Research Paper

In silico Investigation and Biological Evaluation of Synthesized Sulfamethoxazole Derivatives

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A series of 4-[4-(substitutedaryl/heteroaryldiazenyl]-N-(5-methylisoxazol-3-yl)benzene sulphonamide derivatives (4a-4f) were designed and synthesized coupling a mixture of diazotized sulfamethoxazole with six different phenolic and enolic compounds in an *in situ* reaction. The structural environment of synthesis of each molecule was confirmed by Fourier-transform infrared spectroscopy, proton nuclear magnetic resonance and elemental analysis. These derivatives were further screened in various biological assays *in vivo* for analgesic and antiinflammatory activities and *in vitro* for antioxidant and antimicrobial activities. When tested for analgesic activity at a dose of 50 mg/kg, compounds 4-((2-hydroxynaphthalen-1-yl)diazenyl)-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4d) and 4-((4-hydroxy-5-isopropyl-2-methylphenyl)diazenyl)-N-(5-methylisoxazol-3- yl)benzene sulphonamide (4f) showed 58.33 and 57.76 % of pain inhibition, respectively. These two molecules also exhibited significant antioxidant activity at 10 and 50 µg/µl. The compound 4-[(4-hydroxy-2-oxo-2H-chromen-3-yl)diazenyl]-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4a) exhibited antibacterial activity against *Staphylococcus aureus* resistance, *Candida albicans* and *DescriptionCryptococcus neoformans* at a concentration of 31.25 µg/ml. The analgesic action of these synthesised analogues was predicted in molecular docking experiments with a specific target protein, cyclooxgenase-2 of *Mus musculus* and results indicated all tested compounds to exhibit good binding interaction with the active site amino acid of the target enzyme.

Key words: Sulfanilamide, thymol, 4-hydroxy coumarin, antioxidant, antimicrobial, antiinflammatory

Sulfanilamides were established molecules in the field of medicine. Though these are rarely prescribed these days, but in medicinal chemistry the importance of sulfanilamide entity is well-recognised as it continues to offer several therapeutic benefits for drug development[1]. Some 70-80 y ago, a red dye Prontosil, an azo-linked sulfonamide pro-drug was widely used to treat *streptococcal* infections[2]. For antibacterial drug development identification of correct lead candidates is a major challenge. Thus, sulfanilamide analogues continue to offer insights for the development of newer antimicrobials.

Isoxazole is a heterocyclic azole moiety with oxygen and nitrogen atoms in cyclopentadiene ring. Compounds bearing the isoxazole ring serve as an important source for developing useful drug candidates, for treating several infectious diseases. Although, majority of isoxazole derivatives have exhibited immunosuppressant and antiinflammatory activities, sulfonamide containing isoxazole analogues were found to display potent analgesic and antiinflammatory action. The commercially available isoxazole bearing drugs are COX-2 inhibitor, valdecoxib, leflunomide, dihydrofolate synthetase inhibitor, sulfamethoxazole (SMZ), sulfisofurazole and β-lactamase-resistant antibiotics, such as cloxacillin and dicloxacillin to mention some.

Moreover, literature revealed that molecules with a diazenyl (–N=N-) group showed versatile biological properties[3] and also that nitrogen bearing heterocyclic

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compounds possess good biological action[4]. Microorganisms cause infectious disease and due to irrational use of antibiotics, some of the pathogenic organisms have developed resistance against these antimicrobial drugs. Thus, Research efforts are to be focused more towards developing drugs that act against the resistant pathogenic organisms with minimal side effects to create socio-economic benefit. Cellular oxidative stress in biological system due to generation of reactive oxygen species (ROS) leads to alterations in genes through oxidation of nucleic acid, impairment of muscle function by protein denaturation, while lipid peroxidation in cells cause perturbation of homeostasis resulting in cell death. ROS are produced during tissue injury and antioxidants play a vital role during wound healing. Although, inflammation is a defensive mechanism in response to foreign bodies but leads to cellular damage due to producing oxidative stress[5]. Under these circumstances, synthetic and natural antioxidants have a major role to protect cells against oxidative stress[6] . Hence, it is obligatory to develop new drugs at minimum cost, which are effective against resistant organisms and also aid in quick healing of infected wounds through free radical scavenging[7].

Previous studies from our laboratory explored the synthesis and characterisation of phenolic/enolic substituted diazenyl SMZ derivatives[8-14]. To continue these efforts, biological activities of these analogues needs to be evaluated using different models. The present work is conceptualized on the basis of literature to design some hybrid molecules containing sulfonamide, 5-methyl-isoxazole moiety and diazenyl group all together with different phenolic/enolic systems with in these structures. In this drug design attempt it was planned to couple 6 bioactive neutral nucleophiles such as, 4-hydroxy coumarin (4-HC), 8-hydroxy quinoline (8-HQ), salicylic acid, β-napthol, salicylaldehyde and thymol with diazonium salt of SMZ to produce the desired molecules 4a-4f to evaluate the biological actions possessed by these molecules. In addition, molecular docking study was carried out to predict the potential of the molecules to exhibit analgesic and antiinflammatory activities. Thus, all designed molecules were subjected to molecular docking against the cyclooxygenase (COX-2) enzyme, the structure of which was retrieved from the Protein Data Bank.

