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Effects of *Sargassum oligocystum* and *Padina australis* extract on Adipogenesis and Adipolysis in 3T3-L1 Cells

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Abstract

In recent years, the search for natural, safe and effective therapies for the management of obesity has become important. The present study investigated the potential of brown seaweeds *Sargassum oligocystum* and *Padina australis* from Malaysian waters as natural alternatives for the management of obesity. Both seaweeds were macerated using acetone at room temperature for ten hours. The *S. oligocystum* extracts (SE) and *P. australis* extracts (PE) were then applied to 3T3-L1 cells during the differentiation stage and during the mature stage of the adipocyte life cycle to assess the effects of extracts on adipogenesis and adipolysis. Application of SE at 12.5 and 50 µg/ml to the cells decreased adipogenesis by 71.7%, and 84.8%, respectively, while cells treated with 12.5 and 50 µg/ml PE showed 85.7%, and 89.0% adipogenesis respectively, compared to control. Application of SE and PE to mature lipid cells stimulated adipolysis and the release of glycerol into the culture media. Application of SE at 12.5 and 50 µg µg/ml in the cell media induced glycerol release by up to 88.6 and 93.0%, respectively, while PE increased glycerol release up to 92.9 and 95.6% respectively, compared to isoproterenol. This study demonstrates the potential of whole brown seaweed extracts from *S. oligocystum* and *P. australis* collected from Malaysian waters as natural anti- obesity agents. Incorporation of the brown seaweed into the diet as a functional food will be useful for prevention and treatment of obesity.

Introduction

Obesity is fast becoming the next preventable epidemic in the world, with over 11.6 billion of the population being overweight (BMI > 25 kg/m^2), and 400 million more obese (BMI > 30 kg/m^2) (WHO, 2013). Obesity has been reported as a risk factor for several diseases including high blood pressure, diabetes, cardiovascular disease, several cancers, disabilities and death (Douketis *et al*., 2005). The cost of these combined diseases puts a financial strain on healthcare expenditure. In 2006 alone, health care expenditure was estimated at 100 billion dollars per year and is expected to rise to approximately 500 billion dollars per year in 2030 (Douketis *et al*., 2005). Existing solutions for the management of obesity include lifestyle changes, pharmacotherapy and surgery (FDA, 2014). However, these methods do not result in sustained, meaningful and safe weight loss. The potential for the abuse of drugs and serious effects on the nervous system are some major issues with pharmacotherapy (Finkelstein *et al*., 2012), despite it being popular. In this regard, natural products are a potential alternative to current methods for the management of obesity (Han *et al*., 2005; Jaswir *et al*., 2011; Jeon *et al*., 2010; Kang *et al*., 2010).

Bitou and co- researchers (1999) have previously screened marine algae for anti-obesity properties and found all of them to exert some pancreatic lipase inhibitory activity. Maeda and his team (2005, 2007)

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focused on brown seaweeds *Undaria pinnatifida, Hijikia fusiformis* and *Sargassum fulvellum* for anti-obesity activity and found the brown carotenoid fucoxanthin present in the seaweeds to inhibit differentiation of 3T3-L1 preadipocytes into adipocytes. Previous research on the antiobesity potential of brown seaweeds has been carried out on Japanese and Korean seaweeds, where the seaweeds are consumed in high amounts by the population (Burtin, 2003).

It is reported that there are 261 taxa of marine seaweeds in Malaysia divided into 6000 species, grouped into green (Chlorophytes), brown (Pheophytes) and red (Rhodophytes) seaweeds (Phang and Wee, 1991; Chandini *et al.*, 2007). Despite this high number, few studies have been carried out on the uses of this marine resource (Wong and Phang, 2004).

Commercial interest in seaweeds in Malaysia is less directed towards food production and is more focused on the production of alginates such as agars and carrageenan for the international market (Chee *et al.*, 2011; Zemke-White and Ohno, 1999). However, interest in seaweeds as a source of important nutrients has been on the rise, with studies reporting seaweeds as excellent sources of carotenoids, dietary fiber, protein, essential fatty acids and vitamins (Mabeau and Fleurence, 1993). Interest in seaweeds as functional foods is also rising. This study aims to identify the potential of local brown seaweeds *Sargassum oligocystum* and *Padina australis* as anti-obesity agents via their action on adipogenesis and adipolysis stages of the adipocyte life cycle.

