J. Pl. Res. Vol. 17, No. 1, pp 131-138, 2019

Monthly Variation of 10-deacetylbaccatin III Content in *Taxus mairei* (Lemée & H. Lév.) S.Y.Hu

Usha Tandukar*, Sajjad Alam and Pramesh Bhahadur Lakhey
Department of Plant Resources, Thapathali, Kathmandu, Nepal
E-mail:utandukar@yahoo.com

Abstract

Leaves were collected from *Taxus mairei (Lemée & H. Lév.) S.Y. Hu* in each of the twelve months of a year from Godavari, Lalitpur. Extraction of 10-deacetylbaccatin III (10-DAB III) was carried out from powdered leaves samples for each month. Extracted 10-DAB III was estimated using the High Performance Liquid Chromatography (HPLC) method. Statistical comparison of physical yield, purity of the yield and actual yield of 10-DAB III for different harvest months showed significant monthly differences in these parameters. Highest yield (actual / standardized) was observed in June (0.50704%) while lowest yield was observed in April (0.22494%).

Keywords: *Extraction, HPLC, 10-DAB III, Percentage purity yield*

Introduction

Taxus mairei (Lemée & H. Lév.) S.Y.Hu is one of the species of genus *Taxus* belonging to family Taxaceace found in Nepal. According to Bhatta, Poudel, Pandey and Basnet (2017), three morphologically, genetically and ecologically distinct species occurring in Nepal. *Taxus contorta* Griffith, a west Himalayan temperate species, is distributed from Darchula district of Western Nepal to northern belt of Gorkha district in Central Nepal. *Taxus mairei* (Lemée & H. Lév.) S. Y. Hu is found scattered in relatively low lying areas of Kavrepalanchok, Makawanpur and Sindhuli districts of Central Nepal, while *Taxus wallichiana* Zucc., being an east Himalayan species, covers the temperate regions of Eastern to Central Nepal extending from Taplejung to the south east part of Baglung district to west (Bhatta et al., 2017). The leaves and fruits of *Taxus* are antispasmodic, aphrodisiac, emmenagogue and sedative. The leaves are used in the treatment of asthma, bronchitis, hiccough, epilepsy and indigestion (Chopra, Nayar & Chopra, 1986). *T. wallachiana*, commonly known as Himalayan yew, is traditionally used as various preparations and parts of the plant have specific uses. Leaf juice is used to treat cancer and bronchitis; bark and leaf juice is used for asthma, bronchitis and cancer, whereas dried leaves are considered to be useful for asthma, bronchitis, hiccough, epilepsy, diarrhea and headache (Kunwar, Shrestha & Bussman, 2010). A tincture made from the young shoots is used for treatment of feeble and falling pulse, coldness of extremities, headache, giddiness, diarrhea and severe biliousness. Decoctions prepared from the bark is used in the management of pain associated with muscles, joints and rheumatism; from the leaves is used for treating liver problems; from the bark, filtered and mixed with jaggary (a sweetener) is used for hysteria and from the stem is taken early in the morning to treat tuberculosis. Some written evidence suggested antirheumatic, anticatarrhal, insecticidal and wound-healing properties to Himalayan yew and recommend the use of the drug in powder form for treatment of several disorders including vitiated blood, tumors, dermatosis and helminthiasis. Himalayan yew is also an important ingredient of several Ayurvedic formulations such as lavanbhaskar churna, talisadi vati, and sudarshan churna (Sharma & Garg, 2015)

As *Taxus* spp. are the natural sources for paclitaxel, a compound with high market value, extensive phytochemical studies on *Taxus* spp. have been carried out during the last two decades (Wani, Taylor, Wall, Coggon & Mc Phail., 1971; Yukimune, Tabata, Higashi & Hara, 1996; Croteau, Ketchum, Long, Kaspera & Wildung, 2006). Around 160 compounds

have been isolated from *T. mairei*. Most of these compounds are taxane diterpenoids (Li, Huo, Zhang & Shi, 2008). The other compounds mainly include abietanes (Yang, Fang & Cheng, 1998), rearranged abietanes (Yang, Fang & Cheng, 1996), lignans (Ohtsuki, Miyai, Yamaguchi, Morikawa, & Okano, 2011) and phenolic compounds (Yang et al., 1998). In addition, several volatile components were identified in the leaves of *T. mairei*, which could be used as natural and supplementary reagents for the treatment of hypertension (Yang, Zhao, Wang, Yu & Liang, 2012). Polyprenols, which are natural lipids with potential efficacy in the treatment of liver fibrosis, were also isolated from *T. mairei* (Yu et al., 2012).

