Research Article

Anti Alzheimer’s Disease Related Activities of Israeli Medicinal Plants
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Abstract

Medicinal plants may provide a potential source of therapeutic agents against Alzheimer’s disease (AD) and other dementias. In the current study medical plants with an historical/traditional use for improving memory were evaluated for anti-AD activity. Plants were collected mainly from wild species growing in Israel and tested for their protective properties against toxicity of amyloid-beta (Aβ) and a decrease in its secretion by neuronal cells (SHSY5Y and PC12). Of 20 plants tested, 5 plants: Citrullus colocynthis, Cistanche tubulosa, Paronychia argentea, Sisymbrium irio and Origanum dayi, showed rescue of cells from amyloid toxicity of 205%, 177%, 42.5%, 40% and 22.7%, respectively. Citrullus colocynthis and Cistanche tubulosa also demonstrated a decreasing trend of 19% and 25%, respectively in Aβ levels secreted by cells. Two additional plants, Varthemia iphionoides and Anchusa strigosa, showed a decreasing trend in secreted amyloid levels of 32% and 34% and a decrease of 23 and 31% in γ-secretase activity respectively.

These results point to a potential therapeutic use of medicinal plants some of which has a long historical/traditional use for improving memory and should be the basis of further investigation for neurodegenerative diseases such as AD.

INTRODUCTION

Alzheimer’s Disease (AD) is a progressive neurodegenerative disorder occurring predominantly in the aging population and representing the commonest cause of dementia. The risk of AD rises sharply with advancing age currently affecting over 20 million people worldwide [1]. With a further increase in life expectancy the number of AD patients is predicted to increase dramatically.

The main neuro pathological hallmarks of AD in the brain are amyloid plaques and neuro fibrillary tangles (NFTs). Amyloid plaques result from the accumulation of several proteins and an inflammatory reaction around deposits of amyloid-beta (Aβ); NFTs are the aggregated and phosphorylated form of the microtubule-associated tau protein. Inflammatory and apoptotic processes as well as oxidative stress are implicated in the pathogenesis. As these cellular changes progress neurons are lost in the brain [reviewed in [2]]. Although impressive advances in understanding AD have been made over the past decade the mechanisms of brain degeneration remain to be resolved with effective drug therapy still not available. There is thus a real need to find drugs to prevent AD, delay its onset and/or retard its progress. An effective treatment able to postpone onset by an average of five years would reduce associated costs to society by nearly 50% [3].

Medicinal plants constitute an important source of bio-active molecules that have been widely used in the search for effective therapies in many diseases including cancer, infections, rheumatic conditions etc. [4,5]. Until now however the potential of plants as a source of innovative molecules for neurodegenerative diseases has not been widely exploited.

Israel with its varied climate and geography situated in a transition zone between Mediterranean, desert and sub-tropical regions possesses some 2600 plant species belonging to 130 different families ranging from alpine plants in the north to desert and salt plants in the south, many endemic only to the area [6]. With a long history of traditional use spanning millennia the medicinal plants of Israel present a unique opportunity for focused screening based on their ethno-botanical use.

In the current study plants were investigated as part of the Middle Eastern Medicinal Plant Project (MEMP), an ethno-botanical initiative of The Natural Medicine Research Center (NMRC) dedicated to the preservation of traditional knowledge,
domestication, conservation and re-introduction of selected species and focused screening of Israeli flora [7,8].

Ethanol extracts of whole plants traditionally used to enhance memory were evaluated on AD related pathogenesis. Investigations included neuroprotective activity against Aβ peptide in neuronal cell lines, ability to reduce amyloid levels secreted by these cells and reduction in activity of γ-secretase, an enzyme catalyzing cleavage of Aβ from its amyloid precursor protein (APP).

Screening of 20 herbal extracts demonstrated rescue of neuronal cells from amyloid toxicity in 5 extracts, decrease in amyloid levels secreted by treated neuronal cells in 4 extracts, of which 2 were also associated with lower activity of γ-secretase.

These results point to the potential therapeutic value of selected plants found in Israel in the treatment of neurodegenerative disorders particularly against amyloid related pathology in AD.

