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Graphical abstract

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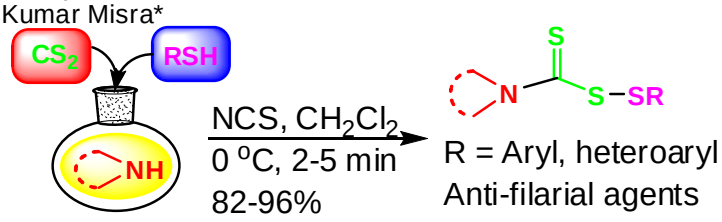

Development of novel anti-filarial agents using carbamo(dithioperoxo)thioate derivatives

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Abstract: A series of novel carbamo(dithioperoxo)thioate derivatives have been prepared in excellent yield using a significantly fast, one-pot three component reaction and experimented for their potential as anti-filarial agents against model filarial nematode *Setaria cervi*. Among 23 compounds (**4a-w**) evaluated for the anti-filarial activities, five compounds (**4a, 4b, 4c, 4d** and **4h**) have shown promising anti-proliferative effects on the juvenile stage microfilariae (mf) as well as in adults in a time and dose dependent manner. Compound **4a** was found most active against oocytes, mf and adult nematods as well as non-cytotoxic to the normal cells. It has been established that the anti-filarial activity of the compounds were observed due to the involvement of reactive oxygen species (ROS) and apoptosis. Several biochemical and microscopic experiments have been carried out to establish the fact that both intrinsic and extrinsic pathways of apoptosis contribute to the compound **4a** mediated death phenomenon of the filarial nematodes.

Key words: carbamo(dithioperoxo)thioate, anti-filarial, *Setaria cervi*, ROS, apoptosis.

Introduction

Lymphatic filariasis (LF) has remained a exigent health hazard and has affected over 110 million people in 51 countries throughout the tropics and sub-tropics of Asia, Africa, the Western Pacific, and parts of the Caribbean and South America (WHO, 2016) [1]. The causative agents of LF are *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* of which *W. bancrofti* and *B. malayi* are majorly responsible for lymphatic filariasis in tropical countries and particularly in India [2]. The immunobiology of the disease and the mode of progression have been so far well documented earlier [3-5]. Diethylcarbamazine (DEC), Ivermectin and Albendazole have remained as the drug of choice but their effectiveness is limited to microfilariae (mf) only [3]. However, combination of DEC and ivermectin

treatment are assisted with systemic and inflammatory adverse reactions [6,7]. DEC and ivermectin showed promising efficacy in controlling the mf in combination to Albendazole [8,9]. The emergence of anthelmintics resistant filarial nematodes is a serious concern to eliminate lymphatic filariasis. Factors that have been considered to mitigate the development of drug resistance in lymphatic filariasis include the combined use of drugs with different modes of action and the long and complex life cycle of filarial nematodes [10]. Currently available therapeutics target the microfilarial stage of the filarial worm and this enhances the opportunity to investigate for effective macrofilarial agents. In search of this, few synthetic compounds such as *trans*-stilbene derivatives [11], *C*-cinnamoyl glycosides [12], 4-oxycoumarin derivatives [13], quinolone derivatives [14], alkaloid derivative [15], a number of natural products [16-18] as well as nanoparticle formulations such as polymer inspired silver nano materials [19], gold nanoparticles [20] have shown promise as potent antifilarial candidates. However, most of the promising antifilarial compounds are not equally effective against mf as well as adult worms. As a consequence, there is a strong need to develop therapeutics, which could be effective against both mf as well as adult worms.

Disulfirams, members of the dithiocarbamate family were introduced in the late 18th centuries to the rubber industries for vulcanization purpose [21]. Later on its therapeutic role as a scabiescide has been established against the intestinal worms too [22]. Disulfirams and their analogs have also been used for discouraging alcohol intake [23]. Apart from these, disulfirams have the potential to control fungal infections [24] and several cancers [25,26]. Inspired by the therapeutic potential of disulfirams, a series of carbamo(dithioperoxo)thioate derivatives have been synthesized and found to have effective therapeutic potential against cancer cells [27]. It has been envisaged that carbamo(dithioperoxo)thioate derivatives could possess anti-filarial activities through anti-proliferative effects on the filarial nematods as they do in the cancer cells. With this proposition, a series of carbamo(dithioperoxo)thioate derivatives were synthesized and evaluated against model filarial nematode *Setaria cervi* (*S. cervi*), which are found in the bovine abdominal cavity. Although, *W. bancrofti* is the major parasite causing filarial infection in humans, it is quite difficult to get the parasite in the laboratory experimental set-up. Whereas, *S. cervi* resembles with *W. bancrofti* in its nocturnal periodicity and antigenic profile and can easily be used as a model parasite for the screening of the compounds for their anti-filarial potential [11,12,28]. The activities of the promising compounds (MIC, IC_{50} and LC_{50} values) against *S. cervi* were validated against *W. bancrofti*. Synthesis of a series of novel carbamo(dithioperoxo)thioate derivatives using a one-pot three

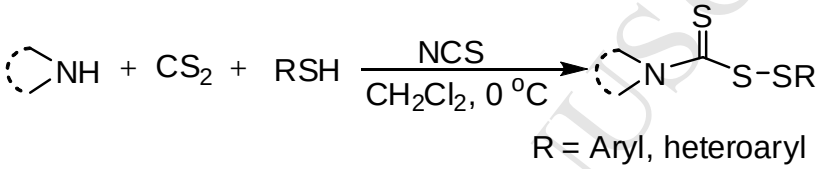
component reaction methodology and its bioevaluation against filarial nematodes is presented in this report. An extensive study at the molecular level on the mechanism of action of the active molecules has also been included in the present study. To the best of our knowledge, this is the first report on the therapeutic potential of carbamo(dithioperoxo)thioate derivatives against lymphatic filariasis.

Results and discussion

Chemistry

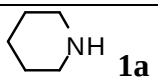
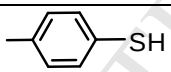
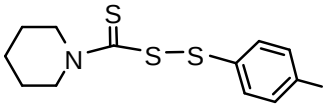
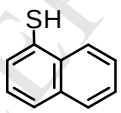
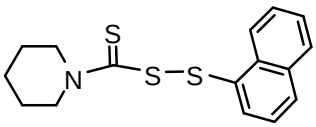
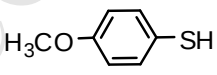
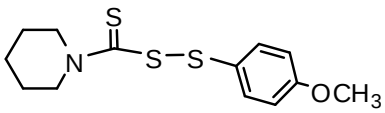
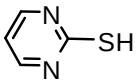
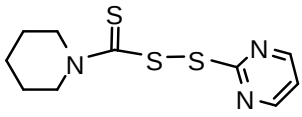
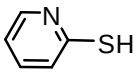
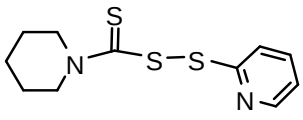
Due to the usefulness of the carbamo(dithioperoxo)thioate derivatives in the drug discovery program, a few reports have appeared in the literature [27,29] for the synthesis of these class of compounds. Earlier, this class of compounds were synthesized by carbon tetrabromide (CBr_4) mediated condensation of amines, carbondisulfide (CS_2) and thiols using a one-pot multi-component reaction condition considering the fact that CBr_4 reacts with thiols to generate sulfenyl bromides, which react with the dithiocarbamate salts to produce the desired compounds. Following literature reported reaction condition [27], piperidine (**1a**) (1.0 equiv.) was treated with CS_2 (**2**) (1.0 equiv.) and *p*-thiocresol (**3a**) (1.0 equiv.) in the presence of CBr_4 (2.0 equiv.) and Et_3N (1.1 equiv.) in CH_2Cl_2 at room temperature. However, the desired product was obtained in low yield together with the formation of disulfide derivative of **3a** in significant quantity. We believed that the aromatic sulfenyl bromide generated *in situ* from **3a** by the treatment with CBr_4 might be very reactive, which reacts with another molecule of thiol (**3a**) to furnish disulfide derivative (self coupling) instead of coupling with a dithiocarbamate salt. In order to overcome this situation, it was decided to carry out the reaction stepwise as well as without using any extra base (Et_3N). At first, the neat equimolar mixture of amine (**1a**) and CS_2 (**2a**) was stirred at room temperature to form the dithiocarbamate salt instantly. The reaction mixture was diluted with CH_2Cl_2 and thiol (**3a**) and CBr_4 were added to it sequentially and it was stirred at room temperature for upto 2 h. Unfortunately, formation of the desired product was not observed after prolonged reaction time. It was decided to use alternative halogenating agents such as *N*-chlorosuccinimide (NCS) and *N*-bromosuccinimide (NBS) etc. for the expected outcome. To our satisfaction, treatment of the reaction mixture with NBS and NCS furnished the expected product in significant short interval of time (2-5 min) at 0 °C in absence of a base (Table S1, supporting information). Although, NCS and NBS were equally effective in terms of the yield of the product formation, NCS has been chosen as the halogen source with the presumption that aryl

