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Research Article

A histopathological comparison of prophylactic effects of Rosmarinic Acid and Oleanolic Acid isolated from Salvia species (sage) in scopolamine induced dementia model

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Abstract

Objective: Alzheimer's disease (AD) is the most common form of dementia, can be created in experimental models by using toxins. Possible therapeutic effects of secondary metabolites Rosmarinic (RA) and Oleanolic acids (OA) obtained from *Salvia* species (sage) were investigated using histopathological and immunohistochemical techniques in experimental Alzheimer's-like dementia model induced by scopolamine.

Study Design: Male BALB/c mice (n: 48) of 3-4 weeks old, divided into 6 groups; control received only saline (i.p.) for 21 days, scopolamine group received 3 mg/kg of scopolamine (i.p.) between 8-21th days. Scopolamine + RA group received 5 mg/kg RA (i.p.) between days 0 and 21 and 3 mg/kg of scopolamine (i.p.) between 8-21th days. Scopolamine + OA group received 5 mg/kg of OA (i.p.) between days 0-21 and 3 mg/kg of scopolamine (i.p.) between 8-21th days. RA group received 5 mg/kg RA (i.p.) between 0-21th days. OA group received 5 mg/kg of OA (i.p.) between 0-21th days. Animals were sacrificed under anesthesia, brain tissues were excised and placed in 10% formaldehyde, embedded in paraffin wax. 5 µm sections were cut, stained for histopathological examinations. Immunoreactivity for Beta-amyloid accumulation was observed. ImageJ 153 software was used to analyze IHC figures. Positive signaling for DAB density was calculated, ANOVA test with the post hoc dunnett's or tukey test were applied, p<0.05 was considered statistically significant.

Results: In scopolamine+RA treated group, tissue degeneration was less compared to the scopolamine group. Scopolamine+OA group revealed signs of pyknosis in neurons. The amyloid beta immunoreactivity was positive in scopolamine group partially positive in scopolamine+OA group but, negative in RA treated scopolamine group. Intensity of signal in scopolamine group was statistically increased compared to control (p=0.0102). Intensity of signal was reduced in Scopolamine+RA and Scopolamine+OA groups, was statistically significant (p=0.0281 and p=0.0362, respectively).

Conclusion: We suggest that pretreatment of RA and OA decreased beta amyloid formation and ameliorated tissue structure but, further studies with different methods are needed to be commercially available.

Keywords: Scopolamine, dementia, Rosmarinic acid, Oleanolic acid, histopathology, Amyloid beta

1. INTRODUCTION

Dementia is defined as a disorder in brain functions. Alzheimer's disease (AD) is the most common form of dementia. Alzheimer's disease is a chronic neurodegenerative disease characterized by progressive memory loss, language skills, disorientation, depression and behavioral disorders^{1,2}. In the pathophysiology of AD, two main brain cell types involved in the immune/inflammatory response in Alzheimer's disease are astrocytes and microglia. The numbers of astrocytes are more in

Alzheimer's patients and these cells are activated to produce prostaglandins that cause inflammation. Active microglial cells produce free radicals damaging cell structure and function³. When exposed to oxidative stress, these active microglia cells form a peptide known as amyloid beta (beta amyloid, Aβ) that kills neurons, which aggregates on the outer surface of neurons in the blood vessels and brain called amyloidosis. Amyloid beta is available in two forms; Aβ-40 and Aβ-42. After the fibrillar structure formed by these two forms, amyloid plaque formation is observed. Amyloid plaque formation causes oxidative damage and

Neurofibrillary tangles (NFTs) formation^{4,5}. Considering the pathophysiology of AD, the key role begins at the point of A β production.

Researchers have revealed that the deterioration in cholinergic function impairs short-term memory and results with memory impairment similar to AD. Scopolamine, a well-known anticholinergic drug, is frequently used for experimental purpose to induce dementia in experimental animal models^{6,7}. Administration of scopolamine produces deficits on tests of visual recognition memory, visuospatial recall, verbal recall, visuospatial praxis, psychomotor speed and visuo-perceptual function^{8,9}. It has been accounted that scopolamine nonselectively blocks the binding sites of acetylcholine (ACh) muscarinic receptors in the cerebral cortex and causes unequal release of ACh which destroys the hippocampus nerves and leads to impairment in learning and memory in a dose-dependent manner in mice¹⁰.

