PHARMACOLOGICAL STUDIES OF THIOCARBAMATE GLYCOSIDES ISOLATED FROM *MORINGA OLEIFERA*

C. JANSAKUL[a], A. WUN-NOI[a], K. CROFT[b] AND L. BYRNE[c]

[a] *Department of Physiology, Faculty of Science, Prince of Songkla University, Hat-Yai, Thailand.*

[b] *Department of Medicine, The university of Western Australia, Nedland, Australia.*

[c] *Department of Organic Chemistry, The University of Western Australia, Nedland, Australia.*

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ABSTRACT

Bioassay directed fractionation of the methanolic extract of dried pods and seeds of Moringa oleifera *resulted in the isolation of a mixture of thiocarbamate glycosides of niazimicin and niaziminin. In the* in vivo *preparation, intravenous injection of the compounds (2-16 mg/kg) caused a decrease in mean arterial blood pressure and heart rate of anesthetized rats in dose dependent manner. Pretreatment of the animals with atropine (1.5 mg/kg) did not modify the hypotensive activity or negative chronotropic activity of the compounds. Pre-treatment of the animals with β-adrenergic receptor antagonist, DL-propranolol (0.6 mg/kg), caused significant reduction in lowering heart rate activity with no changes in hypotensive activity of the compounds.*

In the in vitro *preparation, the thiocarbamate glycosides (0.02-0.6 mg/ml) caused vasodilatation of thoracic aortic rings pre-constricted with phenylephrine both of endothelium-intact and denuded aortic rings. Pre-incubation of the endothelium-intact aortic rings with N^G-nitro-L-arginine (300 mM), a nitric oxide synthase inhibitor, or with DL-propranolol (10 μM), did not abolish the vasodilator activities of the compounds. The thiocarbamate glycosides also caused vasodilatation of the thoracic aortic rings preconstricted with 40 mM KCl.*

The thiocarbamate glycosides caused a decrease in the rate with no changes on force of contraction of isolated rat atria. Pre-incubation of the atria with the thiocarbamate glycosides (0.6 mg/ml) for 40 min, did not modify the positive chronotropic activity of isoproterenol. It, however, caused significant decreased in force of contraction produced by isoproterenol. These results suggested that the hypotensive and negative chronotropic activity of the thiocarbamate gylcosides are unlikely to be due to the stimulation by the compounds of the muscarinic receptors of the parasympathetic or to their action as a β-adrenergic receptor antagonist, but it may act as a Ca^{++}-channel blocker. However, further studies are needed to clarify this possibility.

INTRODUCTION

Moringa oleifera Lam. or Horse-radish tree, belongs to the family Moringaceae. It is a small tree found throughout the tropical parts of the world. Almost every part of the tree are reputed to be used in folk medicine for the treatment of a variety of human ailments. In indian medicine, it is used as a diuretic, antispasmodic, expectorant, cardiotonic and abortifacient[1]. In addition, most parts of the tree are edible, and have been used in the humans diet.

Shukla *et al.*[2,3] reported that the water extract of the roots has antifertility action by changing the duration of rat estrous cycle and also has anti-implantation action in pregnant rats. Eilert *et al.*[4] found that 4 (α-L-Rhamnosyloxy) benzyl isothiocyanate and benzyl isothiocyanate are antibiotic principles isolated from the plant seeds. The roasted seeds contain 4 (α-L-Rhamnosyloxy) phenylacetonitrile , 4-hydroxyphenylacetonitrile and 4-hydroxyphenyl-

acetamide, which showed mutagenic activity in rats[5]. For hypotensive activity, Siddiqi and Khan (1968)[6] reported that crude ethanolic and aqueous extracts of the plant leaves have hypotensive properties, the hypotensive principles of the fraction are niazinin A, niazinin B, niazimicin and niaziminin A&B, which are thiocarbamate glycosides[7].

In Thailand, young pods and seeds of the plant are widely used as vegetable, and some people take the extract of the dried young pods for controlling diabetes and hypertension. Our preliminary study found that the water or methanolic extracts of the young pods and seeds of this plant showed pronounced hypotensive activity in anesthetized rats *in vivo*. Since any extensive pharmacological activities of the pods or seeds of this plant have not yet been investigated, it was of interest to study the pharmacological activities of the isolated active principles from the pods and seeds of the *Moringa oleifera* in relation to its hypotensive activity.

