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Phytochemical and biological activities of *Crataegus sinaica* growing in Egypt

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ABSTRACT

Objective: To evaluate the cardiac activity and hepatoprotection of *Crataegus sinaica (C. sinaica)*. **Methods**: All the isolated compounds were isolated by open-column liquid chromatography (CC) using sephadex LH-20 as stationary phase. Elution of the column was performed with EtOH or MeOH. The phytochemical investigation of the young stem of *C. sinaica* for the first time together with the leaves and flowers lead to the isolation and identification of quercetin, hyperoside, vitexin-2′′-O-rhamnoside, epicatechin, procyanidin B2 and procyanidins C1. **Results:** Rats treated with the low and high dose of *C. sinaica* leaves with flowers extract showed 15% and 17% reduction in the heart rate, and reduction in the ST-segment by 107% and 57%; respectively. The T-amplitude was decreased by 59% of the high dose extract. On the other hand, the young stems and leaves with flowers extracts of *C. sinaica* on primary culture of rat hepatocytes monolayer indicated a hepatoprotection for the total extract, ethyl acetate, butanol, and chloroform fractions at 100 μg/mL, 75 μg/mL, 50 μg/mL, and 25 μg/mL; respectively. **Conclusions:** The results of these chemical and biological studies suggest the use of *C. sinaica* growing in Egypt as a preventive drug against cardiovascular and hepatic diseases. The chemical studies suggest the use of woody young stems as a newly investigated bioactive organ. The extraction of unsaturated fatty acids from the seeds of the plant would serve as a good health and nutritive product.

1. Introduction

Hawthorn, a common name of all plant species in the genus *Crataegus*, is a thorny shrub or small tree[1]. Hawthorn is a member of the *Rosaceae* family and is recognized to have approximately 280 species primarily from northern temperate zones in East Asia, Europe, and eastern North America[2, 3]. *Crataegus sinaica* (*C. sinaica*) grows wildly in the mountains area in Saint Catherine Protectorate, South Sinai, Egypt, and known as Za′rur or Za′rur al-awdiyah[4]. Flavonoids and procyanidins are considered as the most important constituents and primarily responsible for the pharmacological activity of hawthorn[5]. The fruits contain relatively low levels of flavonoids and consist primarily of oligomeric and polymeric procyanidins[6]. Data regarding the young stems and seeds are lacking. Among cardioactive medicinal plants that do not contain typical cardiac glycosides, hawthorn (*Crataegus*) has taken a special position since centuries[7]. *Crataegus* species are famous in folk medicine and are widely used in phytotherapy due to their improvement of the heart function in declining cardiac performance equivalent to stages I and II of the NYHA classification[8-10]. It was deemed important to evaluate the phytoconstituents and bioactivities of *C. sinaica* growing in Egypt. The antiviral and antioxidant activity of some fractions and of a series of flavonoids and proanthocyanidins obtained from *C. sinaica* was valuated[11]. The O-glycosidic flavonoids and oligomeric proanthocyanidins exhibited significant inhibitory activity against herpes simplex virus type 1 (HSV-1), which was shown to be due to an extracellular mechanism for procyanidin C1[12]. However, studies evaluating cardiac activity and hepatoprotection of *C. sinaica* have not done yet.

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2. Materials and methods

2.1. General experiment

TLC was carried out on precoated 0.2 mm layer silica gel F_{254} plates (Merck) developed with ethyl acetate – formic acid – acetic acid – water (30:1.2:0.8:8 v/v). Spots were detected using naturstoff (NA) reagent (2-aminoethyl diphenylborate) 1% w/v in absolute methanol for flavonoids[13], reagent (ii) (vanillin 1% in MeOH and 5% H_2SO_4 in EtOH) for proanthocyanidins analysis[12], UV at 254 and 366 nm for UV detection. UV spectra were measured as diluted samples in MeOH on a Uvikon 931 double beam UV-VIS spectrophotometer in the region of 200-500 nm. FAB-MS spectral analyses in negative or positive mode were performed on a VG 70-SEQ hybrid mass spectrometer. EIMS were recorded on the same instrument by means of a direct insertion probe at ionization energy of 70 ev. ^{1}H-and ^{13}C-NMR spectra were recorded in CD_3OD, or DMSO-d8 on a Bruker DRX-400 instrument. The chemical shifts were reported in values (ppm) with TMS as the internal standard.