In the treatment of AD, several studies have been carried out to prevent the progression of the disease by developing inhibitors as an alternative. Nutrition and food chemistry research in recent years has focused on herbal products with anti-Alzheimer's, anti-cancer and antioxidant potential. As epidemiological evidence confirms that medicinal plants are important sources in reducing the frequency of cancer, diabetes, atherosclerosis etc., obtaining drugs from medicinal plants are being investigated for therapeutic purposes. *Salvia L. (Lamiaceae)* also known as sage, have been used in traditional medicine as a tonic, antirheumatic and chronic pain reliever since ancient times. *Salvia L.*, which contains active substances with antioxidant effect and has more than 1000 species in the world and 100 species are represented in Anatolia, of these 53 are endemic¹¹. Some members of the genus *Salvia L.* have been the subject of intense research due to their different medicinal values such as wound healing^{12,13}. *Salvia* species have a diversity of secondary metabolites giving them antimicrobial and antioxidant property that are used against pathological diseases such as atherosclerosis, brain dysfunctions and cancer¹⁴. Recent studies proved that genus *Salvia* is an important potential source of antioxidants and anticholinesterases. A *Salvia* species, *S. plebeia* is used in traditional medicine in China, Korea, and Japan to treat diseases such as jaundice, inflammation, and hemorrhoids. Pharmacological studies have shown that *S. plebeia* species has a wide range of biological activities such as antityrosinase, antioxidant and antianaphylactic effects¹⁵. *Salvia* species are known for their memory enhancing purposes based on their pharmacokinetic properties^{16,17}. *Salvia* having these qualifications are considered to be of potential value in AD therapy.

The major phytochemicals in flowers, leaves, and stem of *Salvia* are well identified including alkaloids, carbohydrate, fatty acids, phenolic compounds (flavonoids, tannins), steroids, terpenes/terpenoids¹⁸. Rosmarinic acid (RA) (C₁₈ H₁₆O₈) from flavonoids and Oleanolic acid (OA) (C₃₀ H₄₈O₃) from terpenes/terpenoid contents were used for the current study. There are several studies in the literature regarding the effects of these compounds, especially RA. RA beneficially inhibits invasion, angiogenesis and metastasis of tumor cells^{19,20}. In addition to the anti-cancer potential of these compounds, they are used in the areas of cosmetic and meat industries etc., due to their high antioxidant properties so their market value is high. RA is rapidly eliminated from human and rat blood circulation after oral administration and have been reported to exhibit low toxicity in the literature²¹.

In this study, prophylactic and therapeutic effects of secondary metabolites Rosmarinic and Oleanolic acids obtained from *Salvia* species were investigated and compared in mice with Alzheimer's-like dementia induced with scopolamine using histopathological and immunohistochemical techniques.

2. METHODS

2.1. Plant material and extraction procedures

Salvia plant materials were collected from their natural habitats in the Eastern Anatolian Region of Turkey, Diyarbakır (collection date 2018) and air-dried. Aerial parts of the dried plant were macerated with 55–75% v/v EtOH (Merck) in water at 40 °C for 3 h and filtrated²². Stock solutions of Oleanolic and Rosmarinic Acid compounds with analytical purity were prepared and administered at a concentration of 1mg/ml.

2.2. Experimental design and groups

The experimental protocol was approved by the Local Ethical Committee of Health Sciences, Dicle University, Diyarbakır, Turkey (Reg. no. 2018/20). The research was performed in accordance with the ARRIVE guidelines and the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animals (NIH Publications). Experimental animals were obtained from Dicle University Health Sciences Research and Application Center (DUSAM). This study was supported by Dicle University Research Projects Unit (DUBAP) with the Project number (TIP.19.026).

In the experimental protocol, 48 male 3-4 weeks old BALB/c mice were used. The animals were housed in a room with a 12-hour dark, 12-hour light cycle, with 50–70% humidity, at room temperature (25 °C $\pm$ 3 °C), in cages containing up to 8 animals, with free access to water and pellet.

According to the experimental protocol, injections were performed daily at the same time period intraperitoneally (ip). Scopolamine (Scopolamine hydrobromide CAS no: 6533-68-2, Sigma-Aldrich) and, RA and OA extracts dissolved in ethanol were applied 0.2 ml for each as described above.

The groups were created as follows;

Group 1: Control group (n:8) received 0.2 ml of 0.9% saline (i.p.) solution daily for 21 days.

Group 2: Scopolamine group (n:8) received 0.2 ml 0.9% saline (i.p.) solution daily between 0-7th days. 3 mg/kg of scopolamine (i.p.) was administered daily between 8-21th days.

Group 3: Scopolamine + Rosmarinic acid group (n:8) received 5 mg/kg Rosmarinic acid (i.p.) daily between days 0 and 21. Between 8-21th days they received 3 mg/kg of scopolamine (i.p.) daily.

Group 4: Scopolamine + Oleanolic acid group (n:8) received 5 mg/kg of Oleanolic acid (i.p.) between days of 0-21 daily. Between 8-21th days they received 3 mg/kg of scopolamine (i.p.) daily.

