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Evaluation of antioxidant and antiproliferative activities of shogaol and its modulatory effects on key metabolic enzymes

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Abstract

Phenolic compounds are versatile bioactive molecules, particularly in food applications. In this study, the antioxidant capacity of Shogaol, a member of the phenolic class, was evaluated using several methods. Additionally, its inhibitory effects on human carbonic anhydrase I and II (hCA I and II), acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α -glycosidase, and α -amylase enzymes, as well as its antiproliferative effects on A-549 lung cancer, HT-29 colon cancer, MCF-7 and MDA-MB-453 breast cancer cell lines, and the 3T3-L1 mouse fibroblast normal cell line, were investigated. To determine the antioxidant capacity of Shogaol, DPPH and ABTS radical scavenging assays, as well as Cu^{2+} , Fe^{3+} , and FRAP reduction methods, were employed. Its inhibitory effects on hCA I and II, AChE, BChE, α -glycosidase, and α -amylase enzymes were examined. Furthermore, cell counting was performed using the TBE method, and cell viability was measured with the XTT assay to assess the antiproliferative effects of Shogaol on A-549, HT-29, MCF-7, MDA-MB-453, and 3T3-L1 cell lines. Shogaol demonstrated higher antioxidant capacity than BHA, BHT, and Trolox in the FRAP assay (60 $\mu\text{g/mL}$), higher than BHT in the DPPH scavenging assay, and higher than Trolox in the ABTS, Cu^{2+} , and Fe^{3+} reduction assays. Shogaol also exhibited significant inhibitory effects against hCA I, hCA II, AChE, BChE, and α -glycosidase, with IC_{50} values of 75.91, 47.04, 37.44, 24.11, and 35.72 nM, respectively. Moreover, Shogaol showed a more potent antiproliferative effect than the standard chemotherapeutic drug Carboplatin in A-549 lung cancer and HT-29 colon cancer cell lines, and greater efficacy than Docetaxel in the MCF-7 breast cancer cell line.

Keywords Shogaol, Antioxidant activity, Enzyme inhibition, Carbonic anhydrase, α -Glycosidase, Antiproliferative effect

1 Introduction

Free radicals are highly reactive, unstable, and short-lived species that attack surrounding non-radical atoms or molecules, turning them into free radicals as well [1–3]. Free radicals are generated when the balance between oxidants and antioxidants in living organisms is disturbed. Oxidants derived from oxygen are referred to as “reactive oxygen species” (ROS). An antioxidant is a molecule that inhibits the oxidation of other



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molecules, preventing the formation of harmful free radicals. Free radicals can damage cells, proteins, and DNA, leading to aging and diseases. Antioxidants neutralize these reactive species, protecting the body and maintaining cellular health, and are found in foods, vitamins, and enzymes [4, 5]. Antioxidants prevent both the formation of free radicals and the damage they can inflict on biomolecules. In addition, they prolong the shelf life of foods, beverages, and pharmaceuticals by delaying lipid peroxidation [6]. Synthetic antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) are widely used in the food and pharmaceutical industries due to their low cost and high efficiency. These synthetic antioxidants (e.g., BHA, BHT and TBHQ) are manufactured additives used to prolong shelf life by inhibiting lipid oxidation. However, consumer acceptance has declined due to safety concerns, including accumulation in tissues and potential carcinogenicity and toxicity, as well as damage to the DNA [7–9]. Natural antioxidants (e.g., phenolics, vitamin C, tocopherols) occur in foods and help neutralize free radicals, ROS and prevent oxidation processes.

The major natural antioxidants in plants are phenolic compounds and strongly contribute to the antioxidant capacity of many foods. Phenolic compounds are multipurpose bioactive plant metabolites, often termed “secondary metabolites” and neutralize free radicals [10]. Phenolic hydroxyl groups are attached to an aromatic ring, which allows them to donate hydrogen atoms and neutralize free radicals. Their aromatic rings with hydroxyl (–OH) groups enable them to donate hydrogen atoms or electrons to neutralize free radicals and reactive oxygen species, helping to stop chain reactions like lipid peroxidation. Also, their antioxidant activity depends on structure, substitution, glycosylation, and synergistic effects. The higher total phenolic content in plants often correlates with stronger antioxidant activity. They include flavonoids, phenolic acids, tannins, and stilbenes, making them vital for human health [11]. Shogaol is a putative plant-derived phenolic compound and is associated with antioxidant and redox-active properties. While phenolic acids are simpler structures, Shogaol contains a phenolic ring plus an unsaturated side chain, enabling radical scavenging, metal chelation, and modulation of oxidative pathways, contributing to bioactivity. It is a phenolic compound containing one aromatic ring with a carbonyl group and a double bond. In its aliphatic chain, the carbonyl group is located at the third carbon and the double bond at the fourth carbon (Fig. 1). The aliphatic chain also contains a methoxy group at the *meta*- position and a hydroxyl group at the *para*- position. It is a nonvolatile compound and the dehydrated form of 6-gingerol. In addition to 6-Shogaol, homologous forms such as 8-Shogaol and 10-Shogaol also exist. 6-Shogaol can be synthesized starting from vanillin and acetone. 6-Shogaol has received considerable attention due to its antioxidant [12], anticancer [13, 14], and anti-inflammatory [15] properties. It has been shown to induce apoptosis in

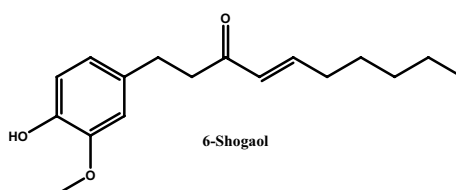


Fig. 1 Chemical structure of 6-shogaol ((4E)-1-(4-hydroxy-3-methoxyphenyl)dec-4-en-3-one)

cancer cells, including lung cancer [16], colorectal cancer [17], hepatocarcinoma [18], and breast cancer [19].

Phenolic antioxidants are known for their ability to scavenge free radicals due to their hydroxyl groups, which donate hydrogen atoms to neutralize ROS [20]. Beyond this, they can interact with enzymes, often inhibiting their activity by binding to active sites or altering enzyme conformation [21, 22]. In some recent studies, it has been reported that a correlation between phenolic content and enzyme inhibition has been widely observed. Accordingly, phenolic compounds with higher antioxidant capacity are often reported to exhibit stronger inhibitory effects on enzymes such as carbonic anhydrase (CA), α -amylase, α -glycosidase, and AChE [23, 24]. While CA inhibition is important for glaucoma therapy, the inhibition of α -amylase and α -glucosidase enzymes is one of the most attractive methods for the clinical treatment of type-2 diabetes (T2DM), which characterized by high blood sugar and insulin resistance. On the other hand, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition holds great promise for the treatment of Alzheimer's disease (AD) as a neurodegenerative disease and the most common form of dementia [25]. Therefore, identifying phenolic inhibitors that have an antioxidant and anticancer ability and act on these enzymes is of great importance.

Carbonic anhydrase (CA) is a metalloenzyme involved in respiration, containing Zn^{2+} in its structure [26, 27]. It catalyzes the conversion of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) in tissue capillaries and HCO_3^- back to CO_2 in pulmonary capillaries. Beyond this role, CA contributes to acid-base balance, cardiovascular regulation, ion exchange, and CO_2 transport [28]. CA inhibitors are used to treat diseases such as epilepsy and glaucoma [29], and modulation of CA activity is important for managing cancer, epilepsy, cardiovascular diseases, diabetes, and AD [30].

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are essential cholinergic enzymes involved in cholinergic neurotransmission. AChE primarily hydrolyzes the neurotransmitter acetylcholine (ACh) at neuronal synapses, ensuring proper nerve signal termination [31, 32]. BChE, also known as pseudocholinesterase, has broader substrate specificity and hydrolyzes various choline-based esters in plasma and tissues. Both cholinergic enzymes play critical roles in maintaining nervous system function and detoxifying xenobiotics [33]. Inhibition of AChE and BChE can lead to increased ACh levels, resulting in enhanced synaptic transmission or toxicity. Therefore, these enzymes are key targets for drugs treating AD and other neurological disorders [34]. AChE is found in the brain, muscles, erythrocytes, and nerve cells, where it hydrolyzes ACh into choline (Ch) and acetates in synaptic spaces [35]. Reduced AChE activity impairs nerve conduction and contributes to AD, leading to brain tissue damage and cognitive decline [36]. Inhibitors of AChE increase ACh levels, improving nerve impulse transmission and partially mitigating AD.

Diabetes mellitus (DM) is a metabolic disorder characterized by elevated blood glucose levels. Inhibition of α -glycosidase and α -amylase, which are secreted by small intestinal cells to break down polysaccharides into monosaccharides, is an important therapeutic strategy for controlling diabetes and hyperglycemia [37]. α -Glycosidase hydrolyzes D-glucose units from the non-reducing ends of α -glycan's in starch and glycogen, performing the final step of starch digestion [38]. On the other hand, α -amylase hydrolyzes α -1,4-glycosidic bonds in starch and is present in saliva and pancreatic secretions across

organisms. It is widely used in the food industry to delay bread staling and extend shelf life and in juice clarification [39].

