Biological and Docking Studies of Sulfonamide Derivatives of 4-Aminophenazone

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Summary: Sulfonamide derivatives of 4-aminophenazone (4APZ) were synthesized and accordingly characterized by spectroscopic techniques. These newly synthesized compounds were examined for their biological activities such as enzyme inhibition, analgesic, antibacterial, antioxidant and DNA interaction. A direct correlation between enzyme inhibition activity and concentration of the compounds was observed both by experimental and molecular docking studies. Analgesic activity of the compounds was investigated by formalin-induced paw licking (FIPL), acetic acid-induced writhing (AIW) and heat conduction methods in mice. Membrane stabilization effect was determined by hypotonicity-induced hemolysis. Bacterial strains, *S. aureus, S. epidermidis, B. subtilis, E. coli, P. aeruginosa, S. mutans* and *A. odontolyticus* were used for investigating the antibacterial potential of the compounds. Antioxidant potential was investigated by Ferric Reducing Antioxidant Power assay (FRAP) and DPPH free radical scavenging method. DNA interaction studies of the synthesized compounds showed weak interaction. Hyperchromic effect was observed along the series and large positive K values were obtained for most of the compounds.

Key Words: Sulfonamide, DNA-interaction, Anti-bacterial activity, Enzyme inhibition, Antioxidant activity, Analgesic activity, Hypotonicity-induced hemolysis.

Introduction

Since the health related issues in our everyday life gained much attention, the prevalence and kinds of life-threatening infections have been increasing correspondingly and have become a serious challenge for the science. A quite alarming associated aspect is the development of the drug-resistance in the bacterial strains against the commercial available drugs. It gets demanding for the development of new and safe chemotherapeutic compounds with lesser side effects. The sulfa drugs contain sulfonamide moiety (–SO_2NH-), which is an important pharmacologically active agent [1]. Compounds containing sulfonamide moiety are frequently used in medicine due to their antibacterial activity [2, 3]. The obstruction of such compounds with the *p*-aminobenzoic acid (PABA) make them an attractive substance to investigate their potential against bacteria. PABA is involved in the biosynthesis of tetrahydrofolic acid, which is important for the bacteria's metabolism. The emergence of drug-resistant strains is also an important reason to evaluate sulfonamide therapy. Sulfonamides are effective against the nocardiosis, methicillin-resistant bacterial infections and for the treatment of urinary tract [4]. Amsacrine and sulfonylhydrazines, sulphonamide derivatives, are antineoplastic agents that are frequently used in cancer chemotherapy [5, 6]. In past, sulfonamide compounds have been used as antibacterial agents [7, 8] anticancer [9, 10] anti-inflammatory, analgesic agents [11], antifungal agents [12] and antiviral agents [13].

4-aminophenazone also known as aminoantipyrine has pyrazolone fragment, which is a biologically active moiety found in several important drugs. Compounds containing pyrazolone possess antibacterial [14], anti-inflammatory, analgesic, antipyretic [15], antifungal [16], antihypertensive [17], anti-HIV [18], and antitumor activities [19]. 4-aminophenazone (an antipyretic drug) has almost no antibacterial activity, while sulfonamides are familiar due to their antibacterial potential. It is worthwhile to synthesize such molecules with fragments that have both the antipyretic and antibacterial activities. The current research was aimed to synthesize sulfonamide derivatives aminophenazone and to evaluate their biological potential.

Experimental

4-Aminophenazone (4APZ), benzenesulfonyl chloride, *p*-toluenesulfonyl chloride, *p*-methoxybenzenesulfonyl chloride, *p*-

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bromobenzenesulfonyl chloride, naphthalen-2-sulfonyl chloride were purchased from BDH, Nutrient Agar & Nutrient Broth (Oxide, USA), Ciprofloxacin (Gift sample from standpharm [pvt] ltd, Lahore). 1,1-diphenyl-2-picrylhydrazyl, DPPH (Sigma Chemical Company. St. Louis, USA), Potassium dihydrogen phosphate (Riedel-de-Haen, Germany) Potassium ferrocyanide (Sigma Aldrich, Sealce, Germany), Sodium hydroxide, Ferric chloride, Dimethyl sulphoxide (Riedel-de-Haen, Germany), Trichloro acetic acid, Ascorbic acid & Formalin (Merck, Darmstadt, Germany), Piroxicam (Hovid, Malaysia). Acetic acid (BDH Laboratories, Poole, England). The bacterial strains, *Staphylococcus aureus (*ATCC 25923), *Esherichia coli (*ATCC 8739), *Bacillus subtilis (*ATCC 6633) *Pseudomonas aeruginosa (*ATCC 27853), *Staphylococcus epidermidis (*ATCC 12228), *Streptococcus mutans (*ATCC 25175), and *Actinomycetes odontolyticus (*ATCC 17929), were procured from Musaji Adam and Sons, Rawalpindi. NMR spectra (300MHz, in DMSO-d_6); Bruker AM-300; d(H) in ppm. Mass Spectra were recorded as ESI (positive) probe.

General procedure for the preparation of Sulfonamides **(1-5)**

A series of sulfonamide derivatives of 4-aminophenazone (4APZ) were prepared by following standard procedure (Scheme 1). In brief, we first prepared the solutions of 4-aminophenazone (4APZ) and different benzene sulfonyl chloride in THF separately, which was followed by mixing of the two solutions. Once, homogenized, the mixtures were refluxed for 3 hours at 60°C. The sulfonamides were precipitated out on cooling to room temperature and were later filtered out accordingly. The precipitates were washed several times with THF and recrystallized by methanol.

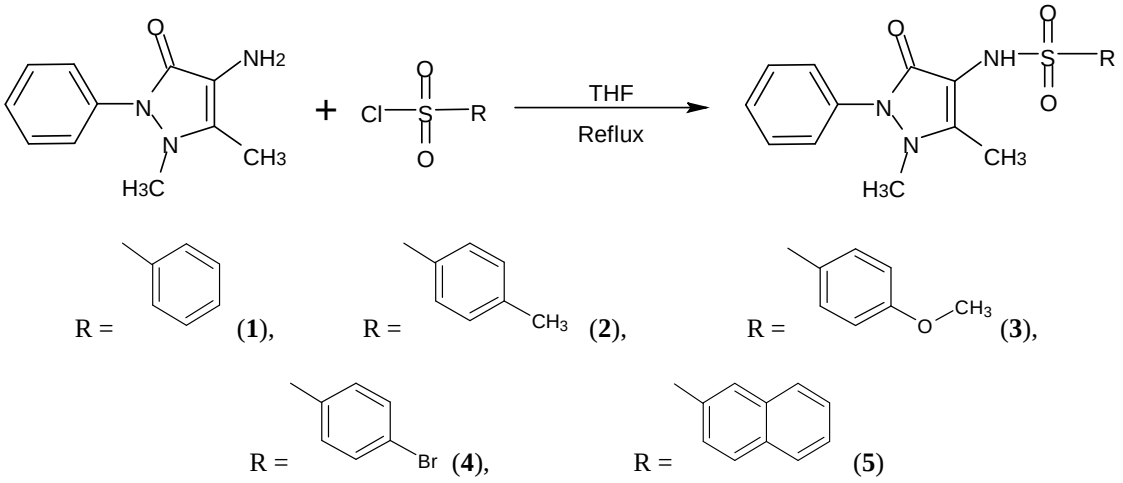
Spectral Data

N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl)benzenesulfonamide **(1)**

Yield: 68%, m.p. 225°C ± 1°C, IR (KBr) υ cm^{-1}: 3314 (N-H), 1617 (C=O), 1271 & 1099 (O_2S-N), 751 (S-N). ^{1}H NMR (300MHz, DMSO-d_6): δ (ppm) 1.75 (3H, s, CH_3), 2.51 (3H, s, CH_3), 6.65-6.72 (3H, m, CH), 7.17-7.30 (3H, m, 3CH) 7.53-7.55 (2H, m, 2CH), 7.92-7.94 (2H, m, 2CH), 7.81 (1H, HN), ^{13}C NMR ($CDCl_3$, 300MHz): δ (ppm) 12.9 (CH_3), 39.7 (CH_3), 113.6 (2CH), 116.5 (C), 119.5 (C), 127.7 (2CH), 129.2 (2CH), 129.6 (2CH), 131.7 (C), 132.1 (CH), 139.8 (C), 136.5 (C), 160.9 (C=O). ES-MS (*m/z*): $[M+H]^+$ 344.6 (41), $[M+H-CH_3]^+$ 329.6 (100), $[M- C_6H_6SO_2]^+$ 202.6 (27), $[M-C_{11}H_{13}N_3O]^+$ 141.6 (21).

