

ROLE OF PECTIN CAPPED SILVER NANOPARTICLES IN EXPERIMENTALLY INDUCED CARCINOMA IN MICE

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ABSTRACT

The search for novel chemopreventive agents to selectively target the tumour cells with negligible toxicity to the host cells constitute an urgent priority. Co-chemotherapy using nano-products and natural products can be fully exploited to the advantage of the cancer patients. In the present study, the anti-tumour potential of fractionated pectin powder (FPP) capped silver nanoparticles (Ag NPs) were explored using Ehrlich ascites carcinoma (EAC) induced mice at two different doses. At the end of the experimental period, parameters including cell viability, haemato-biochemical parameters, antioxidant status, mean survival time (MST) and percentage increase in life span were

recorded. FPP capped Ag NPs considerably restored the abnormal parameters to near normal levels with significant increase in cytotoxicity. The positive role of FPP capped Ag NPs in combating carcinogenesis and increasing the life span of animals suggests that the FPP capped Ag nanoparticle is a potential anti-cancer candidate and can also serve as an adjunct to conventional chemotherapy.

KEYWORDS: Fractionated Pectin Powder (FPP), Silver Nanoparticles (Ag NPs), Fractionated pectin powder capped silver nanoparticles (FPP capped Ag NPs), Ehrlich ascites carcinoma (EAC), Anti-tumour potential.

1. INTRODUCTION

Cancer is an evolutionary process through which dysregulation in select cellular mechanisms confers a clonal advantage, leading to tumour growth and eventually metastasis.^[1] Cancer is an emerging leading cause of death in economically developed and developing countries.^[2]

with the annual cancer cases expected to rise from 14 million in 2012 to 22 million in the next two decades.^[3] According to a recent^[4] estimate, around 5, 55,000 people died of cancer in the year 2010 in India.

Several independent studies have demonstrated that citrus pectin, a complex branched polysaccharide fibre rich in galactoside residues, interacts with galectin-3 and inhibits tumour growth or cancer metastasis *in vivo* and *in vitro* in various types of cancers including prostate, colon, breast, melanoma and multiple myeloma.^[5,6,7,8,9,10,11] Commercially available fractionated pectin powder (FPP) induced significant apoptosis in androgen responsive and androgen independent prostate cancer cells when compared to citrus pectin and Modified Citrus Pectin.^[12]

Conventional chemotherapy has limitations due to non-specific binding, poor pharmacokinetic properties and side effects. Nanoparticles are now being designed to assist therapeutic agents to pass through biologic barriers, to mediate molecular interactions and to identify molecular changes. They have larger surface area with modifiable optical, electronic, magnetic and biologic properties compared to macroparticles.^[13] So nanotechnology can be a better option for targeted delivery. Due to their small size and design, nanoparticles can cross physiologic barriers, delivering drugs in normally inaccessible sites with classical means.^[14] Nanoparticle drug delivery, on the other hand, enhances therapeutic effectiveness and reduces side effects of the drug payloads by improving their pharmacokinetics.^[15]

In this study silver nanoparticles were used with proven antitumour.^[16] antiangiogenic.^[17] and antipermeability effects that acts through inactivation of Src kinase pathway.^[18] Silver nanoparticle complex of α -lipoic acid act as an adjuvant in cancer radiotherapy to protect normal tissues from radiation damages and also enhanced the anti-tumour activity of gamma radiation.^[19] In an *in vitro* study using EAC cell line, FPP capped Ag NPs caused a significant (1.8 fold) reduction in the dose of the nanocomposite in attaining the IC₅₀ value showing that the nanocomposite is a better option than the individual counterparts.^[20]

Pectin is considered as “generally recognized as safe – GRAS” as direct human food ingredients.^[21] and Ag NPs is reported to be a therapeutic agent at varied dose rates.^[16] with LD₅₀ of colloidal AgNPs greater than 5,000 mg/kg body weight.^[22] In an earlier.^[23] study with pectin, LOAEL of 125 mg/kg and NOAEL of 30 mg/kg in 90-day oral toxicity study in rats was documented.

In the present study modified form of pectin (FPP) capped to silver nanoparticles (Ag NPs) was used against carcinogenesis induced in mice to explore its anti-tumour potential.

