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International Journal of Current Research
Vol. 7, Issue, 04, pp.14874-14878, April, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

STUDY THE EFFECT OF PURIFIED FLAVONOID EXTRACTED FROM WILD *LYCIUM BARBARUM* LEAVES ON HEPG-2 CELL CYCLE

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ARTICLE INFO

Article History:
Received 17th January, 2015
Received in revised form
22nd February, 2015
Accepted 10th March, 2015
Published online 30th April, 2015

Key words:

Lycium barbarum flavonoids,
Cytoyoxic assay,
Cell cycle determination

ABSTRACT

The discovery and identification of a new drugs, which can act as anticancer agents had an important goal of researches. Flavonoids from Iraqi wild type *Lycium barbarum* leaves were extracted and identified by the preparative thin layer chromatography (PTLC) technique which were had been shown in a previous study. The study employed to investigate the pathway by which the purified flavonoid extracted from *Lycium barbarum leaves* exerts its cytotoxic effect through cell cycle determination and detecting the target phase of cell cycle that affected by treating cells with different concentrations and determining mechanism by which the flavonoid acts. Result showed that the purified flavonoid affected HepG-2 cell line after 24 hours exposure with different manner for each concentration. At 50µg/ml the purified flavonoid cause significant ($p \leq 0.05$) increase in Phosphohistone-H3 intensity, causing cell cycle arrest in M phase. The higher concentrations (100µg/ml), indeed didn't affect cell cycle at all. However the concentration (25µg/ml) did affect cell cycle of HepG-2 at G_1/S phase after 24 hours treatment but with no significance.

INTRODUCTION

The flora of Iraq, the ancient Mesopotamian land of civilization are intented 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 activities of chemical compounds. There are four classes of plant derived anticancer agents on USA markets including; The catharanthusvinicaalkaloids (vinblastine & vincristine), the epipodophyllotoxine (etopside & teniposide), the taxanes (paclitaxel & doctataxel), and the camptothecinderivatives (topotecan & irinotecan), and others being approved for therapy in last few years (Hury and Wip, 2008). *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 and cell cycle arrest (Cheah *et al.,* 2011). A major constituent of *Lycium* is the 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).

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MATERIALS AND METHODS

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 40ºC .The dried residue was weighted then redisolved in 30 ml 50% ethanol. The obtained extract represented the total flavonoids.

2. Determination of Total Flavonoids

2.1. Quantitative Determination (Wang *et al.,* 2009).

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. The assay was carried on according to Wang and co. (2009) procedure and finally, 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.

2.2. Preparative Thin Layer Chromatography (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, Then TLC chromatography on silica Gf254 was done with the standard (Luteolin, Quercetin, Kaempferol and Rutin). The preparative silica plates was scraped for each band appeared for further detections.

3. Cell cycle Determination

3.1. Cell Cycle Kit (Stilwell *et al.,* 2007)

Cell cycle kit was used for simultaneous quantification of nuclear DNA content to distinguish 2N (G1phase) and 4N(G2/M phase), DNA replication in S phase, and mitosis marker in M phase. The kit allow direct measurement of BrdU(which is bromodeoxyuridine a thymidine analog) incorporation and, mitosis-specific histone H3 phosphorylation, using fixed end- point assay based on immunofluorescence detection in cells. The DNA binding dyeDAP1 is (4^{-},6-diaminidino-2-phenylindole) a fluorescent stain that binds strongly to A-T rich regions in DNA) is used to determine the cell cycle phases through assessment of DNA content of the cells. The total intensity from a DAP1-labeled nucleus, determined on image analysis system such as the Array Scan HCS Reader, is proportional to the nucleus linear range of the dye could vary depending on cell type. The primary antibodies are specific for their targets and have minimal cross-reactivity with other targets. The kit can be used to detect mitotic cells which can be easily detached from the plate.

A- The kit Contents

BrdUdye, BrdU Primary Antibody (mouse), Phosphor-Histone H3 Primary Antibody (rabbit), Dylight 488 conjugated Goat anti-mouse IgG, Dylight 549 conjugated Goat anti-Rabbit IgG, DAP1 Dye, $MgCl_2$, wash buffer, Permeabilization buffer, Blocking buffer, Thin plate sealed

B-Cell Preparation Protocol

- For routine culture of HepG2 cells suspended in EMEM complete medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin were used.
- For BrdU detection, cells were harvested bytrypsinization, diluted with EMEM complete medium to get a density of 5 × 10^4cells/ml.
- For phospho-Histone H3 detection, cells were diluted to a density of 1.0 × 10^5 cells/ml with EMEM complete medium, then 100 µl of the cell suspension per well was transferred into a 96-well microplate.

