SUPPLEMENTRY MATERIAL

GC-MS Analysis of Metabolites From Soxhlet Extraction, Ultrasound-Assisted Extraction and Supercritical Fluid Extraction of *Salacca zalacca* Flesh And Its Alpha-Glucosidase Inhibitory Activity

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Different extraction processes were employed to extract bioactive metabolites from *Salacca zalacca* flesh by a range of aqueous and organic solvents. The highest extraction yield was obtained by 50% ethanol extract of SE (73.18 ± 4.35%), whereas SFE_1 showed the lowest yield (0.42 ± 0.08%). All extracts were evaluated for *in vitro* α-glucosidase inhibitory activity, measured by their IC_{50} values in comparison to that of quercetin, the positive control (IC_{50} = 2.7 ± 0.7 μg/mL). The lowest α-glucosidase inhibitory activity was indicated by water extract of SE (IC_{50} = 724.3 ± 42.9 μg/mL) and the highest activity was demonstrated by 60% ethanol extract by UAE (IC_{50} = 16.2 ± 2.4 μg/mL). All extracts were analysed by GC-MS and identified metabolites like carbohydrates, fatty acids, organic acids, phenolic acids, sterols and alkane-based compounds etcetera that may possess the potential as α-glucosidase inhibitor and may attribute to the α-glucosidase inhibitory activity.

Keywords: *Salacca zalacca*; α-glucosidase; metabolite profiling.

EXPERIMENTAL

Plant material

Different parts (fruits, stems and leaves) of *Salacca zalacca* were collected from a *S. zalacca* farm at Sungai Udang, Melaka, Malaysia at the age of 5 months post pollination. The samples were kept in the herbarium of Kulliyyah of Pharmacy, International Islamic University Malaysia and was identified by voucher specimen no. PIIUM 0269.

Chemicals

The ethanol used in extraction was purchased from Merck KGaA (Massachusetts, United States). The α-glucosidase enzyme was purchased from Megazyme (Wicklow, Ireland), and the substrates 4-nitrophenyl α-D-glucopyranoside (pNPG) as well as the positive control; quercetin was purchased from Sigma-Aldrich (St. Louis, MO).

As for the sample preparation of GC-MS, the pyridine was supplied by Merck, N-methyl-N-(trimethylsilyl)trifluoroacetamidepurum (97%) (gas chromatography grade) and methoxyamine hydrochloride (98%) were purchased from Sigma-Aldrich (St. Louis, MO).

Sample preparation and extraction

Prior to extraction, the flesh was peeled, washed, cut into small pieces and stored at -80°C deep freezer. The frozen fruits were freeze dried to remove all moisture and later were ground into fine powder. All samples were stored at -80°C until further analysis.

Soxhlet Extraction (SE)

10 g of sample was weighed and transferred into a filter paper extraction thimble and loaded onto the main chamber of Soxhlet extractor. Subsequently, the sample was extracted by absolute ethanol, 50% ethanol and water for 6 hours. Rotary vacuum evaporator (Buchi, B-490, Postfach, Flawil, Switzerland) was used to remove the remaining solvent at 40°C and the remaining extracts were kept at −80°C.

Ultrasound-Assisted Extraction (UAE)

5 g of sample was immersed in 100 ml solvent (20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, 100% ethanol and water) in 250 ml conical flask. The sample material was sonicated at 40°C for 30 minutes. The extracts were filtered using Whatman paper no. 1 and solvent was evaporated using a rotary evaporator at 40°C prior to freeze-drying.

Supercritical Fluid Extraction (SFE)

The extraction by supercritical carbon dioxide was conducted using the Waters TAP SFE Biobotanical System (Milford, USA). 20 g of sample was loaded onto the extraction vessel. The extraction was performed for 90 minutes under following condition; CO_2 flow rate: 20 g/min, temperature: 50°C and 60°C, pressure: 300 bar and co-solvent: 10% ethanol.

