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Investigation of Anti-Alzheimer and Anti-diabetic Activity of Callus Culture of Bellevalia edirnensis ÖZHATAY & MATHEW: An Endemic Plant from Turkey

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ABSTRACT

Callus culture, an alternative technique for the production of secondary metabolites, is a plant tissue culture method. In this study, it was aimed to determine the anti-alzheimer and anti-diabetic activity of ethanol extracts of callus tissue obtained from the Edirne hyacinth (*Bellevalia edirnensis* ÖZHATAY & MATHEW), which is endemic and is in danger of extinction. Callus induction started by sterilization of the seeds in 20% bleach and 70% ethanol. Following, it was achieved by culturing in MS medium containing (Murashige & Skoog) GA_3 in 3 different concentrations in addition to the medium supplemented with 7 g/L agar and 30 g/L sucrose. Anti-Alzheimer activity of the obtained calluses were determined using the Ellman method which is a spectrophotometric method. Anti-diabetic activities were determined using the α-glucosidase inhibition method. According to the results obtained, it was found that the callus culture of *Bellevalia edirnensis* ÖZHATAY & MATHEW showed moderate Anti-Alzheimer and Anti-diabetic activity

Keywords: Anti-Alzheimer Activity; Anti-diabetic Activity; *Bellevalia edirnensis*; Callus Culture

Bellevalia edirnensis ÖZHATAY & MATHEW: Türkiye Endemik Bitkisinin Kallus Kültürünün Anti-Alzheimer ve Anti-diyabetik Aktivitesinin Araştırılması

ÖZ

Sekonder metabolitlerin üretimi için alternatif bir teknik olan kallus kültürü, bir bitki doku kültürü yöntemidir. Bu çalışmada, endemik ve nesli tükenme tehlikesi altında olan Edirne sümbülünden (*Bellevalia edirnensis* ÖZHATAY & MATHEW) elde edilen kallus dokusunun etanol ekstraktlarının anti-alzheimer ve anti-diyabetik aktivitesinin belirlenmesi amaçlanmıştır. Kallus indüksiyonu, tohumların % 20 çamaşır suyu ve % 70 etanol içerisinde sterilize edilmesiyle başladı. Ardından 7 g/L agar ve 30 g/L sükroz katkılı besiyerine ek olarak 3 farklı konsantrasyonda (Murashige ve Skogg) GA3 içeren MS besiyerinde kültüre edilerek elde edildi. Elde edilen kallusların Anti-Alzheimer aktivitesi spektrofotometrik bir yöntem olan Ellman yöntemi kullanılarak belirlendi. Anti-diyabetik aktiviteler, α-glukozidaz inhibisyon yöntemi kullanılarak belirlendi.

Anahtar Kelimeler: Anti-Alzheimer Aktivite; Anti-diyabetik Aktivite; *Bellevalia edirnensis*; Kallus Kültürü

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1. INTRODUCTION

Secondary metabolites, like primary metabolites, are not responsible for performing the basic vital activities of the plant, but they have the functions of adapting plants to the environment and pollination, protection and maintaining their generation. Secondary metabolites from plants are divided into three major categories, phenolics, terpenes and alkaloids. As reported in previous studies, secondary metabolites have been reported to be responsible for numerous biological processes, including antioxidant, antimicrobial, anti-inflammatory, anticancer, anti-Alzheimer and antidiabetic (Mazid et al., 2011; Ceylan et al., 2021; Elansary et al., 2019). Turkey has a very wide flora due to climatic conditions and different altitudes. Most of these plants are aromatic and are widely used by the public in the treatment of many diseases. In order to sustain and spread the use of natural and healthy products, it is crucial to support traditionally used plants with new research and scientific publications (Serim et al., 2023; Ceylan & Yeşiloğlu, 2022). It is important to determine the metal contents (toxic compounds) of plants and compounds to be used as food products before activity determinations (Kaya et al., 2010; Kaplan et al., 2009).

The genus Bellevalia Lapeyr is represented by about 80 species, distributed mainly in the Mediterranean region. The genus Bellevalia has 33 species, 21 of which are endemic, in Turkey. As a result, Turkey has a 62.5% endemism rate of this kind. There are three Bellevalia species in Trakya, but only *Bellevalia edirnensis* ÖZHATAY & MATHEW (Asparagaceae) is classified as CR (critically endangered) by the IUCN (International Union for Conservation of Nature) (Uzunhisarcıklı et al., 2013). Plant tissue culture is the process of growing new tissues, plants, or plant products from plant pieces such as whole plants, cells, tissues, or organs in an artificial nutrient media under aseptic conditions. Callus culture, which is a tissuse culture method, is expressed as structures with morphological irregularities formed as a result of the proliferation of organs or tissue parts cut from the primary plant in a semi-solid nutrient medium containing carbon source and plant growth regulators and not losing their division feature. It is stated that plant tissue culture methods have many application areas, as well as a suitable biotechnological approach for the study and synthesis of important secondary metabolites (Zhong, 2001).

