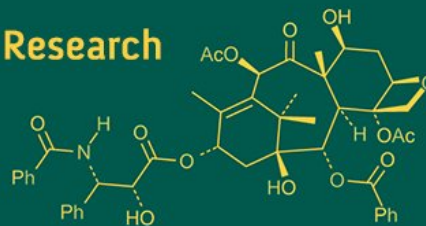


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Evaluation of *in-vitro* antiviral activity of nanocurcumin and nanoeugenol against goat pox

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Abstract

Five treatment combinations-Curcumin, Eugenol, Nanocurcumin, and Nanoeugenol-were taken into consideration. These compounds are known for their medicinal benefits, particularly their antiviral effects against a variety of viruses. They were tested for their antiviral effect against Gopox virus replication *in-vitro*. The Z-average particle size of the nanocurcumin and nanoeugenol products, which were made using the Evaporative Precipitation of Nanosuspension (EPN) and ultrasonication methods, was found to be 64.22 nm and 7.270 nm, respectively, and to be physically stable without any phase separation. All of the medications, including Curcumin, Eugenol, Nanocurcumin, and Nanoeugenol, were determined to be safe for oral dosage up to 2000mg/kg body weight. According to *in-vitro* cytotoxicity tests, the maximum non-toxic doses of curcumin, eugenol, nanocurcumin, and nanoeugenol as well as their combinations were 39.06 g/ml, 78.12 g/ml, 140.62 g/ml, 156.25 g/ml, and 296.875 g/ml, respectively, and the cytotoxic concentration₅₀ values were 290.4±1.419µg/ml, 319.7±1.301µg/ml, 1462±1.0620µg/ml, 432.7±1.1656µg/ml, and 1309±1.2887µg/ml, respectively. All the treatments viz. Curcumin, Eugenol, Nanocurcumin, Nanoeugenol and their combinations were found to have their viral percentage inhibition of 74.88, 79.43, 97.48, 99.55 and 99.85, respectively which are found to be significant at $P < 0.05$ at their MNTDs. Out of which Nanocurcumin, Nanoeugenol and their combinations showed >80% inhibition and their EC₅₀ values found to be 92.47±1.586µg/ml, 54.93±1.1220µg/ml and 52.48±1.1481µg/ml with their therapeutic indices to be 15.81, 7.877 and 24.94, respectively. One step growth inhibition of goatpox virus by Nanocurcumin showed inhibition up to 0 hour post infection, but Nanoeugenol and combination of nanocurcumin and nanoeugenol showed inhibition upto 72 hour post infection.

Keywords: Nanocurcumin, nanoeugenol, *in vitro*, goatpox

Introduction

Goatpox is often a great threat to goats and sheep and characterized by pyrexia and generalised pock lesions with lymphadenopathy causing high mortality (50-100%) and morbidity upto 100% (Bhauprakash *et al.*, 2006a; Babiuk *et al.*, 2008) [2, 7]. It is more severe in young ones compared to adults (Joshiet *et al.*, 1996). The disease is not distinguishable from sheeppox serologically but possible by molecular technique (Hosamani *et al.*, 2004a) [4, 6]. Indigenous sheep and goat exhibit some natural immunity, while the European breeds of sheep and goats are more susceptible to infection with these viruses (Heine *et al.*, 1999). Since its first report by Hansen in 1879 from Norway, goatpox remains to be enzootic in Southwest and Central Asia, Northern and Central Africa, and Indian subcontinent (Hosamani *et al.*, 2004b) [4, 6] and recently in Vietnam, Mongolia and Greece indicating emerging nature of the disease (Babiuk *et al.*, 2008) [2, 7].

In India, first reported case was in 1934 (Ramesh *et al.*, 2009). In India, goatpox is enzootic like other pox viral infections and regular outbreaks have been reported from most of the states (Bhanuprakash *et al.*, 2005) [9]. Major control strategies against goatpox include restriction of movement, test and slaughter and vaccination (Carn *et al.* 1995) [10]. Treatment strategies are almost nil due to lack of effective therapeutics for most of the viral diseases, emergence of the antiviral drug resistance, more of side effects and high cost of some antiviral therapies necessitate finding new effective antiviral compounds (Lemonie *et al.*, 2013) [11]. Hence there is an increasing requirement for new effective antiviral substances of indigenous nature with low toxicity and economically feasible. It has been demonstrated that medicinal plants have wide range of antiviral activity against different viruses. Among them

We have focused on curcumin and eugenol for their anti-viral activities. Curcumin is extracted from turmeric (*Curcuma longa*) plant, a perennial herb belonging to the family Zingiberaceae (Priyadarshini *et al.*, 2014) [36], which is cultivated extensively in south and south-east tropical Asia.

Eugenol, a phytochemical bioactive component with well-defined functional characteristics, is widely found in a variety of herbal plants. Eugenol's ability to scavenge free radicals, prevent the production of reactive oxygen and nitrogen species, increase cyto-antioxidant potential, and disrupt microbial DNA and proteins are some of the main principle mechanistic approaches connected to its therapeutic potential (Khalil *et al.*, 2001) [13]. The body quickly eliminates curcumin and eugenol in their natural form, and it has very little ability to penetrate organs. According to Tsai *et al.* (2011) [14], eugenol and curcumin have a noticeably better penetration and distribution rate in their nano form. In order to evaluate the in-vitro antiviral activity of nanocurcumin and nanoeugenol combined against goitrop, the current investigation was carried out.

Materials and Methods

Preparation of nanocurcumin

Curcumin was purchased from HIMEDIA. All the reagents used were of technical grade. N-Hexane (HPLC grade) and ethanol (99.5-99.8%) were obtained from Mercks.

Evaporative precipitation of nanosuspension

According to the procedure outlined by Siekmann *et al.* (1996) [15], curcumin was dissolved in ethanol before being mixed with an antisolvent (hexane) to create nanocurcumin. The rotary evaporator was used to quickly evaporate the solvent and antisolvent under vacuum to produce the drug particles in the nanosuspension. The nanoparticles were then vacuum dried to thoroughly evaporate all of the liquids. The Solvent to Ant Solvent (SAS) ratios employed ranged from 1:10, 1:15, and 1:20 (v/v), and the drug doses used were 5, 10, and 15 mg/mL. 200-400 ml of hexane were used for every 20 ml of the medication solution.

Preparation of nanoeugenol

Eugenol was procured from SIGMA ALDRICH and Tween-80 was procured from CDH was used for the preparation of nanoeugenol.

Ultrasonication with little modifications

Nanoeugenol was prepared using the method described by Kamel *et al.*, 2015 [16], the eugenol oil (99%, MW164.21; Fluka code 46100), and non-ionic surfactant (Tween 80) were added slowly under gentle stirring until a homogeneous mixture formed. Then water was added drop wise at 37 °C and stirred. This mixture was sonicated using an Ultrasonicator (MLI, Germany) for 10 min at 470 W. The particle size was analyzed by Zeta-sizer (IASST, Guwahati).

Experimental protocol and procedure

Experimental animal

- All experimental protocols were approved by a Institutional Animal Ethical Committee (Approval No. 770/GO/Re/S/03/CPCSEA/FVSc/AAU/IAEC/18-19/693).
- The study is reported in accordance with ARRIVE guideline.

