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Computer-Assisted HPLC Method Development Using DryLab for Determination of Major Phenolic Components in *Corchorus olitorius* and *Vitis vinifera* by using HPLC-PDA-ESI-TOF- MS^n

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Research Article

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ABSTRACT

Computer–assisted HPLC method development is a tool that uses Design-of-Experiment techniques to optimize HPLC methods with minimal time and cost, especially for complex mixtures like natural extracts. *Corchorus olitorius* and *Vitis vinifera* are common edible plants in north Africa that contain a variety of active constituents that have not been completely determined. The aim of this work is to develop and optimize a chromatographic method for the separation and mass spectrometric determination of major components in both herbs. The approach did not use a laboratory prepared mixture of standards, rather it used the extract itself to build the response surface that relates resolution to gradient time and temperature. The predicted optimum conditions are column temperature = 30, 50˚C, gradient time = 100, 25 min from 5% to 100% acetonitrile for both *Corchorus olitorius* and *Vitis vinifera*, respectively at flow rate of 1 mL min^{-1}. These in-*silico* conditions showed capacity factors in range of (88.5-96%) within the fact of complexity of their matrices. Several metabolites were identified in both edible plants using HPLC-PDA-HRESI-MS^n including flavonoids, glycosides and phenolic acids.

INTRODUCTION

In recent years, the physiological functionality of foods has received much attention due to the increasing interest in human health and has been studied in *vitro* and in *vivo* by many researchers, Human consumes and utilizes a variety of vegetable materials in the form of leaves, roots, seeds and fruits. Moreover, there is growing evidence that vegetables and fruits are good sources of natural bioactive molecules, namely some vitamins, carotenoids and phenolic compounds [1]. *Corchorus olitorius Vitis vinifera* are among most popular edible plants in northern Africa and Mediterranean region. They are indigenous in Egypt [2,3].

Corchorus olitorius (mentioned as Molkhyia or Jew's Mallow) is an afro-arabian annual herb. It is rich in a flavonoid content of kaempferol glycosides, rutin and isoquercitrin , a phenolic content 5-caffeoylquinic acid, 3,5-dicaffeoylquinicacid, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-(6-malonylglucoside) and it is considered a vitamins' source of carotenoids, vitamin C, vitamin E, fatty acids and minerals [4-6] . It is reported to have demulcent, diuretic, lactagogue, purgative and tonic properties [6]. Molokhyia is a folk remedy for aches and pains, and swellings. In addition, the leaves are used for cystitis, dysuria,

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fever and gonorrhea. The cold infusion is said to restore the appetite and strength. Furthermore, it is an ingredient of facial creams, lotions, hair tonics and hand creams [7].

On the other hand, *Vitis vinifera* (grape) is a perennial woody vine native to Asia which was introduced in Europe and other continents [8]. *Vitis vinifera* (mentioned as grapevine, European grape) has been used as sort food and beverage, as well as remedy against various complaints in traditional medicine worldwide since ancient times [9]. Leaves of the plant have been used to stop bleeding and to treat inflammatory disorders and pain. Leaves are also recorded to reduce blood glucose levels in diabetics as a folk remedy [10]. The leaves of plant are rich in tannins, flavonoids, procyanidins and also contain organic acids, lipids, enzymes and vitamins [11]. Thus, the determination of the active components in these two extracts is required for the evaluation of its quality searching for further pharmacological applications in order to be a gate for drug discovery from natural source.

Determination of these active components using conventional trial-and-error methods is no longer an effective practice so the use of computer-assisted HPLC method development was adopted for the separation of the phenolics' content followed by their MS determination. DryLab has been in fact previously used for optimization of separation of laboratory prepared mixtures of natural herbal components and naturally obtained extracts [11]. In this study, the elaborated 2-dimensional computer-assisted HPLC method development was based on four basic HPLC runs **(Figures 1 and 2)** at 2 different gradients and 2 different column temperatures to achieve best resolution between the different phenolic components of *Corchorus olitorius and Vitis vinifera* followed by LC-MS identification of these components [12].

