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Cytotoxic, Antioxidant, and Anti-inflammatory Effects of *Crataegus meyeri*

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Abstract

Background: *Crataegus meyeri* A. Pojark is one of Iran›s most well-known medicinal plants. This study aimed to assess the cytotoxic, antioxidant, and anti-inflammatory properties of the methanolic extract of *Crataegus meyeri* (MCM) leaves.

Methods: MCM was prepared from dried leaves. First, the cytotoxic impact of MCM was determined via the MTT test. Then, the anti-inflammatory activity of MCM on the lipopolysaccharide (LPS)-exposed J774A.1 cells was examined by measuring NO production and cyclooxygenase-2 (COX-2) gene expression using the Griess test and quantitative real-time polymerase chain reaction (qRT-PCR), respectively. MCM antioxidant activity was assessed using the FRAP and DPPH tests.

Results: MCM was shown to be non-cytotoxic against J774A.1 cells at ≤125 and ≤62 µg/mL concentrations after 24 and 48 hours, respectively. It also significantly attenuated NO generation in LPS-treated J774A.1 cells at 62 and 125 µg/mL after 24 hours and 62 µg/mL after 48 hours. The extract significantly reduced COX-2 gene expression at concentrations of 62 and 125 µg/mL after 24 hours and at concentrations of 15, 31, and 62 µg/mL after 48 hours. Moreover, the results indicated that MCM at all concentrations exhibited lower antioxidant activity than vitamin C, but the difference was insignificant. Furthermore, MCM could reduce the viability of most of the studied cancer cell lines at concentrations of 250 and 500 µg/mL.

Conclusion: The findings of the present study suggest that MCM may show therapeutic potential for the treatment of inflammatory diseases, a finding that requires clinical trials.

Keywords: *Crataegus meyeri*, Murine macrophage J774A.1 cell line, Anti-inflammation, Antioxidant, Cytotoxicity

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Introduction

Due to their extensive recognition as complementary and alternative treatments, medicinal plants have recently grown in popularity (1). With over 60% of the global population relying on medicinal herbs for medical needs, folk medicine is still widely regarded as the primary healthcare system in numerous regions due to various factors such as different therapeutic effects, accessibility, affordability, and cost-effectiveness (2). In the past decades, numerous studies have proved the effectiveness of medicinal plants and their derivatives in different pathological conditions such as diabetes (3), spinal cord injury (4), liver diseases (5), kidney diseases (6,7), neurodegenerative conditions (8), etc (9-11).

One of the most notable medicinal plants is hawthorn (Crataegus spp.), from the family Rosaceae, estimated to include 150–1200 species (12). Hawthorn is indigenous to the Mediterranean area, which includes Europe, Central Asia, North Africa, and various regions in North America. Many hawthorn species are used in traditional medicine worldwide (13). It has been shown that hawthorn berries have a protective effect on acetic acid-induced colitis in rats due to their anti-inflammatory and antioxidant effects (14). Traditionally, its berries and flowers serve as diuretics and can be utilized to treat kidney problems (15). Hawthorn fruit has been demonstrated to be effective and safe in treating cardiovascular disease and ischemic heart disease (16). Different segments of this plant, especially

the flowers, leaves, and berries, are high in micronutrients and have long been linked to a variety of health, medicinal, and nutraceutical benefits, including antioxidant (17-19), antimicrobial (20-22), anticancer (23), and anti-inflammatory (24) effects. To date, numerous biologically active compounds comprising oligomeric procyanidins and flavone/flavonol types of flavonoids, triterpenoids and sterols, hydroxycinnamic acids, organic acids, lignans, and sugars have been successfully identified in different hawthorn species (25-27). So far, various species of hawthorn have been identified in Iran, including Crataegus meyeri (28). Few studies have focused on the pharmacological effects of C. meyeri; therefore, in the current study, we evaluated the cytotoxic, antioxidant, and anti-inflammatory properties of the methanolic extract of C. meyeri (MCM).

Methods

Sample collection and preparation of MCM

Crataegus meyeri A. Pojark (voucher specimen: KF1627) was collected from Rabor, Kerman, Iran. The samples were dried and the maceration method was employed to prepare MCM using methanol as the solvent (29).

