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FOLATE RECEPTOR TARGETED NARINGENIN GOLD NANOPARTICLES: ITS *IN VIVO* ANTICANCER AND ANTIOXIDANT POTENTIAL IN EHRLICH ASCITES CARCINOMA BEARING SWISS ALBINO MICE

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ABSTRACT

The present study reports the cancer preventing outcome of synthesized biogenic folate receptor targeted naringenin gold nanoparticles (NAR-AuNPs-F) under *in vivo* settings. Ehrlich ascites carcinoma (EAC) is rodent mammary adenocarcinoma having high rate of proliferation along with transplantation potential. Antitumor and antioxidant efficacy of NAR-AuNPs-F was studied in EAC ascites tumor model. NAR-AuNPs-F was treated intraperitoneally at the dose levels of 1.5 and 3 mg/kg body wt. for 14 consecutive days. 5-flurouracil (5-FU) was utilized here as standard drug (20mg/kg body wt.) Tumor volume and tumor cell count, mean survival time, increase in life span, haematological parameters were measured. After treatment with synthesized NAR-AuNPs-F in EAC bearing mice, tumour volume and viable tumour cell count were decreased. Altered parameters such as haematological and antioxidant parameters were restored towards normal. Furthermore the average survival time of the treated animal elevated significantly in compared to the untreated control animals. The NAR-AuNPs-F revealed an extraordinary dose-dependent cytotoxic and antitumor effect on EAC cells *in vivo*. Treatment of mice bearing EAC tumours intraperitoneally with NAR-AuNPs-F showed an anti-proliferative outcome on EAC cells, abridged ascites volume, and upheld mean survival time. These results indicate towards the promising potential of this green synergy of naringenin and its targeted gold nanoparticles in cancer therapeutics.

KEYWORDS: Naringenin, Gold nanoparticles, Folate receptor, Anticancer and Antioxidant potential, Ehrlich ascites carcinoma, Mean survival time.

INTRODUCTION

Cancer is a serious public health burden and its treatment and preventive processes are still challenges in the field of science.[1] The increased death rate and the hazardous side effects of chemo-preventive drugs draw the attention of researchers to develop novel and potent drugs to minimize side effects.[2] Herbal plants and natural phytochemicals have important roles as sources of effective cancer preventing agents. It is noteworthy that about 60% of recently used anticancer compounds are driven from nature.[3]

Usually, chemotherapy has few limitations: a) less solubility in hydrophilic solution, b) toxicity at higher doses, and c) a less circulation half-life.[4] To circumvent these limitations, numerous approaches have been applied in nanoparticles formulation using different drug delivery agents.[5] Amongst those as a drug delivering agent or carrier the AuNPs possess few more advantages, such as ease of synthesis, controlled shape and size, and more biocompatibility.[6,7] Besides, AuNPs show a high surface area and volume ratio -leading to formulate anticancer drugs more using AuNPs than other naïve drugs. Hence, an AuNP-based nano-sized carrier system can decrease the dose-dependent harmful side effect of the conventional anticancer chemotherapy.[8]

Naringenin, a potent bioflavonoid, is present plentifully in the citrus fruits. It has considerable therapeutic efficiency against diseases showed in various previous experimental models.[9] It has also been reported to possess anti-inflammatory, hepato-protective, immune-modulatory, neuro-protective, anticancer and antidiabetic activities.[10] Previous studies documented naringenin preventing motility and proliferation of cells by kinase inhibiting enzyme phosphoinositide-3-kinase (PI3K). Additionally, prevention of the mitogen-activated protein kinase (MAPK) pathways eventually causes suspension of cell growth and cell death.[11] Also, naringenin

prevents the migration of lung cancer cell via suspending MMP-9.[12]

The development of biologically synthesized nanoparticles is a budding branch of nanotechnology. Therapeutic uses of gold nanoparticles (AuNPs) were proved safe and less toxic in drug delivery systems.[6] Gold nano particle became popular in the field of medical research due to their nontoxic record, self-assembly property and improved drug delivery system. Metal nanoparticle synthesis using phytochemicals is popular due to its controlled synthesis of mono dispersed solution, non-toxic, biocompatibility and eco-friendly properties.[14] Green syntheses of nanoparticles using various phytochemicals are safe for biological and clinical uses as well as are eco-friendly and less costly. AuNPs have been used to target folate receptor and folate-conjugated gold nanoparticles are used as a new nanoplatform for targeted cancer therapy as folic acid is a useful ligand to enhance AuNPs endocytosis.[13] It is a challenge to the researchers to discover targeted anticancer therapeutic drug and targeted drug delivery is more effective, less hazardous and are becoming more effective to revolutionize diagnosis and treatment of cancer.[15]

Ehrlich EAC is a transplantable spontaneous (mammary) adenocarcinoma. Ehrlich tumor, a speedily growing carcinoma shows very aggressive characteristics.[16] It is reported to have lack in H-2 histocompatibility antigens and this is apparently the reason behind their rapid growth in host cells.

