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Tanacetum argyrophyllum (K. Koch) Tzvelev var. argyrophyllum ekstresinin *in vitro* antioksidan ve enzim inhibisyon aktivitesi

In vitro antioxidant and enzyme inhibition activity of Tanacetum argyrophyllum (K. Koch) Tzvelev var. argyrophyllum extract

Short title: In vitro biological activity study
Kısa başlık: In vitro biyolojik aktivite çalışmaları

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ABSTRACT
INTRODUCTION: Tanacetum L. belongs to Asteraceae family, is represented by 46 species in Turkey. Tanacetum genus is known for its insecticide and insect repellent effect. Tanacetum argyrophyllum contains sesquiterpene lactone derivatives. These compounds are responsible for various activities, especially cytotoxic, anti-tumor, phytotoxic, antimicrobial, antiviral, and antifungal activity. There are not enough biological activity studies on the plant that are likely to have a wide variety of activities in terms of the compounds it contains. The aim of the present study is to evaluate various biological activities of 80% aqueous methanol extract prepared from aerial parts of T. argyrophyllum var. argyrophyllum that is collected from Sivas province of Turkey.
METHODS: The antioxidant activity of methanol extract was determined by DPPH, ABTS radical scavenging activity, total phenolic, and total flavonoid content tests. The acetylcholinesterase and butyrylcholinesterase inhibition activities were investigated via Ellman's spectrometric method.
RESULTS: Total phenolic content was found as 71.67 mg/GAE g and flavonoid content was 252.25 mg/QE g dry extract weight basis. In this work, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α-glycosidase enzymes were inhibited by the extract of T. argyrophyllum var. argyrophyllum. IC50 values for these enzymes were obtained 266.79 µg/mL for AChE, 176.91 µg/mL for BChE. Also, α-glycosidase activity exhibited a dose-dependent manner with increasing concentration.

DISCUSSION AND CONCLUSION: According to the results, T. argyrophyllum var. argyrophyllum can be used as an ingredient of functional foods as well as herbal products for diabetic and Alzheimer patients.
Keywords: Tanacetum argyrophyllum var. argyrophyllum, antioxidant, acetylcholinesterase, butyrylcholinesterase, antidiabetic
ÖZ
GİRİŞ ve AMAÇ: Asteraceae familyasına ait olan Tanacetum L. Türkiye'de 46 tür ile temsil edilmektedir. Tanacetum cinsi, insektisit ve böcek kovucu etkileriyle bilinmektedir. Tanacetum argyrophyllum, seskiterpen lakton türevi bileşikler içermektedir. Bu bileşikler, özellikle sitotoksik, anti-tümör, fitotoksik, antimikrobiyal, antiviral ve antifungal aktivite olmak üzere önemli aktivitelerden sorumludur. İçerdiği bileşikler açısından çok çeşitli aktivitelere sahip olması muhtemel olan bitki üzerinde yeterli biyolojik aktivite çalışması bulunmamaktadır. Bu çalışmanın amacı, Sivas ilinden toplanan T. argyrophyllum var. argyrophyllum toprak üstü kısımlarından hazırlanan %80 sulu metanol ekstresinin çeşitli biyolojik aktivitelerini değerlendirmektir.
YÖNTEM ve GEREÇLER: Metanol ekstresinin antioksidan aktivitesi, DPPH, ABTS radikal süpürücü aktivite, toplam fenolik ve toplam flavonoid içerik testleri ile değerlendirilmiştir. Asetilkolinesteraz ve butirilkolinesteraz inhibisyon aktivitesi ise Ellman'ın spektrometrik yöntemi ile belirlenmiştir.
BULGULAR: Kuru ekstre ağırlığı bazında toplam fenolik madde miktarı gallik asite eşdeğer 71.67 mg/g ve flavonoit içeriği 252.25 mg/g olarak bulunmuştur. Bu çalışmada, T. argyrophyllum var. argyrophyllum ekstraktı asetilkolinesteraz (AChE), bütirilkolinesteraz (BChE), α-glukozidaz enzimlerni inhibe etmiştir. Enzimlerin inhibisyon değerleri, AChE için IC50 = 266.79 µg/mL, BChE için IC50 = 176.91 µg/mL olarak bulunmuştur. Ek olarak, α-glukozidaz aktivitesi, artan konsantrasyonla doza bağlı olarak artış sergilemiştir.
TARTIŞMA ve SONUÇ: Elde edilen sonuçlara göre, T. argyrophyllum var. argyrophyllum, bitkisel ürünlerin yanı sıra diyabet ve Alzheimer hastaları için fonksiyonel gıdaların hazırlanmasında da kullanılabilir.
Anahtar Kelimeler: Tanacetum argyrophyllum var. argyrophyllum, antioksidan, asetilkolinesteraz, butirilkolinesteraz, antidiyabet

