



Hesperetin-loaded polymeric nanoparticles inhibit proliferation and oxidative stress in Ehrlich ascites carcinoma bearing Swiss albino mice: An *in vivo* study exploring anticancer potential

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Article History

Volume 6 Issue 12, 2024
Received: 25 May 2024
Accepted : 25 June 2024
doi:
[10.48047/AFJBS.6.12.2024.324-333](https://doi.org/10.48047/AFJBS.6.12.2024.324-333)

Abstract

Hesperetin (HSP) is a natural bioflavonoid found abundantly in citrus fruits with promising antioxidant, anti-inflammatory, anti-hypertensive and anti-cancer properties. The aim of the present study was to evaluate the *in vivo* anticancer activity of synthesized HSP loaded PLGA nanoparticles (PLGA-HSP NPs) in Ehrlich Ascites Carcinoma (EAC) cell bearing Swiss albino mice using the standard drug 5-fluorouracil. PLGA-HSP NPs led to a considerable reduction in tumor volume, the number of viable tumor cells, and body weight in EAC tumor-bearing mice, as well as an increase in the mean survival time. PLGA-HSP NPs protect the host tissues from oxidative stress by recovering the antioxidant status in host mice. PLGA-HSP NPs also restored the haematological parameters. The aforementioned finding revealed that PLGA-HSP NPs have a favourable impact on EAC cell bearing Swiss albino mice. Therefore, the findings of the present study indicate that PLGA-HSP NPs may be considered as a competent anticancer agent in future.

Keywords: Hesperetin, PLGA; Ehrlich ascites carcinoma; *In vivo* anticancer activity; Antioxidant status; Hematological parameters.

Introduction

Cancer is a complicated disease worldwide with multiple facets and a high death rate (Janssen, 2018). Around 20 million new cases of cancer were reported globally in 2022, and 9.7 million people have been deceased due to this disease globally (Bray et al., 2024). It is commonly known that cancer is caused by a combination of hereditary and environmental factors. Acquiring knowledge about these variables and the cancer microenvironment can lead to novel targets and strategies for cancer treatment. The only available treatments now are traditional therapies (chemotherapy, radiation, and surgery), despite a great deal of

research and developments in innovative ways (Xiao and Yu, 2021). Chemotherapeutic chemicals are used to treat cancer, but they can severely damage healthy cells and create multiple drug resistance in cancer cells (Nikolaou et al., 2018).

Many naturally occurring chemicals, such as terpenoids, lignans, phenolic acids, tannins, quinones, flavonoids, coumarins, and alkaloids, were shown to contain high antioxidant properties and play crucial roles in the treatment of cancer. Plants were the sources to obtain these compounds (Cai et al., 2004). A flavone mostly found in citrus fruits called hesperetin (4'-methoxy-3', 5,7-trihydroxy flavanone) is pharmacologically safe (Aranganathan et al., 2008; Bai et al., 2017). Hesperetin is most commonly found in citrus fruits like lemon, orange etc. In recent years, a significant number of studies have been carried out on its antibacterial (Kim et al., 2021), anticarcinogenic (Bodduluru et al., 2015), antiviral (Ahmadi et al., 2016), antioxidant, anti-allergic, and anti-inflammatory activities (Khan et al., 2020). HSP is known to be effective against a variety of cancers including hepatocellular carcinoma (Zhang et al., 2015), breast cancer (Palit et al., 2015), colon cancer (El-Deek et al., 2012), prostate cancer (Shirzad et al., 2015), and many others.

Several studies highlighted the fact that nanoparticle-based drug delivery systems could improve the solubility and stability of the encapsulated drugs while maintaining their intracellular therapeutic concentration (Yusuf et al., 2023). Furthermore, other characteristics such as increased drug loading ability, longer half-life with minor systemic toxicity, increased internalization into the tumor via endocytosis, sustained and regulated release of cytotoxic drug over the suitable duration and time along with body excretion are significant for nanoparticles in cancer treatment (Liu et al., 2021). The primary disadvantages of the majority of nanoparticle-based systems are their short shelf life, high surfactant and co-surfactant concentrations, susceptibility to lipid oxidation and transformation, incompatibility with different active agents, and low drug loading efficiency (Badawi et al., 2022).

