Current Trends in Biotechnology and Pharmacy
Vol. 17 (1) 697 - 716, Jan 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.1.11

Evaluation of Anti-cancer Potential of Epigallocatechin and Berberine Loaded Chitosan Nanoparticle in Ehrlich Ascites Carcinoma Bearing Mice

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Abstract

The goal of this study was to determine how the chitosan nanoparticle (EBNP) loaded with berberine and epigallocatechin gallate (ECGC) affected the Ehrlich Ascites Carcinoma (EAC) tumour cell line and its anti- cancer effects. The EAC cells were used to studythe serum biochemical measures, endogenous antioxidants, lipid peroxidation biomarkers (MDA), histopathological, and haematological parameters. The in vitro cytotoxicity test revealed that EBNP's IC50 value was 48.55 µgm. In EAC-bearing mice, biochemical measureslike total protein, creatinine, bilirubin, SGPT, SGOT, and ALP levels increased. Administering EBNP at high and low doses reduced the raised level to the point closest to normal. The haematological parameters like WBC, ESR, and CRP level increased in EAC bearing mice and the administered nanoformulation reduced the level significantly. The liver homogenatein EAC bearing mice showed that anti-oxidant enzymes such as SOD, catalase, GSH, GPXand GST level drastically declined. The EBNP nanoformulation elevated this level significantlyin a dose dependent manner. In mice with tumours, the level of the lipid peroxidation marker MDA increased. The discovery that MDA level decreased in comparison to control demonstrated the effectiveness of the nanoformulation in preventing lipid oxidativedegradation. The 5-Flouro uracil was usedas std for comparison and all the results arestatistically treated to verify its significance at$P<0.05$ level. Based on the histological studieson kidney and liver, EBNP nanoformulation hadreversed the kidney and liver damage.The findings showed that thetumour can be targeted by encapsulatingepigallocatechin gallate (ECGC) + berberinein chitosan. Hence the EBNP nanoformulationcan be used in cancer therapy as possiblepotential drug candidates.

Keywords: berberine, epigallocatechin gallate, EAC, nanoparticle and MTT assay

Introduction

Cancer is one of the leading causes of mortality worldwide [1]. Chemotherapy, surgery, and radiation therapy are the most frequently used cancer treatment modalities [2–5]. The traditional anticancer medications have a variety of disadvantages, such as unfavorable side effects, damage to healthy tissues, and multidrug resistance [6, 7]. Therefore, one of the most current efforts is to find and assess risk-free new chemicals with high specificity and selectivity from natural sourcesthat can kill malignant cells with little to no adverse effects [8–11]. For instance, there has recently been increased interest in the therapeutic potential of alkaloids like berberine for the treatment of a number of disorders. A variety of significant

medicinal plants, including Berberis aquifolium, Berberis aristata, and Tinospora cordifolia, contain berberine, an isoquinoline alkaloid that has long been employed in the Ayurveda, Unani, and Chinese medicine. According to reports, berberine possesses anticancer properties and inhibits the growth of HepG2 cells, teratocarcinoma cells, and brain tumor cells in in vitro. Berberine also prevented HepG2 cells from secreting alpha-fetoprotein. [12, 13]. Berberine sulphate dramatically reduced tumor yield and incidence of tumor-bearing mice in a two-stage mouse skin carcinogenicity trial. [14] Because of its property to damage DNA or RNA through strong complex formation by positively charged nitrogen atom on C-7 position of berberine, this highly prospective chemical has a wide range of pharmacological activities, including anticancer effects. [15-16] Although berberine has been described as a safe and effective chemotherapeutic agent, its effectiveness may be compromised for a number of reasons, most notably due to its extremely poor solubility in water, limited bioavailability, low intestinal permeability, extensive metabolism, and rapid excretion [17]. The second natural substance was also looked for in the study along with berberine. In this regard, green tea was included in the study since it has been shown to be healthy against a number of diseases, including cancer, obesity, diabetes, cardiovascular disorders, and neurodegenerative diseases [18, 19]. These positive effects are thought to be a result of the polyphenolic chemicals (catechins) found in green tea, which have anticancer, antioxidant, and anti-inflammatory characteristics [20]. The most prevalent physiologically active catechin found in green tea is called ()-Epigallocatechin-3-gallate (EGCG) [21]. Antioxidant and anti-inflammatory qualities were discovered to enhance its anticancer effects [22]. EGCG causes apoptosis and reduces cell proliferation in a variety of cancer cell types. One potentially intriguing feature of EGCG is its ability to specifically suppress the proliferation of cancer cells while leaving surrounding normal cells unaffected [23, 24]. Despite these intriguing qualities, there are a number of drawbacks that prevent EGCG from providing full clinical advantages. For instance, reduced oral bioavailability of EGCG is caused by low stability and gastrointestinal tract degradation [25]. Additionally, it is vulnerable to oxidation and transport by cells of efflux [26]. On the one hand, this demonstrates that large dosages of green tea must be consumed in order to effectively concentrate EGCG, which typically causes negative effects. On the other hand, it emphasizes how critical it is to create site-specific delivery methods in order to increase their effectiveness and thereby lower the required dose. According to reports, using nanoparticles for drug administration has potential benefits and outperforms conventional delivery methods [27, 28]. For instance, it has been discovered that utilizing nano carriers of small, ideal size can transport loaded medications to target tissues more precisely and selectively than other delivery methods [29, 30]. Drug nanocarriers with the right size range can pass through tiny blood capillaries and avoid phagocytosis, which in turn prolongs the nanoparticles' circulation time and causes them to be passively stored in the tumor through the increased permeability and retention (EPR) effect. Additionally, the use of nanocarriers in drug delivery enables the controlled release of medications while minimizing any negative side effects [31, 32]. The goal of the current work was to develop a nano formulation employing chitosan as a suitable nano carrier in order to enhance the combination of EGCG and berberine's target-specific effect. Since it was approved by the US FDA and used in innovative medication delivery systems, the chitosan polymer was chosen as a nano carrier. This enables increasing the effectiveness of EGCG + berberine while utilizing the lowest dose possible to prevent adverse effects. Hence we created chitosan nanoparticles filled with EGCG and Berberine and looked into their potential to fight cancer. EBNP was examined in the current work as a potential anti-tumor drug utilizing EAC tumor- bearing mice as the experimental model.

Materials and Methods

Materials

ECGC and berberine were purchased from Sigma Aldrich, India. Chitosan was purchased from Yarrow Chem Products, Mumbai, Maharashtra; India.Tripolyphosphate (TPP) was procured from Sisco Research Laboratories Pvt. Ltd. (SRL), India and 0.45 µm filter papers were purchased from Millipore. Glacial acetic acid of analytical grade and polyetheleneglycol 400 (PEG 400) were purchased from Thermo Fisher Scientific India Pvt Ltd. The injection 5-flouro uracil was obtained from local medical store. Roswell Park Memorial Institute (RPMI) 1640 medium, Fetal Bovine Serum (FBS), antibiotic and antimycotic preparations for cell culture, Trypsin EDTA solution and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide) (MTT) were obtained from Hi Media laboratories Pvt. Ltd., India.

