.4 ($C_{2'/6'}$) and the proton signal at δ_H 6.69 ($H_{2'/6'}$) with carbon signals at δ_C 81.2 ($C_{7'}$) confirmed the presence of benzodioxan moiety for a coumarino-lignan. Other HMBC correlations between the proton signal at δ_H 6.75 (H_5) with carbon signal 144.9 (C_4) confirmed its position. The *trans* relationship of the aryl and methyl groups was deduced from the coupling constant of 8.2 Hz between $H_{7'}$ and $H_{8'}$ as well as from the biosynthetic consideration [29].

From the above data and through comparison with published literature data [5, 29-30], compound **7** is identified as 5'-methoxy propacin (Jatrocin B). This is the first report of the isolation of this compound from *H. sabdiffira*. It was previously isolated from *H. syriacus* [5], *Jatropha gossypifolia*, family Euphorbiaceae [31] and roots of *Mondia whitei,* family Periplocaceae [30].

Compound (**8**) was isolated as white amorphous powder. The UV absorptions maxima of **8** at 327 and 217 nm supported its oxygenated coumaryl skeleton [28]. It was clear from its ^{1}H and ^{13}C-NMR data (Table 1) that it is identical structure to **7** except that the methyl group at $C_{8'}$ is replaced by a hydroxy methylene group as suggested from the carbon signal at δ_c 60.4 ($C_{9'}$). This was confirmed from the two proton signals at δ_H 3.50 ($H_{a9'}$, *dd*, J = 13.3, 3.6 Hz) and 3.80 ($H_{b9'}$, *dd*, J = 13.3, 2.7 Hz). Further confirmation was done using the HMBC correlations (Table 1) that correlated the protons $H_{a9'}$ and $H_{b9'}$ with the carbon signal at δ_C 77.5 ($C_{7'}$) confirming its position.

Thus, compound (**8**) is identified as the known compound aquillochin, which is previously isolated from *H. syriacus* [5] and *H. tiliaceus* [32]. However, this is the first report of its isolation from *H. sabdiffira*.

Compound (**10**) was isolated as white amorphous powder. Its molecular formula is $C_{12}H_{18}O_6$, established on the basis of GC-MS showing molecular ion peak at 258. IR (KBr, ν_{max}) spectrum showed characteristic absorption bands for aliphatic acids at 3084, 2921, 1671, 1426 and 1276 cm^{-1} [21]. ^{1}H-NMR and APT spectra of **10** suggested a symmetric structure as revealed from two proton signals at δ_H 6.71 (s, 2H) and 2.50 (brs, 12H) and four carbon signals at δ_c 175.0 (s, 2C), 167.2 (s, 2C), 134.2 (CH, 2C), 28.9 (CH_2, 6C). The structure was confirmed by ^{1}H-^{1}H COSY and HMBC as 5,8-dihydroxy dodeca-5,7-dienedioic acid. It is the first report for the isolation of this compound from family Malvaceae.

2.1. Biological activity

Different plant extracts and compounds **3, 4**, **5**, **6**, **7**, **8**, **10, 11** and **12** were subjected to free radical scavenging assay for evaluating their antioxidant activity [33]. The assay employs a radical cation derived from ABTS (azino-bis-3-ethyl benzthiazoline-6-sulfonic acid) as a stable free radical to assess antioxidant activity of different extracts. The antioxidant activities of different isolated compounds and extracts are cited in Table 2. It revealed that the phenolic compounds; **7**, **8**, **11** and **12** displayed a high antioxidant activity compared to the aliphatic acids, triterpene and steroids. The activities of the phenolic compounds and the extracts are comparable to the well known; ascorbic acid.

