



The Anticancer Activity of *Cetraria Islandica* (L.) Ach in Breast Cancer Cells Through Crosstalk of Ampk- α 1 and Erk1/2 Signalling

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ABSTRACT

In the present study, we aimed to evaluate the anticancer activities of *Cetraria islandica* (*C. islandica*) extracts on MCF-7 breast cancer cell lines. Cell viability, protein levels, apoptotic cells number, F-actin distribution were measured. Cell viability of MCF-7 breast cancer cells was found to be reduced in a dose-dependent manner. EC₅₀ values of *C. islandica* on MCF-7 cells were found to be 9.2047 E-5 g/ml (cell amount) by using intelligence system. Expressions of p53, caspase 3 and Bcl-2, were shown to be elevated after low doses of extract and diminished after high dose treatments. PPAR- γ protein level was decreased, although AMP-activated kinases- α 1 (AMPK- α 1) protein level was increased in its extract groups. ERK1/2 protein level was also elevated in its extract groups. 125 mg/ml of extract treated cells show a low decrease in actin filament density. MCF-7 cells with *C. islandica* treatment for 24 h increased the apoptotic cell percentage, though the cells-treated with *C. islandica* for 48 h was high necrotic cells percentage. Consequently, the *C. islandica* extract treatment causes to elevate ERK1/2 and AMPK- α 1 protein levels, resulting in PPAR- γ and then triggers the apoptosis by modulation caspase-3 and P53 protein levels. Therefore, *C. islandica* might be a good candidate for anticancer tissue, especially soft tissue tumours.

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Introduction

Cancer is one of the major health problems in recent years, and its pathogenesis and treatment are not completely solved yet. Notably, breast cancer has been reported to scale up among women (Nikhil et al., 2014). There are some strategies to fight against tumors e.g. surgery, hormone therapy (Nikhil et al., 2014) immunotherapy (Manola et al., 2011) chemotherapy (Bakker et al., 2015) and radiotherapy (Hoskin et al., 2016) treatment methods. Although emerging treatment strategies are promising, many of the current methods are not efficient and developing novel therapeutic approaches are a high priority. So far, an enormous number of studies were conducted to identify novel, useful phototherapeutic agents including compounds derived from plants

(Bontempo et al., 2015; Shweta, et al., 2013; Unnati Shah, 2013; Vaithyanathan and Mirunalini, 2016), fungi (Shweta et al., 2013) and lichens (Kosanic, et al., 2013). It has suggested that lichens and their secondary metabolites can be used as an alternative method for the treatment of cancers (Kosanic et al., 2013, 2014) due to its immune stimulation effect (Colak, et al., 2013). Recently, studies intended to show the effects of lichen extracts on various types of cancer are increasing (Coskun et al., 2015; Çelikler Kasimoğulları et al., 2014; He et al., 2010; Yang et al., 2014). One of the most studied lichen species is *Cetraria islandica* (Bessadottir et al., 2015) also known as Iceland Moss (Xu et al., 2016). *C. islandica* belongs to *Parmeliaceae* family, and it has been reported to be used

in the treatment of several diseases (Kartnig, 1987). *C. islandica* have exhibited many of biological activity due to its phytochemical compounds such as (+)-protolicheterinic acid (Bessadottir et al., 2015; Xu et al., 2016). (+)-Proto licheterinic acid has been reported to have antiproliferative effect by using some cancer types such as breast, pancreas, colon and multiple myelomas by stimulation of apoptosis, including inhibition of HSP-70 protein expression and sensitive redox pathways (Xu et al., 2016). Active polysaccharides of water and alkali extracts of *C. islandica* increased the activity in immunological test (Olafsdottir et al., 1999). Also, this lichen species have shown highly neuroprotective and antioxidant activities and highly cytotoxic to U373-MG cancer cells (Roleira et al., 2015). Moreover, in the phytochemical determination tests, protocetraric, vulpinic, pinastric, usnic acids derivatives were determined in this lichen species (Roleira et al., 2015). Protocetraric acid has induced apoptosis in prostate cancer line. Pulvinic Acid has been reported to have an antimalarial effect (Xu et al., 2016).

The peroxisome proliferator-activated receptors (PPAR) is part of nuclear receptor superfamily and has shown three major isoforms, including PPAR α , PPAR β/δ , and PPAR γ . Many studies have worked on PPAR γ . Its activation has been reported to be one of anticancer approach (Sakharkar et al., 2013). PPAR- α regulates fatty acid oxidation, although PPAR- γ plays a significant role in glucose homeostasis (Guo et al., 2010).

An AMP-activated kinase (AMPK) has three component named as alpha, beta, and gamma. Alpha subunits of AMPK is a catalytic element, although beta and gamma are regulators components. Alpha subunit has a threonine at 172 position (Thr¹⁷²) which is the phosphorylated site of AMPK, resulting in the activity of many enzymes and kinases. AMPK is reported to activate by multiple ways such as hypoxia, oxidative stress, ischemia, resulting in elevation of energy production via glycolysis or fatty acid oxidation (Sozio et al., 2011).

The mitogen-activated protein kinases (MAPK) is a serine-threonine kinase family which is a conservative family in all eukaryotes (Mebratu and Tesfaigzi, 2009). The family has been identified four members, including of the extracellular-signal-regulated kinase 1/2 (ERK $\frac{1}{2}$), c-Jun-amino-terminal kinase (JNK), and p38 (Mebratu and Tesfaigzi, 2009; Nishimoto and Nishida, 2006). The MAPK has played a significant role in cell growth and differentiation, cell survival, and apoptosis, gene expression, mitosis, cell motility, metabolism, and embryogenesis. ERK $\frac{1}{2}$ can regulate both cell survival and death associated with pro-apoptotic which is by decreasing pro-apoptotic protein expression and anti-apoptotic pathways which are through increasing anti-apoptotic protein activity (Lu and Xu, 2006). Also, ERK $\frac{1}{2}$ might participate to develop drug resistance against anticancer treatment due to an elevation of P53 transcriptional function, eventually increasing Bcl-2 level (Lu and Xu, 2006).