2. MATERIALS AND METHODS

2.1 CHEMICALS AND MATERIALS

Fractionated pectin powder (FPP) used in this research was obtained from Thorne Research, Dover, U.S.A. Silver nitrate, chitosan, ascorbic acid were obtained from Sigma-Aldrich. 5-fluorouracil and trypan blue were obtained from Himedia. All other chemicals and solvents used were of analytical grade.

2.2 PREPARATION OF NANOPARTICLE AND CHARACTERIZATION OF NANOPARTICLES

Silver nanoparticle and FPP capped AgNPs were prepared using chitosan and ascorbic acid and characterized.^[20] The size of FPP capped AgNPs varied between 35 and 45 nm, whereas FPP size ranged from 15 to 20 nm and Ag NPs size ranged from 20 to 25 nm.

3. IN VIVO STUDY

3.1 Experimental animals

Experiments were carried out with Swiss albino mice of either sex procured from the Unit of Laboratory Animal Medicine, Tamilnadu Veterinary and Animal Sciences University, Madhavaram, Chennai 600 051. The animals were housed in large spacious cages under standard conditions of relative humidity (55 ± 5 %), temperature ($23 \pm 2^\circ\text{C}$) and well ventilated with 12 h light /12 h dark cycle throughout the experimental period. The experiments were conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (190/CPCSEA).

3.2 Experimental induction of EAC & Tumour development in mice

EAC cells were grown in the peritoneal cavity of six to eight weeks old mice by peritoneal transplantation of 0.2 ml cell suspension ($\sim 2 \times 10^6$ cells/mouse) in sterile phosphate buffer saline.^[24] Before injection, viable EAC cells were counted by trypan blue exclusion test.

3.3 EXPERIMENTAL PROTOCOL

A total number of eighty four mice were randomised into experimental and control groups. They were divided into seven groups with twelve animals in each group.

Table No. 1 Experimental Protocol.

Group	Treatment
1.	Control
2.	EAC tumour control
3.	EAC + 5-fluorouracil @ 20 mg/kg b.w
4.	EAC + FPP @ 40 mg/kg b.w
5.	EAC + AgNPs @ 2.6 mg/kg b.w
6.	EAC + FPP capped AgNPs @ 20 mg/kg b.w
7.	EAC + FPP capped AgNPs @ 40 mg/kg b.w

Drug treatments (5-fluorouracil, FPP, Ag NPs, FPP capped AgNPs at 2 predetermined doses were purified with syringe filter and diluted with deionized water) were continued for 14 days through i/p route (directed to the tumour cells) after inoculation of tumour cells on 0th day.

3.3.1 Cell viability assay

Trypan blue exclusion test was conducted to determine the number of viable cells and non viable cells. In brief, EAC cells, harvested from tumour bearing mice from all the groups, were washed twice with phosphate buffer saline (PBS) and mixed with 0.1% trypan blue solution in equal proportion. The number of viable and non viable cells were determined using Neubauer's counting chamber. Upon staining, the viable cells did not take the stain while the non viable cells were stained blue.

3.3.2 Haematological parameters

At the end of experiment, all the animals were sacrificed and blood was collected. RBC, WBC counts and haemoglobin levels were determined by routine clinical laboratory techniques. Absolute leukocyte Count was estimated using leishman stained blood smears. Serum was separated by centrifuging the blood at 7000 rpm for 10 minutes.

3.3.3 Serum biochemistry

At the end of the experiment period, all the animals were sacrificed and blood was collected. Serum was separated by centrifuging the blood at 7000 rpm for 10 minutes.

3.3.3.1 Enzymic indices of cellular integrity

The markers for cellular integrity (alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP) and blood urea nitrogen (BUN)) were estimated in the serum by kinetic method using standard kits (Agappe Diagnostics).

3.3.4 Estimation of antioxidants

Immediately after sacrificing the animals, the liver was excised was used for the estimation of lipid peroxidation (LPO).^[25], reduced glutathione (GSH).^[26] total protein (TP).^[27] superoxide dismutase (SOD).^[28] catalase (CAT).^[29] and glutathione peroxidase (GPX).^[30]

3.4 MEAN SURVIVAL TIME (MST) AND PERCENTAGE INCREASE IN LIFE SPAN (% ILS)

The animals were observed for their mortality daily until their death or up to a maximum of 6 weeks period. Mortality was monitored by recording MST and % ILS as per the following formulae;

$$\text{MST} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{Total no. of mice}}$$

$$\text{ILS \%} = \left\{ \frac{\text{MST of treated group}}{\text{MST of tumour control group}} \right\} - 1 \times 100$$

3.5 STATISTICAL ANALYSIS

The results were expressed as mean \pm standard error (S.E). The differences between groups were assessed by using the Statistical Package for Social Sciences (SPSS) software package for Windows. The effects of treatments were determined by analyzing the data using one-way ANOVA followed by Duncan's multiple comparison test. P values < 0.05 or < 0.01 were considered as statistically significant.

