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Original article

Biosynthesized Platinum Nanoparticles Inhibit the Proliferation of Human Lung-Cancer Cells *in vitro* and Delay the Growth of a Human Lung-Tumor Xenograft *in vivo*

-*In vitro* and *in vivo* Anticancer Activity of bio-Pt NPs-

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Key Words

biosynthesized platinum nanoparticles, complementary and alternative medicine, lung cancer, severe combined immune deficient mice, tumor growth inhibition, xenograft

Abstract

Objectives: Lung cancer remains a deadly disease with unsatisfactory overall survival. Cisplatin, a standard platinum (Pt)-based chemotherapeutic agent, has the potential to inhibit the growth of lung cancer. Its use, however, is occasionally limited by severe organ toxicity. However, until now, no systematic study has been conducted to verify its efficacy with proper experimental support *in vivo*. Therefore, we examined whether biosynthesized Pt nanoparticles (NPs) inhibited human lung cancer *in vitro* and *in vivo* to validate their use in alternative and complementary medicine.

Methods: We evaluated the *in vitro* and the *in vivo* anticancer efficiencies of biosynthesized Pt NPs in a subcutaneous xenograft model with A549 cells. Severe combined immune deficient mice (SCID) were divided into four groups: group 1 being the vehicle control group and groups 2, 3 and 4 being the experimental groups. Once the tumor volume had reached 70 — 75 mm^3, the progression profile of the tumor growth kinetics and the body weights of the mice were measured every week for 6 weeks after oral administration of Pt NPs. Doses of Pt NPs of 500, 1,000 and 2,000 mg/kg of body weight were administered to the experimental groups and a dose of honey was administered to the vehicle control group. The efficacy was quantified by using the delay in tumor growth following the administration of Pt NPs of A549 human-lung-cancer xenografts growing in SCID mice.

Results: The *in vitro* cytotoxicity evaluation indicated that Pt NPs, in a dose-dependent manner, inhibited the growth of A549 cells, and the *in vivo* evaluation showed that Pt NPs at the mid and high doses effectively inhibited and delayed the growth of lung cancer in SCID mice.

Conclusion: These findings confirm the antitumor properties of biosynthesized Pt NPs and suggest that they may be a cost-effective alternative for the treatment of patients with lung cancer.

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1. Introduction

Lung cancer remains a deadly disease with an unsatisfactory overall survival [1, 2]. Though the treatment of patients with lung cancer includes surgery, chemo-

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therapy and radiotherapy, the overall survival remains poor. Moreover, due to the resistance to conventional therapy, the 5-year combined survival rate of patients with lung cancer of all stages is still only 16% [3]. Non-small-cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancers and is the leading cause of tumor-related death worldwide, highlighting the need for more effective treatment strategies [4, 5]. NSCLC is inherently resistant and generally not responsive to initial chemotherapy [6]. Therefore, the identification of potential drugs for treating patients with lung cancer is important. The use of taxanes and platinum (Pt)-based chemotherapy after surgery has been established as the first-line treatment by previous randomized controlled trials [7, 8]. However, cisplatin has nephro-toxicity, gastrointestinal toxicity and neurotoxicity.

Over the past decade, significant progress has been made with respect to the development of novel nanoparticles (NPs) designed for the detection and/or treatment of cancer [9, 10]. Inorganic nanoparticles, notably metal nanoparticles, have raised much interest in research on their material properties, availabilities, capabilities, specific targeting, and sustained release [11]. Biosynthesized nanoparticles are currently employed in the fields of bioremediation, biolabelling, biosensors and several more [12]. For many years, Pt-based molecules have received considerable attention because of their electrocatalytic properties [13, 14]. In medicine, Pt NPs have only been used as catalysts because of their conductivity and reactivity [15]. Few Pt-based nanomaterials have notable therapeutic applications [16]. Functional Pt NPs have shown apoptosis-inducing properties through target-specific pathways [17, 18]. Pt NPs have also been shown to induce death in cervical cancer cells through G2/M phase arrest [19] Gehrke *et al* [20] demonstrated the cytotoxic effect of human colon carcinoma cell lines HT29 and Caco-2 by using different sizes of Pt NPs. Also, evidence for deoxyribonucleic acid (DNA) damage and anti-oxidant response changes associated with Pt-NP treatment *in vitro* exists [21].

