



## Quantitative Analysis and Validation of Hirsutenone and Muricarpone B from Fermented *Alnus sibirica*

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**Abstract** – *Alnus sibirica* (AS) geographically distributes in Korea, Japan, Northeast China and Russia. The bark of this plant had been used for antipyretic, expectorant, anti-phlogistic, antitussive, anti-asthmatic and as a health tea for alcoholism. Recently, we studied various biological activities of AS and the isolated diarylheptanoid. In present study, we conducted fermentation of AS (FAS) and isolated two diarylheptanoid (hirsutenone and muricarpone B). Moreover, we established the validation and contents determinations of the two compounds by HPLC on FAS.

**Keywords** – *Alnus sibirica*, Fermentation, HPLC, Validation, Content

### Introduction

*Alnus sibirica* (AS), geographically distributes in Korea, Japan, Northeast China and Russia, and the bark part had been used as antipyretic, expectorant, antiphlogistic, antitussive, and as a health tea for alcoholism.<sup>1</sup> It was reported that *Alnus* species contain many phenolic constituents (concluding diarylheptanoids,<sup>5</sup> flavonoids,<sup>6,7</sup> and tannins<sup>8-11</sup>) and triterpenoids<sup>12-13</sup> and it exhibits a variety of bioactivities including anti-oxidant activity, anti-inflammation,<sup>14</sup> cytotoxicity,<sup>15</sup> and anti-adipogenic activity.<sup>16</sup>

Fermentation process could make organic compound degrades to new product through oxidation-reduction producing ATP by glycolysis. And it attempted to ferment by herb medicine with various practical fermentation technology in order to obtain maximum therapeutic effect<sup>17</sup> or/and reduce the side effects.<sup>18</sup> Moreover, it is recently under attention as not only herb medicine<sup>19</sup> but also balancing the rumen microorganisms.<sup>20</sup>

On this research, we fermented AS (FAS) with *Lactobacillus plantarum* and isolated two compounds, hirsutenone and muricarpone B. And hirsutenone which was changed to the major component in FAS, and muricarpone B, which was not be found in *Alnus* species plants, were conducted to evaluate the contents and validations as characteristic components of FAS.

### Experimental

**Plant material** – The stems of AS were collected from ‘Kuksabong’, Seoul, Republic of Korea, on January of 2015, certificated by Prof. Lee Min Won (Pharmacognosy Lab, College of Pharmacy, Chung-Ang University). And a voucher specimen was deposited at the herbarium of the college of pharmacy, Chung-Ang University. The botanical specimen ID is 20150816AS.

**Extraction and Fermentation** – AS were extracted with 80% prethanol A at room temperature, and yielded AS extract. The AS extract was fermented (FAS) by using *Lactobacillus plantarum* with MRS broth (6.4% dextrose, 18.2% proteose peptone No.3, 18.2% beef extract, 9.1% yeast extract, 9.1% sodium acetate, 1.8% polysorbate 80, 3.6% amonium citrate, 3.6% dipotassium phosphate, 0.9% magnesium sulfate, and 0.5% manganese sulfate) in 7 days at room temperature.

**Isolation** – FAS was applied to Sephadex LH-20 with the water-methanol gradient (from 10:0 to 0:10) to yield 22 fractions. The fraction 15 was applied to MCI CHP20P column with the water-methanol gradient (from 2:8 to 0:10) to yield hirsutenone (750 mg) and muricarpone B (230 mg).

Hirsutenone [1,7-bis-(3,4-Dihydroxyphenyl)-4-Heptene-3-One] : amorphous brown oil; <sup>1</sup>H NMR (300 MHz, Acetone-d<sub>6</sub>) δ 6.88 (dt, *J* = 16.0, 6.8 Hz, 1H, H-5), 6.74 – 6.67 (m, 4H, H-2', 2'', 5', 5''), 6.51 (m, 2H, 6', 6''), 6.08 (d, *J* = 16.0 Hz, 1H, H-4), 2.82 – 2.40 (m, 8H, H-1, 2, 6, 7).

