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In-vitro and In-vivo Evaluation of Cytotoxic Activity of Rutin Berberine Loaded Polycaprolactone Nanoparticles against EAC Cell Line

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ABSTRACT

Background and Purpose: Rutin, a flavonoid found in various species of plants like buck wheat, citrus fruits, asparagus, apples, berries and tea was found to possess various biological properties like antioxidant, anti-inflammatory, vasodilation, enzyme regulation, anticancer and neuroprotective effects. On the other hand, berberine is an alkaloid found in berberis species, *Hydrastis canadensis and Coptic chinensis* with wide range of properties like anti inflammatory, antioxidant and anti-microbial. Therefore, in this study our goal was to formulate rutin and berberine in combination loaded polycaprolactone nanoparticles and test its invitro and in vivo anticancer potential. Materials and methods: The nanoparticles were formulated using single emulsification method. Their physicochemical properties were gauged using SEM, FTIR, DLS, Drug content and invitro drug release. The antineoplastic activity of the drug loaded nanoparticles in vitro was assessed by MTT assay. The in vivo anti neoplastic activity was studied in female swiss albino mice using the EAC cell lines. Results: in vitro neotoxicity studies revealed that the Rutin berberine loaded PCL nanoparticles were cytotoxic in dose dependent manner as calculated by its percentage cell viability and also showed cellular damage and cell death features of apoptosis. The in vivo neotoxicity study reports as studied by the histopathological evaluation of liver revealed the reservation of hepatocytes with reduction in the necrotic cells and dysplastic hepatocytes showing pattern of recovery with the presence of Kupffer cells and in kidney showing no changes in glomerular dilation and tubular necrosis. Nuclei and nephron cells remained normal with no necrosis infiltration showing the drug loaded nanoparticles possess good anti tumor property. The haematological parameters as measured by Hb level, RBC, WBC, ESR And CRP and biochemical parameters as measured by total protein, creatinine, bilirubin, SGPT, SGOT, ALP, albumin, urea, uric acid, SOD, catalase, GSH, GPx, GST and lipid peroxidase showed that the drug loaded PCL nanoparticles possessed good antineoplastic activity comparable with the standard drug Cisplatin. Conclusion: Rutin berberine loaded PCL nanoparticles are identified as an effective nanoparticulate system in treating the Ehrlich ascites carcinoma paving the way for further scientific research in humans.

Keywords: *Rutin, Berberine, PCL, anticancer activity, EAC (Ehrlich Ascites Carcinoma)*

1. INTRODUCTION

In the pharmaceutical field, nanotechnology has paved the way for the development of faster and more cost-effective treatments. Beyond cost and speed, pharmaceutical nanotechnology offers a unique advantage. Unlike traditional drugs that act systemically, nanotechnology medicines can be directed to the exact location of the affected area. This targeted approach not only enhances the effectiveness of the medicine but also reduces the likelihood of adverse effects [1].

Pharmaceutical nanotechnology plays a crucial role in cancer research, offering a distinctive strategy and comprehensive technology for early diagnosis, prediction, prevention, individualized treatment, and medication. Key priorities in nanotechnology research for pharmaceutical applications include personalized medication therapy and techniques for early disease detection [2].

In the medical field, pharmaceuticals are of paramount importance, and pharmaceutical manufacturing companies must leverage innovation, cutting-edge scientific and engineering expertise, and high-quality management principles to address challenges arising from new discoveries, such as novel drugs and nanotechnology, as well as evolving business models, including individualized therapy and genetically tailored treatment. It is essential for regulatory practices to be effective in managing these advancements [3].

In the modern era, cancer treatment has evolved to become more targeted and specific, aiming to provide greater benefits to patients with reduced side effects compared to traditional methods. The future of cancer treatment is anticipated to be significantly influenced by the development of nanotechnology, which holds the potential for single-agent diagnosis, simultaneous treatment, and real-time monitoring of patient responses to therapy. While still in the research phase, nanotechnology, particularly in the field of breast cancer, is poised for practical application, bridging the gap from laboratory studies to clinical implementation—an exciting prospect that mirrors advancements once thought to be the realm of science fiction [4].

Research on cancer treatment utilizing nanotechnology extends beyond modifying existing drugs to creating entirely new ones made possible by the unique properties of nanomaterials. Nanoparticles, being larger than cells, can accommodate various tiny molecules simultaneously. Ligands, such as DNA or RNA strands, peptides, aptamers, or antibodies, can be added to the nanoparticles' high surface area, making them more functional. These ligands have the potential to act as medicinal agents or regulators of nanoparticle behavior within biological organisms.

The convergence of therapeutic and diagnostic functions, known as "theranostic," becomes achievable through these characteristics, allowing the use of a variety of drugs in therapy. Nanoparticles' ability to absorb and re-radiate energy can be harnessed to damage diseased tissue, similar to laser ablation. Currently, surgery, radiation therapy, and chemotherapy are the main options for cancer patients, each with potential risks of harming healthy tissues or not completely curing the cancer. Nanotechnology equips surgeons with tools to simplify tumor removal during surgery, direct chemotherapy precisely at cancer cells, enhance the effectiveness of radiation therapy, and improve other modern treatment modalities. These advancements have the potential to lower patient risks and increase their chances of survival. Innovative nanoparticle packaging and active pharmaceutical compounds open the door to a broader range of active components, extending beyond those with acceptable pharmacokinetic or biocompatibility behavior.

Owing to the various setbacks of synthetic anticancer drugs many of the herbal anti cancer agents are being explored these days to flaunt their potential in treating various types of cancer. Rutin, a flavonoid found in various species of plants like buck wheat, citrus fruits, asparagus, apples, berries and tea was found to possess various biological properties like antioxidant, anti-inflammatory, vasodilation, enzyme regulation, anticancer and neuroprotective effects. On the other hand, berberine is an alkaloid found in berberis species, *Hydrastis canadensis and Coptic chinensis* with wide range of properties like anti inflammatory, anticancer, antioxidant and anti-microbial.

Converting these plant products in to nanoparticles range and formulating them in to nanoparticles using the biodegradable polymers like polycaprolactone is a challenging task and can be fruitful in treating various cancers.

Ehrlich ascites carcinoma EAC, is a well-established model for studying the tumor biology for the development of anti-tumorigenic agents. EAC cells fill the mouse peritoneum by rapid cell division causing a local inflammatory reaction due to increased vascular permeability resulting in intense edema formation, cellular migration and progressive ascitic fluid formation. *In vitro* and *in vivo* models are important tools for pharmacological investigations. Ehrlich tumors in mice are one of these models, used for more than a century.