It is stated that plant tissue culture methods have many application areas, as well as a suitable biotechnological approach for the study and synthesis of important secondary metabolites (Kurnaz-Yetim et al., 2022; Akkurt et al., 2020).

Alzheimer's disease is defined as the knotting of neurofibrils and the formation of amyloids associated with the loss of cholinergic neurons in regions of the brain related to learning and memory. Acetylcholine level decreases in Alzheimer's patients. One of the ways to treat this is by inhibition of the enzyme responsible for the hydrolysis of acetylcholine (acetylcholinesterase) (Ertaş et al., 2014; Kurnaz-Yetim et al., 2020).

Diabetes mellitus is a complex illness that can cause serious complications. As a result, the treatment incorporates a variety of therapeutic modalities. In diabetic patients, postprandial hyperglycemia occurs after a meal due to glucose absorption from the gastrointestinal tract. In the case of postprandial hyperglycemia, which is typical in diabetics, preventing glucose uptake in the intestines and encouraging glucose uptake in tissues helps reduce the amount of blood glucose (Thilagam et al., 2013). When we look at the literature, it has been reported in the literature that karyosystematic, molecular cytogenetics, pollen mitosis and morphology and cytological and histological studies on the development of the reproductive system related to *B. edirnensis* (Johnson, 2003; Dane, 1999; Dane, 2006). In addition, no study findings regarding the *ex situ* and *in situ* conservation of this endemic and CR category species have been published. In this study, it was aimed to germinate seeds in order to protect the species *in vitro*, production of callus culture by means of embryo culture and onion scale culture and it was aimed to investigate the anti-diabetic and anti-Alzheimer activities of the obtained calluses *in vitro*.

2. MATERIALS AND METHOD

2.1. Chemicals and Spectral Measurements

Acetylcholinesterase (AChE), 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide (AcI) and butyrylthiocholine chloride (BuCl), phosphate buffer (pH 6.8-8.0), galantamine, *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG), genistein were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). α-glucosidase, dimethyl sulfoxide (DMSO), methanol, ethanol were purchased from Merck (Darmstadt, Germany). The BioTek Power Wave XS (USA) 96-well microplate was used for the bioactivity measurements. Utilizing Gen5 Data Analysis software, the measurements and computations of the activity data were assessed.

2.2. Plant Material and Callus Culture Procedure

B. edirnensis seeds were collected from Küçükkarakarlı Village grassland (Tekirdağ Province) in June 2019. The seeds stored at +4 ºC for 3 months were sterilized by first soaking in 20% commercial bleach solution for 15 minutes and then shaking in 70% ethanol solution for 4 minutes. Afterwards, they were rinsed 5 times with sterile distilled water and left to swell for 72 hours. Matured embryos isolated from swollen seeds were transferred to Murashige & Skoog (MS including vitamins, Duchefa Biochemie, M0222) basal nutrient medium (pH 5.8) containing 1 mg/L α-Naphtalene Acetic Acid (NAA, Duchefa Biochemie, N0903), 30 g/L Sucrose (Duchefa Bicohemie, S0809) and 7 g/L Plant Agar (Duchefa Biochemie, P1001). 50 petri dishes each containing 5 embryos were incubated at 4 ºC in the dark for 1 week. Then, cultures were taken into the growth chamber at 25 ± 2 ºC and kept in the dark for 1 month and then in 12/12 h light/dark photoperiod. Callus cultured were subcultured on the same nutrient medium once a month for 6 months. Calluses formed

during this period were transferred to MS basal nutrient medium containing 1 mg/L NAA, 1 mg/L 6-Benzylaminopurine (6-BAP, Duchefa Biochemie, B0904), 30 g/L Sucrose and 7 g/L Plant Agar for biomass increase. Yellowish colored friable callus tissues with a weight of 1 g and above were selected and used for extraction procedures.