Cell Lines

A cell line known as EAC (Ehrlich Ascites Carcinoma) was purchased from NCCS Pune in India. The stock cell was grown in 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 g/ml)-supplemented media at 37°C in a humidified 5% CO_2 environment until confluent.

Animal selection

Female Swiss albino mice 10–12 weeks old weighing 20–25 g were chosen from an inbred colony kept under controlled temperature, humidity, and light/dark cycle (12 hrs.) conditions. Free sterilized food and water were available to the animals. Throughout the experiment, the animals were kept in separate polypropylene cages with bedding made of sterile rice husk that was locally purchased. The Institutional Animal Ethical Committee approved the study.

Acute toxicity studies [33]

According to Organization for Economic Cooperation and Development (OECD) guideline 423, the acute toxicity evaluation of the nano formulation was conducted on mice (25-30 g), using the maximum allowable test dose of 500 mg/kg. Before each trial, all of the animals were kept on an overnight fast with free access to water. Five groups of eight animals each were formed from the animals. The first group acted as a normal control, while the other groups 2, 3, 4, and 5 were treated as test subjects by being given the best batch of formulation intraperitoneally at doses of 50 mg/kg, 100 mg/kg, 200 mg/kg, and 500 mg/kg. Each animal's body weight was assessed prior to administration of the dose, and the dose was calculated in accordance with body weight. For the first four hours following the treatment period, the animals were monitored for any harmful effects. Over the course of three days, additional animals were observed for any toxic effects, behavioral abnormalities, and other indicators like body weight, urine, food intake, water intake, respiration, convulsion, tremor, temperature, constipations, changes in eye and skin colors, etc.

Cell viability assay (MTT assay):

The EAC cell lines were used for the cell viability experiment with optimized EBNP nanoparticles, as per the previously described procedure [34]. In DMEM media supplemented with 10% fetal bovine serum, 100 g/ml penicillin, 200 g/ml streptomycin, and 2 mM L- glutamine, EAC cell lines were cultured as adherent and the culture was kept in a humid environment with 5% CO_2. In order to achieve the necessary treatment concentrations, the formulations and control nanoparticles were dispersed or dissolved in sterile PBS, PEG, and ethanol. The MTT assay, which uses the mitochondrial reduction of yellow MTT tetrazolium dye to a highly pigmented purple formazan product, was used to assess the cytotoxicity of formulations. Formulations with a series of concentrations

were incubated with 1x104 Cells (counted using the Trypan blue exclusion dye method) in 96-well plates for 48 hours at 37 degrees in DMEM with 10% FBS medium. The plates were then incubated at 37° for 4 hours with the aforesaid medium changed with 90 l of fresh serum-free media and 10 l of MTT reagent (5 mg/ml), and the plates were then incubated with 200 µl of DMSO. The absorbance was measured at 570 nm on a microplate reader. The concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically standard graph was plotted by taking concentration of the drug in X axis and relative cell viability in Y axis. The cell viability was calculated by the following formula. Cell viability (%) = Mean OD/Control OD x 100

Tumor model

EAC procured from the National Center for Cell Science, Pune, was maintained and propagated by serial intraperitoneal transplantation of EAC cells in an aseptic environment. Viable EAC cells (2.5 x10^6) were injected intraperitoneally into each animal in an aseptic environment, and the day of tumor inoculation was considered as day 0.

Transplantation of Ehrlich ascites carcinoma (EAC):

National Center for Cell Science, Pune provided the Ehrlich ascites carcinoma (EAC) cells line,which was kept in female Swiss albino mice as tumor cells were extracted from a donor who was 7 days old and had a moderate rate of growth. The vitality of the EAC cells was evaluated. Each mouse had 0.1 ml of newly collected ascites fluid, diluted in 1:5 saline, inserted subcutaneously into the intraperitoneal cavity. Each of the inoculums, where the tumors were produced, had roughly 2.5 X 10^6 cells. [35]
Experimental design: A total number of 40 adult female Swiss albino mice weighing 20-25 g were randomly assorted into 5 groups (n=8/ group) as follows:
Group 1: It was injected (I.P) with sterile saline (0.1 ml / mouse) for 14 days (day after day) acted as normal group.
Group 2: It was inoculated (I.P) with (EAC) cell line, (2.5× 10^6 cells/ 0.1 ml /mouse) once in the first day acted as a negative control.
Group 3: It was inoculated (I.P) with (EAC) cell line, (2.5× 10^6 cells/ 0.1 ml /mouse) + (EBNP 100mg/kg) for 14 days
Group 4: It was inoculated (I.P) with (EAC) cell line, (2.5× 10^6 cells/ 0.1 ml /mouse) + (EBNP 200mg/kg) for 14 days
Group 5: It was inoculated (I.P) with (EAC) cell line, (2.5× 10^6 cells/ 0.1 ml /mouse) +(standard drug 5-FU 3mg/kg) for 14 days
After 14 days, 5 mice in each group were anesthetized and sacrificed to evaluate anti-tumor activity to conduct hematological, biochemical and histopathological assay. The remaining mice were kept alive to estimate the mean survival time (MST) and percent increase in life span (% ILS).

Sample collection

Each group of mice was utilized to collect blood from a tail vein at the end of the experiment. Without using an anticoagulant, blood samples were drawn into sterile test tubes for serum separation, which were then stored for a while and centrifuged for 15 minutes at 3000 rpm. The resultant supernatant was collected and kept at -20°C until biochemical parameters were analyzed. The liver and kidney were prepared for histological investigation after being fixed in a 10% buffered formalin solution at room temperature.

Hematological measurements

The complete blood profilers includes hemoglobin content, red blood cells (RBC), white blood cells (WBC), ESR, CRP was done by using Automated Hematology Analyzer with readymade available kits.

Serum biochemical parameters

All the serum biochemical parameters such as total protein, SGPT, SGOT, bilirubin, ALP, albumin, creatinine, urea and uric acid were evaluated by kit method.

Determination of biochemical parameters in liver homogenate

The liver tissues were homogenized (1%) in 10 mL volumes of 100 mM KH2PO4 buffer containing 1 mM ethylene diamine tetra-acetic acid (EDTA; pH 7.4) and centrifuged at 21 12,000 × g for 30 minutes at 4 ºC. The supernatant was collected and used for the assessment of antioxidant enzymes.

Estimation of MDA

MDA cellular levels were spectrophotometrically determined in the homogenate of cells using the assay kit of Bio diagnostic based on Satoh et al [36].

Estimation of SOD

SOD cellular activity levels were spectrophotometrically determined in liver homogenates according to Nishikimi et al. [37], by using Bio diagnostic kit assay.

