ISSN : 0974 - 7508 Volume 8 Issue 5

Trade Science Inc.

Natural Products

An Indian Journal

Full Paper

NPAIJ, 8(5), 2012 [208-214]

Phytochemical screening, antibacterial, and *Invitro* cytotoxic evaluation of *Cichorium intybus* root extracts indigenous to Iraqi Kurdistan

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Received: 30th March, 2012 ; Accepted: 30th April, 2012

ABSTRACT

The phytochemical screening in the root of *Cichorium intybus* had led to detection of alkaloids, carbohydrates, cardioactive glycosides, steroids, and phenolic compounds. Antibacterial evaluation of petroleum ether, 80% ethanol, and ethyl acetate root extracts at three different concentrations against three gram positive and five gram negative bacteria using agar well diffusion method was carried out, all bacterial strains found to be susceptible for ethyl acetate extract with lowest MIC value (15.625 mg/ml) against Bacillus spp, and Staphylococcus aureus. TLC agar overlay bioautography method resulted in identification of three constituents from ethyl acetate extract with antibacterial activities chlorogenic acid, caffeic acid and kaempferol of which caffeic acid showed the lowest MIC value (0.15 mg/ml) against Escherichia coli, and Proteus spp. In vitro cytotoxic activity of 80% ethanol and ethylacetate extracts at concentrations 0.2, 0.4, 0.5, 0.6, 0.8, 1 mg/ml against human lung adenocarcinoma epithelial cell line (A549) at time intervals 24, 48, and 72hr showed significant decrease in cell viability with increase in concentration of extracts. IC50 for 80% ethanol extract was recorded as (0.7033±0.0472 mg/ml) on 72hr, and for ethylacetate extract as (0.96±0.02 mg/ml) and (0.566±0.01527 mg/ml) on 48 and 72hr respectively.

KEYWORDS

Cichorium intybus;
Cytotoxic activity;
Antibacterial activity;
Phytochemical screening.

INTRODUCTION

Chicory, "Arabic name (Hindiba) Kurdish name (chaqchaqa)", is the common name given to the widely cultivated flowering plant *Cichorium intybus* of the family Asteraceae. The plant is found in Europe, the Middle East as far as Iran, north and south Africa, all of America, Australia and New Zealand[1], it is a world-wide species and commonly found in Kayseri, Turkey[2], also found in Kurdistan, Northern Iraq[3]. The main components of C. intybus are sesquiterpene lactones, such as lactucin, 8-deoxylactucin, lactupicrin and 11b-dihydro-terpene derivatives[4], which are responsible for the bitter taste of this plant. Other components are coumarins, such as cichoriin, es-

culin, umbelliferone, scopoletin and 6,7-dihydroxycoumarin[5], flavone derivatives (apigenin, quercitin)[6], Caffeic acid derivatives chiroric acid, chlorogenic acid, isochlorogenic acid, dicaffeoyl tartaric acid[1], carbohydrates (glucose, fructose and inulin)[7] and vitamins[8]. C. intybus is very popular since antiquity as food and medicine in human life. Green leaves of C. intybus are being eaten as salad, traditionally it has been used for hepatic conditions and liver rejuvenation[9]. Besides this has been a primary component of variety of herbal formulations, especially in cough relief. The roots of C. intybus are roasted ground and mixed with coffee for he benefit of drinkers of that beverage[10]. The root extracts of C. intybus was showed antibacterial activity of against pathogenic bacteria like gram positive (Bacillus subtilis, Staphylococcus aureus and Micrococcus luteus) and gram negative (Escherichia coli and Salmonella typhi) bacteria by in vitro agar well diffusion method[11], the root extract was also found to have potential antifungal activity[12]. In a research in Southern Italy among sixteen edible plants from evaluated for their in vitro antiproliferative properties, using the sulforodamine B (SRB) assay, C. intybus was found with significant activity against amelanotic melanoma C32[13]. It was recorded that ethanolic extract of chicory roots causes significant inhibition of Ehrlich ascites carcinoma in mice at doses ranging from 300-700mg/kg[14]. The root of C. intybus is used traditionally in Kurdistan for the treatment of different diseases, and from literature survey on this plant there are very few reports are available on antibacterial properties of chicory root against the human pathogenic bacteria and its cytotoxic activities, therefore the aim of this study is to do phytochemical screening for the main natural product groups in the root of C. intybus and antibacterial and cytotoxic activities evaluation of different root extracts with bioassay guided identification of the most important active constituents.

