Synthesis and biological evaluation of some pyrazolone based Schiff base derivatives as enzymes inhibitors, antioxidant, and anticancer agents

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Abstract

Drugs with sufficient efficacy for use in the therapy of Alzheimer and other diseases caused by oxidative stress have not yet been produced until today despite the great efforts. Therefore, many people all over the world are experiencing serious health problems due to these diseases nowadays. In the current study, a series of pyrazolone based Schiff base derivatives (**2a-e**) (except **2a**) as target molecules were successfully synthesized for the first time, and then structurally illuminated by using FT-IR, ^1H NMR and ^{13}C NMR. Their inhibition activities on acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase enzymes were extensively tested, respectively. Furthermore, the synthesized molecules were also investigated for antioxidant and anticancer activities. The potential *in vitro* cytotoxic activities of the title molecules were examined on HeLa cancer and 3T3 mouse normal fibroblast cell lines using MTT assay. Our results showed that **1b** (IC_{50} : 9.497 mM) and **2a** (IC_{50} : 30.49 mM) significantly decreased the proliferation of HeLa cells. On the other hand, the apoptotic effect of **1b** and **2a** were investigated with acridine orange/propidium iodide double staining. The apoptotic cell ratios of the molecules treated with **1b** and **2a** were determined as 60 and 64%, respectively. While **2b** was found to be a very active molecule in antioxidant activities assays in ABTS cation radical scavenging (IC_{50} : 17.95 ± 0.47 mM) and CUPRAC ($\text{A}_{0.5}$: 48.73 ± 0.52 mM) activities, **2c** had a very active molecule in AChE, BChE and tyrosinase inhibitory activities with 82.79 ± 1.03 , 91.39 ± 1.06 and 92.60 ± 1.80 inhibition%, respectively. Also, the target molecules (**2a-e**) showed better antioxidant and enzyme inhibitory activities than those of ester derivatives (**1a-e**).

Introduction

Cancer is the deadliest health problem after cardiovascular system diseases. The most basic method employed in the therapy of cancer, which is a preventable and treatable disease, is chemotherapy. Therefore, the discovery and the development of new chemotherapeutics nowadays have become a necessity in order to overcome disadvantages of multidrug resistance [1, 2]. Cervical cancer is known worldwide as the fourth type most prevalent cancer influencing female. It was reported in 2018 that this disease caused almost 570,000 diagnosed cases and 311,000 deaths [3, 4]. In spite of the increasing risk of this type of cancer, no specific treatments are currently available for the patients [2–4]. Extensive tissue damage occurs at the end of most established treatments such as cryotherapy, chemotherapy and surgical excision [5, 6]. In addition, it has been known that the drugs approved for the treatment of cervical cancer by the Food and Drug Administration (FDA) have serious side effects and fall short due to their lack of specificity [7]. Today, the discovery studies of novel drugs that have the potential to be used in the treatment of this disease are ongoing uninterruptedly [8, 9].

Antioxidants, which are molecules to decrease the negative effects of free radicals in living metabolism, have a significant influence in delaying and prohibiting the progression of diverse chronic diseases [10, 11]. In recent years, the search for new drugs with antioxidant effects has increased in drug design

studies, because multifunctional drugs with antioxidant effects are thought to be effective in eliminating free radicals and drug side effects that take place as a result of metabolic events [12–14].

Alzheimer's Disease (AD) is defined as a progressive neurodegenerative disease that causes irreversible loss of cognitive skills and memory. It is the most common cause of dementia in advanced age [15–17]. AD was first defined in 1906 by German psychiatrist and neuropathologist, Alois Alzheimer [18]. This disease is the most prevalent disease among neurodegenerative diseases. Although the prevalence of the disease is 10% over the age of 65, this rate rises to 30% over the age of 85 [19]. In the population over the age of 60, the rate doubles every passing five years. Although it does not depend entirely on gender, it is stated that the risk of AD is higher in females over the age of 85 compared to male [20]. It is predicted that AD will be one of the most serious health problems in the future [21, 22]. Nowadays, inhibition of AChE and BChE enzymes that hydrolyze acetylcholine (ACh) and butyrylcholine (BCh) neurotransmitters have become a therapy option for this disease [23, 24]. For this reason, many researchers have tried to discover new inhibitors of both enzymes for the treatment of AD [25, 26].

Tyrosinase enzyme, which is found in animals, plants, fungi and microorganisms [26], belongs to the polyphenol oxidase (PPO) enzyme class [27]. This enzyme takes the first place among the PPO enzymes that have been best defined and characterized by its structure until now [28]. Tyrosinase enzyme, which is found in almost all living structures, performs different physiological tasks according to the organism type [28]. This enzyme is the main enzyme responsible for the production of melanin pigment in fungi and vertebrates [29]. When there is any injury and tissue damage in plants, it causes browning as a result of enzymatic darkening of phenolic compounds [27, 3, 30]. Tyrosinase not only provides color formation in the pigmentation process in mammals, but it also has a homeostatic effect in preventing UV damage [31–33]. However, in the event that the production pathway of tyrosinase is disrupted for any reason, especially, hyperpigmentation problems often occur. These molecules, used as hypopigmentation agents or tyrosinase inhibitors, can be obtained by chemical or biological means [34].

