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Selective Cytotoxicity of Chemical Composition of *Spirulina platensis* Extract In Endometrial Cells: *In Vitro* and *In Silico* Approach

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ABSTRACT

Microalgae are considered as an ample source of bioactive compounds. These wide variety of chemicals possess promising potential to translate into drug candidates. Hence, the aim of this study is to determine potential anti-cancer effects of *Spirulina platensis* microalgae extract against endometrial cancer cells. The selective cytotoxic effect and cytotoxicity index of *S. platensis* microalgae extract on the Ishikawa endometrial cancer cell line, the determination of DNA fragmentation and the change in gene expression levels of apoptosis-related Bad, Poly-ADP Ribose Polymerase-1 (PARP1), p53, Akt1 and caspase-3 signaling proteins were determined. ECV304 human umbilical vein endothelial cell line was used for a control cell line. Cytotoxic effects of 500 µg mL-1 of S. platensis microalgae extract on Ishikawa and ECV304 cells were determined as 43±4.87 % and 22±1.87 % respectively. In order to delineate specific compounds in the extract, GC-MS analysis was further conducted and seven major compounds revealed to be abundantly present. Since DNA fragmentation induced by *S. platensis* extract might be related interaction of GC-MS identified compounds with PARP1, an *in silico* analysis was further implemented. Results indicated the presence of a possible PARP1 inhibitory mechanism contributing to the apoptotic response. In conclusion, a possible link between a specific compound found in *S. platensis* microalgae extract and the cytotoxicity in endometrial cells was formed through combining *in vitro* and *in silico* approaches. The result of these approaches has contributed to the identification of potential anti-cancer compound candidates found in *S. platensis* microalgae extract.

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Endometriyal Hücrelerde *Spirulina platensis* Ekstresinin Kimyasal Bileşiminin Seçici Sitotoksisitesi: *İn Vitro* ve *İn Siliko* Yaklaşım

ABSTRACT

Microalgae are considered as an ample source of bioactive compounds. These wide variety of chemicals possess promising potential to translate into drug candidates. Hence, the aim of this study is to determine potential anti-cancer effects of *Spirulina platensis* microalgae extract against endometrial cancer cells. The selective cytotoxic effect and cytotoxicity index of *S. platensis* microalgae extract on the Ishikawa endometrial cancer cell line, the determination of DNA fragmentation and the change in gene expression levels of apoptosis-related Bad, Poly-ADP Ribose Polymerase-1 (PARP1), p53, Akt1 and caspase-3 signaling proteins were determined. ECV304 human umbilical vein endothelial cell line was used for a control cell line. Cytotoxic effects of 500 µg mL-1 of S. platensis microalgae extract on Ishikawa and ECV304 cells were determined as 43±4.87 % and 22±1.87 % respectively. In order to delineate specific compounds in the extract, GC-MS analysis was further

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INTRODUCTION

Endometrial carcinoma causes about 90,000 deaths of women each year in worldwide in 2018 (Zhang at al., 2019). Endometrial Cancers (ECs) are separated into estrogen-dependent, equably or highly differentiated type 1 (80% of all cases), and estrogen independent, poorly differentiated type 2 (Hevir-Kene et al., 2015). Estrogen-dependent "ECs" are associated with prolonged exposure to estrogens of exogenous or endogenous origin that is not antagonized by synthetic progestins or progesterone (Hevir-Kene et al., 2015). The underlying mechanism of leading cause of EC is not clear, and treatment options for patients with EC in advanced stages are limited. Thus, novel candidate drugs are in need for treatment of this particular type of cancer (Del Carmen et al., 2011).

Microalgae, different chemical and biological compounds important because of its ability to produce are organisms. Vitamins, pigments, proteins, minerals, lipid and polysaccharides are the main products obtained. Compared to other living sources algae, especially unsaturated fatty acids (PUFA), gamma linoleic acid (GLA), allophycocyanin, c-phycocyanin, such as myxoxanthophyll and zeaxanthin very rich in pigments. *Spirulina platensis* is blue-green a filamentous, spiral-shaped algae. *S. platensis* in cosmetics, medicine, human and animal food widely used in various industries (Koru and Cirik, 2003).

Microalgae species could be considered as a potent source of anti-cancer drug candidates due tremendeously high concentration of bioactive compounds (Atasever-Arslan et al., 2022). Thus, this work was planned to investigate the anti-cancer potential of *S. platensis* microalgae extract on endometrial cancer cells. First, presence of cytotoxicity and apoptosis, as well as related intracellular signaling targets were investigated. Based on results obtained, the compounds present in the microalgae extract that were possibly responsible for the apoptotic effect, were identified. At the final phase, in order to understand the mechanism underlying the apoptotic response to *S. platensis* microalgae extract, *in silico* docking and molecular dynamics studies were conducted for the interaction between PARP1 and the compounds found in *S. platensis* microalgae extract.

In addition, the essential oils of *S. platensis* were analyzed with Gas Chromatography-Mass Spectrometry.

MATERIALS and METHODS

Mammalian Cell Cultures

To analyze cytotoxic potential of *S. platensis* microalgae extract on endometrial cancer cells, Ishikawa endometrial cancer cell line was used. At the same time, ECV304 human umbilical vein endothelial cell line that presenting many features of endothelial cells, was used for a non-cancerous cellular model (Suda et al., 2011).