4. RESULTS

4.1 CARCINOGENIC INDICES

4.1.1 Cell viability assay

There was a significant ($P < 0.05$) decrease in the total cell count in treated groups when compared to EAC control animals. There was a marked ($P < 0.01$) drop in the live cell count and a significant ($P < 0.05$) increase in the dead cell count after treatment with FPP, AgNPs and FPP capped AgNPs in EAC induced animals. FPP capped AgNPs @ 20mg/kg b.w was most effective in decreasing the live cell count and increasing the dead cell count (Table-2).

Table-2.

PARAMETERS	Total cell count* (X10 ⁴)/ml	Viable tumour cell count**(X10 ⁴)/ml	Non viable Cell count* (X10 ⁴)/ml
EAC	2092.80 ^b ±251.84	2067.20 ^c ±247.43	32.00 ^a ±6.53
EAC+5-FU	-	-	-
EAC+FPP	1240.00 ^a ±144.22	1189.33 ^b ±145.6	50.67 ^{ab} ±9.61
EAC+Ag NPs	1160.00 ^a ±112.66	1098.67 ^{ab} ±115.13	61.33 ^{ab} ±9.61
EAC+ FPP capped AgNPs (20mg/kgb.w)	980.00 ^a ±105.19	581.33 ^a ±197.46	148.00 ^c ±58.92
EAC+ FPP capped AgNPs (40mg/kgb.w)	1136.00 ^a ±96.00	842.67 ^{ab} ±177.29	108.80 ^{bc} ±15.51

4.2 HAEMATOLOGICAL PARAMETERS

The haematological parameters of EAC cells bearing mice showed significant changes when compared to normal mice on the 14th day (Table 3). The total WBC count was found to increase with a reduction in the haemoglobin content and total RBC count. Treatments with FPP, AgNPs and FPP capped AgNPs at two predetermined doses significantly restored the altered haemoglobin content ($P<0.05$), RBC ($P<0.05$) and WBC count ($P<0.01$) to near normal levels.

The absolute count of WBC showed that the neutrophils count increased significantly ($P<0.01$) while that of lymphocytes and monocyte count remained unchanged in the EAC control group when compared to normal mice. FPP capped AgNPs at 20 mg/kg b.w and 40 mg/kg b.w reverted these altered parameters significantly to more or less the normal values. But treatment with FPP and FPP capped AgNPs at 40 mg /kg b.w, showed increased neutrophil count. Standard drug 5-fluorouracil significantly restored all the haematological parameters to normal levels.

4.3 COMPROMISED CELLULAR INTEGRITY – BIOCHEMICAL PARAMETERS

The compromised cellular integrity in the untreated EAC-induced mice is depicted in Table No. 4. There was a sharp increase in the activities of ALP, AST, ALT, BUN in the serum of tumour control animals. Significant ($P<0.001$) restoration in the activities of ALP, AST, ALT and BUN was observed on treatment with FPP capped AgNPs @ 20 and 40 mg/kg b.w doses.

4.4 CARCINOGENIC – OXIDATIVE ASSAULT

4.4.1 Antioxidant enzymes and non-enzymes

Table No. 5 presents the activities of the antioxidant enzymes in the liver tissues of experimental animals. Statistically significant inhibition in the activities of antioxidant

enzymes (SOD, CAT and GPX) were observed in EAC-induced animals as against the control animals (Table-5). On treatment with FPP, AgNPs and FPP capped AgNPs at two predetermined doses, the activities of SOD and CAT had a sharp ($P<0.01$) increase when compared to the tumour-induced non treated animals. The status of GPX was significantly ($P<0.05$) elevated in the treated groups in comparison to the tumour induced untreated animals. The level of non-enzymatic antioxidant GSH in liver tissue of control and experimental animals are depicted in Table 5. In untreated EAC induced animals, the level of GSH was significantly ($P<0.01$) decreased as against the control animals. In the treated groups, the levels of GSH were restored to near normalcy.

