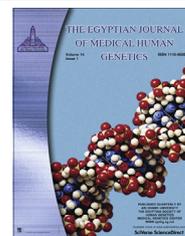




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ORIGINAL ARTICLE

# The antiproliferative effect of mulberry (*Morus alba* L.) plant on hepatocarcinoma cell line HepG2

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## KEYWORDS

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**Abstract** This study aimed to investigate the antiproliferative effect of aqueous and organic extracts of mulberry leaves (*Morus Alba* L.) on human hepatocellular carcinoma HepG2 cell line. Mulberry leaf extracts were prepared using the solvents: water, 50% aqueous MeOH, and 100% MeOH for different time intervals, while the cells treated with dimethyl sulfoxide (DMSO) served as control. The effects of aqueous and organic extracts of *M. alba* L. leaves on HepG2 cell viability, nuclear factor kappa B (NF- $\kappa$ B) gene expression, alfa-fetoprotein (AFP), albumin (ALB), gamma-glutamyl transpeptidase ( $\gamma$ -GT) and alkaline phosphatase (ALP) were measured. The results of the cell viability assays showed that water, 50% aqueous MeOH, and 100% MeOH extracts exhibited a highly significant inhibitory effect on HepG2 cell proliferation which was evidenced by a reduction in viable cell count. The results were confirmed by microscopical examination of cell morphology. Furthermore, the mulberry leaf extracts suppressed the activity of NF- $\kappa$ B gene expression of HepG2 cells compared to the control. Also a highly significant depression occurred at the levels of AFP,  $\gamma$ -GT and ALP in HepG2 cells compared with that of controls in a time dependent manner. By contrast, the mulberry leaf extracts increased the secretion of ALB. Therefore, the conclusion was that the organic and aqueous extracts of mulberry leaves inhibit the growth of HepG2 cells through suppressing the activity of NF- $\kappa$ B gene expression and modulate the biochemical markers.

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## 1. Introduction

The burden of hepatocellular carcinoma (HCC) has been increasing in Egypt with a doubling of the incidence rate in the past 10 years. This has been attributed to several biological

(e.g. Hepatitis B and C virus infection) and environmental factors (e.g. Aflatoxin, AF). Other factors such as cigarette smoking, occupational exposure to chemicals such as pesticides, and endemic infections in the community, as schistosomiasis, may have additional roles in the etiology or progression of the disease [1,2]

According to the World Health Organization (WHO) Egypt has one of the highest incidences of hepatitis C, one of the main causes of liver cancer, in the world. The number of deaths resulting from liver cancer in Egypt had risen from 4% in 1993 to 11% in 2009 [3]

Hepatocellular carcinoma is a preventable disease rather than a curable one, since there is no well-documented effective treatment modality until now. There has been a worldwide trend toward the use of various plants and many efforts are focused on the search for a potential source rich in biologically active compounds. It was reported that some plants exercise various bioactivities, including antioxidant, anti-inflammatory, and anti-diabetic [4].

One of the less studied plants is mulberry (*Morus alba* L.). Mulberry is a fast-growing deciduous plant. *M. alba* L. belongs to Moraceae family, commonly known as white mulberry. *Morus* is the old Latin name; *alba* refers to the white fruits. The leaves are nutritious, palatable and nontoxic [5].

Reports indicate that mulberry leaves contain proteins, carbohydrates, iron, zinc, calcium, magnesium, phosphorous, ascorbic acid, *b*-carotene, vitamins B-1 and D, and folic acid [6]. Also, rutin, quercetin, isoquercetin and other flavonoids in mulberry leaves have been found [7]. The mulberry plant possesses medical benefits, including diuretic, hypoglycemic, antibacterial, antiviral, hypotensive properties and neuroprotective functions [8,9]. Therefore, searching for new natural and nontoxic compounds with the cytotoxic effects against HCC cells is of particular interest.

In this study, we investigated the antiproliferative effect of organic and water extracts of Mulberry leaves (*M. alba* L.) on human hepatocellular carcinoma HepG2 cell line which is a well-differentiated transformed cell line closely related to HCC.

