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Pharmacological Action of Some Chemical Constituents from *Ilex cornuta* Lindl. ex Paxt. for Cardiac Diseases

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ABSTRACT

Fourteen compounds were isolated from the dried leaves of *Ilex cornuta* Lindl. ex Paxt. and were identified based on chemical and spectral evidence, and for which pharmacological tests of compounds 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 [3-β-*O*-(β-D-glucopyranosyl-α-L-arabinopyranosyl)-pomolic acid (28→1)-β-D-glucopyranosyl ester] were carried out to evaluate the effect for cardiac diseases. The results showed that 3,4-dihydroxyl cinnamic acid and daucostorol did not give protection to mice against hypoxia, ventricular fibrillation caused by chloroform and myocardial ischemia induced by pituitrin, Gouguside 4 performed similarly as the former two compounds but protected rats against pituitrin-induced myocardial ischemia. 3,4-dihydroxyl cinnamic acid and daucostorol had no influence on the cardiac rate of apo-cardiac muscle, crown sphygmo-flow and contractility of cardiac muscle of guinea pigs either. Gouguside 4 did not influence the cardiac rate of apo-cardiac muscle, crown sphygmo-flow, but decreased contractility of cardiac muscle of guinea pigs.

Keywords: angiocardiopathy, 3,4-dihydroxyl cinnamic acid, daucostorol, Gouguside 4 [3-β-*O*-(β-D-glucopyranosyl-α-L-arabinopyranosyl)-pomolic acid (28→1)-β-D-glucopyranosyl ester]

INTRODUCTION

Leaves of some *Ilex* species are used to make a widely consumed beverage in China called "*KuDingCha*", "*KuCha*" (Jiangsu New Medical College 1986; Chau and Wu 2006; Heck and Mejia 2007). In addition to its common consumption, *KuDingCha* is also popular for its health benefits. *Ilex cornuta* Lindl. et Paxt. is an evergreen shrub growing in Eastern and Southern China. The dried leaves of *I. cornuta* are traditionally employed as one of the staple *Ilex* species for "*KuDingCha*" or as an ingredient in dietary supplements. In addition, the aqueous decoction made from its leaves is commonly used as a contraceptive, cardiovascular or antibacterial agent (Jiangsu New Medical College 1986; Qin *et al.* 1986; Zhang *et al.* 2003). In order to search for active substances from this plant, chemical constituents need to be isolated and pharmacological tests should be carried out.

Some chemical constituents have been isolated from the leaves of *I. cornuta* (Tsutomu *et al*. 1982; Qin *et al*. 1986, 1988a, 1988b). In this research 14 compounds were isolated and identified from the dried leaves of this plant. It concerns gouguside 1 (pomolic acid 3-β-*O*-α-L-arabinopyranoside); gouguside 2 [3-β-*O*-D-glucopyranoside-pomolic acid (28→1)-β-D-glucopyranosyl ester]; gouguside 4 [3-β-*O*-(β-D-glucopyranosyl-α-L-arabinopyranosyl)-pomolic acid (28→1)-β-D-glucopyranosyl ester]; gouguside 5 (pomolic acid 3-β-*O*-α-L-2′-acetoxyl-arabinopyranosyl-28-*O*-β-D-glucopyranosyl ester, namely cornutaside A); gouguside 6 [3-β-*O*-(β-D-glucopyranosyl-(1→2)-4'-acetoxyl-α-L-arabinopyranosyl)-pomolic acid (28→1)-β-D-glucopyranosyl ester]; gouguside 7 (3-β-*O*-α-L-arabinopyranosyl-pomolic acid 28-*O*-β-D-glucopyranosyl erter); daucostorol; 2,4-dihydroxybenzoic acid; 3,4-dihydroxyl cinnamic acid and five long chain fatty acids. Pharmacological tests of compounds 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 were carried out to evaluate their effect for cardiovascular diseases.

