

Green synthesis, characterization and anticancer potential of platinum nanoparticles Bioplatin

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OBJECTIVE: In the present study, the anticancer potential of platinum nanoparticles Bioplatin is explored and the mode of interactions of Bioplatin with calf thymus DNA and honey was analyzed.

METHODS: Bioplatin was synthesized with the help of green nanotechnology and characterized by particle size, zeta potential and surface morphology. The interaction of Bioplatin with DNA and honey was also checked with the help of circular dichroism spectroscopy and Fourier-transform infrared spectroscopy, respectively. The anticancer potential of Bioplatin was evaluated on peripheral blood mononuclear cells and A375 cells *in vitro* by analyzing results of MTT (3-(4, 5)-dimethylthiazazo(-z-y1)-3, 5-di-phenyltetrazoliumromide), fluorescence microscopic studies and DNA fragmentation assay.

RESULTS: Bioplatin exhibited a small particle size of 137.5 nm and a surface charge of -35.8 mV. Bioplatin interacted with DNA and brought in effective changes in structure and conformation of DNA, and formed a new complex that increased its stability of DNA intercalated with the base pair of DNA. *In vitro* studies demonstrated that Bioplatin arrested cell proliferation, and induced chromatin condensation and internucleosomal DNA fragmentation.

CONCLUSION: Bioplatin induces apoptosis in cancer cells and may have some beneficial effect against human carcinoma. It interacts with DNA, brings stabilization to DNA, and thus prevents the replication of DNA.

KEYWORDS: metal nanoparticles; antineoplastic agents; apoptosis; biosynthetic pathways

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In the last two decades, a number of nanoparticle-based therapeutic and diagnostic agents have been developed for the treatment of cancer, diabetes, pain, asthma, allergy, infections, and so on^[1,2]. These nanoscale agents may provide more effective and more convenient routes of administration, lower therapeutic toxicity, extend the life cycle of the product, and ultimately reduce health-care costs^[3]. Physical parameters such as surface area, particle size, surface charge and zeta potential are very important for providing mechanistic details in the uptake, persistence, and biological activity of nanoparticles inside living cells^[4].

Inorganic metallic nanoparticles such as gold, platinum and silver whose structures exhibit significantly distinct physical, chemical and biological properties, functionally due to their nanoscale size, have elicited much interest^[5]. Pelka *et al*^[6] and Porcel *et al*^[7] reported that platinum nanoparticles (Bioplatin) might be useful in cancer therapy.

Nanotechnology is one of the most promising fields for generating new applications in medicine. However, only a few nanoproducts are currently in use for medical purposes^[8]. Therefore, the main objectives of the present study are to explore: if Bioplatin can selectively arrest proliferation of cancer cells; whether it can induce apoptosis in cancer cells; whether Bioplatin interacts with DNA and prevents DNA replication; whether honey gives extra stability to the nanoparticles and may be used as a drug carrier. The literature review indicates that, this is the first attempt where honey has been used to give stability to the platinum nanoparticles and apparently, it can be successfully used as a capping agent. Bioplatin has been synthesized with the help of green technology without any toxic chemicals^[9]. The characterization studies are inclusive of the physicochemical properties and surface morphology of Bioplatin by analyzing data of particle size, zeta potential, poly dispersity index and atomic force microscopy.

In this study the anticancer (apoptotic) potential of Bioplatin was checked with 3-(4,5)-dimethylthiaziazolide (-z-y1)-3, 5-di-phenyltetrazolium bromide

(MTT) assay, fluorescence microscopic study and DNA fragmentation assay.

1 Materials and methods

1.1 Preparation of Bioplatin Platinum wires (99.9% pure) were preferred as the source of pure metallic platinum. Phase I involved biopurification of the fine platinum metal. Fine platinum metal available in its purest form was used in phase I of the process. Phase II involved particle size reduction of the phase I purified platinum metal. Phase III involved conversion of the phase II product into nontoxic bioplatin of high therapeutic value. Each of these phases was carried out at different temperatures and with the aid of plant materials, namely, *Dolichos biflorous*, *Ocimum sanctum*, *Euphorbia nerifolia* (Snoohi), *Sesbania grandiflora* (Agustyan), *Piper betle* (Nagavalli), *Calospropis procera* (Arkapataram) and *Asteracanpha longifolia* (Kokilaksha) (patent number-WO 2007/031888 A2)^[9].

1.2 Characterization of Bioplatin

1.2.1 Surface morphology study by scanning electron microscopy The surface morphology of the formulated nanoparticle-loaded drug was determined by scanning electron microscopy (SEM, Hitachi S-500, USA) equipped with 15 kV, SEM detector with a collector bias of 300 V. The lyophilized samples were spread over the double-sided conductive tape (12 mm) fixed onto the metallic stud.

1.2.2 Surface morphology study by atomic force microscopy The samples were prepared for atomic force microscopy (AFM) imaging by placing a drop of Bioplatin suspension on a freshly cleaved mica sheet and allowed to dry in the air. We recorded our observation through AFM (VEECO di CP-11) imaging by using AFM in amplitude and tapping modes.

