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Preliminary Study for the Anticancer Activity of Flavonoids Extracted from Wild *Lycium barbarum* Leaves

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Abstract: *The discovery and identification of a new drugs, which can act as anticancer agents had an important goal of researches. This study demonstrates the favorable effects of Iraqi wild type Lycium barbarum active components as anticancer agent. Flavonoids from Lycium barbarum leaves were extracted and identified by the preparative thin layer chromatography (PTLC) technique which was the best to separate several flavonoids among them; Rutin, Quercetin, Kaempferol, luteolin and a major quantity of unknown flavonoid that had been separated and purified to evaluate its biological activity. Results showed that Iraqi L. barbarum leaves contain total flavonoids (11.28 mg/g dried leaves) calculated as Quercetin, and the purified extracted flavonoid showed cytotoxic effect towards both: the primary cell culture of normal hepatic cells (WRL-68), and cancer hepatic cell lines (HepG-2) at 100µg/ml concentration for 24 hours treatment. The High Content Screening (HCS) assay was held only for the purified flavonoid to investigate the mechanism by which the purified flavonoid affected living cells toward apoptosis. The most significant reduction (p≤0.05) in cell viable count was at the concentration 100µg/ml which appear to cause the induction of cell death via mitochondrial pathway for HepG-2 cell line after 24 hours exposure.*

Keywords: *(Lycium barbarum flavonoids, cytoyoxic assay, High content screening Assay)*

1. Introduction

Plant natural products play an important role in chemotherapy, and over 60 available chemotherapeutic agents are plant derived agents(Al-Hilli, 2009). The flora of Iraq, the ancient Mesopotamian land of civilization had interesting about 1500 medicinal plat species which have been recorded in Iraq, and large number of these plants are used for medicinal purpose(Townsend and Guest,1985). Studies are in progress to understand how these compounds may or may not provide protection against toxic, mutagenic and carcinogenic effects of chemical compounds. *Lycium barbarum*, a well-known Chinese traditional medicine and foodstuff, contained different active components which have many proposed pharmacological and biological effects, including anti-aging activity (Chang and So, 2008), immune modulation (Gan *et al.*,2004) and anti-cancer activity (Zhu and Zhang, 2013). The majority of anticancer drugs presently used in clinical settings have been described to induce cell death by apoptosis (Cheah *et al.*,2011).Due to the critical role of apoptosis in tissue homeostasis and cancer development, the modulation of apoptosis has become an interesting target in both therapeutic and preventive approaches in cancer(Zhu and Zhang,2013). All studies and researches on *Lycium barbarum* biological active components were done on the Chinese grown plant, while there is little (if not) researches about the Iraqi wild type plant.

A major constituent of *Lycium barbarum* is flavonoids which comprise a large class of low-molecular-weight plant metabolites ubiquitously distributed in food plants. These dietary antioxidants exert significant antitumor, antiallergic, and anti-inflammatory effects. The molecular mechanisms of their biological effects remain to be clearly understood (Nair *et al.*,2006).On many occasions, traditional herbal medicine systems remain a complicated task for modern researchers as it has thousands of different active ingredients in different proportions. Though these formulations have proven pharmacological activity, they fail to produce for isolated key ingredients. Hence, modern researchers should take the basic concepts of traditional medicines for getting success in their research (Srinivasan and Rajendren,2012).

The present work employed firstly extraction, and identification of the major components from the *Lycium barbarum* leaves, and isolate some of these ingredients aiming to study their cytotoxic effects against cancer and normal cells. The aims could be set in the following projects:

1. Identify the major active components from leaves and of the Iraqi wild *L. barbarum* plant, qualitatively and quantitatively.
2. Isolate the main flavonoids in the leaves of this plants and find a proper technique for their purification, since the main flavonoids of the leaves were still unknown.
3. Investigate the cytotoxic activity of the extracted components towards human cell lines (by MTT assay) .
4. The study also employed the pathway by which the purified flavonoid exerts its cytotoxic effect with HCS assay.