INTRODUCTION

Asteraceae family has approximately 1535 genera around the world and 138 genera and 1186 species in Turkey.[1] The genus *Tanacetum* L. is one of the largest genera in this family and is represented by 46 species, 18 subspecies, and 5 varieties that 26 taxa are endemic to Turkey.[2] Several members of *Tanacetum* include medicinally important taxa. According to recent literature, essential oils and extracts of *Tanacetum* species have anti-inflammatory, antibacterial, antifungal, anti-Alzheimer, and insecticidal effects.[3] Various biological activities are thought to associate with sesquiterpene lactone content of *Tanacetum* species.[4] The interest in the *Tanacetum* species is increasing day by day due to the fact that it is effective against many diseases due to its powerful secondary metabolites such as essential oil, sesquiterpenes, sesquiterpene lactones, flavonoids, coumarins, tannins, and sterols.[5-6.] However, there are not enough studies on the biological activity of *Tanacetum argyrophyllum* var. *argyrophyllum* except for the antimicrobial activity.[7]

Alzheimer's disease (AD) is a neurological disorder characterized by decreased cognitive functions, daily activities, behavioral changes, and psychiatric symptoms.[8] Acetylcholine is a neurotransmitter released from the synapse of the neuron and the biochemical changes in AD are closely related to reduction of acetylcholine levels in the brain neurons.[9] The acetylcholinesterase enzyme has become impressive in drug design studies. The cholinesterase inhibitors are commonly used in the AD therapy , however they can cause serious side effects such as gastrointestinal disturbances, fatigue, or depression.[10] The

drawbacks of these licensed drugs have pushed researchers to find new and potential inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) from plants that have therapeutic properties.

Diabetes mellitus (DM) is a metabolic disease marked with an excessive increase in blood glucose levels, which is regulated by α-glucosidase and α-amylase enzyme. α-amylase initiates carbohydrate digestion by hydrolyzing polysaccharide 1, 4-glycosidic linkages to disaccharides. α-glucosidase converts disaccharides into monosaccharides, which causes blood glucose levels to increase after meals.[11] α-glucosidase inhibitors (AGIs) are a new class of antidiabetic medicines that can control blood sugar level by inhibiting glycosidase competitively and preventing sugar breakdown. However, the AGI drugs are not satisfactory because of fewer in their numbers, lower bioavailability, and have gastrointestinal problems.[12] Therefore, it needs to research out new and safer AGI inhibitor drugs from natural products. In the literature review, there are not enough biological activity studies on the *T. argyrophyllum* var. *argyrophyllum*. Therefore, the aim of this study is to evaluate antioxidant, anti-AChE, anti-BChE, α-glucosidase, and α-amylase activity of *T. argyrophyllum* var. *argyrophyllum* methanol extract *in vitro*. Total phenolic content (TPC) and total flavonoid content (TFC), as well as ABTS and DPPH radical scavenging assays, were used to assess antioxidant capacity. AChE and BChE inhibition methods were used to determine anti-Alzheimer activity. α-glucosidase and α-amylase enzyme inhibition approaches were used to assess anti-diabetic activity.

