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# Research Article
# Pre-purification of an Anticancer Drug (Paclitaxel) Obtained from Nut Husks

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## Abstract

**Background and Objective:** Hazelnut husk is known to contain taxanes, including paclitaxel. There are many methods for purifying paclitaxel, which is sold under the commercial brand name Taxol and is used as a chemotherapy medication. In this study, the effect of precipitation on the purity and yield of taxanes in the purification process of some taxane compounds from hazelnut husk was investigated by using different solvents. **Materials and Methods:** Collected, dried and milled samples of hazelnut husk were treated with methanol (1:15 g $mL^{-1}$) at room temperature for 72 h in order to obtain extracts. The extraction process was repeated 20 times to achieve the desired concentrated stock solution. Each 1 mL sample taken from this stock solution was treated with four different solvents with various quantities of binary mixtures (1:5, 1:10 and 1:20) and then analyzed using HPLC (High-Performance Liquid Chromatography) following filtration. **Results:** The HPLC was used to determine both purity and recovery yield in the stock solution before and after the analysis. Baccatin III has the greatest purity among the paclitaxel derivatives as 10-Deacetylbaccatin III, baccatin III, cephalomannine and paclitaxel, that were evaluated in the present study. **Conclusion:** Thus, the effects of precipitation from extracts containing taxane compounds derived from the hazelnut husk were determined in the pre-chromatographic purification processes.

Key words: Taxan, paclitaxel, 10-Deacetylbaccatin, baccatin III, cephalomannine

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Taxane-class compounds as paclitaxel, 10-Deacetylbaccatin, baccatin III, cephalomannine are one of the most important groups of anticancer agents[1]. Paclitaxel, a member of this class of diterpenoids and known by the commercial brand name Taxol, is approved by the US Food and Drug Administration (FDA) and is used to treat many different types of cancer including breast and ovarian cancers. Paclitaxel shows its antitumor effects by increasing aggregation of microtubules in the cell, preventing depolymerization and thus generating stable microtubule communities[2]. This molecule enhances microtubule stability, thereby inducing apoptosis in tumor cells. Therefore, taxols are used as a drug for ovarian and breast cancer, Kaposi's sarcoma and many other types of cancer for therapeutic purpose[1,3]. Paclitaxel is the sole active ingredient used in the treatment of metastatic breast cancer. Taxane production must be based on biological methods since there is growing need for taxol compounds used as an effective drug in the treatment of ovarian, breast and non-small cell lung cancers. It is extremely important, given the high demand for these drugs and limited sources, to investigate what should be the most efficient way of deriving these components from taxol-containing plants[4,5]. Effective responses to treatment with taxanes were observed in many cancer studies and, as a result, many oncologists adopted taxane components such as paclitaxel as a new treatment method[6,7]. It was first obtained in the USA from the bark of the Pacific yew (*Taxus brevifolia*) and it was an important component of the species. One of the most important developments in organic chemistry was to perform a full synthesis of taxanes from the Yew *Taxus baccata* by Holton *et al*.[8] However, as it has a rather long synthesis period, it could not be evaluated commercially. In extractions obtained from yew, other taxanes have been identified as well as paclitaxel in the extraction. However, it is not yet known which method may be used to obtain the paclitaxel in the most efficient and optimal amount.

Newman and Cragg[9] have recently assessed the usability of secondary metabolites of natural products in drug research. They emphasized that the development of different chemicals and biological processes is crucially important for using these active molecules in new drug syntheses. The paclitaxel present in hazelnut husk is a secondary metabolite of the plant and is usually found in small quantities in the hard shell and husk of the nut. Processes such as extraction, purification and characterization used to separate the secondary metabolites cause this small quantity to decrease gradually[10]. Therefore, various separation and purification processes are required to derive high purity paclitaxel from the appropriate organic extracts while minimizing losses.

The purpose of applying pre- and final purification methods to organic extracts is to derive the high purity paclitaxel, which are present only in small amounts. These methods involve several steps, the most important of which is a well-chosen method used for the pre-purification. Active clay application is one of the methods used to purify paclitaxel from cell culture. This has been accepted as a method that may also be used especially for macro levels as it needs less solvent in the purification process after precipitation and facilitates complex HPLC applications[11].

