

Aryl butenes active against K562 cells and lacking tyrosinase inhibitory activity as new leads in the treatment of leukemia

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ABSTRACT

The inhibitory effects of four series of aryl butene derivatives, active against breast cancer, on the monophenolase activity of tyrosinase, in melanin-free ink from *Sepia officinalis*, have been studied.

Hydroxytamoxifen **1**, ferrociphenol **17** and several aryl butene analogs have shown strong antiproliferative activity on hormone-dependent and hormone-independent breast cancer cells and were evaluated in leukemia K562 cell proliferation. Their potential to induce skin depigmentation by evaluating their anti-tyrosinase activity was also estimated. In order to better rationalize the tyrosinase inhibitory action and the binding mode of the compounds, docking studies were carried out.

Hydroxytamoxifen and some aryl butenes showed strong antiproliferative effects against K562 cells at 1 μ M without showing tyrosinase inhibition.

If phenolic compounds **16** and **17** showed the best antiproliferative activity on K562, Hydroxytamoxifen and compounds **5**, **10**, **20** and **21** have been identified as candidates for further development against chronic myeloid leukemia (CML), and are predicted to not induce depigmentation of the skin, a side effect encountered with imatinib, conventionally used for the treatment of CML.

Keywords: hydroxytamoxifen, ferrociphenol, aryl butenes, imatinib, chronic myeloid leukemia, anti-tyrosinase activity, skin depigmentation.

INTRODUCTION

Hydroxytamoxifen **1**, the active metabolite of tamoxifen and an estrogen receptor (ER) modulator, is the front-line chemotherapeutic agent for patients with hormone-dependent breast cancer [1]. A particular analogue of tamoxifen possessing a *para*-aniline group (compound **20**, Figure 1) was shown selective for leukemia, CNS (central nervous system) cancer, and renal cancer [2]. Tested on a full panel of 60 human tumor cell lines (NCI/DPT), this compound was particularly active against ACHN and SF-539, but also against UACC-62 melanoma, HL-60 (TB), and SR leukemia cell lines with GI₅₀ (growth inhibitory activity) values below 0.2 μM [2]. In another study, the antiproliferative effects of a series of ferrocene-tamoxifen derivatives (compounds **2**, **20**, **15**, **16**, **3**, **5**, **13**, and **14**) were evaluated against HL-60 (human promyelocytic leukemia) cells, with IC₅₀ range of 0.66-8.4 μM [3]. Imatinib (STI571, Gleevec), a methylpiperazine derivative, was previously approved for chronic myeloid leukemia (CML) treatment after a striking efficacy was demonstrated in all stages of the disease [4]. Nevertheless, this drug may induce local or generalized hypopigmentation [5]. More than 40% of patients may be affected by the development of localized or secondarily larger hypopigmented lesions on the integument [6]. Hypopigmentation is time and dose dependent and can lead to generalized but reversible lightening of the skin [5]. Tyrosinase activity was detected on normal fibroblasts and fibroblasts originating from nonlesional generalized vitiligo, treated with 1, 5 or 10 $\mu\text{mol L}^{-1}$ imatinib mesilate. These results suggest that imatinib mesilate could decrease skin pigmentation directly via the inhibition of tyrosinase activity [7].

In this work, hydroxytamoxifen **1**, phenolic compounds **4**, **12**, **16**, **17**, **18** and several aryl butene analogs have been tested against leukemia K562 cells. Their anti-tyrosinase activity was also evaluated in order to find potential replacements for imatinib, with similar efficacy, but lacking imatinib's skin depigmentation side effects. With an aim to explore and rationalized the tyrosinase inhibitory effect of imatinib, hydroxytamoxifen **1** and several aryl butene analogs

in context to experimental data, a molecular docking study was carried out, and possible interactions of these compounds with *Agaricus bisporus* mushroom tyrosinase have been investigated.

