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Research Article

PHYTOCHEMICAL AND CYTOTOXIC STUDIES OF POLYPHENOLIC FLAVONOIDS CONTENTS OF *URTICA DIOICA*

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ABSTRACT
The polyphenolic flavonoids found in several medical plants and herbal remedies containing flavonoids, have been used in folk medicine around the world. The weight of laboratory studies, epidemiological investigation and human clinical trials indicate that polyphenolic chemistry have important effects on cancer chemoprevention and chemo –therapy. *Urtica dioica* (UD) "stinging nettle" has been consumed for centuries as a phyto-medical agent and as a food substance. Although its history associated with alternative remedies was remarkable but the number of its cytotoxic studies are rather scarce. Therefore, more focused phyto and medicinal chemistry studies are required to establish whether such dietary effects of *Urtica dioica* ’s extract can be exploited to achieve even preliminary cytotoxic effect on Hep -2 cell line. The major compounds detected and isolated from the ethanolic extract of the aerial parts of *Urtica dioica* were determined as flavonoids by chromatographic, chemical and spectral (UV, IR) methods. In this paper, the down ward viability -concentration curve of the ethanolic extract of the *Urtica dioica* ‘s methanolic extract, using Hep-2 cell line indicate its positive cytotoxic activity. The promising results will stimulate the full phytochemical and cytotoxic studies of flavonoids for cancer chemoprevention and chemotherapy. We believe that this one cell line study may be contradictory in part, and gives a conclusion that there's still a long way to go until we do a full phytochemical investigation for *Urtica dioica* 's different polyphenolic compounds; several works addressing this matter are referred to predict a full cytotoxic profile of *Urtica dioica*.
Keywords: polyphenolic flavonoids, *Urtica dioica*, cytotoxic activity.

INTRODUCTION

Poly hydroxyl chemistry of flavonoids is characterized by a phenylbenzopyran chemical structure. The general structure includes a C_{15} (C_6-C_3-C_6) skeleton joined to a chroman ring (benzopyran moiety). The helerocyclic benzopyran ring is known as the C ring, the fused aromatic ring as the A ring, and the phenyl constitute as the B ring. The A ring can be of two types a phloroglucinol type that is meta-dihydrorylated[1,2]. The B ring can be monohydroxylated, or the dihydroxylated or vicinal-hihydroxylated. The center heterogcle most commonly exists in one of three forms: pyran pyrilium or γ-pyrone[3]. And they are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavonols, flavanones, and flavanonols (Figure 1)[4,5]

