

Polyphenolic Profiles and Cytotoxic Effect of Iraqi *Morus alba* leaves Ethyl Acetate Extract

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Morus alba, member of the Moraceae family, is a perennial tree utilized in folk medicine, preparing the modern drug, and considered the main food for silkworms. However, data on chemical content in the leaves is still limited; the main objective of this study is to detect the presence and determine the concentration of different polyphenolic constituents in the leaves of the *Morus alba* plant by reverse phase-high performance liquid chromatography (RP-HPLC) and evaluate the cytotoxic effect of ethyl acetate extract of this plant on human breast cancer (AMJ-13) cell line. Phytochemical analysis of the *Morus alba* leaves ethyl acetate extract led to identifying and quantification of six polyphenolic constituents designated as phenolic acids (caffeic, chlorogenic, and p-coumaric acid), catechins (epicatechin) and flavonoids (luteolin and apigenin) in which p-coumaric acid exhibited the highest concentration followed by luteolin (identified for the first time), chlorogenic acid, caffeic acid, apigenin and epicatechin as the least. According to estimates, the ethyl acetate extract with a high concentration of polyphenolic constituents gave the best findings as cytotoxic against breast cancer AMJ-13 cell line with an IC₅₀ value of 129.5 µg/ml.

Keywords: AMJ-13 cell line; Catechins; flavonoids; HPLC; Phenolic acids.

Morus alba L. (of family Moraceae) is a deciduous, monoecious tree of moderate size with 1.8 meters in width and 3 meters in height¹. It is native to Japan, China, and India and is sometimes distributed in other places like North America, Europe, and Africa. It is commonly named white mulberry. *Morus alba* is distributed where silkworms are elevated throughout the world. Its leaf is the main food for silkworms². The leaves of the mulberry are used as powdered juice and tea in Japan. White mulberry is utilized in various parts of the world as a vegetable and is also grown for fruit production in European countries¹. The root bark,

fruit, and leaf of white mulberry have a prolonged history in conventional medicine of Chinese. Its root bark is an expectorant, anti-inflammatory, antitussive, and diuretic agent. The mulberry fruit act as an analgesic and tonic agent. The mulberry leaves can be used for antitussive, pyrolysis treatment, blood cooling, and improvement of eye problems³. As it is rich in minerals and contains metabolizable energy, protein, and small anti-nutritional agents such as tannic acid, mulberry leaves have been considered an alternative source of protein for poultry production. White mulberry is an excellent source of β -carotene, ascorbic acid, and antioxidant compounds like

rutin⁴. Also, this plant contains several active phytochemical compounds such as flavonoids, phytosterols, tannins, sitosterols, triterpenes, saponins, benzofuran derivatives, anthocyanins, anthraquinones glycosides, oleanolic acid, and others⁵. In addition to the nutritive value, the leaves of the mulberry are safe and normal medicinal agents reported to have antimicrobial, antidiabetic, antimutagenic, anticancer, antioxidant, anxiolytic, antistress, anthelmintic, immunomodulatory, nephroprotective, hypocholesterolemic, hepatoprotective⁶⁻⁸, hypouricemic⁹, neuroprotective¹⁰, anti-inflammatory¹¹, and cardioprotective actions¹². To best knowledge, few reports have been published on *Morus alba*, so it sparked our interest in conducting scientific research on the polyphenolic composition and cytotoxic activity of Iraqi *Morus alba* ethyl acetate leaves extract.

MATERIALS AND METHODS

Plant material

Morus alba leaves (Family Moraceae) were cultivated and assembled in Baghdad, Iraq. The plant leaves were washed with tap water (H₂O), dehydrated in shadow (at room temperature), and then ground as powder by mortar and pestle.

Preparation of *Morus alba* extract

Extraction was made with some modifications as process mentioned by Iswantini *et al.* (14). Powder leaves of *Morus alba* (25 g) were macerated with 250 ml of n-hexane for one day and then filtrated. The plant materials left were extracted with 250 ml of aqueous ethanol (ethanol-water 85:15 v/v) by soxhlet apparatus for 12 hrs. The crude extract was filtered and reduced in volumes under vacuum to obtain a dry residue. The dried extract was hanging in the water and fractionated with ethyl acetate (100 ml x 2), as shown in figure 2. The ethyl acetate extract was dried under vacuum to give 0.408 gm, and the percentage yield was 1.632% and then stored for further examination.

Qualitative and Quantitative determination of phenolic compounds (flavonoid, phenolic acid, and catechin) by RP-HPLC

RP-HPLC technique is used in the identification, and quantitative estimation of phenolic acids (caffeic, chlorogenic, and p-coumaric

acid), catechins (epicatechin), and flavonoids (luteolin and apigenin) in the *Morus alba* ethyl acetate extract of leaf part, in which using a mobile phase (Isocratic) consist of acetonitrile: water (1:1). The C18 Column (250 mm x 4.6 mm, with particle size 5 μ m) was utilized as stationary phase. The 20 μ l injection volume and 1.0 ml/min flow rate were used, and the injection concentration was 1 mg/ml. The UV detection was performed at 265 nm (15).

Cytotoxic evaluation of the ethyl acetate extract of *Morus alba*

To evaluate the cytotoxic effect of *Morus alba* ethyl acetate extract of leaf part, breast cancer cell (AMJ13) was taken from an Iraqi patient with breast cancer and maintained in RPMI-1640, supplemented with penicillin (100 unit/mL), streptomycin (100 μ g/mL), and fetal bovine (10%). The cells were passaged using Trypsin-EDTA, incubated at 37 °C, and reseed twice per 7 days at 50% confluence¹⁶.

