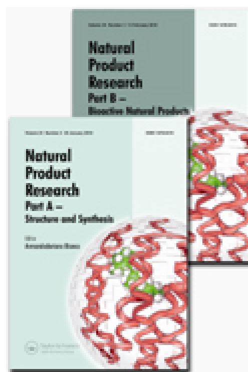

Chemical constituents and HRMS analysis of an Algerian endemic plant-Verbascum atlanticum Batt.- extracts and their antioxidant activity

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Keywords:	Verbascum atlanticum, Phenylpropanoid glycosides, Saponin glycosides, HR-ESI-MS, Antioxidant activity

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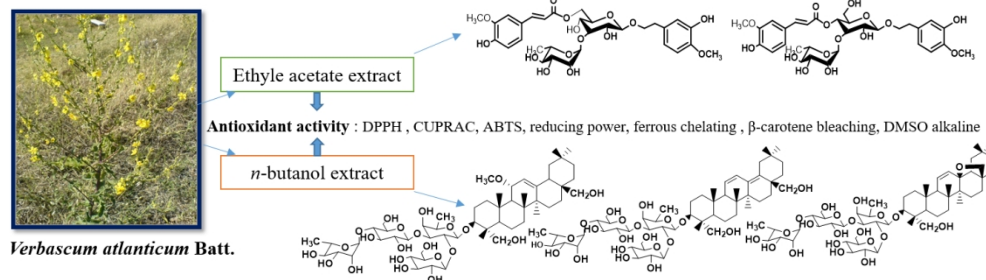

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Chemical constituents and HRMS analysis of an Algerian endemic plant- *Verbascum atlanticum* Batt.- extracts and their antioxidant activity

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Chemical constituents and HRMS analysis of an Algerian endemic plant– *Verbascum atlanticum* Batt. – extracts and their antioxidant activity.

ABSTRACT

This is the first report on the phytochemistry and antioxidant activity of *Verbascum atlanticum* Batt. (Scrophulariaceae) extracts collected from El Kala region, Algeria. The ethyl acetate (VAA) and *n*- butanol (VAB) extracts of *V. Atlanticum* Batt. Were subjected to a phytochemical study which led to identifying nine compounds of fatty acid esters, diterpenes, phenylpropanoid glycosides and saponin glycosides type. In addition, both extracts were analysed by LC-UV-MS and HR-ESI-MS. This later revealed, on the basis of in-house library, the presence of eight other known bioactive microbial metabolites. All these compounds were identified for the first time in *Verbascum atlanticum.* Finally, both extracts were evaluated for their phenolic and flavonoid contents as well as their antioxidant activity. The results showed that the VAA extract, which was richer in flavonoids, had the most antioxidant effect.

Keywords:

Verbascum atlanticum, Phenylpropanoid glycosides, Saponin glycosides, HR-ESI-MS, Antioxidant activity.

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1. Introduction

In this work we investigated *Verbascum atlanticum* Batt. an Algerian endemic plant belonging to *Verbascum* genus (Benaissa et al. 2018). This genus is widely used in folk medicine as an expectorant, mucolytic, sudorific, sedative, diuretic and constipate (Tatli et al. 2003). It is represented by 400 species distributed through the world (Stooted et al. 2018), and it is well known for its variety of iridoids, saponosides and phenyl glycosides (Frezza et al. 2018) are valuable for the taxonomic evaluation of this genus. In the present study, the ethyl acetate (VAA) and *n*-butanol (VAB) extracts of *V. Atlanticum* were subjected to chromatographic separation, this step led to the isolation and identification of nine compounds which belong to fatty acid esters, diterpenes, phenylpropanoid glycosides and saponines glycosides types. LC-UV-MS and HRMS analysis of samples extracts allowed in the identification of eight other known bioactive microbial on the basis of in-house library (Martin et al. 2014). Finally, both extracts were submitted to qualitative and quantitative analysis as well as the evaluation of their antioxidant activity using different *in vitro* model assays.

2. Results and discussion.

Both (VAA) and (VAB) extracts of *V. atlanticum* were chromatographed on silica gel column to give VAA1, VAA15 and VAB fractions respectively. The semi preparative HPLC of subfractionsVAA1-2, VAA15-3 and fraction VAB11 led to the isolation and identification of nine compounds named : (Z,Z,Z)-9, 12, 15-Octadecatrienoic acid, methyl ester (methyl linolenate) (1) (Chabert et al.2006); (Z,Z)- 9, 12-Octadecatrienoic acid, methyl ester (methyl linoleate) (2) (Huang et al. 2015; Díaz et al. 2007); Phytol-1(3) (Alarif et al. 2010); Martynoside (4) (Khodaie et al. 2018) ; Isomartynoside (5) (Calis et al. 1984); *Cis*-martynoside (6) (Skrzypek et al, 1999) ; Ilwensisaponin C (7) (Caliş et al. 1993); Ilwensisaponin B (8) (Mortada et al. 2008) ; Ilwensisaponin A (9) (Caliş et al. 1993) (figure

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1). The chromatograms of isolated compounds (figures 2, 3 and 4) are reported in the Supplementary Material. The spectroscopic data (NMR and MS) of all obtained compounds from our experiments were in good agreement with those reported in literature and are also reported in the Supplementary Material. In addition, all sub fractions obtained from chromatographic separation of both extracts of *V. Atlanticum* were submitted to LC-UV-MS analysis. This step permitted to choose nineteen samples which were injected in HR-ESI-MS. This later allowed to identifying eight other compounds named: Betulinic acid, Erythrodiol, Maslinic acid, 4-formyl-3 (formylmethyl)-4-hexenoic acid, Caffeine, Genistein, Threhalose and 12-hydroxy-8, 10-octadecadienoic acid (Table 1).These bioactives microbials metabolites were included in our in-house library (Martin et al. 2014).The identification of phenyl propanoid glycosides (4), (5), (6) and saponin glycosides (7), (8) and (9) which were previously identified in *Verbascum* species is very interesting from the chematoxanomic aspect of *Verbascum* genus (Frezza et al. 2018). On the other hand, the presence of these compounds is very important under the ethnopharmacological standpoint.

