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Short communication

Synthesis, *in vitro* antioxidant, anthelmintic and molecular docking studies of novel dichloro substituted benzoxazole-triazolo-thione derivatives

R.V. Satyendra [a,1], K.A. Vishnumurthy [a,*], H.M. Vagdevi [b], K.P. Rajesh [c], H. Manjunatha [c], A. Shruthi [b]

[a] Department of P.G. Studies and Research in Industrial Chemistry, Sir M.V. Government Science College, Bommanakatte, Bhadravathi 577302, Karnataka, India
[b] Department of P.G. Studies and Research in Chemistry, Sahyadri Science College (Autonomous) Kuvempu University, Shivamogga 577203, Karnataka, India
[c] Department of P.G. Studies and Research in Biotechnology and Bioinformatics, Jnanasahyadri, Kuvempu University, Shankaraghatta 577451, Karnataka, India

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A B S T R A C T

A novel 6,8-dichloro [1,2,4]triazolo [3,4-*b*] [1,3]benzoxazole-3(2*H*)-thione **4** and its derivatives **5a** and **5b** are synthesized from 5,7-dichloro-2-hydrazinyl-1,3-benzoxazole **3**, obtained by reaction of hydrazine hydrate with ethyl [(5,7-dichloro-1,3-benzoxazol-2-yl)sulfanyl]acetate **2**. The newly synthesized compounds are characterized by analytical ^{1}H NMR, ^{13}C NMR, LC-MS mass spectrometry and elemental analysis. All synthesized compounds are screened for *in vitro* antioxidant and anthelmintic activities. In correlation to anthelmintic activity, compounds are subjected to molecular docking studies for the binding to β-Tubulin, target protein elite to the parasites.

Compounds **3**, **4** and **5a** exhibited potential radical scavenging capacity with good anthelmintic activity. In molecular docking study also, compounds showed minimum binding energy and have good affinity toward the active pocket thus, they may be considered as good inhibitor of β-Tubulin.
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1. Introduction

Helminths infections are a medical and public health problem of high magnitude, both in humans and domestic animals, causing considerable suffering and poor growth. In addition, helminths of livestock pose a serious economic loss, in particular in areas where extensive grazing is practiced. Effective anthelmintic drugs, used to treat and control these infestations, must have selective toxic effects on these parasites. Unfortunately with the increased use of these compounds, anthelmintic resistance has appeared and increased in frequency [1]. If resistance to a particular antehehnintic has occurred, there is clearly a critical need for the development of new effective anthelmintic drugs tolerated by the host. Among the heterocyclic compounds, derivatives of benzoxazole have gained much importance because of its wide applications in medicinal sector. In recent years triazoles are associated with diverse pharmacological activities such as analgesic, antiasthmatic, diuretic, antihypersensitive, anticholinergic, antibacterial, antifungal and anti-inflammatory activity [2–6]. Both triazole and benzoxazole are biologically potent molecules, so it was planned to synthesize new triazole derivatives of benzoxazole and screen against anthelmintic and antioxidant activities. As the intermediates have slight structural resemblance with Albendazole, along with the triazolo derivative, intermediates were also subjected for *in vitro* anthelmintic and antioxidant activities.

Some of the anthelmintic drugs showed activity by binding selectively to β-Tubulin of nematodes, cestodes and fluke, a protein subunit of microtubule and thereby disrupting microtubule structure and function [7–9]. Microtubules are highly dynamic, ubiquitous cellular organelles serving a variety of vital functions including mitosis, motility and transport, in all eukaryotes. Many of these structures exist in a dynamic equilibrium in which assembly and disassembly of the soluble subunits are balanced. In such systems, the drug–tubulin interaction results in a shift of this equilibrium with a net loss of microtubules and accumulation of free tubulin. In view of the crucial roles, that microtubules play in many cellular processes, their drug-induced destruction eventually leads to the death of the organism [9]. *In silico* molecular docking technique play an important role in the drug design and discovery to predict the conformations of each ligand molecule at the active site, hence, the *in silico* (molecular docking) studies of newly synthesized compounds were carried out to predict the β-Tubulin inhibitory activity and results are reported.

* Corresponding author. Tel./fax: +91 8282 270296.
E-mail address: dr.kavishnumurthy@gmail.com (K.A. Vishnumurthy).
[1] Present address: BASF India Ltd., Surathkal-Bajpe Road, Mangalore 575 030, Karnataka, India.

