Effects of a *Cleome viscosa* extract and its constituents on the beating amplitude of myocardial cell sheets in culture

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Abstract

A methanol extract of the whole plant of *Cleome viscosa* sbowed a positive inotropic effect at a concentration of 0.2 mg/ml on the spontaneous beating of cultured myocardial cell sheets prepared from the mouse embryonic heart. Through bioassay-directed fractionation of the extract, stigmast-4-en-6β-ol-3-one and stigmast-4-en-3,6-dione were isolated as positive inotropic substances, which appreciably increased the beating amplitude of the cell sheets at 0.5-100 μM. Since both compounds also inhibited Na^+-K^+ ATP ase activity concentration dependently at 1-100 μM, their positive inotropic effects on the beating of the cell sheets might be caused by inhibition of this enzyme, similar to the case of ouabain.

Key words *Cleome viscosa*, Capparidaceae, cultured myocardial cell, positive inotropic effect, stigmast-4-en-6β-ol-3-one, stigmast-4-en-3,6-dione.

Introduction

A number of crude drugs have been used for treatment of various types of heart diseases in traditional systems of medicine, such as Ayurvedic, Unani and Chinese medicines, but most of them are not fully evaluated for their efficacy with modern techniques of science. We have previously reported that some of the crude drugs and their constituents, such as flavonoids, triterpenes, coumarins, cinnamic acid derivatives and alkyl amides, induce chronotropic and/or inotropic action(s) on the spontaneous beating of cultured myocardial cells.[1-6]

In the present paper, we describe the effects of some crude drugs used for treatment of heart diseases in Ayurvedic medicine, on the spontaneous beating of cultured myocardial cell sheets, and the isolation of cardioactive substances from the extract through bioassay-directed fractionation.

Materials and Methods

Apparatus : Melting points were measured with a Yanagimoto melting point apparatus (Yanagimoto Co., Kyoto, Japan) and are not corrected. Optical rotations were measured with a JASCO DIP 4 automatic polarimeter at 25°C. Calcium, potassium and sodium contents were determined with an atomic absorption spectrometer (Shimadzu, Kyoto, Japan).

Plant materials : Sarama (the roots of *Boerhaavia diffusa* L., Nyctaginaceae), Ehela (the bark of *Cassia fistula* L., Legminosae), Rammanissa (the whole plant of *Cleome viscosa* L., Capparidaceae), Tippili (the fruit-spikes of *Piper longum* L., Piperaceae), Bebila (the whole plant of *Sida veronicaefolia* LAMK., Malvaceae), Kumbuk (the bark of *Terminalia arjuna* WIGHT et ARN., Compositae), Nika (the root of *Vitex*

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negundo L., Verbenaceae) and Inguru (the rhizome of *Zingiber officinale* L., Zingiberaceae) were purchased at the market of Colombo in Sri Lanka, and their botanical sources were determined by Upali Pilapitiya, Bandaranayake Memorial Ayurvedic Research Institute, Sri Lanka. These specimens are deposited at the Museum of Materia Medica of Toyama Medical and Pharmaceutical University, Toyama, Japan.

Chemicals, enzymes and media : Calcium-containing and calcium-free Eagle's minimal essential media (Eagle's MEM) and Dulbeco's calcium-and magnesium-free phosphate buffered saline were products of Nissui Laboratories (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Whittaker M & A Bioproducts (Walkersville, USA) ; *N*-2-hydroxyethyl piperadine-*N'*-2-ethanesulfonic acid (HEPES) from Dojin Laboratories (Kumamoto, Japan) ; ethyleneglycol bis (*β* aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA) and ouabain from Wako Pure Chemical Industries (Osaka, Japan) ; adenosine 5'-triphosphate (ATP), Na^+-K^+ ATPase, collagenase and fibronectin from Sigma Co. (St. Louis, USA) ; trypsin from Difco Co. (Detroit, USA). Standard solutions of Na^+, K^+ and Ca^{2+} were obtained from Wako Pure Chem. Ind. and Nacalai Tesque Co. (Kyoto, Japan). Other reagents used were of special grade.

