ORIGINAL ARTICLE

CONTENTS

Phytochemical analysis of ethanolic leaf extract of Cordia myxa and its anti-Inflammatory and cytotoxic activities

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ABSTRACT

Aim: Testing Cordia myxa extract on colon cancer cell line and caspase-3 gene and COX-2 protein expression.

Materials and Methods: This study used Cordia myxa ethanolic extract at various dosages on SW480 cells. Cell proliferation was measured using MTT, also examined effect of Cordia myxa extract on caspase-3 gene expression using quantitative real-time polymerase chain reaction. The Elisa technique measured cox2 protein.

Results: Cell proliferation tests showed the dose-dependent anticancer effects of Cordia myxa ethanolic extract. Cordia myxa extract also upregulates caspase-3 gene expression and decreases COX-2 protein levels.

Conclusions: Cordia myxa may be a good choice for colon cancer research and anticancer drug development. This plant may cure cancer because of its antioxidant, anti-inflammatory, and anticancer properties.

KEY WORDS: colorectal cancer, apoptosis, inflammation, Cordia myxa

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INTRODUCTION

The glandular epithelial cells of the large intestine often give rise to colorectal (CRC) cancer, a complex solid tumor. Cancer is caused by genetic or epigenetic alterations that favor specific epithelial cells [1]. Colorectal cancer (CRC) is the second leading cause of death and the third most prevalent malignancy globally, with 1.9 million cases and 0.9 deaths in 2020 [2]. Several factors can cause colorectal cancer. If they or their family have cancer, colon polyps, inflammatory bowel diseases, diabetes, or cholecystectomy, colorectal cancer risk increases, according to research. Lifestyle factors are major causes of colorectal cancer [3]. Genetic alteration, including the inactivation of tumor-suppressor genes like adenomatous polyposis coli (APC), which may transform the normal colonic epithelium, and DNA-repair gene inactivation lead to a hyperproliferative stage that activates K-RAS oncogenes in the early adenoma stage, and epigenetic alterations through aberrant chromosomes. Later, the tumor suppressor gene p53 is inactivated, which may be associated with the adenoma-to-carcinoma transition [4]. Plant or drug repurposing is an emerging concept that involves using old drugs or exploring medicinal plants for new treatment indications. Several studies and clinical trials have been conducted on drug repurposing, showing that Dipyridamole, a platelet inhibitor with antithrombotic properties, and Nebivolol, a beta blocker used for high blood pressure and heart failure, have a significant heart-protective effect against doxorubicin-induced cardiotoxicity in rats [5-6]. Among the medicinal herbs, *Cordia myxa* is a medicinal plant used to cure several ailments. Several studies have shown that *C. myxa* is anti-inflammatory, anti-diabetic, antiparasitic, antibacterial, and immunomodulatory [7]. *Cordia myxa*, a Boraginaceae plant, is known as the "Indian cherry and Assyrian plum" for its tasty fruits [8]. The relaxing properties helped minimize coughing and treat respiratory infections and sore throats. The pulp was also an anthelminthic, abscess emollient, and rheumatism pain reliever. A leaf macerate lotion was used for tick bites to treat trypanosomiasis [9]. In health and illness, apoptosis is well-planned and controlled. An understanding of apoptosis is vital to the genesis of many diseases. Cancer is a result of inadequate apoptosis. Multiple paths make apoptosis challenging. Any error in any of these channels might produce malignant cells, tumor spread, and anticancer drug resistance [10].The extrinsic

death receptor and intrinsic mitochondrial pathways are the primary apoptotic pathways [11]. Caspase-3, a cysteine-aspartic acid protease, is encoded by CASP3 [12]. Caspase-3 is crucial to the apoptotic pathway which is activated by extrinsic stimulation [13]. Linked to cancer chronic inflammation contributes to tumor growth, making it a hallmark of cancer [14]. Chronic, unresolved, dysregulated inflammation increases cancer risk and spreads most tumors [15]. Human COX-2, also known as prostaglandin-endoperoxide synthase 2, is encoded by PTGS2. A homodimer comprising 581 amino acids, it weighs 70 kDa. COX-2 converts arachidonic acid to prostaglandins, which are then converted to PGH2. PGH2 is metabolized by tissue-specific synthases into five prostaglandins (PGD2, PGE2, PGF2α), prostacyclin (PGI2), and thromboxane A2 (TXA2) [16]. COX-2 is known to cause inflammation and cancer. Numerous studies showed that Inflammation causes cells to produce COX-2, which they don't ordinarily express. COX-2 aids tumor invasion, angiogenesis, metastasis, and apoptosis [17]. Overexpressing COX-2 is associated to gastrointestinal malignancies, particularly colorectal cancer [18].