DMEM medium (HyClone, 16777-133) content was prepared as recommended by Pirildar et al., 2010. All incubations were applied in a humidified atmosphere containing 5% CO_2 at 37°C. For experimental protocol, ECV304 and Ishikawa cells were detached by 0.5% (v v^{-1}) Trypsin-EDTA solution (Sigma, T3924), washed with Phosphate-Buffered saline two times and resuspended in DMEM medium at 5×10^5 cells mL^{-1} density.

Cytotoxicity Assay

To determine the cytotoxic effect of *S. platensis* microalgae extract on Ishikawa cells was by using MTT (3-(4,5- dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) (Sigma, M-5655) assay (Pirildar et al., 2010; Şeker-Karatoprak et al., 2022).

Aqueous concentrations of *S. platensis* microalgae extract were prepared as 5000, 500, 100, 50, and 10 µg mL^{-1}. For experiments, cell suspension (90 µL) was dispensed into 96-well round-bottom plates containing 10 µL of microalgae extract dilutions. In this way, the last concentrations of *S. platensis* microalgae extract were adjusted to 500, 50, 10, 5, 1 µg mL^{-1}, respectively (Atasever-Arslan et al., 2016). Medium (10 µL) without adding microalgae extracts were used as negative control, while medium consisting of only cells and only extract were solely used as controls for blank optical density (OD) measurements. Further steps of the MTT test were performed according to the protocol recommended by Pirildar et al. (Pirildar et al., 2010) OD of each well was measured on a "Bio-Rad Benchmark Microplate Reader" (Philadelphia, USA). The cytotoxicity test was repeated 6 times. Index of cytotoxicity was calculated with the formula below:

Cytotoxicity index= $1-[OD_{(\text{treated wells})}/OD_{(\text{control wells})}]\times 100$

DNA Fragmentation Assay

To investigate apoptotic effect of *S. platensis* microalgae extract, DNA fragmentation assay was used. Ishikawa cells were incubated for 24 hours with *S.platensis* extract while control groups were incubated with only medium. After 24 hours, cells were taken to eppendorf and the DNA Fragmentation Assay protocols recommended by Arslan et al. and Kaya et al. were performed (Kaya et al., 2016; Arslan et al., 2017). Isolated DNAs were run on 2% agarose gel for 400 minutes with 100V and viewed under laminator (Kaya et al., 2016; Arslan et al., 2017).

Spirulina platensis Culture and Extraction Procedure

S. platensis (UTEX Collection Culture No #LB2340, Cyanophyceae, Texas, USA) was cultivated in Zarrouk's Medium. Instructions for preparing the culture media were retrieved from the "University of Texas at Austin (UTEX)" culture collection of algae. *S. platensis* was extracted by using pure methanol prior to bead-beater homogenization. Methanolic extract was evaporated by a rotavapor and was further dissolved in methanol using an ultrasonic bath, and filtered just before the cytotoxicity tests.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Using a "Shimadzu gas chromatograph (QP5050, NY, USA), equipped with a Rtx®-5MS column (30 m×0.25 mm ID, 0.10 µm film thickness", essential oils of *S. platensis* were analyzed. For a carrier gas nitrogen was used (average flow rate, 1 mL min^{-1}). The protocol recommended by Atasever-Arslan et al. was used for GC-MS analysis (Atasever-Arslan et al., 2016).

Identification of Components

To identificate of oil constituents, "National Institute Standards and Technology (NIST) Library" was used. Component relative percentages were calculated based on GC peak areas without using correction factors (Atasever-Arslan et al., 2015; Atasever-Arslan et al., 2016).

Real-Time PCR (q-PCR)

"Roche LightCycler® FastStart DNA Master SYBR Green I Kit" manifacturer's procedure was used for q-PCR test. We examined expression profiles of p53, Bad, Caspase-3, PARP and Akt1 genes. Gene expression was evaluated by relative quantification normalized to b-actin gene.

Detailed Protocol Used for *in silico* Analysis

The docking and complex selection was done as previously described (Atasever-Arslan et al., 2016). We docked five different compounds found in *S. platensis* microalgae extract, octadecanoic acid (Chemspider ID: 5091), hexadecanoic acid methyl ester (Chemspider ID: 7889), octadecanoic acid methyl ester (Chemspider ID: 7909), triethyl orthoacetate (Chemspider ID: 59606) and thiosemicarbazide (Chemspider ID: 2005980) to the active site of PARP1 (PDB ID: 1WOK) (Iwashita et al., 2005). Experimentally shown inhibitor of PARP1, 3-(4-Chlorophenyl)-5-quinoxalinecarboxamide (Chemspider ID: 571257) was also docked to the active site of PARP1 (Ala 760, Asp 766, Gln 759, Glu 763, Glu 988, Gly 863, Ser 904, Tyr 889, Tyr 896, and Tyr 907) and was used as the positive control (PC) (Iwashita et al., 2005). The Auto Dock Tools package was employed to generate the docking input files of PARP1, five different compounds found in *S. platensis* microalgae extract and the positive control inhibitor. Before docking, PARP1 structure (PDB ID: 1WOK) was energy minimized as follows the protocol the described by Atasever-Arslan et al., 2016. For all other compounds, ligand pose oriented in the active site of PARP1, with the highest predicted binding affinity was selected and used for molecular dynamics simulations. Parameter and topology for each compound and positive control inhibitor were prepared using The CHARMM General Force Field (CGenFF) (Vanommeslaeghe et al., 2010; Yu et al., 2012; Vanommeslaeghe and MacKerell, 2012; Vanommeslaeghe et al., 2012). Molecular dynamics (MD) simulations were implemented using six complex structures. As the positive control, 3-(4-Chlorophenyl)-5-quinoxalinecarboxamide-PARP1 complex was used in MD simulations.

