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**Journal of Chemical and Pharmaceutical Research, 2016, 8(4):207-217**

**Research Article**

**ISSN : 0975-7384**
**CODEN(USA) : JCPRC5**

# Phenolic Compounds from Grape Wastes and their Impact in Neurodegenerative Disease

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**ABSTRACT**

*Twelve phenolic compounds were isolated from Thompson seedless grape and Grenache Noir (GN) wastes. These compounds were identified as quercetin 3-**O-β-D**-glucopyranosyl (1→2)-**O-β-D**-glucopyranoside, quercetin-3-**O-α-L**-rhamnopyranosyl (1→6)-O-β-D-glucopyranoside, quercetin-3-**O-β-D**-glucopyranoside, kaempferol-3-**O-β-D**-glucopyranoside, kaempferol-3-**O-β-D**-galactopyranoside, kaempferol-7-**O-β-D**-glucopyranoside, quercetin, catechin, isorhamnetin, gallic acid, cinnamic acid and ferulic acid. All structures were characterized by spectroscopic analyses and comparisons with the previously reported data. Two of the isolated compounds, gallic and catechin revealed a power full antioxidant activity which due to its hydroxyl groups. The present study has been designed also to explore the possible role of grape pomace extract against aluminium chloride-induced neurotoxicity in rats. Aluminium chloride (70 mg/kg) was administered daily for six weeks that significantly increased cognitive dysfunction and oxidative damage as indicated by a rise in nitrite oxide concentration. Chronic administration of grape pomace extract (13 and 129 mg/kg) daily to rats for a period of 6 weeks significantly improved the memory performance tasks of rats, attenuated oxidative stress (superoxide dismutase and catalase), decreased acetylcholinesterase activity. Results showed that BDNF, Bcl-2 and AChE return to their normal value after administration of the extract. This study demonstrated the neuroprotective potential of grape waste extract in aluminium chloride-induced cognitive dysfunction and oxidative damage.*

**Keywords:** Grape waste, Phenolic compounds, Antioxidant activity, Alzheimer's disease

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## INTRODUCTION

Recently, there has been an increase in the exploitation of the waste materials coming from the wine industry. Wine waste is characterized by the presence of natural antioxidants much safer than synthetic materials. Grapes (*Vitis vinifera*) are the world's largest yield with over than 60 million tons produced per year. About 80% of the total fruit crop is used in wine industry [1]. Pomace represents approximately 20% of the weight of grapes processed. In Egypt, grapes are considered the second important crop after citrus with growing area about 152.5 feddan producing about 200,000 ton fruits, in which pomace represents about 10 to 20 thousand tons/year [2]. Grape pomace is characterized by high-phenolic contents because of poor extraction during wine making, which makes their utilization worthwhile. In recent years, the use of grape seed extracts (GSE) has gained important role as a nutritional supplement due to its antioxidant activity. The by-products obtained after winery exploitation, either seeds or pomaces, are considered a very cheap source for the antioxidant flavanols, which can be used as dietary supplements, or in the production of natural phytochemicals, with important medicinal use and in turn providing

important economic added value [3]. Grape seed is one of the by-products of wine production, accounting for 38-52% of pomace on dry weight basis. It is considered so importance for its high polyphenolic contents which are mostly known for their antioxidant properties. They are also reported to be involved in a wide range of other biological activities [4]. Grape seed contains around 13% of oil with high level of oleic and linoleic acids. Also, it was reported that a total polyphenol content ranging from 59 to 115.5 mg/g as gallic acid [5]. Grape seed oil contains 399.785 mg/kg vitamin (E) depending on variety and environmental conditions [6].

Grape seed oil ranged from 11.8 to 12 %. While in pomace, the oil varied from 3.1 to 9.5% which are rich in oleic and linoleic acids. Alpha-tocopherol was the most abundant tocopherol in the oil, while gama-tocopherols were higher in Thompson seedless and lower in Crimson seedless, red roomy skin and seeds [7]. Phenolic components are considered secondary products that are the derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants. These compounds are one of the most widely occurring groups of phytochemicals, of considerable physiological and biological importance in plants. Phenolic compounds play an important role in growth and reproduction, provides protection against pathogens and predators [8]. Phenolic compounds exhibit a wide range of medicinal uses, such as anti-allergenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects [9, 10].

Therefore, the objectives of this study are the exploitation of by-products from wine making industries and investigation the feasibility of extracting high-value phytochemicals.

## EXPERIMENTAL SECTION

**General:**
NMR experiments were performed on a Bruker AMX 400 instrument with standard pulse sequences operating at 400 MHz in $^{1}H$ NMR and 100 MHz in $^{13}C$ NMR. Chemical shifts are given in δ values (ppm) using tetramethylsilane as the internal standard and DMSO-$d_6$ as solvent at room temperature. UV spectral data was measured on a Shimadzu 240 spectrometer in MeOH. Paper chromatography Whatman 1, using solvent systems A (15% AcOH) and B (*n*-BuOH-AcOH-$H_2O$, 4:1:5, upper layer). Compounds were visualized by exposure to UV light (365 nm), before and after spraying with $AlCl_3$ and Naturestoff-polyethylene glycol reagents.

**Plant material:**
Thompson seedless skin and Grenache noir (GN) waste obtained from "El-Kroom Company", Alexandria Governorate. The collected samples were air dried then ground to a fine powder, grinding was necessary to improve extraction efficiency. The powdered samples were then kept frozen in a -35°C freezer until used.

**Extraction and isolation of phenolics from Thompson and GN waste:**
Powdered Thompson skin and GN waste (5 Kg) were extracted with EtOH (80%, 6L×5) by soaking at room temperature. The combined methanol extracts were concentrated under vacuum at 40 °C to yield 725 and 640 g of residue, respectively. The crude ethanolic extract was suspended in a hot water, left overnight, filtered and was successively partitioned with methylene chloride and *n*-butanol (BuOH) and then evaporated till dryness under vacuum (93 and 89 g, respectively). The two different BuOH fractions were subjected to TLC and paper chromatography in two different systems; A (15% AcOH) and B (*n*-BuOH-AcOH-$H_2O$, 4:1:5, upper layer). Both of them revealed the same chromatographic profile. So, one of them was subjected to isolation the phenolic compounds.