2. METHODOLOGY

2.1. Extraction of Flavonoids from *L .barbarum* Leaves (Harborne,1984)

Areal parts from *Lycium barbarum* grown as a wild plant in Iraq were collected from Al-Jadriya district at University of Baghdad, and classified by the herbarium of the Biology Department, collage of Science at Baghdad University. A quantity of 25 g from *L.barbarum* dried leaves were defatted by soxhlet for 10 hours using 300 ml n-hexane, then the defatted leaves were reflected for another 10 hours after filtration using 200 ml of 2M HCl solution. The filtrate was cooled and transferred to a separatory funnel. The aglycon moiety was extracted three times each with(50 ml) ethyl acetate. The collected ethyl acetate layers were washed with distilled water to get rid of the excess acid then evaporated to dryness by rotary evaporator at 40℃ .The dried residue was weighted then redisolved in 30 ml 50% ethanol. The obtained extract represented the total flavonoids.

2.2. Determination of Total Flavonoids

Quantitative Determination

According to(Wang *et al.,*2009) procedure, Quercetin standard stock solution was prepared(1mg/ml in 50%ethanol), from which serial dilutions were made to get different Quercetin standard solutions with concentration of (0.5,0.25 and 0.1)mg/ml in 50% ethanol. Aliquot of 1ml was transferred from each standard solution and from there dissolved extracted residue into a glass tubes, then 0.75 ml of 5% sodium nitrite solution was added and mixed well and left to stand at room temperature for 5 minutes. To all tubes 1.5 ml of 10% $AlCl_3$ in 50% ethanol was added, shacked well and left to stand at room temperature for another 5 minutes. finally 5ml of 1N NaOH solution was added to all tubes. The absorbance were read at 510nm, and a standard curve was plotted between each concentration and the absorbance, then the amount of total flavonoid was calculated as Quercetin from the equation of straight line that obtained from the plotted curve.

*Preparative TLC as Qualitative Determination (*Simon*et al.,*1998*):*

About 2ml from the leaves extract of *L.barbarum* flavonoid was applied as straight line on silica glass plate of 0.5 cm thickness with aid of syringe of 25 gage needle. Thin layer chromatography was performed on silica gel Gf254 plates using mobile phase of: chloroform: glacial acetic acid: formic acid: (44:3.5:2.5), one spot of 0.1mg/ml ethanol from rutin, kaempferol, quercetin and luteolin standard solutions. The preparative silica plates was scraped for each band appeared for further detections.

3. DETERMINATION OF CYTOTOXICITY

The cytotoxic effect of the extracted flavonoids from *L.barbarum* were investigated according to selected parameters including, MTT assay as a cell functional assay to determine cell viability, high content screening (HCS) technique for cell apoptosis, by treating cells with different concentrations for determining mechanism by which the extracts act.

3.1. MTT Assay(Freshney, 2012; Chih et al., 2004)

To determine the cell viability by colorimetric assay using 3-[4, 5 – dimethylthiazoyl]-2, 5-diphenyltetrazolium bromide (MTT dye), two kinds of cells were employed in this work: The Hepatocellular human carcinoma (HepG-2cell line) and the Normal human hepatic cells (WRL-68).

Briefly, 100 µl cell suspension was added onto the flat-bottomed micro culture plate wells, each line in a separated plate, for the two cell lines and treated then with 100µl flavonoids extract, incubated for 24 hours, centrifuged to remove the dead cells. Aliquot of 100μL from 2mg/ml MTT dye was added

to each well and incubation was continued for a further 4hours, then50μL of solubilization solution of DMSO was added into each well. The experiment was performed in triplicate. After complete solubilization of the dye, the absorbance of the colored solution obtained from living cells were read at 620 nm with an ELISA reader. The mean absorbance for each group of replicates was calculated. Percentage viability of cells exposed to various treatments was obtained as follows:

%Cell Viability= [(Mean Absorbance of treated sample/ Mean Absorbance of non-treated sample)× 100] (Chih *et al.,*2004)**.**

The control was the non-treated cultures in all experiments that contained cells in the medium only. This assay was held at Centre for Natural Product Research and Drug Discovery, Department of Pharmacology, Faculty of Medicine, University of Malaya / Kuala Lumpur, Malaysia.

