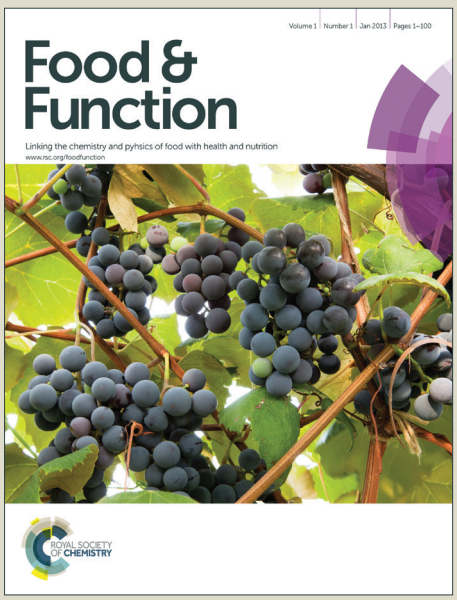
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

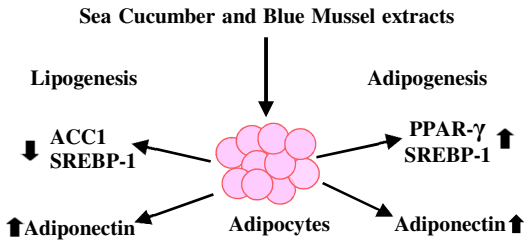
ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Sea Cucumber and Blue Mussel: A new source of phospholipid enriched omega-3 fatty acids with a potential role in 3T3-L1 adipocyte metabolism

Hitesh Vaidya and Sukhinder K Cheema

Sea Cucumber and Blue Mussel methanolic extracts are enriched in n-3 polyunsaturated fatty acids structured in phospholipids, which increases adipogenesis and decreases lipogenesis in 3T3-L1 adipocytes.



Sea Cucumber and Blue Mussel: A new source of phospholipid enriched omega-3 fatty acids with a potential role in 3T3-L1 adipocyte metabolism

Hitesh Vaidya and Sukhinder K Cheema

Affiliation

Department of Biochemistry, Memorial University, St. John's, NL A1B3X9, Canada

Address for Correspondence

Sukhinder Kaur Cheema, PhD
Professor, Department of Biochemistry,
Memorial University, St. John's, NL, A1B3X9, Canada
Tel # 709-864-3987
Fax # 709-864-2422
Email: skaur@mun.ca

Abstract

Omega (n)-3 polyunsaturated fatty acids (PUFA), namely docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are known to reduce the risk of insulin resistance, and ameliorate obesity-associated disorders. DHA and EPA structured in phospholipids form possess superior biological effects compared to triglyceride form available in fish oil. In this study, we have identified that Sea Cucumber (SC) and Blue Mussel (BM) from Newfoundland and Labrador are a rich source of n-3 PUFA structured in phospholipid form. Treatment with SC and BM methanolic extracts (250 and 100μg/mL, respectively) significantly ($p<0.01$) increased triglyceride accumulation in 3T3-L1 adipocytes, along with an increase in the mRNA expression of peroxisome proliferator-activated receptor-γ (37 and 39%, respectively) and adiponectin (57 and 56%, respectively) compared to control cells ($p<0.05$). Only SC extract (250μg/mL) increased the mRNA expression of sterol regulatory element-binding protein-1 (SREBP-1). Treatment with higher concentrations of SC and BM extracts (500 and 750μg/mL, respectively) significantly ($p<0.01$) decreased triglyceride accumulation in 3T3-L1 cells as opposed to an increase in triglyceride accumulation at the lower concentrations. This was due to inhibition of acetyl-CoA carboxylase-1 and SREBP-1 mRNA expression compared to control cells ($p<0.05$). There was no effect of the extracts on the mRNA expression of hormone sensitive lipase or lipolysis, suggesting that the decrease in triglyceride accumulation at higher concentrations is not due to breakdown and release of fat. This is the first report to show that SC and BM are a new source of phospholipid bonded n-3 PUFA, with the potential to target insulin resistance and obesity.

Keywords: Obesity, Insulin resistance, Sea Cucumber, Blue Mussel, N-3 PUFA

Introduction

Obesity is associated with type 2 diabetes and insulin resistance leading to an increase in the production of insulin thereby causing hyperinsulinemic state.[1] Though adipose tissue is the primary organ for fat storage, adipocytes also secret potent adipokines, which include hormones, cytokines and other proteins with specific biological functions.[2] Thus, the development and maintenance of adipocytes is critical for their proper function. The conversion of preadipocytes to mature, fat laden adipocytes, known as adipogenesis; is strictly regulated in animals and humans.[3] It is a multistep process characterized by a sequence of events during which adipoblasts divide until confluent and become mature adipocytes. Peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein-α (C/EBP-α) are the decisive transcriptional factors that play an important role during adipogenesis.[4, 5] These proteins works cooperatively during adipogenesis, and regulate the expression of other adipocyte specific genes.[5, 6]

Several drugs, which decrease insulin resistance have been shown to increase adipogenesis in adipocytes via up regulation of PPAR-γ and adiponectin mRNA expression in obese patients.[7] Marine sources such as fish and fish oil are also gaining attention due to their beneficial effects under diabetic and obese conditions.[8, 9] The beneficial effects of marine sources are mostly due to omega (n)-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[9] The preventive effects of n-3 PUFA against obesity are generally mediated by increasing resting energy expenditure, increasing fat oxidation, and suppressing appetite[10] due to alterations in the expression of genes involved in adipogenesis and lipogenesis.

Besides fish and fish oil, other marine sources such as Sea Cucumber and Blue Mussel are also a rich source of n-3 PUFA.[11, 12] These sources have recently been shown to possess anti-inflammatory, anti-arthritic and lipid lowering effects.[13-15] A recent study has shown that feeding EPA-enriched phosphatidylcholine fraction isolated from Sea Cucumber improved glucose homeostasis by increasing insulin production in streptozotocin induced diabetic rats.[16] Others have also shown that feeding saponins fraction isolated from Sea Cucumber decreased serum triglycerides and glucose concentration in high-fat diet induced obese mouse.[17] Olivera et al[15] also found that Sea Cucumber meals decreased serum triglycerides, cholesterol and low density lipoprotein concentrations in Wistar rats.

Atlantic Ocean with its cold waters is home to Sea Cucumber and Blue Mussel. Studies have shown that cold temperature modifies the lipid composition of marine organisms;[18] however, there is no information on the lipid composition and fatty acid profile of Sea Cucumber and Blue Mussel from the Great Northern Peninsula of the Canadian province of Newfoundland and Labrador (NL). We hypothesized that lipid composition of Sea Cucumber and Blue Mussel from the Northern Peninsula of the Canadian province of NL will have a unique lipid composition. We further hypothesized that methanolic extracts of Sea Cucumber and Blue Mussel will have beneficial effects in insulin resistance and obese condition. Our findings shows for the first time that Sea Cucumber and Blue Mussel from NL are a newer marine source of phospholipid- DHA and EPA, that has a significant effect on the regulation of adipose tissue metabolism in 3T3-L1 cells. Our findings suggest that Sea Cucumber and Blue Mussel has the potential to target obesity and insulin resistance.

