

Cytotoxic Effects of *Glycyrrhiza glabra* L., *Morus nigra* L. and *Urtica urens* L. Extract against the Human Hepatocarcinoma HepG2 and Mouse L₂₀B

Cell Lines

التأثيرات السمية لمستخلصات نباتات عرق السوس والتوت الأسود والحكيك ضد خط الخلايا الكبدية HepG2 و خط خلايا الفأر L₂₀B

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Abstract

The present study aimed to study the cytotoxic effects of three Iraqi traditional medical plants extracts using tissue culture technique on the human hepatocarcinoma HepG2 and mouse cell L₂₀B cell lines. *Glycyrrhiza glabra* L. root, *Morus nigra* L. and *Urtica urens* L. leaves were extracted using 99% ethanol solvent. Seven crude concentrations were prepared by serial dilution, with concentrations of 3.9, 7.81, 15.62, 31.25, 62.5, 125 and 250 mg/ml, respectively. These were added to the microtiter plate containing 1×10^5 cells/well and 200 μ l of the medium. The seven concentrations were used in triplicate to investigate their cytotoxic and anti-proliferative effects. The analysis of chemical composition of these plant extracts were determined by fast liquid chromatography (FLC). The extract of *Urtica urens* L showed the highest potent cytotoxicity in the HepG2 and L₂₀B cell lines, while both *G. glabra* L. and *Morus nigra* L. crude extracts showed the lowest cytotoxicity. All concentrations of crude extracts showed different cytotoxic activity *in vitro*.

Keywords: plant extracts, cytotoxic activity, HepG2, L₂₀B.

الملخص

قيمت الفعالية السمية الخلوية لثلاث نباتات طبية محلية في خطين خلويين هما: خط سرطان الكبد البشري HepG2 وخط خلايا الفأر L₂₀B. تم استخلاص مستخلص جذور عرق السوس واوراق التوت الأسود والحكيك باستخدام المذيب الأيثانولي 99%. وحضرت سبع تراكيز بوساطة التخفيف المتسلسل، وهي 3.9، 7.81، 15.62، 31.25، 62.5، 125 و 250 ملغم/مل على التوالي. أضيفت هذه المستخلصات الى لوحة المايكروليتر التي تحتوي 1×10^5 خلية لكل حفرة و200 ميكروليتر من الوسط الزرع واستخدمت التراكيز السبع في ثلاث مكررات للكشف عن فعاليتها السمية الخلوية وتأثيرها المضاد للتكاثر الخلوي. أيضا حلل وحدد التركيب الكيميائي للمستخلصات النباتية بوساطة كروماتوغرافي السائل السري (FLC). أظهر مستخلص الحكيك أعلى تأثير سمي خلوي للخطين الخلويين (HepG2 و L₂₀B)، بينما أظهر مستخلص كل من عرق السوس و التوت الاسود تأثير سمي خلوي أقل. أظهرت كل التراكيز الخام فعاليه سمييه مختلفه في المختبر.

الكلمات الدالة: المستخلصات النباتية، تأثير سمي، خط الخلايا الكبدية و خط خلايا الفأر

Introduction

Cancer chemoprevention is the use of natural, artificial or biologic compounds to reverse, suppress or avoid the development of invasive cancer. Phytochemicals are attractive increasingly significant sources of chemo preventive agents, chiefly as they can reveal their useful potential at all stages of tumor configuration [1,2]. Among plant secondary metabolites phenols are of persistent interest as many epidemiological studies have shown a reduced risk of incidence of several cancers in individuals with the diet rich in fruits and vegetables [3,4]. In the pharmaceutical industry, improving the early detection of drug-induced hepatotoxicity is essential as it is one of the most important reasons for attrition of candidate drugs during the later stages of drug development [5].

The disease-prevention properties of fruits and vegetables are attributed to the biological activities of the dietary fiber, vitamins, minerals and phytochemicals in the plants, however many studies suggest the

protective effects of fruits and vegetables against chronic diseases are due in large part to the phytochemical content of the plants [6,7].