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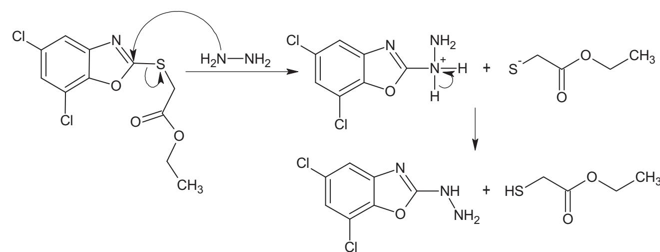
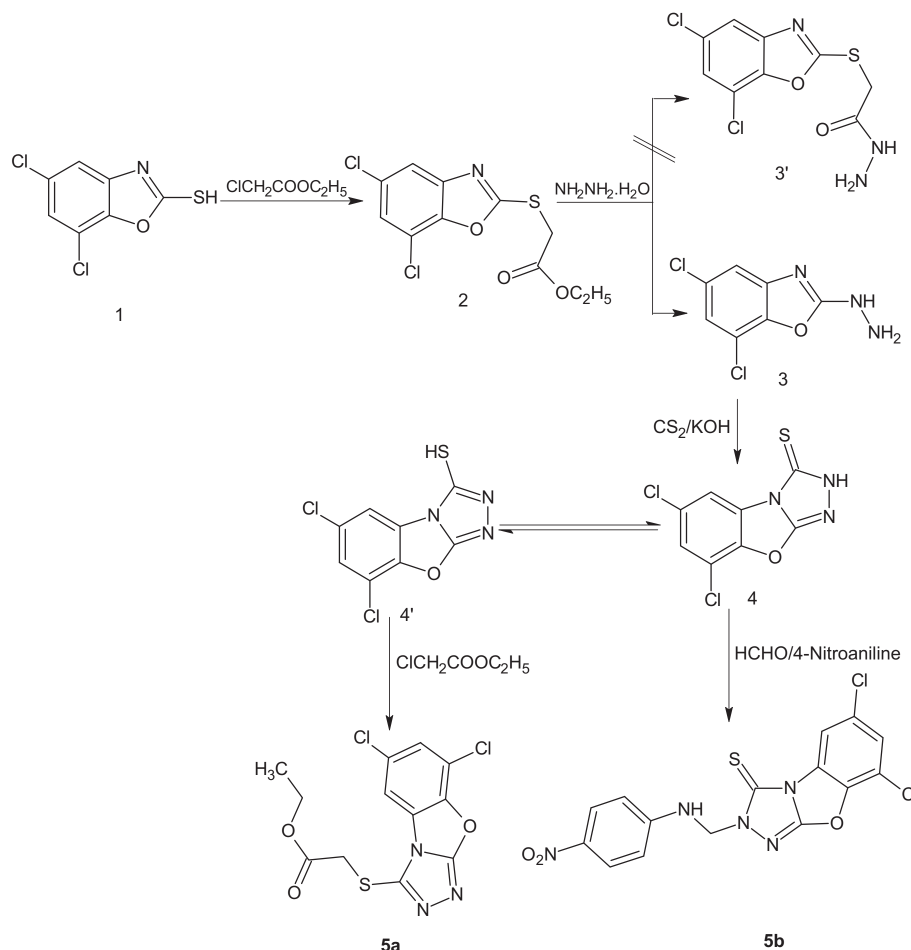
2. Result and discussion

2.1. Chemistry

6,8-Dichloro [1,2,4]triazolo [3,4-*b*] [1,3]benzoxazole-3(2*H*)-thione **4** and its derivatives **5a** and **5b** were synthesized from 5,7-dichloro-2-hydrazinyl-1,3-benzoxazole **3**. Compound **3** was obtained by anomalous reaction of hydrazine hydrate with ethyl [(5,7-dichloro-1,3-benzoxazol-2-yl)sulfanyl]acetate **2** (Scheme 1). Usually thioacetyle hydrazides were formed by reaction of ester derivative of thiols with hydrazine hydrate [10] and thioacetyle hydrazides undergo rearrangement in presence of base leading to the formation of hydrazine derivative [11]. It has been predicted that the presence of the two chlorine atoms on the benzoxazole ring in compound 2 renders the carbon linked to sulfur very electrophilic. Then hydrazine hydrate could possibly react directly at this (C-2) position ejecting ethyl mercaptoacetate. The reaction mechanism is proposed as provided in Scheme 2. Structure of newly synthesized compounds was confirmed by analytical ^{1}H NMR, ^{13}C NMR, LC-MS mass spectrometry and elemental analysis. Absence of singlet at δ 4.31 for 2H of $-CH_2$, presence of broad singlet at δ 4.68 for 2H of $-NH_2$ (D_2O exchangeable) and at δ 9.33 for 1H of $-NH$ (D_2O exchangeable) in ^{1}H NMR spectra of **3** confirmed the structure of the molecule. It was further supported by recording ^{13}C NMR spectrum and the signals appeared in the spectrum accounts for all C-atoms present in molecule **3**. In ^{1}H NMR of **4**, presence of singlet at δ 9.95 for 1H of $-NH$, two singlet at δ 7.12 and δ 7.97 for two aromatic proton confirmed the structure. Presence of weak singlet at δ 13.65 for $-SH$ proton in ^{1}H NMR of **4** indicates tautomeric nature of the compound. The ^{13}C spectra of **4** has indicated signal at δ 183.15 for $-C{=}S$ carbon of triazole ring. The molecule **5a** was confirmed by the appearance of triplet at δ 1.22 for 3H of $-CH_3$ and quartet at δ 4.19 for 2H of $-CH_2$ in its ^{1}H NMR. The signal at δ 169.04 in ^{13}C spectra of **5a** confirmed $-N{=}C{-}S$ carbon of triazole ring. The molecule **5b** was confirmed by the presence of singlet at δ 8.33 for 1H of $-NH$ and doublet at δ 5.70 for 2H of $-CH_2$ in ^{1}H NMR of the molecule. Signal at δ 181.23 for $-C{=}S$ carbon of triazole ring, in ^{13}C NMR, confirmed the formation of Mannich base. The structure of all the molecules was further confirmed by ^{13}C spectrum and LCMS mass spectrometry. Yield, melting point and elemental analysis data are tabulated in Table 1.