3.2. The High Content Screening (HCS)Assay (Diana et al.,2010)

The HCS kit used in this assay allowed simultaneous measurement of five independent parameters that monitor cell health, including cell loss, nuclear size and morphological changes, DNA content, changes in cell membrane integrity and cell permeability, mitochondrial membrane potential, and cytochrome c localization, released from mitochondria. Different concentrations(25, 50 and100)µg/ml of the purified flavonoid from *Lycium* leaves, were used for treatment one cell line: the human liver cancer cell lineHepG-2 for one interval time24 hours (passage number was 5).The assay was carried on at the Centre for Natural Product Research and Drug Discovery, Department of Pharmacology, Faculty of Medicine, University of Malaya Kuala Lumpur, Malaysia

The HCS Kit Contents

Cytochrome C Primary Antibody, DyLight649 Conjugated Goat Anti-Mouse IgG, Mitochondrial Membrane Potential Dye, Permeability Dye, Hoechst Dye, Wash Buffer, Permeabilization Buffer, Blocking Buffer, and thin sealed plate.

Cell Preparation Protocol

For HepG2 cells the protocol is optimized according to instructions of ATCC(American Type Culture collection)Production No.HB-8065).

4. RESULTS

4.1. Flavonoid Extraction

According to our results, the main active components of *Lycium barbarum* leaves have been identified as flavonoids. The results indicated that total flavonoid in 25g *L.barbarum* dried leaves was 281 mg determined as Quercetin (11.28 mg/g of the dried leaves). There is no study about Iraqi wild type *Lycium barbarum* and its active components. However ;*L.barbarum* as a traditional Chinese herb possessing vital biological activities , such as prevention of cancer and age-related macular degeneration, is widely used in Asian countries(Ke*et al.,*2011).

4.2. Flavonoids Determination and Purification

Preparative TLC results, showed that different flavonoids were separated as straight lines indicated by different R_f values. Five layers were scraped and eluted with ethanol; some were detected as Luteolin, Quercetin(gives two spots), Kaempferol and Rutin , in corresponding to standard solutions. There were major flavonoid component which was isolated but still unknown.

4.3. Cytotoxic Effect of the Purified Flavonoid from *Lycium barbarum*

The MTT results shown in Table(1)indicated that 100µg/ml purified flavonoid posses significant cytotoxic effect toward both cell lines treated for 24 hours.

Table1. *Cytotoxic effect of purified extracted flavonoid from Lycium barbarum at Concentration of (100µg/ml) on HepG-2 and WRL68 cells for and 24 hours exposure time.*

Compound	**%Cell Viability Mean**± SE		T-test value
	HepG2	WRL-68	
Flavonoid	46.99 ± 2.56	35.48 ± 2.02	4.262 *
* (P<0.05).			

Plant-derived phenolic compounds, including flavonoids, and phenolic acids, have been included under investigation for their anticancer therapy and chemoprevention properties. Certain mechanisms underlying the differential effects of any flavonoids on tumor versus normal cells have been determined and suggested that flavonoids may simultaneously activate multiple pathways. The study described the establishment of an *in vitro* survival/apoptosis testing system based on detecting these mechanisms by HCS assay and cell cycle phase alteration. This system is able to screen potential chemopreventive or therapeutic agents from (but not limited to) plant-derived compounds based on the pathways differentially activated by these agents.