## 2. Materials and methods

### 2.1. Materials

Dimethyl sulfoxide, phosphate-buffered saline (PBS), and methanol (MeOH) high-performance liquid chromatography HPLC grade (means high quality) were obtained from Fisher Scientific (USA). Trypan blue stain (0.4%), and fetal bovine serum, were from Gibco BRL (USA), Amphotiricin B (Lonza Bio Whittaker), Cell culture medium; RPMI 1640 (Lonza Bio Whittaker).

### 2.2. Methods

#### 2.2.1. Preparation of aqueous and organic extracts of *M. alba* L.

Fresh mulberry (*M. alba* L.) leaves were from highest grade commercially available. The leaves were cleaned, dried in the air without being exposed to heat or, sunlight and ground to a fine powder. The resulting powder was then passed through an 80-mesh sieve and kept in a sealed aluminum bag at 4 °C, till further use.

**2.2.1.1. Preparation of aqueous extracts.** The method described by [10] was followed in preparing the aqueous extract of mulberry leaves. Briefly, 2 g of mulberry leaves powder was soaked in 200 ml of boiling water (W) for 20 min. The mixture was cooled to room temperature before being filtered through Whatman (Maidstone, UK) No. 1 filter paper and lyophilized. The freeze-dried solid extract was stored at -20 °C in plastic tubes and protected from light. The solid extract was redissolved in double distilled water and filtered through a syringe filter (bore size, 0.22 µm) prior to use in all assays

**2.2.1.2. Preparation of organic extracts.** For the preparation of organic extracts, the solvent extraction was carried out according to the method described by [10]. In brief, 2 g of the dried powdered mulberry leaves was extracted separately with 20 ml of various organic solvents (100% MeOH, and 50% aqueous MeOH) for 3 h in the dark at room temperature, on a multimagnetic platform. Each extract was separated by centrifugation (13,000g, 10 min), the supernatant was removed, the residue was resuspended with 20 ml of the same solvent, and the mixture was again separated by centrifugation. The two resulting supernatants were then combined and concentrated under vacuum to dryness, and the residue was stored in the dark at -20 °C. For bioassays, the residue of each extract was redissolved in 1 ml of dimethyl sulfoxide at a concentration of 250 mg/ml and diluted with tissue culture medium before use [8].

#### 2.2.2. Cell viability assays

Human hepatocellular carcinoma cells (HepG2) were purchased from the holding company for biological products (VACSERA); in a semi-confluent 25 ml tissue culture flask (T-25). HepG2 were maintained in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 1% Amphotricin B in the laminar flow hood under complete aseptic conditions, culture medium was changed three times per week. Then, T-25 flasks of completely confluent HepG2 cells were treated with each type of *M. alba* L. extracts and the cells treated with dimethyl sulfoxide (DMSO) only served as control (mulberry extracts were each dissolved in dimethyl sulfoxide at a concentration of 250 mg/ml and diluted with tissue culture medium before use) as follows:

10 ml of the above extracts (treatment) and 10 ml of the tissue culture media were added to each flask, following the sterility rules. The treated flasks were then maintained at 37 °C in a humidified incubator with 95% air and 5% CO<sub>2</sub>.

After 6 h, 24 h, and 48 h respectively all flasks were trypsinized with 1 ml of trypsin-EDTA and counted under the light microscope (100×) using trypan blue dye (0.04%) to count the number of viable cells. The cell suspensions were centrifuged then; the pellet was washed with physiological saline (0.9%) and kept in sterile eppendorffs at -80 °C until the biochemical assays were performed.

#### 2.2.3. Nuclear factor kappa B gene expression RT-PCR analysis

Total RNA was extracted from all of the above cell-lysates using the RNeasy Mini kit (Qiagen, cat. No. 74104, USA) according to the manufacturer's instructions. In order to preserve RNA samples, which are very vulnerable to degradation at room temperature, total RNA was transcribed into cDNA using the reverse transcription system kit (*High Capacity*