N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl)-4-methylbenzenesulfonamide **(2)**

Yield: 76%, m.p. 210°C ± 1°C, IR (KBr) υ cm^{-1}: 3311 (N-H), 1610 (C=O), 1261 & 1095 (O_2S-N), 745 (S-N). ^{1}H NMR (300MHz, DMSO-d_6): δ (ppm) 1.72 (3H, s, CH_3), 2.46 (3H, s, CH_3), 2.34 (3H, s, CH_3), 6.65-6.72 (3H, m, CH), 7.17-7.19 (2H, m, 2CH), 7.33-7.35 (2H, m, 2CH), 7.80-7.82 (2H, m, 2CH), 7.83 (1H, HN), ^{13}C NMR ($CDCl_3$, 300MHz): δ (ppm) 12.9 (CH_3), 24.6 (CH3), 39.4 (CH_3), 113.4 (2CH), 116.3 (C), 119.4 (CH), 127.5 (2CH), 129.3 (2CH), 129.7 (2CH), 131.6 (C), 136.5 (C), 136.7 (C), 141.7 (C), 160.8 (C=O). ES-MS (*m/z*); $[M+H]^+$ 358.4 (37), $[M+H-CH_3]^+$ 343.4 (100), $[M-C_7H_8SO_2]^+$ 202.4 (23), $[M- C_{11}H_{13}N_3O]^+$ 155.4 (20)



Scheme-1 Synthesis of 4-aminophenazone sulfonamide.

N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl)-4-methoxybenzenesulfonamide **(3)**

Yield: 79%, m.p. 250°C ± 1°C, IR (KBr) υ cm^{-1}: 3319 (N-H), 1618 (C=O), 1271 & 1103 (O_2S-N), 752 (S-N). ^{1}H NMR (300MHz, DMSO-d_6): δ (ppm) 1.71 (3H, s, CH_3), 2.46 (3H, s, CH_3), 3.74 (3H, s, CH_3) 6.65-6.72 (3H, m, 3CH), 7.02-7.06 (2H, m, 2CH), 7.16-7.19 (2H, m, 2CH), 7.79 (1H, HN), 7.81-7.83 (2H, m, 2CH), ^{13}C NMR ($CDCl_3$, 300MHz): δ (ppm) 12.6 (CH_3), 39.2 (CH_3), 60.1 (CH^3), 113.1 (2CH), 114.6 (2CH), 116.4 (C), 119.3 (CH), 128.3 (2CH), 129.5 (2CH), 131.7 (C), 131.9 (C), 136.2 (C), 160.5 (C=O), 164.1 (C). ES-MS (*m/z*): $[M+H]^+$ 374.5 (39), $[M+H-CH_3]^+$ 359.5 (100), $[M- C_7H_8SO_3]^+$ 202.5 (23), $[M- C_{11}H_{13}N_3O]^+$ 171.5 (20).

4-bromo-N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl)benzenesulfonamide **(4)**

Yield: 78%, m.p. 243°C ± 1°C, IR (KBr) υ cm^{-1}: 3312 (N-H), 1613 (C=O), 1265 & 1097 (O_2S-N), 748 (S-N). ^{1}H NMR (300MHz, DMSO-d_6): δ (ppm) 1.76 (3H, s, CH_3), 2.49 (3H, s, CH_3), 6.65-6.72 (3H, m, 3CH), 7.15-7.19 (2H, m, 2CH), 7.68-7.72 (2H, m, 2CH), 7.78 (1H, HN), 7.80-7.82 (2H, m, 2CH), ^{13}C NMR ($CDCl_3$, 300MHz): δ (ppm) 12.9 (CH_3), 39.7 (CH_3), 113.6 (2CH), 116.8 (C),119.6 (CH), 126.7 (C), 129.1 (2CH), 129.5 (2CH), 131.1 (C), 131.8 (2CH), 136.1 (C), 138.6 (C), 160.6 (C=O). ES-MS; (*m/z*): $[M+H]^+$ 423.1 (27), $[M+H-CH_3]^+$ 408.1 (100), $[M-C_{11}H_{13}N_3O]^+$ 220.1 (34), $[M-C_6H_5SO_2Br]^+$ 202.1 (39).

N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl)naphthalene-2-sulfonamide **(5)**

Yield: 65%, m.p. 240°C ± 1°C, IR (KBr) υ cm^{-1}: 3321 (N-H), 1625 (C=O), 1271 & 1106 (SO_2-N), 758 (S-N). ^{1}H NMR (300MHz, DMSO-d_6) δ (ppm) 1.73 (3H, s, CH_3), 2.46 (3H, s, CH_3), 6.64-6.70 (3H, m, 3CH), 7.15-7.17 (2H, m, 2CH), 7.30-7.32 (2H, m, 2CH), 7.64-7.68 (2H, m, 2CH), 7.84 (1H, HN), 7.94-7.98 (2H, m, 2CH), 8.31(1H, s, CH), ^{13}C NMR ($CDCl_3$, 300MHz): δ (ppm) 12.4 (CH_3), 39.1 (CH_3), 113.0 (2CH), 116.2 (C), 119.0 (CH), 123.2 (CH), 125.9 (CH) 126.1 (2CH), 128.2 (2CH), 128.4 (CH), 129.3 (2CH), 131.4, (C), 134.0 (C), 136.2 (C), 136.6 (C), 137.0 (C), 160.7 (C=O). ES-MS (*m/z*): $[M+H]^+$ 394.3 (22), $[M+H-CH_3]^+$ 379.3 (100), $[M- C_{10}H_8SO_2]^+$ 202.3 (42) , $[M- C_{11}H_{13}N_3O]^+$ 191.3 (38).

XRD Analysis

Fig. 1 and Fig. 2 display the crystal structures of compounds **3** & **5.** The crystal structures of compounds **1, 2 & 4** are reported elsewhere [20-22]. The details of the crystal data are given in Table-1.

Animals Used

Swiss albino mice (weighing 20-30 g) of either sex have been used in the present study. They have been housed in stainless cages under controlled room temperature (12hrs, light and dark cycle, 25 ± 1ºC; relative humidity 60-70%) in the animal house of the Faculty of Pharmacy, University of Sargodha, Sargodha. They were offered standard pellet diet with fresh portable water *ad libitum* and were handled according to guidelines approved by Local Institutional Ethical Committee NIH. Swiss albino mice were grouped into seventeen groups of six animals each for the evaluation of analgesic activity (Table-2). Oral and intraperitoneal administrations were carried out by following the standard procedure reported by Murtaza *et al*. [15].

Antibacterial Activity

Nutrient agar (27g) was suspended in distilled water (1L) and autoclaved at 121ºC for 15 minutes. After cooling down to ambient temperature, 15 ml of this suspension was poured in separate Petri dishes and were allowed to solidify at room temperature. The Petri dishes were stored in the refrigerator at 4ºC. The pH of the medium was kept around 7.2. In parallel experiments, Nutrient broth (13g) was prepared in similar way and its pH was kept around 6.8. Inoculum was prepared as described by Panthi [23] and its turbidity was adjusted by 0.5 McFarland standard solution [24]. The antimicrobial susceptibility test was carried out by Cup-plate method [25].

Analgesic Activity

5% v/v DMSO was prepared by dissolving 5 ml of DMSO and making final volume upto 100 ml with distilled water. Acetic Acid 0.6% (v/v) solution was prepared by dissolving 0.6 ml of acetic acid in 100 ml of distilled water. Solutions of all test compounds were prepared by dissolving the powdered compounds in 5% DMSO. Analgesic activity by formalin-induced paw licking in mice and acetic acid-induced writhing test in mice (Chemical stimulation) were determined as described in ref. 15.

Table-1: Crystal data and refinement details for compounds **3** & 5.