Schiff bases, namely imines, are usually the condensation products of primary amines with carbonyl compounds under certain conditions [35]. Schiff base derivatives, carrying an azomethine ($-\text{CH}=\text{N}-$) functional group, were first reported by Hugo Schiff [36–38]. These compounds have determined to possess a wide spectrum of biological effects such as anticancer [39], antimicrobial [40, 41], antioxidant [42], tyrosinase [43] and cholinesterase properties, etc. [44]. Apart from these, they are also used as catalysts, intermediates in organic synthesis, pigments and dyes and as polymer stabilizers [37]. In these days, a large number of researchers working in the field of medicinal chemistry are trying to obtain novel enzyme inhibitors, antioxidant and anticancer agents, which will have the desired efficacy, by using the molecules belonging to different classes of organic compounds [42–44]. Schiff base derivatives are also among the compound classes whose biological activities have been studied most when compared to other compound classes.

Pyrazolone, a five-membered heterocyclic compounds that contains two adjacent nitrogen atoms, can be considered as a derivative of pyrazole which has an additional carbonyl ($\text{C}=\text{O}$) group. Since pyrazolone

derivatives have various biological significances such as anti-inflammatory, antipyretic, analgesic, hypoglycemic, antimicrobial, and antioxidant, they attracted a significant attention of medicinal chemists [45, 46]. Schiff bases synthetically derived from 4-aminoantipyrine (4-AAP) are significant pyrazolone analogs, which have multifunctional traits including antifungal, antibacterial, antimalarial, antiviral, anti-inflammatory and antipyretic properties [47, 48]. Nowadays, 4-AAP derivatives are employed as significant biomodel compounds in medicinal and biological fields [49, 50].

The above observations encouraged us to carry out a study targeting the discovery of new enzymes inhibitors, antioxidant and anticancer agents. Thus, it was aimed to investigate anticancer, antioxidant, anticholinesterase and tyrosinase activities of some Schiff base analogs of 4-AAP (**2a-e**) *in vitro* conditions in this study. All molecules obtained within the scope of the study were characterized in detail by spectroscopic methods (FT-IR, ^1H NMR and ^{13}C NMR).

Results And Discussion

Synthesis and characterization

In this study, a series of 4-AAP Schiff base derivatives (**2a-e**) as target molecules were successfully prepared in high purity in two steps according to the pathway shown in Scheme 1. In the first step, the vanillin ester derivatives (**1a-e**) were easily synthesized with **78–86%** yield by the esterification reaction of vanillin with an appropriate benzoyl chloride derivative in a molar ratio of 1:1 in pyridine as solvent. In the second step, target molecules (**2a-e**) were quickly obtained with **79–87%** with yield by treatment of 4-AAP with the corresponding ester derivative in the molar ratio of 1:1 in ethanol as solvent. From these synthesized target molecules, **2b-e** are novel but **2a** are already known in the literature [51]. All reactions were performed by refluxing in 50 mL reaction flasks for 1 h under reflux with magnetic stirrer. The structural elucidation of all synthesized molecules was performed by ^1H , ^{13}C NMR, and FT-IR, respectively.

IR spectra of the Schiff base derivatives (**2a-e**) afforded the C-H stretching bands of the aromatic rings ($3104\text{--}2959\text{ cm}^{-1}$), C = O stretching bands of the pyrazolone ring ($1646\text{--}1635\text{ cm}^{-1}$) and the ester group ($1755\text{--}1743\text{ cm}^{-1}$). In ^1H NMR spectra, C-H proton belonging to the imine group (CH = N), indicating that Schiff base was formed, resonated as a singlet peak at 9.62–9.61 ppm. The methyl groups in the 1st and 5th positions of the heterocyclic pyrazolone ring were observed as two singlets at 3.21–3.20 ppm and 2.49 ppm, respectively. In addition, the protons of the methoxy group were also found to resonate at 3.90–3.85 ppm as a singlet. The protons of the aromatic rings were recorded in the aromatic region between 9.12 and 7.12 ppm with splitting in the form of doublet, triplet, doublet of doublet or multiplet. When ^{13}C NMR spectra of Schiff bases were examined, it was seen that the carbons of the methyl groups on the pyrazolone ring resonated at 35.81–35.78 and 10.31–10.29 ppm, respectively. The signal of the carbon of C = O group of the pyrazolone ring appeared at 160.03–159.99 ppm. The signal of the carbons of CH=N group was observed at 153.85–153.59 ppm. Other aromatic carbon signals were detected as 152.73–110.86 ppm.

Biological evaluation

Cytotoxic effects of the synthesized molecules

All synthesized molecules were examined for anti-tumor potential against HeLa (human cervical carcinoma), and 3T3 mouse fibroblast cell lines (control cell line) using MTT test. IC₅₀ values of all molecules are shown in Table 1. IC₅₀ values of these molecules showed different effects on each cell lines, and those with common effects were screened. Thus, the molecule with a concentration of less than 50 mM and low cell viability among all molecules was chosen as the drug candidate. In order to find potential candidates for the anticancer agents, the molecules cytotoxic to cancer cells and harmless to normal cells were selected. IC₅₀ values of **1b** and **2a** on HeLa cells showed the expected effect. **2e** appeared to have the same anti-survival effect in two cell lines. However, it also showed toxic activity on normal 3T3 cells. Therefore, we selected **1b** and **2a** for concentration activities of IC₅₀ in HeLa cells for ongoing our cellular studies.

