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Induction of apoptosis in HepG2 by *Vitex agnus-castus* L. leaves extracts and identification of their active chemical constituents by LC-ESI-MS

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ABSTRACT

Objective: To evaluate the cytotoxic activity and cytopathological changes of *Vitex agnus-castus* L. (*V. agnus-castus*) leaves extracts and characterize their bioactive chemical constituents.

Methods: The dried leaves powder of *V. agnus-castus* was extracted using 85% methanol (MeOH). The methanolic extract was defatted using petroleum ether and fractionated using ethyl acetate (EtOAc) and butanol (BuOH). The anticancer potential of different extracts was evaluated by neutral red assay, cytopathological changes of apoptosis and caspase-3 expression in hepatoma cell line (HepG2). The chemical constituents of most active extracts were identified using liquid chromatography-electrospray ionisation mass spectrometry analysis.

Results: The butanolic fraction was the most active in inhibiting the proliferation of HepG2 cells [IC_{50} = (13.42 ± 0.17) mg/mL] compared with MeOH extract [IC_{50} = (17.61 ± 0.15) mg/mL) and EtOAc fraction [IC_{50}= (22.51 ± 0.26) mg/mL]. The cytopathological examinations demonstrated the morphology of apoptosis and caspase-3 expression was more evident in HepG2 cells treated with BuOH than cells treated with MeOH and EtOAc. The liquid chromatography-electrospray ionisation mass spectrometry analysis exhibited that the defatted MeOH extract and BuOH fraction had different bioactive secondary metabolites, such as phenolic acids, flavonoids, and iridoids.

Conclusions: The butanolic fraction has higher contents of secondary metabolites than the defatted methanolic extract. The cytotoxic activities, apoptotic changes, and caspase-3 activation may be due to the presence of these bioactive secondary metabolites (iridoids, flavonoid, and phenolic acids) in these extracts. These results would suggest *V. agnus-castus* to be used as an adjuvant in cancer therapy.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most widespread malignancy in the world with a cautiously increasing incidence[1]. HCC is one of the major health problems in Egypt and its incidence is increasing[2]. HCC is a highly aggressive tumor that responds poorly to currently available therapies[3]. It was suggested that the bioactive compounds of some plant extracts are capable of killing liver carcinoma cells[4].

Apoptosis is an important protective mechanism against cancer cells[5]. The inability of the cells to undergo apoptosis may result in tumor drug resistance[6]. Induction of apoptosis is one of the main strategies in anticancer therapy[7]. Caspase-3 plays an important role in apoptosis. Expression of the caspase-3 precursor (pro-caspase-3) alone cannot induce apoptosis and activation requires a death-inducing stimulus, such as cancer chemotherapy[8].

According to the World Health Organization, 80% of the world's population living in rural areas depends on plant-based products for their health care needs[9]. Herbs have been authorized as an alternative form of medicine with less toxicity and are safe for patients[10]. *Vitex agnus-castus* L. (*V. agnus-castus*) is a member of the Verbenaceae family which grows naturally in Southern

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Europe and the Middle East. It has been reported that *Vitex* species show cytotoxic properties against many of human cancer cell lines including ovarian, prostate and breast cancer cell lines[11-14].

Many previous studies have reported that *V. agnus-castus* contains a lot of different bioactive secondary metabolites, such as iridoids, flavonoids, terpenoids, ketosteroids, glycosides and phenolic acids [11-13,15]. Liquid chromatography coupled with mass spectrometry was used to determine the phytochemicals in plant extracts and has been found to be a powerful technique due to soft ionization, which easily enables the analysis of thermally labile, polar and non-volatile compounds. Flavonoids and phenolic groups isolated from different plants inhibited proliferative activity by induction of apoptosis in cancer cells[16,17].

The objectives of the present study was the preparation of the defatted methanol (MeOH) extract of *V. agnus-castus* and its derived fractions [ethyl acetate (EtOAc) and butanol (BuOH)], evaluation of the cytotoxic activity of the extract and fractions against the hepatoma cell line (HepG2) and the identification of their chemical constituents using liquid chromatography-electrospray ionisation mass spectrometry (LC-ESI-MS) analysis.

2. Materials and methods

2.1. Plant material

V. agnus-castus (Family: Verbenaceae) leaves were collected from Menofia Governorate, Egypt in April 2015. The voucher plant sample was characterized by Prof. Dr. Wafaa Amer, professor of plant taxonomy, Faculty of Science, Cairo University and by the taxonomist Theresa Labib, Orman Botanical Garden, Giza, Egypt. The voucher specimen (No. 524) has been deposited in Medicinal Chemistry Laboratory, Theodor Bilharz Research Institute. The leaves of the plant were dried in the shade, finely powdered with an electric mill, and the dry powder was kept away from any moisture for the extraction process.

2.2. Extraction and fractionation process

Finely powdered leaves of *V. agnus-castus* (1.5 kg) were extracted with 7 L of 85% MeOH at room temperature. 85% MeOH extract was filtrated and concentrated to dryness under reduced pressure using a rotatory evaporator for three times. 85% MeOH extract was defatted with petroleum ether, and the aqueous defatted MeOH extract was subjected to fractionation using dichloromethane (CH_2Cl_2), EtOAc and BuOH respectively. The three fractions were concentrated to dryness with a rotatory evaporator. The defatted methanol extract and the three fractions were kept away from any moisture.

2.3. Cytotoxic activity of plant extracts

The cytotoxic activity of the defatted MeOH extract of *V. agnus-castus* as well as EtOAc and BuOH fractions on the HepG2 cell line was determined by neutral red (NR) assay[18]. The cells were cultured in culture media containing RPMI-1640 medium pH 7.4 (Sigma Chem. Co. St. Louis, MO, USA), 20 mL/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma, USA), 3 mL/L 7.5% Na bicarbonate (Gibco, UK), 0.1 mL/L penicillin (10 000 units/mL), streptomycin (10 mg/mL) (Sigma, USA), 10 mL L-glutamine (Gibco, UK) and 20% fetal bovine serum (Hyclone) heat inactivated at 56 ℃ for 30 min. The culture media was filtered, kept at 4 ℃ and stored for no longer than two weeks. 1×10^5 HepG2 cells/mL were seeded in microplates (96-flat bottomed wells, M 129 A Dynatech, USA). About 125 µL of the cell suspension per well were dispensed. A total of 200 µL of phosphate buffered saline (PBS) (without cells) was dispensed into the peripheral wells of the plate (Blank). The plate was then incubated overnight at 37 ℃ in 5% CO_2 incubator. The plant extracts were dissolved in culture medium containing 0.5% (v/v) of dimethyl sulphoxide (DMSO) (PARK, Northampton, UK). About 125 µL of different plant extract concentrations (25, 12.5, 6.25, 3.125 and 1.562 mg/mL) were added to wells in a concentration-dependent manner. Culture medium with 0.5% (v/v) DMSO without plant extracts served as control after incubation for 72 h at 37 ℃ in a humid incubator. The inhibition of cell proliferation was assessed by NR assay. About 250 µL of NR solution were added to all wells after a careful wash with pre-warmed PBS. The NR was carefully removed after 3 h and then rinsed with 200 µL of pre-warmed PBS. After adding 150 µL of 49% water, 50% ethanol and 1% glacial acetic acid to each well, the plate was kept for 15 min in the dark. Absorbance was recorded at 540 nm by spectrophotometer (Spectra max 190 Microplate Reader, Molecular Devices, USA). The assay was repeated in triplicates. Mean ± SEM of three separate experiments for each concentration was calculated.

