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Acetylcholinesterase Inhibitory and Antioxidant Properties of *Euphorbia characias* Latex

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Abstract: The aim of the present study was to evaluate the acetylcholinesterase inhibitory capacity and the antioxidant properties of extracts of *Euphorbia characias* latex, a Mediterranean shrub. We performed a new extraction method involving the use of the trichloroacetic acid. The extract showed high antioxidant activity, was rich in total polyphenolic and flavonoid content and exhibited substantial inhibition of acetylcholinesterase activity.

Keywords: *Euphorbia characias*; acetylcholinesterase inhibitors; antioxidants; flavonoids; free radical-scavenging; polyphenols.

1. Introduction

During the last twenty years antioxidants have gained an importance in foods and drug industries due to the action of antioxidant molecules on oxidative damages and, consequently, on various deseases. It is well known that free radicals are one of the causes of several pathological conditions including Parkinson's and Alzheimer's disease. Moreover, the use of antioxidants in association with inhibitors of acetylcholinesterase (AChE), the fundamental enzyme in the breakdown of acetylcholine, is considered one of the most promising approaches for Alzheimer's disease treatment [1 and references therein].

Many studies have been issued on natural antioxidants and it is therefore very difficult for us to quote them all. As examples, we can cite a review of the most important ones [2 and References therein].

Plants are considered as natural antioxidant resources and antioxidant molecules have been well characterized in the latex of some plants, e.g. *Ficus carica*, the common fig [3]. Latex, a milk sap, is contained inside the laticifers, specialized elongated cells or vessel–like series of cells that permeate various aerial tissues of the plant [4]. Latex is an emulsion with a diversified composition, including alkaloids, terpenoid and phenolic compounds, polymeric substances such as resins and gums, starch, oils, and a large number of proteins and enzymatic activities [4, 5]. Latex provides an important

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contribution to plant defense mechanisms through repelling insects, controlling the growth of microbial phytopathogens and producing toxic effects on herbivores [4, 6].

A large number of phytochemical compounds, isolated from Euphorbiaceae, have been well characterized [7]. Natural antioxidants have been recently found in the latex of some Euphorbiaceae [8-11], and we have selected the Mediterranean spurge *Euphorbia characias*, occurring in vast areas of the Mediterranean basin, as an experimental model to study the complexity of plant latex chemistry. In Sardinia (Italy), *E. characias*, called "lua" in local language, grows luxuriously in various habitats like rocky hillsides, open woods, and around the rivers. This spurge was used as a laxative and, due to its toxicity, by poachers to kill fishes on the rivers.

E. characias is known to contain several biological active compounds as polycyclic diterpenoids [12], bicyclic diterpenes [13], tocopherols, fatty acids and sterols [14]. Moreover, several papers report on the presence, in *E. characias* latex, of proteins that, acting as antioxidant enzymes, could interact with other latex substances to assure some kinds of plant protection against invading pathogens and/or against environmental stresses [15].

The goal of the present work is to explore the antioxidant and acetylcholinesterase inhibitory activities in the latex of *E. characias* to contribute to the knowledge of this plant product that might represent a potential source of natural molecules for treatment of some diseases.

2. Results and Discussion

In this paper we describe a new procedure developed for the extraction of both antioxidant and AChE inhibitory activities from *Euphorbia* latex involving the use of trichloroacetic acid. This procedure was compared to three common extraction methods which make use of organic solvents such as methanol (MeOH), ethanol (EtOH), and petroleum ether/methanol (PE/MeOH).

Total free radicals were determined with 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) assays, total polyphenol content by Folin-Ciocalteau and horseradish peroxidase (HRP) enzymatic method, total flavonoid content by aluminum nitrate method, and AChE activity inhibition by Ellman's method. All the material and the methods used are reported as Supporting Information.

Figure 1A shows the content of free radical-scavenging molecules acting on the $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ radical signals. As observed, a lower amount of antioxidants was detected using $DPPH^{\bullet}$ instead of $ABTS^{+\bullet}$ in all of the four extracts. Moreover, in PE/MeOH extracts, the antioxidant activity was maximal until 3 h had elapsed in end-over-end apparatus, and slowly decreased as the incubation time increased (not shown). In MeOH and in EtOH extracts the maximal activity was seen after 10-12 h incubation.

