Prophylactic Effects of *Syzygium aromaticum* L. and *Cynara Cardunculus* L. Extracts against d 7,12-dimethylbenz(a)anthracene (DMBA)-Induced Colorectal Cancer in Male Albino Rats

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Abstract

Colorectal cancer is the third most familiar cancer worldwide. In recent years, colorectal cancer incidence and mortality have increased dramatically in a number of emerging countries. Spices and flavoring plants, are gaining a lot of interest as a potential source of cancer-fighting substances these days. Clove is a spice and food flavour made from the sun-dried unopened flower bud of the plant *Syzygium aromaticum L.* The *Cynara cardunculus L.* has evidenced also as anticancer protective compounds on different types of cancer. This study aimed to evaluate the protective potential impact of acetonic extract of clove and ethanolic extract of *Cynara cardunculus* L. in a rat model with induced d 7,12-dimethylbenz(a)anthracene (DMBA) for 3 months. Our results demonstrated that *Cynara cardunculus* & *Syzygium aromaticum* extract induced apoptosis via activation of P53 and caspase-3 in addition to a down regulation of Bcl-2. Our results also showed that the two extracts have a significant inhibitory role on NF-κB and IL-1 β levels. Finally, this study suggested that using clove and *Cynara cardunculus* extracts may represent a protective antitumor compound against induced DMBA in rats.

Keywords Colorectal cancer, *Syzygium aromaticum L, Cynara cardunculus*, Apoptosis, 7,12-dimethylbenz[a]anthracene**.**

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Introduction

Colorectal cancer (CRC) is considered the world's second-deadliest cancer and the third-most-common malignancy. For many years, cancer patients have relied on surgery and chemotherapy as their primary treatments.[1] Chemoprevention is a popular therapeutic option for a wide range of cancers. The therapeutic use of natural products as anticancer drugs to prevent or delay cancer development has gotten a lot of interest.[2]

Cloves, *Syzygium aromaticum L,* dried buds, have been used as a spice and in Chinese and Indian medicine for centuries. Oleanolic acid, a pentacyclic triterpene present in a variety of herbs, fruits, and vegetables, appears to be one of the active chemicals found in cloves.[3] It has a wide range of pharmacological and biochemical effects, including anti-inflammatory, cardiovascular, antihyperlipidemic, antioxidant, and hypoglycemic effects.[4, 5]

In the Mediterranean diet, the globe artichoke (*Cynara cardunculus*) is commonly used as food and medicine, caffeoylquinic acid polysaccharide, fatty acids, triterpenes, and sesquiterpenes are among the components found in it.[6] β-amyrin is one of the common pentacyclic triterpenoid molecule found in petroleum ether ethanolic extract of *Cynara cardunculus* hydro ethanolic extract. *In vitro* and *in vivo* studies have shown that β-amyrin has antimicrobial, antidepressant, anti-inflammatory, antinociceptive, and gastroprotective effects.[7]

Cell death is necessary for multicellular organisms' development, tissue homeostasis, and integrity. The most common types of cell death are necrosis and programmed cell death. Programmed cell death, also named as apoptosis, is a genetically programmed process of cell suicide in response to specific signals. Normally, programmed cell death is regulated by several extracellular and intracellular signals that are influenced by the cell's surroundings and intracellular signals. Intrinsic and extrinsic apoptosis are the two types of apoptosis.[8] Pro-apoptotic, anti-apoptotic, and Bcl-2 regulator proteins are part of the Bcl-2 family. Apoptosomes are made up of the apoptotic protease-activating factor-1 and cytochrome C, which cleaves the pro-caspase-9 and subsequently activates caspase-3, resulting in apoptosis.[9]

According to the above mentioned, this study aimed to reveal the protective impact of acetonic extract of clove and hydro ethanolic extract of *Cynara cardunculus* in induced colorectal cancer DMBA rat model our results demonstrated that *Cynara cardunculus* & *Syzygium aromaticum* extracts by activation of P53, Bax and caspase-3 and down regulation of Bcl-2 causes cell apoptosis.

Material and methods

Materials and Extraction Procedure

Clove buds used for the current study was purchased from local market in Zagazig, Egypt. *C. cardunculus* was collected from the garden of Faculty of Pharmacy, Zagazig University. All other chemicals used in this experiment were purchased from sigma Aldrich (St. Louis, MO).

About 500 gm of the air-dried flower buds of Syzygium aromaticum was milled and extracted with acetone till exhaustion. The resulting crude extract was evaporated under vacuum to give (20 gm) of acetone extract (4 % w/w). The air dried powdered aerial parts of *Cynara cardunculus* (2 kg) was extracted with 80% ethanol. The resulting ethanolic extract was evaporated under vacuum to give (200 gm) of alcoholic extract (10 % w/w).

Sample derivatization

The samples were extracted, dried and resuspended in 50 µL of bis(trimethylsilyl)trifluoroacetamide (BSTFA)+ trimethylchloro-silane (TMCS) 99:1 silylation

reagent and 50 µL pyridine for derivatization samples functional groups to trimethylsilyl groups (abbreviated TMS) prior to GC-MS analysis.

Gas chromatography–mass spectrometry analysis (GC-MS)

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness). Analyses were carried out using Hydrogen as the carrier gas at a flow rate of 2.0 ml/min at a splitless, injection volume of 2 µl and the following temperature program: 50 °C for 5 min; rising at 5 °C /min to 100 °C and held for 0 min and rising at 10 °C /min to 320 °C and held for 10 min. The injector and detector were held at 280 °C, 320 °C. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 25-700 and solvent delay 6 min. The mass temperature was 230°C and Quad 150 °C. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Study protocols

Sprague-Dawley male albino rats (n = 40) weighing 200 to 250 g, obtained from the Animal House of the Faculty of Veterinary Medicine, Zagazig University. The rats were randomly divided into 4 groups each group (n=10). Negative control group 1 the animals left without any treatment. Positive control group 2 the induction of CRC to animals was done by DMBA (25 mg/rat) orally, 2 times per week for 3 months.[10] Group 3 the animals were received the extract of clove (50 mg/kg b.wt) for 30 days orally by stomach tube once daily then induced CRC by DMBA.[11] Group 4 the animals were received the extract of *Cynara cardunculus* (500 mg/kg b.wt) for 30 days orally by stomach tube once daily then induced CRC by DMBA.[12] The blood samples were collected from orbital venous plexus. The serum samples were preserved at -20 °C until used. The rat colon tissues were harvested and processed in several steps until used. The study was approved by the Institutional Animal Care and Use Committees Zagazig University (ZU-IACUC) with approval No. ZU-IACUC/2/F/197/2021.

Molecular determinations

Total RNA was extracted using the PureLink® RNA Mini Kit (Catalog numbers: 12183018A). NanoDrop® ND-1000 Spectrophotometer, was used to assess the purity of RNA samples. The High-Capacity cDNA Reverse Transcription Kit, number 4374966, was used to synthesize cDNA. To measure the gene expression, we used a Maxima SYBR Green qPCR Master Mix (2X) kit (cataloug #K0251). β-actin was used to standardize gene expression (Table 1).

Table 1 Primer sequence of apoptotic and anti-apoptotic markers.

Gene	Sequence	Gene bank accession No.
P53	F 5′-AACTATGGCTTCCACCTGGGCTTC-3′ R 5'-TGGTCTTCGGGTAGCTGGAGTGAG-3'	NM_030989
Caspase-3	F 5′-CGATTATGCAGCAGCCTCAA-3′ R 5'-AGGAGATGCCACCTCTCCTT-3'	NM_012922.2
Bcl-2	F 5′-TGCGCTCAGCCCTGTG-3' R 5'-GGTAGCGACGAGAGAAGTCATC-3'	NM_016993
β-actin	F 5′-CGTTGACATCCGTAAAGAC-3′ R 5'-TGGAAGGTGGACAGTGAG-3'	NM_031144.3

ELISA

The serum Nuclear factor kappa B (NF-κB) levels were evaluated by using the rat NF-κB ELISA Kit (number: MBS453975), the serum Interleukin 1 beta (IL-1 β) levels were determined using ELISA Kit for IL-1 β (number: SEA563Ra), the serum carcinoembryonic antigen (CEA) levels were determined using ELISA Kit for CEA (number: SEA150Ra), and the serum Carbohydrate antigen 19-9 (CA 19-9) levels were evaluated by using Rat CA 19-9 ELISA Kit (number: MBS729408), according to the manufacturer's directions.

