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An Investigation of Anticholinesterase and Anticancer Effects of *Verbascum insulare Boiss. Et Heldr.* Extracts Growing in Muş Region

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Abstract

Since the existence of humanity, plants have been used in many treatment methods. Members of the genus *Verbascum*, a member of the Scrophulariaceae family, also known as mullein, have recently received great attention due to their remarkable biological activities and have been evaluated in traditional therapeutic uses against various ailments. In this study, the anticholinesterase and anticancer activities of *Verbascum* member *Verbascum insulare* Boiss. Et Heldr. collected from Muş region were investigated. *V.insulare* leaf ethanol (L-EtOH), leaf purified water (L-PW), root ethanol (R-EtOH), and root purified water (R-PW) extracts were obtained. The anticancer activity of the extracts against HT-29, MCF-7, and L-929 cell lines was examined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, and the anticholinesterase activity of the extracts was determined. It was found that the ethanol extract showed higher anticancer activity against cancer cell lines than the pure water extract and had more effective anticholinergic influences. It is aimed to shed light on the biological effects of *Verbascum insulare* Boiss. Et Heldr, which is thought to be a natural source of bioactive compounds for the development of phytopharmaceuticals targeting oxidative stress-related diseases such as cancer and neurological diseases. No study has been found in the literature on the anticancer and anticholinesterase activity of this plant yet. Therefore, the results obtained from this study will contribute significantly to the development of herbal medicine in the future.

1. Introduction

Nowadays, plants are of great interest all over the world as an effective therapeutic agent for the treatment and prevention of diseases. In addition, plants, which form an important component of foodstuffs, have formed the basis of traditional medicine throughout history. It has been determined that there are approximately 420,000 plant species on earth, and more than 35,000 of these plants are used for medicinal purposes [1]. *Verbascum* genus, a member of the Scrophulariaceae family, also known as "king's candle or mullein" in the flora of Turkey, has 233 species, 185 of which are endemic, and more than 100 hybrids [2]. In traditional Turkish medicine, all aerial parts of *Verbascum* L. (Scrophulariaceae) species are used as a drying agent for wounds such as inflammatory skin disorders. In addition, some species of the plant are reported to have expectorant, mucolytic and sedative properties used in the treatment of respiratory ailments such as bronchitis, tuberculosis, and asthma [3]. *Verbascum nubicum* Murb species showed significant hepatoprotective, anti-inflammatory, and antioxidant effects[4]. *Verbascum bombyciferum* Boiss inhibited several enzymes, including acetylcholinesterase and butyrylcholinesterase[5]. In a study investigating the

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antiproliferative effect of different extracts of *Verbascum sinaiticum* against Hep-2, MCF-7, and Vero cell lines, it was determined that the ethanol extract of the plant exhibited high antiproliferative potential against the tested cell lines [6]. In a study of the phytochemical examination of *Verbascum thapsus*, it was determined that luteolin and 3-O-fucopyranosylsaikogenin F compounds induced apoptosis of A549 lung cancer cells and showed promising antiproliferative activities as a result [7]. Phenolic content of *Verbascum insulare* Boiss. Et Heldr., antimicrobial, antioxidant, and DNA protective activities of different extracts were determined. It was found that ethanolic extract indicated higher antioxidant and antifungal activity and increased DNA stability than pure aqueous extract [2].

Due to the increase in the number of older adults, the prevalence of diseases such as cancer and neurodegenerative disorders has increased greatly in the world in recent years. Alzheimer's disease (AD) is the most common neurodegenerative disease that progresses over time and is very difficult to diagnose, seen in middle-aged and elderly individuals. This disease is characterized by early progressive loss of neuronal cells [8]. Cancer is characterized by inappropriate cell proliferation and resistance to cell death. The common causative factor of AD and cancer is thought to be aging. It is possible to associate cancer and Alzheimer's disease with various mechanisms, and one of these mechanisms is that both diseases increase oxidative stress and thus tumor or neuronal cells become vulnerable [9]. It is known that oxidative stress increases colon and breast cancer types [10]. Consequently, new, safe and effective therapeutic approaches are needed to control these diseases. Considering the above, many *Verbascum* species can be considered promising raw materials for the design of new pharmaceutical products. While many *Verbascum* species have been studied, more research is needed to provide scientifically validated information, particularly on poorly studied species. Our aim with this research article is to investigate the anticholinesterase and anticancer activities of the medicinally important plant *V.insulare* and determine whether it can be used in the pharmaceutical industry.

