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Research Article

Synthesis, spectral characterization and bioactivity studies of some *S*-substituted derivatives of 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol

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ABSTRACT

A new series of 5-(4-Chlorophenyl)-1,3,4-Oxadiazol-2-thiol derivatives was prepared from 4-chlorobenzoic acid (**1**) by converting it successively into corresponding ester (**2**), carbohydrazide (**3**) and 5-(4-Chlorophenyl)-1,3,4-Oxadiazol-2-thiol (**4**). Finally the target compounds, **6a-l**, were synthesized by stirring **4** with different electrophiles, **5a-l**, in DMF using NaH as weak base and activator. The proposed structures of newly synthesized compounds were confirmed by spectroscopic techniques such as ^{1}H-NMR, ^{13}C-NMR, HR-MS and EI-MS. All synthesized compounds were evaluated for their anti-bacterial, antifungal, cytotoxicity and enzyme inhibition activities. The compounds, **6e** and **6g** exhibited significant inhibition activity against acetyl cholinesterase enzyme (AChE) and **6j** moderate activity against butyryl cholinesterase enzyme (BChE). The molecule, **4** exhibited good MIC (minimum inhibitory concentration) value against all the bacterial and fungal strains taken into account.

Key Words: 1,3,4-Oxadiazole, 4-Chlorobenzoic acid, Antimicrobial activity, Cytotoxicity, Enzyme inhibition

INTRODUCTION

1,3,4-Oxadiazoles belong to an important class of five membered heterocyclic ring and are present in a number of biologically active molecules [1]. They undergo a variety of organic reactions such as electrophilic substitution, nucleophilic substitution, thermal and photochemical reactions; and hence medicinal backbone on which a number of valuable molecules have been constructed. This class possesses a wide spectrum of biological activities such as antimicrobial [2], anticancer [3], anticonvulsive [4], anti-inflammatory [5, 6], antitubercular [7], cytotoxic [8], fungicidal [9], hypoglycemic [10], insecticidal [11] and anti-hypertensive agents [12]. Generally acyl hydrazides are used as starting materials for the synthesis of 1,3,4-Oxadiazoles [13].

Cholinesterases are well-known enzymes, present in cholinergic and non-cholinergic tissues as well as in the plasma and other body fluids of animals [14]. These enzymes are divided into two classes on the basis of their specificity for different substrate molecules and inhibitors, acetyl cholinesterase (AChE) or "true cholinesterase" and butyryl cholinesterase (BChE) or pseudo cholinesterase. AChE is commonly found in brain, muscles and erythrocyte membrane whereas BChE is present in liver, intestine, heart, kidneys and lungs [15]. The function of AChE is the hydrolysis of the neurotransmitter acetylcholine at cholinergic synapses [16] and BChE hydrolyzes ester-containing drugs and scavenges cholinesterase inhibitors including potent organo-phosphorus nerve agents before reaching the synaptic targets [17]. It has been reported that the acetylcholine deficiency is associated with Alzheimer's disease [18] which involves selective loss of cholinergic neurons in the central nervous system. At present, one of the major therapeutic strategies to treat Alzheimer's disease is to inhibit the biological activity of AChE. Organophosphates and carbamates are among the most commonly used

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AChE inhibitors and are also employed as insecticides [19].

Hence, it is important to synthesize such pharmacological inhibitors of cholinestrases which are important to control such type of diseases which involve impaired acetylcholine mediated neurotransmission. In protraction of our previous work [20, 21], an attempt has been made to synthesize titled compounds and screen them for their antimicrobial and enzyme inhibition potential.

MATERIAL AND METHODS

General: All the chemical reagents were purchased from Alfa Aesar, Merck and Sigma Aldrich through local suppliers. The solvents were of analytical grade and used without further purification. The reaction completion and purity were confirmed by TLC performed on aluminum plates coated with silica gel G-25-UV_{254}, run by EtOAc and *n*-hexane solvent system and visualized under UV at 254 nm. Melting points were recorded on Griffin-George melting point apparatus by using open capillary tubes and were uncorrected. ^{1}H-NMR spectra were recorded at 400 MHz by Bruker NMR spectrometers in $CDCl_3$, demonstrating chemical shifts in ppm values. ^{13}C-NMR spectrum was recorded at 100 MHz by Bruker NMR spectrometer in CD_3OD. EI-MS spectra were taken by using JMS-HX-110 spectrometer, with data system.

Procedure for the synthesis of ethyl 4-chlorobenzoate (2): 4-Chlorobenzoic acid (**1**; 5g, 0.032 mol) was taken in a 250 mL round bottom (RB) flask and dissolved in absolute C_2H_5OH (20 mL). 1.0 mL of concentrated H_2SO_4 was added gradually into the reaction mixture and set to reflux for 5 hours. The reaction was supervised by thin layer chromatography (TLC) using *n*-hexane and ethyl acetate as solvent system (80:20). At maximal completion of reaction, because of reversibility of reaction, excess of ethanol was evaporated and reaction mixture was poured into a separating funnel containing 50 mL distilled water. Sodium carbonate solution was added to remove free acid from the mixture by setting pH=8-10. Diethyl ether (10 mL) was added to the separating funnel followed by vigorous shaking and allowed to stand till the appearance of two separate layers. The lower aqueous layer was removed and discarded while the upper layer of ether containing ethyl 4-chlorobenzoate (**2**) was collected. Diethyl ether was distilled off to collect liquid ester.

