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PHENOLIC AND ALIPHATIC GLUCOSIDES FROM *CRYPTOSTEGIA GRANDIFLORA* AND CARDIOTONIC ACTIVITY OF CRYPTOSTIGMIN II

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فى دراسة كيميائية لخلاصة الكحول الإيثيلى لأوراق نبات الكربتوستيجيا جراندفلورا المنزرع فى مصر بعد تجزئتها بـ ن هكسان وداى كلورو ميثان تم فصل والتعرف على أربعة جليكوزيدات أثنان منهم جليكوزيدات فينوليه ٢،٤،٦ تراى هيدروكسى بنزوفينون ٢ أ بيتا جلوكوبيرانوزيد وأكانثوزيد ب بالاضافة الى ميجاستجمان اكاريزيد ب١ و ز ٣ هيكسينيل بيتا د جلوكوبيرانوزيد وقد تم التعرف على هذه المركبات بواسطة طرق التحليل الكيميائى والطيفى المختلفة وكذا بالمقارنة بعينات أصيله. هذا وقد أوضحت الدراسة البيولوجية لخلاصة الكحول الإيثيلى ومادة الكربتوستجمين ٢ وهى المكون الأساسى من الكاردينوليدات السابق فصلها من نفس النبات بأن لها تأثير مشابه تماما لتأثير الديجوكسين على عضلة القلب.

From the leaves of Cryptostegia grandiflora, two phenolic glucosides 2,4,6-trihydroxybenzophenone 2-O-β-glucopyranoside and Acanthoside B together with a megastigmane (Icariside B1) and (Z)-3-hexenyl β-D-glucopyranoside have been isolated. Moreover the cardiotonic activities of Cryptostegia extract and Cryptostigmin II, the major cardenolide previously isolated from the same plant leaves were also investigated. The latter showed similar effects to those of Digoxin.

INTRODUCTION

Cryptostegia grandiflora R.Br. (Asclepiadaceae) is an ornamental plant cultivated in Egypt and warm countries.[1-3] From its leaves, few cardenolides (cryptograndoside A, B and digitalinum verum) have been already reported.[4] Recently, six more cardenolides (subalpinosid, 16-O-acetyl-digitalinum verum and cryptostigmin I-IV) have been isolated from the leaves of *C. grandiflora.*[5] The present study deals with the isolation and structure determination of a benzophenone glucoside together with a lignan glucoside and two aliphatic glucosides from the leaves of *C. grandiflora* in addition to investigation of the cardiotonic activity of the leaves extract and cryptostigmin II previously isolated from this plant.

MATERIAL AND METHODS

Plant material

Leaves of *C. grandiflora* R.Br. (Asclepiadaceae) were collected from the trees cultivated in the Experimental Station of faculty of Agriculture, Assiut University, Assiut, Egypt, in March 1997. The plant was identified by Prof. N. El-Keltawi, Department of Horticulture, Faculty of Agriculture, Assiut University, Assiut, Egypt. A voucher specimen is deposited at the Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

General experimental procedures

^{1}H and ^{13}C-NMR (TMS as int. standard): 400 MHz and 100 MHz respectively were recorded on a JEOL JNM α-400 spectrometer. FAB MS spectra were taken on a JEOL JMS-SX 102 spectrometer by direct inlet method at an ionizing voltage of 70 eV. Optical rotations were measured with a Union PM-1 digital polarimeter. HPLC: D-ODS-5 column (20 mm i.d. x 25 cm, YMC) with a Toyo Soda high speed chromatograph HLC-803 D pump and a Tosoh refraction index (RI-8) detector; flow rate of mobile phase 6 ml/min, injection volume 0.8-1.0 ml. The solvent system used on