Table 1. IC ₅₀ values of all synthesized molecules		
IC ₅₀ (μM)		
Compounds	HeLa	3T3
1a	321.78	130.97
1b	9.497	16.73
1c	ND	ND
1d	338.85	84.60
1e	209.69	472.55
2a	30.49	66.7
2b	104.49	1130.57
2c	174.23	40.54
2d	165.32	1416.06
2e	22.71	14.33

The morphological effects of **1b** and **2a** on HeLa cervical cancer cells were examined with images taken at 10x magnification (Fig. 1a). When compared with the control group cells, it was observed that cells incubated with **1b** and **2a** had shrinkage and divergence from each other. After 24 hours of incubation, the viability of HeLa cells incubated with **1b** and **2a** decreased to 48% and 41%, respectively (Fig. 1b, 1c). In a similar study, Teran et al. synthesized Schiff base derivatives of 4-AAP and stated that the IC₅₀ doses of these molecules on mammalian macrophage cells were between 0.11-0.15 mg/mL [52]. When compared with this study, it is seen that (**2a-e**) synthesized in our study are less cytotoxic to normal cells.

Apoptotic effects of the synthesized molecules

Acridine orange and propidium iodide are dyes used to differentiate between living and non-living cells. Acridine orange dyes living cells, and gives them a green fluorescent, while propidium iodide stains dead cells and emits red fluorescence [53]. To investigate the apoptotic effects of **1b** and **2a**, HeLa cells were treated with 10 μ M doses of these molecules. It was found that cells treated with **1b** and **2a** emitted more red fluorescence compared to the control group (Fig.2a). On the other hand, the apoptotic cell ratios of the molecules treated with **1b** and **2a** were 60% and 64%, respectively. In control cells, the rate of apoptotic cells was calculated as 22% (Fig. 2b). These results confirm that the apoptotic pathways of HeLa cells treated with **1b** and **2a** are activated.

Antioxidant activity results

Of synthesized Schiff base derivatives, **2a**, **2b**, **2c** and **2e** showed strong antioxidant activities in ABTS cation radical scavenging and CUPRAC assays, the most active one of which is **2b** with IC_{50} :17.95 $\pm$ 0.47 mM value which is very close to the BHA compound (IC_{50} :17.59 $\pm$ 0.10 mM) used as a standard. The rest of Schiff bases (**2a-e**) had no activity in DPPH free radical scavenging and metal chelating assays with IC_{50} values >1000 μ M (Table 2). While (**2a-e**) showed good activity, (**1a-e**) demonstrated very weak activity. The most active synthesized compound is **2e**. **2e** ($A_{0.5}$:43.75 $\pm$ 0.62 mM) and **2b** ($A_{0.5}$:48.73 $\pm$ 0.52 mM) have better activity than α -TOC ($A_{0.5}$:50.58 $\pm$ 0.39 mM) used as a standard (Table 2). Synthesized molecules demonstrated no activities in DPPH free radical scavenging and metal chelating assays (Table 2).

Table 2 Antioxidant activity results*

Samples	IC ₅₀ values (mM) ^a			A _{0.5} values (mM) ^b
	DPPH Free Radical	ABTS Cation Radical	Metal Chelate	CUPRAC
1a	>1000	>100	>1000	503.13±2.05
1b	>1000	>1000	>1000	>1000
1c	>1000	>1000	>1000	644.17±1.84
1d	>1000	>1000	>1000	545.29±1.28
1e	>1000	>1000	>1000	280.86±1.16
2a	>1000	22.49±0.59	>1000	55.45±0.76
2b	>1000	17.95±0.47	>1000	48.73±0.52
2c	>1000	28.98±0.52	>1000	89.16±0.48
2d	>1000	>1000	>1000	118.52±1.26
2e	>1000	87.16±0.75	>1000	43.75±0.62
BHA	48.37±0.58	17.59±0.10	-	18.44±0.15
α-TOC	16.30±0.79	9.74±0.42	-	50.58±0.39
BHT	354.31±1.23	13.25±0.27	-	27.68±0.24
EDTA	-	-	26.82±0.10	-

*Values are means of three parallel measurement ± Standard deviation, n=3

^aValues were given as IC₅₀ for DPPH free radical, ABTS cation radical scavenging and metal chelating activities

^bValues were given as A_{0.5} for CUPRAC activity

Enzyme inhibitory activity results

For acetylcholinesterase inhibitory activity, galanthamine was used to be the standard with 81.78±0.62% inhibition value. **2c** with 82.79±1.03 % inhibition value showed the best anti AChE activity among all tested compounds. **2d** also showed strong activity in AChE enzyme inhibitory activity (74.35±0.43% inhibition). We can say that (**2a-e**) showed better activity than (**1a-e**) looking at Table 3.

2a, 2c and **2e** demonstrated very strong activity against BChE enzyme with 98.79±1.47, 91.39±1.06 and 84.88±1.08 inhibition %, respectively, better than galanthamine (76.58±1.08 % inhibition) used as standard. Also, it can be seen in Table 3 that (**2a-e**) showed better activity than (**1a-e**).

Kojic acid was used as standard compound in tyrosinase enzyme inhibitory activity with 69.38 ± 0.80 inhibition %. **2c** and **2a** showed very strong tyrosinase enzyme inhibitory activity, with 92.60 ± 1.80 and 74.98 ± 1.33 % inhibition, respectively, which are better than kojic acid. Again, it is possible to say that (**2a-e**) showed better activity than (**1a-e**) (Table 3).

