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Antitumor activity and phenolic profile of *Melissa officinalis* extract against human gastric adenocarcinoma cells

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Abstract: Gastric cancer remains a global health concern, driving the exploration of natural products with anticancer potential. This study investigated the antiproliferative activity and chemical composition of a 70% ethanolic extract from *Melissa officinalis* L. against human gastric cancer cells. The extract was prepared and evaluated for total phenolic content, antioxidant capacity, and flavonoid content. The MTT test checked how well it stopped the growth of human gastric adenocarcinoma (AGS) and normal dermal fibroblast (HDF) cells. Data analysis (SPSS Statistics) determined viable cell percentages and performed regression analysis ($p<0.05$). The extract exhibited significant antiproliferative activity against AGS cells compared to normal cells ($p<0.05$), with decreasing IC50 values (564.3, 258.0 and 122.5 µg/ml) over 24, 48 and 72 hours. It also displayed antioxidant activity (IC50=16.8±1.41µg/ml) and contained substantial phenolics (225.76±4.1 mg GAE/g) and flavonoids (22.36±2.6 mg RUT/g). This study suggests the 70% ethanolic extract of *M. officinalis* effectively suppresses AGS cell growth and possesses promising antioxidant properties, highlighting its potential as a natural source of anticancer and antioxidant agents, deserving further investigation.

Keywords: Antioxidant activity, flavonoids, gastric cancer, *Melissa officinalis*.

INTRODUCTION

Globally, cancer remains a leading cause of mortality, affecting diverse populations with a various type of cancer (Marshall and Wagstaff, 2020). Among various cancers, stomach cancer poses a significant global health burden, accounting for roughly 754,000 fatalities annually worldwide (Sitarz *et al*., 2018, Kalan Farmanfarma *et al*., 2020). In Iran, the situation is particularly concerning, with stomach cancer prevalence exceeding the global average and disproportionately impacting males (Ghaffari *et al*., 2019, Fattahi *et al*., 2022).

Traditional cancer treatment options, such as surgery, chemotherapy, radiation, and hormone therapy, while effective, often come with the unfortunate drawbacks of severe side effects and potential drug resistance. This has necessitated the exploration of alternative approaches, with plant-derived compounds emerging as potentially safer and more effective therapeutic agents (Chehelgerdi *et al*., 2023). These plant-based compounds exhibit diverse anticancer mechanisms, including free radical scavenging, potent antioxidant activity, the ability to induce cell cycle arrest and apoptosis (programmed cell death) and the inhibition of angiogenesis, the process of new blood vessel formation essential for tumor growth (Asadi-Samani *et al*., 2016).

Recent research has shed light on the significant potential of bioactive compounds derived from the flora native to Iran as promising tools in the fight against cancer (Asma *et al*., 2022). Studies suggest a strong correlation between a diet rich in fruits and vegetables and a reduced risk of developing cancer. This link is potentially attributable to the presence of bioactive components like phenolics, known for their free radical scavenging abilities. However, despite the encouraging evidence from dietary studies, comprehensive investigations specifically focused on the antiproliferative effects of *M. officinalis* against AGS human gastric cancer cells remain scarce (Anantharaju *et al*., 2016, Franco *et al*., 2018).

M. officinalis, commonly known as lemon balm, is a member of the Lamiaceae family and originates from the Mediterranean region. While its native range encompasses the Mediterranean, East Asia, Southeast Siberia, and North Africa, it has successfully adapted and thrives globally (Miraj *et al*., 2017).

This medicinal herb holds potential applications in various therapeutic areas, including as an antioxidant and in the management of gastrointestinal disturbances and neurological disorders linked to oxidative stress (Franco

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et al., 2018). Notably, its antioxidant properties are attributed to key components such as rosmarinic acid, alongside other polyphenols like quercetin and caffeic acid (Adomako-Bonsu *et al*., 2017, Sentkowska *et al*., 2015, Zemmouri *et al*., 2019).

This study aims to address the existing gap in research by investigating the *in vitro* antiproliferative activity, polyphenolic content and antioxidant capacity of a 70% ethanolic extract derived from *M. officinalis* against AGS cells. By delving deeper into the potential of this natural extract, this research contributes to the broader exploration of alternative and potentially safer therapeutic approaches.

