Neuromuscular blocking activity of methanolic extract of *Piper sarmentosum* leaves in the rat phrenic nerve-hemidiaphragm preparation

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Abstract

Methanolic extract of *Piper sarmentosum* Roxb. (Piperaceae) leaves was studied for the neuromuscular blocking activity in rat phrenic nerve-hemidiaphragm preparations. The plant extract, at concentrations of 3.2, 4.0, 4.8 and 6.4 mg/ml, exhibited an initially transient increase in twitch tension which was followed by a marked dose-related neurally-evoked twitch depression. The neuromuscular blocking effect produced by the plant extract was compared with *d*-tubocurarine (dTC) and succinylcholine (SCh). The EC₅₀ for neurally-evoked twitch depression of the extract, dTC and SCh was 4.07 mg/ml, 1.1 μM and 15 μM, respectively. The neurally-evoked twitch depression produced by the extract was partially antagonized by tetraethylammonium (TEA) but not by neostigmine (NS). These findings suggested that the plant extract possessed a marked neuromuscular blocking activity at the neuromuscular junction and a possible mechanism which was likely to inhibit neurotransmitter (acetylcholine) release at the presynaptic terminal. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Piper sarmentosum*; Methanolic extract of *Piper sarmentosum*; Neuromuscular blocking activity

1. Introduction

Thailand is endowed with a great diversity of indigenous medicinal plant species, and the Thais have a long tradition of using medicinal herbs and plants in folklore medicine. However, many of the claimed curative properties have not been scientifically proven (Mahidol, 1996). Among the plants, *Piper sarmentosum* Roxb. (syn: *Piper rostratum* Roxb.), a terrestrial herb of the piperaceae family, 1–2 feet high, jointed at the nodes, with thin, dark green and ovate leaves, has been known locally as ‘Cha-plu’ and is widely distributed throughout Thailand (Suvatti, 1978). In

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folk medicine, *P. sarmentosum* has been used as a carminative, an expectorant, and to relieve muscle pain (Pongboonrod, 1976; Li Ning-hon, 1980).

A few studies on pharmacological activities of the *P. sarmentosum* extract have been scientifically investigated. It has been reported that the crude aqueous extract of *P. rostratum* Rox. reduces blood glucose in alloxan-induced diabetic rabbits, and administration of the extract to maturity-onset diabetic patients results in a reduction of blood glucose level (Pongmarutai, 1980). The benzene-soluble fraction of the methanolic extract isolated from the leaves of *P. sarmentosum* showed antimicrobial activity (Masuda et al., 1991). Six chemical constituents of *P. sarmentosum* leaves and fruits, extracted with petroleum ether, were hydrocinnamic acid, β-sitosterol, pellitorine, pyrrole amide, sarmentine and sarmentosine (Niamsa and Chantrapromma, 1983; Likhitwitayawuid et al., 1987; Strunz and Finlay, 1995). From the pharmacodynamic point of view, the muscle-pain relieving property of *P. sarmentosum* has received much attention in studies of the neuromuscular blocking activity. Studies on pharmacological activity of the components of *P. sarmentosum* methanolic extract have not been investigated. However, in vitro preliminary study of the neuromuscular blocking action of crude methanolic extract of *P. rostratum* was first investigated in our laboratory using a rat phrenic nerve-hemidiaphragm preparation as a model, and it was found that the methanolic extract initially produced initially transient twitch potentiation which was then followed by twitch depression (Sunbhanich et al., 1988). However, no prior studies on the neuromuscular blocking action of this medicinal plant extract have yet been systematically reported. Therefore, the purposes of the present study described in this report were to extend the details of the neuromuscular blocking activity, and to investigate possible mechanisms produced by the methanolic extract of *P. sarmentosum* leaves in comparison with standard neuromuscular blocking drugs, d-tubocurarine (dTC) and succinylcholine (SCh).

### 2. Materials and methods

#### 2.1. Plant material

Fresh leaves of *P. sarmentosum* Roxb. (Piperaceae) were collected in the flowering season from the Promkeeree district, Nakhornsithamarat province, Thailand in May 1996, and identified by Department of Biology, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand. A voucher specimen (No. 106) has been kept at the Herbarium and at our laboratory for future reference.