**Isolation of phenolic compounds from BuOH extract of GN waste:**
The dry BuOH extract (50 g) was loaded on a polyamid 6S column chromatography (100 x 5 cm). The column was eluted with $H_2O$, and then $H_2O$-EtOH mixtures of decreasing polarity and 10 fractions (1 L, each) were collected. The major phenolic fractions obtained were combined into five fractions after chromatographic analysis. Fraction A (1.5 g) was fractionated by column chromatography on sephadex LH-20 with aqueous EtOH (0-80 %) for elution to give compounds 1 (11 mg) and 2 (21 mg). Fraction B (2.5 g) was chromatographed on sephadex LH-20 column and EtOH (70%) was used as an eluent to give two major subfractions, then each of them was separately fractionated on another small sephadex LH-20 to yield pure compounds 3 (19 mg) and 4 (3 mg). Using the same procedure, fraction C (3 g) and fraction D (2.4 g) gave chromatographically pure samples 5 (14 mg), 6 (17 mg) and 7 (11 mg). Fraction E (1.3 g) was chromatographed on sephadex LH-20 using aqueous EtOH (80 %) for elution to give pure

compounds 8 (22 mg) and 9 (15 mg). Fraction D (0.9 g) was subjected to sephadex LH-20 column chromatography using aqueous EtOH (70 %) to afford compounds 10 (14 mg), 11 (20 mg) and 12 (9 mg).

**DPPH radical-scavenging ability of isolated compounds:**
Free radical scavenging ability of four Grenache noir waste extract concentrations (5, 25, 50 and 100 µgml-1) was evaluated by the method of Mahakunakorn et al. (2004) [11] using DPPH (1,1-diphenyl-2-picrylhydrazyl). The percentage of inhibition was calculated according to the formula:

% Inhibition = $[(A_{control} - A_{sample}) / A\ control] \times 100$, where A is absorbance.

**Anti-Alzheimer efficiency of grape wastes extract:**
The aqueous alcoholic extracts of Grenache noir seed (GNS) grape wastes were orally administered at two doses (13 and 130 mg/kg) for 90 days started after neurotoxicity induction. $AlCl_3$ (17 mg/kg/day) was used to induce neurotoxicity, Alzheimer in experimental rats in an oral administration rout for 30 days.

**Animals, housing and Experimental design:**
Adult healthy male albino rats (100 rats) weighing 150-180g were obtained from Animal House, National Research Centre, Dokki, Cairo, Egypt. Rats were randomly divided into 10 groups of eight rats each and fed on standard diet and ad libitum water. Animals were acclimatized to the laboratory conditions for one week before starting the experiment. Temperature during housing was adjusted to 24ºC with relative humidity 65±5% and 12/12 h of light/dark cycles. This work was approved by Ethical Committee of National Research Centre, Egypt. Animals were classified into three main groups; the first is -ve control group which orally administered saline for six months. The second main group is +ve control constituted three sub-groups treated as following, sub-group administered $AlCl_3$(17mg/kg/ 30 days and then remained without any treatment ), while the other two sub-groups administered the low and high doses of extract (13 and 130 mg/kg) for five months started after 30days from experiment. The third main group included two sub-groups administered $AlCl_3$ 17mg/kg for 30 days and then administered extracts at the two doses for five months.

At the end of the experiment, rats fasted overnight and then they were sacrificed under anesthesia. Blood samples were withdrawn from the reto-orbital plexus with heparinized tube and centrifuged under cooling at 4000 rpm/20 min at 8ºC and sera were used for biochemical analyses.

**Brain tissue Preparation:**
The whole brain of each rat was rapidly dissected, washed with isotonic saline and dried on filter paper. The brain was weighed and homogenized in ice-cold medium containing 50 mM Tris/HCl and 300 mM sucrose at pH 7.4 to give a 10% (w/v) [12]. This homogenate was centrifuged at 1400×g for 10 min at 4°C then supernatant was stored at –80°C for biochemical analyses.

**Biochemical investigations:**
The biochemical evaluated parameters included evaluation of brain antioxidant status parameters (brain total antioxidant capacity (TAC) according to Koracevic [13], superoxide dismutase activity according to Nishikimi et al. [14] and catalase activity (CAT) according to Aebi [15]), kits were purchased from Biodiagnostic Co., Dokki, Cairo, Egypt, as well as oxidative stress biomarkers including $H_2O_2$concentration by the method of Aebi [15], lipid peroxidation products as Malone dialdehyde (MDA) by method of Satoh [16] with brain nitric oxide concentration by Berkels et al. method [17]. These analyses were accompanied with anti-apoptotic marker (Bcl-2) determination as mentioned by Barbareschi et al. [18] (kits was obtained from Hycult Biotech, Netherelands) and brain derived neurotrophic factor (BDNF) by method of Barakat-Walter [19] (kits was purchased from Hycult Biotech, Netherelands) as well as, cholinergic biomarkers parameters including activities of acetylcholinesterase (AchE) (Den Blaauwen et al. [20]) and cholinesterase ( Ach) (Wheelock et al. [21]), kits were obtained from Quimica Clinica Aplicada S.A. with determination of Acetylcholine concentration (Oswald et al. [22]). Total protein concentration of brain was measured by Lowry et al. Method [23] to express the concentration of different brain parameters per mg protein.