Assessment of MCM cytotoxicity

The cytotoxicity of MCM was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) test. The cell lines were prepared from the Iranian Biological Research Center (Tehran, Iran) (MCF-7, Caco-2, HT-29, A549, and J774A.1) and Pasteur Institute of Iran (Tehran, Iran) (NIH 3T3, PC12, and MDA-MB- 231). After treating the cells with 10 µL of MTT solution, the plates were incubated for 4 hours in an incubator. Then, the medium was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to each well and kept for 1 hour at room temperature. Finally, the optical absorbance was determined using an ELISA reader (Bio-Tek ELX800, USA) at 570 nm and a reference wavelength of 630 nm (30).

Measurement of the anti-inflammatory effects

After reaching 80% confluence, cells were dissociated and 2×106 cells were loaded into each well of a 6-well plate. After one day, 3 mL of culture medium containing varying extract concentrations was added to each well. Then, 5 µg/mL of lipopolysaccharide (LPS) was added to each well. The supernatant was used to measure NO levels, and the cells were used to measure COX-2 expression.

Measurement of NO levels

In order to measure NO levels, the Griess method was used based on a previous study (31). Briefly, 50 µL of the sample was loaded into the wells, and 50 µL of 8% vanadium chloride in 1 M HCl solution and 50 µL of the Griess reagent were added. Then, the plate was placed at 37 °C for one minute, and then absorbance was red at 540 nm.

Quantitative evaluation of cyclooxygenase-2 (COX-2) expression

The RNX-Plus solution (CinnaGen Co., Iran) was used for RNA extraction. Then based on the guidelines of the cDNA Synthesis Kit (Takara Bio Inc., Japan), cDNA was synthesized. For performing quantitative reverse transcription-polymerase chain reaction (qRT-PCR), a reaction mixture included 12.5 µL Maxima SYBR Green/ROX qPCR master mix (Sigma Co., US), 1 µL cDNA template, and 0.3 µL of each primer (Table 1) was prepared. The internal control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (32).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) test

The DPPH test was used to measure MCM's free radical scavenging ability. Briefly, the mixture was prepared by adding 50 µL of varying extract concentrate ions (0–500 µg/mL) to 150 µL of freshly prepared solution of DPPH in methanol. After shaking (60 seconds) and placing the mixture at room temperature (30 minutes), its color change was evaluated at 517 nm utilizing a multi-mode microplate reader (BioTek®, USA). The reaction mixture without the studied compounds was used as the negative control, and vitamin C was used as the positive control. All the procedures were performed in triplicate, and the mean of the results was reported (33).

The ferric-reducing ability of plasma (FRAP) test

To evaluate the reducing power of the MCM extract, 295 µL of FRAP solution was mixed with different concentrations of the extract (5 µL). The solution was maintained for 10 minutes, and the absorbance was determined at 593 nm. The results were mentioned as reduced mM Fe II/g dry weight of the extract. Various concentrations of a solution of FeSO4 from 0.1 to 5 mM were used for calibration curve construction. A similar protocol was performed for vitamin C as the positive control. All the experiments were conducted in triplicate on different days (34).

Statistical analysis

All data are expressed as a standard error of the mean (SD), and one-way analysis of variance (ANOVA) and Tukey's multiple comparisons were employed to compare the groups with GraphPad Prism 8 software. P values ≤ 0.05 were considered statistically significant.

Results

Investigation of MCM cytotoxicity on J774A.1 cells

As shown in Figure 1, none of the concentrations ≤ 125 µg/mL reduced the viability of macrophage cells following 24 hours. After two days, concentrations ≤ 62 µg/mL were non-toxic and did not negatively affect cell viability. Therefore, some of these non-toxic concentrations were

Table 1. Primer sequences for qRT-PCR

Gene	Sense primer (5′→3′)	Anti-sense primer (5′→3′)
Mouse COX2	F: 5′ AACCGCATTGCCTCTGAAT 3′	R: 5′ CATGTTCCAGGAGGATGGAG 3′
Mouse GAPDH	F: 5′ GTCGGTGTGAACGGATTTGGC 3′	R: 5′ GTTGAATTTGCCGTGAGTGGAGTC 3′

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; COX-2: cyclooxygenase-2 .

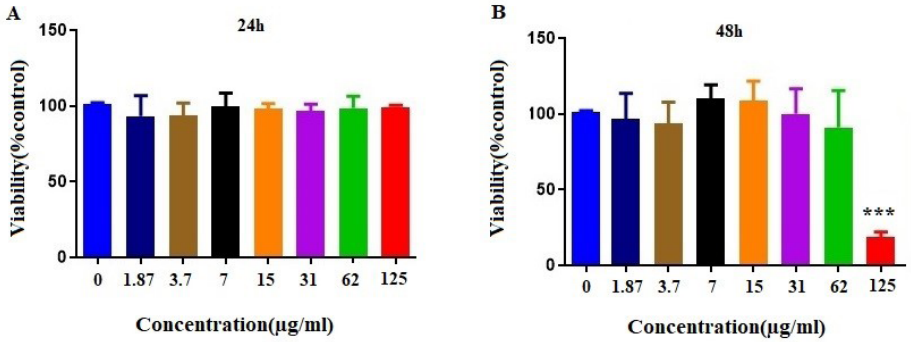

Figure 1. The viability of murine J774A.1 cells following MCM exposure after (A) 24 h and (B) 48 h (*** $P<0.001$)

selected for the subsequent experiments.