In comparison, polymeric nanoparticles exhibit a higher drug loading capacity, comparatively better *in vivo* half-life, and the ability to release drugs under regulated conditions (Kamaly et al., 2016). Polymeric nanoparticles seemed to be one of the most suitable options for drug administration in cancer therapy (Jose et al., 2018). Poly-lactide-co-glycolide (PLGA) is regarded as an excellent polymeric nanocarrier because of its extended drug release profile, biocompatibility, and biodegradability (Singh and Singha, 2021). Hydrophobic groups on the inside and polar groups on the outside of PLGA nanoparticles allow for the efficient encapsulation of both hydrophobic and hydrophilic drugs. Hydrophobic regions on the nanoparticles facilitate the binding of phytochemicals through hydrogen bonding and hydrophobic interactions. In a dispersion system, nanoparticles (NPs) can maintain their stability due to inter-particle repulsions and hydration (Guo et al., 2023).

The focus of present study is to evaluate the *in vivo* anticancer effects of hesperetin encapsulated PLGA nanoparticles in Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice.

Materials and methods

Materials

Hesperetin, Poly (D, L-lactic-co-glycolic) acid (PLGA), poloxamer were purchased from Sigma Aldrich Co, LLC, US. 2,4,4-dithionitrobenzoic acid (DTNB), trichloro acetic acid (TCA), thiobarbutaric acid (TBA), Tris HCl, sodium dodecyl sulfate, potassium dihydrogen phosphate (KH_2PO_4), hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), sulfosalicylic acid (SSA), sodium chloride (NaCl), absolute alcohol, ether, drabkin's diluent solution, RBC and WBC dilution fluid, Leishmann stain, ethylenediamine tetra-acetic

acid (EDTA), trypan blue, 5-fluorouracil (5-FU) and other chemicals were purchased from Merck Ltd., SRL Pvt. Ltd., Mumbai, India and other reagents utilised in the tests were of analytical grade.

Synthesis of hesperetin encapsulated PLGA Nanoparticles

Hesperetin encapsulated PLGA nanoparticles were synthesized previously by a modified nano-precipitation method (Giri et al., 2024). Briefly, PLGA and hesperetin were dissolved in acetone and added dropwise to a water phase containing Millipore water and poloxamer, followed by stirring for 24h. The nanoparticles were then washed, centrifuged, filtered to remove aggregates, and stored at -20°C. Finally, they were lyophilized to obtain freeze-dried nanoparticles. To measure the particle size dynamic light scattering (DLS) of hesperetin-loaded PLGA was performed using Zetasizer Nano ZS instrument (Malvern Instruments, U.K.). UV-Vis and other analyses were performed for characterization of hesperetin-loaded PLGA.

Animal maintenance

Swiss albino female mice weighing 18–25 gm were kept in a pathogen-free environment with free access to food and water, a 12h light-dark cycle, a temperature of $25 \pm 2^\circ\text{C}$ and a humidity of $60 \pm 5\%$. The animals were given a regular pellet diet, and water was available to them sufficiently. The animal experiments were performed by the Institutional Animal Ethical Committee, Vidyasagar University, Midnapore, West Bengal, India, authorized by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of Ministry of Environment, Forests and Climate Change of India with approval number (VU/IAEC/CPCSEA 12/5/2022).

Culture of cell line

Ehrlich Ascites Carcinoma (EAC) cells used in this study were collected from Chittaranjan National Cancer Research Institute (CNCR), Kolkata. EAC was kept alive in female albino Swiss mice by performing weekly intraperitoneal transplanting at a cell concentration of 2×10^6 cells/mouse.

Treatment schedule

Sixty healthy female Swiss albino mice were grouped into six ($n=12$). EAC (2×10^6 cells/mouse) was inoculated intraperitoneally to all mice except the saline control group. Group-I (saline control) received normal saline; Group-II was considered as EAC control. After 24 h of EAC cells inoculation, mice were treated for subsequent 14 days with free HSP (20 mg/kg body weight, Group-III), PLGA-HSP NPs (equivalent dose of 20 mg/kg of HSP, Group-IV) and positive control 5-fluorouracil (5-FU, 20 mg/kg body weight, Group-V). After the last dose and a fasting period of 24 h, six mice from each group were sacrificed by using sodium pentobarbital anesthesia for the evaluation of antitumor activities (El-Naggar et al., 2017; Dey et al., 2022). The rest of the animals from each group were kept for the study of the mean survival time.