Materials and methods

Plant preparation

Fresh *S. oligocystum* and *P. australis* seaweeds were procured from Port Dickson, Negeri Sembilan, Malaysia. The seaweed was rinsed using tap water several times to get rid of dirt and inspected for attaching species. The final rinse was done using distilled water. Washed seaweed were spread on flat surfaces and left to air dry for 1-2 days before moved into a 40°C oven (Memmert Germany) and dried until the weight remained constant. The dried seaweed was kept in an airtight container until ready for grinding. The seaweed was ground using a Waring blender (UK) and sieved using mechanical sieve (Retsch, Germany) 500 µm in size. The ground seaweed was stored in airtight containers at room temperature until ready for extraction.

Extraction

5 g of dried ground seaweed was weighed and placed into a conical flask. 250 ml of acetone was added into the flask, mixed to homogenize and left to macerate for 10 hours at room temperature (26°C ± 2) with occasional stirring. Extraction was carried out under dim lighting to avoid oxidation via direct light. After extraction, the macerate was filtered using Whatman filter paper no.1 into a round bottom flask. The extract was concentrated using a rotary evaporator (BUCHI rotavapor R- 210, Switzerland) at 40°C. The concentrated extract was transferred into pre-weighed bijou bottles and purged with nitrogen to remove final traces of solvent. The final extract was then kept airtight and stored away from light at 4°C until ready to be used for biological studies. Extraction was carried out in triplicate.

Cell culture

ATCC mouse 3T3-L1 preadipocyte cells were obtained from Cell Culture Laboratory, Department of Pharmaceutical Technology, Kulliyyah of Pharmacy, IIUM. The cells were cultured in Dulbecco's modified Eagles medium (DMEM) (Sigma- Aldrich) with 10% fetal bovine serum (FBS) (10.000 u/ml) (Sigma-Aldrich) and 1% penicillin- streptomycin (10.000 ug/ml) (Sigma- Aldrich) at 37°C in an incubator with humidified atmosphere containing 5% CO_2 (Purecell TC, Nuaire USA).

Cell viability assay /MTT Assay

Cells were counted and assessed for viability. The cells were then grown in 96- well plates. Seeding density for the wells was 1.2 x 10^6 cells/ well. Cell viability was assessed using the MTT assay as described previously by Mosmann (1983) with slight modifications. Cells were plated in 96-well culture plates at density of 1 x 10^6 cells/well. After 24 hours incubation, the cells were treated with the extracts diluted with complete media at concentration of 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml, in triplicates. Blank and untreated wells were also included. The plates were incubated for 48 h at 37°C. Then, 10 µl of 3-(4-5 dimethylthiozol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT) (Sigma- Aldrich) prepared to a concentration of 5 mg/ml in phosphate buffer saline (PBS) (Sigma- Aldrich) was added to all the wells. The plates were incubated for 4 h at 37°C. Next, 100 µl DMSO (Sigma- Aldrich) was added to the wells to dissolve the formazan crystals. The plates were left at room temperature for four hours before being read on a microplate reader (NanoQuant infinite M200 Tecan Austria), using a wavelength of 570 nm and a reference wavelength of 630 nm.

Sample preparation

Seaweed extracts were weighed at 1 mg and dissolved completely in 1 % DMSO. The solution was vortexed to ensure proper dissolution of the extract. Then, complete DMEM was added gradually into the solution to 1 ml with vortexing between additions to allow complete dissolution. The extract from *S. oligocystum* was labeled SE, while from *P. australis* was labeled PE. The extracts were stored at 4°C away from light until ready to use.

Cell culture

ATCC mouse 3T3-L1 preadipocyte cells were obtained from Cell Culture Laboratory, Department of Pharmaceutical Technology, Kulliyyah of Pharmacy, IIUM. The cells were cultured in Dulbecco's modified Eagles medium (DMEM) (Sigma- Aldrich) with 10% fetal bovine serum (FBS) (10.000 u/ml) (Sigma-Aldrich) and 1% penicillin- streptomycin (10.000 ug/ml) (Sigma- Aldrich) at 37°C in an incubator with humidified atmosphere containing 5% CO_2 (Purecell TC, Nuaire USA).

Cell growth

Cells were counted and assessed for viability. The cells were then grown in 96- well plates. Seeding density for the wells was 1.2 x 10^6 cells/ well.

Cell viability assay /MTT Assay

Cell viability was assessed using the MTT assay as described previously by Mosmann (1983) with slight modifications.