1.2.3 Particle size determination by dynamic light scattering method The average size and size distribution of the Bioplatin were determined by dynamic light scattering (DLS) using a Zetasizer, Nano-ZS instrument (Malvern Instruments, Southborough, UK). The intensity of the scattered light was detected at 90° to an incident beam. The data



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were analyzed in the automatic mode. Measured size was presented as the average value of 20 runs, with triplicate measurements within each run^[10].

1.3 Fourier-transform infrared spectroscopy analysis

The fourier-transform infrared spectroscopy (FTIR) spectra were obtained after mixing Bioplatin with honey on Perkin Elmer FTIR (400) spectrometer with the samples as potassium bromide pellets.

1.4 Circular dichroism measurements

Circular dichroism (CD) spectra showed changes in the structure of DNA, which were monitored in the region (200 to 320 nm) using 1 cm path length cells (Jasco spectropolarimeter; model J-720; equipped with a computer). DNA concentration of 1.06 mmol/L was used in the experiments. The induced CD spectra resulting from the interaction of the Bioplatin in three concentrations (0, 20 and 40 $\mu\text{mol/L}$) with DNA at 37 $^{\circ}\text{C}$ were obtained by subtracting the CD spectrum of the native DNA and mixture of DNA-Bioplatin from the CD spectrum of the buffer and spectrum of buffer-nanoparticle solution, respectively^[10].

1.5 Cell culture reagents

The reagents, such as Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, neomycin (PSN) antibiotic, trypsin and ethylenediaminetetraacetic acid (EDTA) were procured from the Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from BD Bioscience (USA). All organic solvents used were of high-performance liquid chromatography grade. MTT and all other chemicals used were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

1.6 Cell culture procedure

The A375 cell line obtained from the National Center for Cell Science, Pune, India was grown at 37 $^{\circ}\text{C}$ in 5% carbon dioxide atmosphere in DMEM supplemented with 10% FBS and 1% PSN. For experimental studies, cells were grown to 80% to 90% confluence, harvested with ice-cold buffer saline and plated at desired density and allowed to re-equilibrate for 24 h before any treatment. Peripheral blood mononuclear cells (PBMCs) were also cultured and exposed to Bioplatin to test cytotoxic effects, if any.

1.7 Isolation of PBMCs

Blood from healthy human volunteers was obtained with heparinized syringes and was placed into sterile polypropylene tubes. PBMCs were further isolated by hystopaque 1077 density gradient centrifugation at $400 \times g$ for 30 min at 25 $^{\circ}\text{C}$ (Sigma-Aldrich, St. Louis MO, USA). PBMCs were then washed twice with FBS-free medium (RPMI 1640) at $250 \times g$ for 10 min at 25 $^{\circ}\text{C}$ and adjusted to 10^4 cells per well for analysis.

1.8 Cell treatment with Bioplatin

The amount of different concentrations of Bioplatin was added to each well in triplicates. Different concentrations (25, 50, 75 and 100 $\mu\text{g/mL}$) of Bioplatin were inoculated into grown cells (10^4 cells per well)

and the cell population was determined by optical microscopy at 24, 48 and 72 h, respectively.

1.9 MTT assay

MTT assay^[11] was used to determine energetic cell metabolism by measuring the activity of one of the oxidative enzymes. The dye is reduced in mitochondria by succinic dehydrogenase to an insoluble violet formazan product. A375 cells (10^4 cells per well) were cultured for 24, 48 and 72 h on 96-well microplates. The cells were incubated for 24, 48 and 72 h with or without compounds on test. Then MTT was added. After incubation, formazan crystals were solubilized with acidic isopropanol and the absorbance of the solution was measured at 595 nm using an enzyme-linked immunosorbent assay reader. Cell viability was measured by taking water as 100%. Here, water was the vehicle of the Bioplatin.

1.10 Fluorescence microscopy

In order to detect whether Bioplatin induces apoptosis, staining with 4', 6-diamidino-2-phenylindole (DAPI) and Hoechst 33258 was performed. Cells treated with or without Bioplatin (at 100 $\mu\text{g/mL}$) for 24, 48 and 72 h were stained with 10 $\mu\text{g/mL}$ of DAPI and cells were seen under a fluorescence microscope (Axiscope plus 2, Zeiss, Germany).

1.11 DNA fragmentation assay

Cells were harvested after treatments of different time intervals (24, 48 and 72 h) into extraction buffer (10 mmol/L Tris-HCl pH 7.4, containing 10 mmol/L NaCl, 20 mmol/L EDTA and 1% Triton X-100). Genomic DNA was isolated by digesting the cell extract with 10 $\mu\text{g/mL}$ of proteinase K at 56 $^{\circ}\text{C}$ for 8 to 12 h. DNA was purified by phenol/chloroform precipitated with ethanol and dissolved in Tris-EDTA. Integrity of DNA was analyzed by gel electrophoresis on 1% agarose gels followed by ethidium bromide staining^[10].