All extracts were dried in a freeze dryer (Crist, Beta 1-8 LD Plus, Newtown, Wern, Shropshire, U.K.) to evaporate remaining solvent. All extracts were stored at -80°C for further analysis. The total extraction yields were determined in percentage using the following equation:

$$\textit{Extraction yield (\%)} = [(\textit{Mass of dry extract}) / (\textit{Initial mass of dry sample})] \times 100$$

***In vitro* α-Glucosidase Inhibition Study**

The assay was performed in 96-well microtiter plate using the method described by Sabina et al. (2016) with some modifications. A 5 mg extract was weighed and immersed in 1 mL of dimethyl sulfoxide (DMSO) to prepare the stock solution. 100 µL of 30 mM phosphate buffer (pH 6.5) was added to stock solution to obtain a final assay concentration of 200 µg/mL. 1 mg of quercetin was dissolved in 1 mL of DMSO to make a positive control (Subramanian et al. 2008). The α-glucosidase enzyme (α-glucosidase type 1 from *Saccharomyces cerevisiae*; Megazyme 130301) was dissolved in 50 mM potassium phosphate buffer (pH 6.5). The highest concentration of enzyme solution (0.02 U/well) was used and incubated for 5 min at room temperature. 6 mg of substrate (p-nitrophenyl-α-D-glucopyranose; Sigma-Aldrich,

N1377-5G) was weighed and diluted in 20 mL volume of 50 mM phosphate buffer (pH 6.5). Following that, substrate solution was added into wells and allowed to incubate at room temperature for 15 min. The enzymatic reaction was stopped by adding 2M glycine (pH 10), the stopper reagent. Upon addition of glycine, a bright yellow color was observed as the enzyme hydrolyzed the substrate (pNPG) into p-nitrophenol. The rate of reaction was directly proportional to the enzyme activity. According to the procedure, the 30 mM phosphate buffer (100 µL), plant extracts (10 µL), quercetin (10 µL), α-glucosidase enzyme (15 µL), substrate (75 µL) and glycine (50 µL) were added to a 96-well microtiter plate, to make up a final volume of 250 µL. The absorbance (Abs) was analyzed at 405 nm using a spectrophotometer (NanoQuant/Tecan, Infinite M 200) (Deutschländer et al. 2009). The inhibitory activity was calculated according to the following formula (Wang et al. 2010):

$$\% \text{ Inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

The highest inhibitory activity was further distinguished by analyzing the concentration required to inhibit 50% of the α-glucosidase activity (IC_{50}), which were established graphically. IC_{50} values of all extracts were calculated from the dose response curves by regression analysis and were reported as mean ± standard deviation of three independent assays and each independent experiment was conducted in triplicate.

Derivatization

Derivatization procedure was carried out prior to the analysis (Robinson et al. 2005). 25 mg of the sample extract was immersed in 50 µL of pyridine in a 2 mL centrifuge tube and was sonicated for 10 min at 30°C. After that, 100 µL of methoxyamine HCl (20 mg/mL in pyridine) was added into the sample solution, and then it was vortexed. The mixture was incubated for 2 hr at 60°C with an addition of 300 µL of MSTFA [N-Methyl-N-(trimethylsilyl) trifluoroacetamide] later, followed by another incubation at 60°C for 30 min. Lastly, the sample solutions was filtered and injected into the column for the analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed on derivatized samples of *S. zalacca* flesh extracts, using the Agilent's GC-MS system. A DB-5MS 5% phenyl methyl siloxane column with an inner diameter (ID) of 250 μm and a film thickness of 0.25 μm was used as the GC column for the analysis. The oven temperature was initially set at 160 °C for 5 min, and then increase to a target temperature of 315 °C in 5 min at a rate of 30 °C/min. Helium was used as the carrier gas at a fixed flow rate of 1 mL/min. The injector and ion source temperatures was adjusted to 330 and 250 °C respectively. Subsequently, mass spectra was acquired by a full scan and a monitoring mode with a mass scan range of 50 to 550 m/z after a 6 min of solvent delay. The spectra for each of the chromatogram peaks was then compared with those in the NIST14 database library and the retention time (RT) index of common primary and secondary metabolites. Both of the chromatogram and mass spectra was processed using Agilent ChemStation, Automated Mass Spectral Deconvolution and Identification System (AMDIS) and Agilent's Deconvoluted Reporting Software (DRS).

