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Evaluation of Anticancer Activity of Extracted Flavonoids from Morus Alba Leaves and its interaction with DNA

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ABSTRACT

Morous Alba, known as white mulberry contains many oxidative flavonoids, widely used in the treatment of many diseases like hyperglycemia, inflammation, fever and cancer. In the present study we investigated the interaction of extracted flavonoids from Iranian Morus Alba leaves with DNA as a main target for anticancer drugs. Various spectroscopic techniques (UV/Vis, CD and Fluorescence Spectroscopy) were used to detect the interaction. In vivo studies also were done to confirm the effectiveness of the extracted flavonoids. The spectroscopic results showed that the extracted flavonoids bind to DNA especially to the sugar-phosphate backbone and making DNA conformational changes upon this binding. Experiments on the cancerous mice with solid tumors indicated that the treatment of mice with these extracted flavonoids increased significantly the life span but they did not have any effects on the tumor size reduction. These data suggest that Morus Alba flavonoids may use as an effective natural anticancer drug in the near future.

Key words: Flavonoids; anticancer activity; DNA interaction; Morus Alba

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INTRODUCTION

In last years, research on medicinal plants has been increased. Mulberry plant is one of the traditional herbs used as medicine in China and India. Morus Alba, known as white mulberry is reported to contain many anti oxidative flavonoid compounds. It has also hypoglycemic, hypolipidemic and antioxidant effects. Its leaves show valuable effects because of the presence of steroids, flavonoids, amino acids, vitamins and other trace elements. The leaves have been used for years in china to treat hyperglycemia, inflammation, cough, hypertension, cancer and fever [1-3]. The data indicate that flavonoids that are present in the leaves possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antiviral, antimicrobial and anticancer activities [4-6].

Similarly, anticancer drugs like anthracyclines and their derivatives have also phenolic rings similar to flavonoids. These drugs enter the cell nucleus and bind chromosomal DNA. Intercalation between DNA base pairs leading to inhibition of DNA and RNA synthesis was the first mechanism described for cytotoxicity of anthracyclines [7,8]. As Morus Alba leaves contain flavonoids that are resemble structurally to anthracyclines, chromosomal DNA may be the target of extracted flavonoids too. Therefore, in this research interaction of flavonoids extracted from Iranian Morus Alba (FIMA) leaves with DNA was studied in order to understand the exact mechanism of Morus Alba flavonoids action. The results provide evidence that FIMA binds to backbone of chromosomal DNA and making DNA conformational changes. Also *in vivo* studies showed that FIMA treatment increases the life span of cancerous mice.

MATERIALS AND METHODS

Extraction and purification of flavonoids from mulberry leaves

A modified method of Chen was used for extraction and determination of flavonoids [9]. Dried leaves from Iranian Morus alba were used for extraction of total flavonoids. Powdered material was extracted twice with 70% ethanol solution at 90°C for 2h. After filtration and centrifugation (3000 rpm, 15 min), the solvent was evaporated and the aqueous extract was condensed under reduced pressure. 1 ml of the solution containing flavonoids was mixed with 0.7 ml of 5% (w/w) NaNO2, 10 ml of 30% (v/v) ethanol and 0.7 ml of 10% AlCl3 (w/w) and the mixture was then stirred for 6 min. Then, 5 ml of 1 mol/l NaOH was added. Finally, the NKA-9 macroporous resins were chosen for purification of the separated flavonoids from the crude extract. The purified flavonoids were diluted to 1.5 mg/ml and stored at 4°C before use.

UV/Vis spectroscopy

Calf thymus DNA (50 μg/ml) was dissolved in 10 mM Tris-HCl (pH=7.4) and incubated with different concentrations of extracted flavonoids for 45 min at room temperature. Control samples containing equal volumes of calf thymus DNA in the same buffer were incubated along with the flavonoids treated samples under the same condition. The treated and control samples were then subjected to spectroscopic analysis using Shimadzo UV-160 spectrophotometer. The wavelengths of 210 and 260 nm were selected to detect the extracted flavonoids effect on DNA. Each experiment was repeated at least three times.

