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Original Article

Assessment of Cytotoxic Effect and Phytoconstituents of *Moringa oleifera* Seeds Cultivated in Iraq Against MCF-7 Breast Cancer Cells

Running Title: *Moringa oleifera* Seeds Cytotoxicity

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ABSTRACT

The cytotoxic activity of *Moringa oleifera* (MO) dried seeds against the Michigan Cancer Foundation-7 (MCF-7) cell line was assessed, and its phytoconstituents were screened by reverse-phase high-performance liquid chromatography (RP-HPLC). For this purpose, butanol and ethyl acetate extracts of MO seeds were prepared, and the 3-(4,5-dimethylthiazoline-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on MCF-7 breast cancer cells treated with different concentrations of the ethyl acetate extract (31.25, 62.5, 125, 250, 500, and 1000 µg mL^{-1}). After 72 hours of treatment, cell viability was assessed. The ethyl acetate fraction of MO seeds showed cytotoxic activity with a half-maximal inhibitory concentration (IC_{50}) value of 60 µg mL^{-1} against the MCF-7 cell line. In addition, RP-HPLC analysis showed that caffeic acid, ellagic acid and p-coumaric acid were found in abundance in the butanol fraction, while ferulic acid was found in abundance in the ethyl acetate fraction. Overall, extracts from MO seeds show potential anticancer activity.

Keywords: Michigan Cancer Foundation-7, *Moringa oleifera*, Phenolic compounds, RP-HPLC

INTRODUCTION

Moringa oleifera (MO) is a plant with several different types of organic chemicals, many of which have been used as medicines. They could be used as a model for developing new drugs that work well for many different disorders in the coming years. MO is the most commonly cultivated member of the family, Moringaceae. Moringaceae is a monogeneric family endemic to sub-Himalayan India, Pakistan, Bangladesh, and Afghanistan [1]. MO seeds contain monounsaturated fatty acids and active ingredients such as alkaloids, isothiocyanates (ITCs), thiocarbamates, and glucosinolates (GLs), which give them great potential [2]. They often treat disorders in traditional medicine, such as ulcers, impaired eyesight, and joint discomfort [3]. It has been shown that MO seeds may exhibit antimicrobial, antifungal, antibacterial, and insecticidal activities [4]. Other research reveals the presence of different compounds (about 100 compounds) in the MO extract, among them alkaloids, terpenoids, steroids, saturated and unsaturated fatty acids, aromatic and aliphatic hydrocarbons, and polyphenolic compounds [5]. Additionally, MO seed powder has been shown to successfully protect animals from oxidative stress while also decreasing tissue arsenic contents [6]. Recent research has shown that MO can be used as an anticancer medication. These studies also indicated that this plant extract might suppress cell proliferation, migration, and growth by influencing several cellular signaling pathways [7, 8]. Comparable to selenium's (Se) effect in preventing cancer, MO extract may stimulate cancer death through mitochondria, as shown by research. In vitro studies show that MO extract inhibits the growth of melanoma cells. Both caspase-dependent and -independent apoptotic processes may be triggered by - mediated caspase activation [9, 10]. Also, MO has great promise as a cancer therapy medicine since it may suppress tumor development without interfering with the body's normal physiology or function.

ITC is a natural, tiny chemical generated from glucosinolate precursors in cruciferous vegetables that promotes carcinogen detoxification and inhibits carcinogen activation [11]. The chemical structures of the ITCs that are produced by the biotransformation of MO glucosinolates include a greater amount of sugars, which makes them more stable than other ITCs found in other crops [12]. MIC-1 is an ITC that is especially high in the seeds of MO [13] and is the main phytochemical found in the extraction of MO seeds so far [14, 15]. The study aimed to screen the phytoconstituent of MO and evaluate its cytotoxic effect on MCF-7. A study on MO leaves cultivated in Iraq has identified significant bioactive compounds, such as cryptochlorogenic acid with IC_{50}=20.8 µM against MCF-7 cells, and more than 100 phytochemicals, including phenolics, flavonoids, terpenoids, and alkaloids [16].

Despite extensive research on the general medicinal properties of MO, there is still limited knowledge about the phenolic profiles of its butanol and ethyl acetate fractions and their correlation with anticancer activity, particularly their targeted cytotoxic effects on MCF-7 breast cancer cells. Although MO has been widely studied in different regions, the concentration and diversity of phytochemical composition of seeds cultivated in distinct climatic conditions of Iraq remain largely unexplored. Therefore, this study aims to fill these gaps by evaluating the cytotoxic potential of MO seed extracts cultivated in Iraq and characterizing their phenolic composition to identify active components that may contribute to their anticancer effects.

