

Effects of aerosol formulation to amino acids and fatty acids contents in Haruan extract

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Abstract: Haruan (*Channa striatus*) extract was formulated to aerosol for wound and burn treatment. Haruan extract is containing amino acids and fatty acids that important for wound healing process. The purpose of this study is to observe the effect of formulation and other excipients in the formula to amino acids and fatty acids content in Haruan extract before and after formulated into aerosol. Precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) method is used for amino acids analysis. Fatty acids in Haruan extract were esterified using transesterification method to form FAMES before analyzed using GC. Boron trifluoride-methanol reagent is used for transesterification. Tyrosine and methionine concentrations were different after formulated. The concentrations were decrease. There are six fatty acids have amount that significantly different after formulated into concentrate and aerosol. Contents of these fatty acids were increase. Generally, fatty acids which had content increased after formulated were the long-chain fatty acids. This might be happen because of chain extension process. Saponification and decarboxylation would give the chain extended product. Therefore contents of long-chain fatty acids were increase. Generally, the aerosol formulation did not affect the amino acids concentrations in Haruan extract while some long-chain fatty acids concentrations were increase after formulated into concentrate and aerosol.

Keywords: Aerosol, Haruan, *Channa striatus*, amino acids, fatty acids.

INTRODUCTION

According to Mat Jais (2007) and Zuraini (2006) Haruan contained protein ($78.32 \pm 0.23\%$), lipids ($2.08 \pm 0.08\%$) and 0.265 ± 0.013 mg vitamin A (an essential factor for wound healing) per total lipid. The major amino acids in Haruan were glutamic acid, aspartic acid and lysine, ranging from 9.7% to 21.7%. The lipids were categorised into phospholipids, partial glyceride, cholesterol, fatty alcohol, triglyceride and cholesterol ester. It also has a high content of arachidonic acid (AA) 20:4n-6, and docosahexaenoic acid (DHA) 22:6n-3. Flesh of Haruan contain high arachidonic acids (AA) but almost no eicosapentaenoic acids (EPA), which were hypothesised involved in initiating wound repair of tissue (Mat Jais *et al.*, 1994). Haruan contained 16.2 ± 0.1 % lipid, 1.2% ω -3, 16% ω -6 (Salimon and Rahman, 2008). Omega-3 and omega-6 were required in wound healing process. Generally, the fatty acids of the Malaysian Freshwater fish (included Haruan) showed a higher unsaturated, rather than saturated acid content. The ratio of unsaturated/saturated ranged from 1.2 to 2.3. Haruan has lipid content 2.10g/100g fillet, ratio unsaturated: saturated 1.28 (Endinkeau and Kiew, 1993).

Amino acids in Haruan extract were analysed in this study using high performance liquid chromatography (HPLC) method for separation techniques. There are two

derivatization techniques for spectroscopic detection i.e. postcolumn and precolumn derivatization. Postcolumn techniques should be run on line for maximum accuracy while precolumn techniques can be run either off line or on line. Numerous derivatizing agents have been developed for both techniques (Callejón *et al.*, 2010). Some of them are phenylisothiocyanate (PITC) (Marrubini *et al.*, 2008; Ponce-Soto *et al.*, 2007; Mat Jais *et al.*, 1994; Heinrikson and Meredith, 1984), 9-fluorenylmethyl-chloroformate (FMOC-Cl) (López-Cervantes *et al.*, 2006), o-phthalaldehyde (OPA) (Bartolomeo and Maisano, 2006), ninhydrin (Zakaria *et al.*, 2007; Zuraini *et al.*, 2006; Vidotti *et al.*, 2003), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Callejón *et al.*, 2008; Gam *et al.*, 2005; Hernández-Orte *et al.*, 2003; Wandelen *et al.*, 1997; Díaz *et al.*, 1996), 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) (Bryan *et al.*, 1999), butylisothiocyanate (BITC), 4-dimethyl-aminoazobenzene-4-sulfonyl chloride (DBS) and 1-dimethylamino-naphthalene-5-sulfonyl chloride (Dansyl-Cl) (Aristoy and Toldra, 2004). AQC which introduced by Cohen and Michaudis is the best derivatizing agent for precolumn derivatization techniques which reacts with primary and secondary amines from amino acids, peptides and proteins, broad optimum pH, yielding very stable derivatives with fluorescent properties, which are easily separated by reverse-phase HPLC, no interfere of excess reagent and reaction time is short (Aristoy and Toldra, 2004; Hernández-Orte *et al.*, 2003; Cohen and Michaud, 1993). Precolumn derivatization with 6-aminoquinolyl-N-

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hydroxysuccinimidyl carbamate method reported by Cohen (2005) is used in this study. There are 16 amino acids in Haruan extract which could be analysed using acid hydrolysis. Cystine has to be oxidized with performic acid prior to acid hydrolysis while tryptophane on the other hand has to be hydrolysed using alkali prior to analysis.