Scheme 2. Proposed reaction mechanism for formation of 5,7-dichloro-2-hydrazinyl-1,3-benzoxazole **3**.



Scheme 1. Synthesis of 6,8-dichloro [1,2,4]triazolo [3,4-*b*] [1,3]benzoxazole-3(2*H*)-thione **4** and its derivatives.

Table 1
Characterization data (**1**–**4**,**5a** and **5b**).

Compound no.	MP (°C)	% yield	Elemental analysis found [calculated]		
			C	H	N
1	217–220	84.25	38.18 [38.20]	1.41 [1.37]	6.32 [6.36]
2	41–43	86.35	43.20 [43.15]	2.98 [2.96]	4.60 [4.57]
3	179–181	85.02	38.59 [38.56]	2.27 [2.31]	19.30 [19.27]
4	203–205	80.00	36.98 [36.94]	1.11 [1.16]	16.15 [16.16]
5a	93–95	84.25	41.60 [41.63]	2.59 [2.62]	12.16 [12.14]
5b	195–197	82.20	43.97 [43.92]	2.20 [2.21]	17.10 [17.07]

2.2. Biological evaluation

All the synthesized compounds were screened for biological activities such as *in vitro* antioxidant (total antioxidant capacity, total reductive capability and DPPH radical scavenging activity), *in vitro* anthelmintic and *in silico* β-Tubulin inhibitory activity.

2.2.1. Antioxidant activities

2.2.1.1. Total antioxidant capacity. Total antioxidant capacity of all the synthesized compounds was performed by phosphomolybdenum method as described by Prieto et al., 1999 [12]. Antioxidant capacities are expressed as equivalents of ascorbic acid. Among the tested compounds, **3**, **4**, **5a** and **5b** have shown antioxidant activity as equivalents to ascorbic acid. The results of total antioxidant activity were presented in Fig. 1.

2.2.1.2. Total reductive capability. The reductive ability of compounds was determined as according to the method of Oyaizu, 1986 [13]. Quercetin was used as a standard to compare the activity of synthesized compounds. Compounds **3** and **4** have shown equipotent reductive ability and compounds **5a**, **5b** have shown moderate activity whereas **1** and **2** were less active. The results are presented in Fig. 2.

2.2.1.3. 1, 1-dDiphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. All the synthesized compounds were screened for free radical scavenging activity by DPPH method [14]. All compounds have exhibited free radical scavenging capacity by comparison with the standard Butylated Hydroxytoulene (BHT). Among the tested compounds, **1**, **3**, **4** and **5a** have exhibited promising radical scavenging activity, as compared with standard. The variation exhibited in DPPH scavenging capacity could be attributed to the effect of different substitutions and results are documented in Table 2.

2.3. Anthelmintic studies

All the synthesized compounds were screened for *in vitro* anthelmintic activity against *Pheretima posthuma* owing to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings for preliminary evaluation of anthelmintic activity [15–17]. It is evident from Table 3 that, all compounds have exhibited anthelmintic activity in dose-dependent manner giving shortest time of paralysis and death with 3% concentration. Among the tested compounds, the compound **3**, **4** and **5a** have emerged as highly active against *P. posthuma* whereas other compounds are found to be less active in comparison with standard Albendazole at 1% concentration.

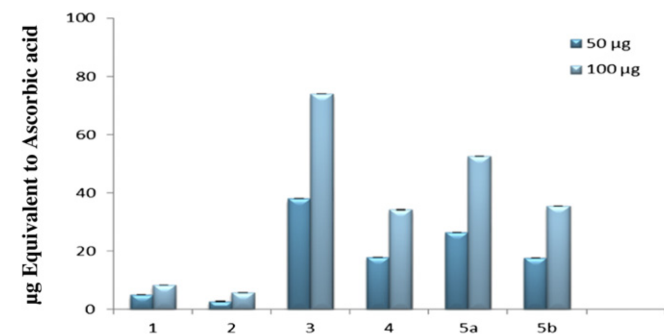


Fig. 1. Total antioxidant capacity of the compounds (**1**–**5b**).

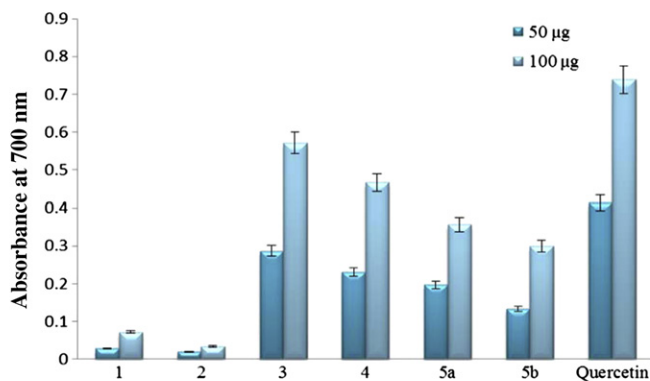


Fig. 2. Total reductive capability of the compounds (**1**–**5b**).

