



Effect of Green Synthesized Magnesium Oxide Nanoparticles Using *Crataegus aronia* L. on Human Colon Cancer – CaCo-2 Cell Line

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ABSTRACT

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Crataegus aronia L. is commonly known as Hawthorn and used in traditional Jordan medicine. In this research, the phytochemical compounds were studied qualitatively and quantitatively using fruit extract of the wild *C. aronia* collected from Jordan. The total phenolic, total tannin, total alkaloid, and total flavonoid contents of the fruit extract of *C. aronia* was determined using standard methods. The antioxidant activity was evaluated using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay. Biogenic synthesis of magnesium oxide nanoparticles (MgONPs) using the plant extract was carried out. The purified nanoparticles were characterized by UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Scanning electron microscopy (SEM), and Transmission electron microscopy (TEM). The cytotoxic effect of the MgONPs against colon cancer (CaCo2) cell line was evaluated using the MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. The MgO nanoparticles had smooth surface and randomly arranged with an average particle size of 20–40 nm. The nanoparticles exhibited cytotoxic activity against colon cancer cell lines with an IC₅₀ value of 98 µg/mL. The study concluded that MgONPs synthesized using fruits of *Crataegus aronia* have potential anticancer activity against CaCo2 cell lines.

Keywords: Nanodrug, MgONPs, *Crataegus aronia*, Colon Cancer, Nanoparticles.

Introduction

Scientists searched for drugs from various sources, such as plants.¹ Plants play an essential role in drug synthesis, and medicinal plants are considered a healthy contributor to human health because of their therapeutic capacities in treating different diseases. The therapeutic capacities of a medicinal plant depend on its phytoconstituents, such as alkaloids, cardiac glycosides, saponins, flavonoids, tannins, volatile oil, steroids, terpenoids, resin, and mucilage.² There is an ongoing worldwide revolution that is mainly premised on the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs.³ According to the World Health Organization, about 80% of the world population relies on traditional medicine for primary health care, and more than 30% of the plant species have been used medicinally. Plant species have long been used as principal ingredients in traditional medicine.⁴ The Flora of Jordan is rich in several medicinal plant species.⁵ Therefore, it was recorded that 20% of the total Flora of Jordan are medicinal plants,⁵ of which are reportedly used in folk medicine and can be used in the pharmaceutical industry.⁵⁻¹⁹ In addition, green synthesis of nanoparticles using plant extracts has been performed, and their bioactivity has been confirmed by *in vitro* and *in vivo* studies.²⁰⁻²¹

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The plant *C. aronia*, commonly called Hawthorn, is a perennial, deciduous, thorny shrub or tree, 2-6 m, dense branches above, leathery leaves, 3-5 lobed, hairy. The flowers are 2 cm in diameter and white, unusual in clusters of up to 10. Fruits are 1-1.5 cm in diameter, yellow and edible. The plant's habitat in Jordan is mostly in the mountains and forests in Irbid, Ajloun, Jerash, Salt, Amman, and Al Karak. The fruits flower in March- May.²² Cancer is a dangerous disease involving abnormal tissue growth that divides uncontrollably and spreads almost anywhere in the body. Radiotherapy, chemotherapy, and surgical excision are the standard approaches used to treat cancer, either separately or in combination. However, these treatment approaches possess significant side effects. Recently, scientists and researchers have been working to discover new cancer treatments using new-featured materials. Nanotechnology can potentially improve current approaches while reducing toxicity and adverse effects associated with traditional therapies.²³ Metal nanoparticles have significantly increased usage in various fields, like electronics, catalysts, medicine, and biotechnology.²⁴ Cancer is one of the leading causes of morbidity and mortality in Jordan. Some studies showed the ethnomedicinal use of plant species by the inhabitants of Jordan for treating colon cancer, and they were inadequately screened for therapeutic/chemopreventive potential and phytochemical findings.²⁵⁻²⁸

This work examined the *in vitro* cytotoxic activities of ethanol, aqueous extract, and nanoparticles synthesized using *C. aronia* against CaCo2 cell lines and evaluated their antioxidant potential using 1,1 diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay.

Materials and Methods

All chemical materials and reagents purchased from commercial sources were of analytical grade. Cancer cell lines (colon cancer CaCo2) and fibroblasts were obtained from the American Type Culture Collection (ATCC) (USA). Other materials include a Binder incubator (Germany), Biotek ELX800 Plate Reader with BioTek's Gen5™

(USA), and Heidolph laborota rotary evaporator (Germany). Double distilled water was used in the preparation of all solutions.

Plant collection and preparation

The fruits of the plant *Crataegus aronia* were collected during spring and summer (2018) and thoroughly dried. The plant was taxonomically identified by Prof. Sawsan Oran (Department of Biological Sciences, Jordan University). A voucher specimen of the collected plant was deposited at Jordan University herbarium (No. 11).

