Egypt. J. Chem. **Vol. 65,** No. 11 pp. 761 - 777 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Cytotoxic, Genotoxic and pro-apoptotic effect of some medicinal plants on the expression of colon cancer-related genes (P53 & Bcl2) in the colorectal cell line and syngenic animal cancer model

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Abstract.

Colorectal cancer (CRC) is the third leading cause of cancer-associated mortality worldwide. Current available chemotherapeutics against CRC have various disadvantages. Medicinal plants are source of many biologically active compound(s) which have potential for development as therapeutic agents against different types of cancer. The aim of the present study was to screen for effective novel therapeutic agents against CRC from natural medicinal plants and herbs.

Method:20 different plants from various species were collected randomly from various areas of Egypt. The cytotoxic and genotoxic effects against HCT-116 colon cancer cells was analyzed. Cytotoxicity was determined by MTT assay and comet assay was used to assess DNA damage. Gene expression was analyzed by qPCR for P53, Bcl2 and Bax.

Results:The results revealed that the methanolic extracts of *Ononis vaginalis* and *Quercus robur L.* showed significant cytotoxic effect against HCT116 cells *in vitro* in monolayer and multicellular cancer spheroids(MCS). Further investigation of methanolic extracts-treated HCT116 cells revealed marked fold increase in the expression of p53, Bax, whereas Bcl2 expression was significantly decreased. Moreover, the methanolic extract of *O.vaginalis* induced more DNA damage to colon cancer cells(HCT-116), the results was confirmed by the visual score which indicated that most of the effect was gradual increase in the length of the comet tail in parallel with a decrease in nuclear DNA content. This variation in DNA damage accounted for the observed genotoxic effect of *O.vaginalis* on HCT-116 cells in-vitro. Also, the methanolic extract of *O.vaginalis* significantly inhibited tumor growth in Syngeneic tumor model in C26 injected mice compared with the vehicle control group. Conclusion: Collectively, the findings of the present study indicated that methanolic extract of *O.vaginalis* was able to decrease proliferation and promote apoptosis in colon cancer cells by inducing the p53/Bax-mediated signaling pathway.Therefore, It may be used as a novel source of new antitumor agent in the treatment of CRC.

Keywords: Colorectal cancer; medicinal plants; cytotoxicity; genotoxicity; Apoptpsis, P53. Bcl2; syngeneic tumor model.

Introduction:

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women worldwide [1]. However, the diagnosis and medical treatment have advanced significantly recently, its prevalence is rising, and the survival rate is still low [2]. CRC ranks third among the leading causes of cancer-related mortality worldwide, with >1.2 million new cases and >600,000 deaths annually [3].

CRC progression is multi-factorial and multistage process that involves the activation of oncogenes and inactivation of tumor suppressor genes. Several studies stated that p53 is a key tumor suppressor gene and an important elements of anticancer defense mechanisms in humans [4,5]. It is believed that the progression of CRC follows mutations of p53 genes [6]. P53 mutations are known to play a critical role in Cell proliferation, Tumor growth and Tissue invasion [7].

Dysregulation of p53 tumor suppressor gene is one of the most frequent events contributing to the transformation of colorectal cancer (CRC), as well as the aggressive and metastatic features of CRC [5]. P53 is a stress-inducible transcription factor, which

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Receive Date: 14 May 2022, Revise Date: 17 July 2022, Accept Date: 01 August 2022

DOI: 10.21608/EJCHEM.2022.138368.6087

regulates a large number of diverse downstream genes to exert regulative function in multiple signaling processes. It induces the expression of proapoptotic Bcl-2 (B-cell lymphoma-2) family proteins, mainly Bax and downregulates the pro-survival Bcl-2, leading to permeabilization of outer mitochondrial membrane and eventually activates apoptosis and cell death pathway. [9- 8].

Currently available chemotherapeutics against CRC have various disadvantages such as high price, toxicity, and low efficacy. These disadvantages highlight the need for exploring safe and effective novel therapeutic agents against CRC [11].

Natural products, including medicinal plants and herbs, have played a significant role in the discovery and development of new drugs and therapeutic agents. Medicinal plants are source of many biologically active compound(s) which have potential for development as therapeutic agents. More than 35,000 plant species have been reported to be used in various human cultures around the world for medical purposes [12]. There is a growing scientific consensus about the impending loss of natural product drug discovery scientists [13].

medicinal plants and herbs are known to contain various phytochemicals, such as flavonoids, polyphenols, polysaccharides, triterpenoids, alkaloids, glycosides, phenols and luteolin glycosides. These phytochemicals were proven that they inhibit tumor cell proliferation and also induce apoptosis. Also, they affect transcription and cell cycle via different mechanisms [14,15].

So, the aim of the present study was to determine the therapeutic effects of different medicinal plants and herbs on colon cancer cells and elucidate the molecular mechanisms underlying the medicinal properties in colon cancer, in order to provide the basis for new pharmaceutical entities for future research in colon cancer treatment.

2.1.Experimental:

2.1.1. Collection of plant material

Plant material used in this study amounts to 20 species as shown in Table 1. Plant specimens were collected randomly. Native species were collected from various areas of Egypt, no special approvals were required for collection of wild plant samples. The identification of cultivated plants was done by Therese Labib [16], and Voucher specimens were deposited in the National Research Center (NRC-Plant Drug Discovery Herbarium), Dokki, Giza, Egypt. The identification of native plants was carried out by Loutfy Boulos, and the nomenclature was done according to [17]

2.1.2. Extraction and preparation of working concentrations:

Preparation of plant extracts were carried out according to the method of [18] with slight modification. The dried plants were grinded into final powder then extracted by maceration with 90% methanol and concentrated to dryness in rotary evaporator at 45°C. The resulting extract was stored in a refrigerator at -20°C until use. The plant extract was dissolved at a concentration of 10mg/500µl DMSO for preparation of stock solution.

2.2. In-Vitro Study:

2.2.1. Determination of anticancer activities (Cytotoxic activity):

2.2.1.1. Cell culture

All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, and Sanford, ME, USA). The cytotoxic effect of the plant extracts was assayed against human carcinoma cell lines HCT-116 (colon), as well as hTERT-RPE1 human normal immortalized epithelial cell line. HCT116 cell cultures were maintained in DMEM, while, hTERT-RPE1 cell line were cultured in DMEM-F12 media with 10 % fetal bovine serum, penicillin (100 U/mL), and streptomycin (2 mg/mL) and maintained at 37 °C in 5% CO_2 and 95% humidity. Cells were passaged using 0.15 % trypsin-versene solution. Cell lines were kindly supplied by Professor *Stig Linder*, Oncology and Pathology department, Karolinska Institute, Stockholm, Sweden, and were formerly obtained from the American Type Culture Collection (ATCC).

2.2.2. Cytotoxicity on HCT-116 colon cancer monolayers:

Cells were plated in 96-well plate at a concentration of (1×10^4) cells/well for HCT-116 and (2×10^4) cells/well for hTERT-RPE1 cell lines. 24h after seeding, media were aspirated and replaced with serum-free culture media containing the extracts at different concentrations (100 µg/ml - 0.78 µg/ml) for 48hrs in triplicates. Doxorubicin served as positive control and DMSO (0.5 %) was served as negative control. The cytotoxicity was evaluated using the MTT assay and according to the following equation: $\%\ cytotoxicity = [1- (AV_x / AV_{NC})]\ x\ 100$ where AV_x: average absorbance of sample well and AV_{NC}: average absorbance of negative control well measured at 595 nm with reference at 690 nm[19].