2.2. High performance liquid chromatography analysis

High performance liquid chromatography(HPLC) analysis was carried out using Agilent 1 200 equipment with quaternary pump, auto sampler injector. The chromatograms were detected with a DAD (80 Hz) for full spectral acquisition. The automated Quaternary LC system was operated and data was managed using Hewlett-Packard model computer. Zobrax Eclipse XDB-C18 Column (Analytical 4.6 ×150 mm; 5 microns) was used at temperature 25 ℃.

2.3. Plant materials

C. sinaica were obtained from Wadi Gebal in Saint Catherine Protectorate (South Sinai, Egypt) in November 2005. Voucher specimens were kindly identified by Prof. Dr. K. H. El-Batanouny, Botany Department, Faculty of Science, Cairo University, Egypt. It was air-dried, then segregated into leaves with flowers, young stems, and fruits; from which seeds were further separated.

2.4. Authentic reference compounds

Quercetin were previously isolated and purified from the leaves of *C. sinaica*[11]. Vitexin-2″-O-rhamnoside & hyperoside were purchased from ROTH. Epicatechin was purchased from Sigma Chemical Co. (St. Louis, MO). B2 and C1 were previously isolated and purified from the leaves of *C. sinaica*[12].

2.5. Extraction and isolation

The air-dried and powdered leaves with flowers and young stems (1 500 g), and fresh fruits (2 000 gm) were extracted exhaustively with 70% and 80% aqueous acetone; respectively at room temperature. The combined filtrates were concentrated in vacuo at 40 ℃ then subjected to lyophilization. Then extracted successively with CHCl3 (300 mL × 3), EtOAc (500 mL × 5), and BuOH (150 mL × 3); respectively. Each fraction was completely dried in vacuo at 40 ℃and the final dry weight was noted.

2.6.Sample preparation for HPLC

Two g of dried powder were extracted 3 times with 200 mL 70% acetone. The extract was reduced to about 10 mL in *vacuo* and then diluted with 20 mL of distilled water and defatted with petroleum ether. The aqueous layer was evaporated to dryness, then dissolved in methanol and transferred to a 25 mL measuring flask and continued to the mark with methanol (solution A). 5 mL of solution A were filtered through a 25 mm filter, pp 0.45 Nylon and diluted to 10 mL with methanol. A 20 μL of this solution was injected.

2.7. Solvent system

Isocratic elution of A: Water/Formic acid (0.05%) (86%) and B: Acetonitrile (14%). Standard authentics concentration: 1 mg/mL (0.8 mL/min flow rate).

2.8. Biological activities analysis

In vivo bioassay using cardiac activity was performed according to the method of Vish Nevskeya[14]. For this purpose 36 rats weighing 250-300 g were used (from the animal house colony of the NRC) and Epinephrine (Epinephrine®) was purchase from Misr for pharmaceutical products, Egypt. It has been established that within 5 minutes of intramuscular injection of 0.8 mL epinephrine in white rats myocardial ischemia appeared. ECG changes appeared in the form of depressed ST-segment, heart rate changes and diminution or inversion of the T-amplitude.

Rats were divided into 5 groups as follow: The 1st group served as control and received distilled water, the 2nd and 3rd group received low dose level (10 mg/kg/day) and high dose level (100 mg/kg/day) of *C. sinaica* leaves with flowers extract; respectively, the 4th and 5th group received low dose level (10 mg/kg/day) and high dose level (100 mg/kg/day) of the leaves with flowers extract standard (*Crataegi Foui Cum Flore* Extract; Siccum, Certa, from Belgium); respectively. The ECG changes were measured using ECG in standard lead II using power lab ECG module.