Material and methods

Sea Cucumber and Blue Mussel extracts

Sea Cucumber and Blue Mussel freeze dried powders were provided by St. Anthony Basin Resources Inc, NL, Canada. Different solvents were used for making Sea Cucumber and Blue Mussel extracts based on the polarity index; methanolic extract was the best compared to chloroform, choloroform:methanol (2:1), methanol:water (2:1) and phosphate buffer saline (PBS). For the extractions, Sea Cucumber and Blue Mussel freeze dried powders were mixed with methanol and stirred overnight at room temperature. Methanolic extracts were vacuum filtered in acid washed glass tubes and evaporated to dryness under nitrogen gas and stored at -20˚C till further analysis.

Lipid composition and fatty acids analysis

Total lipids were separated from the methanolic extract of Blue Mussel and Sea Cucumber by thin layer chromatography (TLC) and identified with coupled flame ionization detector using Iatroscan, followed by multistep development scheme.[19] Phospholipids were separated on TLC plates using hexane: ethyl ether: acetic acid (70:30:2 v/v).[20] Fatty acid analysis of total lipids and phospholipids was determined using gas liquid chromatography as per our previously published method.[21] Data presented only represents one analysis.

Treatment of 3T3-L1 cells

3T3-L1 preadipocytes were obtained from American Type Culture Collection (ATCC # CL-173, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) containing 10% calf serum (Gibco, USA) in a 5% CO_2, humidified environment at 37˚C.

Differentiation of confluent preadipocytes culture (day 0) was induced using DMEM containing 10% fetal bovine serum (FBS), 0.5 mmol/l 3-isobutyl-1-methylxanthine, 1µM insulin, and 1µM dexamethasone in the presence or absence of extracts. A dose response curve was initially established for both Sea Cucumber and Blue Mussel extracts using concentrations range from 25-1000µg/ml of the extract. Sea Cucumber and Blue Mussel extracts were reconstituted in dimethyl sulfoxide (DMSO) and added to the cells; control cells received DMSO. The final concentration of DMSO on cells was less than 0.1%. After 48h, the medium was changed to DMEM containing 10% FBS and 1µM insulin along with the treatments; medium was replaced to maintenance media (DMEM + 10% FBS) every 48h till day 8. On the 8th day, fully differentiated adipocytes were washed with 1X phosphate buffer saline (PBS) and used either for triglyceride accumulation assay or for total RNA extraction for gene expression analysis.

Cell viability measurements

Cell viability was measured using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay with modifications[22] in pre-adipocytes as well as in mature adipocytes treated with different concentrations of Sea Cucumber or Blue Mussel (25-1000µg/ml) for 8 days. For MTT assay in pre-adipocytes, cells were grown in 24 well plates (50,000 cells/well) and incubated for 24h with or without different concentrations of Sea Cucumber or Blue Mussel (25-1000µg/ml). Media was replaced with fresh medium after 24h and 20µl of MTT (5mg/ml) reagent was added to each well and incubated for 2h at 37°C. Media containing MTT reagent was then removed and formazan crystals were solubilized in 250µl of DMSO to measure the coloured solution.

To study the effect of Sea Cucumber and Blue Mussel extracts on cell viability of the mature adipocytes, cells were differentiated and treated with or without different concentrations of Sea Cucumber or Blue Mussel (25-1000µg/ml) for 8 days; medium was replaced every 48h till day 8. On day 8, media was replaced with fresh medium and 20µl of MTT (5mg/ml) was added to each well and incubated for 2h at 37°C. Media containing MTT reagent was then removed and formazan crystals were solubilized in 250µl of DMSO. The coloured solution was quantified at 570nm and cell viability was expressed as a percentage over control.

Triglyceride accumulation assay

Cellular triglyceride accumulation in adipocytes was measured using commercially available triglyceride assay kit (Kit # 236-60, Genzyme, Canada). Briefly, cells were washed twice with 1X PBS, total fat was extracted using Folch method[23] and triglycerides were measured using triglyceride assay kit as per our previously published method.[24]

Lipolysis assay

Lipolysis was assayed by measuring the amount of non-esterified free fatty acids (NEFA) released in the growth media. Fully differentiated adipocytes (Day 8) were treated with Sea Cucumber and Blue Mussel extracts in fatty acid free medium for 24h and NEFAs were measured in the growth medium using NEFA kit (Kit # 999-34691, Wako chemicals, USA). Protein concentration of cell lysate was measured using Lowry method.[25] Data was expressed as mEq of NEFA per mg of protein.

Total RNA isolation and gene expression analysis

Total RNA was isolated from the cells using TriZol method (Invitrogen, USA) and integrity of RNA was confirmed. Multiplex reverse transcription (RT)-polymerase chain reaction (PCR) was

performed using β-actin as the housekeeping gene as per our previous publication.[24] Mouse primer sequences for PPAR-γ, adiponectin, acetyl-CoA carboxylase 1 (ACC1), hormone sensitive lipase (HSL) and sterol regulatory element-binding protein-1 (SREBP-1), glucose trasporter-4 (GLUT-4), PPAR-α and β-actin are given in Table 1. The RT-PCR products were analyzed using Chemi-Imager 4400, normalized to β-actin as the house keeping gene and expressed as arbitrary units.

Statistical analysis

The results were analyzed using One-way analysis of variance (ANOVA) and Dunnett's multiple tests to compare treatment groups with control. A value of $p<0.05$ was considered to be significant. The results were expressed as mean ± SD. The statistical analysis was carried out by using Graph Pad 5.0 software.

Results

Sea Cucumber and Blue Mussel are rich in phospholipids

Different classes of lipids were analyzed in Sea Cucumber and Blue Mussel extracts; namely, hydrocarbon, steryl esters/wax esters, ethyl ketones, methyl ketones, triacylglycerol, free fatty acids, alcohols, sterols, acetone mobile polar lipids and phospholipids (Table 2). It was interesting to note that phospholipids constituted majority of the lipids of Sea Cucumber and Blue Mussel extracts, containing 75.59 and 84.12% of total fat, respectively (Table 2). On the other hand, triacylglycerols were only 2.2% in Sea Cucumber and not detected in Blue Mussel.

Sea Cucumber and Blue Mussel are high in n-3 PUFA

Sea Cucumber and Blue Mussel extracts contained 38% and 56% PUFA of total fatty acids; of which 28% and 49% were n-3 PUFA, respectively. Blue Mussel extract was rich in DHA (21%) compared to Sea Cucumber (0.6%), while Sea Cucumber extract was rich in EPA (25%) compared to Blue Mussel (20%) (Table 3). Sea Cucumber phospholipids fraction contained 57% total PUFA, with 44% EPA and 3.9% DHA, while Blue Mussel phospholipids contained 41% total PUFA, with 20% EPA and 11% of DHA (Table 4). The total n-3 PUFA content of Sea Cucumber and Blue Mussel phospholipids was 50% and 34% with an n-6/n-3 PUFA ratio of 0.14 and 0.22, respectively (Table 4).