Figure 1: Identified compounds of VAA and VAB extracts

Quantitative analysis

Total phenolic and total flavonoid contents of (VAA) and (VAB) extracts of *V. Atlanticum* were determined as gallic acid equivalents (GAEs) and quercetin equivalents (QEs), respectively. Both extracts were rich in phenolic and flavonoid contents. The (VAB) extract exhibited the highest value in polyphenol (416.84±2.0 mg PEs/g extract) while (VAA) presented the highest value in flavonoid content (96.54±3.64 mg QEs/g extract) (Table 2).

Antioxidant activity

It's suitable to use multiple methods of evaluation of antioxidant activity because of the difference in the mechanisms of action of antioxidants (Huang et al., 2005).The antioxidant potential of (VAA) and (VAB) extracts from the aerial parts of *V. Atlanticum* was assessed

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using seven methods and the results are shown in (table 3). In all antioxidant methods used in the present study, including the seven radical scavenging assays (DPPH, ABTS, CUPRAC, Reducing power, Ferrous chelating, β-carotene bleaching and Superoxyde alkaline DMSO) as well as Alkaline DMSO assay (table 3), we observed higher activity results in comparison with all the standards.

The results of the antioxidant capacity measurements by DPPH showed that the (VAA) extract (IC_{50}:19.94±0.06µg/mL) was more active than the (VAB) extract (IC_{50}: 96.20±0.83 µg/mL) compared to standard compounds BHT (IC_{50}: 12.99±0.41µg/mL), α- tocopherol (13.02±5.17µg/mL) and ascorbic acid (13.94±2.81µg/mL).

The obtained values by the ABTS method for both extracts exhibited close activities (IC_{50}: 9.68±0.22; 13.37±0.11 µg/mL respectively). In addition, the extracts indicated better activity that was very close to the activity of α-tocopherol (IC_{50}: 7.59±0.53 µg/mL). However, the extracts exhibited moderate activity in comparison with BHT, BHA and ascorbic acid (IC_{50}: 1.81±0.10; 1.29±0.30; 1.74±0.10µg/mL respectively).In addition, (table 3) showed the cupric reducing antioxidant capacity of *V. atlanticum.* The (VAA) extract exhibited the highest activity (IC_{50}: 11.90±0.71µg/mL) in comparison with standards: ascorbic acid and α-tocopherol (IC_{50}: 12.43±0.09; 19.92±1.46 µg/mL respectively) and moderate compared with BHA (IC_{50}: 3.64±0.19µg/mL), however the (VAB) extract exhibited moderate activity (IC_{50}:24.68±0.93µg/mL). (table 3) showed also the chelating effects of both extracts compared with EDTA as standard on ferrous ions. The (VAA) extract (IC_{50}: 220.20±3.56 µg/mL) showed the highest metal chelating activity in comparison with activity of both extracts. However, none of the extracts had comparable results to EDTA (IC_{50}:8.80±0.47µg/mL).

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2 The reducing power of both extracts of *V. Atlanticum* was presented in (table 3). The
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4 difference between the tested extracts and the control was statistically significant ($p<0.05$).
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6 The (VAA) ($A_{0.50}$: 23.81±0.16μg/mL) was found to be the best reducing agent in comparison
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8 with the (VAB) extract, better than α-tocopherol ($A_{0.50}$: 34.93±2.38μg/mL), moderate
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10 compared with BHA and ascorbic acid ($A_{0.50}$: 7.99±0.87; 6.77±1.15μg/mL respectively).
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15 As known, the β-carotene bleaching method reveals the percentage of inhibition of lipid
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17 peroxidation. In this assay, the highest activityof *V.atlanticum* was exhibited by the (VAA)
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19 extract (IC_{50}:18.80±0.65 μg/mL), which was more active than ascorbic acid (IC_{50}: 52.59±1.98
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21 μg/mL), followed by the (VAB) extract (IC_{50}: 97.72±1.12 μg/mL). The activity of both
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23 extracts is lower than BHT, BHA and α-tocopherol (IC_{50}: 1.05±0.01; 0.90±0.02;
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25 1.79±0.03μg/mL respectively).
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32 The results of Superoxide DMSO alkaline assay were also presented in (table 3).The (VAA)
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34 and (VAB) extracts (IC_{50}:5.04±0.21, 6.46±0.34μg/mL respectively) exhibited the highest
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36 inhibitory activity, even higher than all antioxidants standards used such as ascorbic acid and
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38 α-tocopherol (IC_{50}:7.59±1.16; 31.52±2.22μg/mL respectively).
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44 A literature survey showed that the *Verbascum* genus contains several classes of secondary
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46 metabolites that have interesting biological properties.The phytochemical study of EtOAc and
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48 *n*-BuOH extracts of *V. Atlanticum* allowed the isolation and structural elucidation of nine
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50 compounds as well as eight other compounds identified with HR-ESI-MS belonging to
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52 different classes of secondary metabolites which have several biological activities. This
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54 finding is in good agreement with the literature data. Effectively, all of the identified
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56 compounds are endowed with interesting biological properties; in particular, martynoside (4)
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59 which is a good antioxidant, antiproliferative, cytoxic, antimetastatic, antiestrogenic and
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immunomodulatory (Frezza et al. 2018)]; Isomartynoside (5) is an antioxydand and anti Alzheimer (Kolak et al. 2011); *Cis*-martynoside (6) is an antibacterial (Zajdel et al. 2013); Ilwensisaponin A (9) is an antioxidant, cytotoxic and anticancer(Tatli et al. 2007; Kupeli et al. 2007) while Ilwensisaponin C **(**7) et Ilwensisaponin A (9) present antinoceptive , anti-inflammatory and antimicrobial activities (Kupeli et al. 2007; Tatli et al. 2005). The antioxidant activity of *V. Atlanticum* is in good agreement with the literature data and can be confirmed by the presence of other compounds identified in both extracts such as erythrodiol, genistein, caffein, etc.

4. Conclusion

To our knowledge, this is the first report on the phytochemical study and antioxidant activity of *Verbascum atlanticum* extracts. Nine compounds were isolated and identified using chromatographic separation and NMR spectroscopic data and eight other compounds were characterized by HR-ESI-MS analysis from ethyl acetate and *n*-butanol extracts which are known as taxonomic markers of *Verbascum* genus. All of the identified compounds are new for this species. The EtOAc extract of *V. Atlanticum* exhibited the highest flavonoid content and the highest antioxidant effect in all tests.

