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Research Article

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Design, synthesis and screening of novel 5-substituted-1,3,4-thiadiazol-2-amines and their Schiff bases

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ABSTRACT

In the present study, an attempt was made to synthesize novel 5-substituted-1,3,4-thiadiazol-2-amines and their Schiff bases to evaluate antioxidant, anti-inflammatory and anticancer activities. Various aromatic acids were made to react with thiosemicarbazide and benzaldehyde conventionally to yield the title compounds. The synthesized compounds were screened for antioxidant and anti-inflammatory activity by DPPH radical and carrageenan induced rat paw oedema model respectively. In silico, in vitro and in vivo anticancer activity was carried out respectively by docking, MTT assay and EAC liquid tumor model. Among the compounds tested, TDZS-4 showed promising antioxidant activity with low IC_{50} value (16.2 µg/mL). Further, it also exhibited significant reduction in the inflammation both at 1st and 3rd h. Compounds TDZS-1 and TDZS-2 showed better Mol dock scores indicating their better interaction with 2CIB protein. Compounds which showed well docked configuration with 2CIB had also shown better in vitro cytotoxicity on Hep2 cell lines. Among them, treatment of EAC tumor bearing mice with TDZS-1 was found to be effective. To conclude, Schiff bases showed promising biological activities and the presence of electron withdrawing groupsin the scaffold may assist their activity potential.

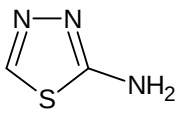
Key words: 1,3,4-thiadiazoles,antioxidant, anti-inflammatory, anticancer activity.

INTRODUCTION

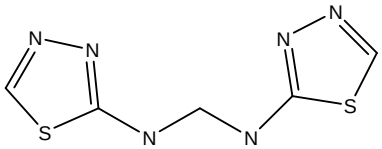
Heterocycles containing nitrogen, oxygen, sulphur and selenium possess varied pharmacological activities[1,2]. Thiadiazole is a 5-membered heterocyclic ring system containing two nitrogen and one sulphur atom. Among the three isomers of thiadiazole, 1,3,4-thiadiazole exhibits wide spectrum of biological activities which could be due to the presence of -N=C=S moiety (3).The detailed literature survey revealed that thiadiazoles are widely exposed to therapeutic world, because of their known anti-HIV [4], anti-inflammatory[5-7] anticancer [8], antitubercular [9] antihypertensive [10,11], antibacterial [12], antifungal [13], antioxidant, antiprotozoal [14], anticonvulsant, diuretic, radio protective, sedative-hypnotic and CNS neurotoxicity [15]activities. Sulphonamide drugs containing 1,3,4-thiadiazoles were reported as antibacterial agents in the market. Further, the drugs such as, acetazolamide and methazolamide are the 1,3,4-thiadiazole derivatives and were reported for their diuretic property. Another drug worthwhile to be mentioned here is sulfamethazole, which was referred for their antimicrobial activity [16].

Furthermore, identification of new compounds for the treatment of cancer is an important undertaking in pharmaceutical research. The unusual ring structure of 2-amino-1,3,4-thiadiazoles and their related compounds with their effect in a number of experimental tumour systems have attracted considerable interest in cancer chemotherapy

[17, 18]. For example, 2-amino-1,3,4-thiadiazole [Compound 1a] for advanced non squamous cervical carcinoma [19] and 2,2'-(methylenediimino)bis-1,3,4-thiadiazole (Compound 1b) for antitumor activity [20].



[Compound 1a]



[Compound 1b]

In the view of the above findings, it was considered worthwhile to synthesize new analogues of 1,3,4-thiadiazole with further modification on 2nd and 5th position in order to optimize the structure activity relationship (SAR) and their potency against inflammation and cancer.

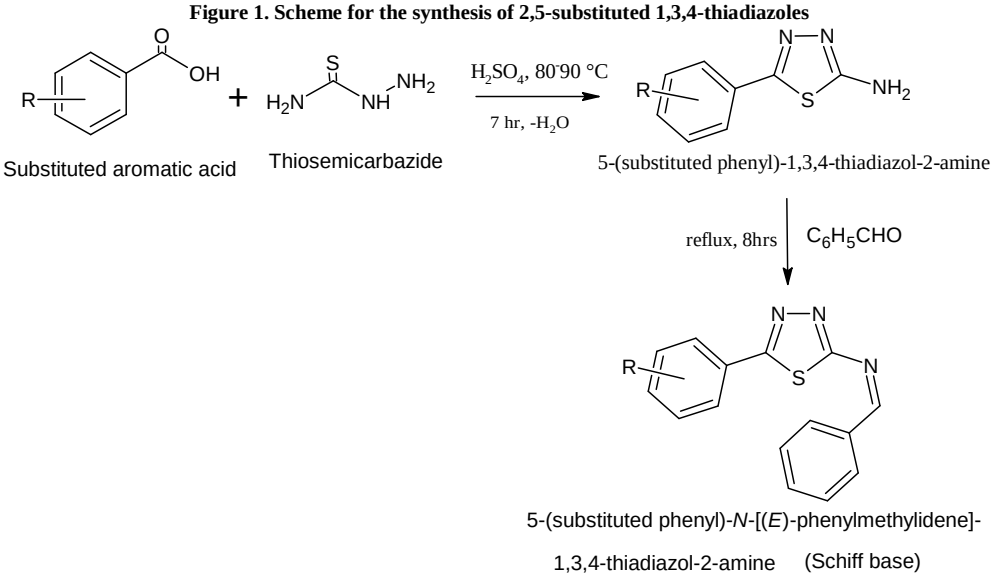
EXPERIMENTAL SECTION

The melting point of the synthesized compounds was determined by open capillary tube method using scientific melting point apparatus and it was uncorrected. The progress of the reaction and the purity of the compounds wereanalyzed by using precoated TLC plates; the solvent system used was Petroleum ether and Ethyl acetate (1:9). The spots were visualized under UV light. Further, the compounds were characterized by using UV absorption spectrophotometer, Shimadzu UV 4650 JAPAN. IR spectra of the synthesized compounds were recorded using Shimadzu FT-IR 8310 JAPAN and KBr press. Proton and Carbon-13 NMR spectra of the synthesized compounds were recorded on Bruker Biospin Avance-300MHz at SAIF, IIT, Chennai. Mass spectra of the synthesized compounds were recorded on Shimadzu MS-MS QP5050 at SAIF, IIT, Chennai.