Cancer, defined as uncontrolled cell proliferation, is a major global health issue with over 200 known types, affecting various tissues and organs. Its development is influenced by hereditary, environmental, behavioral, and biological factors [40]. Environmental factors include radiation, air pollution, chemicals, free radicals, ROS, bacteria, viruses, and UV rays. Genetic factors involve inherited gene mutations, with DNA damage forming the basis of most cancers [41]. Altered genes disrupt apoptosis, differentiation, and cell cycle pathways, eventually resulting in tumor formation [42]. Cancer is biologically important because it reveals how disruptions in cell cycle control, DNA repair, and apoptosis drive uncontrolled proliferation. Studying cancer uncovers mechanisms of mutation, signaling, angiogenesis, and metastasis, guiding biomarker discovery, targeted therapies, immunotherapy development, and preventive strategies that improve survival and public health on a global scale [43].

Upon reviewing the current literature, it is evident that there is a broad spectrum of studies on the Shogaol, demonstrating numerous biological activities. However, there is no study in current literature in which the antioxidant activity of Shogaol has been evaluated using multiple methods and compared with three different antioxidant standards, while also investigating its inhibitory effects on metabolically relevant enzymes associated with major global diseases and comparing these results with clinically established standard inhibitors. The aim of this study is to determine the antioxidant and antiproliferative capacities of Shogaol and to investigate its inhibitory effects on some metabolic enzymes such as hCA I, hCA II, AChE, BChE, α -glycosidase, and α -amylase, which are associated with certain global diseases.

2 Materials and methods

2.1 Chemicals

6-Shogaol, Trolox, neocuprine (2,9-dimethyl-1,10-phenanthroline), α -tocopherol, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH $\cdot$), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), p-nitrophenyl- α -D-glycopyranoside (p-NPG), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), para-nitrophenolate (PNF), acetone, CuCl₂, FeCl₃, K₃[Fe(CN)₆], L-Tyrosine, acetylthiocholine iodide, butyrylthiocholine iodide, and α -amylase were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The remaining unnamed compounds were obtained from either Sigma-Aldrich or Merck.

2.2 Antiproliferative bioassay

2.2.1 Cell lines and culture

Human alveolar basal epithelial (A549), human colorectal adenocarcinoma (HT-29), breast adenocarcinoma (MCF-7), human breast cancer (MDA-MB-453), and mouse fibroblast cell lines were obtained from ATCC (American Type Culture Collection, LGC Promochem, UK). A549, MDA-MB-453, and MCF-7 cells were cultured in EMEM supplemented with L-glutamine and 25 mM HEPES, while HT-29 cells were cultured in McCoy's 5 A medium (BI, USA) supplemented with Na-pyruvate (1%), penicillin-streptomycin (1%), and heat-inactivated fetal bovine serum (FBS, 10%) (BI, USA). Cultures were maintained at 37 °C in a humidified incubator with 5% CO₂ (NUVE, Turkey).

2.2.2 Determination of cell survival

The cytotoxicity of the samples was assessed using the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2 H-tetrazolium-5-carboxanilide (XTT) assay [44]. Succinate dehydrogenase enzymes in the mitochondria of viable cells reduce the tetrazolium salt XTT to a formazan product, which is orange in color and absorbs at 415 nm. Cells were seeded at 100 μ L per well in 96-well plates and incubated in a CO₂ incubator for 24 h. After incubation, cells were treated with FBS, EAE, and ME. For each well, 50 μ L of medium and 50 μ L of sample were added. XTT reagent was added after 24 h of treatment, and the plates were incubated for an additional 8 h. Cellular metabolic activity was then measured at 415 nm using an Epoch Microplate Reader (BioTek, USA). Cytotoxicity results were expressed as IC₅₀ values [45].

2.3 DPPH radical scavenging activity

The DPPH radical scavenging activity of Shogaol was evaluated according to the method described previously [46], with minor modifications [47]. A 0.1 mM DPPH solution in ethanol was prepared and pre-incubated in the dark for 16 h to allow radical stabilization. For the assay, 0.5 mL of the DPPH solution was mixed with 0.5 mL of Shogaol at different concentrations (15–60 μ g/mL) in ethanol. The mixtures were then incubated at 30 °C in the dark for 30 min. After incubation, absorbance was measured at 517 nm. The control consisted of ethanol in place of the sample or reference antioxidants [48].

2.4 ABTS radical scavenging activity

The ABTS[•] radical scavenging activity of Shogaol was determined according to a previously reported method [49]. To generate ABTS radicals, a 2 mM ABTS solution was mixed with 2.45 mM potassium persulfate in equal volumes and allowed to react in the dark at room temperature for 6 h. Before the assay, the absorbance of the resulting ABTS radical solution was adjusted to approximately 1.0 at 734 nm using phosphate-buffered saline (PBS, 0.1 M, pH 7.4). For the assay, 1 mL of the ABTS radical solution was mixed with 3 mL of Shogaol solutions at various concentrations (5–20 μ g/mL), and the absorbance was measured at 734 nm. The control reaction included PBS in place of the sample or standard antioxidants [50].

The percentage of radical scavenging by Shogaol and standard compounds was calculated using the following equation:

$$\text{Percentage scavenging (\%)} = \left(1 - \frac{A_s}{A_c}\right) \times 100$$

where A_c is the absorbance of the control and A_s is the absorbance in the presence of Shogaol or other scavengers.

2.5 Fe³⁺ reducing activity

The Fe³⁺ reducing power of Shogaol was evaluated by monitoring the reduction of Fe³⁺ in the presence of potassium ferricyanide, as described by Oyaizu [51]. In the assay, equal volumes of 0.2 M PBS buffer (pH 6.6) and 1% (w/w) potassium ferricyanide were mixed with 0.75 mL of Shogaol solutions at various concentrations (15–60 μ g/mL). The mixtures were incubated at 50 °C for 30 min, after which 1.25 mL of 10% trichloroacetic acid

(w/w) and 0.25 mL of 0.1% ferric chloride were added. The solutions were thoroughly vortexed, and absorbance was recorded at 700 nm. PBS buffer served as the blank [52].

2.6 Cu²⁺ reducing ability

The Cu²⁺ reducing activity of Shogaol was measured according to the method of Apak et al. [53], using neocuprine and an acetate buffer at pH 7.0. The assay mixture consisted of equal volumes of 10 mM CuCl₂, 7.5 mM neocuproine, and 1.0 M ammonium acetate buffer (pH 6.5) combined with Shogaol solutions at different concentrations (15–60 µg/mL) to a total volume of 2 mL. The mixtures were incubated at 25 °C for 30 min, and the absorbance was then measured at 450 nm. Acetate buffer was used in place of Shogaol as the blank [54].

2.7 FRAP reducing activity

FRAP activity was assessed by mixing 0.5 mL of Shogaol solutions at varying concentrations (15–60 µg/mL in 0.3 M acetate buffer, pH 3.6) with 2.25 mL of FRAP reagent and 2.25 mL of 20 mM FeCl₃, resulting in a total reaction volume of 5 mL. The mixtures were incubated at 37 °C for 30 min, after which absorbance was measured at 593 nm. The FRAP reagent was prepared as previously described [55]. Acetate buffer alone served as the blank.

2.8 Carbonic anhydrase inhibition ability

Carbonic anhydrase (CA) isoenzyme activity was determined spectrophotometrically using p-nitrophenyl acetate as the substrate, following Wilbur and Anderson [56]. The esterase activity of hCA I and hCA II isoenzymes was monitored at 348 nm. For the assay, a reaction mixture of 360 µL of 0.07 mM p-nitrophenyl acetate, 400 µL of 0.05 M Tris-SO₄ buffer (pH 7.4), and 20 µL of enzyme solution was prepared in a total volume of 1 mL. Shogaol concentration increased incrementally until more than 50% inhibition of enzyme activity was observed. Tacrine served as the reference inhibitor, and control consisted of the reaction mixture without Shogaol [57].

2.9 AChE and BChE inhibition ability

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were measured colorimetrically according to Ellman's method [58], using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine / butyrylthiocholine iodide as substrates. Inhibition assays were performed kinetically by combining 50 µL of 10 mM DTNB, 10 mM acetylthiocholine iodide or butyrylthiocholine iodide, and 100 µL of 1 M Tris-HCl buffer (pH 8.0), with absorbance monitored at 412 nm for 5 min. Shogaol concentration gradually increased until enzyme activity was reduced by over 50%. Donepezil was used as the standard inhibitor for AChE and BChE enzymes. Controls contained the same reaction mixture without Shogaol.