Fresh leaves of the plants were cleaned with tap and distilled water, respectively, and air-dried at room temperature; 10 kg of fresh leaves yields 2 kg of dried leaves. The dried leaves were pulverized to a fine powder by an electric blender. The powder was stored in a closed container and kept at room temperature for further extraction.

#### 2.2. Extraction

The *P. sarmentosum* leaves powder was extracted using the method modified from the previous technique described by Niamsa and Chantrapromma, 1983. Briefly, 2 kg of the plant leaves powder was initially extracted using cold extraction by macerating in 20.0 l of methanol and allowed to stand for 7 days at room temperature. The methanol extract was filtered and evaporated at low temperature, under reduced pressure, in a rotavapor. The evaporated methanolic extract was then extracted by *n*-hexane several times to remove hexane-soluble compounds. The methanol extract obtained was evaporated to give dark brown viscosity, oil-like mixture (137.2 ml) which was then extracted with chloromethane: methanol: distilled water (6: 4: 1). Water was added as necessary to separate layers. The brown solution obtained was freeze-dried to give 36.8 g brown powder (1.84% w/w yield) which was stored in a closed bottle and kept in desiccator containing moisture absorbant substance. Then they were kept in a refrigerator at below 4°C.
2.3. Experimental animals

Both sexes of adult Wistar rats, weighing 200–300 g, grown in the animal house of the Faculty of Science at the Prince of Songkla University and fed on standard chow pellets and tap water ad libitum, were used in all experiments. The animal’s rooms were kept at 23–25°C with a 12-h light/dark cycle.

2.4. Preparation of rat phrenic nerve-hemidiaphragm for recording neurally-evoked twitch

The phrenic nerve-hemidiaphragm preparation was dissected, isolated and prepared for recording neurally-evoked twitches based on the technique previously described by Bulbring (1946). A rat was killed by cervical dislocation, decapitation, and left to bleed as much as possible. The chest was opened by cutting the ribs alongside the joints at the sternum. The diaphragm muscle was cut into fan-shaped segments and the phrenic nerve attached to each hemidiaphragm was cleared from surrounding tissues up to the thymus gland and the end of phrenic nerve was immediately cut. The base of the hemidiaphragm was tied with a cotton thread to make a loop in order to be hooked at the bottom of the organ bath. Another thread was tied firmly to the apex of the fan-shaped hemidiaphragm and connected to a force displacement transducer (Grass FT 0.3). The preparation was suspended in a 50-ml double-walled organ bath containing Krebs’ solution aerated with a mixture of oxygen (95%) and carbon-dioxide (5%) and the temperature was kept constant at 37°C. The pH of the solution was adjusted to 7.4. The preparation was equilibrated for 30 min under 2 g tension before the beginning of the experiment. The phrenic nerve was gently drawn through the loop of a platinum-wired bipolar stimulating electrode and stimulated with a square-wave pulse of 0.2 ms duration at a frequency of 0.1 Hz with a supramaximal voltage using a Grass Model S88 stimulator with Grass SIU 5 stimulus isolation unit (Grass International C., Quincy, MA). The isometric contraction of the diaphragm was measured with a force displacement transducer (Grass FT 0.3C) and recorded on a polygraph (Grass International). All drug and testing solutions were added into a 50-ml organ bath containing Krebs’ solution. The twitch tensions were recorded for 60 min or until the steady state was obtained. After each experiment, the preparation was washed every 5 min at least three times, and rested for 30 min. Each preparation was used for no more than two different experiments.

2.5. Preparation of rat hemidiaphragm for recording directly-evoked twitch

The preparation was set up as mentioned previously for the isolation of rat phrenic nerve-hemidiaphragm preparation.

Fig. 1. Neuromuscular blocking activity of methanolic extract of *Piper sarmentosum* (ME, 3.2 and 4.0 mg/ml), *d*-tubocurarine (dTC, 1.0 and 1.5 µM) and succinylcholine (SCh, 10.0 and 25.0 µM) in neurally-evoked twitch of the rat phrenic nerve-hemidiaphragm preparation.
hemidiaphragm for recording neurally-evoked twitches. In addition, one end of a platinum-wired bipolar stimulating electrode was sutured into the diaphragm muscle near the costal margin and another one was attached to the base of the hemidiaphragm. The preparation was then suspended in a 50-ml double-walled organ bath containing Krebs' solution aerated with a mixture of oxygen (95%) and carbon-dioxide (5%). The temperature was kept constant at 37°C by a thermoregulator. The directly-evoked twitch was recorded by supramaximal voltage stimulation at a frequency of 0.1 Hz and 0.2 ms duration. To eliminate the neuromuscular transmission, the preparation was completely blocked by addition of 5 μM dTC (Apisariyakul, 1975) into an organ bath 5 min before the beginning of direct stimulation.