MATERIALS AND METHODS

Plant material and chemicals

Dried leaves of *M. officinalis* were purchased from a local market in Iran in June 2022. Taxonomic identification was confirmed by code (702) registered at the Herbarium of the Medical Plants Research Center, SKUMS, Iran. All chemical compounds were obtained from Sigma-Aldrich.

Cell lines

AGS cells were obtained from the Pasteur Institute of Iran and HDF was acquired from the Cellular and Molecular Research Center, SKUMS, Iran.

Preparation of 70% ethanolic extract

A hundred grams of *M. officinalis* leaves in powdered form underwent maceration with 400mL of 70% ethanol at room temperature for 96 hours. Subsequently, the filtrate obtained after filtration underwent concentration at 40°C under reduced pressure through a rotary evaporator. The resultant extract was refrigerated in aseptic containers awaiting additional utilization (Hashemi *et al*., 2017).

Fourier transform infrared (FT-IR) spectroscopy

Using FT-IR spectroscopy, the vibrational spectrum of the 70% ethanolic extract of *M. officinalis* was analyzed. Spectra were recorded between 400 and 4000cm^{-1} using a PerkinElmer Spectrum 100 (Massachusetts, USA) instrument.

Evaluation of the extract's capacity to scavenge free radicals

The effectiveness of the extract in scavenging free radicals was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method, as modified by Moon and Terao (Baliyan *et al*., 2022). As part of this procedure, varying amounts of the extract were added to a DPPH solution that was dissolved in methanol, with the end goal of reaching a volume of 3.0 milliliters, with the DPPH solution initially containing 0.3mg/mL. The absorbance of the solution was determined at 517nm after 15-minute incubation at ambient temperature, employing a UV-vis spectrophotometer (UNICO 2100, USA). A greater absorbance signifies a diminished capacity to scavenge free radicals. BHT (butylated hydroxytoluene), which had been butylated, functioned as the positive control. Equation 1 was utilized to determine the percentage inhibition of DPPH radical scavenging activity (Gulcin and Alwasel, 2023). To ascertain the IC50 value, which signifies the minimum concentration of the extract needed to scavenge 50% of the initial DPPH concentration, linear regression analysis was applied to graphs illustrating the relationship between extract concentration and antiradical activity (Olugbami *et al*., 2014):

$$\%\ \text{Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \qquad \text{Equation 1}$$

Total phenolic content (TPC)

The Folin-Ciocalteu technique was used to determine the TPC in the *M. officinalis* extract (Stini *et al*., 2024). Briefly, a quick process lasting 3-8 minutes at room temperature involved combining 100µL of a diluted extract with 500µL of 10% (v/v) Folin-Ciocalteu reagent. Following this, a 7.5% (w/v) sodium carbonate solution (volume: 400µL) was introduced into the mixture (Lucas *et al*., 2022). The absorbance was measured at 765 nm using a UV-vis spectrophotometer (UNICO 2100, USA) after 30 minutes in darkness. Each gram of dry plant material was subjected to a gallic acid equivalent (GAE) calibration curve to ascertain the total phenolic content (Molole *et al*., 2022).

Total flavonoid content (TFC)

To ascertain the TFC present in the extract, the aluminum chloride technique was utilized. Briefly, a volume of 500 microliters of diluted extract was combined with precise amounts of methanol, aluminum chloride solution, potassium acetate solution and distilled water (Karimi and Moradi, 2015). The absorbance of the reaction mixture was assessed using a UV-vis spectrophotometer at a wavelength of 415nm. This measurement was conducted after the mixture had undergone incubation at room temperature for forty minutes. Using a standard curve that was generated under the same conditions as the original (Chen *et al*., 2013), the TFC was expressed as milligrams of rutin equivalents (Kumar *et al*., 2017) per gram of dry plant material.

Cell lines and culture conditions

AGS and HDF cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). The medium was supplemented with 10% FBS, 100µg/mL streptomycin, 100 U/mL penicillin and 0.25µg/mL amphotericin B. Cells were cultured at 37°C in a humidified incubator with 5% carbon dioxide (Naderi *et al*., 2020).

Cell culture and treatment

AGS and HDF cells were meticulously placed into 96-well plates (SPL Life Sciences, Korea), ensuring a concentration of 5,000 cells per well, with each well generously filled with 100µL of a comprehensive and nutrient-rich medium. After an incubation span of 24

hours at 37ºC in an atmosphere of 5% CO2, the existing medium was meticulously replaced with varying concentrations of extracts, skillfully blended into fresh medium, each well receiving an ample 100µL. Following this procedure, the cells continued their incubation journey in a controlled environment, maintaining a temperature of 37ºC with 5% CO2 for the subsequent durations of twenty-four, forty-eight and seventy-two hours (Park *et al*., 2015).