Dose dependent effect of Sea Cucumber and Blue Mussel on cell viability and adipogenesis

Treatment of 3T3-L1 preadipocytes with Sea Cucumber and Blue Mussel extracts in the range of 25-1000µg/ml had no effect on cell viability after 24h (Figure 1A and B, respectively) and after day 8 (Figure 1C and D, respectively), suggesting that the extracts had no toxic effects.

Sea Cucumber significantly ($p<0.001$) increased triglyceride accumulation up to a concentration of 250µg/ml compared to control cells, however higher concentrations of Sea Cucumber (500, 750 and 1000µg/ml) dramatically inhibited triglyceride accumulation (Figure 2A). On the other hand, Blue Mussel significantly ($p<0.001$) increased triglyceride accumulation at 50 and 100 µg/ml compared to control cells, while higher concentrations (500, 750 and 1000µg/ml) significantly ($p<0.001$) inhibited triglyceride accumulation in adipocytes compared to control cells (Figure 2B).

A lower concentration of Sea Cucumber and Blue Mussel extracts increased adipogenesis

Since lower concentrations of Sea Cucumber and Blue Mussel (250 and 100μg/ml, respectively) significantly increased triglyceride accumulation in adipocytes (Figure 2A and B) compared to control cells, we measured the gene expression of adipogenic genes (PPAR-γ, Adiponectin, GLUT-4 and SREBP-1). Treatment with Sea Cucumber and Blue Mussel extracts showed a significant increase in PPAR-γ (37 and 39%, respectively) (Figure 3A) and adiponectin (Figure 3B) mRNA expression (57 and 56%, respectively) compared to control cells. Treatment with Sea Cucumber showed 38% increase in the mRNA expression of SREBP-1; surprisingly Blue Mussel extract had no effect on SREBP-1 gene expression (Figure 3C). Treatment with Sea Cucumber and Blue Mussel extracts had no effect on GLUT-4 mRNA expression (data not shown).

Higher concentrations of Sea Cucumber and Blue Mussel extracts decreased lipogenesis and triglyceride accumulation

Since both Sea Cucumber and Blue Mussel methanolic extracts at higher concentrations (500 and 750 μg/ml, respectively) significantly decreased triglyceride accumulation compared to control cells, we investigated whether higher concentrations of Sea Cucumber and Blue Mussel inhibit lipogenesis and/or induced lipolysis. Treatment with a higher concentration of Sea Cucumber and Blue Mussel methanolic extracts inhibited ACC1 mRNA expression compared to control cells (Figure 4A). Additionally, Sea Cucumber decreased the mRNA expression of SREBP-1 (Figure 4B), and increased adiponectin mRNA expression by 60% compared to control cells (Figure 4C), while Blue Mussel extract had no effect. Treatment with both Sea Cucumber and Blue Mussel methanolic extracts had no effects on the mRNA expression of HSL

(Figure 4D) compared to control cells. Furthermore, there was no effect of Blue Mussel or Sea Cucumber methanolic extracts on lipolysis as identified by the release of non-esterified fatty acids (NEFA) in the culture medium (Figure 4E). The mRNA expression of PPAR-α was also not altered after treatment with Blue Mussel or Sea Cucumber (data not shown).

Discussion

N-3 PUFA contained in fish and fish oil are well known to elicit health benefits,[9, 26, 27] however recent studies have shown that supplementation of n-3 PUFA structured in phospholipids form exert stronger biological effects compared with the triglycerides form found in fish and fish oil.[28] Phospholipids form has been shown to augment the bioavailability of DHA and EPA in both rodents[29] and humans.[30] Krill oil is the best example where n-3 PUFA are structured in phospholipids form;[31] supplementation of Krill oil showed superior effects to modulate inflammation as well as inhibiting fat accumulation in rodents[32-34] compared to fish oil. Previous studies have shown that Blue Mussel (*Mytilus edulis)*[19, 35] and Sea Cucumber (*Cucumaria frondosa)*[11] from Newfoundland and Labrador are a rich source of n-3 PUFA. However, it is not known whether n-3 PUFA of Blue Mussel and Sea Cucumber are in triglyceride or phospholipids form. We are reporting for the very first time that Sea Cucumber and Blue Mussel from the Great Northern Peninsula of the Canadian province of Newfoundland and Labrador are a rich source of n-3 PUFA structured in phospholipids form.

We have found that Sea Cucumber contained 75.59% of total lipids as phospholipids, while Blue Mussel contained 84.12% of the total lipids as phospholipids. Alkanani et al.,[35] also found a similar amount of phospholipids in Blue Mussels from NL, however there is no data available for phospholipids content of Sea Cucumber from NL. We are the first to show that Sea Cucumber is a rich source of EPA structured in phospholipids form; however Sea Cucumber is not a rich

source of DHA. It was also interesting to find that phospholipids-EPA of Blue Mussel was lower compared to Sea Cucumber, however Blue Mussels phospholipids-DHA was higher compared to Sea Cucumber. It has been previously reported that Blue Mussels from New Zealand contained 13% EPA and 21% DHA of total lipids,[12] however Blue Mussels from NL contained 20% EPA and 20.7% DHA demonstrating that Blue Mussels from Newfoundland has a better nutritional value compared to the New Zealand variety. Furthermore, n-3 PUFA in Blue Mussel and Sea Cucumber from NL are structured in phospholipids form. Studies have shown that cold temperature modifies the lipid composition of marine organisms,[18] thus the higher content of EPA and DHA, in both total lipids and phospholipids of Blue Mussel and Sea Cucumber from NL is most likely due to the colder temperatures of Atlantic Ocean surrounding Newfoundland. Krill oil has been shown to contain 18.5% EPA and 9% DHA of total lipids,[36] whereas our findings showed that Blue Mussel contained 20% EPA and 20.65% DHA of total lipids, thus Blue Mussel is a richer source of these fatty acids compared to Krill oil. The phospholipids-EPA and –DHA content of Krill oil has been reported to be 28% and 13% respectively.[36] The phospholipids-EPA and –DHA content of Blue Mussel in our study was found to be 20% and 11% respectively, whereas Sea Cucumber phospholipids-EPA was 44.4%. Thus, cold water Blue Mussel and Sea Cucumber from Newfoundland are compatible with Krill oil as a new source of n-3 PUFA structured in phospholipids form.