Chemistry

Totally ten novel 5-substituted-1,3,4-thiadiazol-2-amine derivatives were synthesized according to the scheme given in Figure 1. Various substituted aromatic acid (0.15 mol), concentrated sulphuric acid (sp. gr. 1.84, 25 mL), and thiosemicarbazide (0.125 mol) were slowly heated to 80-90°C on a thermostatically controlled water bath for 7 h. After cooling, the content was poured onto crushed ice. The acid was neutralized with the help of 10 % ammonia solution. The crude precipitate, 5-(substituted phenyl)-1,3,4-thiadiazol-2-amines that got separated was filtered, washed several times with distilled water, dried and recrystallized from 50 % ethanol. [21, 22]. The same was dissolved (0.01 mol) in 20 mL of glacial acetic acid. To that, the solution of substituted aromatic aldehyde (0.01 mol) in glacial acetic acid was added drop wise with stirring and the reaction mixture was heated to reflux for 8 h.After completion of the reaction, the content was poured onto crushed ice; the product thus obtained was filtered, washed with water and recrystallized from ethanol[23].

Figure 1. Scheme for the synthesis of 2,5-substituted 1,3,4-thiadiazoles



Substituted aromatic acid — Thiosemicarbazide — 5-(substituted phenyl)-1,3,4-thiadiazol-2-amine

5-(substituted phenyl)-*N*-[(*E*)-phenylmethylidene]-1,3,4-thiadiazol-2-amine (Schiff base)

The spectral data of the representative compounds were as follows:

4-(5-amino-1,3,4-thiadiazol-2-yl)-2-nitrophenol (TDZ-1):
Yield: 68%; m.p. 205 ºC; IR (KBr) cm^{-1}: 665.4 (C-S), 842.89 (N-O), 1510.26 (C=N), 1620.21 (NO_2), 1323.17 (C-N), 3425.58 (NH_2); ^{1}H NMR (DMSO) δ ppm: δ 3.5(singlet), δ 8.8-9.3(multiplet); ^{13}C NMR (DMSO) δ ppm: δ 136 (NO_2 carbon), δ 109-167 (aromatic carbons); MS: *m/z* 238 (M^+), 138($C_6H_5NO_3$), 61(CH_3NS).

5-(4-methoxy-3-nitrophenyl)-1,3,4-thiadiazol-2-amine (TDZ-2):
Yield: 60%; m.p. 210 ºC; IR (KBr) cm^{-1}: 1637.56 (NO_2), 1504.48 (C=N); ^{1}H NMR (DMSO) δ ppm: δ 3.3 ppm (singlet), δ 6.4-8.0 ppm (multiplet), δ 4.8 ppm (singlet); MS: *m/z* 252 (M^+), 206($C_9H_8N_3OS$), 190($C_9H_6N_2OS$), 71(C_2H_2NS), 236($C_9H_6N_3O_3S$).

5-(3,5-dinitrophenyl)-1,3,4-thiadiazol-2-amine (TDZ-3):
Yield: 62%; m.p. 215 ºC; IR (KBr) cm^{-1}: 1635.64 (NO_2), 1506 (C=N), 3273.2 (NH_2); ^{13}C NMR (DMSO) δ ppm: δ 110-170 ppm (aromatic carbons); MS: *m/z* 257 (M^+), 165($C_7H_7N_3S$), 205($C_8H_3N_3O_2S$), 148($C_7H_4N_2S$), 175($C_8H_5N_3S$).

5-(2-amino-4-chlorophenyl)-1,3,4-thiadiazol-2-amine (TDZ-5):
Yield: 70%; m.p. 231 ºC; IR (KBr) cm^{-1}: 3277 (NH_2), 1510 (C=N), 1109.07 (C-Cl), 1635.64 (NO_2); MS: *m/z* 255 (M^+), 194($C_8H_4ClN_2S$), 159($C_8H_3ClN_2S$), 110(C_6H_3Cl).

2-nitro-4-(5-{[(Z)-phenylmethylidene]amino}-1,3,4-thiadiazol-2-yl)phenol (TDZS-1):
Yield: 42%; m.p. 160 ºC;IR (KBr) cm^{-1}: 3390 (OH), 1543 (NO_2), 1582 (C=N), 1311.6 (C-N); MS: *m/z* 326 (M^+), 280($C_{15}H_{10}N_3OS$), 163($C_7H_5N_3S$), 138($C_6H_4NO_3$).

***5-(4-methoxy-3-nitrophenyl)-N-[(Z)-phenylmethylidene]-1,3,4-thiadiazol-2-amine*(TDZS-2):** Yield: 39%; m.p. 168 ºC; IR (KBr) cm^{-1}: 1313.52 (C-N), 1618.28 (C=N), 1541.12 (NO_2); MS: *m/z* 340 (M^+), 295($C_{15}H_9N_3O_2S$).

5-(2-amino-4-chlorophenyl)-N-[(Z)-phenylmethylidene]-1,3,4-thiadiazol-2-amine* (TDZS-*5): Yield: 34%; m.p. 173 ºC; IR (KBr) cm^{-1}: 3439 (NH_2), 1647 (NO_2); MS: *m/z* 314 (M^+), 299($C_{15}H_{10}ClN_3S$); ^{1}H NMR (DMSO) δ ppm:δ 2.5 ppm (singlet), δ 1.8 ppm (singlet), δ 7.3-8.5 ppm (multiplet).

