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Research Article

ANTI-CHIKUNGUNYA ACTIVITY OF GREEN SYNTHESIZED SILVER NANOPARTICLES USING *CARICA PAPAYA* LEAVES IN ANIMAL CELL CULTURE MODEL

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ABSTRACT

Objective: The aim of the present study is to synthesized silver nanoparticles (AgNPs) of *Carica papaya* L. leaves and evaluation of their anti-Chikungunya activity *in vitro*.

Methods: The AgNPs were prepared using *C. papaya* leaves extract in 1:4 ratio. Synthesized AgNPs were characterized using ultraviolet-visible spectroscopy, Fourier-transform infrared spectroscopy, and scanning electron microscopy. The cytotoxicity of plant NP and 50% tissue culture infective dose of Chikungunya virus (CHIKV) were determined before antiviral assay by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. After that, the maximum non-toxic dose (MNTD) and ½MNTD were calculated. The absorbance values were detected using a microplate reader at 595 nm. Median tissue culture infective dose ($TCID_{50}$) dose was calculated using the Reed and Muench method. *In vitro* antiviral activity was performed to CHIKV using NP MNTD, ½MNTD, and calculated $TCID_{50}$ dose of CHIKV.

Results: The MNTD and ½MNTD of *C. papaya* AgNPs were found to be 125 and 62.5 µg/ml, respectively. The MNTD and ½MNTD brought about 39% and 52% of CHIKV inhibition, respectively, when compared to virus control. The infected cell viability increased (14%) when treated with plant AgNPs at ½MNTD.

Conclusion: There are no antiviral agents available to treat CHIKV. The medicinal plants and their metabolites are the most important source for the invention and development of new drugs against many types of disease. In view of the rapid expansion of CHIKV at the global level, there is an urgent need to develop newer anti-Chikungunya drugs with unique drugs target.

Keywords: Chikungunya virus, Cytopathic effect, Medicinal plants, Maximum nontoxic dose, Silver nanoparticles.

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INTRODUCTION

Chikungunya virus (CHIKV) is a serious global health problem due to its high mortality and morbidity, especially in Africa and Asia. The vectors of CHIKV are the same as for dengue virus, i.e., *Aedes aegypti* and *Aedes albopictus* mosquitoes. CHIKV is a single-stranded, non-segmented, and linear positive sense RNA virus. It belongs to the genus *Alphavirus* of *Togaviridae* family [1]. Clinical symptoms include high fever, headaches, severe back pain, joint pain, fatigue, and rash for 4–7 days [2]. CHIKV was first time isolated in 1952–1953 from Tanzania [3]. In India, the first outbreak was reported from Kolkata in 1963 while subsequent epidemics in Tamil Nadu, Maharashtra, and Andhra Pradesh during 1964–1965. There is no antiviral agent available to treat CHIKV. There is a strong need to investigate and develop noble antiviral agents against chikungunya, which will help in curbing the infection and controlling severe outbreaks.

Nanoparticles (NPs) and their use for human healthcare and medicine have been an exciting area of nanotechnology. Their size ranges from 1 to 100 nm and they have unique electrical optical and biological properties. Metals such as Ag, Au, Al, Zn, C, Fe, and Cu have been usually used for the synthesis of NPs [4]. Medicinal plants-based NPs are simple, efficient, less harmful, and eco-friendly [5]. Due to the high surface area-to-volume ratio, NPs can interact with the pathogenic microbes efficiently. Especially, AgNPs have shown a greater response in the field of nanomedicines. AgNPs have extensive applications in drug delivery, diagnostics, bio-sensing, and tissue engineering [6,7].

Carica papaya is commonly called paw-paw (papaya). It is a member of the family *Caricaceae*. *C. papaya* contains two active compounds chymopapain and papain. It also contains anticancer, antiviral, and antimicrobial activities [8,9]. The present study focused on the biological synthesis of AgNPs from *C. papaya* and evaluation of their antiviral activity against CHIKV *in vitro*.