MATERIALS AND METHODS

Isolation of chemical constituents

Five kg powder of dried leaves of *I. cornuta* was extracted three times with 70% ethanol solution. The combined ethanol extracts were concentrated under vacuum in a Rotavapor to a proper volume to obtain a total extract. The concentrated extract was dispersed in four times its volume of water, then extracted with petroleum ether, dichloromethane, ethyl acetate (EtOAc) and *n*-butanol, respectively. The EtOAc-extract that weighed 28.5 g was evaporated *in vacuo* and was subjected to column chromatography on silica gel, using a solvent gradient system from petroleum ether to EtOAc with gradually increasing polarity. The eluted portions were monitored by TLC and similar fractions were combined into nine fractions. The fraction eluted by the mixture of petroleum ether: EtOAc (6:4) was further subjected to a silica gel column with the petroleum ether: EtOAc gradient system to yield pure white crystals (3, 4-dihydroxyl cinnamic acid, 1:1).

The *n*-butanol extract that weighed 129 g was dissolved in methanol and treated with diethyl ether to get two parts. The diethyl ether soluble parts weighed 28 g and was evaporated and subjected to column chromatography on silica gel, using a solvent gradient system from $CHCl_3$ to MeOH with gradually increasing polarity to obtain daucostorol (8:2). The diethyl ether insoluble fraction that weighed 21.3 g was subjected to column chromatography on silica gel and the fraction eluted by $CHCl_3$:MeOH: H_2O (10:4:1) was recrystallized with a mixed solvent of diethyl ether and MeOH to yield pure gouguside 4.

Animals

ICR mice weighing 18-22 g or 25-32 g, Sprague-Dawley rats weighing 180-220 g and guinea pigs weighing about 300 g were used in all experiments, which were supplied by the Center of Experimental Animal, China Pharmaceutical University.

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Original Research Paper

Experiments on mice against hypoxia

Mice of both sexes weighing 18-22 g were divided into eight groups of 10 mice each at random. Group 1 served as the control and received normal saline only. Group 2 was *i.g.* administered with propranolol (Changzhou Kangpu Medicine Industry Co., Ltd, 10 mg/Table) at a dose of 20 mg/kg body weight. The other six groups were *i.v.* treated with 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 at doses of 20 and 40 mg/kg body weight, respectively. Five minutes after administration, the mice were put in an airtight jar (60 mL) with 5 g Soda-lime to absorb the CO_2 and H_2O, and the survival time was recorded.

Experiments on mice against ventricular fibrillation

Mice of both sexes weighing 25-32 g were divided into eight groups of 10 mice each at random. Group 1 served as the control and received normal saline only. Group 2 was *i.g.* administered with propranolol at a dose of 20 mg/kg body weight. The other six groups were *i.v.* treated with 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 at doses of 4, 20 and 40 mg/kg body weight, respectively. Five minutes after the administration, the mice were put in an airtight jar (125 mL) with cotton soaked by chloroform. The ratio of resistance to ventricular fibrillation was observed after thoracotomy as soon as the mice stopped breathing.

Experiments on Sprague-Dawley rats in myocardial ischemia

SD rats of both sexes weighing 180-220 g were divided into eight groups of 5 rats each at random. Group 1 served as the control and received normal saline only. Group 2 was *i.g.* administered with propranolol at a dose of 20 mg/kg body weight. The other six groups were *i.v.* treated with 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 at doses of 4, 20 and 40 mg/kg body weight, respectively. Five minutes after the administration, each animal was anesthetized intraperitonally with 20% urethan (Shanghai Caoyang Second Middle School Chemical Plant, 500 g/flash) at a dose of 1.2 g/kg body weight. Then the rats were connected to an II-link electrocardiograph and the femoral vein was catheterized for injection of pituitrin (Nanjing Xintian Biomedicine Co., Ltd, 6 U/mL) at a dose of 0.75 u/kg within 10 sec. The II-link electrocardiographs were recorded at the times of 30 seconds before the injection and 1, 3, 5, 10, 20 minutes after the injection, and the height of the T Wave and the p-p interphase were calculated.