Biological evaluation

Antioxidant activity-DPPH radical scavenging assay

The assay was carried out in a 96 well microtitre plate. 100 μL of DPPH solution was added to 100 μL of each of the test sample of concentrations 250, 125, 62.5, 31.25 and 15.125 μg/mL and the standard solution i.e., ascorbic acid, separately in each well of the microtitre plate. The plates were incubated at 37ºC for 20 minutes and the absorbance of each solution was measured at 540 nm, using Enzyme Linked Immuno Sorbent Assay (ELISA) microtitre plate reader. The absorbance of solvent control containing the same amount of methanol and DPPH solution was measured as well. The experiment was performed in triplicate and % scavenging activity was calculated using the formula given below. IC_{50} (Inhibitory Concentration) is the concentration of the sample required to scavenge 50 % of DPPH free radicals and it was calculated from the graph, % scavenging Vs concentration [24].

$$\% \text{ Inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100$$

Antioxidant activity-Nitric Oxide scavenging assay

The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (PBS, 0.5 mL) and 0.5 mL of each test sample or ascorbic acid in DMSO were incubated separately at 25ºC for 150 minutes. After incubation, 50 μL of the reaction mixture containing nitrate was removed and 100 μL of sulphanilamide reagent was added and allowed to stand for 5 minutes for completion of diazotization.Then, 100 μL of naphthyl ethylene diamine dihydrochloride was added and allowed to stand for 30 minutes in diffused light at room temperature. The absorbance of these solutions was measured at 540 nm using ELISA microtitre plate reader. The absorbance of solvent control containing the same amount of DMSO, sodium nitroprusside, sulfanilamide and NEDD reagents was measured as well. The experiment was performed in triplicate and % scavenging activity was

calculated using formula given below. IC_{50} is the concentration of the sample required to scavenge 50 % of nitrite ions and it was calculated from the graph, % scavenging Vs concentration [25].

$$\% \text{ Inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100$$

***In vivo* Anti-inflammatory activity Carrageenan induced rat hind paw oedema model**

Selection of animals

Wistar rats inbred at Central Animal Research Facility, Manipal University, Manipal 576104 were used in the study.

Acute toxicity study

Acute toxicity studies were conducted to determine the safe dose by up and down stair case method. Animals were divided into groups each consisting of 6 animals. Drug suspension was prepared using 0.25 % carboxy methyl cellulose (CMC). Test compounds were given orally as suspension at a single dose of 500, 1000 and 2000 mg/kg body weight. Control group was treated with 0.25 % CMC. Animals were continuously observed for 7 days for behavioral changes, toxicity and mortality [26].

Procedure

0.25 % CMC was selected as vehicle to suspend the standard drug and test compounds. The rats were starved for 18 h prior to the experiment. The animals were divided into 7 groups, each containing of six animals. Oedema was induced in the left hind paw of all rats by subcutaneous injection of 0.1 mL of 1 % w/v carrageenan in 0.9 % saline into their footpads. The 1st group was kept as control and was given 0.25 % CMC. The 2nd to 6th groups were given an aqueous suspension of the synthesized compounds at 100 mg/kg body mass. And, the 7th group (standard) was administered Diclofenac (10 mg/kg body mass). All the test compounds and the standard drug were administered orally 1 h before the carrageenan injection. The paw volume of each rat was measured using a digital plethysmometer (UGO Basil, Italy), immediately after the carrageenan injection (0 h) and at 1st and 3rd h of the post administration of carrageenan injection. The increase in the paw volume was calculated for each group and the results were expressed as the mean increase in the paw volume ± S.E.M. Results were analyzed statistically using one-way ANOVA followed by multiple comparison by post-hoc Dunnett's test. The minimum level of significance was fixed at $p < 0.01$. The percentage inhibition of paw volume for each test group was calculated using the following equation [27].

Percentage of inhibition (%) = 100 (1- (x/y))

Where
x = mean increase in paw volume of treated animals
y = mean increase in paw volume of control animals

Docking studies

Molecular docking is a tool of computational investigation of ligand binding to a receptor. It predicts the affinity and activity of the molecules on their protein targets [28]. The X-ray crystal structure of the protein, 2CIB [29] was downloaded from the RSCB Protein Data Bank (RSCB PDB) for the docking studies. Molegro Virtual Docker (MVD) program was used in docking simulation to investigate the potential binding modes of compounds TDZ 1-5 and TDZS 1-5 on the catalytic site of cytochrome P450 14α-demethylase (PDB, 2CIB) as the target enzyme. This study rendered useful information to design 2-amino-1,3,4-thiadiazole as inhibitors of cytochrome P450 14α-demethylase.

Experimental

The molecules in the workspace must be in a suitable format before they are involved into the docking process. Hence, the 3D conformations of the molecule and the enzyme were generated, then, they were imported into the MVD software. Then, the potential binding site for the protein was narrowed down by selecting the option 'Detect Cavities'. Then, the docking simulation was performed by invoking the 'Docking Wizard' option. While the simulation was running, the energy of the best pose (the pose with the lowest energy) was observed on the 'Graph tab page'. The graph showed the docking score as a function of number of iterations performed by the docking search algorithm. When the docking run finishes, the poses found can be imported into MVD by dragging and dropping the Docking results icon onto the MVD application.

After importing the Docking Results file, the Pose Organizer will appear showing the poses found. The pose with highest moldock score was added to the workspace. The possible H-bond interactions seen in the ligand-receptor complex were identified and their bond length and total energy along with the atoms and amino acids involved in the interaction were noted down. The molecule with highest Moldock score value was considered to be more potent comparative to others.

In vitro anticancer activity- MTT assay

Exponentially growing cells were harvested from T-25 mL flask and a stock cell suspension was prepared. A 96-well flat bottom tissue culture plate was seeded with $5x10^4$ cells/mL in medium and supplemented with 10 % FBS. Then, the plate was incubated at 37ºC for 24 h in 5 % CO_2 atmosphere. A partial monolayer was formed after 24 h; the supernatant liquid was flicked off and to this 100 μL of different drug concentrations (1000 μg/mL, 500μg/mL, 250μg/mL and 125μg/mL) was added. The cells in the control group received no treatment. The plates were then incubated at 37ºC for 3 days in 5 % CO_2 atmosphere. After the treatment for 72 h, drug containing media was removed and the plates were washed twice with 100 μL of PBS. To each well of the 96 well plate, 100 μL of MTT reagent (stock: 2 mg/mL) was added and incubated for 4 h at 37 ºC. Plates were centrifuged at 2000 RPM for 10 minutes and inverted on tissue paper to remove the media. To solubilize formazan crystals in the wells, 100 μL of isopropanol was added to each well and incubated at 37ºC for 30 minutes. The optical density (OD) was measured by an ELISA plate reader at 540 nm.

