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Optimization of polyphenol extraction from *Vitis vinifera* L. leaves, antioxidant activity and its correlation with amelioration effect on AlCl₃-induced Alzheimer's disease

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ABSTRACT

This research focused on optimization of polyphenol extraction from *Vitis vinifera* L. (cv. Flame Seedless) leave based on 2,2-diphenyl-1-picrylhydrazyl (DPPH•) scavenging capability in additional discussion of structure-activity relationship according to its amelioration effect on AlCl₃-induced Alzheimer's disease. The highest extraction yield was found for acidified aqueous solvents. While the highest total phenolic and flavonoid contents were found for aqueous solvents. The hydroacetic extract showed significantly superior content in total phenolic and flavonoid accompanied by highest DPPH scavenging capability than other tested extracts. High-Performance Liquid Chromatography with Diode-Array Detection (HPLC/DAD) analysis of extracts led to identifying catechin, rutin, isoquercetin, quercitrin, apigenin-7-*O*-glucoside, quercetin, kaempferol and myricetin for the first time in *V. vinifera* L. (cv. Flame Seedless) leaves. In addition, quercetin-3-*O*-β-D-arabinopyranoside was isolated for the first time from *V. vinifera* L., detected as a major flavonoid in all extracts. This study emphasized the role of solvents played in polyphenol extraction and antioxidant activity from *V. vinifera* L. (cv. Flame Seedless) leaves. The hydroacetic extract mainly composed of flavonoidal compounds which could ameliorate AlCl₃-induced cerebral damages and neurocognitive dysfunction. This may lead to the development of new nutraceutical and pharmaceutical agents used as anti-oxidative stress and neuroprotective agent on Alzheimer and aging diseases.

Keywords: *Vitis vinifera*, hydroacetic extract, quercetin derivatives, antioxidant, structure-activity relationship.

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1. INTRODUCTION

Polyphenols were the most important bioactive phytochemicals because they exerted a multiplicity of biological activities and beneficial health effects including inhibition of some degenerative diseases for a human such as cardiovascular diseases, cancers, reducing plasma oxidation stress and slowing aging [1]. Several

studies recorded that the polyphenol could improve memory decline, motor, and cognitive performance as well as oxidative status in the cerebellum [2].

Vitis (grapevines) is a genus of 60 species, among of them *Vitis vinifera*, belonging to family Vitaceae [3]. *Vitis vinifera* leaves are used in traditional food (dolmathes) in some

Mediterranean countries. They are composed of a wide range of polyphenols compounds including anthocyanins, hydroxycinnamoyl tartaric esters, flavonol, flavan-3-ols, and organic acids [4]. Due to their astringent and hemostatic properties, they are used in traditional medicine for treatment of diarrhea, hemorrhage, varicose veins, hemorrhoids, an inflammatory disorder, pain, hepatitis, and free radical related diseases [5]. Also, they showed antioxidant, anti-inflammatory, analgesic, antipyretic, hepatoprotective, spasmolytic, hypoglycemic, vasorelaxant and antimicrobial activities [4, 6]. Moreover, they have ameliorated disturbances in cholinergic and dopaminergic neurotransmissions as well as oxidative stress and dyslipidemia in Al-induced Alzheimer rats [7, 8].

Here in, the study deal with the optimization of the polyphenolic extraction method from *V. vinifera* L. (cv. Flame Seedless) leaves including their antioxidant activity and chemical composition to clarify the principle for selection of hydroacetic extract for treatment $AlCl_3$ neurotoxicity in our previous *vivo* studies. As well as its chemical composition versus our previous neuroprotective on Alzheimer and aging diseases studies, the structure-activity relationship will be discussed.

2. MATERIAL AND METHODS

2.1. Plant Material

Leaves of red grape (*Vitis vinifera* L.) cv. Flame Seedless, belonging to the family *Vitaceae*, were collected during lush vegetation period in May 2015 from Elzomor Farm, Alexandria road. A voucher specimen is deposited under the number A 09 at Pharmacognosy Department, National Research Centre. The leaf petioles were carefully manually separated and leaves were dried at room temperature under the shaded condition, and then coarsely powdered and stored in polyethylene plastic bags in a dry place.

2.2. Chemicals

Phenolics and flavanoids were purchased from Sigma-Aldrich Co. USA such as pyrogallol, gallic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, caffeic acid, vanillic acid, gantisic acid, cinnamic acid, syringic acid, and sinapinic acids. Flavanol: Catachin; flavonol: quercetin, kaempferol, myricetin; flavonol glycoside: rutin. Flavanone glycoside: Hesperidin. Flavone: Apigenin, apigenin-7-glucoside. While hyperoside (quercetin-3-*O*-galactoside), isoquercetin (quercetin-3-*O*-glucoside), quercetin-3-*O*-rhamnoside, kaempferol-3-*O*-rhamnoside, and *trans*-resveratrol were obtained from the Department of Phytochemistry and Plant Systematics, National Research Center, Dokki, Giza, Egypt.

