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NEW PURIFICATION APPROACH AND THE ANTICANCER ACTIVITY OF PHENOLIC COMPOUNDS FROM *MANGIFERA INDICA* LEAVES CULTIVATED IN IRAQ

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ABSTRACT. Cancer continues to be a primary cause of death globally, prompting the investigation of effective natural therapeutic agents. *Mangifera indica* L. (mango) leaves are notable for their abundant bioactive compounds, especially polyphenols. This study investigated the extraction, purification, and anticancer properties of polyphenolic compounds from *M. indica* L. leaves cultivated in Iraq. The research employed systematic extraction procedures using methanol followed by purification through Sephadex LH60 column chromatography. Phytochemical analysis and High-Performance Liquid Chromatography (HPLC) characterization revealed significant concentrations of bioactive compounds, with mangiferin (49.8 mg/L) being the predominant polyphenolic constituent, alongside other compounds including apigenin (40.5 mg/L), ferulic acid (36.5 mg/L), and kaempferol (28.9 mg/L). The cytotoxic potential of methanolic extract of *M. indica* diphenyltetrazolium bromide (MTT) assay. The results demonstrated that the cytotoxic effect is dependent on dose, with maximum cell death (68.33%) observed at 1000 µg/mL concentration. The extract exhibited a moderate cytotoxic effect with a half-inhibitory concentration (IC_{50}) value of 132.4 µg/mL. The observed anticancer activity is attributed to the synergistic effects of various polyphenolic compounds, particularly mangiferin, which triggers apoptosis through both intrinsic and extrinsic pathways. These findings suggest that *M. indica* leaf extract could be a promising source of natural anticancer agents.

Keywords: *Mangifera indica L., mangiferin, MTT, HRT-18*

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INTRODUCTION

The rising cancer incidence in the world has heightened the quest for new therapeutic agents available in nature, especially those found in plants [1]. For many years, medicinal plants have been valued for their rich content of bioactive substances, and phenolic compounds, in particular, are now viewed as strong prospects for use in creating new anticancer therapies [2]. One such plant is the *Mangifera indica* L. (mango), which is a member of the family Anacardiaceae and has attracted considerable attention in the scientific world because of its rich phytochemical composition and its traditional usage as a medicine [3]. *M. indica* L. is considered one of the key tropical fruits in the world, which is assumed to have Asian roots [4]. Human beings use both the ripe and the unripe mangoes in pickles, juice, oils, nectar, powder, sauce, cereal flakes and jam [5]. Mango fruit, peel, and flesh are known to be highly rich in fiber, vitamins C and A, essential amino acids, and polyphenols [6]. Mango seeds have been termed as a great source of polyphenols [7]. Although consumption of mango fruit as a food item is widely common, various parts of mango trees have been used in medicine since ancient times, predominantly in the Southeast Asian countries, with recent uses in Iraq [8]. Although the fruit of *M. indica* L. is a valuable source of economic growth; its leaves were traditionally used to treat diabetes [9], hypertension [10] and other inflammatory diseases [11].

Phenolic compounds represent one of the most homogenous groups of plant secondary metabolites [12] that include simple phenols, phenolic acids, flavonoids and more complex polyphenolic compounds [13]. They consist of one or more aromatic rings that have hydroxyl groups, which give them antioxidant potential [14]. The phytochemical research on *M. indica* L. leaves has revealed a number of phenolic groups, such as mangiferin, gallic acid, quercetin and different gallotannins [15].

The anticancer effects of phenolic compounds have been shown to have a promising future based on several mechanisms such as antioxidant activity, cell cycle control, apoptosis and cellular pathways control and modulation [16]. The molecular pathway that uses phenolic compounds to exert an anticancer effect is usually a multifaceted one. These are neutralization of reactive oxygen species [17], inhibition of pro-inflammatory mediators [18], control of cell cycle checkpoint proteins [19] and activation of caspase-dependent apoptotic pathway [20]. Kim *et al* demonstrated that the ethanolic extract of *M. indica* peel can trigger apoptosis in human cervical cancer HeLa cells through upregulation of apoptosis-related proteins expression, including Bax, Bcl-2, Bid, and caspases (3, 8, and 9) [21].

This study presents the first comprehensive characterization of Iraqi-cultivated *M. indica* leaf polyphenols, employing two-step Sephadex LH60 purification, HPLC analysis and cytotoxicity against HRT-18 colon cancer cells, expanding *M. indica* therapeutic applications while validating Middle Eastern cultivar bioactivity potential.