Formula	$C_{18}H_{19}N_3O_4S$ (3)	$C_{21}H_{19}N_3O_3S$ (5)
Formula Weight	373.42	393.45
Crystal system	Monoclinic	Orthorhombic
Space Group	$P2_1/c$	$Pca2_1$
a, b, c (Å)	5.2252 (4), 21.8248 (17), 15.5817 (13)	19.3074 (14), 9.8179 (6), 20.5570 (11)
~, , ¥ (deg)	90, 93.241 (4), 90	90, 90, 90
V (Å^3)	1774.1 (2)	3896.7 (4)
Z	4	8
°$_{calc}$ (g/cm^3)	1.398	1.341
(mm^{-1})	0.21	0.19
F(000)	784	1649
Crystal size (mm)	0.38 × 0.20 × 0.18	0.38 × 0.28 × 0.24
Temperature (K)	296(2)	296(2)
λMoK~ (Å)	0.71073	0.71073
θ range (deg)	1.607 – 28.013	1.981–27.00
h, k, l limits	-6:4, -28:28, -20:20	-24:17, -11:12, -26:17
Reflections; collected/ Uniq.	16556/4260[R(int) = 0.0526]	17323/7436 [R(int) = 0.0566]
Reflections: observed [$I>2\sigma(I)$]	2636	4238
T_{min}, T_{max}	0.924, 0.963	0.931, 0.956
Data/restraints/parameters	4260 / 0 /238	7436 / 1/474
Goodness-of-fit on F^2	1.016	0.966
R indices [$I > 2\sigma(I)$]	R_1 = 0.0528 ; wR_2 = 0.0986	R_1 = 0.0553 ; wR_2 = 0.1142
Largest diff. peak, hole (e Å^{-3})	0.404, -0.390	0.261, -0.260

Table-2: Animal group ID, compound administered and the dose amount.

Group ID	Sample	Amount (mg/kg)	Group ID	Sample	Amount (mg/kg)
G2	Ciprofloxacine	5	G10	Comp. 3	225
G3	Comp. 1	200	G11	Comp. 3	250
G4	Comp. 1	225	G12	Comp. 4	100
G5	Comp. 1	250	G13	Comp. 4	150
G6	Comp. 2	100	G14	Comp. 4	200
G7	Comp. 2	150	G15	Comp. 5	100
G8	Comp. 2	200	G16	Comp. 5	150
G9	Comp. 3	200	G17	Comp. 5	200

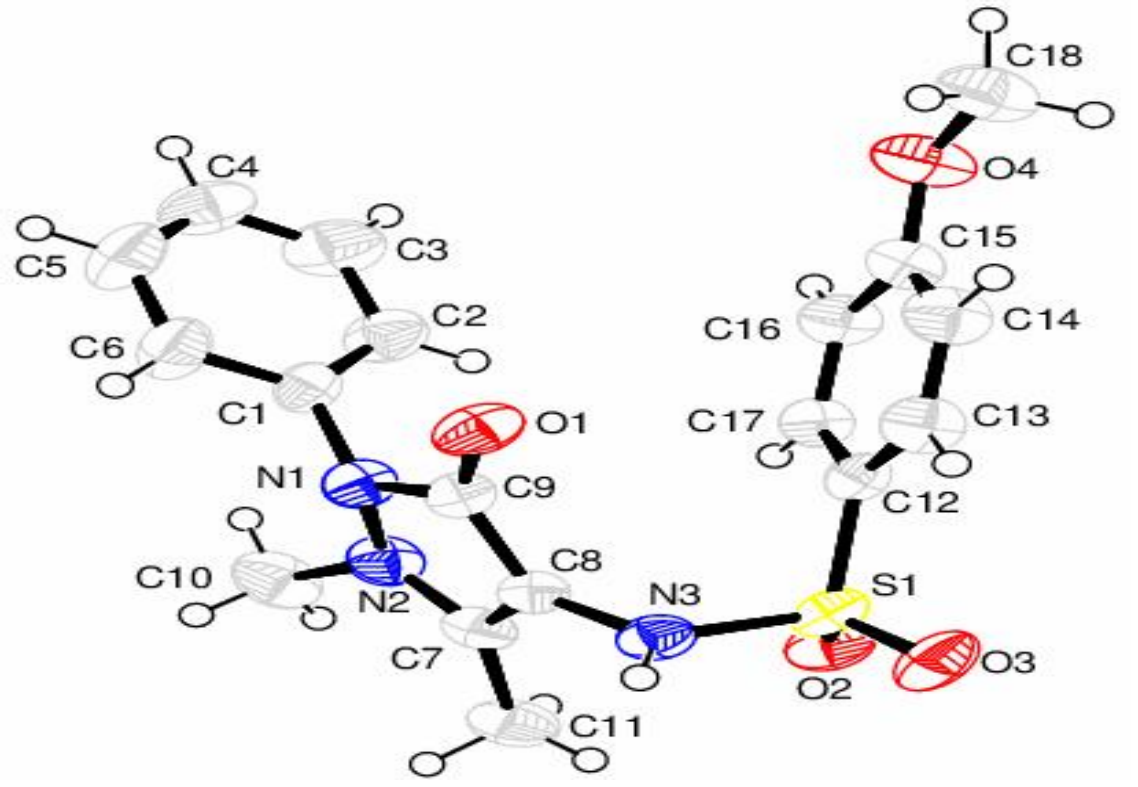


Fig. 1: ORTEP drawing of **3** with atomic numbering scheme.

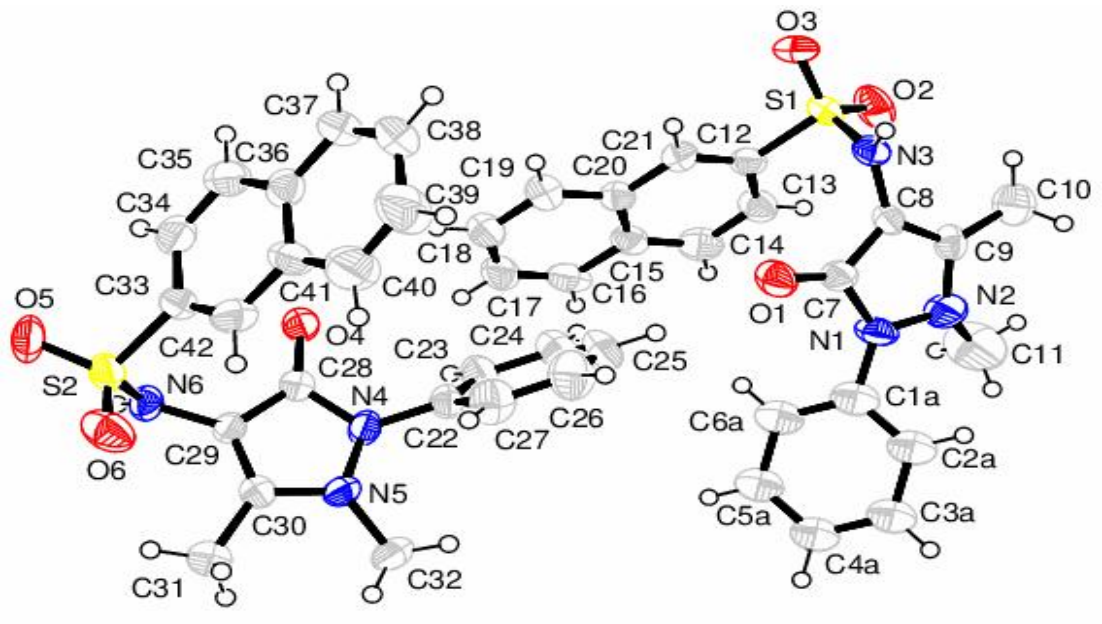


Fig. 2: ORTEP drawing of 5 with atomic numbering scheme.

Percentage inhibition of writhing was calculated using the following formula:

$$\%\ inhibition = \frac{W(control) - W(test)}{W(control)} \times 100$$

where

$W_{(control)}$ = Mean no. of writhings for control group.

$W_{(test)}$ = Mean no. of writhings for test groups treated with different doses

Heat Conduction Method

The heat conduction method described by Kulkarni [26] was employed to determine the analgesic activity of sulfonamide derivatives. G1 was as untreated control and G2 was used as piroxicam (5mg/kg orally) treated control. Remaining groups (G2−G17) were treated with different doses of sulfonamide derivatives. After 15 minutes, 30 minutes, 1 hour, 2 hours and 3 hours, the tail of mice was dipped up to 5cm into hot water (58ºC). The time duration was recorded as the mice withdraw its tail out from hot water. A time interval of 10 sec was maintained as cut off time to avoid the burning of mice tail. The time (in second) required for flicking the tail of mice was recorded to measure the efficiency of the compounds to noxious stimulus.