Preparation of ethanol and aqueous extracts.

The fruits of *Crataegus aronia* were air-dried at room temperature in the dark for approximately four weeks and then ground to a fine powder. To prepare the extract, 10 g of fruit powder was macerated in 100 mL of absolute ethanol and distilled water (1:10 ratio). The extracts (aqueous and ethanol) were kept at 20°C under frequent agitation for 72 h. Afterwards, the extracts were filtered through Whatman No. 1 filter paper and concentrated to dryness under reduced pressure with a rotary evaporator. The crude extracts obtained were stored at -20°C in an airtight container until further use.¹⁹

Preparation of MgONPs from C. aronia extract.

About 100 g of the freeze-dried *C. aronia* fruit was placed in 200 mL of deionized water, which was heated for 1 hr at 80°C. The extract was filtered using filter paper. The synthesis of *C. aronia* MgONPs was carried out using 20 mL of the *C. aronia* extract in 180 mL of 0.1 M aqueous Mg₂SO₄ solution. The mixture was stirred and heated at up to 80 °C for different durations, and the colour changed from white to light brown. The brown-coloured solid obtained was heated to 450 °C in a calcination process to form cream-white coloured MgO nanoparticles. The NPs obtained were well dispersed in deionised water by sonication and used for characterisation studies and other experiments.²⁵

Qualitative Determination of Phytochemicals

Test for Tannins

The fruit extract (1 mL) was combined with 10 mL of deionised water and then treated with 3 drops of ferric chloride. A greenish-brown precipitate was observed.²⁹

Test for Steroids

The fruit extract (1 mL) was mixed with an equal volume of chloroform, and a few drops of concentrated sulfuric acid were added. A brown ring appeared at the interface.³⁰

Test for Alkaloids

The fruit extract (2 mL) was mixed with 2 mL of diluted hydrochloric acid, and then a few drops of Wagner's reagent were added to the test tube. A reddish-brown precipitate was observed.³¹

Test for Flavonoids

To 2 mL of a 2.0% NaOH solution and 2 mL of plant extract were added. A concentrated yellow colour was formed, which became colourless after adding 2 drops of diluted acid to the mixture.³²

Test for Phenols

To 1 mL of the fruit extract, 2 mL of distilled water and a few drops of 10% ferric chloride were added. A formation of a green colour was observed.³⁰

Test for Amino acid

A few drops of the ninhydrin reagent were added to 2 mL of the fruit extract, and the mixture was heated for 5 minutes. There was the formation of a purple colour.³⁰

Test for Protein

The fruit extract (2 mL) was mixed with 1 mL of NaOH solution (40%) and 2 drops of copper sulfate solution (1%). The solution turns from blue to purple.³¹

Quantitative Determination of Phytochemicals

Determination of Total Phenolic Content

The total phenolic content of the extract was performed using the Folin-Ciocalteu assay. 1 mL Folin-Ciocalteu phenol reagent was combined with 1 mL of the extract (1 mg/mL), then 13 mL of deionized distilled water and 10 mL of 7% sodium carbonate solution were added to the mixture after five minutes and shaken well. The solutions were kept at room temperature for 90 minutes in the dark. In the same way as previously mentioned, a set of reference standard solutions of gallic acid (20–100 µg/mL) were prepared. The absorbance of all samples was measured at 760 nm using a UV/ Visible spectrophotometer. The determination was done in triplicate. Using the calibration curve, the total phenol content was determined and presented as milligrams of Gallic acid equivalents /g of dried extract.³³

Determination of Total Tannin Content

The total tannin content of the extract was performed using the Folin-Ciocalteu assay. About 0.1 mL of the sample extract (1 mg/mL) was added to 7.5 mL of distilled water, 0.5 mL of Folin-Ciocalteu phenol reagent, and 1 mL of 35% sodium carbonate solution, and diluted to 10 mL with distilled water. The solutions were shaken well and kept at room temperature for 30 minutes. In the same way as previously mentioned, a set of reference standard solutions of gallic acid (20–100 µg/mL) were prepared. The absorbance of all samples was measured at 725 nm using a UV/ Visible spectrophotometer. The determination was done in triplicate. Using the calibration curve, the total tannin content was determined and presented as milligrams of Gallic acid equivalents/g of dried extract.³⁴

Determination of Total Flavonoid Content

The total flavonoid content of the extract was performed using the Kostić *et al.* method.³⁵ 2% AlCl₃ dissolved in methanol was mixed with the extract (1 mg/mL) in a ratio of 1:1. The solutions were shaken well and kept at room temperature for 1 hour. In the same way as previously mentioned, a set of reference standard solutions of quercetin (20–100 µg/mL) were prepared. The absorbance of all samples was measured at 420 nm using a UV/ Visible spectrophotometer, and all tests were performed in triplicate. Using the calibration curve, the total flavonoid content was determined and presented as milligrams of quercetin equivalents/ g of dried extract.

