BRIEF REPORT

REVISED Fractionation of *Averrhoa bilimbi* hexane extract corresponding to brown adipocytes stimulation [version 2; peer review: 2 approved with reservations]

Previous title: Isolation of active *Averrhoa bilimbi* phytochemicals corresponding to brown adipocytes stimulation

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Abstract

Averrhoa bilimbi is a fast-growing tree widely found in countries of tropical Asia. Due to easy accessibility and traditional knowledge, various parts of this plant are adopted as folk medicine and a natural health remedy. Recently, beneficial effects of bilimbi in combating obesity including its potential antihyperlipidemic and hypoglycemic activities have been discovered. This paper reports the successive isolation and purification of bioactive compounds from the leaf of bilimbi that corresponds to brown adipocyte activation. Bilimbi ethanolic extract underwent bioassay-guided partitioning and fractionation. The n-hexane partition exhibited highest brown adipogenesis potential via adipomyocytes differentiation. Further isolation of this active partition yielded 10 fractions. Active fractions with the highest brown adipogenesis potential were further evaluated via the adipomyocytes assay. Chemical structures of the constituents were elucidated by gas chromatography-mass spectrometry (GC-MS). Major phytochemicals in the n-hexane partition include hexadecanoic acid, phytol, 9-Octadecenoic acid (Z)- and squalene.

Keywords

Averrhoa bilimbi, obesity, brown adipocytes, white adipocytes, adipomyocytes

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REVISED Amendments from Version 1

We are pleased to submit this revised article with an updated title "Fractionation of Averrhoa bilimbi hexane extract corresponding to brown adipocytes stimulation". The article has been revised according to the reviewers' comments. No changes were made to figures and dataset. The word "isolation" has been removed and replaced by "extraction" and "fractionation" to better indicate the work that had been carried out in this study. Tables 1 and 2 have been updated. "Mass spectrum" has been replaced with "GC Chromatogram" in the figure 5-8's legends. The content has also been rephrased to reflect the current findings and potential future work.

Any further responses from the reviewers can be found at the end of the article

1. Introduction

Averrhoa bilimbi, also known as bilimbi by locals, is widely found in many countries of tropical Asia including Malaysia, the Philippines, Indonesia, and India. Bilimbi is a fast growing and long-lived plant species that is commonly 16-33 feet tall.¹ It belongs to the same Oxalidaceae family as the carambola or star fruit. Scientific studies have been conducted on various parts of this plant, with medicinal benefits including antioxidant, hepatoprotective, anti-cancer, wound healing, anti-diabetic, anti-hyperlipidemic, anti-hypertensive, anti-hypercholesterolemic, anti-ulcerative colitis effects, as well as attenuated hyperglycemia-mediated oxidative stress and anti-thrombotic activities.²⁻⁵ Its beneficial effects in brown adipogenesis activation, a phenomenon that combats obesity, has also been recently reported.⁶

There are two main types of adipose tissue. White adipose tissue (WAT) is the primary site of fat storage and its amount increases in obesity. Brown adipose tissue (BAT) on the other hand plays specific roles in promoting energy expenditure and maintaining body temperature via heat generation. Although BAT and WAT are originated from distinct lineages, the plasticity of WAT allows brown adipocyte-like cells to emerge upon appropriate and adequate stimulation. This process is termed "browning" or "beiging" of WAT, which induces thermogenesis in cellular and animal models.⁷⁻⁹ Fundamental to the development of obesity is an imbalance between caloric intake and energy expenditure. The induced BAT development from WAT may help to increase energy consumption as well as reduce adverse effects of WAT to improve metabolic health. The stimulation of BAT development and activity can also be strategised to combat obesity.

Although the molecular effects of bilimbi in stimulating BAT activity to combat diet-induced obesity has been investigated recently, the corresponding phytochemicals to the reported pharmacological effects remain poorly studied. Here, we identify the phytochemicals of plant partition, and its active fractions that induce BAT associated adipocytes differentiation and enhance brown adipogenesis activities.

2. Methods

2.1 Plant extractions and fractions with Flash Column Chromatography

Bilimbi leaves were sampled from an orchard with the owner's permission. The botanical authentication of the specimen was conducted by botanists of the School of Biological Sciences, Universiti Sains Malaysia (USM) and a voucher specimen 11738 was deposited in the herbarium of USM. Fresh leaves were dried in an oven at 45°C until the percentage yield of moisture content value was less than 10%. Dried leaves were then ground to a fine powder using a grinder then weighed, recorded and kept in a plastic bag. The powder was soaked in ethanol (EtOH) for 3 days at room temperature. After filtration with Whatman No. 1 filter paper, the solvent was removed under reduced pressure at 40°C using a rotary evaporator (Buchi, Switzerland) to obtain the crude ethanolic extract. The weight and percentage yield were recorded, stored in an amber bottle, labelled and kept refrigerated until further use. Next, the crude was sequentially extracted with organic solvents, namely *n*-hexane (*n*-Hex), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) with increasing polarity respectively. The filtrate was then concentrated and dried under reduced pressure at 40°C using a Buchi rotary evaporator. The remaining aqueous layer was evaporated and lyophilised in a freeze-dryer. The weight and percentage of each yield was recorded and kept refrigerated. A flow diagram showing the step-by-step extraction and partitioning processes is depicted in Figure 1.

After the bioactivity of these extracts was confirmed in cell-based assays, 200 g of dried leaves was soaked in 15 L of *n*-Hex to yield 12 g of extract. Fractionation was conducted with normal phase silica gel flash column chromatography. Mobile phases in the separation consisted of gradient of *n*-Hex, EtOAc, and methanol.

2.2 High-Performance Thin Layer Chromatography (HPTLC)

HPTLC has advanced separation efficiency and detection limits compared to conventional TLC Chromatography. The pre-coated silica gel Merck, TLC silica gel plates 60 F 254, 200 × 100 mm were used. 2 µl of the sample solution was applied on the plate using a CamagLinomat V automatic sampler applicator in the form of bands (length: 5 mm, width: 1 mm, distance between two bands: 10 mm) by using a 100 µl Camag Microtitre Syringe (Hamilton, Bonaduz,

Figure 1. Representation of continuous procedure of extraction, partitioning and fractionation of *Averrhoa bilimbi*.