Ferric Reducing Antioxidant Power Assay

Antioxidant activity of test compounds was investigated by reducing power assay method (Oyaizu, 1986) [27]. Solutions of varied concentrations of ascorbic acid as standard and test compounds in deionized water were prepared. To these solution, phosphate buffer (2.5 ml of 200 mmol/L at pH 6.6) and 2.5ml of 1% KFe (CN) were mixed and incubated at 50°C for 20 minutes. Then 1% TCA (2.5 ml) was added and centrifuged at 3000 rpm for 5 minutes. The supernatant (2.5ml) was diluted by distilled water (2.5ml) and 0.1% of ferric chloride (0.5ml) was added. The absorbance of the mixture was measured at 700nm.

DPPH Free Radical Scavenging Activity

Antioxidant activity of test compounds was also examined by using DPPH, following the procedure reported by Khan *et al*. [28]. In brief, DPPH (4.3mg) was first dissolved in methanol (3.3ml) in an amber bottle covered with Al foil to avoid the contact with light. In a separate experiment, DPPH solution (150µl) was added in methanol (3ml) and absorbance (at λ=517 nm) was recorded immediately as a control. The solutions of different concentrations of test compounds were taken and DPPH (150 µl) was added to each sample. The absorbance at λ=517 nm was noted after 15 minutes interval on UV-Visible spectrophotometer. Methanol was used as blank. The capability to scavenge the DPPH radical was calculated using the following formula.

DPPH scavenged (%) =

$$\frac{\text{Absorbance of control-Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

Membrane Stabilization Effect

In order to determine the membrane stabilization activity, the procedure was adopted as described earlier [15]. For this purpose, the Erythrocyte suspension was prepared by following the method reported by Olajide *et al.* [29] with some modifications. The percentage membrane stabilizing activity was determined by using the following relationship;

$$\%\ inhibition\ of\ haemolysis = \frac{A_1 - A_2}{A_1} \times 100$$

where, A_1 = Absorption of the control solution and A_2 = Absorption of test sample solution.

Enzyme Inhibition

The well known Ellman method [30] was used with slight modifications to determine the enzyme inhibition activity of the synthesized compounds. Sample solutions of synthesized compounds were prepared in concentrations of 0.9 mM, 1 mM, 2 mM, 4 mM, 6 mM. Buffer solution (1.35 ml) was diluted by the addition of sample solutions (50 µl) followed by the addition of 50 ml each of Butyrylthiocholine chloride ($C_9H_{20}ClNOS$) substrate and the Butyrylcholinesterase (BuChE) enzyme. The solutions were allowed to stay for about 20 minutes after vigorous shaking. Spectrophotometric absorbance was checked at 412 nm and %age inhibition was calculated by following formula;

$$Percentage\ Inhibition = \frac{E - S}{E} \times 100$$

Molecular Docking Studies for Enzyme Inhibition Activity

To manifest the interaction of synthesized compounds with butyrylcholinesterase, the newly synthesized compounds in the present study were also investigated by Molecular docking studies. The protein was exported from Protein Data Bank (PDB ID 1p0p). The molecular structures (3D) of synthesized compounds were generated through ChemUltra3D 8.0. PacthDock online molecular docking server was used for computational analysis using default settings and was provided with protein receptor and synthesized derivatives ligand files. The protein structure was cleaned by removing the solvent molecules. The lowest atomic connection energy (ACE) conformation was selected as the binding mode [31].

DNA Interaction Studies

The synthesized compounds were tested for their ability to interact with the calf thymus DNA by using UV-Vis absorption spectroscopic method. Stock solution of DNA was prepared by the dissolution of an apt amount of DNA in 10% methanol and the concentration of DNA in this solution was determined with the help of Beer-Lambert's law $A = \varepsilon cl$ ($\lambda_{max} = 260\ nm$, $\varepsilon_{DNA} = 6600\ M^{-1}cm^{-1}$). The solution was stored in refrigerator and was not used for more than 4 days. Stock solutions of synthesized compounds were prepared in 90% methanol. The concentration of the compounds was kept constant and absorption titrations were performed by continuously increasing the concentration of DNA to both the compound and the reference solutions. The effect of DNA absorbance was minimized with the help of reference solution. The solutions were allowed to stand for 30 minutes at room temperature. Spectra were recorded using a double beam spectrophotometer [32].

Acute Toxicity and Behavioural Pattern Studies

Toxic effects of the tested compounds were preliminarily screened by keeping the mice under keen observation for twelve hours daily for the following week. The symptoms including awareness, CNS, mood, excitation, muscle tone, posture, body weight, reflexes food consumption, motor activity and motor in coordination were recorded for seven days. During the next two weeks, any mortality occurred was also recorded [33].

Results and Discussions

Chemistry

Sulfonamides were prepared by the reaction of 4-aminophenazone with benzenesulfonyl chloride, 4-tolunesulfonyl chloride, 4-methoxybenzenesulfonyl chloride, 4-bromobenzenesulfonyl chloride and naphthalen-2-sulfonyl chloride (scheme 1). The reaction was monitored through TLC and products were characterized by IR, NMR, MS and XRD. A single absorption peak appeared in the range of 3311−3320 cm^{-1} can be ascribed to the stretching of –NH (for **1**-**5**) indicated the conversion of $–NH_2$ group to –NH group, which was further supported by the

absorption peaks appeared within the ranges of 1271−1261 & 1106−1095 cm^{-1} due to the O_2S-N (for **1**-**5**). ^1H NMR and ^{13}C NMR spectra of the compounds (**1**-**5**) confirmed their structures. The additional peaks at δ= 1.71 ppm and δ= 3.74 ppm in ^1H NMR spectra of compounds **2** and **3** respectively can be ascribed to the presence of methyl and methoxy groups in these compounds. Similarly, ^{13}C NMR spectra of compounds (**1**-**5**) confirm the number of carbons. In EI-MS spectra of (**1**-**5**), M+H peak appeared for all compounds confirming the formation of the corresponding products. The XRD data also confirmed the structure of the newly synthesized compounds.

Antibacterial Activity

Antibacterial activity of compounds (**1**-**5**) at three different concentrations, i.e. 1000 µg/ml, 500 µg/ml and 250 µg/ml was determined by cup-plate method against *S. aureus, S. epidermidis, B. subtilis, E. coli, P. aeruginosa, S. mutans* and *A. odontolyticus*. Among these, the *S. aureus* causes various cholic diseases and throat infections while *S. mutans* and *Actinomyces* species are involved in dental procedures and oral abscesses. *S. epidermidis* strains are often resistant to antibiotics, including methicillin, penicillin and amoxicillin and are a risk for the patients with compromised immune system. *P. aeruginosa* usually infects the urinary tract, pulmonary tract, wounds, burns and also involved other blood infections. *E. coli* can cause serious food poisoning. High efficiency of (**1**-**5**) was observed at highest tested concentration (1000 µg/ml). The results of antibacterial activity are tabulated in (Table-3). Compound **5** showed excellent results at (1000 µg/ml) against 4 strains *i.e. S. aureus* (IZD=27 mm), *B. subtilis* (IZD= 21mm), *E. coli* (IZD= 29mm) and *P. aeruginosa* (IZD=30 mm) and it would be a better choice for curing infections caused by these strains. Compound **2** at the same concentration exhibited maximum activity against three strains *B. subtilis* (IZD=21 mm), *S. mutans* (IZD=28 mm) and *S. epidermidis* (IZD=24 mm). Compound **3**(1000 µg/ml) showed preeminent effect against *A. Odontolyticus* with IZD=31 mm comparable to that of the reference drug (IZD=32 mm). Compound **3** and **4** at concentration 1000 µg/ml showed best results against *B. subtilis* **(**IZD=21 mm) and *A. odontolyticus* (IZD=31 mm) respectively. All the tested compounds have exhibited significant ($P<0.05$) activities against gram positive and negative bacterial strains.

Analgesic Activity

Experimental animal models were employed for the evaluation of analgesic activity of the tested compounds, which include formalin-induced paw licking, acetic acid-induced writhing and heat conduction methods. These methods are quite useful to determine the anti-nociceptive mechanisms involved. The formalin and acetic acid methods (chemical stimuli) have been used to evaluate the central and peripheral mediated pain, while heat conduction method (thermal stimuli) was applied for centrally mediated pain. Histamine starts inflammatory reaction while bradikinin along with other mediators leads to peripheral and central sensitization phenomenon involved in pain [34]. It has been suggested that the pain research has not only explored the molecular and neuronal basis of pain pathways of healthy individuals but also provided insights into function and plasticity of the pain system during clinically relevant pains. These new insights into pain processing will significantly alter researchers approach to pain control and the development of new analgesics [35].

