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The Anti-Cancer Effects of Mustard Oil Through the Targeting of Oncoprotein Transcription Factors in Colon Cancer Cells

Kareem El khashab[1,2]; Samir El-Masry[1]; Hany Khalil[1]*

[1]Department of Molecular Biology, Faculty of Biotechnology, University of Sadat City, El-Sadat City, Egypt.

[2]Medical Laboratory Department, Faculty of Applied Health Science, Nile Valley University, Al-Fayoum, Egypt.

Abstract

Colon cancer, which originates in the large intestine (colon), is often aggressive due to late-stage diagnoses, resulting in poor prognosis and high mortality rates. In this study, we explore the biological functions and potential anticancer effects of several natural oils, including camphor oil, mustard oil, and rosemary oil, in colon cancer Caco-2 cells. We monitored cell viability and the number of surviving cells after treatment with these oils, comparing them to control groups treated with DMSO. Additionally, Lactate Dehydrogenase (LDH) production was measured as an indicator of necrosis following treatment. The relative gene expression levels of NFKB, CMYC, P53, and Caspase 3 (CAS3) were also analyzed using Quantitative Real-Time PCR (QRT-PCR) to understand their roles in cell proliferation and Programmed Cell Death (PCD). Notably, mustard oil treatment led to a significant, dose-dependent decrease in the viability of Caco-2 cancer cells, while having minimal impact on the viability of Normal Colon Cells (NCM-460), in contrast to camphor and rosemary oils. Furthermore, only the treatment of Caco-2 cells with 6.1 µL/mL mustard oil significantly reduced the number of living cancer cells, with little effect on the number of live normal cells. In contrast, LDH production was notably increased in cancer cells treated with mustard oil for 24 hours, indicating cell damage. We also observed a significant reduction in the expression of both NFKB and cMyc in Caco-2 cells treated with 6.1 µL/mL mustard oil, compared to other treatments and control cells. On the other hand, the expression of P53 and CAS3 genes was significantly elevated in mustard oil-treated Caco-2 cells, accompanied by an overproduction of interleukin 1 alpha (IL-1α) and IL-1β, whereas their expression was reduced following treatment with the other oils. Additionally, IL-4 and IL-10 levels were increased over time after mustard oil treatment, suggesting an anti-inflammatory effect. In conclusion, treatment with mustard oil at various concentrations promoted Programmed Cell Death (PCD), exhibited anti-inflammatory effects, and inhibited the expression of the critical oncogenes NFKB and cMyc in Caco-2 cells. These findings suggest that mustard oil may have potential as a therapeutic agent for colon cancer.

Keywords: Colon cancer; Mustard oil; Transcription factors; Programmed cell death; Anti-inflammation.

Introduction

The colon, also known as the large intestine, is responsible for extracting water and salt from solid waste. The remaining waste then passes through the rectum and is expelled from the body through the anus. Colon cancer is the third leading cause of cancer-related deaths in worldwide [1]. This disease continues to be a focal point for researchers aiming to discover new treatments for this widespread condition. Additionally, due to the onset of side effects and the development of drug resistance, there is an urgent need to identify new and more effective therapies for the treatment of colon and Colorectal Cancer (CRC) [2,3].

Several cellular signaling have been identified in colon cancer such as Protein Kinas C (PKC) signaling pathway [4]. PKC is a versatile family of serine/threonine kinases that regulate a

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Correspondance: *Hany Khalil, Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, 79 Sadat City, Egypt. Tel: + 2048- 2601265/2601267; Email: hany.khalil@gebri.usc.edu.eg*

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variety of cellular processes, such as cell growth, differentiation, and apoptosis. Activation of the PKC pathway helps protect cells from apoptosis and regulates several key cellular functions [5]. Furthermore, normal cell replication involves mechanisms such as Cyclin-Dependent Kinases (CDKs) and the Epidermal Growth Factor Receptor (EGFR) [6]. Specifically, EGFR activates downstream signaling pathways, including the pro-oncogene families RAS-RAF-MEK-ERK and AKT-PI3K-mTOR [7]. In cancer cells, overexpression of EGFR and mutations in proteins such as KRAS, BRAF, and PIK3CA are associated with dynamic changes that lead to drug resistance, altered oncogenic behavior, and malignant transformation [8,9]. Targeting EGFR with anticancer drugs inhibits the signal transduction pathways necessary for cancer cell growth, proliferation, and resistance to cell death [10-13]. Among these therapies are tyrosine kinase inhibitors like Sorafenib (SOR), also known as Nexavar, which targets Raf-1 activation and inhibits its downstream effects in hepatocellular carcinoma [14]. The nuclear factor KB (NFKB) is a family of transcription factors involved in regulating a wide range of cellular processes. While NFKB is most commonly associated with immune responses and inflammation, increasing evidence suggests that it also plays a crucial role in oncogenesis [15]. NFKB controls the expression of genes involved in key processes such as cell proliferation, migration, and apoptosis, all of which are essential for cancer development and progression. Aberrant or persistent NFKB activation has been observed in numerous human cancers. In recent years, many studies have focused on understanding the functional outcomes of NFKB activation and it's signaling pathways. As a result, NFKB has emerged as a promising therapeutic target for cancer treatment [16].