2.3. Chemicals and Spectral Measurements

The spectrophotometric technique created by Ellman et al. was slightly modified to evaluate the inhibitory activities of acetyl-cholinesterase (Ellman, et al., 1961). The reaction's substrates were acetylthiocholine iodide and DTNB was employed to gauge the anticholinesterase activity. Methanol was used to dissolve each callus culture in order to create stock solutions at a concentration of 4000 g/mL. The following ingredients were combined and incubated for 15 minutes at 25 ºC: 150 microliters of 100 mM sodium phosphate buffer (pH 8.0), 10 g/mL of sample solution, 20 µL of AChE enzyme solution, and 10 g/mL of DTNB. Acetylthiocholine iodide 10 g/mL was then added to start the reaction. The callus culture's final concentration in solution was 10, 25, 50, and 100 g/mL. The production of yellow 5-thio-2-nitrobenzoate anion with a wavelength of 412 nm, which occurs when DTNB reacts with thiocholine produced by the enzymatic hydrolysis of acetylthiocholine iodide, served as a marker for the hydrolysis of these substrates. The samples and controls were dissolved in methanol, which served as a solvent. Galantamine was used as standard.

2.4. Chemicals and Spectral Measurements

The callus culture was evaluated for the inhibition of Saccharomyces cerevisiae's -glucosidase using a technique somewhat modified by Tsujii et al. (Tsujii et al., 1996). In a nutshell, a 40 L solution of -glucosidase (3.0 U/mL, dissolved in phosphate buffer, pH 6.8) was pre-incubated at 37 ºC for 30 min with 10 L of each callus culture in DMSO. P-nitrophenyl-D-glucopyranoside (p-NPG; final concentration 0.5 mM) was added to the mixture to start the enzymatic reaction, which continued for another 30 minutes. Monitoring the p-nitrophenol produced from p-NPG at 405 nm allowed researchers to ascertain the -glucosidase activity. The positive control utilized was genistein.

3. RESULTS AND DISCUSSION

3.1. Anti-Alzheimer Activity

Acetylthiocholine iodide is utilized as the substrate and acetylcholinesterase enzyme is employed as the inhibitor of acetylcholinesterase activity. The reaction is based on the formation of the yellow 5-thio-2-nitrobenzoate anion with DTNB of the thiocholine formed as a result of the acetylcholine iodide

decomposition by the acetylcholinesterase enzyme and its spectrophotometric measurement at 412 nm. The standard is galantamine, an isolated alkaloid substance from the Galanthus plant. Ethanol was used as a control. Anticholinesterase activity was calculated as % inhibition relative to control using the following equation.

% inhibition = $(A_{control} - A_{sample} / A_{control}) \times 100$

$A_{control}$ = Ethanol absorption

A_{sample} = Callus culture extracts

Callus tissue cultures showed anti-Alzheimer activity in direct proportion to the increase in concentration (Figure 1). According to Table 1, although galantamine was used as a standard, it did not show 100% inhibition. Therefore, assuming 100% inhibition of galantamine, callus culture extracts showed moderate anti-alzheimer's activity relative to galantamine.

Table 1: Anticholinesterase inhibition of callus culture extracts and standard.

Concentration μg mL^{-1}	% inhibition of callus culture extracts	% inhibition of galantamine
10	18.87±1.11	84.81±0.27
25	26.16±0.27	83.67±0.17
50	34.14±0.54	83.09±0.23
100	48.86±1.42	81.75±0.76

Values are given as the mean and standard deviation of three parallel measurements.

3.2. Anti-diabetic Activity

A class of medications used to treat diabetes prevents the conversion of carbohydrates into glucose by blocking the enzymes -amylase and -glucosidase, which are involved in the breakdown of carbohydrates in metabolism. Extracted callus tissue cultures were made according to the method described for inhibition of α-glucosidase from *Saccharomyces cerevisiae*. Genistein was used as a standard. The control group was analyzed by replacing samples with phosphate buffer. α-glucosidase inhibition activity was calculated as % inhibition relative to control using the following equation.

% inhibition = $(A_{control} - A_{sample} / A_{control}) \times 100$

$A_{control}$ = Phosphate buffer

A_{sample} = Callus culture extracts

Callus tissue cultures showed anti-diabetic activity in direct proportion to the increase in concentration (Figure 1). According to Table 2, although genistein was used as a standard, it did

