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Candelariella vitellina extract triggers *in vitro* and *in vivo* cell death through induction of apoptosis: A novel anticancer agent

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ABSTRACT

Candelariella vitellina is common green-yellow lichen found on barks, wood, and rocks in Japanese forests. To investigate the mechanism of its anticancer potential, *C. vitellina* (80% MeOH/H_2O) extract was prepared. High-performance liquid chromatography–high-resolution electrospray ionization mass spectrometry analysis revealed seven new compounds and 11 natural compounds of terpenes and polyketides. *In vitro* cytotoxicity analysis of Caco-2 cells exhibited an IC_{50} of 125 ± 4.1 μg/mL. No significant cytotoxicity was observed *in vitro* in normal human peripheral lymphocytes. Both the IC_{25} and IC_{50} were determined to explore the potent anticancer potential in this study. *C. vitellina* exhibited a mitochondrial P53-independent apoptotic effect with negative P53 expression and an elevated *BAX/BCL2* ratio as well as upregulated *CASP3* mRNA expression. Similarly, *in vivo* analysis showed the same pattern of anticancer potential but was dependent on the *P53* expression. Furthermore, *C. vitellina* induced antioxidative conditions *in vitro* and *in vivo*. The decreased invasion of tumor cells *in vivo* and increased apoptotic features *in vitro* and *in vivo* suggest the moderate to strong apoptotic anticancer potential of *C. vitellina*. However, further studies are needed to determine the extent and mechanism of action on different cell lines to support the anticancer properties of this lichen.

1. Introduction

Colorectal cancer (CRC) occurs in the colon- and rectum-lining cells. CRC is the third most prevalent cancer worldwide after lung and breast cancers in both genders and is the fourth leading cause of mortality (Nabil et al., 2016). In Egypt, studies have shown that CRC was detected in 11–15% of patients who underwent colonoscopy and diagnosed in 29–31% of patients aged 40 years or younger (Gado et al., 2014). Epidemiological studies reported the risk factors of CRC as obesity, alcohol consumption, increasing age, smoking, family history, and dietary factors (El-Khadragy et al., 2018). In an *in-vitro* experiment, the Caco-2 cell line was used to evaluate the efficacy of anticancer agents for treating colon cancer. The carcinoma colon (Caco-2) cell line originated from human colonic adenocarcinoma and shows similarities with small intestinal enterocytes (Plackal Adimuriyil George et al., 2015). These cells develop the typical morphology of enterocytes with distinct apical and basolateral membrane domains, microvillus, and tight junctions (Awortwe et al., 2014).

Although synthetic drugs can suppress tumor growth, alternative strategies are urgently needed to overcome several limitations of treating CRC including the metastasis of cancerous cells, which is the leading cause of mortality and morbidity, increasing the sensitivity of the immune system response, and reducing inflammation caused by cancer (Hanahan and Weinberg, 2011).

Alternative natural therapies derived from plants or microorganisms have emerged to overcome the side effects of chemotherapy and for use as chemo-preventive agents in anticancer studies (El-Nabi et al., 2018; Elkhateeb et al., 2018). Over 60% of currently used anticancer drugs originated from natural sources (Cragg et al., 2009).

Lichens represent a mutualistic relationship between microalga and fungus. They are used in medicine for many purposes such as for treating bronchitis, spleen enlargement, asthma, heart and stomach

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disorders, and vomiting (Salgado et al., 2017). They are a valuable source of many natural classes with varying biological potentials including antiviral, antifungal, analgesic, antipyretic, antioxidant, and anticancer effects (Molnar and Farkas, 2010; Shrestha and Clair, 2013). *Candelariella vitellina* is a lichen that inhabits trees and woods. No studies have evaluated the biological activities of this organism. In this study, the total metabolites of *C. vitellina* were examined as *in vitro* inhibitors in the Caco-2 cell line and anti-Ehrlich solid tumor development *in vivo*. This study was conducted to explore the cytotoxic levels of *C. vitellina* extract.

2. Materials and methods

2.1. Material collection and identification

The lichen was cut from the rocks and trees in Hakozaki Higashi-ku Fukuoka-shi. Japan and identified as *C. vitellina* by the Mycological Society of Japan.

2.2. Extraction method

The sample was extracted with 80% methanol several times and the total extract was evaporated and concentrated at 40 °C with a rotary evaporator (BÜCHI, Flawil, Switzerland).

2.3. LC-HRMS analysis

A mass spectrometry (MS) system (LTQ XL/LTQ Orbitrap Discovery, Thermo Fisher Scientific, Waltham, MA, USA) was used to obtain high-resolution mass spectrometric data; this device was coupled to a high-performance liquid chromatography (HPLC) system (Accela PDA detector, Accela PDA auto sampler, and Accela pump, Thermo Fisher Scientific). The conditions were applied as previously described (Elkhateeb et al., 2018).

2.4. In vitro study

2.4.1. Maintenance of Caco-2 cell line

The human colon cancer cell line (Caco-2; passage 19) provided by VACSERA (Giza, Egypt) was cultured in 5 mL of complete growth medium (Lonza, Basel, Switzerland), which was RPMI-1640 medium supplemented with 10% fetal calf serum, 1% penicillin, and streptomycin. The cells were incubated in humidified 5% CO_2 atmosphere at 37 °C in T25 culture flasks at a density of 2×10^4 cells/cm^2. The medium was changed every 48 h. Cell confluence (80%) was confirmed with an inverted microscope. All *in vitro* experiments were performed 24 h post-seeding to prevent cell differentiation.

2.4.2. Assessment of growth inhibition using neutral red uptake assay

To evaluate the maximal half inhibitory concentration (IC_{50}), 1×10^4 of Caco-2 cells in a volume of 100 µL were seeded into each well of a 96-well plate. After reaching 70% confluence, the cells were exposed to different concentrations (0–1000 µg/mL) of *C. vitellina* for 24 h. After incubation, neutral red dye (4 mg/mL) in fresh serum-free medium was added and the cells were incubated for 3 h. The cells were washed with phosphate-buffered saline then distained using neutral red de-staining solution (50% ethanol 96%, 1% glacial acetic acid, and 49% deionized water). The absorbance was measured at 540 nm with a microtiter plate reader spectrophotometer (Repetto et al., 2008). All determinations were performed as three independent experiments in triplicate. The cell growth inhibitory rate was calculated using the following equation:

$$\text{Inhibition\%} = (1 - OD_{test}/OD_{Control}) \times 100$$

The 50% inhibitory concentration (IC_{50}) of the *C. vitellina* extract on Caco-2 cells was determined by polynomial regression analysis with Microsoft Excel.

For further mechanistic investigation, Caco-2 cells were treated with dimethyl sulfoxide (3 µl/mL), 5-fluorouracil (5-FU, 1.5 µg/mL), and the IC_{25} and IC_{50} of *C. vitellina* extract for 24 h. Cells treated with vehicle served as a negative control and the treatments were applied in triplicate.

2.4.3. Caco-2 cell examination by inverted microscopy

Caco-2 cell were examined with an Olympus BX41 microscope (Tokyo, Japan) at × 400 magnification and morphological changes were digitally evaluated.