Consequently, studies on the anticancer effects of this lichen species are very limited and remain as elusive. Also, identifying novel alternative therapeutics against cancers, especially the breast cancer, is of keen interest

for the developing effective treatment methods. Moreover, the little is known about which *Cetraria islandica* counteracts proliferation on the cancer cell through intracellular effect. Accordingly, in the present study, our hypothesis was to evaluate that the anticancer activity of extracts of *Cetraria islandica* on MCF-7 breast cancer cell lines is associated with enhancing ERK $\frac{1}{2}$, resulting in increasing of AMPK, which inhibits to PPAR- γ and finally initiate of apoptosis.

Materials and Methods

Preparation of Extracts

In the development of extracts, *Cetraria islandica* samples were air-dried, and standard Soxhlet extraction method was followed. Ethanol was aspirated by using rotary evaporator then extracts were air-dried at room temperature to eliminate remaining ethanol residues.

Cell Culture

MCF7 (HTB-22) breast cancer cells were purchased from ATCC (American Type Culture Collection, VA, USA). For the cultivation of cells, Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich, MO, USA) were used. Mediums were supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, MO, USA). For appropriate culturing conditions cells were preserved in the air, 95%; carbon dioxide (CO₂), 5% conditions at 37°C. After the extraction of *C. islandica*, the concentrations were determined by different dose, including 1, 5, 25, 125 μ g/ml. However, according to our cell viability results (Figure 1), the 1 and 125 μ g/ml named as C1 and C4 were used in the current study because last three doses (5, 25, 125 μ g/ml) had shown a similar effect. After proved to have without the toxic effect of dimethyl sulfoxide (DMSO), the control group was exposed to only DMSO.

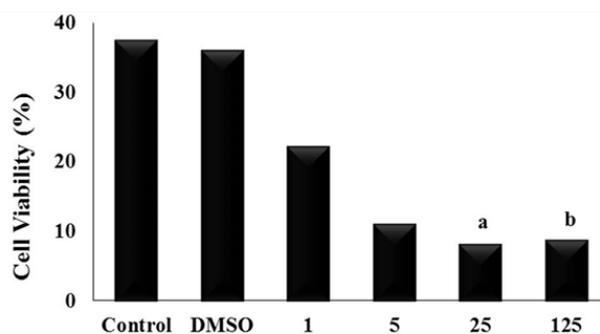


Figure 1 Anti-proliferative effects of *C. islandica* extract on MCF-7 breast cancer cells in MTT cell viability assay.

Cells were treated with extracts for 24 hours. DMSO: dimethyl sulfoxide. Control: the group was not exposed any chemical, just incubated only with medium, DMSO: the group was treated medium with DMSO, 1: the group was incubated with 1 mg/ml *C. islandica* extract, 5: the group was incubated with 5 mg/ml *C. islandica* extract, 25: the group was incubated with 25 mg/ml *C. islandica* extract, 125: the group was incubated with 125 mg/ml *C. islandica* extract. A: P<0.05 vs Control and DMSO groups, b: P<0.05 vs 25 group.

Determination of Cell Viability by MTT Assay

MTT (3- [4,5- dimethylthiazole- 2- yl]- 2,5- diphenyl-tetrazolium bromide) were used to measure cell viability of MCF-7 cells. Briefly, cells were detached using 3.0 mL of Trypsin-EDTA solution (Sigma-Aldrich, MO, USA) after reaching 70-80% confluency and seeded in 24-well plates and incubated for 24 hours. After 24 hours, varying dilutions of (1, 5, 25, 125 µg/ml) extracts were applied, and cells incubated for 24 hours. Control cells only treated with the growth medium not supplemented with FCS. After 24 hours of incubation, the supernatants were replaced with 1 mg/ml MTT (Sigma) dissolved in growth medium and incubated at 37°C until a purple precipitate was visible. Then, supernatants were removed, and MTT absorbed by cells were dissolved adding dimethyl sulfoxide (DMSO) (Sigma-Aldrich, MO, USA). Plates were read at 570 nm using an Epoch spectrophotometer (BioTek Instruments, Winooski, VT).

Dynamic Monitoring of Cell Proliferation by Using the xCELLigence System

Real-Time monitoring of cell viability was achieved by a using the quantitative measurement of the cell proliferation in xCELLigence system. MCF7 cells seeded in an E-plate 16 (ACEA Bioscience Inc.) at a density of 100000, 50000, 25000, 12500, 6250, 3125 and 1562 cells/well and cell indexes were recorded for 24 h.

Determination of Protein Expression by Western Blot

After experimental groups had been carried out, lysates were isolated from groups by using RIPA buffer (Sigma). For the western blot analysis, total 50 µg protein was mixed with sample loading reagent and subjected to denaturation for 5 minutes. Proteins were resolved on 12% polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). For immunoblotting membranes were incubated with the blocking buffer (5% BSA) and treated with primary and alkaline phosphatase-conjugated secondary antibodies. Signal generation was achieved by using BCIP/NBT substrate solution. Following antibodies were utilized in the western blotting experiments; Caspase-3, P53, Bcl-2 primary antibodies (Abcam, Inc., Cambridge, MA); AMPK, PAMPK (Abcam); ERK½, PERK½ (Cell Signalling), AIF (Millipore).