It had initially been difficult and laborious to achieve paclitaxel extraction with high-yield by only solvent extraction and chromatography in the purification processes. Moreover, chromatography-based methods, for example HPLC, without the pre-purification revealed that other substances other than the paclitaxel were present in the end product. Formation of these different components in addition to paclitaxel reduced the purity and yield of the paclitaxel[12]. A final purification process should therefore be applied after a pre-purification process in order to produce large quantities of taxol and related derivatives with high purity in the organic solvents.

For this purpose, this study aimed to evaluate the amount and yield as a result of precipitation of impurities by using the solvent effect of the pre-purification processes. In the present study, binary mixtures of different quantities of acetone, hexane, dichloromethane (DCM) and pentane solvents were initially treated with the prepared stock solution and then the impurities were precipitated. Precipitation analyzes were performed by HPLC for purity and recovery of paclitaxel, cephalomannine, baccatin III and 10-Deacetylbaccatin III molecules.

## MATERIALS AND METHODS

**Plant material:** Hazelnut nut husks samples from different regions in Turkey (Giresun, Trabzon, Sakarya, Samsun, Düzce) at different altitudes (0-250 m, 250-500 m, 500+m) were collected. A hazelnut nut husk was collected August and September, 2014.

These samples were dried without the sun. The collected samples were ground to powder and it was eliminated so as to be smaller than 80 mesh. Dried and sieved samples are wrapped with aluminum foil on the outside of the glass bottle (to be dark) and are stored in cold.

**Taxane extraction:** A 100 g sample of hazelnut husks was extracted at 1500 mL methanol ($\geq$99.9% analytical standard

from Sigma Aldrich) at room temperature for 72 h. All of the chemicals were taken of analytical grade from Sigma Aldrich company. The extraction processes were repeated 20 times to provide the main enriched and concentrated stock solutions. The second stock solution was prepared by taking about 30 mL of solvent from each of the stock solutions, then by dissolving them in 30 mL of dichloromethane (DCM). The precipitation effect studies were carried out by taking 1 mL of this second stock solution.

For hard hazelnut nut husks, 100 g sample was extracted at 1500 mL methanol at room temperature for 72 h and for hazelnut husk. The main stock solutions were obtained by repeating these processes 20 times and enriching and then concentrating them. About 30 mL of solvent was taken from cache of the main stock solutions obtained and evaporated, then the second stock solution was prepared by dissolving them in 30 mL of Dichloromethane (DCM) and the adsorbent effect studies were carried out by taking 1 mL of this stock solution.

**Precipitation processes:** The stock solution was prepared by taking 30 mL of the solution, evaporating and then dissolving in 30 mL of acetone for precipitation experiments. The solvent precipitation effect was examined using 1 mL of this stock solution. The solvent mixtures used for the precipitation effect experiments were prepared in the ratios of acetone-hexane (1:5), acetone-pentane (1:7.5), DCM-hexane (1:10) and DCM-pentane (1:20). Hexane or pentane at the indicated ratios was added to 1 mL of the stock solution containing acetone and it was observed that precipitation had occurred. The solution was centrifuged at 600 rpm for about 5 min to increase the amount of precipitation. The supernatant was then decanted and the precipitate was dried under vacuum. When dry, the precipitate was put into vials containing 1 mL of methanol for the HPLC analyses.

After 30 mL of the solution prepared for precipitation experiments were removed and evaporated, the stock solution was prepared by dissolving in 30 mL of acetone and the solvent precipitation effect was examined by taking 1 mL of this stock solution. In the precipitation effect experiments, mixtures of acetone-hexane, acetone-pentane, DCM-hexane and DCM-pentane were prepared, 1:5, 1:7.5, 1:10 and 1:20 (mL: mL). About 1 mL of the acetone stock solution is taken, hexane or pentane is added at the specified ratios and precipitation is observed. To increase amount of precipitation, centrifuge at 600 rpm for almost 5 min is applied. The supernatant is then decantated and the precipitate is completely dried under vacuum. The precipitate reaching the supernatant is vialled with 1 mL of methanol for the application of HPLC analyzes.

