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Biotransformation of Andrographolide using Tropical Versus Psychrotolerant Fungi as Biocatalyst

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ABSTRACT

Andrographolide, a labdane diterpenoid compound, is known to have a wide range of biological properties, including anti-inflammatory, antibacterial, anticancer, antidiabetic, antimalarial, and hepatoprotective effects. Biotransformation of andrographolide is a promising method for discovering new drugs to treat various diseases. This study aims to ferment andrographolide with chosen fungal strains and to subject the cultured extract to HPLC analysis. The HPLC profiles between two selected fungi of different biotopes were compared to evaluate their ability to perform biotransformation reactions. To perform this study, andrographolide was fermented using *Beauveria bassiana* ATCC 74040, a tropical fungus, and R3-2 SP 17, a psychrotolerant fungi. The extraction was carried out after 4, 8, and 12 days of fermentation of andrographolide with each chosen fungi strain. The HPLC profile of the resulting extracts was compared and analyzed with the HPLC profiles of starting material, positive and negative controls. The appearance of a new peak in the HPLC profiles of the resulting extracts indicated the presence of a biotransformed metabolite of andrographolide. After comparing the HPLC profile of the resulting extract between tropical and psychrotolerant fungus, it is indicated that the psychrotolerant fungus, R3-2 SP 17, showed more promising results in the discovery of new chemical derivatives. This study would aid people, especially in the pharmaceutical field, discover a new drug to overcome infectious diseases and cancer.

INTRODUCTION

The immense chemical diversity and abundant biodiversity in natural products have established them as the foremost source of medicinal compounds. Examples of noteworthy natural products include aspirin, obtained from the bark of willow trees, digoxin, extracted from the flowers of Digitalis lanata, and morphine, derived from opium [1]. Over the last few decades, there has been a significant rise in the

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utilization of natural products as crucial components in drug discovery, specifically focusing on their potential in combating cancer and infectious diseases [2]. Natural products, which encompass a diverse range of compounds derived from various natural sources, such as animals, plants, and minerals, undergo specific transformations leading to the production of biologically active molecules. These molecules are subjected to rigorous analysis to evaluate their efficacy in treating human ailments.

In drug research and development, natural products present numerous advantages. When compared to synthetic compound libraries, they exhibit distinct characteristics such as increased molecular mass, a higher presence of sp3 carbon and oxygen atoms, reduced nitrogen and halogen atoms, a more significant number of H-bond acceptors and donors, enhanced hydrophilicity, and enhanced molecular rigidity [2]. Consequently, these features of natural products are highly sought after by researchers seeking suitable lead compounds in drug research and development activities.

Andrographolide is a compound derived from the herbaceous plant Andrographis paniculata, which has a long history of medicinal use in Chinese and Indian civilizations. It is commonly known as the "king of bitter" but also known as kalmaegh in India and biner kalomegh in Bangladesh [3, 4]. This plant is widely distributed in tropical and subtropical regions of Asia, Southeast Asia, and India. Andrographolide is predominantly obtained from this plant due to its intricate structure and the challenges associated with its chemical synthesis. [5]. The extraction of andrographolide, a labdane diterpenoid, is commonly carried out from the stem and leaves of Andrographis paniculata.

Throughout history, microbial transformation has been employed in drug research and development to discover new derivatives of existing drugs. Microbial transformation involves a parent compound's structural and chemical modification to generate alternative derivatives, utilizing microorganisms as biocatalysts. Notably, Xin et al. [6] highlighted the advantages of this approach over organic synthesis, specifically its exceptional stereo and regio-selectivity. Fungi's ability to produce extracellular enzymes is a remarkable attribute that has attracted much attention at the industrial enzyme level. These enzymes are produced within the cell and released to break down macromolecules into smaller ones [7]. The characterization of microorganisms is influenced by their specific habitats and climates, leading to the generation of a diverse range of novel active metabolites. Tropical fungi, found in tropical or subtropical regions and capable of tolerating temperatures as high as 18 °C [8], contribute to this diversity.

Conversely, extreme environments such as Antarctica offer a distinct source of microorganisms that have the potential to serve as biocatalysts in microbial transformation. Psychrotolerant fungi, which thrive in cold environments below 10 °C, have adapted to these harsh conditions by producing secondary metabolites that enhance their characteristics, including an adaptable structure, increased turnover, and innate catalytic efficiency at low temperatures [9]. The variation in habitat and climate thus plays a crucial role in shaping the characteristics of microorganisms, providing a rich resource for exploring novel active metabolites in research activities.

The biotransformation of andrographolide is a significant approach in drug discovery to treat various diseases. In a study conducted by He et al. [10], it was discovered that derivatives of andrographolide exhibited promising anti-proliferative effects against human leukemia (HL-60), human colon cancer (HCT-116), and human breast cancer (MCF-7). This demonstrates the effectiveness of employing fungi as biocatalysts in microbial transformation to explore and develop novel drug derivatives. To date, no published studies have compared the biological approaches for the biotransformation of andrographolide using tropical and psychrotolerant fungi as biocatalysts. Consequently, this report presents a microbial transformation study of andrographolide utilizing two distinct fungi: Beauveria bassiana, a tropical fungus, and R3-2 SP17, a psychrotolerant fungus.

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MATERIALS AND METHODS

Media Preparation

The biotransformation experiments for *Beauveria bassiana* and R3-2 SP 17 were conducted under the following standardized conditions. A fermentation medium was prepared according to a specific protocol, which involved precise measurements of the following ingredients: glucose (10.0 g), peptone (5.0 g), KH2PO4 (5.0 g), glycerol (10.0 mL), yeast extract (5.0 g), NaCl (5.0 g), and 1.0 L of purified water [11, 12]. The ingredients were thoroughly mixed by stirring until complete dissolution was achieved. The resulting medium was then uniformly distributed into nine conical flasks, each containing 50 mL of the medium in a 250 mL conical flask. To prevent contamination, the openings of the flasks were sealed using cotton and aluminum. All the flasks containing the positive and negative control media were sterilized in an autoclave at 120 °C for 15 minutes.

Inoculation of Fungi

Aseptic techniques were employed to transfer a small piece of each fungus from the pure desired culture and introduce it into four conical flasks containing 50 mL of sterile medium. This process was repeated for both fungal strains, resulting in eight conical flasks. All inoculation procedures were conducted within a biohazard safety cabinet to prevent contamination. The same procedure was then repeated for the remaining fungi. All conical flasks were incubated in an orbital shaker, set at 140 rpm and a temperature of 28 °C, for a duration ranging from four to twelve days. Throughout the experiment, thorough aseptic measures were followed to ensure the integrity and reliability of the results.

Feeding of Substrate

A small quantity of andrographolide (Figure 1) was dissolved in 1 mL of acetone. Subsequently, using a micropipette, 250 µL of the solution was carefully added to each flask, including the positive control and excluding the negative control. The entire inoculation procedure was conducted within a biohazard safety cabinet to maintain a sterile environment and prevent contamination. For each fungal strain, the andrographolide was fed into three conical flasks, while the remaining flask served as a negative control, containing only media and fungus. Subsequently, one conical flask from the three previously inoculated flasks for each fungus was selected for extraction after a four-day incubation period. This procedure was repeated after eight and twelve days using a different type of fungi. The flasks and the controls were then placed on an orbital shaker and incubated at 28 °C for 4 to 12 days.

Extraction

After an eight-day incubation period, one of the conical flasks containing the substrate was selected for extraction for each fungus. The culture medium underwent filtration and subsequent washing with ethyl acetate to separate the mycelium from the broth. The aqueous medium obtained was then subjected to a three-time extraction process using ethyl acetate to generate an extract. This extract was subsequently dried and filtered using anhydrous sodium sulfate. The filtered extract was then subjected to a rotary evaporator at 94 rpm and a temperature of 40 °C. The same extraction process was repeated with another conical flask after 12 days of incubation for each fungus. Additionally, the procedure was replicated for both the negative and positive controls after a 12-day fermentation period. Finally, the dried extract was washed with methanol and transferred to vials, ensuring the lids remained open to facilitate complete drying.