Determination F-Actin Distribution

Cells cultured on coverslips were subjected to various concentrations of *Cetraria islandica* extract and subjected to F-actin staining. Fluorescently-labelled phalloidin

(Alexa Fluor® 595 phalloidin, Thermo Fisher Scientific) was used to visualize and quantitate actin filaments in MCF-7 breast cancer cells. For the nuclear counter stain, DAPI (Thermo Fisher Scientific) was used.

Assessment of Apoptosis by Flow Cytometry

For apoptosis analysis, cells were seeded at a 1x10⁶/ml density to 6-well plates, and efficient dose of *Cetraria islandica* extract applied for 24 and 48 hours. PE Annexin V Apoptosis Detection Kit I (Becton Dickinson, Pharmingen, UK) were used to analyse induction of apoptosis in cells. The analysis was carried out using Beckman Coulter flow cytometry.

Statistical Analysis

Statistical analysis of data was achieved using GraphPad Prism (v6.02) and SPSS (version 20) software. Multiple analyses of variance (ANOVA), followed by a post hoc protected Tukey test, were used for comparison among different groups. To analyse the parameters within the group to figure out the time table (24 and/or 48 hours) and therapeutic dose of its, independent t-test was used after p value was corrected according to the number of comparisons. For all statistics p values were two-tailed, and P<0.05 was accepted as statistically significant.

Results

The Effect of Cetraria Islandica Extract on Cell Viability and Proliferation In MCF-7 Cells

To determine dose-dependent (1, 5, 25, 125 µg/ml) anti-proliferative effects of ethanol extract of *Cetraria islandica*, we used MTT cell viability assay and xCELLigence Real-Time Cell viability monitoring. Cell viability of MCF-7 cells was significantly diminished after extract treatments as revealed in both methods. In MTT assay, cell viability of MCF-7 breast cancer cells was found to be reduced in a dose-dependent manner. 1 mg/ml and 5 mg/ml doses of extracts did not show much effect on cell proliferation (Figure 1). However, a significant level of attenuation of cell viability was observed at a dose of 25 and 125 mg/ml. Suggesting effective treatment must be higher than 25 mg/ml. As consistent with MTT assay results, cell viability was found to reduce significantly in MCF-7 cells associated with a dose-dependent manner after extract treatments xCELLigence Real-Time Cell viability monitoring system. EC50 values of *C. islandica* on MCF-7 cells were found to be 9.2047 E-5 g/ml (9.2047×10⁻² mg/ml) (Figure 2).

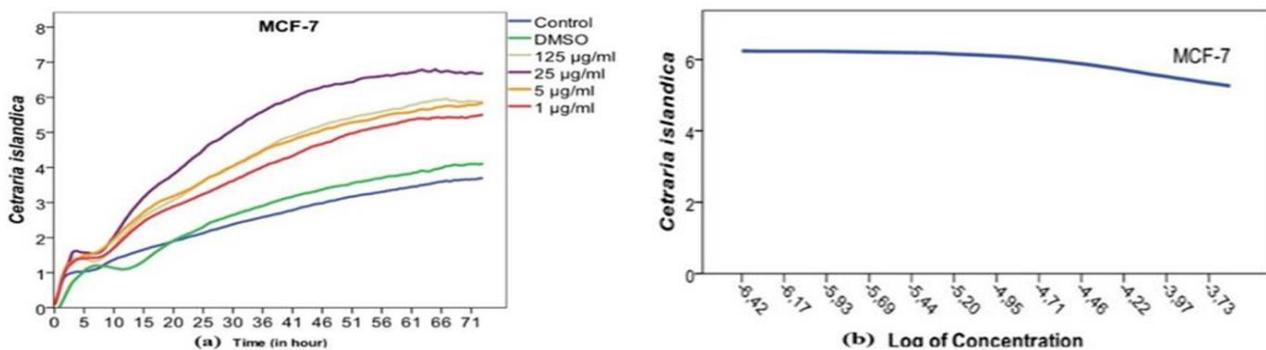


Figure 2 Anti-proliferative effects of *C. islandica* extract on MCF-7 breast cancer cells (A), and HUVEC (B) in xCELLigence Real-Time cell viability monitoring system. Cells were dynamically monitored for 24 hours.