The focus of this paper is to evaluate the *in vivo* antitumor and antioxidant efficacy of folate modified naringenin gold nanoparticles in Ehrlich ascites carcinoma bearing Swiss albino mice.

MATERIALS AND METHODS

Chemicals and reagents

Naringenin was purchased from Sigma Aldrich. Analytical grade hydro-chloroauric acid (HAuCl4), sodium borohydrade (NaHB4), tri sodium citrate, and potassium bromide (KBr) were procured from Merck India, Ltd., Mumbai, India. EDTA, Tris buffer, titron X-100, phenol, chloroform, iso-amyl alcohol, ethidium bromide (EtBr),5,5'-Dithio-bis-(2-nitrobenzoic Acid) (DTNB) and 2-vinylpyridine were purchased from Merck Millipore (India) Pvt. Ltd., Mumbai. All other chemicals were from Merck Ltd., SRL Pvt. Ltd., Mumbai, Himedia India, Ltd., and Mumbai, India.

Synthesis and characterization of folate receptor targeted biogenic gold nanoparticles using Naringenin

Gold nanoparticles of naringenin (NAR-AuNPs) were synthesized by treating naringenin in hydrochloroauric acid at room temperature in our laboratory.[6,17] Finally folate receptor targeted biogenic gold nanoparticles was synthesized by the mixture of folic acid (FA) and N-(3-dimethyl –aminopropyl N- ethylcarbodimide hydrochloride (EDAC), and then centrifuged at 13,000 rpm and air dried.[17]

Zetasizer Nano ZS instrument (Malvern Instruments, U.K.) was used to measure dynamic light scattering (DLS) of NAR-AuNPs-F. The polydispersity index (PDI) was also determined for the detection of the particle size distribution. Fourier transforms infrared spectroscopy (FTIR) analysis of the dried NAR-AuNPs-F sample was performed by FTIR spectrometer (Perkin Elmer Spectrum Express-1.03.00) to detect the presence of different biomolecules.[17,18]

Animal maintenance

8-10 weeks old female Swiss albino mice of 18-24 g weight were used and given standard diet and water *ad libitum* under controlled environment in 12 h light/dark cycle. EAC cells were collected from the Chittaranjan National Cancer Institute, Kolkata, India and were cultured in these mice in 1×10^6 cells /ml concentration.[19]

Approval for the study was given by the Institutional Animal Ethical Committee (IAEC), under the registration of CPCSEA, Govt. of India (approval No. IEC/7-14/C14-16).

EXPERIMENTAL DESIGN

In vivo anticancer study

One hundred two female Swiss albino mice were grouped into nine groups and only group I had six mice. Group II to group IX - each had twelve mice; six mice for the evaluation of survival time and six for the assessment of all biochemical and histological parameters. EAC cells at 1×10^6 cells /ml/mice were inoculated in each mouse of group II to group IX and this day was considered as 'Day zero'. The treatments were given to the mice by the following way for 14 consecutive days.

Group I: Saline control
Group II: EAC control
Group III: EAC + NAR (50mg/kg body weight)
Group IV: EAC + NAR (100mg/kg body weight)
Group V: EAC + NAR-AuNPs (2.5mg/kg body weight)
Group VI: EAC + NAR-AuNPs (5mg/kg body weight)
Group VII: EAC + F-NAR-AuNPs (1.5 mg/kg body weight)
Group VIII: EAC + F-NAR-AuNPs (3 mg/kg body weight)
Group IX: EAC + 5-FU (20 mg/kg body weight)

The changes in body weight were recorded regularly from the very first day to the final day.[19] Blood sample from six mice from each group were collected by cardiac puncture for estimating haematological parameters. For studying the parameters for tumour regression, ascites fluid was collected from the peritoneal cavity.[19] For the renal and hepatic oxidative stress biomarkers and

histological examination, tissues from the mice liver and kidney were collected.

Change in Body weight

The growth rate of tumor was recorded by checking changes in daily body weight from the very first day till the final day.[18,19]

Studies on host survival time and increase life span

The mean survival time (MST) and Increase life span (ILS) were calculated (Jacob and Latha, 2013) by the following equations.

Mean survival time = (Day of 1st death + Day of last death)/2,

Increase life span (ILS) (%) = [(Mean survival time of treated group/mean survival time of control group) - 1] x 100

Tumour volume

At the time of sacrifice, injected normal saline were aspirated from the peritoneal cavity of mice aseptically. The tumor volume was calculated by the following way:

Tumor volume = Volume of saline and tumor cells (ml) – Volume of saline (ml).

