# NATURAL PRODUCTS AS SOURCES OF POTENTIAL ANTIAMYLOIDOGENIC AGENTS

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***Abstract*— Natural products have played a dominant role in the discovery of leads for the development of drugs aimed at the treatment of human diseases. Moreover, they may serve as lead compounds for the synthesis of potential therapeutic agents against several diseases. In this study, we present an integrated approach towards the evaluation of the antiamyloidogenic activity of isolated components from the stigmas of saffron, combining isolation of bioactive components from saffron, real-time *in vitro* screening for noncovalent association with A*β* by ESI MS and cell viability assays. This is a suitable approach for selecting compounds for the ensuing *in vivo* studies, and it may provide insights into the design and synthesis of novel compounds for the prevention, or treatment of Alzheimer's Disease (AD).**

***Keywords: Natural products, Alzheimer's Disease, beta amyloid peptide, electrospray tandem mass spectrometry***

## I. INTRODUCTION

Natural products have played a dominant role in the discovery of leads for the development of drugs aimed at the treatment of human diseases. In addition, *Mother Nature* has provided an inspiration to organic and medicinal chemists to devise ingenious syntheses that are structural mimics of natural compounds, which may serve as potential therapeutic agents against several diseases, such as cancer and neurodegenerative diseases. Neurodegenerative diseases are progressive diseases that produce important health-related direct and indirect cost to the world. Alzheimer's disease (AD) is the most common cause of senile dementia in our ageing society, and it is characterized by a gradual and harmful decline in cognitive and non-cognitive function. AD is the fourth main cause of death affecting more than 35 million people worldwide whereas it is projected to almost quadruple by 2050 [1]. Deaths from AD have increased 68 percent between 2000 and 2010, while those from other major diseases, including heart disease, stroke and HIV continue to experience significant declines. In addition to the human suffering caused by the disease, AD is creating an enormous strain on the health care system, families and the country's budget.

The direct and indirect economic cost associated with the disease in 2010 was estimated at more than $600 billion worldwide [2]. Estimated costs include direct medical care and social support costs, as well as indirect costs, such as home-based long-term care services, and the value of lost economic productivity in AD patients. The prevalence of dementia increases strongly with age and it is projected that the costs of dementia could more than double by 2040 as the nation's population continues to grow older, assuming that the age-specific occurrence rate of the disease remains constant [2]. The constantly rising cost of caring for people with AD and other dementias has emphasized the immediate need to halt and reverse this trend.

The pathogenesis of AD has not yet been clarified and the understanding of the disease mechanism remains elusive. Nevertheless, it is widely believed that abnormal accumulation and aggregation of disease-specific proteins lead to neurodegeneration. It has been proposed that the beta amyloid peptide (A*β*), abnormal tau protein or probably both play critical role in the development of the disease. In particular, abnormal accumulation and aggregation of the A*β* peptide lead to the formation and cerebral deposition of amyloid plaques, whereas aggregation of hyper-phosphorylated tau protein results in neurofibrillary tangles (NFTs). These senile plaques and neurofibrillary tangles have been recognized as the two main pathological hallmarks of AD. These changes are also associated with increased levels of oxidative stress, inflammation, and nerve cell death [3]. Even though the correlation between protein aggregation

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and nervous system degeneration is mostly unknown, these disease-specific aggregated proteins and peptides have apparent diagnostic and even therapeutic implications [4]. Despite the immense research efforts that have been put over the past years on the characterization of AD and the development of disease-modifying therapeutic approaches, there is still *no cure* for AD. Moreover, initiation of AD pathology is estimated to start several (10-15) years prior to the onset of clinical symptoms; thus, making imperative the discovery of diagnostic markers for AD [5] as the early symptoms of AD are rather subtle.