2.10 α-Glycosidase inhibition ability

The α-glycosidase inhibitory assay was performed using p-nitrophenyl-α-D-glycopyranoside (p-NPG) as the substrate, according to a previously reported protocol [59]. Briefly, various concentrations of Shogaol were prepared in phosphate buffer (0.1 M, pH 6.9). The enzyme reaction was initiated by adding 20 µL of α-glycosidase solution

to the buffer, followed by the addition of 50 μ L of p-NPG. The increase in absorbance was monitored at 405 nm over a period of 3 min, using a blank containing phosphate buffer in place of the sample. A control assay lacking Shogaol was included to represent 100% enzyme activity. Acarbose served as the positive control inhibitor, and the control reaction consisted of the complete assay mixture without Shogaol.

2.11 α -Amylase inhibition ability

The α -amylase inhibitory effect of Shogaol was evaluated following the method described by Tao et al. [60], with slight modifications. Briefly, the starch substrate was prepared by dissolving 3 g of starch in 150 mL of 0.4 M NaOH and heating the mixture at 98 °C for 10 min. After cooling to room temperature, the pH was adjusted to 6.9, and the volume was adjusted to 100 mL using distilled water. For the assay, a reaction mixture containing 35 μ L of the starch solution, 35 μ L of phosphate buffer (pH 6.9), and 15 μ L of Shogaol solutions were prepared and pre-incubated at 37 °C for 20 min. Subsequently, 20 μ L of α -amylase solution was added, and the mixture was further incubated for an additional 20 min under the same conditions. The reaction was stopped by adding 50 μ L of 0.1 M HCl, and the absorbance was recorded at 580 nm.

2.12 IC₅₀ values determination

In this study, IC₅₀ values represent the concentration of Shogaol required to inhibit 50% of enzyme activity. Values were calculated using a non-linear regression model based on normalized responses to Shogaol [61].

2.13 Molecular docking studies

The X-ray crystallography structures of AChE (PDB ID: 4EY7) [62], BChE (PDB ID: 6T9P) [63], α -glycosidase (PDB ID: 5NN8) [64], α -amylase (PDB ID: 2QV4) [65], CA II (PDB ID: 3HS4) [66] and CA I (PDB ID: 1AZM) [67] were downloaded from the “Protein Data Bank” website (PDB) with resolutions of 2.35 Å, 2.70 Å, 2.45 Å, 1.97 Å, 2.70 Å and 2.00 Å, respectively [68]. To investigate the binding affinities of the Shogaol compound at the active sites of the target enzymes, the 3D version of the chemical structure of the ligand was downloaded from PubChem [69]. Enzyme and ligand structures were optimized and loaded with AutoDock-Tools (v1.5.7, The Scripps Research Institute, San Diego, CA). Then the molecular docking protocol was applied to the determined targets using AutoDock Vina 1.1.2 [70]. The best docking scores and binding interactions are analyzed by BIOVIA Discovery Studio (Accelrys Software Inc., San Diego, CA, USA, 2012).

3 Results and discussion

The DPPH radical scavenging method is a commonly used assay to evaluate the free radical scavenging activity of antioxidants. The DPPH radical is stable, long-lived, and dark blue-violet in color. It is a nitrogen-centered radical with a maximum absorbance at 517 nm [71]. It is commercially available and does not need to be generated before experimentation. In contrast, another radical, ABTS, must be produced prior to use. However, ABTS radicals are more reactive than DPPH radicals [72]. The DPPH $\cdot$ radical is reduced when it reacts with an antioxidant capable of donating a hydrogen atom, forming the non-radical DPPH-H. As a result, the dark blue-violet color changes to light

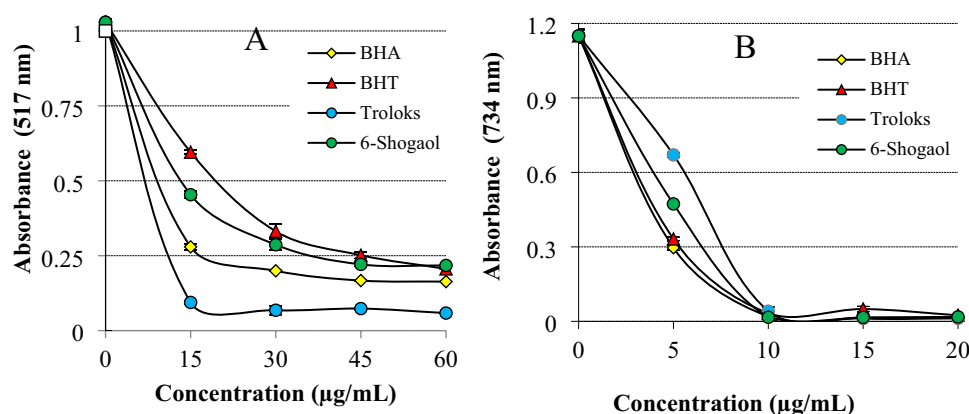


Fig. 2 **A** DPPH• scavenging activities of Shogaol and standards; **B** ABTS•⁺ scavenging activities of Shogaol and standards

Table 1 Half maximal inhibition concentration values (IC_{50} , $\mu\text{g/mL}$) of shogaol for DPPH•, ABTS•⁺ scavenging activities

Antioxidants	DPPH• scavenging		ABTS• ⁺ scavenging	
	IC_{50}	r^2	IC_{50}	r^2
BHA	18.45	0.6119	4.48	0.6752
BHT	28.11	0.8534	4.98	0.6888
Trolox	10.88	0.5263	6.70	0.8101
Shogaol	24.79	0.7355	5.61	0.7502

ABTS 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), DPPH 1,1-diphenyl-2-picryl-hydrazyl, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

yellow, and the absorbance at 517 nm decreases. A limitation of this method is that other compounds can also absorb light at wavelengths near 517 nm [73]. For instance, anthocyanins show strong absorption in the 500–550 nm range. Therefore, when interpreting results, it is necessary to assess possible spectral interference. The DPPH• scavenging activity of Shogaol was observed to increase with concentration. As seen Fig. 2A; Table 1, Shogaol was found to be more effective than BHT but less effective than Trolox and BHA at concentrations between 15 and 45 $\mu\text{g/mL}$. The IC_{50} values for DPPH• scavenging activity at 30 $\mu\text{g/mL}$ were as follows: Trolox (λ_{517} : 10.88 $\mu\text{g/mL}$), BHA (λ_{517} : 18.45 $\mu\text{g/mL}$), Shogaol (λ_{517} : 24.79 $\mu\text{g/mL}$), and BHT (λ_{517} : 28.11 $\mu\text{g/mL}$).

Recently, the DPPH radical scavenging activity of many biologically active phenolic compounds has been reported [73]. The IC_{50} values for DPPH• scavenging activity of Shogaol and the standards at a concentration of 30 $\mu\text{g/mL}$ are as follows: Trolox (λ_{517} : 10.88 $\mu\text{g/mL}$), BHA (λ_{517} : 18.45 $\mu\text{g/mL}$), Shogaol (λ_{517} : 24.79 $\mu\text{g/mL}$), and BHT (λ_{517} : 28.11 $\mu\text{g/mL}$). IC_{50} values reported in the literature for various compounds for DPPH• scavenging activity: curcumin, 34.90 $\mu\text{g/mL}$ [74], L-adrenaline, 30.60 $\mu\text{g/mL}$ [75], quercetin, 31.41 $\mu\text{g/mL}$; gallic acid, 16.13 $\mu\text{g/mL}$; p-coumaric acid, 48.85 $\mu\text{g/mL}$ [76], gingerol, 17.33 $\mu\text{g/mL}$ [77] and capsaicin, 34.65 $\mu\text{g/mL}$ [78]. Shogaol exhibited stronger DPPH• scavenging activity than compounds such as BHT, curcumin, quercetin, p-coumaric acid, capsaicin, and synephrine, but lower activity than gingerol and gallic acid.

The ABTS•⁺ scavenging method is suitable for both lipophilic and hydrophilic compounds. The principle of the method is based on the generation of ABTS radicals through the reaction of ABTS with $K_2S_2O_8$, and the subsequent reduction in absorbance of the ABTS•⁺ cation radical at 734 nm [79]. The ABTS•⁺ scavenging activity of Shogaol

was found to be more effective than that of Trolox at a concentration of 5 µg/mL but lower than those of BHA and BHT. At concentrations of 10, 15, and 20 µg/mL, both the standards and Shogaol completely eliminated the ABTS radicals. The IC₅₀ values of the ABTS^{•+} scavenging activities of Shogaol and standard antioxidants at a concentration of 5 µg/mL are as follows: BHA (λ_{734} : 4.588 µg/mL), BHT (λ_{734} : 4.980 µg/mL), Shogaol (λ_{734} : 5.611 µg/mL), and Trolox (λ_{734} : 6.707 µg/mL) (Fig. 2B). The ABTS^{•+} radical scavenging effects of many phenolic compounds have also been determined and compared with standard antioxidants. The IC₅₀ values reported in the literature for ABTS^{•+} scavenging activity are, 22.56 µg/mL for p-coumaric acid, 14.35 µg/mL for quercetin, 13.75 µg/mL for ellagic acid, 11.50 µg/mL for ferulic acid, 6.93 µg/mL for L-adrenaline [75], 11.14 µg/mL for gallic acid [76], 4.33 µg/mL for capsaicin [78], 22.35 µg/mL for octopamine [80]. Shogaol demonstrated higher ABTS^{•+} scavenging activity than compounds such as Trolox, quercetin, p-coumaric acid, ferulic acid, octopamine, ellagic acid, and phenylephrine, but lower activity than capsaicin, synephrine, and gingerol.

