Phytomedicine 21 (2014) 159–163

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Production of high titre antibody response against Russell's viper venom in mice immunized with ethanolic extract of fruits of *Piper longum* L. (Piperaceae) and piperine

P.A. Shenoy[a,*], S.S. Nipate[a,**], J.M. Sonpetkar[a], N.C. Salvi[b], A.B. Waghmare[b], P.D. Chaudhari[a]

[a] *Department of Pharmacology, Progressive Education Society's Modern College of Pharmacy, Sector No. 21, Yamunanagar, Nigdi, Pune 411044, Maharashtra, India*

[b] *Haffkine Bio-Pharmaceutical Corporation Ltd., Pimpri, Pune 411018, Maharashtra, India*

ARTICLE INFO

Article history:
Received 19 May 2013
Received in revised form 7 July 2013
Accepted 9 August 2013

Keywords:
Russell's viper
Doboia russelii
Piper longum
Piperine
Antivenom

ABSTRACT

Piper longum L. fruits have been traditionally used against snakebites in north-eastern and southern region of India. The aim of the study was to assess the production of antibody response against Russell's viper venom in mice after prophylactic immunization with ethanolic extract of fruits of *Piper longum* L. and piperine. The mice sera were tested for the presence of antibodies against Russell's viper venom by *in vitro* lethality neutralization assay and *in vivo* lethality neutralization assay. Polyvalent anti-snake venom serum (antivenom) manufactured by Haffkine Bio-Pharmaceutical Corporation Ltd. was used as standard. Further confirmation of presence of antibodies against the venom in sera of mice immunized with PLE and piperine was done using indirect enzyme-linked immunosorbent assay (ELISA) and double immunodiffusion test. Treatment with PLE-treated mice serum and piperine-treated mice serum was found to inhibit the lethal action of venom both in the *in vitro* lethality neutralization assay and *in vivo* lethality neutralization assay. ELISA testing indicated that there were significantly high ($p < 0.01$) levels of cross reactions between the PLE and piperine treated mice serum and the venom antigens. In double immunodiffusion test, a white band was observed between the two wells of antigen and antibodies for both the PLE-treated and piperine-treated mice serum. Thus it can be concluded that immunization with ethanolic extract of fruits of *Piper longum* and piperine produced a high titre antibody response against Russell's viper venom in mice. The antibodies against PLE and piperine could be useful in antivenom therapy of Russell's viper bites. PLE and piperine may also have a potential interest in view of the development of antivenom formulations used as antidote against snake bites.

Introduction

Occurrence of injury and lethality due to bites of venomous snake has been a major sociomedical problem throughout the world. Envenomation caused by snakes leads to many pathophysiological alterations like haemorrhage, necrosis, nephrotoxicity, cardiotoxicity, inflammation and death (Theakston and Reid 1983). Russell's viper (*Doboia russelii*, Viperidae) snake is considered to be in the category of highest medical importance in India as it is a highly venomous snake which is commonly widespread and causes numerous bites resulting in high levels of morbidity, disability or mortality (World Health Organization 2010). Presently the conventional polyvalent anti-snake venom serum (antivenom) is the only available medical antidote for the treatment of snake bites. However, it does not provide sufficient protection to combat with venom induced local effects, and often leads to precipitation of adverse hypersensitivity reactions, because they are large protein molecules (Thwin et al. 2010). In our laboratory, we have made consistent efforts to study the immunopotentiation capacity and safety of various adjuvants used in the production of safe and potent antivenom considering the importance of antivenom in victims of envenomation (Waghmare et al. 2009). In our laboratory, we have also been standardizing the techniques by which the antibodies in general can be quantified during their production (Salvi et al. 2010). Number of plants has been recommended in Indian traditional medicine to be beneficial in treatment of snake bites

Abbreviations: Antivenom, conventional polyvalent anti-snake venom serum; ELISA, enzyme-linked immunosorbent assay; i.p., intraperitoneal; LD_{50}, median lethal dose; PBST, phosphate buffered saline containing 0.1% Tween 80; PLE, ethanolic extract of fruits of *Piper longum* L. (Piperaceae); TMB, 3,3′,5,5′-tetramethylbenzidine.

* Corresponding author. Tel.: +91 9764221010.
** Corresponding author. Tel.: +91 9421061097.
E-mail addresses: priyank.shenoy@rediffmail.com (P.A. Shenoy), sonynipate@rediffmail.com (S.S. Nipate).

0944-7113/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.phymed.2013.08.017

(Nadkarni 1976; Kapoor 2005). Many plant species have also been proved to possess good anti-snake venom properties (Dey and De 2012; Machiah et al. 2006; Da Silva et al. 2012; Samy et al. 2012; Tan et al. 2009). Such herbal antagonists can play an important role, along with antivenom, in neutralizing snake venom induced toxicities (Gomes et al. 2007). Herbal drugs have not only been proved to possess neutralizing abilities against snake venom but also that these herbal constituents may generate antibodies which may cross react with venom components (Meenatchisundaram and Michael 2010; Tan et al. 2009; Aguiyi et al. 1999). The ancient usage of seeds of *Mucuna pruriens* (Family: Farbacea) in the Plateau State, Nigeria, as an oral prophylactic anti-snake venom drug in traditional medicine has also been confirmed scientifically (Aguiyi et al. 1999, 2001).