The inhibition activity (%) = $[(A_{Control} - A_{Sample})/(A_{Control} - A_{Blank})] \times 100$

Where A is the absorption at wavelength 540 nm, control is the untreated cells and blank is media without cells.

2.4. Cytopathological examinations

Cultured HepG2 cells were trypsinized, washed in PBS, pH = 7.4 and collected in a tube. The samples were centrifuged at a rate of 1200–1500 r/min for 15 min using Shandon Cytospin (Thermo Fisher Scientific, Waltham, Massachusetts). The cell pellet was spread on positively charged glass slides. Slides were immediately fixed in 95% ethanol for 24 h. The slides were stained with hematoxylin and eosin (H & E)and immunohistochemistry for caspase 3.

2.5. Immunohistochemical studies

Unstained sections were processed for immunostaining with caspase 3 monoclonal antibodies using a standard 3-layer protocol, as previously described[19]. The smears were dehydrated with 100%, 95%, and 70% ethanol. Smears were washed in PBS, 200 µL of caspase 3 primary antibodies (Novus Biological, USA) were added to each slide (at a dilution of 1:100) and incubated for 24 h in a humid chamber at 4 ℃. Smears were rinsed in PBS, incubated for 30 min with the secondary biotinylated antibody followed by avidin-peroxidase complex for another 30 min according to the manufacturer's instructions (Universal Detection Kit, Novus Biological, USA). The samples were incubated with

diaminobenzidine until the brown color developed within 5 min, washed in distilled water, and counterstained with Mayer's hematoxylin for 60 s. The procedure was done at room temperature. In addition, negative controls in which the primary antibody was omitted and replaced by PBS were also included.

Caspase-3 antigen was expressed as brown cytoplasmic staining. Apoptotic index was calculated according to Duan *et al.*[20].

$$\text{Apoptotic index (\%)} = \frac{\text{Active caspase 3} - \text{Immunopositive cells number}}{\text{Total cells number of 10 high power fields}} \times 100$$

2.6. LC-ESI-MS Analysis

The defatted MeOH extract and BuOH fraction of *V. agnus-castus* (5 mg/mL) solution was prepared using high performance liquid chromatography (HPLC) analytical grade solvent mixture of $CH_3CN/MeOH/H_2O$ (1:1:2; v/v/v), filtered using a membrane disc filter (0.45 μm) then subjected to LC-ESI-MS analysis. This analysis was carried out using HPLC system (Waters Alliance 2695, Waters, USA) and mass analyzer (Waters 3100). Samples injection volumes (20 μL) were injected into the HPLC instrument equipped with reverse phase C-18 column (Phenomenex 250 mm, 5 μm particle size). Sample mobile phase was prepared by filtering using 0.45 μm filter membrane disc and degassed by sonication before injection. Mobile phase elution was made with the flow rate of 0.4 mL/min using gradient mobile phase comprising two eluents: eluent A is H_2O acidified with 0.1% formic acid and eluent B is $CH_3CN/MeOH$ (1:1, v/v) acidified with 0.1% formic acid. Elution was performed using the following gradient: 5% B, 0–5 min; 5%–10% B, 5–10 min; 10%–50% B, 10–55 min; 50%–95% B, 55–65 min; 5% B, 65–70 min. The parameters for analysis were carried out using negative ion mode as follows: source temperature 150 ˚C, cone voltage 50 eV, capillary voltage 3 kV, desolvation temperature 350 ˚C, cone gas flow 50 L/h, and desolvation gas flow 600 L/h. Mass spectra were detected in the ESI negative ion mode between *m/z* 50–1 000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time (Rt) and mass spectrum with reported data.

2.7. Statistical analysis

The statistical analysis was made using the SPSS for Windows 13.0 for determination the IC_{50}. The data were presented as mean ± SEM. Apoptotic index results were assessed by the One-way analysis of variance (ANOVA) and values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cytotoxic assay

Inhibition effect of the defatted MeOH extract, EtOAc and BuOH fractions of *V. agnus-castus* on the HepG2 was evaluated using the neutral red cytotoxicity assay. The proliferation of HepG2 cells was inhibited by the three extracts in a concentration-dependent manner. BuOH fraction was more active in the inhibition of proliferation of HepG2 cells [IC_{50} = (13.42 ± 0.17) mg/mL] than the defatted MeOH extract [IC_{50} = (17.61 ± 0.15) mg/mL]. EtOAc fraction had the lowest inhibition of proliferation activity in HepG2 cells [IC_{50} = (22.51 ± 0.26) mg/mL] as shown in (Figure 1). DMSO showed no inhibition activity.

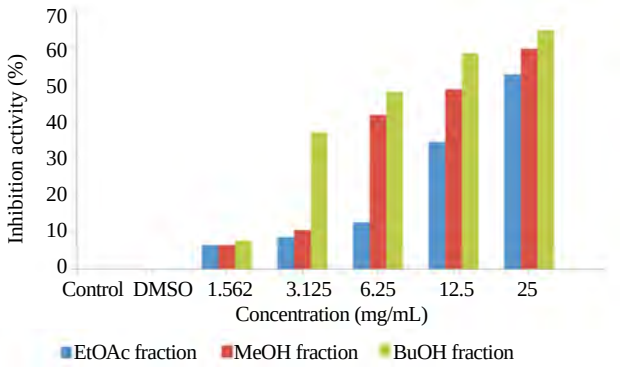


Figure 1. The inhibition of proliferation activity in the HepG2 cell line of the defatted MeOH extract of *V. agnus-castus* as well as EtOAc and BuOH fractions using the neutral red cytotoxic assay.

3.2. Cytopathological examination

Cytospin smear from untreated HepG2 cells stained with H & E exhibited neoplastic polyhedral shape hepatocytes consisting of glands and clusters with large nuclei and an increased nucleocytoplasmic ratio (Figure 2). HepG2 cells treated with the three *V. agnus-castus* extracts showed apoptotic changes included cell shrinking, condensation and fragmentation of nuclear chromatin and membrane blebbing. The apoptotic changes were more evident in HepG2 cells treated with BuOH fraction than the defatted methanolic extract of *V. agnus-castus* and EtOAc fraction (Figure 2).