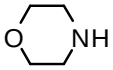
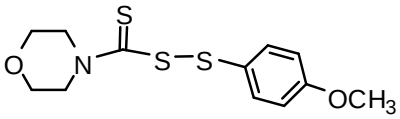
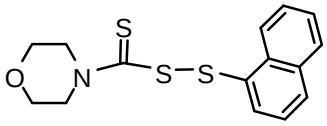
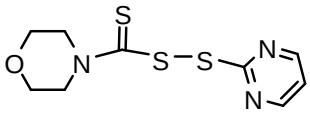
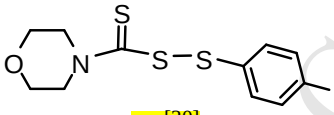
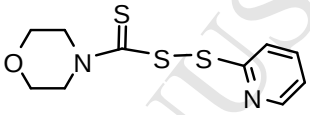
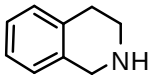
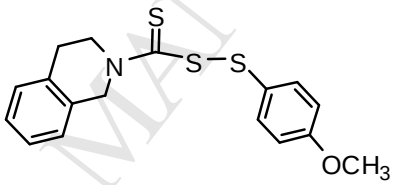
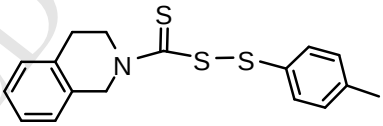
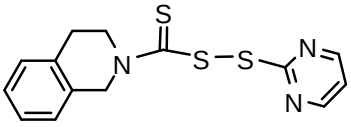
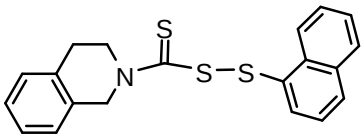
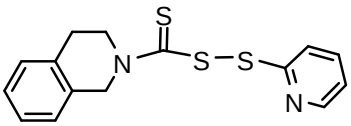
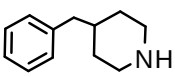
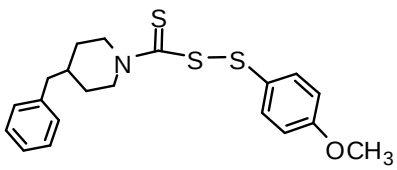
sulfenyl chloride might be less reactive than aryl sulfenyl bromide which allows formation of the desired product reducing the chance of disulfide formation. Optimizing the reaction condition, it was observed that treatment of **1a** (1.0 mmol) with CS_2 (1.1 mmol) followed by addition of thiol (**3a**) and NCS (1.1 mmol) in CH_2Cl_2 furnish desired compound **4a** in 92% yield at 0 °C in 2 min (Scheme 1). Among several commonly used organic solvents, CH_2Cl_2 was found superior and used for the reaction. Following the optimized reaction condition, a series of carbamo(dithioperoxo)thioate derivatives (**4a-w**) were prepared in excellent yield by a significantly fast reaction of a variety of amines (**1a-e**) with CS_2 (**2**) and aromatic thiols (**3a-e**) in one-pot (Table 1). All synthesized compounds were characterized using NMR and mass spectral analysis.

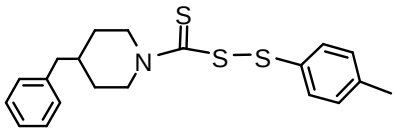
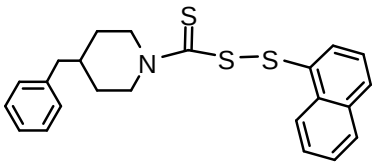
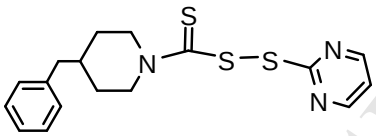
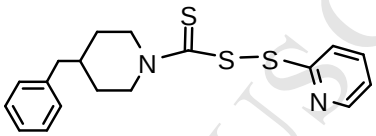
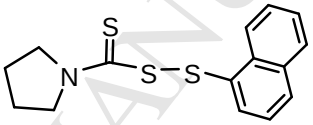
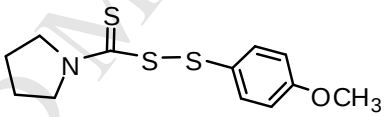
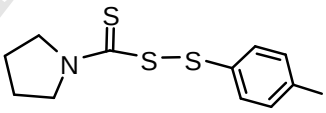


Scheme 1: Synthesis of carbamo(dithioperoxo)thioate derivatives by NCS mediated multi-component reaction of amines, CS_2 and thiols in one-pot.

Table 1: Synthesis of carbamo(dithioperoxo)thioate derivatives in one-pot.

Sl. No.	Amine	Thiol	Product	Time (min)	Yield (%)
1	 **1a**	 **3a**	 **4a**	2	92
2	**1a**	 **3b**	 **4b**	5	90
3	**1a**	 **3c**	 **4c**	2	90
4	**1a**	 **3d**	 **4d**	5	88
5	**1a**	 **3e**	 **4e**	5	86

6	 **1b**	**3c**	 **4f**	2	95
7	**1b**	**3b**	 **4g**	5	90
8	**1b**	**3d**	 **4h**	5	90
9	**1b**	**3a**	 **4i** [30]	2	92
10	**1b**	**3e**	 **4j**	5	90
11	 **1c**	**3c**	 **4k**	2	92
12	**1c**	**3a**	 **4l**	2	96
13	**1c**	**3d**	 **4m**	5	87
14	**1c**	**3b**	 **4n**	5	88
15	**1c**	**3e**	 **4o**	5	85
16	 **1d**	**3c**	 **4p**	2	94

17	**1d**	**3a**	 **4q**	2	86
18	**1d**	**3b**	 **4r**	5	84
19	**1d**	**3d**	 **4s**	5	82
20	**1d**	**3e**	 **4t**	5	86
21	 **1e**	**3b**	 **4u**	5	92
22	**1e**	**3c**	 **4v**	2	95
23	**1e**	**3a**	 **4w**	2	96

Biology

The fundamental effects of the synthesized compounds (**4a-w**) on cellular viability and mortality of microfilaria of *S. cervi* were conducted through relative movability (RM) assessment and inhibition of proliferation (MTT) assay. The observation from the MTT assay is in terms of MIC (Minimum Inhibitory Concentration), IC_{50} and LC_{50} are presented in Table 3. The elementary data collected from these *in vitro* experiments showed that among the compounds tested (**4a-w**), five compounds (**4a, 4b, 4c, 4d** and **4h**) were found to be promising lead compounds against different developmental stages (oocytes, mf and adults) of *S. cervi* and compound **4a** had shown its maximum effectiveness due to its significant low MIC, IC_{50} and LC_{50} values 0.358 ± 0.02 µg/mL, 0.708 ± 0.021 µg/mL and 3.89 ± 0.18 µg/mL

respectively in comparison to the standard drug Ivermectin (IVM) (MIC, IC_{50} and LC_{50} values 3.12 ± 0.78 µg/mL, 6.25 ± 0.14 µg/mL and 16.57 ± 0.21 µg/mL respectively) against mf (Table 2 and Figure 1a).

Table 2: Evaluation of anti-filarial activity of the synthesized carbamo(dithioperoxo)thioate derivatives.

Compound	MIC (µg/mL)	IC_{50} (µg/mL)	LC_{50} (µg/mL)
4a	**0.358 ± 0.02**	**0.708 ± 0.021**	**3.89 ± 0.18**
4b	**0.583 ± 0.006**	**2.15 ± 0.049**	**6.49 ± 0.21**
4c	**2.30 ± 0.075**	**4.61 ± 0.146**	**6.94 ± 0.09**
4d	**2.39 ± 0.061**	**4.75 ± 0.11**	**5.10 ± 0.05**
4e	8.69 ± 0.02	24.32 ± 0.035	97.28 ± 0.08
4f	6.69 ± 0.27	20.08 ± 0.74	80.32 ± 0.28
4g	7.41 ± 0.654	22.23 ± 0.54	88.92 ± 0.38
4h	**0.97 ± 0.019**	**5.50 ± 0.655**	**6.17 ± 0.10**
4i	6.63 ± 0.21	19.91 ± 0.043	79.64 ± 0.034
4j	15.88 ± 0.054	47.66 ± 0.025	190.64 ± 0.02
4k	10.46 ± 0.065	31.40 ± 0.06	125.63 ± 0.32
4l	10.70 ± 0.48	32.12 ± 0.038	128.48 ± 0.45
4m	10.36 ± 0.83	31.08 ± 0.054	124.32 ± 0.67
4n	10.90 ± 0.342	32.71± 0.286	130.82 ± 0.39
4o	8.26 ± 0.64	24.79 ± 0.53	99.18 ± 0.076
4p	20.70 ± 0.3	62.11 ± 0.012	248.45 ±0.065
4q	1.22 ± 0.116	19.65 ± 0.187	22.54 ± 0.33
4r	20.23 ± 0.054	60.70 ± 0.25	242.82 ± 0.34
4s	19.97 ± 0.048	59.92 ± 0.056	239.68 ± 0.7
4t	19.71 ± 0.054	59.13 ± 0.86	236.53 ± 0.45
4u	7.52 ± 0.053	22.57 ± 0.346	90.31 ± 0.487
4v	8.08 ± 0.039	24.25 ± 0.06	97.01 ± 0.15
4w	7.86 ± 0.569	23.58 ± 0.765	94.32 ± 0.067
Ivermectin (IVM) (Standard drug)	**3.12 ± 0.78**	**6.25 ± 0.14**	**16.57 ± 0.21**

From the trypan blue dye exclusion test death percentages for each of the compounds was measured and seemed to increase dose dependently in both mf and adults. The synthetic compounds were much effective in combating mf and adult of *S. cervi* in a dose and time dependent manner as was evident from the MTT reduction assay thus confirming their role as emerging novel anti-filarials. The percentage of both mf and adult cells of *S. cervi* that were inhibited were calculated from the MTT reduction test and found to increase dose dependently in both mf and adult *S. cervi* after 24 h of treatment (Figure 1b and 1c). A dose dependent increase in the death percentage in adult nematodes was also evident **(Figure 1c)**. In addition, when the cytotoxic effect of the five potent compounds (**4a, 4b, 4c, 4d** and **4h**) were evaluated in normal human lung fibroblast cell line i.e. WI-38, it was observed that compounds **4a** and **4d** had the comparatively low IC_{50} values 19.83±2.50 μg/mL and 10.61±1.19 μg/mL respectively against WI-38 cell lines (Figure 1f) in comparison to Ivermectin (IVM) 39.91±1.48 μg/mL**.** It is noteworthy that compound **4d** and IVM showed almost similar cytotoxic effect (respectively 5 fold and 6 fold lower cytoxicity against the normal cell line (WI-38) as compared to *S. cervi*). Whereas, compound **4a** showed significantly lower cytotoxicity (25 fold against the normal cell line (WI-38) as compared to *S. cervi*), which is 4 fold better than IVM itself. Hence, **4a** has been considered as the best lead amongst the tested compounds (**4a-w**) and chosen for its further biological studies at the molecular level. The dose dependent accrual in the conversion of nitroblue tetrazolium salt (NBT) into dark formazan crystals proved the involvement of reactive oxygen species (ROS) [31] in the treated samples of both mf and adult (Figure 1d, 1e). Therefore it can be assumed that the death of the parasite may be mediated through increased reactive oxygen species (ROS) especially the superoxide anions ($0_2^{\cdot-}$).