MATERIALS AND METHODS

CELL LINE CULTURE

Colon cancer cell line SW480 was supplied by the Iran Center of Cancer and Medical Genetics Research (ICCMGR). It was derived from the colorectal adenocarcinoma epithelial cells of 50 years 50-year-old Caucasian male. Cells were grown in RPMI-1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and were kept at 37°C with 5% CO2 in a fully humidified environment.

PREPARATION OF PLANT EXTRACTS

Collected leaves of cordia myxa were cleaned, shade-dried, and powdered. The dried coarse powder was subjected to Soxhlet extraction with ethanol to get the crude extract. The appearance of colorless solvent in the siphon tube was taken as the termination of extraction. The extract was filtered and then concentrated to dryness in a rotary evaporator under reduced pressure and controlled temperature. The extract was then kept in a sterile bottle, under refrigerated conditions, until further use [19]. The stock solution of the extract was prepared by dissolving 50 mg of each extract in 10 ml of serum-based medium (5 mg/ml), then filtered through a 0.2 µm- Millipore filter. From this stock, serial dilutions were made resulting in six concentrations (1000, 500, 250, 125,6.5, and 3.25 µg/ml).

PHYTOCHEMICAL SCREENING OF PLANT EXTRACTS

DETERMINATION OF TOTAL ALKALOIDS CONTENT

A fraction of both extracts was mixed with 2 N HCl and subsequently filtered. A separatory funnel was used to transfer 1 ml of each solution, which was then rinsed with 10 ml of chloroform. The pH of every solution was modified to a neutral state using 0.1 N NaOH. Next, each solution was supplemented with 5 ml of BCG solution and 5 ml of phosphate buffer. After shaking the mixtures, the resulting complexes were extracted using chloroform through vigorous shaking. The samples were gathered and placed into a 10-milliliter flask. Afterward, they were combined with chloroform until the volume was reached. The measurement of complex absorbance in chloroform was conducted at a wavelength of 470 nm [20].

DETERMINATION OF TOTAL PHENOLIC CONTENT

The Folin-Ciocalteu procedure was used to determine the quantity of total phenolics present in the extracts. Samples of 2 ml volume were placed inside test tubes, following which 1.0 mL of Folin-Ciocalteu's reagent and 0.8 mL of sodium carbonate 7.5% were included. The tubes were combined and left undisturbed for 30 minutes. The measurement of absorption at 765 nm was conducted using a UV-visible spectrophotometer. The overall amount of phenolic compounds was measured in milligrams of Gallic acid equivalents (GAE) per gram of dry substance [21].

DETERMINATION OF TOTAL TANNINS CONTENT

To determine the tannin content or Proanthocyanidin levels, 400µL of the extract was mixed with 3 mL of a vanillin in methanol solution and 1.5 mL of concentrated hydrochloric acid. A 500 nm wavelength was used to measure the absorbance after it had been incubated for 15 minutes. The produced color can be measured quantitatively and represented as tannic acid equivalents [22].

DETERMINATION OF TOTAL FLAVONOIDS CONTENT

The colorimetric technique using aluminum chloride was employed to determine the flavonoids. The extracts (0.5 ml) were combined individually with 2.8 ml of distilled water, 0.1 ml of 10% aluminum chloride, 1.5

Table 1. HPLC system components

	Component	Model or version	Company and origin
1	Binary high-pressure gradient pump	P6.1L	Knuaer, Germany
2	Diode array detector	DAD 2.1L	Knuaer, Germany
3	Sample loop (20 µl) and injector	D1357	Knuaer, Germany
4	Analyses and system control software	Claritychrom, V 7.4.2.107	Dataapex, Czech Republic

ml of methanol, and 0.1 ml of 1 M potassium acetate. After 30 minutes at room temperature, the reaction mixture's absorbance was calculated using a UV/Visible spectrophotometer at 415 nm. Quercetin was used as the standard in the calibration curve [23].

