RESEARCH PAPER

Synthesis and Characterisation of Piperazine Based Ligands, Metal Complexes and Their In Vitro and In Vivo Activity

Rohan Phantosh Tembare[1*], Ritu M. Gilhotra[2], Prashant Kumar Dhakad[3]

[1,2,3]Gyan Vihar School of Pharmacy, Suresh Gyan Vihar University, Jaipur, Rajasthan 302017, India

Corresponding Author:

Rohan Phantosh Tembare, Gyan Vihar School of Pharmacy, Suresh Gyan Vihar University, Jaipur, Rajasthan 302017, India

Email: rohantembare18@gmail.com

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ABSTRACT

Cancer is not a contemporary affliction, with evidence tracing back to 3000 BC; yet, its comprehension and management have markedly progressed since the era of Hippocrates, particularly in the 21st century. Notwithstanding global advancements, cancer continues to pose a significant health challenge, resulting in 9.6 million fatalities in 2018 as reported by the World Health Organization, with an increasing prevalence in emerging nations such as India due to restricted healthcare access and delayed diagnosis. In India, oral and breast cancers are the most common, primarily due to tobacco consumption and insufficient awareness, resulting in elevated mortality rates. This study assesses the biological activity of synthesized compounds via in vitro antibacterial, antifungal, and anticancer assays, including MTT-based cytotoxicity on PC-3 cell lines. Additionally, in vivo anticancer efficacy was evaluated utilizing a rabbit xenograft model, accompanied by biochemical, histological, immunohistochemical, and pharmacokinetic assessments. The research seeks to ascertain the therapeutic potential and effectiveness of the optimized chemical in cancer therapy. The synthesized derivatives had modest antibacterial and antifungal activity, with substances S1 and S4 exhibiting consistent efficiency against several microbial strains. Of all compounds, S1 emerged as the most promising contender, exhibiting substantial in vitro anticancer activity with more cytotoxicity (80.71%) than the conventional medication Relugolix in PC-3 cell lines. In vivo studies corroborated the anticancer efficacy of S1, demonstrating diminished tumor volume, enhanced biochemical and histological markers, and a comparable pharmacokinetic profile with increased bioavailability, suggesting its viability as a therapeutic agent.

Keywords: In-vivo anticancer activity, In-vitro anticancer activity, Antibacterial activity, Antifungal activity, Piperazine Derivatives etc.

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Introduction

There are some misconceptions about cancer, one of them is that people often think that cancer is a disease of modern world. But there are a lot of records and evidence available that suggest that cancer has been present in the world since 3000 BC. Although, the word cancer was later given by the Greek physician Hippocrates in 460 − 370 BC. Some of the most reliable evidence are found in the human mummies in Egypt. When science started progressing in 16th century, perception towards cancer also started to change. When the modern microscope came in the world, scientist understood the real structure of cancer and this led to the surgical treatment of cancer. Gradually the treatment techniques improved over a long period and 21st century is proved to be the most remarkable time for treatment of cancer. Scientist developed more lifesaving treatment in the century such as robotic surgery and nanotechnology. Almost all countries have patients who suffer from cancer, but as far as statistics is concerned the prevalence of cancer is found higher in developed countries like U.S.A., Canada and Australia. But developing countries like China, India, and Bangladesh are not also far behind in this scenario. Countries with huge population are facing rapid growth in the number of cancer patients every year and the poor health infrastructure of these countries make this problem even worse. Experts believe that in most of developing countries, we never get the exact data of the sufferer of cancer as huge number of people do not have access to the government medical help. According to the data of World Health Organisation (WHO), cancer was responsible for almost 9.6 million deaths in 2018 and 70% of these deaths were found in the low or middle income countries or we can say in the developing or undeveloped countries. Countries like Australia, New Zealand, Ireland,

Hungary report highest rate of new cancer patients every year on per 100, 000 people. India is a huge country with a very dense population. Being a developing country, it is trying to provide healthcare facility to every citizen. Having said that, it cannot be denied that the government suffers to give high standard medical facilities to people. As far as cancer is concerned, India reported 1.1 million cases in 2020. There is a significant growth in number of cancer patients over the years in India. While WHO warned Indian government that in the near future one in every fifteen people in India will die of cancer and one in every 10 Indian will develop cancer in lifetime. In India, cancer of oral cavity causes 25% cancer cases. Oral cancer is the most common cancer in India, it accounts for 16% cancer patients in the country. The excessive use of tobacco is responsible for most of the oral cancer in India. Despite several awareness program conducted by government of India, oral cancer cases are growing day by day as the people keep on consuming tobacco and hence keep on getting caught by this deadly disease. Other most common cancer found in India is breast cancer, it accounts for 25% to 32% of female cancers in the country. Only 60% of women who suffer from breast cancer survive. The main reason of high death rate of this type of cancer is lack of awareness among the people. It is estimated that 50% of the breast cancer patients are diagnosed in stage 3 or 4. So lack of early diagnosis causes so many deaths due to cancer in India. As health infrastructure is improving day by day, it is believed that cancer patients will get appropriate treatment in the country. [1-4]

1. Materials And Method

1.2 Materials

- **Procurement of chemicals and solvents**

The required chemicals to carryout designed synthetic schemes were procured from local chemical supplier (Thermosil Fine Chem industries). All the chemicals were of laboratory grade. The starting chemicals i.e. tert-butyl piperazine -1-carboxylate and other chemicals and solvents such as DMF, 1 ,4 dioxane, dimethoxy boronic acid , sodium carbonate, HCl were procured.

- **Experimental animals**

The In-vivo study was conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India) guidelines and approved by Institutional Animal Ethics Committee (IAEC), - Invitox R and D Institute, Pune.

To carry out the study white albino rabbits weighing 2.5-3kg were used. The rabbits were purchased from verified local suppliers and kept in animal house at – Invitox R and D Institute, Pune. The temperature of the cage was maintained at 25°C. The rabbits were divided into 04 groups each containing 04 rabbits.

Animals required:

Table 1- Animals used in study

Species / Common name	Rabbits
Age / Weight / Size	2.5-3 kg
Gender	Male
No. to be used	4

2.1 Biological Activity

In vitro activity study [5-10]

Synthesis and Characterisation of piperazine based ligands, metal complexes and their in vitro and in vivo activity.

a) Antibacterial activity against different bacteria by Well plate diffusion Method is characterized against synthesized 10 derivatives.

The inoculum of the microorganism was derived from the bacterial cultures. Fifteen milliliters of nutrient agar (Hi Media) were dispensed into sterile Petri dishes and permitted to cool and harden. 100 µl of bacterial strain broth was pipetted and uniformly dispersed over the medium with a spreading rod until it dried completely. Wells with a diameter of 6mm were created using a sterile cork borer. Solutions of all substances (100, 200, 300 µg/ml) in DMSO were formulated. 100 µl of plant extract solutions was added to the wells. The Petri plates were incubated at 37°C for 24 hours. Streptomycin (1 mg/ml) was utilized as a positive control, while DMSO served as the negative control. The antibacterial activity was assessed by measuring the widths of the zones of inhibition, with all determinations conducted in triplicate.

b) Antifungal activity against Candida albicans and Aspergillus nigar

Antifungal efficacy Stock solution for antifungal efficacy: Each chemical for the antifungal investigation was solubilized in DMSO at a concentration of 5 mg/ml and refrigerated until further use. The antifungal activity of the compounds were assessed using the agar well diffusion assay. The assay was conducted following the methodology of Hufford et al. (1975). Sabouraud dextrose agar (Hi Media) was utilized for fungal cultivation. A media with an acidic pH (pH 5.5 to 5.6) and a reasonably high glucose concentration (40%) is created by combining SDA. Sabouraud dextrose and distilled water were autoclaved at 121°C for 15 minutes. Twenty-five milliliters of molten SDA medium at 450°C was aseptically placed into each sterile 100mm × 15mm Petri dish. Fungal spores were suspended in normal saline to achieve a volume of 1 ml and subsequently enumerated using a hemocytometer (Neubauer chamber). After the agar solidified, 8mm wells were created using a sterile cork borer. Subsequently, 0.1 ml (100 µl) from each stock solution of the compounds, with a final concentration of 5 mg/ml, was added to each well, and the plates were incubated for 24 hours at 29°C. Registered with the Ministry of Micro, Small and Medium Enterprises (MSME): UAN-MH29D0010740 Registered with CPCSEA under the number 2114/PO/Re/S/20/CPCSEA Each petri dish had two wells supplemented with DMSO and a reference antifungal agent. Streptomycin (1 mg/ml) dissolved in DMSO serves as the negative and positive control, respectively. The antifungal activity was quantified by the diameter (mm) of the clear zone indicating growth inhibition.