MATERIALS AND METHODS

Chemicals

2,2'-diphenyl-1-picryl-hydrazyl (DPPH), 2,2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), butylated hydroxy anisole (BHA), α-glucosidase (from *Saccharomyces cerevisiae*, Type I, lyophilized powder), acetylcholinesterase (from *Electric eel* type-VI-S,EC 3.1.1.7) and butyrylcholinesterase (from horse serum, EC 3.1.1.8) enzymes, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide (AChI), butyrylthiocholine chloride (BChC), and galantamine were supplied from Sigma Chemicals Co (St. Louis, MO, USA). All of the other chemicals used were of analytical grade.

Plant Materials

The plant material was gathered from a natural habitat during the flowering period. Locality data of collection: Turkey- B6 Sivas: Şerefiye to Suşehri, before of Abdiağa Çeşmesi, steep slope, 39° 59' 41,2" N, 37° 43' 45,7" E, 1529 m, 27 June 2016, M. Tekin 1739. The dried specimens were preserved at the Sivas Cumhuriyet University, Faculty of Science Herbarium (CUFH), Department of Biology, Sivas, Turkey. Taxonomical identification was done based on the Flora of Turkey (Davis, 1966) by botanist Dr. Mehmet Tekin, Trakya University (Edirne-Turkey), Department of Pharmaceutical Botany.

Preparation of the extract

The plant's aerial parts were dried at room temperature. Then dried materials were grounded to powder using a laboratory type mill and macerated with methanol: water = 80:20, v/v for 24 h at room temperature. The extracts were filtered and the solvent was removed by rotary evaporation at 40 °C. This extraction process was repeated with the residue three times (24 h x 3). Extracts obtained from the process were held at +4˚C until they were used.

Antioxidant activity

Determination of Total Phenolic Content

The total phenolic content (TPC) was determined as gallic acid equivalent according to the procedure.[13] In a test tube, 2.5 mL of 7.5% sodium carbonate solution and 2.5 mL of 0.1N

Folin-Ciocalteu reagent were combined with 500 µL of test solution (2 mg/mL). After vortexing, the tubes were left in the dark for 2 hours and the absorbance was measured with a UV-vis spectrophotometer (Shimadzu, UV-VİS 1800, Japan) at 730 nm. The extract's total phenolic content was calculated as mg gallic acid equivalents per gram of dry extract.

Estimation of Total Flavonoid Content

The total flavonoid content (TFC) was measured by a colorimetric method using aluminum chloride according to the procedure of Yang et al.[14], and the results were given in milligrams of quercetin equivalents per gram of dry extract. TFC were calculated using an equation derived from normal concentration-response graph of quercetin.

DPPH radical scavenging activity

The DPPH radical scavenging capacity of extracts and standards (BHA and BHT) was evaluated using the previously described method with slight modifications.[15] 100 µL of various concentrations of test samples were combined with 100 µL of methanol containing DPPH (0.1Mm) and incubated at 25ºC for 30min in the dark. The absorbance was read with a UV-vis spectrophotometer at 517 nm. The % of inhibition was found according to the formula:

% Inhibition= $(A_0-A_t)/A_0*100$,

Where A_0 represents the absorbance of control without sample and A_t represents the absorbance of test solution

ABTS radical scavenging assay

The assay of $ABTS^{\bullet+}$ radical cation decolorization was used to assess the radical scavenging activity of extracts according to the method of Re et al.[16]. The stock solution of $ABTS^{\bullet+}$ was generated by mixing 2.45 mmol/L potassium persulfate with 7 mmol/L ABTS. The mixture was stood for in the dark at room temperature for 12-16 h. Diluting the stock with methanol yielded an $ABTS^{\bullet+}$ working solution the absorbance was 0.70 ±0.02 at 734 nm. 100 µL sample solution was combined with 100 µL of $ABTS^{\bullet+}$ working solution and incubated for 7 min at 25°C. A UV–vis spectrophotometer set to 734 nm was used to read the absorbance of the mixture.