Statistical analysis

All data were expressed as mean ± SD. The results of extraction yield and IC_{50} of extracts were assessed with Minitab 16 (Minitab Inc., State College, PA) by one-way analysis of variance (ANOVA) with Tukey's comparison test. A 95% confidence interval was used and all data were considered significantly different when $P < 0.05$.

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TABLE S1: Comparison of extraction yield and α-glucosidase inhibitory activity of *S. zalacca* extracts under different extraction conditions

Extraction condition	Extraction yield (%)	α-Glucosidase inhibitory activity, IC_{50} (μg/mL)
SE_0% Ethanol	33.7 ± 3.9^{b}	724.3 ± 42.9^{e}
SE_50% Ethanol	73.18 ± 4.35^{g}	110.9 ± 12.5^{bc}
SE_100% Ethanol	50.37 ± 1.81^{de}	89.8 ± 6.0^{bc}
UAE_0% Ethanol	46.27 ± 3.64^{cd}	663.1 ± 48.3^{d}
UAE_20% Ethanol	57.28 ± 2.38^{f}	103.7 ± 8.2^{bc}
UAE_40% Ethanol	52.30 ± 1.60^{ef}	83.8 ± 11.0^{bc}
UAE_60% Ethanol	49.2 ± 2.63^{de}	16.2 ± 2.4^{a}
UAE_80% Ethanol	42.08 ± 1.62^{c}	23.8 ± 1.6^{a}
UAE_100% Ethanol	33.41 ± 2.18^{b}	19.9 ± 2.9^{a}
SFE_1 (No co-solvent, 50°C)	0.42 ± 0.08^{a}	27.9 ± 14.8^{a}
SFE_2 (10% Ethanol, 50°C)	0.67 ± 0.04^{a}	26.7 ± 1.9^{a}
SFE_3 (10% Ethanol, 60°C)	0.51 ± 0.04^{a}	17.6 ± 1.5^{a}
Quercetin	ND	2.7 ± 0.7^{a}

Each value in the table is represented as mean ± SD (n = 3).
Values in each column with different superscript letters are significantly different ($P < 0.05$). ND: Not determined.

TABLE S2: Metabolites identified in SE extracts

No	RT	Similarity Index (%)	Molecular weight	Molecular formula	Tentative metabolites	Relative extracts
1	20.032	90	180.16	$C_6H_{12}O_6$	D-Psicofuranose	SE_E50
2	20.638	87	180.16	$C_6H_{12}O_6$	D-(-)-Fructofuranose	SE_E50, SE_E100
3	25.719	91	180.16	$C_6H_{12}O_6$	L-(-)-Sorbose	SE_E50, SE_E100
4	26.89	95	180.16	$C_6H_{12}O_6$	α-D-Glucopyranose	SE_E50, SE_E100
5	27.056	90	180.16	$C_6H_{12}O_6$	D-Glucose	SE_E50, SE_E100
6	27.862	80	164.16	$C_6H_{12}O_5$	L-Rhamnose	SE_E50, SE_E100
7	28.308	99	170.12	$C_7H_6O_5$	Gallic acid	SE_E50
8	28.685	93	180.16	$C_6H_{12}O_6$	Beta-D-Glucopyranose	SE_E50, SE_E100
9	29.331	99	256.42	$C_{16}H_{32}O_2$	Palmitic acid	SE_E50, SE_E100
10	29.685	93	180.16	$C_6H_{12}O_6$	Myo-Inositol	SE_E50, SE_E100
11	30.457	99	282.46	$C_{18}H_{34}O_2$	11-Octadecenoic acid	SE_E50
12	30.657	99	284.48	$C_{18}H_{36}O_2$	Stearic acid	SE_E50
13	32.817	95	342.30	$C_{12}H_{22}O_{11}$	D-(+)-Trehalose	SE_E50, SE_E100
14	37.601	91	342.30	$C_{12}H_{22}O_{11}$	Sucrose	SE_E50, SE_E100
35	23.124	80	180.16	$C_6H_{12}O_6$	Beta-D-Galactofuranose	SE_E100