The present study aims at applying biosynthesized Pt NPs for their *in vivo* anticancer activity, an area in which the number of available reports is limited. Plants have been utilized extensively by mankind since the advent of civilization, and use of plant products in nano-biotechnology has grown tremendously in recent years. Hence, a new effective Pt-based chemotherapeutic agent with less toxicity and non-cross-resistance is needed for the treatment of patients with lung cancer. Herein, an attempt is, therefore, made to use biosynthesized Pt NPs as an effective alternate method for treating patients with lung cancer.

The present study is a continuation of an earlier work and is carried out to assess the anti-tumor activity of Pt NPs against a human lung-cancer xenograft model. As human tumor xenografts are frequently used as predictive preclinical models for anticancer drug activity in humans [22], we chose this model for anti-tumor efficacy. The cytotoxic effect of Pt NPs against A549 cells was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The progression profiles of tumor growth kinetics and the body weights of the severe combined immune deficient mice (SCID) mice used in this study were measured every week. We also investigated tumor growth inhibition after oral administration of biosynthesized PT NPs. Nevertheless, the efficacy of any chemo-modulatory agent is not scientifically validated until it has been examined through pre-clinical or clinical trials. This is our first approach at an evaluation of the anti-tumor effect of orally-administered Pt NPs in mice bearing A549 lung tumor xenografts, given that most Pt-based chemotherapeutic drugs are administered intravenously.

2. Materials and Methods

Pt NPs were synthesized through green technology and characterized based on particle size, zeta potential and surface morphology [23]. The recovered NP sample was used for cytotoxicity and anti-cancer studies. Cell culture reagents were purchased from Himedia laboratories Pvt. Ltd. (India). Fetal bovine serum (FBS) was purchased from Invitrogen (US).

The human lung-cancer cell line A549 was obtained from the National Centre for Cell Science (NCCS), sub-cultured, and then used to determine cell cytotoxicity after exposure to the drug. The cells were cultured in Dulbecco modified eagle medium (DMEM) supplemented with 10% FBS at 5% CO_2 and 37°C. At 85% confluence, the cells were harvested using 0.25% trypsin and seeded in 96-well plates. The cells were allowed to 70% attach to the surface prior to treatment. A stock solution of Pt NPs 10 mg/mL was prepared in vehicle and was diluted to appropriate concentrations for *in vitro* study. For the *in vivo* experiment, Pt NPs was administered as an oral suspension at a dose volume of 10 mL/kg based on animal's weight.

This study was carried out in two steps. In the first set of experiments, *in vitro* cytotoxicity was measured. Then, based on the *in vitro* results, we evaluated the *in vivo* anticancer activity in a second set of experiments. The cytotoxicity of Pt NPs in human lung-cancer cells was determined by using MTT assays. The MTT assay measures the cell proliferation rate and conversely, the reduction in cell viability, when metabolic events lead to apoptosis or necrosis [24]. The yellow compound MTT is reduced by mitochondrial dehydrogenases to the water-insoluble blue formazan compound, depending on the viability of the cells. Cells (2×10^4 cells/mL) were grown on microtiter plates in 96-well microplates with different concentrations (50, 100 and 200 μg/mL) of Pt NPs for a period of 48 hours. After the treatment period, the cells were allowed to react with MTT for a period of 3 - 4 hours in the dark at 37°C. By the end of the incubation period, dark purple formazan crystals had formed. These crystals were dissolved in an organic solvent (e.g., isopropanol), and the absorbance at 595 nm was measured spectrophotometrically. The experiment was repeated at least three times. To determine the cell viability, we calculated the percent viability as % viability = [(Optical Density {OD} of treated cell - OD of blank)/(OD of vehicle control - OD of blank)] × 100. The absorbance for vehicle-treated cells was considered as 100%. The results were determined based on three independent experiments.