Table 1. ^{1}H and APT spectral data for compound **7** and **8**

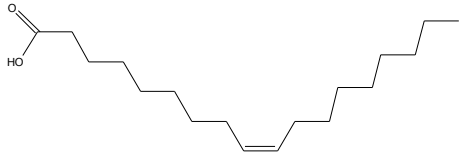
	7			8		
C/H No.	^{13}C*	^{1}H*	HMBC	^{13}C*	^{1}H*	HMBC
2	160.8, s	------		161.7, s	------	
3	113.3, CH	6.22, d (9.6)	2, 10	113.5, CH	6.28, d (9.6)	2, 10
4	144.9, CH	7.81, d (9.6)	2, 5, 9	144.7, CH	7.64, d (9.6)	2, 5, 6
5	100.8, CH	6.75, s	4, 6, 9	100.2, CH	6.50, s	4, 6,7, 9
6	145.9, s	------		146.1, s	------	
7	137.9, s	------		137.9, s	------	
8	132.0, s	------		132.1, s	------	
9	138.5, s	------		138.6, s	------	
10	111.7, s	------		111.5, s	------	
11	55.7, CH_3	3.74, s	6	56.3, CH_3	3.82, s	6
1'	126.6, s	------		126.0, s	------	
2', 6'	105.4, CH	6.69, s	1', 2', 3', 4', 5', 6', 7'	104.6, CH	6.61, s	1', 2', 3', 4', 5', 6', 7'
3', 5'	148.3, s	------		147.7, s	------	
4'	136.4, s	------		135.7, s	------	
7'	81.2, CH	4.65, d (8.2)	1', 2', 6', 8'	77.5, CH	4.95, d (8.2)	1', 2', 6', 8'
8'	74.0, CH	4.29, m		78.8, CH	4.04, dd (8.2, 1.4)	
9'	16.6, CH_3	1.16, d (6.4)	7', 8'	60.4, CH_2	3.50, dd(13.3, 3.6) 3.80, dd (13.3, 2.7)	7'
10', 11'	55.9, CH_3	3.74, s	3', 5'	56.3, CH_3	3.82, s	3', 5'

*The chemical shift (δ) is expressed in ppm and coupling constant (*J*) in Hz.
^{13}C and ^{1}H are measured in $CDCl_3$- CD_3OD at 100 MHz and 400 MHz, respectively.

Table 2. Results of antioxidant screening of different extracts and compounds of *H. sabdariffa*

Fractions /Compounds	% inhibition
Hexane	19.85
Methylene chloride	86.14
Ethyl acetate	84.83
Total	86.42
Compound **3**	4.30
Compound **4**	2.05
Compound **5**	2.99
Compound **6**	2.62
Compound **7**	80.84
Compound **8**	80.14
Compound **10**	2.43
Compound **11**	88.57
Compound **12**	86.89
Ascorbic acid (standard)	87.26

Figure (1). Structures of isolated compounds



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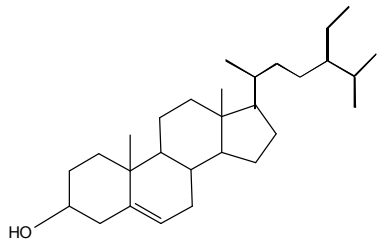

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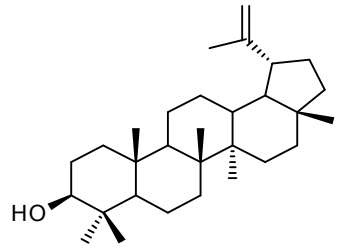

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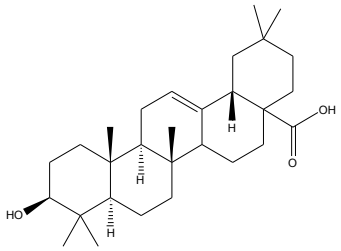

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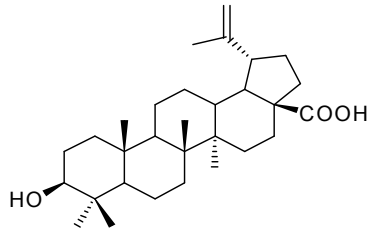

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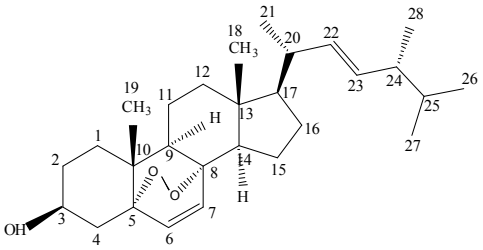