METHODS

Collection of plant sample

C. papaya L. plant was identified from the herbal garden of M.D University, Rohtak, Haryana, India, and the specimen was deposited in the Department of Genetics, with voucher No. MDU 3201. Fresh, healthy, and well-grown leaves of *C. papaya* were collected. Leave were washed properly under tap water and air dried at room temperature for 1 weak and pulverized. About 40 g of leaves of *C. papaya* were added into separate 250-mL Erlenmeyer flasks along with 100 mL of sterile double-distilled water and heated for 25 min. After cooling, the extract was filtered through the Whatman filter paper No.1 and filtrate was stored at 4°C until use further.

Green synthesis of silver NPs (AgNPs) using *C. papaya* leaf extract

The 20 ml of *C. papaya* extract was treated with 80 ml of 1 mM, $AgNO_3$ aqueous solution and was incubated at room temperature for 24 h. The color of the solution changed from yellow to yellowish brown within 10–15 min of mixing, showing the reduction of $AgNO_3$ to Ag+ ions. After proper incubation, the solution was centrifuged at 12,000×*g* for 15 min at room temperature followed by washing with double distilled water to remove unbounded capping materials. The procedure was repeated in 3 times and the final solution was lyophilized and stored as powdered form at 4°C.

Characterization of green synthesize AgNPs
Characterization of *C. papaya* AgNPs was done using ultraviolet-visible (UV-Vis) spectroscopy, Fourier transforms infrared (FTIR) spectroscopy, and scanning electron microscopy (SEM).

UV-Vis spectroscopy
UV-Vis absorbance spectral analysis of the aqueous suspension of AgNPs was carried out using a spectrophotometer (UV-1700, Shimadzu Corp., Japan). The absorbance spectrum of the sample was taken at 300–600 nm range of wavelength with a resolution of ± 1 nm. The double distilled water was used as a blank reference.

FTIR spectroscopy
FTIR spectroscopy was done to identify the biomolecules formation during the reduction of the silver nitrate to the silver of green AgNPs of *C. papaya* leaves. The chemical compositions and potential biomolecules of the AgNPs were analyzed by the FT-IR spectrum, recorded using an ALPHA FT-IR spectrometer (Bruker, Germany). Percentage transmittance of the FT-IR spectrum was recorded in the region of 4000–500 cm^{-1}.

SEM for the size of NPs
Morphology of *C. papaya* AgNPs was calculated through SEM of the aqueous suspension of AgNPs. The sample was prepared by mounting AgNPs suspension on specimen stubs and coated with gold by an ion sputter coater (Hitachi Model-E1010, Japan). The SEM observation was performed using EVO 18 special edition SEM (Carl Zeiss Inc., Germany).

Maintenance of vero cell line and virus propagation
Vero cell lines were obtained from the National Center for Cell Sciences, Pune, India. Cells were maintained in the DMEM (Sigma-Aldrich, St Louis, USA) media in the T-25 cell culture flask at 37°C in 5% CO_2 as per standard tissue culture procedure. DMEM media supplemented with 10% fetal bovine serum (Gibco, New York, USA). The culture medium was supplemented with 1% of penicillin-streptomycin (Gibco, New York, USA) as antibiotic and sodium bicarbonate (Merck, Kenilworth, USA) to maintain the neutral pH. The cells become fully confluent after 4–5 days after that trypsinized by TPVG for further subculture. CHIKV was used in the study and propagated in Vero cells. The virus was harvested after observation of cytopathic effect (CPE) after 7 days post-infection. The virus aliquots were collected and stored at −80°C until used.

Determination of maximum non-toxic dose (MNTD)
As for the plant extract cytotoxicity evaluation, the Vero cells (1.5×10^3) were seeded into 96 well flat bottom plate (Nunc, Thermo Fisher Scientific, USA) and incubated overnight at 37°C in 5% CO_2 incubator. Briefly, 90% confluent Vero cells were treated with different serially diluted conc. (1000–31.25 µg/ml) of *C. papaya* NP in triplicates. DMEM media were used as a negative control. After incubation of approximately 5 days, 3-(4, 5–Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT], 20 µl) dye was added in 96 wells. After 3 h incubated the plate, MTT was discarded followed by 100 µl of DMSO (Sigma-Aldrich, USA) was added as per well to stop the reaction. Absorbance reading was taken using a microplate reader (Bio-Rad, USA) at 595 nm. Percentage of cell cytotoxicity was calculated by absorbance values of plant NPs with the microplate reader.