Preparation of myocardial cell sheets : According to the methods of Goshima and Tonomura[7] and Namba *et al.*[1] the heart taken from a 14-16 day old embryo of ICR mouse (Sankyo Laboratories, Tokyo, Japan) was digested with a mixture of collagenase (0.025 % ; 77 units/ml) and trypsin (0.125 % ; 313 units/ml) in calcium and magnesium free phosphate buffered saline for 10 min at 37°C. The same volume of Eagle's MEM supplemented with 10 % FCS (standard medium) was added to stop the digestion. The cells were collected by centrifugation at 200 × g and resuspended in the standard medium. Fibroblast-like cells were removed by the method of Polinger.[8] The myocardial cells were seeded onto a glass cover strip coated with fibronectin in a 35 mm plastic Petri dish, and incubated at 37°C under an atmosphere of 5 % CO_2-95 % air for 48 h, leading to the formation of a single cell layer.

Preparation of water and methanol extracts : One hundred grams of each pulverized plant material were extracted with boiling MeOH (500 ml×2) or water (500 ml×2) for 3 h. After filtration, the solutions were evaporated *in vacuo* to give the respective extracts.

Extraction and fractionation of the whole plant C. viscosa : The whole plant of *C. viscosa* (2.5 kg) was extracted two times with boiling MeOH (1,200 ml) for 3 h for each extraction. The combined MeOH solutions were evaporated *in vacuo* to give a residue (MeOH extract ; 50.7 g). The residue was suspended in water (500 ml) and successively extracted with hexane (500 ml×2), EtOAc (500 ml×2) and BuOH (500 ml×2). After evaporating each solvent, hexane-, EtOAc- and BuOH-soluble fractions were obtained in yields of 10.3, 11.0 and 9.5 g, respectively. The hexane-soluble fraction was applied to a column of silica gel (3.0×300 cm) and eluted with hexane-$CHCl_3$ (10 : 2, 10 : 4, 10 : 6, 10 : 8 and 1 : 1) and then with $CHCl_3$. Each eluate (50 ml each) was collected and monitored with TLC (solvent system, hexane-$CHCl_3$=1 : 1). The $CHCl_3$-eluate was further applied to a column of charcoal-celite (2.5×30 cm), which was eluted with MeOH-$CHCl_3$ (4 : 1, 3 : 1, 2 : 1 and 1 : 1, 50 ml each) to give stigmast-4-en-3-one (**1**) (54 mg), stigmast-4-en-6*β*-ol-3-one (**2**) (30 mg), stigmast-4-en-3,6-dione (**3**) (133 mg) and stigmast-5-en-3*β*-ol-7-one (**4**) (18 mg).

Stigmast-4-en-3-one (1) : White prisms from hexane. mp. 94-97°C. $[\alpha]_D$+59.8° (*c*=0.04, MeOH).

Stigmast-4-en-6β-ol-3-one (2) : White prisms from hexane. mp. 189-193°C. $[\alpha]_D$+35.8° (*c*=0.03, MeOH).

Stigmast-4-en-3,6-dione (3) : Colorless needles from hexane. mp. 102-105°C. $[\alpha]_D$-22.2° (*c*=0.02, MeOH).

Stigmast-5-en-3β-ol-7-one (4) : White prisms from hexane. mp. 98-102°C. $[\alpha]_D$-31.1° (*c*=0.04, MeOH).

Measurement of the beating rate (BR) and the beating amplitude (BA) : The BR and the BA of cultured myocardial cell sheets were measured as reported previously.[9] Briefly, the cell sheet which adhered to the cover glass was mounted upside-down on a small chamber in an acrylic plate. The chamber was filled with a standard medium, which was then replaced with a new standard medium containing an extract, its fractions or its constituents dissolved in DMSO. The final concentration of DMSO was 1 %.

The beating of the cell sheet was observed under a phase-contrasted microscope (Olympus IMT-2) at 37 ±1°C. The BR was monitored by a photo-electric recording method and expressed as relative value to an initial BR (average 140±15 beats/min, n=9) observed just before application of a sample. For measuring the BA, cell images observed through the microscope were monitored with a TV camera (Olympus FCD-10) and recorded with a video-camera cassette recorder (National AC-6300). The recorded images were displayed on a picture monitor (NEC PC-KD 854) and were analyzed with a personal image analyzer (PIAS LA-500) equipped with a microcomputer (NEC PC-9801). Several cell images with high light intensity were selected and their central positions were recorded at 0.07 sec intervals. Displacement of each central position, caused by spontaneous beating of the cell sheet, was monitored. The BA was measured as a distance between peak and trough in the oscillogram.

