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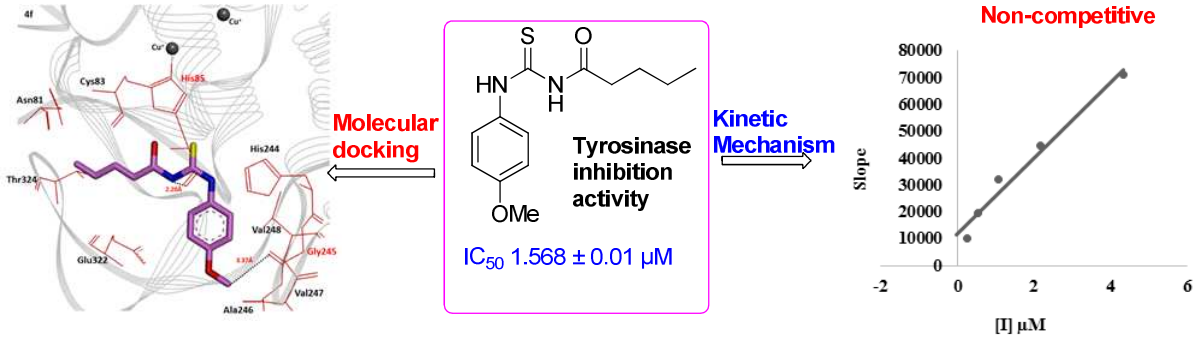
Graphical abstract



Design, Synthesis, kinetic mechanism and molecular docking studies of novel 1-Pentanoyl-3-arylthioureas as inhibitors of mushroom tyrosinase and free radical scavengers

Fayaz Ali Larik[a], Aamer Saeed[*a], Pervaiz Ali Channar[a], Urooj Muqadar[a], Qamar Abbas[b], Mubashir Hassan[b], Sung-Yum Seo[b] , Michael Bolte[c]

[a] *Department of Chemistry, Quaid-I-Azam University, Islamabad 45320, Pakistan.*

[b]*Department of Biological Sciences, College of Natural Sciences, Kongju National University, 56 Gongjudehak-Ro, Gongju, Chungnam 314-701, Republic of Korea*

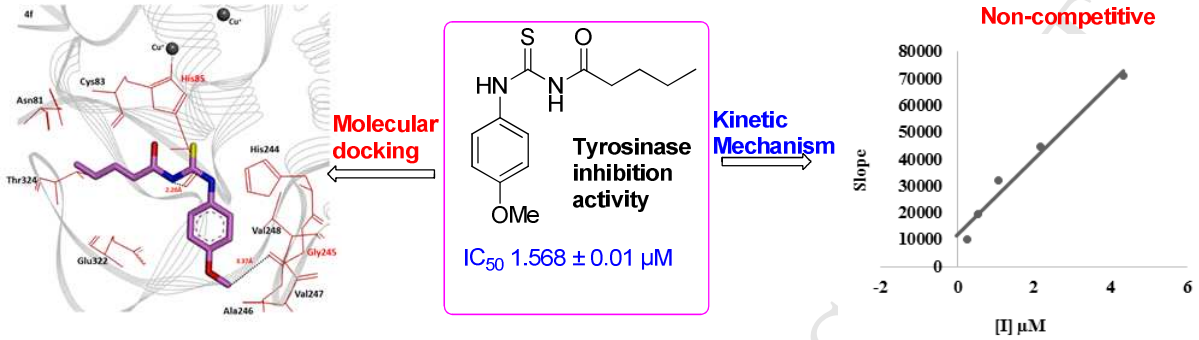
[c]*Institut für Anorganische Chemie, J.W.-Goethe-Universität, Max-von-Laue-Str.7, D-60438 Frankfurt/Main, Germany.*

[*] Email: aamersaeed@yahoo.com, Tel +92-51-9064-2128; Fax: +92-51-9064-2241

Highlights

- A small library of novel 1-Pentanoyl-3-arylthioureas (**4a-4j**) synthesized.
- Mushroom tyrosinase inhibition and free radical scavenging activity were evaluated
- Most of the compounds show excellent activity, particularly **4f** higher than the standard.
- The kinetic mechanism proposed **4f** is non-competitive inhibitor of mushroom tyrosinase.
- Molecular docking, druglikeness, Ramachandran graph, Chemo-informatics and Lipinski's rule were studied.

Graphical abstract



Abstract

A series of novel 1-pentanoyl-3-arylthioureas was designed as new mushroom tyrosinase inhibitors and free radical scavengers. The title compounds were obtained in excellent yield and

characterized by FTIR, ^{1}H NMR, ^{13}C NMR and X-ray crystallography in case of compound (**4a**). The inhibitory effects on mushroom tyrosinase and DPPH were evaluated and it was observed that 1-Pentanoyl-3-(4-methoxyphenyl) thiourea (**4f**) showed tyrosinase inhibitory activity (IC_{50} 1.568 ± 0.01 mM) comparable to Kojic acid (IC_{50} 16.051 ± 1.27 mM). Interestingly compound **4f** exhibited higher antioxidant potential compared to other derivatives. The docking studies of synthesized 1-Pentanoyl-3-arylthioureas analogues were also carried out against tyrosinase protein (PDBID 2ZMX) to compare the binding affinities with IC_{50} values. The predicted binding affinities are in good agreement with the IC_{50} values as compound (**4f**) showed highest binding affinity (-7.50 kcal/mol) compared to others derivatives. The kinetic mechanism analyzed by Line-weavere Burk plots exhibited that compound (**4f**) inhibit the enzyme inhibits the tyrosinase non-competitively to form an enzyme inhibitor complex. The inhibition constants Ki calculated from Dixon plots for compound (**4f**) is 1.10 µM. It was also found from kinetic analysis that derivative **4f** irreversible enzyme inhibitor complex. It is proposed on the basis of our investigation that title compound (**4f**) may serve as lead structure for the design of more potent tyrosinase inhibitors.

Keywords: Thiourea derivatives, Synthesis, Mushroom Tyrosinase inhibitor, Kinetic mechanism, Molecular docking, Crystal structure.

1. Introduction

It is believed that the browning of fruits, vegetables and beverages poses serious threat to agro-economic countries [1]. The food industry has recently witnessed loss of quality during

postharvest handling and processing of fruits and vegetables [2]. The plausible mechanism of browning of food is well recognized can be enzymatic or non-enzymatic in origin [3]. The enzymatic browning is catalyzed by tyrosinase (copper containing multifunctional glycosylated enzyme) also known as polyphenol oxidase (PPO; 1,2 benzenediol oxygen oxidoreductase; EC 1.10.3. l), polyphenolase, phenolase, catechol oxidase, cresolase, or catecholase is widely found in nature [4]. PPO is typically present in the majority of microorganisms, plants and animals. In plants, tyrosinase acts by oxidizing mono phenols into diphenols (monophenolase activity) and the oxidation of *o*-diphenols into *o*-quinones (diphenolase activity) followed by quinones into dark brown pigments [5]. These results in deterioration of flavor, color, and nutritional quality and continue during postharvest handling and marketing of vegetables [6]. In fungi and vertebrates, tyrosinase catalyzes the rate-limiting step in the formation of the pigment melanin from tyrosine [7]. Melanin is responsible for skin color and plays an important role in the protection of the skin against UV light induced damage [8]. During the melanin biosynthesis pathway, tyrosinase (EC 1.14.18.1) is the rate-limiting enzyme that hydroxylates L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and L-DOPA is further oxidized to the corresponding *o*-dopaquinone [9, 10].These phenomena have encouraged researchers to seek new potent tyrosinase inhibitors, for use in cosmetics and foods.