Procedure for the preparation of 4-chlorobenzohydrazide (3): To a solution of ethyl 4-chlorobenzoate (**2**; 5 mL, 0.025 mol) in methanol (20 mL) in a 250 mL RB flask, hydrazine hydrate (2.5 mL, 0.050 mol) was added drop wise and mixture was allowed to stir at room temperature for 8 hours. The reaction progress was observed by TLC using *n*-hexane and ethyl acetate solvent system (40:60). After complete reaction, excess of solvent was evaporated and ice cold water was added along with shaking till precipitation. White colored precipitates of carbohydrazide **3** were filtered and washed with cold distilled water.

Synthesis of 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol (4): 4-Chlorobenzohydrazide (**3**; 4g, 0.024 mol) was dissolved in C_2H_5OH (20 mL) in a 250 mL RB flask at 28 ^{o}C and then solid KOH (1.34g, 0.024 mol) was dissolved on reflux. Carbon disulphide (3.70 mL, 0.048 mol) was poured into the reaction mixture drop wise at 28 ^{o}C and was allowed to reflux again for 6 hours. At the end of reaction, observed by TLC using *n*-hexane and ethyl acetate solvent system (70:30), excess of ethanol was evaporated. Excess of ice cold distilled water was added followed by addition of dilute HCl till constant pH of 4-5. Light peach colored precipitates of **4** were filtered and washed with distilled water.

General procedure for the synthesis of *S*-substituted derivatives of 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol (6a-l): The compound 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol (**4**; 0.1g, 0.00047 mol) was dissolved in *N*,*N*-dimethyl formamide (DMF, 5-10 mL) in a 100 mL RB flask. Solid NaH (0.005g) was added and mixture was stirred for half an hour. Then different electrophiles, alkyl/aralkyl halides, **5a-l**, were added in equimolar ratios and further stirred for 3-5 hours. The reaction was monitored by TLC using *n*-hexane and ethyl acetate solvent system (80:20). After complete reaction, ice cold distilled water was added and the products were collected by filtration or solvent extraction.

Spectral Characterization of the synthesized compounds: Physical characteristics of the synthesized derivates, **6a-l**, are mentioned in table 1 while the NMR and EI-MS spectral data of the molecules is given below.

5-(4-Chlorophenyl)-1,3,4-Oxadiazol-2-thiol (4): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.88 (brd, J = 8.8 Hz, 2H, H-2' & H-6'), 7.55 (brd, J = 8.8 Hz, 2H, H-3' & H-5'); ^{13}C-NMR (CD_3OD, 100 MHz): δ (ppm) 180.1 (C-2), 161.5 (C-5), 139.3 (C-4'), 130.6 (C-3' & C-5'), 128.7 (C-2' & C-6'), 123.0 (C-1'); HR-MS: $[M]^+$ 212.6257 (Cacld for $C_8H_5ClN_2OS$; 212.6698); EI-MS (*m/z*): 214 $[M+2]^+$, 212 $[M]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-(ethylthio)-1,3,4-Oxadiazole (6a): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.92 (d, *J* = 8.8 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.4 Hz, 2H, H-3' & H-5'), 3.30 (q, *J* = 7.2 Hz, 2H, H-1''), 1.50 (t, *J* = 7.2 Hz, 3H, H-2''); HR-MS: $[M]^+$ 240.7108 (Cacld for $C_{10}H_9ClN_2OS$; 240.7668); EI-MS (*m/z*): 242 $[M+2]^+$, 240 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$, 29 $[C_2H_5]^+$.

5-(4-Chlorophenyl)-2-(isopropylthio)-1,3,4-Oxadiazole (6b): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.93 (d, *J* = 8.4 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.4 Hz, 2H, H-3' & H-5'), 3.96 (sep, *J* = 6.8 Hz, 1H, H-1''), 1.51 (d, *J* = 6.8 Hz, 6H, CH_3-2'' & CH_3-3''); HR-MS: $[M]^+$ 254.7375 (Cacld for $C_{11}H_{11}ClN_2OS$; 254.7698); EI-MS (*m/z*): 256 $[M+2]^+$, 254 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$, 43 $[C_3H_7]^+$.

5-(4-Chlorophenyl)-2-(allylthio)-1,3,4-Oxadiazole (6c): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.92 (d, *J* = 8.8 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.4 Hz, 2H, H-3' & H-5'), 5.99-5.95 (m, 1H, H-2''), 5.40 (brd, *J* = 17.2 Hz, 1H, H_b-3''), 5.21 (brd, *J* = 9.6 Hz, 1H, H_a-3''), 3.91 (d, *J* = 7.2 Hz, 2H, H-1''); HR-MS: $[M]^+$ 252.72125 (Cacld for $C_{11}H_9ClN_2OS$; 252.798); EI-MS (*m/z*): 254 $[M+2]^+$, 252 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$, 41 $[C_3H_5]^+$.

