BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, December 2022. Vol. **19**(4), p. 895-915

Design, Synthesis, Computational Docking and Biological Evaluation of Novel 4-Chloro-1,3-Benzoxazole Derivatives as Anticancer Agents

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http://dx.doi.org/10.13005/bbra/3040

(Received: 01 October 2022; accepted: 10 December 2022)

An efficient, cost effective and ecologically safe method for the design of series of novel 2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[phenylmethylidene] acetohydrazides 5(a-j) have been synthesized by fusing 2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl] acetohydrazide with substituted aromatic aldehyde. The prepared compounds were characterized via LC-MS, IR, 1H NMR, 13C NMR and C, H, N analysis technique. All the synthesized compounds were evaluated for biological potency, which includes antimicrobial, antifungal, antioxidant and anticancer activities. The compounds 5a, 5b, 5d, 5e, 5g and 5h showed appreciable antimicrobial, MIC and antioxidant activity. Further, it was also noticed that the prior mentioned compounds showcased more than 70% of cell viability. We also performed molecular docking for all the synthesized compounds and examined their binding affinities to the anticancer receptor 2A91 to qualitatively elucidate their anticancer activity. The data generated from the molecular modeling and the values obtained from the biological screening were correlated.

Keywords: Antimicrobial; Antioxidant; Benzoxazole; molecular docking; PDB: 2A91.

As the practice of medicinal chemistry has evolved over time, it has dedicated its entire existence to discovering and developing new remedies for diseases [1]. Furthermore, medicinal chemistry has always emphasized on re-establishing a connection between chemical structure and pharmacological activity. Besides heterocyclic compounds contributed the most to the invention of new medications and were extensively studied in clinical aspects. Benzoxazole derivatives being an integral part of the heterocycle family, have momentous pharmacological potentialities in the field of medicinal chemistry.

In research, benzoxazole finds its uses as a starting material for the synthesis of larger bioactive molecules. It has been found within the chemical structures of pharmaceutical medicines, like *Flunoxaprofen*. Despite the fact that as a heterocycle, its aromatic character makes it moderately stable, it possesses reactive sites,

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which allow functionalization [2]. The basic aim of the synthetic and medicinal chemistry was to synthesize the compounds that results in high yields with greatest purity and show excellent activity as therapeutic agents with minimal toxicity. Eminent among these are anti-histaminic[3], antifungal[4], cyclooxygenase Inhibiting[5], anti-tumor[6], anti-ulcer[7], anticonvulsant[8], hypoglycemic[9], anti-inflammatory[10,11], anti-tubercular activity[12], anti-parasitics[13], herbicidal[14], antiviral[15], anti-allergic and anthelmintic activities[16]. Also, they have a number of optical applications such as photoluminescents, whitening agents and in dye lasers [17] and are also used as organic brightening agents and organic plastic scintillators [18].

The quest for new antimicrobial and antioxidant agents lacking side effects persists to be an active area of research in medicinal chemistry. Despite the development of new and important drugs, their cost was out of the reach of commoners. As a result, these changes have accentuated the urgent need for new, increasingly powerful, less expensive and safe antimicrobial agents. The current effort is intended for the design, synthesis, and investigation of novel benzoxazoles derivatives, with hydrazide serving as the parent molecule, based on the aforementioned facts. The synthesised derivatives of *2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[phenylmethylidene] acetohydrazides* 5(a-j) were tested for their antioxidant and cytotoxic activities as well as antibacterial activity against a number of chosen bacteria and fungi. To understand the binding affinity of produced derivatives with the active receptor sites, a molecular docking research was conducted.

EXPERIMENTAL

Materials and Instrumentation

An electrically heated apparatus was used to measure melting points that were uncorrected by placing the sample in a glass capillary sealed at one end. The ^{1}H NMR and ^{13}C NMR measurements were conducted via a Bruker at 400MHz at MIT, Manipal, Karnataka, India, with tetramethylsilane (TMS) as an internal standard and chemical shifts are expressed as 5ØýÞvalues (ppm). Analysis of elements like C, H, and N were performed by a Perkin-Elmer 2400 Series analyzer. At Centralized Instrumentation Facility of Mysore University, Karnataka, India, molecular weights of unknown compounds were characterized using LC-MS spectroscopy. A Shimadzu Fourier Transform Infrared (FT-IR Nicolet-5700) spectrometer was used to procure the FT-IR spectra of the compounds. A thin layer chromatography (TLC) method was used to examine the completion of the reaction using silica gel coated on aluminium sheets (silica gel 60 F254). Solvents and reagents of commercial grade were employed for synthesis purpose and Table 1 enlists the yields, melting points, molecular formula and molecular weight of the compounds.

RESULTS AND DISCUSSION

Design and synthesis of novel 4-chloro-1,3-benzoxazole derivatives

Preparation of 4-chloro-1,3-benzoxazole-2-thiol (2)

Methanol (50ml) and potassium hydroxide (1.1 eq) were combined and agitated for 10 minutes to start the reaction. Next, a measured amount

Table 1. Physical data of synthesized compounds 5(a-j) comprising of molecular formula, molecular weight, percentage of carbon, hydrogen, nitogen, melting point and percentage of yield

Compounds	Mol.formula	Mol.wt	Found(Calculated)%			%	M.P
			C	H	N	Yield	(°C)
5a	C16H11N3Cl2O2S	380.24	50.54(50.56)	2.92(2.94)	11.05(11.07)	81	184
5b	$C_{16}H_{11}N_4ClO_4S$	390.8	49.17(49.21)	2.84(2.86)	14.34(14.36)	76	206
5c	$C_{18}H_{17}N_4ClO_2S$	388.8	55.59(55.62)	4.48(4.50)	14.41(14.43)	74	214
5d	$C_{17}H_{14}N_3ClO_4S$	391.8	52.11(52.13)	3.60(3.63)	10.72(10.74)	78	216
5e	$C_{16}H_{11}N_4ClO_4S$	390.8	49.17(49.21)	2.84(2.86)	14.34(14.36)	75	206
5f	$C_{16}H_{11}N_3BrClO_2S$	422.94	45.25(45.27)	2.61(2.63)	9.89(9.91)	83	186
5g	$C_{16}H_{12}N_3ClO_3S$	361.8	53.11(53.14)	3.34(3.36)	11.61(11.64)	78	230
5h	$C_{17}H_{14}N_3ClO_3S$	375.83	54.33(54.36)	3.75(3.78)	11.18(11.20)	82	216
5i	$C_{17}H_{14}N_3ClO_3S$	375.83	54.33(54.36)	3.75(3.78)	11.18(11.20)	79	210
5j	$C_{19}H_{18}N_3ClO_5S$	435.88	52.35(52.37)	4.16(4.18)	9.64(9.66)	76	204

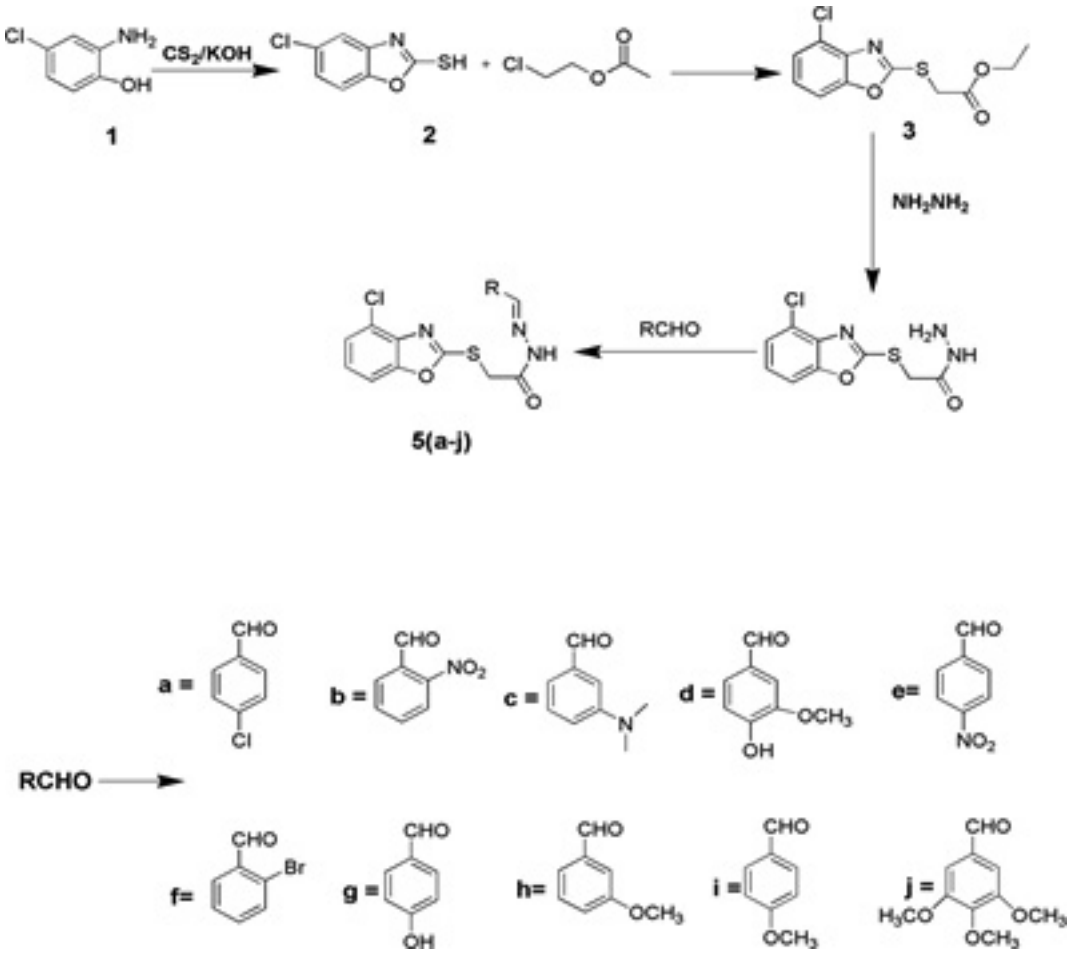
of carbon di sulphide (1.1 eq) was slowly added at room temperature. As the aforementioned reaction mass was still being stirred, 4-chloro-2-aminophenol was added and simultaneously refluxed for 6 hours on a water bath. TLC was used to monitor the reaction till it was finished. On purpose, reaction mass was added to ice-cold water, which was then acidified with glacial aceticacid. Finally the procured solid was further filtered, dried and recrystallized [19]. Yield (95%), M.P.1980C -199^{0}C. MS:m/z = 185.93 and (M+2) = 187.93.