Investigation of the anti-inflammatory effects of MCM

MCM effect on COX-2 gene expression

After exposure of the cells to varying concentrations of the extract and LPS (5 µg/mL), the expression levels of COX-2 were assessed using qRT-PCR. LPS considerably enhanced the expression of COX-2 compared to the untreated control. MCM (62 and 125 µg/mL after 24 hours and 15, 31, and 62 µg/mL after 48 hours) decreased the expression of COX-2 in LPS-exposed cells (Figure 2).

MCM effect on NO generation

The J774A.1 cells were exposed to different concentrations of MCM and LPS (5 µg/mL). LPS markedly increased NO generation compared to the control. As observed in Figure 2, MCM reduced the amount of NO induced by LPS at a concentration of 62 and 125 µg/mL after one day and 62 µg/mL after two days compared to the LPS group. However, after 48 hours, the extract at lower concentrations than 62 µg/mL could not reduce the amount of NO compared to the LPS group.

Investigation of the antioxidant activity of MCM

The antioxidant properties of MCM were investigated utilizing DPPH and FRAP assays. Based on the DPPH assay, the free radical scavenging activity of MCM at different concentrations was lower than vitamin C, but the difference was insignificant. Similarly, according to the results of the FRAP assay, MCM extract at all concentrations exhibited lower antioxidant activity than vitamin C. However, the difference was insignificant compared to similar concentrations (Figure 3).

Investigation of the antiproliferative effects of MCM

The antiproliferative effects of the MCM extract (0–500 µg/mL) on fibroblast NIH/3T3 and some cancerous cells were examined. Figure 4 depicted that the MCM extract attenuated the viability of A594, Caco-2, HT-29, MCF-7, MDA-MB231, 3T3, and PC-12, in a dose-dependent fashion. It appears that MCM at concentrations of 500 and 250 µg/mL had more potent effects on cancerous cell lines.

Discussion

Using medicinal herbs to treat different diseases dates back to the dawn of humankind. Previous studies have shown the effectiveness of plants in various diseases (35-37). The findings of the current investigation demonstrated that different concentrations of MCM attenuated LPS-stimulated COX-2 and NO production. It has been established that NO is a crucial inflammatory response mediator. During an inflammatory reaction, the level of NO, a highly reactive compound, is enhanced. This excess NO interacts with the superoxide radical to induce cytotoxic effects and damage the tissues (38,39). Therefore, it is possible to treat inflammatory illnesses by reducing NO levels. Pro-inflammatory genes, such as COX-2, are upregulated in macrophages in response to inflammation and other inflammatory components, including NO. These cytokines are crucial in the development of inflammatory disorders (40). In our work, the extract suppressed the gene expression of COX-2 at concentrations of 125 and 62 µg/mL after 24 hours and at concentrations of 62,31 and 15 µg/mL after 48 hours.

In a study by Li et al, the anti-inflammatory properties of the water fraction extract of Crataegus pinnatifida Bunge were shown. It was reported that these effects might be mediated by the downregulated expression of COX-2, IL-1β, IL-6, and TNF-α genes in LPS-treated murine macrophage RAW 264.7 cells (41). According to the present study, the extract has antioxidant properties comparable to vitamin C. The research conducted by Ekin