MATERIALS AND METHODS

Plant materials

C. intybus roots were collected from the mountains of Choman district, Erbil, Kurdistan region in Iraq, during July 2010. The roots were washed thoroughly with tap water, and dried in air for seven days. They were authenticated by the department of Pharmacognosy College of pharmacy, Hawler Medical University, and Department of Biology, College of education, University of Salahaddin and voucher (Aveen1) was kept at department of Pharmacognosy College of pharmacy, Hawler Medical University.

Preliminary phytochemical screening

Ten g dried powdered plant materials were extracted with 80% ethanol (200 ml) using soxhlet extractor for 3hr, the extract obtained by filtration through Buckner funnel, and evaporated to dryness by rotary evaporator[15]. Preliminary phytochemical investigation for the important natural product groups were carried out following the standard procedures[16, 17, 18]

Preparation of root extracts for antibacterial and cytotoxic assay

One hundred g of the dried powdered roots were extracted with 1 L petroleum ether using Soxhlet extractor for 3hr[15]. The extract was filtrated, concentrated *in vacuo*, yielded 1g petroleum ether extract (PE). The residual plant materials were dried and re-extracted with 1L 80% ethanol for 3 hr yielded 39.76 g total extract (TE) on drying *in vacuo*. 20 g of TE extract were hydrolyzed by dissolving in 10 ml (5%) HCl and refluxing for 1hr, liquid-liquid fractionation using ethyl acetate (5x20 ml) resulted 1.436 g organic fraction (OF) on drying *in vacuo*.

Determination of antibacterial assay

Microorganisms

A total of eight bacteria were kindly provided by biology department, Science College, Salahaddin University. *Bacillus spp, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Proteus spp, Enterobacter spp, Pseudomonas aeruginosa.*

Agar well diffusion assay

The antibacterial activity of the root extracts were determined using agar well diffusion method

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[19]. TE, OF, and PE extracts were evaluated at three different concentrations. 125, 250, and 500 mg against *Bacillus spp, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Proteus spp, Enterobacter spp, Pseudomonas aeruginosa.* 10% DMSO and 20% tween80 were used as negative controls, and streptomycin antibiotic used as a positive control in concentration of (10 µg\ml) in distilled water. The antibacterial activity was evaluated by measuring the diameter of inhibition zone (DIZ) surrounding of the tested bacteria. DIZ was expressed in millimeters. All tests were performed in triplicate.

Bioautography agar overlay assay

The antibacterial activity of the crude and chromatographic fractions was ascertained by bioautography using agar overlay method[20], this technique was used to evaluate antibacterial activity of OF separated on thin layer chromatography plates (Merck, silica gel GF254) and reference substances kaempferol (Ka), chlorogenic (Ch), and caffeic (Ca) (Chromadex, USA) against *Bacillus spp, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus spp, Enterobacter spp, Pseudomonas aeruginosa.* 10 mg OF extract, 1 mg each of Ka, Ch, and Ca were dissolved separately in 1ml ethyl acetate and 1 ml ethanol respectively. 10 µl each of OF, Ka, Ch, and Ca were applied on two TLC plates (10x8 cm) one for bioautography and the other used as a reference plate. Both plates were developed to a distance of 10 cm in the same tank using [toluene: ethyl acetate: formic acid: water (15:90:5:5)] as a mobile phase. The plates were run in duplicate, the reference plate was observed under UV at 254 and 366 nm and subsequently sprayed with the 5% ferric chloride reagent. Streptomycin (0.5 mg/ml) in distilled water 10 µl was applied on an appropriate place on all the TLC plates as a positive control. The bacterial strains were grown on nutrient broth at 37 °C for 24hr. Agar suspensions were prepared by addition of 1 ml bacteria broth to test tube containing 9 ml muller hinton agar at 45 °C using hotplate and shaker. The developed and dried TLC plates were placed in sterile circle Petri dish (12x12 cm). The agar was spread over the TLC plates along the line of fixed plant extracts and the digalski spatula was drawn in one direction across the plate, resulting in a 1 mm agar layer, and a lid is placed over the containers, after solidification the petridishes were sealed off with parafilm and incubated at 37 °C for 18 to 24hr. Bioautograms were sprayed with aqueous solution of 2 mg/ml Dimethylthiazolyl diphenyl tetrazolium bromide (MTT), after 15 min. spots showing any inhibition were noted. The test was performed in triplicate. All these procedures were carried out at room temperature and Laminar air flow hood.