MATERIAL AND METHODS

Plant Material

Fresh MO seeds were collected from a local farm in Baghdad and identified by Dr. Zainab Abed Aoun, a plant taxonomy specialist at the University of Baghdad (Fig. 1).

Fig. 1 Fresh *Moringa oleifera* pods and seeds

Preparation of Plant Extract

Five hundred grams of fresh MO seeds were washed with water to remove external materials, then air-dried in the shade for several weeks until completely dry. The dried seeds were then ground into a fine powder using an electric blender and weighed for extraction.

Phytochemical Component Extraction Using the Soxhlet Apparatus

First, 50g of powdered MO seeds were soaked in n-hexane for 48 hours to remove the fat, after which they were dried at room temperature. A Soxhlet apparatus was used to extract the defatted plant components. The powder was put in a thimble, and 800 mL of 85% aqueous ethanol was used as a solvent for 15 hours. A rotary evaporator was used to evaporate the solvent at a lower pressure, producing a dry extract that weighed about 11 g. The residue was suspended in 300 mL of water. Then it was partitioned progressively with chloroform, ethyl acetate, and n-butanol (three 300-mL containers each) for each fraction. After being dried over anhydrous sodium sulfate, the residues from the first two fractions were filtered [17] and then evaporated until they were completely dry. Each fraction was given its weight and was afterward allocated for further analysis (Fig. 2).

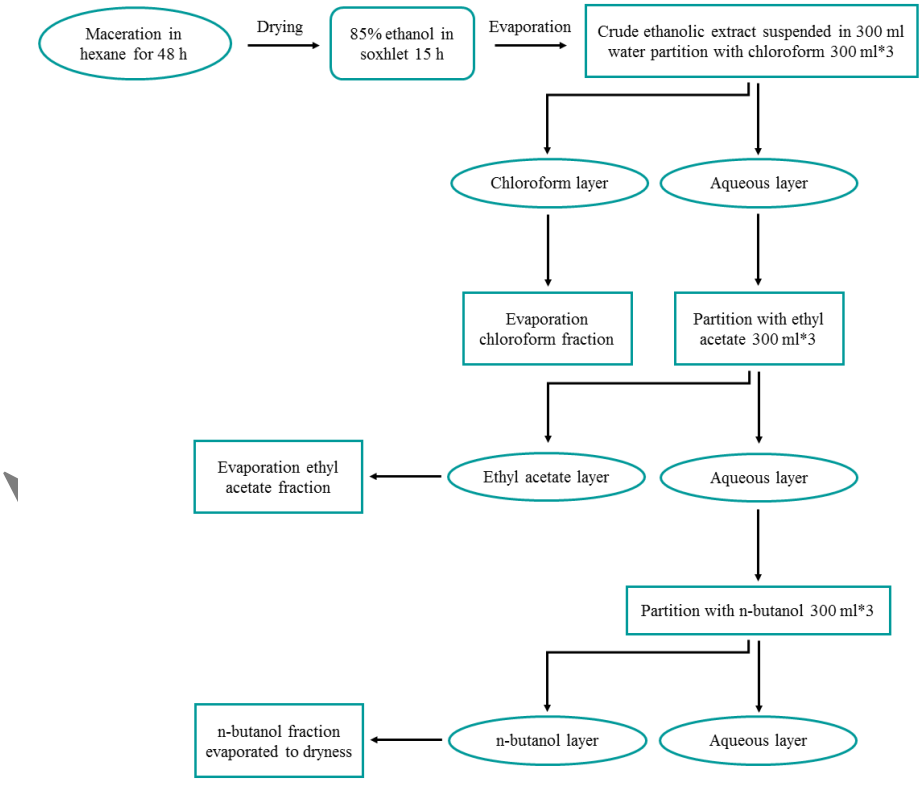


Fig. 2 Extraction scheme of *Moringa oleifera* phytoconstituent cultivated in Iraq

It is acknowledged that solvent partitioning in liquid-liquid extraction is not an absolute process; thus, certain phytochemical compounds, depending on their differential but sometimes partial solubility in multiple solvents, may be distributed between fractions. Therefore, some

phenolic compounds might be detected in more than one fraction due to their varying polarity and affinity, as confirmed by sensitive RP-HPLC analysis.

Phytochemical Screening of MO by Using RP-HPLC

Evaluations were conducted on both qualitative and quantitative scales to identify phytochemicals. That is present in ethyl acetate and butanol fractions of seeds using RP-HPLC at the Ministry of Science and Technology/Environmental and Water Research Department. The qualitative identification was made by comparing the samples' retention times and real standards under certain chromatographic conditions. Based on the standards, four constituents were found. For quantitative measurements, calibration curves were made by plotting the area under the curve (AUC) versus four concentration levels of the standards (x-axis). The concentration of the analyst was found by using an equation for a straight line ($y = MX + c$), where c represents the intersection with the y-axis and m represents the slope of the line.