The major proteinaceous component found in post mortem analyzed plaques is the A$\beta$ peptide, mainly in the A$\beta$1-40 and A$\beta$1-42 forms, which is a cleavage product of the precursor protein, Amyloid Precursor Protein (APP). In the process of amyloidosis, the A$\beta$ peptide misfolds and aggregates to form an initial nucleus, comprising of a small number of A$\beta$ molecules, followed by a rapid elongation stage after which incorporates new A$\beta$ molecules. The presumed central role of the amyloid plaques in AD pathogenesis led to the development of the *amyloid cascade hypothesis* [6]. In fact, A$\beta$ oligomers can either lead to neuronal death caused by the toxicity of insoluble amyloid fibrils [6], and/or disruption of synaptic function and memory failure caused by small soluble A$\beta$ oligomers [7]. Among the proposed underlying mechanisms to justify A$\beta$'s neurotoxicity, oxidative stress and neuroinflammation have been credited as the principal pathways of neurodegeneration [8,9]. In view of the suggested mechanistic link between oxidative stress, inflammation and neurodegeneration [10], neuroprotection by plant-derived and dietary antioxidants may offer a motivating therapeutic route for protection against the risk of AD [11-13]. In particular, plants from the Mediterranean basin (a global biodiversity "hot-spot" [14], in which only the Southern part of Greece offers 6,000 plants species and 1,200 endemic [15]) are worth to be investigated.

In this study, we present an integrated approach towards the evaluation of the antiamyloidogenic activity of isolated components from the stigmas of saffron (*Crocus sativus* L.), which could lead to novel aggregation inhibitors for the prevention, or treatment of AD. Numerous health-promoting properties of saffron have been reported such as antidepressant [16,17], anti-inflammatory [18], antiproliferative action towards specific cancer cell-lines [19,20], and reducing blood pressure effects [21]. We employed novel nano electrospray ionization (ESI) mass spectrometry (MS)-based methodologies to assess the noncovalent interactions between the A$\beta$ peptide and natural products derived from *Crocus sativus* L. ESI MS (developed by John Fenn; 2002 Nobel prize in Chemistry) is an ideal method to elucidate macromolecular structures and study their interactions with small molecules or other macromolecules [22]. That, in turn, may shed some light towards understanding their biological function and developing new therapeutics [23]. ESI MS was used for the screening of several bioactive compounds in terms of binding to A$\beta$, by noncovalent interactions, and the study of their relative binding strength. This *in vitro* screening was complemented with cell viability assays using differentiated neuronal SH-SY5Y cells to assess any potential toxic effects of the selected substances. The detection of the noncovalent interactions between the A$\beta$ peptide and the natural product/ligand could provide some insights into the mechanisms of AD pathology, as well as the identification of potential anti-amyloidogenic agents that can be employed towards prevention or even treatment of AD.

## II. MATERIALS AND METHODS

### A. Sample Preparation for ESI MS Screening

An aliquot of a freshly prepared A$\beta$ (1-40) 50 μM solution (average $M_r$ 4329.9; Bachem AG, Bubendorf, Switzerland) in deionized water was mixed with equimolar aqueous solution of the ligand (50 μM). Oleuropein (OE) ($M_r$ monoisotopic: 540.18) was isolated from olive leaves of *Olea europaea* (var. koroneiki), according to a previously described procedure [24], and was generously donated from Professor Skaltsounis Laboratory, School of Pharmacy, National and Kapodistrian University of Athens. The purity was checked by HPLC and NMR and was greater than 99%. Major biologically active components of *Crocus sativus* L. were extracted, separated and isolated from dried stigmas of saffron flowers provided by Cooperative De Safran Krokos (Kozani, Greece) by semi-preparative HPLC as previously described [25]. Mass spectral analysis was carried out on a Waters Premier quadrupole reflectron time-of-flight (QqTOF) instrument equipped with an ESI source in the positive ion mode. Accurate mass measurements of the crocus-derived bioactive components was performed on-line on the QqTOF high-resolution MS using the leukine-enkephaline standard as a lock mass. Structure characterization of the crocus-isolated components was carried out by ESI MS in combination with low-energy CID/tandem MS of the sodiated $[M+Na]^+$ molecular ions on the QqTOF and/or a Bruker 3D-ion trap ($MS^n$, n= 2,3) instrument as previously described [25].

