Original Article

Proteomic Approach Reveals the Activation of Apoptosis by *Padina gymnospora* in Oral Cancer Cell Line

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ABSTRACT

Aim/Background: Cancer is still known to be a devastating disease condition. Even after getting advancements in multimodality therapies, the success rate with respect to patient survival is very limited. Hence there is an emerging need to find alternative medication strategies for cancer treatment. Recently seaweeds are getting their significance due to its tremendous medicinal properties. Among different category of seaweeds, *Padina gymnospora* which is a brown seaweed is known to possess many important bioactivities including antimicrobial activity, anti-oxidative property and anti-inflammatory. Role of this seaweed in treating oral cancer cells is not much explored in detail. Hence, this study aims to understand the significance of *Padina gymnospora* in oral cancer cell line – SAS. **Materials and Methods:** The ethanol extract of Padina was treated to oral cancer cell line – SAS and incubated for 24 hr. After incubation, MTT assay was performed to find the efficiency of the extract. Further the cells were taken for protein extraction and subjected to SDS PAGE, two-dimensional electrophoresis (2D) coupled with tandem mass spectrometry approach. The differentially expressed proteins were further identified and subjected to bioinformatic analysis. **Results and Conclusion:** It was observed that pro apoptotic proteins including BIK, BAK1, BID, BAD and CASP8 were highly upregulated in the Padina treated samples compared with untreated control samples. This data clearly demonstrates that *Padina gymnospora* induces the apoptosis in the oral cancer cells. This study paves us the way to explore more about the therapeutic potential of *Padina gymnospora* in treating the oral cancer cells.

Keywords: *Padina gymnospora*, SAS oral cancer cell line, Apoptosis, 2D electrophoresis, Mass spectrometry, MTT Assay, Seaweed.

INTRODUCTION

Cancer is known to be the second leading cause of death globally. Reports suggests that among all cancers, head and neck based cancers accounts for more than 4% with more that 4% mortality rate.[1] Generally, carcinogenesis is defined to be a combination of different level of intricate and multiple process. To manage the cancer process in a better way, it is very important to understand the process behind the uncontrollable cell proliferation and diminished cell death. Apoptosis is known to be specifically designed systemized process which results in cell death.

Though we have many improvements in therapeutic strategies but the search for an effective treatment strategy is still in process. Routine conventional strategies for cancer treatment include surgery, radiation and chemotherapy but these methods of strategies still lack in complete metastasis prevention and fatality rate. Hence, there is always an emerging need to identify novel and effective alternative treatments for cancer. Seaweed is one among the naturally available source for effective alternative medicine.

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Generally marine based macro algae are categorized and called as seaweed. Based on their nutrient components and chemical composition, they are sub-grouped as green algae (chlorophyta), brown algae (phaeophyta) and red algae (rhodophyta). Among them, red and brown algae play a major role in human dietary zone. It has been commonly consumed by mankind. Seaweed possesses different kind of medically significant components such as antibiotics, anticoagulants, antiulcer products and laxatives. Many reports described about their significant role in wound healing process and its role as antioxidants anti-bacterial agents. Very few reports are available to describe about their pro apoptotic property in cancer process. Hence this study aims to understand the efficacy of brown seaweed called *Padina gymnospora* in treating the oral cancer cells.

MATERIALS AND METHODS

Collection of Brown Algae

The whole plant of Anisomeles malabarica was collected around College of Life Sciences, Lanzhou University. China The collection was made during the period of June 2018 – August 2018. Polythene bags were used to collect plant materials in fresh conditions. All the collected plants were trimmed off with scissor. Leaves were separated from the plant twigs and shade dried at room temperature. The dried leaves were powdered mechanically using electrical stainless-steel blender and sieved using thin nylon mesh to get fine powder.

The collected plant samples were identified morphologically and were rinsed with water to remove epiphytes and necrotic parts. The plants were again washed with tap water to remove any associated debris and shade dried at room temperature (28±2°C) for 5-8 days or until they are brittle. After completely drying, the plant materials were ground to a fine powder using electrical blender and then immediately subjected to extraction using ethanol.

Cell Culture

The SAS cell lines were procured from the College of Life Sciences, Lanzhou University. China. Dulbecco`s Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO_2 at 37°C.

MTT Assay

The cytotoxicity of selected plant materials on SAS cells was determined by the method of Mosmann,[2] The yellow 3-4,5dimethylthiozol-2-Yl)-2,5diphenyltetrazolium bromide (MTT) is reduced by mitochondrial dehydrogenase of viable cells yielding a measurable purple formation product. Viable cells contain NAD(P) H-dependent reductase, which reduce the MTT reagent to formazon, with a deep purple colour. Formazon crystals are then dissolved using solubilizing solution and absorbance is measured at 500-600 nm by plate reader.

