

## New Cyclolignans from *Origanum glandulosum* Active Against $\beta$ -amyloid Aggregation

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**Abstract:** *Origanum glandulosum* Desf is an endemic flavoring herb widely distributed in North Africa that is commonly used in traditional medicine. This oregano species is rich in essential oils but little is known about its phenolic composition. In the present study, a crude extract of *O. glandulosum* was prepared in order to isolate and investigate its neuroprotective potential to inhibit  $\beta$ -amyloid peptide (A $\beta$ ) aggregation. The three major compounds of the extract were isolated: rosmarinic acid and two cyclolignans in *Origanum* genus, globoidnan A and a new derivative named globoidnan B. Rosmarinic acid and globoidnan A showed significant anti-aggregative activity against  $\beta$  amyloid aggregation (IC<sub>50</sub> 7.0 and 12.0  $\mu$ M, respectively). In contrast, globoidnan B was found to be less active.

**Keywords:** *Origanum glandulosum*; oregano; cyclolignans;  $\beta$ -Amyloid aggregation; NMR. ©2014 ACG Publications. All rights reserved.

### 1. Introduction

*Origanum* genus is widely distributed throughout the Mediterranean region and numerous species, subspecies and hybrids have been described [1,2]. Belonging to the *Origanum* genus, *O. glandulosum* Desf is an endemic widely distributed taxon in North Africa (Algeria and Tunisia) that is commonly used as a spice as well as in traditional medicine. *O. glandulosum* Desf is rich in essential oils, but little is known about its phenolic composition. To our best knowledge, phenolic constituents of *O. glandulosum* extract have not been previously investigated by modern analytical methods.

The genus *Origanum* is rich in essential oils and polyphenols that have antimicrobial, antioxidant and other bioactive properties [3-5]. Several studies indicate that polyphenols present in high amounts in natural products could play a preventive role in the incidence of age-related neurological disorders. These findings have been supported by epidemiological studies [6,7] and confirmed by *in vitro* and *in vivo* studies [8,9]. With regard to Alzheimer's disease (AD), Hamaguchi *et al.* have shown that rosmarinic acid inhibits  $\beta$ -amyloid peptide (A $\beta$ ) aggregation *in vitro* and

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*in vivo* [10]. Rosmarinic acid derivatives, which are plant secondary metabolites belonging to the class of hydroxycinnamic acid esters, are prominent constituents of the *Origanum* species [4,5,11]. Thus oregano extracts could be of therapeutic value in AD prevention. AD is the most common type of senile dementia and is characterized by the abundance of extracellular deposition of A $\beta$ . Because A $\beta$  aggregation promotes pro-inflammatory responses and activates neurotoxic pathways leading to dysfunction and death of brain cells [12], many researchers favor therapeutic approaches that target formation, deposition and clearance of A $\beta$  fibrils [10,13-18].

The present study was undertaken to (i) identify and quantify the major phenolic constituents of *O. glandulosum* extract by high-performance liquid chromatography (HPLC), mass spectrometry (MS) and NMR analysis; (ii) evaluate *in vitro* activity of the extract on A $\beta$  aggregation in AD prevention; (iii) evaluate the inhibitory potential of the pure compounds against A $\beta$  fibril formation.

## 2. Materials and Methods

### 2.1. Reagents and standards

The A $\beta$  (25-35) (A $\beta$ <sub>25-35</sub>) peptide sequence corresponding to residues of the human wild type sequence was purchased from Bachem California (Torrance, CA). Except for petroleum ether, which was of synthesis grade, all organic solvents used were of HPLC grade. Methanol and ethanol were purchased from Carlo Erba (Val de Reuil, France). Acetonitrile, ethyl acetate and petroleum ether were purchased from Scharlau Chemie (Sentmenat, Spain). Curcumin (Cur) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Water was purified using the Elga (Bucks, UK) water purification system with a resistivity of no less than 18 M $\Omega$ /cm. NMR experiments were performed in deuterated acetone-*d*<sub>6</sub> purchased from Euriso-top (Gif-sur-Yvette, France).

### 2.2 Plant material

The material consisted of the aerial part of Algerian *Origanum glandulosum*. Samples were collected from Boukhelifa in the city of Bejaia (north-eastern Algeria), when the plant is highly present. Botanical identification was made on a herbarium specimen by the Botany Laboratory of Bejaia University (Bejaia, Algeria). The samples were well dried in the shade and away from moisture, then ground into powder for analysis.