RESULTS AND DISCUSSION

Extraction of *M. Indica* L. Leaves

The leaves of *M. indica* L. were subjected to a series of extraction and purification processes for the isolation of polyphenols. Results in Table (1), exhibit that the resulted yield from the final purification step of *M. indica* methanolic extract (MI-ME) was 1.3 g. All fractions collected from Sephadex LH60 (2 × 30 cm) final purification step were detected positive for the presence of polyphenols.

Table 1. The total weight and yield of following *M. indica* L. leaves extraction and purification.

Steps	Weight	Yield (%)
M. indica L. Leaves	50 g	----
Methanolic Extraction	3.8 g	7.6
Sephadex LH60 (2 × 20 cm)	2.6 g	68.4
Sephadex LH60 (2 × 30 cm)	1.3 g	50.0

Sephadex LH60 is a size exclusion medium that is based on hydroxypropylated dextran, which offers an effective means of purifying phenols in alcoholic extracts of plants. Its unique cross-linked structure and optimal distribution of pore sizes make it highly efficient in separation of polyphenolic chemicals in terms of their molecular weights. The hydrophobic nature of LH60 enables strong interactions with phenolic compounds in an operation in organic solvents like ethanol or methanol [22].

Phytochemical Analysis of MI-ME

The crude MI- ME was measured quantitatively on the content of polyphenols, quantities of alkaloids and flavonoids. The crude MI-ME according to results was positive in polyphenols because of the reaction of

the bluish green color. The appearance of yellow color reaction was followed by the detection of flavonoids in MI-ME. Secondly, the presence of alkaloids was also detected by the presence of both white and brown precipitates that were obtained after the addition of Mayer and Wagner reagents, respectively.

M. indica L. has numerous bioactive chemicals that are mainly polyphenols and terpenoids in leaves [23]. Its main phenolic compounds include mangiferin (xanthone C-glycoside), gallic acid, quercetin glycosides as well as catechins [24]. In addition, triterpenes cycloartenol, friedelin and lupeol are very abundant in the leaves. Analysis of phytochemicals always indicates that it contains alkaloids, tannins, saponins, and flavonoids [25]. These chemicals vary in their concentration according to environmental factors, maturity of the leaves and methods of extracting them. The active components make the leaves supplement the already known antioxidant, anti-inflammatory, and antibacterial activity.

HPLC analysis revealed that the majority of purified MI-ME contained polyphenolic phytochemical constituents (Table 2). These phenolic compounds include apigenin, chlorogenic acid, ferulic acid, gallic acid, mangiferin, kaempferol, quercetin and rosmarinic acid with different concentration range between (20.6 for quercetin to 49.8 ppm for mangiferin).

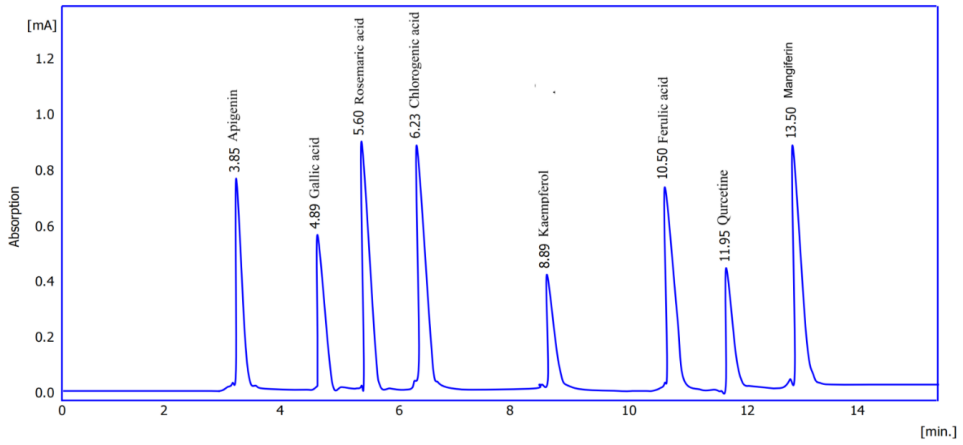
Table 2. Mean ± SD concentration (mg/L) of polyphenolic compounds HPLC quantified using HPLC (*n* = 3).