MATERIALS AND METHODS

Reagents

L-tyrosine was purchased from Fluka Biochimika. Dimethyl sulfoxide (DMSO) was obtained from Carlo Erba. The synthesis, anti-proliferative and/or antimicrobial activity of compounds **1**[8], **2** [9], **3** [10], **4** [11], **5** [12], **6** [13], **7** [13], **8** [10], **9** [10], **10** [10], **11** [13], **12** [14], **13** [15], **14** [16], **15** [16], **16** [17], **17** [9], **18** [18], **19** [19], **20** [20] and **21** [10] have been previously reported (Figure 1).

Antitumor effect

Cell culture

The K562 leukemia cell line was gifted by the Laboratory of Cell Biology (Faculty of Pharmacy, Marseille). The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and a 1% mixture of penicillin (100 IU/ml) and streptomycin (100 µg/ml), incubated at 37°C in a humid atmosphere of 5% CO₂ / 95% air. The number of passages was between 30 and 40. Population doubling-time is about 28 h for K562.

Proliferation assay

A total of 5×10^3 cells were plated in a 96-well plate and incubated for 24 h. The following day (D₀), 100 µL of the culture medium containing the compounds to be tested diluted in DMSO, were added to the plates (5 wells for each compound). Cells were gently washed once with 1% PBS and fixed with 1% glutaraldehyde/PBS (10 min, 20°C). After three days (D₅), cells were stained with 0.1% crystal violet (30 min, 20°C). The excess dye was removed by three washes in running tap water. The absorbance of each well was measured at 655 nm

with a Biorad microplate reader. The results were expressed as the percentage of proteins versus the control. Experiments were performed at least in duplicate.

Tyrosinase inhibition

Enzyme extraction and purification

Tyrosinase was prepared from the cephalopod mollusk *Sepia officinalis*. Specimens were collected from the gulf of Gabes, Tunisia. The ink sacs were removed from fresh individuals and the ink was collected and stored at 4°C. The ink was centrifuged at 20,000 rpm for 4 h, and the black pellet was discarded while the uncolored supernatant was saved. A phenyl sepharose column equilibrated with 0.5 mM ammonium sulfate and 50 mM sodium phosphate buffer pH 7 was used to elute the supernatant using a linear gradient of 0.5-0 mM ammonium sulfate in 50 mM sodium phosphate (pH 7.0). The active fractions were then collected. One unit (U) of enzymatic activity was defined as the amount of enzyme transforming 1 μmol of L-DOPA per minute. All steps were carried out at 4°C. The protein concentration was determined using the Bradford method with bovine serum albumin as control [21].

Effect of inhibitors on the enzyme activity

Tyrosinase catalyzes two distinct reactions : hydroxylation of monophenols to *o*-diphenols (monophenolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). The reaction medium (1 mL) contained 2 mM L-Tyr in 50 mM sodium phosphate buffer (pH 6.8). The inhibitor was dissolved in 3.3% DMSO as a final concentration and 3.3% DMSO without inhibitor was used as a negative control. The substrate (L-Tyr), the inhibitors and the enzyme fraction were both mixed after a pre-incubation period of 5 min at 58°C. Monophenolase activity in *Sepia* tyrosinase was then spectrophotometrically monitored at 58°C by dopachrome formation at 475 nm on a Shimadzu spectrophotometer.

Molecular Docking studies

The 3D structure of *A. bisporus* tyrosinase (receptor) was retrieved from the Protein Data Bank (PDB ID: 2Y9X) [22]. All ligand molecules were built through Chem-Sketch software (ACD Labs) and energetically minimized. The control drug (**Imatinib**) and some representatives of the various series of aryl butenes derivatives like (compound **8**, **16**, **17**, and **18**) were imported to docking studies set up using Molegro Virtual Docker 2010.4.1.0 (MVD) software and all missing bond orders, hybridization states, and angles then were assigned [23]. The MVD program used to perform the docking study applies the MolDock algorithm, which is based on a new hybrid search algorithm, called guided differential evolution that combines the differential evolution optimization technique with a cavity prediction algorithm [23]. The following parameters were used for the guided differential evolution algorithm: molecular surface (expanded van der Waals), maximum number of cavities ($n = 5$), cavity volume (44.544), probe size (1.20), maximum number of ray checks ($n = 16$), minimum number of ray hits ($n = 12$), grid resolution (0.30), population size = 100, max evaluations = 2500.

