Screening for paclitaxel and other taxanes in kernel and shell of Corylus avellana (Hazelnut)

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Abstract
Interestingly, paclitaxel was found in shells and leaves of hazelnut plant Corylus avellana. The aim of this present work was to verify whether hazelnut kernel contained paclitaxel. Hazelnut kernels were obtained from two places (local market and Oregon, USA) were analyzed by HPLC-MS. Paclitaxel and other taxanes 10-deacetylbaccatin III, Baccatin III, 10-deacetyl-7-xylosylcephalomannine, 10-deacetyl-7-xylosylpaclitaxel, 10-deacetylpaclitaxel, 7-xylosylpaclitaxel, Cephalomanine, 10-deacetyl-7-epipaclitaxel, 7-epi-paclitaxel were analyzed based on m/z value of molecular ion but none was found except for molecular ion of m/z=854 in the crude extract of kernel from a local market and shell from Oregon, US. The molecular ion was suspected belonging to 7-epi-paclitaxel, an isomer of paclitaxel. 7-epi-paclitaxel could be present in the extract of kernel and shell of tested varieties in this study. Paclitaxel was not detected it could have degraded during sampling process or the tested varieties did not produce paclitaxel or was too low to be detected.

Keywords: Paclitaxel, taxanes, Taxus brevifolia, Corylus avellana, HPLC-MS

1. Introduction
Paclitaxel is the active ingredient in the anticancer drug taxol marketed by Bristol-Myers Squibb Company (New York, N.Y). Taxol has been approved for treatment of ovarian and breast cancer, Kaposi’s sarcoma and non-small cell lung cancer [1]. It is also in under investigation for treatment of several cancers in combination with other chemotherapeutic agents. Several other uses for paclitaxel have been identified, including treatment for psoriasis, polycystic kidney disease, multiple sclerosis, and Alzheimer’s disease [2]. As an antimitotic cytotoxic agent, paclitaxel increases microtubule stability in cells and causes inhibition of many cell functions and the interruption of the cell cycle [3].

Paclitaxel was originally derived from the bark of the Pacific yew, Taxus brevifolia. This species is slow growing and taking over a hundred years for a young yew to mature. Moreover, the yield of paclitaxel in yew tree is low, usually between 0.004% and 0.1% on a dry weight basis [4]. As more applications of paclitaxel has been found in recent years, the demand for paclitaxel keeps increasing. Intense efforts in searching for alternative methods for the production of paclitaxel have been addressed. The total synthesis of paclitaxel has been accomplished [5, 6] but this is not a cost effective method to obtain paclitaxel due to the complex structure of the molecule. The current method to produce paclitaxel for clinical use is semi-synthesis, starting from a natural precursor, 10-deactylbaccatin III obtained from different yew tissues with a relatively good yield [7].

Plant cell cultures from different Taxus species are considered one of the promising approaches to provide a stable supply of paclitaxel and related compound generally named taxanes [8]. Not only from plants tissues, paclitaxel has been found in culture medium of Taxomyces andreanae, a new fungus isolated from Taxus brevifolia branches [9]. In the past, Taxus species and endophytic fungi were considered the only sources that produce paclitaxel and taxanes. However, hazelnut Corylus avellana has been reported as a paclitaxel producing species. Paclitaxel and taxanes were detected by HPLC-MS from plant extract of leaves and shells of Corylus avellana [10, 11]. Hazelnut cell cultures also produced paclitaxel and taxanes under controlled condition [12]. This result suggests that hazelnut possesses the ability for taxanes production which was considered to be a pathway specific to Taxus. To date, there has been no report on the presence of paclitaxel in hazelnut kernels.

Hazelnuts are commonly used as an ingredient in confectionary products, as raw material for pastry and chocolate industry. Hazelnuts are rich in fat (60% fresh weigh) with oleic acid being the major fatty acid [13]. Hazelnut has also been reported to serve as a good source of essential minerals, amino acids, and the B complex vitamins [14]. The aim of this present study was to explore the possibility of paclitaxel being present in hazelnut kernel
2. Material and Method
Paclitaxel standard was purchased from Sigma-Aldrich, Singapore. Methanol and acetonitrile HPLC grade were from Tedia (Ohio, USA). Methanol and hexane used for extraction were from River Bank Chemical Pte Ltd, Singapore. Hazelnut kernels were obtained from two places: locally purchased and grown in Oregon, US (purchased form Freddy Guys Hazelnut, Oregon, US). Kernels from Oregon, USA grown kernels (100 g) were extracted in 250 ml MeOH 0.01% acetic acid for 2 days in the dark. Solvent was removed under reduced pressure. The residue was re-dissolved in small amount of MeOH. An aliquot (1 mL) was filtered through a 0.45 μm pore size nylon membrane before going for analysis.