Determination of median tissue culture infective dose ($TCID_{50}$)
CHIKV stock was characterized by infectious titer in terms of 50% $TCID_{50}$/mL. Briefly, Vero cells (5×10^3) were seeded into a 96-well plate and incubated at 37°C with 5% CO_2 incubator under cell culture procedure for 24 h. Then, 100 µl of ten-fold serially diluted CHIKV was propagated into the Vero cells for 1 h and incubated at 37°C with 5% CO_2 incubator. Then, 100 µl of fresh medium (DMEM) was added into each 96 well and the plate was incubated for 4–5 days for CPE. Then, the plate was observed under an inverted microscope (Motic AE31, USA) and CPE was determined. The highest dilution of the virus, which demonstrated >50% CPE was considered to calculate $TCID_{50}$/mL of the virus stock using the Reed-Muench method [10].

***In vitro* anti-Chikungunya assay**
In vitro anti-Chikungunya assay, the Vero cell was treated with the 100 µL of calculated MNTD and half of the MNTD value of *C. papaya* NPs with 100 µL of CHIKV at 10^3 $TCID_{50}$/mL into 96 well except in the wells marked as cell control. The plate setup included cell control, virus control, and AgNP-treated cells. The plate was incubated on ice for 45 min with gentle shaking after every 10 min. The plate was sealed with parafilm and incubated at 37°C in 5% for 1 h. After incubation, the inoculum was aspirated from each well without disturbing cell layer. 200 µl of virus growth medium was added into each 96 well, and the plate was incubated at 37°C in 5% CO_2 incubator. Infected cells treated with plant AgNPs were determined using MTT assay as described above. The experiment was repeated twice and triplicates were included each time.

Data analysis
The data were analyzed using GraphPad Prism version 7.04 (GraphPad Software, CA, USA). Data are expressed as means and standard deviations.

RESULTS

Synthesis and characterization of *C. papaya* AgNPs

UV-Vis spectroscopy

The color of extract changed from light yellow to yellowish brown after addition of silver nitrate solution (Fig. 1). This color change was observed due to the reduction of Ag+ ions in the aqueous solution of $AgNO_3$ into AgNPs. The UV spectra of papaya NP were recorded in the range of 300–600 nm. A broad surface plasmon resonance band was observed in between the wavelengths of 400–440 nm (Fig. 2) indicate the confirmation of AgNPs synthesis.

Scanning electron microscopy

The plant AgNPs were also characterized for their shape and size by SEM. Fig. 3 shows the SEM images of the plant AgNPs along with their size. The average size of *C. papaya* AgNPs was found to be in

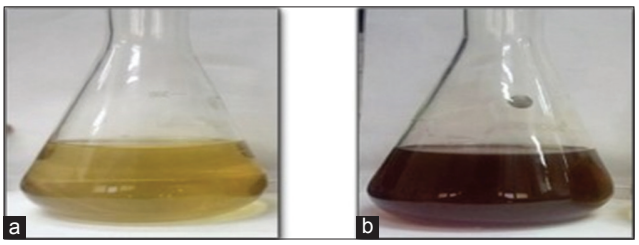

Fig. 1: (a) Represents filtered leaf extract of *Carica papaya* with 1 mM $AgNO_3$ (b) dark brown color represents the formation of green silver nanoparticles of *C. papaya* leaves extract

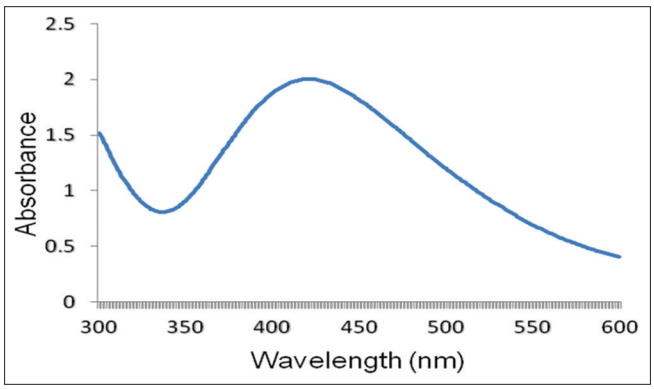

Fig. 2: Ultraviolet spectroscopy graph of silver nanoparticles synthesized from *Carica papaya* L. leaves

the range of 64–151 nm (Fig. 3). The shape was found in spherical in form.