RP-HPLC Parameters for Ethyl Acetate and Butanol Fractions

RP-HPLC analysis of the ethyl acetate and butanol fractions was performed using a SYKAM LC C18 column (25 cm × 4.6 mm, 5 µm particle size). The mobile phase consisted of methanol (eluent A) and 1% (v/v) formic acid in water (eluent B), with a gradient of 40% B from 0-6 minutes and 50% B from 7-15 minutes. The flow rate was set at 1 mL min^{-1}. Detection was carried out using a UV detector at a wavelength of 280 nm. The injection volume was 50 µL with a sample concentration of 1 mg mL^{-1}. Samples analyzed included the ethyl acetate and butanol fractions of the seeds, and caffeic acid, ellagic acid, ferulic acid, and p-coumaric acid were used as standard compounds.

Chemicals and Reagents Used

The chemicals and reagents used in this experiment are listed in Table 1.

Table 1 The Compounds and reagents utilized in the investigation

No.	Items	Company	Country
1	MTT stain	Bio-World	USA
2	DMSO	Santacruz biotechnology	USA
3	Trypsin/EDTA	Capricorn	Germany
4	Fetal bovine serum	Capricorn	Germany
5	RPMI 1640	Capricorn	Germany

Instruments Used

The instruments used in this experiment are listed in Table 2.

Table 2 The instruments utilized in the investigation

No.	Item	Company	Country
1	Laminar flow hood	K & K Scientific Supplier	Korea
2	Microtiter reader	Gennex Lab	USA
3	Incubator	Cypress Diagnostics	Belgium
4	Micropipette	Cypress Diagnostics	Belgium
5	Cell culture plates	Santa Cruz Biotechnology	USA

Maintenance of Cell Cultures

One often used cell type for cancer research is MCF-7. Cell growth was done using minimum essential medium (MEM) including 10% foetal bovine serum, 100 g mL^{-1} of streptomycin, and 100 U mL^{-1} of penicillin. To accomplish cell passage, cells were reseeded at 50% confluence twice weekly during 37°C incubation using trypsin-EDTA [18].

Combination Cytotoxicity Assays

To assess whether or not the substance was cytotoxic, on 96-well plates, an MTT experiment to determine the vitality of the cells was carried out [19]. The seeding density for cell lines was 1×10^4 cells per well; the drug under investigation was put into contact with the cells and observed for its effects. After twenty-four hours had passed or the creation of a confluent monolayer, following a treatment period of 72 hours, the vitality of the cells was evaluated by taking away the medium first and then adding 28 µL of a solution of MTT at a concentration of 2 mg/mL and incubating the cells for 1.5 hours at 37 degrees Celsius. The crystals in the wells were solubilized by adding 130 µL of DMSO (Dimethyl Sulfoxide) and incubating the plate at 37 °C for 15 minutes while shaking after the MTT solution had been removed [20]. Absorbency was measured using a microplate reader set to 492 nm (test wavelength); the experiment was repeated three times. The formula that was used to assess the level of cytotoxicity (the suppression of cell growth) with the use of correct absorbance is below [21]:

$$\% \text{ Cytotoxicity} = (100 \times (\text{control} - \text{sample}))$$

Statistical Analysis

GraphPad Prism 6 was used for statistical analysis using an unpaired t-test [22]. The mean of three duplicates ± standard deviation was used to represent the values [23]. The mean percentage of inhibition across many treatment doses was compared using a one-way analysis of variance (ANOVA). Regression analysis was applicable to ascertain the value of IC50 since the percent inhibition was less than 0.05, which can be considered significant.

RESULTS

HPLC analysis

The qualitative and quantitative profiling of phenolic compounds in the ethyl acetate and butanol fractions of MO seeds cultivated in Iraq was conducted using HPLC. Retention times of the target compounds—p-coumaric acid, ellagic acid, caffeic acid, and ferulic acid—were compared with those of authentic standards under identical chromatographic conditions to ensure reliable identification. The analysis revealed that p-coumaric acid, ellagic acid, and caffeic acid were significantly more concentrated in the butanol fraction, while ferulic acid was predominantly found in the ethyl acetate fraction (Tables 3 and 4; Fig. 3-5).