Switzerland). A constant application rate of 150 nl/s with mobile phase of *n*-hexane: ethyl acetate (80:20, v/v) was adopted. The plate was then placed in the mobile phase, and ascending development was performed to a distance of 8.5 cm, the plate was then air dried and performed densitometry scanning at 250 nm. Images of the TLC plates were captured using the Camag's TLC Visualizer documentation system. The system provided illumination with white light (remission, transmission or a combination of both), where the exposure of RT white was optimised at 1.482 s, R 254 at 0.185 s, and R 366 at 0.877 s.

2.3 Cell culture and adipocyte differentiation

C2C12 myoblast was purchased from the American Type Culture Collection (Manassas, VA, USA) and was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Adipocyte

differentiation in the myoblast was initiated after the cells reached confluent by adding 0.5 mM isobutyl methylxanthine (IBMX), 1 μ M dexamethasone (DEX), 1 μ g/ml insulin, and 1 μ M rosiglitazone (ROSI). Fresh differentiation medium containing DMEM, 10% FBS, 1 μ g/ml insulin and 1 μ M ROSI was replaced two days later. These cells were then stabilised in DMEM containing 10% FBS for another two days until the formation of oil droplets was observed.⁸

2.4 Adipocyte determination by fluorescent dye staining

Cells were washed once with PBS and fixed with 4% paraformaldehyde for ten minutes. Lipid droplets produced by adipocytes were stained by Nile Red solution and were visualised using the IN Cell 2200 Analyzer High Content Screening System (GE Healthcare, PA, USA) at an excitation wavelength of 460 nm.¹⁰

2.5 GC/MS analyses and identification of components

The GC/MS analyses of non-polar hexane partition extract and active fractions were carried out using the Agilent 5977A Series GC/MSD system with an HP-5ms ultra inert column (30 m $\times$ 0.25 mm id, 0.25 μ m film thicknesses). Spectroscopic detection by CG/MS involved an electron ionisation system which utilised high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with a flow rate of 1 ml/min. The initial GC oven temperature was set as 110°C for two minutes and programmed with increasing rate of 10°C/min to 200°C in five minutes. Finally, the temperature was increased to 250°C at 10°C/min in five minutes and held at that temperature for 13 minutes. The split ratio was 1:25 with a scan range of 40 to 550 amu.¹¹ Hexane fractions (1 μ l, 1 mg/ml) and partition extract (3 μ l, 10 mg/ml) diluted in *n*-Hex were injected into the GC/MS via an auto-injector. Compounds present in the partition extract and active fractions were analysed by the mass fragmentation pattern. The National Institute of Standards and Technology (NIST) 14 and Wiley 10 library (W10N14.L) were used for the identification of compounds with spectral similarity score higher than 60%.¹²

3. Results

3.1 Biological Assay-Guided Fractionation of *A. bilimbi* Extract

Four partitioned extracts were in the yield: *n*-Hex, EtOAc, *n*-BuOH and H₂O. The yield of each component is indicated in Figure 1.

3.2 Flash Column Chromatography and High-Performance Thin Layer Chromatography (HPTLC)

Flash column chromatography was conducted on the partition extract with an equilibration solvent ratio of *n*-Hex and EtOAc (95:5). A Hi Flash Column with a size of 3 L (46 $\times$ 130 mm silica gel) was used. With a flow rate of 60 ml/min, a total of 80 fractions were collected and spotted on a plate, employing the HPTLC. Plate visualisation was done under short (254 nm) and long wavelength (366 nm) ultraviolet lights, as well as white light illumination. Fractions with similar separation profiles were pooled. A total of ten fractions were collected with respective TLC profiles as shown in Figure 2.

3.3 Differentiation of adipocytes in Myf5 lineage precursor cells

The partitioned extracts and fractions corresponded to the growth stimulation of brown adipocyte were investigated via cell-based study. The myf5-positive characteristics of C2C12 murine myoblast allow the co-development of mature myotubes and brown adipocytes upon appropriate stimulations. ROSI was served as the positive control. Adipogenesis was observed on day four after treatment with 1 μ M ROSI. A similar phenomenon was found in 200 μ g/ml EtOH and 100 μ g/ml *n*-Hex treated cells. Myotube development was observed in all treated cells. All positively treated cells showed intracellular lipid droplet production after 7 days of incubation. These lipid droplets were captured by Nile Red staining and imaged at 20 $\times$ magnification (Figure 3). Among the four partitioned extracts tested, *n*-Hex exhibited the highest amount of lipid droplet production.

A bioassay guided fractionation technique was used to identify the active components present, as well as to evaluate the corresponding phytochemicals in brown adipocyte activation.

The cell-based study was then repeated on ten *n*-Hex fractions yielded from the HPTLC fractionation. Cell morphology and lipid droplet accumulation was observed for seven days with treatments by ten fractions. Myotube development was observed in all treated cells. Adipocyte differentiation was also observed in f5, f6, f8, f9 and f10 treated cells, with lipid droplet formation as depicted in Figure 4. Massive death was observed in f7 treated cells on day 3, and cell death was also found in f6 treatment from day 5 onwards. DMSO was used to replace bilimbi and ROSI in non-treated cells. These negative control cells were supplemented with adipogenesis culturing media and were fully developed into myotubes.

3.4 Gas-Chromatography/Mass-Spectrometry (GC/MS)

A GC/MS based qualitative analysis was performed on *n*-Hex and subsequently three active fractions, namely f8, f9, and f10, to determine the types of compounds present (Figure 5-8). Major constituents identified are presented in

Figure 2. Thin layer chromatography plates showing separation of compounds of f1 – f10 using mobile phase *n*-Hex:EtOAc (80:20, v/v). Two batches (1 & 2) of fractionation samples were collected and run on the same plates (A) TLC plate image viewed under UV 254 nm. (B) TLC plate image viewed under UV 366 nm. (C) TLC plate image viewed under white light illumination.

Figure 3. Differentiation of Myf5 lineage precursor cells upon stimulants. Lipid droplets formation upon adipogenesis upon treatment by (A) 200 μ g/ml EtOH, (B) 1 μ M ROSI, (C) 100 μ g/ml *n*-Hex, (D) 100 μ g/ml EtOAc, (E) 100 μ g/ml *n*-BuOH and (F) H₂O were stained by a Nile Red solution. Treatment of myoblasts with adipogenesis stimulants facilitated myocytes and adipocytes co-differentiation.