5-(4-Chlorophenyl)-2-((2-bromoethyl)thio)-1,3,4-Oxadiazole (6d): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.92 (d, *J* = 8.8 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.4 Hz, 2H, H-3' & H-5'), 3.77 (brt, *J* = 6.4 Hz, 2H, H-2''), 3.70 (brt, *J* = 6.4 Hz, 2H, H-1''); HR-MS: $[M]^+$ 319.6065 (Cacld for $C_{10}H_8BrClN_2OS$; 319.6987); EI-MS (*m/z*): 321 $[M+4]^+$, 319 $[M+2]^+$, 317 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$,107 $[C_2H_4Br]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-((2-methylbenzyl)thio)-1,3,4-Oxadiazole (6e): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.91 (d, *J* = 8.4 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.4 Hz, 2H, H-3' & H-5'), 7.39 (brd, *J* = 7.6 Hz, 1H, H-4''), 7.22-7.15 (m, 3H, H-3'', H-5'' & H-6''), 4.54 (s, 2H, H-7''), 2.44 (s, 3H, CH_3-1''); HR-MS: $[M]^+$ 316.8063 (Cacld for $C_{16}H_{13}ClN_2OS$; 316.8675); EI-MS (*m/z*): 317 $[M+2]^+$, 315 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$, 105 $[C_8H_9]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-((2-chlorobenzyl)thio)-1,3,4-Oxadiazol (6f): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.90 (d, *J* = 8.4 Hz, 2H, H-2' & H-6'), 7.61 (dd, *J* = 7.2, 2.0 Hz, 1H, H-3''), 7.44 (d, *J* = 8.8 Hz, 2H, H-3' & H-5'), 7.39 (dd, *J* = 7.6, 1.6 Hz, 1H, H-6''), 7.23-7.19 (m, 2H, H-4'' & H-5''), 4.61 (s, 1H, H-7''); HR-MS: $[M]^+$ 337.2247 (Cacld. for $C_{15}H_{10}Cl_2N_2OS$; 337.2775); EI-MS (*m/z*): 340 $[M+4]^+$, 338 $[M+2]^+$, 336 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 125 $[C_7H_6Cl]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-((3-chlorobenzyl)thio)-1,3,4-Oxadiazole (6g): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.90 (d, *J* = 8.8 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.4 Hz, 2H, H-3' & H-5'), 7.34-7.24 (m, 4H, H-2'', H-4'' to H-6''), 4.46 (s, 1H, H-7''); HR-MS: $[M]^+$ 337.2247 (Cacld for $C_{15}H_{10}Cl_2N_2OS$; 337.2775); EI-MS (*m/z*): 340 $[M+4]^+$, 338 $[M+2]^+$, 336 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 125 $[C_7H_6Cl]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-((4-chlorobenzyl)thio)-1,3,4-Oxadiazole (6h): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.89 (d, *J* = 8.4 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.8 Hz, 2H, H-3' & H-5'), 7.39 (d, *J* = 8.4 Hz, 2H, H-3'' & H-5''), 7.29 (d, *J* = 8.4 Hz, 2H, H-2'' & H-6''), 4.46 (s, 1H, H-7''); HR-MS: $[M]^+$ 337.2247 (Cacld for $C_{15}H_{10}Cl_2N_2OS$; 337.2775); EI-MS (*m/z*): 340 $[M+4]^+$, 338 $[M+2]^+$, 336 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 125 $[C_7H_6Cl]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-((4-bromobenzyl)thio)-1,3,4-Oxadiazole (6i): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.89 (d, *J* = 8.4 Hz, 2H, H-2' & H-6'), 7.46 (d, *J* = 8.8 Hz, 2H, H-3' & H-5'), 7.43 (d, *J* = 8.8 Hz, 2H, H-3'' & H-5''), 7.32 (d, *J* = 8.4 Hz, 2H, H-2'' & H-6''), 4.44 (s, 1H, H-7''); HR-MS: $[M]^+$ 381.6764 (Cacld for $C_{15}H_{10}ClBrN_2OS$; 381.6989); EI-MS (*m/z*): 385 $[M+4]^+$, 383 $[M+2]^+$, 381 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 170 $[C_7H_6Br]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-((4-fluorobenzyl)thio)-1,3,4-Oxadiazole (6j): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.90 (d, *J* = 8.8 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.8 Hz, 2H, H-3' & H-5'), 7.42 (d, *J* = 8.4 Hz, 2H, H-3'' & H-5''), 7.00 (d, *J* = 8.4 Hz, 2H, H-2'' & H-6''), 4.47 (s, 1H, H-7''); HR-MS: $[M]^+$ 320.7786 (Cacld for $C_{15}H_{10}ClFN_2OS$; 320.7815); EI-MS (*m/z*): 322 $[M+2]^+$, 320 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$,109 $[C_7H_6F]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-((2-phenylethyl)thio)-1,3,4-Oxadiazole (6k): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.92 (d, *J* = 8.4 Hz, 2H, H-2' & H-6'), 7.46 (d, *J* = 8.4 Hz, 2H, H-3' & H-5'), 7.32-7.22 (m, 5H, H-2'' to H-6''), 3.52 (t, *J* = 7.2 Hz, 2H, H-8''), 3.15 (t, *J* = 7.2 Hz, 2H, H-7''); HR-MS: $[M]^+$ 316.8062 (Cacld for $C_{16}H_{13}ClN_2OS$; 316.8698); EI-MS (*m/z*): 318 $[M+2]^+$, 316 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 111 $[C_6H_4Cl]^+$, 105 $[C_8H_9]^+$, 76 $[C_6H_4]^+$.