- In this present study mice were only used for acute toxicity study. Acute toxicity study was carried out according to OECD (Organization of Economic Co-operation and Development) 423 guideline.

A total of 54 numbers of Wistar albino rats and mice weighing 120-150g and 25-30g were procured from Animal house, Department of Pharmacology and Toxicology, AAU. All the animals were kept in polypropylene cages in a small group of 6 rats per cage. Animals were fed with standard balanced ration and clean drinking water *ad libitum* and were maintained in a standard laboratory conditions (12:12 hour light/ dark cycle at ambient temperature ranging between (22-25 °C). Any kind of anesthesia/ euthanasia were not used for this study.

Experimental design

Acute oral toxicity

The study was carried out according to OECD (Organization of Economic Co-operation and Development) 423 guidelines. The nanocurcumin & nanoeugenol at different doses i.e, 300 and 2000 mg/kg body weight was administered to the mice and the animals were observed for behavioural changes, body weight gain and mortality up to 24 hours and animals were further observed for 14 days to record body weight changes and mortality (Gopi *et al.*, 2016) [17].

Cell lines

African green monkey kidney cell line i.e; VERO cell line (Passage no. 51) obtained from cell line repository, Department of Microbiology, College of Veterinary Science, Khanapara, Guwahati, Assam was used in the present study.

Maintenance of vero cells

Vero cells passage 51 were obtained from cell line repository, Dept of Microbiology, College of Veterinary Science, A.A.U, Khanapara, Guwahati and propagated using minimum essential media with Earle's salts (EM/EBSS, CELL-lone), supplemented with 8% Fetal Bovine Serum (FBS; Sigma Aldrich). Antibiotic solution (combination of penicillin, Streptomycin, Amphotericin B; Hyclone) was added to avoid contamination of the growth medium @ of 100 units per ml for maintaining the cells. L-glutamine at 3% was added to Growth Media for better maintenance of cell morphology. For maintaining the cells and infected cells, maintenance medium containing MEM with 2% FBS, 3% L-glutamine and 100 units per ml antibiotic was used.

Subculture of vero cell lines

Cell culture flask (25cc or 75cc) with 100% confluent monolayer of Vero cells were selected for subculturing. The growth media was discarded and the monolayer was washed with EMEM. Then 750µl for 25cc and 2.5µl for 75cc of 0.25% trypsin with 2% EDTA solution was added and kept at room temperature for 2-3 minutes. Later trypsin was discarded and the flask was gently tapped to detach the cells. The cells were split in 1:2 ratio from 25cc and 1:3 ratio from 75cc flask.

Virus

Source of virus

Procured from the goatpox virus isolate repository, Department of veterinary microbiology, College of Veterinary Science, Khanapara, Guwahati.

Isolation of goatpox virus

0.75ml of isolated virus was inoculated into the 70-80% confluent Vero monolayer grown in 75cc cell-culture flasks. The flask was kept for adsorption for 1hr at 37 °C with 5% CO₂ and with gentle shaking every 15 min to ensure uniform adsorption. After 1hr of adsorption, MM containing 2% FBS was added (20ml) and flask was incubated at 37 °C and observed for 72 hrs for CPE (Cytopathic Effect) of the virus. The flasks were harvested by repeated freezing and thawing after the development of 70-80% CPE. Then the harvested cell culture fluid from the flasks were transferred to cryovials and stored at -20 °C until use.

Cell cytotoxicity testing

Determination of maximum non-toxic concentration/dose (MNTC/MNTD)

Cell cytotoxicity testing of each active principle and nanopreparations (i.e, curcumin, nanocurcumin and eugenol, nanoeugenol) based on cellular morphological changes was carried out according to Goncalves *et al.* (2005) [37] with slight modifications.

Determination of cell cytotoxicity₅₀ (CC₅₀/CyC₅₀)

Dye exclusion method

Concentration of active principle and nanoparticles (i.e, curcumin, nanocurcumin and eugenol, nanoeugenol) inducing 50% of the cell lysis or cell death (CC₅₀ or CyC₅₀) was determined as per Tolo *et al.* (2006) [38]. Total, viable cells and viable cell percentage were calculated using the following formulas.

Total cells/ml = total cells in a square × dilution factor × 10⁴

Viable cells/ml = viable cells in a square × dilution factor × 10⁴

% of viable cells = 100 × (viable cell count/ total cell count)

Dilution factor = 2

The concentration of the curcumin, nanocurcumin and eugenol, nanoeugenol reducing the cell viability by 50% (CC₅₀ or CyC₅₀) was determined from a curve colligating percent cell viability to concentration of the nanoparticles.

Virus estimation assay

Determination of tissue culture infective dose₅₀ (TCID₅₀)

TCID₅₀ was performed following guidelines of virology laboratory manual. Cell culture fluids were collected from Vero cell adapted positive field isolates. A volume of 0.9 ml of media was dispensed into 5 tubes and labeled. 0.1 ml of the positive cell culture fluid was added to the first tube. The content of the first tube were mixed by repeated pipetting. Then 0.1 ml was withdrawn and transferred to the second tube and same procedure was followed until all the dilutions (10⁻¹ to 10⁻⁵). Vero cell monolayer was grown in 24 well cell culture plate for 24 hours. 100µl of inoculum from different dilutions were added to all the wells from row 1-5 and row 6 was kept as control. The plate was kept in CO₂ incubator and

maintained. It was monitored for 4-5 days and gradual development of CPE was recorded in the following manner:

- No CPE = (-)
- About 50% of cells exhibiting CPE = (+)
- About 75% of cells exhibiting CPE = (++)
- The monolayer was totally destroyed = (+++)

TCID₅₀ was calculated by Karber's method (Veterinary virology, 1981) as per the following formula:

- $\text{Log}_{10}\text{TCID}_{50} = L - d (s - 0.5)$ (L = Log_{10} of most concentrated virus dilution tested,
- d = Log dilution factor, s = Sum of proportions).

Virus inhibition assays

Cytopathic Effect (CPE) reduction assay

Antiviral activity was determined by reduction of virus titres using TCID₅₀ determinations according to Goncalves *et al.* (2005) [37]. TCID₅₀ was calculated by Karber's method (Veterinary virology, 1981) as per the following formula: $\text{Log}_{10}\text{TCID}_{50} = L - d (s - 0.5)$, (L = Log_{10} of most concentrated virus dilution tested

d = Log dilution factor, s = Sum of proportions).

1. The experiment was carried out in triplicates.
2. The antiviral activity was expressed as percentage of inhibition (PI) using antilogarithm values of TCID₅₀.

PI = 1 (T antilogarithm/ C antilogarithm). The active principle and nanoparticles was considered active when the PI is >80%, at its MNTC.

Effective concentration₅₀ (EC₅₀)

Modified end point assay was followed as described by Berghe *et al.* (1986) for nanocurcumin, nanoeugenol and their combination. TCID₅₀ was calculated by Karber's method (Veterinary virology, 1981) as per the following formula:

$\text{Log}_{10}\text{TCID}_{50} = L - d (s - 0.5)$, (L = Log_{10} of most concentrated virus dilution tested.

d = Log dilution factor, s = Sum of proportions).