c) In vitro anticancer activity [11-16]

MTT assay: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. PC-3 human prostate adenocarcinoma cells were cultured in RPMI-1640 media at 37ºC with 5% CO2 in a humidified incubator for 6-7 hours. Cells were extracted, enumerated (3×10^4 cells/ml), placed to a 24-well plate, and incubated for 24 hours before the introduction of the test chemical. Serial dilutions of test samples were created by dissolving compounds in DMSO and subsequently diluting with RPMI-1640 medium to achieve a final concentration of pure medication and optimum formulation of 120 µl. Stock solutions of the samples were prepared. Ten microliters of sample and ninety microliters of cell lines were cultured for seventy-two hours. MTT solution at 5 mg/ml was diluted in 1 ml of Phosphate Buffer Solution (PBS), and 10 µl of this solution was added to each of the 24 wells. The wells were encased in aluminum foil and incubated at 37ºC for four hours. The solution in each well containing medium was unbent MTT, and dead cells were removed using suction, followed by the addition of 150µl of DMSO to each well. The optical density was measured with a microplate reader (spectrophotometer) at 595 nm. DMSO utilized as a control. Controls and samples were evaluated and repeated for each concentration, with three repetitions for each cell line. Following a 24-hour incubation of test materials, cytotoxicity on the cancer cell lines was assessed with the MTT assay. The cytotoxicity was determined by comparing the absorbance of the samples to that of the control. The data were subsequently employed to determine the concentration of synthetic product necessary to achieve a 50% reduction (IC50) in growth (cell number) in PC-3 cell lines.

Cell viability (%) = Mean OD/Control OD x 100

d) In vivo anticancer activity [17]

Animals: - Induction and Treatment of Human Prostate Carcinoma Epithelial Cell Line

The experimental design using CWR22Rr1 cell tumor xenografts entailed detaching the cells from culture dishes via trypsinization, followed by collection, washing, and resuspension in RPMI 1640. CWR22Rr1 cells were utilized to develop a rabbit model. We established a chemically induced CWR22Rr1 cell animal model (IRDI/IAEC/M02/07/2024-25). This model utilized 24 male albino rabbits, each weighing between 2-3 kg, acquired by a modified induction technique. CWR22Rr1 cells were started by an injection of s.c. with 1 × 10^6 CWR22Rr1 cells combined with 50 µL RPMI and 50 µL Matrigel

(Collaborative Biomedical Products, Bedford, MA) in the right and left flanks of each rabbit. administered at a dosage of 0.5 mL/kg of body weight, as previously delineated, dissolved in a normal saline solution. Subsequent to initiation, all rabbits underwent a 3-day fasting period followed by a single day of re-feeding, serving as a mitotic proliferative stimulus. In vivo research aim to replicate authentic human scenarios, positing that individuals are more inclined to ingest up to S1 (120mg/kg). Body weight, dietary intake, and hydration levels were documented biweekly during the study. Upon the initiation of xenograft growth, their dimensions were assessed biweekly. The tumor volume was determined using the formula

Tumor Volume = 0.5238 × L1 × L2 × H

L1 represents the long diameter, L2 represents the short diameter, and H represents the height of the tumor. This formula is developed from an equation for computing the volume of a hemi-ellipsoid, the geometric object that most closely approximates the shape of tumors. one week post-Diethyl nitrosamine (DEN) administration, rabbits received six doses of prostate cancer epithelial cells for a duration of six weeks to facilitate the progression of prostate carcinoma. Following the induction of CWR22Rr1 cell tumor xenografts for 6 weeks, the treatment continued for an additional 6 weeks.

Group 1: Non-induced rabbits (control group).

Group 2: CWR22Rr1 cells induced rabbits.

Group 3: CWR22Rr1 cells induced rabbits treated with tab methotrexate (2.5 mg/ kg).

Group 4: CWR22Rr1 cells induced rabbits treated with S1 (120mg/kg)

In the control group (Group 1), animals received distilled water (5 mL/kg body weight) and underwent a single saline injection during the trial period. Relugolix was obtained as API at 98% purity. S1 combinations were administered via gavage at a daily dosage of 120 mg/kg, as previously documented.

After one week of DEN treatment followed by six weeks of 2-Acetylaminofluorene (2-AAF) administration, rabbits were subjected to anti-cancer treatment during six weeks. At week 13, blood samples were collected through marginal ear vains and animals were then sacrificed.

The body weights of rabbits were weekly recorded. Ratio of total body weight changes (%) = (Final weight–Original weight)/Original weight × 100.

Liver index (%) = Liver weight/Final body weight × 100.

- **Evaluation of Serum Biochemical Parameters [18-19]**

  Blood samples were obtained at the time of sacrifice in serum tubes (vacutainer) and centrifuged at 3000 rpm for 20 minutes at 4 ◦C to isolate serum. The serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), total cholesterol (TC), triglycerides (TG), conjugated bilirubin, C-reactive protein (CRP), interleukin-6 (IL-6), lactate dehydrogenase (LDH), and protein induced by vitamin K absence-II (PIVKA-II) were assessed utilizing ELISA kits for rabbits procured from BioSource USA, adhering to the protocols outlined in the internal kit documentation.

- **Evaluation of Liver Homogenate Biomarkers [20]**

  Liver tissues were homogenized with a buffer (1:10, w/v) comprising 100 mM KCl, 100 mM potassium buffer (pH 7.4), and 1 mM EDTA for 90 seconds, followed by centrifugation of the homogenates at 10,000× g for 30 minutes at 4 ◦C to get supernatants. The supernatants were utilized to quantify the concentrations of glutathione (GSH) and the liver cancer biomarker α-fetoprotein (AFP) employing ELISA rabbit kits obtained from Preclinical Bio Lab, Pune, in accordance with the kit instructions. The protein concentration of the aforementioned supernatant was determined using the method developed by Lowry et al.

- **Histopathological Staining [21- 22]**

  Liver tissue specimens preserved in 10% neutral-buffered formalin were dried with ethanol, cleaned with xylene, and embedded in paraffin to create tissue blocks. The specimens were sectioned (4–5 μm), and the slides were either stained with hematoxylin and eosin (H & E) or utilized for immunostaining.

- **Immunohistochemistry [23-24]**

Immunohistochemical staining for Ki67 was conducted using the Benchmark GX machine with Standard Cell Conditioning (CC1) on 4 µm paraffin sections mounted on positively charged slides, incubated for 1 hour at 60 °C. Sections were treated for 16 minutes at 37 °C with the pre-diluted primary antibody, rabbit anti-Ki-67 (clone 30-9). The antigen-antibody interaction was identified using 3, 3-diaminobenzidine (DAB) with an Ultra View Universal DAB Detection Kit. The sections were counterstained with Hematoxylin for 4 minutes, dehydrated in a graded series of ethanol, cleaned with xylene, and mounted with DPX.

- **Pharmacokinetic study**
  A pharmacokinetic research was conducted on the consumption of STD and the optimized medication.

