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In Vivo Biological Evaluation of Novel Synthesized 6, 7-Dimethoxy-N-Aryl-4-Amino Quinazoline Derivative for Colorectal Cancer

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Abstract: The novel 6, 7-dimethoxy-N-aryl-4-amino quinazoline derivative was synthesized by the nucleophilic substitution process. In these process aryl amines and 6, 7-dimethoxy-4-chloroquinazoline was reacted with each other. Synthesized compound SERB-111(N2-(6,7-dimethoxy-3,8a-dihydroquinazolin-4-yl)-N8, N8, 3-trimethyl-phenazine-2,8-diamine) was the most potent inhibitors of HCT116 cell line with sub-micromolar IC_{50} values (7.95 µg/ml). The target compound was screened to evaluate their inhibitory effect on human cholchicine binding site. The tested SERB-111 compound was forwarded for calculation of LD_{50}, acute and sub-acute toxicity on rats. This SERB-111 was assessed for *in vivo* estimation and evalution of stability in human biological fluid (plasma). The synthesized novel quinazoline derivative (SERB-111) was found stable in human plasma.

Keywords: Quinazoline, Acute and sub-acute toxicity**,** Median Lethal Dose, Colorectal Cancer

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Introduction

Colorectal cancer is a multifaceted disease. In the world, it is the second most prevalent cause of cancer-related fatalities and the third most commonly diagnosed type of cancer. Uncertainty surrounds the cause of colorectal cancer. Patients with colorectal cancer frequently exhibit only non-specific symptoms or no typical clinical manifestations during the early stages of the disease, which leads to a low early diagnosis rate even though early diagnosis can greatly improve the prognosis. Various therapy approaches are available, contingent on the tumor's stage and the

patient's features. These consist of molecular targeted therapy, radiation, chemotherapy, surgery, immunotherapy, and other treatments (Duan *et al*., 2022). The field of medicinal chemistry is essential for the creation of drugs that preserve and enhance human health. Designing chemical entities to stop the growth of microorganisms that come into touch with people on a daily basis is also crucial (Katke *et al*., 2011; Kim *et al*., 2017). Quinazoline is the most commonly experienced hetero-cyclic core in the medicinal research owing to its wide spectrum of pharmacological potential and a huge numbers of its derivatives have been referred to be salient structural synthons for numerous physiological relevance and pharmaceutical applications. These include usage in drug design and medicinal researches as they have been reported to display antiviral (Long *et al*., 2015), anti-HIV (Vijayakumar *et al*., 2013), anti-cancer (Marvania *et al*., 2011), anti-microbial (Bedi *et al*., 2004), anti-fungal (Xu *et al*., 2007), anti-inflammatory (Alafeefy et al. 2010) and anti-convulsant (Jatav *et al*., 2008) activities, etc. Over the last 50 years, about 500000 natural and synthetic chemical compounds have been tested for anticancer activity but only about 25 of these are in wide use at present (Lichota *et al*., 2018) and it provides an indication of difficulties for treatment methods of this problem. In the treatment of cancer and in many diseases, quinazolines play an important role for design and synthesis as parent moiety at colchicine binding site. Novel derivative, 6, 7-dimethoxy-N-aryl-4-amino quinazoline was synthesized by interaction of aryl amine and 6, 7-dimethoxy-4-chloroquin-azoline. The compound SERB-111 was screened for their HCT116 (colorectal cancer cell line) with sub-micromolar IC_{50} values, LD_{50}, acute and sub-acute toxicity on rat, *in vivo* estimation and evaluation of stability in human biological fluid (plasma).

Materials and Methods

Synthesis of quinazoline derivative:

Novel derivative, 6, 7-dimethoxy-N-aryl-4-amino quinazoline was synthesized by nucleophilic substitution processes (Gabriel, 1903; Liu *et al*., 2007). Aryl amine and 6, 7-dimethoxy-4-chloroquinazoline was interacted during this process. Methyl 3,4-dimethoxy benzoate was prepared by O-methylation of phenol with dimethyl sulfate and then, it was esterified with methanol. In this reaction, methyl 2-nitro-3,4-dimethoxybenzoate was produced from the initial chemical reaction and during the process, 70% conc. nitric acid in acetic acid at 40-50°C was added. After hydrolysis of nitro ester, the nitro group was reduced to the amino group with the help of $SnCl_2$ at 80°C. Finally, 2-amino-3,4-dimethoxybenzoic acid was formed. The synthesized compound was cyclized by formamide and chlorinated by phosphorus oxychloride. The formation of 4-chloro-6,7-dimethoxyquinazoline is described in Figure 1 (Scheme 1). This classical method was used for the synthesis of SERB-111 (N2-(6,7-dimethoxy-3,8a-dihydroquinazolin-4-yl)-N8, N8, 3-trimethylphenazine-2,8-diamine) (Liu *et al*., 2007). The process comprised of mixing of 2-propanol (100 ml) and K_2CO_3 (1.9 N, 10 ml in distilled water) in a solution of 4-chloro-6,7 dimethoxyquinazoline (0.25 mmol) and aryl amine (0.25 mmol). The reaction was completed under reflux at 80°C (24 h) and expressed in Figure 2 (Scheme 2).

In vitro anti-cancer activity of SERB-111:

One of the most popular techniques for *in vitro* cytotoxicity screening is the sulforhodamine B (SRB) assay which was created in 1990. The assay depends on SRB's capacity to bind to proteins in cells that have been trichloroacetic acid-fixed on tissue culture plates (TCA). The bright pink aminoxanthene dye SRB has two sulfonic groups that, in mildly acidic conditions, bind to basic amino-acid residues and, in mildly basic conditions, dissociate. The observed amount of dye taken from stained cells is precisely proportional to the mass of the stained cells since SRB's binding is stoichiometric. Several biological investigations concerning colon cancer proliferation and related inhibitors employ HCT116 cells. For performing SRB assay, the