MATERIALS AND METHODS

1. Isolation of thiocarbamate glycosides

The plant was identified by Assist. Prof. Choathip Purintavaragul, Department of Biology, Faculty of Science, Prince of Songkla University, where a voucher specimen has been deposited. Isolation of the thiocarbamate glycosides were modified from Jansakul's method.[8] Dried young pods and seeds of *Moringa oleifera* were minced and extracted with warm (60°C) 70 % methanol (in water) for 3 hrs twice. The clear solution was decanted and concentrated under vacuum with a rotary evaporator. The concentrated solution was partitioned with water-saturated n-butanol. The n-butanol phase was collected and evaporated to dryness *in vacuo* and lyophilized. Dried powder of the n-butanol fraction was separated by silica gel column chromatography using a mixture of ethylacetate and methanol (8:2, V:V) as a mobile phase. Five fractions were separated. The hypotensive fraction (3rd fraction) was further separated by column chromatography, eluting the substances with gradient of chloroform and methanol (v:v). Five fractions were obtained; the third fraction, which showed pronounce hypotensive activity, was further purified by HPLC, using a reverse phase C_{18} column, and a mixture of MeOH : H_2O: acetic acid = 55:45:0.01 as a mobile phase. Eluted substances were detected with UV-detector (wavelength, 245). Four peaks were detected, however, only two major peaks were collected, namely peak 1 and peak 2. Both of these peaks showed a hypotensive property on i.v. injection in anesthetized rats. However, only peak 2 which is the main fraction, could be isolated in sufficient amount for chemical structure elucidation.

The chemical structure elucidation of the isolated compound, was done at the Department of Organic Chemistry, The University of Western Australia. The NMR spectrums of the original substance as well as from the modified compounds by acetylation of the OH functional groups suggested that the peak 2 compound was a mixture of niazimicin and niaziminin A&B, the same compounds which were isolated from leaves of this plant reported by Faison *et al.*[7] They are thiocarbamate glycosides: niazimicin, O-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate and niaziminin A&B, O-ethyl 4-[(4'-O-acetyl-α-L-rhamnosyloxy) benzyl] thiocarbamate.

2. Pharmacological studies of the thiocarbamate glycosides

In vivo preparation

Female Wistar rats in estrus were anesthetized with Nembutal (60 mg/kg). A polyethylene catheter was cannulated through the right common carotid artery and connected to a pressure

transducer and polygraph for monitoring blood pressure and heart rate. The animal was then equilibrated for 1 hr. The dose-response relationship to the thiocarbamate glycosides or distilled water vehicle (0.1 ml) injected through left jugular vein, were determined.

Effects of the thiocarbamate glycosides on blood pressure and heart rates after blocking with atropine or propranolol

After equilibration of animals for 25 min, atropine (1.5 mg/kg) or DL-propranolol (0.6 mg/kg) was injected through the jugular vein. After 20 min re-equilibration, dose- response relationship to intraveneous injection of the thiocarbamate glycosides was determined.

***In vitro* preparation**

The female rats were killed by cervical dislocation. The atria, both the left and the right were excised and mounted immediately in a 20 ml organ bath. For thoracic aorta, two adjacent rings were cut, and the endothelium removed from one by mechanical disruption by method of Jansakul *et al.*[10] The thoracic aortic rings were placed in organ baths and attached to isometric force transducers and the signals recorded on a polygraph. The organ bath contained Kreb's Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ $7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at 37°C, and continuously bubbled with 95% O_2 and 5% CO_2.

Prior to addition of drugs, tissues were equilibrated for 60 min under resting tension of 0.5 g for atria and 1.0 g for thoracic aorta. The Kreb's solution was replaced every 10-20 min.

After equilibration, the presence of functional endothelium of the thoracic aorta was tested as follows. The aortic ring was preconstricted with $3x10^{-6}$ M phenylephrine for 5-8 min (by which time the response had plateaued), and dilator responses to 10^{-6}M acetylcholine recorded. Eighty to ninety per cent vasodilatation to acetylcholine occurred with endothelium-intact rings.

Effects of the thiocarbamate glycosides on thoracic aortae *in vitro*

After 40 min re-equilibration, cumulative dilator response to the thiocarbamate glycosides of the thoracic aortic ring preconstricted with $3x10^{-6}$M phenylephrine was studied. Following several washings, only the thoracic aortic rings with endothelium-intact was re-equilibrated with N^G-nitro-L-arginine (L-NOARG, $3x10^{-4}$ M) for 30 min, a second cumulative dilator responses to the thiocarbamate glycosides was obtained in the presence of L-NOARG. In another experiment, thoracic aortic rings without endothelium were pre-incubation with 10^{-5} M DL-propranolol for 40 min, and cumulative dilator responses to thiocarbamate glycosides of the thoracic aortic ring preconstricted with $3x10^{-6}$ M phenylephrine was studied in the presence of DL-propranolol.

In a further set of experiments, after 40 min re-equilibration, the thoracic aortic rings with endothelium-intact was incubated with L-NOARG for 30 min. Then cumulative dilator responses to thiocarbamate glycosides of the thoracic aortic ring preconstricted with 40 mM KCl was performed in the presence of L-NOARG.

Effects of thiocarbamate glycosides on rate and force of isolated atrial contraction *in vitro*

After 40 min equilibration, the cumulative dose-response relationships of the thiocarbamate glycosides on the atrial rate and force were studied. In a second experiment, cumulative dose-response relationship to isoproterenol was studied. Follow several washing and re-equilibration for 40 min, the atria was incubated with 0.6 mg/ml of the thiobarbamate

glycosides, a second cumulative dose-response relationship to isoproterenol in the present of the thiocarbamate glycosides was obtained.

