RJC

RASĀYAN *J. Chem.*
Vol. 10 | No. 4 |1391-1401 | October - December | 2017
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

INVESTIGATION OF SOME CHEMICAL CONSTITUENTS, CYTOTOXICITY AND ANTIOXIDANT ACTIVITIES OF *Beta Vulgaris* var. *altissima* CULTIVATED IN EGYPT

S. S. El-Hawary[1], F. M. Hammouda[2], W. A. Tawfik[2], H. A. Kassem[1], K. A. Abdelshafeek[2] and S. S. El-Shamy[3*]

[1]Pharmacognosy Department, Faculty of Pharmacy, Cairo University,
Kasr El-Aini Street, 11562 Cairo, Egypt.
[2]Phytochemistry Department, National Research Centre (NRC), 12311 Dokki, Cairo, Egypt.
[3]Pharmacognosy Department, Faculty of Pharmacy,
Modern University for Technology and Information, Cairo, Egypt.
*E-mail : s_elshamy@hotmail.com

ABSTRACT

The phenolic compounds were isolated for the first time from the alcoholic extract of the non-flowering aerial parts of *Beta vulgaris* var. *altissima* (sugar beet) and were identified as: Apigenin, Luteolin, Apigenin 7-*O*-di-glucoside, Luteolin 4'-*O*-rhamnoside, Caffeoyl-6-(3,4-dihydroxy benzoyl) *β*-D-glucoside, Apigenin 5-*O*-rhamnoside, Isoscutellarein 7-*O*-glucosyl- 8-*O*-xyloside and 5, 7, 5'-trihydroxy-3'-methoxy flavone 4'-*O*-glucoside. The structures of the isolated compounds were elucidated through chromatographic and spectroscopic measurements. The *n*-hexane extract of the plant was fractionated to fatty acid methyl esters and unsaponifiable matter. The gas-liquid chromatography analysis of fatty acids methyl esters proved the presence of 13 fatty acids of which eicosenoic acid $C_{20:1}$ (41.38%) was the main acid, while the gas liquid chromatography of the unsaponifiable matter substantiated the presence of a mixture of 28 components in which campesterol (23.85%), *n*-triacontane C_{30} (12.71%) and cholesterol (8.77%) were the major components. The ethyl acetate fraction exhibited the highest antioxidant activity (62.65%) with effective concentration 57.2 µg/ml using radical scavenging activity. The percent proliferation of human hepatocellular carcinoma was reduced to 69.5% and 72.4% on treatment with the ethyl acetate and the methanol extracts respectively at concentration 100 µg/ml.

Keywords: *Beta vulgaris*, Chenopodiaceae, phenolic compounds, fatty acids, antioxidants, HepG2.

INTRODUCTION

Beta vulgaris subspecies *vulgaris* var. *altissima* (BVVA) (family Chenopodiaceae) which is known as sugar beet is native to the coasts of the Mediterranean, widely cultivated in Europe and America.[1,2] Chenopodiaceae family contains about 1400 species in 105 genera.[3] Beets were cultivated to use their roots in food while the foliages were fed to domestic animals. Napoleon was the first to pronounce that beet can be used as an alternative for sugar production.[4]

In traditional Chinese medicine, the roots of sugar beet have been listed as a medicinal herb, which has been known to exhibit sedative and emmenagogue-like effects.[5] It is used as a vegetable and food garnish in Japanese-style dishes.[6] In Egypt, BVVA was cultivated in 1982 in an area of 16943 feddan increased to 235259 at 2008 with a mean production of 50700 tons sugar.[7] The roots of sugar beets have been used industrially as a raw material for sugar. The aerial parts of sugar beets are used in feeding animals.[8]

The fixed oil of the leaves of sugar beet contains undecane and undecane 2,6-dimethyl and that of the fruit contains hexadecanoic acid methyl ester and 9-octadecenoic acid,[9] while the hexane extract of the seeds contains squalene, β-amyrine, cycloartenol, 24-Methylenecycloartanol, cholesterol, 24-Methylenecholesterol, campesterol, stigmasterol, ergost-7-en-3β-ol, stigmast-9(11)-en-3β-ol, spinasterol, sitosterol, stigmasterol, 22-dihydrospinasterol and avenasterol.[10] The sugar beet root was found to contain inositol, galactinol, and dehydrodiferulates (arabinan and galactan side chains esterified by ferulic acid

http://dx.doi.org/10.7324/RJC.2017.1041936

residues.[11, 12] It was found that BVVA contains flavonoids as quercetin, quercetin 3-*O*-galactopyranoside, quercetin 3-*O*-*β*-D-glucopyranoside, vitexin,3-hydroxytryptamine, betagarin, betavulgarin, 5,2'-dihydroxy-6,7-methylene dioxy-isoflavone, dihydrooroxylin A,3,5-dihydroxy-6,7-methylene dioxy-flavanone, 2',5-dihydroxy-6,7-methylene dioxy-isoflavone and 5-hydroxy-6,7-methylene dioxy-flavone.[13,-17]
In addition to phenolic acids as gallic, caffeic, vaneltic, coumaric, ferulic and cinnamic acids. [13] Many saponins were identified as 3-*O*-*β*-D-glucopyranosyl-3*β*-hydroxy-olean-12-en-28-oic acid, the methyl ester of oleanolic acid, 3-*O*-*β*-D-glucuronopyranosyl-3*β*-hydroxy-olean-12-en-28-oic acid, 3-*O*-[*β*-D-gluco-pyranosyl-(1-2)-(*β*-D-xylopyranosyl-(1-3)]-*β*-D-glucuronopyranosyl-3*β*-hydroxyolean-12-en-28-oic acid, 3-*O*-[*β*-D-glucopyranosyl-(1-2)-(*β*-D-xylopyranosyl-(1-3))-*β*-D-glucurono-pyranosyl]-28-*O*-*β*-D-glucopyranosyl-3*β*-hydroxy-olean-12-en-28-oic, betavulgarosides I, II, III, IV, V, VI, VII, VIII, IX and X.[6, 18-23] The saponin fractions of the leaves and roots of BVVA decreased the glucose in the blood of rats.[6, 22-24] *Beta vulgaris* is also known for having antioxidant, antihepatotoxic and anti-inflammatory activity.[2, 13, 25]
It was interesting to investigate the lipid and flavonoidal content, the antiproliferative activity and the antioxidant activity of the non-flowering aerial parts of sugar beet growing in Egypt. The precise nature of the bioactive components of the non-flowering aerial parts of BVVA responsible for its antioxidant activity still, remains unclear.