2.4.4. Fluorescent acridine orange/ethidium bromide (AO/EB) double staining

To assess the evidence of apoptosis, necrosis, and viability of cells, double staining of cells by AO/EB was conducted as described previously by Liu et al. (2015). Briefly, 4 µL of treated and control cultured cells as described in the growth inhibition assay were transferred to glass slides and stained with 1 µL of AO/EB stain solution (100 µg/mL AO and 100 µg/mL EB) and the slides were immediately examined with a fluorescent microscope (Olympus BX41). Two hundred cells in each of five randomly selected fields were counted and representative photos were captured. In this method, viable cells were green-colored cells with intact structures and dead cells were orange to red-colored. In contrast, the apoptotic cells showed a blebbed cytoplasmic membrane and nuclear fragmentation.

2.4.5. Immunocytochemical staining

Caco-2 cells were grown on coverslips until they reached 65% confluence and then incubated for 24 h with various treatments. The Caco-2 cells were processed for immunocytochemical reactions with an avidin biotin complex immunoperoxidase technique (Hsu et al., 1981) using P53 (tumor suppressor protein), BCL2 (anti-apoptotic protein) and Ki-67 (cell proliferation marker) anti-human monoclonal antibodies (Glostrup, Hovedstaden, Denmark). Caco-2 (200 cells) were examined in five randomly chosen fields. The mean percentage of positive cells in all groups was calculated as the immunocytochemical score (positive cells/total number of counted cells) × 100. Cells were examined (× 400) and images were acquired under a light microscope (Olympus BX41).

2.5. In vivo study

2.5.1. Induction of Ehrlich tumor

Ehrlich ascites carcinoma (EAC) cells were proliferated in the peritoneal cavity of female mice through intraperitoneal (i.p.) injections of 10^6 cells in 0.2 mL of PBS/mouse and transferred every 5 days to new mice. The mice were monitored daily and cell viability was evaluated using the trypan blue dye exclusion method (Metwally et al., 2014). A model of solid Ehrlich carcinoma (SEC) was generated, with 2.5×10^6 of Ehrlich carcinoma cells implanted subcutaneously into the right thigh of the hind limb of mice.

2.5.2. Animals and experimental design

Female Swiss albino mice, 8–12 weeks old and weighing 20 ± 5 g, were housed in plastic cages at a controlled temperature (25 ± 2 °C) with a 12-h light/darkness cycle and free access to standard pellet diet and water in the animal house of the National Organization for Drug Control and Research.

Mice (7 per group) were randomly divided into three groups (control, 5-FU, and *C. vitellina*). At four days after tumor inoculation, the mice were subjected to different treatments for 5 day as follows: EAC-bearing mice injected with 0.2 mL physiological saline (0.9% NaCl) and served as control mice, EAC-bearing mice injected intraperitoneally with 5-FU at 20 mg/kg body weight and EAC-bearing mice

Table 1
LC-HRMS analysis of *C. vitellina* extract.

Accurate *m/z*	Quasi-form	Suggested formula[a]	Tentative identification[b]	Chemical class	Activity
249.11246	$[M+H]^+$	$C_{14}H_{16}O_4$	Dechloromycorrh- izin A	Terpenes	Antimicrobial (Stadler et al., 1995)
331.15442	$[M+H]^+$	$C_{19}H_{22}O_5$	Hericenone A	Terpenes	Cytotoxic (Rama Rao and Reddy, 1992)
361.16473	$[M+H]^+$	$C_{20}H_{24}O_6$	Chaetoquadrin A	polyketide	No reported activity
417.15454	$[M+H]^+$	$C_{22}H_{24}O_8$	Comazaphilone C	polyketide	Antibacterial, cytotoxic (Gao et al., 2011)
417.11768	$[M+H]^+$	$C_{21}H_{20}O_9$	3-O-(α-D-Ribofuranosyl)-questin	polyketide	Strong antioxidant (Li et al., 2009)
279.15936	$[M+H]^+$	$C_{16}H_{22}O_4$	Acrostalidic acid	Terpenes	Antifungal (Hosoe et al., 1999)
401.12302	$[M+H]^+$	$C_{21}H_{20}O_8$	Hormothamnione	polyketide	Cytotoxic (Gerwick et al., 1986)
382.12869	$[M+H]^+$	$C_{21}H_{19}NO_6$	Arnottianamide	polyketide	Cytotoxic (Yang et al., 2009)
371.11279	$[M+H]^+$	$C_{20}H_{18}O_7$	Avermutin	polyketide	No reported activity
291.06540	$[M+H]^+$	$C_{18}H_{10}O_4$	No hits	–	
323.09152	$[M+H]^+$	$C_{19}H_{14}O_5$	Demethoxyviridin	Terpenes	No reported activity
405.19070	$[M+H]^+$	$C_{22}H_{28}O_7$	Scytalidic acid	Terpenes	No reported activity
437.21674	$[M+H]^+$	$C_{23}H_{32}O_8$	No hits	–	
416.17020	$[M+H]^+$	$C_{22}H_{25}NO_7$	Ovellin B	Terpenes	Potent cytotoxic (Belofsky et al., 1998)
321.07587	$[M+H]^+$	$C_{19}H_{12}O_5$	4′-Hydroxyphlebiarubrone	polyketide	No reported activity
311.09174	$[M+H]^+$	$C_{18}H_{14}O_5$	Ligustrone B	polyketide	No reported activity
343.11789	$[M+H]^+$	$C_{19}H_{18}O_6$	Atrovenetin	polyketide	No reported activity
462.17529	$[M+H]^+$	$C_{23}H_{27}NO_9$	No hits	–	
448.19626	$[M+H]^+$	$C_{23}H_{29}NO_8$	No hits	–	
476.13373	$[M+H]^+$	$C_{26}H_{21}NO_8$	No hits	–	
355.28442	$[M+H]^+$	$C_{21}H_{38}O_4$	Ceriporic acid B	Terpenes	Antioxidant (Rahmawati et al., 2005)
439.35672	$[M+H]^+$	$C_{30}H_{46}O_2$	Ganoderol A	Terpenes	Cytotoxic (Chen et al., 2017)
455.35144	$[M+H]^+$	$C_{30}H_{46}O_3$	Ganoderiol F	Terpenes	Cytotoxic (Chen et al., 2017)
457.36728	$[M+H]^+$	$C_{30}H_{48}O_3$	Ganodermatriol	Terpenes	Cytotoxic (Liu et al., 2012)
469.36700	$[M+H]^+$	$C_{31}H_{48}O_3$	Fomefficinic acid A	Terpenes	Cytotoxic (Shen et al., 2013)
267.12289	$[M+H]^+$	$C_{14}H_{18}O_5$	Papyracon D	Terpenes	Antibiotic, cytotoxic (Shan et al., 1996)
394.12833	$[M+H]^+$	$C_{22}H_{19}NO_6$	Citropone A	polyketide	No reported activity
502.32721	$[M+H]^+$	$C_{28}H_{43}N_3O_5$	Beauverolide D	Cyclic peptides	No reported activity
516.34253	$[M+H]^+$	$C_{29}H_{45}N_3O_5$	Beauverolide E	Cyclic peptides	No reported activity
520.33917	$[M+H]^+$	$C_{28}H_{45}N_3O_6$	No hits (New Beauverolide)	–	No reported activity
522.35516	$[M+H]^+$	$C_{28}H_{47}N_3O_6$	No hits (New Beauverolide)	–	No reported activity
564.34235	$[M+H]^+$	$C_{33}H_{45}N_3O_5$	Beauverolide F	Cyclic peptides	No reported activity
530.35887	$[M+H]^+$	$C_{30}H_{47}N_3O_5$	Beauverolide A	Cyclic peptides	No reported activity
590.35996	$[M+H]^+$	$C_{35}H_{49}N_3O_5$	Beauverolide C	Cyclic peptides	No reported activity

[a] Using XCalibur 3.0 software.
[b] Derelication of the extracted molecular formula using Dictionary of Natural Products (DNP 23.1, 2015 on DVD) and Reaxys online database.

administered orally with *C. vitellina* at 150 mg/kg body weight. The experiments were approved by the Committee of Research Ethics for Laboratory Animal Care, Department of Zoology, Faculty of Science, Helwan University (approval no, HU2017/Z/02) and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, 8th edition (NIH Publication No. 85-23, revised 1985).