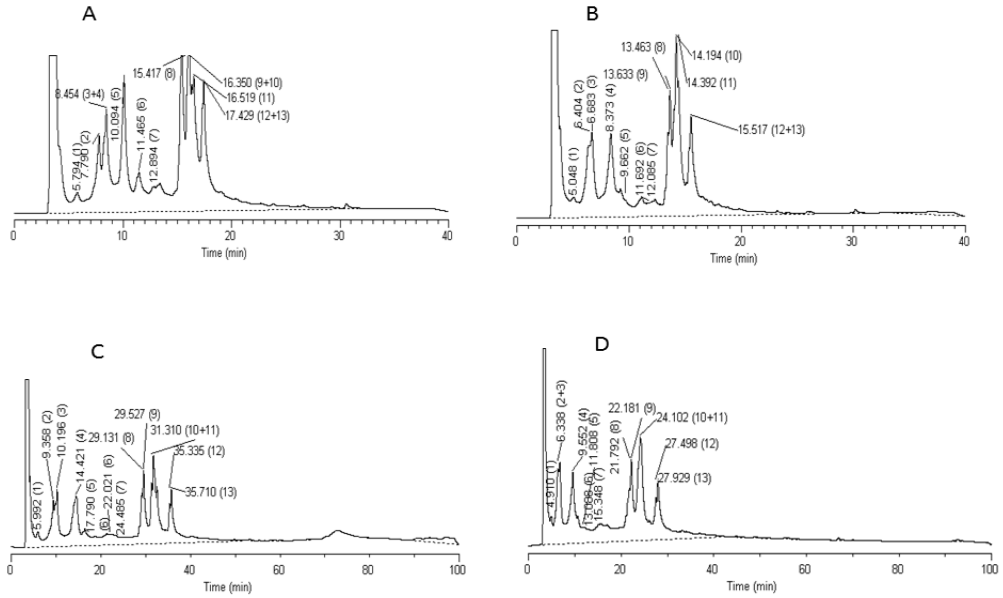

Figure 1. Four basic runs at different tG/T conditions for the aqueous alcohol extract of *Corchorus olitorius* using 25 mM phosphate buffer pH 2.6 as solvent A and acetonitrile as solvent B (A) 30 min/60°C; (B) 100 min/60°C; (C) 30 min/30°C; (D) 100 min/30°C.

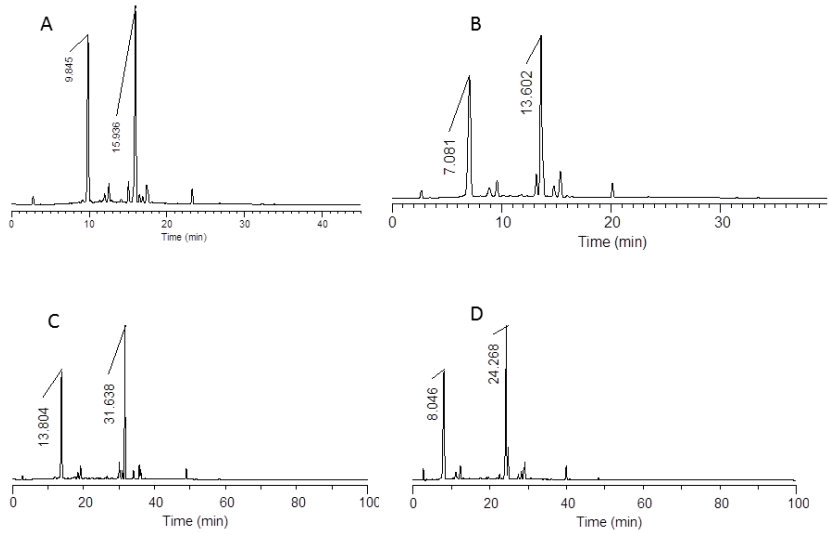

Figure 2. Four basic runs at different tG/T conditions for the aqueous alcohol extract of Vitis vinifera using 25 mM phosphate buffer pH 2.6 as solvent A and acetonitrile as solvent B (A) 30 min/60°C; (B) 100 min/60°C; (C) 30 min/30°C; (D) 100 min/30°C.