Therefore the present study focused in developing rutin and berberine nanoparticles using the biodegradable polymer PCL and and evaluating its in vitro and in vivo anticancer potential.

2. MATERIALS AND METHODS:

Rutin and berberine were purchased from Sigma Aldrich, Polycaprolactone was purchased from SRL Chemicals India. All the other reagents used in this study is of Laboratory grade.

FORMULATION OF RUTIN-BERBERINE POLYCAPROLACTONE NANOPARTICLES BY SINGLE EMULSIFICATION METHOD:

100 mg of polycaprolactone was dissolved in 5 ml of methylene chloride to obtain a transparent solution and then added dropwise to 1% PVA solution in water and sonicated 10 times using ultrasonic processor at 200 W under ice bath condition. The resulting primary emulsion was added at a controlled rate 2 ml/min into 0.5% aqueous PVA solution in which 50 mg of rutin and 50 mg of berberine are dissolved. To eliminate any excess drugs and unentrapped polymer, the solution was subjected to centrifugation. This process involved centrifuging the solution at 12,000 rpm for 20 minutes. After centrifugation, the resulting nanoparticles were subjected to freeze drying to remove any remaining moisture. The RB-PCL NPs were stored in a vacuum desiccator for preservation [5,6]. This method was employed to produce RB-PCL nanoparticles, offering a means to enhance the stability and bioavailability of rutin and berberine for various potential applications.

CHARACTERIZATION OF NANOPARTICLES:

The nanoparticles were evaluated for its morphology using SEM analysis, zeta potential using zeta sizer nano instrument, drug content using the HPLC method, in vitro drug release, % encapsulation efficiency and stability.

SEM Analysis:

Morphological characterization of the nanoparticles was performed using SEM. To enhance the conductivity of the samples and to ensure accurate imaging, a thin layer of carbon was coated onto the samples. SEM utilizes an electron beam that passes through the sample, generating signals containing information about the sample's surface topography, composition, and other properties. The aim was to visually inspect and understand the physical characteristics and surface morphology of the nanoparticles in the optimized formulation. SEM provides high-resolution imaging, allowing for detailed examination of nanoparticle structures. The technique reveals information about the surface topography and composition of the nanoparticles. Coating the samples with carbon is a common practice in SEM to ensure optimal electron beam conductivity and prevent charging effects. The electron beam passing through the sample generated signals containing valuable information about the nanoparticles characteristics. The morphological characterization using SEM is instrumental in gaining insights into the physical attributes of the nanoparticles. It provides a visual representation of their size, shape, and surface features. This information is crucial for validating the success of the optimization process and understanding the structural properties that may impact the performance of the nanoparticles in various applications [7,8].

Zeta potential analysis:

Zeta potential was determined utilizing a Zeta sizer Nano instrument equipped with a He-Ne laser, operating at a maximum power of 4 mW, and offering a measurement precision of 0.12 m•cm/V•s. The colloidal suspension, confined within quartz cuvettes, underwent meticulous examination using the Zeta sizer Nano instrument. The NIST SRM1980 standard material of reference provided a benchmark for accurate zeta potential determination. The standard deviation, along with the recorded zeta potential values, offered insights into the stability and electrokinetic behaviour of the colloidal system. This detailed zeta potential analysis, integrating both standard material reference and advanced instrumentation, contributes to a comprehensive understanding of the aqueous system's characteristics and holds significance in diverse scientific and industrial applications [9-12].

Estimation of Drug Content

The determination of drug content in Rutin-Berberine PCL nanoparticles was conducted using an HPLC method. The process involved specific steps to ensure accurate measurement and quantification.

Sample Preparation*:* Approximately 50 mg of PCL nanoparticles taken. The nanoparticles were mixed with ethanol, and the mixture was vortexed for five minutes to facilitate thorough mixing. Ultracentrifugation was employed to separate the components. The samples were subjected to ultracentrifugation for 30 minutes at 4°C and 20,000 rpm.

A developed HPLC method utilizing a gradient technique was applied. Acetonitrile and 0.1% phosphoric acid was used as mobile phase with the flow Rate of 1 mL/min. C_{18} reverse-phase column was used with UV Detector (Berberine: 230 nm and Rutin: 256 nm). The amount of free (untrapped) drugs (Rutin and Berberine) in the supernatant was determined. The drug content (DC) for the prepared nanoparticles was calculated using the formula:

DC (%) = (total amount of drug taken –Untrapped free drug)/weight of NPs × 100

The equation allows for the quantification of the percentage of drug content in the nanoparticles, providing insights into the efficiency of drug encapsulation. The use of HPLC ensures high precision and accuracy in drug content determination. Ultracentrifugation is a crucial step in separating the untrapped drugs from the nanoparticles, facilitating accurate analysis. The application of the described methodology, including ultracentrifugation and HPLC analysis, ensures a reliable and

precise estimation of drug content in the Rutin-Berberine nanoparticles. This information is vital for assessing the encapsulation efficiency and overall quality of the prepared nanoparticles [13-15].

Drug Entrapment Efficiency

The entrapment efficiency (EE) of nanoparticles was evaluated through an indirect method, involving the quantification of unentrapped drugs within the nano-suspension. This process utilized High-Performance Liquid Chromatography (HPLC) to determine the amount of berberine and rutin that remained unentrapped. In the experimental procedure, approximately 50 mg of the PCL nanoparticles (NPs) were dispersed in ethanol, followed by vortexing for 5 minutes to ensure thorough mixing. Subsequently, ultracentrifugation at 20,000 rpm at 4°C for 30 minutes was employed to separate free, unentrapped drugs from the nanoparticle suspension. The supernatant liquid, containing the unentrapped drugs, was carefully collected. The collected supernatant underwent suitable dilution with methanol, and the diluted solution was subjected to HPLC analysis to determine the concentration of unentrapped drugs (berberine and rutin). The entrapment efficiency was then calculated using the formula:

EE (%) = (total amount of drug taken –unentrapped free drug) / total amount of drug × 100

Entrapment efficiency is a critical parameter reflecting the efficacy of a drug delivery system, indicating the proportion of the drug successfully encapsulated within the nanoparticles. By quantifying the unentrapped drugs using HPLC analysis, this method provides valuable insights into the effectiveness of the formulation in encapsulating berberine and rutin within the nanoparticles. Such information is crucial for optimizing drug delivery systems and ensuring the controlled release of therapeutic agents [16].