2.5.3. Evaluation of tumor volume (TV) of SEC

Tumor volume was evaluated on days 1 and 5 after the different treatments using a Vernier caliper (Tricle, Jiangsu, China). The volume of the developed tumor mass was calculated as follows:

$$TV\ (mm^3) = 4\pi(A^2 \times B \times 0.5)$$

where A is the shortest tumor diameter, B the longest tumor diameter, and π equals 3.14 (Metwally et al., 2014).

2.5.4. Microscopic examination of SEC tissues

The solid tumor was harvested, fixed in neutral-buffered formalin (10%) for 24 h, and embedded in paraffin, followed by sectioning into 4–5-μm sections. Sections were stained with hematoxylin and eosin (H& E) then examined with an Olympus BX 41 microscope.

2.5.5. Immunohistochemical staining of SEC tissues

Immunohistochemical detection of P53, Bcl2 and Bax proteins was performed with an avidin biotin complex immunoperoxidase technique (Hsu et al., 1981) in 4–5-μm SEC sections. All sections were analyzed with Image J software (NIH, Bethesda, MD, USA) and the score was divided into four groups based on the percentage of positively stained tumor cells as follows: 0 (negative expression, 0–15%); 1+ (weak expression, 16–30%); 2+ (moderate expression, 31–60%), and 3+ (strong expression, 61–100%).

2.5.6. Oxidative stress markers in SEC tissues

To assess lipid peroxidation (LPO), nitric oxide (NO), and glutathione (GSH) in Caco-2 cells and SEC tissues, Caco-2 cells or SEC tissues were washed with PBS and homogenized in 50 mmol of Tris–HCl buffer (pH 7.4), and the protein concentration in the supernatants was determined using the Lowry method (1951). The supernatants were used to determine LPO as described by Ohkawa et al. (1979), NO as previously reported by Green et al. (1982), and GSH as described by Ellman (1959).

2.5.7. Quantitative real-time polymerase chain reaction

To assess *BCL2*, *BAX*, and *CASP3* mRNA expression, total RNA was isolated from Caco-2 cells and SEC tissues using an RNeasy Plus Minikit (Qiagen, Hilden, Germany). cDNA was synthesized using the RevertAid™ H Minus Reverse Transcriptase (Fermentas, Waltham, MA, USA). Real-time PCR was performed using Power SYBR® Green (Life Technologies, Carlsbad, CA, USA) on an Applied Biosystems 7500 system (Foster City, CA, USA). Relative values of gene expression were normalized to *GAPDH*. Primer sequences and accession numbers of genes used in the current study are shown in Supplementary material: Table (S1).

2.5.8. Statistical analysis

Data are reported as the mean ± standard deviation (SD). Data for multiple variable comparisons were analyzed by one-way analysis of variance. To compare the significance of differences between groups, Duncan's test was used as a post hoc test using the statistical package

SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA) and a probability level of $P < 0.05$ was considered significant.

3. Results

3.1. LC-HRMS analysis of C. vitellina extract

Liquid chromatography (LC)-high-resolution mass spectrometry (HRMS) analysis of *C. vitellina* extract (Supplementary material: Fig. S1) revealed the presence of approximately 34 metabolites from diverse chemical classes (Table 1). Tentative identification of the quasi-molecular $[M+H]^+$ ion peaks and their molecular formulae indicated 27 known metabolites and 7 potentially new compounds for which no natural hits were found for such formulae in the Dictionary of Natural products Version 23.1 or Reaxys online databases. The most identified compounds represented different chemical classes of natural products, mainly terpenes, polyketides, and cyclic peptides. A literature search indicated that nine of the identified metabolites exhibited moderate to strong cytotoxic effects against different cancer cell lines, four with strong antimicrobial effects, and two with strong antioxidant effects. However, 11 of the identified compounds had no reported biological activity.

3.2. In vitro study

3.2.1. Assessment of cytotoxicity in Caco-2 cells

The 50% inhibitory effect of *C. vitellina* on Caco-2 cells after 24 h of an ascending range of incubations was determined by the neutral red uptake assay; we found an IC_{50} value of 125 ± 4.1 µg/mL. Inverted phase contrast examination revealed cellular shrinkage and irregular cell shapes following *C. vitellina* treatments (Fig. 1). Interestingly, *C. vitellina* showed no or low cytotoxicity towards normal human peripheral lymphocytes (Supplementary material: Fig. S2).

3.2.2. Assessment of morphological changes by AO/EB fluorescent double staining in Caco-2 cells

AO/EB fluorescent dual staining can be used to visualize apoptosis-associated changes in cell membranes during programmed cell death. This method can also precisely recognize cells in different stages of apoptosis (Liu et al., 2015). In the current study, no obvious apoptosis with normal intact morphology with bright green staining (Fig. 1) was detected in untreated or dimethyl sulfoxide-treated Caco-2 cells groups. However, early stage apoptosis (yellowish green nuclear staining), late stage apoptosis (orange nuclear staining), and necrosis (orange-red staining) were significant ($P < 0.05$) with *C. vitellina* incubations at high concentration compared to control Caco-2 cells (Fig. 2).

3.2.3. Immunocytochemical assessment of apoptosis and proliferation of Caco-2 cells

Antioxidants have anticancer properties such as the suppression of tumor initiation, tumor promotion, and cell transformation mediated by apoptosis induction through different pathways (Liu et al., 1998). Treatment with *C. vitellina* (125 and 65 µg/mL) had anti-proliferation activity and induced apoptosis in Caco-2 cells. However, *C. vitellina* downregulated Ki-67 (a proliferation protein marker) and BCL2 (an anti-apoptotic protein) expression (Fig. 1). Positive cell counting revealed a significant ($P < 0.05$) concentration-dependent decrease in both proteins (Fig. 3). In contrast, P53 (a tumor suppressor protein) expression showed negative immuno-reactivity in both control and treated Caco-2 cells.

3.2.4. Altered mRNA expression of apoptosis markers in Caco-2 cells

The effect of *C. vitellina* treatment on the mRNA expression levels of *BCL2*, *BAX*, and *CASP3* by was investigated by qRT-PCR. *C. vitellina* proportionally altered *BCL2*, *BAX*, and *CASP3* gene expression after 24 h of treatment in Caco-2 cells with elevated concentrations (Fig. 4). Compared to untreated cells, *BCL2* levels were significantly ($P < 0.05$) downregulated with increasing *C. vitellina* concentrations, whereas *BAX* and *CASP3* were upregulated. Moreover, the *BAX/BCL2* ratio showed significant elevation with *C. vitellina* treatment compared to the untreated control. These results suggest that *C. vitellina* treatment induced changes in the expression of apoptosis-related genes, thereby increasing the likelihood of apoptosis in Caco-2 cell lines.