TABLE S3: Bioactive metabolites identified in active UAE extracts

No	RT	Similarity Index (%)	Molecular weight	Molecular formula	Tentative metabolites	Relative extracts
1	20.032	90	180.16	$C_6H_{12}O_6$	D-Psicofuranose	E60, E80, E100
2	20.638	87	180.16	$C_6H_{12}O_6$	D-(-)-Fructofuranose	E60, E80, E100
15	21.175	91	192.17	$C_6H_8O_7$	Citric acid	E60, E80
19	23.215	81	180.16	$C_6H_{12}O_6$	D-Psicopyranose	E80
16	23.387	94	180.16	$C_6H_{12}O_6$	D-(+)-Talofuranose	E60, E80
17	25.719	91	180.16	$C_6H_{12}O_6$	D-(-)-Fructose	E60, E80, E100
20	25.742	91	150.13	$C_5H_{10}O_5$	D-(+)-Xylose	E80
18	26.53	91	195.17	$C_6H_{13}NO_6$	Fructose oxime	E60, E100
4	26.89	95	180.16	$C_6H_{12}O_6$	α-D-Glucopyranose	E80
5	27.056	90	180.16	$C_6H_{12}O_6$	D-Glucose	E60, E80, E100
8	28.685	93	180.16	$C_6H_{12}O_6$	Beta-D-Glucopyranose	E60, E80, E100
9	29.331	99	256.42	$C_{16}H_{32}O_2$	Palmitic acid	E60, E80, E100
10	29.685	93	180.16	$C_6H_{12}O_6$	Myo-Inositol	E60, E80, E100
12	30.657	99	284.48	$C_{18}H_{36}O_2$	Stearic acid	E60, E100
13	32.817	95	342.30	$C_{12}H_{22}O_{11}$	D-(+)-Trehalose	E60, E80, E100
21	37.195	99	414.71	$C_{29}H_{50}O$	Beta-Sitosterol	E100
14	37.601	91	342.30	$C_{12}H_{22}O_{11}$	Sucrose	E60, E80, E100

TABLE S4: Bioactive metabolites identified in all SFE extracts

No	RT	Similarity Index (%)	Molecular weight	Molecular formula	Tentative metabolites	Relative extracts
15	21.175	91	192.12	$C_6H_8O_7$	Citric acid	SFE_2, SFE_3
3	25.719	91	180.16	$C_6H_{12}O_6$	L-(-)-Sorbose	SFE_2, SFE_3
4	26.89	95	180.16	$C_6H_{12}O_6$	α-D-Glucopyranose	SFE_2, SFE_3
5	27.056	90	180.16	$C_6H_{12}O_6$	D-Glucose	SFE_2, SFE_3
22	27.525	91	180.16	$C_6H_{12}O_6$	D-Galactose	SFE_2, SFE_3
23	27.925	98	164.16	$C_9H_8O_3$	4-Coumaric acid	SFE_2, SFE_3
7	28.308	99	170.12	$C_7H_6O_5$	Gallic acid	SFE_2, SFE_3
9	29.331	99	256.42	$C_{16}H_{32}O_2$	Palmitic acid	SFE_2, SFE_3
10	29.685	93	180.16	$C_6H_{12}O_6$	Myo-Inositol	SFE_2, SFE_3
24	30.017	99	180.16	$C_9H_8O_4$	Caffeic acid	SFE_2, SFE_3
11	30.457	99	282.46	$C_{18}H_{34}O_2$	11-Octadecenoic acid	SFE_2
25	30.474	95	280.45	$C_{18}H_{32}O_2$	Linoleic acid	SFE_2, SFE_3
26	30.508	99	282.46	$C_{18}H_{34}O_2$	Oleic acid	SFE_2, SFE_3
12	30.657	99	284.48	$C_{18}H_{36}O_2$	Stearic acid	SFE_2, SFE_3
27	30.731	70	282.46	$C_{18}H_{34}O_2$	9-Octadecenoic acid	SFE_2
28	31.36	99	338.65	$C_{24}H_{50}$	Tetracosane	SFE_2, SFE_3
29	31.703	83	180.16	$C_6H_{12}O_6$	D-Lactose	SFE_2