Table 3 Enzyme inhibition activities results*

Comp.	AChE	BChE	Tyrosinase
1a	NA	14.77 ± 0.39	12.68 ± 0.08
1b	15.34 ± 0.08	20.96 ± 1.08	16.17 ± 0.90
1c	7.32 ± 0.00	50.00 ± 0.85	NA
1d	NA	NA	NA
1e	58.30 ± 1.04	53.06 ± 0.71	21.74 ± 0.57
2a	47.01 ± 0.57	98.79 ± 1.47	74.98 ± 1.33
2b	49.44 ± 0.93	41.56 ± 0.60	25.11 ± 0.80
2c	82.79 ± 1.03	91.39 ± 1.06	92.60 ± 1.80
2d	74.35 ± 0.43	63.29 ± 0.37	6.98 ± 0.09
2e	46.16 ± 0.75	84.88 ± 1.08	38.09 ± 0.54
Galantamine^a	81.78 ± 0.62^f	76.58 ± 1.08	-
Kojic acid^b	-	-	69.38 ± 0.80

*All enzyme inhibition activity values were given as inhibition % at 200 mg/mL

^a Standard compound for AChE and BChE

^b Standard compound for Tyrosinase

NA: Not Active

Conclusion

In this study, a series of Schiff base derivatives of 4-AAP (**2a-e**) were successfully prepared, fully characterized by some spectral techniques; and their antioxidant activities were examined by using four different methods, respectively. The inhibitory effects of the synthesized molecules against AChE, BChE, and tyrosinase enzymes were extensively screened. According to the results obtained from all methods applied, **2b** showed the best antioxidant activity. (**2a-e**) generally showed better antioxidant activity than (**1a-e**), except for DPPH free radical scavenging and metal chelating activity. **2c** exhibited a very potent

activity in AChE and tyrosinase inhibitory activities. In addition, according to the results achieved from all procedures used, **2a** showed the best enzyme inhibitory activities. Besides these, the cytotoxic effects of these molecules were also evaluated on HeLa human cervical cancer and 3T3 mouse fibroblast cell lines. Compared to other compounds, **1b** and **2a** showed the highest cytotoxic effect on HeLa cells.^{f2e}

Materials And Methods

Chemicals and instrumentations

Unless otherwise stated, all chemicals used in this study were obtained from the representatives of Aldrich and Sigma-Aldrich Companies, and employed without further purification. Melting points of the synthesized molecules were determined in open capillary tubes by using a melting point apparatus (Barnstead IA9100 Electrothermal Digital Melting Points Apparatus), and uncorrected. Characterization of all compounds was performed by using Fourier Transform Infrared Spectrometer (FTIR Agilent Cary 630 with ATR), and ¹H and ¹³C NMR spectra (Bruker Avance 500 MHz spectrometer by using DMSO-*d*₆ as solvent and tetramethylsilane (TMS) as an internal standard at 500 MHz and 125 MHz, respectively.

General procedures for biological studies

Anticancer Activity

The synthesized molecules were investigated for their anticancer activities using the cervical cancer cell line (HeLa). Mouse normal fibroblasts cell line (3T3) was used as a control cell. The anticancer activity was evaluated using MTT assay [54]. HeLa cancer cells were maintained in DMEM medium (including 100 U/mL penicillin, 100 mg/mL streptomycin, 10% FBS and 2 mM L-glutamine). 3T3 mouse normal fibroblast cells were also maintained in DMEM medium. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Firstly, cells were removed from the flask bottom with trypsin-EDTA solution (0.25%, Invitrogen) and seeded into 96-well plates (1×10⁴ cells/well). The cells were incubated for 24 hours to adhere to the bottom of the wells. After the adherence, cell viability was determined for all compound effects. The different concentrations (1, 10, 100, 1000 mM) of each molecule were added in each well and incubated for 24 h, respectively. Then, the mediums of the cells were discharged, and cells were washed with PBS. 100 µL fresh medium added to each well. After this step, a 10 µL MTT solution (5mg/mL) was added to each well. Cells were also incubated for 4 h in growth condition for labeling the cells. At the end of the incubation, the medium in the wells was discharged and 100 µL of DMSO was added to each well to dissolve the formed formazan dye. The absorbance of color change from formazan precipitate solubility was measured at 570 nm using ELISA reader (Epoch, Biotek, USA). MTT assay was carried out triplicate. Besides, IC₅₀ values of the molecules on HeLa and 3T3 cells were calculated by using AATbio IC50 calculator.

PI/AO Double Staining

PI/AO double staining was performed to determine the apoptotic effect of the synthesized molecules on HeLa cells. A density of 1×10^5 HeLa cells were firstly seeded in a six well plate. After 24 h of incubation, cells were treated with an IC_{50} concentration of **1b** and **2a**. Control cells were maintained with PBS buffer. After incubating for 24 h, cells were washed twice with DPBS; and 2 mL of fresh medium was added onto the cells. Subsequently, 10 $\mu\text{g/mL}$ acridine orange and 10 $\mu\text{g/mL}$ propidium iodide were added into cell medium after 24 h of incubation. Cells were incubated for 10 min for staining. Then, staining cells were washed with DPBS to remove the excess dye; and 2 mL of fresh medium was also added onto the cells. Images of cells were captured under a fluorescence microscope (Olympus BX51, Japan).

Antioxidant methods

In this study, antioxidant activity of all synthesized molecules were determined according to slightly modified modern versions of previously reported methods. For the determination of antioxidant activity of each title molecule, four different methods known in the literature and frequently used were preferred. These methods are 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation radical decolorisation assay, metal chelating activity and Cupric Reducing Antioxidant Capacity (CUPRAC) assay. IC_{50} values in the current study were determined by a concentration-inhibition graph. $A_{0.5}$ values were computed by a concentration-absorbance graph.