DETERMINATION OF TOTAL GLYCOSIDE CONTENT

10 mL of newly made Baljet's reagent (95 mL of 1% picric acid + 5 mL of 10% NaOH) was combined with 10% of each plant's extracts. Once the mixture had been mixed for one hour, 20 milliliters of distilled water were added, and a UV/VIS spectrophotometer was used to detect the absorbance at 495 wavelengths. Milligrams of securidaside per gram of dried extracts were the standard of measurement for total glycosides [24].

DETERMINATION OF VITAMIN C CONTENT

A small amount of bromine water was added to the filtrated sample solution until the solution took on color, signifying that the conversion of ascorbic acid to dehydroascorbic acid had been completed. The clear solution was then obtained by adding a few drops of thiourea solution to eliminate the excess bromine. Next, the oxidized ascorbic acid and 2,4-ditrophenyl hydrazine solution were thoroughly added to all standards. Total vitamin C is measured by spectrophotometric means using a coupling reaction between vitamin C and 2,4-ditrophenyl hydrazine dye [25].

DETERMINATION OF VITAMIN E CONTENT

To span the range of the calibration curve (0.5 – 28 µg/ml vitamin E), a serial dilution was prepared in a 10 mL calibrated flask starting with a concentration of 100 µg/mL of vitamin E solutions. Subsequently, 2 mL (100 µg/mL) of [FeNH4(SO_4)2. 12H_2O] and 4 mL (100 µg/mL) of K3Fe(CN)6 were added. The solutions were then adjusted to pH = 4 and diluted with methanol to the appropriate level. Measure the absorbance at 743 nm against the reagent blank after 10 minutes [26].

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF PLANT EXTRACTS

PRINCIPLE

According to the HPLC concept, a liquid phase (mobile phase) is pushed through a porous material (stationary phase) at a greater pressure in a column where the sample solution injected into it. The solute adsorbs on the stationary phase according to its affinity for the stationary phase, which is the separation principle that is used. The separation process can occur in four distinct ways, depending on the characteristics of the stationary phase (Table 1) [27].

PROCEDURE

The sample was separated using a C18 column from Knauer, Germany, with dimensions of 250 x 4.6 mm, a particle size of 5 µm, and a pore size of 80 Å The mobile phase consists of a 1% aqueous acetic acid solution (Solvent A) and acetonitrile (Solvent B). The flow rate was set to 1 ml/min, the column temperature was maintained at 28°C, and the injection volume was 20 µl. Gradient elution was conducted by altering the ratio of solvent B to solvent A (Table 2). HPLC chromatograms were detected using a photodiode array UV detector at wavelengths (210 nm) .

The detection of compounds was performed by matching the retention time and absorbance spectrum of each standard. The concentration was calculated by serial concentrations of external standard materials (ten standards are used) to build a calibration curve between concentration and its equivalent peak area.

PREPARATIONS OF SAMPLES FOR MTT ASSAY

When cell growth in the flask became monolayer before it reached the exponential time, monolayer cells were harvested and re-suspended with a serum-free medium in a concentration of $5*10^5$ cells/ml and seeded in a 96-well cell culture plate. Since the cell growth reached 80%, the wells were treated with six concentrations (1000, 500, 250, 125, 62.5, and 31.25 µg/ml) of *Cordia myxa* extract to evaluate its cytotoxic effects on the SW480 cell line during a 24-hour incubation period. Three wells were used for each concentration.

Table 2. The gradient program

Time (min)	Mobile A (%)	Mobile B (%)	Flow rate ml/min
0	90	10	1ml/min
28	60	40	1ml/min
39	40	60	1ml/min
60	10	90	1ml/min

MOLECULAR STUDY BY REAL-TIME PCR

SAMPLE PREPARATION

To create a monolayer with 80% confluence, 12.5 ml cell culture flasks are seeded with Sw480 cells and cultured for 24 hours. Following that, 6 ml of the experimental drug-containing media was added to the flask, and the old medium was discarded. In addition, a flask treated with media alone was used as the control group. Every flask underwent a 24-hour incubation period. Trypsinization was used to separate the cells in each flask after the incubation time. Next, two milliliters of media were added to the centrifuge tube containing the cells. To get a pellet of cells, centrifugation was performed for five minutes at 13,000 rpm.

