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In-vitro inhibition of BVDV, a surrogate model for HCV, using novel gold nanoparticles

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Abstract

In this study, the in-vitro cytotoxic effect and antiviral properties of gold nanoparticles (AuNPS) against cytopathic strain (NADL) bovine viral diarrhea virus (BVDV)were evaluated. AuNPSwas previously reported to possess in-vitro antiviral properties against HIV and multistrains of influenza virus.Citrated AuNPS of 7±2 nm were prepared and PEG functionalized. Evaluation of the cytotoxicity of prepared AuNPS did not show toxic effects to MDBK cells with concentrations of 2 and 4 ppm. The antiviral activity of AuNPS was evaluated by the inhibition of the cytopathic effect on infected MDBK cells by means of (MTT) based colorimetric assay and was found that 4 ppm is the optimum concentration for virus inhibition. Results of the in- vitro antiviral activity and cytotoxicity showed that prepared nanoparticles has limited in-vitro toxic effect at concentration of 4 PPM also has strong affinity and reasonable inhibitory effect on BVDV.

Keywords: BVDV, nanotechnology, gold nanoparticles, antiviral

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Introduction

Viruses belonging to the Flaviviridae family cause clinically significant diseases in humans and animals. This family includes three genera;Pestivirus [including bovine viral diarrhea virus (BVDV)], Flavivirus [including yellow fever virus (YFV), dengue virus, and West Nile virus (WNV)], and Hepacivirus [including hepatitis C virus (HCV)]. Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle andis endemic in cattle herds in most parts of the world. It is the prototype virus of the genus *Pestivirus*, which also includes *Hog Cholera Virus* (HCV) and *Border Disease Virus* (BDV), the etiologic agents of Classical Swine Fever and Sheep Fever; respectively **(Paton et al., 1995).**

BVDV is considered to be a valuable surrogate virus model for identifying and characterizing antiviral agents to be used against HCV**(Buckwold et al., 2003 and Yamane et al., 2009).**BVDV is a small enveloped virus with a diameter of about 40 nm, and is classified into BVDV genotype 1 ($BVDV_1$) and BVDV genotype 2 ($BVDV_2$) Viruses from either $BVDV_1$ or $BVDV_2$ genotypes have shown characteristic phenotypes in cell culture and are classified as non-cytopathic or cytopathic biotypes **(Donis and Dubovi, 1987 and Ridpath et al., 1994).**BVDV has been associated with significant economic losses throughout the world. Although several prevention and control measures for BVDV have been adopted, the disease remains prevalent. Bovine persistently infected by non-cytopathic BVDV are immunotolerant to the virus. They have infectious virions in all body secretions and can develop a potentially fatal form of BVDV

known as mucosal disease resulting from the genetic recombination between non-cytopathic and cytopathic BVDV(**Bolin et al., 1991 and Salim et al., 2010**).

Vaccination adopted as a method of control causes concern due to the viral characteristics of antigenic and genetic diversity(**Kalaycioglu et al., 2007**), therefore, the discovery of new antiviral agent has been the aim of previous researchers (**Paeshuyse et al., 2007, Tabarrini et al., 2006 and Salim et al., 2010**).

Hepatitis C virus (HCV), a member of the hepacivirus genus, family Flaviviridae, is a major cause of human hepatitis throughout the world. The World Health Organization estimates that 130 to 150 million people are chronically infected with HCV. Improved therapies are urgently needed to manage the disease burden caused by HCV. Unfortunately, study of HCV has been hampered by the inability to propagate the virus efficiently in cell culture. However, HCV shares many molecular and virologicalsimilarities with pestiviruses. Both HCV and BVDV have single strand RNA genomes (approximately 9,600 and 12,600 nucleotides in length, respectively) that encode about nine functionally analogous gene products (**Rice, 1996**). Thus, comparative studies with the pestiviruses have greatly facilitated our understanding of HCV. Pestiviruses, in fact, has been adopted by many investigators as a model and surrogate for HCV(**Buckwold et al., 2003 and Yamane et al., 2009**).In view of the economic impact of pestivirus diseases and the important relationship between Pestiviruses and HCV, we sought to identify inhibitors of Pestivirus attachment and replication. Here, we report on a potent and selective nanoparticles inhibitor of Pestivirus attachment and replication.