2. Results

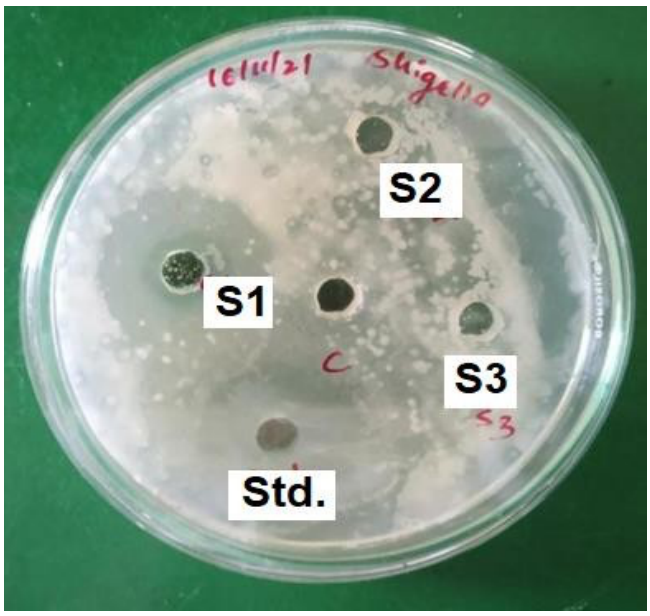
2.1 Biological Activity

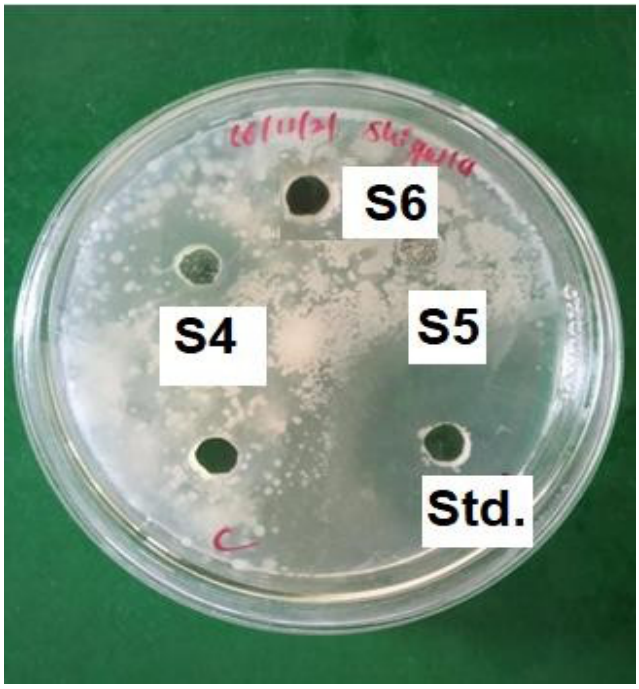
2.1.1 Antibacterial activity

Table 2. Antimicrobial Activity of 10 derivatives against *Shigella*

Sr. No	Samples	Concentration (mg/ml)	Zone In Diameter (mm)
1	Control	-	0
2	Standard (Streptomycin)	1	20
3	S1	1	17
4	S2	1	-
5	S3	1	-
6	S4	1	9
7	S5	1	-
8	S6	1	
9	S7	1	
10	S8	1	
11	S9	1	
12	S10	1	

Result: At the concentration 1 mg/ml, the SAMPLES S1, S4 showed moderate activity against *Shigella*


S2
S1
S3
Std.



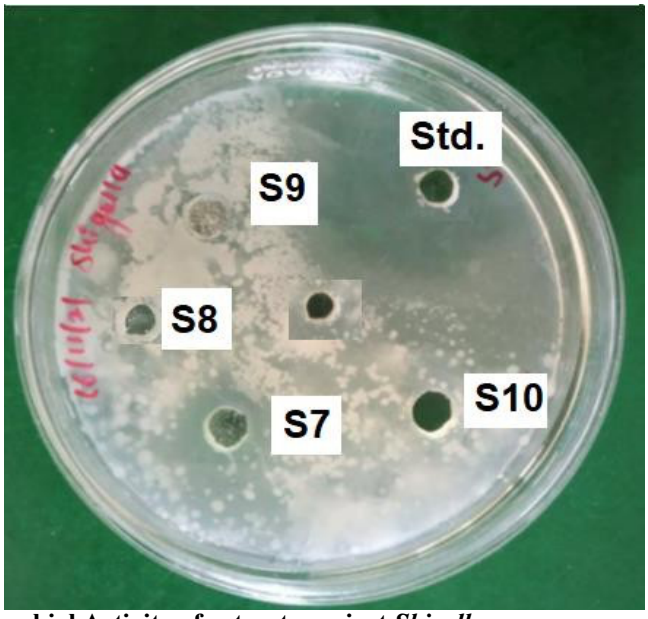


Figure 1. Antimicrobial Activity of extracts against *Shigella*

Table 3. Antimicrobial Activity of 5 extracts against *E. coli*

Sr.No	Samples	Concentration (mg/ml)	Zone In Diameter (mm)
1.	Control	-	0
2.	Standard (Streptomycin)	1	19
3.	S1	1	13
4.	S2	1	-
5.	S3	1	-
6.	S4	1	9
7.	S5	1	-

Result: At the concentration 1 mg/ml, the samples S1, S4 showed moderate activity against E.coli

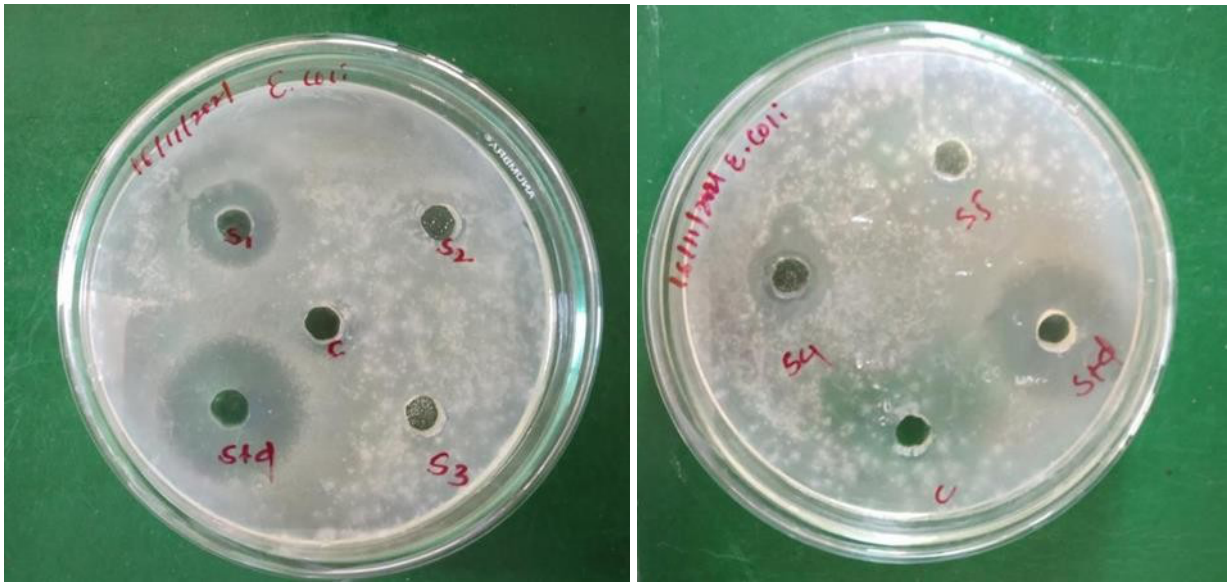


Figure 2. Antimicrobial Activity of 5 extracts against *E. coli*

Table 4. Antimicrobial Activity of 5 extracts against *Bacillus subtilis*

Sr.No	Samples	Concentration (mg/ml)	Zone In Diameter (mm)
1.	Control	-	0

2.	Standard (Streptomycin)	1	29
3.	S1	1	16
4.	S2	1	-
5.	S3	1	-
6.	S4	1	7
7.	S5	1	-

Result: At the different concentrations sample S1, S4 showed moderate activity against ***Bacillussubtilis***