The following drugs were used: phenylephrine chloride, DL-propranolol, L-NOARG, isoproterenol and acetylcholine chloride were obtained from sigma, U.S.A.

Statistical analysis

Vasodilator activity by the thiocarbamate glycosides of thoracic aorta was calculated as a percentage of the induced tension which existed at the start of a relaxant concentration-effect experiment.

Other data are expressed as means ± s.e.mean of 4-8 experiments (n = 4-8), and tests of significant made using student's paired or unpaired t-test or one-way ANOVA. In all cases, a p value of 0.05 or less was considered statistically significant.

RESULTS

The effects of thiocarbamate glycosides on mean arterial blood pressure (MAP) and heart rate(H.R.) are shown in Fig.1. Basal mean arterial blood pressure and heart rate of anesthetized rats both of control and experiment groups are similar (water vehicle control group, MAP=149.3±5.6 mmHg, H.R.=401.3±9.4 beats/min, n=4; experiment group, MAP=148.7±8.1 mmHg, H.R.=417.5±12.0 beats/min, n=7). The thiocarbamate glycosides (2-16 mg/kg) caused a decrease in mean arterial blood pressure and heart rate in anesthetized rats in a dose dependent manner, while water vehicle (0.1 ml) did not have any effects on blood pressure or heart rate. The lowest dose of the thiocarbamate glycosides (2 mg/kg) caused a decrease in mean blood pressure of about 22.2±4.4 mmHg and decrease in heart rate to 11.4 ±1.5 beats/min and the highest dose (16 mg/kg) caused decrease in blood pressure of about 77.9±9.8 mmHg and decrease in heart rate of 65.0±9.8 beats/min. There were no signs of acute toxicity such as internal bleeding of liver, lung, gastrointestinal tract or urinary bladder, at any doses of the thiocarbamate glycosides studies.

The effects of atropine, a non-specific muscarinic recepter antagonist; DL-propranolol, a non specific adrenergic receptor antagonist on the lowering of mean arterial blood pressure and heart rate by the thiocarbamate glycosides are shown in Fig. 2. Blocking muscarinic receptor by atropine or blocking β-adrenergic receptor by DL-propranolol did not significantly modified the lowering effect of blood pressure by the thiocarbamate glycosides. In case of the heart rate, however, blocking the β-adrenergic receptors by DL-propranolol, causes a diminishing in lowering of heart rate by the thiocarbamate glycosides.

Fig. 3 shows the effects of the thiocarbamate glycosides on the vasodilatation of thoracic aortae in the *in vitro* preparation. The thiocarbamate glycosides caused a dose-dependent vasodilatation of the thoracic aortic ring which had been preconstricted with phenylephrine ($3x10^{-6}$ M), both the rings with and without endothelium. Pre-incubation of the endothelium-intact aortic rings with L-NOARG ($3x10^{-4}$ M), a nitric oxide synthase inhibitor or pre-incubation the endothelium-denuded aortic rings with the DL-propranolol, a non-specific β-adrenergic receptor antagonist, did not modified any effects of the thiocarbamate glycosides on vasodilatation of pre-constricted thoracic aortic rings with phenylephrine.

Thiocarbamate glycosides also caused vasodilatation in a dose dependent manner of the aortic rings preconstricted with KCl (Fig. 4). However, at higher concentration of the glycosides (0.6 mg/ml), the vasodilatation of the rings preconstricted with phenylephrine were greater than those which were pre-constricted with KCl.

