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Original Research Article

Anti-Cancer Properties of Clove Bud Essential Oil in Colon Cancer Cell Line

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Abstract

Colon cancer is a type of cancer that begins in the large intestine (colon). Its aggression is owing to late diagnosis so poor prognosis and higher mortality rate are reported. Colon cancer has become a thorny research region that requires more examination in cellular pathways involved in its emergency. Here we aim to investigate the possible anticancer properties of the clove oil as natural and popular oil in colon cancer CaCo-2 cells. Accordingly, cell viability rate and number of survived cells were monitored in response to the clove oil treatment compared with the black seed oil and DMSO-treatment. Lactate dehydrogenase (LDH) production was also considered as an indicator for necrotic event following treatment. The relative gene expression of *Raf-1*, *MEK*, and *ERK* was detected as proliferation effectors that linked to the expression profile of the tumor suppressor genes *PTEN* and *TP53* using quantitative real-time PCR (qRT-PCR). Interestingly, here we identified Raf-1 pathway as a potential targeted singling by the clove oil resulted in controlling colon cancer cell proliferation. The current study elucidates the anticancer activities of the clove oil in colon cancer division through restoring the relative gene expression of *PTEN* and *TP53* accompanied by over-production of interleukin 1 alpha (IL-1α) and IL-1β. Accordingly, treatment of the CaCo-2 cells with different concentrations of the clove oil increased programmed cells cell death (PCD) and inhibited the proliferation singling when compared with the black seed oil. Collectively, our results demonstrate that controlling of Raf-1 activity and PCD by clove oil treatment provide evidence for its anticancer effect in colon cancer cells.

Keywords: Colon cancer cell line, clove oil, black seed oil, Raf-1 signaling, proinflammatory cytokines.

INTRODUCTION

The colon, or large intestine, is where the body draws out water and salt from solid wastes. The waste then moves through the rectum and exits the body through the anus. Colon cancer is the third most common cause of cancer-related death in the U.S. (Xi and Xu, 2021). Until now colon cancer remains a hot area for researchers to inspect and find a new solution for this endemic disease. Furthermore, because of the occurrence of side effects and the emergence of drug resistance, there is an urgent need to find new and more effective drugs for colon cancer and colorectal cancer (CRC) treatment (Maher *et al*., 2020).

Several cellular signaling have been identified in colon cancer such as protein kinase C (PKC) signaling pathway (Islam *et al*., 2018). PKC is a multifunctional family of serine/threonine kinase that modulates various cellular events such as cell growth, differentiation and cell death. The activation of PKC signaling pathway protects the cells from apoptosis and regulates several cellular processes (Black and Black, 2013). Noteworthy, the mechanism by which the normal cells can successfully replicate includes cyclin-dependent kinases (CDKs), and the epidermal growth factor receptor (EGFR) (Wee and Wang, 2017). The EGFR particularly stimulates downstream targets of the pro-oncoproteins family such as RAS-RAF-MEK-ERK and AKT-PI3K-mTOR signaling pathways (Zhang and Liu, 2002). In cancer cells, the overexpression of EGFR and the mutant proteins KRAS, BRAF, PIK3CA have been linked to a dynamic change that occurs in cancer cells leading to drug resistance, distinct oncogenicity alteration, and malignancy transformation (Sobani *et al*., 2016; Balcik-Ercin *et al*., 2020). Targeting EGFR

by anticancer drugs blocks the signal transduction pathways required to control cancer cell growth, proliferation, and resistance to cell death (Wykosky *et al*., 2011; Xie *et al*., 2020; Hamouda *et al*., 2021; Taher *et al*., 2021). Among these drugs are tyrosine kinase inhibitors such as Sorafenib (SOR), also known as Nexavar, which targets Raf-1 activation and blocks its downstream activity in hepatocellular carcinoma (HCC) (Ben Mousa, 2008).