Accumulating evidence has shown that numerous natural products possess effective anti-CRC properties and may serve as alternative chemotherapy agents for CRC treatment [17]. Mustard oil, which has been used as a traditional medicinal herb for thousands of years, is known for its antiseptic, antibacterial, antifungal, and antiviral properties. However, its potential anticancer activity remains underexplored [18]. Similarly, rosemary oil has been widely utilized for promoting health and combating disease, with well-documented antioxidant, anti-inflammatory, and antibacterial activities [19]. Another herbal compound, thymol, derived from essential oils such as thyme, has various biological effects, including antioxidant, anti-inflammatory, antinociceptive, antiseptic, and antifungal properties [20]. However, many active herbal agents, such as camphor and thymol, have low water solubility [21]. Based on this, in this study, we investigated the anticancer properties of several natural oils, including mustard, camphor, and rosemary oils, in vitro, by assessing their potential anticancer effects on colon cancer cells. Notably, we also aimed to generalize the molecular mechanisms and regulatory networks through which mustard oil exerts its anticancer effects on cell proliferation and Programmed Cell Death (PCD).

Materials and methods

Preparing oil extract: The natural oils (camphor, mustard, and rosemary oil) were first filtered using a Mills-GP Filter (0.22 µm, Sigma, USA) and collected in clean, sterilized Eppendorf tubes. Various concentrations of the oils were prepared in dimethyl sulfoxide (DMSO), including 100 µL/mL, 50 µL/mL, 25 µL/mL, 12.5 µL/mL, and 6.125 µL/mL. The final extracts were incubated at 4°C until further use.

Cell lines: The colon cancer cells (Caco-2 cell line) and normal colon epithelial cells (NCM-460), sourced from the Egyptian VacSera company, were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, ThermoFisher, USA). The medium was supplemented with 4 mM L-glutamine, 4 mM sodium pyruvate, and 5% heat-inactivated Bovine Serum Albumin (BSA). The cells were maintained in 75 ml cell-culture flasks and incubated at 37°C with 5% CO_2 condition [22]. Cell imaging was performed using an inverted microscope with a Zeiss A-Plan 10X objective.

Cytotoxic concentration 50% (CC_{50}): The prepared oil extracts were assessed for their cytotoxic effects, and the potential CC_{50} values in Caco-2 and NCM-460 cell lines were determined. The cells were cultured in 96-well plates at a density of 10,000 cells per well and incubated in a CO_2 incubator at 37°C. They were then exposed to varying concentrations of each extract (50-6.125 µL/mL) and incubated overnight. Cell viability and the cytotoxic concentration were evaluated using the MTT cell growth assay kit (Sigma-Aldrich, Germany), which measures the amount of formazan dye produced, with absorbance measured at 570 nm [23].

Lactate Dehydrogenase (LDH) production: The LDH assay kit (ab65393, Abcam, USA) was used to measure LDH production in the media collected from cultured cells. Cells were seeded in a 96-well plate and incubated overnight. Afterward, the cells were treated with 6.125 µL/mL in a total volume of 200 µL, with three replicates for each oil treatment. Cells treated with DMSO at the same volume and replicate count were used as the control. The treated cells were incubated for 24 hours. Following the manufacturer's instructions, 100 µL of the cultured media was combined with 100 µL of the LDH reaction mix and incubated for 30 minutes at room temperature. LDH activity was measured using a plate reader at OD450 nm. The relative LDH production was calculated by dividing the absorbance mean values of the treated cells by the mean values of nontreated cells (NT), resulting in the fold change in LDH production [24,25].

Cell proliferation assay: The oils were evaluated for their effects on cell morphology and the number of viable cells in both Caco-2 and NCM-460 cells. Cells were cultured in 6-well plates at a density of 100,000 cells per well and incubated at 37°C in a CO_2 incubator. Each treatment was applied to the cells at a concentration of 6.125 µL/mL, with a total volume of 2 mL per well, followed by overnight incubation. Cell images were captured using an inverted microscope to observe any changes in cell morphology. The number of surviving cells was determined by removing the old media, washing the cells with PBS, trypsinizing the attached cells, and then counting the cells using a hemocytometer. The number of surviving cells after treatment was manually recorded [26].