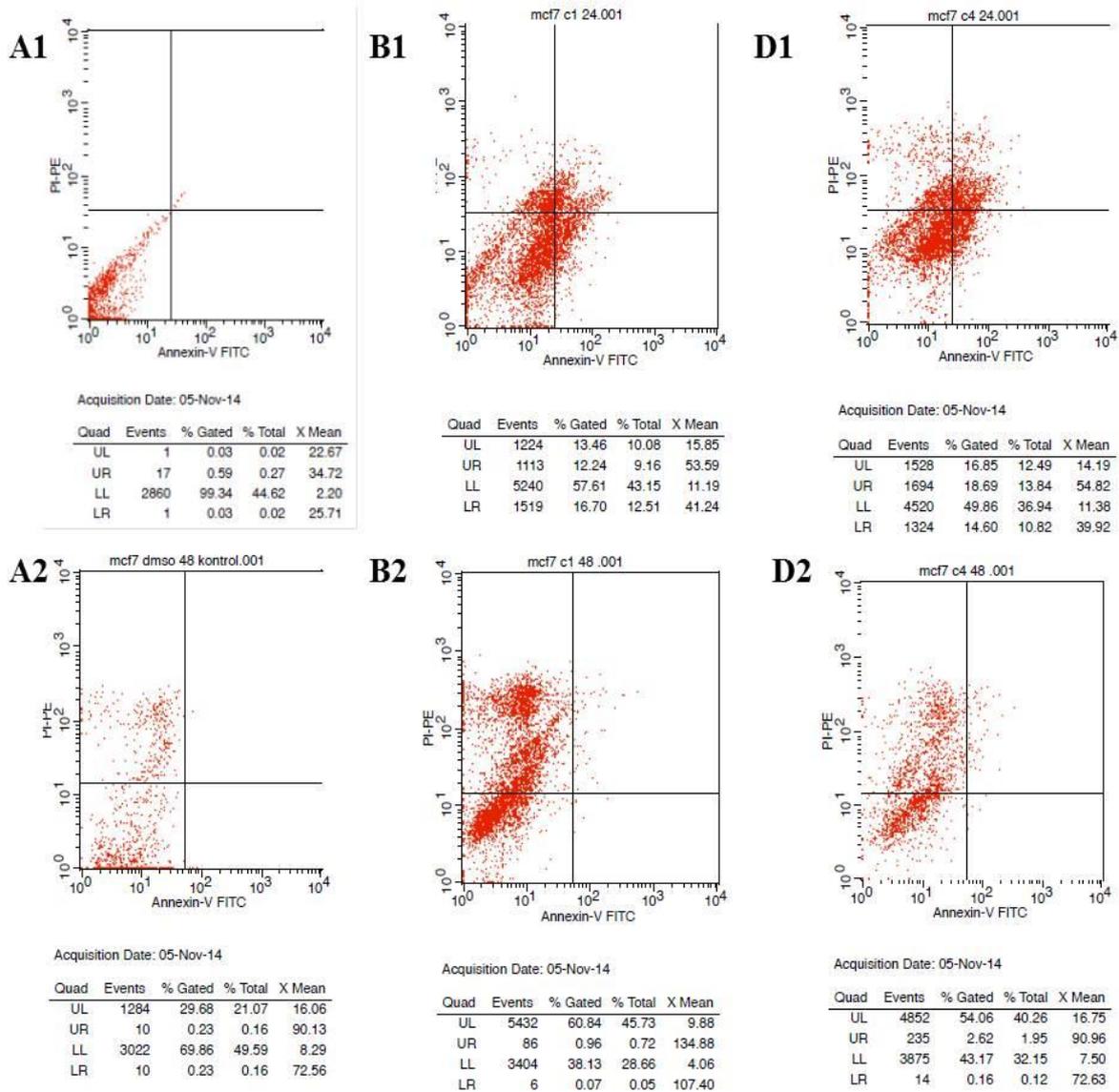


Figure 3 Flow cytometric analysis of Annexin V and propidium iodide (PI)staining: A1 and A2 Control groups 24H and 48H, respectively, B1 and B2 C1 groups 24H and 48H, respectively, D1 and D2 C4 groups 24H and 48H, respectively. C1: 1 µg/ml concentration of *C. islandica* extract, C4: 125 µg/ml concentration of *C. islandica* extract.

The Effect of Cetraria Islandica Extract on Apoptosis In MCF-7 Cells

AnnexinV/PI staining was performed to test apoptosis induction in breast cancer cells after extract treatments due to allowing to distinguish early, late apoptosis and necrotic cell. Cells were treated with concentrations of 1 (C1), 125 (C4) µg/ml extracts for 24H and 48 H and subjected to in flow cytometric analysis. After 24 h treatment period, C1 and C4 have shown the similar apoptotic effect which gave rise to increased early and late apoptosis (Figure 3B1 and 3B2). However, 48 h treatment has been determined differently than 24 H treatment which is elevated to necrotic cell number (Figure 3D1 and 3D2). Even though control groups has been found to increase necrotic cell count which is much less than C1 and C4 after 48 h treatment (Figure 3A1; 3B2 and Figure 4).

The Effect of Cetraria Islandica Extract on Some Apoptotic Protein Expressions In MCF-7 Cells

p53, caspase 3, AIF (apoptosis inducing factor) and Bcl-2 proteins were determined for assessment of apoptosis. AIF plays a significant role in apoptosis pathway which is independent caspases. 1 and 125 µg/ml concentration of its extract caused to increase AIF protein levels. Although exposed to 1 µg/ml concentration of its extract in MCF-7 gave rise to an increase of P53 protein levels, 125 µg/ml concentration of its extract led to attenuate to P53 protein level. Exposed to 125 µg/ml concentration of its extract caused to decrease caspase-3 protein level, but not exposed to 1 µg/ml concentration. Bcl-2 which is one of anti-apoptotic protein created to increase at one µg/ml concentration but decrease at 125 µg/ml concentration (Figure 5).