Sample Preparation Method for Proteomic Analysis

After the treatment of the oral cancer cell line with *Padina gymnospora* for 24 hr, the cells were scraped and solubilized with 2DE lysis buffer comprising of 7M urea, 2M thiourea, 4% w/v 3- [(3-holamidopropy/ dimethy-ammonia]-1- propane sulfonate (CHAPS) (Pierce, Rockford, IL, USA), 0.2% ampholite 4 - 7 (Amersham, USA), 100 mM PMSF and 100 mM DTT. The lysate was sonicated with 40% amplitude for 15 cycles with intermitted 10 sec brake. Following the process, the lysate was centrifuged at 12,000 rpm for 30 hr at 4°C and the supernatant was collected and protein was estimated using Bradford assay.

Two-dimensional Gel Electrophoresis (2DE)

The protein concentration used for 2DE analysis is 150 ug. The sample was solubilized in rehydration buffer and passive rehydration was performed for a minimum of 12 hr. Further focusing of the strips were done at a temperature of 20°C using the IPGphor IEF apparatus (GE Healthcare) as per the following protocols: 500 V for 1hr Step and hold; 1000 V for 1 hr Gradient; 8000 V for 3 hr Gradient; 8000 V for 8 hr Step and hold. The strips were further equilibrated and processed further of second dimensional analysis. The protocol of Candiano and his group (2004),[3] was followed for coomassie blue staining. The gels were fixed in 40% methanol and 10% acetic acid in water for an hour. After washing 3 times in water, for 10 min each, gels were stained overnight in Coomassie stain containing 10% phosphoric acid, 10% ammonium sulphate, 0.12% Coomassie Blue G-250 and 20% methanol. Gels were then de-stained in water.

Processing of 2de Spots For Maldi Tof Mass Spectrometry

Differentially expressed proteins were selected from the 2D gels and processed further for In gel trypsin digestion as described earlier.[4] The extracted peptides were analyzed through mass spectrometry using

SHIMADZU MALDI 7090 (TOF/TOF MS) as described earlier.[5] The acquisition parameters were as follows the lyophilised samples were diluted in 8uL of 50:50 (ACN:Water in 0.1 FA). The analysis was carried out in Reflectron Mode with the mass range of 1-5000 Da, eliminating the Matrix ions through setting up the ion-gate blanking mass of 700 Da (ions below the 700mz were not acquired by the system). The laser energy was set to 35-45 (based upon the sample) with the laser beam diamter of 100 micron and pulsed extraction at 1500mz mass (Foccusing ions at a range). Data were processed with Gaussian smoothing method: Value set for processing was 3. Peak delimetring method for determining the peak Mass: Threshold Centroid 25% is used for the processing. Mono isotopic mass picking was from 700-3000 range with minimum two isotopes.

Gene Ontology by Panther Software and Protein Interaction by String

Analysis of functional enrichment of differentially regulated proteins was performed in PANTHER software,[6] and protein interaction studies were performed using STRING database.[7] The combined list of official gene symbols corresponding to the identified proteins was used for input.

RESULTS AND DISCUSSION

Brown algae known to synthesize different kind of secondary metabolites, especially these are used for their survival in the time of stressful condition. To manage these situations, they undergo a process in developing new biological components that possess various functional activity. The major well-known components include fiber, protein, polyphenol, terpene, fatty acids, minerals and vitamins.[8-10] All these components were analysed further to in the field of therapeutic purposes and reported that it plays a major role in improving the immune system, in preventing vascular diseases and eventually even in inhibiting the cancer metastasis.[11] To understand more about the effect of these seaweed, we treated the SAS oral cancer cell line with a well-known brown alga named *Padina gymnospora*.

The cell viability assay was performed, and the data was represented in Figure 1. The data suggest that the extract of the *Padina gymnospora* showed increased percentage of inhibition compared to the untreated control with the IC_{50} of 221 ug/ml. This clearly demonstrate that bioactive components synthesized by the brown algae possess efficient activity to induce apoptosis in the oral cancer cell line. Further to understand the mechanism behind this apoptosis process, proteomics analysis was performed between seaweed extract treated sample versus untreated control sample. Figure 2a represents the SDS PAGE profile of the proteins extracted from the oral cancer cell line upon treatment with seaweed extract. The protein bands clearly show a differentially regulated pattern in comparison with the untreated samples. Figure 2b depicts the two-dimensional electrophoresis (2DE) analysis for the