On the other hand, accumulated evidences have demonstrated that many natural products possess effective anti-CRC effects and may serve as alternative chemotherapy agents for CRC treatment (Huang *et al*., 2019)**.** Cloves (*Syzygium aromaticum*) have been used as a traditional Chinese medicinal herb for thousands of years. Cloves possess antiseptic, antibacterial, antifungal, and antiviral properties, but their potential anticancer activity remains unknown (Alexa *et al*., 2022). Likewise, the black seed (*Nigella sativa*) herb and oil have been used in Asia, Middle East and Africa to promote health and fight disease. The antioxidant, anti-inflammatory, and antibacterial activities of *Nigella sativa* extracts are well known (Ahmad *et al*., 2013). Particularly, Thymoquinone (TQ) is a promising dietary chemopreventive agent found in the black seed. Its anticancer activities were attempted in HCT-116 human colon cancer cells to identify its potential molecular mechanisms of action. It has been reported that TQ can inhibit the growth of colon cancer cells which was correlated with G1 phase arrest of the cell cycle (Gali-Muhtasib *et al*., 2004).

Accordingly, in this study, we investigated the anti-cancer properties of some natural products including clove oil and black seed oil *in-vitro* through assessing the potential anticancer activity of these compounds in colon cancer cells. Notably, we generalize the experiment-based molecular mechanisms and the regulatory networks whereby the clove oil exerted anticancer effects on cell proliferation and programmed cell death (PCD).

MATERIALS AND METHODS

Preparing natural oils

Natural oils, clove (*Syzygium aromaticum)* and black seed oil (*Nigella sativa)*, were firstly filtered by using Millx-GP Filter, 0.22µm (Sigma, USA) and collected in clean and sterilized eppendorf tubes. Different concentrations from oils were prepared in dimethyle sulfoxide (DMSO) including 50µl/ml, 100µl/ml, 150µl/ml, 200µl/ml, and 250µl/ml. The final extract was incubated at 4°C until used.

Cell lines

Colon cancer cells (CaCo-2 cell line) were grown in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 4 mM L-glutamine, 4 mM sodium pyruvate, and 5% of heat-treated bovine serum albumin (BSA). The cells were cultured in 75ml cell-culture flask and were incubated at 37°C under 5% CO_2 condition (Khalil *et al*., 2017; Abd El Maksoud *et al*., 2019). The imaging of cultured cells was determined by using inverted microscopy with a Zeiss A-Plan 10X.

Cytotoxic concentration 50% (CC_{50})

The prepared oil extracts were tested for their cytotoxic effect and the potential CC_{50} in CaCo-2 cells line was calculated. Accordingly, the cells were cultured in 96-well plates in a density of $10X10^3$cells/well and were incubated in CO_2 incubator at 37°C. The cells were then treated with different concentration of each indicated extract (50-250µl/ml) followed by overnight incubation. The cell viability rate and cytotoxic concentration were monitored by using MTT cell growth assay kit (Sigma-Aldrich, Germany), based on the amount of formazan dye which has been measured by measuring absorbance at 570nm.

Lactate dehydrogenase (LDH) production

LDH assay kit (Abc-65393) was used to assess LDH production in the fluid media that collected from cultured-treated cells. According to the manufacture procedures; 100µl of lysed cells was incubated with 100µl LDH reaction mix for 30min at room temperature. LDH activity was quantified by a plate reader at OD450nm. The relative LDH production was calculated by dividing the absorbance mean values of treated cells by the mean values of RPMI media (mock values) indicated by fold change of LDH production (Khalil, 2012; Khalil *et al*., 2019).

Enzyme-linked immunosorbent assay (ELISA)

ELISA assay used for the quantification analysis of the released tumor necrosis factor-alpha (TNF-α) and interleukin-1 alpha (IL-1α) using human ELISA kits (Abcam 46042 and Abcam 181421, respectively). CaCo-2 cells cultured in 96-well plates were overnight incubated. Then the cells were treated with either 100µl/ml of each indicated extract followed by incubation period of (0, 6, 12, 24, 36, 48, and 72 hrs). At each time point, the cells were lysed using 1X cell lysis buffer (Invitrogen, USA) then 100 µl of the lysed cells were transferred into the ELISA plate reader and were incubated for 2 hrs at RT with 100 µl control solution and 50µl 1X biotinylated antibody. Then 100 µl of 1X streptavidin-HRP solution was added to each well of samples and incubated for 30 min in the dark. 100 µl of the chromogen TMB substrate solution was added to each well of samples and incubated for 15 min at RT away from the light. Finally, 100 µl stop solution was added to each well of samples to stop the reaction. The absorbance of each well was measured at 450 nm (Abd El Maksoud *et al*., 2019).