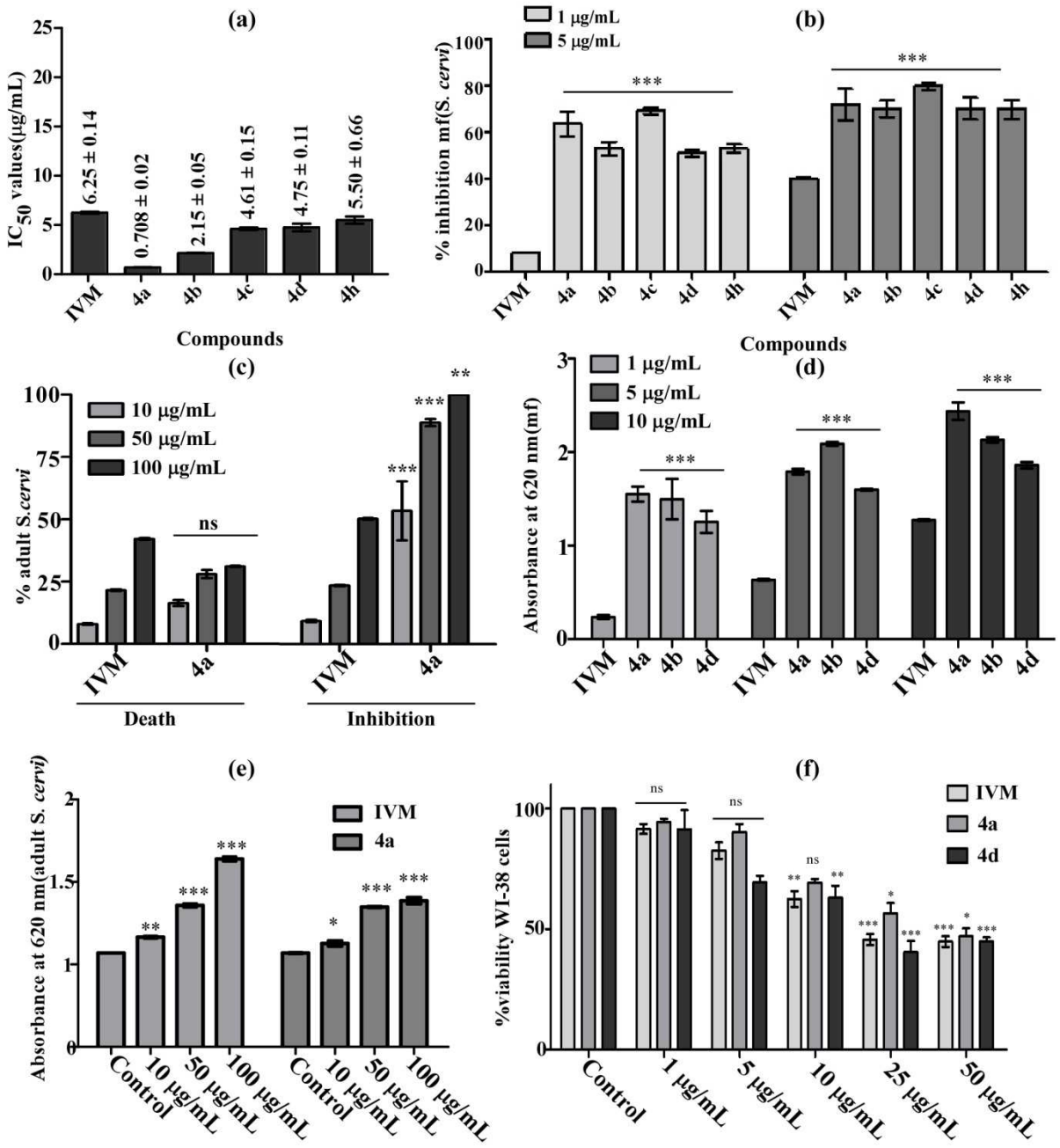


Figure 1. Evaluation of antifilarial potential of the compounds 4a, 4b, 4c, 4d and **4h along with Ivermectin (IVM).** (a) Comparative IC_{50} value of the projected compounds compared to the standard **IVM.** (b) Percentage inhibition in compound **4a, 4b, 4c, 4d, 4h** along with **IVM** treated microfilaria S. *cervi* (c) Death and inhibition percentage of adult *S. cervi* treated with compound **4a** and **IVM**. (d) Dose dependent evaluation in the absorbance at 620 nm, indicating generation of increased superoxide anions in microfilaria S. *cervi.* (e) **4a** and **IVM** treated adult worm of *S. cervi* indicating generation of increased superoxide anions. (f) Showing the effect of Compound **4a**, **4d** and **IVM** on normal human lung fibroblast cell line WI-38. All the data represent minimum of three independent experiment and the bar graph shows ±SEM (*p <0.05**p < 0.01, ***p < 0.001, ns = not significant).

Apoptosis or programmed cell death is of prime importance during the development, immune regulation and in defense against pathogens. In order to keep consistency in an organism, cell death and cell survival and/or proliferation are linked intimately through several interconnected molecular aspects [32]. It is a vital process in multi-cellular organisms for getting rid of spoiled and unwanted cells to maintain tissue homeostasis [33]. Apoptosis is the fastest occurring phenomenon among the several cell death pathways typically induced within the same tissue, while other forms, like necrosis or autophagy are visible only when apoptosis is inhibited [34,35]. There are several distinct characteristic features of apoptosis which can be designated as the hallmarks such as chromatin condensation, shrinkage and loss of cellular attachment towards the substratum, blebbing of cell membrane etc.[36,37] During apoptosis, outward translocation of phosphatidylserine (PS) takes place, which is a phospholipid normally embedded inside the plasma membrane. This outwardly translocated PS exert a pull on macrophages, and are efficiently phagocytosised [36,37]. Both extrinsic and intrinsic pathways of apoptosis are present in the filarial nematodes as evidenced by Mukherjee *et al*., 2016 [11]. It was decided to further determine the specific branch on which these novel compounds are working in causing death in the filarial parasites. Interestingly, the Hoechst 33342 (Sigma, USA) stained mf has confirmed that chromatin condensation had occurred in all samples after treatment with compounds **4a, 4b, 4c, 4d** and **4h**. However, maximum chromatin condensation was observed in the **4a** treated samples (5 µg/mL) in comparison to the control (Figure 2a). Moreover, quite importantly the compound **4a** showed its promising effect within 3 h of treatment on mf as evident from the phase contrast microscopy (Figure 2b). The stain specifically binds to the condensed chromatin of apoptotic cells more intensely than that of normal cells, which is regarded as the hallmark of apoptotic cell death further strengthens the hypothesis of cell death in the treated filarial nematodes. Acridine orange (AO) and ethidium bromide (EtBr) double staining technique added extra pillars to confirm the apoptotic death mechanism of the compound **4a** (1 and 5 µg/mL) treated oocytes and mf. AO is a vital dye and can move into both live and dead cells whereas the EtBr can penetrate only where the membrane integrity has been lost. Increase in the intensity of orange fluorescence due to EtBr in the **4a** (1, 5 µg/mL) treated samples further corroborate with the finding that membrane rupture enhances with the increase of the dose (Figure 2c).