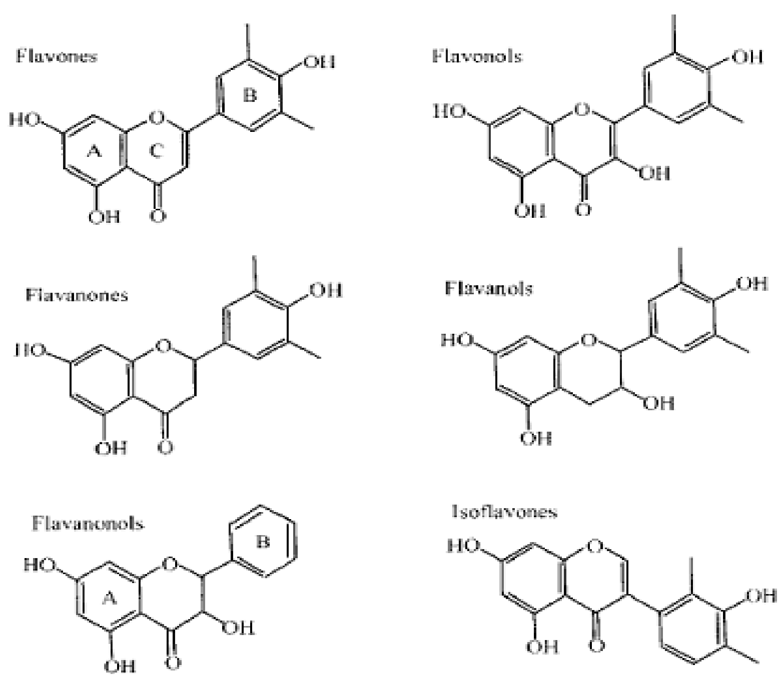


Figure 1: Chemical Structures of flavonoid family

Without a doubt, the area of knowledge of the vast world of polyphenols from their rich chemistry to their extensive list of pharmacology has experienced an increasing popularity in the past years, as represented in (Figure 2)[6]. Increasingly, flavonoids are becoming the subject of medical research. They have been reported to possess many useful properties, including anti inflammatory, enzyme inhibition, vascular and cytotoxic antitumor activity[7], but the antioxidant activity is, without doubt, the most studied one attributed to flavonoids. This well established antioxidant activity of flavonoids is also responsible for other biological activities in which the prevention of oxidative stress is beneficial. For example, the anticancer activity of some compounds is due to their ability scavenge free radicals, thus avoiding the early stages of cancer promotion. These polyphenolic compounds display a remarkable spectrum of biological activities including those that might be able to influence processes that are dysregulated during cancer development. These include, for example, anti allergic, anti-inflammatory, antioxidant, anti mutagenic, anti carcinogenic and modulation of enzymatic activities[8-11]. They may therefore have beneficial health effects and can be considered possible chemo preventive or therapeutic agents against cancer [12,13]. About 60 % of currently used anticancer agents are derived in one way or another from natural sources. Indeed, the natural products have played, and continue to play in a dominant role in the discovery of leads for the development of conventional drugs for the treatment of the most human disease. The search for anticancer agent from plant sources started in earnest in 1950s with the discovery and development of vinca alkaloids, and the isolation of the cytotoxic podophyllotoxins[11]. *Urtica dioica* or Stinging nettle has been consumed for centuries as a phyto-medical agent and as a food substance. Its active constituents, known to contain flavonoids: flavonol glycosides including isorhamnetin, kaempferol and quercetin; Quercetin has demonstrated significant anti-inflammatory activity because of direct inhibition of several initial processes of inflammation[14]. Kaempferol inhibited proliferation of malignant human cancer cell lines, including A431, SK-MEL-5 and SK-MEL-28, and HCT-116. These results indicate that targeting RSK2 with natural compounds, such as kaempferol, might be a good strategy for chemo preventive or chemotherapeutic application[15]. The flavonoids kaempferol and quercetin seems to act synergistically in reducing cell proliferation of cancer cells, meaning that the combined treatments with quercetin and kaempferol are more effective than the additive effects of each flavonoid[16,17]. All flavonoid glycosides showed high intracellular killing activity[18].

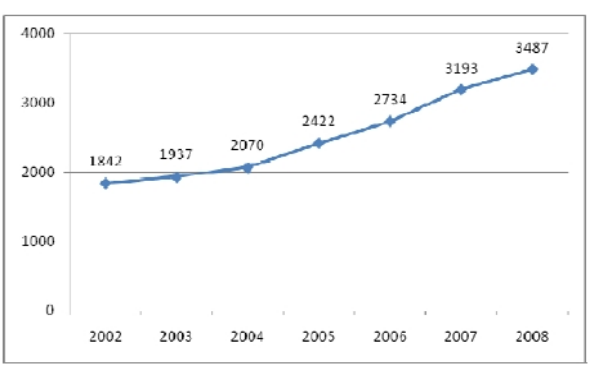


Figure 2: Evolution in the number of papers with the keyword "phenolics"

Many epidemiological studies have suggested that there is a link between the consumption of some foods and drinks with a high phenolic content and the prevention of some diseases[19, 20], whereas the revision carried out by Block and coworkers[21] showed that, of 156 epidemiological studies, 128 stated that consumption of fruit and vegetables was inversely related to the risk of acquiring cancer. Among the properties of phenolic compounds, they have been found to protect plants against oxidative damage and may have the role in humans[22,23]. These discoveries prompted us to initiate a phytochemical investigation to well-known folk medicinal plant *Urtica dioica*, *F. Urticaceae*. In addition we have found no literature on the effect of *Urtica dioica*'s extract on HEP-2 cell line, although some studies do exist on the activity of *Urtica dioica* on other cell lines as indicated previously.