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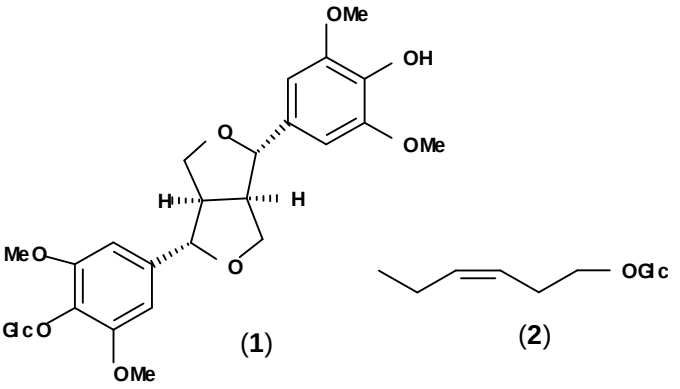
ODS column was 18% MeCN. Silica gel 60 Merck (70-230 mesh) and Diaion HP 20 (Mitsubishi) were used for column chromatography. Silica gel 60 precoated plates F-254 and HPTLC using RP-18 precoated plates, F-254 s (Merck) were used for TLC analysis.

Extraction and Isolation

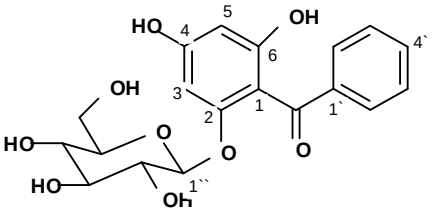
The air-dried powdered leaves of *C. grandiflora* (500 mg) were extracted with 70% EtOH. The dried ethanolic extract (125 g) was suspended in H_2O and defatted successively with n-hexane and CH_2Cl_2. The aq. fr. was applied to a column of Diaion HP-20 and eluted with H_2O, 40% MeOH, 80% MeOH, MeOH and acetone successively. The 40% MeOH eluate was chromatographed by silica gel CC using CH_2Cl_2-MeOH-H_2O (75:25:2) and finally with (65:35:5) to give 7 fractions. Fraction 1 (1.66 g) was separated on silica gel column using CH_2Cl_2-MeOH-H_2O (85:15:1 - 65:35:5) and HPLC using ODS column with 18% MeCN to afford compounds **1** (white powder, 20 mg) and **2** (white powder, 11 mg). Fraction 5 (289 mg) was subjected to HPLC using ODS column and 18% MeCN to give compound **3** (white powder, 105 mg). Fraction 7 (87 mg) was separated by HPLC under the same conditions of fractions 1 and 5 to afford compound **4** (white powder, 12 mg).

A part of the residue obtained from the total ethanolic extract was used for the biological study and had been dissolved in normal saline.



(**1**)

(**2**)



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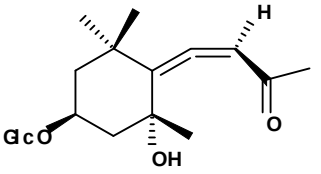
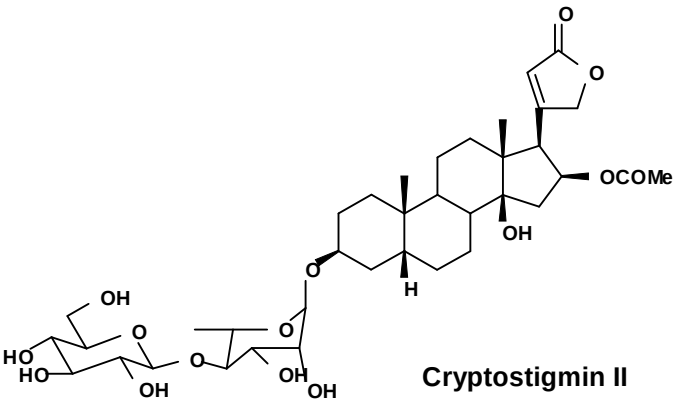


(**4**)



Cryptostigmin II

Compound (1)*:* Was obtained as white powder (71 mg), $[\alpha]_D^{25}$ –25.2º (*c* 0.48, MeOH), had the molecular formula C_{28} H_{36} O_{13}, negative FAB – MS showed molecular ion peak at m/z 579 $[(M-1)]^-$ and a peak at m/z 417 [(M-1) – glucose] $^-$. 400 MHz ^{1}H-NMR (C_5D_5N): δ 3.79 (6H, s, 3``, 5``, OMe), 3.82 (6H, s, 3`,5`, OMe), 3.92 (2H, d, *J*= 2.1, 4 & 8 β), 4.22 (2H, m, 4 & 8 α), 4.96 (1H, m, Glc-1), 6.92 (2H, s, 2``,6``), 6.94 (2H, s, 2`, 6`). 100 MHz ^{13}C-NMR (C_5D_5N) as cited in Table 1.