By Formalin-Induced Paw Licking in Mice

Sulfonamide derivatives (**1**-**5**) were examined for their analgesic effect by "formalin-induced paw licking method" to exploit the analgesic potential. This method measures the behavioural index (licking) and the results obtained showed that all tested compounds exhibit a dose dependent analgesic response. The groups of animals treated with compound **2**, **4** and **5** at dose 200 mg/kg took shortest time to lick their paws that indicated high analgesic efficiency of these compounds. Compound **1** and **3** at dose 250 mg/kg were also found active (Table-4). The inhibition of the analgesic effects by tested compounds (**1**-**5**) was observed higher in G5, G8, G10, G14, G17 than the group treated with standard drug G2 (Fig. 3). Formalin has the ability to produce neurogenic as well as inflammatory pain as formalin measures characteristic response of biphasic pain (peripheral and centrally mediated response). This chemical causes immediate and intense stimulation of C fibre and produce diverse behavioural response demonstrated by licking of mice's paw. The results of test compounds examined on two phases have suggested that compounds may have both peripheral as well as central effect [36].

Table-3: Zones of inhibitions provided by compounds (1-5) at three different concentrations against different bacterial strains

Inhibition Zone diameter (IZD) in mm (Means ± S.E.M)							
Samples (µg/ml)	***S. aureus***	***B. subtilis***	***S. mutans***	***E. coli***	***P. aeruginosa***	***S. epide-rmidis***	***A.odont-Olyticus***
Reference (5)	**34±1.52**	**30±2.02**	**32±0.88**	**33±1.52**	**35±2.08**	**36±2.08**	**32±0.88**
Comp-1(250)	**8±0.33**	**16±0.57**	**11±0.66**	**7±0.57**	**7±0.57**	**15±0.88**	**9±0.57**
Comp-1(500)	**8±1.15**	**17±0.33**	**19±0.88**	**9±0.33**	**9±0.57**	**17±0.66**	**11±0.88**
Comp-1(1000)	**12±0.57**	**17±0.88**	**24±1.2**	**11±0.5**	**12±0.88**	**22±0.57**	**14±1.2**
Comp-2(250)	**7±0.57**	**13±0.88**	**17±2.08**	**10±0.57**	**8±0.88**	**12±0.88**	**10±0.57**
Comp-2(500)	**7±0.33**	**20±0.57**	**22±0.88**	**13±0.57**	**11±0.57**	**20±0.66**	**15±0.88**
Comp-2(1000)	**8±0.66**	**21±0.33**	**28±0.88**	**15±0.66**	**13±1.15**	**24±1.85**	**27±1.45**
Comp-3(250)	**6±0.33**	**12±0.88**	**17±1.15**	**10±0.57**	**9±0.33**	**16±0.33**	**10±0.57**
Comp-3(500)	**8±0.33**	**16±1.2**	**22±0.66**	**14±0.57**	**11±0.57**	**20±0.33**	**12±0.33**
Comp-3(1000)	**12±0.33**	**21±1.15**	**25±1.52**	**17±1.15**	**13±0.33**	**23±1.15**	**14±0.88**
Comp-4(250)	**9±0.66**	**9±0.57**	**14±0.57**	**10±0.57**	**9±0.33**	**16±1.15**	**21±1.15**
Comp-4(500)	**10±0.88**	**12±0.88**	**21±1.0**	**14±1.15**	**13±0.57**	**19±0.33**	**26±0.66**
Comp-4(1000)	**14±0.57**	**20±0.88**	**24±0.57**	**20±0.88**	**15±2.08**	**22±0.88**	**31±1.15**
Comp-5(250)	**20±0.57**	**12±0.88**	**14±1.15**	**15±1.52**	**15±2.08**	**16±1.2**	**14±1.2**
Comp-5(500)	**22±0.88**	**16±0.66**	**20±0.57**	**22±0.88**	**22±0.88**	**22±0.66**	**21±0.57**
Comp-5(1000)	**27±2.18**	**21±1.2**	**26±1.73**	**29±0.57**	**30±0.88**	**22±0.88**	**27±0.66**

Table-4: The time (in second) of licking to determine Analgesic effects of the compounds (1-5) by formalin-induced paw licking in mice

Sr. No. Group ID	1	2	3	4	5	6	Mean ± SEM
G1	**205**	**176**	**198**	**219**	**187**	**165**	**191.6 ± 8.0**
G2	**83**	**49**	**34**	**56**	**31**	**42**	**49.1 ± 7.7**
G3	**131**	**43**	**143**	**176**	**149**	**119**	**127 ± 18.5**
G4	**32**	**44**	**67**	**98**	**64**	**31**	**56 ± 10.4**
G5	**23**	**34**	**47**	**56**	**21**	**42**	**37.1 ± 5.6**
G6	**112**	**92**	**68**	**104**	**79**	**118**	**95.5 ± 7.9**
G7	**72**	**91**	**58**	**54**	**75**	**67**	**69.5 ± 5.4**
G8	**21**	**39**	**43**	**23**	**19**	**29**	**29 ± 4.0**
G9	**68**	**110**	**59**	**70**	**56**	**112**	**79.1 ± 10.2**
G10	**52**	**67**	**19**	**43**	**35**	**65**	**46.8 ± 7.5**
G11	**26**	**39**	**24**	**31**	**66**	**17**	**33.8 ± 7.1**
G12	**176**	**143**	**76**	**114**	**146**	**69**	**120.7 ± 17.2**
G13	**62**	**58**	**84**	**77**	**65**	**98**	**74 ± 6.2**
G14	**46**	**32**	**49**	**37**	**28**	**32**	**37.3 ± 3.4**
G15	**76**	**63**	**101**	**73**	**89**	**68**	**78.3 ± 5.7**
G16	**33**	**32**	**74**	**36**	**50**	**54**	**46.5 ± 6.6**
G17	**12**	**19**	**37**	**28**	**37**	**41**	**29 ± 4.7**

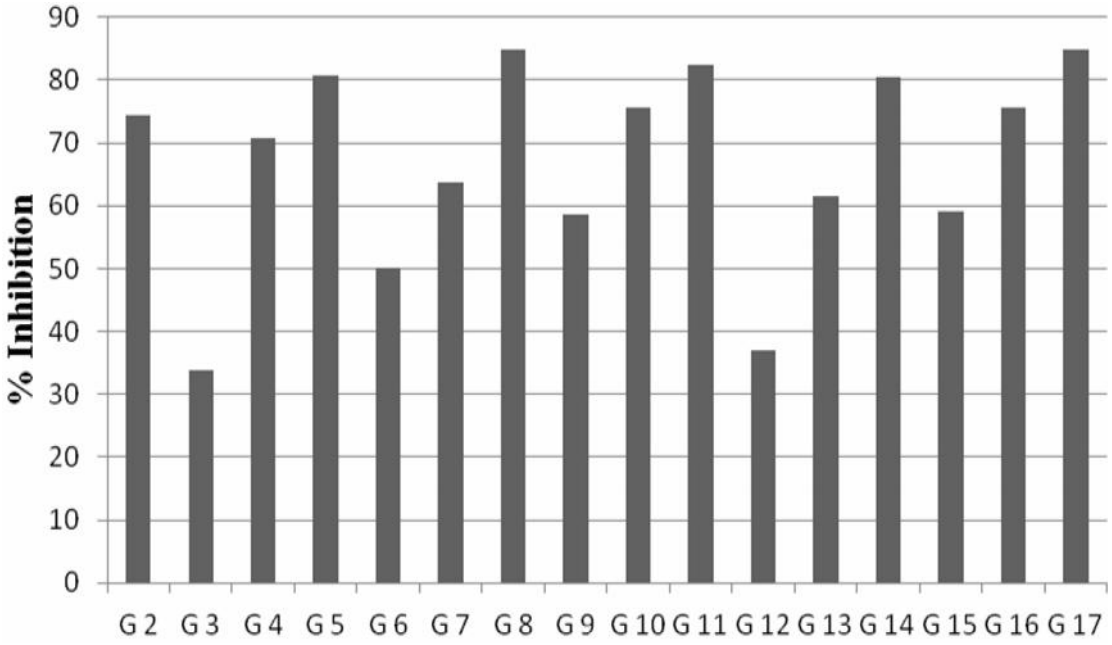


Fig. 3: The percentage inhibition of Analgesic effects (formalin-induced paw licking) in mice by the different doses of compounds (**1**-**5**).