2.3. General procedure

Nuclear magnetic resonance (NMR) experiments were recorded on a Jeol EX-500 spectroscopy (JOEL Inc., Tokyo, Japan): 500 MHz (1H NMR) and 125 MHz (^{13}C NMR). UV spectra were obtained using Shimadzu model-2401 CP spectrophotometer (Shimadzu Inc., Tokyo, Japan). Silica gel 60 F₂₅₄ was performed using developing system ethyl acetate: methanol: formic acid: acetic acid: water (80:10:1:1:8 v/v/v/v/v). Paper chromatography (PC, descending) Whatman No. 1 mm and 3 mm papers, was performed using solvent systems; 15% acetic acid (acetic acid: water, 15:85 v/v), BAW (*n*-butanol: acetic acid: water, 4: 1: 5 v/v/v, upper layer). Complete acid hydrolysis (2N HCl, 3 hours, 100 °C) was carried out and followed by paper co-chromatography with authentic samples to identify the aglycone and sugar moieties.

HPLC analysis was carried out using Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector (DAD). The analytical column was an Eclipse XDB-C18 (150 x 4.6 μ m; 5 μ m) with a C18 guard column (Phenomenex, Torrance, CA).

The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The gradient was programmed as follows: 0-5 min, 100% B (isocratic step); 30 min, 100- 85% B (linear gradient); 20 min, 85-50% B (linear gradient); 5 min, 50- 0% B (linear gradient); 5 min, 0 - 100% B (linear gradient) at a flow rate of 0.8 ml min⁻¹. The injection volume was 20 µL and peaks were monitored simultaneously at 280, 320 and 360 nm for the benzoic acid, cinnamic acid derivatives, and flavonoid, respectively. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

2.4. Preparation of phenolic extracts

Initially, the *Vitis vinifera* L. (cv. Flame Seedless) leaves were defatted using petroleum ether (40-60 °C). The defatted powder were soaked in dark flasks separately for 30 min using eight different solvent mixtures containing acetone/ water (80: 20, v/v) (Extract 1), acetone/ water/ HCl (80: 19: 1, v/v/v) (Extract 2); ethanol/ water (80: 20, v/v) (Extract 3), ethanol/ water/ HCl (80: 19: 1 v/v/v) (Extract 4); methanol/ water (80: 20, v/v) (Extract 5), methanol/ water/ HCl (80: 19:1 v/v/v) (Extract 6); water (80: 20, v/v) (Extract 7), water/ HCl (99: 1 v/v) (Extract 8) at a solid to solvent ratio of 1:10 (w/v). The extraction process was repeated three times and the filtrate was combined and then evaporated under vacuum in rotary-evaporator at 37 °C; the remaining water solution was lyophilized [9].

2.5. Estimation of total phenol and flavonoid contents

The determination of total phenol and flavonoid contents were described in Borai et al., 2017 [7].

2.6. Determination of DPPH[•] radical scavenging activity

Free radical scavenging capacity was determined spectrophotometrically using stable 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) as mentioned in Borai et al., 2017 [7].

2.7. Data of Compound 1

UV λ_{\max} (nm): MeOH (260, 302s, 360); NaOH (274, 328, 402); AlCl₃ (276, 305s, 430); AlCl₃/HCl (270, 363, 405); acetic acid (272, 322s, 381); acetic acid/Na borate (263, 377). ¹H-NMR (DMSO-d₆, 500 MHz) 6.19 (1H, d, *J* = 2.0 Hz, H-6), 6.37 (1H, d, *J* = 2.0 Hz, H-8), 6.96 (1H, d, *J* = 8.6 Hz, H-5'), 7.45 (1H, d, *J* = 2.1 Hz, H-2'), 7.66 (1H, d, *J* = 2.1 and 8.6 Hz, H-6') and 5.18 (1H, d, *J* = 5.75 Hz). ¹³C-NMR (DMSO-d₆, 125 MHz): δ 157.1 (C-2), 134.1 (C-3), 177.6 (C-4), 162.5 (C-5), 99.2(C-6), 167.4(C-7), 93.6 (C-8), 156.7 (C-9), 103.9 (C-10), 120.9 (C-1'), 115.9 (C-2'), 146.6 (C-3'), 148.7 (C-4'), 114.5 (C-5'), 121.7(C-6'), 104.9(C-1''), 73.9 (C-2''), 74.2 (C-3''), 69.3 (C-4''), 67.9 (C-5''). The ¹H-NMR and ¹³C-NMR data were in agreement with that of quercetin-3-*O*- β -D-arabinopyranoside [10].

2.8. Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) program, version 14 (IBM software, NY, USA). Values are expressed as mean \pm standard deviation of triplicate assays. Data were analyzed by one-way analysis of variance (ANOVA). The difference was considered significant where *P* < 0.05.

3. Results and Discussion

3.1. Optimization of polyphenol extraction

To optimize polyphenols extraction characterized by the high antioxidant potential in consideration of *Vitis vinifera* L. (cv. Flame seedless) leaves as a source of natural antioxidant

[11]. The extraction process occurred at room temperature in dark flask for 30 min extraction time to avoid the chance of oxidation and hydrolyzation of phenolics, using 80% aqueous solvents to enhance extraction yield as well as addition of hydrochloric acid (1%) to the extraction solvent which enhanced the degradation of plant material, increased the rate of extraction of polyphenols from the matrix and minimized degradation reactions of flavonoids [9].

