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THE CYTOTOXIC EFFECT OF CHELIDONIUM ALKALOIDS ON CELLS ISOLATED FROM ADULT RAT LIVER

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ABSTRACT

Alkaloids of the aerial parts of chelidonium were extracted in the salt form, and their aqueous solutions were prepared in different concentrations. Rat hepatocytes were obtained by liver perfusion. The alkaloidal solutions were added to suspensions of hepatocytes in petri dishes and the mixtures were incubated. Two types of controls have been used; in one type, no alkaloidal extract was added to the media, and in the other, alkaloidal extract of datura which has no cytotoxic activity was added to the hepatocytes. Intracellular LDH activity as well as the activity of leaked LDH into the media, the glucose uptake by the cells, and the glycogen contents of the cells were determined after incubation.

The results indicate that 0.05 ml of the alkaloidal solution of chelidonium has no detectable effect on LDH activity during a 240 minute incubation period. With 0.1 ml doses, detectable changes were observed only after 240 minutes of incubation. When 0.2 ml doses were used, the intracellular LDH activity was lowered by 3.23,6.79 and 30.89 percent after 60,120 and 240 minutes of incubation respectively, as compared with the controls. The activity of leaked LDH into the media
duration of incubation was increased. Determination of glucose in different media showed that the uptake of this sugar by the hepatocytes incubated with chelidonium decreased as the dose and incubation periods increased. On the other hand, as the glycogen content of the hepatocytes incubated with chelidonium was the same as that of the controls, we believe that the hepatocytes lost their viability in the presence of chelidonium-derived cytotoxic alkaloids.

Key Words: *Chelidonium majus; Papaveraceae*; Cytotoxic effect; Rat hepatocytes.

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INTRODUCTION

Chelidonium majus L. (Fam. : *Papaveraceae*) is a perennial medicinal herb which grows wildly throughout Europe, Western Asia, and North Africa, with a preference for rubble.[1,2] In Iran, it grows in the northern parts of the country[3] (Fig. 1).

Chelidonium contains alkaloids (including

Table I. Changes of intracellular LDH activity when media containing different amounts of datura or chelidonium alkaloids were incubated for 60, 120, and 240 minutes at 37°C.

			Enzyme activity (units/μg DNA)			
			at the beginning of incubation	after 60 minutes of incubation	after 120 minutes of incubation	after 240 minutes of incubation
		Ordinary control	172.8	160.9	148.6	118.8
Volume (ml) of added extract	Datura	0.05	172.8	160.8	148.5	118.8
		0.10	172.7	160.9	148.5	118.8
		0.20	172.8	160.9	148.4	118.7
	Chelido-nium	0.05	172.7	160.3	145.8	111.2
		0.10	172.6	159.6	143.2	100.7
		0.20	172.6	155.7	138.5	82.1

Note:
- The number of cells in each petri dish was 20×10^6, and separate petri dishes were used for each course of incubation.
- Each result represents the mean of four experiments.

chelidonine, sanguinarine and coptisine)[2,4] and mineral salts.[3] It has a long history in Europe as being useful for the treatment of colonic polyposis, by enema. Topically, the juice of the plant has been used in tinea, eczema, warts, papillomas, condylomas, nodules of nursing mothers, and malignant tumors of the skin.[2,4] It has been shown that extracts from this plant are inhibitory for sarcoma 180 and Ehrlich mouse carcinoma.[5,6]

Since there have been some reports indicating that the alkaloids of chelidonium have cytotoxic activity,[4,6] especially against Eagles KB carcinoma of the nasopharynx in cell culture[4], we decided to prepare the alkaloidal extract of the plant growing in Iran and check it's cytotoxicity on adult rat hepatocytes.

MATERIAL AND METHODS

Plant Material

The plant material used in this investigation was derived from the aerial parts of chelidonium, collected in Roodbar jungle during the flowering period (the end of spring). Voucher specimens were identified and authenticated by the Biology Department of the Faculty of Sciences, University of Isfahan. The collected fresh aerial parts of the plant were dried in the shade.

To check the cytotoxic activity of chelidonium, two types of controls have been used; in one type (ordinary control), no alkaloidal extract was used, and in the other, alkaloidal extract of the plant *Datura stramonium L.* (Fam. : *Solanaceae*) which has no cytotoxic activity,[7,8] was added to the media. Standard datura plants which had been cultivated in Isfahan were obtained from Amin Chem. Pharm. Co.