1.12 Statistical analysis

Statistical analysis using Software SPSS 11.0 version was conducted. All results were expressed as mean \pm standard error of three independent experiments. Homogeneity of the different series was tested by post hoc analysis followed by the LSD one-way analysis of variance to demonstrate significant statistical differences, if any. $P < 0.05$ value was considered statistically significant.

2 Results

2.1 Characterization of Bioplatin

2.1.1 Particle size and polydispersity index

DLS data showed that the mean diameter of Bioplatin was 137.5 nm with polydispersity index (PDI) of 0.380. See Figure 1.

2.1.2 Surface charge

In the present study, it was observed that zeta potential of the drug was -35.8 mV (Figure 2). High absolute value of zeta potential indicates high electrical charge on the surface of the nanoparticles, which can cause strong repellent forces among particles to prevent aggregation of nanoparticles.

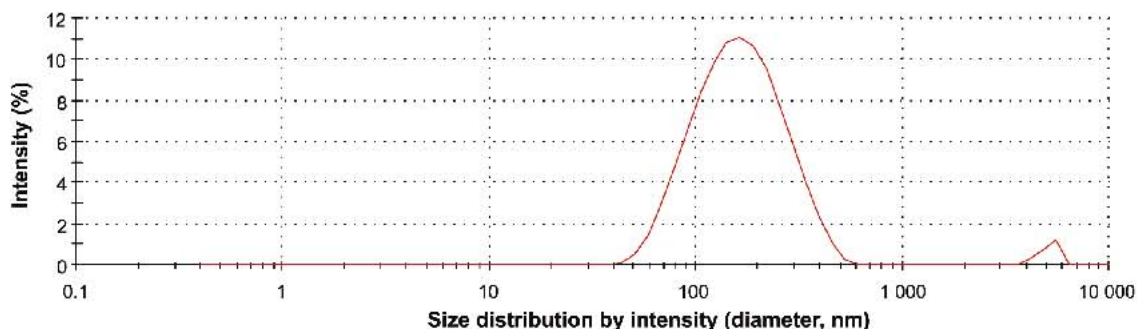


Figure 1 Average particle size obtained from dynamic light scattering data for Bioplatin

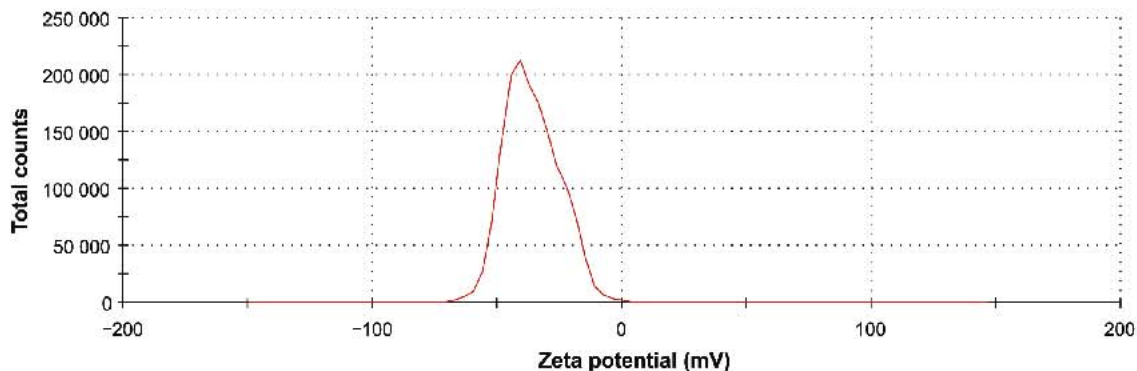


Figure 2 Zeta potential of Bioplatin

2.1.3 Surface morphology observed by SEM Figure 3 displays the cubic shape of Bioplatin without crack (cracks generally created during the process of mechanical shearing).



Figure 3 Surface morphology of Bioplatin observed by scanning electron microscopy (30.0 kV, ×100 000)

2.1.4 Surface morphology observed by AFM The structure of the nanoparticles plays an important role in determining their adhesion to and interaction with cells. The features of morphology of drug under AFM are shown in Figure 4. AFM image of Bioplatin shows the smooth surface of nanoparticles without any noticeable pinholes or cracks.

2.2 CD studies From the CD spectra, we observed a positive band at 275 nm due to base stacking and a negative band at 245 nm due to change in helicity (Figure 5).

2.3 FTIR analysis In the present study we got spectral band at 1 638. 41/cm. This band arose due to carboxyl (-COOH) stretch and amine (N-H) deformation in amide linkage of the protein. The very strong band at 1 058. 66/cm arose from dialkyl (C-O-C) symmetric stretching and C-O-H (alcohol) bending vibration of protein in honey. The intense band at 2 928. 40 and 3 427. 70/cm was due to hydroxyl (-OH) and alkanes present in honey. See Figure 6.