This study attempted to prepare and characterize AuNPsthen evaluate in-vitro AuNPs cytotoxicity and antiviral activity against BVDV as surrogate model for HCV.

Material and Methods

Cells and Viruses:

Madin-Darby bovine kidney (MDBK) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% horse serum (DMEM-HS) at 37°C in a humidified, 5% CO_2 atmosphere. BVDV-1 (strain NADL) was propagated and titrated according to **Reed and Muench(1938).**

Synthesis of Gold Nanoparticles:

Colloidal gold nanoparticles were synthesized by the optimization of classical Citrate Reduction method according to**Turkevich et al. (1951).**

Surface modification of Gold Nanoparticles:

Surface modification of gold nanoparticlesnecessary to increase its colloidal stability in physiological fluid was performed by adding poly –ethylene glycol (PEG) according to the method described by **Alcantar et al.(2000) .**

Characterization of Gold Nanoparticles:

Prepared gold nanoparticles were characterized by using UV-Vis Spectrophotometer (PERKIN-ELEMER Lambda 40 B double beam), Zetasizer (Malvern, UK, and Model: Zetasizernano series (Nano ZS), Size range 0.6:6000 nm.), and high Resolution Transmission Electron Microscopy(Tecnai, FEI, Netherland) according to **Liu and Lu (2006).**

Cytotoxicity of Gold Nanoparticles by MTT assay:

Prior to using the synthesized gold nanoparticles for anti-viral assay, cytotoxicity of nanoparticles was determined using Vero cell lines by MTT (a tetrazole) assay. MDBK cell lines were trypsinized, suspended in minimal essential medium and counted using trypan blue exclusion dye according to **Mosmann(1983).**

Determination of AuNPs antiviral activity and interaction of viral particles with AuNPs(Kesarkar et al., 2012):

To study the mode of action of Nanoparticles with BVDV, we used two different ways:

Method I: In the first method, the viral particles were interacted with the gold NPSfor 15 minutes and then allowed tointeract with the MDBK cells. 25 µl of viral suspension (10^6 TCD_{50}/ml) was added with 25 µl of the different concentrations of the synthesized gold NPS in PCR tubes and incubated for 15 minutes at 37°C. The concentrations of AuNPSused (2, 4, 6 and 8 ppm) were based on the results of the cytotoxicity assay done for the Nanoparticles showing minimumcytotoxicity.

Method II: In the second method, the viral particles were first allowed to infect MDBK cells and the AuNPSwere added to the suspension afterwards. A volume of 25µl of viral suspension (10^6 TCD_{50}/ml) was added to different wells of tissue culture plate containing 5 x 10^5cells/ml of MDBK cells and incubated for 1 hr in CO_2 incubator. After 1 hr of incubation,2, 4, 6 or 8ppm of Nanoparticles were added and the wells were incubated for 72 hr at 37°C in CO_2 incubator.

The following two controls were also kept parallel for incubation.

1. Triple control containing only cells and viral suspension.
2. Triple control containing only cells.

After incubation, 10µl (5mg/ml) MTT reagent was added in each well and incubated at 37°C for 4 hrs in CO_2 incubator.Then 100 µl of 0.1N acidified*iso*-propanol was added to each well and kept in dark for 30 min at room temperature. The well plates were then kept on a shaker for 1 min and OD was taken at 530 nm wavelength. The average values from triplicate readings were determined and the average value for the blank was subtracted.Absorbances against number of cells/ml were plotted.