Inhibitory activity against Na^+-K^+ ATPase : Na^+-K^+ ATPase activity was measured by the method of Jones *et al.*[10] A mixture containing 50 mM histidine, 3 mM $MgCl_2$, 1 mM Tris/EGTA, 100 mM NaCl, 10 mM KCl and 3 mM Tris/ATP (pH 7.4) with or without a test sample, was incubated for 60 min at 37°C and released inorganic phosphate (*Pi*) was determined by the method of Baginski *et al.*[11]

Measurement of sodium, potassium and calcium contents : Contents of inorganic elements were determined by atomic absorption spectrometry after the extracts were heated with concentrated HNO_3.

Statistical analysis : The data are shown as mean ±S.E.M. and statistical significance was evaluated by the Dunnett method.

Results and Discussion

For *in vitro* evaluation of some crude drugs used for cardiac diseases in traditional Ayurvedic medicine, water and methanol extracts of eight different crude drugs were tested for their effects on the beating amplitude (BA) and the beating rate (BR) using a myocardial cell assay system. Of the crude drug extracts examined, a methanol extract of the whole plant of *Cleome viscosa* L. significantly increased the beating amplitude, while water and methanol extracts of the bark of *Cassia fistula* L. and a methanol extract of the bark of *Terminalia arjuna* WIGHT et ARN. tended to increase the BA at a concentration of 0.2 mg/ml, 10 min after the test samples were applied to the culture medium (Table I). Under the conditions, a control (1 % DMSO) showed no appreciable change in the BA and BR, while 10 μM ouabain induced a signifi-

Table I Effects of water and MeOH extracts of Ayurvedic medicines on the beating amplitude (BA) of cultured myocardial cell sheets in a standard medium containing 2.1 mM calcium ions.

Crude drug	Relative BA (%)	
	Water extract	MeOH extract
Control (1 % DMSO)	103.4±2.4	103.5±1.7
Ouabain (1.0×10^{-5}M)	118.7±2.1**	119.4±20**
Roots of *Boerhaavia diffusa*	106.8±7.9	101.3±4.5
Bark of *Cassia fistula*	112.1±3.9	110.7±3.2
Whole plant of *Cleome viscosa*	107.3±3.1	117.3±3.5*
Fruit-spikes of *Piper longum*	98.6±4.1	89.9±4.1
Whole plant of *Sida cordifolia*	97.3±6.8	104.8±3.4
Bark of *Terminalia arjuna*	103.1±4.2	114.5±4.1
Roots of *Vitex negundo*	100.9±5.1	103.7±6.3
Rhizomes of *Zingiber officinale*	101.2±7.3	99.1±5.3

The BA measured in a standard medium (initial BA) is defined as 100 % and the BA at 10 min after replacing the medium with a new standard medium containing 1 % DMSO with or without a test sample is represented as percentage of the initial BA. The concentration of crude drug extracts was 0.2 mg/ml. The values of relative BA are expressed as mean±S.E.M. of five independent experiments. Significantly different from control : *, $p<0.05$; **, $p<0.01$.

cant inotropic action.

On the other hand, water extracts of *C. fistula* (bark) and *T. arjuna* (bark) and methanol extract of *C. fistula* (bark), *C. viscosa* (whole plant), *S. cordifolia* (whole plant) and *Z. officinale* (rhizome) significantly decreased the BR up to the levels of approx. 80-90 % of the initial value (Table II). Under the conditions, a positive control of norepinephrine significantly increased the BR (132-135 % of the initial value) at 10 μM.

Since the spontaneous beating of myocardial cell sheets is appreciably influenced by extra-cellular ion concentrations, Na^+, K^+ and Ca^{2+} contents of the extracts were determined by atomic absorption spectrometry. Of these extracts, the water extract of *Z. officinale* (rhizomes) showed the highest ion content (Ca^{2+}, 30.0×10^{-5} mol/g ; K^+, 117.6×10^{-5} mol/g ; Na^+, 77.5×10^{-5} mol/g) (Table III). At a concentration of 0.2 mg/ml in the above experiment, the extract included 0.06 mM Ca^{2+}, 0.24 mM K^+ and 0.16 mM Na^+, which were quite low in concentration, compared to the respective ions present in the standard medium (2.1 mM Ca^{2+}, 5.36 mM K^+ and 116 mM Na^+). Therefore, the effects of inorganic elements originated from the extracts may be negligible to the spontaneous beating of the cell sheets.

To isolate the BA-stimulating substances from the whole plant of *C. viscosa*, the methanol extract was partitioned between water and various organic solvents (Fig. 1), and the fractions were assayed for

Table II Effects of the water and MeOH extracts on the beating rate (BR) of cultured myocardial cell sheets in a standard medium containing 2.1 mM calcium ions.

