Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Ethnopharmacology 97 (2005) 527–533
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Inhibition of *Naja kaouthia* venom activities by plant polyphenols

Pimolpan Pithayanukul[a,*], Pakatip Ruenraroengsak[a], Rapepol Bavovada[b], Narumol Pakmanee[c], Rutt Suttisri[b], Suwipa Saen-oon[d]

[a] *Faculty of Pharmacy, Mahidol University, Bangkok, Thailand*
[b] *Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand*
[c] *Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand*
[d] *Faculty of Science, Chulalongkorn University, Bangkok, Thailand*

Received 15 July 2004; received in revised form 26 November 2004; accepted 17 December 2004

Abstract

Plant polyphenols from the aqueous extracts of *Pentace burmanica*, *Pithecellobium dulce*, *Areca catechu* and *Quercus infectoria* were tested for their inhibitory activities against *Naja kaouthia* (NK) venom by in vitro neutralization method. The first three extracts could completely inhibit the lethality of the venom at 4 LD_{50} concentration and the venom necrotizing activity at the minimum necrotizing dose while also inhibited up to 90% of the acetylcholinesterase activity of NK venom at much lower tannin concentrations than that of *Quercus infectoria*. The ED_{50} of plant tannins in inhibiting NK venom activities varied according to condensed tannins and their content in the extracts. Molecular docking of the complexes between α-cobratoxin and either hydrolysable or condensed tannins at their lowest energetic conformations were proposed. The anti-venom activities of these plant polyphenols by selectively blocking the nicotinic acetylcholine receptor and non-selectively by precipitation of the venom proteins were suggested.

Keywords: Plant polyphenols; Tannins; Anti-snake venom; *Naja kaouthia* venom; α-Cobratoxin; Nicotinic acetylcholine receptor

1. Introduction

Application of medicinal plants with anti-snake venom activities might be useful as first aid treatment for victims of snake bite (Pithayanukul et al., 2004). This is of particular importance especially in local areas where antivenin is not readily available. Tannin is one snake venom antidote found widely distributed in the plant kingdom (Ribereau-Gayon, 1972; Haslam, 1989; Okuda et al., 1991). However, there was no previous report on its use against snake bite in Thailand. Tannin has been used for several medicinal purposes, i.e., as astringent, wound healing aid, anti-burns and anti-inflammation. The presence of tannin produces tannin-protein complex under which the natural healing processes can occur, loss of fluid is reduced and external toxins are prevented from re-absorption (Haslam, 1989). Tannins from plants have been shown to interact with snake enzyme systems. For example, aqueous extract of the dried roots of *Mimosa pudica* displayed significant inhibitory effect on the lethality, myotoxicity and enzyme activities of *Naja kaouthia* venom (Mahanta and Mukherjee, 2001). The protective effect of the extract of *Mucuna pruriens* root against *Echis carinatus* venom was demonstrated in vivo (Guerranti et al., 2001). Tannin from the extract of young persimmon fruit, *Diospyros kaki*, has a strong detoxifying effect on the venoms of several snake species, as well as on a number of bacterial toxins (Okonogi and Hattori, 1978). It also inhibited the swelling produced in the feet of mice by *Laticauda semifasciata* venom and improved the survival rate of mice injected with the venoms of this snake and *Trimeresurus flavoviridis*

Abbreviations: AC, areca catechu L; MND, minimum necrotizing dose; NK, *Naja kaouthia* venom; PB, *Pentace burmanica* kurz; PD, *Pithecellobium dulce* Benth; QI, *Quercus infectoria* Olivier

* Corresponding author. Tel.: +66 2 6448694; fax: +66 2 6900756.
E-mail addresses: pypph@hotmail.com, pypph@mahidol.ac.th (P. Pithayanukul).

0378-8741/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2004.12.013

(Okonogi et al., 1979). Both neurotoxic and haemorrhagic venoms were neutralized by the tannin. The effects were so significant that the authors had recommended the extract from young persimmon fruit as a washing agent for the emergency treatment of snakebites. *Diospyros kaki* tannin was reported as being water-soluble condensed tannin made up of catechin and gallocatechin units connected by carbon–carbon bonds and having an approximate molecular weight of 13.8 kDa (Mors, 1991). The in vitro detoxifying effect of another plant extract, that of the leaves of *Guiera senegalensis*, against the venoms of two common northern Nigerian species, *Echis carinatus* and *Naja nigricollis*, was also suggested as due to its tannin content (Abubakar et al., 2000).