Fluorescence Spectroscopy

The measurements were performed on Carry Eclipse fluorescence spectrophotometer. All samples were made in 10 mM Tris-HCl (pH=7.4) and quartz fluorescence cell of 1 cm path length was used in the experiment. The spectra were recorded between 350-400 nm after excitation at 370 nm. Each experiment was repeated at least three times.

Circular dichroism experiment

Circular dichroism (CD) experiment was performed using CD spectrometer model 215 (Aviv instrument INC). The far-UV CD spectra of DNA in 10 mM Tris-HCl (pH=7.4) and in the absence and presence of various concentrations of extracted flavonoids were recorded in the range of 220-300 nm with a spectral resolution of 1 nm. The scan speed was 20 nm/min and the response time was 0.33030 sec with a band width of 1 nm. Quartz cell with a path length of 10 mm was used and all measurements were carried out at 25°C. Results are expressed as molar ellipticity expressed as [θ], in $deg \times cm^2 \times dmol^{-1}$.

***In vivo* studies**

A LL2 cell line (derived from transplantable murine Lewis lung carcinoma) was grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 2mM glutamine, 100μg/ml streptomycin and 100 IU/ml penicillin. Ten normal female BALB/c mice (inbred, 20–30 gr) that were 8–10 weeks old selected for tumor transplantation. The animals were received from the Pasteur Institute (Tehran, Iran) maintained under standard temperature (22±0.5 °C) and light in conditions (12-h light/12-h darkness) with free access to food and water. All experiments were executed in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Ethics Committee - Iran University of Medical Sciences (IR.IUMS.REC 2015/12460).

The lung tumor xenograft was established by subcutaneous injection of two million LL2 cells (in PBS) in the right flank region of the mice. The therapeutic studies were performed when xenografts volume reached 50 mm3 in all mice. Then FIMA treatment was done in 5 cancerous mice and intravenous injection of 50 mg/mL of FIMA was done via the tail vein. 5 mice were used as controls and injected with phosphate buffer saline (PBS). Injection was repeated in every 3 days. The size of the tumors was measured in 3 dimensions every 3 days with an electronic slide caliper until the tumor volumes exceeded 1000 mm (9th day). Tumor volume was calculated as:

$$V = \frac{4}{3}\pi \times r_1 \times r_2 \times r_3,$$

Where r_1, r_2, and r_3 are tumor radii in 3 dimensions, expressed in cubic millimeters [10].

RESULTS AND DISCUSSION

FIMA binds to DNA backbone

In recent years many studies have been done to determine the components of Morus Alba leaves; the focus was on the antioxidative and hypoglycemic effects [11,12].

In the present study an experiment was designed using calf thymus DNA incubated in the presence and absence of the various concentrations of FIMA. As is seen in figure 1, at low concentrations of FIMA (up to 30 μg/ml) a slight decrease in the

absorbance at 260 nm is observed but at higher FIMA values the absorbance is remained unchanged. But on the other hand, the absorbance at 210 nm is considerably decreased when FIMA concentrations is increased. As previous data indicate that absorbance changes of DNA spectrum at 210 and 260 nm upon drug binding is due to the binding of the drug to DNA backbone and DNA base pairs respectively [13]; so the results obtained here show that FIMA binds specially to the DNA backbone (sugar and phosphate) instead of binding to DNA base pairs. These findings are in contrast to the interaction of most anthracyclines like daunomycin with DNA showing that they bind chiefly to DNA base pairs instead of DNA backbone [14,15].

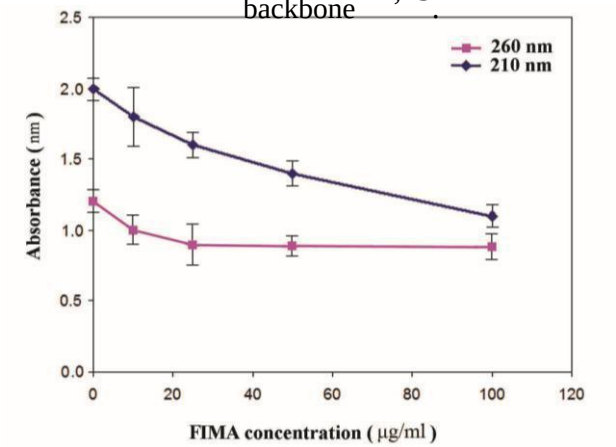

Figure 1: Absorbance changes of calf thymus DNA at 210 (diamond) and 260 nm (square) upon FIMA binding. The reaction was carried out in 10 mM Tris-HCl (pH=7.4) and incubation time was 45 min. Results are average of 3 individual experiments.