**HPLC analysis:** The HPLC analyses of DCM stock solution and precipitated samples were performed. Analyses were carried out using a HPLC Reverse Phase (RP) phenyl-hexyl column (250×4.6 mm, 5 μm particle size). The mobile phases were performed in a gradient program (ACN: $H_2O$-25:75, 75:25, 40 min) at a flow rate of 1.00 mL $min^{-1}$ at room temperature. The injection volume was 20 μL and peak values were determined at 227 nm using a UV detector. Four molecules (paclitaxel, cephalomannin, baccatin III and 10-Deacetyl baccatin III) were used as standards.

These analyses provided two important data sets. Both quantities and purity values of paclitaxel and its derivatives were measured in the stock and after the processing. Thus, it was shown whether the process led to any loss of material in measuring the effect of precipitation on purification.

## RESULTS

Precipitation results showed that the amount of paclitaxel and its derivatives in hazelnut husk was very small. Although the amounts of taxan compounds as paclitaxel, 10-Deacetylbaccatin, baccatin III, cephalomannine seems less, these kind of compounds obtained from natural products may be associated with the annual amount of production efficiency change. In this study, precipitation for using pre-purification technique was very effectively from both of purity and recovery from this naturel plants of hazelnut husk. As shown in Table 1, the purity of paclitaxel was found to be highest in acetone/hexane (1:20 ratio) as a result of the precipitation experiments performed for hazelnut husk.

Figure 1 illustrates that the highest and lowest purity of paclitaxel derivatives was observed in acetone/hexane (1:7.5) and dichloromethane/hexane (1:7.5), respectively. Baccatin III has the highest purity among the paclitaxel derivatives (10-Deacetylbaccatin III, Baccatin III, Cepholamine and paclitaxel) that were evaluated in the present study. As can be seen in the diagram in Fig. 2, the purity of baccatin III, another taxane component in the husk, was found greatest in DCM/hexane (1:5 ratio) and acetone/hexane (1:10 ratio). Table 2 and Fig. 3 show that minimal loss of paclitaxel material occurred in acetone/pentane and acetone/hexane mixtures. The diagram in Fig. 4 shows that the use of acetone/pentane (1:5 ratio) and acetone/hexane (1:20 ratio) mixtures led to a minimal loss of material in the case of Baccatin III.

