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# Quantification of quercetin, isorhamnetin and ferulic acid in dry extract of *Anoectochilus setaceus* Blume from Vietnam

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**Abstract**
*Anoectochilus setaceus* Blume is a precious medicinal plant that contains many compounds with high pharmacological effects. In this study, the dry extract of *A. setaceus* collected in Vietnam was first created by fermentation from *Saccharomyces cerevisiae* strain and using high-performance liquid chromatography (HPLC) method to analyze the contents of some compounds in this dry extract. The results of the study determined that the contents of quercetin, isorhamnetin and ferulic acid in the dry extract of *A. setaceus* were 106.8 µg, 187.2 µg and 51.9 µg respectively. This has shown that the dry extract of *A. setaceus* that we produced has a very high pharmacological effect in supporting the treatment of human diseases.

**Keywords:** quantification, dry extract, *Anoectochilus setaceus*, quercetin, isorhamnetin, ferulic acid

## 1. Introduction
*Anoectochilus* genus in Vietnam currently has 12 species reported, including *Anoectochilus setaceus* Blume [1]. This species is known not only for its ornamental value but also its medicinal value. According to traditional Chinese medicine, *A. setaceus* is used to treat diabetes, cancer, cardiovascular and liver diseases [2-7]. Studies have shown that *A. setaceus* contains many compounds with high pharmacological effects such as quercetin, isorhamnetin and ferulic acid [8-9]. Specifically, quercetin is a flavonoid that improves cardiovascular health, reduces bad cholesterol and increases good cholesterol, reduces plaques in the arteries to help prevent strokes and some cardiovascular diseases [10-11]. Isorhamnetin prevents cell damage in the body and acts as a powerful antioxidant. Thanks to its ability to inhibit cell proliferation and promote cell apoptosis, isorhamnetin is considered an active substance in preventing cancer [12-14]. Moreover, ferulic acid has been proven to have anti-inflammatory, analgesic, anti-UV radiation and anti-arterial blockage (antithrombotic) effects, as well as neutralize free radicals (anti-free radical) and enhance the body's immunity [15-16].

Fermentation is considered a new technology, with many advantages compared to the traditional technology to extract valuable active ingredients from medicinal herbs such as steroids saponin, flavonoid, phenolic, carotenoids, alkaloids,... in herbs. It is used to replace extraction using water or alcohol to improve extraction efficiency and increase the diversity of bioactive substances in the extract, because many new substances can be formed under the action of enzymes in microbial strains used in fermentation [17-19]. In fact, there have not been many studies that produce dry extracts of *A. setaceus* species. Given the medicinal values of *A. setaceus* for humans, it is necessary to produce a dry extract of *A. setaceus* through fermentation and determine the content of the compounds in this dry extract. Therefore, the aims of this study are to (1) use the *Saccharomyces cerevisiae* strain to produce dry extracts of *A. setaceus* through fermentation and (2) determine the contents of quercetin, isorhamnetin and ferulic acid in the dry extracts of *A. setaceus* obtained by high-performance liquid chromatography (HPLC) method.

## 2. Materials and Methods
### 2.1 Plant Materials
*Anoectochilus setaceus* was collected in Thanh Hoa, Vietnam in March 2018. These are fresh samples with enough roots, stems and leaves. It was determined by Assoc. Prof. Dr. Tran Minh Hoi (Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology). These fresh samples were collected, stored in paper bags with silica gel, then moved to the laboratory and kept at -20°C before extraction.

### 2.2 Chemicals and Equipment
*Saccharomyces cerevisiae* fermentation strain was provided by the Institute of Biotechnology, Vietnam Academy of Science and Technology. Saccharomyces cerevisiae culture medium includes 5g $(NH_4)_2SO_4$, 1g $KH_2PO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2.2H_2O$, 0.1g NaCl, 1g yeast extract, 30g glucose, 1000g water, PBS buffer. NanoDrop-1000 absorption spectrometer (ND-1000, USA). Shimadzu HPLC high-performance liquid chromatograph (Japan).