4.4.2 Lipid peroxidation (LPO)

There was a steep increase in the extent of LPO in the liver of EAC tumour control group as against the normal control animals. The administration of FPP capped AgNPs at the two predetermined doses provided a remarkable counter effect, as observed by the low levels ($P<0.01$) of LPO in the EAC tumour induced animals (Table 5).

4.5 MEAN SURVIVAL TIME (MST) AND INCREASE IN LIFE SPAN (ILS)

MST of tumour control and treated mice were evaluated based on direct observation for a period of 6 weeks, where the day of death of each animal was recorded. The results show that there is significant ($P<0.01$) increase in MST among all the treated groups when compared to EAC control groups. 5-fluorouracil treated groups showed excellent results in increasing the MST but there were no significant differences between 5- fluorouracil and treatment with FPP capped AgNPs @ 20 and 40 mg/kg b.w doses in increasing the lifespan. Similar results were obtained in the case of % ILS where all the treated groups outperformed in comparison to the tumour control groups but there were no significant differences between the treated groups (Fig. 1).

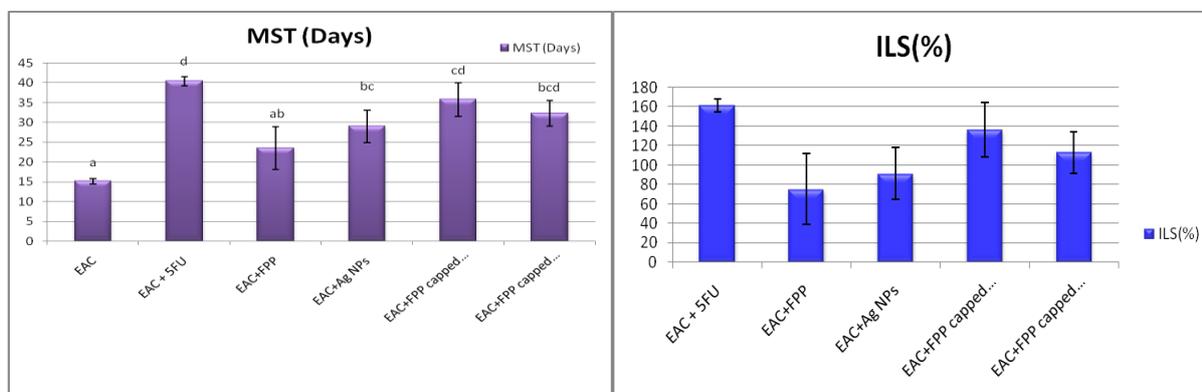


Fig.1 Effect of 5-fluorouracil, FPP, nAg particles, nAg particles tagged FPP on MST (A) and ILS% (B). Data represented as mean \pm SE of six independent experiments.**

Table-3: Effect of drugs on haematological parameters of EAC bearing mice.

PARAMETER	Hb*(g%)	RBC*(x10 ⁶ / μ l)	WBC** (x10 ³ / μ l)	Neutrophil** (x10 ³ / μ l)	lymphocyte (x10 ³ / μ l)	Monocyte (x10 ³ / μ l)
CONTROL	14.07 ^b ±0.61	7.54 ^b ±0.16	5.82 ^a ±0.96	1.78 ^a ±0.27	3.92±0.78	0.10±0.045
EAC	10.42 ^a ±0.58	5.33 ^a ±0.20	16.74 ^c ±3.47	10.93 ^c ±2.01	5.47±1.60	0.18±0.068
EAC+5 FU	13.82 ^b ±1.12	7.42 ^b ±0.51	6.92 ^{ab} ±1.30	2.75 ^{ab} ±0.56	4.03±0.73	0.06±0.024
EAC+FPP	11.03 ^a ±1.07	7.02 ^b ±0.59	12.72 ^{bc} ±2.07	10.02 ^c ±2.47	2.32±0.73	0.16±0.081
EAC+nAg	11.48 ^{ab} ±0.79	6.68 ^b ±0.58	12.05 ^{bc} ±2.11	5.91 ^{abc} ±1.47	5.89±1.00	0.13±0.016
EAC+FPPcappedAgNPs@20mg/kbw	12.08 ^{ab} ±0.71	7.25 ^b ±0.33	11.65 ^{abc} ±1.86	6.92 ^{bc} ±1.77	4.34±1.41	0.26±0.099
EAC+FPPcappedAgNPs@40mg/kbw	13.04 ^{ab} ±0.59	7.34 ^b ±0.59	12.64 ^{bc} ±1.33	8.52 ^c ±1.38	3.92±1.38	0.11±0.029

Table-4 - Effect of drugs on markers of cellular integrity – (ALT, AST, ALP, BUN)**.