FT-IR result analysis

The FT-IR spectra of lyophilized extract AgNPs are shown in Table 1, in which the spectrum of *C. papaya* AgNPs showed prominent transmittance bands (Fig. 4). This absorbance peak values can be compared to the IR spectrum list on Google (Table 1).

MNTD assay

MNTD of green synthesized that AgNPs of *C. papaya* leaves extracts were calculated by serial dilution in Vero cell line followed by MTT assay. Microplate reader provides the numerical value from each 96 well have different concentration of NP. The blank and control value recorded were 0.036 and 0.92, respectively. The MNTD and ½MNTD of *C. papaya* AgNPs were calculated as 125 µg/ml and 62.5 µg/ml, respectively. A graph was plotted among cell viability versus *C. papaya* plant NP concentration (Fig. 5).

$TCID_{50}$ assay

$TCID_{50}$ was carried out to determine the concentration of CHIKV, which induce CPE in 50% of the cells. Under observation, the CHIKV infected cells undergo structural changes, such as syncytia and blabbing, when seen under the inverted microscope. $TCID_{50}$ value was calculated as 10^3 $TCID_{50}$/mL for antiviral assay.

Determination of antiviral property of AgNPs

The antiviral assay was performed to determine the antiviral effect of AgNPs on the CHIKV. The anti-CHIKV activity of AgNPs of the plant was measured in terms of inhibition of CPE as well as the increase of percentage cell viability. However, on treating the CHIKV infected cells with AgNPs of *C. papaya* at MNTD and ½MNTD, an increase in the percentage of cell viability was observed by MTT assay. *C. papaya* AgNPs at MNTD and ½MNTD brought about 39% and 52% inhibition of CPE when compared to virus control (Fig.6). The half of the MNTD of *C.papaya* AgNPs showed considerable inhibition of CHIKV with increased cell viability of 14% in comparison to virus control while MNTD is not significantly increased the cell viability.

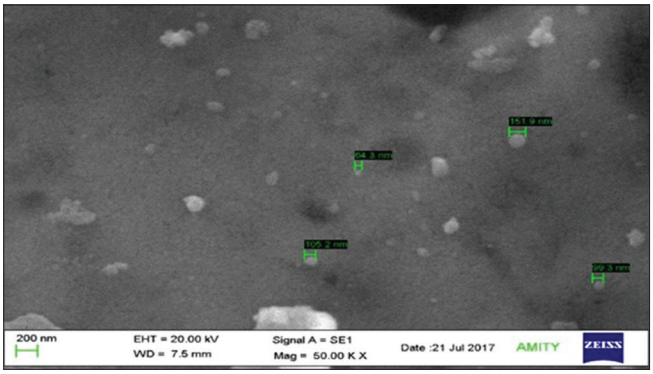

Fig. 3: Scanning electron micrographs of synthesized silver nanoparticles from *Carica papaya* leaves