### 2.3 Research Methods
#### 2.3.1 Fermenting *A. setaceus* with *Saccharomyces cerevisiae* fungal strain
In this study, we used fermentation technology to produce the extract of *A. setaceus*, the fungal strain used to obtain the extract is *Saccharomyces cerevisiae* instead of the traditional extraction technology using water or alcohol to improve extraction efficiency and increase the diversity of

bioactive compounds in extracted products. *Saccharomyces cerevisiae* grows at high temperatures, mainly hovering on the surface, temperature of fermentation is from 10 - 25ºC. After the culture period at 25-30ºC for three weeks, we centrifuge 300 rpm or settle at 4°C to obtain cell biomass.
The obtained cell biomass was washed twice with PBS buffer (pH = 7.5). The cell biomass was reconstituted in PBS buffer (pH = 7.5) and added to the flask (note: the fermentation tank needs a gas outlet when necessary during fermentation) containing *A. setaceus* dried and cut small (the best size is 1 x 1 mm), add distilled water at the rate of 3 liters per 1 kg of material. The yeast cells are supplemented to reach $10^3$ cells per 1 liter. To speed up fermentation, we can add 2g glucose per 1 liter of water. Close the fermentation vessel tightly and store it in the dark, after 5-7 days, the fermenting solution is filtered through a cloth or filter paper and concentrated to the remaining volume of 1/10 of the original volume on the vacuum rotary device at low pressure and a temperature at 60-70ºC. Then add adjuvants (Maltodextrin 30%, Lactose 20%, talcum powder 10%) and dry at low temperature to collect dry extract of *A. setaceus*.

### 2.3.2 Quantify quercetin, isorhamnetin and ferulic acid in the dry extract of *A. setaceus*

Quantify quercetin, isorhamnetin and ferulic acid in the dry extract of *A. setaceus* using high performance liquid chromatography (HPLC) with Shimadzu HPLC device (Japan).

#### 2.3.2.1 Sample preparation

*Standard solution*: For each compound (quercetin ($C_{15}H_{10}O_7$), ferulic acid ($C_{10}H_{10}O_4$) and isorhamnetin ($C_{16}H_{12}O_7$)), accurately weigh 5.0 mg, into a 5 mL volumetric flask. Add 3 mL of methanol, ultrasound until completely dissolved, quantify sufficient methanol obtained from the standard solution of the substances (quercetin ($C_{15}H_{10}O_7$), ferulic acid ($C_{10}H_{10}O_4$) and isorhamnetin ($C_{16}H_{12}O_7$)) at a concentration of 1 mg/mL. From this solution, dilute with methanol to obtain a series of standard solutions with lower concentrations. All of these solutions were filtered through 0.45 µm cellulose acetate membrane prior to HPLC analysis.
*Testing solution*: Accurately weigh 100 mg of *A. setaceus* dry extract into a 100 mL volumetric flask, add 50 mL of methanol, weigh and record the mass, let stand for 10 minutes. Reflux extraction with water for 2 hours; cool to room temperature, weigh the flask and add methanol until reaching initial mass. Filter through 0.45 µm cellulose acetate membrane filter, obtain the testing solution.

#### 2.3.2.2 Chromatographic conditions

*Chromatographic conditions for quantification of quercetin, isorhamnetin:* Equipment: HPLC Shimadzu; Column Rp18; 250 x 4.6 mm; 5µm. Column series: H16-248657; Column protection: Rp18; 4x3 mm; Column temperature: 40ºC; Detector: PDA, λ = 370 nm; Flow rate: 1.5 mL/min; Autosampler: 4ºC; Injection volume: 50 µL; Mobile phase: ACN: 0.5% phosphoric acid = 72:28 (%).
*Chromatographic conditions for quantification of ferulic acid*: Equipment: HPLC Shimadzu; Column Rp18; 250 x 4.6 mm; 5µm. Column series: H16-248657; Column protection: Rp18; 4x3 mm; Column temperature: 40ºC; Detector: PDA, λ = 313 nm; Flow rate: 1.5mL/min; Autosampler: 4ºC; Injection volume: 20 µL; Mobile phase: ACN: Buffer $CH_3COONa$ 0.05M pH 4.0 = 12:88 (%).