The reducing capacity of bioactive compounds can be determined by the reduction of $\text{Fe}[(\text{CN})_6]^{3-}$ to $\text{Fe}[(\text{CN})_6]^{2-}$. When Fe^{3+} is added to the reduced product, a Prussian Blue complex ($\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$) is formed, which shows strong absorbance at 700 nm [81]. The yellow color of the test solution changes to shades of blue or green depending on the reducing strength of the antioxidant [82]. An increase in absorbance indicates greater complex formation and thus higher reducing ability. The reducing power of a compound serves as an important indicator of its potential antioxidant activity [83]. The Fe^{3+} reducing capacities of Shogaol and the standards at 30 µg/mL were as follows: BHA (λ_{700} : 3.390 ± 0.037) > BHT (λ_{700} : 3.170 ± 0.167) > Shogaol (λ_{700} : 1.511 ± 0.055) > Trolox (λ_{700} : 1.491 ± 0.069) (Fig. 3B; Table 2). Absorbance values reported in the literature for Cu^{2+} reducing capacity include: 1.084 for gingerol [77] 0.864 for olivetol [84] at 30 µg/mL; 1.556 for quercetin and 0.506 for p-coumaric acid [76], and 0.757 for capsaicin [78] at 10 µg/mL. Shogaol exhibited greater Fe^{3+} reducing capacity than compounds such as Trolox, gingerol, and olivetol.

The CUPRAC method is based on the reduction of Cu^{2+} to Cu^+ by antioxidants in the presence of neocuprine, a heterocyclic compound and chelating agent, in an aqueous ethanolic medium (pH 7.0). The initial light blue color turns yellow-orange after the reaction, and the resulting complex shows maximum absorption at 450 nm. The CUPRAC reagent has a lower redox potential and greater stability than other oxidative reagents. This method allows for the determination of the antioxidant activity of both hydrophilic and lipophilic compounds [84]. The Cu^{2+} -reducing capacity of Shogaol was found to be higher than that of Trolox but lower than those of BHA and BHT at all tested concentrations. According to the Fig. 3A, the Cu^{2+} -reducing capacities of Shogaol and the standards at 30 µg/mL were as follows: BHA (λ_{450} : 1.297 ± 0.052 µg/mL) > BHT (λ_{450} : 1.295 ± 0.023 µg/mL) > Shogaol (λ_{450} : 1.041 ± 0.025 µg/mL) > Trolox (λ_{450} : 0.909 ± 0.047 µg/mL). It was observed that the Fe^{3+} -reducing capacity of Shogaol was more effective than Trolox at a concentration of 30 µg/mL and slightly lower than BHA and BHT at 15 µg/mL. Absorbance values reported in the literature for Cu^{2+} -reducing capacities of various compounds are as follows: 0.343 for gingerol [77], 1.314 for olivetol at 30 µg/mL [83], 1.590 for quercetin and 0.918 for p-coumaric acid [76], 0.195 for octopamine [95], and 0.402 for capsaicin at 10 µg/mL [78]. Shogaol exhibited greater Cu^{2+} -reducing capacity than Trolox and gingerol but lower than olivetol. The CUPRAC method, as noted, relies

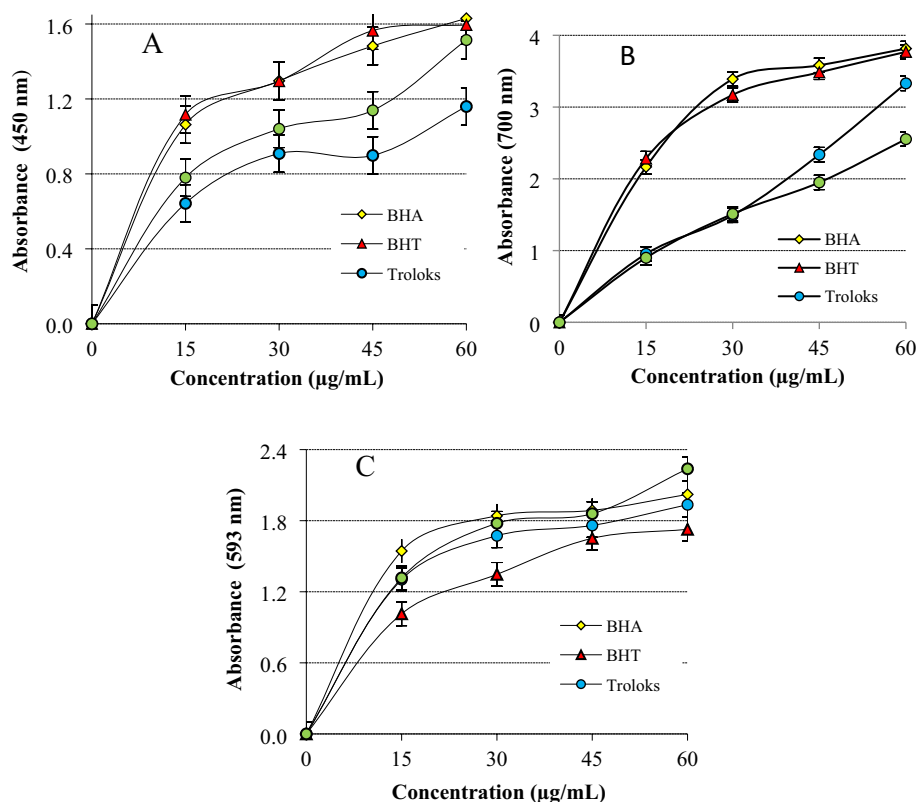


Fig. 3 **A** Cu^{2+} reducing activity of Shogaol and standards; **B** Fe^{3+} reducing activity of Shogaol and standards; **C** According to FRAP method, Fe^{3+} reducing activity of Shogaol and standards

Table 2 Cu^{2+} (30 $\mu\text{g/mL}$), Fe^{3+} (30 $\mu\text{g/mL}$) and Fe^{3+} (FRAP; 60 $\mu\text{g/mL}$) reducing activities of Shogaol and of standard antioxidants

Antioxidants	Cu^{2+} Reducing		Fe^{3+} Reducing		FRAP reducing	
	λ_{450}	r^2	λ_{700}	r^2	λ_{593}	r^2
BHA	1.297 ± 0.052^b	0.8073	3.390 ± 0.037^c	0.8173	2.022 ± 0.158^c	0.6918
BHT	1.295 ± 0.023^b	0.7749	3.170 ± 0.167^c	0.8199	1.727 ± 0.004^a	0.8523
Trolox	0.909 ± 0.047^a	0.8447	1.491 ± 0.069^a	0.9918	1.935 ± 0.018^b	0.7679
Shogaol	1.041 ± 0.025^a	0.8976	1.511 ± 0.05^b	0.9836	2.238 ± 0.087^d	0.8351

FRAP Ferric reducing antioxidant power, ABTS 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), DPPH 1,1-diphenyl-2-picryl-hydrazyl, BHA butylated hydroxyanisole, BHT butylated hydroxytoluene

*Results indicated by separate letters showed statistically significant differences ($p < 0.05$)

on the reduction of Cu^{2+} by antioxidants in the presence of neocuprine—a heterocyclic chelating agent—in an aqueous ethanolic medium (pH 7) [84].