*RNA-to-cDNA Master Mix*, Applied Bio systems). The resultant cDNA was diluted 10 times in double distilled H<sub>2</sub>O and kept at -20 °C for RT-PCR analysis. RT-PCR was performed using a Real time PCR 7500 fast thermal cycler (Applied Biosystems, USA). The quantitative RT-PCR was briefly carried out in a 10 µl final volume containing the following: 5 µl (2x) SYBR Green, 1 µl master mix (Qiagen, USA), 1 µl of 5 µM forward primer and reverse primer, respectively, and 3.0 µl diluted cDNA. After an initial denaturation step at 95 °C for 15 min, temperature cycling was initiated. Each cycle consisted of denaturation at 94 °C for 15 s, annealing at 60 °C for 25 s, and elongation at 72 °C for 20 s. A total of 45 cycles was performed. Each run was completed with a melting curve analysis in order to confirm the specificity of amplification and lack of primer dimers. Mouse β-actin was amplified as the control for normalizing the quantities of transcripts of each of the above genes. Primer pairs for NF-κB, and β-actin were designed using the primer design program (primer 3 software version 1.0). Forward and reverse primer sequences for the respective genes and their corresponding amplicon size are listed in Table 1.

The expression differences for NF-κB, between the control and treated cells were calculated by normalizing with β-actin gene expression according to the following formula [11]:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

$$= 2^{-\{[CT(\text{control})\text{gene X-CT}(\text{control})\text{actin}] - [CT(\text{activated})\text{gene X-CT}(\text{activated})\text{actin}]\}}$$

#### 2.2.4. Biochemical assays

At 6, 24, 48 time intervals hours after treatment, culture medium was harvested for measuring the contents of AFP, albumin, γ-GT and the activities of alkaline phosphatase.

- The concentrations of AFP were measured using an ELISA Dia. Metra s.r.i kit, Italy, by the manufacturer's instructions according to the method described by [12].
- The albumin content was determined using (Linear Chemicals, S.L., Spain) assay kit according to the method of *Wolf RL.*, following the manufacturer's instructions [13].
- γ-GT was determined using (Linear Chemicals, S.L., Spain) assay kit according to the method of *Tietz N.W.*, following the manufacturer's instructions [14].
- The activities of alkaline phosphatase were determined using (Linear Chemicals, S.L., Spain) assay kit according to the method of *Young D.S.*, following the manufacturer's instructions [15].

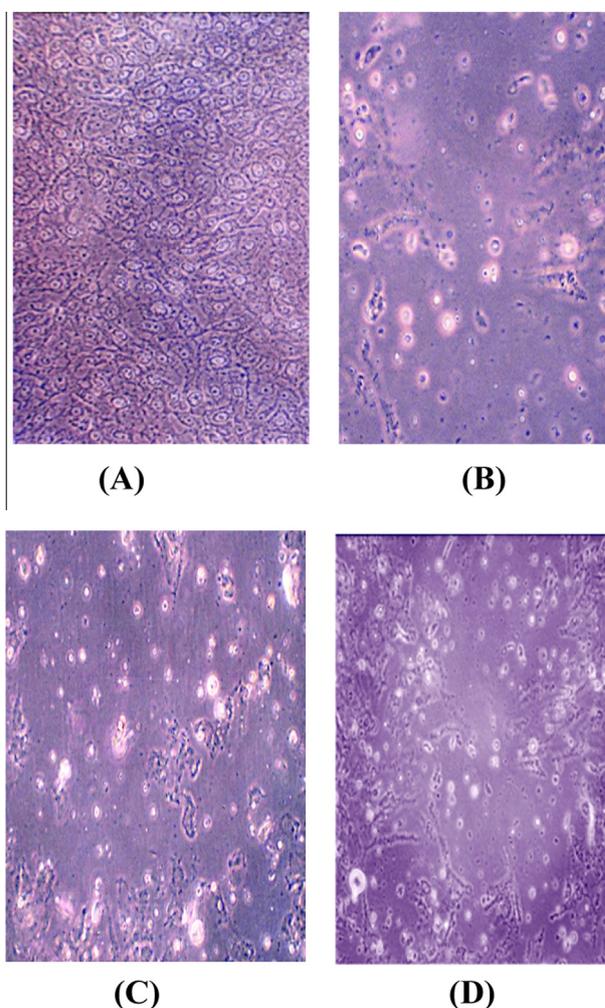
#### 2.2.5. Statistical analysis of data

The statistical analysis was performed using the computer program SPSS version (16) using the arithmetic mean  $\bar{x}$ , standard deviation (SD), and one-way analysis of variance (ANOVA) followed by the Tukey's test for comparison between groups. A probability (*p*) value less than 0.05 indicated a significant difference.