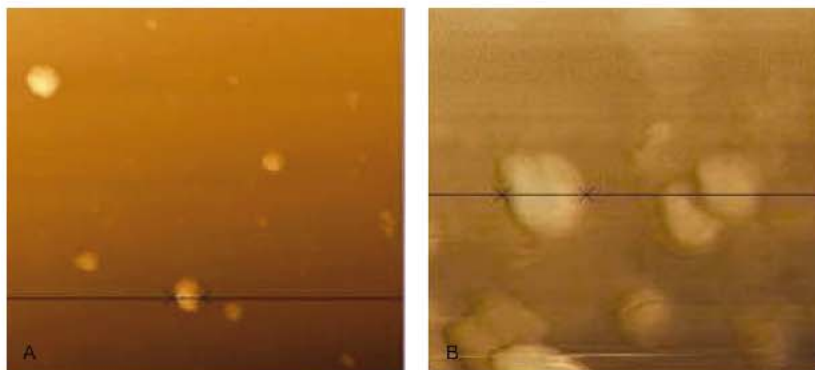


Figure 4 Surface morphology observed by atomic force microscopy in two different scanning modes

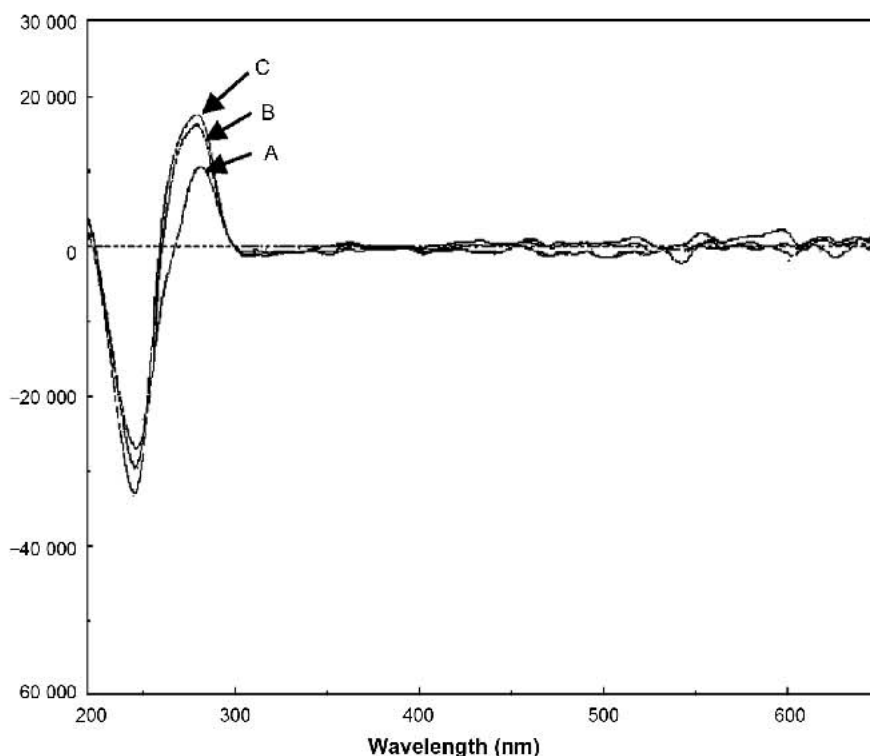


Figure 5 Circular dichroism spectra of calf thymus DNA incubated with Bioplatin
 A: Calf thymus DNA; B: Calf thymus DNA plus 20 $\mu\text{mol/L}$ Bioplatin; C: Calf thymus DNA plus 40 $\mu\text{mol/L}$ Bioplatin.

