BRIEF REPORT

Isolation of active *Averrhoa bilimbi* phytochemicals corresponding to brown adipocytes stimulation [version 1; peer review: 2 approved with reservations]

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v1 First published: 18 May 2021, 10:398
<https://doi.org/10.12688/f1000research.52197.1>
 Second version: 08 Dec 2021, 10:398
<https://doi.org/10.12688/f1000research.52197.2>
 Latest published: 18 Jan 2022, 10:398
<https://doi.org/10.12688/f1000research.52197.3>

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

This article is included in the Plant Science gateway.

Open Peer Review

Approval Status

	1	2
version 3		
(revision)		
18 Jan 2022		view
version 2		
(revision)		
08 Dec 2021		view
version 1		
18 May 2021	view	view

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Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the Fundamental Research Grant Scheme of the Ministry of Higher Learning of Malaysia (FRGS/1/2019/STG05/MOSTI/I4).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Hamzah MF, Amanah A and Lau WK. **Isolation of active Averrhoa bilimbi phytochemicals corresponding to brown adipocytes stimulation [version 1; peer review: 2 approved with reservations]** F1000Research 2021, 10:398 <https://doi.org/10.12688/f1000research.52197.1>

First published: 18 May 2021, 10:398 <https://doi.org/10.12688/f1000research.52197.1>

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 ethanolic 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, 200g of dried leaves was soaked in 15L 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 Microlitre Syringe (Hamilton, Bonaduz, 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). Rosiglitazone, a PPARgamma agonist with known browning activities on both classic white adipocytes and inducible brown adipocytes,

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

was used as the positive control.^{10,11} Adipocyte differentiation in the myoblast was initiated after the cells reached confluent by adding 0.5mM isobutyl methylxanthine (IBMX), 1µM dexamethasone (DEX), 1µg/ml insulin, and 1µM rosiglitazone (ROSI). Fresh differentiation medium containing DMEM, 10% FBS, 1µg/ml insulin and 1µM 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.

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.

2.5 GC/MS analyses and identification of components

The GC/MS analyses were carried out using the Agilent 5977A Series GC/MSD system with an HP-5ms ultra inert column (30 m × 0.25 mm id, 0.25 µm 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. Hexane fraction (1 µL, 1 mg/mL) and extract (3 µL, 10 mg/mL) diluted in n-Hex were injected into the GC/MS via an auto-injector. Phytochemical constituents were identified based on the comparison of their mass spectra of the MassHunter Library WION 14.L.

3. Results

3.1 Biological Assay-Guided Fractionation of Bilimbi Extract

Four partitioned extracts were in the yield: n-Hex, EtOAc, n-BuOH and water extracts. 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 plant extract with an equilibration solvent ratio of n-Hex and EtOAc (95:5). A Hi Flash Column with a size of 3L (46 × 130mm 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 1µM ROSI. A similar phenomenon was found in 200 µg/ml EtOH and 100 µg/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× 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 [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- (phenylseleno) -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, 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.

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 $\mu\text{g/ml}$ EtOH, (B) 1 μM ROSI, (C) 100 $\mu\text{g/ml}$ n-Hex, (D) 100 $\mu\text{g/ml}$ EtOAc, (E) 100 $\mu\text{g/ml}$ 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.

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

Figure 5. Mass spectrum of *A. bilimbi* n-Hex.

Figure 6. Mass spectrum of *A. bilimbi* n-Hex fraction f8.

Figure 7. Mass spectrum of *A. bilimbi* n-Hex fraction f9.

Figure 8. Mass spectrum 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
3.140	Benzene, 1,4-diethyl-	C10H14	0.34	119
3.675	2-Methyl-3-hydroxypyrrol-4(4H)-one	C6H6O3	0.11	126.1
5.156	N-Methyl-N-nitro-4-t-butylaniline	C11H16N2O2	0.06	147.1
5.342	(E)-(S)-(+)-6-Hydroxy-8-phenyl-2-pivaloxyoct-7-enyl pivalate	C24H36O5	0.11	131
5.569	Neopentyl 2,2-dimethylpropanoate	C10H20O2	0.11	71.1
5.723	5-Isopropyl-2-methylpyrrole-3-carbonitrile	C9H12N2	0.13	133
5.942	Naphthalene, 2-methyl-	C11H10	0.67	142.1
6.169	Naphthalene, 1-methyl-	C11H10	0.43	142.1
6.990	(3-Phenyl-2-propynylidene) cyclopropane	C12H10	0.06	154.1
7.331	Naphthalene, 1,4-dimethyl-	C12H12	0.55	156.1
7.524	Naphthalene, 2,3-dimethyl-	C12H12	0.50	156.1
7.655	Benzeneethanimidic acid, .alpha.-phenyl-, ethyl ester	C16H17NO	0.06	167
7.980	2-Naphthalenol, 1,2-dihydro-1,1-dimethyl-,acetate	C14H16O2	0.16	141.1
8.419	Benzeneethanimidic acid, .alpha.-phenyl-, ethyl ester	C16H17NO	0.10	168.1
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)	C17H24N2	0.07	162.1
8.936	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	C11H16O2	0.41	111.1
9.028	Octanoic acid	C8H16O2	0.08	73
9.532	Pentane, 3-bromo-	C5H11Br	0.02	71.1
10.073	2-Propanone, 1-(5-ethyl-2-furanyl)-	C9H12O2	0.09	109.1
10.644	2-Bromo-1-nonen-3-ol	C9H17BrO	0.08	123.1
10.821	Phenol, 2-(1-phenylethyl)-	C14H14O	0.08	71.1
10.956	1-Heptyl-1H-(1,2,3)-triazole-N-[[2'-(hydroxyethoxy)ethylamino]ethyl]-4-carboxamide	C14H27N5O2	0.05	74
11.825	Acetonyl decyl ether C13H26O2 8	C13H26O2	0.02	57.1
12.383	(2S,5R)-2-Isopropyl--5methylhept-6-en-1-ol	C11H22O	0.52	68.1
12.456	2-Pentadecanone, 6,10,14-trimethyl-	C18H36O	0.60	43
12.728	7,9-Dodecadien-1-ol, acetate, (E,Z)-	C14H24O2	0.17	81.1
13.231	(E,E)-6,10,14-trimethyl-5,9,13-pentadecatrien-2-one	C18H30O	0.20	69.1
13.646	(E,E)-6,10,14-trimethyl-5,9,13-pentadecatrien-2-one	C18H30O	0.08	69.1
13.691	Hexadecanoic acid, methyl ester	C17H34O2	0.28	74
14.336	Hexadecanoic acid	C16H32O2	7.87	73
17.794	Phytol	C20H40O	14.82	71.1
18.399	9-Octadecenoic acid (Z)-	C18H34O2	19.69	55.1
20.132	(2S,5R)-2-Isopropyl--5methylhept-6-en-1-ol	C11H22O	0.59	68.1
22.192	geranyl linalyl ester	C20H34O	0.13	69.1
23.396	geranyl linalyl ester	C20H34O	0.24	69.1
26.281	Di-(2-ethylhexyl) phthalate	C24H38O4	2.75	149.1
30.355	(E) and (Z)-7,8-Dimethoxycarbonyl-6,9-di(4-methoxyphenyl)-6,8-tetradecadiene	C32H42O6	0.03	69.1
32.864	Squalene	C30H50	100.00	69.1
35.158	Dodecane, 2,6,10-trimethyl-	C15H32	3.92	57.1
36.713	delta.-Tocopherol	C27H46O2	1.52	402.3
37.210	3-Cyclohexen-1-one, 4-(1,5,9-trimethyl-4,8-decadienyl)-, (Z)-(+)-	C19H30O	1.10	69 *