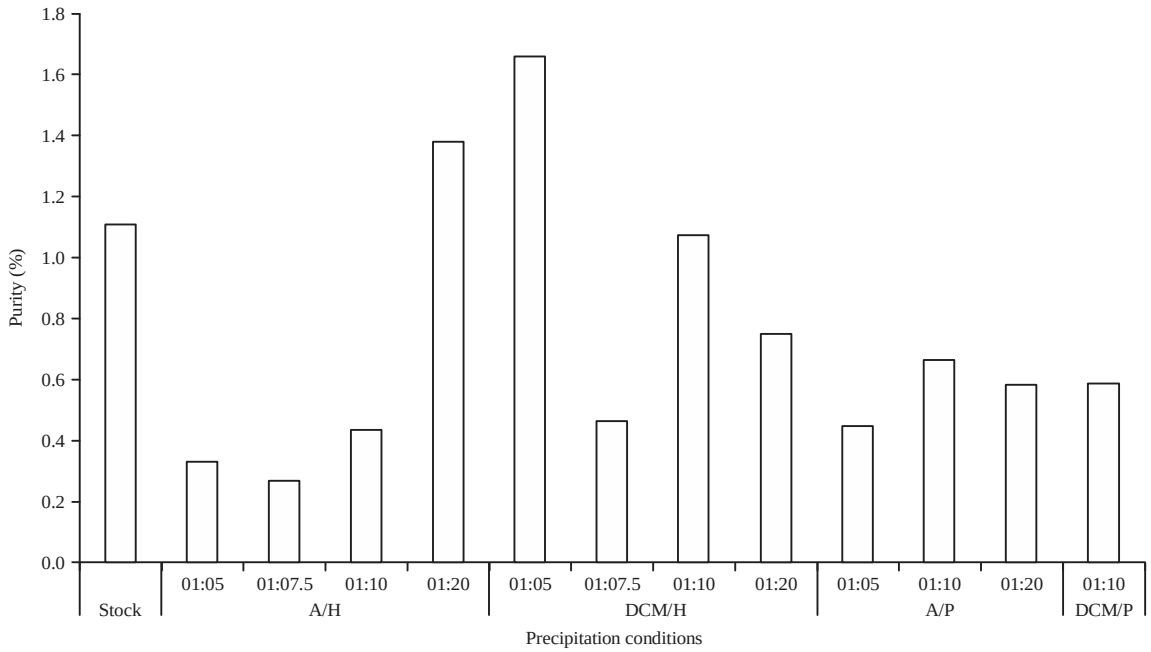


Fig. 1: Purity efficiency after precipitation for paclitaxel

Table 1: Purity result of taxan compounds by precipitation (%)

| Precipitation system | Ratio | 10-Deacetylbaccatin III | Baccatin III | Cephalomannine | Paclitaxel |
|---|---|---|---|---|---|
| Acetone/Hexane | 1:5 | ND | 0.3334 | 0.2027 | 0.0464 |
| Acetone/Hexane | 1:7.5 | ND | 0.2706 | 0.0325 | 0.0097 |
| Acetone/Hexane | 1:10 | ND | 0.4386 | 0.0409 | 0.0053 |
| Acetone/Hexane | 1:20 | 0.6675 | 1.3847 | 0.9540 | 1.0264 |
| Dichloromethane/Hexane | 1:5 | 1.2235 | 1.6632 | 0.1814 | 0.1378 |
| Dichloromethane/Hexane | 1:7.5 | 0.5342 | 0.4683 | 0.1176 | 0.0567 |
| Dichloromethane/Hexane | 1:10 | 1.2931 | 1.0769 | 0.0438 | 0.0491 |
| Dichloromethane/Hexane | 1:20 | 0.5542 | 0.7558 | 0.0438 | 0.0218 |
| Acetone/Pentane | 1:5 | 0.7684 | 0.4522 | 0.0604 | 0.0108 |
| Acetone/Pentane | 1:10 | ND | 0.6668 | 0.2556 | 0.1746 |
| Acetone/Pentane | 1:20 | 0.6024 | 0.5857 | 0.0985 | 0.0182 |
| Dichloromethane/Pentane | 1:10 | 0.5227 | 0.5933 | 0.0465 | 0.0132 |
| Dichloromethane stock | | 0.6624 | 1.1139 | 0.9908 | 0.2465 |

ND: Not determined

Table 2: Yielding efficiency from hazelnut husk by precipitation (ppm)

| Precipitation system | Ratio | 10-Deacetylbaccatin III | Baccatin III | Cephalomannine | Paclitaxel |
|---|---|---|---|---|---|
| Acetone/Hexane | 1:5 | ND | 6.5053 | 5.6765 | 1.0089 |
| Acetone/Hexane | 1:7.5 | ND | 4.6129 | 3.9476 | 0.9745 |
| Acetone/Hexane | 1:10 | ND | 6.8192 | 5.9985 | 0.6067 |
| Acetone/Hexane | 1: 20 | ND | 17.5186 | 4.0028 | 1.1589 |
| Dichloromethane/Hexane | 1:5 | 1.2598 | 12.9822 | 2.0881 | 1.2321 |
| Dichloromethane/Hexane | 1:7.5 | 1.5122 | 4.0342 | 2.2045 | 0.8721 |
| Dichloromethane/Hexane | 1:10 | 3.1044 | 6.6508 | 0.8581 | 0.7466 |
| Dichloromethane/Hexane | 1:20 | 1.5909 | 11.8227 | 2.1290 | 0.8236 |
| Acetone/Pentane | 1:5 | 0.8152 | 19.4588 | 11.2397 | 1.5605 |
| Acetone/Pentane | 1:10 | ND | 2.0414 | 0.8615 | 0.0939 |
| Acetone/Pentane | 1:20 | 0.7030 | 6.2659 | 3.3376 | 0.6263 |
| Dichloromethane/Pentane | 1:10 | 1.6675 | 16.0899 | 4.0480 | 0.8933 |
| Dichloromethane stock | | 17.6451 | 58.4206 | 79.4178 | 2.4370 |