In the traditional system of medicine, the roots and rhizomes of *G. glabra* have been in clinical use for centuries. Studies have shown that the extract of it has a growth inhibitory effect on breast cancer [8]. Methanol extract *G. glabra* roots have shown good antioxidant activity [9]. Glycyrrhizin acid found in *G. glabra* L. (licorice) is a strong modulator of nontoxic oxidative mutagens and a potent scavenger of free radicals [10]. Also, *Morus nigra* fruits are source of secondary metabolites like flavonoids and anthocyanin which considered as an excellent antioxidant [11]. Beside, Leaves extracts of *U. urens* contain appreciable levels of polyphenols that have antioxidant action and radical scavengers [12,13]. The *U. urens* extract had a therapeutic action in different studies; preserving liver cirrhosis and fibrosis protect them against oxidative stress [14] and have anti-carcinogenic effects [15,16]. The aim of this study was to evaluated the cytotoxic effects of *Glycyrrhiza glabra* L., *Morus nigra* L., and *Urtica urens* L. extracts against HepG2 and L₂₀B cell lines *in vitro*.

Materials and Methods

Plant materials

Plant materials were collected from various parts of Baghdad -Iraq. Authentication of plant materials was carried out at the herbarium of the Department of Biology, College of Sciences/ Baghdad University/Iraq. The plant materials were rinsed thoroughly with tap water to remove extraneous contaminants and cut into small pieces, oven-dried at 50°C until stability of dry weight was observed, and then grounded into powder with an electric-grinder to prepare it for extraction [17].

Preparation of crude extracts

Extraction was carried out by different steps

- 1- Macerating (100 g) of plant materials in 500 ml of 99% ethanol in (25-30°C) for 7 days in stoppered flasks.
- 2- Filtration the extracted solvent and separated through Whatman No. 1 filter paper.
- 3- Evaporation the extracts under reduced pressure using rotary evaporation.
- 4- Weighed the crude extracts powder and stored at 4°C until used [18].
- 5- Dissolved the crude extracts powder in Dimethylsulphoxide (DMSO) in order to prepare different concentrations for use in cytotoxic assay [19].

Cytotoxic assay experiment

Preparation of stock solution

- 1- To prepare 400 mg extract /1 ml medium stock solution mixing 400 µl of crude extract with 10 µl of DMSO, with the volume being completed up to 1 ml using a serum free medium [20, 21].
- 2- Serial dilution prepared , with concentrations of 3.9, 7.81, 15.62, 31.25, 62.5, 125 and 250 mg/ml, respectively and added in triplicate to the microtiter plate containing 1×10^5 cells/well and 200 µl of the medium.

Culture of cell lines

HepG2 hepatocarcinoma and L₂₀B (a mouse cell line that expresses the genes for human cellular receptor for *polio viruses*) Cells were cultured in DMEM medium supplemented with 10% foetal bovine serum, L-glutamine. Cells were grown as a monolayer at 37°C with 5% CO₂. The experiments were performed when cells were in the logarithmic phase of growth [22]. Cell line was incubated with different concentrations of each plant extract. The nine concentrations were used in triplicate to investigate their cytotoxic and anti-proliferative effects. A complete medium was used as negative control [22,23]. This experiment done at the Biotechnology Research Center / Al Nahrain University.

Neutral red assay

After incubating the cells with the extracts for 48 hours, the wells are washed with PBS and a freshly prepared neutral red solution (0.01 g of the dye is dissolved in 100 ml of PBS, and used immediately). (100µl/well) is added and incubated for 2 hrs. Finally, wells were washed again with PBS to remove excessive dye. An elution buffer (100 µl/well) is added and the absorbance was measured using an ELISA reader at a wavelength of 492 nm. The percentage of inhibition rate (IR) is calculated according to the formula: (%IR) = $\frac{[\text{absorption at 490 nm for control} - \text{absorption at 490 nm for volatile oil}]}{\text{absorption at 490 nm for control}} \times 100$

[24,25].

Analysis of chemical composition of the plant extracts by FLC

The analysis of the chemical composition was made by fast liquid chromatography (FLC). FLC consists from a mobile phase which is polar and consists of a mixture of solvents such as water and acetonitrile, while the stationary phase comprises of a column which is usually stainless steel and packed with silica particles, a sample of 50µl was injected into the mobile phase and it passes along the stationary phase, the time taken for a sample to pass through the system is recorded as its retention time RT that is one of the characteristic used to identify the compound, all the compound were separated and identified using FLC with separation conditions C-18, 3 µm particle size, 50× 4.6 mm internal diameter of the column, detection U.V. set at 280 nm, flow rate 1.4 ml/min. and 30 C°. Mobile phase was (0.1 % acetic acid and acetonitrile with linear gradient from 0-100% B in 10 min). Phenolic compounds and, deionized water: methanol 40:60 V/V or alkaloids and 0.1% acetic acid in deionized water: acetonitrile (20-80V/V). The area under a peak is used for calculating the concentration of a sample as the following formula was used:

$$\text{Conc. of sample } (\mu\text{g.ml}^{-1}) = \frac{\text{Area of the sample}}{\text{Area of the standard}} \times \text{Standard conc.} \times \text{Dilution factor}$$