Determination of Total Alkaloid Content

The total alkaloid content of the extracts was determined using the Madhu *et al.* method.³⁶ 1 mL of sample extract (1 mg/mL) was added to 5 mL pH 4.7 phosphate Buffer and 5 mL Bromocresol Green (BCG) solution. 1- 4 mL of chloroform was added to the mixture and shaken vigorously. In the same way as previously mentioned, a set of reference standard solutions of Atropine (20–100 µg/mL) were prepared. The absorbance of all samples was measured at 470 nm using a UV/ Visible spectrophotometer, and all tests were performed in triplicate. Using the calibration curve, the total alkaloid content was determined and presented as milligrams of atropine equivalents/ g of dried extract.

Determination of Antioxidant activity

The antioxidant activity of the plant extracts was evaluated using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay previously described by Chan *et al.* One milliliter (1 mL) of different dilutions of plant extracts (50 – 800 µg/mL) were mixed with 1 mL of 0.1 mM DPPH (in ethanol), the solutions were shaken well and then

allowed to stand at room temperature for 30 min.³⁷ The absorbance of the sample was measured at 517 nm using a UV/Visible spectrophotometer. Ascorbic acid was used as positive control. All tests

were performed in triplicate. To determine the percentage of DPPH radical scavenging activity, the formula below was used:

$$\% \text{ DPPH scavenging Activity} = [(A \text{ blank} - A \text{ Sample}) / A \text{ blank}] \times 100$$

Where A blank is the absorbance of the control and A Sample is the absorbance of the extract. The values for the IC₅₀ were calculated using a linear regression analysis.

Nanoparticles Characterization techniques

MgONPs were examined by X-ray diffractometer (Shimadzu XRD-6000) equipped with Cu K α radiation $\lambda = 1.5405$ over a wide range of Bragg angles ($20^\circ \leq 2\theta \leq 80^\circ$) using Debye Scherrer equation:

$$D = (K \cdot \lambda) / (\beta \cdot \cos(\theta))$$

Where λ is the X-ray wavelength, θ the diffraction angle, and k a constant usually equal to 1, λ denotes the wavelength, β denotes the full width at half maximum (FWHM).

FTIR analysis of MgONPs and *C. aronia* fruit extract

FTIR Spectra for *C. aronia* fruit extract and MgONPs were obtained in the 4000–500 cm⁻¹ range with IR-Prestige-21 Shimadzu FTIR spectrophotometer.

SEM, TEM, and GC-MS analysis

Scanning electron microscopic (SEM) analysis of MgONPs was done using the TESCAN VEGA 3 SEM machine. Transmission electron microscopic (TEM) analysis of MgONPs was done using Morgagni FEI Holland with a megaview 3 digital camera. 200 mL of suspension was added to a 200 mesh coated grid for 2 min, the suspension was removed, and the grid was dried at room temperature. The *C. aronia* fruit extract was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis using methanol as solvent.

In vitro cytotoxic activity of MgONPs

CaCo2 and fibroblast (normal) cell lines were seeded into 96-well culture plates at a density of 1×10^4 . After the 24 h incubation, the medium was replaced with fresh medium. Different concentrations (200, 100, 50, 25, 12.5, and 6.25 $\mu\text{g/mL}$) of *C. aronia* MgONPs were then added to the cell cultures to obtain the final concentration of MgONPs and incubated for 24 h at 37°C. Zinc oxide (ZnONPs) was used as the positive control (0.1 g/mL). After the incubation, 100 μL of MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma) was added, and then cells were incubated for 3 h. The absorbance was measured at 570 nm and 630 nm. The percentage of survival cells was detected by using the following formula:

$$\text{Survival \%} = 100 - (AC - AT) / AC \times 100$$

IC₅₀ values were detected as the concentrations that exhibited 50% inhibition of proliferation on any tested cell line.

Statistical analysis

Graph pad prism, version 5.02 statistical programs, was used. The results were considered to be significant if $p < 0.05$. Data are expressed as the mean \pm SD (Standard Deviations). One-way analysis of variance (ANOVA) was used for statistical analysis, and the Dunnett test for means deference was used to calculate cyto-selectivity by comparing means of IC₅₀ for the plant extracts and magnesium oxide nanoparticles against the cancer cell lines vs. the mean IC₅₀ of the noncancerous cell lines.