MATERIALS AND METHODS

Sample preparation

Urtica dioica, aerial part was collected from Mosul area (Mosul-Iraq) and authenticated in the biology department-Education college in Mosul University.

Phytochemical study

Extraction and separation

A 200 g of the dried aerial parts of *Urtica dioica* was crushed to form a coarse greenish-red powder and then macerated with ethanol (3 x 300 ml) by successive overnight soaking with stirring by magnetic stirrer. The combined extracts after filtration, were evaporated under reduced pressure. The exudates was dissolved in minimum quantity of acidified (0.1 % Hcl) water (50 ml) and then shaken with ethyl acetate (3 x 100 ml) using separator funnel. The ethyl acetate fractions were collected together and evaporated under reduced pressure to give a reddish brown gum (4.5 g). Ethyl acetate residue (4.5 g) was subjected to silica gel column chromatography and successively eluted with chloroform-methanol (95:5 to 80:20) to give 5 fractions. Fraction IV was subsequently chromate graphed on a small silica gel column and eluted with heptane-ethyl acetate (90:10 to 75:25) to give yellow oil (compound I, 105 mg). Fraction V (350 mg) was rechromato graphed on a silica gel column and eluted with heptane-acetone (95:5 to 50:50) to give (compound II, 115 mg). These separated fractions were purified, detected and purified as flavonoids by thin layer chromatography using silica gel plates, and developed by using solvent system: Heptane: ethyl acetate (8:2) and heptane: acetone (5:5), but not identified exactly.

The detection of Flavonoid was detected by

Spectral detection

- By using the ultraviolet instrument, in this experiment the ultraviolet light gave a fluorescent spot, this is a characteristic of flavonoids by giving yellow spots.
- By measuring the λ max using ultraviolet instrument gave wave lengths peak at 250 nm, 290 nm and 350 nm, this is a characteristic for flavonoids, and using IR instrument gave peaks characteristics at 3417 cm^{-1} phenolic OH,1646 cm^{-1} for carbonyl, 3099 cm^{-1} for aromatic hydrogen and 2925 cm^{-1} for aliphatic hydrogen.

Chemical detection

- For confirming our results, we use spray reagent: 10 % sulfomolybdic acid in alcohol, this reagent sprayed on the plate, and then the plate heated with hot air, gave bluish-purple spots, characteristic of phenolic compounds.

Another detection by spraying with vanillin reagent (saturated vanillin in ethanol) and dried with hot air, gave a blue color, this was also an indicator of phenolic compounds.

- The alcoholic extract (5 ml, corresponding to 1 g of plant material) was treated with a few drops of concentrated Hcl and magnesium salt (0.5 g). The presence of flavonoids was indicative if pink or magenta-red color developed within 3 minutes[24].
- Flavonoids commonly used reagent for the alcohol solution of aluminum chloride, its color theory for the flavonoid molecules often contain 5 OH 4-keto, 3 OH 4-keto, adjacent hydroxide groups of these structural elements, and Al^{3+} to form a yellow fluorescent complex. In addition, there ammonia fumigation, alcohol solution of sulfuric acid, iodine vapors smoked and other methods[25].
- Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids[26].
- Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicates the presence of flavonoids[27].

