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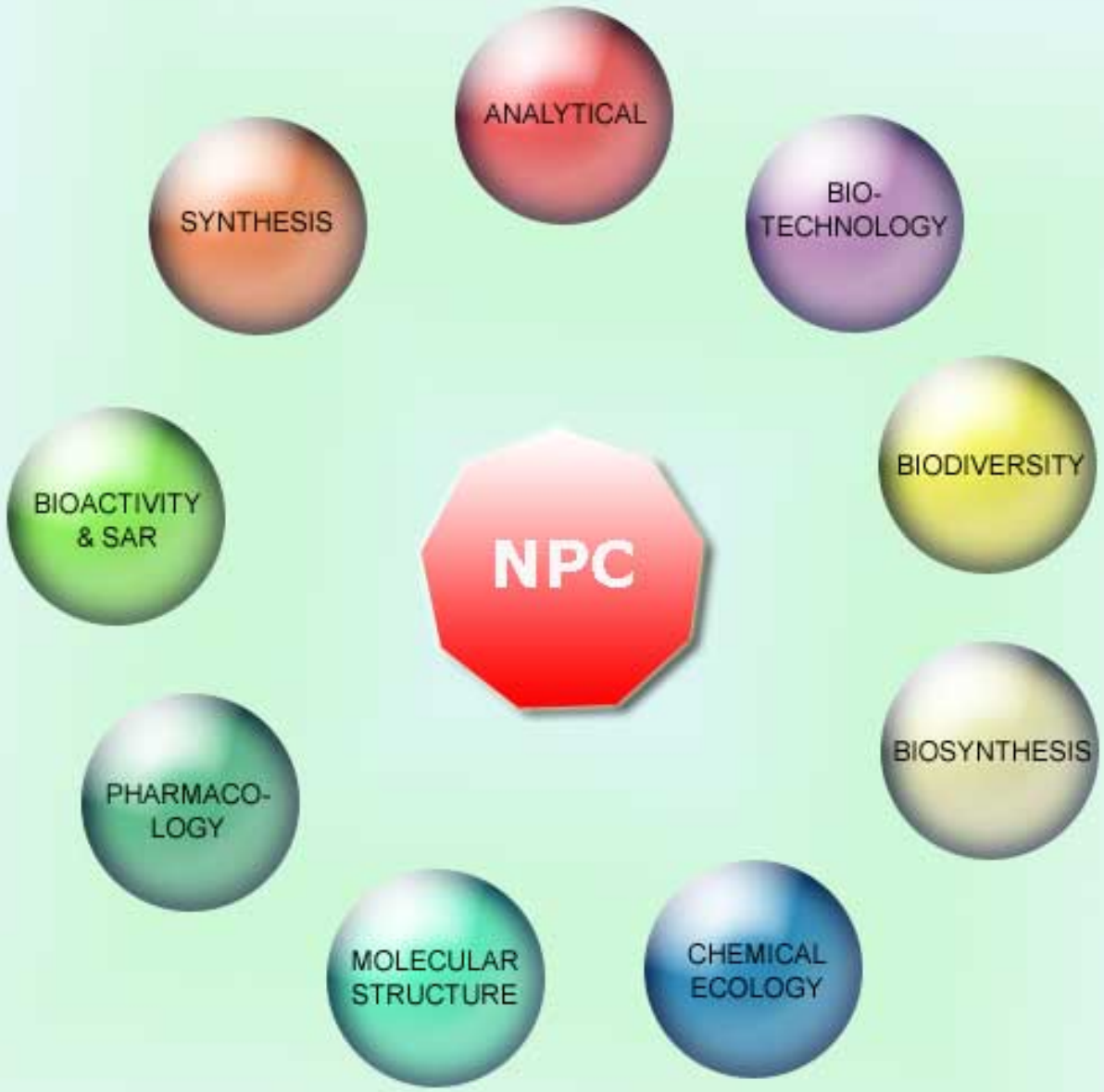