2.9. Hepatoprotection and cytotoxicity using MTT colorimetric assay

Primary culture of rat hepatocytes was prepared according to method[15], modified by Kiso[16], using Westar male rats (250-300 g), obtained from the animal house of the NRC. Culture medium composed of RPMI-1640 medium(100 mL), supplemented with 10% inactivated (56 ℃ for 30 min) fetal calf serum (FBS), penicillin-streptomycin (PS) (0.01 g/mL), insulin (0.7 g/mL) and dexamethasone (4 g/mL). All items were purchased from Sigma-aldrich Company. Inocula of 4×10^6 cells/mL were seeded into sterile plastic 96-well plates and incubated in CO_2-Incubator at 37 ℃, under 5% CO_2 in air for 22-24 hrs. All buffers were freshly prepared and sterilized at 121℃ for 30 min. before use. Four extract and fractions (total acetonic extract and EtOAc, BuOH and $CHCl_3$ fractions) from both the leaves with flowers and young stems extracts of *C. sinaica* were tested for their potential cytotoxic and hepatoprotective activity.

Evaluation of hepatoprotective activity undergo by preparing different concentrations from each of the extracts of *C. sinaica*, starting from 25 μg/mL and increasing concentrations in ascending order by dissolving in DMSO (1% maximum concentration). For each concentration, three replicates were carried out, in addition to controls which were: cell control (*i.e.*, cells only), 50% of cell control, negative control: 20 mM paracetamol (*i.e.*, cells + paracetamol), positive control: 50 μg/mL Sylimarin (*i.e.*,

cells + sylimarin + paracetamol).

In order to determine LC_{50}, different concentrations were prepared for each of the extracts. The range of concentrations used started from 100 μg/mL followed by increasing concentrations in ascending order up to the concentration 1000 μg/mL to determine the concentration that induce death for half the number of cells. *C. sinaica* extracts were dissolved in dimethylsulphoxide (DMSO) (1% maximum concentration). For each concentration, three replicates were carried out, in addition to controls which were: Cell control (*i.e.*, cells only), 50% of cell control

Evaluation of the effect of the different extracts on cultured hepatocytes was obtained by calculating the absorption of the cell viability with respect to control cells (cells only without addition of *C. sinaica* extracts). Each experiment was carried out twice to confirm validity of results. A graph was plotted with x-axis showing different concentrations of extracts used, y-axis showing absorbance percentage of viable cells.

3. Results

3.1. Bioactivites

3.1.1. Cardiovascular activity

All groups showed no significant effect for the ECG changes after one week administration of the drug. In the positive control group heart rate was reduced by 27% and the ST-segment was reduced by 247%.

Rats treated by the low and high dose of *C. sinaica* leaves with flowers extract showed 15% and 17% reduction in the heart rate, and reduction in the ST-segment by 107% and 57% respectively. The T-amplitude was decreased by 59% of the high dose extract. On the other hand, the rats treated by the low and high dose of the leaves with flowers extract standard (*Crataegi Foui Cum Flore* Extract) showed 27% and 22% reduction in the heart rate and reduction in the ST-segment by 111% and 42%; respectively. The T-amplitude was decreased by 41% of the high dose standard (Table 1, Figure 1 & 2).

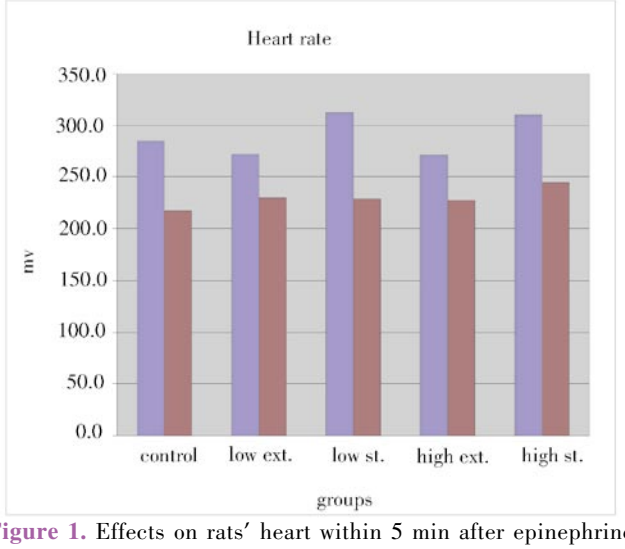

Figure 1. Effects on rats' heart within 5 min after epinephrine injection.