For the *in vivo* xenograft experiment, five- to seven-week-old female, SCID mice were obtained from Laboratory An-

imal Research Services (LARS), Navi Mumbai, India. The animals were maintained in cages with filter paper covers under controlled atmospheric conditions. Cages, covers, bedding, food and water were changed and sterilized weekly. Animals were handled in a sterile manner in a laminar down-flow hood. This experiment was performed according to the guidelines for Animal Experimentation (CPCSEA) at Reliance Life Science, Navi Mumbai, Maharashtra, India, and had been approved by the Animal Care and Use Committee of the same.

Murine tumor models were used as previously described [25, 26]. For the *in vivo* experiment, A549 cell line was obtained from the ATCC (American Type Culture Collection). A549 cells (5×10^6 cells/mouse) in 0.2 mL of phosphate buffered saline were injected subcutaneously into the flank region of the SCID mice on day 0, after which the mice were observed daily to look for the occurrence of a tumor (Fig. 1). The mice were inspected for tumor formation twice per week, and the tumor size was measured by using a vernier caliper. After the tumors had reached a mean volume of approximately 70 — 75 mm^3, the mice were randomly assigned to one of four groups (6 mice per group), and the following treatments were administered: (a) honey (the vehicle control group) and (b) 500 $mg \cdot kg^{-1}$ body weight (bw), (c) 1,000 $mg \cdot kg^{-1}$ bw, and (d) 2,000 $mg \cdot kg^{-1}$ bw of Pt NPs (experimental groups).

Based on the outcomes of an acute oral toxicity study in Swiss albino mice (data not published), we knew that Pt NPs were safe up to 5,000 mg/kg bw. Thus, in this study, we used doses of 500, 1,000, 2,000 mg/kg bw for evaluating anticancer activity. Each animal received the treatment through gavage once daily for six weeks. The mice were closely monitored before the tumors were removed. Animals were weighed weekly until the completion of the study. The mice were examined for overt signs of any adverse drug-related side effects. Acceptable toxicity for cancer drugs was defined, based on National Cancer Institute (NCI) guidelines, as a mean BW loss of the group of < 20% during the study period and not more than one toxic death among ten related animals [27]. The *in vivo* anti-tumor activity assessment was checked by determining the length and the width of the tumor by using a vernier caliper. Tumor growth was monitored and recorded twice weekly to evaluate the progression profile of tumor growth kinetics. Tumor volume was calculated by using the following formula: tumor volume (mm^3) = (w2 × l)/2, where w = width (in mm) and l = length (in mm) of the tumor.

The percentage growth inhibition (% tumor growth inhibition, TGI) was calculated as follows: [100 × (tumor volume of vehicle control - tumor volume of treated group)] ÷ tumor volume of the vehicle control. The day on which the average size of the tumor of the vehicle control group reached the size of the tumor in the treated group on the last day gave the tumor growth delay (TGD).

Statistical comparisons were made by using the student's *t*-test. Results were expressed as means ± standard deviations (SDs). *P*-values of less than 0.05 were considered significant.

3. Results

The present study aimed to demonstrate the anti-cancer activity of Pt NPs. The first set of experiments was carried out to explore the cytotoxic effect of the dose of Pt NPs on A549 cells *in vitro*. In the second set of experiments, anticancer activity was evaluated *in vivo*. Female SCID mice were subcutaneously injected with A549 cells, and the mice were observed until the tumor volume had reached 70 — 75 mm^3. Then, the mice were divided in the four groups, and each animal was treated with either honey or a different dose of Pt NPs as described above and shown in Fig. 1.

