*ejpmr, 2020,7(9), 75-81*

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

# POTENT ANTIOXIDANT AND ACETYLCHOLINESTERASE INHIBITION ACTIVITIES OF THE ESSENTIAL OIL OF SALVIA LIBANOTICA GROWN IN LEBANON

**Wassim El Basset*, Hussein Kanaan, Sahar Azar, Layal AlHajjar, Marianne Haddad**

Laboratory of Chemical Synthesis and Extraction of Polysaccharides from Seaweed, School of Pharmacy, Lebanese University, Hadath, Lebanon.

***Corresponding Author: Wassim El Basset**
Laboratory of Chemical Synthesis and Extraction of Polysaccharides from Seaweed, School of Pharmacy, Lebanese University, Hadath, Lebanon.

**Article Received on 14/07/2020** **Article Revised on 04/08/2020** **Article Accepted on 24/08/2020**

**ABSTRACT**

*Salvia libanotica* is an endemic plant of the Mediterranean region widely used in Lebanon as a traditional remedy. The purpose of this study was to extract the EO from the leaves of *Salvia libanotica* growing in four random Lebanese sites via hydrodistillation, to determine the effect of the region on EO's yield as well as on the chemical composition studied by Gas Chromatography/ Mass Spectrometry analysis and to examine its antioxidant and anti-Alzheimer effects via DPPH and Ellman's methods respectively. Results showed that the EO yield varied between 0.22-1.2% according to region, and the chemical composition revealed a total of 22 compounds in all leaves' oil extracts with a difference in the relative abundance of some components. Moreover, the EO showed a significant antioxidant and AChE inhibitory activities reaching a maximum of 92.88% and 71.24% respectively. This supports the plant's use as preventive remedy against oxidative stress and Alzheimer's disease.

**KEYWORDS:** *Salvia libanotica*, Essential Oil, Chemical composition, Antioxidant, Anticholinesterase.

## 1. INTRODUCTION

Oxidative Stress (OS) is defined as an imbalance between free radicals known as "Oxidants" and the counteracting "Antioxidant System" resulting in an accumulation of Reactive Oxygen Species (ROS) and eventually cell death.[1] Moreover, OS is thought to promote and enhance inflammation via multiple processes and signaling pathways linking it with many diseases with a special focus on Neurodegenerative Diseases, among which stands "Alzheimer's Disease" (AD).[2] AD is considered as a debilitating disease of elderly in which prevention seems difficult.[3] However, some studies have shown the benefits of antioxidants on AD but more evidence is still needed.[4] In addition to OS, the Cholinergic Nervous System plays an important role in the pathogenesis of AD as a malfunction or deficiency in its main neurotransmitter A (ACh) seems to be associated with cognitive symptoms, hence why inhibiting the key enzyme responsible for its breakdown such as Acetylcholinesterase (AChE) appears to play a fundamental role in decreasing AD severity.[5] Sources of antioxidants are variable with the most important being plants due to their high content of flavonoids and polyphenols.[6] In addition, plants also play an inevitable role in AD as they were shown to be an AChE inhibitor.[7] Also acting as a vital source of drugs; plants are distributed throughout the planet; and a vast variety (estimated about 10% of world's higher plants) is found in the Mediterranean Bassin.[8] Sage, being a part of such floral diversity, is an old plant widely used in traditional medicine. It belongs to the genus *Salvia*, which comes from the Latin name *"Salvare"* i.e. to heal.[9] This genus is the largest member of Lamiacea or Mint family, containing between 900-1000 species worldwide.[10,11] Among the most important species are *Salvia officinalis* also known as common sage, *Salvia libanotica* also known as *Salvia fruticosa* or *Salvia triloba*.[10-13] *Salvia libanotica*, also native to the Mediterranean region is predominantly found in Lebanon where it grows wildly in different sites.[12] It is still regarded as a traditional remedy against many ailments as inflammation (tonsillitis, bronchitis, …), and diabetes,[14] giving it its popularity and importance in Lebanon.[12] Due to its countless benefits and properties, this plant caught the attention of researchers worldwide and several studies were conducted in this regard on various *S. libanotica* extracts and EO to investigate their phytochemical constituents and their biological activities, but no study, to our knowledge shed the light on the therapeutic benefits of EO extracted from *leaves* of *S. libanotica* cultivated in Lebanon. Therefore, in this context, we investigated in this study the in-vitro effect of the EO extracted from leaves of *S. libanotica* grown in Lebanon on oxidation and AChE activity as well as the effect of the geographical factor on EO's yield and chemical composition.