Results

Synthesis and Characterization of AuNPS:

In the present experiments, trisodium citrate served the dual role of both a reducing agent and a stabilizer. Trisodium citrate initially acts as a reducing agent to reduce the Au (III) ions to Au (0) then acts as the stabilizing agent by forming a layer of citrate ions over the AuNPs surface.

a) UV-Visible Absorption Spectroscopy:

The absorption spectrum for the solution of citrated AuNPs is characterized by the excitation of the Plasmon resonance in the neighborhood of 518 nm (Fig.,1).

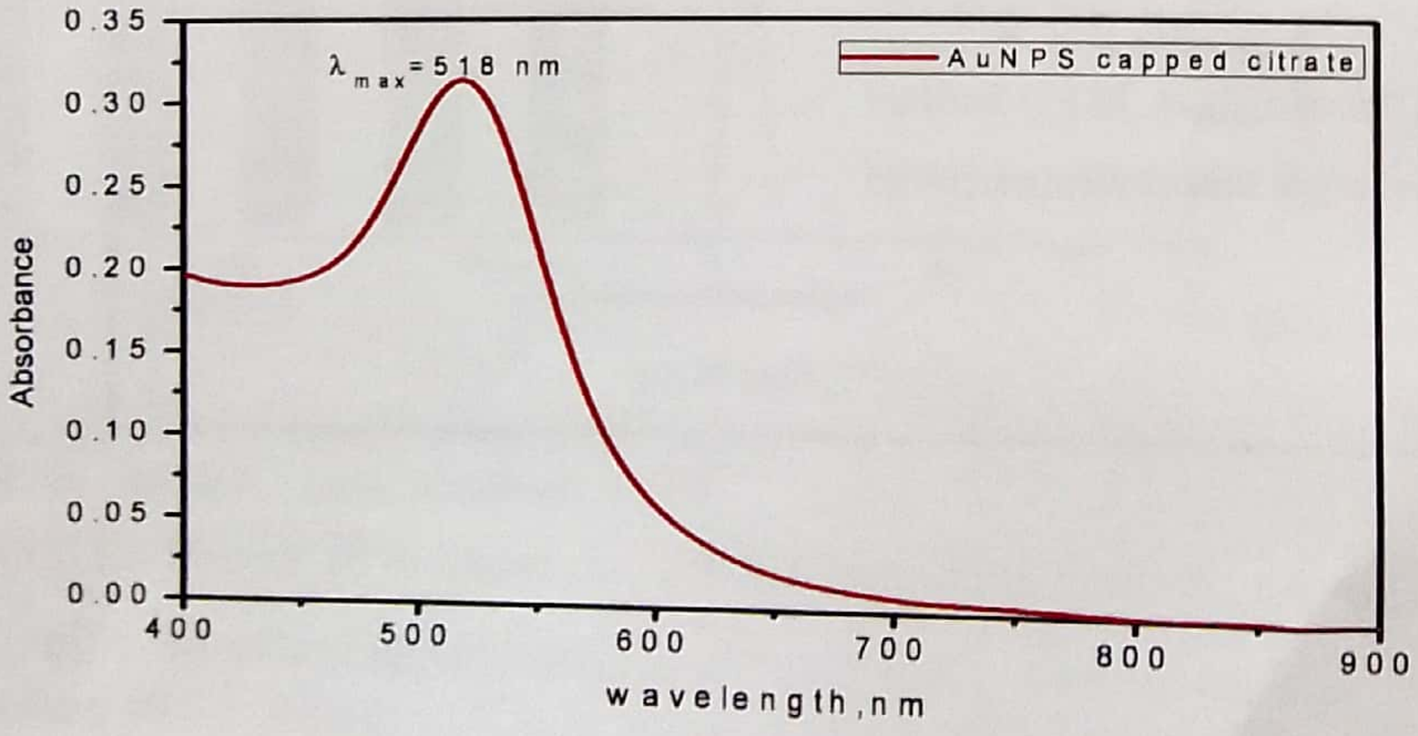

Fig (1): Spectrophotometer results of AuNPS characterization showing peak absorption at wave length 518nm

b)Transmission Electron Microscopy (TEM) imaging:

The size, shape and size distribution of the prepared AuNPS have been measured by TEM imaging. A representative TEM image of these particles is given in Fig (2, a and b).The particles are mostly spherical. The average size (diameter) of particles, measured on the TEM images was7± 2nm.

c) Zeta potential:

The zeta potential of AuNPS @ citrate was found to be around -12.3 mV Fig (3), verifying that AuNPS were negatively charged and stable.