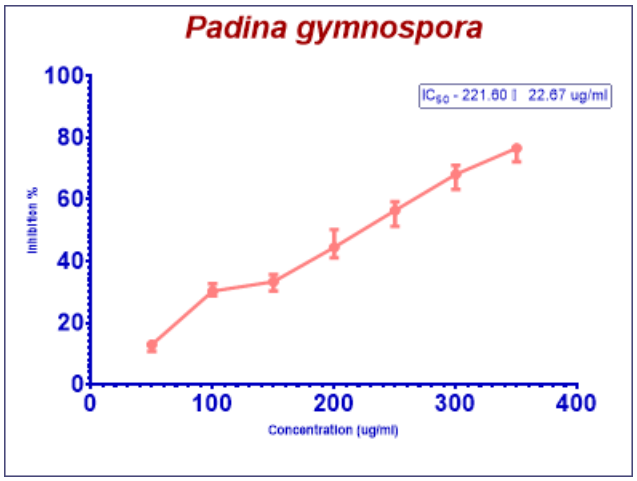
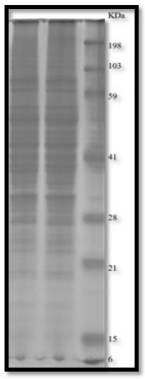
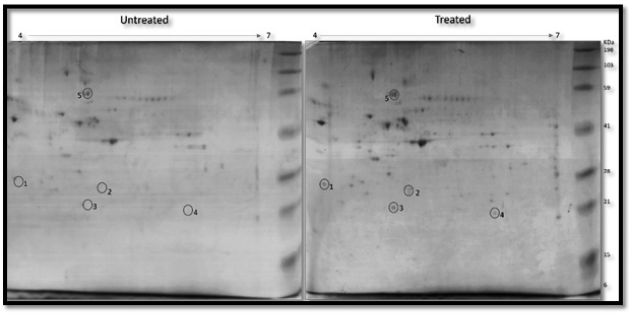


Figure 1: MTT assay with IC_{50} concentration for *Padina gymnospora* in SAS cell line. The analysis was performed with triplicates.



2a



2b

Figure 2: a) SDS PAGE analysis for untreated (U) and treated (T) SAS oral cancer cells; b) 2DE for untreated (U) and treated (T) SAS oral cancer cells separated in pI range of 4-7. The differentially expressed protein spots were labelled.

Table 1: Protein Identification details with relevant information.						
Match ID	**Gene ID**	**Accession No**	**Protein Name**	**Mol Weight**	**pI**	**Regulation status in treated samples**
1	BIK	Q13323	Bcl-2-interacting killer	18.015	4.21	Up Regulated
2	BAK1	Q16611	Bcl-2 homologous antagonist/killer	23.409	5.66	Up Regulated
3	BID	P55957	BH3-interacting domain death agonist	21.995	5.25	Up Regulated
4	BAD	Q92934	Bcl2-associated agonist of cell death	18.392	6.6	Up Regulated
5	CASP8	Q14790	Caspase-8	55.391	4.99	Up Regulated

extracted proteins obtained from oral cancer cell lines upon treatment with seaweed extract. Image analysis of these 2DE gels revealed that 20 proteins spots were differentially regulated, among which 5 spots were upregulated in the seaweed treated samples compared with untreated control samples. These data evidently described that the seaweed treated samples showed a statistically differential expression of the protein profile completely getting altered. To further understand about the protein mechanism that were altered, the differentially expressed proteins were analysed by mass spectrometry analysis. The proteins identified by the mass spectrometry analysis were represented in Table 1. To note interestingly, most of the differentially regulated proteins were found to be pro apoptotic proteins. This gives us the strong understanding that the seaweed extract induced the apoptotic pathway in the oral cancer cell line upon treatment. Further to know more about the differentially expressed proteins, gene ontology analysis was performed and represented in Figure 3.

In most of the cancer conditions, the ratio of cell growth and cell death is getting altered and this effect results in abnormal cell growth.[12-13] Specifically in oral cancer condition, most of the cases shows p53 mutation and this eventually results in the check point control failures. As a consequence of this, the process of apoptosis was also interrupted, and this led to the replication of daughter cells with defective genome.[14] These chromosomal aberrations and deposition of mutations in crucial genes that encoded to important proteins or oncoproteins which in control proliferation and cell death will eventually lead to induction of neoplastic formation.[15-16] These important genes include pro apoptotic genes that encodes for caspases, BAD, BAX, BIK, BAK, BID and TNF receptors.[14]

In oral cancer process, the main reason for the clumping for newer cells was occurring because of increased cell proliferation, decreased turnover in cell population, or sometimes it might be a combined result of both effects. Most of the cancers escape the apoptosis process, as a result they achieved to have an increased survival process.[17] Caspases play a major role in the