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**Table 1.** Analysis condition of HPLC

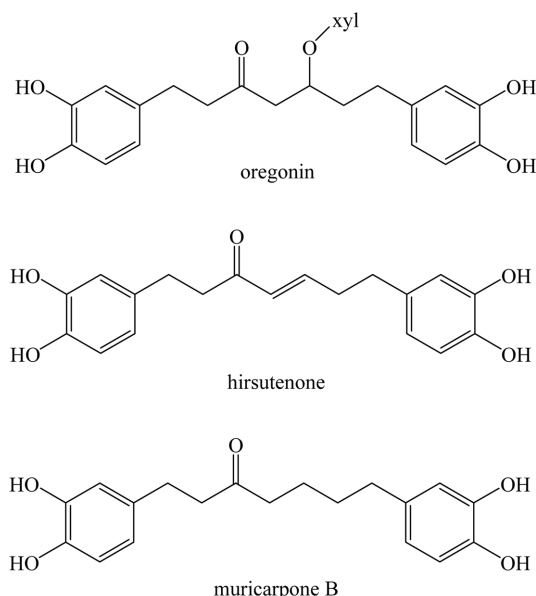
Column	Thermo® Synchronis C <sup>18</sup> HPLC column (5 µm, 250 × 4.6 mm)						
Detector	Waters 112 UV/VIS (280 nm)						
	0 min	20 min	35 min	38 min	45 min	47 min	55 min
H <sub>2</sub> O	95	75	60	0	0	95	95
ACN	5	25	40	100	100	5	5

Muricarpone B [1,7-bis-(3,4-Dihydroxyphenyl)-4-Heptene]: amorphous brown oil; <sup>1</sup>H NMR (600 MHz, Acetone-d<sub>6</sub>) δ 6.68 (d, *J* = 7.2, 1H, H-5'), 6.68 (d, *J* = 7.2, 1H, H-5''), 6.66 (d, *J* = 2.1 Hz, 1H, H-2'), 6.64 (d, *J* = 2.1 Hz, 1H, H-2''), 6.46 (dd, *J* = 7.2, 2.1, 1H, H-6''), 6.44 (dd, *J* = 7.2, 2.1, H-6'), 2.7-2.58 (br, 4H, H-1, 2), 2.42 (d, *J* = 7.2 Hz, 2H, H-7), 2.39 (d, *J* = 7.2 Hz, 2H, H-4), 1.48 (m, 4H, H-5, 6); <sup>13</sup>C NMR (151 MHz, Acetone-d<sub>6</sub>) δ 210.27(C-3), 144.83 (C-3''), 144.75 (C-3'), 143.09 (C-4''), 142.87 (C-4'), 133.78 (C-1''), 132.81 (C-1'), 119.31 (C-6''), 119.21 (C-6'), 115.30 (C-2''), 115.27 (C-2'), 115.08 (C-5''), 114.99 (C-5'), 44.05(C-2), 42.12(C-4), 34.70 (C-7), 31.03 (C-5), 28.97(C-1), 23.08 (C-6).

**HPLC analysis** – High-performance liquid chromatography (HPLC) (Waters 600 system (USA)), Thermo® Synchronis C<sup>18</sup> (5 µm, 4.6 × 250 mm) Column was used for quantitative surveys of the compounds content. The mobile phase consists of solvents A (H<sub>2</sub>O) and B (ACN) were described and filtered by Whatman® membrane filters (0.2 µm, diam. 47 mm) (Table 1).

**Validation** – For set up the HPLC analysis method of main compounds from FAS, we used validation guide line that was published by KFDA (Korea Food and Drug Administration), including specificity, linearity, limit of quantitation (LOQ), accuracy and precision. [Specificity: to confirm if the compounds were separated well from other compounds. Linearity: to solute the main compounds (hirsutenone and muricarpone B) of FAS by MeOH and dilute to 4 concentrations (2000, 1000, 500, 250 µg/mL) and to calculate the linear regression equation (*y* = *ax* + *b*, *y*: area of peak on HPLC, *x*: concentration of compound) and the volume of *R*<sup>2</sup> (the linearity is valid when *R*<sup>2</sup> is greater than 0.99). The limit of Quantitation (LOQ): to calculate the LOQ by using the formula  $LOQ = 10 * \sigma/S$  ( $\sigma$ : deviation of *b*, *S*: average of *a*) that is a calculation of LOQ published by KFAD. The accuracy and precision: in 4 concentrations (2000, 1000, 500, 250 µg/mL) of compounds should be tested by HPLC intraday and interday, 3 times respectively (accuracy: ± 10%, precision: < 2).