5-(4-Chlorophenyl)-2-((3-phenylpropyl)thio)-1,3,4-Oxadiazole (6l): ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 7.91 (d, *J* = 8.8 Hz, 2H, H-2' & H-6'), 7.45 (d, *J* = 8.8 Hz, 2H, H-3' & H-5'), 7.28-7.25 (m, 3H, H-3'' to H-5''), 7.19 (d, *J* = 7.6 Hz, 2H, H-2'' & H-6''), 3.28 (t, *J* = 7.6 Hz, 2H, H-9''), 2.79 (t, *J* = 7.6 Hz, 2H, H-7''), 2.17 (quin, *J* = 7.6 Hz, 2H,

H-8"); HR-MS: $[M]^+$ 330.8335 (Cacld for $C_{17}H_{15}ClN_2OS$; 330.8675); EI-MS (*m/z*): 332 $[M+2]^+$, 330 $[M]^+$, 211 $[C_8H_5ClN_2OS]^+$, 139 $[C_7H_4ClO]^+$, 119 $[C_9H_{11}]^+$, 111 $[C_6H_4Cl]^+$, 76 $[C_6H_4]^+$.

Cholinesterase assay: The AChE and BChE inhibition activity was performed according to Ellman method [22] with slight modifications. Total volume of the reaction mixture was 100 µL. It contained 60 µL Na_2HPO_4 buffer with concentration of 50 mM and pH 7.7. 10 µL test compound (0.5 mM $well^{-1}$) was added, followed by the addition of 10 µL (0.005 unit $well^{-1}$ AChE or 0.5 unit $well^{-1}$ BChE) enzyme. The contents were mixed and pre-read at 405 nm. Then contents were pre-incubated for 10 min at 37 ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM $well^{-1}$ substrate (acetylthiocholine iodide for AChE or butyrylthiocholine chloride for BChE), followed by the addition of 10 µL DTNB (0.5 mM $well^{-1}$). After 15 min of incubation at 37 ºC absorbance was measured at 405 nm using 96-well plate reader Synergy HT, Biotek, USA. All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM $well^{-1}$) was used as a positive control. The percent inhibition was calculated by the help of following formula,

$$\text{Inhibition } (\%) = \frac{Control - Test}{Control} \times 100$$

IC_{50} values were calculated using EZ–Fit Enzyme kinetics software (Perrella Scientific Inc. Amherst, USA).

Cytotoxicity assay: The cytotoxicity was studied by the brine-shrimp cytotoxic assay method as described earlier [23, 24]. Artificial sea water was prepared using sea salt 34 gL^{-1}. Brine shrimp (*Artemiasalina*) eggs (Sera, Heidelberg, Germany) were hatched in shallow rectangular dish (22x32 cm) under constant aeration for 48 hours at room temperature. After hatching, active shrimps free from eggs were collected from brighter portion of the hatching chamber and used for the assay. Ten shrimps were transferred to each vial using Pasteur pipette vial containing 5 mL of artificial sea water with 200, 20, 2 and 0.2 μgmL^{-1} final concentration of test compound from their stock solution. The vials were maintained under illumination at room temperature 25 °C to 28 °C. After 24 hours, the number of surviving shrimp was counted. Experiment was performed in triplicate. Data was analyzed with Finney computer program to determine LD_{50} (Lethal Dose that killed 50% of shrimps) values.

Antibacterial assay: Test compounds were screened to determine their antibacterial activity against six bacterial strains; two gram positive bacteria, *Staphylococcus aureus* (ATCC 6538) and *Micrococcus luteus* (ATCC 10240) and four gram negative bacteria, *Escherichia coli* (ATCC 15224), *Enterobacter aerogenes* (ATCC 13048), *Bordetella bronchiseptica* (ATCC 4617) and *Salmonella typhi* **(**ATCC 14028**)** by using disc diffusion method [25, 26]. The organisms were cultured in nutrient broth at 37 °C for 24 hours. One percent broth culture containing approximately 10^6 colony-forming units (CFU/ml) of test strain was added to nutrient agar medium at 45 °C and poured into sterile petri plates. The medium was allowed to solidify. Five microliters of the test compound (40mg/mL in DMSO) was poured on 4 mm sterile paper discs and placed on nutrient agar plates respectively. In each plate DMSO served as negative control and standard antibacterial drugs Roxithromycin (1mg/mL) and Cefexime (1mg/mL) served as positive control. Triplicate plates of each bacterial strain were prepared. The plates were incubated at 37 °C for 24 hours. The antibacterial activity was determined by measuring the diameter of zones showing complete inhibition (mm).