## 2. MATERIAL AND METHODS

### 2.1. Plant collection

The samples from wild growing *Salvia fruticosa* were collected in the time period extending from February 2019 till March 2019 during their pre-flowering stage from four different Lebanese sites: Harissa (550 m) (SL HA), Deir Koubel (300m) (SL DK), Aramoun (730m) (SL AR) and Wadi Chahrour (300m) (SL WC) and were identified by Pr. Kanaan and each were labeled with a specific voucher number SL for Salvia Libanotica followed by two-letter initials for the region. After collection, the leaves were dried at room temperature for 3 days to facilitate EO extraction.

### 2.2. Extraction of the essential oil

The EOs from dried S. Libanotica leaves were obtained by Hydrodistillation procedure using a Clevenger-type apparatus. Briefly, 30g of S. Libanoticas' dried leaves were subjected to hydro-distillation using distilled water as a solvent. The extraction lasted for 3 hours after which the oil volume in the burette was collected and stored in the refrigerator until usage.

### 2.3. Gas-chromatography/mass spectroscopy analysis

Fifty µl of volatile oil sample were diluted to 250 µl by hexane. One µl from this solution was then used for *Gas Chromatography (GC)* analysis. GC analysis was carried out on a Shimadzu GC 2010 with FID detector and a DB23 capillary column (60m x 0.25 mm; film thickness 0.25 um). The carrier gas was helium with a flow rate of 0.72 ml/min. The oven temperature for the first 4 min was kept at 60 ˚C and then increased at a rate of 4˚C/min until reached to the temperature of 250˚C and kept on for 5 minutes. The injector and detector temperature were set at 250˚C.

The data output from the detector appears as a line graph (chromatogram), with the amount of compound detected shown against the retention time. The volatile compounds appear as peaks on the graph. The relative abundance for each component is thus calculated as follows:

$$\% \text{ Relative Abundance} = \frac{\text{Area for each component}}{\text{Sum of Areas}} \times 100$$

### 2.4. Antioxidant activity: DPPH Assay

Different concentrations of S. Libanotica leaves' EOs were prepared by dilution in methanol into a final volume of 1ml. After that, for each EO dilution tube prepared, 1ml of DPPH was added and it was incubated for 30 minutes in darkness. A blank (2 ml of methanol), a negative control tube (1ml methanol added to 1 ml DPPH) and a positive control (1ml of ascorbic acid at 100 µg/ml added to 1ml DPPH) were also prepared. Finally, the absorbance was measured at 517 nm using a UV–visible spectrometer. The experiment was performed in triplicate

The DPPH scavenging activity is calculated as follows:

**DPPH scavenging capacity (%)=**

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

### 2.5. In-vitro AChE inhibition assay

Twenty µl of different EO concentrations (ranging from 1% till 20%) were placed in their corresponding test tubes and 50 µl of 1U/mL of AChE were added to each tube alongside 830 µl of Tris-HCl buffer (pH 8). The tubes were left for incubation at room temperature for 15 minutes. A negative control (50 µl methanol added to 50 µl AChE) and a positive control (50 µl of Galantamine at 10 µM added to 50 µl of AChE) were also prepared and treated similarly to determine their absorbance. After the incubation, 150 µl AChI and 950 µl DTNB were added to each tube. Finally, the reaction mixture was left for a 30-minute incubation period at room temperature, and the absorbance was measured at 412 nm using a UV–visible spectrometer. The experiment was performed in triplicate for leaves' EO extract.

The percentage of AChE inhibition is calculated as follows:

**Percentage of enzyme inhibition (%) =**

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

### 2.6. Statistical analysis

The data obtained in the study were expressed as Mean ± SEM and were analyzed using student's t- test compared to the control. A value is considered significant if $P \leq 0.05$.

## 3. RESULTS

### 3.1. EO's yield

The yields of EOs (%w/w) obtained from *S. libanotica* leaves from the four regions of collection (Harissa, Deir Koubel, Aramoun and Wadi Chahrour) are summarized in table 1 below where they are ranked from the highest to the lowest percentage.