2.6. Preparation of rat sciatic nerve for recording stimulus-evoked action potential

The stimulus-evoked action potentials were recorded in the isolated rat sciatic nerves. Both right and left sciatic nerves were dissected out as long as possible. The nerve was mounted in a three compartment chamber.

A bipolar stimulating electrode was placed under the proximal end of the nerve and the recording electrode was placed under the distal end on the other side of the central compartment. The nerve was constantly stimulated at a frequency of 0.3 Hz with a rectangular wave pulse of 0.5 ms duration and supramaximal voltage using MacLab® System (AD Instruments Pty, Australia). All nerve action potentials were displayed on a computer monitor and recorded for the analysis.

2.7. Determination of inorganic ions in the plant extract

The concentrations of Na⁺, K⁺ and Ca²⁺ in 4.0 mg/ml solution of the plant extracts were measured using inductively coupled plasma atomic emission spectroscopy (Varma, 1991).

2.8. Drugs and solutions

All experiments were performed in Krebs’ solution of the following composition (mM): NaCl, 188; KCl, 5; KH2PO4, 1.2; MgSO4, 1; NaHCO3, 25; Glucose, 11 and CaCl2, 2.5. Krebs’ solution was aerated with a gas mixture of 95% oxygen.
and 5% carbon dioxide to a pH 7.2–7.4. The testing solution of methanol extract was prepared by dissolving the leaf extract powder in Kreb’s solution to make the final concentration of 400 mg/ml and then clarified by centrifugation at 2500 rpm for 30 min. The supernatant was kept in a refrigerator at 4°C and used as a stock testing solution for the neuromuscular blocking activity. The reference drugs, dTC and SCh, (non-depolarizing and depolarizing neuromuscular blocking agent, respectively), were purchased from Sigma (St. Louis, MO). The reversal agents used were the reversal anticholinesterase, neostigmine methylsulphate (NS) and a drug acting on excitable cells, tetraethylammonium (TEA). All standard drug solutions were freshly prepared in each day by dissolving in Kreb’s solution.

Methanolic extract testing solutions were added to an organ bath containing 50 ml Kreb’s solution to give the desired concentrations (3.2, 4.0, 4.8 and 6.4 mg/ml).

2.9. Data and statistical analysis

Twitch tension was expressed as a percentage of the control values in each experiment. All data are presented as the mean ± S.E.M. of six experiments. Differences between sets of data were tested by a two-tailed paired Student’s t test or analysis of variance (ANOVA) followed by Duncan’s multiple range test with the level of significance set at P < 0.05.

3. Results

3.1. Effects of the plant extract on neurally- and directly-evoked twitch

The methanolic extract at concentrations of 3.2, 4.0, 4.8 and 6.4 mg/ml elicited an initially transient increase in twitch tension and was followed by a profound dose-dependent manner of twitch depression on neurally-evoked twitch which progressed to complete neuromuscular blockade (Figs. 1 and 2). The extract had no depressive action on directly-evoked twitches. The dose-response curves of the plant extract, dTC and SCh were compared to their neuromuscular blocking activities at 30 min as shown in Fig. 3. The EC50 of the extract, dTC and SCh on neurally-evoked twitch were 4.07 mg/ml, 1.1 μM (1.1 × 10−3 mg/ml) and 15 μM (1.5 × 10−2 mg/ml), respectively. The neuromuscular blocking potency was in order as follows; dTC > SCh > ME.

3.2. Possible mechanisms of the plant extract

3.2.1. Interaction of the plant extract with antagonists

The neuromuscular blockade produced by the extract at concentration 4.0 mg/ml was not antagonized by the reference antagonist, NS (5 μM) while the neuromuscular blockade produced by dTC (1 μM) was antagonized by NS (5 μM).
However, TEA (1 mM) partially antagonized the neuromuscular blockade produced by the extract (Fig. 4).

Synergistic effect of neuromuscular blockade between the extract and dTC was also observed (data not shown).

