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Antiacetylcholinesterase Activitie of Sweet Orange (*Citrus Sinensis*) Essential Oil from Algeria

Hicham Boughendjioua[1,*], Abdarraouf Hafsa[1] and Alaeddine Kadach[1]

[1] *Department of Natural Sciences. High School Professors Technological Education, Skikda, 21000, Algeria.*

**Author to whom correspondence should be addressed: Email: boughendjioua.hicham@yahoo.com Mobile: +2136 99 25 93 37, Fax: +21338 95 96 84.*

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Abstract: Presently the search for new drugs from natural resources is of growing interest to the pharmaceutical industry. Natural products have been the source of new drugs since ancient times. Plants are a good source of secondary metabolites which have been found to have beneficial properties. The purpose of this research was to evaluate the chemical composition by gas chromatography coupled with mass spectrometry (GC/MS) and acetylcholinesterase inhibitory activity was also determined of sweet orange (*Citrus sinensis*) essential oil.
The main components of the essential oil were Limonene (89.547 %), β.-Myrcene (4.106 %), β.-Phellandrene (1.670 %), α.-Pinene (1.312 %).
The results of these studies clearly demonstrate that orange may represent a valuable source of new secondary metabolites with high diverse biological activities, in particular for finding new natural acetylcholinesterase AChE inhibitors, with an (IC_{50} = 35.07 ± 2.47 µg. mL^{-1}) for the essential oil compared to (IC_{50} = 6.27 ± 1.15 µg. mL^{-1}) for Galantamine.

Keywords: essential oil, *Citrus sinensis*, GC/MS, antiacetylcholinesterase.

I. Introduction

Alzheimer's disease (AD) is a multifactorial disease that affects a significant portion of the population and its incidence has grown over the years due to the increasing proportion of elderly people in the world population. Factors such as formation of senile plaques and neurofibrillary tangles, reduction of acetylcholine levels (by inhibiting the enzyme acetylcholinesterase) and oxidative phenomena are related to the development and/or progression of AD [01, 02]. The acetylcholinesterase (AChE), an enzyme inhibitor associated with AD, is widely detected by Ellman's test. According to the principle of the method of Ellman *et al*., (1961) [01], the reaction with the thiol has been shown to be suiciently rapid so as not to be rate limiting in the measurement of the enzyme and in the concentrations used does not inhibit the enzymatic hydrolysis [01, 02].
Some AChE inhibitors are found naturally in medicinal plants. Reversible cholinesterase inhibitors are currently used in clinical trials for treatment of Alzheimer's disease [03]. The treatment is based on the inhibition of AChE, which hydrolyzes acetylcholine, increasing their availability to cholinergic transmission [04].
The food industry is growing significantly every year. Annually, large quantities of fruits and vegetables are grown to meet the population demand. Among them, the orange (*Citrus sinensis*) production was 70.45 Mt in 2013. After consumption, the remaining orange peel is considered as a

waste. From an economic and environmental perspective, the extraction of orange essential oil is a high value-added option for the valorization and use of the orange peel [05].

II. Materials and Methods

II. 1. Plant materials

The plant material was collected in December 2017 from location on Azzaba, Skikda city (North-east of Algeria). The plant was taxonomically identified at Department of Natural Sciences, High School Professors Technological Education, Skikda, Algeria by Dr. Hicham Boughendjioua. Specie name is according to International Plant Name Index (IPNI).

II. 2. Isolation of the essential oil

Obtaining essential oil was carried out by cold expression (physical process), it's the simplest processes applied only to Citrus fruit [06], this extraction does not change the composition of the oil [07]. The product obtained is called gasoline, because it does not undergone any chemical modification [06, 08]. The obtained essential oil was stocked at 4 °C until further analyses.

II. 3. Chromatography Analysis

The GC-MS (Agilent 7890B) analysis was performed using a Hewlett Packard 5973-6800 system operating in EI mode (70 eV) equipped with a split/splitless injector (250 °C), a split ratio 1/50, using a fused silica HP-5 MS capillary column (30 m × 0.25 mm (i.d.), film thickness: 0.25 µm. The temperature program for the HP-5 MS column was from 50°C to 260 °C at a rate of 2 °C/min. Helium was used as a carrier gas at a flow rate of 0.5 ml/min. Injection volume of the sample was 0.2 µl. The identification of the components was conducted in an IS system managing a library of spectrum NIST11.L, The GC/MS analysis was performed at the Biotechnology Research Center (BRC), (Constantine, Algeria).

