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Hypotensive and cardio-chronotropic constituents of *Tinospora crispa* and mechanisms of action on the cardiovascular system in anesthetized rats

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ABSTRACT

Ethnopharmacological relevance: Tinospora crispa has been used in folkloric medicine for the control of blood pressure. We previously found that an extract of *Tinospora crispa* stems decreased the mean arterial blood pressure (MAP) with a transient decrease, followed by an increase in the heart rate (HR) in rats.
Aim of the study: To identify the active components of the *Tinospora crispa* extract and investigate the mechanisms of action on blood pressure and heart rate in anesthetized rats.
Materials and methods: The active components of *Tinospora crispa* extract were separated by column chromatography and a preparative HPLC. The effects and mechanisms of the active compounds on blood pressure and heart rate were studied in anesthetized, normal and reserpinized rats *in vivo*.
Results: 5 active compounds: adenosine, uridine, salsolinol, higenamine and tyramine were isolated. Adenosine decreased MAP and HR and this effect was inhibited by DMPX (A_{2A} adenosine receptor antagonist). Uridine increased MAP and decreased HR and this was inhibited by suramin but not by DMPX. Salsolinol decreased the MAP and HR and this was inhibited by phentolamine but not by ICI-118,551 (β_2-adrenoceptor antagonist) or atropine. In reserpinized rats, salsolinol had a hypertensive effect that was inhibited by prazosin and phentolamine, but not by atenolol, and caused an increase in HR that was inhibited by atenolol, but not by prazosin or phentolamine. Higenamine decreased the MAP with an increase in HR. The hypotensive effect was inhibited by ICI-118,551 or atenolol, whereas the increase in HR was not inhibited by ICI-118,551. Atenolol inhibited the increase in HR at a small dosage of higenamine but potentiated it at a higher dosage. In reserpinized rats, a small dosage of higenamine tended to potentiate the effect but at a higher dosage it caused inhibition. ICI-118,551 significantly inhibited this hypotensive effect. Tyramine caused an increase in MAP and HR and these effects almost disappeared in reserpinized rats.
Conclusions: The results demonstrate that these 5 compounds from *Tinospora crispa* acted in concert on the cardiovascular system of anesthetized rats. Salsolinol, tyramine and higenamine acted via the adrenoreceptors, whereas uridine and adenosine acted via the purinergic adenosine A_2 and P_2 receptors to decrease blood pressure with a transient decrease of HR followed by an increase.

1. Introduction

Tinospora crispa (L.) Miers ex Hook. F. & Thoms (*Tinospora rumphii* Boerl or *Tinospora tuberculata* Beumee) or Borapet in Thai, has been used in Thai traditional medicine for many purposes including: as an antipyretic, an antidiabetes agent, for treating internal inflammation, reducing thirst, increasing appetite, cooling down the body temperature and for maintaining good health (Kongsaktrakoon et al., 1994; Dweck and Cavin, 2006). In Indonesia (Borneo) it has been used to treat diabetes, hypertension, and lumbago (Dweck and Cavin, 2006). However, scientific investigations to test these therapeutic claims are scarce. Noor and Ashcroft (1989)

Abbreviations: CH_2Cl_2, dichloromethane; CH_3OH, methyl alcohol; $CHCl_3$, chloroform; DMPX, 3,7-dimethyl-1-propargylxanthine; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; HR, heart rate; i.p., intraperitoneal; i.v., intravenous; MAP, mean arterial blood pressure; MPLC, moderate pressure liquid chromatography; NMR, nuclear magnetic resonance.

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were the first to investigate the antihyperglycemic activity of an aqueous extract from the stems of the *Tinospora crispa* in chemically induced hyperglycemic rats. They found that it caused a reduction in blood glucose level in moderately diabetic rats. This effect was probably due to an active component that stimulated the release of insulin via modulation of the ß-cell Ca^{2+} concentration (Noor and Ashcroft, 1998). However, the hypoglycemic activity of a capsulated dried stem powder of the *Tinospora crispa* (orally 3 g/day) was not detected in diabetic humans after having been taken for 6 months (Sangsuwan et al., 2004). For the cardiovascular system, Mokkhasmit et al. (1971) conducted a pharmacological screening of some Thai medicinal plants and found that a crude alcohol extract from the stems of *Tinospora crispa* caused an increase in blood pressure with a decrease in heart rate in anesthetized dogs. Later Kongkathip et al. (2002) isolated cycloeucalenone and cycloeucalenol from its crude hexane and chloroform extract respectively, and found that only cycloeucalenol produced a slight increase in the force of spontaneous contraction of the right atrium, but caused a decreased in the force of contraction of the left atrium.

We (Praman et al., 2011) have recently demonstrated that a crude n-butanol extract from the stems of *Tinospora crispa* exerted a hypotensive activity, as well as both negative and positive chronotropic effects in anesthetized rats. The hypertensive effect was demonstrated only after the store of norepinephrine had been depleted by reserpine. These results also indicated that there were at least three different active components involved in the mechanisms responsible for these effects. This included: (1) one compound that acted via the β_2-adrenoceptors to cause a decrease in blood pressure, and the β_1- and β_2-adrenoceptors to cause an increase in heart rate, (2) another acting via α-adrenoceptors to cause an increase in blood pressure and heart rate, and (3) the third acting by some other pathway in addition to those acting via the adrenoceptor and muscarinic receptors to cause a decrease in blood pressure and/or heart rate. In the present study we aimed to isolate the active substances from the n-butanol extract of the stems of the *Tinospora crispa* that were responsible for those effects on blood pressure and heart rate, as well as to assess the mechanism of each of the compounds involved.