Preparation of ethyl [(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]acetate (3)

Ethyl chloroacetate was added drop wise in the presence of K_2CO_3 after completely dissolving the 2-mercaptothiozole in acetone upon continuous stirring in a reaction flask. For nearly 4-5 hours the resultant mixture was refluxed and poured over freezing water. The obtained semisolid was washed repeatedly with water. The formed crystals after filtration were washed completely with water and dried which was further recrystallized from ethanol [20]. Yield (95%), M.P. 198^{0}C -199^{0}C.

Synthesis of 2-[(4-chloro-1,3-benzoxazol-2-yl) sulfanyl]acetohydrazide (4)

The flask containing 20 ml of methanol along with the compound 3 were stirred continuously for 15 min. The ester was added upon continuous by stirring for nearly 15 min. The hydrazine hydrate was added slowly to the above mentioned mixture which was agitated for 3 hours to get the desired product. The obtained semisolid compound was filtered and washed with



Scheme 1. Synthesis of substituted of *2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[phenylmethylidene] acetohydrazide* derivatives

pet ether. Finally the compound was collected after drying [21]. ^{1}HNMR (DMSO-d_6,äppm): 7.347-7.725 (m,3H,Ar-H), 4.342 (d,2H,S-CH_2), 4.080 (s,2H,NH_2), 9.414(s,1H,NH); MS: m/z = 257.96.

General procedure for the synthesis of2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[phenylmethylidene]acetohydrazides 5(a-j)

To an ethanolic solution (20ml), the hydrazide compound (1eq) and aromatic aldehyde (1.1eq) was added and stirred for 2-3 mins. To this mixture 2-3 drops of glacial acetic acid was added and refluxed on water bath for about 6 hours. After the completion of reaction the resultant product was added to the ice cold water and filtered, dried and recrystallized from ethanol to obtain pure product [22].

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(4-chlorophenyl)methylidene]acetohydrazide (5a)

IR (KBr,cm^{-1}): 3282 (N-H), 2362 (Ar-CH), 1681 (O=C-NH), 1450 (C=C), 1250 (C=N), 746 (C-S), 681 (C-Cl); ^{1}HNMR (DMSO-d_6,äppm):

Table 2. Antibacterial activity of synthesized compounds 5(a-j) using the agar well diffusion method against Gram-positive bacteria, specifically *Staphylococcus aureus* and *Bacillus subtillus,* and Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumonia*

Compound	*K.pneumoniae*	*P. aeruginosa*	*B .subtilis*	*S .aureus*
5a	19±0.81	18±0.94	16±1.24	18±0.94
5b	15±1.24	14±0.42	15±0.42	15±0.94
5c	14±0.94	13±1.24	12±0.94	13±0.47
5d	16±0.47	15±0.81	15±1.24	16±0.71
5e	17±0.71	16±0.94	15±0.81	17±0.94
5f	18±1.24	17±0.71	16±0.42	15±0.71
5g	18±0.42	16±0.81	15±1.24	16±0.81
5h	15±0.94	14±1.24	14±0.81	13±1.24
5i	13±1.24	13±0.47	14±0.42	13±0.94
5j	10±0.81	10±0.94	10±0.81	11±0.94
STD	23±0.42	20±0.47	19±0.94	21±0.47

*STD=*Chloramphenicol compound* =250 5Øg/ml
*Each value is expressed as the mean ± SD of three replicates for the zone of inhibition.

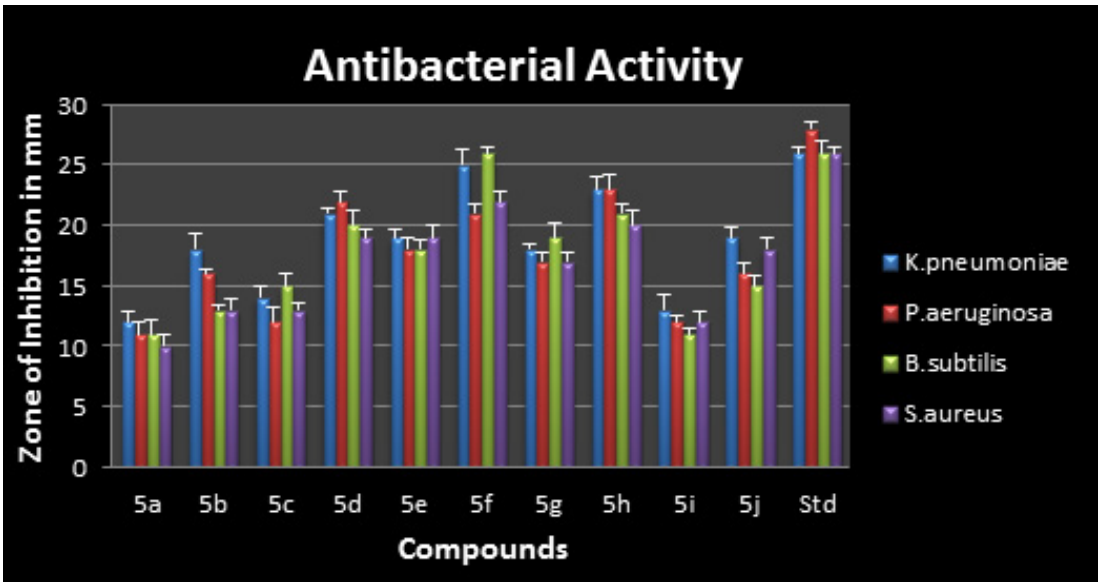


Fig. 1. Antibacterial activity bar graph representing the zone of inhibition of synthesized compounds 5(a-j)

7.14-7.6 (m,7H,Ar-H), 3.82 (d,2H,S-CH_2), 8.0 (bs,1H,CH), 8.0(bs,1H,NH); ^{13}C-NMR(DMSO-d_6,äppm): 173, 165.0, 151.4 143.0, 140.5, 136.6, 131.9, 130.6, 130.6, 129.0, 129.0, 125.8, 125.3, 123.8, 108.8, 40.9; MS: m/z = 380.2.

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(4-nitrophenyl)methylidene]acetohydrazide (5b)

IR(KBr,cm^{-1}): 3280(N-H), 2367(Ar-CH), 1688(O=C-NH), 1452(C=C), 1328(C-NO_2), 1252(C=N), 745(C-S), 680(C-Cl,Ar-H)); 7.14-8.2 (m,7H), 3.82(d,2H,S-CH_2), 8.0(s,1H,CH), 8.1(bs,1H,NH); ^{13}C-NMR(DMSO-d_6,äppm): 173, 165.0, 151.4, 148.2, 143.0, 140.5, 135.0, 131.9, 132.0, 130.1, 126.3, 125.8, 125.3, 123.8,121.2, 108.8, 40.9; MS: m/z=390.8.

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(3-trimethylphenyl)methylidene] acetohydrazide (5c)

IR(KBr,cm^{-1}): 3284(N-H), 2809(N-CH_3), 2360(Ar-CH), 1692(O=C-NH), 1449(C=C), 1261(C=N), 744(C-S), 682(C-Cl); ^{1}HNMR(DMSO-d_6,äppm):6.6-7.27(m,7H,Ar-H), 3.82(d,2H,S-), 2.85(t,3H,CH_3), 8.0(bs,1H,CH), 8.1(s,1H,NH);

Table 3. Antifungal activity of synthesized compounds 5(a-j) using the sabouraud dextrose agar diffusion method against fungal strains Gram positive fungi *Candida albicans, Cryptococcus neoformans* and Gram negative fungus *Aspergillus niger, Pencillium*

Compound	*C. albicans*	*C.neoformans*	*A.niger*	*Penicillium*
5a	24±0.47	22±0.94	23±1.24	19±0.81
5b	23±0.81	19±1.69	18±0.94	15±0.47
5c	20±1.24	17±0.47	16±0.81	13±0.94
5d	20±0.94	18±0.81	20±0.47	16±1.24
5e	21±1.88	20±0.94	21±0.47	18±0.81
5f	24±1.69	20±0.81	22±0.94	18±1.24
5g	23±0.81	19±0.47	21±1.24	17±0.94
5h	19±0.47	17±1.24	20±1.88	19±0.81
5i	19±0.94	15±1.24	18±0.81	16±1.88
5j	18±1.24	15±0.94	14±0.81	15±1.69
Std	27±0.47	28±0.81	29±1.24	25±0.94

*STD=Chloramphenicol compound
*Each value is expressed as the mean ± SD of three replicates for the zone of inhibition.