Determination of anticholinesterase inhibition activity

The inhibitory activities of AChE and BChE were determined using Ellman's spectrophotometric method[17] with minor modifications. In a 96 well-plate, 0.14 mL of 0.1mM sodium phosphate buffer (pH 8.0), 0.02 mL test sample, and 0.02 mL AChE (0.22U/ml) or BChE (0.1 U/mL) solution were combined and incubated at 25°C for 15 min before adding 10 µL of 0.5 mM 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction was started with the addition of 10 µL of 0.71 mM AChI or 0.2 mM BChC to the mixture. The substrate's hydrolysis was estimated at 412 nm. By comparing the reactions of samples to a blank, the percentage inhibition of AChE/BChE enzymes were calculated.

Antidiabetic activity

Inhibition of α-glucosidase

The inhibitory activity of extract against α-glucosidase was measured using the method reported by Abirami[18]. 50 µL of test samples were combined with 0.1mL of 0.1M phosphate buffer (pH 6.8) and 0.1mL of 1 U/mL α-glucosidase solution and incubated for 5 min at 25°C. After pre-incubation, 0.1mL of 5 mM *p*-nitrophenyl-α-*D*-glucopyranoside solution was applied to the reaction mixture which was then incubated for 10 min at 25ºC. The absorbance at 405 nm was then measured, and the percent inhibition of α-glucosidase estimated.

α-amylase inhibititory activity

The inhibitory activity of extract against α-amylase was investigated using the method reported by Ademiluyi A. et al.[19]. After boiling 0.5 g potato starch in 0.1 L phosphate buffer

(pH 6.8) for 5 min, the substrate was cooled to 25 ºC. In 96-well plate, 20 µL of samples in different concentration, 50 µL of 0.1 M phosphate buffer (pH 6.8) and 10 µL of 2 U/ mL *α*-amylase solution were applied, and the mixture was incubated at 37 ºC for 15 min. Following the preincubation, 20 µL of starch solution was added. Then 100 µL of 3,5-Dinitrosalicylic Acid (DNSA) was added to the mixture as coloring reagent and left in hot water for 10 min. Then the absorbance was read by a microplate reader (Epoch, USA) at 540 nm. As a positive control, acarbose was used.

Statistical analysis

The results are presented as mean ± standard deviation (SD) of three parallel measurements. The analysis of variance was carried out with Graphpad prism software 7.0 (USA). Significant differences between means were determined using the Student's t-test with $P < 0.05$ considered significant.

RESULTS AND DISCUSSION

As part of our study on the biological activity of medicinal plants growing in Sivas province (Turkey), the *in vitro* antioxidant, anticholinesterase, and antidiabetic potential of an endemic species *T. argyrophyllum* var. *argyrophyllum* were investigated. The present research is the first report on the different biological activity investigations of *T. argyrophyllum* var. *argyrophyllum* except for its essential oil.

In vitro antioxidant activity

The antioxidant activity of the aqueous methanol extract prepared from the aerial part of *T. argyrophyllum* var. *argyrophyllum* was evaluated using different *in vitro* methods. Total flavonoid and phenolic contents, as well as DPPH free radical and ABTS cation radical decolorization methods were used to assess the antioxidant activity. The results were given in Figure 1. Considering the antiradical activity, *T. argyrophyllum* var. *argyrophyllum* aqueous methanol extract exerted a remarkable DPPH scavenging effect, even stronger than standard compound BHT from 250 to 1000 µg/mL concentration. As for ABTS radical-scavenging effect, the *T. argyrophyllum* var. *argyrophyllum* aqueous methanol extract demonstrated concentration-dependent activity but shows lower radical scavenging activity than reference compound BHT.