Experiments on coronary blood flow of guinea pigs

Guinea pigs of both sexes weighing about 300 g were knocked at the head to death and the hearts were excised rapidly and immersed in cold Krebs-Henseleit buffer solution (4°C). The heart was attached to a Langendorff apparatus *via* the aorta for retrograde perfusion with Krebs-Henseleit buffer solution. The perfusion was equilibrated with 95% O_2 and 5% CO_2, maintained at 37°C and pH 7.4. Perfusion pressure was maintained at 80 cm H_2O. Different concentrations of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 were injected to the perfusion system and the effluent was connected to a force transducer (JZ101 muscle force transducer, Gaobeidian, Hebei, China) to a LMS-2B physiological recorder (type LMS-2B, Chengdu Electronical Instrument Co., China). Salvia injection (Shanghai Bio-chemical Medicine Company, 3 g/2 mL) and Deslanoside injection (Shanghai First Pharmaceutical Factory, 0.4 mg/2 mL) were used as control drugs. The heart rate was continuously monitored. Coronary flow was measured by timed collection of coronary effluent.

Declaration of animal ethics

The experimental method, observational index and executing animal method conform to the Administration Rule of Laboratory Animal of Institute of Botany, Jiangsu Province and the Chinese Academy of Sciences.

RESULTS

Pharmacological effect of three compounds isolated from *I. cornuta* to mice against hypoxia

From **Table 1**, it can be concluded that the survival time of mice in groups with 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 respectively with different doses was not significantly prolonged. On the contrary, 20 mg/kg of propranolol significantly prolonged the survival time of mice in anoxic condition.

Pharmacological effect of three compounds isolated from *I. cornuta* to mice against ventricular fibrillation caused by chloroform

Table 2 shows that the ratio of resistance to ventricular fibrillation of propranolol group was 100%, that of the control group and compound groups were all zero. It could be concluded that 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 did not afford protection to mice against ventricular fibrillation caused by chloroform.

Pharmacological effect of three compounds isolated from *I. cornuta* to p-p interphase of rats against myocardial ischemia induced by pituitrin

In **Tables 3** and **4**, it is shown that after *i.v.* administration of pituitrin, the height of the T Wave of the control group significantly declined and the p-p interphases were significantly prolonged, which indicated that administered serious myocardial ischemia appeared in rats. The rats that suffered from myocardial ischemia to which 20 and 40 mg/kg of gouguside 4 was *i.v.* administered, the height of the T Wave was significantly lowered. The administration of 40 mg/kg gouguside 4 also significantly extended the p-p interphase, which implied that gouguside 4 could provide protection to rats against myocardial ischemia induced by pituitrin. 3,4-dihydroxyl cinnamic acid and daucostorol showed no pro-

Table 1 Pharmacological effect of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 isolated from *I. cornuta* to mice against hypoxia.

Group	Dose (mg/kg)	Survival time (min)
control	--	21.2 ± 4.264
propranolol	20	46.7 ± 11.605**
3,4-dihydroxyl cinnamic acid	20	20.2 ± 4.050
	40	23.0 ± 3.887
daucostorol	20	21.7 ± 4.762
	40	21.4 ± 6.004
gouguside 4	20	22.8 ± 3.120
	40	22.5 ± 3.808

Statistical analysis of the difference of the means was calculated using Student's *t*-test.
Results are reported as mean ± standard error of the mean. ** p<0.01: Compared with control.

Table 2 Pharmacological effect of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 isolated from *I. cornuta* to mice against ventricular fibrillation caused by chloroform.

Group	Dose (mg/kg)	№ of mice with ventricular fibrillation	Ratio of resistance to ventricular fibrillation (%)
control	--	10	0**
propranolol	20	10	100
3,4-dihydroxyl cinnamic acid	20	10	0**
	40	10	0**
daucostorol	20	10	0**
	40	10	0**
gouguside 4	20	10	0**
	40	10	0**

Statistical analysis of the difference of the means was calculated using Student's χ^2-test.
Results are reported as mean ± standard error of the mean. ** p<0.01: Compared with propranolol.

Table 3 Pharmacological effect of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 isolated from *I. cornuta* to height of T wave of rats against myocardial ischemia caused by pituitrin.