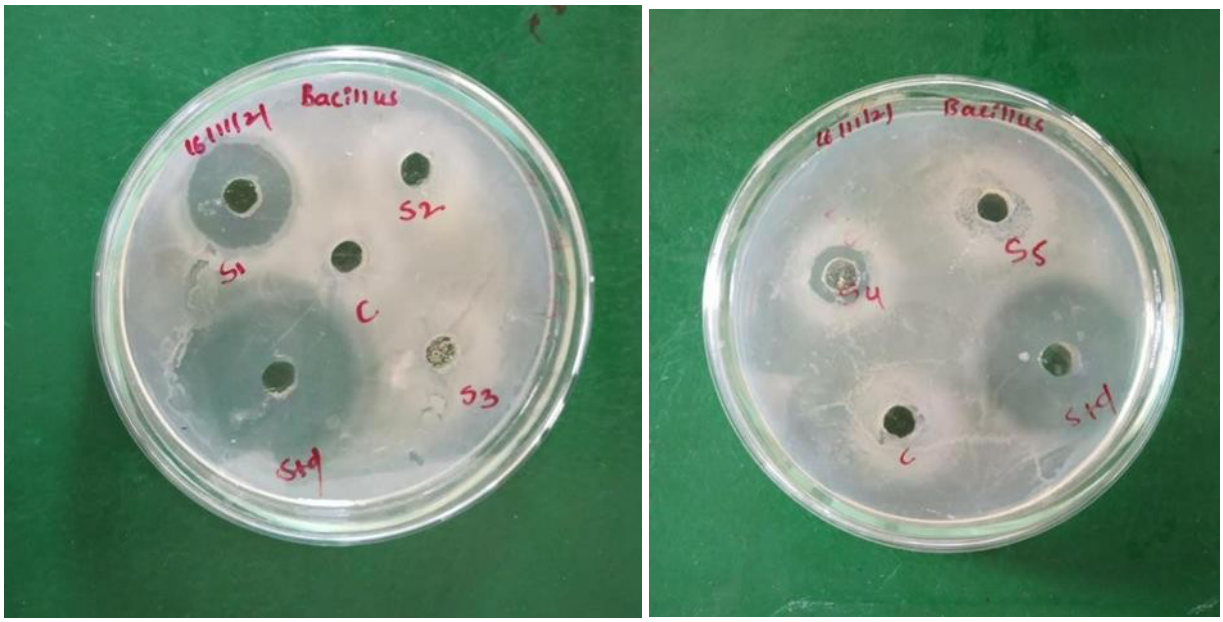


Figure 3. Antimicrobial Activity of 5 extracts against *Bacillus subtilis*

Table 5. Antimicrobial Activity of 4 extracts against *Styphilococus aureus*

Sr.No	**Samples**	**Concentration (mg/ml)**	**Zone In Diameter (mm)**
1.	Control	-	0
2.	Standard (Streptomycin)	1	22
3.	S1	1	14
4.	S2	1	-
5.	S3	1	-

6.	S4	1	7
7.	S5	1	-

Result: At the different concentrations sample S1, S4 showed moderate activity against **Styphilococus aureus**

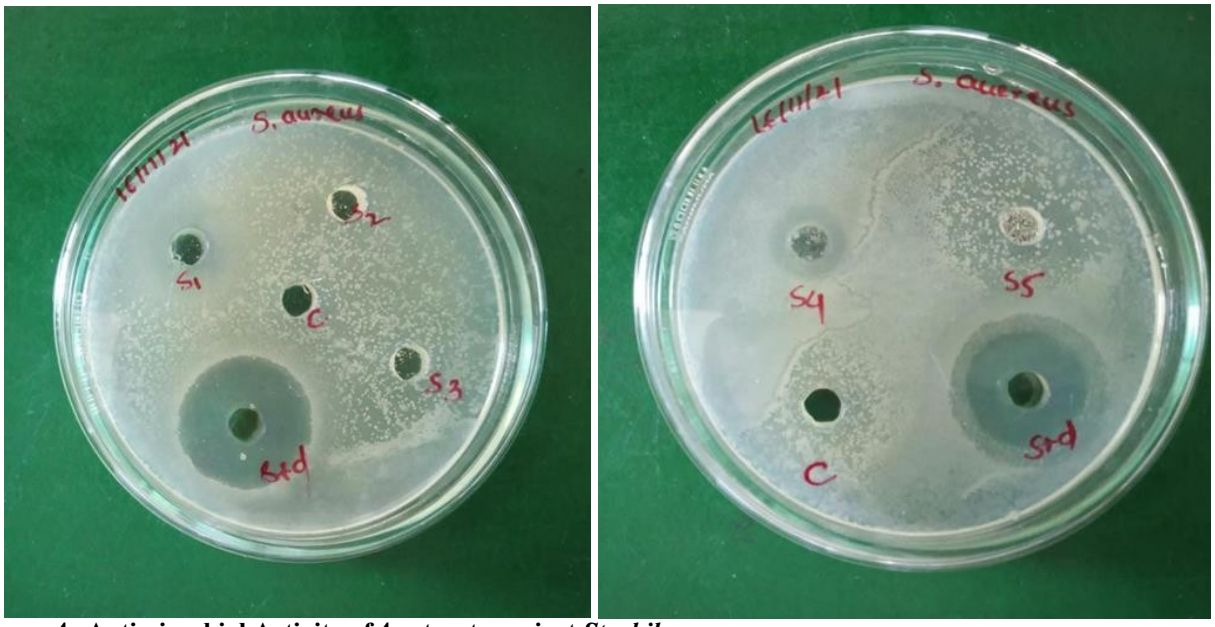


Figure 4. Antimicrobial Activity of 4 extracts against *Styphilococus aureus*

2.1.2 Antifungal activity

Table 6. Antifungal Activity of Synthetic compounds against candida albicans and aspergillus nigar

Sr.No	**Samples**	**Conc. (mg/ml)**	**Zone In Diameter (mm) against *Candida albican***	**Zone In Diameter (mm) against *Aspergilllus nigar***
1.	Control	-	00	00
2.	Standard (Streptomycin)	1	16	17
3.	S1	1	08	06
4.	S2	1	06	07

5.	S3	1	01	06
6.	S4	1	04	03
7.	S5	1	04	04

Conclusion: The antifungal activity of the synthetic compounds studied on candida albicans, in which compounds showed moderate activity.