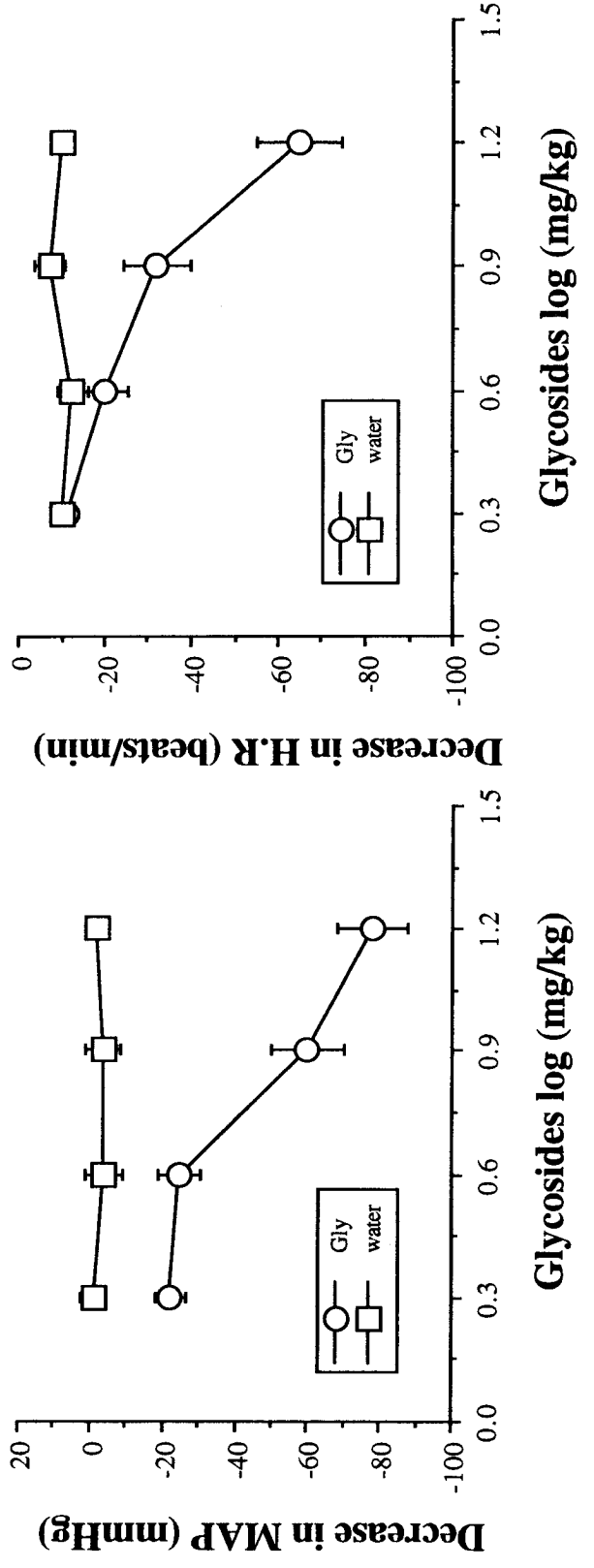

Fig. 1 Effects of thiocarbamate glycosides on blood pressure (left) and heart rate (right) in anesthetized rats compared to those of distilled water injection. Each point represents of the mean±s.e.mean of 4-7 experiments.

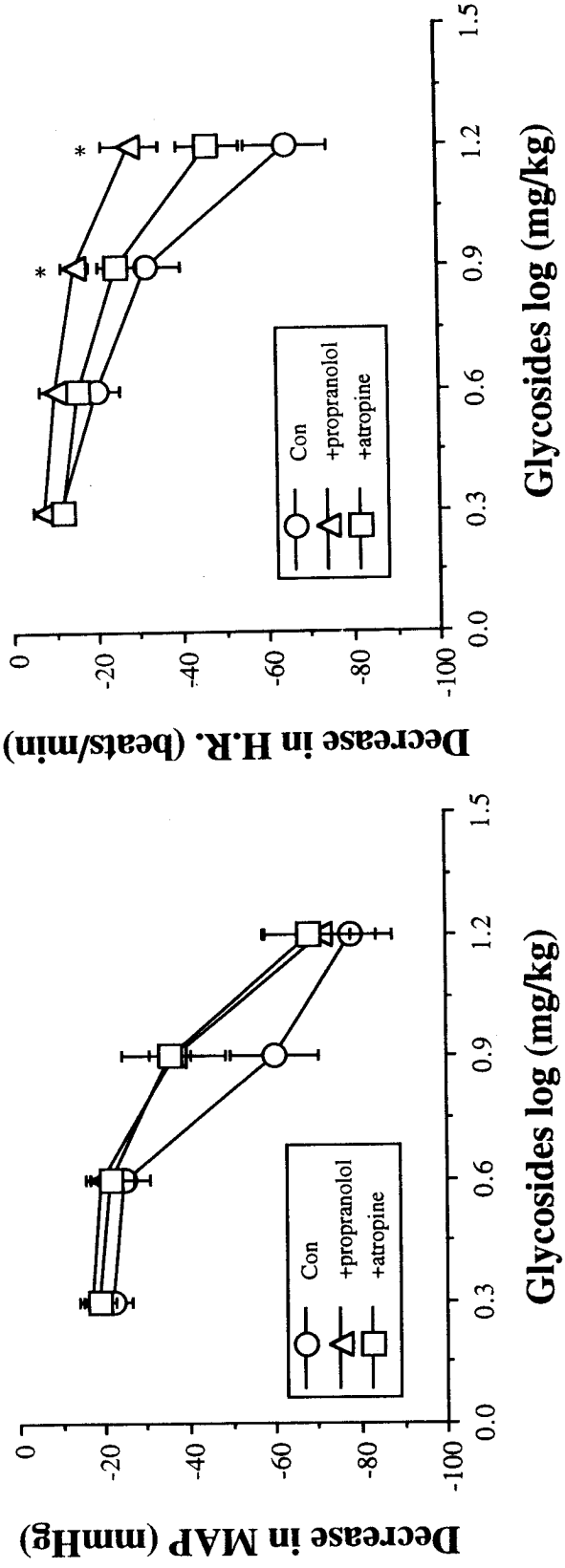

Fig. 2 Effects of atropine and DL-propranolol on the decrease in blood pressure(left) and heart rate (right) of the thiocarbamate glycosides. Each point represents of the mean±s.e.mean of 6 experiments. * significantly higher that those of control.

