Antioxidant, Antidiabetic, Anticholinergic, and Antiglaucoma Effects of Magnofluorine

Lokman Durmaz 1, Hatice Kiziltas 2, Leyla Guven 3, Hasan Karagecili 4, Saleh Alwasel 5 and Ilhami Gulcin 6,*

Abstract: Magnofluorine, a secondary metabolite commonly found in various plants, has pharmacological potential; however, its antioxidant and enzyme inhibition effects have not been investigated. We investigated the antioxidant potential of Magnofluorine using bioanalytical assays with 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS**), N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD**), and 1,1-diphenyl-2-picrylhydrazyl (DPPH*) scavenging abilities and K3[Fe(CN)6] and Cu2+ reduction abilities. Furthermore, we compared the effects of Magnofluorine and butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), α-Tocopherol, and Trolox as positive antioxidant controls. According to the analysis results, Magnofluorine removed 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals with an IC50 value of 10.58 µg/mL. The IC50 values of BHA, BHT, Trolox, and α-Tocopherol were 10.10 µg/mL, 25.95 µg/mL, 7.059 µg/mL, and 11.31 µg/mL, respectively. Our results indicated that the DPPH scavenging effect of Magnofluorine was similar to that of BHA, close to that of Trolox, and better than that of BHT and α-tocopherol. The inhibition effect of Magnofluorine was examined against enzymes, such as acetylcholinesterase (AChE), α-glycosidase, butyrylcholinesterase (BChE), and human carbonic anhydrase II (hCA II), which are linked to global disorders, such as diabetes, Alzheimer’s disease (AD), and glaucoma. Magnofluorine inhibited these metabolic enzymes with Ki values of 10.251.94, 5.991.79, 25.411.10, and 30.563.36 nM, respectively. Thus, Magnofluorine, which has been proven to be an antioxidant, antidiabetic, and anticholinergic in our study, can treat glaucoma. In addition, molecular docking was performed to understand the interactions between Magnofluorine and target enzymes BChE (D: 6TYP), hCA II (A:3HS4), AChE (B:4EY7), and α-glycosidase (C:5NN8). The results suggest that Magnofluorine may be an important compound in the transition from natural sources to industrial applications, especially new drugs.

Keywords: Magnofluorine; phenolic compound; butyrylcholinesterase; antioxidant activity; carbonic anhydrase; acetylcholinesterase; α-glycosidase

1. Introduction

The oxidation of biological and food molecules leads to the creation of free radicals, resulting in the deterioration of cells and foods [1]. Methods such as air locking, temperature reduction, and the addition of antioxidant substances during processing, transportation, and storage are generally used to prevent oxidation of such components. The biological role of antioxidants is to neutralize cellular free radicals and reactive oxygen species (ROS) that have a negative effect on living organisms. Antioxidants terminate the oxidation of free radicals [2]. Antioxidant-rich molecules can be naturally created in humans or taken into the body exogenously with dietary components and food supplements [3]. However,
the addition of antioxidants to the medium is the most convenient and economical method for processing oils and foods. What are these antioxidants? In terms of food, antioxidants are natural or synthetic substances that delay or completely prevent deterioration due to the autoxidation of fats and oils, even at low concentrations [4,5]. From a biological point of view, antioxidant compounds can protect the metabolism from the dangerous effects of oxidative stress and ROS [6,7]. Oxidative stress is a relatively new notion that has lately gained popularity in medicine and fundamental sciences [8]. Further, antioxidants prevent some chronic diseases, including diabetes mellitus (DM), cancer, cataract, and cardiovascular disorders [9,10]. They can neutralize free radicals and ROS and terminate damage to the biomolecules found in cells and tissues [11–13]. Although commercially used synthetic antioxidants have negligible toxicity at the prescribed doses, natural antioxidants have a higher demand and are safer [14–16].

Recently, it has been reported that antioxidants inhibit enzymes such as butyrylcholinesterase (BChE), acetylcholinesterase (AChE), α-amylase, carbonic anhydrase, and α-glycosidase, which are associated with diseases such as type 2-DM (T2DM), Alzheimer’s disease (AD), and glaucoma [15–17]. Thus, antioxidants can help treat the above-mentioned disorders [18]. Further, antioxidants play an important role in the prevention of T2DM and AD [19–21]. Some recent and basic scientific studies have revealed a link between T2DM and AD [22,23]. Therefore, AChE inhibitors (AChEIs) are known to cure AD. However, numerous drugs, including tacrine, have some hepatotoxic effects [24,25]. Among them, tacrine has some undesired effects, such as nausea, weight loss, vomiting, agitation, stomach upset, skin rash, chills, and diarrhea [26]. Thus, there is a need for the design and development of novel α-glycosidase and AChE inhibitors of natural origin [27,28].

Alkaloids are natural and active herbal metabolites containing one nitrogen atom in their chemical structure [29]. They are derived from benzylisoquinolines by removing two hydrogen atoms from two benzene nuclei, resulting in the formation of a 9,10-dihydrophenanthrene structure known as Magnofluorine, which is enzymatically synthesized in a few steps from dopamine and 4-hydroxyphenylacetate [30]. Among them, Magnofluorine is important from a biological point of view. Magnofluorine has multiple biological effects, such as anti-inflammatory, immunomodulatory, anticancer, antiallergic, cardiovascular, antibacterial, anti-osteoporotic, antiviral, and antifungal activities [31]. It can pass through the brain–blood barrier and affect the central nervous system. However, no study has investigated the antioxidant and enzyme inhibition effects of Magnofluorine.