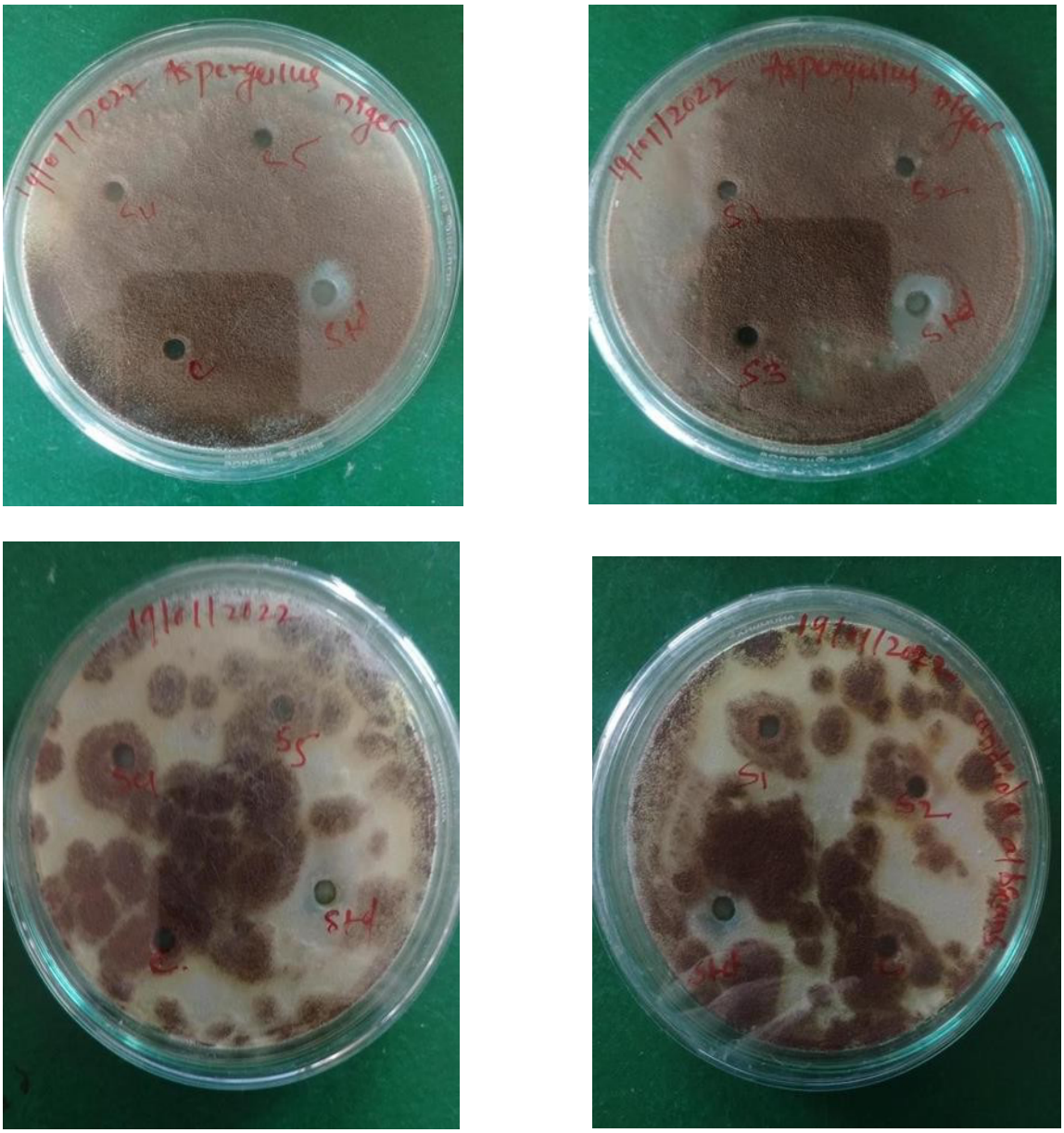


Figure 5. Zone of inhibition – Antifungal activity

A total of 10 derivatives were produced and subsequently put to first antimicrobial screening to assess their biological efficacy of these 5 derivatives had notable antibacterial activity, suggesting that particular structural characteristics within this group enhance biological efficiency.

Synthesis and Characterisation of piperazine based ligands, metal complexes and their in vitro and in vivo activity.

The preliminary antimicrobial screening served as a first filter, facilitating the exclusion of less effective compounds and concentrating subsequent research on the most promising possibilities. Among these five active derivatives, one compound was chosen as the lead molecule due to its somewhat superior activity profile. The chosen derivative was subsequently assessed for anticancer efficacy, exhibiting significant cytotoxic potential. The justification for choosing this compound is based on: Its superior biological activity relative to other derivatives. The potential for similar or overlapping pathways between antibacterial and anticancer activity, such as interactions with cellular targets, DNA, or enzymes.
Its capacity to function as a lead molecule for dual biological action.
The sequential screening method—from 10 synthesized derivatives to 5 active antimicrobial agents, culminating in 1 leading anticancer candidate—guarantees a targeted, efficient, and logical drug discovery process, reducing resource expenditure while enhancing the likelihood of discovering a potent therapeutic agent.

2.1.3 In vitro anticancer activity

From MTT assay, after treatment with various concentrations of Relugolix (120µg/ml) parameters like percentage cell viability, percentage cytotoxicity was compared with untreated (control) cells. Decrease in cell viability and increase in cytotoxicity by Relugolix was observed in PC-3 cell line in dose manner, but a significant decrease in cell viability ($p < 0.001$) was observed for dose Pure drug Relugolix (120µg/ml) & S1 (120µg/ml) produced significant growth inhibition. The growth inhibitory activity of S1 was more significant as the concentration of pure drug Relugolix (120µg/ml) & S1 (120µg/ml) increases in case of PC-3 cell lines.

Table 7. In vitro cytotoxic activity of pure drug Relugolix (120µg/ml) & S1 (120µg/ml) on PC-3 cell line

Concentration of Relugolix (µg/ml)	**% of cell viability**	**% of cytotoxicity**
Control	100	-------
Pure drug Relugolix (120µg/ml)	30.872	69.128
S1 (120µg/ml)	19.985	80.715

2.1.4 In vivo anticancer Activity

- **Effect of S1 Treatment on CWR22Rr1 cells Induced rabbits**

In the two-stage CWR22Rr1 cells induction model protocol, initiation and promotion steps are important in developing CWR22Rr1 cells where promotor induces clonal expansion of initiated cells. To improve CWR22Rr1 cells development in animal models, exposure to a tumor promoter, such as 2-acetylaminofluorene (2-AAF), often helps inducing the formation of altered hepatocytes foci (AHF) and hyperplastic nodules that would ultimately develop into CWR22Rr1 cells . Fasting-refeeding and employing 2-AAF after 2 weeks of using DEN are reported as mitotic proliferative stimuli. In the present model, initiation is followed by a mitotic proliferative stimulus (fasting and re-feeding) during treatment with promoting agent such as 2-Acetyl Amino fluorene (2-AAF) that induces selective proliferation of the initiated cell population over non-initiated cells in the target tissue. Although feed deprivation for three days is considered as a severe stress and itself may lead to body weight loss, it reduces the death that results from other models, such as cutting a piece of the liver, and we have established this model in our previous experiments to induce liver cancer.

- **Effect of S1 on Liver and Body Weights**

Changes in total body weights (TBWs) for the non-induced (Group 1) and CWR22Rr1 cells induced (group 2) rabbits were recorded from week 1 to week 6 of the experimental period (Table). TBWs were significantly ($p <$

0.05) decreased in the CWR22Rr1 cells rabbits as compared to the control group (Table). After 6 weeks post-CWR22Rr1 cells induction, the ratio of total body weight increase was 40.51% in the control group and only 28.16% in the CWR22Rr1 cells group. CWR22Rr1 cells induced a significant weight loss compared to the control.

Table 8. Body weight (grams) and ratios of body weight changes (%) during 6 weeks after CWR22Rr1 cells induction in different studied groups.

Variables	Group 1 (Control)	Group 2 (CWR22Rr1 cells)
Total body weight at 1st week (grams)	2524.15± 6.85	2457.63 ± 11.69
Total body weight at 2nd week (grams)	2578.25± 8.18	2311.25 ± 9.17
Total body weight at 3rd week (grams)	2644.11 ± 12.78	2654.44 ± 13.85
Total body weight at 4th week (grams)	2847.55 ± 22.16	2847.58 ± 12.82
Total body weight at 5th week (grams)	2945.22 ± 25.03	2947.29 ± 18.94
Total body weight at 6th week (grams)	3021.78 ± 31.38	2987.96 ± 22.05
Ratio of total body weight changes (%)	19.71	21.57

Data were expressed as mean ± standard deviation. Significance was made using one-way ANOVA test followed by least significant test. a: significance versus Group 1. Significance at $p < 0.050$.Animal treatment started at week 7. The initial TBWs in CWR22Rr1 cells HCC, G3, G4 groups were significantly decreased versus control group (G1) ($p < 0.05$) (Table). Meanwhile, the final TBWs were significantly increased upon treatment with Tab methotrexate (G3), S1 (G4). The TBW increase was comparable to the normal group for all treatments and significantly higher than that of the non-treated group ($p < 0.05$) (Table). Anti-tumor treatments succeeded to restore the animal's body weight at week 13. The liver index showed no significant changes in the different studied groups ($p < 0.05$). Meanwhile, as compared to the control animals (Table)

Table 9. Changes in animal body

Data	Group 1 (Control)	Group 2 (CWR22Rr1 cells)	Group 3 (Induced CWR22Rr1 cells + Tab. methotrexate)	Group 4 (Induced CWR22Rr1 cells CWR22Rr1 cells CWR22Rr1 cells + S1)
Initial body weights at 7th Week	3028.54± 4.12	3012.44 ± 10.25	3011.47 ±6.56	2988.26 ± 10.47
Final body weights at 13th week	3112.45 ± 31.38	3204.69 ± 22.05	3108.14 ± 5.26	3159.26 ± 4.87
Ratio of total increase of body (%)	19.71	21.57	19.88	24.49
Liver weights (grams)	22.14 ±1.47	22.92 ± 1.47	22.65 ± 1.74	21.55 ± 1.65

Liver index (%)	0.73 ±0.78	0.76 ± 1.05	0.72 ± 1.11	0.77 ± 1.23

- **Effect of S1 on Biochemical Parameters**

Liver function tests were analyzed for the different studied groups (Table). The serum levels of ALT, AST, ALP, and conjugated bilirubin were significantly increased upon CWR22Rr1 cells induction (G2). Treatment with Relugolix and to a lesser extent with Relugolix combination could reduce the enzymatic activity to levels close to those of normal animals. The treatment with Relugolix was more effective in restoring the serum level of liver enzymes. Treatment with Relugolix partially reduced these activities whereas adding Relugolix improved its ability to recover serum enzymes normal levels.