Both the standard drug and the tested extracts showed a protective effect on the heart especially in the high dose level. These results prove the efficacy of *Crataegus* growing in Egypt and possibility of using it therapeutically in cardiovascular diseases.

3.1.2. Hepatoprotection and cytotoxicity

Data were subjected to analysis of variance and treatment means were compared by an approximate Student's *t*-test ($P<0.05$). All treatments in experiments described consisted of three replicates.

The results of leaves with flowers, as well as, young stems extracts of *C. sinaica* indicated that they exhibited hepatoprotection for total extract, ethyl acetate, butanol, and chloroform fractions at 100 μg/mL, 75 μg/mL, 50 μg/mL, and 25 μg/mL; respectively (Figure 3 & 4, Table 2 & 3).

The viability assay was applied with a broad range of concentrations of the studied extracts (from 100-1000 mg/mL) on monolayer of rat hepatocytes. It revealed that with the given extracts, increasing absorbance values were observed with increasing concentrations from 100 μg/mL to 1 000 μg/mL in monolayer culture of primary rat hepatocytes except in the $CHCl_3$, however no cytotoxicity was observed microscopically in the cellular systems, as shown in Table 4, 5 and Figure 5, 6.

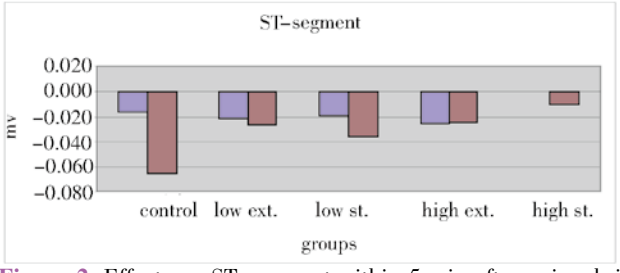

Figure 2. Effects on ST-segment within 5 min after epinephrine injection.

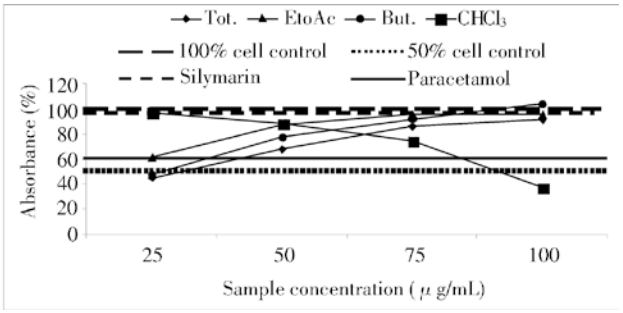

Figure 3. Viability of rat hepatocyte after treatment with different concentrations of leaves extracts followed by treatment with paracetamol using MTT colorimetric assay.

*Each point represents the Mean±SD (n=3).

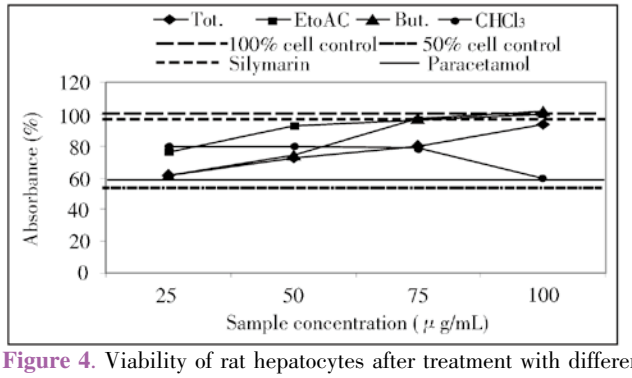

Figure 4. Viability of rat hepatocytes after treatment with different concentrations of branch extracts followed by treatment with paracetamol using MTT colourimetric assay.

*Each point represents the Mean±SD (n=3).