Antifungal assay: Antifungal activity of test compounds was studied against five fungal strains; *Mucor species* (FCBP 0300), *Aspergillus niger* (FCBP 0198), *Aspergillus flavus* (FCBP 0064), *Aspergillus fumigatus* (FCBP 66) and *Fusarium solani* (FCBP 0291) by using disc diffusion method [27-29]. The organisms were cultured on SDA at 28 °C for 24 hours. Autoclaved broth culture (3 mL) was allowed to cool down to 45 °C and poured into sterile petri plates. The medium was allowed to solidify. 5 µL of the test compound (40mg/mL in DMSO) was poured on 4 mm sterile paper discs and placed on SDA plates respectively. The discs supplemented with DMSO were used as negative and positive control, respectively. Plates were incubated at 28 °C for seven days and fungal growth was determined by measuring growth diameter (mm) and growth inhibition was calculated with reference to the negative control.

Statistical analysis: All the measurements were executed in triplicate and statistical analysis was performed by Microsoft Excel 2010. Results are presented as mean ± sem.

RESULTS AND DISCUSSION

The designed *S*-substituted derivatives of 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol, **6a-l**, were synthesized in awesome amounts (scheme 1) and evaluated for their antibacterial, antifungal, anti-enzymatic and cytotoxic activities. The reaction

procedures with conditions and spectral characterization of synthesized molecules are discussed in detail in the experimental section.

Chemistry: In the presented work, 4-chlorobenzoic acid (**1**) was converted into ethyl 4-chlorobenzoate (**2**) on reaction with ethanol in the presence of concentrated sulfuric acid, which was treated with hydrazine hydrate to form 4-chlorobenzohydrazide (**3**). The cyclization of **3** with carbon disulfide in basic medium resulted into 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol (**4**) and finally its derivatives, **6a-l**, were prepared by reacting **4** with different alkyl halides, **5a-l**, as outlined in scheme 1. The parent molecule, 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol (**4**) was synthesized in good yield with light peach color. The molecular formula $C_8H_5ClN_2OS$ was established with the help of EI-MS, by counting the number of protons in ^{1}H-NMR spectrum and by ^{13}C-NMR for the verification of quaternary carbons. The EI-MS data showing molecular ion peak at *m/z* 212, also gave a distinct peak at *m/z* 111 for the fragment $[C_6H_4Cl]^+$ and the other prominent fragment ion peak was observed at *m/z* 101 for 1,3,4-Oxadiazole ring. The mass fragmentation pattern of 5-(4-chlorophenyl)-2-((2-phenylethyl)thio)-1,3,4-Oxadiazole (**6k**) was also sketched for further detail (figure 1). In its ^{1}H-NMR spectrum, the signals in aromatic region appeared at δ 7.55 (d, J = 8.8 Hz, 2H, H-3' & H-5') and 7.88 (d, J = 8.7 Hz, 2H, H-2' & H-6') which were assigned to the protons of the di-substituted ring derived from 4-chlorobenzoic acid. In its ^{13}C-NMR (BB and DEPT) spectrum, the signals appeared at δ 180.1 (C-2) and 161.5 (C-5) for the quaternary carbon atoms of the Oxadiazole ring. Thus, the structure of parent **4** was established as 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol.
Similarly, on the basis of spectral evidences from EI-MS and ^{1}H-NMR, as described in experimental section, the structures of other derivatives, **6a-l**, were elucidated. The physical characteristics of all the synthesized molecules are given in table 1.

Enzyme inhibition activity: The screening of these synthesized compounds against acetyl cholinesterase (AChE) and butyryl cholinesterase (BChE) enzymes (cholinesterases) revealed that these molecules exhibited variable inhibitory potential as shown by their IC_{50} values (table 2). The IC_{50} results against acetyl cholinesterase (AChE) enzyme demonstrated that only two among the synthesized molecules, 5-(4-chlorophenyl)-2-((2-methylbenzyl)thio)-1,3,4-Oxadiazole (**6e**) and 5-(4-chlorophenyl)-2-((3-chlorobenzyl) thio)-1,3,4-Oxadiazole (**6g**) were found to be the most potent inhibitors having IC_{50} value of 64.61±0.07 µmoles/L and 66.81±0.34 µmoles/L respectively relative to eserine, a reference standard, with IC_{50} value of 0.04±0.001 µmoles/L. The activity of **6e** might be attributed to the presence of methyl substituted aralkyl group at ortho position in the molecule while that of **6g** to the electron withdrawing chloro group at *para* position of *S*-substituted benzyl group. 5-(4-Chlorophenyl)-2-(isopropylthio)-1,3,4-Oxadiazole (**6b**) found to be lowest inhibitor having IC_{50} value of 220.15±0.08 µmoles/L relative to the reference standard. Against butyryl cholinesterase (BChE) enzyme, only 5-(4-chlorophenyl)-2-((4-fluorobenzyl)thio)-1,3,4-Oxadiazole (**6j**) was the most potent inhibitor having IC_{50} value of 145.11±0.14 µmoles/L, relative to eserine, a reference standard, with IC_{50} value of 0.85±0.0001 µmoles/L, probably due to the substitution of 4-fluorobenzyl group. The molecule among the series, 5-(4-chlorophenyl)-2-((3-phenylpropyl)thio)-1,3,4-Oxadiazole (**6l**) was found to be the lowest inhibitor having IC_{50} value of 256.22±0.08 µmoles/L. All other compounds showed inhibitory potential against butyryl cholinesterase between these two extreme values.