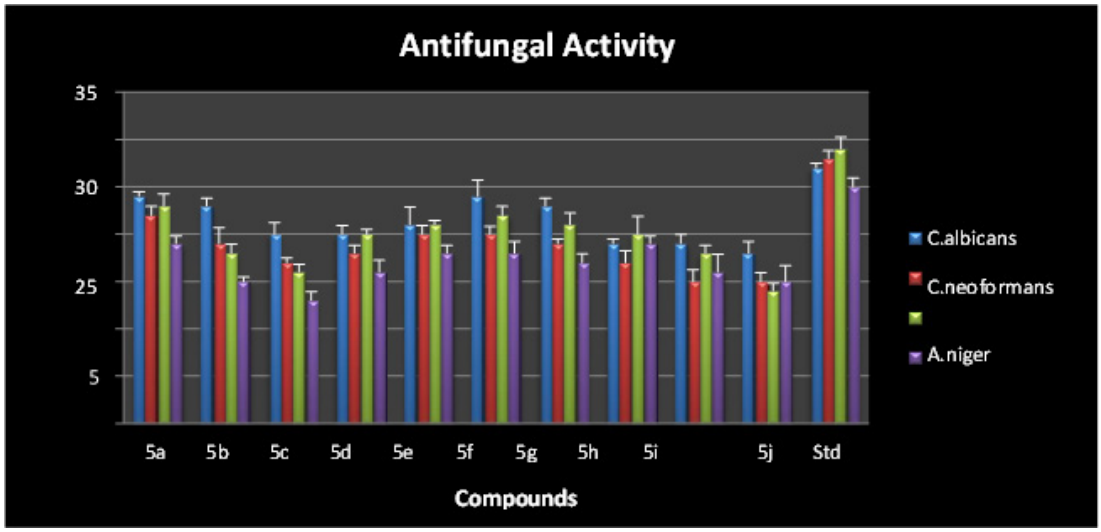


Fig. 2. Antifungal activity bar graph representing the zone of inhibition of synthesized compounds 5(a-j)

Table 4. MIC of synthesized Compounds 5(a-j) using serial dilution technique at different concentrations (100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml) against two bacterial strains *K.pneumoniae and B.subtilis*

Compounds	Concentration	*K.pneumoniae*	*B.subtilis*
5a	100mg/ml	19±0.94	13±0.81
	50mg/ml	18±1.24	11±0.94
	25mg/ml	16±0.81	11±1.24
	12.5mg/ml	14±0.47	10±0.81
	Standard	24±1.69	22±0.94
5b	100mg/ml	16±1.88	12±0.94
	50mg/ml	13±0.94	12±1.24
	25mg/ml	11±0.47	10±0.81
	12.5mg/ml	11±1.24	10±0.94
	Standard	24±0.81	22±0.47
5c	100mg/ml	13±1.69	14±0.81
	50mg/ml	13±1.24	11±0.94
	25mg/ml	12±0.81	11±1.24
	12.5mg/ml	11±0.47	10±0.94
	Standard	24±0.94	22±0.81
5d	100mg/ml	15±1.69	18±0.47
	50mg/ml	15±1.88	18±0.94
	25mg/ml	12±0.47	14±0.94
	12.5mg/ml	11±0.94	12±1.24
	Standard	24±0.81	22±1.24
5e	100mg/ml	17±1.69	14±0.47
	50mg/ml	15±0.47	14±0.94
	25mg/ml	12±0.81	11±1.24
	12.5mg/ml	12±0.94	11±0.47
	Standard	24±1.24	22±0.94
5f	100mg/ml	17±1.69	11±0.94
	50mg/ml	16±1.88	11±0.47
	25mg/ml	14±0.47	10±0.94
	12.5mg/ml	13±0.94	10±0.81
	Standard	24±0.81	22±1.24
5g	100mg/ml	16±1.24	16±0.47
	50mg/ml	15±0.47	13±0.94
	25mg/ml	14±1.69	10±1.24
	12.5mg/ml	12±0.81	10±0.94
	Standard	24±0.94	22±1.24
5h	100mg/ml	14±1.88	14±0.94
	50mg/ml	12±0.47	11±0.81
	25mg/ml	11±0.81	11±0.47
	12.5mg/ml	11±0.94	11±1.24
	Standard	24±1.24	22±0.94
5i	100mg/ml	12±0.81	15±1.69
	50mg/ml	11±0.94	12±0.47
	25mg/ml	11±1.69	10±0.81
	12.5mg/ml	11±0.47	10±0.94
	Standard	24±0.94	22±1.24
5j	100mg/ml	10±1.88	16±0.81
	50mg/ml	10±1.24	14±0.47
	25mg/ml	11±0.94	11±1.24
	12.5mg/ml	11±0.81	11±0.94
	Standard	24±0.47	22±0.81

*Std = Ascorbic acid

*Each value is expressed as the mean ± SD of three replicates for the zone of inhibition.

Table 5. MIC of synthesized Compounds 5(a-j) using serial dilution technique at different concentrations (100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml) against two fungal strains C.albicans and A.niger

Compound	Concentration	*C.albicans*	*A.niger*
5a	100mg/ml	19±0.81	12±0.94
	50mg/ml	16±0.94	12±1.24
	25mg/ml	15±1.24	10±0.81
	12.5mg/ml	14±0.81	11±0.47
	Standard	25±0.94	27±1.69
5b	100mg/ml	16±0.94	11±1.88
	50mg/ml	15±1.24	11±0.94
	25mg/ml	13±0.81	10±0.47
	12.5mg/ml	11±0.94	10±1.24
	Standard	25±0.47	27±0.81
5c	100mg/ml	13±0.81	13±1.69
	50mg/ml	12±0.94	13±1.24
	25mg/ml	11±1.24	10±0.81
	12.5mg/ml	11±0.94	11±0.47
	Standard	25±0.81	27±0.94
5d	100mg/ml	15±0.47	17±1.69
	50mg/ml	14±0.94	15±1.88
	25mg/ml	12±0.94	14±0.47
	12.5mg/ml	12±1.24	13±0.94
	Standard	25±1.24	27±0.81
5e	100mg/ml	17±0.47	14±1.69
	50mg/ml	15±0.94	13±0.47
	25mg/ml	14±1.24	11±0.81
	12.5mg/ml	12±0.47	11±0.94
	Standard	25±0.94	27±1.24
5f	100mg/ml	17±0.94	10±1.69
	50mg/ml	16±0.47	10±1.88
	25mg/ml	14±0.94	10±0.47
	12.5mg/ml	13±0.81	10±0.94
	Standard	25±1.24	27±0.81
5g	100mg/ml	16±0.47	15±1.24
	50mg/ml	15±0.94	13±0.47
	25mg/ml	13±1.24	12±1.69
	12.5mg/ml	12±0.94	12±0.81
	Standard	25±1.24	27±0.94
5h	100mg/ml	14±0.94	14±1.88
	50mg/ml	14±0.81	14±0.47
	25mg/ml	12±0.47	11±0.81
	12.5mg/ml	11±1.24	10±0.94
	Standard	25±0.94	27±1.24
5i	100mg/ml	14±1.69	15±0.81
	50mg/ml	13±0.47	13±0.94
	25mg/ml	11±0.81	11±1.69
	12.5mg/ml	11±0.94	11±0.47
	Standard	25±1.24	27±0.94
5j	100mg/ml	12±0.81	18±1.88
	50mg/ml	11±0.47	15±1.24
	25mg/ml	10±1.24	12±0.94
	12.5mg/ml	10±0.94	12±0.81
	Standard	25±0.81	27±0.47

*STD=*Fluconazole Compound*

*Each value is expressed as the mean ± SD of three replicates for the zone of inhibition.

^{13}CNMR(DMSOd_6,äppm):173, 165, 151.4, 149.7, 143.0, 140.5, 134.7, 129.8, 125.3, 123.8, 118.7, 116.6, 111.6, 108.8, 40.9,40.3; MS: m/z=388.8.

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(3-methoxy,4-hydroxyphenylmethylidene] acetohydrazide (5d)

IR(KBr,cm^{-1}): 3280(N-H), 2367(Ar-CH),1688(O=C-NH), 1452(C=C), 1328(C-NO_2), 1252(C=N), 745(C-S), 680(C-Cl); ^{1}HNMR(DMSO-d_6,äppm):12.071(bs,1H,-NH), 9.531(s,1H,OH), 8.076(s,1H,-CH), 7.504-6.816(m,6H,Ar-H), 4.006(d,2H,S-CH_2), 3.812(s,3H,-OCH_3); ^{13}CNMR(DMSOd_6,äppm):161.05, 149.20, 148.43, 147.31, 146.41, 128.69, 125.90, 121.86, 121.11, 116.44, 115.97, 110.78, 109.56, 56.04 ;MS: m/z=391.27, (M+1)=392.14.

Table 6. Antioxidant activity of synthesized compounds 5(a-j) using DPPH methods at different concentrations (400µg/ml, 200µg/ml, 100 µg/ml, 50µg/ml, and 25µg/ml)

Compound	Scavenging activity of different Concentration (*µg/ml*) in%				
	400µg/ml	200µg/ml	100µg/ml	50µg/ml	25µg/ml
5a	97.86±0.28	95.21±0.41	92.16±0.7	87.45±0.48	86.15±0.32
5b	94.27±0.8	93.11±0.39	89.05±0.25	84.15±0.56	82.33±0.75
5c	83.88±0.57	80.56±0.79	78.56±0.91	75.35±0.87	72.22±0.25
5d	92.25±0.66	90.16±0.45	88.19±1.13	84.27±0.22	82.06±0.15
5e	95.19±0.73	95.82±0.52	90.15±0.78	86.12±0.61	84.34±0.52
5f	96.18±0.38	95.61±0.76	91.42±0.48	86.71±0.64	85.23±0.17
5g	94.54±0.53	93.25±0.18	92.79±0.31	85.98±0.34	84.63±0.26
5h	88.82±0.45	82.09±0.55	81.91±0.83	79.25±0.14	76.41±0.31
5i	86.91±0.36	81.11±0.43	80.08±0.51	77.42±0.3	75.33±0.37
5j	84.02±1.16	81.23±0.13	79.5±0.69	76.25±0.65	74.14±0.41
Std	98.68±0.31	96.72±0.77	94.29±0.54	90.12±0.43	88.38±0.38

*Std = Ascorbic acid

*Each value is expressed as the mean ± SD of three replicates for the zone of inhibition.