Adipogenesis of 3T3-L1 cells and Oil red O staining

Adipogenesis assay was carried out using the components inside the Adipogenesis Assay Kit (item no. 10006908, Cayman Chemical Company, U.S.A). 48 h after cell confluence, differentiation was initiated using differentiation medium I containing IBMX, dexamethasone and insulin. In treatment groups, insulin was replaced with extracts at different final concentrations in the media (0, 12.5, 50 µg/ ml). After 48 h, the medium was replaced with complete DMEM. The media was changed every two days till day 14. On day 15, media was removed from the wells. Oil Red O staining protocol was carried out following kit guidelines. Stained cells were visualized using a microscope. After extracting dye from the cells, the stain was quantified by reading the absorbance at 490 nm with Infinite200 micro plate reader device fitted with Tecan i-control 1.6.19.2 software application (NanoQuant, Austria).

Glycerol release assay

Adipolysis assay was carried out using the components inside the Adipolysis Assay Kit (item no. 10009381, Cayman Chemical Company, U.S.A). 3T3-L1 cells were differentiated using a standard protocol for adipocyte differentiation described above. The media was changed every two days till day 14. On day 14, the media was replaced with complete media containing extracts at final concentrations of 0, 12.5, and 50 µg/ml, in triplicates. Isoproterenol solution was added into the positive control wells and DMEM to the negative control wells. The cells were incubated for another 48 hours. After 48 hours, 25 µl of cell culture supernatants were collected from each well and transferred into a new 96-well plate. 100 µl of diluted Free Glycerol Assay Reagent was added to the wells and mixed well, then incubated for 15 minutes. Absorbance was read at 540 nm using Infinite200 micro plate reader device fitted with Tecan i-control 1.6.19.2 software application.

Results and discussion

This study aimed to compare the adipogenesis and adipolysis activity of 3T3-L1 cells with and without the addition of *Sargassum oligocystum* (SE) and *Padina australis* extracts (PE).

Cell viability was assessed using the MTT assay as described previously by Mosmann (1983) with slight modifications. Seaweed extracts were not cytotoxic to the 3T3-L1 cells when applied for 24 and 48 h at concentrations selected (data not shown). Our results were in line with previous results showing seaweed compounds to not be toxic to the cells (Beppu *et al.*, 2009; Iio *et al*., 2010; Asai *et al.*, 2004).

The images of the cells on day 14 and oil- red O stained cells treated with insulin, PE and SE during the adipocyte differentiation stage are shown in Figure 1, 2 and 3 and compared against cells with no differentiation. Oil drops were observed in most of the cells in both control and treatment. Treatment with the seaweed extracts led to reduced adipogenesis in 3T3-L1 adipocytes compared to positive control. Cells treated with positive control insulin showed the absorbance of 0.237 A, after differentiation and lipid staining.

To determine the inhibitory effects of SE and PE extracts on adipocyte differentiation, 3T3-L1 preadipocytes were differentiated in the differentiation medium alone or differentiation medium with PE and SE (0 - 50 µg/ml) during adipocyte differentiation for 14 days. 3T3-L1 cells were fully differentiated by 14 days, and the accumulation of lipid droplets was visualized with a microscopic inspection and Oil Red

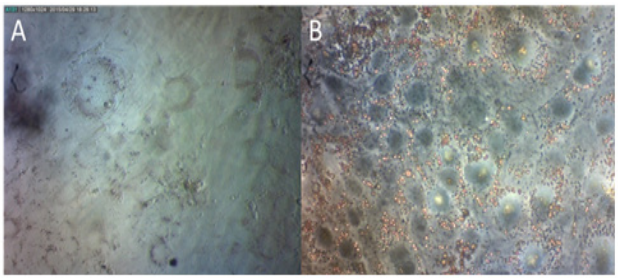

Figure 1. Photos of 3T3-L1 cells (A) cells with no treatment (insulin or extract) added did not form clearly visible adipocyte cells. Meanwhile, (B) shows cells stained with Oil Red O stain after given positive treatment with insulin.

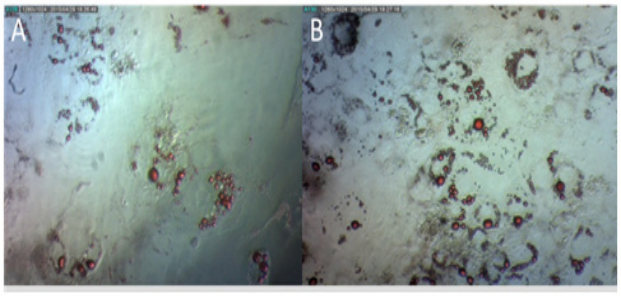

Figure 2. Photos of 3T3-L1 adipocytes on day 14 of post-confluence treated with (A) 12.5 µg/ml and (B) 50 µg/ml SE during the preadipocyte differentiation stage