Minimum inhibitory concentration (MIC)

The microplate serial dilution method[21] modified by Masoko et al., [22]was used to determine the MIC. Concentrations of 125-0.975 mg/ml were tested for extracts and 1.25–0.009 mg/ml for pure compounds against *Staphylococcus aureus, Bacillus spp, Escherichia coli, Pseudomorrous aeruginosa, Proteus Spp., Klebsiella pneumoniae,* and *Enterobacter spp.* Streptomycin was used as a control.

Cytotoxic activity

MTT assay was carried out according to the method described by Mosmann[23]. In metabolically active cells, MTT is reduced by the mitochondrial enzyme succinate dehydrogenase to form insoluble purple formazan crystals that are subsequently solubilized, and the optical density (OD) measured spectrophotometrically. This technique was used to evaluate cytotoxic activity of TE and OF against human lung adenocarcinoma epithelial cell line A549 cells. Concentrations of TE and OF extracts (200-1000 µg/ml) were prepared. Cancer cells A549 were seeded into each well of a 96-well microplate each containing 200 µl of the growth medium RPMI containing 10% FBS (at the seeding density of 30000 cell/cm2). After 24hrs, the cells either treated with serial concentrations of extract (200-1000 µg/ml) over different incubation periods (24, 48 and 72hrs), or remained as untreated controls (also vehicle only is added to the wells at the used concentration present in the extract solution. The cells were left in the CO2 incubator at 37 ˚C and the media was replenished every other day. On the day of MTT experiment, the media was replaced with 200 µl

fresh media containing 50µl of MTT solution (2 mg/ml in PBS) and they were incubated for an additional 4hr at 37 ^{o}C. After incubation period media/MTT mixture was carefully removed and 200 ml of DMSO plus 25 ml of Sorenson's glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added to each well and the cell viability determined by measuring the absorbance of each well after 10 seconds shaking, employing a microplate reader (Biotek, ELx 800, USA) at 570 nm. The cytotoxicity was obtained by comparing the absorbance between the samples and control. The values were then used to calculate the concentration of plant extracts required to cause a 50% reduction (IC50) growth (cell number) of A549 cell on the different time points studied.

Cell Viability % = Mean OD/ Control OD X 100

Calculations and statistics

All experiments were performed in three replicates. The results were expressed as mean of triplicate ± SD., for agar well diffusion, MIC and cytotoxicity. The differences in mean values were analyzed by student's t-test, $p < 0.05$ was considered statistically significant. IC50 values were calculated for cytotoxicity from the dose response curves obtained by plotting the percentage of inhibition versus the concentrations. All statistical analyses were carried out with Microsoft excel.

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening of plants is an important aspect for the scientific verification of folklore claim with regard to the utility of plants. *C. intybus* showed the presence of a number of important phytochemical natural product groups that supported the data recorded previously for alkaloids, carbohydrates, coumarins, steroids, terpenes, flavanoids, and tannins[11], while anthraquinone glycosides and saponins were found absent. cardioactive glycosides were recorded for first time in *C. intybus* root.