Cell viability assay (MTT)

After the designated incubation period, the existing medium was carefully aspirated, making room for the addition of 50µL of MTT solution (1mg/mL in PBS) to each well. Subsequently, a 4-hour incubation at 37°C allowed the formazan crystals to develop. To dissolve these crystals efficiently, 100µL of DMSO was added per well and agitated on a shaker (IKA Company, Germany) for 15 minutes. The absorbance of the resulting solution was then measured at 492 nm using a microplate reader (Stat Fax 2100). It's noteworthy that every experiment was meticulously conducted in triplicate to ensure the reliability and consistency of the results (Jadhav *et al*., 2012).

Cell survival calculation

The cell survival percentage for both cell lines was calculated (Naderi *et al*., 2020) using Equation 2:

$$\text{Cell Survival (\%)} = \frac{A_{treated}}{A_{control}} \times 100 \qquad \text{Equation 2}$$

Where A treated and A control are the absorbance values of treated and control cells, respectively. The IC50 values represent the concentration at which 50% inhibition of cell growth occurs.

STATISTICAL ANALYSIS

GraphPad Prism (Version 7.05) and SPSS Statistics (Version 26.0) were utilized to carry out the statistical analysis. A level of significance of five percent ($p<0.05$) was determined to be established and group differences were compared using a t-test. For visual representation, error bars indicating mean ± SD were included. Furthermore, the determination of IC50 values involved the utilization of the probit model and the regression formula ($y = mx + c$).

RESULTS

FT-IR Spectral Analysis

Fig. 1 illustrates the FT-IR spectrum of the 70% ethanolic extract of *M. officinalis*, displaying distinct peaks characteristic of various functional groups. These peaks correspond to previously reported compounds present in *M. officinalis*, including triterpenes (oleanolic and ursolic acids), volatile compounds (citronellal, geranial, geraniol and neral), phenolic acids (caffeic, chlorogenic and rosmarinic acids) and flavonoids (lutein, quercetin and rhamnocitrin) (Petrisor *et al*., 2022).

The presence of these compounds potentially contributes to the observed antioxidant activity of the extract. The FT-IR data further supports the presence of these bioactive constituents. FT-IR peaks whereas 750, 822, 936 and 990 cm^{1}: These regions exhibit small peaks likely corresponding to various mono-substitution (ortho, meta, para) patterns observed in the aromatic rings of the identified compounds. 690-900cm^{-1}: This region shows bending vibrations of the C-H group, aiding in identifying the type of substitution. 1000-1300cm^{-1}: Strong absorptions in this region indicate the presence of ester groups present in oleanolic acid, rosmarinic acid, chlorogenic acid, quercetin, rhamnocitrin, and lutein. Additionally, overlapping peaks represent C-O stretching frequencies arising from acidic groups in ursolic acid, oleanolic acid, rosmarinic acid, caffeic acid, and chlorogenic acid. 1475-1600cm^{-1}: This region displays characteristic paired peaks corresponding to stretching vibrations of aromatic rings present in the various identified compounds. 1667-2000cm^{-1}: Overtones and combination bands associated with aromatic rings are observed in this region. 2939 and 2897cm^{-1}: These peaks represent sp2 and sp3 C-H stretching vibrations in aromatic and aliphatic rings, respectively, present in chlorogenic acid, lutein, oleanolic acid, quercetin, rosmarinic acid, rhamnocitrin, and ursolic acid. 2840 cm^{-1}: This peak indicates the presence of aldehyde groups found in geranial and citronellal. 3400-3600cm^{-1}: A broad peak in this region signifies the O-H stretching vibrations present in all identified compounds

Evaluation of Antioxidant Properties

The *M. officinalis* extract was tested for its antioxidant potential, total phenolic content (TPC), and total flavonoid content (TFC). The extract exhibited a TPC of 225.74±1.41mg GAE/g and a TFC of 22.36±2.61 mg RUT/g. These results suggest the presence of significant amounts of antioxidant compounds.