Alkaloid Extraction

125 ml of 96 percent ethyl alcohol and 25 ml of 0.2N hydrochloric acid were added to a 50 gm sample of air dried, coarsely milled aerial parts of chelidonium.The mixture was shaken for one hour. The filtrate was condensed, and the residue dissolved in 25 ml of distilled water and shaken with chloroform. Finally, the aqueous layer was filtered and used for the preparation of different alkaloidal concentrations which were representatives of 100, 200, and 400 mg dried plant material per ml. The presence of alkaloids in the final extracts was identified by TLC and application of Dragendorff's reagent. Alkaloids of datura were also extracted using the same procedure described above.

Preparation of Rat Hepatocytes

The rat hepatocytes were prepared by using Suzangar and Dickson's method.[9] Adult rats, preferably males, 5-6 months old, and weighing 200-300 gm were

Table II. Changes of LDH activity in the media containing different amonnts of datura or chelidonium alkaloids after incubation for 60, 120, and 240 minutes at 37°C.

			Enzyme activity (units/mg protein)			
			at the beginning of incubation	after 60 minutes of incubation	after 120 minutes of incubation	after 240 minutes of incubation
		Ordinary control	7500.4	8282.1	9101.7	10175.3
Volume (ml) of added extract	Datura	0.05	7501.4	8275.0	9102.4	10177.0
		0.10	7501.1	8296.0	9102.6	10177.3
		0.20	7501.0	8289.0	9103.0	10177.8
	Chelido-nium	0.05	7502.3	8327.6	9210.6	10622.1
		0.10	7501.7	8349.4	9316.6	11281.6
		0.20	7501.9	8600.5	9518.1	123656.7

Note:
- The number of cells in each petri dish was 20×10^6, and separate petri dishes were used for each course of incubation.
- Each result represents the mean of four experiments.

used. The animals were anesthetized with diethyl ether. The liver was perfused at room temperature with warm buffered EDTA (pH= 7.4)[10] via the inferior vena cava until blanching occurred. The perfused liver was then excised, washed with perfusion fluid, gently blotted with filter paper, weighed, and transferred to a petri dish containing 10 ml of dispersion solution.[10]

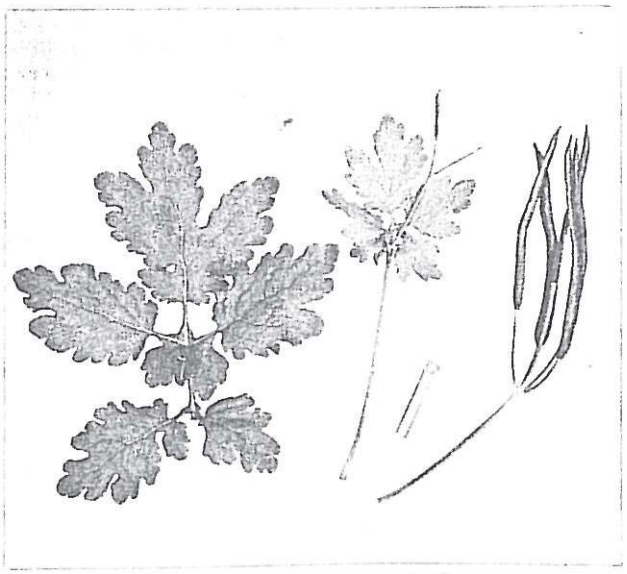

Fig. 1. *Chelidonium majus L.*

The liver was cut into several small pieces, then another aliquot of dispersion solution was added. A modified Potter-Elvehjem hemogenizer was used for the dispersion of the tissues and preparation of cell suspension. The cell suspension was filtered through an 80 mesh stainless steel sieve, followed by a 200 mesh sieve to remove connective tissue and cell clumps, and centrifuged for 1 minute at 150 g. The supernatant was discarded and the cells were suspended again in dispersion solution and centrifuged. A known number of cells were resuspended in ringer-bicarbonate buffer (RBB), and the obtained suspension was used for the cytotoxicity studies.

Assessment of Hepatocyte Viability

Viability of the hepatocytes was assessed using morphological assays (i.e. membrane blebbing, volume, and cytoskeletal assays).[11]

Addition of Alkaloidal Extracts and Incubation of Hepatocytes

Sterile petri dishes were used for the incubation of hepatocytes. A known number of cells was transferred to each petri dish, then a sufficient amount of RBB was added. In order to study the degree of glucose uptake, RBB containing 10 mM glucose was used. Enough oxygen was blown to each suspension. Then, after cover-

Table III. Changes of glucose concentration in the media containing different amounts of datura or chelidonium alkaloids after incubation for 60, 120, and 240 minutes at 37°C.