The % yield was determined by the following equation

$$\frac{\text{Mass of the E.O (g)}}{\text{Mass of the Dried Leaves (g)}} * 100$$

As the table shows, the highest yield was for *S. libanotica* collected from Harissa site, which had 1.2% w/w yield of essential oil, followed directly by Deir Koubel with a yield equal to 0.86%. Aramoun had the lowest yield which was equal to 0.22. Given these results, EO extracted from *S. libanotica* collected from Harissa was used to further evaluate the pharmacological properties.

**Table 1: EO yields according to the area of collection.**

| Site | Yield (%w/w) |
|---|---|
| Harissa | 1.2 |
| Deir Koubel | 0.86 |
| Wadi Chahrour | 0.75 |
| Aramoun | 0.22 |

### 3.2. EO chemical composition

The chemical composition of the EO is shown in table 2. There wasn't a significant difference in the number and type of components between the regions chosen, but a difference in the relative abundance of some components was noted. In fact, a total of 22 components were identified in all leaves' oil extracts. These components can be classified based on their structure into Monoterpene Hydrocarbons (MH), Oxygen-Containing Monoterpenes (OMT), Sesquiterpene Hydrocarbons (SH) and Oxygen-Containing Sesquiterpenes (OST). OMT were the dominant fraction representing approximately 41% of all tested samples, followed by MH representing 27% of all tested samples. Among these 22 components, some were found predominately high. A component was considered predominant if it was Found in amounts higher than 3% (Cvetkovikj *et al.*, 2015). The majority of these predominant components are common for all four regions such as: Eucalyptol (34.53-42.01%), Caryophyllene (5.44-11.08%), Beta-Myrcene (7.61-10.79%), Beta-Pinene (7.19-9.36%), Camphor (3.658-8.265%), Alpha-Terpineol (4.53-5.34%) and Alpha-Pinene (3.43-5.32%). Specific components not common to all four Regions were also found: Camphene (4%) in EO extracted from *S. libanotica* collected from Harissa, p-menth-1-en-8-ol in EO extracted from *S. libanotica* collected from Harissa (3.90%) and Deir Koubel (3.21%), Alpha-Humulene (3.5%) in EO extracted from *S. libanotica* collected from Aramoun and finally Viridiflorene (4.28%) in EO extracted from *S. libanotica* collected from Aramoun (Table 2).

**Table 2: Relative abundance of different components of S. Libanotica EOs according to the area of collection.**

| Component Name | RT | Relative Abundance of Studied Areas (%) | | | | Component Type |
|---|---|---|---|---|---|---|
| | | Deir Koubel | Aramoun | Wadi Chahrour | Harissa | |
| Alpha-Pinene | 9.40 | 5.32 | 3.43 | 4.51 | 5.06 | MH |
| Camphene | 9.85 | 2.59 | 1.81 | 2.37 | 4.002 | MH |
| Beta-Pinene | 10.73 | 9.36 | 7.21 | 7.19 | 8.92 | MH |
| Beta-Myrcene | 11.25 | 9.46 | 10.79 | 9.42 | 7.61 | MH |
| Eucalyptol | 12.91 | 34.53 | 38.63 | 42.01 | 36.81 | OMT |
| Gamma-Terpinene | 13.65 | 1.61 | 1.22 | 0.78 | 1.23 | MH |
| Terpinolene | 14.68 | 1.25 | 0.44 | 0.47 | 0.49 | MH |
| Linalool | 15.15 | 0.98 | 0.24 | 0.45 | 0.08 | OMT |
| Thujone | 15.32 | 1.61 | 1.11 | 1.99 | 2.69 | OMT |
| Thujone beta | 15.6 | 1.34 | 0.83 | 0.88 | 1.50 | OMT |
| Camphor | 16.64 | 6.59 | 3.65 | 6.83 | 8.26 | OMT |
| Cis-Pinocamphone | 17.04 | 0.73 | 0.32 | 0.42 | 0.37 | OMT |
| p-menth-1-en-8-ol | 17.30 | 3.21 | 2.07 | 3.01 | 3.90 | OMT |
| Terpinen-4-ol | 17.58 | 1.54 | 1.18 | 1.30 | 1.25 | OMT |
| Alpha-Terpineol | 18.05 | 5.12 | 4.54 | 5.34 | 4.54 | OMT |
| Alpha-Terpinyl Acetate | 22.05 | 1.25 | 0.13 | 1.06 | 0.59 | OMT |
| Caryophyllene | 23.80 | 5.44 | 11.08 | 7.28 | 8.49 | SH |
| Alpha-Humulene | 24.5 | 1.98 | 3.50 | 1.69 | 1.4 | SH |
| Longifolene | 25.30 | 1.47 | 1.12 | 0.36 | 0.33 | SH |
| Viridiflorene | 25.49 | 2.35 | 4.28 | 1.28 | 1.30 | SH |
| Ledol | 27.75 | 1.29 | 2.20 | 1.03 | 0.86 | OST |
| Caryophyllene oxide | 28.05 | 0.85 | 0.10 | 0.23 | 0.17 | OST |