13	32.817	95	342.30	$C_{12}H_{22}O_{11}$	D-(+)-Trehalose	SFE_2, SFE_3
30	33.023	95	240.47	$C_{17}H_{36}$	Heptadecane	SFE_2, SFE_3
31	34.103	99	394.76	$C_{28}H_{58}$	Octacosane	SFE_2, SFE_3
32	35.418	99	386.65	$C_{27}H_{46}O$	Cholesterol	SFE_1, SFE_2, SFE_3
33	36.38	99	400.68	$C_{28}H_{48}O$	Campesterol	SFE_1, SFE_2, SFE_3
34	36.561	94	412.69	$C_{29}H_{48}O$	Stigmasterol	SFE_1, SFE_2, SFE_3
21	37.195	99	414.71	$C_{29}H_{50}O$	Beta-Sitosterol	SFE_1, SFE_2, SFE_3
14	37.601	91	342.30	$C_{12}H_{22}O_{11}$	Sucrose	SFE_2, SFE_3
36	32.143	93	240.48	$C_{17}H_{36}$	Heptadecane	SFE_1
37	32.56	93	308.59	$C_{22}H_{44}$	1-Docosene	SFE_1
38	33.00	93	282.56	$C_{20}H_{42}$	Eicosane	SFE_1
39	31.908	98	390.56	$C_{24}H_{38}O_4$	Di-N-Octyl phthalate	SFE_1

TABLE S5: The composition of identified metabolites of all extracts

	Percentage of bioactive metabolites present in all extracts (%)		
Type of compounds	**SE**	**UAE**	**SFE**
Carbohydrates	73	76	28
Fatty acids	20	12	21
Phenolic acids	7	-	7
Organic acids	-	6	10
Sterols	-	6	14
Alkane	-	-	21

TABLE S6: Reported bioactive metabolites with α-glucosidase inhibitory activity

No	Compound	IC_{50} (μg/ml)	Plant	Reference
1	Citric acid	20 (100% inhibition)	*Rosa laevigata*	Ye et al. 2010
2	Palmitic acid	> 400 8.9 ± 2.1 (per 10 μg/ml) 21.3	*Weston wheat germ* *Taxus sumatrana* *Arctium lappa L.*	Liu et al. 2011 Artanti et al. 2012 Miyazawa et al. 2005
3	Stearic acid	5.3 ± 1.6 (per 10 μg/ml) 22.2	*Taxus sumatrana* *Arctium lappa L.*	Artanti et al. 2012 Miyazawa et al. 2005
4	Linoleic acid	> 200 17.9	*Weston wheat germ* *Arctium lappa L.*	Liu et al. 2011 Miyazawa et al. 2005
5	Oleic Acid	> 400 97.4 ± 5.3 (per 10 μg/ml) 3.43 64.2	*Weston wheat germ* *Taxus sumatrana* *Stichopus japonicas* *Arctium lappa L.*	Liu et al. 2011 Artanti et al. 2012 Nguyen & Kim 2015 Miyazawa et al. 2005
6	9-Octadecenoic acid	4.2	*Stichopus japonicas*	Nguyen & Kim 2015
7	Gallic acid	0.88	*Terminalia superba*	Wansi et al. 2007
8	Stigmasterol	34.2 ± 3.7 (% inhibition) at 50 μg/ml	*Dillenia indica*	Kumar et al. 2013
9	Beta-Sitosterol	52.5 ± 2.4 (% inhibition) at 50 μg/ml	*Dillenia indica*	Kumar et al. 2013