RESULTS AND DISCUSSIONS

Antiproliferative Effect

A selection of substances (hydroxytamoxifen **1**, phenolic compounds **4**, **12**, **16**, **17**, **18** and several aryl butene analogs) shown in Figure 1 was evaluated *in vitro* against the K562 cell line by following the growth of K562 cells in the absence or in the presence of 1 μM of each compound in culture medium, Figure 2. Hydroxytamoxifen **1**, which has an IC_{50} of 29 μM (Table 1) against hormone-independent breast cancer cells MDA-MB-231, inhibited 95% of K562 cells at a concentration of 1 μM (Figure 2). Compounds **16** and **17** also express antiproliferative activity against K562 cells, with 93% and 89% of growth inhibition, respectively. In a lesser extent, **15** and **18** demonstrated weaker antiproliferative activities against K562 cells with 5% and 23% inhibition at a concentration of 1 μM , respectively. These

activities could be correlated to the structure (Figure 1) of these compounds, and presence of a ferrocenyl group and –OH substitution probably promote antitumor activity against these cells. Compound **15** previously reported effective against leukemia cell line (HL-60) with IC₅₀ of 1.04 μM [3], but was not inhibitory on K562 cells in this study. The relevance of ferrocene to the antiproliferative activity towards K562 is furthermore validated by the inhibition of 86% recorded for compound **12**, despite the change of ferrocene position. Besides, the presence of ferrocene for the compounds possessing amino chains (**1**, **4** and **5**) does not seem to be very important. For example, the ferrocene analogue of hydroxytamoxifen **4**, inhibited 87% of K562 cells, slightly less effective than hydroxytamoxifen (95%) itself. The importance of the O(CH₂)₃NMe₂ group is also reflected in the antiproliferative effect (78% inhibition) by compound **5**, possessing two amino chains and a ferrocenyl group. Finally, ferrocene bearing compounds **20**, with an NH₂ substituent, and **21**, with an COO^tBu substituent, were active with inhibition of 79 and 72% of cells, respectively.

Anti-Tyrosinase Activity

Within series 1 (Compounds 7-11), **8** showed a non-competitive inhibition of tyrosinase of *S. officinalis* with an IC₅₀ of 0.025 mM (Unpublished results). Changing the size of the amido substitution is accompanied by the loss of tyrosinase inhibition, Table 1. Adding or removing a CH₃ function to the skeleton of **8** leads respectively to **9** and **7**, provoking a decline in tyrosinase inhibition from 63% to around 39%. The addition of two CH₃ groups gives compound **10**, which is only weakly active against tyrosinase (7% inhibition). On the other hand, the replacement of a phenyl ring by a ferrocenyl group increases the inhibition from 12% for **11** to 39% for **7**. Ferrocene therefore contributes significantly to the inhibitory potency of the compounds against tyrosinase.

For series 2 (Compounds 15-21), **16** and **17** were competitive tyrosinase inhibitors with respective IC₅₀ values of 0.028 and 0.02 mM (Unpublished results) and inhibition of 65% and

94% of activity at 0.04 mM (Table 1). In this series, –OH substitution is essential for tyrosinase inhibition. Indeed, compounds **15** (unsubstituted), **20**, (NH₂) and **21** (COO^tBu) were not active against tyrosinase with inhibitory values at 0.02 mM of only 29%, 8% and 2%, respectively. Similarly, compounds **18** and **19** showed inhibition values at 0.04 mM of 22% and 44%, respectively, far lower than their ferrocene counterparts **16** (65%) and **17** (94%). This result confirms the importance of ferrocene for conditioning anti-tyrosinase activity of the compounds.