Paclitaxel is naturally present in small amounts in the bark of the species of genus *Taxus* which are very slow growing plants. To tackle the problems encountered obtaining paclitaxel from natural source, enormous efforts have been given to develop a more sustainable source of paclitaxel including total and semi-synthetic approaches, biotechnological and bioprocess engineering methodologies (Kim, 2004; Jeon, Mun & Kim, 2006; Khosroushahi et al., 2006). One of the common ways to obtain paclitaxel is its semi-synthesis from a precursor, named 10-deacetylbaccatin III (10-DAB III) which is present in larger amounts in the same plants and is mainly located in the needles. Also 10-DAB III has been used in the semi-synthesis of taxotere which is twice as active as paclitaxel as an antitumor agent (Dziedzic, Vesely & Cordova, 2008). The removal of the leaves from the tree has no effect on the "health" of the tree and the leaves are regenerated relatively quickly, so it is unnecessary to cut down the trees to obtain the bark. The conversion of 10–DAB III to Taxol is thus an excellent option for the large scale and economic synthesis of Taxol (Paclitaxel) (Nautiyal, 2014). This study was carried out to compare the amount of 10-DAB III in leaf samples of *T. mairei* harvested in different months of a year in order to identify the best suited harvest month for maximum yield of 10-DAB III.

Materials and Methods

Chemicals and Reagents

Acetonitrile, Ethyl acetate, Distilled water, Calcium carbonate, Sodium sulphate, filter papers and Standard 10-deacetylbaccatin III.

Sample collection and processing

Leaves of *T. mairei* were collected once a month for 12-months-duration starting from August 2015 to July 2016 from the Utilisation and Pilot Plant Section of Department of Plant Resources located at Godawari, Lalitpur, Nepal (27.58922 N, 85.38138 E, 1528 m asl). The samples were identified by National Herbarium and Plant Laboratories, Godavari, Nepal. The collected samples were dried in shade for 30 days and powered by grinding. Extraction was carried out in each month samples.

Extraction of 10-deacetylbaccatin III (10 –DAB III)

To 125 ml distilled water, 25 g of powdered sample was added. The mixture was sonicated for 1 hour then filtered. The filtrate was extracted with 90 ml of ethyl acetate for three times. The combined organic phases were washed with 0.1M sodium carbonate solution then with demineralized water and finally dried over sodium sulphate. The combined organic extract was concentrated in rotary vacuum evaporator to obtain a dried extract which was dissolved in acetonitrile. The extract dissolved in acetonitrile was kept overnight at 4°C in a refrigerator. The crystalline precipitate, so obtained was separated by filtration. The physical yield was measured by weighing the crystalline precipitate (Margraff, 1995). The percentage physical yield was calculated using the following formula:

$$\text{Percentage physical yield} = \frac{\text{physical yield}}{\text{weight of sample i.e. 25 g}} \times 100\%$$

Estimation of percentage purity of the extracted 10-DAB III by HPLC method (High Performance Liquid Chromatography)

The percentage purity of extracted 10-deacetylbaccatin III (10-DAB III) in the physical

yield was estimated using C18 column with water/acetonitrile (70:30 v/v) at flow rate of 1 $mL.min^{-1}$ and detection wavelength of 227 nm with PDA detector system (Ghassempour et al., 2010), using as the reference standard,10-DAB III manufactured by Tokyo Chemical Industry Co. Ltd 4-10-1 Nihonbashi, Chuo-ku, Tokyo 103-0023, Japan (purity 99.7 %, Lot no 4U7XH).

Calculation of Standardized/Actual Percentage yield of 10-DAB III

The standardized percentage yield of 10-DAB III from the monthly harvests were calculated using the following formula:

$$\text{Standardized/actual percentage yield of 10 – DAB III} = \frac{\text{Percentage physical yield} \times \text{Percentage purity from HPLC}}{100} \%$$

Statistical analysis

The physical yields, percentage purities and standardized/actual percentage yields of 10-DAB III for harvest months were statistically analyzed and compared using SPSS 20.0