MATERIALS AND METHODS

All chemicals (Merck Specialties Ltd., and Hi-Media Laboratories Pvt. Ltd., Mumbai, India) used were of synthetic and analytical grade. Melting points (°) were determined using the open capillary method (Elico) and were uncorrected. The IR spectra, molecular mass, ^{1}HNMR spectra and elemental analysis were carried out on Jasco FT/IR 4100, Japan, Shimadzu, Bruker ^{1}H NMR, 400 MHz and Perkin Elmer 2400, respectively. The UV absorption (λ_{max}) maximum was measured on a Jasco V-630 spectrophotometer

In silico investigation:

COX-2 of *Mus musculus* with PDB ID: 1CX2 was retrieved from Protein Data Bank (PDB) and docked against the proposed molecules with Arugus Lab 4.0 individually. The protein-ligand interaction was carried out by Discovery Studio Visualizer 3.1 software.

Synthesis of the proposed molecules (4a-4f):

The synthetic procedures of compounds (4a-4f) have been previously reported (fig. 1)[8-12]. A cold solution of sodium nitrite was added dropwise to an aqueous solution of the desired SMZ with concentrated hydrochloric acid; the temperature of the reaction mixture was maintained at 0-5°. When addition was completed, the solution was to stand a few minutes with occasional stirring to complete diazotization. Then it was poured into an ice cold solution of individual phenolic/enolic compounds (2a-2f) in 20 % sodium hydroxide the reaction mixture was stirred and kept overnight in a refrigerator. The final precipitate obtained was filtered and recrystallized from hot ethanol.

Spectral characterization:

The spectral data of individual analogues 4a-4f was previously reported. 4-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)diazenyl]-*N*-(5-methylisoxazol-3-yl) benzene sulphonamide (4a)- red colour; yield, 72 %; R_f; 0.8, mp (°); 225-228; UV/Vis (λ_{max}, nm, CH_3OH): 403; IR (KBr, γ, cm^{-1}): 3179 (O-H str.), 1726 (C=O str.), 1617 (-C=C- str.), 2925 (CH_2 str.), 1506 (-N=N-), 1390, 1157 (SO_2 str.); ^{1}HNMR ($CDCl_3$ δ ppm, 400 MHZ): 2.43 (s, 3H, CH_3), 7.93 (d, coumarin H-5), 7.654 (m, coumarin H-6), 7.65 (m, coumarin H-7), 7.42 (d, coumarin H-8), 5.62 (s, 1H, isoxazol-4-yl H); LC-MS (% area); 100; *m/z*; 427.18 (M+1); analysis for $C_{19}H_{13}N_4O_6S$: Calcd % C, 53.52; H, 3.31; N, 13.14;S,7.52; found %: C, 53.47; H, 3.36; N, 13.21;S,7.49.

4-((8-hydroxyquinolin-5-yl)diazenyl)-*N*-(5-methylisoxazol-3-yl)benzene sulphonamide (4b)- orange colour; yield 92 %; R_f; 0.8, mp (°); 220-223; UV/Vis (λ_{max}, nm, CH_3OH): 403; IR (KBr, γ, cm^{-1}): 3297 (O-H str.), 1616 (-C=C- str.), 2847 (CH_2 str.),

