Microwave-assisted extraction of Taxol and 10-deacetylbaccatin III from the leaves and branches of red pine (*Taxus wallichiana* Zucc.)

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ABSTRACT

Taxol has been by far the most well-known worldwide as an effective anticancer natural drug. With the great treatment abilities at low concentration, Taxol is a considerable interest of many scientists in various fields. The commercial products of Taxol can be isolated directly from Taxus species or can be synthesized from 10-deacetylbaccatin III (10-DAB III) or baccatin III (BC III), which are known as precursors of Taxol, by using semisynthetic methods. In this study, the extraction of Taxol and 10-DAB III from the leaves and branches of red pine cultivated in Lam Dong Province, Vietnam was carried out. Some traditional methods such as Soxhlet, maceration as well as the modern methods such as microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE) were used to extract and evaluate the extraction efficiency. The concentration of 10-DAB III experienced at over 90% as compared to maceration and the amount of Taxol accounted for approximately 80% of Soxhlet. MAE was thus more suitable for recovering both Taxol and 10-DAB III than the others because of its short time and less solvent consumption. Besides, four parameters including solvent nature (MeOH and EtOH), extraction time, material/solvent ratio (1:10, 1:15, 1:20 and 1:25) and microwave power (40W, 240W and 440W) were investigated the effects of these elements on the content of 10-DAB III and Taxol. The results illustrated that the optimal conditions providing 95.85% 10-DAB III were as follows: 240W, 1:15 ratio and 20 minutes for extraction. To get the highest amount of Taxol (79.83%), extraction was

subjected at these conditions concluding 40W, 1:25 ratio and 20 minutes. Diaion, NP-silicagel were used to enrich taxol and 10-DAB III from methanol extract and chloroform extract, respectively. The highest concentration of Taxol and 10-DAB III constituted at 0.64% and 19.76% with NP-silicagel (PE: aceton, 7:3).

Keywords: *Taxol, 10-DAB III, Microwave assisted extraction (MAE), Taxus wallichiana Zucc.*

1. INTRODUCTION

Taxol, a natural diterpenoid, was first isolated from the bark of Taxus brevifolia by Wall and Wani in 1965. 6 years later, the structure of this promising anti-cancer compound got published [1]. In 2000, Taxol became the best-selling cancer drug. It serves as an effective inhibitory against ovarian, breast, lungs and skin cancers [1-3]. Other medical uses includes antiepileptic, anti-inflammatory, antipyretic, analgesic and antimicrobial activities [4]. Besides, the bark was used as a plaster on bone, as well as for relief from headache [5], and the leaves and bark extracts are used for the treatment of bronchitis, asthma, poisonous insect bites [3]. According to many studies, Taxol concentration is only about 0.001 to 0.05% [1,6,7] whereas the global demand is nearly 800-1000 kilograms per year [8]. Semi-synthesis is an alternative; many studies from Denis and Greene (1988), Ojima (1992) elucidated that 1 kg of 10-DAB III and BC III could be converted into about 0.6 to 0.7 kg of Taxol. Nonetheless, source of these precursors is limited to only one Taxaceae species, called genus Taxus spp.

Taxol, 10-DAB III and other taxoids have been isolated from yew tree species with some conventional methods such as maceration, ultrasonic-asisted extraction, microwave-assisted extraction, pressurised liquid extraction, solid-phase extraction, Soxhlet [9, 10]. Despite of simple procedures, these methods have many disadvantages like low selectivity, large solvent consumption and long extraction time. In recent years, the application of microwave for extraction of constituents from plant has shown tremedous research interest and potential due to its highlighted advantages as efficient and friendly environmental extraction, high extraction rate and selectivity, and less solvent consumption [11]. MAE has been used for the extraction of some natural products such as ginger [12], citrus lemon [13], triterpenoid compounds in olive leaves [14], mangosteen [15], etc. As a result, MAE is an alternative, powerful extraction method especially for thermosensitive compounds.

It has been documented that the selectivity as well as the extraction yield of MAE can be improved by adjusting the operating parameters [16]. Therefore, evaluating the effects of process parameters on desirable responses is highly necessary. It will provide not only a better understanding of the overall extraction process but also useful data for the optimization and scale up. In this work, the optimization methodology was used to study the effects of operating conditions, i.e. the solvent nature, extraction time, material/solvent ratio and power on the recovery and concentration of Taxol and 10-DAB III from leaves and branches of *Taxus wallichiana* Zucc.