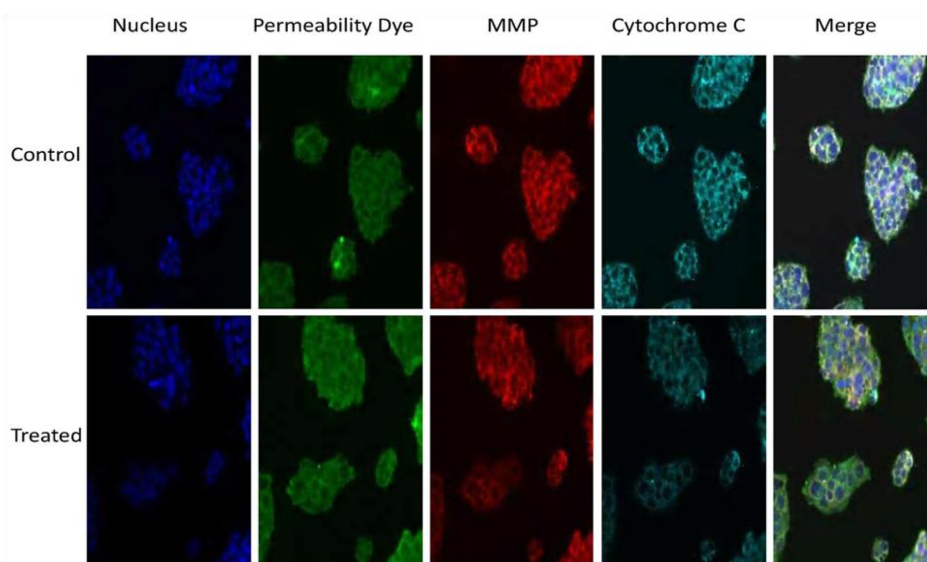
Effect of the Purified Flavonoid on Apoptosis

The HCS assay was held to investigate the effect of the purified flavonoid on the mechanism of apoptosis and the cell cycle arrest. Accordingly, Table(2) included cell permeability, cell count, nuclear intensity, mitochondrial membrane potential and cytochrome C level as a parameter for detecting apoptosis changes during 24 hours exposure, at various concentrations on HepG-2 cell line.

Table2. *Effect of purified flavonoid on physiological parameters of HepG-2 Cell Line Treated for 24 hours. MMP: is the Mitochonderial membrane potential*

Purified Flavonoid Concentration (μg/ml)	**Parameter tested**				
	Cell permeability (fluorescentintensity)	**Cell count**(cell number)	**Nuclear intensity** (fluorescentintensity)	**MMP***** (fluorescentintensity)	**cytochrome C** (fluorescentintensity)
25	145.14 ±15.23 b	1318.5± 18.54 a	216.75 ±9.26a	469.0 ±7.93b	442.77 ±11.72b
50	158.64 ±10.08 b	1203.50 ±23.76 b	204.92± 16.32a	498.0±9.51ab	432.50 ±6.84 b
100	197.24 ±12.76 a	1086.50± 34.26 c	160.14± 13.53b	430.0 ±7.43c	549.40 ±12.54a
Control	162.14 a	1687.5 a	207.43 a	521.33 a	416.26 b
LSD Value	93.04 NS	147.33*	14.87 *	34.35 *	51.70 *
* (P≤0.05). Means of different letters within the same column represented a significant differences					