The yield, total phenol, total flavonoid contents and DPPH[•] scavenging activity were summarized in (Table 1), the yield of extracts by various solvents decreased in the following order: Acidified 80% methanol > acidified 80% acetone > 80% methanol > acidified 80% ethanol > 80% ethanol > 80% acetone > acidified water > pure water. It was noticed that the highest yields were found for acidified aqueous solvents may be other compounds have been extracted and contributed to higher yield.

In contrast, the highest total phenolic content in *Vitis vinifera* leaves were obtained with 80% acetone > 80% methanol > 80% ethanol > acidified 80% ethanol > acidified 80% acetone > acidified 80% methanol > acidified water > water (Table 1). Katalinic *et al.* (2013) reported that the total phenol content in *V. vinifera* L. leaves ranged from 9.4 to 23.4 g GAE/kg of dry leaves which was less than that of our sample (12.51- 50.25g GAE/kg according to the extraction solvent) and also found that the leaf-picked in September contain higher total phenolic compound than that of May [6]. Our sample was picked in May, to avoid any chemical additive such as pesticides and fertilizer, which could cause stress, led to change in its phenolic composition.

Regards to flavonoid content expressed as catechin, the total flavonoid contents ranged from 19.43- 189.97 mg CE/g extract, were the predominant phenolic compounds in the tested

Vitis vinifera leaves (Table 1). The results of total phenol and flavonoid contents were different from other reported on phenolic content in *Vitis vinifera* which depended on extraction process, solvent, time of picking leaves and varieties [6, 11]. The DPPH[•] scavenging activity assay revealed that all extracts showed antioxidant activity (Table 1). The hydroacetic, hydromethanolic and hydroethanolic extracts gave significantly the highest antioxidant activity than the acidified of the same solvents except for water extract. 80% acetone extract exhibited the most antioxidant activity followed by 80% methanol extract, 80% ethanol extract compared to other extracts (Table 1). Generally, the total phenolic contents can be used as an indicator in assessing the antioxidant activity of *V. vinifera* L. leaves.

3.2. The chemical composition of polyphenol extracts

Phenol composition of all extracts was determined qualitatively and quantitatively by HPLC/DAD and the results were summarized in (Table 2). Eighteen phenolic compounds were identified in *Vitis vinifera* L. (cv. Flame Seedless) leaves, in addition, a major unidentified flavonoid (compound 1). HPLC analysis revealed the presence of hydroxybenzoic acid derivatives (gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, and vanillic acid), hydroxycinnamic acid derivatives (chlorogenic acid, ferulic acid, and sinapic acid). The majority of phenolic acids in *V. vinifera* L. leaves occurred as an esterified form [12], that explains the absence of free phenolic acids such as caffeic, *p*-coumaric and cinnamic acids or presence of phenolic acids in low concentrations such as gallic and vanillic acids.

On the other hand, rutin, isoquercetin, quercitrin, and apigenin-7-glucoside were found as dominant flavonoidal glycosides in all tested extracts. While, aglycone flavonoids; (+)

catechin, quercetin, myrectein, and keampferol were detected in the minor content. The stilbene; resveratrol was also detected in trace amount (3.08-48.25 $\mu\text{g/g}$ extract) in a more polar

acidified extract of *V. vinifera* L. leaves but less than that reported by Katalinic *et al.* 2013 depending on the variety and the phenophase [6].

Table 1. The yield, total phenol, total flavonoid contents and antioxidant activity of *Vitis vinifera* L. (cv. Flame Seedless) different extracts

Extract	Yield (W/W %)	T. phenols (mg GAE/g extract)	T. flavonoids (mg CE/g extract)	DPPH (mgTE/g extract)
1	19.25 \pm 1.23	217.53 \pm 0.08	189.97 \pm 0.07	6496.99 \pm 0.01
2	44.59 \pm 1.07	112.67 \pm 0.01	93.46 \pm 0.04	1898.98 \pm 0.03
3	19.84 \pm 1.34	116.97 \pm 0.0	102.56 \pm 0.03	4416.68 \pm 0.00
4	32.39 \pm 0.99	114.88 \pm 0.03	68.34 \pm 0.01	769.83 \pm 0.005
5	22.98 \pm 1.42	149.12 \pm 0.06	87.50 \pm 0.05	5467.52 \pm 0.01
6	44.99 \pm 1.19	93.82 \pm 0.09	52.94 \pm 0.07	1748.54 \pm 0.02
7	12.10 \pm 1.81	42.97 \pm 0.03	19.43 \pm 0.08	714.71 \pm 0.01
8	18.34 \pm 1.92	68.20 \pm 0.06	50.95 \pm 0.07	1810.61 \pm 0.005

Data are presented as mean \pm SD (n=3). All extracts are significant difference in each group at $p < 0.05$ except yields of extracts 2 and 6 are non-significant. Extract 1: acetone/ water (80: 20, v/v%), Extract 2: acetone/ water/ HCl (80: 19: 1, v/v/v%); Extract 3: ethanol/ water (80: 20, v/v%), Extract 4: ethanol/ water/ HCl (80: 19: 1 v/v/v %); Extract 5: methanol/ water (80: 20, v/v%), Extract 6: methanol/ water/ HCl (80: 19: 1 v/v/v%); Extract 7: water (80: 20, v/v%), Extract 8: water/ HCl (99: 1 v/v%). mg GAE: mg of gallic acid; mg CE: mg of catechin; mg TE: mg of trolox.