Fatty acids should be converted into fatty acid methyl esters (FAMES) for GC analysis. Fatty acids in Haruan extract were esterified using transesterification method to form FAMES. Boron trifluoride-methanol reagent (12-14%) is the most often used for transesterification of all types of lipids (AOAC, 2000; Eder, 1995; Liu, 1994). Derivatization of fatty acids for GC analysis is carried out to increase the volatility of the substances, to improve separation and to reduce tailing (Brondz, 2002; Liu, 1994). GC is the most widely used technique for determining the fatty acid profile (Petrovic *et al.*, 2010).

The purpose of this study is to observe the effect of formulation and other excipients in the formula of amino acids and fatty acids content in Haruan extract before and after formulated into aerosol.

MATERIALS AND METHODS

Materials, reagents and solvents

Haruan extract was supplied by Major Interest Sdn. Bhd. (Malaysia). Reagents and solvents used for amino acids

analysis were hydrochloric acid (R&J Chemicals, Malaysia), α -aminobutyric acid (AABA) (Sigma-Aldrich, USA), Waters AccQ Fluor reagent (Borate buffer, AQC powder, Acetonitrile), C-18 AccQ-Tag column (3.9 $\times$ 150 mm), Waters AccQ Concentrated Eluent A, Amino acid standard Pierce (ThermoFisher, USA), Acetonitrile (HPLC grade) (J&T Bakers, Germany). Reagents and solvents used for fatty acids analysis were GC Column 19091J-436 HP-5, 60 meters, ID 0.250 mm, film 0.25 μ m, serial No. US8620925J (Made in USA), FAME mixture (catalog No. 47885-U) (Supelco, USA), potassium hydroxide and anhydrous sodium sulphate (R&M Chemicals, UK), commercially 50% boron trifluoride in methanol (Merck, Germany), dichloromethane (Fluka, USA), methyl nonadecanoate (Fluka, USA), hydrogen and helium gas (MOX, Malaysia). Samples for amino acid analysis were 1 g Haruan extract, 20 g concentrate of formula E2 (contained 1 g Haruan extract) and 20 g aerosol of formula E2 (contained 1 g Haruan extract). Samples for fatty acid analysis were 200 mg freeze-dried Haruan extract from 5 g Haruan extract, 100 g concentrate and 100 g aerosol. Concentrate and aerosol were formed as film prior used in analysis.

Amino acids analysis

Hydrolysis Samples

1 gram (contain protein approximately 40 mg) Haruan extract was hydrolysed with 5 ml HCl 6 M in an electric oven for 24 hours at 110°C. The sample was then cooled to room temperature and 400 μ l of internal standard

Table 1: Instrument and experimental condition of HPLC for amino acid analysis

Instrument	Water System (e2695 separation module) equipped with quaternary pump delivery system, auto sampler, column heater
Detector	Waters 2475 Multi λ fluorescence, λ_{em} = 248 nm, λ_{ex} = 395 nm
Processing Software	Empower System
Injection volume	5 μ L
Column	AccQ-Tag column, 3.9 μ m, 150 x 4.6 mm (Waters, USA)
Column Temperature	38°C
Mobile phase	Eluent A (acetate phosphate buffer), Eluent B (acetonitrile), Eluent C (Milli-Q water)
Flow rate	1 mL/min

Table 2: Gradient table for amino acids analysis using HPLC

Time (Min)	Flow rate (ml/min)	%A	%B	%C	Curve
Initial	1.0	100.0	0.0	0.0	-
0.50	1.0	99.0	1.0	0.0	6
18.0	1.0	95.0	5.0	0.0	6
19.0	1.0	91.0	9.0	0.0	6
29.5	1.0	83.0	17.0	0.0	6
33.0	1.0	0.0	60.0	40.0	11
36.0	1.0	100.0	0.0	0.0	11

solution (AABA, 2.5 $\mu\text{mole/ml}$) was added. The content of the tube were quantitatively transferred to a 100 ml clean volumetric flask and diluted to 100 ml with distilled water. After through mixing, 1 ml of dilute sample was filtered, and 10 μl filtrate was placed in a derivatizing tube (Cohen, 2005; Gam *et al.*, 2005).