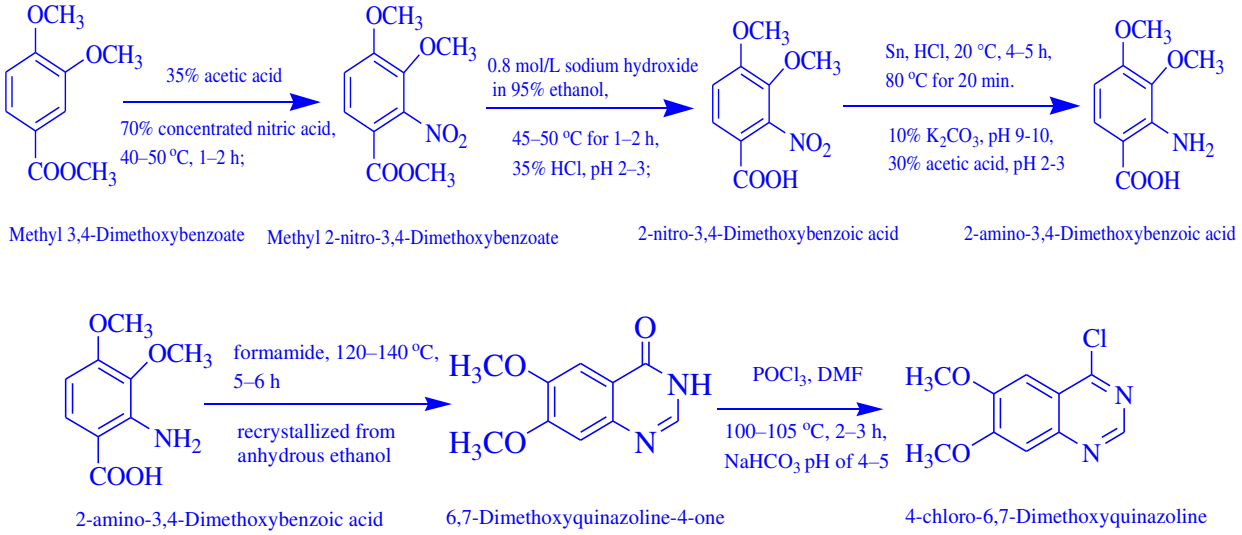

Fig. 1: Scheme 1: Preparation of 4-chloro-6,7-dimethoxyquinazoline from methyl 3,4-dimethoxybenzoate.

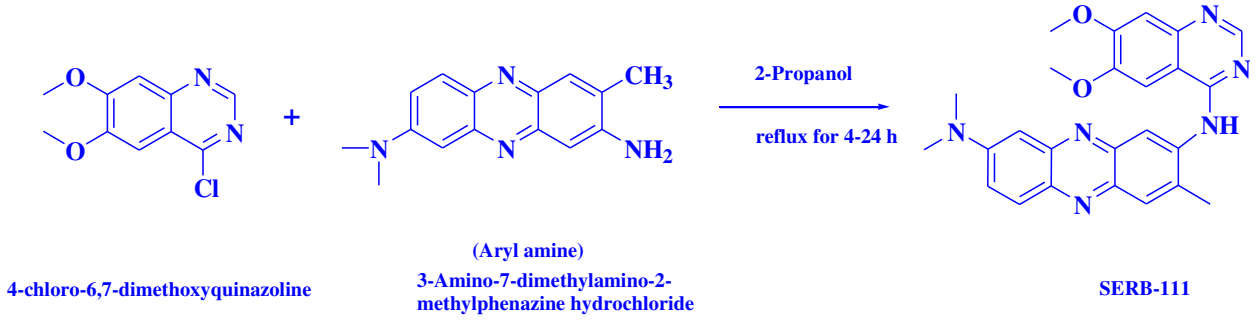

Fig. 2: Scheme 2: Preparation of SERB-111.

procedure was followed as given below (Denekamp, 1984; NCI, https://www.cancer.gov, 2019).

Methodology of SRB assay for anticancer activity offTable solution preparation:

The treatment solution for the assay was prepared in enough amount to be used for triplicates in 96-well plates (50 µl per replicate; final volume 100 µl). The treatment solution was prepared in aqueous media/or solvent of choice (e.g., Dimethyl sulphoxide).

(B) *Cell suspension preparation*:

The culture medium was removed from the cell monolayers and the cells were washed with sterilized physiological buffer solution (PBS). PBS was removed and 1 ml (100 mm plates) of 0.25% (wt./vol.) trypsin was added to evenly colour the cell-growth surface. Further, it was incubated at 37°C for 5 min or until cells started to dissociate. In next phase, trypsin was inactivated with 10 volumes of culture medium containing physiological buffer solution. It was mixed up and down to obtain a homogenous single cell suspension. The cell suspension was transferred to a sterile Falcon tube. The cell concentration was counted in a hemocytometer chamber under a microscope using 1:1 mixture of cell suspension and 0.4% w/w trypsin blue solution to determine cell viability prior to cell seeding. Besides this, in alternate method, the cells were spin down in order to wash trypsin and resuspended in growth

medium. The viability of cells in terms of health was assessed by checking trypsin blue staining. The cell concentration was adjusted with growth medium (10% PBS) to obtain an appropriate cell seeding density per well in a volume of 50 µl (96-well format). For this, the cell density was maintained as $2x10^4$. The cell suspension was transferred into a sterile reagent reservoir to make it easier to pipette with a multichannel pipette.

(C) *Treatment exposure*:

The treatment solution was prepared by mixing the treatment solutions prepared as above by pipetting. A 50 µl of the solution was dispensed into each well. The cell suspension prepared as bove method was mixed thoroughly and 50 µl was added to each well already containing treatment solutions. The cell solution was added directly to the bottom of the well and avoiding to the touch the well. It was adopted to avoid the ring effects and to ensure the even cell distribution in the bottom of the plate. The three well in the plate containing only solvent contained cell suspension for an untreated or vehicle control and were set aside. The three wells in the plate containing only medium were also left for background subtraction. The plate was incubated at 37°C in a humidified incubator with 5% CO_2 until plate was to be read.

(D) *Cell fixation and staining*:

A 25 µl (96-well format) of cold 50% (w/v) TCA was added to each well directly to medium supernatant and the plates were incubated at 4°C for 1 h. In this process, mixing was not required as this could lead to some cells detaching from the bottom of the well. The plates were washed four times by submerging the plate in a tub with slow-running tap water and the excess water was removed by gently tapping the plate into a paper towel. After the last wash, the plate was allowed to air dry at room temperature. Afterwards, a 50 µl (96-well format) of 0.04 (w/v) SRB solution was added to each well. It was ensured that the solution was in direct contact with bottom of the well and there were no bubbles in between. Further, the plates were left at room temperature for 1 h and then quickly rinsed four times with 1% (v/v) acetic acid (200 µl for 96-well format) to remove unbound dye. The plate was allowed to air-dry at room temperature. The washings were done quickly and homogenously across the entire plate. The washings were performed by multi-channel pipette and injecting the solution indirectly into the well using the walls of the well.

(E) *Absorbance measurement*:

A 50 µl to 100 µl of 10 mM Tris base solution (pH 10.5) was added to each well and the plate was shaken on an orbital shaker for 10 min to solubilize the protein-bound dye (approximately). The absorbance was measured at 510 nm in a microplate reader.

(F) *Data analysis*:

For the data analysis, background absorbance was subtracted from each well plate. The following formula was applied for determination of the proportion of cell-growth inhibition normalizing treatments to miRNA negative control. The sample SERB-111 was fed to HCT116 cells at various dosages, a dose-dependent reduction in cell viability was seen. The sample's IC_{50} value was determined by following expression:

$$\%\ cell\ growth = \frac{As}{Anc} x100$$

Where As= Absorbance of sample; Anc = Absorbance of negative control or untreated sample.