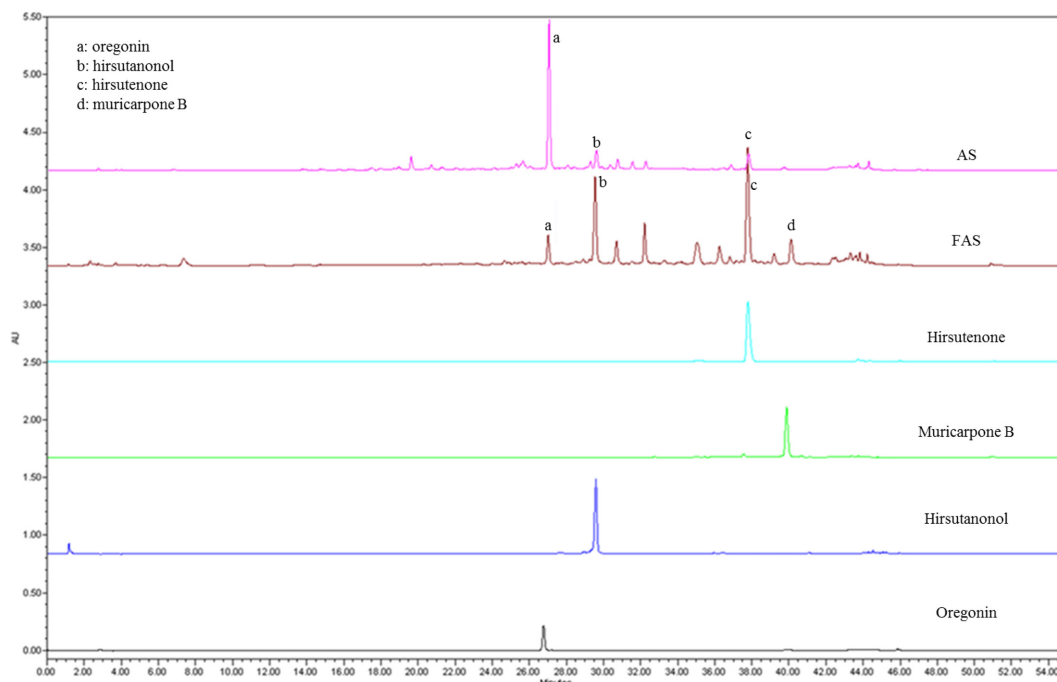
**Content** – We calculated the contents of hirsutenone and muricarpone B from FAS by using the linear regression equation.

**Fig. 1.** The structures of main components of FAS, oregonin, hirsutenone and muricarpone B.

## Results and Discussion

We fermented AS with *Lactobacillus plantarum* yielded FAS and hirsutenone<sup>21</sup> and muricarpone B<sup>22</sup> were isolated from FAS. (Fig. 1) The result of HPLC showed that oregonin (a), which is a major component present in AS, was decreased obviously. On the other hand, aglycone types of diarylheptanoid including hirsutenone (b), hirsutanonol (c) and muricarpone B (d) which are minor components in AS or not found from AS, were increased (Fig. 1, 2). It is probably due to the fermentation not only to cleave the aglycone and sugar, but also led to produce new material through some other oxidation or/and reduction. In addition, aglycones like hirsutenone showed more potent biological activities than its glycoside, oregonin. Thus, fermentation might be a way to develop the bioactivities of original plants.

The contents and the validations of hirsutenone (b) and muricarpone B (d) in FAS were evaluated. The content of hirsutenone which has various biological activities<sup>23-26</sup> was increased almost 15-fold compared with AS and muricarpone B, which not been isolated from AS, was



The standard oregonin (a) and hirsutanonol (c) were isolated from previous work.<sup>15</sup>

**Fig. 2.** HPLC pattern of FAS, AS and the specificity of hirsutenone and muricarpone B.

**Table 2.** Content of hirsutenone and muricarpone B in extract of FAS and AS

Compound	FAS (%)	AS (%)
Hirsutenone	28.70	2.74
Muricarpone B	6.72	—

detected by HPLC (Fig. 2).

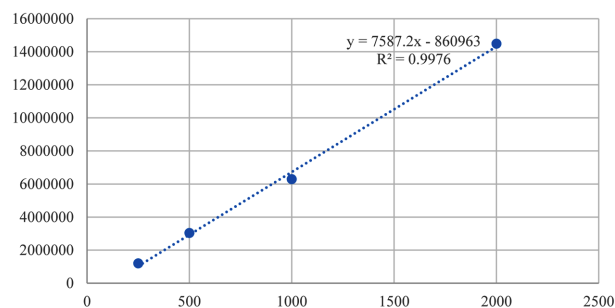
**Content** – HPLC pattern of FAS and AS had been studied by HPLC analysis with a good separation (Fig. 2). The content of hirsutenone and muricarpone B in FAS were 28.7% and 6.72%. (Table 2).