**MATERIALS AND METHODS**

**Plants**

**Plant Selection:** Plants were selected for screening using a focused method based on their historical/traditional ethno botanical use. Information was obtained by data mining techniques and was derived from a variety of sources. These included the ethno botanical database of NMRC containing the traditional and folk medicine use of over 500 Israeli medicinal plants (SS unpublished data).

In addition the following sources were also consulted: medieval pharmacopoeias (texts listing herbs, minerals, animal products and their medical effects and usages) and medical encyclopaedias (describing diseases with therapeutic recommendations including drugs useful for the problem). These texts translated when necessary by author (HP) from Latin, Hebrew and Arabic sources range from the 1st -16th century. Any reference to a plant or remedy being used in a way that indicates it could have psychotropic or neurological effects particularly a memory improving effect e.g. "against loss of memory" was noted. To evaluate the relative reliability of this information and provide a criteria for inclusion in focused screening (below), plants drugs described in these texts were assessed in the following way (by author HP, unpublished data): (a) the frequency with which authors mention particular plants/drugs; (b) the plants/drugs mentioned by most of the authors; (c) the exact indication for which they were used (which will show if a plant/drug has a specific effect against the given problem, for example, against memory loss or if its effect could be assumed to be more general e.g. improving the patient’s general physical condition); (d) the percentage of the texts the plant/drug in question is mentioned as having the quality or effect sought; (e) how highly the quality in question is stressed in the text (i.e. how often it appears in the same description); (f) how continuously the drug is referenced between different texts over time.

On the basis of these assessments, a list of plant species / drugs and/or plant genus were compiled as being the most likely to possess potentially psychotropic, neurologic and/or anti-amnesic properties.

**Plant collection:** The majority (18) of the plants selected by the above method for screening were collected from wild sources (Judean Hills) in Israel. Two species (extract #114 and extract #63) were harvested from domesticated wild plants at the MEMP cultivation site, Kibbutz Ketura in Israel’s southern Arava region. Domestication of these plants derived originally from seeds obtained from wild sources took place without the use of fertilizers or pesticides using shaded net houses and drip irrigation.

All plants from wild and domesticated sources were identified by botanists Dr. Ori Fragman-Sapir and Ms. Hagar Leschner (see acknowledgements) with voucher herbarium specimens deposited at NMRC officers in Hadassah Medical Organization, Jerusalem.

**Plant Extracts (Ethanol extraction)**

All reagents used in the study were purchased from Sigma (St. Louis, MO) unless otherwise stated. Freshly harvested plants were air dried at room temperature and extracted with ethanol (50% v/v, 10v per gram weight) by vigorous stirring in a covered beaker for 24 h at room temperature after which the process was repeated.

The supernatant of both extracts was filtered and ethanol evaporated in a chemical hood for 4 days with the evaporated extract frozen at -70°C followed by lyophilization till dryness. The dried extract was kept at 4°C. Stock solutions of the plant extracts (200 mg/ml) were prepared by weighing the powder and dissolving in 10% dimethyl sulfoxide/ phosphate buffered saline (DMSO/PBS). The solution was divided to aliquots and kept at -20°C, until used.

**Cells and cell viability assessment**

Human neuroblastoma cells SHSY5Y cells (10⁵ cells/ml) were grown in RPMI medium supplemented with L-Glutamine 1%, FCS 10% and Gentamicine 50mg/ml in CO2 10%. PC12 pheochromocytoma of the rat adrenal medulla cells (10⁵ cells/ml) were grown in DMEM, FCS 7.5%, horse serum 7.5%, Penstrep 1% and L-Glut 1%. The cell reagents were purchased from Biological Industries Israel Beit Haemek Ltd. Cells were exposed or non-exposed to Aβ_{1-42} (10μM) (amyloid was first incubated at 37°C for 1h to allow its aggregation). Cells exposed to the Aβ_{1-42} were treated concomitantly with each of the extracts dissolved in 10% DMSO or with vehicle only as controls and further incubated overnight. In one set of experiments the extract was added to the cells at the following different time points for comparison: 2h before exposure to amyloid, concomitantly with amyloid, or 2h after amyloid was added. In all other experiments amyloid and plant extracts were administered to the cells concomitantly. After an overnight incubation cells were washed and lysed and cell viability estimated by the Methyl Thiazolyl Tetrazolium bromide (MTT) assay (a sensitive measure of the normal metabolic status of cells particularly mitochondria and reflecting early cellular redox changes), expressed as Optical Density (OD) units at 595 nm as previously tested by us [9].