## RESULTS AND DISCUSSION

BuOH fraction of Grenache noir seed (GNS) waste (50 gm) was subjected to polyamide 6S and sephadex LH-20 columns chromatography to afford 12 pure compounds characterized with different spectral analyses as follows:

**Quercetin 3-O-β-D-glucopyranosyl (1→2)-O-β-D-glucopyranoside (1)**
$^1$H NMR (400 MHz, DMSO-d$_6$): δ d 4.57 (1H, d, *J* = 7.5 Hz, H-1'"), 5.65 (1H, d, *J* = 7.5 Hz, H-1"), 6.18 (1H, d, *J* = 2.0 Hz, H-6), 6.38 (1H, d, *J* = 2.0 Hz, H-8), 6.84 (1H, d, *J* = 8.1 Hz, H-5'), 7.52 (1H, d, *J* = 2.0 Hz, H=2'), 7.67 (1H, dd, *J* = 8.1, 2.0 Hz, H-6'), 12.69 (1H, br *s*, OH). $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ "d 59.9 (C-6'"), 60.7 (C-6"), 67.5 (C-4'"), 69.5 (C-4"), 73.3 (C-3"), 74.4 (C-2'"), 75.8 (C-3'"), 76.5 (C-5"), 76.7 (C-5'"), 80.8 (C-2"), 93.4 (C-8), 98.4 (C-1"), 98.6 (C-6), 103.8 (C-10), 104.3 (C-1'"), 115.3 (C- 5'), 115.9 (C- 2'), 121.1 (C-1'), 122.1 (C-6'), 133.0 (C-3), 144.8 (C-3'), 148.5 (C-4'), 155.4 (C-2), 156.2 (C-9), 161.2 (C-5), 164.1 (C-7), 177.4 (C-4)" [24].

**Quercetin-3-*O*-α-L-rhamnopyranosyl (1→6)-*O*-β-D-glucopyranoside (rutin) (2)**
$^1$H NMR (400 MHz, DMSO-d$_6$): δ "6.20 (1H, *d*, *J* = 1.8 Hz, H-6), 6.39 (1H, d, *J* = 2.2 Hz, H-8), 7.66 (1H, d, *J* = 1.8 Hz, H-2'), 6.86 (1H, d, *J* = 8.0 Hz, H-5'), 7.60 (1H, dd, *J* = 8.0, 1.8 Hz, H-6'), 5.35 (1H, d, *J* = 7.8 Hz, H-1"), 3.25-3.47 (4H, m, H-2", H-3", H-4", H-5"), 3.38 (1H, m, H*a*-6"), 3.80 (1H, d, *J* = 10.5 Hz, H*b*-6"), 4.40 (1H, d, *J* = 1.8 Hz, H-1'"), 3.63 (1H, dd, *J* = 3.5, 1.5 Hz, H-2'"), 3.53 (1H, dd, *J* = 9.5/3.5 Hz, H-3'"), 3.28 (1H, *m*, H-4'"), 3.44 (1H, *m*, H-5'"), 1.11 (3H, *d*, *J* = 6.0 Hz, CH3-6'")". $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ "156.4 (C-2), 133.2 (C-3), 177.3 (C-4), 161.2 (C-5), 98.7 (C-6), 164.1 (C-7), 93.6 (C-8), 156.6 (C-9), 103.9 (C-10), 121.1 (C-1'), 115.2 (C-2'), 144.7 (C-3'), 148.4 (C-4'), 116.2 (C-5'), 121.5 (C-6'), glucose: 101.2 (C-1"), 74.1 (C-2"), 76.4 (C-3"), 69.9 (C-4"), 75.8 (C-5"), 67.0 (C-6"), rhamnose: 100.7 (C-1'"), 70.5 (C-2'"), 70.3 (C-3'"), 71.8 (C-4'"), 68.2 (C-5'"), 17.7 (C- 6'")" [25].

**Quercetin -3-*O*-β-D-glucopyranoside (3)**
$^1$H NMR (400 MHz, DMSO-d$_6$): δ "7.70 (1H, d, *J* = 1.9 Hz, H-2'), 7.68 (1H, dd, *J* = 8.5, 1.9 Hz, H- 6'), 6.86 (1H, d, *J* = 8.5 Hz, H-5'), 6.36 (1H, d, *J* = 2.2 Hz, H-8), 6.17 (1H, d, *J* = 2.2 Hz, H-6), 5.32 (1H, d, J = 7.3 Hz, H-1"), 3.85-3.30 (6H, sugar protons)".$^{13}$C NMR (100 MHz, DMSO-d$_6$): δ "156.2 (C-2), 133.3 (C-3), 177.5 (C-4), 161.2 (C-5), 98.7 (C-6), 164.1 (C-7), 93.5 (C-8), 156.3 (C-9), 104.0 (C-10), 121.2 (C-1'), 115.5 (C-2'), 144.8 (C-3'), 148.5 (C-4'), 116.2 (C-5'), 121.6 (C-6'), glucose: 100.8 (C-1"), 74.1 (C-2"), 76.5 (C-3"), 70.0 (C-4"), 77.6 (C-5"), 61.0 (C-6")" [26].

**Kaempferol-3-O-β-D-glucopyranoside (4)**
$^1$H NMR (400 MHz, DMSO-d$_6$): δ "8.03 (2H, d, *J* = 8.6 Hz, H-2', 6'), 6.87 (2H, d, *J* = 8.6 Hz, H-3', 5'), 6.36 (1H, d, *J* = 2.3 Hz, H-8), 6.17 (1H, d, *J* = 2.3 Hz, H-6), 5.33 (1H, d, *J* = 7.6 Hz, H-1")". $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ "178.1 (C-4), 164.1 (C-7), 161.2 (C-5),160.2 (C-4'), 157.6 (C-2), 157.2 (C-9), 134.0 (C-3), 130.9 (C-2'), 130.9 (C-6'), 120.9 (C-1'), 114.7 (C-3'), 114.7 (C-5'), 104.2 (C-10), 98.7 (C-6), 93.5 (C-8), 102.7 (C-1"), 74.4 (C-2"), 77.0 (C-3"), 70.0 (C-4"), 76.7 (C-5"), 61.2 (C-6")" [27].