Results and Discussion

Table 1 shows that tannins, steroids, alkaloids, flavonoids, phenols, and amino acids were present in both aqueous and ethanol extracts, while proteins were absent in both extracts. In the quantification assay for different phytochemicals, the TPC in the *C. aronia* L. fruit extracts was obtained from the calibration curve ($y = 0.0053x - 0.0245$, $R^2 = 0.9927$) of gallic acid (20-100 $\mu\text{g/mL}$) (Figure 1A). The results were expressed in mg of gallic acid equivalents per gram of plant extract weight. The total phenolic content in the ethanol fruit extract of *C. aronia* L. was 37.006 ± 1.75 mg gallic acid equivalence/ g of fruit extract, and the aqueous fruit extract of *C. aronia* L. was 32.043 ± 1.03 mg gallic acid equivalence/ g of fruit extract (The result is the mean of three tests \pm standard deviation). Also, the total tannin content of the extracts was quantified from the calibration curve ($y = 0.0045x - 0.0117$, $R^2 = 0.9956$) of gallic acid (20-100 $\mu\text{g/mL}$) (Figure 1B). The results were expressed in mg of gallic acid equivalents per gram of plant extract weight. The total tannin content in the ethanol fruit extract of *C. aronia* L. was 29.63 ± 3.00 mg Gallic acid equivalence/ g of fruit extract, and the aqueous fruit extract of *C. aronia* L. was 20.29 ± 1.00 mg Gallic acid equivalences/ g of fruit extract (The result is the mean of three tests \pm standard deviation). Similarly, the alkaloidal contents of the extracts were determined from the calibration curve ($y = 0.0017x - 0.0024$, $R^2 = 0.9943$) of atropine (20-100 $\mu\text{g/mL}$) (Figure 1C). The results were expressed in mg of atropine equivalents per gram of plant extract weight. The total alkaloid content in the ethanol fruit extract of *C. aronia* L. was 14.74 ± 1.480 mg atropine equivalence/ g of fruit extract, and the aqueous fruit extract of *C. aronia* L. was 10.23 ± 1.176 mg atropine equivalence/ g of fruit extract (The result is the mean of three tests \pm standard deviation). The total flavonoid content of the extracts was determined using the calibration curve ($y = 0.0134x + 0.0632$, $R^2 = 0.9898$) of quercetin (20-100 $\mu\text{g/mL}$) (Figure 1D). The results were expressed in mg of quercetin equivalents per gram of plant extract weight. The total flavonoid content in the ethanol fruit extract of *C. aronia* L. was 2.72 ± 0.25 mg quercetin equivalence/ g of fruit extract, and the aqueous fruit extract of *C. aronia* L. was 0.507 ± 0.121 mg quercetin equivalence/ g of fruit extract (The result is the mean of three tests \pm standard deviation). The Antioxidant activities of the ethanol and aqueous extracts of the *C. aronia* L. fruit, as measured by DPPH assay, are shown in Figure 2. The result showed that the IC₅₀ value of DPPH scavenging activity of the ethanol extract was 382.39 $\mu\text{g/mL}$ and for the aqueous extract was 504.225 $\mu\text{g/mL}$, were lower than the IC₅₀ of the ascorbic acid (5.48 $\mu\text{g/mL}$). The GC-MS analysis of the extracts was in agreement with the results of the qualitative and quantitative determination of the phytoconstituents for *C. aronia* extracts, as shown in Figure 3 and Table 1. The results showed the presence of tannins, steroids, alkaloids, flavonoids, phenols, and amino acids. On the other hand, proteins were absent.