3.2.5. Effect of C. vitellina on oxidative status in Caco-2 cells

To evaluate the oxidative status in Caco-2 cells, LPO, NO, and GSH were investigated after treatment with *C. vitellina*. After 24 h of exposure, LPO was significantly decreased at 125 µg of *C. vitellina* treatment whereas the NO level and GSH content were significantly ($P < 0.05$) increased with increasing extract concentrations (Fig. 5). In contrast, 5-FU treatment led to oxidative stress.

3.3. In vivo study

3.3.1. Effect of C. vitellina on SEC volume

The average SEC volume in the control group increased rapidly (15-fold) on day 4 post-tumor implantation. Interestingly, both *C. vitellina* (150 mg/kg) and 5-FU (20 mg/kg, i.p.) treatments significantly decreased the tumor volume compared to the control SEC group. However, approximately 80% tumor growth inhibition was observed with *C. vitellina* treatment whereas only 69.8% inhibition was observed with 5-FU treatment, compared to the untreated SEC volume (Fig. 6).

3.3.2. Effect of C. vitellina treatment on SEC histopathological changes

After staining with H&E, cross-sectional images of SEC showed cellular infiltration and large tumor cells between the muscle fibers in SEC-bearing animals. Treatment with either 5-FU or *C. vitellina* resulted in decreased tumor cell invasion and more abundant necrotic lesions (Fig. 7). Interestingly, mitotic activity was also decreased and apoptotic bodies were increased in treated animals.

3.3.3. Apoptosis markers expression in SEC tissues

Immunohistochemistry analysis was conducted to determine the effect of *C. vitellina* on apoptosis-related proteins. We found that *C. vitellina* administration induced upregulation of Bax and P53 and downregulation of Bcl2 compared to the control (Fig. 8). Moreover, *C. vitellina* treatment significantly elevated ($P < 0.05$) the Bax/Bcl2 ratio by approximately 3-fold compared to the control (Fig. 8).

3.3.4. Altered mRNA expression of apoptosis markers in SEC tissues

We evaluated the underlying molecular anticancer mechanisms of *C. vitellina* by qRT-PCR. Compared to the untreated control SEC, *C. vitellina* was observed to have induced significant downregulation of *BCL2* mRNA and upregulation of *BAX* and *CASP3* mRNA expression (Fig. 9). Consistent with the immunohistochemical findings, apoptosis was stimulated, as indicated by an 8-fold increase in the *BAX/BCL2* ratio compared to that in the control group. Taken together, our data provide insight into the molecular mechanisms underlying *C. vitellina*-induced apoptosis in SEC.

3.3.5. Assessment of oxidative status in SEC tissues

Oxidative stress markers in SEC tissues following treatment with *C. vitellina* or 5-FU were evaluated. Our results revealed that treatment with *C. vitellina* significantly ($P < 0.05$) increased GSH and markedly decreased LPO and NO. Thus, *C. vitellina* may play a critical role in restraining oxidative stress. However, 5-FU treatment led to increased LPO and NO levels and reduced GSH content in SEC tissue (Fig. 10).

4. Discussion

Currently, the pharmaceutical industry has focused on using natural resources to obtain active compounds for the preparation of potential

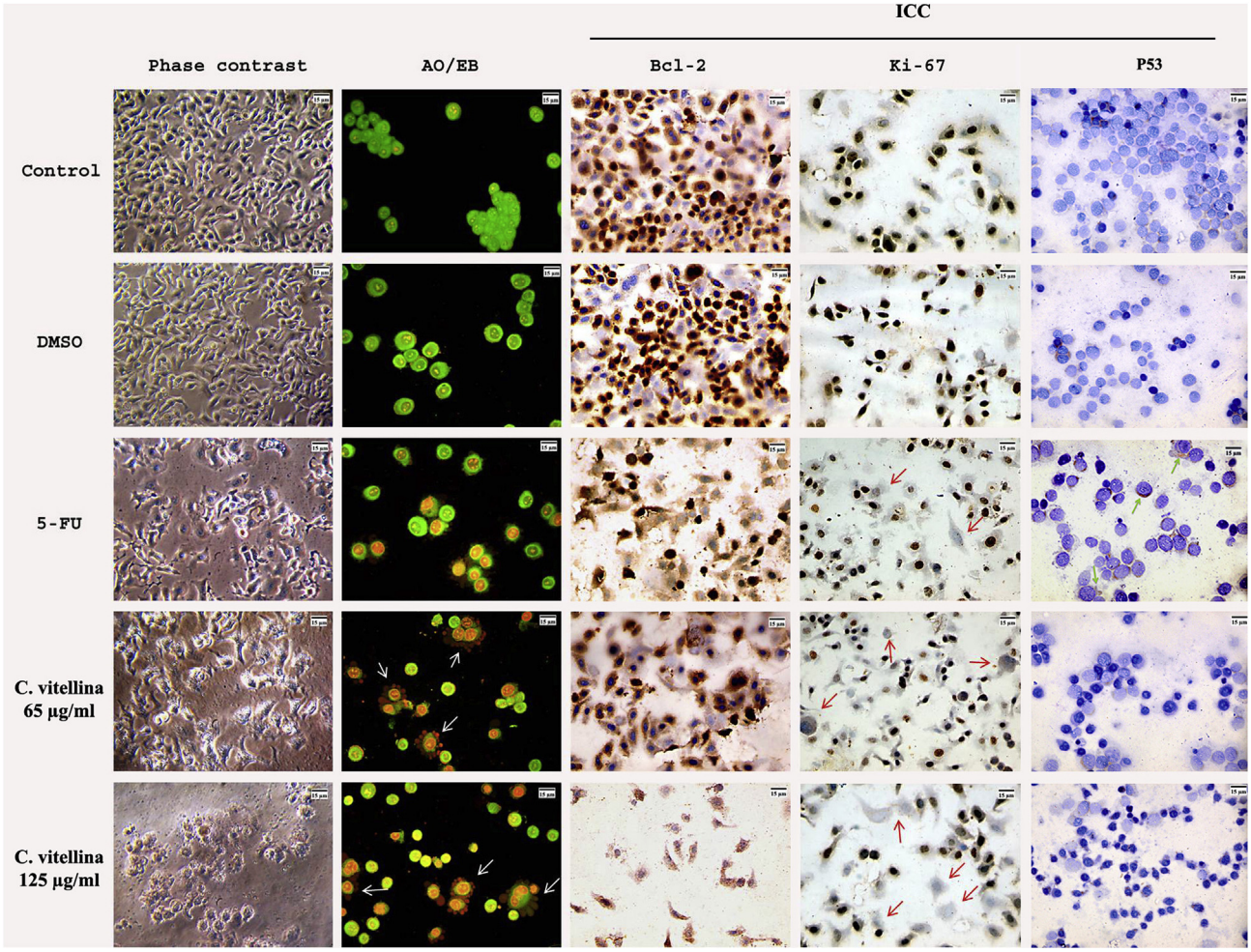

Fig. 1. Representative photomicrographs of treated and untreated Caco-2 cells after 24 h. Observed cellular morphological changes by light inverted microscopy (phase contrast) in addition to assessment of viability and cell death stages as detected by acridine orange/ethidium bromide (AO/EB) fluorescent double staining showed marked membrane blebbing (white arrows) and apoptotic nuclear fragmentation as a hallmark of apoptosis and reddish necrotic nuclei, whereas viable cells had a green intact structure. Immunocytochemical (ICC) reactivity was evaluated for BCL2 protein expression (-ve cells, black arrows) as well as Ki-67 (-ve cells, red arrows), whereas P53 expression was nearly negative following all treatments except cytoplasmic localization in cells undergoing apoptosis in the 5-FU group, green arrows). 5-FU: 5-Fluorouracil (1.5 µg/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

