

Inhibition of carbonic anhydrase and cholinesterase enzymes by acetone extract of *Bryoria capillaris* (Ach.) Brodo & D.Hawksw.

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Abstract: In traditional medicine, people commonly consume *Bryoria capillaris* (BC) as flour and tea, particularly in Northeast Anatolia, where it is one of the most prevalent lichen species. This study aimed to obtain an acetone extract of BC and investigate its inhibitory effects on carbonic anhydrase (CA) I, CA II, acetylcholine esterase (AChE), and butyrylcholine esterase (BChE) enzymes. We determined IC₅₀ values of BC for each enzyme to measure the level of inhibition. The IC₅₀ values for CA I and CA II were 8.77 µg/mL and 7.56 µg/mL, respectively. Acetazolamide, a specific CA I and II inhibitor, had IC₅₀ values of 1.65 and 0.016 µg/mL, respectively. The IC₅₀ values of BC for AChE and BChE were 7.96 and 8.58 µg/mL, respectively. Galantamine had IC₅₀ values of 4.68 and 16.07 µg/mL for AChE and BChE, respectively. These results indicate that BC extract has a high potential to provide new drug candidates for all the tested enzymes, particularly for BChE.

1. INTRODUCTION

A lichen is usually described as the iconic example of symbiosis, but it is a minuscule ecosystem. A fungus and one or more photosynthetic partners comprise the prominent symbiosis. Other parties, such as lichenicolous fungi, bacteria, and even invertebrates, can live under or within this symbiosis. Thus, the symbiosis in question can become complicated beyond its disposition.

The secondary metabolites produced by this complex structure have unique natures. Most of these secondary metabolites are not synthesized by other organisms, even by the fungal partner cultured outside the symbiosis. Although about 1000 secondary metabolites have been isolated from lichens so far (Elix, 2014; Furmanek *et al.*, 2022b), the number of those tested for their biological activities is relatively small. However, the number of studies on the bioactivities of

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lichen-derived secondary metabolites has increased significantly in recent years (Adenubi *et al.*, 2022).

Bryoria capillaris (Ach.) Brodo & D. Hawksw. (BC) is a pendulous, filamentous, and widespread lichen found wrapped around tree trunks and branches in the coniferous and deciduous forests of the world. It is commonly known as "horsehair lichen" (Smith, 2009). Researchers reported antibacterial (Karagoz *et al.*, 2018; Tas *et al.*, 2019; Yılmaz Sarıözlü *et al.*, 2016), antifungal (Furmanek *et al.*, 2022a, 2022b, 2022c), anti-cancer (Goncu *et al.*, 2020; Ozturk *et al.*, 2021; Tripathi *et al.*, 2022; Varol, 2018), antioxidant and antigenotoxic (Tas *et al.*, 2017; Tufan-Cetin & Cetin, 2021; Turkez *et al.*, 2014) effects of BC.

Enzyme inhibitors play a vital role in the management of various medical conditions (Şentürk, 2017). Carbonic anhydrases (CAs, EC 4.2.1.1) are a family of metal-containing enzymes that catalyze the conversion of carbon dioxide to bicarbonate and protons. These enzymes are found in a wide range of living organisms and are encoded by eight different gene families (Akocak & Supuran, 2019; Supuran, 2023). In humans, 15 forms of the alpha-CA gene family have been identified. Among these, the CA I and CA II isoforms are particularly common in all tissue types. The study of how carbonic anhydrase activity can be modulated is of great importance for the development of new treatments for a wide range of clinically significant diseases (Supuran, 2008; Yaseen *et al.*, 2016). For example, inhibitors of specific forms of the enzyme, such as CA I and II, have been used to create new drugs for conditions such as epilepsy, edema, and glaucoma. Therefore, the discovery of new inhibitors of carbonic anhydrase isoenzymes holds great promise as a potential therapeutic strategy (Arslan *et al.*, 2020; Özil *et al.*, 2019; Yaseen *et al.*, 2016).

Acetylcholinesterase (AChE) is a particularly important enzyme, as it plays a crucial role in regulating the signaling process within the cholinergic system. AChE breaks down acetylcholine (ACh), a neurotransmitter that is involved in memory formation and the functioning of motor neurons. Inhibiting the activity of AChE can have a significant impact on the treatment of diseases related to the cholinergic system (Comert Onder *et al.*, 2022; Hampel *et al.*, 2018).