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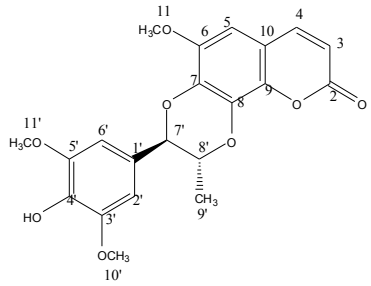

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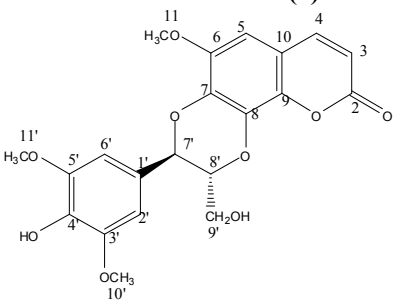

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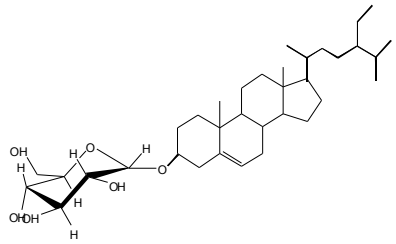

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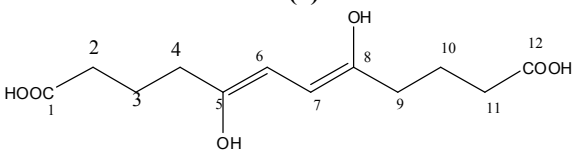

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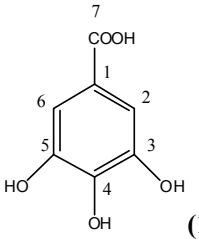

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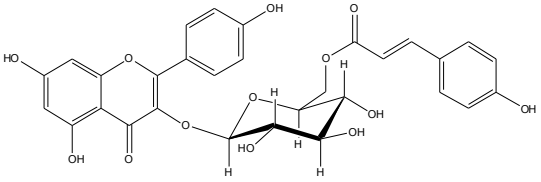

(12)

3. Experimental

3.1. General

^{1}H and ^{13}C NMR spectra were recorded on JOEL Spectrophotometer (400 and 100 MHz for ^{1}H and ^{13}C respectively), Bruker DPX-400 spectrometer. Melting point apparatus (Fisher-johns scientific Co., USA). GC-MS was carried out on (JOEL JMS-600 spectrometer, Japan). ABTS (2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) from Sigma Chemicals Co., St. Louis, USA. Ascorbic acid (Cevarol®) tablets from Memphis Pharmaceutical Co., Cairo, Egypt. All other chemicals used were of analytical grade.

3.2. Plant material

Hibiscus sabdariffa waste represented by all the aerial parts except the calyx and epicalyx was collected in December 2010 from crops grown at Faculty of Pharmacy fields. The plant was identified by Prof. Ibrahim Mashaly, Systematic Botany Department, Faculty of Sciences, Mansoura University. A voucher specimen is kept in Pharmacognosy Deptartment, Faculty of pharmacy, Mansoura University.

3.3. Extraction and isolation procedures

Dried powdered plant (3500 g) was percolated with MeOH till exhaustion at room temperature. The combined extracts were collected and evaporated to dryness under reduced pressure at 40 ^{o}C. The residue, 193 g, was suspended in distilled water and extracted successively with pet. ether, methylene chloride and EtOAc. The different extracts were evaporated under reduced pressure to obtain pet. ether fraction (fraction A, 40g), methylene chloride fraction (fraction B, 16g) and EtOAc fraction (fraction C, 14.5g).

3.3.1. Isolation of compounds

Fraction A was subjected to silica gel column chromatography and eluted with n-hexane – EtOAc gradient (0:30%). Similar fractions were pooled based on similar R_f values. These fraction are purified by chromatographic and repeated crystallization to afford oleic acid **1** (50 mg), β-sitosterol **2** (75 mg), lupeol **3** (15 mg), oleanolic acid **4** (16 mg), betulinic acid **5** (6 mg) and 5α, 8α-Epidioxyergosta-6,22-dien-3β-ol **6** (16 mg).