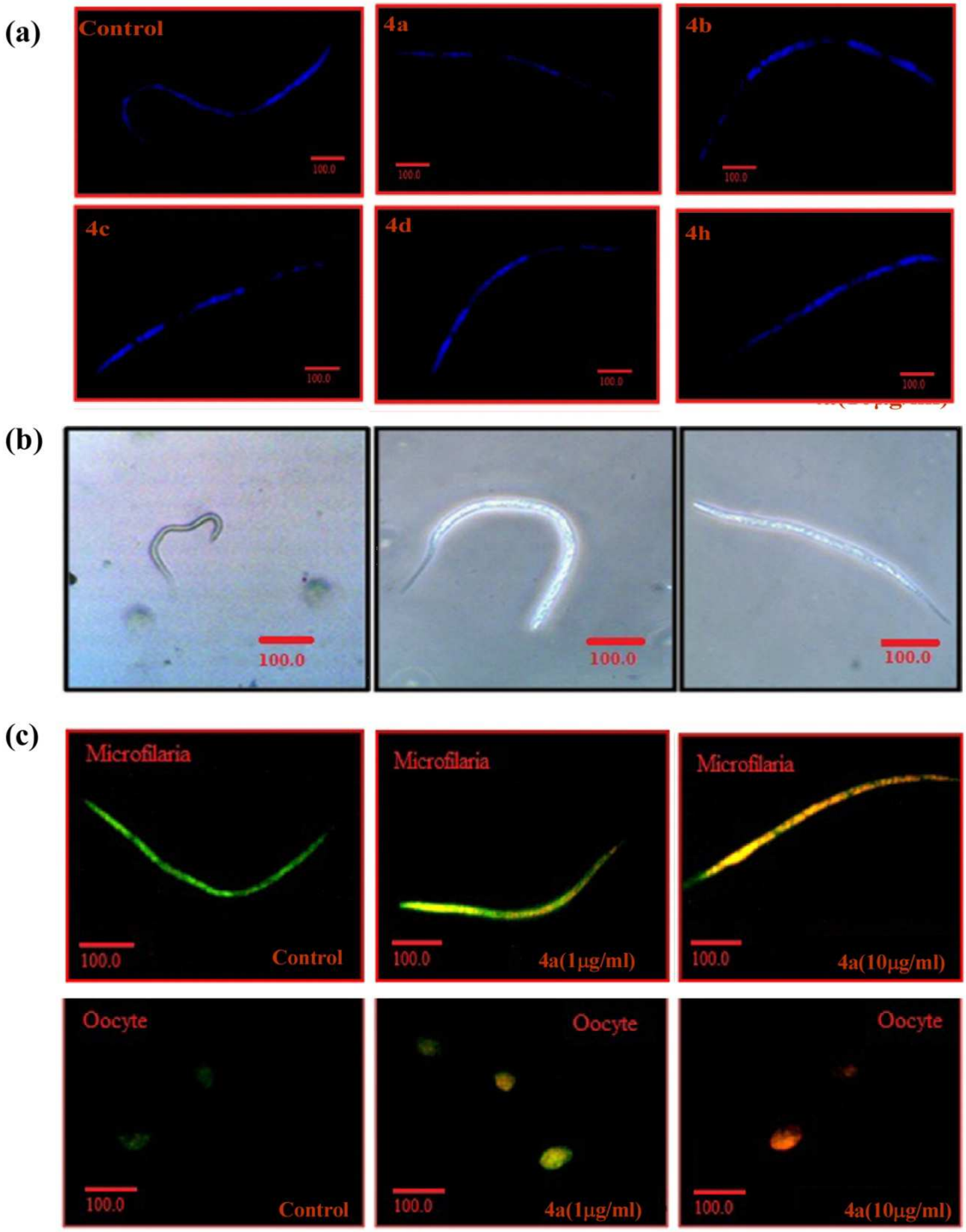


Figure 2: Microscopical studies of mf and oocytes and treated with compounds. (a) Hoechst stained images of *S. cervi* mf. The DNA fragmentation and chromatin condensation were found in all the treated parasites but absent in control. (b) The compound **4a** treated nematodes appeared as blue due to the incorporation of trypan blue inside the body in comparison to the control one in a dose dependent manner; Magnification 20X; (c) Acridine orange (AO) and ethidium bromide (EtBr) double staining of mf (upper panel) and oocytes (Lower panel).

In addition to the observed results mentioned above, apoptotic incidence was confirmed through the flow cytometric data of the **4a** (50 µg/mL) treated oocytes and mf. The entire plot area has been divided into four quadrants UL (necrotic cells), UR (late apoptosis), LL (viable cells) and LR (early apoptotic cells) [38]. After 6 h of treatment with compound **4a** (50 µg/mL) 24.6% oocytes showed apoptotic morphology among which 13.3% (LR) in early stage of apoptosis and 11.3% (UR) in the late apoptotic phase. However, same dose of **4a** caused 31.44% apoptotic characteristics in mf of which 15.82% late apoptotic (UR) and 15.62% in the early phase of the apoptotic event (LR) (Figure 3a). The flow cytometric data were also accounted as the strongest evidence for establishing apoptotic process. Moreover the fluorescence microscopic images of the **4a** treated oocytes (1, 10 µg/mL) showed green fluorescence of FITC labeled Annexin-V, which binds to the phosphatidylserine (PS) of the cell membrane that normally resides in the inner leaflet of the plasma membrane, and is translocated outward due the disintegrity of the cellular membrane. Propidium iodide usually excluded by the intact cell, intercalates with the base pairs of the double stranded DNA when it gets entry inside as a consequence of membrane rupture (Figure 3b).

The apoptosis mediated death of compound **4a** treated *S. cervi* was further confirmed by the expression of EGL-1, CED-3 and CED-9 proteins using western blot experiments after treatment of compound **4a** in a dose dependent (10 µg/mL, 50 µg/mL and 100 µg/mL) manner for 24 h. EGL-1 and CED-3 proteins are pro-apoptotic in nature whereas; CED-9 is anti-apoptotic, which is homologous to proto-oncogene Bcl-2 [39]. EGL-1 shows its pro-apoptotic function in conjugation with BH3 motif that are present in death promoting Bcl-2/CED-9 family members [40,41]. During apoptosis events the activation of CED-3 protein forms cysteine protease complex that leads to the death of the nematodes. Changes in the protein expression patterns clearly showed the dose dependent increase in the pro apoptotic proteins EGL-1 and CED-3. On the contrary, anti apoptotic protein CED-9 expression was decreased in a dose dependent manner (Figure 3c). Collectively these data suggested that compound **4a** induced apoptosis in *S. cervi* involving EGL-1, CED-3 and CED-9 proteins.

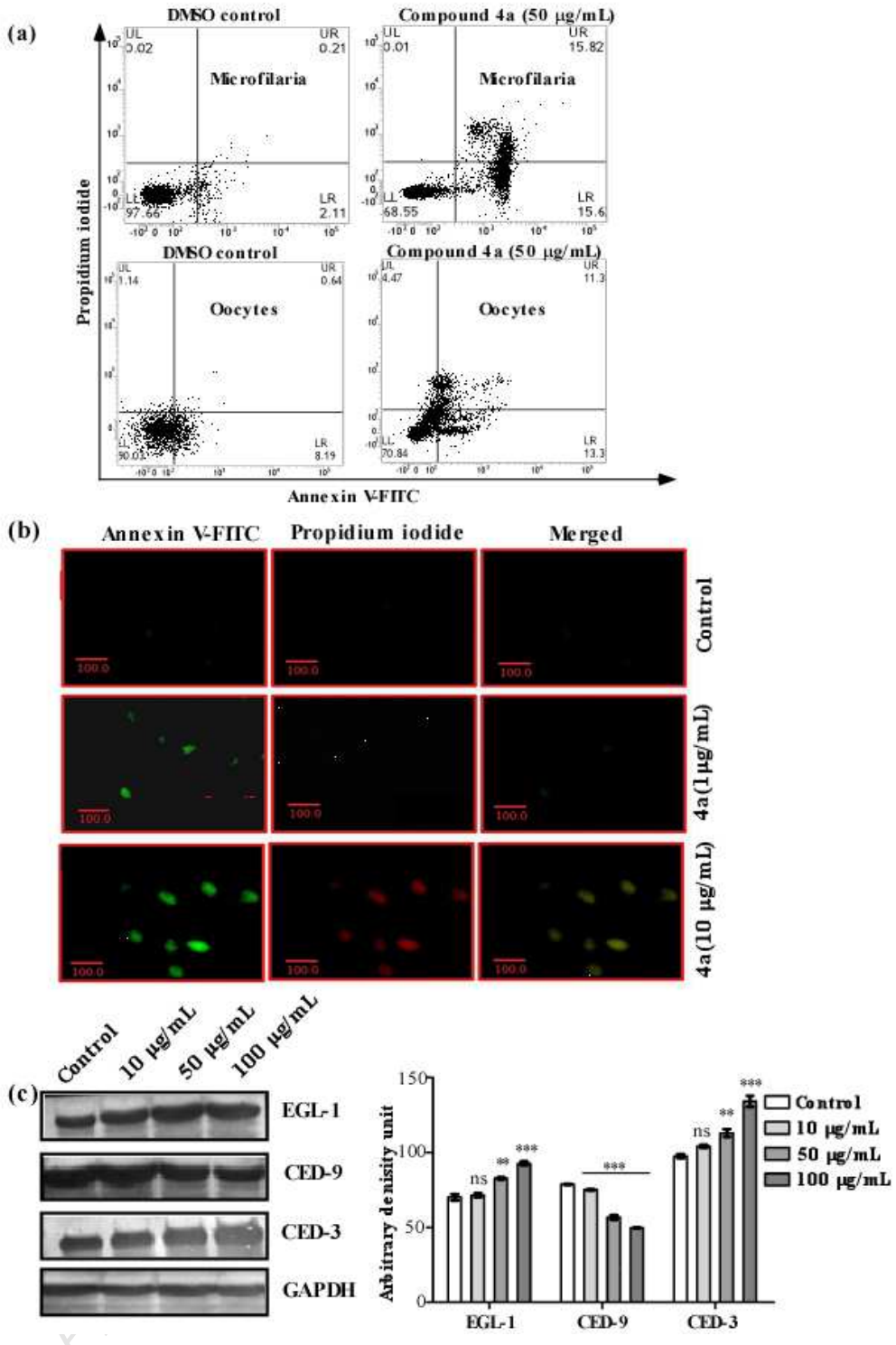


Figure 3. Evaluation of anti-apoptotic potential of compound 4a in S. *cervi* (a) Annexin-V FITC and PI labeling confirmed induction of apoptosis in mf (upper panel) and oocytes (lower panel) of S. *cervi* treated with compound **4a** (50 µg/ml); (b) Fluorescence microscopy showing concentration dependent increase in Annexin-V FITC and PI oocytes population in response to compound **4a;** (c) Western immune-blot demonstrating apoptogenic change in CED-3, CED-9 and EGL-1 with respect to the loading control GAPDH along with pixel density. All the data represent minimum of three independent experiment and the bar graph shows ±SEM (*p <0.05**p < 0.01, ***p < 0.001, ns = not significant).