Results and Discussion

The results of the extraction of 10-deacetylbaccatin III (10-DAB III) from leaves samples of *T.mairei* in the monthly basis are given in Table 1. Statistical analyses indicated that there were significant differences between percentage physical yields, percentage purities and standardized/actual percentage yields for different harvest months at $p<0.05$ level of significance [for percentage physical yields $F(11,24)= 394.926$, $p=0.000$; for purity percentages $F(11,24)= 185.991$, $p=0.000$; for standardized/actual percentage yields $F(11,24)= 503.221$, $p=0.000$]. The percentage physical yield of 10-DAB III crystals from the samples harvested in the month of July was the highest, in the month of June was the second highest while the least percentage physical yield was observed in the month of October (Table 1, Figure 5). However, when the percentage purities of 10-DAB III crystals extracted from monthly harvests were determined by comparison with the standard 10-DAB III using HPLC method, the crystals extracted from August harvest showed the highest purity of 76.74549%, while the least purity of 35.31482% was observed in the crystals extracted from July harvest (Table 1, Figure 4). Hence, on calculating standardized/actual percentage yield of 10-DAB III for each month, the highest value was observed for the June harvest (0.50704%) while the lowest value was observed for April harvest (0.22494%) (Table 1, Figure 5)

Calibration curve of standard 10-DAB III is given in Figure 1 with R^2value of 0.9998. Chromatograms of 10-DAB III of standard and sample (extracted 10-DAB III) are given in Figure 2 and Figure 3 respectively. Retention time of 10–DAB III was observed to be 4.2 minutes. In the chromatogram of sample 10-DAB III, other two peaks were also observed. The identification of those compounds was not under scope of this study. Nevertheless, they contribute as impurities in extracted sample. The variation in the percentage purity of extracted 10–DAB III seems to be due to these impurities. Mean percentage purity yield of extracted 10-DAB III with months is plotted in bar graph in Figure 4. Mean percentage physical yield and mean standardized/actual percentage yield with month is plotted in line graph which shows clear comparison of these parameters for the extracted 10-deacetylbaccatin III in Figure 5.

In this study, the standardized/actual percentage yield of 10-DAB III for June harvest is about 0.50704% w/w which is the maximum percentage yield in overall months without considering the amount of 10-DAB III lost in the mother liquor. The percentage yield in this study was determined by considering only the precipitated (crystallized) 10-DAB III. The limitation of the study is not being able to estimate the 10-DAB III in the mother liquor which may ultimately contribute to the increase in the yield of the 10-DAB III.

Margraff (1995) reported that the percentage yield of 10-DAB III content in *Taxus baccata* L. by HPLC was 0.08% (that is 400mg of 10-DAB III in 500g of foliage) including the estimation of 10-DAB III in

the mother liquor. During this study, when the method developed by Margaff (1995) was applied for extraction of 10-DAB III from leaf samples of another species of *Taxus*, i.e .*T.mairei* collected from Godawari, Lalitpur, Nepal (27.58922 N, 85.38138 E, 1528 m asl), the maximum percentage yield of 10-DAB III was found to be 0.50704% while minimum percentage yield was found to be 0.22494%. Hence, the leaf samples of *T. mairei* analyzed during this study yielded more 10-DAB III in comparison to *T. baccata* when extracted and analyzed by the same method. It can be concluded that 10-DAB III content varies with species of *Taxus*. According to Mroczekand Glowniak (2001) , relatively high concentration of 10-DAB III (i.e about 0.06%) in comparison to other taxoids from *T. baccata* var. *elegantissima* and *T. baccata* var *aurea* by Solid Phase Extraction-High Performance Liquid Chromatography (SPE-HPLC) method with mobile phase consisting of acetonitrile and water in gradient elution. Zarek and Waligórski (2009) determined 10-DAB III concentration in *T. baccata* needles collected from four different population of southern Poland by using micellar electrokinetic chromatography method and found that mean concentrations of 10-DAB III in the samples collected from Cisy w Nowej Wsi, Cisowa Góra, Zadni Gaj and Cisynad Liswart[1] were 0.135 mg.g^{-1}d.w. (0.0135 %), 0.185 mg.g^{-1}d.w. (0.0185%), 0.143 mg.g^{-1}d.w.(0.0143 %) and 0.150 mg.g^{-1}d.w.(0.0150 %)) respectively. Wianowska et al. (2009) used four types of solvent extraction methods (ultrasound and microwave assisted extraction, pressurized liquid extraction, and extraction in the Soxhlet apparatus) for paclitaxel, cephalomannine, and 10-deacetylbaccatin from *T. baccata* twigs and reported pressurized liquid extraction (PLE) as the most effective extraction method for taxoids. HPLC was used for the analysis of the extracts. The greatest yields were obtained by multiple PLE, in which the yield in methanol as solvent was 0.1470 mg.g^{-1} dry wt of sample (0.01470%) and in ethyl acetate as solvent was 0.0742 mg.g^{-1} dry wt of sample (0.00742%). This suggests that solvent and extraction process also contribute in the yield of 10-DAB III.