Quantitative real time PCR (qRT-PCR)

The quantification analysis of genes expression was detected by using qRT-PCR in which the cellular total RNA was obtained using TriZol

(Invitrogen, USA) and was purified by using RNA purification kit (Invitrogen, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using M-MLV reverse transcriptase (Promega, USA). The quantification analysis of mRNA expression of Raf-1, MEK, ERK, PTEN, and p53 was achieved using QuantiTect-SYBR-Green PCR Kit (Qiagen, USA) and the specific primers listed in table 1. The housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression level was used for normalization in the real-time PCR data analysis. The PCR reaction system conatined 10 µl SYBR green, 0.5 µl RNase inhibitor (50 U/µl), 0.2 µM of each primer, 2 µL of synthesized cDNA, and nuclease-free water up to a final volume of 25 µL. The following PCR conditions were used; 94°C for 5 min, 40 cycles (94°C for 30 sec, 60°C for 15 sec, 72°C for 30 sec) (Khalil *et al*., 2019; El-Fadl *et al*., 2021).

Data analysis

All histogrames and charts were prepared by Microsoft Excel. Delta-Delta Ct analysis was used in the quantification analysis of mRNA delivered from qRT-PCR assay based on the following equations: (1) delta-Ct = Ct value for gene- Ct value for GAPDH, (2) (delta–delta Ct) = delta Ct value for experimental –delta Ct for control), (3) Quantification fold change = ($2^{-\text{delta-delta ct}}$) (Rao *et al*., 2013; Khalil H *et al*., 2017). The student's two-tailed t-test was used for statistical analysis. P-value ≤ 0.05 was considered statistically significant.

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

Description	Primer sequences 5'-3'
Raf-1-forward	TTTCCTGGATCATGTTCCCCT
Raf-1-reverse	ACTTTGGTGCTACAGTGCTCA
MEK1- forward	GACCTGCGTGCTAGAACCTC
MEK1- reverse	TCTGGACGCTTGTAGCAGAG
ERK1- forward	CCTGCGACCTTAAGATTTGTGATT
ERK1- reverse	CAGGGAAGATGGGCCGGTTAGAGA
PETN- forward	TGCGGTGACATCAAAGTAGAG
PETN- reverse	CTCTGGTCCTGGTATGAAGAATG
P53- forward	GCGAGCACTGCCCAACAACA
P53- reverse	GGTCACCGTCTTGTTGTCCT
GAPDH- forward	TGGCATTGTGGAAGGGCTCA
GAPDH- reverse	TGGATGCAGGGATGATGTTCT

RESULTS

CaCo-2 cell viability influenced by clove seed oil compared with black seed oil

To investigate the potential cytotoxic effect of clove oil in cell viability rate, MTT assay and relative LDH production were achieved in Caco-2 that pretreated with different concentrations of either clove oil or black seed oil. As shown in Figure 1A and Table 2, the mean absorbance values revealed that cell viability rate of Caco-2 cells was markedly decreased in a dose-dependent manner of clove oil treatment, while showed constant rat in response to black seed oil treatment. Furthermore, the relative LDH production was significantly increased in cancer cells treatment with clove oil (150µl/ml) for 24 hrs compared with other control-treated cells (Figure 1B and Table 3). Together, these data suggest the potential anticancer properties of clove oil and excluding the possible anticancer activities of the black seed oil.

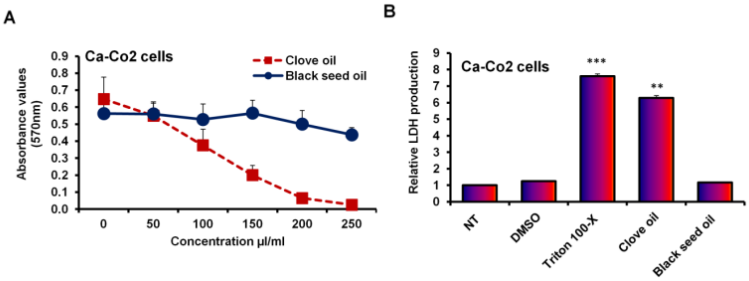

Figure 1: Cytotoxicity of each of Clove oil and Black seed oil on Caco-2 cells; A: Absorbance values at 570nm in relation to concentration (µl/ml). B: Relative LDH production in relation to nontreated (NT) Caco-2 cells and those treated with each of clove oil, black seed oil, DMSO and Triton 100-X. Error bars indicate standard deviation (SD) of four different replicates. Student's two-tailed *t*-test was used to determine the significance of differentiated values. (**) indicates ≤0.01 and (***) indicates the *P* ≤0.001