Colchicine binding site for treating cancer:

The eukaryotic cytoskeleton's main components, microtubules, have remained mostly unchanged throughout evolution. They play a role in cell structure, maintenance, intracellular transport, and cellular motility. Most significantly, microtubules are in-charge of separating the chromosomes during mitosis, which is crucial in the context of the development of anti-cancer small-molecule drugs (Thorpe *et al.*, 2003). *Colchicum autumnale*, sometimes referred to as the autumn crocus or "poisonous meadow

saffron," is the source of colchicine, a tricyclic alkaloid. *Colchicum autumnale* has been originally mentioned as a medicinal component approximately three thousand years ago, and colchicine has been used as a medication ever since (Orellana and Kasinski, 2016; Shaik *et al*., 2020). It effectively prevents cellular mitosis and is a highly toxic anti-cancer. Colchicine may also alter the voltage-dependent anion channels found in mitochondrial membranes (Vichai and Kirtikara, 2006), which would restrict the metabolism of mitochondria in cancer cells (Orellana and Kasinski, 2016). The Colchicine Binding Site (BS) is most important for the control of DNA division into two daughter cells and occurs during the fifth phase of a typical cell cycle, which is called mitosis (Fig. 3) (O'Connor and Adams, 2010).

In vivo studies of SERB-111:

Determination of acute toxicity and LD_{50} of SERB-111:

The determination of LD_{50}, or the dose that has been shown to be deadly (producing death) to 50% of the tested group of animals, takes acute toxicity into account. When assessing and evaluating the hazardous properties of all substances, the measurement of acute oral toxicity is frequently the first screening step (Akhila, 2007). In this study, it was carried out in accordance with OECD 423 criteria for acute oral toxicity studies and LD_{50} value was calculated as per "up and down" or "staircase" method. Both of these studies serve as the foundation for classification and labeling; provide initial information on the mode of toxic action of a substance; help to determine the dose of a new compound; aid in dose determination in animal studies; and assist in determining LD_{50} values, which provide a variety of index values of potential types of drug activity. The "up and down" or "staircase" method is a straight forward way to determine the real LD_{50}.

In this method, two rats were administered a certain amount of test compound and monitored for 24 h to see if any of them died (Baß *et al*., 1982; Ghosh, 1984). In this toxicology, the research examines the hazardous effects that a medicine or chemical has on animals over the course of a short or lengthy period of time. Paracelsus stated more than 500 years ago that the toxicity is dose-dependent. However, median lethal dosage (LD_{50}), which Trevan first proposed in 1927 and has since undergone numerous revisions and it is also used to estimate short-term hazardous effects. Research on medicinal plants and the study of patent medicines both involve the investigation of acute toxicity. Although LD_{50} is still used and accepted in some regions of the world, its application has been substantially decreased. Furthermore, since euthanasia of test animals may cover up some toxicity symptoms of the test agents, animals on which LD_{50} tests are performed should be allowed to die to see the final effects of the test drug or chemical. As a result, the scientific method of studying the toxicity of medications and chemicals is essential for the discovery and development of drugs as well as the identification of possible toxicants (Saganuwan, 2017). However, the lethal dose for humans may range from 1 to 1000% above or below the stated LD_{50} (Robert and William, 1987).

Methodology:

The acute toxicity and LD_{50} was determined (Gawai *et al*., 2019, Kyhoiesh *et al*., 2021) by simple up and down or staircase method. Two rats were injected with a particular dose and observed for a period of 24 h for any mortality. The subsequent doses were then increased by 1.5 if the dose was tolerated, and decreased by a factor of 0.7 if it was lethal. By this, a dose range was thus determined. The approximate standard error (S.E.) of LD_{50} value was obtained by the formula (Miller and Tainter, 1944):

$$Approximate\ S.E.\ of\ LD50 = (Log\ LD84 - Log\ LD16)/\sqrt{2N}$$

Where N is the total number of animals employed in each group (N=10).

Determination of SERB-111 sub-acute toxicity:

According to OECD 407 recommendations, the

oral toxicity was carried out. In this study, the body weight of the four groups of animals was determined weekly on the initial (0), 9th, 18th, and 28th days. The first one was the control group; Group I was SERB-1; Group II was SERB-2; and the last group named as Group III was SERB-3.

Estimation of synthesized derivative (SERB-111) in biological fluid:

A novel derivative of the quinazoline was synthesized by nucleophilic substitution processes. In this process, the synthesized compound was marked as SERB-111 (N2-(6,7-dimethoxy-3,8a-dihydroquinazolin-4-yl)-N8,N8,3-trimethylphenazine-2,8-diamine). This SERB-111 was assessed for *in vivo* estimation and evalution of stability in human biological fluid (plasma). Afterwards, the synthesized SERB-111 was used in sustained release matrix tablet formulation to be applied as colon-specific drug delivery system (CDDS) for treatment of colon cancer proliferation and inhibition of the growth of HCT116 cells. For *in vivo* estimation, HPLC assay method was applied for the determination of SERB-111 in human plasma at selected concentration (Helmy and Bedaiwy, 2013).

Instrumentation:

The analysis of SERB-111 was performed in liquid chromatographic system composing of Waters HPLC with 515 pump, 2489 UV-VIS detector and a manual Rheodyne 7125i injector equipped with a 20 µl loop (Waters, USA). The data was acquired and processed using Empower (TM) software. Other instruments used in this study include: Bath sonicator, Balance, Magnetic stirrer, Centrifuge, pH meter, Vortex tube shaker, micro-pipettes and micro liter syringe.

Procedure:

The separations were performed at isocratic mode with mobile phase composed of a mixture of 0.02 M phosphate buffer (pH 3.5), acetonitrile, methanol, and triethanolamine (TEA) (50:40:10:0.1%, v/v/v/v) at ambient temperature maintained on Xbridge C18 column (250 mm×4.6mm), 5µm as particle size (Waters, USA). The flow rate was kept at 1.0 ml/min with a total run time of 16 min. The absorbance wavelength was set at 240 nm for the analyte of interest (Blaszczak-Swiatkiewicz and Mikiciuk-Olasik, 2006; Helmy and Bedaiwy, 2013; Sharma, 2016). The conditions for HPLC estimation for SERB-111 are shown in Table 1.

Standard solutions:

For the construction of calibration curves, drug stock solution (500 µg/ml) was prepared by dissolving SERB-111 in methanol. The stock solution was further diluted in methanol to prepare seven working solutions of SERB-111 with concentrations of (10 µg/ml) for spiking human plasma samples. Standard solutions were stored at 4°C. The prepared standard stock solution (10 µg/ml) was used for SERB-111 analysis in human plasma.