Measurement of oxidative stress markers

The activity of glutathione peroxidase (GPx) was by kits of Biodiagnostic, catalogue number: GP 2524) (Paglia and Valentine, 1967). GSH R activity was determined using a Biodiagnostic kit (number: GR 25 11) (Beutler *et al.,* 1963). Superoxide dismutase (SOD) was evaluated spectrophotometrically at 560 (Sun *et al.,* 1988).

Immunohistochemistry assay

Sections of paraffin-embedded tissues were cut, with 3 % H2O2 in PBS, and then incubated overnight with the indicated antibodies (COX-2 and VEGF) at 4 °C then conjugation done by horseradish peroxidase, and the signal was detected using a DAB kit.

Histopathology

The tissues of the rats were obtained and preserved with formaldehyde solution (10 %) after they were dissected then; the tissues were fixed in paraffin blocks and blemished with haematoxylin–eosin.

Statistical analysis

The results were presented as mean ± SEM (Standard Error of Mean). One-way analysis of variance (ANOVA) was performed to determine the impact of the four groups on the various biochemical markers, followed by Tukey's Honestly Significant Difference (Tukey's HSD) test as a post hoc test.

Results

GC-MS analysis

Table 2 illustrated that clove contain major constituents which are eugenol, malic acid, gallic acid, palmitic acid, alpha Linolenic acid, β-Amyrin, α-Amyrin, Lupeol trimethylsilyl ether and Oleanolic acid. However, there were differences in term of percentage of composition between them.

Table 2 Chemical compositions in major constituents of clove

Peak	RT	Name	Formula	Area	Area %
1	18.517	Eugenol TMS	C13H20O2Si	769344732	50.28
2	19.117	Malic acid, 3TMS derivative	C13H30O5Si3	188539453	12.32
3	24.323	Gallic acid, 4TMS derivative	C19H38O5Si4	184529141	12.06
4	24.884	Palmitic Acid, TMS derivative	C19H40O2Si	37743242	2.47
5	26.341	alpha-Linolenic acid, TMS derivative	C21H38O2Si	19675460	1.29
6	34.304	β-Amyrin, TMS derivative	C33H58OSi	80449465	5.26
7	34.507	α-Amyrin, TMS derivative	C33H58OSi	37195840	2.43
8	35.251	Lupeol trimethylsilyl ether	C33H58OSi	11324434	0.74
9	35.706	Oleanolic acid 2TMS	C36H64O3Si2	201177995	13.15

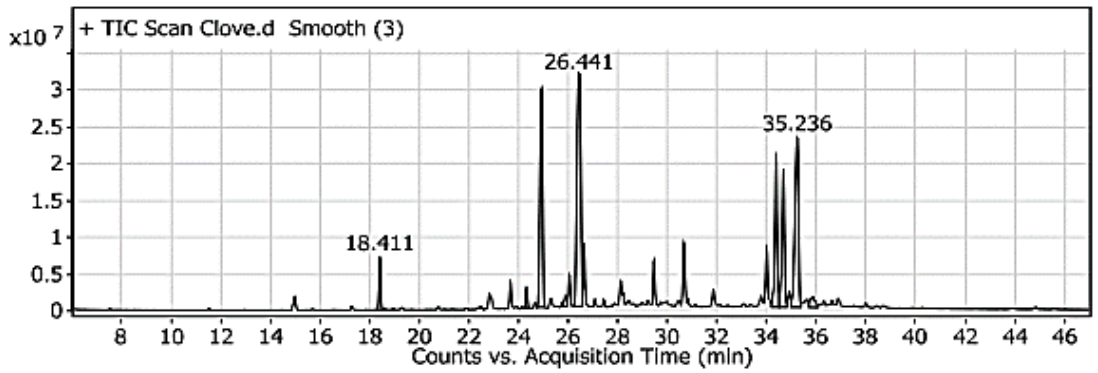

Fig. 1 Typical gas chromatogram of clove.

Table 3 Integration Peak List

Peak	Start	RT	End	Height	Area	Area%
1	18.298	18.411	18.692	7141468.81	36414438.72	10.41
2	18.873	18.927	19.035	101851.55	429954.57	0.12
3	24.246	24.331	24.429	2776892.76	12856694.26	3.67
4	24.74	24.93	25.077	29683033.4	235906036	67.41
5	25.81	25.896	25.97	1473473.18	10353371	2.96
6	26.208	26.441	26.584	31670433.4	349935193.1	100
7	34.188	34.386	34.515	20276454.57	142517292.1	40.73
8	34.515	34.682	34.818	18812332.34	136952168.3	39.14
9	34.993	35.236	35.395	23056849.5	247293909.9	70.67
10	35.706	35.865	36.086	1444479.15	20937697.93	5.98

Table 4 illustrated that *Cynara cardunculus* contain major constituents which are eugenol, malic acid, gallic acid, palmitic acid, alpha Linolenic acid, β-Amyrin, α-Amyrin, Lupeol trimethylsilyl ether and Oleanolic acid. However, there were differences in term of percentage of composition between them.

Table 4 Chemical compositions in major constituents of *Cynara cardunculus*

Peak	RT	Name	Formula	Area	Area %
1	18.411	Eugenol TMS	C13H20O2Si	36414439	3.05
2	18.927	Malic acid, 3TMS derivative	C13H30O5Si3	429954.57	0.04
3	24.331	Gallic acid, 4TMS derivative	C19H38O5Si4	12856694	1.08
4	24.93	Palmitic Acid, TMS derivative	C19H40O2Si	235906036	19.76
5	25.896	Linoleic acid ethyl ester	C20H36O2	10353371	0.87
6	26.441	.alpha.-Linolenic acid, TMS derivative	C21H38O2Si	349935193	29.32
7	34.386	.beta.-Amyrin	C30H50O	142517292	11.94
8	34.682	.alpha.-Amyrin, TMS derivative	C33H58OSi	136952168	11.47
9	35.236	Lupeol trimethylsilyl ether	C33H58OSi	247293910	20.72
10	35.865	Oleanolic acid	C30H48O3	20937698	1.75

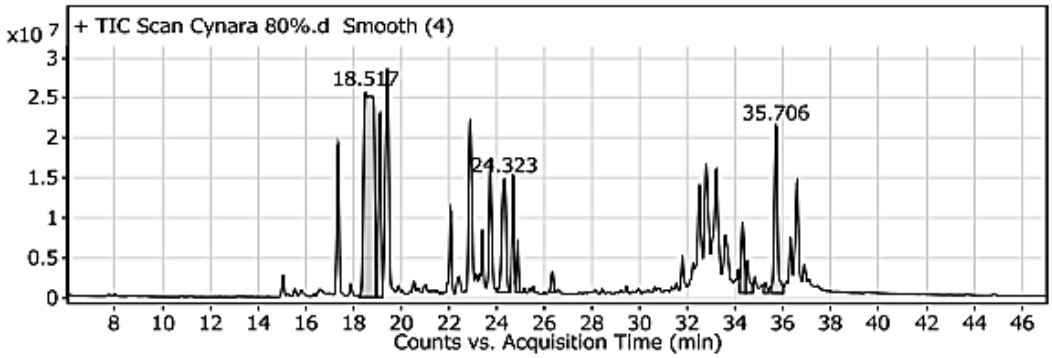

Fig. 2 Typical gas chromatogram of *Cynara cardunculus*.