2.4 Shell

Extraction method 4: Shells (50 g) were extracted in 250 ml MeOH 0.01% acetic acid for 2 days in the dark [11]. Solvent was removed under reduced pressure. The residue was re-dissolved in small amount of MeOH. An aliquot (1 mL) was filtered through a 0.45 μm pore size nylon membrane before going for analysis.

Extraction method 5: Shells (200 g) were extracted in hexane to defat. The defatted residue was then extracted in 1000 mL EtOH for 1 day in the dark. Solvent was evaporated till dryness. The residue was re-dissolved in MeOH. An aliquot was dilute with water (1:1 v/v MeOH: H2O). The solution was passed through C-18 Sep-Pak (Phenomenex, Torrance, CA) cartridge to remove salt then was eluted by MeOH [10]. The cartridge was first washed with methanol before conditioning with water. 20mL of diluted sample was added into the cartridge. The cartridge was washed with 40mL water to remove polar compounds such as mineral and carbohydrate. Compounds that bonded to packed material in the column were then eluted out with 40mL MeOH. 1mL was collected and analysed.

Extraction method 6: Shells (200 g) were extracted in 1000 mL MeOH 0.01% acetic acid for 1 day in the dark. Solvent was removed under reduced pressure. The residue was re-dissolved in 50 mL ethyl acetate. The solution was then passed through silica gel column (25 mm x 2.5 mm internal diameter) conditioned in hexane. The column was eluted with mixture of hexane and ethyl acetate with increasing amount of ethyl acetate: (I) 500 mL 100% Hexane; (II) 500 mL Hexane: EA 4:1; (III) 600 mL Hexane: EA 1:1; (IV) 500 mL Hexane: EA 2:3; (V) 500 mL Hexane: EA 1:4; (VI) 500 mL Ethylacetate 100%. Solvent in fraction (II) (III) (IV) and (V) were removed and the residues were dissolved in small amount of MeOH. Aliquots (1 mL) of each fraction were analysed.

3. Results and Discussion
Paclitaxel and other taxanes have been detected from shell and leaves of Corylus avellana obtained from Turkey and Italy [10, 11]. In those studies, plant materials were extracted in ethanol at 4°C for 16 h or in methanol 0.01% acetic acid for 2 to 3 days in the dark. In this study, hazelnut kernels were first extracted with hexane to remove non-polar components and the defatted residues were then extracted with 0.01% acetic acid methanol. Hazelnut shells were extracted with ethanol or 0.01% acetic acid methanol. Three different purification steps (Amberlite XAD-4, SPE C18, silica gel) were applied to remove highly polar or non-polar components. Table 1 shows
a summary all extraction methods used in this study. Paclitaxel standard solution was prepared in methanol and analysed by HPLC. The retention time of paclitaxel standard was 30-30.2 min (Figure 1a). Crude extracts of dry hazelnut, fresh hazelnut and shell were analysed by HPLC and the chromatograms showed an unknown peak with a retention time 29.8-30.1 min (Figure b-d) which was very similar to retention time of standard paclitaxel. Column chromatography was used to remove unwanted compounds which were either too polar such as salt or too non-polar such as oil. Amberlite XAD4 resin was used to purify the extract of kernels K1. Shell extract was purified by two methods: reverse phase C18 SPE and normal phase silica gel. Amberlite XAD4 is a polyimide adsorbent which adsorbs hydrophobic molecules from polar solvent. Polar compounds from dry nut extract such as minerals which did not bind to the adsorbent were washed out with water. HPLC chromatogram of purified extract by XAD4 showed signal enhancement of some peaks compared to the crude extract (Figure 2a) in the range of retention time from 13 min to 40 min. C18 SPE cartridge was a useful technique for purification. Neither non-polar components nor highly polar impurities were seen in the semi-purified fraction collected from the cartridge (Figure 2b) when compared to the crude extract (Figure 1d). In silica gel column chromatography, mixture of hexane and ethyl acetate at increasing ratio of ethyl acetate were used as eluents and is shown in Figure 3(a-d). At first hexane 100% was used to remove highly non-polar components. Components with higher polarity were eluted later. In all the purified extract, a unidentified (Unknown) compound with similar retention time as paclitaxel standard in HPLC chromatogram was retained.