AChE is located in the postsynaptic membrane of neurons and is responsible for breaking down the neurotransmitter ACh to terminate signal transduction. BChE, on the other hand, is primarily produced in the liver and is found in various bodily fluids and tissues, such as blood plasma and in the central and peripheral nervous systems. Clinical studies have demonstrated that inhibitors of AChE can boost ACh levels at cholinergic synapses and enhance cholinergic activity. While ACh is primarily broken down by AChE, BChE is believed to have only a minor role in regulating ACh levels in the brain. However, it has been found to play a crucial role in drug metabolism and the removal of toxins from the body. Targeted inhibitors can be used to treat motor neuron diseases such as dementia, myasthenia gravis, and Alzheimer's disease by decreasing the activity of AChE and BChE (Başaran *et al.*, 2022; Comert Onder *et al.*, 2022; Hampel *et al.*, 2018).

In this study, inhibitory effects of BC acetone extract against cholinesterase enzymes (AChE and BChE) and carbonic anhydrases (CA I and II) were investigated in order to establish a steady biochemical basis of some of its therapeutic actions.

2. MATERIAL and METHODS

2.1. Lichen Material

BC was collected from Uzunoluk forest in Oltu county of Erzurum province, Turkey, in 2011. The lichen was identified according to literature (Smith, 2009). After drying in the shade, and removal of debris and foreign material, 10 g of BC was ground into a coarse powder and macerated with acetone (3 x 100 ml) at room temperature. Extracts were filtered and pooled

together. Acetone was removed in a rotary evaporator under reduced pressure at 40 °C. The residue (crude extract) weighed 200 mg (yield 2%).

2.2. Enzymes and Substrates

The enzymes and substrates used in this study were obtained from Sigma-Aldrich Company (USA, Lot numbers are as follows; AChE C1682, BChE B4186, CA I C4396, CA II C6624, 4-nitrophenyl acetate [NPA] N8130, 5,5'-Dithiobis-(2-Nitrobenzoic Acid) [DTNB] D218200, acetylthiocholine iodide A5771, S-butrylthiocholine chloride B3128).

2.3. Carbonic Anhydrase I And II Inhibition

The activity of these isoenzymes was measured using spectrophotometry by observing the change in absorbance at 348 nm as 4-nitrophenyl acetate (NPA) was converted to 4-nitrophenolate (NP) over 3 minutes at 25°C. The reaction mixture consisted of 1.4 mL of 50 mM Tris-SO₄ buffer at pH 7.4, 1 mL of 3 mM NPA, 0.5 mL of water, and 0.1 mL of enzyme solution, for a total volume of 3.0 mL (Verpoorte *et al.*, 1967).

2.4. Cholinesterase Enzymes Inhibition

The assay system employed comprised a sample of the BC acetone extract, with a volume ranging from 5 to 60 mL, in conjunction with 200 mL of buffer, specifically 1 M Tris-HCl buffer for the assay of AChE and PB for the assay of BChE, both at a pH of 8.0. Additionally, 50 mL of 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) (0.5 mM) and 50 mL of acetylthiocholine iodide/S-butrylthiocholine chloride (10 mM) were incorporated, as well as 10 mL of the enzyme, with a concentration of 0.28 U/mL for the AChE assay and 0.32 U/mL for the BChE assay (Ellman *et al.*, 1961). The reaction was initiated by adding the enzyme, and the system was prepared at room temperature within a quartz cuvette. A control was also performed, consisting of all the afore mentioned chemicals, except for the inhibitor.

2.5. General Enzyme Inhibition Studies

Inhibition activities of BC acetone extract on CA I/II, AChE, and BChE were defined using the spectrophotometric methods. In this analysis, acetazolamide (AZA) for CA I/II, galantamine (GAL) for AChE/BChE were used as reference molecules.

In this study, stock solution of the extract under investigation was prepared by dissolving it in dimethyl sulfoxide to achieve a concentration of 1 mg per mL. The resulting stock solution was then meticulously diluted one thousand fold with distilled water. To evaluate the inhibitory activity of these extract on the enzymes under examination, measurements were conducted at seven distinct concentrations. The methodology employed in this study has been previously described in detail in prior studies (Arslan *et al.*, 2020; Cavdar *et al.*, 2019; Özil *et al.*, 2019).

3. RESULTS

IC₅₀ values obtained for BC acetone extract is summarized in [Table 1](#) and [Figures 1-4](#).

Table 1. IC₅₀ values obtained with BC acetone extract and reference drugs for the enzymes tested.

Enzyme	BC Extract	Reference molecules
CA I ^a	8.77 ± 0.12 µg/mL	1.65 ± 0.03 µg/mL
CA II ^a	7.56 ± 0.10 µg/mL	0.016 ± 0.001 µg/mL
AChE ^b	7.96 ± 0.09 µg/mL	4.68 ± 0.31 µg/mL
BChE ^b	8.58 ± 0.11 µg/mL	16.07 ± 1.04 µg/mL

^aAcetazolamide was used as a specific inhibitor and reference drug for CA I and II isoenzymes.