2.2.4. Cytotoxicity bioassay on HCT116 and hTERT-RPE1 multicellular Spheroids:

The multicellular cancer spheroids were utilized for screening of active extracts. The Generation of 3D-multicellular spheroids model was done following the method of Ivascu and Kubbies [20] with slight modification. HCT116 human colon carcinoma cell line was recruited as model for in vitro 3D tumours generation. A cell suspension (10000 cells/well) were centrifuged in poly-HEMA coated 96-round bottom plates at 1000 ×g for 10 minutes. Cells were incubated for 5 days in a standard growth medium until the formation of compact spheroids of about

500-µm diameter (Figure3a&b) medium was changed every 48 h.

Test extracts were added in triplicates to a final concentration of 100 ppm and were incubated for 120 h. 1 µM final concentration Doxorubicin was used as positive control and 1 µl DMSO was used as a negative control. At the end of incubation, cytotoxicity was determined using the acid phosphatase method [21]. After washing twice with 250 µl PBS buffer, 100 µl of 0.1M sodium acetate, 0,1% Triton X-100, p-nitrophenylphosphate (Pierce Biotechnology Inc, Rockford, IL) were added to each well and incubated for 1.5 hours at 37°C. after incubation, 10 µl 1N NaOH stop solution was added to each well, and absorbance was read at 405 nm. Cytotoxicity was calculated according to the following equation: [1-(av(x))/(av(NC))]*100. Where: Av: average, X: absorbance of sample, NC: absorbance of the negative control.

2.2.5.2. RNA isolation

The assay was done following the procedure of Oliveira et al [22] .In brief, HTC-116 cells were seeded on a six-well plate at a concentration of 3×10^5 cells/well. After a 24-h incubation, the IC_{50} dose of plant extract was applied to each well excluding the control well. Total RNA was isolated according to the Trizol reagent protocol. The cells were collected and centrifuged at 2500rpm for 15 minutes. The total RNA isolation kit (RNeasy extraction kit) was used to isolate RNA while TranScriba First Strand cDNA Synthesis kit (BIORAD) is used to obtain cDNA, as per the manufacturer's instructions.

2.2.5.3. Reverse transcription (RT) reaction:

The RT-PCR analysis was performed using specific primers for the gene. To identify gene expressions, the results of the selected gene's expressions were normalized to the beta-actin housekeeping gene (Table.3.). The RT reaction was started by incubation at 50 °C for 45min for cDNA synthesis and followed by real-time PCR amplification cycles (95°C for 10 sec and 60 °C for 60 sec, 40 cycles) in a Rotor-Gene 3000 (Corbett Robotics, Australia). Negative control was also used in each run to access the specificity of primers and possible contamination [22].

2.2.5.4. Gene expression analysis by quantitative Real-Time PCR method(qPCR)

P53, BCL-2, BAX, gene expression analyses were performed using the Step One Plus quantitative RT-PCR system (AppliedBiosystems, USA) with respect to SYBR Green (Thermo-Scientific, USA) method [23].

2.2.5.5. Assessment of DNA damage using the comet assay

The comet formation test was used to assess genotoxic damage of HCT-116 by plant extract at IC_{50} values. The comet assay was done according to the method of (Basri et al., [24] with slight modification. HCT 116 cell suspension at concentration of 5 x 10^4 cells/ml was seeded in 6-well microtitre plate and incubated for 24 h. The cells were then treated with extract and incubated. At the end of incubation period (30 minutes), cells were washed twice with PBS solution then trypsinized The solutions were centrifuged at 2500 rpm for 10 min. A low-molecular-weight genomic DNA was extracted as described before (Yawata et al., 1998). The supernatant contained the fragmented DNA was extracted with an equal volume of neutral phenol-chloroform-isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 µg/mL ethidium bromide (Sigma Aldrich, USA) at 20 µg/ml. The extent of DNA damage was recorded under a fluorescent microscope (Biochrom ASYS, UK).

2.3. In Vivo approach

2.3.1. Animals

For *Acute toxicity* study: adult seventy eight (78) male white albino mice weighing (25-30g) were used for the study. For *Syngeneic mice mode*: Fifteen (15) Balb/c male mice (seven-to 9-week-old) were recruited. All animals were procured from *Schistosome Biological Supply Centre* (SBSC) at *Theodore Bilharz Research Institute* (TBRI), Imbaba. All the animals were acclimatized for a week under standard husbandry conditions. The animals were housed in polypropylene cages (45×24×15cm), maintained under room temperature of 25 ± 2ºC and 12 h light/ dark cycles. The animals had free access to a standard pellet diet and normal tap water was available ad libitum to the animals throughout the experimental period. The standard pellet diet contained 24% protein, 4% fat, 4.5% fiber, and 2% vitamin. Animal handling and experimental procedures were approved by the NRC Animal Ethics Committee in accordance with the guidelines provided by the CPCSEA and World Medical Association Declaration of Helsinki on Ethical Principles for studies involving experimental animals. The study was carried out in compliance with the ARRIVE guidelines.

2.3.1. Acute toxicity and determination of median lethal dose LD_{50}.

2.3.1.1. Sample size determination:

The total number of mice(N) required for the acute toxicity study was calculated by G*power program was 80. To achieve the experiment, the mice were randomly(according to weight) allocated in to different groups, each with an equal number of mice (n=6).The inputs required to output the total sample size were the number of the experimental groups, the significance of the probability, the size effect, the power of the experiments, and the applied statistical analysis. In this manner, the significant levels were at alfa 0.05(□=0.05), the power of the experiment was 0.95, and one-way analysis of variances was applied. Following computation, the total sample

size was (80), which were then divided into groups of six mice (n=6) each.

2.3.1.2. Acute toxicity study

To determine acute toxicity, the protocol reported by Wilbrandt [25]was followed with slight modifications. After one week of acclimatization, animals were divided randomely into different groups, each group of 6 mice(n=6). Two plant extracts that showed promising anticancer activity were selected. The methanolic extracts of (*Ononis vaginalis* and *Quercus robur*) were administered by oral gavage at different doses (100, 250, 500,1000,2000, and 3000 mg/kg body weight) to a total of 6 healthy mice for each concentration. Animals were given the highest concentration (3000mg/Kg. body weight) first then were observed continuously for the initial four hours followed by further monitoring for 20 hours for signs of any toxicity/lethality. After that, the second concentration (2000 mg/Kg. body weight) was introduced and the signs of toxicity/lethality was recorded. The step was repeated with the next two concentrations (1000 and 500 mg/Kg. body weight) until no further death signs was observed, hence, the remaining concentrations (100 and 250 mg/kg body weight) were not tested. One group (n=6) was given orally the respective amount of DMSO and left as control. At the end of experiment, the untreated animals were left for breading purposes.

2.3.1.3. determination of LD_{50}:

the LD_{50} promising extracts were determined on uninfected mice to determine the threshold toxic dose. LD_{50} of each extract was thus determined using the method described by Wilbrandt [25]. LD_{50} of each extract was calculated according to the formula:

$LD_{50} = Dm - \Sigma (Zxd)/ n$

Where Dm is the minimum dose that kills all animals in the group;

Z is the mean of dead animals in two successive groups;

d is the constant factor between two successive groups;

n is the number of animals of each group; and

Σ is the sum of (Zxd).