**Aβ_{1-42} levels secreted by the cells (ELISA)**

To determine the level of Aβ_{1-42}, secreted by cells, the medium of the SHSY5Y cells grown with the tested plant extracts or with
vehicle only (controls) were collected and submitted to ELISA (Aβ1−42 kit, Covance Laboratories Inc., Chantilly, Virginia). The results were normalized for protein concentration in the cell lysates, determined with BCA protein assay.

**Preparation of detergent-solubilized membrane fractions and γ-secretase activity assays**

Secretase activity was determined in membrane fractions of the cells grown with or without plant extracts using Fluorescence Resonance Energy Transfer (FRET) assay based on published protocols [9,10] with some modifications, as follows: The cells in 10 cm dishes (3 dishes per sample) were collected and re-suspended in homogenization buffer (50 mm Hepes, pH 7.0, 250 mm sucrose, 5 mm EDTA) containing complete protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). The re-suspended cells were homogenized by Tissue Grinders homogenizer and the cell debris and nuclei removed at 3,000 × g for 10 min. The supernatants were then centrifuged at 100,000 × g for 1 h. The membrane pellets were solubilized with 1% digitonin or CHAPSO in homogenization buffer for 90 min on ice and then centrifuged at 100,000 × g for 1 h. The protein concentration of the supernatant was determined with BCA protein assay reagent. CHAPSO-solubilized membrane fractions were diluted to 0.25% CHAPSO and then incubated with 8 μm peptide γ-secretase substrate, Fluorogenic (Millipore, Billerica, Massachusetts) in the absence or presence of the γ-secretase inhibitor DAPT (10 μM) at 37 °C overnight. After incubation, reactions were centrifuged at 16,100 × g for 15 min and placed on ice. Supernatants were transferred to a 96-well plate (Black, Nunclon, Nunc) and fluorescence measured using a plate reader (Fluorstar Galaxy) with excitation wavelength at 320 nm and emission wavelength at 460 nm. The results were normalized for protein concentration in the cell lysates.

**Statistical analysis**

Results are presented as Mean ± SEM. The unpaired t-test was used for comparing results of MTT assay, amyloid levels and enzyme activity assays between study groups (p values of ≥ 0.05 were considered significant).

**RESULTS**

**Herbal extracts demonstrating neuroprotective activity against amyloid toxicity**

A total of 20 plant extracts were screened for potency in rescuing cells from toxicity of Aβ1−42 peptide exposed concomitantly with each extract. Of these 5 extracts (#63, #106, #120, #107 and #114) showed anti-amyloid neuroprotective properties some of them presenting a very robust statistically significant effect of increased cell viability (177-205%), while others demonstrated a more modest trend of neuroprotection (22.7−42.5%).

These included the following: extract #63 (Citrullus colocynthis (L) Schrad) showed a robust dose dependent effect of rescuing SHSY5Y cells, as follows: while exposure to amyloid reduced cell viability from 1.167±0.17 to 0.465±0.048 OD units (p=0.014), treating the amyloid exposed cells with the plant extract increased cell viability to: 1.28±0.069, 1.42±0.127 (205% ), 1.047±0.153 and 0.979±0.007 for concentrations of 0.01, 0.1, 1, and 5μg/ml (p=0.0001, p=0.04, p=0.015, p=0.002 respectively), reaching OD values similar and even higher to cells not exposed to amyloid (Figure 1a). A similar protective effect in higher concentrations of extract #63 was demonstrated in PC12 cells: exposure to amyloid reduced cell viability from 0.797±0.053 to 0.362±0.053 (p=0.0005); treating cells with extract #63 increased cell viability to 0.585±0.046 (p=0.005) and 0.452±0.066 for concentrations of 30 and 60μg/ml, respectively.