Group	Dose (mg/kg)	Height of T Wave (mv)						
		Before administration	After administration					
			30 sec	1 min	3 min	5 min	10 min	20 min
control	--	0.226±0.043	0.138±0.124	0.106±0.098#	0.120±0.082#	0.144±0.062#	0.178±0.070	0.254±0.083
propranolol	20	0.310±0.069	0.298±0.090*	0.302±0.063**	0.282±0.069**	0.262±0.047**	0.274±0.067	0.306±0.062
3,4-dihydroxyl cinnamic acid	20	0.260±0.093	0.294±0.134	0.238±0.084	0.228±0.093	0.220±0.051	0.204±0.089	0.222±0.090
	40	0.244±0.058	0.290±0.119	0.208±0.072	0.206±0.093	0.214±0.106	0.248±0.069	0.278±0.067
daucostorol	20	0.300±0.068	0.272±0.200	0.216±0.145	0.210±0.098	0.222±0.064	0.212±0.056	0.292±0.068
	40	0.274±0.034	0.166±0.164	0.158±0.193	0.190±0.137	0.232±0.058	0.242±0.024	0.282±0.033
gouguside 4	20	0.296±0.071	0.226±0.123	0.256±0.100*	0.292±0.138*	0.246±0.073*	0.288±0.078*	0.302±0.061
	40	0.250±0.071	0.242±0.179	0.234±0.058*	0.266±0.076*	0.274±0.054**	0.240±0.061	0.272±0.054

Statistical analysis of the difference of the means was calculated using Student's *t*-test.
Results are reported as mean ± standard error of the mean. *p<0.05; **p<0.01: Compared with control.

Table 4 Pharmacological effect of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 isolated from *I. cornuta* to p-p interphase of rats against myocardial ischemia caused by pituitrin.

Group	Dose (mg/kg)	p-p interphase (s)						
		Before administration	After administration					
			30 sec	1 min	3 min	5 min	10 min	20 min
control	--	0.146±0.012	0.206±0.071	0.216±0.064*	0.220±0.044**	0.228±0.036**	0.205±0.027**	0.206±0.082
propranolol	20	0.186±0.047	0.257±0.046*	0.254±0.055	0.259±0.028*	0.247±0.031*	0.234±0.053	0.206±0.038
3,4-dihydroxyl cinnamic acid	20	0.176±0.045	0.222±0.046	0.226±0.038	0.237±0.044	0.234±0.041	0.201±0.031	0.178±0.036
	40	0.185±0.053	0.247±0.044	0.238±0.034	0.230±0.030n	0.228±0.031	0.202±0.046	0.206±0.048
daucostorol	20	0.194±0.060	0.211±0.054	0.225±0.053	0.229±0.047	0.227±0.051	0.217±0.045	0.177±0.040
	40	0.174±0.046	0.232±0.059	0.230±0.039	0.276±0.077*	0.238±0.044	0.241±0.048	0.191±0.052
gouguside 4	20	0.164±0.034	0.203±0.068	0.191±0.042	0.195±0.048	0.198±0.042	0.202±0.031	0.188±0.022
	40	0.153±0.023	0.196±0.036	0.273±0.082*	0.247±0.058**	0.238±0.040**	0.218±0.051*	0.157±0.023

Statistical analysis of the difference of the means was calculated using Student's *t*-test.
Results are reported as mean ± standard error of the mean. *p<0.05; **p<0.01: Compared with control.

Table 5 Pharmacological effect of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 isolated from *I. cornuta* to coronary blood flow of guinea pigs.