Furthermore, pyrogallol was found in both alcoholic extracts (1.753 and 2.466 mg/g extract) which was previously detected in red grape leaves (sultana cultivar) [13].

Vitis vinifera L. (cv. Flame Seedless) hydroacetic extract constituted mainly of flavonoid compounds (95.20% of total identified phenolic compounds), especially quercetin derivatives (88.99%) which had scavenging capacity more than other extracts (Table 1). So, the 80% acetone was the best solvent for the extraction of polyphenols with the highest antioxidant activity. This prompted us to follow up the fractionation process with chloroform, ethyl acetate, *n*-butanol and water to determine the antioxidant responsibility. The yield of fractions decreased in the following order: *n*-butanol (47.98 w/w % of acetone extract) > ethyl acetate (32.24 w/w %) > water (11.20 w/w %) > chloroform (8.58 w/w %). Whereas, the ethyl acetate fraction was significantly highest DPPH scavenging ability (3688.68 mg TE/g) than other tested fractions; water (1881.31 mg TE/g

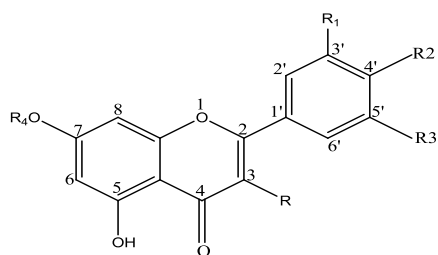
fraction), chloroform (1642.54mg TE/g fraction) and *n*-butanol (1152.13mg TE/g fraction) but less than its native extract (6496.99mgTE/g extract). Therefore, the antioxidant activity of 80% acetone extract was due to the synergistic property of the phenolic compounds. According to Pinelo *et al.* (2005); acetone is being the best solvent for flavonols and flavan-3-ols, while methanol is the best solvent for anthocyanins, hydroxycinnamic and ellagic acid derivatives [9]. Phenolic profile of extracts showed quercetin glycosides as the main compounds which were in concordant with Katalinic *et al.* 2013; Hmamouchi *et al.*, 1996 and Dresch *et al.*, 2014 [6, 14, 15]. The obtained HPLC data were mutually supportive of TLC. Extracts and fractions of 80% acetone extract, as well as available standard phenolics and flavonoids, were co-chromatographed on Silica gel 60 F₂₅₄ using developing system ethyl acetate: methanol: formic acid: acetic acid: water (80:10:1:1:8 v/v/v/v/v). As a result of the comparison of R_f values, and color changes with NH₄OH, FeCl₃ and AlCl₃ reagents.

Table 2. Quantification and qualification of polyphenol in different extracts of *Vitis vinifera* L. (cv. Flame Seedless) leaves

	Compound	λ_{\max} / nm	Rt	Extracts (mg/g extract)							
				1	2	3	4	5	6	7	8
1	Pyrogallol	280	4.32	nd	nd	1.753	nd	2.466	nd	nd	nd
2	Gallic acid	280	5.32	nd	nd	0.155	nd	0.091	0.071	nd	nd
3	Protocatechuic acid	280	10.20	0.063	nd	nd	nd	nd	nd	nd	nd
4	<i>p</i> -Hydroxybenzoic acid	280	15.34	0.350	0.149	0.134	nd	0.107	0.077	1.277	nd
5	Gentisic acid	280	17.40	0.128	nd	nd	nd	nd	nd	nd	nd
6	catechin	280	18.49	1.401	0.374	4.268	nd	6.577	0.284	nd	nd
7	Chlorogenic acid	320	20.90	0.031	1.276	nd	0.385	0.074	0.871	nd	nd
8	Vanillic acid	280	25.14	0.162	0.049	0.049	nd	0.110	1.387	nd	nd
9	Ferulic acid	320	33.08	0.083	nd	0.079	nd	0.075	0.027	nd	nd
10	Sinapic acid	320	34.21	0.043	nd	0.034	nd	0.027	nd	nd	nd
11	Quercetin-3- <i>O</i> -rutinoside	360	36.06	8.275	1.318	3.246	0.241	5.276	1.011	2.370	0.074
12	Quercetin-3- <i>O</i> -glucoside	360	36.56	3.691	0.397	2.660	0.106	2.401	0.309	1.400	1.680
13	Compound 1	360	37.50	12.123	2.897	7.188	0.694	7.045	1.622	4.22	1.670
14	Quercetin-3- <i>O</i> -rhamonoside	360	38.46	0.137	0.011	0.091	0.004	0.076	0.011	0.028	0.003
15	Apigenin-7- <i>O</i> -glucoside	360	38.80	0.075	0.281	0.050	0.741	0.039	0.533	0.072	0.142
16	Myricetin	360	39.71	0.519	0.155	0.366	nd	0.286	nd	nd	nd
17	Resveratrol	320	40.60	nd	nd	nd	0.048	nd	0.004	nd	0.003
18	Quercetin	360	43.36	0.470	1,766	0.365	0.659	0.342	1.684	nd	nd
19	Kaempferol	360	46.31	0.198	0.519	0.084	0.258	0.078	0.459	nd	nd

Extract 1: acetone/ water (80: 20, v/v %), Extract 2: acetone/ water/ HCl (80: 19: 1, v/v/v %); Extract 3: ethanol/ water (80: 20, v/v %), Extract 4: ethanol/ water/ HCl (80: 19:1 v/v/v %); Extract 5: methanol/ water (80: 20, v/v %), Extract 6: methanol/ water/ HCl (80: 19: 1 v/v/v %); Extract 7: water (80: 20, v/v %), Extract 8: water/ HCl (99: 1 v/v %).