EXPERIMENTAL

Plant Material

The non-flowering aerial parts of *Beta vulgaris* subsp. *vulgaris* var. *altissima* (BVVA) (sugar beet) were collected from a farm in Tanta governate, Egypt, in March 2013. The plant was kindly identified by Professor Doctor Ibrahim El-Garf, Botany Department, Faculty of Science, Cairo University, a specimen of the plant was placed in the herbarium of the National Research Centre, Dokki, Cairo, Egypt.

The GLC (Gas-liquid chromatography) conditions for the (FAME) Fatty acid methyl esters

FAME was analyzed on GC HP-6890. Column HP-5 (phenyl methyl siloxane) 30 m (0.32 mm × 0.25 mm). Temperature program: Initial temperature 70 ºC, Initial time two minutes, program rate 8 ºC/min., final temperature 270 ºC, final time 27 minutes, injection temperature: 270 ºC, flame ionized detector, T=300 ºC, flow rate of carrier gas N_2: 30 ml/ minute, H_2: 35 ml/ minute, air: 300 ml/ minute.

The GLC (Gas-liquid chromatography) conditions for the unsaponifiable matters (Unsap)

The unsaponifiable matter was analyzed on column HP-1 (methyl siloxane) 30 m (0.53 mm × 2.65 mm) Temperature program: Initial temperature 60 ºC, Initial time two minutes, program rate 10º C/min, final temperature 280 ºC, final time 0.5-hour, injection temperature: 260 ºC, flame ionized detector, T=300 ºC, flow rate of carrier gas N_2: 30 ml/ minute, H_2: 35 ml/ minute, air: 300 ml/ minute.

Extraction of the lipid content

About 500 g of the air-dried powdered non-flowering aerial parts of BVVA were macerated in n-hexane for 3 days with continuous shaking. The solvent was evaporated under reduced pressure at 40ºC yielding 2.98 g.

Saponification of n-hexane extract

About 2 g of the hexane extract was saponified as mentioned in (Finar, 1973), [26] to give a brown residue from the unsaponifiable matter (0.91 g) and the saponifiable matter (free fatty acids, 0.52 g). The total fatty acid fraction was methylated according to British Pharmacopoeia (1973) to afford the fatty acid methyl esters.[27]

Extraction, isolation, and purification of flavonoids

About 2.5 kg of the air-dried powdered non-flowering aerial parts of *Beta vulgaris* subsp. *vulgaris* var. *altissima* (sugar beet) were first defatted with hexane (3 × 3L) till exhaustion. The hexane extracts were gathered and concentrated under vacuum at 35 ºC to yield a semisolid yellowish residue (4.98 g). The defatted powder was percolated in 80 % methanol till exhaustion. The methanolic extracts were gathered and concentrated under vacuum to yield a dark brown gummy residue (374.42 g). A portion of the alcoholic

extract (300 g) was suspended in hot distilled water (600 ml), cooled, filtered and the aqueous filtrate was fractionated with successive portions of methylene chloride (3 × 500 ml), ethyl acetate (4 × 500 ml) and finally with *n*-butanol (5 × 500 ml). All the portions were combined and concentrated under vacuum to yield brown residue weighing 12.77 g, 6.5 g and 39.93 g, respectively.

The ethyl acetate residue (1 g) was subjected to preparative paper chromatography (3MM) *n*-butanol: acetic acid: water (3: 1: 1) as the mobile phase. Two main bands were cut into small pieces and eluted separately by 90% methanol. The two bands were further purified separately on Sephadex column (1 × 25 cm) using 100% methanol giving two pure compounds (1) (1 mg) and (2) (3 mg). The *n*-butanol residue (16 g) was dissolved in the least amount of 70% aqueous methanol and introduced onto the top of polyamide column (5 × 75 cm). Gradient elution was carried out using distilled water and decreasing the polarity by 10% stepwise addition of methanol. Fractions of 1 L each were collected. Fraction I eluted by 20% methanol was further fractionated on a small polyamide column (2 × 40 cm). Gradient elution was carried out using distilled water and decreasing the polarity using methanol, starting with 100% distilled water up to 45% methanol to give compound (3) (10 mg). Fraction II eluted by 40% methanol was further purified on a small Sephadex column (2 × 40 cm) eluted with 50% methanol to afford compound (4) (4 mg). Fraction III eluted with 50% methanol was divided into two portions. The first portion of fraction III- was subjected to preparative paper chromatography (3MM, 20% acetic acid) as mobile phase, two main bands were cut and eluted separately by 90% methanol. The two bands were further purified on a Sephadex column (1 × 25 cm) using 90% methanol giving two pure compounds (5) (12 mg) and (6) (7 mg). The second portion of fraction III- was applied on a Sephadex-LH 20 column (3 × 40 cm) using 90% methanol as mobile phase, then subjected to PPC (3MM, 20% acetic acid) as an irrigating solvent, two main bands were cut and eluted separately by 90% methanol. The two bands were passed through a Sephadex column (1 × 25 cm) using 90% methanol as the mobile phase to give (7) (11 mg) and (8) (7 mg), respectively.