Figure 4. Differentiation of Myf5 lineage precursor cells upon stimulants. Lipid droplet formation upon treatment by *n*-Hex fractions (f1 – f10) and 1 μ M ROSI were stained by a Nile Red solution. *n*-Hex fractions with adipogenesis stimulating components facilitated myocyte and adipocyte co-differentiation. Cells treated with 0.5% DMSO served as negative control (-ve) and demonstrated a complete process of myogenesis.

Tables 1 & 2. Studies on f6 & f7 were discontinued after the demonstration of cytotoxic effect in these treated cells, as shown in **Figure 4**.

The database information search via the MassHunter Library WION 14.L revealed the presence of nine predominant compounds in f8, ten in f9, and five in f10. (1.alpha., 4.alpha., 4a.alpha., 10a.alpha.) -1, 4, 4a, 5, 6, 7, 8, 9, 10, 10a -decahydro- 1, 4, 11, 11-tetramethyl-1, 4-methanocycloocta [d] pyridazine and i-propyl 3- (phenylamino) -2- (phenyl-seleno) -3- (phenyl) propanoate were detected in all the three fractions whereas nonanal and hexadecanoic acid were commonly present in f9 and f10. The high abundance compounds include 3-nonen-2-one; 2(4H)-Benzofuranone, 5, 6,

Figure 5. GC Chromatogram of *A. bilimbi* n-Hex.

Figure 6. GC Chromatogram of *A. bilimbi* n-Hex fraction f8.

Figure 7. GC Chromatogram of *A. bilimbi* n-Hex fraction f9.

Figure 8. GC Chromatogram of *A. bilimbi* n-Hex fraction f10.

Table 1. Major chemical components identified in *A. bilimbi* n-Hex partition using GC/MS.

RT	Name	Molecular formula	Area (%)	m/z (base peak)	Mass (DB)	Score (%)
3.140	Benzene, 1,4-diethyl-	C ₁₀ H ₁₄	0.17	119	134	89.18
3.675	2-Methyl-3-hydroxypyran-4(4H)-one	C ₆ H ₆ O ₃	0.05	126.1	126	85.49
5.156	N-Methyl-N-nitro-4-t-butylaniline	C ₁₁ H ₁₆ N ₂ O ₂	0.02	147.1	218	81.39
5.342	(E)(S)(+)-6-Hydroxy-8-phenyl-2-pivaloxyoct-7-enyl pivalate	C ₂₄ H ₃₆ O ₅	0.04	131	404	82.3
5.569	Neopentyl 2,2-dimethylpropanoate	C ₁₀ H ₂₀ O ₂	0.04	71.1	172	96.06
5.723	5-Isopropyl-2-methylpyrrole-3-carbonitrile	C ₉ H ₁₂ N ₂	0.07	133	148	88.79
5.942	Naphthalene, 2-methyl-	C ₁₁ H ₁₀	0.35	142.1	142	96.6
6.169	Naphthalene, 1-methyl-	C ₁₁ H ₁₀	0.21	142.1	142	95.8
6.990	(3-Phenyl-2-propynylidene)cyclopropane	C ₁₂ H ₁₀	0.03	154.1	154	86.65
7.331	Naphthalene, 1,4-dimethyl-	C ₁₂ H ₁₂	0.29	156.1	156	96.54
7.524	Naphthalene, 2,3-dimethyl-	C ₁₂ H ₁₂	0.41	156.1	156	97.16
7.655	Benzeneethanimidic acid, .alpha.-phenyl-, ethyl ester	C ₁₆ H ₁₇ NO	0.020,02	167	239	83.82
7.980	2-Naphthalenol, 1,2-dihydro-1,1-dimethyl-,acetate	C ₁₄ H ₁₆ O ₂	0.05	141.1	216	84.08
8.419	Benzeneethanimidic acid, .alpha.-phenyl-, ethyl ester	C ₁₆ H ₁₇ NO	0.04	168.1	239	95.76
8.683	(1'.alpha.,4'.alpha.,4a'.alpha.,5'.beta.,8'.beta.,8a'.alpha.,)1',4',4a',5',8',8a'-hexahydro-1',4'-dimethylspiro (cyclohexane-1,9'-[1,4:5,8]dimethanophthalazine)	C ₁₇ H ₂₄ N ₂	0.01	162.1	256	87.66
8.936	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	C ₁₁ H ₁₆ O ₂	0.18	111.1	180	87.13
9.028	Octanoic acid	C ₈ H ₁₆ O ₂	0.02	73	144	92.95
9.532	Pentane, 3-bromo-	C ₅ H ₁₁ Br	0.01	71.1	150	85.53
10.073	2-Propanone, 1-(5-ethyl-2-furanyl)-	C ₉ H ₁₂ O ₂	0.01	109.1	152	84.74
10.644	2-Bromo-1-nonen-3-ol	C ₉ H ₁₇ BrO	0.05	123.1	220	85.19
10.821	Phenol, 2-(1-phenylethyl)-	C ₁₄ H ₁₄ O	0.03	71.1	198	88.26
10.956	1-Heptyl-1H-(1,2,3)-triazole-N-([2'-(hydroxyethoxy)ethylamino]ethyl)-4-carboxamide	C ₁₄ H ₂₇ N ₅ O ₂	0.01	74	297	85.11
11.825	Acetonyl decyl ether C ₁₃ H ₂₆ O ₂₈	C ₁₃ H ₂₆ O ₂	0.01	57.1	214	88
12.383	(2S,5R)-2-Isopropyl-5methylhept-6-en-1-ol	C ₁₁ H ₂₂ O	0.62	68.1	170	82.65
12.456	2-Pentadecanone, 6,10,14-trimethyl-	C ₁₈ H ₃₆ O	0.77	43	268	80.23