Name	Mean Concentration ± SD (mg/L)
Apigenin	40.5±4.73
Chlorogenic acid	22.5±1.62
Ferulic acid	36.5±2.66
Mangiferin	49.8±5.09
Kaempferol	28.9±2.41
Quercetin	20.6±2.83
rosmarinic acid	24.6±3.37
Gallic acid	25.7±2.19

SD: Standard Deviation

The leaves of *M. indica* L. are characterized by a substantial array of polyphenolic chemicals, with mangiferin as the principal C-glucosylxanthone, Phytochemical investigations have identified substantial levels of flavonoids, including quercetin, kaempferol derivatives, and gallotannins [26]. Figure (1) presents the HPLC results, indicating the presence of several phenolic acids—including gallic, protocatechuic, and ellagic acids—that contribute to

the extract's antioxidant activity. [27]. Recent research employing liquid chromatography/mass spectrometry has discovered new polyphenolic chemicals, such as benzophenone derivatives and intricate gallotannins [24].



Result chromatography Table (Uncal - F:\ sample 1)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.85	7542.59	795.85	13.25	13.24	0.20	Apigenin
2	4.89	5214.98	586.49	9.59	9.33	0.12	Gallic acid
3	5.60	15985.64	850.49	15.98	15.48	0.25	Rosemaric acid
4	6.23	16995.24	850.66	15.77	15.74	0.25	Chlorogenic acid
5	8.89	2011.45	395.44	7.35	7.65	0.12	Kaempferol
6	10.50	9630.31	784.11	14.56	14.26	0.20	Ferulic acid
7	11.95	2589.67	390.65	7.44	7.30	0.14	Qurcetine
8	13.50	17854.66	854.99	15.59	15.42	0.25	Mangiferin
	Total	77824.78	5508.86	100.00	100.00		

Figure1. HPLC Analysis several phenolic acids.

The polyphenolic concentrations identified in this study demonstrate both consistency and variation compared to previously reported values from different geographical origins. Zhang et al., 2012 reported that mangiferin contents analyzed in *M. indica* from China were 5.04 to 18.95 mg/g, confirming mangiferin as the predominant xanthone across cultivars [28]. In another study, the concentration of both gallic acid and quercetin showed great variation in different mango cultivars from Spain with 16-500 µg/mL and 16-800 µg/mL, respectively [29].

The inclusion of these bioactive chemicals enhances the extract's therapeutic potential, demonstrating notable antioxidant, anti-inflammatory, and antibacterial properties. The synergistic interactions among these polyphenolic chemicals augment their biological performance, especially in free radical scavenging and enzyme inhibitory activities.

Cytotoxic Activity of Purified MI-ME Against HRT-18 Cancer Cells

The potential anticancer activity of purified MI-ME rich with phenolic compounds was investigated using MTT cytotoxic assay against HRT-18 cell line. Figure (2) shows the effect of MI-ME on the HRT-18 cell line at different concentrations. The results indicate that the cytotoxicity of MI-ME against HRT-18 cells increases by increasing the concentration in dose-dependent pattern. The highest killing activity observed at 1000 µg/mL concentration, with a value of 68.33% and the lowest cytotoxicity was at concentration 31.2 µg/mL, with a value of 17.54%. Both concentrations, 1000 and 500 µg/mL, showed no significant differences in inhibiting HRT-18 cell viability, however, significant ($p < 0.05$) differences were observed with 250, 125, 62.5 and 31.2 µg/mL. The increase in cytotoxicity with increasing dose indicates the toxic effect of the MI-ME on the cell viability.

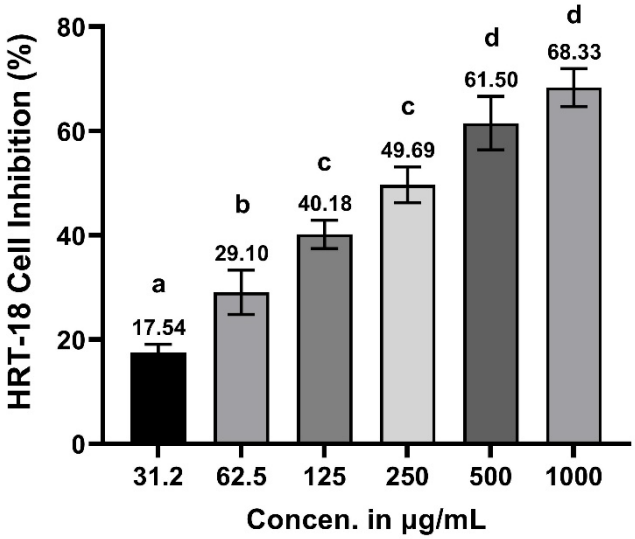


Figure 2. Cytotoxic activity of MI-ME against HRT-18 cell line. Different letters (a, b, c and d) considered significantly different at $p < 0.05$.