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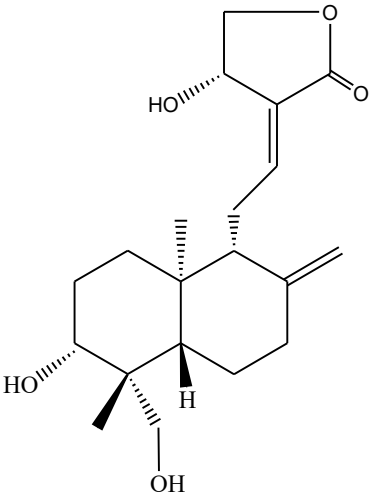

Fig. 1. Chemical structure of andrographolide.

HPLC Analysis and Identification of Biotransformed Products

The presence of biotransformed products was determined through HPLC analysis by identifying the peaks observed in the chromatogram. The HPLC method employed in this study adhered to the Standard Operating Procedures (SOP) established by Sharifah Nurfazilah, Siti Hajar Sadiran, Associate Prof. Dr. Sadia Sultan, and J.F.F Weber for the Faculty of Pharmacy, UiTM Puncak Alam, Selangor.

Sample Preparation

The dried extract, except for andrographolide, was dissolved in 100 % methanol (MeOH), while andrographolide was dissolved in a mixture of 90 % methanol:10 % water. The solution was filtered using a 0.45 µm nylon membrane filter and transferred to an autosampler vial.

Mobile Phase Preparation

A mixture of 900 mL of deionized water (Solvent A) and 900 mL of acetonitrile containing 1 mL of 90 % formic acid (Solvent B) was prepared for the mobile phase. Before use, both the deionized water and the acetonitrile with formic acid were subjected to ultrasonic degassing for 30 minutes.

HPLC Method Set-Up

The pump had been set up with a 1 mL/minute flow rate, and a. Gradient elution type was selected for the analysis. The pump was configured with a flow rate of 1 mL/minute with maximum and minimum pressure of 300 and 5 bars, respectively. The analysis was performed using gradient elution.

Table 1. Gradient elution concentration

Time (minutes)	Solvent A (deionized water), %	Solvent B (Acetonitrile), %
0	90	10
5	90	10
10	60	40
15	30	70
20	0	100
30	0	100
35	90	10
37	90	10

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Sample Analysis

The HPLC analysis of the samples was conducted at a flow rate of 1 mL/minute, with a column temperature of 30 °C. An injection volume of 10 µl was used for each sample. The detection wavelengths were set at 254 nm and 280 nm.

Comparison of HPLC Profiles

The HPLC profiles of all obtained extracts were analyzed and compared with those of the negative and positive controls. The results of the positive and negative controls were used as references to identify any potential biotransformation metabolites by comparing the peaks observed in the control chromatograms with those in the experimental chromatograms.

RESULTS AND DISCUSSION

High-Performance Liquid Chromatography (HPLC) Profile

HPLC, a type of column chromatography involving the high-pressure pumping of a sample (analyte) dissolved in a solvent (mobile phase) through an immobilized chromatographic packing material, was employed in the research [13]. All the obtained extracts, including the starting material (andrographolide), negative control (media with specific fungus strain), and positive control (media with andrographolide), were subjected, and analyzed by using HPLC. The resulting extract chromatograms were examined using 254 nm and 280 nm wavelengths, whereby each peak was compared with the corresponding peaks in the chromatograms of the starting material, negative control, and positive control. This comparison aimed to identify potential biotransformation metabolites or products by discerning the presence of peaks in the chromatographic profiles of the control and experimental samples.

HPLC profile of andrographolide (starting material)

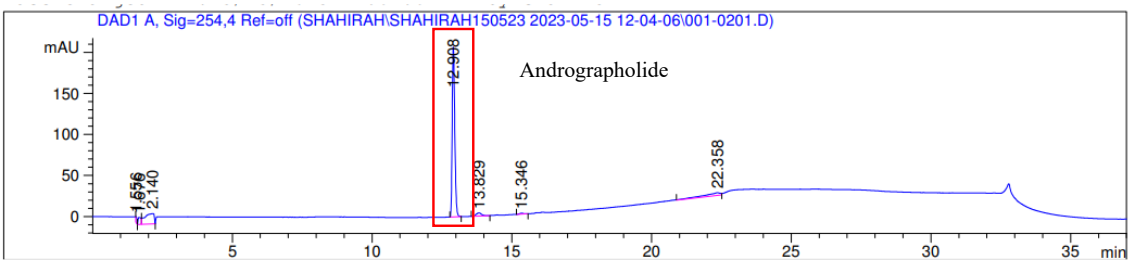

Fig. 2. Andrographolide HPLC chromatogram. The chromatogram at 254 nm demonstrates the appearance of a prominent peak with a retention time of 12.908 minutes, which refers to the starting material.

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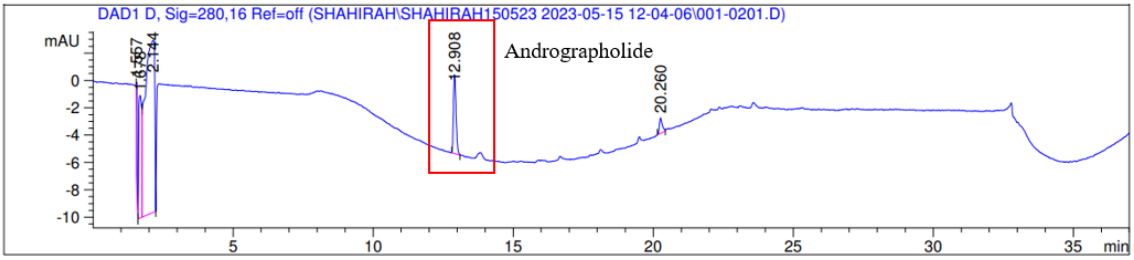

Fig. 3. Andrographolide HPLC chromatogram. The chromatogram at 280 nm demonstrates the appearance of a prominent peak with a retention time of 12.908 minutes, which refers to the starting material.

HPLC profile of positive control (media with andrographolide)

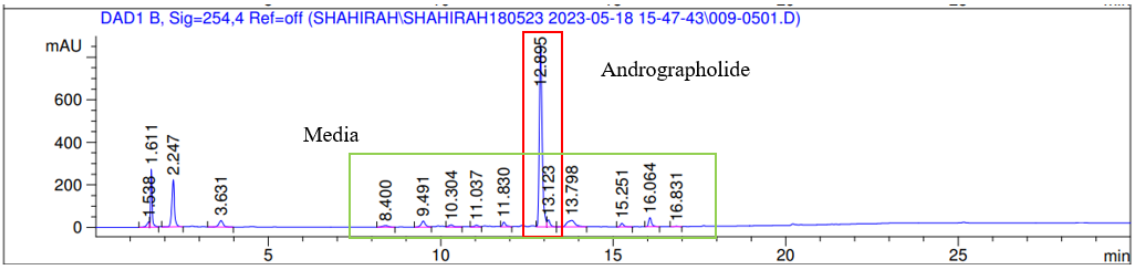

Fig. 4. HPLC profile of positive control at 254 nm. The chromatogram demonstrates the appearance of a peak with a retention time of 12.895 minutes, which refers to the starting material. The presence of minor peaks with retention time in the 5 to 20 minutes refers to the experiment's media.