On 96-well plates, the MTT test was performed, and at 1×10^4 cells per well, the cell line was seeded¹⁷. Cells were treated with the serial concentrations of ethyl acetate fraction (tested compound) when a confluent monolayer was done (after 24 hrs.). After treatment with 72 hrs, the viability of the cell was determined through the elimination of the intermedia, a solution of MTT (28 μ L) was inserted, and the cells were incubated for about 1.5 hrs. at 37 °C. After the solution of MTT was eliminated, the residual crystal was dissolved in the wells through an addendum of Dimethyl Sulphoxide (130 μ L), then incubated for about 15 min (at 37 °C) with stirring¹⁸. The test was done in triplicate, and the absorbance



Fig. 1. Photo of Iraqi *Morus alba* plant

was computed at 492 nm (wavelength of the test) on a microplate reader. The cell growth inhibitory rate (the cytotoxicity percentage) was determined according to the next equation¹⁹:

$$\% \text{ Cell viability} = (\text{absorbance of treated cell} / \text{Absorbance of non-treated cell}) \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \text{cell viability}$$

Statistical analysis

Data obtained statistically utilizing the unpaired T-test were analyzed with Graph Pad

Prism 6. For the triplicate measurements, values were given as mean \pm SD⁽²⁰⁾.

RESULTS AND DISCUSSION

Qualitative and Quantitative determination of phenolic compounds by RP-HPLC

The qualitative estimation of phenolic acids, flavonoids, and catechins in the *Morus alba* ethyl acetate extract by RP-HPLC was made

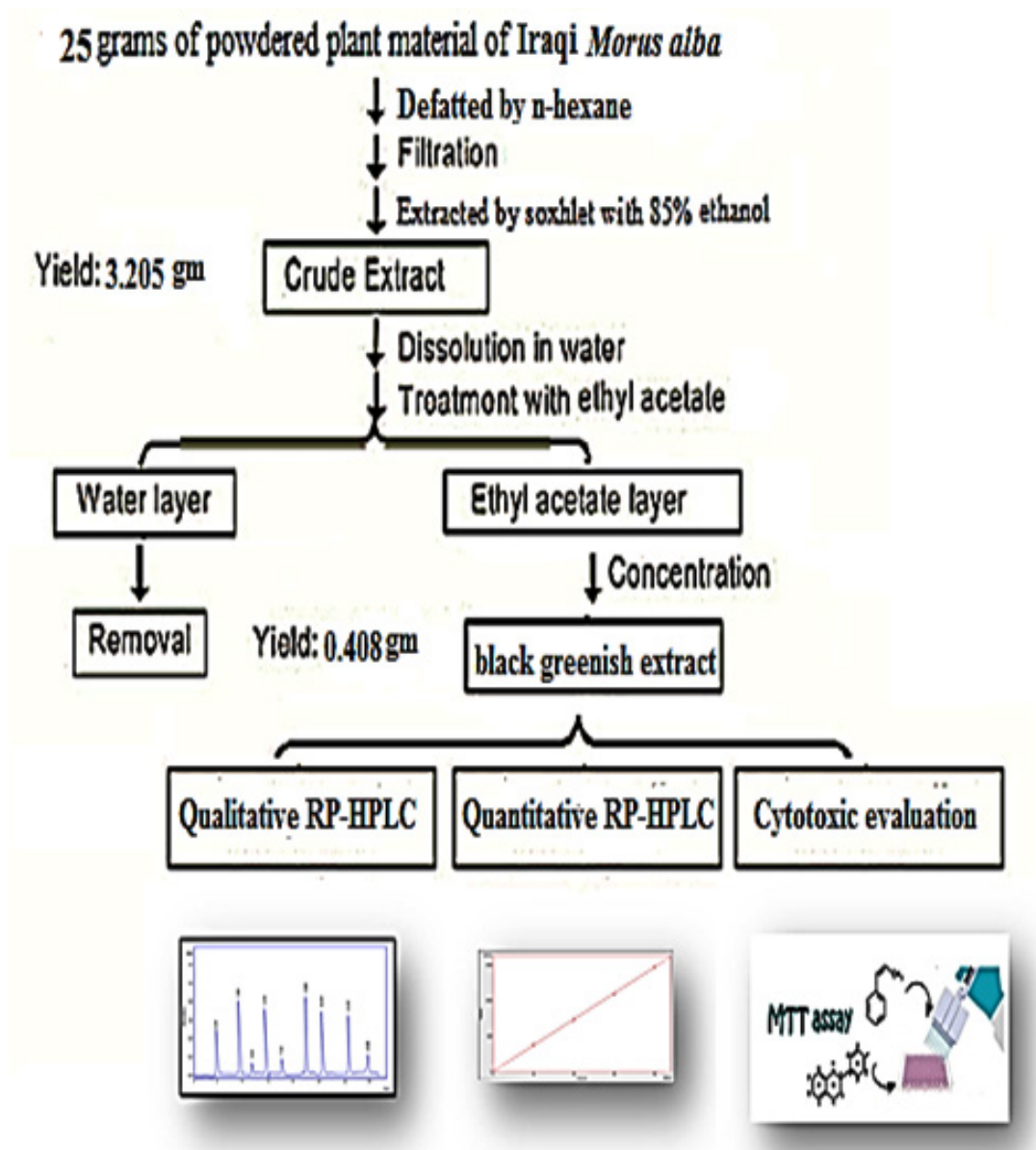


Fig. 2. Scheme of extraction, polyphenolic determination, and cytotoxic evaluation of Iraqi *Morus alba*

Table 1. Concentration (ppm) of two flavonoids, three phenolic acids, and one catechin in *Morus alba* leaves ethyl acetate extract

Active constituents	Concentration (ppm)
Caffeic Acid	942.31
P-Coumaric Acid	1530.29
Chlorogenic Acid	1025.36
Apigenin	494.59
Luteolin	1142.94
Epicatechin	484.21

by matching the retention time of six standards (caffeic, chlorogenic, and p-coumaric acid, apigenin, luteolin, and epicatechin) with plant extract at identical chromatographic condition.