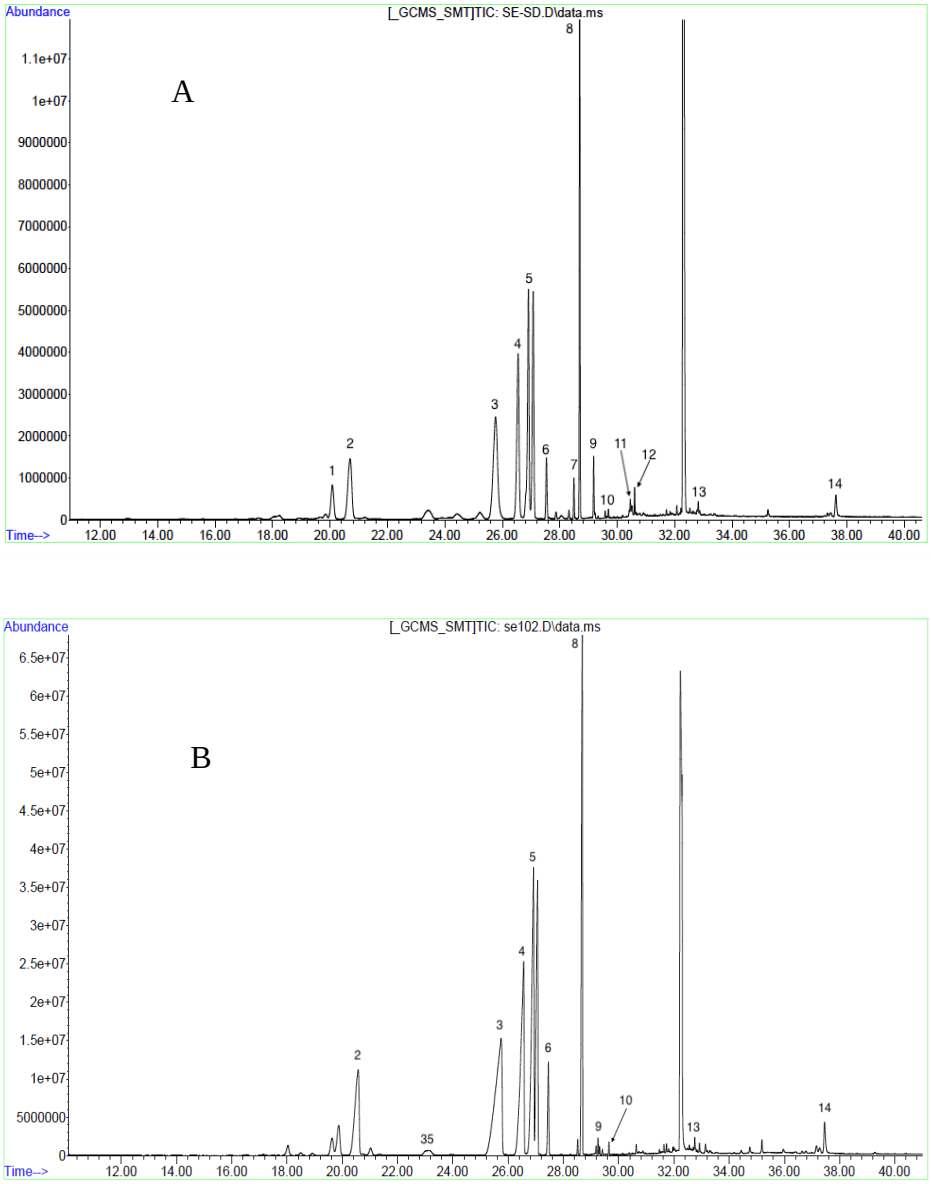

FIGURE S1(a): GC-MS chromatogram (TIC) of metabolites in SE_E50 (A) and SE_E100 (B) extracts from flesh of *S. zalacca*