Then each of the calculated tumor volume is compared with tumour control group.[18]

Tumor cell count

The ascitic fluid was taken aseptically from peritoneal cavity of each mouse and diluted 100 times with phosphate buffer saline. Then a drop of the diluted cell suspension was charged in Neubauer counting chamber and the numbers of tumour cells were counted in the 64 small squares.[18]

Evaluation of haematological parameters

Red blood cell (RBC) count

A Neubaur hemocytometer was used to count the total amount of red blood cells. 1:200 blood sample diluting ratio was used. For counting, RBC dilution fluid was utilized and charged in the hemocytometer chamber.[20]

White blood cell (WBC) count

In a 1:200 ratio, the blood sample was diluted. In the haemocytometer chamber, WBC dilution fluid was charged in order to count the cells. The four huge squares at each corner of the chamber were the only ones counted under a microscope.[20]

Determination of haemoglobin

Using the cyanmethemoglobin technique, the percentage of haemoglobin was determined. First, 5ml of Drabkin's solution was placed into a glass test tube, and 20 µl of blood was added. The optical density (OD) of each sample was measured at 540 nm.[21]

ANTIOXIDANT PARAMETERS

Estimation of malondialdehyde (MDA) content

Firstly, one ml of liver and kidney homogenate (20 mg/ml phosphate buffer) was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of acetate buffer (20% pH 3.5) and 1.5 ml of aqueous solution of thiobarbituric acid (0.8%). After heating of that mixture at 95°C for 60 min red pigment was formed. After that 5 ml of *n*-butanol-pyridine mixture (15: 1) was was used for its extraction and centrifuged at 5000 rpm at room temperature for 10 min. The optical density was noted at 535 nm.[22]

Estimation of reduced glutathione (GSH)

At first, 200 µl of liver and kidney homogenate was mixed with100 µl sulfo salicylic acid and the mixture was centrifuged at 3000 rpm for 10 min. Then 1.8 ml of DTNB was mixed with 200 µl of supernatant. The supernatant was shaken well and the optical density was noted at 412 nm.[23]

Estimation of superoxide dismutase (SOD)

For the estimation of superoxide dismutase (SOD) of liver and kidney homogenate, 10 µl of homogenate, 100 µl of 2 mM pyrogallol and 2 ml of buffer mixture (50 mM TrisHCl,10 mM hydrochloric acid (HCl) and 1 mM EDTA) were poured in glass cuvette and the absorbance was measured at 420 nm for 3 min.[24]

Estimation of catalase (CAT)

Estimation of catalase was performed by the method of Aebi, 1974.After mixing of 1 ml of 30mM H_2O_2 and 1.9ml of 15mM PBS in 0.1ml of homogenate, readings were taken in the spectrophotometer at 30 sec interval at 240nm.[25]

Histopathology study

For histopathological examinations, organs such as liver, kidney were collected from sacrificed animals of all the groups. The collected organs were weighed and dipped into 10% neutral buffered formalin for preservation. Then the tissues were dehydrated in graded alcohols and embedded in paraffin. Tissues were cut in five micron thickness and the sections were stained with haematoxylin and eosin (H and E) for histopathological study.[26]

Statistical analysis

All the experiments were done in triplicate manner. The results were expressed as Mean ± SEM. Comparisons between the means of the control and treated groups were calculated by using the one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA), $p<0.05$ as a limit of significance.

RESULTS

Synthesis and characterization of folate receptor targeted biogenic gold nanoparticles using naringenin

The folate receptor targeted biogenic gold nanoparticles using naringenin (NAR-AuNPs-F) was synthesized and characterized in our laboratory.[17]

Effect of NAR-AuNPs-F on tumor growth and body weight change of tumor bearing EAC-bearing mice

Body weight of the mice increases significantly in EAC control group compared to control group, but in NAR-AuNPs and NAR-AuNPs-F treated groups at dose level of 5 and 3 mg/kg bwt the body weight was decreased significantly compared to EAC control group (Figure 1).

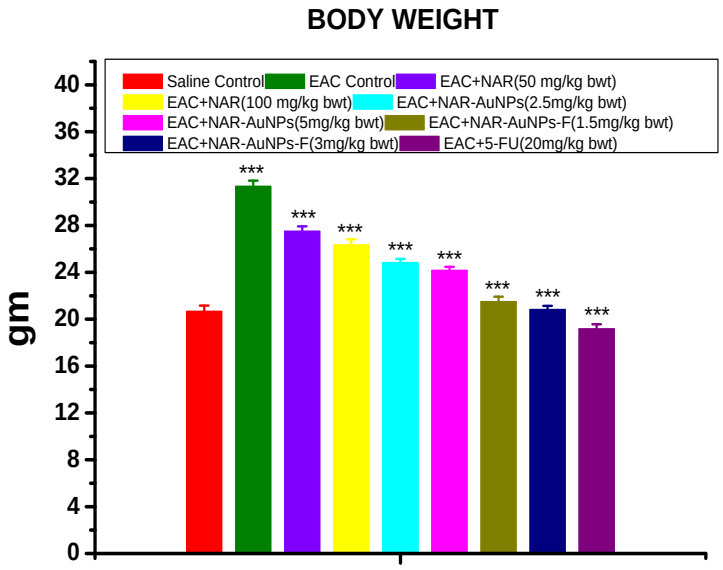

Figure 1: The effect of NAR, NAR-AuNPs, NAR-AuNPs-F on change of body weight of EAC bearing mice. Data are expressed as Mean± SEM. '*' indicates p<0.001; probability values are determined in respect of saline control.**