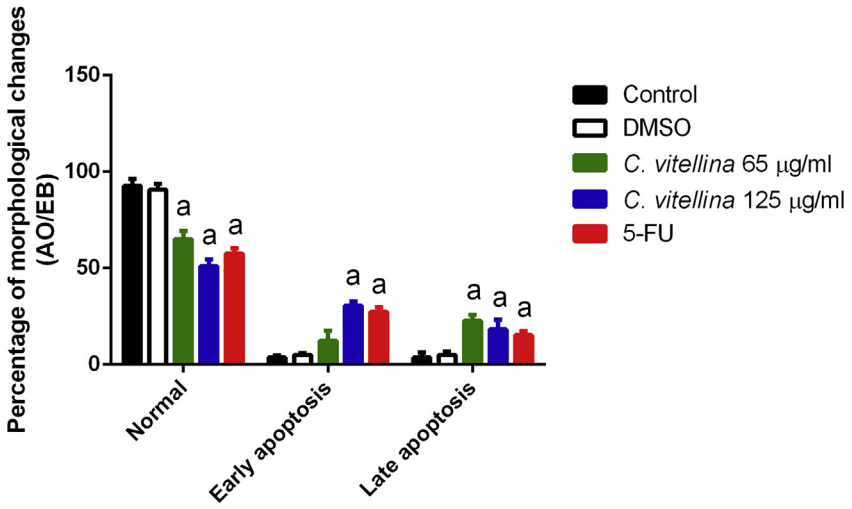

Fig. 2. *In vitro* pro-apoptotic effect of *C. vitellina* on human colon cancer cell line (Caco-2). Decreased viability of cells and increased morphological alterations towards apoptosis induction were observed after acridine orange/ethidium bromide (AO/EB) double fluorescent staining for treated and untreated cells after 24 h. Data represents the means of three independent experiments; bars, standard deviation and a: significant with respect to the control ($P < 0.05$). 5-FU: 5-fluorouracil (1.5 µg/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

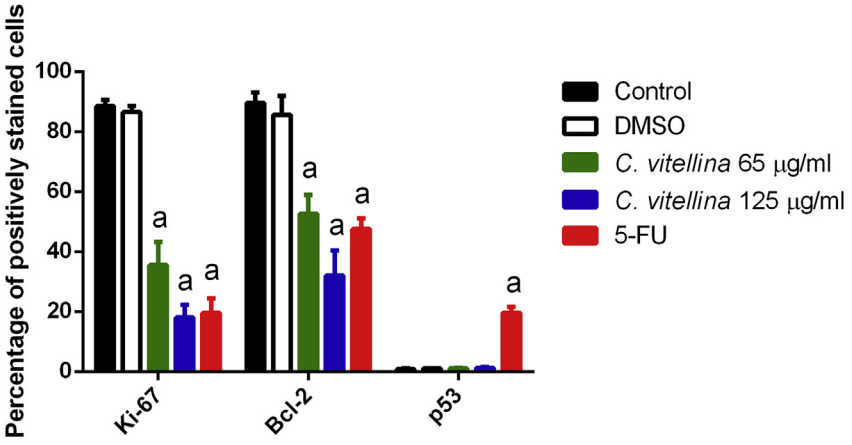

Fig. 3. Effect of *C. vitellina* on the percentage of positive immunocytochemical reactive cells for BCL2, Ki-67, and P53 protein expression shows upregulation dependent on elevated doses in the control and treated Caco-2 cells after 24 h. Data represent the means of ten different fields; bars, standard deviation and a: significant with respect to the control ($P < 0.05$). 5-FU: 5-fluorouracil (1.5 µg/mL).

anti-cancer drugs (Prada-Gracia et al., 2016). Lichens have been used for centuries in folk medicines and many cultures have used lichens to treat a variety of ailments as part of their traditional medicines. Lichens are effective against various cancer cell lines both in crude (Ren et al., 2009) and purified form (Backorova et al., 2011). Furthermore, lichen metabolites are strongly cytotoxic and can terminate cell proliferation at micro-molar concentrations (Einarsdottir et al., 2010). Although lichens have been reported to show anti-cancer activity, no reports have described the biological properties of *Candelariella* of the *Lecanoraceae* family. Hence, this study was conducted to examine the anti-cancer properties of *C. vitellina* against the Caco-2 cell line and solid Ehrlich tumor. The results clearly indicated that the crude extract of *C. vitellina* showed a significant proportional increase in anti-cancer effect on Caco-2 cell viability at increasing concentrations. Monitoring the morphology of cells treated with different concentrations of *C. vitellina* revealed that cells showed abnormal characteristics and began to detach in response to extract exposure. These changes eventually resulted in cell death.

In vivo examinations confirmed the cytotoxicity and anticancer effectiveness of *C. vitellina*. SEC tissues and other tumors tend to contain high levels of reactive oxygen species (ROS) owing to high metabolic rates that result in oxidative stress, which may enhance cancer progression and resistance to therapies (Burdon, 1995; Kumari et al., 2018). ROS are important signaling molecules that induce cellular proliferation and cause damage to DNA and macromolecular components, which may lead to cancer. Thus, ROS are thought to play a multifaceted role in tumor initiation, progression, and maintenance by promoting oncogenesis. There is evidence that decreasing intracellular