			Glucose concentration (µg/mg protein)			
			at the beginning of incubation	after 60 minutes of incubation	after 120 minutes of incubation	after 240 minutes of incubation
		Ordinary control	1590	1400	1280	970
Volume (ml) of added extract	Datura	0.05	1570	1370	1250	1000
		0.10	1550	1360	1240	980
		0.20	1600	1420	1220	950
	Chelidonium	0.05	1560	1390	1240	990
		0.10	1610	1410	1300	1100
		0.20	1625	1500	1420	1290

Note:
- The number of cells in each petri dish was 20×10^6, and separate petri dishes were used for each course of incubation.
- Each result represents the mean of four experiments.

ing, the petri dishes were incubated for 10 minutes at 37°C. Chelidonium and datura alkaloidal extracts were added in different concentrations to the petri dishes and the final volume of the suspension in each petri dish was adjusted for 60,120 and 240 minutes at 37°C.

Separation of Hepatocytes from other Medium Contents

After incubation, the content of each petri dish was centrifuged for 1 minute at 150 g. The supernatant was then separated, and the precipitated cells were washed with three aliquots of normal saline. Some of the washed cells were frozen to facilitate their breakdown and make them ready for DNA estimation. Other portions of the washed cells were used for the assay of LDH as well as the determination of glycogen and protein contents.

Estimation of DNA

Frozen cells were left at room temperature to thaw, then their DNA content was estimated by Burton's method.[12] A standard curve of DNA was plotted using solutions having different concentrations of DNA.

Assay of Lactate Dehydrogenase (LDH)

This enzyme was assayed colorimetrically using Sigma Kits. The method is based on the reverse (pyruvate-lactate) reaction, and the velocity of the reaction is dependent on LDH activity. An excess known quantity of the substrate (pyruvate) is usually added to samples having LDH, and in the presence of NADH, the substrate will be reduced to lactic acid. Unreduced pyruvate is then reacted with 2,4-dinitrophenylhydrazine to form the corresponding phenylhydrazone, which has a golden color at alkaline pH. The color of pyruvic 2,4-dinitrophenylhydrazone is measured at 400-550 nm.[13] A standard curve was drawn using different concentrations of pyruvate and measuring the color of the relevant phenylhydrazone derivative.

Determination of Protein

Protein determinations were carried out by the method of Lowry et al.[14] A stock solution of bovine serum albumin was used to draw the standard curve.

Determination of Glycogen

Glycogen was determined by using the colorimetric micromethod of Kemp and Kits Van Heiningen.[15] A stock solution of standard glucose was used to draw the standard curve.

Determination of Glucose

Amounts of glucose were determined in the media after incubation, using the orthotoluidine method.[13] A

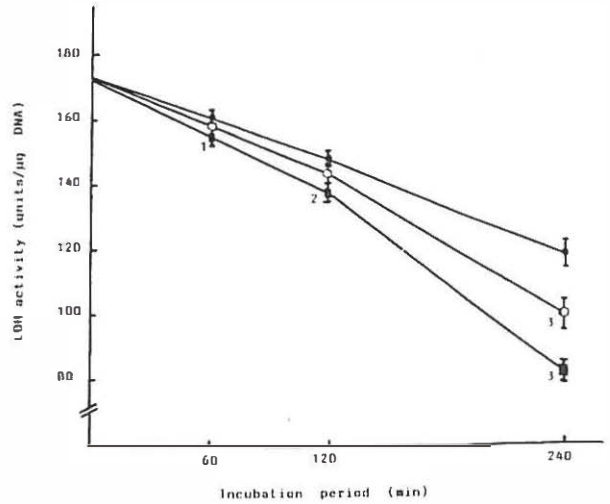

Fig. 2. Effects of different doses of chelidonium alkaloidal extracts on intracellular LDH activity after 60, 120 and 240 minutes of incubation.
(●) Control
(○) 0.10 ml alkaloidal extract
(■) 0.20 ml alkaloidal extract
1: P< ●. ●5 2: P< ●. 01 3: P< ●. 005