DPPH free radical scavenging assay

The DPPH radical scavenging activity of Schiff base derivatives (**2a-e**) and vanillin ester derivatives (**1a-e**) was measured by a spectrophotometric method [55]. This procedure was based on the reduction in ethanol solution of DPPH. For this, 2, 5, 10 and 20 μL of 1 mM stock solutions of each molecule were firstly prepared, and then each of the prepared solutions was completed to 40 μL with DMSO, respectively. Afterwards, 160 μL of 0.1 mM of DPPH solutions was added into each well in the microplate, separately. Then, the resulting solution was allowed to react for about 30 min at room temperature in the dark. Finally, the absorbance was measured at 517 nm against a blank, respectively. In this study, in order to calculate the inhibition of DPPH in percent (I %), the following formula was utilized:

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

where A_{control} is the absorbance of the control reaction (containing all reagents except for the tested molecules), and A_{sample} is the absorbance of the tested molecules. BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and α -TOC (α -Tocopherol) in this process were employed as positive control. All tests were repeated three times one after the other.

ABTS cation radical scavenging assay

The inhibition of decolorisation percent of $\text{ABTS}^{\cdot+}$ cation radical of Schiff base derivatives (**2a-e**) and vanillin ester derivatives **1a-e** in this study was determined the inhibition percentage as a function of time

and concentration, and then the results obtained were assessed by comparison with BHT, BHA and α -TOC molecules employed as standards[56]. For this, 2, 5, 10 and 20 μ L of 1 mM stock solutions of each molecule were firstly prepared, and then each of the prepared solutions was completed to 40 μ L with DMSO, respectively. Afterwards, these solutions were added to each well respectively, and then, 160 μ L of 7 mM ABTS solutions were added into each well in the microplate, separately. After that, the mixture was allowed to react for about 6 min at room temperature. Finally, the absorbance was measured at 734 nm. In this study, in order to calculate ABTS cation radical decolorisation activity as inhibition%, the following formula was utilized:

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

where A is the absorbance. All tests were repeated three times one after the other.

Metal chelating activity

Metal chelating ability of Schiff base derivatives (**2a-e**) and vanillin ester derivatives (**1a-e**) was investigated according to the method of Dinis et al., [57]. For this, 2, 5, 10 and 20 μ L of 1 mM stock solutions of each molecule were pipetted to each well, and then each of the samples were completed to 188 μ L with DMSO and then 4 μ L of 2 mM ferrous (II) chloride was added to the resulting solution, separately. After these processes, the reaction was initiated by the adding 8 μ L of 5 mM ferrozine to the obtained solution. The mixture was allowed to react for about 10 min at room temperature. Finally, the absorbance was measured at 562 nm against a blank, respectively. The results obtained were stated as percentage of inhibition of the ferrozine-Fe²⁺ complex formation. In order to calculate the percentage inhibition of the ferrozine-Fe²⁺ complex formation, the following formula was utilized:

$$\text{Metal chelating ability (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

where A is the absorbance. EDTA in this process was employed as a positive control. All tests were repeated three times one after the other.

CUPRAC assay

This method consists of the reduction of Cu(II)-neocuproine into its colored form Cu(I)- neocuproine chelate in the presence of antioxidant molecules [58]. For this, 2.5, 6.25, 12.5, and 25 μ L of 1 mM stock solutions of each molecule were pipetted to each well and then 61 μ L of 10 mM CuCl₂, 61 μ L of 7.5 mM neocuproine, and 61 μ L of 1 M of NH₄OAc solutions were added into prepared solutions at different concentrations, respectively. After obtaining the complex during the experiment, the absorbance was carefully measured at 450 nm. Finally, A_{0.5} values obtained from a concentration-absorbance graph were compared with standard molecules, BHA, BHT and α -tocopherol. All tests were repeated three times one after the other.

Enzyme inhibition activity assays

Anti-tyrosinase activity and anti-cholinesterase assay of all prepared molecules in this study were investigated according to modified modern versions of the earlier reported methods.

Anti-cholinesterase assay

The inhibitory effect of Schiff base derivatives (**2a-e**) and vanillin ester derivatives (**1a-e**) on AChE and BChE enzymes activities in the current study was determined according to slightly altered spectrophotometric method of Ellman et al., [59]. For this, all synthesized molecules were firstly dissolved in DMSO to obtain stock solutions at 4 mM concentration. Afterwards, aliquots of 150 μ L of 100 mM sodium phosphate buffer (pH 8.0), 10 μ L of sample solution and 20 μ L BChE (or AChE) solution were mixed, and then, the resulting solution was incubated for about 15 min at 25 °C. After these processes, 10 μ L of Ellman's reagent [5, 5'-dithiobis (2-nitrobenzoic acid), DTNB] was added to the solution. Finally, the reaction was initiated by the addition of 10 μ L of butyrylthiocholine iodide (or acetylthiocholine iodide) as substrate to the obtained solution. After 30 min, the absorbances were measured at 412 nm. The final solution of tested molecules was 200 μ L. In this study, in order to calculate the percentage of both enzyme inhibitions, the following formula was utilized:

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

where A is the absorbance. Galantamine in this process was employed as a positive control. All tests were repeated three times one after the other.

Anti tyrosinase activity

Anti-tyrosinase activity of Schiff base derivatives (**2a-e**) and vanillin ester derivatives (**1a-e**) was determined according to the method designed by Hearing and Jimenez [60]. For this, all synthesized molecules were firstly dissolved in DMSO to obtain stock solutions at 4 mM concentration. Afterwards, aliquots of 150 μ L of 100 mM sodium phosphate buffer (pH 6.8), 10 μ L of sample solution and 20 μ L tyrosinase solution were mixed, and then, the resulting solution was shaking for 3 minutes and incubated for 10 min at 37 °C. After these processes, 20 μ L of DOPA solution which is used as substrate was added to the mixture. After 10 min at 37 °C, the absorbances were measured at 475 nm. In order to calculate the percentage of all enzyme inhibitions, the following formula was utilized:

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

where A is absorbance. Kojic acid for the positive control was employed as an inhibitor. All tests were repeated three times one after the other.