**Kaempferol- 3-O-β-D-galactopyranoside (5)**
$^1$H NMR (400 MHz, DMSO-d$_6$): δ "8.06 (d, *J* = 8.7 Hz, H-2' , 6' ), 6.86 (d, *J* = 8.5 Hz, H-3', 5'), 6.42 (d, *J* = 1.8 Hz, H-8), 6.19 (d, *J* = 1.9 Hz, H-6), 5.39 (d, *J* = 7.6 Hz, H-1"), 3.20-3.83 (sugar- H)". $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ "d 60.15 (C-6"), 67.81 (C-4"), 71.17 (C-2"),73.07 (C-3"), 75.74 (C-5"), 93.68 (C-8), 98.77 (C-6), 101.52 (C-1"), 103.84 (C-1'), 115.07 (C-3', 5'), 120.89 (C-1'),130.84 (C-2', 6'), 133.17 (C-3), 156.16 (C-2), 156.41 (C-9), 159.91 (C-40), 161.19 (C-5), 164.52 (C-7), 177.38 (C-4)" [28].

**kaempferol-7-O-β-D-glucopyranoside (6)**
$^1$H NMR (400 MHz, DMSO-d$_6$): δ "7.98 (2H, d, *J* = 8.6, H-2′,6′); 6.9 (2H, d, *J* = 8.6, H-3′,5′); 6.64 (1H, d, *J* =2.0, H- 8); 6.42 (1H, d, *J* = 2.0, H-6); 5.05 (1H, d, *J* =7.5, H-1″), 3.20-3.83 (sugar- H)". $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ "147.5 (C-2), 136.2 (C-3), 176.4 (C-4), 160.2 (C-5), 98.9 (C-6), 162.4 (C-7), 94.5 (C-8), 155.9 (C-9), 104.3 (C-10), 121.2 (C-1'), 129.8 (C-2'), 115.4 (C-3'), 159.4 (C-4'), 115.4 (C-5'), 129.7 (C-6'), 99.8 (C-1"), 73.4 (C-2"), 77.3 (C-3"), 69.7 (C-4"), 76.7 (C-5"), 60.7 (C-6")" [29].

**Quercetin (7)**
$^1$H NMR (400 MHz, DMSO-d$_6$): δ "7.74 (1H, d, *J* = 2.1 Hz, H-2'), 7.62 (1H, dd, *J* = 8.3, 2.1 Hz, H-6'), 6.88 (1H, d, *J* = 8.3 Hz, H-5'), 6.39 (1H, d, *J* = 2.0 Hz, H-8), 6.18 (1H, d, *J* = 2.0 Hz, H-6)". $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ

"d 93.2 (C-8), 98.0 (C-6), 102.9 (C-10), 115.0 (C-2'), 115.4 (C-5'), 119.8 (C-6'), 121.8 (C-1'), 135.5 (C-3), 144.9 (C-3'), 146.7 (C-2), 147.5 (C-4'), 156.0 (C-9), 160.6 (C-5), 163.8 (C-7), 175.7 (C-4)" [30].

**Catechin (8)**

$^1$H NMR (100 MHz, DMSO-$d_6$): δ "6.84 (1H, d, *J* = 2.0 Hz, H-2'), 6.77 (1H, dd, *J* = 8.1 Hz, H-5'), 6.71 (1H, dd, *J* = 2.0, 8.1 Hz, H-6'), 5.92 (1H, d, *J* = 2.4 Hz, H-8), 5.85 (1H, d, *J* = 2.4 Hz, H- 6), 4.56 (1H, d, *J* = 8.0 Hz, H-2), 3.97 (1H, ddd, *J* = 8.0, 8.0, 4.8 Hz, H-3), 2.85 (1H, dd, *J* = 4.8, 16.0 Hz, H-4), 2.50 (1H, dd, *J* = 8.0, 16.0 Hz, H-4)". $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ "27.7 (C-4), 66.3 (C-3), 80.9 (C-2), 93.9 (C-6), 95.1 (C-8), 114.5 (C-2'), 115.1 (C-5'), 18.4 (C-6') and other aromatic carbons showed peaks at *δ* of 99.1, 130.6, 144.6, 144.8, 155.3, 156.1 and 156.4" [31, 32].

**Isorhamnetin (9)**

$^1$H NMR (400 MHz, DMSO-$d_6$): δ "7.92 (1H, d, *J* = 1.6 Hz, H-2'), 7.60 (1H, dd, *J* = 1.6, 8.4 Hz, H-6'), 6.91 (1H, d, *J* = 8.4 Hz, H-5'), 6.40 (1H, *J* = 1.8 Hz, H-8), 6.20 (1H, d, *J* = 1.8 Hz, H-6), 3.92 (3H, s, $OCH_3$)". $^{13}$C NMR (100MHz, DMSO-$d_6$): δ "148.61 (C-2), 135.62 (C-3), 175.72 (C-4), 156.05 (C-5), 98.11 (C-6), 163.71 (C-7), 93.53 (C-8), 160.32 (C-9), 102.91 (C-10),121.84 (C1'),111.56 (C2'), 146.43 (C3'), 147.21 (C4'),115.33 (C5'),121.64 (C6'), 55.63 ($OCH_3$)" [33].

**Gallic acid (10)**

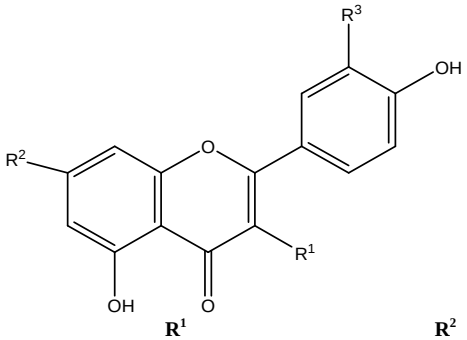
$^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.1, 3.3, 2.0, 1.2. $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 169.1 (C-1), 144.9 (C-4, 5), 138.2 (C-5), 120.6 (C-2), 108.9 (C-3, 7) [34].