The percentage growth inhibition was calculated using the following formula.

$$\text{Percentage growth inhibition} = 1 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right) \times 100$$

The IC_{50} value obtained was the concentration of sample required to inhibit the growth of 50 % of viable cell population [30].

In vivo anticancer activity - EAC liquid tumor model **[31]**

Selection of animals

Swiss albino mice inbred at Central Animal Research Facility, Manipal University, Manipal 576104 were used in the study.

Liquid tumor model

The EAC cells originally obtained from Amala Cancer Research Center, Thrissur, Kerala, India, were maintained and propagated by serial intraperitoneal transplantation in adult Swiss Albino mice. The ascitic carcinoma bearing mice (Donor) was taken 12-15 days after tumor transplantation. The ascitic fluid was drawn using an 18-gauge needle into sterile syringe. A small amount was tested for microbial contamination. Tumor viability was determined by trypan blue exclusion test and cells were counted using haemocytometer. The ascitic fluid was suitably diluted in normal saline to get a concentration of 10 $x10^6$ cells/mL. From this stock suspension, 0.25 mL (2.5 million cells) was injected intraperitoneally to obtain ascitic tumor. The mice were weighed on the day of tumor inoculation and then alternatively for 14 days. The animal care and handling was carried out in accordance to guidelines issued by the Institutional Animal Ethics Committee.

Acute toxicity study

Acute toxicity studies were conducted to determine the safe dose by up and down stair case method. Animals were divided into groups consisting of 6 animals in each group. Drug suspension was prepared using 0.25 % CMC. Test compounds were given orally as suspension at a single dose of 500, 1000 and 2000 mg/kg body weight. Control group was treated with 0.25 % CMC. Animals were continuously observed for the first 72 h and then for 7 days for behavioural changes, toxicity and mortality [26]. The animals treated with 1000 mg/kg of the test samples were found be safe. So, $1/10^{th}$ of the safer dose was selected for the activity

Procedure

The compounds which showed significant *in vitro* anticancer activity were further selected for *in vivo* anticancer activity. The animals were weighed and divided into eight groups, each containing of 6 animals. EAC cells (2.5 million cells) were injected by intraperitoneal route to each mouse of each group except the normal control (Group

I). This was taken as day 0. Treatment with test compounds and cisplatin (standard) were given alternatively for 7 days from day 1. On 14th day, after 24 h of the last dose, half the numbers of animals from each group were sacrificed for studying the following parameters.

Parameters monitored

% increase in weight as compared to day "0" weight

Upon weighing the animals on the day of inoculation and after once in 2 days of the post inoculation period, the increase in weight was calculated as follows

$$\left(\frac{\text{wt on respective day}}{\text{wt on day 0}} - 1\right) \times 100$$

Mean survival time (MST) and Increase in lifespan (% ILS)

Total number of days an animal survived from the day of tumor inoculation was counted. Subsequently, the Mean survival time was calculated. The % ILS was calculated as follows.

$$\left(\frac{\text{MST of treated group} - \text{MST of control group}}{\text{MST of control group}}\right) \times 100$$

An enhancement of life span by 25 % or more than that of the control was considered as an effective anticancer response.

Haematological parameters

On 14th day, after sacrificing the animals, blood samples were collected in order to find out the red blood cells (RBC), white blood cells (WBC) and Haemoglobin (Hb) counts which are the important parameters to assess the effect of the synthesized compounds on EAC cells bearing tumour mice.

RESULTS AND DISCUSSION

Chemistry

Totally, ten novel 2,5-substituted-1,3,4-thiadiazole derivatives were synthesized (Table 1) and characterized by UV, IR, ^{1}HNMR, ^{13}C NMR and Mass spectral analysis.

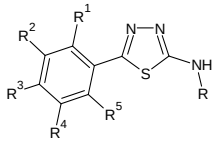


Table 1. List of the 1,3,4-thiadiazole derivatives synthesized

Code	R_1	R_2	R_3	R_4	R	Mol. formula	Molecular wt.
TDZ-1	-	NO_2	OH	-	H	$C_8H_6N_4O_3S$	238
TDZ-2	-	NO_2	OCH_3	-	H	$C_9H_8N_4O_3S$	252
TDZ-3	-	NO_2	-	NO_2	H	$C_8H_5N_5O_4S$	267
TDZ-4	OH	-	-	Cl	H	$C_8H_6ClN_3OS$	228
TDZ-5	NH_2	-	Cl	-	H	$C_8H_7ClN_4S$	227
TDZS-1	-	NO_2	OH	-	Phenylmethylidene	$C_{15}H_{10}N_4O_3S$	326
TDZS-2	-	NO_2	OCH_3	-	Phenylmethylidene	$C_{16}H_{12}N_4O_3S$	340
TDZS-3	-	NO_2	-	NO_2	Phenylmethylidene	$C_{15}H_9N_5O_4S$	355
TDZS-4	OH	-	-	Cl	Phenylmethylidene	$C_{15}H_{10}ClN_3OS$	316
TDZS-5	NH_2	-	Cl	-	Phenylmethylidene	$C_{15}H_{11}ClN_4S$	315

All compounds showed characteristic peaks as expected. The IR spectra of all the 1,3,4-thiadiazole derivatives were recorded in the 900-3600 cm^{-1} range on Shimadzu FT-IR 8310 JAPAN using KBr pellets. The absorption bands in

the regions 1515-1582 cm^{-1} and 1500-1540 cm^{-1} indicated the presence of C=N and NO_2 groups, respectively. The aromatic C=C stretching showed a peak in the region 1570-1605 cm^{-1} and the presence of C-N stretch was observed in the range between 1310-1320 cm^{-1}. In addition, compounds containing O-H group exhibited a peak at 3150 cm^{-1}. The ^1H NMR spectra were recorded on Bruker Biospin Avance-300 MHz using *d*-DMSO as solvent. The chemical shifts were reported as parts per million downfield from tetramethylsilane (Me_4Si). The protons of the amino group in the TDZ series showed a singlet between δ 3.3 - 3.5 ppm; Whereas, in TDZS-5 a singlet was observed at δ 2.5 ppm. Further, ^{13}C NMR spectrum of TDZ-1 and 3 were taken to confirm their identities. The results were found satisfactory.