RNA EXTRACTION

TransZol Up Plus RNA Kit was used for the isolation of total RNA from cells according to the manufacturer's protocol**.**

PRIMER PREPARATION

The lyophilized version of the primers was provided. Each primer was dissolved in 300 µl of nuclease-free water following the "Macrogen Company instructions" to produce a final stock concentration of "100 pmol/µl." This inventory was kept at -20 °C. The working primer solution (10 pmol/µl) was created by mixing 10µl of primer stock solution with 90 µl of nuclease-free water.

COMPLEMENTARY DNA SYNTHESIS

EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit was used to produce complementary DNA (cDNA) by reverse transcription using mRNA as a template, reverse transcriptase enzyme, and a thermostable primer, complementary to the 3' end of the RNA template for quantifying gene expression analysis using real-time PCR according to the manufacturer's protocol.

EVALUATION OF GENE EXPRESSION BY RT-PCR

Quantify CASP3 gene expression in untreated cells (control group) and cells treated with 500 and 1000 µg/ml of *Cordia myxa* extract.

PREPARATION OF SAMPLES FOR ELISA ASSAY

48-culture plates were seeded with Sw480 cells. The plate was then placed in an incubator for 24 hours at 37°C and 5% CO_2. Following the medium's removal, tested compounds were applied to the wells. Three wells were treated for each concentration. In addition, eight wells served as controls by receiving no treatment at all from any experimental medicine. The plate was then left to incubate for a full day. Following the completion of the exposure time, each well's supernatant was pipetted into a sterile 5-milliliter sterile tube, which was then kept at -20°C. The COX2 content was determined in untreated cells (control group) and cells treated with *Cordia myxa* extract at concentrations of 100, 500, and 1000 µg/ml.

STATISTICAL ANALYSIS

The program GraphPad Prism version 8.4.3 (GraphPad program, La Jolla, CA, USA) was used to analyze the data in this study. Unless otherwise noted, all data were expressed as mean ± Standard Error Mean (SEM). The data was analyzed using One-Way Analysis of Variance (ANOVA) and the Bonferroni multiple comparison test. For all tests, statistical significance was determined at a significance level of $P < 0.05$.

RESULTS

PHYTOCHEMICAL SCREENING

The *Cordia myxa* extracts underwent quantitative phytochemical screening, which revealed the presence of many classes of chemicals, including tannins, flavonoids, alkaloids, polyphenols, glycosides, vitamin C, and vitamin E. The screening was conducted using established techniques (Table 3).

PHENOLIC PROFILE OF CORDIA MYXA EXTRACTS BY HPLC

The chemical composition of *Cordia myxa* extract was analyzed using high-performance liquid chromatography (Table 4).

Table 3. Phytochemical screening results of Cordia myxa crude plant

Chemical compound	*Cordia myxa*	
Alkaloids	3.5904	atropine mg/g
Polyphenol	18.072	Gallic acid mg/g
Tannins	3.8103	tannic acid mg/g
Flavonoids	11.808	Rutin mg/g
C	10.932	ascorbic acid mg/g
E	1.656	vitamin E mg/g
Glycosides	4.6	Securidaside mg/g

Table 4. The main composition of Cordia Myxa extracts

Phenolic compounds	Concentration (µg/mg)
Caffeic acid	728
Chlorogenic acid	214
Silybin	81
Naringin	126
Rutin	9932
Gallic acid	114

CYTOTOXIC EFFECT OF CORDIA MYXA EXTRACT ON SW480 CELL LINE

The data from the study showed that there was a significant decrease in the viability for all concentrations except with the lower two concentrations (62.5 µg/ml and 31.25 µg/ml) which caused an insignificant variation in the viability in comparison with the control (Table 5, Fig. 1). Also, the present study found an IC_{50} of 500 µg/ml of the extract inhibits 50% of SW480 cells.

Serum-starved cells were incubated at concentrations of 31.25 62.5, 125,250, 500, and 1000 µg /ml of cordia myxa extract for 24 hours. Cell viability was measured using an MTT assay. One-way ANOVA followed by the Bonferroni multiple comparison test was used for analysis. Data are presented as mean ± SEM.