### B. Cell Viability Assays

To assess the effects of the selected substances on neuronal cells, the human neuroblastoma derived SH-SY5Y cell line was selected. *In vitro* viability assays of the compounds of interest were performed using the differentiated neuronal SH-SY5Y cells to determine any potential toxic effects of the selected substances. Upon differentiation to neurons, following 6-day exposure to 10μM retinoic acid, they were treated with a range of different concentrations (1 μM to 1mM) of each selected substance. Different incubation times were assessed for each concentration (24, 48 and 72 hours). Since the selected substances were dissolved in DMSO, and given its potential effect on cell viability, all measurements were normalized to those of differentiated SH-SY5Y cells exposed for the same time period to the same concentrations of DMSO, in the absence of a test substance. The successful differentiation to neurons was confirmed by western blotting and immunofluorescence using antibodies against the neuronal markers MAP2, TUJ1 and NEUN. The effect of the selected substances on differentiated SH-SY5Y cell viability was assessed using the WST-1 assay. For statistical purposes all samples were performed in biological triplicates.

## III. RESULTS AND DISCUSSION

### *A. Extraction, Isolation, Scale up Process and Structure Determination Steps*

Chemical analysis of *Crocus sativus* L. stigmas has shown the presence of unusually polar carotenoids (crocins), picrocrocin and safranal. In the characterization procedure of the crocus-derived active components, we have used dried stigmas of saffron flowers exhibiting a moisture level of 10-12 %. Extraction of stigmas has been conducted simply by stirring dried herb (50 mg) with 10 mL $CH_3OH/H_2O$ (50%, v/v), at ambient temperature in the dark for 24 h, as previously reported [25]. The extracts have been analyzed and quantitated by high performance liquid chromatography (HPLC) on a C8 reversed phase column (3μm, 150 mm x 2.1 mm) employing a gradient elution program of acetonitrile and 0.05 % aqueous TFA. Saffron contains a range of water-soluble carotenoids, the so-called crocins that are glucosyl esters of crocetin. The crocins of the crude extract have been separated and isolated by semi-preparative HPLC and Centrifugation Partition Chromatography (CPC). The semi-preparative HPLC system was equipped with a C18 reversed phase column and the isolation of crocins was carried out with a gradient elution program of $H_2O$/acetonitrile, whereas detection of the eluted crocin components was performed at the wavelengths of 250, 325 and 440 nm. Alternatively, CPC and/or Accelerated Solvent Extraction (ASE) was also employed to purify the major and some of the minor crocins on a preparative scale since large amounts of the pure compounds (crocins) are required in order to evaluate the pharmacological activities. The purity of all isolated crocus-derived compounds prior to scale up process has been checked by both high-resolution MS and NMR analysis.

Generally, crocins are glycosyl esters of crocetin a water-soluble carotenoid dicarboxylic acid. Crocetin mainly occurs in *all-trans* form however the *cis*-isomers have been also identified. Likewise, crocins could be divided according to the number and the position of the β-L-glucopyranosides attached to the central carotenoid unit, comprising crocetin mono- and bis-ester glycoside compounds with R1 and R2 substituents being mono- (G), bi- (GB) and tri-saccharide (GT and NP) moieties [25] (**Figure 1**). For the structural elucidation of glycosylated crocetins (i.e., crocins) from the stigmas of saffron (*Crocus sativus* L.) six derivatives containing one up to five glucosyl residues with maximum number of three glucoses per glycosylation site (glucose, gentiobiose, gentiotriose and neapolitanose) were isolated and purified by semi-preparative HPLC.