The fact that CWR22Rr1 cells elevated liver enzymes ALT, AST, and ALP is indicative of a liver damage upon CWR22Rr1 cells induction. Treatment with Relugolix alone or combined with Relugolix reduced efficiently the elevated liver enzyme's levels compared to Relugolix alone. A similar effect was observed for total protein concentration that increased for CWR22Rr1 cells induced animals and was restored totally for Relugolix treatment and partially for the other treatments. Total cholesterol and triglycerides, CRP, LDH and IL-6 serum levels in CWR22Rr1 cells animals significantly increased indicating clear liver damage and inflammation induction. Relugolix alone or combined with Relugolix markedly reduced concentrations of those markers compared to control levels. Administration of Relugolix alone significantly deceased the levels but in a less effective manner as compared to Relugolix or Relugolix mix. Expectedly, the levels of the Serum tumor marker PIVKA-II, liver tumor marker AFP and oxidation stress enzymes (GSH and MDA) significantly increased upon CWR22Rr1 cells induction. The various treatments attenuated the levels of PIVKA-II that remained however higher than the control. S1 displayed a better performance than Relugolix PURE DRUG alone in attenuating tumor makers and oxidation parameters. The Relugolix AND S1 succeeded to lower conjugated bilirubin, LDH and AFP as compared to Relugolix alone ($p < 0.05$) (Table).

- **Histopathological and Immunostaining Changes Induced by Relugolix H & E stain of the control group displayed a normal liver structure.**

However, CWR22Rr1 cells HCC Animals exhibited a marked liver histological damage indicated by wide distribution of tumor cells and nodules, hemorrhage, angiogenesis, or vascular invasion, hyper cellularity, and lymphocytic infiltration in many places (Figure). The tumor nodules were of trabecular and solid patterns with irregular demarcation, basophilic and coagulative cytoplasm. Many tumor nodules were vascularized. Clear cells variant of tumor nodules, cytologic atypia, mitotic figures, Mallory-bodies within the tumor cells, and unpaired arteries were noticed. Reduced number of portal triads and bile ducts were also noticed. Bile production was frequently observed. The clear-cell variant of CWR22Rr1 cells HCC, characterized by clear cytoplasm was observed in some parts of the tumor. The tumor cells often have an increased cell size but show regular nuclei without atypia.

Table 10. Measured parameters in different studied groups

Data	**Parameters**	**Group 1**	**Group 2 (CWR22Rr1 cells)**	**Group 3 (Induced CWR22Rr1 cells + Relugolix pure drug)**	**Group 4 (Induced CWR22Rr1 cells + S1)**
Serum	ALT (U/L)	16.99 ± 3.98	79.80 ± 10.35	21.00 ± 5.66	37.00 ± 4.80
	AST (U/L)	20.36 ± 4.72	107.10 ± 8.96	26.00 ± 7.38	55.20 ± 25.37
	ALP (U/L)	43.80 ± 3.56	134.40 ± 14.47	45.20 ± 6.34	98.40 ± 22.07

	TP (mg/mL)	6.91 ± 0.78	10.98 ± 0.95	7.52 ± 0.76	9.36 ± 0.48
	Conjugated bilirubin (mg/dL)	0.31 ± 0.05	1.81 ± 0.31	0.38 ± 0.13	0.98 ± 0.08
	TC (mg/dL)	121.20 ± 12.99	254.40 ± 35.56	124.60 ± 8.08	197.80 ± 11.58
	TG (mg/dL)	73.80 ± 3.11	144.00 ± 38.97	76.20 ± 4.15	118.60 ± 9.37
	CRP (mg/dL)	7.24 ± 1.27	26.60 ± 6.11	10.92 ± 3.46	19.38 ± 2.88
	IL-6 (pg/mL)	4.99 ± 0.79	23.34 ± 5.53	6.16 ± 1.43	14.75 ± 1.77
	LDH (U/L)	158.00 ± 19.20	448.40 ± 83.25	214.00 ± 43.78	337.20 ± 93.08
	Serum tumor marker PIVKA-II (mAU/mL)	3.50 ± 0.55	17.34 ± 2.05	7.34 ± 1.63	13.36 ± 1.24
Tissue homogenates	GSH (ng/mg proteins)	17.90 ± 5.37	2.08 ± 0.65	10.84 ± 2.07	5.82 ± 2.53
	MDA (nmol/mg proteins)	0.40 ± 0.11	1.63 ± 0.31	0.77 ± 0.23	1.19 ± 0.23
	Homogenate tumor marker AFP (ng/mg proteins)	13.64 ± 2.71	72.12 ± 10.87	22.84 ± 2.18	52.60 ± 10.16

Data were expressed as mean ± standard deviation. Significance was made using one-way ANOVA test followed by least significant test. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: alkaline phosphatase; TP: total protein; TC: total cholesterol; TG: triglyceride; CRP: C-reactive protein; IL-6: interleukin-6; LDH: lactate dehydrogenase; PIVKA-II: Protein induced by vitamin K absence-II; GSH: glutathione; MDA: malonaldehyde; AFP: α fetoprotein. a: significance versus Group 1. b: significance versus Group 2; c: significance versus Group 4. Significance at $p < 0.050$.

However, when rats were treated with Relugolix pure drug methotrexate, S1, the hypercellularity and tumor cells and nodules, as well as hemorrhage and vascular invasion were markedly reduced. In the S1 group particularly, the cytoplasm was eosinophilic, and the portal areas reappeared. The tumor nodules reduced in number and size with increased apoptotic cells. However, the S1 group distinctly exhibited extensive fat vesicles in the liver tissue. The cytoplasm was basophilic in many areas with marked reduction in tumor nodules. In the animals treated with the (S1) the liver tissue exhibited color differentiation, marked reduction in tumor nodules with increased apoptosis and necrosis and decreased number of fat vesicles. Sinusoids reappeared between hepatocytes strands.

- **Immune Histochemical Staining of Ki67**

  Ki-67 protein in the nucleus is associated with cell proliferation. In CWR22Rr1 cells HCC patients, high levels of Ki-67 are usually indicative of tumor aggressiveness such as an advanced tumor stage. Ki-67 was therefore proposed as an independent prognostic factor for surgically resected CWR22Rr1 cells HCC. Our results show increased Ki67 expression in CWR22Rr1 cells HCC animals compared to the other groups (Figure).

- **Average Tumor Volume**

At the end of the experiment, the mice were euthanized, tumors were excised from the mice and weighted. The tumor volumes (Table) were measured according to the formula below:

Table 11. The tumor volume of Tumor cancer cells in the four treatment groups

Group	Tumor Volumes
Negative control group	18.47 ± 1.8 cm^3
Positive control group	2.14 ± 0.8 cm^3
Induced CWR22Rr1 cells + Relugolix	6.31 ± 1.48 cm^3
Induced CWR22Rr1 cells + S1	2.63 ± 1.78 cm^3