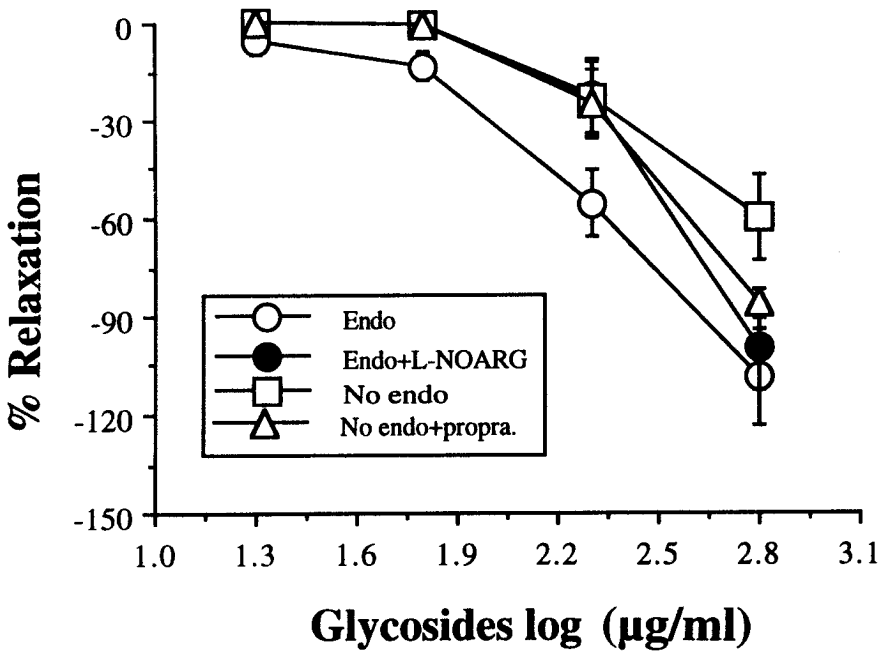

Fig. 3 Effects of thiocarbamate glycosides on vasodilatation of endothelium-intact (endo) or denuded (no endo) thoracic aortae in the absence and after blocking with L-NOARG or DL-propranolol (propra.). Each point represents of the mean±s.e.mean of 5-7 experiments.

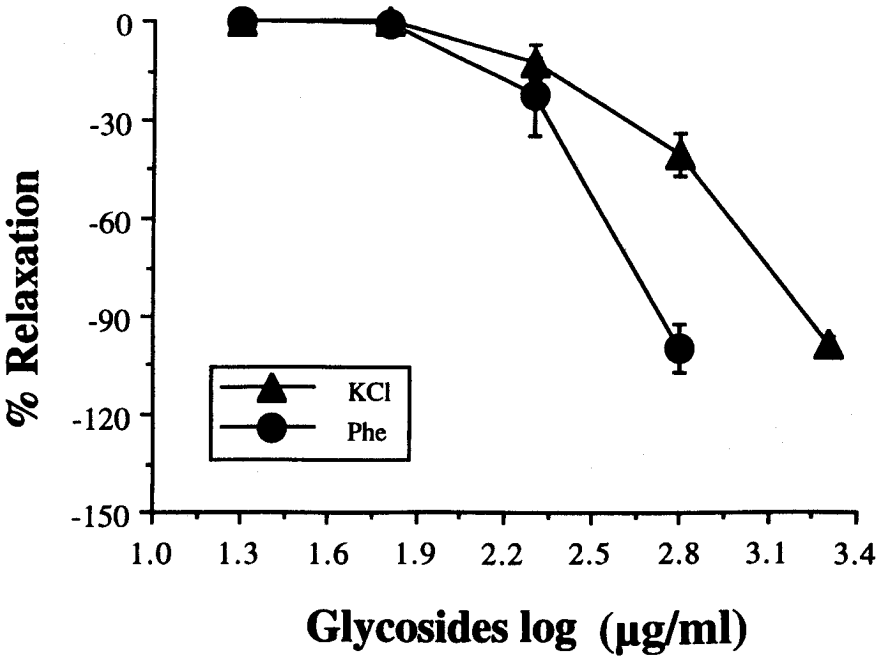

Fig. 4 Effects of thiocarbamate glycosides on vasodilatation of endotheliam-intact thoracic aortic rings preconstricted with phenylephrine or KCl after blocking with L-NOARG. Each point represents of the mean±s.e.mean of 5-7 experiments.