Derivatization of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)

Ten microliters filtrate of the above acid hydrolysis was added with 70 μl of AccQ Fluor Borate Buffer (Waters Corporation, USA). The sample tube was vortexed briefly prior to adding of 20 μl of reconstituted AccQ Fluor Reagent to the sample tube. After vortexing for several seconds, the sample tube was left to stand for 1 minute at room temperature and then heated in oven at 50°C for 10 min. The mixture was transferred to LVI vial before injection to HPLC (Callejón *et al.*, 2008; Cohen, 2005; Wandelen *et al.*, 1997).

Fatty acids analysis

Base hydrolysis (saponification) and esterification process to produce fatty acid methyl esters (FAMES)

Haruan extract was freeze-dried in a Vacuum Freeze Dryer (Hetovac) prior used as sample for fatty acids analysis. Approximately 200 mg freeze-dried Haruan extract (equal with 5g liquid Haruan extract) was weighed accurately and put into a hydrolysis tube and 2 ml 0.5 M methanolic-potassium hydroxide was added. The mixture was refluxed for 10 to 15 minutes (until the oil globules have gone). Two millilitres of a 14% boron trifluoride in methanol solution was added and boiling was continued for another 5 minutes. Two millilitres of dichloromethane was added and boiling was continued for further 2 minutes. The boiled contents were allowed to cool and 3 ml of saturated NaCl was added. The tube was then stoppered and was shaken vigorously for 15 seconds while solution was still tepid. The content was let to settle and the organic layer was taken and transferred to other test tube. A small amount of anhydrous sodium sulphate was added and the mixture was then shaken well to clear the sample. The clear solution (organic layer) was transferred to vial and added with 200 μl of internal standard solution. Mixture of the sample and internal standard in vial was dried under nitrogen. The dried sample was reconstituted with 1 ml dichloromethane prior analysis. A 1 μl of the reconstituted sample was injected to GC apparatus equipped with Flame Ionization Detector (FID) (AOAC, 2000; Eder, 1995; Liu, 1994).

For concentrate and aerosol, prior to the analysis, fatty acids were extracted from the sample using 30 ml chloroform for 72 hours at room temperature. Films and non-lipid material were removed by filtration. Chloroform was removed in a stream of nitrogen prior to the analysis (Petrovic *et al.*, 2010; Basconcillo and McCarry, 2008; Bond *et al.*, 2005; Seppänen-Laakso and Laakso, 2002).

Column specification and optimization of instrumental conditions

Table 3: Experimental condition of GC-FID to analyze FAMES

Column	GC Column J&W Scientific Agilent Technologies 19091J-436 HP-5, 60 meters, ID 0.250 mm, film 0.25 μm , Serial No. US8620925J (Made in USA)
Inlet temperature	300°C
Injection volume	1 μl
Split ratio	1:50
Carrier gas	Helium
Head pressure	Constant pressure mode 230 kPa at 50°C, 33 cm/s at 50°C
Oven temperature	50°C for 2 min, 15 °C/min to 225°C, 10°C /min to 300°C, hold for 0.5 min
Detector temperature	320°C
Detector gases	Hydrogen: 60 kPa Air: 50 kPa Helium make up gas: 100 and 230 kPa

RESULTS

Amount of amino acid before and after formulated into aerosol have tabulated in table 4. Only tyrosine and methionine concentrations were different after formulated into concentrate and aerosol.

Amount of fatty acid before and after formulated into aerosol have tabulated in Table 5. There are six fatty acids have amount that significantly different after formulated into concentrate and aerosol i.e. lauric acid, arachidic acid, eicosapentaenoic acid, erucic acid, lignoceric acid and nervonic acid.

DISCUSSION

Haruan extract contain amino acids and fatty acids that important for wound healing process. Haruan extract have been formulated into aerosol for drug delivery system to wound and burn treatment (Febriyenti *et al.*, 2011; Febriyenti *et al.*, 2008). Evaluation of the film properties from concentrate of aerosol had been done in other study (Febriyenti *et al.*, 2010). A good formulation should not affect the quantity of the active ingredient. The quantity of the active ingredient should be the same before and after formulated. In Table 4, concentration of tyrosine and methionine were decrease after formulated. Generally, the aerosol formulation did not affect the amino acids concentrations in Haruan extract.

Contents of six fatty acids in Haruan extract were increase after formulated. Generally, fatty acids which had content

increased were the long-chain fatty acids. This might be because of chain extension process. This process might be due to the extraction process of fatty acids from samples prior to analysis and other compound in formula. Saponification and decarboxylation would give the chain extended product. This is an efficient route to C₂₀ polyenes, not easily isolated from natural sources, starting from readily available C₁₈ sources (Basconcillo and McCarry, 2008; Bond et al., 2005; Scrimgeour, 2005;

Seppänen-Laakso and Laakso, 2002). Therefore, the content of long-chain fatty acids was increased.