Cytotoxicity: The results of Brine Shrimp cytotoxicity assay revealed that all tested compounds ranged in LD_{50} values from 101.2 to 0.46 μgL^{-1}. The compounds, **6a**, **6b**, **6c**, **6i** and **6k** showed highly significant cytotoxic activity with LD_{50} values of 0.57, 0.46, 0.55, 0.67 and 0.50 μgL^{-1}, respectively. The remaining molecules might be further tested for their application in drug designing program because of low toxicity.

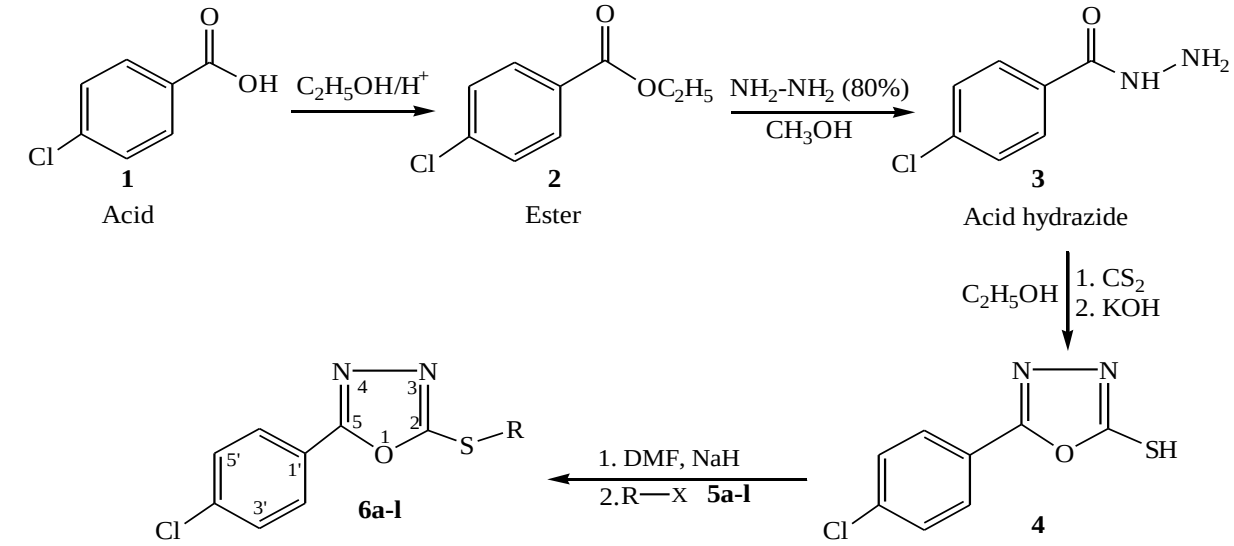
Antibacterial activity: To study the antibacterial activity of all the synthesized molecules, six bacterial strains were taken into account, as described in the assay and the results are presented in the form of MIC values in table 3. The compounds, **4** and **6d** were found to possess significant activity against all the six bacterial strains. The molecules, **6c** and **6f** exhibited activity against *B. bronchiseptica* & *E. aerogenes*; and **6b, 6e, 6h** and **6i** against *E. aerogenes* only.

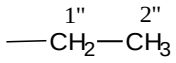
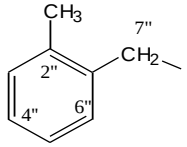
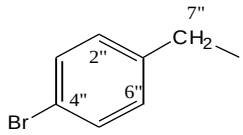
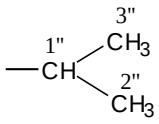
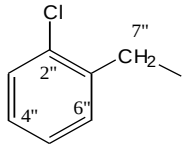
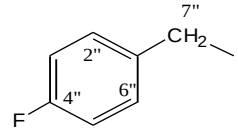
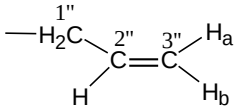
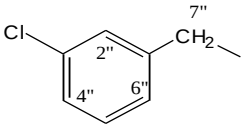
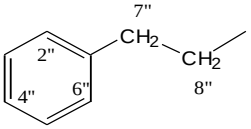
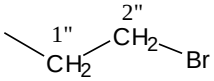
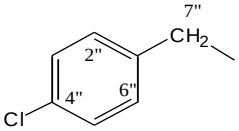
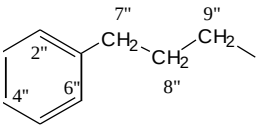
Antifungal activity: The results of antifungal activities in the form of MIC values are also summarized in table 3. The compound **4** also exhibited good antifungal activity against all the tested fungal strains in addition to antibacterial activity. Also the molecule, **6a** executed significant antifungal activity against *A. fumigatus* and *F. solani* while **6b** and **6d** were found to be active against *A. flavus* and *F.solani*, respectively.