Flavonoids Hydrolysis

The flavonoid glycosides were subjected to acid hydrolysis.[28, 29]

Apparatus

UV-visible spectrophotometer: Shimadzu UV-2401-PC. Jeol NMR: 1H-NMR-600 MHz, 13C-NMR-125 MHz. Mass analyzer in Thermo scientific GCMS, model (ISQ LT) using Thermo X-Caliber Software

Investigation of the antioxidant activity: (DPPH) 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay

The methanol extract, hexane fraction, methylene chloride fraction, ethyl acetate fraction, *n*-butanol fraction and some of the isolated compounds from the non-flowering aerial parts of *Beta vulgaris* subsp. *vulgaris* var. *altissima* were prepared in DMSO as 10x stocks from each test concentration (between 6.25-100 µg/ml) and were sonicated in an ultrasonic water bath. Samples producing radical scavenging activities equal to or higher than 50% at 100 µg/ml in the preliminary screening were extra tested and the EC_{50} (concentration of the extract producing 50% scavenging of DPPH radicals) determined using non-linear regression analysis of the dose % AA relationship plotted on GraphPad Prism® V6.0software. The radical scavenger (quercetin) was used as positive control. The radical scavenging activity procedures are based on the modified method (Hamed, 2009) which is based essentially on previously published literature (Nara *et al*., 2006).[30, 31] The antioxidant activity percent (% AA) was calculated according to the following equation:

% Antioxidant activity DPPH (% AA) = 100 x (OD 540 (blank) - OD 540 (sample))/ (OD 540 (blank)) (1)

Eqn.-1. Calculation of the % AA for DPPH assay. OD540 (blank) and OD540 (sample) are the averages of duplicate determinations of the corrected readings of blank and sample at 540 nm, respectively.

Cell culture

The reagents and the materials were purchased from LONZA, Germany. Human hepatocellular carcinoma cell line HepG2 (ATCC®) was sustained as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, 100 U/ml penicillin, 10% FBS and 100 µg/ml streptomycin

sulfate. Monolayers were passaged at 70-90% confluence using trypsin-EDTA solution. The cells were incubated in a humidified CO_2 incubator with 5% CO_2 at 37 °C.

Cell proliferation assay

HepG2 cells (10,000 cells/well) were seeded in 96-well plates in a total volume of 200 µl and were left overnight to form a semi-confluent monolayer. The mitochondrial dehydrogenases of metabolically active cells reduced the MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide dye to insoluble formazan crystals. [32] The monolayers were treated for 48 hours with vehicle (DMSO, 0.1% v/v), tested samples (6.25-100 µg/ml) and the positive control doxorubicin HCl. After the treatment for 48 hours, MTT solution in PBS (5 mg/ml) was added to all the wells and were incubated for 90 minutes. The formazan crystals formation was confirmed using phase contract microscopy. 100 µl/ well DMSO was added to dissolve the formazan crystals. The absorbance was recorded at 492 nm against the number of blank cells on Sunrise™ microplate reader (Tecan Austria Gmbh, Grödig, Austria). Cell proliferation was calculated comparing the average OD values of the control wells and those of the samples, both were represented as % proliferation (control proliferation=100%). IC50 values (concentration of sample causing 50 % loss of cell proliferation of the vehicle control) were calculated using the dose-response curve fit non-linear regression correlation using GraphPad Prism® V6.0 software.

RESULTS AND DISCUSSION

The data in Table-1 of the gas-liquid chromatography analysis of the unsaponifiable matter of the non-flowering aerial parts of BVVA revealed the presence of 18 compounds. The relative percent of the hydrocarbons, sterols, and terpenes are 61.43%, 34.25% and 4.32%, respectively. The major components are campasterol (23.85%), *n*-triacontane C_{30} (12.71%) and cholesterol (8.77%). These data are in disagreement with Elghandour *et al.* as they reported the presence of undecane and undecane 2,6-dimethyl as the most abundant hydrocarbons while tetradecane and pentadecane as the lower abundant hydrocarbons concerning the oil fraction of the leaves.[9]

The gas-liquid chromatography analysis of the fatty acid methyl esters (FAME) of the non-flowering aerial parts of BVVA (Table-2) revealed the presence of 13 compounds. The saturated and the unsaturated fatty acid methyl esters are 21.15% and 78.85%, respectively. The major acids are eicosenoic acid $C_{20:1}$ (41.38%) and lignoceric acid (9.19%). The monounsaturated fatty acids, di-unsaturated fatty acids, and poly-unsaturated fatty acids constitute 47.41%, 7.48% and 23.96%, respectively. It is reported that the oil fraction of the fruits contained hexadecanoic acid methyl ester and 9-octadecenoic acid (Z) methyl ester as major fatty acids methyl esters. Also Schmidt *et al*., 1994 reported that the hexane extract of the seeds contains squalene, β-amyrine, cycloartenol, 24-methylenecycloartanol, cholesterol, 24-Methylenecholesterol, campesterol, stigmasterol, ergost-7-en-3β-ol, stigmast-9(11)-en-3β-ol, spinasterol, sitosterol, stigmasterol, 22-dihydrospinasterol and avenasterol.[10]

Table-1: GLC analysis of the unsaponifiable matter of BVVA

R_t(min)	Component	Relative percentage
11.67	*n*-Pentadecane C_{15}	1.59
12.97	*n*-Hexadecane C_{16}	0.71
13.96	*n*-Heptadecane C_{17}	0.82
16.56	*n*-Nonadecane C_{19}	6.09
18.22	*n*-Eicosane C_{20}	2.36
18.78	*n*-Heneicosane C_{21}	6.0
19.96	*n*-Docosane C_{22}	1.58
22.09	*n*-Tetracosane C_{24}	7.25
22.99	*n*-Pentacosane C_{25}	1.57
24.34	*n*-Hexacosane C_{26}	3.73
25.73	*n*-Heptacosane C_{27}	5.85
26.34	*n*-Octacosane C_{28}	5.61
26.83	*n*-Nonacosane C_{29}	5.54

27.67	*n*-Triacontane C_{30}	12.71
29.94	Cholesterol	8.77
32.51	Campesterol	23.85
35.71	β-Sitosterol	1.63
37.46	α-Amyrine	4.32
Hydrocarbons		61.43%
Sterols		34.25%
Terpenes		4.32%