All complexes, composed of ~35000 atoms were placed in water boxes with dimensions of 75x65x75 Å3 respectively. Then all systems were ionized to a neutral state. The resulting systems were used in MD simulations using the NAMD program (Phillips et al.,

2005) with the CHARMM 22 parameters (MacKerell et al., 1998; Brooks, et al., 2009) which contained correction map for backbone atoms (Feig et al., 2003; MacKerell et al., 2004). Water molecules within the system were treated explicitly using the TIP3P model (Jorgensen et al., 1983). An isothermal–isobaric (NpT) ensemble was used in molecular dynamics simulations with periodic boundary conditions, and the long-range Coulomb interactions were computed using the particle-mesh Ewald algorithm. Pressure was maintained at 1 atm and temperature was maintained at 310 K using the Langevin pressure and temperature coupling. A time step of 2 fs was used in all MD simulations. The systems were fully energy minimized in 20,000 steps and carefully equilibrated under constant temperature and volume for 0.5 ns. Then they were heated gradually from 10 K to 310 K in 30 ps before production runs. The production were lasted for 5 ns and repeated two times. Visual molecular dynamics (VMD) (Humphrey et al., 1996) was used for the analysis of trajectories and the visualization of structures. Stability analysis of each complex was done through calculation of the root mean square displacements (RMSD) of backbone atoms of each protein molecule, excluding hydrogens. Distance from center of mass of each compound to the center of mass of the active site of PARP1 was calculated in all complexes and compared to that of positive control in order select compound that reside in active site of PARP1 similar to positive control. Furthermore, distances vs. distance distribution analysis of the distances measured were also analyzed in order select the most plausible candidate that possibly acts as PARP1 inhibitor.

Statistical Analysis

For statistical analysis, "Statistical Package for the Social Sciences (SPSS) Software" was used. Results were expressed as the mean ± standard deviation (SD). Statistical differences were assessed by Student's unpaired t-test, with $p < 0.05$ as statistically significant.

RESULTS and DISCUSSION

Spirulina platensis Extract's Cytotoxic Activity on Ishikawa Endometrial Cancer Cells

Initially, cytotoxic effect of *S. platensis* microalgae extract on Ishikawa cells and ECV304 endothelial cell were investigated by MTT colorimetric assay. For this purpose, Ishikawa cell line was treated with different concentrations (500, 50, 10, 5, 1 µg mL^{-1}) of *S. platensis* microalgae extract. Results pointed out selectively elevated cytotoxicity of *S. platensis* microalgae extract on Ishikawa endometrial adenocarcinoma cell line, compared with non-cancerous cell line (Figure 1). More specifically, at 500 µg mL^{-1} concentration, *S. platensis* microalgae extract displayed 43±4.87 % cytotoxic activity on Ishikawa cells while same dose of the extract showed 22±1.87 % cytotoxic activity on non-cancerous ECV304 endothelial cells.

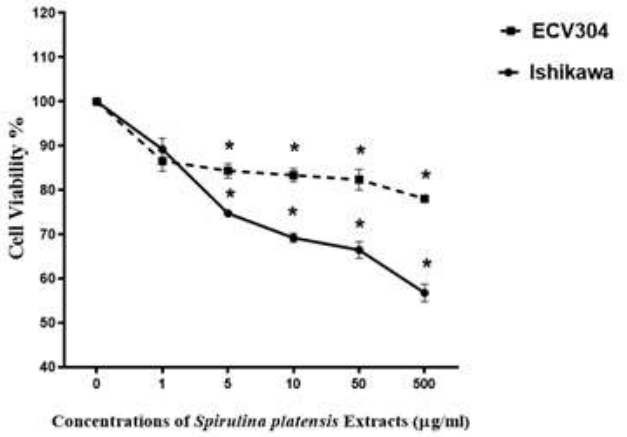

Figure 1. S. platensis extract's cytotoxic activity against Ishikawa and ECV304. The cytotoxicity test was repeated 6 times for every concentration of S. platensis microalgae extracts. A statistically significant cytotoxic effect of S. platensis extract against Ishikawa endometrial cancer cells was shown at 500, 50, 10, 5 µg mL^{-1} concentrations ($p<0.05$).

Şekil 1. S. platensis ekstresinin Ishikawa ve ECV304'e karşı sitotoksik aktivitesi. Sitotoksisite testi, S. platensis mikroalg ekstraktlarının her konsantrasyonu için 6 kez tekrarlanmıştır. S. platensis ekstraktının Ishikawa endometrial kanser hücrelerine karşı istatistiksel olarak anlamlı sitotoksik etkisi 500, 50, 10, 5 µg mL^{-1} konsantrasyonlarında gösterilmiştir ($p<0.05$).