CONCLUSION

All the molecules were synthesized in awesome amounts and evaluated for biological activities with

better results. The biological activities are critical to the presence of different substituents in the molecule, as shown by the results (table 2, table 3). So it can be concluded that the discussed activities varied upon the type and position of the substituent at 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol core. The cytotoxic results have also been processed to evaluate the cytotoxicity of the synthesized molecules and found a few of them toxic up to some extent and others with much less toxicity. Overall most of the synthesized molecules remained good inhibitors of cholinesterase enzymes, the key agents for Alzheimer's disease and so the cytotoxic behavior can be utilized to check out their affectivity and importance as new drug candidates. These molecules in addition to enzyme inhibition activity, also demonstrated the antibacterial and antifungal activities. Hence these might be considerable for the drug designing program by the pharmaceutical industries.



Comp.	-R	Comp.	-R	Comp.	-R
6a		**6e**		**6i**	
6b		**6f**		**6j**	
6c		**6g**		**6k**	
6d		**6h**		**6l**	

Scheme 1: Outline for *S*-substituted derivatives of 5-(4-chlorophenyl)-1,3,4-Oxadiazol-2-thiol (**4**)

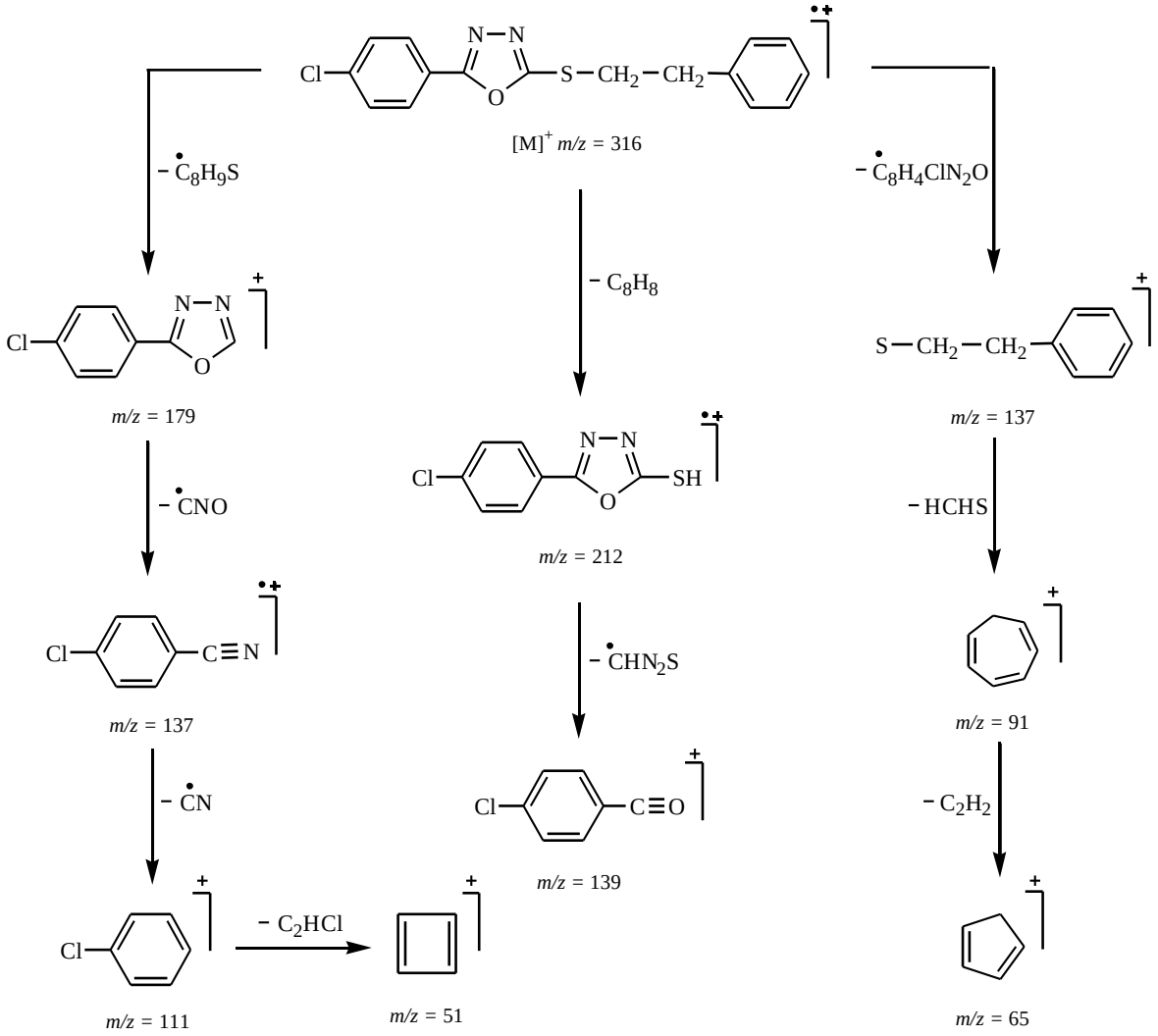


Figure 1: Mass fragmentation pattern of 5-(4-Chlorophenyl)-2-((2-phenylethyl)thio)-1,3,4-Oxadiazole (**6k**)