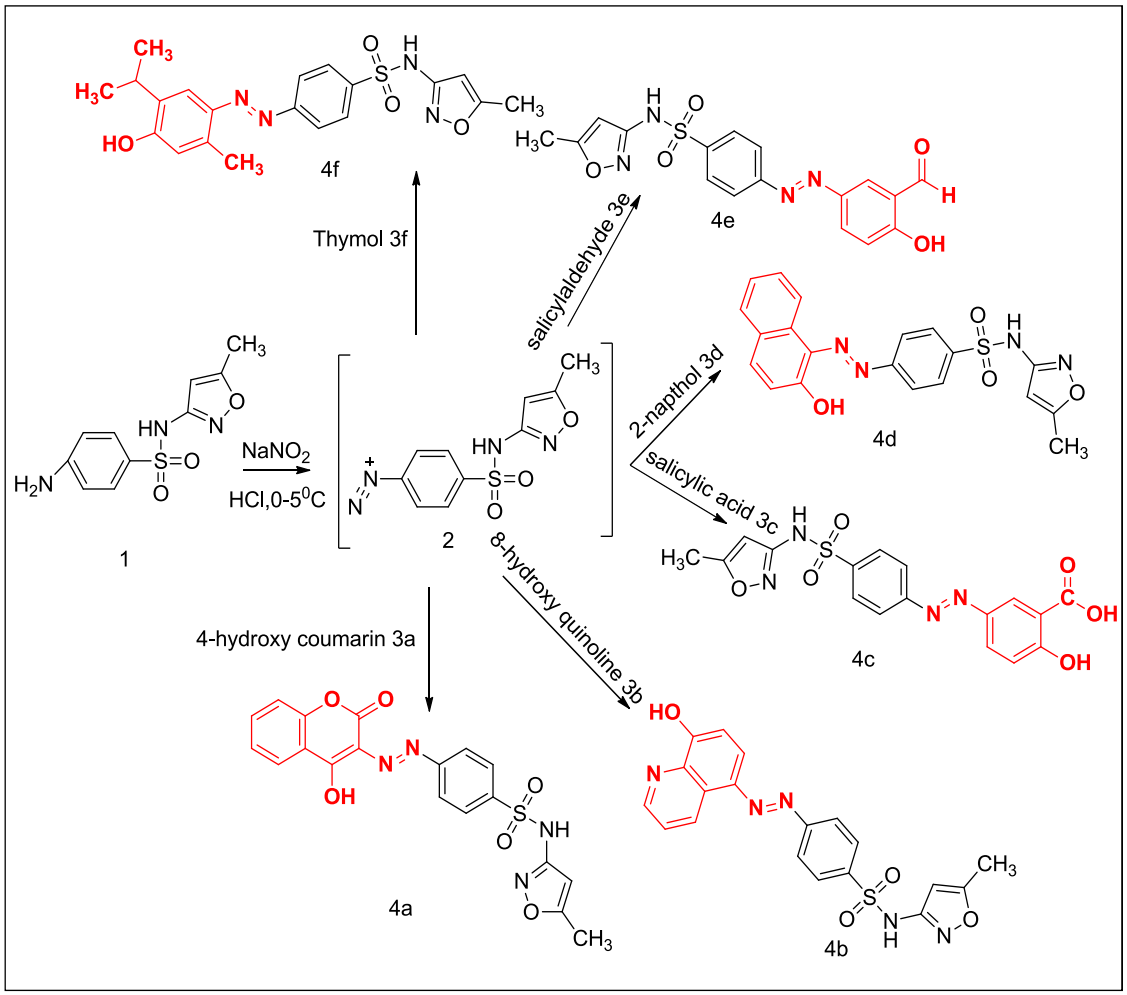


Fig. 1: Synthesis of sulfamethoxazole analogues 4a-4f

1573 (C=N str.), 1506 (-N=N-), 1341 1170 (SO_2str.); ^{1}HNMR (DMSO-d_6, δ ppm, 400 MHZ): 2.83 (s,3H,CH_3), 8.01-8.05 (m,4H,ArH), 9.28 (d, quinolinyl H-2), 7.63 (d, quinolinyl H-3), 8.03 (d, quinolinyl H-4), 8.07 (d, quinolinyl H-6), 7.26 (d, quinolinyl H-7), 6.28 (s,1H,isoxazol-4-yl H); LC-MS (% area); 100; *m/z*; 410.30 (M+1); Analysis for $C_{19}H_{15}N_5O_4S$: Calcd % C, 55.74; H, 3.69; N, 17.11 S, 7.67; found %: C, 55.72; H, 3.73; N, 17.13; S,7.85.

2-hydroxy-5-((4-(N-(5-methylisoxazol-3-yl) sulfamoyl)phenyl)diazenyl)benzoic acid (4c)- Grey colour powder; yield 73 %; R_f; 0.8, mp (°); 227-230; UV/Vis (λ_{max}, nm, C_2H_5OH): 370; IR (KBr, γ, cm^{-1}): 3461 (N-H str.), 1668 (C=O str.), 1614 (-C=C- str.), 2922 (CH_2 str.), 1573, (C=N str.), 1510 (-N=N-), 1315, 1170 (SO_2str.); ^{1}HNMR (DMSO-d_6, δ ppm, 400 MHZ): 7.25-8.01 (m,7H,ArH), 2.43 (s, 3H, CH_3), 11.67 (s, IH, OH), 12,11 (sb, 1H,COOH), 6.17 (s, 1H, isoxazol-4-yl H); LC-MS (% area); 100; *m/z*; 403.18 (M+1); analysis for $C_{17}H_{14}N_4O_6S$: Calcd % C, 50.75; H, 3.51; N, 13.92; S,7.97; found %: C, 50.47; H, 3.55; N, 13.96; S,7.89.

4-((2-hydroxynaphthalen-1-yl)diazenyl)-*N*-(5-methylisoxazol-3-yl)benzene sulfonamide (4d)- Orange red colour powder; yield 90 %; R_f; 0.8, mp (°); 170-178; UV/Vis (λmax, nm, C_2H_5OH): 470; IR (KBr, γ, cm^{-1}): 3251 (O-H/NH str.), 1511, 1607 (C=C str.),1460 (-N=N-), 1385 (O-H bend.), 1316, 1159 (SO_2 str.),1212 (C-O str.), 956 (S-N str.); ^{1}H NMR ($CDCl_3$, δ ppm, 400 MHZ): 17.49 (s, 1H, SO_2NH), 16.00 (1H, OH), 7.85-8.34 (m, 4H, ArH), 7.14-7.85 (6H, naphthyl H), 6.26 (s, 1H. isoxazol-4yl H), 2.37 (s, 3H, CH_3); LC-MS (% area); 97; *m/z*; 409.68 (M+1); analysis for $C_{20}H_{16}N_4O_4S$: Calcd % C, 58.84; H, 3.97; N, 13.71; S,7.86; found %: C, 50.81; H, 3.95; N, 13.72; S,7.85.