Effect of NAR-AuNPs-F on mean survival time, increase life span in EAC bearing mice

Treatment with NAR-AuNPs-F at the doses of 1.5 and 3 mg/kg body wt increased the mean survival time (MST) (Figure 2) by 39.75±1.1, 36.75±2.05 days, respectively compared to EAC control group. SEM.

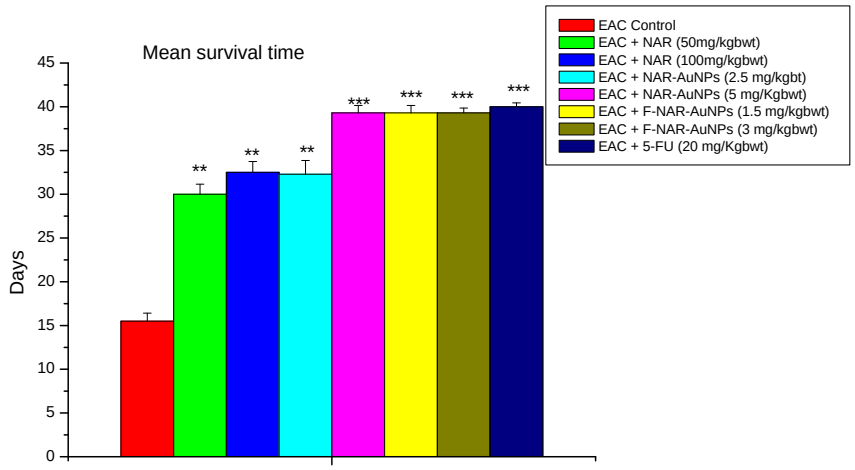

Figure 2: The effect of NAR, NAR-AuNPs, and NAR-AuNPs-F on Mean survival time by Kaplan Meier method in EAC bearing mice. Data are expressed as Mean± SEM. '' indicates significantly difference at p<0.01; '***' indicates p<0.001; probability values are determined in respect of EAC control.**

Effect of NAR-AuNPs-F in tumor volume in EAC bearing mice

NAR-AuNPs-F treatment significantly reduced tumor volume (Figure-3) compared to tumor control mice.

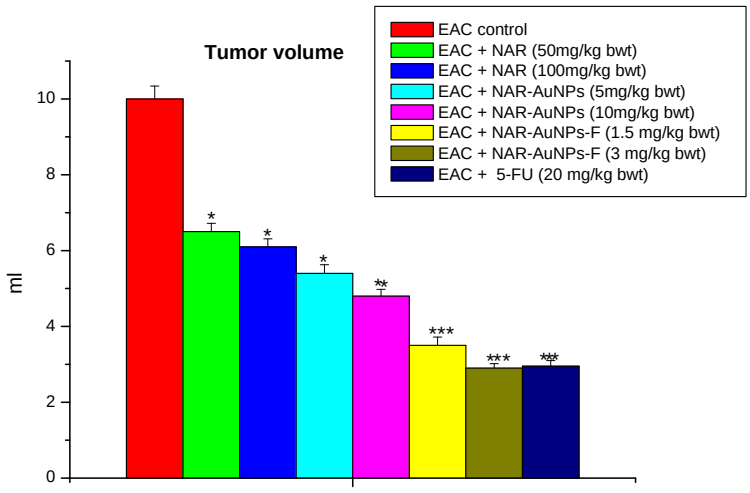

Figure 3: The effect of NAR, NAR-AuNPs and NAR-AuNPs-F on tumor volume in EAC bearing mice. Data are expressed as Mean ± SEM. Probability values are given in asterisks. '*' indicates significantly difference at p<0.05; '' indicates significantly difference at p<0.01; '***' indicates p<0.001; probability values are determined in respect of EAC control.**