Crude drug	Relative BR (%)	
	Water extract	MeOH extract
Control (1 % DMSO)	100.0±0.8	100.0±0.8
Norepinephrine (1.0×10^{-5}M)	132.1±4.3**	134.5±3.4**
Roots of *B. diffusa*	97.0±2.5	97.3±2.1
Bark of *C. fistula*	89.7±4.4*	88.1±4.6*
Whole plant of *C. viscosa*	97.4±1.8	78.2±5.5**
Fruit spikes of *P. longum*	99.5±1.7	105.8±3.1
Whole plant of *S. cordifolid*	96.5±2.3	83.3±4.7*
Bark of *T. arjuna*	89.7±3.0*	97.3±2.5
Roots of *V. negundo*	97.1±2.3	100.5±2.8
Rhizomes of *Z. officinale*	92.1±3.0	79.5±3.4**

The BR measured in a standard medium (initial BR) is defined as 100 % and the BR at 10 min after replacing the medium with a new standard medium containing 1 % DMSO with or without a test sample is represented as percentage of the initial BR. The concentration of crude drug extracts was 0.2 mg/ml. The values of relative BR are expressed as mean±S.E.M. of five independent experiments. Significantly different from control : *, $p<0.05$; **, $p<0.01$.

Table III Ion contents of various extracts used in this experiment.

Crude drug	Ion content (10^{-5} mol/g)					
	Water extract			MeOH extract		
	Na^+	K^+	Ca^{2+}	Na^+	K^+	Ca^{2+}
Roots of *B. diffusa*	6.0	8.3	1.9	0.0	4.1	0.0
Barks of *C. fistula*	52.8	19.2	12.8	36.4	14.2	1.8
Whole plants of *C. viscosa*	6.8	17.6	1.5	0.9	2.2	0.0
Fruit-spikes of *P. longum*	4.8	22.6	3.2	0.0	0.0	0.0
Whole plants of *S. cordifolia*	5.8	14.6	0.7	0.6	0.7	0.4
Barks of *T. arjuna*	5.0	13.3	3.8	2.6	1.5	0.0
Roots of *V. negundo*	39.9	106.7	28.4	5.5	30.7	0.1
Rhizomes of *Z. officinale*	77.5	117.6	30.0	65.0	55.6	14.5

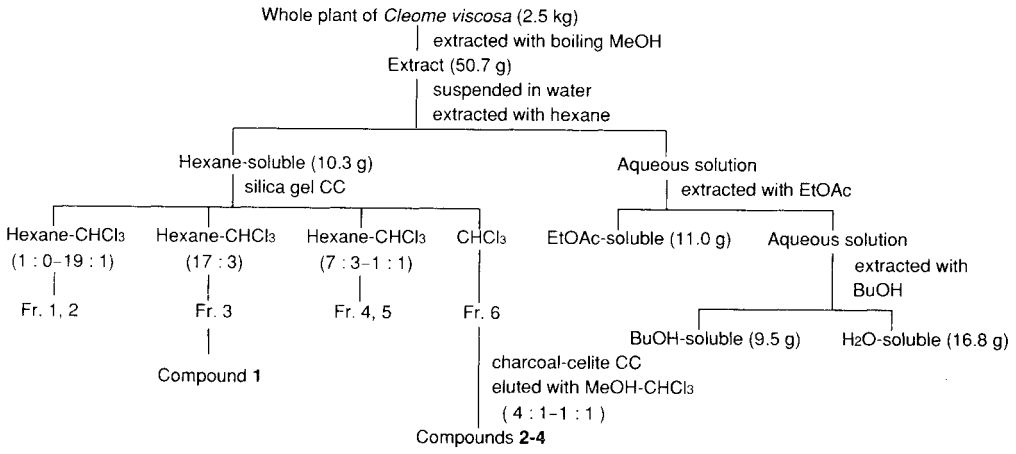

Fig. 1 Fractionation of a methanol extract of *C. viscosa*.