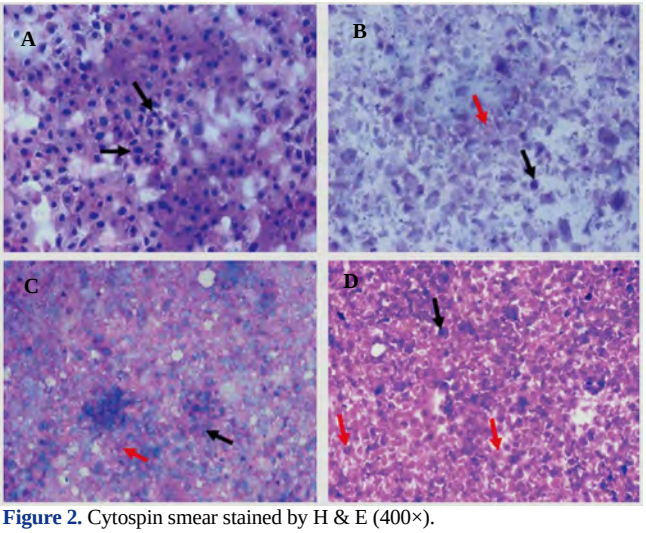


Figure 2. Cytospin smear stained by H & E (400×).
A: Untreated HepG2 cells showing alarge number of neoplastic hepatocytes consisting of groups of cells with enlarged nuclei and increased nucleocytoplasmic ratio (arrow); B: HepG2 cells treated with the defatted MeOH extract of *V. agnus-castus* showing a large number of hepatocytes with central nuclei (black arrow) and with scattered apoptotic and degenerative changes (red arrow); C: HepG2 cells after treatment with BuOH fraction from *V. agnus-castus* showing scattered shrinking hepatocytes with central nuclei (black arrow) and with scattered apoptotic and degenerative changes (red arrow); D: HepG2 cells after treatment with EtOAc fraction from *V. agnus-castus* showing a reduced number of small shrinking hepatocytes with central nuclei (black arrow) and with scattered apoptotic changes (including cell shrinking, condensation and fragmentation of nuclear chromatin, and membrane blebbing) and degenerative changes (red arrow).

3.3. Immunohistochemistry detection of caspase-3

In untreated HepG2 cells, no positive cells for caspase-3 were detected. In extract treated cells, it was observed that a caspase-3 expression was detected in the cells showing apoptotic changes. The apoptotic index was calculated from the relative count of positive cells expressing caspase-3 to the total number of cells. The apoptotic index of HepG2 cells treated with BuOH fraction of *V. agnus-castus* (33.16 ± 1.94) was significantly higher ($P < 0.01$) than the apoptotic index of HepG2 cells treated with the defatted MeOH extract (22.50 ± 2.58) or treated with EtOAc fraction (10.00 ± 2.28) respectively. An apoptotic index of 0% for untreated HepG2 cells was observed, as shown in Figure 3.

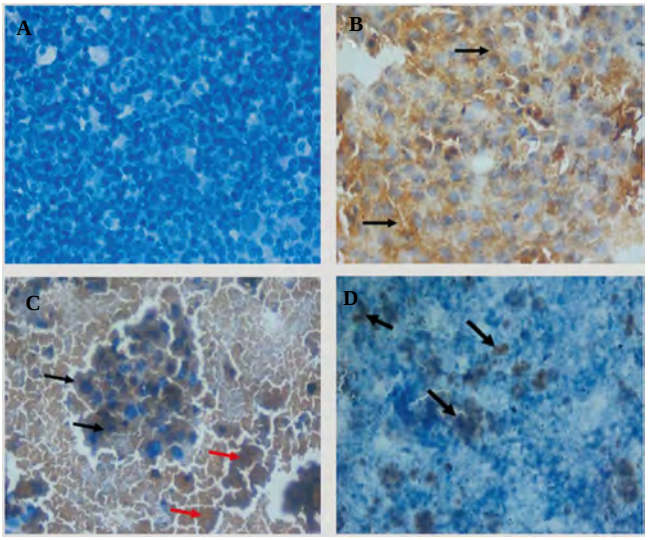


Figure 3. Immunohistochemical cytospin smear (caspase-3, 3, 3'-diaminobenzidine, 400×).
A: Untreated HepG2 cells showing a large number of neoplastic hepatocytes consisting of groups of cells with enlarged nuclei and increased nucleocytoplasmic ratio, negative staining for caspase-3; B: HepG2 cells treated with the defatted MeOH extract of *V. agnus-castus* showing positive expression of caspase 3 as brownish cytoplasmic coloration in the hepatocytes with apoptotic changes (arrows); C: HepG2 cells after treatment with BuOH fraction from *V. agnus-castus* showing positive expression of caspase 3 as brownish cytoplasmic coloration in the hepatocytes with apoptotic changes (arrows), positive expression in apoptotic hepatocytes (red arrow); D: HepG2 cells after treatment with EtOAc fraction from *V. agnus-castus* showing positive expression of caspase-3 as brownish cytoplasmic coloration in the hepatocytes with central nuclei and with apoptotic changes (arrows).

3.4. LC-ESI-MS identification of defatted MeOH extract and BuOH fraction of V. agnus-castus

The most active cytotoxic extracts (defatted MeOH extract and BuOH fraction) of *V. agnus-castus* were analyzed by LC-ESI-MS. A total of 32 compounds were observed in the negative LC-ESI-MS profile of the defatted MeOH extract (Table 1 and Figure 4) whereas 37 compounds were detected in BuOH fraction (Table 2 and Figure 5). Each compound was identified by comparison of its *m/z* value and its fragmentation pattern with standard and reported data.

Table 1
Tentative identification of certain compounds in the defatted MeOH extract of *V. agnus-castus* by LC-ESI -MS.

Compound No.	Rt (min)	Molecular weight	$[M-H]^-$	MS fragments	Proposed compounds
1	2.08	502	501	403, 273, 288, 159, 131	Unidentified
2	2.42	282	281	129, 113	Oleic acid
3	2.84	684	683	341, 179, 161	Caffeic acid hexoside dimer
4	3.00	192	191	127, 93, 85	Quinic acid
5	3.17	534	533	191 (100%), 127, 93, 85	Quinic acid derivative
6	19.95	138	137	93 (100%), 65	*p*-Hydroxybenzoic acid
7	22.96	708	707	353, 191 (100%), 93, 85	5-*O*-Caffeoylquinic acid dimer
8	24.63	516	515	353, 191, 179, 135	1,3-Di-*O*-caffeoylquinic acid
9	25.21	628	627	465, 303, 179	Taxifolin-di-*O*-hexoside
10	25.88	516	515	353, 191, 179, 173, 137	4,5-Di-*O*-caffeoylquinic acid I
11	29.14	496	495	357, 281, 213, 137	2′-*p*-Hydroxybenzoyl mussaenosidic acid (Negundoside I)
12	29.72	496	495	357, 281, 213, 137	2′-*p*-Hydroxybenzoyl mussaenosidic acid (Negundoside II)
13	31.98	448	447	357, 327, 285	Luteolin-8-C-glucoside (Orientin)
14	32.56	466	465	285, 137	Agnuside
15	34.06	678	677	515, 353, 191, 179, 161	1,3-Di-*O*-caffeoylquinic acid-*O*-hexoside
16	34.73	496	495	357, 281, 213, 137	2′-*p*-Hydroxybenzoyl mussaenosidic acid (Negundoside III)
17	35.40	442	441	281, 137 (100%), 93	*p*-Hydroxybenzoic acid-*O*-hexosyl-rhamnoside
18	35.65	432	431	341, 311 (100%), 283	Apigenin-8-C-glucoside (Vitexin)
19	36.65	448	447	285, 179	Luteolin-*O*-hexoside
20	37.82	462	461	285, 179	Luteolin-*O*-glucuronide
21	38.40	516	515	353, 191 (100%), 179, 135	3,5-Di-*O*-caffeoylquinic acid
22	39.49	194	193	133 (100%), 62	Ferulic acid
23	41.33	516	515	353, 191, 179, 173, 137	4,5-Di-*O*-caffeoylquinic acid II
24	41.91	544	543	357, 185, 151	6′-*O*-(6,7-Dihydrofoliamenthoyl) mussaenosidic acid (Agnucastoside B)
25	42.41	542	541	339, 295, 163, 119	6′-*O*-foliamenthoylmussaenosidic acid (Agnucastoside A)
26	45.34	462	461	315, 285, 161	Kaempferol-*O*-glucuronide
27	46.25	504	503	375, 193	Unidentified
28	48.59	866	865	681, 503, 179, 113	Unidentified
29	49.43	286	285	151, 133	Kaempferol
30	50.26	684	683	521, 179, 161	Rosmarinic acid di-*O*-hexoside
31	52.27	712	711	503, 441, 173, 113	Unidentified
32	57.53	344	343	329, 301, 286, 181	Penduletin