Fraction B was subjected to silica gel column chromatography and eluted with n-hexane – methylene chloride gradient (0:100%) followed by methylene chloride - MeOH (0:100%). The latter elution afforded fractions which were collected based on similar R_f values. These fraction are purified by chromatographic and repeated crystallization methods to afford 5'-methoxy propacin **7** (7 mg), Aquillochin **8** (25 mg) and β- sitosterol glucoside **9** (45 mg).

Fraction C (14.5 g) was subjected to silica gel column using n-hexane- EtOAc (50:100%), and EtOAC - MeOH (0:60%). Similar fractions were pooled based on similar R_f values. Collected fractions were subjected to purification through chromatography and repeated crystallization to afford 5,8-dihydroxy dodeca-5,7-dienedioic acid **10** (38 mg), gallic acid **11** (10mg) and trans tiliroside **12** (7 mg).

3.3.2. Identification of the isolated compounds

5α,8α-epidioxyergosta-6,22-dien-3β-ol (6), It was shown as single spot (R_f 0.29) on precoated silica gel plates GF_{254} using 30% EtOAC /pet. ether and colored violet upon spraying with vanillin/ H_2SO_4 spray reagent and heating at 110 ˚C for 1 min., m.p. 182-185 ˚C. ^{1}H-NMR ($CDCl_3$, 400 MHz, δ ppm): 3.94 (m, H-3), 6.49 (*d*, *J* = 8.2 Hz, H-6), 6.23 (*d*, *J* = 8.2 Hz, H-7), 0.81 (s, H-18), 0.86 (s, H-19), 0.98 (*d*, *J* = 6.4 Hz, H-21), 5.24 (*dd*, *J* = 15.3, 7.4 Hz, H-22), 5.14 (*dd*, *J* = 15.1, 8.2 Hz, H-23), 0.89 (*d*, *J* = 6.8 Hz, H-27). ^{13}C-NMR ($CDCl_3$, 100 MHz, δ ppm): 34.8 (C-1), 30.2 (C-2), 66.6 (C-3), 37.0 (C-4), 82.2 (C-5), 135.5 (C-6), 130.9 (C-7), 79.6 (C-8), 51.1 (C-9), 37.0 (C-10), 23.5 (C-11), 39.4 (C-12), 44.6 (C-13), 51.7 (C-14), 20.7 (C-15), 28.7 (C-16), 56.3 (C-17), 13.0 (C-18), 18.3 (C-19), 39.8 (C-20), 21.0 (C-21), 135.3 (C-22), 132.3 (C-23), 42.8 (C-24), 33.1 (C-25), 19.7 (C-26), 20.0 (C-27), 17.6 (C-28).

5'-methoxy propacin (7), white amphorous powder, m.p. 243-245˚C; UV λ_{max} (MeOH) 321, 223; IR (KBr, ν_{max}): 3408, 2924, 1711, 1614, 1522, 1116, 1040 cm^{-1}; ^{1}H and ^{13}C NMR, see table 1.

Aquillochin (8), white amphorous powder, m.p. 255-257˚C; UV λ_{max} (MeOH) 327, 217; IR (KBr, ν_{max}): 3392, 2924, 1699, 1614, 1524, 1118, 1030 cm^{-1}; ^{1}H and ^{13}C NMR, see table 1.

5,8-dihydroxy dodeca-5,7-dienedioic acid (10), white amphorous powder**,** m.p. 189-191 ˚C IR (KBr, ν_{max}): 3084, 2921, 1671, 1426, 1276 cm^{-1}**.** ^{1}H-NMR ($CDCl_3$, 400 MHz, δ ppm): 6.71 (s, H-6, 7), 2.50 (s, H-2, 3, 4, 9, 10, 11). ^{13}C-NMR ($CDCl_3$, 100 MHz, δ ppm): 175.0 (C-1, 12), 167.2 (C-5, 8), 134.2 (C-6, 7), 28.9 (C- 2, 3, 4, 9, 10, 11).

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