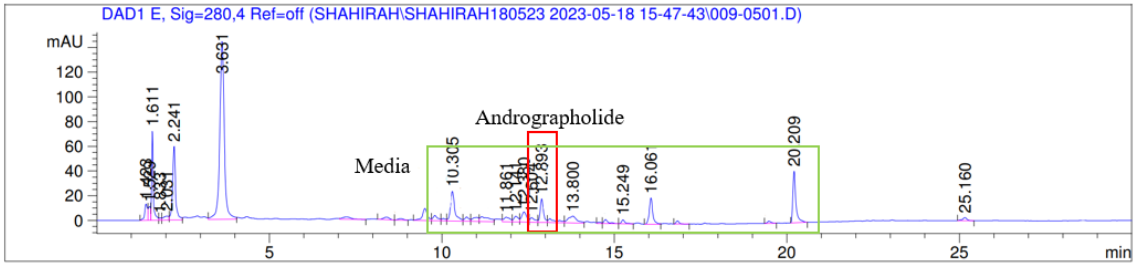

Fig. 5. HPLC profile of positive control at 280 nm. The chromatogram demonstrates the appearance of a peak with a retention time of 12.893 minutes, which refers to the starting material. The presence of minor peaks with retention time in the 5 to 25 minutes refers to the experiment's media.

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PLC profile of negative control of *Beauveria bassiana*

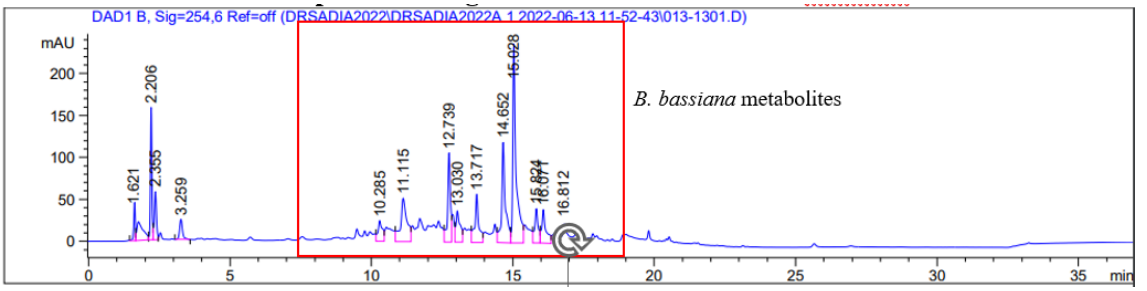

Fig. 6. Negative control of *B. bassiana* HPLC chromatogram at 254 nm. The chromatogram shows the appearance of peaks. The highlighted part in the above HPLC profile with a retention time range from 10 minutes to 20 minutes refers to the experiment's media and *B. bassiana* metabolites.

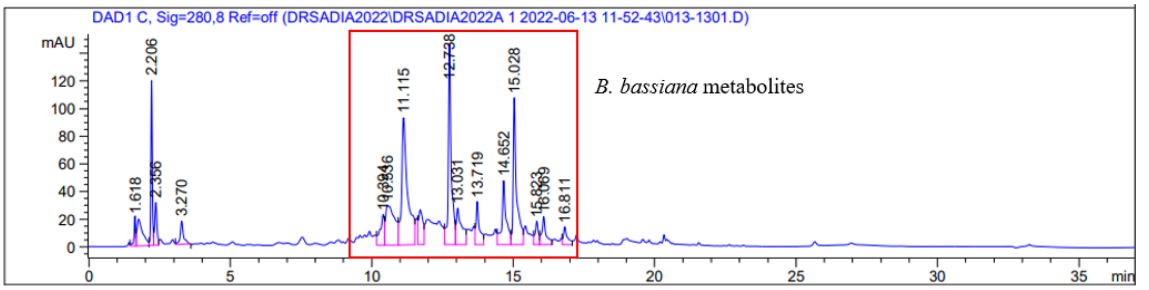

Fig. 7. Negative control of B. bassiana HPLC chromatogram at 280 nm. The chromatogram shows the appearance of peaks. The highlighted part in the above HPLC profile with a retention time range from 10 minutes to 20 minutes refers to the experiment's media and *B. bassiana* metabolites.

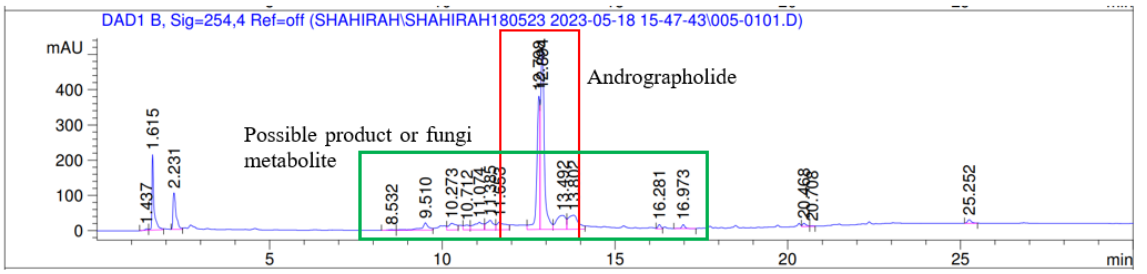

Fig. 8. HPLC profiles of *B. bassiana* of 8 days fermentation at 254 nm. The peak with a retention time of 12.894 minutes refers to the starting material. The green highlighted part shows the appearance of minor peaks with a retention time range from 5 minutes to 25 minutes, which may refer to possible biotransformed andrographolide and fungi metabolites.

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HPLC profile of an extract of andrographolide and *Beauveria bassiana* fermentation at 12 days

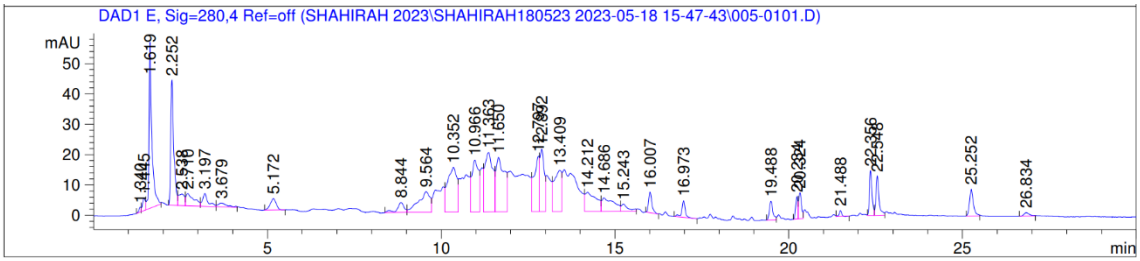

Fig.9. HPLC profiles of *B. bassiana* of 8 days fermentation at 280 nm. The peak with a retention time of 12.797 minutes refers to the starting material. The green highlighted part shows the appearance of minor peaks with a retention time range from 5 minutes to 25 minutes, which may refer to possible biotransformed andrographolide and fungi metabolites.

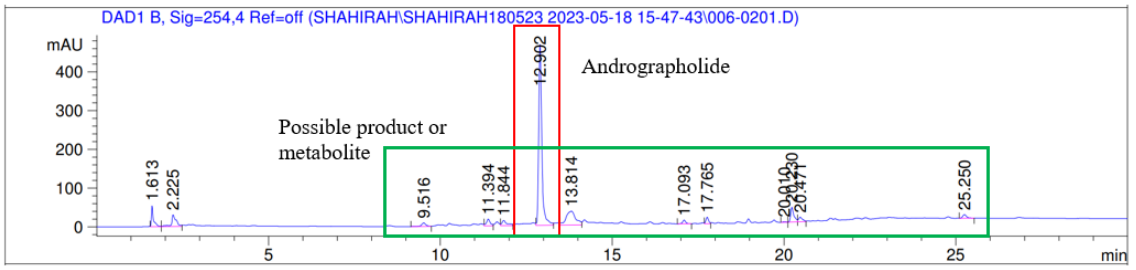

Fig. 10. HPLC profiles of *B. bassiana* of 12 days fermentation at 254 nm. The prominent peak appeared at 12.902 minutes and may be referred to as the starting material. The green highlighted part shows the appearance of minor peaks with a retention time range from 5 minutes to 25 minutes, which may refer to possible biotransformed andrographolide and fungi metabolites.