### 3. Results

#### 3.1. *M. alba* L. extracts inhibited the growth of and caused morphological changes in HepG2 cells: Fig. 1

The results indicated that *M. alba* L. extracts inhibited the proliferation of HepG2 cells in a time-dependent manner at 6 h, 24 h, and 48 h post-treatment using water, 50% aqueous MeOH, and 100% MeOH extracts respectively as the % of survival rates which have significantly decreased with the progress of time compared with the untreated control cells. After 6 h 100% methanol extract showed highly significant depression than the other extracts. However all the treatments showed highly significant decrease after 48 h. Microscopic observations of cell morphology show that *M. Alba* L. extracts induced morphological changes in HepG2 cells toward more mature forms of hepatocytes (using an inverted light microscope) (see Fig. (A–D)).



**Table 1** Sequence of specific primers.

Primer	Forward	Reverse	Amplicon size (bp)
NF-κB	gcgtacacattctctggggagt	cgaagcaggagctatcaac	178
β-Actin	agccatgtacgtagccatcc	gctgtggtggtgaagctgta	222

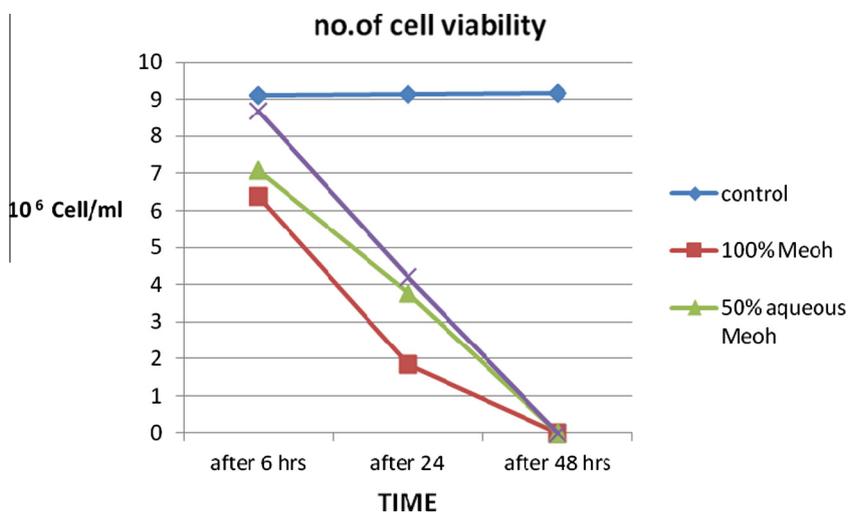


Figure 1 Time course responses of *Morus alba* extracts on HepG2 cell viability.

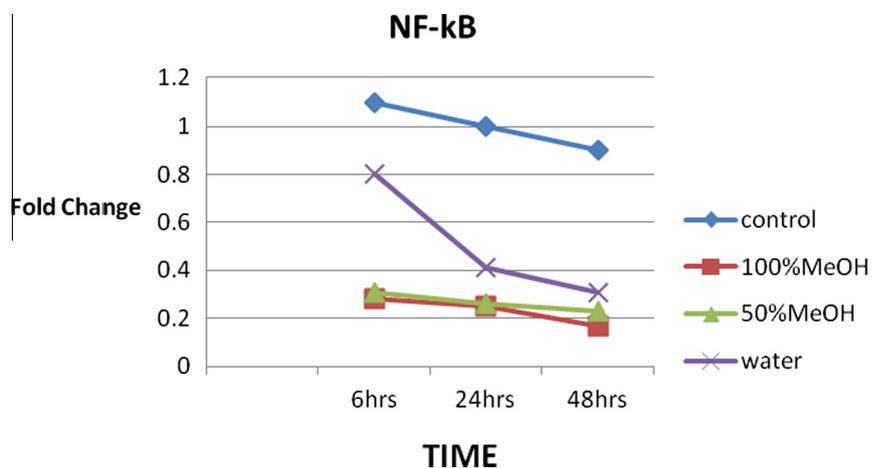


Figure 2 The effects of aqueous and organic extracts of *Morus alba* L. leaves on Nuclear factor kappa B gene expression.