Table 5 Integration Peak List

Peak	Start	RT	End	Height	Area	Area%
1	18.244	18.517	18.973	25550111.05	769344731.9	100
2	18.973	19.117	19.231	22951451.05	188539453	24.51
3	24.019	24.323	24.558	14114301.84	184529141.4	23.99
4	24.786	24.884	25.009	6200945.48	37743241.96	4.91
5	26.178	26.341	26.476	2565680.8	19675459.68	2.56
6	34.181	34.304	34.439	8632733.18	80449465.03	10.46
7	34.439	34.507	34.735	4071970.24	37195840.29	4.83
8	35.182	35.251	35.387	1304879.02	11324434.32	1.47
9	35.486	35.706	36.017	20797730.36	201177994.8	26.15

Effect of *Syzygium aromaticum* and *Cynara cardunculus* extracts on P53, Caspase-3 and Bcl-2 gene expressions.

The mRNA levels of apoptotic and anti-apoptotic markers (P53, Caspase-3, and Bcl-2) in male albino rats exposed to clove and *Cynara cardunculus* extracts for 4 months (one month before induction of colorectal cancer and 3 months during the induction) were analyzed using real-time PCR. We also discovered that both extracts significantly changed the mRNA levels of these indicators in animals. The normalization of target gene expression data was done using housekeeping genes. The β-actin gene is found in all cells and is one of the most widely used in gene expression data comparisons. P53 is required for the induction of growth arrest after DNA damage.[13] It was revealed that there was highly statistically different among groups in P53 value. G1 (negative control group) revealed the lowest P53 reading and there was significant elevation of P53 in group G2 (positive control group), while the largest value was found in the group treated

with extract of clove (50 mg/kg b.wt) that was significantly increased than G1, G2 and group treated with the extract of *Cynara cardunculus* (500 mg/kg b.wt) (Fig. 3A).

We also explored the influence of clove and *Cynara cardunculus* extracts on Caspase-3 and Bcl-2 mRNA expression. Caspase-3 and Bcl-2 have long been thought to be important in regulating cellular fate in damaged cells.[13] Increased Caspase-3 expression can cause programmed cell death, whereas increased Bcl-2 protein expression protects cells from apoptosis. In our study, the expression pro-apoptotic Caspase-3 gene in the negative control group had revealed the lowest caspase reading while it was significantly increased in positive control group. The level of Caspase-3 gene expression was raised in the group treated with extract of clove in comparison with G1, G2 and group treated with the extract of *Cynara cardunculus* (500 mg/kg b.wt), this means that the group treated with extract of clove can induce high caspase-3 gene. Increased P53 gene expression in cancer cells promotes an increase in Bax gene expression, which leads to increased membrane permeability and cytochrome c release. In this way, clove and *Cynara cardunculus* extracts thought that they led to apoptotic death to cancer cells in the induced colorectal cancer DMBA rat model (Fig. 3B).

In reverse anti-apoptotic Bcl-2 gene expression was significantly the highest readings in the positive control group while in the group treated with extract of *Cynara cardunculus* showed non-significant difference than the group treated with the extract of clove. There was significant decrease in the negative control group than the group treated with clove extract and the group treated with *Cynara cardunculus* extract. As a result of these findings, we hypothesized that our extracts could trigger the apoptotic pathway by eliminating Bcl-2 gene barriers. Our findings indicated that our extracts produced a consistent up-regulation of P53, Caspase-3, and down-regulation of Bcl-2, which could be one of the molecular mechanisms by which our extracts may initiate the intrinsic apoptotic pathway (Fig. 3C).

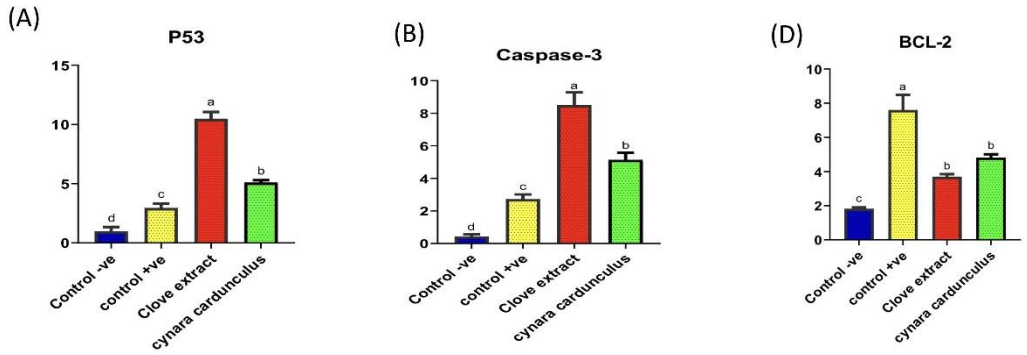

Fig. 3 Analysis of PCR product of apoptotic and anti-apoptotic markers in DMBA-induced colorectal cancer in male albino rats by using real time PCR. **(A)** The level of P53 gene expression was the largest value in the group treated with extract of clove in comparison with G1, G2 and group treated with the extract of *Cynara cardunculus.* **(B)** The level of Caspase-3 gene expression was raised in the group treated with extract of clove in comparison with G1, G2 and group treated with the extract of *Cynara cardunculus.* **(C)** The level of Bcl-2 gene expression was significantly the

highest readings in the positive control group, while in the group treated with extract of *Cynara cardunculus* showed non-significant difference than the group treated with the extract of clove and significant decrease in the negative control group.

Effect of *Syzygium aromaticum* and *Cynara cardunculus* extracts on serum NF-κB levels

NF-κB controls the expression of genes associated with a variety of activities important in cancer growth and progression, including proliferation, migration, and apoptosis. Many human cancers have been found to have abnormal or constant NF-κB activation. Inhibitors of Apoptosis (IAPs) and several members of the anti-apoptotic Bcl-2 family are also induced by NF-κB. Through direct suppression of effector caspases (caspases-3, -6, -7, and 9) the IAPs decrease apoptosis produced by both extrinsic and intrinsic routes, whereas the anti-apoptotic members of the Bcl-2 family counteract the activity of some pro-apoptotic members.[14] We found that there was a highly statistically different among groups in NF-κB value (Fig. 4A). G2 revealed the highest NF-κB reading, while the group treated with extract of *Cynara cardunculus* showed a non-significant difference than the group treated with clove extract. But the group treated with extract of *Cynara cardunculus* showed a significant change than G2, also the group treated with clove extract showed a significant change in comparison with G2. While there was a significant decrease in NF-κB value in the negative control group in comparison with the group treated with clove extract and the group treated with *Cynara cardunculus* extract. Overall, these results indicated that clove and *Cynara cardunculus* extracts have a significant inhibitory role in NF-κB levels.

Effect of *Syzygium aromaticum* and *Cynara cardunculus* extracts on serum IL-1β levels

In numerous tumour forms, NF-κB and the presence of several cytokines, such as IL-1β, are increased.[15] This comes in agreement with our results that revealed that there was a highly statistically difference among groups in IL-1β value (Fig. 4B). G2 revealed the highest IL-1β reading and this proved that IL-1β increased cancer cell proliferation as in several studies and significant with all groups, the group treated with *Cynara cardunculus* extract showed a significant difference than the group treated with clove extract. While there was a significant decrease in IL-1β value in the negative control group in comparison with the group treated with clove extract and the group treated with *Cynara cardunculus* extract. And this also supports our above results that both clove and *Cynara cardunculus* extracts have a significant inhibitory role in IL-1β levels and a protective role against cancer.

Effect of *Syzygium aromaticum* and *Cynara cardunculus* extracts on serum CEA levels

CEA acted as selectin ligands, allowing colon cancer cells to spread more easily. It is found in a high percentage of carcinomas in a variety of other organs. CEA is involved in tumour metastasis, which has a significant impact on prognosis, and it may be linked to a variety of cancer outcomes.[16] Our data revealed that there was a highly statistically different among groups in CEA value (Fig. 4C). G2 revealed the highest CEA reading and was significant with all groups, the group treated

with *Cynara cardunculus* extract showed a significant difference than the group treated with clove extract. While there was a significant decrease in CEA value in the negative control group in comparison with groups treated with clove and *Cynara cardunculus* extracts.