Effective concentration reducing CPE by 50% (EC₅₀) in comparison with virus controls was estimated from data plots by plotting different percentage of inhibition, according to Serkedjieva *et al.* (1998) [39] and expressed as the mean of 3 independent experiments. Therapeutic Index (TI) was calculated as the ratio of CC₅₀/EC₅₀.

One step growth curve

To determine the stage at which the extract has effect on the replication of GTPV (Yamasaki and Tayaga, 1980).

PI was calculated with the formula

PI = 1 (T antilogarithm/ C antilogarithm).

Dose-response study

Vero cell monolayer (48 hr old) were washed with EMEM and infected with 10 fold dilution of GTPV at 50µl/well (log 10) with each dilution in triplicates. Then various concentration of extract in 50µl/well were added to each virus dilution in triplicates and appropriate controls were maintained. Reduction in titre in the treated and untreated virus controls was determined.

Result

The present study was conducted to evaluate the antiviral effect of nanocurcumin and nanoeugenol against goatpox in-vitro. The nanocurcumin was prepared from active principle

curcumin by Evaporative precipitation of nanosuspension method, had the particle size of 64.22nm, while original curcumin had the particle size of 4706nm. The nano-eugenol was prepared from active principle eugenol by Ultrasonication and particle size 97.65nm, while original eugenol was 7.27nm.

Acute toxicity studies of Nanocurcumin and Nano-eugenol

The acute oral toxicity potential of nanocurcumin & nano-eugenol was conducted on female mice (300mg/kg and 2000mg/kg doses) did not show any clinical signs of toxicity and mortality for 24 hours. There were no significant changes in the clinical signs, body weights, gross

pathological changes and mortality in the period of study. These results suggested that it is safe.

Maximum non-toxic concentration/dose (MNTC/MNTD)

The maximum non-toxic dose (MNTD) was determined by confirming no visual changes in the Vero cells i.e, granulation of the cell, cell integrity, and morphology and cell death. The Table 1 represents the MNTDs of various treatment groups. The MNTD for Curcumin was found to be 39.06µg/ml, Nano-curcumin was 140.62µg/ml, Eugenol was 78.12µg/ml, Nano-eugenol was 156.25µg/ml and combination of Nanocurcumin and Nano-eugenol was 296.875µg/ml. The MNTDs of the each suspension were used for the antiviral efficacy testing.

Table 1: Maximum Non-Toxic Dose (MNTD) (µg/ml) of various treatment groups

Drug	MNTD (µg/ml)
Curcumin	39.060
Nanocurcumin	140.62
Eugenol	78.120
Nano-eugenol	156.25
Nanocurcumin+ Nano-eugenol (Combination)	296.875

Cytotoxic concentration₅₀ (CC₅₀)

The cell death was determined by rounding, granulations, loss of intra-cellular matrix, clumping and finally detachment of the cells from the surface were the major manifestations. The Cytotoxic Concentration₅₀ (CC₅₀) was determined by measuring the cell viability using trypan blue and the concentration showing 50% of the cell death was considered (Fig. 1 to 5). The Table 2 represents CC₅₀ value

for Curcumin, Nano-curcumin, Eugenol, Nano-eugenol and combination of Nanocurcumin and Nano-eugenol. The CC₅₀ value for Curcumin was found to be 290.4±1.419µg/ml, Nanocurcumin was 1462±1.0620µg/ml, Eugenol was 319.7±1.301µg/ml, nano-eugenol was 432.7±1.1656µg/ml, and the combination of nanocurcumin and nano-eugenol was 1309±1.2887µg/ml.

Table 2: Cytotoxicity Concentration₅₀ values of different treatment groups used for antiviral assay

Drug	Cytotoxicity Concentration ₅₀ (CC ₅₀) (µg/ml)	95% Confidence Interval (CI) (µg/ml)	Goodness of Fit (R ²)	Degrees Of Freedom (df)
Curcumin	290.4±1.419	242.9 to 347.1	0.9629	32
Nanocurcumin	1462±1.0620	986.2 to 2207	0.9657	29
Eugenol	319.7±1.301	252.6 to 404.6	0.9466	29
Nano-eugenol	432.7±1.1656	313.7 to 596.8	0.9072	29
Nanocurcumin+ Nano-eugenol (Combination)	1309±1.2887	1028 to 1666	0.9188	32

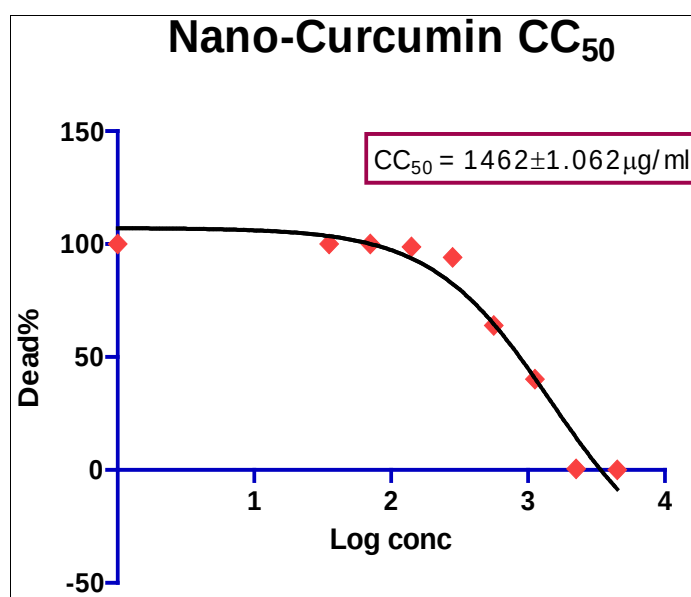
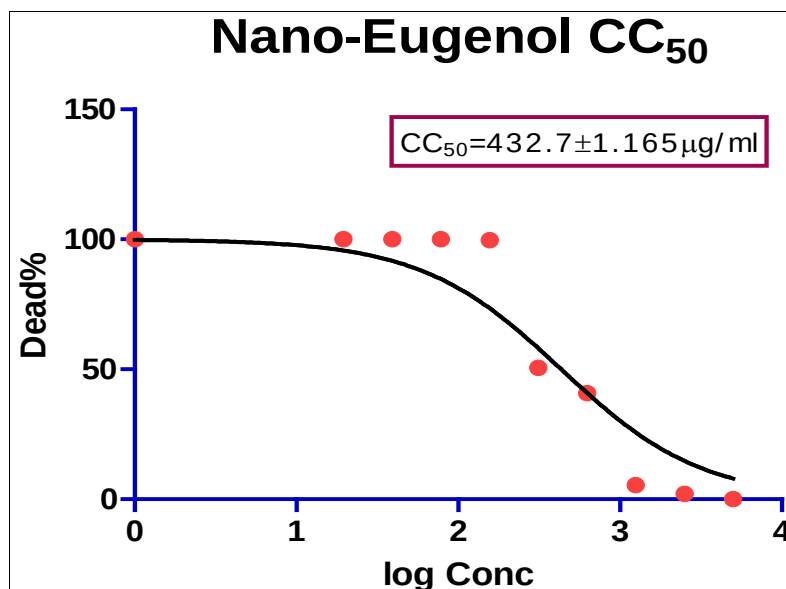
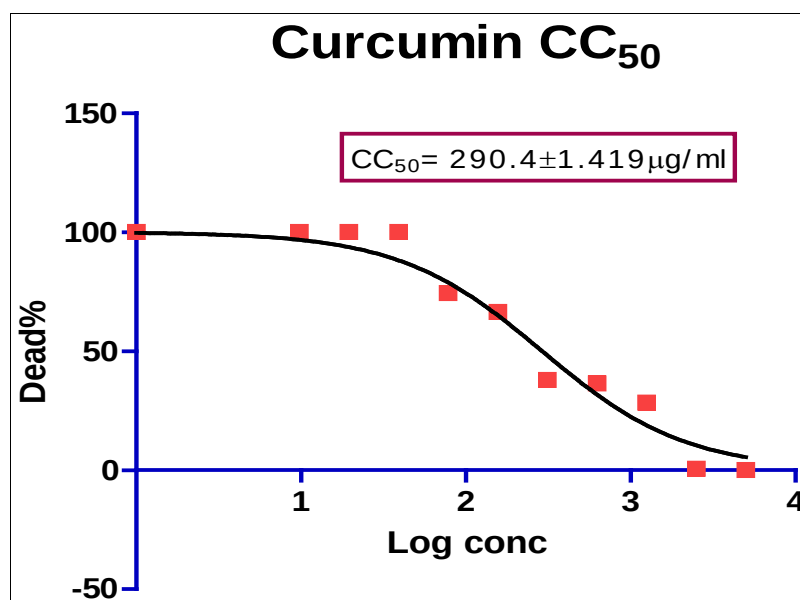
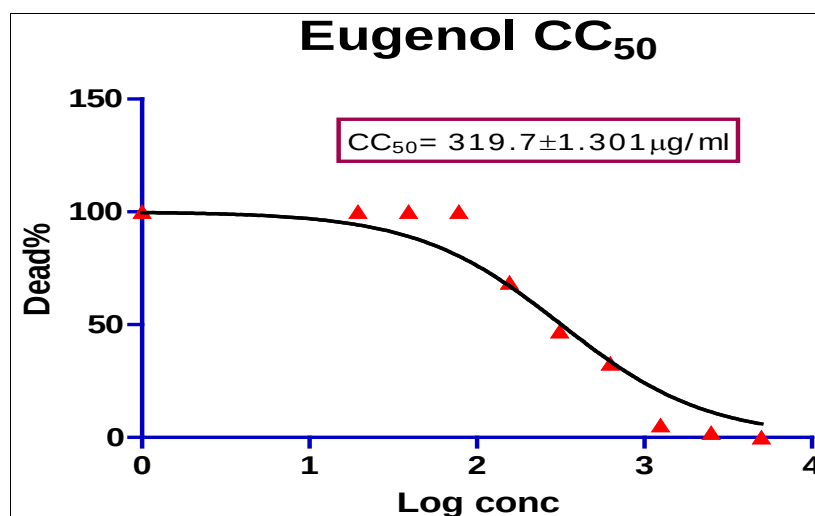