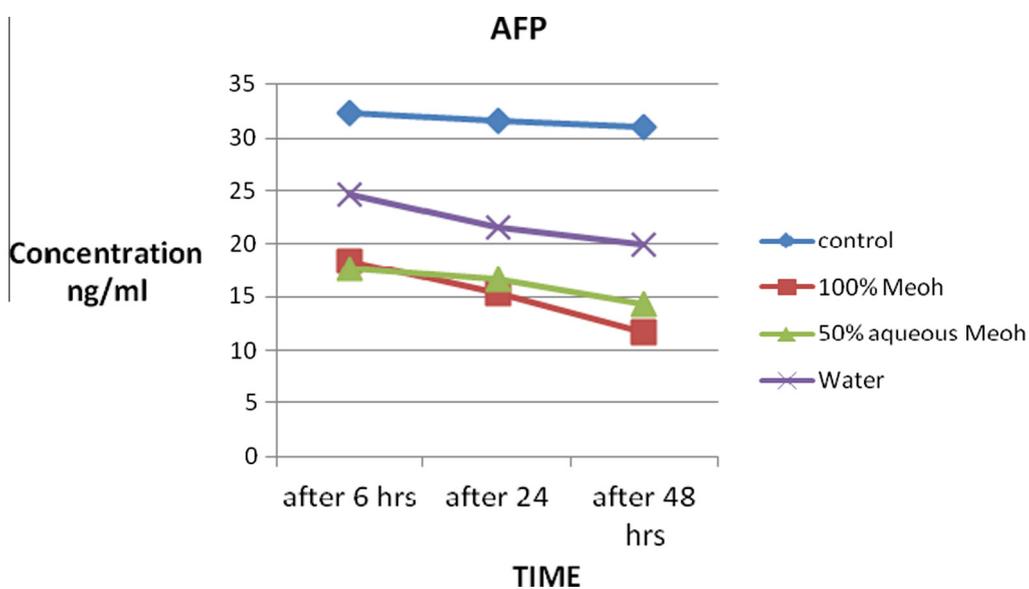


Figure 3 The effects of aqueous and organic extracts of *Morus alba* leaves on alpa-fetoprotein.

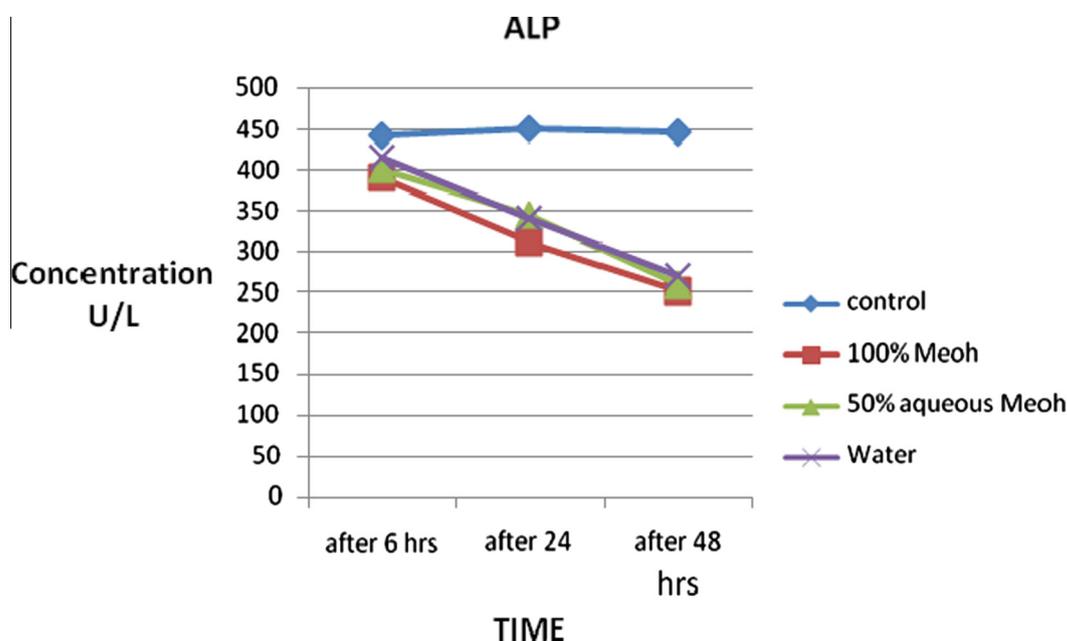


Figure 4 The effects of aqueous and organic extracts of *Morus alba* L. leaves on alkaline phosphatase.