Similar results that confirm the observations could also be found in the literature. *C-phycocyanin* isolated from *S. platensis* microalgae extract has been shown to suppress colon carcinogenesis (Saini et al., 2014). Also anti-proliferative effect of *S. platensis* microalgae extract on pancreatic cancer cells has been previously discussed (Konickova et al., 2014). Another study demonstrated the anti-viral effect of *S.platensis* extracts (Kok et al., 2011). Overall, the results clearly indicated that chemical constituents of *S. platensis* microalgae extract may be responsible for its highly selective cytotoxic effect on Ishikawa cells, in depth analyses for understanding possible DNA fragmentation, composition of the extract and changes in expression of genetic marker, were conducted at the next steps.

Apoptotic Activity of *Spirulina platensis* Extract on Ishikawa Cells

Since selective cytotoxicity of *S. platensis* extract was evidently present in Ishikawa cells, potential extract induced apoptotic activity was examined at the next

step. In order to reveal apoptotic activity of *S. platensis* microalgae extract on Ishikawa endometrial cancer cells, DNA fragmentation assay was utilized since fragmented DNA is a widely established hallmark of apoptosis. For implementation of the assay, Ishikawa cells were treated with *S. platensis* microalgae extract for 24 hours and Ishikawa cells treated with only medium were used as negative control. Genomic DNA stability of Ishikawa cells incubated with *S. platensis* microalgae extract was compared with genomic DNA stability of control cells under same conditions. Results indicated an increased apoptotic DNA fragmentation in Ishikawa cells which were incubated with *S. platensis* microalgae extract, validating results obtained from cytotoxicity analysis (Figure 2).

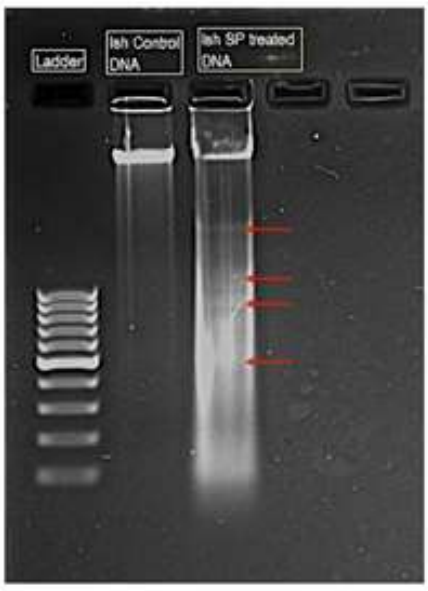

Figure 2. DNA fragmentation, a hallmark of apoptosis, induced by S. platensis microalgae extract in Ishikawa cells. DNA fragmentation was repeated 3 times; a representative gel picture is shown.

Şekil 2. *Ishikawa hücrelerinde S. platensis mikroalg ekstresi tarafından indüklenen, apoptozun ayırt edici özelliği olan DNA parçalanması. DNA parçalanması 3 kez tekrarlandı; temsili bir jel resmi gösterilmektedir.*

Determination of The Essential Oil Composition of *Spirulina platensis* Extract

Next, the essential oil content of *S. platensis* microalgae extract was investigated to reveal potentially responsible compound for the apperant evidence on cytotoxicity and DNA fragmentation. GC-MS analysis was utilized to determine the compounds of *S. platensis* microalgae extract's essential oils. Table 1 exhibits the percentage composition of *S. platensis'* essential oils of the extract, which consists of 42 compounds.

Out of 42, the seven main compounds found predominantly are given below: "Octadecanoic acid, methyl ester (40.67 %), hexadecanoic acid, methyl ester (Cas) methyl palmitate (17.83 %), 9,12-octadecatrienoic acid (Z, Z)-, methyl ester, (8.45 %), hydrazinecarbothioamide (3.75 %), ethane, 1,1,1-triethoxy- (CAS) Triethyl orthoacetate (5.53 %), 6,9,12-octadecatrienoic acid, methyl ester (CAS) methyl 6,9,12-octadecatrienoate (4.72 %), octadecanoic acid (4.32 %)". Same or similar compounds to the ones identified in this study, has shown wide variety of biological activity in different biological systems. Takeara et al., 2008 showed that *Didemnum psammatodes* extract including "Methyl myristate, methyl palmitate, methyl stearate, palmitic acid and stearic acid" has cytotoxic activity against human leukemia cells. Octadecanoic acid identified as one the main compounds found in extracts of the algea is a saturated fatty acid with an 18-carbon chain and also defined as stearic acid. Khan et al., 2013 showed that steraic acid, as an ester derivative, inhibits the growth of human breast cancer cells. In another study, it was shown that *Salvia verbenaca* including hexadecanoic acid as the main constituent, inhibited the growth of human melanoma cell line M14 cells (Russo et al., 2015).

The other compounds that were also detected in significant amounts (above 1%) were listed as follows: "9-hexadecenoic acid, methyl ester, (Z)-, l-(+)-ascorbic acid 2,6-dihexadecanoate, benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester, phenol, 2,4-bis (1,1-dimethylethyl)- (CAS), p-dioxane-2,3-diol".