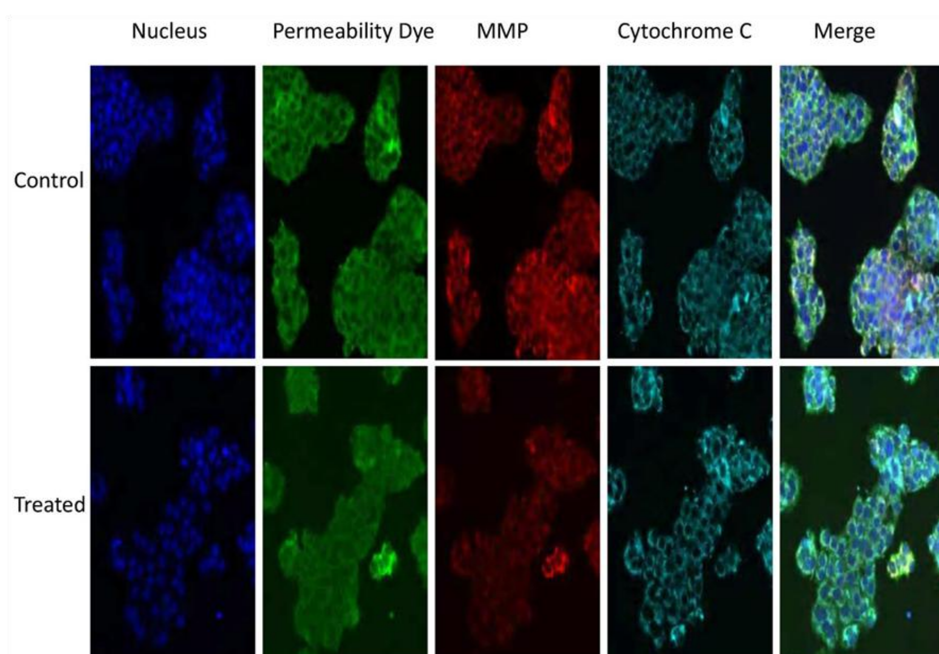
(A)



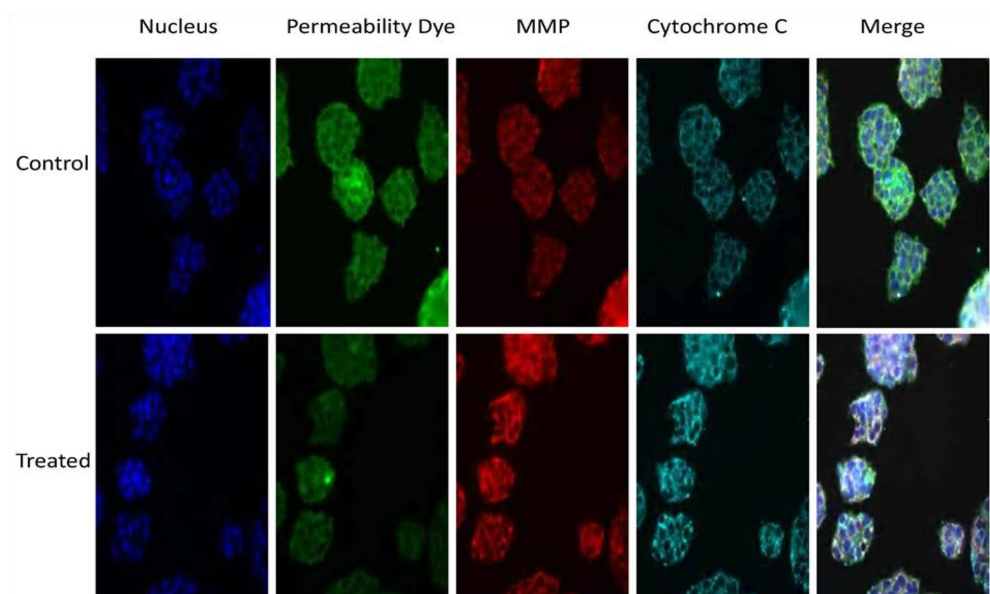
Screening potential drugs cytotoxicity is an essential aspect of new drug discovery, since cytotoxicity is a complex process affecting multiple parameters and pathways. As shown in Table(2), The HepG-2 cell line treated with purified flavonoid at different concentrations showed a remarkable induction of cell death(apoptosis).The toxic effect on HepG-2 cell viability increased with the concentration elevation. The most significant reduction($p<0.05$) in cell count was at the concentration 100μg/ml. Results showed that at high doses, the purified flavonoid operate upon increase cell permeability in comparison to other concentrations and to control(but with no significance between all concentrations and control).This means that high doses of the flavonoid increased cell membrane blebbing leading to increase cell membrane permeability. Moreover; apoptosis involved chromatin condensation and

nuclear fragmentation then decreasing in nuclear intensity as the fragmentation increased , here in case of high flavonoid doses ,the induction of cell death was showed to be via mitochondrial pathway since mitochondrial membrane potential (MMP) value and cytochrom C extra-nuclear level were affected significantly($p \leq 0.05$) by high concentration(100 µg/ml), in relative to control reading . While lowering in flavonoid concentrations appeared to affect nuclear intensity, mitochondrial membrane potential, and cytochrom C level but with no significance. The following figure(1)represents fluorescent views of HepG-2 cells treated with the purified flavonoid at different concentrations ; 100µg/ml,50µg/ml, and 25µg/ml.