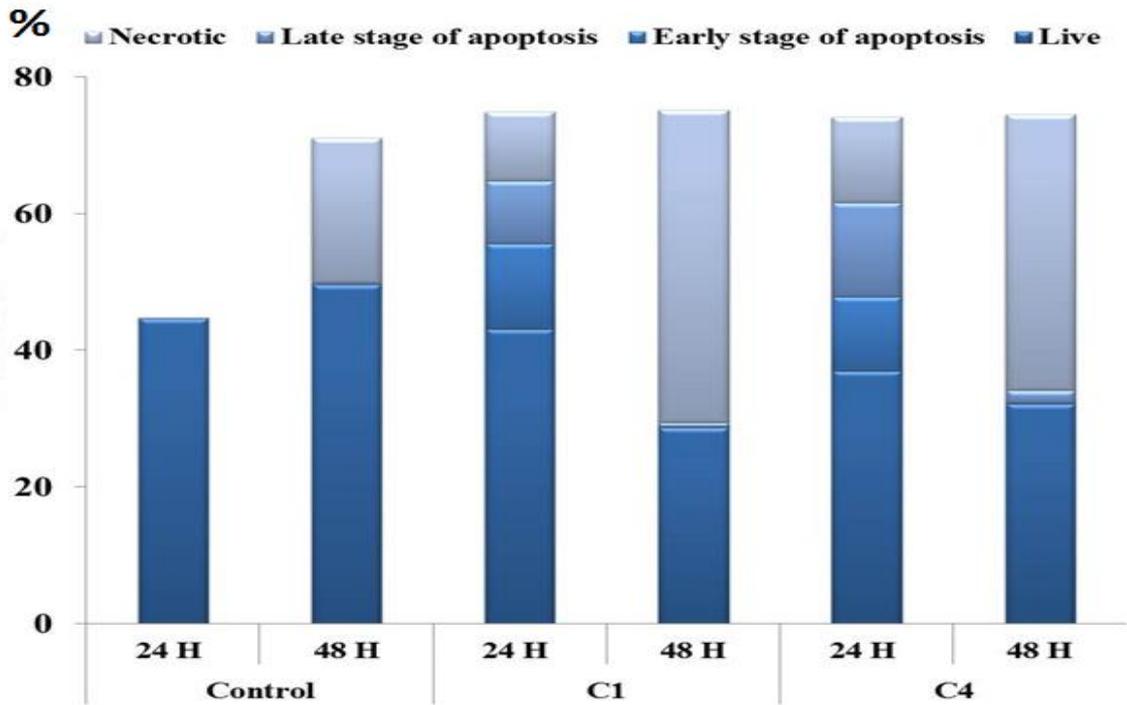


Figure 4 Percentage of flow cytometric analysis of Annexin V and propidium iodide (PI)staining: A1 and A2 Control groups 24H and 48H, respectively, B1 and B2 C1 groups 24H and 48H, respectively, D1 and D2 C4 groups 24H and 48H, respectively. C1: 1 µg/ml concentration of *C. islandica* extract, C4: 125 µg/ml concentration of *C. islandica* extract.

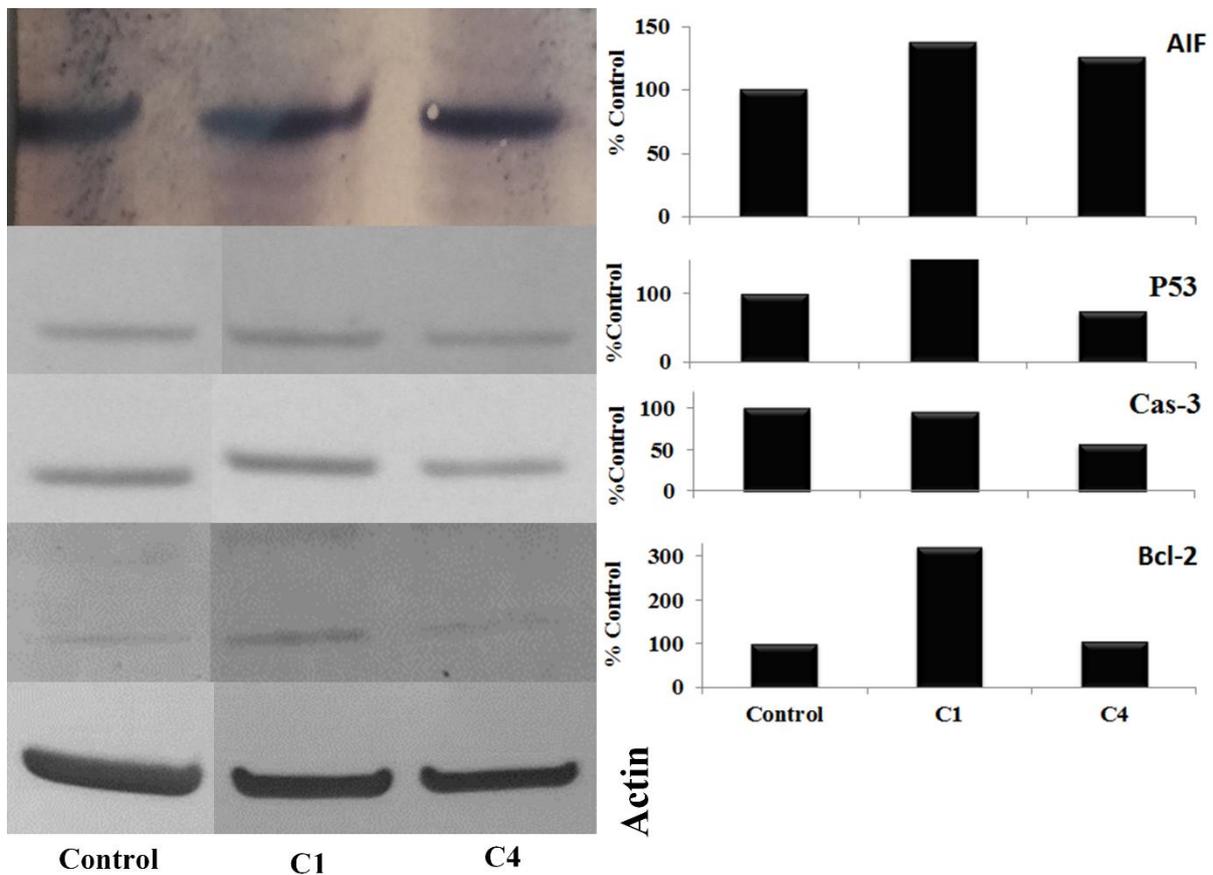


Figure 5 Expression levels of apoptotic and antiapoptotic proteins after extract treatments.C1: 1 µg/ml concentration of *C. islandica* extract, C4: 125 µg/ml concentration of *C. islandica* extract. Beta-Actin was used as a reference protein. ImageJ was used for analysing.