We investigated whether these extracts will affect triglyceride accumulation in 3T3-L1 cells. There was a dose dependent increase in triglyceride accumulation with both Sea Cucumber and Blue Mussel extracts, where Sea Cucumber at a concentration of 250µg/ml showed the highest triglyceride accumulation, while a lower concentration of Blue Mussel (100µg/ml) induced the highest triglyceride accumulation. These differences in the concentrations of Blue Mussel and

Sea Cucumber on fat accumulation is likely due to differences in their fatty acid composition; Sea Cucumber contained very high amounts of EPA, whereas Blue Mussel contained high amounts of both EPA and DHA. It has previously been shown that a combination of DHA and EPA has a stronger biological effect compared to EPA alone.[37-39]

Triglyceride accumulation is an indicator of adipogenesis, thus we investigated if treatment with Sea Cucumber and Blue Mussel extracts altered the expression of genes involved in adipogenesis. PPAR-γ is a nuclear receptor and a key gene involved in adipogenesis.[40] Treatment with Sea Cucumber and Blue Mussel extracts at a concentration of 250 and 100μg/ml, respectively increased 37 and 39% PPAR-γ gene expression compared to control cells, which corresponds to an increase in fat accumulation in 3T3-L1 cells. Treatment with Sea Cucumber also showed 38% increase in the mRNA expression of SREBP-1, a transcription factor that regulates the mRNA expression of PPAR-γ. Others have also shown that expression of SREBP-1 and PPAR-γ increased concomitantly during differentiation to control adipogenesis.[41] Treatment with Blue Mussel showed no change in SREBP-1 mRNA expression, however there was an increase in PPAR-γ mRNA expression, suggesting that Blue Mussel extract has a direct effect on PPAR-γ. Others have shown that fatty acids act as a direct ligand for PPAR-γ expression.[42] Long chain n-3 PUFA are known to increase the mRNA expression of PPAR-γ,[42] however it was interesting to note in our study that the effect of the extracts was dose dependent. A similar type of dose dependent effect of arachidonic acid has been reported, where lower concentrations increased PPAR-γ mRNA expression in 3T3-L1 adipocytes, while higher concentrations decreased PPAR-γ mRNA expression.[43] Adipose tissue PPAR-γ gene also regulates production and secretion of adipokines such as adiponectin and leptin, important mediators of insulin action on peripheral tissues.[44] Moreover, adiponectin mRNA expression is dramatically induced during

adipogenesis process in the presence of PPAR-γ agonist.[45] Treatment with both Sea Cucumber and Blue Mussel extracts significantly up regulated adiponectin mRNA expression (57 and 56%, respectively) compared to the control cells. In a recent study, Krill oil supplementation in the diets of rodents caused an increase in the mRNA expression of adiponectin and also plasma adiponectin concentrations.[46] Our findings demonstrate that Sea Cucumber and Blue Mussel extracts significantly increase adipogenesis via up-regulating adipogenic genes, thus implicating the importance of Blue Mussel and Sea Cucumber in targeting insulin resistance.

Although Blue Mussel and Sea Cucumber increased triglyceride accumulation and the expression of genes involved in adipogenesis at the lower concentrations, the higher concentrations caused a dramatic decrease in triglyceride accumulation. A decrease in triglyceride accumulation may be due to inhibition of lipogenesis or an increase in lipolysis. We measured the effects of both Sea Cucumber and Blue Mussel extracts at the higher concentrations (500 and 750μg/ml, respectively) on lipolysis and lipogenesis in 3T3-L1 cells. The mRNA expression of ACC1, the rate limiting enzyme for de-novo fat synthesis [47], was inhibited by Sea Cucumber and Blue Mussel extracts at the higher concentration compared to control cells. Furthermore, treatment with Sea Cucumber decreased the mRNA expression of SREBP-1 in adipocytes suggesting that inhibition of ACC1 is likely regulated by SREBP-1. Similar findings have been reported after supplementing with Krill oil in rodents that inhibited ACC-1 and SREBP-1 mRNA expression.[34, 46, 48] Surprisingly, treatment with Blue Mussel extract did not change the mRNA expression of SREBP-1; Sea Cucumber is rich in EPA while Blue Mussel is rich in DHA. Studies have shown that treatment with EPA decreased hepatic SREBP-1 expression in C57BL/6J mice,[49] however DHA supplementation significantly decreased mRNA expression of ACC-1, with no effect on SREBP-1 gene expression.[50] Thus, the

differential effect of Sea Cucumber and Blue Mussel on SREBP-1 gene expression is likely due to differences in the n-3 PUFA composition of these extracts. Treatment with Sea Cucumber extract also showed an increase in the mRNA expression of adiponectin, however, there was no effect of Sea Cucumber and Blue Mussel extracts on the mRNA expression of PPAR-γ at the higher concentrations (data not shown). Our findings suggest that the inhibition of ACC1 and an increase in adiponectin mRNA expression is independent of PPAR-γ; however the mechanisms need to be further investigated. We also investigated whether inhibition of triglyceride accumulation by higher concentrations of Sea Cucumber and Blue Mussel extracts is due to an increase in β-oxidation or an increase in lipolysis. An up-regulation of PPAR-α gene has been shown to increase fatty acid release from the adipocytes followed by β-oxidation.[51] There was no effect of Blue Mussel or Sea Cucumber on the mRNA expression of PPAR-α or NEFA concentrations in the medium, suggesting that a decrease in fat accumulation is not due to an increase in lipolysis or β-oxidation. We also measured the mRNA expression of HSL, an enzyme responsible for the release of free fatty acids from adipose tissue.[52] There was no change in the HSL mRNA expression, confirming that these extracts had no effect on lipolysis. Although inhibition of ACC1 and SREBP-1 will account for a decrease in triglyceride accumulation in 3T3-L1 cells, it does not explain the dramatic inhibition of fat accumulation. Thus, a potent lipid lowering effect of Sea Cucumber and Blue Mussel extracts may involve other molecular targets apart from ACC1 and SREBP-1 to be investigated in our future studies.

Overall, findings from this study established that Sea Cucumber and Blue Mussel are new marine sources of n-3 PUFA structured in phospholipids. Our studies also demonstrate that Sea Cucumber and Blue Mussel methanolic extracts have differential effects on fat accumulation when used at higher or lower concentrations. At lower concentrations, these extracts increased

adipogenesis, reflecting their potential to improve insulin resistance. Furthermore, Sea Cucumber and Blue Mussel methanolic extracts, when used at higher concentrations, inhibited triglyceride accumulation suggesting their potential role in obesity. These effects of Sea Cucumber and Blue Mussel extracts on adipogenesis and lipogenesis are comparable to those reported for Krill oil, where n-3 PUFA are also structured in phospholipids form. Thus, we are the first to report that Sea Cucumber and Blue Mussel are a rich source of n-3 PUFA structured in phospholipids, and has the potential to target insulin resistance and adipose tissue metabolism.

Acknowledgment: We would like to acknowledge the financial support from St. Anthony Basin Resource Inc. (SABRI), Newfoundland and Labrador, Canada, for this research. We would also like to thank SABRI for providing Blue Mussel and Sea Cucumber powders.