Enzyme-Linked Immunosorbent Assay (ELISA): The ELISA assay was employed to quantify the released interleukin-1 alpha (IL-1α) using human ELISA kits (ab181421, Abcam, USA). For the assessment of IL-1β in treated Caco-2 cells, human ELISA kits (ab46052, Abcam, USA) were utilized. Additionally, the production levels of IL-4 and IL-10 were measured using human ELISA kits ab46058 and ab46059 (Abcam, USA), respectively. Caco-2 cells

were cultured in 96-well plates at a density of 10,000 cells per well and incubated overnight. Following this, the cells were treated with 6.125 µL/mL of each specified extracted oil, as described previously, and incubated for various time points (0, 6, 12, 24, 36, 48, and 72 hours). At each designated time point, cells were lysed using 1X cell lysis buffer (Invitrogen, USA). Subsequently, 100 µL of the lysed cells were transferred into an ELISA plate, incubated for 2 hours at Room Temperature (RT) with 100 µL of the control solution and 50 µL of the 1X biotinylated antibody. Afterward, 100 µL of the 1X streptavidin-HRP solution was added to each well and incubated in the dark for 30 minutes. Following this, 100 µL of the TMB chromogen substrate solution was added to each well, and incubation continued for 15 minutes at RT, shielded from light. Finally, 100 µL of stop solution was added to halt the reaction. Absorbance was measured at 570 nm for each sample [27].

Quantitative Real Time PCR (QRT-PCR): Gene expression was quantified using qRT-PCR. Total cellular RNA was isolated with Trizell (Invitrogen, USA) and purified using an RNA purification kit (Invitrogen, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using M-MLV reverse transcriptase (Promega, USA). mRNA expression levels of NFκB, cMyc, P53, and CAS3 were quantified using the QuantiTect-SYBR-Green PCR Kit (Qiagen, USA), with the specific primers detailed in (Table 1). Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used as the housekeeping gene for normalization in real-time PCR analysis. The PCR reaction mixture included 10 µL SYBR green, 0.5 µL RNase inhibitor (50 U/µL), 0.2 µM of each primer, 2 µL cDNA, and nuclease-free water to a final volume of 25 µL. PCR was performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 15 seconds, and 72°C for 30 seconds [28,29].

Data analysis: The histograms and charts were generated using Microsoft Excel. Quantification of mRNA, derived from the qRT-PCR assay, was performed using Delta-Delta Ct analysis, following these equations: (1) delta-Ct = Ct value for the gene minus the Ct value for GAPDH, (2) delta-delta Ct = delta Ct value for the experimental group minus the delta Ct for the control group, and (3) Quantification fold change = 2^(-delta-delta Ct). Statistical significance was determined using the Student's two-tailed t-test, with a P-value ≤ 0.05 considered significant [30,31].

Table 1: Oligonucleotides sequences used for mRNA quantification of indicated genes.

Description	Primer sequences 5'-3'
NFκB-forward	CGCAAAAGGACCTACGAGAC
NFκB-reverse	TGGGGGAAAACTCATCAAAG
cMyc-forward	TGAGGAGACACCGCCCAC
cMyc-reverse	ATCGATTTCTTCCTCATCTTC
CAS3-forward	ATGGAAGCGAATCAATGGA
CAS3-reverse	TGTACCAGACCGAGATGTC
P53-forward	GCGAGCACTGCCCAACAACA
P53-reverse	GGTCACCGTCTTGTTGTCCT
GAPDH-forward	TGGCATTGTGGAAGGGCTCA
GAPDH-reverse	TGGATGCAGGGATGATGTTCT

Results

Caco-2 cell viability decreased by mustard oil compared with other oil treatment: To assess the potential cytotoxic effects of mustard oil on cell viability, an MTT assay was conducted using Caco-2 cells pretreated with varying concentrations of mustard oil, camphor, or rosemary oil. The treated cells were incubated for 24 hours following the treatment. As shown in (Figure 1A), the mean absorbance values (570 nm) indicated a significant, dose-dependent decrease in the viability of Caco-2 cells following mustard oil treatment, while the cell viability remained stable in response to camphor and rosemary oil treatments. Notably, a concentration of 6.125 µL/mL of mustard oil resulted in approximately 50% inhibition of cell viability within 24 hours, suggesting that this concentration represents the Cytotoxic Concentration 50% (CC50) for mustard oil on Caco-2 cells. Additionally, the viability of NCM-460 cells remained unaffected by the oil treatments, regardless of the concentration used (Figure 1B). These findings suggest that mustard oil may have potential as an anti-proliferative agent in colon cancer cells, with minimal toxicity to normal colon epithelial cells.