Estimation of CAT

Catalase activity was determined using the method of Bears and Sizer 1952. [38]

Estimation of Glutathione peroxidase (GPX)

GPx activity was measured at 37 °C by the method of Batist G et al. [39]

Estimation of GST

GST was estimated by the method Lila Moatamedi Pour et al [40]

Estimation of GSH

The cellular levels of GSH were estimated in cell homogenate spectrophotometrically, using the assay kit provided by Bio diagnostic. [41]

Evaluation of Tumor volume: The volume of the EAC was detected by measuring tube in milliliters (ml)

Determination of Percentage increase in life span (%ILS): Mean survival time (MST) and

%ILS for each group were calculated according to the following formula, MST= (no. of days to 1st death + no. of days to last death)/2

%ILS= [(MST of drug group / MST of control group)-1] × 100

Histopathological studies of liver and kidney

Histological studies were conducted using the industry-reported standard methods. The samples were collected, fixed, and then embedded in paraffin blocks to create sections with hematoxylin and eosin stains that were 5 mm thick. In a nutshell, ethanol was used to prepare the samples for dehydration. They were cleaned with xylene next, then at 58 degrees Celsius, they were soaked with liquid paraffin wax and imbedded in paraffin blocks. The samples were then cut into 5 m sections using a Rotary Microtome (Leica RM2255) and stained using Microm HMS7 and Hematoxylin and Eosin. A light microscope was used to view the stained slices (Olympus CX21) **Statistical *analysis***

The experimental results are expressed as mean ± std deviation. The data were analyzed by ANOVA ($p<0.05$) and means separated by Duncan's multiple range tests (by SPSS version 21software).

Results and Discussion

Acute toxicity study

There was no change in body weight, toxicological symptoms, or mortality at the levels of 50 mg/kg b.w., 100 mg/kg b.w., or 200 mg/kg b.w., however 2 animals died at 500 mg/kg. Therefore, the therapeutic dosages for the biological evaluation were 100 mg/kg and 200 mg/kg.

MTT assay

The EBNP treatment of the EAC cell line at doses ranging from 6.25µ g/ml to 100 µg/ml resulted in a notable loss in cell viability, with values as high as 82% at 6.25 µg and 16.36% at 100µ g/ml. (Fig. 1 in tab 1). This findings showed that the EAC cell line's viability is 80% (cell death is 20%) after exposure to the nano

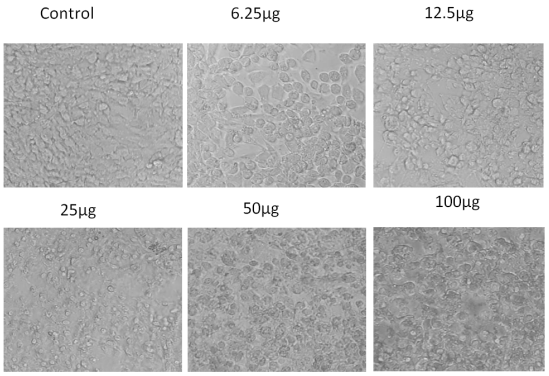

Fig. 1: Effect of EBNP on EAC cell line

formulation. Cell viability ranges from 63% at the median dose to 16% at the high dose of 100 g/ml (cell death is 84%).From the STD calibration graph, it was found that IC_{50} of the EBNP was 48.55µg/ml.[fig2]

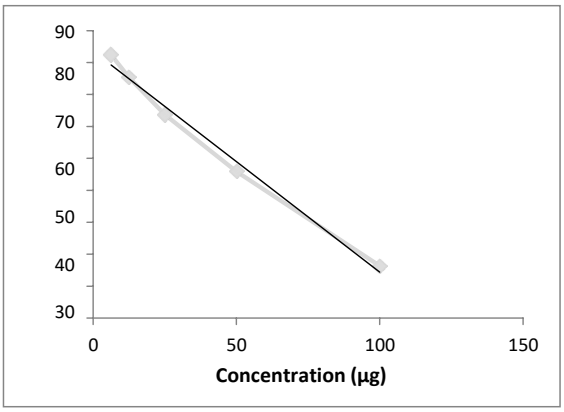

Fig. 2: cell viability graph

to 1.58U/ml in both the low and high dose nano formulations, but low dose mice bearing EAC displayed 1.04U/ml as compared to EAC bearing control mice, i.e. 0.40U/ml. The GST level drastically reduced with EAC mice and elevated in the mice administration with nano formulation. %).[tab 5 & fig 6]

GPX, also known as glutathione peroxidase, is an antioxidant enzyme (GPX). It serves a protective purpose by converting H2O2 into H2O + O2. It can be inferred that the GPX activity was observed to be 24.16% in normal mice and 10.03% in EAC-bearing mice. This information demonstrated that tumor-bearing animals have lower GPX levels than do normal

Table 1:- MTT Assay

Conc (EBNP)	
In µg	% viability
6.25	82.48232522
12.5	75.38622676
25	63.68159204
50	45.95443833
100	16.36554072

Estimation of Anti-oxidants

SOD, an internal antioxidant, had a notable impact on the EAC cell line. The results showed that EBNP dramatically enhanced the level of SOD in EAC carrying mice at both low and high doses. In comparison to the control, the greater dose of 200 mg/kg b.w. considerably raised the level of SOD. For instance, 5-Fluorouracil was employed as a reference, and the SOD activity at higher doses was 120U/mm as opposed to low doses 112U/mm. This result clearly indicated that EBNP has significant antioxidant effect. [tab 2 & fig 3]

From the table 3 & figure 4 it was observed that catalase activity was increased with respect to dose. The higher dose of EBNP has shown 41.3% catalase activity when a low dose had shown 38.46%. The EAC bearing mice demonstrated decreased levels of catalase i.e., 26.83 as compared to control group 42.56. Catalase activity was markedly increased in EBNP-treated animals in a dose-dependent manner, proving the substance's antioxidant properties. At the P0.05 level, each result was statistically significant. The GSH is also endogenous non enzymatic defense mechanism play key role to control oxidative damage of these tissues. The resultobtained from the activity data indicated that EBNP elevated the level of GSH to 65.70%, 62.43% from high dose to low dose respectively as compared to EAC treated mice (39.56%).[tab 4 & fig 5]

Glutathione S-transferase, or GST , is a key enzyme in the detoxification of xenobiotics. When EBNP was tried on mice carrying EAC, the results were encouraging. In mice treated with 100g/ml of EBNP, the GST level increased

SOD(U/ml)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	132.9	72.2	128.5	109.4	119.4
	135.3	74.4	127.2	115.2	119.2
	134.2	69.9	129.9	111.8	121.6
Mean	134.13	72.16	128.5	112.13	120.06
S.D	1.2	2.25	4.25	2.91	1.33

Tab 2: Estimation of SOD
EBNP 100 µg = EBC-Low dose EBNP 200 µg = EBC-high dose

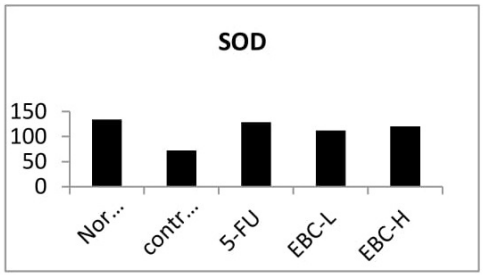

Fig 3: Bar graph representation of SOD

Catalase (U/ml)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	42.4	28.8	31.4	36.3	39.8
	43.2	26.3	41.1	39.6	42.2
	42.1	25.4	43.9	39.5	41.9
Mean	42.56	26.83	42.01	38.46	41.3
S.D	0.56	1.76	1.5	1.87	1.3