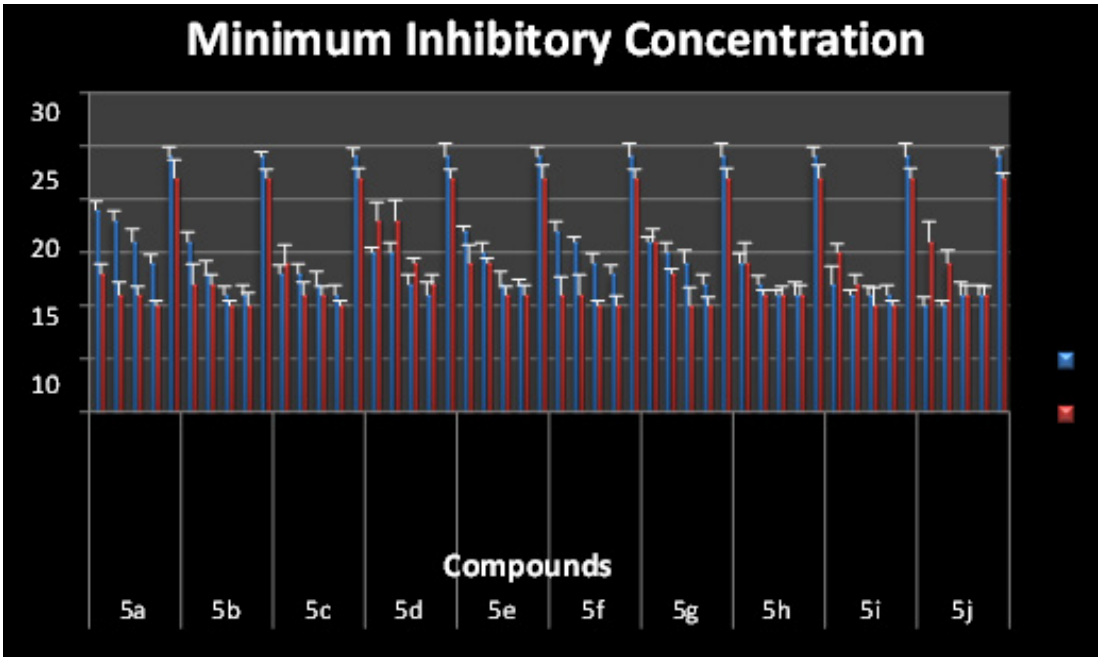


Fig. 3. MIC of antibacterial activity bar graph representing the zone of inhibition of synthesized compounds 5(a-j).

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(4-nitrophenyl)methylidene]acetohydrazide (5e)

IR(KBr,cm^{-1}): 3280(N-H), 2367(Ar-CH), 1688(O=C-NH), 1452(C=C), 1328(C-NO_2), 1252(C=N), 745(C-S), 680(C-Cl); 7.14-8.2(m,7H,Ar-H), 3.82(d,2H,S-CH_2), 8.0(s,1H,CH), 8.1(bs,1H,NH); ^{13}C-NMR(DMSO-d_6,äppm):173, 165.0, 151.4, 148.2, 143.0, 140.5, 135.0, 131.9, 132.0, 130.1, 126.3, 125.8, 125.3, 123.8, 121.2, 108.8, 40.9; MS: m/z =390.8

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(2-bromophenyl)methylidene] acetohydrazide (5f)

IR (KBr,cm^{-1}): 3285 (N-H), 2366 (Ar-CH), 1683 (O=C-NH), 1450 (C=C), 1252 (C=N), 744(C-

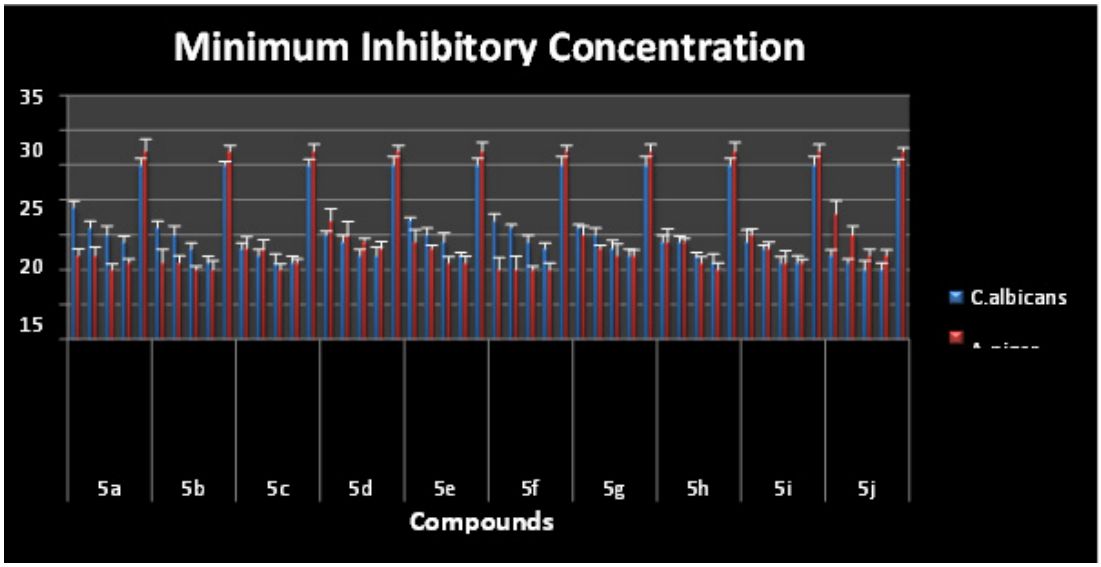


Fig. 4. MIC of antifungal activity bar graph representing the zone of inhibition of synthesized compounds 5(a-j)

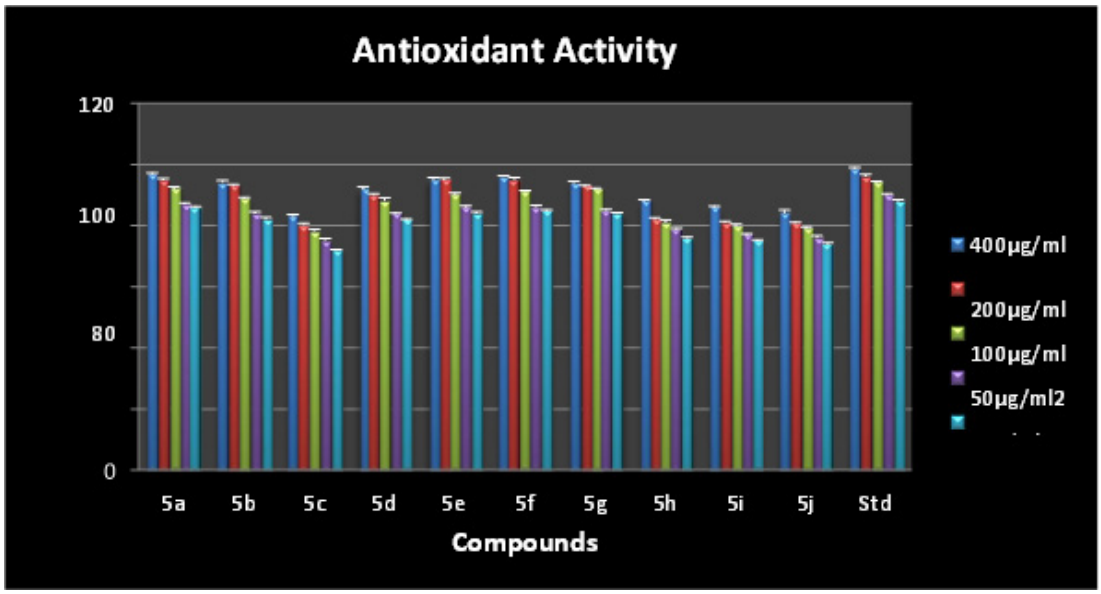


Fig. 5. Antioxidant activity bar graph representing the percentage of antioxidant potency of synthesized compounds 5(a-j).