The literature survey reveals that researchers are devoted to design and synthesize novel tyrosinase inhibitors such as Seo et al.[11] reviewed the importance of mushroom tyrosinase and defined its biochemical character and inhibition and activation by several inhibitors from natural and synthetic origin. Zhu et al.[12] also reported that tyrosinase enzyme has also been linked to Parkinson's disease. Loizzo et al.[13] reviewed the tyrosinase inhibitors from both natural and

synthetic origin for their use in food and cosmetic industry. Khan also reviewed the molecular design of tyrosinase inhibitors from synthetic origin [14].

Recently, Chang et al reported that thio-containing compounds could react with dopaquinone, to form colorless products [15,16]. Then the melanogenetic process is slowed down, until all the scavengers are consumed. Thus, the development of safe and effective tyrosinase inhibitors is of great concern in the medical, food and cosmetic industries.

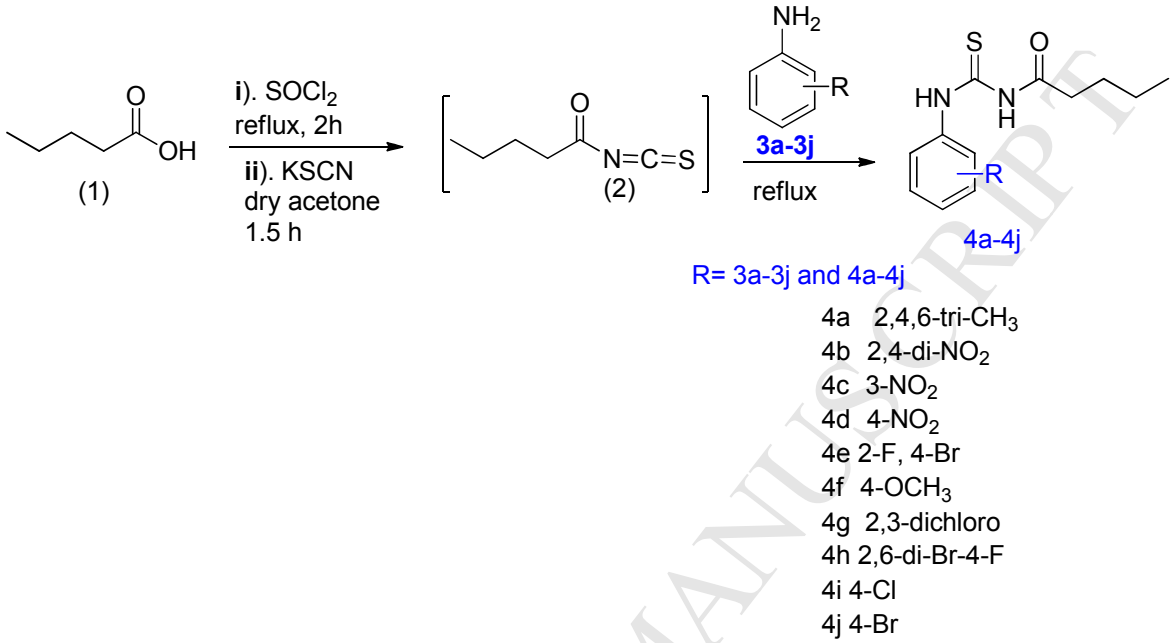
Our research group has fine contributions in the field of medicinal chemistry and recently we have designed and synthesized novel enzyme inhibitors [17-19]. The above mentioned literature survey prompted us to synthesize thio-containing compounds in order to be utilized as safe and effective inhibitors of tyrosinase enzyme.

Aryl thioureas found a wide diversity of applications in heterocyclic syntheses, metal complexes and molecular electronics and exhibit a vast array of biological activities [20-22]. Arylthioureas containing both carbonyl and thiocarbonyl groups can coordinate to metals using both sulphur and oxygen atoms, the presence of these hard and soft donor sites offer a huge bonding potential for metals such as copper [23,24]. Thiourea possess more than one binding sites it provoked us to design and synthesize novel 1-Pentanoyl-3-aryl thioureas as the potent inhibitors of tyrosinase enzyme.

2. Results and discussion

2.1 Chemistry

A novel series of thioureas derivatives (**4a-j**) were synthesized by forming isothiocyanate intermediate using the well-known protocol (treating potassium thiocyanate in dry acetone for 30 min and followed by the addition of acid chloride, after cooling the reaction mixture different aromatic substituted amines were incorporated) in scheme 1.



Scheme 1. Synthetic path to novel 1-pentanoyl-3-arylthioureas (**4a-4j**)

2.2 Spectroscopic characterization

The synthesized aromatic substituted 1-pentanoyl-3-aryl thioureas have been characterized on the basis of spectroscopic data.^{1}H and ^{13}C NMR were recorded in deutrated DMSO-d_6 solvent. Three different types of protons gave distinct signals in ^{1}H NMR, two N-H protons at 11.87 ppm and 11.34 ppm appeared singlet, most deshielded signals because of intra molecular hydrogen bonding, in ^{1}H NMR intramolecular hydrogen bonding shifts signals towards higher ppm value justifying presence of thio core in thiourea, signals at 7-8 ppm region clearly indicated the aromatic ring. Two types of methyl groups are present, one methyl group was attached with acyl

group that appeared at 2.35 ppm and other methyl group which is directly attached to aromatic ring appeared slightly shielded than the methyl protons attached with acyl group. In all of the synthesized thioureas, the –NH proton situated in between phenyl ring and C=S, is maximum deshielded due to the intramolecular hydrogen bonding and thus gave a singlet at around 12 ppm and the –NH proton located in between the C=O and C=S is deshielded to a lesser extent thus providing a singlet in the region of 10-11 ppm [8,15-17]. The aromatic ring protons gave their signals between 7 and 8 ppm. In ^{13}C NMR, carbon bonded with sulfur provided a strong signal at about 180 ppm and the carbonyl carbon appeared in the range of 172-175 ppm. Signals for aromatic carbons appeared in the region between 120-140 ppm and the signals for ipso carbons were found weak in intensity due to less number of hydrogen. In FTIR, -NH of thioureas gave a broad band above 3200 cm^{-1} due to the intramolecular hydrogen bonding between the oxygen of the carbonyl and -NH. Just around 3000 cm^{-1} Ar-H stretch was present and carbonyl group appeared as an intense band in the region of 1700-1600 cm^{-1}. C=S was available between 1050 cm^{-1} and 1250 cm^{-1} for all the compounds.

2.3 Bioassay for tyrosinase inhibition activity

1-Pentanoyl-3-arylthioureas have been designed to evaluate their inhibitory effects on mushroom tyrosinase activity. Kojic acid a competitive tyrosinase inhibitor was used as standard for comparison purpose. The aryl thioureas tyrosinase inhibitors as described in the preceding section are of special interest because of their high activity (IC_{50} < 1.578 mM). Novel aryl thioureas derivatives (**4a-4j**) have been synthesized by incorporation of substituted anilines to isothiocyanate. The synthesis 1-pentanoyl-3-arylthioureas was carried out to explore the role electron donating and electron withdrawing groups in tyrosinase inhibition. It has been exposed

from our bioassay results (Table 1) that the major determining factor of inhibitory activity is the position and not the number of the electron donating groups.

Table 1 The inhibitory effects of compounds **(4a-4j)** on mushroom tyrosinase

Compound	**Tyrosinase inhibition activity IC_{50} ± SEM (µM)**
4a	19.658 ± 1.25
4b	10.079 ± 0.87
4c	16.599 ± 0.98
4d	7.986 ± 0.21
4e	6.461 ± 0.05
4f	1.568 ± 0.01
4g	7.750 ± 0.87
4h	19.537 ± 1.25
4i	2.316 ± 0.08
4j	3.371 ± 0.05
Kojic acid	16.051 ± 1.27

SEM = Standard error of the mean; values are expressed in mean ± SEM.