Glowniak, Mrocze and Hajnos (1999) used different combined methods including LLE/TLC/HPLC, SPE/TLC/HPLC and SPE/HPLC for the determination of common taxoids (10-deacetylbaccatin III, paclitaxel, baccatin III and cephalomannine) in different *Taxus* species including *T. baccata* and its varieties (var. *aurea*, var. *elegantissima*), *T. media* var *hicksii*, *T. cuspidata* and *T. brevifolia* and concluded that the quantities of taxoids were dependent on plant origin, type of plant organs and also on vegetative period. Glowniak, Mroczek and Zobel (1999) studied seasonal concentrations of four taxoids in fresh needles and stems of *T. baccata* during late autumn-spring period (November to April) and concluded that epigenetic factors - date of collection (and thus phylogenesis) and kind of plant tissue determine taxoid levels.

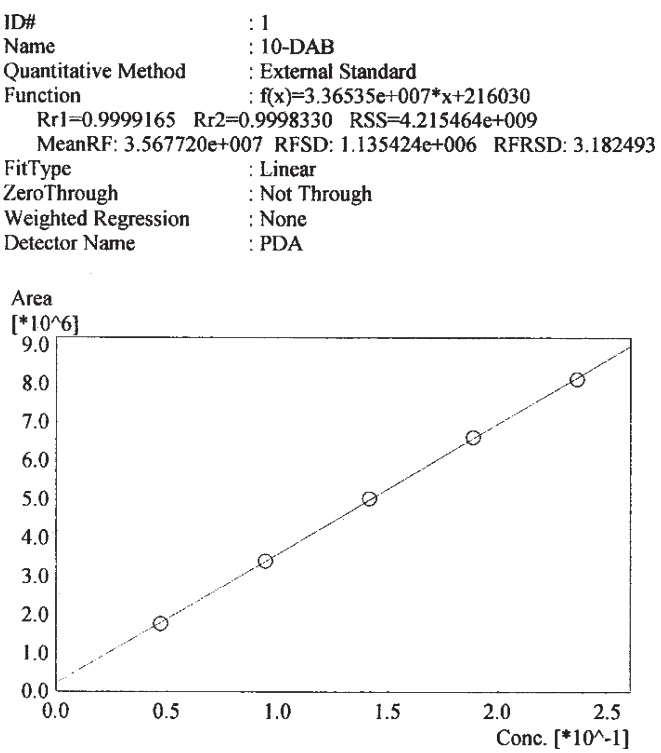

Figure 1: Calibration curve of 10-deacetylbaccatin III standard

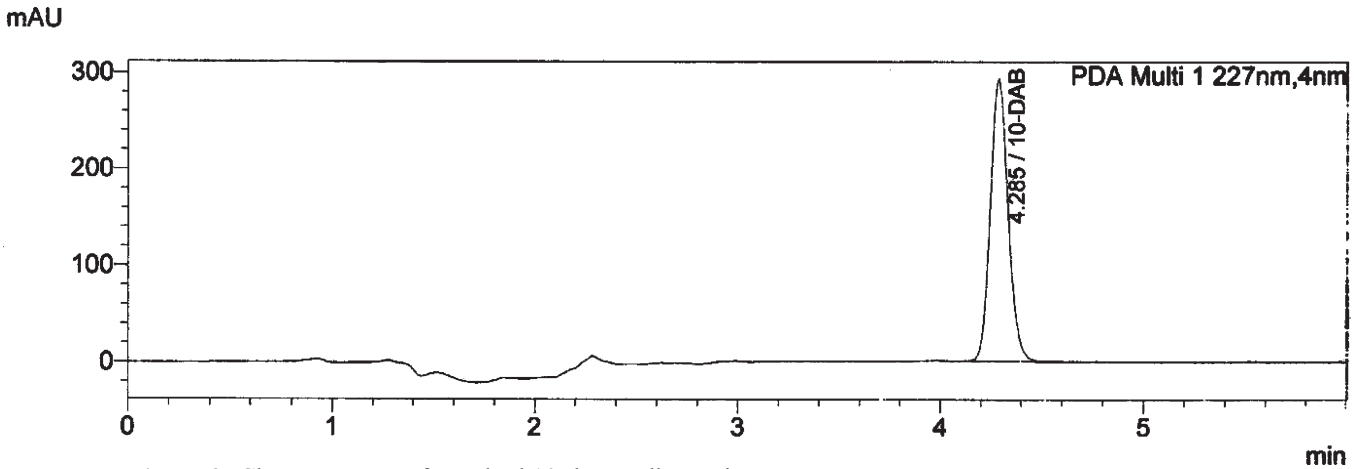

Figure 2: Chromatogram of standard 10-deacetylbaccatin III

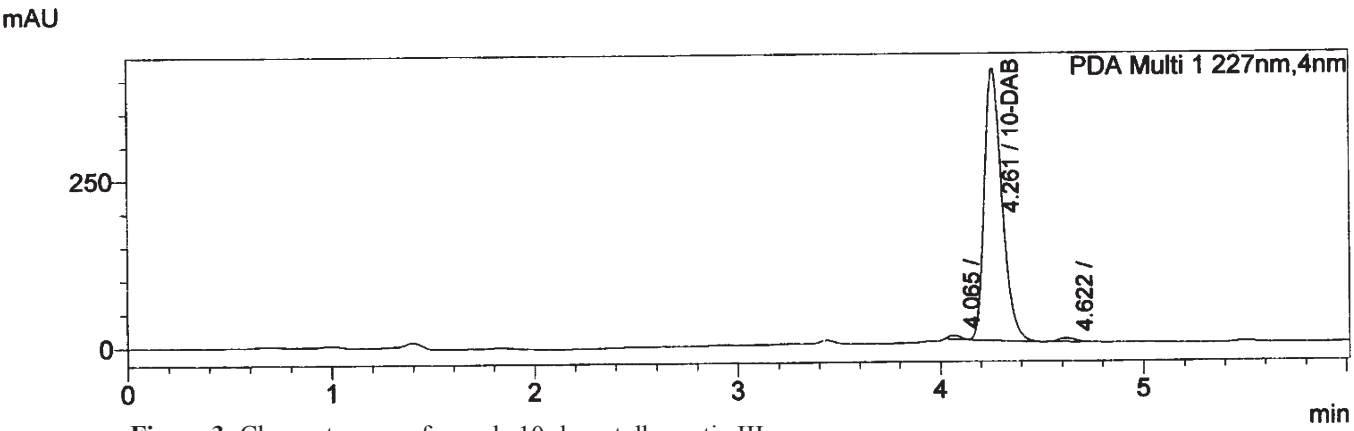

Figure 3: Chromatogram of sample 10-deacetylbaccatin III

Table 1: Monthly variation of yield of 10-deacetylbaccatin III

