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Full Length Research Paper

Promising antioxidant and cytotoxic activities of the aqueous ethanolic extract of carob leaves

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Plants are endless reservoir for several phytochemicals including antioxidant and cytotoxic drugs. The objective of this study was the investigation of the antioxidant and the cytotoxic activities (against Vero and HEp-2 cell lines) of the aqueous ethanolic extract of carob leaves. The extract inhibited xanthine oxidase at IC_{50} of 244 µg/ml. There is a marked cytotoxic activity of crude aqueous extract of carob on both mammalian cell lines (Vero and HEp-2) and the effect is more obvious on the human cell line (HEp-2). The results revealed that the crude aqueous extract of carob leaves could be a promising source for antioxidant as well as antitumor bioactive agents.

Key words: Carob leaves, aqueous ethanolic extract, *Ceratonia siliqua*, antioxidant, xanthine oxidase, cytotoxicy.

INTRODUCTION

The carob tree (*Ceratonia siliqua* L. Fabaceae) has been widely cultivated in Mediterranean countries for years. A multitude of publications have reported the physiological effects of carob fruits but very few ones for carob leaves. Carob fruits are given to prevent vomiting in infants, while the mucilage is given to thicken the stomach content (Samuelsson, 1999; Duke, 2002). The intake of carob fibre was found to reduce LDL and total cholesterol concentrations (Law et al., 1994; Zunft et al., 2003). Extracts from pods and leaves of carob were tested for their ability to inhibit cell proliferation of mouse hepatocellular carcinoma cell line (T1). Results of the two extracts, showed a marked alteration of T1 cell proliferation in a dose-related fashion reaching the maximal effect at a concentration of 1 mg/ml (Corsi et al., 2002).

Xanthine dehydrogenase and not xanthine oxidase is the natural genetically coded protein in the body. In tissues NADH is the natural substrate of xanthine dehydrogenase, but under ischemic conditions xanthine dehydrogenase is deproteinised and converted to xanthine oxidase (Halliwell et al., 1993). Upon return to aerobic conditions (reperfusion), an oxidative burst due to interaction of xanthine oxidase with hypoxanthine or xanthine releases copious amounts of superoxide, which in the presence of suitably chelated iron can catalyse the formation of the highly reactive hydroxyl radical (McCord, 1987). The aqueous ethanolic extract of carob leaves inhibited the hydroxylation of salicylic acid by reactive oxygen species (ROS) in a dose-dependent manner (IC_{50} = 308) (Eldahshan, 2006). So, the extract was assessed for its ability to inhibit the xanthine oxidase enzyme itself. There is increasing demands for anticancer therapy (Yanno et al., 2005). Therefore, the current study undertook assessing the probable cytotoxic activity of crude aqueous ethanolic extract of carob leaves. Currently, there are general trends designed to simplify and hasten the cytotoxicity testing which implicate the reduction in the use of laboratory animals and to supplement (Abraham et al., 2004; Malonne et al., 2000) or totally supplant the *in-vivo* cytotoxicity studies with *in-vitro* cytotoxicity assays. This is done partly in response to a societal call on humane grounds, hence cultured tissues and cells are nowadays increasingly used (Byrd et al., 2000; Gilmour, 2004). Furthermore, such test systems are likely faster, simpler, reliable and less expensive and augment the variety of studies, especially