II. 4. Acetylcholinesterase activity

Acetylcholinesterase (AChE) activities were measured with a slight modification of the spectrophotometric method described by Ellman *et al*., (1961) [01].
AChE electric eel was used, while acetylthiocholine iodide was used as substrates. DTNB [5,5-dithio-bis (2-nitrobenzoic)] was used for the measurement of cholinesterase activity. Ethanol was used as a solvent to dissolve test compounds 0 and controls. Briefly, 150 µl of sodium phosphate buffer (100 Mm, pH 8.0), 10 µl of a sample solution dissolved in ethanol at different concentrations and a volume of 20 µl AChE (5.32 × 10-3 U) was mixed and incubated for 15 min at 250 °C, then 10 µl of DTNB (0.5 mM) were added. Then, the reaction was initiated by the addition of 20 µl of acetylthiocholine iodide (0.71 mM). The hydrolysis of these substrates was followed spectrophotometrically by the formation of a yellow color of the methyl 5-thio-2-nitrobenzoate anion, depending on the result of the DTNB reaction with thiocholine, released by the enzymatic hydrolysis of the acetylthiocholine iodide, respectively, at a wavelength of 412 nm, using a 96-well microplate reader (Spectra Max PC 340, Molecular Devices, USA). Measurements and calculations were evaluated using PRO Softmax v 5.2 software. Percent inhibition of AChE was determined by comparison of sample reaction rates against the control sample (ethanol in phosphate buffer, pH 8) using the formula (E-S) / E × 100 where E is the activity of the enzyme without a test sample, and S, is the activity of the enzyme with the test sample. The experiments were performed in triplicate. Galantamine was used as the reference compound.

$$\textbf{Inhibition (\%)} = A_{\text{controle}} - A_{\text{Huille essentielle}} / A_{\text{controle}} \times 100$$

III. Results
III. 1 Essential oil analysis

Sweet orange oil has a sweet, fresh and tangy smell, is yellow to orange in color and watery in viscosity. Qualitative and quantitative analysis by gas chromatography coupled with mass spectrometry (GC/MS) of the essential oil identified 115 compounds (**Table 1**). The essence of sweet orange (*Citrus sinensis*) consists mainly of (Principal constituents): Limonene (89.547%), β.-Myrcene (4.106%), β.-Phellandrene (1.670%), α.-Pinene (1.312%). totaling approximately 98.396%.
These results are in agreement with references that mentioned the percentage of limonene as 90% in the peels of *Citrus sinensis* [09].

Table 1. *Chemical composition of sweet orange (Citrus sinensis) essential oil (Principal constituents).*

No.	Compounds	Retention time (min)	%
1.	**α.-Pinene**	11.053	**1.312%**
2.	**β.-Phellandrene**	13.797	**1.670%**
3.	**β.-Myrcene**	15.299	**4.106%**
4.	α.-Phellandrene	16.090	0.165%
5.	3-Carene	16.405	0.210%
6.	**Limonene**	18.405	**89.547%**
7.	δ.-Terpinene	20.329	0.239%
8.	Cyclooctane	21.388	0.159%
9.	1,6-Octadien-3-ol	23.415	0.257%
10.	α.-Terpineol	29.840	0.352%
11.	Decanal	31.178	0.251%
12.	Naphthalene	49.899	0.128%
	Total		**98.396**

In literature some reports were found on the composition of Citrus peel essential oils all over the world.

The composition of the laboratory-prepared Turkish bitter orange oil (*Citrus aurantium* L.) samples (bitter orange fruits cultivated two different area) were studied. The volatile fraction was analyzed by GC and GC/MS, and 29 components were identified in the oil. The results obtained were compared with those in the literature for Italian and Spanish bitter orange oil. The identified components were grouped into six classes (monoterpene hydrocarbons, sesquiterpenes, oxygenated compounds, carbonyl compounds, alcohols, and esters). Variations in the oil composition relative to the fruit provenance, the percentage of each component and six classes, were also described. It was found that the oil composition varied according to the fruit provenance. The main components of the oil were limonene (93.68-94.32%), myrcene (1.73-1.86%), linalyl acetate (1.17-1.32%), linalool (0.33-0.46%), (β-pinene (0.40-0.57%) and α-pinene (0.39-0.45%) [10].