Table 1. Continued

RT	Name	Molecular formula	Area (%)	m/z (base peak)	Mass (DB)	Score (%)
12.728	7,9-Dodecadien-1-ol, acetate, (E,Z)-	C ₁₄ H ₂₄ O ₂	0.03	81.1	224	83.35
13.231	(E,E)-6,10,14-trimethyl-5,9,13-pentadecatrien-2-one	C ₁₈ H ₃₀ O	0.08	69.1	262	95.1
13.646	(E,E)-6,10,14-trimethyl-5,9,13-pentadecatrien-2-one	C ₁₈ H ₃₀ O	0.14	69.1	262	82.77
13.691	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	0.27	74	270	80.53
14.336	Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	4.85	73	256	94.92
17.794	Phytol	C ₂₀ H ₄₀ O	8.37	71.1	296	96.69
18.399	9-Octadecenoic acid (Z)-	C ₁₈ H ₃₄ O ₂	8.09	55.1	282	82.61
20.132	(2S,5R)-2-Isopropyl-5methylhept-6-en-1-ol	C ₁₁ H ₂₂ O	0.29	68.1	170	83.91
22.192	geranyl linalyl ester	C ₂₀ H ₃₄ O	0.13	69.1	290	86.59
23.396	geranyl linalyl ester	C ₂₀ H ₃₄ O	0.15	69.1	290	88.18
26.281	Di-(2-ethylhexyl)phthalate	C ₂₄ H ₃₈ O ₄	1.48	149.1	390	93.94
30.355	(E) and (Z)-7,8-Dimethoxycarbonyl-6,9-di(4-methoxyphenyl)-6,8-tetradecadiene	C ₃₂ H ₄₂ O ₆	0.06	69.1	522	82.83
32.864	Squalene	C ₃₀ H ₅₀	56.31	69.1	410	95.56
35.158	Dodecane, 2,6,10-trimethyl-	C ₁₅ H ₃₂	2.01	57.1	212	85.48
36.713	delta.-Tocopherol	C ₂₇ H ₄₆ O ₂	1.51	402.3	402	83.16
37.210	3-Cyclohexen-1-one, 4-(1,5,9-trimethyl-4,8-decadienyl)-, (Z)-(+,-)-	C ₁₉ H ₃₀ O	0.59	69.1	274	80.3

Table 2. Major chemical components identified in *A. bilimbi* fractions using GC/MS.

RT	Name	Molecular formula	Area (%)	m/z (base peak)	Mass (DB)	Score (%)
Fraction 8						
3.935	Tri-o-trimethylsilyl, N-trifluoroacetyl derivative of Terbutaline	C ₂₃ H ₄₂ F ₃ NO ₄ Si ₃	12.34	73	537	80.64
5.581	3-nonen-2-one	C ₉ H ₁₆ O	28.01	43	140	67.06
5.815	1-[3-(Hydroxycyclohexenyl)]ethanone	C ₈ H ₁₂ O ₂	6.32	43	140	80.42
6.694	(1R*,2R*)-N,N-Diethyl-2-hydroxy-2-methyl-3-oxacyclopentanecarbamide	C ₁₁ H ₁₉ NO ₃	3.22	157.1	213	74.27
6.727	1-(N-Phenylcarbamoyl)-1,3-diphenylurea	C ₂₀ H ₁₇ N ₃ O ₂	4.24	93	331	70.01
8.526	(1.alpha.,4.alpha.,4a.alpha.,10a.alpha.)-1,4,4a,5,6,7,8,9,10,10a-decahydro-1,4,11,11-tetramethyl-1,4-methanocyclooctal[d]pyridazine	C ₁₅ H ₂₆ N ₂	6.07	191	234	84.49
15.019	3-Acetyloxypropyl 2,3,4,6-tetra-O-methyl- α -L-[(5-D)gulopyranoside	C ₁₅ H ₂₇ DO ₈	4.12	88	336	84.57
19.061	spiro[2.4]heptane-5-carboxaldehyde	C ₈ H ₁₂ O	9.15	79	124	78.37
26.298	i-Propyl 3-(phenylamino)-2-(phenylseleno)-3-(phenyl)propanoate	C ₂₄ H ₂₅ NO ₂ Se	22.34	149	439	90.7
Fraction 9						
3.527	Nonanal	C ₉ H ₁₈ O	2.14	57	142	99.18
8.326	Phenol, 2-(1-phenylethyl)-	C ₁₄ H ₁₄ O	1.03	57	198	93.01
8.526	(1.alpha.,4.alpha.,4a.alpha.,10a.alpha.)-1,4,4a,5,6,7,8,9,10,10a-decahydro-1,4,11,11-tetramethyl-1,4-methanocyclooctal[d]pyridazine	C ₁₅ H ₂₆ N ₂	1.14	191	234	84.31
10.657	11-Acetoxy-8(12)-drimen-7.alpha.-ol	C ₁₇ H ₂₈ O ₃	1.50	123	280	77.4
10.833	Phenol, 2-(1-phenylethyl)-	C ₁₄ H ₁₄ O	0.66	85	198	92.08
11.628	2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-6-hydroxy-4,4,7a-trimethyl-, (6S-trans)-	C ₁₁ H ₁₆ O ₃	11.46	111.1	196	73.16
14.319	Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	10.95	73	256	67.32
18.367	(12R)-(9Z)-12-hydroxy-9-octadecenoic acid	C ₁₈ H ₃₄ O ₃	48.12	55	298	71.92
26.298	i-Propyl 3-(phenylamino)-2-(phenylseleno)-3-(phenyl)propanoate	C ₂₄ H ₂₅ NO ₂ Se	9.77	149	439	93.47
Fraction 10						
3.527	Nonanal	C ₉ H ₁₈ O	5.66	57	142	80.88
5.337	[(6a.alpha.,6b.alpha.,10a.beta.,10b.alpha.)-6a,6b,7,8,9,10,10a,10b-octahydro-10b-hydroxy-5-methylbenzo[3,4]cyclobuta[1,2-c]quinolin-6(5H)-one	C ₁₆ H ₁₉ NO ₂	3.61	175.2	257	79.34
8.526	(1.alpha.,4.alpha.,4a.alpha.,10a.alpha.)-1,4,4a,5,6,7,8,9,10,10a-decahydro-1,4,11,11-tetramethyl-1,4-methanocyclooctal[d]pyridazine	C ₁₅ H ₂₆ N ₂	9.29	191	234	88.44
14.319	Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	55.64	73	256	80.86
26.298	i-Propyl 3-(phenylamino)-2-(phenylseleno)-3-(phenyl)propanoate	C ₂₄ H ₂₅ NO ₂ Se	19.29	149	439	90.78

7, 7a-tetrahydro -6- hydroxy-4, 4, 7a-trimethyl-, (6S-trans)-; hexadecanoic acid; (12R)-(9Z)-12-hydroxy-9-octadecenoic acid; spiro [2.4] heptanes-5-carboxaldehyde; and i-propyl 3 -(phenylamino) -2- (phenylseleno) -3- (phenyl) propanoate.