The Effect of Cetraria Islandica Extract on Some Intracellular Signal Protein Expressions In MCF-7 Cells

The peroxisome proliferator-activated receptors- γ (PPAR- γ) was shown two bands in the current study. Interesting C1 caused to increase PPAR- γ protein levels more than C4. AMPK- α 1 protein levels were elevated at one $\mu\text{g/ml}$ concentration of its extract but not 125 $\mu\text{g/ml}$ concentration. When phosphorylated AMPK (PAMP) was to determine, both concentrations (1 and 125 $\mu\text{g/ml}$) led to increasing PAMPK protein levels. ERK $\frac{1}{2}$ protein levels were increased at 125 $\mu\text{g/ml}$ treatment but not one $\mu\text{g/ml}$ concentration of its extract, although phosphorylated

ERK $\frac{1}{2}$ (PERK $\frac{1}{2}$) caused to decrease at 125 $\mu\text{g/ml}$ treatment not one $\mu\text{g/ml}$ concentration of its extract (Figure 6).

The Effect of Cetraria Islandica Extract on Actin Filaments Distribution in MCF-7 Cells

Immunofluorescence imaging of actin filaments was revealed that 125 $\mu\text{g/ml}$ of extract treated cells show a low decrease in actin filament density. At the same time, 1 mg/ml of extract treated cells showed a reduction in the actin filaments as compared to Control and DMSO control groups (Figure 7).

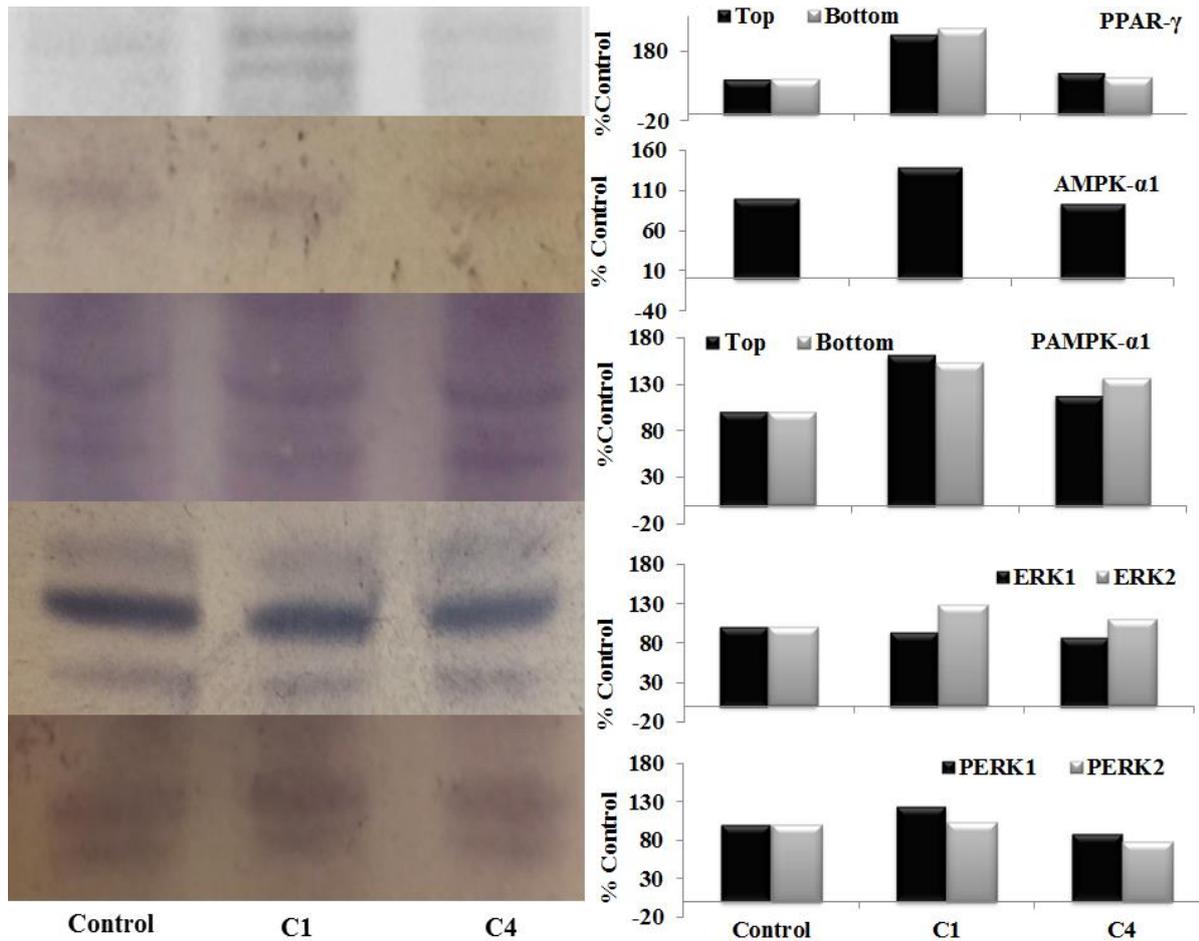


Figure 6 Expression levels of PPAR- γ , ERK $\frac{1}{2}$, P ERK $\frac{1}{2}$, AMPK- α 1, PAMPK- α 1 proteins after extract treatments. C1: 1 $\mu\text{g/ml}$ concentration of *C. islandica* extract, C4: 125 $\mu\text{g/ml}$ concentration of *C. islandica* extract. Beta-Actin was used as a reference protein. ImageJ was used for analysing

Discussion

Several lines of evidence from our current study have supported the hypothesis: elevation of ERK $\frac{1}{2}$ causes to enhance AMPK, especially phosphorylated PAMPK, resulting in a decline of PPAR- γ . Then modulation of P53, AIF and caspase levels end up increasing apoptosis (Figure 8).