Compound (2): was obtained as white powder (methanol), (11 mg), $[\alpha]_D^{17}$ –33.7º (*C*= 0.92, MeOH), had the molecular formula $C_{12}H_{22}O_6$, ^{1}H-NMR (C_5D_5N): δ 0.81 (3H, t, *J*= 7.6 Hz, H-6), 1.91 (2H b.q, *J*= 7.2 Hz, H-5), 5.41 (2H, m, H-3, H-4), 2.43 (2H, b. q, *J*= 6.8 Hz, H-2) and 4.84 (1H, d, *J*= 7.8 Hz, Glc-1). 100 MHz ^{13}C-NMR (C_5D_5N) as cited in Table 1.

Compound (3): $[\alpha]_D^{17}$ –67.2º (c 5.33, MeOH). HRFAB-MS (negative): $C_{19}H_{20}O_9$–H, found: 391.3441 $[M-H]^-$ calcd: 391.3490. ^{1}H-NMR (400 MHz, DMSO): δ= 7.70 (2H, m, H-3`,5`), 7.56 (1H, m, H-4`), 7.45 (2H, m, H-2`,6`), 6.15 (1H, d, *J*= 1.9 Hz, H-3), 6.07 (1H, d, *J*= 1.9 Hz, H-5) and 4.74 (1H, d, *J*= 7.6 Hz, Glc-1). ^{13}C-NMR 100 MHz (DMSO) as cited in Table 1.

Table 1: 100 MHz ^{13}C-NMR data of compounds **1**,**2**,**4** in (C_5D_5N) and **3** in (DMSO).

C. No.	1	2	3	4	
Aglycone 1	54.8	69.3	108.7	36.2	
2	86.2	28.3	157.0	48.0	
3		133.5	94.3	71.8	
4	72.0	125.5	160.3	47.0	
5	54.7	20.8	96.7	71.2	
6	86.2	14.3	157.1	119.7	
7			C=0 194.9	209.6	
8	72.0			100.4	
1`	135.4		138.7	C-9	197.7
2`	104.8		128.3	C-10	26.4
3`	153.8		129.0	C-11	31.0
4`	138.3		132.5	C-12	29.1
5`	153.9		129.0	C-13	31.9
6`	104.7		128.3		
1``	132.0				
2``	104.8				
3``	149.2				
4``	137.0				
5``	149.2				
6``	104.7				
OCH_3	57.0 57.6				
β-glucopyranose 1	104.9	104.7	100.7	103.0	
2	75.9	75.2	73.2	75.4	
3	78.2	78.5	76.6	78.6	
4	71.5	71.7	69.5	71.6	
5	78.5	78.6	77.1	78.3	
6	62.5	62.8	60.7	62.7	

Compound (**4**): was obtained as white powder, (12 mg), $[\alpha]_D^{17}$ –60.0º (*c* 1.0, MeOH), had the molecular formula $C_{19}H_{30}O_8$. 400 MHz ^{1}H-NMR (C_5D_5N): δ 1.06 (3H, s, H-13), 1.48 (3H, s, H-11 or H-12), 1.50 (3H, s, H-11 or H-12), 2.17 (3H, s, H-10), 5.87 (1H, s, H-8), 5.09 (1H, d, *J*= 7.6 Hz, Glc-1). 100 MHz ^{13}C-NMR (C_5D_5N) as cited in Table 1.