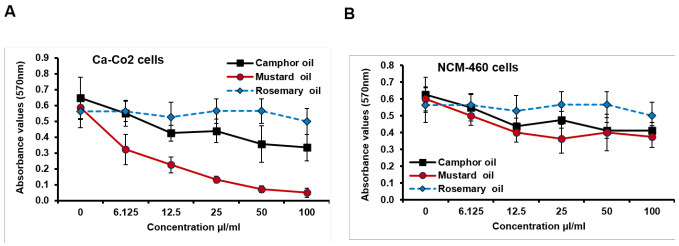

Figure 1: Caco-2 and NCM-460 cell viability upon oil treatment. **(A)** Absorbance measurements at 570 nm in Caco-2 cells in response to treatment with varying concentrations of mustard, camphor, or rosemary oils. **(B)** Absorbance measurements at 570 nm in NCM-460 cells in response to treatment with varying concentrations of mustard, camphor, or rosemary oils. Cells were cultured in 96-well plates in triplicates and treated with the specified concentrations of each oil for 24 hours. Error bars represent the Standard Deviation (SD) from three independent experiments.

Mustard oil altered the morphology of colon cancer cells, decreased the number of viable cells, and boosted LDH production: Cell morphology and cell survival were evaluated in treated cells with camphor oil, mustard oil, or rosemary oil (6.125 µL/mL) using an inverted microscope. Caco-2 and NCM-460 cells were seeded in 6-well plates at a concentration of 100,000 cells per well and incubated overnight. When cells reached approximately 70% confluency, they were treated with 6.125 µL/mL of each oil in duplicate. For controls, the same volume of DMSO was used for one group, while another group remained untreated (NT). Notably, Caco-2 cells showed significant alterations in morphology, with disturbed proliferation after treatment with mustard oil compared to other treated groups. In contrast, the morphology of NCM-460 cells (normal colon cells) showed little to no change in response to the oil treatments or control treatment (Figure 2A). The number of viable Caco-2 cells significantly decreased only after mustard oil treatment, while the number of surviving NCM-460 cells remained unchanged across all oil treatments, including mustard oil, compared to the control (Figure 2B). These results highlight the effects of mustard oil on Caco-2 cells. Furthermore, the relative LDH production was significantly higher in Caco-2

cells treated with mustard oil (6.125 µL/mL) for 24 hours, whereas there were minimal changes in normal NCM-460 cells compared to other control-treated cells (Figure 2C). In conclusion, these findings suggest that mustard oil may have potential anticancer properties, while camphor and rosemary oils appear to lack significant anticancer effects.

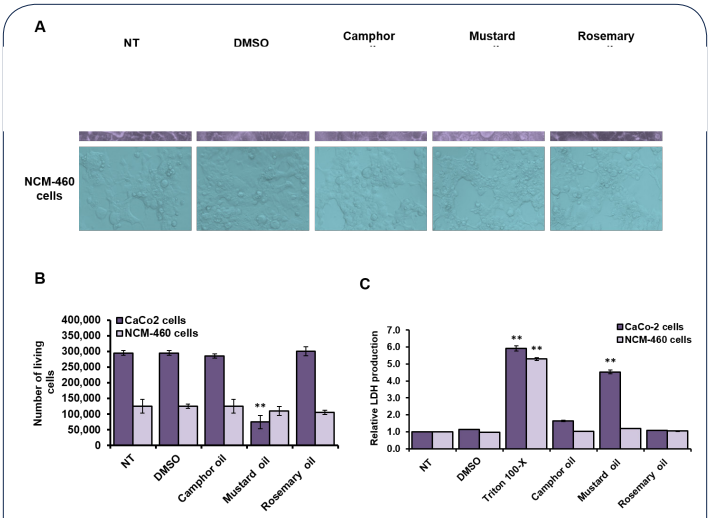

Figure 2: Caco-2 and NCM-460 cell morphology and survival upon treatment with indicated oils. **(A)** The morphology of representative colon cancer and normal cells, observed under inverted microscopy, shows the cell viability of both untreated (NT) cells and those treated with camphor, mustard oil, rosemary oil, or DMSO. **(B)** The number of surviving cells treated with the indicated oils, compared to DMSO treatment and untreated cells. **(C)** Relative LDH production in relation to untreated (NT) cells and those treated with camphor, mustard oil, rosemary oil, DMSO, or Triton X-100. Error bars represent the SD of four independent replicates. Statistical significance was determined using a two-tailed Student's t-test, with (**) indicating p≤0.01.