Table 2: Cell viability rate of oil-treated cells

Treatment	Concentration (µl/ml)	Mean Absorbance (570nm)	Standard deviation	Student two tails t-test	*P*-values
Clove oil	**0.0¥**	0.65	0.01		
	50	0.55	0.08	0.25	> 0.05
	100	0.38	0.10	0.013	≤ 0.01**
	150	0.20	0.06	0.007	≤ 0.01**
	200	0.07	0.03	0.001	≤ 0.01**
	250	0.03	0.02	0.001	≤ 0.01**
Black seed oil	**0.0¥**	0.56	0.01		
	50	0.56	0.08	0.96	> 0.05
	100	0.53	0.08	0.63	> 0.05
	150	0.57	0.07	0.97	> 0.05
	200	0.50	0.09	0.37	> 0.05
	250	0.44	0.15	0.06	> 0.05

¥: DMSO treatment (Control)
**: Highly significant values

Table 3: Relative LDH production in treated CaCo-2 cells

	NT	DMSO	Triton 100-X	Clove oil	Black seed oil
Mean absorbance	0.044	0.055	0.33	0.28	0.05
STD	0.01	0.02	0.15	0.15	0.01
Relative LDH production	1	1.26	7.60	6.29	1.17
P values		0.3159	0.009**	0.02*	0.68

Clove oil amended cancer cell morphology and the number of living cells

Cell morphology and the number of survived cells were assessed in both treated cells with clove oil or black seed oil (150µl/ml) using an inverted microscope. Markedly, the cell morphology revealed a disturbing proliferation of Caco-2 cells treated with the clove oil compared with the cell morphology of other treated cells (Figure 2A). The number of living cells significantly decreased in response to clove oil treatment only in colon cancer cells. Meanwhile, the same concentration of the black seed oil showed typical number of living cells when compared with control-treated cells (Figure 2B and Table 4). These findings demonstrate the effect of clove oil treatment in the Caco-2 cells.

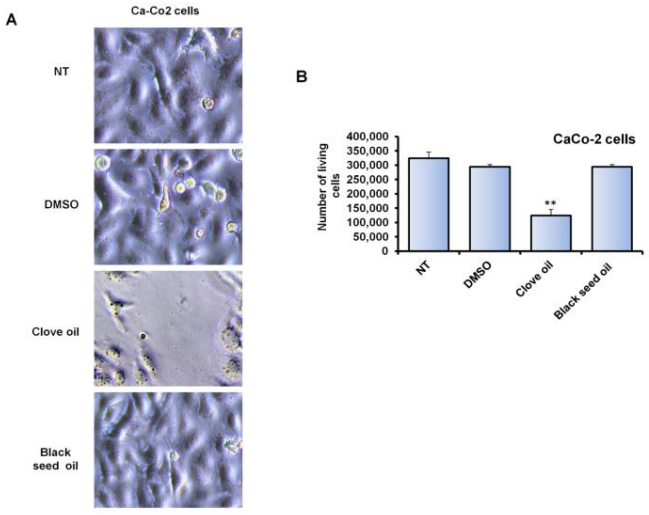

Figure 2: Caco-2 cell morphology and number of survived cells upon treatment with Clove seed oil and Black seed oil; A: Representative cell morphology indicated by inverted microscopy reveals the cell viability of both nontreated (NT) Caco-2 cells and those treated with black seed oil or clove seed oil or DMSO. B: Number of survived Caco-2 cells treated with black seed oil or clove seed oil or DMSO compared with Caco-2 nontreated cells. Error bars indicate SD of two independent experiments. Student's two-tailed *t*-test was used to determine the significance of differentiated values. (**) indicates *P* ≤0.01