Analysis of the chemical composition was made by injecting 20µl of the extract of each sample in FLC for identification. The conditions of separation were listed in Table (1). The peaks were detected by UV detector. The analysis was carried out in the laboratories of Ministry of Science and Technology [26].

Table (1): Conditions of Fast Liquid Chromatographic used for analysis of phenolic compounds of the plants extracts.

Parameter	Characteristic
Type of Column	C-18
Column dimensions	3µm particle size (50×4.6 mm ID)
Flow Rate	1.4 ml / min
Detector	UV spectrophotometer at 280 nm
Volume injection sample	20 µl
Mobile phase	Solvent A : 0.1% phosphoric acid in deionized water .Solvent B 20:80 V/V , 0.1% phosphoric acid in deionized water : acetonitrile HPLC grade , linear gradients 0% B-100% B.
Temperature	30°C

Statistical Analysis

The results obtained were statistically analysed using SAS software (version 17; SAS Inc., Chicago, IL, USA) [27].

Results and Discussion

The Cytotoxicity assay

The ethanol extracts showed clear inhibitory action against the proliferation of the hepatocellular carcinoma cell line after 48 hrs. The ethanol extract resulted in a dose dependent decrease in cell viability. The sensitivity of the ethanol extract was more in higher dosage especially with *G. glabra* and *U. urens* extract Table (2). Treatment with the *G. aglabra* extract (250 µg/mL) decreased the cell viability of hepatocellular carcinoma HepG2 with value of 92.1 %IR after 48 hrs. and further decreased to 88.00 %IR for *U. urens* extract after 48 h compared to the control cells, respectively Table (2).

Table (2): The cytotoxic effect expressed as the inhibition rate percentage (%IR) for different concentrations of the extracts after 48 hours exposure of the HepG2 cell line.

Concentration mg/ml	Inhibition rate percentage (%IR) HepG2 cell line			Mean
	<i>Glycyrrhiza glabra</i> extract	<i>Morus nigra</i> extract	<i>Urtica urens</i> extract	
Control	0.00	0.00	0.00	0.00
31.25	14.0	10.5	37.0	20.3
62.5	37.5	56.5	50.0	48
125	52.0	21.5	70.00	47.3
250	92.0	41.5	88.00	73.3
Mean	39.1	26	49	
LSD value	4.31*	3.51*	4.90*	

Also, all these extracts have same cytotoxic effects against L20B cells line. Treatment with the *U. urens* and *G. aglabra* extracts (250 µg/mL) decreased the cell viability of hepatocellular carcinoma and L₂₀B cells line value of 93.00 and 92.00 %IR after 48hrs. respectively, further decreased to 41.8% IR after 48hrs and for *M.*

nigra extract compared to the control cells Table (3). These results may be attributed to their contents of polyphenols, flavonoids, anthocyanin's, ellagitannins, and vitamin C. It is the phytochemicals that are responsible for many of the biological activities of their crude extracts, including antioxidant, reduce inflammatory and anticancer properties [11,12,13,15].

Table (3): The cytotoxic effect expressed as the inhibition rate percentage (%IR) for different concentrations of extracts after 48 hours exposure of the L₂₀B cell lines.