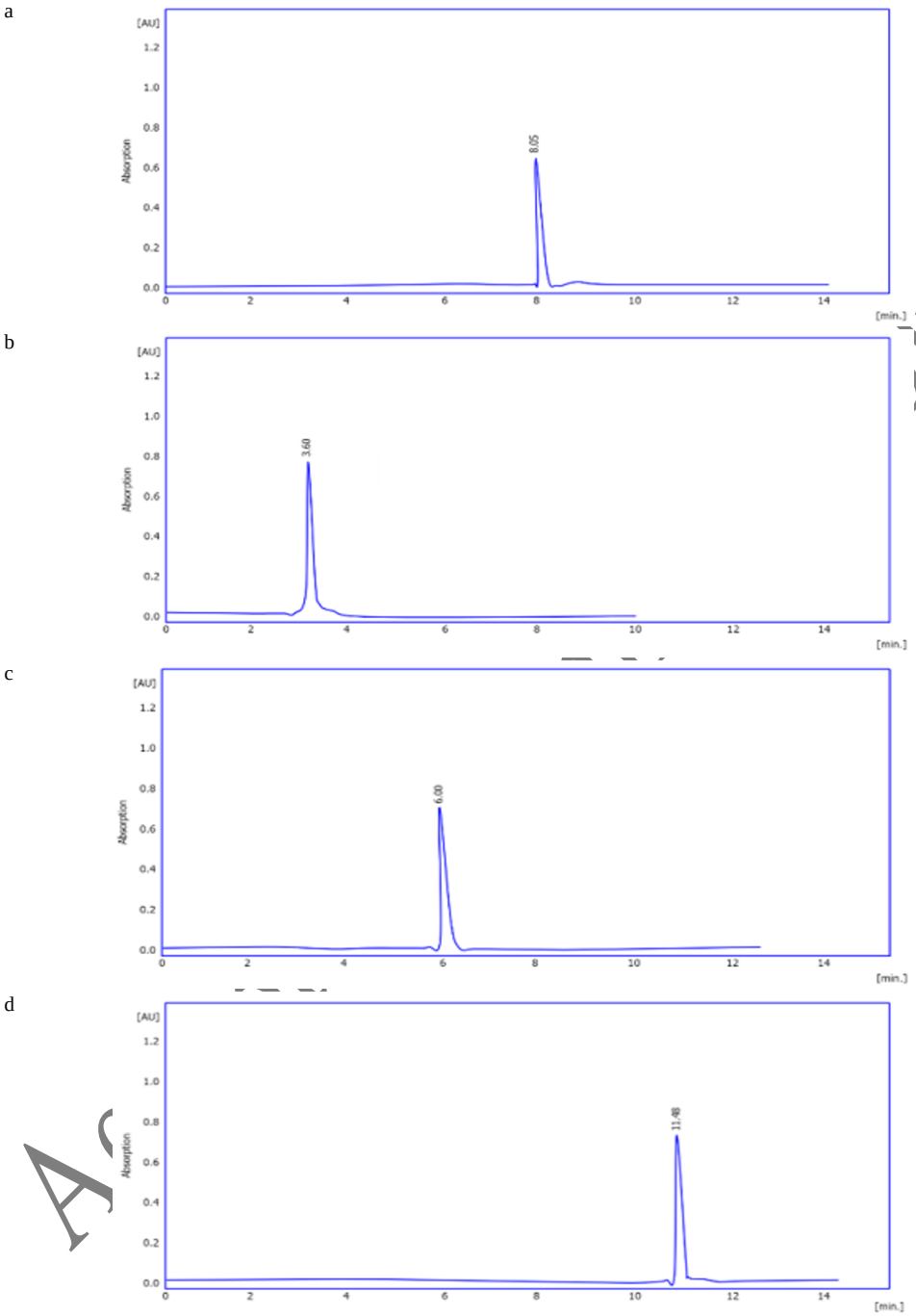


Fig. 3 HPLC chromatogram for a: coumaric acid standard, b: caffeic acid standard, c: ferulic acid standard, and d: ellagic acid standard