Histopathological study:

The liver, heart, spleen and kidney were removed and weighed. For histological study, these organs were kept in a fixation medium containing 10% buffered formalin. The tissues were routinely processed with paraffin embedding method, sections were cut at 6 µm and stained with hematoxylin-eosin (HE).

Plasma sample preparation:

The solvent was evaporated, and 0.25 ml of blank plasma was added to tube to form a set of calibration standards with concentrations of 20 µg/ml. The sample was vortexed, and then 0.25 ml of 6% trichloroacetic acid was added, shaken for one min, and centrifuged at 3000 rpm for 7 min. The supernatant was separated and 20 µl was injected into the HPLC.

Results and Discussion

Synthesis of 6, 7-dimethoxy-N-aryl-4-amino quinazoline derivative:

The synthesis of 6, 7-dimethoxy-N-aryl-4-amino quinazoline has been described Figure 1 (scheme 1) and Figure 2 (scheme 2).

N-aryl-4-amino quinazoline derivative was

Table 1: Optimized chromatographic conditions for SERB-111

S. No.	Parameters	Condition
1	Stationary phase	Xbridge C18 column (250 mm × 4.6 mm)
2	Mobile phase	0.02M phosphate buffer (pH-3.5), acetonitrile, methanol, and TEA (50:40:10:0.1%, v/v/v/v)
3	Flow rate	1.0 ml/min
4	Detector	2489 UV-VIS detector
5	Injection volume	20µl
6	Detection wavelength	240 nm
7	Retention time	5.72 min

synthesized by nucleophilic substitution reactions of methyl 3,4-dimethoxybenzoate which was prepared by O-methylating the phenol with dimethyl sulfate and later esterified with the methanol. The methyl 2-nitro-3,4-dimethoxy-benzoate was synthesized by adding concentrated 70% nitric acid into acetic acid at a temperature of 40-50°C. It was hydrolyzed by using nitro ester and with the use of tin dichloride at 80°C. Afterwards, this nitro group was reduced into an amino group and the resultant product was 2-amino-3,4-dimethoxybenzoic acid. For cyclization of the previously synthesized compound, phosphorus oxychloride and formamide were used and chlorination was done with dimethyl formamide. 4-chloro-6, 7-dimethoxyquinazoline was obtained as described in Figure 1 (Scheme 1). To obtain the final product SERB-111 (N2- (6,7-dimethoxy-3,8a -dihydroquinazolin-4 -yl) -N8, N8, 3- trimethylphenazine-2,8-diamine); a 0.25 mmol of 4-chloro-6,7 dimethoxyquinazoline and 0.25 mmol of aryl amine were reacted under a basic catalyst along with K_2CO_3 (1.9 N, 10 ml in distilled water) and 2-propanol (100 ml) as solvent. It was recrystallized with ethanol. The chemical processes have been depicted in Figure 2 (Scheme 2).

Pharmacological evaluation:

In vitro assay anticancer assay of SERB-111 against HCT116 cells:

Baseline absorbance was taken out of each test plate for the data analysis. The ratio of cell-growth inhibition normalizing therapies to miRNA negative control was calculated using the provided formula. When several doses of the sample SERB-111 were given to HCT116 cells, a dose-dependent decrease in cell viability was observed. The IC_{50} value for the sample was calculated to be 7.95 g/ml.

Mechanism of action SERB-111 at colchicine Binding site:

The study of microtubules that are present in the human cell, have colchicine binding site between the two α and β tubulin monomers and had been employed as a research tool. Colchicine is recognized to have a similar mode of action to anti-cancer medications like vinca alkaloids. It stabilizes microtubule structures at low doses and destabilizes them at high concentrations (Lange, 2001). Colchicine binding site has various benefits to creating cancer medicines that target cancer cells indirectly by attacking the tumour vasculature, according to a consensus that has emerged in recent years as a result of newly available information about the environment of solid tumors (Maldonado *et al*., 2010, Dominguez-Brauer *et al*., 2015). In cancer, various research groups have been concentrating on the quinazoline and benzothiadiazine scaffold during the past few years. This had inspired us to investigate quinazoline's anticancer properties (McLoughlin and O'Boyle, 2020).

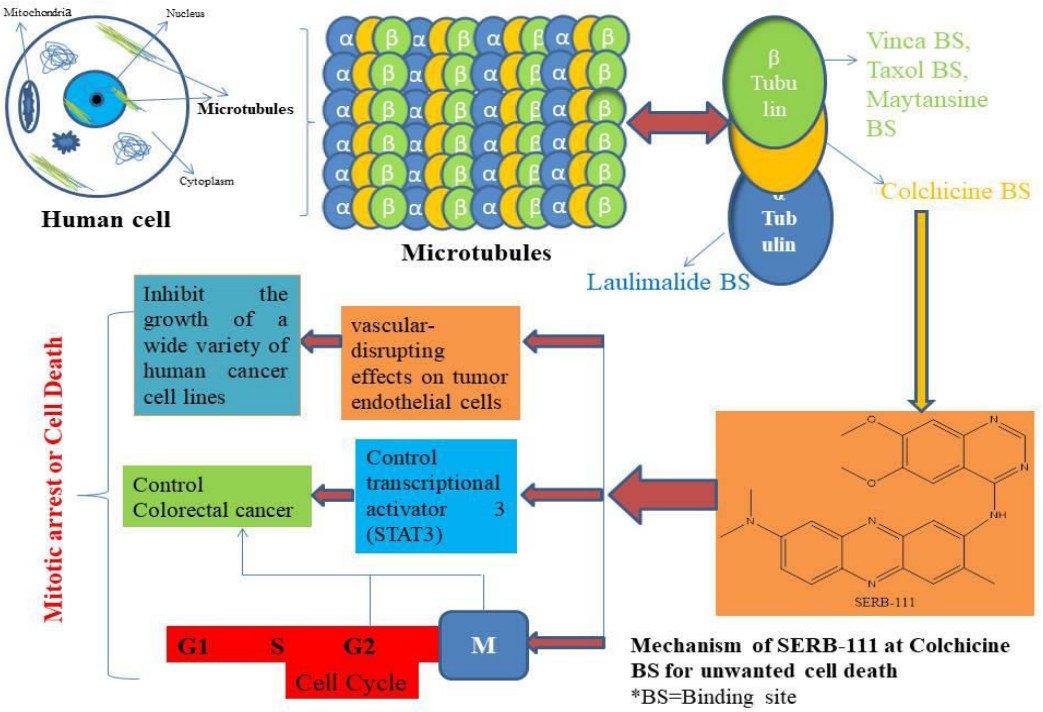

Fig. 3: Mechanism of novel SERB-111 derivative at Colchicine BS.