***In vitro* Drug Release Studies**

In the investigation of *in vitro* drug release, phosphate buffer pH 7.4 served as the dissolution media. The study utilized nanoparticles loaded with drugs (50 mg), and a dissolution apparatus was employed to simulate drug release under controlled conditions.

A total of 900 mL of phosphate buffer was introduced into the dissolution apparatus, maintaining the pH within specified limits. The temperature was carefully regulated at 37±1°C to replicate physiological conditions, and the rotation speed was set at 100 rpm for consistency. The study spanned over a 24-hour period, with sampling occurring at specific intervals: 0th hr, 1 hr, 2 hrs, 3 hrs, 4 hrs, 6 hrs, 8 hrs, and 10 hrs.

At each time point, 1 mL of samples was withdrawn, and an equal volume of freshly prepared buffer solution was introduced to replace the withdrawn sample, ensuring sink conditions. The concentration of the released drug in the withdrawn samples was analyzed using High-Performance Liquid Chromatography (HPLC). The quantification was achieved through the calibration curve method, establishing a reliable correlation between HPLC results and known concentrations.

These *in vitro* drug release studies provide essential insights into the release kinetics of drugs from nanoparticles. The controlled conditions and systematic sampling strategy allow for a comprehensive understanding of the drug delivery system's performance over time. The HPLC analysis, coupled with the calibration curve method, ensures accurate and quantitative assessment of drug release, contributing to the evaluation of the drug delivery system's effectiveness [17,18].

Stability Studies

To assess the stability of the PCL nanoparticles, a rigorous stability study was conducted. The nanoparticles were carefully housed in borosilicate glass vials and sealed securely. Over a 90-day period, these vials were subjected to storage under controlled conditions, including room temperature at 40°C±2°C with a relative humidity (RH) of 70%. Variations in humidity were also introduced, ranging at ±5% RH. Observations and analyses were carried out at crucial intervals, specifically at the 30th day, 60th day, and 90th day of storage. The stability parameters evaluated included drug content, drug release, and any changes in the physical appearance of the nanoparticles. The drug content assessments aimed to identify potential deviations from the initial drug content, serving as an indicator of stability issues. Monitoring the drug release profile over time provided insights into the sustained release characteristics of the nanoparticles. Additionally, careful observation of any alterations in the physical appearance, such as changes in colour, texture, or other visible characteristics, was a key aspect of the stability evaluation. The meticulous observation and analysis of each sample at the designated time points allowed for the identification of variations that could signify changes in the stability of the nanoparticles over the storage duration. Stability studies are paramount for ensuring the reliability and performance of drug delivery systems over an extended period, aiding in the identification of potential formulation issues or degradation. The outcome of these stability studies contributes to the assessment of the nanoparticles' shelf-life and overall stability under different storage conditions [17,18].

Cell Culture

EAC cell lines were procured for the *in vitro* anti-cancer studies. These cells play a crucial role in assessing the cytotoxicity potential of the nanoparticles.

MTT Assay for Cytotoxicity Studies

The MTT assay was conducted to evaluate the cytotoxic potential of the PCL nanoparticles. This involved creating samples of nanoparticle suspensions with concentrations ranging from 6.25 to 100 µg/ml. Cell viability count was assessed after the MTT assay to determine the cytotoxicity potential of the nanoparticles. The procedure involved the use of various materials and methods. For the MTT experiment, samples of a suspension of nanoparticles were created using serial dilutions that ranged from 6.25 to 100 µg/ml. EAC (Ehrlich-Lettre Ascites-E) cell lines was obtained from NCCS. The cell lines were stored in media that had been grown. In order to nourish the cells in culture, 10% inactivated Foetal Bovine Serum (FBS) was added. Medium was incubated until confluent in a humidified atmosphere of 5% CO_2 at 37°C with antibiotics such as penicillin (100 IU/ml) and streptomycin (100 µg/ml). Each well of 96-well plates was filled with around 100 µl of a cell suspension with a density of 1×10^4 cells per well, and the plates were incubated for 24 hours. The partial monolayer in the cell suspension was examined after 24 hours. There was a removal of the supernatant. The monolayer was rinsed and then dipped one again in the medium. About 100 µl of test material, with concentrations ranging from 6.25 to 100 µg/ml, was added to the complete monolayer in microtiter plates. The plates were then incubated for 24 hours at 37°C in a 5% CO_2 environment. After incubation, 1 mg/ml of MTT in PBS was transferred to every well, and the resulting supernatant solutions were discarded. The plate was kept in a 37°C/5% CO_2 incubator for 4 hours. After the supernatant had been drained from the plate, 100 ml of DMSO was added to disintegrate the forms of formazan that had formed. The absorbance was measured at 570 nm using a microplate reader. The common medication cisplatin underwent the same process. The following formula was used to get the cell viability percentage [19-28].

% of viability = Sample absorbance/Control absorbance x 100

***In vivo* Anticancer Studies**

The *in vivo* anticancer activity of the RB-PCL nanoparticles was investigated using Swiss albino mice as the tumor model. Tumors were induced in the mice through inoculation with EAC. Female Swiss albino mice, aged 10 to 12 weeks and weighing 20 to 25 g, were obtained from a controlled in-bred colony. The mice were maintained under regulated conditions, including a temperature of approximately 25°C, 50% humidity, and a light/dark cycle of 12 hours. Throughout the experiment, the mice had free access to sterilized food and water. A group of 8 animals was accommodated in each polypropylene cage, which was filled with sterile paddy husk procured locally and used as bedding throughout the experiment. This bedding material ensured a conducive environment for the well-being of the mice during the study. The *in vivo* anticancer studies involved the administration of the optimized nanoparticles to the Swiss albino mice with induced tumors. These studies contribute crucial insights into the potential therapeutic efficacy of the nanoparticles in a living organism, paving the way for a deeper understanding of their anti-cancer properties and possible applications in cancer treatment [19-28].

Induction of Tumors in Recipients of Transplanted Ehrlich Ascites Carcinomas:

For tumor induction, fresh ascites fluid, diluted at a ratio of 1:5 with saline, was subcutaneously injected into each mouse model. The average number of cells in the inoculums that led to tumor formation was approximately 2.5×10^{-6}.

