10. SUMMARY & CONCLUSION

The present work deals with generation of a small combinatorial library of 22500 ligands using virtual screening method of thiadiazole scaffold. MPO was selected as target for the study and the designed ligands were screened for their ADMET properties using Qikprop, binding affinity of ligands was calculated by protein ligand binding energy (ΔGbind kcal/mol) approach and molecular docking was carried out at three levels (HTVS, SP and XP) and 40 top MPO inhibitors were identified and based on literature survey results 18 were selected for synthesis.

The synthesis was carried out in two steps by thermal condensation of heterocyclic amines with carbon disulphide and hydrazine hydrate in presence of sodium carbonate and chloroacetic acid to yield heterocyclic amine substituted thiosemicarbazides followed by condensation and cyclisation with aryl or unsaturated aryl carboxylic acids in presence of phosphoryl chloride to yield 2,5-disubstituted-1,3,4-thiadiazoles. Synthesized compounds were purified by column chromatography and characterized using IR, $^1$H NMR, $^{13}$C NMR, Mass and XRD.

Pharmacophore modeling was done for the synthesized compounds (Phase Module) to identify the common features for good activity. The optimization processes identified DRRR (1 H-Bond Donor and 3 Ring Aromatics) as common pharmacophore constraints and from the QSAR set of 18 compounds compound 3350, 3800, 4250, 4700 and 5150 were found to have good fitness score between 2.5-3.00.

The synthesized compounds were analyzed for their free radical scavenging property at concentration (31.25, 125, 500 and 1000µg/ml) by DPPH, ABTS and TAA. Among all the compounds tested compounds 3350, 3800, 4250, 4700 and 5150 showed effective good to moderate free radical scavenging activities compared with standard
ascorbic acid at IC50 range - 16 to 29(µg/ml) for DPPH, 50 to 68(µg/ml) for ABTS and 201 to 271 (mm eq) for total antioxidant activity. Among the tested 18 compounds top 5 (Compounds 3350, 3800, 4250, 4700 and 5150) were selected for further enzymatic and non-enzymatic antioxidant study which was carried by both computational and in-vitro screening methods.

**Enzymatic and non-enzymatic antioxidant study**

The selected compounds 3350, 380), 4250, 4700 and 5150 were screened for their enzymatic and non-enzymatic potential using computational studies (docking and Protein ligand binding energy (ΔGbind kcal/mol) calculation) with selected enzymatic targets viz Human Catalase (IDGH) and Glutathione-S-Transferase (3GUS). The results showed compound 3350 and 5150 with increased glide score and increased binding affinity (ΔG kcal/mol) of 81.366 for (5150) and 85.331 for (3350) with target Human Catalase and -73.821 for (5150) and -58.431 for (3350) with target Glutathione-S Transferase. Among all the compounds tested, compound 5150 displayed increased binding affinity when compared to standard ascorbic acid -37.31 for Human Catalase and -34.13 for Glutathione S Transferase.

The *in-vitro* enzymatic and non enzymatic antioxidant study was performed on compounds 3350, 3800, 4250, 4700 and 5150 at a concentration of 100µg/ml by performing specific enzyme antioxidant namely (catalase, superoxide dismutase and glutathione peroxidase) and non specific antioxidant activity (vitamin C & vitamin E) . The enzymatic and non enzymatic antioxidant studies have shown compound 3350 to be equipotent and compound 5150 with increased activity when compared with standard ascorbic acid. All the values were expressed as average of triplicates in terms of mean ± SEM. Compound 5150 displayed maximum enzymatic antioxidant activity for (catalase, SOD and GST) as (82.41± 1.49, 0.83±0.344, 139.1±2.82) when compared with
compound 3350 (63.42±3.08, 0.79±0.045, 123±3.401) and ascorbic acid standard (59.17 ±2.2, 0.81±0.233, 127.4±1.713). Similarly compound 5150 showed maximum activity against non enzymatic antioxidants vitamin C and vitamin E in mg/gm (7.6±0.696, 0.74±0.040) compared to compound 3350 showing (5.4±0.737, 0.69±0.163) and standard ascorbic acid showing (6.1±0.614, 0.64±0.163). The results proved 5150 and 3350 with excellent antioxidant profile compared with standard ascorbic acid.

Compound 3350 and 5150 showed maximum antioxidant potential and were selected for simultaneous computational and in-vitro screening methods against oxidative stress induced diseases.

**Anti inflammatory study**

Human 5-Lipoxigenase and TGF beta type I were selected as inflammatory targets, compound 3350 and 5150 were subjected to molecular docking and ligand binding affinity studies. The docking score was found to be maximum for standard Diclofenac (Table 33, 35) but the ligand binding affinity studies have suggested both compounds displayed good binding affinity with compound 5150 showing maximum ($\Delta G_{bind} = \text{kcal/mol}$) against Human 5-Lipoxigenase (-75.263) and TGF beta type I(-68.5) compared to Diclofenac standard (-55, -41).