### 2.3 Extraction of plant samples

A portion of the finely powdered material from *Origanum glandulosum* (100 g) was first extracted three times with 70% methanol (MeOH, 2 L) during a 24-h period at ambient pressure and temperature. After removal of MeOH under reduced pressure at 35°C, the aqueous phase was defatted with petroleum ether (0.8 L) and then extracted with ethyl acetate (EtOAc). Extraction was carried out until a colorless extract was obtained. The EtOAc solutions were concentrated and lyophilized.

### 2.4 Instrumentation

The lyophilized extract was dissolved in 50% methanol and chromatographed using HPLC-DAD. The chromatography apparatus, an Agilent 1200 from Agilent Technologies (Santa Clara, CA, USA), was equipped with a degasser, a binary pump, an autosampler module, a column heater/selector and a UV–visible-DAD detector from the same provider. The *Origanum* extract was passed through a 0.45  $\mu$ m Millipore membrane before chromatographic separation at 23°C with a 250  $\times$  4 mm i.d., 5  $\mu$ m, Prontosil 120-5-C18-AQ reverse-phase column, Bischoff (Leonberg, Germany). Water 0.1% TFA (solvent A) and acetonitrile 0.1% TFA (solvent B) were used as mobile phases. The gradient elution program was as follows (v/v): 0 min 10% B, 5 mn 10% B, 10 mn 15% B, 60 mn 22% B, 62 mn 100% B linear for 10 min, followed by 10 min for re-equilibration. The detection wavelengths were set at 260 and 320 nm. This HPLC was coupled to an Esquire 3000+ ion trap mass spectrometer using an ESI source from Bruker Daltonics (Billerica, MA, USA). The HPLC output

flow was split with a passive splitter with an average 1:100 ratio depending on the flow solvent, viscosity and rate. Nitrogen drying gas was set at 9.0 L/min and 350°C, nebulizer pressure was set at 27 psi. ESI-MS parameters (positive mode): HV capillary – 4100 V, end plate offset – 500 V, capillary exit 134.3 V, skimmer 40 V, trap drive 59.3, scan 25000  $\mu$ s, rolling average 2 and trap averages 5.

Final purification was performed by semi-preparative HPLC (Varian, model ProStar 210 coupled with a diode array detector ProStar 335) with a Varian Microsorb 100 C18 Dynamax reverse-phase column (250 x 21.4 mm, 5  $\mu$ m particle size) at room temperature. The flow rate was 15 mL/min. The solvent system and the gradient program used were the same as previously described. The eluted compounds were manually collected according to the visualization of the UV profile.

NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (600 MHz for  $^1\text{H}$  and 151 MHz for  $^{13}\text{C}$  experiments) at 300 K in the acetone- $d_6$  deuterated solvent. Spectra were referenced to the signal of acetone- $d_6$  at  $\delta_{\text{H}}$  2.06 and  $\delta_{\text{C}}$  29.9 ppm.

Accurate masses were measured on a microTOF-Q mass spectrometer (Bruker, Bremen, Germany). Sample solutions were infused at 3  $\mu$ L/min. Electrospray ionization in negative mode was used. Each spectrum was calibrated with a 10 mM lithium formiate solution using enhanced cubic calibration model of microTOF control software (Bruker).

### 2.5 Measurement of inhibitory activity by UV-visible spectroscopy

A $\beta_{25-35}$  peptide stock solution of 1 mM was prepared by solubilizing the lyophilized A $\beta_{25-35}$  peptide in sterile water at 4°C, then by sonication for 1 min. The peptide stock solution was aliquoted and stored at –20°C. All steps were carried out at 4°C to prevent A $\beta$  polymerization.

The detailed method for measuring the inhibitory activity on A $\beta$  aggregation was given in a previous report [19]. Briefly, UV-visible measurements were used to search for inhibitors of A $\beta$  fibril formation. Stock solutions of A $\beta_{25-35}$  at 1 mM were prepared by solubilizing the lyophilized A $\beta$  by vortexing briefly in sterile water at 4°C, then by sonicating for 10 min. Extract and pure polyphenols were solubilized in MeOH solution to a concentration of 1 mg/mL. Stock solutions were aliquoted and stored at –20°C. UV-visible spectrometry was performed on a Cary 300 spectrophotometer (Varian, USA). To study A $\beta$  fibril inhibition, experiments were carried out by using a reaction mixture containing 80  $\mu$ L phosphate buffer (10 mM final concentration), 10  $\mu$ L A $\beta_{25-35}$  (100  $\mu$ M final concentration) and 10  $\mu$ L tested compound, pH 7.2.