Studies on body weight and mean survival time

The daily weight variations of each mouse ($n=6$) were recorded. The mean survival time (MST) was determined by applying the following equations to the daily death rates that were recorded for a period of six weeks in order to determine how long the host mouse was able to live (Choudhury et al., 2010).

$$\text{MST} = (\text{Day of first death} + \text{Day of last death})/2$$

Determination of tumor volume

At the 15th day, 2 ml of normal saline solution was injected into the intraperitoneal cavity of each mouse. After that, the peritoneal fluid was removed aseptically from the peritoneal cavity of each mouse, and the volume of the tumor was determined using the following formula (Bepari et al., 2015).

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

Measurement of tumor cell count

EAC cells were removed aseptically from the intraperitoneal cavity of each animal and then the sample was diluted one hundred times using phosphate-buffered saline (pH 7.4). After that, a drop of the diluted cell solution was charged on the chamber of the hemocytometer, and the number of tumor cells in each of the 64 small squares was counted (Saha et al., 2011).

Hematological parameters

Red blood cell (RBC) count

The Neubauer hemocytometer was used to count the total red blood cells. RBC dilution fluid was used to dilute the blood 1:200 before it was charged into the hemocytometer chamber for counting (Wintrobe, 1967).

White blood cell (WBC) count

Blood was charged into the hemocytometer chamber after being diluted in a 1:20 ratio with a WBC dilution solution. Under a microscope, large squares from each of the four corners of hemocytometer chamber was counted (Wintrobe, 1967).

Determination of haemoglobin percentage

The cyanmethaemoglobin method was used to estimate the haemoglobin percentage. First, 5 ml of Drabkin's solution was placed in a test tube with 20 µl of blood added. At 540 nm, the optical density of the sample was determined (Dacie and Lewis, 1975).

Evaluation of Antioxidant parameters

Malondialdehyde (MDA)

The hepatic and renal MDA contents were determined (Ohkawa et al., 1979) by mixing 1 ml of each tissue homogenate (20 mg/ml phosphate buffer), 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of acetate buffer (20% pH 3.5), and 1.5 ml of an aqueous solution of 0.8% thio-barbituric acid. After heating the mixture for 1 h at 95°C, a red pigment was produced. This pigment was extracted using 5 ml of a mixture of n-butanol and pyridine (15:1) and centrifugation was done at room temperature at 5000 rpm for 10 min. At a wavelength of 535 nm, the absorbance of the supernatant was measured.

Reduced glutathione (GSH)

In short, 200 µl of tissue homogenate was mixed with 100 µl of sulfosalicylic acid, and the mixture was centrifuged at 3000 rpm for 10 min. The residue was taken and mixed with 1.8 ml of DTNB with proper shaking. At 412 nm, the reading was noted (Griffith, 1981).

Superoxide dismutase (SOD)

The SOD activity was determined by measuring the percentage of inhibition of the pyrogallol auto-oxidation by SOD (Marklund et al., 1974). In a spectrophotometric cuvette, 2 ml of buffer mixture (50 mM Tris HCl, 10 mM hydrochloric acid in the presence of 1 mM EDTA), 100 µl of 2 mM pyrogallol and 10 µl of tissue homogenate were mixed and the absorbance was measured for 3 min at 420 nm using a spectrophotometer (UV-245 Shimadzu, Japan).

Catalase (CAT)

By mixing 0.1 ml of tissue homogenate with 1 ml of 30 mM H₂O₂ and 1.9 ml of 15 mM PBS, the amount of catalase was determined (Aebi, 1974). The spectrophotometer was used to record the readings at 240 nm at 30 sec intervals.

Statistical analysis

All the experiments were done in a triplicate manner. The results were stated as Mean ± standard deviation (S.D.). Comparisons between the means of the control and treated groups were analysed by using the one-way analysis of variance (ANOVA) (Origins 8.5 (Origin Lab, Northampton, USA)). All statistical significance was considered at $p < 0.05$.