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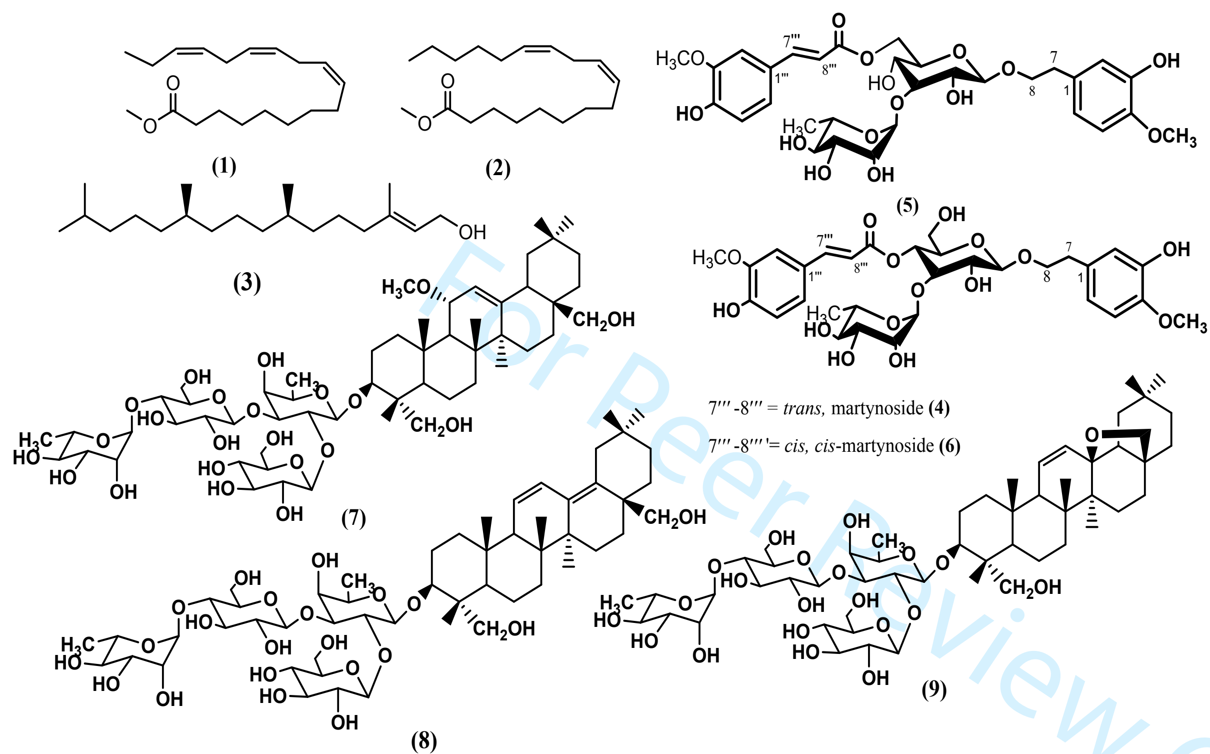

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Supplementary material

Chemical constituents and HRMS analysis of an Algerian endemic plant- *Verbascum atlanticum* Batt.- extracts and their antioxidant activity

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ABSTRACT

This is the first report on the phytochemistry and antioxidant activity of *Verbascum atlanticum* Batt. (Scrophulariaceae) extracts collected from El Kala region, Algeria. The ethyl acetate (VAA) and *n*- butanol (VAB) extracts of *V. Atlanticum* Batt. Were subjected to a phytochemical study which led to identifying nine compounds of fatty acid esters, diterpenes, phenylpropanoid glycosides and saponin glycosides type. In addition, both extracts were analysed by LC-UV-MS and HR-ESI-MS. This later revealed, on the basis of in-house library, the presence of eight other known bioactive microbial metabolites. All these compounds were identified for the first time in *Verbascum atlanticum.* Finally, both extracts were evaluated for their phenolic and flavonoid contents as well as their antioxidant activity. The results showed that the VAA extract, which was richer in flavonoids, had the most antioxidant effect.

Keywords:

Verbascum atlanticum, Phenylpropanoid glycosides, Saponin glycosides, HR-ESI-MS, Antioxidant activity.

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I.Experimental part

I.1. Extraction and isolation

The aerial parts of *V. Atlanticum* Batt. were collected from the region of El-Kala (East of Algeria) in May 2013 which were dried and pulverized into powder using a grinder. The powdered material (817g) was extracted with several solvents in cold, for 48 hours, firstly in petroleum ether, then in chloroform, in ethyl acetate, in methanol and finally in *n*-butanol. Different extracts were obtained by evaporation of the filtrate in vacuum (Rotavapor R-210 büchi, Switzerland).

Ethyl acetate (VAA) (10g) and *n*-butanol (VAB) (7g) extracts of *Verbascum atlanticum* Batt. were subjected to chromatography separation on silica gel columns eluted with gradient of methanol in chloroform. (VAA) afforded two fractions namedVAA1 (150.7mg)and VAA15 (361.2mg) in the ratios of 98.5:1.5, and 50:50 respectively. (VAB) extract, gave the fractionVAB11 (1467mg) at 45% of methanol. The fraction VAA1 was separated on silica gel column (150mg, 31cm x 1cm) with isocratic mixture of hexane/ ethyl acetate (5:1) where subfraction VAA1-2 (50mg) was collected. The fraction VAA15 was eluted over Sephadex LH-20 with MeOH. The subfraction named VAA15-3 (6.5mg) was collected. The subfractionVAA1-2 (100mg/ml dissolved in Methanol) was chromatographed using a Gilson FX-281 on column ZorbaxRX-C8 (5µ, 9.4 x 250 mm) HPLC with the flow of 3.6ml/min. The separation was performed with gradient of acetonitrile (5-100 %) in water for 46min. The chromatograms were recorded at 210 nm and 280 nm. Three compounds were run with retention times 36.4min, 37.5min and 38.75min respectively. Quantitative analysis of these compounds was showed as follow: compound **1** (29 mg), compound **2** (4.2mg) and compound **3** (0.8 mg). Subfraction VAA15-3 (13mg/ml) was separated on the same HPLC instrument on column ZorbaxSB-C18 (5µ, 9.4 x 250 mm), used acetonitrile in water in the ration of 20-50% at UV wave 280nm for 50min, that led to three compounds **4** (1mg) at 17.75min,**5** (0.7mg) at 18.70min and **6** (0.8mg) at 19.49 min. Fraction VAB11 underwent several separation by HPLC in Gilson FX-281 in the same conditions described previously. One time on column Zorbax RX-C8 (5µ, 9.4 x 250 mm) with the gradient system (5-100% ACN in H_2O) and two times on column ZorbaxSB-C18 (5µ, 9.4 x 250 mm) firstly with the system (10-80% ACN) and finally with (10-60% ACN) at 280 nm to yield the compounds **7** (7.5mg) at 27.50min, **8** (3.4mg) at 31min and **9** (1.2mg) at 35.75min.