Effect of *Syzygium aromaticum* and *Cynara cardunculus* extracts on serum CA 19-9 levels

CA 19-9 is an e-selectin ligand that plays a crucial role in cancer cell adherence to endothelial cells. In gastrointestinal malignancies, it has been employed as colon tumour marker. It could also be due to an increase in some benign disorders.[17] According to that our results demonstrated that there was a highly statistically different among groups in CA 19-9 value (Fig. 4D). G2 revealed the highest CA19-9 reading and was significant with all groups, the group treated with clove extracts showed a non-significant difference than the group treated with *Cynara cardunculus* extract. While there was a significant decrease in CA 19-9 value in the negative control group in comparison with the group treated with clove extract.

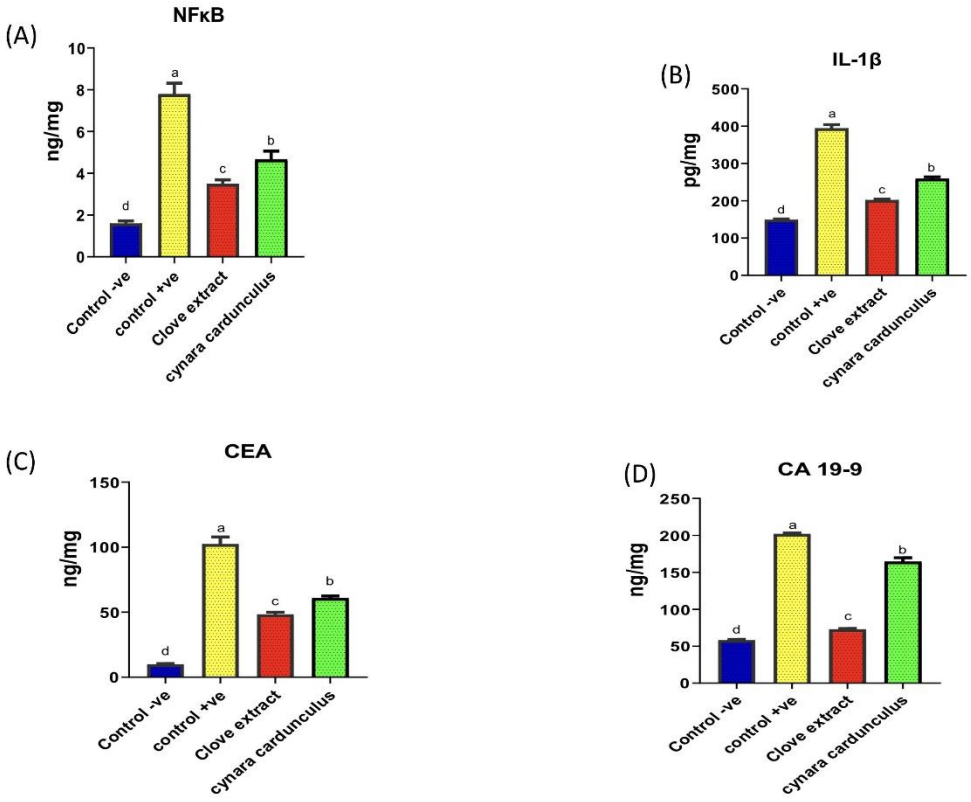

Fig. 4 Analysis of some markers in DMBA-induced colorectal cancer in male albino rats by using ELIZA. **(A)** The level of NF-kB in the group treated with extract of *Cynara cardunculus* and the group treated with clove extract showed a significant change than G2. **(B)** IL-1β level in the group treated with clove extract showed a significant difference than the group treated with *Cynara cardunculus* extract. **(C)** The highest CEA reading was in G2 and was significant with all groups. **(D)** The highest CA19-9 reading was in G2 and was significant with all groups.

Effect of *Syzygium aromaticum* and *Cynara cardunculus* extracts on oxidative stress markers (GPx, GSH R, SOD)

GSH R is an important non-protein thiol that acts in conjunction with GPx to protect cells against cytotoxic and carcinogenic chemicals by scavenging reactive oxygen species. By conjugating xenobiotics, carcinogens, free radicals, and peroxides with GSH R, GPx enzymes also help to detoxify xenobiotics, carcinogens, free radicals, and peroxides, protecting cells and organs from carcinogen-induced damage. GSH R has been found to be sequestered by tumour cells in order to meet the demands of the growing tumour.[18] As shown in (Fig. 5A) our results demonstrated that there was a highly statistically different among groups in GPx value. G2 revealed the lowest GPx reading. GPx significantly increased in the group treated with *Cynara cardunculus* extract and the group treated with clove extract than G2 group.

Also, results revealed that there was a highly statistically different among groups in GSH R value (Fig. 5B). Positive control group revealed the lowest GSH R reading. There were a significant increase of GSH R values in the group treated with *Cynara cardunculus* extract and also the group treated with clove than G2 group. The highest significant value in negative control group than other groups. So, our results strongly suggested that administration of clove and *Cynara cardunculus* extracts significantly inhibited cancer incidence and enhanced enzymic and non-enzymic antioxidant concentrations.

The obtained results of SOD revealed that there was highly statistically difference among groups in SOD value (Fig. 5C). G2 revealed the highest SOD reading and significant with all groups, the group treated with *Cynara cardunculus* extract showed non-significant difference than the group treated with clove extract. While there was significant decrease in SOD value in control negative group with the group treated with clove extract and the group treated with *Cynara cardunculus* extract.

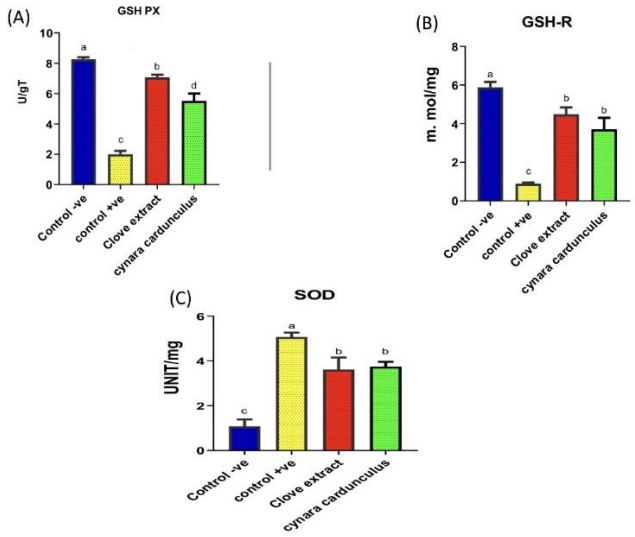

Fig. 5 Analysis of oxidative stress markers (GPx, GSH R, SOD) in DMBA-induced colorectal cancer in male albino rats. **(A)** G2 revealed the lowest GPx reading and significantly increased of GPx in the group treated with clove extract and the group treated with *Cynara cardunculus* extract.

(B) Positive control group revealed the lowest GSH R reading and there was a significant increase of GSH R in the group treated with clove extract and also the group treated with *Cynara cardunculus* extract than G2 group. **(C)** G2 revealed the highest SOD reading and significant with all groups, the group treated with clove extract showed non-significant difference than the group treated with *Cynara cardunculus* extract.

Immunohistochemistry assay

Negative control group (Fig. 6A) showed negative immune expression of COX-2. Meanwhile, an intense expression of COX-2 was observed in positive control group (Fig. 6B). The groups treated with clove (Fig. 6C) and *Cynara cardunculus* extracts (Fig. 6D) showed moderate amounts of COX-2 in colon wall.