In series 3 (Compounds 1-6), hydroxytamoxifen **1** does not inhibit tyrosinase at 0.04 mM (3% inhibition). At this dose, the substitution of the phenyl group by ferrocene in compound **2** increased the tyrosinase inhibition to 25% (Table 1). This level of inhibition is preserved for the ferrocene compound **3** bearing a citrate at the R2 position (20%), and **4**, having a longer amine chain than **2** (36%) (Table 1). Interestingly, compound **5**, with two amino substituents, presented a low inhibitory activity of 6%, while **6**, also with two amino substituents but lacking ferrocenyl and phenyl groups, registers 44% inhibitory activity at 0.04 mM. These results confirm the need for an –OH function and a ferrocenyl group for the product to have an inhibitory effect (Table 1).

In series 4, compounds **12**, **13** and **14** contain ferrocene and phenol group at variable positions. While compounds **12** and **13** presented an inhibitory activity of 29% and 23%, respectively, **14** had an inhibitory activity of only 8% (Table 1). This weak inhibitory activity of compound **14** compared to **6** (44%), benzophenone (31%), 4-hydroxybenzophenone (43%), and 4,4'-dihydroxybenzophenone (57%), at 0.04 mM, highlights the importance of a triaryl structure in addition to ferrocene and an OH function for a strong inhibitory activity.

Docking Study

With an aim to explore and rationalize the tyrosinase inhibitory effect of the compounds in context to experimental data, a molecular docking study was carried out, and possible interactions of these compounds with *Agaricus bisporus* mushroom tyrosinase have been investigated. Results of the docking studies have been compiled in Table 2.

The highest MolDock score (-150.16) obtained in case of standard drug Imatinib indicated that it must be best fit into the cavity comparatively and found to have interactions with Asn 81, His 85, and Thr 84 amino acid residues. However, other aryl butene derivatives used in this study showed interactions with the Asn 81, Thr 84, His 61, His 85, His 94, His 244, His 296, and Asn 320 amino acid residues with MolDock score between -112.8 to -144.16. Best poses of the compounds selected for study and standard drug along with the hydrogen bond interactions, distances along with the structural features involved are presented in Table 2 and Figure 3.

Hydroxytamoxifen **1** was shown effective for the treatment of hormone-dependent breast cancer, with an IC_{50} of 31 μ M on MCF-7 [24] and a similar IC_{50} value of 29 μ M against hormone-independent MDA-MB 231 tumor cells [12]. For tyrosinase from *S. officinalis* used in this work, a 606 amino acid protein was found with accession number "CAC82191.1". A homologous tyrosinase has been found in humans, consisting of 534 amino acids with accession number "AAA61244.1" with an E-value of 1e-07 and an identity of 25%. Inhibitors of *S. officinalis* tyrosinase thus are likely to be active against human tyrosinase.

In this work, hydroxytamoxifen **1** was able to inhibit 95% of K562 cells at a concentration of 1 μ M. This result makes hydroxytamoxifen **1** an excellent candidate for the treatment of leukemia, especially if we take into consideration its weak inhibiting potential of tyrosinase (3%) which may prevent skin depigmentation, a side effect encountered with the conventional CML treatment, imatinib.

The results here obtained against K562 cells open a new chemotherapeutic use of hydroxytamoxifen and derivatives. Indeed it has been reported that ferrociphenol **17** possesses a certain selectivity for leukemia with a GI_{50} of 0.2 μ M [2], which was confirmed in this present work with 89% inhibition of the K562 cells at 1 μ M. Nevertheless, this compound also appears to be a tyrosinase inhibitor and may therefore cause depigmentation of the skin during its chemotherapeutic use, giving no advantages over imatinib. From this point of view, compounds **20** and **21** having respectively 79 and 72% proliferation inhibition of K562 at 1 μ M, and 2 and 8% inhibition of tyrosinase activity at 0.02 mM, are better candidates for the treatment of leukemia than phenolic compounds **16** and **17** having respectively 93 and 89% inhibition of K562 at 1 μ M, and 45 and 60% inhibition of tyrosinase at 0.02 mM. Compounds **20** and **21**, while slightly less active than **16** and **17** at a dose of 1 μ M against K562 leukemia cells, are less likely to induce skin depigmentation as a side effect of treatment.