In addition, to support the above data, Mass spectra were recorded on the Shimadzu MS-MS QP5050. The molecular ion peak of the reference compounds, TDZ-1, 2, 3, 5 and TDZS-1, 2, 5 were observed respectively at m/z, 238, 252, 267, 225, 326, 340 and 314 and the mass spectral data of the compounds were satisfactory. Further, in the MS spectra of compound TDZ-1, the peak at m/z 84 represents the thiadiazole fragment, and the loss of amino group from the molecular ion peak produced a peak at m/z 222. In addition, the peak produced at m/z 60 indicated the base peak formed due to $CNSH_2$ fragment. In the MS spectra of compound TDZ-2, the peak at m/z 206 represented the loss of nitro group. The MS spectra of the compound TDZ-3 shown a peak at m/z 205 represented the loss of nitro and amino group. Compound TDZ-5 shown MS spectra with a peak at m/z 194 indicating loss of 2 amino groups from the molecule. Moreover, TDZS-1 shown mass spectra with a base peak at m/z 163 by a 5-cyclopenta-1,3-dienyl-[1,3,4]thiadiazole-2-ylamine fragment. Further, TDZS-5 showed a peak at m/z 299 due to loss of amino group. Hence, all the above spectral data confirmed the proposed structure of the synthesized compounds.

Biological evaluation

In vitro Antioxidant Activity-DPPH radical method

All the synthesized compounds with concentrations ranging from 15.625-250 μg/mL were tested for their antioxidant activity by DPPH radical method. The potency of the test compounds was determined based on their IC_{50} values.

Table 2. Percentage DPPH radical scavenging activity

Code	% DPPH radical scavenging (µg/mL)					
	250	125	62.5	31.25	15.625	IC_{50}
TDZ-1	85.5094	76.4531	55.2553	35.801	24.1753	74.9920
TDZ-2	63.7982	57.7745	39.9310	23.263	12.1123	148.283
TDZ-3	40.0594	24.8386	15.1652	8.3082	5.21910	309.500
TDZ-4	53.1111	35.1648	30.5448	18.190	13.2612	221.753
TDZ-5	87.3913	87.9671	66.6922	44.565	28.7966	39.6147
TDZS-1	90.1625	91.8150	84.5674	63.604	43.0959	57.3655
TDZS-2	82.9422	88.1673	89.4737	87.809	70.9763	> 250
TDZS-3	68.6222	57.6465	55.2124	46.385	34.4610	76.4404
TDZS-4	85.8303	76.5125	62.9795	53.710	40.8092	16.1930
TDZS-5	84.7473	83.3630	68.2426	47.968	30.0792	33.4973
Ascorbic acid	57.9300	56.4500	54.5300	53.340	49.5700	15.4500

Among the compounds tested, TDZS-4, bearing 5-chloro-2-hydroxy group at the 5th position on the 1,3,4-thiadiazole scaffold exhibited free radical scavenging activity better than all other compoundsand it exhibited 85.8 % radical scavenging activity at 250 μg/mL, as shown in Table 2. Whereas, compound TDZ-5 and its Schiff base TDZS-5, having 2-amino-4-chlorophenyl substitution at 5th position exhibited 50 % radical scavenging activity at 33.5 and 39.6 μg/mL, respectively.

Further, Schiff bases containing 4-hydroxy-3-nitro and 3,5-dinitro substituted phenyl group at the 5th position showed the antioxidant activity with IC_{50} at 57.4, 76.4 μg / mL, respectively. However, the rest of the synthesized compounds were found to have 50 % radical scavenging activity at > 100 μg/mL concentrations.

Thus, the results revealed that, the presence of electron withdrawing groups such as, chloro, hydroxyl and amino substituted phenyl ring at the 5th position on the parent structure would be the probable reason for their increased activity. In addition, it was also observed that, Schiff bases, TDZS 1-5 showed better radical scavenging activity than that of their intermediates TDZ 1-5. This observation revealed that, the presence of substituted amino group at the 2nd position played a crucial role in determining the activity probably because of their increased aromaticity.

In vitro antioxidant activity-Nitric oxide scavenging method

To substantiate the antioxidant activity of the synthesized compounds, nitric oxide scavenging assay was performed with concentrations ranging from 1000-31.25 μg/mL.

Table 3. Nitric oxide radical scavenging activity

Code	% Nitric oxide Scavenging (µg/mL)						
	1000	500	250	125	62.5	31.25	IC_{50}
TDZ-1	23.7500	9.8040	-	-	-	-	>1000
TDZ-2	34.3750	18.137	12.2892	5.2154	-	-	>1000
TDZ-3	25.6250	15.931	8.91566	9.7506	-	-	>1000
TDZ-4	22.5000	11.275	-	-	-	-	>1000
TDZ-5	19.5833	12.500	1.68675	-	-	-	>1000
TDZS-1	10.4308	8.1250	7.59804	6.0241	5.5556	1.1038	>1000
TDZS-2	18.3333	9.3137	6.74699	7.4830	5.0773	3.5556	>1000
TDZS-3	17.5904	16.327	13.9583	8.3333	7.5556	1.9868	>1000
TDZS-4	22.4490	21.556	20.0000	14.128	10.294	8.3333	>1000
TDZS-5	10.6250	-	-	-	-	-	>1000

'-' *no nitric oxide scavenging activity*

From the results showed in Table 3, it was clear that, all the synthesized compounds were found to have 10 – 34 % antioxidant activity at 1000 μg / mL and their IC_{50} were expected to be at > 1000 μg/mL. Hence, this investigation revealed that, none of the synthesized compounds were found to exhibit profound scavenging of nitrites.

In vivo anti-inflammatory activity

The compounds with significant antioxidant activity were selected for *in vivo* anti-inflammatory activity.The dose of the compounds was determined by performing the acute toxicity study on *Wistar* rats by up and down staircase method at a single oral dose of 500, 1000 and 2000 mg/kg body weight. Results showed that, there were no toxic manifestations up to 1000 mg/kg body weight. Thus, 1/ 10^{th} of the higher safe doseswere considered for the *in vivo* anti-inflammatory activity.