**Cinnamic acid (11)**

$^1$ H NMR (400 MHz, DMSO-$d_6$): δ 12.43 (1H, s), 7.67(2H, m), 7.59 (1H, d, J = 16.4 Hz), 7.42 (d, J=2.8 Hz, 3H), 6.53 (d, J = 16.0Hz, 1H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ: "167.7, 144.0, 134.4, 130.3, 128.9, 128.2, 119.4" [35].

**Ferulic acid (12)**

$^1$H NMR (400 MHz, $CDCl_3$): δ: 3.98 (3H, s, H-4'), 6.34 (1H, d, *J*=15 Hz, H-2'), 6.97 (1H, d, *J*=9 Hz, H-6), 7.14 (1H, dd, *J*=8 and 2 Hz, H-5), 7.09 (1H, d, *J*=2 Hz, H-3), 7.75 (1H, d, *J*=15 Hz, H-1'). $^{13}$C NMR (100 MHz, $CDCl_3$) δ: "55.98 (C-4'), 109.48 (C-5), 114.39 (C-2), 114.78 (C-2'), 123.57 (C-3), 126.68 (C-4), 146.81 (C-1'), 147.05 (C-6), 148.37 (C-1), 171.36 (C-3')" [36].




| Compound | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **1** | glucopyranosyl (1-2) glucopyranoside | OH | OH |
| **2** | rhanmnopyranosyl (1-6) glucopyranoside | OH | OH |
| **3** | glucopyranoside | OH | OH |
| **4** | glucopyranoside | OH | H |
| **5** | galactopyranoside | OH | H |
| **6** | OH | glucopyranoside | H |
| **7** | OH | OH | OH |
| **9** | OH | OH | $OCH_3$ |

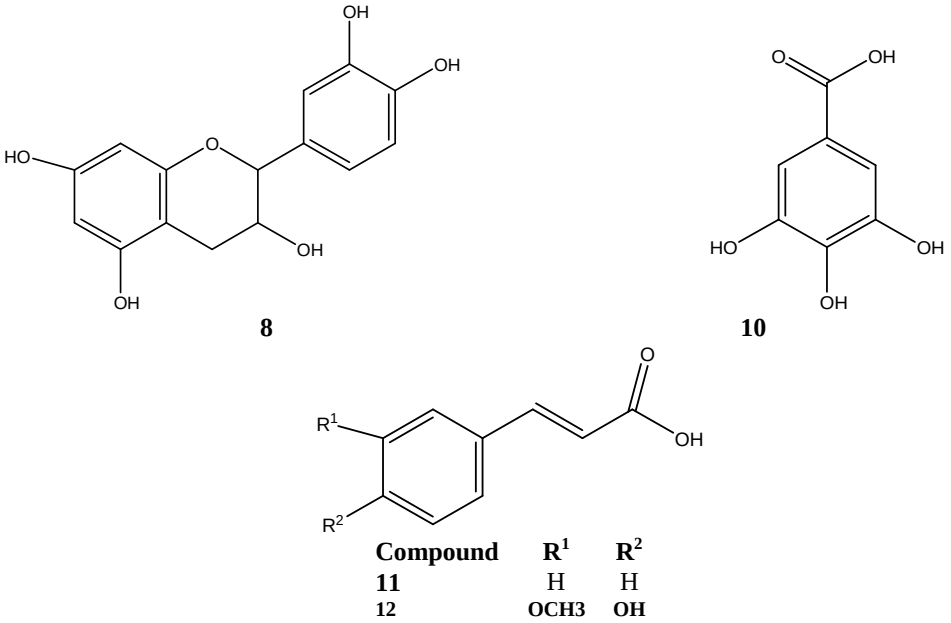



**Fig 1. Chemical structure of the isolated compounds (1-12)**

**Antioxidant activity:**

The antioxidant activity of the pure isolated compounds at different concentrations (5-100 µg/ml) was assessed against DPPH radical. Only kampferol-3-O-β-D- galactopyranoside obtained in trace amount which enable us to estimation its antioxidant activity.

**Table 1. Antioxidant activity (%) of isolated compounds against DPPH radical at different concentrations**

| Compound | Conc. (µg/ml) | | | | $IC_{50}$ |
|---|---|---|---|---|---|
| | 5 | 25 | 50 | 100 | |
| Ferulic acid | 28.8 ± 0.7$^{e}$ | 49.0 ± 0.3$^{e}$ | 66.7 ± 0.5$^{d}$ | 93.2 ± 1.3$^{b}$ | 25.5 |
| Rutin | 26.7 ± 0.1$^{f}$ | 36.2 ± 0.4$^{g}$ | 45.8 ± 0.8$^{f}$ | 65.8 ± 1.3$^{d}$ | 54.5 |
| Cinnamic acid | 12.5 ± 0.2$^{h}$ | 25.8 ± 0.5$^{j}$ | 25.8 ± 0.1$^{j}$ | 34.7 ± 1.7$^{i}$ | 181 |
| Isorhamnetin | 32.8 ± 0.3$^{b}$ | 57.8 ± 0.2$^{c}$ | 89.2 ± 0.3$^{b}$ | 100.0 ± 0.0$^{a}$ | 21.6 |
| Catechin | 36.3 ± 0.3$^{a}$ | 99.0 ± 0.9$^{a}$ | 100 .0 ± 0.1$^{a}$ | 100.0 ± 0.0$^{a}$ | 6.9 |
| Gallic acid | 36.4 ± 0.1$^{a}$ | 75.3 ± 0.2$^{b}$ | 100 .0 ± 0.1$^{a}$ | 100.0 ± 0.0$^{a}$ | 6.86 |
| Quercetin | 32.8 ± 0.1$^{c}$ | 55.6 ± 0.53$^{d}$ | 86.7 ± 0.6$^{c}$ | 100.0 ± 0.0$^{a}$ | 7.61 |
| Quercetin-diglu | 27.0 ± 0.4$^{f}$ | 30.3 ± 0.06$^{i}$ | 36.7 ± 0.03$^{i}$ | 55.8 ± 0.05$^{f}$ | 68.2 |
| Kaempferol-3-glu | 30.0 ± 0.1$^{d}$ | 34.2 ±0.22$^{h}$ | 40.8 ± 0.02$^{g}$ | 58.3 ± 0.12$^{e}$ | 61.2 |
| Quercetin-3-glu | 26.5 ± 0.15$^{f}$ | 30.0 ± 0.0$^{i}$ | 38.3 ± 0.68$^{h}$ | 47.8 ± 0.45$^{g}$ | 105 |
| Kaempferol-7-glu | 25.2 ± 0.55$^{g}$ | 31.0 ± 0.33$^{i}$ | 37.5 ± 0.09$^{hi}$ | 42.9 ± 0.2$^{h}$ | 117 |
| *Resveratrol | 29.4 ± 0.01$^{de}$ | 47.0 ± 0.9$^{f}$ | 64.2 ± 0.11$^{e}$ | 84.1 ± 0.4$^{c}$ | 26.6 |
| *Ascorbic acid | 14.5 ±0.63 | 72.3 ±2.24 | 86.2 ±2.17 | 90.2 ±2.5 | 17.3 |
| *BHT | 15.4 ±0.58 | 76.9 ±1.04 | 87.5 ±1.54 | 94.1 ±2.03 | 16.3 |
| *LSD 0.01 | 0.73 | 1.07 | 0.9 | 1.6 | |