The FRAP method is a typical electron-transfer-based assay. In an acidic medium, the ferric 2,4,6-tripyridyl-s-triazine complex ($[\text{Fe}^{3+}-(\text{TPTZ})_2]^{3+}$) is reduced by antioxidants to the dark blue ferrous complex ($[\text{Fe}^{2+}-(\text{TPTZ})_2]^{2+}$). This complex shows absorbance at 593 nm. The FRAP method can be applied to liquids, foods, plant extracts, spices, vegetables, beverages, and juices. It is a simple, rapid, inexpensive, and powerful method that does not require specialized equipment. However, a limitation of the FRAP method is that it cannot measure the antioxidant capacity of compounds that react with Fe^{2+} or those containing $-\text{SH}$ groups [85]. According to the FRAP assay results, the Fe^{3+} -reducing activity of Shogaol was higher than that of all standards at 60 $\mu\text{g/mL}$ and was more effective than BHT and Trolox, being very close to BHA at 45

$\mu\text{g/mL}$. The Fe^{3+} -reducing activities of Shogaol and the standards at 60 $\mu\text{g/mL}$ were as follows: Shogaol (λ_{593} : 2.238 ± 0.087) > BHA (λ_{593} : 2.022 ± 0.158) > Trolox (λ_{593} : 1.935 ± 0.018) > BHT (λ_{593} : 1.727 ± 0.004) (Fig. 3C). IC_{50} values reported in the literature for Fe^{3+} -reducing activity (FRAP method) are as follows: 1.260 for gingerol [77], 1.260 for olivetol at 30 $\mu\text{g/mL}$ [83], and 0.397 for octopamine [80]. Shogaol exhibited stronger Fe^{3+} -reducing activity in the FRAP assay than compounds such as Trolox, BHT, gingerol, and olivetol.

Carbonic anhydrase (CA) is an enzyme that catalyzes the reversible conversion of carbon dioxide and water into carbonic acid, which subsequently dissociates into bicarbonate and hydrogen ions. It plays a crucial role in maintaining the acid–base balance in blood and tissues. Found in red blood cells, kidneys, and other organs, CA helps regulate pH and facilitates the transport of carbon dioxide. Enzymes are also involved in respiration and ion transport and hold significant medical relevance in the treatment of conditions such as glaucoma and epilepsy [26]. Following purification using Sepharose-4B–L-Tyrosine–sulfanilamide affinity chromatography, the hCA I isoenzyme was purified 243-fold with a yield of 40.67%, and its specific activity was determined as 1.367 EU/mg. The hCA II isoenzyme was purified 309-fold with a yield of 81.92%, and its specific activity was found to be 1.546 EU/mg (Table 3). The IC_{50} values of the esterase activities of both hCA I and hCA II isoenzymes were found to be 75.91 nM (r^2 : 0.9613) and 47.04 nM (r^2 : 0.9265), respectively.

Lineweaver–Burk plots were generated using the results obtained from assays conducted with five different concentrations of p-nitrophenyl acetate as the substrate and three different concentrations of the sample to determine the K_i values of the hCA I and hCA II isoenzymes [86]. In the resulting plots, the lines corresponding to the control and the three sample concentrations intersected on the x-axis. This indicates that Shogaol acts as a non-competitive inhibitor for both hCA I and hCA II isoenzymes. The K_i values for hCA I were found to be 41.71 nM, 42.00 nM, and 42.63 nM for I_1 , I_2 , and I_3 , respectively, with a mean K_i value of 42.11 nM (Fig. 4A). The K_i values for hCA II were determined as 31.36 nM, 28.40 nM, and 28.67 nM for I_1 , I_2 , and I_3 , respectively, with a mean K_i value of 29.47 nM. Shogaol was found to exert an inhibitory effect on hCA I and hCA II isoenzymes, expressed by IC_{50} values of 75.91 nm and 47.04 nm, respectively (Fig. 4B). IC_{50} and mean K_i values on hCA I and hCA II isoenzymes of Shogaol and some compounds from the literature are given in Table 4.

It was observed that the inhibitory effect of Shogaol on the hCA I isoenzyme was higher than that of phenolic compounds such as carvacrol, olivetol, phloretin, and chrysin, but lower than that of the positive control acetazolamide and compounds such as gingerol, naringin, and resveratrol. Similarly, the inhibitory effect of Shogaol on the hCA

Table 3 Enzyme unit, specific activity, %yield and purification coefficient results for hCA I and hCA II isoenzymes purified by affinity chromatography from haemolysate obtained from human erythrocyte

Purification steps	Activity (EU/mL)	Volume (mL)	Protein (mg/mL)	Total activity (EU)	Total protein (mg)	Specific activity (EU/mg)	Yield (%)	Purification fold
Haemolysate	236	18	39485.68	4248	710,742	0.005	100	1
hCA I	216	8	158	1728	1264	1.367	40.67	273
hCA II	580	6	375	3480	2250	1.546	81.92	309

hCA I / human carbonic anhydrase I isoenzyme, hCA II / human carbonic anhydrase II isoenzyme

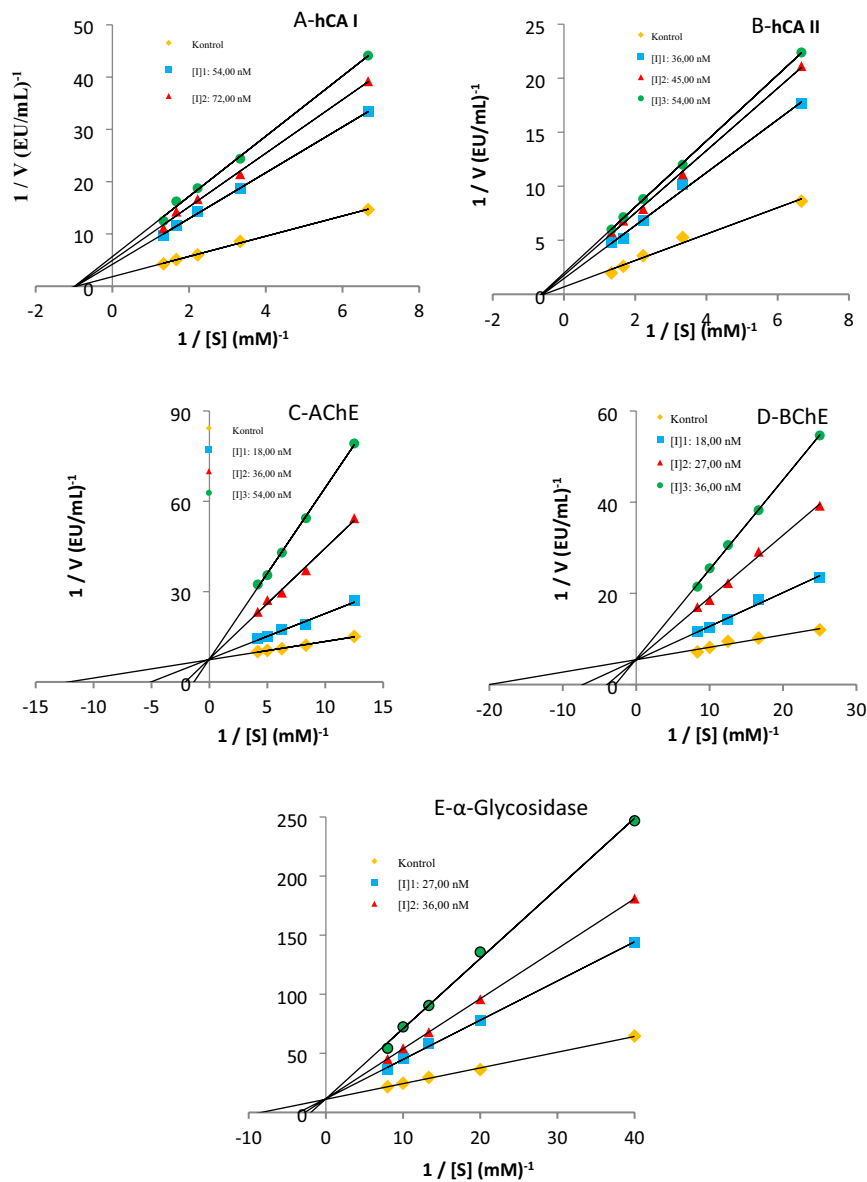


Fig. 4 Lineweaver-Burk graph drawn to find the K_i values of Shogaol for hCA I (A); hCA II (B), AChE (C), BChE (D), α -glycosidase (E) enzymes

Table 4 Inhibition type, IC_{50} and K_i values of Shogaol on hCA I and hCA II isoenzymes

Enzyme	IC_{50} (nM)	r^2	Average K_i (nM)	Inhibition type
hCA I	75.91	0.9613	42.11	Non-competitive
hCA II	47.04	0.9265	29.47	Non-competitive
AChE	37.44	0.9748	8.95	Competitive
BChE	24.11	0.9527	7.60	Competitive
α -Glycosidase	35.72	0.9654	16.83	Competitive
α -Amylase	402.90	0.9928	—	—

hCA I human carbonic anhydrase I isoenzyme, *hCA II* human carbonic anhydrase II isoenzyme, *AChE* Acetylcholinesterase enzyme, *BChE* Butyrylcholinesterase enzyme

II isoenzyme was higher than that of phenolic compounds such as carvacrol, zingerone, olivetol, chrysin, and resveratrol, but lower than that of the positive control acetazolamide and compounds such as gingerol and naringin.