Treating amyloid exposed cells with extract #63 2 h before exposure to amyloid also showed robust neuroprotection (while exposure to amyloid reduced cell viability from 0.818±0.184 to 0.308±0.019 (p=0.039), treating cells with this extract increased cell viability to 0.647±0.029 (p=0.001) and 0.389±0.019 (0.007) at concentrations of 30 and 60μg/ml, respectively). A smaller neuroprotective trend was demonstrated even when treatment with extract #63 was initiated 2 h following exposure to amyloid (while exposure to amyloid reduced cell viability from 0.711±0.05 to 0.335±0.048, treatment with 30μg/ml of the extract increased viability to 0.394±0.054).

These results suggest that the protective effect of plant extract #63 may have potential therapeutic activity although its effect seems more pronounced when used as a prophylactic.

All other extracts were tested for rescuing cells from amyloid toxicity exposed concomitantly with each extract.

Extract #106 (Cistanche tubulosa (Schenk) Hook, f) rescued amyloid exposed SHSY5Y cells from cell viability of 0.198±0.003 (relative to 0.368±0.023 of cells not exposed to amyloid, p=0.005) to 0.433±0.044, 0.549±0.085 (177%) and 0.569±0.092 at concentrations of 1, 5, and 50 μg/ml (p=0.001, p=0.018, p=0.027) respectively (Figure 1b).

Extract #120 (Paronychia argentea Lam) showed a protective effect against amyloid with cell viability increased from 0.439±0.032 (relative to 0.850±0.1 of cells not exposed to amyloid, p=0.013) to 0.626±0.032 (42.5%) and 0.571±0.109 when treated with 1 and 10μg/ml of the extract; and from 0.781±0.097 under amyloid exposure to 1.014±0.096 (p=0.042) when treated with 50μg/ml of the extract.

Extract #107 (Sisymbrium irio L) rescued amyloid exposed cells from cell viability of 0.927±0.06 (relative to 1.379±0.015 in cells not exposed to amyloid, p=0.06) to 1.023±0.09 in 1µg/ml extract; and of 0.992±0.06 to 1.392±0.131 (40%) (p=0.019) to 1.023±0.09 in 1µg/ml of the extract.

Extract #114 (Origanum dayi Post) showed a dose response trend of protecting cells against amyloid toxicity although not reaching statistical significance: from cell viability 0.308±0.04 under amyloid exposure (relative to 0.451±0.052 in cells not exposed to amyloid, p=0.024) treated cells showed an increase of viability to 0.335±0.027, 0.378±0.008 (22.7%), 0.365±0.075, 0.368±0.036, 0.318±0.069 and 0.335±0.027 at concentrations of 0.01, 0.1, 1, 10, 50μg/ml respectively.

**Herbal extracts associated with reduced levels of amyloid secretion from cells**

Screening 8 plant extracts for their ability to reduce the levels...
Figure 1a The herbal extracts #63 and #106 rescued the SHSY5Y from Aβ toxicity in MTT assay. (a) Aβ exposed cells showed significantly lower viability than cells not exposed to Aβ (\(p=0.014\)). Aβ exposed cells treated with extract #63 showed significant increase in viability relative to Aβ exposed cells treated with vehicle only [controls (Aβ cont)], at concentrations of 0.01, 0.1, 1 and 5 µg/ml (\(*_{1} p=0.0001, *_{2} p=0.04, *_{3} p=0.015, *_{4} p=0.002\), respectively.

Figure 1b Aβ exposed cells showed significantly lower viability than cells not exposed to Aβ (\(p = 0.005\)). Aβ exposed cells treated with extract #106 showed significant increase in viability relative to Aβ exposed cells treated with vehicle only, at concentrations of 1, 10 and 50 µg/ml (\(*_{1} p = 0.011, *_{2} p = 0.018, *_{3} p = 0.027\), respectively).

of the Aβ₁₋₄₂ peptide secreted by the SHSY5Y cells, revealed that 4 of them showed trends in reducing amyloid levels in the medium of cells grown with the extracts.