## 3. Results and Discussion

### 3.1. Structure elucidation

To assess whether the extracts contained a large number of different compounds, and in an attempt to identify these compounds, HPLC analysis was carried out with the EtOAc extract of *O. glandulosum* (Fig. 1). The analytical chromatogram of the extract presented three major peaks. The phenolic compounds corresponding to these peaks were isolated and their structures were elucidated by 1D- and 2D-NMR and MS data.

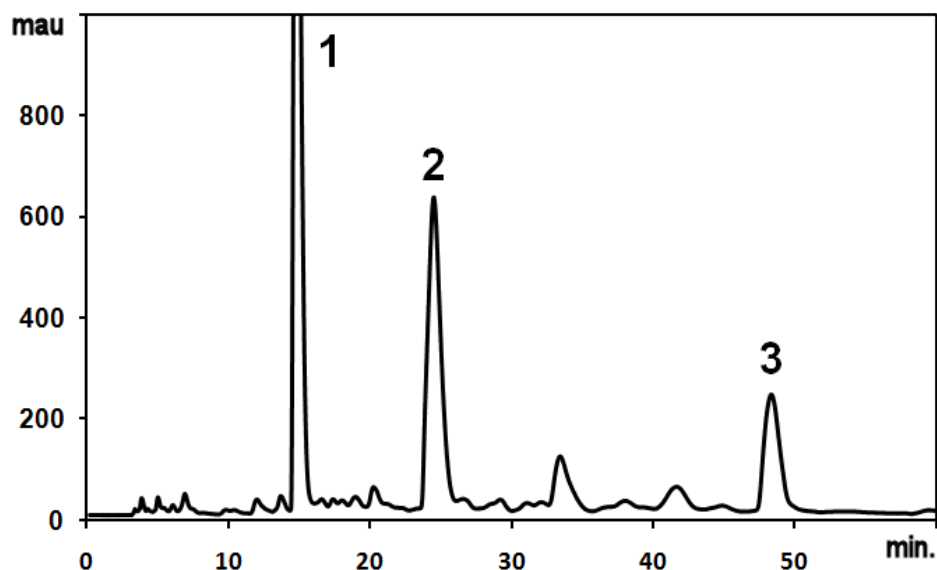


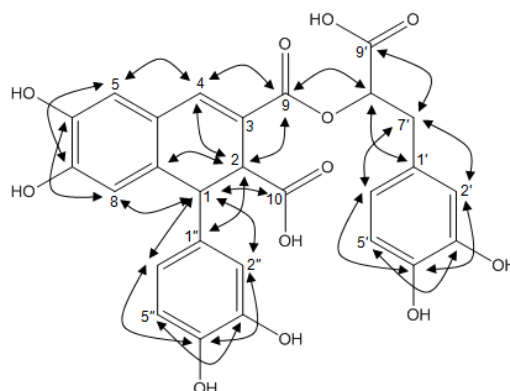
Figure 1. HPLC chromatogram of *O. glandulosum* extract.

Globoidnan B (**1**), peak  $t_R = 15.1$  min, 208 mg/100g fresh weight (FW), was obtained as a brown, amorphous powder, UV(MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) 236 (3.23), 370 (4.26) nm (Fig. 2). The molecular formula  $C_{27}H_{22}O_{12}$  was deduced from a quasi-molecular ion peak in the ESI-HRMS mass spectrum at  $m/z$  537.1033  $[M-H]^-$  (required 537.1038 for  $C_{27}H_{21}O_{12}$ ). The  $^1H$ - and  $^{13}C$ -NMR data (Table 1) are consistent with a highly aromatized molecule, as the  $^{13}C$ -NMR chemical shifts suggested that 21 of the 27 carbons were aromatic. COSY and HMBC correlations identified resonances consistent with a 1,2,3-trisubstituted dihydronaphthalene-6,7-diol moiety ( $\delta_C$ : 148.3, 144.7, 138.7, 131.5, 124.4, 122.6, 116.8, 116.7, 47.6, 45.9 ppm;  $\delta_H$ : 7.61, 6.95, 6.67, 4.49, 3.92 ppm) as well as an alkylated 3,4-catechol ( $\delta_C$ : 145.3, 144.5, 128.6, 121.6, 117.2, 115.7, 74.0, 37.4 ppm;  $\delta_H$ : 6.82, 6.73, 6.64, 5.15, 3.07, 3.05 ppm) and a mono-substituted 3,4-catechol ( $\delta_C$ : 145.3, 145.0, 136.0, 119.3, 115.7, 115.2 ppm;  $\delta_H$ : 6.68, 6.50, 6.45 ppm). These data, along with the observation of three carbonyl  $^{13}C$  resonances ( $\delta_C$ : 173.2, 170.0, 166.5 ppm) consistent with the presence of ester and carboxylic acid parts, accounted for all of the available double bond equivalent.