2. Material and Method

2.1. The Extraction and Collection of Plant Samples

V.insulare plant was collected during the vegetation period in 2018 from Guzeltepe village of Mus province. The identification of the collected plant samples according to the Flora of Turkey was done by Murat Kurşat (Bitlis, Turkey). The plant samples were turned into herbarium material (Y. Alan: 4913) and stored in Muş Alparslan University, Central Research Laboratories Application and Research Center. The leaves and roots of the plant were removed and left to dry in the shade. *V.insulare* leaf ethanol (L-EtOH), leaf purified water (L-PW), root ethanol (R-EtOH), and root purified water (R-PW) extracts were prepared according to our previous study [2].

2.2. Cholinesterase Inhibition Assay

Acetylcholinesterase (AChE) from Electric eel (*Electrophorus electricus*) and butyrylcholinesterase (BChE) from equine serum were obtained from Sigma- Aldrich (St. Louise, MO). The inhibitory effects of *V. insulare* extracts against AChE and BChE were measured using a slight modification of Ellman's spectrophotometric method [11] as described previously [12] and using commercially available galantamine as the reference compound. Acetylthiocholine iodide (AChI), butyrylthiocholine iodide (BChI) were used as the substrate in the reaction, 5′dithiobis-2 -nitrobenzoic acid (DTNB) was used as Ellman's reagent were purchased from Sigma - Aldrich. The absorbance of the reaction mixture was measured at 412 nm three times within 5 min of the start of the reaction on a Thermo Fisher Scientific Multiskan Go Finland, and the results are reported as mean ± standard deviation. Activity (%) was plotted to determine the inhibitory effects of *V. insulare* extracts on AChE and BChE. IC_{50} values were obtained by activity (%) versus compound plots.

2.3. The Application of Extracts to Cell Lines

Human colon cancer cell line (HT-29), human breast cancer cell line (MCF-7), and healthy mouse fibroblast cell line (L-929) were used. Dulbecco's Modified Eagle Medium (DMEM) was used as the medium for these cell lines. *V. insulare* extracts were prepared in the DMEM at concentrations of 0.1, 0.2 and 0.5 mg/mL, and the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was applied to determine their cytotoxicity [13]. For the MTT test, $5x10^3$ cells were seeded in 100 µL of the medium in each well of 96 well plates. The inoculated cells were incubated in a 5% CO_2 incubator for 24-48 hours. 100 µl of the diluted extracts in the medium was added to the cell lines. Only 100 µl of the medium

was added to the cells in the control wells. Samples were incubated for 24 hours. After incubation, the medium in the wells was removed with the help of a vacuum pump. 10 µl of MTT solution and 90 µl of medium were added to each well and placed in a CO_2 incubator at 37℃ for 4 hours. After 4 hours, the medium containing MTT was removed from the 96 well plate. 100 µl of Dimethylsulfoxide (DMSO) was added to each well, and their optical density (OD) was measured with a microplate reader (Thermo Fisher Scientific Multiskan Go, Finland) at 540 nm wavelength. A cell line medium without a sample was used as the control group. The average absorbance values obtained by reading the control wells was taken, and this value was accepted as 100% viable cells. The % inhibition rates of the cells were calculated with the help of the following formula.

$$\%\ \text{inhibition} = 1 - \left(\frac{\text{OD}_{\text{Sample}}}{\text{OD}_{\text{Control}}}\right) \text{x}100$$

2.4. Statistical Analysis

The % inhibition results of the cell culture and the cholinesterase inhibitory activity were compared among themselves using the t-test following One Way ANOVA. Those with $p<0.05$ were statistically significant, and the statistical significance level was indicated with the symbol "*". In this respect, $P<0.05$ (significant); *, $P<0.01$ (very significant); **, $P<0.001$ and $P<0.0001$ (extremely significant); ***-**** and $P>0.05$ (not significant), ns.

3. Results and Discussion

3.1. Anticholinergic Activity

Today, the inhibition of cholinesterases has emerged as an encouraging approach to alleviate the symptoms of Alzheimer's disease. Therefore, inhibition of AChE and BChE enzymes is one of the main goals of researchers. Various cholinesterase inhibitors such as galantamine, tacrine, and rivastigmine are produced synthetically. However, these drugs have side effects such as nausea, diarrhea, dizziness, and vomiting [14]. For these reasons, the discovery of natural and safe enzyme inhibitors has become inevitable. Therefore, the enzyme inhibition properties of *V. insulare* extracts tested against AChE and BChE enzymes were examined by comparing them with galantamine(Table 1 and Figure 1).