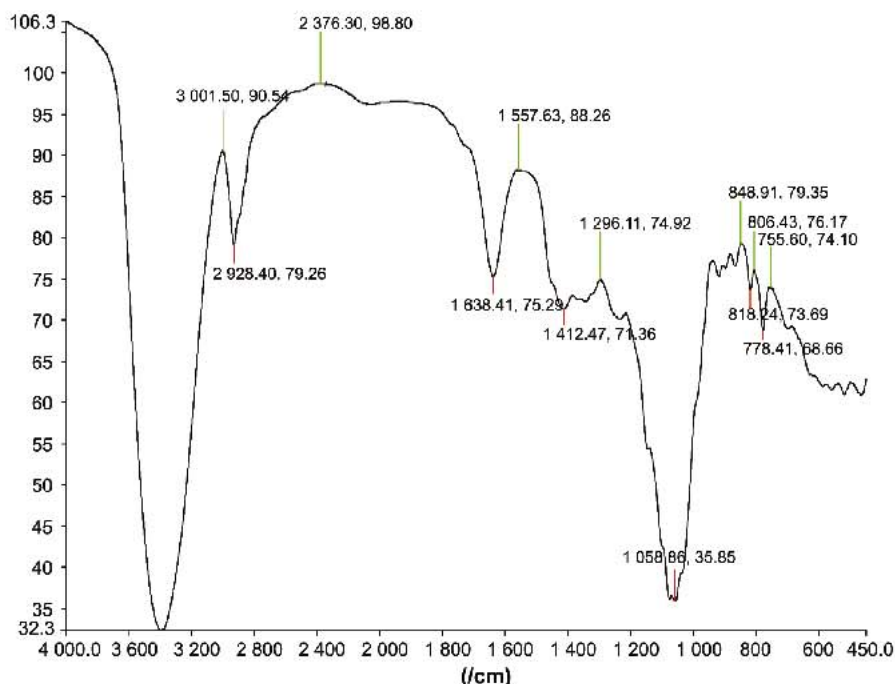


Figure 6 Fourier-transform infrared spectra of Bioplatin mixed with honey

2.4 Percentage of cell viability of PBMCs and A375 cells Bioplatin showed no or negligible cytotoxic effect on PBMCs. The highest dose (100 $\mu\text{g/mL}$) of Bioplatin showed 92%, 89.5% and 86.8% cell viability in 24, 48 and 72 h respectively in PBMCs (Figure 7A). Lower dose showed 2% to 8% cytotoxicity in different time intervals in PBMCs. Data on percentage of cell viability in A375 as a result of treatment with different non-cytotoxic doses

(25, 50, 75 and 100 $\mu\text{g/mL}$) of Bioplatin at 24, 48 and 72 h have been furnished in Figure 7B. All results were expressed with vehicle control as 100%. It would be evident from the data that, as compared to their vehicle (water) control, the percentages of cell viability were dramatically reduced in Bioplatin ($P < 0.05$ or $P < 0.01$); there was a linear decrease along with the increase in concentration and incubation time of the Bioplatin.

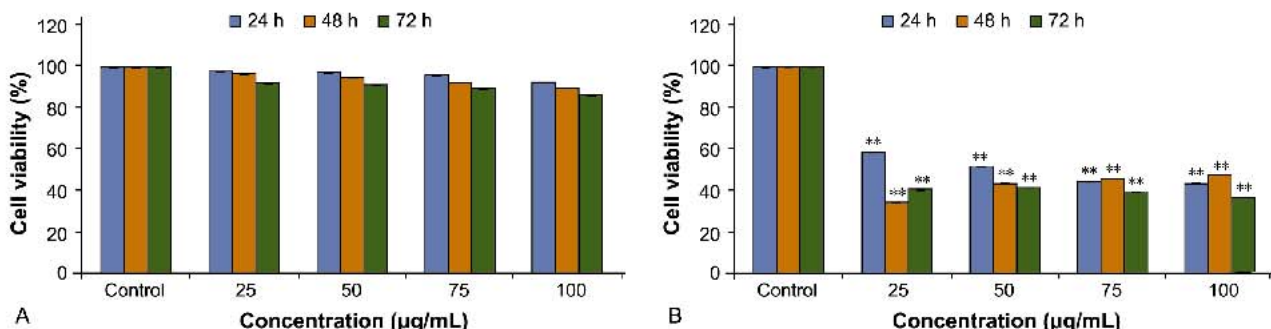


Figure 7 Cell viability of Bioplatin with different concentrations against PBMCs (A) and A375 cells (B) tested by MIT assay. All results were expressed with vehicle control as 100%. Data are represented as mean ± standard deviation, $n = 3$; * $P < 0.05$, ** $P < 0.01$, vs control group. PBMC: peripheral blood mononuclear cell; MTT: 3-(4,5)-dimethylthiazazo(- π -y1)-3,5-di-phenyltetrazolium bromide.

2.5 Fluorescence microscopic study

2.5.1 DAPI staining Untreated A375 cells did not take positive staining with DAPI and showed no cells with visible chromatin condensation. However, with Bioplatin treatment, cells with chromatin condensation appeared to increase in number along with the increase of time intervals (Figure 8A). Average fluorescence intensity of cells after 24, 48, and 72 h of 100 µg/mL Bioplatin treatment was significant higher than that of the control group ($P < 0.01$). See Figure 8B.

2.5.2 Hoechst staining To examine the biological findings of apoptosis in A375 cells induced by Bioplatin, Hoechst staining was performed. As shown from Hoechst staining, some nuclei of Bioplatin-treated cells exhibited typical features of apoptosis such as nuclear condensation. See Figure 9.

2.6 DNA fragmentation assay DNA fragmentation data as revealed from the DNA fragmentation in agarose gel electrophoresis were presented in Figure 10. As compared to the control, the fragmentation in Bioplatin-treated groups appeared to

be more smeared, indicating more fragmentation of DNA suggestive of greater degree of apoptosis.