Table 4: Number of survived cells upon treatment

	DMSO	Clove oil	Black seed oil
Mean	295000	125000	295000
STD	7071.07	21213.20	7071.07
P values	0.198	0.011*	0.198

STD: Standard deviation of three independent experiments

*: Indicates significant P values ≤ 0.05

**: Indicates high significant *P* values ≤0.01

Clove oil targets Raf/MEK/ERK singling pathway in treated colon cancer cells

To assess the biological role of the clove oil in regulating cancer cell proliferation, the expression profile of cell proliferation effectors was monitored in treated Caco-2 cells. Accordingly, the relative expression of *Raf-1*, *MEK*, and *ERK* gene was monitored in treated cells using qRT-PCR with a satisfactory, sensitive, and specific assay indicated by the melting curve showed in Figure 3A. Interestingly, the relative expression of all indicated effectors was dramatically down-regulated in cells treated with the clove oil (150µl/ml) compared to control cells and cells treated with the black seed oil (Figure 3B and Figure 4A and B). The statistical analysis represented in Table 5 indicated that the depletion of *Raf-1*, *MEK*, and *ERK* gene expression was highly significant as *P* values were equal to 0.01 in all cases. Together, these data suggest that clove oil can regulate the expression of *Raf-1* and inhibits the activation of Raf/MEK/ERK singling pathway in cancer treated cells.

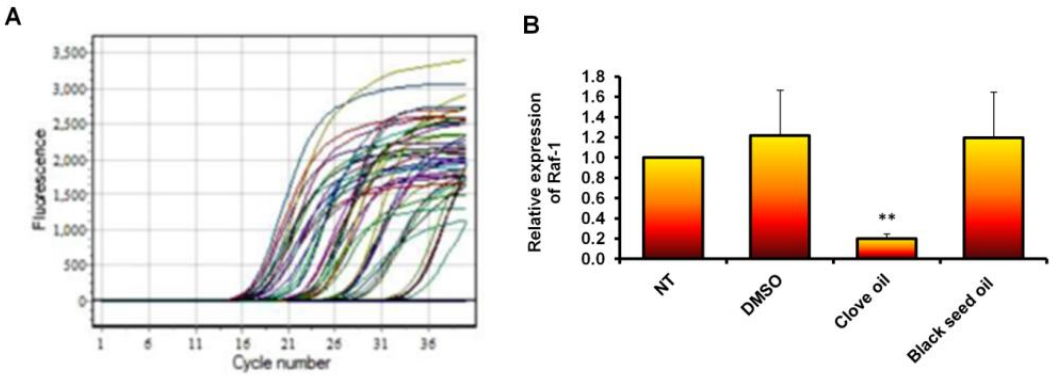

Figure 3: Relative gene expression of Raf-1 as a proliferation effector; (A) Melting curve of each amplified sample using QuantiTect-SYBR-Green PCR kit and qRT-PCR indicate the cycle threshold (CT) of each investigated genes (B) The mRNA expression level of *Raf-1* gene was quantified as indicated by fold change in treated Caco-2 cells compared with untreated cells (NT) and DMSO-treated cells. Error bars indicate the SD of two independent experiments. The student's two tailed *t*-test was used to determine the significance of differentiated Ct values. (*) indicates *P-values* ≤0.05, and (**) indicates the *P* ≤0.01.