The enhanced activity of the compounds could be attributed to the slight structural resemblance with standard drug. Both albendazole and benzoxazole have benzene ring fused with five membered heterocyclic ring which contains two hetero atoms but differs in one hetero atom and side chains. However, based on the results it is not appropriate to arrive at the conclusion of structure activity aspects of the moieties and further evaluation is necessary for their clinical use.

2.4. Molecular docking studies

In correlation to *in vitro* anthelmintic activity it is thought worthwhile to carryout *in silico* studies to support the *in vitro* activity. Automated docking was used to assess the orientation of inhibitors bound in the active pockets of β-Tubulin. A Lamarckian genetic algorithm method, implemented in the program AutoDock4.2, was employed. Fig. 3 showed structure of modeled β-Tubulin (PDB ID: 1OJ0) [18]. The molecular docking of ligand molecules **1**–**4**, **5a** and **5b** with β-Tubulin revealed that, all the compounds have exhibited the bonding with one or the other amino acids in the active pockets which is showed in Fig. 4. The ligands were designed and the structure was analyzed using ChemDraw Ultra 6.0. 3D coordinates of ligand molecules were prepared using PRODRG server (http://davapc1.bioch.dundee.ac.uk/prodrg/). The protein structure file (PDB ID: 1OJ0) taken from PDB (www.rcsb.org/pdb) was edited by removing the hetero atoms, adding C-terminal oxygen. Fig. 5 shows the *in silico* active pocket prediction of amino acids of protein β-Tubulin involved in binding

Table 2
DPPH radical scavenging activity of synthesized compound.

Sl. no.	Mol. no.	Percentage of inhibition
1	**1**	32.113 ± 0.72
2	**2**	9.488 ± 0.051
3	**3**	81.520 ± 0.59
4	**4**	74.850 ± 0.14
5	**5a**	65.670 ± 1.54
6	**5b**	17.980 ± 0.98
7	**BHT**	16.214 ± 0.678

Each value represents mean ± SE; $n = 3$.

Table 3
Anthelmintic activities of compounds.

Compounds/parameters	Concentration [%]	**1**	**2**	**3**	**4**	**5a**	**5b**	**Albendazole**
Paralytic time in (min)[a]	1%	8.22 ± 1.70	6.61 ± 0.81	4.42 ± 0.67	5.48 ± 1.09	5.92 ± 0.69	7.95 ± 1.01	5.37 ± 1.08
	2%	7.27 ± 1.34	5.87 ± 0.90	3.57 ± 0.50	4.29 ± 0.92	4.74 ± 0.72	6.58 ± 1.23	–
	3%	3.20 ± 0.74	5.28 ± 1.02	3.08 ± 0.39	3.82 ± 0.72	4.00 ± 0.53	5.97 ± 1.05	–
Death time in (min)[a]	1%	9.05 ± 1.68	7.23 ± 1.16	4.77 ± 0.54	6.48 ± 0.82	6.13 ± 0.94	8.33 ± 0.85	7.20 ± 1.95
	2%	7.83 ± 1.57	6.37 ± 1.36	4.22 ± 0.66	5.49 ± 1.01	5.95 ± 0.91	7.07 ± 0.96	–
	3%	5.95 ± 1.13	5.75 ± 1.11	3.23 ± 0.42	4.12 ± 0.77	4.85 ± 1.10	6.64 ± 1.00	–

[a] Mean ± SEM, n = 6.

with the ligand obtained from PDB sum. For docking calculations, Gasteigere-Marsili partial charges were assigned to the ligands and nonpolar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The Lamarckian genetic algorithm and the pseudo-Solis and Wets (pSW) methods were applied for minimization, using default parameters. Theoretically all the ligand molecules showed encouraging binding energy. Among the six molecules, docking of β-Tubulin with **3**, **4** and **5a** revealed that their binding energy were −8.37 kJ mol^{-1}, −7.05 kJ mol^{-1} and −7.22 kJ mol^{-1} respectively and it is considered as good inhibitor of β-Tubulin. In *in vitro* studies also **3**, **4** and **5a** has emerged as active against *P. posthuma*. It can be predicted that the activity may be due to inhibition of β-Tubulin of helminths and interfering with microtubule dynamics, consequently disturbing microtubule-based processes.

3. Conclusion

The data reported herein indicates that compound **3**, **4** and **5a** has emerged as potentially active compounds as anthelmintic and antioxidant compounds. These molecules have shown significant results as compared to standard drug. In molecular docking studies ligand molecules showed minimum binding energy and increased affinity with the protein and it was found that hydrogen bond formation with amino acid residues of active pocket may be responsible for the anthelmintic activity as referred to Albendazole.

According to these results, we can conclude that compounds **3**, **4** and **5a** appears to be the most interesting compound among the newly synthesized and seem potentially attractive as anthelmintic drug.