(B)



(C)



Several plant-derived bioactive agents exhibited induction of apoptosis in number of experimental models of carcinogenesis(Taraphdar *et al.*,2001).Apoptosis: a key event in many biological processes, was very different from another type of cell death, known as necrosis which followed massive tissue injury. Whereas necrosis involved the swelling and rupture of the injured cells, apoptosis involved a specific series of events that lead to the dismantling of internal contents of the cell(Wayne *et al.*,2009).There are two major pathways that lead to apoptosis, both of which culminate in a common death program: The mitochondrial(intrinsic)pathway and the death receptor(extrinsic) pathway. The

mitochondrial pathway involved the induction of specialized protein that induce mitochondrial leakiness, leading to release of cytochrom c which binds to a protein called apoptosis-activating factor-1(APaf-1) a death-inducing protein. In the death receptor pathway, the triggering of cell surface receptors of the TNF-receptor family results in activation of common death pathway (Abbas *et al.,* 2008).

Apoptotic death occurred in two phases. During the ***latent phase***the cell looks morphologically normal but was actively making preparation for death and the duration of this phase ranged from a few hours to several days. ***The execution phase*** was characterized by a series of dramatic structural and biochemical changes that culminate in fragmentation of cell into membrane –enclosed apoptotic bodies, this phase lasts about an hour during which the following morphologic and physiological changes occurred: loss of microvilli and intercellular junction, shrinkage of the cytoplasm, dramatic changes in cytoplasmic motility with activation of violent blebbing, loss of plasma membrane asymmetry, with the distribution of phosphatidylserine being randomized so that it appeared in the outer membrane leaflet, hypercondensation of chromatin and its collapse against the nuclear periphery, and the explosive fragmentation of the cell into apoptotic bodies, all these changes were investigated by action of specific set of death-inducing proteases. The apoptotic bodies caused cells that ingested them to secrete anti-inflammatory cytokine, as a result, apoptotic death didn't lead to an inflammatory response (Thomas *et al.,*2008).

5. CONCLUSION

Flavonoids posses anticancer effect . Proposed mechanisms by which flavonoids may prevent cancer and act as tumor toxic agent explained by one or more of the following mechanisms:

Antioxidant: through which flavonoids act to; Scavenge free radicals and reduce oxidative stress, inhibit tumor cell proliferation and oncogene expression, induce the following: tumor suppress gene expression, cell differentiation (Mata-Greenwood *et al.*,2001) cell-cycle arrest and apoptosis (Ren *et al.,* 2001). The other mechanism is byinhibiting signal transduction pathways, through **Enzyme induction and Enhancing detoxification**(phase II enzymes) such as glutathione Peroxidase, catalase, superoxide dismutase, or through **Enzyme inhibition** (phase I enzyme to block activation of carcinogens) such as cyclooxygenase-2, induce nitric oxide synthase and xanthine oxide enzyme(Mutoh *et al.,*2000), or by **Enhancing the immune functions** as anti-angiogenesis(Tosetti *et al.,*2002) inhibit cell adhesion and invasion, prevent DNA binding , and finally flavonoids possess **Antibacterial and antiviral effects**(Ren *et al.,*2011). Many experiences strengthen the notion that flavonoid could be used as anticancer agent, and todate few clinical studies have demonstrated that these bioflavonoids retain anticancer properties in human *in vivo*. To investigate the mechanism by which the purified flavonoid possessed it's cytotoxic effect, HCS assay and cell cycle phases that may be altered bythis component were employed.

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