Mustard oil affects the oncoprotein transcription factors NFκB and cMyc in colon cancer cells that are treated: To investigate the biological influence of mustard oil in regulating cancer cell proliferation, the expression profiles of the transcription factors NFKB and cMyc were analyzed in treated Caco-2 cells. Caco-2 cells were seeded in 6-well plates at a density of 100,000 cells per well and incubated overnight. The cells were then treated with 6.125 µL/mL of each oil in duplicate. For controls, one group was treated with the same volume of DMSO, while another group remained untreated (NT). The relative expression of NFKB and cMyc genes was measured in the treated cells using qRT-PCR, with a sensitive and specific assay providing fold-change results as shown in (Figure 3A,B). Notably, NFKB expression was significantly down-regulated in the mustard oil-treated cells compared to both the control and the other oil-treated groups (Figure 3A). Statistical analysis (Table 2) revealed that the decrease in NFKB expression was highly significant, with a P-value of 0.01. Similarly,

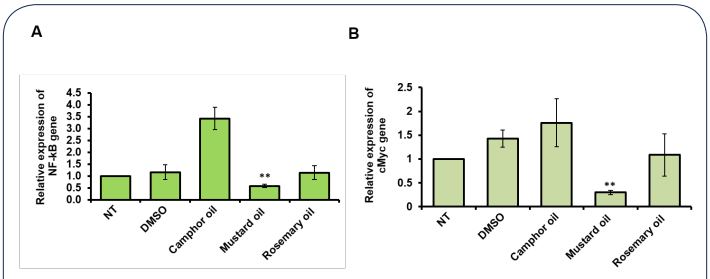

Figure 3: Relative Gene Expression of Transcription Factors in Caco-2 Treated Cells. **(A)** The mRNA expression levels of the NFKB gene were quantified as fold changes in treated Caco-2 cells compared to untreated (NT) and DMSO-treated cells. Error bars represent the Standard Deviation (SD) from two independent experiments. **(B)** The mRNA expression levels of the cMyc gene were quantified as fold changes in treated Caco-2 cells compared to untreated (NT) and DMSO-treated cells. Error bars represent the SD from two independent experiments. The student's two-tailed t-test was applied to assess the significance of the differentiated Ct values. Asterisks (**) indicate a p-value ≤ 0.01.

Table 2: The results of a Delta-delta Ct analysis, which determined the fold change in NFKB expression levels in Caco-2 cells that were pretreated with the indicated oils.

	GAPDH		NFκB		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Fold change Ct1	Fold change Ct2	Mean Fold change	SD	P Value
	Mean Ct1	Mean Ct2	Mean Ct1	Mean Ct2									
NT	19.19	19.73	29.32	29.21	10.13	9.48	0.00	0.00	1.000	1.000	1.000	0.000	
DMSO	20.30	19.80	30.52	28.82	10.22	9.02	0.09	-0.46	0.940	1.376	1.158	0.309	0.54
Camphor	19.46	18.16	27.96	25.73	8.50	7.57	-1.63	-1.91	3.087	3.755	3.421	0.473	0.018
Mustard	20.15	19.35	30.96	29.76	10.81	10.42	0.68	0.93	0.624	0.523	0.574**	0.071	0.013
Rosemary	20.05	19.18	29.76	28.76	9.71	9.58	-0.42	0.10	1.338	0.932	1.135	0.287	0.575

Table 3: The results of a Delta-delta Ct analysis, which determined the fold change in cMyc expression levels in Caco-2 cells that were pretreated with the indicated oils.

	GAPDH		cMyc		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Fold change Ct1	Fold change Ct2	Mean Fold change	SD	P Value
	Mean Ct1	Mean Ct2	Mean Ct1	Mean Ct2									
NT	19.190	19.730	29.785	28.940	10.595	9.210	0.000	0.000	1.000	1.000	1.000	0.000	
DMSO	20.300	19.800	30.261	28.630	9.961	8.830	-0.634	-0.380	1.552	1.301	1.427	0.177	0.076
Camphor	19.456	18.800	28.970	27.520	9.514	8.720	-1.081	-0.490	2.116	1.404	1.760	0.503	0.166
Mustard	20.150	19.348	32.670	30.160	12.520	10.812	1.925	1.602	0.263	0.329	0.296**	0.047	0.002
Rosemary	20.050	19.180	30.160	28.763	10.110	9.583	-0.485	0.373	1.400	0.772	1.086	0.444	0.810

cMyc expression was markedly reduced in the mustard oil-treated cells compared to the controls and the other oil-treated cells (Figure 3B). The statistical analysis (Table 3) confirmed that the reduction in cMyc expression was also highly significant, with a P-value of 0.01. These results suggest that mustard oil plays a role in regulating the expression of NFKB and cMyc transcription factors, which in turn may influence the proliferation signals in colon cancer cells.

Mustard oil stimulates PCD in cancer treated cells via restoring P53 expression: To assess the impact of mustard oil in the PCD process, the expression profiles of P53 (a tumor suppressor gene) and CAS3 (a key effector in apoptotic signaling) were analyzed in treated Caco-2 cells. Notably, P53 gene expression was significantly upregulated, showing a 3.5-fold increase in cells treated with mustard oil compared to both control-treated cells and those treated with the other oil (Figure 4A). Statistical analysis (Table 4) revealed that the increase in P53 gene expression was

Table 4: The results of a Delta-delta Ct analysis, which determined the fold change in P53 expression levels in Caco-2 cells that were pretreated with the indicated oils.