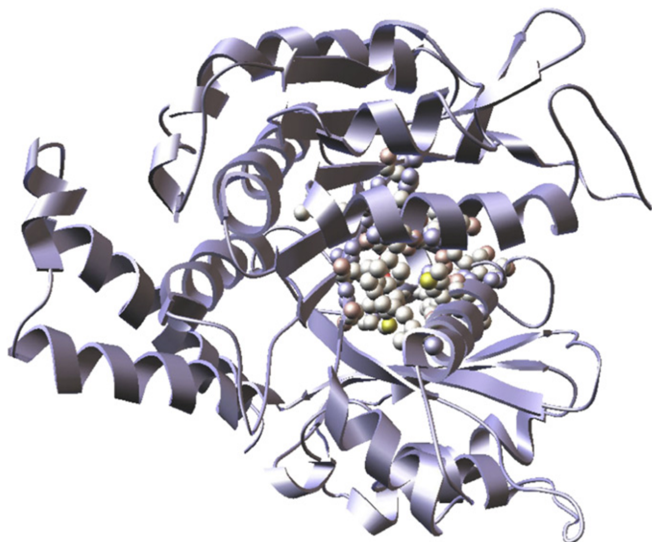


Fig. 3. Structure of β-Tubulin (PDB ID: 1OJ0).

4. Experimental

4.1. Chemistry

Melting points were recorded on electrothermal melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 400 MHz spectrometer IISc, Bangalore, Karnataka, India. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as internal standard. LC-MS were obtained using C 18 column on Shimadzu, LCMS 2010A, Japan. The FT-IR spectra of compound were taken in KBr pellet (100 mg) using Shimadzu Fourier transformed infrared (FT-IR) spectrophotometer. Column chromatography was performed using a silica gel (230–400 mesh). Elemental analysis was carried out using Vari-oMICRO V1.7.0 (Elemental Analysersysteme GmbH). Silica gel GF254 plates from Merck were used for TLC and spots located either by UV, dipping in potassium permanganate solution.

The chemicals were purchased from Sigma–Aldrich Co. and solvents for column chromatography were of reagent grade and were purchased from commercial source.

4.1.1. *5,7-Dichloro-1,3-benzoxazole-2-thiol (**1**)*

A mixture of 2-amino-4,6-dichlorophenol (0.1 mol), potassium hydroxide (0.1 mol) and methanol (100 ml) was taken in a round bottomed flask and carbon disulphide (0.1 mol) was added drop wise to the mixture with constant stirring in ice cold condition. Then the reaction mixture was refluxed for 8 h, poured onto crushed ice and acidified with acetic acid (pH 6). The separated product was filtered, dried and recrystallized from ethanol. The structure was assigned to 5,7-dichloro-1,3-benzoxazole-2-thiol **1** by melting point and standard ^{1}H NMR Data.

4.1.2. *Ethyl [(5, 7-dichloro-1,3-benzoxazol-2-yl)sulfanyl]acetate (**2**)*

Equimolar quantity of 5,7-dichloro-1,3-benzoxazole-2-thiol **1** (0.1 mol) and ethyl chloroacetate (0.1 mol) was taken in dry acetone (40 ml) containing anhydrous potassium carbonate (5 g) and refluxed on water bath for 10 h. Then the reaction mixture was poured onto crushed ice, solid product thus obtained was filtered, dried and recrystallized from ethanol. IR (KBr, ν_{max} cm^{-1}): 1595 (C=N), 1737 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.77 (s, H, C-6), 7.61 (s, H, C-4), 4.31 (s, 2H, −S−CH_2 proton), 4.16 (q, 2H, J = 8 Hz, CH_2 protons of ester), 1.19 (t, 3H, J = 8 Hz, −CH_3). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 167.94 (C=O), 163.16, 147.90, 143.91, 130.78, 124.96, 117.77, 116.16, 62.89 (O−CH_2), 34.85 (S−CH_2), 14.59 (CH_3). MS (LCMS): m/z 306 [M+], 308 [M+2], 310 [M+4].

4.1.3. *5,7-Dichloro-2-hydrazinyl-1,3-benzoxazole (**3**)*

A mixture of ethyl [(5,7-dichloro-1,3-benzoxazol-2-yl)sulfanyl] acetate **2** (0.1 mol) and hydrazine hydrate (0.2 mol) in methanol (30 ml) was stirred for 30 min. The obtained solid was filtered, dried and recrystallized from dimethyl formamide. IR (KBr, ν_{max} cm^{-1}): 1575 (C=N), 3356 (N−H). ^{1}H NMR (DMSO, 400 MHz): δ 9.33 (s, 1H, NH, disappeared on D_2O exchange), 7.29 (s, H, C-6), 7.17 (s, H, C-4), 4.68 (s, 2H, NH_2, disappeared on D_2O exchange). ^{13}C