The TLC profile showed the ethyl acetate fraction composed mainly of flavonoids nearly similar to its native extract. TLC profile showed rutin ($R_f = 0.25$), compound 1 ($R_f = 0.31$), isoquercetin ($R_f = 0.53$), quercitrin ($R_f = 0.63$), quercetin ($R_f = 0.92$) and kaempferol ($R_f = 0.98$), which were detected previously in various varieties of *Vitis species* [14, 15], but they were detected here for first time in *V. vinifera* L. (cv. Flame Seedless) leaves.



Compounds	R	R ₁	R ₂	R ₃	R ₄
Quercetin	OH	OH	OH	H	H
Quercetin-3-O-rhamnoside	O-rhamnoside	OH	OH	H	H
Quercetin-3-O-arabinoside	O-arabinoside	OH	OH	H	H
Quercetin-3-O-glucoside	O-glucoside	OH	OH	H	H
Rutin	O-rhamnoglucoside	OH	OH	H	H
Apiginin-7-O-glucoside	H	H	OH	H	glucose
Kampferol	OH	OH	OH	OH	H
Myricetin	OH	OH	OH	OH	H

Compound 1 was isolated from ethyl acetate fraction as a yellowish amorphous powder. The UV spectra of diagnostic shift reagents revealed the presence of free hydroxyl groups at C-5, C-7, C-3', and C-4' and substituted at position 3 [14]. The ¹H NMR (DMSO-d₆) spectrum showed a pair of a meta coupled doublets (δ 6.19 and 6.37, $J = 2.0$ Hz) which attributed to H-6 and H-8 on the phloroglucinol A-ring and an ABX system (δ 6.96, $J = 8.6$ Hz for H-5'; 7.45, $J = 2.1$ Hz for H-

2'; 7.66, $J = 8.6$ and 2.1 Hz for H-6') for the catechol B-ring protons. In addition, ¹³C NMR (DMSO-d₆) spectrum showed the signals at δ 157.1, 134.1, 177.6 and 103.9 were typical of C-2, C-3, C-4 and C-10 of a flavonol moiety. The anomeric proton was observed at δ 5.18 (1H, d, $J = 5.75$ Hz) and C-1'' and C-4'' appeared at δ 104.9 and 69.3, respectively, revealing the presence of β -arabinopyranoside moieties [10]. Acid hydrolysis of compound 1 afforded quercetin and arabinose moieties which were confirmed by TLC co-chromatography with authentic samples. So, compound 1 was recognized as quercetin-3-O- β -D-arabinopyranoside (guajaverin) according to its spectral and literature data [10]. It is worthy to be mention that it is the first time for isolation quercetin-3-O- β -D-arabinopyranoside from *Vitis species*.

DPPH free radical scavenging by flavonoids is highly dependent on the number and location of the hydroxyl moieties, the presence of an ortho-catechol (3',4'-OH), free 3-OH and 2,3-double bond in conjugation with a 4-oxo function in ring C. In addition to the glycosylation model (C-glycosides or O-glycosides) which were played critical role in antioxidant activity [11]. Flavonoids with both 3-OH and 3,4-catechol were reported to be 10-fold antioxidant activity more than the corresponding catechol and 3-OH free flavonoids [16]. Bernardi et al., (2007) reported that quercetin-3-O- β -D-arabinopyranoside, which detected in the current study as a major identified flavonoid, was able to scavenge DPPH radicals higher than hyperoside, isoquercitrin, and quercetin-3-methyl-ether that explained the antioxidant activity was depended on substitution on 3-OH [17]. Otherwise, the antioxidant activity of phenolic acids depended on the number and orientation of hydroxyl groups relative to the electron-withdrawing CO₂H, CH₂CO₂H, or (CH)₂CO₂CH functional group

[18]. This explained that all phenolic acids; hydroxyphenylacetates (metabolites of quercetin) and hydroxybenzoates demonstrated lower TEAC (Trolox Equivalent Activity Capacity) values than flavonoids and hydroxycinnamates [16].