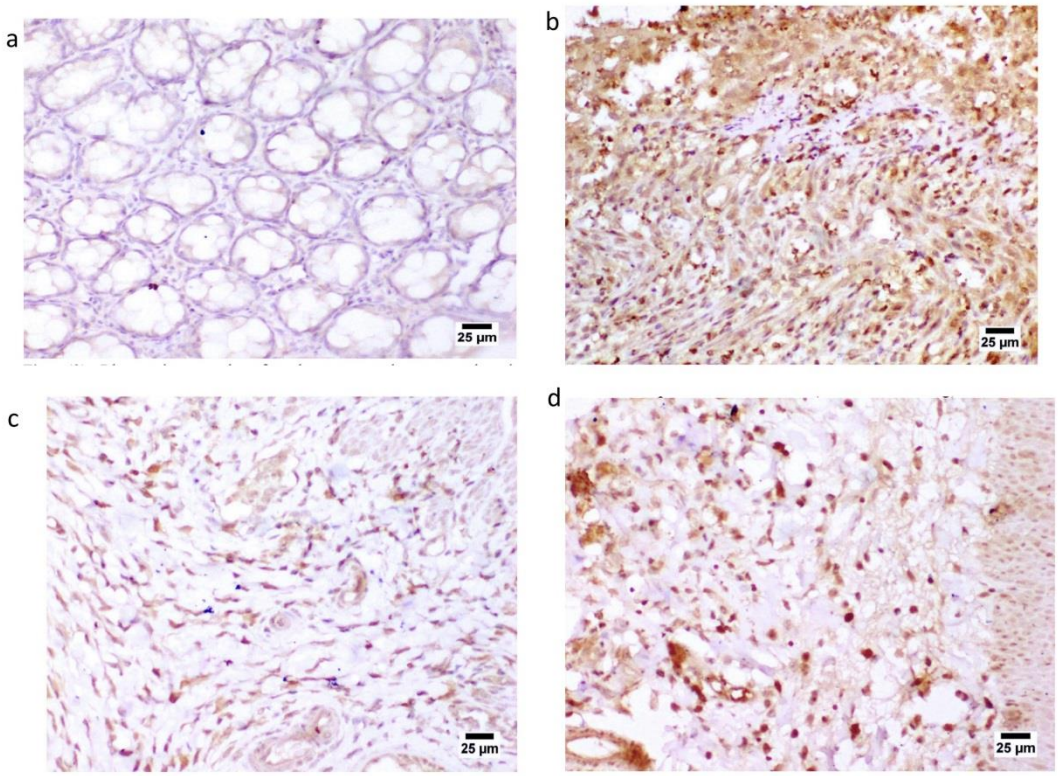

Fig. 6 Immunohistochemical evaluation in rat colon. **(A)** Photomicrograph of colon, negative control group showing negative expression of COX-2 (Immune staining). **(B)** Photomicrograph of colon, positive control group showing positive expression of COX-2 (Immune staining). **(C)** Photomicrograph of colon, the group treated with clove extract showing moderate expression of COX-2 (Immune staining). **(D)** Photomicrograph of colon, the group treated with *Cynara cardunculus* extract showing moderate expression of COX-2 (Immune staining).

Positive immune staining for VEGF in colon sections from negative control group (Fig. 7A) was very limited to negative amounts. Marked increase in VEGF positive staining was observed in positive control group (Fig. 7B) meanwhile, moderate VEGF expression was detected in the group

treated with *Cynara cardunculus* extract (Fig. 7C). Marked decrease of VEGF positive staining was shown in the group treated with clove extract (Fig. 7D).

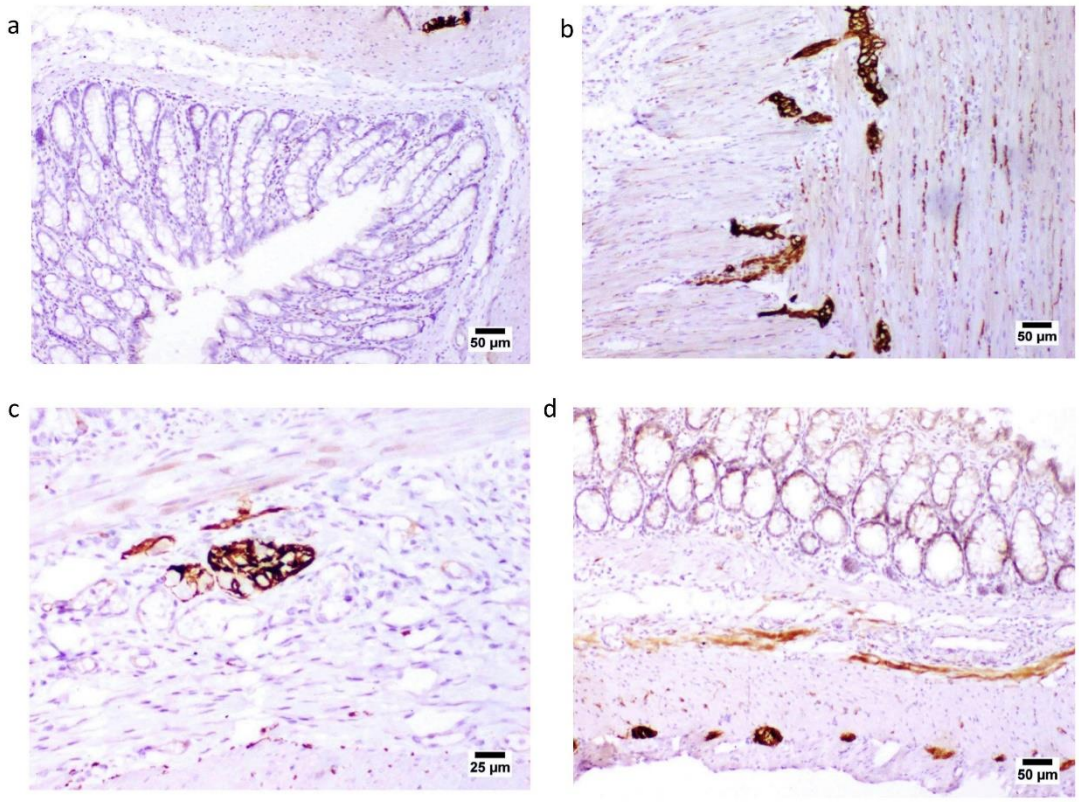

Fig. 7 Immunohistochemical evaluations in rat colon. **(A)** Photomicrograph of colon, negative control group showing very limited to negative of VEGF (Immune staining). **(B)** Photomicrograph of colon, positive control group showing increased expression of VEGF (Immune staining). **(C)** Photomicrograph of colon, the group treated with *Cynara cardunculus* extract showing moderate expression of VEGF (Immune staining). **(D)** Photomicrograph of colon, the group treated with clove extract showing mild expression of VEGF (Immune staining).

Histopathology

Microscopic examination of colon tissue samples from negative control group (Fig. 8A) revealed normal histology of colon with normally abundant mucous glands. On contrary, colon samples from positive control group (Fig. 8B) showed serious histopathological alterations, colon mucosa was completely necrosed with extensive ulceration and heavy mononuclear inflammatory cells infiltration. Mucous glands of colon were depleted. Dysplasia of gland with frequent mitosis was also observed. Some invasive carcinomas were frequently observed in submucosa. Submucosa suffered from marked inflammatory edema with existence of numerous dilated blood vessels. Mild improvement was detected in the group treated with *Cynara cardunculus* extract (Fig. 8C), some of the examined colon sections were apparently normal while some others showed ulcerative colitis manifested by complete destruction in colon mucosa including glands and heavy inflammatory cells infiltration. Dysplasia was noticed in some glands with development of groups of altered

glands. The group treated with clove extract (Fig. 8D) showed marked improvement as most of the examined sections were apparently normal except for few individuals that exhibited mild inflammatory edema in both mucosa and submucosa.