References

1. J. Steinberger and S. R. Daniels, *Circulation*, 2003, **107**, 1448-1453.
2. R. F. Morrison and S. R. Farmer, *J Nutr*, 2000, **130**, 3116S-3121S.
3. B. M. Spiegelman, *Diabetes*, 2013, **62**, 1774-1782.
4. E. D. Rosen and O. A. MacDougald, *Nat Rev Mol Cell Biol*, 2006, **7**, 885-896.
5. E. D. Rosen and B. M. Spiegelman, *Annu Rev Cell Dev Biol*, 2000, **16**, 145-171.
6. E. D. Rosen, C. J. Walkey, P. Puigserver and B. M. Spiegelman, *Genes Dev*, 2000, **14**, 1293-1307.
7. P. F. Pilch and N. Bergenhem, *Molecular pharmacology*, 2006, **70**, 779-785.
8. E. K. Lund, *Food Chem*, 2013, **140**, 413-420.
9. J. Oehlenschlager, *Int J Vitam Nutr Res*, 2012, **82**, 168-176.
10. K.-H. Kim and Y. Park, *Annual Review of Food Science and Technology*, 2011, **2**, 237-257.
11. Y. Zhong, M. A. Khan and F. Shahidi, *J Agric Food Chem*, 2007, **55**, 1188-1192.
12. K. Murphy, B. Mooney, N. Mann, P. Nichols and A. Sinclair, *Lipids*, 2002, **37**, 587-595.
13. B. D. Fredalina, B. H. Ridzwan, A. A. Abidin, M. A. Kaswandi, H. Zaiton, I. Zali, P. Kittakoop and A. M. Jais, *Gen Pharmacol*, 1999, **33**, 337-340.
14. U. Grienke, J. Silke and D. Tasdemir, *Food Chem*, 2014, **142**, 48-60.
15. L. Olivera-Castillo, A. Davalos, G. Grant, N. Valadez-Gonzalez, J. Montero, H. A. Barrera-Perez, Y. Chim-Chi, M. A. Olvera-Novoa, V. Ceja-Moreno, P. Acereto-Escoffie, J. Rubio-Pina and R. Rodriguez-Canul, *PLoS One*, 2013, **8**, e79446.
16. S. Hu, L. Xu, D. Shi, J. Wang, Y. Wang, Q. Lou and C. Xue, *Journal of bioscience and bioengineering*, 2013.
17. X. Hu, Z. Li, Y. Xue, J. Xu, C. Xue, J. Wang and Y. Wang, *J Med Food*, 2012, **15**, 909-916.
18. T. Farkas and S. Herodek, *J Lipid Res*, 1964, **5**, 369-373.
19. A. Both, C. C. Parrish, R. W. Penney and R. J. Thompson, *Aquatic Living Resources*, 2011, **24**, 295-301.
20. K. A. Balogun and S. K. Cheema, *Neurochemistry International*.
21. K. Chechi, G. R. Herzberg and S. K. Cheema, *Prostaglandins Leukot Essent Fatty Acids*, 2010, **83**, 97-104.
22. D. C. Marks, L. Belov, M. W. Davey, R. A. Davey and A. D. Kidman, *Leuk Res*, 1992, **16**, 1165-1173.
23. J. Folch, M. Lees and G. H. Sloane Stanley, *J Biol Chem*, 1957, **226**, 497-509.
24. H. Vaidya, R. K. Goyal and S. K. Cheema, *Phytother Res*, 2013, **27**, 624-627.
25. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J Biol Chem*, 1951, **193**, 265-275.
26. T. Nicholson, H. Khademi and M. H. Moghadasian, *Food & function*, 2013, **4**, 357-365.
27. N. Siriwardhana, N. S. Kalupahana and N. Moustaid-Moussa, *Advances in food and nutrition research*, 2012, **65**, 211-222.
28. M. Rossmeisl, Z. M. Jilkova, O. Kuda, T. Jelenik, D. Medrikova, B. Stankova, B. Kristinsson, G. G. Haraldsson, H. Svensen, I. Stoknes, P. Sjovall, Y. Magnusson, M. G. Balvers, K. C. Verhoeckx, E. Tvrzicka, M. Bryhn and J. Kopecky, *PLoS One*, 2012, **7**, e38834.
29. M. Cansell, F. Nacka and N. Combe, *Lipids*, 2003, **38**, 551-559.
30. K. C. Maki, M. S. Reeves, M. Farmer, M. Griinari, K. Berge, H. Vik, R. Hubacher and T. M. Rains, *Nutrition research*, 2009, **29**, 609-615.
31. J. M. Kwantes and O. Grundmann, *Journal of dietary supplements*, 2014.
32. V. R. Ramprasath, I. Eyal, S. Zchut and P. J. Jones, *Lipids Health Dis*, 2013, **12**, 178.
33. N. F. Vigerust, B. Bjorndal, P. Bohov, T. Brattelid, A. Svardal and R. K. Berge, *Eur J Nutr*, 2013, **52**, 1315-1325.
34. A. Ferramosca, L. Conte and V. Zara, *Journal of Animal Physiology and Animal Nutrition*, 2012, **96**, 295-306.

35. T. Alkanani, C. C. Parrish, R. J. Thompson and C. H. McKenzie, *Journal of Experimental Marine Biology and Ecology*, 2007, **348**, 33-45.
36. P. Araujo, H. Zhu, J. Breivik, J. Hjelle and Y. Zeng, *Lipids*, 2014, **49**, 163-172.
37. L. J. Schwellenbach, K. L. Olson, K. J. McConnell, R. S. Stolcpart, J. D. Nash, J. A. Merenich and G. Clinical Pharmacy Cardiac Risk Service Study, *Journal of the American College of Nutrition*, 2006, **25**, 480-485.
38. R. C. Block, L. Kakinami, M. Jonovich, I. Antonetti, P. Lawrence, N. Meednu, P. CalderonArtero, S. A. Mousa, J. T. Brenna and S. Georas, *Prostaglandins Leukot Essent Fatty Acids*, 2012, **87**, 143-151.
39. E. M. Tillman, R. A. Helms and D. D. Black, *JPEN. Journal of parenteral and enteral nutrition*, 2012, **36**, 36-42.
40. M. Gurnell, *Clinical endocrinology*, 2003, **59**, 267-277.
41. L. Madsen, R. K. Petersen and K. Kristiansen, *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 2005, **1740**, 266-286.
42. A. Georgiadi and S. Kersten, *Advances in Nutrition: An International Review Journal*, 2012, **3**, 127-134.
43. Evanthia Nikolopoulou, M. Parker and M. Christian, presented in part at the Society for Endocrinology BES 2011, Birmingham, UK, 11 April 2011 - 14 April 2011, 2011.
44. U. Kintscher and R. E. Law, *Am J Physiol Endocrinol Metab*, 2005, **288**, E287-291.
45. N. Maeda, M. Takahashi, T. Funahashi, S. Kihara, H. Nishizawa, K. Kishida, H. Nagaretani, M. Matsuda, R. Komuro, N. Ouchi, H. Kuriyama, K. Hotta, T. Nakamura, I. Shimomura and Y. Matsuzawa, *Diabetes*, 2001, **50**, 2094-2099.
46. S. Tandy, R. W. S. Chung, E. Wat, A. Kamili, K. Berge, M. Griinari and J. S. Cohn, *Journal of Agricultural and Food Chemistry*, 2009, **57**, 9339-9345.
47. L. Tong, *Cell Mol Life Sci*, 2005, **62**, 1784-1803.
48. L. Burri, K. Berge, K. Wibrand, R. K. Berge and J. L. Barger, *Frontiers in genetics*, 2011, **2**, 45.
49. A. Sato, H. Kawano, T. Notsu, M. Ohta, M. Nakakuki, K. Mizuguchi, M. Itoh, T. Suganami and Y. Ogawa, *Diabetes*, 2010, **59**, 2495-2504.
50. H. Y. Lin, C. C. Chen, Y. J. Chen, Y. Y. Lin, H. J. Mersmann and S. T. Ding, *BioMed research international*, 2014, **2014**, 310981.
51. K. Schoonjans, B. Staels and J. Auwerx, *J Lipid Res*, 1996, **37**, 907-925.
52. F. B. Kraemer and W.-J. Shen, *Journal of Lipid Research*, 2002, **43**, 1585-1594.