HPLC profile of an extract of andrographolide and *Beauveria bassiana* fermentation at 12 days

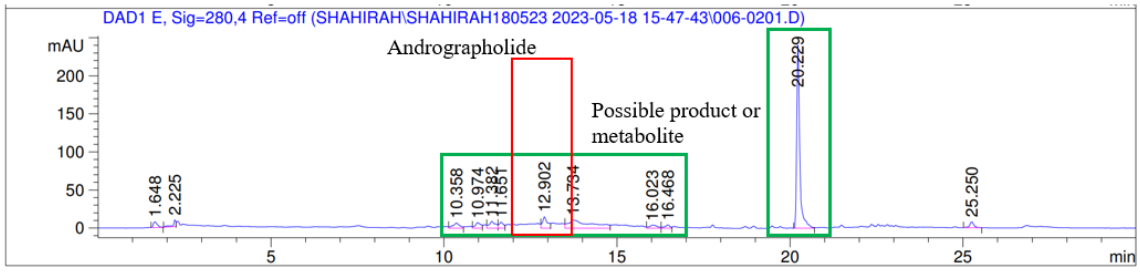

Fig. 11. HPLC profiles of *B. bassiana* of 12 days fermentation at 280 nm. The peak at a retention time of 12.902 minutes may be referred to as the starting material. The green highlighted part shows the appearance of minor peaks with a retention time range from 5 minutes to 25 minutes, which may refer to possible biotransformed andrographolide and fungi metabolites. A prominent peak at 20.229 minutes may be the *B. bassiana* metabolites or biotransformed andrographolide.

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HPLC profile of negative control of R3-2 SP 17

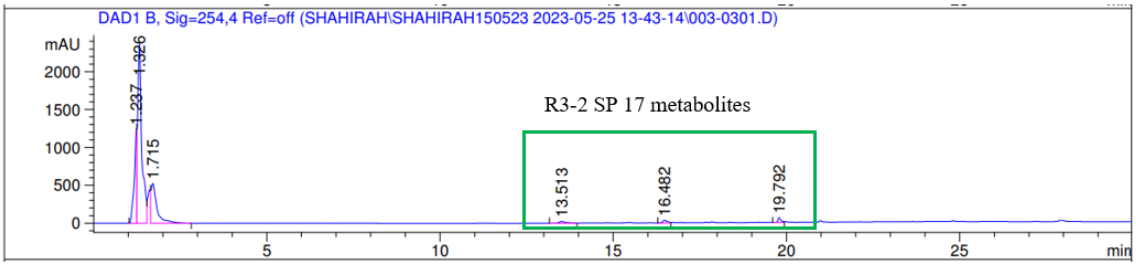

Fig. 12. Negative control of R3-2 SP 17's HPLC chromatogram at 254 nm. The highlighted green part in the chromatogram gives the appearance of peaks with a retention time in the range of 10 to 20 minutes, which may be referred to as the R3-2 SP 17 metabolites.

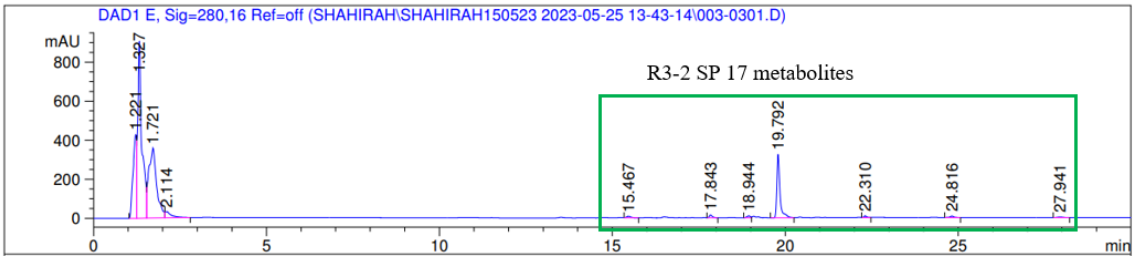

Fig. 13. Negative control of R3-2 SP 17's HPLC chromatogram at 280 nm. The chromatogram demonstrates the appearance of a prominent peak at 19.792 minutes. The highlighted green part in the chromatogram gives the appearance of peaks with a retention time range of 15 minutes to 25 minutes, which may refer to the experiment's media and fungi metabolites.

HPLC profile of an extract of andrographolide and *R3-2 SP 17* fermentation at eight days

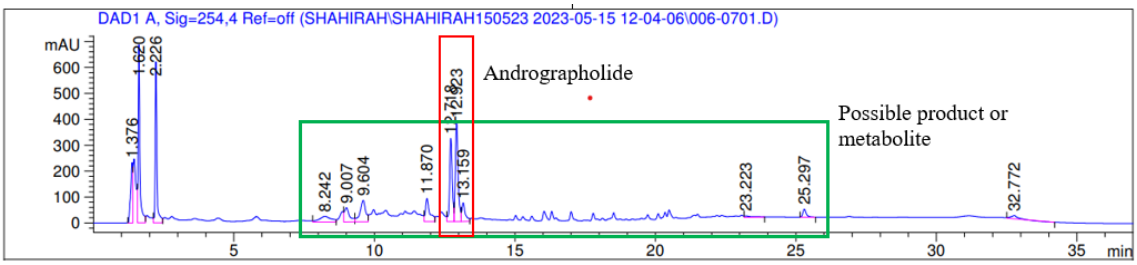

Fig. 14. The appearance of the peak with a retention time of 12.923 minutes may be referred to as the starting material. The highlighted green part in the chromatogram at 254 nm gives the appearance of peaks with a retention time range from 5 minutes to 25 minutes that may be referred to as the biotransformed andrographolide or the fungi metabolites. The peak 12.718 minutes of retention may be biotransformed andrographolide or the fungi metabolite.

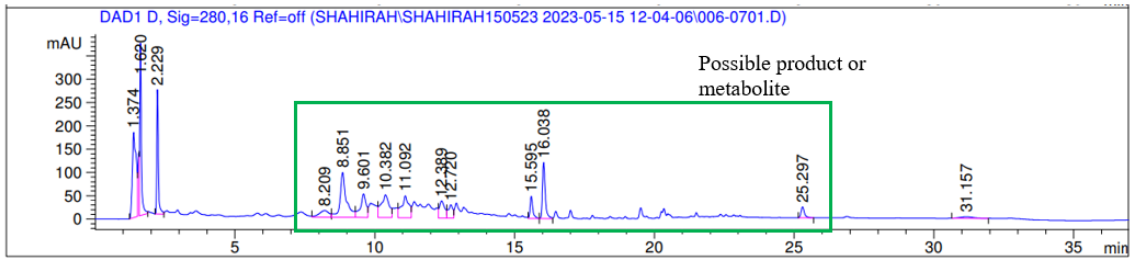

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Fig. 15. The highlighted green part in the chromatogram at 280 nm gives the appearance of peaks with a retention time range from 5 minutes to 25 minutes that may be possibly referred to as the biotransformed andrographolide or the fungi metabolites. Both retention times of 8.851 and 16.038 minutes give a prominent peak.