Table 7. Binding energies and types of binding interaction of synthesized compounds 5(a-j) on the anticancer receptor, PDB code 2A91

Compounds Code	H-bond	Pi-Lone pairinteraction	Docking score	Pi-alkylinteraction	Alkyl-AlkylInteraction
5a	TYR29 THR8 THR8T HR8A SN38T HR8G LU40	TYR29AS N38THR8 LEU39TY R62TYR62 ASN38	-311.09	LEU415 TYR62 LEU39	TYR62GLU4 0LEU64LEU 39ASN281A RG411
5b	LYS11 ASP9A RG26A SP23A RG77A SP55A RG82G LU58A RG122 ASP19 0ARG1 22 GLU189 ARG136 ASP97A RG167A SP144	THR8 TYR29 THR8 GLY41 8ARG 13ASN 417	-317.99	TYR62 TYR62 LEU39	HIS236 GLN218 LYS34 8GLU3 84GLU 383AS N406G LY418
5c	SER442 GLY412 GLY7T YR29G LY7AS N38TH R8GLY 418 LEU39 TYR29	GLY418 SER442 THR8T YR29G LY418	-303.30	THR8 TYR6 2TYR 62	LEU39 VAL63
5d	GLY418 LEU415 SER442 SER442 GLY7G LY7AS N38TH R8 GLY418	GLY7THR 8ASN38LE U415SER44 2	-310.17	TYR62	TYR62 LEU39
5e	LYS11 TYR6 2ASN 38AS N38T HR8A SN38	GLY418 LEU415 SER442 ARG41 1SER44 2	-316.21	GLY418 LEU415 GLY418 TYR62	TYR62 LEU41 5TYR6 2LEU3 9

	ASN3 8 GLN85				
5f	GLY7 GLN3 6THR8A SN38TH R8THR8	MET10 LYS11 LEU12 ARG13 LEU14	-295.31	PRO18 GLU1 9THR2 0	GLY7A SN38LE U39LEU 415SER 442
	ASP9ASN417				
5g	ASN38 LEU39 TYR62 GLU40 ASN38 TYR62 ASN38 TYR62 GLU40 TYR62 GLY41 8LEU415	ARG411 GLY412 GLY7A SN38TH R8GLY 418	-314.58	TYR62A RG411G LY418T YR62	TYR62 LEU39
5h	GLY7 THR8 ASP9 MET1 0 LYS11 LEU12 ARG13	LEU14 PRO15 ALA16 SER17 PRO18	-318.29	THR8G LY418A RG411S ER442 ARG411 TYR62	TYR62 GLY41 8ASN38
5i	THR8 ASN3 8ASN 38TH R8LE U39T YR62 GLU4 0ASN 38TY R62A SN38 TYR6 2GLU 40 TYR62	TYR29 GLN3 0GLY 31CYS 32GL N33V AL34 VAL3 5	-322.59	GLY7 TYR29 TYR62 TYR62	TYR62 LEU64 LEU39 VAL63
5j	THR8 ASP9 MET10 LYS11 LEU12 ARG13 LEU14 PRO15	ASN68 GLN6 9VAL 70ARG 71GLN 72VA L73PR O74	-320.29	PHE87 GLU8 8ASP8 9ASN 90TY R91	THR165 ASN166 ARG16 7SER16 8

S), 685 (C-Cl), 601 (C-Br); ^{1}HNMR (DMSO-d_6,äppm): 11.837 (bs,1H,-NH), 8.697 (s,1H,-CH), 8.197-7.349 (m,7H,Ar-H), 4.687 (d,2H,S-CH_2),3.812;^{13}C-NMR (DMSOd_6,äppm): 168.52, 166.68, 163.37, 150.73, 146.76, 143.60, 143.15, 133.91, 132.61, 130.79, 129.54, 124.91, 123.88, 118.67, 112.07, 35.35; MS:m/z = 423.86,(M+2) = 425.85.

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(4-hydroxyphenyl)methylidene] acetohydrazide(5g)

IR(KBr,cm^{-1}): 3445(O-H), 3287(N-H), 2368(Ar-CH), 1686(O=C-NH), 1455(C=C), 1253(C=N), 749(C-S), 682(C-Cl); ^{1}HNMR(DMSO-

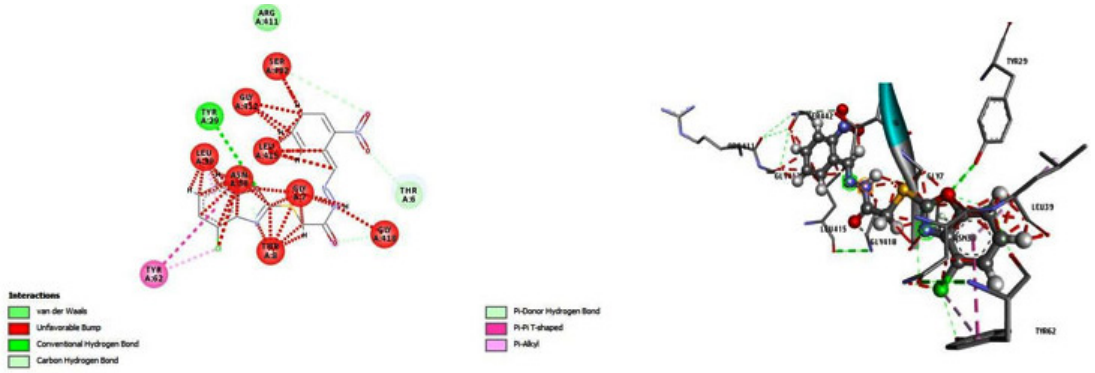


Fig. 6. 2D and 3D bonding interactions of receptor 2A91with compound 5a

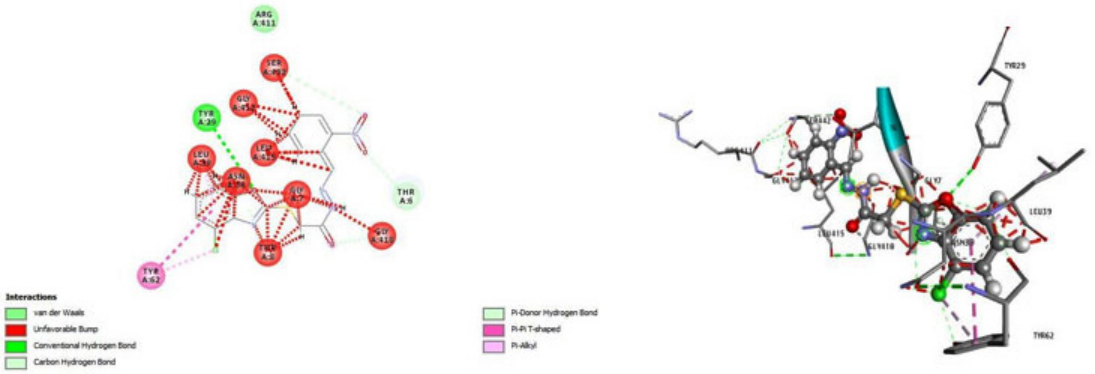


Fig. 7. 2D and 3D bonding interactions of receptor 2A91 with compound 5b

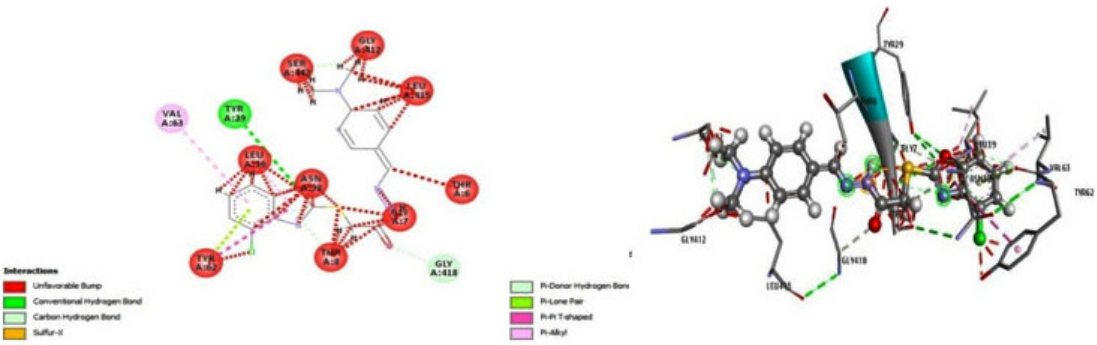


Fig. 8. 2D and 3D bonding interactions of receptor 2A91 with compound 5c

d_6,äppm): 12.071(bs,1H,-NH), 9.531(s,1H,OH), 8.1 (s,1H,-CH), 6.82-7.27(m,7H,Ar-H), 3.82(d,2H,S-CH_2); ^{13}C-NMR(DMSO-d_6,äppm): 173, 160.8, 165.0, 151.4, 143.0, 140.5, 130.6, 126.4, 125.8, 125.3, 123.8, 116, 108.8, 40.9; MS:m/z =361.8.

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(3-methoxyphenyl)methylidene] acetohydrazide (5h)

IR(KBr,cm^{-1}):3289(N-H), 2864(-OCH_3), 2366(Ar-CH), 1684(O=C-NH), 1454(C=C), 1259(C=N), 751(C-S), 688(C-Cl); ^{1}HNMR(DMSO-