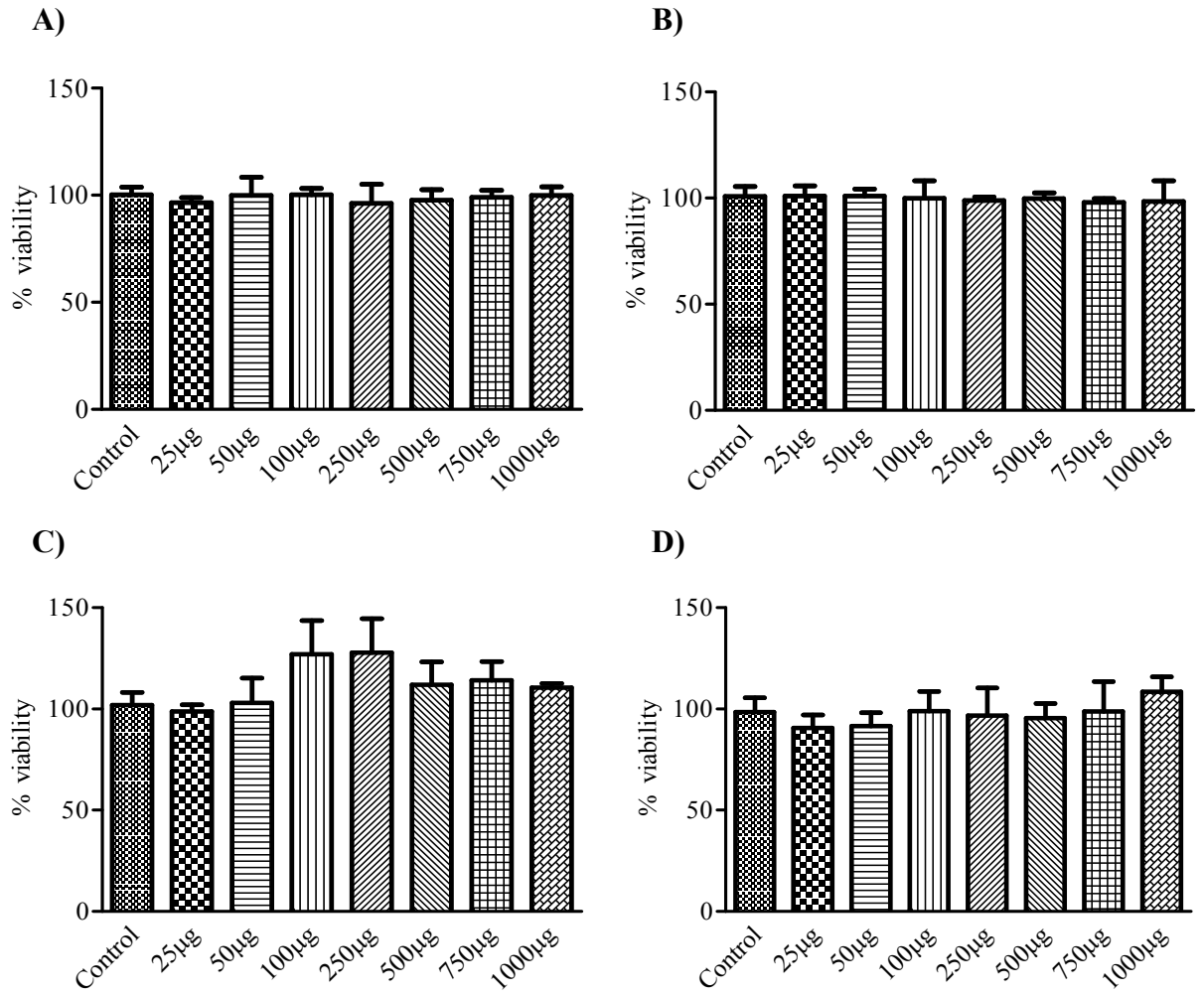


Figure 1 Effect of Sea Cucumber and Blue Mussel methanolic extracts on viability of 3T3-L1 pre-adipocytes and mature cells. 3T3-L1 preadipocytes were incubated with different concentrations of Sea Cucumber and Blue Mussel methanolic extracts (25-1000µg/ml) for 24h (A and B, respectively) or differentiated and treated till day 8 (C and D, respectively) as explained in the methods section. Cells were incubated with MTT reagent for 2h at 37˚C in fresh media. After incubation, media was removed and formazan crystals were dissolved in 250µl diethyl sulfoxide; the coloured solution was quantified at 570nm. Cell viability was expressed as a percentage over control (n=3, mean±SD).

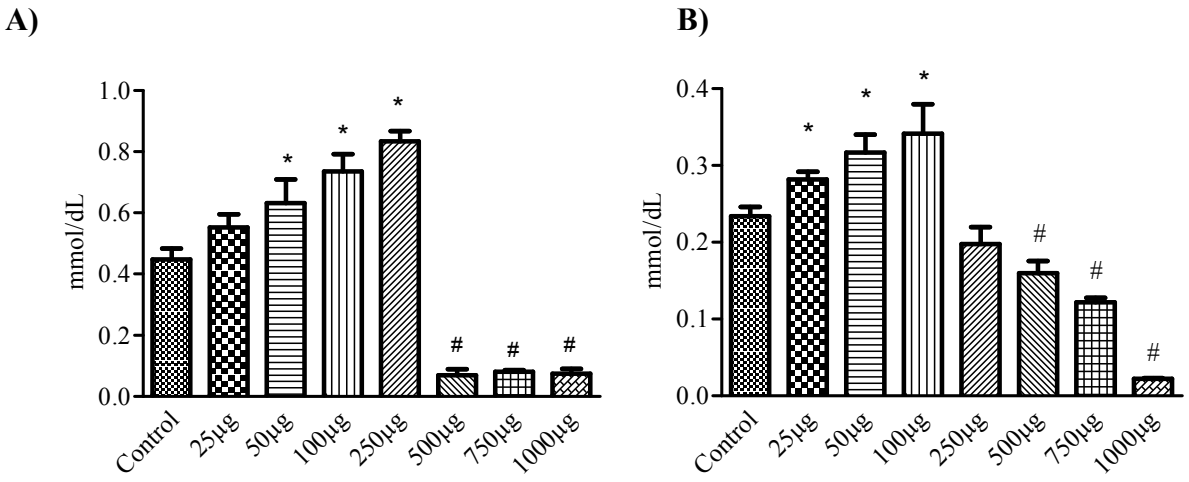


Figure 2 Dose dependent effect of Sea Cucumber (A) and Blue Mussel (B) methanolic extracts on triglyceride accumulation. Total fat was extracted on day 8 after treatment with Sea Cucumber and Blue Mussel extracts and triglycerides were measured using commercially available kit as explained in the methods section. Results were analyzed using One-way analysis of variance (ANOVA) and Dunnett's multiple tests was performed to check statistical significant effects. Each bar represents mean ± SD, n=3. Asterisk (*) indicates significantly ($p<0.05$) different from control cells.