Evaluation of the effects of Digoxin, Cryptostigmin II and *Cryptostegia* extract on blood pressure and heart rate[6,7]

Adult male rabbits (1.5-2.5 kg average weight) were used. The rabbits were anesthetized with intraperitoneal injection of 25% urethane solution in a dose of 6.4 ml/kg. Their tracheae were cannulated for artificial respiration when necessary. The right femoral vein was cannulated for intravenous injection of the tested samples. The arterial blood pressure was recorded via the carotid artery which was connected to a PT 400 Blood Pressure Transducer and an amplifier of a two-channel oscillograph MD2 (Bioscience, Kent, UK). The transducer was then calibrated and the electrocardiographic changes were simultane-ously recorded using standard lead II. The changes in blood pressure and heart rate of rabbits were recorded before and at 15, 30, 60 and 90 min after intravenous injection of the different doses from the sample solution in normal saline. The obtained results are recorded in Tables 1 and 2.

Evaluation of the effects of Digoxin, Cryptostigmin II and *Cryptostegia* extract on the contractility of the isolated perfused rabbit's heart

The isolated hearts were prepared for perfusion according to Langendorff (1895)[7] as follows: the rabbits were injected with heparin (1000 units/kg) into the ear vein and 30 min later, the rabbits were sacrificed and the heart with at least 1 cm of attached aorta was removed as quickly as possible and placed in a dish of Lock-Ringer solution at 37º. The aorta was cut just below the point where it divides and the heart was transferred to the perfusion apparatus which contains two bottles, one for the unmedicated perfusion fluid and the second for the perfusion fluid that contains the required concentration of digoxin, *Cryptostegia* extract and cryptostigmin II. The aorta was tied onto the glass cannula and the perfusion fluid was an oxygenated lock. Ringer solution was warmed to 37º by means of a water jaket adjusted at 38º.

Table 2: Time course of changes in the mean arterial blood pressure of rabbits receiving different dose levels of Digoxin, *Cryptostegia* extract and Cryptostigmin-II intravenously.

Time	Mean arterial blood pressure (mmHg)								
	Digoxin			*Cryptostegia* extract			Cryptostigmin II		
	0.5 mg/kg	1.0 mg/kg	1.5 mg/kg	2.5 mg/kg	5.0 mg/kg	7.5 mg/kg	0.5 mg/kg	1.0 mg/kg	1.5 mg/kg
0	105±7.5	105±5.7	105±3.4	105±4.2	105±2.7	108±9.5	105±4.8	105±2.4	100±5.3
15 min	108±4.5	110±2.4	110±2.1	105±2.6	108±4.2	110±2.1	110±2.0	100±8.6	95±8.7
30 min	105±8.6	105±8.2	102±5.7	105±2.8	102±7.9	105±3.7	105±5.9	110±2.1	105±2.7
60 min	105±7.0	105±9.2	104±4.6	105±6.9	103±9.1	105±1.8	110±9.8	115±8.8	105±8.2
90 min	105±6.5	105±3.8	102±6.8	105±2.2	105±3.5	105±7.2	110±4.6	105±2.9	105±3.8

Each figure represents the mean five experiments ± standard error.

The rate of flow of the perfusion fluid was kept constant at 3 ml/min by adjusting the pressure exerted on the fluid by means of a mercury manometer connected to the perfusion system. A thread was attached to the ventricle by a hook and the other end of the thread was passed over pulley wheels and was attached to the displacement transducer connected to the Oscillograph 400 2C. The contractions of the perfused hearts were recorded at a constant chart speed. Normal contraction was recorded during a period of 20 min until it was stabilized and become consistent. Then, the Lock-Ringer's solution containing the definite concentrations of the investigated samples was started to perfuse the hearts and recording the myocardial contractility every 5 min for a period of 30 min. The percentage change in the amplitude of normal myocardial contractility after perfusion with the tested samples was calculated. The results are given in Table 3.