Months	Percentage Physical Yield of extracted 10-deacetylbaccatinIII (Mean±SEM) (w/w)	Percentage Purity of Extacted 10-deacetylbaccatin III from HPLC (Mean±SEM) (w/w)	Standardized/Actual Percentage yield of 10-deacetylbaccatin III (Mean±SEM) (w/w)
August 2015 (Shrawon)	0.47263±0.00333^{cde}	76.74549±0.92353^{f}	0.36266±0.00182^{e}
September 2015 (Bhadra)	0.51303±0.01293^{ef}	70.65153±1.09137^{de}	0.36219±0.00365^{e}
October 2015 (Ashoj)	0.398107±0.00659^{a}	74.2736±0.77285^{ef}	0.29559±0.00221^{bc}
November 2015 (Kartik)	0.712759±0.00951^{g}	61.28112±1.12199^{c}	0.43660±0.00423^{f}
December 2015 (Mansir)	0.507867±0.01059^{ef}	71.66805±0.84269^{de}	0.36380±0.00351^{e}
January 2016 (Poush)	0.428213±0.00059^{abc}	71.72553±0.99970^{de}	0.30713±0.00408^{c}
February 2016 (Magh)	0.450989±0.00447^{bcd}	69.06962±0.36334^{d}	0.31151±0.00407^{c}
March 2016 (Falgun)	0.538699±0.00993^{f}	62.75801±0.92168^{c}	0.33791±0.00287^{d}
April 2016 (Chaitra)	0.416335±0.00814^{ab}	54.06496±0.96140^{b}	0.22494±0.00066^{a}
May 2016 (Baisakh)	0.478567±0.00577^{de}	58.82231±0.30380^{c}	0.28153±0.00462^{b}
June 2016 (Jestha)	0.838936±0.01305^{h}	60.46008±0.73560^{c}	0.50704±0.00311^{g}
July 2016 (Ashad)	1.015855±0.01744^{i}	35.31482±0.46464^{a}	0.35859±0.00196^{e}

The values in each column followed by different superscripts are significantly different at 5% level of significance as shown by ANOVA test followed with Tukey HSD test.