Table-2: Results of GLC analysis of the fatty acids methyl esters of BVVA

R_t (min)	Component (FAME)	Relative percentage
8.88	Capric acid $C_{10:0}$	1.89
11.28	Lauric acid $C_{12:0}$	6.22
12.81	Myristic acid $C_{14:0}$	1.02
14.32	Myristoleic acid $C_{14:1}$	2.07
15.69	Palmitic acid $C_{16:0}$	1.43
18.57	Stearic acid $C_{18:0}$	1.4
19.04	Oleic acid $C_{18:1}$	1.31
20.20	Linoleic acid $C_{18:2}$	7.48
21.82	α-Linolenic acid $C_{18:2}$	17.46
23.91	Eicosenic acid $C_{20:1}$	41.38
25.27	Arachidonic acid $C_{20:4}$	6.5
27.16	Lignoceric acid $C_{24:0}$	9.19
28.49	Nervonic acid $C_{24:1}$	2.65
Saturated fatty acid methyl esters		21.15%
Unsaturated fatty acid methyl esters		78.85%
Mono-unsaturated fatty acid methyl esters		47.41%
Di-unsaturated fatty acid methyl esters		7.48%
Poly-unsaturated fatty acid methyl esters		23.96%

The phenolic compounds were isolated from the ethyl acetate and *n*-butanol fractions of the aqueous alcoholic extract using different chromatographic techniques and were identified as follow:

Compound (1): Apigenin

The compound was isolated as a dull yellow powder with deep brown color in UV light, which was turned to yellowish green after spraying with $AlCl_3$. The compound was identified through comprising with authentic apigenin on PC (3MM) *n*-butanol: acetic acid: water (3: 1: 1) as the mobile phase. It has same R_f value as that of authentic the authentic apigenin (0.8).[28] Also, the EI Mass spectrum gave a molecular ion peak M^+ at m/z = 270 corresponding to the molecular formula $C_{15}H_{10}O_5$.

Compound (2): Luteolin

The compound was isolated as a yellow powder with deep violet color in UV light, which was turned to yellowish green after spraying with $AlCl_3$. The compound was identified through spotting with authentic apigenin on PC (3MM) *n*-butanol: acetic acid: water (3: 1: 1) as the mobile phase. It has same R_f value as that of authentic the authentic luteolin (0.86). [28] Also, the EI Mass spectrum gave a molecular ion peak M^+ at m/z = 286 corresponding to the molecular formula $C_{15}H_{10}O_6$.

Compound (3): Apigenin 7-*O*-di-glucoside

The compound was isolated as a yellowish powder with yellow color in UV light, which was turned to brown after spraying with $AlCl_3$. It was found to contain di-sugar moieties because of its high R_f value (0.78) in 15% acetic acid. The partial acid hydrolysis of compound (3) yielded apigenin-7-*O*-glucoside and

glucose, while the complete acid hydrolysis yielded glucose as a sugar and apigenin as an aglycone. [33] The UV spectra of this flavonoid in methanol and different shift additives (NaOMe, $AlCl_3$, $AlCl_3$/HCl, NaOAc and NaOAc/H_3BO_3) proved that, it is a flavone type structure like apigenin with glucosilation at C7, where there is no bathochromic shift in band-II of NaOAc spectrum more than 5 nm (4nm) relative to the methanol. It gave band-I at 334 nm which is shifted to 392 nm with increasing in intensity after addition of NaOMe which proves the presence of free OH at C4'. The ^{1}H-NMR showed signals at δ in ppm= 7.95 (*d*, 2H, H2' and H6'), 7.8 (2H, *d*, *J*=8 Hz, H5' and H3'), 6.53 (1H, *d*, H8), 6.18 (1H, *s*, H3), 6.16 (1H, *d*, H6), Two sugar moieties: 5.03 (1H, *d*, H1''), 4.3 (1H, *d*, H1'''), 3.68 - 2.9 (sugar protons, *m*). The EI mass spectrum gave a molecular ion peak M^+ at m/z = 594 related to the molecular formula $C_{27}H_{30}O_{15}$. The other fragments like 270 [M^+- 2 glucose], 242 [M^+- (CO)], 152 and 164. So, compound (3) can be identified as Apigenin-7-*O*-di-glucoside.[34]

Compound (4): Luteolin 4'-*O*-rhamnoside

This compound was isolated as a yellow powder and appeared as yellowish-orange spot after spraying with $AlCl_3$ under UV light. Its behavior on the cellulose plate in different solvents proved its glycosidic nature. The UV spectra of this compound proved the flavone nature of this compound by the exhibition of band-I at 342 nm in methanol which is bathochromically shifted on the addition of NaOMe with low intensity, indicating the absence of free OH at C4'. The presence of a free OH group at C7 was proved through the bathochromic shift of band-II in NaOAc spectrum relative to methanol spectrum from 270 nm to 280 nm. The acid hydrolysis of the compound (4) yielded rhamnose as a sugar part and luteolin as an aglycone. [33] The ^{1}H-NMR displayed δ in ppm: 7.94 (*d*, 2H, H2', H6'), 7.8 (*d*, 2H, *J*=8 Hz, H5', H3'), 6.18 (1H, *s*, H3), 6.56 (1H, *d*, H8), 6.19 (1H, *d*, H6), 1.1 (3H, *d*, methyl of rhamnose), sugar moiety: 5.26 (1H, *d*, H1'), 2.5 - 3.5 (sugar protons, *m*), these data were in accordance with that reported by Markham. [28] The EI mass spectrum gave a molecular ion peak M^+ at m/z = 432 related to the molecular formula $C_{21}H_{20}O_{10}$. The other fragments like 286 [M^+-rhamnose], 258 [M^+-CO)], 152 and 134. All these data substantiated that the structure of compound (4) can be assigned as Luteolin 4'-*O*-rhamnoside.[35]