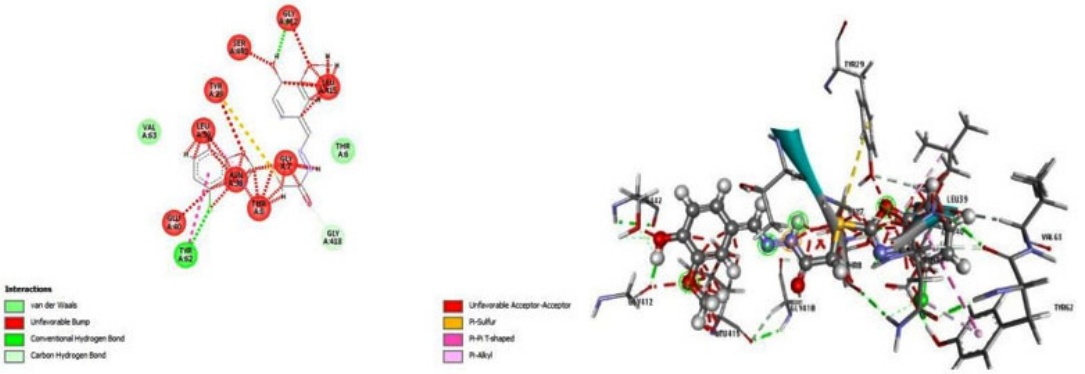


Fig. 9. 2D and 3D bonding interactions of receptor 2A91 with compound 5d

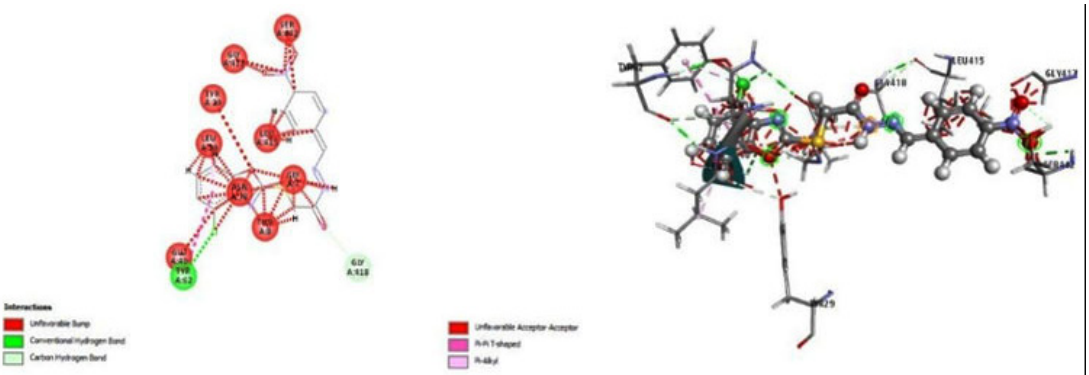


Fig. 10. 2D and 3D bonding interactions of receptor 2A91 with compound 5e

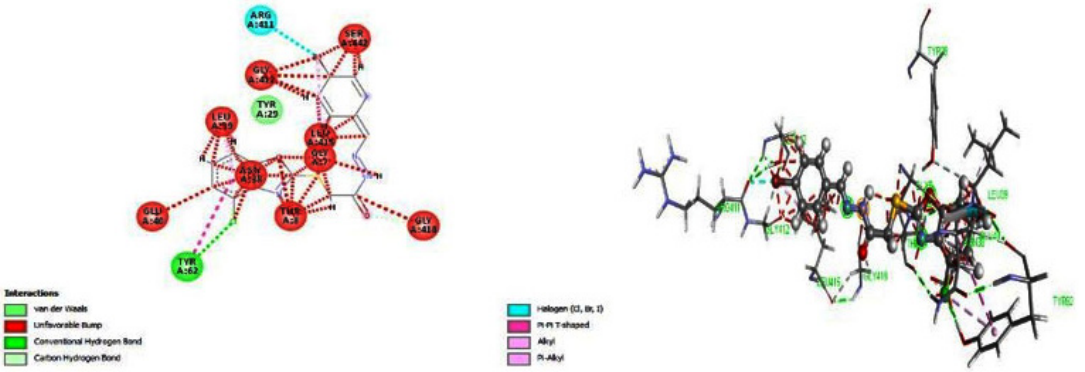


Fig. 11. 2D and 3D bonding interactions of receptor 2A91 with compound 5f

d_6,äppm): 12.071(bs,1H,-NH), 9.531(s,1H,OH), 8.1(s,1H,-CH), 6.82-7.27 (m,7H,Ar-H), 3.82 (d,2H,S-CH_2), 3.73(3H,-OCH_3); ^{13}C NMR(DMSO-d_6,äppm): 173, 163, 145.8, 143.0, 134.3, 130.2, 130.2, 126.6, 126.1, 122.3, 121.6, 114.8, 114.4, 114.4, 53.9, 34.4; MS: m/z=375.4.

2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(4methoxyphenyl)methylidene]acetohydrazide (5i)

IR(KBr,cm^{-1}): 3286(N-H), 2866(-OCH_3), 2369(Ar-CH), 1683(O=C-NH), 1450(C=C), 1255(C=N), 752(C-S), 680(C-Cl); ^{1}HNMR(DMSO-d_6,äppm): 11.771(bs,1H,-NH), 8.163(s,1H,-CH), 8.137-6.979(m,7H,Ar-H), 4.670(d,2H,S-CH_2), 3.776(3H,-OCH_3); ^{13}CNMR(DMSO-d_6,äppm): 168.32, 166.25, 159.98, 150.57, 144.41, 135.74, 130.40, 129.35, 124.64, 120.0, 118.51, 116.33, 113.18, 112.22, 111.91, 55.642; MS: m/z=375.95, (M+2)=377.95.

2-[(4-chloro-1,3-benzoxazol-2-yl) sulfanyl]-N'-[(3,4,5-trimethoxy phenyl) methylidene] acetohydrazide (5j)

IR(KBr,cm^{-1}):3278(N-H), 2860(-OCH_3), 2371(Ar-CH), 1677(O=C-NH), 1457(C=C),

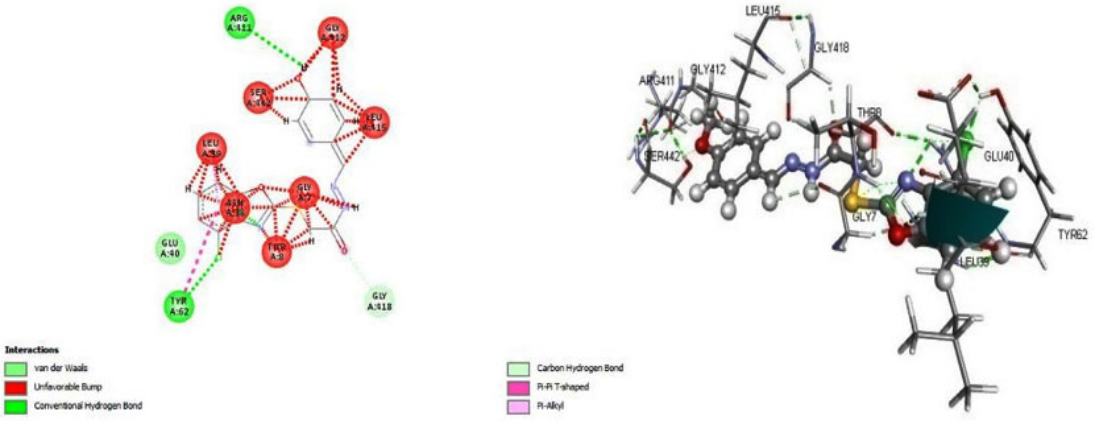


Fig. 12. 2D and 3D bonding interactions of receptor 2A91 with compound 5g

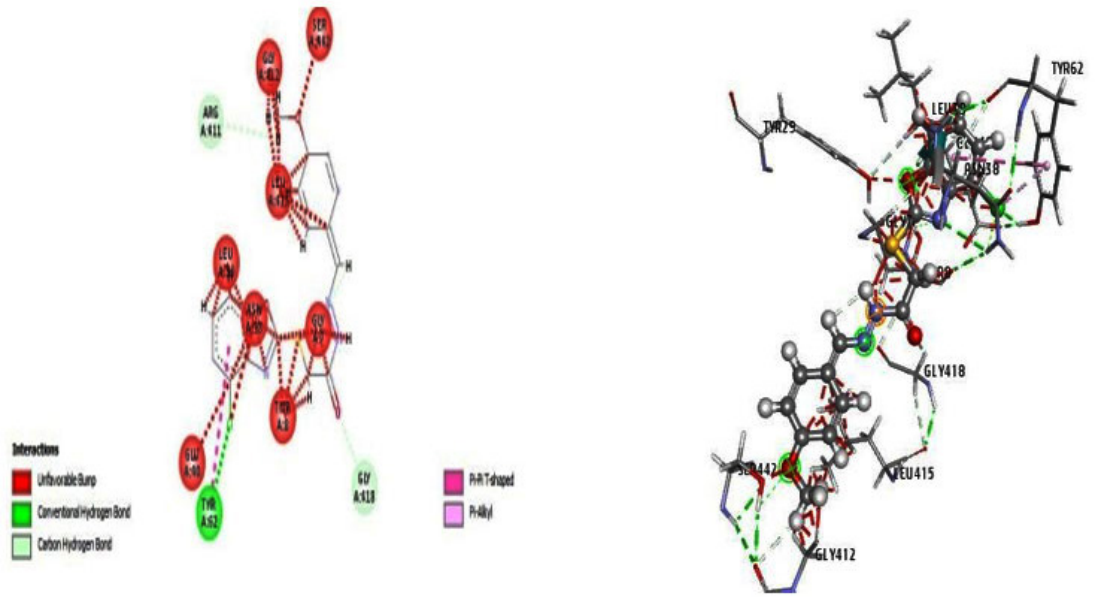


Fig. 13. 2D and 3D bonding interactions of receptor 2A91 with compound 5h

1249(C=N), 750(C-S), 686(C-Cl); ^{1}HNMR(DMSO-d_6,äppm):11.771(bs,1H,-NH), 8.163(s,1H,-CH), 7.27-6.6(m,5H,Ar-H), 3.82(2H,S-CH_2), 3.73(9H,-OCH_3); ^{13}CNMR(DMSO-d_6,äppm):173, 165.0, 150.9, 150.9, 151.4, 143.0, 141.5, 140.5, 128.1, 125.8, 125.3, 123.8, 108.8, 106. 7, 106.7, 56.5, 56.2, 40.9 ; MS: m/z=435.88

Biological Activities of novel 4-chloro-1,3-benzoxazole derivatives

Antibacterial Activity of compounds 5(a-j)

Novel benzoxazole derivatives were synthesized and tested for antibacterial activity by using the agar well diffusion method against Gram-positive bacteria, specifically *Staphylococcus aureus* and *Bacillus subtillus,* and Gram-negative bacteria *Pseudomonas aeruginosa* and *Klebsiella pneumonia* [23]. The 24 hour old Mueller-Hinton broth culture of test bacteria was swabbed on sterile Mueller-Hint on agar plates with the help of sterile cotton swab, which was continued by punching wells of 6mm with the aid of sterile cork borer. To the corresponding specified wells, the standard drug (Chloramphenicol, 1mg/mL of sterile distilled water), compounds 5(a-j) (250 *µg/ml* in 10% DMSO) and control (10% DMSO) were added. The plates were left to stand for nearly 30 minutes and incubated for 24 hour at 37^{0}C in upright position and the zone of inhibition was observed and enlisted in Table 2 and represented in Figure1.