#### 2.3.2.3 Setting a standard curve

*Standard curve of quercetin*: Dissolve exactly 25 mg of standard quercetin into a 50mL volumetric flask, dissolve and add sufficient MeOH to obtain a solution with a quercetin concentration of about 500 µg/mL (1). Absorb 1.2 mL of dd (1) dissolved in 20 mL MeOH (~ 30 µg/mL). Mix up a range of quercetin standard solutions of 1.5 - 15 µg/mL. Carry out the chromatography, taking the peak response at each concentration. Construct linear regression equation (standard concentration curve).
*Standard curve of isorhamnetin*: Dissolve exactly 5 mg of standard isorhamnetin (Sigma) into a 10 mL volumetric flask, dissolve and add sufficient MeOH to obtain a solution with isorhamnetin concentration of about 500 µg/mL (2). Aspirate 1 mL of solution (2) dissolved in a 25 mL flask containing MeOH (20 µg/mL) (3). Aspirate 2 mL of solution (3) in a 10 mL MeOH flask (4 µg/mL). Mix a series of isorhamnetin standard solutions of 0.2 - 2 µg/mL. Carry out the chromatography, taking the peak response at each concentration. Construct linear regression equation (standard concentration curve).
*Standard curve of ferulic acid*: Dissolve exactly 20 mg of ferulic acid standard into a 20 mL volumetric flask, dissolve and add sufficient MeOH to obtain a solution with ferulic acid concentration of about 1000 µg/mL (1). Aspirate 1 mL of solution (1) dissolved in 10mL MeOH (~ 100 µg/mL) (2). Aspirate 1 mL of solution (2) into a 10 mL flask, ad sufficient MeOH (~ 10 µg/mL). Mix a series of ferulic acid standard solutions with a concentration of 1 - 5 µg/mL. Carry out the chromatography, taking the peak response at each concentration. Construct linear regression equation (standard concentration curve).

## 3. Results and Discussion

### 3.1 Results of quercetin determination

#### 3.1.1 Results of quercetin determination in standard samples

In the survey concentration range of 1.5 - 15 µg/mL, the regression equation obtained is Y = 168544x - 20357. Correlation coefficient $R^2 = 0.9999$. The coefficients a and b are significant with 95% confidence. The results in Table 1 and Figure 1 show that the quercetin content in the standard sample from S1 to S5 through the test is very stable and linear according to the concentration of quercetin. This result ensures the reliability for us to quantify quercetin in *A. setaceus* dry extract samples.

**Table 1:** Results of quercetin determination in standard samples

| Sample | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| Concentration (µg/mL) | 1.5 | 3 | 6 | 12 | 15 |
| CC1 | 225255 | 496074 | 978191 | 1971054 | 2483795 |
| CC2 | 218315 | 487278 | 1001059 | 2021000 | 2482451 |
| CC3 | 223587 | 498848 | 998319 | 2030356 | 2540226 |
| *Average* | *222386* | *494067* | *992523* | *2007470* | *2502157* |

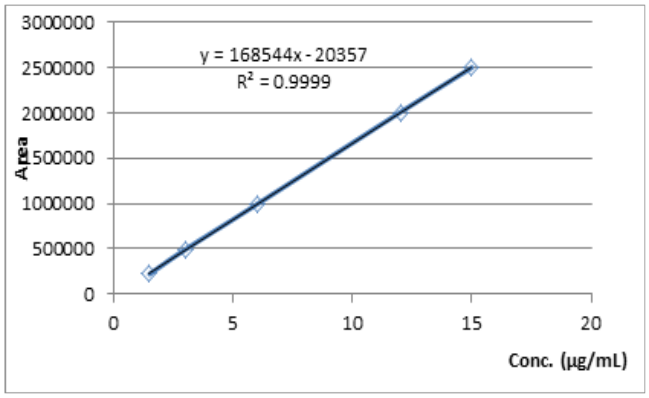


**Fig 1:** Line showing the correlation between peak area and quercetin concentration

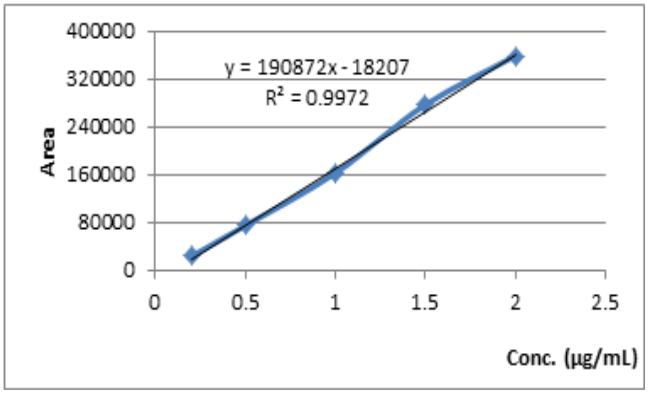


**Fig 2:** Line showing the correlation between peak area and isorhamnetin concentration

### 3.1.2 Results of quercetin determination in test samples

*A. setaceus* dry extract was prepared to form a test solution (see method). This test solution is further diluted 20 times. We used the 20-time diluted solution and the original test solution for chromatographic analysis. The peak area under the corresponding curve of quercetin compared to the standard curve is shown in Table 2.