DISCUSSION

CHIKV is a global health problem due to severe break bone fever. There are no commercial antiviral drugs available in the market to fight against CHIKV. Various clinical trials are now in progress for the development of the chikungunya vaccine. Medicinal plants extract and their derivatives have a capacity to fight against many types of disease due to their therapeutic properties [11,12]. Verma *et al*. (2008) have demonstrated that *Swertia chirata* was very effective against herpes simplex virus [13]. Sharma *et al*. (2018) have demonstrated that plants AgNPs of *Andrographis paniculata, Phyllanthus niruri,* and *Tinospora cordifolia* showed potent inhibitory activity to CHIKV [14]. Utilization of medicinal plant material for the synthesis of green AgNPs has been revolutionized in recent years for antiviral activity. Literature suggests that there are several mechanisms by which AgNPs could inhibit viruses by (1) interaction of AgNPs with DNA and interruption of DNA replication and translation and (2) block cellular factor and the viral vector which help in viral replication [15]. The mechanism of action of AgNPs is ambiguous in HIV-1 [16], hepatitis B virus [17], and H1N1 virus [18]. These can also enhance the antiviral activity of certain chemical antiviral drugs, i.e., acyclovir [19]. *C. papaya* contains various compounds such as alkaloids, diterpenoid, lactones, aliphatic compounds, and steroids. Nanomaterials had proven to be more efficient for drug delivery [20]. The NPs can be used as an antibacterial, antiviral, antifungal, and anticancer agent. The biocompounds in leaf extract having the carbonyl groups are believed to be reducing the silver ions and converting to unsaturated carbonyl groups by auto-oxidizing [15,21]. The FT-IR analysis of *C. papaya* leaves-AgNPs showed two sharp absorption peaks at 1640 cm^{-1} and 3359 cm^{-1} indicating a possible interaction between proteins and AgNPs. The absorption peak at 1640 cm^{-1} could be due to amide bond coming from carbonyl group of a protein and peak at 3359 cm^{-1} may be due to -OH group present in alcohols and phenolic [22,23]. The MNTD of plant NPs does not show any change in cell viability while half MNTD provides an additional 14% viability increase. The results revealed that at a lower concentration of plant NP (½MNTD) may provide a good therapeutic effect to CHIKV. The previously reported studies showed that the extract of papaya leaves increases the blood platelet of patients [24,25]. Green AgNPs

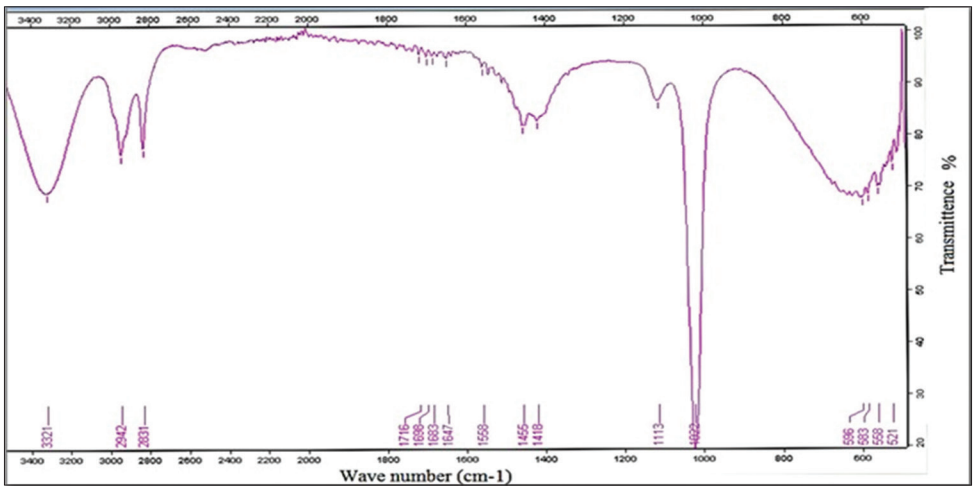

Fig. 4: Fourier transforms infrared transmittance graph of lyophilized *Carica papaya* silver nanoparticles

Table 1: FT-IR spectrum of *C. papaya* AgNPs in the region of 4000–500 cm^{-1}

Wavenumbers (cm^{-1})	Chemical bond	Phytoconstituent	Peaks observed (AgNPs)
3200–3600	O-H Stretching	Alcohol, Phenol	3321
2800–3000	C-H Stretching	Alkanes	2942, 2831
1700–1725	C=O Stretching	Aldehyde, Ketone, Carboxylic acid	1716
1680–1700	C=O Stretching	Aryl ketone	1698
1665–1685	=C-H Stretching	Alkene	1683
1620–1680	C=C Stretching	Aromatic, Alkenyl	1647
1550–1640	N-H bending	Amide	1558
1400–1600	C=C Stretching	Aromatic, Alkenyl	1455, 1418
1080–1360	C-N Stretching	Amine	1113
1000–1300	C-O Stretching	Primary, Secondary alcohol	1113, 1022
500–600	C-Br Stretching	Alkyl-halides	596, 583, 558, 521