2. MATERIAL AND METHODOLOGY

2.1. Materials, chemicals and equipment

Leaves and branches of red pine were collected from VIMEDIMEX Company, Da Lat, Lam Dong province, Vietnam. The materials then went through dust removal and dried at ambient temperature for several days. After that, material with 8.36% of humidity was milled and stored in the dark bottle at room temperature until use.

Organic solvents including ethanol, methanol, n-hexane, chloroform and distilled water were bought from China. All chemicals were analytical grade, with purification level higher than 99%. Taxol and 10-DAB III standards' purification were 98.35% and 98.4%, purchasing from Sigma-Aldrich, respectively.

The equipment included microwave (Sanyo EM-S2086W), supersonic bath (Power Sonic 410), a set of Soxhlet apparatus, filtering evaporator, rotating evaporator (model R-215, BUCHI Labortechnik AG, Switzerland), ultraviolet – visible spectrophotometer (PG Instruments T70) and high performance liquid chromatography (HPLC) were used in this study.

2.2. Extraction procedure

The experimental carried out some conventional and modern techniques such as ultrasound-assisted extraction (UAE), maceration, and microwave-assisted extraction (MAE) and Soxhlet. Each 10 g of material and appropriate amount of solvent (MeOH or EtOH) was loaded into extractor for all methods. Extraction by Soxhlet method conducted 200 mL of solvent during 3.5 hours while UAE method used 100 mL of solvent per 30 minutes with total of 180 minutes extraction. Extraction by maceration consumed up 500 mL of solvent/day within 5 days.

For extraction by using MAE, the parameters comprising extraction time, solvent, microwave energy (40, 240 and 440 W) and material/ solvent ratio (1:10, 1:15, 1:20 and 1:25 g/ml) were investigated. In this study, 5 g of dried material and appropriate amount of solvent were extracted during 50 minutes. The solution was removed and the new solvent was added every 5 minutes. For all methods, with the exception of Soxhlet methods, the experiments were set at room temperature. The solvent was boiled and refluxed in the Soxhlet extractor at 65 ± 5^{o}C. Depending on the method used, the extract was taken and the new same amount of solvent was added after a specific amount of time. This process was repeated until the solution was completely colourless. The solution was filtered through a Whatman (No.1) filter paper, then the filtrate was concentrated under reduced pressure at 45^{o}C using a rotary vacuum evaporator. Residue was weighed, dissolved in methanol. Prior to analysis by HPLC, this methanol solution was filtered through 0.45 µm membrane filter.

2.3. Enrichment process

Firstly, 400 g of dry material were immersed in MeOH during 48 h at room temperature. The methanol extracts were pooled and were concentrated under reduced pressure. The residue was soaked with pure water and extracted with n-hexane then discarding n-hexane. After that, continued to extract with CHCl3. The chloroform extract was combined and concentrated under reduced pressure at 45^{o}C. Methanol extract and chloroform extract was accurately weighed and transferred to

volumetric flask with methanol. Next, these solutions were analysis by HPLC to determine the concentration of Taxol and 10-DAB III.

In an attempt to increase the concentration of 10-DAB III and Taxol, enhancement procedure conducted as follows:

- After concentration in vacuum evaporator of MeOH extract, part of the residue was subjected to diaion column chromatography eluting with 98g of silicagel, eluting solvent of the mixture of MeOH and water from 10% to 100% of MeOH.

- Besides, chloroform extract was subjected to 50 g of silicagel column chromatography eluting with PE: Acetone mixture from 0% to 100% of acetone.

- All obtained solutions were diluted in MeOH for HPLC analysis.

2.4. Determination the concentration of Taxol and 10-DAB III

High performance liquid chromatography (HPLC) was carried out on a Agilent 1100 series system (USA) including Quaternary pump, online vacuum degasser, Agilent auto sampler, Thermostatted Column Compartment, diode-array detector (DAD) and Agilent LC Chemstation 32bit software to analysis the concentration of Taxol and 10-DAB III.

The chromatographic separation was accomplished with an Eclipse XDB C18 column (150mm x 4.6mm, I.D., 5µm) at room temperature. The mobile phase was the mixture of acetonitrile, MeOH and H2O, the flow rate was adjusted at 1.25 mL/min, the injection volume was 5µL. Each run was followed by an equilibration time of 45 minutes. The chromatogram was determined at 232 nm and 228 nm for 10-DAB III and Taxol by a diode-array detector, respectively.