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Vasoconstrictor and Inotropic Effects Induced by the Root Bark Extracts of *Anthocleista schweinfurthii*

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The present study was undertaken to investigate the cardiovascular effect of three extracts from the root bark of *Anthocleista schweinfurthii* Gilg.: an aqueous extract (A_E), a dichloromethane extract (DCMR) and a fraction enriched in cardiac glycoside type compounds (CARDAN). In isolated perfused frog heart, bolus injection of the extracts produced a positive inotropic effect. The responses to A_E and DCMR, but not to CARDAN, were depressed by propranolol. In isolated rat aorta, DCMR produced a transient increase in contractile tension while A_E and CARDAN induced a sustained constriction. A_E vasoconstrictor effect was abolished by phentolamine, while contraction evoked by CARDAN was antagonized by verapamil. In aortic rings contracted in low K^+ media, the addition of K^+ evoked a relaxation, which was abolished by ouabain, depressed by DCMR but not affected by either A_E or CARDAN. These observations indicate that *Anthocleista schweinfurthii* contains substances that promote vasoconstriction and increase cardiac contraction. The effect of DCMR was only partially mediated by inhibition of the Na^+ pump while the mechanism of action of A_E and CARDAN was distinct from the inhibition of the Na^+,K^+-ATPase pump, but could involve adrenergic receptors, or either direct or indirect activation of L-type calcium channels.

Keywords: *Anthocleista schweinfurthii*, Gentianaceae, vasoconstriction, inotropic action.

Anthocleista schweinfurthii Gilg. is a big tree of tropical regions. Leaves, stem and root barks of this plant are eaten by bonobos, an endemic pygmy chimpanzee of the Democratic Republic of Congo and also used in folk medicine to treat several disorders (malaria, cancers, venereal diseases, bacterial diseases) [1-2]. Little information is available about the chemical composition of this plant. A phytochemical screening indicated that some extracts of the root bark were positive to Kedde reagent, considered as specific for cardiac glycosides (mainly cardenolides) [3], so were likely to contain compounds with possible cardiotoxicity due to inhibition of the Na^+,K^+-ATPase pump [4]. As there is no report of cardiovascular activity of extracts of *A. schweinfurthii*, the aim of this study was to evaluate the vascular and the inotropic effects of three extracts of this species, and the potential implication of the Na^+,K^+-ATPase pump in their effect.

TLC screening of the root bark aqueous extract (A_E) and the dichloromethane extract (DCMR) revealed the presence of phenolic compounds (xanthones), triterpenoids and steroids, which were not present in a fraction enriched in cardio-active compounds (CARDAN). Positive pink and purple spots (visible) with Kedde reagent attested the presence of digitalis-like compounds in all extracts, but the TLC spots were more intense in CARDAN than in the other extracts. Digitoxin gave purple spot with Kedde reagent, but none of the extracts showed a Kedde positive spot at a similar Rf as digitoxin.

All extracts exhibited a positive inotropic effect in the isolated frog heart without affecting the heart rate, with CARDAN being the most effective (Figure 1A). Under the same conditions the known positive inotropic agents ouabain (1mM) and adrenaline (1mM) increased cardiac contraction by 46.3±1.3% (n=3) and 72.1±0.7% (n=3), respectively.

The inotropic effect of A_E and DCMR was depressed by the beta-adrenergic receptor antagonist propranolol