Concentrations Mg/ml	Inhibition rate percentage (%IR) L ₂₀ B cell line			Mean
	<i>Glycyrrhiza glabra</i> extract	<i>Morus nigra</i> extract	<i>Urtica urens</i> extract	
Control	0.00	0.00	0.00	0.00
31.25	44.5	30.5	67.0	47.3
62.5	57.5	39.5	70.5	55.6
125	72.0	41.5	81.00	64.8
250	92.0	41.8	93.00	75.3
Mean	53.2	30.6	62.2	
LSD value	5.01*	3.59*	5.96*	

Chemical identification of plant crude extracts using FLC

FLC chromatograms in Table (4) and Figures (1- 3) were used as a references for quality control in future experiments. Commonly found in these plants, the phenolic compounds were used as markers in our experiments. Pure compounds, namely gallic acid, chlorogenic acid, catechin, epicatechin, caffeic acid, vanillic acid, vanillin, coumaric acid, ferulic acid and quercetin, were used as marker compounds. Results of FLC of the *U. urens*, *G. glabra* and *M. nigra* extracts referred to present of phenols. The action activity of phenol extracts depends upon their active components. However, the biological effect is often due to a synergic action between the compounds [28]. Thus, the extracted phenols in this study were analyzed in a screening for the most active compounds (Chlorogenic acid, Caffeic acid and Gallic acid) that most topics and studies referred that antimicrobial activity due to them. According to the results of the profile FLC, this showed that the total concentrations of phenolic compounds were the highest in *U. urens* than of those in other tested extracts. The *M. nigra* extracts showed the lowest inhibitory action against hepatocarcinoma HepG2 and L₂₀B cell lines which could primarily due to its values of the phenolic compounds which were the lowest than in the other extracts in this study.

When phenolic compounds represent one of the most important allelochemicals as [29] referred that phenols are very active substances. Nakai *et al.* [30] reported that plant extracts from *Cyperus alternifolius* and *Cana generalis*, had active inhibitory effects against cyanobacteria due to release allelochemicals and they screened for these allelochemicals. they found 9 phenolic compounds [resorcinol, 3-hydroxy benzoic acid, 4-hydroxy benzoic acid, (4-hydroxyphenyl) acetic acid, vanillic acid, protocatechic acid, p-coumaric acid, gallic acid, and ferulic acid], in which anti-cyanobacterial activity were involved, vanillic acid, protocatechuic acid, p-coumaric acid and gallic acid. Rice [31] reported eight phenolic compounds produced by plants inhibited the growth of *Anabaene* sp. and *Lyngbya* sp. which were chlorogenic acid, coumaric acid, gallic acid, iso chlorogenic acid, scopoletin, α -naphthol, tannic acid and hydroxybenzaldehyde, thus, chlorogenic acid, coumaric acid, gallic acid were recorded in most extracts in this study and they may played the inhibitory role of these extracts against hepatocarcinoma HepG2 and L₂₀B cell lines.

Table (4): Types and concentrations of phenols in plant extracts

Plant extracts/ Phenol	Chlorogenic acid $\mu\text{g/ml}$	Caffeic acid $\mu\text{g/ml}$	Gallic acid $\mu\text{g/ml}$	Glabridin $\mu\text{g/ml}$	di-Caffeic acid $\mu\text{g/ml}$	OH-benzoic acid $\mu\text{g/ml}$	D-coumaric acid $\mu\text{g/ml}$	Total concentration $\mu\text{g/ml}$
<i>U. urens</i>	116.802	82.871	155.49	-----	-----	43.00	-----	398.163
<i>G. glabra</i>	-----	111.93	-----	218.83	-----	-----	66.85	397.61
<i>M. nigra</i>	125.22	23.93	-----	-----	91.03	-----	41.96	282.14

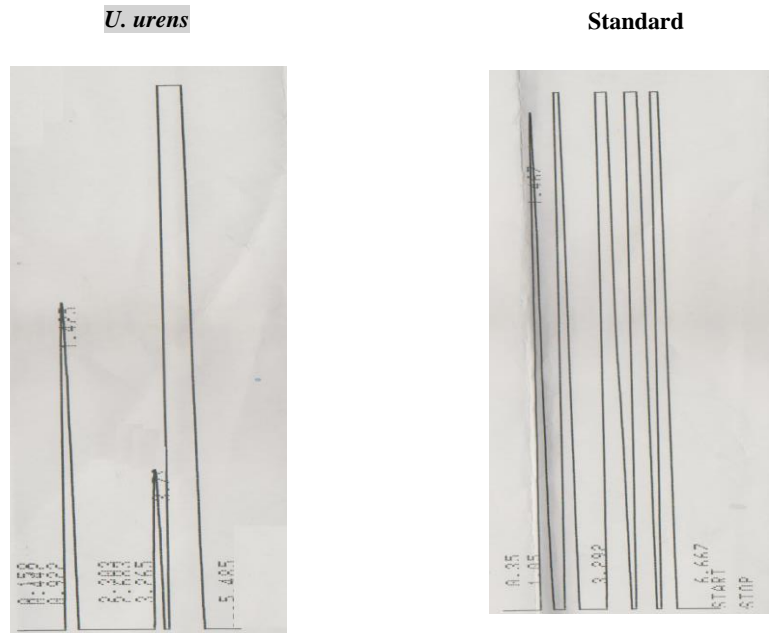
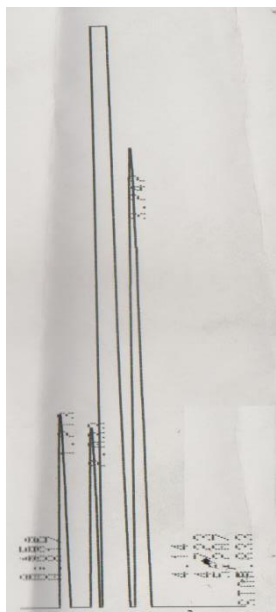