TLC for the qualitative study of Flavonoids

Thin layer chromatography of flavonoids in qualitative research currently used with the standard R_f values and literature control. The R_f value of spot shape whether the fluorescence color and features are exactly the same, can make a preliminary conclusion may be the same compound[28]. The two isolated flavonoids suppose to be kaempferol and quercetin, in corresponding position of fluorescent spots, was the same color as reviewed in literature, when the UV Lamp (365 nm) under review, on other hand the silica gel GF254, developing solvent toluene, chloroform, acetone (40:25:35) for TLC analysis, one of the R_f value of quercetin, $R_f = 0.50$, and the value of kaempferol, $R_f = 0.72$, was the same color as reviewed in literature[29].

Cytotoxic Screening

Materials

Bovine serum (BS), phosphate buffered saline (PBS), pH 7.2, Hep-2 cell line and all other solutions and media for cytotoxic study were leanly provided by the Iraqi center for cancer and Medical Genetics Research (ICCMGR).

Sample for cytotoxic study

A 0.1 g of the methanolic extract of *Urtica dioica* was dissolved in 10 ml of serum free medium (SFM). The solution sterilized by filtration through sterile 0.2 um Millipore filtration unit, stored at -20˚c.

Cell line synthesis for cytotoxic study

Confluent monolayer was treated, the growth medium was decanted off and the cell sheet washed twice with PBS. 2-3 ml of trypsin-versene were added to the cell sheet after approximately 30 seconds most of the trypsin was poured off and the cell incubated at 30˚c until they had detached from the flask. After wards, 200 µL of cells in growth medium were added to each well of a sterile 96-well micro titration plate. The plate were seated with a self-adhesive filter, lid placed on and incubate at 37˚c in 5 % CO_2 humidity's atmosphere incubator, when the cells are exponential growth, i.e. after log phase, the medium was removed and serial dilution of the compound (50, 250, 125 and 62.5) µg/ml under assay in SFM were added to the well. There duplicate were used for each concentration of the methanolic extract. The cell line was exposed to cisplatin (EBEWE-Austeria Europe) as a reference (positive control) and the 3 columns used as negative control are cells treated with SFM only. Afterwards, the plates were re incubated at 37˚c in humidified, 5 % CO_2 atmosphere. For the selected exposure time (48 h) then the medium was decanted off the cells in the wells were washed by gently adding and removing 0.1 ml PMS two times, after that the washed-well exposed to diluted formalin 0.1 ml/ well for 2 hours then crystal violet 50 ml/well was added after 30 minutes. They were washed twice with PBS and left to dry[30]. The viable cell count was calculated using the following formula[31]:

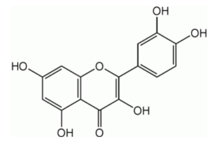
$$c = n \times d \times 10^4$$

Where c = cell concentration (cell/ml), n = number of cell counted and d= dilution factor (=10)

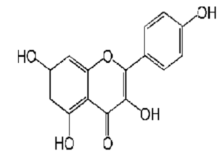
The results were expressed as percentage of viability which was calculated as the percentage of the mean of absorbance compared to the negative control[32]. IC_{50}, which is the lowest concentration that kills 50 % of cells[33], was calculated according to Wilson[34]. The data were analyzed using statistical software SPSS 10.0 for windows. Significance between control and samples was determined using students t-test. P value ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