The results of RP-HPLC revealed the existence of phenolic acids (caffeic, chlorogenic, and p-coumaric acid), catechins (epicatechin), and flavonoids (luteolin and apigenin) in the plant leaves; in which the retention time of these detected compounds in the plant extract was identical with their standards, as shown in figures 3-9.

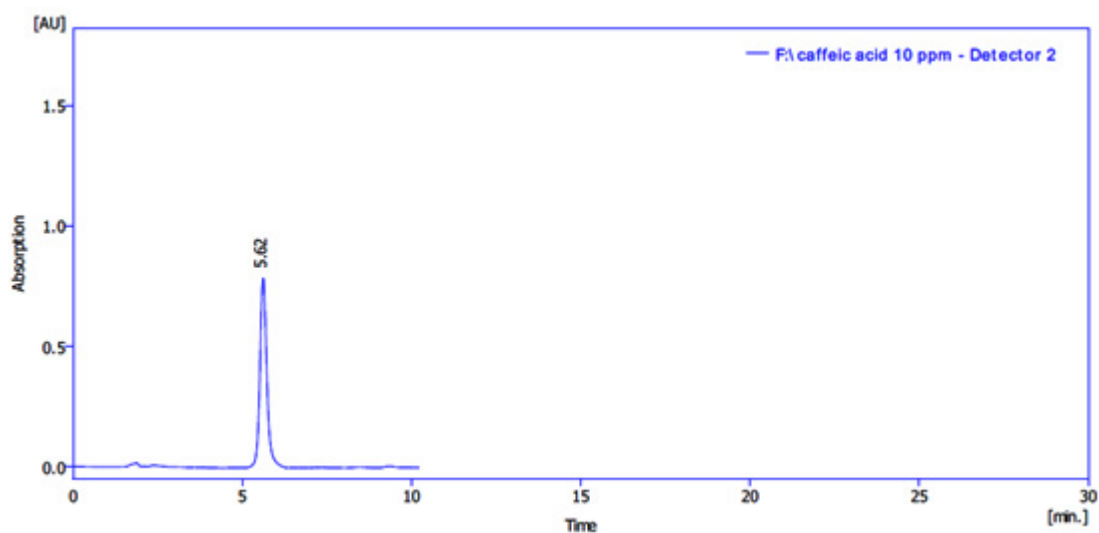


Fig. 3. HPLC chromatogram of std. caffeic acid

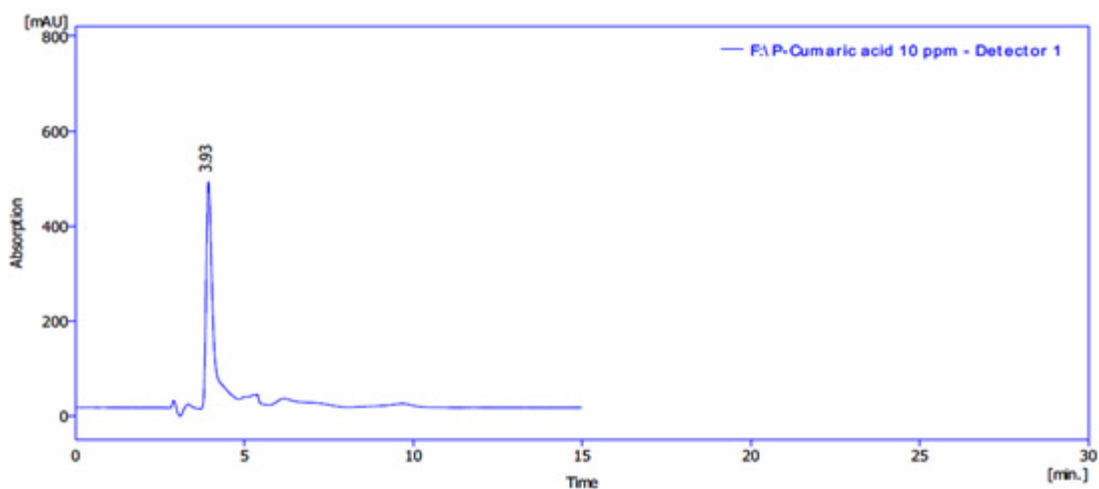


Fig. 4. HPLC chromatogram of std. p-coumaric acid

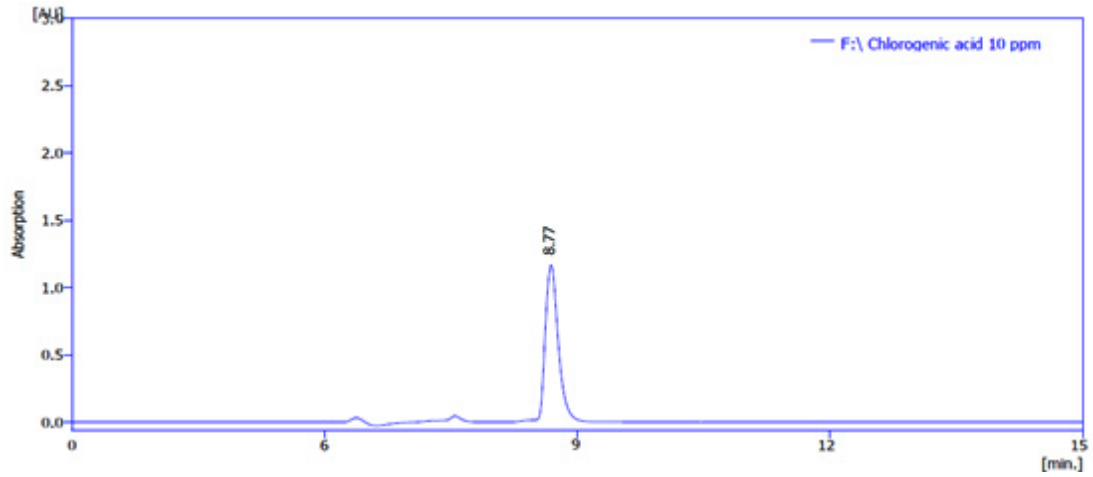


Fig. 5. HPLC chromatogram of std. chlorogenic acid

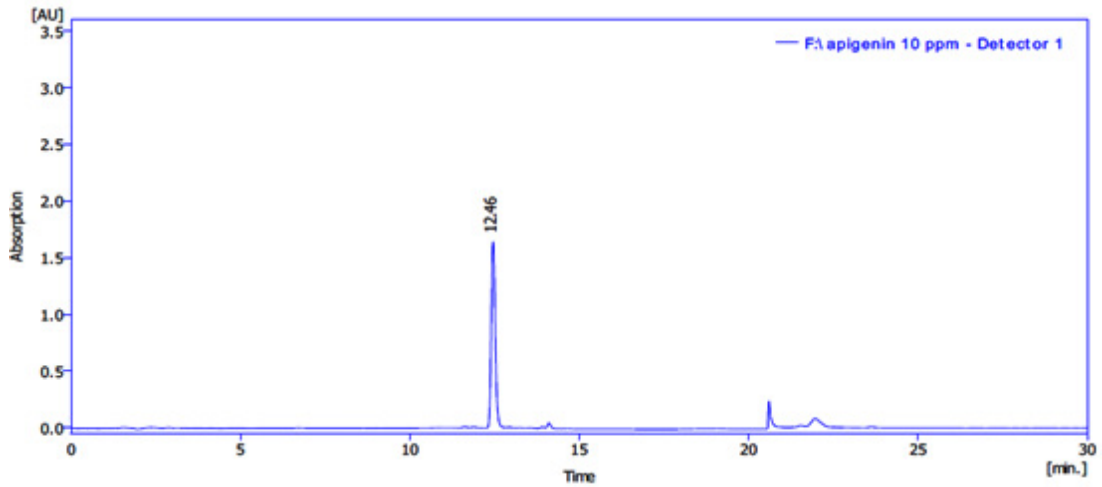


Fig. 6. HPLC chromatogram of std. apigenin

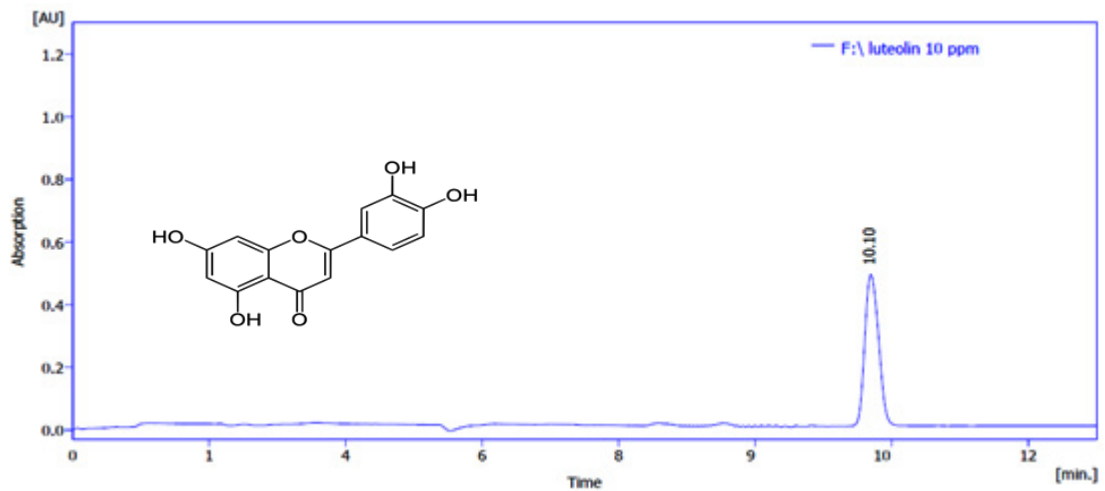


Fig. 7. HPLC chromatogram of std. Luteolin

The quantitative estimation of caffeic, chlorogenic, and p-coumaric acid, apigenin, luteolin, and epicatechin were achieved by utilizing a calibration chart, which a series of diluted solutions of each standard was constructed from 100 ppm as stock solution. The concentrations of these compounds in the *Morus alba* ethyl acetate extract were measured by a straight-line equation obtained after plotting the concentration of serial dilutions of each standard versus the area under the curve, as shown in figures 10-15.

The results show that the concentration of p-coumaric acid was higher than the concentration of other constituents in the sample (ethyl acetate extract), and the amounts of phenolic acids are

higher than that of flavonoids in *Morus alba* leaves, as shown in table (1).

Cytotoxic assay

The cytotoxic assay was done to investigate the cytotoxicity of *Morus alba* leaves ethyl acetate extract on the human tumor cell line, breast ductal carcinoma AMJ-13 cells, by the MTT test.