Cholinesterases are versatile enzymes that have attracted considerable attention due to their molecular structure and essential roles in the cholinergic system. AChE hydrolyzes the excess acetylcholine accumulated after neuronal communication [81]. Excessive degradation of ACh leads to decreased levels of this neurotransmitter in the brain, resulting in impaired nerve conduction. This can cause various neurological disorders such as memory loss, language difficulties, psychological disturbances, and impairments in perception, abstract thinking, judgment, learning, and problem-solving skills. Inhibitors of the AChE enzyme increase the concentration of ACh by preventing its breakdown, thereby facilitating nerve impulse transmission. In this way, AD symptoms can be partially alleviated [86]. The IC_{50} values of Shogaol for AChE and BChE enzymes were determined as 37.44 nM (r^2 : 0.9748) (Fig. 4C) and 24.11 nM (r^2 : 0.9527) (Fig. 4D), respectively (Table 4). On the other hand, tacrine, used as clinical inhibitor for both cholinergic enzymes, demonstrated that the IC_{50} value of 15.06 nM for AChE enzyme.

Shogaol showed a higher inhibitory effect on AChE than phenolic compounds such as zingerone, carvacrol, phloretin, capsaicin, and lower than the positive control tacrine and compounds such as gingerol, olivetol, naringin, resveratrol. It is seen that the inhibition effect of Shogaol on the BChE enzyme is higher than phenolic compounds such as carvacrol, zingerone, chrysin, resveratrol, naringin and lower than the positive control tacrine and compounds such as gingerol and olivetol.

Lineweaver-Burk graph was generated with the results obtained by working with 5 different concentrations of acetylcholine iodate used as a substrate and three different Shogaol concentrations to find the K_i value of the AChE and BChE enzymes. In the graph obtained, it was seen that the control and 3 different sample concentration lines coincided with the y-axis. From this, it was determined that Shogaol acts as a competitive inhibitor for both the AChE and BChE enzymes. K_i values for AChE were found 12.74, 7.44, 6.67 nM for I_1 , I_2 and I_3 , respectively, and mean K_i : 8.95 nM. K_i values for BChE were found 10.22, 6.64, and 5.96 nM for I_1 , I_2 and I_3 , respectively, and average K_i : 7.60 nM. However, Tacrine, was used as clinical inhibitor for cholinergic enzymes, demonstrated that the IC_{50} value of 16.75 nM for BChE enzymes. These results clearly showed that Shogaol had higher BChE inhibitor potency than that of Tacrine, which is used to treat the symptoms of mild to moderate AD.

α -Glycosidase enzymes hydrolyze starch and glycogen molecules. With the reactions catalyzed by these enzymes, carbohydrates are broken down into glucose and fructose molecules. Glucose and fructose are absorbed from the intestine and pass into the blood [86]. When inhibitors of the α -glycosidase enzyme inhibit the enzyme, carbohydrate digestion time is prolonged, and glucose absorption is reduced. As a result, the amount of blood sugar decreases. Diabetes can be treated by taking advantage of this situation [79]. The IC_{50} values of Shogaol towards α -glycosidase and α -amylase enzymes were found to be 35.72 nM (r^2 : 0.9654) and 402.90 nM (r^2 : 0.9928), respectively (Fig. 4E). α -Amylase is an enzyme that breaks down starch into simpler sugars like maltose and glucose. It is found in human saliva and pancreatic juice. α -Amylase plays a key role in digestion and is widely used in food, textile, and brewing industries due to its starch-degrading ability.

Cancer is one of the deadliest diseases today. Among the factors which provoke cancer are radiation, chemicals, ROS, UV rays, race, gender, age, malnutrition, cigarette-alcohol use. The fact that a definitive treatment has not yet been found reveals how much cancer threatens human health. Scientists are increasingly researching the treatment of this disease. An important part of these studies is to investigate the cytotoxic effects of phenolics on cancer cells. Because it has been reported in studies that phenolics inhibit cancer cells, even in the initial stage or in advanced stages, show cytotoxic effects and reduce the risk of cancer [87, 88]. The XTT method was used to determine the antiproliferative effect of Shogaol compound against cancerous cell lines [89]. The XTT method is based on the reduction of XTT, which is a heterocyclic tetrazolium salt, to the formazan product, which absorbs at 415 nm. Succinate dehydrogenase enzymes in the mitochondria of living cells reduce the XTT tetrazolium salt to the formazan product, which is orange in color and absorbs at 415 nm. The ring with four nitrogen atoms in the center of the XTT salt opens after reduction. The resulting orange colored XTT formazan product absorbs at 415 nm in direct proportion to the number of active cells [90].

Docking studies were performed to analyze the binding modes of Shogaol and to elucidate its inhibition mechanisms. According to the molecular docking results, Shogaol exhibited strong binding affinities toward six enzyme targets, including AChE, BChE, CA I, hCA II, α -glycosidase, and α -amylase (Table 5). The Shogaol-hCA I complex showed a binding affinity of -5.4 Kcal/mol (Table 5). The interactions included three hydrogen bonds with Thr199, Gln92, and His200, a π -sigma interaction with Leu198, and several alkyls and π -alkyl interactions involving Trp209, His94, His119, Tyr204, Leu198, Val143, and Ala135. A π - π stacked interaction was also observed with His200 (Fig. 5D; Table 5). Shogaol also exhibited a high binding affinity toward CA II, with a docking score of -6.2 Kcal/mol (Table 5). The molecule formed three hydrogen bonds with His64, Glu239, and Tyr7. Additionally, the aromatic ring participated in an amide- π stacked interaction with Tyr7, a π -anion interaction with Glu239, a π -alkyl interaction with Lys170, and an alkyl interaction with Phe231 (Fig. 5E; Table 5).

Among these, Shogaol - AChE complex showed the highest binding affinity, with a docking score of -8.4 Kcal/mol (Table 5). The interactions between Shogaol and AChE were characterized by three hydrogen bonds with residues Ser203 and Gly121, as well as several alkyls, π -alkyl, and π - π T-shaped interactions with residues Trp286, Tyr341, Val294, Phe338, Phe297, and His447. Additionally, the aromatic ring of Shogaol formed two π - π T-shaped interactions with His447 and Trp86 (Fig. 5A; Table 5). The Shogaol-BChE complex exhibited a docking score of -7.8 Kcal/mol (Fig. 5B; Table 5). The interactions included two hydrogen bonds with His438 and Glu197, two π - π stacked interactions with Trp82 and His438, and multiple alkyls and π -alkyl interactions involving Leu286, Phe398, His438, and Trp82/Trp231 (Fig. 5B; Table 5). The binding affinity of the Shogaol- α -glycosidase complex was calculated as -6.0 Kcal/mol. The complex displayed three π -alkyl interactions with residues Leu678, Trp376, and Phe649 (Fig. 5C; Table 5).

The binding affinity of the Shogaol- α -amylase complex was determined as -6.6 Kcal/mol (Table 5). The interactions included three hydrogen bonds with Glu233, Arg195, and Asp300; two π -anion interactions with Asp197 and Asp300; and several alkyls and π -alkyl interactions involving Trp59, Leu162, and His305 (Fig. 5E; Table 5). Based on the docking scores, Shogaol demonstrated effective binding and inhibitory potential toward

all six target enzymes. These computational findings are in strong agreement with the *in vitro* results obtained in this study.

Molecular docking was performed using single inhibitor structures of the target enzymes. These static conformations do not fully represent protein flexibility or dynamic conformational changes that may occur under physiological conditions, potentially affecting ligand binding modes. The docking protocol primarily considers ligand flexibility, while the protein is treated as rigid. Side-chain rearrangements and induced-fit effects upon Shogaol binding are therefore not fully captured. Also, the binding energies obtained from AutoDock Vina are estimations based on empirical scoring functions. These scores may not accurately reflect true binding free energies or correlate quantitatively with experimental IC_{50} or K_i values. Docking studies were performed on isolated enzyme structures and do not consider cellular context, post-translational modifications, protein–protein interactions, or differences among enzyme isoforms *in vivo*.

While investigating the antiproliferative effect of Shogaol against the A-549 human lung cancer cell line and the MCF-7 and MDA-MB-453 human breast cancer cell lines, the chemotherapeutic drugs including mitoxantrone, carboplatin, and docetaxel were used as positive controls. A graph of percentage cell proliferation versus concentration was plotted using the data obtained from absorbance measurements at 415 nm, and IC_{50} values were calculated from the resulting curves. As seen in Table 6, the IC_{50} values of Shogaol and the standard chemotherapeutic drugs against the A-549 human lung cancer cell line were found to be as follows (in increasing order of IC_{50}): Mitoxantrone (2.74 μM) < docetaxel (4.35 μM) < Shogaol (11.49 μM) < carboplatin (62.93 μM). For the MCF-7 human breast cancer cell line, the IC_{50} values were determined as follows: Mitoxantrone (2.55 μM) < carboplatin (16.36 μM) < Shogaol (51.31 μM) < docetaxel (74.61 μM). For the MDA-MB-453 human breast cancer cell line, the IC_{50} values were found as follows: Mitoxantrone (2.17 μM) < docetaxel (4.58 μM) < carboplatin (22.63 μM) < Shogaol (62.42 μM).