Piper longum L. (Piperaceae) is a herbal drug, traditionally known in India as "Pippali", which is very popular for its traditional uses in menstrual pain, gonorrhoea, sleeping problems, respiratory tract infections, tuberculosis and arthritis. The diuretic, analgesic, anxiolytic, muscle relaxing effects, immunomodulatory and antitumor effects of the drug are also well known (Sunila and Kuttan 2005). *Piper longum* is traditionally known for its use in actual snakebites in north-eastern and southern region of India (Nadkarni 1976; Kapoor 2005). In our own previous study, we have found that ethanolic extract of fruits of *Piper longum* L. (Piperaceae) possesses good anti-snake venom activities against Russell's viper venom and we have also characterized piperine as the active chemical compound responsible for the anti-snake venom activity of the extract (Shenoy et al. 2013). The objective of the present study was to assess the production of antibody response against Russell's viper venom in mice after prophylactic immunization with ethanolic extract of fruits of *Piper longum* (PLE) and piperine.

Materials and methods

Snake venom

Russell's viper venom was obtained from in-house snake farm of Haffkine Bio-Pharmaceutical Corporation Ltd., Pune, India and was preserved at 4 °C. The venom was dissolved in saline before use and subjected to centrifugation at 2000 rpm for 10 min. The above supernatant was used for different immunological studies. All the venom concentrations were expressed in terms of dry weight.

Plant material

Dried fruits of *Piper longum* were obtained commercially from M/s. Gopal Govind Lokhande (Dealers in Ayurvedic and Unani medicines), Pune, India. The specimen was authenticated by P.G. Diwakar, Joint Director, Botanical Survey of India, Western Regional Centre, Pune, Maharashtra, India and a voucher specimen (V.NO.PASPIL1) was deposited.

Animals

Animals were purchased from National Institute of Biosciences, Pune, Maharashtra, India. Healthy Swiss albino mice weighing about 18–22 g were used for the present study. The animals were housed in well ventilated cages, maintained under standard conditions of light, temperature and humidity (12 h light and 12 h dark cycle; 25 ± 3 °C; 35–60% humidity) and were fed with standard diet and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) and the permitted species of animals involved in the research were handled and experimented in strict accordance with guidelines and procedures for animal experimentation as prescribed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Preparation of ethanolic extract of fruits of Piper longum L. (PLE)

Piper longum fruits were chopped, powdered and macerated three times with 99.97% ethanol at 50–60 °C for 30 min and then filtered. The filtrate was collected and evaporated to obtain a residue. PLE thus obtained was subjected to phytochemical analysis (Pithayanukul et al. 2005).

Drugs and treatment

For administration in mice, PLE was resuspended with 2% gum acacia in 0.9% saline (Sunila and Kuttan 2005). Pure piperine was commercially obtained from Yucca Enterprises, Dombivli, Maharashtra, India. It was dissolved in rice bran oil for administration in mice (Bhutani et al. 2009). Polyvalent anti-snake venom serum (standard antivenom) manufactured by Haffkine Bio-Pharmaceutical Corporation Ltd. was administered to animals by intraperitoneal (i.p.) route at concentrations in accordance with its label claim of neutralization capacity after adjusting volume with water for injection if required (Shirwaikar et al. 2004). The label claim of neutralization capacity of standard antivenom is that 1 ml of reconstituted antivenom neutralizes 0.6 mg of Russell's viper venom. The selection of dose of PLE and piperine administered in experimental animals was based on the safety data of these drugs obtained from toxicity studies conducted by us in our own previous study (Shenoy et al. 2013).

Determination of median lethal dose (LD_{50}) of venom

Lethality of venom in mice was studied by i.p. administration of different concentrations of venom to groups ($n = 6$) of mice. The LD_{50} of venom was then calculated by analysis of deaths occurring within 24 h of venom injection (Theakston and Reid 1983).

Immunization protocol

Group of mice ($n = 6$) was subcutaneously injected with 750 mg/kg PLE. Fourteen days later mice were re-injected with half of the initial dose (375 mg/kg). On day 30 mice were bled and after coagulation and centrifugation, the sera (PLE-treated mice serum) were collected and preserved at −20 °C. Similar protocol was followed for immunization of mice with pure piperine, using initial dose of 4.5 mg/kg and subsequent half dose of 2.25 mg/kg to obtain piperine-treated mice serum. Sera of the mice not immunized with any drug (untreated-mice serum) were used as negative control in experiments (Aguiyi et al. 1999).

In vitro lethality neutralization assay

Four groups of mice ($n = 6$) were used for the assay. First group was injected i.p. with a mixture of 0.5 ml venom solution and 0.5 ml PLE-treated mice serum incubated at 37 °C for 1 h. Second group was injected i.p. with a mixture of 0.5 ml venom solution and 0.5 ml piperine-treated mice serum incubated at 37 °C for 1 h. Third group was injected i.p. with a mixture of 0.5 ml venom solution and 0.5 ml untreated mice serum (negative control) incubated at 37 °C for 1 h. Fourth group was injected i.p. with a mixture of 0.5 ml venom solution and standard antivenom (positive control) incubated at 37 °C for 1 h. In all the four groups, the mixture of venom and serum/antivenom administered was such that it incorporated LD_{50} of venom. Dead mice were counted after 24 h (Aguiyi et al. 1999; Tan et al. 2009).