Pharmacological - Experimental Design:

Female Swiss albino mice, weighing 20-25 g each, were randomly divided into 7 groups (n=8) with the following allocations:

Group (1) - Normal: Given sterile saline (0.1 ml/mouse) intraperitoneally once daily for 14 days.

Group (2) - Tumor Control: Injected with EAC cell line (2.5×10^6 cells/0.1 ml/mouse) intraperitoneally.

Group (3) - Rutin and Berberine Treatment: Treated with Rutin and Berberine at 10 mg/kg for 14 days.

Group (4) - RB–PCL NPs Treatment: Treated with RB–PCL NPs at 50 mg/kg for 14 days.

Group (5) - Standard Drug Cisplatin Treatment: Treated with the standard drug Cisplatin at 2 mg/kg for 14 days.

After the 14-day treatment period, the anti-tumor activity was assessed. In each group, 8 mice were anesthetized, sacrificed, and blood was drawn for subsequent hematological, biochemical, and histological tests. This comprehensive experimental design provides valuable insights into the potential anti-tumor effects of the various treatments on mice with transplanted Ehrlich ascites carcinomas [29-38].

Sample collection:

Sample preparation for serum biochemistry

At the end of the 14th day, the blood of the all the 8 animals in a group were collected. Samples of blood were withdrawn from the tail vein. The samples were collected in plain sterile containers without anticoagulant for serum separation. The obtained blood samples were centrifuged for 15 minutes at 3000 rpm after being held stationary for a while. Once the reaction was complete, the supernatant was removed and stored at -20°C for further biochemical analysis.

Biochemical in serum samples were tested for Serum glutamate pyruvate transaminase (SGPT) and Serum glutamate oxaloacetate transferase (SGOT), Alkaline phosphatase (ALP), Total protein, Albumin, Urea, Uric Acid & Creatinine and

Bilirubin.

Immediately after sacrificing the animal, tumor tissues were taken, weighed from groups. Following incubation in a 10% buffer formalin solution for 30 minutes at room temperature, tumour tissue specimens were processed for histological evaluation [19-28].

Hematological parameters

At the end of the 14th day blood was collected from 6 mice from each group. Blood samples were collected in EDTA containers and subjected to analysis of hematological parameters like Hb level, PCV, RBC, WBC and Differential leucocytes count.

Collection of Blood Samples

On day 14 of treatment, blood was collected from the orbital sinuses of the mice using EDTA-treated, sterile test tubes. In order to calculate packed cell volume (PCV), the micro hematocrit technique was used. The hemoglobin concentration (Hb) was determined using an automated blood biochemistry analyzer based on the cyanmethemoglobin procedure. Using automated hematology analyzer, red blood cell count (RBC) and total white blood cell count (WBC) were determined. Leishman technique was adopted to determine Differential leucocytes count of the mice in all groups.

Biochemical estimation:

Preparation of Liver Tissue Homogenate

Liver Tissue of the treated and untreated groups of mice was homogenized using a Teflon homogenizer. 1g of wet liver tissue to 10 times w/v of 0.05 M ice-cold phosphate buffer (pH 7.4) was homogenized. The homogenate was then combined 1:1 using 10% Tri chloroacetic acid. For 20 minutes, at 15000 rpm and 4°C, the mixture was centrifuged in a cooling chamber. Separating the supernatants required centrifugation at 5000 rpm, 4°C, for 10 minutes. The enzymes GST, SOD, catalase, and ALP were measured in the second supernatant.

Preparation of Kidney Tissue Homogenate

Kidney Tissue isolated from treated and untreated groups of mice were separately homogenized using a Teflon homogenizer. 1g of wet kidney tissue to 10 times w/v of 0.05 M ice-cold phosphate buffer (pH 7.4) was homogenized. The homogenate was then combined 1:1 using 10% Tri chloroacetic acid. For 20 minutes, at 15000 rpm and 4°C, the mixture was centrifuged in a cooling chamber. The supernatants were then centrifuged again at 5000 rpm and 4°C for 10 minutes to remove any remaining debris. GST, SOD, catalase, and TBARS estimations were made using the second supernatant.

Histopathology evaluation

Liver

The remaining portion of the same liver tissue was dissected and fixed in 10% formal saline embedded in paraffin, sectioned and stained with Hematoxylin and Eosin (H&E) [29-38].

Kidney

The remaining kidney tissue was divided into sections, embedded in paraffin, and preserved in 10% formalin. For inspection under light microscopy, Hematoxylin and eosin were used to stain the kidney slices before they were put on a slide.

3. RESULTS AND DISCUSSION

Formulation of Rutin Berberine PCL Nanoparticles:

Rutin berberine PCL nanoparticles were formulated successfully using the single emulsification technique.

Characterization of Rutin Berberine PCL Nanoparticles:

Scanning electron microscopy

The characterization studies using scanning electron microscopy showed the nanoparticles had spherical form and the average size of the formulated nanoparticles was 97.3 nm as shown in figure 1.

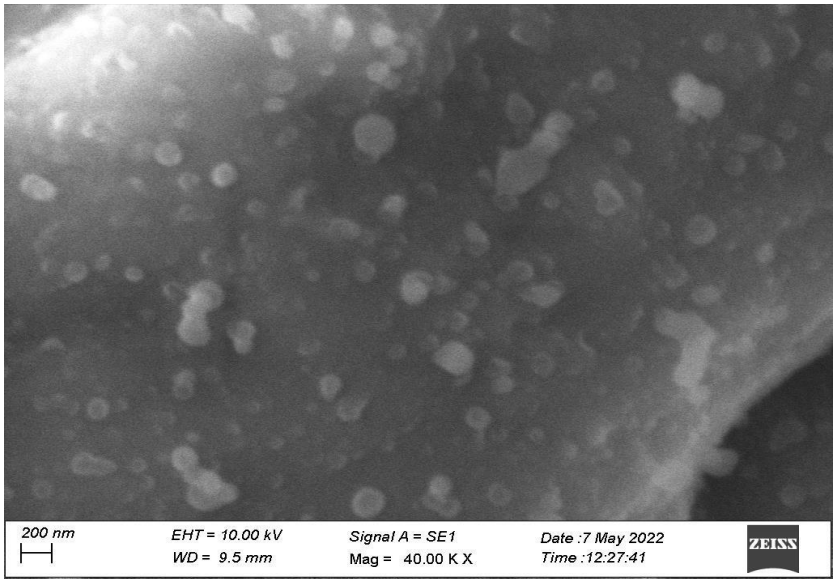

Figure 1: SEM Image of RB-PCL-Nanoparticles

Zeta Potential:

Zeta potential is described as electrical potential between the medium and the layer of fluid attached to the dispersed particle. It is the measurement of the magnitude of the charge repulsion or attraction between the particles and is the most important elementary variable which is known to affect stability. The zeta potential of the formulated nanoparticles was found to be -6.41 mV as shown in figure 2. The higher the zeta potential the particle aggregation will be reduced by the repulsive force and more stable the nanoparticles. This shows the stability of the formulated nanoparticles as indicated by high zeta potential value.