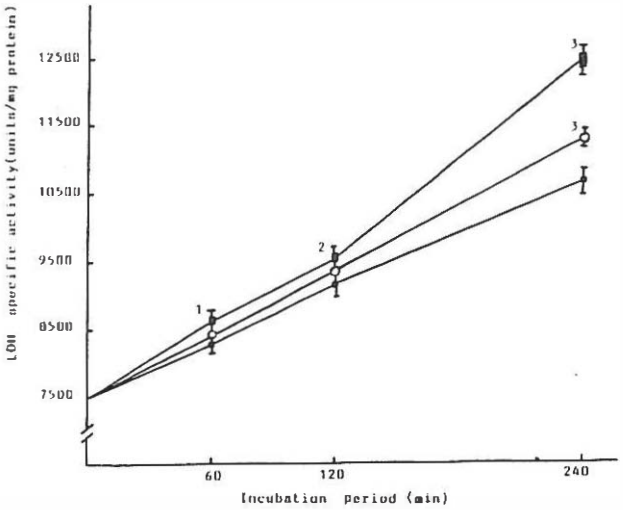

Fig. 3. Effects of different doses of chelidonium alkaloidal extracts on LDH activity in the media after 60,120 and 240 minutes of incubation.
(●) Control
(○) 0.10 ml alkaloidal extract
(■) 0.20 ml alkaloidal extract
1: P< 0.05 2: P< 0.025 3: P< 0.005

standard curve was plotted using different concentrations of glucose prepared from a stock solution.

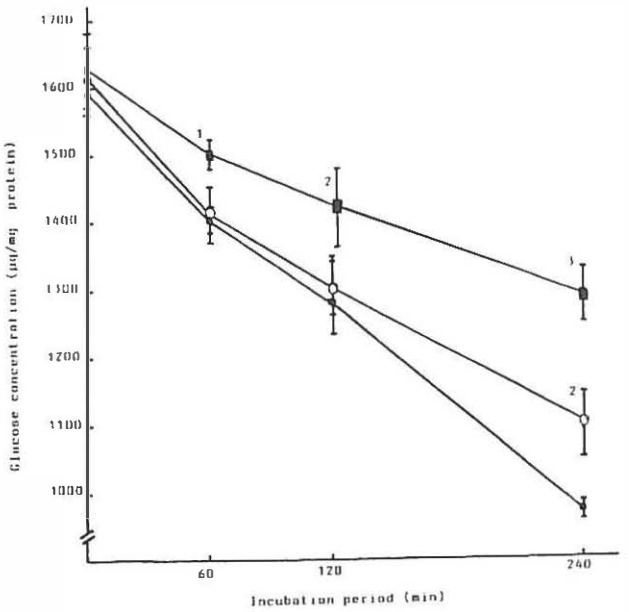

Fig. 4. Effects of different doses of chelidonium alkaloidal extracts on glucose concentrations in the media after 60, 120 and 240 minutes of incubation.
(●) Control
(○) 0.10 ml alkaloidal extract
(■) 0.20 ml alkaloidal extract
1: P< 0.05 2: P< 0.01 3: P< 0.005

RESULTS

Number and Morphology of Cells obtained from Liver Perfusion

The number of cells obtained when buffered EDTA was used for liver perfusion was 33.5×10^6 cells per gram of liver. Results of hepatocyte viability assessment indicated that 80 percent of the cells were morphologically intact.

Intracellular LDH Activity

A: Effects of Datura Alkaloidal Extracts

Intracellular LDH activity was decreased in the ordinary control media after incubation, since some cells die in the experimental conditions. Thus, after 240 minutes of incubation, the enzyme activity decreased from 172.8 units/μg DNA to 118.8 units/μg DNA (i.e. there was a 31.25 percent reduction). On the other hand, media to which 0.05,0.1 and 0.2 ml of datura alkaloidal extract were added showed no change in LDH activity after incubation for 60, 120 and 240 minutes as compared with the ordinary controls (Table I).

B: Effects of Chelidonium Alkaloidal Extracts

Changes of intracellular LDH activity when different concentrations of alkaloidal exracts of chelidonium

Table IV. Changes of cellular glycogen content when media containing different amounts of datura or chelidonium alkaloids were incubated for 60, 120, and 240 minutes at 37°C.

			Glycogen content (µg/mg protein)			
			at the beginning of incubation	after 60 minutes of incubation	after 120 minutes of incubation	after 240 minutes of incubation
		Ordinary control	32	30	30	29
Volume (ml) of added extract	Datura	0.05	30	31	29	29
		0.10	32	28	30	31
		0.20	33	34	33	30
	Chelido-nium	0.05	32	33	33	32
		0.10	29	29	28	29
		0.20	30	32	30	29

Note:
- The number of cells in each petri dish was 20×10^6, and separate petri dishes were used for each course of incubation.
- Each result represents the mean of four experiments.

were used are shown in Table I. The results indicate that cells to which 0.05ml of the extract was added and were incubated for 60,120 and 240 minutes, as well as cells to which 0.1 ml of the extract was added and were incubated for 60 and 120 minutes showed no significant change in intracellular LDH activity as compared with the controls. However, when the latter cells were incubated for as long as 240 minutes, they showed a 15.23 percent decrease in their intracellular LDH activity as compared with the controls. Cells to which 0.2 ml of the extract was added and were incubated for 60,120 and 240 minutes showed prominent reductions in their intracellular LDH activity. These reductions were 3.23 , 6.79 and 30.89 percent after 60,120 and 240 minutes of incubation respectively (Fig.2).