Compounds 4, 5, 7, 8, 10, 15, 21 and 23 (Rt = 3.00, 3.17, 22.96, 24.63, 25.88, 34.06, 38.40 and 41.33 min, respectively) on the defatted MeOH extract (Table 1 and Figure 4) and compounds 3, 7, 8, 16, 17, 22, 23 and 25(Rt = 3.00, 22.87, 23.88, 33.81, 34.15, 38.15, 38.74 and 41.66 min, respectively) in BuOH fraction (Table 3 and Figure 5) were identified as quinic acid derivatives. Compounds 6 and 17 (Rt = 19.95 and 35.40 min) in the defatted MeOH extract and compound 6 in BuOH fraction (Rt = 19.87 min) were identified as hydroxybenzoic acid derivatives. Also, compounds 3, 22 and 30 (Rt = 2.84, 39.49 and 50.26 min, respectively) in the defatted MeOH extract and compounds 2, 5, 26, 30 and 33(Rt = 2.83, 18.95, 41.91, 45.25 and 46.67 min, respectively) in BuOH fraction were identified as hydroxycinnamic acid derivatives.

Compounds 13 and 18 (Rt = 31.98 and 35.65 min, respectively) in the defatted MeOH extract (Table 1 and Figure 4) and compounds 14 and 19 (Rt = 31.98 and 35.82 min, respectively) in BuOH fraction (Table 2 and Figure 5) were identified as *C*-glycosyl flavonoids. On the other hand, compounds 9, 19, 20, 26, 29 and 32 (Rt = 25.21, 36.65, 37.82, 45.34, 49.43 and 57.53

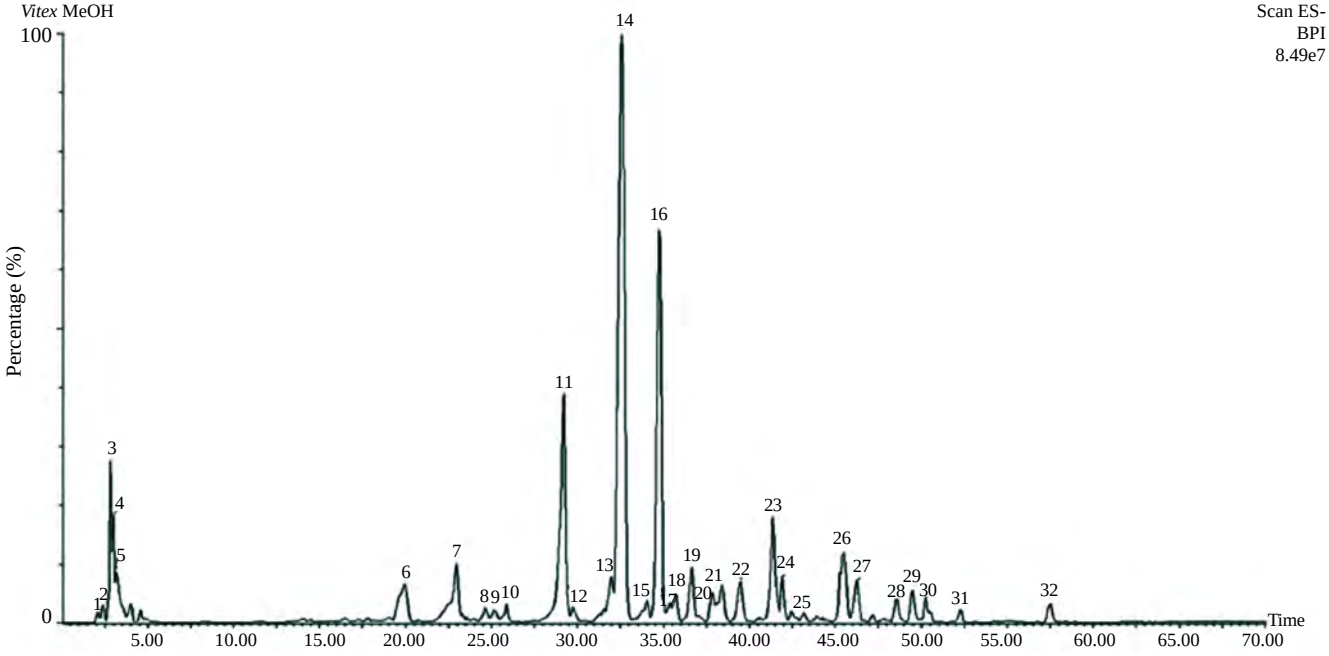


Figure 4. LC-ESI-MS total ion current chromatogram of the defatted MeOH extract of *V. agnus-castus* at the negative ion mode.