4-((3-formyl-4-hydroxyphenyl) diazenyl)-*N*-(5-methylisoxazol-3-yl) benzene sulfonamide (4e)- Grey colour powder; yield 90 %; R_f; 0.8, mp (°); 170-175; UV/Vis (λ_{max}, nm, C_2H_5OH): 450; IR (KBr, γ, cm^{-1}): 3442 (O-H str.), 2915 (CH str.), 1660 (C=O str.), 1615 (C=C str.), 1478 (-N=N-), 1305, 1175 (SO_2 str.), 1137 (C-O str.), 901 (S-N str.); ^{1}HNMR (DMSO-d_6, δ ppm, 400 MHZ): 11.42 (s, 1H, OH), 11.02 (s, 1H, SO_2NH),

10.03 (s, 1H, CHO), 8.18-8.23 (m, 4H, Ar H), 8.01 (d, 1H, salicylaldehyde H-6), 7.98 (s, 1H, salicylaldehyde H-2), 7.53 (d, 1H, salicylaldehyde H-5), 6.24 (s, 1H, isoxazole H-4), 2.38 (s, 3H, =C-CH_3); LC-MS (% area); 100; *m/z*; 386.22 (M+1); analysis for $C_{17}H_{14}N_4O_5S$: Calcd % C, 52.83; H, 3.67; N, 14.51;S,8.29; found %: C, 50.85; H, 3.68; N, 14.52; S, 8.33.

4-((4-hydroxy-5-isopropyl-2-methylphenyl)diazenyl)-*N*-(5-methylisoxazol-3-yl)benzene sulphonamide (4f)- Brick red colour powder; yield 84 %; R_f; 0.8, mp (°); 304-310; UV/Vis (λmax, nm, 1,4-dioxane): 409; IR (KBr, γ, cm^{-1}): 3489, 3163 (O-H str.), 2961 (CH_2 str.), 1619 (C=C str.), 1466 (-N=N-), 1341, 1129 (SO_2 str.), 899 (S-N str.). ^{1}H NMR (DMSO-d_6, δ ppm, 400 MHZ): 9.75 (s, 1H, SO_2NH), 9.56 (s, 1H, OH), 7.95-8.50 (m, 4H, Ar H), 7.85 (s, 1H, thymol H-6), 6.95 (s, 1H, thymol H-3), 6.80 (s, 1H, isoxazolyl H), 2.97 (m, 1H, CH (CH_3)$_2$), 2.38 (s, 3H, methyl), 1.25 (d, 6H, CH (CH_3)$_2$; LC-MS (% area); 100; *m/z*; 415.18 (M+1); analysis for $C_{20}H_{22}N_4O_4S$: Calcd % C, 57.85; H, 5.29; N, 13.45; S,7.68; found %: C, 57.96; H, 5.55; N, 13.52; S,7.74 (Table 1, fig. 2)

Pharmacological evaluation:

Acute oral toxicity of the synthesised molecules was determined using recommended procedure as prior OECD guideline No. 420. The animals were orally administered with the test molecules at the doses of 5, 50, 300 and 2000 mg/kg at an interval of 24 h between of each dose. The entire study was carried out as per CPCSEA and IAEC guidelines (registration number 1171/C/08/CPCSEA and Ref. No. 60/SPS/IAEC/SOAU).

In vivo acetic acid-induced writhing method was carried out with a little modification to evaluate the analgesic activity[15]. The group I was treated as a negative control and the group II was as positive control and was administered orally 100 mg/kg acetylsalicylic acid (ASA). Animals from groups III-XIV were orally administered with test molecules (4a-4f) at the dose level of 25 and 50 mg/kg. After 1 h of the administrations, all the groups were treated with 0.6 % v/v acetic acid solution (1 ml/100 g) intra peritoneally and the onset and number of writhings was noted. Finally percent of analgesic activity was calculated as follows, % analgesic activity=mean writhing count (control−treated group)/mean writhing count of control group×100. The reaction time was expressed as mean±SEM. The statistical analysis was done by one way-ANOVA followed by Dunnett's *t*-test.

Eddy's hot plate (Techno) was used to induce pain by thermal stimuli and to measure the response latencies as per method first described by Eddy and Leimback[13] with a slight modification and the instrument was adjusted to 55±0.5° and the basal reaction time of each animal were recorded before administering either the test or standard compounds. The reaction time for all the groups was measured at an interval of 60 min for 3 h after administration of test molecules and finally the percent analgesic activity (reaction time) was determined. Carrageenan-induced rat hind paw oedema method was followed with a slight deviation using the Ugo Basile plethysmometer (7150). All treatments were given orally I h before the injection of carrageenan. Percent inhibition of inflammation was calculated using the formula, % inhibition = 100 (1-Vt/Vc), where Vc represents oedema volume in control and Vt oedema volume in group treated with test molecules.