ND: Not determined

The consequences are important due to the fact that baccatin III is a precursor compound in the semi-synthesis of paclitaxel. In the results obtained, the highest positive effect on the purification of

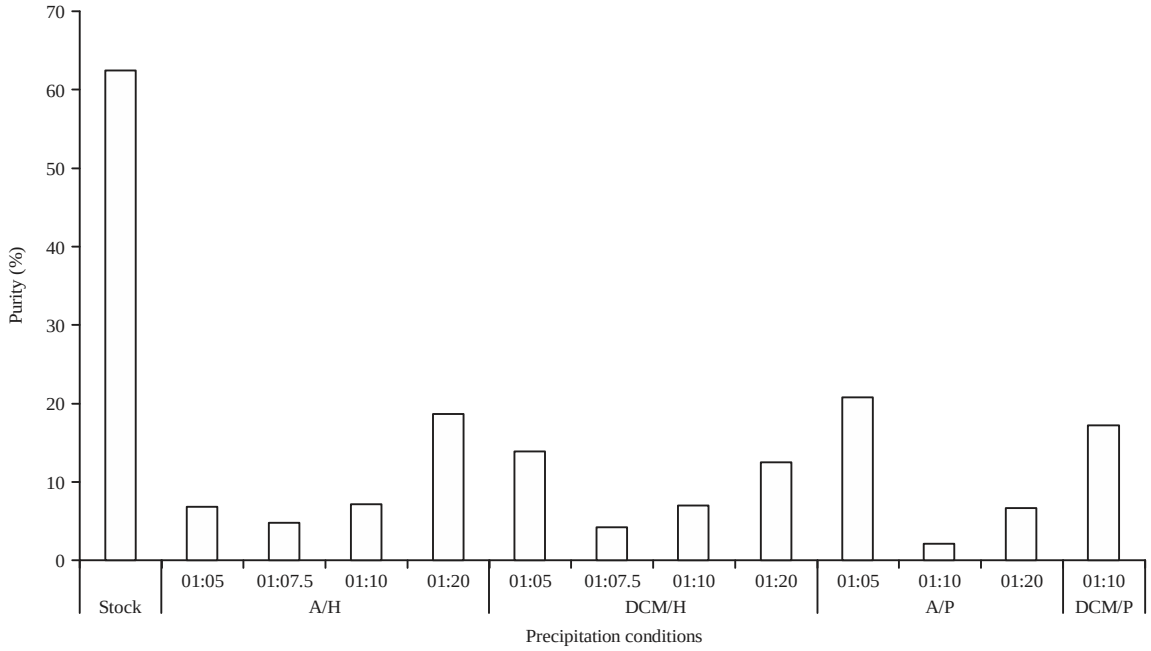


Fig. 2: Purity efficiency after precipitation for Baccatin III

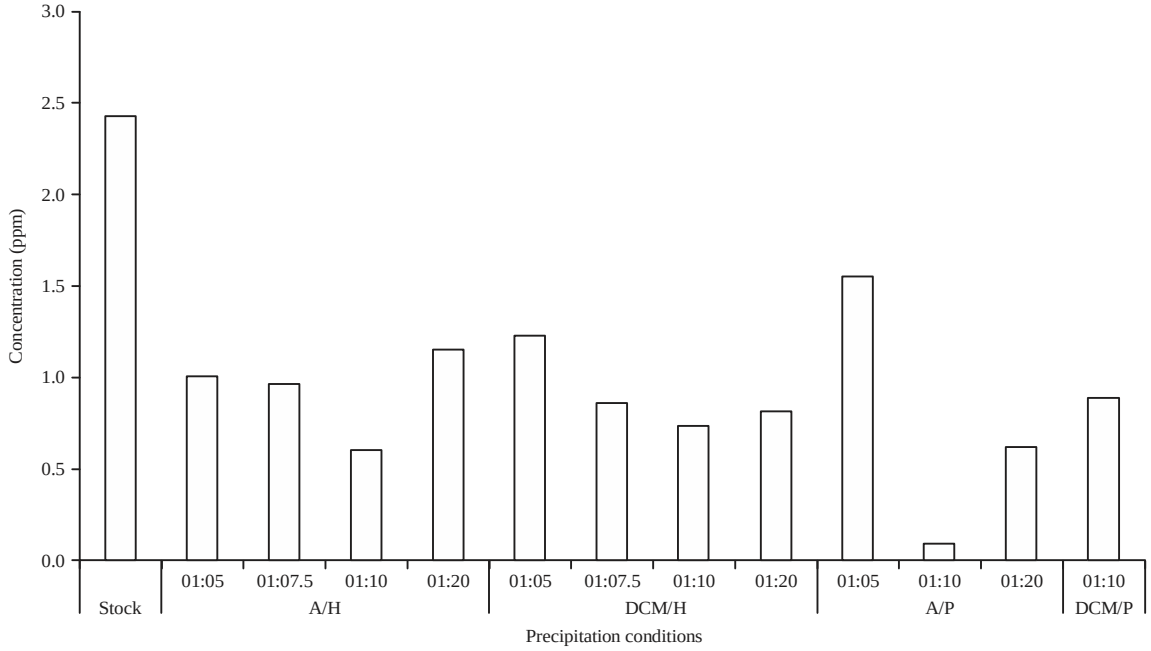


Fig. 3: Efficiency of paclitaxel amount after precipitation

baccatin III in the hazelnut husk was determined in all components.

## DISCUSSION

Turkey alone accounts for 65% of hazelnut production in the world. About 500 thousand t of hazelnuts are produced every year. In addition to this amount, approximately 250 thousand t of brown hard-shelled hazelnuts have been put onto the domestic market. Consequently an enormous amount of husk waste is produced annually.

This amount of waste has increased interest in the presence of paclitaxel and its precursor baccatin III compounds in hazelnut husk[13,14]. The amount of paclitaxel