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MATERIAL AND METHODS

Sample Preparation

Plant material of aerial parts of *Corchorus olitorius* and leaves and their stalks of *Vitis vinifera* and were collected from the Zoological Garden, Giza, Egypt, in May 2015. The authenticity of species was confirmed by Prof. Dr. Abdel Salam Mohamed Al-Nowiahi, Professor of Taxonomy, Faculty of Science, Ain-Shams University, Egypt. The aerial parts (1Kg) were exhaustively extracted with distilled water (4L). The extract was evaporated in *vacuo* at low temperature till dryness followed by ethanol extraction. The ethanol soluble extract was evaporated in *vacuo* till dryness.

HPLC Equipment and Softwares

All HPLC experiments were performed using Thermo Finnigan Spectrasystem, UK (consisting of a P2000 at flow rate 1mL min^{-1}, UV detector at 220 nm and 330 nm, AS 3000, Rheodyne injection valve with 20µl loop autosampler and Chromquest acquisition software). Standards and extracts were applied on the same RP stationary phase: Hypersil Gold Octadecylsilane column (250 mm, 4.5 mm, 5 µm) for all determinations (method development with UV, detector as well as MS detector). The modeling software was DryLab2010 software package, (Molnár Institute for Applied Chromatography, Berlin, Germany). For Optimization of analytical conditions 4 basic experiments consisted of two linear gradient runs using a mobile phase gradient of 5-100 % acetonitrile (ACN) in 30 and 100 min, each at two different temperatures: 30˚C and 60˚C, to explore the effect of temperature on the elution behavior of the phenolics' components. The 2 components of the mobile phase were ACN and phosphate buffer (pH 2.6; 25 mM). The obtained chromatograms were imported to PeakMatch software (in AIA format) for peak tracking. The calculated dwell volume for the system was 2.3 mL. All standards used to confirm the chemical structure of compounds upon using UV detection in **Table 1** were purchased from Sigma– Aldrich (Germany).

Table 1. Peak assignments using HPLC/PDA/ESI-qTOF-MS^n of metabolites detected in hydroalcohol extract from aerial parts of *Corchorus olitorius*.

Peak #	Retention time(min)	Compound	Formula	$[M-H]^-$	Fragments	λ max (nm)	References
1	2.7	Quinic acid	$C_7H_{12}O_6$	191.17	191	310	[17]
2	8.24	Chlorogenic acid	$C_{16}H_{18}O_9$	353.33	191	326	Std.
3	17.22	Caffeoylquinic derv.	$C_{18}H_{18}O_9$	353.25	191,179	328	[18]
4	50.8	1,5 dicaffeoyl quinic acid	$C_{25}H_{24}O_{12}$	515.33	353,191,179,161	330	[19]
5	51.3	3,5dicaffeoyl quinic acid	$C_{25}H_{24}O_{12}$	515.33	353,191,179,173	330	[18]
6	53.3	Quercetin3-*O*- galactoside (hyperoside).	$C_{21}H_{20}O_{12}$	463.25	301	256/358	Std.
7	53.3	Quercetin3-β-glucoside (Isoquercitrin)	$C_{21}H_{20}O_{12}$	463.25	301	256/358	[19]
8	58.01	Quercetin3-(6 (malonylglucoside)	$C_{24}H_{22}O_{15}$	549.08	505,301	265/357	[5]
9	63.01	Dicaffeoyl quinic acid derv.	$C_{20}H_{18}O_9$	515.25	353,179	328	[20]

HPLC/MS Conditions

Analyses were performed using HP 1100 liquid chromatography equipped with Photodiodoarray detector and 1100 Mass spectrometer detectors; the interface was an HP 1100 MSD API-electro spray. All the instruments were from Agilent Technology (Palo Alto, CA, USA). The MS analyses were carried out in faculty of Pharmacy, Humboldt-University, Berlin, Germany, using negative high resolution electrospray ionization mode (HRESI), negative ion mode, 10L min^{-1} dry gas flow at temperature 350˚C with a fragmentation voltage range 80-150 V, capillary voltage of +4 kV , scans at *m/z* 100-1000 equipped with Varian Polaris 3 C18-Ether , 3 µm 150 x 1 mm with a guard column, the solvents were (A) 2 % Acetic acid (pH 2.6) and (B) 80% methanol, 2% acetic acid, pH 2.6. The gradient elution was from 5% to 50% B at 30˚C in *Corchorus olitorius* extract and 7-27%B at 40˚C in *Vitis vinifera* extracts at a flow rate of 100 µl min^{-1} and the sample injection volume was of 20 µl.