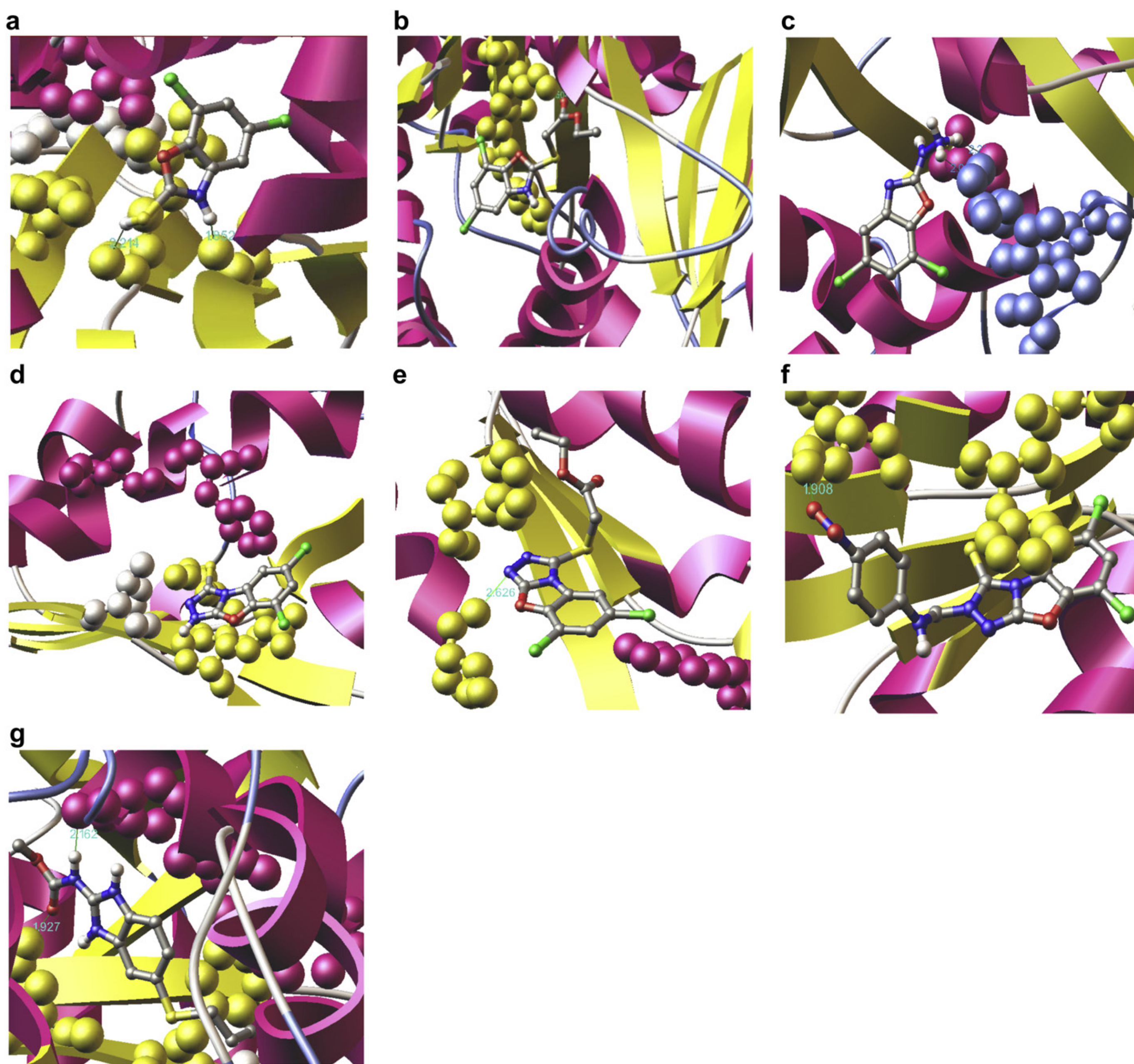


Fig. 4. Binding mode of ligand molecule, a. 1 with β-Tubulin, b. 2 with β-Tubulin, c. 3 with β-Tubulin, d. 4 with β-Tubulin, e. 5a with β-Tubulin, f. 5b with β-Tubulin, h. Albendazole with β-Tubulin.

NMR (DMSO, 400 MHz): δ 166.63, 146.58, 144.41, 129.33, 120.48, 115.25, 113.93. MS (LCMS): *m/z* 218 [M+], 220 [M+2], 222 [M+4].

4.1.4. 6,8-Dichloro [1,2,4]triazolo [3,4-b] [1,3]benzoxazole-3(2H)-thione (*4*)

The compound 2-[(5,7-dichloro-1,3-benzoxazol-2-yl)sulfanyl] acetohydrazide **3** (0.003 mol) was dissolved in a solution of potassium hydroxide (0.006 mol) in water (2 ml) and ethanol (20 ml). Carbon disulfide (2 ml) was then added under stirring and the reaction mixture was heated under reflux for 8 h. The reaction mass was poured onto crushed ice and neutralized with acetic acid. The separated product was filtered, washed with water, dried and recrystallized from ethanol to get **4**. IR (KBr, ν_{max} cm^{-1}): 1295 (C=S), 1570 (C=N). ^{1}H NMR (DMSO, 400 MHz): δ 9.95 (s, 1H, NH proton), 7.97 (s, H, C-7), 7.12 (s, H, C-5). ^{13}C NMR (DMSO, 400 MHz): δ 183.15 (C=S), 157.16, 141.84, 132.66, 124.52, 122.40, 122.33, 117.41. MS (LCMS): *m/z* 260 [M+], 262 [M+2], 264 [M+4].