EFFECT OF CORDIA MYXA EXTRACT ON CASP3 GENE EXPRESSION IN SW480 CELL LINE

The real-time PCR data analysis reveals a significant variation in CASP3 level between the control group and treated SW480 cells with *Cordia myxa* extract at concentrations of 1000 µg /ml. However, there was no significant increase at 500 µg /ml concentration (Table 6, Fig. 2).

Serum-starved cells were incubated at concentrations of 500, and 1000 µg /ml of cordia myxa extract for 24 hours. Gene expression of CASP3 was measured using the RT-PCR technique. One-way ANOVA followed by

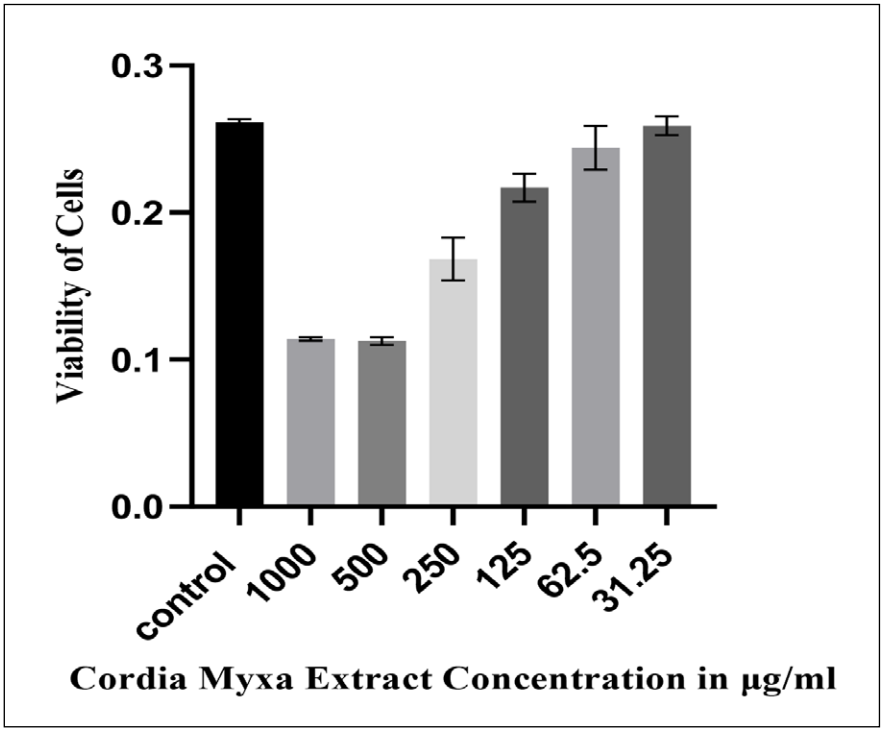

Fig. 1. Effect of Cordia myxa extract on the viability of SW480 cells.

Table 5. Effect of Cordia myxa extract on the viability of SW480 cells

Concentration (µg/ml)	Mean ± SEM	P Value
1000	0.1475 ± 0.01023	<0.0001 ****
500	0.1488 ± 0.01023	<0.0001 ****
250	0.09317 ± 0.01023	<0.0001 ****
125	0.04450 ± 0.01023	0.0026 **
62.5	0.01750 ± 0.01023	0.6316 ns
31.25	0.00250 ± 0.01023	>0.9999 ns

ns: non-significant, **P<0.01 vs. control , ****P<0.0001 vs. control

Table 6. Effect of Cordia Myxa Extract on CASP3 Gene Expression

Concentration (µg/ml)	Mean ± SEM	P Value
500	-0.4977 ± 0.2573	0.1885 ns
1000	-2.578 ± 0.2573	<0.0001 ****

ns: non-significant, ****P<0.0001 vs. control

Table 7. Effect of Cordia myxa extract on the COX-2 level

Concentration (µg/ml)	Mean ± SEM	P Value
100	-1.521 ± 0.7961	0.2405 ns
500	6.368 ± 0.7961	<0.0001 ****
1000	11.91 ± 0.7961	<0.0001 ****

ns: non-significant, ****P<0.001 vs. control

the Bonferroni multiple comparison test was used for analysis. Data are presented as mean ± SEM.