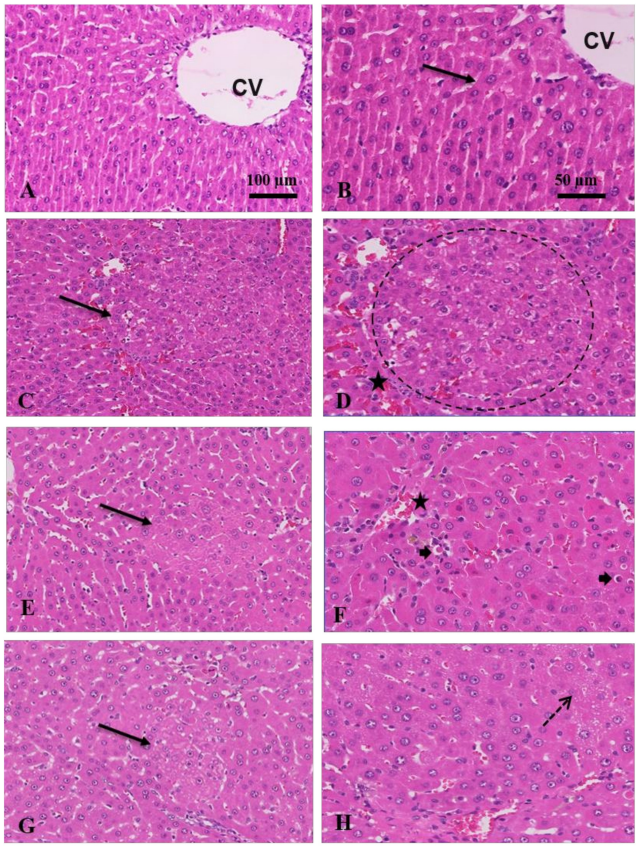


Figure 6. Anti-tumor effect of treatment: (A) control, untreated shows normal hepatocytes; (B) intact cell membrane (arrow), and central vein (CV); (C) CWR22Rr1 cells , a moderate increase in cell density (arrow); (D) wide distribution of haemorrhage (star) and tumor nodules (circle) with >3 cell strand thickness; (E) methotrexate, eosinophilic cytoplasm with marked reduction in tumor nodules (arrow), cellular density; (F) and haemorrhage (star), increase in apoptosis (arrowheads); (G) S1 reduced tumor nodules (arrow); (H) with marked increased in fat vesicles or steatosis (dot arrow)

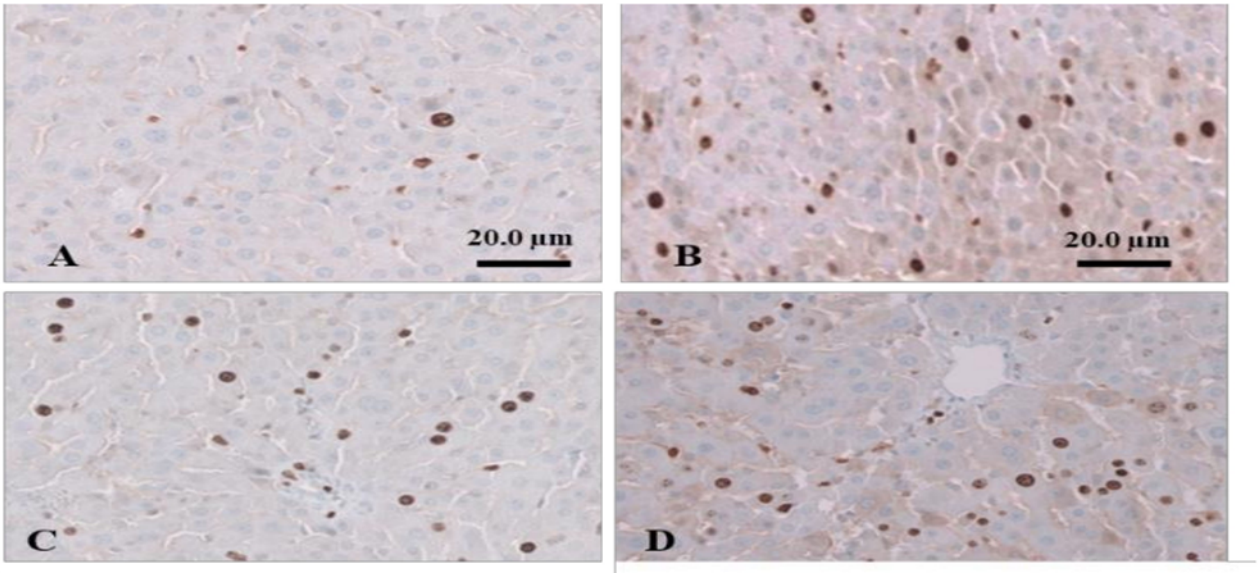


Figure 7. Antitumor effect of methotrexate and S1 on cell proliferation. Representative images of immunohistochemical staining with Ki-67 in control group (A). CWR22Rr1 cells group (B). Pure drug Relugolix -treated group (C). S1 -treated group (D), and S1 treated group shows the quantitative analysis of Ki-67 immunoreactive cells in 10 fields of each section of the Ki-67 positive foci and quantitative region analysis of the Ki-67–positive foci ×100 magnification (Scale bar 20 µm). The values were evaluated by one-way ANOVA followed by Dennett's t-test compared to the CWR22Rr1 cells - induced group. Data are represented as mean ± SEM. The letter (a) is used for $p < 0.05$ vs. control group and the letter (b) is used for $p < 0.05$ vs. CWR22Rr1 cells group.

In-vivo bioavailability of drug from synthesized entity in comparison with pure drug in experimental animal.

Tissue distribution of drug from synthesized entity in comparison with pure drug in experimental animal.

- **PK data analysis**

Plasma concentration vs. time data of Relugolix was analysed by Pk solver version 2.0 to derive various pharmacokinetic parameters, viz., AUC0-t, AUC0-∞, Cmax, tmax and t½.

Table 12. PK data analysis

Standard	Relugolix API
AUC Calculation Method	Linear Trapezoidal

Table 13. Summary Table- Input Variable

Time In Hr	Plasma Drug Conc. Mcg/ml	Log Plasma Drug Conc. Mcg/ml
0	0	0
0.15	4.12	0.614897216
0.5	3.22	0.507855872
1	12.47	1.095866453
2	10.48	1.020361283
4	9.55	0.980003372
8	13.57	1.132579848
12	11.48	1.059941888
20	10.25	1.010723865
24	8.44	0.926342447

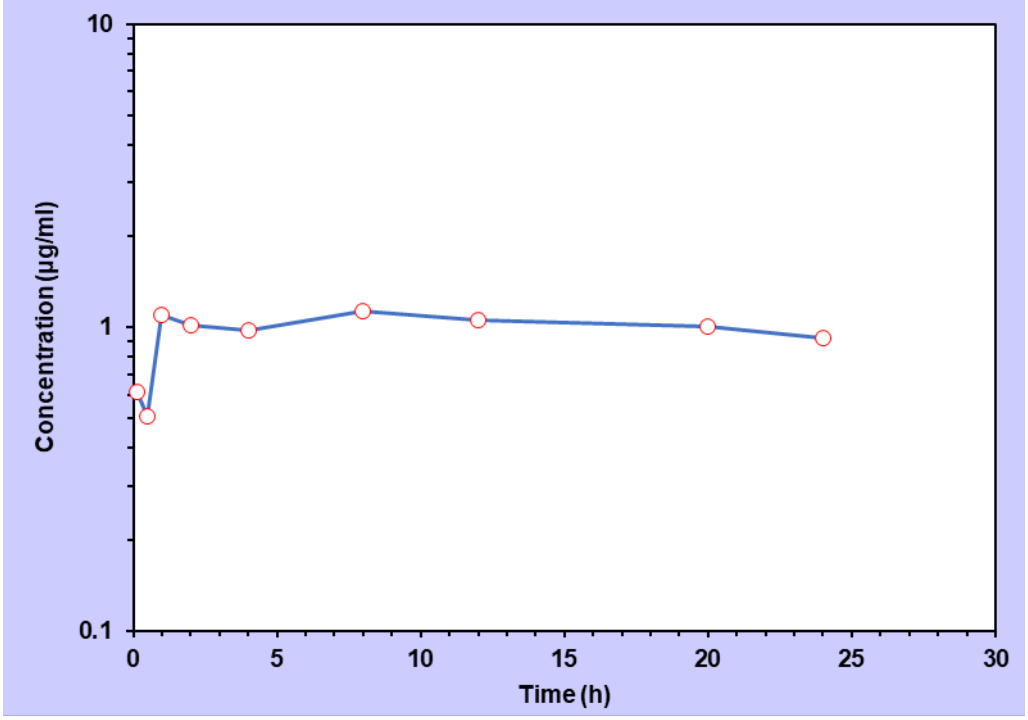
Table 14. Summary Table- Output

Time	Conc	ln(C)	AUC	AUMC	R	R_adj
0	0		0	0		
0.15	0.61489722	-0.4863002	0.04611729	0.00691759		
0.5	0.50785587	-0.6775576	0.24259908	0.06749603		
1	1.09586645	0.09154533	0.64352966	0.40494463		
2	1.02036128	0.02015676	1.70164353	1.97323914		
4	0.98000337	-0.0201993	3.70200819	7.93397519		
8	1.13257985	0.12449808	7.92717462	33.8952797	0.9708718	0.91388817
12	1.05994189	0.05821408	12.3122181	77.4551626	0.9364756	0.75397294
20	1.01072387	0.01066677	20.5948811	209.190282		
24	0.92634245	-0.0765113	24.4690137	294.083675		