*Data are represented as mean ± S.D.*

*Statistical analysis is carried out by two way analysis of variance and IC50 calculated using SPSS program Unshared letters between brackets were significant value between groups.*

**Standard antioxidant*

The present data in Table (1) revealed that, catechin was found to had the highest antioxidant activity than other isolated compounds (99 % at 25 µg/ml), followed by gallic acid (100% at 50 µg/ml) then isorhamnetin and quercetin (100% at 100 µg/ml) and had more activity than ascorbic acid, BHT and resveratrol which used as positive controls (90.15, 94.1 and 84% at 100µg/ml, respectively), and this activity was concentration dependent.

The scavenging activity of flavonoids can be arranged in the following order: catechin > gallic > isorhamnetin > quercetin> BHT > ferulic > ascorbic > resveratrol > rutin > kaempferol-3-glucooside > quercetin-di-glucoside > quercetin-3-glucoside > kaempferol-7-glucoside.

The relatively high scavenging activity of catechin could be due to the presence of B ring catechol and three free hydroxyl groups leading to high radical scavenging and strong radical absorption [37].

"The B-ring in catechin structure strongly enhances lipid peroxidation inhibition. This arrangement is a salient feature of the most potent scavengers of peroxyl, superoxide, and peroxynitrite radicals. For example, the peroxyl radical scavenging ability of luteolin substantially exceeds kaempferol; both have identical hydroxyl configurations, but kaempferol lacks the B-ring catechol. Peroxynitrite scavenging by catechin is mainly ascribed to its B-ring catechol. Oxidation of a flavonoid occurs on the B-ring when the catechol is present, yielding a fairly stable ortho-semiquinone radical through facilitating electron delocalization. Flavones lacking catechol or O-trihydroxyl (pyrogallol) systems form relatively unstable radicals and are weak scavengers. The ability to quench singlet oxygen seems to be in relation with the chemical structure of catechin, with the presence of the catechol moiety on ring B and the presence of a hydroxyl group activating the double bond on ring C. Group at the 3–OH position in catechin, effectively quench the secondary products of lipid peroxidation. The antioxidant activity of gallic acid may be due to the presence of three hydroxyl groups as hydrogen donor, exhibits exceptional antioxidant activity. The low antioxidant activity of kaempferol among the flavonolaglycones could be attributed to the presence of a single hydroxyl group in the B ring which apparently makes little contribution even in the presence of the conjugated double bond system and the 3-OH group. Similar results have been mentioned by Lien et al. [38]".

"Quercrtin is found to be more active than its glycosides, these results hold true with those of Rice-Evans et al., (1996) [39] who found that, glycosilated flavonoids lose their activity in comparison with aglycones. The superiority of quercetin in inhibiting both metal and nonmetal-induced oxidative damage is partially ascribed to its free 3-OH substituent, which is thought to increase the stability of the flavonoid radical. The torsion angle of the B-ring with respect to the rest of the molecule strongly influences free radical scavenging ability. Substitution of 3-OH by a methyl or glycosyl group completely abolishes the activity of quercetin and kaempferol against carotene oxidation in linoleic acid. It is postulated that B-ring hydroxyl groups form hydrogen bonds with the 3-OH, aligning the B-ring with the heterocycle and A-ring. Eliminating this hydrogen bond affects a minor twist of the B-ring, compromising electron delocalization capacity".

The antioxidant activity of flavonoids depends strongly on the number and position of hydroxyl groups in the molecule. Dihydroxylated B-ring (catechol structure), presence of unsaturation and of 4-oxo function in the C-ring which are also presumed to increase the antioxidant capacity [40].

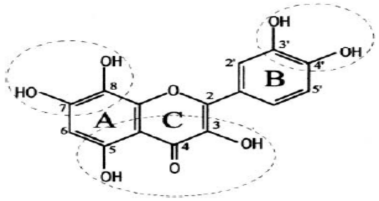



**Fig. 2. Structural features of flavonoids with a high radical scavenging activity**

The suggested mechanism by which the phenolic acids exert their antioxidant activity against DPPH radicals is probably due to their chemical structures as shown in Figure (2). The antioxidative activity of polyphenols is generally imputed to their hydroxyl groups, but it is not the only factor in determining the potency of their activities [41].

**Oxidative stress and Alzheimer:**

In view of the oxidative theory of ischemia-induced cell death and injury, increasing interest has been focused on natural products that can ameliorate delayed neuronal death due to cerebral ischemia [42].

The results of table (2) show the effect of grape seed extracts (GSE) on brain oxidative stress markers represented by $H_2O_2$, nitric oxide and MDA levels in Al-intoxicated rats.