^bGalantamine was used as a specific inhibitor and reference drug for AChE and BChE enzymes (Faraone *et al.*, 2019).

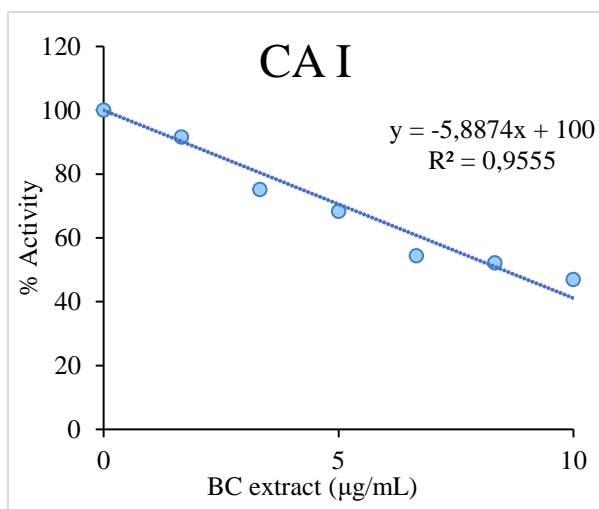


Figure 1. IC50 plot for CA I and BC extract.

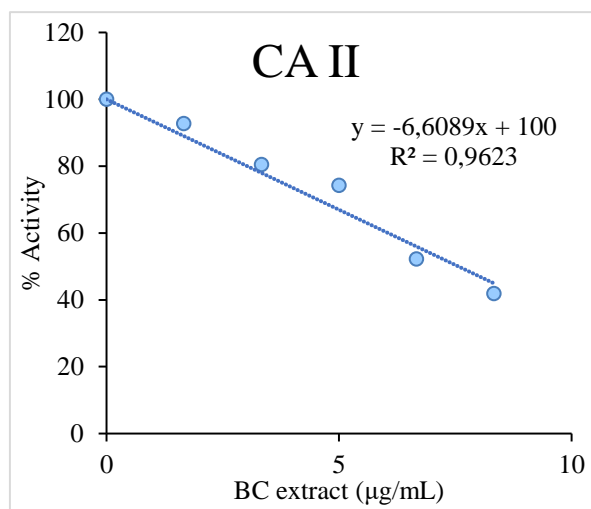


Figure 2. IC50 plot for CA II and BC extract.

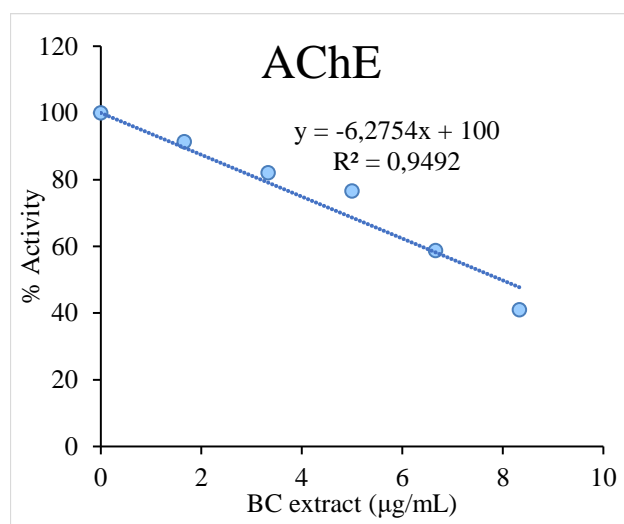


Figure 3. IC50 plot for AChE and BC extract.

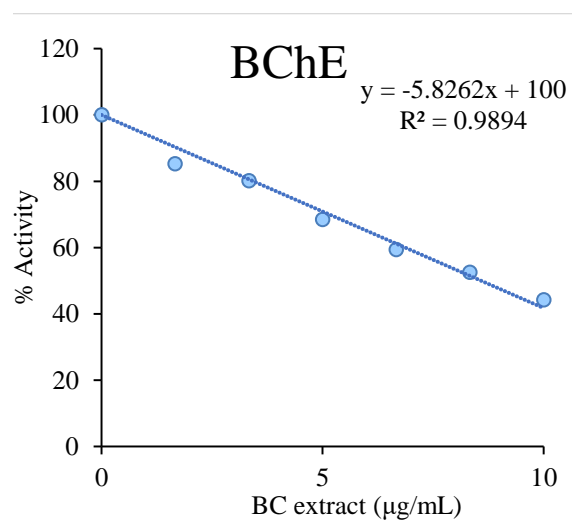


Figure 4. IC50 plot for BChE and BC extract.