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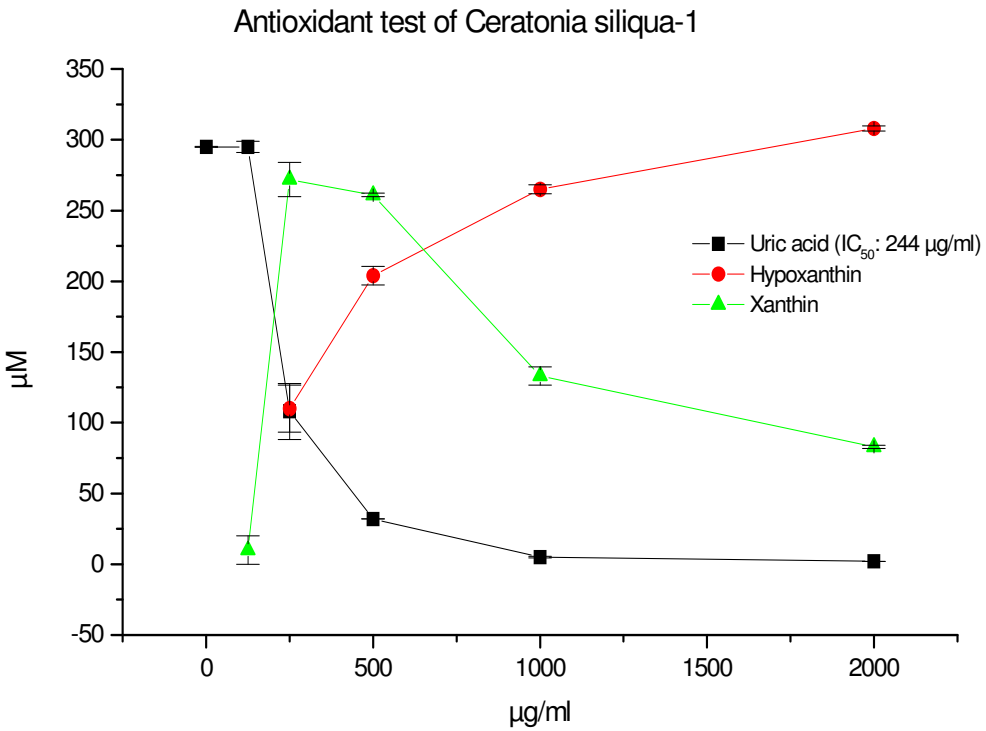

Figure 1. Inhibition of xanthine oxidase by the aqueous ethanol extract of carob leaves in xanthine oxidase assay.

those related to the mechanism of toxicity (Frank, 1996; Freshney, 2000).

EXPERIMENTAL

Reagents

Xanthine, hypoxanthine, xanthine oxidase and EDTA were obtained from Merk (Darmstadt, Germany). Epithelial cells of African green monkey kidney (Vero cell line) and human epithelial cells of larynx (HEp-2 cell line) were obtained from Vacsera, Egypt.

Glutaraldehyde and trypan blue were obtained from Fluka chemie GmbH, Buchs, Germany. While, crystal violet and glacial acetic acid were obtained from El-Nasr Pharmaceutical Chemicals Co. (ADWIC), Cairo, Egypt.

Cisplatin, cyclophosphamide and doxorubicin HCl were the products of KUP under technical assistance of United Douglas Pharm. Co., USA. Bleomycin was obtained from Nippon Kayakudo, LTD, Tokyo, Japan. Dacarbazine citrate was supplied by Gesellschaft fur Klinische Spezial Praparate GmbH, Fehlandtstrasse Hamburg, Germany. While, calcium folinate was obtained from Mayne Pharma Plc, Warwickshire, UK.

Preparation and extraction protocol

Carob leaves were collected from Orman garden, Giza, Egypt. It was authenticated by Prof. Dr Abdel Salam El Noyehy, Prof. of Taxonomy, Faculty of Science, Ain Shams University, Cairo, Egypt. Voucher specimens were deposited at the herbarium of Pharmacognosy department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt. The plants were dried in shade and reduced to a fine powder. Powder of the air dried leaves of carob leaves were extracted by 70% ethanol on cold till exhaustion. The solvent was distilled of in Rota vapour at 55°C till dryness. The extract was dried till constant weight in vacuum desiccators over anhydrous calcium chloride. Different concentrations of the completely dried aqueous extract of carob leaves were prepared in normal saline solution. The aqueous ethanolic extract of carob leaves was sterilized by filtration through a Millipore membrane filter (0.45 µm). The extract was kept at 4°C and warmed in a water bath at 37°C before its use in the cytoxic activity.

Hypoxanthine/xanthine oxidase assay

Antioxidant activity was determined according to the method of (owen et al., 2000). The reaction mixture consisted of 2.76 ml of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH = 10.0), 0.06 ml of 10 mM xanthine and 0.06 ml of sample dissolved in DMSO. The reaction was started by addition of 0.12 ml Xanthine oxidase (0.04 units) and the absorbance at 293 nm was recorded for 90 seconds. Rate of uric acid formation was calculated from the proportional increase in absorbance. The inhibition profile of xanthine oxidase by the aqueous ethanolic extract is depicted in Figure 1.