Fig 1: Graph representing the CC₅₀ of nanocurcumin

Fig 2: Graph representing the CC₅₀ of nano EugenolFig 3: Graph representing the CC₅₀ of curcuminFig 4: Graph representing the CC₅₀ of eugenol

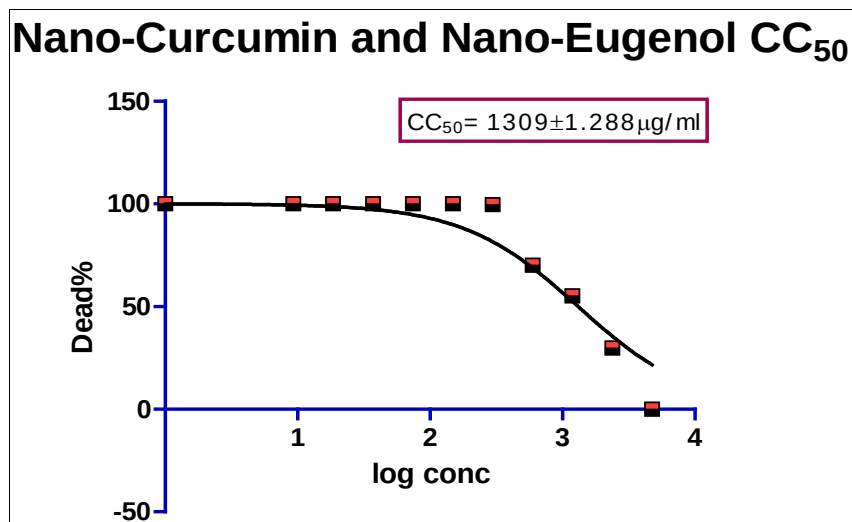


Fig 5: Graph representing the CC_{50} of nanocurcumin+nano Eugenol combination

Tissue culture infective dose₅₀ (TCID₅₀)

The TCID₅₀ for Goatpox virus sample no. 106 was calculated by Spearman-Kärber's method. The CPE was confirmed by rounding of the cells, loss of intracellular

matrix and clumping, and vacuole formation and graded accordingly by the extent of CPE exhibited (Fig. 6). The log TCID₅₀ for this particular sample was 4.75 and this was taken as virus control for the further antiviral studies.

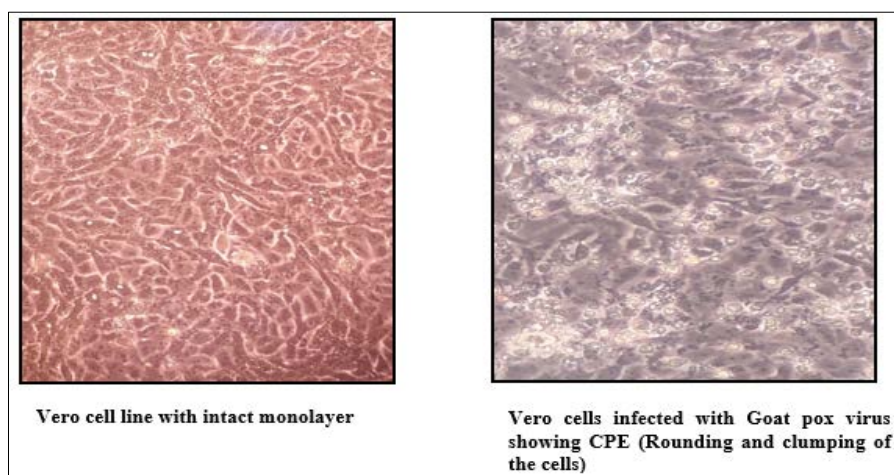


Fig 6: TCID₅₀ for GTPV-106

Table 3: Visual Comparison (VC) of the Proposed Method Across Five Test Cases (10-1 to 10-5)

VC	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
+++	+++	+++	+++	++	--
+++	+++	++	++	+++	++
+++	+++	+++	+++	--	+
+++	+++	++	--	++	++

Abbreviations: VC = Virus Control; +++ = Complete cytopathic effect; ++ = Moderate cytopathic effect; + = Mild cytopathic effect; = No cytopathic effect

Virus inhibition assay / cytopathic effect reduction assay

The MNTDs of each suspension were used for the antiviral efficacy testing. Antiviral activity was determined by reduction of virus titers using antilogarithm of treatment and control TCID₅₀ values as per the protocol. The virus titre in virus control was 4.75 and the reduction in the virus titre was seen when it was treated with curcumin, nanocurcumin, eugenol, nano Eugenol and combination of nanocurcumin and nano Eugenol. The reduction in virus titre with curcumin was 3.5 with 74.88 percentage of inhibition which was significant at $P < 0.05$. The reduction in virus titre with

nanocurcumin was 2.5 with 97.48 percentage of inhibition which was significant at $P < 0.05$. The reduction in virus titre with eugenol was 3 with 79.43 percentage of inhibition which was significant at $P < 0.05$. The reduction in virus titre with nano Eugenol was 1.75 with 99.55 percentage of inhibition which was significant at $P < 0.05$. The reduction in virus titre with combination of nanocurcumin and nano Eugenol was 1.25 with 99.85 percentage of inhibition which was significant at $P < 0.05$ which is represented in Table 3.