	GAPDH		P53		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Fold change Ct1	Fold change Ct2	Mean Fold change	SD	P Value
	Mean Ct1	Mean Ct2	Mean Ct1	Mean Ct2									
NT	20.12	20.73	28.52	29.81	8.40	9.08	0.00	0.00	1.00	1.00	1.00	0.00	
DMSO	19.13	19.12	27.52	28.12	8.39	9.00	-0.01	-0.08	1.01	1.06	1.03	0.04	0.33
Camphor	20.60	18.80	29.96	28.73	9.36	9.93	0.96	0.85	0.51	0.55	0.53	0.03	0.00
Mustard	19.55	19.48	25.96	26.76	6.41	7.28	-1.99	-1.80	3.97	3.47	3.72**	0.35	0.01
Rosemary	19.24	20.88	28.76	30.76	9.52	9.88	1.12	0.80	0.46	0.57	0.52	0.08	0.01

Table 5: The results of a Delta-delta Ct analysis, which determined the fold change in CAS3 expression levels in Caco-2 cells that were pretreated with the indicated oils.

	GAPDH		CAS3		Δ Ct1	Δ Ct2	Δ- Δ Ct1	Δ- Δ Ct2	Fold change Ct1	Fold change Ct2	Mean Fold change	SD	P Value
	Mean Ct1	Mean Ct2	Mean Ct1	Mean Ct2									
NT	20.12	20.73	29.23	28.11	9.11	7.38	0.00	0.00	1.00	1.00	1.00	0.00	
DMSO	19.13	19.12	28.10	27.03	8.97	7.91	-0.14	0.53	1.10	0.69	0.90	0.29	0.67
Camphor	20.60	18.80	28.97	26.52	8.37	7.72	-0.74	0.34	1.67	0.79	1.23	0.62	0.65
Mustard	19.55	19.48	26.11	24.12	6.56	4.64	-2.55	-2.74	5.86	6.68	6.27**	0.58	0.01
Rosemary	19.24	20.88	28.16	28.76	8.92	7.88	-0.19	0.50	1.14	0.71	0.92	0.31	0.76

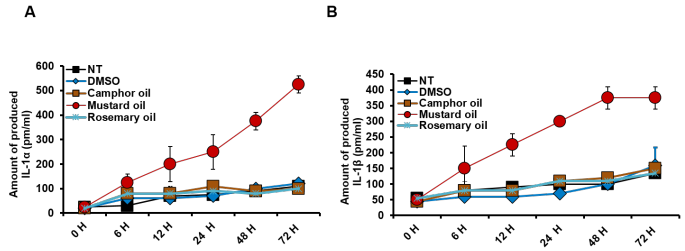

Figure 5: Inflammatory cytokine levels in treated Caco-2 cells. **(A)** The concentration of IL-1α (pm/mL) in the culture media of Caco-2 cells exposed to 6.125 µL/mL of camphor, mustard, or rosemary oil at the specified time points, compared to DMSO-treated cells. **(B)** The concentration of IL-1β (pm/mL) produced by Caco-2 cells subjected to 6.125 µL/mL of camphor, mustard, or rosemary oil at the indicated time points, in comparison to DMSO-treated cells. Error bars represent the Standard Deviation (SD) from three replicate experiments.

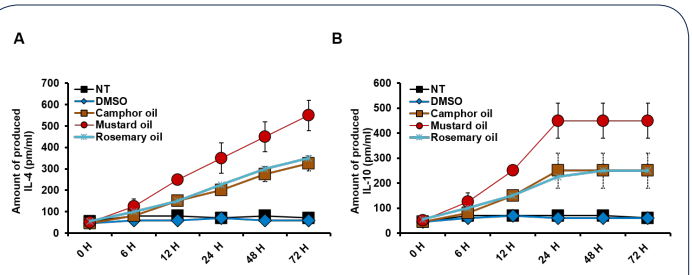

Figure 6: Anti-inflammatory cytokine levels in treated Caco-2 cells. **(A)** The concentration of IL-4 (pm/mL) in the culture media of Caco-2 cells exposed to 6.125 µL/mL of camphor, mustard, or rosemary oil at the specified time points, compared to DMSO-treated cells. **(B)** The concentration of IL-10 (pm/mL) produced by Caco-2 cells subjected to 6.125 µL/mL of camphor, mustard, or rosemary oil at the indicated time points, in comparison to DMSO-treated cells. Error bars represent the Standard Deviation (SD) from three replicate experiments.

highly significant, with a P-value of 0.01. Additionally, CAS3 gene expression was significantly upregulated, reaching a 6-fold increase in mustard oil-treated cells compared to the control and other oil treatments (Figure 4B). The statistical analysis (Table 5) showed that this increase in CAS3 expression was also highly significant, with a P-value of 0.01. Overall, these findings suggest that mustard oil treatment effectively enhances P53 expression, which may contribute to the stimulation of apoptotic signaling, as evidenced by the overexpression of CAS3 in colon cancer cells.