Polyphenol content, in MeOH, EtOH, PE/MeOH, and TCA extracts, is reported on Figure 1B. A comparable amount of polyphenols was obtained in the four extracts using the Folin-Ciocalteau method, whereas the peroxidase enzymatic method showed that the polyphenol amount was, in MeOH and EtOH extracts, respectively 7 and 3 folds higher than that determined in PE/MeOH and TCA extracts. Figure 1C shows the flavonoid content. The QE value appeared similar in the MeOH, EtOH and TCA extracts and sensitively lower in PE/MeOH extract.

Euphorbia latex extracts exhibited acetylcholinesterase inhibitory activity. Figure 1D shows the content of AChE inhibitors in the four extracts expressed as galanthamine equivalent molarity. There were only few differences between the three extracts made by organic solvents and the activity in all the extracts was not incubation time dependent. The AChE inhibitor amount was approximately ten folds higher in TCA extract than in the other ones.All the obtained results are summarized on Table 1 (Supporting Information).

The GC/MS chromatographic profile of a TCA latex extract showed nine different phenolic compounds, analyzed as their stable TBDMS derivatives, and identified through the GC/MS chromatogram of a standard mixture (Figure 1 Supporting Information). Each molecule has been characterized by EI-MS and the spectral data are reported in Table 2 (Supporting Information). Due to the high number of hydroxyl groups, quercetin and myricetin were analyzed as TMS derivatives (Figure 2 Supporting Information) instead of TBDMS derivatives and the relative spectral data are reported in Table 2 (Supporting Information).

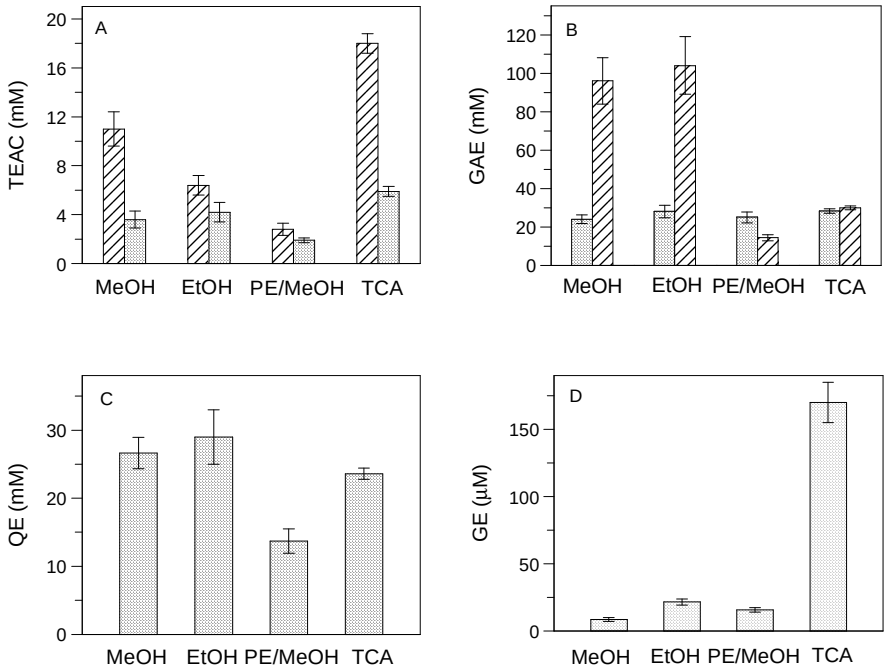

Figure 1. Free radical-scavenging, polyphenol, flavonoid, and AChE inhibitor content in four different extract from *E. characias* latex.