Antibacterial activity

The results of antibacterial screening, TABLE 1, showed that all the three plant extracts possessed antibacterial activity against tested bacteria. The extracts showed more activity against Gram positive bacteria than Gram negative bacteria. Among different extracts studied, OF showed higher degree of inhibition, followed by TE then PE. This activity was demonstrated by previous work done which showed that the ethyl acetate was the most active when antibacterial activity of ethyl acetate, water, and ethanol extracts of *C. intybus* were investigated[24].

TE at three different concentrations showed pronounced growth inhibition, at the lowest concentration (125 mg/ml) exhibited greater activity against *Bacillus spp* and *Staphylococcus aureus*, moderate inhibition on *Escherichia coli* and *Enterobacter Spp*, with no activity on *Pseudomonas aeruginosa*, *Proteus spp*, and *Klebsiella pneumoniae* at the highest concentration (500 mg/ml). OF exhibited greater activity at the lowest concentration on *Proteus spp*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and

TABLE 1 : Antibacterial activities of TE, OF, PE extracts at three concentrations (500,250 and 125 mg/ml)

Bacteria	Inhibition zone diameter (mm)									Streptomycin 10µg
	TE			OF			PE			
	500mg	250mg	125mg	500mg	250mg	125mg	500 mg	250mg	125mg	
Bacillusp	15± 0.15	14± 0.25	13±0.1	20.2±0.2	13.7±0.25	10.8±0.15	11.1±0.17	8.1± 0.21	7.1±0.17	30
Staphylococcsaureus	14.7±0.28	13.1±0.24	10±0.14	17±0.24	15.8±0.24	14.9±0.03	13.1±0.21	10.2±0.35	8.1±0.21	30
Staphylococcs epidermidis	9.1± 0.24	---	---	11.8±0.17	---	---	---	---	---	28
Escherichia coli	12± 0.07	10± 0.17	9± 0.35	19±0.35	15±0.17	11± 0.35	10.2±0.35	8.1± 0.17	7.2±0.35	28
Enterobacter Spp	10.2±0.35	9.5± 0.14	9± 0.07	17±0.14	13±0.07	8.5± 0.07	---	---	---	28
Pseudomonas aeruginosa	---	---	---	23.5±0.51	17.1±0.7	12.8±0.7	---	---	---	28
Proteus Spp	---	---	---	20±0.07	18.1±0.21	15.7±0.35	---	---	---	30
Klebsiela Pneumoniae	---	---	---	16.7±0.35	15.1±0.17	9.8± 0.21	---	---	---	28

Each values represents the mean of triplicate ± SD
(---) No inhibition

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TABLE 2 : MIC values of TE, OF, PE, Ka, Ch, and Ca against different types of bacteria

Samples→ Bacteria↓	MIC (mg/ml)						
	TE	OF	PE	Ka	Ch	Ca	Streptomycin
Bacillus Spp	15.625	31.25	125	0.31	0.31	0.31	<0.019
Staphylococcus aureus	15.625	31.25	15.625	---	1.25	0.62	0.039
Escherichia coli	62.5	62.5	62.5	0.31	0.31	0.15	<0.019
Enterobacter spp	62.5	62.5	ND	---	---	0.62	0.039
Pseudomonas aeruginosa	ND	31.25	ND	---	---	0.31	0.039
Proteus Spp	ND	31.25	ND	---	---	0.15	<0.019
Klebseilla Pneumoniae	ND	62.5	ND	---	---	1.25	0.039

(ND): Not done (--) : No antibacterial activity at the highest concentration tested

Bacillus spp. PE extract showed antibacterial activity only on three types of bacteria at the lowest concentration *Staphylococcus aureus*, *Bacillus Spp*, and *Escherichia coli*. Nandogopal and Kumari study showed that petroleum ether extract of chicory had antibacterial activity may be due to the presence of volatile oils and fatty acids content[11].