C-Procedure for BrdU Incorporation and Phospho-Histone H3 Analysis

According to Cellomics® Cell Cycle Kit I from Thermo Scientific company/USA protocol

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. Effect of the Purified Flavonoid on Cell Cycle Phases Alteration

The effect of different concentrations (25,50,100) µg/ml from purified flavonoid on the cell cycle phases alteration was shown in Table (3).

Results shown in Table (3) and Figure (2) declared that regardless of the concentration there was no significant differences between them in respect to control. The decrease in BrdU uptake following treatment taken as indicator for G1/S phase arrest, while the increase in the percentage of cells expressing of phospho-histone H3 following treatment,

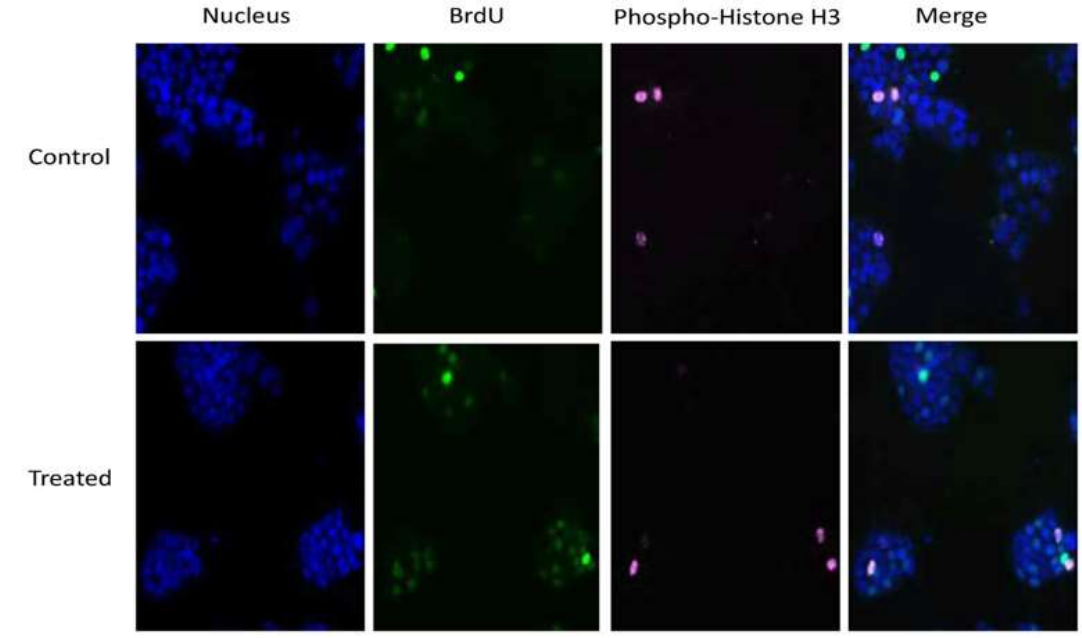
indicated the drug induced cell cycle arrest in M phase (Cheah *et al.,* 2011). However the purified *Lycium* flavonoid affected HepG-2 cell line after 24 hours exposure with different manner for each concentration. At 50µg/ml the purified flavonoid cause significant ($p \leq 0.05$) increase in Phosphohistone-H3 intensity, causing cell cycle arrest in M phase. The higher concentrations (100µg/ml), indeed didn't affect cell cycle at all. However the concentration (25µg/ml) did affect cell cycle of HepG-2 at G_1/S phase after 24 hours treatment but with no significance.