3.2.2. Effects of the plant extract on compound action potentials

The methanolic plant extract at concentration of 4.0 mg/ml which represented for the testing concentration did not significantly affect rat sciatic nerve compound action potentials ($P > 0.05$).

3.2.3. Effects of $K^+$ on twitch tension

The effects of $K^+$ at concentration of 9 mM which was equivalent to the amount of $K^+$ in the plant extract (4.0 mg/ml) did not produce both neurally- and directly-evoked twitch depression.

4. Discussion

The purposes of this report are to study the neuromuscular blocking activity of the methanolic extract of the $P. sarmentosum$ leaves and a preliminary intent to characterize its traditional remedies in order to validate the use of this plant in relieving of muscle pain by the Thai’s native doctors in folk medicine.

The present results obtained indicate that the methanolic extract of the leaves of $P. sarmentosum$ (in the dose examined) possesses a profound neuromuscular blocking activity like other plants or neuromuscular blocking agents. However, the potency of the extract to produce neuromuscular blockade was less than reference drugs, dTC and SCh. The EC$_{50}$ of the extract, dTC and SCh to produce neuromuscular blockade are 4.07 mg/ml, 1.1 $\mu$M, and 15 $\mu$M, respectively. In this study, the methanolic extracts decreased the neurally-evoked twitch without having the direct depressive effect on nerves or muscles. Moreover, the
concentration of K⁺ (9 mM) which was equivalent to the amount of K⁺ in the plant extract at concentration of 4.0 mg/ml did not produce both neurally- and directly-evoked twitch depression. Thus the extract certainly acted somehow on neuromuscular junction.

The neuromuscular blocking agents are classified either as non-depolarizing (competitive) agents, of which dTC is an example, or as depolarizing agents, such as SCh. The depolarizing and non-depolarizing agents are used widely to achieve muscle relaxation during anesthesia. In our investigation we found that the methanolic leaves extract of *P. sarmentosum* elicited a marked dose-related neuromuscular blocking activity at neuromuscular junction of skeletal muscle (rat phrenic nerve-hemidiaphragm preparations). Possible mechanisms of the extract at neuromuscular junction were examined. Since the plant extracts did not have a direct depressive action on an isolated rat sciatic nerve or a diaphragm muscle, therefore, neuromuscular transmission at synapses was likely to be interfered by the extract. The mechanism of action of the extract was also postulated by studying the antagonistic effect of NS and TEA on the neuromuscular blockade produced by dTC, SCh and the extract. The possible site of action of the extract at neuromuscular junction was probably located at presynaptic terminal as the present evidences suggest that the neuromuscular blockade produced by the extract was partially antagonized by TEA but not by NS. TEA, a ganglionic blocking drug, competes with ACh at cholinergic receptor in autonomic ganglion. Furthermore, it also acts on several excitable tissues (Hajiwara and Watanabe, 1955; Kuperman and Okamoto, 1965, 1966.). The twitch potentiating effect of TEA is due to enhancement of ACh release at the neuromuscular junction (Chantaratham, 1974; Collier and Exley, 1963). TEA, an ant-curare agent, also antagonizes the neuromuscular blockade produced by dTC and pancuranium (Apisariyakul, 1975; Kensler, 1950). In the present study, TEA partially produced an antagonistic effect on the neuromuscular blockade caused by the methanolic extract of *P. sarmentosum*. Therefore, the neuromuscular blocking activity of the plant extract is probably resulted from its action at presynaptic site by inhibiting the ACh release from nerve terminal. It is widely accepted that dTC blocks neuromuscular transmission by competitive inhibition with ACh at nicotinic cholinergic receptor at motor end-plate. However, some investigators reported that curare reduced the ACh release from motor nerve ending (Hubbard et al., 1969). Therefore, the possible mechanism of the extract was likely to have the neuromuscular blocking activity similar to that of dTC. However, in this study, the mechanism of action of the plant extract which exhibited neuromuscular blocking activity could only be partially investigated. The in vivo study on the neuromuscular blocking activities of the plant extract in animals will be carried out in our laboratory to achieve its more details neuromuscular blocking activities and mechanisms of action. Furthermore, other parts of the plant e.g. roots and stems would be also investigated for their neuromuscular blocking activities in comparison with the plant leaves extract. However, the results presented here may help to establish the scientific basis for the use of *P. sarmentosum* remedies as a muscle relaxant to relieve muscle ache in Thai folklore medicine.

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