3 Discussion

Nanobiotechnology is an emerging field that has made its contribution to all spheres of human life. Bioplatin has been synthesized with traditional method of Ayurveda with the help of different plants. Presumably, the plant extracts provide some coating material to the bare nanoparticles and thereby reduce their toxic nature. It appears that Bioplatin is 137.5 nm in size and therefore it could enter into the cell very fast. It was reported in literature that smaller nanoparticles would have greater ease of entry and durability in the tumors^[12]. It was suggested that large particles (< 5 µm) would be taken up via the lymphatics and small particles (< 500 nm) can cross the membrane of epithelial cells through endocytosis^[13]. As platinum is in Bioplatin, so it diffuses through the cell much more rapidly. Bioplatin inhibits the growth of cancer cells *in vitro* due to its small size and

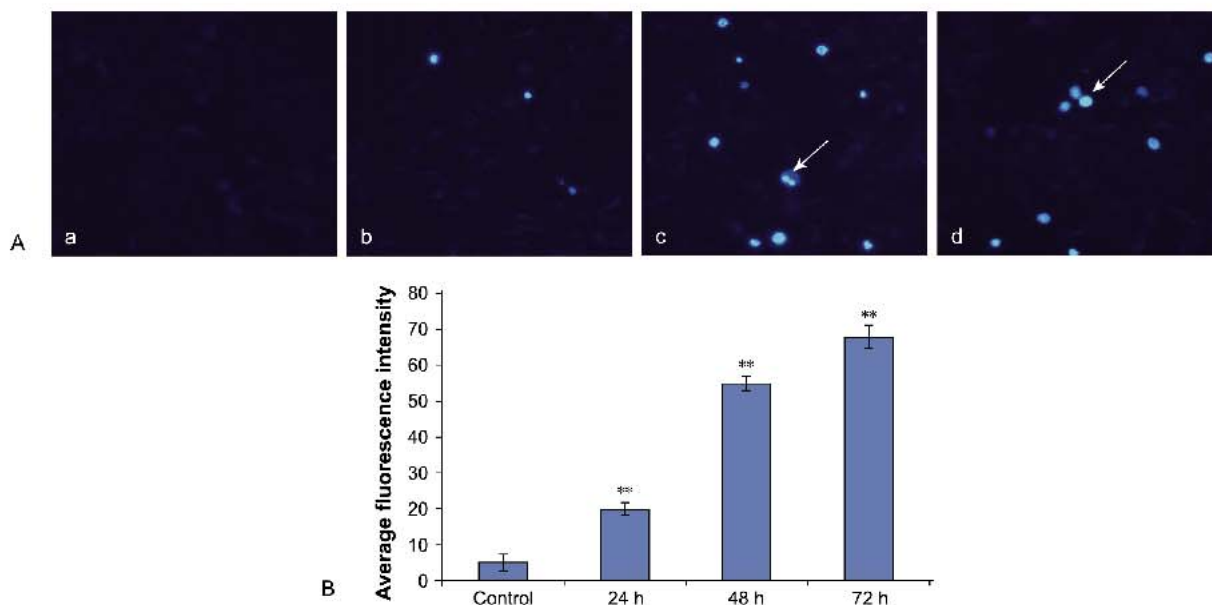


Figure 8 Fluorescence photomicrographs of A375 cells stained with DAPI

A: Cells were stained with DAPI (a: Control; b to d: 24, 48, and 72 h after 100 µg/mL Bioplatin treatment). The cells were harvested at different time intervals and the blue fluorescence was monitored by fluorescence microscopy ($\times 10$). B: Histogram represents average fluorescence intensity of cells after DAPI staining. Data are represented as mean ± standard error, $n = 3$; ** $P < 0.01$, vs control group. DAPI: 4',6-diamidino-2-phenylindole.