Group	Dose (mg/kg)	Coronary blood flow (%)							
		Before administration	After administration						
			1 min	2 min	3 min	4 min	5 min	10 min	15 min
control	--	0	1.38±1.10	1.16±0.65	1.16±0.90	0.95±0.96	1.80±1.07	1.48±0.93	1.92±1.09
Salvia injection	150.0	0	17.72±1.83**	28.24±11.93**	21.80±5.59**	18.36±8.02**	11.27±8.86**	11.27±7.46*	3.87±6.58
Deslanoside injection	0.01	0	3.26±2.17	2.46±2.87	2.72±1.31	3.32±2.86	3.66±1.30**	3.66±3.30	4.70±3.51
3,4-dihydroxyl cinnamic acid	1.50	0	2.45±1.71	3.01±1.82	4.10±3.01	4.63±2.21	5.32±1.99	5.32±1.54	5.85±4.89
	3.00	0	5.64±10.28	6.72±11.63	6.67±9.88	4.94±9.70	6.19±11.58	6.19±10.16	8.44±8.68
daucostorol	1.00	0	2.91±2.55	2.64±1.96	5.67±5.37	6.71±3.43	3.61±3.88	3.61±3.45	2.53±1.78
	2.00	0	1.33±0.83	2.34±1.72	2.91±4.19	3.87±2.05	6.35±2.51	6.35±5.83	7.32±6.86
gouguside 4	0.04	0	1.41±0.95	2.73±1.66	2.01±1.69	3.91±1.52	2.75±1.46	2.75±2.71	3.10±0.95
	0.08	0	4.61±3.27	4.90±4.71	2.43±3.48	3.16±2.28	2.32±1.65	1.07±0.64	1.70±1.23

Statistical analysis of the difference of the means was calculated using Student's *t*-test.
Results are reported as mean ± standard error of the mean. *p<0.05; **p<0.01: Compared with control.

Table 6 Pharmacological effect of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 isolated from *I. cornuta* to heart rate of apo-cardiac muscle of guinea pigs.

Group	Dose (mg/kg)	Heart rate (times/min)							
		Before administration	After administration						
			1 min	2 min	3 min	4 min	5 min	10 min	15 min
control	--	200.4±44.8	198.0±45.1	198.0±46.1	199.2±45.4	199.2±44.4	199.2±44.4	199.2±44.4	200.2±40.4
Salvia injection	150.0	210.0±47.4	204.0±51.4	202.8±48.1	208.8±44.8	206.4±45.0	202.8±40.6	205.2±42.9	207.6±42.6
Deslanoside injection	0.01	241.2±20.5	240.0±28.1	248.4±22.3	240.0±18.5	236.4±19.7	246.0±24.0	236.4±21.5	237.6±22.7
3,4-dihydroxyl cinnamic acid	1.50	190.4±54.2	188.0±51.5	189.2±50.9	194.0±40.5	192.8±41.8	191.6±48.1	186.8±47.8	192.8±50.8
	3.00	183.2±46.3	188.4±51.9	177.6±37.1	175.2±35.8	178.8±41.2	180.0±37.7	177.6±39.5	177.6±41.0
daucostorol	1.00	189.6±49.0	184.8±52.5	183.6±53.9	182.4±50.5	180.0±52.1	177.6±53.9	175.2±52.0	175.2±51.5
	2.00	188.4±74.4	182.4±77.6	178.8±74.5	181.2±74.7	184.8±74.9	183.6±75.5	178.8±74.5	182.4±73.8
gouguside 4	0.04	212.8±23.6	210.4±37.1	210.4±34.5	212.8±31.4	215.2±36.8	211.6±44.2	210.4±40.2	213.2±41.5
	0.08	218.4±22.7	213.6±19.7	214.8±20.1	211.2±17.7	211.2±17.7	214.8±21.4	208.8±22.2	211.2±19.6

Statistical analysis of the difference of the means was calculated using Student's *t*-test.
Results are reported as mean ± standard error of the mean. p>0.05: Compared with the data before administration.

tection to rats against myocardial ischemia caused by pituitrin.

Pharmacological effect of three compounds isolated from *I. cornuta* to coronary blood flow of guinea pigs

From **Tables 5** and **6**, it can be concluded that administration of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 in different doses, respectively, did not influence crown sphygmo-flow and cardiac rate of apo-cardiac muscle of guinea pigs. As shown in **Table 7**, treatment of gouguside 4 at doses of 0.04 and 0.08 mg/kg both significantly decreased the contractility of cardiac muscle of guinea pigs at 1 min after its administration. Deslanoside of 0.01 mg/kg dose also significantly increased the contractility of cardiac muscle of guinea pigs at 1 and 2 min after administration.