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

RT	Name	Molecular formula	Area %	Mass (DB)	m/z	Fraction
3.527	Nonanal	C ₉ H ₁₈ O	14.1, 19.8	142	57	9,10
3.935	Tri-o-trimethylsilyl, N-trifluoroacetyl derivative of Terbutaline	C ₂₃ H ₄₂ F ₃ NO ₄ Si ₃	38.1	537	73	8
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	11.8	257	175.2	10
5.581	3-nonen-2-one	C ₉ H ₁₆ O	95.7	140	43	8
5.815	1-[3-(Hydroxycyclohexenyl)]ethanone	C ₈ H ₁₂ O ₂	36.9	140	43	8
6.694	(1R*,2R*)-N,N-Diethyl-2-hydroxy-2-methyl-3-oxacyclopentanecarbamide	C ₁₁ H ₁₉ NO ₃	17.5	213	157.1	8
6.727	1-(N-Phenylcarbamoyl)-1,3-diphenylurea	C ₂₀ H ₁₇ N ₃ O ₂	18.1	331	93	8
8.326	Phenol, 2-(1-phenylethyl)-	C ₁₄ H ₁₄ O	8.9	198	57	9
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-methanocycloocta [d]pyridazine	C ₁₅ H ₂₆ N ₂	25.4, 8.7, 24.0	234	191	8,9,10
10.657	11-Acetoxy-8(12)-drimen-7.alpha.-ol	C ₁₇ H ₂₈ O ₃	6.7	280	123	9
10.833	Phenol, 2-(1-phenylethyl)-	C ₁₄ H ₁₄ O	4.8	198	85	9
11.628	2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-6-hydroxy-4,4,7a-trimethyl-, (6S-trans)-	C ₁₁ H ₁₆ O ₃	64.6	196	111.1	9
14.319	Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	67.3, 100	256	73	9,10
15.019	3-Acetyloxypropyl 2,3,4,6-tetra-O-methyl-.alpha.,L-(5-D) gulopyranoside	C ₁₅ H ₂₇ DO ₈	14.3	336	88	8
18.367	(12R)-(9Z)-12-hydroxy-9-octadecenoic acid	C ₁₈ H ₃₄ O ₃	100	298	55	9
19.061	spiro[2.4]heptane-5-carboxaldehyde	C ₈ H ₁₂ O	100	124	79	8
26.298	i-Propyl 3-(phenylamino)-2-(phenylseleno)-3-(phenyl)propanoate	C ₂₄ H ₂₅ NO ₂ Se	89.6,37.5, 47.4	439	149	8,9,10

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 phytocomponents that are responsible for the program. Major phytocomponents in the n-Hex partition include hexadecenoic acid, phytol, 9-Octadecenoic acid (Z)- and squalene, which agree with an earlier report.¹² 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.¹³ 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.¹⁴ (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.^{15,16} 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.¹⁷ 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.¹⁸

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 metabolites, including capsenoid, curcumin, quercetin, and resveratrol, without major adverse events.¹⁹ 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.^{20,21} 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%.²² 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 UCP1.²³ 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. Fatty acids that were found in the n-Hex extract and fractions are potential BAT thermogenic agents. Other major phytocompounds including phytol, squalene, and terbutaline, are believed to enhance lipolysis and thermogenesis of adipocyte tissues.

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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Version 1

Reviewer Report 08 July 2021

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

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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

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 reworded 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 reworded 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

¹ Graduate School for International Development and Cooperation (IDEC), Hiroshima University, Higashi-Hiroshima, Japan

² 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.

References

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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:

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".....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

- 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.

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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