Cytotoxicity assay using Vero and HEp-2 cell lines

Maintenance of Vero cell line and HEp-2 cell lines

Vero or HEp-2 cell lines were grown in 50 ml sterile tissue culture flasks (Nunclon, Denmark) and subcultured every 96 h as follows:

The old MEM was removed and the mammalian cells were rinsed

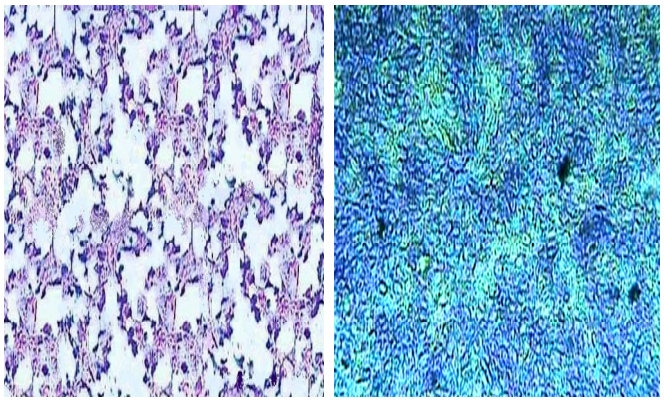

Figure 2(a). Cytotoxic effect of crude aqueous extract of carob on Vero cell line (100X), **(b)** Control Vero cell line (100X).

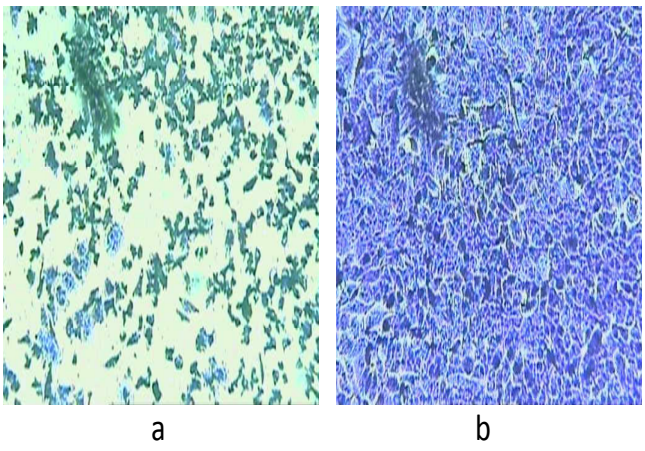

Figure 3(a). Cytotoxic effect of crude aqueous extract of carob on HEp-2 cell line (100X), **b.** Control HEp-2 cell line (100X).

once with 0.5 ml of 0.25% trypsin solution (VACSERA, Cairo, Egypt). After discarding the rinse, the mammalian cells were detached by incubating with an additional 0.5 ml of the same trypsin solution at 37℃ for 3 - 5 min. After cell detachment, 10 ml of fresh MEM supplemented with 5% fetal bovine serum (FBS) were added to the flask; the cell suspension was then aspirated and dispensed trypsinized and suspended in fresh MEM supplemented with 10% fetal bovine serum (FBS) to count of 10^4 - 10^5 living cell/ml. The count of living cells was confirmed using haemocytometer (Shanghai, China) after staining with trypan blue solution (0.4 g % in phosphate buffered saline). Aliquots of 200 µl of mammalian cell suspensions were transferred to tissue culture flasks each containing 5 ml MEM supplemented with 10% foetal bovine serum (FBS). The flasks were then incubated at 37℃ in presence of 5% CO_2 and humid atmosphere for 48 h to form a confluent monolayer.