Quantitative structure activity relationship analysis

In addition to the biochemical assays, structure activity relationship (3D-QSAR) analyses were also performed using Accelry's discovery studio [42] to understand the structural features of the test compounds those elicit anti-filarial activity. In order to analyze the anti-filarial activity, four features pharmacophore model has been developed [43] using data set compounds (23 molecules) that were categorized into training set (16 molecules) and test set (7 molecules) by considering their drug like characters and ADMET properties [44]. Almost all data set compounds complied with Lipinski's rule [45] except 7 molecules where AlogP value exceeds 5.6 and those molecules were taken in test set. The statistical significance of this model is given in Table 3. The training set molecules have shown higher fit value, correlation coefficient and lower RMSD value which denote the fitting of active pharmacophoric features in the training set molecules with high integrity. The correlation between the experimental IC_{50} and estimated IC_{50} values of training set and test set molecules are shown in Figure 4A. Overlaying of molecules confirmed that carbamo(dithioperoxo)thioate moiety is present in the skeleton of all molecules is the essential feature of eliciting anti-filarial activity. All compounds have heterocyclic ring in one end of carbamoaromatic rings and heterocycle or aryl group at the other end (Figure 4B). Mapping of the most active compounds **4a** and **4b** with the pharmacophore model has shown excellent interactions to all pharmacophoric features (Figure 4C and 4D). Whereas, mapping of the least active compounds **4j** and **4s** were exhibited constraints in interactions with hydrogen bond acceptor features (Figure 4E and 4F). The activity of the compounds may be reduced when heterocyclic group present in both end of carbamo(dithioperoxo)thioate moiety, substituted heterocycle with aryl group and bulkier aryl group on the other end that deter the hydrogen bond acceptor features due to reduction of electron density on the carbamo(dithioperoxo)thioate functional groups. Whereas heterocycle fused with aryl group exhibited moderate activity. Heterocycle fused with aryl group and bulkier aryl group on the other end improve the hydrophobicity features.

Table 3: Statistical Result of 3D-QSAR Model

Training set	**Test set**
N1 = 16	N2 = 7
Corr. Coefficient = 0.9467	Corr. Coefficient = 0.7351
RMSD = 0.1455	RMSD = 0.2376
Max. fit value = 8.5094	Max. fit value = 7.897
Weight = 1.2139	Weight = 1.3361

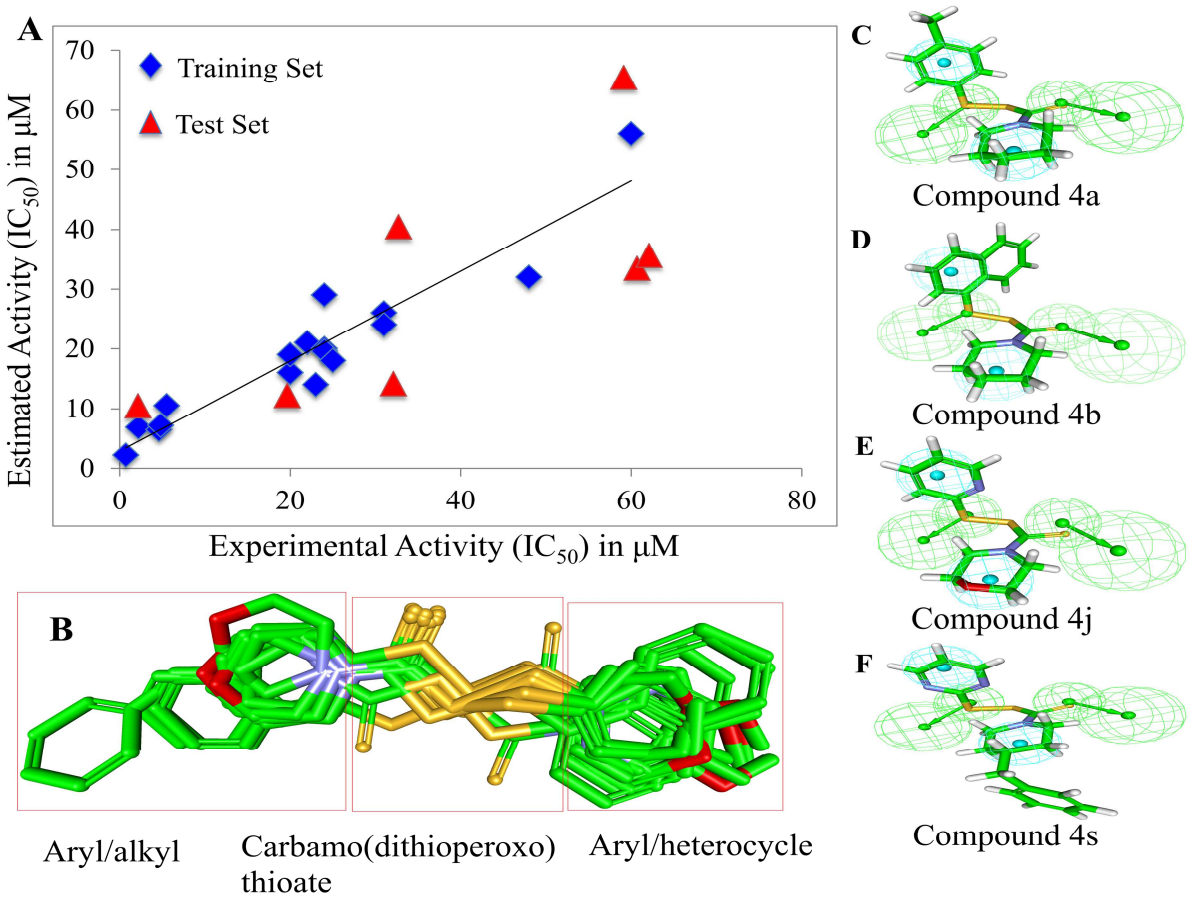


Figure 4: 3D QSAR analysis of carbamo(dithioperoxo)thioate derivatives with anti-filarial activities. (A) Regression plot representing the statistical analysis with reference to the predicted IC_{50} value and theoretical IC_{50} values; (B) Alignment of the molecules showing conserved carbamo(dithioperoxo)thioate moiety and variable alkyl or aryl/heteroaryl groups; (C and D): Pharmacophoric mapping of active compounds (**4a** and **4b**); (E and F): Pharmacophoric mapping of inactive compounds (**4j** and **4s**).

Conclusion

In summary, a series of novel carbamo(dithioperoxo)thioate derivatives have been synthesized in excellent yield using NCS mediated one-pot multicomponent reaction of amines, CS_2 and thiols. The synthesized compounds were evaluated for their potential to act as anti-filarial agants against bovine *S. cervi* microfilaria (mf) and oocytes using a number of biological assays. Among twenty three tested compounds six compounds showed promising anti-filarial activities to be considered as promising leads. However, based on the significant

less cytotoxic nature on the normal cells, effect of compound **4a** on the mf and adult filarial nematods has been further studied in cellular level. It has been established that compound **4a** showed significant detrimental effect on the oocytes, mf and adult worms following apoptotic mechanism, which were justified by the chromatin condensation and DNA fragmentation and differential protein expressions. A 3D-QSAR study has also been carried out to establish the structure-activity relationships of active compounds, which supported the biological and microscopic experimental findings. Collectively these experimental findings suggest that carbamo(dithioperoxo)thioate derivatives and particularly compound **4a** has tremendous filaricidal potential and can be considered for further development for the advancement of anti-filarial therapy.

Experimental

General methods: All reactions were monitored by thin layer chromatography over silica gel coated TLC plates. The spots on TLC were visualized by warming ceric sulphate (2% $Ce(SO_4)_2$ in 2N H_2SO_4) sprayed plates on a hot plate. Silica gel 230-400 mesh was used for column chromatography. ^1H and ^{13}C NMR spectra were recorded on Bruker Avance 500 MHz spectrometer using $CDCl_3$ as solvent and TMS as internal reference unless stated otherwise. Chemical shift values are expressed in δ ppm. ESI-MS were recorded on a Micromass mass spectrometer. Elementary analysis was carried out on Carlo Erba analyzer. Biological experiments were carried out in a Shimadzu UV-2401PC spectrophotometer. Commercially available grades of organic solvents of adequate purity are used in many reactions. Solvents of highest purity grade were purchased from Merck India and Milli-Q water (Milli-Q Academic with 0.22 μm Millipak R-40) was utilized for the assays. Antibiotics, fetal bovine serum (FBS) (Gibco), Hoechst 33342, Acridine orange, Ethidium bromide were purchased from Sigma-Aldrich, USA, and AnnexinV-FITC Apoptosis Detection Kit were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). RPMI-1640, MTT, NBT were obtained from Hi-Media Laboratories, Mumbai, India. Egl-1, Ced-9 and Ced-3 primary antibodies, alkaline phosphatase conjugated secondary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA). GAPDH primary antibodies were purchased from BioBharati Life Science Pvt. Ltd., India.

***Typical experimental condition for the preparation of carbamo(dithioperoxo)thioate derivatives*:** Piperidine (100 µL, 1.01 mmol) was added drop wise to CS_2 (65 µL, 1.07 mmol) and the mixture was stirred for 2 min and diluted with anhydrous CH_2Cl_2 (2 mL). To the

reaction mixture was added *p*-thiocresol (125 mg, 1.0 mmol) and it was cooled to 0 °C. To the cooled reaction mixture was added NBS (200 mg, 1.12 mmol) and the reaction mixture was allowed to stir at 0 °C for 2 min. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and successively washed with 5% $Na_2S_2O_3$ (10 mL), H_2O (10 mL). The organic layer was dried (Na_2SO_4) and concentrated. The crude product was purified over SiO_2 using hexane-EtOAc as eluant to give pure compound **4a** (260 mg, 92%). Compounds (**4b-w**) were prepared following the similar reaction condition (Table 1).