The *in-vitro* anti inflammatory studies of Compound 3350 and 5150 were evaluated by their effect on HRBC membrane stabilization method (Heat and Hypotonicity induced hemolysis) at concentrations ranging from (100-500μg/ml). Both compounds 3350 and 5150 show concentration dependant increase in protective effect on both heat and hypotonic saline-induced erythrocyte lysis. Compound 5150 showed significant percentage stabilization of 80-85% when compared to standard Diclofenac 75-80%. 

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Anti arthritis study

Tumor Necrosis factor alpha and p38 MAP kinase were selected as targets for arthritis. The computational studies showed compound 5150 with nearly equivalent glide score of (-4.3 and -7.04) compared to standard (-5.9 and -7.35) for the target TNF alpha and p38 MAP kinase. The ligand binding affinity studies have suggested compound 5150 with increased binding affinity of -73.67 with p38 MAP kinase compared to -67.34 for Diclofenac.

The in-vitro anti arthritic studies of compound 3350 and 5150 was evaluated by their percentage inhibition of protein denaturation and protease inhibition at concentrations ranging from (100-500 μg/ml). The percentage inhibition of protein denaturation and protease inhibition was found to be between 91%-92% for compound 5150, 84.85% for compound 3350 compared to 86.87% for Diclofenac sodium. These findings suggest compound 5150 to show increased anti arthritic activity on par with Diclofenac studies.

Anti cancer study

Aurora Kinase A and Aurora Kinase B were selected as targets for cancer study. The computational studies showed compound 5150 with increased glide score -5.6 and ligand binding affinity -73.05 against Aurora kinase B were compound 3350 showed increased glide score -5.9 and ligand binding affinity -68.19 against Aurora Kinase A.

The in-vitro cytotoxicity of the compound 3350 and compound 5150 was evaluated by MTT cell proliferation assay against human cancer cell lines namely PC3 (Prostate Cancer), T47D (Breast Adenocarcinoma), HepG2 (Liver Cancer) and A549 (Lung Cancer) at serially fivefold dilutions (62.5, 125, 250, 500 and 1000 μg/ml). The cytotoxicity values showed both the compounds to be active on all the four cell lines with IC_{50} values in the range of 190-70 μg/ml. Compound 3350 showed increased cytotoxicity
against HepG2 Cell lines (81.6 μg/ml) were as compound 5150 showed increased cytotoxicity against PC3 cell line (70.1 μg/ml).

**Anti hyperlipedemic study**

HMG CoA reductase and Squalene synthase were selected as targets for hyperlipedemia. The computational studies showed both the compounds with decreased glide score against HMG CoA Reductase when compared with gallic acid. Compound 5150 showed increase glide score (-7.2) against Squalene synthase compared to gallic acid -6.6. The protein ligand binding studies presented both the compounds with excellent binding affinity with HMG CoA Reductase and Squalene synthase. Compound 5150 showed maximum (ΔGbind = kcal/mol) for HMG CoA Reductase (-44.57) and squalene synthase (-81.097) compared with standard gallic acid. (-16.24, -49.789). The results show compound 5150 to be the most stable conformer for both HMG CoA Reductase and Squalene synthase.

The *in-vitro* anti hyperlipedemic activity was studied by estimation of Cholesterol solubilization technique and determination of bile acid binding capacity. Compound 5150 showed significant inhibition towards cholesterol inhibition 37.4±0.23 compared to 27.26±0.71 for gallic acid and increased bile acid inhibition 27.55±2.32 (Glycodeoxycholic acid), 31.43±2.31 (Taurocholic acid) and 6.52±0.88 (Taurode Oxycholic acid) compared to standard cholestyramine 28.4±2.4, 30.3±1.35, 5.65± 0.76.

**Anti diabetic study**

Aldose Reductase and Protein tyrosine phosphatase were selected as targets for diabetic study. The molecular docking studies suggested both the compounds with decreased glide score compared to selected standard Glibenclamide. The ligand binding affinity studies showed compound 5150 with increased binding affinity of -77.43 (Aldose
Reductase) and -58.43(PTP-1β) compared to compound 3350 but less than standard (-91.36, -91.71).

The *in-vitro* anti-diabetic activity of compounds 3350 and compounds 5150 was carried out at concentration of (100-500 μg/ml) by study of percentage increase of glucose uptake by yeast cells and percentage inhibition of the amount of glucose remaining in the medium after a specific time. A dose dependant increase in the percentage inhibition of glucose uptake in yeast cells was observed. Compound 5150 exhibited significantly higher activity 96.3% than compound 3350 with 82.2% at 500 μg/ml when compared to standard Metronidazole 82.5%. Alpha-amylase inhibition method showed compound 5150 shows good activity as compared to compound 3350 but significantly less activity when compared with Glibenclamide standard drug.

**Anti atherosclerotic study**

Human Neutrophil Elastase and Human Phospholipase A2 were taken as targets for study. The molecular docking studies had shown compound 5150 with increased glide score (-5.74, -3.41) against sPLA2 & HNE compared to compound 3350 (-4.43, -3.06). The protein ligand binding studies showed compound 5150 with increased binding affinity of ( -58.79 , -64.56 ) for sPLA2 & HNE compared to compound 3350 (-50.78, -48.02).