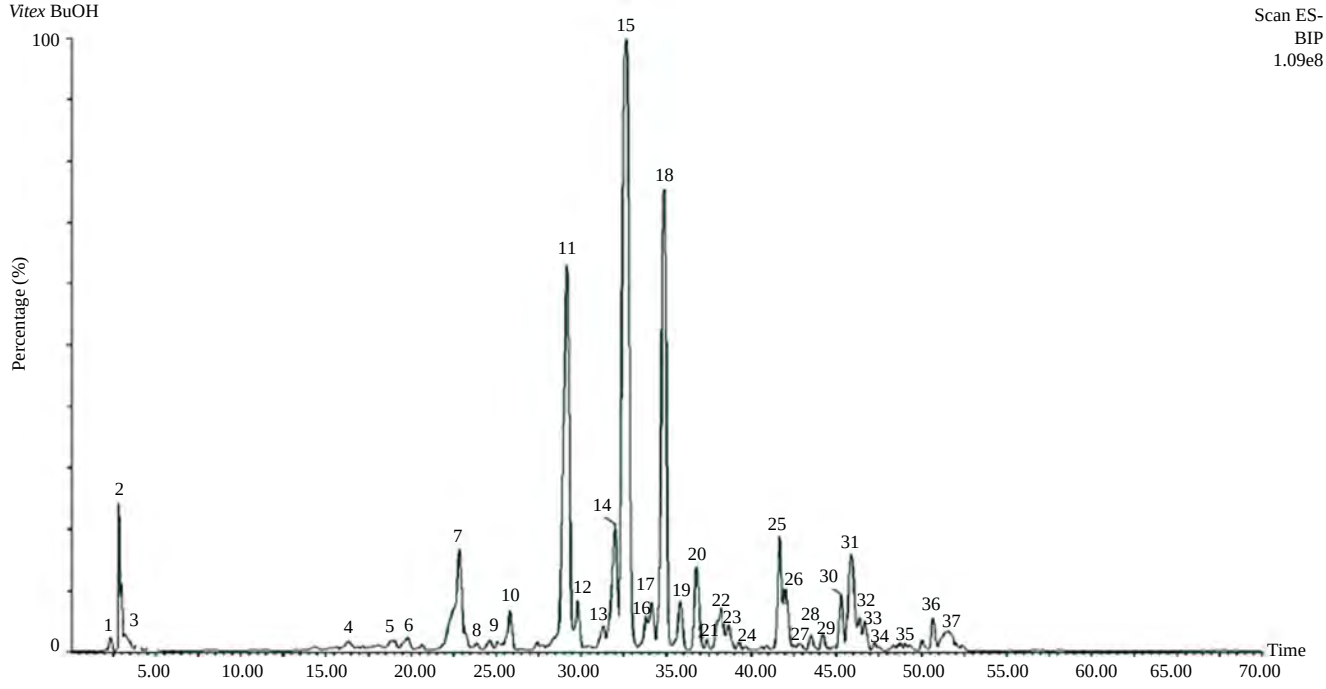
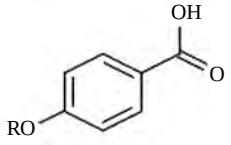
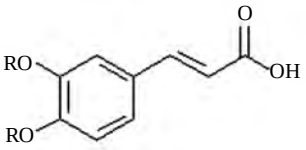


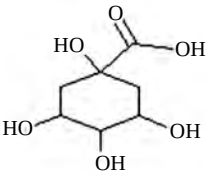
Figure 5. LC-ESI-MS total ion current chromatogram of *V. agnus-castus* BuOH fraction at the negative ion mode.



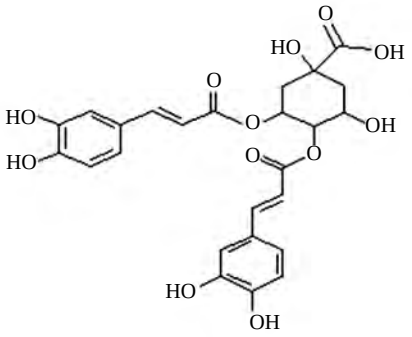
p-Hydroxybenzoic acid-*O*-hexosyl-rhamnoside;
(R = Hexose-*O*-rhamnose)



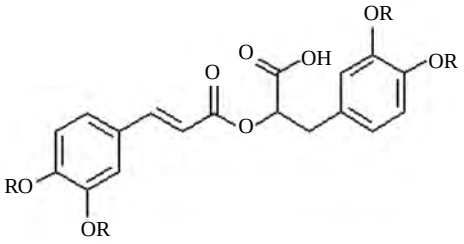
Caffeic acid-*O*-hexoside;
(1R = Hexose, 1R = H)



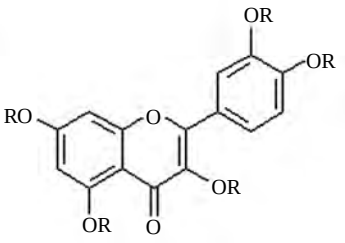
Quinic acid



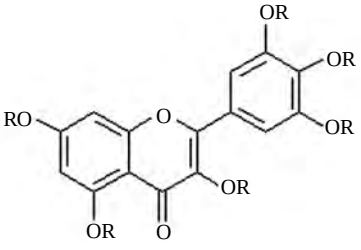
4,5-*O*-di-caffeoylquinic acid



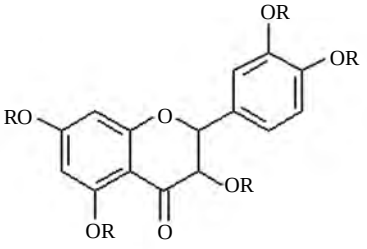
Rosmarinic acid-di-*O*-hexoside
(2R = Hexose, 2R = H or 1R = Hexose-*O*-hexose, 3R = H)



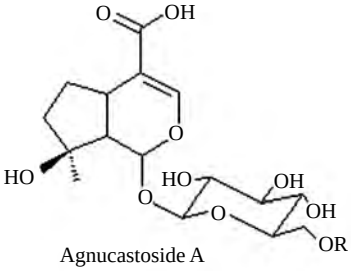
Quercetin-*O*-hexoside
(1R = Hexose, 4R = H)



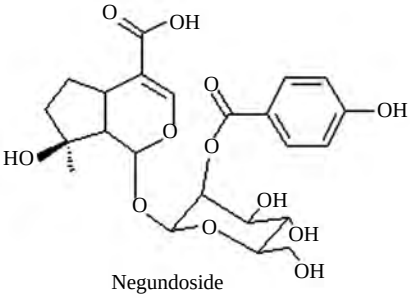
Myricetin-*O*-pentoside-glucuronide
(1R = Pentose, 1R = Glucuronic acid, 4R = H or 1R = Pentoside-*O*-glucuronide, 5R = H)



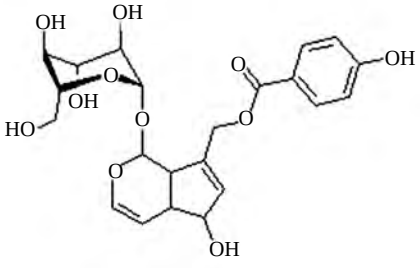
Taxifolin-*O*-hexoside
(1R = Hexose, 4R = H)
Taxifolin-di-*O*-hexoside
(2R = Hexose, 3R = H or 1R = Hexose-*O*-hexose, 4R = H)



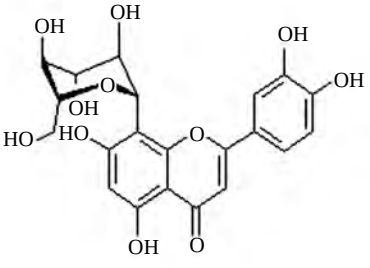
Agnucastoside A
(R = Foliamenthoyl)
Agnucastoside B
(R = 6,7-dihydrofoliamenthoyl)