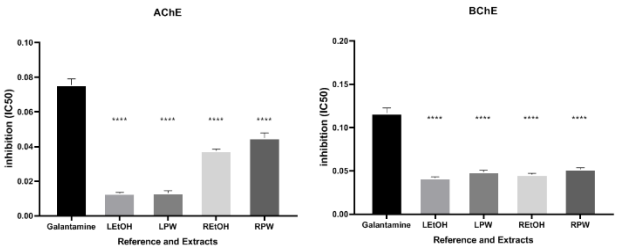
Table 1. AChE/BChE inhibition assays for LEtOH (ethanolic leaf), LPW (aqueous leaf pure), REtOH (ethanolic root) and RPW (aqueous root pure) extracts of *V.insulare*

Extracts	AChE inhibition IC_{50}	BChE inhibition IC_{50}
LEtOH	0.013±0.001	0.042±0.002
LPW	0.014±0.002	0.049±0.003
REtOH	0.038±0.002	0.046±0.002
RPW	0.044±0.005	0.052±0.003
*Galantamine	0.078±0.006	0.117±0.010

*Galantamine was used as a positive control for Cholinesterase enzymes and determined as µM levels. Cholinesterase inhibitory activity of the extracts was tested against AChE and BChE at 500 µg/mL concentration.

All extracts of *V. insulare* exhibited potent AChE and BChE inhibitory activity at 500 µg/mL. In addition, these extracts showed a better AChE and BChE inhibitory effect than galantamine, a cholinesterase inhibitor used as a therapeutic agent in the treatment of Alzheimer Demands. Galantamine which is used as standard cholinesterase inhibitor was found to have IC_{50} values of 0.078±0.006 µmol/L and 0.117±0.010 µmol/L, respectively, against AChE and BChE enzymes. LEtOH extract of *V. insulare* showed a stronger inhibitory effect for both enzymes than galantamine and other extracts. When the extracts and galantamine activities were compared, it was determined that the extracts showed a highly significant difference from galantamine in general ($P<0.0001$). It has also been reported that many *Verbascum* species inhibit cholinesterase enzymes. For example, in the study of Angeloni et al., aqueous and methanolic extracts of *Verbascum bombyciferum* were obtained using different techniques such as HAE (homogenizer assisted), MAC (maceration), and infusion was found to exhibit anticholinesterase effects. They found that especially water extracts (maceration: 0.30 ± 0.08 mg GALAE/g and homogenizer assisted extraction: 0.28 ± 0.02 mg GALAE/g) were more weaker inhibitors of AChE when compared to methanolic extracts (homogenizer assisted extraction: 1.89 ± 0.15 mg GALAE/g and maceration: 2.02 ± 0.20 mg GALAE/g) [5]. It was found that aqueous and methanolic extracts of *V. oocarpum* showed an anticholinesterase effect, and the inhibition range varied between 66.7 ± 1.2 and 28.8± 0.5 at 200 µg/mL. As a result, it was determined that the plant extracts had moderate AChE and BChE inhibitory activities [15]. Verbascoside isolated from the aqueous extract of *V. mucronatum* Lam was found

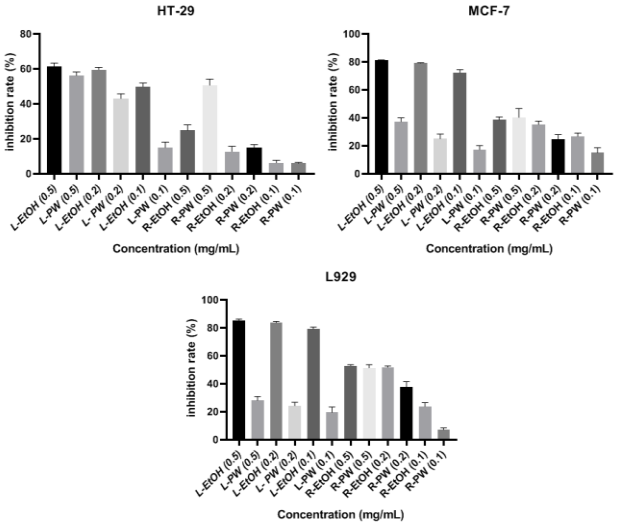
to inhibit cholinesterase enzymes. According to their data, verbascoside exhibited moderate inhibition of AChE compared to galantamine but was ineffective against BChE [16].



In a study to determine the anticholinesterase activity of extracts of two endemic *Verbascum* species using five different solvents, the hexane extract of (*V. oocarpum)* one species (AChE = 2.35 and BChE = 4.68 mg GALAE/g) and the ethyl acetate extract of (*V. euphraticum)* the other species of (AChE = 1.96 and BChE = 3.29mg GALAE/g) were reported to show high anticholinesterase activities. In this context, cholinesterase inhibition assays can offer the potential therapeutic value of many *Verbascum* species to control neurodegenerative complications [17].