RESULTS AND DISCUSSION

Computer-Assisted HPLC Method Development Using Dry Lab

An attempt was made to create a complete separation of the phenolic content of hydro-alcohol extract of *Corchorus olitorius* and *Vitis vinifera.* The main problem in separation of complex natural mixtures (e.g. plant extracts) is finding systems which have different selectivity to allow effective separation especially when investigated compounds have closely related structures. Computer-assisted HPLC method development on reversed stationary phase can be performed in one, two or three dimensions of space, where each dimension represents one chromatographic factor. Separation of complex plants extract usually fails with the one dimensional approach where only %B is varied isocratically with monitoring of resolution, coelution of closely related compounds is inevitable [13]. Separation of the compounds of interest in the present study, phenolics in *Corchorus olitorius* and *Vitis vinifera* represents a challenging scenario due to this similarity in structures. Hence a two dimensional response surface (resolution map) was attempted by varying gradient time (t_G) with temperature (T) Since peaks necessarily vary in retention factors between the four runs, peak matching among different chromatograms needed to be performed using peak areas as attributors for the identity.

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Aided with the obtained resolution map **(Figures 3 and 4)**, the predicted chosen optimum conditions were gradient elution 5-50% buffer-acetonitrile in 100 min at T=30˚C at a flow rate of 1 mL min^{-1} and detection at 330 nm and gradient elution 7-27%, 25 mM phosphate buffer pH 2.6 -acetonitrile, t_G 30 min, T=50˚C, at 1 mL min^{-1}, detected at 220nm for *Corchorus olitorius* and *Vitis vinifera* respectively.

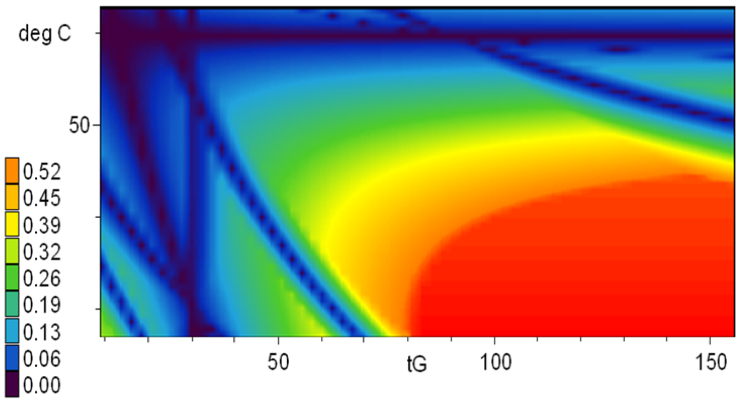

Figure 3. Two dimensional resolution map for separation of 13 phenolic components of the aqueous alcohol extract of *Corchorus olitorius,* tG versus T to change in resolution R_s showing different resolution values for the critical peak pair. Blue areas show the least resolution which increases to the maximum at the red areas.

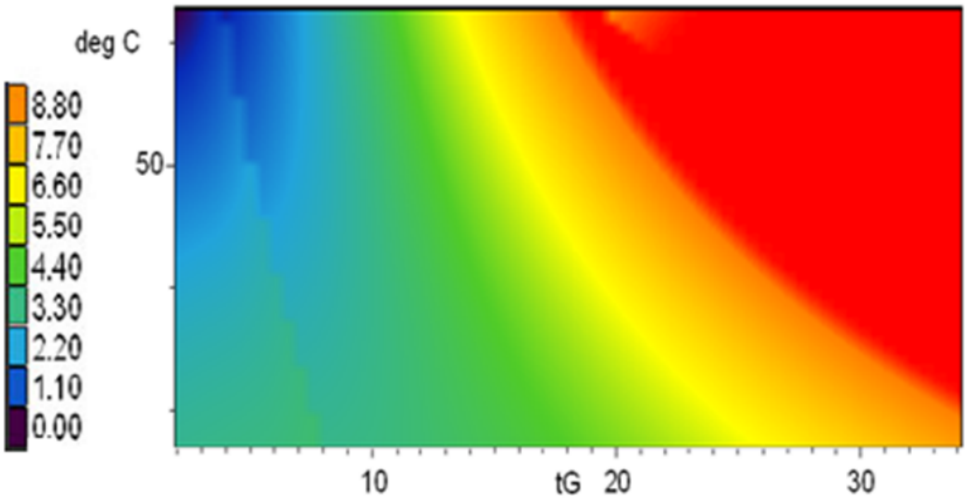

Figure 4. Two dimensional resolution map for separation of phenolic components of the aqueous alcohol extract of Vitis vinifera, tG versus T to change in resolution Rs showing different resolution values for the critical peak pair. Blue areas show the least resolution which increases to the maximum at the red areas.