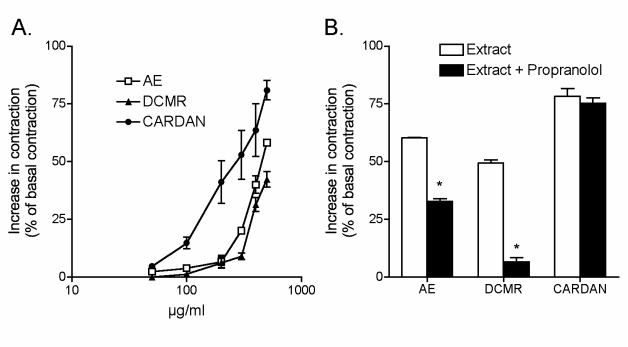

Figure 1: Inotropic effect of *A schweinfurthii* extracts in isolated frog heart preparation.
A. The extracts of *A. schweinfurthii* were bolus injected into the perfusion solution. Data are mean values ± SEM from 3 determinations.
B. The hearts were treated with propranolol (10 µM) before bolus injection of the extracts at 500 µg/mL into the perfusion solution. Data are means ± SEM from 3 determinations. *P<0.05 compared to extract alone.

(10 µM) by 46 % and 87 %, respectively. The effect of CARDAN was unaffected by propranolol (Figure 1B).

Vascular activity of these extracts was tested on isolated rat aorta. A_E and CARDAN (320 µg/mL) produced a rapid, intense and sustained contraction: maximal tension was 87.3±0.7 % (n=3) and 93.8±2.1% (n=3) of the contraction evoked by high KCl solution, respectively (data not shown). The contraction evoked by A_E was completely reversed by phentolamine (1 µM), whereas the contractile response to CARDAN was inhibited by the addition of verapamil (1 µM) into the bath solution. DCMR also produced a rapid vasoconstriction reaching 64.9±1.4% (n=3) of the contraction evoked by high KCl solution, but this effect was transient and contraction relaxed spontaneously (data not shown). The addition of *A. schweinfurthii* extracts on the plateau of the contraction evoked by either high KCl solution or noradrenaline produced a further increase in tension (data not shown), which indicated that these extracts also exhibited potent vasoconstrictive properties.

It is known that cardiac glycosides produce an increase in the contractile force of the heart by inhibiting Na^+,K^+-ATPase [5]. In order to investigate the involvement of the Na^+ pump in the positive inotropic action of *A. schweinfurthii* extracts, aortic rings were pre-contracted in 0 mM KCl for 10 min, which inhibits the activity of the Na^+ pump, and thereafter contracted by the addition of either noradrenaline (1 µM) or *A. schweinfurthii* extracts (320 µg/mL) into the bath solution. The addition of K^+ into the solution then re-activates the Na^+ pump and relaxes the artery, probably following the hyperpolarization of the cell membrane.

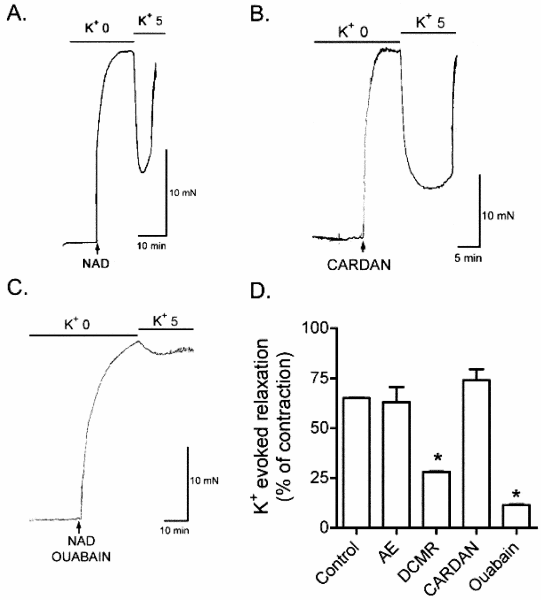

Figure 2: Effect of *A. schweinfurthii* extracts on the relaxation evoked by the addition of K^+ on isolated rat aorta bathed in low K^+ solution.
A - C Typical recordings of the effect of the addition of 5 mM K^+ on the contraction evoked by noradrenaline (NAD – 1 µM, A), the cardiac glycoside-rich fraction from *A. schweinfurthii* (CARDAN, 320 µg/mL, B), or NAD plus ouabain (C) in 0 K^+ solution.
D. Mean values of the relaxation evoked by the addition of 5 mM K^+ in the presence of 320 µg/mL of aqueous extract (A_E), dichloromethane extract (DCMR), cardiac glycoside type-rich fraction (CARDAN) from *A. schweinfurthii* and ouabain (1 mM). Data are means ± SEM from 3 determinations. * P<0.05 compared to control.