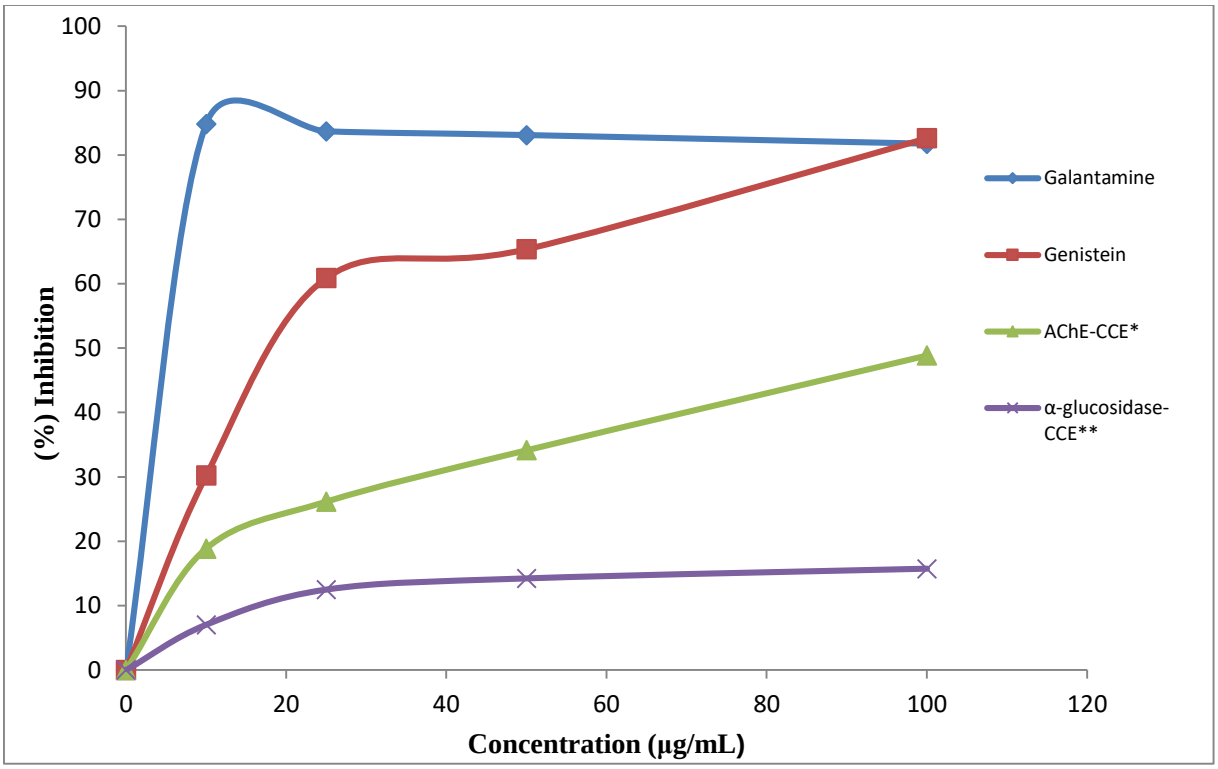
not show 100% inhibition. Therefore, assuming 100% inhibition of genistein, callus culture extracts showed moderate anti-alzheimer's activity relative to genistein.

Table 2: α-glucosidase inhibition of callus culture extracts and standard.

Concentration µg mL^{-1}	% inhibition of callus culture extracts	% inhibition of genistein
10	7.03±1.11	30.21±0.27
25	12.48±0.27	60.90±0.17
50	14.21±0.54	65.38±0.23
100	15.72±1.42	82.62±0.76

Values are given as the mean and standard deviation of three parallel measurements.

In previous studies, IC_{50} values in the cytotoxic activity of *Bellevalia longipes* ranged from 0.62 to 5.35 µM (El-Elimat et al., 2022). In another study, MIC values of antimicrobial activity of different extracts *Bellevalia eigii* were between 17 to 25 µg/mL (Alali et al., 2015).



*: Anticholinesterase inhibition of callus culture extracts, **: α-glucosidase inhibition of callus culture extracts

Figure 1: % inhibition of callus culture extracts and standards.

4. CONCLUSION

Callus tissue culture, which is used in the production of secondary metabolites, is an alternative method to traditional cultivation and chemical methods for the production of cosmetic, pharmaceutical or agriculturally valuable compounds. Production of secondary metabolites by callus tissue culture method is advantageous in many aspects. Secondary metabolites obtained by callus tissue culture from *B. edirnensis* ÖZHATAY & MATHEW plant showed moderate anti-Alzheimer and anti-diabetic activity. The data obtained from this study showed that the secondary metabolites obtained from the *B. edirnensis* plant by tissue culture method can be used in the isolation of the new active ingredient, in extracts or for commercial purposes.

CONFLICT OF INTEREST STATEMENT

Conflicts of Interest/Competing Interests There is no conflict of interest between authors and any kind of organisation.

CONTRIBUTIONS OF AUTHORS

B.C.: Conceptualization, methodology, software, validation, formal analysis, investigation, resources, writing-original draft preparation and editing.

S.D.: Conceptualization, methodology, formal analysis, investigation, resources, writing-original draft preparation.

E.A.: Conceptualization, formal analysis, investigation, resources, writing-original draft preparation.

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