Sample Name: RBC-PCL	Mean	Area (%)	St Dev (mV)
Zeta potential (mV): -6.41	Peak 1: -6.41	100	4.96
Zeta deviation (mV): 4.96	Peak 2: 0.00	0.0	0
Conductivity (mS/cm): 0.182	Peak 3: 0.00	0.0	0

Result quality: Good

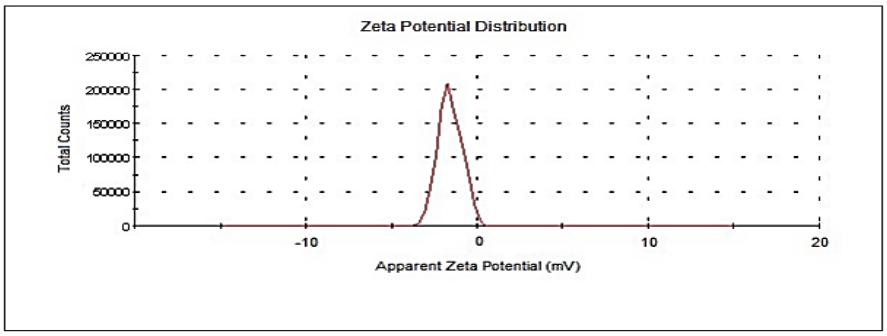

Figure 2: Zeta potential distribution RB-PCL Nanoparticles

Drug content:

The drug content of the rutin berberine loaded PCL nanoparticles was found to be 99.12% and 99.7 % for rutin and berberine respectively . The nanoparticles contained the highest amount of medication content. The HPLC are shown in figure 3 and 4 respectively.

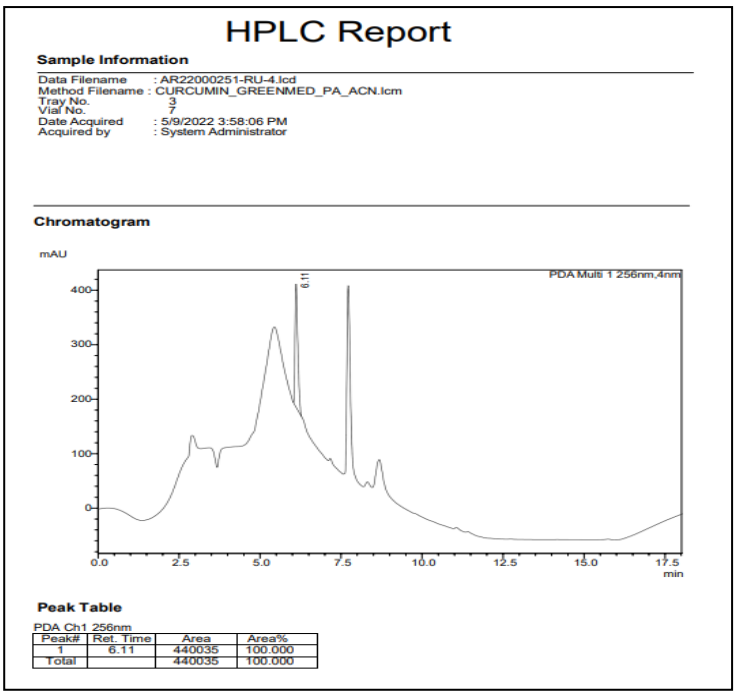

Figure 3 : RB-PCL- NPs % Rutin content -256nm

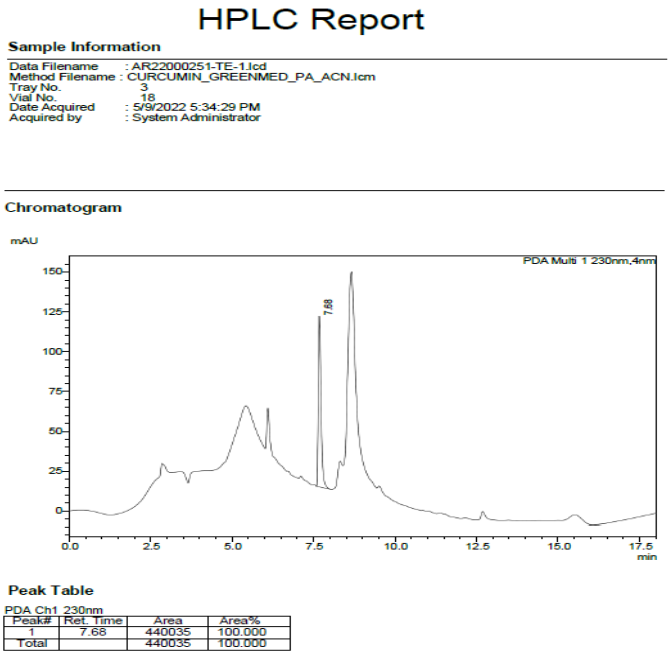

Figure 4: RB-PCL- NPs % Berberine content -230nm

Drug entrapment efficiency:

EE has a great influence on the success of the formulation development. It is necessary the drugs are encapsulated in the NDDS, so the objective of the DDS is attained. The % EE of berberine and rutin was determined by HPLC analysis. Berberine was detected at 230 nm and rutin at 256 nm. In RB-PCL-NPs; Berberine and rutin were successfully encapsulated with an efficiency of 97.82 and 82.81 % respectively. The chromatograms are shown from figure 5.