Further analysis of the HMBC spectrum revealed several diagnostic correlations (Fig. 2). The HMBC correlations H-2''/C-1 and H-6''/C-1 as well as H-2/C-1'' established that the mono-substituted 3,4-catechol was attached at the C-1 position of the 1,2,3-trisubstituted dihydronaphthalene-6,7-diol. The HMBC correlations H-2/C-9 and H-4/C-9 placed an ester carbonyl at C-3 of the 1,2,3-trisubstituted dihydronaphthalene-6,7-diol. The HMBC correlation H-1/C-10 was consistent with a carboxylic acid at the C-2 position, and hence established the substitution pattern of the 1,2,3-trisubstituted dihydronaphthalene-6,7-diol moiety as shown.

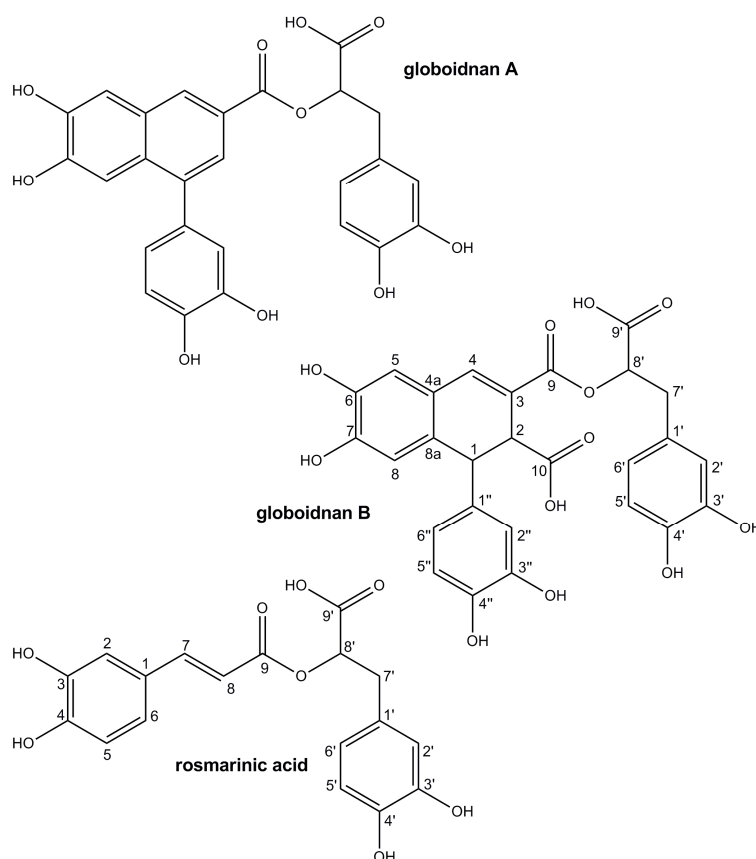
The point of attachment for the 3-(3,4-dihydroxyphenyl) lactic acid moiety to the 1,2,3-trisubstituted dihydronaphthalene-6,7-diol at C-3 was determined through the observed HMBC correlation H-8'/C-9 and confirmed by the NMR chemical shift data for C-8' ( $\delta_C$ : 74.0 ppm;  $\delta_H$ : 5.15 ppm), which was consistent with an ester methine. Finally, the remaining carboxylic acid functionality have to be attached at position C-8' as indicated by the HMBC correlation H-7'/C-9', leading to the proposed structure.