Statistical analysis

The results of the antioxidant, anti-cholinesterase and tyrosinase activity assays in this work are stated as the mean $\pm$ SD of three parallel measurements. The statistical significance was forecasted by utilizing a Student's t-test, where p value < 0.05 was considered significant.

Experimental procedure for synthesis of title molecules (1a-e) and (2a-e)

General procedure for synthesis of ester derivatives of vanillin (1a-e)

A mixture of vanillin (5 mmol) and an appropriate benzoyl chloride derivative (5 mmol) in pyridine (20 mL) in 100 mL two-neck round-bottomed flask equipped with a magnetic stirrer, reflux condenser and thermometer were heated under reflux at 115 °C for 1 h with continuous stirring [61]. After completion of the reaction, the mixture was cooled to room temperature and poured into 100 mL of icy water. Afterward it was held for 6 h at room temperature, the formed precipitate was filtered off, washed with 50 mL of distilled water, dried under air suction, and crystallized from ethanol to get pure ester derivative.

Spectral data for all compounds (1a-e)

4-Formyl-2-methoxyphenyl benzoate (1a)

White solid, yield: 81 %, m.p. 75-76 °C (lit. [62] m.p. 75-76 °C). FT-IR (ATR): $\nu_{\max}$, (cm⁻¹), 3061, 2960 (C-H_{arom}), 2830, 2735 (C-H_{aldehyde}), 1732 (C=O_{ester}), 1697 (C=O_{aldehyde}), 1594 (C=C). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 10.02 (s, 1H, CH_{aldehyde}), 8.17–8.12 (m, 2H, H_{arom}), 7.78 (t, *J*= 7.5 Hz, 1H, H_{arom}), 7.80–7.76 (m, 4H, H_{arom}), 7.54 (d, *J*= 8.0 Hz, 1H, H_{arom}), 3.87 (s, 3H, OCH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 192.55 (C=O_{aldehyde}), 164.07 (C=O_{ester}), 152.13, 144.77, 135.75, 134.78, 130.40, 129.56, 128.69, 124.39, 124.12, 112.44 (C_{arom}), 56.59 (OCH₃).

4-Formyl-2-methoxyphenyl 2-nitrobenzoate (1b)

White solid, yield: 78 %, m.p. 121-122 °C (lit. [63] m.p. 111.0-112.5 °C). FT-IR (ATR): $\nu_{\max}$, (cm⁻¹), 3075, 2960 (C-H_{arom}), 2826, 2736 (C-H_{aldehyde}), 1747 (C=O_{ester}), 1696 (C=O_{aldehyde}), 1599 (C=C_{arom}), 1526 (NO_{2asym}), 1345 (NO_{2sym}). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 10.02 (s, 1H, CH_{aldehyde}), 8.20–8.10 (m, 2H, H_{arom}), 7.99–7.93 (m, 2H, H_{arom}), 7.71–7.66 (m, 2H, H_{arom}), 7.52 (d, *J*= 8.0 Hz, 1H, H_{arom}), 3.93 (s, 3H, OCH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 192.55 (C=O_{aldehyde}), 162.63 (C=O_{ester}), 151.95, 148.59, 143.77, 136.11, 134.42, 134.33, 130.94, 124.95, 124.94, 124.06, 123.76, 112.82 (C_{arom}), 56.77 (OCH₃).

4-Formyl-2-methoxyphenyl 3-nitrobenzoate (1c)

White solid, yield: 83 %, m.p. 125-126 °C (lit. [64] m.p. 114-115 °C). FT-IR (ATR): $\nu_{\max}$, (cm⁻¹), 3087, 2933 (C-H_{arom}), 2842, 2740 (C-H_{aldehyde}), 1740 (C=O_{ester}), 1686 (C=O_{aldehyde}), 1595 (C=C_{arom}), 1528 (NO_{2asym}), 1344 (NO_{2sym}). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 10.03 (s, 1H, CH_{aldehyde}), 8.81–8.76 (m, 1H, H_{arom}), 8.62–8.60 (m, 1H, H_{arom}), 8.56–8.54 (m, 1H, H_{arom}), 7.94 (t, *J*= 8.0 Hz, 1H, H_{arom}), 7.72–7.64 (m, 2H, H_{arom}), 7.60 (d, *J*= 8.0 Hz, 1H, H_{arom}), 3.93 (s, 3H, OCH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 192.57 (C=O_{aldehyde}), 162.43 (C=O_{ester}), 151.93, 148.58, 144.27, 136.37, 136.01, 131.60, 130.20, 129.20, 124.74, 124.29, 124.09, 112.60 (C_{arom}), 56.69 (OCH₃).

4-Formyl-2-methoxyphenyl 4-nitrobenzoate (1d)

White solid, yield: 86 %, m.p. 188-189 °C (lit. [64] m.p. 189-190 °C). FT-IR (ATR): $\nu_{\max}$ (cm⁻¹), 3109, 2948 (C-H_{arom}), 2848, 2754 (C-H_{aldehyde}), 1744 (C=O_{ester}), 1697 (C=O_{aldehyde}), 1596 (C=C_{arom}), 1521 (NO_{2asym}), 1347 (NO_{2sym}). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 10.03 (s, 1H, CH_{aldehyde}), 8.45–8.36 (m, 4H, H_{arom}), 7.70–7.65 (m, 2H, H_{arom}), 7.59 (d, *J* = 8.0 Hz, 1H, H_{arom}), 3.88 (s, 3H, OCH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 192.56 (C=O_{aldehyde}), 162.68 (C=O_{ester}), 151.90, 151.26, 144.31, 136.00, 134.04, 131.91, 124.65, 124.27, 124.09, 112.59 (C_{arom}), 56.68 (OCH₃).