2.3.1 Free radical scavenging

All of the synthesized compounds **(4a-4j)** were evaluated for DPPH free radical scavenging ability. The compound **4f** showed good 80% scavenging potency, other nine compounds did not show significant radical scavenging potential even at high concentration (100µg/mL) Figure 1

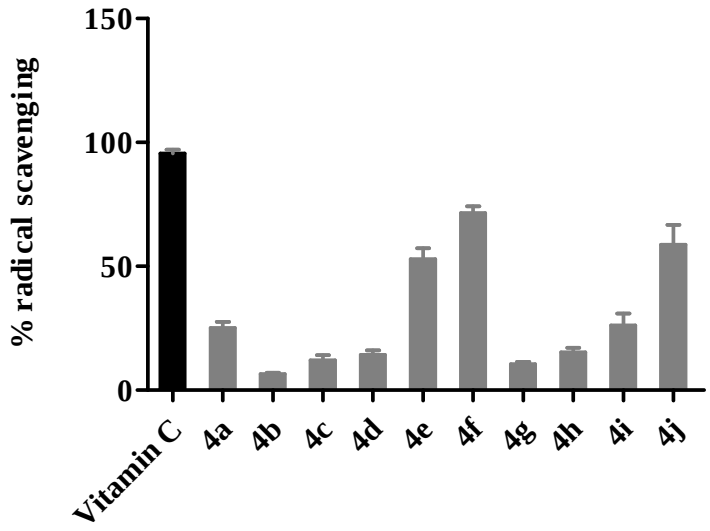


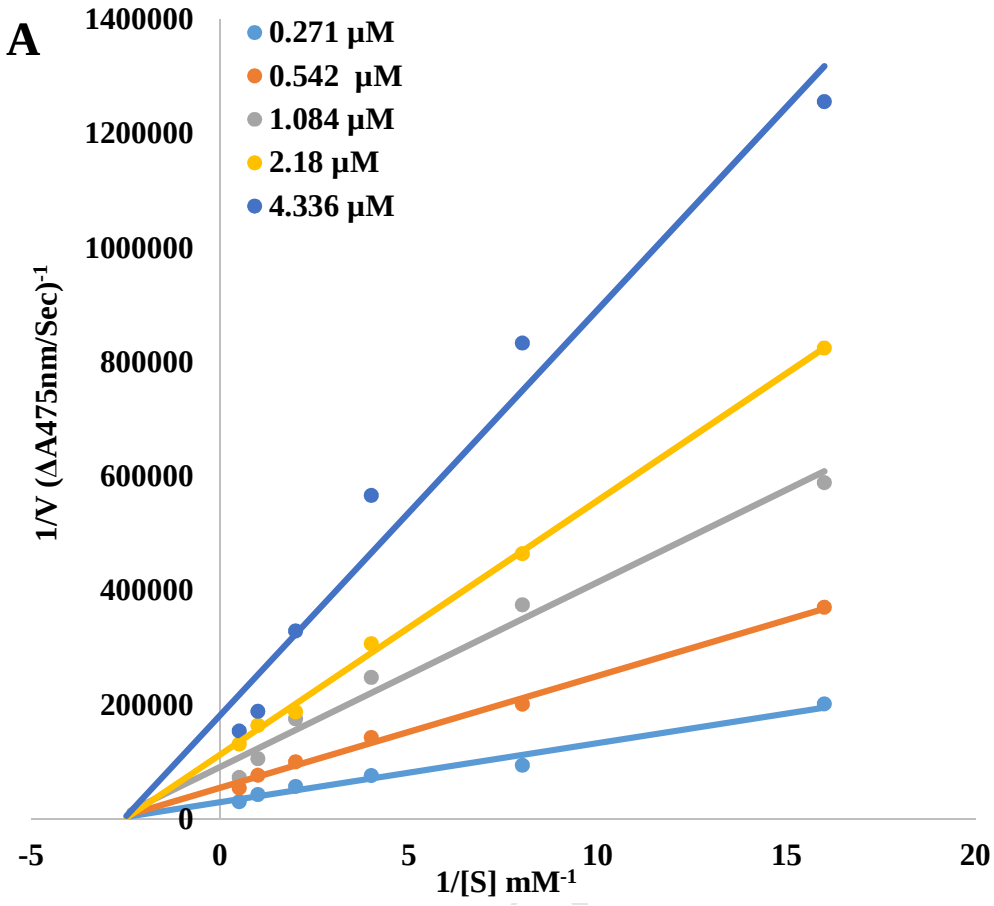
Figure.1 The % radical scavenging activity of synthetic inhibitors. The values are expressed in mean ± SEM. All inhibitors and Vitamin C concentration were 100µg/mL. SEM= standard error of mean

2.4 Kinetic Mechanism

To understand the inhibitory mechanism of synthetic compounds on mushroom tyrosinase inhibition kinetic study were performed. Based upon our IC_{50} results we select our most potent compound **4f** to determine their inhibition type and inhibition constant. The kinetic results of the enzyme by the Line weaver-Burk plot of 1/V versus 1/[S] in the presence of different inhibitor concentration gave a series of straight lines, the result of compound **4f** showed that all of which intersected at the same point on the x-axis (Figure 2A). The analysis showed that 1/Vmax increased to new value while that of a Km remains the same as a result increase in the concentration of compound **4f**. This behavior indicated that compound **4f** inhibits the tyrosinase non-competitively to form an enzyme inhibitor complex. Second plot (Figure 2B) of slope against concentration of **4f** showed EI dissociation constant.

Table 2. The kinetic parameters of the mushroom Tyrosinase for L-DOPA activity in the presence of various concentration of **4f** compound.

Compound	Concentration (µM)	1/Vmax (ΔA/Sec)	Km (mM)	Inhibition Type	*Ki* (µM)
4f	0.271	28571.428	0.389		
	0.542	52380.952	0.389		
	1.084	90476.190	0.389	Non-competitive	1.10
	2.18	109523.809	0.389		
	4.336	185714.285	0.389		


A
0.271 µM
0.542 µM
1.084 µM
2.18 µM
4.336 µM
1/V (ΔA475nm/Sec)-1
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1400000
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1000000
800000
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-5
0
5
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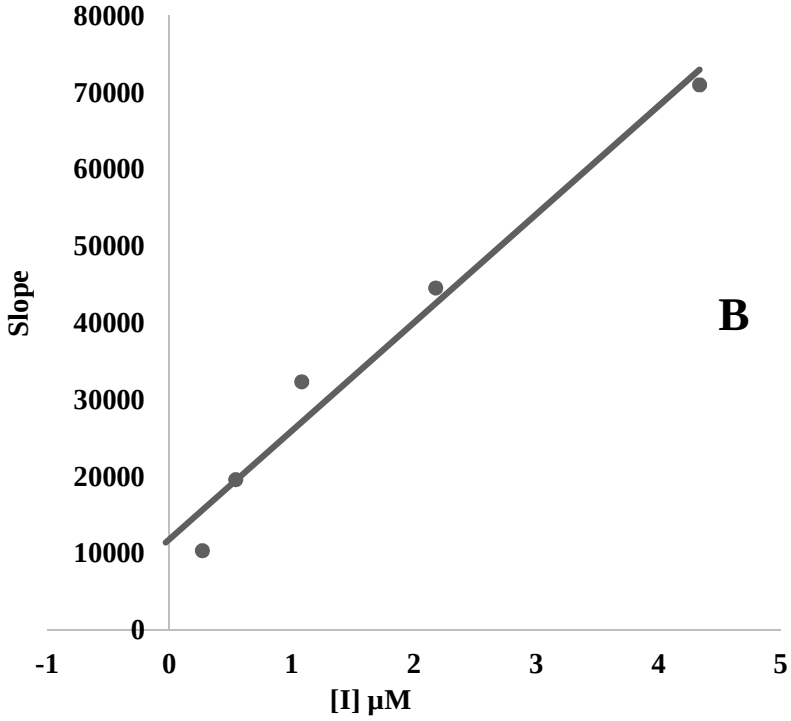
15
20


B
Slope
[I] µM
80000
70000
60000
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40000
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20000
10000
0
-1
0
1
2
3
4
5

Figure 2. Lineweaver-Burk plot for inhibition of tyrosinase in the presence of various concentrations of compound **4f. (A).** The insets represent the plot of the slope versus inhibitor **4f** to determine the inhibition constant (**B**). The lines were drawn using linear least square fit.