The IC_{50} which represents the concentration of purified MI-ME that inhibits cell growth by 50%. As illustrates in Figure (3), the calculated IC_{50} was 132.4 µg/mL, indicating that the extract exhibits a moderate cytotoxic effect on the HRT-18 cells line.

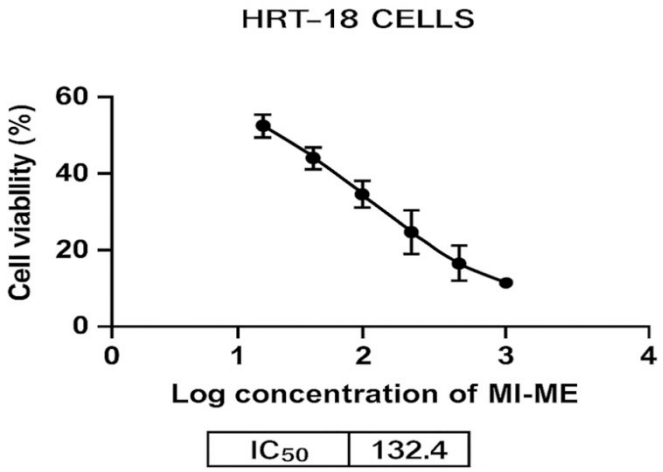


Figure 3. IC_{50} of purified MI-ME on HRT-18 cell line.

The cytotoxic properties of *M. indica* L. leaf extract have been thoroughly investigated, revealing notable antiproliferative effects due to its abundant polyphenolic content [30]. A study by Yehia and Altwaim indicated the cytotoxic activity of ethanolic extract of *M. indica* collected from Saudi Arabia with IC_{50} of 41.2 and 44.7 µg/mL against MCF-7 and HeLa cell lines [31]. While mango cultivars from Costa Rica showed a moderate cytotoxic activity against AGS, HepG2 and SW620 cancer cell lines with IC_{50} range of 138–175 µg/mL [32]. Mangiferin, the principal bioactive molecule, demonstrates specific cytotoxicity towards diverse cancer cell types via several routes. Research indicates that these polyphenols induce apoptosis through both intrinsic and extrinsic mechanisms, as seen by elevated activity of caspase-3, -8, and -9 [29]. The antiproliferative potential of gallotannins derived from mango seed extract against a spectrum of cancer cell lines, including liver, breast, and leukemia was investigated [33]. Vermerris and Nicholson indicated that proanthocyanidins and tannins have the potential to prevent digestive and other internal organ cancers [33]. The combined activities of quercetin derivatives and gallotannins augment the extract's antiproliferative capacity via ROS-mediated pathways and DNA fragmentation [30].

Many studies have also indicated an investigation the effect of an extract on other breast cancer cells lines like MDA-MB-231 and MCF-7. Also, Phenolic compounds of *M. indica* extract shown inhibition of proliferation of cancer cell, sometimes involving the induction of cell cycle arrest and apoptosis of many cancer cell lines types like Lung Cancer cell cines (A-549), Leukemia: Cell lines (Molt-4 and HL-60), Cervical Cancer (HeLa cells), Prostate Cancer (LnCap) and Gastric Cancer (AGS).

CONCLUSION

This study provides new insights including: (1) first comprehensive phytochemical characterization of Iraqi *M. indica* cultivar, (2) innovative two-step Sephadex LH60 purification methodology, and (3) first anticancer evaluation against HRT-18 colon cancer cells. Findings correlate with established mangiferin dominance and polyphenolic diversity while revealing regional quantitative variations and expanding therapeutic applications. The findings in this study indicate potential anticancer activity of methanolic extract of *M. indica* leaves but further investigation into other cancer models and *in vivo* systems are needed.

MATERIALS AND METHODS

Methanolic Extraction of *M. INDICA* L. Leaves (MI-ME)

The leaves of *M. indica* L. were collected from local mango farms in April 2024. The leaves were washed with tap water to remove impurities and air dried. The dried leaves crushed and powdered using electrical blender then 50 g of dried leaves were mixed with 450 mL from absolute methanol in ratio of 1:9 respectively. The mixture was subjected to constant mixing using shaker incubator (120 rpm) at room temperature for one week. After the end of maceration, the plant parts removed from the extract by filtration using filter paper (Whatman No. 1). The solvent was removed under vacuum by evaporation in rotary evaporator at 40°C, and the dried crude extract was stored at 4°C in refrigerator.