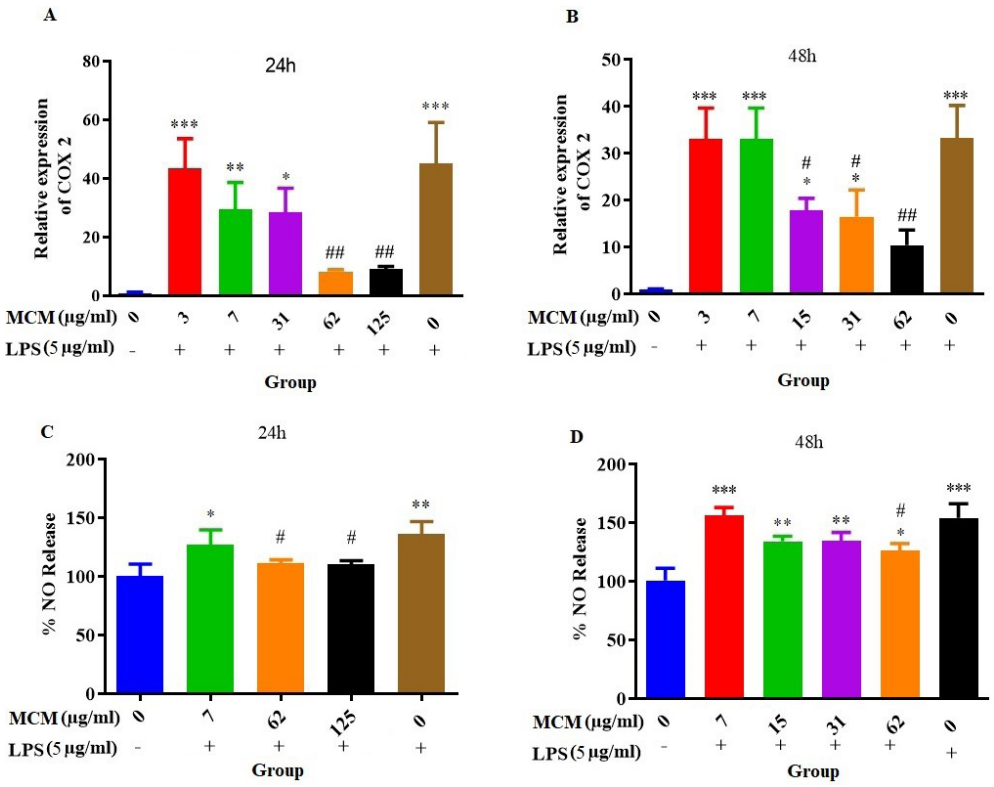

Figure 2. Investigation of the anti-inflammatory properties of MCM. The impact of the extract on the expression of the COX-2 gene by LPS-treated J774A.1 cells after (A) 24 h, (B) 48 h. The impact of MCM on NO generation by LPS-treated J774A.1 cells after (C) 24 h, (D) 48 h. MCM: methanol extract of Crataegus meyeri; LPS: lipopolysaccharide; NO: nitric oxide; COX-2: cyclooxygenase-2 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control; # $P<0.05$, ## $P<0.01$ vs. LPS) (n=3)

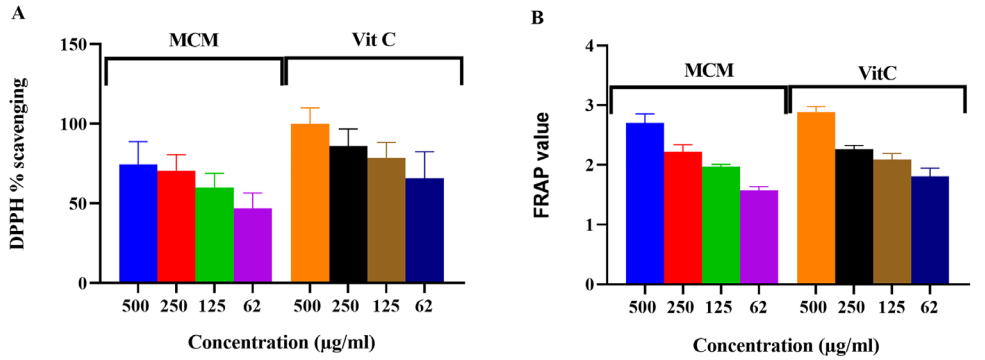

Figure 3. Investigation of the antioxidant properties of MCM. (A) DPPH assay; (B) FRAP assay. MCM: methanol extract of Crataegus meyeri

et al indicated that the leaf extract of C. meyeri exhibited strong antioxidant properties regarding DPPH's radical-scavenging effects, metal chelating effects, and total antioxidant activity, which were consistent with the results of our study (42). This potent antioxidant activity could be due to hawthorns' high phenolic and flavonoid content, which could contribute to cell defense against free radical damage (42,43).

Furthermore, current results revealed that MCM reduced the viability of all studied cancer cell lines in a dose-dependent manner. Consistently, Ma et al indicated that homogeneous polysaccharide isolated from hawthorn exhibited cytotoxic activity against HCT116, a human colon cancer cell line (44). In another study, Maldonado-Cubas et al demonstrated that the petroleum ether extracts isolated from the leaves and stems of Mexican hawthorn (Crataegus gracilior) exhibited cytotoxic effects against breast cancer cells. They suggested that the antitumor effects of hawthorn were mediated by two components, β-sitosterol and tocopherol (45).