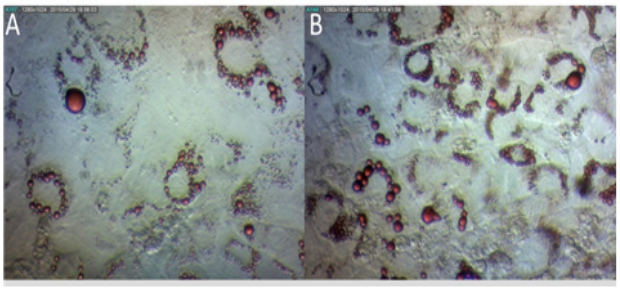

Figure 3. Photos of 3T3-L1 adipocytes on day 14 of post-confluence treated with (A) 12.5 µg/ml and (B) 50 µg/ml PE during the preadipocyte differentiation stage

O staining. The classic Oil Red O staining for lipid droplets was used as an indicator of the degree of adipogenesis, quantified in absorbance after the dye was extracted from the lipid droplet.

Cells treated with differentiation medium with insulin replaced with 0, 12.5, and 50 µg/ml SE showed 64.14%, 71.7%, and 84.8% adipogenesis, respectively, as compared with control as shown in Figure 4. Meanwhile, cells treated with differentiation medium with insulin replaced with 0, 12.5, and 50 µg/ml PE showed 64.14, 85.7%, and 89.0% adipogenesis, respectively, compared to control. While overall adipocyte differentiation was lower compared to control, increased dose of the seaweed extracts increased differentiation in cells treated with both extracts.

The images of the cells on day 14 and oil- red O stained cells treated with insulin, PE and SE during the adipocyte differentiation stage are shown in Figure 1, 2 and 3 and compared against cells with no differentiation. Oil drops were observed in most of the cells in both control and treatment.

Adipogenesis is the increase in mass of adipose tissue via increase in cell size, cell number, or both. It is implicated in the development of obesity (Sakai *et al*., 2006). It is sometimes referred to as adipocyte proliferation, and leads to increase in white adipose tissue mass, which characterizes the development of obesity (Sakai *et al*., 2006). Reduction in adipocyte proliferation inhibits the development of obesity-related insulin resistance (Sakai *et al.*, 2006).

One of the key strategies for the management of obesity is decreasing the adipocyte number via control of differentiation, or adipogenesis. Several studies report the ability of natural compounds to inhibit adipogenesis by controlling the transcription factors responsible for differentiation (Yun, 2010). The extracts were added into the media to test for effect against the adipogenesis of the 3T3-L1 cells. Inhibition of adipogenesis is a marker for antiobesity activity. The amount of inhibition can be quantified by the amount of dye uptake into the lipid cells using the Oil Red O staining method. The Oil Red O dye allows for easier observation of the cells.

These results indicated that seaweed extracts SE and PE inhibited cell differentiation in 3T3-L1 cells compared to control, although adipogenesis increased with increased concentration of extract. Both extracts did not exert cytotoxicity during 3T3-L1 differentiation. The results suggest that brown seaweeds might effectively inhibit adipocyte differentiation in 3T3-L1 adipocytes when applied in low doses.

Regulation of adipolysis as strategy for the management of obesity

Adipolysis (also known as lipolysis) is the process of triglyceride mobilization into free fatty acids (FFA) and glycerol (Duncan *et al.*, 2007). Mechanisms regulating lipolysis are closely related to obesity, insulin resistance, dyslipidemia and diabetes (Carmen and Victor, 2006). Targeting the mature lipid cells via stimulation of adipolysis may be an important strategy to reduce fat stores in the body (Langin, 2006; Rayalam *et al.*, 2008).

To determine the stimulatory effects of SE and PE extracts on glycerol release in mature 3T3-L1 cells, mature 3T3-L1 adipocytes were incubated in medium alone or medium containing isoproterenol or medium containing the extracts (0 – 50 µg/ml) for 48 h. After 48 h, lipid metabolism and the release of