2.5 Structure Activity relationship

Structure Activity Relationships (SAR) are relations between the molecular structure and biological or physicochemical activity of chemicals. The SAR allows to design and modify the structure in order to obtain effective drugs. A series of 1-pentanoyl-3-aryl thioureas was designed and synthesized as potent inhibitors of tyrosinase enzyme. The pharmacophore of thiourea comprised of three parts (Figure 3).

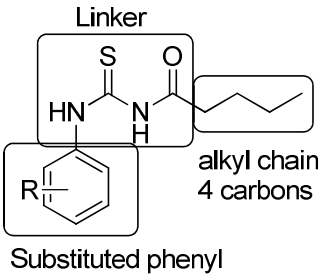

Figure 3. Design of novel 1-pentanoyl-3-arylthioureas (**4a-4j**)

The results inhibitory effects of compounds revealed that the compound **4f** was found to most potent in the series and exhibited approximately 15 time higher inhibition compared to standard Kojic acid. In compound **4f,** the methoxy group at para-position could facilitate to occupy the whole pocket and lead to stronger hydrophobic interactions (figure 4). The compound **4a** bears three methyl groups which creates crowding/steric hindrance and was found to be least potent inhibitor in the series.

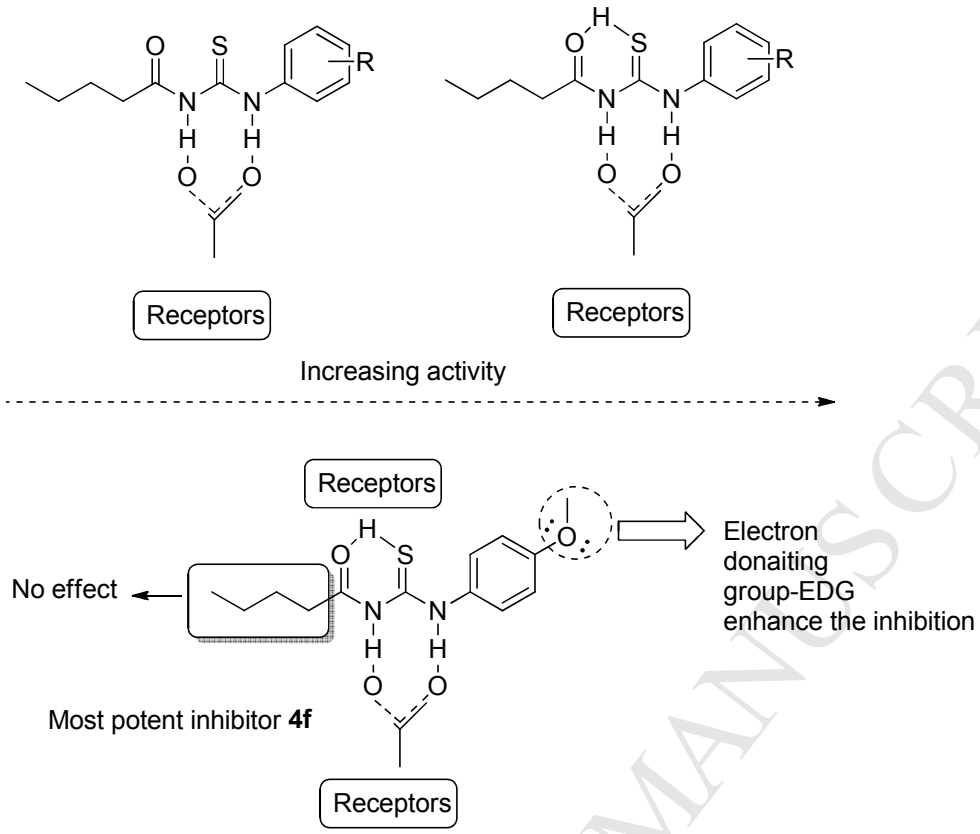

Figure 4. Structure activity relationship and the plausible binding sites

3. Docking studies

3.1 Structural assessment of target protein

Mushroom tyrosinase (*Agaricus bisporus*) is a class of oxidoreductase copper containing protein comprises 391 residues. The structural architecture of mushroom tyrosinase showed that, it consists of 39% helices (154 residues) and 14% β sheets (57 residues) and 46% coils (180 residues). The X-Ray diffraction study confirmed its resolution 2.78Å, R-value 0.238 and unit cell crystal dimensions like length and angles of coordinates. The unit cell length values were observed for a=103.84, b=104.82 and c=119.36 with angles 90°, 110.45° and 90° for all α, β and γ dimensions respectively. The Ramachandran plots and values indicated that 95.90% of residues were in favored regions and 100.0% residues were lies in allowed regions (Figure. S14).

3.2 Chemo-informatics properties and Lipinski's rule of synthesized compounds (4a-4j)

The predicted properties such as molar volume and refractivity, density, polarizability and surface tension were evaluated by computational approach. Literature study showed a standard

value for molar refractivity (40 to 130), molecular weight (160 to 480) and number of atoms (20 to 70) [25]. Results showed that **4a-4j** predicted values are better than standard values. Moreover, the Lipinski's rule of five (RO5) results showed that compounds (**4a-4j**) possess HBA, HBD and log*P* values which are significantly justified its drug like behavior. Moreover, their molecular weight of **4f** (266.11 g/mol) was also much better than standard value (<5000g/mol). The RO5 justifies that molecules with poor absorption are more likely to have- more than 5 HBD, MWT over 500, log*P* over 5 and more than 10 HBA. However, there are plenty of examples available for RO5 violation amongst the existing drugs [26]. In overall results, these predicted values justify the significance of the **4f** synthesized compound as good candidate molecule (Table. 2).

3.3 Molecular docking analyses

The docked complexes of all the synthesized compounds (**4a-4j**) were analyzed on the basis of lowest binding energy values and hydrogen bonding analyses. Results showed that **4f** was the most active compound with best binding energy value (-7.50 kcal/mol) compared to others derivatives. The graphical depiction of 4f docking complex is mentioned in Figure 5.