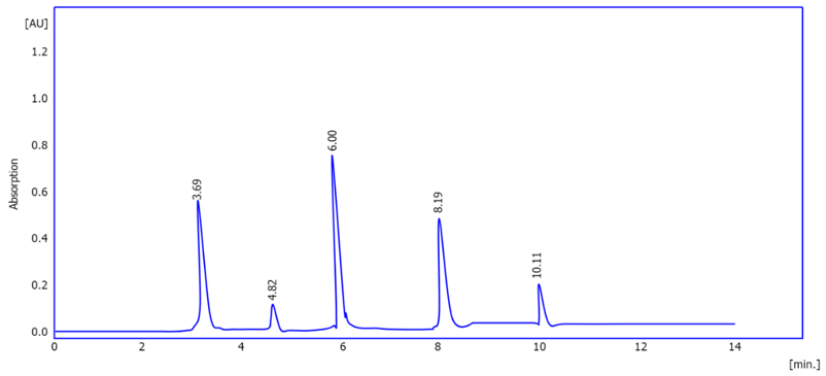


Fig. 4 HPLC chromatogram of ethyl acetate fraction of *Moringa oleifera* seeds

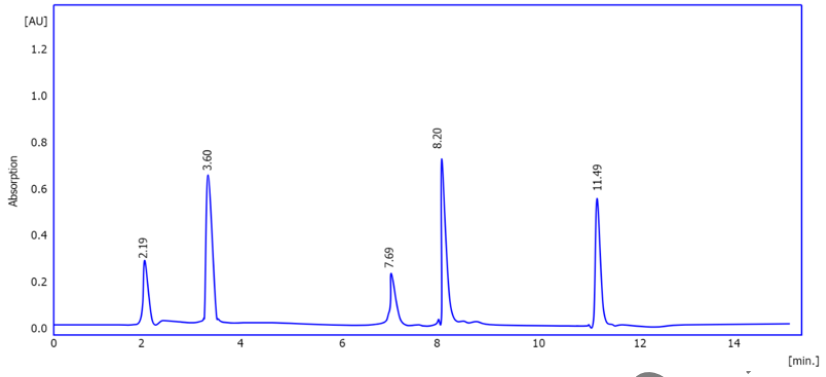


Fig. 5 HPLC chromatogram of *Moringa oleifera* seeds butanol fraction

Table 3 Retention time value of ethyl acetate fractions and butanol fractions of *Moringa oleifera* compared to caffeic acid, ellagic acid, ferulic acid, and p-coumaric acid standards.

Standard name	Rt value of standard	Ethyl acetate fraction	Butanol fraction
Caffeic acid	3.60	3.69	3.60
p-coumaric acid	8.05	8.19	8.20
Ferulic acid	6.00	6.00	7.69
Ellagic acid	11.48	10.11	11.49

Minor shifts in retention times compared to pure standards can occur due to matrix effects, solvent composition, and chromatographic conditions in complex plant extracts. These shifts do not necessarily negate the identity of the compounds but are within acceptable experimental variability for RP-HPLC analysis.
Additionally, some compounds may appear in more than one fraction due to partial solubility overlap and partitioning behavior inherent to the solvents used.

Using a linear equation to graph the area under the curve (AUC) against four concentration levels for every one of the standards—p-coumaric acid, caffeic acid, ellagic acid, and ferulic acid—we could determine the concentration of phenolic phytoconstituents in the ethyl acetate and butanol fractions. This is done through the given calibration curve (Fig. 6-9).