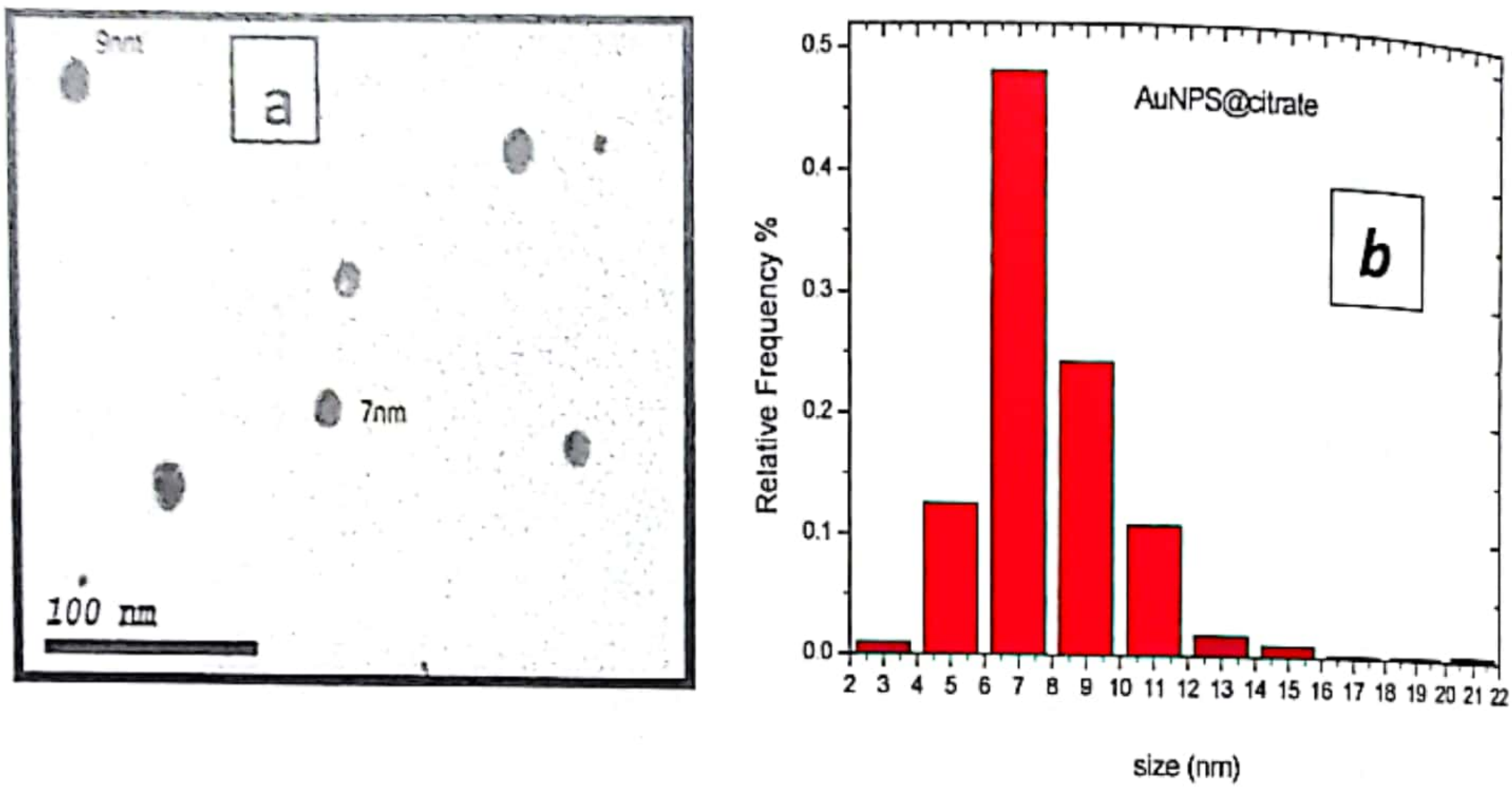

Fig. (2): a-TEM image showing spherical shape of the prepared AuNPS
b- Histogram chart providing the size distribution of synthesized AuNPS capped citrate.