Many of scientists have focused on alternative natural resources for against cancer agent in recent years (Khazir et al., 2014; Unnati Shah, 2013) although many of those studies were not elucidated the underlying molecular mechanism of alternative natural resources. It is of great interest to determine in which pathway these natural extracts or individual compounds show their effects on

cancer cells (Remila et al., 2015; Tagne et al., 2014). Although many plant species used in previous studies, currently various lichen species have gained much consideration because of anticancer activities (Kosanic et al., 2013; Kosanić et al., 2014). Specific chemical components of these natural sources provide novel therapeutics in the treatment of cancers. In the light of this knowledge, anticancer activities of ethanol extract of *C. islandica* in our study, which is an important lichen species and bears significant biological activities (Bessadottir et al., 2015; Fernandez-Moriano et al., 2015; Olafsdottir et al., 1999), were assessed in breast cancer cell lines. At first, the effects of *C. islandica* on cell

viability MCF-7 breast cancer cells were tested by MTT assay. The resulting data suggested that particularly in a high concentration of *C. islandica* viability of breast cancer cells significantly diminished. Fernandez-mariano found that >10 mg/ml *C. islandica* extract was active in MCF-7 cells. Similar to these findings in our study, an effective dose of *C. islandica* extract was found to be >25 mg/ml in MCF-7 cells (Fernandez-Moriano et al., 2015). It should be noted that differences in effective doses can be linked to the number of passages of cells and extraction methods. Also, the proliferation of cells was also evaluated by xCELLigence dynamic cell monitoring method, and appropriate cell density was determined in our study. The highest 125 mg/ml and the lowest 1 mg/ml application dose were chosen to better comparisons in our study.

Furthermore, to assess whether the MCF-7 cells undergo apoptotic or necrotic cell death we used AnnexinV/PI staining method in flow cytometry. After extract treatments, the small fractions of MCF-7 cells experienced apoptotic pathway. Necrotic cell death was observed to be increased in 24 h and 48 h treatments. As the flow cytometry analysis revealed reduced cell viability in MTT and xCELLigence methods not because of an apoptotic cell death. Moreover, protein expression of apoptotic and antiapoptotic members was also demonstrated in our study. Interestingly, while protein expressions of p53, caspase-3 and Bcl-2 were found to be

increased in low dose extract treatments, they were considered to be diminished nearly control levels in high dose extract treatments. Suggesting that these can result from the first response to extracts and after returning to its initial state. Previous studies reported that *C. islandica* extract is not effective in caspase-3 activity (Fernandez-Moriano et al., 2015). In our study, small expression changes were observed, yet it seems to be not active on caspase-3 activity. In consistent with the flow cytometry results, as the apoptosis not induced in these cells, p53 activity was also not much altered by extract treatments. Immunofluorescence results showed that while low dose extract treatments were not effective on F-actin structure, a slight decrease was observed in actin filament density in high doses. One of the previous studies was stated that *C. islandica* could be a scavenger of superoxide radical (Gulcin et al., 2002) based on the ingredient of lichen and linoleic acid. Lichen has also prevented mutagenesis and carcinogenesis in human (Colak et al., 2013).

The distinguished the current study from previous studies is that the anticancer effect of *C. islandica* is underlined molecular mechanism which is elucidated yet, although many studies stated its anti-cancer impact. Therefore, our hypothesis was to evaluate that the anticancer effect of *C. islandica* associated with ERK $\frac{1}{2}$, resulting in increasing of AMPK, which inhibits to PPAR- γ and eventually initiates of apoptosis.