Calibration equations were calculated as $Y = 0.0066x + 0.1146$ ($R^2 = 0.9981$) for total phenolic content generated from gallic acid and $Y = 0.0023x + 0.1247$ ($R^2 = 0.9971$) for total flavonoid contents prepared from quercetin. Results of total phenol and flavonoid contents of the extract were shown in Figure 1. The aqueous methanol extract obtained from the aerial part of *T. argyrophyllum* var. *argyrophyllum* was found to contain the total phenol amount (71.67 mg/GAE g) as gallic acid equivalent and total flavonoid content (25.225 mg/QE g) as quercetin equivalent on dry weight basis of the extract. Wu et al. identified phenolic compounds of *T. parthenium* and 3,5-, 4,5- and 3,4-di-*O*-caffeoylquinic acids were characterized as major compounds with potent antioxidant activity of the plant.[20] In another study, methanol extracts of three different *T. densum* subspecies were screened for antioxidant activities and positive relation was found between total phenolic content and antioxidant activity. [21] In this case, flavonoid and phenolic compounds which may be found in methanol extract are supposed to be responsible for the antioxidant activity of *T. argyrophyllum* var. *argyrophyllum*.

Anticholinesterase inhibition assay

The acetylcholinesterase and butyrylcholinesterase inhibitory activities of *T. argyrophyllum* var. *argyrophyllum* aqueous methanol extract were determined by Ellman's

spectrophotometric method. The extract was tested at different concentrations (10, 100, 250, 500, 1000, and 2000 µg/mL) and the concentration-enzyme inhibition graph was generated and compared with the standard compound galantamine (Figure 2). Lower IC_{50} value indicates higher enzyme inhibition activity. The IC_{50} values of the extract were obtained 266.79 µg/mL for AChE, 176.91 µg/mL for BChE. As can be seen from the antioxidant activity results, enzyme inhibition activity seems to be in accordance with total phenol and flavonoid content. Wszelaki et al. observed for the methanolic and hexane extracts of *Tanacetum parthenium* Sch. Bip. was ranging from 32.4% to 40.9% against AChE and BuChE at a concentration of 400 µg/mL.[22] Erdogan et al. investigated different *Tanacetum* taxa for their cholinesterase inhibitory activity, found that the leaf of *T. argenteum* (Lam.) Willd. subsp. *flabellifolium* (Boiss. & Heldr.) Grierson had the highest inhibition of 96.68% at 100 µg/mL concentration against AChE, while *T. argyrophyllum* var. *argyrophyllum* showed the best inhibition of 63.81% against BChE.[23] However, parthenolide, the main constituent of *Tanacetum* taxa has demonstrated lower inhibition activity against both enzymes. It can be concluded that the major compound parthenolide found in most of *Tanacetum* taxa may not be an active principle for anticholinesterase activity or it can exert its activity by synergistic effect with other secondary metabolites. The AChE inhibitory activity was tested on the essential oil of *T. densum* (Labill.) Heywood ssp. *sivasicum* Hub. - Mor. & Grierson and *T. mucroniferum* Hub. - Mor. & Grierson, pure oil showed 100% inhibition, however dilutions demonstrated lower inhibition.[24] In another study on *T. vulgare* L. root, polyacetylenes were identified as active zone for antibacterial, antioxidant, and AChE inhibitory effects by HPTLC combined with in situ effect-directed analysis and spectrometric techniques.[25]

Antidiabetic activity assay

Inhibition of α-amylase contributes to improving the symptoms of type 2 diabetes by delaying or cutting glucose absorption as a result of slowing starch digestion. While the primary goal of α-amylase inhibition is to reduce the rate at which maltose and glucose are produced from starch, this enzyme can slow down the function of α-glucosidase by eliminating the substrate.[26] The *T. argyrophyllum var. argyrophyllum* aqueous methanol extract inhibited both α-glucosidase and α-amylase activity *in vitro* in a concentration-dependent manner (IC_{50} of 234.77 ± 1.76 µg/mL and 806.68 ± 2.36 µg/mL, respectively) in comparison with the positive control acarbose (Figure 3). A previous study on antidiabetic and enzyme inhibition properties of *T. praeteritum* (Horw.) Heywood essential oil revealed that the oil has noteworthy inhibitory activity with IC_{50} of 0.89 ± 0.13 mg/mL against porcine pancreatic α-amylase.[27] In another study, *T. poteriifolium* ethyl acetate extract exhibited inhibition on α-glucosidase (23.67 mmol acarbose equivalent (ACAE)/g extract)[28] *T. nubigenum* which is a Himalayan Medicinal plant from India, showed potent stimulation of glucose uptake with +61.2% in C_2Cl_2 myotubes. Also, ethanol extract of T. *nubigenum* decreased blood glucose level significantly in STZ induced Sprague-Dawley rats (15.5%).[29]