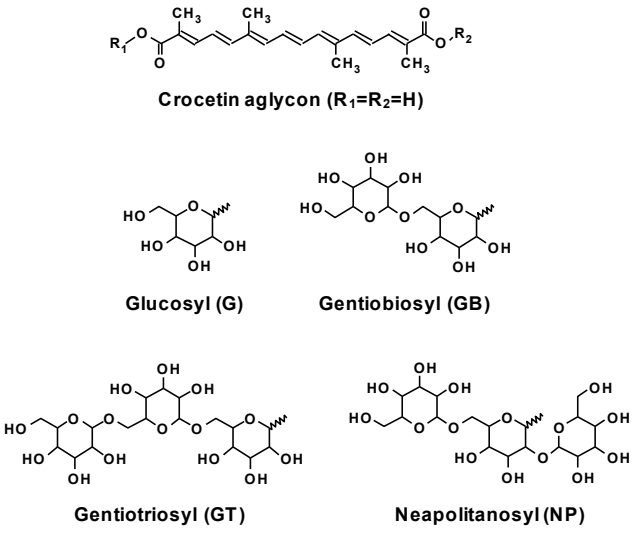


**Figure 1**. Chemical structures of selected crocin ester glycosides isolated from the stigmas of *Crocus sativus* L. The variable substituents $R_1$ and $R_2$ on the crocetin refer to the mono-, bi- and tri-saccharide moieties G, GB, GT and NP.

The major *Crocus sativus* L.-derived bioactive constituents are crocetin mono- and bis-ester glycoside compounds, containing mono- and bi-saccharides (crocins). ESI MS was employed in combination with low-energy CID/tandem MS of the sodiated $[M+Na]^+$ crocin precursor ions utilizing the QqTOF MS and/or a 3D-ion trap ($MS^n$, n= 2,3) instrument [25]. This work enabled the structural elucidation of the aforementioned glycoconjugates by multi-stage MS and focused on the identification of new crocins in saffron with special emphasis on the differentiation of positional crocetin glycoside isomers. The latter was especially important in distinguishing between the two different penta-glucosylated crocetins containing the isomeric trisaccharides gentiotriose (GT) and neapolitanose (NP) by CID-$MS^3$ analysis.

### *B. ESI MS Screening Experiments*

The formation of 1:1 noncovalent complexes of A*β* with certain antioxidants such as oleuropein (OE) and melatonin (M) has been previously demonstrated by ESI MS [26,27], whereas their interaction with the hydrophobic region of the peptide has been reported by ESI MS mapping studies [28]. Similar 1:1 noncovalent interactions between the A*β* peptide and the main crocus components were also observed in this study, as shown in the deconvoluted ESI mass spectrum of A*β* and the bi-saccharide containing crocin TC2 (**Figure 2).** Nevertheless, it should be noted that the observation of these noncovalent interactions was only possible only after careful optimization of the experimental parameters especially in terms of the entrance potential in the MS ion source, the ligand concentration, the pH and the presence of organic modifiers in the solution, which affect conformational changes of the A*β* peptide.

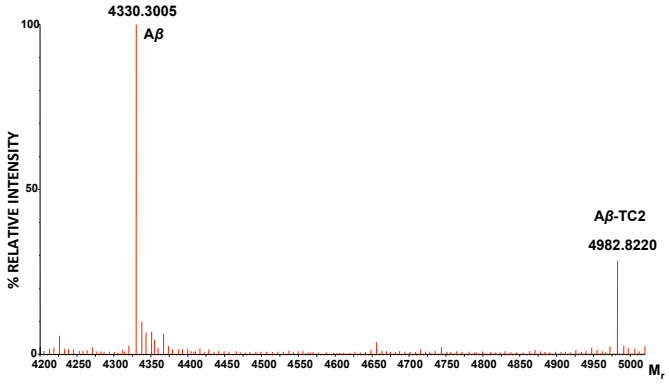


**Figure 2**. The deconvoluted ESI mass spectrum of the A*β*–TC2 noncovalent complex acquired on a QqTOF mass spectrometer.