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I.2. HPLC analysis conditions:

A sample of 0.5mg/ml dissolved in methanol from VAA and VAB extracts were analysed by LC-UV-MS and HR-ESI-MS. LC-UV-MS analysis was performed on an Agilent 1100 single quadrupole LC-MS system, using a Zorbax SB-C8 column (2.1 × 30 mm, 5 µm), maintained at 40 °C and with a flow rate of 300 µL min^{-1}. Solvent A consisted of 10% acetronitrile and 90% water with 1.3 mM trifluoroacetic acid and ammonium formate and solvent B was 90% acetronitrile and 10% water with 1.3 mM trifluoroacetic acid and ammonium formate. The gradient started at 10% B and went to 100% B in 6min, kept at 100% B for 2min, and returned to 10% B for 2 min to initialize the system. Full diode array UV scans from 100 to 900 nm were collected in 4 nm steps at 0.25 s/scan. The eluting solvent was ionized using the standard Agilent 1100 electrospray ionization source adjusted to a drying gas flow of 11 L min^{-1} at 325 °C and a nebulizer pressure of 40 psig. The capillary voltage was set to 3500 V. Mass spectra were collected as full scans from 150 m/z to 1500 m/z, with one scan every 0,77s, in both positive and negative modes. HR-ESI-MS spectra were acquired using a Brukermaxi QTOF mass spectrometer coupled to the same HPLC system as described above. The mass spectrometer was operated in positive ESI mode. Database searching was performed using an in-house-developed application where the DAD (UV−vis) spectra, retention time, and positive and negative mass spectra of the samples are compared to the corresponding UV-LC-MS data of known microbial metabolites stored in the proprietary database (Fundación MEDINA reference library containing annotated metabolite data obtained under identical conditions to those for the samples under analysis; the library includes 380 fungal metabolites and 450 metabolites from bacteria and actinomycetes) (Martin et al. 2014)

I.3. Antioxidant activity

I.3.1. Chemicals and reagents:

The measurements and calculations of the antioxidant activity results were carried out on a 96-well microplate reader, Perkin Elmer Multimode Plate Reader En Spireat Center of biotechnology Research, Constantine Algeria. The reagents 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylatedhydroxylanisole (BHA), Butylatedhydroxyltoluene (BHT), α- Tocopherol, Ascorbic acid, β-carotene, linoleic acid, polyoxyethylenesorbitanmonopalmitate(Tween-40),Neocuproine,2,2'-azino-bis (3-ethylbenzothiazoline -6sulfonicacid) diammonium salt (ABTS), Trichloroacetic acid (TCA), Potassium ferricyanide, 3-(2-Pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt (Ferrene), Ethylenediaminetetraacetic acid

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(EDTA), Nitro blue tetrazolium (NBTT), Dimethyl sulfoxyde (DMSO) were obtained from Sigma Chemical Co.(Sigma-Aldrich GmbH, Stern-heim, Germany), Sodium Carbonate, Aluminum Nitrate, Iron (III) chloride ($FeCl_3$), Iron (II) chloride, Sodium bicarbonate, Copper (II) chloride, Potassium persulfate, Potassium acetate, were obtained from Biochem Chemopharma, All other chemicals and solvents were of analytical grade.

I.3.2. Determination of total phenolic and flavonoid contents

The total phenolic content **(TPC)** of VAA and VAB extracts of *V. atlanticum* was determined spectrophtometrically following the Folin–Ciocalteu (Singleton 1977), while the total flavonoid content **(TFC)** was determined spectrophtometrically (Tel et al. 2013).

I.3.3. Determination of antioxidant activity

DPPH scavenging activity

The free radical-scavenging activity was determined spectrophotometrically by the DPPH assay (Blois 1958). Briefly 40 µl of MeOH solution of the sample at various concentrations was added to 160 µl DPPH (0.1 mM). The reaction mixture was shaken vigorously and the absorbance of remaining DPPH was measured at 517 nm after 20 min. BHA and BHT were used as antioxidant standards for comparison of the activity. The scavenging capability of DPPH radical was calculated using the following equation

$$\text{DPPH scavenging effect}(\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

The results were given as IC_{50} value (µg/mL) corresponding the concentration of 50% inhibition.

ABTS scavenging activity

The spectrophotometric analysis of the $ABTS^{\bullet+}$ scavenging activity was determined according to the method of Re et al. (1999). After preparation of the oxidation solution of ABTS, the $ABTS^{\bullet+}$ solution was diluted to get an absorbance of 0.700 ± 0.020 at 734nm with water. Then, 160µL of ABTS solution were added to 40µL of sample solution in methanol at different concentrations. After 10 min, the absorbance was measured at 734 nm. Methanol was used as a control. BHA and BHT were used as antioxidant standards for comparison of the activity. The results were given as the IC_{50} (µg/mL).

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Cupric reducing antioxidant capacity activity (CUPRAC)

The cupric reducing antioxidant capacity was determined according to the CUPRAC method (Apak et al. 2004). In each well the reaction mixture containing 40µL of sample solution and 50µL of a copper (II) chloride solution, 50µL of neocuproine alcoholic solution, and 60µL of ammonium acetate aqueous buffer at pH 7 was added to give a final volume of 200µL. After 30 min, the absorbance was measured at 450nm. Results were recorded as absorbance compared with the absorbance of BHA and BHT, which were used as antioxidant standards.