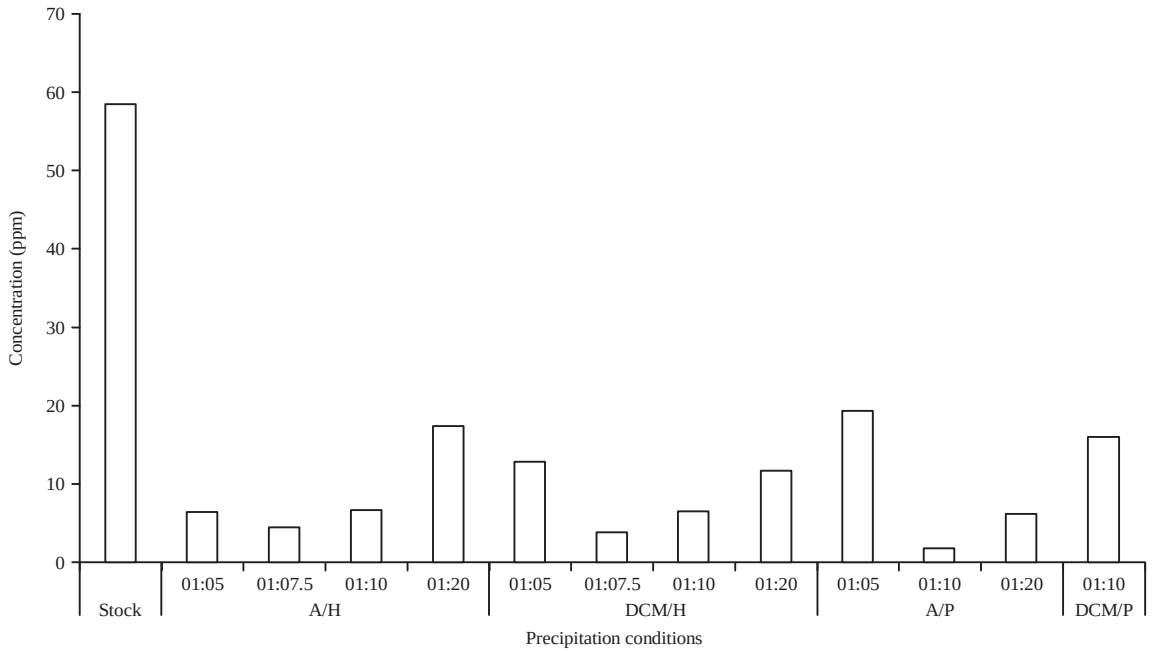


Fig. 4: Efficiency of Baccatin III amount after precipitation

produced by *Taxus* species, which is an important paclitaxel source, is not sufficient for the world demand because *Taxus* species are becoming increasingly scarce and yew plants are slow-growing and rather difficult to grow from seed. For this reason, its production is carried out by fermentation and cell culture using alternative biotechnological methods[15] that bring additional costs and time. In this regard, hazelnut husks are potential alternative sources as they are rich in paclitaxel and its precursor baccatin III. Paclitaxel belongs to a broad class of diterpenoids known as taxanes. Taxol, which has been used as an anticancer drug, has paclitaxel as the active ingredient.

Paclitaxel is a secondary metabolite of Hazel that is not responsible for plant growth[7]. 10-Deacetylbaccatin, which is an intermediate for taxol and baccatin III, which is a precursor to paclitaxel, are other natural products in hazelnut husk, which are used as starting materials in the semi-synthesis[4]. In this study, better results were achieved in terms of purity and the amount of recovered substance especially for baccatin III, which is a precursor to paclitaxel. It has been reported in the literature that the use of chromatographic methods in preliminary purification or methods of purifying crude extract by direct HPLC without preliminary purification were very expensive. This also pushed up the costs in industrial production[16,17].

The cost of production could be reduced by pre-processing before the final HPLC process was applied[16]. Jang *et al.*[15] reported that the use of hexane precipitation was advantageous to the pre-purification of plant cell cultures (>98%) for paclitaxel elution. The purity level of taxane compounds was found to be low (>10%) in the raw paclitaxel extracts. Moreover, the presence of tar and wax components in the medium after the liquid-liquid extraction further reduced the purity efficiency and final HPLC led to various problems resulting in entrainment. The presence of these interferences, such as tar and wax structures, also shortened the lifetime of the column material in HPLC analysis[18]. A precipitation process performed during pre-purification and before applying HPLC ensures that such problems are eliminated.