Determination of the median lethal dose

The median lethal dose (LD_{50}) and its 95% fiducial limits were calculated for *Cryptostegia* extract and cryptostigmin II. Groups of 6 male adult albino mice (20-28 g each) housed under the same conditions were intraperitoneally injected with graded doses of the extract (10 mg. 20 mg. 40 mg and 80 mg) and cryptostigmin II (2 mg, 3 mg, 4 mg and 8 mg). The mortality was determined 24 hours later in each group of the animals. Then the LD_{50} results (mg/kg) were recorded as follows: 21.37±3.52 for *Cryptostegia* extract and 5.75±0.82 for cryptostigmin II.

RESULTS AND DISCUSSION

The ethanolic extract of the leaves of *C. grandiflora* was defatted with n-hexane and CH_2Cl_2 and the aqueous layer was subjected to a column chromatography of Diaion HP-20. The 40% methanol eluate was repeatedly chromatographed on columns of silica gel and HPLC to afford four glucosides (**1**-**4**).

^{1}H-NMR spectrum of compound (**1**) showed one anomeric proton for β-glucopyranose at δ 4.96, four OCH_3 groups at δ 3.82 and the other signals are similar to those reported for Liriodendrin.[8] ^{13}C-NMR spectrum revealed the presence of one anomeric carbon for β-glucopyranose at 102.9 ppm, four aromatic carbons at 104.7 and 104.8 ppm, and four methoxylated aromatic carbons at 153.9 ppm. ^{13}C-NMR spectrum for the aglycone revealed the upfield shift of C-4` and c-4``, the upfield shifts of the other aromatic carbons and the other signals are very similar to those reported for (+)-syringaresinol.[9] This compound was previously isolated from the bark of *Eucommia ulmoides* Oliv. (Eucommiaceae).[10]

Table 3: Time course of changes in the heart rate of rabbits receiving different dose levels of Digoxin, *Cryptostegia* extract and Cryptostigmin II intravenously.

Time	Heart rate (beat/min)								
	Digoxin			*Cryptostegia* extract			Cryptostigmin II		
	0.5 mg/kg	1.0 mg/kg	1.5 mg/kg	2.5 mg/kg	5.0 mg/kg	7.5 mg/kg	0.5 mg/kg	1.0 mg/kg	1.5 mg/kg
0	310±26	300±24	300±20	290±25	280±18	280±22	280±20	290±15	300±28
15 min	310±20	240*±20	220*±18	300±22	275±20	220*±20	290±22	290±20	260*±25
30 min	310±22	230*±18	210*±20	300±25	275±25	220*±18	290±26	290±20	260*±22
60 min	310±25	230*±20	220*±16	290±28	280±24	240*±20	290±24	290±23	240*±20
90 min	310±18	230*±20	220*±21	290±24	280±23	240*±22	290±20	290±15	260*±24

Each figure represents the mean five experiments ± standard error.
* Significant difference ($P < 0.01$) Vs control values.

^{1}H-NMR spectrum for compound (**2**) showed multiplet signal at δ 5.41 for 2H at positions 2 and 3; quartet signal at δ 1.91 *J*= 7.2 Hz for 2H at positions 4 and 5, triplet signal at δ 0.81 *J*= 7.6 Hz for 3H at position 6 and a doublet signal at δ 4.84, *J*= 7.8 Hz for anomeric proton.

The ^{13}C-NMR spectrum revealed the presence of twelve carbon atoms, six of them were at δ 104.7, 75.2, 78.5, 71.7, 78.6 and 62.8 which are characterisitc for β-glucopyranoside and the other six carbon atoms were characteristic for the presence of olefinic carbons between C-3 and C-4 at δ 133.5 and 125.5, in addition to oxygenated methine carbon at δ 69.3 corresponding to C-1 and at δ 20.8 corresponding to C-5 and one signal for methyl group at δ 14.3 ppm. It can be concluded that the structure is in full agreement with that reported for (Z)-3-hexenyl β-D-gluco-pyranoside.[11]