FIMA causes hypochromisity in the DNA fluorescence profile

The florescence emission spectra obtained from the interaction of FIMA with calf thymus DNA is also shown in figure 2. DNA, in the absence of FIMA exhibit emission spectrum with a maximum intensity at 375 nm. Addition of FIMA to the DNA solution reduces the fluorescence intensity of the DNA without any red shift in the emission maxima (I_{max}) as FIMA concentration is increased. The reduction in fluorescence emission of DNA is due to quenching of its functional groups. It is important to note that the fluorescence results presented here are very similar to those previously reported for daunomycin and idarubicin as anthracycline drugs. They also induced reduction of fluorescence emission intensity upon binding to DNA [14,16].

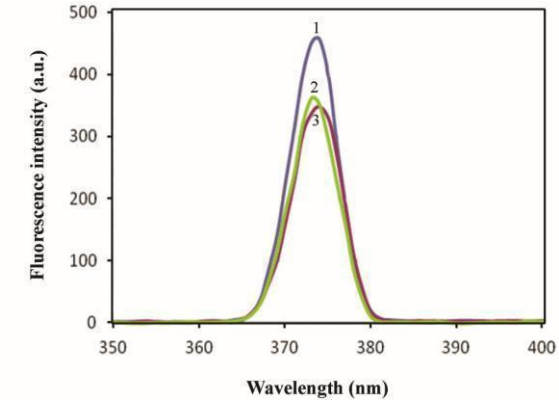

Figure 2: Fluorescence emission spectra of calf thymus DNA in the presence and absence of various concentrations of FIMA. All samples were prepared in10 mM Tris-HCl (pH=7.4). Excitation was at 370 nm. Spectra 1-3 are 0, 50 and 100 µg/ml of FIMA respectively.

FIMA causes DNA conformational changes

To obtain further information about the binding of FIMA to DNA, we compared the CD spectrum of DNA in the presence and absence of FIMA. As shown in figure 3, DNA displays a CD spectrum with negative and positive extremes at 245 and 275 nm respectively. FIMA treatment caused a gradual decrease in the intensity of the peaks, which implies the conformational changes of DNA upon FIMA binding. These results are in agreement with CD reports for anthracycline drugs; they alter the structure of DNA too [14].

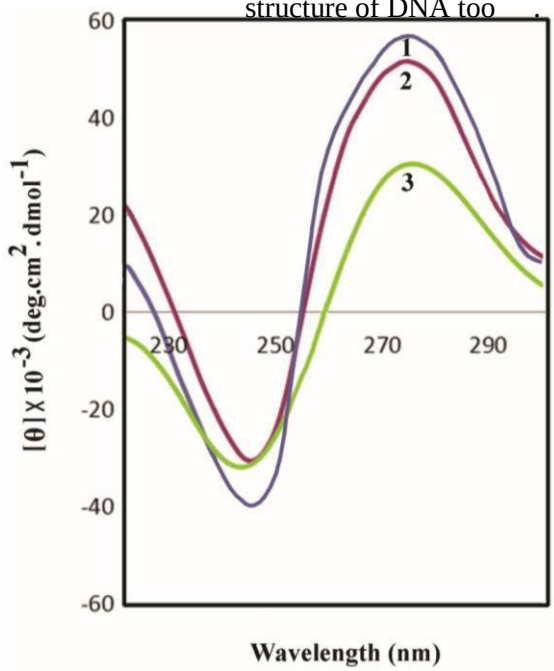

Figure 3: Far UV CD spectra of DNA in10 mM Tris-HCl (pH=7.4) in the absence and presence of FIMA. 1-3 are 0, 50 and 100 µg/ml of FIMA respectively.