4-Formyl-2-methoxyphenyl 3,5-dinitrobenzoate (1e)

White solid, yield: 80%, m.p. 162-163 °C (lit. [65] m.p. 163-164 °C). FT-IR (ATR): $\nu_{\max}$ (cm⁻¹), 3109, 2948 (C-H_{arom}), 2848, 2754 (C-H_{aldehyde}), 1744 (C=O_{ester}), 1697 (C=O_{aldehyde}), 1596 (C=C_{arom}), 1521 (NO_{2asym}), 1347 (sym., NO₂). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 10.03 (s, 1H, CH_{aldehyde}), 9.13 (t, *J* = 2.0 Hz, 1H, H_{arom}), 9.08 (d, *J* = 2.0 Hz, 2H, H_{arom}), 7.72–7.63 (m, 4H, H_{arom}), 3.89 (s, 3H, OCH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 192.56 (C=O_{aldehyde}), 160.99 (C=O_{ester}), 151.79, 149.08, 143.92, 136.20, 131.53, 129.92, 124.21, 124.05, 123.88, 112.75 (C_{arom}), 56.74 (OCH₃).

General procedure for synthesis of target molecules (2a-e)

A mixture of 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one (1 mmol) and the corresponding ester derivative (1 mmol) was dissolved in anhydrous ethanol (10 mL) in 50 mL two-neck round-bottomed flask equipped with a magnetic stirrer, reflux condenser and thermometer. The reaction mixture was heated gently with continuous stirring at 80 °C under reflux for 2 h. After the completion of the reaction, the mixture was allowed to cool to room temperature, and then, the obtained crude product was removed by filtration, washed several times with petroleum ether. The residue was crystallized from ethanol to give the target molecule.

Spectral data for all compounds (2a-e)

4-[[[4-(benzoyloxy)-3-methoxyphenyl]methylene]amino]-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (2a)

Light yellow powder, yield: 81%, m.p. 206-207 °C (lit. [51] m.p. 195-197 °C). FT-IR (ATR): $\nu_{\max}$ (cm⁻¹), 3065, 3037 (C-H_{arom}), 2936, 2839 (C-H_{aliph}), 1743 (C=O_{ester}), 1646 (C=O_{pyrazolone}), 1579, 1484 (C=C_{arom} and C=N_{imine}). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 9.61 (s, 1H, CH_{imine}), 8.17–8.11 (m, 2H, H_{arom}), 7.79–7.74 (m, 1H, H_{arom}), 7.65–7.60 (m, 3H, H_{arom}), 7.56–7.52 (m, 2H, H_{arom}), 7.46 (dd, *J* = 8.0, 1.5 Hz, 1H, H_{arom}), 7.41-7.37 (m, 3H, H_{arom}), 7.34 (d, *J* = 8.0 Hz, 1H, H_{arom}), 3.85 (s, 3H, OCH₃), 3.20 (s, 3H, –N-CH₃), 2.49 (s, 3H, =C-CH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 164.39 (C=O_{ester}), 160.03 (C=O_{pyrazolone}),

153.85 (C=N_{imine}), 152.73, 151.74, 141.34, 137.24, 135.03, 134.62, 130.33, 129.65, 129.53, 129.01, 127.42, 125.13, 123.81, 120.71, 116.64, 110.86 (C_{arom}), 56.34 (OCH₃), 35.81 (–N-CH₃), 10.31 (=C-CH₃).

4-[[[4-(2-nitrobenzoyloxy)-3-methoxyphenyl]methylene]amino]-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (2b)

Light yellow powder, yield: 87%, m.p. 229-230 °C. FT-IR (ATR): $\nu_{\max}$, (cm⁻¹), 3069, 2959 (C-H_{arom}), 2937, 2870 (C-H_{aliph}), 1750 (C=O_{ester}), 1635 (C=O_{pyrazolone}), 1571, 1494 (C=C_{arom} and C=N_{imine}), 1529 (NO_{2asym}), 1348 (NO_{2sym}). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 9.61 (s, 1H, CH_{imine}), 8.20–8.15 (m, 1H, H_{arom}), 8.12–8.08 (m, 1H, H_{arom}), 7.97–7.92 (m, 2H, H_{arom}), 7.65 (d, *J* = 1.5 Hz, 1H, H_{arom}), 7.57–7.52 (m, 2H, H_{arom}), 7.48 (dd, *J* = 8.0, 1.5 Hz, 1H, H_{arom}), 7.40–7.37 (m, 3H, H_{arom}), 7.32 (d, *J* = 8.0 Hz, 1H, H_{arom}), 3.90 (s, 3H, OCH₃), 3.20 (s, 3H, –N-CH₃), 2.49 (s, 3H, =C-CH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 162.99 (C=O_{ester}), 159.99 (C=O_{pyrazolone}), 153.61 (C=N_{imine}), 152.75, 151.57, 148.63, 140.49, 137.71, 135.00, 134.29, 134.26, 130.89, 129.65, 127.46, 125.28, 125.18, 124.90, 123.14, 120.69, 116.54, 111.13 (C_{arom}), 56.51 (OCH₃), 35.77 (–N-CH₃), 10.30 (=C-CH₃).