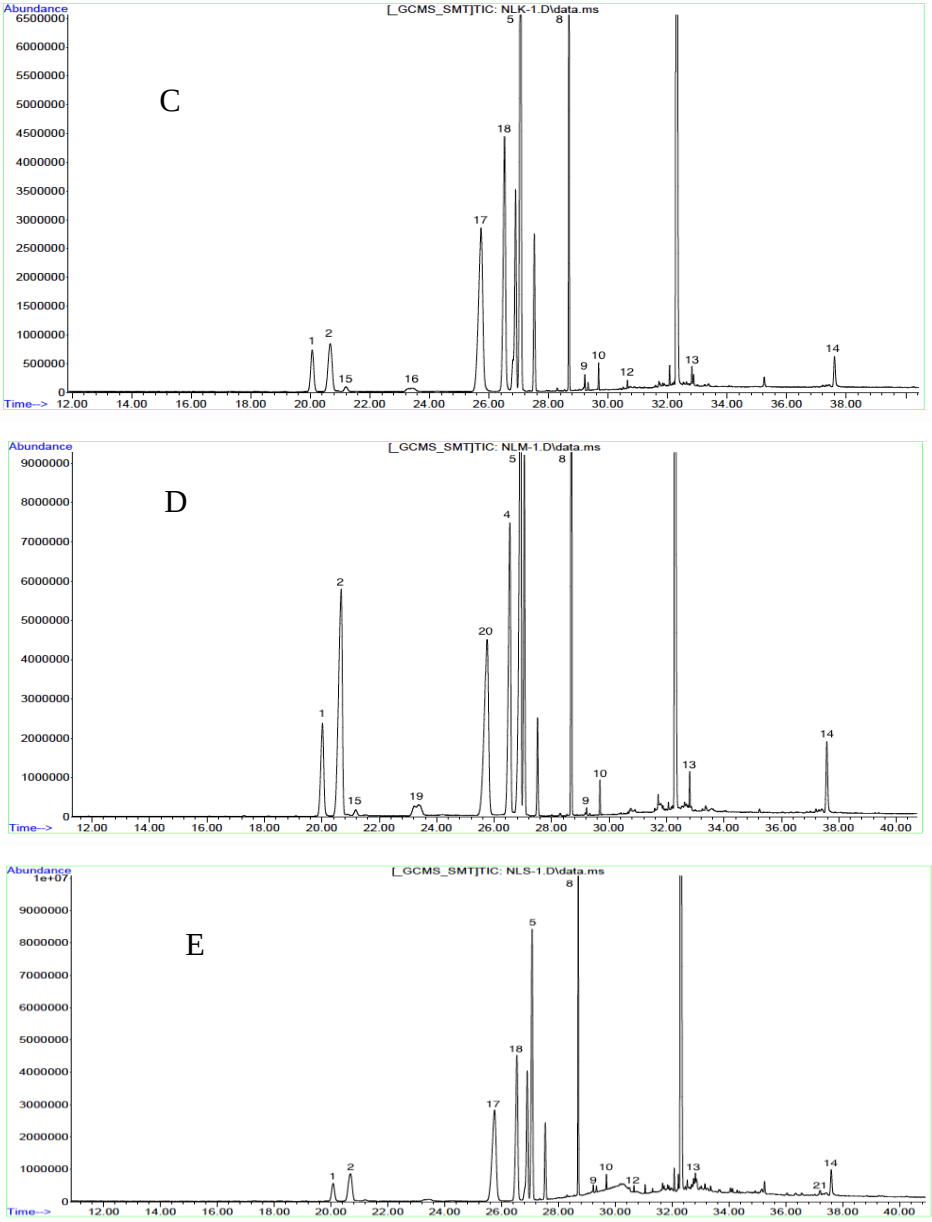

FIGURE S1(b): GC-MS chromatogram (TIC) of metabolites in UAE_E60 (C), UAE_E80 (D) and UAE_E100 (E) extracts from *S. zalacca*