Table 4: Antiviral effect of curcumin, nanocurcumin, eugenol, nanoeugenol, and combination of nanocurcumin+nanoeugenol against goatpox (TCID₅₀)

Treatment	Infectivity titer of GTPV (TCID ₅₀ /ml)		Percentage Inhibition (PI)
	Virus control	Virus + Treatment	
Curcumin	4.75	3.5	74.88
Nano-curcumin	4.75	2.5	97.48
Eugenol	4.75	3	79.43
Nano-eugenol	4.75	1.75	99.55
Nano-curcumin + Nano-eugenol	4.75	1.25	99.85

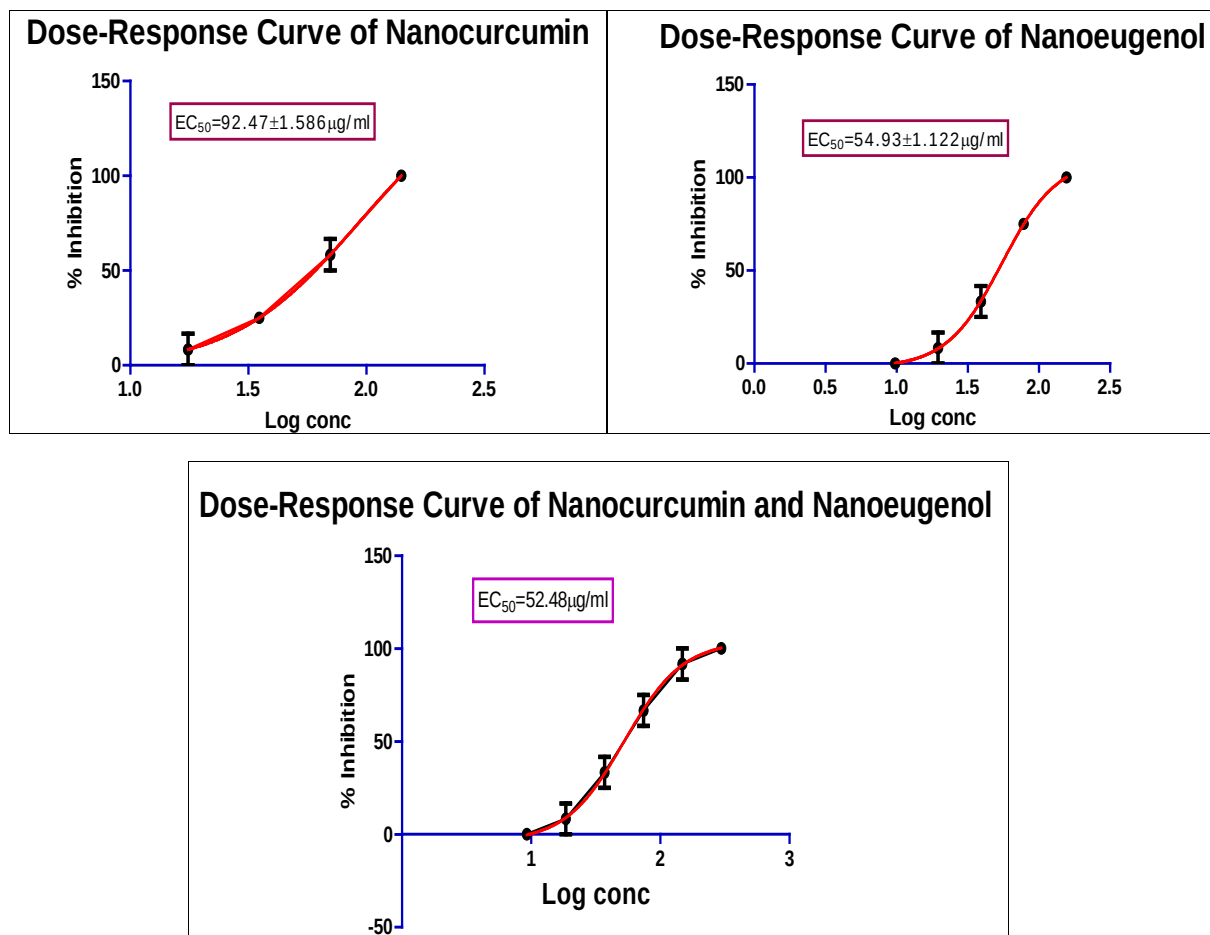
Effective concentration₅₀ (EC₅₀)

The doses used were within and around MNTD of each suspension for the antiviral efficacy testing. The concentration at which the drug inhibited 50% of the viral CPE was considered as EC₅₀ and was determined using Dose-Response Curve. The Table 4 represents the EC₅₀ of

various treatment groups. The EC₅₀ of Nanocurcumin was found to be 92.47±1.586µg/ml, Nanoeugenol was found to be 54.93±1.1220µg/ml and combination of Nano-curcumin+Nano-eugenol was found to be 52.48±1.1481µg/ml (Fig.7).

Table 5: Effective Concentration₅₀ of nanocurcumin, nanoeugenol and combination of nanocurcumin+ nanoeugenol

Treatment	Effective Concentration ₅₀ (EC ₅₀) (µg/ml)	95% Confidence Interval (µg/ml)	Goodness Of Fit (R ²)	Degrees Of Freedom (df)
Nano-curcumin	92.47±1.586	10.91 to 783.6	0.9465	8
Nano-eugenol	54.93±1.1220	40.74 to 74.07	0.9639	11
Nano-curcumin + Nano-eugenol	52.48±1.1481	37.38 to 73.67	0.9419	14

**Fig 7:** Graphs representing EC₅₀ of different treatment groups**Therapeutic index (TI)**

The Table 5 represents the therapeutic index of various treatment groups. The therapeutic index of Nanocurcumin

was found to be 15.81, Nanoeugenol was found to be 7.877, combination of Nanocurcumin+Nanoeugenol was found to be 24.94.

Table 6: Therapeutic indices of different treatment groups

Treatment groups	Therapeutic index
Nanocurcumin	15.81
Nano Eugenol	7.877
Nanocurcumin+Nano Eugenol	24.94

One step growth curve

One step growth curve was conducted to ascertain the stage of viral inhibition by the different treatment groups. The stages of virus multiplication are attachment and adsorption, penetration, un-coating, early protein synthesis, DNA replication, late protein synthesis, assembly and release.