Results and Discussion

Cancer is characterized by unchecked cell division and the capacity of these cells to infect other tissues, either directly through invasion into nearby tissues or indirectly through metastasis into distant regions (Freemantle et al., 2007). Traditional plants may have served as valuable sources for creating novel anticancer agents and may offer an effective alternative in cancer therapy.

Due to the quick and steady formation of Ehrlich ascites carcinoma, EAC-bearing control mice showed a marked rise in body weight. Treatment with PLGA-HSP NPs significantly reduced the body weight of mice compared to that of EAC control mice demonstrating the inhibition of tumor cell progression (Figure 1A). When compared to the EAC control group, MST of the PLGA-HSP NPs and 5-FU treated groups were seen to be considerably improved ((Figure 1B).). A consistent, quick increase in ascitic tumor volume was seen in mice carrying EAC tumors. The immediate nutritional supply for tumor cells is ascitic fluid, and a rapid rise in ascitic fluid with tumor growth would be a way to meet this nutritional need (Prasad and Koch, 2014). The tumor volume and tumor cell count were significantly decreased by PLGA-HSP NPs and 5-FU treatment compared to the EAC control group (Figure 4C & D). The body weight loss in the treated tumor-bearing mice may be due to a decrease in tumor volume (Kim et al., 2016).