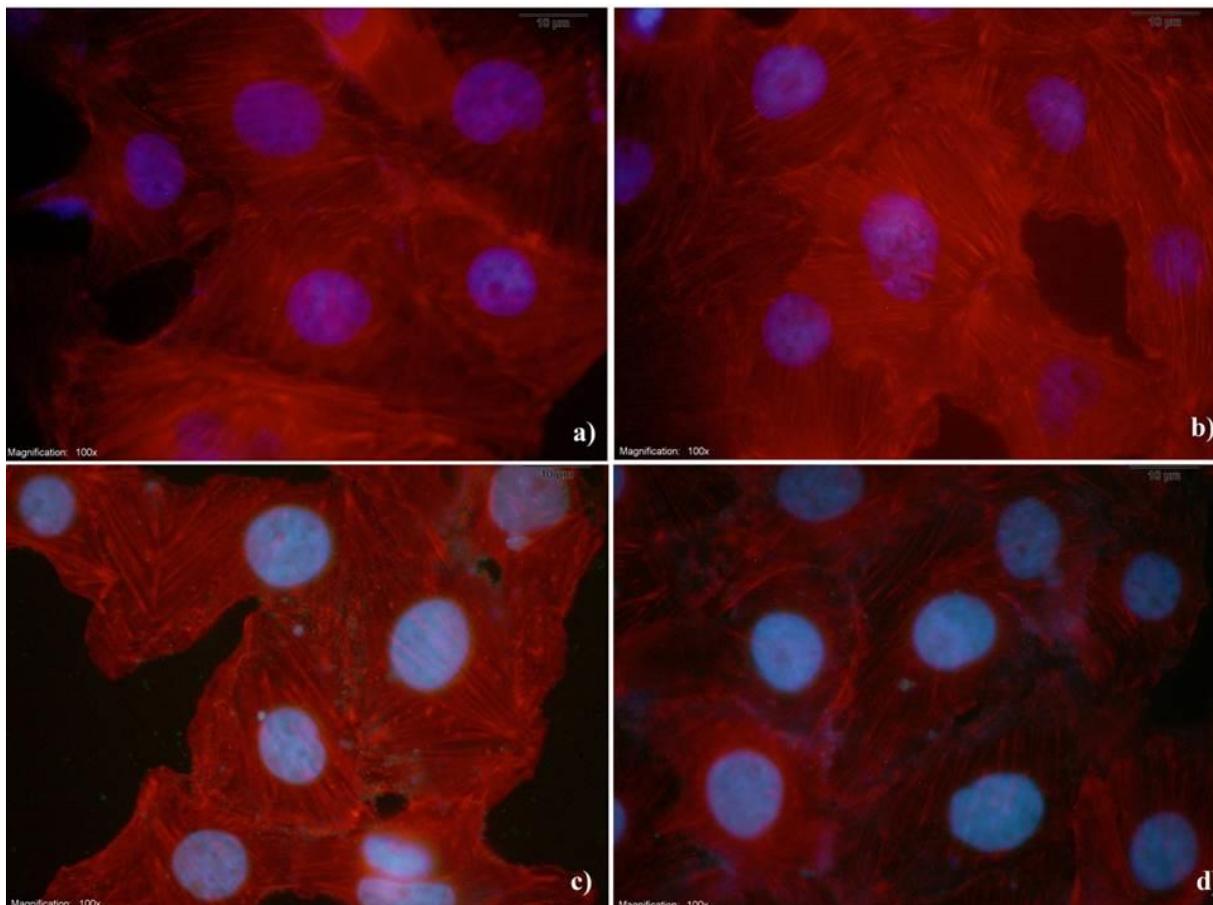


Figure 7 Immunofluorescence imaging of actin filaments (F-actin) after extract treatments: a) Control, b) DMSO, c) C1, d) C4. C1: 1 µg/ml concentration of *C. islandica* extract, C4: 125 µg/ml concentration of *C. islandica* extract.

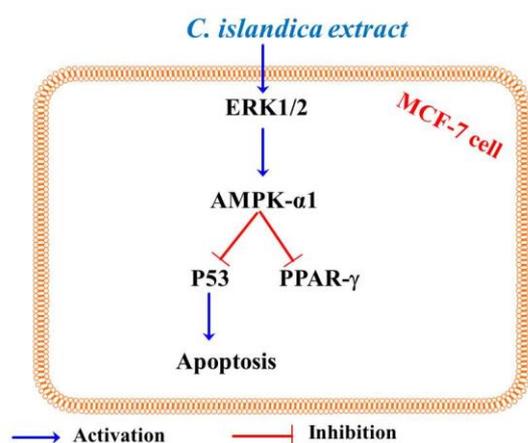


Figure 8 Graphical abstract of the current study

AMPK has been reported to probably enhance its activity in the cancerous cell due to the necessity of enormous energy (Carling et al., 2012). AMPK associated with MAPK to trigger to or cell survival or death pathway (Hadrich et al., 2016). ERK $\frac{1}{2}$ has a significant role in cell survival, but P38 plays a crucial role in cell death (Yan et al., 2016; Yen et al., 2016). Phosphorylated AMPK has been reported to decrease PPAR- γ . AMPK has an anti-inflammatory impact as well. One of a recent study was indicated that AMPK gave rise to decline P38 MAPK, resulting in inhibition of inflammatory pathway (Zhang et al., 2016). Interestingly, there was reported a positive correlation between AMPK- α and ERK $\frac{1}{2}$ in a recent study (Güven et al., 2016). ERK $\frac{1}{2}$ is related to attenuate apoptosis in some cell line. Moreover, ROS could modulate survival and death pathway by activation of MAPK (Yan et al., 2016). How ERK $\frac{1}{2}$ could be modified by *C. islandica* may relate with containing lichen and linoleic acid due to their scavenging of superoxide (Gulcin et al., 2002). There is an interesting link between ERK $\frac{1}{2}$ and AMPK. The current study result consists of the relationship which means that ERK $\frac{1}{2}$ could regulate of shuttling of AMPK between nucleus and cytoplasm. Activation of ERK $\frac{1}{2}$ accumulates AMPK in the cytoplasm (Kodiha et al., 2007).

Since breast cancer cell has been reported to increase PPAR- γ expression, that was why PPAR- γ protein expression was evaluated in the present study as well. Moreover, phosphorylation of mitogen-activated kinases (MAPK) which are ERK $\frac{1}{2}$, P38, and JNK can inhibit PPAR- γ . ERK $\frac{1}{2}$ has been reported to affect P53 as well (Kole et al., 2016). It plays a significant role in the regulation of cell proliferations and differentiation, and as a tumour suppressor as well. However, it has also been reported to be dysregulated by some cancer type such as breast, prostate, colon, and lung (Herrera et al., 2015). PPAR- γ is a mystery to be oncogenic or tumour suppressor activity (Nikhil et al., 2014).

Conclusion

Taken together, the results of the current study suggest that *C. islandica* bears anti-cancer effects on MCF-7 breast cancer cells. Its anticancer effect might relate with the ERK $\frac{1}{2}$ protein level, resulting in modulation of

AMPK- α 1 protein level, then restoration of PPAR- γ protein level. Finally, those effects cause to trigger apoptosis (Figure 7). Also, compounds isolated from lichen species can be more efficient in the induction of apoptotic cell death in these cancer cells. Several compounds found in these extracts can mask the anti-cancer effects of effective cancer compounds. Therefore, in the future studies, it is of great interest to isolate biologically active compounds of *C. islandica*.

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Conflict interest

The authors have not conflict interest

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