While investigating the antiproliferative effect of Shogaol against the HT-29 human colon cancer cell line and the 3T3-L1 mouse fibroblast normal cell line, the chemotherapeutic drug carboplatin was used as a positive control. A graph of percentage cell proliferation versus concentration was plotted using the data obtained from absorbance measurements at 415 nm, and IC_{50} values were calculated from the resulting curves. The IC_{50} values of Shogaol and the standard chemotherapeutic drug against the HT-29 human colon cancer cell line were found as follows: Shogaol (8.38 μM) < carboplatin (46.16 μM). The IC_{50} values of Shogaol and carboplatin against the 3T3-L1 mouse fibroblast normal cell line was determined as follows: carboplatin (21.32 μM) < Shogaol (124.35 μM). It was observed that Shogaol exhibited the highest antiproliferative effect on the HT-29 colon cancer cell line, with an IC_{50} value of 8.38 μM . Shogaol demonstrated greater activity than carboplatin in the A-549 and HT-29 cell lines, and higher activity than docetaxel in the MCF-7 cell line. The higher antiproliferative effectiveness of Shogaol against A-549, HT-29, and MCF-7 cell lines compared with some standard drugs may indicate its potential as an anticancer agent (Fig. 6).

The IC_{50} values of Shogaol and the standard chemotherapeutic drug carboplatin against the 3T3-L1 mouse fibroblast normal cell line was found to be 124.35 μM and 21.32 μM , respectively. Shogaol exhibited a much lower antiproliferative effect than carboplatin on the 3T3-L1 normal cell line (Table 6). These results are highly valuable for

Table 5 Molecular interactions ofShogaol with AChE (B: 4EY7), BChE (C:6T9P), α-glycosidase (E: 5NN8), α-amylase (2QV4), Carbonic anhydrase I (F: 1AZM), and Carbonic anhydrase II (D: 3HS4)

Complex	Docking scores (Kcal/mol)	Types of interactions	Interacting residues	2D docking poses of shogaol and the enzymes
AChE (4EY7) -Shogaol	- 8.4	Hydrogen bonding alkyl π alkyl π-π T-shaped	SER:203; GLY: 121 TRP:286; TYR:341; VAL:294 PHE:338, 297; HIS:447 HIS:447; TRP:86	
BChE(6T9P) -Shogaol	- 7.8	Hydrogen bonding π-π stacked alkyl π alkyl	HIS:438; GLU:197 TRP:82; HIS:438 TRP:231; TRP-82 LEU:286; PHE:398; HIS:438; TRP:82	

Table 5 (continued)

Complex	Docking scores (Kcal/mol)	Types of interactions	Interacting residues	2D docking poses of shogaol and the enzymes
α -Glycosidase (5NN8)-Shogaol	- 6.0	π alkyl	LEU:678; TRP:376; PHE:649	
α -Amylase (2QV4)-Shogaol	- 6.0	Hydrogen bonding π - π T-shaped π anion π alkyl	ARG:600; ASP:518, 404 TRP:481 ASP:616 HIS:674; TRP:516,618; LEU:650,678	
	- 6.6	Hydrogen bonding π -anion alkyl π alkyl	GLU:233; ARG:195; ASP:300 ASP:197; 300 TRP:59; LEU:162 HIS:305; TRP:58	

Table 5 (continued)

Complex	Docking scores (Kcal/mol)	Types of interactions	Interacting residues	2D docking poses of shogaol and the enzymes
hCA I (1AZM) -Shogaol	- 5.4	Hydrogen bonding alkyl π alkyl π sigma π - π stacked	THR:199; GLN:92; HIS:200 TRP:209; HIS:94; 119; TYR:204 LEU:198; VAL:143; ALA:135 LEU:198 HIS:200	
hCA II (3HS4) -Shogaol	- 6.2	Hydrogen bonding π alkyl Alkyl Amide- π stacked π anion	HIS:64; GLU:239; TYR:7 LYS:170 PHE:231 TYR:7 GLU:239	

hCA /human carbonic anhydrase I isoenzyme, hCA II/human carbonic anhydrase II isoenzyme, hCA Acetylcholinesterase enzyme, hCA Butyrylcholinesterase enzyme

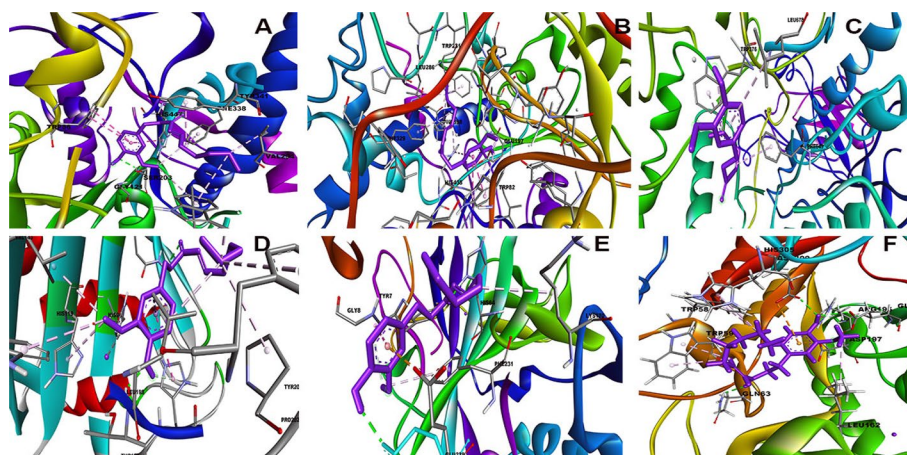


Fig. 5 3D docking poses of Shogaol and the enzymes AChE (A), BChE (B), α -glycosidase (C), CA I (D), CA II (E), and α -amylase (F)

Table 6 Half maximal inhibition concentration (IC_{50} , μ M) values of Shogaol and different chemotherapeutic drugs on various cancer (A-549, HT-29, MCF-7, MDA-MB-453) and normal cell (3T3-L1) lines

Compounds	A-549	HT-29	MCF-7	MDA-MB-453	3T3-L1
Shogaol	11.49 ± 0.92^d	8.38 ± 0.70^a	51.31 ± 1.44^c	62.42 ± 1.44^d	124.35 ± 2.15^b
Carboplatin	62.93 ± 1.54^c	46.16 ± 0.80^b	16.36 ± 0.60^b	22.63 ± 0.70^c	21.32 ± 1.06^a
Docetaxel	4.35 ± 0.36^b	–	74.61 ± 1.35^d	4.58 ± 0.44^b	–
Mitoxantrone	2.74 ± 0.75^a	–	2.55 ± 0.44^a	2.17 ± 0.52^a	–
<i>Selectivity Index (SI) values</i>					
Shogaol	10.82	46.16	16.36	22.63	124.35
Carboplatin	0.33	8.38	51.31	62.42	21.32

* Results indicated by separate letters showed statistically significant differences ($p < 0.05$)

cancer drug development, as ideal anticancer agents are expected to selectively target and kill cancer cells while causing minimal damage to healthy, normal cells. This property is referred to as the “selectivity index.” The selectivity index values of Shogaol and carboplatin against the HT-29 colon cancer cell line were calculated as 14.83 and 0.46, respectively. Accordingly, Shogaol was found to be 32.24 times more selective than carboplatin. Moreover, Shogaol also appeared to be more selective than carboplatin against other tested cell lines. These findings provide valuable insights for future studies and indicate that Shogaol may be an effective pharmacological agent.

