Journal of Clinical Pharmacology and Therapeutics

ISSN: 2766-8347

Research Article

Evalution of Antiviral Activity of Combination of Nanocurcumin and Nanoeugenol against Goat Pox

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Abstract

Curcumin, Eugenol, Nanocurcumin, Nanoeugenol and their combinations were known for their medicinal values especially their anti-viral effects against various viruses. Nanocurcumin and Nanoeugenol prepared by Evaporative Precipitation of Nanosuspension (EPN) and ultrasonication method had Z-average particle size of 64.22 nm and 7.270 nm, respectively which was found to be physically stable without any phase separation. The nano-ointment was prepared by the combination of nano curcumin and nanoeugenol and applied on skin lesions of positive goat pox cases which resulted in better and faster healing. Results indicated that combination of nanocurcumin and nanoeugenol had the highest antiviral effect with greater therapeutic index.

Keywords: Nanocurcumin; Nanoeugenol; Goatpox

Introduction

Goatpox and Sheeppox are systemic infectious disease, highly contagious, OIE (World Organisation for Animal Health) notifiable and economically important trans boundary viral diseases of goat and sheep, respectively, caused by Goatpox Virus (GTPV), Sheeppox Virus (SPPV) and Lumpy Skin Disease Virus (LSDV) of the genus *Capripoxvirus*, sub-family Chordopoxvirinae of family Poxviridae [1,2]. Both the diseases are posing significant economic threat globally and more particularly in developing countries like India. Capripox Viruses (CaPVs) usually have no host preference as they infect both sheep and goats [3]. However, sheeppox and goatpox viruses are considered as different entity in India [4,5] and recently confirmed in other countries [6]. SPPV cause severe disease in sheep and relatively mild disease in goats, just opposite behaviour noticed with GTPV but some strains produce equally severe disease in both the species [7-9]. In India, first reported case was in 1934 [10]. In India, goatpox is enzootic like other pox viral infections and regular outbreaks have been reported from most of the states [11]. In Assam and other North Eastern States, recent outbreaks of goatpox from different parts with high morbidity and mortality were recorded for the first time, indicating the emerging nature of the disease in this part of the country (Unpublished data, Department of Veterinary Microbiology, College of Veterinary Science, Khanapara, A.A.U. 2016). The disease has been reported for the first time in Assam and as such, no systematic vaccination policies and treatment strategies are being followed so far against this disease. 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* [12], which is cultivated extensively in south and south-east tropical Asia.

Eugenol is a phytogenic bioactive component is frequently found in diversified herbal plants possessing well defined functional attributes. The main principle mechanistic approaches associated with the therapeutic potential of eugenol include its free radical scavenging activity, hindrance of reactive oxygen species' generation, preventing the production of reactive nitrogen species, enhancement of cyto-antioxidant potential and disruption of microbial DNA and proteins [13]. The normal form of curcumin and eugenol is rapidly eliminated from the body and its organ penetration property is very low [14]. Reported that the nano form of curcumin and eugenol have significantly higher penetration and distribution rate. Hence, aim of this present study was to evaluation of antiviral activity of nanocurcumin and nanoeugenol combination against goatpox.

Materials and Methods

Preparation of nanocurcumin

Evaporative precipitation of nanosuspension: Nanocurcumin was as per the method described by [15], The solution of original curcumin was prepared in ethanol and then a nanosuspension was formed by adding hexane (antisolvent). Drug particles in the nanosuspension were obtained by quick evaporation of the solvent and antisolvent, under vacuum using a rotary evaporator. This was followed by vacuum drying of the nanoparticles to completely evaporate all the solvents. The drug concentrations used were 5 mg/mL, 10 mg/mL, 15 mg/mL and the Solvent to Antisolvent (SAS) ratios were varied from 1:10, 1:15 and 1:20 (v/v). For 20 ml of the drug solution, 200 ml-400 ml hexane was used.