The MIC values, TABLE 2, showed that *Bacillus spp* and *Staphylococcus aureus* were the most sensitive pathogens against TE (MIC 15.625 mg/ml), OF (MIC 31.25 mg/ml), while *Escherichia coli* and *Enterobacter spp* ranked next in sensitivity against TE and OF (MIC, 62.5 mg/ml). PE achieved stronger MIC value against *Staphylococcus aureus* (15.625 mg/ml). MIC value of methanolic extract of *C. intybus* leaves was recorded (0.010 mg/ml) against *Staphylococcus aureus* and *Bacillus subtilis*, (0.075 mg/ml) against *Bacillus cereus*, while ethanolic extract shown no activity against those strain of bacteria[25].

TLC agar overlay bioautography

Bioautography is a very convenient and simple way of testing plant extracts, partially purified fractions and pure substances for their effects on both human pathogenic and plant pathogenic microorganisms. It can be employed in the target-directed isolation of active constituents[26].

The bioautography assay was applied to seven types of bacteria which was useful in the separation and identification of three bioactive compounds from OF separated on TLC plate. Microbial growth inhibition appeared as yellow zones around constituents with antibacterial activity against a violet background. The agar overlay bioautography method results were confirmed the results obtained by agar well diffusion method. TLC results showed the presence of a number of separated phenolic constituents in OF extract, of which chlorogenic acid (R_f,0.2), caffeic acid (R_f,0.54) and kaempferol (R_f,0.66) were identified by comparing R_f values and color properties with reference substances, Figure 1. Ca showed clear inhibition zones against all types of bacteria tested, Ch showed activity against *Bacillus spp*, *staphylococcus aureus*, and *Escherichia coli*, while Ka was active against *Bacillus spp* and *Escherichia coli*. Ca showed the strongest antibacterial activity against *Escherichia coli*, and *Proteus spp* (MIC, 0.15 mg/ml). *Bacillus spp* and *Escherichia coli* showed best susceptibility towards Ka and Ch (MIC, 0.31 mg/ml). Caffeic acid previously isolated from *C. intybus* showed antibacterial activity against *Staphylococcus aureus*, and

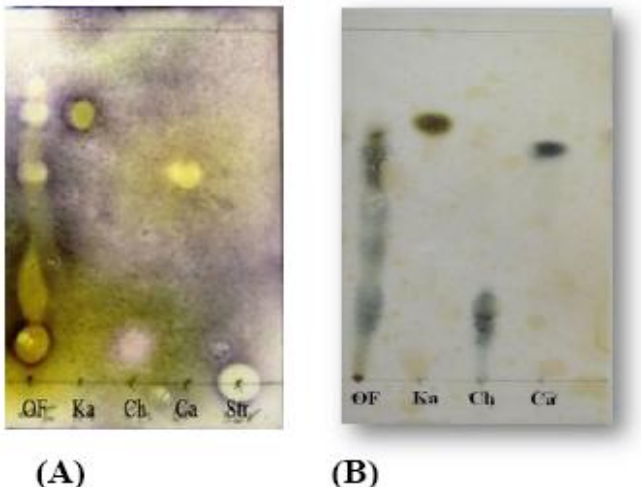

Figure 1 : (A) Bioautographic assay with Bacillus spp and (B) TLC plate sprayed with 5% ferric chloride reagent viewed in VIS.