AD is a rapidly progressing neurological illness with behavioral changes, forgetfulness, memory loss, and impaired language and cognition [32]. AChE decomposes acetylcholine (ACh) to acetate (CH$_3$COO$^-$) and choline (Ch), while BChE catalyzes the breakdown of butyrylcholine (BCh) to butyrate and Ch [33]. Of the two enzymes, AChE hydrolyzes several ChEs in the body, pancreas, blood, CNS, and liver [34], and it is found in erythrocyte membranes, nerves, muscle, CNS, peripheral tissues, and cholinergic, non-cholinergic, sensory, and motor fibers. In addition, BChE is mainly related to the glial and endothelial cells in the brain [33]. Many recent studies have examined the nature of AChE inhibition to elucidate the effect of ACh receptor binding [35]. However, the exact physiological role of another cholinergic enzyme, BChE, has not been completely elucidated. When AChE activity begins to decline during AD progression, BChE has been shown to functionally complement this enzyme and play a prominent role. However, it is well-known that selective inhibitors of the enzymes are important in designing active drugs against neurodegeneration, and they play an important part in AD treatment. Therefore, dual co-inhibitors of AChE/BChE enzymes can be a promising therapeutic alternative to treat AD [36].

Carbonic anhydrases (CA) are metalloenzymes, which catalyze water and carbon dioxide (CO$_2$) to reversibly hydrate into protons (H$^+$) and bicarbonate (HCO$_3^-$) and metabolize acid-base concentrations having Zn$^{2+}$ in their active side structures [37,38]. Additionally, they maintain fluid equilibrium throughout the body, especially in the eyes, stomach, and kidneys. The high intraocular pressure (IOP) associated with glaucoma can be alleviated or treated using carbonic anhydrase inhibitors (CAIs) [39,40]. Thus, hCA II inhibition
is a critical mechanism of action in treating glaucoma-related IOP reduction [41]. It is well-known that glaucoma is one of the main causes of blindness worldwide. It is also conjectured that the number of people suffering from glaucoma worldwide will reach 120 million by 2040 [36,42]. Clinically, laser, pharmacological therapies, and surgery are the main treatment methods for glaucoma. Thus, it is important to use CAIs topically to prevent their undesired effects.

We conducted this study to investigate the antioxidant abilities of Magnofluorine using bioassays such as Fe$^{3+}$ and Cu$^{2+}$ reduction, DMPD$^{•+}$, ABTS$^{•+}$, and DPPH$^{•}$ scavenging abilities and test the purported inhibitory abilities of Magnofluorine towards BChE, hCA II, AChE, and α-glycosidase associated with glaucoma, AD, and T2DM diseases.

2. Results

A variety of methodologies and activity assessments are used to prevent oxidation using antioxidants. As seen in Figure 1A and Table 1, Magnofluorine showed effective Fe$^{3+}$-reducing ability ($p < 0.01$). The reducing effect of Magnofluorine and the positive controls increased in a concentration-dependent manner (30 µg/mL) for the tested materials. The Fe$^{3+}$ reducing ability of Magnofluorine and the positive controls decreased as follows: BHA ($\lambda_{700}: 2.347, r^2: 0.9086$) > Trolox ($\lambda_{700}: 2.119, r^2: 0.9586$) > Magnofluorine ($\lambda_{700}: 0.967, r^2: 0.9938$) > α-Tocopherol ($\lambda_{700}: 0.957, r^2: 0.9863$) ≥ BHT ($\lambda_{700}: 0.952, r^2: 0.9154$). The results demonstrated that the Fe$^{3+}$ reduction ability of Magnofluorine is better than that of BHT and α-Tocopherol, but lower than that of Trolox and BHA. Additionally, previous studies have reported the Fe$^{3+}$-reducing absorbance values to be 0.278 ($r^2: 0.9567$) [12], 2.769 ($r^2: 0.9945$) [43], 0.739 ($r^2: 0.9778$) [3], 0.432 ($r^2: 0.9981$) [44], 2.509 ($r^2: 0.9906$) [45], and 2.428 ($r^2: 0.9474$) [46] for similar quantities of usnic acid, caffeic acid, coumestrol, uric acid, CAPE, and tannic acid, respectively.

The Cu$^{2+}$ reduction ability of Magnofluorine and the positive controls (30 µg/mL) is shown in Figure 1B and Table 1. Further, the Cu$^{2+}$-reducing ability of Magnofluorine was dose-dependent (10–30 µg/mL). The Cu$^{2+}$-reducing ability of Magnofluorine and the positive controls decreased as follows: BHA ($\lambda_{450}: 2.216, r^2: 0.9928$) > BHT ($\lambda_{450}: 2.044, r^2: 0.9937$) > Trolox ($\lambda_{450}: 1.548, r^2: 0.9305$) > α-Tocopherol ($\lambda_{450}: 0.816, r^2: 0.9897$) > Magnofluorine ($\lambda_{450}: 0.458, r^2: 0.9938$). Additionally, the absorbance values of the same concentration of natural phenolics such as usnic acid, coumestrol, resveratrol, eugenol, olivetol, and taxifolin are 0.277 ($r^2: 0.9836$) [12], 0.739 ($r^2: 0.9778$) [3], 0.085 ($r^2: 0.8403$) [47], 0.762 ($r^2: 0.9957$) [48], 1.314 ($r^2: 0.9682$) [49], and 0.750 ($r^2: 0.9550$), respectively [50].