The anti atherosclerotic activity of compound 3350 and compound 5150 was evaluated by measuring the effect of compounds on serum HDL-Paroxonase activity and HDL oxidation at concentration (0-100μg/ml). A concentration dependent inhibitory effect of purified PON on HDL oxidation was observed when this lipoprotein was pretreated with compound 5150 than reference and compound 3350. Compound 5150 (14 ± 1*) showed maximum decrease in serum paroxonase activity compared to trolox standard (14 ± 5*) and compound 3350 (18 ± 6*). Conversely the HDL Oxidation -
Malonaldehyde increases with increase in concentration of test samples. Compound 5150 and compound 3350 showed increased HDL oxidation (37.3 ± 0.8*, 22.3 ± 0.2*) compared to trolox (20.7 ± 0.2*).

**Neuroprotective study**

Acetyl Cholinesterase and Butryl Cholinesterase were selected as targets for computational study. Both the compounds 3350 and 5150 showed good compatible glide score of -8.26, -8.57 compared to trolox (-8.51) for Acetyl Cholinesterase. Compound 5150 displayed increased ligand binding affinity (-58.62, -49.62) against both Acetyl Cholinesterase and Butryl Cholinesterase compared to compound 3350 (-54.68, -33.39).

The *in-vitro* neuroprotective effect was performed based on effect of compounds at concentration of 1, 5, 10 μg/ml on dose-dependent glutamate induced oxidative toxicity by determining the percentage of MTT reduction upon incubation of HT22cells. To determine the cytotoxic potential of compound 3350 and 5150 cell viability of HT22 cell and BV2 cells was evaluated. Dose dependent decrease in cellular viability was observed. Compound 5150 showed maximum decrease in cellular viability of (75.18, 86.51%) in HT22 and BV2 cells compared to (64.61, 67.69%). The effective dose of compound 5150 on HT22 and BV2 cells was found as

- HT22-ED$_{50}$ (μM)-3.1 μM (5150) - 3.7 μM (Trolox)
- BV2 -ED$_{50}$-(5150)-2.17 μM and 3.14 μM (Trolox)

Both the compounds were found to be least toxic and showed increased cell viability but compound 5150 showed increased cell viability than standard trolox and doxorubicin.
Effect of compound 5150 on ht22 murine hippocampal cells against endoplasmic stress induced apoptosis

The simultaneous in-vitro and computational study showed compound 5150 with pronounced results against selected oxidative stress induced diseases compared to their corresponding standards. Compound 5150 displayed maximum in-vitro neuroprotective effect at a micromolar concentration of 3.6 compared to trolox at 4.5. ER stress has increasingly come into focus as a factor contributing to neuronal injury. The effects of 5150 on apoptotic death of HT22 mouse hippocampal neuronal cells induced by thapsigargin (TG) and brefeldin A (BFA), two representative ER stress inducers was carried out by measuring Cell viability (MTT), Apoptosis (Sub G1 DNA content), Reactive oxygen species (ROS) production - flow cytometry, expression level of activation and cleavage of apoptosis associated proteins (caspase 12, Caspase 3 and PARP), phosphorylation status of ER stress-associated proteins (p38, JNK, and ERK) and were analyzed by Western blot analysis and Mitochondrial Membrane Potential by staining with 3, 3'-dihexyl oxacarbocyanine iodide (DiOC6)-flow cytometry.

Compound 5150 reduced Thapsigargin and Brefeldin-A induced apoptosis of HT22 cells by reducing ROS accumulation, activation and cleavage of apoptosis-associated proteins, such as caspase-12 and -3 and poly (ADP-ribose) polymerase. Compound 5150 also reduced the Thapsigargin and Brefeldin-A -induced expression of ER stress-associated proteins, mitogen-activated protein kinases, such as p38, JNK, and ERK. Compound 5150 prevent TG (62.8 – 82.4%) and BFA (71.4 – 86.1%) induced mitochondrial damage through antioxidant activity when compared with NAC.

All the studies of in-vitro and computational study have shown compound 5150 with desired activity as anti inflammatory, anti arthritic, anti cancer, anti
hyperlipedemic, anti diabetic, anti arthrosclerosis and neuroprotective activity in a dose dependant manner.

The IUPAC name of the screened Compound 5150 is N-(4-((Z)-phenyl diazenyl) phenyl)-5-((Z)-styryl -1,3,4-thiadiazol-2-amine

![Chemical structure of Compound 5150]

In specific, compound 5150 showed good Neuroprotective effect at ED_{50} of 3.1 \mu M in HT22 cell line. Compound 5150 showed neuro protective effect for ER stress-induced apoptosis in HT22 neuronal cells. Compound 5150 could be concluded to protect HT22 neuronal cells against ER stress-induced apoptosis by reducing CHOP induction as well as reactive oxygen species accumulation and mitochondrial damage.

**Compound 5150 appear to be a promising drug candidate for development as a potential therapeutic and neuroprotective agent for various neurodegenerative disorders.**