By Acetic Acid-Induced Writhing Method

Acetic acid-induced writhing method is a sensitive procedure for peripherally mediated analgesic effect produced by constriction of abdominal muscles. The dose dependent analgesic effects were observed for all compounds (**1**-**5**). The lowest mean number of writhes by the animals treated with compounds **2**, **4** and **5** at dose 200 mg/kg were 1.16, 1.83 and 2.50 respectively that indicated a quick relief in pain. For compound **1** and **3** at dose 250 mg/kg, this number was 3.16 and 1.0 respectively. Compound **3** at the dose of 250 mg/kg was found to be the most potent analgesic among all tested compounds (Table-5). The pain produced by acetic acid is due to liberation of endogenous mediators such as histamine, prostaglandins, bradykinin, serotonin and substance P, which causes nerve ending stimulation. The abdominal constriction is caused by the local peritoneal receptors [37]. This method is associated with prostanoid and lipoxygenase pathway. The significant reduction in number of writhes by tested compounds would suggest that these compounds act by peripherally mediated analgesic mechanism via inhibition of production and release of prostaglandins as well as other endogenous substances. The inhibition of the analgesic effects by tested compounds (**1**-**5**) was observed higher in G8, G11, G14 and G17 than the group G2 treated with standard drug (Fig. 4).

Table-5: Analgesic activity of compounds (1-5) by acetic acid-induced writhing method (No. of writhes).

Sr. No. Group ID	1	2	3	4	5	6	Means ±SEM
G1	21	19	23	26	28	19	22.6 ± 1.5
G2	4	0	0	3	6	2	2.50 ± 0.9
G3	17	15	15	14	10	12	13.83 ± 1.0
G4	8	9	8	6	5	8	7.33 ± 0.6
G5	6	3	0	4	0	6	3.16 ± 1.1
G6	17	14	14	13	14	16	14.66 ± 0.8
G7	9	8	7	9	8	7	8.00 ± 0.4
G8	0	0	2	2	0	3	1.16 ± 0.5
G9	18	13	14	13	14	16	14.66 ± 0.8
G10	8	7	9	7	6	7	7.33 ± 0.4
G11	0	2	0	0	0	4	1.00 ± 0.7
G12	15	13	10	10	7	7	10.33 ± 1.3
G13	12	8	6	6	8	6	7.66 ± 0.9
G14	4	0	2	4	1	0	1.83 ± 0.7
G15	1	7	21	1	1	2	14.33 ± 1.6
G16	9	6	5	7	6	9	7.00 ± 0.7
G17	1	3	0	5	6	0	2.50 ± 1.0

By Heat Conduction Method

In order to determine the centrally mediated analgesic effect these tested compounds were subjected to "Tail flick assay or heat conduction method". This method is selective for centrally mediated analgesic effect *e.g.* for opioid analgesics. It was observed that the test compounds, even at maximum doses did not produce analgesic response; suggesting that tested compounds did not show centrally mediated analgesic effect because they did not enhance the latency of tail flick [38].

Antioxidant Activity

Oxidative stress leads to the generation of ROS (Hydroxyl radical, peroxy radical and superoxide). These ROS are involved in the pathophysiology of certain diseases including cancer, atherosclerosis, diabetes mellitus, Alzheimer's diseases, cardiovascular events, ageing and inflammation. All the test compounds have been found to possess significant antioxidant potential by free radical scavenging activity (DPPH) and ferric reducing activity [39], suggesting that the compounds exhibit analgesic activity as free radicals are also involved in the pathophysiology of neuropathic pain and various pathological conditions [40].

By DPPH Free Radical Scavenging Activity

Antioxidant activity of sulfonamide derivatives (**1**-**5**) was determined by DPPH free radical scavenging assay. The mean %age inhibition by different concentration of sulfonamides was calculated. Compound **3** (1000 µg/3.3 ml) showed excellent antioxidant activity as the mean %age inhibition was 82%, which was very close to standard (ascorbic acid) 84%. Compound **4** and **5** also showed good antioxidant activity. Comparatively poor antioxidant activity was observed in case of **1** (Table-6).

By Ferric Reducing Power Assay Method

Antioxidant activity of tested compounds was also determined by ferric reducing power assay. Compound **4** (1000 µg/11.5 ml) showed excellent antioxidant activity as compared with all other test compounds. Compounds **1**, **2** and **3** showed comparable antioxidant activity with mean absorbance of 0.83, 0.81 and 0.78 respectively. Compound **5** showed mean absorbance of 0.40 and was proved to have comparatively weak antioxidant potential (Table-7).

Hypotonicity Induced Haemolysis

The tested compounds were also subjected to membrane stabilizing activity as erythrocyte membrane is similar to lysosomal membrane and this

test established the fact that the stabilization of erythrocyte membrane resembles the stabilization of lysosomal membrane. The results showed dose dependent efficiency of the tested compounds (Table-8). The haemolysis may result from shrinkage of red blood cells due to osmotic loss of fluid components and intracellular electrolytes. The destabilization of membranes may involve in the inflammatory as well as in nociception. The tested compounds may impede the processes responsible for stimulation or enhancer of efflux of intracellular components. Compound **5** showed maximum efficiency followed by compounds **2**, **4**, **3** and **1** at their highest doses with absorbance values 0.07, 0.111, 0.115, 0.127 and 0.138 respectively (Table-8). Highly significant results with inhibition above 70% were observed in G8, G11, G14 and G17. The highest value of percentage inhibition 84.7% was observed in the case of G17 (Fig. 5).

Enzyme Inhibition

The synthesized compounds were evaluated for their ability to inhibit the activity of butyrylcholinesterase by using the well-known Ellman method with slight modifications [41]. Butyrylthiocholine chloride and butyrylcholinesterase were used as substrate and enzyme respectively whereas Galantamine was used as positive control. The results are summarized in Table-9.

A direct correlation between the concentration and inhibitory activity of the synthesized compounds can be inferred from the results (Fig. 6). However, all the synthesized compounds were less active than the positive control Galantamine.

Table-6: Measurement of DPPH scavenging activity of compounds (1-5) at λ=517 nm (Percent inhibition ± SEM)

Sr. No.	Amount of compound µg/3.3ml	Standard (Ascorbic acid)	Comp.1	Comp. 2	Comp.3	Comp.4	Comp.5
1	500	60.31±0.66	31.93±0.21	48.75±5.97	52.56±0.28	50.32±2.29	58.25±0.32
2	600	62.62±0.32	34.4±0.43	50.05±0.48	61.65±0.29	60.46±0.42	62.85±0.46
3	700	68.79±0.44	35.91±0.22	52.24±0.53	66.54±0.34	67.18±4.28	68.06±0.39
4	800	75.53±1.07	39.05±0.38	55.26±0.47	74.79±0.23	66.27±0.51	75.58±0.32
5	900	79.86±1.21	45.22±0.06	58.25±0.43	78.93±0.54	74.51±0.33	78.49±0.27
6	1000	83.63±0.33	47.47±0.27	61.7±1.22	81.63±0.51	75.98±0.39	78.08±0.69

Table-7: Measurement of reducing power activity of compounds (1-5) at λ=700nm (amount in µg dissolved in 11.5 ml of solvent) (Mean absorbance ± SEM)

Sr. No.	Amount of compound (µg)/11.5 ml	Standard	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5
1	31.25	0.63±0.002	0.644±0.004	0.602±0.001	0.608±0.006	0.614±0.003	0.4±0.001
2	62.5	0.765±0.008	0.701±0.001	0.634±0.002	0.645±0.001	0.655±0.009	0.433±0.003
3	125	0.802±0.005	0.716±0.003	0.687±0.002	0.707±0.001	0.684±0.004	0.435±0.007
4	250	0.749±0.001	0.757±0.001	0.762±0.028	0.748±0.006	0.757±0.003	0.502±0.002
5	500	0.983±0.002	0.8±0.001	0.771±0.001	0.761±0.001	0.77±0.001	0.748±0.002
6	1000	1.146±0.029	0.83±0.004	0.807±0.001	0.785±0.002	0.851±0.001	0.399±0.298

Table-8: Effect of compounds (1-5) on hypotonicity induced haemolysis.