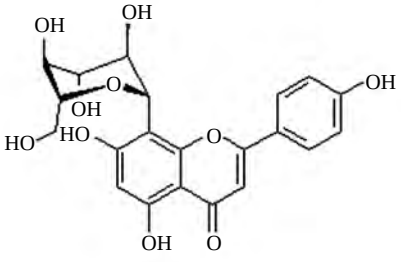
Negundoside



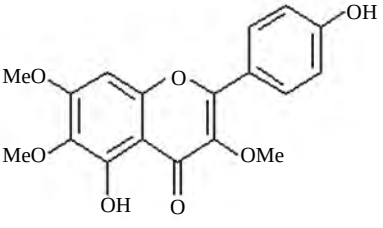
Agnuside



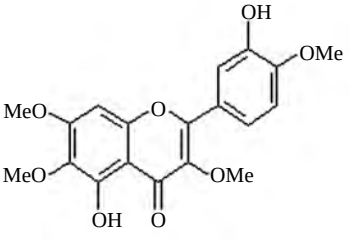
Orientin



Vitexin



Penduletin



Casticin

Figure 6. Structures of some of the identified compounds presented in the defatted MeOH extract and BuOH fraction of *V. agnus-castus*.

min, respectively) in the defatted MeOH extract and compounds 4, 9, 13, 20, 28, 31, 32, 35 and 37 (Rt = 16.36, 24.63, 31.31, 36.82, 43.50, 45.84, 46.34, 50.01 and 51.35 min, respectively) in BuOH fraction were identified as *O*- glycosyl flavonoids.

Compounds 11, 12, 14, 16, 24 and 25 (Rt = 29.14, 29.72, 32.56, 34.73, 41.91 and 42.41 min, respectively) in the defatted MeOH extract (Table 1 and Figure 4) and compounds 11, 12, 15, 18, 24, 27 and 36 (Rt = 29.14, 29.80, 32.56, 34.81, 39.24, 42.08 and 50.68 min, respectively) in BuOH fraction (Table 2 and Figure 5) were identified asiridoids derivatives.

Table 2
Tentative identification of certain compounds in BuOH fraction of *V. agnus-castus* by LC-ESI-MS.

Compound No.	Rt (min)	Molecular weight	$[M-H]^-$	MS fragments	Proposed compounds
1[a]	2.33	282	281	129, 113	Oleic acid
2[a]	2.83	684	683	341, 179	Caffeic acid-*O*-hexoside dimer
3	3.00	534	533	341, 191 (100%), 85	Feruloylquinic acid derivative
4	16.36	464	463	301, 273, 179, 151	Quercetin-*O*-hexoside
5	18.95	462	461	281, 239, 179, 137, 93	Caffeic acid derivative
6[a]	19.87	138	137	93 (100%), 65	*p*-Hydroxy benzoic acid
7[a]	22.87	708	707	353, 191 (100%), 93, 85	5-*O*-Caffeoylquinic acid dimer
8	23.88	354	353	191, 179 (100%), 173, 135, 93, 85	4-*O*-Caffeoylquinic acid
9[a]	24.63	628	627	465, 303, 197	Taxifolin-di-*O*-hexoside
10	25.80	494	493	459, 395, 281, 137	Unidentified
11[a]	29.14	496	495	357, 281, 137	2′-*p*-Hydroxybenzoyl mussaenosidic acid (Negundoside I)
12[a]	29.80	496	495	357, 281, 137	2′-*p*-Hydroxybenzoyl mussaenosidic acid (Negundoside II)
13	31.31	628	627	493, 317, 197, 151	Myricetin-*O*-pentosyl-*O*-glucuronid
14[a]	31.98	448	447	357, 327, 297	Luteolin-8-C-glucoside (Orientin)
15[a]	32.56	466	465	303, 285, 137	Agnuside
16	33.81	678	677	515, 353	3,4,5-Tri-*O*-caffeoylquinic acid
17[a]	34.15	678	677	515, 353, 191, 179, 135	1,3-Di-*O*-caffeoylquinic acid hexoside
18[a]	34.81	496	495	357, 281, 239, 137	2′-*p*-Hydroxybenzoyl mussaenosidic acid (Negundoside III)
19[a]	35.82	432	431	341, 311 (100%), 283	Apigenin-8-C-glucoside (Vitexin)
20[a]	36.82	448	447	285, 179	Luteolin-*O*-hexoside
21	37.40	706	705	459, 347, 173, 137	Unidentified
22[a]	38.15	516	515	353, 191, 179, 173, 137	4,5-Di-*O*-caffeoylquinic acid I
23[a]	38.74	516	515	353, 191 (100%), 179, 135	1,3-Di-*O*-caffeoylquinic acid
24[a]	39.24	542	541	357 (100%), 183, 151	6′-*O*-foliamenthoylmussaenosidic acid (Agnucastoside A)
25[a]	41.66	516	515	353, 191, 179, 173, 137	4,5-Di-*O*-caffeoylquinic acid II
26	41.91	798	797	635 (100%), 455, 179, 173	Caffeoyl-1,3,4-tri-*O*-galloyl-hexsoide
27[a]	42.08	544	543	357, 185	6′-*O*-(6,7-Dihydrofoliamenthoyl) mussaenosidic acid (Agnucastoside B)
28	43.50	932	931	465, 303, 179	Taxifolin-*O*-hexoside dimer
29	44.17	706	705	521, 285, 179, 161	Unidentified
30	45.25	522	521	359, 179, 135	Rosmarinic acid-*O*-hexoside
31[a]	45.84	462	461	285, 161	Kaempferol-*O*-glucuronide
32	46.34	636	635	461, 285, 161	Kaempferol-*O*-di glucuronide
33[a]	46.67	684	683	521, 359, 179	Rosmarinic acid di-*O*-hexoside
34	47.26	712	711	503, 285	Unidentified
35[a]	50.01	286	285	151, 133, 61	Kaempferol
36	50.68	684	683	521, 503, 343, 271, 179	7-*O*-Trans-*p*-coumaroyl-6'-O-trans-caffeoyl-8-epiloganic acid (Agnucastoside C)
37	51.35	374	373	358, 343	Casticin

[a]: Compounds present in the defatted MeOH extract and BuOH fraction.

4. Discussion

4.1. Cytotoxic and apoptotic effect of V. agnus-castus extracts

Induction of apoptosis is an important goal for cancer chemotherapy[21]. In previous studies, *V. agnus-castus* extracts exhibited cytotoxic effect against several types of human cancer cells[22,23]. In the current study, the anticancer potential of *V. agnus-castus* extracts was evaluated by cytotoxicity assay, cytopathological changes of apoptosis and caspase-3 expression in HepG2 cells. The antiproliferative effect and induction of apoptosis of the defatted MeOH extract of *V. agnus-castus* as well as EtOAc and BuOH fractions were measured in HepG2 *in-vitro*. HepG2 cells represent an experimental *in-vitro* model to study hepatocellular carcinoma. *In-vitro* cytotoxicity assay has become a primary screening method for evaluating the anticancer properties of various compounds and plant extracts[22].