4.1.5. Ethyl [(6,8-dichloro [1,2,4]triazolo [3,4-b] [1,3]-benzoxazol-2-yl)sulfanyl]acetate (*5a*)

Equimolar quantity of 6,8-dichloro [1,2,4]triazolo [3,4-b] [1,3] benzoxazole-3(2H)-thione **4** (0.1 mol) and ethyl chloroacetate (0.1 mol) was taken in dry acetone (40 ml) containing anhydrous potassium carbonate (5 g) and refluxed on water bath for 8 h. Then the reaction mixture was poured onto crushed ice, solid product thus obtained was filtered, dried and recrystallized from ethanol to obtain **5b** IR (KBr, ν_{max} cm^{-1}): 1579 (C=N), 1735 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.39 (s, H, C-7), 7.18 (s, H, C-5), 4.19 (q, 2H, *J* = 8 Hz, CH_2 protons of ester), 4.03 (s, 2H, –S–CH_2 proton), 1.22 (t, 3H, *J* = 6.8 Hz,–CH_3). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 171.54 (C=O),

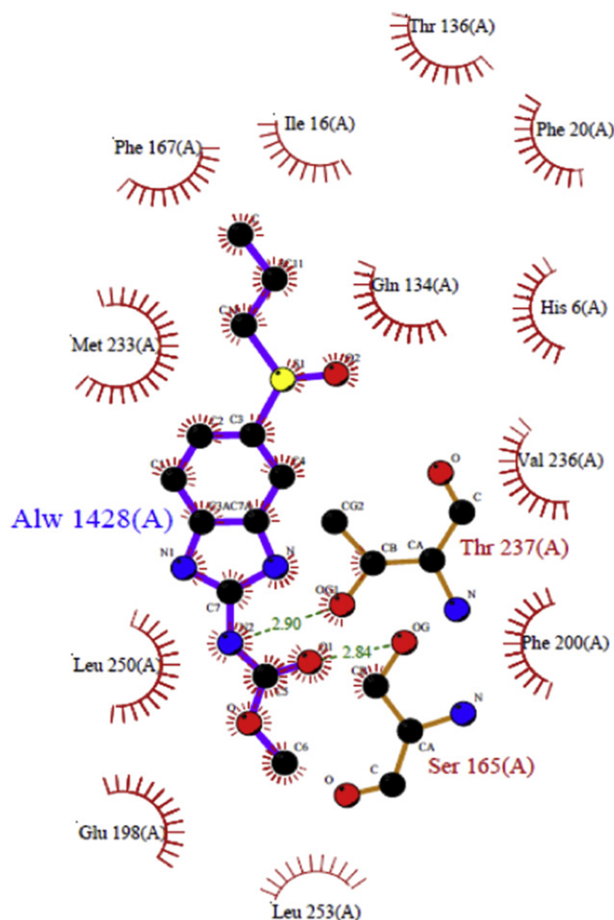


Fig. 5. Ligplot results for β-Tubulin. Sowing the binding of ligand Alw (*Methyl 5-(propylsulfinyl)-1h-benzimidazol-2- ylcarbamate [Albendazole sulphoxide]*) on A-chain amino acids present in an active pocket of β-Tubulin.

169.04 (N=C–S), 151.76, 141.04, 132.68, 129.85, 122.14, 116.28, 115.81, 62.00 ($O-CH_2$), 33.48 ($S-CH_2$), 14.15 (CH_3). MS (LCMS): m/z 346 [M+], 348 [M+2], 350 [M+4].

4.1.6. 6,8-Dichloro-2-{[(4-nitrophenyl)amino]methyl} [1,2,4] triazolo [3,4-b] [1,3]benzoxazole-3(2H)-thione (**5b**)

Formaldehyde (0.1 ml) was added to a stirred solution of 6,8-dichloro [1,2,4]triazolo [3,4-b] [1,3]benzoxazole-3(2H)-thione **4** (0.0025 mol) in absolute ethanol (10 ml). The ethanolic solution of (10 ml) of 2-chloroaniline (0.0025 mol) was added portion wise to the reaction mixture and stirred overnight at room temperature. The precipitate formed was filtered, dried and recrystallized with ethanol to obtain **5b**. IR (KBr, ν_{max} cm^{-1}): 1245 (C=S), 1576 (C=N), 3345 (N–H). 1245 (C=S). ^1H NMR (DMSO, 400 MHz): δ 8.33 (s, H, NH proton, disappeared on D_2O exchange), 7.06–8.08 (m, 6H, aromatic proton), 5.70 (d, J = 8 Hz, 2H, $-CH_2$ protons). ^{13}C NMR (DMSO, 400 MHz): δ 181.23 (C=S), 153.96, 153.75, 141.88, 138.66, 132.26, 126.92, 124.59, 122.83, 122.31, 117.31, 113.08, 57.33 (CH_2). MS (LCMS): m/z 410 [M+], 412 [M+2], 414 [M+4].

4.2. Biological activities

4.2.1. Antioxidant activities

4.2.1.1. Total antioxidant capacity. The test compounds (300 µl) in absolute alcohol at two different concentrations (50 µg and 100 µg) were taken in separate test tubes. To this, 3 ml of reagent mixture containing 4 mM ammonium molybdate, 0.6 M sulfuric acid and 28 mM of sodium phosphate was added. Test tubes were kept for incubation at 95 °C for 90 min and allowed to cool. Absorbance of the content of each test tube was measured at 695 nm (Systronics, PC based double beam spectrophotometer 2202) against a blank. Antioxidant capacity of each compound is expressed as equivalents of ascorbic acid. Ascorbic acid equivalents are calculated using standard graph of ascorbic acid. Test was performed in triplicate, and the results were averaged.

4.2.1.2. Total reductive capability. The 1 ml of test compound solution at two different concentrations (50 µg and 100 µg) in absolute alcohol was mixed with phosphate buffer (2.5 ml, 0.2 mol/L, pH 6.5) and potassium ferricyanide (2.5 ml, 1%). Then the mixture was incubated at 50 °C for 20 min. At the end of the incubation, trichloroacetic acid (2.5 ml, 10%) was added to the mixture, which was centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was collected and mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank. Increased absorbance of the reaction mixture indicated increased reducing power. Test was performed in triplicate, and the results were averaged.