Group 5: Rosmarinic acid group (n:8) received 5 mg/kg Rosmarinic Acid (i.p.) daily between 0-21th days.

Group 6: Oleanolic acid group (n:8) received 5 mg/kg of Oleanolic acid (i.p.) daily between 0-21th days.

2.3. Anesthesia and Surgical Procedures

At the end of experiments, all subjects were sacrificed by cervical decapitation under ether anesthesia. The cerebral samples (hippocampal and cortical tissue) were taken immediately. The excised tissues were taken in 10% formaldehyde for routine histological tissue preparation and finally embedded in paraffin blocks.

2.4. Hematoxylin Eosin examination

Tissue sections of 5 μ m thickness were cut with a microtome (Leica, Germany) and taken on slides, then placed into xylene for 2x15 min. After the decreasing alcohol series, slides were taken in hematoxylin stain (Sigma, US) for 6 min. Washed in running tap water for 5 min, they were kept in eosin for 4 min. Immediately after 1 min. increasing alcohol series, then slides were incubated in xylene for 2x15 min. Finally, sections were mounted with entellan (Sigma, US) for histopathological examinations.

2.5. Immunohistochemical Procedure

Remaining sections taken from paraffin blocks were processed for deparaffinization, passed through alcohol series and taken into distilled water. Slides were dried without damaging the tissue then marked with Dakopen (Dako, Glostrup, Denmark). In order to inhibit tissue endogenous peroxidase, 3% hydrogen peroxide was applied for 20 min. Ultra V block (TA-125-UB, Thermo Fisher Scientific, US) was dropped onto the sections for 8 min prior to the addition of the primary antibodies, which were incubated at +4° C overnight (Beta Amyloid Antibody (cat # 71-5800) Thermo Fischer Scientific, US, 1:100). Sections were washed with biotinylated secondary antibody (Thermo Fischer Scientific, US) for 14 minutes. Streptavidin peroxidase (Thermo Fischer Scientific, US) was dropped onto sections. Chromogen DAB (Thermo Fischer Scientific,

US) was applied and counter stained with Harris hematoxylin and slides were mounted with entellan. Sections were examined under Light microscope (Zeiss, Imager A2, Germany), and the levels of amyloid beta accumulation were compared.

2.6. Signal quantification and statistical analysis

Signal quantification for positive beta amyloid accumulation was done by Image J 153 software by analyzing IHC figures. DAB density was calculated by setting image threshold and measuring signals. Statistical analysis was performed with SPSS IBM 24.0 software. Differences of means between groups were calculated by one-way ANOVA test and then with the post hoc dunnett's or tukey test were applied for multiple comparison test. The $p < 0.05$ level was considered statistically significant in the results.

3. RESULTS

3.1. Histopathological findings

Hematoxylin-eosin stained sections of control group cerebral tissues revealed normal neuron structures and cerebral tissue integrity (Figure 1.A). In scopolamine group, signs of pyknosis were remarkable especially in neuron structures. Degenerations were observed in areas of tissue structure. Presence of amyloid plaque structure was found in the areas close to the neurons (Figure 1.B). RA showed improvements in areas of tissue degeneration in the sections of scopolamine+RA group. Structure of amyloid plaques was not observed and protective activity in the structure of neurons was prominent (Figure 1.C). The tissue integrity was improved in neuron structures thanks to protective activity of OA (Figure 1.D). However, OA has seemed to be less protective than RA from the histopathological point of view (Figure 1.E).

Figure 1. A) Control group; A neuron in normal structure (black arrow). B) Scopolamine group; A degenerated neuron (black arrow), tissue degeneration (red arrow), amyloid plaque structure (orange arrow). C) Scopolamine + RA Group; A neuron (black arrow) and degenerated tissue (red arrow) showing improvement compared to the scopolamine group. D) Scopolamine + OA Group; tissue degeneration (red arrows) and a pycnotic neuron. E) RA Group; A neuron in normal structure (black arrow) and F) OA Group; A neuron in normal structure (black arrow).

3.1. Immunohistochemical findings

Beta amyloid expressions in cerebral tissue of all groups were shown in Figure 2. The expression of amyloid beta in the control group was negative. On the other hand, the presence of neurons with positive structure of scopolamine

group amyloid beta antibody staining was detected (Figure 2 A, B). When the sections of RA and OA groups were compared for amyloid beta expression, it was clearly decreased due to the protective activity of RA. We can suggest that the protective activity of OA is not effective as much as RA (Figure 2 C, D).