Table 7 Pharmacological effect of 3,4-dihydroxyl cinnamic acid, daucostorol and gouguside 4 isolated from *I. cornuta* to cardiac contractility of guinea pigs.

Group	Dose (mg/kg)	Cardiac contractility (mm)							
		Before administration	After administration						
			1 min	2 min	3 min	4 min	5 min	10 min	15 min
control	--	2.62±0.90	2.62±0.94	2.64±1.06	2.66±1.04	2.68±1.02	2.66±1.08	2.56±0.90	2.70±1.00
Salvia injection	150.0	2.70±1.57	2.36±1.45	2.38±1.38	2.24±1.02	2.24±1.00	2.30±1.09	2.10±1.16	2.14±1.23
Deslanoside injection	0.01	4.38±1.92	7.46±2.23*	7.26±1.82*	6.28±1.98	5.92±1.92	5.42±1.82	4.76±1.78	4.38±1.79
3,4-dihydroxyl cinnamic acid	1.50	3.50±1.84	3.34±1.42	3.42±1.66	3.40±1.65	3.36±1.64	3.20±1.62	3.08±1.70	3.04±1.52
	3.00	2.72±1.41	2.54±1.36	2.48±1.19	2.48±1.34	2.52±1.56	2.38±1.60	2.38±1.36	2.40±1.53
daucostorol	1.00	4.70±2.55	4.48±2.31	4.82±2.70	4.64±2.66	4.58±2.72	4.62±2.71	4.56±2.79	4.54±2.80
	2.00	4.20±2.95	4.24±3.08	4.38±3.03	4.30±3.05	4.24±2.84	4.16±2.77	3.92±2.73	3.94±2.79
gouguside 4	0.04	5.72±2.40	2.62±1.68*	3.56±1.84	3.60±1.73	3.76±2.04	3.76±2.16	4.14±2.14	3.78±2.02
	0.08	3.96±1.60	1.52±1.06*	2.60±1.78	2.80±1.72	3.04±1.82	3.08±1.89	3.16±1.76	2.92±1.89

Statistical analysis of the difference of the means was calculated using Student's *t*-test.
Results are reported as mean ± standard error of the mean. *p<0.05; **p<0.01: Compared with control.

DISCUSSION

The dried leaves of *I. cornuta* are a traditional Chinese medicine for the treatment of dizziness, hypertension and sore throat (Qu 1997), and also used in folklore for fertility regulation and weight loss (Lü *et al*. 1997). Previous phytochemical investigation of *I. cornuta* showed that leaves of this plant are a rich source of triterpenoids and flavonoids as well as their corresponding glycosides (Nakanishi *et al.* 1982; Qin *et al.* 1986; Wu *et al.* 2005; Li *et al.* 2006). Some researchers found that the methanolic extract of leaves of *I. cornuta* increased coronary blood flow in the isolated blood-perfused dog heart preparation (Zhu *et al*. 1994). The saponin-containing fractions from leaves of this plant have been reported to possess various important pharmacological effects such as the increase of coronary blood flow and anti-hematoblastic coagulation activities (Qin *et al.* 1986, 1988c).

In this research work, we found that 3,4-dihydroxyl cinnamic acid and daucostorol could not provide protection to mice against hypoxia and ventricular fibrillation caused by chloroform and myocardial ischemia caused by pituitrin. Gouguside 4 exerted a similar effect as the former two compounds but showed protection to rats against pituitrin-induced myocardial ischemia. 3,4-dihydroxyl cinnamic acid and daucostorol could not inflect the cardiac rate of apo-cardiac muscle, crown sphygmo-flow and contractility of cardiac muscle of guinea pigs, either. Gouguside 4 could not inflect the cardiac rate of apo-cardiac muscle, crown sphygmo-flow, but could decrease the contractility of cardiac muscle of guinea pigs. These results confirmed previous conclusions about pharmacological activities for cardiovascular diseases, but also indicated that these compounds in the present tests were not the key active components. Further research work on the isolation of chemical constituents and their assessment of pharmacological activity of *I. cornuta* leaves needs to be carried out.

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