Analytical data of the synthesized compounds:

***p*-Tolyl piperidine-1-carbo(dithioperoxo)thioate (4a)**: Yellow oil; IR (neat): 1486.7 (N-C=S), 1277.6 (C-N), 1106.6 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.47 (d, J = 9.0 Hz, 2 H, Ar-H), 7.05 (d, J = 9.0 Hz, 2 H, Ar-H), 4.30-3.85 (m, 4 H, -CH_2N), 2.30 (s, 3 H, CH_3), 1.75-1.66 (m, 6 H, -CH_2-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 194.8 (*C*=S), 138.0-129.6 (Ar-C), 55.3, 51.9, 26.3 (2 C), 25.6, 21.2 (*C*H_3); ESI-MS: 306.0 $[M+Na]^+$; Anal. Calcd. for $C_{13}H_{17}NS_3$ (283.47): C, 55.08; H, 6.04; N, 4.94; found: C, 54.90; H, 6.18; N, 4.70.

Naphthalen-1-yl piperidine-1-carbo(dithioperoxo)thioate (4b): Yellow oil; IR (neat): 1487.2 (N-C=S), 1279.0 (C-N), 1103.4 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.04-7.40 (m, 7 H, Ar-H), 4.35-3.92 (m, 4 H, NCH_2-), 1.75-1.70 (m, 6 H, -CH_2-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 194.4 (*C*=S), 133.4-126.3 (Ar-C), 55.4, 52.0, 26.3 (2 C), 25.6; ESI-MS: 342.0 $[M+Na]^+$; Anal. Calcd. for $C_{16}H_{17}NS_3$ (319.50): C, 60.15; H, 5.36; N, 4.38; found: C, 60.00; H, 5.50; N, 4.15.

***p*-Methoxyphenyl piperidine-1-carbo(dithioperoxo)thioate (4c)**: Yellow oil; IR (neat): 1488.7 (N-C=S), 1282.7 (C-N), 1108.2 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.60 (d, J = 9.0 Hz, 2 H, Ar-H), 6.80 (d, J = 9.0 Hz, 2 H, Ar-H), 4.36-3.87 (m, 4 H, NCH_2-), 1.75-1.65 (m, 6 H, -CH_2-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.1 (*C*=S), 160.3-114.4 (Ar-C), 55.2 (2 C, NCH_2, OCH_3), 51.9 (NCH_2), 26.2 (2 C), 25.6; ESI-MS: 322.0 $[M+Na]^+$; Anal. Calcd. for $C_{13}H_{17}NOS_3$ (299.47): C, 52.14; H, 5.72; N, 4.68; found: C, 52.00; H, 5.90; N, 4.50.

Pyrimidin-2-yl piperidine-1-carbo(dithioperoxo)thioate (4d): Yellow oil; IR (neat): 1486.7 (N-C=S), 1280.7 (C-N), 1107.2 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.59-8.55 (m, 2 H, Ar-H), 7.08-7.05 (m, 2 H, Ar-H), 4.35-4.10 (m, 4 H, NCH_2-), 1.78-1.67 (m, 6 H, -CH_2-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 191.7 (*C*=S), 156.6-116.9 (Ar-C), 54.3 (NCH_2), 51.1

(NCH_2), 26.2 (2 C), 25.6; ESI-MS: 294.0 $[M+Na]^+$; Anal. Calcd. for $C_{10}H_{13}N_3S_3$ (271.42): C, 44.25; H, 4.83; N, 15.48; found: C, 44.10; H, 5.00; N, 15.25.

Pyridin-2-yl piperidine-1-carbo(dithioperoxo)thioate (4e): Yellow oil; IR (neat): 1488.9 (N-C=S), 1278.4 (C-N), 1110.2 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.43-7.04 (m, 4 H, Ar-H), 4.32-4.05 (m, 4 H, NCH_2-), 1.76-1.68 (m, 6 H, -CH_2-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 191.4 (*C*=S), 157.9-118.4 (Ar-C), 54.3 (NCH_2), 51.1 (NCH_2), 25.2 (2 C), 24.6; ESI-MS: 293.0 $[M+Na]^+$; Anal. Calcd. for $C_{11}H_{14}N_2S_3$ (270.43): C, 48.85; H, 5.22; N, 10.36; found: C, 48.70; H, 5.30; N, 10.50.

***p*-Methoxyphenyl morpholine-4-carbo(dithioperoxo)thioate (4f)**: Yellow oil; IR (neat): 1489.0 (N-C=S), 1112.9 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.61 (d, *J* = 9.0 Hz, 2 H, Ar-H), 6.80 (d, *J* = 9.0 Hz, 2 H, Ar-H), 4.32-3.90 (m, 4 H, OCH_2), 3.77 (s, 3 H, OCH_3), 3.76-3.73 (m, 4 H, NCH_2-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 196.9 (*C*=S), 160.5-114.5 (Ar-C), 66.2 (2 C, OCH_2), 55.2 (OCH_3), 51.5 (2 C, NCH_2); ESI-MS: 324.0 $[M+Na]^+$; Anal. Calcd. for $C_{12}H_{15}NO_2S_3$ (301.44): C, 47.81; H, 5.02; N, 4.65; found: C, 47.65; H, 5.20; N, 4.80.

Naphthalen-1-yl morpholine-4-carbo(dithioperoxo)thioate (4g): Yellow oil; IR (neat): 1464.9 (N-C=S), 1280.5 (C-N), 1111.2 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.05-7.43 (m, 7 H, Ar-H), 4.34-3.98 (m, 4 H, OCH_2-), 3.77-3.74 (m, 4 H, NCH_2-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 196.1 (*C*=S), 133.3-126.6 (Ar-C), 66.3 (2 C), 53.4, 51.4; ESI-MS: 344.0 $[M+Na]^+$; Anal. Calcd. for $C_{15}H_{15}NOS_3$ (321.48): C, 56.04; H, 4.70; N, 4.36; found: C, 55.90; H, 4.90; N, 4.05.

Pyrimidin-2-yl morpholine-4-carbo(dithioperoxo)thioate (4h): Yellow oil; IR (neat): 1472.6 (N-C=S), 1281.3 (C-N), 1113.5 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.67-8.64 (m, 2 H, Ar-H), 7.14-7.11 (m, 1 H, Ar-H), 4.35-3.90 (m, 4 H, OCH_2-), 3.88-3.74 (m, 6 H, NCH_2-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 186.5 (*C*=S), 157.6-117.9 (Ar-C), 66.1 (2 C, OCH_2), 52.9 (2 C, NCH_2); ESI-MS: 296.0 $[M+Na]^+$; Anal. Calcd. for $C_9H_{11}N_3OS_3$ (273.39): C, 39.54; H, 4.06; N, 15.37; found: C, 39.40; H, 4.22, N, 15.05.

***p*-Tolyl morpholine-4-carbo(dithioperoxo)thioate (4i)**: Yellow oil; IR (neat): 1488.9 (N-C=S), 1114.4 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47 (d, *J* = 9.0 Hz, 2 H, Ar-H), 7.07 (d, *J* = 9.0 Hz, 2 H, Ar-H), 4.30-4.05 (m, 4 H, -OCH_2), 3.79-3.74 (m, 4 H, -NCH_2), 2.31 (s, 3 H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 196.6 (*C*=S), 138.5-129.7 (Ar-C), 66.3 (2 C,

O*C*H$_2$), 52.9, 51.1, 21.2 (*C*H$_3$); ESI-MS: 308.0 [M+Na]$^+$; Anal. Calcd. for $C_{12}H_{15}NOS_3$ (285.44): C, 50.49; H, 5.30; N, 4.91; found: C, 50.30; H, 5.45; N, 4.75.

Pyridin-2-yl morpholine-4-carbo(dithioperoxo)thioate (4j): Yellow oil; IR (neat): 1489.9 (N-C=S), 1280.2 (C-N), 1108.2 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.44-7.04 (m, 4 H, Ar-H), 4.30-4.05 (m, 4 H, OC*H*$_2$-), 3.79-3.72 (m, 4 H, NC*H*$_2$-); ^{13}C NMR (125 MHz, $CDCl_3$): δ 190.4 (*C*=S), 157.9-118.4 (Ar-C), 66.3 (O*C*H$_2$), 52.8 (ON*H*$_2$), 51.1 (NC*H*$_2$); ESI-MS: 295.0 [M+Na]$^+$; Anal. Calcd. for $C_{10}H_{12}N_2OS_3$ (272.41): C, 44.09; H, 4.44; N, 10.28; found: C, 43.92; H, 4.60; N, 10.00.

***p*-Methoxyphenyl 3,4-dihydroisoquinoline-2(1*H*)-carbo(dithioperoxo)thioate (4k)**: Yellow oil; IR (neat): 1491.8 (N-C=S), 1108.2 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.64 (d, *J* = 9.0 Hz, 2 H, Ar-H), 7.23-7.12 (m, 4 H, Ar-H), 6.78 (d, *J* = 9.0 Hz, 2 H, Ar-H), 5.29-5.00 (m, 2 H, NC*H*$_2$), 4.41-4.10 (m, 2 H, NC*H*$_2$), 3.76 (s, 3 H, OC*H*$_3$), 3.00-2.96 (m, 2 H, C*H*$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.8 (*C*=S), 160.4-114.5 (Ar-C), 55.7 (NCH_2), 55.2 (OCH_3), 51.9 (NCH_2), 48.2 (CH_2); ESI-MS: 370.0 [M+Na]$^+$; Anal. Calcd. for $C_{17}H_{17}NOS_3$ (347.52): C, 58.75; H, 4.93; N, 4.03; found: C, 58.60; H, 5.10; N, 4.20.

***p*-Tolyl 3,4-dihydroisoquinoline-2(1*H*)-carbo(dithioperoxo)thioate (4l):** Yellow oil; IR (neat): 1472.1 (N-C=S), 1280.0 (C-N), 1107.1 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.49-7.04 (m, 8 H, Ar-H), 5.29-5.10 (m, 2 H, NC*H*$_2$), 4.40-4.13 (m, 2 H, NC*H*$_2$), 3.00-2.96 (m, 2 H, C*H*$_2$), 2.30 (s, 3 H, C*H*$_3$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.8 (*C*=S), 157.8-127.7 (Ar-C), 55.8 (N*C*H$_2$), 52.1 (N*C*H$_2$), 48.3 (*C*H$_2$), 21.0 (*C*H$_3$); ESI-MS: 354.0 [M+Na]$^+$; Anal. Calcd. for $C_{17}H_{17}NS_3$ (331.52): C, 61.59; H, 5.17; N, 4.23; found: C, 61.45; H, 5.30; N, 4.00.