Table 3. Effect of purified flavonoid on cell cycle of HepG-2 cell line

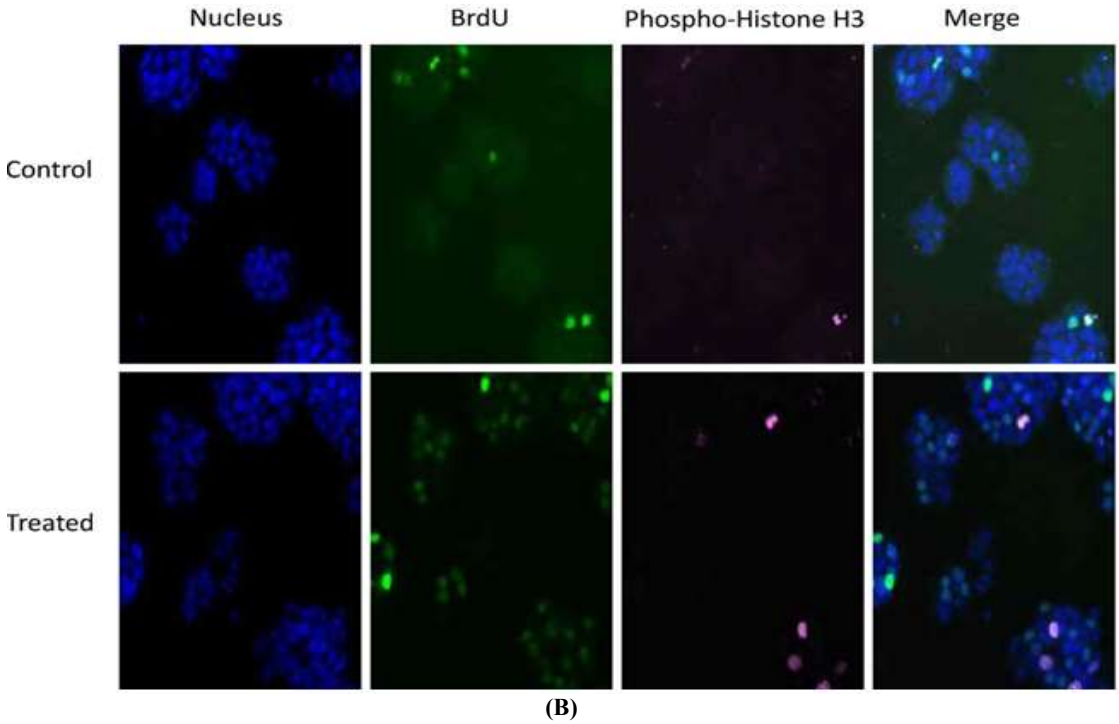
Flavonoid Concentration (µg/ml)	Cells Viable % in relative to controlMean ± SE		LSD Value
	Brdu Dye Intensity(S phase Cells Viable %)	Phospho-HistonH3Intensity (M phase Cells Viable %)	
25	96.06 ± 3.81 a	97.166 ± 4.61 b	4.79 NS
50	100.29 ± 4.3 a	107.57 ± 5.29 a	11.04 NS
100	99.88 ± 3.76 a	94.076 ± 3.92 b	8.652 NS
LSD Value	6.42 NS	7.21 *	-----

* ($P \leq 0.05$), NS: Non-significant.

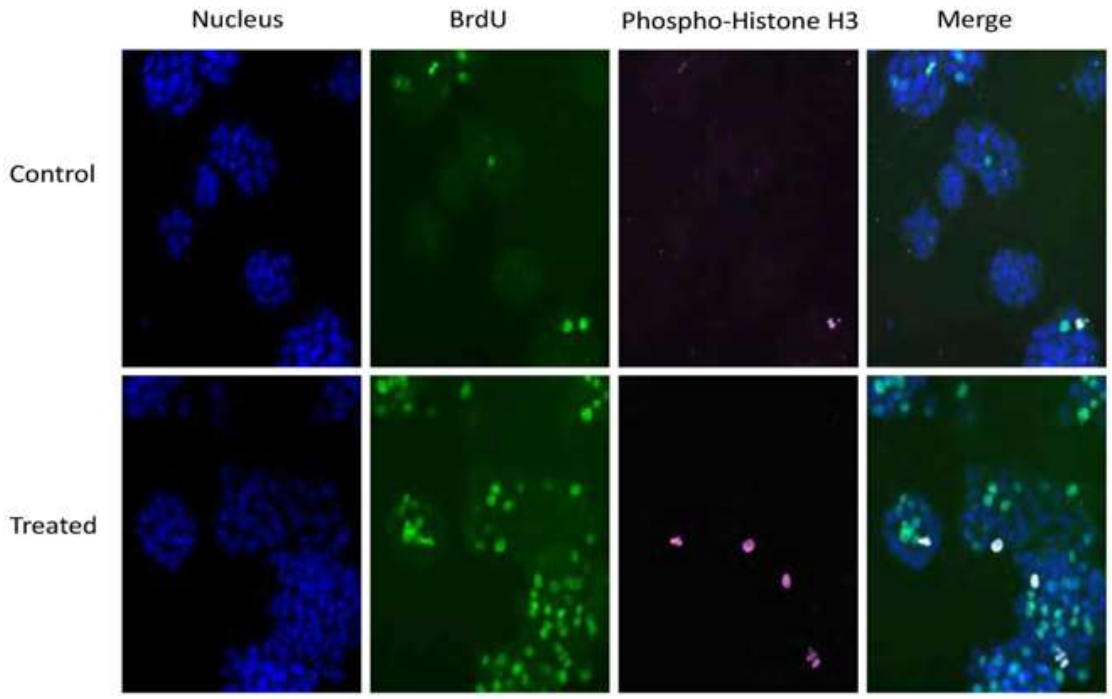
The following stained Figures (2) declare the effect of different flavonoid concentrations on cell cycle