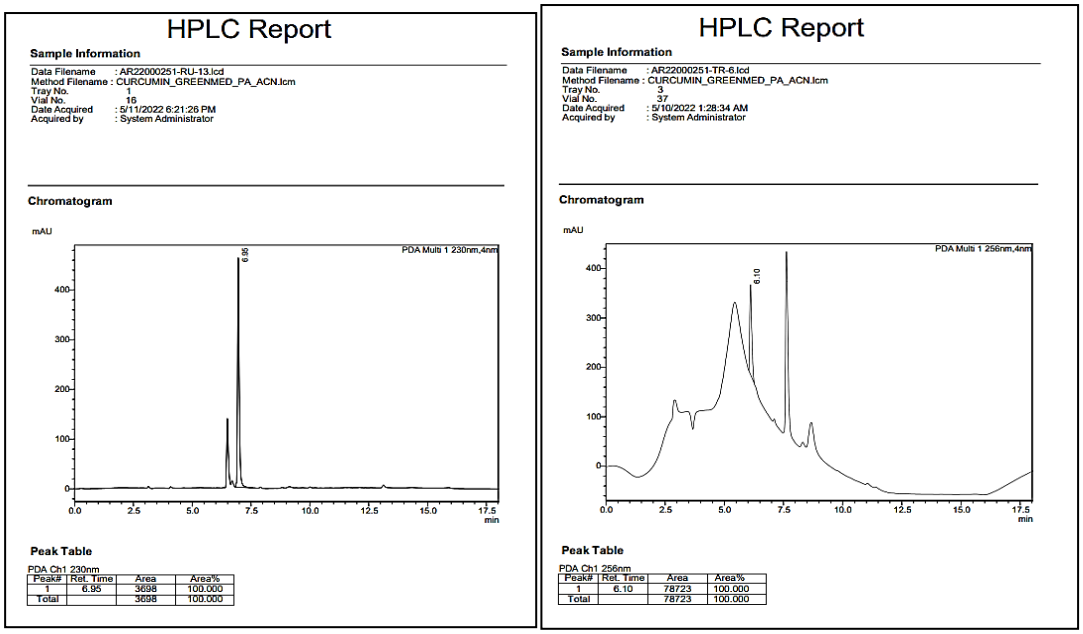

Figure 5: RB-PCL- NPs HPLC chromatogram for-untrapped berberine and Rutin at 230nm

***In vitro* Drug Release of Rutin and Berberine in RB PCL NPs:**

The release profile of berberine and rutin-loaded PCL nanoparticles is shown graphically in the figure 6. The pattern demonstrates that the PCL nanoparticle releases berberine and rutin in amounts of 94.92 and 94.22 %, respectively, By 4 hrs, 50% of berberine and rutin is released from RB-PCL-NPs. But both the drugs are released gradually from the nano particles. The HPLC peak area of the drugs was interpolated in the linearity chart of the respective drugs and the % drug release pattern was determined for both the drugs in the nanoparticles. As a result, the medicine was released at a rate of 70 % over the first six hours, increasing to 80 % in eight hours after a sustained release of 50 to 55 % in four hours. This release pattern can be supported by the following criteria: The burst release may have been brought on by the drug that was surface-adsorbed, and the extended pattern might have been brought about by the gradual degradation of the carrier matrix. The sluggish drug diffusion out of the polymer matrix contributes to the continuous release pattern.

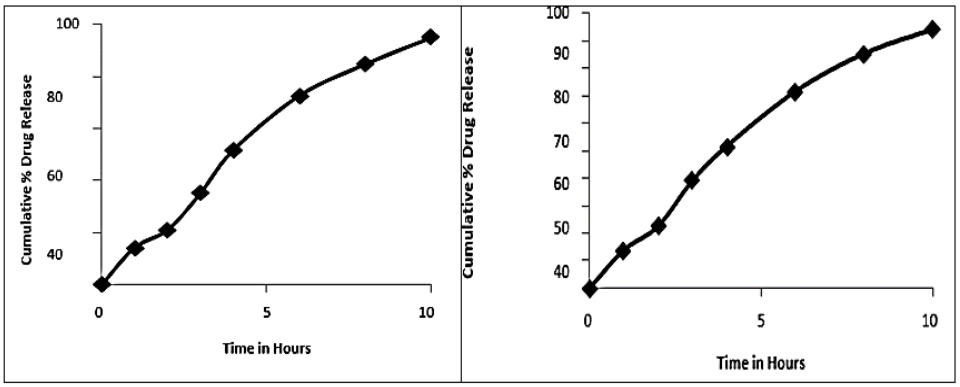

Figure 6: *In vitro* drug release study Rutin and Berberine from RB-PCL NPs

Stability Studies:

Stability tests on nanoparticles loaded with drugs were based on the results of the physical characters of NPs on storage for up to 90days. It also deals with the drug content and drug release pattern on 0, 30, 60 and days of storage. The physicochemical properties of RB-PCL- NPs studied for 90 days under refrigeration are compiled in Table 1. Physical characteristics including color and appearance did not significantly change. It shows that the NPs are physically compatible. The % of Rutin-content and Berberine content in the drug loaded nanoparticles decreased from are 99.12% to 93.93 % and 99.70 % to 94.18 % respectively. The % drug release of rutin and berberine from the NPs decreased from 96 % to 92 % and from 94% to 90 % from 0 day to 90 days respectively. From the results of the percentage of drug release and tpercentage of

drug content, it can be concluded that RB-PCL-NPs should be kept chilled throughout storage.

Table 1: Summary of Stability Study Results under Different Storage Conditions

Drugs	Parameters	Days		
		After 30 days	After 60 days	After 90 days
Berberine	Color and appearance	No change	No change	No change
	% Drug content	97.52±1.67	96.85±1.55	94.18±1.87
	% Drug release	94.93.±2.61	92.62±1.98	90.49±1.53
Rutin	Color and appearance	No change	No change	No change
	% Drug content	95.85±1.48	94.42±1.80	93.93±2.76
	% Drug release	96.92±1.97	94.73±2.64	92.51±1.45

MTT Assay of RB-PCL NPs :

The % cell viability in RB-PCL NPs treated EAC cell lines showed a gradual decrease in number of living cells in from 78.16 % to 5.99 % of cell viability with the increase in concentration rutin berberine loaded PCL NPs. The nanoparticles are cytotoxic from an initial concentration of 6.25 µg/ ml and show a gradual increase in cytotoxicity and found to be maximum at a dose of 100 µg/ ml. The data is tabulated in table 2. The microscopic images of the treated and untreated (control) cell lines are shown in figure 7. The IC_{50} value of RB-PCL NPs was determined and found to be 20.81µg/ml as shown in table 3. The morphological alterations of the control EAC cell lines and the treated cell lines after treatment with increasing levels of the NPs was studied under microscope. The microscopic images are shown in figure 8. The control cell lines were found adhering to surface of the tubes and also with neighbouring cells. The cells were intact and were of normal shape and cell death characters of apoptosis like shrinking in size, rounding, membrane blebbing and the cells loses contact with the adjacent cells. Compared to the control cell lines the treated cell lines show typical damage to cell structure and the cell damages increased with concentration of drug loaded PCL nanoparticles.