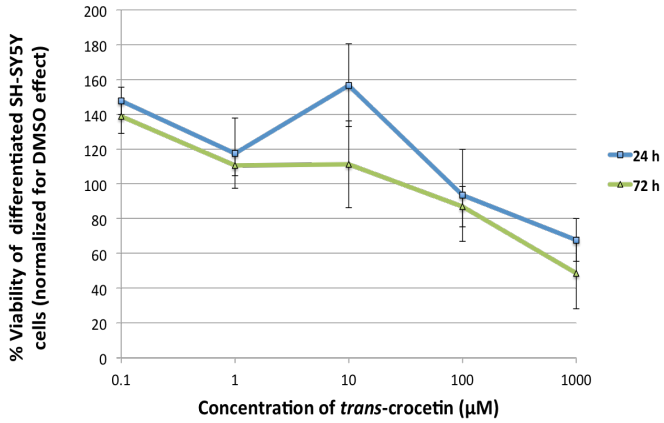


**Figure 3**. Percent viability of differentiated SH-SY5Y cells following exposure to different concentrations of *trans*-crocetin. Since *trans*-crocetin is diluted in DMSO the measurements were normalized to the viability of differentiated SH-SY5Y cells exposed to the same concentration of DMSO alone.

The noncovalent complexes of A*β* with OE and crocins showed considerable stability even under experimental conditions, which usually do not favor noncovalent interactions, e.g., high organic modifier content and elevated entrance potential. The specificity of these noncovalent interactions was also evaluated at low concentration levels, i.e., 5-100 μM, where the occurrence of nonspecific aggregation in the gas-phase can be prevented. For example, the ESI signals corresponding to the noncovalent complex of A*β* with OE and crocins were present for all concentration levels, thus indicating a very specific interaction. On the contrary, ESI MS analysis of the A*β* – quercetin glycoside solution yielded no signals corresponding to the formation of a noncovalent complex [13]; thus showing the specificity of the interactions of the A*β* peptide with OE, M and the *Crocus sativus* L.- derived components.

In another study, the binding strength of the aforementioned interactions was assessed by conducting ESI tandem MS experiments. In this study the +5 charged ion of the noncovalent complex was selected and subjected to collision-induced dissociation in the QqTOF mass spectrometer. It was clearly shown that it takes more energy to dissociate the A*β* signal with OE and crocins than that of the A*β* with M and *trans*-crocetin, thus demonstrating the higher binding strength of the former noncovalent interactions over the latter.

### C. Cell Viability Assays

At the cellular level, OE and *trans*-crocetin did not appear to have a toxic effect at concentrations up to 10μM. Similar results were obtained at both 24 and 72 hours of incubation with each substance. It should be noted that *trans*-crocetin appear to modestly enhance cell proliferation at 24 hours of incubation with concentrations between 0.1 and 10 μM. Cell viability appears to be compromised for higher concentrations, especially during prolonged incubation time as shown for *trans*-crocetin (**Figure 3**).

Following these preliminary cell viability studies, PC12 cells producing hyper-phosphorylated tau along with PC12 wild type will be exposed to different concentrations of the selected compounds and cell proliferation/viability assays will be performed. Thus *in vitro* scanning of the compounds of interest towards the inhibition of APP misprocessing and A*β* generation, as well as the abnormal tau hyper-phosphorylation will be performed. Therefore, the potential inhibitory role of the crocus-isolated compounds on A*β* production and key molecules of APP misprocessing (e.g., C99, sAPPβ), as well as enzymes that regulate APP sequential cleavage (e.g., BACE-1, presenilin and nicastrin) and tau hyper-phosphorylation will be monitored.

## IV. CONCLUSION

This work demonstrates that natural products may be effective in inhibiting A*β* fibrillogenesis without limiting neuronal cell viability at low concentrations. This integrated strategy combining isolation of bioactive components from saffron, real-time *in vitro* screening for noncovalent association with A*β* by ESI MS and cell viability assays is a suitable approach for selecting compounds for the ensuing *in vivo* studies. In addition, this methodology may shed some light into the AD pathogenesis. Finally, these results may provide insights into the design and synthesis of novel compounds, which could act as protective or even therapeutic agents against AD. These natural products and derivatives thereof can be eventually exploited, in the form of nutraceuticals towards the prevention and/or treatment of AD. The potential use of nutraceuticals will also have a positive impact to preserve and enhance the environment and natural resources, and it will provide a stimulus for extensive cultivation of some of these plants in the originating countries.

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