The extracts produced an increase in contractile tension equivalent to the response to noradrenaline. In control rings, the addition of KCl (5mM) into the solution evoked a rapid relaxation of 65±0.1% (n=3) of the contractile tension (Figure 2A). This relaxation was nearly abolished in the presence of ouabain (1mM), confirming the role of Na^+,K^+ -ATPase in this process (Figure 2C). A_E and CARDAN did not affect the relaxation evoked by KCl (Figure 2B). In the presence of DCMR, the relaxation was inhibited by 57 % (n=3) (Figure 2D).

Taken together these results suggest that the inhibition of the Na^+ pump could partially explain the inotropic activity of the dichloromethane extract of *A. schweinfurthii*, while the inotropic effect of the aqueous extract and of a fraction enriched in cardiac glycoside-type compounds was not related to the Na^+,K^+-ATPase, at least under these experimental conditions. However, other mechanisms could be responsible for inotropic activity, such as stimulation of adrenergic receptors, which increases cAMP level, PKA activation and Ca^{2+} channel activity [6]. The inotropic effect of A_E and DCMR was, respectively, partially and completely inhibited by propranolol and the vasoconstriction evoked by A_E was reversed by phentolamine. These observations suggest that the

effect of these extracts could be mediated by the activation of adrenergic receptors.

The inotropic effect of CARDAN was unaffected by pre-treatment with propranolol, and its vasocontractile activity was not blocked by phentolamine. These observations allow the exclusion of the activation of adrenergic receptors by CARDAN. However, the aortic contraction evoked by this extract was abolished by the calcium channel blocker verapamil, suggesting that CARDAN could act by activating calcium channels. The effect of CARDAN could then be similar to the reported effects of calcium channel activators: Schramm *et al*. [7] observed that the dihydropyridine Ca^{2+} channel agonist BAYK8644 produces a positive inotropic response *in vitro* by stimulating calcium influx in cardiac muscle. BAYK8644 also enhances calcium influx in other tissues and cells, including vascular smooth muscle, leading to vasoconstriction [8]. The vascular activity of $A_{E,}$, DCMR and CARDAN presented some similarities to the vasocontractile response evoked by bufadienolides [9]. Bagrov [10] described the cardiac glycoside bufalin with a vasoconstrictor activity antagonized by phentolamine suggesting the involvement of alpha-adrenoceptors. The vasocontractile response to A_E was also antagonized by phentolamine, suggesting that this extract could contain a bufalin-like compound.

Further investigations are necessary to identify the compounds in *A. schweinfurthii* extracts that are responsible for these effects and to determine the mechanism of their action.

Experimental

Plant material: Root bark of *Anthocleista schweinfurthii* (Gilg.) was collected in Kinshasa (DRC) in August 2007. The plant was identified at INERA, University of Kinshasa. A voucher specimen has been deposited in the national botanic garden of Belgium (Voucher N°BR0000009752597)

Extraction and fractionation: Dichloromethane extract (DCMR): Air dried and powdered root bark of *A. schweinfurthii* (500 g) was macerated with 95% EtOH for 48 h at room temperature in the dark. After evaporation of the solvent under reduced pressure at 30°C, the dried residue (61.5g) was suspended in 600 mL H_2O and fractioned by partition with light petroleum (3x600 mL, 60°-80°C), then dichloromethane (3x600 mL). The extract was concentrated under vacuum giving 3.9 g of DCMR extract, which was dissolved in ethanol for the tests.

Aqueous extract (A_E): The aqueous extract was prepared by boiling 50 g of dried and powdered root bark in 500 mL distilled water for 20 min. After filtration, the decoction was lyophilized, giving A_E (1.3 g). This was diluted in physiologic solution for *in vitro* experiments on the day of the experiment.