The extract's proficiency in scavenging free radicals was corroborated through the DPPH assay. It demonstrated a concentration-dependent capability in scavenging DPPH radicals, yielding an IC50 value of 16.8±1.41µg/mL, as outlined in table 1. This value compares favorably to the standard antioxidant, BHT, which had an IC50 value of 26.33±2.77µg/mL.

Antiproliferative activity

The 70% ethanolic extract of *M. officinalis* was evaluated for its anatiproliferative activity against AGS and HDF cell lines. Different quantities of extract were applied to cells for twenty-four, forty-eight and seventy-two hours, and the MTT test was utilized to evaluate the viability of the cells (fig. 2).

Table 1: Comparative free radical scavenging activity of *M. officinalis* extract and BHT against DPPH*

Sample	Concentration (µg/mL)	DPPH Radical Scavenging Activity % (± SEM)	IC50 (µg/mL)
M. officinalis extract	100	96.05 ± 0.05	16.8 ± 1.41
	50	95.00 ± 1.30	
	25	85.00 ± 0.80	
	12.5	47.70 ± 0.01	
	6.25	20.00 ± 1.50	
	3.125	17.00 ± 1.02	
	1.56	8.00 ± 0.07	
BHT	50	90.80 ± 1.50	26.33 ± 2.77
	40	78.30 ± 1.20	
	30	55.50 ± 0.70	
	20	40.09 ± 1.70	
	10	22.00 ± 1.06	

The results are reported as the mean value ± SEM derived from three independent trials.

Table 2: IC50 values of the M. officinalis extract for AGS and HDF cell lines

Cell line	AGS (µg/ml)		HDF (µg/ml)	
Exposure time (h)	IC_{50}	CI95%*	IC_{50}	CI95%*
24	564.3	(516.7-616.3)	738.8	(615.9-886.3)
48	258	(250.3-266)	700.7	(558.8-878.6)
72	122.5	(119.2-125.9)	549.1	(458.3-657.8)

* 95% confidence interval (CI 95%)