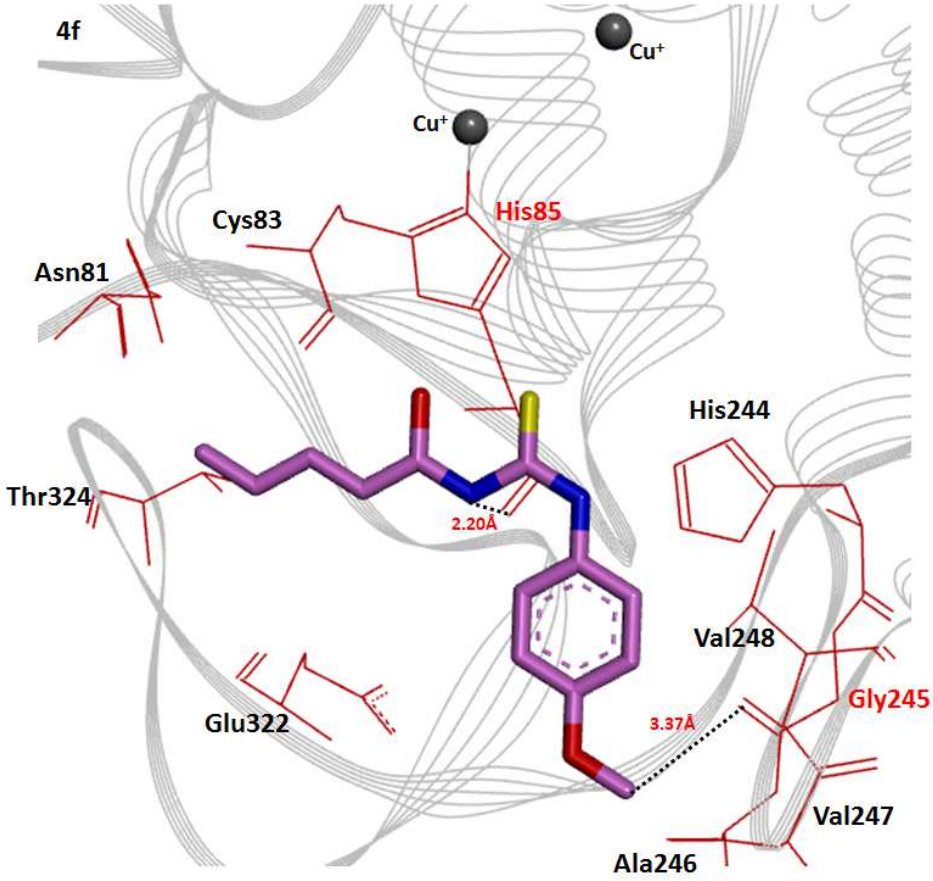

Figure 5. Docking interaction between **4f** and mushroom tyrosinase. The **4f** is mentioned in purple color with nitrogen, oxygen and sulphur functional groups in blue, red and yellow colors. The target protein is highlighted in line ribbon format with light gray color. The active binding site amino acids are highlighted in red color. Two copper ions are also mentioned in black color. Two hydrogen bonds were observed between **4f** and receptor amino acids like His85 and Gly245 with bonding distances 2.20Å and 3.37Å, respectively. The black dotted lines show the binding distance in angstrom (Å).

The bonding analysis of all compounds against mushroom tyrosinase showed that **4f** compound is directly interacts with active residues of targeted protein. The structure activity relationship analysis shows that **4f** builds two hydrogen bonds at specific residues His85 and Gly245 against target protein. The carbonyl nitrogen of benzene ring of **4f** interacts with His85, while methyl group of benzene ring interacts with Gly245 having bonds lengths 2.20Å and 3.37Å,

respectively. Literature study also justified that these interacted residues are significant in the downstream signaling pathways [27]. The graphical representations of all other interacted compounds (**4a**-**4e** and **4g**-**4j**) against the target protein are mentioned in (Supplementary Figure. **S15**-**S24**). Moreover, comparative energy binding analysis showed that **4f** has good energy values (-7.50 kcal/mol) as compared other derivatives. The other candidate compounds (**4a**-**4e** and **4g**-**4j**) also possess good binding energy values -6.70, -6.60, -7.30, -7.60, -6.60 and -7.00, -6.70, -6.70, -6.60 Kcal/mol binding energy values respectively (Figure S25). Here, it is noticeable that most of candidates compounds bind in the active binding regions but little fluctuated with their conformational positions. The computational and bioassay studies showed that **4f** is the most active and significant compound as compared to other synthesized compounds.

Literature study showed that different mushroom tyrosinase inhibitors can be further employed for human tyrosinase inhibitions [28]. The computational and assay based approach depicts the significance of these synthesized ligands in tyrosinase inhibition activity.

4. Conclusion

The 1,3-disubstitued thioureas (**4a-4j**) having aryl and alkyl are synthesized. The molecular docking investigation revealed that thiourea derivative (**4f**) possesses maximum binding affinity (-7.50 kcal/mol) with the target protein (PDBID 2ZMX). The wet lab results are in good agreement with the calculated docking scores. The most potent activity against mushroom tyrosinase was exhibited by the compound (**4f**) having IC_{50} 15.2 mM. The presence of the methoxy group at para-position on phenyl ring which play very significant role in the tyrosinase inhibition activity. The kinetic analysis of compound (**4f**) shown that it is non-competitive type inhibitor with Ki value 1.10 mM and formed an irreversible drug receptor complex with

mushroom tyrosinase. It was concluded from our results that the title compound (**4f**) may serve as lead structure for the design of more potent tyrosinase inhibitors.

5. Experimental part

5.1 General methods and materials

Melting points were recorded using a digital Gallenkamp (Tokyo, Japan) model MPD.BM 3.5 apparatus and are uncorrected. ^{1}H NMR spectra were determined as $CDCl_3$ solutions at 300 MHz using a Bruker AM-300 spectrophotometer using TMS as an internal reference and ^{13}C NMR spectra were determined at 75 MHz using a Bruker 75 MHz NMR spectrometer in DMSO solution. FTIR spectra were recorded on an FTS 3000 MX spectrophotometer. Mass Spectra (EI, 70 eV) on a MAT 312 instrument, and elemental analyses were conducted using a LECO-183 CHNS analyzer.

5.2 General method for the synthesis of 1-pentanoyl-3-arylthiourea (4a-j).

All chemicals, organic solvents and reagents were purchased from Sigma-Aldrich, Fluka and E. Merck. Organic solvents, acetone (99.9 % pure), and *n*-hexane (95 % pure) were distilled purified & dried according to reported methods. Potassium thiocyanate (98% pure), pentanoic acid (99 % pure), 4-nitroaniline (99 % pure), 4-chloro-3-nitroaniline (99 % pure), 2-methyl-4-nitroaniline (99 % pure) and 2-methoxy-4-nitroaniline (99 % pure) were used without further purification.

5.2.1 General procedure for the synthesis of title compounds

A freshly prepared solution of pentanoyl chloride was synthesized from pentanoic acid by treating the reaction mixture with thionylchloride.The acid chloride synthesized (10 mmol) in anhydrous acetone (50 ml) was added dropwise to a suspension of potassium thiocyanate (10

mmol) in acetone (30 ml) at room temperature. The reaction mixture was refluxed and cooled to room temperature. A solution of the substituted anilines (10 mmol) in anhydrous acetone (20 ml) was added dropwise and the reaction mixture was refluxed further for 2-3 h. On completion of reaction (TLC) the mixture was poured into cold water and the precipitated thioureas were filtered and recrystallized from methanol.

5.3 Experimental Data

5.3.1 1-Pentanoyl-3-(2,4,6-trimethylphenyl)thiourea (4a)

White crystalline solid, Yield=90%, M.P=199 ^{o}C, R_f=0.52 (n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3184.2 (N-H, stretching), 2982.5 (Aromatic C-H, stretching), 1679.2 (C=O, stretching), 1573 (N-O, bending), 1509.5 (C-C, stretching), 1411.8 (C-H, bending), 1332.89 (N-O, bending), 1255.4 (C=S, stretching). ^{1}H NMR (DMSO-d_6); δ 11.77 (s, 1H, NH), 11.56 (s, 1H, NH), 6.94 (2H Ar-H), 2.24 (s, 6H Ar 2x CH_3),2.10 (s, 3H Ar-CH_3); 2.3 (t, 2H, *J*=2.4), 2.0 (2H,quintet, *J*= 1.8), 1.9 (sex,2H, *J*=2.1),1.4 (t, 3H, *J*= 2.5); ^{13}C NMR: δ 180 (C=S), 172.9 (C=O), 136.9, 135.0, 133.8, 128.9 (Ar-C) ,28(CH_3, Ar), 25(CH_3, Ar), 22 (CH_3-Ar), 40, 24, 20, 17 (CH_3) (Anal.Calcd. for $C_{14}H_{20}N_2OS$: C, 62.99; H, 7.82; N, 12.85; S, 13.57 found: C, 63.97; H, 7.84; N, 12.84; S, 13.58.