In the present study daily administration of $AlCl_3$ to rats showed significant elevation in all oxidative stress biomarkers ($H_2O_2$, NO and MDA) when compared with control group. The significant elevation of brain NO level after $AlCl_3$ administration in rats is in agreement with the finding results reported by Guix *et al*. [43]., they found that cerebellar levels of inducible NOS (nitric oxide synthase) was elevated following both short and long-term Al administration in rats.

It is obvious from the Table (2) that treatment of (Al) intoxicated rats with GSE produced significant decrease in brain $H_2O_2$, NO and MDA levels. These remarkable effects of GSE may be related to the inhibitory effect of monoamine oxidase activity in the brain as described by Mizutani *et al*. [44]., which contribute this activity as a mechanism by which resveratrol could reduce oxidative stress, production of $H_2O_2$ and lipid peroxidation.

**Table 2. Effect of GSE treatment on brain oxidative stress parameters in $AlCl_3$ intoxicated rats**

| Groups | MDA nmol/mg protein | $H_2O_2$ brain mmol/mg protein | NO umol/mg protein |
|---|---|---|---|
| Control | 2.62 ±0.06$^{f}$ | 8.71 ±0.09$^{d}$ | 92.0±1.95$^{e}$ |
| GSE (low dose), +ve control | 2.7 ±0.05$^{ef}$ | 7.41 ±0.04$^{e}$ | 86.6 ±0.56$^{ef}$ |
| GSE (high dose), +ve control | 2.45 ±0.05$^{g}$ | 6.63 ±0.24$^{f}$ | 51.96 ±2.9$^{f}$ |
| $AlCl_3$-intoxicated control | 4.7 ±0.05$^{a}$ | 33.2 ±0.02$^{a}$ | 302.3 ±2.4$^{a}$ |
| Intoxicated treated with GSE at low dose | 3.18 ±0.13$^{c}$ | 10.5 ±0.09$^{b}$ | 185.0 ±6.1$^{c}$ |
| Intoxicated treated with GSE at high dose | 2.63 ±0.09$^{f}$ | 9.61 ±0.08$^{c}$ | 128.5 ±1.55$^{d}$ |
| Sig. | 0 | 0 | 0 |
| $P< 0.01$ | ** | ** | ** |

*Low dose (13 mg/kg) and high dose (129 mg/kg).*
*Data are represented as mean ± S.D. of 10 male rats/group.*
*Statistical analysis is carried out by one way analysis of variance using SPSS program.*
*Unshared letters between brackets are significant value between groups, where P is the level of significance.*

**Antioxidant enzymes:**

Antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and total antioxidant capacity (TAC) were determined in rat's brain, and the data were compiled in Table (3).

The results in Table (2) revealed that, all tested defense enzymes CAT, SOD and TAC were decreased in $AlCl_3$-intoxicated control by 46, 91.4 and 60.1 %, respectively. However, the level of control defense enzymes not changes during treatment with different grape extracts in comparison with control (untreated group). CAT increased due to treatment with GSE, SOD and TAC nearly unchanged compared with -ve control.

**Table 3. Effect of GSE treatment on brain antioxidant status in $AlCl_3$ intoxicated rats**

| Groups | SOD U/mg protein | CAT U/mg protein | TAC mmol/mg protein |
|---|---|---|---|
| Control | 2.8 ±0.03$^{a}$ | 9.9 ±0.12$^{e}$ | 13.9 ±0.67$^{a}$ |
| GSE (low dose), +ve control | 2.8 ±0.03$^{a}$ | 12.5 ±0.01$^{b}$ | 14.7±0.15$^{a}$ |
| GSE (high dose), +ve control | 2.5 ±0.11$^{c}$ | 11.6 ±0.01$^{c}$ | 16.0 ±0.2$^{a}$ |
| $AlCl_3$-intoxicated control | 1.5 ±0.01$^{d}$ | 0.9 ±0.03$^{g}$ | 5.6 ±0.18$^{b}$ |
| Intoxicated treated with GSE at low dose | 2.8 ±0.00$^{a}$ | 11.9 ±0.06$^{b}$ | 13.7 ±0.42$^{a}$ |
| Intoxicated treated with GSE at high dose | 2.8 ±0.06$^{a}$ | 10.7 ±1.5$^{d}$ | 15.9 ±0.17$^{a}$ |
| Sig. | 0 | 0 | 0 |
| $P< 0.01$ | ** | ** | ** |

*Low dose (13 mg/kg) and high dose (129 mg/kg).*
*Data are represented as mean ± S.D. of 10 male rats/group.*
*Statistical analysis is carried out by one way analysis of variance using SPSS program.*
*Unshared letters between brackets are significant value between groups, where P is the level of significance.*

The results obtained in this concern are in agreement with the results obtained by Aly *et al*., [45], Mahdy *et al*., [46], and Naidu *et al.*, [47]. They reported that, the daily administration of $AlCl_3$ induced significant inhibition in brain SOD and CAT enzymes. GSE brought an increase in CAT and TAC. Considering SOD, Richardson [48] reported a decrease or reduction in SOD from 25% to 35% activity in AD frontal cortex, hippocampus and cerebellum.

**Determination of BDNF and Bcl-2 expression:**
BDNF (Brain-derived neurotrophic factor) is critical for the survival and maintenance of sympathetic and sensory neurons vital to learning, memory, and higher thinking. BDNF itself is important for long-term memory [49]. Without the nerve growth factor, the sympathetic and sensory neurons will undergo apoptosis.