Effect of NAR-AuNPs-F in tumor cell count of tumor bearing mice

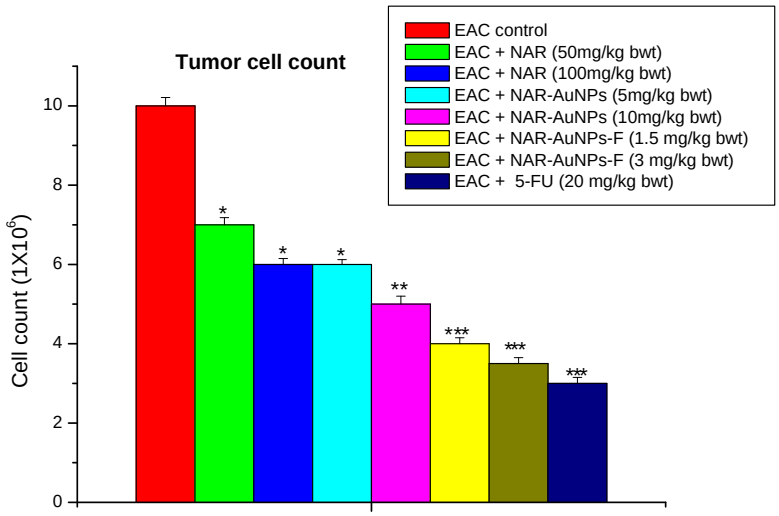

Figure 4: The effect of NAR, NAR-AuNPs, NAR-AuNPs-F on tumor cell count in EAC bearing mice. Data are expressed as Mean ± SEM. '*' indicates significantly difference at p<0.05; '' indicates significantly difference at p<0.01; '***' indicates p<0.001; probability values are determined in respect of EAC control.**

Effect of NAR-AuNPs-F on haematological parameters

The hematological parameters were altered significantly (p<0.001) after 14 days of treatment with NAR-AuNPs-F when compared to EAC (Table-1) control group. It was found that total WBC count was increased significantly in EAC control group whereas, the hemoglobin percentage and RBC count were decreased significantly in the tumor control group. After treating with NAR-AuNPs-F for 14 days, at 1.5 and 3 mg/kg in case of EAC bearing mice, hematological parameters were brought back towards normal level. These results suggested that NAR-AuNPs-F have protective role on haemopoietic system. However, the standard drug 5-FU (20 mg/kg body weight) exhibited significant (p< 0.001) results in all these haematological parameters.

Table 1: Effect of NAR, NAR-AuNPs and F-NAR-AuNPs on haematological parameters on EAC bearing mice.

Haematological Parameters	Saline Control	EAC Control	EAC + NAR (50 mg/kg bwt)	EAC + NAR (100 mg/kg bwt)	EAC + NAR-AuNPs (2.5 mg/kg bwt)	EAC + NAR-AuNPs (5mg/kg bwt)	F-NAR-AuNPs (1.5mg/kg bwt)	F-NAR-AuNPs (3mg/kg bwt)	EAC + 5-FU (20 mg/kg bwt)
Hb percentage	12.5±0.1	8±0.15a***	10.5±0.15 a*b*	11.5±0.1 a* b**	12.3±0.17 b***	12.4±0.15 b***	12.5±0.1b***	12.3±0.12b***	12.7±0.1 b***
Total RBC count (×10^6 mm^3)	6.5±0.05	1.7±0.05 a***	5.5±0.05a* b***	6±0.05 a*b***	6±0.01 b***	6.±0.002 b***	6±0.15b***	5.9±0.1 b***	5.8±0.07 a* b***
Total WBC count (/µl)	4100±30	8900±100a***	6000±50 a**b**	5500±40 a**b***	5300±50 a**b ***	4900±24 b***	4800±50b***	4600±50b***	4500±30 b***

Data are expressed as Mean± SEM (n=6). 'a*' represents significant difference at (p<0.05), 'a**' represents significant difference (p<0.01), 'a***' represents significant difference at (p<0.001) compared to saline control. 'b*' represents significant difference (p<0.05), 'b**'represents significant difference at (p<0.01) and 'b***'represents significant difference (p<0.001) compared to EAC control.

Effect of NAR-AuNPs-F on antioxidant biomarkers

The level of lipid peroxidation in liver and kidney tissue was significantly increased in tumor control mice when compared to saline control mice. After administration of NAR-AuNPs-F (1.5 and 3 mg/kg bwt) in case of EAC bearing mice, lipid peroxidation levels were significantly decreased when compared with tumor control mice. Similarly, reduced glutathione level (GSH) was changed in tumor control compared to saline control, restored to the near normal values after treatment with NAR-AuNPs-F as well as 5-FU. In tumor control mice there were significant reduction in antioxidant enzymes like super oxide dismutase (SOD), catalase which were significantly improved by the treatment of NAR-AuNPs-F as well as 5-FU. GPx and GST levels both were decreased significantly in tumor control group, NAR-AuNPs-F treatment increased significantly.