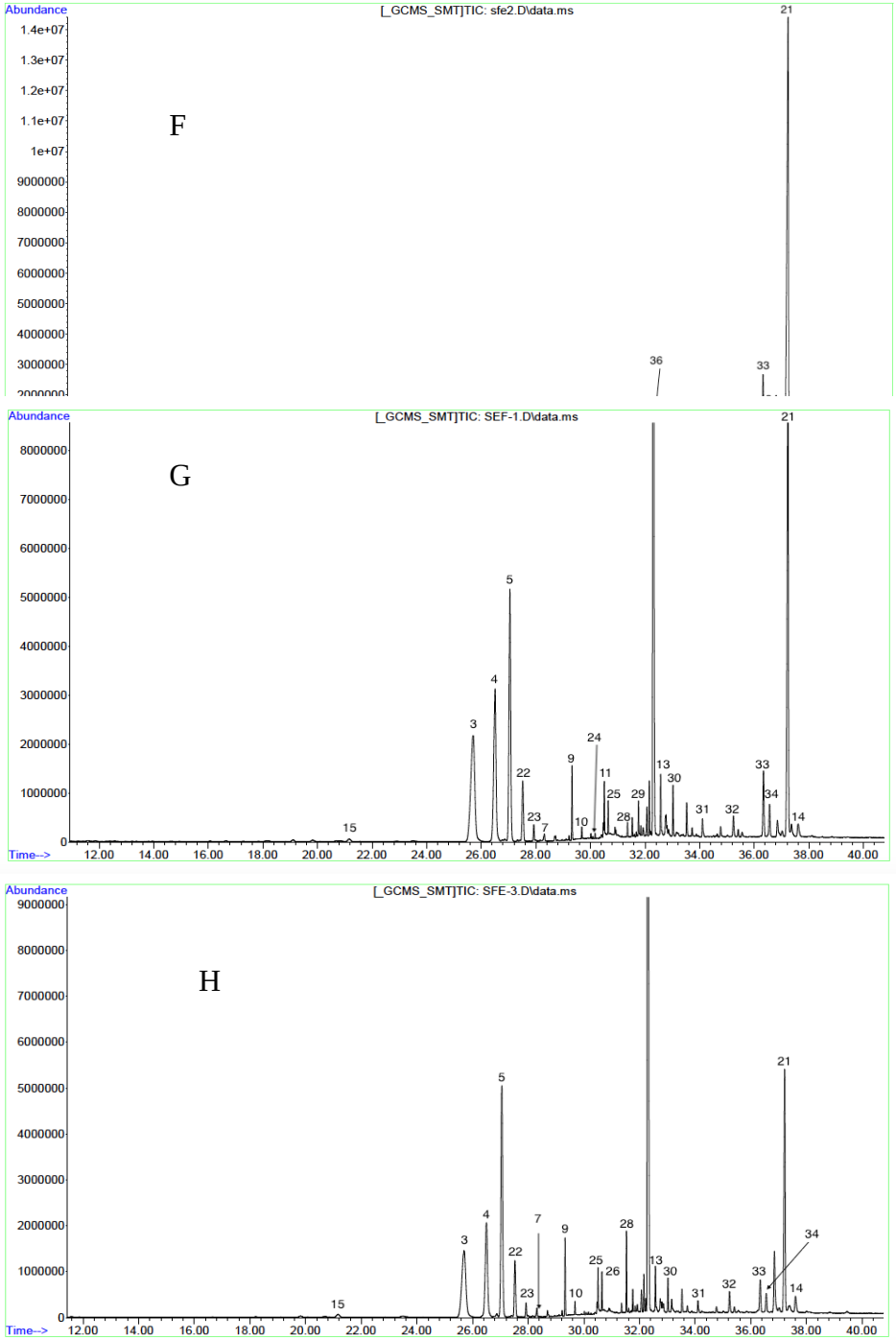

FIGURE S1(c): GC-MS chromatogram (TIC) of metabolites in SFE_1 (F), SFE_2 (G) and SFE_3 (H) extracts from *S. zalacca*.