The molecular formula of compound **3** was deduced as $C_{19}H_{20}O_9$ from HR FAB-MS spectrometry (see experimental section). The ^{13}C-NMR spectrum and DEPT experiment of **3** (Table 1) displayed the presence of one unsubstituted β-glucopyranosyl unit from the signals at δ_C 100.7 (C-1``), 73.2 (C-2``), 76.6 (C-3``), 69.5 (C-4``), 77.1 (C-5``) and 60.7 (C-6``) together with 7 methines (δ_C 132.5, 129.0 for 2 carbons, 128.3 for 2 carbons, 96.7 and 94.3) and 6 quaternary carbon signals (δ_C 194.9, 160.3, 157.1, 157.0, 108.7 and 138.7) for the aglycone. The two chemically equivalent methines at δ_C 129.0 (C-3`,5`) as well as those at δ_C 128.3 (C-2`,6`) together with the methine at δ_C 132.5 (C-4`) suggested a presence of monosubstituted benzene.[12] From $^1$H-NMR spectrum of **3**, the multiplet signal at $\delta_H$ 7.56 for one proton (H-4`) together with the two multiplets for two pairs of chemically equivalent protons at δ_H 7.45 (H-2`,6`) and 7.70 (H-3`,5`) confirmed the presence of a monosubstituted benzene. On the other hand the three quaternary carbons at δ_C 160.3 (C-4), 157.1 (C-6) and 157.0 (C-2) were assigned for three oxygenated aromatic carbons due to their downfield shifts.[13] In the ^{1}H-NMR spectrum of **3**, the two doublets at δ_H 6.07 (H-5) and 6.15 (H-3) with a coupling constant 1.9 Hz each, indicated the presence of two meta coupled aromatic protons. This was confirmed from the two methine carbons at δ 94.3 (C-3) and 96.7 (C-5) in the ^{13}C-NMR spectrum. Therefore the second benzene ring was determined as tetrasubstituted benzene. Moreover, The quaternary carbon signal at δ 194.9 was very clear for a ketonic carbonyl connecting the two benzene rings that was confirmed from the two quaternary carbons at δ 108.7 (C-1) and 138.7 (C-1`) in the ^{13}C-NMR spectrum. Therefore, the aglycone of **3** was identified as 2, 4, 6-trihydroxybenzophenone. In the ^{1}H-NMR spectrum of **3**, the doublet signal at δ 4.74 with J (constant) 7.6 Hz for the anomeric proton of the glucosyl residue indicated its β configuration. The attachment of the glucopyranosyl unit to C-2 of the aglycone was esablished by 2D NMR spectral analyses. The HMQC spectral data of **3** revealed the correlations between each carbon and its directly attached protons while H-H COSY interpreted the proton-proton couplings. The HMBC spectral analysis of **3** (Fig. 1) displayed significant correlation peaks between H-3,5,2`, 3`,5`,6` and C=O; H-3, 5 and C-1; H-6`, 2` and C-1` as well as between H-1 of the glucopyranosyl moiety and C-2 of the aglycone. Consequently, the structure of compound **3** was identical to that of 2,4,6-trihydroxybenzo-phenone 2-O-β-glucopyranoside.[14,15] This is the first report for ^{1}H-, ^{13}C-, 2D NMR and HR-FAB-MS spectral data for compound **3.**

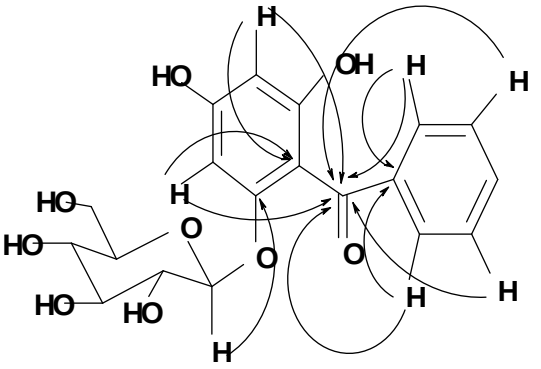


Fig. 1: Significant HMBC correlations of compound **3**.