Nanocurcumin

The nanocurcumin was administered at different hours of pre and post-infection to ascertain the stage of viral inhibition. Treatment with nanocurcumin was given pre-infection with goatpox virus and 100% inhibition was seen. Treatment with nanocurcumin at 0th hour post infection, 99.85% inhibition was seen. The rest 24, 48, 72, 120, 144 hour post infection the inhibition was nil.

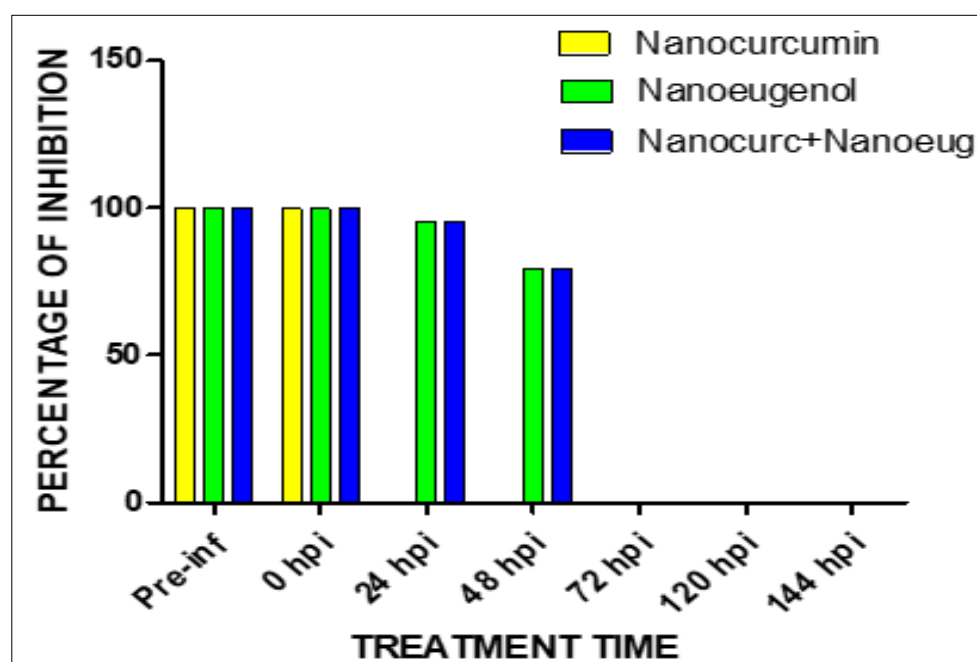
Nano Eugenol

The nano Eugenol was administered at different hours of pre and post-infection to ascertain the stage of viral inhibition. Treatment with nano Eugenol was given pre-infection with goatpox virus and 100% inhibition was seen. Treatment

with nano Eugenol was given in 0th hour post infection and 99.74% inhibition was seen. The 24 h.p.i 98.58% was seen and 48 and 72 h.p.i, 95.53 and 79.431% was seen respectively. No inhibition at 120 and 144 hour post infection.

Combination of Nanocurcumin+Nano Eugenol

The Combination of Nanocurcumin+Nano Eugenol was administered at different hours of pre and post-infection to ascertain the stage of viral inhibition. Treatment with Combination of Nanocurcumin+Nano Eugenol was given pre-infection with goatpox virus and 100% inhibition was seen. Treatment with Combination of Nanocurcumin+Nano Eugenol was given in 0th hour post infection and 100% inhibition was seen. The 24 h.p.i 100% was seen and 48 and 72 h.p.i, 95.53 and 79.431% was seen. No inhibition at 120 and 144 hour post infection. The comparison of all the treatments with their percentage of inhibition at different time of pre and post infection is represented in graph (Fig.8).

**Fig 8:** Graph representing percentage of inhibition of goatpox virus at different hour of pre and post infection**Dose-response curve**

When the dose was increased from the lowest dose which exhibited CPE inhibition to the MNTD, there was a graded increase in the percentage of inhibition (PI) of the Goatpox viral replication.

Nanocurcumin

Percentage Inhibition (PI) was represented in Fig. 9 as 8.58% at 80µg/ml, followed by 79.431% at 100µg/ml, 99.85% at 120µg/ml and 100% at 140µg/ml.

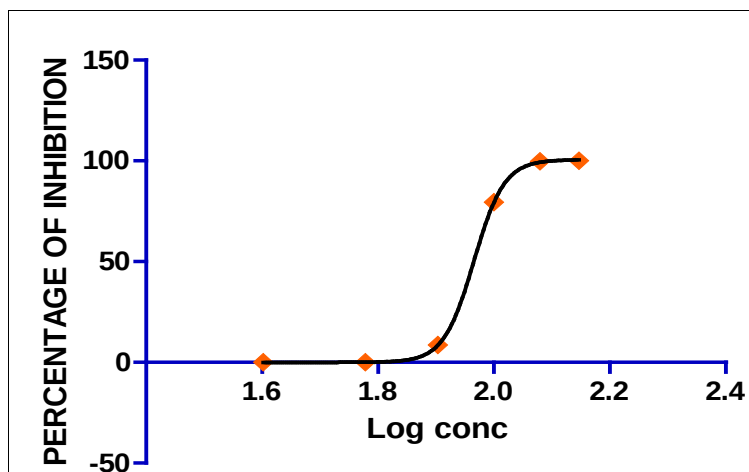


Fig 9: Dose-response curve of nanocurcumin

Nano Eugenol

Percentage Inhibition (PI) was represented in Fig. 10 as 8.58% at 60 μ g/ml, followed by 79.431% at 80 μ g/ml,

98.58% at 100 μ g/ml, 99.85% at 120 μ g/ml and 100% at 140 μ g/ml and 160 μ g/ml.

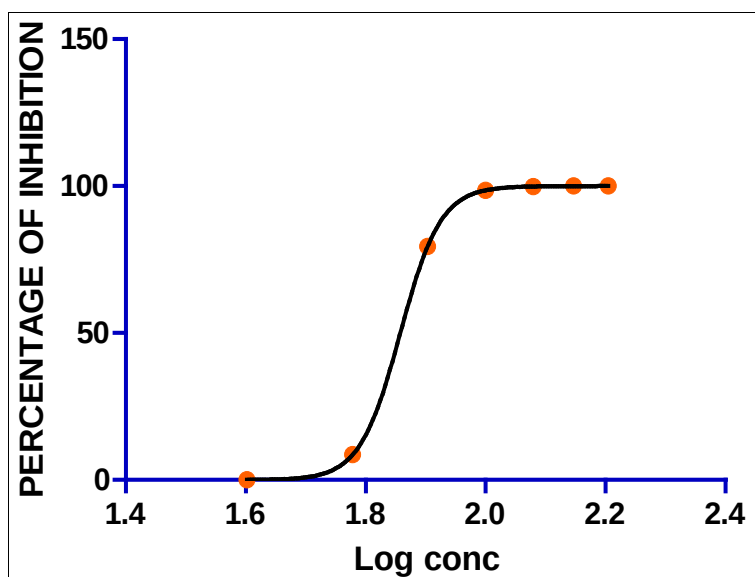


Fig 10: Dose-response curve of nano eugenol

Combination of Nanocurcumin+Nano eugenol

Percentage Inhibition (PI) was represented in Fig.11 as 8.58% at 50 μ g/ml, followed by 79.431% at 100 μ g/ml,

95.53% at 150 μ g/ml, 98.58% at 200 μ g/ml, 99.74% at 250 μ g/ml and 100% at 300 μ g/ml.