Fig. (1): FLC profile of phenols in *U. urens*.

G. glabra



Standard curve

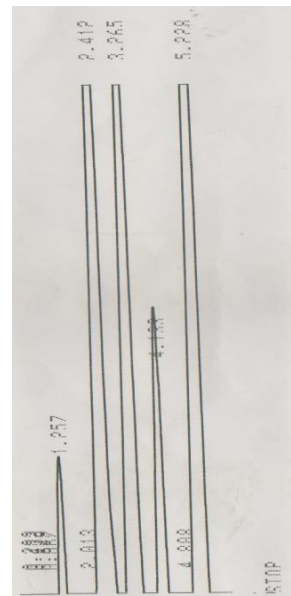


Fig. (2): FLC profile of phenols in *G. glabra*.

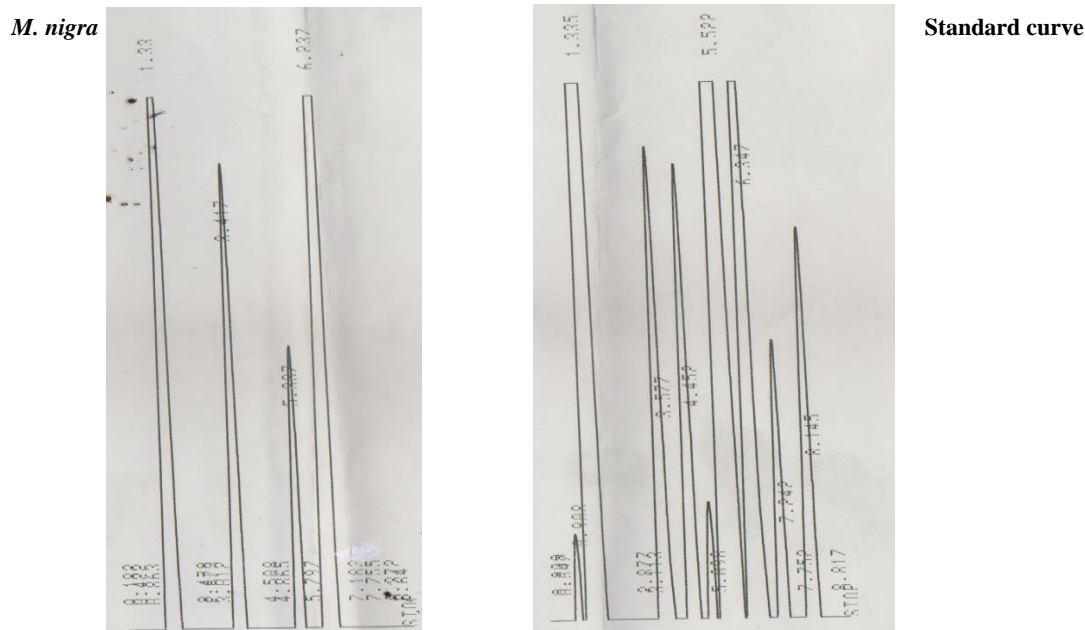


Fig. (3): FLC profile of phenols in *M. nigra*

Conclusions

The crude extract of the *Glycyrrhiza glabra* L., *Morus nigra* L. and *Urtica urens* L. have the ability to inhibit the growth activity and reduce the proliferation of cell lines used in this study.

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