$$\textit{Relative Abundance} = \frac{Area\ of\ each\ component}{Sum\ of\ areas} * 100$$

*MH: Monoterpene Hydrocarbon, OMT: Oxygenated Monoterpene, SH: Sesquiterpene, OST: Oxygenated Sesquiterpene*

### 3.3. Antioxidant effect of EO

The DPPH assay results showed that the scavenging activity of Salvia leaves' oil is maximal at the lowest EO concentration (1%) (Figure 1); at this concentration, the scavenging activity was equal to 92.88% and was no more different from that of the positive control ascorbic acid at 100 $\mu$g/mL (94.39%) (P=0.17). As the EO concentration increased from 1% to 15%, the antioxidant effect decreased dose-dependently from 92.94% to 77.93% respectively but still significantly high at all concentrations compared to control (P<0.001, Figure 1). This indicates the strong antioxidant activity of Salvia leaves' EO.

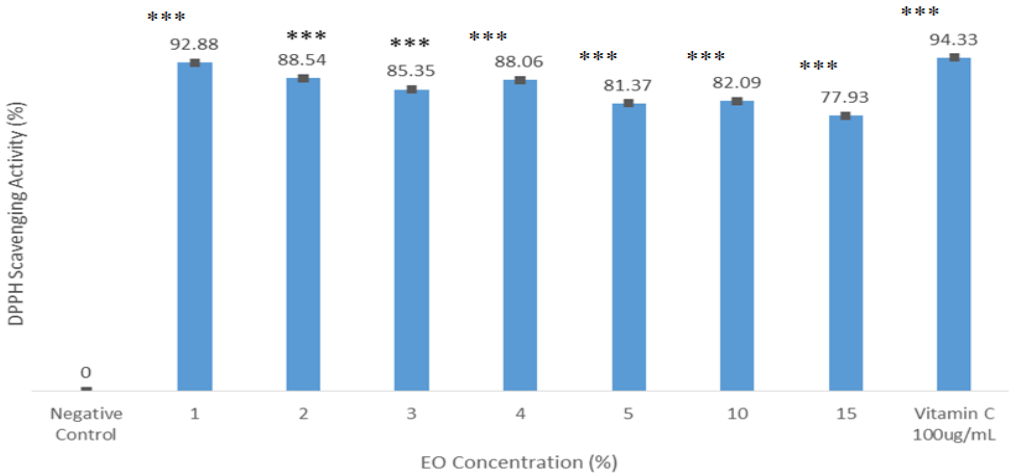


**Figure 1: DPPH scavenging activity of S. Libanotica's EO.**
*****: P<0.001 compared to negative control.**

### 3.4. In-vitro AChE inhibition activity of EO

Inhibitory concentration data revealed a maximal AChE inhibition (71.24%) at 20% EO concentration (P<0.001). This inhibition percentage was not different from that seen with the positive control Galantamine at 10$\mu$M (77.07%; P=0.23) as shown in figure 2. As the concentration of the EO decreased from 15% to 1%, the AChE inhibition decreased from 69.73% to 43.58% but still significantly high at all concentrations compared to control (P<0.001, Figure 2). This indicates the strong AChE inhibition activity of Salvia leaves' EO.