The relative stereochemistry of compound **1** was determined by ROESY experiments and by coupling constant analysis. The relationship between H-1 and H-2 is *trans* on the basis of the small  $J$  value (2.1 Hz) between these two protons [20,21]. This relationship was confirmed by the NOE correlations between H-2/H-2'' and H-2/H-6''. The stereochemistry of C-8' was deduced from comparison with literature [20,21]. This compound is a novel ester composed of (3,4-dihydroxyphenyl) lactic acid and 1-(3,4-dihydroxyphenyl)-6,7-dihydroxy-1,2-dihydronaphthalene-2,3-dicarboxylic acid, named globoidnan B. Interestingly, this is the first report of this type of cyclolignan from *Origanum* genus.



**Figure 2.** Selective HMBC correlations of Globoidnan B (**1**).

Rosmarinic acid (RA) (**2**), peak  $t_R = 24.4$  min, 188 mg/100g fresh weight (FW), was obtained as a white amorphous powder, UV(MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 290 (3.32), 328 (3.42) nm (Fig. 3). The molecular formula  $C_{18}H_{16}O_8$  was deduced from a quasi-molecular ion peak in the ESI-HRMS mass spectrum at  $m/z$  359.0768  $[M-H]^-$  (required 359.0772 for  $C_{18}H_{15}O_8$ ). Rosmarinic acid was identified by its observed MS, HRMS,  $^1H$ - and  $^{13}C$ -NMR data (Table 1) and by comparison with the literature [21,22].



**Figure 3.** Structure of Globoidnan B (**1**), Rosmarinic acid (**2**) and Globoidnan A (**3**).

Globoidnan A (**3**),  $t_R = 48.5$  min, 120 mg/100g FW, was obtained as a brown amorphous powder, UV(MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 221 (4.12), 358 (3.56) nm. The molecular formula  $C_{26}H_{20}O_{10}$  was deduced from a quasi-molecular ion peak in the ESI-HRMS mass spectrum at  $m/z$  491.0983  $[M-H]^-$  (required 491.0984 for  $C_{26}H_{19}O_{10}$ ). The  $^1H$ - and  $^{13}C$ -NMR data are summarized in Table 1. The NMR data were found to be very similar to Globoidnan B, except for the naphthalene moiety. COSY and HMBC correlations identified resonances consistent with a 1, 3-disubstituted naphthalene -6,7-diol moiety ( $\delta_c$ :

149.3, 145.5, 139.5, 131.1, 128.9, 127.0, 124.9, 123.9, 112.1, 108.9 ppm;  $\delta_{\text{H}}$ : 8.33, 7.70, 7.45, 7.36 ppm) as well as a mono-substituted 3,4-catechol and a 3-(3-4-dihydroxyphenyl) lactic acid moiety.

HMBC correlations H-2''/C-1 and H-6''/C-1 as well as H-2/C-1'' established that the monosubstituted 3,4-catechol was attached at the C-1 position, and the HMBC correlations H-2/C-9 and H-4/C-9 placed an ester carbonyl at C-3 of the 1,3-disubstituted naphthalene -6,7-diol. Finally, the HMBC correlation H-8'/C-9 indicated the attachment point between the 1,3-disubstituted naphthalene-6,7-diol and the 3-(3-4-dihydroxyphenyl) lactic acid moieties, leading to the proposed structure of globoidnan A. While this cyclolignan has been previously reported in *Eucalyptus globoidea* [23], this is the first report in the *Origanum* genus to our knowledge.

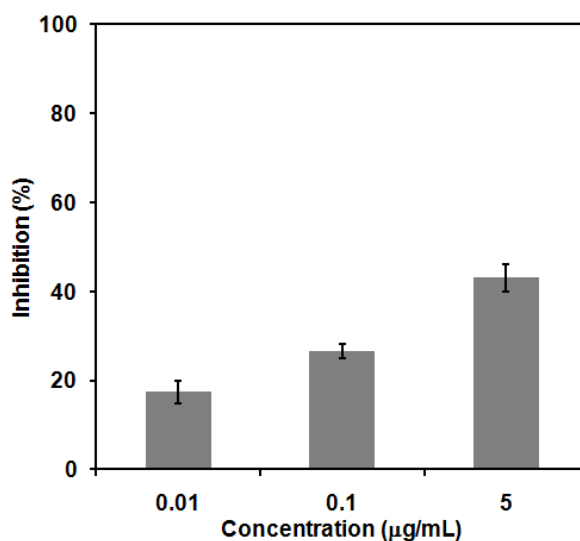
**Table 1.**  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data of Globoidnan B (1), Rosmarinic acid (2) and Globoidnan A (3).