2. Materials and methods

2.1. Plant material

Stems of *Tinospora crispa* (10 kg) were collected (January–May 2005–7) from Phangnga Province, Thailand. Botanical identification of the plant was carried out by Prof. Poungpen Sirirugsa, Department of Biology, Prince of Songkla University, Thailand, where a voucher specimen has been deposited (Collecting No. 2548-02).

2.2. Preparation of the Tinospora crispa extract and isolation of the cardioactive substances from stems of Tinospora crispa

Preparation of *Tinospora crispa* extract followed Praman et al. (2011). Briefly air-dried stems of *Tinospora crispa* were simmered in hot filtered water for a period of 3 h. The clear solution was collected and heated at 50 °C to reduce the volume to 30%. The concentrated solution was partition extracted with chloroform, followed by ethyl acetate, and finally with n-butanol. The three organic extracts were evaporated under reduced pressure, and assayed for hypotensive activity. Only the n-butanol fraction showed a hypotensive effect in anesthetized rats (10 and 30 mg/kg, i.v.).

Bioassay guided fractionation of the *Tinospora crispa* n-butanol extract (400 g) via column chromatography on silica gel 100 (0.063–0.200 mm, 850 g) using step gradient elution from 100% $CHCl_3$ to 100% CH_3OH, gave 5 fractions (A1–A5). At dosages of 3–10 mg/kg (i.v. injection), fractions A1–A3 had no activity on blood pressure and heart rate. Fraction A4 caused a decrease in blood pressure and heart rate, while fraction A5 caused a decrease in blood pressure with an increase in heart rate.

The hypotensive and cardioactive fraction, A5, was chromatographed on a silica gel reversed phase C_{18} column using step gradient elution from 10% CH_3OH/H_2O to 100% CH_3OH (10% increments using 2.5 L for each step), to yield 8 subfractions (A5.1–A5.8). The subfraction A5.8 was identified as borapetoside D (2.03 g). The subfractions A5.3–5.7 were further chromatographed on silica gel 60 (9:1 $CHCl_3$:CH_3OH), silica gel reverse phase C_{18} (step gradient 10–100% CH_3OH:H_2O) or sephadex G-15 (50% CH_3OH/H_2O). Each active fraction was finally purified via C_{18} reverse phase HPLC (using a CSC-Inertsil 150A/ODS2, 5 μm 25 cm × 0.94 cm column). Eluting respectively, with 100% H_2O, 5%, 15% or 17% CH_3OH, or 0.5% acetonitrile gave the 5 compounds: uridine (108.3 mg), salsolinol (123 mg), tyramine (12 mg), higenamine (326.4 mg) and litcubinine (88.4 mg).

The fraction A4, a hypotensive fraction, was chromatographed on silica gel to yield 3 subfractions, A4.1–A4.3. The subfractions A4.1 and A4.3 were purified via C_{18} reverse phase HPLC, with 5% and 10% acetonitrile as eluant, to give adenine (51.6 mg) and syringin (2.78 g), respectively. The subfraction A4.2 was chromatographed on silica gel reverse phase C_{18} with gradient elution from 5% to 10% CH_3OH/H_2O to give adenosine (431.6 mg).

Fraction A3 was further chromatographed on silica gel 60, with gradient elution from CH_2Cl_2 to CH_3OH, to yield the 3 subfractions A3.1–3.3. The subfractions A3.1 and A3.2 appeared to contain the same compounds as fraction A4, and no further purification was undertaken. The subfraction A3.3 was fractionated on silica gel reverse phase C_{18} with gradient elution from H_2O to CH_3OH to yield syringin (2.78 g) and borapetoside B (3.65 g). The fractions A1 and A2 were chromatographed on silica gel 60 with gradient elution from CH_2Cl_2 to CH_3OH, to give subfractions A1/2.1–2.3. Final purification of the subfraction A1.2 was achieved with moderate pressure liquid chromatography (LiChroprep® RP_{18}, 40–63 μM, Merck), using a gradient of 5–10% CH_3OH/H_2O at a flow rate of 2.5 ml/min, to give borapetoside E (2.96 g). Subfractions A2.1 and A2.3 were found to contain the same compounds as A1 and A3 so no further purification was undertaken. Finally, subfraction A2.2 was chromatographed on silica gel reversed phase C_{18}, with gradient elution from H_2O to CH_3OH, to obtain borapetoside A (2.68 g) (see Fig. 1).

The structures of the pure compounds (Fig. 2) were assigned based on comparison of the mass spectra (low and high resolution ESI-QIT-MS recorded on a Bruker-Hewlett Packard 1100 Esquire-LC system mass spectrometer), and NMR spectra (recorded on a Bruker AV-600 spectrometer with a 5 mm CPTCI cryoprobe) with the literature (Graziano et al., 1971; Fukuda et al., 1983, 1985, 1986, 1993; Son et al., 1991; Lee et al., 1996; Martin et al., 1996; Munkombwe et al., 2003; Ahmad et al., 2011; Nagasawa et al., 2011) and standards.

2.3. Pharmacological effects of the compounds isolated from the Tinospora crispa extract on blood pressure and heart rate

Adult female Wistar rats in estrus (220–280 g) were supplied from the Animal House, Faculty of Science, Prince of Songkla University. They were maintained in a controlled environment (24–26 °C), with a 12 h light/dark cycle and allowed access to standard food and tap water *ad libitum*. The preparation of the animals followed the Prince of Songkla University guidelines for the approved Care and Use of Experimental Animals. Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Their tracheal tube was cannulated with a polyethylene tube to facilitate spontaneous respiration. The systemic blood pressure was recorded from