Cardiac glycosides type rich fraction (CARDAN): The classical method for the purification of cardiac glycosides from Digitalis leaves described in the European Pharmacopea was used [11]. Briefly, heterosides were extracted from the dried material with EtOH (50%v/v). Pigments were precipitated with lead acetate and, after filtration and removal of excess lead, the remaining solution was extracted with chloroform. From 600 g root bark, 4.1 g of cardiac glycoside-type rich extract was obtained.

Phytochemical screening: Phytochemical screening was performed to determine the main components of the extracts following the methods described by Farnsworth [12-14]. Tests were conducted for alkaloids, phenolic compounds, tannins, flavonoids, anthocyanins, leucoanthocyanins, quinonic derivatives, polysaccharides, triterpenoids, glycosides and cardiotonic-like compounds. The presence of cardiac glycoside type compounds was confirmed by TLC analysis performed on silicagel $60F_{254}$ layers (Darmstadt, Germany). Each extract (10 µL) was applied to the plates in narrow bands and developed in a saturated TLC chamber using toluene/ethyl acetate/methanol (80:18:2) as mobile phase. After development, the plate was dried and the components were first detected under UV light (366 nm) and then by spraying the plate with Kedde reagent (3,5-dinitrobenzoic acid 2% in ethanol 96%), followed by an aqueous solution of potassium hydroxide 5% [3]. Digitoxin was used as standard.

Animals: The experimental procedures were approved by the Research Ethics Committee of Louvain Drug Research Institute of the Université catholique de Louvain. Male Wistar rats (200-250 g) were used for isolated aortic rings experiments. Frogs (*Rana esculenta*) weighing 25-40 g were used for isolated heart experiments.

Isolated aortic rings contraction: Aorta isometric contraction was measured as described [8]. Briefly, rings of rat thoracic aorta were mounted in a organ chamber filled with warmed (37°C) physiologic solution (in mM: NaCl 122, KCl 5.9, $CaCl_2$ 1.25, $MgCl_2$ 1.25, $NaHCO_3$ 15, glucose 11), bubbled with 95% O_2 and 5% CO_2. The rings were first contracted with high-KCl solution (in mM: NaCl 27, KCl 100, $CaCl_2$ 1.25, $MgCl_2$ 1.25, $NaHCO_3$ 15, glucose 11). Relaxation with

acetylcholine (10^{-6}M) was used to check the functional integrity of the endothelium. Aortas were thereafter challenged with either noradrenaline or the extracts. The amplitude of the contraction induced by the extracts was compared with that produced by 100 mM KCl solution in the same artery ring. The activity of the Na^+,K^+-ATPase was estimated in aortic rings pretreated with nitro-L-arginine (10^{-4}M), an inhibitor of nitric oxide synthase and first exposed to 0 K^+ media for 10 min. The aortas were contracted with either noradrenaline or the tested extracts, with or without ouabain (1mM). The relaxation evoked by the addition of K^+ (5mM) in the bath was expressed as a percentage of the contraction measured before the addition of K^+.

Isolated frog heart: Frogs were stunned by a blow on the head and then pithed. The heart was dissected out and perfused in Frog-Ringer's solution at room temperature (22-25°C) (in mM: NaCl 111, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.8, $NaHCO_3$ 15) using a cannula inserted into the inferior vena cava. The apex of the heart was fixed to a lever connected to an isotonic transducer (Havard). The volume of solution in the cannula was adjusted to 2 mL. Tested compounds, extracts or the same volume of solvent were added into the cannula. The injected volume never exceeded 10 µL to avoid solvent effect. Inotropic effect was expressed as a percentage of the baseline contraction.

Data analysis: Data were expressed as means ± SEM and were analyzed for statistical significance by one-way ANOVA, followed by Bonferroni's test using GraphPad Prism. The level of significance was set at $P<0.05$.

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