4. DISCUSSION and CONCLUSION

B. capillaris contains barbatolic acid and alectorialic acid as major compounds (Culberson, 1969; Culberson, 1970; Culberson *et al.*, 1977). Previously, Areche *et al.* (2022) reported that barbatolic acid isolated from *Himantormia lugubris* was an effective inhibitor of AChE and BChE (IC50 values 17.42 ± 0.03 and 23.95 ± 0.02 µg/mL, respectively). In our study BC extract presented a better inhibition of these enzymes, suggesting that synergistic effects may occur due to presence of other chemical compounds.

In another study (Yañez *et al.*, 2023) barbatolic acid was estimated to be a moderate inhibitor of Cytochrome P450 (CYP) isoenzymes with calculated binding energies ranging between -6 to -9.2 kcal/mol. Authors concluded that tested lichen substances, including barbatolic acid, could exert antioxidant activity by interacting with the CYP system. As oxidative stress is implied in so many pathological conditions (Liguori *et al.*, 2018), antioxidant molecules or mixtures, the BC extract in our case, may provide a means of preventing or treating such conditions.

Methanol extracts of different mushroom, plant and honey samples were obtained by Sahin *et al.* (2012). In the study, IC50 studies on CA I/II were performed with these extracts. The

authors determined that the IC₅₀ values of the extracts for CA I were between 0.52 and 36.66 µg/mL. The IC₅₀ values of these extracts for the CA II enzyme were found to be between 0.49 and 24.02 µg/mL. In our study the IC₅₀ value of BC for the CA I isoenzyme was determined as 8.77 µg/mL. This value suggests a very effective inhibition value when compared to one of the strongest known inhibitors, AZA (IC₅₀: 1.65 µg/mL). For CA II isoenzyme, the IC₅₀ value was determined as 7.56 µg/mL, which is seen to be a moderately effective result when compared to AZA.

CA inhibitors (CAIs) have established roles as diuretics and antiglaucoma drugs. However, recent findings indicate that CAIs promise potential as novel anti-obesity, anticancer and anti-infective drugs (Supuran, 2008). According to our results, *Bryoria capillaris* may provide molecules that serve as effective CAIs, therefore deserves further investigation on this field.

The IC₅₀ of the BC extract tested against the AChE enzyme was found to be 7.96 µg/mL. For the reference molecule galantamine, the AChE IC₅₀ value was determined as 4.68 µg/mL. The IC₅₀ value of BC extract inhibitory activity on BChE enzyme was determined as 8.58 µg/mL, and the IC₅₀ of galantamine was found as 16.07 µg/mL. It was determined that BC extract has an effective inhibition for cholinesterase enzymes.

Butyrylcholinesterase acts as a backup for acetylcholinesterase by hydrolyzing acetylcholine that has diffused out of nerve synapses (Lockridge *et al.*, 2011). In management of Alzheimer's disease (AD), first choice for the treatment is the AChE inhibitor. However, AChE inhibitors have some flaws, such as insufficient long-term treatment effect and dose limitations. Recent studies revealed that BChE inhibitors or double inhibitors (molecules that inhibit both AChE and BChE) have better effects on AD, and the side effects are lower than those of specific AChE inhibitors. Dual target cholinesterase inhibitors have become a new hot spot in the research of anti-AD drugs (Zhou & Huang, 2022). In the patient with AD, a potent selective BChE inhibitor may produce significant increases of brain ACh levels without triggering severe peripheral or central cholinergic adverse effects (Giacobini, 2001). In this perspective, BC extract presents high potential as a source of anti-AD molecules as it inhibits AChE comparable to galantamine, and BChE better than galantamine.

In conclusion, the present study demonstrates that BC extract has a significant therapeutic potential due to its inhibition effects on CA I, CA II, AChE, and BChE enzymes. The IC₅₀ values obtained in this study indicate that the extract has a moderate to high level of inhibition on these enzymes. These findings suggest that the extract of BC could be a promising candidate for the development of new drugs for the treatment of various diseases related to these enzymes, such as Alzheimer's disease and other neurodegenerative disorders. However, further studies are required to evaluate the safety and efficacy of this extract by *in vivo* models and to determine the active compounds responsible for its inhibition effects. Additionally, it is important to consider traditional usage and toxicity of BC, before any clinical application. In general, this study provides a scientific basis for the traditional use of BC and highlights the importance of further research in this area to fully understand the therapeutic potential of this lichen species.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship Contribution Statement

Yalçın Karagöz: Field research, material collection, extraction, manuscript preparation. **Naim Uzun:** Extraction, enzyme assays. **Bayram Alparslan:** Extraction, enzyme assays. **Murat Şentürk:** Enzyme assays, manuscript preparation.

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