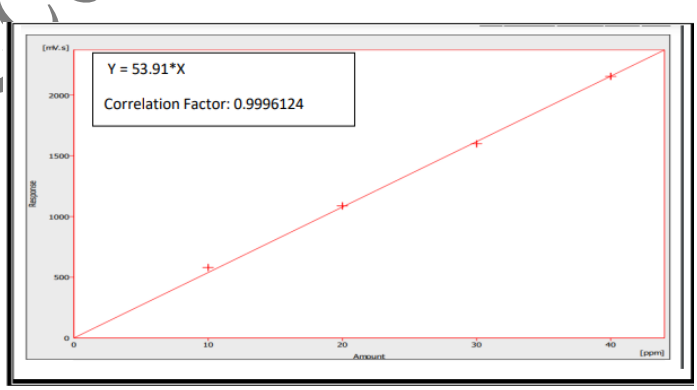


Fig. 6 calibration curve for caffeic acid

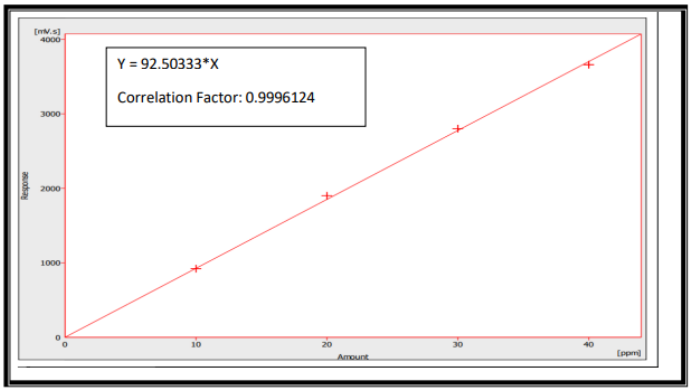


Fig. 7 calibration curve for ferulic acid

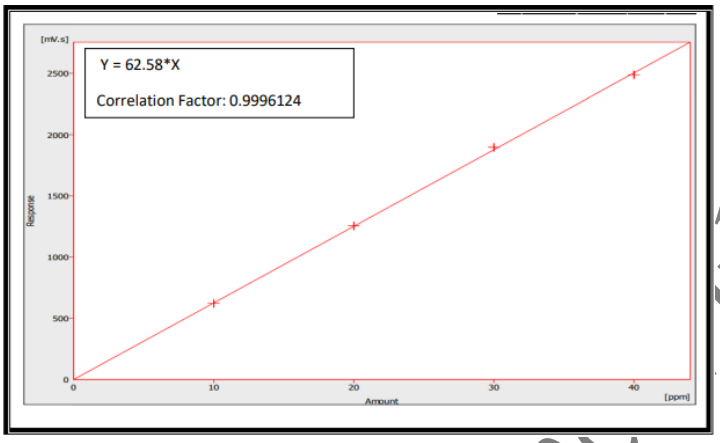


Fig. 8 calibration curve for p-coumaric acid

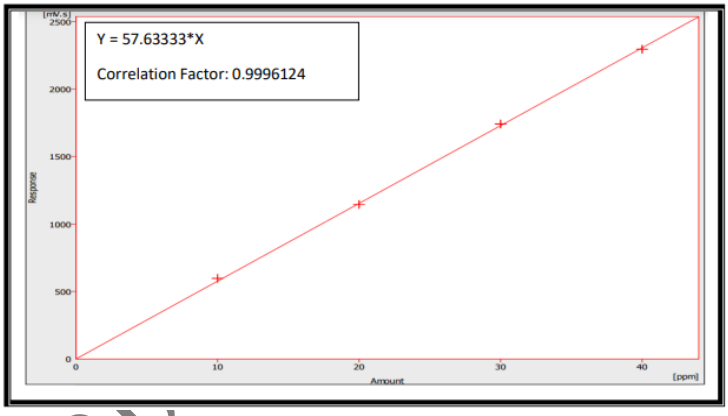


Fig. 9 calibration curve for ellagic acid

Table 4 The kind and quantity of the various phytoconstituents found in Moringa oleifera seeds

Ethyl acetate fraction		Butanol fraction	
Phytoconstituent	Concentration (µg mL^{-1})	Phytoconstituent	Concentration (µg mL^{-1})
Ferulic acid	269	Ferulic acid	92.4
Ellagic acid	62.44	Ellagic acid	442.6
p-coumaric acid	344.4	p-coumaric acid	425
Caffeic acid	368.2	Caffeic acid	554.1

Cytotoxicity

Treatment with increasing concentrations of the ethyl acetate extract of MO seeds resulted in a dose-dependent inhibition of MCF-7 cell proliferation. The IC50 value was calculated to be 60.7 µg mL-1 (Fig. 10). A dose-dependent reduction in cell viability indicates that higher concentrations of the extract led to stronger cytotoxic effects. Further data in Table 5 and Fig. 11 also showed the reverse correlation between extract concentration and MCF-7 cell survival. With increasing the concentration of the MO seed extract (from 1.494 to 3.000 µg mL^{-1}) the mean viability of the candidate cells gradually decreased and standard deviation provided an evidence of good reproducibility between replicates. Fig. 11 illustrates this trend graphically.

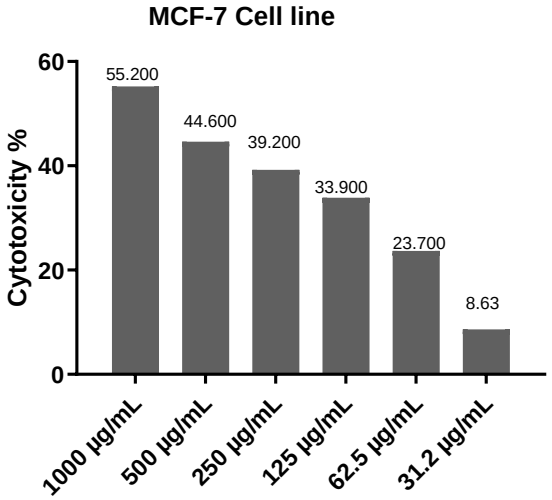


Fig. 10 Cytotoxic effect of *Moringa oleifera* seeds ethyl acetate fraction with gradient concentration on MCF-7 cells

Table 5 Effect of concentration on cell viability

concentration	Cell viability			Mean viability	Standard deviation (SD)
	A:1	A:2	A:3		
	0.815	0.896	0.898		
1.494	0.793	0.793	0.798	0.870	0.045
1.796	0.661	0.673	0.656	0.795	0.003
2.097	0.558	0.598	0.567	0.663	0.008
2.398	0.522	0.511	0.552	0.547	0.016
2.699	0.453	0.498	0.487	0.525	0.026
3.000	0.381	0.393	0.394	0.479	0.025