Figure 3: Bioinformatic analysis for the differentially expressed proteins.

process of apoptosis, and it possess unique activities in oral cancer.[18] Studies reported that Bax expression was significantly correlated with grading of oral cancer by histological mode of classification,[19] but in other reports, it was stated that Bak expression was more productive in identifying the prognosis prediction in oral cancer.[20] In our study, we observed that key proteins involved in the apoptosis process were showing increased expression upon treatment with seaweed extract in oral cancer cell line. In the chain of consequences of pathological process, it is reported that Bcl-2 plays a major as well as a significant role in the carcinogenesis process. It possess a unique synergistical effect with both oncogenes and tumor suppressor gene.[21] In most of the cases, it is observed that Bcl-2 positive expression is always linked to increased tumor grade with increased mitotic index.[22]

CONCLUSION

Thus, our study clearly demonstrates that the *Padina gymnospora* seaweed extract possess bioactive compounds that induce apoptosis in oral cancer cells. This proteomics-based study implies the significance of the seaweed extract on oral cancer cell line. Further it had shed the light on the induced apoptosis mechanism by seaweed components in oral cancer cell line. Further investigation would help us more in understanding which should investigated further in the aspect of therapeutic strategies. This would benefit the existing cancer treatment modality and might show an added advantage for better treatment management.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