C	1, $\delta_{\text{C}}$	1, $\delta_{\text{H}}$ , J(Hz)	2, $\delta_{\text{C}}$	2, $\delta_{\text{H}}$ , J(Hz)	3, $\delta_{\text{C}}$	3, $\delta_{\text{H}}$ , J(Hz)
1	45.9	4.49 <i>d</i> (2.1)	126.6	-	139.5	-
2	47.6	3.92 <i>d</i> (2.1)	114.1	7.17 <i>d</i> (2.1)	123.9	7.70 <i>d</i> (1.8)
3	122.6	-	145.3	-	127.0	-
4	138.7	7.61 <i>s</i>	147.8	-	128.9	8.33 <i>d</i> (1.8)
4a	124.4	-			124.9	-
5	116.7	6.95 <i>s</i>	115.1	6.86 <i>d</i> (8.1)	112.1	7.45 <i>s</i>
6	148.3	-	121.8	7.05 <i>dd</i> (2.1, 8.1)	149.3	-
7	144.7	-	145.8	7.56 <i>d</i> (16.0)	145.5	-
8	116.8	6.67 <i>s</i>	113.9	6.31 <i>d</i> (16.0)	108.9	7.36 <i>s</i>
8a	131.5	-			131.1	-
9	166.5	-	165.6	-	166.6	-
10	173.2	-				
1'	128.6	-	128.1	-	128.9	-
2'	117.2	6.82 <i>d</i> (2.0)	116.3	6.85 <i>d</i> (2.1)	117.2	6.94 <i>d</i> (2.0)
3'	145.3	-	144.6	-	145.5	-
4'	144.5	-	143.7	-	144.6	-
5'	115.7	6.73 <i>d</i> (8.1)	114.9	6.75 <i>d</i> (8.1)	115.8	6.79 <i>d</i> (8.1)
6'	121.6	6.64 <i>dd</i> (2.0, 8.1)	120.6	6.67 <i>dd</i> (2.1, 8.1)	121.6	6.76 <i>dd</i> (2.0, 8.1)
7'	37.4	3.05 <i>dd</i> (7.2, 14.4)	36.7	3.03 <i>dd</i> (8.5, 14.4)	37.5	3.17 <i>dd</i> (8.5, 14.4)
		3.07 <i>dd</i> (5.2, 14.4)		3.13 <i>dd</i> (4.3, 14.4)		3.24 <i>dd</i> (4.3, 14.4)
8'	74.0	5.15 <i>dd</i> (5.3, 7.2)	73.0	5.22 <i>dd</i> (4.3, 8.5)	73.9	5.39 <i>dd</i> (4.3, 8.5)
9'	170.0	-	170.3	-	171.2	-
1''	136.0	-			133.0	-
2''	115.2	6.50 <i>d</i> (2.2)			117.6	6.99 <i>d</i> (2.1)
3''	145.3	-			145.4	-
4''	145.0	-			145.4	-
5''	115.7	6.68 <i>d</i> (8.1)			116.0	7.00 <i>d</i> (8.0)
6''	119.3	6.45 <i>dd</i> (2.2, 8.1)			128.9	6.82 <i>dd</i> (2.1, 8.0)

### 3.2. Neuroprotective activity of *O. glandulosum* extract

Given the central importance of A $\beta$  aggregation in the pathogenesis of AD, particular interest is currently being shown in research aimed at therapeutic developments that target amyloid production, aggregation, clearance and toxicity. Reports that describe the effects of polyphenols on Alzheimer's A $\beta$  aggregation *in vitro* are already available. Using a routine *in vitro* assay based on UV-visible measurements and electron microscopy, we recently reported that resveratrol derivatives inhibit the aggregation of the peptide [19]. For this assay we used the A $\beta$ <sub>25-35</sub> peptide fragment that preserves the properties of neurotoxicity and aggregation of the entire peptide [24]. Using this method, the activity of the EtOAc extract of *Origanum glandulosum* was examined. The *O. glandulosum* EtOAc was tested at concentrations between 0.01 to 5  $\mu\text{g/mL}$ . The extract reduced A $\beta$  fibril formation in a dose-

dependent manner (Fig. 4). The inhibitory properties of major phenolic constituents were evaluated to evaluate their influence in the anti-aggregative properties of the global extract.