The amino compounds **4** and **5** have not been previously tested for their anti-leukemia activity and they also showed a good antiproliferative effect of respectively 87 and 78% on K562, and 36 and 6% inhibition of tyrosinase activity at 0.04 mM. At the same concentration, phenolic compounds **16** and **17** show strong tyrosinase inhibition (65 and 94%, respectively), which makes **4** and **5** less favorable towards skin depigmentation, and more useful as therapeutic agents.

Although amides **7**, **8**, **9**, **10** and **11** were not tested here against the leukemia cell line K562, **7** and **8** are the most active against MDA cell line with IC_{50} values of 0.65 and 1.2 μ M respectively. Their strong inhibitory activities against tyrosinase challenges us as to their use for the treatment of breast cancer and guided us to the selection of the product **10** which has an IC_{50} of 5.7 μ M against the MDA cell line and shows a 6% tyrosinase inhibition at a concentration of 0.04 mM.

Standard drug used in the docking study found to possess MolDock Score -150.16. On the other hand, compound **18** was found to possess only -112.08. This large difference in the docking scores also strengthens the hypothesis that the proposed compounds have very less inhibition towards tyrosinase. However, the replacement of a phenyl ring by a ferrocenyl group increases the MolDock score from -112.08 for **18** to -117.21 for **16**. So that it can be concluded here that ferrocene contributes significantly towards the inhibition. Compounds **16** and **17** have MolDock scores values of -117.21 and -127.71 depicted that hydroxyl group substitution is essential for tyrosinase inhibition. Indeed, changing the size of the amido substitution or adding or removing a CH₃ function to the skeleton accompanied by the loss of tyrosinase inhibition, compound **8** showed inhibition of tyrosinase with MolDock Score value – 144.16. Furthermore, a lack of direct interaction between the ligands and the copper ions of PDB: 2Y9X is also supported by our inhibition data which indicates that in case of compounds **16**, **17**, and **18** behave as competitive inhibitors.

CONCLUSION

Hydroxytamoxifen and some aryl butenes showed strong antiproliferative effects against K562 cells at 1 μ M without showing tyrosinase inhibition.

Compounds, **10**, **20**, **21** and **5** were judged to be least likely to induce depigmentation of the skin, a side effect encountered with imatinib, conventionally used for the treatment of CML, than the phenolic analogs (**16** and **17**), which makes them the preferred candidates for the development of future anti-leukemia drugs.

LIST OF ABBREVIATIONS

CML : chronic myeloid leukemia

ER : estrogen receptor

DMSO : Dimethyl sulfoxide

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Tables

Table 1:

Compound	Tyrosinase Inhibition (%)		Antitumoral activity
	0.02 mM	0.04 mM	IC ₅₀ MDA (μM)
1	-	3±0.43	29 ^[10]
2	-	25±0.81	0.5 ^[26]
3	-	20±2.21	-
4	-	36±0.59	0.5 ^[16]
5	-	6±1.72	0.45 ^[10]
6	34±0.85	44±1.11	-
7	-	39,5±0.37	0.65 ^[18]
8	41± 1.43	63±0.71	1.2 ^[16]
9	-	38±2.25	2.02 ^[16]
10	0	7±1.75	> 1 ^[16]
11	9±1.65	12±0.91	55.4 ^[16]
12	18±2.28	29±3.12	13 ^[6]
13	13±1.54	23±0.86	28 ^[6]
14	4±1.85	8±2.19	4.15 ^[20]
15	29±0.69	-	7.54 ^[25]
16	45±0.81	65± 1.16	1.13 ^[25]
17	60±0.46	94± 0.22	0.60 ^[25]
18	6±2.55	22±1.95	-
19	34±0.58	44±0.31	-
20	8±1.38	-	0.8 ^[18]
21	2±0.96	-	-