Some other fatty acid derivatives such as "Hexadecanoic acid, octadecenoic acid, L-(+)-ascorbic acid 2, 6-dihexadecanoate", isolated from *S. glomerata*, were shown to control pathogenic bacteria, fungus, virus and anti-tumor activities (Karthikeyan et al., 2014). In another study in the literature, MDA-MB-231 cancer cells treated with Vallaris glabra leaf extract which contains stearic acid displayed an apoptotic effect (Wong et al., 2014). He and colleagues found that Zanthoxylum nitidum including "Spathuleno, n-hexadecanoic acid, ar-tumerone, oleic acid and hexanoic acid" did not displayed any cytotoxic activity against human liver cancer Huh-7 and normal IEC-6 cells. Wei and colleagues (Wei et al., 2011) demonstrated that *Peperomia pellucida* leaf extract including "2-naphthalenol, decahydro-, Hexadecanoic acid, methyl ester and 9,12-Octadecadienoic acid (Z, Z)-, methyl ester (17.61%)" as major compounds has drug potential in breast cancer treatment. Cytotoxicicity of the essential oils might have differences depending on the characteristics of cancerous cells.

These results clearly demonstrated that *Spirulina platensis* microalgae extract contained various types of bioactive compounds including fatty acids that could have roles attributable to the cytotoxic effect and DNA fragmentation presented in endometrial cancer cells.

Thus, antagonistic or synergistic relationship among the essential oils should be investigated in future studies. Nevertheless, in order to deleneate which constituent of the extract is responsbile for interacting with intracellular signaling proteins that could lead to cytotoxicity and DNA fragmentation, expression of genetic markers were evaluated and further *in silico* modelling was conducted based on the data obtained.

Table 1. Chemical compounds (in %) of essential oils of Spirulina platensis
Tablo 1. Spirulina platensis'in uçucu yağlarının kimyasal bileşikleri (%)

Compounds	*rt*	*S. platensis (%)*
p-Dioxane-2,3-diol	3.293	1.8
Phenol, 2,4-bis(1,1-dimethylethyl)- (CAS)	11.123	2.3
Hydrazinecarbothioamide	3.450	5.23
3,3-Dimethoxy-2-butanone	4.283	0.13
Oxime-, methoxy-phenyl-_	4.917	0.43
Ethane, 1,1,1-triethoxy- (CAS) Triethyl orthoacetate	5.867	5.53
1-Tetradecene	10.018	0.53
Hexadecanoic acid, methyl ester (CAS) Methyl palmitate	14.162	17.83
1-Hexadecene (CAS) Cetene	11.710	0.23
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester (CAS)	11.842	0.54
di(Butoxyethyl)adipate	12.295	0.28
Heptadecane	12.535	0.23
1-Hexadecene (CAS) Cetene	13.218	0.40
Tetradecanal (CAS) Myristaldehyde	13.433	0.14
1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	13.867	0.16
9-Hexadecenoic acid, methyl ester, (Z)-	14.037	3.75
Tetradecanoic acid, methyl ester (CAS) Methyl myristate	12.737	0.72
l-(+)-Ascorbic acid 2,6-dihexadecanoate	14.398	3.83
Heptadecanoic acid, methyl ester (CAS) Methyl heptadecanoate	14.858	0.93
6,9,12-Octadecatrienoic acid, methyl ester (CAS) methyl 6,9,12-octadecatrienoate	15.323	4.72
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	15.433	8.45
Octadecanoic acid, methyl ester	15.658	40.67
Octadecanoic acid	15.947	4.32
Tetradecanamide	16.186	0.89
7,10,13-Eicosatrienoic acid, methyl ester	16.994	0.01
11,13-Eicosadienoic acid, methyl ester (CAS) Methyl 11,13-Eicosadıenoate	17.117	0.21
Eicosanoic acid, methyl ester (CAS) Arachidic acid methyl ester	17.304	0.74
Tetratriacontane	17.814	0.56
13-Docosenamide, (Z)-	17.670	0.27
Heneicosane	18.487	0.56
Docosanoic acid, methyl ester	18.701	0.82
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	18.929	0.18
Pentacosane	19.133	0.83
Eicosanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	19.238	0.42
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	19.656	2.49
Tetracontane	19.806	0.66
Tetracosanoic acid, methyl ester (CAS) Methyl lignocerate	20.056	0.41
Pentatriacontane	20.537	0.67
Eicosanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester (CAS)	20.633	0.78
2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	20.883	0.37
Tetracontane	21.321	0.61
Tetracontane	22.221	0.94

rt, retention time (min).