Table 1: Physical data of the synthesized compounds, **6a-l**

Compound	Physical State	Color	Mol. Formula	Mol. Weight	Melting Point (°C)	% Yield
6a	Solid	Pistachio	$C_{10}H_9ClN_2OS$	240.0	75-76	73%
6b	Solid	Light Lemon	$C_{11}H_{11}ClN_2OS$	254.0	56.5-57.5	78%
6c	Solid	Ivory colour	$C_{11}H_9ClN_2OS$	252.0	60-61	80%
6d	Solid	Off white	$C_{10}H_8BrClN_2OS$	318.0	96-98	78%
6e	Solid	Lemon yellow	$C_{16}H_{13}ClN_2OS$	316.0	106-107	81%
6f	Solid	Pistachio	$C_{15}H_{10}Cl_2N_2OS$	336.0	110-111	73%
6g	Solid	Light green	$C_{15}H_{10}Cl_2N_2OS$	336.0	96-97	71%
6h	Solid	Off white	$C_{15}H_{10}Cl_2N_2OS$	336.0	145-146	77%
6i	Solid	Off white	$C_{15}H_{10}BrClN_2OS$	380.0	147-148	82%
6j	Solid	Lemon yellow	$C_{15}H_{10}ClFN_2OS$	320.0	115-116	72%
6k	Solid	Light yellow	$C_{16}H_{13}ClN_2OS$	316.0	119-120	72%
6l	Solid	Lemon yellow	$C_{17}H_{15}ClN_2OS$	330.0	140-142	71%

Table 2: Enzyme inhibition and cytotoxicity of *S*-substituted derivatives of **4**

Compound	AChE		BChE		Cytotoxicity assay
	Inhibition (%) Conc./well (0.5 mM)	IC_{50} (µM)	Inhibition (%) Conc./well (0.5 mM)	IC_{50} (µM)	LD_{50} (µg/ml)
4	49.13±0.52	-	55.91±0.56	>400	101.2
6a	47.39±0.45	-	57.51±0.41	>400	0.57
6b	64.99±0.88	220.15±0.08	62.25±0.81	>400	0.46
6c	81.43±0.26	152.71±0.11	66.65±0.22	175.26±0.15	0.55
6d	52.03±0.31	>300	45.11±0.37	-	1.02
6e	95.94±0.16	64.61±0.07	66.39±0.11	172.11±0.07	1.16
6f	86.27±0.18	149.21±0.05	65.25±0.15	199.61±0.14	15.66
6g	94.97±0.11	66.81±0.34	64.42±0.14	188.11±0.34	8.53
6h	61.51±0.72	>300	54.89±0.71	>400	20.32
6i	59.57±0.71	>300	60.42±0.33	>400	0.5
6j	74.27±0.71	132.91±0.04	72.24±0.11	145.11±0.14	10.25
6k	54.55±0.41	>300	58.45±0.43	>400	0.67
6l	67.70±0.42	219.22±0.15	62.01±0.21	256.22±0.08	1.25
Control	**Eserine 91.29±1.17**	**0.04±0.001**	**Eserine 82.82±1.09**	**0.85±0.0001**	**-**

Note: IC_{50} values (concentration at which there is 50% enzyme inhibition) of compounds were calculated using EZ–Fit Enzyme kinetics software (Perella Scientific Inc. Amherst, USA).
AChE = Acetyl cholinesterase. BChE = Butyryl cholinesterase

Table 3: Antimicrobial activity of *S*-substituted derivatives of **4**

Compound	Antibacterial Activity MIC (µg/mL)						Antifungal Activity MIC (µg/mL)		
	S. aureus	***E. coli***	***B. bronci***	***M. leuteus***	***S. typhi***	***E. aerogenes***	***F. solani***	***A. flavus***	***A. fumigatus***
4	150	150	100	100	100	50	50	100	100
6a	200	-	150	200	-	100	200	-	200
6b	-	-	-	-	-	100	-	200	-
6c	-	-	200	-	-	25	-	-	-
6d	200	-	200	200	200	200	200	-	-
6e	-	-	-	-	-	150	-	-	-
6f	-	-	150	-	-	150	-	-	-
6g	-	-	200	-	-	-	-	-	-
6h	-	-	-	-	-	100	-	-	-
6i	-	-	-	-	-	200	-	-	-
6j	-	-	-	-	-	-	-	-	-
6k	-	-	-	-	-	-	-	-	-
6l	-	-	-	-	-	-	-	-	-

Note: The antibacterial activity was determined by measuring the diameter of zones showing complete inhibition (mm). MIC = Minimum Inhibitory Concentration

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