To check the cytotoxic effect of Pt NPs on A549 cancer cells, we cultured A549 cells in the presence of different concentrations of Pt NPs. Treatment of A549 cells with Pt NPs (50 — 200 μg/mL) for 48 hours reduced cell viability 71.48% ($P < 0.01$) at 50 μg/mL, 66.57% ($P < 0.001$) at 100 μg/mL, and 65.15% ($P < 0.01$) at 200 μg/mL (Fig. 2). These findings indicate that Pt NPs have a dose-dependent cytotoxicity effect on A549 cells.

To determine whether biosynthesized Pt NPs have potential therapeutic value for the treatment of patients with a lung carcinoma, we further tested the inhibition caused by biosynthesized Pt NPs of A549 lung tumor growth in the SCID mice xenograft model. After treatment, the efficacy was assessed based on BW, tumor volume, TGI, TGD, and tumor weight.

To monitor drug toxicity, we measured the BW of each animal weekly. An analysis of BW variations generally defined the adverse effects of the different therapy regiments. No statistically significant changes in BW were observed when any of the treated group of animals was compared with the vehicle control group (Table 1).

The progression profile of tumor growth kinetics was measured. In comparison with the vehicle control, all treated groups exhibited lower tumor volumes. The mid (1,000 mg/kg bw)- and the high (2,000 mg/kg bw)-dose groups showed significantly ($^*P < 0.05$) lower tumor volumes from day 13, but the low-dose group (500 mg/kg bw) did not. On days 40 and 42, even though the volumes of the tumors in the mid- and the high-dose animals were lower, that difference was not statistically significant (Table 2).

Fig. 3 summarizes the percent tumor size inhibition at various time points, demonstrating that tumor growth inhibition in the various treatment groups yielded a maximum effect. TGD was also evaluated. In comparison with the vehicle control group, tumor inhibition was observed in all treated groups, except the low-dose group, up to day 13. At the end of the treatment period, the percent inhibitions, in comparison to vehicle control group, for the low-, mid- and high-dose groups were 37%, 66% and 59%, respectively (Fig. 3). In comparison to the vehicle control group, the tumor growth delay was 11 — 14 days for the low-dose group and 20 — 23 days for both the mid-dose and the high-dose groups (Fig. 3). The tumor weights of all the mice in the four groups were measured on day 42. When compared with the vehicle control group, the tumor weights were observed to be lower in all treated groups. The difference in tumor weight was significant in the mid-dose group ($^*P < 0.05$) when compared to the vehicle con-