IMPACT OF CORDIA MYXA EXTRACT ON COX-2 LEVELS FOR SW480 CELL LINE

The study's data demonstrated that the COX-2 level decreased significantly for the two concentrations (1000 and 500 µg/ml), However, in comparison to the control, the COX-2 level decreased somewhat at the 100 µg/ml concentration (Table 7, Fig. 3).

Serum-starved cells were incubated at concentrations of 100, 500, and 1000 µg /ml of *Cordia myxa* extract for 24 hours. COX-2 level was measured using a human COX2 ELISA kit. One-way ANOVA followed by the Bonferroni multiple comparison test was used for analysis. Data are presented as mean ± SEM.

DISCUSSION

Cancer is a significant worldwide health concern that is projected to overtake heart disease in terms of impact. Chemotherapy has been a prominent therapeutic intervention since World War I, but it induces several detrimental side effects. In the last two decades, there has been a rise in the development of treatments derived from plants, herbs, and vegetables that have the potential to prevent or decrease the occurrence of cancer. Scientists suggest prioritizing the use of botanicals for secure and efficient anticancer therapies, as opposed to synthetic alternatives [28]. The study demonstrates that *Cordia myxa* leaves extract contains a variety of phytochemical substances, including alkaloids, polyphenols, tannins, flavonoids, vitamin C, vitamin E, and glycosides in different quantities. These components may contribute to the anti-proliferative action of the extract. The study demonstrates that *Cordia myxa* extract effectively suppresses the proliferation of SW480 cells, and the cytotoxicity of the extract escalates proportionally with the dosage. The findings align with the study conducted by [29], which showed a clear cytotoxic effect on human colon cancer HCT116 and SW480 cell lines in a dose-dependent manner. Furthermore, other studies discovered that the application of *Cordia myxa* ethanolic extract to MCF7 human breast cancer cell line and A549 lung carcinoma epithelial cells resulted in a substantial reduction in cell viability, with the extent of this effect depending on the dosage [30]. One approach to treating cancer is to exert control over or perhaps halt the unregulated proliferation of cancer cells. Utilizing the cell's intrinsic apoptotic pathway is an exceedingly efficient approach. Furthermore, focusing on apoptosis is the most effective non-invasive therapy [31]. Caspases are enzymes responsible for carrying out apoptosis, a process of programmed cell death. Among these enzymes, caspase-3 is a commonly activated protease that is crucial for initiating, transmitting, and enhancing intracellular signals that lead to apoptosis [32]. The real-time PCR data analysis reveals a significant increase (P <0.0001) in casp3 level between the control group and treated SW480 cells with *Cordia myxa* extract at high concentrations. This finding supports [33] conclusion that the Cordia species extract triggers apoptosis in the MCF-7 breast cancer cell line by upregulating casp8 expression and downregulating bcl2 expression. Another study has shown that following treatment of a human cervical cancer cell line with *Cordia* species extract, a rise in apoptotic cells displaying typical features and noticeable DNA fragmentations, key indicators of apoptosis, was detected. So the extract of *Cordia myxa* leaves demonstrates anticancer properties by causing cell death in sw480 cells by the activation of apoptosis, which is facilitated by an increase in casp3 levels. The extract's polyphenolic components may contribute to these effects because of its combined antioxidant and pro-oxidant characteristics. The relationship between inflammation and the formation of tumors is widely recognized and has been extensively supported by genetic, pharmacological, and epidemiological research in the past