3. RESULT AND DISCUSSION

3.1. The effect of extraction method on the concentration of Taxol and 10-DAB III

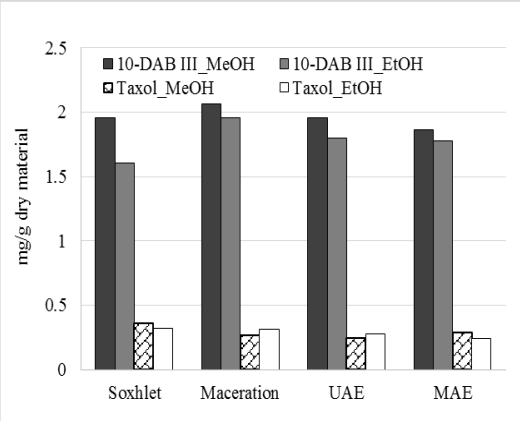

Figure 1. The concentration of 10-DAB III and Taxol

The concentration of 10-DAB III and Taxol obtained by using various extraction methods were shown in Figure 1. It was apparently from Figure 1 that the extraction efficiency of MeOH was better than EtOH. The highest content of 10-DAB III (2.06 mg/g dry weight) and Taxol (0.36 mg/g dry weight) were obtained by extracting using maceration and Soxhlet method, respectively. For MAE method, the amount of Taxoids compounds were about 91% and 81% of the one obtained by maceration and Soxhlet method.

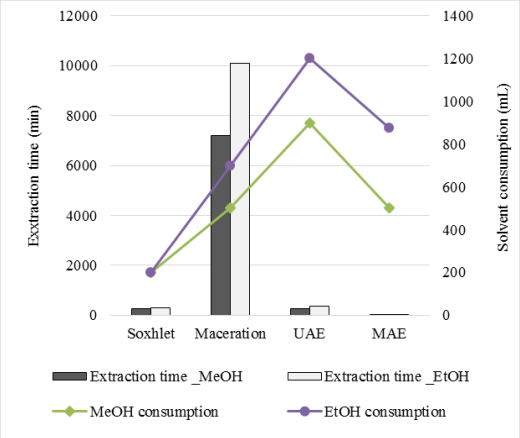

Figure 2. The extraction time and solvent consumption of different methods

Besides, the extraction time and the quantity of solvent were also considered in this study. Figure 2 illustrated the consumption of time and solvent of four extraction methods. Although MAE consumed the second-highest for both of the solvents, it is the most time-efficient. Time consumption was the main disadvantage of other methods. Maceration spent the longest extraction time of 10080 minutes; Soxhlet spent 280 minutes, UAE spent 360 minutes while the extraction by MAE only costed 35 minutes. Significant time-saving and relatively high yield promoted MAE as the most appropritate method for Taxol and 10-DAB III extractions.

3.2. Optimization of MAE conditions

3.2.1 Effect of microwave power on the content of methanolic and ethanolic extract

The effect of microwave energy were strongly dependent on the nature of solvent (as shown in Figure 3). It was clearly seen that the amount of methanolic extract was higher than ethanolic extract. In detail, the highest amount of methanolic and ethanolic extracts were 0.51 and 0.40 g/g dry material at the maximum of power and ratio, 440W and 1:25 (w/v). On the contrary, at the lowest power of 40W and ratio of 1:10 (w/v), the amount of methanolic and ethanolic extracts were the lowest, 0.38 and 0.26 g/g dry material, respectively.

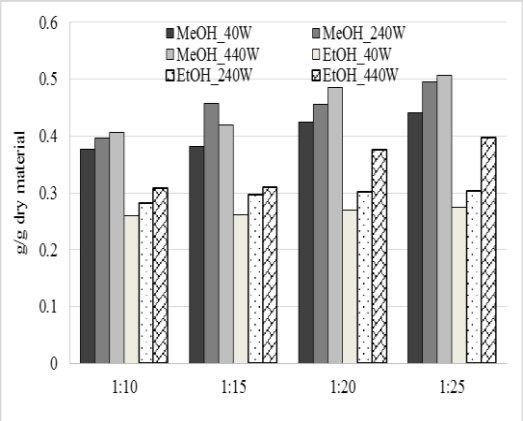

Figure 3. The amount of methanolic extract and ethanolic extract at power of 40W, 240W and 440W