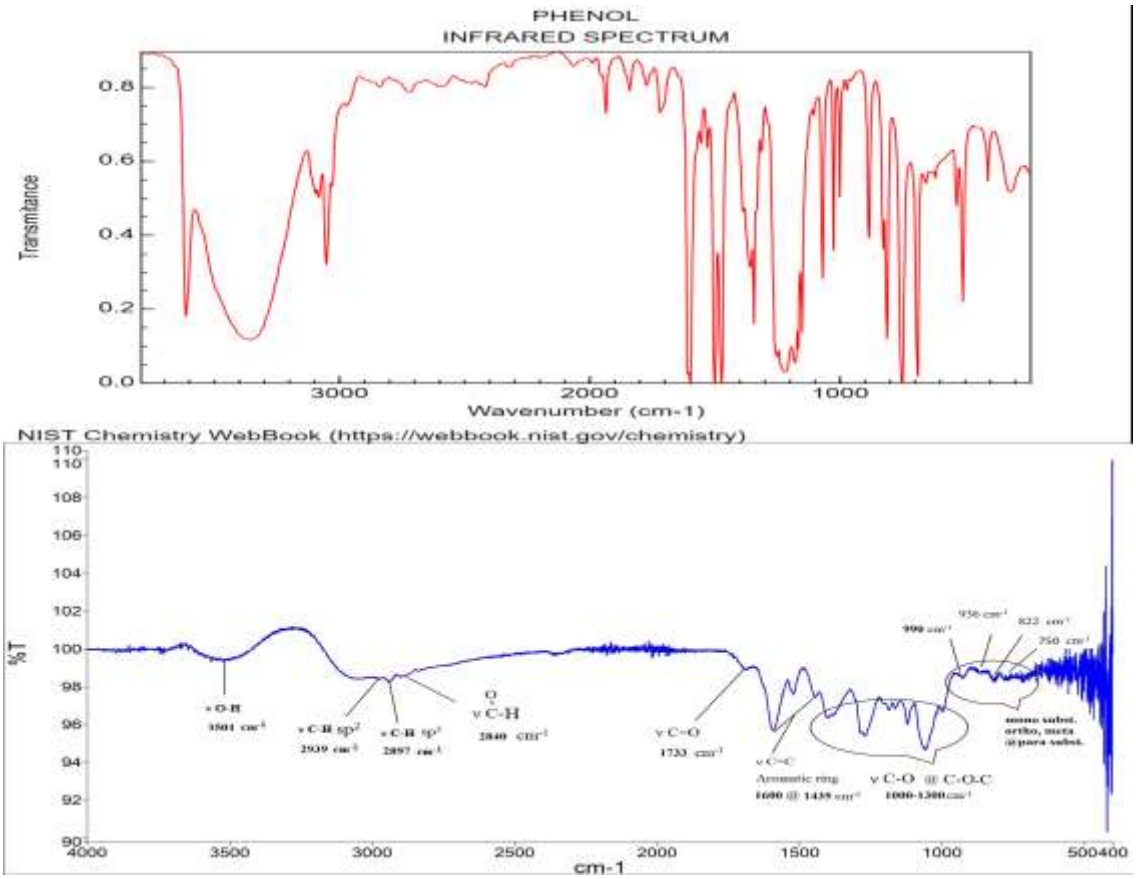

Fig. 1: A) standard FT-IR of phenol, B) FT-IR spectrum of the 70% ethanolic extract of *M. officinalis*.

The extract demonstrated a reduction in cell viability that was dose-dependent for both of the cell lines. A difference with statistical significance ($p<0.01$) was observed in the effects of the extract on both cancer cells and normal cells, as evidenced by the results of the probit regression analysis, with consistently lower IC50 values observed for AGS cells compared to HDFs across all time points (table 2). This suggests a selective antiproliferative effect against the cancer cell line.

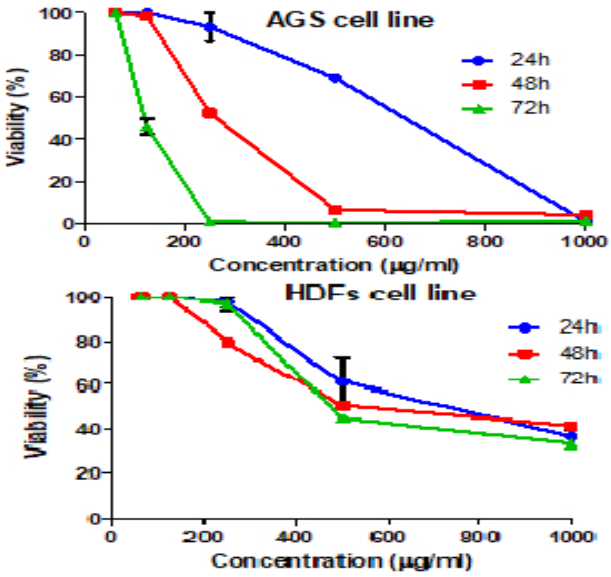

Fig. 2: The *M. officinalis* extract exhibits dose-dependent inhibition of AGS and HDF cell proliferation after 24, 48, and 72 hours. Values represent mean ± SD of n = 3 experiments. * Statistical analysis: t-test; $p < 0.01$ compared to control.

DISCUSSION

Our study investigated the *in vitro* antiproliferative activity, polyphenolic content and antioxidant capacity of a 70% ethanolic extract from *M. officinalis* against AGS human gastric cancer cells.

The *M. officinalis* extract exhibited dose-dependent antiproliferative activity against AGS cells, aligning with previous research highlighting the potential of phenolic compounds in cancer prevention and treatment (Briguglio *et al*., 2020). These compounds exhibit notable bioactivity, including anti-inflammatory, anticarcinogenic, antimutagenic, and antioxidant effects. These bioactivities play a crucial role in various cellular processes, including the induction of apoptosis, regulation of gene expression linked to development and cancer metabolism, inhibition of DNA binding and cell adhesion and the hindrance of cell migration, proliferation and differentiation (Srivastava *et al*., 2016). Our study aligns with this growing body of research, providing further evidence for the potential of *M. officinalis* extracts as promising candidates for gastric cancer prevention and treatment (Zhao *et al*., 2022b).

The results from the *in vitro* antioxidant testing using the DPPH free radical scavenging assay (fig. 2). As expected, the *M. officinalis* extract onstrating greater free radical scavenging ability (table 1). This finding aligns with previous research highlighting the antioxidant potential of *M. officinalis* extracts (Petrisor *et al*., 2022).

Fig. 1 depicts the antiproliferative activity of the *M. officinalis* extract against the AGS human gastric cancer cell line. The graph demonstrates a clear dose-dependent decrease in cell viability with increasing extract concentrations. This visually confirms the extract's potential to inhibit the proliferation of gastric cancer cells.