In a study by Wang et al. [91], the IC_{50} value of chlorogenic acid against the A-549 cell line was found to be 150 μ M. Kabala-Dzik et al. [92] reported that IC_{50} values after 24 h of incubation on the MCF-7 cell line as 102.98 μ M for caffeic acid and 56.39 μ M for caffeic acid phenyl ester using the XTT assay. Chou et al. determined the IC_{50} value of quercetin after 48 h of incubation on the MCF-7 cell line as 92.4 μ M [93]. Luna-Dulcey et al. found the IC_{50} values of 6-gingerol after 48 h of incubation to be 985.8 μ M for the MCF-7 cell line, 599.4 μ M for the MCF-10 A cell line, and 404.5 μ M for the MDA-MB-231 cell line [94]. Rezaei-Seresht et al. reported that IC_{50} values after 48 h of incubation on the MCF-7 cell line as 170 μ g/mL for caffeic acid, 18.5 μ g/mL for gallic acid, and 19.4 μ g/mL for tamoxifen citrate using the MTT method [95]. It was reported that IC_{50} values of 150 μ M for gallic acid and 80 μ M for curcumin after 24 h of incubation on the MDA-MB-231 cell line using the MTT assay [96]. Sales et al. reported IC_{50} values after

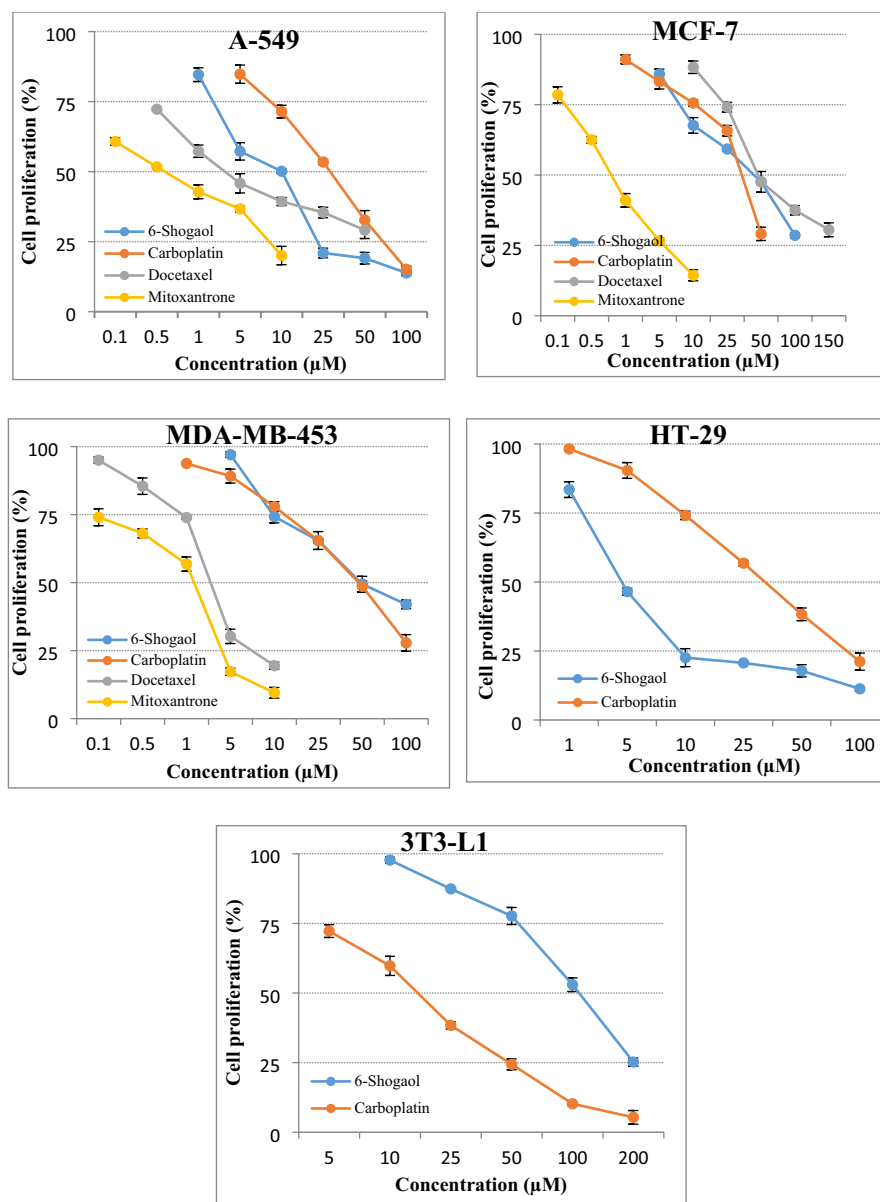


Fig. 6 Dose-dependent antiproliferative effects of shogaol, carboplatin, docetaxel and mitoxantrone on A-549 human lung cancer, MCF-7 and MDA-MB-453 human breast cancer cell lines and HT-29 human colon cancer and 3T3-L1 mouse fibroblast normal cell lines

24 h of incubation on both MCF-7 and MDA-MB-231 cell lines as 40 μ M for octylgallate and 80 μ M for gallic acid, respectively [97].

When compared with the compounds listed above for their antiproliferative effects against various cancer cell lines, Shogaol demonstrates stronger antiproliferative activity against the studied cell lines. These findings suggest that Shogaol possesses potent antiproliferative potential. Its strong antiproliferative effect on cancer cell lines, combined with its high selectivity toward normal cells, is particularly significant. This supports the hypothesis that Shogaol has promising pharmacological potential. Furthermore, its high selectivity index reinforces the idea that natural compounds tend to have fewer side effects and are more suitable for therapeutic use.

Although this study provides comprehensive data on the antioxidant, enzyme inhibition, molecular docking, and antiproliferative activities of Shogaol, several limitations should also be considered. First, biological evaluations were mainly based on *in vitro* experiments, which do not fully reflect physiological complexities such as bioavailability, metabolism, and systemic toxicity. Therefore, the translational relevance of the obtained findings needs to be validated through *in vivo* studies. Second, although strong inhibitory effects were observed against multiple enzymes, isoform selectivity and potential off-target effects, particularly for enzyme families with widespread physiological roles, were not examined in detail. Similarly, antiproliferative activity studies were limited to established cancer cell lines, which do not adequately represent tumor heterogeneity, the tumor microenvironment, or immune system interactions present under *in vivo* conditions. Third, molecular docking analyses were performed using static protein structures and empirical scoring functions, which limits accurate representation of protein flexibility, solvent effects, and binding thermodynamics. Thus, docking results should be interpreted as qualitative supportive evidence rather than definitive indicators of binding strength. Finally, the study primarily focused on biochemical and cellular outcomes, while the detailed molecular mechanisms and signaling pathways underlying the observed effects were not investigated.

4 Conclusion

Living a healthy life is one of the most important issues for all humanity. All countries in the world act with the understanding that “health is everything” and make great efforts to maintain public well-being. Since the early ages of humankind, various treatments have been used against diseases, especially herbal medicines. With the development of technology in the last century, synthetic drugs have been produced instead of natural ones, and their use has greatly increased. However, further research has revealed that, in addition to their benefits, synthetic drugs have many side effects and can be harmful to the body. Therefore, researchers have focused on discovering natural compounds from natural sources that are safe and effective for therapeutic use. In this study, we used the compound Shogaol, a natural bioactive molecule and one of the main components of ginger, to determine its antioxidant capacity against oxidants and free radicals, its enzyme inhibition activity against several metabolic enzymes, and its antiproliferative effects against certain cancer cell lines. As a result of the study, it was observed that Shogaol exhibits strong antioxidant capacity, shows high inhibitory effects against hCA I, hCA II, AChE, BChE, α -glycosidase and α -amylase enzymes, and demonstrates effective antiproliferative activity against A-549, HT-29, and MCF-7 cell lines. Shogaol showed higher antioxidant activity than the standard compounds at a concentration of 60 $\mu\text{g/mL}$ in the FRAP assay. In the ABTS radical scavenging assay, its activity was comparable to that of the standards. Moreover, Shogaol displayed more effective inhibition against hCA I, hCA II, AChE, BChE, α -glycosidase and α -amylase enzymes than many compounds reported in the literature. Shogaol also acted much more selectively than the reference drug Carboplatin on the studied cell lines. In the HT-29 cell line, the selectivity index of Shogaol was determined to be 14.83, whereas that of Carboplatin was 0.46. This result indicates that Shogaol is approximately 32 times more selective. With this high selectivity, Shogaol has the potential to be a promising natural agent for chemotherapy applications. Based on all these results, it can be concluded that Shogaol is a natural compound with high

antioxidant capacity, strong enzyme inhibition potential, and effective anticancer activity, suggesting that it may serve as a valuable drug candidate for the treatment of various diseases, particularly cancer. The antioxidant, antiproliferative, and enzyme inhibition results constitute the most important and critical findings of the study. In addition, the following can be done in future studies. In vivo and toxicity studies should be conducted to validate the antioxidant, enzyme inhibitory, and antiproliferative effects of Shogaol and to assess its safety profile. Pharmacokinetic and bioavailability studies are needed to better understand the absorption, metabolism, and therapeutic potential of Shogaol. Advanced computational approaches, such as molecular dynamics simulations and free energy calculations, should be employed to complement docking results and account for protein flexibility. Mechanistic and structure–activity relationship studies may help elucidate the molecular pathways involved and enable the development of more potent Shogaol derivatives.

Author contributions

IY performed the experiments IG critically analyzed the data, wrote the manuscript drafts. All authors contributed to the manuscript preparation.

Funding

This research did not receive any financial grant from private, public and commercial agencies.

Data availability

Data supporting the results of this study are available upon request.

Declarations

Ethics approval and consent to participate

This study did not involve human participants or animal experiments requiring ethical approval.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 1 December 2025 / Accepted: 16 February 2026

Published online: 13 March 2026

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