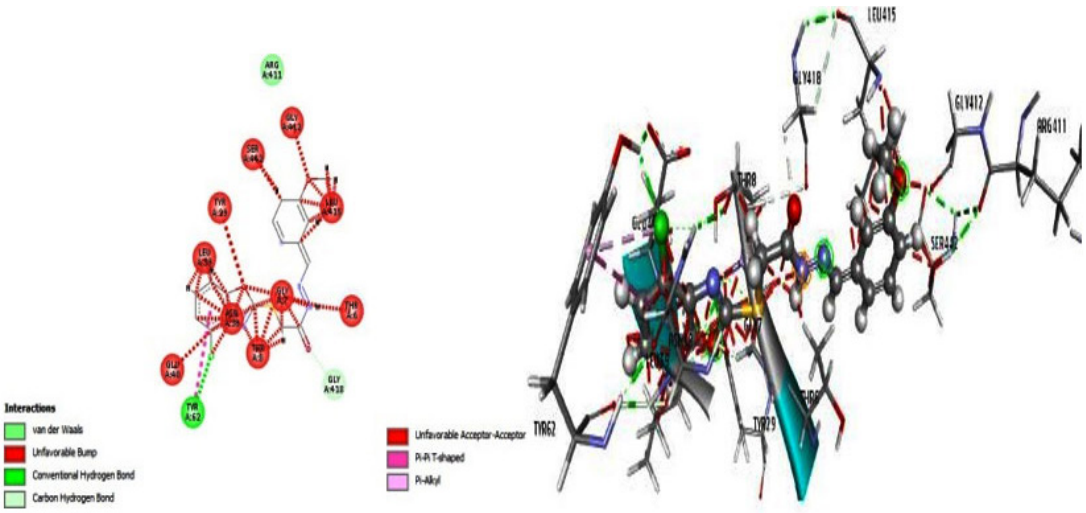


Fig. 14. 2D and 3D bonding interactions of receptor 2A91 with compound 5i

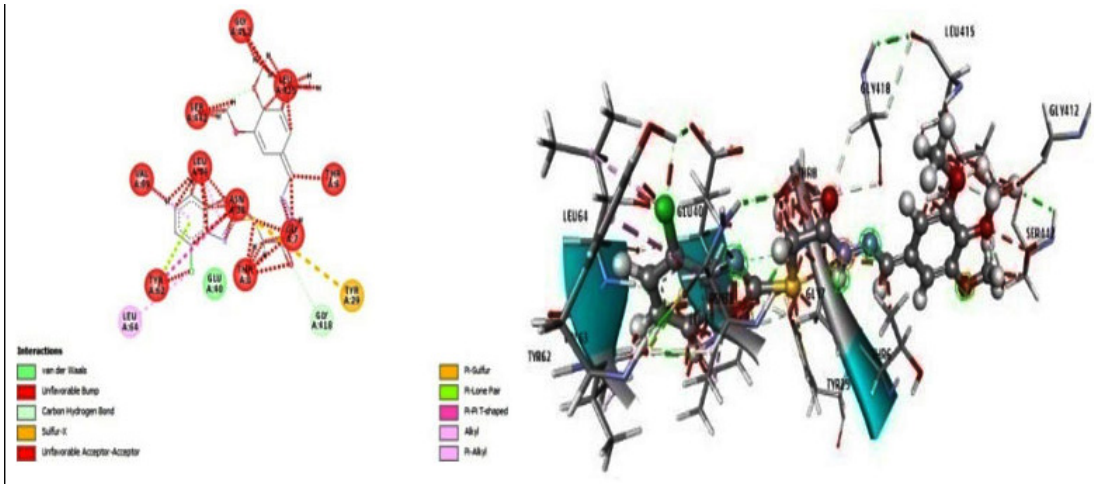


Fig. 15. 2D and 3D bonding interactions of receptor 2A91 with compound 5j

Antifungal activity of compounds 5(a-j)

Antifungal activity of the compounds 5(a-j) were evaluated against fungal strains Gram positive fungi *Candida albicans, Cryptococcus neoformans* and Gram negative fungus *Aspergillus niger, Pencillium* using the sabouraud dextrose agar diffusion method[23]. Wells were prepared (9 mm diameter) with a sterile cork borer. The standard medication (fluconazole, 100 g/mL of sterile distilled water) and control (10% DMSO) were added to the individually labelled wells. To these wells, compounds 5(a-j) (250 µg/mL of 10% DMSO) and control (10% DMSO) were added and the plates were permitted to cool for an hour to facilitate the diffusion. At 37 ºC, the plates were then incubated for 48 hours. At the final of the incubation period, the diameter of the zone of inhibition around the wells was estimated using vernier callipers and observed data are indexed in Table 3 and shown in Figure 2.

Minimum Inhibitory Concentration (MIC)

All the synthesized compounds have undergone testing for antibacterial and antifungal activity. Using the serial dilution technique, the Minimum inhibitory concentration (MIC) of the synthesized compounds 5(a-j) were calculated. The data of minimum inhibitory concentration for antibacterial and antifungal are presented in Table 4 and Table 5. Synthesized compounds were tested for their ability to inhibit the growth of bacterial and fungal strains at different concentrations that is 100, 50, 25, and 12.5 g/mL. The MIC zone of inhibition for antibacterial and antifungal activity of the compounds 5(a-j) are displayed in Figure 3

Table 8. *In-vitro* cytotoxicity of synthesized compounds (5a, 5b, 5d, 5g, 5i) against MCF-7 cell lines

Compound	*MCF-7t5r*					
	400	200	100	50	25	12.5
5a	21±0.47	23±0.11	26±1.25	28±0.57	32±0.22	39±0.11
5b	22±1.15	26±1.52	29±0.19	31±0.47	36±0.65	43±1.74
5d	23±0.65	25±0.33	28±1.15	30±1.52	35±0.47	41±0.17
5g	25±0.57	28±1.15	30±0.22	36±0.58	39±0.18	45±1.25
5i	28±0.90	30±0.13	34±1.15	39±0.47	45±1.24	51±0.33

NegativeControl 100

*Each value is expressed as mean ± SD of three replicates for the zone of inhibition

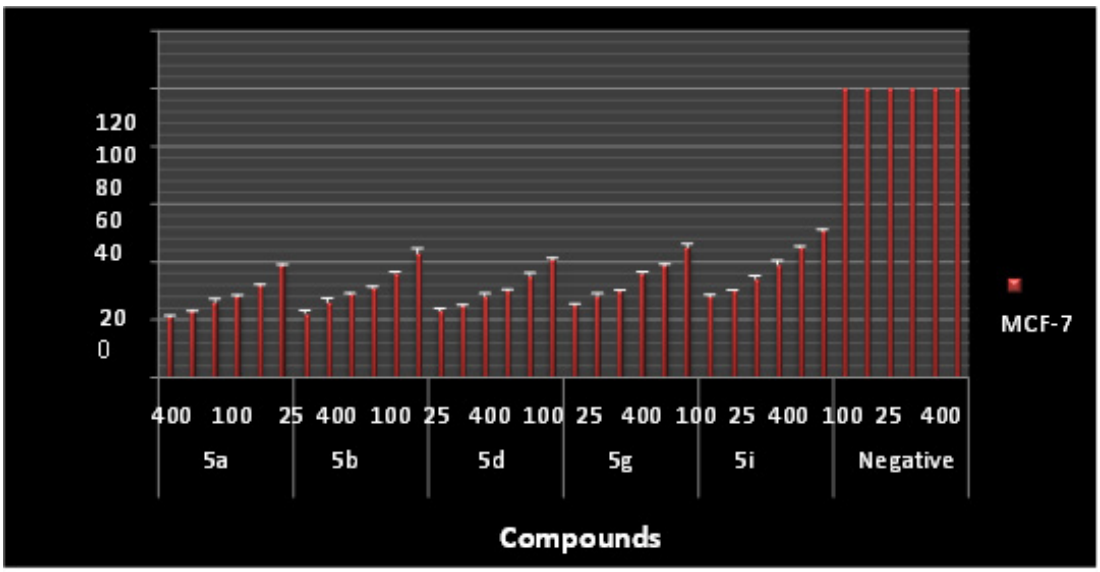


Fig. 16. *In-vitro* cytotoxic potency of synthesized compounds

and Figure 4. . All of the synthesized compounds had promising MIC values against bacterial and fungal strains [24].

Antioxidant Activity (DPPH Assay)

The ability of synthetic compounds 5(a-j) and ascorbic acid(standard) to scavenge free radicals was assessed based on their ability to do so with regard to the DPPH free radical. Different concentrations of the compounds as well as the standard (5, 10, 15, 20 and 25 mg/ml) were prepared in methanol. In clean and clearly labeled test tubes, 3 ml of DPPH solution (0.002% in methanol) was blended with 05, 10, 15, 20 and 25 mg/mL of different concentrations of synthesized compounds and standard individually. Methanol was added to the solution to bring it up to 4 mL.

Table 9. IC_{50} values of synthesized compounds (5a, 5b, 5d, 5g, 5i) against MCF-7 cell lines

Compounds	MCF-7 $IC_{50}(\mu g/mL)$
5a	15.28±0.65
5b	17.63±0.58
5d	13.68±1.74
5g	10.66±1.15
5i	08.77±1.52
Paclitaxel (Positive control)	0.32±0.65

A UV-Visible Spectrophotometer was used to measure the optical density at 517 nm after the tubes had been incubated at room temperature in the dark for 30 minutes. We measured the absorbance of the DPPH control. The Results are graphically represented in Figure 5 and summarised in Table 6. Using the formula, the scavenging activity was determined.

$$\text{Scavenging activity (\%)} = A\text{-}B/A\times 100$$

Where A is the absorbance of DPPH and B is the absorbance of DPPH in standard combination [25].