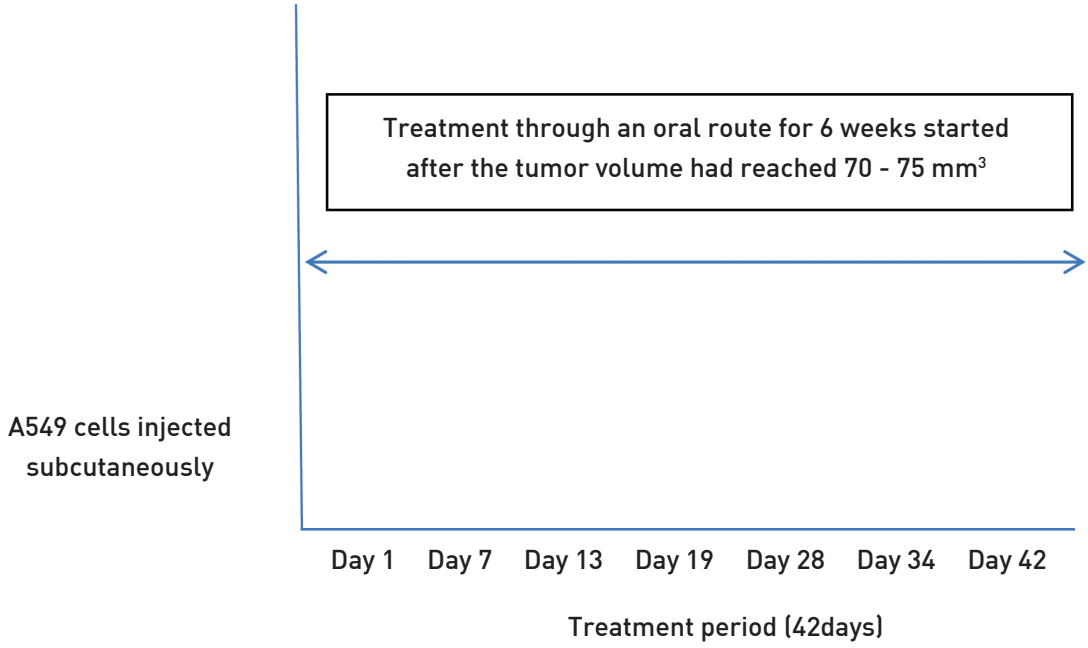

Figure 1 5×10^6 A549 cells in 0.2 mL were injected subcutaneously into SCID mice to create lung tumor. Animals were monitored twice a week till the appearance of palpable tumor (mostly 4 weeks). Once the tumor size reaches 70 — 75 mm^3, animals were selected for dosing for a period of 6 weeks (42 days). Honey (n = 6), or 500 $mg \cdot kg^{-1}$ (n = 6), 1,000 $mg \cdot kg^{-1}$ (n = 6) and 2,000 $mg \cdot kg^{-1}$ (n = 6) biosynthesized PtNPs were administrated orally every day for six weeks.

SCID, severe combined immune deficient mice; PtNPs, platinum nanoparticles.