The newly synthesized quinazoline-based ligand (SERB-111) was used for the targeting of tubulin colchicine binding site in microtubules. This SERB-111 agent had targeted three positions in the cell. Firstly, it has exhibited vascular-disrupting effects on tumor endothelial cells, in addition to inhibit the proliferation of a wide range of human cancer cell lines and secondly, controlling of transcriptional activator STAT3 for treatment of colorectal cancer. Yet, certain medications directly cause apoptosis. The majority of anticancer treatments had stopped the growth of cancer cells at a specific cell cycle stage, lastly the results were either apoptosis or necrosis. Data from cell cycle study clearly showed that the SERB-111 was the chemical arresting HCT116 cells at the G2/M phase. The novel synthesized compound (SERB-111) exhibited vascular-disrupting effects on tumor endothelial cells, in addition to inhibit the proliferation of a wide range of human cancer cell lines (Figure 3). As a result, it was regarded as vascular-disrupting agents (VDAs) (Shagufta and Ahmad, 2017).

LD_{50} determination of SERB-111:

The LD_{50} value obtained from Miller and Tainter method was found to be 50.12 mg/kg, p.o. i.e., LD_{50} is equals to 50.12±11.99 mg/kg, p.o. The oral dose taken to assess pharmacological effect was 5 mg/kg, p.o. The point value of LD_{50} was determined by Miller and Tainter method (1944) (Ghosh, 1984), i.e., by using probit-log scale method (Tables 2, 3). The probit values of 84 and 16 were obtained from the Probit table (Table 2). The Log-LD_{50} values for the probits were obtained from the line on the graph Figure 4 and 5 (Miller and Tainter, 1944).

The observed percentage mortality was converted into probit referring to the probit table (Table 3). The values thus obtained were plotted against log dose. The LD_{50} value and its standard error were determined from the above graph, if the line was straight enough (Table 2). Transformation of percentages to probit was done based on the table of probits (Table 3).

Figure 5 for 96% was determined from the point where the horizontal line on top and the vertical line on the left, 90 and 6, converge. The

Table 2: Transformation of percentage to Probits (Ghosh, 1984)

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.25	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	5.61	4.64	4.67	4.67	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.91	4.92	4.92	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.18	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.44	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

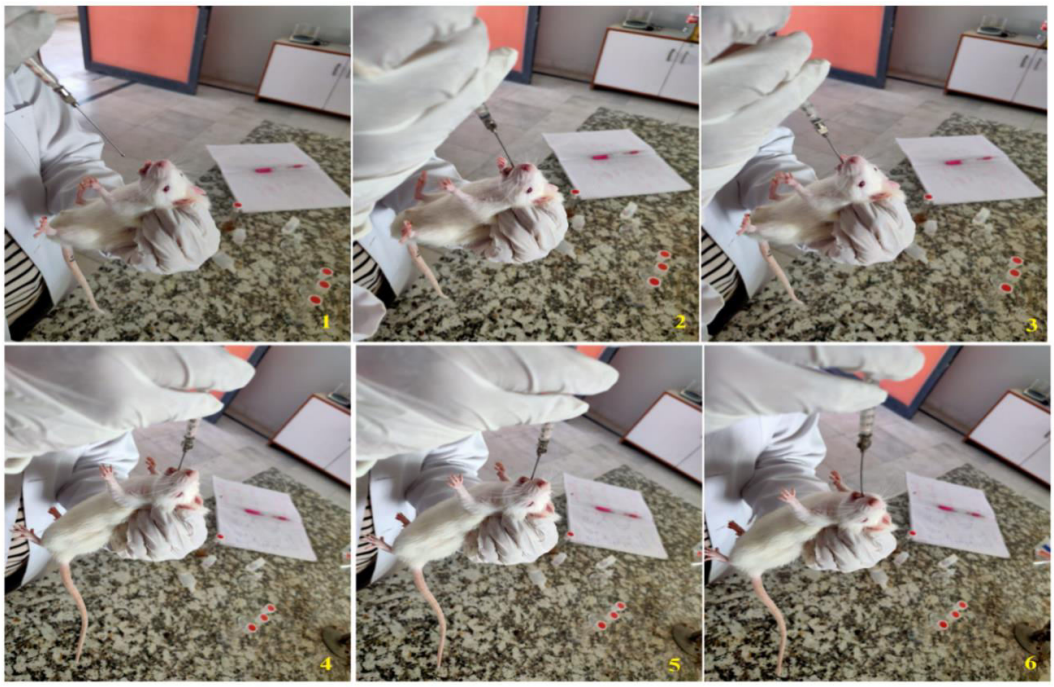

Fig. 4: SERB-111 oral dose injected to rats under simple up and down or staircase method for LD_{50} and acute toxicity calculation (images-1-6).

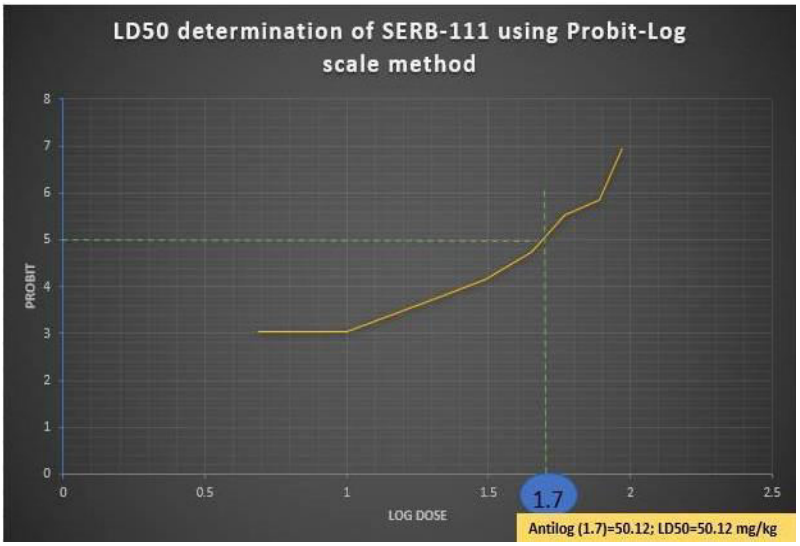

Fig. 5: SERB-111 LD_{50} determination by graphical method.