Table 4. Effect of the selected compounds on mean increase in the paw volume

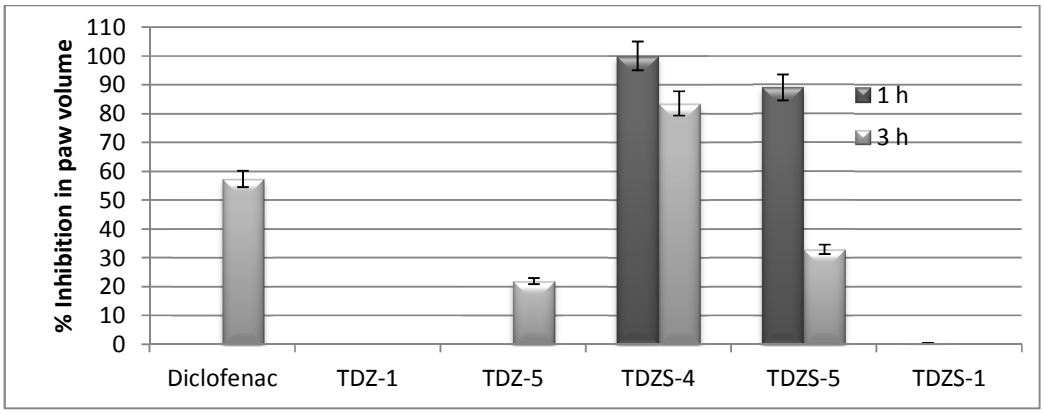
Code	Dose (mg/kg body weight)	Mean increase in the paw volume	
		1 h	3 h
Control	0.25 % CMC	0.147±0.037	0.307±0.048
Diclofenac Sodium	10	0.273±0.074*	0.057±0.130**
TDZ 1	100	0.307±0.012**	0.340±0.110
TDZ-5	100	0.260±0.067*	0.213±0.100
TDZS 4	100	0.000±0.012**	0.03±0.030**
TDZS 5	100	0.033±0.027**	0.183±0.035
TDZS 1	100	0.100±0.058	0.203±0.140

** P < 0.05 **P < 0.01*

Among the compounds tested, TDZS-4 having 5-chloro-2-hydroxy group and TDZS-5, having 4-chloro-2-amino group at the 5^{th} position in the 1,3,4-thiadiazole scaffold showed significant reduction in the carrageenan induced inflammation at the 1^{st} h with 100 % and 89.13 %, respectively and they also showed the mean increase in the paw volume about 0.0±0.012 and 0.033±0.027 mL, indicating the significant reduction of the oedema when compared with that of the control and the standard, as shown in Table 4. However, the other compounds did not show any activity at the 1^{st} h, as depicted in Figure 2.

Results at the 3^{rd} h inferred that, TDZS-4, bearing 5-chloro-2-hydroxy substitution at the 5^{th} position had shown 83.5 % reduction against inflammation with significant reduction in the paw volume (0.03±0.03 mL), when compared to diclofenac which had shown 57.3 % inhibition with paw volume 0.057±0.130 mL. Whereas, compounds TDZS-5 and TDZ-5 exhibited less protection against inflammation and their percentage anti-inflammatory was found to be 32.9 % and 21.95 %, respectively. However, the rest of the other compounds did not show any significant activity against the inflammation. Further, the results showed in table 2 and 3 established the positive correlation between the anti-inflammatory and the DPPH radical scavenging potential of the synthesized compounds. Thus, the above observations indicated that, the presence of the phenyl ring containing electron withdrawing groups at the 5^{th} position of 1,3,4-thiadiazole system might be responsible to exert significant anti-inflammatory activity.

Figure 2. The percentage protection of the selected compounds against the inflammation



Docking studies

Docking study on the receptor interaction was performed to investigate the potential binding modes of compounds TDZ 1-5 and their Schiff bases, TDZS 1-5.

Table 5. Docking studies on 2CIB protein

Code	Docking Parameters				
	Moldoc Score (Grid)	Moldoc score	Rerank score	No. of H-bond interactions	Amino acids involved in the H-bond interaction
TDZ-1	-108.326	-100.048	-78.3772	4	Gln 181, Pro 101, Arg 308
TDZ-2	-113.239	-108.488	-85.9166	4	Arg 308, Asn 171, Val 104
TDZ-3	NI	NI	NI	NI	NI
TDZ-4	-94.6906	-93.6148	-71.6483	7	Gln 149, Gln 181, Phe 102, Val 182
TDZ-5	-97.2475	-93.7244	-75.1659	4	Gln 181, Gln 149
TDZS-1	-142.090	-140.172	-113.715	9	Arg 308, Val 182, Gln 181
TDZS-2	-137.501	-134.020	-113.447	10	Arg 308, Val 182, Gln 181
TDZS-3	NI	NI	NI	NI	NI
TDZS-4	-121.413	-122.360	-96.4144	6	Ile 180, Val 182, Thr 183, Gln 181, Val 104
TDZS-5	-129.294	-131.056	-106.934	7	Ile 180, Pro 101, Val 182,

NI : No interaction

Molegro Virtual Docker (MVD) program was used in docking simulation to predict the binding affinity of the compounds on the protein, cytochrome P-450 14α-demethylase (PDB ID: 2CIB). Different poses of each ligand were screened and marked based on their MolDock score. The binding site for 2CIB enzyme was located majorly at residues Arg 308, Val 182, Gln 181 and rarely at Val 104, Gln 149, Ile 180, Thr 183, Pro 101 and Asn 171.

As shown in Table 5, the MolDock score values of the compounds TDZS-1 and TDZS-2, having 3-nitro-4-hydroxy and 3-nitro-4-methoxy group at the 5^{th} position respectively in the 1,3,4-thiadiazole scaffold were found to be at -140.172 and -134.02, and were far greater than the other compounds indicating that both the compounds were having effective binding potential than the others. The binding interaction of TDZS-1 with 2CIB was shown in the Figure 3. This compound showed maximum number of H-bond interactions between the nitrogen of 3-NO_2 groupof the phenyl ring with C=NH of Arg 308 and the nitrogen atom of 2-NH_2 with NH_2 of Val 182.