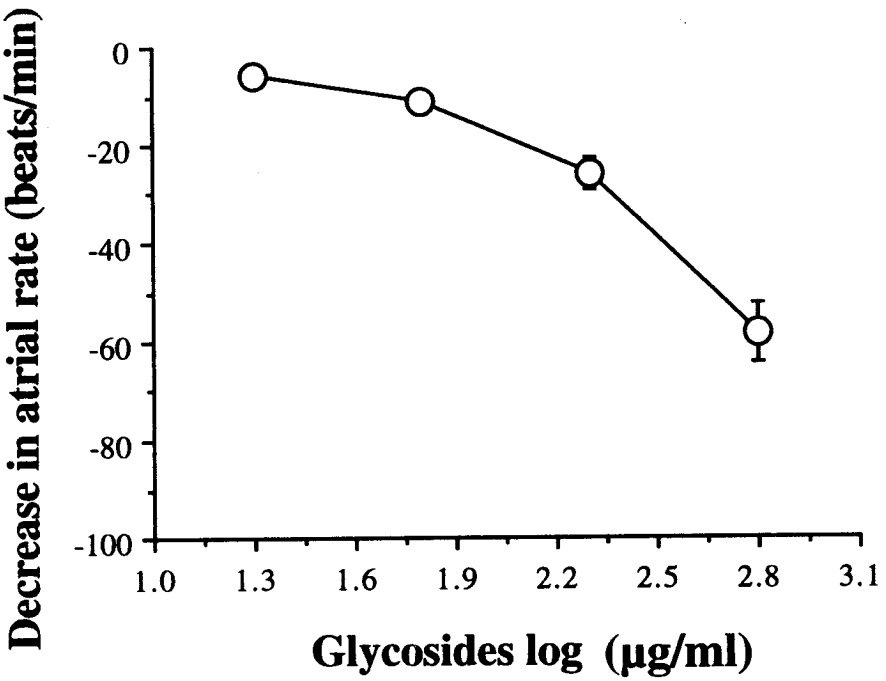

Fig. 5 Effects of thiocarbamate glycosides on the rate of atrial contraction of the isolated atria. Each point represent of the mean±s.e.mean of 6 experiments.

The effects of the thiocarbamate glycosides on rate of contraction of isolated atria, *in vitro* preparation, are shown in Fig. 5. The thiocarbamate glycosides caused a dose dependent decrease in rate of atrial contraction without any effect on the force of contraction (data not shown). Isoproterenol, a non-specific β-adrenoreceptor agonist, caused a dose dependent increase in both rate and force of contraction of the isolated atria. Pre-incubation the atria with the thiocarbamate glycosides (0.6 mg/ml) did not alter the dose-response relationship for positive chronotropic effect of the isoproterenol. For the force of atrial contraction, the thiocarbamate glycosides caused a significantly decrease in force of contraction produced by the Isoproterenol (Fig. 6).

DISCUSSION

The thiocarbamate glycosides caused decrease in both blood pressure and heart rate in a dose dependent manner in anesthetized rats without any signs of acute toxicity. These findings are similar to those reported by Gilani *et al.* (1994)[11] for the same compounds which were isolated from leaves of this plant. The finding that atropine, a muscarinic receptor antagonist, did not block the hypotensive and negative chronotropic activities of the compounds, suggested that a possible involvement of the compounds on muscarinic receptor activation could be ruled out. This finding is also consistent with those of Gilani *et al.* (1994)[11].

The lowering in blood pressure and heart rate activities of the thiocarbamate glycosides may act as a β-adrenoreceptor antagonist of the cardiovascular system. Therefore, other experiments were performed in the *in vivo* preparation by pretreatment the animals with propranolol (0.6 mg/kg) to block the β-adrenergic receptors of both at the blood vessels and the heart. It was expected that if the compounds behave as the β-adrenergic receptor antagonist, the hypotensive and negative chronotropic activities of the compounds would be diminished.