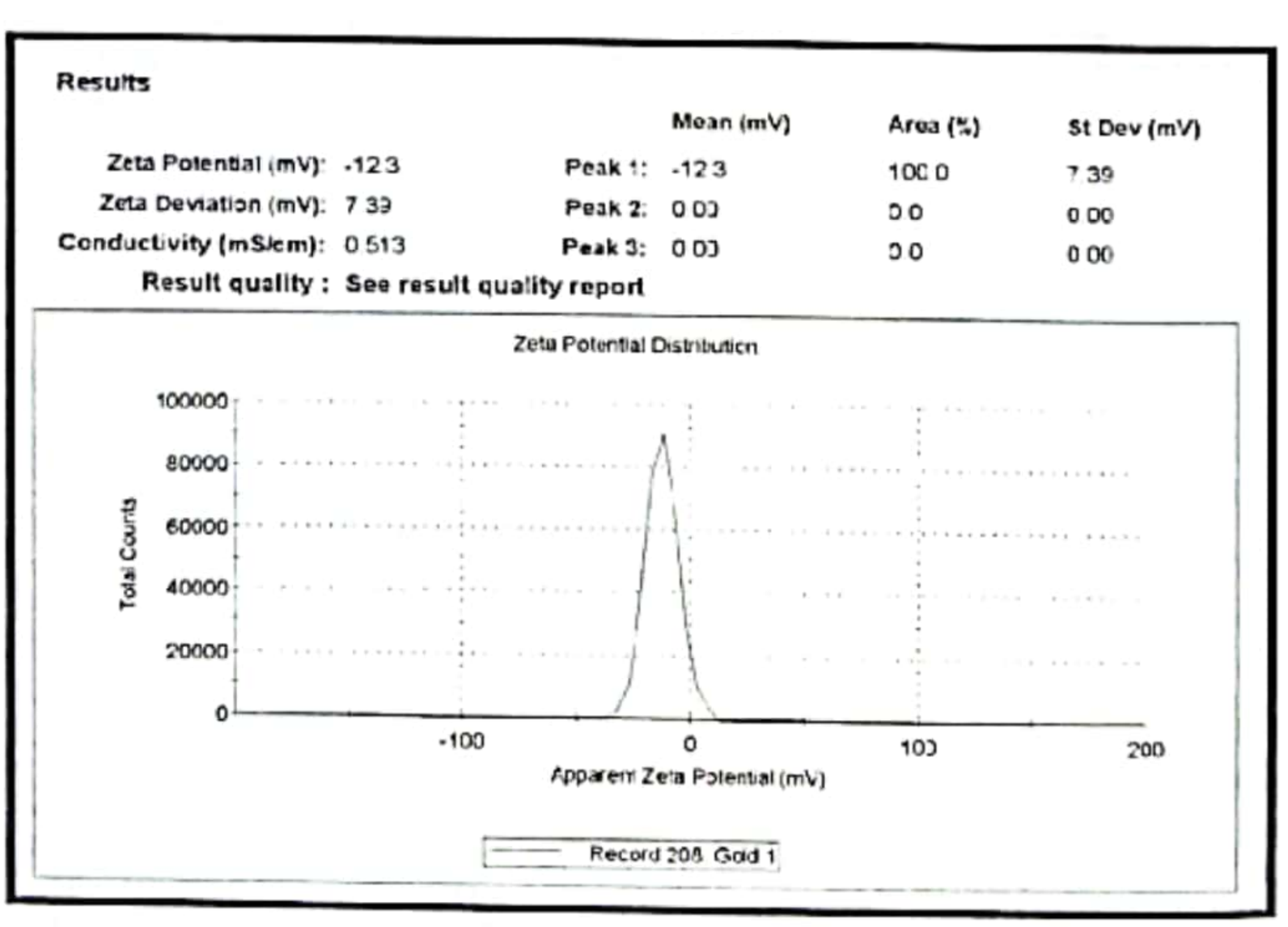

Fig (3): The zeta potential of citrated AuNPS was found to be around -12.3 mV

Results of cytotoxicity assay of prepared AuNPS by MTT (tetrazolium) assay:

Data in table (1) and Fig. (4) demonstrate the mean absorbance of the different concentrations ofAuNPSwhich represent the amount of MTT formazine formed intracellularlyand reflect the degree of cell viability (opposite of cytotoxicity) compared to cell control.

Results, showed that the degree of cell cytotoxicity was directly proportional to concentration of the usedAuNPS. The maximum nontoxic concentrations (MNTCS) of the used AuNPS were 2 and 4 ppm.