HPLC profile of an extract of andrographolide and R3-2 SP 17 fermentation at 12 days

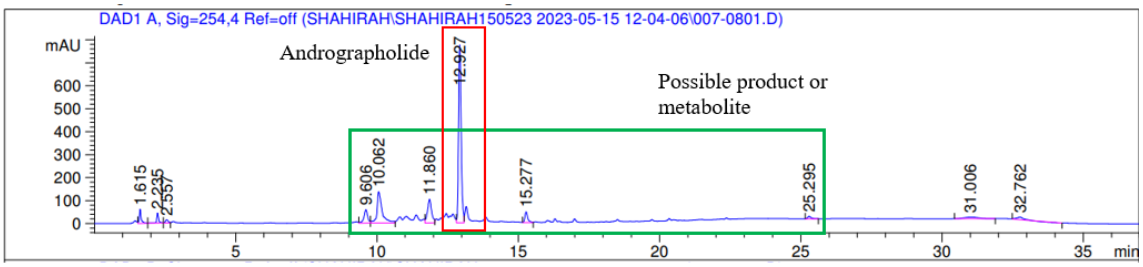

Fig. 16. The peak at a retention time of 12.927 minutes may be the starting material. The highlighted green part in the chromatogram at 254 nm gives the appearance of peaks with a retention time range from 10 minutes to 25 minutes that may be referred to as the biotransformed andrographolide or the fungi metabolites.

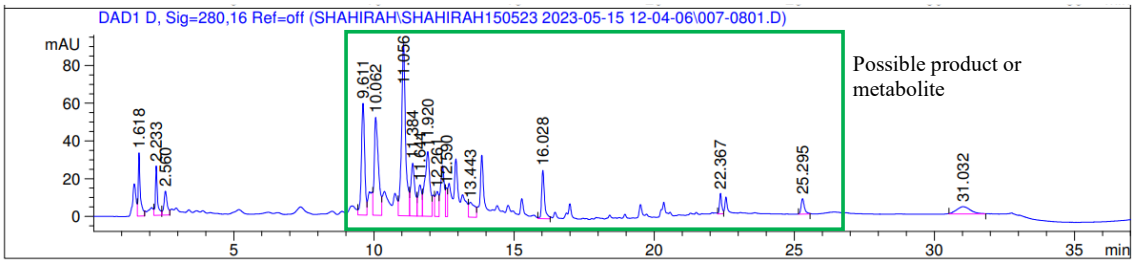

Fig. 17. The highlighted green part in the chromatogram at 280 nm gives the appearance of peaks with a retention time range from 10 minutes to 25 minutes that may be referred to as the biotransformed andrographolide or the fungi metabolites. At a retention time of 11.056 minutes, it has the most intense peak, followed by 9.611 and 10.062 minutes.

Comparison of High-Performance Liquid Chromatography (HPLC) Profiles.

Microbial transformation of andrographolide using *Beauveria bassiana* and *R3-2 SP 17* is the first reported here. Reverse-phase high-performance liquid chromatography is a chromatographic technique used to analyze and separate compounds. It operates based on the principle of differential partitioning between a non-polar stationary phase and a polar mobile phase. This HPLC is the inverse of normal phase chromatography, which involves the polar mobile phase (water/acetonitrile) and the non-polar stationary phase [14].

The extracts, which may contain biotransformed andrographolide and fungal secondary metabolites, were dissolved and injected into the column during the separation process. The non-polar stationary phase interacts more strongly with hydrophobic or non-polar compounds, leading to longer retention and slower elution. On the other hand, polar compounds have weaker interactions with the stationary phase and elute faster with shorter retention times [13–15].

In HPLC, a gradient elution method is often employed to optimize the separation. The composition of the mobile phase, where the ratio of water and acetonitrile, was changed during the separation process. Initially, as a more polar mobile phase is used, it favors the elution of polar compounds [13]. As the separation progressed, the concentration of the acetonitrile was increased, making the mobile phase less polar. This change in mobile phase composition promotes the elution of less polar compounds with longer

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retention times. The acetonitrile acts as an organic modifier to alter the polarity and eluent strength, hence controlling the selectivity of the separation [16].

The eluted components are detected using a diode array detector (DAD), which measures the absorbance or response of the compounds at specific wavelengths [17]. The results are displayed as a chromatogram, providing information on the retention times and intensities of the eluted components. Therefore, to identify the potential biotransformed andrographolide, the chromatograms of the extracted sample were compared with those of the starting material, negative controls, and positive controls. Hence, an additional peak exclusively observed in the sample chromatograms indicated the presence of the biotransformation metabolite.

Biotransformation of Andrographolide by Beauveria bassiana.

Table 2 summarizes the retention times for the peak observed in the HPLC profile of andrographolide biotransformation conducted using *Beauveria bassiana*. The corresponding figures, such as Figures 2 to 11, are referenced in the table. The starting material, andrographolide, was subjected to fermentation with *B. bassiana* for 8 and 12 days and then extracted. Notably, there needs to be more prior research on the biotransformation of andrographolide using *B. bassiana*.

Table 2. Biotransformation of the andrographolide by Beauveria bassiana.

Extract		Major peak retention time (min)	Minor peak retention time (min)
Starting material (Andrographolide)	254nm	12.908	13.029, 15.246, 22.358
	280nm	12.908	20.260
Positive Control (media with andrographolide)	254nm	12.895	8.400, 9.491, 10.304, 11.037, 11.830, 13.123, 13.796, 15.251, 16.064
	280nm	10.305, 12.893, 16.061, 20.209	11.861, 12.141, 12.380, 12.604, 13.800, 15.249
Negative Control (media with B. bassiana)	254nm	11.115, 12.739, 13,717, 14.652, 15.028	10.285, 13.030, 15.824, 16.071
	280nm	11.115, 12.738, 14.652, 15.028	10.394, 10.536, 13.301, 13.719, 15.823, 16.069, 16.811
Extraction on Day 8	254nm	12.798, 12.894	9.510, 11.653, 13.492, 13.802
	280nm	10.352, 10.966, 11.363, 11.650, 12.797, 12.892, 16.007, 16.793, 19.488, 20.324, 22.356, 22.548, 25.252	8.844, 9.564, 13.409, 14.212, 14.686, 15.243, 21.488
Extraction on Day 12	254nm	12.902	9.516, 11.394, 11.844, 13.814, 17.093, 17.765, 20.230, 25.250
	280nm	20.229	10.358, 10.974, 11.382, 11.651, 12.902, 13.734, 16.023, 16.468, 25.250

However, some research used B. bassiana as a biocatalyst for the biotransformation of stemodin and stemodinone, which are diterpenes [18]. The HPLC profiles of the extracted product revealed peaks with retention times ranging from 5 to 20 minutes. The presence of minor peaks in both positive and negative control HPLC profiles suggests that these peaks are the media component. The chromatogram profiles of the positive control (Figure 4 and Figure 5) display multiple peaks and have peaks that appeared at the same retention time (12.895 and 12.893) for both 254 nm and 280 nm. The negative and positive controls were used as a reference for comparing the sample extractions from *B. bassiana* on Days 8 and 12. In the starting material profiles, a significant peak with a retention time of 12.908 minutes was observed for both

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wavelengths, indicating the presence of the starting material, which is andrographolide (Figures 2 and 3). The starting material was also compared to the sample extraction.

A comparison between the negative and positive controls and the sample extraction from the eighth day revealed additional peaks. It can be observed at a retention time of 12.798 and 12.894 min at 254 nm (Figure 8) and 10.352, 10.966, 11.363, 11.650, 12.797, 12.892, 16.007, 16.793, 19.488, 20.324, 22.356, 22.548 and 25.252 min for 280 nm (Figure 9). There are identical major peaks that appeared at 254 nm and 280 nm, which are 12.798 and 12.797 min, respectively. The minor peaks appeared more at 280 nm than 254 nm, as shown in Table 2. More peaks at 280 nm but not at 254 nm can be attributed to the possibility that the compound is detectable only at 280 nm but not at 254 nm.