The chemical composition of cold-pressed peel essential oils of common sweet oranges (*Citrus sinensis* L. Osbeck) from Uganda and Rwanda were analyzed by GC and GC-MS and a total of 51 and 55 volatile chemical components were identified respectively. The major chemical groups were monoterpene hydrocarbons (94.4 and 97.3%), terpene alcohols (1.4 and 1.0%), aliphatic aldehydes (1.6 and 0.8%) and terpene aldehydes (1.4% and trace). The main compounds were limonene; 87.9 and 92.5 %, myrcene; 2.4 and 2.0%, α-pinene; 0.5 and 2.4%, linalool; 1.2 and 0.9%, octanal; 1.3 and 0.6% and decanal, 0.2%. Some of the compounds present in the Ugandan oil, including iso-amyl acetate, iso-safrole, iso-amyl isovalerate, (E)-sabinene hydrate and methyl caprate were absent in Rwandan oil. The Rwanda Citrus oil contains methyl isobutyrate, α-copaene, myrcenol, neryl acetate, geranyl propionate and perilly alcohol which were not detected in Uganda Citrus oil [11].

A yield of essential oil of 5.23% v/w, with a concentration of 74.43% of limonene, 4.27% of p-myrcene, 3.26% of sabinene, 1.54% of β-pinene, and 1.54% of linalool [05].

III. 2. Acetylcholinesterase (AChE) Inhibitory Activity

The essential oil showed a high activity (IC_{50} = 35.07 ± 2.47 µg.mL^{-1}) against AChE compared to the galantamine standard (IC_{50} = 6.27 ± 1.15 µg.mL^{-1}) (**Table 2, Figure 1**).

Table 2. *Acetylcholinesterase inhibitory activity.*

Extracts	Acetylcholinesterase inhibitory activity							
	3.125 µg	**6.25 µg**	**12.5 µg**	**25 µg**	**50 µg**	**100 µg**	**200 µg**	**IC_{50} µg/mL^{-1}**
Essential oil [a]	NA	9.91±0.16	14.98±3.86	37.72±0.16	68.14±3.81	87.77± 2.86	97.63±1.61	35.07±2.47
Galantamine [b]	35.93±2.28	43.77±0.00	68.50±0.31	80.69±0.41	85.78±1.63	91.80±0.20	94.77±0.34	6.27±1.15

a Values expressed are means ± S.D. of three parallel measurements.
b Reference compounds.