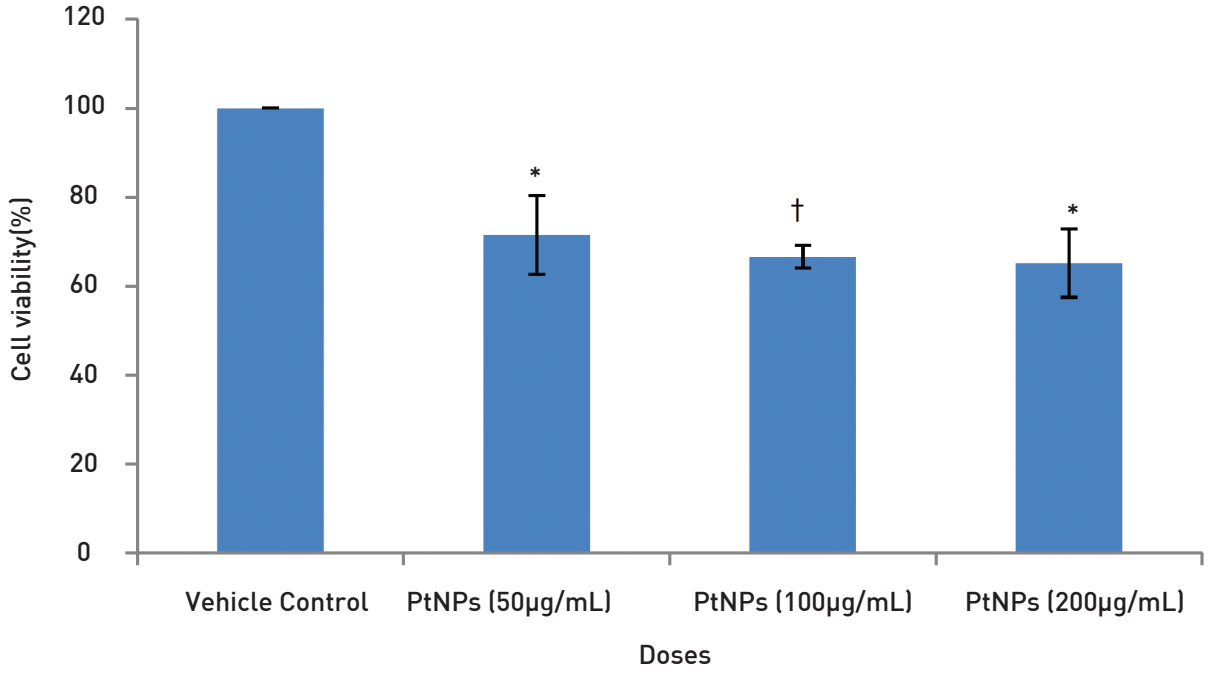

Figure 2 Cytotoxicity of biosynthesized platinum nanoparticles in A549 cells exposed to different concentration (50, 100, 200 μg/mL) of drug for 48 hours. At the indicated time point, cell viability was measured by MTT assay. The absorbance for vehicle treated cells was considered as 100%. $^*P < 0.01$ and $^\dagger P < 0.001$ *vs.* vehicle control group.

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

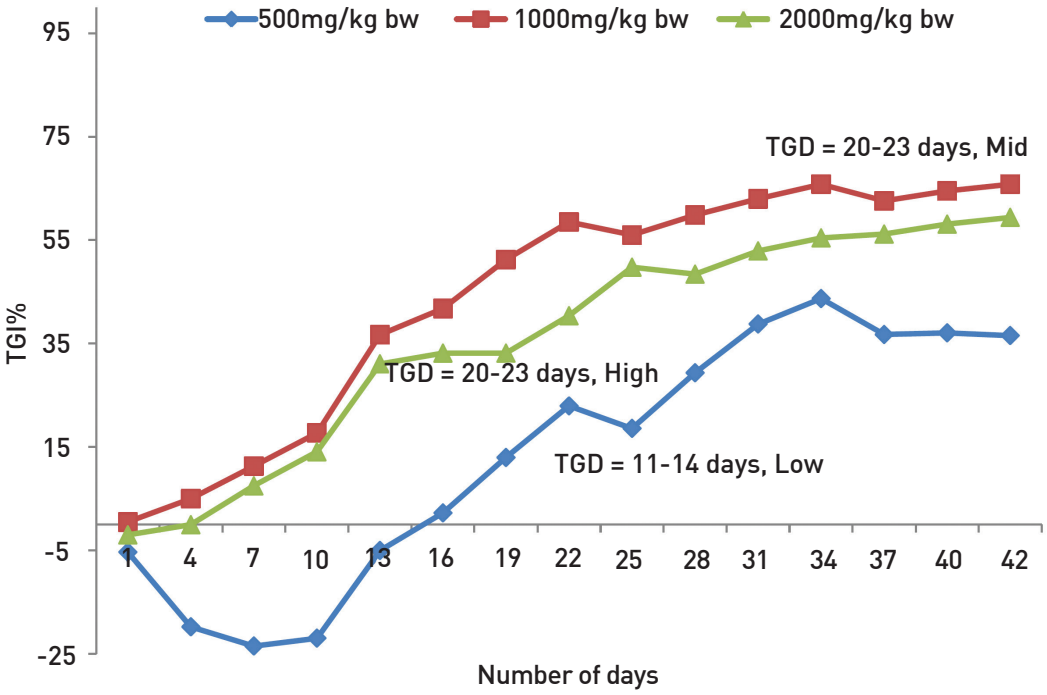

Figure 3 Effects of biosynthesized PtNPs on tumor growth inhibition and tumor growth delay. Data was analyzed against vehicle control.

PtNPs, platinum nanoparticles.

Table 1 Effects of biosynthesized PtNPs on BW. Data was analyzed against vehicle control

Series	BW (gm) in days						
	1	7	14	21	28	35	42
Vehicle Control	19.9 ± 1.42	20.32 ± 1.33	21.07 ± 1.34	21.6 ± 1.44	22.09 ± 1.7	22.69 ± 2.5	23.34 ± 4.1
500 mg/kg bw	18.63 ± 2.19	19.13 ± 1.87	19.62 ± 1.65	20.53 ± 1.53	21.15 ± 1.27	21.4 ± 1.83	21.6 ± 2.02
1,000 mg/kg bw	21.22 ± 1.83	21.33 ± 1.85	21.49 ± 1.97	21.85 ± 1.9	22.9 ± 1.95	23.09 ± 2.12	22.94 ± 1.95
2,000 mg/kg bw	19 ± 2.78	19.33 ± 2.78	19.76 ± 2.76	19.98 ± 2.77	20.66 ± 2.93	20.65 ± 2.93	20.05 ± 1.95

PtNPs, platinum nanoparticles; BW, body weight.