Panel A: Free radical-scavenging content is expressed as Trolox equivalent antioxidant capacity (TEAC) determined by ABTS (▨) and DPPH (▩) assays.
Panel B: Polyphenol amount is expressed as gallic acid equivalents (GAE). GAE is determined by Folin Ciocalteu (▩) and horseradish peroxidase (▨) methods.
Panel C: The amount of flavonoids is expressed as quercetin equivalents (QE).
Panel D: The amount of AChE inhibitor is expressed as galanthamine equivalent molarity (GE).
The reported values are the mean of triplicate experiments.

By comparison with the National Institute of Standards and Technology (NIST) spectral database, several other compounds were identified: 2-hydroxy-propanoic acid, 2,3-dihydroxy-propanoic acid, 3-hydroxy-propanoic acid, 2-hydroxy-3-methyl-butanoic acid, 4-hydroxy-butanoic acid, 2-hydroxy-esanoic acid, 3-phenylpropenoic acid, 3-hydroxy-3-phenylpropenoic acid.

Some conclusions are immediately obvious from the experimental findings. The use of TCA implies a rapid extraction instead of 3-12 h incubation time using MeOH, EtOH, and PE/MeOH.

There are significant differences in the four extraction methods in terms of content of the free radicals-scavenging molecules, and the highest value is obtained using TCA. When PE/MeOH is used, the antioxidant activity increases during the first three hours and slowly decreases as the incubation time increases. The time-incubation sensitivity of these antioxidants in PE/MeOH could be, in itself, not particularly surprising, but reporting this effect seems important since it might produce conflicting experimental results.

Using $DPPH^{\bullet}$ the free radical-scavenging content, compared with the results obtained by $ABTS^{\bullet+}$, was lower in all the four extracts. This might indicate a different sensitivity in the method, that was higher for $ABTS^{\bullet+}$ than $DPPH^{\bullet}$. Nevertheless, the amount of free radical scavenging detected using $DPPH^{\bullet}$, was higher in TCA extract than in the other ones.

Very few differences are seen in the polyphenol amount in all the four extracts when determined by Folin-Ciocalteau method. Surprisingly, the polyphenol amount in MeOH and in EtOH extracts, detected by HRP enzymatic method, was about 4-fold higher than the amount determined by the Folin-Ciocalteau method. This result was different to that obtained in PE/MeOH extract where the polyphenol content, detected by HRP enzymatic method, was about 2-fold slower than the amount detected by Folin-Ciocalteau method.

At the moment we do not know why there are high differences in the amount of polyphenols detected by the two methods. There is a clear obviousness that the use of a unique solvent as extraction method could lead to a misleading determination of antioxidants. It could be due to unspecific interferences depending on the solvent used. Thus, controversial results on the antioxidant determination could be due to a troublesome combination of several of the above drawbacks that could be easy solved using TCA, a fast and highly reproducible method. Moreover, TCA extract allows us to obtained the highest value in terms of AChE inhibitory activity.

GC-MS analysis confirms the presence in TCA extract of several compounds known as antioxidant molecules (compounds 2-9 in Figure 1 Supporting Information), two known flavonoids (Figure 2 Supporting Information), all the compounds identified by NIST (as above reported), precursor of acetylcholinesterase inhibitors (compounds 2, 6, 8, 9 in Figure 1 Supporting Information) and a true acetylcholinesterase inhibitor (compound 7 in Figure 1 Supporting Information) [16].

The results of this research indicate that *E. characias* latex exhibits antioxidant activities determined as total content of free-radical scavenging, polyphenol and flavonoid molecules and AChE inhibitory activities. Our results contribute to the knowledge of the components of this plant product. Therefore, the contemporary presence of antioxidant molecules and enzymatic proteins acting as antioxidants in *Euphorbia* latex leads us to hypothesize a single or a joined action of these important substances directly or indirectly involved in plant defense mechanisms.

An important approach to the treatment of Alzheimer disease is directed to the inhibition of AChE to enhance the acetylcholine level in the brain. Moreover, it is well known that free radicals are one of the causes of several diseases and, between these, Alzheimer type dementia. The contemporary presence in the *Euphorbia* latex of both an antiradical activity and the inhibitory effect of AChE might be due to the presence of a troublesome combination of phenolic compounds leading the TCA extracts a promising source for applications in the pharmaceutical field.

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