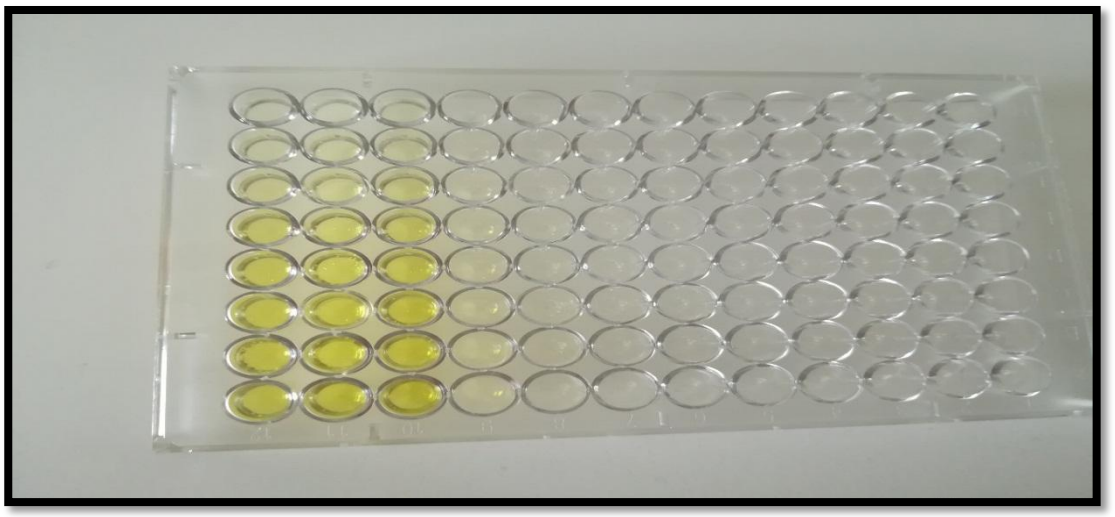

Figure 1. *Color development of acetylcholinesterase activity.*

The anticholinesterase activity of extracts from *Citrus limon* (lemon) leaves compared to galantamine, a drug widely used in the treatment of AD, and other species used in popular medicine in northeast Brazil are reported in a phytochemical screening [11]. Another study described inhibition of a fraction of the ethyl acetate extract from the leaves of *Citrus limon*, which was isolated from the active fraction named two coumarins: 5,8-dimethoxy-psoralen and 5,7-dimetoxi-coumarin. In vitro studies indicate IC_{50} of 5.8 µg mL^{-1} (95% conidence) and in vivo studies with male Swiss mice showed inhibition of 30.09–30.06% for the enzyme AChE compared to neostigmine, a drug used in the treatment of AD [12].

In vitro and in vivo assays with the essential oil of *Citrus sinensis* L. Osbeck (orange) against AChE enzyme indicated that there was a significant decrease in AChE activity in the hippocampal region of mice in in vivo tests. In vitro testing of Citrus sinensis essential oil showed a value inhibition concentration with IC_{50} = 63 µg mL^{-1} whereas for the standard (neostigmine) was obtained as IC_{50} value = 1.87 µg mL^{-1}. For the antioxidant, activity was a significant 20% reduction in the hippocampus of mice treated with 150 mg kg^{-1} on lipid peroxidation, thereby reducing oxidative stress and nitrite content, these doses showed a significant reduction in all groups, suggesting a neuroprotective effect against brain injury [3].

Studies on the species as *Citrus medica* L. cv. Diamante (cidra) demonstrated anticholinesterase activity, which can be explained by the high amount of monoterpenes present in the skin of the fruit [13]. According to studies 17 monoterpenoids with p-methane skeleton was reported, the AChE inhibitory activity of compounds such as γ-terpinene and terpinen-4-ol arrive at 22.6 and 21.4% at 1.2 mM, respectively. Other terpenes such as limonene and linalool present in 164 µg mL^{-1} concentration, inhibition of the 27 and 37%, respectively. The same activity is presented to study the species such as *Citrus hystrix* (Combava), which caused 10% inhibition of AChE enzyme and that this action was

related to the presence of acyclic and monocyclic monoterpenes such as citronellal and β-phellandrene, respectively, present in the essential oil extracted from the leaves of this plant [14].

The structural diversity of terpenoids that exert inhibitory activity of AChE is difficult to predict the potential structure activity relationship. But it is known that some features, such as the presence of a hydrophobic ligand, may be associated with greater effectiveness in the inhibition, since the active site of AChE is known to be susceptible to hydrophobic interactions. The monoterpenes consist of a hydrocarbon skeleton that can be cyclic (α-pinene) or acyclic (linalool), a feature that may also influence their AChE inhibitory activity. For a bicyclic monoterpene skeleton pinene or carene, the potential of AChE inhibition was associated with the position of the double bond [13]. The presence of terminal oleins ($H_2C=CH_2$) resulted in decreased inhibition of AChE, as well as the presence of an oxygenated functional group [04].

IV. Conclusion

The aim of the present study was to explore the essential oil from upper part of the pericarp of *Citrus sinensis* and its use as a candidate drug in the formulation pharmaceuticals for the prevention and/ or treatment of AD.
The essential oil of *Citrus sinensis* results show a significant pharmacological effect in the inhibition of AChE enzyme activity, with an (IC_{50} = 35.07 ± 2.47 µg. mL^{-1}) for the essential oil compared to (IC_{50} = 6.27 ± 1.15 µg. mL^{-1}) for Galantamine, and this action of great interest in the development of new phytomedicine.
The results confirm that AChE inhibitors as alternatives for preparation of phytomedicines are used in therapeutic treatment of AD, being plants the principal source of these inhibitors.

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