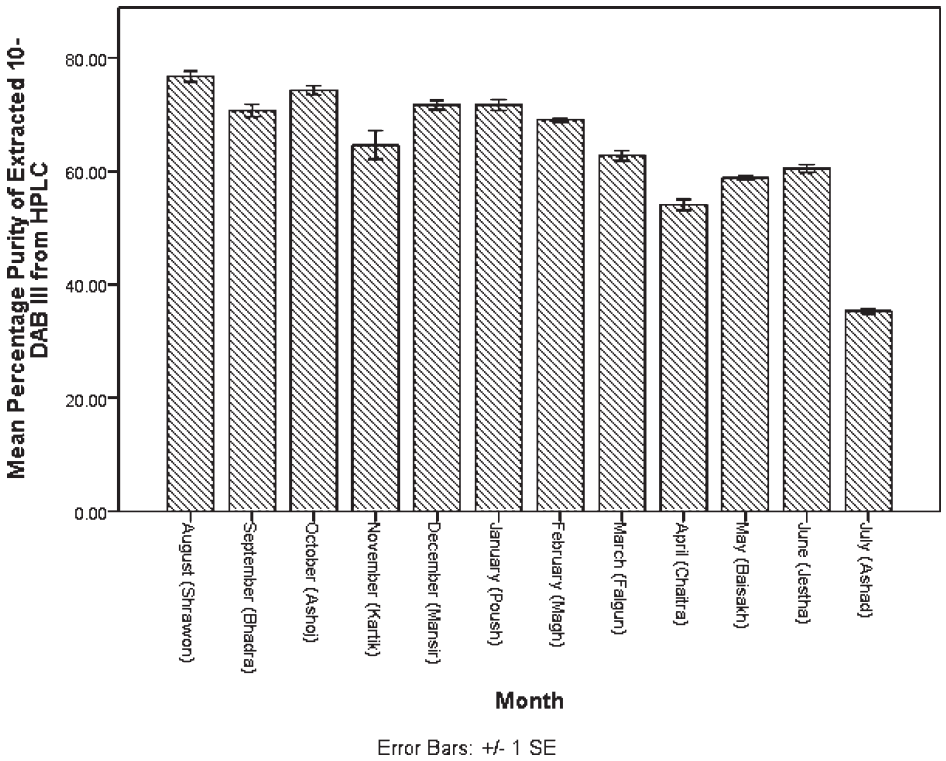

Figure 4: Comparison of monthly mean percentage purity of extracted 10-deacetylbaccatin III from HPLC