FIMA treatment increases the life span of cancerous mice

In this study, we measured the size of tumor in two groups (control and treated mice) until the tumor volumes exceeded 1000 mm as described in material and methods section. As it can be seen from figure 4, the size of tumor in two groups is increased, so in the ninth day, the tumor volume average in the control and in the treated group were 925 mm^3 and 753 mm^3 respectively. Of course the growth of tumors in the mice continued after ninth day. The data shows that FIMA treatment does not stop or slow down tumor growth because mean volume tumor in 2 groups did not show any meaningful differences; the calculated *p value* was also greater than 0.05.

Also the survival rate of mice was calculated and summarized in figure 5. As shown in the figure, the drug (FIMA) has significant impact on the survival rate of cancerous mice (the calculated *p value* was less than 0.05) because the control mice that did not receive any FIMA could survive up to 9 days, but the treated mice could survive up to 26 days (three times more). Furthermore, treated mice have 100% viability up to 17 days but control group has this amount of viability up to 3 days.

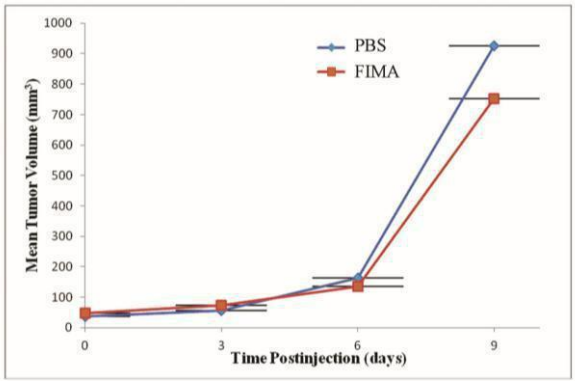

Figure 4: Comparison of the mean tumor volume in treatment and control groups. Each group consists of 5 mice.

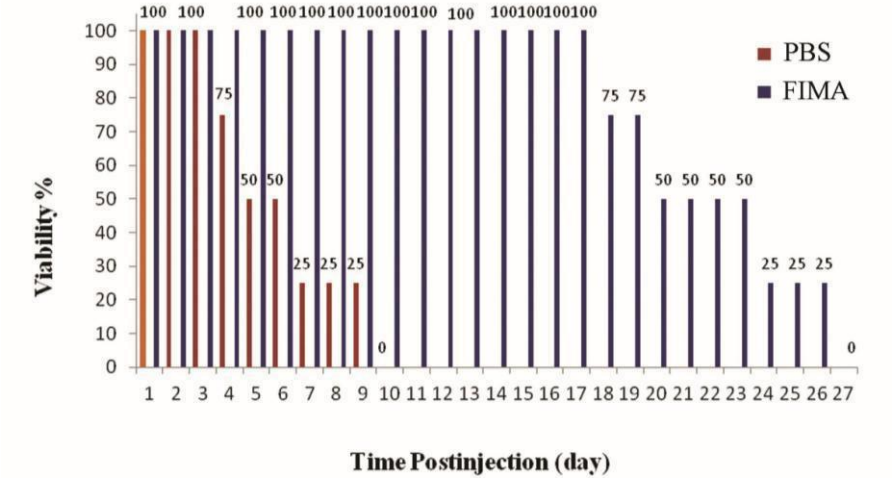

Figure 5: Survival rate of control and treated mice.

CONCLUSIONS

The data presented here suggest that FIMA binds to DNA backbone as absorbance reduction at 210 nm shows. Also FIMA causes DNA conformational changes upon binding as fluorescence intensity reduction of the DNA and gradual decrease in the intensity of the DNA CD peaks show. Also treatment of cancerous mice with FIMA has a significant impact on the survival rate; despite it does not stop or slow down the tumor growth. It is suggested that further studies has to be done in order to understand the exact FIMA mechanism of action. Also FIMA can be used in combination with other anticancer drugs to increase the life span of patients in the near future.

ABBREVIATIONS

FIMA: flavonoids extracted from Iranian Morus Alba; CD: Circular dichroism

COMPETING INTERESTS

The authors declare that they have no competing interests.

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