Pyrimidin-2-yl 3,4-dihydroisoquinoline-2(1*H*)-carbo(dithioperoxo)thioate (4m): Yellow oil; IR (neat): 1490.3 (N-C=S), 1108.2 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.57-7.06 (m, 7 H, Ar-H), 5.35-5.20 (m, 2 H, NC*H*$_2$), 4.48-4.23 (m, 2 H, NC*H*$_2$), 3.10-2.96 (m, 2 H, C*H*$_2$), 2.30 (s, 3 H, C*H*$_3$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 192.3 (*C*=S), 168.3-113.2 (Ar-C), 54.8 (N*C*H$_2$), 51.1 (N*C*H$_2$), 47.3 (*C*H$_2$); ESI-MS: 342.0 [M+Na]$^+$; Anal. Calcd. for $C_{14}H_{13}N_3S_3$ (319.46): C, 52.63; H, 4.10; N, 13.15; found: C, 52.47; H, 4.25; N, 13.32.

Naphthalen-1-yl 3,4-dihydroisoquinoline-2(1*H*)-carbo(dithioperoxo)thioate (4n): Yellow oil; IR (neat): 1489.9 (N-C=S), 1110.2 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.05-7.08 (m, 11 H, Ar-H), 5.29-5.12 (m, 2 H, NC*H*$_2$), 4.43-4.15 (m, 2 H, NC*H*$_2$), 3.05-2.96 (m, 2 H, C*H*$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.1 (*C*=S), 133.3-126.4 (Ar-C), 55.8 (N*C*H$_2$), 52.1

(N*C*H$_2$), 48.3 (*C*H$_2$); ESI-MS: 390.0 [M+Na]$^+$; Anal. Calcd. for $C_{20}H_{17}NS_3$ (367.55): C, 65.36; H, 4.66; N, 3.81; found: C, 65.20; H, 4.80; N, 3.55.

Pyridin-2-yl 3,4-dihydroisoquinoline-2(1*H*)-carbo(dithioperoxo)thioate (4o): Yellow oil; IR (neat): 1488.8 (N-C=S), 1280.7 (C-N), 1108.2 (C=S) cm^{-1};^{1}H NMR (500 MHz, $CDCl_3$): δ 8.46-7.07 (m, 8 H, Ar-H), 5.30-5.20 (m, 2 H, NC*H*$_2$), 4.43-4.28 (m, 2 H, NC*H*$_2$), 3.15-2.98 (m, 2 H, C*H*$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.1 (*C*=S), 148.4-118.4 (Ar-C), 54.8 (N*C*H$_2$), 52.2 (N*C*H$_2$), 48.4 (*C*H$_2$); ESI-MS: 341.0 [M+Na]$^+$; Anal. Calcd. for $C_{15}H_{14}N_2S_3$ (318.48): C, 56.57; H, 4.43; N, 8.80; found: C, 56.40; H, 4.56; N, 8.58.

***p*-Methoxyphenyl 4-benzylpiperidine-1-carbo(dithioperoxo)thioate (4p):** Yellow oil; IR (neat): 1487.7 (N-C=S), 1280.7 (C-N), 1107.4 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.62-7.03 (m, 9 H, Ar-H), 3.76 (s, 3 H, OC*H*$_3$), 3.26-3.10 (m, 2 H), 2.65-2.50 (m, 2 H, NC*H*$_2$), 1.98-1.60 (m, 2 H), 1.50-1.20 (m, 5 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ195.1 (*C*=S), 139.4-114.5 (Ar-C), 55.6 (OC*H*$_3$), 54.5, 42.7, 38.1 (2 C), 31.2; ESI-MS: 412.0 [M+Na]$^+$; Anal. Calcd. for $C_{20}H_{23}NOS_3$ (389.59): C, 61.66; H, 5.95; N, 3.60; found: C, 61.50; H, 6.12; N, 3.40.

***p*-Tolyl 4-benzylpiperidine-1-carbo(dithioperoxo)thioate (4q):** Yellow oil; IR (neat): 1489.8 (N-C=S), 1279.9 (C-N), 1107.6 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ7.48-7.03 (m, 9 H, Ar-H), 2.67-2.50 (m, 4 H), 2.31 (s, 3 H, C*H*$_3$), 1.96-1.60 (m, 2 H), 1.50-1.20 (m, 5 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ195.1 (*C*=S), 139.4-114.5 (Ar-C), 54.4, 42.7, 38.1 (2 C), 32.2, 21.2; ESI-MS: 396.0 [M+Na]$^+$; Anal. Calcd. for $C_{20}H_{23}NS_3$ (373.59): C, 64.30; H, 6.21; N, 3.75; found: C, 64.18; H, 6.38; N, 3.50.

Naphthalen-1-yl 4-benzylpiperidine-1-carbo(dithioperoxo)thioate (4r): Yellow oil; IR (neat); 1487.2 (N-C=S), 1255.9 (C-N), 1103.7 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.04-7.09 (m, 12 H, Ar-H), 3.26-3.10 (m, 2 H), 2.58-2.54 (m, 2 H), 1.96-1.80 (m, 3 H), 1.50-1.20 (m, 4 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ194.6 (*C*=S), 139.4-125.8 (Ar-C), 54.6, 51.1, 42.6, 38.1, 32.2 (2 C); ESI-MS: 432.0 [M+Na]$^+$; Anal. Calcd. for $C_{23}H_{23}NS_3$ (409.63): C, 67.44; H, 5.66; N, 3.42; found: C, 67.27; H, 5.80; N, 3.20.

Pyrimidin-2-yl 4-benzylpiperidine-1-carbo(dithioperoxo)thioate (4s): Yellow oil; IR (neat); 1486.7 (N-C=S), 1256.5 (C-N), 1114.4 (C=S) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.58-7.05 (m, 8 H, Ar-H), 2.68-2.56 (m, 4 H), 1.96-1.78 (m, 3 H), 1.50-1.36 (m, 4 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ192.9 (*C*=S), 157.7-118.0 (Ar-C), 42.6 (2 C), 38.1 (2 C), 30.9 (2

C); ESI-MS: 384.0 $[M+Na]^+$; Anal. Calcd. for $C_{17}H_{19}N_3S_3$ (361.54): C, 56.47; H, 5.30; N, 11.62; found: C, 56.30; H, 5.46; N, 11.45.

Pyridin-2-yl 4-benzylpiperidine-1-carbo(dithioperoxo)thioate (4t): Yellow oil; IR (neat): 1487.4 (N-C=S), 1256.8 (C-N), 1107.4 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.45-7.05 (m, 9 H, Ar-H), 3.35-3.16 (m, 2 H), 2.60-2.51 (m, 2 H), 2.05-1.78 (m, 3 H), 1.55-1.30 (m, 4 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 192.0 (*C*=S), 158.0-118.4 (Ar-C), 53.5, 50.2, 41.5, 37.1, 30.9 (2 C); ESI-MS: 383.0 $[M+Na]^+$; Anal. Calcd. for $C_{18}H_{20}N_2S_3$ (360.56): C, 59.96; H, 5.59; N, 7.77; found: C, 59.80; H, 5.75; N, 7.55.

Naphthalen-1-yl pyrrolidine-1-carbo(dithioperoxo)thioate (4u): Yellow oil; IR (neat): 1486.7 (N-C=S), 1257.5 (C-N), 1114.4 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 8.08-7.43 (m, 7 H, Ar-H), 3.99-3.95 (m, 2 H), 3.81-3.77 (m, 2 H), 2.15-1.98 (m, 4 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 191.3 (*C*=S), 133.4-126.8 (Ar-C), 56.8, 50.5, 26.6, 24.2; ESI-MS: 328.0 $[M+Na]^+$; Anal. Calcd. for $C_{15}H_{15}NS_3$ (305.48): C, 58.98; H, 4.95; N, 4.59; found: C, 58.84; H, 5.10; N, 4.40.

***p*-Methoxyphenyl pyrrolidine-1-carbo(dithioperoxo)thioate (4v):** Yellow oil; IR (neat): 1486.9 (N-C=S), 1256.2 (C-N), 1110.4 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.65 (d, *J* = 9.0 Hz, 2 H, Ar-H), 6.79 (d, *J* = 9.0 Hz, 2 H, Ar-H), 3.96-3.92 (m, 2 H), 3.79 (s, 3 H, OCH_3), 3.72-3.68 (m, 2 H), 2.11-1.96 (m, 4 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 191.9 (C=S), 160.4-114.4 (Ar-C), 56.9, 55.2 (OC*H*$_3$), 50.4, 26.6, 24.2; ESI-MS: 308.0 $[M+Na]^+$; Anal. Calcd. for $C_{12}H_{15}NOS_3$ (285.44): C, 50.49; H, 5.30; N, 4.91; found: C, 50.32; H, 5.46; N, 4.72.

***p*-Tolyl pyrrolidine-1-carbo(dithioperoxo)thioate (4w):** Yellow oil; IR (neat): 1487.7 (N-C=S), 1255.5 (C-N), 1111.4 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.52 (d, *J* = 9.0 Hz, 2 H, Ar-H), 7.09 (d, *J* = 9.0 Hz, 2 H, Ar-H), 3.96-3.94 (m, 2 H), 3.76-3.71 (m, 2 H), 2.31 (s, 3 H, CH3), 2.11-1.96 (m, 4 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 191.9 (C=S), 138.3-128.4 (Ar-C), 56.7, 50.4, 26.6, 24.2, 21.2; ESI-MS: 292.0 $[M+Na]^+$; Anal. Calcd. for $C_{12}H_{15}NS_3$ (269.45): C, 53.49; H, 5.61; N, 5.20; found: C, 53.34; H, 5.78; N, 5.00.