5.3.2 1-Pentanoyl-3-(2,4-dinitrophenyl)thiourea (4b)

Yellow crystalline solid , Yield=89%, M.P=255 ^{o}C, R_f=0.68 (n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3185.3 (N-H, stretching), 2996.4 (Aromatic C-H, stretching), 1685.3 (C=O, stretching), 1575 (N-O, bending), 1509.7 (C-C, stretching), 1417.3 (C-H, bending), 1345.97 (N-O, bending), 1259.3 (C=S, stretching); ^{1}H NMR (DMSO-d_6); δ 11.61 (s, 1H, NH), 11.41 (s, 1H, NH), 7.35-6.9 (m, 3H Ar-H),2.4 (t, 2H, *J*=2.8), 1.8 (quintet,2H,*J*=2.1), 1.3 (sex, 2H, *J*=1.8), 0.9 (t,3H, *J*=2.2);

^{13}C NMR: δ 182.3 (C=S), 175.6 (C=O), 140.9, 137.1, 135.1, 131.5, 127.7, 125.5 (Ar-C), 37, 24, 22,17 (CH_3), Anal.Calcd. for $C_{14}H_{18}N_4O_5S$:C, 47.15; H, 4.17; N, 15.62 S, 9.04 found: C, 48.17; H, 5.78; N, 16.65 S, 10.05

5.3.3 1-Pentanoyl-3-(3-nitrophenyl)thiourea (4c)

Yellow crystalline solid, Yield=98%, M.P=215 ^{o}C, R_f=0.61 (n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3199.1 (N-H, stretching), 2984.1 (Aromatic C-H, stretching), 1685.8 (C=O, stretching), 1589 (N-O, bending), 1509.8 (C-C, stretching), 1419.7 (C-H, bending), 1337.87 (N-O, bending), 1299.2 (C=S, stretching). ^{1}H NMR (DMSO-d_6); δ 12.80 (s, 1H, NH), 11.88 (s, 1H, NH), 7.89-7.32 (m, 4H, ArH),2.4 (t, 2H, *J*=2.5), 2.0 (2H,quintet, *J*=2.3),1.8 (sex,2H, *J*=2.1),1.5 (t,3H, *J*=1.8) ;^{13}C NMR: δ 189.1 (C=S), 170.5 (C=O), 146.4, 139.2, 136.7, 132.2, 129.2, 127.6 (Ar-C), 40.5, 24.5, 20.4,16.4, (CH_3), Anal.Calcd. for $C_{11}H_{13}N_3O_3S$: C, 49.17; H, 4.76; N, 15.59; S, 12.41 found: C, 50.19; H, 5.77; N, 16.58; S, 13.43

5.3.4 1-Pentanoyl-3-(4-nitrophenyl)thiourea (4d)

White crystalline solid , Yield=90%, M.P=199.5 ^{o}C, R_f=0.71 (n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3185.2 (N-H, stretching), 2998.3 (Aromatic C-H, stretching), 1691.2 (C=O, stretching), 1581 (N-O, bending), 1507.7 (C-C, stretching), 1415.8 (C-H, bending), 1337.51 (N-O, bending), 1297.2 (C=S, stretching). ^{1}H NMR (DMSO-d_6); δ 11.78 (s, 1H, NH), 11.59 (s, 1H, NH), 7.75-6.92 (m, 4H, ArH), 2.4 (t, 2H, *J*=2.8), 2.0 (2H,quintet, *J*=2.4),1.8 (sex,2H, *J*=1.9),1.5 (t,3H, *J*=1.5) ^{13}C NMR: δ 183 (C=S), 173.5 (C=O), 137.1, 135.5, 129.7, 128.2, 126.2 (Ar-C), 40.3,

23.4, 20.2, 18.8; Anal.Calcd. forC11H13ClN$_2$OS: C, 51.71; H, 5.45; N, 10.17; O, 7.08; S, 12.04 found: C, 52.30; H, 6.55; N, 11.27; S, 13.08.

5.3.5 1-Pentanoyl-3-(4-bromo-2-fluorophenyl)thiourea (4e)

Yellow crystalline solid , Yield=89%, M.P=205 ^{o}C, R_f=0.68 (n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3185.3 (N-H, stretching), 2996.4 (Aromatic C-H, stretching), 1685.3 (C=O, stretching), 1575 (N-O, bending), 1509.7 (C-C, stretching), 1417.3 (C-H, bending), 1345.97 (N-O, bending), 1259.3 (C=S, stretching). ^{1}H NMR (DMSO-d_6); δ 11.61 (s, 1H, NH), 11.41 (s, 1H, NH), 7.35-6.9 (m, 3H Ar-H),2.4 (t, 2H, *J*=2.4), 2.0 (2H,quintet, *J*=2.1),1.8 (sex,2H, *J*=1.8),1.5 (t,3H, *J*=1.4) ; ^{13}C NMR: δ 182 (C=S), 175.6 (C=O), 140.9, 137.1, 135.1, 131.5, 127.7, 125.5 (Ar-C), 40.2 22.3 18.5, 14.4 Anal.Calcd. for $C_{11}H_{12}BrFN_2OS$:C, 41.15; H, 3.77; N, 9.62 S, 11.04 found: C, 42.17; H, 4.78; N, 10.65, S, 12.05

5.3.6 1-Pentanoyl-3- (4-methoxyphenyl)thiourea (4f)

Brown crystalline solid , Yield=89%, M.P=180 ^{o}C, R_f=0.80(n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3179.5 (N-H, stretching), 2985.5 (Aromatic C-H, stretching), 1675.5 (C=O, stretching), 1569 (N-O, bending), 1504.2 (C-C, stretching), 1416.2 (C-H, bending), 1337.61 (N-O, bending), 1292.8 (C=S, stretching). ^{1}H NMR (DMSO-d_6); δ 11.99 (s, 1H, NH), 11.77 (s, 1H, NH), 7.12 (d, 2H, *J*= 7.6 Hz ArH), 6.83(d,2H, *J*= 7.6 Hz ArH), 3.88 (s,3H),2.4 (t, 2H, *J*=2.6), 2.0 (2H,quintet, *J*=2.4),1.8 (sex,2H, *J*=2.1),1.5 (t,3H, *J*=1.8) ;^{13}C NMR: δ 181.3 (C=S), 169.2 (C=O), 131.1, 130.1, 127.2, 124.8 (Ar-C), 40.5, 23.7, 18.9, 12.5 Anal.Calcd. for $C_{12}H_{16}N_2O_2S$: C, 57.58; H, 6.41; N, 11.51; S, 12.31 found: C, 58.53; H, 7.45; N, 12.52; S, 13.33.

5.3.7 1-Pentanoyl-3-(2,3-dichlorophenyl)thiourea (4g)

White crystalline solid , Yield=90%, M.P=190 ^{o}C, R_f=0.53(n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3189.1 (N-H, stretching), 2994.1 (Aromatic C-H, stretching), 1695.3 (C=O, stretching), 1586 (N-O, bending), 1508.4 (C-C, stretching), 1416.5 (C-H, bending), 1336.57 (N-O, bending), 1298.5 (C=S, stretching). ^{1}H NMR (DMSO-d_6); δ 11.80 (s, 1H, NH), 11.66 (s, 1H, NH), 7.71-6.88 (m, 3H, ArH), 2.4 (t, 2H, *J*=2.6),2.0 (2H,quintet, *J*=2.3), 1.8 (sex,2H, *J*=1.8),1.5 (t,3H, *J*=1.5) ;^{13}C NMR: δ 181.3 (C=S), 171.7 (C=O), 137.4, 136.2, 131.7, 129.2, 127.2 (Ar-C),40.8, 21.7, 18.7, 14.7, Anal.Calcd. for $C_{11}H_{12}Cl_2N_2OS$: C, 45.03; H, 4.15; N, 9.75; S, 11.15 found: C, 46.09; H, 5.05; N, 10.75; S, 12.17.