Sr. No. Group ID	1	2	3	Mean ± SEM
G1	0.512	0.462	0.397	0.457 ±0.033
G2	0.361	0.302	0.276	0.313 ±0.025
G3	0.261	0.253	0.262	0.258 ±0.002
G4	0.176	0.212	0.163	0.183 ±0.014
G5	0.162	0.151	0.102	0.138 ±0.018
G6	0.331	0.342	0.321	0.331 ±0.006
G7	0.213	0.201	0.278	0.230 ±0.023
G8	0.095	0.126	0.112	0.111 ±0.008
G9	0.321	0.312	0.301	0.311 ±0.005
G10	0.243	0.213	0.252	0.236 ±0.011
G11	0.105	0.123	0.154	0.127 ±0.014
G12	0.331	0.327	0.304	0.32 ±0.008
G13	0.176	0.215	0.187	0.192 ±0.011
G14	0.111	0.102	0.134	0.115 ±0.009
G15	0.201	0.214	0.241	0.218 ±0.011
G16	0.143	0.163	0.132	0.146 ±0.009
G17	0.097	0.073	0.042	0.070 ±0.015

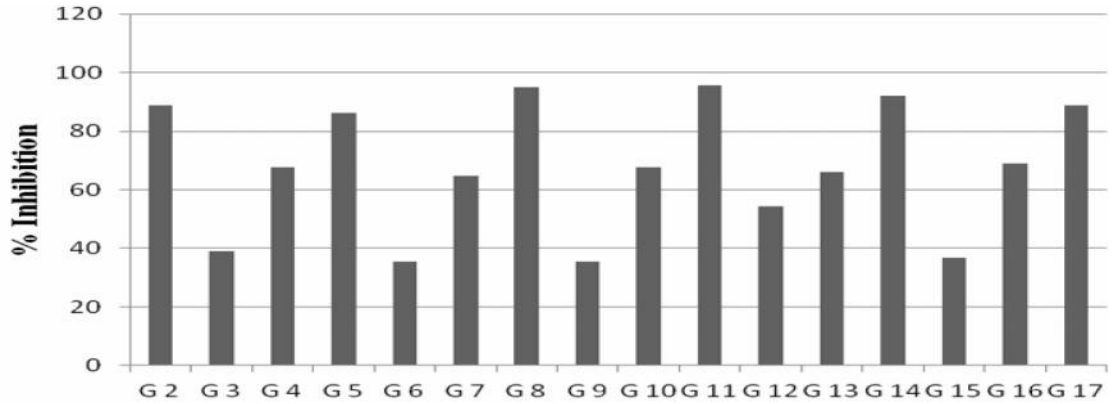


Fig. 4: The percentage inhibition of Analgesic effects (acetic acid-induced writhing method) in mice by different doses of compounds (**1**-**5**).

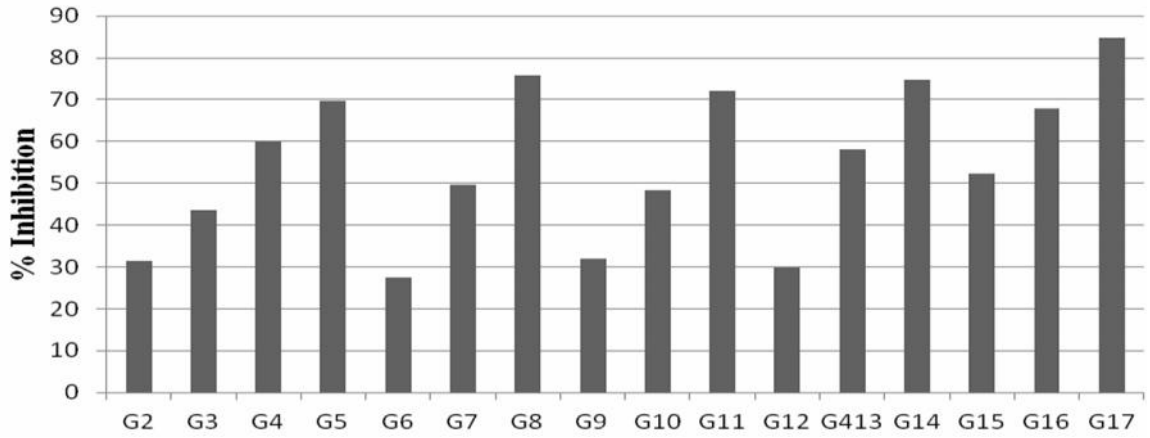


Fig. 5: The percentage inhibition of hemolysis by different doses of compounds (**1**-**5**) on hypotonicity induced haemolysis

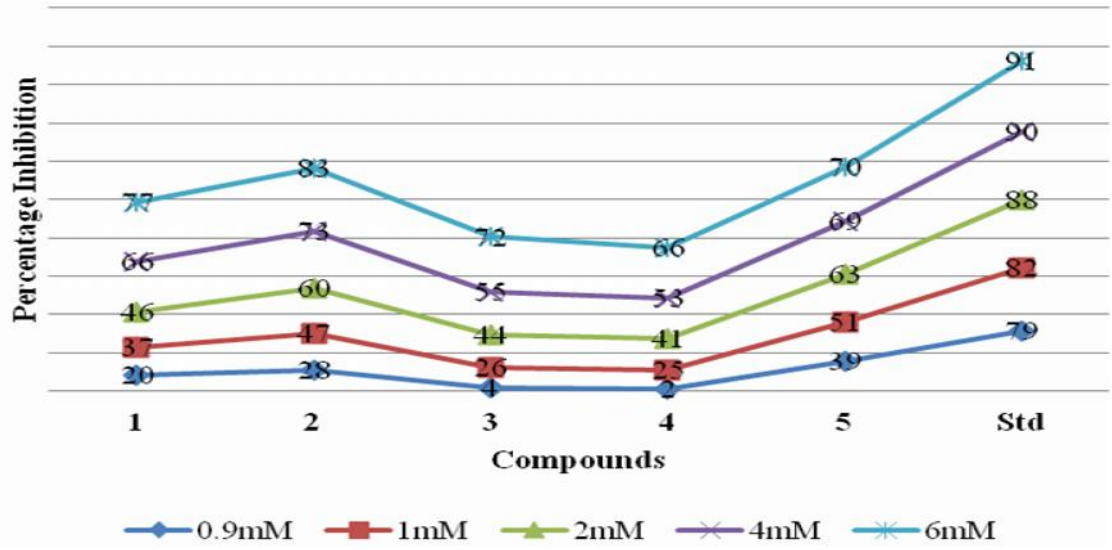


Fig. 6: Enzyme Inhibition Activity of Synthesized Compounds **(1-5).**

Molecular Docking Studies for Enzyme Inhibition Activity

Large shape complementarity scores were produced for all the synthesized compounds by the docking program indicating the formation of stable sulfonamide/enzyme complexes (Table-10). The molecular docked pose of the compound **5** is shown in Fig. 7.

The compound **5** sits suitably inside the binding pocket of the enzyme and is found to form three hydrogen bonds of moderate to weak bonding strength. One hydrogen bond is formed between sulfonamide O of the compound with OG of serine (287) of one chain (chain A) of protein (O(18)…H-O(OG): 2.92Å) the second hydrogen bond is formed between the other sulfonamide O and OH(3) of glycerol (604) (O(16)…H-O(3): 2.62Å) while the third hydrogen bond is formed between the same sulfonamide O with OH (2) of glycerol (604) (O(16)…H-O(2): 3.61Å). The results of experimental studies (Table-9) are reinforced by this computational analysis (Table-10).

Table-9: Percentage Inhibition (IC_{50}) of Synthesized Compounds.

Compound	IC_{50} (mM)
1	**1.24**
2	**3.13**
3	**0.99**
4	**2.4**
5	**3.49**

Table-10: Shape Complementarity Scores of 4-Aminophenazone Derivatives.