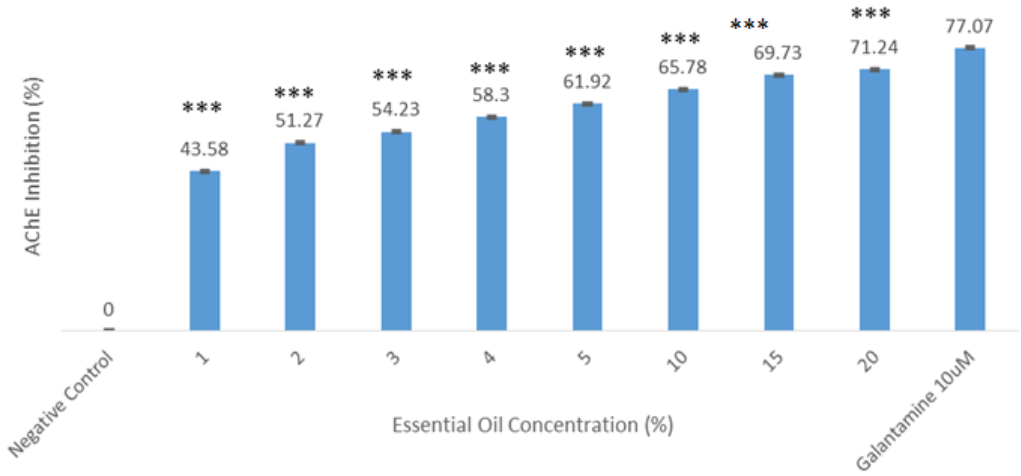


**Figure 2: AChE inhibiting potential of S. Libanotica's EO.**
*****: P<0.001 compared to negative control.**

## 4. DISCUSSION

The objective of this study was to extract the EO of *Salvia libanotica*'s leaves taken from four random Lebanese regions, to study the effect of region on EO's yield and chemical composition and to test the EO's antioxidant and anti-AChE activities.

Results showed that the EO yield varied greatly according to region, reaching its highest in Harissa with 1.2% and its lowest in Aramoun with 0.22%. This fluctuation due to region is in contrast with Zgheib *et al.*, (2019) who showed no geographic influence on the EO yield.[15] In addition, our yield seems lower than that of Zgheib *et al.*, (2019) where it reached a maximum of 5.2%.[15] Different factors can explain these differences such as the method of extraction, the solvent used for extraction, the amount of time put into extraction, the volume of water added, the degree of comminution of the leaves and the state of use of the leave (freshly used or dried),[15,16] in addition to the vegetative state which is different and can affect the EO yield. Moreover, the extraction yield is also affected by the application of fertilizers and the season of harvest.[16-18] Knowing that Harissa has a dry climate can explain the reason why it had the highest yield as EO yield seems to be greater during dry weather.[12]

As for the chemical composition, results showed that EO components did not vary from one region and the other. In fact, a total of 22 compounds were detected in all

regions and this number was in accordance with a study done by Karik *et al.*, (2018) in Izmir Turkey on *S. libanotica* where 22 components were also identified.[11] However, this number disagrees with other studies; for example, 29 components were identified by Zgheib at al.[15] in *S. libanotica* grown in Lebanon which can be due to many factors related to the plant itself (geographical region and season of cultivation) as well as the method of extraction.[17,19,20] However, the percentage of each component varied from one region and the other but whether this variation in the relative abundance was significant or not remains to be elucidated.

Furthermore, in our study, Eucalyptol was the major most abundant component present in all four regions (34.53-42.01%) which is in full agreement with other studies on *S. libanotica* conducted in different countries.[20-23] However, other studies found α-thujone as a major compound.[24,25] This difference in the EO major components can be a result of the intrinsic and extrinsic factors. On one hand, among different Salvia species as investigated by Karik *et al.*, (2018) *S. libanotica* oil was primarily composed of Eucalyptol (57.18%) and β-pinene (8.20%).[11] On the other hand, between the same salvia specie as investigated by Zgheib *et al.*,[15] in which they found high amounts of camphor, camphene and α- pinene in Ansariyeh EO and high amounts of camphor, *trans*-anethole, *trans*-sabinene hydrate and α-terpinyl acetate in Harissa EO. Both of our findings agree that we have different distribution of EO components among regions, but disagree when it comes to Harissas' EO composition, because Trans-anethole and trans-sabinene hydrate seen in their study weren't present in ours; this could be due to a difference in the harvesting season as already mentioned. [17,20] This also disagrees with Cvetkovikj *et al.*, (2015) who investigated the chemical composition and EO yield of *S. libanotica* in nine region of Balkan area and found no significant difference in their chemical composition.[19]