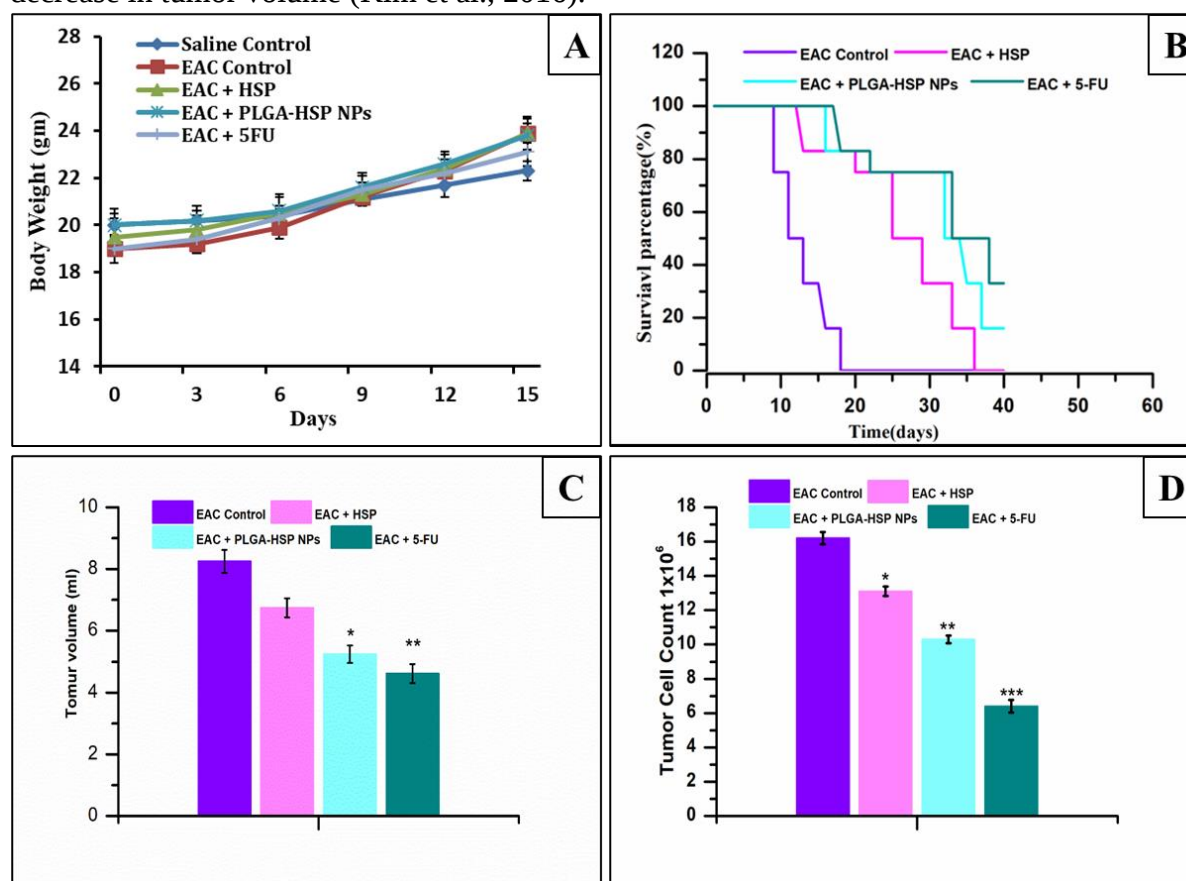


Figure 1. shows the anti-tumor activity of HSP, PLGA-HSP NPs, and 5-FU on body weight (A), mean survival time (B), tumor volume (C), and tumor cell count (D) in Swiss albino mice with EAC cells. The results are given as Mean $\pm$ SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Ascites cancer frequently exhibits myelosuppression and anemia. Elevated WBC counts and decreased hemoglobin percentage and RBC counts were seen in EAC control mice. Increasing levels of WBC are a result of a heightened immunological response, and anemia in cancer patients is mostly caused by poor iron absorption or increased hemolysis (Abd El-Gawad et al., 2021). Total RBC count and hemoglobin content of EAC control group was found to be

significantly lower and their total WBC count was substantial increased compared to saline control mice. The hemoglobin content, RBC and WBC counts returned to nearly normal after administration of PLGA-HSP NPs and it reveals the effectiveness of the PLGA-HSP NPs in defending the hemopoietic system (Table 1).

Table 1. The hematological parameters in EAC-bearing Swiss Albino mice.

Hematological Parameters	Saline Control	EAC Control	EAC + HSP	EAC + PLGA-HSP NPs	EAC + 5-FU
RBC ($\times 10^6/\text{mm}^3$)	6.19 $\pm$ 0.09	2.87 $\pm$ 0.01***	3.38 $\pm$ 0.44##	3.9 $\pm$ 0.02###	4.83 $\pm$ 0.04###
Total WBC ($\times 10^3/\text{mm}^3$)	5.25 $\pm$ 0.06	8.77 $\pm$ 0.09**	6.31 $\pm$ 0.06##	5.83 $\pm$ 0.11###	5.65 $\pm$ 0.05###
Haemoglobin (gm %)	12.88 $\pm$.33	7.74 $\pm$ 0.16***	10.41 $\pm$ 0.04##	10.79 $\pm$ 0.03##	11.84 $\pm$ 0.10###

The data are expressed as Mean $\pm$ SD (n=6). ‘**’ and ‘##’ show a significant difference at $p < 0.01$; ‘***’ and ‘###’ show a significant difference at $p < 0.001$. The EAC Control is compared to the Saline control. The treated groups are compared to the EAC Control using one-way ANOVA.

Free radicals are recognized to have a role in the development of cancer (Okada, 2002). Excessive formation of free radicals results in oxidative stress, which can incite lipid peroxidation *in vivo* and damage macromolecules like lipids. Increased lipid peroxidation contributes to tissue aging. The byproduct of lipid peroxidation, MDA, is more abundant in carcinomatous tissue than in normal tissue (Yagi, 1987; Rahman et al., 2020). In this study, it was found that the treatment with PLGA-HSP NPs in EAC-bearing mice reduced the level of MDA (Figure 2A) like the standard drug 5-FU.

GSH is a powerful antioxidant system component that is found in larger concentrations in normal tissues and at lower concentrations in cancerous tissues. It also plays a significant function in the neoplastic process (Rahman et al., 2020). As an endogenous antioxidant system, it is crucial for free radical scavenging. It should be highlighted that the reduced glutathione level in the EAC control group may be caused by an excess of free radicals using it, but in the treatment group, the GSH level was significantly higher compared to the untreated EAC-bearing mice (Figure 2B).