Along with these, 8 other minor interactions were seen. Similarly, compound TDZS-2 was also found to exhibit better binding profile; besides the minor interactions, this compound exerted 2 major interactions, one was between the methoxy groupand the -NH_2 group of Arg 308 (-2.2 kcal / mol) and the another was in between of the amino group and the Val 182 residue (-0.035 Kcal/mol) as depicted in Figure 4. Whereas, the compounds TDZS-5, TDZS-4, TDZ-2 were identified with moderate activity. The above docking results revealed that, Schiff bases, TDZS 1-5 were more effective in binding with 2CIB when compared to intermediates TDZ 1-5. Further, the compounds having high binding profile with the receptor 2CIB were also speculated to exhibit similar *in vitro* anticancer activity.

Figure 3. Binding orientation of docked TDZS-1 with 2CIB active residues Gln 181, Arg 308 and Val 182

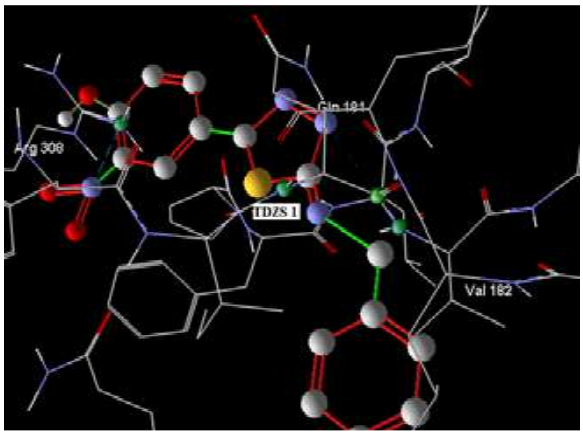
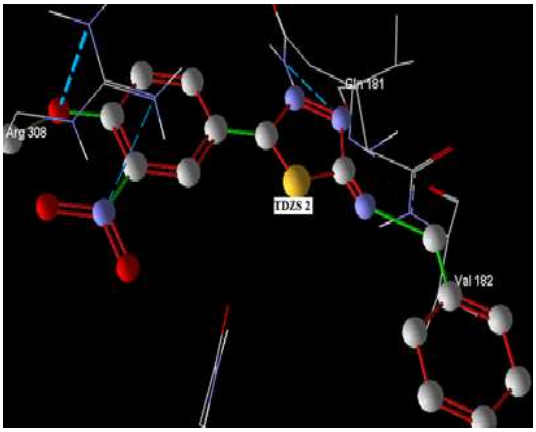


Figure 4. Docked TDZS-2 illustrating H- bonding with the residues Arg 308, Gln 181 and Val 182 of 2CIB



In vitro anticancer activity

All the ten synthesized compounds were screened for their *in vitro* anticancer activity by MTT assay on Hep2 cell line (laryngeal cancer) on different concentrations in the range 1000-125 μg/mL. Among the tested compounds, TDZS-5 and TDZS-4 showed the 50 % inhibition of Hep2 cells growth at a lower concentration. Their IC_{50} values were found to be at 109.99 and 118.424 μg/mL, respectively as represented in Table 6. Compounds TDZ-5, TDZ-3 and TDZ-4 showed moderate growth inhibition of Hep2 cells with IC_{50} at 143.94, 163.82 and 184.7 μg/mL, respectively. However, compounds TDZS-1, TDZS-2 and TDZS-3 were found to exhibit the 50 % cytotoxicity at < 125 μg/mL. Schiff bases, TDZS 1-5 showed better cytotoxicity on Hep2 cell line when compared with that of TDZ 1-5, where they showed 50 % cytotoxicity between 143.9 – 163.8 μg/mL. Further, the *in vitro* cytotoxic potency of the compounds was evidenced by their molecular interaction with the receptor 2CIB. Thus, the above results revealed that all the synthesized *Schiff* bases i.e., TDZS 1-5 were more active when compared to their corresponding free 2-NH_2 derivatives. These results indicated that the substitution on the 2-amino group of the compound was important to induce cytotoxicity.

Table 6. In vitro anticancer activity of synthesized compounds on Hep2 cell lines by MTT assay

Code	% Cytotoxicity				
	1000 (µg/mL)	500 (µg/mL)	250(µg/mL)	125 (µg/mL)	IG_{50} (µg/mL)
TDZ 1	87.83	88.03	78.01	38.84	227
TDZ 2	88.33	87.38	62.18	37.63	208.87
TDZ 3	87.08	86.98	80.06	36.83	163.818
TDZ 4	88.43	86.22	70.55	6.880	184.7
TDZ 5	87.93	87.48	78.46	44.75	143.94
TDZS 1	88.53	88.63	87.03	81.82	< 125
TDZS 2	86.22	86.45	84.90	72.40	< 125
TDZS 3	86.00	86.78	85.67	71.34	< 125
TDZS 4	87.44	86.33	78.31	52.14	118.424
TDZS 5	87.44	86.99	84.84	62.80	109.99

In vivo anticancer activity

The results revealed that, the Schiff bases, TDZS 1-5 were more cytotoxic among the compounds tested. Hence, they were taken further, to test their potency *in vivo* by EAC model. The effects of the compounds TDZS 1-5 on tumour volume, packed cell volume, tumour weight, mean survival time, increase in life span and haematological parameters were compared with control animals to determine their efficacy.

On 14^{th} day, animals were weighed before and after collecting the peritoneal fluid to calculate the tumor weight.

Table 7. Effect of the selected compounds on the parameters tumour volume, tumour weight and packed cell volume of the EAC cells bearing Swiss Albino mice (mean±S.E.M, n=6)

Code	Tumor weight (g)	Tumor volume (mL)	Packed cell volume (mL)
Control	9.5±1.5	10.7±1.40	7.2±0.700
Cisplatin	6.5±0.5	8.55±0.85	3.45±0.35
TDZS 1	6.5±0.5	7.60±0.80	3.25±0.05
TDZS 2	7.5±0.5*	8.0±0.000	3.95±0.15
TDZS 3	9.5±0.5	9.00±2.60	7.85±3.05
TDZS 4	6.0±0.0	9.05±0.15	4.1±0.100
TDZS 5	8.5±0.5	9.65±0.95	5.25±0.45

**P < 0.05 **P < 0.01 ***P < 0.001*

The results shown in Table 7 indicated the effect of compounds on the tumor weight reduction. Among the five tested compounds, TDZS-4 and 1 exhibited more reduction in tumor weight. Whereas, compound TDZS-3, having 3,5-dinitro substituted phenyl group at the 5^{th} position did not show any significant reduction in the tumor weight as compared with the control. However, the rest of the other two compounds reduced the tumor weight moderately.