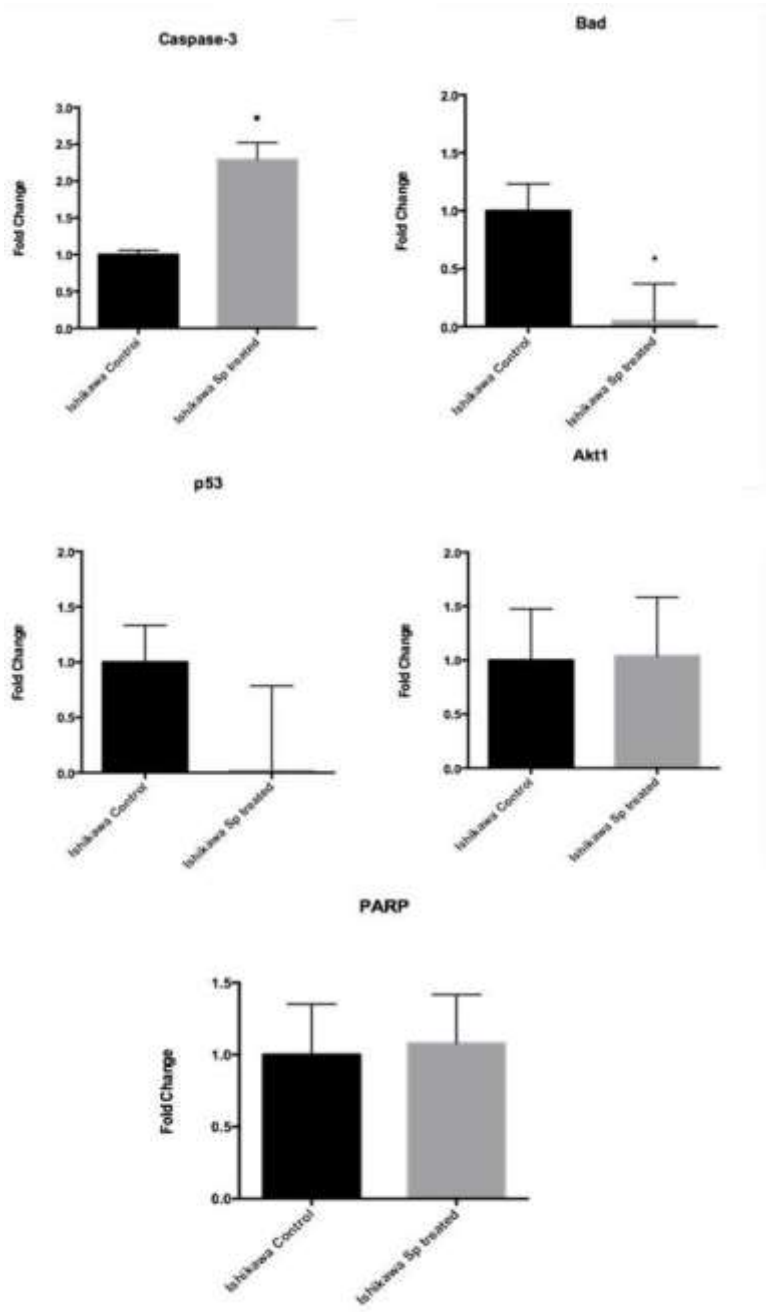

Figure 3. q-PCR analysis of apoptosis related genes. S. platensis microalgae extract (Ishikawa Sp Treated) treatment of Ishikawa cells induced statistically significant increase in caspase-3 gene expression, simultaneous to downregulation of Bad gene expression compared with non-treated control (Ishikawa Control). Rest of the apoptosis related genes did not show any significant change in expression ($p<0.05$).

Şekil 3. Apoptozla ilgili genlerin q-PCR analizi. Ishikawa hücrelerinine S. platensis mikroalg ekstresi (Ishikawa Sp ile muamele edilmiş) uygulanması, kaspaz-3 gen ekspresyonunda istatistiksel olarak anlamlı bir artışa, işlem uygulanmamış kontrol (Ishikawa Kontrolü) ile karşılaştırıldığında Bad gen anlatımında aşağı regülasyonuna neden oldu. Apoptozla ilişkili diğer genlerin anlatımında anlamlı bir değişim gerçekleşmedi ($p<0.05$).

Evaluation of Apoptotis Related Genetic Markers

In order to confirm DNA fragmentation and to understand its mechanism induced the algae extract, q-PCR test was applied to examine expression profiles of apoptosis related genes p53, Bad, Caspase-3, PARP and Akt1 in Ishikawa cell line. Results demonstrated a statistically significant increase in caspase-3 expression, which was accompanied by downregulation of Bad expression, in extract treated cells compared to non-treated control (Figure 3). Other apoptosis related genetic markers p53, Akt1 and PARP mRNA expressions did not show any statistically significant change upon extract treatment as shown in Figure 3. Although, these results validate that apoptotic

machinery was triggered as shown by simultaneous upregulation of caspase-3 and DNA fragmentation, the underlying mechanism induced by algae extract was not clear.

A marker of caspase-3 activation, caspase-3 cleavage has also been previosuly shown through *S. platensis* microalgae extract treatment in some cancer cell lines. Since downregulation of Bad gene expression was also present simultaneous to increased caspase-3, it was deduced that activation of extrinsic apoptotic pathway was a more probable event, compared with extrinsic pathway. Moreover, there were not any gene expression changes for the markers Akt1, p53 and PARP. While all these data suggested that underlying apoptotic mechanism might be more related to extrinsic pathways instead of intrinsic apoptotic pathways, targeting of PARP1 activity by a particular constituent found in the extract was also suspected due to observation of DNA fragmentation and previouuly shown link between DNA fragmentation and PARP1. Thus, *in silico* analysis was conducted to investigate presence of such mechanism.