**Table 4. Effect of GSE treatment on brain antiapoptotic protein (Bcl-2) and neurotrophic factor (BDNF) in $AlCl_3$ intoxicated rats**

| Groups | BDNF(Pg/mg protein) | Bcl-2 (Pg/mg protein) |
|---|---|---|
| Control | 99.6±1.6 [a] | 52.8 ±1.15[e] |
| GSE (low dose),+ve control | 87.8±1.6 [b] | 60.6 ±1.07[c] |
| GSE (high dose),+ve control | 100±1.0 [a] | 90.5 ±0.96[a] |
| $AlCl_3$-intoxicated control | 50.5±1.2[e] | 35.0 ±1.05[f] |
| Intoxicated treated with GSE at low dose | 75.3±0.5[c] | 57.7 ±0.9[d] |
| Intoxicated treated with GSE at high dose | 93.8±1.2[ab] | 89.2 ±1.68[a] |
| Sig. | 0 | 0 |
| $P<$ 0.01 | ** | ** |

*Low dose (13 mg/kg) and high dose (129 mg/kg).*
*Data are represented as mean ± S.D. of 10 male rats/group.*
*Statistical analysis is carried out by one way analysis of variance using SPSS program.*
*Unshared letters between brackets are significant value between groups, where P is the level of significance.*

Table (4) show the results of BDNF and Anti-apoptotic protein Bcl-2 brain concentration in Al-toxicated rat as compared to those treated with grape seeds extraction as well as those neither Al-toxicated nor treated with GSE (mean ± SD).

Bcl-2 levels showed significant increases in comparison the -ve control and +ve control, such curative and improving effect of grape wastes extract may be suggested. On the other hand values of BDNF in $AlCl_3$ intoxicated groups due to treating with extracts of grape seeds investigated but it did not reached the normal level -ve control. The data in Table (4) show that, administration of $AlCl_3$ in rats led to significant reduction in brain Bcl-2 expression (35.0 Pg/mg) as well as BDNF levels in $AlCl_3$-intoxicated control (50.5 Pg/mg) compared with those in control rats (52.8 and 99.6 Pg/mg, respectively). However, the levels of Bcl-2 in most treatments with GSE were insignificant especially at low concentration when compared with control but this level was increased with high concentration treatment of each extracts.

**Determination of Acetyl cholinesterases (AChE):**
The data in Table (5) demonstrate that aluminum administration to rats induced significant elevation in brain AchE activity and significant reduction in brain Ach level in $AlCl_3$-intoxicated control as compared to control rats (untreated) (AchE: 854.8 and 602.01 U/mg, respectively; Ach: 67.3 and 91.1 µmol/mg, respectively), while GSE administration produced significant decrease in brain AchE activity associated with significant increase in brain Ach level in Al-intoxicated rats.

The results revealed that, aluminum treatment increased the AChE activity in serum with 3558.1 U/L/min in $AlCl_3$-intoxicated control, in comparison with untreated group (control), 1759.5 U/L/min. AChE activity in serum was influenced by GSE, which could partially reverse learning and memory deficit induced by Al. From this work, it is speculated that GSE amelioration of learning and memory deficit induced by Al is related to its inhibition of the expression of AChE in blood and brain. "These results obtained in this concern are in agreement with the results obtained by Kaizer *et al*. [50], who found that, Al exposure increased AchE activity via allosteric interaction between Al and the peripheral anionic site of the enzyme molecule, leading to the etiology of AD pathological deterioration". Al exerts cholinotoxic effects by blocking the provision of acetyl-CoA, which is required for Ach synthesis or by impairing the activities of choline acetyl transferase (ChAT) itself [51]. With respect to cholinergic markers, the present results showed significant increase in brain activity of AchE with concomitant decrease in Ach level in Al-intoxicated rats. Zheng *et al*. [52] reported an increased AchE activity in Al-overloaded rats. Treatment of Al-intoxicated rats with GSE produced significant decrease in brain AchE activity accompanied with significant increase in brain Ach level in comparison with Al-intoxicated control group. It has been demonstrated that GSE significantly increases Ach release in the hippocampus [53]. Thus, the promoting effect of GSE on Ach release in the hippocampus may be one mechanism for its memory enhancing effect [54].

## CONCLUSION

Considering the positive results of this study about GSE and its efficacy in attenuating AD-type pheno-type, this natural compound is immediately available to be tested in AD clinical settings to prevent or treat AD. More long-term studies should be undertaken to determine their beneficial effects in slowly developing neurodegenerative disorders. Clinical studies are urgently warranted in order to support this attractive hypothesis.

**Table 5. Effect of GSE treatment on brain and serum acetylcholinesterase (AchE), choline esterase and acetylcholine (Ach) in $AlCl_3$ intoxicated rats**

| Groups | CHE U/mg brain protein | Ach(µmol/mg brain protein) | AChE (U/mg brain protein) | AChE U/L/min sera |
|---|---|---|---|---|
| Control | 291.3 ±2 [b] | 91.1 ±2.6[e] | 602.01 ±3[c] | 1759.5 ± 24[cde] |
| GSE (low dose),+ve control | 180 ±2[f] | 95.4 ±4.5[d] | 498.72 ±5.3[f] | 1876.8 ± 70[cd] |
| GSE (high dose), +ve control | 141.9 ±2.5[g] | 101 ±4.0[c] | 415.7 ±5.8[j] | 1728.22 ± 89[e] |
| $AlCl_3$-intoxicated control | 727.3 ±3.56 [a] | 67.3 ±0.6[g] | 854.85 ±4.3[a] | 3558.1 ± 54[a] |
| Intoxicated treated with GSE at low dose | 143.04 ±1.71[g] | 100 ±5.0[c] | 536.12 ±6.2[e] | 2166.14 ± 54[b] |
| Intoxicated treated with GSE at high dose | 45.785 ±1.12[h] | 128.0 ±3.0[a] | 446.88 ±6[h] | 1900.26 ± 47[c] |
| Sig. | 0 | 0 | 0 | 0 |
| P< 0.01 | ** | ** | ** | ** |

*Low dose (13 mg/kg) and high dose (129 mg/kg).*
*Data are represented as mean ± S.D. of 10 male rats/group.*
*Statistical analysis is carried out by one way analysis of variance using SPSS program.*
*Unshared letters between brackets are significant value between groups, where P is the level of significance.*

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