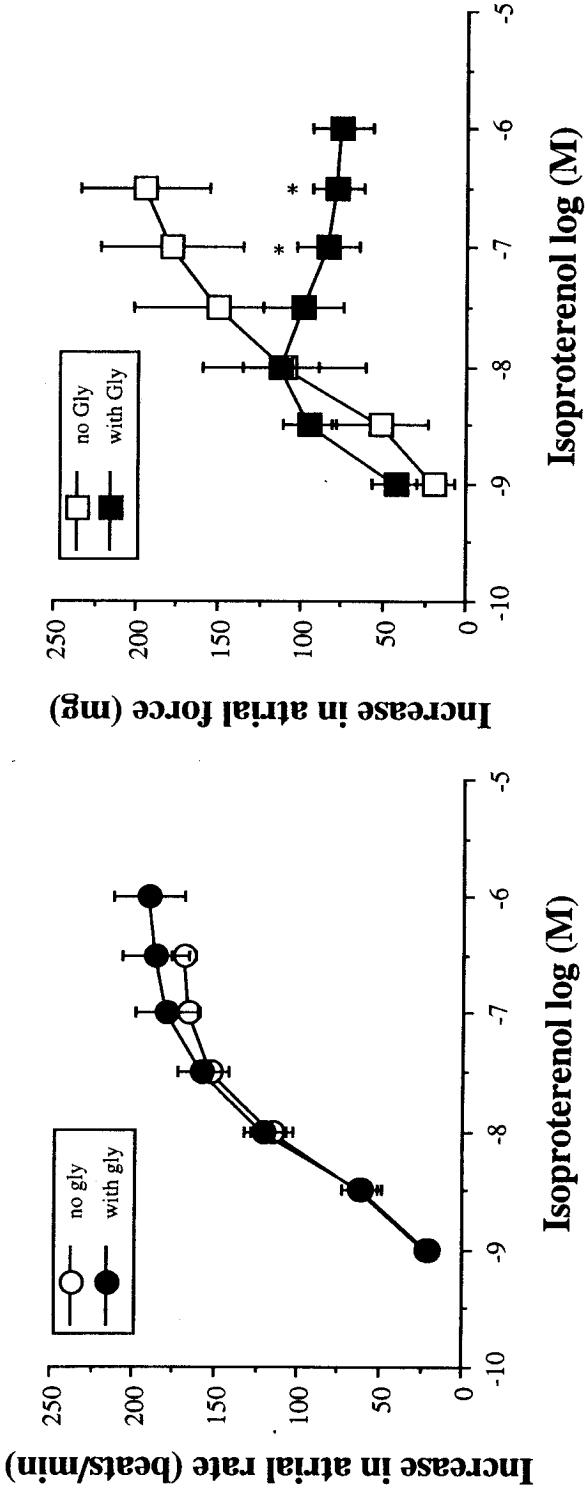

Fig. 6 Effects of isoproterenol on the rate (left) and the force (right) of contraction of isolated atria in the absence or presence of thiocarbamate glycosides (0.6 mg/ml). Each point represent of mean ±s.e.mean of 6 experiments. * significantly lower than those of wihout glycosides.

As shown in Fig. 2, pretreatment the animals with propranolol did not alter the hypotensive activities of the compounds. It, however, caused significantly diminishing in the decrease in heart rate of the compounds to all doses studied. This indicates that the thiocarbamate glycosides may play a role as a β-adrenergic receptor antagonist.

The hypotensive and negative chronotropic activities of the thiocarbamate glycosides *in vivo* in rats may act as a direct effect to cause vasodilatation and/or decrease in atrial rate or by indirect action through another system. In order to prove this possibility, experiments were performed with an *in vitro* preparation using isolated thoracic aortae and atria. As shown in Fig.3, thiocarbamate glycosides caused vasodilatation of thoracic aortic rings pre-constricted with phenylephrine of both endothelium-intact and denuded vessels. These results suggest the compounds should have on direct effects at blood vessels and the atria. In the endothelium-intact thoracic aortic rings, vasodilator effect of the compound was higher than in endothelium-denuded aortic rings at all doses of the glycosides studied except the lowest concentration (0.02 mg/ml). The possible explanation for this may be the glycosides stimulate release of nitric oxide, a potent vasodilator substance from vascular endothelium[12], which partly contribute to the vasodilator effect of the compounds. In order to test this possibility, the thoracic aortic rings with endothelium-intact were pre-incubation with the L-NOARG, a nitric oxide synthase inhibitor, for 40 min and the dose-response relationship to the glycosides was then again performed in the presence of the L-NOARG. As shown in Fig. 3, the nitric oxide synthase inhibitor caused a significant shift in the vasodilator response of the glycosides to the same extent as those of without endothelium. These results suggest that the glycosides also stimulates release of the nitric oxide from the vascular endothelium to potentiate the vasodilator activity to the compounds.

The direct vasodilator activity of the glycosides may act through the β-adrenoreceptor of the vessel or act as a Ca^{++}-channel blocker. To investigate the first possibility, the denuded thoracic aortic rings were pre-incubated with propranolol 40 min before obtaining the dose response relationship to the glycosides. Results showed that the propranolol did not alter the dose-response vasodilatation of the glycosides. Thus, the possibility for the glycoside acting through the β-adrenoreceptor of the blood vessels is unlikely.

Vasoconstrictor responses to KCl *in vitro* is the result of a depolarizing-induced transmembrane influx of calcium ions and this inward current of calcium ions is inhibited by calcium channel blockers[13,14,15]. In order to prove this possibility, the thoracic aortic rings with endothelium-intact were pre-incubated with L-NOARG for 40 min to inhibit the nitric oxide synthase activity. A comparison was made of the percentage relaxation produced by the glycosides of the rings preconstricted with 40 mM KCl with those preconstricted with phenylephrine, which stimulates contraction partly dependent on extracellular calcium and partly dependent on intracellular calcium stores[16]. As shown in Fig. 4, the thiocarbamate glycosides caused vasodilatation in a dose-dependent manner of the rings preconstricted with 40 mM KCl and phenylephrine. Besides this, at higher concentrations of the glycosides, percentage relaxation obtained from the rings preconstricted with phenylephrine was greater than that of those preconstricted with KCl. These results suggest that the thiocarbamate glycosides may act as a Ca^{++}-channel blocker.