Therefore, at the next step, a molecular dynamics based computational approach was taken into account to link the molecules identified in the extract to previously identified mechanism of DNA fragmentation.

In silico Analysis of PARP1 Active Site as a Potential Target of the Compounds Found in *Spirulina platensis* Microalgae Extract

It is previously known that PARP1 is directly involved in poly-ADP ribosylation dependent inhibition of DNA fragmentation factor 40 (DFF40), which is one of the factors responsible for DNA degradation in apoptosis (West et al., 2005). It is also well established that, PARP1 is cleaved by caspase-3 under apoptotic conditions (Lazebnik et al., 1994; Nicholson et al., 1995). Based on these background data and on the observations that there was specific DNA fragmentation event, as well as increased caspase-3 expression upon extract treatment, it was hypothesized that PARP1 may be targeted by the compounds in *S. platensis* microalgae extract. In order to test this hypothesis, an *in silico* ligand-protein docking and subsequent molecular dynamics simulations were conducted. Five of the compounds found in *S. platensis* microalgae extract were docked to PARP1 active site (PDB ID: 1WOK) (Iwashita et al., 2005) and simulated for 5 ns using molecular dynamics. All simulations were stable through 5 ns, thus analyzed further in detail (Figure 4A). Distance analysis indicated that distance from center of mass of the compound to the center of mass of the PARP1 active site were similar or lower compared with the positive control only in simulations containing hexadecanoic acid methyl ester (green) and Octadecanoic acid methyl ester (blue) (Figure 4B). This distance was mostly variable in other complexes obtained with other compounds docked into PARP1 active site (Figure 4B). In detail analysis of these distances using distance vs. distance distribution graphs clearly indicated that octadecanoic acid methyl ester has highest match to the positive control (Figure 4C). Orientation of positive control and octadecanoic acid methyl at ester at 5th ns of MD simulations also supported the findings of distance and distance distribution analysis (Figures 4D and 4E).

Hence it was deduced that octadecanoic acid methyl ester is the most plausible candidate for inhibition of PARP1 catalytic activity based on this in *silico* findings. If the hypothesis of PARP1 inhibition by the compounds found in *S. platensis* is valid, *in silico* analysis indiated that this event was most likely mediated by octadecanoic acid methyl ester. It is also particularly important to note here that octadecanoic acid methyl ester constitutes almost 40% of the extract, a fact that also supports *in silico* observations.

CONCLUSION

The underlying motivation of this study was to identify anti-cancer potential of *S. platensis* methanolic extract, which may lead to the discovery of important candidate anti-cancer compounds to be used as novel anti-cancer therapy options in future. In this regard, a potentially active molecule, which has anti-cancer effect, from *S. platensis* microalgae extract was identified by combining experimental and computational approaches.

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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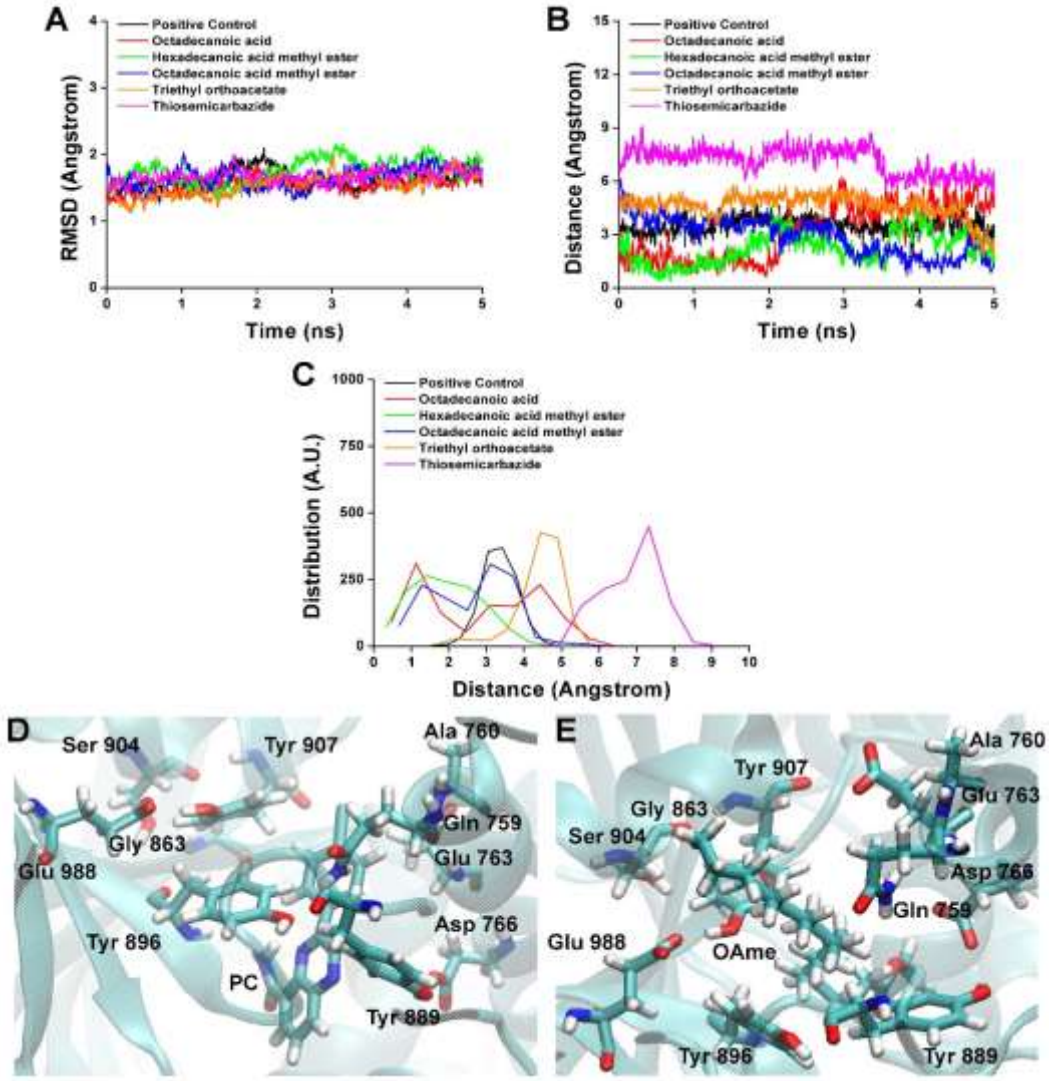