Table 2:

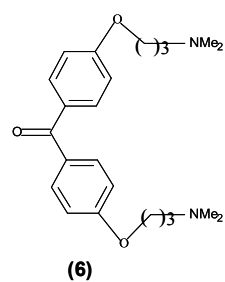
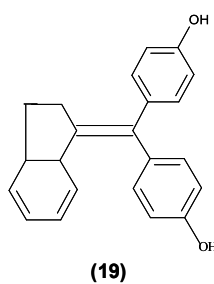
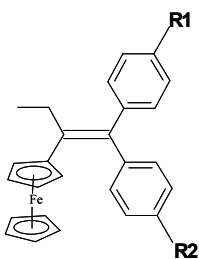
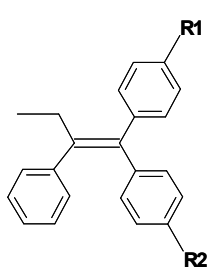
Sr. No.	Compounds	MolDock Score	No. of interactions	Distance (Å)	Amino Acids involved	Molecular Structure
1.	Compound 08	-144.16	01	2.47	Asn 81	O atom NHCOCH ₂ CH ₃
2.	Compound 16	-117.21	04	2.99	His 296	O of OH (R ₁)
				2.82	His 61	O of OH (R ₁)
				2.93	His 94	O of OH (R ₁)
				3.18	His 85	O of OH (R ₁)
3.	Compound 17	-127.71	03	3.15	His 244	O of OH (R ₁)
				3.04	Thr 84	O of OH (R ₂)
				2.58	Asn 320	O of OH (R ₂)
4.	Compound 18	-112.08	01	2.88	His 85	O of OH (R ₁)
5.	Imatinib (stand.)	-150.16	03	2.98	Asn 81	N of bridged NH
				3.07	His 85	N of Pyridyl
				2.72	Thr 84	N of Pyridyl

Legends Tables:

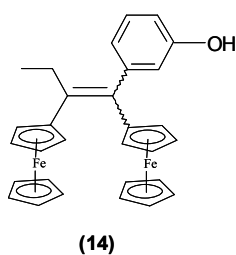
Table 1: Activity of the compounds on tyrosinase and MDA-MB 231 cells

Table 2: Docking score with the hydrogen bond interactions with amino acids and structural features involved.

Figures



(1): R1=OH; R2=O(CH₂)₂NMe₂
 (11): R1=NHAc; R2=H
 (18): R1=OH; R2=H



(2): R1=OH; R2=O(CH₂)₃NMe₂
 (3): R1=OH; R2=Amino citrate
 (4): R1=OH; R2=O(CH₂)₄NMe₂
 (5): R1=R2=O(CH₂)₃NMe₂
 (7): R1=NHAc; R2=H
 (8): R1=NHCOEt; R2=H
 (9): R1=NHCO^tPr; R2=H
 (10): R1=NHCO^tBu; R2=H
 (15): R1=R2=H
 (16): R1=OH; R2=H
 (17): R1=R2=OH
 (20): R1=NH₂; R2=H
 (21): R1=R2=OCO^tBu