**Figure 4.** Effect of ethyl acetate (EtOAc) extract of *Origanum glandulosum* on A $\beta$ <sub>25-35</sub> fibril inhibition. Reaction mixtures containing 100  $\mu$ M A $\beta$ <sub>25-35</sub>, 10 mM phosphate buffer, pH 7.2, and various concentrations of EtOAc extract were incubated at 15°C for 5 h. Means and SD of three independent experiments are shown.

### 3.3. Neuroprotective activity of major compounds

To evaluate the effect of the major phenolic compounds of the extract, a primary evaluation of the inhibition percentage of A $\beta$  fibril formation was performed at a concentration of 10  $\mu$ M. Its inhibitory effect was compared to that of curcumin as a standard [25]. The compounds exhibiting an inhibitory activity at least equal to that of curcumin were further analyzed to determine their IC<sub>50</sub> values. The inhibition percentages and the IC<sub>50</sub> values are summarized in Table 2.

**Table 2.** Inhibition of A $\beta$ <sub>25-35</sub> fibrils formation.

Compound	Inhibition (%)	IC <sub>50</sub> ( $\mu$ M)
Curcumin	43 $\pm$ 3	10 $\pm$ 2
Globoidnan A	40 $\pm$ 4	12 $\pm$ 2
Globoidnan B	20 $\pm$ 3	-
Rosmarinic acid	53 $\pm$ 4	7 $\pm$ 2

Globoidnan A (IC<sub>50</sub> 12.0  $\mu$ M) and RA (IC<sub>50</sub> 7.0  $\mu$ M) exhibited efficient inhibition of A $\beta$  aggregation. Both exhibited inhibitory activity in the same order of magnitude as curcumin. These results are in agreement with those of Ono *et al.* indicating that curcumin, nordihydroguaiaretic acid and RA have similar anti-amyloidogenic activity [13]. In contrast, globoidnan B is less active. The activity of globoidnan B is two-fold lower than that of globoidnan A. Globoidnan A differs from B only by the presence of carboxylic acid, indicating a specific interaction between globoidnan A and A $\beta$ .

RA is an ester of caffeic acid 3,4-dihydroxyphenyllactic acid and is commonly found in species of the Lamiaceae. It has several biological activities such as antioxidant, anti-inflammatory, antimutagenic, antibacterial, and antiviral [11]. In AD pathogenesis, numerous reports indicate that RA can modulate various processes. It reduces A $\beta$ -induced neurotoxicity in PC12 cells including reactive oxygen species formation, lipid peroxidation, DNA fragmentation, caspase-3 activation, and tau ( $\tau$ ) protein hyperphosphorylation [26], and inhibits fibril formation [13, 18]. Globoidnan A is a cyclolignan found for the first time in the *Origanum* genus in this study. It was first isolated from *Eucalyptus globoidea* and reported to inhibit HIV integrase [23]. Our results show its ability to inhibit the progress of A $\beta$  aggregation to a degree almost comparable to that of curcumin.

RA and globoidnan A are the main compounds responsible for the results obtained in this work. It is very difficult to compare the inhibition activities of the pure compounds with that of the whole extract. Taking HPLC analysis into account, a quantification of the compounds within the extract indicates that RA, globoidnans A and B represent 23, 15 and 26% respectively of the whole extract. The highest tested concentration of the extract is 5  $\mu$ M (Inhibition 45%) which corresponds to 0.4, 0.4 and 0.7  $\mu$ g/mL of RA, globoidnans A and B, respectively in the extract. Pure compound were tested at 10  $\mu$ M. This concentration corresponds to 4.9  $\mu$ g/mL of globoidnan A (Inhibition 40%). Therefore, the inhibitory activity of the extract is more pronounced than that of the pure compound. This finding could be attributed to other compounds not extracted or to an additive/synergistic effect.

#### 4. Conclusions

To conclude, using 1D-and 2D NMR and MS analyses the major constituents of *O. glandulosum* extract were determined to be rosmarinic acid, a known lignan globoidnan A and a new lignan globoidnan B. Rosmarinic acid is prominent constituent of *Origanum* species. However, this is the first report of the presence of cyclolignans in this genus. Although globoidnan A has been previously identified in *Eucalyptus globoidna*, globoidnan B is a new cyclolignan derivative isolated from nature for the first time in this study. Interestingly the present findings suggest that *O. glandulosum* extract and constituents inhibit A $\beta$  fibrils formation. The high concentration of RA and cyclolignan derivatives may at least partially explain the anti-aggregative potential of the extract as proved by our experimental results.

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