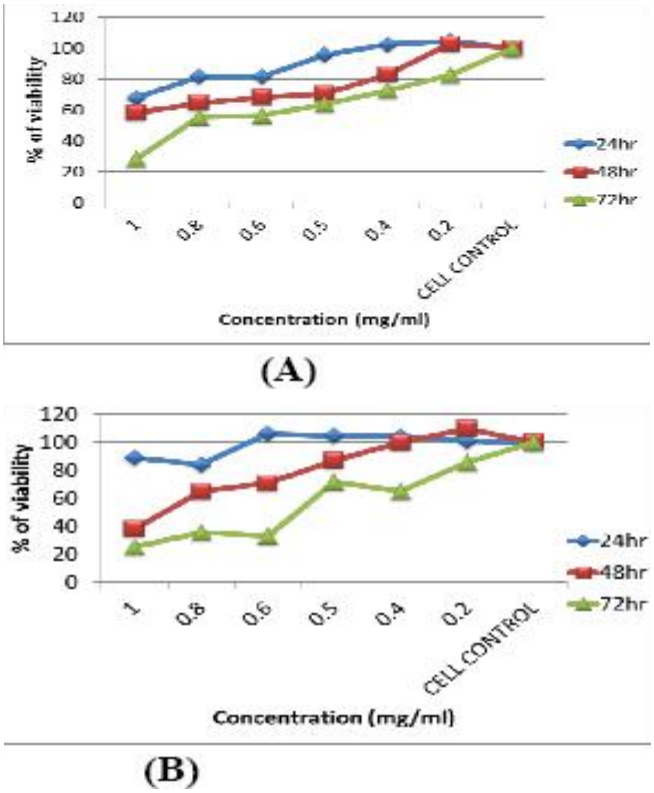

Figure 2 : Viability of A549 cells exposed to A-TE extract; B-OF extract

Escherichia coli while there was no activity on *Pseudomonas aeruginosa*[27,28].

Cytotoxic activity

For both types of TE and OF extracts, decrease in cell count was observed with increase in concentration of extracts, Figure 2. The results of cytotoxic evaluation of different concentrations of TE extract showed significant difference ($p<0.05$) between concentration and cell control at 1 mg/ml with in 24hr, at 1, 0.8, 0.6, 0.5 mg/ml with in 48hr and 1, 0.8, 0.6, 0.5, 0.4 mg/ml with in 72hr. Also there was a significant difference between different time intervals, between 24 and 48hr at concentration 0.8, 0.5, 0.4 mg/ml, between time interval 24 and 72hr at all tested concentration, while between 48 and 72hr only at 1 and 0.2mg/ml. While in case of OF extract the significant difference between different concentration and cell control with in same time interval were observed at 1, 0.8, 0.6 mg/ml with in 48 and 72hr, and there was a significant difference between time interval of 24 and 48hr at concentrations of 1, 0.6 mg/ml. All tested concentrations were shown significant difference between time intervals 24 and 72hr, 48 and 72hr. From the percentage of cell viability against different concentration of TE and OF extracts, IC50 values for both types of extracts at different interval periods were calculated, TABLE 3.

TABLE 3 : IC_{50} values of. *Cichorium intybus* root extracts

Extracts	IC_{50} mg/ml		
	24hr	48hr	72hr
TE	---	---	0.703±0.047
OF	---	0.96±0.02	0.566±0.015

All values are mean ± SD

There were previous investigations which have demonstrated that *C. intybus* can be cytotoxic *in vitro* to several human tumor cells. In an investigation on 16 Algerian plants for their cytotoxic activity, methanolic extract of *C. intybus* leaves was showed remarkable cytotoxic activity against human amniotic epithelial cell line (FL-cell) with IC50 value 15 µg/ml while for ethanolic extract IC50 value >1000 µg/ml[25]. While in other study significant cytotoxic activity was exerted by *C. intybus* root extract on amelanotic melanoma cells C32 cell line (30.8% of inhibition at 100 µg/ml), human breast cancer cells MCF-7 (12.65 % of inhibition at 100 µg/ml), renal cell adenocarcinoma ACHN (14.93% of inhibition at 100 µg/ml), and no actitivity against human prostate cancer cells LNCaP was observed[13]. Survey of the literature revealed that there was no studies on the cytotoxic activity of *C. intybus* against human lung adenocarcinoma epithelial cell line (A549 cells), the exposure of cancer cells to TE and OF induced a significant reduction in the conversion of MTT, which means a cellular disintegration and cytotoxicity reflected from the parallel dose and time dependent decrease of the absorbance measured. As determined by MTT assay the minimum cell viability were 67.76%, 58.12%, 28.26% with TE and 89.16%, 38.32%, 25.40%, with OF after 24, 48, 72hr respectively, which was observed at the highest dosage 1mg/ml and longest exposure duration. It was concluded that the percentage of cytotoxicity increased with increasing concentration as well as exposure duration and OF extract

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was more active than TE extract against the selected cancer cell.

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