That led us to select 80% acetone extract for evaluation of its neurotherapeutic effects on both healthy old aged rats and against aluminum chloride (AlCl_3) neurotoxicity rats and the results were reported in Borai et al., 2017 and Rizk et al., 2018 [7, 8]. Our previous studies observed that the administration of *Vitis vinifera* L. (cv. Flame Seedless) hydroacetic extract (100 mg/kg b.wt. daily for 21 days) to healthy old aged rats showed significant decrease in time to achieve the task as compared to the negative control group (T-maze behavior stress) [7, 8]. This result accompanied with a significant decrease in serum homocysteine (Hcys) level as well as significant decrease in tail length of comets, DNA intensity (tail intensity) and tail moment were detected as compared to the negative control group [7]. Meanwhile, hydroacetic extract showed significant reduction in amyloid β ($\text{A}\beta$), malondialdehyde (MDA), nitric oxide (NO) and protein carbonyls (PC) levels as well as significant reduction in total cholesterol (TC), triacylglycerols (TAG) and sphingomyelins (SM) levels as compared to the negative control group. On the other hand, the orally administration of hydroacetic extract to healthy old aged rats showed insignificant change in acetylcholine (ACh)/ acetylcholinesterase (AChE), dopamine levels (DA), serum Total Antioxidant Capacities (TAC), total lipid (TC), phospholipids level (PL), phosphatidylcholine (PCh) levels and brain total protein content (TP) in healthy negative control rats [8]. These results confirmed by histopathological examination of brain sections which showed well-preserved histoarchitecture without any histopathological findings in the cerebral cortex [7].

Preceding results demonstrated that the administration of AlCl_3 (17 mg/kg body weight daily for 4 successive weeks) which known as an environmental toxin, widely used for induction in Alzheimer disease (AD) rat model to explore the etiology and therapeutic strategies of AD [19]. It induced neurotoxicity in aged rats causing neuroinflammation, extracellular Ab generation, and aggregation, and neurofibrillary tangles in addition to oxidative stress through multiple mechanisms led to imbalance between oxidants and antioxidants with a significant decrease in brain/serum (ACh), (DA), (TAC) and brain-derived neurotrophic factor (BDNF) and (PL) serum levels [20]. Also, it exhibited a significant increase of brain/serum (AChE), (IL-6), (Hcys), (MDA), (NO), (PC), ($\text{A}\beta$), and lipid profile including (TC), (TL), (TAG) as well as (SM) and (PCh) as compared to control values. Interestingly, the treatment with hydroacetic extract (100 mg/kg b.wt. daily for 21 days) was ameliorated disturbances in cholinergic and dopaminergic neurotransmissions as well as oxidative stress and dyslipidemia in Al-induced Alzheimer rats [7, 8].

As results finding in our both previous study, the hydroacetic extract was ameliorated the biochemical parameters of oxidative stress, endothelial dysfunction, and inflammation not only in AD induced rats but also in aged normal rats by quenching free radicals. Post-administration of *V. vinifera* L. (cv. Flame Seedless) hydroacetic extract to Al exposed rats, the amelioration in both biochemical and neurobehavioral effects were interestingly observed by modulation of the biochemical markers, histopathological and T-maze studies. That study was compared with Rivastigmine (0.3 mg/kg b.wt. daily for 21 days) as reference drug which clinically used as acetylcholine inhibitor to treat mild and moderate Alzheimer disease.

These modulations proved that ability of polyphenol extract to reach the brain by crossing the gastrointestinal tract (GIS) and blood brain barrier (BBB) as well as exerting memory enhancing the effect. In addition, it could be combated the oxidative stress caused by AlCl_3 [7]. This finding result approved by various *vivo* studies supported that flavonoids, particularly quercetin glycosides as main of polyphenol in the hydroacetic extract, are able to be absorbed after oral administration. Generally, flavonols are found mostly in their glycoside forms and traces of aglycone. The bioavailability of flavonoid depended on type and position of sugar moieties that was influenced by the absorption of flavonoid glycosides [21]. The flavonoid monoglycosides absorbed faster from the small intestine by glucose transporter than flavonoids diglycosides such as rutin which was absorbed through colon after microbial degradation [16]. Subsequently, flavonoid glycosides underwent cleavage of glycoside bond in enterocytes followed by conjugating either with glucuronide, methyl or sulfate groups exerted flavonoid metabolites which were absorbed from enterocytes into the portal circulation. The accumulation of these flavonoid metabolites in the endothelial cells led to deconjugate glucuronide derivatives liberating aglycone forms which may be capable of entering glial cells and thus the brain. Moreover, flavonols and flavanols could cross the BBB with transcellular and paracellular diffusions which influenced by their lipophilicity [22]. Quercetin is also a substrate for permeability-glycoprotein efflux carriers from the brain interstitial fluid to endothelium [23]. Ishisaka et al (2011) demonstrated that orally administration of quercetin (50 mg/kg body wt) was accumulated in its metabolite form (methylquercetin) in the brain tissue of rats and also attenuated the increased oxidative stress in the hippocampus and striatum of rats exposed to chronic forced swimming [24].

3.3. *Vitis Vinifera* L. (cv. Flame Seedless) hydroacetic extract acts as anti-oxidative stress

Several mechanisms of antioxidant action of flavonoids were reported including suppression of intracellular reactive oxygen species (ROS) production either by inhibition of enzymes or by chelating trace elements, scavenging ROS, and regulation or protection of antioxidant defenses [25]. This activity depended on flavonoid configuration, substitution, and a total number of hydroxyl groups substantially which influenced radical scavenging and metal ion chelation abilities [25]. These activities of flavonoids were attributed to its electron-donating due to its high number of hydroxyl substitutions. The oxidation of 3', a 4'-catechol structure in the B ring of flavonoids formed a fairly stable orthosemiquinone radical which firmly enhanced inhibition of lipid peroxidation due to its act as strong scavengers for reactive oxygen and nitrogen species (ROS and RNS). Also, the capacity of flavonoids to chelate metal ions (iron, copper, etc.) was influenced by the presence of hydroxyl groups either at position 3 and oxygen on carbon 4 or at position 5 and oxygen on carbon 4 in pair for preventing oxidative stress which involved in neuronal plaque formation and lipid peroxidation [18].