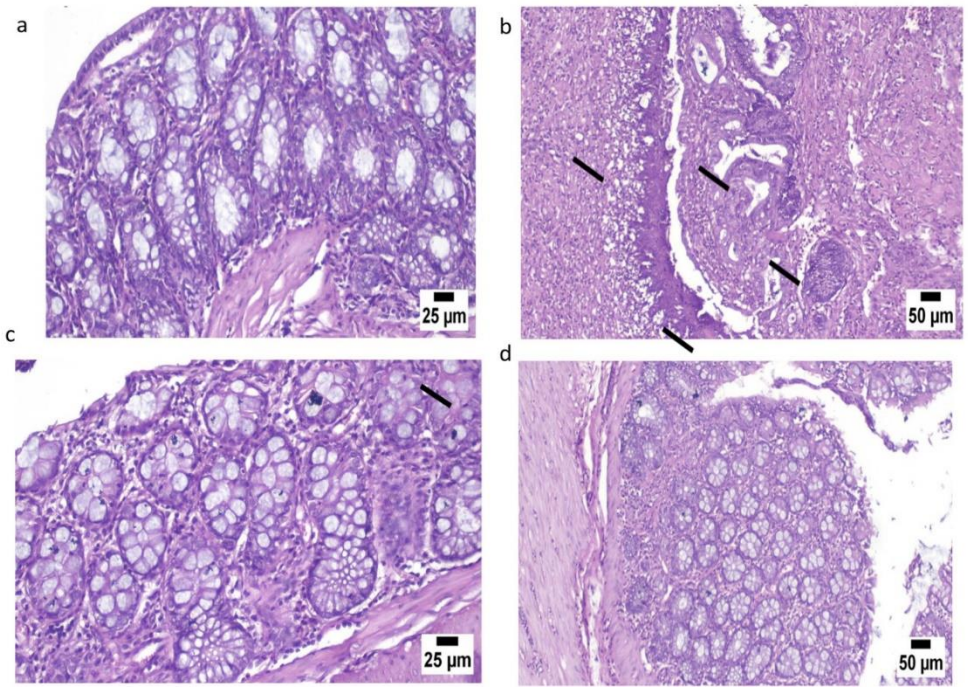

Fig. 8 Histopathological evaluation in rat colon. **(A)** Photomicrograph of colon, negative control group showing normal colon (H&E). **(B)** Photomicrograph of colon, positive control group showing dysplasia in glands with cystic dilatation (H&E). **(C)** Photomicrograph of colon, the group treated with *Cynara cardunculus* extract higher magnification showing apparently normal glands (H&E). **(D)** Photomicrograph of colon, the group treated with clove extract higher magnification showing apparently normal colon wall (H&E).

Discussion

Animal studies provide a reliable model for assessing new synthetic or natural chemicals as anticancer agents. The importance of plant functional foods in terms of health promotion and disease prevention, including cancer, has long been recognized. Importantly, most plants' secondary metabolites are relatively non-toxic and have a benign profile in the human body.[19] We investigated the anticancer protective properties of the clove and *Cynara cardunculus* extracts in CRC model in this work.

In the current study we reported that the clove and *Cynara cardunculus* extracts can enhance powerful pro-apoptotic effect in rat induced cancer model and also affect the cell proliferation. When a cell commits to apoptosis, the balance between proteins that mediate cell death, such as P53 and Bax, and proteins that promote cell survival, such as Bcl-2, is well understood. P53 is one of these proteins that has been shown to aid apoptosis in many malignancies. Caspase-3 is the apoptotic pathway's ultimate executor. The activation of procaspase-3 requires proteolytic

conversion of its inactive zymogen into active p17 and p19 subunits. Bax causes mitochondrial cytochrome c to be released, and the released cytochrome c activates caspase-3.[20]

In this study, we showed that extracts of clove and *Cynara cardunculus* can increase P53 expression. P53 has been proven to cause apoptosis by triggering genes in response to stress signals. The Bcl-2 protein forms heterodimers with the Bax protein in vivo, and the molar ratio of Bcl-2 to Bax determines whether apoptosis is induced or inhibited in a variety of tissues. In our study, treatment with clove and *Cynara cardunculus* extracts lowered Bcl-2 expression while increasing P53 and caspase-3 levels in two separate groups. The following reports backed up and validated our findings.

Following the application of a chemocarcinogen to a rat model, dried flower buds of cloves were fed at various doses through the diet for 13 weeks. After high-dose clove treatment, Bcl-2, Ki67, VEGFA, CD24, and CD44 expression in cancer cells in rats decreased, but Bax and caspase-3 expression increased. In vitro studies also revealed that clove extract has antiproliferative and pro-apoptotic effects in MCF-7 cells, with up-regulation of pro-apoptotic proteins P53, caspase-3, and Bax, and down-regulation of anti-apoptotic protein Bcl-2, as well as growth-promoting proteins cyclooxygenase-2, cMyc, and H-ras. Clove buds were found to have a substantial anticancer impact in vivo and in vitro in a mammary carcinoma model.[21] Another study using MCF-7 and MDA-MB-231 cells found that clove extract increased executioner caspase activity in a dose-dependent manner [22].

Human cancer cell lines were used to study the effects of clove on cell proliferation, cell cycle distribution, and apoptosis. Treatment with clove extract boosted $p21^{WAF1/Cip1}$ and g-H2AX protein expression while decreasing cell cycle-regulated protein expression. These findings showed that clove extract could be a promising new therapeutic herb for the treatment of colorectal cancer, with oleanolic acid being one of the bioactive components.[23] This is in line with another study, which found that cloves are natural compounds with high cytotoxicity against MCF-7 cells, making them interesting candidates for anticancer drug development.[23] Clove also has a chemo preventive impact by inhibiting the production of anti-proapoptotic genes such as Bcl-2, VEGFA, and CD44.[24]

The antitumoral effect of *Cynara cardunculus* extract was also tested on human breast cancer cells (MDA-MB-231), with cell proliferation, cell cycle, and Akt molecular signaling all being assessed. Furthermore, the antioxidant activity of this extract significantly reduced MDA-MB-231 cellular viability, causing cell cycle arrest at G2 phase and inhibiting Akt phosphorylation at serine 473, indicating that the Cynara cardunculus extract has significant antitumoral and antioxidant potential, and this study supports our findings.[25]

The findings of our study are highly validated by earlier research. A study was conducted to test the apoptotic and cytotoxic effects of Cynara cardunculus extract against DLD1, and the results revealed that in the presence of our artichoke extracts, pro-apoptotic (Bax) gene expression and a cell cycle inhibitor (p21) were elevated. In contrast to the control group, anti-apoptotic Bcl-2 gene expression was lowered. Furthermore, DNA fragmentation data revealed DLD1 cell death

through apoptosis.[26] Zhang and Kim[27] discovered that *Cynara cardunculus* extract was harmful to a human lung cancer cell line in a specific way (A549). They discovered that increasing the extract concentration suppressed A549 development.