Omega-3 and omega-6 fatty acids were important fatty acids in wound healing process. Omega-6 is required for production of prostaglandin which could induce inflammation in the early phase of wound healing process. But if the omega-6 is too many, it could prolong the inflammatory phase and prolong the wound healing

Table 4: Amount of amino acid in Haruan extract, concentrate and aerosol formula E2, Mean $\pm$ SD, n = 6

No.	Amino Acid	Amount (mg) amino acid/1 g Haruan extract in:		
		Haruan extract	Concentrate	Aerosol
1	Aspartic acid	2.5558 ^a $\pm$ 0.6183	2.6123 ^a $\pm$ 1.2239	2.0178 ^a $\pm$ 0.5653
2	Serine	1.4251 ^a $\pm$ 0.3031	1.5183 ^a $\pm$ 0.6835	1.2946 ^a $\pm$ 0.2541
3	Glutamic acid	4.5565 ^a $\pm$ 1.1293	4.6910 ^a $\pm$ 2.2730	3.7010 ^a $\pm$ 1.0491
4	Glycine	6.0078 ^a $\pm$ 1.1711	6.2085 ^a $\pm$ 2.9078	5.5486 ^a $\pm$ 1.0066
5	Histidine	0.5529 ^a $\pm$ 0.1344	0.5349 ^a $\pm$ 0.2838	0.4847 ^a $\pm$ 0.1101
6	Arginine	3.0240 ^a $\pm$ 0.5615	3.8922 ^a $\pm$ 1.6661	2.5202 ^a $\pm$ 0.7248
7	Threonine	1.1298 ^a $\pm$ 0.2686	1.2675 ^a $\pm$ 0.5903	1.0373 ^a $\pm$ 0.2207
8	Alanine	3.3387 ^a $\pm$ 0.7086	3.5479 ^a $\pm$ 1.5780	2.9272 ^a $\pm$ 0.6421
9	Proline	2.9134 ^a $\pm$ 0.5873	2.9181 ^a $\pm$ 1.5027	2.6022 ^a $\pm$ 0.5700
10	Tyrosine	0.2410 ^b $\pm$ 0.0994	0.0633 ^a $\pm$ 0.0790	0.0127 ^a $\pm$ 0.0140
11	Valine	0.9574 ^a $\pm$ 0.2373	1.0872 ^a $\pm$ 0.5303	0.8674 ^a $\pm$ 0.2174
12	Methionine	0.6972 ^b $\pm$ 0.2128	0.2421 ^a $\pm$ 0.1715	0.1962 ^a $\pm$ 0.0716
13	Lysine	2.0568 ^a $\pm$ 0.5342	2.3595 ^a $\pm$ 1.0632	1.7373 ^a $\pm$ 0.4604
14	Isoleucine	0.6725 ^a $\pm$ 0.1810	0.7645 ^a $\pm$ 0.3784	0.6004 ^a $\pm$ 0.1556
15	Leucine	1.6501 ^a $\pm$ 0.4390	1.8740 ^a $\pm$ 0.8724	1.4830 ^a $\pm$ 0.3655
16	Phenylalanine	1.0120 ^a $\pm$ 0.2505	1.0829 ^a $\pm$ 0.4948	0.9325 ^a $\pm$ 0.1837

Means within a row with a different letter are significantly different (P<0.05)

Table 5: Amount of fatty acid in Haruan extract, concentrate and aerosol formula E2, Mean $\pm$ SD, n = 6