Preparation of nanoeugenol

Ultrasonication with little modifications: Nanoeugenol was prepared using the method described by Kamel, et al. 2014 [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

Citation: Namitha A, Sarma J, Debnath S, Hazarika A. Evalution of Antiviral Activity of Combination of Nanocurcumin and Nanoeugenol against Goat Pox. J Clin Pharmacol Ther. 2023;4(2):1044.

Publisher Name: Medtext Publications LLC

Manuscript compiled: May 04th, 2023

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by Zetasizer (IASST, Guwahati).

Stability of nanosuspension

The stability analysis was performed for both nanocurcumin and nanoeugenol by centrifuging the 10% nanoemulsion at 3,500 rpm for 30 min. The stability was further checked at both refrigerator temperature (4°C) and room temperature (25°C). Three freeze-thaw cycles were also checked. Stability assay was also performed by storage for 3 months.

Preparation of nano-ointment with combination of nanocurcumin and nanoeugenol

The concentration of the nanocurcumin and nanoeugenol for the ointment was selected according to the *in-vitro* studies where 100% virus growth was inhibited. Ointment was prepared according to the standard protocol. The 99.09 g of petroleum base was taken of the ointment slab. 0.281 g of nanocurcumin and 0.625 g of Nanoeugenol was added to it and properly mixed with the ointment spatula. The resultant was properly bottled in sterile containers for dispatch.

Experimental protocol and procedure

Animals: The study was performed in accordance with the guidelines for the use and care of laboratory animals by Institutional Animal Ethical Committee (Approval No. 770/GO/Re/S/03/CPCSEA/FVSc/AAU/IAEC/18-19/693). A total of 54 numbers of Wistar albino rats and mice weighing 120 g-150 g and 25 g-30 g 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°C-25°C).

Acute dermal irritation test

Acute dermal irritation test was conducted on the albino rats to test the dermal irritation of the combination of nanocurcumin and nanoeugenol ointment preparation with 10 days of induction period and 25 days of challenge dose as per OECD guide line no. 406. The combination of nanocurcumin and nanoeugenol ointment was applied on the dorsal shaved part and responses were scored "0" based on the observation of the area at 60 min, 24, 48, 72 hours from the application and continued to observe up to 10 days (induction period).

Result

The present study was conducted to evaluate the antiviral effect of nanocurcumin and nanoeugenol combination against goatpox. The nanocurcumin was prepared from active principle curcumin as per the protocol. The nanocurcumin solution prepared with the resultant was turbid and pale yellow in colour. The nanocurcumin was dispersed in the aqueous phase (sterile distilled water) in 1:100 ratios (10% solution). The particle size was analyzed by particle size analyzer (Zeta-sizer). The Z-average of Curcumin was estimated to be 4706 nm. The Z-average of nanocurcumin was estimated to be 64.22 nm. The size of the nanocurcumin was found to be physically stable after centrifugation at 10,000 g for 30 min and kept at room temperature for 1 month. Nanosuspension was kinetically stable with no phase separation. This shows that the formulated nanocurcumin survived the stability tests. The nanoeugenol was prepared from active principle eugenol as per the protocol. The nanoeugenol solution prepared with Tween 80 base was turbid and milk white in colour. The nanoeugenol was dissolved with solvent Tween 80 in 1:1 ratio and dispersed in the aqueous phase (sterile distilled water) (10% solution). The particle size was analyzed by particle size analyzer (Zeta-sizer). The Z-average nanoeugenol particle size was 97.65 nm and the Z-average of Eugenol was 7.27 nm. The size of the nanoeugenol was found to be physically stable after centrifugation at 10,000 g for 30 min and kept at room temperature for 1 month. Nanosuspension was kinetically stable with no phase separation of oil and water. This shows that the formulated nanosuspension survived the stability tests. Acute dermal irritation test was conducted on the albino rats to test the dermal irritation of the combination of nanocurcumin and nanoeugenol ointment preparation with 10 days of induction period and 25 days of challenge dose as per OECD guide line no. 406. The combination of nanocurcumin and nanoeugenol ointment was applied on the dorsal shaved part and responses were scored "0" based on the observation of the area at 60 min, 24, 48, 72 hours from the application and continued to observe up to 10 days (induction period). There was no eschar or erythema and no edema in the study period. Challenge dose was given till 25 days, still no erythema and edema was observed.