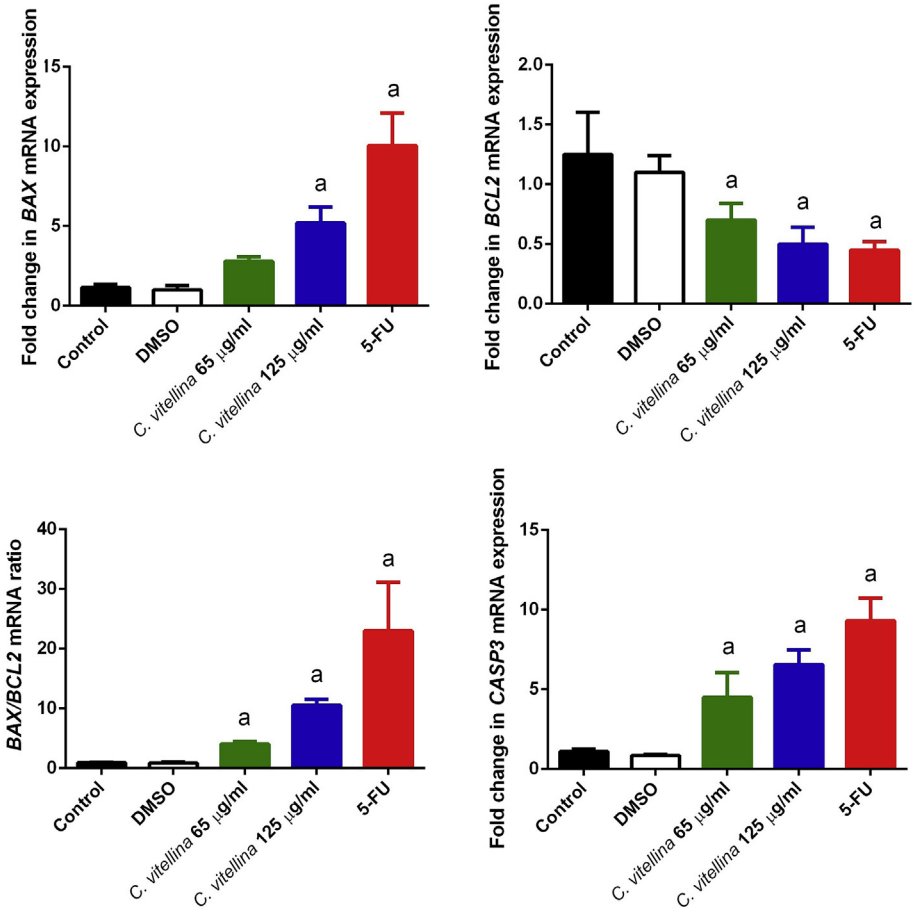

Fig. 4. Effect of *C. vitellina* on relative mRNA alterations of *BAX*, *BCL2*, and *CASP3*, apoptosis related gene expression. Furthermore, increased *BAX/BCL2* ratio depended on elevated doses in Caco-2 cells. Data (means of three assays) were normalized to the *GAPDH* mRNA level and expressed as fold-induction relative to the mRNA level in the control. Bars, standard deviation and a: significant with respect to the control ($P < 0.05$). 5-FU: 5-fluorouracil (1.5 µg/mL).

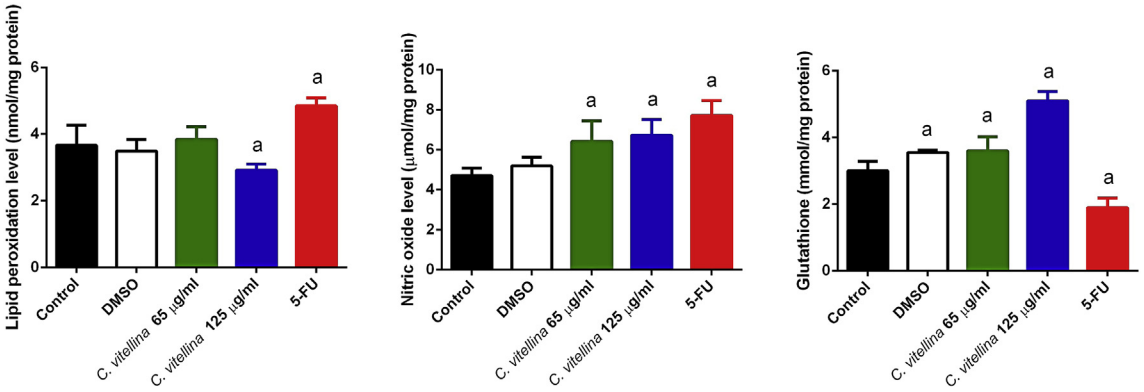

Fig. 5. Effect of *C. vitellina* on the levels of oxidative stress markers in treated and control Caco-2 cells after 24 h. Data represent the means of three independent experiments; bars, standard deviation and a: significant with respect to the control ($P < 0.05$). 5-FU: 5-fluorouracil (1.5 µg/mL).

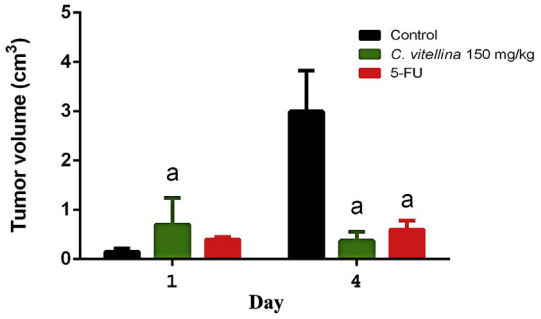

Fig. 6. Effect of *C. vitellina* on the solid Ehrlich tumor volume after four days of treatment. Increased development of tumor was significantly observed. Data represents means (n = 7); bars, standard deviation and a: significant with respect to the control ($P < 0.05$). 5-FU: 5-fluorouracil (20 mg/kg, i.p.).

ROS by treatment with antioxidants reduces cellular proliferation and cancer development (Erker et al., 2005). Antioxidant molecules exhibited cytotoxicity against cancer cells and anticancer activity in experimental animals (Rahman et al., 2017). Antioxidants protect the normal cell cycle, inhibit the proliferation or induction of apoptosis, invasion of tumors, and prevention of angiogenesis, and suppress inflammation (Barrera, 2012). Decreased antioxidant levels in cells causes an increase in the production of free radicals and damage DNA, proteins, and lipids (Mates et al., 1999). In the present study, *C. vitellina* reduced solid tumor growth and cancer cell viability. Furthermore, it reduced lipid peroxidation and NO, effectively increased the antioxidant defense system, exerted cytotoxicity against Caco-2 cancer cells, and induced murine solid tumors, suggesting the potential use of *C. vitellina* extract to inhibit intracellular free radicals induced by oxidative stress (Barrera, 2012). A strong antioxidant polyketide; 3-O-(α-D-ribofuranosyl)-questin (Li, et al. 2009) and potent antioxidants terpene, ceriporic acid B (Rahmawati et al., 2005), in *C. vitellina* extract