The two enzymes found in the system that scavenges free radicals are catalase (CAT) and superoxide dismutase (SOD). Primary function of these two enzymes is to protect the body from hydrogen peroxide and superoxide anions. It has also been documented that the growth of tumors inhibits SOD and CAT activity (Sun et al., 1989; Rahman et al., 2020). In the present study, a decrease in the levels of SOD and CAT was observed in EAC-control mice. In treatment with synthesized PLGA-HSP NPs in Swiss albino mice, SOD and CAT levels increased in comparison to the EAC control like the standard drug 5-FU (Figure 2C & D). In this study, it was established that PLGA-HSP NPs have antioxidant and free radical scavenging abilities.

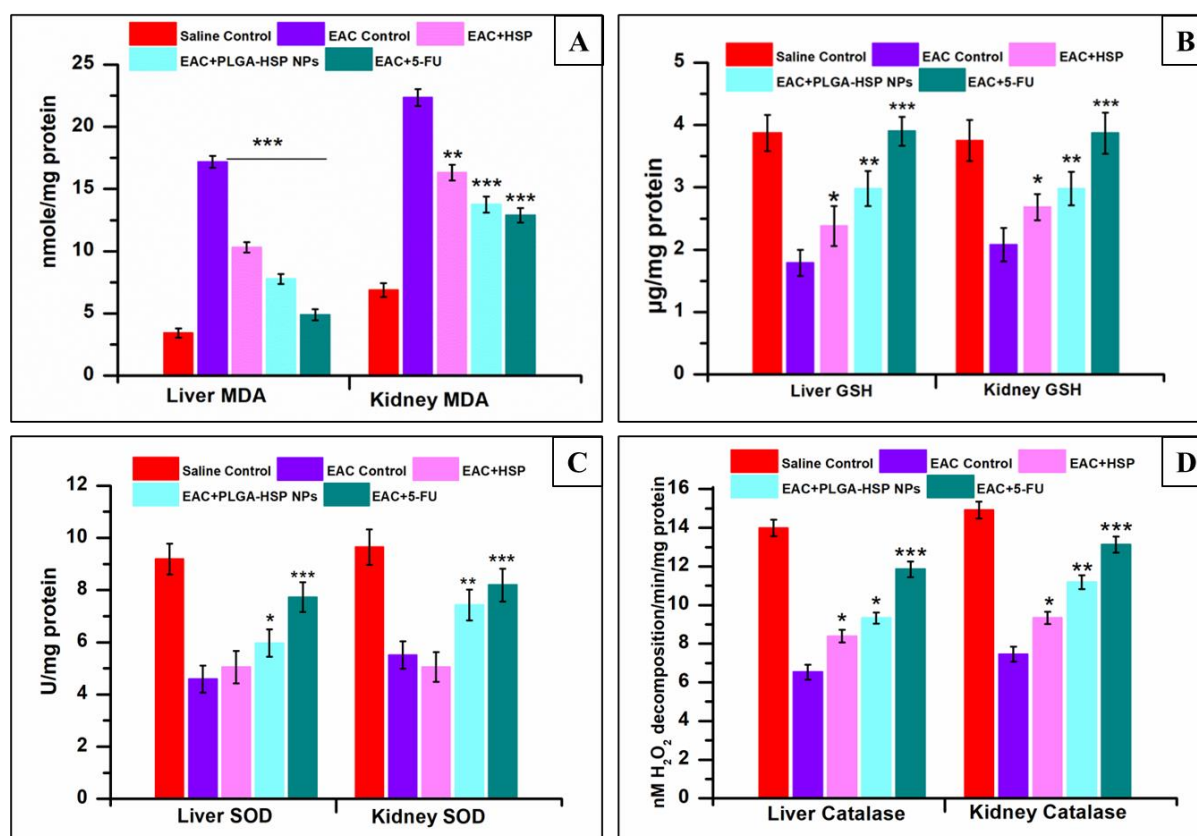


Figure2. Study of *in vivo* liver and kidney antioxidant parameters after the treatment with HSP, PLGA-HSP NPs, and PLGA-HSP-TF NPs. MDA (A), GSH (B), SOD (C) and Catalase (D). The results are given as Mean $\pm$ SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 6$, compared to EAC control).

Conclusion

Cancer is a devastating fatal disease all over the world and scientists are still trying to find an effective way to combat this disease. This study reported that PLGA-HSP NPs have significant antioxidant, and growth inhibition activity to cancer cells. So, PLGA-HSP NPs may be considered as a potent anticancer agent.

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