Table 2: MTT assay - Cell viability- NP2 (RB-PCL-NPs) treated group

S.No	Concentration µg/ml	% Cell Viability
1	6.25	78.16 ± 0.005
2	12.5	69.16 ± 0.004
3	25	52.72 ± 0.003
4	50	18.17 ± 0.005
5	100	5.99 ± 0.005
6	Control	100
7	IC_{50}	20.81µg/ml

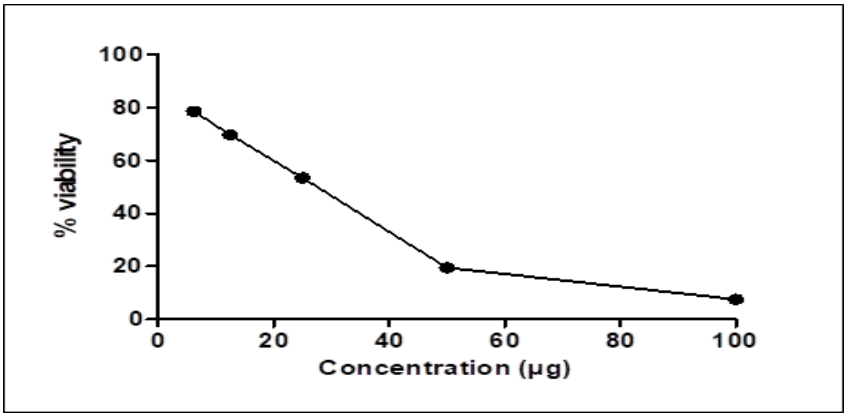

Figure 7: Cell viability Vs Concentration- NP2 treated group

Table 3: log (inhibitor) vs. normalized response of NP2

S. No	log(inhibitor) vs. normalized response -- Variable slope	
1	Best-fit values	
2	Log IC_{50}	1.473
3	Hill Slope	-2.91
4	IC_{50}	29.7
5	Log IC_{50}	0.009451
6	Hill Slope	0.1627
7	Log IC_{50}	1.452 to 1.493
8	Hill Slope	-3.261 to -2.558
9	IC_{50}	28.34 to 31.13
10	Degrees of Freedom	13
11	R^2	0.9944
12	Absolute Sum of Squares	128.9
13	Sy.x	3.149
14	Number of points Analysed	15

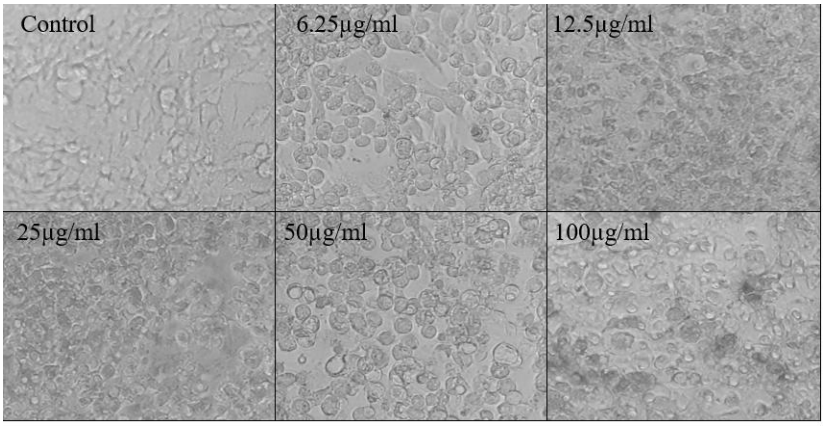

Figure 8: MTT assay - cell viability of EAC cell line using RB-PCL-NPs

Haematological Parameters

The haematogical parameters like HB level, RBC, WBC, ESR and CRP level were analysed for the action of induced tumour followed by treatment. The blood samples were obtained from all the 8 animals in each group and used for the study. Whole blood was used for the study. The results are tabulated in table 4. The HB level of the RB PCL NPs treated animal showed normal level of Haemoglobin. The RBC content of the animals treated with RB PCL NPs showed normal RBC count of 7.4 million. It is similar to the normal levels. The WBC count was although somewhat higher than normal but similar to the standard drug treated group. May be an increase in the dose level of the drugs in the PCL NPs may have a good effect. The ESR of the RB PCL NPs treated animals was higher than the normal control but closer to standard drug Cisplatin treated groups. It may be due to the residual tumour effect in the animals. CRP is a classical acute-phase reactant protein that can bind with and activate cell surface receptors, such as FcγRI/II and FcαRI. C-reactive protein (CRP) acts as a biomarker reflecting different degrees of inflammation. Many reports have suggested that there is a close relationship between CRP and various cancers. CRP has been widely used not only as a predictive marker of infection, cardiovascular diseases and hypertension, but also as a risk marker of various cancers. In this study the CRP level is 11.56, the highest in tumour control group II. It is the least 1.83 in the Normal group. The animals in RB PCL NPS treated group showed a decreased CRP level

of 3.26 when compared to the tumour control group showing a CRP level of 11.56. The standard drug control group cisplatin treated showed a CRP level of 2.33 comparatively. The ESR of the RB PCL NPs treated animals was higher than the normal control but closer to standard drug Cisplatin treated groups. It may be due to the residual tumour effect in the animals.

Table 4: Haematological parameters- *in vivo* anticancer studies for Nanoparticles

Group name	Hb Level* gm/100ml	RBC Count*	WBC Count*	ESR*	CRP*
Normal	14.76±±0.37	8.96±0.802	7.66±0.503	9.03±0.115	1.83±0.251
Tumour Control	7.83±0.602	4.23±0.450	19.16± 0.251	19.53±0.832	11.56±0.776
Rutin& Berberine (RS)	9.76±0.351	6.76±0.416	13.76± 0.907	13.66±0.709	3.96±0.208
RB-PCL-NPs	11.43±0.907	7.43±0.321	11.93± 0.635	12.63±0.305	3.26±0.351
Standard (Cisplatin)	10.06±0.907	8.03±0.230	10.53± 0.351	10.83±0.850	2.33±0.115

(*n=3. Average of three replicate determination; ± standard deviation)