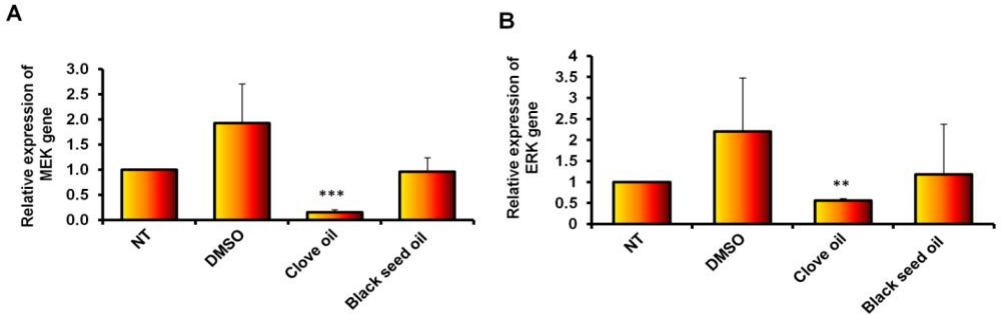

Figure 4: Expression profile of MEK and ERK as down-stream targets of Raf-1 effector; (A) Quantification of the mRNA expression level of the *MEK1* gene indicated by fold change in treated Caco-2 cells compared with untreated cells (NT) and DMSO-treated cells. (B) The mRNA expression level of *ERK* gene was quantified as indicated by fold change in treated Caco-2 cells compared with untreated cells (NT) and DMSO-treated cells. Error bars indicate the SD of two independent experiments. The student's two tailed *t*-test was used to determine the significance of differentiated Ct values. (*) indicates *P-values* ≤0.05, and (**) indicates the *P* ≤0.01

Table 5: Quantification analysis of Raf-1, MEK, and ERK in oil-treated cells

Genes	Condition	Expression fold changes	Standard deviation	Student two-tails t-test	*P*-values
Raf-1	NT	1.00	0.00		
	DMSO	1.22	0.45	0.56	> 0.05
	Clove oil	0.20	0.04	0.001	< 0.01**
	Black seed oil	1.20	0.45	0.60	> 0.05
MEK	NT	1.00	0.00		
	DMSO	1.92	0.78	0.23	> 0.05
	Clove oil	0.15	0.05	0.001	< 0.01**
	Black seed oil	0.95	0.28	0.85	> 0.05
ERK	NT	1	0.00	0.83	
	DMSO	2,2	1.2	0.25	> 0.05
	Clove oil	0.5	0.04	0.005	< 0.01**
	Black seed oil	1.1	0.21	0.84	> 0.05

The clove oil stimulates programmed cell death in cancer treated cells

To validate the impact of the clove oil in the programmed cell death (PCD), the expression profile of *PTEN* and *P53*, as tumor suppressor genes, was detected in treated Caco-2 cells. Notably, the relative gene expression of both *PTEN* and *P53* was strongly upregulated in cells treated with the clove oil compared to control-treated cells and cells treated with the black seed oil (Figure 5A and B). The statistical analysis in Table 6 indicated that the increasing levels of *PTEN* and *P53* gene expression were highly significant as *P* values were equal to 0.01. Collectively, these data suggest that clove oil treatment has the ability to restore the expression of *PTEN* and *P53* to stimulate programmed cells death in treated cells.

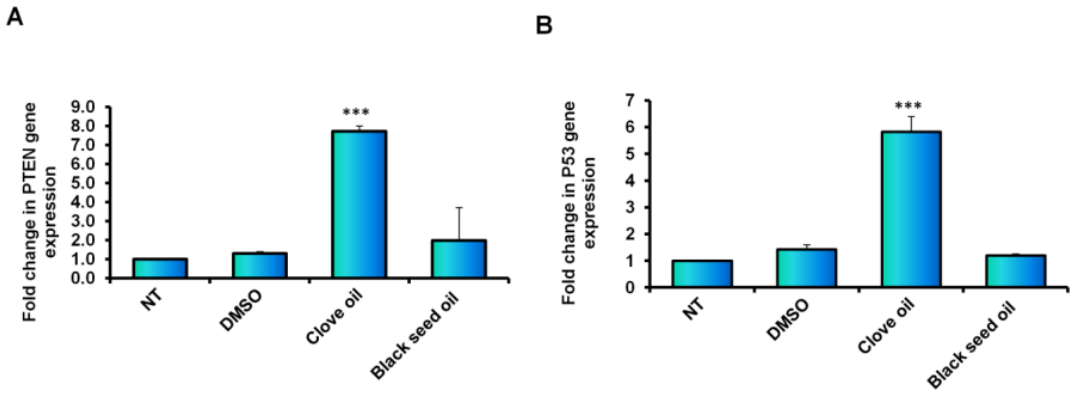

Figure 5: Expression profile of PETN and P53 as apoptosis regulators; (A) Quantification of the mRNA expression level of the *PTEN* gene indicated by fold change in treated Caco-2 cells compared with untreated cells (NT) and DMSO-treated cells. (B) The mRNA expression level of *TP53* gene was quantified as indicated by fold change in treated Caco-2 cells compared with untreated cells (NT) and DMSO-treated cells. Error bars indicate the SD of two independent experiments. The student's two tailed *t*-test was used to determine the significance of differentiated Ct values. (*) indicates *P-values* ≤0.05, and (**) indicates the *P* ≤0.01