The complex nature of *Corchorus olitorius* extract is due to the presence of a variety of minor components (more than 10 compounds) together with the numerous major compounds of interest, a large number of runs had to be performed before finally obtaining the satisfactory reproducible runs with regard to all 13 peaks. The choice of runs for introduction in the peak match process was a challenging decision: there was always a slight retention time deviation between some pairs of peaks in different runs even between the most reproduced ones. We believe that this factor was a decisive factor that might have led to some inconsistency between the predicted-experimental chromatograms **(Figures 5 and 6)**. Nevertheless, error in prediction was in the narrow range of ± 3 min quite similar values to other studies in the field [14]. In addition, it should be noted that the selectivity (peak order) did not change significantly in the experimental versus predicted and this provides valuable information about the specificity of the method. In these previous published studies a synthetic mixture of authentic reference compounds was used for peak matching to generate the resolution map - rather than the natural extract itself as performed in our study - which offered better reproducibility and eased the matching process [13,14]. Nevertheless, in these 2 studies, the accuracy of prediction was still unsatisfactory because of –*in our opinion*- the ignorance of the inevitable difference between the prepared synthetic mixture and the mystery of the natural extract composition. In that later case, unexpected peaks (that are not part of the synthetic mixture) appear only in the experimental chromatogram and one is fortunate their retention factor did not coincide with the targeted peaks [14].

To study the effect of changing the gradient time (t_G) and the column temperature (T) on the resolution of the critical peak pair (i.e. peak pair with the least resolution), the 4 basic runs were exported to DryLab software in AIA format, after peak tracking was accomplished for subsequent modeling. The “critical resolution map” was automatically computed by the software. Virtually, the best resolution could be obtained at the working conditions expressed in the lower right side of the resolution map for

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Corchorus olitorius, **(Figure 3)**. These conditions can be summarized as follows: a linear gradient (5–50% B) in 100 min at 30˚C and a flow rate of 1 mL min^{-1}, while for *vitis vinifera* the best resolution can be obtained at the upper right side of the resolution map, **(Figure 4)**. These conditions can be summarized as follows: a linear gradient (7-27% B) in 30 min at 50˚C and a flow rate of 1 mL min^{-1}. Although conditions vary slightly between UV and MS detection regarding temperature and B composition, yet results still lie in the robust area with optimum resolution which is proven by similarity of chromatograms obtained from both detectors.

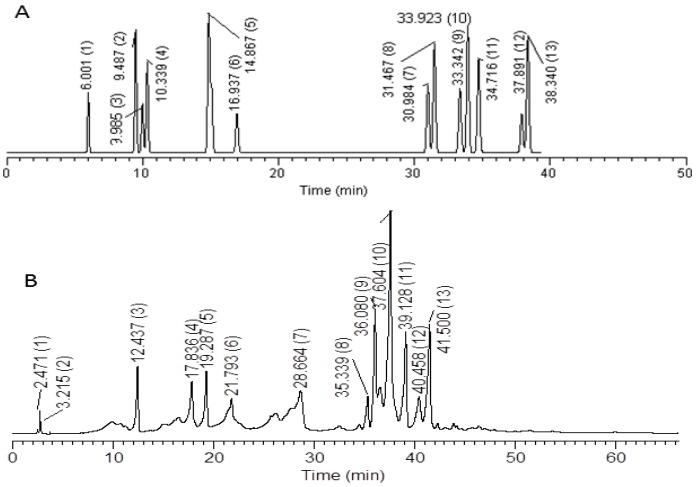

Figure 5. Predicted chromatogram using Dry Lab for an optimum separation conditions for components of the aqueous alcohol extract of *Corchorus olitorius* containing phenolics. The predicted conditions were found to be gradient elution 5-50%B. Mobile phase A: 25 mM phosphate buffer pH 2.6, mobile phase B: acetonitrile, tG 100 min, T=30˚C at 1 ml min^{-1}, detected at 330 nm. (B) Experimental chromatogram for the same extract at the *predicted* optimum conditions.