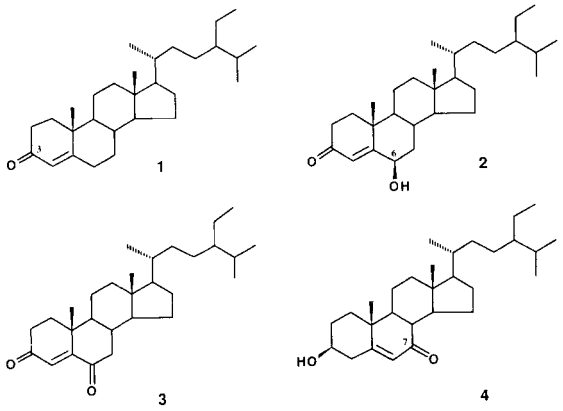

Fig. 2 Structures of compounds isolated from *C. viscosa*.

their effects on the BA of the cell sheets. Both the hexane-soluble and the BuOH soluble fractions were shown to increase the BA up to approx. 120 % and 110 % of control, respectively, at a concentration of 0.02-0.2 mg/ml (Table IV), while the EtOAc-soluble fraction and the remaining aqueous fraction were inactive. The hexane-soluble was further fractionated by column chromatography on silica gel and charcoal-celite to give four steroidal compounds. These were identified as stigmast-4-en-3-one (**1**), stigmast-4-en-6β-ol-3-one (**2**), stigmast-4-en-3,6-dione (**3**) and stigmast-5-en-3β-ol-7-one (**4**) by spectroscopic comparisons of the ^{1}H-NMR, ^{13}C-NMR and mass

Table IV Effects of a MeOH extract of *C. viscosa* and its fractions on the beating amplitude (BA) of cultured myocardial cell sheets.

Material	Concentration (mg/ml)	Relative BA (%)
Control (1 % DMSO)		99.2±1.9
Ouabain (1.0×10^{-5}M)		114.1±2.1**
MeOH extract	0.02	110.9±2.0*
	0.20	111.3±2.1*
Hexane soluble	0.02	123.6±3.5**
	0.20	125.4±4.1**
EtOAc soluble	0.02	99.2±4.3
	0.20	107.3±4.2
BuOH-soluble	0.02	111.2±1.9*
	0.20	111.0±2.3*
Water soluble	0.02	96.9±2.1
	0.20	98.2±2.2

The BA measured in a standard medium (initial BA) is defined as 100 % and the BA at 10 min after replacing the medium with a new standard medium containing 1 % DMSO with or without a test sample is represented as percentage of the initial BA. The values of relative BA are expressed as mean±S.E.M. of five independent experiments. Significantly different from control : *, $p<0.05$; **, $p<0.01$.

spectra with those of reported data[12] and of authentic **2** and **3**, which were synthesized from sitosterol.[13-15]

Of these steroids, **2** and **3** significantly enhanced the BA of the cell sheets at a concentration of 10 μM, while **1** and **4** were found to be inactive (Table V). Under the conditions as ouabain increased the BA concentration-dependently, **2** and **3** also increased the BA at concentrations of 0.5 100 μM (Fig. 3). In addi-

Table V Effects of compounds from *C. viscosa* on the beating amplitude (BA) of myocardial cell sheets.

	Relative BA (%)
Control (1% DMSO)	98.7±1.1
Ouabain	115.8±1.1**
Stigmast-4-en-3-one (**1**)	100.7±2.1
Stimast-4-en-6β-ol-3-one (**2**)	114.7±3.1**
Stigmast-4-en-3,6-dione (**3**)	115.8±2.3**
Stigmast-5-en-3β-ol-7-one (**4**)	101.2±1.7

The concentration of each compound was 10 μM. The values of relative BA at 10 min after teratment with test samples are expressed as mean±S.E.M. of five independent experiments. Significantly different from control : **, $p<0.01$.

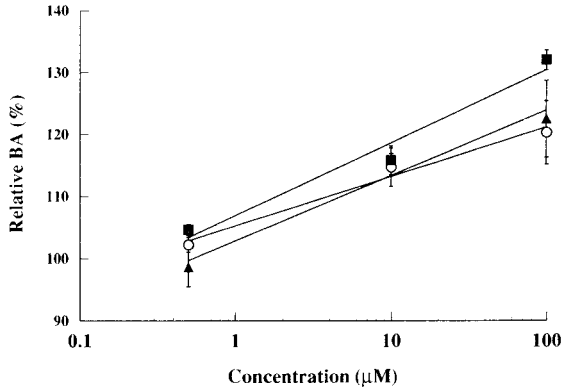

Fig. 3 Effects of stigmast-4-en-6β-ol-3-one (**2**), stigmast-4-en-3,6-dione (**3**) and ouabain on the beating amplitude at various concentrations.
The beating amplitude was measured under the same conditions described in the legend to Table V except for concentrations of test samples. ■, ouabain ; ▲, stigmast-4-en-6β-ol-3-one (**2**) ; ○, stigmast-4-en-3,6-dione (**3**)

tion, all of compounds **1**-**4** did not appreciably influence the BR at concentrations of 0.5-10 μM, in contrast with the case of *Ganoderma* sterols and triterpenes that induced not only a positive inotropic action but also negative chronotropic action on the beating of myocardial cell sheets.[3]