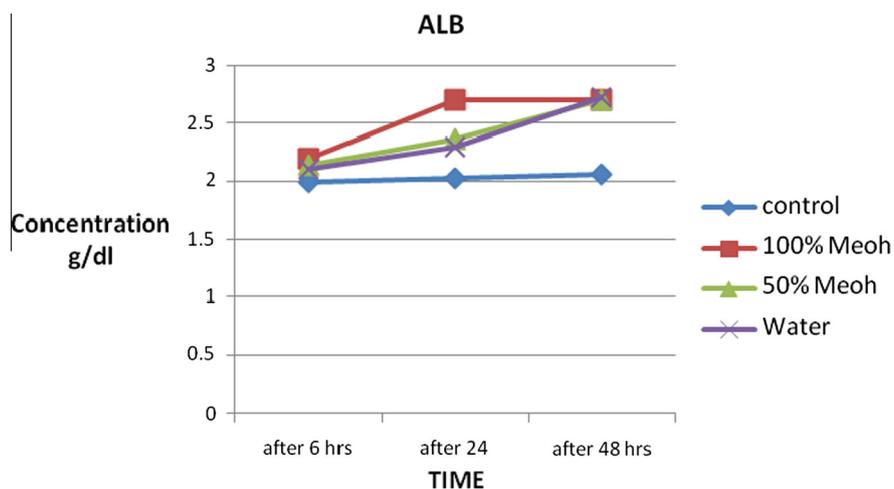


Figure 5 The effects of aqueous and organic extracts of *Morus alba* L. leaves on albumin.

### 3.2. *M. alba* L. extracts inhibited the activity of nuclear factor kappa B gene expression of HepG2 cells

Fig. 2 shows a time dependent inhibition in the activity of Nuclear factor kappa B gene expression of HepG2 cells treated with 100% MeOH, 50% aqueous MeOH, and water extracts of *M. alba* L. Leaves which was evident by depression in the fold change of NF- $\kappa$ B gene compared with the control.

### 3.3. *M. alba* L. extracts induced changes in AFP and albumin secretion and in $\gamma$ -GT and alkaline phosphatase activities in HepG2 cells

Fig. 3 shows a highly significant depression ( $p < 0.001$ ) in the levels of AFP in HepG2 cells treated with water extract, 100%

methanol extract, and 50% aqueous methanol extract respectively compared with that of the control.

Fig. 4 shows insignificant depression ( $p > 0.05$ ) in the levels of ALP in HepG2 cells treated with water extract, 100% methanol extract, and 50% aqueous methanol extract respectively at 6 h compared with that of controls, but after 24 h and 48 h significant elevation occurred at the level of ALB ( $p < 0.01$ ) compared to that of the control.

Fig. 5 shows a highly significant depression ( $p < 0.001$ ) in the levels of  $\gamma$ -GT in HepG2 cells treated with water extract, 100% methanol extract, and 50% aqueous methanol extract respectively compared with that of the control.

Fig. 6 shows a highly significant depression ( $p < 0.001$ ) in the levels of ALP in HepG2 cells treated with water extract, 100% methanol extract, and 50% aqueous methanol extract respectively compared with that of the controls.