Upon comparing the HPLC chromatogram data of the sample extract containing andrographolide with *B. bassiana* on Day 12, significant observations were made where there are additional minor peaks were discovered with retention times of 17.093 and 17.765 min at 254 nm (Figure 10). Some peaks are proximately present at the same retention time, such as 11.394, 11.844, and 13.814 at 254 nm (Figure 10) and 11.382, 11.651, and 13.734 at 280 nm for Day 12 of extraction (Figure 11). Interestingly, a decrease in the peak intensity was also detected with a retention time of 12.902 min, high at 254 nm but minor at 280 nm. There is also a rise in intensity for the peak at 20.230 min (254 nm) when observed at 280 nm (20.229 min). This phenomenon can be attributed to variations in the compound's absorbance characteristics [19]. Additionally, all these additional peaks present in the sample extract on Day 8 may indicate the existence of biotransformed andrographolide or the secondary metabolite of the fungi. Moreover, results that showed peaks with retention times of more than 15 minutes indicate that the possible compounds are less polar than the starting material (andrographolide).

Biotransformation of Andrographolide by R3-2 SP 17.

The summarization of the retention time for the peak in the HPLC profile of the andrographolide biotransformation by *R3-2 SP 17* is shown in Table 3, concerning Figures 2 to 5 and 12 to 19. Currently, no research involves psychrotolerant fungi as biocatalysts for the biotransformation of andrographolide. The resulting product extract's HPLC profiles revealed the peaks with retention time ranging from 5 to 20 minutes. The minor peaks presented in the HPLC profiles of positive and negative controls are indicated as a media component. Intriguingly, the peak presented at negative control of *R3-2 SP 17* with retention times 19.792 is high at 280 nm (Figure 13) but not at 254 nm (Figure 12). Hence, the *R3-2 SP 17* metabolites absorbance is preferred at 280 nm rather than 254 nm.

On comparing the sample extract of andrographolide with *R3-2 SP 17* with HPLC chromatogram data on Day 8, at 12.718 min, a new central peak was identified at 254 nm (Figure 14). Furthermore, the peak intensity with the retention time of 16.038 min is high at 280 nm on Day 8 (Figure 15). More extra peaks were discovered after comparing with the HPLC profile of the sample extract at 12 days of fermentation (Figure 16 and Figure 17). The presence of a significant peak with a retention time of 12.927 min (254 nm) diminished at 254 nm. However, additional peaks appeared at 280nm with retention times of 9.611, 10.062, 11.056, 11.920, and 16.028 min (Figure 17). Therefore, it is likely that the fungus could still perform the biotransformation of the starting material into new derivatives even after 12 days of fermentation, leading to the discovery of additional peaks.

Apart from that, the intensity of the peak with a retention time of 10.062 min appeared significant at 280 nm (Figure 17) but minor at 254 nm (Figure 16) on Day 12. Meanwhile, the peaks in the sample extract on the Day 4 chromatogram with a retention time of 9.601, 11.063, and 13.856 min for a wavelength of 280 nm disappeared on Day 8. The unexpected discovery suggests that the reduction in the peak's intensity and the disappearance of the peak between Day 8 are because, during the fermentation periods, the fungus might consume the compounds as an intermediate compound in the production of a new product or act as an elicitor for the fungus, causing it to generate secondary metabolites and enhance its production. The

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compounds can also be used as an energy source or degraded by the fungus itself, thus causing peak reduction or disappearance [20].

These findings align with the research conducted by Choudhary et al. [20], which indicates that endophytic fungi can produce a diverse range of secondary metabolites capable of transformation, degradation, and detoxification. It appears that *R3-2 SP 17*, the specific microorganism used in the study, can transform andrographolide into higher and lower polarity compounds, as evidenced by peaks with retention times exceeding that of the starting material. These results are consistent with another study involving the same microorganism, where the C-3 ester group of ethynodiol diacetate was hydrolyzed to form an OH group [11]. These findings suggest that *R3-2 SP 17* can potentially convert the initial andrographolide into metabolites with increased polarity.

Table 3. Biotransformation of the andrographolide by R3-2 SP 17.

Extract		Major peak retention time (min)	Minor peak retention time (min)
Starting material (Andrographolide)	254nm	12.908	13.029, 15.246, 22.358
	280nm	12.908	20.260
Positive Control (media with andrographolide)	254nm	12.895	8.400, 9.491, 10.304, 11.037, 11.830, 13.123, 13.796, 15.251, 16.064, 16.831
	280nm	10.305, 12.893, 16.061, 20.209	11.861, 12.141, 12.380, 12.604, 13.800, 15.249
Negative Control (media with R3-2 SP 17)	254nm	-	13.513, 16.482, 19.792
	280nm	19.792	15.467, 17.843, 18.944, 22.310, 24.816, 27.941
Extraction on Day 8	254nm	12.718, 12.923,	9.007, 9.604, 13.159, 25.297
	280nm	16.038	8.851, 9.601, 10.382, 11.092, 12.389, 12.720, 15.595, 25.297
Extraction on Day 12	254nm	12.927	9.606, 10.062, 11.860, 15.277
	280nm	9.611, 10.062, 11.056, 11.920, 16.028	11.384, 12.261, 12.590, 22.367, 25.295

Comparison and Summary of Biotransformation of Ciprofloxacin Using Two Fungal Strains: *Beauveria bassiana* and R3-2 SP 17.

Based on the summary of the biotransformation of andrographolide by *Beauveria bassiana* and *R*3-2 *SP 17* displayed under Table 4, some possible biotransformation reactions can be proposed. The existence of a few polar compounds based on the retention time of the biotransformation of andrographolide by *R3-2 SP 17* suggests that the structural modification of the andrographolide may occur through hydroxylation (Figure 21) [21]**.** Furthermore, the production of less polar or non-polar of the biotransformed andrographolide derivatives is likely produced by both fungi. Therefore, andrographolide may undergo methylation or dehydroxylation reaction (Figure 20) [10, 22–24]. Besides, methylation of the hydroxyl group into primary alcohol was observed in the biotransformation of andrographolide with *B. bassiana* [25].

Therefore, it can be summed up that the *R3-2 SP 17* strain is more effective in biotransforming the andrographolide as a comparison between the two fungi strains' ability to metabolize andrographolide was made. This is because after comparing the HPLC profiles of both fungi, additional peaks were observed at the *R3-2 SP 17* chromatogram even after 12 days of fermentation. Furthermore, these psychrotolerant fungi produced more new or additional peaks. They generated polar and non-polar compounds based on the HPLC profile of an extract of andrographolide with *R3-2 SP 17* compared to *B. bassiana*. The current findings align with previous research, which demonstrated the capability of the *R3-2 SP 17* fungi to generate a polar compound via a hydrolysis reaction [11]. Meanwhile, the Antarctic fungus produced a known

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biotransformation metabolite of ethynodiol diacetate, Wan Yusop et al.'s study [11]. They revealed an enhanced metabolite yield within a shorter timeframe and using fewer substrates compared to previous research techniques. Therefore, further investigation is warranted to determine the extent to which the *R3-2 SP 17* fungi can produce biotransformed andrographolide derivatives.

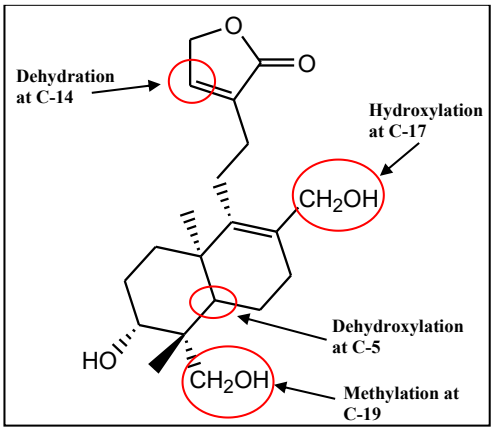

Fig. 20. Andrographolide and its proposed hydroxylation, dehydroxylation, and methylation reactions.