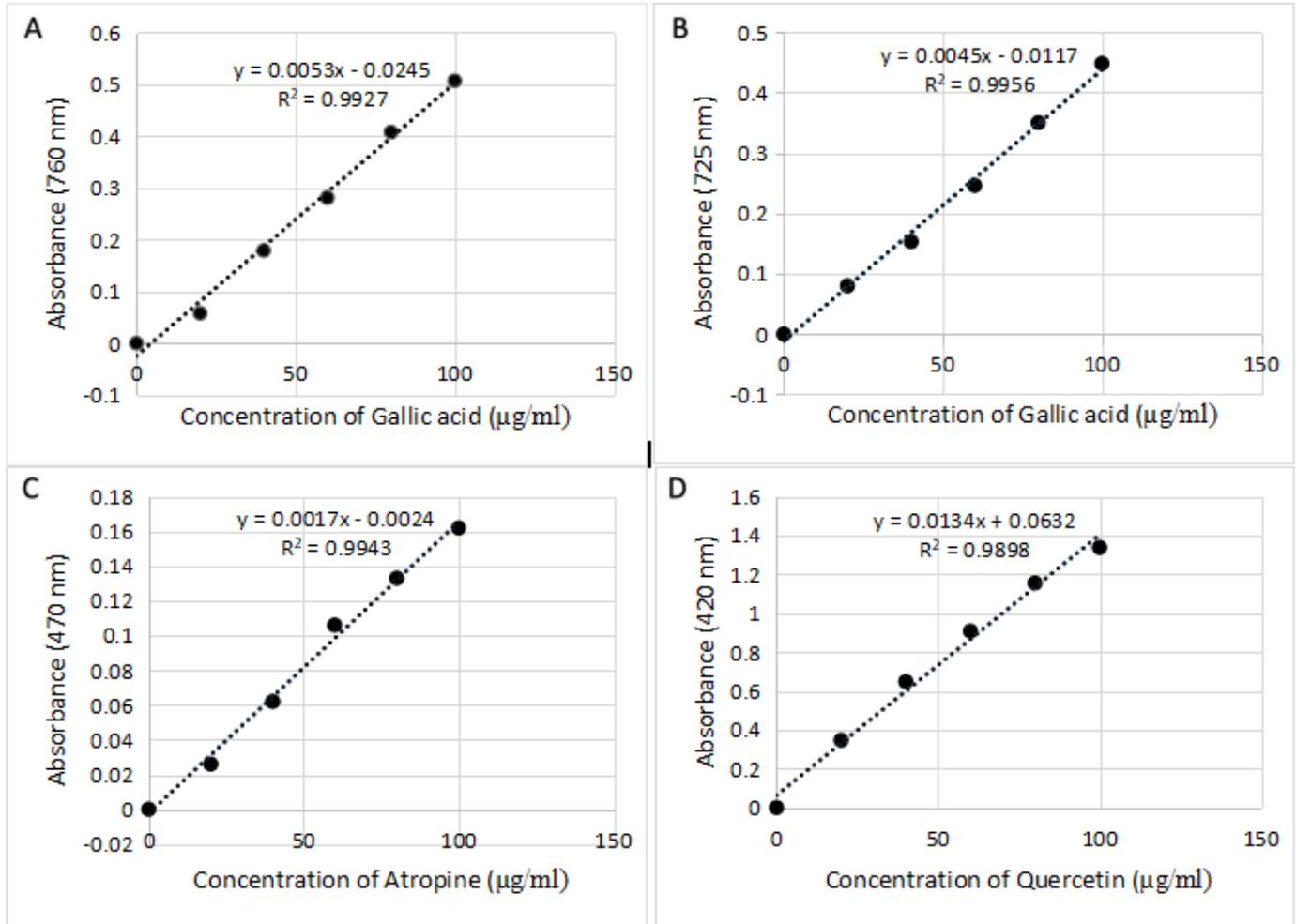


Figure 1: Calibration curve for (A) phenol Content, (B) tannin Content, (C) Alkaloid Content, (D) flavonoid Content.

Table 1: Qualitative phytochemical constituents of *C. aronia* L. extracts

Phytochemical constituent	Aqueous extract	Ethanol extract
Tannins	+	+
Steroids	+	+
Alkaloids	+	+
Flavonoids	+	+
Phenols	+	+
Amino acids	+	+
Proteins	-	-

+ = Present, - = Absent

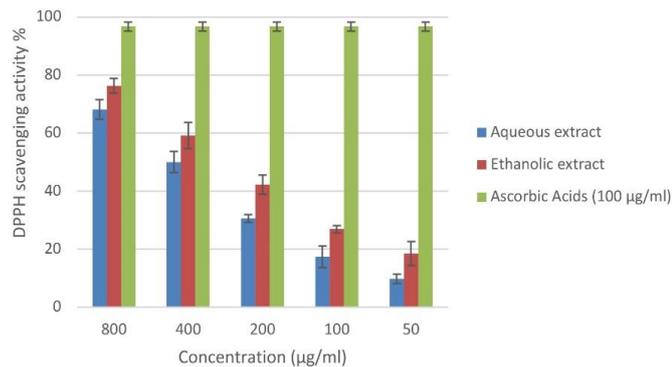


Figure 2: Antioxidant activity of *C. aronia* L. ethanol and aqueous extracts at different concentrations. The percentage of DPPH scavenging activity is shown. Ascorbic acid was used as positive control at 0.1 mM. The result was expressed as mean \pm standard deviation