Figure 2. A) Control group; Negative amyloid beta expression (black arrow). B) Scopolamine group; Positive amyloid beta expression (red arrow). C) Scopolamine + Rosmarinic Acid Group; Negative amyloid beta expression (black arrow). D) Scopolamine + Oleanolic Acid Group; Negative amyloid beta expression (black arrow) and Positive amyloid beta expression (red arrow). E) Rosmarinic Acid Group; Negative amyloid beta expression in neurons (black arrow) F) Oleanolic Acid Group; Negative amyloid beta expression in neurons (black arrow)

3.2. Statistical findings

Positive beta-amyloid antibody signal was quantified by ImageJ 153 software. Scopolamine group showed the highest density of measured signal, while control group resulted in lowest score. Intensity of signal in scopolamine was

statistically increased compared to control group ($p=0.0102$). Intensity of signal was reduced in Scopolamine + Rosmarinic Acid and Scopolamine + Oleanolic Acid groups, and this decrease was statistically significant ($p=0.0281$ and $p=0.0362$, respectively). Results were shown in Table 1 and Figures 3.

Table 1. Positive beta-amyloid signaling by all groups.

Groups	Total area	Mean+SD	P value
Control group	2073600	0.103±5.119	
Scopolamine group	2073600	3.521±25.759	0.0102*
Scopolamine + Rosmarinic Acid	2073600	1.092±4.842	0.0281**
Scopolamine + Oleanolic Acid	2073600	1.788±8.565	0.0362***
Rosmarinic Acid	2073600	0.166±6.512	
Oleanolic Acid	2073600	0.204±7.206	

Note: Area represents the size of the IHC image. Mean grey value gives the quantified signal of antibody of interest. * Scopolamine vs control; ** Scopolamine + Rosmarinic Acid vs Scopolamine; ***Scopolamine + Oleanolic Acid vs Scopolamine

Figure 3. Graphical illustration of Table 1. Positive beta-amyloid signaling for groups

Sc: Scopolamine, RA: Rosmarinic acid, OA: Oleanolic acid.

4. DISCUSSION

Aging population is increasing day by day so researchers have to encounter with a significant rise in the incidence of age-related neurodegenerative diseases one of which is dementia. Several toxins are being used in experimental models to examine dementia based on their mechanism of action for learning and memory impairment such as scopolamine, which mimics the disease pathology of Alzheimer's disease like dementia²³.

The knowledge regarding the chemical composition and health-related benefits of *Salvia* species widely used in complementary and traditional medicine, are considerable. *Salvia* species contain compounds that have antioxidant, antidementia, anti-inflammatory, anticancer, antinociceptive, antimicrobial, antimutagenic, hypoglycemic and hypolipidemic effects¹⁸. A review study on potential cognitive enhancing and protective effects of *Salvia* summarizes that 50 µL of *S. lavandulaefolia* essential oil, administered 1–3 times per day over a 3-week period, statistically significant reductions in caregiver-rated neuropsychiatric symptoms, and improvements in attention over the 6-week period has been reported^{24,16}. The medicinal plant *Salvia* has been used for Alzheimer's disease²⁵. In another randomized placebo-controlled trial study on patients with Alzheimer's disease for the effects of *S. officinalis* on memory and cognitive functions a 60 drops/day of alcoholic extract for week 16 has resulted with an improvement of cognitive functions²⁶. Clinical and animal studies presented that *S. officinalis* advanced cognitive performance in patients with cognitive impairment or dementia²⁷.

Our histopathological results with hematoxylin-eosin staining, in scopolamine group, depicted remarkable signs of pyknosis in neuron structures. Degenerations in areas of the tissue structure and presence of amyloid plaque structure was found in the areas close to the neurons (Figure 1.B). In scopolamine+RA group, no amyloid plaques were observed (Figure 1.C). OA treated scopolamine brain tissue sections showed tissue integrity and improvements in neuron structures (Figure 1.D). However, OA has seem to be less protective than RA from the histopathological point of view (Figure 1.) In immunohistochemical analysis, the presence of neurons with positive structure of scopolamine group amyloid beta antibody staining was detected (Figure 2 A, B). When the sections of Scopolamine +RA and Scopolamine +OA

groups were compared with ImageJ analysis and statistically analysed for amyloid beta immunoreactivity, it was found to be decreased due to the prophylactic activity of RA (Table 1, Figure 3). We suggest that the protective activity of OA is not as effective as RA in scopolamine administered groups (Figure 2 C, D and Table 1).

CONCLUSION

From a histopathological perspective, *Salvia* extracts RA and OA seems to decrease amyloid plaques formation. Yet, we observed that RA had a more comprehensive protective activity than OA in the scopolamine-induced Alzheimer like dementia experimental mice model. RA and OA might be promising components for developing therapeutic agents to ameliorate dementia but, their long term effects require investigation.

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Conflict of Interest

The authors have no conflict of interest.

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