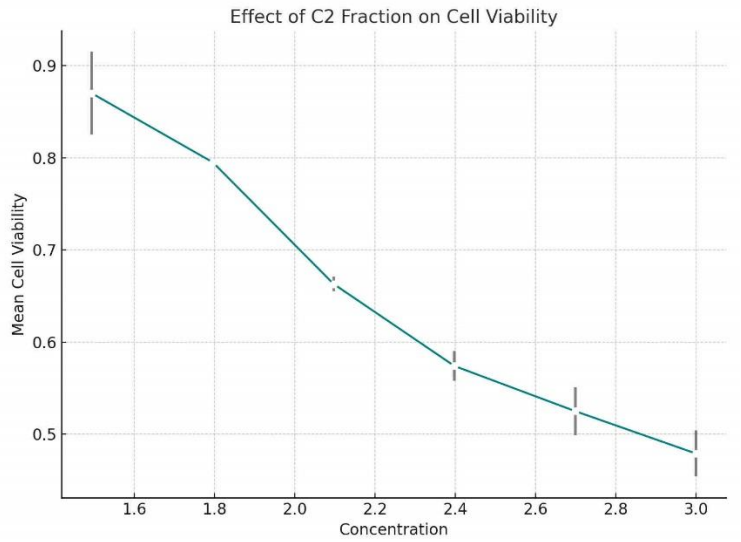


Fig. 11 Effect of concentration on cell viability with error bars

As shown in Fig. 12, the percentage of growth inhibition of MCF-7 cells increased with rising concentrations of the MO seed extracts, supporting the dose-response relationship and highlighting the extract's potential anticancer efficacy. Fig. 13 presents the optical density (OD) values obtained from the MTT assay following treatment of MCF-7 cells with varying concentrations of MO seed extract. A noticeable decline in OD values was recorded as the concentration increased, consistent with decreased cell viability. Fig. 14 shows the effect of MO seed extracts on MCF-7 cells, confirming their significant cytotoxic activity and supporting their potential as a source of bioactive compounds for anticancer therapies.

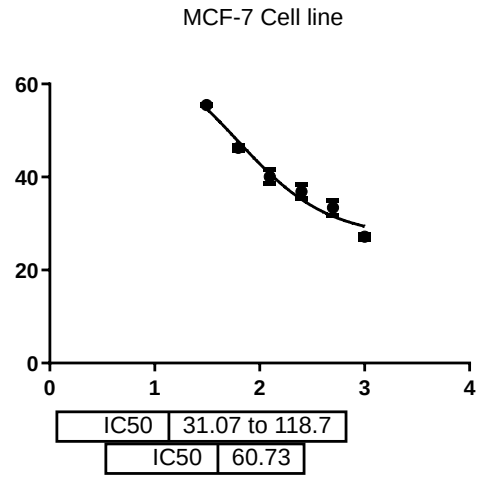


Fig. 12 Inhibition concentration of *Moringa oleifera* seeds on MCF-7 cell line

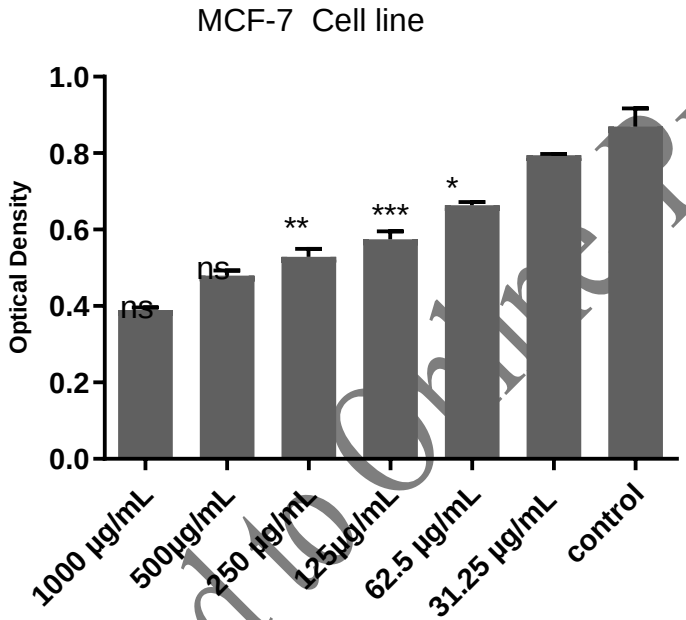
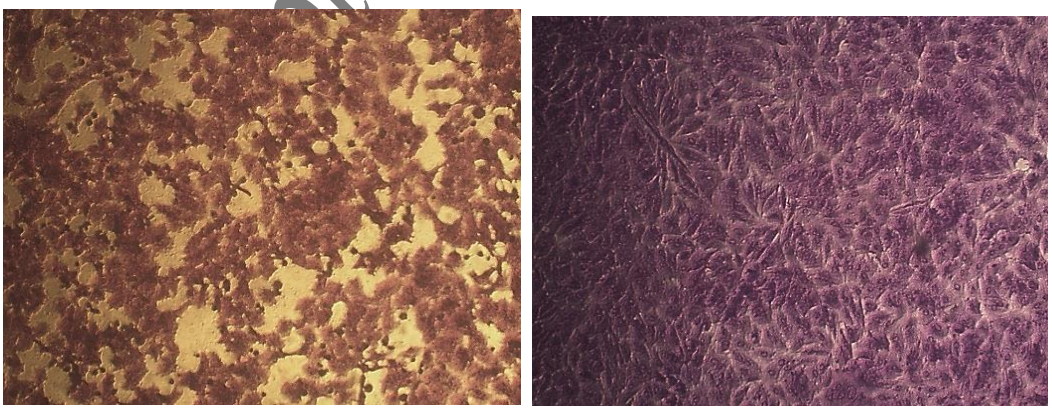