***In vitro* radical scavenging capacity:**

The antioxidant activity of the prepared isoxazole derivatives were measured using the 2,2-diphenyl-

TABLE 1: PHYSICAL CHARACTERISTIC DATA OF SULFAMETHOXAZOLE ANALGUES 4A-4F

Comps.	Heteroaryl	M. formula	*m/z*	Rf	mp (°)	Color	Yield (%)
4a	4-[(4-hydroxy-2-oxo-2H-chromen-3-yl)diazenyl]-*N*-(5-methylisoxazol-3-yl)benzene sulphonamide	$C_{19}H_{14}N_4O_6S$	427.00	0.8	260-270	Bright yellow	75
4b	4-((8-hydroxyquinolin-5-yl) diazenyl) -*N*- (5-methyl isoxazol-3-yl) benzene Sulphonamide	$C_{19}H_{15}N_5O_4S$	410.30	0.8	220-223	Brick red	92
4c	2-hydroxy-5-((4-(*N*-(5-methylisoxazol-3-yl) sulfamoyl) phenyl) diazenyl) benzoic acid	$C_{17}H_{14}N_4O_6S$	403. 00	0.8	227-230	Grey	73
4d	4-((2-hydroxynaphthalen-1-yl)diazenyl)-*N*-(5-methylisoxazol-3-yl) benzene sulphonamide	$C_{20}H_{16}N_4O_4S$	409.30	0.8	170-174	Orange red	90
4e	4-((3-formyl-4-hydroxyphenyl) diazenyl)-*N*-(5-methylisoxazol-3-yl) benzene sulfonamide	$C_{17}H_{14}N_4O_5S$	386.22	0.8	170-172	Grey	90
4f	4-((4-hydroxy-5-isopropyl-2-methylphenyl)diazenyl)-*N*-(5-methylisoxazol-3-yl)benzenesulphonamide	$C_{20}H_{22}N_4O_4S$	414.14	0.8	304-310	Brick red	84

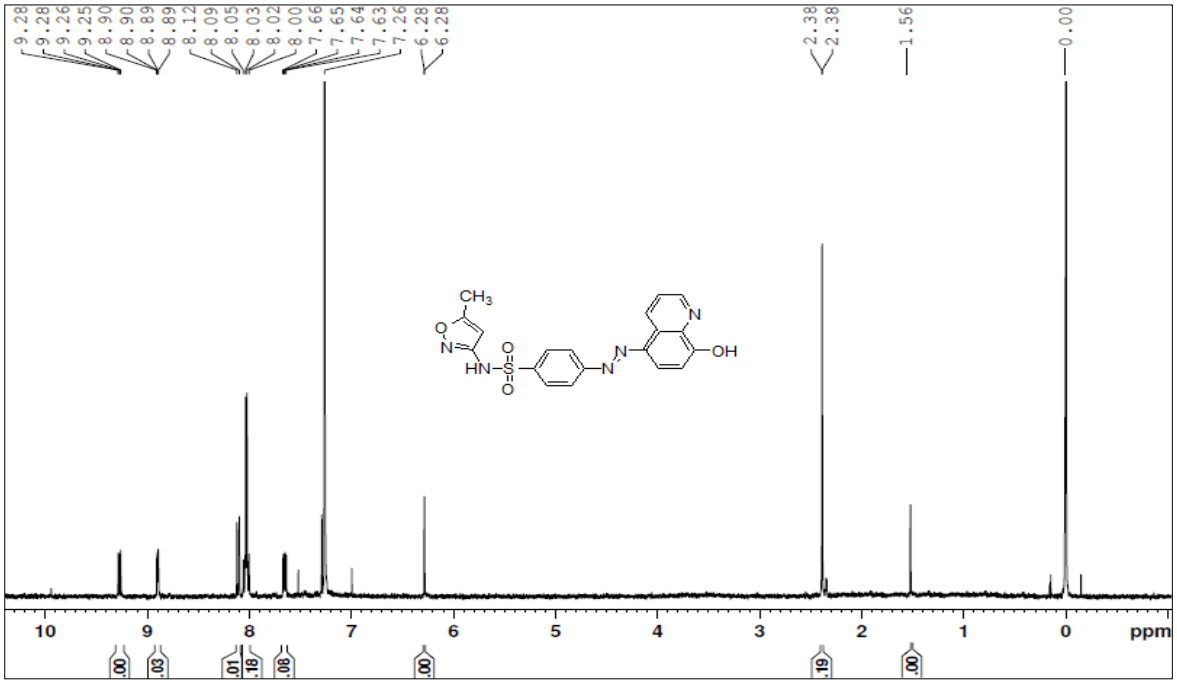


Fig. 2: [1]HNMR of 4-((8-hydroxyquinolin-5-yl)diazenyl)-N-(5-methylisoxazol-3-yl)benzene sulfonamide (4b)

1-picrylhydrazyl (DPPH) assay procedure with some modifications[6] at several concentrations, the absorbance of the test molecules and the standard butylated hydroxy toluene (BHT) was measured at 517 nm on a UV/Vis spectrophotometer and the antioxidant activity was calculated. A mixture of DPPH and methanol was considered as control. All experiments were carried out in triplicate. Followed by Dunnett's post hoc test, the IC_{50} values were expressed as mean±SD. Inhibition (%) = $[(A_{cont} - A_{test})/A_{test}]\times100$, A_{cont} = absorbance of control, A_{test} = absorbance of the test and standard samples, The IC_{50} value was graphically determined.