Reducing power activity

The reducing power of the tested extracts was determined according to the method of (Bouratoua et al. 2017). In order to determine the reducing power activity, 10µl of serial diluted sample were added into a 96 well round-bottomed plate. Following this, 40µL of 0.2M phosphate buffer (pH 6.6) and 50µL of potassium ferricyanide (1%), were added to each well and the plate was incubated at 50 °C for 20min. Finally, 50µL of TCA (10%), (40µL) of distilled water and 10µL of ferric chloride (0.1%), were added into each well in order to measure the reducing power activity. After, the absorbance was measured at 700nm. Higher absorbance of the reaction mixture indicates greater reducing power.

Ferrous ions chelating activity

The chelating activity was established by using 96-well microliter plate in accordance with the reported method (Decker & Welch 1990) with slight modifications. The Iron dichloride (0.2mM $FeCl_2$) was prepared in distilled water. The extracts were dissolved in methanol (MeOH) and all samples (80µL) together with the Iron dichloride solution (40 µL) were mixed in the plate. Afterwards, 80µL of the ferene iron reagent (0.5 mM) was added for initiation of the reaction and the mixture was incubated for 10 min. After complete incubation, the absorbance was measured at 593 nm. Each assay for all samples was carried out in triplicate.

β-carotene bleaching activity

The β-carotene bleaching activity of our extracts was evaluated using the β-carotene-linoleic acid system described by (Marco, 1968). Thus, a solution of β-carotene (0.5 mg) in 1 ml of chloroform is added to 25µl of linoleic acid and 200µl of Tween 40. After evaporation in vacuum of the chloroform, 50 ml of Hydrogen peroxide H_2O_2 are added under vigorous agitation. The absorbance of the solution is adjusted to 0.8-0.9 nm. Amounts of 160µl of this

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solution are added to 40µl of solution of the studied extracts at different concentrations. The absorbance was measured at 470 nm. The emulsion system was incubated for 2 h at 50 ºC. A blank, devoid of β-carotene, was prepared for background subtraction. BHA and BHT were used as standards.

The bleaching rate (R) of β-carotene was calculated according to the following equation:

$$R = \frac{\ln\frac{a}{b}}{t}$$

Where: ln = natural log, a = absorbance at time zero, b = absorbance at time t (120 min).

The antioxidant activity (AA) was calculated in terms of percent of inhibition relative to the control, using the following equation:

$$AA\ (\%) = \frac{R_{Control} - R_{Sample}}{R_{Control}} \times 100$$

Superoxide DMSO alkaline activity

Superoxide was generated according to the alkaline DMSO method (K. Elizabeth and M.N.A. Rao (1990)), the reduction of NBT by superoxide was determined in the presence and absence of extracts. To the reaction mixture containing NBT (1 mg/mL solution in distilled water) and 40µl of sample at various concentrations was added to 130 µl alkaline DMSO (1.0 ml DMSO containing, 5mMNaOH in 0.1 ml water) to give a final volume of 200 µL and the absorbance was measured at 560 nm using microplate reader. The decrease in the absorbance at 560 nm with antioxidants indicated the consumption of generated superoxide.

Statistical analysis

All data on bioassays activity tests were the average of triplicate analyses. The data were recorded as mean ± standard deviation. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by student's-t test, p values <0.05 were regarded as significant.