The *Morus alba* leaves ethyl acetate extract revealed a cytotoxic effect verse the AMJ-13 cell line with a maximum cytotoxic activity at 1000 $\mu\text{g/mL}$ and a minimum cytotoxic activity at 31.2 $\mu\text{g/mL}$ as shown in figure 16 and table 2. This effect depends on the concentration.

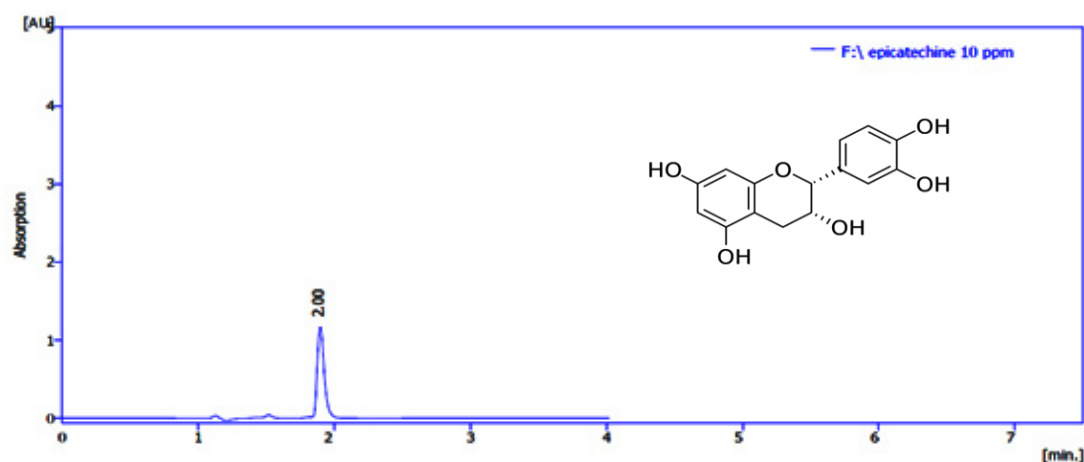


Fig. 8. HPLC chromatogram of std. epicatechin

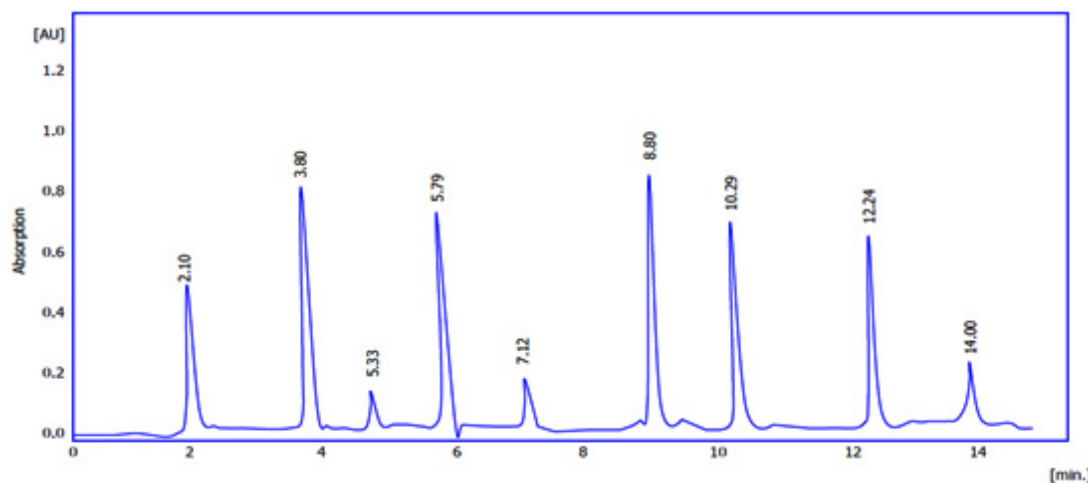


Fig. 9. HPLC chromatogram of *Morus alba* ethyl acetate extract

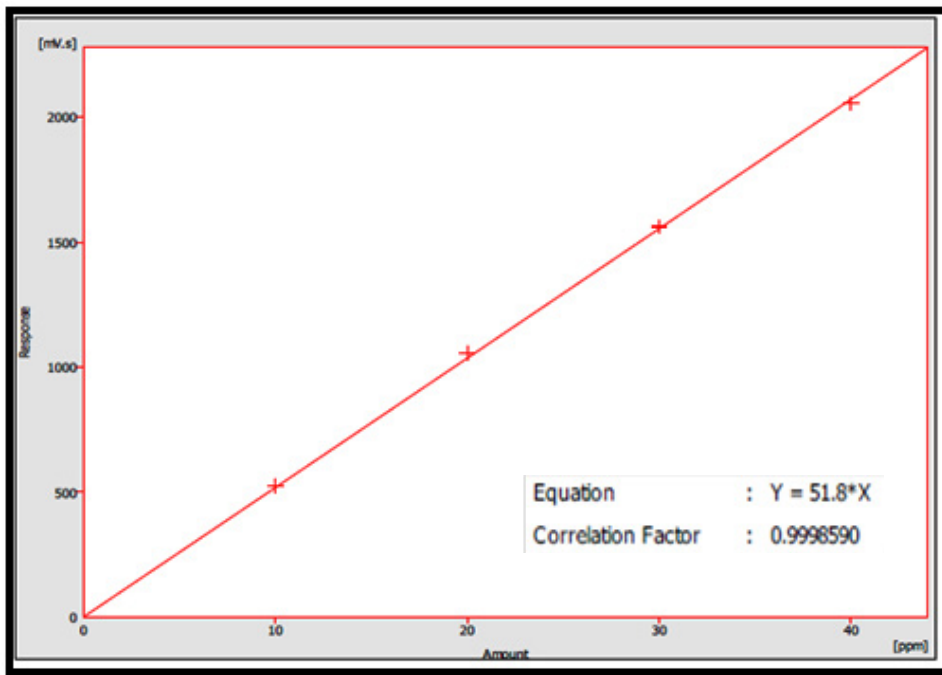


Fig. 10. RP- HPLC calibration curve of epicatechin

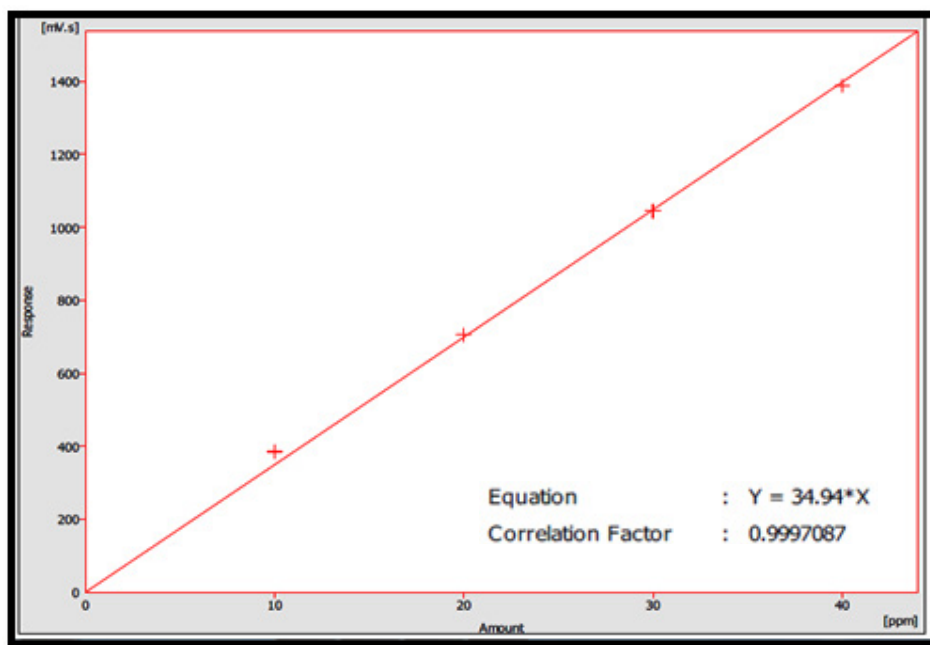


Fig. 11. RP- HPLC calibration curve of p-coumaric acid

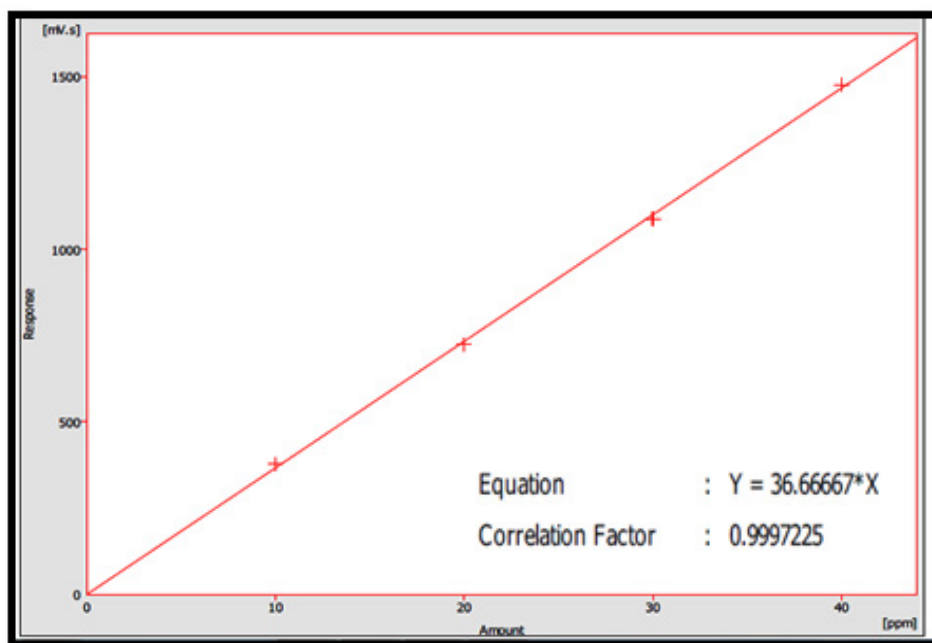


Fig. 12. RP- HPLC calibration curve of caffeic acid

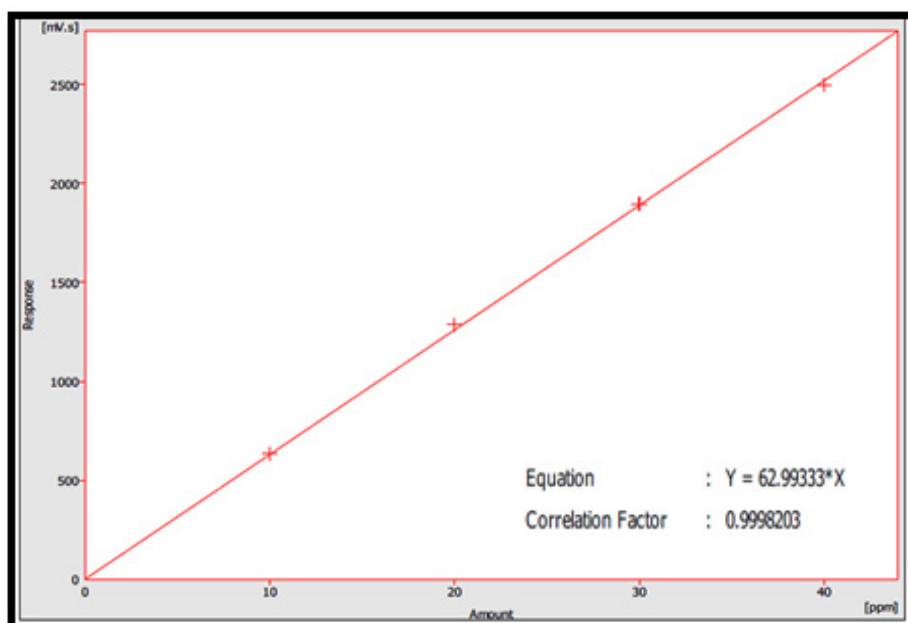