C. papaya: Carica papaya, AgNPs: Silver nanoparticles, FT-IR: Fourier transforms infrared

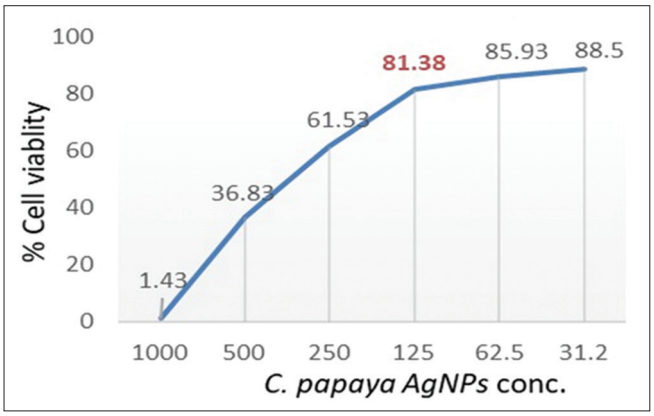

Fig. 5: Cell viability evaluation of plant silver nanoparticles on Vero cells and determination of maximum non-toxic dose (MNTD) and ½MNTD

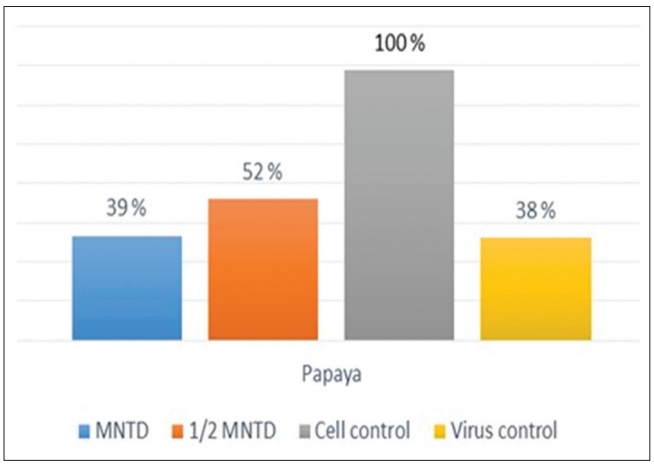

Fig. 6: Antiviral effect of *Carica papaya* silver nanoparticles on Chikungunya virus in Vero cells. The percentage values denote cell viability

of *C. papaya* leaf are more powerful due to their good conductivity, stability, and antimicrobial activity. Although *C. papaya* leaf extract does not much interfere with virus replication, increases the platelets which help in patient management. *C. papaya* contains many secondary compounds, i.e., alkaloids, flavonoids, amino acids, and phenolic compounds. *C. papaya* leaf extract is widely used for the treatment of dengue and Chikungunya fever in India and other tropical countries. Much biomedical research has been conducting that the higher dose of papaya leaf extract has no side effect [26]. Many other studies have been demonstrated the value of medicinal plants [27,28]. Based on related literature, it is recommended that papaya leaf extract to be given from the 1st day of dengue and Chikungunya fever concurrently help to reduce the viruses and also increases blood platelets of patients.

CONCLUSION

AgNPs were successfully synthesized from aqueous extracts of *C. papaya*. The AgNPs synthesized from *C. papaya* showed antiviral activity against CHIKV when tested on Vero cells. The biosynthesis of AgNPs using plant extract is simple, efficient, cost-effective, and an environmentally friendly alternative to develop broad-spectrum antiviral agents which could provide the alternative for the treatment of viral diseases against which have no specific antivirals agents are available. The half MNTD of plant NP potentially inhibited the CHIKV than MNTD value on Vero cells. *C. papaya* leaf extract does not much interfere with virus replication but increases the blood platelets of patients.

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AUTHORS' CONTRIBUTIONS

Sulochana Kaushik and Parneet performed experimentation work such as plant material collection and NPs synthesis. Vikrant Sharma and Sulochana Kaushik maintained cell line and performed the antiviral experiment. Jaya Parkash Yadav and Samander Kaushik were involved in conceptualization, study design, and manuscript draft preparation.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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