LDH Activity in the Media

A: Effects of Datura Extracts

When different doses of datura alkaloidal extracts were used, no significant changes of LDH activity in the media were observed as compared with the ordinary controls.

B: Effects of Chelidonium Extracts

The 0.05 ml dose of the extract had no significant effects on LDH activity in the media after 60,120 and 240 minutes of incubation (Table II). On the other hand, the 0.1 ml dose of the extract caused a significant increase in the enzyme activity only after 240 minutes of incubation. This increase was 10.87 percent as compared with the controls.

The 0.2 ml dose of the extract caused significant increases in LDH activity of the media after different periods of incubation. These increases were 3.84 , 4.57 and 21.52 percent after 60,120 and 240 minutes of incubation respectively (Fig.3).

Glucose Uptake

A: Effects of Datura Extracts

Changes of glucose concentration in the media to which different doses of datura alkaloids were added are shown in able III. In ordinary control media, the cells were able to consume the sugar, and after 240 minutes of incubation, the glucose content was reduced from 1590 to 970 µg/mg protein. As shown in Table III, datura alkaloids had no significant effect on the glucose uptake of the cells in the media as compared with ordinary controls.

B: Effects of Chelidonium Extracts

The 0.05 ml dose of the extract after 60,120 and 240 minutes of incubation, as well as the 0.1 ml dose after 60 and 120 minutes incubation caused no significant changes in glucose uptake as compared with the con-

trols. After 240 minutes of incubation, the latter dose caused reduction in glucose contents from 1610 µg/mg protein at the beginning of incubation to 1100 µg/mg protein. This means that 13.40 percent of the glucose had not been consumed during 240 minutes of incubation as compared with the controls. The 0.2 ml dose of the extract caused reductions of glucose contents from 1625 µg/mg protein at the beginning of incubation to 1500,1420 and 1290 µg/mg protein after 60,120 and 240 minutes of incubation respectively. This means that 7.14,10.93 and 32.99 percent of the glucose content had not bee
incubation as compared with the relevant controls.

Glycogen Content

There were no differences in the glycogen contents of cells incubated in the presence of datura as well as chelidonium alkaloids as compared with the ordinary controls (Table IV).

DISCUSSION

In this investigation, intracellular LDH activity was determined in order to be used as an index of cytotoxicity in the media of rat hepatocytes, since retention of LDH in the cells is an indication of living hepatocytes, while leakage of this enzyme indicates their death.[7,16] Decrease in intracellular LDH activity of the media in which chelidonium alkaloids were added (Table I and Fig. 2) was confirmed when the activity of leaked LDH into the media was determined (Table II and Fig. 3). The increase in cell permeability may simply reflect changes in the level of cytotoxic material inside the cells.[7]

Since one should not use only a single test in order to check the cytotoxicity of a material,[17] glucose uptake was also determined to check the viability of cells to which chelidonium alkaloids were added. The results of this determination confirmed the results obtained from the assays of intracellular and leaked LDH activities, i.e. the glucose uptake by the cells to which 0.1 ml of chelidonium alkaloidal extract was added and were incubated for 240 minutes, as well as the glucose uptake by the cells incubated in the presence of 0.2 ml of chelidonium extract was decreased.

Whether the decrease in glucose uptake by the cells in the presence of chelidonium alkaloids is recompensated by the intracellular glycogen or not, and whether the reduced cellular consumption of glucose in the presence of chelidonium alkaloids results from reduced cellular metabolic activity and cellular death or not, are two questions which could be answered by determining the glycogen contents of the incubated hepatocytes in different media. Results of this determination indicate no difference between the controls and cells incubated in the presence of chelidonium alkaloids. Use of glucose for the production of energy is an indicator of cell viability, and the reduction in glucose uptake in the presence of chelidonium alkaloids is an indicator of the cytotoxic activity of these alkaloids.

Finally, the results of this investigation indicate that the cytotoxic activity of chelidonium alkaloids is dose dependent, i. e. when the amount of the added cytotoxic alkaloids to the hepatocytes was increased, the cytotoxic activity also increased. On the other hand, the duration of incubation of the hepatocytes in the presence of the cytotoxic alkaloids also acted as a factor affecting the cytotoxic activity, because when the incubation period was increased, the cytotoxic activity was also found to increase.

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