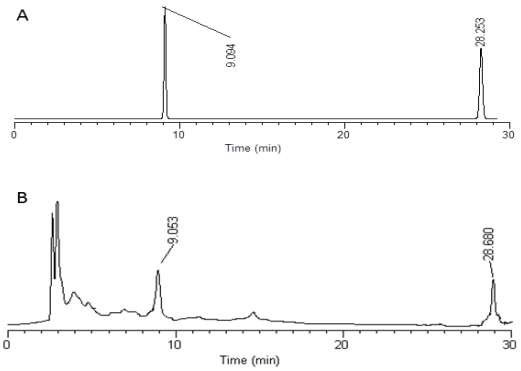

Figure 6. Predicted chromatogram using DryLab® for a optimum separation conditions for components of the aqueous alcohol extract of *Vitis vinifera* containing phenolics. The predicted conditions were found to be gradient elution 7-27%B. Mobile phase A: 25 mM phosphate buffer pH 2.6, mobile phase B: acetonitrile, tG 30 min, T=50˚C, at 1 ml min^{-1}, detected at 330 nm. (B) Experimental chromatogram for the same extract at the predicted optimum conditions.

However, in *Vitis vinifera*, prediction was significantly more accurate (± 0.6 min) because of the limitation of the targeted compounds to 2 in this extract instead of 13 in the *Corchorus olitorius* extract **(Figure 6)**.

Our method used a linear gradient to separate the phenolic content of *Corchorus olitorius* in contrast to previously reported stepwise gradient; linear gradient is easier to control and hence offers a more robust method. Furthermore, our method was able to separate and identify 13 compounds which represent a larger number of phenolic compounds compared to other methods. Excellent resolution was obtained between all peaks of interest, even the critical peak pair (pair of peaks with the least resolution) where Rs was more than 1. While other reported methods [13,14] were not able to exceed 0.5 for the critical peak pair. All these features in our developed method represent an improvement in the separation of the phenolics' content in *Corchorus olitorius* leaves.

The method reported in this work for separation of phenolics from *Vitis vinifera* leaves is the first of its kind a- interestingly- no other methods were reported about the same content of the herb [15,16] **(Figures 7 and 8)**.

Identification of Phenolics' Content by HPLC/PDA/HRESI/MS^n

The method development strategy was employed to produce a MS compatible separation for this phenolics' mixture to make a less complicated mixture that would still give good peak shape and acceptable resolution of the compounds. The structural identification of each compound was carried out mainly on the basis of its UV-Vis spectrum, retention time on reverse phase and

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MS spectra obtained by applying different fragmentation energies with HRESI technique. Moreover, the use of standard reference compounds and / or laboratory extracts helped to complete the identification. All the identified compounds are listed in **Table 1** and the results are discussed for each nutraceutical.

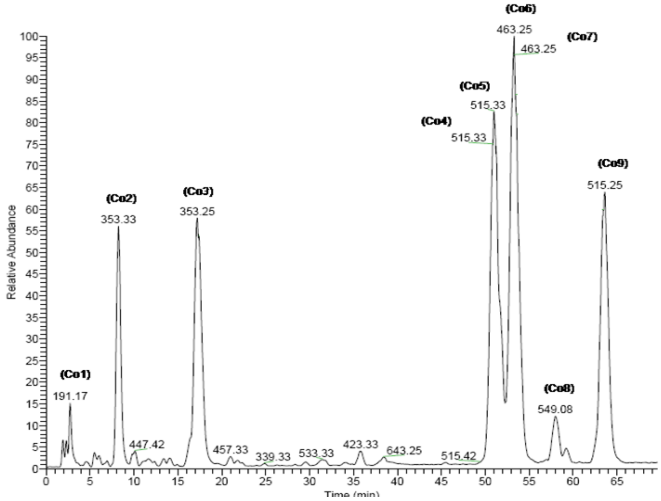

Figure 7. Negative LC/ESI/mass spectrum of phenolics from the aqueous alcohol extract of*Corchorus olitorius.*