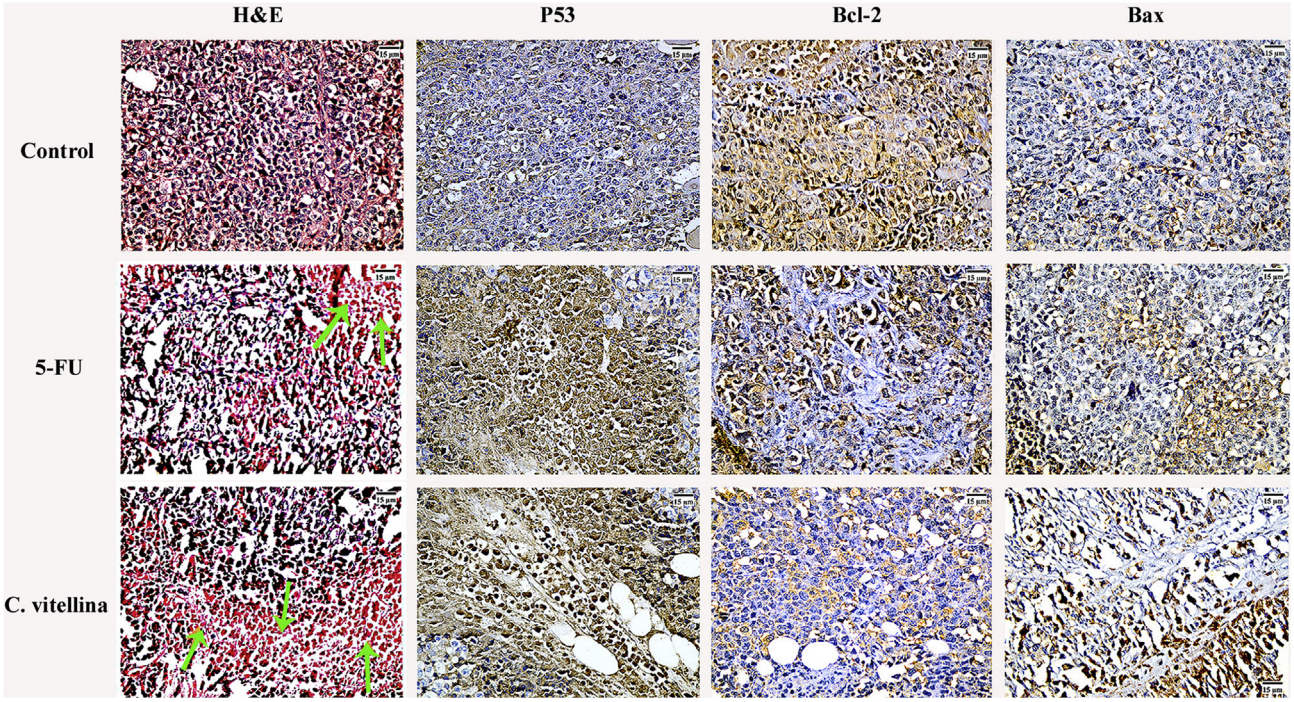

Fig. 7. Representative photomicrographs of control and treated solid Ehrlich tumor sections taken from different treatment groups and stained with hematoxylin and eosin (H&E) showing extensive necrotic areas (arrows); sections were processed for immunohistochemistry, and the development of brown color revealed upregulated nuclear localization of P53 and cytoplasmic localization of Bax protein expression whereas downregulation of Bcl2 cytoplasmic localization (n = 7). 5-FU: 5-fluorouracil (20 mg/kg, i.p.). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

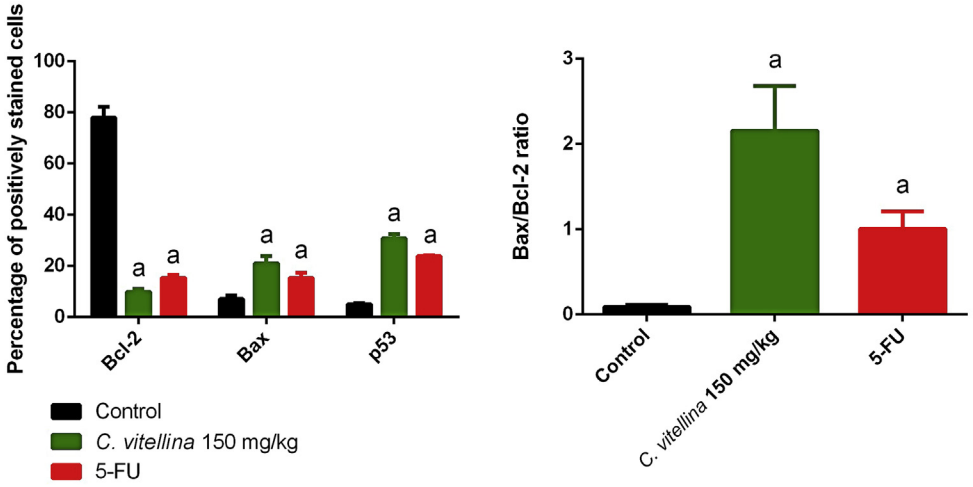

Fig. 8. Effect of *C. vitellina* on the percentage of positively stained cells of apoptosis-related proteins as detected by immunohistochemistry in control and treated SEC tissues (left panel). Increased Bax/Bcl2 ratio after *C. vitellina* treatment (right panel). Data represent the means (n = 7) of ten different fields; bars, standard deviation and a: significant change with respect to the control ($P < 0.05$). 5-FU: 5-fluorouracil (20 mg/kg, i.p.).

(Table 1) may affect the antioxidant status. Furthermore, 1.0 mg/mL C. *vitellina* extract exhibited 2,2-diphenyl-1-picrylhydrazyl scavenging activity of 99.5 ± 0.166% (Unpublished data).

To investigate the pro-apoptotic pathway of *C. vitellina* extract, we evaluated the changes in apoptosis regulatory genes upon *C. vitellina* treatment. Apoptosis is an important cell death mechanism that does not trigger an inflammatory response and results in collateral destruction of normal cells in the surrounding microenvironment. In the present study, we observed a significant increase in the *BAX/BCL2* mRNA expression ratio as well as *CASP3* upregulation, whereas P53 protein expression appeared to be negative *in vitro*. It is well-established that Bax is positively regulated by P53 protein and negatively controls Bcl2

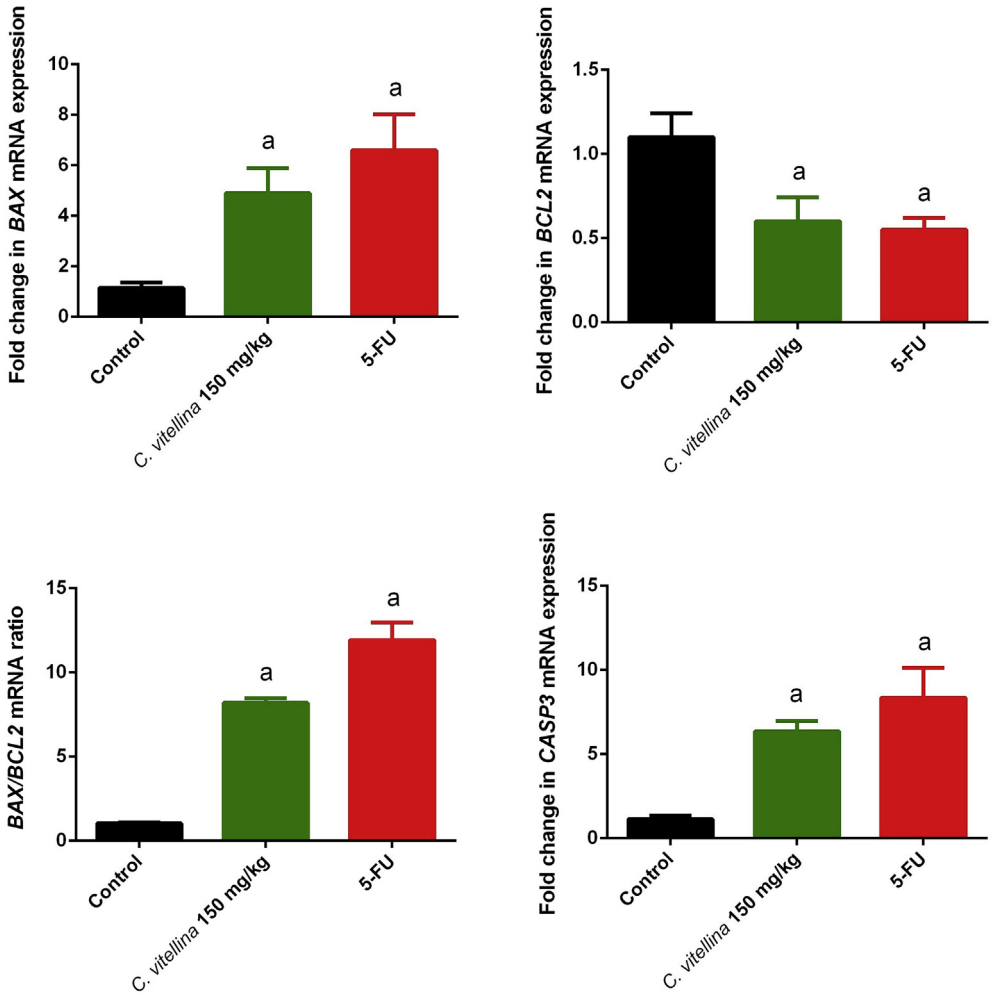

Fig. 9. Effect of *C. vitellina* on relative expression of apoptosis-related gene, and *BAX/BCL2* ratio in ESC tissues isolated from mice-bearing solid Ehrlich tumor. Data (means of three assays) were normalized to the *GAPDH* mRNA level and expressed as fold-induction relative to the mRNA levels in the control. Bars, standard deviation and a: significant change with respect to the control ($P < 0.05$). 5-FU: 5-fluorouracil (20 mg/kg, i.p.).