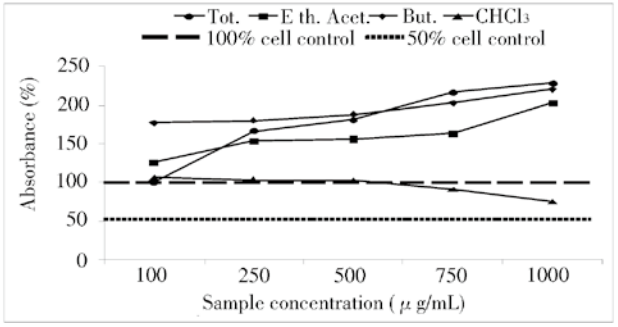

Figure 5. Viability of rat hepatocytes after treatment with different concentrations of the leaves extracts using MTT colorimetric assay.
*Each point represents the Mean±SD (n=3).

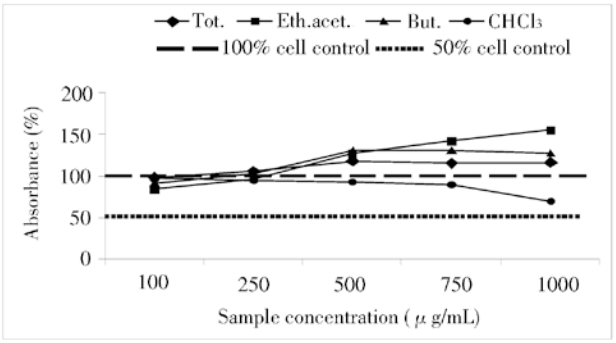

Figure 6: Viability of rat hepatocytes after treatment with different concentrations of the young stems extracts using MTT colourimetric assay.
*Each point represents the Mean±SD (n=3).

Table 1
Effects on rats within 5 min after epinephrine injection.

Item			Control	Low dose ext.	Low dose st.	High dose ext.	High dose st.
Heart rate	Value	Before injection	284.28±28.07	272.24±32.91	312.34±25.42	271.62±17.13	310.17±17.99
		After injection	217.36±19.60	229.87±30.08	228.69±4.95 *	245.07±9.66 *	238.05±25.73
		Percentage change	−27%	−15%	−27%	−17%	−22%
ST-segment	Value	Before injection	−0.016±0.016	−0.020±0.018	−0.021±0.017	0.000 47±0.010 00	−0.016±0.010
		After injection	−0.066±0.037	−0.027±0.020	−0.035±42.060	−0.009 63±0.009 70	−0.024±0.012
		Percentage change	−247%	−107%	−111%	−57%	−42%

* P< 0.05: significantly different from its basal result. (Paired t-test two sided).

Table 2
Hepatoprotection of different concentrations of *C. sinaica* leaf fractions.

Sample concentration μ g/mL	Absorbance percentage (Mean ± SD)			
	Total acetonic extract	EtOAc fraction	BuOH fraction	$CHCl_3$ fraction
25	44.8±1.2	61.3±1.9	47.1±0.8	96.9±2.7
50	67.6±3.5	87.1±4.8	76.9±4.3	88.9±2.1
75	58.2±4.5	95.1±7.1	92.0±5.8	74.7±3.0
100	91.1±5.1	95.1±5.8	104.4±5.4	36.8±1.3
100% cell control		100±4.0*		
50% cell control		50.6±1.1*		
Paracetamol		59.1±0.7*		
Silymarin		96±7.0*		

*Absorbance percentage relative to control (= 100% cells; 50% cells; Silymarin, 50 μ g/mL and paracetamol, 20 mM.

Table 3
Hepatoprotection of different concentrations of *C. sinaica* branch fractions.