CONCLUSION

According to the literature review, the present study is the first investigation on *T. argyrophyllum var. argyrophyllum* in terms of antioxidant and enzyme inhibition activity. This study showed that the aqueous methanol extract possessed polar compounds, which are rich with phenolic and flavonoid compounds, have strong antiradical, acetylcholinesterase, butyrylcholinesterase, and α-glucosidase inhibition activity as well as demonstrated moderate α-amylase inhibitory activity. This work will provide important scientific data for further phytochemical and biological activity-guided studies on the polar extract of *T. argyrophyllum var. argyrophyllum* for identifying active principles that are attributed to the strong activity. However, to identify the active phytochemicals responsible for the antioxidant and enzyme

inhibition activity, it needs to perform bioactivity-guided chromatographic fractionation and isolation.

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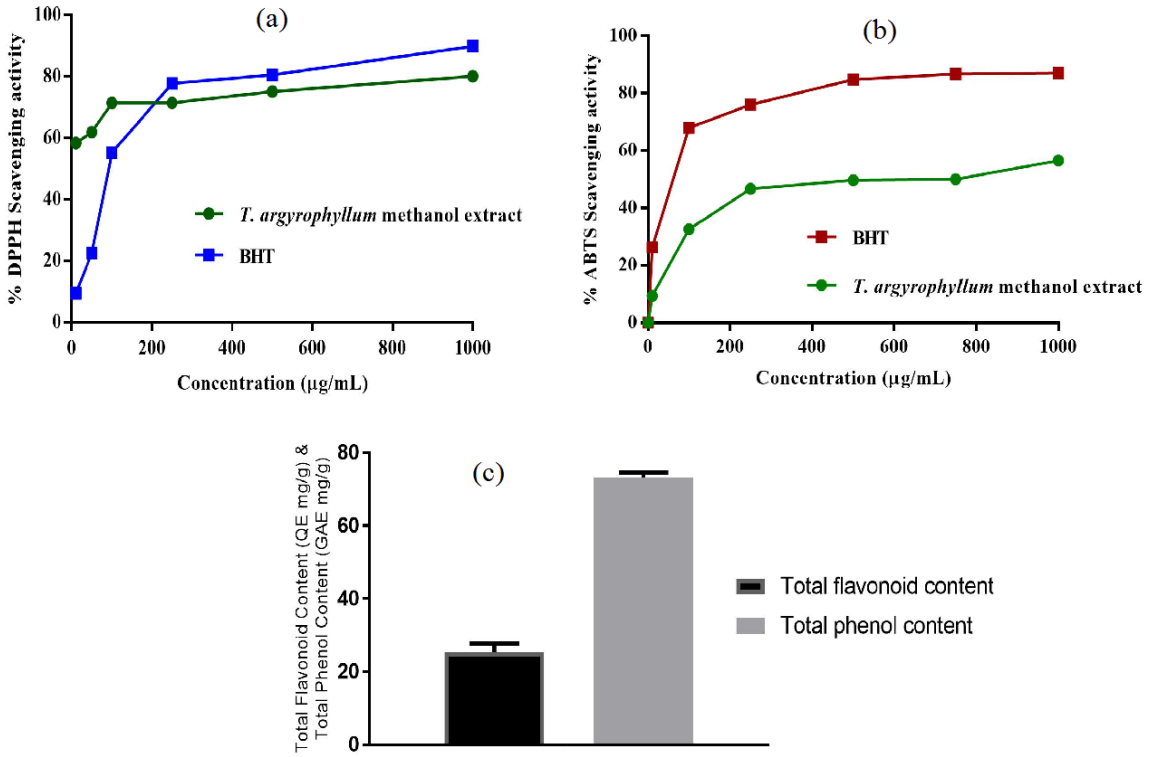