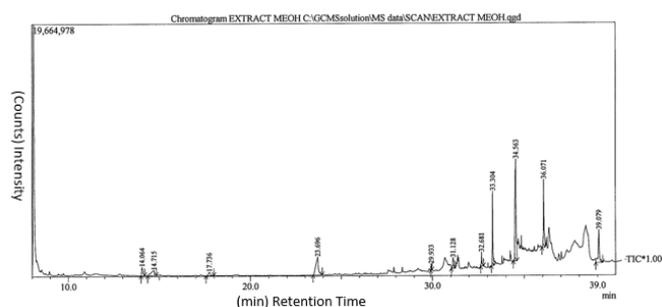


Figure 3: GC Chromatogram of *C. aronia* fruits methanol extract

Results of the FTIR analysis of the extracts of *C. aronia* fruits revealed extensive peaks around 3396 cm^{-1} as shown in Figure 4A. The peaks at approximately 2924 and 1242 cm^{-1} are due to the C–H asymmetric stretching vibration. The peak located at 1537 cm^{-1} suggested the presence of phenolic groups.³⁸ The absorbance peak at about 1743 cm^{-1} , 1689 cm^{-1} , and 1522 cm^{-1} were attributed to carboxyl and carboxylate vibration. These functional groups act as dispersing, capping, and stabilising agents for MgONPs during synthesis.³⁹ FT-IR spectrum indicated a new chemistry linkage on the surface of MgONPs. This suggests that *C. aronia* extract can bind to MgONPs.²⁷ Likewise, the FTIR spectrum of synthesized MgONPs Figure 4B showed strong bands at 446 cm^{-1} , attributed to the vibrations of elongation and deformation of vibratory MgO. The strong absorption peak at 3699 cm^{-1} corresponds to the O–H stretching mode arising from water absorption on the surface of MgONPs and the N–H stretching absorption of amines. The structural changes in FTIR spectra indicated the capping and stabilisation of MgONPs via coordination with OH, –NH, C=O, and C=N.²⁸

X-ray diffraction analysis result of the synthesized MgONPs is illustrated in Figure 5A. The diffraction peaks with 2θ values of 36.74 , 42.92 , 62.18 , 74.46 , and 78.33 correspond to reflections from (111), (200), (220), (331), and (222) planes Bragg reflections, respectively. The assigned peaks at $2\theta = 20.58^\circ$, 29.08° , and 30.54° are related to the crystalline and amorphous organic phase of *C. aronia*. This is supported by the XRD pattern of *C. aronia*, Figure 5B, which showed peaks at $2\theta = 29.06^\circ$ and 30.32° .

Also, the scanning electron microscopic analysis micrographs of synthesized MgONPs presented information on the size and shape of individual nanoparticles.⁴⁰ Figure 6A shows the smooth surface and random arrangement of MgO nanoparticles within an average size of 20–40 nm. The formation of the MgO mesoporous structure can be attributed to the presence of *C. aronia* on the substance of nanoparticles.⁴¹ Similar results were obtained in this study according to

the TEM (Transmission Electron Microscopy), shown in Figure 6B, using a Zeta Potential Analyzer with a MgONPs size of 23–41 nm approximately. The charge was approximately (-30), which means that the result of the nanoparticles was good (because it was a way of zero, and the contribution was homogenous). Moreover, this supports our result of the effect of MgONPs on Colon Cancer, shown below in Figures 7 and 8 as a novel treatment.

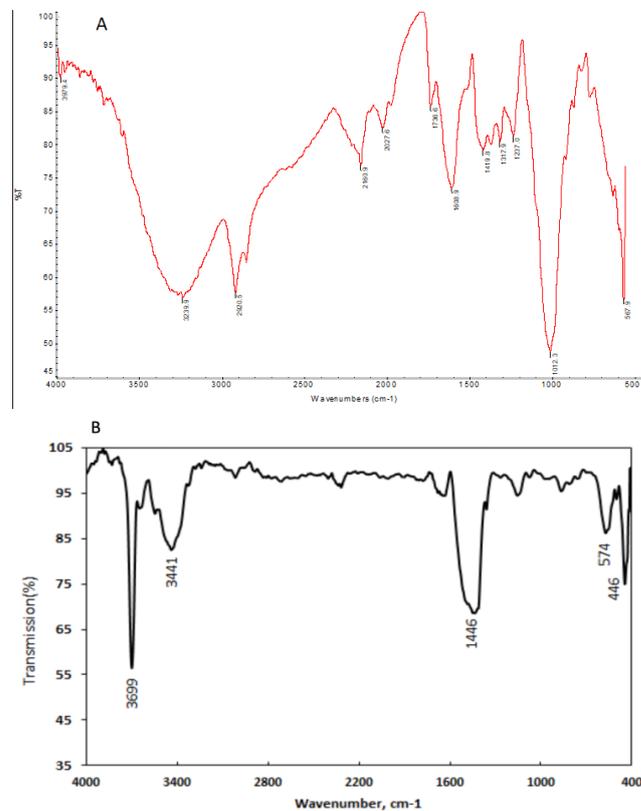


Figure 4: Fourier-transform infrared (FTIR) spectra for (A) *C. aronia* and (B) MgONPs

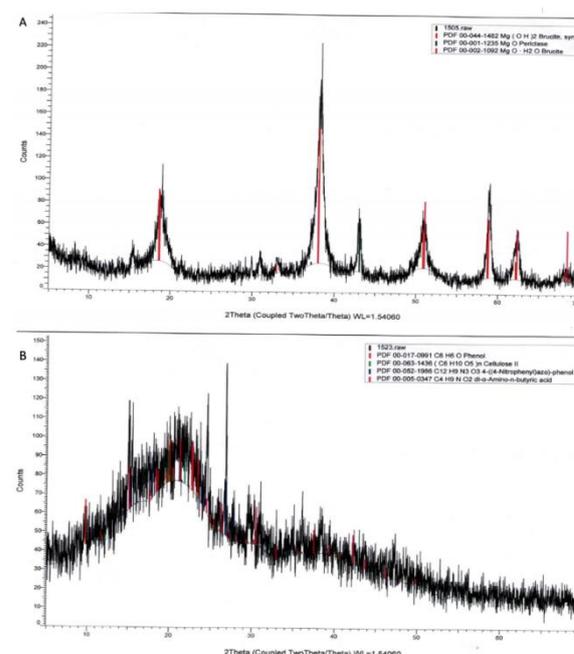


Figure 5: X-ray powder Diffraction (XRD) patterns of (A) MgONPs, (B) *C. aronia* fruit