Figure 1. Fe$^{3+}$ (A) and Cu$^{2+}$ (B) ions reducing abilities of Magnofluorine and standards.
The radical scavenging ability of Magnofluorine was determined using the antioxidant assays, DPPH\(^*\), ABTS\(^{**}\), and DMPD\(^{**}\) methods. Magnofluorine exhibited considerable DPPH\(^*\), ABTS\(^{**}\), and DMPD\(^{**}\) scavenging activities. The results exhibited that Magnofluorine significantly scavenged the DPPH radical in a concentration-dependent manner (10–30 μg/mL). In the DPPH\(^*\) removal studies, the IC\(\text{50}\) value for Magnofluorine was found to be 10.58 μg/mL (\(r^2\): 0.9908) (Table 2 and Figure 2A). In contrast, the IC\(\text{50}\) values were calculated as 7.059 μg/mL (\(r^2\): 0.9614), 10.10 μg/mL (\(r^2\): 0.9015), 11.31 μg/mL (\(r^2\): 0.9642), and 25.95 μg/mL (\(r^2\): 0.9221) for Trolox, BHA, α-tocopherol, and BHT, respectively.

In this context, the IC\(\text{50}\) value was calculated to be 3.30 μg/mL [45], 6.96 μg/mL [47], 16.06 μg/mL [48], 17.77 μg/mL [49], 20.0 mg/mL [51], 30.6 μg/mL [52], 34.9 μg/mL [53], 49.50 μg/mL [12], and 77.00 μg/mL [50] for CAPE, resveratrol, eugenol, olivetol, silymarin, L-Adrenaline, curcumin, usnic acid, and taxifolin, respectively.

ABTS radicals have higher reactivity than DPPH radicals. As seen in Table 2 and Figure 2B, Magnofluorine effectively eliminated ABTS radicals (IC\(\text{50}\): 27.61 μg/mL, \(r^2\): 0.9006). Additionally, the IC\(\text{50}\) values of 5.07 μg/mL for BHA (\(r^2\): 0.9356), 6.16 μg/mL for Trolox (\(r^2\): 0.9692), 6.99 μg/mL for BHT (\(r^2\): 0.9350), and 8.73 μg/mL for α-tocopherol (\(r^2\): 0.9015), were reported. The results exhibited that Magnofluorine had lower ABTS radical scavenging effects when compared to all of the positive controls as standard antioxidants.

### Table 1. Fe\(^{3+}\) and Cu\(^{2+}\) reduction abilities of Magnofluorine and positive controls at 30 μg/mL concentration.

| Antioxidants | Fe\(^{3+}\) Reducing | Cu\(^{2+}\) Reducing |
|--------------|---------------------|---------------------|
|              | \(\lambda\) (700 nm) | \(r^2\) | \(\lambda\) (450 nm) | \(r^2\) |
| BHA          | 2.347               | 0.9086            | 2.126             | 0.9928 |
| BHT          | 0.952               | 0.9154            | 2.044             | 0.9937 |
| Trolox       | 2.119               | 0.9586            | 1.548             | 0.9305 |
| α-Tocopherol | 0.957               | 0.9863            | 0.816             | 0.9897 |
| Magnofluorine| 0.967               | 0.9938            | 0.458             | 0.9729 |

![Figure 2](image-url) 

**Figure 2.** Radical scavenging effects of Magnofluorine and positive controls. (a). DPPH\(^*\) scavenging ability, (b). ABTS\(^{**}\) scavenging ability, (c). DMPD\(^{**}\) scavenging ability.
Figure 3. Lineweaver–Burk graphs of Magnofluorine towards acetylcholinesterase (AChE) enzyme (a), butyrylcholinesterase (BChE) enzyme (b), carbonic anhydrase II isoenzyme (CA II) (c), and α-glycosidase (d).

As seen in Table 2 and Figure 3C, the IC\textsubscript{50} value for the DMPD\textsuperscript{**} removal of Magnofluorine (IC\textsubscript{50}: 15.26 μg/mL, r\textsuperscript{2}: 0.9966) had lower DMPD\textsuperscript{**} removal than that of Trolox (IC\textsubscript{50}: 4.33 μg/mL, r\textsuperscript{2}: 0.9447), α-tocopherol (IC\textsubscript{50}: 7.11 μg/mL, r\textsuperscript{2}: 0.9509), BHT (IC\textsubscript{50}: 8.72 μg/mL, r\textsuperscript{2}: 0.9375), and BHA (IC\textsubscript{50}: 11.99 μg/mL, r\textsuperscript{2}: 0.9580), which were utilized as reference antioxidants in antioxidant studies. Lower IC\textsubscript{50} values indicate higher DMPD\textsuperscript{**} removal effects.