Biochemical parameters

Essential parameters like urea level, uric acid level, Total protein, bilirubin level, total protein, albumin level was determined to study the effect of the tumour induction on the internal organs as well as the effect of the drug loaded nanoparticles on the decrease of tumour. The results are presented in table 5 . The results of the RB loaded PCL nanoparticle treated groups were compared with the normal and control group and also with the standard drug cisplatin treated groups. The results show that RB-PCL-NPs treated animals showed approximately the same results as that of standard drug treated group of animals and also similar to that of the normal control group of animals, showing that the nanoparticles possessed good anti cancer activity. The total protein level of RB PCL NPs treated animals was found to be 7.6 g/dl that is near to the standard drug treated group of about 8.1g/dl. The creatinine level of RB PCL NPs treated groups was found to be 3.3 mg/dl and that of standard drug was 2.9 mg/ dl, which showed that the RB PCL NPs regained the kidneys. The bilirubin level was nearly or more or less at 3 mg /dl same as that for the standard drug treated groups shown in table 5. The SGOT and SGPT levels of the RB PCL NPs treated groups was found to be 48.8 u/L and 34.50 u/L) were near to that of standard drug treated groups (45.03 u/L and 32.46 03u/L). When compared to the standard drug Cisplatin the NPs treated groups gave similar effect.

Table 5: Biochemical parameters *in vivo*anticancer studies for Nanoparticles (Serum 1)

Group name	Total protein (g/dL)	Creatinine (mg/dL)	Bilirubin (mg/dL)	SGPT (U/L)	SGOT (U/L)
Normal	8.6±0.17	1.4±0.1	2.3±0.1	23.96±1.35	43.6±1.743
Tumour Control	3.4±0.20	4.1±0.20	6.3±0.15	59.96±1.22	95.43±2.076
Rutin& Berberine(RS)	6.6±0.25	3.2±0.15	3.9±0.15	38.26±1.10	51.33±1.550
RB-PCL-NPs	7.6±0.20	3.3±0.15	3.3±0.15	34.50±1.35	48.8±1.351
Standard (Cisplatin)	8.1±0.20	2.9±0.15	3.2±0.15	32.46±1.28	45.03±2.324

***n=3. Average of three replicate determinations; ± standard deviation**

Superoxide dismutase (SOD) is a vital enzyme that is entirely responsible for the elimination of superoxide radicals. The superoxide dismutase family plays an essential physiological role in mitigating toxic effects of ROS. SODs, providing the first defending line against superoxide radicals, have been studied in human cancers. SOD dismutase converts the superoxide anion into one oxygen molecule and one hydrogen peroxide molecule, which is detoxified into water by glutathione peroxidase. During carcinogenesis and tumour progression, the level of NO activity increases and the level of SOD decreases. These NO and SOD levels might also serve as therapeutic targets and a guide for prognosis in patients suffering from such a malady. Carcinogenesis is observed to be suppressed, both *in vitro* and *in vivo* studies due to the over expression of

antioxidant enzymes, many studies have showed that significant decrease in SOD enzymatic activity in a variety of human cancers. Thus, any increase in the levels of oxidative stress promote tumour formation and cancer incidence. Increase in the activity of antioxidant enzymes like SOD, CAT, GSH and GPx can be observed in RB loaded PCL NPs. The results are tabulated in table 6 and 7. The results show that the RB-PCL-NPs treated animals show an increase in activity of the anti-oxidant enzymes, similar to that of the standard drug (cisplatin) treated animals group. The lipid peroxidation assay of NPs is based on TBARS assay. It was found that the lipid peroxidation or removal of TBARS or MDA to be 0.13 in normal healthy mice cells. Similarly, the RB loaded PCL NPs show similar results in TBARS assay less than 0.3. But tumour control groups show 1.93 in lipid peroxide assay. Inhibition of lipid peroxide was found to be 0.24 for RB-PCL-NPs treated groups which is comparable to that of standard proving that RB PCL nanoparticles show good anticancer activity.

Table 6: Biochemical parameters *in vivo*anticancer studies for Nanoparticles (Serum-2)

Group name	ALP(U/L)	Albumin (g/dL)	Urea (mg/dL)	Uric acid (mg/dL)
Normal	26.96±1.90	5.53±0.208	2.1±0.173	15.46±0.378
Tumour Control	45.86±0.60	2.33±0.152	5.4±0.21	36.03±2.59
Rutin& Berberine (RS)	35.46±0.832	3.93±0.152	3.06±0.152	27.66±1.58
RB-PCL-NPs	33.73±1.106	4.33±0.152	2.76±0.115	25.53±0.832
Standard (Cisplatin)	31.63±0.642	4.76±0.305	2.43±0.152	21.86±0.305

***n=3. Average of three replicate determination; ± standard deviation**

Table 7: Biochemical parameters *in vivo*anticancer studies for NPs (Liver Homogenates)

Group name	SOD	Catalase	GSH	GPX	GST	Lipid peroxidase
Normal	130.86±3.08	41.1±1.15	63.83±1.16	21.13±0.56	1.45±0.15	0.13±0.01
Tumour Control	72.13±0.47	23.16±1.19	35.6±1.25	9.83±0.35	0.22±0.01	1.92±0.04
Rutin& Berberine (RS)	112.33±6.70	30.76±1.20	51.63±1.05	12.66±0.56	0.75 ±0.03	0.28±0.02
RB-PCL-NPs	118.03±1.48	35.26±1.00	55.4±0.80	17.73±0.35	0.87±0.02	0.24±0.01
Standard (Cisplatin)	121.56±1.95	37.06±1.86	59.5±0.70	19.20±0.60	0.96±0.037	0.17±0.01

4. CONCLUSION

The research is aimed to formulate a good and novel drug delivery system for the drugs berberine and rutin. These two drugs have been already proved to have antitumour property. The research works aims at developing a formulation that could increase the bioavailability of the drugs well as effciency of the drug to impart its activity. Newer trends are being built to enhance the therapeutic efficacy of drugs. That too, Herbal drugs has to be formulated to get a better activity. RB PCL NPS was formulated using single emulsification technique and were physicochemically characterized. In vitro studies were performed by MTT assay method on EAC cell lines. The *invivo* studies were performed on mice induced with cancer by EAC cell lines. In vivo evaluation was based on assessment of haematological parameter, biochemical parameters and was analysed for the formulated RB PCL NPs. The report of the above studies prove that the rutin and berberine loaded PCL nanoparticle can prove to be an effective novel treatment regime for treating cancers of various types, paving way for further preclinical and clinical studies.

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