Table (1): Results of cytotoxicity assay (MTT) of AuNPS on MDBK cells by Microplate reader absorbance.

Group	AuNPS concentration (PPM)	Mean (OD)
AuNPS Citrated	8	0.17 ± 0.04 b
	6	0.20 ± 0.02 b
	4	0.28 ± 0.03 a
	2	0.28 ± 0.02 a
Cells only (control)	(0)	0.27 ± 0.04 a

values with diffrent superscripts letters significant at P ≤0.05

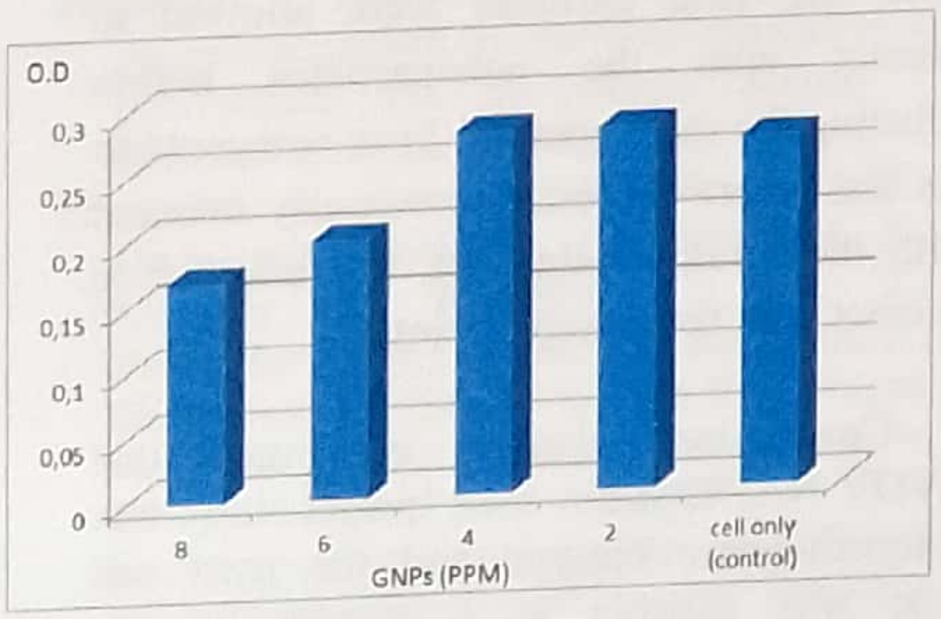

Fig. (4): Results of cytotoxicity assay (MTT)of AuNPS on MDBK cells (method 1) by Microplate reader absorbance.