According to the enzyme inhibition results (Table 3), Magnofluorine effectively inhibited the cholinergic enzymes AChE and BChE with Ki values of 10.25 ± 1.94 and 2.47 ± 0.70 nM, respectively (Table 3 and Figure 3A,B). Additionally, Tacrine, as a clinical drug, had a Ki value of 5.99 ± 1.79 nM (Figure 3B) and 2.43 ± 0.92 nM for BChE and AChE (Figure 3A), respectively.

Table 2. IC\textsubscript{50} (μg/mL) values for DPPH\textsuperscript{*}, DMPD\textsuperscript{**}, and ABTS\textsuperscript{**} scavenging of Magnofluorine and standard antioxidants.

| Antioxidants   | DPPH\textsuperscript{*} Scavenging | ABTS\textsuperscript{**} Scavenging | DMPD\textsuperscript{**} Scavenging |
|----------------|----------------------------------|------------------------------------|-----------------------------------|
|                | IC\textsubscript{50}            | r\textsuperscript{2}             | IC\textsubscript{50}            | r\textsuperscript{2}             | IC\textsubscript{50}            | r\textsuperscript{2}             |
| BHA            | 10.10                          | 0.9015                            | 5.07                             | 0.9356                          | 11.99                           | 0.9580                          |
| BHT            | 25.95                          | 0.9221                            | 6.99                             | 0.9350                          | 8.72                            | 0.9375                          |
| Trolox         | 7.05                           | 0.9614                            | 6.16                             | 0.9692                          | 4.33                            | 0.9447                          |
| α-Tocopherol   | 11.31                          | 0.9642                            | 8.37                             | 0.9015                          | 7.11                            | 0.9509                          |
| Magnofluorine  | 10.58                          | 0.9908                            | 27.61                            | 0.9006                          | 15.16                           | 0.9966                          |
Table 3. Inhibition values of Magnofluorine against α-glycosidase (α-Gly), carbonic anhydrase isoenzyme II (CA II), butyrylcholinesterase (BChE), and acetylcholinesterase (AChE) enzymes.

| Compounds       | CA II  | α-Gly  | CA II | AChE | BChE | α-Gly |
|-----------------|--------|--------|-------|------|------|-------|
| Magnofluorine   | 26.03  | 0.9313 | 10.01 | 0.9429 | 8.71 | 0.9825 |
| Acetazolamide * | 8.37   | 0.9825 | -     | -    | -    | -     |
| Tacrine **      | -      | 5.97   | 0.9706 | 8.37 | 0.9846 | -     |
| Acarbose ***    | -      | -      | -     | -    | -    | 22,800 |

* Acetazolamide (AZA) is a standard for CA II inhibition. ** Tacrine (TAC) is a standard for AChE inhibition. *** Acarbose (ACR) is a standard for α-glycosidase inhibition [54].

Furthermore, Magnofluorine had an efficient inhibition profile against the α-glycosidase as a proteolytic enzyme with a Kᵢ value of 30.56 ± 3.36 nM (Table 3 and Figure 3c). It was reported that Acarbose exhibited an α-glycosidase enzyme with an IC₅₀ value of 22,800 nM [54]. As seen in Table 3 and Figure 3d, Magnofluorine demonstrated a potent CA II inhibition effect (Kᵢ: 25.41 ± 1.10 nM) when compared to Acetazolamide (Kᵢ: 4.41 ± 0.35 nM) as a strong and clinical CA II inhibitor.

According to the docking scores, Magnofluorine molecules effectively inhibited all enzymes, and these results were found to be compatible with the results of in vitro studies of this research. The molecular interactions of Magnofluorine with hCA II (A:3HS4), AChE (B:4EY7), BChE (D: 6T9P), and α-glycosidase (C:5NN8) are given in Table 4 and Figure 4.

Table 4. Molecular interactions of Magnofluorine with α-glycosidase (α-Gly, C:5NN8), human carbonic anhydrase isoenzyme II (hCA II, A:3HS4), butyrylcholinesterase (BChE, D: 6T9P), and acetylcholinesterase (AChE, B:4EY7) enzymes.

| Complex            | Docking Scores (kcal/mol) | Types of Interactions | Interacting Residues           |
|--------------------|---------------------------|-----------------------|--------------------------------|
| hCA II (3HS4)-Magnofluorine | -8.2                     | Hydrogen bonding     | Trp5, His64, His96, Val121, Val143, Leu198, Phe131, His94 |
|                    |                           | Hydrophobic interactions | π-π T-shaped                  |
| AChE (4EY7)-Magnofluorine | -9.5                     | Hydrogen bonding     | Trp86, Tyr337, His447          |
|                    |                           | Hydrophobic interactions | π-π stacked, π-cation           |
| BChE (6T9P)-Magnofluorine | -9.8                     | Hydrogen bonding     | Gly116, Gly117, Ser198, Trp82 Phe329 |
|                    |                           | Hydrophobic interactions | π-π stacked, π-cation, π sigma |
| α-Gly (5NN8)-Magnofluorine | -7.2                     | Hydrogen bonding     | Trp481, Trp376, Phe525, Phe649 |
|                    |                           | Hydrophobic interactions | π-π stacked, π anion           |

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Figure 4. (A). The 2D and 3D interactions of BChE (6T9P) and Magnofluorine, (B). The 2D and 3D interactions of Carbonic anhydrase (II) enzyme (3HS4) and Magnofluorine, (C). The 2D and 3D interactions of AChE enzyme (4EY7) and Magnofluorine, (D). The 2D and 3D interactions of α-glycosidase enzyme (5NN8) and Magnofluorine.