Table 3: Determination of LD_{50} by Miller and Tainter method (1944) (Miller & Tainter, 1944)

Groups	**Dose (mg/kg), p.o.**	**Log dose**	**Dead/Total**	**% Dead**	**Corrected %**	**Probit**
1.	10	1	0/10	0	2.5	3.04
2.	31	1.49	2/10	20	20	4.16
3.	45	1.65	4/10	40	40	4.75
4.	60	1.77	7/10	70	70	5.52

Table 4: Body weight of rat (g) during subacute toxicity study

Group	Dose	Weight (g)			
	(mg/kg), p.o.	Initial day (Mean±S.D.)	9th day (Mean±S.D.)	18th day (Mean±S.D.)	28th day (Mean±S.D.)
Control	--	134.21 ±4.01	139.54 ± 5.24	145.56 ± 4.78	147.21 ± 4.85
SERB-111 (S1)	0.5	138.23 ± 5.22	145.36 ± 5.78	149.25 ± 6.21	153.54 ± 5.74
SERB-111 (S2)	0.5	142.22 ± 6.24	148.26 ± 5.00	152.21 ± 5.89	155.87 ± 5.81
SERB-111 (S3)	0.5	140.25 ± 3.22	146.28 ± 3.89	150.66 ± 4.86	153.24 ± 4.01

Table 5: Hematological parameters in subacute toxicity study

Parameters	Unit	Control	SERB-111 (S1)	SERB-111 (S2)	SERB-111 (S3)
Haemoglobin (Hb)	g/l	141.88 ± 4.31	138.87 ± 4.02	143.31 ± 2.27	149.52 ± 3.51
Total red blood cells (RBC's)	10^{12}/l	8.01 ± 1.23	7.87 ± 1.00	7.20 ± 1.26 ns	7.49 ± 1.22 ns
Packed cell volume (PCV)	L/L	0.45 ± 0.01	0.48 ± 0.00	0.43 ± 0.02 ns	0.42± 0.01 ns
Mean corpuscular volume (MCV)	Fl	54.25 ± 0.01	54.20 ± 0.78	55.81 ± 0.11 ns	55.01 ± 0.06 ns
Mean corpuscular haemoglobin (MCH)	Pg	19.55 ± 1.25	18.55 ± 1.42	18.45 ± 0.14 ns	17.07 ± 0.24 ns
Mean corpuscular haemoglobin concentration (MCHC)	g/l	322.68 ± 4.02	320.22 ± 4.83	313.11± 0.23 ns	310.71 ± 0.44 ns
Total white blood cells (WBC's)	10^9/l	8.52 ± 0.99	9.59 ± 1.54	7.49 ± 0.19 ns	7.33 ± 0.29 ns
Neutrophils	%	12.98 ± 1.41	12.17 ± 1.87	12.60 ± 1.11 ns	12.03 ± 1.79 ns
Lymphocytes	%	85.41 ± 1.02	82.02 ±1.01	86.51 ± 1.13 ns	86.33 ± 1.00 ns
Monocytes	%	2.38 ± 0.00	2.31 ± 0.07	2.61 ± 0.65 ns	2.17 ± 0.31 ns
Eosinophils	%	1.01 ± 0.01	0.09 ± 0.02	0.04± 0.02	0.08± 0.02 ns
Platelet count	10^9/L	877.25±10.52	967.40±12.10	841.11±41.11ns	840.08±53.33ns

Values are expressed as Mean ± SEM (n=10; for each group); p> 0.05 using one-way ANOVA followed by Tukey's Multiple Comparison test; ns: Not significant.

value against 90 and 7 + 90 and 8 was obtained if a decimal was present, such as in the case of 97.5, and the average of the two was used to determine the probit. The probit value and dose logarithm were plotted. The LD_{50} was defined as the dose equal to 50% or probit 5 (Ghosh, 2008).

Sub-acute toxicity determination of SERB-111:

The prescribed medicine (SERB-111) dose was determined to be safe and all of the rat's body weight (g) during oral subacute toxicity, hematological parameters, biochemical parameters, and histological observations were found to be normal. Hematological and histological analyses revealed that the prescribed dose was safe and free from any morbidity or mortality. In the oral subacute toxicity study, the rats' body weight increased steadily throughout the duration of the investigation, as shown in Table 4. The body weight of rats in the control group (147.21 ± 4.85), as well as in other groups I (153.54 ± 5.74), II (155.87 ± 5.81), and III (153.24 ± 4.01), had showed a gradual increase during the study (Table 4).

The effects of subacute administration of SERB-111 (groups like S1, S2, and S3) on hematological parameters arte presented in Table 5. Most of the hematological parameters like hemoglobin, total RBCs, RDW, WBCs, neutrophils, lymphocytes, monocytes, and platelet count, in treated rats were not significantly different from the control with the exception of minor variations in certain parameters (Table 5).

The effects of subacute administration of SERB-111 on biochemical parameters are presented in Table 6. The SERB-111 had no effect on serum electrolytes (Na^+, K^+ and Cl^-). The kidney

Table 6: Biochemical parameters in subacute oral toxicity study

Parameter	Unit	Control	SERB-111 (S1)	SERB-111 (S2)	SERB-111(S3)
Sodium	mmol/l	136.00 ± 0.33	135.35 ± 0.84 ns	133.96 ± 0.56 ns	133.20±0.66 ns
Potassium	mmol/l	6.37 ± 0.08	6.55 ± 0.09 ns	6.59 ± 0.04 ns	6.67 ± 0.11 ns
Chloride	mmol/l	102.53 ± 0.05	102.20 ± 0.07 ns	103.11 ± 0.03 ns	102.00±0.01 ns
Urea	mmol/l	6.55 ± 0.27	6.22 ± 0.17 ns	6.98 ± 0.18 ns	6.86 ± 0.10 ns
Creatinine	µmol/l	47.30 ± 0.52	45.17 ± 0.69 ns	45.16 ± 0.61 ns	45.79 ± 0.49 ns
Uric acid	mol/l	0.16 ± 0.04	0.15 ± 0.05ns	0.13 ± 0.09 ns	0.15 ± 0.02 ns
Total protein	g/l	68.50 ± 0.90	68.30 ± 0.86 ns	69.00 ± 1.14 ns	68.81 ± 0.90 ns
Albumin	g/l	36.67 ± 0.03	37.21 ± 0.03 ns	38.77 ± 0.01 ns	38.92 ± 0.03 ns
Globulin	g/l	31.83 ± 0.05	31.09 ± 0.09 ns	30.23 ± 0.09 ns	29.89 ± 0.07 ns
Albumin/globulin ratio		1.15 ± 0.00	1.19 ± 0.01 ns	1.28 ± 0.01 ns	1.30 ± 0.01 ns
Alkaline phosphatase (ALP)	u/l	133.53 ± 5.17	134.74 ± 9.12 ns	133.65±10.92 ns	135.22±10.33ns
AST	u/l	73.10 ± 3.91	74.40 ± 4.02 ns	74.21 ± 3.29 ns	77.00 ± 5.24 ns
ALT	u/l	49.68 ± 1.17	46.21 ± 2.42 ns	46.27 ± 2.71 ns	50.10 ±1.23 ns
Bilirubin (Total)	mg/dl	0.35 ± 0.01	0.31± 0.00 ns	0.34 ± 0.01 ns	0.45 ± 0.02 ns

Values are expressed as Mean ± SEM (n=10; for each group); P > 0.05 using one-way ANOVA followed by Tukey's Multiple Comparison test; ns: Not significant.