The thiocarbamate glycosides caused a decrease in atrial rate in a dose dependent manner, without any effect on the force of atrial contraction (Fig. 5). This result is different from those reported by Gilani *et al.* (1994)[11], which found the compounds caused decrease in both rate and force of the isolated guinea pig atria. The reason for this may be due to the difference in animal species used.

As the results of the *in vivo* experiments suggest that the negative chronotropic effect of the glycosides may involve a β-adrenoceptor antagonistic effect of the compounds. In order to confirm this possibility, another experiment was done in the isolated atria. Isoproterenol, a β-adrenoceptor agonist caused an increase in both rate and force of the atrial contraction. Pre-incubation of the atria with thiocarbamate glycosides (0.6 mg/ml) for 40 min, however, did not alter the positive chronotropic effect of the isoproterenol. Rather, it appeared to potentiate the force of atrial contraction at lower concentration of the isoproterenol, but it did significantly attenuate the atrial contraction produced by the isoproterenol at higher concentrations. Thus, it is unlikely that the thiocabamate glycosides have a β-adrenergic antagonistic effect on the rat atria.

In conclusion, the thiocarbamate glycosides have hypotensive and negative chronotropic activities in the rats. The mechanisms for these effects are unlikely to involve the compounds acting through the muscarinic receptors or acting as a β-adrenoceptor antagonist of the cardiovascular system. However, the compounds play partly to stimulate release of the nitric oxide from the vascular endothelium to potentiate the vasodilator activity of the compounds. As the compounds caused vasodilatation of skin smooth muscle pre-constricted with phenylephrine or KCl and attenuated inotropic activity of the isoproterenol, it is suggested that the compounds may partly act as a Ca^{++}-channel blocker. However, further studies are needed to clarify this possibility.

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บทคัดย่อ

โดยอาศัยวิธีทางเคมีและทดสอบฤทธิ์ของสารโดยชีววิธี สามารแยกสารผสมของ niazimicin และ niaziminin ซึ่งเป็น thiocarbamate glycosides ได้จากผลมะรุมแห้ง การศึกษาแบบ *in vivo* โดยการฉีดสาร thiocarbamate glycosides (2-16 มก./กก.) เข้าทางหลอดเลือดดำของหนูแร็ทสลบ มีผลทำให้ลดความดันโลหิตและลดอัตราการเต้นของหัวใจ ความแรงในการลดความดันโลหิตและการลดอัตราการเต้นของหัวใจแปรผันโดยตรงกับขนาดของสารที่ฉีดให้แก่สัตว์ทดลอง การยับยั้ง cholinergic receptor ด้วย atropine (1.5 มก./กก.) ไม่มีผลทำให้เกิดการเปลี่ยนแปลงความแรงในการลดความดันโลหิตและการลดอัตราการเต้นของหัวใจโดย thiocarbamate glycosides แต่อย่างใด การยับยั้ง β-adrenergic receptor ด้วย DL-propranolol (0.6 มก./กก.) ไม่มีผลทำให้เปลี่ยนแปลงความแรงในการลดความดันโลหิต แต่มีผลทำให้การลดอัตราการเต้นของหัวใจโดย thiocarbamate glycosides ลดน้อยลง

การศึกษาแบบ *in vitro* พบว่า thiocarbamate glycosides (0.02-0.6 มก./มล.) มีผลทำให้หลอดเลือดแดงทรวงอกที่มีการหดตัวอยู่ก่อนแล้วด้วย phenylephrine เกิดการคลายตัวไม่ว่าหลอดเลือดจะยังมี endothelium อยู่หรือไม่ก็ตาม และผลดังกล่าวไม่สามารถยับยั้งได้ด้วย N^G-nitro-L-arginine (300 mM) หรือ DL-propranolol (10 μM) นอกจากนี้ thiocarbamate glycosides ยังสามารถทำให้เกิดการคลายตัวของหลอดเลือดแดงทรวงอกที่มีการหดตัวอยู่ก่อนแล้วด้วย KCl ผลต่อกล้ามเนื้อ atrium พบว่า thiocarbamate glycosides มีผลยับยั้งอัตราการหดตัวได้เองของกล้ามเนื้อ atrium การ incubate กล้ามเนื้อ atrium ด้วย thiocarbamate glycosides (0.6 มก./มล.) นาน 40 นาที ไม่มีผลทำให้มีการเพิ่มอัตราการหดตัวได้เองของกล้ามเนื้อ atrium โดย isoproterenol เปลี่ยนแปลง แต่มีผลทำให้การเพิ่มความแรงในการหดตัวของกล้ามเนื้อ atrium โดย isoproterenol ลดน้อยลง จากผลการทดลองดังกล่าวชี้ให้เห็นว่า กลไกในการออกฤทธิ์ของ thiocarbamate glycosides ในการลดความดันโลหิตและลดอัตราการเต้นของหัวใจไม่ได้เป็นผลมาจากการกระตุ้นของสารผ่านทาง muscarinic receptor ของประสาทพาราซิมพาเธติค หรือมีผลเป็น β-adrenergic receptor antagonist