The peritoneal fluid which is present in the peritoneal cavity is the direct source for the tumor cells [32]. Thus, the decrease in the tumor volume indicates the anticancer activity of the test compounds. In this study, compounds TDZS-1 and TDZS-2, bearing 4-hydroxy-3-nitro and 4-methoxy-3-nitro phenyl substitution at the 5^{th} position on the 1,3,4-thiadiazole system reduced the total tumor volume better than the cisplatin treatment and their total tumor volume was measured as 7.6±0.8 and 8.0±0.0 mL, respectively. Whereas, the cisplatin treated animals showed 8.55±0.85 mL. However, the other compounds exhibited moderate activity when compared with the standard. Thus, the reduction in the tumor volume could serve as a sign of antitumor activity.

To substantiate the above results, the viable and non-viable tumor cells were counted by treating the peritoneal fluid with the trypan blue dye. The viable cells will not take the dye, whereas, the non-viable cells will be stained as violet. The effect of compounds on the viable and non-viable cell count was showed in Table 8.

Table 8.Effect of the selected compounds on viable and non-viable cell count

Code	viable cells	non-viable cells
Control	56.25	1.75
Cisplatin	22.75	3.50
TDZS 1	27.50	3.50
TDZS 2	40.25	3.00
TDZS 3	47.00	1.75
TDZS 4	44.75	2.00
TDZS 5	50.50	1.75

The results revealed that, all the selected compounds showed significant increase in the ratio of non-viable to viable cells compared to control. Particularly, the effect of compound TDZS-1 on tumor bearing mice was found to be more or less equivalent with that of the cisplatin treatment; the non-viable and viable cell counts of the TDZS-1 treatment was found to be 3.5 x 10^6 and 27.5 x 10^6 cells / mouse, respectively. Cisplatin, being the standard, exhibited significant anticancer activity with 3.5 million non-viable cells and 22.75 million viable cells / mouse. Further, compound TDZS-2, 4-methoxy-3-nitro substituted 1,3,4-thidiazole system showed better activity when compared with the rest of the compounds. The viable and non-viable cell count for this compound was calculated as 40.25 and 3.0 million cells, respectively. Whereas, the other three compounds such as, TDZS-3, 4 and 5 were not found to show great increase in the non-viable cell count.

Anemia is one of the major side effects reported in the cancer chemotherapy. So, in addition, the effects of the compounds on haematological parameters were also studied and the results were shown in Table 9. On 14^{th} day, blood samples were collected by retro orbital puncture and analyzed for the haematological parameters.

Tumor bearing mice (Group I) showed, the decrease in the RBC and Haemoglobin (Hb) levels, whereas, the WBC count was found to be increased. The results shown in the Table 9 indicated that, the RBC counts of the compound treated animals were increased. Particularly, compound TDZS-1 treatment showed RBC cell count of 6.65±0.05 million cells, whereas, in the control group, it was found to be 3.7±0.2 million cells. Moreover, the Hb levels were

also increased and it was observed as 8.3±0.1 and 6.6±0.1 g/dL, respectively for TDZS-1 and control, which signifying the anticancer effect of TDZS-1. However, other compounds also exhibited promising activity.

Table 9. Effect of the selected compounds on the haematological parameters of the EAC cells bearing Swiss Albino mice (mean±S.E.M, n=6)

Code	RBC	Hb	WBC
Control	3.7±0.2	6.60±0.10	38.2±0.95
Cisplatin	6.8±0.0***	8.45±0.05***	23.0±1.0**
TDZS 1	6.7±0.1***	8.30±0.10***	23.2±1.1**
TDZS 2	6.1±0.1***	7.80±0.10**	27.2±1.8*
TDZS 3	5.5±0.3***	6.80±0.60**	29.3±0.4
TDZS 4	5.7±0.3***	7.50±0.20**	30.6±0.6
TDZS 5	5.1±0.1**	6.85±0.05*	31.5±1.4

**P < 0.05 **P < 0.01 ***P < 0.001*

The reduction in WBC cell counts also an important parameter to assess the anticancer potential of the compounds. Here again, compound TDZS-1 showed greater reduction in the WBC cell count when compared with that of the control. Further, it also showed similar effect as cisplatin treatment (Group II). The WBC cell count for this compound, TDZS-1 was observed as $23.2\pm1.1x10^3$ as shown in Table 9.

Table 10.Effect of the selected compounds on Mean Survival Time (MST) and % Increase in Life Span (ILS)

Code	MST	% ILS
Control	11.5	-
Cisplatin	19.5	69.6
TDZS 1	19.0	65.2
TDZS 2	18.5	60.9
TDZS 3	17.0	47.8
TDZS 4	17.5	52.2
TDZS 5	15.5	34.8

The anticancer potential was further assessed by the parameters MST and % ILS. Results shown in Table 10confirmed the promising anticancer activity of TDZS-1. It showed increase in life span of 65.2 % which was comparable with standard (69.6 %). From the above results, it was found that among the synthesized Schiff bases, TDZS-1 was the most potential compound against cancer.

CONCLUSION

To conclude, an attempt was made to synthesize some novel 5-substituted-1,3,4-thiadiazol-2-amine derivatives by conventional method. This method provides us a simple and powerful tool for the synthesis of large number of multifunctional 1,3,4-thiadiazol-2-amines. All the synthesized compounds were evaluated for antioxidant, anti-inflammatory and anticancer activities. From the biological studies, it was well understood that most of the 1,3,4-thiadiazol-2-amines were showing promising anticancer and antioxidant properties. Out of all the tensynthesized compounds,TDZS-4 showed potent antioxidant activity by DPPH radical scavenging method with IC_{50} at 16.2 μg/mL. The same compound also showed significant anti-inflammatory activity. Docking results revealed that, the compounds TDZS-1 and TDZS-2 exhibited better Moldock scores. Similar results were obtained in *in vitro* cytotoxicity assay on Hep2 cell lines, where, they showed IC_{50} at <125 μg/mL. Further, these two compounds showed promising *in vivo* anticancer activity on EAC cells bearing mice.

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