Fig. 13. RP- HPLC calibration curve of chlorogenic acid

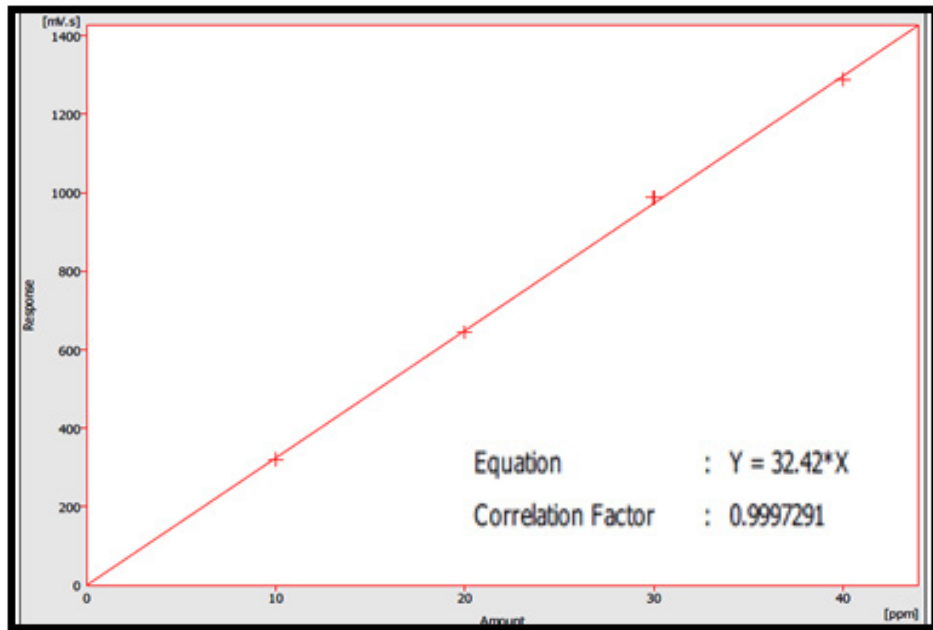


Fig. 14. RP- HPLC calibration curve of luteolin

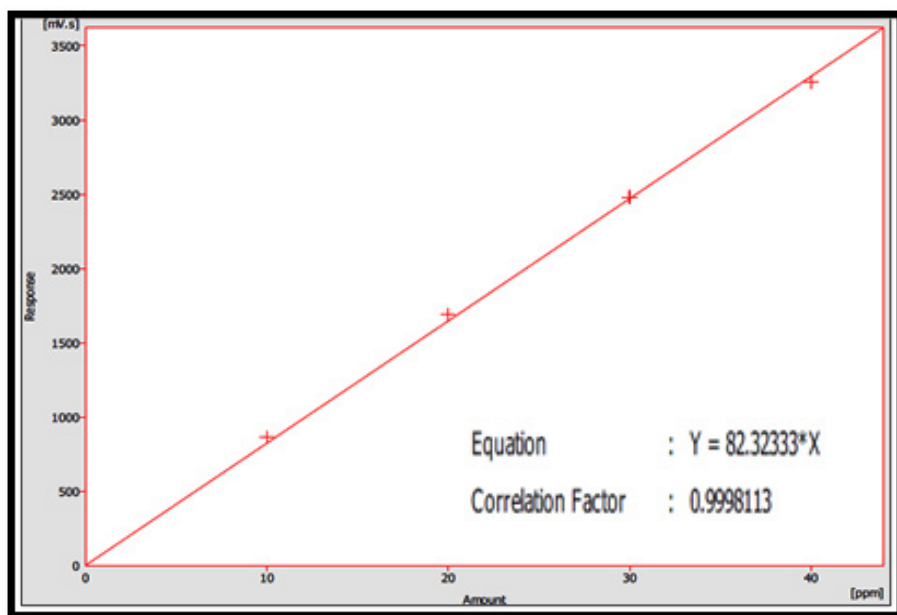


Fig. 15. RP- HPLC calibration curve of apigenin

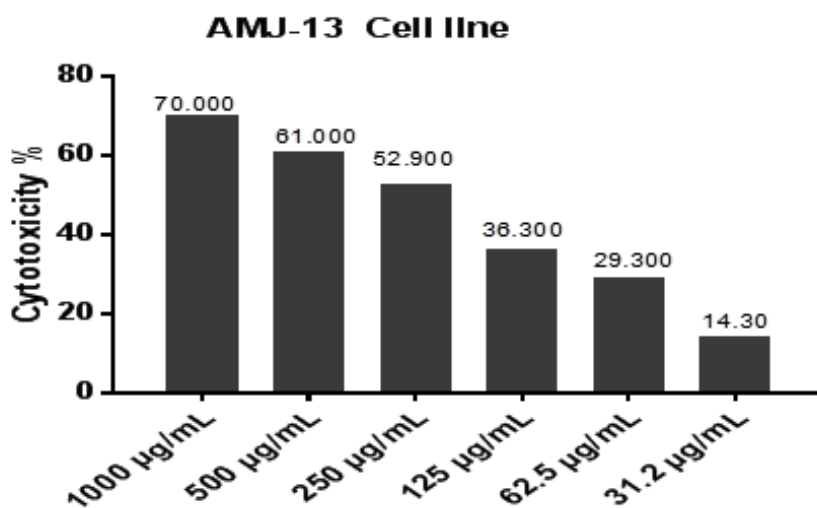


Fig. 16. Cytotoxic effect of *Morus alba* ethyl acetate extract on AMJ13 cell growth

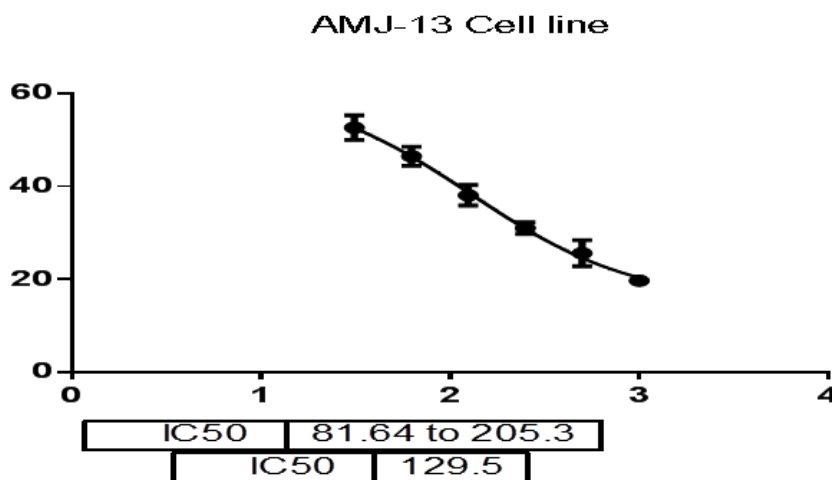


Fig. 17. IC₅₀ of *Morus alba* ethyl acetate extract on AMJ-13 cell line

Table 2. Percentage of cytotoxicity (%) of *Morus alba* ethyl acetate extract at several concentrations

Concentration	1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL	62.5 µg/mL	31.2 µg/mL
% Cytotoxicity	70.00	61.00	52.900	36.300	29.300	14.30