Results of in-vitroAuNPSantiviral activity using MTT assay:

Results in table (2) and Fig.(5) demonstrate the changes in mean absorbance of different AuNPS concentrations on BVDV infected MDBK cells compared to cell control and virus infected cell control. Results revealed that treatment of the virus with AuNPS was effective in method (1) than in method (2) as far as cell viability is concerned and the highest cell viability was observed at 4 ppm AuNPSconcentration.

Table (2): Results of antiviral assay (MTT) of AuNPSon MDBK against BVDV by microplatereader.

Group	AuNPS concentration (ppm)	Mean(OD) method(1)	Mean(OD) method(2)
AuNPS	8	0.17±0.04bc	0.11±0.08c
	4	0.27±0.05a	0.11±0.02c
	6	0.20±0.07ab	0.11±0.05c
	8	0.17±0.01c	0.10±0.08c
+ve virus control	0	0.11±0.02c	
Cells control	0	0.27±0.04a	

values with diffrent superscripts letters significant at P≤ 0.05

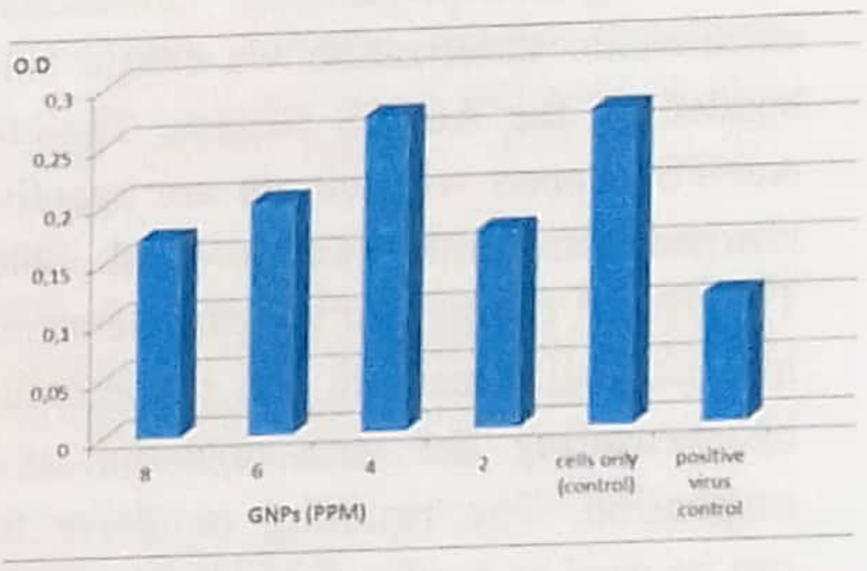

Fig. (5): Results of antiviral assay (MTT) method (1) of AuNPSon MDBK against BVDV by Microplate reader absorbance

Discussion

The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials. AuNPS have unique and well defined physical and chemical properties which can be manipulated

suitably for desired applications. Moreover, their potent antimicrobial efficacy due to large surface area to volume ratio(Rai and Rai, 2011).

A primary goal of AuNPS synthesis for practical applications is to produce monodispersed nanoparticles with a well-defined shape. Therefore, careful selection of the reducing agent and stabilizer are critical steps, which can be more easily controlled when the nanoparticles are synthesized(Liu and Lu, 2006). For these experiments, trisodium citrate served the dual role of both a reducing agent and a stabilizer. Trisodium citrate initially acts as a reducing agent to reduce the Au (III) ions to Au (0) then acts as the stabilizing agent by forming a layer of citrate ions over the AuNPs surface, inducing enough electrostatic repulsion between individual particles to keep them well dispersed in the medium and prevents aggregation or further growth of the particles(Liu and Lu, 2006). The colloidal gold solution proved to be very stable in time, no spectral change has been observed a year long after preparation. This indicates electrostatic stabilization via citrate anions bonded on the AuNPS surface. Therefore, AuNPS capped with citrate are negatively charged composites (Chah et al., 2005). The overall charge that the particle acquires in a particular medium can be determined by measuring the zeta potential of the suspension. The resulting repulsive force can be used to predict the colloidal stability and agglomeration state of nanoparticles (Wang et al., 2008). The zeta potential of citrated AuNPS was found to be around -12.3 mV, verifying it was charged and stable.