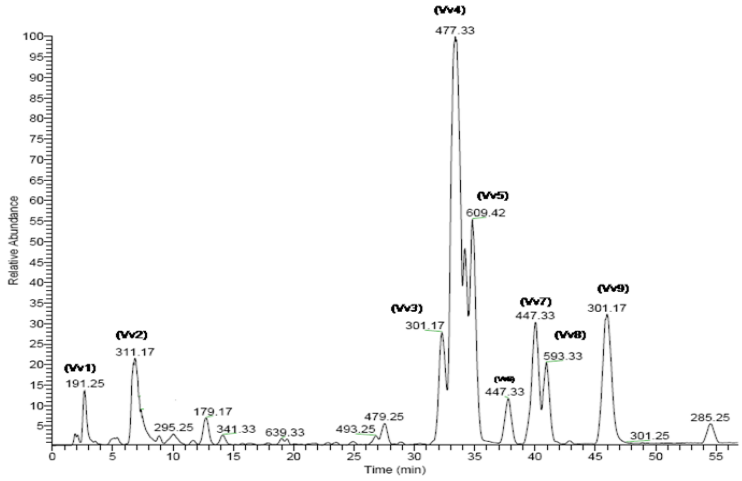

Figure 8. Negative LC/ESI/mass spectrum phenolics from the aqueous alcohol extract of *Vitis vinifera.*

Corchorus olitorius extract showed phenolic components as highlighted in **Table 1** that were confirmed by the presence of several caffeoyl derivatives (peaks 2 - 5 and 9) as major constituents of the aqueous alcohol extract [17]. Chlorogenic acid (peak 2) showed the deprotonated molecule $[M\text{-}H]^-$ (*m/z* 353) and the fragment peak ion corresponding to the deprotonated quinic acid (*m/z* 191), the chlorogenic acid was further confirmed when the authentic standard was subjected to HPLC under the same conditions, appeared on the same retention time and showed complete overlapped peak [18]. Peak 3 showed mass spectral data consistent with those reported to 4-caffeoyl quinic derivatives as the fragment peak of (*m/z* 173) is specific for 4 substituent caffeoyl quinic derivatives [19,20]. The difference between the two positional isomers namely 1,5 dicaffeoyl quinic acid (peak 4) and 3,4 dicaffeoyl quinic acid (peak 5) based on the difference in fragmentation pattern, although both of them gave the same expected molecular ion $[diCQ\text{-}H^+]^-$ at *m/z* 515 as in case of 1,5 dicaffeoylquinic acid (peak 4) MS base peak $[diCQ\text{-}cinnamate\text{-}H^+]^-$ at *m/z* 353 and absence of (*m/z* 335) which is indicative to 5 substituent caffeoyl quinic derivatives while 3,4 dicaffeoylquinic acid was distinguished by its unique pattern in which MS^3 got a strong ion (<50% of base peak) at *m/z* 179 and (*m/z* 173) peak 9 was proven to be of the dicaffeoyl derivative due to its fragmentation pattern [21]. The different sequence of elution was not as expected due to those caffeoylquinic acid derivative with a greater number of free equatorial hydroxyl groups in the quinic acid residue are more hydrophilic than those with a greater number of free axial hydroxyl groups [21]. Many flavonol *O*-glycosides were found in *Corchorus olitorius* such as quercetin 3-*O*-galactoside (hyperoside); peak 6, the spectra showed both the deprotonated molecule $[M\text{-}H]^-$ of the glycoside and the ion corresponding to the deprotonated aglycone $[A\text{-}H]^-$. The fragment ion at (*m/z* 301) is formed by loss of the glucose or galactose moiety from the glycosides. No ions characteristic of the sugar part were observed in the negative ion mode as the (*m/z* 179) appeared due to the loss of ring B of flavonol structure, in addition to comparative study

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was done to the authentic standard material and that was the only way to differentiate from quercetin 3-*O*-glucoside (isoquercitrin) which exhibited the same mass spectrum [22,23]. In addition to quercetin 3-*O*-(6-malonylglucoside) which gave two prominent fragment ions at *m/z* 505 and 301.