3. Discussion

Antioxidants, even when found at extremely low concentrations in foods or the human body, can delay, limit, or completely block the oxidative processes, thus, enhancing food quality [55,56]. Of these, the most crucial function of an antioxidant molecule is reduction, shown by its electron-withdrawing capacity [57–59]. Antioxidant molecules scavenge free radicals and ROS by donating electrons and reducing themselves. Antioxidants can bind free radicals and protect biomaterials against oxidation, which are widely used to delay or prevent food oxidation [60]. The effectiveness of antioxidant compounds such as Magnofluorine depends on several factors, including temperature, structural features, sensitive substrate oxidation, concentration, presence of synergistic and pro-oxidants, and physical conditions [61]. Figures 1 and 2 and Tables 1 and 2 depict the antioxidant activities of Magnofluorine, which were determined using different chemical-based methodologies. The most putative ones are reducing abilities [62]. So far, different chemical assays combined with extremely sensitive and automated detection technologies have been utilized for the evaluation of antioxidant activity using unique approaches, including radical scavenging activity, metal chelation, and reduction potential. Reduction ability directly measures the transfer of electrons from an antioxidant to free radicals or transfer of hydrogen atoms. Additionally, the reduction potential of an antioxidant can be measured using several bioassays [63]. The addition of Magnofluorine to the Fe³⁺ solution enhances the formation of Prussian blue Fe₄[Fe(CN)₆], which has a maximum absorbance at 700 nm [64]. Therefore, the determination of high absorbance at this wavelength indirectly reflects the reducing ability of antioxidant molecules. The simplified Fe³⁺ reducing ability mechanism of Magnofluorine is illustrated as follows:
Magnofluorine + Fe$^{3+}$ → Oxidized Magnofluorine + Fe$^{2+}$ (1)

\[
\text{Fe}^{2+} + [\text{Fe(CN)}_6]^{3-} \rightarrow \text{Fe}[\text{Fe(CN)}_6]^{-}\tag{2}
\]

As shown in Table 1 and Figure 2A, the reducing activity increased with the increase in Magnofluorine concentration (μg/mL). In Magnofluorine reduction, ([Fe(CN)$_6$]$^{3-}$) complex under the action of ferric trichloride is easily reduced to (Fe[Fe(CN)$_6$]$^{-}$) [65]. Higher the absorbance value, the better the reducing ability. Magnofluorine promises antioxidant activity owing to the hydroxyl groups (-OH) linked to aromatic rings in its backbone [66,67]. It was observed that Magnofluorine had a higher reducing ability as compared to standard molecules. The Cu$^{2+}$ reducing assay (CUPRAC assay), which was devised in the early 2000s, has been used to determine the antioxidant ability of pure molecules [68]; however, it has already been changed for different assays for determining the antioxidant ability based on reduction of cupric (Cu$^{2+}$) to cuprous ions (Cu$^{+}$). Similar to other methods, this method uses a ligand to form a copper–ligand complex to measure absorbance. The ligand used for this purpose is the Neocuproine (2,9-dimethyl-1,10-phenanthroline) complex [69]. In the reduction experiment, reactive aromatic -OH groups of polyphenols such as Magnofluorine were oxidized to the corresponding quinones and reduced to Cu$^{2+}$-Neocuproine. In this way, the reduced Cu$^{+}$-Neocuproine complex with an intense yellow–orange color is formed [70].

The radical scavenging activities of Magnofluorine were assessed with DMPD•+, ABTS•+, and DPPH• radical scavenging activities, which were developed on different approaches providing evidence about free radicals and antioxidant agents [71]. Thus, the use of radical removing activity is quite easy for determining the antioxidant abilities of compounds [72]. The results exhibited that Magnofluorine had a similar DPPH- removing activity as compared to α-Tocopherol and BHA, but lower than Trolox and better than BHT. The results showed that Magnofluorine had a more effective DPPH free-radical-scavenging ability. In the DPPH test, the reduction of stable radical DPPH to yellow DPPH$_2$ by Magnofluorine is used to measure the antioxidant ability of a molecule to act as a hydrogen atom or electron donor [73]. In light of this information, the possible mechanisms of Magnofluorine and DPPH radicals are illustrated in Figure 5 and the structure of Magnofluorine.

![Figure 5. Proposed possible DPPH radical scavenging mechanism of Magnofluorine.](image-url)
Following the interaction of Magnofluorine and DPPH·, the radicals disappear after accepting an electron (e−) or hydrogen radical (H·) from Magnofluorine to become DPPH2· based on an electron transfer reaction [74]. The mechanism of the DPPH radical scavenging of Magnofluorine has not been previously reported. Additionally, the best information on this subject is that the radicals generated from the phenolic groups in Magnofluorine are stabilized due to resonance structures. In this way, a Magnofluorine molecule scavenges the two DPPH radicals and switches to a diketonic structure, and these radicals also switch to the neutral form. For comparison, the IC50 values (µg/mL) were calculated to be 0.83 [50], 1.94 [49], 25.95 [3] 6.93 [52], 6.96 [47], 7.84 [48], 8.62 [51], 9.80 [45], 10.41 [12], and 18.07 [53] for taxifolin, olivetol, coumestrol, L-Adrenaline, resveratrol, eugenol, silymarin, CAPE, usnic acid, and curcumin, respectively.