Mustard oil promotes the production of IL-1α and IL-1β in colon cancer cells: To investigate the molecular interactions that may regulate PCD in Caco-2 cells treated with mustard oil, a time-course experiment was conducted to assess the production of proinflammatory cytokines, specifically IL-1α and IL-1β, using an ELISA assay. Treatment with mustard oil at a concentration of 6.125 µL/mL resulted in a time-dependent increase in IL-1α production, reaching 500 pm/mL, while cells treated with other

oils showed similar IL-1α levels to control-treated cells (Figure 5A). Similarly, IL-1β production significantly increased in mustard oil-treated cells, reaching approximately 350 pm/mL, whereas cells treated with other oils exhibited lower IL-1β concentrations over time, similar to control-treated cells (Figure 5B). These findings suggest that mustard oil may play a role in modulating cytokine levels, which are known to be critical in regulating PCD in colon cancer cells.

Mustard oil promotes the production of anti-inflammatory cytokines IL-4 and IL-10 in treated cells: IL-4 and IL-10 are known to play critical roles in regulating inflammation within cells. Therefore, we assessed their production levels in Caco-2 cells treated with mustard oil and compared them to the levels induced by camphor and rosemary oils in a time-course experiment using an ELISA assay. Our results indicated that treatment with mustard oil at a concentration of 6.125 µL/mL led to a time-dependent increase in IL-4 production, peaking at 500 pm/mL at 72 hours post-treatment. In comparison, camphor and rosemary oil treatments also resulted in a marked increase in IL-4 levels over time, though the peak was only 300 pm/mL at 72 hours (Figure 6A). Similarly, mustard oil treatment at the same concentration also caused a time-dependent increase in IL-10 production, reaching 400 pm/mL at 24 hours and continuing at this elevated level through 72 hours post-treatment. While camphor and rosemary oils also induced significant increases in IL-10 production up to 24 hours post-treatment, their levels plateaued at 200 pm/mL by that time (Figure 6A). These findings highlight the anti-inflammatory effects of mustard oil, as well as camphor and rosemary oils, suggesting that mustard oil may regulate inflammatory events through the Programmed Cell Death (PCD) of colon cancer cells.

Discussion

While scientists have made significant progress in understanding the development of colon cancer, much more research and data are still needed. Colon cancer has multiple causes, and various signaling pathways play a role in its progression. Recently, new tools and techniques have been developed to aid in the analysis of cellular signaling and gene expression associated with colon cancer [32,22]. In this study, we aimed to explore the effects of natural oils, including mustard, camphor, and rosemary oils, on regulating colon cancer development with minimal cytotoxicity. We focused on their impact on the cellular immune response in Caco-2 colon cancer cells. Our findings provide evidence for the potential anticancer properties of mustard oil, demonstrating its ability to inhibit the transcription factors NFKB and cMyc and induce PCD in treated Caco-2 cells. This is achieved through the stimulation of IL-1α and IL-1β, with minimal toxic effects on normal colon epithelial cells compared to camphor and rosemary oils.

Mustard oil, derived from Brassica nigra, an annual herb grown primarily in the Mediterranean region and various Southeast Asian countries, has long been used in traditional medicine to treat conditions such as neuralgia, spasms, alopecia, snakebites, epilepsy, toothaches, and several forms of cancer. This plant is also known for stimulating hair growth, while mustard flour serves as an effective antiseptic. The seeds of B. nigra contain about 4% isothiocyanates (including sinigrin and myrosin), with allyl isothiocyanate making up more than 90% of these compounds. Additionally, the seeds are rich in protein (around 30%), fixed oil (27%), and other compounds such as inositol, lecithin, albumins, and mucilage [33]. Brassica vegetables, including mustard, have demonstrated cancer-preventive and therapeutic properties against a wide range of cancers, including ovarian, colon, bladder, lung, and breast cancers [34]. In line with this, extracts of B. nigra, from ethanol, hexane, and ethyl acetate, have shown antiproliferative activity against human hepatocellular (HepG2), cervical (HeLa), Colorectal (HCT), and breast carcinoma (MCF-7) cells. Moreover, B. nigra extract has been found to protect HepG2 cells from benzo [a] pyrene-induced DNA damage, likely through the induction of detoxification enzymes [35]. Allyl isothiocyanate, a compound found in mustard seeds, has also demonstrated cytotoxic effects on lung and bladder cancer cells [36,37]. Further studies indicate that dietary mustard seeds can suppress azoxymethane-induced colon adenomas in mice and dimethylhydrazine-induced colorectal carcinomas in rats [38,39]. Additionally, allyl isothiocyanate has been shown to inhibit the growth of Ehrlich ascites tumors in mice through pro-apoptotic and anti-angiogenic mechanisms [40]. Sinigrin, the main phytochemical in B. nigra, has also been reported to inhibit diethylnitrosamine-initiated hepatocarcinogenesis in rats [41].