Since Beach *et al.*[16] and Akera *et al.*[17] have reported that there is a correlation between the Na^+-K^+ ATPase inhibition and the positive inotropic effect of cardiac glycosides, we investigated the effects of these compounds against Na^+-K^+ ATPase. Under such conditions as ouabain inhibited Na^+-K^+ ATPase activity by 52% at a concentration of 10 μM, **2** and **3** inhibited the activity by 23-25% at the same concentration (Table VI). However, **1** and **4** showed no remarkable inhibition against this enzyme. Furthermore, inhibitory effects of **2** and **3** on Na^+-K^+ ATPase were found to increase in potency depending on their concentrations in a range of 1-100 μM. These findings revealed that the inotropic effects induced by **2** and **3** might be associated with the Na^+-K^+ ATP ase inhibition as those by ouabain and other cardiac glycosides.

Table VI Effects of compounds from *C. viscosa* on Na^+ K^+ ATPase activity.

	Inorganic phosphorus release (nmol/mg protein/h)
Control (1% DMSO)	17.3±0.2
Ouabain	8.3±0.2**
Stigmast-4-en-3-one (**1**)	17.1±0.7
Stigmast-4-en-6β-ol-3-one (**2**)	13.3±0.5**
Stigmast-4-en-3,6-dione (**3**)	12.9±0.4**
Stigmast-5-en-3β-ol-7-one (**4**)	17.3±0.6

The concentration of each compound was 10 μM. The reaction mixture was incubated for 60 min at 37 C and released inorganic phosphate was determined. The vaues are expressed as mean±S.E.M. (n 5). Significantly different from control (1% DMSO) : **, $p<0.01$.

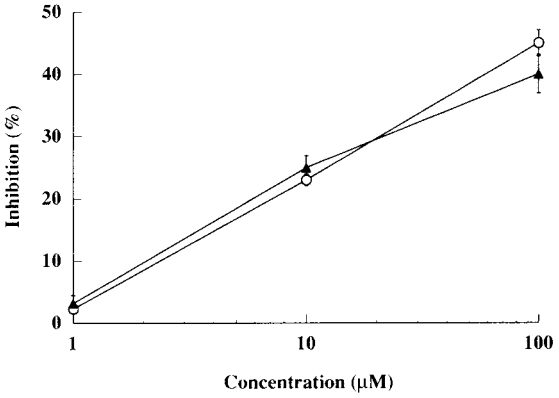

Fig. 4 Inhibitory effects of stigmast-4-en-6β-ol-3-one (**2**) and stigmast-4-en-3,6-dione (**3**) on Na^+ K^+ ATPase activity at various concentrations.
Inhibitory effects were determined under the same conditions described in the legend to Table VI except for concentrations of **2** and **3**. ▲, stigmast-4-en-6β-ol-3-one (**2**) ; ○, stigmast-4-en-3,6-dione (**3**)

The roots, seeds and whole plant of *C. viscosa* are traditionally used as cardiac stimulants[18] and the present *in vitro* experiment verified in part the possible efficacy of the drug for heart failures. Thus, the cultured myocardial cell system provides a useful tool for evaluation of cardio-active crude drugs and their components.

和文抄録

アーユルヴェーダ薬物である *Cleome viscosa* の全草から得たメタノールエキスは，マウスの胎仔心臓から調製した培養心筋細胞シートの自動拍動に対して，0.2 mg/ml の濃度で陽性変力作用を示した。この生物検定を指標にエキスの分画を行い，stigmast-4-en-6β-ol-3-one, stigmast-4-en-3,6-dione を活性物質として単離した。これら化合物は 0.5-100 μM の濃度で細胞シートの振幅を濃度依存的に増加させた。両化合物とも上記濃度範囲で Na^+-K^+ ATPase を濃度依存的に阻害することから，ウアバインと同様にこの酵素を阻害することにより陽性変力作用を引き起こしたものと思われる。

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