Table 2 Progression profile of tumor growth kinetics

Series	Measurement of Tumor volume (mm^3) in days							
	1	4	7	10	13	16	19	22
VC	70.43 ± 27.69	92.22 ± 30.53	129.50 ± 41.21	180.1 ± 66.4	281.16 ± 82	368.94 ± 104.75	494.2 ± 169.17	684.19 ± 285.16
Low	74.20 ± 20.31	110.48 ± 33.49	159.95 ± 55.49	219.7 ± 92.89	295.34 ± 119.38	360.74 ± 161.16	430.23 ± 247.02	527.36 ± 303.52
Mid	70.11 ± 18.74	87.62 ± 21.07	114.93 ± 24.76	148.22 ± 38.96	178.03 ± 45.06*	214.99 ± 61.03*	241.07 ± 53.57*	283.92 ± 49.56*
High	71.85 ± 5.99	92.27 ± 7.69	119.84 ± 14.57	154.79 ± 27.42	193.84 ± 31.46*	246.7 ± 54.93*	294.72 ± 74.85*	343.84 ± 88.27*

(Continued)

Series	Measurement of Tumor volume (mm^3) in days						
	25	28	31	34	37	40	42
VC	787.98 ± 368.92	957.89 ± 475.2	1158.47 ± 572.74	1288.05 ± 688.12	1356.71 ± 789.82	1467.77 ± 927.32	1546.81 ± 1027.95
Low	641.63 ± 371.7	677 ± 323.78	709.6 ± 278.73	725.53 ± 265.85	858.46 ± 415.51	924.12 ± 449.9	981.84 ± 482.02
Mid	347.12 ± 50.73*	384.89 ± 68.09*	429.21 ± 82.23*	440.51 ± 120.43*	507.67 ± 220.26*	521.22 ± 231.02	529.1 ± 216.29
High	406.58 ± 92.88*	451.27 ± 110.63*	515.49 ± 147.87*	548.56 ± 174.96*	594.89 ± 191.39	615.45 ± 202.63	628.28 ± 211.79

VC, vehicle control; Low dose, 500 mg/kg bw; Mid dose, 1,000 mg/kg bw; High dose, 2,000mg/kg bw; BW, body weight.

*$P < 0.05$ *vs*. vehicle control group.

Table 3 Effects of biosynthesized PtNPs on tumor tissue weight. *$P < 0.05$ *vs*. vehicle control group

Vehicle Control	500 mg/kg bw	1,000 mg/kg bw	2,000 mg/kg bw
2.77 ± 1.82	1.48 ± 0.7	0.88 ± 0.45*	1.09 ± 0.43

PtNPs, platinum nanoparticles; BW, body weight.

trol (Table 3).