Table 15. Calculation Results

Parameter	Unit	Value
Lambda_z	1/h	0.011239152
t1/2	h	61.67255226
Tmax	h	8
Cmax	µg/ml	1.132579848
Tlag	h	0
Clast_obs/Cmax		0.817904758
AUC 0-t	µg/ml*h	24.46901373
AUC 0-inf_obs	µg/ml*h	106.8900414
AUC 0-t/0-inf_obs		0.228917619
AUMC 0-inf_obs	µg/ml*h^2	9605.57334

MRT 0-inf_obs	h	89.86406229
Vz/F_obs	(mg)/(µg/ml)	41.61972628
Cl/F_obs	(mg)/(µg/ml)/h	0.467770424



Graph 1. Time in (min) Vs Concentration (µg/ml)

- **PK data analysis**

Plasma concentration vs. time data of S1 was analysed by Pk solver version 2.0 to derive various pharmacokinetic parameters, viz., AUC0-t, AUC0-∞, Cmax, tmax and t½.

Table 16. PK data analysis

Synthesized Entitey	S1
AUC Calculation Method	Linear Trapezoidal

Table 17. Summary Table- Input Variable

Time In Hr	**Plasma Drug Conc. Mcg/ml**	**Log Plasma Drug Conc. Mcg/ml**
0	0	0
0.15	5.44	0.7355989

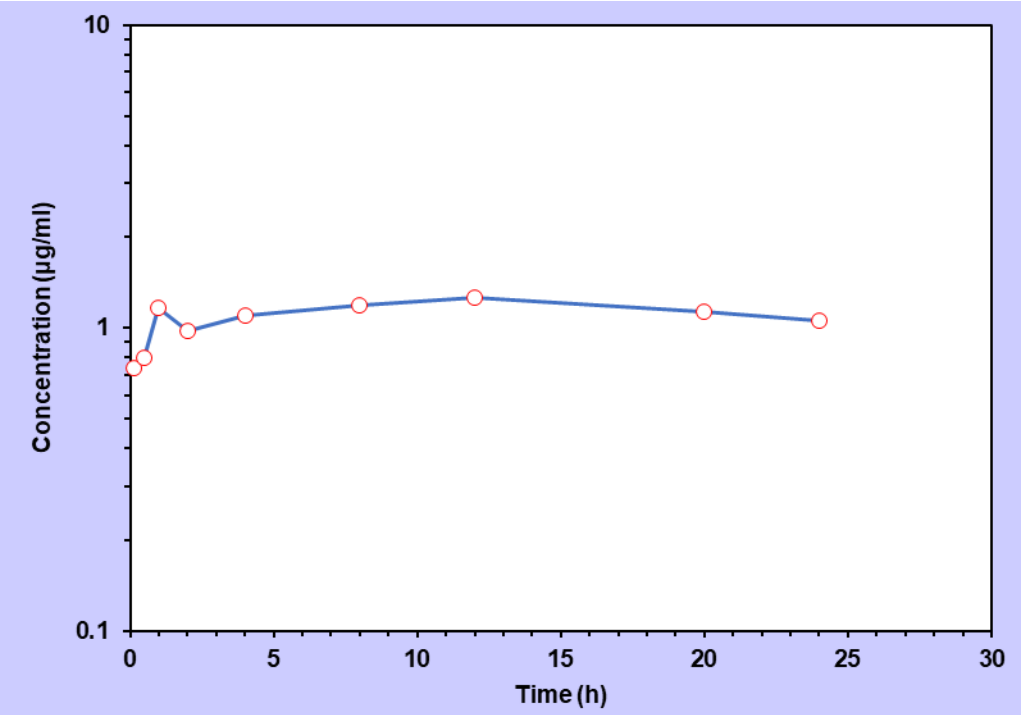
0.5	6.23	0.794488047
1	14.78	1.169674434
2	9.58	0.981365509
4	12.47	1.095866453
8	15.48	1.189770956
12	17.99	1.255031163
20	13.56	1.13225969
24	11.47	1.059563418

Table 18. Summary Table- Output

Time	Conc	ln(C)	AUC	AUMC	R	R_adj
0	0		0	0		
0.15	0.7355989	-0.3070703	0.05516992	0.00827549		
0.5	0.79448805	-0.2300573	0.32293513	0.09710266		
1	1.16967443	0.15672545	0.81397575	0.48883228		
2	0.98136551	-0.0188103	1.88949572	2.055035		
4	1.09586645	0.09154533	3.96672769	8.40123184		
8	1.18977096	0.17376081	8.53800251	36.2044988		
12	1.25503116	0.2271604	13.4276067	85.361582	0.9978227	0.99130036
20	1.13225969	0.12421536	22.9767702	236.183853		
24	1.05956342	0.05785695	27.3604164	332.333285		

Table 19. Calculation Results

Parameter	Unit	Value
Lambda_z	1/h	0.013931408
t1/2	h	49.75428073
Tmax	h	12
Cmax	µg/ml	1.255031163
Tlag	h	0
Clast_obs/Cmax		0.844252676
AUC 0-t	µg/ml*h	27.36041637
AUC 0-inf_obs	µg/ml*h	103.4161477
AUC 0-t/0-inf_obs		0.264566192
AUMC 0-inf_obs	µg/ml*h^2	7616.970557
MRT 0-inf_obs	h	73.65359013
Vz/F_obs	(mg)/(µg/ml)	34.70456774
Cl/F_obs	(mg)/(µg/ml)/h	0.48348349



Graph 2. Time in (min) Vs Concentration (µg/ml)

Table 20. Calculation Results

Parameter	Unit	Value	Value
Lambda_z	1/h	0.011239152	0.013931408
t1/2	h	61.67255226	49.75428073
Tmax	h	8	12
Cmax	µg/ml	1.132579848	1.255031163
Tlag	h	0	0
Clast_obs/Cmax		0.817904758	0.844252676
AUC 0-t	µg/ml*h	24.46901373	27.36041637
AUC 0-inf_obs	µg/ml*h	106.8900414	103.4161477
AUC 0-t/0-inf_obs		0.228917619	0.264566192
AUMC 0-inf_obs	µg/ml*h^2	9605.57334	7616.970557
MRT 0-inf_obs	h	89.86406229	73.65359013
Vz/F_obs	(mg)/(µg/ml)	41.61972628	34.70456774
Cl/F_obs	(mg)/(µg/ml)/h	0.467770424	0.48348349

Oral route of administration was studied in order to evaluate their impact on Relugolix bioavailability and release profiles over 24 hrs for Relugolix API and S1. Relugolix API and S1 were administered orally at 50 mg/kg to determine the absolute bioavailability. The Cmaxs were approximately equal for formulation as well as API after oral administration of equivalent dose levels, but the AUC from 0 to infinity (AUC0-inf) values were comparable. The AUC0-inf for the Relugolix API after oral administration reached 24.46µg/ml/h and for S1 reached 27.36µg/ml/h. The absolute bioavailability of Relugolix API and formulation, calculated after 24hrs, ranged between 24 and 28% after oral administration.

Conclusion

Synthesis and Characterisation of piperazine based ligands, metal complexes and their in vitro and in vivo activity.

The study concluded that cancer continues to pose a substantial global health concern, especially in developing nations such as India, where delayed diagnosis and restricted healthcare access exacerbate mortality rates. The work systematically identified S1 as a potent lead chemical with significant antibacterial, antifungal, and anticancer properties by synthesis, antimicrobial screening, and anticancer evaluation. In vitro and in vivo studies, along with pharmacokinetic analysis, reveal that S1 exhibits superior therapeutic efficacy and equivalent or enhanced performance relative to the conventional drug Relugolix, underscoring its promise as a unique candidate for cancer treatment.

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