In spite of the reported antiviral activity exposed by AuNPS against multistrain of avian influenza virus (Sametband et al., 2011)and HIV(Kesarkar et al., 2012 and Vijayakumar and Ganesan, 2012), no information including AuNPs antiviral activity against BVDV has been reported.

Since long time tetrazolium salts based screening techniques as (MTT) are considered quick and inexpensive for the evaluation of in-vitro cytotoxicity and antiviral activity of AuNPs and other products (Sametband et al., 2011 and Kesarkar et al., 2012).

The importance of performing cytotoxicity assay at the beginning of work was necessary for safety of AuNPS use as anantiviral agent in the future. Therefore, cytotoxicity assay was carried out for prepared AuNPS on MDBK cell line.

Results showed that the maximum non-toxic concentrations of prepared AuNPS were 2 and 4ppm,which totally agree with Sametband et al. (2011)and Kesarkar et al. (2012).

The titer of BVDV was measured by the conventional microculture infectivity titration method (MCVT) (i.e. based on CPE development observation). Result revealed that the virus stock used in this experiment has a titer of 10^6TCID$_{50}$/mL.Antiviral activity evaluation of prepared AuNPS against BVDV showed greater activity in the test when the viral particles were allowed to interact with the nanoparticles before infecting the cells (method 1) as compared to the test where the cells, previously infected with the virus (method 2), not allowed to interact with the nanoparticles.

Conclusion: Results confirmed that BVDV NADL strain used in this study was cytopathogenic biotype and the used cell type was suitable as a theater for the following in vitro virus inhibition assays. BVDV can be a surrogate model for HCV on MDBK cells in the antiviral testing procedures. AuNPS at 4ppm concentration were found to be effective as virus

neutralizing candidate when allowed to interact with cells.

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الملخص العربى

في هذه الدراسة، تم تحضير جسيمات الذهب النانوية المجرّدة والمقترنة بالأجسام المضادة لمستقبلات عامل نمو النسيج الطلائي باستخدام طريقة الاختزال الكيميائي بفعل أملاح السترات. تم بعد ذلك التعرف على خصائص الجسيمات المحضرّة باستخدام مقياس الطيف الضوئي، المجهر الإلكتروني ومقياس زيتا ؛ وذلك لتحديد حجم الجسيمات والتعرف على شكلها ومعرفة شحنتها قبل استخدامها.

تم استخدام الجسيمات المحضرّة لعمل تجربتين ، التجربة الأولى لتقييم مدى سمية جسيمات الذهب النانوية ذات حجم يقارب٧ نانومتر لخلايا مادن دربى بوفين كيدنى باستخدام الملاحظه التغيرات الخلويه وتاكيد النتائج باختبار م.ت.ت.

أما التجربة الثانية فكانت لتحديد قدره الجسيمات النانونيه على تثبيط فيروس الاسهال البقرى فى المعمل على خلايا مادن دربى بوفين كيدنى باستخدام ملاحظه التغيرات الخلويه وتاكيد النتائج باختبار م.ت.ت..

وقد اظهرت نتائج الدراسة مايلى

جسيمات الذهب النانوية المحضرة كانت ذات حجم يقارب ٧ نانومتر وذات شكل كروي لدى فحصها بالمجهر الإلكتروني وكانت قمة الامتصاص الضوئي لمطول جزيئات الذهب عند طول موجي 535 نانومتر باستخدام مقياس الطيف الضوئي.

جسيمات الذهب النانوية زات الحجم ٧ نانوميتر غير سامه للخلايا عند تركيز ٤ جزء فى المليون.

جسيمات الذهب النانونيه لها القدرة على تثبيط فيروس الاسهال البقرى عند تركيز ٤ جزء فى المليون عند خلطها بالفيروس قبل العدوى على الانسجه.