4. Discussion

Brown adipocytes and myocytes, precursor cells that give rise to skeletal muscle, are developed from a common adipomyocyte precursor. White adipocytes on the other hand are derived from pericytes that embedded within the vascular vessel walls. In this study, a straightforward cell-based assay was adopted based on the unique characteristics of precursor cells, where the adipomyocytes containing a mixture of brown adipocytes and myocytes, or solely the myocyte differentiation program, was activated via its initiation factors. Gene program commitments in myoblast-brown adipocyte transition were observed in bilimbi treated cells and the up-regulation of PGC-1 α , UCP1 and PRDM16 proteins which influenced the cellular metabolism and mitochondrial function.⁶

Our observations in this study showed that bilimbi *n*-Hex partition was able to induce a higher cellular response in signalling the co-development of brown adipocytes and myocytes than its crude extract. The induction of brown adipocyte differentiation indicates the presence of active phytochemicals that are responsible for the program. Major phytochemicals in the *n*-Hex partition include hexadecanoic acid, phytol, 9-Octadecenoic acid (Z)- and squalene, which agree with an earlier report.^{13,14} Bioassay-guided fractionations yielded some promising bilimbi fractions from the *n*-Hex partition which further enhanced the brown adipogenesis program. Major chemical compounds of these fractions were then isolated and are summarised in Table 2. Some of these compounds are highly related to the obesity research in the past few years. For instance, Terbutaline is a β_2 -adrenergic receptor agonist that has been recognised to increase lipolysis and thermogenesis.¹⁵ 2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-6-hydroxy-4,4,7a-trimethyl-, (6S-trans)- is a compound that has been found to ameliorate hyperglycaemia, dyslipidaemia, and obesity in high-fat diet-fed mice.¹⁶ (12R)-(9Z)-12-hydroxy-9-octadecenoic acid, also known as ricinoleic acid, is an unsaturated omega-9 fatty acid and hydroxyl acid. Ricinoleic acid is linked to the activation of EP3 prostanoid receptor for prostaglandin E2 regulations in adipogenesis and lipolysis of WAT.^{17,18} i-Propyl3-(phenylamino)-2-(phenylseleno)-3-(phenyl) propanoate, detected in all the three bioactive fractions, is a short-chain fatty acid which regulates gut hormone release, suppresses food intake, and protects against diet-induced obesity.¹⁹ Hexadecanoic acid, also known as palmitic acid, is a controversial component in obesity research, where over-accumulation may result in dyslipidaemia and other obesity associated diseases.²⁰

Evolutionary processes in natural products enhance their structural diversity and shape the pathways of secondary metabolite production. Many of these metabolites play important roles as the starting points for drug discovery in both traditional and modern medicines. Besides, plants are used in the form of spices and herbs based on their natural antioxidant values. Several plant-derived nutraceuticals are found to regulate BAT activity and induced WAT metabolism, including capsaenoid, curcumin, quercetin, and resveratrol, without major adverse events.²¹ Food ingredients that could increase BAT activity include chili peppers, turmeric, thyme, cinnamon, garlic, onion, green tea, mulberry, and cocoa. Their white-to-brown adipose tissue conversion and calorie burning properties are identified through *in vitro* and *in vivo* models.^{22,23} Nevertheless, the involvement of bilimbi fractions and their bioactive compounds in BAT program enhancement are reported for the first time in this study.

It is estimated that 40-50g of active BAT can increase daily energy expenditure in humans by 20%.²⁴ This could be implemented either by enhancing the activity of BAT or by increasing the number of WAT browning. Dietary components have been shown to increase thermogenic capacity by augmenting BAT activity via several mechanisms of action. Capsaicin that is commonly found in red peppers has been shown to activate ADRB3, a member of the G protein-coupled receptor family, that induces adenylyl cyclase after activation, triggering a cascade of responses in the brown adipogenic program. Resveratrol extracted from grapes and berries on the other hand activates SIRT1 to recruit the BAT program via a series of PRDM16 pathway modulators including PPAR γ , C/EBP β , PGC-1 α , and UCP1.²⁵ Bilimbi, in our studies, acted similarly by up-regulating the expression of relevant proteins which influenced the cellular metabolism and mitochondrial function in adipocyte browning. Therefore, the molecular mechanism of action behind each active compound of bilimbi should be further studied to constitute a therapeutic option for obesity treatment in the future.

5. Conclusions

In summary, *n*-Hex extract of bilimbi activated the adipocyte program of adipomyocytes. Several bilimbi fractions showed strong activity in brown adipocyte differentiation from adipomyocytes. Further compound isolation and purification steps are necessary to confirm their biological effects in brown adipocyte stimulation.

Data availability

All data underlying the results are available as part of the article and no additional source data are required.

Author contributions

F.H., A. A. and W.K.L. conceived and designed the experiments, collected and analysed the data; the manuscript was written by W.K.L. and reviewed by F.H. & A.A.

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Open Peer Review

Current Peer Review Status: ? ?

Version 2

Reviewer Report 23 December 2021

<https://doi.org/10.5256/f1000research.79169.r102268>

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Nor Akmalazura Jani

Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Cawangan Negeri Sembilan, Kampus Kuala Pilah, Kuala Pilah, Malaysia

Title

Ok.

Abstract

"This paper reports the successive isolation and purification of bioactive compounds from the leaf of bilimbi that corresponds to brown adipocyte activation."

- Terms "isolation and purification" are incorrect since the authors did not get pure compounds.

"n-hexane"

- *n* should be in italic.

"Further isolation of this active partition yielded 10 fractions."

- Change 'isolation' with fractionation.

"Chemical structures of the constituents were elucidated by gas chromatography-mass spectrometry (GC-MS)."

- Chemical constituents in the active fractions were determined by gas chromatography/mass spectrometry (GC/MS).