No.	Fatty Acid		Amount (μ g) fatty acid / 5 g Haruan extract in:		
			Haruan extract	Concentrate	Aerosol
1	Lauric Acid	C12:0	1.84 ^a $\pm$ 0.80	4.81 ^b $\pm$ 2.05	4.91 ^b $\pm$ 2.39
2	Myristic Acid	C14:0	26.69 ^a $\pm$ 12.26	37.68 ^a $\pm$ 18.39	41.57 ^a $\pm$ 26.13
3	Myristoleic Acid	C14:1	16.08 ^a $\pm$ 4.37	13.67 ^a $\pm$ 5.45	11.77 ^a $\pm$ 5.29
4	Palmitic Acid	C16:0	246.83 ^a $\pm$ 100.35	385.39 ^a $\pm$ 232.37	360.20 ^a $\pm$ 231.24
5	Palmitoleic Acid	C16:1	75.32 ^a $\pm$ 32.99	72.45 ^a $\pm$ 67.64	73.80 ^a $\pm$ 73.56
6	Heptadecanoic Acid	C17:0	22.45 ^a $\pm$ 6.95	15.50 ^a $\pm$ 8.89	17.68 ^a $\pm$ 9.16
7	Heptadecenoic Acid	C17:1	5.41 ^a $\pm$ 2.86	3.49 ^a $\pm$ 1.56	6.61 ^a $\pm$ 5.69
8	Stearic Acid	C18:0	53.55 ^b $\pm$ 37.84	9.15 ^a $\pm$ 3.47	11.66 ^a $\pm$ 5.67
9	Elaidic Acid (trans)	C18:1n-9	17.49 ^a $\pm$ 5.74	32.72 ^a $\pm$ 18.06	31.27 ^a $\pm$ 19.44
10	Oleic Acid (cis)	C18:1n-9	36.53 ^a $\pm$ 10.81	32.58 ^a $\pm$ 14.21	45.83 ^a $\pm$ 29.21
11	Arachidic Acid	C20:0	4.11 ^a $\pm$ 1.97	30.32 ^b $\pm$ 9.63	27.83 ^b $\pm$ 14.01
12	Eicocenoic Acid	C20:1	6.71 ^a $\pm$ 2.52	11.77 ^a $\pm$ 5.96	12.93 ^a $\pm$ 9.01
13	Arachidonic Acid	C20:4n-6	48.22 ^a $\pm$ 20.25	30.63 ^a $\pm$ 20.28	29.08 ^a $\pm$ 22.99
14	Eicosapentaenoic Acid	C20:5n-3	12.44 ^a $\pm$ 4.62	37.10 ^b $\pm$ 14.06	35.36 ^b $\pm$ 18.06
15	Erucic Acid	C22:1n-9	2.86 ^a $\pm$ 1.76	23.05 ^c $\pm$ 8.41	14.07 ^b $\pm$ 2.26
16	Docosahexanoic Acid	C22:6n-3	28.59 ^a $\pm$ 8.87	44.38 ^a $\pm$ 25.75	35.62 ^a $\pm$ 23.22
17	Lignoceric Acid	C24:0	3.33 ^a $\pm$ 1.25	68.95 ^b $\pm$ 40.44	74.02 ^b $\pm$ 29.80
18	Nervonic Acid	C24:1	5.33 ^a $\pm$ 1.79	58.73 ^b $\pm$ 21.92	54.43 ^b $\pm$ 35.37

Means within a row with a different letter are significantly different (P<0.05)

Table 6: Five major rating of fatty acids in Haruan from references and in Haruan extract

No.	(Mat Jais <i>et al.</i> , 1998)	(Mat Jais <i>et al.</i> , 1994)	(Zuraini <i>et al.</i> , 2006)	(Endinkeau and Kiew, 1993)	(Salimon and Rahman, 2008)	Haruan extract
	Mucus	Fillet	Fillet	Fish oil	Fillet	Water extract
1.	C18:2n-6	C16:0	C16:0	C16:0	C18:1	C16:0
2.	C18:1n-9	C22:6n-3	C20:4n-6	C18:1	C16:0	C16:1
3.	C16:0	C18:1	C18:0	18:4n-6	C18:2n-6	C18:0
4.	C18:0	C20:4n-6	C22:6n-3	C18:0	C16:1	C20:4n-6
5.	C20:4n-6	C18:0	C18:1n-9	18:6n-3	C14:0	C18:1n-9
% of total fatty acid						
Omega-3	2.05	17.72	15.18	2.66	1.24	6.68
Omega-6	34.00	12.70	19.02	4.33	15.13	7.86

process. Omega-3 is required to decrease production of prostaglandin (Calder, 2005; Simopoulos, 2002). The results in Table 6 showed that Haruan extract contained omega-6 more than omega-3. Ratio between omega-3 and omega-6 fatty acids was 0.85. Omega-6 fatty acid is needed as inflammatory inducer in the early phase of wound healing process.

CONCLUSION

Aerosol formulation did not affect the amino acids concentrations in Haruan extract. However there were six fatty acids have their amount significantly different after formulated into concentrate and aerosol i.e. lauric acid, arachidic acid, eicosapentaenoic acid, erucic acid, lignoceric acid and nervonic acid. Contents of these fatty acids were increased. Fatty acids which had content increased after formulated were the long-chain fatty acids. This might due to the extraction process of fatty acids from concentrate and aerosol prior to analysis and other compound in formula.

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