Table 6: Quantification analysis of PTEN, and P53 in oil-treated cells

Genes	Condition	Expression fold changes	Standard deviation	Student two-tails t-test	*P*-values
PTEN	NT	1.00	0.00		
	DMSO	1.3	0.09	0.051	> 0.05
	Clove oil	7.7	0.02	0.001	< 0.01**
	Black seed oil	1.98	0.17	0.50	> 0.05
P53	NT	1.00	0.00		
	DMSO	1.4	0.17	0.07	> 0.05
	Clove oil	5.8	0.56	0.006	< 0.01**
	Black seed oil	1.2	0.05	0.06	> 0.05

The clove oil stimulates the production of TNF-α and IL-1α in colon cancer cells

To figure out the connection between the clove oil treatment and the production of proinflammatory cytokines in treated cells, TNF-α and IL-1α were considered in a time-course experiment using ELISA assay. Interestingly, the clove oil treatment showed a high production level of both TNF-α and IL-1α in a time-dependent manner. In contrast, the production level of both cytokines was strongly reduced in treated cells with the black seed oil and control-treated cells (Figure 6A and B). These findings reveal the role of clove oil in regulating the levels of inflammatory cytokines produced from treated cells and further validate its ability to stimulate PCD in colon cancer cells.

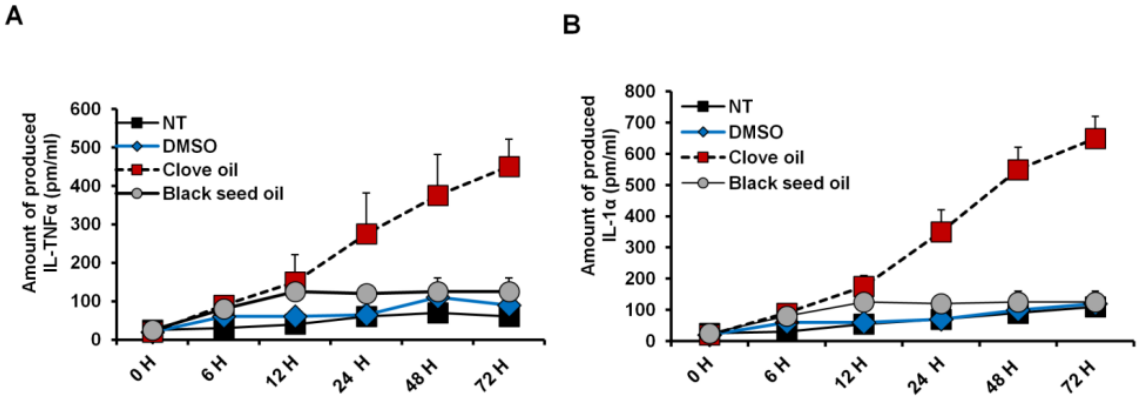

Figure 6: Levels of produced inflammatory interleukins in treated cells; (A) The concentration of produced TNF-α (pm/ml) in the fluids media of Caco-2 cells that were subjected to 150µl/ml of the clove oil or black seed oil at the indicated time points compared with DMSO-treated cells. (B) Level of produced IL-1α (pm/ml) from Caco-2 cells that were subjected to the same concentration of either clove oil or black seed oil for the indicated time points compared with DMSO-treated cells. Error bars reveal the SD of three replicates

DISCUSSION

Although scientists start underling the development of colon cancer cells, indeed, a lot of information and studies are required. Particularly, the colon cancer evidently has different causes, and there are definitely various signaling cascades are involved in cancer development. Recently, new tools and various techniques have been established to facilitate the analysis of cellular signaling and gene expression indicated in colon cancer cells. In this study, we aim to provide novel and efficacious natural oil including clove oil that can regulate colon cancer development with the minimum cytotoxic effects based on its influence on the cellular immune response in the colon cancer CaCo-2 cells. Interestingly, our findings provide evidence for the potential anticancer properties of the clove oil with the ability to induce PCD in treated CaCo-2 cells via stimulation of IL-1α and TNF-α.