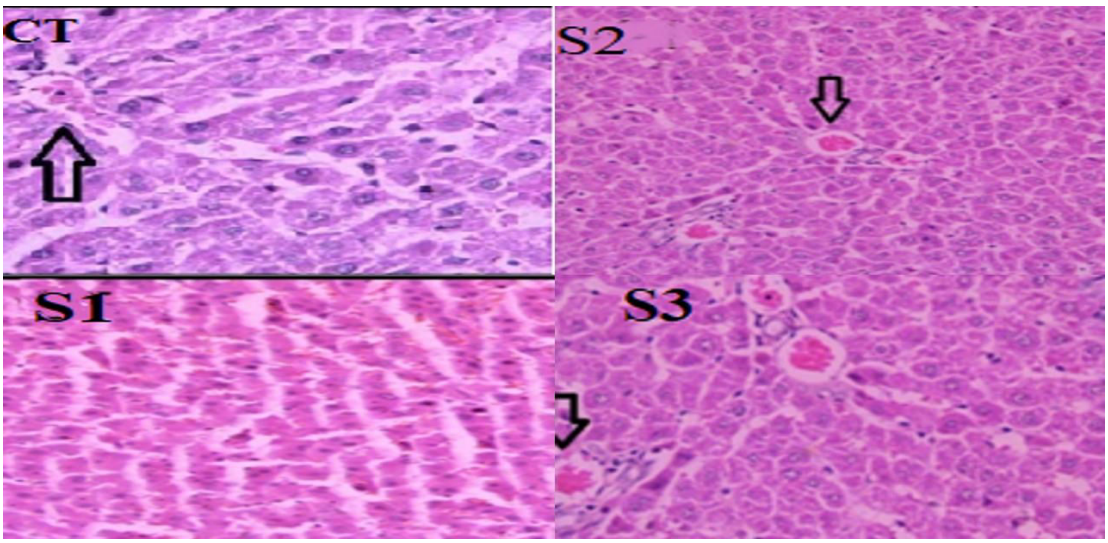

Fig. 6: Photomicrographs of liver histopathology of animals after administration of SERB-111 as oral dose (Magnification: 20x).

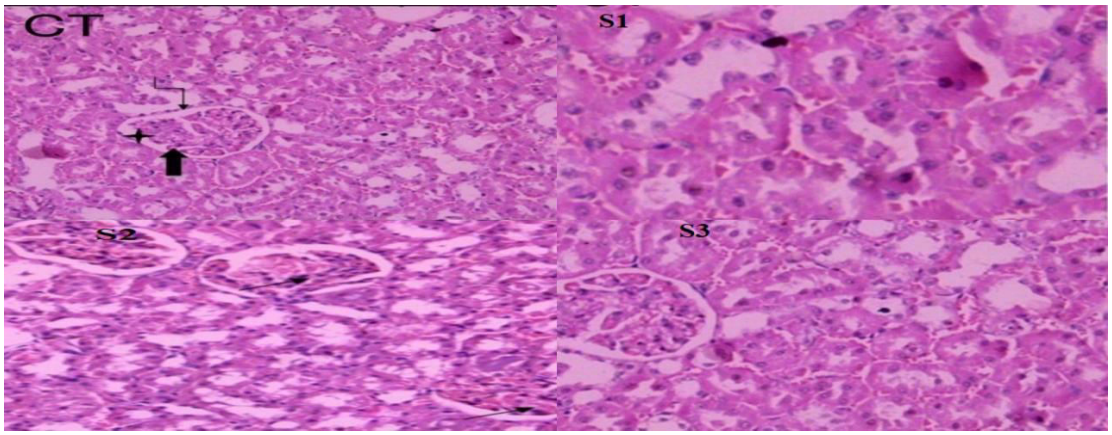

Fig. 7: Photomicrographs of kidney histopathology of animals after administration of SERB-111 as oral dose (Magnification:20x).

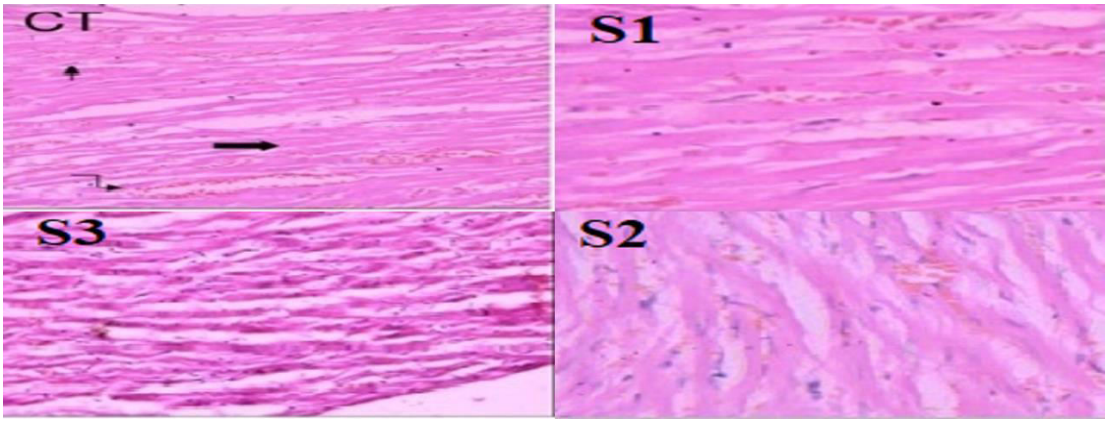

Fig. 8: Photomicrographs of heart histopathology of animals after administration of SERB-111 as oral dose (Magnification:20x).