Mass spectra of paclitaxel standard showed that the main molecular ion of paclitaxel is [M+H]+ (m/z=854) is shown in Figure 4a. The presence of adduct ion of paclitaxel with sodium [M+Na]+ (m/z=876) was also observed. Ion fragments of paclitaxel molecule were labelled with asterisk in the MS spectra. The molecular ions and ion fragments are necessary to identify the presence of paclitaxel. Mass spectra of the unknown peak found from the dry hazelnut, fresh hazelnut and shell extract did not have any similar ion patterns compared to the standard (Figure b, c, d). Therefore the suspected unknown compound was not paclitaxel.

Standard addition is a useful method to verify the whether the unknown peak was paclitaxel. Paclitaxel standard was spiked in sample extract and the solution was analysed by HPLC. Figure 5 shows that the unknown peak and standard peaks could be separated. There was no overlapping between two peaks. Together with the ion chromatogram, result from standard addition method confirmed that the unknown peak was not paclitaxel. The Unknown peak had almost similar retention time to paclitaxel therefore the unknown might have structure related to paclitaxel. Mass spectra of the Unknown (Figure 4 b-d) showed a common peak of m/z=696 in kernels and shells extract. However, in the mass spectra of paclitaxel standard, there was no ion with m/z=696. Therefore, the unknown was not break-down product of paclitaxel.

In Ottagio et al., 2007 (11) and Hoffman et al., 2009 (10), an unknown peak with quite similar retention time with paclitaxel was not reported. HPLC and MS results of shell extract were not showed in Hoffman et al., 2009 (10). The unknown peak may have generated false positive results. In Ottagio et al., 2007 (11), extracted ion currents at m/z=854 of standard and shell extract showed a good match in retention time. This confirmed the presence of paclitaxel in shell extracts.

Besides paclitaxel, other taxanes (Figure 6) have been detected qualitatively and quantitatively in hazelnut shells (10, 11). Paclitaxel and other taxanes are members of Taxus alkaloid family. They share the same core structure. The differences are at the functional groups at position C7, C10 and C13 of the chemical structure. Those derivatives can be used as natural precursor in the semi-synthesis of paclitaxel.

In this recent work, taxanes were searched through the total ion chromatogram of kernels (K1 and K2) and shells extract based on the presence of adduct ions of those six taxanes. Beside the presence of ion m/z=854 at t retention time 27.11 (Figure 7 a-c). It has been reported that 7-epi-pacltaxel has a lower chromatographic polarity than paclitaxel, therefore 7-epi-paclitxel elutes after paclitaxel from the reverse phase HPLC column (15). The reduced polarity of 7-epimer-paclitxel is thought to be due to hydrogen bonding between the 7-alpha hydroxyl group and the acetate moiety in the 4-position. The result from Figure 7a-c suggest that the m/z=854 at t=34 to 35 min could be 7-epi-paclitaxel and is found in the kernels K1 and shell.

Paclitaxel converts primarily to 7-epi-paclitaxel, the thermodynamically more stable isomer, upon heating in the dry state and in organic solvents (15). 7-epi-pacltaxel is a thermodynamic more stable isomer of paclitaxel due to the hydrogen bonding between the C7α-OH and C4α-acetate acyl oxygen (16). The proposed mechanism for the epimerization at C7 requires transfer of the hydroxyl hydrogen from C7 to the C9 carbonyl together with aldehyde formation at C7, ring cleavage between C7 and C8, double- bond formation between C8 and C9, and hence enol formation at C9. Electron density in the C8-C9 alkene attacks the aldehyde carbonyl in either face of the C7 carbon, forming either paclitaxel or 7-epi-paclitaxel (17).