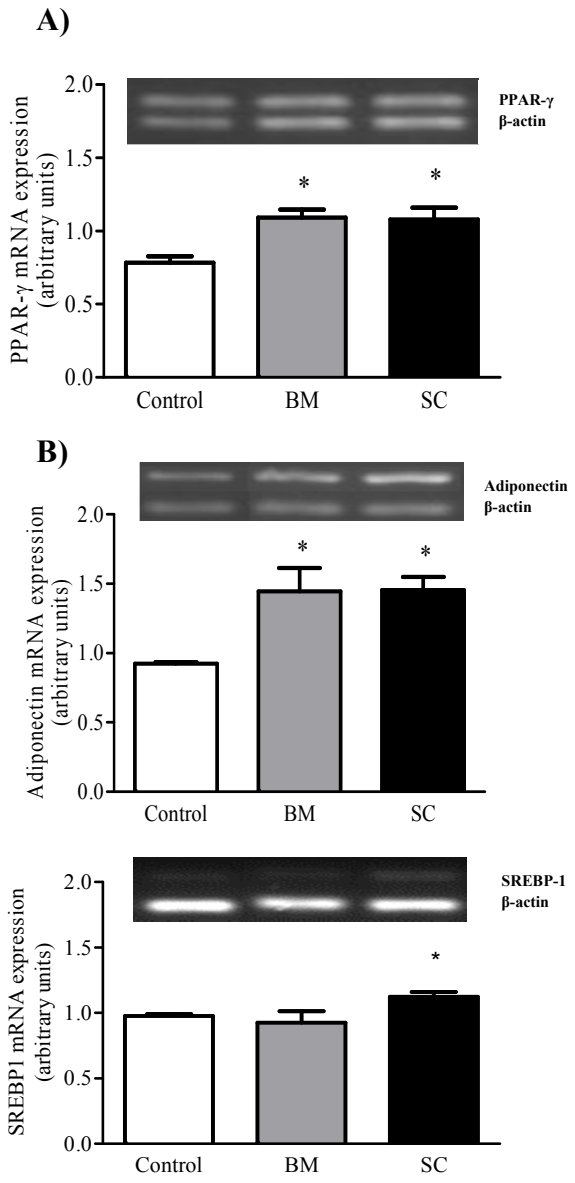


Figure 3 Effects of Sea Cucumber (SC) and Blue Mussel (BM) methanolic extracts on adipogenic gene expression. 3T3-L1 cells were treated with SC and BM extracts at 250µg/ml and 100µg/ml doses respectively till day 8**.** Total RNA was isolated on day 8 and the mRNA expression of PPAR-γ (A), Adiponectin (B) and Sterol Regulatory Element-Binding Protein-1 (SREBP-1) (C) were measured, normalized to β-actin and expressed as arbitrary units. Results were analyzed using One-way ANOVA followed by a Dunnett's multiple tests to compare BM or SC treatment with control. Each bar represents mean ± SD, n=3. Asterisk (*) indicates significantly ($p<0.05$) different compared to control cells and "ns" indicates non-significant compared to control cells.