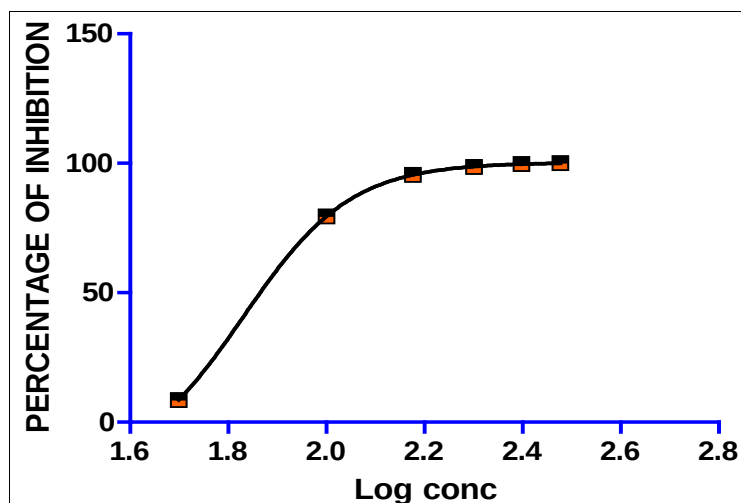


Fig 11: Dose-response curve of nanocurcumin+nano eugenol

Discussion

The present study was conducted to evaluate the anti-viral effect of the curcumin, nanocurcumin, eugenol, nano-eugenol and combination of nanocurcumin+nano-eugenol in-vitro against Goatpox virus in Vero cell lines. The resultant nanocurcumin was prepared by Evaporative Precipitation of Nanosuspensions (EPN) method from the curcumin had the particle size of 64.22nm, while the original curcumin had the particle size of 4706nm and the nano-eugenol was prepared by ultrasonication of eugenol with surfactant Tween-80 had the particle size of 7.27nm, while the original eugenol had the particle size of 97.65nm. The nanocurcumin with various advantages in drug delivery and medicinal values were compared with Siekmann *et al.* (1996) [15] studied nanoparticles by two methods: Antisolvent Precipitation With A Syringe Pump (APSP) and Evaporative Precipitation of Nanosuspension (EPN) and resultant particle size was 330nm and 150nm respectively. Tsai *et al.* (2011) [14] prepared Curcumin-loaded PLGA nanoparticles (C-NPs) by the high-pressure emulsification-solvent evaporation method. The resultant nano-eugenol with better drug delivery and various medicinal values especially antiviral effect were compared with Kamel *et al.* (2015) [16] who prepared eugenol oil emulsion with commercial eugenol (>99%) with non-ionic surfactant (Tween 20) and water by ultrasonication for 10min at 700W and the resultant particle size was in range of 50-110nm (Z-avg:80nm) and shape was spherical. Acute oral toxicity studies were conducted in the mice for nanocurcumin and nano-eugenol at 300mg/kgbw and 2000mg/kgbw. There were no mortality and no change in behaviour and body weight reported in the 14 day study period. The safe nanocurcumin on oral treatment was compared with the studies of Gopi *et al.* (2016) [17] where they conducted acute oral toxicity on curcuminoids and concluded it to be safe. Bisht *et al.* (2007) [18] concluded that the highest dose 2000mg/kgbw and lowest dose 50mg/kgbw of nanocurcumin did not show any signs of toxicity and mortality and it's safe for oral administration. Reddy *et al.*, (2005) [19] conducted acute oral toxicity on mice with nanocurcumin and concluded its safety at 2000mg/kgbw. The safe nano-eugenol on oral treatment was compared with Reddy *et al.*, (2005) [19] found no signs of toxicity, behavioral changes and death when rats were treated with eugenol of doses 200, 600, and 2000 mg/kgbw. The Maximum Non-Toxic Dose (MNTD) was determined by confirming no visual changes in the Vero cells. The calculated MNTD for Curcumin, Nano-curcumin, Eugenol, and Nano-eugenol was found to be 39.06µg/ml, 140.62µg/ml, 78.12µg/ml, 156.25µg/ml respectively, while the combination of Nanocurcumin and Nano-eugenol was 296.875µg/ml. These doses did not produce any morphological changes and cell cytotoxicity in the Vero cells and hence this dose was considered for the further antiviral studies. Use of curcumin, nanocurcumin, eugenol and nano-eugenol against goatpox is very scanty and very feeble work have been done in this regard. Hence our results were compared with Flores *et al.* (2016) [20] who tested curcumin on against Herpes Simplex Virus 1 and 2 in Verocells and MNTC was found to be 30µM and below. Hence the doses below 30µM were chosen for anti-viral studies. Gandapu *et al.*, (2011) [21] described that both curcumin and nano-curcumin were non-toxic in the lower concentration but at 25µM concentration sol-curcumin was

extremely toxic while the nanocurcumin at the same concentration was less toxic significantly. In our present study we have considered the active principles rather than the aqueous or ethanolic extraction of the plants and it is used on a different cell lines. This could be the probable reason for the change in the calculated MNTD values from the range described in the other research. It is proven that curcumin dissolved in ethanol is more toxic to the cells when compared to the nanocurcumin which was prepared by EPN method which was supported by Gandapu *et al.*, (2011) [21] who said curcumin is more toxic on cell lines than nanocurcumin. The same reason can be quoted for the eugenol being more toxic than the nano-eugenol. There are very scanty or no work have been done with the active principle of curcumin and eugenol, and nanocurcumin and nano-eugenol antiviral activity against goatpox. Hence in our present study we found that all the treatments chosen caused very less adverse effects and cell cytotoxicity to the vero cells and can be considered as an antiviral drugs at their particular MNTDs for the further evaluation of the antiviral potency. The Cytotoxic Concentration₅₀ (CC₅₀) was determined by measuring the cell viability using trypan blue and the concentration showing 50% of the cell death was considered. The calculated CC₅₀ values of curcumin and nanocurcumin were compared with Khosropanah *et al.*, (2016) [22] who reported that the proliferation of MDA-MB231 cell line and the overall 50% cell death occurred at 53.18µg/ml and 23.25µg/ml with 48hr treatment for curcumin and nanocurcumin respectively. The IC₅₀ curcumin after 24hr, 48hr and 72hr for MDA-MB231 cell line was 79.58µg/ml, 53.18µg/ml and 30.78 µg/ml, whereas for nanocurcumin was 37.75 µg/ml, 23.25 µg/ml and 12.99 µg/ml respectively. Zandi *et al.* (2010) [23] found the 50% cytotoxic concentration (CC₅₀) of the curcumin, gallium-curcumin and Cu-curcumin on Vero cell line showed to be 484.2µg/mL, 255.8µg/mL and 326.6 µg/mL respectively which was tested for antiviral activity against herpes simplex virus type 1 (HSV-1). Lin *et al.* (2012) [40] demonstrated IC₅₀ levels of nanoencapsulated curcumin ranged from 7.9µM (CT26 cells) to 30µM (A549 cells) and the curcumin resulted in lower IC₅₀ for all the cell lines ranging from 1.0 µM to 1.7 µM. Much wider range of safety was observed with the nanoencapsulated curcumin. In case of curcumin resistant cells, the IC₅₀ of nanoencapsulated curcumin for 2 parental cell lines rose from 8 µM to more than 20 µM. Singh *et al.* (2010) [24] studied di-O-tryptophanylphenylalanine curcumin and di-O-decanoyl curcumin revealed remarkable antiviral activity against VSV and FIPV/FHV with EC₅₀ values of 0.011 µM and 0.029µM, respectively. The calculated CC₅₀ values of eugenol and nano-eugenol were compared with Bhanuprakash *et al.*, 2006b [1] tested *O. sanctum* for its antiviral activity against GTPV in vero celllines and found CC₅₀ value to be 3749±1.2µg/ml (Dye exclusion method) and 3849.7±0.50µg/ml (OD_{540/630}). The MNTDs of the each suspension were used for the antiviral efficacy testing. Antiviral activity was determined by reduction of virus titers using antilogarithm of treatment and control TCID₅₀ values as per the protocol. The virus titre in virus control was 4.75 and the reduction in the virus titre was seen when it was treated with curcumin, nanocurcumin, eugenol, nano-eugenol and combination of nanocurcumin and nano-eugenol and the titre was found to be 3.5, 2.5, 3, 1.75 and 1.25 with 74.88, 97.48, 79.43, 99.55 and 99.85 percentage of inhibition