Fig. 13 Optical density of *Moringa oleifera* seeds on the MCF-7 cancer cell line



MCF-7 (TREATED) MCF-7 (UN-TREATED)

Fig. 14 Effect of *Moringa oleifera* seeds on the MCF-7

DISCUSSION

The MO plant is commonly consumed as food and traditional medicine in several Asian countries, and hence it is named "the Miracle Tree" [24]. Various phenolic compounds (which include ellagic acid, p-coumaric acid, ferulic acid and caffeic acid) were present in the RP-HPLC chromatogram of MO seeds. The seeds of the MO plant were removed with aqueous (water) and 80% ethanol. Based on their

polarity to distinct fractions, the crude ethanolic extract was further fractioned using different solvents (chloroform, ethyl acetate, and n-butanol). Water was used for the extraction because it dissolved all polar compounds. Ethanol was chosen because it has higher solubility, is good at removing phytochemicals from plants, and tends to give relevant compounds, as shown in the previous MO [25]. Different solvents were used to separate parts of the crude ethanolic extract from concentrating, improving the active compounds' purity, and eliminating any unwanted interferences [26].

Results showed reduced proliferation of MCF-7 breast cancer cells when exposed to the ethanol extract of MO seeds. The MO seed extract greatly slowed the cell line's growth because the seeds have high phenolic content and strong antioxidant properties. Additionally, the essential oil that is extracted from the seeds of MO has potent cytotoxic effects on MCF7. RP-HPLC analysis was performed to qualify and quantify phenolic acids (caffeic acid, ferulic acid, ellagic acid, and p-coumaric acid). The butanol fraction contained higher levels of ellagic acid, p-coumaric acid, and caffeic acid, while the ethyl acetate fraction was richer in ferulic acid.

Phenolic acids from MO extracts have demonstrated anticancer effects through various mechanisms, including inhibition of genotoxic molecules, the stifling of mutagen-transforming enzymes' activity, and the regulation of enzymes like heme-containing phase I and carcinogen-detoxifying phase II enzymes [27-29]. The findings of this study align with previous research on the leaves of Iraqi-grown MO, which similarly reported cytotoxic effects against MCF-7 breast cancer cells [16], supporting the therapeutic potential of different plant parts.

It is important to highlight that solvent partitioning is rarely absolute and compounds with intermediate polarity or partial solubility in multiple solvents may distribute across more than one fraction. This distribution is influenced by several factors including solvent polarity, compound structure, and experimental conditions. Therefore, the presence of caffeic acid, ellagic acid, and p-coumaric acid in both butanol and ethyl acetate fractions is consistent with their chemical properties and observed chromatograms. Variations in retention times observed are within acceptable margins due to matrix effects and chromatographic conditions and do not compromise compound identification. Future studies including complementary analytical techniques such as LC-MS could further validate these assignments.

CONCLUSION

In this study, phytochemicals were extracted from MO seeds cultivated in Iraq, and the ethanolic extract was fractionated to evaluate their cytotoxic effects on MCF-7 breast cancer cells. The ethyl acetate extract exhibited significant anti-proliferative activity with an IC_{50} value of 60.7 $\mu g\ mL^{-1}$. This cytotoxicity is likely attributed to the high phenolic content in the seeds of MO. However, the findings are limited to in vitro experiments, and further in vivo studies are needed to confirm the therapeutic potential and safety profile of these fractions. Future research should also investigate the underlying molecular mechanisms, such as apoptosis induction, oxidative stress modulation, and signaling pathway interference, to better understand the anticancer effects of MO seed phytochemicals.

Although the current study used RP-HPLC for phenolic compound identification in different solvent fractions, limitations related to solvent partitioning and chromatographic variability are acknowledged. Further advanced analytical methods such as LC-MS or NMR would provide more definitive characterization of phytoconstituents. Nonetheless, the observed cytotoxic activity correlates with the phenolic content demonstrated here, supporting the potential anticancer role of MO seed extracts.

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