Conclusion

According to the current research findings, MCM exerts strong inhibitory effects on LPS-stimulated NO generation and COX-2 expression in J774A.1 cells, and it showed an antioxidant effect comparable to vitamin C at the same concentration. It also has a desirable antiproliferative impact against cancer cell lines at concentrations of 250

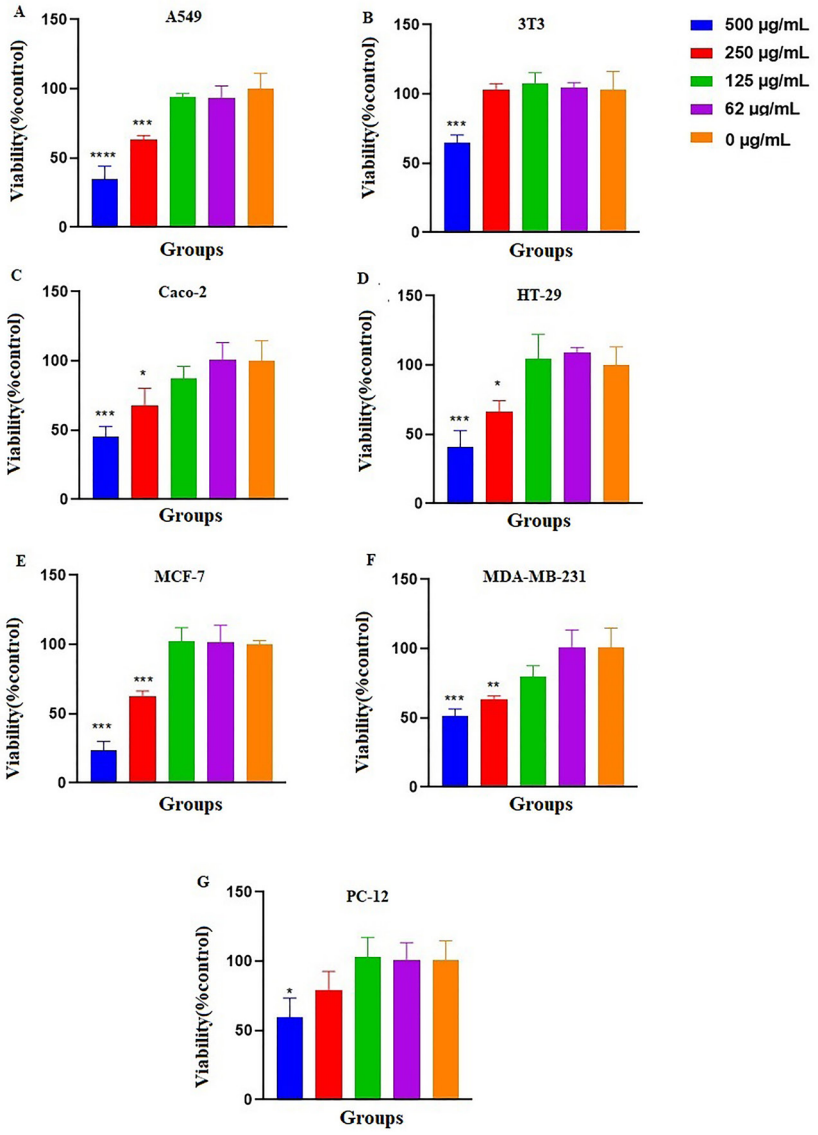

Figure 4. Effect of MCM cytotoxicity on the A594 (A), 3T3 (B), Caco-2 (C), HT-29 (D), MCF-7 (E), MDA-MB231 (F), and PC-12 (G) cell lines using the MTT assay. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$)

and 500 (µg/mL). Therefore, it may exhibit therapeutic potential for treating inflammatory diseases and cancer. It is recommended to assess the expression of genes upstream of the inflammatory pathway, such as NF-κB, and other pathways involved in inflammation, such as the MAPK pathway. Moreover, hawthorn leaf fractions, fruit extract, and its fractions should be isolated in future studies for further investigation of the effects of C. meyeri.

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Competing Interests
The authors declared no conflict of interest.

Ethical Approval
The ethics committee of Kerman university of medical sciences approved the protocol of current study (Ethical code: IR.KMU.REC.1402.357).

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