Tab 3: Estimation of catalase

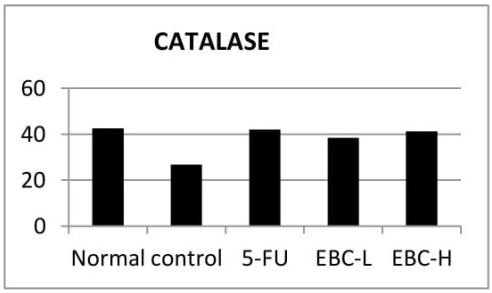

Fig 4 : Bar graph representation of catalase

GSH (mmol)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	69.4	39.2	65.2	62.1	66.4
	69.8	39.4	65.7	63.8	66.2
	67.6	40.1	67.9	61.4	64.5
Mean	68.93	39.56	66.23	62.43	65.7
S.D	1.17	0.47	1.91	1.23	1.04

Tab 4: Estimation of GSH

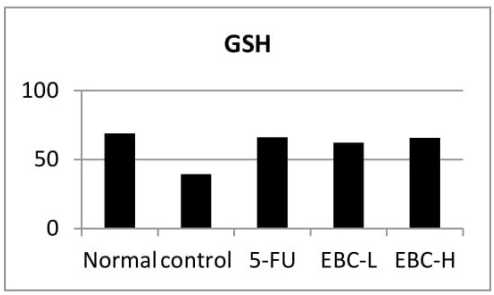

Fig 5: Bar graph representation of GSH

GSTµmol)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	2.37	0.46	1.96	1.02	1.48
	2.26	0.37	1.99	1.04	1.82
	2.43	0.38	1.89	1.08	1.46
Mean	2.35	0.4	1.94	1.04	1.58
S.D	0.08	0.049	0.03	0.03	0.2

Tab 5: Estimation of GST

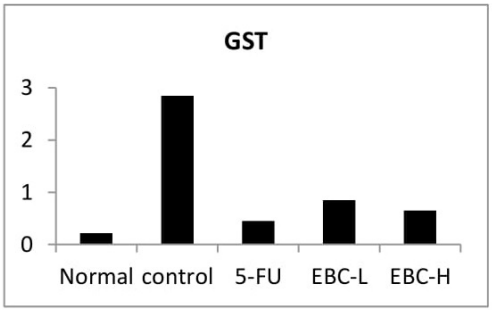

Fig 6: Bar graph representation of GST

GPX(U/L)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	24.3	9.8	23.6	19.3	22.3
	25.3	9.9	23.9	21.8	23.2
	22.9	10.4	23.1	23.6	21.9
Mean	24.16	10.03	23.86	21.56	22.46
S.D	1.2	0.32	0.87	2.15	0.66

Tab 6: Estimation of GPX

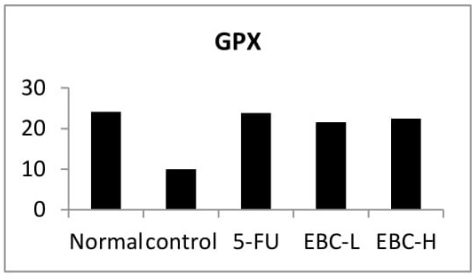

Fig 7: Bar graph representation of GPX

MDA (µMol)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	0.19	2.79	0.44	0.99	0.46
	0.22	2.94	0.45	0.82	0.68
	0.25	2.83	0.46	0.75	0.82
Mean	0.22	2.85	0.45	0.85	0.65
S.D	0.03	0.07	0.03	0.12	0.18

Tab 7: Estimation of MDA

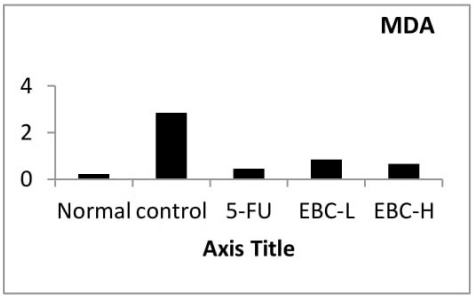

Fig 8: Bar graph representation of MDA

mice. The level of GPX was enhanced by the nano formulation EBNP at low and high dose levels, respectively, by 21.56 and 22.46%.All the experimental results are statistically significant at PC 0.05. %).[tab6 & fig 7]

Lipid oxidation occurs as a result of lipid oxidative damage. A marker called MDA is typically used to assess the degree of lipid peroxidation. When lipid peroxidase is present, the level of MDA will undoubtedly increase. Our experimental findings unequivocally demonstrated that EBNP had the ability to suppress the production of MDA. The normal mouse left untreated had produced 0.22 µmole of MDA. Due to the tumor load, this level increased significantly in mice with EAC. The test findings showed that MDA levels were reduced to 0.85–0.65 µmol % at low and high doses of nano formulation. [Fig. 8 & Tab. 7]

Serum Biochemical parameter

When compared to normal mice, EAC-bearing animals had significantly different levels of total protein, creatinine, bilirubin, SGPT, SGOT, and ALP, according to the biochemical analysis of blood serum. The trial results made it abundantly evident that the nano formulation increased the level of the declining biochemical parameters. It is significant to note that the levels of the aforementioned biochemical markers rose in EAC-bearing animals. In EAC-bearing mice, the total protein level dropped from 9.03 to 3.83 compared to the control group. The total protein was enhanced by the nano formulation into 8.46 and 8.86 respectively at moderate and high doses. All the experimental results are statistically significant at P<0.05. %).[tab 8 & fig 9]

The nano formulation decreased the level of creatinine from 3.3 to 2.8 as compared to the EAC bearing control.[tab 9 & fig 10] Bilirubin level drastically increased in EAC bearing mice as compared to normal mice (2.86). .[tab 10 & fig 11] The nano formulation decreased the level

ESTIMATION OF BIOCHEMICAL PARAMETERS

Total Protein (g/dL)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	9.2	3.6	9.6	8.2	8.9
	8.8	4.1	9	8.4	9.2
	9.1	3.8	8.4	8.8	8.5
Mean	9.03	3.83	9	8.46	8.86
S.D	0.2	0.25	0.54	0.3	0.35

Tab 8: Estimation of Total Protein

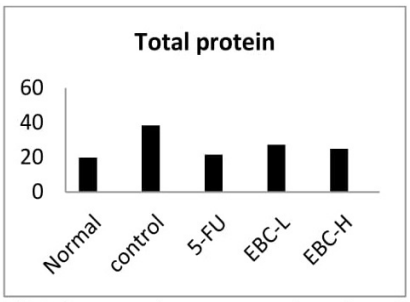

Fig9: Bar graph representation of total protein

Creatinine (mg/dL)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	0.9	3.9	1.6	3.2	2.2
	1.1	3.5	1.8	3.6	2.4
	1.4	3.8	2.0	3.1	2.8
Mean	1.13	3.73	1.8	3.3	2.46
S.D	0.25	0.2	0.35	0.26	0.3