The major phenolic compound in *Vitis vinifera*; **Table 2** peak 4 was identified as quercetin-3-*O*-glucuronide having UV maximum absorption bands at 260 and 350 nm with depronated molecule (*m/z* 447) and MS^2 at *m/z* 301 besides the MS^3 at *m/z* 151 showed that a quercetin aglycone. The second major compound found as peak 2 presented the cinnamic-type UV spectrum, [*M*-H]- molecular ion at *m/z* 311 and fragment ions at *m/z* 179 which coincided with the mass of caffeic acid, and was thus identified as caftaric acid [24].

Table 2. Peak assignments using HPLC/PDA/ESI-qTOF-MSn of metabolites detected in hydroalcohol extract from leaves of *Vitis vinifera*.

Peak #	Retention time(min)	Compound	Formula	$[M-H]^-$	Fragments	λ max (nm)	References
1	2.69	Quinic acid	$C_7H_{12}O_6$	191.17	191	310	[17]
2	6.8	unidentified	$C_8H_{12}O_7$	311.17	179	320	
3	32.28	Hesperitin	$C_{16}H_{14}O_6$	301.17	157	265/354	[26]
4	33.3	Quercetin-glucuronide	$C_{21}H_{18}O_{13}$	477.33	301,179,151	260/350	Std.
5	34.8	Quercetin3-*O*-rutinoside (rutin).	$C_{27}H_{30}O_{16}$	609.42	300	265/354	[21], std.
6	37.8	Luteolin7-*O*-hexoside.	$C_{21}H_{20}O_{11}$	447.33	285	260/348	[19]
7	40	Cyanidin3–*O*- glucoside.	$C_{21}H_{21}O_{11}$	447.33	287	220/230	[22]
8	40.9	kaempferol coumaroyl hexoside.	$C_{27}H_{30}O_{16}$	593.33	284	265/356	[23]
9	45.9	Quercetin	$C_{15}H_{10}O_7$	301.17	179	255/320	[24]

Quercetin -3-*O*- rutinoside (rutin) (peak 5) was one of the main flavonol glycosides identified in *vitis vinifera*, UV absorption bands at 260 and 345 nm, it exhibited the fragment ion peaks typical to those of glycosyl derivatives (losses of terminal sugars), with ions at *m/z* 463 $[M-H-Rha]^-$ and *m/z* 301 $[M-H-Rha-Glu]^-$; this latter ion corresponds to the $[M-H]^-$ ion of the aglycone quercetin. This information concludes that, compound 5 is quercetin -3-*O*- rutinoside, and this conclusion was supported by comprising its ESI-MS^n spectra with those of an authentic sample. Two glycosides were also detected and identified according to their UV spectral data and ESI fragmentation pattern as luteolin 7-*O*-glucoside (peak 6) and the anthocyanin monoglucoside (cyanidin 3–*O*-glucoside); peak7 [22,23]. Compound 8 was identified as kaempferol coumaroyl glucoside with UV maximum absorption at 250/356 nm, (MS *m/z* 594, MS/MS *m/z* = 284) [25,26]. Compound 3 was identified as hesperitin which exhibits parent peak at *m/z* 301(M-1) which is formed by cleavage of glycoside link in hesperidine and supported by ion peak at *m/z* 157, however, according to some authors hesperitin should be eluted after quercetin (peak 9) in a reversed phase column [27]. In this study, results showed the opposite trend where quercetin (peak 9) eluted after hesperitin (peak 3), furthermore, these results similar to the reported one [28].

The identification of such compounds seems to be limited within the complexity of both extracts, but this study is directing towards the major compounds in each extract and to be correlated with the biological activity as antinflammatory and anticancer which was discussed in another publication [29].

CONCLUSION

The present study represents the development of a real robust method to establish a chromatographic profile of the aqueous alcohol extract for *Corchous olitorouis* and the first established chromatographic profiles of the aqueous alcohol extract for *Vitis vinifera*, with the aid of DryLab software which allows the chromatographer to develop a robust HPLC method for more efficiently and faster separation of complicated extracts than the traditional trial and error approach. Based on the results obtained from DryLab, the identification of the major phenolic compounds of both entitled plants were performed be adopting LC-PDA-HRESI-MS^n.

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