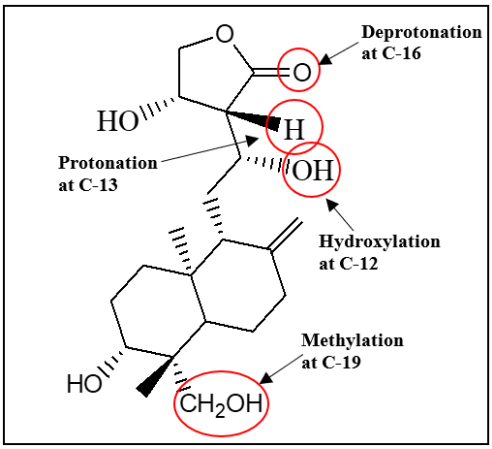

Fig. 21. Andrographolide and its proposed deprotonation, protonation, hydroxylation, and methylation reactions.

The current findings align with previous research, which demonstrated the capability of the *R3-2 SP 17* fungi to generate a polar compound via a hydrolysis reaction [11]. Meanwhile, the Antarctic fungus produced a known biotransformation metabolite of ethynodiol diacetate, Wan Yusop et al.'s study [11]. They revealed an enhanced metabolite yield within a shorter timeframe and using fewer substrates compared to previous research techniques. Therefore, further investigation is warranted to determine the extent to which the *R3-2 SP 17* fungi can produce biotransformed andrographolide derivatives.

This study's experimental work and gathered data revealed that the psychrotolerant fungus provides several benefits, including accelerated microbial growth rates, heightened enzymatic activities, and improved catalytic efficiency [26]. The study results showed a consistent decrease in the peak intensity of

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the starting material with each day of sample extraction. This observation suggests that the fungi may have contributed to the degradation or transformation of andrographolide, which aligns with previous research indicating that certain fungi could partially or wholly degrade the starting material [27–31].

Also, the appearance of a new peak in the HPLC profile of a resulted extract could be either a biotransformed product with a comparison between the starting material, negative and positive controls chromatogram profiles, or the peak also can be secondary metabolites that are essential for the development, survival, and adaption of fungus.Furthermore, the disappearance of peak or diminished peak intensity in the HPLC profile could be employed as an intermediate compound consumed to produce a new product with an increased peak and a fungal elicitor [32]. The fungi can also use or devour it as an energy source. Moreover, the finding implies that among time-course studies for 8 and 12 days, the eight days is more efficient for *B. bassiana*, whereas 12 days of fermentation is efficient for *R3-2 SP 17* to produce more high-yield metabolite.

Table 4: Summarization of biotransformation of andrographolide.

Fungi		Day 8	Day 12
Beauveria bassiana	**254nm**	• An additional major peak was observed. (Retention times of 12.798 min). • Additional minor peaks were observed. (Retention times of 9.510, 11.653, 13.492, 13.802 min).	• Additional minor peaks were observed. (Retention times of 11.394, 11.844, 13.814, 17.093, 17.765, and 20.230)
	280nm	• More additional major peaks observed. (Retention time of 10.352, 10.966, 11.363, 11.650, 12.797, 12.892, 16.007, 16.793, 19.488, 20.324, 22.356, 22.548, 25.252 min)	• An increase in peak intensity compared to 254nm on Day 12 was observed (Retention time of 20.229 min) • The additional minor peaks were observed (Retention time of 10.358, 10.974, 11.382, 11.651, 12.902, 13.734, 16.023, 16.468 min)
R3-2 SP 17	**254nm**	• An additional major peak was observed. (Retention time of 12.718 min). • Additional minor peaks were observed. (Retention time of 9.007 and 13.159 min).	• An additional minor peak was discovered. (Retention times of 15.277 min)
	280nm	• An additional major peak was observed. (Retention time of 16.038 min). • Additional minor peaks were observed. (Retention time of 10.382, 11.092, 12.389, 12.720, and 15.595 min).	• The intensity of the peak at 9.611 min increased slightly more than on Day 8 (9.601 min). • Additional peaks were discovered. (Retention times of 10.062, 11.056, 11.920, and 16.028)

CONCLUSION

In conclusion, the microbial transformation of andrographolide was conducted using two types of fungi: the tropical fungus *Beauveria bassiana* and the psychrotolerant fungus *R3-2 SP 17*. Based on the observed peaks, the microbial transformation reactions proposed in this study encompass methylation, hydroxylation, protonation, deprotonation, dehydroxylation, or hydroxylation. Conversely, the disappearance or decrease in intensity of a peak in the HPLC profile of the resulting extract can indicate an intermediate molecule in the development of new derivatives or the formation of a compound with an amplified peak intensity, possibly acting as a fungal elicitor. This also concludes that Day 8 for *B.bassiana* and Day 12 for *R3-2 Sp 17* are identified as the optimal fermentation duration for effectual biotransformation of andrographolide. *R3-2 SP17* is more effective in andrographolide biotransformation as new peaks emerge on Day 12.

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AUTHOR'S CONTRIBUTION

Nur Shahirah Shaharudin and Muhammad Hatta Syahiran Abdul Halim performed the bench work and compiled all the data. Dr Sadia Sultan conceptualized the central research idea, provided the theoretical framework, designed the research work, and assisted in preparing and writing the manuscript. Dr. Gurmeet Kaur Surindar Singh, Dr. Syed Adnan, and Prof. Teh Lay Kek helped revise and improve the write-up. All authors read and agreed to the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted without any self-benefits or commercial or financial conflicts and declare the absence of conflicting interests with the funders.

REFERENCES

[1] Mathur, S., & Hoskins, C. (2017). Drug development: Lessons from nature. *Biomedical Reports*, *6*(6), 612–614. https://doi.org/10.3892/br.2017.909

[2] Atanasov, A.G., Zotchev, S.B., Dirsch, V.M., & Supuran, C.T. (2021). Natural products in drug discovery: advances and opportunities. *Nature Reviews Drug Discovery*. Nature Research. https://doi.org/10.1038/s41573-020-00114-z

[3] Jiang, M., Sheng, F., Zhang, Z., Ma, X., Gao, T., Fu, C., & Li, P. (2021). Andrographis paniculata (Burm.f.) Nees and its major constituent andrographolide as potential antiviral agents. *Journal of Ethnopharmacology*, *272*. https://doi.org/10.1016/j.jep.2021.113954

[4] Tundis, R., Patra, J.K., Bonesi, M., Das, S., Nath, R., Das Talukdar, A., Das, G., Loizzo, M.R. (2023). Anti-Cancer Agent: The Labdane Diterpenoid-Andrographolide. *Plants*, *12*(10), 1969. https://doi.org/10.3390/plants12101969

[5] Yan, Y., Fang, L.H., & Du, G.H. (2018). Andrographolide. In *Natural Small Molecule Drugs from Plants* (pp. 357–362). Springer Singapore. https://doi.org/10.1007/978-981-10-8022-7_60

[6] Xin, X. lan, Su, D. hai, Wang, X. jie, & Yuan, Q. peng. (2009). Microbial transformation of dehydroandrographolide by Cunninghamella echinulata. *Journal of Molecular Catalysis B: Enzymatic*, *59*(1–3), 201–205. https://doi.org/10.1016/j.molcatb.2009.02.015

[7] Zainal ariffin, Z. (2020). Aspergillus sydowii Strain SCAU066 and Aspergillus versicolor Isolate BAB-6580: Potential Source of Xylanolytic, Cellulolytic and Amylolytic Enzymes. *Science Letters*, *14*(2), 15. https://doi.org/10.24191/sl.v14i2.9539

[8] Hyde, K., Cai, L., & Jeewon, R. (2005). Tropical Fungi. 93–115. https://doi.org/10.1201/9781420027891.ch5