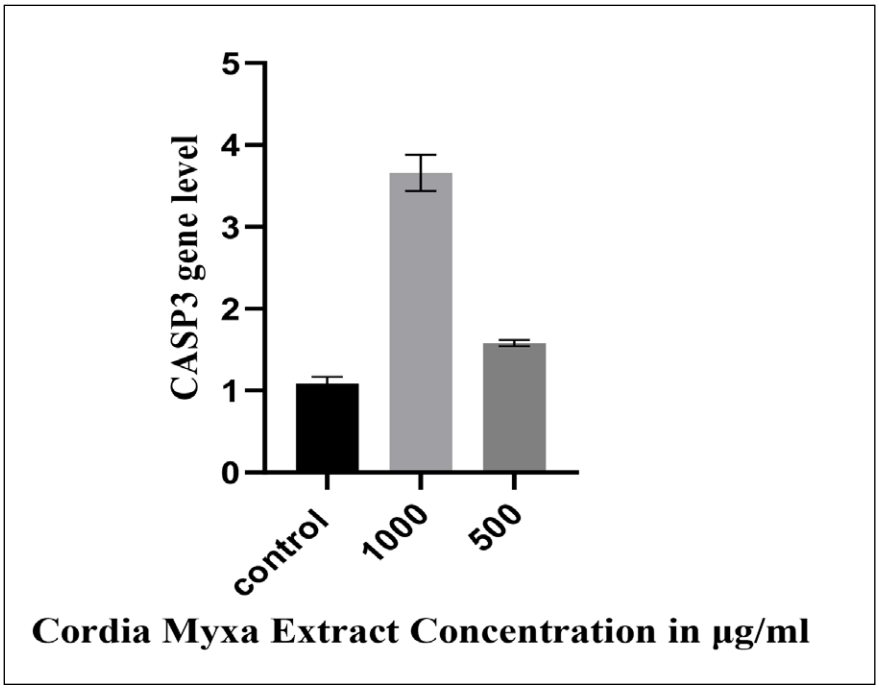

Fig. 2. Effect of Cordia myxa extract on CASP3 gene expression for SW480 cells.

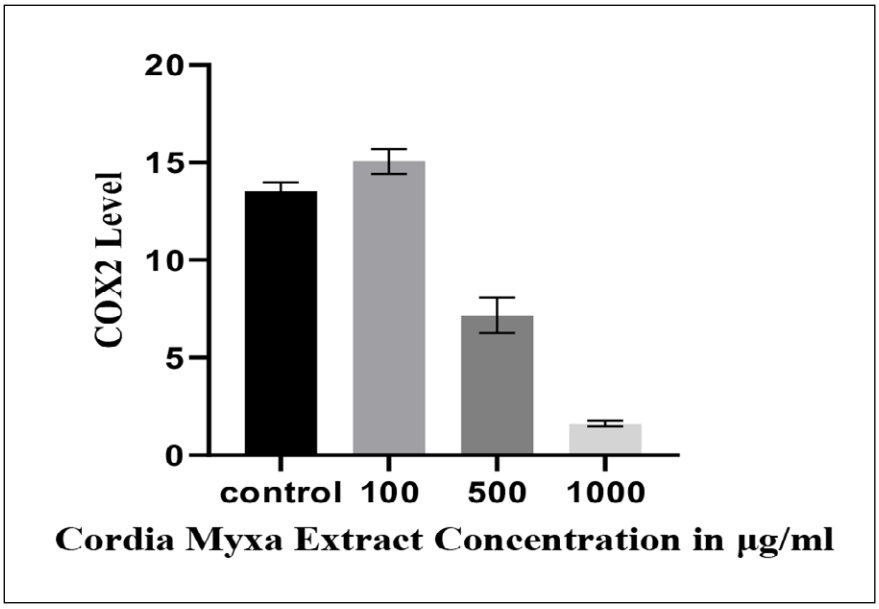

Fig. 3. Effect of Cordia myxa extract on COX-2 level for SW480 cells.

decade. Inflammatory bowel disease significantly increases the risk of developing colon cancer [34]. In the present study, the data demonstrated that the COX-2 level decreased significantly for the two concentrations (1000 and 500 µg/ml) in comparison to the control. As a result, Cordia myxa extract can act as a COX-2 inhibitor with anti-inflammatory properties. This finding aligns with the research conducted by [35] which showed that cordia myxa extracts may act as analgesic and anti-inflammatory agents via COX-2 mechanisms in experimental mice models. The ethanolic leaf extract of Cordia myxa was found to include alkaloids, glycosides, flavonoids, phenols, vitamin C, vitamin E, and tannins, as determined by the phytochemical analysis. Hence, it may be inferred that the extract's anti-inflammatory properties may be attributed to its bioactive components, particularly flavonoids. The anti-inflammatory and anti-cancer action of leaves may be influenced by the polyphenols and flavonoids present in them.

CONCLUSIONS

Cordia myxa shows promise as a suitable option for developing anticancer medications and warrants more research as a prospective candidate for studying colon cancer. This plant may have potential uses in cancer treatment due to its activities as an antioxidant, anti-inflammatory, and anticancer.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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