Cytotoxicity assay

The cytotoxicity assay on Vero and HEp-2 cell lines was carried out according to the method described by Daikoku et al. (1989), with some modifications. For this study, 96–well tissue culture plates (Nunclon, Denmark) were seeded with (200 µl/well) Vero cell suspension (3 - 5 × 10^4 cells/ml). The plates were incubated at 37℃ in humidified 5% CO_2 incubator till a confluent monolayer was obtained (after 48 h) as determined by inverted microscope (Hund Wetzlar, Helmut Hund GmbH, Germany). The liquid medium above the monolayer was aspirated and the cells were washed once and incubated with MEM, containing 1% FBS (200 µl/well) for further 24 h.

After incubation, the remaining medium in each well was aspirated and replaced by 180 µl of MEM with 1% FBS and the volume was completed to 200 µl using aqueous extract of carob or one of the cytotoxic drugs (calcium folinate, cisplatin, bleomycin SO_4, dacarbazine citrate, doxorubicin HCl and cyclophosphamide). Twelve wells for each concentration of the tested cytotoxic drug or aqueous extract of carob against Vero or HEp-2 cell lines. The tissue culture plates were then incubated for 24 h at 37℃ in a humidified 5% CO_2 incubator. After incubation, plates were removed and the remaining liquid in each well was aspirated followed by the addition of 110 µl of 1% glutaraldehyde solution (as a fixative). After 15 min contact time at room temperature the glutaraldehyde solution was aspirated and 100 µl of 0.1% crystal violet solution was added and the plates were left for further 30 min. The plates were then inverted for draining off crystal violet solution. The plates were submerged in water and the excess stain was removed with continuous slow flow of water for 15 min. The plates were then air dried. The stain was eluted with 10% glacial acetic acid in distilled water (100 µl /well) and absorbance of each well was measured spectrophotometrically at 590 nm with an ELISA micro–plate reader (Meter Tech. E 960, USA). The mean values and standard deviation (STD) for each concentration of different test agents were calculated using IBM compatible. The control was similarly treated in parallel except that the cytotoxic agents were replaced with normal saline solution.

The concentrations tested for aqueous extract are (6.25, 12.5, 25, 50 mg/ml), while for cytotoxic drugs [calcium folinate or cyclophosphamide (1.25, 2.5, 5, 10 mg/ml), cisplatin (0.125, 0.25, 0.5, 1.0 mg/ml), bleomycin SO_4 (1.5, 3, 6, 12 mg/ml), dacarbazine citrate (4.5, 9, 18, 36 mg/ml) and doxorubicin (0.25, 0.5, 1.0, 2.0 mg/ml).

Percentage of cytotoxicity was calculated according to the formula described by Zheng et al. (2000):

Percentage of cytotoxicity = {(A – B) / A} × 100%

Where, A = absorbance of the control; and B = absorbance of the test at 590 nm.

The cytotoxic effects of the crude aqueous extract of carob leaves on vero cel line and HEP-2 cell line are depicted in Figures 2 and 3

RESULT

Antioxidant activity

The aqueous ethanol extract of carob leaves exhibited An antioxidant capacity through inhibition of xanthine oxidase, at IC_{50} value of 244 µg/ml.

Cytotoxic activity

The dose used of the aqueous ethanolic extract of carob leaves was compared with the therapeutic dose of each antitumor agent. The cytotoxic activity of the aqueous

Table 1. Cytotoxic activities of aqueous extract of carob as compared to that of some anticancer drugs against Vero and HEp-2 cell lines.