The clinical cases of goatpox were taken in the Eastern India to study the antiviral potency of the nano-ointment preparations. The clinical cases were confirmed as goatpox by the history and clinical signs exhibited as per the different literatures. Total of 12 cases were taken from different districts of Assam and Orissa. Out of which 2 were kept as control (without treatment) and 10 positive cases were taken for treatment with combination nano-ointment.

The combination nano-ointments prepared was used for the treatment of the goatpox topically on daily basis till the lesions are healed. Based on the type of lesion the healing period differed as represented in Table 1 (Figure 1-4).

Discussion

The present study was conducted to evaluate the anti-viral effect of the nanocurcumin and nanoeugenol against Goatpox virus. The resultant nanocurcumin was prepared by Evaporative Precipitation of Nanosuspensions (EPN) method from the curcumin had the particle size of 64.22 nm, while the original curcumin had the particle size of 4706 nm and the nanoeugenol was prepared by ultrasonication of eugenol with surfactant Tween-80 had the particle size of 7.27 nm, while the original eugenol had the particle size of 97.65 nm. The nanocurcumin with various advantages in drug delivery and medicinal values were compared with Kakran et al. [15] who studied nanoparticles by two methods: Antisolvent Precipitation with a Syringe Pump (APSP) and Evaporative Precipitation of Nanosuspension (EPN) and resultant particle size was 330 nm and 150 nm respectively. Tsai et al. [14] prepared Curcumin-loaded PLGA Nanoparticles (C-NPs) by the high-pressure emulsification-solvent evaporation method. The resultant nanoeugenol with better drug delivery and various medicinal values especially antiviral effect were compared with Kamel et al. [16] who prepared eugenol oil emulsion with commercial eugenol (>99%) with non-ionic surfactant (Tween 20) and water by ultrasonication for 10 min at 700 W and the resultant particle size was in range of 50 nm-110 nm (Z-avg:80 nm) and shape was spherical.

Acute dermal irritation test

The nanocurcumin, nanoeugenol and combination of nanocurcumin and nanoeugenol ointment was applied on the dorsal shaved part and responses were scored "0" based on the observation

Table 1: Treatment with the Combination Nano-Ointment on Positive Clinical Cases.

Positive Cases	Before Treatment	After Treatment
CONTROL	Pox lesions in the ears and around the eyes – Day 3Development of pox lesion in the untreated animal on Day 10	-NA-
CASE-1	Presence of papules in the ears and around the eye with mucopurulent nasal discharges	Healing of the papules in the treated animal with combination of nano curcumin and nan-eugenol ointment - 10days post treatment
CASE-2	Presence of papules all over the body in the infected animal.	Healing of the papules in the treated animal with combination ointment 18 days post treatment
CASE-3	Ulceration in the perianal area of the infected animal	Healing of the ulcers in the animals treated with combination of nano curcumin and nan-eugenol 15 days post treatment.

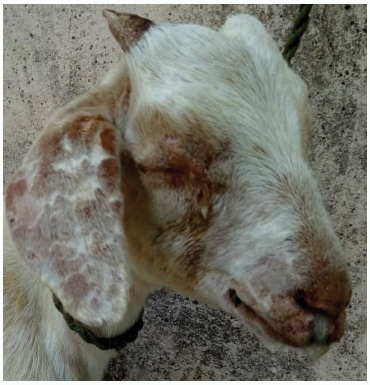

Figure 1: Presence of papules in the ears and around the eye with mucopurulent nasal discharges.

Figure 2: Healing of the papules in the treated animal with combination of nano curcumin and nan-eugenol ointment.