5.3.8 1-Pentanoyl-3-(2,6-dibromo-4-fluorophenyl)thiourea (4h)

Brown solid, Yield=95%, M.P=209 ^{o}C, R_f=0.55 (n-Hexane: Ethyl acetate 1:1) FTIR *υ* (cm^{-1}) 3189.5 (N-H, stretching), 2991.8 (Aromatic C-H, stretching), 1689.8 (C=O, stretching), 1580 (N-O, bending), 1509.3 (C-C, stretching), 1414.3 (C-H, bending), 1382.19 (N-O, bending), 1257.8 (C=S, stretching). ^{1}H NMR (DMSO-d_6); *δ* 11.76 (s, 1H, NH), 11.55 (s, 1H, NH), 7.50 (2H Ar-H),2.4 (t, 2H, *J*=2.7),2.0 (2H,quintet, *J*=2.5), 1.8 (sex,2H, *J*=2.2),1.5 (t,3H, *J*=1.4); ^{13}C NMR: *δ* (ppm) 185 (C=S), 178.9(C=O), 139.9,136.0, 134.8, 130.9 (Ar-C),40, 23, 20, 15 (CH_3), Anal.Calcd. for $C_{11}H_{11}Br_2FN_2OS$:C, 29.24; H, 1.92; N, 7.57; S, 8.67 found: C, 30.26; H, 2.33; N, 8.59; S, 9.69

5.3.9 1-Pentanoyl-3-(4-chlorophenyl)thiourea (4i)

Light Yellow solid , Yield=95%, M.P=188 ^{o}C, R_f=0.58 (n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3186.2 (N-H, stretching), 2994.3 (Aromatic C-H, stretching), 1687.2 (C=O, stretching), 1571 (N-O, bending), 1507.1 (C-C, stretching), 1415.3 (C-H, bending), 1337.91 (N-O, bending), 1295.3 (C=S, stretching). ^{1}H NMR (DMSO-d_6); δ 11.77 (s, 1H, NH), 11.55 (s, 1H, NH), 7.73 (d, 2H, *J*= 7.8 Hz ArH), 7.47(d,2H, *J*= 7.8 Hz ArH);2.3 (t, 2H, *J*=2.8),2.0 (2H,quintet, *J*=2.4), 1.9 (sex,2H, *J*=2.1),1.4 (t,3H, *J*=2.2); ^{13}C NMR: δ 187 (C=S), 170.1 (C=O), 134.1, 132.5, 128.7, 126.5 (Ar-C),40.4 24.5, 21.8, 18.5, Anal.Calcd. for $C_{11}H_{13}ClN_2OS$:C, 51.25; H, 5.98; N, 12.25; S, 14.02 found: C, 52.28; H, 6.95; N, 13.23; S, 15.06.

5.3.10 1-Pentanoyl-3-(4-bromophenyl)thiourea (4j)

White crystalline solid , Yield=95%, M.P=183 ^{o}C, R_f=0.47(n-Hexane: Ethyl acetate 1:1) FTIR υ (cm^{-1}) 3189.5 (N-H, stretching), 2995.5 (Aromatic C-H, stretching), 1685.5 (C=O, stretching), 1579 (N-O, bending), 1509.8 (C-C, stretching), 1419.5 (C-H, bending), 1338.93 (N-O, bending), 1296.5 (C=S, stretching). ^{1}H NMR (DMSO-d_6); δ 11.79 (s, 1H, NH), 11.65 (s, 1H, NH), 7.66 (d, 2H, *J*= 7.7 Hz ArH), 7.43(d,2H, *J*= 7.7 Hz ArH)2.4 (t, 2H, *J*=2.7), 2.0 (2H,quintet, *J*=2.4),1.8 (sex,2H, *J*=1.9),1.5 (t,3H, *J*=1.4) ;^{13}C NMR: δ 182 (C=S), 169.2 (C=O), 132.1, 131.1, 128.2, 125.8 (Ar-C),40, 25, 20, 18, Anal.Calcd. for $C_{11}H_{13}BrN_2OS$:C, 43.55; H, 4.38; N, 9.26; S, 10.71 found: C, 43.57; H, 4.39; N, 9.29; S, 10.75.

5.4 Mushroom tyrosinase inhibition assay

The mushroom tyrosinase (Sigma Chemical, USA) inhibition was performed following our previously reported methods [9,10]. In detail, 140 µL of phosphate buffer (20 mM, pH 6.8), 20

µL of mushroom tyrosinase (30 U/mL) and 20 µL of the inhibitor solution were placed in the wells of a 96-well micro plate. After pre-incubation for 10 min at room temperature, 20 µL of L-DOPA (3,4-dihydroxyphenylalanine, Sigma Chemical, USA) (0.85 mM) was added and the assay plate was further incubated at 25 °C for 20 min. Afterward the absorbance of dopachrome was measured at 475 nm using a micro plate reader (OPTI Max, Tunable). Kojic acid was used as a reference inhibitor and phosphate buffer was used as a negative control. The amount of inhibition by the test compounds was expressed as the percentage of concentration necessary to achieve 50 % inhibition (IC_{50}). Each concentration was analyzed in three independent experiments. The IC_{50} values were determined by the data analysis and graphing software Origin 8.6, 64-bit.

The % of Inhibition of tyrosinase was calculated as following

Inhibition (%) = [(B-S)/B] ×100

Here, the B and S are the absorbance's for the blank and samples.

5.5 Kinetic analysis of the inhibition of tyrosinase

On the basis of IC_{50} we select most potent **4f** compound for kinetic analysis. A series of experiments were performed to determine the inhibition kinetics of **4f** by following the already reported method. [9,10]. The inhibitor concentrations for **4f** are 0.271, 0.542, 1.084, 2.18 and 4.336 µM. Substrate L-DOPA concentration was between 0.0625 to 2 mM in all kinetic studies. Pre-incubation and measurement time was the same as discussed in mushroom tyrosinase inhibition assay protocol. Maximal initial velocity was determined from the initial linear portion of absorbance up to five minutes after addition of enzyme at a 30s interval. The inhibition type on the enzyme was assayed by Lineweaver–Burk plots of inverse of velocities (1/*V*) versus the inverse of substrate concentration 1/[S] mM^{-1}. The EI dissociation constant *Ki* was determined by secondary plot of 1/*V* versus inhibitor's concentrations.

5.5 Free radical scavenging assay

Radical scavenging activity was determined by modifying already reported method [9,10] by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The assay solution consisted of 100 µL of DPPH

(150 µM), 20 µL of increasing concentration of test compounds and the volume was adjusted to 200 µL in each well with DMSO. The reaction mixture was then incubated for 30 minutes at room temperature. Ascorbic acid (Vitamin C) was used as a reference inhibitor. The assay measurements were carried out by using a micro plate reader (OPTI $_{Max}$, Tunable) at 520 nm. The reaction rates were compared and the percent inhibition caused by the presence of the tested inhibitors was calculated. Each concentration was analyzed in three independent experiments run in triplicate.

5.6 Molecular docking

5.7 Retrieval of receptor protein from PDB

The crystal structure of mushroom tyrosinase (PDBID: 2Y9X) was retrieved from the Protein Data Bank (PDB) (http://www.rcsb.org). The energy minimization of selected protein was done by the conjugate gradient algorithm and Amber force field in UCSF Chimera 1.10.1 [25]. The stereo-chemical properties, Ramachandran graph and values [29] of mushroom tyrosinase were assessed by Molprobity server [30], while the hydrophobicity graph was generated by Discovery Studio 4.1 Client [31]. The protein architecture and statistical percentage values of helices, beta-sheets, coils and turns were accessed by VADAR 1.8 [32].

5.8 Candidate structures

The synthesized candidate molecules (**4a-4j**) were sketched in drawing ACD/ChemSketch tool. The designed ligand molecules were further visualized and minimized by UCSF Chimera 1.10.1 in PDB format. The drug assessment properties of these compounds were accessed by various computational tools like Molinspiration (http://www.molinspiration.com/) and Molsoft (http://www.molsoft.com/). Lipinski's rule of five was justified by using Molsoft and Molinspiraion tools. Furthermore, different molecular properties such as molar refractivity, density, surface tension and polarizability were also accessed by chemsketch.