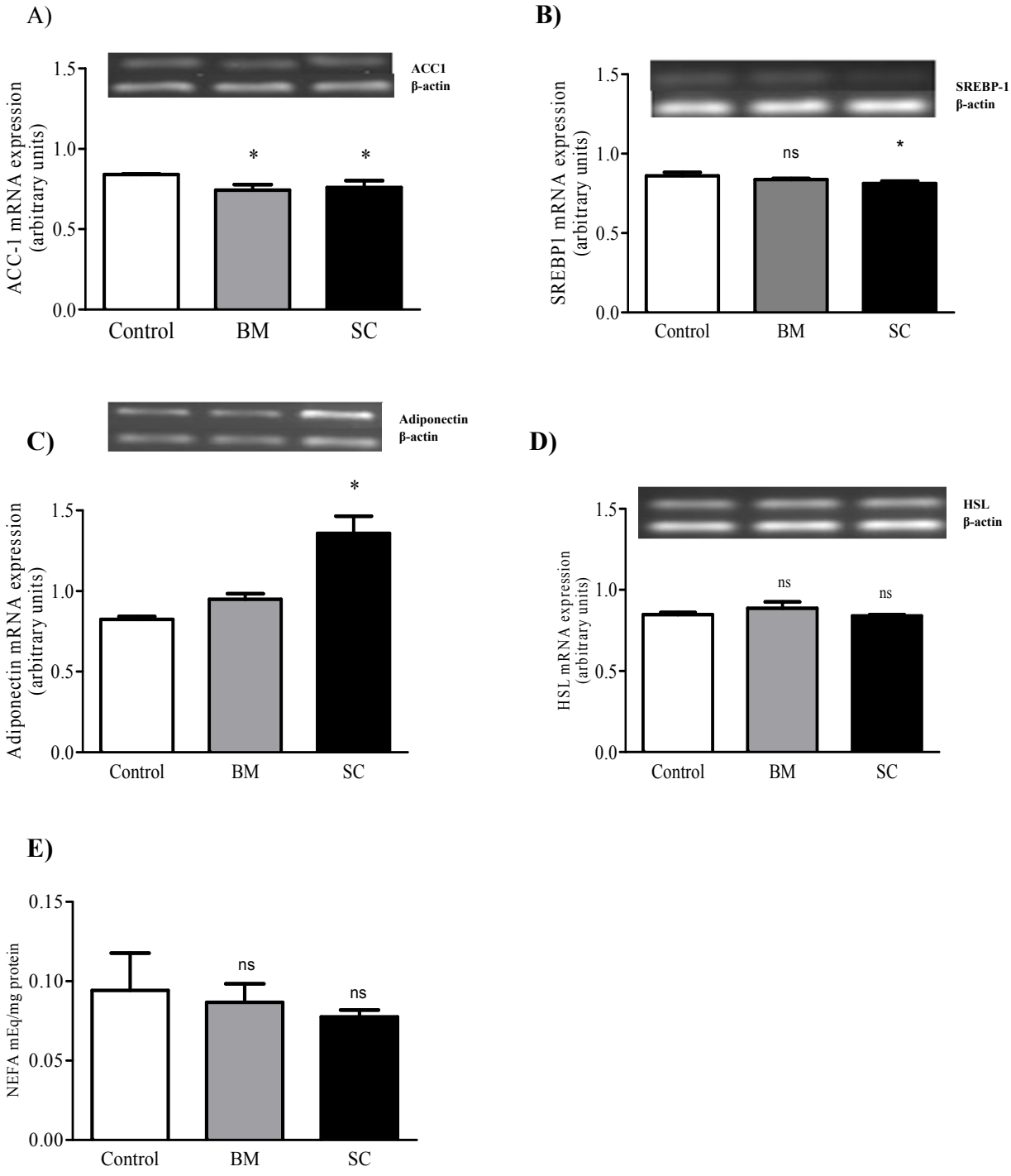


Figure 4 Effects of Sea Cucumber (SC) and Blue Mussel (BM) methanolic extracts on lipogenesis and lipolysis. 3T3-L1 cells were treated with SC and BM extracts at 500µg/ml and 750µg/ml concentrations, respectively till day 8. Total RNA was isolated on day 8 and the mRNA expression of Acetyl-CoA carboxylase 1 (ACC-1) (A), Sterol Regulatory Element-Binding Protein-1 (SREBP-1) (B), Adiponectin (C) and hormone sensitive lipase (HSL) (D)

were measured, normalized to β-actin and expressed as arbitrary units. Lipolysis was determined by measuring non-esterified fatty acids (NEFA) concentration in cell culture medium (E). Results were analyzed using one-way ANOVA followed by a Dunnett's multiple tests to compare BM or SC treatment with control. Each bar represents mean ± SD, n=3. Asterisk (*) indicates significantly ($p<0.05$) different compared to control cells and "ns" indicates non-significant compared to control cells.

Table 1 Sequence of primers (Mus musculus) used for reverse transcription-polymerase chain reaction

Primers (GenBank ID)	Forward	Reverse
Acetyl-CoA carboxylase 1 (XM006531951.1)	5'-GGACCACTGCATGGAATGTTA-3'	5'-TGAGTGACTGCCGAAACATCTC-3'
Adiponectin (NM009605.4)	5'-GCCCAGTCATGCCGAAGA-3'	5'-TCTCCAGCCCCACACACTGAAC-3'
Glucose transporter-4 (GLUT-4) (AB008453.1)	5'-GATTCTGCTGCCCTTCTGTC-3'	5'-ATTGGACGCTCTCTCTCCAA-3'
Hormone Sensitive Lipase (HSL) (U08188.1)	5'-AGACACCAGCCAACGGATAC-3'	5'-GGGCATAGTAGGCCATAGCA-3'
Peroxisome proliferator activated receptor-γ (U01664.1)	5'-GAGCTGACCCAATGGTTGCTG-3'	5'-GCTTCAATCGGATGGTTCTTC-3'
Peroxisome proliferator activated receptor-α (BC016892.1)	5'-AGGCAGATGACCTGGAAAGCT-3'	5'-ATGCGTGAACTCCGTAGTGG-3'
Sterol regulatory element-binding protein-1 (SREBP-1) (BC056922.1)	5'-TAGAGCATATCCCCCAGGTG-3'	5'-GGTACGGGCCACAAGAAGTA-3'
β-actin (BC138614.1)	5'-ATGGTGGGAATGGGTCAGAAG-3'	5'-CACGCAGCTCATTGTAGAAGG-3'

Table 2 Lipid composition of Sea Cucumber and Blue Mussel methanolic extract

Lipid Class	Sea Cucumber (%)	Blue Mussel (%)
Hydrocarbon	1.11	1.13
Steryl esters/Wax esters	1.84	ND
Ethyl ketones	2.05	2.14
Triacylglycerols	2.27	ND
Free fatty acids	5.20	0.15
Alcohols	2.41	3.74
Sterols	3.97	3.67
Acetone mobile polar lipids	5.52	7.18
Phospholipids	75.59	84.12

Table 3 Fatty acid composition of total lipids of Sea Cucumber and Blue Mussel

Fatty Acids (%w/w)	Sea Cucumber	Blue Mussel
Saturated Fatty acids (SFA)		
C14:0	1.75	1.58
C15:0	0.81	0.82
C16:0	5.38	14.8
C18:0	2.75	3.43
C20:0	0.5	0.03
C22:0	0.41	0.02
Total SFA	11.6	20.68
Monounsaturated Fatty acids (MUFA)		
C14:1	0.42	0.04
C16:1n-7	17.45	5.63
C16:1n-5	0.87	0.39
C18:1n-9	2.3	2.38
C18:1n-7	4.04	2.86
C20:1n-11	2.27	1.09
C20:1n-9	0.98	5.72
C20:1n7	0.39	1.48
C24:1	1.36	ND
Total MUFA	32.72	23.38
Polyunsaturated Fatty acids (PUFA)		
n-6 PUFA		
C18:2n-6	0.4	1.68
C18:3n-6	0.27	0.08
C20:2n-6	0.34	0.87
C20:3n-6	ND	0.1
C20:4n-6	1.38	3.57
C22:4n-6	5.02	0.12
C22:5n-6	ND	0.35
n-3 PUFA		
C18:3n-3	0.11	1.41
C18:4n-3	0.71	2.9
C20:4n-3	0.15	0.29
C20:5n-3	25.35	20.1
C22:5n-3	0.26	1.46
C22:6n-3	0.63	20.65
Total PUFA	37.79	56.2
Total n-3	27.59	48.75
Total n-6	7.45	6.48
n-6/n-3	0.27	0.13

Table 4 Phospholipids fatty acid composition of Sea Cucumber and Blue Mussel

Fatty Acids (% w/w)	Sea Cucumber	Blue Mussel
Saturated Fatty acids (SFA)		
C14:0	2.14	3.56
C16:0	3.76	18.03
C18:0	6.78	6.87
Total SFA	12.68	28.46
Monounsaturated Fatty acids (MUFA)		
C16:1n-7	0.57	2.92
C18:1n-9	5.08	3.24
C18:1n-7	1.52	3.12
C20:1n-9	2.01	3.8
Total MUFA	9.18	13.08
Polyunsaturated Fatty acids (PUFA)		
n-6 PUFA		
C18:2n-6	0.44	3.18
C18:3n-6	0.37	1.19
C20:4n-6	1.68	3.01
C22:4n-6	1.23	ND
C22:4n-6	3.49	ND
C22:5n-6	ND	ND
n-3 PUFA		
C18:3n-3	0.18	1.06
C18:4n-3	0.77	0.83
C20:4n-3	0.34	0.7
C20:5n-3	44.4	19.7
C22:6n-3	3.9	11.24
Total PUFA	56.8	40.9
Total n-3	49.59	33.53
Total n-6	7.21	7.38
n-6/n-3	0.14	0.22