Figure 4. In silico dynamics of octadecanoic acid methyl ester docked into the active site of PARP1, showed similar distance and distance distribution pattern, compared with the positive control. Five of the major compounds, determined in section 3.4., was docked into the active site of PARP1 (PDB ID: 1WOK) and simulated for 5 ns. To be able to assess the comparability in between docking and molecular dynamics procedures, a positive control (PC) PARP1 inhibitor (3-(4-Chlorophenyl)-5-quinoxalinecarboxamide) was also docked into PARP1 active site and simulated in the same time frame. Active site residues of PARP1 was selected based on the previous literature and determined as Ala 760, Asp 766, Gln 759, Glu 763, Glu 988, Gly 863, Ser 904, Tyr 889, Tyr 896, and Tyr 907. A) All of the simulations displayed stability throughout 5 ns, indicating suitability for further analysis. B) The distance between center of mass of the docked compounds and the center of mass of the active site residues were lower or similar to that of positive control in the simulations containing hexadecanoic acid methyl ester (green) and octadecanoic acid methyl ester (blue) for 5ns. For the rest of the compound-PARP1 complexes, this measured distance was higher than the positive control during significant amount of the simulation time. C) Distance vs. distance distribution analyses of the distances measured in B, revealed that octadecanoic acid methyl ester had highly similar distance distribution to that of positive control, in the active of PARP1 during 5 ns. A.U.: Arbitrary units D & E) Orientation of positive control compound in the active site of PARP1 at the 5th ns of molecular dynamics simulations (D) compared with the orientation of octadecanoic acid methyl ester (OAme) in the active site of PARP1 (E).

Şekil 4. PARP1'in aktif bölgesine yerleştirilen oktadekanoik asit metil esterin in siliko dinamikleri, pozitif kontrol ile karşılaştırıldığında benzer mesafe ve mesafe dağılım modeli gösterdi. Bölüm 3.4'te belirlenen ana bileşiklerden beşi, PARP1'in aktif bölgesine yerleştirildi (PDB ID: 1WOK) ve 5 ns için simüle edildi. Yerleştirme ve moleküler dinamik prosedürleri arasındaki karşılaştırılabilirliği değerlendirebilmek için, PARP1 aktif bölgesine bir pozitif kontrol (PC) PARP1 inhibitörü (3-(4-Klorofenil)-5-kinoksalinkarboksamid) de yerleştirildi ve aynı zaman diliminde simüle edildi . PARP1'in aktif bölge kalıntıları, önceki literatüre göre seçildi ve Ala 760, Asp 766, Gln 759, Glu 763, Glu 988, Gly 863, Ser 904, Tyr 889, Tyr 896 ve Tyr 907 olarak belirlendi. A) Hepsi simülasyonlar, 5 ns boyunca kararlılık göstererek daha fazla analiz için uygun olduğunu gösterdi. B) 5ns için yerleştirilmiş bileşiklerin kütle merkezi ile aktif bölge kalıntılarının kütle merkezi arasındaki mesafe, hekzadekanoik asit metil ester (yeşil) ve oktadekanoik asit metil ester (mavi) içeren simülasyonlarda pozitif kontrolünkinden daha düşük veya benzerdi. Bileşik-PARP1 komplekslerinin geri kalanı için, ölçülen bu mesafe, simülasyon süresinin önemli bir kısmı sırasında pozitif kontrolden daha yüksekti. C) B'de ölçülen mesafelerin mesafeye karşı mesafe dağılımı analizleri, oktadekanoik asit metil esterin, 5 ns boyunca PARP1 aktifinde pozitif kontrolünkine oldukça benzer mesafe dağılımına sahip olduğunu ortaya çıkardı. A.U.: Arbitrary birimler D & E) PARP1'in (E) aktif bölgesindeki oktadekanoik asit metil esterin (OAme) oryantasyonu ile karşılaştırıldığında moleküler dinamik simülasyonlarının (D) 5. ns'sinde PARP1'in aktif bölgesindeki pozitif kontrol bileşiğinin oryantasyonu.

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