4-[[[4-(3-nitrobenzoyloxy)-3-methoxyphenyl]methylene]amino]-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (2c)

Yellow powder, yield: 85%, m.p. 229-230 °C. FT-IR (ATR): $\nu_{\max}$, (cm⁻¹), 3072, 2970 (C-H_{arom}), 2941, 2867 (C-H_{aliph}), 1747 (C=O_{ester}), 1645 (C=O_{pyrazolone}), 1575, 1462 (C=C_{arom} and C=N_{imine}), 1531 (NO_{2asym}), 1344 (NO_{2sym}). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 9.61 (s, 1H, CH_{imine}), 8.81–8.76 (m, 1H, H_{arom}), 8.62–8.54 (m, 2H, H_{arom}), 7.93 (t, *J* = 8.0 Hz, 1H, H_{arom}), 7.66 (d, *J* = 1.5 Hz, 1H, H_{arom}), 7.57–7.53 (m, 2H, H_{arom}), 7.48 (dd, *J* = 8.0, 1.5 Hz, 1H, H_{arom}), 7.41–7.37 (m, 4H, H_{arom}), 3.86 (s, 3H, OCH₃), 3.20 (s, 3H, –N-CH₃), 2.49 (s, 3H, =C-CH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 162.76 (C=O_{ester}), 160.00 (C=O_{pyrazolone}), 153.71 (C=N_{imine}), 152.73, 151.53, 148.58, 140.91, 137.57, 136.33, 135.00, 131.58, 130.50, 129.66, 129.06, 127.47, 125.18, 124.69, 123.69, 120.70, 116.57, 110.96 (C_{arom}), 56.41 (OCH₃), 35.78 (–N-CH₃), 10.30 (=C-CH₃).

4-[[[4-(4-nitrobenzoyloxy)-3-methoxyphenyl]methylene]amino]-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (2d)

Yellow powder, yield: 79%, m.p. 235-237 °C. FT-IR (ATR): $\nu_{\max}$, (cm⁻¹), 3104, 3011 (C-H_{arom}), 2940, 2867 (C-H_{aliph}), 1740 (C=O_{ester}), 1645 (C=O_{pyrazolone}), 1579, 1491 (C=C_{arom} and C=N_{imine}), 1525 (NO_{2asym}), 1347 (NO_{2sym}). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 9.62 (s, 1H, CH_{imine}), 8.45 – 8.41 (m, 2H, H_{arom}), 8.40 – 8.36 (m, 2H, H_{arom}), 7.66 (d, *J* = 1.5 Hz, 1H, H_{arom}), 7.57 – 7.53 (m, 2H, H_{arom}), 7.48 (dd, *J* = 8.0, 1.5 Hz, 1H, H_{arom}), 7.42 – 7.38 (m, 4H, H_{arom}), 3.87 (s, 3H, OCH₃), 3.21 (s, 3H, –N-CH₃), 2.49 (s, 3H, =C-CH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 163.00 (C=O_{ester}), 159.99 (C=O_{pyrazolone}), 153.71 (C=N_{imine}), 152.73, 151.50,

151.18, 140.95, 137.56, 135.00, 134.36, 131.84, 129.65, 127.45, 125.16, 124.63, 123.66, 120.70, 116.58, 110.95 (C_{arom}), 56.40 (OCH₃), 35.78 (–N-CH₃), 10.29 (=C-CH₃).

4-[[[4-(3,5-dinitrobenzoyloxy)-3-methoxyphenyl]methylene]amino]-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (2e)

Yellow powder, yield: 80%, m.p. 227-228 °C. FT-IR (ATR): ν_{max} (cm⁻¹), 3095, 3071 (C-H_{arom}), 2968, 2876 (C-H_{aliph}), 1755 (C=O_{ester}), 1641 (C=O_{pyrazolone}), 1573, 1488 (C=C_{arom} and C=N_{imine}), 1544 (NO_{2asym}), 1344 (NO_{2sym}). ¹H NMR (500 MHz; DMSO-*d*₆, ppm): δ 9.61 (s, 1H, CH_{imine}), 9.12 (d, *J*= 2.0 Hz, 1H, H_{arom}), 9.07 (t, *J*= 2.0 Hz, 2H, H_{arom}), 7.67 (s, 1H, H_{arom}), 7.54 (t, *J*= 7.5 Hz, 2H, H_{arom}), 7.50 (d, *J*= 8.0 Hz, 1H, H_{arom}), 7.44 (d, *J*= 8.0 Hz, 1H, H_{arom}), 7.41-7.37 (m, 3H, H_{arom}), 3.87 (s, 3H, OCH₃), 3.21 (s, 3H, –N-CH₃), 2.49 (s, 3H, =C-CH₃). ¹³C NMR (125 MHz; DMSO-*d*₆, ppm): δ 161.30 (C=O_{ester}), 159.98 (C=O_{pyrazolone}), 153.59 (C=N_{imine}), 152.76, 151.39, 149.08, 140.60, 137.83, 135.00, 131.79, 129.87, 129.66, 127.47, 125.19, 123.76, 123.58, 120.67, 116.54, 111.07 (C_{arom}), 56.46 (OCH₃), 35.78 (–N-CH₃), 10.30 (=C-CH₃).

Declarations

Conflict of Interest: The authors declare that they have no conflict of interest.

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Figures

Figure 1

a) The morphological images of HeLa cells treated with IC50 concentrations of 1b and 2a b) Cell viability measurement of HeLa cell lines after treated with 1b (1-1000 μM) for 24 h c) Cell viability measurement of HeLa cell lines after treated with 2a (1-1000 μM) for 24 h.

Figure 2

a) AO/PI double staining of 1b and 2a for 24 h treatment in HeLa cells b) Percentage of AO/PI double staining of 1b and 2a for 24 h treatment in HeLa cells

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