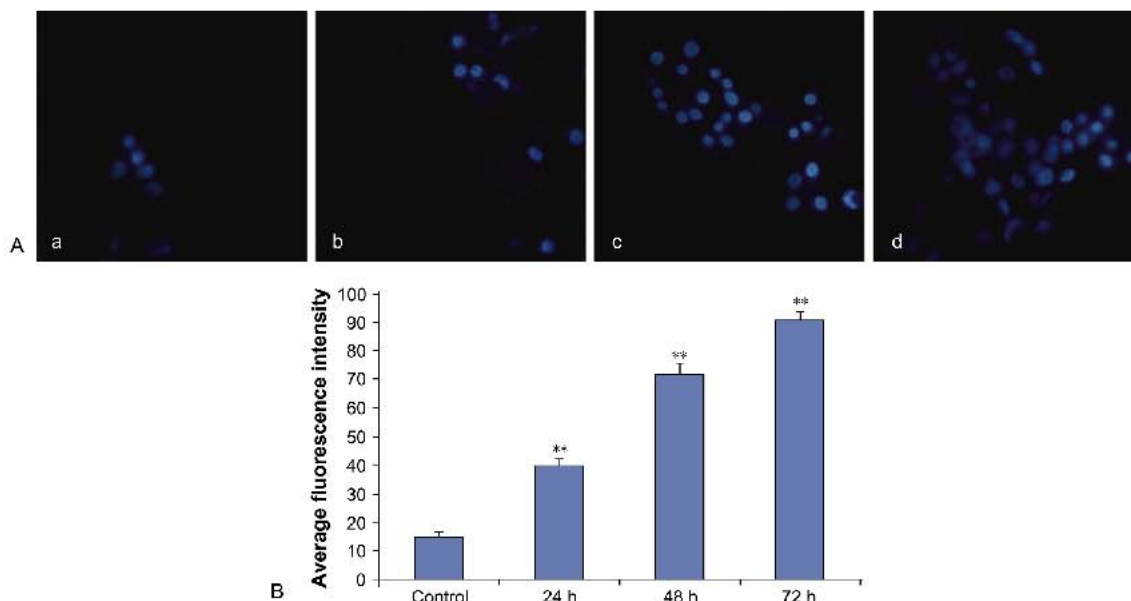


Figure 9 Fluorescence photomicrographs of A375 cells stained with Hoechst 33258

A: Cells were stained with Hoechst 33258 (a: Control; b to d: 24, 48, and 72 h after 100 $\mu\text{g}/\text{mL}$ Bioplatin treatment). The cells were harvested at different time intervals and the blue fluorescence was monitored by fluorescence microscopy ($\times 10$). B: Histogram represents average fluorescence intensity of cells after Hoechst 33258 staining. Data are represented as mean \pm standard error, $n=3$; ** $P<0.01$, vs control group.

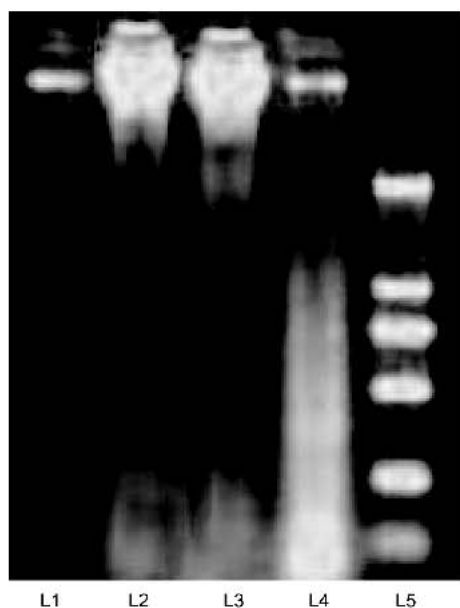


Figure 10 DNA fragmentation after treatment of cells with Bioplatin in different time intervals

A375 cells were treated with 100 $\mu\text{g}/\text{mL}$ Bioplatin for 24, 48 and 72 h and Genomic DNA was extracted and DNA was separated on 1% agarose gels and stained with ethidium bromide. L1: Control DNA; L2: DNA from treated cells (24 h); L3: DNA from treated cells (48 h); L4: DNA from treated cells (72 h); L5: A standard DNA ladder.

faster mobility. Zeta potential is one of the most important physicochemical characteristics of nanoparticles^[14]. High absolute value of zeta potential of Bioplatin indicates high electrical charge on the surface of the nanoparticles, which can cause strong repellent forces among particles to prevent aggregation of nanoparticles^[15]. The negative surface charge of Bioplatin prevents aggregating and increases its stability into the medium.

The CD spectra of the Bioplatin with double-stranded DNA can provide us with useful information concerning the nanoparticles-nucleotide interaction^[10]. CD spectroscopy is useful for diagnosing changes in DNA morphology during drug-DNA interactions, as the positive band due to base stacking (275 nm) and the negative one due to right-handed helicity (245 nm) are quite sensitive to the mode of DNA interactions with small molecules^[16]. The changes in the CD signals of DNA observed on interaction with drugs may often be assigned to the corresponding changes in the DNA structure^[17]. From the results, it can be suggested that Bioplatin can significantly change the helicity conformation of DNA and then induce the alteration of nonplanar and tilted orientations of DNA bases, resulting in the changes of DNA base stacking, and acts as an intercalator. Intercalation proves that Bioplatin arrests the cell division which is the main criteria of growth inhibition of cancer cells.

From the results of fluorescent staining and DNA fragmentation assay, the authors concluded that Bioplatin enters into nucleus, induces chromatin condensation and induces apoptosis through DNA fragmentation. The amide I band of protein is expected to occur as prominent infrared radiation bands around 1 650/ cm^{-1} ^[18]. Ongoing study reflects the same pattern of signal at 1 638.41/ cm^{-1} (Figure 6). This band arises due to carboxyl stretch in amide linkage of the protein. Actually strong band at 1 058.86/ cm^{-1} arises from C-O-C symmetric stretching and C-O-H bending vibration of protein in honey. From the results, it was concluded that honey acts as a stabilizing agent and capping agent and may be used as a suitable carrier. In the

future, more studies are required to observe the biological effects of honey. It is felt that the comparative studies should be conducted to observe the role of honey in the bioavailability of nanoparticles and its suitability as carrier for drug administration.