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II. Results

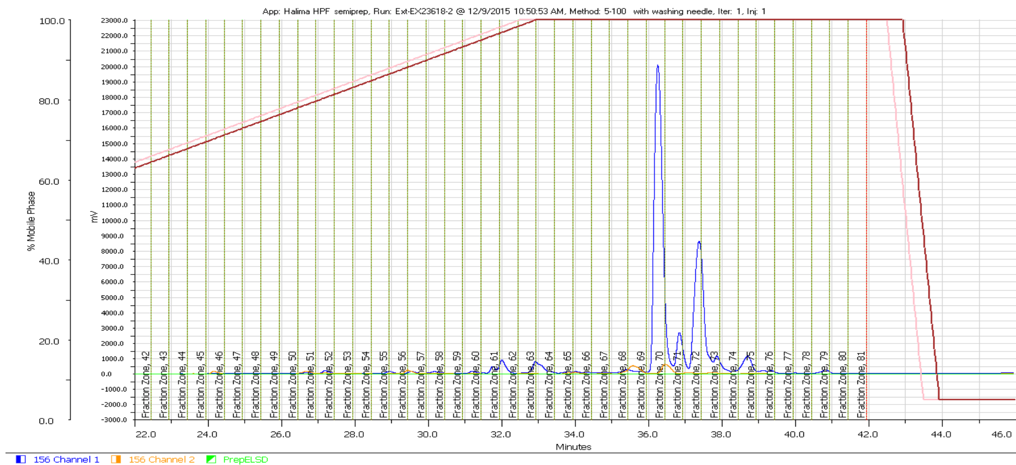

Figure 2.Chromatogram of compounds 1, 2, and 3

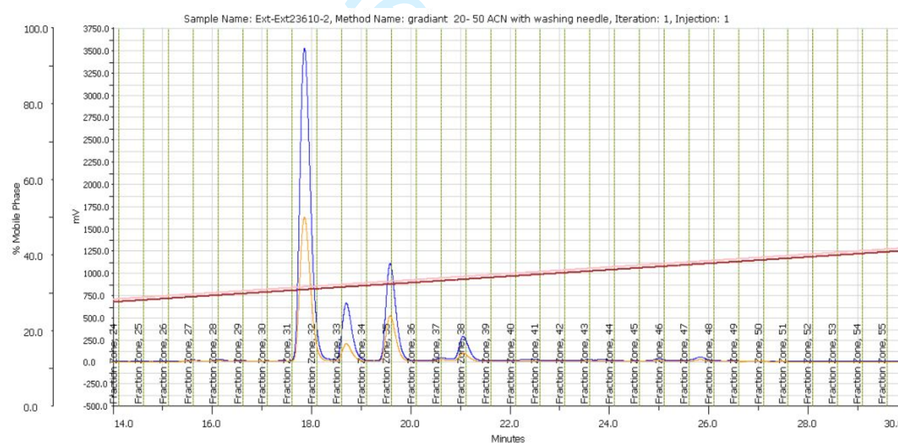

Figure 3. Chromatogram of compounds 4, 5, and 6

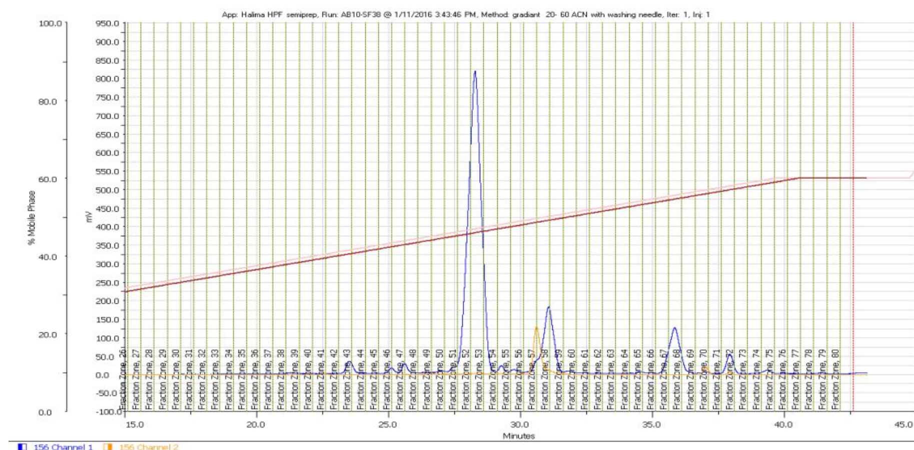

Figure 4. Chromatogram of compounds 7, 8, and 9

NMR data:

NMR spectra were recorded on a Bruker Advance III spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) and on a Bruker Fourier 300 spectrometer (Bruker Billerica, Massachusetts, MA, USA).

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(Z, Z, Z)- 9, 12, 15-Octadecatrienoic acid, methyl ester (1):HR-ESI-MS: *m/z* = 292.2553; molecule formula $C_{19}H_{32}O_2$, ^{1}HNMR ($CDCl_3$, 500MHz) δ: 5.34 (6H, m); 3.66 (3H, s); 2.79 (4H, t, *J*= 5.9 Hz); 2.30 (2H, t, *J*= 7.5 Hz); 2.06(4H, m); 1,61 (2H, m); 1,34 (8H, brl); 0.96 (3H, t, *J*= 7.5 Hz).(Chabert, 2006)

(Z, Z)-9,12-Octadecadienoic acid, methyl ester (2):HR-ESI-MS: m/z =294.2553; molecular formula $C_{19}H_{34}O_2$, ^{1}HNMR ($CD_3OD/CDCl_3$, 500MHz): δ : 5.29 (4H, m); 3.64 (3H, s), 2.71 (2H, t, *J*= 6.4 Hz); 2.24(2H, t, *J*= 7.57 Hz), 1,98 (4H, q, *J*= 6.8 Hz), 1.55 (2H, m), 1,24 (12H, br); 0.83 (3H, t, *J*=6.9 Hz). ^{13}CNMR (125 MHz): δ 130.0 (C-10, C-13); 127.8 (C-9, C-12); 51.4 (OCH_3); 34.01 (C-2); 31.5 (C-16); 28.8-29.8 (C4, C-5, C-6, C-7, C-15); 27.1 (C-8; C-14); 24.9 (C-11); 24.8 (C-3); 22.4 (C-17); 14.1 (C-18).(Huang, 2015; Díaz, 2007).

Phytol-1 (3) : HR-ESI-MS $[M-H]^+$ =295.2553, ^{1}HNMR ($CDCl_3$, 500MHz): δ 5.34 (1H, t, *J*= 6.3Hz); 4.14(2H, d, *J*= 6.8 Hz);1.97 (2H, t, *J*=9.5Hz); 1.64 (3H, s);1.51 (1H, m), 1,04-1.43 (16H, m); 0.85 (3H, d, *J*= 6.4 Hz); 0.84 (3H, d, *J*=6.4 Hz); δ 0.84 (3H, d, *J*= 6.0 Hz); 0.83 (3H, d, *J*=6.7 Hz).^{13}CNMR (125MHz) δ: 123.6 (C-2); 59.4 (C-1); 39.9 (C-4); 39.3 (C-5); 22.7 (C-19); 19.6 (C-16, C-18); 16.0 (C-20); 13.9 (C-17). (Alarif, 2010)

Martynoside (4): LC-UV-MS: *m/z* = 651.5 $[M-H]^+$, molecular formula $C_{31}H_{40}O_{15}$

^{1}H NMR (500 MHz, CH_3OH-*d4*) δ: 7.69 (1H, d, *J* =15.9, H-7'''); 7.22 (1H, d, *J*=1.6 Hz, H-2'''); 7.10 (1H, dd, *J* =1.6 Hz & 8.2 Hz, H-6'''); 6.86 (1H, d, *J* =8.2 Hz, H-5'''); 6.84 (1H, d, *J* = 8.0 Hz, H-5); 6.76 (1H, d, *J* =1.8 Hz, H-2); 6.70 (1H, dd, *J* =1.8 Hz & 8.0 Hz, H-6); 6.40 (1H, d, *J* =15.9, H-8'''); 5.22 (1H, d, *J*=1.35 ,H-1''); 4.94 (1H, t, *J* =9.3 Hz, H-4'); 4.40 (1H, d, *J* =7.9 Hz, H-1'); 3.99 (1H, m, H-5''); 3.92 (1H, m, H-2''); 3.89 (3H, s, OCH_3); 3.84 (3H, s, OCH_3); 3.83 (1H, m, H-3'); 3.75-4.07 (2H, m, H-8); 3.56(1H, m, H-2'); 3.54-3.63 (2H, m, H-6'); 3,54 (1H, m, H-3''); 3.42 (1H, t, *J*=7,41 Hz, H-5') ; 3.30 (1 H, m, H-4''); 2.84 (2H, 1H, dt, *J* =7.4 Hz & 2.4 Hz, H-7); 1.12 (1 H, d, *J*=6.2 H-6''); ^{13}CNMR (125 MHz, CH_3OH-*d4*) δ (ppm): 146.4 (C-7'''); 122.7 (C-6'''); 119.4 (C-6), 115.33 (C-2), 114.3 (C-5), 113.4 (C- 8'''), 111.3 (C-5'''), 109.4 (C-2'''), 102.7 (C-1'), 101.1 (C-1''), 70.2 (C-8), 54.8 (2 OCH_3), 35.2 (C-7), 16.5 (C-6'').(Khodaie, 2018)