Oxidative stress increased during the aging process, and antioxidant status may significantly influence the effects of oxidative damage associated with advancing age [26]. *V. vinifera* L. (cv. Flame Seedless) hydroacetic extract (100 mg/kg b.wt. daily for 21days) can modulated the oxidative biomarker of healthy aged animal which played a protective role by reducing brain oxidative enzymatic activity such as nitric oxidase (NO), protein oxidation (PC) and lipid peroxidation (MDA). This result was in accordance with previous reports that quercetin, myricetin, apigenin and kaempferol played an

important role in altering the progression of neurodegenerative diseases by protection against oxidative stress. In addition, quercetin significantly attenuated amyloid β -induced cytotoxicity, protein oxidation, lipid-peroxidation, and apoptosis as well as its potential benefit for conditions involving mitochondrial dysfunction [27]. In addition, quercetin can ameliorate Al neurotoxicity, which decreased ROS levels, mitochondrial DNA oxidation and citrate synthase activity in both hippocampus and corpus striatum regions [27]. Furthermore, it prevented Al-induced translocation of cytochrome c, up-regulated Bcl-2, and down-regulated Bax, p53 and caspase-3 activation. In addition, it increased MnSOD activity as well as reduced DNA fragmentation, increased the mitochondrial DNA copy number and mitochondrial content in the regions of rat brain.

3.4. *Vitis Vinifera* L. (cv. Flame Seedless) hydroacetic extract acts as acetylcholinesterase (AChE) inhibitor

The hydroacetic extract showed a significant decrease in brain/serum (AChE) and a significant increase in brain/serum (ACh), hence, it may act as an AChE inhibitor. The beneficial effect of AChE inhibitor is an enhancement of cholinergic transmission in the brain, reducing the aggregation of β -amyloid and the formation of the neurotoxic fibrils in AD which was achieved by *Vitis Vinifera* hydroacetic extract [28]. The inhibition activity of AChE was increased depending on the hydroxylation on both rings A and B, the dehydrogenation of the C2–C3 double bond which increased both the affinity for AChE and Aches inhibition. In addition, molecular property-affinity relationship revealed that the hydrogen bond force of flavonoid molecule played an important role in binding flavonoids to AChE [28]. However, the glycosylation decreased the AChE inhibitory

activities of flavonoids and lowered the affinities for AChE depending on the conjunction site and the type of sugar moiety. In this regard, it is important to take into consideration that glycosylated quercetin, kamferol, and myricetin derivatives can undergo hydrolysis in the gastrointestinal tract as aforementioned.

3.5. *Vitis Vinifera* L. (cv. Flame Seedless) hydroacetic extract acts as amyloid beta ($A\beta$) inhibitor

V. vinifera L. (cv. Flame Seedless) hydroacetic extract showed reduction of serum amyloid beta ($A\beta$) concord with results of Stefani and Rigacci, (2013) and Crimi et al.,(2016) [29, 27] which reported that quercetin, myricetin, kampferol, and apigenin could be counteracted the formation and aggregation of $A\beta$ fibrils and protected the neurons against $A\beta$ -induced toxicity. The inhibition of $A\beta$ fibril growth depended on ortho catechol and increasing the number of hydroxyl groups on the B and C ring, which improved the anti-aggregation effect of flavonoids [30]. On the other hand, the halt of the fibrillization process of $A\beta$ fibrils was inhibited by the interaction between flavonoid aromatic and aromatic residues of the $A\beta$ sequence which competed with polypeptide monomers for interaction with growing fibrils [31]. Moreover, myricetin reduced $A\beta$ 1–40 and $A\beta$ 1–42 productions and exhibited an anti-tau effect (a target protein which is another major plaque-forming factor for AD) and this activity showed previously by *V. vinifera* L. flavonoids [32].

3.6. *Vitis Vinifera* L. (cv. Flame Seedless) hydroacetic extract acts as monoamine oxidase MAO inhibitor

Toxicity of Al caused a decrease of dopamine level either by oxidation stress and promoted the aggregation of α -synuclein led to interfere with dopamine-binding receptors in the brain cortex and striatum. The decreased level of dopamine

and altered cholinergic function might be attributed to increasing MAO activity that led to increased degradation of dopamine [7]. Flavonoids; quercetin, kaempferol, myricetin, and rutin acted as MAO inhibitor [33]. Mercer et al., (2005) reported that polyphenols catechin, quercetin, and others were found to protect mesencephalic dopamine neurons from apoptosis due to oxidative stress [34].

Kaempferol has a neuroprotective effect which regulated the expression levels of proteins, such as Bcl-2, Bid, apoptosis-inducing factor, and mitogen-activated protein kinase. While apigenin-7-glucoside inhibited the overexpression of COX-2 and iNOS in age- and LPS-induced amnesia [27].

