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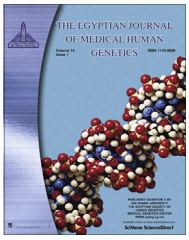

ORIGINAL ARTICLE

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

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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-κB) gene expression, alfa-fetoprotein (AFP), albumin (ALB), gamma-glutamyl transpeptidase (γ-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-κB gene expression of HepG2 cells compared to the control. Also a highly significant depression occurred at the levels of AFP, γ-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-κB gene expression and modulate the biochemical markers.

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

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(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), Amphotircin B (Lonza Bio Whittaker), Cell culture medium; RPMI 1640 (Lonza Bio Whittaker).

2.2. Methods

2.2.1. Prepration 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 (13,000*g*, 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_2.

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 eppindorffs 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*