FTIR analysis revealed functional groups characteristic of phenolic compounds in the extract, potentially contributing to its observed bioactivities (fig. 2). The identification of these functional groups through FTIR analysis supports the potential mechanisms by which the *M. officinalis* extract might exert its effects.

While the precise mechanisms underlying the observed antiproliferative effects of *M. officinalis* extract warrant further investigation, several potential pathways can be explored based on the identified bioactive compounds warrant further investigation. The presence of phenolic acids and flavonoids suggests the extract might exert its anticancer effects through. The presence of phenolic acids and flavonoids suggests the extract might exert its anticancer effects through various mechanisms, including (i) modulation of cell cycle progression and induction of apoptosis in gastric cancer cells, (ii) inhibition of angiogenesis and metastasis, and (iii) suppression of oxidative stress and DNA damage. Additionally, the presence of terpenes like carnosic acid and carnosol might contribute through their anti-inflammatory and pro-apoptotic properties, potentially targeting specific pathways relevant to gastric cancer (Christopoulou *et al*., 2021, Zhao *et al*., 2022a).

The current investigation focused on assessing the *in vitro* antioxidant testing, polyphenolic content, and antiproliferative properties of an extract derived from M. officinalis, aligning with previous research that highlights its potential therapeutic benefits (Miraj *et al*., 2017). Our findings revealed a dose-dependent decrease in the cell viability of AGS, a human gastric cancer cell line, upon the application of the *M. officinalis* extract. The extract, composed of phenolic and flavonoid components, exhibited superior antioxidant activity compared to BHT. The extraction process of *M. officinalis*, attributed to its chemical components, has been associated with diverse therapeutic effects, including antibacterial, antioxidative, and anticancer properties. The unique properties of this

substance can be traced back to the presence of various terpenes. Among the monoterpenes contributing to these characteristics are citronellal, geranial, geraniol, linalool, and neral. Additionally, the sesquiterpenes caryophyllene and caryophyllene oxide contribute to these properties (Petrisor *et al*., 2022). Herbal extracts, particularly those rich in phenolic compounds like *M. officinalis* extract, have been increasingly recognized for their preventive and therapeutic potential in managing various diseases (Anantharaju *et al*., 2016).

The aromatic plant *M. officinalis* emerges as a promising source of natural antioxidants, making it a readily available resource. Furthermore, its potential as a prospective food supplement adds to its value. The investigation examined various extracts produced by *M. officinalis* using petroleum ether, chloroform, ethyl acetate, n-butanol and aqueous solvents, emphasizing their ability to combat free radicals, microbes, and exhibit antiproliferative properties (Stoyanova *et al*., 2023, Moacă *et al*., 2018). Our findings further support this growing body of evidence, suggesting the potential of *M. officinalis* extracts for various applications.

M. officinalis possesses diverse medicinal properties attributed to various bioactive compounds, including the ones identified in our study, such as caffeic acid derivatives and flavonoids (Ieri *et al*., 2017, Draginic *et al*., 2022). Several studies support the potential of rosemary extract and its bioactive compounds for cancer treatment (Allegra *et al*., 2020, Sirajudeen *et al*., 2024). Franco *et al*. (2010) investigated the molecular mechanisms underlying the anticancer effects of rosemary extract, rich in phenolic compounds, highlighting its potential therapeutic applications (Franco *et al*., 2018). Further research specifically focused on rosmarinic acid, a key caffeic acid derivative in rosemary, exploring its anticancer properties and potential mechanisms of action (Shrestha *et al*., 2016, Ijaz *et al*., 2023). Additionally, others evaluated the anticancer activity of rosemary extract using both *in vitro* and *in vivo* models, providing further evidence for its promising role in cancer therapy (Karimi *et al*., 2017, Pérez-Sánchez *et al*., 2019). These studies collectively contribute to the growing body of research supporting the potential of rosemary and its constituents for cancer treatment.

These findings contribute to the understanding of the mechanisms underlying the observed antiproliferative activity and highlight the potential of *M. officinalis* as a source of natural therapeutic agents.

CONCLUSION

This study provides preliminary evidence supporting the potential of the 70% ethanolic extract of *M. officinalis* as a source of natural antioxidants with antiproliferative activity against human gastric cancer cells. Further investigations are warranted to explore its full potential, including elucidating the specific bioactive compounds responsible for the observed effects and evaluating the *in vivo* efficacy and safety of the extract in relevant preclinical models.

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