In general, there was a significant increase in the amount of extracts according to an increase of microwave power energy (from 40W to 440W) and ratio of material/solvent (from 1:10 to 1:25, w/v) for both type of solvents (MeOH and EtOH). These results can be explained by some previous researches [16, 18]. In MAE, the extraction efficiency depends on the dissipation factor (tanδ) which evaluates the ability of the solvent to absorb microwave energy and convert it to heat to the surrounding molecules. It is caculated by the equation: $\tan\delta = \varepsilon''/\varepsilon'$ where ε'' is the dielectric loss which indicates the efficiency of converting microwave energy into heat; ε' is the dielectric constant which is the measure of the ability to absorb microwave energy. According to theory, methanol absorbs much microwave than ethanol due to higher its ε' value (ε'=32.6 for methanol and ε'=24.3 for ethanol) [16]. Moreover, the methanol's overall heating efficiency is also

much better than ethanol since the tanδ values of methanol and ethanol were 0.64 and 0.25, respectively [19].

With a constant amount of material, the larger the solvent volume consumed, the better the extraction capacity was observed because of increasing in mass transfer process which helped to release 10-DAB III and Taxol into solvent in a more efficient manner.

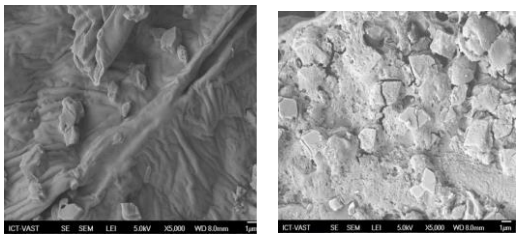

Figure 4. The SEM result of red pine's sample before and after extraction by Microwave-assisted at zooming ratio 1:5000

Scanning electron micrographs of raw and treated material were presented in Figure 4. The cell walls of MAE's sample were completely broken. The principle of heating by MAE is based on two simultaneous mechanisms, dipolar rotation and ionic conduction. With a frequency of 2,450 MHz, the electrical component of the wave changes 4.9x109 times per second resulted in rapid heating. This phenomenon supported to destroy the structures of cells efficiently and enhanced the efficiency of mass transfer. The rapid rupture of cell walls lead to improve diffusive capacity of compounds from insight cell to solvent. Therefore, the extraction of Taxol and 10-DAB III finished by MAE in very short time [19].

3.2.2 Effect of microwave power and ratio material/solvent on the concentration of 10-DAB III and Taxol.

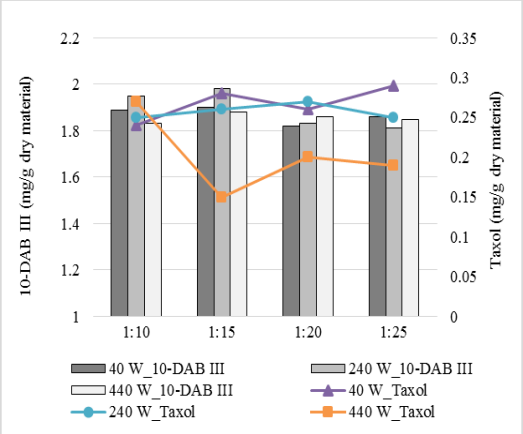

Figure 5. The concentration of 10-DAB III and Taxol in methanolic extracts at different ratios and microwave power levels

The contents of 10-DAB III and Taxol extracted by MAE at different conditions were clearly depicted in Figure 5. Overall, the contents of 10-DAB III and Taxol obtained from branches and leaves of red pine *Taxus wallichiana* Zucc. were stable with an increase of ratio material/solvent and microwave power excluding at power of 440W, the content of Taxol decreased with a rise of ratio maerial/solvent. Especially, a percentage increase was observed in Taxol content, about 17% from 0.24 to 0.28 mg/g dry weight at ratio of 1:10 to 1:15. Whilst 10-DAB III content increased only 2.7%, from 1.83 mg/g at 1:10 to 1.88 mg/g at 1:15. It could be explained that the larger quantity of solvent was consumed, the easier transferring of Taxoids from material to solution was occurred. On the other hand, the higher microwave energy leads to the expansion and rupture of cell walls, then liberated analytes into the solvent. Additionally, high temperature decreased the viscosity of solvent, interaction of material and solvent were promoted. Thus, the diffusive rate of solvent raised and extraction

speed improved [19]. In fact, both the power level and the amount of solvent strongly affected to 10-DAB III and Taxol contents.