"9-Octadecenoic acid (Z)-"

- *Z* should be italic. Octadecanoic acid; Alphabet "O" should be written in lowercase.

Introduction

The authors have mention phytochemicals in *Averrhoa bilimbi* in the Discussion section. However, I think it would be better if the authors can state major group of phytochemicals such as alkaloids, terpenoids etc. that are present in the plant.

Results

3.2 Flash Column Chromatography and High-Performance Thin Layer Chromatography (HPTLC)

- I think it would be better if you can include a TLC plate which visualized with vanillin sulfuric acid reagent in results section. In addition, you also can add the methodology of

visualization of TLC plate with vanillin sulfuric acid reagent as well as some discussion in discussion section.

3.4 Gas-Chromatography/Mass-Spectrometry (GC/MS)

- Please check area (%) for compound at retention time 1.655 min.
- Please standardize decimal points for score value (two decimal point).

Discussion

Second paragraph:

"Major chemical compounds of these fractions were then isolated and are summarised in Table 2."

- Please revise term "isolated". I think the sentence should be "The major chemical compounds in the active fractions were listed in Table 2".

Note: The manuscript needs to be proofread before it can be resubmitted.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Natural product chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 Jan 2022

Wai Kwan Lau

Abstract

- Terms "isolation and purification" were removed and replaced throughout the whole manuscript as they might cause confusion to readers.
- The authors opt to retain the writing style of chemical names according to the GC/MS database provided by the manufacturer.

Introduction

- Major phytochemical groups of bilimbi have been included and cited.

Results

- A vanillin reagent developed TLC exp which was conducted based on reviewer's request during first revision has been included as part of the Results in this revision.
- Retention time 1.655 min as requested by reviewer was not found, which could be a typo from the reviewer. However, a duplicate data entry at RT 7.655 min has been corrected.
- The score values in Table 1 & 2 have been standardised to two decimal points as requested.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 08 July 2021

<https://doi.org/10.5256/f1000research.55440.r87939>

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Nor Akmalazura Jani

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² Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Cawangan Negeri Sembilan, Kampus Kuala Pilah, Kuala Pilah, Malaysia

Title

The word "isolation" is incorrect since the authors does not obtain any pure compounds from *Averrhoa bilimbi*. Therefore, the authors should revise the title of manuscript.

Abstract

"This paper reports the successive isolation and purification of bioactive compounds from the leaf of bilimbi that corresponds to brown adipocyte activation. Bilimbi ethanolic extract underwent bioassay-guided partitioning and fractionation. The n-hexane partition exhibited highest brown adipogenesis potential via adipomyocytes differentiation. Further isolation of this active partition yielded 10 fractions. Active fractions with the highest brown adipogenesis potential were further evaluated via the adipomyocytes assay."

- The authors performed the bioassay-guided fraction approach to afford the active fraction corresponds to brown adipocyte activation. Thus, the terms "isolation and purification" are incorrect. I think the word "fractionation" is more suitable.

"Chemical structures of the constituents were elucidated by gas chromatography-mass spectrometry (GC-MS)."

- GC-MS is used to identify the presence of compounds within a test sample. Basically, the chemical structures of organic compounds are elucidated by spectroscopic techniques including NMR, IR, MS and UV. I think the authors should revise the above-mentioned sentence.

The authors can include the active fractions as well as compounds detected by GC/MS from the active fractions. I think the compounds identified from the *n*-hexane partition are not that necessary to be included in the abstract.

Introduction

Please include literature review on the phytochemicals of *Averrhoa bilimbi*.

Methods**2.1 Plant extractions and fractions with Flash Column Chromatography**

- Figure 1: Please rearrange yield of F1 until F10.

Results

Please change title for section 3.1 to "Biological Assay-Guided Fractionation of *A. bilimbi* Extract".

3.2 Flash Column Chromatography and High-Performance Thin Layer Chromatography (HPTLC)

- The TLC plates were visualized under UV (254 and 366 nm) and white light illumination. However, based on Tables 1 and 2, most of the detected compounds are weakly- or not chromophore in which their spot maybe could not detected by the UV light and/or white light illumination. I think the authors can use a TLC visualization reagent such as vanillin sulfuric acid reagent to detect weakly- and non-chromophore compounds to see the whole TLC profile of the extracts and fractions. Reference:

<https://link.springer.com/content/pdf/bbm%3A978-3-662-02398-3%2F1.pdf>.

3.4 Gas-Chromatography/Mass-Spectrometry (GC/MS)

- Please revise the title of Figures 5-8. The title should be GC chromatogram of (tested sample) not mass spectrum of (tested sample). A mass spectrum is an intensity versus m/z (mass-to-charge ratio) plot representing a chemical analysis, while GC chromatogram is a plot of an intensity/abundance versus retention time.
- Please revise the percent area of each compound in the *n*-hexane partition. The total percent should not exceed 100%.
- Please use subscript to write number of atoms in the molecular formula. For example, H₂O.
- Why does the m/z of the detected compound not match with the exact molecular weight of the compound?
- Why were the major compounds in the *n*-hexane partition (i.e., hexadecanoic acid, phytol, 9-Octadecenoic acid (Z)- and squalene) not detected in any active fractions?
- Explain why the majority of compounds in the *n*-hexane partition and active fractions were not similar.

Discussion

Second paragraph: Major chemical compounds of these fractions were then isolated and are summarised in Table 2.

- Please revise the term "isolated". I think the sentence should be "The major chemical compounds in the active fractions were listed in Table 2".

Conclusions

I think the authors should revise the statement related to the detected compounds in the *n*-hexane partition and active fractions. I recommended to add further study such as isolation of compound from the active fractions.

Technical

n-hexane and *n*-butanol: the alphabet "n" should be written in italic (*n*-hexane, *n*-butanol, *n*-Hex, *n*-BuOH).

Please give a space between number and unit. For example, 20 g instead of 20g.

Please add a comma before the word "whereas". For example: "____, whereas".

The term "GC-MS" should be written consistently either as "GC-MS" or "GC/MS".

The abbreviation of litre should be written as “L” in capital letter.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Natural product chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Nov 2021

Wai Kwan Lau

Reviewer 2

Title

The word “isolation” is incorrect since the authors does not obtain any pure compounds from *Averrhoa bilimbi*. Therefore, the authors should revise the title of manuscript.