Compound (5): Caffeoyl-6-(3,4-dihydroxy benzoyl) *β*-D-glucoside

This compound was obtained as an amorphous whitish powder and appeared as sky blue spot under UV light. The EI Mass molecular ion peak M^+ at m/z: 478 which corresponds to the molecular formula $C_{22}H_{22}O_{12}$. The fragment at m/z: 163 confirmed the presence of hexose (glucose) moiety. The other fragment at m/z: 154 proved the presence of di-hydro benzoic acid moiety, while the fragment at m/z: 179, 163, 154, 78 and 66 proved the presence of caffeoyl moiety. In addition to fragments of di-hydroxy benzoic acid: 71, 139, 141 and 169. The ^{1}H NMR spectrum was recorded in CD_3OD and showed signals for two sets of aromatic protons at (δ 6.81 and 7.45 ppm, J=8.7 Hz) and (6.65 and 6.94 ppm, J=8.7 Hz), trans-olefinic protons (J=15.9 Hz) at δ 7.63 and 6.34 ppm, and sugar protons (anomeric proton at δ 4.73 ppm).[33] All these data confirmed the identification of compound (5) as caffeoyl-6-(3,4-dihydroxy benzoyl) *β*-D-glucoside.[36]

Compound (6): Apigenin 5-*O*-rhamnoside

The compound was isolated as faint yellow amorphous powder and appeared as yellowish-orange spot after spraying with $AlCl_3$ under UV light. Its behavior on the cellulose plate in different solvents proved its glycosidic nature. The flavone nature of this compound was proved by the exhibition of band-I at 331 nm in methanol, which is bathochromically shifted to 391nm on the addition of NaOMe with high intensity, indicating the presence of free OH at C4'. The presence of a free OH group at C7 was proved through the bathochromic shift of band-II in NaOAc spectrum relative to methanol spectrum (from 271 nm to 279 nm). The EI mass spectrum gave a molecular ion peak (M^++H) at m/z = 417 related to the molecular formula $C_{21}H_{20}O_9$. The acid hydrolysis yielded rhamnose as a sugar and apigenin as an aglycone. The fragment 270 [M^+-rhamnose] proved that the aglycone is apigenin and the sugar part is deoxyhexose moiety. The other fragments at 242 [M^+- CO], 152 and 117. The structure of compound (6) can be elucidated as apigenin 5-*O*-rhamnoside.[37]

Compound (7): Isoscutellarein 7-*O*-glucosyl, 8-*O*-xyloside

This compound was isolated as a white powder and as greenish yellow spot after spraying with $AlCl_3$ under UV light. It appeared as a glycosidic in nature through its chromatographic behavior different solvents. Its UV spectra in methanol and other different shift reagents confirmed its flavone nature (band-I at 330 nm in methanol) with the presence of free OH at C4', in addition to the absence of a free OH group at C7. The ^{1}H-NMR displayed δ in ppm: 7.97 (2H, *d*, H2', H6'), 7.82 (2H, *d*, *J*=8 Hz, H5', H3'), 6.58 (1H, *d*, H8), 6.25 (1H, *s*, H3), 6.22 (1H, *d*, H6), sugar moiety: 5.13 (1H, *d*, H1'"),*5* (1H, *d*, H1"), 3-4.5 (sugar protons, *m*). The ^{13}C-NMR displayed in ppm: 184 (C4), 164 (C2), 162.89 (C4'), 162.71 (C7), 130 (C2', C6'), 129.8 (C-8), 123.6 (C1'), 117(C3', C5'), 106.27 (C1"), 103.56 (C3), 100.56 (C10), 99.30 (C6), 77.37 (C5"), 75.22 (C2"), 70.95 (C4"), 62.9 (C6"). The EI mass spectrum gave a molecular ion peak (M^+-H) at m/z = 579 fit with the molecular formula $C_{26}H_{28}O_{15}$. The other fragments like 285 [M^+- (hexose + pentose)], 257 [M^+-(CO + 1)], 168 and 118. The acid hydrolysis confirmed the presence of glucose and xylose moieties and isoscutellarein as aglycone where it gave M^+-1 at m/z: 285, thus, compound (7) can be identified as Isoscutellarein 7-*O*-glucosyl, 8-*O*-xyloside.[38]

Compound (8): 5, 7, 5'-trihydroxy-3'-methoxy-flavone 4'-*O*-glucoside

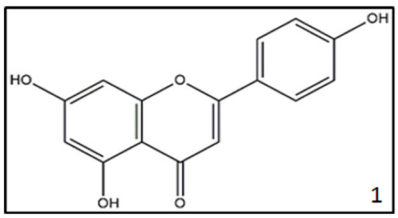
This compound was isolated as an amorphous whitish powder and appeared as yellowish green spot after spraying with $AlCl_3$ under UV light. Its behavior on the cellulose plate in different solvents proved it's glycosidic nature. The UV spectra in methanol and different shift reagents are (λ_{max}/nm): (MeOH) 260, 270sh, 338, 346; (NaOMe) 275, 385(low intensity); ($AlCl_3$) 266, 300_{sh}, 348; ($AlCl_3$/HCl) 276, 336; (NaOAc) 275, 387; (NaOAc/H_3BO_3) 369, 357, which prove the flavone nature of this compound with the absence of free OH at C4'. The presence of a free OH group at C7 was proved through the bathochromic shift of band-II in NaOAc spectrum relative to methanol spectrum from 260 nm to 275 nm. [28] The ^{1}H-NMR spectrum displayed different signals at δ in ppm: 7.68 (1H, *d*, *J*=3-6 Hz, H2'), 7.60 (1H, *d*, *J*=3-6 Hz, H6'), 6.9 (1H, *s*, H3), 6.41 (1H, *d*, H8), 6.19 (1H, *d*, H6), sugar moiety: 5.35 (1H, *d*, H1"), 3.93 (3H, *s*, of the methoxy group), 3-3.5 (sugar protons, *m*), these data were in accordance with that reported by Markham. [28] The EI mass spectrum gave a molecular ion peak M^+ at m/z = 478 related to the molecular formula $C_{22}H_{22}O_{12}$. The other fragments like 316 [M^+-hexose], 258 [M^+-(OCH_3 + CO)], 152 and 164. The acid hydrolysis yielded glucose as sugar and an aglycone which was confirmed through its UV and mass spectra where it proved the presence of the sugar moiety at C-4' through NaOMe spectrum. All these data proved that compound (8) can be identified as 5, 7, 5'-trihydroxy-3'-methoxy-flavone 4'-*O*-glucoside. [39] All the structures of the isolated and identified compounds were shown in Fig.-1.