Cytotoxic agent or aqueous extract	Concentration (mg/ml)	Percentage of cell line destruction	
		Vero (±STD)	HEp-2 (±STD)
Aqueous extract of carob	6.25	24.34 ± 1.19	44.6 ± 2.2
	12.50	35.03 ± 2.87	72.2 ± 3.2
	25.00	45.16 ± 4.46	84.1 ± 5.8
	50.00	54.25 ± 3.18	90.6 ± 3.7
Calcium folinate	1.3	8.81 ± 0.57	5.53 ± 0.75
	2.5	22.59 ± 3.38	11.01 ± 1.43
	5.0	30.47 ± 2.08	19.42 ± 4.84
	10.0	38.11 ± 2.99	39.73 ± 3.86
Cisplatin	0.13	12.50 ± 0.71	6.00 ± 1.41
	0.25	18.58 ± 2.24	12.16 ± 3.05
	0.50	25.40 ± 1.28	29.82 ± 1.87
	1.00	27.20 ± 4.52	44.27 ± 4.63
Bleomycin SO_4	1.5	6.68 ± 0.54	9.28 ± 1.25
	3.0	13.16 ± 1.65	20.28 ± 3.23
	6.0	18.94 ± 2.74	26.16 ± 1.64
	12.0	24.09 ± 1.54	34.90 ± 5.51
Dacarbazine citrate	4.5	6.53 ± 0.61	22.94 ± 1.33
	9.0	16.51 ± 2.13	32.19 ± 3.09
	18.0	25.45 ± 1.21	43.46 ± 2.06
	36.0	36.35 ± 3.89	48.59 ± 5.08
Doxorubicin HCl	0.25	5.53 ± 0.66	17.29 ± 1.82
	0.50	9.26 ± 1.22	23.34 ± 3.31
	1.00	22.16 ± 3.15	81.58 ± 3.64
	2.00	33.79 ± 2.05	83.99 ± 5.64
Cyclophosphamide	1.25	14.77 ± 0.39	27.53 ± 3.57
	2.50	21.71 ± 1.57	35.99 ± 2.81
	5.00	26.93 ± 2.73	43.40 ± 1.99
	10.00	35.23 ± 3.15	47.27 ± 4.63

Values are mean ± STD.

ethanolic extract of carob on Vero and HEp-2 cell lines was evaluated according to the method described by Daikoku et al. (1989), and the results (Table 1) were compared with six well-known antitumor agents (calcium folinate, cyclophosphamide, cisplatin, bleomycin SO_4, dacarbazine citrate and doxorubicin).

The IC_{50} of the tested aqueous extract is lower than that of dacarbazine citrate on Vero cell line. On the other hand, the IC_{50} of the tested extract is significantly lower than that of calcium folinate, bleomycin SO_4 and cyclophosphamide and prominently much lower than that of dacarbazine citrate but, seven folds higher than that of cisplatin.

DISCUSSION

Antioxidant compounds scavenge free radicals such as peroxide or hydroperoxide and thus reduce the level of oxidative stress and slow/prevent the development of complications associated with oxidative stress-related diseases.

The aqueous ethanolic extract of carob leaves inhibited xanthine oxidase at low IC_{50} reflecting a strong antioxidant activity. This is the first scientific report of cytotoxic activity of carob leaves where the results of the cytotoxicity assays (Table 1) revealed that, there is marked cytotoxic activities of crude aqueous extract of

Table 2. Inhibition concentration 50% (IC_{50}) for aqueous ethanolic extract of carob as well as some anticancer drugs against Vero and HEp-2 cell lines.

Cytotoxic agent or aqueous extract	IC_{50} (mg/ml) against:	
	Vero	HEp-2
Aqueous extract of carob	38	7.5
Calcium folinate	> 10.00	> 10.00
Cisplatin	> 1.00	> 1.00
BleomycinSO4	> 12.00	> 12.00
Dacarbazine citrate	> 36.00	≥ 36.00
Doxorubicin HCl	> 2.00	0.72

carob on both mammalian cell lines (Vero and HEp-2) and the effect is more obvious on the human cell line (HEp-2). These results indicate that the sensitivity of HEp-2 cell line for cytotoxic drugs is higher than that of Vero cell line for the same cytotoxic agents.

The significantly low IC_{50} (Table 2) of crude aqueous extract of carob compared to that of calcium folinate, bleomycin SO_4, cyclophosphamide and dacarbazine citrate, suggests that the purified active constituent(s) will have much lower IC_{50}.

Carob is an important source of food in tropical regions, but at present the leaves are discarded. These waste products which contain a lot of phenolics appear to have real potential low-cost source of antioxidant and cytotoxic drug.

So, the availability and cheapness of carob leaves which considered as waste products in Egypt suggest that such plant could be a promising low cost and potential natural source for antioxidant as well as antitumor bioactive agents.

Conclusion

This study could offer scientific basis for the further in-depth evaluation of the aqueous ethanolic extract of carob leaves for separation of different constituents of crude aqueous extract of carob and assessing the cytotoxic activity for each compound as well as evaluation of the clinical relevance of the obtained data. This may be a useful area for future study.

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