Cells treated with extract #123 (Varthemia iphionoides Boiss & Blanche) showed amyloid levels as expressed by Relative Light Units (RLU) of 49065.84±6858.36, 52922.57±18107.16 and 59396.84±15677.59 for 1, 10 and 50 µg/ml of extract, respectively vs. 72357.65±20595.59 RLU in vehicle-treated controls (a decrease of 32.19%, 26.84% and 17.93% respectively).

At similar concentrations cells treated with extract #124 (Anchusa strigosa Banks & Sol) also showed lower amyloid levels (decrease of 27.06%, 34.38%, and 11.20%, respectively relative to vehicle-treated controls) (Figure 2).

Some beneficial trends were also detected when cells were treated with 0.1, 1, 10 and 50 µg/ml of extract #106 (Cistanche tubulosa) [decrease of 25.36% (\(p=0.044\)), 10.32%, 16.87%, and 21.95%, respectively, relative to vehicle-treated controls] as well as with 0.01, 0.1, 1, 10 µg/ml of extract #63 (Citrullus colocynthis) (decrease of 19.09%, -2.28%, 6.23% and 13.54% respectively, relative to vehicle-treated controls).

Further confirmation for a decrease in secretion of amyloid by cells treated with plant extracts was obtained by demonstrating reduced activity of the enzyme catalyzing formation of Aβ₁₋₄₂, presented below.

Plant extracts associated with reduced γ-secretase activity

The decrease demonstrated above in Aβ levels secreted by neuronal cells treated with selected plant extracts suggests that
Figure 2 The herbal extracts #123 and #124 reduced the level of Aβ₁₋₄₂ secreted by the SHSY5Y cells, detected by ELISA. Trends were detected showing that at concentrations of 1-50 µg/ml extract #123 and extract #124 reduced the Aβ level relative to vehicle treated control cells (cont) (by 32% at 1µg/ml, and by 34% at 10µg/ml, respectively).

Figure 3 The herbal extracts #123 and #124 reduced the activity of the γ-secretase in SHSY5Y cells, detected by FRET analysis. At concentrations of 10µg/ml, extract #123 and extract #124 reduced each the γ-secretase activity relative to vehicle treated control cells (cont) by 23% (⁎ p=0.003) and 31% (∗ p=0.04), respectively. For comparison we used the γ-secretase inhibitor DAPT, which reduced the enzyme activity by 45% (⊥p=0.02), relative to vehicle treated control cells.

these extracts may interfere with the formation of this toxic peptide. To test this possibility activity of γ-secretase (catalyzing cleavage of APP to generate Aβ peptide following cleavage by β-secretase in the amyloidogenic pathway) was tested in the SHSY5Y cells treated with 2 plant extracts that were associated with a decrease in Aβ levels: #123 (Varthemia iphionoides) & #124 (Anchusa strigosa).

A significant decrease in enzyme activity was demonstrated: cells treated with 10µg/ml of extract #123 showed a lower secretase activity: 76.73%±2.5% of activity in vehicle-treated control cells (p=0.003) (decrease of 23.27%). In cells treated with extract #124 activity was 68.89%±3.08% (decrease of 31.11%) (p=0.04) (Figure 3a).

By comparison, reduced γ-secretase activity was demonstrated using γ-secretase inhibitor DAPT [55.51%±12.42% of activity in vehicle-treated control cells (p=0.025) (decrease of 44.49%)].

DISCUSSION

These results point to the largely unexplored therapeutic potential of selected plants with activity against amyloid-pathology associated with AD. Significant effects were demonstrated both prior to and concomitantly with exposure to amyloid-toxicity.