4.2.1.3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Free radical scavenging activity of the test compounds were carried based on the scavenging activity of stable DPPH. Test compounds in absolute alcohol (20 µg ml^{-1}) were added to each test tubes and volume was made up to 4 ml using absolute alcohol. To this 3 ml of 0.004% DPPH in 95% ethanol was added and the mixtures were incubated at room temperature under dark condition for 30 min. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm. Radical scavenging activity was calculated using the formula: % of Radical scavenging activity = $([(A_{control} - A_{test})/A_{control}]) \times 100$, where $A_{control}$ is the absorbance of the control sample (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution + test compound). The DPPH radical scavenging activity of BHT (final concentration, 20 µg/4 ml) was also assayed for comparison. Test was performed in triplicate, and the results were averaged.

Table 4
In silico ADMET properties of synthesized compound.

Mol. no.	Mutagenic	Tumerogenic	Irritation	Reproductive effect	cLog P	Solubility	Drug likeness	Drug score
1	No	No	No	No	3.69	−4.81	−3.87	0.35
2	No	No	High risk	No	5.69	−5.35	−7.33	0.14
3	No	No	No	No	1.96	−4.61	−4.9	0.38
4	No	No	No	No	4.03	−5.2	1.84	0.57
5a	No	No	No	No	3.89	−4.9	−2.25	0.34
5b	Medium risk	No	No	No	4.72	−6.98	−8.25	0.15
Albendazole	Yes	No	No	Yes	3.48	−4.1	−2.11	0.15

Table 5
Molecular docking results with β-Tubulin.

Molecule	Binding energy	Intermol energy	Torsional energy	RMS	H-bonds	Bonding	Bond length (Å)
1	−6.7	−7.0	0.3	0.0	2	1::DRG1:HAG:β-tubulin:A:SER165:O	2.214
						1::DRG1:HAA:β-tubulin:A:GLN134:O	1.852
2	−2.43	−3.92	1.49	0.0	1	2::DRG1:OAM:β-tubulin:A:SER165:HG	1.504
3	−8.37	−7.97	0.6	0.0	2	3::DRG1:HAA:β-tubulin:A:VAL236:O	2.001
						3::DRG1:HAC:β-tubulin:A:THR237:OG1	2.215
4	−7.05	−7.05	0.0	0.0	0	–	–
5a	−7.22	−8.71	1.49	00	1	5a1::DRG1:NAJ::β-tubulin:A:SER165:O	2.626
5b	−2.04	−1.18	1.19	00	1	5b::DRG1:OAZ: β-tubulin:A:SER165:HG	1.908
Albendazole	−8.47	−9.67	1.19	0.0	2	Albendazole::DRG1:HAB:β-tubulin:A:VAL236:O	2.162
						Albendazole::DRG1:OAC:β-tubulin:A:SER165:HG	1.927

4.3. *Anthelmintic activity*

The anthelmintic assay was carried out as per the method of Ajayieoba et al. (2001) with appropriate modifications [19]. *P. posthuma* of nearly equal size (6 ± 1 cm) were collected from Vermicompost manufacturing farm. Worm type was identified at the Agriculture Research Station, Shivamogga, Karnataka. The worms were acclimatized to laboratory conditions before experimentation. The earth worms were divided into three groups of six each. Albendazole diluted with normal saline solution to obtain 1% (m/V) served as standard and is poured into Petri dishes. The synthesized compounds were dissolved/suspended in minimal quantity of tween 80 and diluted to prepare three concentrations of 1%, 2% and 3% (m/V) of each compound. Normal saline served as a control. The time taken for complete paralysis and death was recorded. The mean paralysis time and mean lethal time were calculated for each compound (each reading was taken in triplicate). The time taken for worms to become motionless was noted as paralysis time. To ascertain death, each worm was frequently subjected to external stimuli that stimulate and induce movement in earth worms, if alive.

4.4. *Molecular docking studies*

The synthesized molecules were subjected for molecular docking by calculating the minimum energy to inhibit the target protein involved in the microtubule formation. The ligands were drawn in ChemDraw Ultra 6.0 assigned with proper 2D orientation (ChemOffice package) and the structure of each ligand was analyzed by using Chem-3D Ultra 6.0 (ChemOffice package) and was checked for the error in bond order. ADMET property was achieved (Table 4) through Organic chemistry portal (http://www.organic-chemistry.org/prog) web-based application for predicting *in silico* ADMET. Energy of the molecules was minimized using Dundee PRODRG2 Server. Then the file was opened in SPDB viewer and C-terminal Oxygen was added using fit module property. Active pockets were identified and ligplot of PDB Sum provided in the external links of PDB for the proteins was downloaded from PDB. CASTp (Computed Atlas of Surface Topography of proteins) server was used to cross-check the active pockets on target protein molecules. Autodock V4.2 was used to perform Molecular Docking. The docking results for ligand molecules against β-Tubulin [PDB Id: 1OJ0], showed minimum binding energy, inhibition constant, intermolecular energy with 0.0 RMS as documented in Table 5.

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