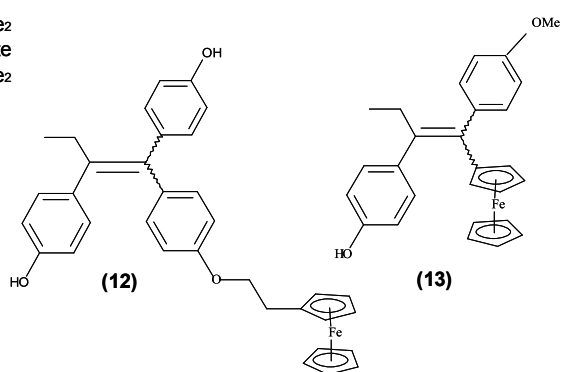


Figure 1

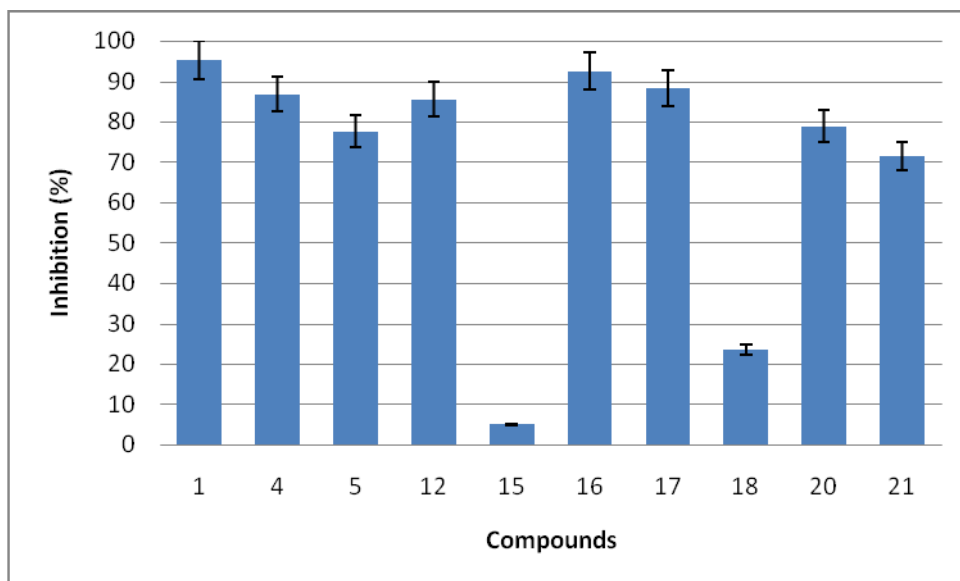


Figure 2

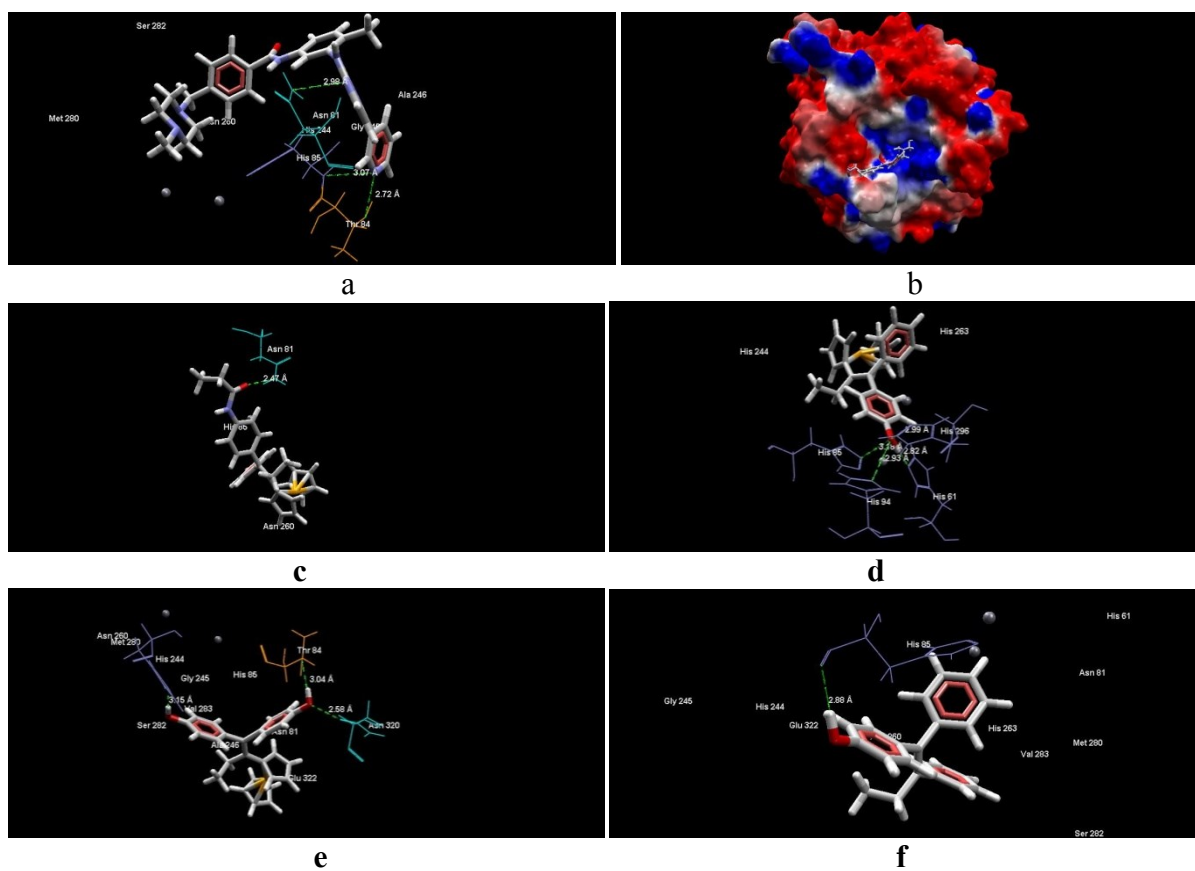


Figure 3

Legends Figures:

Figure 1 : Compounds used in this study

Figure 2 : Antiproliferative activity of several compounds on K562 cell line.

Figure 3 : Molecular interaction of different compounds with tyrosinase receptor.

- a) Binding mode of Standard drug (Imatinib) into the binding site of tyrosinase pocket. It has Moldock score -150.16 and forms 3 hydrogen bonds shown as green dotted lines, showing one hydrogen bond between N of bridged NH with Asn 81 of distance 2.98 Å, two hydrogen bonds between N of Pyridyl with His 85 and Thr 84 of distances 3.07 Å and 2.72 Å respectively.
- b) Electrostatic Surface Potential of entrance to di-copper centre showing standard drug (Imatinib) modelled into the binding site of tyrosinase pocket PDB: 2Y9X
- c) Binding mode of compound (8) into the binding site of tyrosinase pocket. It has Moldock score -144.16 and forms one hydrogen bond shown as green dotted line, between O atom of $\text{NHCOCH}_2\text{CH}_3$ with Asn 81 of distance 2.47 Å.
- d) Binding mode of compound (16) into the binding site of tyrosinase pocket. It has Moldock score -117.21 and forms 4 hydrogen bonds shown as green dotted lines, showing interactions between O of OH (R_1) of aryl butene moiety with His 296, His 61, His 94, His 85 of distances 2.99, 2.82, 2.93 and 3.18 Å, respectively.
- e) Binding mode of compound (17) into the binding site of tyrosinase pocket. It has Moldock score -127.71 and forms 3 hydrogen bonds shown as green dotted lines, showing one hydrogen bond between O of OH (R_1) of aryl butene with His 244 of distance 3.15 Å, two hydrogen bonds between O of OH (R_2) of aryl butene with Thr 84 and Asn 320 of distances 3.04 Å and 2.58 Å respectively.
- f) Binding mode of compound (18) into the binding site of tyrosinase pocket. It has Moldock score -112.08 and forms one hydrogen bond shown as green dotted lines, showing one hydrogen bond between O of OH (R_1) of aryl butene with His 85 of distance 2.88 Å