Compound	ACE	Shape Complementarity Score
1	**-306.22**	**4398**
2	**-274.26**	**4502**
3	**-271.88**	**4638**
4	**-281.54**	**4492**
5	**-365.41**	**4752**

ACE = Atomic Connection Energy

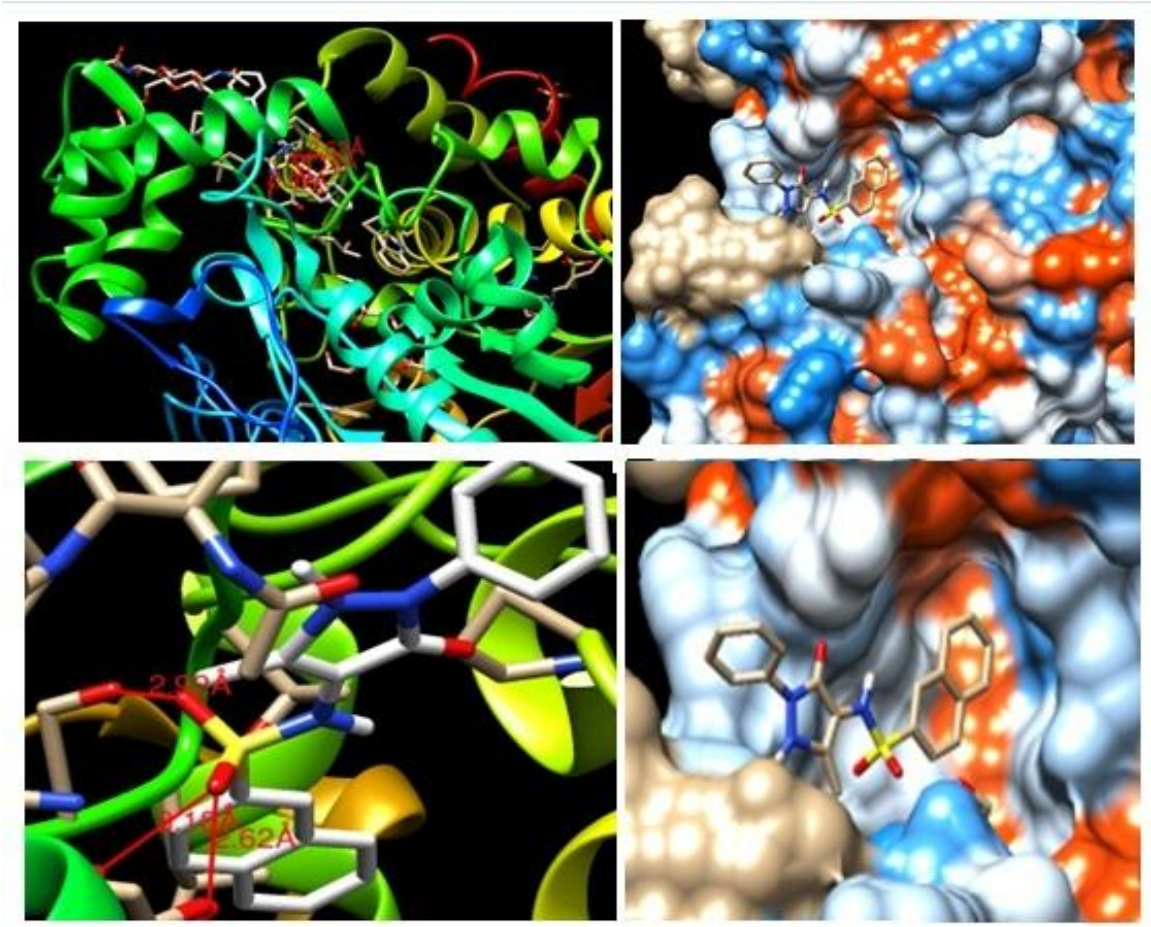


Fig. 7: Molecular Docked Structure of **5** with BuCHE

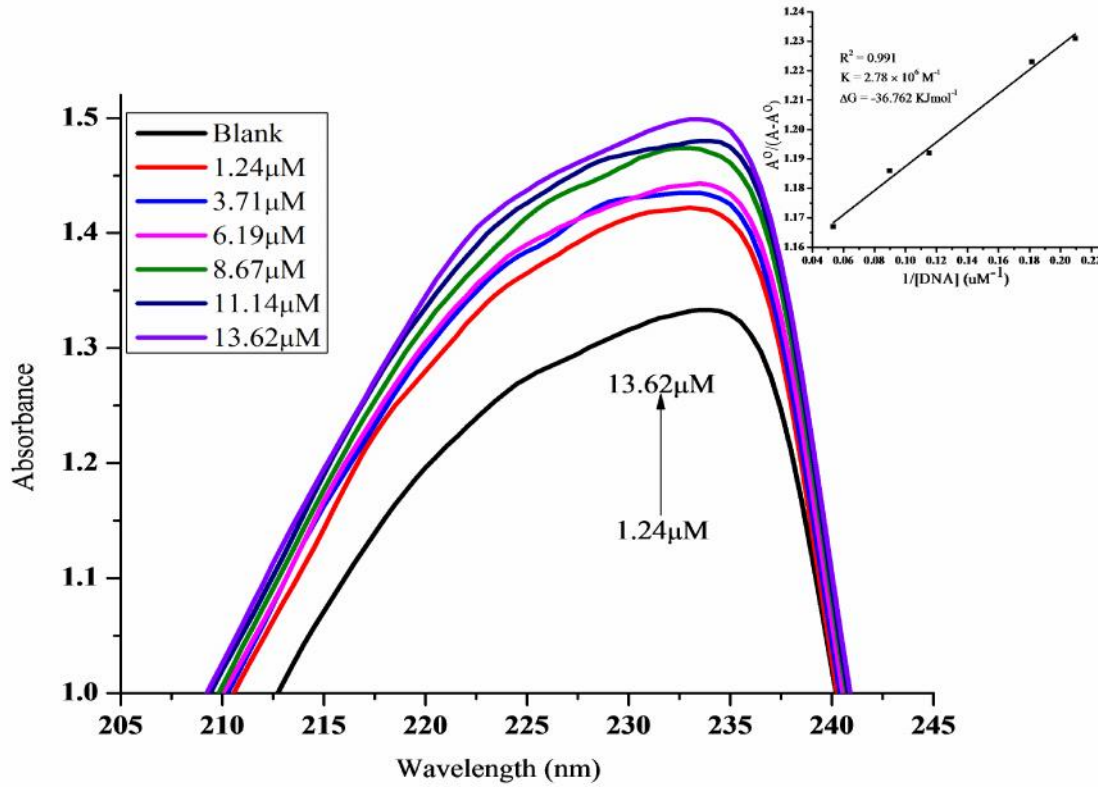


Fig. 8: Electronic spectra for compound 5 upon addition of increased concentration of CTDNA. Arrow heads indicate the change in absorption intensity upon increasing the DNA concentration. (Inset: Plot of $A_0/A\text{-}A_0$ versus 1/[DNA]).

DNA Binding Interactions

The synthesized compounds were tested for their DNA binding ability with the help of UV-Vis absorption method. Most of the compounds showed large positive *K* values providing clear evidence for the stability of sulfonamide/DNA complexes. *K* values were calculated with the help of Benesi-Hildebrand equation [42]. Negative values of Gibbs free energy (ΔG) as calculated by using the formula ΔG = -RTln*K* indicated the spontaneity of binding of sulfonamide with that of calf thymus DNA.

$$\frac{A_o}{A - A_o} = \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} + \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} \times \frac{1}{K[DNA]}$$

Table-11: DNA Binding Constant and Free Energy Values for the Synthesized Compounds.

Compound	Binding Constant (K)	Free Energy (ΔG)
1	1.1E+06	-34.465
2	8.12E+05	-36.067
3	9.5E+05	-34.10
4	Inactive	Inactive
5	2.78E+06	-36.762

A change in absorption intensity of the compound **5** was observed as shown in Fig. 8. Hyperchromic effect was observed along the series except for the compound **4**, which could not show remarkable DNA binding activity. The absorption spectra help in suggesting the groove binding of the compounds as hyperchromic effect is usually associated with the groove binding of the compounds and hypochromic effect may be associated with intercalative and/or groove binding [43].

Statistical Analysis

The results are expressed as means ± SEM. The difference between the assayed values of synthetic compounds was analyzed using one-way ANOVA method. Results with $P<0.05$ were considered as statistically significant, while those with $p<0.01$ were regarded as highly significant.

Conclusion

Drug development is considered as pronounced concept applied in therapeutic disciplines. Its effectiveness lies in its innovative approach regarding the development of efficacious, novel and safe compounds of biological importance. Keeping in

view all the parameters required for drug designing, a wide range of antibiotics have been introduced in the past. In order to improve efficacy with minimum side effects kept the search for modification of parent compounds ongoing. From the above discussion, it can be concluded that the synthesized sulfonamide derivatives possess significant enzyme inhibition, antibacterial, and analgesic as well as antioxidant properties. The results therefore encouraged that the clinical trials of these compounds must be experienced. However, further studies are needed to pin point the site and exact mechanisms of above compounds and to establish the safety and efficacy profile for FDA approval.

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