The cytotoxic effect of *C. aronia* MgONPs was evaluated in CaCo2 cancer cell lines and fibroblasts. The cytotoxic effect on cell growth was examined at different concentrations (200, 100, 50, 25, 12.5, and 6.25 $\mu\text{g}/\text{mL}$),⁴²⁻⁴³ and the results were expressed as inhibitory concentration (IC_{50}). The IC_{50} value of *C. aronia* MgONPs was observed at the concentration of 98 $\mu\text{g}/\text{mL}$ against CaCo2 cancer cell lines, and 230 $\mu\text{g}/\text{mL}$ in fibroblasts normal cell lines, and the standard ZnONPs was found at 4.45 $\mu\text{g}/\text{mL}$ in CaCo2 cancer cell lines and 6.01 $\mu\text{g}/\text{mL}$ in fibroblasts normal cell lines. The results show no cytotoxic effects on the *C. aronia* MgONPs treated in noncancerous cell lines of fibroblasts. In CaCo2, the percentage of cancer cell growth inhibition was found to be high with the increasing concentrations of *C. aronia* MgONPs. The

IC_{50} values showed that the cytotoxic effect of the biosynthesized *C. aronia* MgONPs against CaCo2 cells was more potent than the noncancerous cell line (Figures 7 and 8).

In the Arab world, *C. aronia* is used to treat different health problems related to cardiovascular diseases and different types of cancers. In a study investigating the effects of the combination of hydroalcoholic extract of *C. aronia* and cisplatin on the A549 human lung cancer cell line, the combination exhibited a cytotoxic effect on these cells.⁴⁴ In addition, the combination stimulates apoptosis by downregulation of different proteins, such as Bcl2.⁴⁵ In our study, *C. aronia* MgONPs exhibited a cytotoxic effect against CaCo2 cancer cell lines with 98 $\mu\text{g}/\text{mL}$. This indicated that it could be a potential treatment against CaCo2 cancer cell lines with minimal side effects on normal cells.

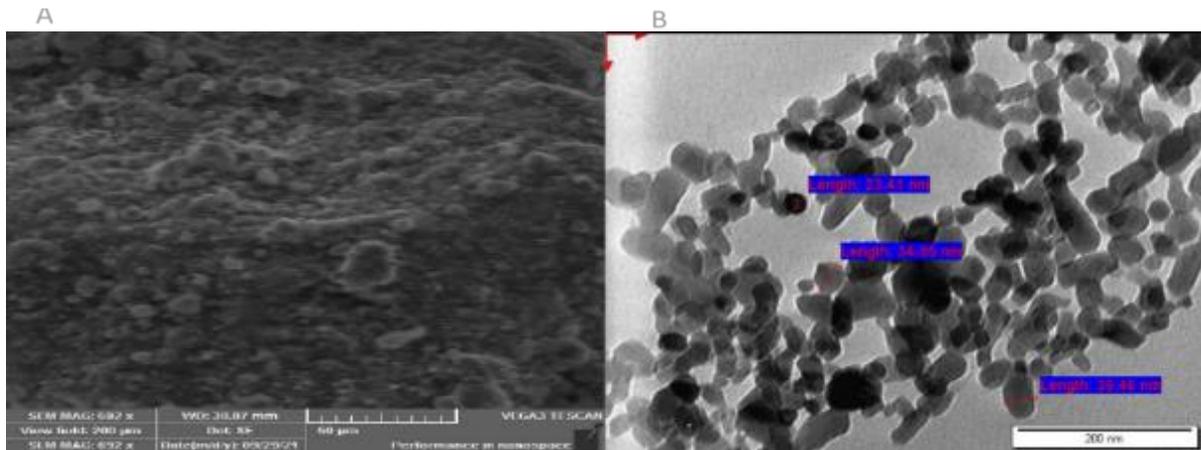


Figure 6: (A) Scanning Electron Microscopy (SEM) image of MgONPs, (B) Transmission electron microscopy (TEM) image of MgONPs