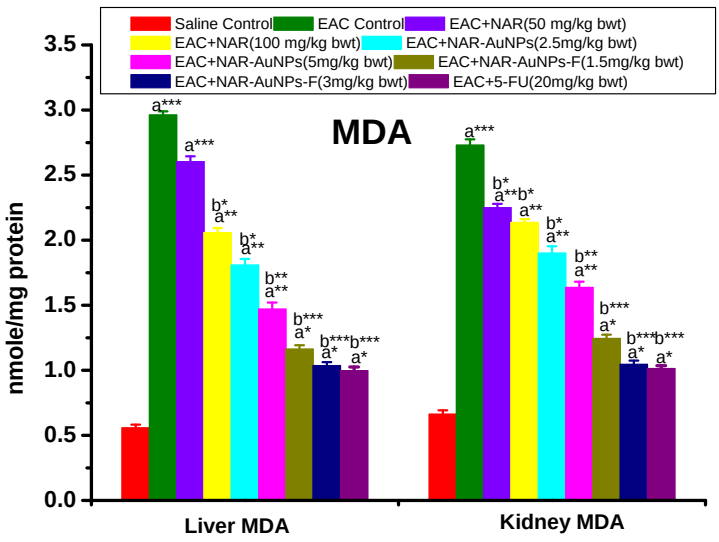

Figure 5: shows the effect of NAR, NAR-AuNPs and NAR-AuNPs-F on liver and kidney MDA after 15 days treatment in EAC bearing mice. Data are expressed as Mean± SEM (n=6). a*'represents significant difference at (p<0.001) compared to saline control; 'b**' represents significant difference at (p<0.01); 'b***''represents significant difference at (p<0.001) compared to EAC control.**

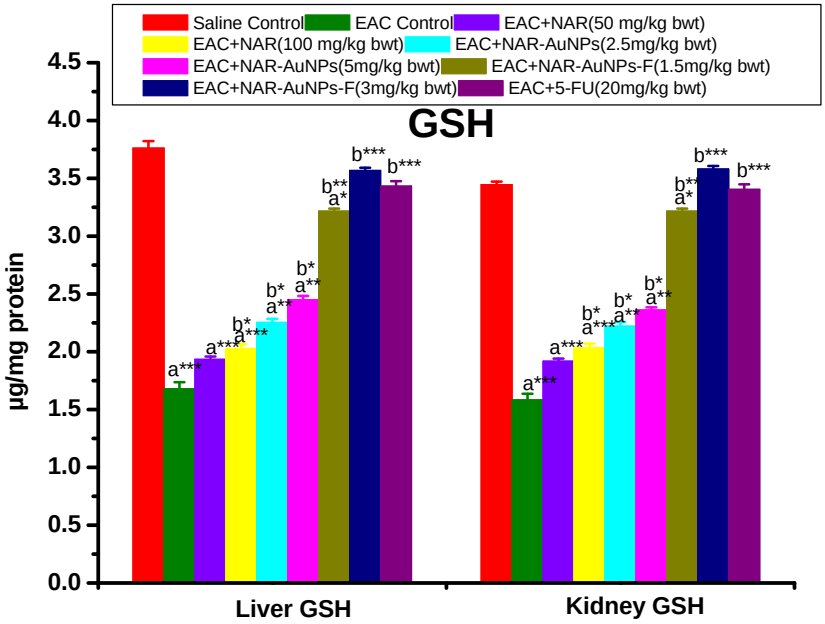

Figure 6: Shows the effect of NAR-AuNPs -F on liver and kidney GSH after 15 days treatment in EAC bearing mice. Data are expressed as Mean± SEM (n=6). 'a*' represents significant difference at (p<0.05), 'a' represents significant difference (p<0.01), 'a***' represents significant difference at (p<0.001) compared to saline control. 'b*' represents significant difference (p<0.05), 'b**'represents significant difference at (p<0.01) and 'b***'represents significant difference (p<0.001) compared to EAC control.**