**Table 2:** Results of quercetin quantification in test samples

| Sample | Peak area (Area) | 20-time diluted sample concentration (µg/mL) | Initial sample concentration (µg/mL) |
|---|---|---|---|
| 1 | 376134 | 5.277 | 105.5 |
| 2 | 376134 | 5.277 | 105.5 |
| 3 | 389540 | 5.463 | 109.3 |
| Average | 380603 | 5.339 | 106.8 |

The results in Table 2 show that the quercetin content in the samples ranged from 105.5 to 109.3 µg/mL, the average being 106.8 µg/mL. This also shows that *A. setaceus* in Vietnam contains quercetin, which is one of the compounds that reduce bad cholesterols and increase good cholesterols, helping avoid strokes and heart attacks [10, 11, 20]. This result was also confirmed by Wang *et al*. (2011) and He *et al*. (2005a) [8, 9].

## 3.2 Results of Isorhamnetin Determination

### 3.2.1 Results of isorhamnetin determination in standard samples

In the survey concentration range of 0.2 – 2.0 µg/mL, the regression equation obtained is Y = 190872x - 18207. Correlation coefficient $R^2$ = 0.9972. The coefficients a and b are significant with 95% confidence. The results in Table 3 and Figure 2 show that the isorhamnetin content in the standard sample from S1 to S5 through the test is very stable and linear according to the concentration of isorhamnetin. This result ensures the reliability for us to quantify isorhamnetin in *A. setaceus* dry extract samples.

**Table 3:** Results of isorhamnetin determination in standard samples

| Sample | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| Concentration (µg/mL) | 0.2 | 0.5 | 1.0 | 1.5 | 2.0 |
| CC1 | 21944 | 78732 | 160507 | 279643 | 373492 |
| CC2 | 25531 | 73212 | 160945 | 272075 | 372500 |
| CC3 | 23447 | 74312 | 169414 | 283469 | 335275 |
| *Average* | *23641* | *75419* | *163622* | *278396* | *360422* |

### 3.2.2 Results of isorhamnetin determination in test samples

*A. setaceus* dry extract was prepared to form a test solution (see method). This test solution is further diluted 20 times. We used the 20-time diluted solution and the original test solution for chromatographic analysis. The peak area under the corresponding curve of isorhamnetin compared to the standard curve is shown in Table 4.

**Table 4:** Results of isorhamnetin quantification in test samples

| Sample | Peak area (Area) | 20-time diluted sample concentration (µg/mL) | Initial sample concentration (µg/mL) |
|---|---|---|---|
| 1 | 153703 | 9.007 | 180.1 |
| 2 | 164868 | 9.592 | 191.8 |
| 3 | 162852 | 9.486 | 189.7 |
| Average | 160474 | 9.361 | 187.2 |

The results in Table 4 show that the isorhamnetin content in the samples ranged from 180.1 to 191.8 µg/mL, with an average of 187.2 µg/mL. This shows that *A. setaceus* in Vietnam contains isorhamnetin, which is one of the anticancer compounds because of its ability to not only inhibit cell proliferation but also cause cell apoptosis [12, 13, 14, 20, 21, 22]. This result was also confirmed by Wang *et al*. (2011) and He *et al*. (2005b) [8, 23].

## 3.3 Results of ferulic acid determination

### 3.3.1 Results of ferulic acid determination in standard samples

In the survey concentration range of 1.0 – 5.0 µg/mL, the regression equation obtained is Y = 72272x – 5278.8. Correlation coefficient $R^2$ = 0.9996. The coefficients a and b are significant with 95% confidence. The results in Table 5 and Figure 3 show that the ferulic acid content in the standard sample from S1 to S5 through the test is very stable and linear according to the concentration of ferulic acid. This result ensures the reliability for us to quantify ferulic acid in *A. setaceus* dry extract samples.