5.9 Molecular Docking

The molecular docking experiment was employed on all synthesized ligand molecules (**4a-4j**) against mushroom tyrosinase using diverse PyRx tool [33]. The grid box parametric dimension values (X=61.0781, Y=56.3001 and Z=63.1015) with spacing 1.0Å were adjusted to attain the

finest binding conformational pose of protein-ligand molecules. The maximum docking poses (100 numbers of run) for each docking were adjusted. All compounds were docked separately against the crystal structure of mushroom tyrosinase and the obtained docked complexes were further evaluated on lowest binding energy (Kcal/mol) value. The binding analysis and graphical depiction was done by Discovery Studio (4.1).

6. X-ray Crystallography studies

Figure 6 shows the molecular structure of (**4a**) and Table S1 as supplementary material summarizes the main crystallographic parameters. The molecular conformation is stabilized by an Intramolecular N-H…O hydrogen bond. The crystal packing shows centosymmetric dimers connected by N-H…S hydrogen bonds, which are packing in stacks parallel to the a axis.

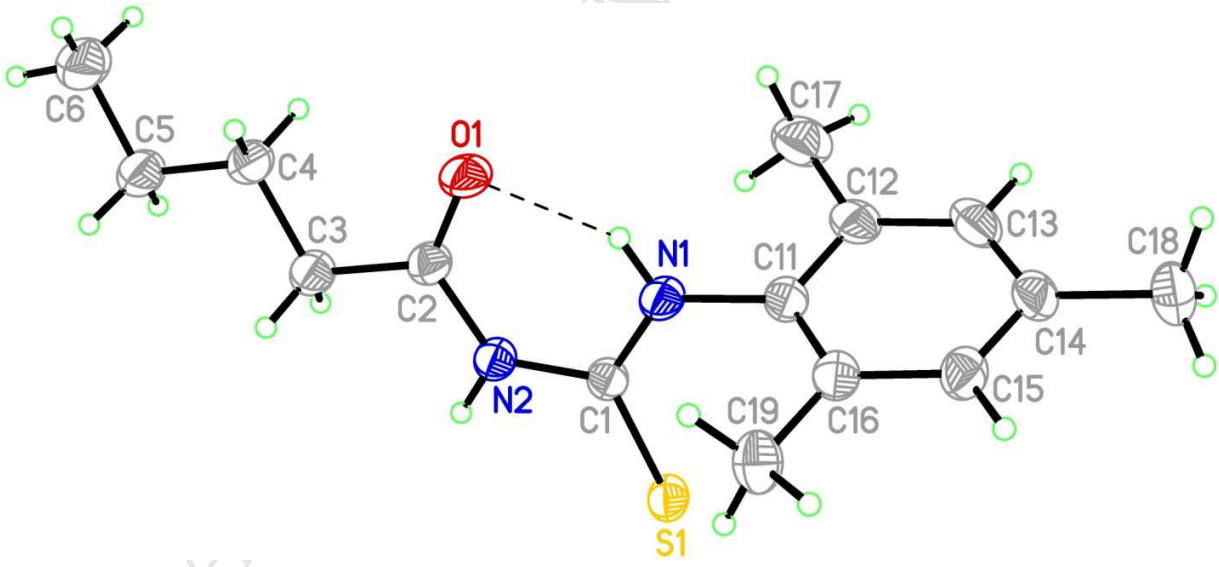

Figure 6. Crystal structure of **4a**

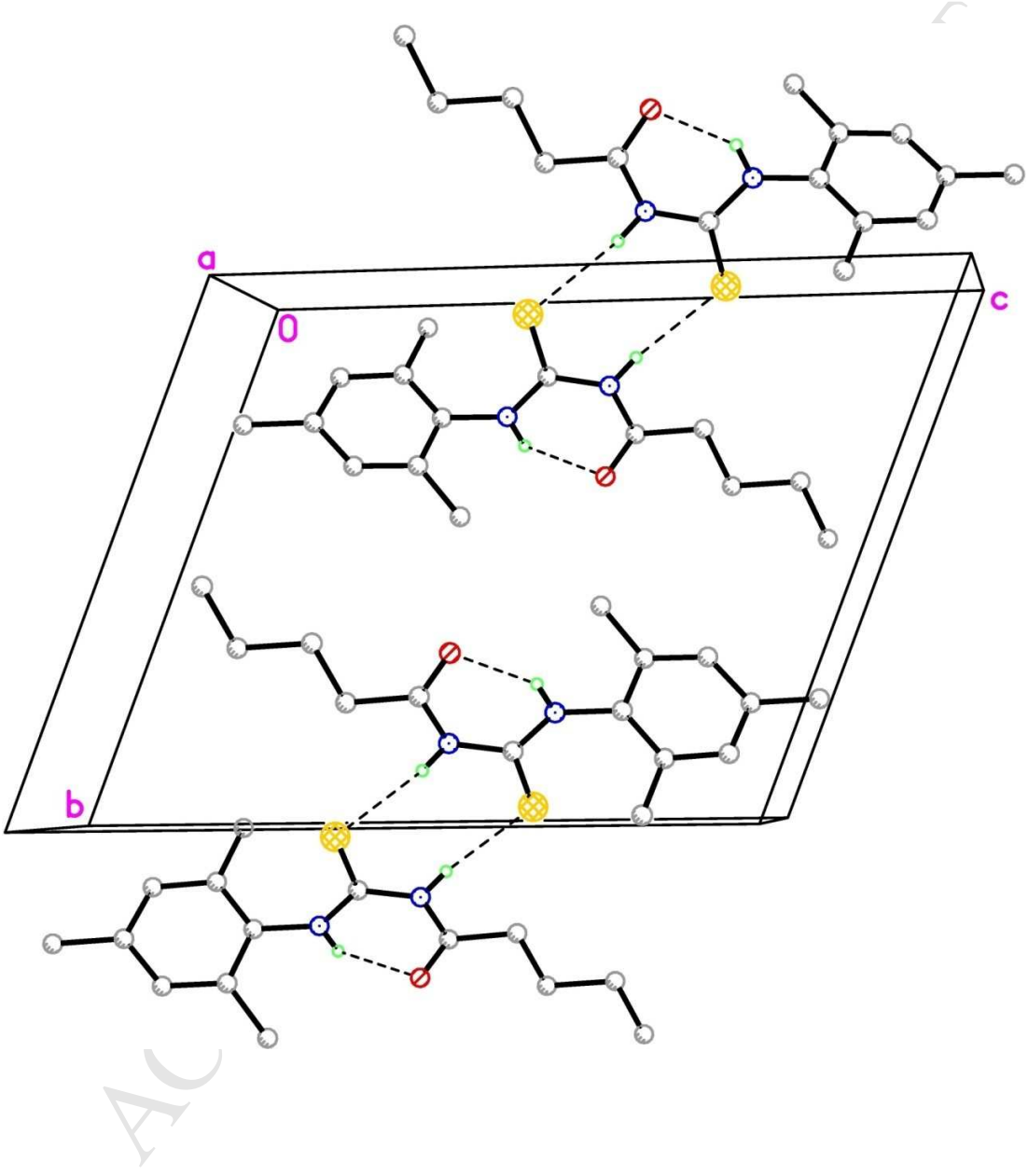

Figure 7. Crystal Packing diagram for **4a** viewed along a-axis

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Conflict of Interest

The authors declare no any conflict of interest

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Highlights

- A small library of novel 1-Pentanoyl-3-arylthioureas (**4a-4j**) synthesized.
- Mushroom tyrosinase inhibition and free radical scavenging activity were evaluated
- Most of the compounds show excellent activity, particularly **4f** higher than the standard.
- The kinetic mechanism proposed **4f** is non-competitive inhibitor of mushroom tyrosinase.
- Molecular docking, druglikeness, Ramachandran graph, Chemo-informatics and Lipinski's rule were studied.