Separation and Purification of Polyphenols from MI-ME

Column chromatography of the polyphenols was conducted based on the method outlined by Harborne [34]. The dried powder of MI-ME obtained from extraction method was dissolved in 5 mL methanol and introduced into an open glass column (2 × 20 cm) filled with Sephadex LH60 previously equilibrated with 70% methanol. The elution was collected in 25 separated tubes each tube was filled with 5 mL of the eluent at a rate of 0.5 mL/min. All fractions were tested for $FeCl_3$ (1%) solution as a colorimetric method for polyphenols identification. Only positive tubes for $FeCl_3$ were collected and dried in dry oven at 40°C. The dried powder was subjected to a second round of purification using Sephadex LH60 (2 × 30 cm) column using the same conditions. A total of 20 tubes were collected and all tubes were tested for $FeCl_3$ 1% solution for polyphenols identification, and only the positive tubes were collected and dried.

Phytochemical Analysis

1. Detection of Polyphenols

Aliquot of 3 mL of sample was mixed with 2 mL of 1% $FeCl_3$, %; the appearance of bluish green color indicated the presence of phenols.

2. Detection of Alkaloids

An amount of 0.5 g of MI-ME was dissolved in 2.5 mL of distilled water acidified with 4% hydrochloric acid and 0.5 mL of the mixture was tested in a watch glass with each of the following reagents. First, Mayer reagent (1.36 g $HgCl_2$ and 5.0 g KI in 100 mL DW), the appearance of white precipitate indicated the presence of alkaloids. Second, Wagner reagent (2.5 g iodine and 12.5 g KI in 250 mL DW), the appearance of brown precipitate indicated the presence of alkaloids.

3. Detection of Flavonoids

Flavonoids were detected according to the method previously described [35]. In brief, one gram of MI-ME was suspended in 1 mL of 95% ethanol (Solution A). One volume of solution A was mixed with 1 volume of solution B (consist of 1 mL of ethyl alcohol (50%) and 1 mL of 50% potassium hydroxide). The appearance of yellow color after mixing of equal amounts of solution (A) and (B) indicated the presence of flavonoids.

High Performance Liquid Chromatography (HPLC)

MI-ME (10 µL) was automatically injected, and chromatographic separation was performed on a Poroshell 120 EC-C18 column (100 × 2.1 mm, 1.9 µm) with a flow rate of 0.3 mL/min. The mobile phase was made up of water with 0.5% formic acid (solvent A) and acetonitrile and methanol (80:20, v/v) as solvent B. Gradient elution was performed as follows: 0-1 min at 15% B to 20% B, 12 min at 50% B, 15 min at 60% B, 17.0-19.0 min at 95% B, and 19.0-20.0 min at 15% B, with a total run time of approximately 1 hour. The column temperature remained at 40°C.The study was performed in both positive and negative ionization modes, employing dynamic multiple reaction monitoring with a dwell time of 0.02 second. Each analysis was repeated three times.

Cell Line

HRT-18 colon cancer cell line was kindly provided from Biotechnology Research Center – Al-Nahrain University, Baghdad, Iraq.

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA) media supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37°C and 5% CO_2 environment. When cells reached 80% confluency, a brief trypsinization was performed for 2 – 3 min followed by culturing into a new flask [36].

MTT Experiment

A uniform volume of HRT-18 cell suspension (2×10^4 cells/mL) was added to the selected wells of a 96-well sterile tissue culture plate. The filter-sterilized (using Millipore Filter, 0.22 µm) MI-ME polyphenols (Dissolved in DMSO) was added to the wells of a microtiter plate to obtain final concentrations ranging from 0 to 1000 µg/mL in DMEM. The cells exposed to purified polyphenols of MI-ME were incubated in a CO_2 incubator at 37°C with 95% humidity for a duration of 24 h. After incubation, DMEM (100 µl) was removed from each well, and 20 µL of freshly prepared MTT (5 mg/mL in distilled water) was added. The wells were then incubated for 4 h at 37°C in a CO_2 incubator. Subsequently, the DMEM with MTT was entirely removed. The purple product generated by the cells in each well was obtained by adding dimethyl sulfoxide (100 µL/well), and the absorbance at 570 nm was measured with an Enzyme Linked Immunosorbent Assay (ELISA) reader [37]. Each concentration was tested in triplicate, and the IC_{50} was calculated following the MTT assay using GraphPad Prism (version 6) through the following equation:

$$Y= D+A-D / 1+10^{(X-logC) B}$$

Where Y: response, X: dose, D, A, C and B are constants.

STATISTICAL ANALYSIS

The data obtained was statically analyzed using one-way ANOVA with GraphPad Prism 6. The values were presented as the mean ± SD of triplicate measurements. Significant differences were adjusted at $p < 0.05$.

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