respectively which was significant at $P < 0.05$. This results were supported by antiviral actions of curcumin, nanocurcumin, eugenol, nanoeugenol and the combination of nanocurcumin and nanoeugenol in various different viruses. Barthelemy *et al.* (1998)^[27] studied Curcumin and its derivatives (reduced curcumin, allyl-curcumin, and tocopheryl-curcumin) revealed 70% to 85% inhibition in Tat protein trans-activation of HIV-1 LTR measured by β -galactosidase activities of HeLa cells which in HIV-1 LTR was fused to the indicator of *lacZ* gene. Tocopheryl-curcumin demonstrated the most inhibition activity with 70% inhibition at 1nM compared to 35% inhibition of curcumin at this concentration. The doses used for the antiviral efficacy testing were within and around MNTD of the each suspension. The concentration at which the drug inhibited 50% of the viral CPE was considered as EC_{50} and was determined using Dose-Response Curve. These results were compared with the antiviral effect and EC_{50} of curcumin and eugenol, since there almost no works on antiviral effect of nanocurcumin and nanoeugenol and their combination. Kutluay *et al.* (2008)^[26] Plaque assay was performed for the anti-viral activity of the curcumin. Curcumin reduced the number of plaques on vero cell monolayer by 3 folds. Mean plaque size after 3 days was 0.64 ± 0.10 mm when compared with untreated 0.73 ± 0.13 mm. *In vitro* replication of HSV-2 could be decreased by curcumin with ED_{50} value of 0.32mg/mL. Therapeutic index is the ratio between cytotoxicity and the inhibitory capacity of the substance (CC_{50}/EC_{50}). These results were compared with Ichsyani *et al.* (2017)^[41] who found the SI of curcumin to be 2.56 when treated for Dengue on Huh7it-1 cells. Padilla *et al.* (2014) found the SI to be 4.8 when used on the Vero cells for antiviral study of curcumin on Dengue virus type-2. The preparations was administered at different hours of pre and post-infection to ascertain the stage of viral inhibition. Treatment with nanocurcumin was given pre-infection with goatpox virus and 100% inhibition was seen. Treatment with nanocurcumin at 0th hour post infection, 99.85% inhibition was seen. The rest 24, 48, 72, 120, 144 hour post infection the inhibition was nil. Curcumin has anti-viral effect, but their biochemical mechanisms of anti-viral effect has been incompletely defined (Li *et al.*, 1993; Sui *et al.*, 1993; Bruke *et al.*, 1995; Mazumder *et al.*, 1995 and Bourne *et al.*, 1999)^[93]. Maximum PI (100% and 99.83%) of GTPV was observed, when the extract was added simultaneously and after 0 hpi. This indicated that the nanocurcumin might have the direct virucidal activity, when it is administered simultaneously and can also inhibit one or more steps of viral replication after the adsorption/entry. Treatment with nanoeugenol was given pre-infection with goatpox virus and 100% inhibition was seen. Treatment with nanoeugenol was given in 0th hour post infection and 99.74% inhibition was seen. The 24 h.p.i 98.53% was seen and 48 and 72 h.p.i, 95.53 and 79.431% was seen respectively. No inhibition at 120 and 144 hour post infection. Bhanuprakash *et al.* (2006a)^[1] studied one step growth curve for *A. Arabica* and *E. jambolana* on vero cells against goatpox virus and found they are virucidal and inhibited the viral replication after entry upto some extent. This indicated that the nanoeugenol might have the direct virucidal activity, when it is administered simultaneously and can also inhibit one or more steps of viral replication after the adsorption/entry upto 72 hours of post infection. Treatment with Combination of Nanocurcumin+Nanoeugenol was given

pre-infection with goatpox virus and 100% inhibition was seen. Treatment with Combination of Nanocurcumin+Nanoeugenol was given in 0th hour post infection and 100% inhibition was seen. The 24 h.p.i 100% was seen and 48 and 72 h.p.i, 95.53 and 79.431% was seen. No inhibition at 120 and 144 hour post infection. This indicated that the nanoeugenol might have the direct virucidal activity, when it is administered simultaneously and can also inhibit one or more steps of viral replication after the adsorption/entry upto 72 hours of post infection. This proves that all our preparations have a potent virucidal activity and can be considered as potent antiviral agent against goatpox virus. There was graded increase in response i.e, Percentage of Inhibition (PI) of virus replication as the concentration of the treatments were increased. Percentage Inhibition (PI) of nanocurcumin was 8.58% at 80 μ g/ml, followed by 79.431% at 100 μ g/ml, 99.85% at 120 μ g/ml and 100% at 140 μ g/ml and nanoeugenol was 8.58% at 60 μ g/ml, followed by 79.431% at 80 μ g/ml, 98.58% at 100 μ g/ml, 99.85% at 120 μ g/ml and 100% at 140 μ g/ml and 160 μ g/ml, while the combination of nanocurcumin and nanoeugenol was 8.58% at 50 μ g/ml, followed by 79.431% at 100 μ g/ml, 95.53% at 150 μ g/ml, 98.58% at 200 μ g/ml, 99.74% at 250 μ g/ml and 100% at 300 μ g/ml. Such graded increase in dose response of all the treatments were supported with the reports by Mokbel *et al.* (2017) who tried different concentration of EON (125, 250, 500 μ g/ml) on banana bunchy top virus, which inhibited the virus in graded response (94.81%, 95.5% and 96.66% respectively). PI was 36.9 at 31.25 μ g/ml, 60.1 at 62.5 μ g/ml, 84.2 at 125 μ g/ml, 96.9 at 250 μ g/ml, 93.9 at 500/1000 μ g/ml and 99.68 at 2000 μ g/ml for *E. jambolana* against goatpox was reported by Bhanuprakash *et al.* (2006a)^[1]. In the present study, CPE assays indicated the potential inhibition of the goatpox virus replication by all the preparations. The dose response study as anticipated, signaled that the higher concentration has more inhibitory effects than the lower ones.

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Conflict of interest

The author declared no conflict of interest.

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Author's contribution

All the authors read and approved the final manuscript.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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