Tab 9: Estimation of Creatinine

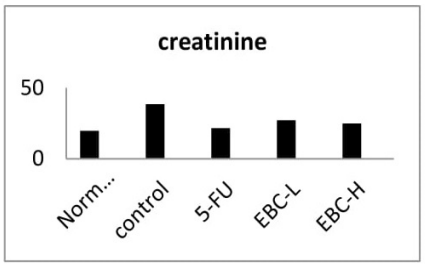

Fig 10: Bar graph representation of creatinine

Bilirubin (mg/dL)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	2.9	7.1	3.0	3.8	4.2
	2.8	6.8	3.4	4.1	3.9
	2.9	6.9	3.2	4.3	3.8
Mean	2.86	6.93	3.2	4.06	3.96
S.D	0.05	0.15	0.2	0.25	0.2

Tab 10: Estimation of Bilirubin

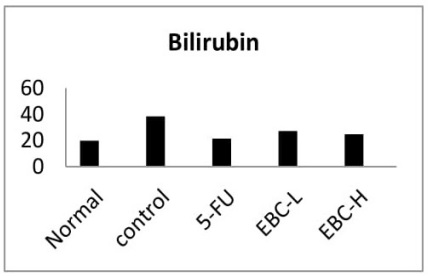

Fig 11: Bar graph representation of bilirubin

SGPT (U/L)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	28.6	65.4	34.2	39.6	40.3
	29.2	68.5	33.2	40.1	38.8
	32.1	65.2	36.4	42.3	38.5
Mean	29.96	66.36	34.6	40.66	39.2
S.D	1.87	1.85	1.53	1.43	0.96

Tab 11: Estimation of SGPT

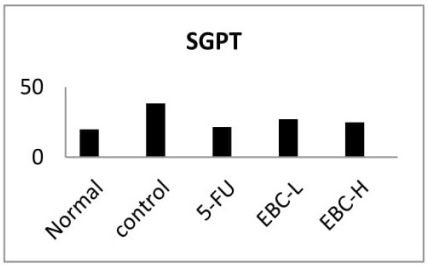

Fig 12: Bar graph representation of SGPT

SGOT (U/L)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	46.8	101.2	48.1	48.7	50.3
	49.6	98.2	47.9	50.2	48.8
	48.4	97.9	48.0	52.8	49.6
Mean	48.26	99.1	48.0	50.56	49.56
S.D	1.4	1.82	0.85	2.07	0.75

Tab 12: Estimation of SGOT

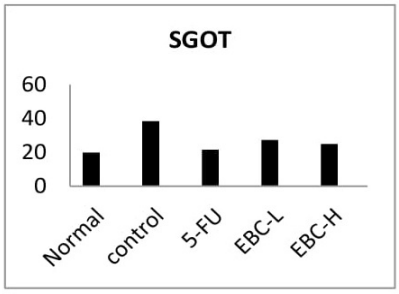

Fig 13: Bar graph representation of SGOT

ALP (U/L)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	32.2	53.5	33.18	39.3	35.5
	31.8	52.9	33.89	38.6	36.5
	32.4	53.3	33.02	37.8	37.2
Mean	32.13	53.23	33.36	38.56	36.4
S.D	0.3	0.3	0.92	0.75	0.85

Tab 13: Estimation of ALP

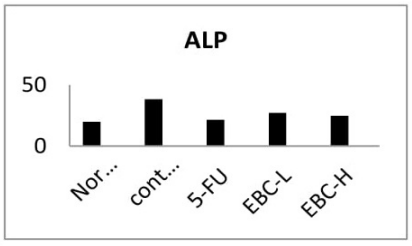

Fig 14: Bar graph representation of ALP

Albumin (g/dL)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	9.2	3.1	8.6	6.2	7.5
	9.6	3.3	8.2	6.7	7.2
	9.9	3.7	8.6	6.4	7.6
Mean	9.56	3.36	8.46	6.43	7.43
S.D	0.35	0.3	0.23	0.25	0.2

Tab 14: Estimation of Albumin

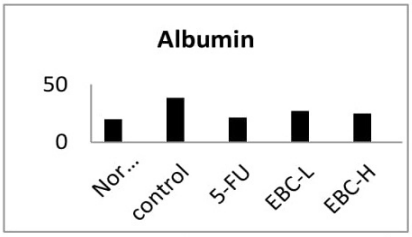

Fig 15: Bar graph representation of album

Urea (mg/dL)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	2.2	5.2	2.7	2.9	2.8
	2.4	4.9	2.6	3.3	2.9
	2.8	5.6	2.5	3.2	3.1
Mean	2.46	5.23	2.6	3.13	2.93
S.D	0.3	0.35	0.3	0.2	0.15

Tab 15: Estimation of Urea

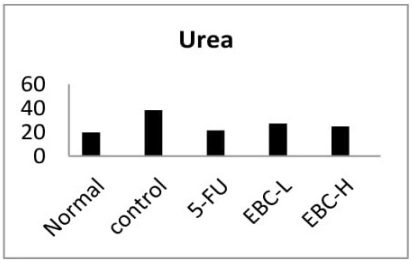

Fig 16: Bar graph representation of urea

Uric acid (mg/dL)					
	Normal	control	5-FU	EBNP 100 µg	EBNP 200µg
	21.2	39.4	21.6	27.3	24.9
	19.4	38.2	21.7	26.8	25.1
	18.9	37.9	21.3	27.6	24.6
Mean	19.83	38.5	21.53	27.23	24.86
S.D	1.2	0.79	0.86	0.4	0.25

Tab 16: Estimation of Uric acid

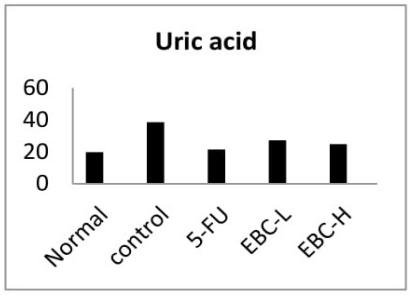

Fig 17: Bar graph of uric acid

of bilirubin from 6.93 to 3.96. Similarly the SGPT level increased in EAC bearing control whereas the nano formulation decreased these levels from 66.36 to 39.2. .[tab 11 & fig 12] In a similar fashion the elevated level 99.1 was observed in EAC bearing control as compared to the normal (48.26). However the nano formulation reduced the level of SGOT remarkably from 99.1 to 49.56. .[tab 12 & fig 13] The EAC bearing mice had shown the elevated ALP level at 53.23.[tab 13 & fig14] The ALP was reduced by the nano formulation to 36.4, which is less than the standard. The albumin level change often runs counter to the biochemical signals listed above. The albumin level typically decreases in pathological conditions. Therefore, the results of our study were consistent with the experimental evidence that has already been published. However, the level enhanced in EAC carrying mice was dramatically raised by the Nano formulation. Albumin levels were lower in EAC-bearing animals than in control mice. [Fig. 15 & tab 14]. The urea and uric acid levels were reduced by the nano formulation in a dose-dependent manner, with EBNP at higher doses reducing uric acid levels to 24.86 and 29.3 mg/dL for urea.[tab 15 , 16 & fig 16, 17] In all the experimental procedures 5-F-Uracil was used for comparison. All the experimental results are statistically significant at P< 0.05.