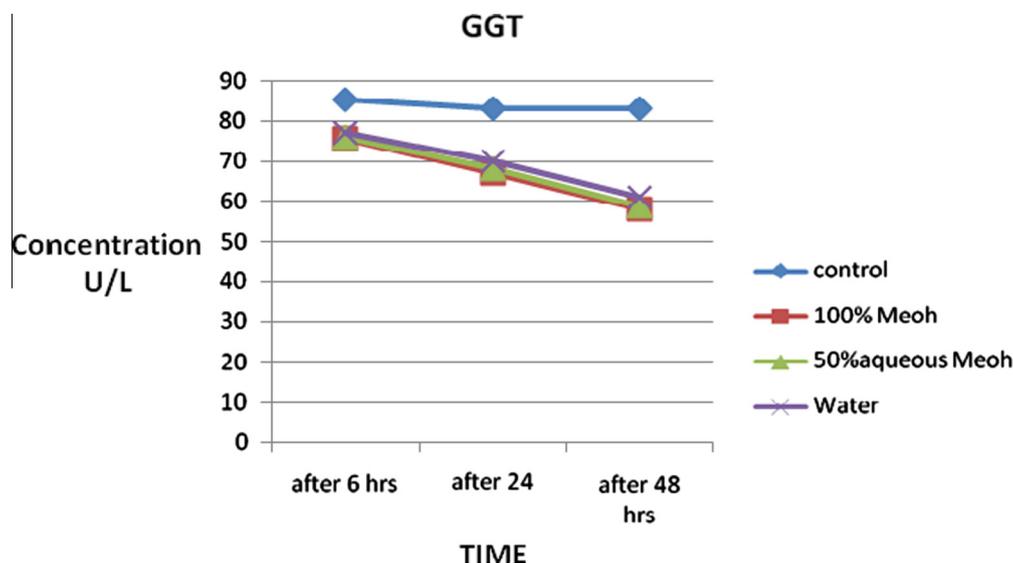


Figure 6 The effects of aqueous and organic extracts of *Morus alba* L. leaves on gamma-glutamyl transpeptidase.

#### 4. Discussion

Various plants have been found to inhibit tumor growth and cause a phenotype reversion in certain cancers. Recently, natural plants have received much attention as sources of biological active substances including antioxidants, antimutagens and anticarcinogens [16].

The results of the Cell viability assays showed that water, 50% aqueous MeOH, and 100% MeOH extracts exhibited a highly statistically significant ( $p < 0.001$ ) inhibition on HepG2 cell proliferation which were evidenced by a reduction in the viable cell count. The organic extracts had much stronger inhibitory activity than the water extract after 6 h and 24 h but they had the same effect after 48 h. Therefore, the mulberry extracts of 50% aqueous MeOH and 100% MeOH had the most effective inhibiting effect on the growth of HepG2 cells. These results are in agreement with the previous work on leukemia cells, showing that mulberry leaf extracts can inhibit cell proliferation and induce cell cycle arrest in the G2 = M phase and induce apoptosis in leukemia cell lines [17]. Also, these results were in agreement with that of Wanlaya et al. [8], who demonstrated that Mulberry leaf extracts are able to inhibit human leukemia cells (HL-60) and B16 mouse melanoma cells.

Morphological examination showed that *M. alba* L. extract-treated cells were clearly discerned by their rounded shapes compared to the polygonal shapes of control cells, suggesting possible growth-arresting and apoptosis-inducing effects of *M. alba* L. extracts [18].

Nuclear factor-kappa B (NF- $\kappa$ B) is a transcription factor and antagonist of apoptosis during liver regeneration and is closely related to the formation and development of hepatocellular carcinoma [19]. NF- $\kappa$ B is kept in an inactive state in the cytoplasm by the members of the inhibitor of NF- $\kappa$ B (I $\kappa$ B) family [20]. NF- $\kappa$ B is activated by free radicals, inflammatory stimuli, cytokines, carcinogens, tumor promoters, endotoxins, g-radiation, ultraviolet (UV) light, and X-rays [21]. Several dietary agents are natural chemopreventive agents that have been found to be potent inhibitors of NF- $\kappa$ B [22].