GROUPS	ALT (IU/L)	AST(IU/L)	ALP (IU/L)	BUN (mg/dl)
CONTROL	112.50 ^a ±13.19	62.33 ^a ±27.33	35.00 ^{abc} ±8.19	23.99 ^{ab} ±2.86
EAC	213.66 ^b ±9.73	187.66 ^c ±17.27	78.00 ^d ±1.13	55.95 ^c ±2.50
EAC+ 5 FU	108.00 ^a ±6.70	146.50 ^{bc} ±16.46	50.50 ^{bc} ±9.50	30.80 ^b ±3.59
EAC+FPP	204.00 ^b ±29.51	137.83 ^{bc} ±23.22	53.33 ^c ±8.33	26.86 ^{ab} ±1.02
EAC+nAg	198.50 ^b ±10.95	154.50 ^c ±25.71	37.83 ^{abc} ±5.83	20.53 ^a ±1.15
EAC+FPPcapped AgNPs(20mg/kbw)	127.00 ^a ±25.25	67.66 ^a ±4.10	27.50 ^a ±1.82	25.65 ^{ab} ±2.26
EAC+FPPcapped AgNPs(40mg/kbw)	147.66 ^a ±14.03	84.0 ^{ab} ±22.11	31.50 ^{ab} ±5.38	26.21 ^{ab} ±2.22

Table-5: Effect of drugs on antioxidant enzymes, glutathione content and lipid peroxidation in the liver of EAC bearing mice.

Groups	SOD** (U/mg protein)	CAT** (U/mg protein)	GPX* (μ M of GSH utilized/min/mg protein)	GSH** (mg of reduced GSH/g of tissue)	LPO** (μ M of MDA/g tissue)
CONTROL	6.47 ^b ±0.59	23.17 ^c ±0.59	9.83 ^a ±1.25	10.31 ^b ±2.12	143.05 ^a ±12.35
EAC	1.85 ^a ±0.18	10.08 ^a ±0.67	4.53 ^a ±0.26	5.63 ^a ±0.82	350.43 ^b ±78.88
EAC+5 -FU	3.30 ^a ±0.11	23.00 ^c ±0.38	9.76 ^a ±0.72	11.93 ^b ±0.82	219.27 ^a ±33.52
EAC+FPP	2.38 ^a ±0.82	16.92 ^b ±1.73	6.88 ^{ab} ±0.88	12.46 ^b ±0.33	118.65 ^a ±17.29
EAC+nAg	6.61 ^b ±0.94	20.24 ^{bc} ±1.59	8.96 ^a ±0.92	10.52 ^b ±0.41	200.47 ^a ±29.12

EAC+FPPcappedAgNPs (20mg/kgb.w)	6.21 ^b ±1.1 5	23.22 ^c ±2.79	9.90 ^a ±1.41	10.37 ^b ±0.67	181.88 ^a ±27.71
EAC+FPPcappedAg NPs (40mg/kgb.w)	6.02 ^b ±1.6 3	23.06 ^c ±2.68	9.49 ^a ±2.53	10.67 ^b ±0.40	153.54 ^a ±23.66

Effect of 5-FU, FPP, Ag NPs, FPP capped AgNPs at two predetermined doses on oxidative stress and haemato-sero biochemical markers. Data represented as mean ± SE of six independent experiments (**P<0.01, * P<0.05). Mean bearing different superscripts between the drugs treatments differ significantly.

5. DISCUSSION

5.1 ANTITUMOUR POTENTIALS

5.1.2 Cytotoxicity

In the present study, significant reduction in the viable and non viable cell count, FPP capped AgNPs showed better results than their individual counterparts.