Solution composition, solution pH, reduced pressure, contact with glass surface, and/or trace amount of catalyst on the solvent used in extraction process are among the possible factors affecting paclitaxel degradation in methanol. Reduced pressure during rotary evaporation may enhance paclitaxel degradation by removing the relative volatile methyl ester side chain from the solution. Indeed, in this study, the crude extract samples were concentrated, typically by rotary evaporation. However, this result was not showed in the crude extract of kernels K2 as well as in the purified extract of all samples. The ion peak 854 shown in the Figure 7b, c could be random adduct ion of some other molecules in the extract. Future work needs to be done to confirm the presence of paclitaxel in kernels and shells and with standards of 7-epi-paclitaxel.

7-epi-paclitaxel found in hazelnut shell (10, 11) could be either the secondary metabolite produced from the plant itself or the product from the degradation of paclitaxel during extraction process. The degradation of paclitaxel through sample preparation procedure reduces the original amount of paclitaxel in plant tissues. Cell cultures from stems, leaves and kernels Corylus avellana were reported to contain taxanes. Paclitaxel, 10-deacetyl paclitaxel and 10-
deacetyl-baccatin III were the main taxanes identified from media recovered from suspension cell culture. It was suggested that hazelnut possesses the enzyme for paclitaxel production. The study showed the recovery of paclitaxel in hazelnut cell culture grown in controlled condition was similar to that found in yew cultures. However, in this study, paclitaxel was not detected from the extracted of kernels tissues. Perhaps as a secondary metabolite is influence by the growing conditions and in this case the amount of paclitaxel produced by plants in nature was too low to be detected. Hazelnut shells from plants grown at different places: Turkey and Italy have been reported to produce paclitaxel [10, 11]. The studies have shown that the amounts of paclitaxel obtained from plant collected at different places and different seasons are varied. Paclitaxel was not detected in the green shell of hazelnut plant from Turkey [10]. Plant secondary metabolites, as well as paclitaxel, are generally influences qualitatively and quantitatively by many factors, including season and growing conditions. Hazelnut samples in this study which had different origin compared to that used in past studies, might not possess pathway producing paclitaxel or even if it has, the amount of paclitaxel produced could be very low.

Since the first report of recovery of paclitaxel in Corylus avellana by Hoffman et al. in 1998 [18], the number of published research on this field is not great. The two most significant papers are from Ottaggio et al. in 2007 [11] and Hoffman et al. in 2009 [10] which reported on the recovery of paclitaxel and other taxanes from leaves and shell of Corylus avellana obtained from Turkey and Italy. These two varieties may produce paclitaxel in significant amount. Overall an unknown peak with almost similar retention time with paclitaxel standard was found in HPLC chromatogram of kernels and shells extract. However, MS result and standard addition method verified that the Unknown was not paclitaxel. HPLC and LC/MS are two compatibles methods in analysing plant extracts where the complex matrix can generated false positive result. In this study, paclitaxel was not detected in kernel and shells of Corylus avellana. The identity of ion peak with m/z=854 at later retention time than paclitaxel standards suggested the presence of 7-epi-paclitaxel in the plant extract. More research is needed.

Fig 1: HPLC chromatogram of a) paclitaxel standard, b) locally purchased kernel, c) U.S.A grown kernels, d) U.S.A grown shells.
Fig 2: HPLC chromatograph of locally purchased methanolic extract of a) XAD-4 column purification of kernel and b) solid phase extraction (SPE C-18) of shell.

Fig 3: HPLC chromatogram using different eluents with increasing amount of ethyl acetate (EA) using in silica gel chromatography to purify hazelnut shell extract.
Fig 4: Mass spectra of a) paclitaxel standard. \([M+H]^+\) (m/z=854) was the main molecular ion, b) hazelnut kernels locally purchased, c) hazelnuts grown in Oregon, USA and d) hazelnut shells.
Fig 5: HPLC chromatogram of (a) Kernels K2 extracted spiked with paclitaxel standard and (b) hazelnut shell extract spiked with paclitaxel standard.

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Fig 6: Structure of paclitaxel and other taxane derivatives
4. References
13. Amaral J et al., Characterization of several hazelnut (Corylus avellana L.) cultivars based in chemical, fatty acid and sterol composition. European Food Research and Technology, 2006; 222(3):274-280.
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