Finally, **Table 3** showed the relative therapeutic effect of *Vitis vinifera* L. leaves hydroacetic extract compared to Rivastigmine as acetylcholine inhibitor which ameliorated $AlCl_3$ neurotoxicity as mentioned in Bori et al., 2017 and Rizk et al., 2018 [7, 8]. The hydroacetic extract showed superior lipid peroxidation, oxidative/nitrosative stress, antioxidant, and hepatic protein synthesis activities on MDA, NO, TAC, and TP, respectively, over that of Rivastigmine. Furthermore, it exhibited higher cholinergic and dopaminergic activities when compared with Rivastigmine. In the other hand, it showed good amyloidogenic, oxidative stress and proinflammatory cytokine activities when compared with Rivastigmine.

4. CONCLUSION

Our study highlighted the effective solvents to extract the bioactive polyphenolic content from *Vitis vinifera* L. (cv. Flame Seedless) leaves by an easily available extraction method, low cost and simple technique in parallel with the antioxidant capacity to characterize the bioactive extracts aiming for selection of the promising

extract to pursue the evaluation on $AlCl_3$ -induced Alzheimer's disease in rat. To the best of our knowledge, no quantitative and qualification analysis of polyphenols especially flavonoids of *Vitis vinifera* L. (cv. Flame Seedless) leaves have been performed. Among vinification wastes, grapevine *Vitis vinifera* L. leaves which also have been consumed as food in Middle East nations. flavonoids; quercetin, kaempferol, myricetin and quercetin derivatives as well as antioxidant properties of a different variant of *Vitis* species were reported in several studies. The current study proved that hydroacetic extract of *Vitis vinifera* L. (cv. Flame Seedless) leaves was appropriate with a significant content of flavonoids known as Vitamin P exhibiting the highest antioxidant capacity than other tested extracts. It is worthy to be mention that quercetin-3-O- β -D-arabinopyranoside was isolated for the first time from *Vitis vinifera* L. species. While, catechin, rutin, isoquercetin, quercitrin, apigenin-7-O-glucoside, quercetin, kaempferol, and myricetin were identified for the first time in cultivar *V. vinifera* L. (cv. Flame Seedless) leaves. The hydroacetic extract of *Vitis vinifera* L. is considered as a diverse target to act as a multifunction drug for counteracting the multiple factors implicated in $AlCl_3$ -induced cerebral damages and neurocognitive dysfunction including the neuroinflammation, oxidative stress, amnesic, and hyperlipidemia. Also, it acts as AChE inhibitor, elevating ACh and suppression IL-6, and consequently increasing BDNF level.

From these interesting results, *Vitis vinefiera* L. (cv. Flame Seedless) hydroacetic extract has both protective and therapeutic impacts in neurodegenerative diseases and this may lead to new development of promising drugs for prevention and treatment of neurodegenerative diseases.

So, further preclinical and clinical studies may be conducted for preparing a new neuroactive

pharmaceutical formulation alone or in combination with other drugs such as rivastigmine, which is used in treating neurodegenerative diseases, in order to improve their therapeutic effect as well as overcome their side effects. Concerning its anti-oxidative stress,

hydroacetic extract could be applied as supplementary, nutraceutical and functional food preparations in mitigating other health problems such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, and cardiovascular diseases.

Table 3. Relative therapeutic effect of *Vitis vinifera* L. (cv. Flame Seedless) hydroacetic extract to the therapeutic effect of Rivastigmine as acetylcholine inhibitor drug

Functions	Biomarkers	Relative therapeutic effect
Cholinergic neurotransmission	Acetylcholine (ACh) (serum)	71.27
	Acetylcholine (ACh) (brain)	86.85
	Acetylcholine esterase (AChE) (serum)	79.58
	Acetylcholine esterase (AChE) (Brain)	136.01
Dopaminergic neurotransmission	Dopamine (DA)	78.47
Neurotrophic factor	Brain-derived neurotrophic factor (BDNF)	102.80
Amyloidogenic	Amyloid- β (A β)	69.31
Lipid peroxidation (oxidative stress)	Serum malondialdehyde (MDA)	125.09
Oxidative/nitrosative stress	Serum Nitric oxide (NO)	113.81
Oxidative stress	Protein carbonyl (PC) in brain	57.12
Antioxidant	Total antioxidant capacities (TAC)	136.62
Proinflammatory cytokine	Interleukin-6 (IL-6) increased	73.50
Endothelial dysfunction	Total Homocysteine (Hcys)	82.15
DNA damage	Tailed %	56.57
	Untailed%	40.48
	Tail DNA%	31.78
Lipoidal Profile	Serum total cholesterol (TC)	192.51
	Serum triacylglycerols (TAG)	108.54
	Total lipid (TL)	330.19
Phospholipid composition (Sphingolipid metabolism)	Phosphatidylcholine (Pch)	93.84
	Sphingomyelins (SM)	88.32
	Phospholipids (PL)	102.45
Hepatic protein synthesis	Brain total protein (TP)	150.45

*The activity was evaluated according to the therapeutic activity related to Rivastigmine activity >75%, high; 75-50%, good; 50-25%, normal; < 25%, weak activity.

Conflict of interest

The author declares that there are no conflicts of interest.

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