In conclusion, the overall results indicated that Bioplatin synthesized by the green nanotechnology has the potential to bind with ds-DNA and intercalate with base pair of DNA. Another aspect of the study was that honey brings extra stability to Bioplatin and acts as a capping agent. Structural and conformational changes of DNA due to the interaction of Bioplatin possibly result in the accentuation of the anticancer power of these nanoparticles and the *in vitro* results also reflect that Bioplatin induces apoptosis in cancer cells. This encouraging result would provide ample useful information to design a much better anticancer compound using nanoparticles with lower side effects in the furtherance of anticancer research.

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5 Competing interests

The authors declare that they have no competing interests.

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纳米铂金颗粒 Bioplatin 的绿色合成、特征及其抗肿瘤作用

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目的:研究纳米铂金颗粒的抗癌作用并分析其与小牛胸腺 DNA 及蜂蜜的相互作用。

方法:使用绿色纳米技术合成纳米铂金颗粒 Bioplatin 并确定其物理特性如颗粒大小、电动电位及表面形态学。使用圆二色光谱分光光度法及傅里叶变换红外光谱技术以小牛胸腺 DNA 和蜂蜜作为靶点检测药物-DNA 相互作用。使用噻唑蓝、荧光显微镜及 DNA 片段分析法检测其对外周血单核细胞及 A375 细胞的体外抗癌作用。

结果:Bioplatin 的纳米直径为 137.5 nm,表面电荷为 -35.8 mV。Bioplatin 与 DNA 相互作用后对 DNA 的结构产生了明显的作用,使其发生改变,并形成了一种能够提高 DNA 稳定性的新的化合物。体外实验表明 Bioplatin 能够抑制细胞增殖,引起细胞核染色质凝固和 DNA 核小体断裂。

结论:Bioplatin 能够引起肿瘤细胞凋亡,因此具有一定的抗肿瘤作用。它能够与 DNA 相互作用,增加 DNA 的稳定性,从而抑制 DNA 的复制。

关键词:金属纳米粒子;抗肿瘤药;细胞凋亡;生物合成途径

第四届世界中西医结合大会首轮通知

第四届世界中西医结合大会将于 2012 年 10 月在天津举行,大会的主题是“结合创新·持续发展”。会议将总结和交流第三届世界中西医结合大会成功举行后五年来,世界结合医学(包括中西医结合)领域所取得的新成就、新经验和新趋势,探索结合医学在基础研究、临床研究和药学研究等方面促进学术创新和成果转化的特点与模式,研讨结合医学可持续发展的途径、方法和前景,并对推动结合医学可持续发展的政府干预、政策引导、医疗体制改革、社会认知等支持因素进行有益的讨论。

这是全世界结合医学的实践者、研究者、教育者和政策制定者的又一次历史性盛会,必将促进结合医学在不断继承和创新中实现可持续发展。会议将通过(大会发言、分组发言)卫星会议、墙报、展览会形式展现会议内容,并用中文与英文(会议提供同声翻译)进行报告。

会议时间和地点 会议时间:2012 年 10 月 20~22 日,10 月 19 日(周五)报到;地点:天津市万丽天津宾馆(天津市河西区宾水道 16 号,电话 022-58223388)。

大会组织 主办单位:中国中西医结合学会;承办单位:天津市中西医结合研究院、天津市中西医结合学会、天津市中西医结合医院(天津市南开医院)。

征文内容 (1)对结合医学学术地位与作用的再认识,以及对新形势下发展结合医学的思路、途径和方法的理论探讨与经验总结;(2)展示五年来结合医学在临床研究、基础研究、药学研究、教学研究、学科建设、政策研究等方面取得的代表性成果;(3)结合医学各临床学科新诊疗经验的总结和分析,中西医结合新技术、新方法的推介与评价,以及实现科研成果向临床应用转化的新经验与新模式;(4)中西医结合优势病种临床诊疗路径的实践经验和临床共性问题的探讨;(5)对结合医学的未来研究,以及其他促进结合医学发展的相关研究。

征文要求 (1)论文原则上应为未在其他杂志正式发表或其他学术会议上发表的论文。(2)来稿务请注明作者工作单位、职务、职称、通讯地址(含邮政编码)和手机号码。(3)论文要求全文 4 000 字以内,顺序为文章题目、作者单位、邮政编码、作者姓名、中英文摘要和正文。摘要应不少于 400 字且需要中英文双语,大会可接受英文代译。代译费每篇人民币 100 元。欲了解更详细的论文格式要求请登录中国中西医结合学会网站 www.caim.org.cn。(4)论文采用 WORD 文档格式,标题为 4 号黑体字,正文为小 4 号宋体字。电子稿件请以附件方式发送至 zxyjhdh@163.com 或 zxyjhdh@yeah.net。来邮必复,如未见回复,请再次发送。纸质版稿件请邮寄至天津市和平区南京路 98 号(邮政编码 300040)天津市中西医结合学会。信封左下角注明“第四届世界中西医结合大会投稿”字样,并随信附寄含电子版 U 盘或光盘。截稿日期为 2012 年 7 月 31 日。

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