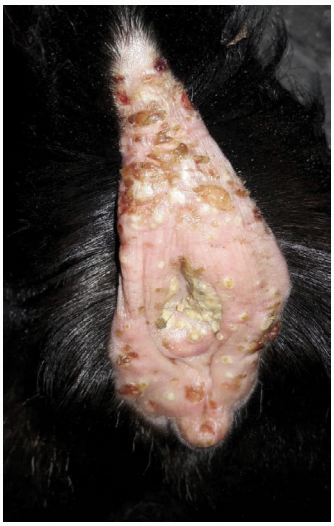

Figure 3: Ulceration in the perianal area of the infected animal.

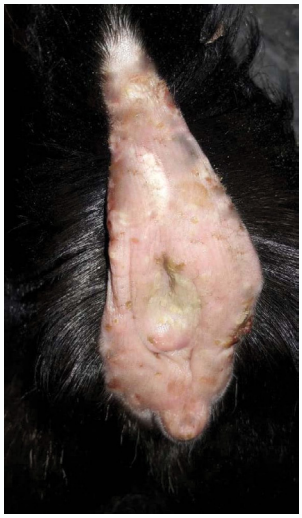

Figure 4: Healing of the ulcers in the animals treated with combination of nanocurcumin and nanoeugenol 15 days post treatment.

of the area at 60 min, 24, 48, 72 hours from the application and continued to observe up to 14 days (induction period). There was no eschar or erythema and no edema in the study period. This protocol was supported by Marzulli and Maibach [17] who tested nano-ointments of some herbal medicines for their stability and dermal irritation [18]. Tested some nano and microparticulate chitosan-based systems for topical antiviral drug delivery. McKeough et al. [19] did comparision of new topical treatments for herpes labialis: efficacy of penciclovir cream, acyclovir cream, and n-docosanol cream against experimentally induced herpes infection. On dermal test it was slightly irritant to the guinea pigs. The nano-ointment preparation did not cause any irritation and was considered safe for topical application of the ointment on the skin.

Clinical cases treated by nano-ointment preparation

The clinical cases of goatpox were taken in the Eastern India to study the antiviral potency of the nano-ointment preparations. The clinical cases were confirmed as goatpox by the history and clinical signs exhibited as per the different literatures. Total of 12 positive cases were taken from different districts of Assam and Orissa. Out of which 2 were kept as control (without treatment) and 10 positive cases were taken for treatment with combination nano-ointment. The combination nano-ointments prepared was used for the treatment of the goatpox topically on daily basis till the lesions are healed. Based on the type of lesion the healing period differed. This results were supported by the studies of Ungphaiboon et al. [20] who did clinical trial of clear liquid soap containing 0.5% w/v ethanol extract of *C. longa* rhizome on HIV patients reduced the wound infections and 100% decrease in itching symptom and it also affected the abscess to convert to dryness scabs (78.6%) within 2 weeks. Topical application

of eugenol delayed the development of herpes virus induced keratitis in mouse model [21]. Since the goatpox virus is epitheliotropic virus, no much systemic adverse effects and the goatpox is usually complicated by secondary bacterial infection and maggot infestation, we ought to select topical application of the treatment in the form of ointment. This resulted in very good healing of the lesions with faster rates when compared to the untreated cases. Hence we could say that our preparation showed better efficacy in the goatpox treatment.

Conclusion

Nanocurcumin and Nanoeugenol was found to be safer for topical application. Nanocurcumin, Nanoeugenol and their combination was found to be safer than the curcumin and eugenol. The Nano-ointment preparation showed faster healing in clinical cases as compared to control group.

Acknowledgement

We are thankful to Dept of Pharmacology & Toxicology, College of Veterinary Science, Assam Agricultural University, and Khanapara for providing facility for carrying out research work.

Funding

This work was funded by College of Veterinary Science, Assam Agricultural University, Khanapara.

Animal Welfare and Ethics Statement

The animal experimentation was carried out according to the Committee for the Purpose of Control and Supervision of experimental animals (CPCSEA) guideline and Institutional Animal Ethical Committee Approved all the procedure for investing experimental pain in conscious animals.

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