[9] Hughes, K.A., Misiak, M., Ulaganathan, Y., & Newsham, K.K. (2018). Importation of psychrotolerant fungi to Antarctica associated with wooden cargo packaging. *Antarctic Science*, *30*(5), 298–305. https://doi.org/10.1017/S0954102018000329

[10] He, X., Zeng, X., Hu, H., & Wu, Y. (2010). Cytotoxic biotransformed products from andrographolide by Rhizopus stolonifer ATCC 12939. *Journal of Molecular Catalysis B: Enzymatic*, *62*(3–4), 242–247. https://doi.org/10.1016/j.molcatb.2009.11.002

[11] Nurfazilah Wan Yusop, S., Imran, S., Ilham Adenan, M., Ashraf, K., & Sultan, S. (2021). Biocatalytic modifications of ethynodiol diacetate by fungi, anti-proliferative activity, and

acetylcholinesterase inhibitory of its transformed products. *Steroids*, *171*. https://doi.org/10.1016/j.steroids.2021.108832

[12] Yusop, S.N.W., Imran, S., Adenan, M.I., & Sultan, S. (2020). Medroxyprogesterone derivatives from microbial transformation as anti-proliferative agents and acetylcholinesterase inhibitors (combined in vitro and in silico approaches). *Steroids*, *164*. https://doi.org/10.1016/j.steroids.2020.108735

[13] Petrova, O.E., & Sauer, K. (2017). High-Performance Liquid Chromatography (HPLC)-Based Detection and Quantitation of Cellular c-di-GMP. 33–43. https://doi.org/10.1007/978-1-4939-7240-1_4

[14] Kumar, Y., Mumtaz, S.M., & Ahmad, M. (2018). HPLC: Principle and Maintenance with Application. *International Journal of Trend in Scientific Research and Development*, *Volume-2*(Issue-5), 1618–1626. https://doi.org/10.31142/ijtsrd17134

[15] Vidushi, Y., Meenakshi, B., & Bharkatiya, M.B. (2017). A review of HPLC method development and validation. https://doi.org/10.26479/2017.0206.12

[16] Alghoul, Z.M., Ogden, P.B., & Dorsey, J.G. (2017). Characterization of the polarity of subcritical water. *Journal of Chromatography A*, *1486*, 42–49. https://doi.org/10.1016/j.chroma.2016.12.072

[17] Papadoyannis, I.N., & Gika, H.G. (2004). Peak Purity Determination with a Diode Array Detector. *Journal of Liquid Chromatography & Related Technologies*, *27*(6), 1083–1092. https://doi.org/10.1081/jlc-120030180

[18] Buchanan, G.O., & Reese, P.B. (2001). Biotransformation of diterpenes and diterpene derivatives by Beauveria bassiana ATCC 7159. *Phytochemistry*, *56*(2), 141–151. https://doi.org/10.1016/S0031-9422(00)00403-9

[19] van der Doelen, G.A., van den Berg, K.J., Boon, J.J., Shibayama, N., René de la Rie, E., & L. Genuit, W.J. (1998). Analysis of fresh triterpenoid resins and aged triterpenoid varnishes by high-performance liquid chromatography–atmospheric pressure chemical ionization (tandem) mass spectrometry. *Journal of Chromatography A*, *809*(1–2), 21–37. https://doi.org/10.1016/S0021-9673(98)00186-1

[20] Choudhary, M., Gupta, S., Dhar, M.K., & Kaul, S. (2021). Endophytic Fungi-Mediated Biocatalysis and Biotransformations Paving the Way Toward Green Chemistry. *Frontiers in Bioengineering and Biotechnology*. Frontiers Media S.A. https://doi.org/10.3389/fbioe.2021.664705

[21] Wetzstein, H.G., Stadler, M., Tichy, H.V., Dalhoff, A., & Karl, W. (1999). Degradation of ciprofloxacin by basidiomycetes and identification of metabolites generated by the brown rot fungus Gloeophyllum striatum. *Applied and Environmental Microbiology*, *65*(4), 1556–1563. https://doi.org/10.1128/aem.65.4.1556-1563.1999

[22] Xin, X.-L., Deng, S., Zhang, B.-J., Huang, S., Tian, Y., Ma, X.-C., An, L., Shu, X., Yao, J.-H., & Cui, X. (2011). Microbial Transformation of Deoxyandrographolide by *Alternaria Alternata* AS 3.4578. *Natural Product Communications*, *6*(6), 1934578X1100600. https://doi.org/10.1177/1934578X1100600609

[23] Patil, H.S., Jadhav, D.D., Paul, A., Mulani, F.A., Karegaonkar, S.J., & Thulasiram, H. V. (2018). Regioselective and efficient enzymatic synthesis of antimicrobial andrographolide derivatives. *Bioorganic and Medicinal Chemistry Letters*, *28*(6), 1132–1137. https://doi.org/10.1016/j.bmcl.2018.01.007

[24] Handayani, R., Dinoto, A., & Bintang, M. (2022). Novel microbial transformation of Andrographis paniculata by Aspergillus oryzae K1A. *Biodiversitas*, *23*(1), 110–117. https://doi.org/10.13057/biodiv/d230114

[25] Parshikov, I.A., Moody, J.D., Heinze, T.M., Freeman, J.P., Williams, A.J., & Sutherland, J.B. (2002). Transformation of cinoxacin by Beauveria bassiana. *FEMS Microbiology Letters*, *214*(1), 133–136. https://doi.org/10.1111/j.1574-6968.2002.tb11336.x

[26] Trytek, M., & Fiedurek, J. (2005). A novel psychrotrophic fungus, Mortierella minutissima, for D-limonene biotransformation. *Biotechnology Letters*, *27*(3), 149–153. https://doi.org/10.1007/s10529-004-7347-x

https://doi.org/10.24191/sl.v18i1.24791

[27] Rusch, M., Kauschat, A., Spielmeyer, A., Römpp, A., Hausmann, H., Zorn, H., & Hamscher, G. (2015). Biotransformation of the Antibiotic Danofloxacin by *Xylaria longipes* Leads to an Efficient Reduction of Its Antibacterial Activity. *Journal of Agricultural and Food Chemistry*, *63*(31), 6897–6904. https://doi.org/10.1021/acs.jafc.5b02343

[28] Prieto, A., Möder, M., Rodil, R., Adrian, L., & Marco-Urrea, E. (2011). Degradation of the antibiotics norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products. *Bioresource Technology*, *102*(23), 10987–10995. https://doi.org/10.1016/j.biortech.2011.08.055

[29] Čvančarová, M., Moeder, M., Filipová, A., & Cajthaml, T. (2015). Biotransformation of fluoroquinolone antibiotics by ligninolytic fungi – Metabolites, enzymes and residual antibacterial activity. *Chemosphere*, *136*, 311–320. https://doi.org/10.1016/j.chemosphere.2014.12.012

[30] Rusch, M., Spielmeyer, A., Zorn, H., & Hamscher, G. (2018). Biotransformation of ciprofloxacin by Xylaria longipes: structure elucidation and residual antibacterial activity of metabolites. *Applied Microbiology and Biotechnology*, *102*(19), 8573–8584. https://doi.org/10.1007/s00253-018-9231-y

[31] Manasfi, R., Chiron, S., Montemurro, N., Perez, S., & Brienza, M. (2020). Biodegradation of fluoroquinolone antibiotics and the climbazole fungicide by Trichoderma species. *Environmental Science and Pollution Research*, *27*(18), 23331–23341. https://doi.org/10.1007/s11356-020-08442-8

[32] Zhai, X., Jia, M., Chen, L., Zheng, C., Rahman, K., Han, T., & Qin, L. (2017). The regulatory mechanism of fungal elicitor-induced secondary metabolite biosynthesis in medical plants. *Critical Reviews in Microbiology*, *43*(2), 238–261. https://doi.org/10.1080/1040841X.2016.1201041

https://doi.org/10.24191/sl.v18i1.24791