3.2.3 Effect of microwave power and ratio material/solvent on the recovery yield of 10-DAB III and Taxol

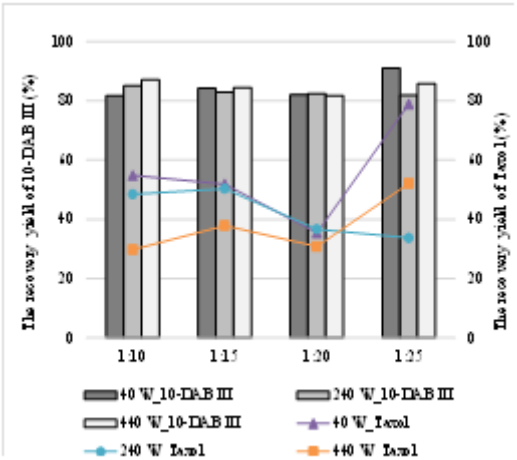

Figure 6. The recovery yield of 10-DAB III and Taxol (using MeOH) at different ratios and microwave power levels

The recovery yield (Y%) was the amount pure product recovered (M) divided by the maximum amount (M_{max}) that product presented in dry material multiplied by 100%.

$$Y\% = \frac{M}{M_{max}} \times 100\% \qquad (1)$$

From Figure 6, it was obviously seen that the recovery yield of 10-DAB III was over 88% and peaked at 95.85% at ratio of 1:15 and 240 W. In general, the recovery yield of 10-DAB III slightly increased with an increase of power level and ratio material/solvent .

Similar to 10-DAB III, it also indicated that the recovery efficiency of Taxol varied from 41.48% to 79.83%. When extraction set at 440 W, the extraction efficiency decreased with increasing ratio. The optimal conditions for recovering Taxol (79.83%) were at 40W, ratio of 1:25.

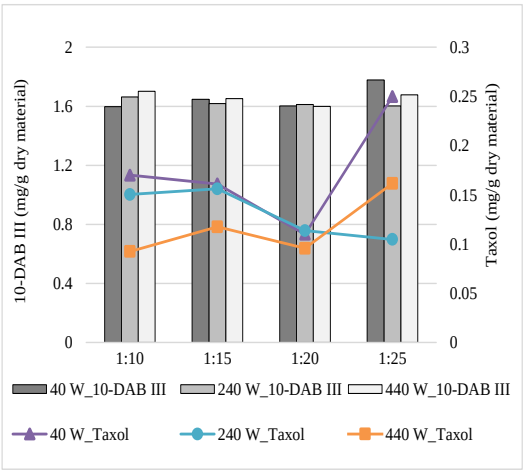

Figure 7. The concentrations of 10-DAB III and Taxol in ethanolic extracts at different ratios (w/v) and microwave power levels

From Figure 7, there was a slight fluctuation in 10-DAB III content and a strong fluctuation occurred in Taxol. There was an upward trend in Taxol content from 1:10 to 1:15 before went down at 1:20 and rebounded at 1:25. At the same ratio of 1:25, the highest content of 10-DAB III and Taxol accounted for 1.78 mg/g and 0.25 mg/g material which was recorded at 440W and 40 W, respectively. In addition, it can be clearly seen that the content of Taxoids went up at a higher power with a fixed ratio.

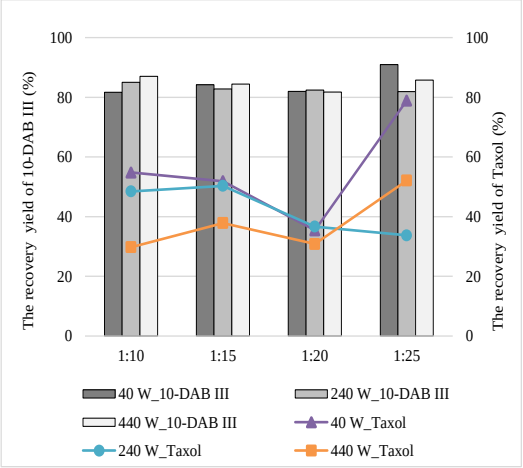

Figure 8. The recovery yields of 10-DAB III and Taxol (using EtOH) at different ratios (w/v) and microwave power levels

Solvent nature and volume strongly played key factors for the extraction efficiency. The recoveries of 10-DAB III and Taxol from ethanolic extracts were much lower than those from methanolic extracts. A range from 81.69% to 90.95% for 10-DAB III and from 29.77% to 78.91% for Taxol were obtained. The optimal MAE conditions using EtOH were power of 40 W, ratio of 1:25 for the highest recovery of both 10-DAB III and Taxol.