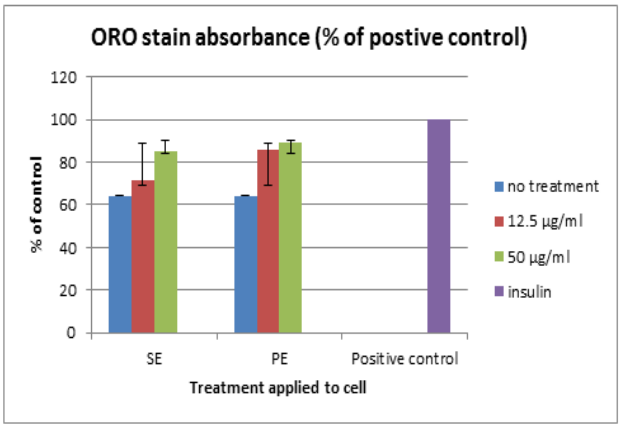

Figure 4. Mean ORO absorbance percentage, out of control. Treatment with insulin positive control was considered to result in 100% adipogenesis; absorbance was 0.237 A. SE showed higher adipogenesis-inhibiting results with lower lipid accumulation (71.7, 84.8%) compared to PE (85.7, 89%). Adipogenesis increased in both extracts with increased dose.

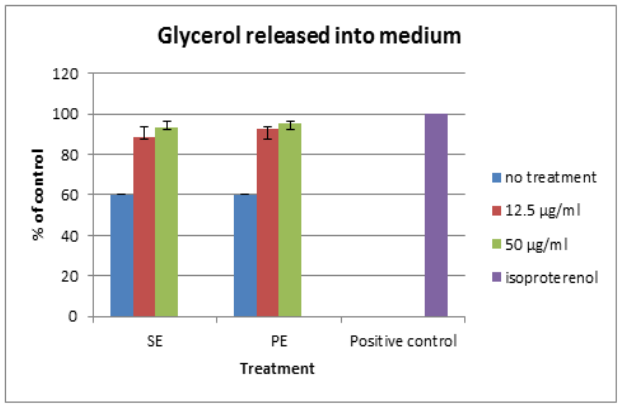

Figure 5. Mean glycerol released into the medium from cells, as a percentage out of control. Treatment with isoproterenol positive control was considered to result in 100% adipolysis; absorbance was 0.237 A. PE showed higher adipolysis- stimulating results (92.9, 95.6%) compared to SE (88.6, 93.0%). Adipolysis increased in both extracts with increased dose

glycerol and FFAs was observed. Glycerol release into the media was quantified.

Treatment of mature 3T3-L1 adipocytes with SE and PE led to increased glycerol release from the lipid cells, as shown in Figure 5. Cells treated with positive control isoproterenol released 31.96 ± 21.01 µg/ml glycerol into the media. In cells treated with 0, 12.5 and 50 µg/ml SE and PE, glycerol released was 39.89, 88.58, and 92.99%, and 39.89, 92.7, and 95.56%, respectively, compared to the control. The results show that brown seaweed extracts exert pro-adipolytic activity through metabolism of the lipids in the cell into glycerol and triglycerides. The results also suggest that SE and PE effectively promote adipolysis in a dose- dependent manner. Treatment with SE showed higher amounts of glycerol release compared to PE.

Fucoxanthin- rich extracts of the brown seaweeds *Undaria pinnatifida* and *Petalonia binghamiae* have been shown to increase lipid metabolism *in vivo* (Maeda *et al*., 2005, 2009; Kang *et al*., 2012). However, as far as the authors' knowledge, this is the first report of brown seaweed extracts stimulating adipolysis *in vitro*. Plant foodstuffs that stimulate adipolysis of 3T3-L1 cells were reported by Niwano *et al.* (2008) but did not include brown seaweeds. Studies on adipolysis in vitro have focused on isolated compounds from brown seaweeds fucoidan and fucoxanthin.

Exploring new uses for brown seaweeds is important for Malaysia as a means to add value to existing resources and capitalize on the benefits of local plants. The anti-obesity attributes of brown seaweed extracts will allow it to be used as a functional food and may be incorporated as an important part of the diet.

Conclusion

The two brown seaweeds *Sargassum oligocystum* and *Padina australis* showed interesting anti-adipogenic and pro- adipolytic activity on in vitro model for obesity. Treatment with SE was more effective compared to PE for the inhibition of adipogenesis, while PE was more effective for the stimulation of adipolysis compared to SE. Isolation of the active compounds and elucidation of the cellular and molecular mechanisms underlying the action of the brown seaweed extracts would be the next step to further increase our understanding of the anti-obesity activity of whole brown seaweeds. Additionally, in vivo studies would help further identify the effectiveness of the extracts. This is the first such study describing the pro- adipolytic activity of brown seaweed extracts via stimulation of lipid metabolism *in vitro*. The study points out the benefits of using whole brown seaweed extracts in future anti-obesity remedies.

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