Thai cobra, *Naja kaouthia* (also known as *Naja naja siamensis*), is the most dangerous cobra in Thailand. They can be found in almost every part of the country. Local necrosis is the most common consequence of cobra bite and approximately half of the victims bitten developed local tissue necrosis, which is quite difficult to treat (Pakmanee et al., 1993). The skin necrotic area may vary from a few to 600 cm^2. Extensive skin loss may take several months to heal (Reid, 1964). Therefore, this study aimed to determine the potential of some selected medicinal plants commonly found in Thailand in inhibiting cobra venom activities.

2. Materials and methods

2.1. Plant materials

The barks of *Pentace burmanica* Kurz. (PB) (Tiliaceae) and *Pithecellobium dulce* Benth. (PD) (Mimosaceae) were collected from Nakornratchasima province. The seeds of *Areca catechu* L. (AC) (Palmae) and nutgalls (leaf galls) of *Quercus infectoria* Olivier (QI) (Fagaceae) were purchased from local market. The specimens were identified by R. Bavovada and voucher specimens (RB 02010, RB 02048, RB 02035 and RB 02024) have been deposited at the Museum of Natural Medicine, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2.2. Extraction and tannin content analysis

Plant materials were chopped and macerated at least three times with 50% aqueous ethanol at 50–60 °C for 30 min. After partial removal of the solvent under reduced pressure, the extracts were further defatted with hexane, then evaporated at 80–90 °C to dryness. The extracts were tested for the presence of tannins by reacting with ferric chloride test solution. Identification of the tannin types within the extracts was based on the development of blue–black and greenish colors for hydrolysable tannins and combination of condensed and hydrolysable tannins, respectively (Evans, 1996).

Tannin content of the extracts was analyzed following the modified AOAC method (Kakiuchi et al., 1986; Haslam, 1989; Okuda et al., 1991). First, the calibration curve of aqueous tannic acid solutions of known concentrations was prepared. Folin–Ciocalteu's phenol reagent (5 ml) and 20% sodium carbonate solution (10 ml) were added to each tannic acid standard solution to make 100 ml. The solutions were kept at room temperature (30 °C) for 30 min before measuring their absorbance at 760 nm by spectrophotometer. Clear, aqueous solutions of the crude extracts (0.1%, w/v) were prepared and each 1.0 ml of the test solutions was mixed with Folin–Ciocalteu's phenol, 20% sodium carbonate solution and distilled water to 100 ml. The mixtures were kept at room temperature for 30 min before measuring their absorbance at 760 nm. The percentages of tannin in the extracts were calculated from the calibration curve.

2.3. Animals and venom

Swiss albino mice of both sexes weighing about 18–20 g were used for the anti-lethal effect test. Male Sprague–Dawley rats weighing about 250–280 g were used for the inhibition of necrotizing effect test. The experiments were performed according to the international and Mahidol University's guidelines on animal studies. Lyophilized *Naja kaouthia* (NK) venom was provided by Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand.

2.4. Tests for anti-snake venom activities

2.4.1. Inhibition of lethality

The median lethal dose (LD_{50}) of NK venom was determined according to the method described by Theakston and Reid (1983). The venom, in 0.2 ml of physiological saline, was injected into the tail vein of mice. Triplicate experiments were carried out in six mice for each venom dose. The LD_{50} was calculated from the number of death occurring within 24 h of venom injection (Reed and Muench, 1938) with the confidence limit at 95% probability (Pizzi, 1950). Inhibition of lethality by QI, PD, PB and AC extracts was determined against 4 LD_{50}s of NK venom by the in vitro neutralization method. Different doses of the extracts were pre-incubated with the venom at 37 °C for 1 h and centrifuged at 18,110 × *g* for 10 min before 0.2 ml of the supernatant was injected intravenously through the tail vein of mice. Six mice were used for each tested dose of the extract and the experiments were performed in duplicate. The extracts, the venom and physiological saline alone were used as controls. Death and survival of mice were recorded for 24 h and the median effective doses (ED_{50}s) of the extracts, producing 50% survival of the mice against 4 LD_{50}s of NK venom, were calculated according to Reed and Muench (1938).

2.4.2. Inhibition of necrotizing activity

The methods of Kondo et al. (1960) and Theakston and Reid (1983) were followed in order to determine the minimum necrotizing dose (MND) which is defined as the smallest amount of venom that can cause a necrotic lesion of 5 mm