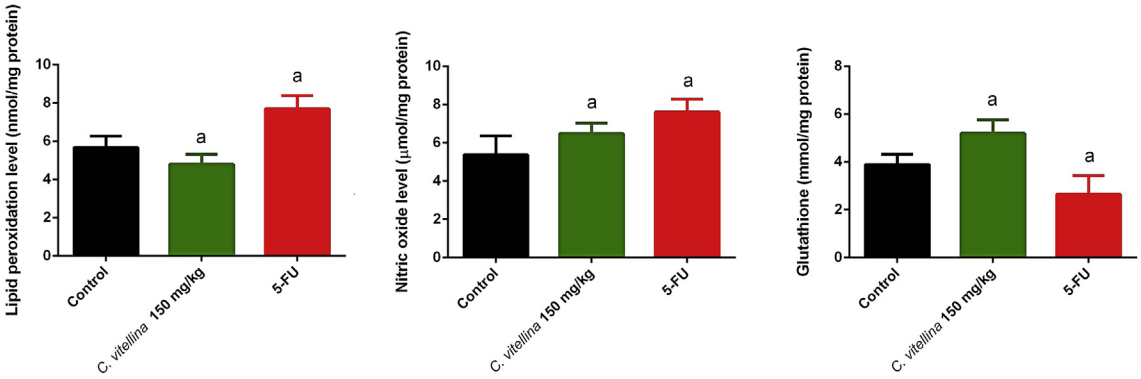

Fig. 10. Effect of *C. vitellina* on oxidative stress markers levels in solid Ehrlich tumor tissues after 4 days of treatment. Data represent the means (n = 7); bars, standard deviation and a: significant change with respect to the control ($P < 0.05$). 5-FU: 5-fluorouracil (20 mg/kg, i.p.).

expression (Kumar et al., 2017). Apoptosis is a well-controlled process involving changes in the expression of an array of genes. Bcl2 family proteins have an important role in regulating cell apoptosis (Burlacu, 2003). Over-expression of pro-apoptotic molecules, such as Bax, can accelerate cell apoptosis (Abdel Moneim, 2016). Inhibition of Bcl2 protein expression and their anti-apoptotic functions can help to increase the efficiency of chemotherapeutic agents.

In the current study, upregulation of P53 protein expression was detected *in vivo*, which may be related to oxidative stress (Barrera, 2012). P53 is a nuclear transcription factor activated in response to oxidative stress to promote apoptosis by regulating numerous downstream effectors. Following activation, the cell cycle is arrested for DNA repair to restore normal cell function (O'Brate and Giannakakou, 2003). The *C. vitellina*-induced antioxidation may have increased P53 levels and subsequently led to non-phase specific cell cycle arrest in SEC cells. Furthermore, antioxidants may enhance P53 DNA binding activity (Liu et al., 1998). Thus, *C. vitellina* extract induced apoptosis *in vitro* through a P53-independent mitochondrial intrinsic pathway, whereas it affected the P53-dependent apoptotic pathway *in vivo*. However, 5-FU is DNA-damaging anticancer drugs and DNA damage triggers P53 activation (Vousden, 2002). Interestingly, cytoplasmic localization of P53 protein in Caco-2 cells was observed following 5-FU treatment. This may be associated with a poor drug response and therefore P53 cannot perform nuclear functions in colorectal carcinoma (Bosari et al., 1995). Furthermore, some P53 can be localized in mitochondria to induce apoptosis in a transcription-independent manner (Marchenko et al., 2000; Mihara et al., 2003) or by causing endoplasmic reticulum stress to prevent P53-dependent apoptosis via the glycogen synthase kinase-3β pathway (Qu et al., 2004).

In the current study, *C. vitellina* showed more potent antitumor activity *in vivo* than *in vitro* according to the *P53* expression. The putative anticancer compounds in the extract may be metabolized by mice, which may lead to the generation of different bioactive compounds or activate some compounds of *C. vitellina* that are inactive under the cell culture conditions. Furthermore, these metabolites may have enhanced the expression of some silenced genes as resulted in increased *P53* mRNA of solid tumors in mice in our study. This may support the different levels of tumor growth inhibitory effects observed. More potent effects of the metabolites of some drugs have been detected in previous studies. For example, *N*(1)-acetyl-*N*(2)-formyl-5-methoxykynuramine) and *N*(1)-acetyl-5-methoxykynuramine, which are metabolites of melatonin, are more powerful electron donors than melatonin itself (Abdel Moneim et al., 2015). Additionally, abiraterone is metabolized to Δ4-abiraterone and showed better antitumor effect than the parent molecule. In the current study, the greater inhibitory effect of *C. vitellina* on tumor growth *in vivo* may have been caused by metabolites in the extract. However, additional studies to explore the metabolism of *C. vitellina* extract are needed to clarify the impact of biotransformation on the efficacy of *C. vitellina* extracts *in vivo*.

The results suggest that the anti-proliferative and pro-apoptotic effects of *C. vitellina* extract can be attributed to the secondary metabolites identified by LC-HRMS analysis of *C. vitellina* extract. Numerous studies have reported the inhibitory effects of the following terpenes as secondary metabolites of *C. vitellina*; Hericenone A (Rama Rao and Reddy, 1992), Ovellin B (Belofsky et al., 1998), Ganoderol A and Ganoderiol F (Chen et al., 2017), Ganodermatriol (Liu et al., 2012), and Fomefficinic acid A (Shen et al., 2013). In contrast, cytotoxicity was attributed to compounds with a polyketides structure such as Hormothamnione (Gerwick et al., 1986), Arnottianamide (Yang et al., 2009), and Comazaphilone C (Gao et al., 2011) (Table 1).

5. Conclusion

Candelariella vitellina extract exhibited antioxidant and pro-apoptotic effects on Caco-2 cells and Ehrlich solid tumor. The decrease in the viability of the colon cancer cell line Caco-2 and reduction in Ehrlich solid tumor volume after treatment with *C. vitellina* extract were demonstrated. These effects may occur through the action of the various secondary metabolites and other phytochemical constituents in the extract. The present results also demonstrated that secondary metabolites in *C. vitellina* may function as anticancer molecules, indicating their potential for developing new drug alternatives. However, further studies to explore the metabolism of *C. vitellina* extract are needed to clarify the impact of biotransformation on the efficacy of *C. vitellina* extracts *in vivo* and determine which compound in the extract is responsible for the anticancer effects.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.003.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.003.

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