Cell cycle arrest is another major mechanism that has been found to contribute to the anticancer effects of numerous well-known medicines. As we mentioned before that β-amyrin is the most common pentacyclic triterpenoid molecule found in petroleum ether ethanolic extract of *Cynara cardunculus*. β-amyrin showed substantial anticancer efficacy against Hep-G2 cancer. The anticancer effects were linked to the dose-dependent stimulation of apoptosis and G2/M cycle arrest. β-amyrin has also been shown to activate the p38 and JNK signaling pathways. In response to the β-amyrin treatment, the expression of Bax was elevated while that of Bcl-2 was lowered, according to the results of western blotting.[28]

The NF-kB family of transcription factors regulates immunological and inflammatory responses and protects cells against apoptosis in response to cellular stress. A variety of signaling pathways can activate the NF-kB pathway, causing NF-kB proteins to translocate from the cytoplasm to the nucleus and trigger the expression of certain cellular genes. Through transcriptional dysregulation of pro-apoptotic or anti-apoptotic proteins, NF-kB is implicated in the apoptotic mechanism. Increased Bcl-2 expression decreases apoptosis in resistant cells, altering the Bcl-2: Bax ratio and inhibiting apoptosis, and modulating caspase-8 to further affect Bcl-2, resulting in decreased apoptosis.[29]

Our results demonstrated that the group treated with the clove extract and the group treated with the *Cynara cardunculus* extract have a significant inhibitory role in NF-κB and IL-1β levels. The results of the current study are well supported with previous studies such as many cells, including macrophages, NK cells, monocytes, and neutrophils, express IL-1β. Apoptotic pathways are activated in cells exposed to genotoxic stimuli when IL-1β levels are high. Several studies have shown that clove has anti-inflammatory properties, able to modulate inflammatory markers like COX-2, nitric oxide, iNOS, and NF-kB, it inhibits the production of IL1 β, IL-6, and IL-10.[30]

The anti-inflammatory effect of oleanolic acid was high, suppressing NO, PGE2 generation, NF-κB expression, and stimulating Nrf2 expression. MafK expression and MafK-mediated p65 acetylation were both dramatically reduced by oleanolic acid. These findings suggested that oleanolic acid, as an NF-kB inhibitor, could be employed in therapeutic applications to treat oxidative stress-related disorders.[31] In a variety of tumour models, oleanolic acid has been shown to inhibit NF-κB activation as well as signal transducer and activator of transcription 3, caspases, VEGF, and poly (ADP-ribose) polymerase.[32]

β-amyrin treatment reduces pancreatic inflammation and tissue injury by inhibiting neutrophil infiltration, TNF-α and IL-6 cytokine production, and TNF-α and iNOS expression.[33] In a mouse model of steatohepatitis, the globe artichoke-related avoidance of hepatic oxidative stress and lipid metabolism problem was linked to decreased IL-1β production, which is linked to the beginning of osteoarthritis.[34]

The glycoprotein CEA is the most widely used blood-based CRC molecular marker, and it has proven to be an effective tool for patient monitoring. Examining CEA expression before surgery seems sensible from a prognostic standpoint, especially in individuals with metastases.[16] However, the main drawback of utilizing CEA as a CRC marker is its link to other malignancies, such as ovarian cancer, and benign diseases (inflammatory bowel disease). Other serum markers, such as CA19-9, CA125, and serum ferritin, have been employed as indicators for CRC diagnosis, post-operative surveillance, and therapy effects monitoring.[17, 35] Due to the above-mentioned studies, we evaluated the level of CEA, CA 19-9 in the serum of tumor induced rats and the results revealed that G2 had the highest CEA and CA 19-9 reading, the group treated with clove extracts showed a significant difference than the group treated with *Cynara cardunculus* extracts. While there was a significant decrease in CEA and CA 19-9 value in the negative control group in comparison with groups treated with clove and *Cynara cardunculus* extracts. As far we know no previous studies revealed the impact of clove and *Cynara cardunculus* extracts on the levels of CEA and CA 19-9.

Antioxidant and apoptotic actions are regarded crucial features of anti-cancer drugs because of their ability to modify cellular levels of reactive oxygen species. The normal to malignant transition happens when ROS levels rise and do not drop, revealing and modifying the gene expression profile under oxidative stress.[36] In this way, stressed cells are supposed to activate the two primary cellular response systems, antioxidant defense systems and apoptotic response pathways; we found that oral administration of clove and Cynara cardunculus extract, which is high in antioxidants (flavonoids), reduced GPx, GSH R, and SOD levels in rats with CRC.

Due to the components of *Syzygium aromaticum L.* (clove), which include tannins, flavonol glycosides, and volatile phenolic oils, clove has a high level of antioxidant activity among other plants (eugenol, acetyl eugenol). Clove phytochemical components have potent antioxidant, antiproliferative, antibacterial, antiseptic, and anti-inflammatory effects, making it a powerful cancer chemo preventive.[3]

According to previously mentioned, the obtained results of our work revealed that there was a highly statistically different among groups in GPx, GSH R and SOD values. G2 revealed the lowest GPx, GSH R reading and significantly increased of GPx, GSH R in the group treated with clove extract and the group treated with the *Cynara cardunculus* extract increased than G2 followed by the highest significant value in control negative group than other groups. While the G2 revealed the highest SOD reading and significant with all groups. While there was significant decrease in SOD value in control negative group. These results supported by the following studies.

Administration of a reasonable quantity of cloves in the diet has been proposed to prevent oxidative stress caused by hyperlipidemia. Oxidative damage was observed in hyperlipidemic rats. All these enzymes SOD, CAT, GPX and GST showed a marked elevation in activity in liver and kidneys of hyperlipidemic group rats. The activities of these enzymes were found to increase further significantly in group of rats co-administered with clove extract.[37] Clove extract decreases blood sugar, oxidative stress, plasma cholesterol, triglycerides, and LDL levels, according to the findings of another study. DSA also reduces oxidative stress and improves liver tissue damage, which helps

to minimize diabetes-related tissue damage by enhancing antioxidant enzymes (SOD, CAT, and GPx) and decreasing oxidative stress. Clove plant can be used as an efficient herbal therapy in decreasing and curing diabetes-related liver tissue disorders as a result of these therapeutic characteristics.[38]

GSH R may reduce the production of free radicals by creating transient metal catalysts, inhibiting chain processes, lowering ROS levels, and increasing antioxidant enzyme levels (SOD, CAT, GPx, and GST). Clove extract supplementation increased SOD, GPx, and GSH R and decrease MDA levels.[39]

High-fat diet-induced hepatotoxicity in rats was recovered by *Cynara* spp leaf extract administration, as demonstrated by the restoration of hepatic enzymes such as serum Ornithine Carbamoyl Transferase levels, according to Ben Salem et al.[40] ROS, GSH R, SOD, GPx and glutathione peroxidase were all improved by the artichoke leaf extract. Another study used carbon tetrachloride to cause acute hepatotoxicity in rats, and they detected changes in SOD, GPx, and CAT activity in tissue, which were alleviated by aqueous extracts of artichoke leaves and pulp.[41] Kaymaz et al.[42] used alpha-amanitine to cause hepatotoxicity in rats. When treated with the aqueous extract of artichoke 1500 mg/kg, they saw a restoration of different oxidative stress indices (MDA, SOD, CAT, and GPX). The presence of cynarine, a hepatoprotective and regenerating phenolic molecule, was responsible for these positive benefits. These results support our theory also.

In this work, the immunohistochemistry approach was used to assess the mechanism of action of clove. Clove's anticancer effects on rat mammary gland carcinoma cells are assumed to be attributable to the pro-apoptotic, antiproliferative, anti-angiogenic, and antioxidant capabilities of a range of plant bioactive compounds. Apoptosis is known to be triggered by the activation of caspase-3, an executioner protease. The VEGF-ligand/receptor kinase's signaling is critical for the formation of new blood vessels. COX-2 expression is upregulated in the majority of colon, prostate, lung, breast, esophageal, pancreatic, and gastric tumours, and it's linked to a higher stage, a higher grade, and a worse prognosis.[43] In rat mammary carcinomas in vivo, we discovered that the groups treated with clove extract and the *Cynara cardunculus* extract lowered the expression of VEGF, an important signal molecule produced by cells that promotes vasculogenesis and angiogenesis. In addition, the clove extract and *Cynara cardunculus* extract groups revealed moderate quantities of COX-2 in the colon wall, while the negative control group showed negative immunological expression of COX-2 and VEGF. Meanwhile, the positive control group showed a high level of COX-2 and VEGF expression.