3.2. The Cytotoxicity of Extracts against Cancer and Healthy Cell Lines

V.*insulare* extracts at different concentrations (0.1, 0.2, and 0.5 mg/mL) were treated with HT-29, L-929, and MCF-7 cells for 24 hours, and the % inhibition values of the cell lines were calculated with the measured absorbance spectrophotometrically. The MTT test determined the inhibition of cell proliferation. The % inhibition graphs of cell lines are given in Figure 2.



L-EtOH extract at 0.2 and 0.1 mg/mL concentrations against the HT-29 cell line displayed a highly significant difference compared to L-PW. In addition, it was determined that the R-PW extract displayed a highly significant difference from the R-EtOH extract. L-EtOH extract at all concentrations displayed a highly significant difference compared to L-PW against MCF-7 and L-929 cell lines. At the same time, it was determined that the 0.2 and 0.1 mg/mL concentrations of R-EtOH extract against MCF-7 and L-929 cell lines showed a highly significant difference compared to R-PW. When evaluated in general, it was determined that the antiproliferative properties of ethanol extracts were higher than those of pure water extracts in all cell lines. It is known that plants have been used in cancer treatment since ancient times. The compounds found in the extracts obtained from the plants used for this purpose show significant anticancer activity[18]. In previous studies, it has been reported that these compounds found in extracts obtained from plants have anticancer effects [19].

Although there are studies on species belonging to different *Verbascum* genus in the literature, no anticancer study has been found on the *V.insulare* species used. A study reported that the methanolic extract obtained from the *Verbascum* genus had a cytotoxic effect against the melanoma cell line but was ineffective against the ovarian cell line [20].

IIiescu et al. determined that the methanolic extract from the aerial parts of *Verbascum nigrum* did not affect the cytotoxicity of HaCaT cells but showed a significant effect on A431 cells [21]. The effects of extracts obtained from plants vary according to cell lines. The data obtained in this study also vary according to cell lines, as in previous studies. Tatlı and Akdemir compared the cytotoxic effect of methanol and ethyl acetate extracts of some *Verbascum* species in Turkey and reported that ethyl acetate extract showed a stronger cytotoxic effect against cancerous cell lines [20]. In a different study, the cytotoxicity of *V. sinaiticum* Benth flower extracts prepared with different solvents against mammary adenocarcinoma MCF-7 was examined, and it was seen that ethanol extract showed the best cytotoxicity [6]. It was also reported that the hydroethanolic leaf extract of the same plant showed the best cytotoxic effect against HepG2 and MRC-5 cell lines [22]. The cytotoxic effect of methanol and hydromethanol extracts obtained from *V. calvum* were compared, and it was reported that the methanol extract displayed important antiproliferative effects against A-549 lung cancer cell line [23]. The plant extracts obtained using different solvents consist of different compounds.

For this reason, it has been determined that the extracts exhibit different anticancer activities in studies. According to the results of this study, it was determined that ethanol extract showed a better antiproliferative effect than pure water extract. This study supports the view that the cytotoxic effect may be due to the compounds contained in the extract, as in previous studies.

4. Conclusion and Suggestions

It is known that the damage caused by oxidative stress in cells and tissues may play a role in the development of many diseases such as Alzheimer's and cancer in the organism. In the key enzyme inhibition theory, which is effective for the management of neurological diseases such as Alzheimer's, inhibition of these enzymes can alleviate existing symptoms. For this purpose, the presence of natural inhibitors for cholinesterase inhibition is very important. In this study, anticancer activity of *V.insul*are, a species belonging to the genus *Verbascum*, against human colon cancer cell line (HT-29), human breast cancer cell line (MCF-7), and healthy mouse fibroblast cell line (L-929), and enzyme (AChE, BChE) inhibitory effects were investigated for the first time. It was determined that the ethanol extract from the extracts obtained by using different plant parts and solvents showed better anticholinesterase activity and antiproliferative properties than the pure water extract. In addition, leaf ethanol extract showed the best activity for both biological activity tests. This is thought to be due to the fact that the extracts prepared in different parts of the plant and in different solvents contain different compounds. It is anticipated that future studies will isolate and purify the active compounds and investigate their effects on a wider variety of cancer cells and enzymes. However, *in vivo* tests, namely more studies, are needed to explore the full mode of action of the active compounds in herbal medicine development and to test the safety of the extracts used here.

Contributions of the authors

Corresponding author: Data curation, writing (original draft, review & editing), investigation, visualization methodology, *in vitro* studies and interpretation of experimental results.
Coauthor: Methodology, investigation, visualization, literature review, *in vitro* studies, and interpretation of experimental results.

Conflict of Interest Statement

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

Research and publication ethics compiled within the study.

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