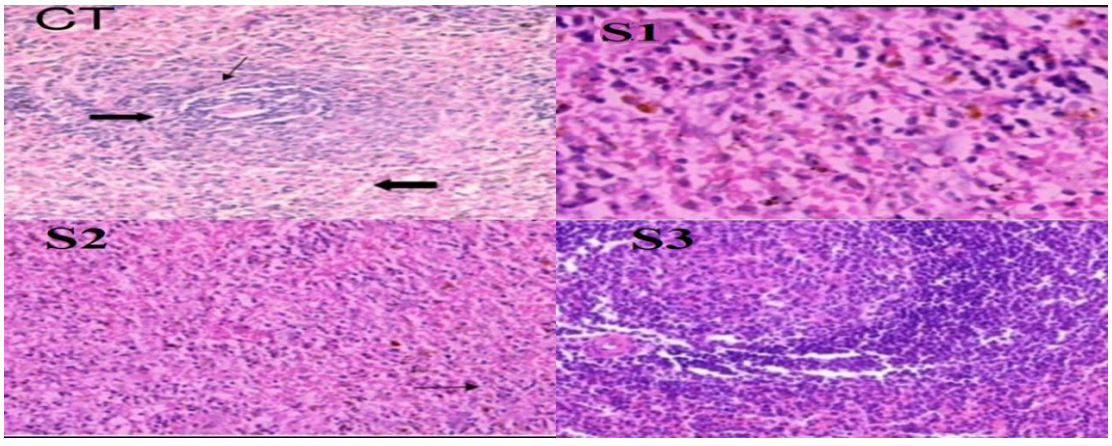

Fig. 9: Photomicrographs of spleen histopathology of animals after administration of SERB-111 as oral dose (Magnification:20x).

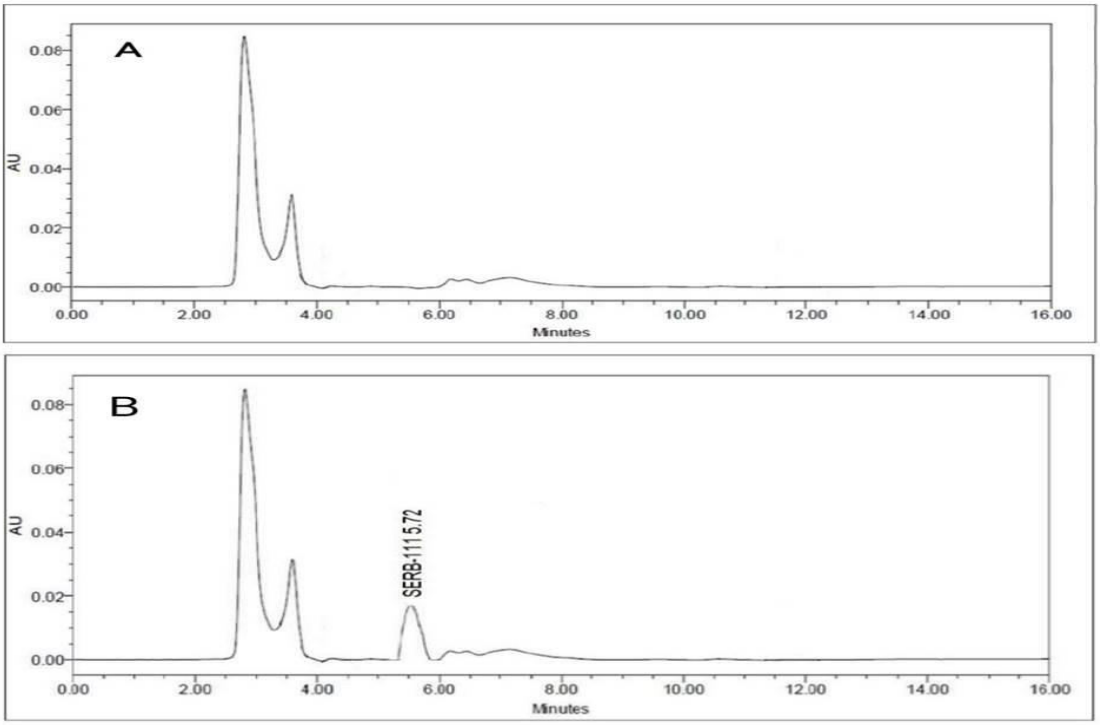

Fig. 10: Chromatograms for blank plasma sample (A) and blank plasma spiked with SERB-111 (B) having concentration of 10 µg/ml.

function parameters like urea, creatinine and uric acid had not revealed any significant changes ($p>0.05$). No statistically significant differences in the liver function parameters (ALT, AST, and ALP) were noted ($p>0.05$) with the exception of slight variations. Also, no relevant changes were found in total protein, albumin, and globulin (Table 6).

Histopathology of different organs:

(A) *Histopathology of Liver*:

The liver image of rat of control group (CT) showed normal portal triads (up arrow) with normal hepatocytes (S1) (Fig. 6). The liver of rat treated with SERB-111 (S1) showed a central vein (arrow) and portal triads (right arrow) showing normal architecture. Liver of SERB-111 treated rat (S2) showed mild congestion at the portal vein (down arrow) with normal surrounding hepatocytes. The liver of SERB-111 treated rat (S3) also showed mild congestion at the portal vein (down arrow) with normal surrounding hepatocytes. All the phomicrographs were acquired at 20x.

(B) *Histopathology of kidney*:

Histopathological examination of the kidney of rats in subacute toxicity (CT) (Fig. 7). The photomicrograph of kidney of control rat showed glomeruli (elbow arrow-connector); capillaries (up arrow) and Bowman's space (four-point star). The kidney of treated rats also showed normal tubules. The kidney of SERB-111 (S1) treated animal group revealed mild congestion at glomerular capillaries (arrow). The kidney of SERB-111 treated rats (S2) showed mild congestion at glomerular capillaries (arrow). However, the kidney of SERB-111 treated rat (S3) was observed with normal structure. All the photomicrographs of control and treated animal groups were acquired at 20x.

(C) *Histopathology of heart*:

Histopathological examination of the hearts of rats in subacute toxicity was observed by photomicrographs of heart cells of animals of control and treated groups (Fig. 8). The photomicrographs of heart of control group rats (CT) revealed normal architecture of the myocardium with capillaries (elbow arrow connector), an intercalated disc (up arrow), and

muscles. In photomicrographs of heart of SERB-111 treated rats (S1), normal architecture of the myocardium was observed. In treated rats with SERB-111 (S2), the photomicrographs showed normal architecture of myocardium with slight congestion of blood vessels (left arrow). The photomicrographs of heart of SERB-111 treated rat showed normal architecture of the myocardium.

(D) *Histopathology of spleen*:

Histopathological examination of the spleen of rats in subacute toxicity was observed by photomicrographs (Fig. 9). The spleen of control group of rats ((CT) showed the central artery (arrow); white pulp (right arrow) and red pulp (left arrow). The photomicrographs of spleen of rats treated with SERB-111 (S1) showed slight congestion of red pulp (up arrow). (S2) The spleen of rats treated with SERB-111 (S3) revealed mild congestion in the red pulp (arrow). The photomicrographs were acquired at 20x.

Estimation of Synthesized derivative (*SERB-111*):

After the HPLC estimation of novel compound SERB-111 in human plasma fluid, the retention time was found to be 5.72 min. The synthesized novel quinazoline derivative (SERB-111) was found stable in human plasma. The observed graphs of blank plasma and standard SERB-111 are shown in Figure 10.

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