2.3.2. Syngeneic mice model

2.3.2.1. Experimental procedure:

The syngeneic animal model was performed following the method of Hoffman R. et al[26] with slight modification. The C26 cell line was selected to induce the colon cancer tumor model. The obtained tumor has several characteristics that make it a suitable experimental animal model for human colon cancer studies. After harvesting, the cells were loaded in a 1-ml tuberculin syringe with 18-G hypodermic needle, to take up cells, if necessary. Make sure to remove all air bubbles from the syringe column. One hundred microliters of C26 cell suspension (5×10^6) in culture medium was injected subcutaneously into the right rear flank of each animal. After approximately 3 weeks, when the subcutaneous tumors had grown to >100 mm^3, the xenografted mice were randomly divided into 3 groups (n=5 per group). Control group (g1); treated with vehicle only. Group 3 and 4: are plant extract treated groups (*Ononis vaginalis* or *Quercus robur* extract). In plant extract treated group the mice were intragastrically administered *Ononis vaginalis* or *Quercus robur* extract (at 216 and 192 mg/kg per ,1/10 $LD_{50,}$ respectively) dissolved in 5 mL of 1% CMC in PBS/kg three-time weekly for 28 to 30 days, while the control group mice were gavaged with saline. The diameter of the tumor was measured weekly by a vernier caliper to calculate the tumor volume (V=L x S x S/2 where V, tumor volume; L, long diameter; and S, short diameter). Mice were sacrificed after injection by pentobarbital (50mg/kg IP). The formed tumor masses were isolated and prepared for Immuno-histochemical analysis.

2.3.2.2. Immuno-histochemical

The excised tumor specimens were removed, subsequently snap-frozen in liquid nitrogen and prepared for TUNEL staining (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeled method) [24]. The frozen sections (7-mm) were then treated with 4% paraformaldehyde for 30 min and incubated with 0.3% H_2O_2 for 20 min. The sections were then permeabilized with 0.1% Triton X-100 for 2 min and dyed with TUNEL reaction mixture (5 µl TdT+45 µl fluorescein-dUTP) for 60 min. Images were immediately captured using fluorescence microscopy.

Statistical analysis:

Microsoft Excel and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) were used for statistical analysis and plotting graphs of in-vitro cell line studies. The sample size was calculated by G*Power software (g*power computer program version3.1.9.4)

The in-vivo data were analysed by using SPSS Software Version 22. All data were done in triplicate and were expressed as the mean ± S.E.M. from three different experiments. One way ANOVA followed by followed by post hoc Dunnett's test, was used to compare the differences among the groups. All *p*-values less than 0.05 ($p < 0.05$) were considered as statistically significant.

3.Results

3.1. In vitro cytotoxicity activity

3.1.1. Cytotoxicity on HCT116 and hTERT-RPE1 monolayers:

The screening of 20 methanolic plant extracts on HCT116 and hTERT-RPE1 monolayer cell lines is illustrated in Figure 2. It was observed that 8 extracts

showed more than 65% cytotoxicity at 100 µg/ml against HCT-116 colon cancer cell line. Meanwhile, The screening on hTERT-RPE1 cell line monolayer at 100 µg/ml indicated that only two plant extract showed low cytotoxicity(<20% cytotoxicity) .These extract were *Ononis vaginalis* and *Quercus robur L.* with 15% and 2.5% cytotoxicity respectively.

The two methanolic plant extracts were subjected to dose response study at different concentrations to calculate their IC_{50}. It was found that the IC_{50} for *Quercus robur L.* and *Ononis vaginalis* extracts were 90.1 and 58.0 µg/ml respectively.

3.1.2. Cytotoxicity screening on HCT116 and hTERT-RPE1 multicellular Spheroids

Unlike classical cellular monolayer, three dimensional cultures known as multicellular tumor spheroids(MCS) provides a more reliable system to investigate the *in vivo* drug efficacy. Therefore, the active extracts against HCT116 cancer cells on the 2D culture with low cytotoxic effect on the normal cell line were selected for further characterization in the environment of three-dimensional culture using HCT116 and hTERT-RPE-1 multicellular spheroids (MSC) that were developed successfully in our laboratory and results are represented in Figure 3. In this study, the two promising extracts (*Quercus robur L.* and *Ononis vaginalis*) were screened on HCT116 and RPE1 spheroids to assess their penetration power. The results displayed in table 2 indicated that the *Ononis vaginalis* and *Quercus robur L.* extract showed 94±5.3% and 64.6±1.2% cytotoxicity onHCT-116 cancer cell line respectively. Regarding normal cell line, they showed 4.7±0.6% and 10.5±0.5 respectively.

3.2.2. Gene expression analysis by real-time (qPCR):

RT-PCR Results of Control and plant extracts treated cells were observed in terms of gene expression analysis using RT-PCR after total RNA isolation; cDNA synthesis using the cDNA Synthesis kit. Effects of plant extracts on RT-PCR analysis in the present study demonstrated that the real time PCR showed a significant fold increase in the *P53* mRNA level in HCT116 cell line treated with *O.vaginalis* extract in comparison with untreated cells (11.5±0.05,P<0.05). While *Q. robur* extract had moderate effect on P53(8.9±0.023 ,P<0.05). In the same manner, exposure of cells to promising plant extracts for 24 h resulted in a marked fold decrease in the protein expression of anti-apoptotic Bcl-2 (0.22±0.006 for *Q. robur* and 0.5±0.005 for *O.vaginalis*, at P<0.05). Meanwhile, the expression of pro-apoptotic Bax protein. Was significantly associated with a fold increase (8.3±.0083 for *Q. robur* and 5.0785±.01286 for *O.vaginalis*, at P<0.05) Figure 4.

3.2. In-Vivo approach:

3.2.1. Short term toxicity and determination of median lethal dose (LD_{50})

Concerning *Ononis vaginalis* extract most of the animals (5 animals out of 6) were dead at a dose of 3000 mg/kg body weight. While there were no negative changes in the behavioral patterns of animals were observed as a decrease in food intake, weight loss, and decrease in the activity at doses 1000, to 100mg/kg body weight with median lethal dose LD_{50} 2166.7 mg/kg (Table: 4). In case of *Quercus robur* extract six, five, and 3 animals were dead at concentrations of 3000, 2000, and 1000 mg/kg, respectively. There were no any negative changes in the behavioral patterns of animals were observed at other concentration with LD_{50} is 1291.7 mg/kg. (Table:5)

3.2.3. Assessment of genotoxic activity using alkaline comet assay

The genotoxic effects of the two plant extracts on HCT-116 cells were illustrated in figures (5&6) and table (2). Figure(5) presented the tail moment of HCT-116 cells after treatment with the two plant extract(*O.vaginalis* and *Q.robur)* at 100µg/ml (IC_{50} value) for 30 min. One-way ANOVA analysis displayed a significant (P< 0.05) increased of HCT 116 cell tail moment at 100µg/ml (IC_{50} value) for both methanolic plant extracts when compared to the negative control (8.75±0.48A.U.) which were 26.50±0.65A.U and 18±1.2A.U, respectively. From Figure 5 it could be observed that the methanolic extract of *O.vaginalis* induced more DNA damage to colon cancer cells(HCT-116), these results was confirmed by the visual score of the DNA damage illustrated in figure 6.

Also, classification of the comets represented in table 2 indicated that most of the effect was in classes 2&3. Comet categories are defined by the size and fluorescence intensity of the head and tail as well as tail length. The differences among classes reflect the extent of DNA damage. Class 0, normal nucleus (very little DNA damage); class 1, halo around the nucleus (slight DNA damage); class2&3 , gradual increase in the length of the comet tail in parallel with a decrease in nuclear DNA content(varying DNA damage). This data accounted for the genotoxic effect of *O.vaginalis* on HCT-116 cells in-vitro.