As presented in Table-3 and Fig.-2, the study of the antioxidant activity of different fractions of the BVVA extract as well as the isolated compounds revealed that, the ethyl acetate fraction has the highest antioxidant activity among all samples recording a DPPH scavenging of 62.6% of solvent control. In addition, the *n*-butanol fraction and methylene chloride fraction cause lower scavenging percentage, recording 39.6 and 25.6% scavenging, respectively. In contrast, the isolated phenolic compounds recorded weak DPPH scavenging; this can be attributed to the absence of ortho di-hydroxy system in ring B of their flavonoid structure.[40] However, the antioxidant activity of the *n*-butanol fraction may be due to the synergistic effect of these compounds. The data in Table-4 and Fig.-3 proved that, the percent of HepG2 cell line proliferation was not affected significantly by the methanolic extract, hexane, methylene chloride, ethyl acetate and *n*-butanol fractions of BVVA at concentrations less than 50 µg/ml. However, the percent of HepG2 proliferation was reduced to 69.5% and 72.4% on treatment with the ethyl acetate and the methanol extract respectively at concentration 100 µg/ml. The *n*-butanol fraction reduced the percent of HepG2 proliferation to 79.3% at concentration 100 µg/ml.

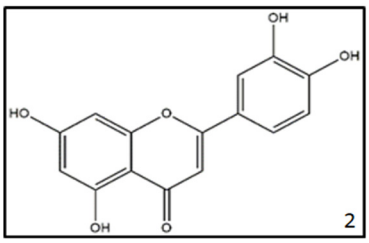
CONCLUSION

8 phenolic compounds (apigenin, luteolin, apigenin 7-*O*-di-glucoside, luteolin 4'-*O*-rhamnoside, caffeoyl-6-(3,4-dihydroxy benzoyl) *β*-D-glucoside, apigenin 5-*O*-rhamnoside, isoscutellarein 7-*O*-glucosyl, 8-*O*-xyloside and 5, 7, 5'-trihydroxy-3'-methoxy-flavone 4'-*O*-glucoside) were isolated for the first time from the non-flowering aerial parts of *Beta vulgaris* var. *altissima* (BVVA) (sugar beet) cultivated in Egypt. The

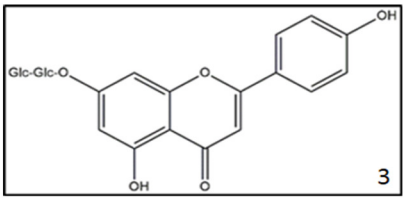
GLC analysis of the unsaponifiable matter of the non-flowering aerial parts of BVVA revealed the presence of 18 compounds in which the main compound is campesterol (23.85%). The GLC analysis of the fatty acid methyl esters of the non-flowering aerial parts of BVVA revealed the presence of 13 compounds in which the major acid is eicosenoic acid $C_{20:1}$ (41.38%). The highest antioxidant activity was exhibited by the ethyl acetate fraction (62.65%). The highest antiproliferative activity against HepG2 was exhibited by the methanol extract of BVVA.



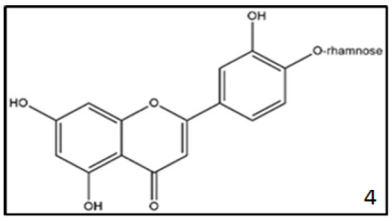
Apigenin



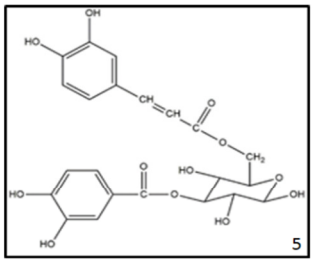
Luteolin



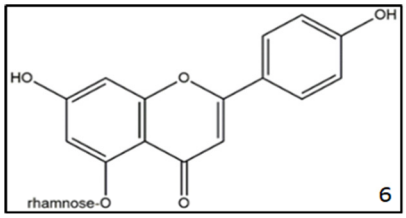
Apigenin 7-*O*-diglucoside



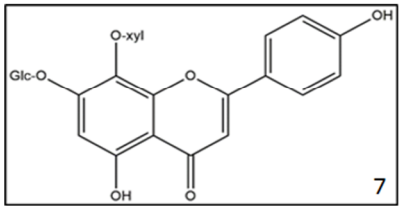
Luteolin 4'-*O*-rhamnoside



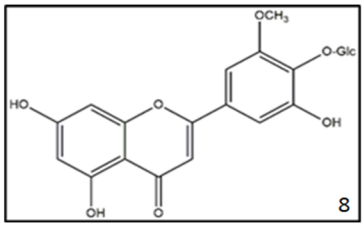
Caffeoyl-6(3,4-dihydroxy benzoyl) β-D-glucoside



Apigenin 5-*O*-rhamnoside



Isoscutellarein 7-*O*-glucosyl, 8-*O*-xyloside



5,7,5'-trihydroxy-3'-methoxy-flavone 4'-*O*-glucoside

Fig.-1: Chemical structures of isolated compounds from BVVA

Table-3: Antioxidant activity using DPPH radical scavenging assay

Sample	% DPPH scavenging activity
Methanolic extract	11.96
Hexane fraction	3.6
Methylene chloride fraction	25.56
Ethyl acetate fraction	62.66
n-Butanol fraction	39.57
Compound 3	3.41