Isomartynoside (5): HR-ESI-MS :*m/z* = 652.2366[M]$^{+}$, molecular formula $C_{31}H_{40}O_{15}$, ^{1}HNMR (500 MHz, CH_3OH-*d4*) δ:7.65 (1H, d, *J* =15.9, H-7''');7.18 (1H, d, *J*=1.6 Hz, H-2'''); 7.04 (1H, dd, *J* =1.6 Hz & 8.2 Hz, H-6'''); 6.81 (1H, d, *J* =8.2 Hz, H-5'''); 6.71 (1H, d, *J* =1.8 Hz, H-2); 6.69 (1H, d, *J* = 8.2 Hz,H-5); 6.64 (1H, dd, *J* =8.2 Hz & 1.8Hz, H-6); 6.40 (1H, d, *J* =15.9, H-8'''); 5.20 (1H, d, *J* =1.2 Hz, H-1''); 4.02(1H, dd, *J* =9.6 Hz & 6.2 Hz, H-4'); 4.36 (1H, d, *J* =7.9 Hz,H-1'); 3.96 (2H, m, H-5'', H-2''); 3.88 (3H, s, OCH_3); 3.77 (3H, s, OCH_3); 3.72 (2H,1H, dd, *J* =9.5 Hz & 3.4 Hz, H-8); 4.52-4.39 (2H, m, H-6'); 2.82 (2H, t, *J*=7.6, H-7); 1.28 (1H, d, *J*=6.2, H-6''); ^{13}CNMR (125MHz, CH_3OH-*d4*) δ: 146.1 (C-7'''); 122.9 (C-6'''); 119.8 (C-6); 115.6 (C-2); 115.0 (C-5); 113,9 (C-8'''); 111.5 (H- 5'''); 110.5 (C-2'''); 102.9 (C-1'); 101.2 (C-1''); 70.7 (C-8); 54.6 (2 OCH_3); 35.1 (C-7); 16.3 (C-6''). (Calis**,** 1984)

Cis-martynoside (6) : HR-ESI-MS :*m/z* = 652.2366[M]$^{+}$, molecular formula $C_{31}H_{40}O_{15}$, ^{1}HNMR (500 MHz, CH_3OH-*d4*) δ:7.89 (1H, d, *J*=1.8 Hz, H-2′′′); 7.17 (1H, dd, *J* =1.8 Hz & 8.0 Hz, H-6′′′); 6.96 (1H, d, *J* =13, H-7′′′); 6.84 (1H, d, *J* =8.0 Hz, H-5′′′); 6.79 (1H, d, *J* = 8.2 Hz, H-5); 6.75 (1H, d, *J* =2.0 Hz, H-2); 6.71 (1H, dd, *J* =2.0 Hz & 8.2 Hz, H-6); 5.82 (1H, d, *J* =13, H-8′′′); 5.18 (1H, d, *J* =1.4 Hz, H-1′′); 4.38 (1H, d, *J* =7.9 Hz, H-1′); 3.91 (3H, s,OCH_3); 3.83 (3H, s,OCH_3); 3.74,4.06 (2H, m, H-8);; 3.53-3.63 (2H, m, H-6′); 2.82 (2 H, m, H-7); 1.18 (1 H, d, *J*=6.2 H-6′′); ^{13}CNMR (125 MHz, CH3OH-*d4*) δ: 146.3 (C-7′′′); 126,1 (C-6′′′), 119.8 (C-6), 115.8 (C-2), 114.3 (C-5), 114.3 (C-8′′′), 113.8 (C-2′′′), 111.6 (C-5′′′), 102.7 (C-1′), 101.1 (C-1′′), 70.2 (C-8), 54.8 (2 OCH_3), 35.2 (C-7), 16.6 (C-6′′).(Skrzypek, 1999).

Ilwensisaponin C(7): LC-UV-MS : *m/z* = 1128 [M+Na]$^{+}$,^{1}HNMR (300 MHz, DMSO-*d6*) δ: 5.27 (1H, d, *J*= 3.5 Hz, H-12); 4.69 (1H, brs, H-1 *α*-L-rhamnose); 4.66(1H, d, *J*=7.8 Hz, H-1 *β*-D-glucose'); 4.52 (1H, d, *J*=7.8 Hz, H-1 *β*-D-glucose) 4.32 (1H, d, *J*=8.0 Hz, H-1 *β*-D-fucose); 3.76 (1H, dd, *J*= 8.6Hz & 3.5 Hz, H-11); 3.44 (1H, m, H-3); 1.64 (1H, d, *J*=8.6, H-9); 3.11 (3H, s, OCH_3); 1.17 (3H,s, H-27); 1.12 (3H, d, *J*=6.9Hz, H-6 rhamnose); 1.11 (3H, d, *J*=6.5 Hz, H-6 fucose); 1.00 (3H, s, H-25); 0.91 (3H, s, H-26); 0.88 (3H,s, H-29); 0.86 (3H, s, H-30); 0.63 (3H, s, H-24); ^{13}CNMR (75 MHz, DMSO-*d6*) δ: 148.9 (C-13); 122.1 (C-12); 103.9 (C-1 glucose); 103.5 (C-1 fucose); 102.3 (C-1 glucose'); 100.9 (C-1 rhamnose); 81.0 (C-3); 75.3 (C-11); 67.9 (C-28); 62.8 (C-23); 53.5 (OCH_3); 51.6 (C-9); 33.3 (C-29); 25.1 (C27); 23.9 (C-30); 18.2 (C-26); 18.2 (C-6 fucose); 17.3 (C-25), 17.0 (C-6 rhamnose); 12.8 (C-24). (Caliş 1993).