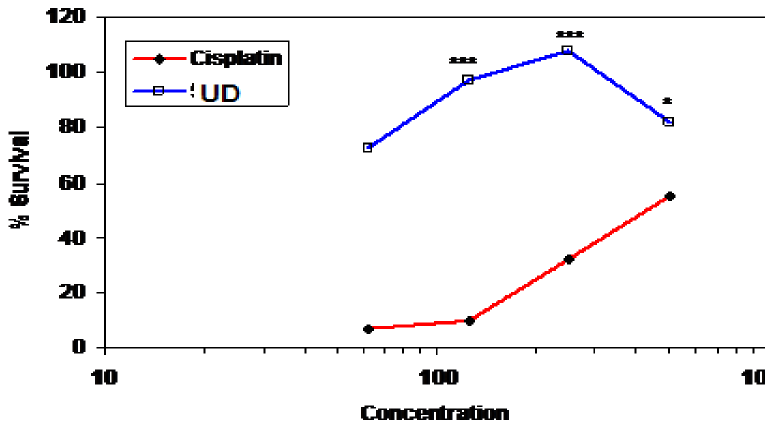
The % viability of HEP-2 cell line exposed to *Urtica dioica* extract is dramatically downward after at 250 µg/ml indicate a high sensitivity of HEP-2 cell to methanolic extract of flavonoids comparing with resistant with Cisplatin (positive control), this agree with the weight of the epidemiological evidence for a protective effect of flavonoids against cancer in impressive. A growing number of epidemiological studies suggest that high flavonoids intake may be correlated with a decreased rise of cancer. Flavonoids, chemically are electron donors. They serve as derivatives of conjugated ring structure and hydroxyl groups that have the potential to function as antioxidants *in vitro* cell culture or cell free systems by scavenging superoxide anion, singlet oxygen, lipid peroxy-radicals, and/or stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species. It was suggested that the synergy of anti proliferative and antioxidant activities of *Urtica dioica* 's polyphenolic-rich was contributing to its chemo preventive potential. Several flavonoids have been demonstrated to be present in areal part extract *urtica dioica*. Although very little literature data were available about the cytotoxic effect of whole polyphenolic *Urtica dioica* 's extract, it's possible to relate why *Urtica dioica* extract in the present study were active cytotoxic agents, it's important to acknowledge that specific *Urtica dioica* polyphenolic contents with high purity levels enhanced their effectiveness as cytotoxic agent against human numerous cell. Overall results showed that the percentage of inhibition by the crude extract against Hep-2 cell line, did not exceed 50 % at any tested concentration, therefore no IC_{50} was registered, thus reflecting that *Urtica dioica* extract was not active against Hep-2, however the 72 h. test period was recommended over the 48 h to avoid false negative indications of cytotoxic activity, this was because some bioactive compounds, particularly these that inhibit cell proliferation may need longer time to exert their cytotoxicity[35]. Thus a shorter incubation period will result in failure to discriminate the potential cytotoxic activity of the chemicals. Two flavonoids were separated by column chromatography and identified by TLC R_f value, IR, UV and supposed to be Quercitine and Kaempferol. Therefore, possible to conclude that the cytotoxic effect of the aerial part of *urtica dioica* may be due to its content of flavonoids and/or phenolic compounds.



Quercetin



Kaempferol



*Significant difference from cisplatin at p<0.05 and *** at p<0.001

Figure 3: Graphical representation of concentration – dependent effect of *Urtica dioica* ' extract on Hep-2 cell treated for 48 h

Table 1: The cell survival as a percentage of the control for the HEP-2cell line, when the cell was treated with *Urtica dioica*'s extract

Concentration (µg/10 ml)	% of Survival (Mean ± SD)	
	Cisplatin	UD
62.5	6.77 ± 1.27	72.34 ± 49.89
125	10.15 ± 1.88	97.28 ± 8.87
250	32.42 ± 4.29	101.79 ± 11.85
500	54.88 ± 4.60	70.93 ± 8.70

It was observed that the methanolic extracts inhibited the highest content of phenolic compounds this was consistent with findings that reported that phenolic compounds were more easily dissolve in methanol, because the solvent has more polarity[36,37]. Generally, the more hydroxyl groups, the phenolic compound has, the greater antioxidant potential. This is because the substitution of the hydroxyl group alongside the presence of electron donating groups tends to increase the antioxidant potential of phenolic compounds.

CONCLUSION

We believe that this one cell line study may be contradictory in part, and gives a conclusion that there' s still a long way to go until we do a full phytochemical investigation for *Urtica dioica* 's different polyphenolic compounds; several works addressing this matter are referred to predict a full cytotoxic profile of *Urtica dioica*.

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