Kim *et al.*[19] reported that the purity level of paclitaxel obtained from *Taxus chinensis* using the hexane precipitation method in the pre-purification stage increased by 70%. Hexane precipitation-one of the pre-purification methods used to derive paclitaxel from methanol-extracted extracts-and subsequent purification by HPLC considerably reduced the total cost. This precipitation method is a simple and efficient procedure for the pre-purification of paclitaxel and the effect of its use on both purity and yield before performing HPLC has been recorded in the literature. There are many methods of separation from complex mixtures that are recommended for the isolation and purification of the secondary metabolites, which are known to have diverse biological activities and have also been tested for their potential uses as drugs[20].

It is believed that selecting the most suitable purification techniques and bringing purity to high levels will allow considerable progress in the pharmaceutical industry. In this study, findings achieved better results both in terms of an increase in purity and recovery through the precipitation method, which is the pre-purification method we applied to derive paclitaxel from the hazelnut husk. This study reports for the first time that the effect of precipitation on purity and minimizing loss of the starting compound "Baccatin III" is highly effective, especially when taxols are obtained in the synthetic pathway.

## CONCLUSION

In our study paclitaxel, a notable anticancer drug, was obtained with very positive results both in terms of increase in purity and recovery by the precipitation method, which is a pre-purification method for paclitaxel in the samples obtained from hazelnut nut husk. When taxol is obtained synthetically, the purity of Baccatin III as a precursor for the semisynthesis of the paclitaxel and the effect of precipitation on minimizing matter loss are very good and this is revealed for the first time in this study.

## SIGNIFICANCE STATEMENT

The cure for many cancer types has not been found yet in today's world and the treatments for many cancer types are extremely expensive. The use of plants increase in cancer treatment and therefore the isolation of many natural compounds were performed by using plants. This is because in present study taxanes are searched which are derived from hazelnut husks and used as a potent anticancer drug that using efficiently precipitation techniques.

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