Extract #106 (Cistanche tubulosa (Schenk) Hook, f) associated in this study with both a neuroprotective effect and reduction in amyloid secretion belongs to a worldwide genus of 22
holoparasitic desert plants in the family Orobanchaceae. Plants in this genus are widely used in traditional and folk medicine particularly in China (where 5 species are found) forming an important component in a number of Chinese medicines including those used in treating “amnesia” and memory problems. Previous chemical analysis of *C. tubulosa* as well as several other plants in the genus indicate the main constituents are essential oils, phenyl ethanoid glycosides (PhGs), iridoids, lignans, aldilols, oligosaccharides and polysaccharides [11]. *Cistanche* species have demonstrated a variety of activities including vasorelaxation [12], hepatoprotective [13], sedative (*C. deserticola*) [14] anti-allergic [15], anti-nociceptive and anti-inflammatory [16], hypcholesterolemia [17] and anti-oxidant [18]. Recently the potential of *Cistanche* species to improve memory has indicated that echinacoside and acteoside fractions isolated from *C. tubulosa* are associated with memory-improving effects in SAMP/B mice and scopolamine-induced amnesia [19,20]. Until now the mechanism of this effect has not been fully evaluated, although in other *Cistanche* species (*C. Herba*) induction of nerve growth factor (NGF) in PC12 cells [21] suggests that this may be one possible mode of action.

Extract #63 (*Citrullus colocynthis* (L) Schrad) also demonstrated both neuroprotection and a reduction in amyloid secretion activity. A member of the family *Cucurbitaceae* the fruit and roots of this small perennial creeping herb has been used medicinally since ancient times for infections, jaundice, diabetes urinary problems, nerve pain and particularly as a powerful purgative [22]. Phytochemical analysis has indicated tertiary or quaternary alkaloids, glycosides, saponin as well as amino acids, and proteins [23]. No relevant studies however could be found on the memory enhancing or anti-Alzheimer’s activity of *C. colocynthis*, although previous studies have demonstrated numerous other activities including anti-tumor based on the presence of *Cucurbitacin* glycosides [24], cytotoxic and anti-leismanial activity [22], anti-oxidant [25] and immune stimulating [26].

The two extracts #123 (*Varthemia  iphionoides Boiss & Blanche*) and #124 (*Anchusa strigosa Banks &Soo*) were both associated in the current study with reduced amyloid secretion and γ-secretase activity. *Varthemia  iphionoides* a common herb in the Eastern Mediterranean in the family *Compositae* (*Asteraceae*), is a bushy perennial with aromatic stems [27]. Its aerial parts, commonly used as an aqueous extract in folk medicine for diabetes and gastrointestinal disorders [28] have exhibited anti-fungal, anti-spasmodic, anti-platelet, cytotoxic, anti-oxidant and anti-bacterial activity [27,29,30].We believe however that this is the first study to demonstrate a potential effect of *V.  iphionoides* on pathological elements associated with AD.

*Anchusa strigosa* a herbaceous, perennial plant with stiff spiny hairs is a member of the *Borage (Boraginaceae)* family that includes some 27–30 species mainly distributed in the Mediterranean basin and Middle East [31]. Chemical studies have shown that *Anchusa strigosa* contains aliphatic hydrocarbons, oil, proteins, pyrrolizidine alkaloids and polyphenols. Pharmacological effects include gastric protective, anti-microbial, hypotensive and anti-diabetic activity [32]. Although a memory-enhancing effect in chronically stressed mice has been found in a related species *Anchusa italic* [32] no studies until now have demonstrated an anti-Alzheimer’s effect for *Anchusa strigosa*.

Three other species in this study were also associated with neuroprotection against amyloid toxicity: #120 (*Paronychia argentea Lam*), #107 (*Sisymbrium irio L*) and #114 (*Origanum dayi Post*). Of these only *Paronychia argentea* has demonstrated potentially relevant activity with acetyl-cholinesterase inhibitory activity, the key enzyme in the breakdown of acetylcholine, and considered a promising strategy for the treatment of neurological disorders such as AD [33].

The wide spectrum of effects noted in this study, including protection against amyloid cellular toxicity as well as inhibition of enzymatic pathways involved in amyloid formation makes several of these plants both interesting and potentially valuable in the treatment and prevention of AD. Further research is needed to analyze the active constituents responsible for these effects and evaluate their in-vivo efficacy and toxicity. Using whole plant extracts as novel preventative and/or therapeutic agents in AD should also be considered in view of the increasing attention natural products have received in the last decades for their potential in treating many diseases and the significantly shorter time and lower costs required for them to become commercially available.

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