3.2.2. Effect of methanolic plant Extract on tumor growth inhibition and apoptosis in CD balb/C mice (Syngeneic mice model):

To verify the effect of active methanolic plant extracts on HCT116 cells *in vivo*, our lab utilized a murine colon adenocarcinoma cancer model. The model was established successfully by implanting Colon26 (3×10^5) cells subcutaneously into the right flank of male BALB/c mice as previously described. CD balb/C mice were then treated with LD_{50} concentration of both *Ononis vaginalis* (2166.7 mg/kg, Figure7 (a-e). Based on tumor volume measurements, the methanolic extract of *Ononis vaginalis* exerted an inhibitory effect on C26 tumor growth as indicated by a decrease in tumor volume by almost 93% (at concentration 2166.7 mg/kg) compared with the vehicle control group (Fig. 8 g

and h). TUNEL staining of tumor sections showed manifestation of apoptosis which confirmed that the formed mass is a tumor not an inflammation (Figure7-f)

Table (1): plant names under investigation

	B-no	Plant name	part	family	kind
1	338	*Euphorbia dendroides L.*	L	Euphorbiaceae	W
2	1246	*Putranjiva roxburghii Wall.*	H	Euphorbiaceae	C
3	1722	*Anacardium occidentale L.*	Br	Anacardiaceae	C
4	1728	*Derris elliptica (Wallich) Benth.*	Br	Fabaceae	C
5	1807	*Washingtonia filifera*	Br	Arecaceae	C
6	1709	*Pleiogynum solandri*	Fr	Anacardiaceae	C
7	1770	*Adenanthera pavonina*	L+Br	Fabaceae	C
8	1800	*Ruta graveolens*	H+Fl	Rutaceae	C
9	1803	*Acacia seyal*	L+Br	Fabaceae	C
10	1802	*Acer negundo*	Bark	Sapindaceae	C
11	71	*Ficus misoranus*	Br	Moraceae	C
12	75	*Cordia holstii*	L	Boraginaceae	C
13	122	*Rumex dendatus*	weed	Polygonaceae	W
14	142	*Silene rubella*	weed	Caryophyllaceae	W
15	166	*Opuntia chrisa-cretics*	Br	Cactaceae	C
16	182	*Retama raetam*	H	Fabaceae	W
17	217	*Hyoscyamus boveanus*	H	Solanaceae Juss	C
18	319	*Ononis vaginalis (C.D. 2-4-99)*	H	Fabaceae	W
19	337	*Anithacanthus virgularis L.*	L&Br	Acanthaceae	W
20	549	*Quercus robur L. (C.D. 23-8-99)*	Br	Beech family	W

*w: ,c: ,H: , L: , Br: ,Fl: , Fr: ,

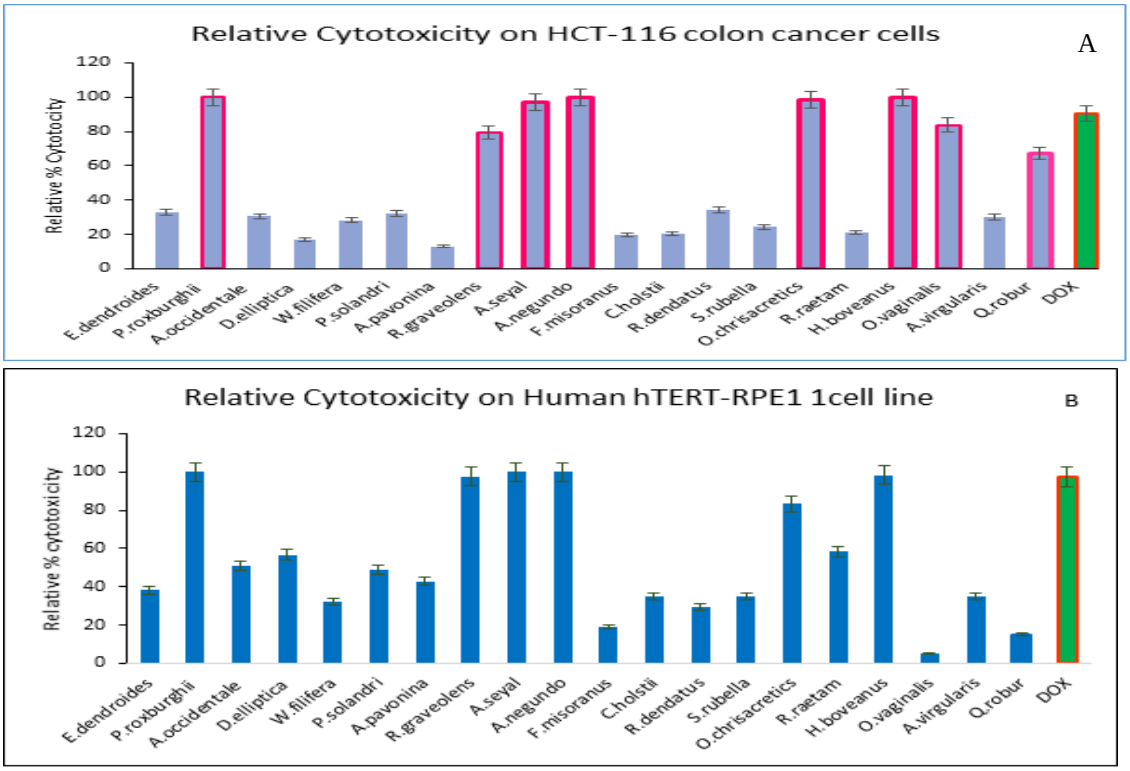

Figure 2: Average % cytotoxicity of the methanolic extract of different plants and doxorubicin (DOX) on cell viability/proliferation on human colon cancer cells, HTC116(A) and hTRET-RPE1 Normal cell line(B) after 48 h, evaluated by MTT assay. Values are expressed as mean ± SD, n = 3 at a concentration of 100µg/ml.

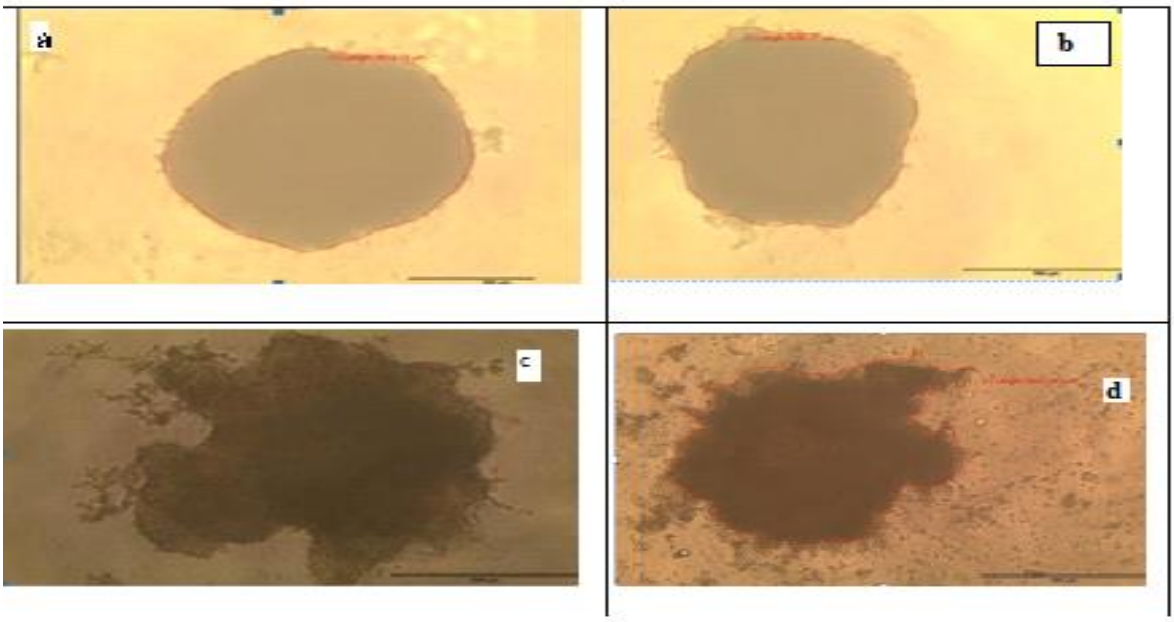

Figure3 :a&b: Phase-contrast microscope image of HCT-116 spheroids of size = 500µm diameter showing the outer proliferating layer and the inner hypoxic core, c: exposed to methanolic extract of *Ononis vaginalis* at (100µg/ml) and d:exposed to methanolic extract of *Quercus robur* at (100µg/ml) versus cisplatin (50µM). Control untreated spheroids were cultured in plain media. Imaging in 96-well microplates was carried out manually on Olympus CKX41- inverted phase-contrast microscope, cellsens software. Treatment of cells with plants methanolic extract for 7 days resulted in significant loss of morphology and integrity as indicated by the red line referring to the outer layer of the spheroid (proliferating layer).