(A)



(B)



(C)

Figure 2. Effect of purified flavonoid on HepG-2 cell cycle arrest treated for 24 hours.
A: HepG-2 cell line treated with100µg/ml *Lycium* extracted flavonoid
B: HepG-2 cell linetreated with50µg/ml *Lycium* extracted flavonoid;
C: HepG-2 cell linetreatedwith25µg/ml *Lycium* extracted flavonoid, Merge represents all parameters

In all eukaryotic cells, passage through the cell cycle is a highly regulated process involving ordered transition of G0/G1 to S and G2/M phases (John, 2002). The non-replicating cells are generally stopped in the G0/G1 stage, when cells begin to replicate, the DNA synthesis happen in S phase. Finally the cells divide in the G2/M stage. Growth factors or others external events affect the rate of cell proliferation are ultimately likely to act by controlling cell cycle progression (Yao *et al.,*2003). Assessment of the proliferative state of a cell population had become an important parameter in drug discovery research, particularly in evaluating cancer therapeutics and in determining the health of a cell population during recent studies. A traditional method for detection of cell proliferation is measurement of [^{3}H] thymidine incorporation as cells enter S phase. This technology is slow, labor-intensive and has several limitations, including the handling and disposal of radioisotopes. A well established alternative to [^{3}H]thymidine uptake uses bromodeoxyuridine (BrdU), a thymidine analog, to replace [^{3}H] thymidine (Stilwell *et al.*, 2007). When cells are pulsed with BrdU, it is incorporated into newly synthesized DNA strands of actively proliferating cells. The incorporation of BrdU into cellular DNA may then be detected using anti-BrdU antibodies, allowing assessment of the population of cells which are synthesizing DNA (Thomas, 2007). This provides an indication of cell proliferation rate and a measurement of the number of cells in S phase during the pulse cellular processes that influence proliferation. Phospho-histone H3 (Ser 10) is expressed in the nuclei of cells during M phase (mitosis). Induction of apoptosis and/or inhibition of cell proliferation are highly correlated with the activation of a variety of intracellular signaling pathways leading to arrest the cell cycle in the G1, S, or G2/M phase of the cell cycle. Since mitosis is accompanied by phosphorylation of histone H3 on serine 10, the presence of phosphorylated histone H3 (Ser10) indicates that a cell is mitotic (Hans and Dimitrov, 2001). Upon exit from mitosis, a global dephosphorylation of histone H3takes place. These phosphorylation and dephosphorylation events are well characterized and accordingly, phospho-histone H3 nuclear expression is widely used as a measure of mitotic index (the percentage of cells in M phase) in flowcytometer or HCS applications (Gasparri *et al.,* 2007). Due to its important regulatory role in cell proliferation and neoplastic cell transformation, histone H3 has been identified as a crucial target for cancer chemotherapy (Dong and Bode, 2006)and increasingly being used as a tool in drug discovery. Cell cycle regulation and its modulation by various plant-derived agents have gained widespread attention in recent years (Barabasz *et al.*, 2006) for example in onestudy, apoptosis induced panduratin A (a component extracted from *Chinese ginger*) is presumably a consequence of either G2/M block or the DNAdamagedue to abnormal mitotic arrest (Cheah *et al.,* 2011). Different compound can affect cell cycle at different phases. Aphidicolin (an inhibitor of DNA polymerase) and etoposide (a DNA topoisomerase II inhibitor) are capable of arresting the cell cycle at G1/S and S/G2 phases, respectively. Nocodazole (a microtubule depolymerization agent) and paclitaxel (a microtubule stabilizer) are each capable of arresting the cell cycle at G2/M phase. Flavonoids uptake can affect cell cycleof human colon adenocarcinoma cells (Caco-2) by decreasing in G_1-phase cell associated with an accumulation in G_2/M phase (Salucci *et al.,* 2002). Many studies were done to investigate *Lycium* effects toward different cell lines. In a study by Hu *et al*. (1994) about the

effect of *L.barbarum* active components on cervical cancer cells, found the number of S phase cells decreased from 56 to 49%, and the number of G0/G1 phase cells increased from 16 to 33% (Hu *et al*., 1994). These results suggest that multiple mechanisms are responsible for the anticancer effects of *L.barbarum*, depending on the different type of cancer and the plant extract component which may candidate as anticancer agent.

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