Response to reviewer:

The Title section has been rephrased to “fractionation”.

Abstract

"This paper reports the successive isolation and purification of bioactive compounds from the leaf of bilimbi that corresponds to brown adipocyte activation. Bilimbi ethanolic extract underwent

bioassay-guided partitioning and fractionation. The n-hexane partition exhibited highest brown adipogenesis potential via adipomyocytes differentiation. Further isolation of this active partition yielded 10 fractions. Active fractions with the highest brown adipogenesis potential were further evaluated via the adipomyocytes assay."

- The authors performed the bioassay-guided fraction approach to afford the active fraction corresponds to brown adipocyte activation. Thus, the terms "isolation and purification" are incorrect. I think the word "fractionation" is more suitable.

"Chemical structures of the constituents were elucidated by gas chromatography-mass spectrometry (GC-MS)."

- GC-MS is used to identify the presence of compounds within a test sample. Basically, the chemical structures of organic compounds are elucidated by spectroscopic techniques including NMR, IR, MS and UV. I think the authors should revise the above-mentioned sentence.

The authors can include the active fractions as well as compounds detected by GC/MS from the active fractions. I think the compounds identified from the *n*-hexane partition are not that necessary to be included in the abstract.

Response to reviewer:

The Abstract section has been reworded to extraction and fractionation accordingly. The last sentence which describes compounds identified from the *n*-hexane partition have been removed.

Introduction

Please include literature review on the phytochemicals of *Averrhoa bilimbi*.

Response to reviewer:

This was included in the second paragraph of Discussion section where two references were cited (Gunawan *et al*, 2013; Saini, 2016). "Major phytocomponents in the n-Hex partition include hexadecenoic acid, phytol, 9-Octadecenoic acid (Z)- and squalene, which agree with earlier reports."

Methods

2.1 Plant extractions and fractions with Flash Column Chromatography

- Figure 1: Please rearrange yield of F1 until F10.

Response to reviewer:

Figure 1 has been rearranged to align the label and yield of f1 - f10.

Results

Please change title for section 3.1 to "Biological Assay-Guided Fractionation of *A. bilimbi* Extract".

3.2 Flash Column Chromatography and High-Performance Thin Layer Chromatography (HPTLC)

- The TLC plates were visualized under UV (254 and 366 nm) and white light

illumination. However, based on Tables 1 and 2, most of the detected compounds are weakly- or not chromophore in which their spot maybe could not detected by the UV light and/or white light illumination. I think the authors can use a TLC visualization reagent such as vanillin sulfuric acid reagent to detect weakly- and non-chromophore compounds to see the whole TLC profile of the extracts and fractions. Reference: <https://link.springer.com/content/pdf/bbm%3A978-3-662-02398-3%2F1.pdf>.

3.4 Gas-Chromatography/Mass-Spectrometry (GC/MS)

- Please revise the title of Figures 5-8. The title should be GC chromatogram of (tested sample) not mass spectrum of (tested sample). A mass spectrum is an intensity versus m/z (mass-to-charge ratio) plot representing a chemical analysis, while GC chromatogram is a plot of an intensity/abundance versus retention time.
- Please revise the percent area of each compound in the *n*-hexane partition. The total percent should not exceed 100%.
- Please use subscript to write number of atoms in the molecular formula. For example, H_2O .
- Why does the m/z of the detected compound not match with the exact molecular weight of the compound?
- Why were the major compounds in the *n*-hexane partition (i.e., hexadecanoic acid, phytol, 9-Octadecenoic acid (Z)- and squalene) not detected in any active fractions?
- Explain why the majority of compounds in the *n*-hexane partition and active fractions were not similar.

Response to reviewer:

Title for section 3.1 has been reworted accordingly.

3.2 Flash Column Chromatography and High-Performance Thin Layer Chromatography (HPTLC)

A TLC plate with vanillin sulfuric acid reagent development has been provided as follows:

Hex F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 Phy Sq

Hex: n-Hex extract

F1 – F10: Fractions

Phy – Standard Phytol Isomers

Sq – Standard Squalene

3.4 Gas-Chromatography/Mass-Spectrometry (GC/MS)

1. Figure 5 – 8 have been reworted accordingly.

2. The % area of each compound has been revised and tabulated in Table 1 & 2.
3. Revision has been made on the molecular formula according to the format as requested, as tabulated in Table 1 & 2.
4. m/z refers to the base peak and mass DB refers to the molecular weight of compound.
5. From the TLC plate developed under vanillin sulfuric acid reagent, these compounds did not present in F8-F10 and therefore were not being detected in GC/MS. Standard compounds of squalene and phytol isomers have been purchased to be spotted with the hexane partition extract and fractions as references. The squalene compound was only seen in F1 and F2.
6. Some of the compounds in hexane extract are weakly or not detected compared to fractions because during fractionation the extract was fractionated and concentrated. Besides, major compounds in hexane extract in this study could be present in other fractions.

Discussion

Second paragraph: Major chemical compounds of these fractions were then isolated and are summarised in Table 2.

- Please revise the term "isolated". I think the sentence should be "The major chemical compounds in the active fractions were listed in Table 2".

Response to reviewer:

The second paragraph of the Discussion section has been reworded accordingly.

Conclusions

I think the authors should revise the statement related to the detected compounds in the *n*-hexane partition and active fractions. I recommended to add further study such as isolation of compound from the active fractions.

Response to reviewer:

The Conclusion section has been rephrased.

Technical

n-hexane and *n*-butanol: the alphabet "n" should be written in italic (*n*-hexane, *n*-butanol, *n*-Hex, *n*-BuOH).

Please give a space between number and unit. For example, 20 g instead of 20g.

Please add a comma before the word "whereas". For example: "_____, whereas".

The term "GC-MS" should be written consistently either as "GC-MS" or "GC/MS".

The abbreviation of litre should be written as "L" in capital letter.

Response to reviewer:

Corrections have been made accordingly.

Competing Interests: No competing interests were disclosed.