Antioxidants reduce the blue–green color of ABTS•+; this reaction was then followed by absorbance measurement at 734 nm. Another radical elimination method used in this study was the DMPD•+ scavenging ability. In this antioxidant method, antioxidant molecules transfer an H atom to DMPD radicals, removing the existing color and causing the solution to lighten [75]. The IC50 value for the DMPD•+ removing ability was more effective as compared to that reported previously. For instance, the IC50 (µg/mL) values were 9.5 [47], 10.04 [48], 12.81 [3], 15.6 [53] 19.25 [49], 26.70 [48], 33.00 [12], 34.5 [53], and 173.25 [50] for resveratrol, eugenol, coumestrol, L-Adrenaline, olivetol, CAPE, usnic acid, curcumin, and taxifolin, respectively.

Further, Magnofluorine was more than two times more effective than Tacrine against AChE, and it had a similar inhibition ability against Tacrine against BChE. On examining the results, we observed that the selectivity index (AChE/BChE; 1.15) was in favor of AChE. In some recent clinical studies, some putative inhibitors of AChE, including rivastigmine, donepezil, and tacrine, have been used in the early stages of AD treatment. Tacrine was later clinically proven to have hepatotoxicity. Therefore, although tacrine is effective in the treatment of AD, it has been withdrawn from clinical trials [66]. Additionally, the K_i value of some molecules for AChE inhibition were calculated as 3.39 nM [12], 23.80 [3], 0.518, and 0.322 nM for usnic acid, coumestrol, and CAPE [40]. Moreover, K_i values (µg/mL) for AChE were recorded as 5.13 [49] and 16.70 [39] for olivetol and taxifolin, respectively.

T2DM is a common metabolic disorder originating from high blood glucose levels. Therefore, recent studies have focused on the inhibition of α-glucosidase that controls carbohydrate digestion [76]. Magnofluorine had a K_i value of 30.56 ± 3.36 nM towards α-glycosidase (Table 3 and Figure 3C). The results clearly exhibited that Magnofluorine had efficient α-glycosidase inhibition ability as compared to acarbose (IC50: 22,800 nM) [54]. The results also demonstrated that Magnofluorine had a more efficient K_i value as compared to acarbose as an effective starch blocker [77].

Since phenolic compounds have slightly acidic properties, they lose protons (H+) from their hydroxyl groups and form highly soluble phenolate anions in water. It is known that phenolic compounds can efficiently inhibit CA isoenzymes due to the presence of functional groups such as phenolic -OH, -OCH3, and -COOH groups in their scaffolds [78]. They inhibit CA II isozymes that coordinate to Zn2+ in the active cavity of CA. CA II isoform is involved in the protection of body fluids [79]. As shown in Table 3 and Figure 3D, when profiling the assay against cytosolic and predominant hCA II isoform, Magnofluorine had a K_i value of 25.41 ± 1.10 nM. In comparison, AZA demonstrated a K_i value of 4.41 ± 0.35 nM against cytosolic and dominant hCA II isoenzyme, which exist everywhere in the cells and tissues [80].

Docking studies were followed by an analysis of the binding modes to understand the inhibition mechanisms. According to docking scores, Magnofluorine exhibited high binding affinity with all enzyme targets (Figure 4 and Table 4). The binding affinity of the Magnofluorine-BChE (6T9P) complex was calculated to be −9.8 kcal/mol (Table 4). Magnofluorine formed three hydrogen bonds with Gly116, Gly117, and Ser198 residues in the active site of the BChE. Furthermore, the Magnofluorine-BChE complex showed hydrophobic interactions with Trp82 and Phe329 residues, π−π stacked interactions with
Trp82 and Phe329, \( \pi \) cation interaction with His438, and \( \pi \) sigma interaction with Trp231 (Figure 4a). The binding affinity of the Magnoflorine-hCA II (A:3HS4) complex was calculated to be \(-8.2\) kcal/mol (Figure 4b). Magnoflorine was shown to bind to the active site via two H-bond interactions between Asn62 and Thr200. Hydrophobic interactions with Trp5, His64, His96, Val121, Val143, Leu198, Phe131, \( \pi - \pi \) stacked interactions with Tyr337 and Phe338, and also the \( \pi - \pi \) T-shaped interaction with His94 were formed (Figure 4b). Magnoflorine was placed in the active site of the enzyme AChE (PDB code: 4EY7). Figure 4c represents the 3D and 2D interactions of Magnoflorine-AChE, and the docking score was calculated to be \(-9.5\) kcal/mol (Table 4). Magnoflorine was shown to bind to the active site via two H-bond interactions between its hydroxyl groups and the active site amino acids Tyr124 and Asp74. Moreover, hydrophobic contacts with Trp86; Tyr337; His447, \( \pi - \pi \) stacked interactions with Tyr337; Phe338, and also the \( \pi \) cation interaction with Trp86 were formed (Figure 4c). Magnoflorine-\( \alpha \)-glycosidase (5NN8) complex’s docking score was calculated as \(-7.2\) kcal/mol (Table 4). The interactions in the Magnoflorine binding mode comprised two H bonds with Arg600 and Asp282 active site amino acids, four hydrophobic interactions with Trp481, Trp376, Phe525, Phe649, and a \( \pi \) anion interaction with Asp616 (Figure 4d). According to the docking scores, it has been determined that the Magnoflorine effectively inhibited all of the studied enzymes, and these results were found to be compatible with the results of in vitro studies of this research.