5.2 HAEMATOLOGICAL PARAMETERS

In the present study, in EAC cells bearing mice all haematological parameters were found to be significantly altered when compared to the normal mice. This is in accordance with the previous reports.^[31,32] which document that myelosuppression and anaemia have been frequently observed in ascites carcinoma. Anemia encountered in ascites carcinoma is mainly due to iron deficiency, either by hemolytic or myelopathic conditions which finally lead to reduced RBC number.^[33] The altered haematological parameters almost restored towards normal values when treated with FPP, Ag NPs and FPP capped Ag NPs at both the predetermined doses. But there was neutrophilia in all treated groups, especially in FPP and FPP capped Ag NPs (40mg/kg.b.w) groups, although the neutrophil count in treated groups was lesser than the tumour control group. Neutrophilia observed in the present study in the treatment groups might be the tumour associated neutrophils (TAN) that infiltrate in the tumour microenvironment and assume tumour cytotoxic phenotype to destroy the tumour cells. The results corroborate the reports of Fridlender and Albelda, (2012).^[34] Neutrophils are also a source of anti angiogenic mediators, such as elastase, which promotes the degradation of VEGF-A, bFGF and α -defensins.^[35, 36, 37]

5.3 MARKERS OF CELLULAR INTEGRITY - SERUM BIOCHEMISTRY

The increased levels of serum AST, ALT, ALP and BUN activities in the EAC tumour induced mice may indicate the leakage of these cytosolic enzymes into the circulatory system due to hepatocellular damage during EAC propagation.

Treatment with FPP, AgNPs and FPP capped AgNPs at the two predetermined doses significantly reversed the alterations to near normal levels. This might be due to the underlying hepato- and nephro-protective mechanisms of FPP, AgNPs and FPP capped AgNPs at the two predetermined doses. The improvement in the liver and kidney functions due to nanocomposite administration at the two predetermined doses are in agreement with EI-Batal (2012)^[38] who documented that the markers of cellular integrity (ALT AST, serum creatinine and urea activities) in EAC bearing mice treated with MCP and selenium were restored significantly.

5.3 ANTIOXIDANT STATUS

Reactive oxygen species (ROS) can accelerate DNA damage, stimulate pro-carcinogenesis, initiate LPO, inactivate antioxidant enzyme systems and can modulate the expression of genes related to tumour promotion.^[39, 40] It has been reported that antioxidants can inhibit proliferation of cancer cells.^[41] FPP capped Ag NPs at two predetermined doses significantly reduced the elevated levels of LPO and increased the GSH level. Thus FPP capped AgNPs might have induced the detoxifying enzymes that would have scavenged the ROS in EAC bearing mice. On the other hand, it was earlier.^[42] reported that tumour growth inhibits the free radical scavenging systems, SOD and CAT. Similar findings were observed in the present investigation with EAC bearing mice. The administration of FPP capped AgNPs at two predetermined doses increased the SOD and CAT levels in a dose dependent manner, which may indicate the antioxidant and free radical scavenging property of FPP capped AgNPs. The improvement in the liver antioxidant status by FPP capped Ag NPs at two predetermined doses are in agreement with EI-Batal (2012).^[38] who reported that liver antioxidant status in EAC bearing mice restored to near normal levels when treated with MCP in combination with selenium.

5.4 MEAN SURVIVAL TIME (MST) AND INCREASE IN LIFESPAN (ILS)

The reliable criteria for the establishment of efficacy and quality of any anticancer drug are prolongation of life span of the animals and decrease in WBC.^[43, 44] Andreani *et al.*, (1983).^[45] suggested that an increase in the life span in the ascites bearing animals by 25% is

indicative of significant drug activity. In the present study, treatment with FPP capped Ag NPs at the two predetermined doses significantly increased MST and % ILS as against the controls. The prolongation of survival of animals is directly related to the decrease in viable cell count and ascitic fluid accumulation.^[46] Treatment with FPP capped AgNPs significantly increased lifespan of tumour induced animals highlighting the positive potentials of the nanocomposite in alleviating the tumour burden and enhancing life expectancy.

6. CONCLUSION

The above results signify the antitumour efficacy of the FPP capped AgNPs which can be attributed to the decrease in the survivability of cancer cells and reduction of oxidative stress. Hence this study clearly demonstrates that FPP capped AgNPs had anti-tumour effects at both tested doses indicating the beneficial effects of targeted drug delivery. The two tested doses of FPP capped AgNPs had equipotent effects indicating that the anticancer effect could be obtained with the optimal dose (lower dose). Due to restoration of various altered oxidants, haematological and biochemical variables by the nano formulation, it is obvious that FPP capped AgNPs maintain better health status, accounting for holistic approach to cancer. Further rigorous studies are needed to elucidate the concrete mechanisms of antitumour activities of FPP capped AgNPs on the entire apoptotic pathway underlying carcinogenesis.

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