Table (2): percent cytotoxicity of monolayer (2D) and multicellular spheroids (3D) against HCT116 colon cancer cell line and hTERT-RPE-1normal cell line

Treatment	**% Cytotoxicity**			
	2D		**3D**	
	HCT-116	**hTERT-RPE-1**	**HCT-116**	**hTERT-RPE-1**
∗*O.vaginalis* (100 µg/ml)	83.8±0.3	5.2±0.4	94±5.3	4.7±0.6
∗*Q. robur* (100 µg/ml)	67.5±1.7	15.3±0.8	64.6±1.2	10.5±0.5
∗∗Doxrubocin(	91.4±4	94.9±2.3		
∗∗Cisplatin (50 µM)			37.26 ± 3.01	42.12 ± 3.35

∗Results are represented as mean ±SD of 3 individual treatments.

∗∗Doxorubicin was used as positive control drug in the2D study, while Cisplatin was used as positive control drug in the 3Dsystem.

Table 3: Primer Sequence of different genes

Gene	**Forward strand**	**Reverse strand**
p53	5'-CCCCTCCTGGCCCCTGTCATCTTC-3'	5'-GCAGCGCCTCACAACCTCCGTCAT-3'
Bcl-2	5’-CCTGTG GAT GAC TGA GTA CC-3’	5’-GAGACA GCC AGG AGA AAT CA-3’
Bax	5’-GTTTCA TCC AGG ATC GAG CAG-3’	5’-CATCTT CTT CCA GAT GGT GA-3’
B-actin	5’-GTGACATCCACACCCAGAGG-3’	5’-ACAGGATGTCAAAACTGCCC-3’

Table 2: DNA damage in colon tumor cell lines treated with O.vaginalis and Q.robur

Treatment	**No. of cells**		**Class****			
	Analyzed*	**Comets**	**0**	**1**	**2**	**3**
HCTControl (-ve)	400	**35**	91.25%	5.50%	1.75%	1.50%
O.vaginalis	400	**106**	**73.50%**	**7%**	**10.50%**	**9%**
Q.robur	400	**72**	82%	3.75%	7.75%	6.50%

*: Number of cells examined per a group, **: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus.

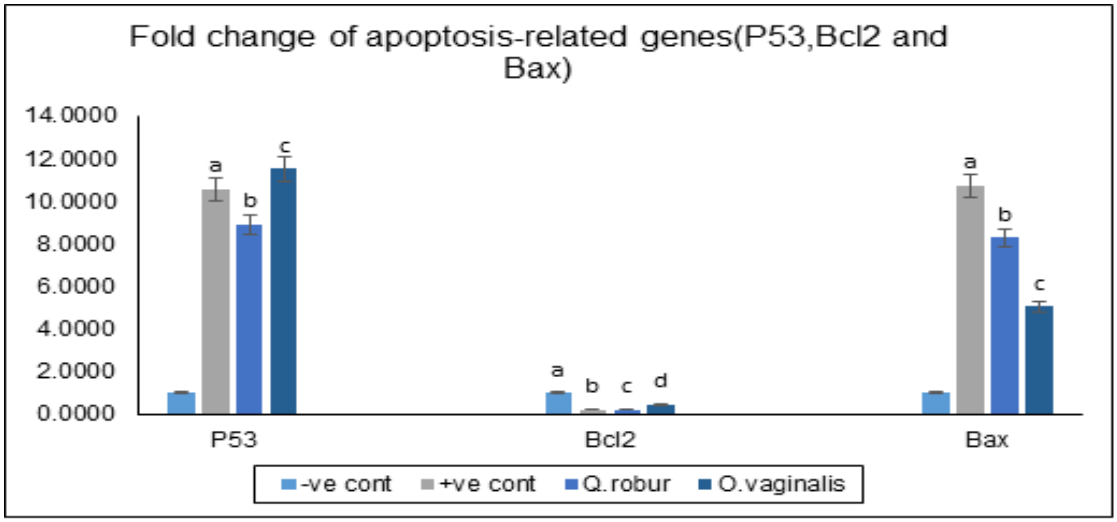

Figure 4: Gene expression pattern of HCT116 cells treated with methanolic plant extracts for 24hr. using real-time polymerase chain reaction. Fold change difference in the mRNA expression of apoptosis related genes (P53, Bcl2 and Bax) relative to beta-actin mRNA expression was calculated. Data are represented as mean ±SD of 3 individual treatments. Statistical evaluation was done by applying One Way-ANOVA followed by LSD test. Different letters indicate significant difference at ($p<0.05$).

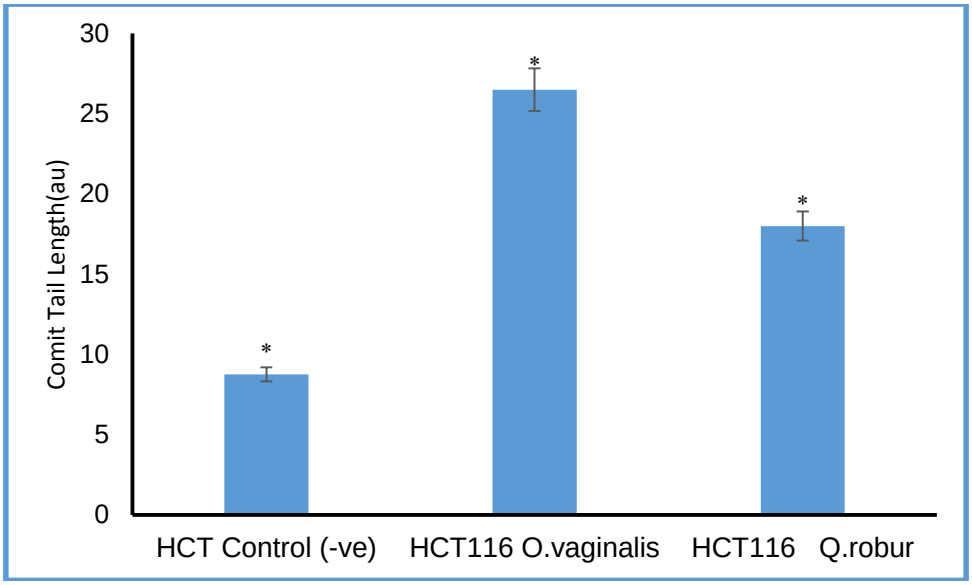

Figure 5. Comet Score(AU) indicating DNA damage in HCT 116 cells after 30 min of treatment. Cells were treated with methanolic extract from *O.vaginalis* and *Q.robur*.DNA damage expressed as the mean percentage of DNA in the comet tail according to the alkaline comet assay. Four hundred (400) cells were analysed for each treatment. Data are expressed as the mean AU of triplicates from 3 different experiments ± S.E.M (*)—statistically significant differences compared to the control (ANOVA ($p < 0.05$).AU stands for arbitrary units.