Hematological parameter analysis

Hematological markers such hemoglobin content, RBC, WBC, ESR, and CRP were assessed.The hemoglobin (Hb) level in EAC bearing mice was 7.56 as compared to the normal mice. The mice treated with EBNP nano formulation had shown elevated level of Hb i.e. 10.4 and 11.2. The standard drug 5-F-uracil also elevated the Hb level but lesser than the normal mice. [tab 17 & fig 18]
According to the experimental results, RBC levels in EAC-bearing mice were 4.46 percent lower than those in control animals (10 million). The nano formulation had shown increased level of RBC i.e. 6.36 and 8.03 million whereas the standard drug had shown 5.46 million RBC. This data proved that the nano formulation has the potential to elevate the RBC level. [Tab 18 & Fig 19]

As far as WBC levels are concerned, illness conditions will often cause WBC levels to rise. Therefore, compared to normal mice with 8.6 million WBC, EAC-bearing mice increased their WBC level to 22.1 million. It's interesting to note that the Nano formulation reduced WBC levels, which were 14.5 and 10.4. The standard drug also reduced the level of WBC as compared to control 8.66 .[tab 19 & fig 20] From the experimental data EAC bearing mice elevated the level of ESR up to 21.1mm/hr. as compared to control 11.3mm/hr. %).[tab 20 & fig 21]

The EBNP treated mice had shown marked decrease of ESR i.e. 13.6 and 11.4. In similar way CRP level drastically increased in EAC bearing mice i.e. 12.1 as compared to

Estimation of Hematological Parameters

Hb (mg/dL)				Mean	S.D
Normal	13.8	14.9	13.5	14	0.73
Control	8.4	7.2	7.1	7.56	0.72
EBNP 100 µg	10.6	10.6	10.1	10.4	0.28
EBNP 100 µg	11.9	10.8	11.1	11.2	0.56
5-FU	12.2	12.1	12.9	12.4	1.15

Tab 17: Estimation of Hb

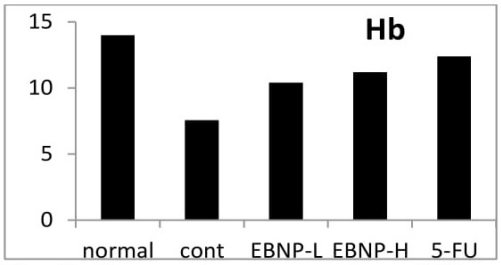

Fig 18: Bar graph representation of Hb

	RBC (10^{12}/L)			Mean	S.D
Normal	9.6	10.7	9.8	10	0.58
Control	4.4	4.1	4.9	4.46	0.40
EBNP 100 µg	6.3	6.6	6.2	6.36	0.20
EBNP 100 µg	7.4	7.8	8.9	8.03	0.77
5-FU	8.4	8.1	8.9	8.46	0.40

Tab 18: Estimation of RBC

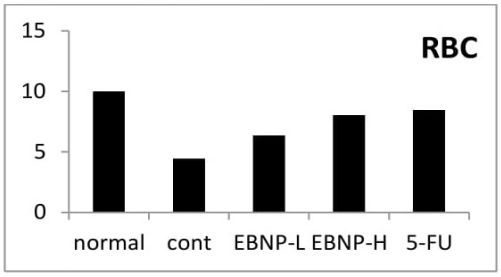

Fig 19: Bar graph representation of RBC

WBC (10^9/L)				Mean	S.D
Normal	8.4	8.3	9.1	8.6	0.43
Control	21.2	22.4	22.9	22.1	0.87
EBNP 100 µg	14.5	13.9	15.2	14.5	0.65
EBNP 100 µg	9.7	11.3	10.3	10.4	0.80
5-FU	8.3	8.9	8.8	8.66	1.00

Tab 19: Estimation of WBC

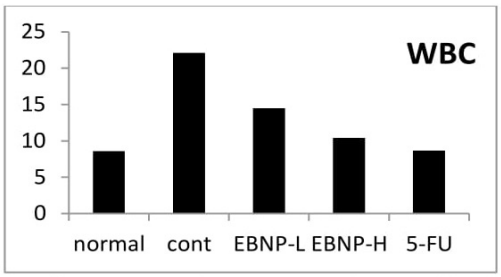

Fig 20: Bar graph representation of WBC

ESR (mm/h)				Mean	S.D
Normal	10.4	11.3	12.4	11.3	1
Control	21.4	22.5	19.6	21.1	1.46
EBNP 100 µg	14.3	12.6	13.9	13.6	0.88
EBNP 100 µg	11.3	10.6	12.4	11.4	0.9
5-FU	9.8	10.2	10	10	0.75

Tab 20: Estimation of ESR

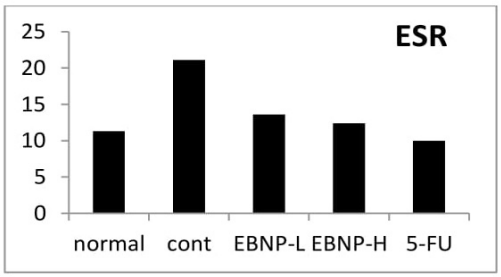

Fig 21: Bar graph representation of ESR

normal. The nano formulation treated mice had shown remarkably decreased level of CRP i.e. 3.56 and 2.93. All the results are statistically significant at P value <0.05. %).[tab 21 & fig22]

Tumor Volume and life span

Tumor volume measurements revealed that EAC-bearing mice had larger tumor volumes (7.4 ml), compared to animals who received EBNP at low and high doses, which showed 4.3 ml and 2.43 ml of tumor volume, respectively. But the standard medication 5-F-uracil significantly decreased volume to 1.96ml. [tab 22 & figure 23]As far as life span of the treated mice is concerned, EAC bearing mice life span is 32 days as compared to normal mice i.e., 122 days. It's interesting to note that both formulations extended the mice's life span from 32 days to 44 days and 73 days. However std drug had increased more significantly i.e., 100 days. These results confirmed the anticancer effect of EBNP's on mice model with statistically significant result (P <0.05). %).[tab 23 & fig 24]

Histological changes in kidneys

The kidneys in the control group had a typical structure, according to the histological investigation. The typical kidney segment displays a glomerulus with its normal endothelium and a normal renal cortex. The nuclei of the nephron cells were plainly visible, and there was no lymphocyte bruising, necrosis, or infiltration. Control group: The tubules in the control group exhibit significant necrosis and extensive infiltration. Degeneration of the glomerulus and focal diffuse tubular degeneration also observed. The mice treated with low dose EBNP nanoparticle shows intact glomerulus with endothelium and urinary capsule. The urinary space was small, but not constricted and aggregations of cells were observed in some part of the section. At higher dose, the juxta-glomerular apparatus and urinary capsule appeared normal. Mild aggregation of cells and mild constriction of urinary space were observed standard drug (5-F -uracil 3mg/kg): The kidney tissue section showed that std drug treated group showed moderate glomerular and tubular damages with mild necrosis.[fig 25]