4. Materials and Methods

4.1. Chemicals

Magnoflorine (\( \geq 98\% \) (HPLC)), acetylcholinesterase, butyrylcholinesterase, \( \alpha \)-glycosidase, \( p \)-nitrophenyl-\( D \)-glucopyranoside, \( \alpha \)-tocopherol, acetylcholine iodide, butyrylcholine iodide, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), \( N, N \)-dimethyl-\( p \)-phenylenediamine dihydrochloride and 1,1-diphenyl-2-picrylhydrazyl were obtained from Sigma-Aldrich GmbH (Steinheim, Germany). For the antioxidant activity, Magnoflorine was dissolved in ethanol; however, for enzyme inhibition studies, Magnoflorine was dissolved in DMSO due to the possible inhibition effects of ethanol.

4.2. Antioxidant Assays

The Fe\( ^{3+} \) reducing ability of Magnoflorine was investigated and compared with the reduction abilities of the positive controls. Different concentrations of dissolved Magnoflorine (10–30 \( \mu g/mL \)) were mixed with 2 mL of sodium phosphate buffer (200 mM, pH 6.6) and 1 mL \( K_2Fe(CN)_6 \) (1%), and the solution was incubated at 50 \(^\circ\)C for 25 min. The reaction was terminated by adding 1 mL of trichloroacetic acid (TCA, 10%). Following this, 0.5 mL of newly prepared \( FeCl_3 \) (0.1%) was transferred, and the absorptions were measured at 700 nm. Deionized water was used as the blank control. The Cu\( ^{2+} \) reduction ability of Magnoflorine was realized at 450 nm according to previous procedures \[81\]. Magnoflorine was prepared at diverse concentrations (10–30 \( \mu g/mL \)) and added to 250 \( \mu L \) CuCl\( _2 \) solution (10 mM), 250 \( \mu L \) neocuproine solution prepared in ethanol (7.5 mM), and 250 \( \mu L \) acetate buffer (1.0 M). Finally, after 20 min, absorbances were recorded at 450 nm \[68\].

The DPPH radical scavenging activity of Magnoflorine was estimated according to the Blois method at 517 nm \[82\]. Briefly, 0.2 mL of Magnoflorine in different concentrations of ethyl alcohol (10–30 \( \mu g/mL \)), 0.2 mL of DPPH solution (0.3 mM) in methanol, and 0.6 mL ethanol were added to test tubes, and the tubes were incubated at 37 \(^\circ\)C for 30 min. The DPPH radical scavenging activities were assessed by measuring the absorbance at 517 nm after keeping in the dark for 30 min \[81\]. Further, the ABTS radical scavenging activity of Magnoflorine was determined. ABTS solution (7.0 mM) was prepared using \( K_2S_2O_8 \) (2.45 nM), and the absorbance of the control was set to 0.700 ± 0.025 at 734 nm on dilution with buffer solution (0.1 M and pH 7.4). Finally, 1 mL of ABTS radicals was transferred to different concentrations of Magnoflorine (10–30 \( \mu g/mL \)), and the absorbance was recorded at 734 nm. The control included only ABTS radical solution \[82\]. The DMPD radical removing ability of Magnoflorine was determined at 505 nm according to a
previous method [83]. Briefly, 0.2 mL of FeCl₃ (50 mM) and 1 mL of DMPD solution were added to the buffer (pH 5.3, 100 mM). The concentrations of all of the samples were 10–30 µg/mL. The total volume was adjusted to 0.5 mL using deionized water. An aliquot (1 mL) of DMPD radicals was added, and the absorbance was recorded at 505 nm. The radical scavenging results were expressed as half maximal scavenging concentrations (IC₅₀, µg/mL) [84].

4.3. Anticholinergic Assay

The inhibition effect of Magnofluorine on AChE from Electrophorus electricus was realized according to a previous study [85]. AChI and BChI were used as substrates for enzymatic reactions. An aliquot (0.1 mL) of Tris/HCl buffer (pH 8.0, 1.0 M) and different concentrations of Magnofluorine (10–30 µg/mL) were added to 50 µL of the AChE/BChE solution (5.30 × 10⁻³ EU), and the mixture was incubated at 20 °C for 20 min. Following this, 50 µL of 5,5′-dithio-bis 2-nitro-benzoic acid (DTNB, 0.5 mM) and acetylcholine iodade (AChI)/butyrylcholine iodade (BChI) were added and enzymatic reactions were initiated. The AChE/BChE activities were spectrophotometrically determined at 412 nm [62].