The inhibition of cell proliferation was measured by neutral red cytotoxicity assay. The three extracts of *V. agnus-castus* inhibited HepG2 cell proliferation. The BuOH fraction was more active in the inhibition of human hepatoma HepG2 cells than the defatted MeOH extract, whereas the EtOAc fraction was less active. This activity may be attributed to the difference in percent of the active constituents in the tested extracts. Cytopathological changes were examined in cytospin smears in parallel samples of untreated and treated HepG2 cells with different extracts. Treating HepG2 cells with *V. agnus-castus* extracts showed changes in cell morphology which were consistent with an apoptotic mechanism cell death[23]. The apoptotic changes in HepG2 cells recorded higher activity when treated with the BuOH fraction than cells treated with the defatted MeOH extract and the EtOAc fraction. This was confirmed by the cytopathology changes. Many previous studies revealed that different plant extracts had antiproliferative activity and apoptosis in cancer cell lines[11,16,21,24].

The results indicated that the apoptotic changes in cancer cell lines treated with *V. agnus-castus* extracts are associated with elevated expression of caspase-3. These results are in agreement with the results obtained by Imai *et al*.[24] and Park *et al*.[25]. The authors observed that the apoptosis induction accompanied activation of caspase-3 when the human colon carcinoma cells were treated with *Vitex* extracts. The present results proved that *V. agnus-castus* extracts could induce apoptosis in the HepG2 cell line through caspase-3 activation.

Owing to the high anticancer activity of the defatted MeOH

extract of *V. agnus-castus* and BuOH fraction, the two extracts were submitted to LC-ESI-MS analysis for identification of the active constituents of these extracts.

4.2. LC-ESI-MS identification of the defatted MeOH extract and BuOH fraction of V. agnus-castus

The identification of the resulted peaks (compounds) from the LC-ESI-MS analysis in this current work was based on their main molecular ions and their fragmentation pattern as well as taking into accounts the previously reported results. The results revealed that the phenolic acid derivatives, iridoid glycosides, and flavonoid glycosides represented the major components of the defatted MeOH extract and BuOH fraction.

4.2.1. Identification of phenolic acid derivatives

Compound 4 in the defatted MeOH extract had a deprotonated molecule in negative ion mode $[M-H]^-$ ion at *m/z* 191 and base peak at *m/z* 127$[M-H-H_2O-CO_2]^-$, therefore, this compound was identified as quinic acid; while compound 5 in the same extract had $[M-H]^-$ ion at *m/z* 533 and base peak at 191 (100%). Therefore, it was identified as quinic acid derivative[26,27]. Also, compound 7 in the defatted MeOH extract and compound 7 in BuOH fraction gave a deprotonated molecule $[2M-H]^-$ of *m/z* 707 yielded two fragments; *m/z* 353 and 191 (100%) $[M-H-162]^-$. Therefore, this compound was identified as 5-*O*-caffeoylquinic acid dimer. Compound 8 in the defatted MeOH extract and compound 23 in BuOH fraction showed $[M-H]^-$ ion at *m/z* 515 and the same fragmentation pattern of *m/z* 353[M-H-162 (caffeic acid)]$^-$, *m/z* 191[M-H-2 × 162 (2 caffeic acid units)]$^-$. Therefore, these compounds were characterized as 1,3 di-*O*-caffeoylquinic acid. Compounds 10 and 23 in the defatted MeOH extract along with compounds 22 and 25 in BuOH fraction gave $[M-H]^-$ ion at *m/z* 515 and yielded ions at *m/z* 353[M-H-caffeic acid]$^-$, 191 (quinic acid), 179 of caffeic acid, 173 and 137 characteristic for 4,5 di-*O*-caffeoylquinic acid. Compound 8 in BuOH fraction showed $[M-H]^-$ ion at *m/z* 353 and other fragment peaks; *m/z* 191[M-H-caffeic acid]$^-$, 179 (100%), 173, 135 and 85, so, it was identified as 4-*O*-caffeoylquinic acid[28]. Compound 15 in the defatted MeOH extract and compound 17 in BuOH fraction showed $[M-H]^-$ ion at *m/z* 677 and other fragments at *m/z* 515[M-H-162 (hexose)]$^-$, 353, 191, 179, 161, therefore, these compounds were interpreted as 1,3 di-*O*-caffeoylquinic acid hexoside. Compound 21 in the defatted MeOH extract exhibited $[M-H]^-$ with *m/z* 515 and other fragments; *m/z* 353, 191 (100%), 179, 135. So, this compound was identified as 3,5 di-*O*-caffeoylquinic acid. Compound 3 in BuOH fraction, exhibited $[M-H]^-$ ion at *m/z* 533 and produced fragment peaks at *m/z* 341, 191 (100%) and 85. Therefore, this compound was identified as feruloylquinic acid derivative. Compound 16 in BuOH fraction showed $[M-H]^-$ ion at *m/z* 677 and fragment peaks at *m/z* 515[M-caffeic acid]$^-$, 353[M-H-2 caffeic acid]$^-$, so compound 16 was interpreted as 3,4,5 tri-*O*-caffeoylquinic acid[29,30].

Compound 6 in the defatted MeOH extract and compound 6 in BuOH fraction showed $[M-H]^-$ ion at *m/z* 137, and base peak with *m/z* 93$[M-H-CO_2]^-$. This reflected that the two compounds were *p*-hydroxybenzoic acid. Compound 17 in the defatted MeOH extract showed $[M-H]^-$ ion at *m/z* 441 and two major fragments; *m/z* 281$[M-H-160]^-$ and 137$[M-H-160-144]^-$ which meant it lost hexose and rhamnose units. So, this compound was identified as hydroxybenzoic acid-*O*-hexosyl-rhamnoside[13,30,31].

Compound 3 in the defatted MeOH extract and compound 2 in BuOH fraction exhibited *m/z* 683$[2M-H]^-$ and produced other fragments; *m/z* 341$[M-H]^-$ and *m/z* 179[M-H-162 (hexose)]$^-$. So, these compounds were identified as caffeic acid hexoside dimer. Compound 5 in BuOH fraction showed $[M-H]^-$ ion at *m/z* 461 and other fragments; *m/z* 281, 239, 179 and 137 characteristics for caffeic acid derivatives[32-34]. Compound 26 in BuOH fraction showed $[M-H]^-$ ion at *m/z* 797 and other fragments at *m/z* 635 (100%) [M-H-162 (hexose)]$^-$. Therefore, this compound was characterized as caffeoyl-1,3,4-tri-*O*-galloyl hexoside[35]. Compound 22 in defatted MeOH extract showed $[M-H]^-$ with *m/z* 193 and base peak at *m/z* 133 (100%) $[M-H-H_2O-CO_2]^-$. Therefore, this compound was identified as ferulic acid[12,36]. Compound 30 in BuOH fraction exhibited $[M-H]^-$ ion at *m/z* 521 and other fragment peaks; *m/z* 359[M-H-162 (hexose)]$^-$, 179 and 135. Therefore, this compound was interpreted as rosmarinic acid-*O*-hexoside. Compound 30 in the defatted MeOH extract and peak 33 in BuOH fraction exhibited $[M-H]^-$ ion at *m/z* 683 and yielded ions at *m/z* 521[M-H-162 (hexose)]$^-$ and 359[M-H-2 × 162 (hexose)]$^-$. So, the two compounds have the same structure and are identified as rosmarinic acid-di-*O*-hexoside[37].