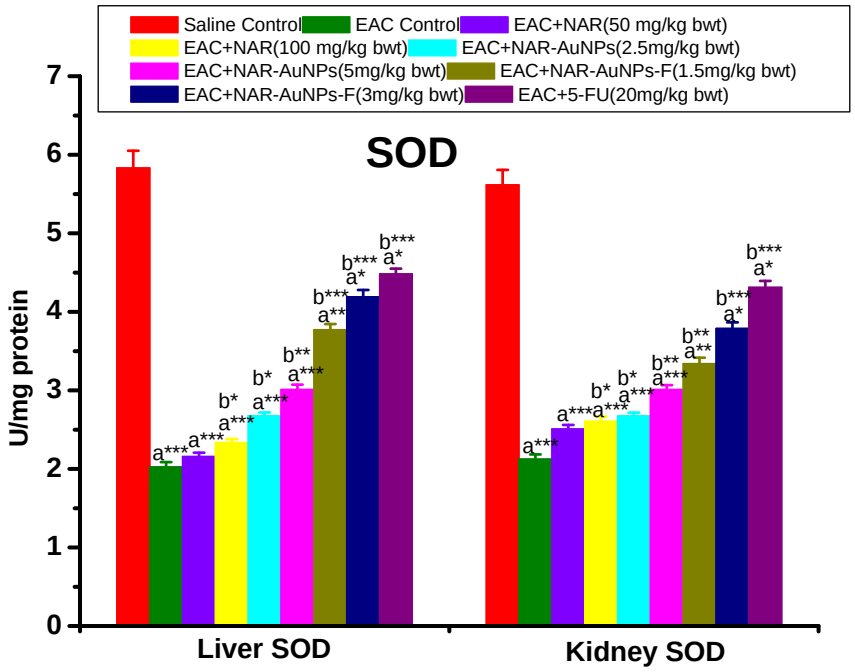

Figure 7: Shows the effect of NAR-AuNPs-F on liver and kidney SOD after 15 days treatment in EAC bearing mice. Data are expressed as Mean± SEM (n=6). 'a*' represents significant difference at (p<0.05), 'a' represents significant difference (p<0.01), 'a***' represents significant difference at (p<0.001) compared to saline control. 'b*' represents significant difference (p<0.05), 'b**'represents significant difference at (p<0.01) and 'b***'represents significant difference (p<0.001) compared to EAC control.**

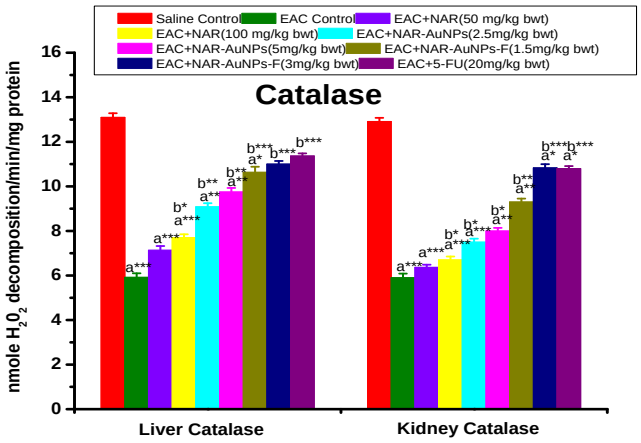

Figure 8: Shows the effect of NAR, NAR-AuNPs, and NAR-AuNPs-F on liver and kidney catalase after 15 days treatment in EAC bearing mice. Data are expressed as Mean± SEM (n=6). 'a*' represents significant difference at (p<0.05), 'a' represents significant difference (p<0.01), 'a***' represents significant difference at (p<0.001) compared to saline control. 'b*' represents significant difference (p<0.05), 'b**'represents significant difference at (p<0.01) and 'b***'represents significant difference (p<0.001) compared to EAC control.**

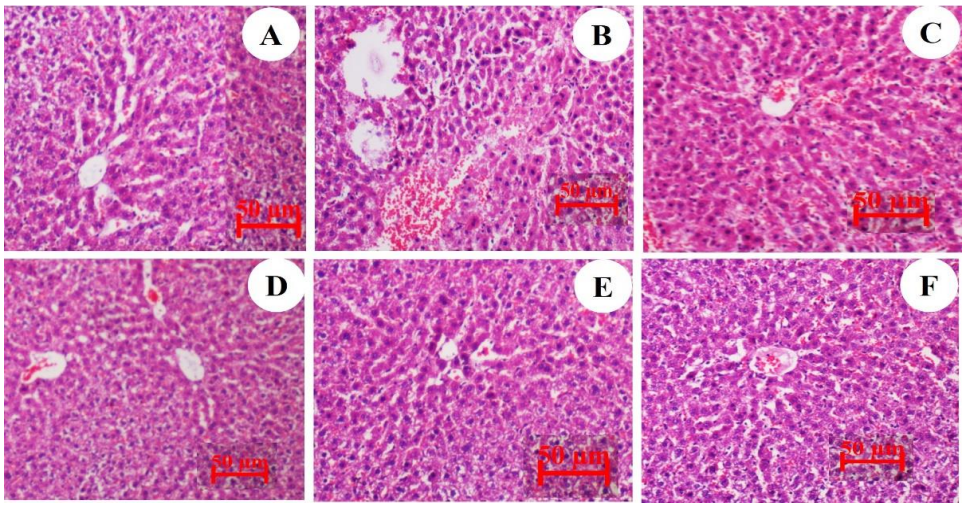

Figure 9: Histological alterations of liver after treatment of NAR, NAR-AuNPs, F-NAR-AuNPs in EAC bearing Swiss albino mice. A) Saline control, B) EAC control, C) EAC + NAR (100 mg/kg body wt), D) EAC + NAR-AuNPs (5mg/kg body wt), E) EAC + F-NAR-AuNPs (3mg/kg bwt), F) EAC + 5-FU (20 mg/kg body wt).