Collection and in vitro treatment procedure of parasites

Motile adult *Setaria cervi* worms (males and females) were collected from the peritoneal cavity of freshly slaughtered cattle (*Bovis indica* Linn.) and brought into the laboratory in

normal saline (0.85%). The worms were repeatedly washed in PBS (50 mM, pH 7.0) in order to remove the extraneous materials. The mature female worms were kept in PBS (50 mM, pH 7.0) at 37 °C in humidified chamber till dissection. Compounds were dissolved in Dimethyl sulfoxide (DMSO) (Merck, Germany) with gentle heating and mild sonication. Treatments of mf and adults were given in 24 well plates (Tarson, India) and 60mm plates (Tarson, India) respectively maintaining proper sterility of the culture condition as well as media (RPMI-1640) (Himedia). Culture media was supplemented with 10% Fetal Bovine Serum (FBS) (Gibco). *In vitro* treatment of mf and adults were done following the method of Kaushal *et al*. [46] and Mukherjee *et al*.[47] The doses were standardized on the basis of the MIC and LC_{50} values obtained. The entire treatment setup was placed inside CO_2 incubator (New Brunswick, Germany) with 5% CO_2 flow for 24 hrs and repeatedly monitored for activity.

Assessment of *in vitro* parasite viability through MTT assay

Parasite viability was quantitatively assessed by performing 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT, Sigma) reduction assay [48]. After stipulated time of incubation with the compounds and succinct microscopic observations, mf and adult worms were carefully removed from culture RPMI-1640 and washed twice with PBS at 7500 rpm at 25°C for 5 mins. Approximately 1×10^3 mf and adult worms were suspended in 0.3 ml of 0.25 mg/ml of MTT (Himedia, India) followed by incubation at 37 °C for 2 hrs in dark. The dark blue crystals of formazan thus formed by the mitochondrial dehydrogenase enzyme were then solubilized in 100 µL of DMSO in a 96-well microtiter plate. The absorbance intensity was measured with the help of a microtiter plate reader (Biorad, USA) at a 595-nm wavelength keeping DMSO as a blank. The percent inhibition calculation was carried out following the method of Nayak *et al*. [49]. Each experiment was repeated for at least four times and the results were used to calculate mean ± SE [50]. The LC_{50} values of the compounds were calculated by using Origin Pro 8.0 software.

Microfilaricidal activity *in vitro* and relative movability (RM) study

Based on the LC_{50} data, compounds with maximum efficacy were chosen and triggered for determining the *in vitro* microfilaricidal efficiency. The compounds were evaluated through relative movability assessment of the treated mf with respect to the control. Mf of *S. cervi* (~1×10^5 mf/ well) were incubated with the compounds in a series of doses in a 24-well plate (Tarsons, India). The entire experiment was repeated thrice in triplicate manner. The movement of the worms was examined microscopically at every 6 h interval up to 24 h. The

movements of the worms were categorized depending on different stages and a score was given such as 0 for the dead, 4 for highly motile mf, 3 (moderately active and motile); 2 (slightly active and motile); 1 (less active but response to stimuli) following the method of Mukherjee *et al*.[47] The IC_{50} and Minimum inhibitory concentration (MIC) values were calculated using the Origin Pro 8.0 software. The obtained results were used to determine mean ± SE.

Trypan blue dye exclusion test

Upon treatment with the compounds, compound **4a** caused rapid mobility loss within 3 h of treatment. In order to confirm whether the parasites have become paralyzed or died, the entire culture was vigorously mixed to homogeneity and finally 100µL of culture media was taken out containing adequate number of parasites through pipetting and transferred into fresh media followed by shifting the plate in the CO_2 incubator. Viability assessment of *S. cervi* microfilariae was checked primarily by trypan blue, because it can stain dead cells blue and live one transparent. The microfilariae viability (%) was determined by dividing the number of live ones by the total number of microfilariae following the method of Mukherjee *et al*.[47]

Evaluation of cytotoxicity against human normal lung fibroblast cell line WI-38

In order to carry out this experiment 2.0×10^4 WI-38 cells were grown in 48 well plates for 24 h. Post incubation cells were treated with varying concentrations (0-50 µg/mL) of the compounds for 24 h. Later, 5% FBS containing phenol red free RPMI with MTT (400 µL; 0.5 mg/mL) was added to each well and incubated at 37 °C for 4 h in a humidified incubator containing 5% CO_2. The purple colored formazan crystals formed in the wells were dissolved in DMSO and their absorbance was measured at 570 nm with a microplate reader (Multiscan GO).

Assessment of generated superoxide anion through NBT assay

Following requisite time of incubation with the selected compounds (**4a, 4b, 4c, 4d** and **4h**) the cultures of mf and only **4a** treated adult were collected and centrifuged at 7500 rpm for 12 mins at 25 °C and the supernatants were discarded and the parasites pellets were washed thoroughly in PBS (50 mM, pH 7.0). The parasites pellets were incubated in 2% nitroblue tetrazolium salt (NBT) solution at 37 °C for 2 h. The tetrazolium salt reacts with the generated superoxide anion and forms formazan crystals. After stipulated time the pellets

were again centrifuged and washed with PBS following one methanol wash. The crystals were dissolved in a mixture of 240 µL of 2M KOH and 480 µL of DMSO with placid shaking for 10-15 mins at room temperature [47]. The absorbance was measured at 620 nm using a micro-plate reader (BioRad, USA).

Assessment of chromatin condensation through Hoechst 33342 staining in mf

Chromatin condensation in the treated mf was detected microscopically by nuclear staining with Hoechst 33342 [12]. Briefly after treatment with the compound, the mf were washed in PBS (50 mM, pH 7.2) and were fixed in paraformaldehyde. Then the treated and untreated mf were stained with 1 mg/mL Hoechst 33342 stain and incubated for 5 mins followed by a gentle wash in PBS. Poly-L-Lysine coated glass slides were prepared and mf were fixed on to it and caught in the fluorescence microscope at 490 nm excitation (Leica, Germany).

Double staining of oocyte and mf

AO/EtBr double staining was performed following the method described in Roy *et al*.[17] Briefly 1:1 ratio of AO and EtBr from the stock of 100 mg/mL in 0.1 M PBS was prepared and added on the oocyte and mf. The suspensions were taken on to clean glass slides, covered by cover glass and photographed under fluorescence microscope (Dewinter, Italy).

Fluorescence microscopy of Annexin V-FITC and PI stained oocytes

Oocytes were collected from the gravid females of *S. cervi* and they were cultured in RPMI-1640 supplemented with FBS and treated with the test compound **4a** at 1 µg/mL and 10 µg/mL doses for stipulated time period. After proper incubation period oocytes were properly washed and suspended in PBS (50 mM, pH 7.2). The suspension was centrifuged at 7500 rpm, at 25 °C for 5 mins and incubated with Annexin V- FITC and PI (Sigma Aldrich, MO, USA) following the manufacturer's guideline followed by photography under inverted fluorecence microscope (Leica, Germany) and Las-X software.

Flow cytometry analysis of oocyte and mf

In order to study quantitatively the apoptotic effects of the targeted compounds on *S. cervi*, oocytes (~1×10^3) and mf (1×10^4) were treated with two different concentrations (50 and 100 µg/mL) of compound **4a** and cultured for 8 h and 16 h at 37 °C for oocyte and mf respectively. The treated oocyte and mf were obtained following centrifugation and washing with PBS twice accordingly and resuspended in PBS (50 mM, pH 7.0) at 4 °C overnight.

Oocyte and mf were both stained with AnnexinV-FITC Apoptosis Detection Kit (Sigma, USA) following Mukherjee *et al*.[11]. Quantitative flowcytometry analysis was further done through BD FACSDiva version 6.1.2.

Western blotting of the apoptogenic protein in the adults of *S. cervi*

Profiling of nematode specific apoptogenic proteins i.e. EGL-1, CED-3 and CED-9 were assessed using western blot experiments with GAPDH as loading control [29]. Briefly, proteins from control and treated adult nematodes (100 µg) were estimated following Lowry *et al*. [51]. Each proteins (100 µg) resolved by 12.5% SDS-PAGE, transferred on poly vinylidene difluoride (PVDF) membranes, blocked with 10% skimmed milk and incubated with different primary antibodies overnight at 4 °C. The membranes were thereafter washed and incubated with alkaline phosphatse conjugated secondary antibody for 6 h. Alterations in the expressions of the said apoptogenic proteins were examined by specific substrate BCIP/NBT (Amresco, USA).

Quantitative structure activity relationship (QSAR) analysis

In order to elucidate a quantitative structure-activity relationship, the molecular structures were compared with the IC_{50} values obtained from the experimental data. 3D-QSAR module of Accelry's discovery studio [42] was used for deriving the atomic analysis. All molecular structures were prepared and minimized using Maestro GUI software. All 23 molecules were categorized into test set and training set. The model divides the space of grids into uniform-sized cubes that reflect the biological relationship using structural features, corresponding through positive (blue) and negative (red) contours. To reflect statistically significant results the characterization of molecules was performed using independent variables for structural components. Models were built up using partial least-squares regression (PLS).

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Development of novel anti-filarial agents using carbamo(dithioperoxo)thioate derivatives

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Research highlights:

- Carbamo(dithioperoxo)thioate derivatives were prepared using one-pot reaction.
- Synthetic compounds were evaluated for their potential as antifilarial agents.
- Five compounds were found promising anti-filarial agents against *Setaria cervi*.
- Biochemical and microscopic experiments were carried out for the best compound.
- Anti-filarial potential of active compounds rely on ROS and apoptosis pathway.