Cancer cells often exhibit overactive cell proliferation signals, including the MAPK pathway and autophagy, along with an overexpression of oncoproteins and elevated secretion levels of pro-inflammatory cytokines such as IL-1α and IL-1β [42-45]. Our results support the role of mustard oil in regulating cancer cell proliferation by decreasing the expression of transcription factors like NFκB, while promoting the overexpression of tumor suppressor P53 and apoptosis-related CAS3 in treated Caco-2 cells. The Raf/MEK/ERK pathway, a critical signal transduction route for receptor tyrosine kinases and the small GTPase Ras, plays an essential role in regulating cell survival, cycle progression, and differentiation. Specifically, the Ser/Thr kinase Raf (c-Raf-1, Raf-B, or Raf-A) activates MEK1 and MEK2, which in turn phosphorylate the ERK1 and ERK2 kinases [46]. his pathway also influences the activity of various apoptotic proteins, including Bim, Bax, CAS9, CAS3, and P53, ultimately modulating autophagosomal formation and contributing to programmed cell death [47,48]. Mechanistically, mustard oil may influence TP53 mRNA expression by activating the mitochondrial-mediated apoptotic pathway, which leads to the upregulation of Bax (Bcl-2 associated x protein). This, in turn, triggers the expression of CAS9 and CAS3 at both the mRNA and protein levels [33]. The cytotoxic effects of mustard oil on cancer cells could be attributed to the overproduction of pro-inflammatory cytokines IL-1α and IL-1β, which are indicators of necrosis. To assess the level of cell death, the LDH assay, also known as the LDH release assay, is commonly used to evaluate plasma membrane damage. LDH, a stable enzyme present in all cells, is rapidly released into the culture medium when the plasma membrane is compromised or when the cell undergoes lysis. When exposed to a cytotoxic compound, cells may either cease to grow and divide or undergo cell death via necrosis or apoptosis. In our study, the LDH production in the culture media of the Caco-2 cell line treated with mustard oil showed a variable increase. As shown in (Table 3) and (Figure 1B), untreated Caco-2 cells and those treated with other oils, DMSO, or Triton X-100 displayed minimal differences. However, a significant increase in LDH levels was observed in Caco-2 cells treated with mustard oil, with a highly significant increase noted in cells treated with Triton

X-100, a known toxic compound. No significant increase in LDH levels was found in Caco-2 cells treated with DMSO or other oils.

Conclusion

This study investigated the biological functions and potential anticancer effects of several natural oils, including camphor oil, mustard oil, and rosemary oil, on colon cancer Caco-2 cells. We assessed cell viability and the number of surviving cells following treatment with these oils, comparing the results with control groups treated with DMSO. Additionally, Lactate Dehydrogenase (LDH) production was measured as an indicator of necrosis after treatment. To understand the role of specific genes in cell proliferation and PCD, we analyzed the relative expression levels of NFκB, cMyc, P53, and caspase 3 (CAS3) using qRT-PCR. The results showed that mustard oil treatment significantly reduced the viability of Caco-2 cancer cells in a dose-dependent manner, with minimal impact on the viability of Normal Colon Cells (NCM-460), unlike camphor and rosemary oils. Notably, treatment with 6.1 µL/mL of mustard oil led to a marked decrease in the number of survived cancer cells, while sparing normal cells. Moreover, LDH production was significantly elevated in mustard oil-treated Caco-2 cells after 24 hours, indicating cell damage. Gene expression analysis revealed that mustard oil treatment significantly decreased the expression of NFκB and cMyc in Caco-2 cells, compared to other treatments and control cells. Additionally, the expression of P53 and CAS3 genes was notably increased in mustard oil-treated Caco-2 cells, accompanied by an overproduction of IL-1α and IL-1β, while the other oils resulted in reduced levels of these cytokines. Furthermore, the levels of IL-4 and IL-10 increased over time after mustard oil treatment, suggesting an anti-inflammatory effect. In conclusion, mustard oil treatment at varying concentrations promoted PCD, exhibited anti-inflammatory effects, and inhibited the expression of key oncogenes NFKB and cMyc in Caco-2 cells. These findings indicate that mustard oil may hold potential as a therapeutic agent for colon cancer.

Declarations

Authors' contributions: K.E. performed the experiments. S.M. helped conceptualize experiments, interpret data, and prepare the manuscript. H.K designed the research plan, supervised overall research, provided and interpreted data, organized, and wrote the manuscript.

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