Reviewer Report 01 June 2021

<https://doi.org/10.5256/f1000research.55440.r85583>

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Yusuf Andriana

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⁴ Indonesian Institute of Sciences, Jakarta, Indonesia

Hamzah *et al.* studied the isolation of active *Averrhoa bilimbi* phytochemicals corresponding to brown adipocytes stimulation. Before indexing, several points need to be clarified:

Abstract:

- The authors stated "This paper reports the successive isolation and purification of bioactive compounds from the leaf of bilimbi that corresponds to brown adipocyte activation".
- I think the authors couldn't say "isolation and purification" because in their study, they only yielded fraction (mixture of compounds) not pure compound. I suggest to use the term "fractionation".
- ".....bioactive compounds from the leaf of bilimbi that corresponds to brown adipocyte activation" - in this case the authors did not know exactly which compounds were responsible/linked to brown adipocyte activation. The authors just checked the mixture compounds (in F8,F9,F10), the major compounds in F8-10 didn't correspond with brown adipocyte activation, except the authors check single compounds in F8-10.

Introduction:

- Please add information about bioactive compounds that have been reported from *Averrhoa bilimbi*.

Methods:

- For Cell culture and adipocyte differentiation, Adipocyte determination by fluorescent dye staining, GC/MS analyses and identification of components were needed to add citations,

from where did the authors obtain the method, did the authors develop the methods by themselves?

- For GC-MS, the authors should explain why they use the HP5-MS column as the column was a non-polar column, it is better to use more general columns such as DB5-MS, because in Figure 3, ethanol extract seems to show lipid droplet formation upon similar with the standard (ROSI) and hexane extract. I mean maybe the polar compounds might be also responsible for this activity.

Results:

- In GC-MS result, it is better to present Retention Index, so please change retention time to retention index following NAST.
- In hexane extract, Phytol (14.82%), 9-Octadecanoic acid-Z- (19.69%), and Squalene (100.00%) (Table 1) - How can % area be more than 100%?
- It seems phytol, 9-Octadecanoic acid-Z-, Squalene were the major compounds, but why in the F8,9,10 they weren't detected?
- I think you should conduct GC-MS analysis at least twice and present as mean $\pm$ standard deviation, to make your research more producible.

Conclusion:

- Did you detect, squalene, and terbutaline in F8,9,10? There is no strong evidence to say that these compounds enhance lipolysis and thermogenesis of adipocyte tissues.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: natural product chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Nov 2021

Wai Kwan Lau

Reviewer 1

Hamzah *et al.* studied the isolation of active *Averrhoa bilimbi* phytochemicals corresponding to brown adipocytes stimulation. Before indexing, several points need to be clarified:

Abstract:

- The authors stated "This paper reports the successive isolation and purification of bioactive compounds from the leaf of bilimbi that corresponds to brown adipocyte activation".
- I think the authors couldn't say "isolation and purification" because in their study, they only yielded fraction (mixture of compounds) not pure compound. I suggest to use the term "fractionation".

".....bioactive compounds from the leaf of bilimbi that corresponds to brown adipocyte activation" - in this case the authors did not know exactly which compounds were responsible/linked to brown adipocyte activation. The authors just checked the mixture compounds (in F8,F9,F10), the major compounds in F8-10 didn't correspond with brown adipocyte activation, except the authors check single compounds in F8-10.

Response to reviewer:

The Title and Abstract sections have been reworded to extraction and fractionation accordingly. The Abstract has also been rewritten to better reflect the current findings of this study.

Introduction:

- Please add information about bioactive compounds that have been reported from *Averrhoa bilimbi*.

Response to reviewer:

This was included in the second paragraph of Discussion section where two references were cited (Gunawan *et al.*, 2013; Saini, 2016). "Major phytochemicals in the n-Hex partition

include hexadecenoic acid, phytol, 9-Octadecenoic acid (Z)- and squalene, which agree with earlier reports.”

Methods:

- For Cell culture and adipocyte differentiation, Adipocyte determination by fluorescent dye staining, GC/MS analyses and identification of components were needed to add citations, from where did the authors obtain the method, did the authors develop the methods by themselves?
- For GC-MS, the authors should explain why they use the HP5-MS column as the column was a non-polar column, it is better to use more general columns such as DB5-MS, because in Figure 3, ethanol extract seems to show lipid droplet formation upon similar with the standard (ROSI) and hexane extract. I mean maybe the polar compounds might be also responsible for this activity.

Response to reviewer:

1. References for cell culture, adipocyte differentiation and adipocyte determination methods (Sharma *et al*, 2014; Melhem *et al* 2013) have been included.
2. References for GC/MS method and analysis (Azeem, 2015; Suluvoy *et al*, 2017) have also been cited.
3. The GC/MS detection focused on hexane extract and fractions which in this study showed best biological activity for brown adipocyte activation. The non-polar column was used because hexane is a non-polar solvent which extract non-polar extracts and compounds. Please refer to Section 2.5 for further elaboration of the method.

Results:

- In GC-MS result, it is better to present Retention Index, so please change retention time to retention index following NAST.
- In hexane extract, Phytol (14.82%), 9-Octadecanoic acid-Z- (19.69%), and Squalene (100.00%) (Table 1) - How can % area be more than 100%?
- It seems phytol, 9-Octadecanoic acid-Z-, Squalene were the major compounds, but why in the F8,9,10 they weren't detected?
- I think you should conduct GC-MS analysis at least twice and present as mean $\pm$ standard deviation, to make your research more producible.

Response to reviewer:

The GC/MS results which were reported based on the database NIST-WILEY have been re-tabulated in Table 1.

Standard compounds of squalene and phytol isomers are purchased for this purpose. The standard compounds are spotted with the hexane partition extract and fractions on a TLC plate as shown in the figure below to confirm the GC/MS analysis. It is indicated that squalene and phytol compounds do not or minutely present in F8-F10 and therefore were not being detected in the GC/MS.

Hex F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 Phy Sq

Hex: n-Hex extract

F1 – F10: Fractions

Phy – Standard Phytol Isomers

Sq – Standard Squalene

Conclusion:

- Did you detect, squalene, and terbutaline in F8,9,10? There is no strong evidence to say that these compounds enhance lipolysis and thermogenesis of adipocyte tissues.

Response to reviewer:

These compounds have been removed from the Conclusion section and only their potential functions are being discussed in the Discussion section.

Competing Interests: No competing interests were disclosed.

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