S. libanotica hydroalcoholic extracts, as an overall, possess several therapeutic effects such as herbicidal, anti-inflammatory, antioxidant, anticancer, antibacterial, antiviral, and antifungal activities and different properties were previously elucidated.[14,15,20] Nevertheless, to our knowledge, no studies on the EO of *S. libanotica* grown in Lebanon investigated the pharmacological properties of its EO emphasizing on the importance of this study.

The first investigated property was the antioxidant activity evaluated by the DPPH Assay which showed a significant antioxidant effect at all concentrations when compared to the negative control ($p<0.001$). This potent antioxidant effect was demonstrated on one hand in other studies regarding *S. libanotica* leaves extracts and EO as seen with Nasreddine *et al.*, (2018) and Papageorgiou *et al.*, (2008) respectively;[26,27] on other hand this was also demonstrated with other species such as *S. lanigera* EO, *S. euphratica* and *S. eremophila* EOs.[28-31] This potency could be attributed to Eucalyptol (36.81%) (32), α-Pinene (5.06%), Caryophyllene (8.49%),[33-35] Alpha-Terpineol α-T (4.54%),[36] and β-myrcene (7.61%)[37] as the biological properties of any EO vary according to the major components present, their types and their concentration.[38] However, the "reverse effect" displayed by our EO and defined as a negative correlation between the concentrations used and its scavenging potential effect disagrees with other studies conducted on *S. libanotica* which showed a dose-dependent increase in the antioxidant effect. Nevertheless, this effect of antioxidant transforming to a pro-oxidant is seen with well-known antioxidants such as ascorbic acid (vitamin C) where it acts as an antioxidant at low concentrations and becomes pro-oxidant at high and even at pharmacological concentrations.[39-41]

Since our EO displayed a good antioxidant potential, it was appealing to us to see if it offers a beneficial effect on Alzheimer's disease (AD) as the latter is initiated by oxidative stress enhancing AD's main causative factors (amyloid β (Aβ) peptide, and neurofibrillary tangles of hyperphosphorylated τ proteins).[42] Hence, the second investigated property was the AChE inhibition activity via Ellman's method described previously. The EO showed a significant dose dependent increase in AChE inhibition at all concentrations ($p<0.05$) reaching a maximum inhibition of 92.88% at 20% which is in agreement with other work that studied the anticholinesterase activity of *S. libanotica* EO performed on non-Lebanese *S. Libanotica*.[43,44] Other Salvia species EO such as *Salvia chionantha*'s were also tested for the AChE inhibiting potential and followed the same trend in inhibition.[29] Moreover, *Salvia officinalis* various extracts were evaluated in clinical settings on healthy subjects and others with cognitive impairment and showed a very promising outcome.[45] Likewise, this highly beneficial effect is due to various mechanisms such as the inhibition of amyloid-beta protein aggregation and the inhibition of the enzyme acetylcholinesterase. The AChE inhibition activity seen with the EO can also be attributed to the main components' activity such as Eucalyptol, Alpha-Pinene and lastly Camphor,[46,47] which were previously shown to exhibit an anticholinesterase activity. Eucalyptol and camphor were found to be inhibitors of the enzyme AChE with Eucalyptol being more potent than Camphor with IC50 respectively of 2.27 µM and 21.43 µM. [46] Molecular docking revealed that these compounds bind to key amino acids in the catalytic domain of AChE, similar to standard drugs.[46]

## 5. CONCLUSION

In summary, the present work showed a geographic factor influence on the EO yield and the percentage of chemical components of *Salvia libanotica* grown in Lebanon. Moreover, and for the first time, it showed a potent antioxidant and anti-AChE effects of *S. libanotica* EO supporting its use as a remedy for oxidation and its related diseases as well as Alzheimer's disease.

**6. FUNDING**

The authors would like to thank the president of the Lebanese University Prof. Fouad Ayoub for the financial support (Grant No. EPALL/104/21/LU).

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