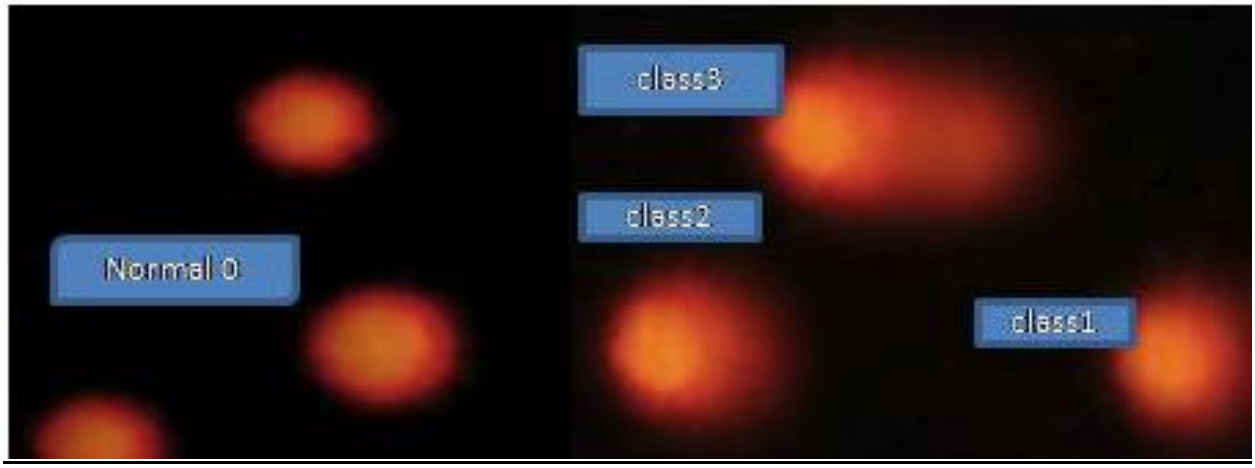

Figure 6: Photo of HCT 116 cell stained with ethidium bromide under fluorescent microscope at 30 min representing Visual score of DNA damage (classes 0, 1, 2 and 3) using alkaline comet assay in colon cancer cell lines exposed to methanolic extract from O.vaginalis and Q.robur. Comet categories are defined by the size and fluorescence intensity of the head and tail as well as tail length. The differences among classes reflect the extent of DNA damage. Class 0,normal nucleus(very little DNA damage); class 1,halo around the nucleus(slight DNA damage); classes 3&4, gradual increase in the length of the comet tail in parallel with a decrease in nuclear DNA content(varying DNA damage).

Table 4: Lethal dose LD50 of *Ononis vaginalis* methanol extract

Dose (mg / kg b.wt.)	Number of mice	Number of dead mice	Z	d	(Z)x(d)
100	6	0	0	100	0
250	6	0	0	150	0
500	6	0	0	250	0
1000	6	1	0.5	500	0
2000	6	2	1.5	1000	1500
3000	6	5	3.5	1000	3500

LD_{50} of *Ononis vaginalis* methanol extract = 2166.7 mg/kg

Table 5: Lethal dose LD_{50} of *Quercus robur* methanol extract (*Qr*)

Dose (mg / kg b.wt.)	Number of mice	Number of dead mice	Z	d	(Z)x(d)
100	6	0	0	100	0
250	6	0	0	150	0
500	6	0	0	250	0
1000	6	3	1.5	500	750
2000	6	5	4	1000	4000
3000	6	6	5.5	1000	5500

LD_{50} of *Quercus robur* methanol extract = 1291.7 mg/kg

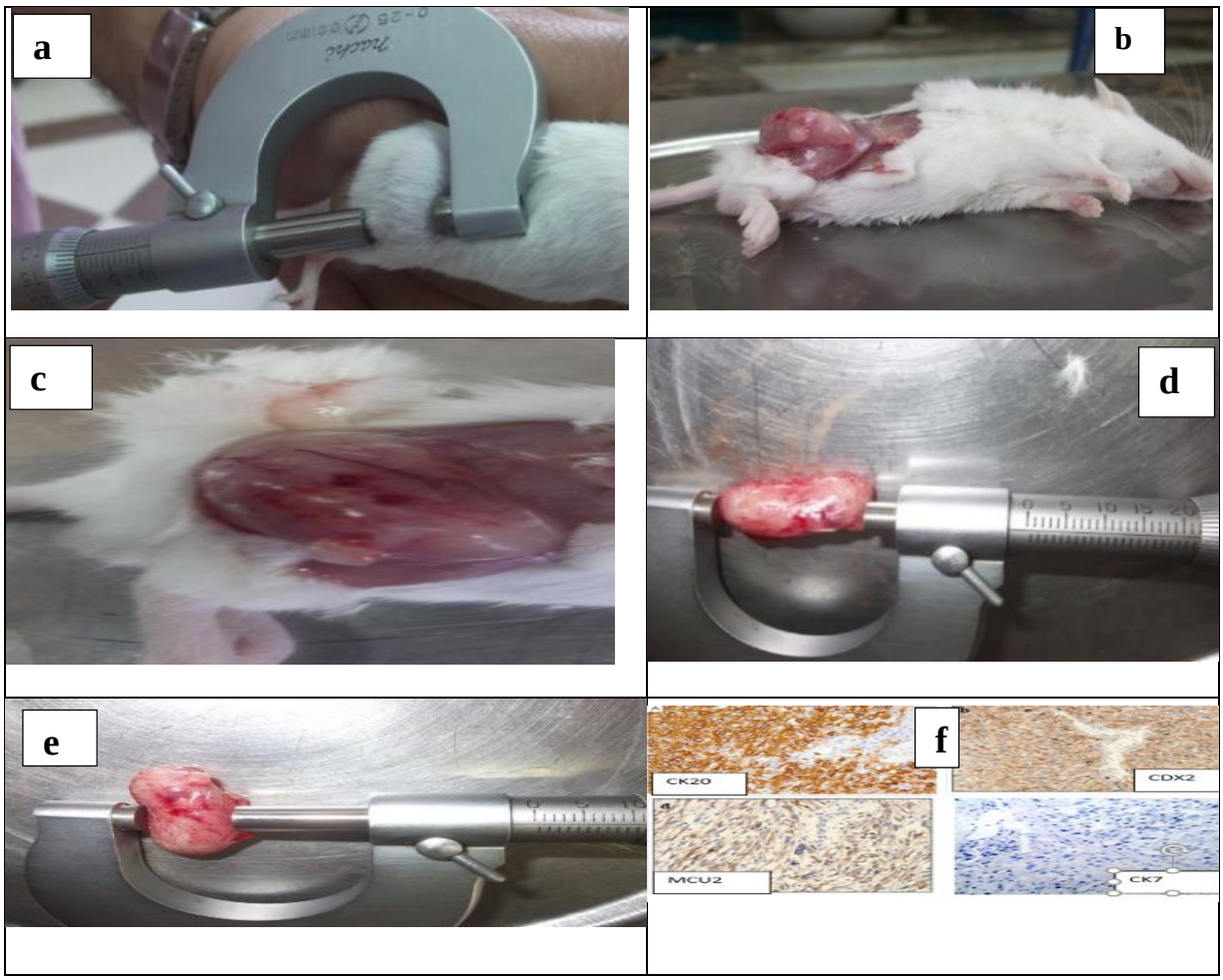

Figure 7. Tumor formation in balb/c mice injected with C26 cells, (a) Tumor formation and (b,c) tumor diameter in Balb/c mice after C26 cell injection on the day of harvest. (d,e) tumor diameter in Balb/c mice after C26 measured by caliber. The volume of tumor mass ranged from 2cm -2.5cm length and 1.1cm -1.5cm width. The diameter of the tumors was measured by vernier calipers: Tumor volume= long diameter x short diameter x (short diameter/2); n=3. (f)Immunohistochemical markers for colorectal cancer a histopathological study proved that the growing mass is perfect cancer, not an inflammation.