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Ilwensisaponon B(8): HR-ESI-MS *m/z* = 1072.5828 $[M]^+$, molecular formula $C_{54}H_{88}O_{21}$, ^{1}HNMR (300 MHz, CH_3OH–*d4*) δ: 6.41 (1H, dd, *J*= 2.3 Hz, 10.5 Hz, H-11); 5.59 (1H, d, *J*= 10.5 Hz, H-12); 4.85(1H, d, *J*=7.6 Hz, H-1 *α*-L-rhamnose); 4.85 (1H, d, *J*=7.6 Hz, H-1 *β*-D-glucose'); 4.64 (2H, d, *J*=7.7 Hz, H-1, H-1 *β*-D-glucose) 4.47 (1H, d, *J*=7.8 Hz, H-1 *β*-D-fucose); 0.98 (3H, s, H-27); 0.95(3H, s, H-24); 0.94 (3H, s, H-30); 0.78 (3H, s, H-25); 0.72 (3H, s, H-29); 0.71 (3H, s, H-26); ^{13}CNMR (75 MHz, CH_3OH –*d4*) δ (ppm): 136.2 (C-13), 134.5 (C-18), 125.7 (C-11), 125.0 (C-12), 103.7 (C-1 glucose), 103.3 (C-1 fucose), 102.1 (C-1 glucose′), 101.5 (C-1 rhamnose); 83.0 (C-3), 62.3 (C-23); 63.2 (C-28); 54.3 (C-9); 31.3 (C-30), 23.4 (C-29); 19.5 (C-27); 16.7 (C-25); 16.4 (C-26); 15.4 (C-24). (Mortada 2008).

Ilwensisaponon A(9): HR-ESI-MS *m/z*= 1072.5823$[M]^+$, molecular formula $C_{54}H_{88}O_{21}$, ^{1}HNMR (300 MHz, CH_3OH–*d4*) δ: 5.96 (1H, d, *J*= 10.4 Hz, H-12); 5.35 (1H, dd, *J*= 10.4 Hz, 2.7 Hz, H-11); 4.64 (2H, d, *J*=7.8 Hz, H-1 *α*-L-rhamnose & H-1 *β*-D-glucose'); 4.49 (2H, d, *J*=7.8 Hz, H-1 *β*-D-glucose, H-1 *β*-D-fucose) 4.49 (1H, d, *J*=7.8 Hz, H-1); 1.11(3H, s, H-26); 1.02 (3H, s, H-29); 0.99 (3H,s, H-27); 0.97 (3H,s, H-29); 0.91 (3H,s, H-30); 0.74 (3H, s, H-24); ^{13}CNMR (75 MHz, CH_3OH–*d4*) δ (ppm): 136.2 (C-12), 129.1 (C-11), 104.3 (C-1 fucose), 103.8 (C-1 glucose), 103.3 (C-1 glucose′) 103.2 (C-1 rhamnose), 87.2 (C-13), 80.3 (C-3), 78.4 (C-28), 60.7 (C-23), 53.4 (C-9); 51.1 (C-18), 31.4 (C-29), 29.3 (C-30 & C26), 22.3 (C-25), 15.5 (C-27), 13.0 (C-24). (Caliş 1993).

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Table 1. Identified compounds by HPLC/MS and HRESI-MS using standards

N°	Fraction	Formula	Retention time (min)	Molecular mass m/z	ESI MS fragments	Compound
1	VAA1-3	$C_{18}H_{32}O_3$	5.8	296.2344	No fragment appeared	12-Hydroxy-8. 10-octadecadienoic acid
2	VAA5	$C_{30}H_{48}O_3$	6.36	456.3589	456.3589,421.3229,432.3613,356.2906,434.3769	Betulinic acid
3		$C_{30}H_{50}O_2$	6.94	442.38	442.38,420.3981,460.3906	Erythrodiol
4	VAA10	$C_{30}H_{48}O_4$	5.54	489.3812	489.3812,472.3537,280.239,258.2572,262.2283	Maslinic acid
5	VAA15-3	$C_9H_{12}O_4$	0.51	184.0728	184.0728,162.0909,166.0625,206.0551,144.0805	4-Formyl-3-(formylmethyl)-4-hexenoic acid
6	VAA16	$C_8H_{10}N_4O_2$	0.5	216.0604	216.0604,194.078,276.081,180.0626,162.0524	Caffeine
7	VAB8	$C_{15}H_{10}O_5$	3.3	270.0518	270.0518,650.4012,752.3738,328.2237,537.3645	Genistein
8	VAB11	$C_{12}H_{22}O_{11}$	0.48	342.1154	342.1154	Trehalose

Table 2. Total phenolic and total flavonoid contents

Extract	Total phenolic content (µgGAE/ml)	Flavonoids content (µg QE/ml)
Ethyl acetate extract	390.44±1.6	96.54±3.64
Butanol extract	416.84±2.0	83.31±3.83

Table 3. In *vitro*IC_{50} and $A_{0.50}$values of antioxidant essays of *Verbascumatanticum*.

Extracts	DPPH assay	ABTS assay	CUPRAC assay	Reducing Power assay	Ferrous Chelating assay	*β*-carotene bleaching assay	Alkaline DMSO assay
	IC_{50} µg/mL	IC_{50} µg/mL	$A_{0.50}$ µg/mL	$A_{0.50}$ µg/mL	IC_{50} µg/mL	IC_{50} µg/mL	IC_{50} µg/mL
EtOAc	19,94±0,06	9,68±0,22	11.90±0.71	23.81±0.16	220.20+3.56	18.80±0.65	5,04±0,21
BuOH	96,20±0,83	13,37±0,11	24.68±0.93	82.57±1.33	NA	97.72±1.12	6,46±0,34
BHT	12.99±0.41	1.81±0.10	9.62±0.87	>200	NA	1.05±0.01	>200
BHA	6.14±0.41	1.29±0.30	3.64±0.19	7.99±0.87	NA	0.90±0.02	>200
α- tocopherol	13.02±5,17	7.59±0.53	19.92±1.46	34.93±2.38	NA	1.79±0.03	31.52±2.22
Ascorbic acid	13.94±2.81	1.74±0.10	12.43±0.09	6.77±1.15	NA	52.59±1.98	7.59±1.16
EDTA	NT	NT	NT	NT	8.80±0.47	NT	NT

NT: not tested, NA: not active, NT: not tested, NA: not active

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