4. Discussion

Although progress has been made in the management of advanced lung cancer, many challenges still remain. Chemotherapy is the primary treatment for patients with an advanced NSCLC. The response rate in patients with a NSCLC from treatment with cisplatin alone is about 20% and in combination with a second agent improves to about 26% [28]. However, recent reports suggest that no significant improvement in survival is likely to occur in those patients [29, 30]. The Pt-based compounds cisplatin and carboplatin belong to the group of most frequently used anti-cancer drugs in clinical practice. Their use, however, is occasionally limited by severe organ toxicity, especially neuro-and nephrotoxicity. In the present study, we assessed the *in vitro* and the *in vivo* activities of biosynthesized Pt NPs against human lung cancer. Nanotechnology has the prospective to modernize cancer therapy [31], and the cytotoxicity of biosynthesized Pt NPs against A549 cells was confirmed by *in vitro* studies. A series of different doses, 50 μg/mL, 100 μg/mL, and 200 μg/mL were used, and the results showed that Pt NPs dose-dependently reduced the viability of A549 cells (Fig. 2). Based on the *in vitro* results, we performed *in vivo* experiments by subcutaneously xenografting SCID mice with human lung- carcinoma cells from the A549 cell line. This is the first time investigation on the treatment of human lung-cancer xenografts by using biosynthesized Pt NPs. As shown in Fig. 3, Pt NPs exhibited a 66% tumor growth inhibition at 1,000 mg/kg bw. Of note is that the mid and the high doses (1,000 and 2,000 mg/kg bw) of Pt NPs significantly inhibited the growth of lung cancer from day 13 (*$P < 0.05$ *vs*. vehicle control). Among the three treated groups, the group that received 1,000 mg/kg bw was observed to maintain the greatest amount of antitumor activity as the tumor volumes and the tumor weights were reduced significantly (Tables 2, 3). Also of note is that the mid and the high doses of Pt NPs inhibited the growth of tumors and delayed the progression of tumor growth more significantly than the low dose did. This suggested a significant inhibitory and tumor suppression effect of Pt NPs on tumor growth in SCID mice bearing A549 lung-tumor xenografts. These results are very encouraging and consistent with our *in vitro* data.

An analysis of BW variations generally defined the adverse effects of the different therapy regiments. No significant differences were observed among the four groups (Table 1). Biosynthesized Pt NPs have been shown to achieve therapeutic cure against lung-cancer xenografts through oral administration. Our findings indicate that orally-administered Pt NPs therapeutically had no side effects even at the high dose. BW measurements indicated that the animals were able to tolerate bio Pt NPs well even at the highest dose of 2,000 mg/kg bw. The effective dose found in this study seems to be high compared to those of other Pt-based anticancer drugs. However, considering the safety profile of this drug as observed in an acute toxicity study (data not published), these doses seem to be in the safe range. This drug is currently in the early phase of exploratory clinical trials, and different doses are being tested on patients with advanced stage solid tumors. The outcome from these studies will help to determine an effective dose in humans for further confirmatory trials.

Ayurveda, a 5000-year-old traditional Indian system of

medicine, is believed to have been in existence from time immemorial. Evidence exists for the use of drugs derived from minerals, vegetables and animal products [32]. The dependence of human life on nanotechnology emerged naturally from ayurveda. Though modern science started exploring the term "Nano" in the 21st century, ayurvedic medicinal systems already had been using noble metals, such as gold, silver etc., in nano form as bhasmas for various medical applications [33]. Because NPs are more biocompatible than conventional therapeutics, they play an important role, due to their bio-availability and compatibility with conventional therapeutics, in the treatment of patients with diseases like cancer [34]. Metal-based drugs were prepared by transmutation of base metals into noble ones and were used with plant extracts meant to eradicate the toxic effects of the metal [35, 36]. Our novel biosynthesized nano-Pt obtained through the ayurvedic process is less toxic and has a higher bio-availability. The antitumor efficacy of biosynthesized Pt NPs for treating lung cancer in SCID mice lends support for its use in various traditional systems of medicine. The activity of biosynthesized Pt NPs in A549 human lung-carcinoma cells *in vitro* and in mouse xenografts provides the rationale for clinical use of this orally-administered agent in patients with lung cancer as most of the other Pt-based chemotherapeutic drugs are administered intravenously and have side effects.

5. Conclusion

Our results show that biosynthesized Pt NPs have antitumor activity *in vitro* and *in vivo* against lung cancer. The results of this study are encouraging enough to undertake further clinical trials involving patients with lung cancer so that the effects of biosynthesized Pt NPs as a potent anti-lung-cancer agent and its safe use by complementary and alternative medicine (CAM) practitioners can be verified.

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Conflict of interest

The authors declare that there are no conflict of interest.

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