4.4. Antidiabetic Assay

The inhibitory effect of Magnofluorine against α-Glycosidase was determined according to the methods described by Tao et al. [54] and Hashmi et al. [86] using p-nitrophenyl-D-glucopyranoside (p-NPG) as the substrate. Firstly, 75 µL of phosphate buffer (pH 7.4) was mixed with 5 µL of sample and 20 µL of α-glycosidase enzyme solution (0.15 U/mL) in phosphate buffer (pH 7.4). Following a short period of incubation (10 min), 50 µL of p-nitrophenyl-D-glucopyranoside (p-NPG) in phosphate buffer (5 mM, pH 7.4) was added and incubated at 37 °C, and absorbance was measured at 405 nm [87]. One unit is the quantity of α-glycosidase, which hydrolyzes 1.0 mol substrate per minute (pH 7.4) [88]. The α-amylase inhibition effects of Magnofluorine were determined as described previously [89]. Principally, 1 g starch was dissolved in 50 mL NaOH solution (0.4 M) and heated at 80 °C for 20 min. After cooling, the pH was adjusted to 6.9, and the volume was adjusted to 100 mL using distilled water. Next, 35 µL of starch solution, 35 µL of phosphate buffer (pH 6.9), and 5 µL of the Magnofluorine solutions were mixed. After incubation at 37 °C for 20 min, 20 µL of enzyme solution was added and incubated again for 20 min. The reaction was completed by adding 50 µL of 0.1 M HCl, and absorbance was measured at 580 nm.

4.5. Antigliaucoma Assay

The CA II isozyme was purified from human erythrocytes using Sepharose-4B-Tirozyne-sulfanylamide affinity column chromatography [90]. Further, it was precipitated, and the serum was separated and adjusted with solid Tris to pH 8.7. The sample was then loaded to the affinity chromatography column and equilibrated with Tris-Na₂SO₄/HCl (pH 8.7, 22 mM/25 mM). CA II was eluted with sodium acetate/NaClO₄ (0.5 M, pH 5.6, 25 °C) [91]. Protein quantity during the purification study was determined using the Bradford method [92]. Bovine serum albumin was used as the standard protein [93]. The purity of CA II was controlled using SDS-PAGE [94]. During purification and inhibition of CA II, esterase activities were performed following the change in absorbance at 348 nm [95].

4.6. Inhibition Parameters

The IC₅₀ values were calculated from activity (%) versus Magnofluorine plots [96]. First, the enzyme inhibitions were studied at different Magnofluorine concentrations. The obtained values were plotted as % activity against Magnofluorine concentrations. Then, the Magnofluorine concentrations, which cause 50% enzyme inhibition (IC₅₀), were calculated from these graphs. The Kᵢ values and other parameters were calculated from Lineweaver-Burk graphs [97] as described priorly [98]. The Kᵢ values were taken out from this graph [75].
All of the analyses were independently conducted in triplicate, and the results are expressed as mean values ± SD.

4.7. Molecular Docking Studies

The crystal structures of the AChE (PDB ID:4EY7) [99], α-glycosidase (PDB ID:5NN8) [100], BchE (PDB ID:6TPP) [101], and hCA II (PDB ID:3HS4) [102] enzymes were downloaded from the “Protein Data Bank” website with resolutions of 2.35 Å, 2.45 Å, 2.70 Å, and 1.10 Å, respectively [103]. The structures of these enzymes were optimized in AutoDock-Tools 1.5.7 [104]. The 3D version of the chemical structure of Magnofluorine was downloaded from the pubChem database (https://pubchem.ncbi.nlm.nih.gov/ accessed on 2 August 2022). Structure optimization and the most stable conformations of the ligands were determined with AutoDockTools. Then, the PDBQT file of the ligands was prepared. AutoDock-Tools program was used for docking. The binding interactions were analyzed with BIOVIA Discovery Studio Visualizer v21.1.0.20298 (Accelrys Software Inc., San Diego, CA, USA, 2012, https://pubchem.ncbi.nlm.nih.gov/ accessed on 2 August 2022) and PLIP [105].

4.8. Statistical Analysis

Statistical analyses were performed by unpaired Student’s t-test using the statistical program of IBM SPSS Statistics 20. The results were recorded as means with their standard deviation (SD). p < 0.05 was the minimum significance level.

5. Conclusions

In this study, Magnofluorine exhibited an efficient antioxidant profile as compared to the standards including BHA, BHT, α-Tocopherol, and Trolox. Furthermore, Magnofluorine, which possesses a wide spectrum of biological activities, was found to neutralize ROS and free radicals by donating a hydrogen atom or electron to free radicals. The results obtained from this study showed that Magnofluorine, a safer natural phenolic antioxidant, can be used to prevent or delay the formation of lipid autoxidation. In this way, it can extend the shelf-life of materials processed in the pharmaceutical and food industries and maintain their nutritional quality for a long time. Additionally, Magnofluorine was tested against some metabolic enzymes, including acetylcholinesterase, α-glycosidase, butyrylcholinesterase, and carbonic anhydrase isomerase II, which are linked to some common and global diseases, such as epilepsy, diabetes, Alzheimer’s disease, and glaucoma. Finally, the results contributed to the evidence that Magnofluorine has biological effects such as anticholinergic, antidiabetic, and antiglaucoma effects. Thus, it can be used in the treatment of diseases after approval by in vivo and clinical studies.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are not available from the authors.

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