Microbiological evaluation:

The antimicrobial activity of the synthesized molecules was investigated according to the agar well diffusion method using the nutrient agar and Sabouraud dextrose agar medium (HI-Media) for bacteria and fungi, respectively. The antimicrobial diffusion test was performed using a cell suspension of about 1.5×10^6 CFU/ml employing a McFarland turbidity standard No. 0.5[14]. The microbial strains *K. pneumonia* (MTCC 109) *Candida albicans* (MTCC 3017) and *C. neoformans* (MTCC 3019) were procured from the Institute of Microbial Technology and Gene bank (IMTECH), Chandigarh, India. *Escherichia coli* and *Staphylococcus aureus* resistant to norfloxacin, ofloxacin, and ampicillin were isolated from the urine sample collected from UTI patients at the IMS, SUM Hospital, Bhubaneswar, India. Amoxicillin and fluconazole was used as reference standards against bacterial and fungal strains, respectively. The lowest concentration of the test compounds inhibiting visible growth for bacteria and fungi was termed as minimum inhibitory concentration (MIC). Test solutions of synthesized molecules were prepared suing the two-fold dilution method at a concentration level ranging from 500-31.25 µg/ml using DMF to evaluate the MIC.

Statistical analysis:

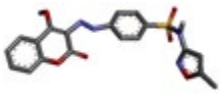
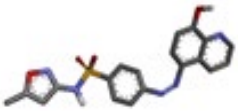
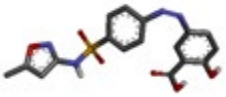
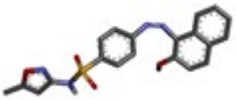
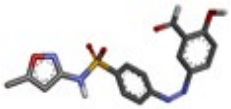
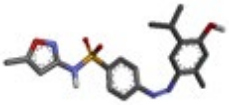
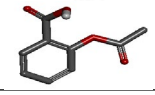
Dunnett's post hoc test and Dunnett's *t*-test were used and the observed data subjected to one way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

A series of phenolic/enolic substituted azosulfamethoxazoles 4a-4f were synthesized by the *in situ* reactions between diazotised SMZ and 6 different individual phenolic and enolic compound under mild conditions. All the proposed isoxazole derivatives were synthesized, physical properties and spectral interpretation were made (Table 1), which were similar to those reported earlier[8-12].

The research work was aimed to theoretically validate the binding of the synthesized SMZ derivatives (4a-4f**)** using molecular docking against COX-2 protein of *Mus musculus* as depicted in Table 2. The docking score and molecule interactions (fig. 3) were obtained in least binding energy of the compound 4d and 4f at value -12.980 and -12.386 kcal/mol with

TABLE 2: CONVERTED 3D- STRUCTURES AND DOCKING SCORES OF INDIVIDUAL SMZ ANALGUES 4A-4F AGAINST TARGET ENZYME CYCLOOXYGENASE-2

Comps.	Chemical name	3D- structure	Docking score (kcal/mol)	Interaction with amino acid of target enzyme during docking
4a	4-[(4-hydroxy-2-oxo-2H-chromen-3-yl)diazenyl]-N-(5-methylisoxazol-3-yl) benzene sulphonamide		-10.958	LYS83, PRO8, VAL89, LEU93, ILE112, TYR115, TYR122, PHE35
4b	4-((8-hydroxyquinolin-5-yl) diazenyl) -N- (5-methyl isoxazol-3-yl) benzene Sulphonamide		-10.652	PHE200, VAL295, LEU391, PHE395, LEU408, TYR409, VAL444
4c	2-hydroxy-5-((4-(N-(5-methylisoxazol-3-yl) sulfamoyl) phenyl) diazenyl) benzoic acid		-11.386	MET196, PHE200, GLN203, VAL291, PHE292, LEU294, VAL295, LEU298, LEU391, PHE395, LEU408
4d	4-((2-hydroxynaphthalen-1-yl) diazenyl)-N-(5-methylisoxazol-3-yl) benzene sulfonamide		-12.980	LYS83, VAL89, LEU93, ILE112, TYR115, VAL116, SER119, ARG120, TYR122
4e	4-((3-formyl-4-hydroxyphenyl) diazenyl)-N-(5-methylisoxazol-3-yl) benzene sulfonamide		-11.159	MET196, PHE200, GLN203, VAL291, LEU294, VAL295, LEU298, HIS388, LEU391, PHE395, LEU408
4f	4-((4-hydroxy-5-isopropyl-2-methylphenyl)diazenyl)-N-(5-methylisoxazol-3-yl) benzenesulphonamide		-12.386	PHE200, GLN208, VAL295, LEU391, PHE395, PHE404, LYS405, PHE407, LEU408, TYR409, VAL444, VAL447
Acetyl salicylic acid	2-acetoxybenzoic acid		-10.256	PHE210, ASN375, ILE377, ALA378, PHE381