Histological changes in liver

The hepatocytes demonstrated a normal cytoplasm with a large nucleus in the control group. In normal liver, normal hepatic architecture with hepatic cords, sinusoids and kupffer cells and polygonal shaped nuclei with central veins were observed. The cytoplasm was clearly visible with endoplasmic reticulum, golgi apparatus and mitochondria and ribosome. Hepatocytes with uneven size, shape, and border were visible in the control liver. Additionally, there were liver sinusoids, a hemorrhagic zone around the major vein, and signs of necrosis. The hepatocyte- containing liver sections had a relatively restrained morphology at low doses. In this group, therewere fewer necrotic cells and dysplastic hepatocytes, demonstrating a pattern of healing. Kupffer cells that were active were also found. Additionally, spherical nuclei, large nucleoli, and eosinophilic granular cytoplasm were seen. The nanoparticle-treated mice had normal portal veins, fewer hepatocytes, and decreased renal tubular degeneration. A noticeable thickening and inflammatory cell infiltration associated with degeneration, besides the potent congestion in the portal vein, mild focal necrosis in hepatocytes and parenchymatous degeneration was observedin std drug treated mice.[fig 26]

As per OECD Guideline 423, acute toxicity study was carried out. At the level of 50mg/kg, 100 mg/kg and 200mg/kg, there was no change in body weight and toxicological signs and mortality whereas 2 animals died at 500mg/kg.b.w. Thus 100mg/kg and 200mg/kg were selected as therapeutic doses. Tetrazolium salt reduction is now recognized as a trustworthy method for assessing cell growth. Dehydrogenase enzymes help metabolically active cells reduce the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) to produce

CRP mg/dL				Mean	S.D
Normal	2.1	1.9	2.8	2.26	0.47
Control	12.6	11.5	12.4	12.1	0.58
EBNP 100 µg	3.9	3.2	3.6	3.56	0.35
EBNP 100 µg	2.8	2.9	3.1	2.93	0.15
5-FU	2.4	2.2	2.3	2.3	0.3

Tab 21: Estimation of CRP

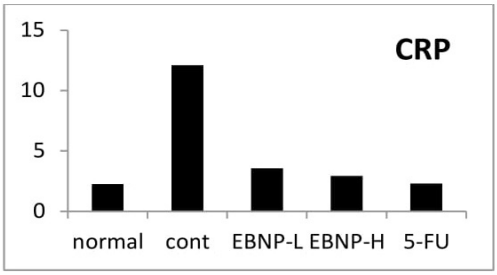

Fig 22: Bar graph representation of CRP

Tumor volume (ml)					
	ml			Mean	S.D
Control	6.8	7.6	7.9	7.43	0.56
EBNP 100 µg	4.2	4.8	3.9	4.3	0.45
EBNP 100 µg	2.4	2.2	2.7	2.43	0.25
5-FU	1.8	1.9	2.2	1.96	0.2

Tab 22: Estimation of Tumor volume

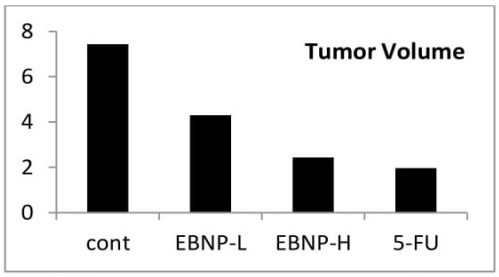

Fig 23 Bar graph representation of tumor volume

Days		Mean		**S.D**	
Normal	125	123	119	122.33	3.05
Control	28	33	36	32.33	4.04
EBNP 100 µg	41	48	44	44.33	3.51
EBNP 100 µg	73	79	68	73.33	5.5
5-FU	103	107	90	100	2.51

Tab23: Estimation of life span

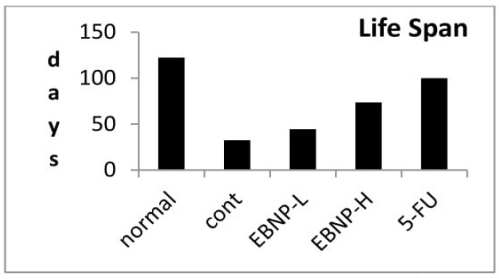

Fig 24: Bar graph representation of lifespar

reducing equivalents like NADH and NADPH. Using a spectrophotometric technique, the resultant intracellular purple formazan may be solubilized and measured. Based on the results obtained, it was found that $IC_{50\ of}$ the nano formulation was found to be 48.55 µgm. Reactive oxygen species produced by the animal body, including superoxide (O_2+), hydroxyl (OH), per- hydroxyl (HO_2), nitric oxide (NO), and hydrogen peroxide (H_2O_2), cause oxidative stress, which damages cells and may result in a number of clinical consequences. [42] When this occurs, the body's defensive mechanisms both enzymatic and non-enzymatic metabolize free radicals and their chemical byproducts in order to reduce the oxidative burden and its related consequences. It was therefore intriguing to determine whether EBNP affects both the enzymatic and non- enzymatic defense system. After administering the EBNP to the mice for 14 days, the amount of glutathione (GSH), a marker of the non-enzymatic system, and the specific activity of enzymes involved in free radical metabolism were assessed in the liver. Superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione

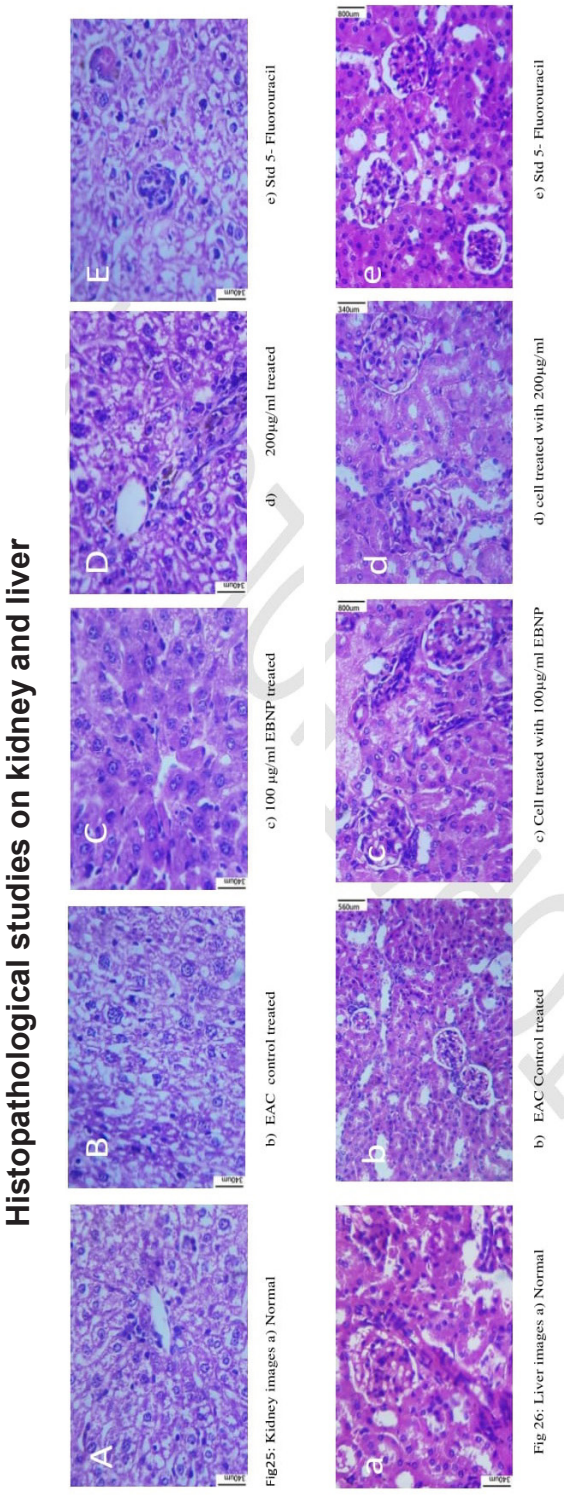

Fig25: Kidney images Fig 26: Liver images

S-transferase (GST), and GSH were among the specific enzymes whose activity was improved by the EBNP. As a result, SOD is probably going to dismutate O2 - to H_2O_2, and then H_2O_2 to water and molecular oxygen by catalase and GPX. Glutathione Reductase helps recycle and make glutathione accessible for the antioxidant activity of GPx by reducing glutathione disulphides (GSSG) to GSH [43].Since GSH is known to be free radical scavenger [44], its increased level contribute and enhance the free radical scavenging potential of EBNP. Apart from the scavenging of free radicals, the EBNP augmented the metabolism of free radicals and restored the antioxidant level. It was supported by the lowered levels of peroxidative damage in the liver, under similar circumstances.

MDA has long been employed as the main marker of lipid peroxidation. MDA can bind to proteins and DNA to produce adducts, and it has been demonstrated that this is true even in healthy human liver tissue [45]. These adducts may be formed as a result of lipid peroxidation at a base level, and an elevated level of adducts would be a sign of a pathological situation. Due to tissue injury, the amount of MDA in the liver increased. The radical scavenging ability of EBNP, which was also consistent with the literature, can be associated with the EBNP administration's drop in MDA level in a dose-dependent way. Since intracellular enzymes are released into the blood following hepatic injury, serum levels of SGOT and SGPT are frequently utilized as indicators of hepatic damage [46]. Some studies found those breast cancer patients' serum levels of SGOT and SGPT significantly increased. It can be deduced that an increase in the rate of gluconeogenesis may be the cause of the considerable elevation in liver transaminases. These findings would suggest that EAC-bearing mice have some hepatocellular damage, and that chemotherapy may exacerbate this damage [47]. ALP is a sensitive indicator of both the progression of hepatocellular injury and minor biliary obstruction. According to the data, a significant portion of EAC-bearing mice with subsequent

liver and/or bone metastases hadabnormal ALP [48]. According to the research, tumor cells harm the liver and disrupt the hepatic cell's normal functioning, which affects the levels of blood enzymes like SGPT, SGOT, and ALP. The increased biochemical indicators were returned to normal range following EBNP treatment at both doses, demonstrating protection against tumor cell-induced hepatotoxicity. Animal models with abnormal total protein and albumin levels show liver impairment. The majority of the proteins in blood serum, including more than half of all proteins, are found in serum albumin [49]. Reduced serum albumin (SA) level is used as an independent prognostic indicator in breast cancer [50 51] The EBNP-treated mice restored the total protein and albumin level to normal, which was consistent with earlier literature, but these levels fell in the EAC- bearing positive control group. This might be the mechanism underlying the lack of a statistically significant change in the serum levels of total proteins observed in this investigation. The fact that albumin and other proteins, collectively known as globulins, make up total serum protein and that it is known that oxidative stress, such as the stress associated with cancer, can change the serum albumin concentration, may be the cause of this rise in total serum protein concentration. Additionally, as the plasma moves through the tissues, it picks up proteins that have been liberated from their original places as a result of specific physiological occurrences,

such as tissue remodeling, trauma, and cell death, which raises the total protein concentration in the blood. In general, the EAC control group has higher levels of bilirubin, urea, and uric acid than the normal group. Previous theories have suggested that oxidative stress aids in the development of cancer. According to Krishna et al., untreated females with breast cancer had much higher uric acid levels than those who receive treatment, which may be related to elevated oxidative stress. In this investigation, we came to the conclusion that elevated serum uric acid levels may be caused by their protective effects in response to elevated oxidative stress. Due to bilirubin scavenging during oxidative load or oxidative stress in female breast cancer patients, the amount of bilirubin was slightly above or within normal range. [52] The increase in blood urea concentration was attributed by the author to the catabolic action of the tumor, which increased urea production. Our results demonstrated a very highly significant increase in serum creatinine concentration in tumor bearing mice. These results were similar to previous report which was observed that, serum creatinine level showed a significant increase in mice-bearing Ehrlich ascites carcinoma due to muscle necrosis Hematological parameters are used to assess their implications in various cancers [53]. The blood components such as RBC, WBC, Hb, ESR and CRP where used as indicator to find out the physiological changes before and after administration of EBNP. The EAC bearing mice had expressed the decreased level of Hb, RBC with concomitant elevation of WBC, ESR and CRP. The administered EBNP at 50 and 100µg/ml reversed the imbalanced hematological profile to normalcy. Therefore, it is corroborated that EBNP did not considerably modify the blood profile but rather returned the abnormally changed level to normalcy. The Nano formulation with lower and higher doses boosted RBC and Hb levels while lowering WBC, ESR, and CRP levels. Studies on kidney and liver histopathology demonstrated how EBNP has a protective impact. It is clear from the tumor volume results that EBNP reduced the level of tumor volume that was increased in EAC carrying mice. The data on life expectancy showed that EBNP treated mice had a significantly longer life expectancy. These two findings demonstrated the anticancer potential of EBNP

Conclusion

Through both in vitro and in vivo experiments, we have successfully investigated the anti-cancer potential of EBNPs in the current work. To create nanoparticles, the two active moieties, berberine and epigallocatechin,

were mixed and placed onto chitosan polymer. Acute toxicity tests on the EBNP formulation revealed that doses of 100 and 200 mg kg b.w. would be safe for all in vivo tests. The results of the MTT experiment showed that the EBNP formulation was cytotoxic at 48.55 gm. The detailed analysis of the hematological parameters revealed that the Nano formulation has the ability to restore the changed parameter to normalcy. Similar to this, EBNP restored the liver's biochemical parameters to levels that were close to normal. The altered enzymatic and non-enzymatic antioxidant level was also brought to normalcy. Studies conducted in-vivo on EAC-bearing mice also showed that EBNP has anticancer potential, as evidenced by a reduction in tumor burden and an extension of mean survival time when compared to the other treatment groups. The decreased tumor burden and enhanced survival of mice in EBNPs treated group might be a consequence of the combined anticancer effects of epigallocatechin and berberine constituents of the Nano formulation. The protective effect of EBNP was convincingly established through histopathology tests on the liver and kidney by correcting the altered histological alterations in mice models. The aforementioned experimental data allow for the conclusion that EBNP did, in fact, demonstrate a substantial anti-cancer capability in the EAC carrying mouse model. Therefore, this nano formulation may be a substitute for cytotoxic traditional chemotherapeutics as a chemo preventative strategy.

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