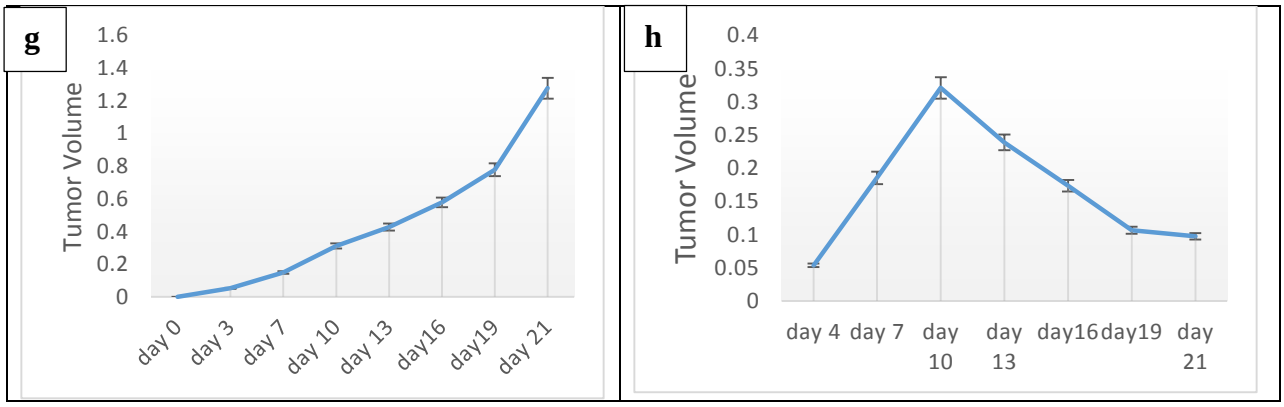

Figure 8: Tumor formation in balb/c mice injected with C 26 cells. Tumor volume was evaluated and calculated in control group treated with saline(g) vesus plant extract treated group(h) for 28 days

Discussion:

Seeking for new effective anticancer agent is a challenge for both researchers and patients. The development of side effects and drug resistance remains an obstacle to be solved. Plant-based anticancer agents continues to grab attention of most scientists as it thought to be likely more effective in the cancer treatment [27]. In the present study, the cytotoxic, genotoxic and gene modulatory effects of methanolic extracts of some medicinal plants against human colorectal cancer cell HCT 116 were investigated. From the MTT assay, the methanolic extracts of O.vaginalis and Q.robur showed high cytotoxic effect against HCT-116 cell line after 48 h of treatment at 100ug/ml as compared to the positive control drug(DOX) . On the other hand, both exerted very low cytotoxic effect against the human normal cell line (hTERT-RPE-1). These effects may be related to the presence of natural compounds that have the ability to affect only cancer not normal cell lines [28]. Our findings are in agreement with a previous study [24] which reported that acetone extract from the stem bark of C. odontophyllum displayed the highest cytotoxic effect against HCT 116 cells. Also, several studies had reported that the cytotoxic activity of a plant against cancer cells is based on their phytochemical properties [24,29,30,]. As mentioned before, plants are known to contain plethora of natural compounds, mainly flavonoid and phenolic compounds, that possess diverse biological activities such as anti-inflammatory, anti-allergic, antimicrobial, anticarcinogenic and antitumor effects [29,31]. Polyphenolic compounds are known to contribute to the antitumor activity of most methanolic plant extracts [32]. Based on the obtained results, the methanolic extract of (O.vaginalis and Q.robur) were selected for further investigations, as both of them displayed potent cytotoxic effect on colon cancer cells, meanwhile, they were safe to normal cells. In general, an anticancer agents to be effective, it should have to execute the cancer cells and at the same time will not exert any toxic effect towards the normal cells [28, 33]. Our results are in agreement with results of a similar study which stated that the extracts of *Eugenia jambolana* Lam. were able to inhibit the growth of breast cancer cell lines (MCF-7 and MDA-MB-231) but did not affect the growth of normal breast cells (MCF10A) [34]. The mechanisms underlying the antitumor activity of these cytotoxic extracts on cells with uncontrolled growth may be related to the inhibition of increased nucleic acid synthesis and suppression of the abnormal metabolic pathways [35].The cancer cells depend mainly on aberrations in apoptosis signaling pathways in order to proliferate and devide. This also accounts for the cytotoxic selectivity of anticancer agents towards cancer not normal cells [36].

The methanolic plant extracts were screened against the multicellular cancer spheroids. Treatment of cells with plants methanolic extract for 7 days resulted in significant loss of morphology and integrity of the outer layer of the spheroid (proliferating layer) as confirmed by the acid phosphatase test. Un like the usual cellular monolayer, three-dimensional culture systems known as multicellular cancer spheroids, which are performed completely in our laboratory, represent a more decisive method to elucidate the efficacy of new anticancer drug entities and reveal their penetration though solid tumor mass in vivo. It was stated that the MCS are an excellent model for testing drugs as the formed tumor mass is of great resemblance to the in vivo ones [37 &38]. Moreover, The HCT-116 MCS are composed of two distinguished layers. The outer one is active proliferating layer, while, the inner is a non-dividing hypoxic center. The presence of proliferating cells near to blood supply and quiescent cells far from blood supply is a characteristic of clinically diagnosed solid tumors. Following chemotherapy, the quiescent subpopulations cells regrow during the recovery period of cancer patients, causing a problem to cure cancer. Accordingly, the spheroids model is of great benefit for in vitro screening for agents that can eradicate this specific non-dividing resistant subpopulation of cells. In addition, the spheroids model can select drugs that can penetrate the solid tumor, which is a significant feature of a successful antineoplastic agent [39 & 40].

The active methanolic extracts of (O.vaginalis and Q.robur) display significant cytotoxic effect on the formed spheroids as compared to Cisplatin(at 50uM). In a previous study, it was reported that treatment of spheroids with cisplatin (50 μM for 7 days) induced apoptosis to the outer layers of the spheroids but did not eliminate the cells, and that was related to its poor penetration power. In the present study, both plant extracts showed cytotoxic effect on the HCT-116 spheroid [28]. To confirm the safety of the selected extracts, a counter screening on normal cell line (hTERT-RPE-1) spheroids was utilized [18].

Moreover, in the present study, the extracts of (*O.vaginalis* and *Q.robur*)showed potent cytotoxic activity against colon cancer cells in both mono layer and multilayer cancer spheroids. These findings prompted us to further investigate the mechanisms underlying this effect on molecular basis.

Based on the results of the Comit test, the cytotoxic effect induced by (*O.vaginalis and Q.robur*) methanolic extract against HCT 116 cells may be caused by DNA damage and the recorded genotoxicity might be the cause of cell death via apoptosis. Alkaline comet assay was used to detect genotoxic effect of test

compound by measuring its DNA damage at single cell that can be observed under a fluorescent microscope whereas a comet head (nucleus) and its tail (DNA fragments) can be seen [40]. The percentage of DNA in tail and tail moment represent the most precise outcome for the degree of damage [42]. In this study, the methanolic extract from (O.vaginalis and Q.robur)showed significant ($p < 0.05$) DNA damage in HCT 116 cells after 30 min of treatment. The ultimate goal for most anticancer agents is to induce highly damaged-DNA in cancer cells and through variable mechanisms and different types of DNA lesion [43]. DNA is the key target for most cytotoxic anticancer drugs as the most vital cell cycle phase is DNA replication. Cytotoxic anticancer drugs may act directly through reactive metabolites or indirectly through the incorporation into DNA nucleotide analogues or by blockade of DNA-metabolizing functions such as DNA polymerase or topoisomerase. Damage may cause disruption of transcription or replication of the DNA where it can lead to cell death or aging [44]. An example of genotoxic agent that binds to DNA and abrupt the replication is doxorubicin. Doxorubicin binds to DNA through intercalation between specific bases and thus prevents DNA synthesis [45].