Figure 1. Total antioxidant activity results of *Tanacetum argyrophyllum var. argyrophyllum* aqueous methanol extract by DPPH, ABTS, TPC, and TFC method

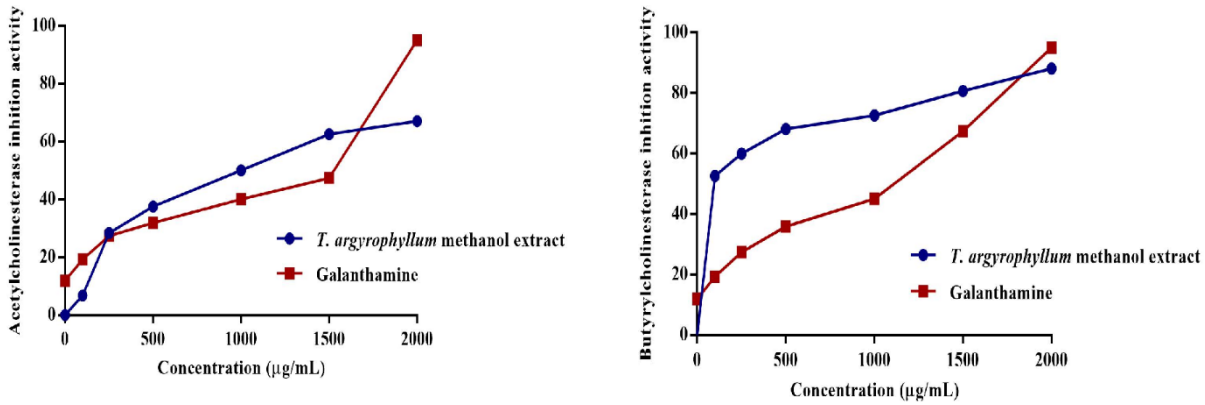

Figure 2. Acetylcholinesterase and butyrylcholinesterase inhibitory results of *Tanacetum argyrophyllum* var. *argyrophyllum* aqueous methanol extract with reference compound galanthamine

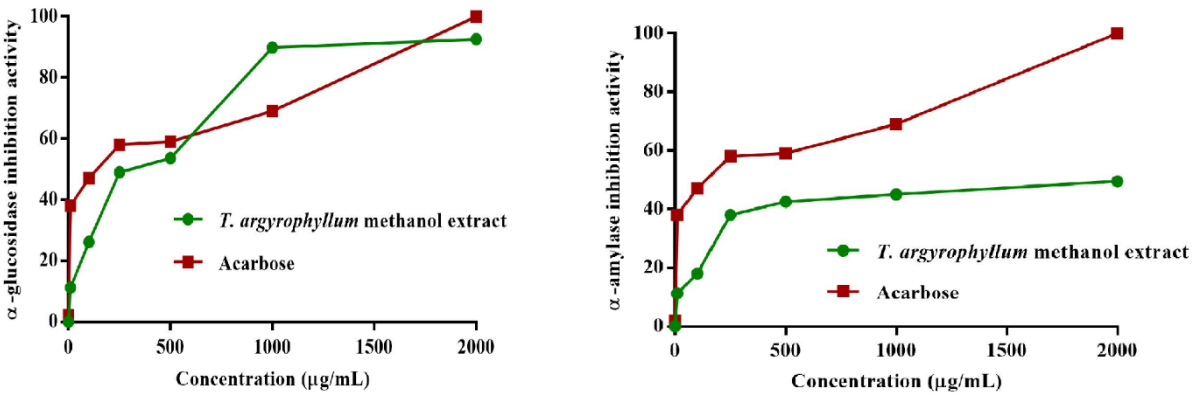

Figure 3. α-glucosidase and α-amylase inhibitory results of *Tanacetum argyrophyllum* var. *argyrophyllum* aqueous methanol extract with reference compound acarbose