The major oil found in clove (*Syzygium aromaticum*) which known as clove oil has been reported as a relevant protocol in herbal medicine to reduce pain and endorse healing, in addition to utilizing in the fragrance and flavouring industries (Cortés-Rojas *et al*., 2014). Clove oil contains two major agents, kwon as eugenol and β-caryophyllene, which represent 78% and 13% of the oil components, respectively (Prashar *et al*., 2006). Clove oil with these agents is generally considered as safe composition; however some *in-vitro* studies demonstrated the potential cytotoxic effect of both on human fibroblasts and endothelial cells. The cytotoxicity of clove oil was reported at concentrations of 0.03% (v/v), with up to 73% attributable to eugenol effect, while β-caryophyllene did not exhibit any cytotoxic properties, indicating that other safe components exist within the clove oil together with the harmful compounds (Prashar *et al*., 2006).

Indeed, in cancer cells, the cell proliferation signals such as MAPK and autophagy are over-stimulated accompanied by overexpression of several oncoproteins and high secretion levels of the pro-inflammatory cytokines such as IL-1α and TNF-α (Galluzzi *et al*., 2015; Abd El Maksoud *et al*., 2019; Elimam *et al*., 2020; Zappavigna *et al*., 2020). Our findings confirm the regulatory role of clove oil on cancer cell proliferation via reducing the expression profile of Raf-1 and its downstream effectors, MEK-1 and ERK factors, parallel with the overexpression of both tumor suppressor genes *TP53* and *PTEN* in treated CaCo-2 cells. Noteworthy, the Raf/MEK/ERK pathway is serving as a key signal transducer of receptor tyrosine kinases and the small GTPase, Ras. The Ser/Thr kinase Raf (c-Raf-1, Raf-B, or Raf-A) activates the downstream kinase MEK1 and MEK2 which, in turn, phosphorylate Ser/Thr kinases ERK1 and its homolog ERK2 (Shaul and Seger, 2007). The Raf/MEK/ERK pathway has key roles in regulating cell survival, cell cycle progression, and differentiation. Furthermore, the Raf/MEK/ERK pathway can also modulate the activity

of many proteins involved in the apoptotic signal such as Bim, Bax, casp-9, casp-3, and p53 which, in turn, regulates autophagosomes formation resulted in programmed cell death (McCubrey *et al*., 2007; Khalil *et al*., 2020).

Mechanistically, the effect of clove oil on *PTEN* and *TP53* mRNA may be related to activation of mitochondrial-mediated apoptotic pathway via increasing the expression of Bax (Bcl-2 associated x protein) which in turn stimulates the expression of casp-9 and casp-3 on mRNA and protein level (Li *et al*., 2011). Such cytotoxic effect of the clove oil on cancer cells may due to over-production of IL-1α, and TNF-α as a pro-inflammatory cytokines and indicators for necrotic event. In this way, the LDH assay, also known as LDH release assay, is a cell death cytotoxicity assay used to assess the level of plasma membrane damage in a cell population. LDH is a stable enzyme, present in all types of the cells, which is rapidly released into the cell culture medium upon damage of the plasma membrane or cell lysis. When treated with a cytotoxic compound, living cells may face one of two fates. They could either stop growing and dividing or die through either of two distinct processes; necrosis or apoptosis. Importantly, we found that the LDH production in the fluid media of the Caco-2 cell line was variably increased. Data in Table (3) and Figure (1B) showed that the nontreated Caco-2 cells and those treated with DMSO, Triton 100-X, Clove oil and Black seed oil had relative cytotoxicity values of 1.00, 1.26, 7.60, 6.29 and 1.17, respectively. This result clearly showed significant increase of LDH in Caco-2 cells treated with clove oil and highly significant increase due to Triton-100X treatment, as a toxic compound. Meanwhile, insignificant increase in relative LDH were produced in Caco-2 cells treated with either DMSO or black seed oil.

Authors' Contributions

Walaa Salah performed the experiments. Salwa E. Mohamed assisted in designing the research plan and data analysis. Adel A. Guirgis and Shaden Muawia helped conceptualize experiments, interpret data, and prepare the manuscript. Hany Khalil designed the research plan, supervised overall research, provided and interpreted data, organized, and wrote the manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest: All authors declare that there are no conflicts of interest.

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