It is stated that medicinal plant-derived extracts contain various compounds that can act as antitumor compounds and have apoptosis-inducing properties in cancer cells [46]. A previous study suggested that apoptosis was the primary cell death of HCT 116 cells after treatment with ethanol extract from sporophyll of Undaria pinnatifilda [47]. Moreover, plant derived anticancer drug plays a vital role in the treatment of cancer [48]as the synthetic ones such as alkylating and antimetabolite agent affects normal cell and develop many serious side effects [49]. Apoptosis or programmed cell death has been the aim for treatment of cancer during the different phases of tumor growth [50]. During apoptosis, apoptotic bodies undergo phagocytosis and will not submit itself to the inflammation process and may not cause disturbance towards nearby cell [38]. Apoptosis signalling pathways are divided into two mechanisms which are those that involved the mitochondria known as the intrinsic pathway or those that signals through death receptors namely the extrinsic pathway [51].

Cell apoptosis is regulated by several proteins on different levels of cell . P53 is a tumor suppressor protein that controls the cell cycle pathway [52]. It acts as a checkpoint in the cell cycle which allows cells to repair DNA damage at any point in the cycle. Also, P53 regulates many other genes that's involved in G1phase arrest. In the present study, treatment of HCT-116 colon cancer cells with methanolic extracts of (*Ononis vaginalis and Quercus robur*) induces significant fold increase in the P53 protein expression. Our data were in agreement with a previous study by Basu A. and Haldar S [53]who stated that A lot of DNA-damaging drugs that induce apoptosis,can prompt the expression of P53 protein.

Another important genes that are involved in the apoptotic pathway are the *bcl2* family. They are involved in the manipulation of cell survival after cytotoxic stress and considered key regulators of the mitochondrial-mediated apoptosis pathway. The family comprised of several proteins with pro-apoptotic (Bax) and anti-apoptotic(Bcl2) mode of action. The level of anti-apoptotic versus proapoptotic and their balance is the core for determining the resistance of cell to apoptosis [54].

In the present study, treatment of cancer cells with methanolic extracts resulted in upregulation of Bax protein expression meanwhile, the expression of Bcl2 was downregulated. These results indicated that plant extracts induces apoptosis through Bax-initiated mitochondrial-mediated pathway.

Since the anti-proliferative effect of many naturally occurring cancer chemo-preventive agents is tightly linked to their ability to induce apoptosis [55].

Numerous DNA-damaging drugs that trigger programmed cell death also induce p53 expression. The mechanism of p53-mediated cell death is possibly associated with its function as transcriptional modulator and this may be mediated by activation or suppression of the transcription of other genes. p53 has been shown to induce cell cycle arrest at the G1–S transition. This action of p53 has been ascribed to its ability to induce the expression of a cellular gene p21WAF1 which encodes a 21 kDa inhibitor of cyclin-dependent kinases [56].

Our findings demonstrate that promising plant extracts have an anti-proliferative impact on HCT116 cells, that could be the result of the induction of apoptosis, cell cycle arrest, and/or the inhibition of growth.

Syngeneic animal tumor model is one of the most used methods to evaluate the therapeutic effects of treatments on colorectal cancer. It is a simple, easily accessible with high tumor growth rates. The model is established by implanting murine tumor cells, from induced-colorectal cancer animals, into healthy animals with the same genetic material [57].

Because solid tumors *in vivo* harbor both quiescent and proliferating cells, so that successful treatment would likely rely on a combination of compounds targeting these distinct cell populations [58].

In the present work, we managed to establish a murine colon adenocarcinoma tumor model in male BALB/C. The investigated tumor sections indicated manifestation of apoptosis which confirmed that the formed mass is a tumor not an inflammation. Based on tumor volume measurements and considering the in-

vitro results, it could be concluded that the methanolic extract of *Ononis vaginalis* exerted an inhibitory effect on C26 tumor growth as indicated by the recorded decrease in tumor volume.

Several previous studies have utilized this model[59]. In a study by Tao and colleagues, they used a human colon cancer cell line, HCT-116, to evaluate the anticolorectal cancer activity of Weichang'an, a Chinese herbal medicine, with 5-fluorouracil. The cells were injected subcutaneously in male BALB/C mice axilla, and after tumor growth, they were transplanted into the cecum. It was observed that the tested drug the growth of colon cancer and prevented inhibited hepatic metastases [60].

As mentioned earlier, numerous studies have evaluated the potential anticancer effects of individual compounds including flavonoids. Flavonoids, a group of polyphenols found in several medical plants, also exhibited antitumor activity in vivo in different human cancer cells including CRC cells [61,62]. Interestingly, flavonoids can interact synergistically with other polyphenols in the treatment of cancer to induce apoptosis [63]. In the same context, the plant Inula viscosa leaves contain, among other compounds, sesquiterpens, flavonoids and phenols with proven anticancer activity in vivo [64].

These data, together with our in vivo results, strengthen the assumption that the different compounds composing the extract probably have a synergistic effect on the pronounced inhibition of tumor growth in mice.

Conclusion:

In conclusion, the methanol extract of (*Ononis vaginalis*) exerted an inhibitory effect on the growth of HCT116 in vitro andC26 cells in vivo with mild cytotoxic effect against hTERT-RPE-1 normal cells. The inhibitory mechanism was associated with cell cycle arrest at the G0/G1 phase by induction of p53 expression in the cultured colon cancer cell line. The increase of *P53* gene specific RNA probably suggests a mechanism of extract- induced cell death and cytotoxicity. The extracts also triggered apoptosis by down regulating the expression of Bcl-2 proteins and increased expression of Bax mRNA expression. Taken together, these findings demonstrated that methanolic extracts of (*Ononis vaginalis*)suppressed the proliferation and enhanced the apoptosis of colon cancer cells; thus, it may serve as a potential natural source of novel drug leads that could be applied in the treatment and prevention of colon cancer with no harmful side effects.

As far as known, no studies were found that examine the antitumor effect in vivo of two selected plant extract in the present study. However, the detailed molecular mechanisms and exact phytochemical composition remain unclear and further investigations are required.

Abbreviations:

DNA: deoxyribonucleic acid; RNA: Riboxyribonucleic acid; IC50: half maximal inhibitory concentration; LD_{50} MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HCT116: human colon carcinoma cell line; RT-PCR using Real-Time PCR, CRC: Colorectal cancer, hTERT-RPE1: immortalized human retinal epithelial cell line that stably expresses human telomerase reverse transcriptase (hTERT); MCS: multicellular cancer spheroids. Bcl-2 :(B-cell lymphoma-2) family proteins; DMSO:Dimethylsulfoxide;TUNEL staining (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeled method); LD_{50} :Leathal Dose that causes 50% death.

Declarations:

- **Competing interests**

The authors declare that they have no competing interests.

- **Acknowledgements**

The authors would like to express their deepest gratitude to the National Research Center, Pharmacognosy Department for their educational and supervision assistance .

- **Funding**

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

- **Ethics approval**

The study was approved for the use of animals and human cell lines by the Medical Research Ethics Committee(MREC), National Research Centre, Dokki, Egypt

- **Availability of data and materials:**

The datasets generated during and/or analyzed during the current study are not publicly available due to [privacy issues] but are available from the corresponding author upon reasonable request.

- **Consent for publication:**

Not applicable

- **Authors' contributions:**

KM and ZAE: conceived the original idea, planned the experiments, contributed to the interpretation of the results, supervised the project, took the lead in writing the manuscript and Editing. AM, DM, KI, RT, MO, MR, MS: carried out the experiments, contributed to sample preparation, in-vitro and in-vivo experiments. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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