Compound 4	10.93
Compound 5	1.86
Compound 6	3.86
Compound 7	6.23

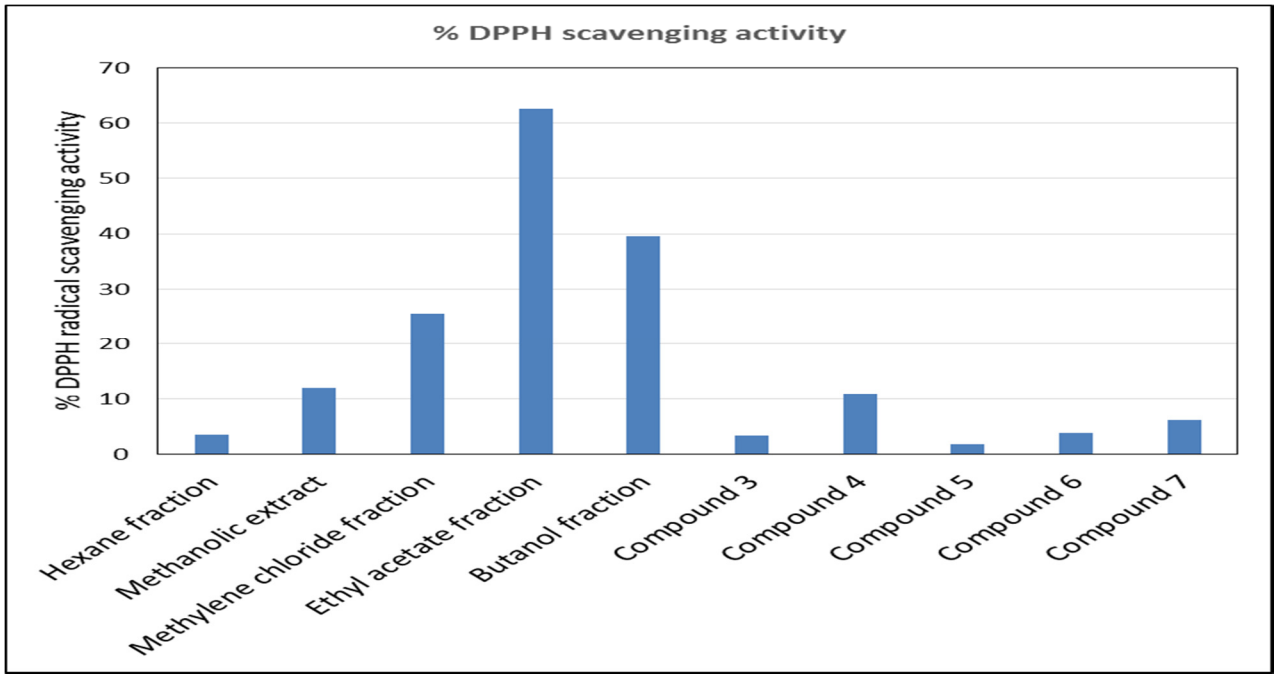


Fig.-2: Antioxidant activity using DPPH radical scavenging assay

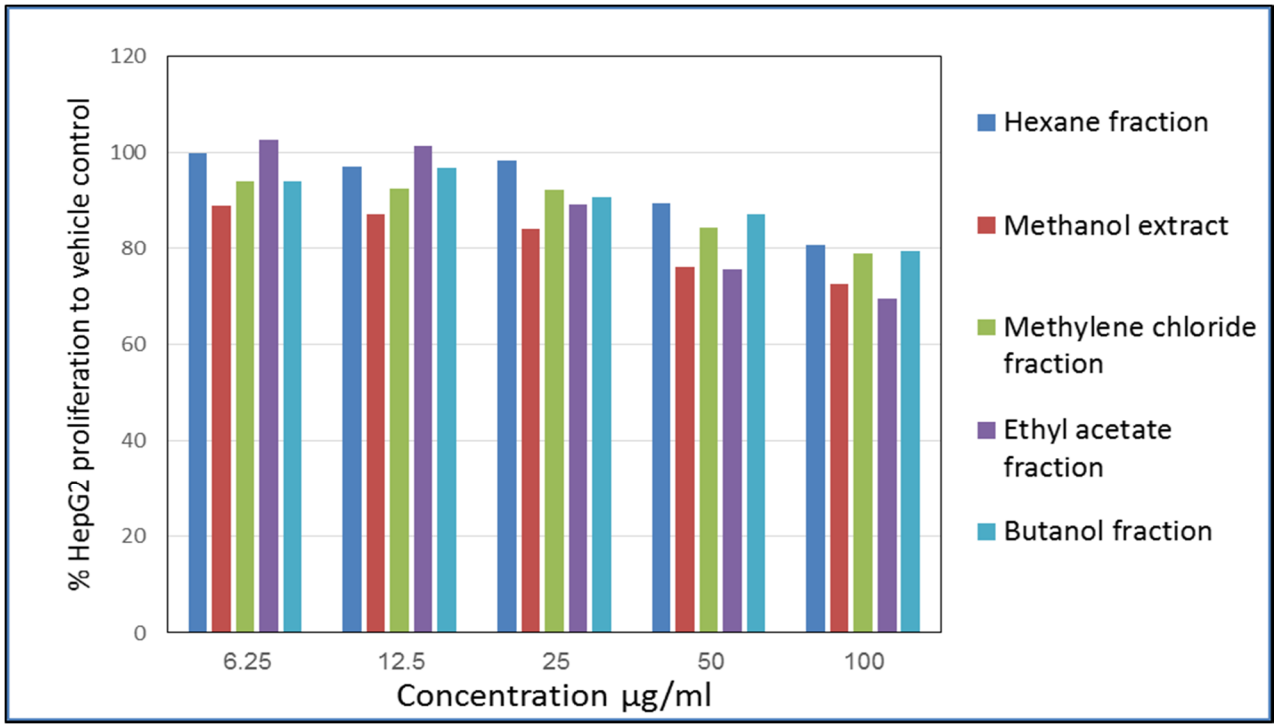


Fig.-3: Antiproliferative activity against Human Hepatocellular carcinoma cell line

Table-4: Antiproliferative activity against Human Hepatocellular carcinoma cell line

Concentration µg/ml	6.25	12.5	25	50	100
Hexane fraction	88.9	87.2	84.1	76	72.4
Methanol extract	99.7	96.9	98.2	89.5	80.5
Methylene chloride fraction	93.9	92.5	92.3	84.3	78.9
Ethyl acetate fraction	102.5	101.4	89.3	75.5	69.5
n-Butanol fraction	93.9	96.7	90.7	87.1	79.3