RT-PCR analysis showed that the mulberry leaf extracts suppressed the constitutive activity of NF- $\kappa$ B gene expression of HepG2 cells with treatment of water, 50% aqueous MeOH, and 100% MeOH extracts which effectively induced tumor growth when compared to the control. *M. alba* L. extracts may block one or more steps in the NF- $\kappa$ B signaling pathway such as the signals that activate the NF- $\kappa$ B signaling cascade, translocation of NF- $\kappa$ B into the nucleus, DNA binding of the dimers, or interactions with the basal transcriptional machinery as Bharat and Shishir [23]. This result was in agreement with Jenq-Chang et al. who reported that *M. alba* L. extracts inhibited the NF- $\kappa$ B-regulated expression of genes involved in cell proliferation, anti-apoptosis, and invasion [24]. These data suggested that the *M. alba* L. extract most likely reduced apoptotic effects by suppressing NF- $\kappa$ B activity and NF- $\kappa$ B regulated transcription of genes involved in cell survival. Also, these results were in agreement with those of Marcello and Lakita who demonstrated that the inhibition of NF- $\kappa$ B activity might constitute an effective strategy to be a preferential method for the treatment of HCCs [25].

AFP is a carcinoembryonic protein and a very important marker of primary hepatocellular carcinoma. Elevation of the AFP level of abnormally high values occurs in several malignant diseases, as in primary hepatocellular carcinoma. High  $\gamma$ -GT activity is also a marker of hepatocarcinoma: its activity is extremely low in the adult liver and gradually increases during the oncogenesis of liver cancer. On the other hand, Albumin is a protein secreted by mature hepatocytes and its secretion is one of the markers of hepatocyte differentiation. Also liver disorders are probably the most common reason for alkaline phosphatase elevation.

As regards to AFP results, a highly significant depression ( $p < 0.001$ ) was seen in the levels of AFP in HepG2 cells treated with water, 50% aqueous MeOH, and 100% MeOH extracts respectively compared with those of controls. Also these results showed that organic extracts, induced higher activity than the water extract. Bredaki et al. stated that the AFP secretion was reduced following *M. alba* L. treatment and suggested that *M. alba* L. extracts can induce the normal

differentiation of HepG2 cells and may reverse the malignant phenotype of HepG2 cells [26].

This study revealed that HepG2 cells treated with water, 50% aqueous MeOH, and 100% MeOH extracts showed an insignificant depression ( $p > 0.05$ ) in the levels of albumin (ALB) in HepG2 cells at 6 h compared with those of controls, but after 24 h and 48 h it showed significant elevation ( $p < 0.01$ ) compared to the control. Andrzej et al., state that HepG2 cells were observed to be able to produce albumin, a major component of blood plasma produced in the liver. The albumin level in blood dramatically decreases during liver insufficiency and may serve as an indicator of the HepG2 cell proliferation inhibition [27].

The present study showed that a highly significant depression ( $p < 0.001$ ) was seen in the levels of  $\gamma$ -GT in HepG2 cells treated with water, 50% MeOH, and 100% MeOH extracts respectively compared with those of controls. Furthermore the treatment with 100% methanol extract and 50% methanol extract relatively induced higher activity than the water extract in a time dependent manner. These results were in agreement with *Notas* who reported that *M. alba* L. extracts may reverse the phenotype of human hepatocarcinoma cells through the induction of their differentiation toward more mature forms of hepatocytes [28].

Regarding Alkaline phosphatase (ALP), the results of the current study showed that highly significant depression ( $p < 0.001$ ) was seen in the levels of ALP in HepG2 cells treated with water, 50% MeOH, and 100% MeOH extracts respectively compared with those of controls. Similar results have been reported by [18] with K562 and the HL-60 cell line. Based on these they suggested a regulatory role of the extract of *M. alba* L. on the ALP activity. The results of the present study came in disagreement with the results obtained by Hanachi et al. who reported that the level of ALP in the treated cell was increased 4-fold which might be due to the damage of the cells, causing it to be leaky [29].

In conclusion, the present study demonstrates the effects of aqueous and organic extracts of *M. alba* L. leaves on the proliferation of human hepatocellular carcinoma cells. It demonstrates that organic and aqueous extracts of *M. alba* L. Leaves especially the 100% MeOH extract, inhibit cell proliferation, modulate the biochemical markers of differentiation and malignancy, and induce cell morphological changes toward more mature forms of hepatocytes of HepG2 cells. These findings altogether show that the organic and aqueous extraction of *M. alba* L. leaves are of value for further exploration as a potential anticancer agent. The exact mechanism should be further investigated in future studies.

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