#### Validation

**Specificity:** Compare the HPLC chromatogram of FAS with the standard compounds (hirsutenone and muricarpone B), the retention time of hirsutenone and muricarpone B were confirmed at 37.87 min and 40.40 min, respectively. From the FAS HPLC chromatography, hirsutenone and muricarpone B were separated well with other compounds (Fig. 2).

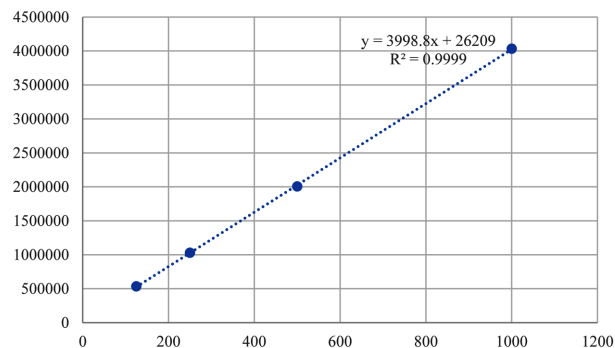
**Linearity:** In the linear regression equation of hirsutenone,  $y = 7587.2x - 860963$  and  $R^2$  was 0.9976. And in the linear regression equation of muricarpone B,  $y = 3998.8x + 26209$  and  $R^2$  was 0.9999. Both the  $R^2$  of hirsutenone and muricarpone B were greater than 0.99, thus the linearity of hirsutenone and muricarpone B were confirmed (Fig. 3, 4; Table 3).

linearity of hirsutenone



**Fig. 3.** Linearity of hirsutenone.

linearity of muricarpone B



**Fig. 4.** Linearity of muricarpone B.

**Limit of Quantitation (LOQ):** The LOQ of hirsutenone and muricarpone B were 76.12  $\mu\text{g/mL}$  and 4.49  $\mu\text{g/mL}$ ,

**Table 3.** Linear ranges and LOQ of hirsutenone and muricarpone B

Compound	Linear range (µg/mL)	Response slope (a)	Response Factor (b)	Correlation Coefficient (R <sup>2</sup> )	LOQ (µg/mL)
Hirsutenone	250~2000	7587.2	-860963	0.9976	76.12
Muricarpone B	125~1000	3998.8	26209	0.9999	4.49

**Table 4.** Precision and accuracy of hirsutenone and muricarpone B

Compounds	Conc. (µg/mL)	Accuracy (%)		Precision (c.v., %)	
		Inter-day	Intra-day	Inter-day	Intra-day
Hirsutenone	2000	98.86	98.83	0.08	0.07
	1000	105.73	105.71	0.33	0.35
	500	97.49	97.32	1.07	0.90
	250	91.20	91.56	1.99	1.42
Muricarpone B	1000	99.90	99.82	0.11	0.15
	500	100.49	100.85	0.57	0.77
	250	99.70	99.82	1.19	0.77
	125	99.39	98.35	1.76	1.5

respectively, which is satisfying the KFAD validation guide line (Table 3).

**Accuracy and Precision:** The precision of hirsutenone and muricarpone B were 0.07~1.99 and 0.11~1.76, respectively. Both the precision of two compounds were less than 2, thus the precision of hirsutenone and muricarpone B were confirmed. The accuracy of hirsutenone and muricarpone B were 91.20%~105.73% and 98.35%~100.85%, respectively. Both the accuracy of two compounds were within the range of 90%~110%, thus the accuracy of hirsutenone and muricarpone B were confirmed (Table 4).

### Conclusion

We fermented AS and isolated hirsutenone and muricarpone B. Moreover, we established the HPLC validation of hirsutenone and muricarpone B, and calculated the content of two compounds as 28.7% and 6.72% respectively from FAS, and the accuracy and precision of the HPLC analysis was in the range of 80~120% and less than 2%, respectively, showed the developed HPLC analysis method is an accurate and a good reproducibility. These results of HPLC analysis method of hirsutenone and muricarpone B might be a good database for further research.

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