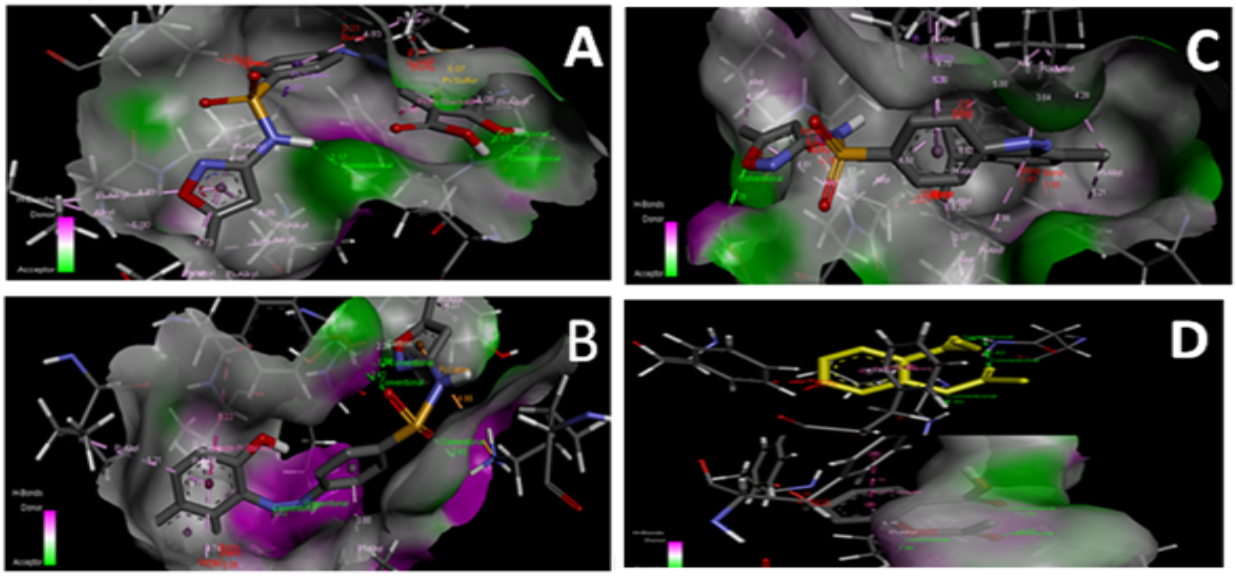


Fig. 3: Docking images captured by the software discovery studio visualizer
Docking images captured during interaction of 1CX2 with SMZ derived molecules A. 4c, B. 4d, C. 4f and D. standard acetylsalicylic acid, respectively

highest binding affinity to COX-2 and compared to standard those of ASA at –10.256 kcal/mol. Compound 4-((2-hydroxynaphthalen-1-yl)diazenyl)-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4d**)** binds to several amino acids, LYS83, VAL89, LEU93, ILE112, TYR115, VAL116, SER119, ARG120 and TYR122 of the active site of COX-2.

The absorption spectra of the molecule 4-((4-hydroxy-2-oxo-2H-chromen-3-yl)diazenyl)-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4a) gave the largest

bathochromic shift 425 nm with isopropanol in comparison to other solvents. Molecule 4a and 4c showed the maximum wave length (λ_{max}) with ethanol at 420 and 370 nm, respectively; the molecule 4b, 4d and 4e showed at 403.8, 480 and 360 nm with methanol, respectively, while the molecule 4f showed λ_{max} at 409 nm with1 4-dioxane. The molecule 4d showed maximum bathochromic shift with methanol, ethanol, DMSO and THF in comparison to other molecules.

Results of acute oral toxicity study indicated that the synthesized isoxazole derivatives were safe up to 2000 mg/kg with no mortality, no toxic symptoms and no gross behavioural changes observed in Wistar rats. In the acetic acid-induced model in the control group, acetic acid produced 52.8±5.3 writhes in 10 min of observation period and the standard ASA (100 mg/kg) inhibited pain by 63.63 % and compounds (4a-4f) produced 16.09, 13.25, 44.88, 58.33, 36.553 and 57.76 % pain inhibition at 50 mg/kg dose level. Among which the 2-naphthol and thymol coupled diazotized isoxazole molecules 4d and 4f reported with significant percent of pain inhibition 58.33 and 57.76 % respectively at a dose of 50 mg/kg body weight. In radiant heat model, the negative control group of animal showed 4.03±0.18 of reaction time by the end of 60 min, while the compound 4d showed significant analgesic activity by the end of 120 and 180 min for a reaction time of 6.7±0.59 and 6.3±0.5. The compound 4f noticed with reaction time of 5.8±0.52 and 5.7±0.5 by the end of 120 and 180 min, respectively. The maximum percent inhibition of pain produced by the test compounds at a dose of 50 mg/kg were for compound 4d (80), 4f (62.85), 4e (51.42) and 4a (48.57) and for the standard (77.14). However, in both acetic acid-induced writhing and radiant heat models molecules 4d and 4f exerted significant analgesic activity. The molecules 4d and 4f significantly ($p<0.05$) inhibited the inflammatory oedema when compared to negative control by the end 120 min of carrageenan injection.