3.3. Enrichment process

The comparison of Taxoids contents between crude methanolic extract and chloroform extract were illustrated in Figure 9. After n-hexane-chloroform purification step, both the purified proportion of 10-DAB III and Taxol remarkably increased and reached to 4.57%, 0.28%, which were 10 times for 10-DAB III and 2.5 times for Taxol higher than in crude methanolic extracts. Purification step showed that n-hexane and chloroform effectively removed non-polar components. Because the polarity of these solvents is closed to the polarity of lipids and other non-polar compounds, these impurities were thus easily removed and the contents of 10-DAB III and Taxol also grew considerably. The significant difference in the amount of 10-DAB III between crude methanolic extract and in extract after purification two-steps was up to 10 times due to the different polarity of 10-DAB III and Taxol.

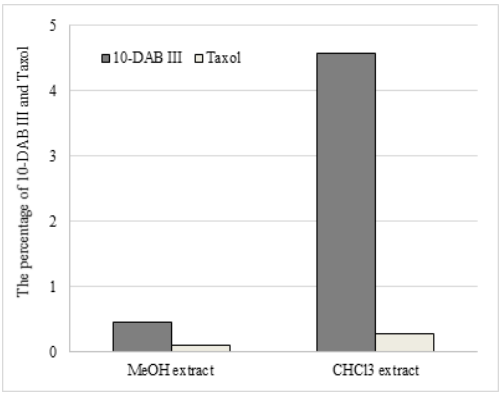

Figure 9. The percentage of 10-DAB III and Taxol in crude methanol extract vs. chloroform extract

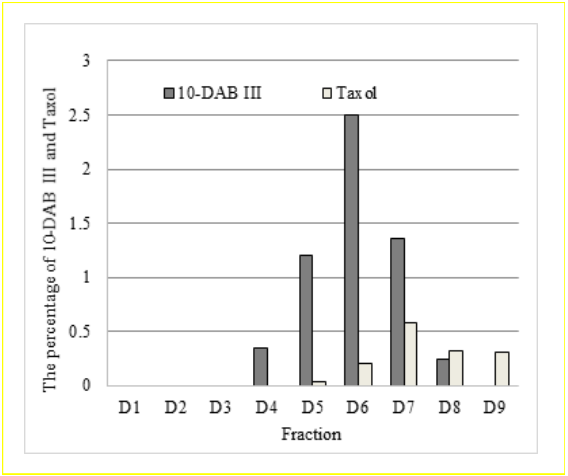

Figure 10. The percentage of 10-DAB III and Taxol after SPE using diaion column

Because of the low taxoids contents and the presence of various impurities (waxes and

chloropylls) [21], for the purpose of 10-DAB III and Taxol purification, the solid-phase extraction process was conducted with specially prepared cartridges with diaion and NP-silicagel. After eluting throughout diaion column by the mixture of H_2O: MeOH, nine fractions were collected. The highest percentage of 10-DAB III was observed at 2.5% at elution fraction H_2O: MeOH (40:60). At fraction H_2O: MeOH (20:80), the highest percentage of Taxol was 0.59%.

Two different types of SPE partitioning were performed and compared. As can be seen from Figure 11 that NP-silicagel sorbent column was good for cleaning and partitioning efficiency. The results suggested that when the column was directly washed with the mixture of PE:acetone (P4), the highest recoveries of 10-DAB III and Taxol were accounted for 19.76%, 0.64%, respectively.