The IC₅₀ stimulating 50% inhibition of cell growth to the tested sample was 129.5 µg/ml (figure 17). AMJ13 cell Morphology after treatment and before treatment with *Morus alba* leaves ethyl acetate extract were observed in figure 18.

The phytochemical investigation revealed the existence of polyphenolic constituents in the *Morus alba* ethyl acetate extract. Phenolic compounds owned free radicals scavenging and antioxidant properties. It has been shown

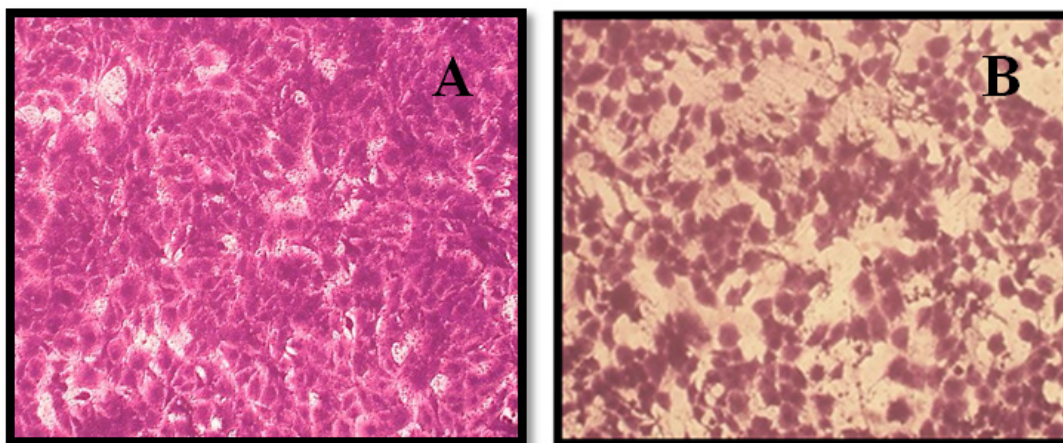


Fig. 18. Morphology of AMJ-13 cell A: before treatment, B: After treatment

anticancer effects affecting several main elements back to apoptosis, cell proliferation, metastasis, and angiogenesis²¹.

CONCLUSION

This study showed that the phytochemical found in leaves extract of Iraqi *Morus alba* could be considered an important source of medicine and vital for good health. In this study, six polyphenolic compounds have been identified, some of them for the first time, in the leaves extract of *Morus alba* by RP-HPLC. The result shows that the RP-HPLC method can be adopted for qualitative and quantitative determinations of phenolic acids, flavonoids, and catechins in *Morus alba* dried leaves extract. In addition, the dried leaves ethyl acetate extract of this plant was active against breast cancer AMJ13 cell line with IC₅₀ value of 129.5 µg/ml, and the good cytotoxic property of white mulberry is mainly related to the existence of polyphenolic compounds and other related compounds.

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Conflict of Interest

There is no conflict of interest.

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