Molecular docking study

The reported approach was used to complete the molecular docking study [26, 27]. The In-Silco molecular docking procedure was used on the anticancer receptor, PDB code 2A91, the Protein Data Bank (PDB; *http://www.rcsb.org/pdb*) provided the receptor's crystal structure. Prior to screening, the water molecules and heteroatoms were eliminated. Utilizing the protein preparation module of the HEX modelling package 8.0, the receptor structure was built before being used in the docking investigation. During the protein preparation, all hetero and water molecules were removed from the crystal structure except water molecules within 5Å from the ligand. The 3D structure of each ligand together with the receptor binding interactions were visualised to optimise

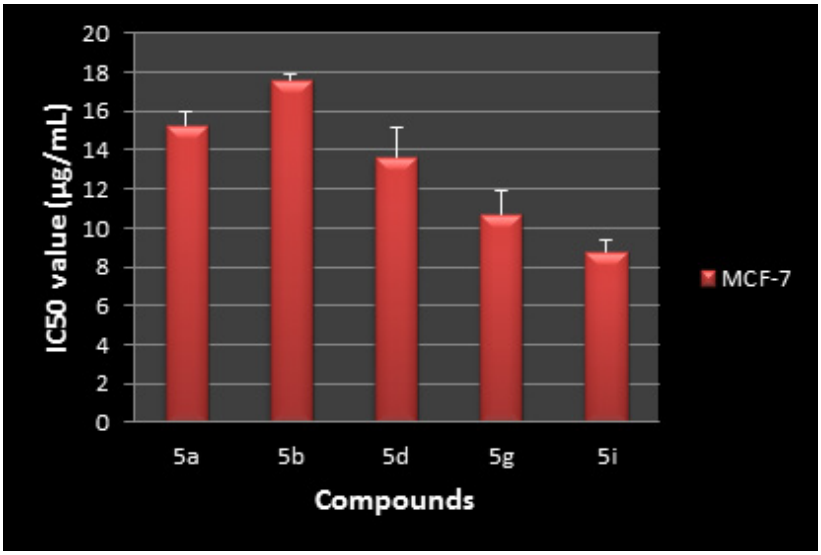


Fig. 17. IC_{50} values of synthesized compounds against MCF-7 cell line in comparison with Paclitaxel (Positive control)

quality by discovery studio 3.2. The results of the *In-silico* molecular docking provide important information on the capacity of recently synthesised drugs to attach to the receptor active sites. Thus, we performed a wet study of anticancer activity using the acquired docking values as a reference. The findings of binding scores of synthesized compounds 5(a-j) are indexed in Table 7 and the 2D and 3D binding orientation of prepared compounds 5(a-j) with receptor 2A91is displayed in Figure 6 to Figure 15.

Anticancer activity [Cell preparation and cell viability]

The in-vitro anticancer activity of the synthesized compounds 5(a-j) were assessed using *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT) against an human cancer cell line MCF-7 (cancer breast)[28]. The assay was observed to be entirely relying on the decrease of the tetrazolium salt via mitochondrial dehydrogenase of viable cells in order to produce a blue formazan product dissolved in DMSO, which was measured at 570nm. With the aid of graph Pad Prism Version 5.1, IC_{50} (µM) data of synthesized compounds were estimated and Paclitaxel was utilized as positive control. The human cancer cell lines were procured from National Centre for Cell Science, Pune, India and Dulbecco's Modified Eagle Medium(DMEM) with low glucose (Cat No-11965-092, Gibco, Invitrogen) was used to culture the cell lines enhanced with 10% fetal bovine serum(CatNo-10270106,Gibco,Invitrogen) and 1% antimycotic (Cat No-15240062, Thermo fisher Scientific) was used for cell culture. Untreated cells were considered as control. The results of the anti-cancer screening are indexed in Table 8 and represented in Figure 16. In addition to this, IC_{50} values of synthesized compounds were also estimated, which are indexed in Table 9 and depicted in Figure 17.

The cells were cultivated in a 96-well flat-bottom microplate and stored overnight at 37ºC in 95% humidity and 5% CO_2. Different sample concentrations (400, 200, 100, 50, 25, 12.5ìg/*ml*) were treated. For an additional 48 hours, the cells were incubated and the wells were washed twice with PBS. Further, 20*iL* of the MTT staining solution was introduced to individual well and plates were incubated at 37ºC. After 4 hours, 100 mL of DMSO was added to each well to dissolve the formazan crystals, and using a microplate reader, the absorbance at 570 nm was measured. The following formulae were used to calculate the cytotoxicity:

$$\text{Cytotoxicity (\%)} = 1 - \frac{\text{Mean absorbance of test compound}}{\text{Mean absorbance of -ve control}} \times 100$$

Cell viability % = 100 - Cytotoxicity %

DISCUSSIONS

A cyclized product of chloro substituted 1, 3-benzoxazole-2-thiol 2 compound has been prepared from 4-Chloro-2-amino-phenol by treating it with carbon disulphide and potassium hydroxide in the presence of methanol [19].The SH group which is present in the compound 2 was undergo substitution reaction with ethyl chloroacetate with the addition of acetone to produce thio ether product ethyl [(4-chloro-1, 3-benzoxazol-2-yl) sulfanyl] acetate 3 [20]. A further treatment of compound 3 with hydrazine hydrate led to the formation of peptide or amide bond formation by the elimination of ethyl alcohol to ptoduce an intermediate 2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]acetohydrazide 4 [21]. ^{1}H NMR characterized compound 4 as having two singlets at ä 4.490 and ä 9.414 ppm due to the presence of -NH_2 and -NH protons respectively. As a result of reacting intermediate 4 with varied aromatic aldehydes, the NH_2 group in the product 4 reacts with aldehyde to produce imine(C=N) bond through condensation reaction, derivatives *2-[(4-chloro-1, 3-benzoxazol-2-yl) sulfanyl]-N'-[-phenylmethylidene] acetohydrazides* 5(a-j) have been obtained [22]. The newly synthesized molecules displayed intense absorbance band at 1660cm^{-1} for -NH and 1692 cm^{-1} for -C=O groups in IR spectrum and the ^{1}H NMR revealed a peak at ä 11.836 (bs, -NH) justifying the disappearance of NH_2 proton and the formation of new ring by insertion reaction [29]. In addition, the mass peak also correlated with the molecular weight of the synthesized molecules.

Studies have also been performed on the synthesized molecules 5(a-j) for their antibacterial, antifungal, MIC, antioxidant and cytotoxic activity. Based on the results of antibacterial and antifungal studies, few compounds have demonstrated potent zone of inhibitions, as shown in Table 2 and Table 3 and Figures 1 and 2. Comparatively to

standard drugs Chloramphenicol and Fluconazole, compounds 5a, 5b, 5d, 5e, 5g and 5h displayed marked zones of inhibition against bacteria and fungi. At different concentrations, the compounds were explored for their Minimum Inhibitory Concentration (MIC) to determine their distinct zones of inhibition against bacteria and fungi and Tables 4 and 5 and Figures 3 and 4 illustrate the results of this analysis. A marked zone of inhibition was noted for compounds 5a, 5b, 5d, 5e, 5g and 5h against gram positive and gram negative bacteria at four various concentrations (*100g/ml, 50g/ml, 25g/ml and 12.5g/ml)*. In spite of concentration differences, chloro, nitro, methoxy and hydroxy substituted benzoxazole derivatives showed significant efficacies. This observation is favoured by antioxidant activity, which was done with effective free radical scavenge as outlined in Table 6 and Figure 5 respectively. The derivatives 5(a-j) exhibited powerful free radical scavenging properties.

In order to become better acquainted with the binding energies and types of binding interactions of the prepared compounds, molecular docking was performed on the synthesized compounds. Compared to the rest of the prepared compounds, the synthesized compound 5i possessed admirable binding scores (-322.59 kcal/mol). The binding score obtained from the molecular docking study and also by considering the similar structures of newly prepared compounds, where only the position of substituent differs, few of the selected compounds were screened for their cytotoxic activity against MCF-7 cell line and the observations are tabulated in Table 8 and represented in Figure 16 [30]. Following the binding scores of docking study and considering that the only difference between newly prepared compounds is the position of the substituents, few compounds were selected for cytotoxic testing against MCF-7 cells. At the least concentration of 12.5 g/mL, both compounds 5g and 5i displayed impressive inhibitory activity of 45% and 51%, respectively. Also, compound 5i demonstrated potential activity for MCF-7 cell line with an IC_{50} value of 8.77*μg/mL*

CONCLUSION

Current work comprises of series of the synthesis of novel *2-[(4-chloro-1,3-benzoxazol-2-yl)sulfanyl]-N'-[(-phenylmethylidene] acetohydrazide* 5(a-j) derivatives. The expected target molecules were prepared, structurally confirmed by using IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis. They were also subjected to various biological activities, which includes antimicrobial, antioxidant and *in-vitro* cytotoxic activity. Among the synthesized compounds 5g and 5i were found to exhibit increased potency and considered as potential molecules for further toxicological development of drugs.

ACKNOWLEDGEMENT

The authors are thankful to the Directorate of minorities, Bangalore, Karnataka, India, for financial support. The authors are grateful to the Principal, Sahyadri Science College, Shivamogga for providing the necessary research facilities. We are also grateful to Sophisticated Analytical Instruments Facility, Mysore University, Karnataka India, MIT Manipal for providing ^{1}HNMR, ^{13}C NMR and Mass spectral facilities.

Conflict of interest:

There is no conflict of interest.

Funding Sources

There is no funding sources.

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