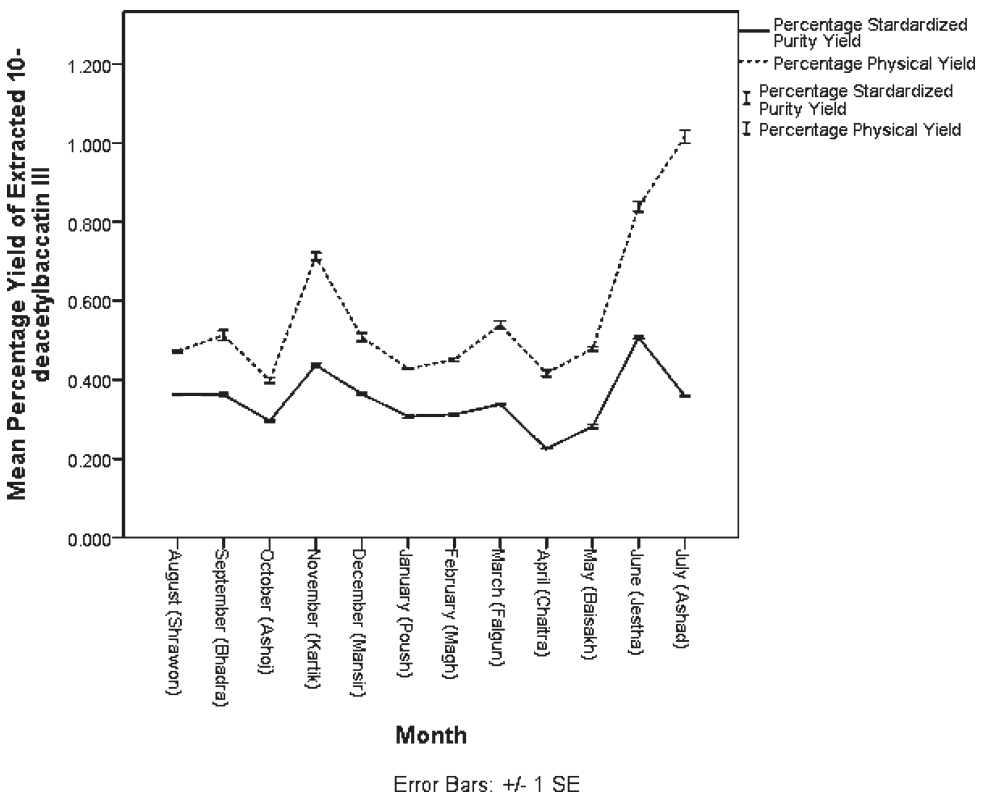

Figure 5: Monthly variation in percentage physical yield (w/w) and standardized/actual percentage (w/w) yield of extracted 10-deacetylbaccatin III

Conclusion

The reported method based on aqueous extraction followed by liquid-liquid extraction was used for the extraction of 10-deacetylbaccatin III (10-DAB III) from leaf samples of *T. mairei*. Aqueous extraction was performed successfully followed by estimation of 10-DAB III in the extracted samples using High Performance Liquid Chromatography (HPLC) method. Chromatogram of the sample showed three distinct peaks in which the major peak was of 10-DAB III and other peaks were of unknown compounds. These unknown compounds were not identified and contributed to the impurities in the isolated 10-DAB III.

The results obtained from this study indicate that 10-deacetylbaccatin III (10-DAB III) content of *T. mairei* leaves vary significantly with harvest months of a year. The most suitable month for the collection of samples for 10-DAB III was found to be June showing highest percentage yield. Similarly, November, showing second highest percentage yield, may also be suitable for the harvest of *T. mairei* leaves for 10-DAB III extraction. Further researches are necessary to compare extraction methods for the identification of high yielding methods and to compare the 10-DAB III yield from different species of *Taxus* indigenous to Nepal.

Acknowledgements

This study was carried out under the planned program of DPR Utilization and Pilot Plant Section of Department of Plant Resources. The authors are grateful to the Mr. Sanjeev Kumar Rai, Director General, Ms. Jyoti Joshi Bhatt, Deputy Director General and Former Deputy Director General Ms. Sushma Upadhyaya, Department of Plant Resources. Heartfelt gratitude are also due to the chief of Instrument Section Mr. Tara Datt Bhatt, the chief of Procurement Section Mr. Krishna Prasad Humagain and all the staff members of Department of Plant Resources for providing necessary facilities to carry out this research work.

References

Bhatta, G.D., Poudel, R.C., Pandey, T.R., & Basnet, R. (2017). *Yews of Nepal*. Godawari, Nepal: National Herbarium and Plant Laboratories (NHPL).

Chopra. R.N., Nayar. S.L. & Chopra. I.C. (1986). *Glossary of Indian Medicinal Plants* (Including the Supplement). New Dehli, India: Council of Scientific and Industrial Research.

Croteau, R., Ketchum, R.E., Long, R.M., Kaspera, R., & Wildung, M.R. (2006). Taxol biosynthesis and molecular genetics. *Phytochem. Rev.*, *5*, 75–97.

Dziedzic, P., Vesely, J., & Cordova, A. (2008). Catalytic asymmetric synthesis of the docetaxel (taxotere) side chain: organocatalytic highly enantioselective synthesis of esterification ready-α-hydroxy-β-amino acids. *Tetrahedron Lett.*, *49*, 6631-6634.

Ghassempour, A., Rezadoost, H., Mashouf, A., Aboul-Enein, H.Y., Spengler, B., & Rompp, A. (2010). Monitoring of paclitaxel, taxine B and 10-deacetylbaccatin in *Taxus baccata* L. by nano LC-FTMS and NMR spectroscopy. *Chromatographia*, *72*, 833–839.

Glowniak, K., Mroczek, T., & Hajnos, M. (1999). Modern methods for chromatographic determination and isolation of 10-deacetylbaccatin III and other taxoids from *Taxus* species. *Acta Poloniae* Supplement vol. *56*.