The ^{1}H-NMR spectrum of compound (**4**) exhibited four singlet methyl signals at δ 1.06 (3H) at 13, 1.48 (3H) at 11 or 12, 1.50 (3H) at 11 or 12 and 2.17 (3H) at 10, the last one being due to a methyl ketone. An anomeric proton

signal at δ 5.09 (1H, d, *J*= 7.6 Hz) and an olefinic proton signal at δ 5.87 (1H, s) at 8.

^{13}C-NMR spectrum showed the presence of two carbinol carbon signals at 71.8 (d) and 71.2 (s). The former was shifted downfield by 6.2 ppm in ^{13}C-NMR spectrum of the aglycone and the later shifted downfield by only 1.0 ppm., this indicated the presence of glucosidation at position C-3.

These results led us to conclude that, the structure is in full agreement with that reported for Icariside B_1.[16,17]

The structure determination was based on comparison of their physical chemical and spectral data (^{1}H, ^{13}C-NMR and FAB-MS) with those reported for acanthoside B, (Z)-3-hexenyl B-D-glucopyranodside, 2,4,6-trihydroxybenzo-phenone 2-O-β-Glucopyranoside and icariside B_1.

The cardiotonic activity of Cryptostigmin II, the major cardenolide previously isolated from the leaves of *C. grandiflora* as well as the plant extract were investigated in comparison with digoxin. From the data reported in Table 2, the intravenous administration of different doses of digoxin, *C. grandiflora* extract and Cryptostigmin II showed no change in the arterial blood pressure during the period of investigation (90 min). On the other hand, the intravenous administration of digoxin in doses of 1.0 and 1.5 mg/kg decreased the heart rate of rabbits. This reduction was started after 15 min of administration (Table 3). From the results reported in the same Table, no change in the heart rate was observed after administration of the extract in doses of 2.5 and 5 mg/kg but the decrease in the heart rate was obvious at a dose of 7.5 mg/kg of the extract after 15 min of the intravenous administration. Moreover, no change in the heart rate was observed with doses of 0.5 and 1.0 mg/kg of Cryptostigmin II, but a significant decrease in the heart rate was clear when the dose increased to 1.5 m/kg after 15 min of administration. On the other hand, recording the contractility of the isolated perfused rabbit's heart every 5 min for a period of 30 min showed that digoxin produced no change at a concentration level of 0.25 mg/ml while the increase in the contractility was observed when the concentration levels were inreased to 0.5 and 1.0 mg/ml during the investigation period (Table 4). From the same Table, it's evident that the extract didn't exhibit any effect on the contractility at a concentration level of 2.5 mg/ml while it increased the contractility significantly when the concentration levels were increased to 5.0 and 7.5 mg/ml Cryptostigmin II revealed significant increase in the contractility at a concentration level of 1 mg/ml that is more potent than that of digoxin at the same concentration level after 15 and 30 min of administration.

Table 4: Effect of different concentrations of Digoxin, *Cryptostegia* extract and Cryptostigmin II on the contractility of the isolated rabbit's heart at different time intervals.

Time	Contractility (% of change from control)								
	Digoxin			*Cryptostegia* extract			Cryptostigmin II		
	0.25 mg/ml	0.5 mg/ml	1.0 mg/ml	2.5 mg/ml	5.0 mg/ml	7.5 mg/ml	0.5 mg/ml	1.0 mg/ml	1.5 mg/ml
5.0 min	2.0	22*±2.1	30*±2.8	2.0	50*±4.2	25*±1.2	2.0	27*±1.8	20*±1.8
10 min	2.0	34*±3.0	40*±3.7	2.0	57*±3.8	37*±2.2	2.0	43*±2.8	30*±2.8
15 min	3.0	33*±2.8	50*±4.5	2.5	96*±7.2	42*±3.1	3.0	66*±5.2	35*±2.9
30 min	3.0	33*±3.1	50*±4.5	3.0	96*±8.1	42*±3.8	3.0	66*±4.6	35*±2.1

Each figure represents the mean five experiments ± standard error.
* Significant difference (P < 0.01) Vs control values.

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