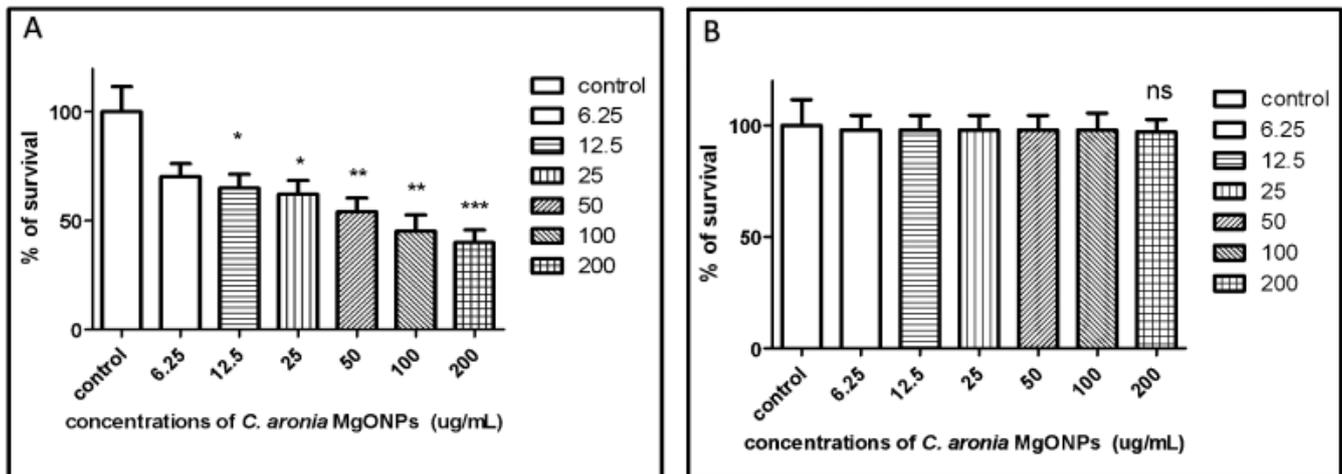


Figure 7: The cytotoxic effect of *C. aronia* MgONPs against (A) CaCo2 cell lines, (B) Fibroblasts (normal) cell lines after 24 h incubation

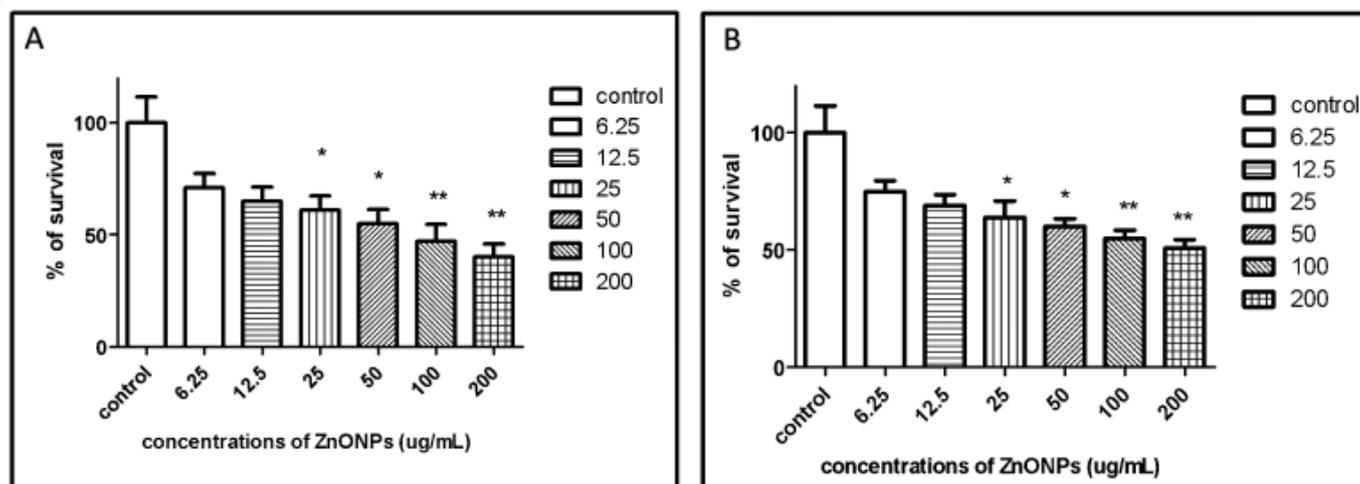


Figure 8: The cytotoxic effect of ZnONPs $\mu\text{g/mL}$ against (A) CaCo2 cell lines and (B) Fibroblasts (normal) cell lines after 24 hrs incubation

Conclusion

Cytotoxic effects of the aqueous and ethanol extracts of *C. aronia* were assessed against colon cancer cell lines (CaCo2). The ethanol extract of *C. aronia* demonstrated a pronounced cytotoxicity against CaCo2. The MgONPs synthesized using *C. aronia* fruits exhibited cytotoxicity against CaCo2. In contrast, it was not cytotoxic against noncancerous cells. This study concluded that MgONPs synthesized using *C. aronia* have promising activity against colon cancer and could further be developed as a nano-drug.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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