REFERENCES

1. R. N. Chopra, S. L. Nayar and I. C. Chopra, Glossary of Indian medicinal plants, CSIR, New Delhi, (1956).
2. N. P. S. Prejapali and A. Sharma, A Handbook of Medicinal Plant, 5th Ed., Publishing House, (2003).
3. L. Watson and M. Dallwitz, The families of flowering plants: descriptions, illustrations, identification and information retrieval, University of New Orleans, (1999).
4. B. Neelwarne and S. B. Halagur, Red Beet: an overview, Red Beet Biotechnology, Springer, p.1-43(2013).
5. T. Murakami, H. Matsuda, M. Inadzuki, K. Hirano and M. Yoshikawa, *Chemical and pharmaceutical bulletin*, **47**, 1717(1999)
6. M. Yoshikawa, T. Murakami, M. Kadoya, H. Matsuda, O. Muraoka, J. Yamahara and N. Murakami, *Chemical and Pharmaceutical Bulletin*, **44**, 1212(1996)
7. A. Refat and M. Ghaffar, *Australian Journal of Basic and Applied Sciences*, **4**, 1641(2010)
8. M. Al-Dosari, S. Alqasoumi, M. Ahmed, M. Al-Yahya, M. N. Ansari and S. Rafatullah, *Farmacia*, **59**, 669(2011)
9. M. S. Afifi, M. A. Amer, S. R. Gedara, S. M. Elghandour, *Journal of Environmental Sciences*, **44**, 161(2015)
10. J. Schmidt, C. Kuhnt and G. Adam, *Phytochemistry*, **36**, 175(1994)
11. R. J. Brown and R. F. Serro, *Journal of the American Chemical Society*, **75**, 1040(1953)
12. M-C. Ralet, G. André-Leroux, B. Quéméner and J-F. Thibault, *Phytochemistry*, **66**, 2800(2005)
13. S. E. El-gengaihi, M. A. Hamed, D. H. Aboubaker and H. M. Abdel-tawab, *International Journal of Pharmacy and Pharmaceutical Sciences*, **8**, 281(2016)
14. J. Geigert, F. Stermitz, G. Johnson, D. Maag and D. Johnson, *Tetrahedron*, **29**, 2703(1973)
15. R. Gardner, A. Kerst, D. Wilson and M. Payne, *Phytochemistry*, **6**, 417(1967)
16. H. Chui, Y. Arakawa, S. Ueda, M. Kuroda and M. Izawa, *Phytochemistry*, **25**, 281(1985)
17. H. Takahashi, T. Sasaki and M. Ito, *Bulletin of the Chemical Society of Japan*, **60**, 2261(1987)
18. C. A. Marsh and G. A. Levvy, *Biochemistry Journal*, **63**, 9(1956)
19. H. M. Bauserman and P. C. Hanzas, *Journal of American Society of Sugar Beet Technology*, **9**, 295(1957)
20. C. L. Ridout, K. R. Price, G. Parkin, M. G. Dijoux and C. Lavaud, *Journal of Agricultural and Food Chemistry*, **42**, 279(1994)
21. G. Massiot, M-G. Dijoux, C. Lavaud, L. Le Men-Olivier, J. D. Connolly and D. M. Sheeley, *Phytochemistry*, **37**, 1667(1994)
22. M. Yoshikawa, T. Murakami, M. Kadoya, H. Matsuda, J. Yamahara, O. Muraoka and N. Murakami, *Heterocycles*, **8**, 1621(1995)
23. M. Yoshikawa, T. Murakami, M. Kadoya, J. Yamahara and H. Matsuda, *Chemical and Pharmaceutical Bulletin*, **46**, 1758(1998)
24. T. Kushiro, M. Shibuya and Y. Ebizuka, *Excerpta Medica International Congress Series*, p. 421-428(1998).
25. J. A. Duke, Handbook of Medicinal Herbs, CRC press (2002).
26. I. L. Finar, Organic Chemistry, 6th Ed., Longman group Ltd, England (1973).
27. British Pharmacopoeia, The pharmaceutics Press, London (1973).

28. K. R. Markham, Techniques of flavonoid identification, Academic Press, London, (1982).
29. A. A. Shahat, K. A. Abdel-Shafeek and H. A. Husseiny, *Natural Product Sciences*, **12**, 122(2006)
30. A. Hamed, Investigation of multiple cytoprotective actions of some individual phytochemicals and plant extracts, The University of Nottingham, United Kingdom (2009).
31. K. Nara, T. Miyoshi, T. Honma and H. Koga, *Bioscience,Biotechnology and Biochemistry*, **70**, 1489 (2006)
32. T. Mosmann, *Journal of Immunological Methods*, **65**, 55(1983)
33. T.J. Mabry, K. R. Markham and M. B. Thomas, The systematic identification of flavonoids. Springer (1970).
34. A. M. El-Hawiet, S. M. Toaima, A. M. Asaad, M. M. Radwan and N. A. El-Sebakhy, *Revista Brasileira de Farmacognosia*, **20**, 860(2010)
35. J. Intekhab, M. Aslam and H. Khalid, *American Journal of Plant Sciences*, **2**, 657 (2011)
36. T. Sarg, A. Abdel-Ghani, R. Zayed and M. El-Sayed, *Journal of Natural Products*, **5**, 10(2012)
37. O. Purev, F Pospíšil and O Motl, *Collection of Czechoslovak Chemical Communications*, **53**, 3193(1988)
38. Y. C. Teles, C. C. R. Horta, M. D. F. Agra, W. Siheri, M. Boyd, J. O. Igoli, A. I. Gray and M. D. F. V. De Souza, *Molecules*, **20**, 20161(2015)
39. S. Numonov, S. Usmanova and H. Aisa, *Chemistry of Natural Compounds*, **49**, 511(2013)
40. K. E. Heim, A. R. Tagliaferro and D. J. Bobilya, *The Journal of Nutritional Biochemistry*, **13**, 572(2002)

[RJC-1936/2017]