4.2.2. Identification of flavonoid derivatives

The diagnostic mass fragmentation pattern from LC-ESI-MS in the negative ion mode at 317, 301 and 285 was interpreted to be aglycones as myricetin, quercetin, and kaempferol, respectively. The neutral losses of 162, 146 and 132 mass units allowed for the interpretation of hexoside (glucose or galactose), deoxyhexoside (rhamnose), and pentoses (xylose or arabinose), respectively[27].

The results from the LC-ESI-MS analysis of the defatted MeOH extract *V. agnus-castus* and BuOH fraction showed the presence of a mixture of flavonoid compounds. Compound 13 in the defatted MeOH extract and compound 14 in BuOH fraction gave $[M-H]^-$ ion at *m/z* 447 which produced other fragment peaks; *m/z* 357, 327 and 285. So, these compounds were identified as luteolin-8-C- glucoside (Orientin). Compound 18 in the defatted MeOH extract and compound 19 in BuOH fraction exhibited the same $[M-H]^-$ ion at *m/z* 431 and other fragment ions; *m/z* 341, 311 (100%) and 283. The two compounds were identified as apigenin-8-C-glucoside (Vitexin)[29,33,37-39]. Compound 4 in BuOH fraction showed $[M-H]^-$ ion at *m/z* 463 and base peak of quercetin at *m/z* 301[M-H-162 (hexose)]$^-$. So, this compound was characterized as quercetin-*O*-hexoside. Compound 9 in the defatted MeOH extract and compound 9 in BuOH fraction exhibited $[M-H]^-$ ion at *m/z* 627 and other fragment peaks at *m/z* 465[M-H-162 (hexose)]$^-$ and 303[M-H-2 × 162 (hexose)]$^-$. Therefore, the two compounds characterized as taxifolin- di-*O*-hexoside. Also, compound 28 in BuOH fraction showed $[2M-H]^-$ ion at *m/z* 931 of taxifolin-di-*O*-hexoside dimer[34]. Compound 13 in BuOH fraction exhibited $[M-H]^-$ ion at *m/z* 627 and gave fragment peaks; *m/z* 493[M-H-134 (pentose)]$^-$ and 317[M-H-134 (pentose)-176 (glucuronic acid)]$^-$. Therefore, this compound was interpreted as myricetin-*O*-pentosyl-glucuronide. Compound 19 in the defatted MeOH extract and compound 20 in BuOH fraction exhibited $[M-H]^-$ ion at *m/z* 447 and gave fragments ions at *m/z* 285[M-H-162 (hexose)]$^-$ and 179. Therefore, this compound was characterized as luteolin-*O*-hexoside. Compound 20 in the defatted MeOH extract showed $[M-H]^-$ ion at *m/z* 461 and other fragment peaks at *m/z* 285[M-H-176 (glucuronic

acid)]$^-$ and 179 characteristic for luteolin-*O*-glucuronide[29,39,40]. Compound 29 in the defatted MeOH extract and compound 35 in BuOH fraction exhibited the same [M-H]$^-$ ion at *m/z* 285 and other peaks at 151 and 133. So, the two compounds were identified as kaempferol aglycone. Compound 26 in the defatted MeOH extract and peak compound 31 in BuOH fraction gave [M-H]$^-$ ion at *m/z* 461 and another fragment at *m/z* 285[M-H-176 (glucuronic acid)]$^-$ for kaempferol-*O*-glucuronide. Compound 32 in BuOH fraction exhibited [M-H]$^-$ with *m/z* 635, and fragment peaks; *m/z* 461[M-H-174 (glucuronic acid)]$^-$ and 285[M-H-174 (glucuronic acid)-176 (glucuronic acid)]$^-$, this compound was interpreted as kaempferol-di-*O*-glucuronide[29,33,37]. Compound 32 in the defatted MeOH extract showed [M-H]$^-$ ion at *m/z* 343 and other peaks at *m/z* 329, 301, 286 and 181, which produced characteristics for penduletin. Also, compound 37 in BuOH fraction exhibited [M-H]$^-$ ion at *m/z* 373 and other fragment ions; *m/z* 358 and 343 for casticin[12,29,33,39,41].

4.2.3. Identification of iridoids derivatives

Iridoids belong to the class of secondary metabolites; they are bicyclic monoterpenes containing a pyran ring in their structure and may include a sugar moiety[42]. In the present work, the typical LC-ESI-MS chromatograms of the defatted MeOH extract and BuOH fraction of *V. agnus-castus* exhibited some iridoid compounds. Compounds 11, 12 and 16 in the defatted MeOH extract and compounds 11, 12 and 18 in BuOH fraction gave [M-H]$^-$ ion at *m/z* 495 and the characteristic fragment peaks; *m/z* 375, 281, 213 and 137. This reflected that this compound was 2′-*p*-hydroxy benzoyl mussaenosidic acid (negundoside)[43-45]. Compound 14 in the defatted MeOH extract and compound 15 in BuOH fraction showed the same molecular ion peak [M-H]$^-$ ion at *m/z* 465 and yielded other fragment peaks at *m/z* 303, 285 and 137, which produced characteristics foragnuside[29,33,37,44,46]. On the other hand, compounds 25 and 24 in the defatted MeOH extract and compounds 24 and 27 in BuOH fraction gave [M-H]$^-$ ions at *m/z* 542 and 543, respectively. Including the fragmentation data, the chromatograms reflected the structure of the two compounds as 6′-*O*-foliamenthoylmussaenosidic acid (agnucastoside A) and 6′-*O*-(6,7-dihydroxy foliamenthoyl mussaenosidic acid (Agucastoside B), respectively. Also, compound 36 in BuOH fraction represented [M-H]$^-$ ion at *m/z* 683 and had characteristic fragmentation data of 7-*O*-trans-*p*-coumaroyl-6′-*O*-trans-caffeoyl-8-epiloganic acid (Agucastoside C)[47,48].

It may be concluded that from the above results, the LC-ESI-MS analysis of the defatted MeOH extract *V. agnus-castus* and BuOH fraction demonstrated the presence of three major bioactive secondary metabolites, *viz.* phenolic acids, flavonoids, and iridoids. It could be concluded that the anticancer activity of each of the tested extracts of *V. agnus-castus* is attributed to the presence of phenolic acids, flavonoids, and iridoids. These bioactive secondary metabolites can induce apoptosis in the HepG2 cell line through caspase-3 activation. The antiproliferative and apoptotic properties of *V. agnus-castus* extracts would suggest its use as an adjuvant in cancer therapy. Further isolation and evaluation of each bioactive constituent are highly recommended.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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