In a study of rat tumours, immunohistochemistry revealed an increase in cytoplasmic and nucleic caspase-3 expression in the clove-treated group when compared to the control group. When compared to controls, higher doses of clove dramatically reduced Bcl-2, Ki67, VEGFA, and MDA expression. In comparison to the control, the expression of Bax and VEGFR-2 in treated cancer cells did not alter.[21]

In vitro and in vivo, an oleanolic acid derivative was found to decrease HBV activity. Because HBV can cause liver fibrosis, its effect on chronic CCl4 fibrosis was investigated.[44] Histology and immunohistochemical examination showed that daily intraperitoneal treatment of oleanolic acid (14-28 mg/kg, ip for 11 weeks) reduced the development of chronic CCl4 (9 weeks)-induced liver fibrosis. TGF-1 expression was likewise reduced by oleanolic acid. Oleanolic acid is protective against CCl4-induced chronic fibrosis and cirrhosis when taken simultaneously.[45]

During DMBA-induced colorectal cancer in rat distinct histopathological changes could be identified as serious histopathological alterations; colon mucosa was completely necrosed with extensive ulceration and heavy mononuclear inflammatory cells infiltration. Mucous glands of colon were depleted. Dysplasia of gland with frequent mitosis was also observed. Some invasive carcinomas were frequently observed in submucosa. Submucosa suffered from marked inflammatory edema with existence of numerous dilated blood vessels. Clove extract was found to inhibit or delay this progression, some of the examined colon sections were apparently normal while some others showed ulcerative colitis manifested by complete destruction in colon mucosa including glands and heavy inflammatory cells infiltration. Dysplasia was noticed in some glands with development of groups of altered glands. While in the group treated with *Cynara cardunculus* extract showed marked improvement as most of the examined sections were apparently normal except for few individuals that exhibited mild inflammatory edema in both mucosa and submucosa. Thus, histopathological examination in a CRC rat model suggests that clove and *Cynara cardunculus* extracts played a protective function in limiting carcinogenesis and preventing or delaying the formation of early lesions through preventative intervention.

In CDDP-treated mice, histological examination of liver tissue validated the oleanolic acid's hepatoprotective activity. The mice given a single CDDP injection showed more toxicity, as seen by liver necrosis, vacuolization, and dilatation and congestion of the central vein. Because of the repeated doses of CDDP, the animals given with and NPs form of oleanolic acid showed less toxicity, vacuolization, and hydrophobicity than the other groups, implying that OA has a better hepatoprotective impact against CDDP-induced liver toxicity.[46]

SECs, KCs, and CD4+ Th cells were activated following concanavalin A- injection, resulting in increased levels of cytokines, edoema, and necrosis in hepatocytes, according to another study. The greatest increases occurred at 8 hours, and oleanolic acid significantly reduced the activity of these transaminases. The intermediately dosed animals (40 mg/kg) showed the greatest reduction in concanavalin A-dependent alterations. Furthermore, the oil group's liver tissue had well-preserved hepatic architecture and entire liver lobules. When compared to treatment with concanavalin A- alone, the oleanolic acid pretreatment group showed narrowed necrotic areas, slight congestion, and milder lymphocytic accumulation. Western blotting and TNF-α, IL-1β, and IL-6 immunohistochemistry analysis also confirmed that oleanolic acid significantly reduced the production of TNF-α, IL-1β, and IL-6 on a protein level. These findings imply that oleanolic acid pretreatment can reduce concanavalin A-induced liver damage in mice. [46]

Several studies suggested the daily doses for human as follow 2.5 mg/kg of clove and 300 mg per day.[47, 48] Taken together, these findings imply that the clove extract group and the *Cynara cardunculus* extract group are viable lead molecules for the treatment of CRC cancer.

Conclusion

In conclusion, our study showed that clove and *Cynara cardunculus* extracts are potent cytotoxic agents for various cancer cells, particularly CRC cells, and that they can induce apoptosis. Suppression of NF-kB and IL 1β was one putative mechanism of action. The clove and *Cynara cardunculus* extracts also induce apoptosis by activating P53 and caspase-3 and decreasing Bcl-2 expression. These findings suggest that the clove and *Cynara cardunculus* extracts could be used as an antitumor compound against various cancer cells depending on their sensitivity to it. More research is being done to determine whether NF-κB targets such IAPs are involved. Furthermore, it's probable that pretreatment with these substances makes cells more sensitive to traditional chemotherapeutic agents, and that they could represent new CRC therapy methods. Because of its rapid and precise production, as well as its high bioavailability and targeting, nanotechnology has shown to be effective in virtually every aspect of human and animal health. This study brings up new possibilities for low-toxicity oral-delivery formulations, as well as ensuring minimal intestinal toxicity if unintentionally consumed, and improving the bioviability of the extracts.

Author's Contribution

All authors contributed equally.

Conflict of interests

The authors declare no conflict of interests.

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Fig. legends

Fig. 1	Typical gas chromatogram of clove.
Fig. 2	Typical gas chromatogram of *Cynara cardunculus.*
Fig. 3	Analysis of PCR product of apoptotic and anti-apoptotic markers in DMBA-induced colorectal cancer in male albino rats by using real time PCR. (A) The level of P53 gene expression was the largest value in the group treated with extract of clove in comparison with G1, G2 and group treated with the extract of Cynara cardunculus. (B) The level of Caspase-3 gene expression was raised in the group treated with extract of clove in comparison with G1, G2 and group treated with the extract of Cynara cardunculus. (C) The level of Bcl-2 gene expression was significantly the highest readings in the positive control group, while in the group treated with extract of Cynara cardunculus showed non-significant difference than the group treated with the extract of clove and significant decrease in the negative control group.
Fig. 4	Analysis of some markers in DMBA-induced colorectal cancer in male albino rats by using ELIZA. (A) The level of NF-kB in the group treated with extract of Cynara cardunculus and the group treated with clove extract showed a significant change than G2. (B) IL-1β level in the group treated with clove extract showed a significant difference than the group treated with Cynara cardunculus extract. (C) The highest CEA reading was in G2 and was significant with all groups. (D) The highest CA19-9 reading was in G2 and was significant with all groups.
Fig. 5	Analysis of oxidative stress markers (GPx, GSH R, SOD) in DMBA-induced colorectal cancer in male albino rats. (A) G2 revealed the lowest GPx reading and significantly increased of GPx in the group treated with clove extract and the group treated with Cynara cardunculus extract. (B)

	Positive control group revealed the lowest GSH R reading and there was a significant increase of GSH R in the group treated with clove extract and also the group treated with Cynara cardunculus extract than G2 group. (C) G2 revealed the highest SOD reading and significant with all groups, the group treated with clove extract showed non-significant difference than the group treated with Cynara cardunculus extract.
Fig. 6	Immunohistochemical evaluation in rat colon. **(A)** Photomicrograph of colon, negative control group showing negative expression of COX-2 (Immune staining). (**B)** Photomicrograph of colon, positive control group showing positive expression of COX-2 (Immune staining). (**C)** Photomicrograph of colon, the group treated with clove extract showing moderate expression of COX-2 (Immune staining). (**D)** Photomicrograph of colon, the group treated with *Cynara cardunculus* extract showing moderate expression of COX-2 (Immune staining).
Fig. 7	Immunohistochemical evaluations in rat colon. (**A)** Photomicrograph of colon, negative control group showing very limited to negative of VEGF (Immune staining). (**B)** Photomicrograph of colon, positive control group showing increased expression of VEGF (Immune staining). (**C)** Photomicrograph of colon, the group treated with *Cynara cardunculus* extract showing moderate expression of VEGF (Immune staining). (**D)** Photomicrograph of colon, the group treated with clove extract showing mild expression of VEGF (Immune staining).
Fig. 8	Histopathological evaluation in rat colon. (**A)** Photomicrograph of colon, negative control group showing normal colon (H&E). **(B)** Photomicrograph of colon, positive control group showing dysplasia in glands with cystic dilatation (H&E). **(C)** Photomicrograph of colon, the group treated with *Cynara cardunculus* extract higher magnification showing apparently normal glands (H&E). (**D)** Photomicrograph of colon, the group treated with clove extract higher magnification showing apparently normal colon wall (H&E).