**Table 5:** Results of ferulic acid determination in standard samples

| Sample | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| Concentration (µg/mL) | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 |
| CC1 | 67544 | 139350 | 210907 | 279505 | 366017 |
| CC2 | 67694 | 136852 | 211987 | 282357 | 357443 |
| CC3 | 71250 | 139617 | 207384 | 282137 | 353020 |
| *Average* | *68829* | *138606* | *210093* | *281333* | *358827* |

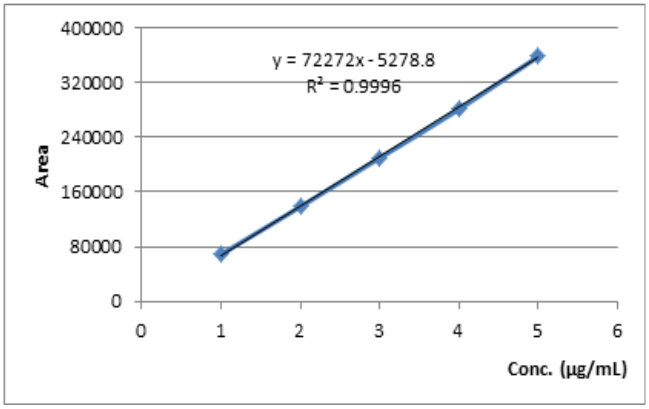


**Fig 3:** Line showing the correlation between peak area and ferulic acid concentration

### 3.3.2 Results of ferulic acid determination in test samples

*A. setaceus* dry extract was prepared to form a test solution (see method). This test solution is further diluted 20 times. We used the 20-time diluted solution and the original test solution for chromatographic analysis. The peak area under the corresponding curve of ferulic acid compared to the standard curve is shown in Table 6.

**Table 6:** Results of ferulic acid quantification in test samples

| Sample | Peak area (Area) | 20-time diluted sample concentration (µg/mL) | Initi.al sample concentration (µg/mL) |
|---|---|---|---|
| 1 | 180197 | 2.6 | 51.3 |
| 2 | 189895 | 2.7 | 54.0 |
| 3 | 177179 | 2.5 | 50.5 |
| Average | 182424 | 2.6 | 51.9 |

The results in Table 6 show that the ferulic acid content in the samples ranged from 50.5 to 54.0 µg/mL, the average being 51.9 µg/mL. This shows that *A. setaceus* in Vietnam contains ferulic acid, which is one of the compounds that have anti-inflammatory, analgesic, anti-clogging (antithrombotic) effects, and can neutralize free radicals (anti-freeradical) and enhance the immunity of the human body [15, 16, 20, 22]. This result was also confirmed by Lin *et al*. (1993), He *et al*. (2005a) and Xu *et al*. (2015) when studying the medicinal compounds and values of *A. setaceus* [9, 20, 24].

## 4. Conclusions

The fermentation process using *Saccharomyces cerevisiae* strain to create dry extract has been successfully applied to *Anoectochilus setaceus* for the first time. The process is simple and can be applied to the production of dry extract of *A. setaceus* on an industrial scale. The contents of quercetin, isorhamnetin and ferulic acid in this dry extract were determined by high-performance liquid chromatography (HPLC). In the dry extract of *A. setaceus* produced by fermentation, quercetin content ranged from 105.5 to 109.3 µg/mL, averaging at 106.8 µg/mL; isorhamnetin content ranged from 180.1 to 191.8 µg/mL, averaging at 187.2 µg/mL and ferulic acid content ranged from 50.5 to 54.0 µg/mL, averaging at 51.9 µg/mL. The present data would certainly help to ascertain the potency of *A. setaceus* dry extract for use in nutraceutical manufacturing.

## Author contributions

Conceptualization, L.D.C.; methodology, L.D.C.; investigation, L.D.C., H.T.T.H. and N.T.K.; formal analysis, L.D.C. and B.B.T.; data curation, L.D.C. and L.Q.H.; writing-original draft preparation, L.D.C.; writing-review and editing, L.D.C. and B.B.T.; supervision, L.Q.H.; project administration, L.D.C. All authors agreed with the final version of the manuscript.

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## Conflicts of interest

The authors declare no conflict of interest.

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