Sample concentration (μ g/mL)	Absorbance percentage (Mean±SD)			
	Total acetonic extract	EtOAc fraction	BuOH fraction	$CHCl_3$ fraction
25	61.7±0.2	76.6±0.7	61.2±0.9	80.3±2.9
50	72.3±1.4	92.6±2.2	75.0±0.9	79.8±2.3
75	80.3±1.5	96.3±1.7	97.3±2.4	78.7±2.9
100	93.6±1.2	100.5±5.4	101.6±2.1	59.6±1.1
100% cell control		100 ±1.1*		
50% cell control		53.2 ±2.1*		
Paracetamol		58.0±1.8*		
Silymarin		99.5±1.0*		

*Absorbance percentage relative to control [(= 100% cells, 50% cells, Silymarin (50 μ g/mL) and paracetamol (20 mM)].

Table 4
Hepatotoxicity of different concentrations of *C. sinaica* leaves fractions.

Sample concentration (μg/mL)	Absorbance percentage (Mean ± SD)			
	Total acetonic extract	EtOAc fraction	BuOH fraction	$CHCl_3$ fraction
100	100.0±3.8	126.8±2.1	177.4±15.3	106.9±8.2
250	167.2±5.0	154.1 ±7.9	180.3±8.6	103.8±7.3
500	181.3±8.6	157.3±8.3	187.5±9.6	102±6.2
750	216.7±8.6	163.3 ±7.1	202.6±10.8	90.8±4.4
1 000	227.9±12.3	203.3±18.5	220.7±10.6	75.7±2.0
100% cell control		100.0±6.8*		
50% cell control		54.4±5.6*		

*Absorbance percentage relative to control (= 100% and 50%)

Table 5
Hepatotoxicity of different concentrations of *C. sinaica* branch fractions.

Sample concentration (μg/mL)	Absorbance percentage (Mean ± SD)			
	Total acetonic extract	EtOAc fraction	BuOH fraction	$CHCl_3$ fraction
100	98.0±5.8	84.0±1.4	90.8±4.5	97.4±6.3
250	105.4±6.2	96.4±7.2	101.4±3.6	94.8±4.6
500	117.4±3.6	126.8±8.8	131.2±3.4	92.8±6.3
750	115.1±7.2	257.0±4.6	129.4±4.4	89.0±3.1
1 000	116.7±7.9	155.3±9.0	128.0±2.9	69.5±6.0
100% cell control		100.0±5.8*		
50% cell control		52.1±1.2*		

*Absorbance percentage relative to control (= 100% and 50%).

4. Discussion

The need for drugs originating from plant materials, and directed for preventing serious diseases as cardiovascular and hepatic diseases is a national need. In this study, the identification of *C. sinaica* compounds was elucidated by means of TLC, FAB-MS, NMR spectroscopy and co-chromatography with HPLC. The cardiovascular and hepatoprotective activities of different fractions obtained from *C. sinaica* leaves with flower and the young stems extracts were investigated.

The main active constituents such as hyperoside, vitexine-2″-O-rhamnoside, epicatechin, procyanidin B2 and procyanidin C1 were isolated and identified from the young stems and confirmed by comparison with previously reported data[11,17]. The presence of those main active constituents explains the biological results discussing the similarity of bioactivities of both *C. sinaica* and other species used abroad in phytopharmaceuticals. These new findings allow the use of young stems beside the leaves of *C. sinaica* biologically.

The increase of absorbance values in some samples was not false negative result for cytotoxicity, as microscopic examination confirmed the viability of the cultured hepatocytes.

The revealed data showed no cytotoxicity exerted by the leaves or young stems of *C. sinaica* in the given range, but leaves had greater safety margin. Also, the chloroformic extract in both parts had a decreasing safety margin with increased concentration. For the hepatoprotection, almost complete hepatoprotection occurred for all fractions of leaves and young stems taking in consideration that the chloroformic extracts always showed prominent at low doses and protection decreased with increased concentration. These new findings may have great impact for future use of *C. sinaica* for the first time in hepatic disorders.

In conclusion, the results of these chemical and biological studies suggest the use of *C. sinaica* growing in Egypt as a preventive drug against cardiovascular and hepatic diseases. The chemical studies suggest the use of woody young stems as a newly investigated bioactive organ. The extraction of unsaturated fatty acids from the seeds of the plant would serve as a good health and nutritive product.

Conflict of interest statement

We declare that we have no conflict of interest.

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