Glowniak, K., Mroczek, T., & Zobel A. M. (1999). Seasonal changes in the concentrations of four taxoids in *Taxus baccata* L. during the autumn-spring period. *Phytomedicine*, *6*(2), 135-140.

Jeon, S.I., Mun, S., & Kim, J.H. (2006). Optimal temperature control in fractional precipitation for paclitaxel pre-purification. *Proc. Biochem.*, *41*, 276–280.

Khosroushahi, A.Y., Valizadeh, M., Ghasempour, A., Khosrowshahli, M., Naghdibadi, H., Dadpour, M.R., & Omidi, Y. (2006). Improved Taxol

production by combination of inducing factors in suspension cell culture of *Taxus baccata*. *Cell Biol. Int.*, *30*, 262–269.

Kim, J.H. (2004). Prepurification of paclitaxel by micelle and precipitation. *Proc. Biochem.*,*39*, 1567–1571.

Kunwar, R.M., Shrestha, K.P., & Bussmann, R.W. (2010). Traditional herbal medicine in far-west Nepal: a pharmacological appraisal. *J Ethnobiol Ethno med*., *6*, 35.

Li, C., Huo, C., Zhang, M. & Shi, Q. (2008). Chemistry of Chinese yew, *Taxus chinensis* var. *mairei*. *Biochem. Syst. Ecol.*, *36*, 266–282.

Margraff, R. (1995). *United States Pat 5,393-896*. Retrieved from https://patents.google.com/patent/US5736366A/en.

Mroczek, T., & Glowniak, K. (2001), Solid-phase extraction and simplified high-performance liquid chromatographic determination of 10-deacetylbaccatin III and related taxoids in yew species. *Journal of Pharmaceutical and Biomedical Analysis*, *26*, 89–102.

Nautiyal, O.H. (2014). Determination of impurities generation in 10-DAB by XRD, HNMR and C-NMR on Storage for 10 years. *Journal of Chemistry and Materials Research*, *1*(2), 45-51.

Ohtsuki, K., Miyai, S., Yamaguchi, A., Morikawa, K., & Okano, T. (2011). Biochemical characterization of novel lignans isolated from the wood of *Taxus yunnanensis* as effective stimulators for glycogen synthase kinase3b and the phosphorylation of basic brain proteins by the kinase in vitro. *Biol. Pharm. Bull.*, *35*, 385–393.

Sharma, H., & Garg, M. (2015). A review of traditional use, phytoconstituents and biological activities of Himalayan yew *Taxus wallichiana*. *J Integr Med*., *13*(2), 80–90.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., & McPhail, A.T. (1971). Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Amer. Chem. Soc.*, *93*, 2325–2327.

Wianowska, D., Hajnos, M. L., Dawidowicz, A. L., Oniszczuk, A., Waksmundzka-Hajnos, M., & G³owniak, K. (2009). Extraction methods of 10-deacetylbaccatin III, paclitaxel, and cephalomannine from *Taxus baccata*L. twigs: a comparison. *Journal of Liquid Chromatography & Related Technologies*, *32*(4), 589-601, doi: 10.1080/10826070802671622.

Yang, S., Fang, J. and Cheng, Y. (1996), Taxanes from *Taxus mairei*. *Phytochemistry*, *43*, 839–842.

Yang, S., Fang, J., & Cheng, Y. (1998). Diterpenes from *Taxus mairei*. *Phytochemistry*, *49*, 2037–2043.

Yang, W., Zhao, Z., Wang, L., Yu, S., & Liang, Z. (2012). Control of hypertension in rats using volatile components of leaves of *Taxus chinensis* var. *mairei*. *J. Ethnopharmacol.*, *141*, 309–313.

Yu, J., Wang, Y., Qian, H., Zhao, Y., Liu, B., & Fu, C. (2012). Polyprenols from *Taxus chinensis* var. *mairei* prevent the development of CCl4-induced liver fibrosis in rats. *J. Ethnopharmacol.*, *142*, 151–160.

Yukimune, Y., Tabata, H., Higashi, Y., & Hara, Y. (1996). Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. *Nat. Biotechnol.*, *14*, 1129–1132.

Zarek, M., &Waligórski, P. (2009). Determination of 10-deacetylbaccatine III in *Taxus baccata* needles by micellar electrokinetic chromatography. *Herba Polonica*, *55*(2), 25-35.