The antioxidant activity of the isoxazole derivatives was investigated using the DPPH assay and the results were compared with those of standard BHT. The IC_{50} values of 4-[(4-hydroxy-2-oxo-2H-chromen-3-yl)diazenyl]-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4a), 4-((8-hydroxyquinolin-5-yl)diazenyl)-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4b), 2-hydroxy-5-((4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)diazenyl)benzoic acid (4c), 4-((2-hydroxynaphthalen-1-yl)diazenyl)-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4d), 4-((3-formyl-4-hydroxyphenyl)diazenyl)-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4e), 4-((4-hydroxy-5-isopropyl-2-methylphenyl)diazenyl)-N-(5-methylisoxazol-3-yl)benzene sulphonamide (4f) and the standard BHT were, 30±0.78, 52±1.13, 59.7±0.03, 38±1.4, 48±0.89, 40±0.59 and 31±0.70 µg/µl, respectively. However, compound (4a) exhibited IC_{50} at the lowest concentration level of 30±0.78 µg/µl in comparison to other compounds tested. The molecule 4d and 4f showed significant antioxidant activity at 10 and 50 µg/µl, with % inhibitions of 36.53±0.02 and 61.41±0.04 when compared to the standard.

Compounds 4a and 4e showed significant antimicrobial activity against *S. aureus*$_{(res)}$ and *C. neoformans* in comparison to amoxicillin and fluconazole, respectively, whereas compounds 4b-4d showed significant antibacterial activity against *E. coli*$_{(res)}$ in comparison to standard (Table 3). The inhibitory property of the isoxazole derivatives was determined in the concentration range of 500-31.25 µg/ml to find out the MIC values in µg/ml. The compound 4a have been exhibited more potential antibacterial activity by inhibiting the growth of *S. aureus*$_{(res)}$, *C. Albicans* and *C. neoformans* at a concentration level of 31.25 µg/ml. The reported MIC results against different microbial strains by the compound 4b would be considered as highly effective antimicrobial compound

TABLE 3: ANTIMICROBIAL ACTIVITY OF SMZ ANALGUES 4A-4F AGAINST DIFFERENT MICROBIAL STRAINS

Comps.	*E. coli*$_{(res)}$	*K. pneumonia*	*S. aureus*$_{(res)}$	*C. albicans*	*C. neoformans*
4a	-	-	24.00 ± 0.63*	24.17 ± 0.75	31.50 ± 0.84*
4b	20±2*	15.83±1.17	25.33±1.97*	8.83±1.33	17.5±2.17
4c	22±1.55*	25±1.1*	-	21±2.83	21.17±0.98
4d	15.67±1.21*	13±2.19	15.17±2.23	-	14.17±0.75
4e	-	-	28.17±1.47*	-	18.17±2.32*
4f	9.83±1.84	9.57	9.41	-	7.83±1.17
RA/RA[1]	12.67±1.51	15.33±1.97	13±1.67	19.33±4.68	24.17±1.94

Results expressed in mean±SD of zone of inhibitions in mm (n=6), data were analyzed using One Way ANOVA followed by Dunnett's Post Hoc test, statistical significance at *p<0.05 in comparison to the reference antibiotic (RA), - no zone of inhibition, *E. coli*$_{(res)}$- *Escherichia coli* (resistant), *K. pneumonia- Klebsiella pneumonia*, *S. aureus*$_{(res)}$- *Staphylococcus aureus* (resistant), *C. albicans- Candida albicans* ,*C. neoformans- Cryptococcus neoformans*

when compared to other synthesized isoxazole derivatives. However, all the synthesized molecules showed potential fungal inhibitory property against *C. neoformans.*

The resultant docking score of the molecules suggested that the molecules 4d and 4f would be potent COX-2 inhibitors. It was also found that from the *in vivo* evaluations that the molecule 4d and 4f showed highest analgesic and antiinflammatory activity among all compounds tested; its action could be due to linking 5-methylisoxazolyl moiety to 2-naphthol and thymol, respectively. Literature supports that isoxazole derived molecules have shown to possess the inhibitory property of COX-2 and also revealed that the nitrogen heterocyclic molecules bearing –N=N- are responsible for inhibition of COX[14]. In present study, the synthesized molecules contained diazenyl function group along with isoxazole nucleus and sulphanilamide together in their structures. Overall structural activity relationships (SAR) study of all analogues had suggested that the presence of phenolic/enolic hydroxyl, diazenyl group and nitrogen containing heterocyclic rings in their structures could be responsible for exhibiting the antioxidant, antimicrobial and antioxidant property.

A series of isoxazolyl derivatives were prepared by azo-coupling reactions and evaluated to investigate their various biological actions. The result suggested that the analogue 4d and 4f exhibited significant analgesic, antioxidant and antimicrobial activities in comparison to standard drugs. Furthermore, the plausible binding sites of these synthesized derivatives could be designated through molecular docking. Thus, SARs of synthesized derivatives suggested that the presence of 5-methylisoxazolyl moiety and a phenolic system, could yield potential leads for developing new therapeutic agents.

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