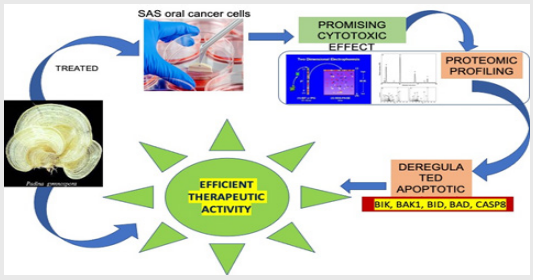
ABBREVIATIONS

MTT: 3-4,5dimethylthiozol-2-Yl)-2,5diphenyltetrazolium bromide; **BCL-2:** B-cell lymphoma 2; **BAD:** BCL2 Associated Agonist Of Cell Death; **BAX:** Bcl-2-associated X protein; **BIK:** BCL2 Interacting Killer; **TNF:** Tumor necrosis factor; **BID:** BH3 interacting domain death agonist; **2DE:** Two-dimensional electrophoresis; **DMEM:** Modified Eagle Media; **FBS:** Fetal Bovine Serum; **NAD(P):** Nicotinamide adenine dinucleotide phosphate; **SAS:** Squamous cell carcinoma; **SDS-PAGE:** Sodium dodecyl sulphate–polyacrylamide gel electrophoresis; **BAK1:** BCL2 Antagonist/Killer 1; **CASP8:** Caspase 8; **PMSF:** Phenylmethylsulfonyl fluoride; **DTT:** Dithiothreitol.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424. doi: 10.3322/caac.21492, PMID 30207593.
2. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983/12/16;65(1-2):55-63. doi: 10.1016/0022-1759(83)90303-4, PMID 6606682.
3. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, *et al*. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis. 2004/06/03;25(9):1327-33. doi: 10.1002/elps.200305844, PMID 15174055.
4. Ananthi S, Chitra T, Bini R, Prajna NV, Lalitha P, Dharmalingam K. Comparative analysis of the tear protein profile in mycotic keratitis patients. Mol Vis. 2008;14:500-7. PMID 18385783.
5. Sivagnanam A, Thangasamy B, Nagarajan V, Ravi SG, Madhesh JC, Perumal MA, *et al*. Comparative proteomic analysis reveals novel biomarkers for gastric cancer in South Indian Tamil population. Comb Chem High Throughput Screen. 2022;25(8):1361-73. doi: 10.2174/1386207324666210603120320, PMID 34082671.
6. Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, *et al*. DAVID: Database for annotation, visualization, and integrated discovery. Genome Biol. 2003;4(5):P3. doi: 10.1186/gb-2003-1184-1189-r1160, PMID 12734009.
7. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, *et al*. STRING v10: Protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 2015;43((Database issue)):D447-52. doi: 10.1093/nar/gku1003, PMID 25352553.
8. Burckhardt IC, Gozal D, Dayyat E, Cheng Y, Li RC, Goldbart AD, *et al*. Green tea catechin polyphenols attenuate behavioral and oxidative responses to intermittent hypoxia. Am J Respir Crit Care Med. 2008/02/16;177(10):1135-41. doi: 10.1164/rccm.200701-110OC, PMID 18276944.
9. Formagio AS, Kassuya CA, Neto FF, Volobuff CR, Iriguchi EK, Vieira Mdo C, *et al*. The flavonoid content and antiproliferative, hypoglycaemic, anti-inflammatory and free radical scavenging activities of Annona dioica St. Hill. BMC Complement Altern Med. 2013/01/15;13:14. doi: 10.1186/1472-6882-13-14, PMID 23311341.
10. Namvar F, Mohamad R, Baharara J, Zafar-Balanejad S, Fargahi F, Rahman HS. Antioxidant, antiproliferative, and antiangiogenesis effects of polyphenol-rich seaweed (*Sargassum muticum*). BioMed Res Int. 2013/10/01;2013:604787. doi: 10.1155/2013/604787, PMID 24078922.
11. Zhang H, Zhang M, Yu L, Zhao Y, He N, Yang X. Antitumor activities of quercetin and quercetin-5′,8-disulfonate in human colon and breast cancer cell lines. Food Chem Toxicol. 2012/02/09;50(5):1589-99. doi: 10.1016/j.fct.2012.01.025, PMID 22310237.
12. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature. 2001/05/18;411(6835):342-8. doi: 10.1038/35077213, PMID 11357141.
13. Scully C, Field JK, Tanzawa H. Genetic aberrations in oral or head and neck squamous cell carcinoma (SCCHN): 1. Carcinogen metabolism, DNA repair and cell cycle control. Oral Oncol. 2000/05/04;36(3):256-63. doi: 10.1016/s1368-8375(00)00007-5, PMID 10793327.
14. Stoll C, Baretton G, Ahrens C, Löhrs U. Prognostic significance of apoptosis and associated factors in oral squamous cell carcinoma. Virchows Arch. 2000;436(2):102-8. doi: 10.1007/pl00008207, PMID 10755598.
15. Regezi JA, Jordan RC. Oral cancer in the molecular age. J Calif Dent Assoc. 2001/10/02;29(8):578-84. PMID 11577668.
16. Scully C. Genetic aberrations in oral or head and neck squamous cell carcinoma 2: Chromosomal aberrations. Oral Oncol. 2000/07/19;36(4):311-27. doi: 10.1016/S1368-8375(00)00021-X.
17. Kaufmann SH, Gores GJ. Apoptosis in cancer: Cause and cure. BioEssays. 2000/11/01;22(11):1007-17.doi:10.1002/1521-1878(200011)22:11<1007::AID-BIES7>3.0.CO;2-4, PMID 11056477.
18. Coutinho-Camillo CM, Lourenço SV, Nishimoto IN, Kowalski LP, Soares FA. Caspase expression in oral squamous cell carcinoma. Head Neck. 2011/07/15;33(8):1191-8. doi: 10.1002/hed.21602, PMID 21755562.
19. Loro LL, Vintermyr OK, Liavaag PG, Jonsson R, Johannessen AC. Oral squamous cell carcinoma is associated with decreased bcl-2/bax expression ratio and increased apoptosis. Hum Pathol. 1999/09/24;30(9):1097-105. doi: 10.1016/s0046-8177(99)90229-0, PMID 10492046.
20. Baltaziak M, Koda M, Barwijuk-Machała M, Musiatowicz B, Duraj E, Kańczuga-Koda L, *et al*. The role of Bak expression in apoptosis of the oral squamous cell carcinoma (OSCC) and metastases to lymph nodes (LNMs). Rocz Akad Med Bialymst. 2004;49;Suppl 1:14-5. PMID 15638360.
21. Polverini PJ, Nör JE. Apoptosis and predisposition to oral cancer. Crit Rev Oral Biol Med. 1999;10(2):139-52. doi: 10.1177/10454411990100020201, PMID 10759418.
22. Sulkowska M, Famulski W, Sulkowski S, Reszeć J, Koda M, Baltaziak M, *et al*. Correlation between Bcl-2 protein expression and some clinicopathological features of oral squamous cell carcinoma. Pol J Pathol. 2003/06/24;54(1):49-52. PMID 12817880.

PICTORIAL ABSTRACT



SUMMARY

To analyze the efficacy of the Padina in oral cancer cells, the ethanol extract of Padina was treated to oral cancer cell line – SAS and further incubated for 24 hr. MTT analysis revealed that Padina possess anticancer activity against oral cancer cells. Proteomics analysis using 2D electrophoresis coupled with mass spectrometry found that pro apoptotic proteins including BIK, BAK1, BID, BAD and CASP8 were highly upregulated in the Padina treated samples compared with untreated control samples. These data strongly suggest that Padina has the therapeutic potential against the oral cancer cells.

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