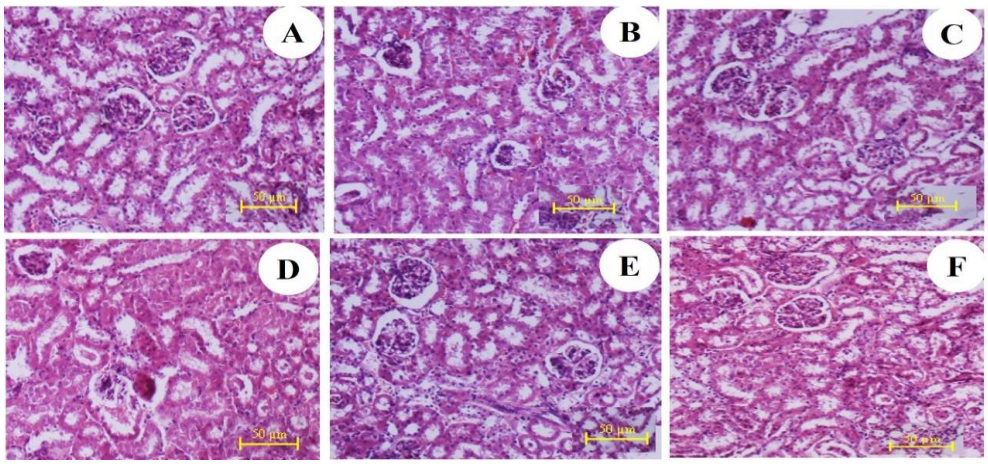

Figure 10: Histological alterations of kidney after treatment of NAR, NAR-AuNPs, F-NAR-AuNPs in EAC bearing Swiss albino mice. A) Saline control, B) EAC control, C) EAC + NAR (100 mg/kg body wt), D) EAC + NAR-AuNPs (5mg/kg body wt), E) EAC + F-NAR-AuNPs (3mg/kg bwt), F) EAC + 5-FU (20 mg/kg body wt).

DISCUSSION

The body weight of NAR-AuNPs-F treated mice was decreased compared to control EAC bearing mice. Tumor volume and tumor cell count also decreased in NAR-AuNPs-F -treated tumor bearing mice. This reduction could be positively correlated with the ascites fluid volume or tumor volume. Ascites fluid provides the nutritional source for tumor cells.[18,27] Prolongation of life span is a reliable criterion to evaluate an anticancer drug.[28] With reliability to this, average life span of NAR-AuNPs-F treated mice increased considerably. Reduction in body weight and ascites fluid volume as well as prolongation of lifespan indicates the restorative prognosis of cancer.[29]

Tumor growth can cause antioxidant disturbances in tissues. Malondialdehyde (MDA) is an end product of oxidative degeneration has been reported to be higher in cancer tissues.[30,31] The levels of MDA were found to be rapidly increased in tumor induced animals and returned back near to normal after treatment of NAR-AuNPs-F. Intracellular protection against free radicals, peroxide and toxic compounds is provided by glutathione. Tumor development noticeably decreased cellular GSH, which may occur due to oxidative stress.[32] Increased free radicals in tumour cells causes reduced levels of GSH for the effective conversion of GSH to GSSG.[31] Significant increment in liver and kidney GSH was attained by the treatment of NAR, NAR-AuNPs and NAR-AuNPs-F, representing the protecting role of NAR, NAR-AuNPs and NAR-AuNPs-F in EAC bearing mice. The levels of catalase (CAT), superoxide dismutase (SOD) were decreased in EAC bearing mice and increased significantly near to normal after treatment with NAR-AuNPs-F.

Histopathological studies also showed the level of damage to liver cells in EAC bearing mice. Histopathological studies also revealed the recovery of liver architecture after treatment of NAR-AuNPs-F in EAC bearing mice. In this study, tumour induced mice treated with NAR-AuNPs-F showed that kidney histoarchitecture was almost similar to that of normal group. From this observation it could be understood that NAR-AuNPs-F induces apoptosis in tumor cells without affecting the normal cells.

CONCLUSION

NAR-AuNPs-F produced cytotoxicity in Ehrlich ascites carcinoma (EAC) cells showing IC_{50} value of 5 µg ml^{-1} respectively and showed non-toxic nature to mice lymphocytes up to the level of 25 µg ml^{-1}. The cytotoxic effect of NAR-AuNPs-F was correlated with elevation of ROS, decrease in mitochondrial membrane potential, DNA fragmentation, cell cycle arrest, imbalance of apoptotic proteins. The antitumor activity noticed in mice model strongly may be due to the hemoprotective, hepatoprotective and antioxidant properties of NAR-AuNPs-F.

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