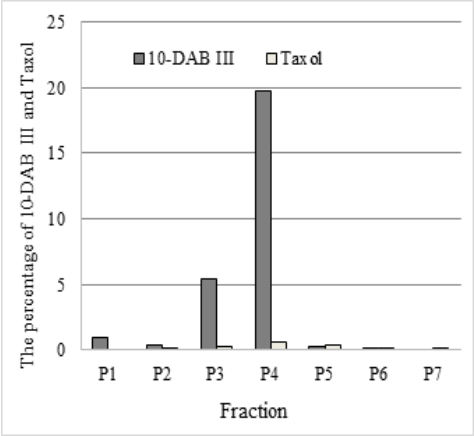

Figure 11. The percentage of 10-DAB III and Taxol after SPE partioning using NP-silicagel chromatography column

After conducted by SPE process, the concentration of both 10-DAB III and Taxol increased remarkably. Especially, 10-DAB III content rose from 4.57% to 19.76% by using diaion column. In term of Taxol, a rising in Taxol proportion ranged from 0.28% to 0.64%. In 2006, 300µg/g dry material of 10-DAB III (from stems of Taxus baccata L.) and 50µg/g dry material of Taxol (from fried needles of *Taxus baccata* L.) were reported by Glowniak and Mroczek [22]. They washed by CH_3OH-H_2O (50:50), dichloromethane prior to using Thin-layer chromatographic (TLC) and quantitiative determination by RP-HPLC (C18) (30% ACN:70%H_2O for 10-DAB III and 50%ACN:50%H_2O for Taxol).

4. CONCLUSION

In general, the study applied SPE and HPLC combination enrichment procedure to improve the yield of 10-DAB III and Taxol extracted from leaves and branches of *Taxus Wachilliana* Zucc. The highest obtained concentrations were 19.76% (10-DAB) and 0.64% (Taxol). Between methanol and ethanol, the first solvent is more appropriate for the extraction of Taxoids; and optimum condiitions were 20 minutes, 240W microwave power and at the ratio of 1:15.

Trích ly Taxol và 10 - deacetylbaccatin III bằng phương pháp vi sóng từ lá và cành cây Thông đỏ (*Taxus wallichiana* Zucc.)

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TÓM TẮT

Taxol được biết đến từ rất lâu như một loại thuốc tự nhiên chữa ung thư hiệu quả. Với khả năng điều trị tuyệt vời ở liều lượng thấp, Taxol đang thu hút nhiều nhà khoa học trên thế giới nghiên cứu. Các sản phẩm Taxol thương mại có thể được phân lập trực tiếp từ loài Taxus hoặc có thể được tổng hợp từ 10-deacetylbaccatin III (10-DAB III) hoặc từ baccatin III (BC III), được biết như tiền chất của Taxol, bằng phương pháp bán tổng hợp. Trong nghiên cứu này, trích ly Taxol và 10-DAB III từ lá và cành cây Thông đỏ được trồng tại Lâm Đồng, Việt Nam sẽ được nghiên cứu. Một vài phương pháp trích ly truyền thống như Soxhlet, ngâm dầm cũng như các phương pháp hiện đại như trích ly có hỗ trợ vi sóng (MAE), trích ly có hỗ trợ siêu âm (UAE) được thực hiện để đánh giá hiệu quả. Nồng độ của 10-DAB III đạt hơn 90% được so sánh với phương pháp ngâm dầm và lượng Taxol chiếm khoảng 80% khi chiết bằng Soxhlet. Vì vậy, MAE phù hợp hơn trong việc trích ly cả hai hợp chất Taxol và 10-DAB III so với các phương pháp khác bởi vì thời gian trích ngắn và tiêu tốn dung môi ít. Bên cạnh đó, bốn thông số bao gồm loại dung môi (MeOH và EtOH), thời gian trích, tỉ lệ nguyên liệu/dung môi (1:10, 1:15, 1:20 và 1:25) và mức năng lượng sử dụng (40W, 240W và 440W) đã được khảo sát những ảnh hưởng của các nhân tố này đến hàm lượng 10-DAB III và Taxol thu được. Kết quả cho thấy điều kiện tối ưu để thu được 95.85% 10-DAB III như sau: 240W, tỉ lệ 1:15 và thời gian trích là 20 phút . Để đạt được hàm lượng Taxol cao nhất (79.83%), việc trích ly phải diễn ra tại mức năng lượng 40W, tỉ lệ 1:25 trong 20 phút. Cột Diaion, NP-silicagel tương ứng được dùng để làm giàu hợp chất 10-DAB III từ dịch chiết bằng methanol và cloroform. Nồng độ cao nhất của Taxol và 10-DAB III là 0.64% và 19.76% với cột NP-silicagel (PE: aceton, 7:3).

Từ khóa: *Taxol, 10-DAB III, Trích ly có hỗ trợ vi sóng (MAE), Taxus wallichiana* Zucc.

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