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Research Article

Comparisons between Hydro and Steam Distillation Processes to Extract *Prunella Vulgaris* Volatile Compounds, and their Anti-oxidative Activities

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Abstract

Objective The aim is to verify our earlier suggestion which accounted for the dynamics of volatile compounds extraction from the herb *Prunella vulgaris* (PV) using steam distillation. Then, the anti-oxidative property of PV is explored.

Methods Because we suggested earlier that the inefficient extraction using steam distillation was due to the obstruction of a mass of herb in the steam flow path, we used hydro distillation which tried to eliminate this obstacle. We used GC-MS to characterize the volatile compounds extracted, and thus compare the extraction efficiency. Then, treating the cancer cells from the SCC154 cell-line with the distillate, the cancer cell cytotoxicity was assessed using the colorimetric test reagent, the Cell Counting Kit-8. To assess the anti-oxidative activity of the PV distillate, Folin-Ciocalteu reagent was used.

Results We successfully showed that the removal of the obstacle, formed by the mass of herb in the steam flow path, enhanced the efficiency of volatile compound extraction. Also, we showed that the PV distillate did not exhibit anti-oxidative activity.

Conclusions Hydro distillation is a more efficient method than steam distillation to extract volatile compounds from the PV herb. However, mild heating did not provide sufficient energy to the convection of the boiling water to move the floating herb; the obstacle still existed and limited the efficiency of extraction also. For another issue of the antioxidant effect of the volatile compounds from PV, it was studied using the Folin-Ciocalteu reagent. It showed that the PV volatile compounds did not possess antioxidant property.

Keywords: *Prunella vulgaris*, *Xia Ku Cao*, Chinese Medicine, steam distillation, hydro distillation, anti-oxidative effect

1. INTRODUCTION

Prunella vulgaris (PV) is a perennial low-growing plant which can be found in many regions in different continents worldwide¹. In many cultures, Indians, Chinese, and Native Americans, preparations of the plant in the forms of salves, teas, decoction² are used in the treatments of various minor ailments such as wounds and inflammation.

From a survey of literature, research works on PV extended back to 3 decades ago³. They can be categorised into three areas: the phytochemical area, the agricultural area, and the pharmacological area. A detailed discussion of these categories of research works can be found in the previous article of the author⁴.

Most research efforts were, however, spent in the pharmacological area. Investigations were to explore the effects of PV on various pathogenic sources. Elaborated discussions of these various investigations in the pharmacological area were given in these previous articles^{4,5,6} and will not be repeated here.

Among research efforts in PV, very few efforts were spent on the investigation of volatile organic compounds (VOCs), however. The reason may be that the traditional method to prepare medicinal treatment for patients is by decoction (the

boiling of herbs in water, which extracts the chemical ingredients into water). In this process, all volatile compounds evaporate away; thus, less attention has been given to the volatile compounds in previous studies. The few research efforts on PV VOCs concentrated on the analysis to identify the chemical composition in the plant^{7,8,9}.

In this project, we used distillation to extract the volatile compounds from PV. In previous works of this research project^{5,6}, we reported on the dynamics of how the volatile compounds were extracted during the whole distillation process. An important result⁵ was that the PV distillate had a cytotoxic activity against the cancer cells from the cancer cell line SCC154, in a dosage dependent manner.

Another issue⁶ investigated was on the aging effect of the PV distillate. We showed that the aging of the distillate (for as long as 8 weeks) did not alter much the cell cytotoxicity of the distillate on the SCC154 cancer cells.

In the first part of this article, we explore the difference between the two distillation processes: steam distillation and hydro distillation. The purpose of doing this comparison was to verify a postulate we put forward in the previous article⁵ which explained the dynamics of the steam distillation process: The mass of herb hanging in the way of the passing steam from the boiling water below impeded the steam flow. Steam

with the volatile compounds extracted re-condensed and dropped back to the boiling water below. This reduced the efficiency of the extraction of volatile compounds from the herb.

In the second part of this article, we report the exploration of the anti-oxidative effect of the PV herb.

2. MATERIAL AND METHODS

2.1 Materials

Dried spica of PV, imported from China, was used in the experiments throughout the whole project. Other materials include rosmarinic acid for the authentication procedure, the tetrazolium salt-based colorimetric reagent, Cell Counting kit-8, and the cancer cell line, SCC154, for the cell cytotoxicity tests. The usage of them was elaborated in the previous articles^{5,6}.

To assess the anti-oxidative activity of the PV distillate, Folin-Ciocalteu (FC) reagent, purchased from Sigma-Aldrich (product number: 47641-100-F), was used.

2.2 Methods

2.2.1 The Distillation Processes

To assess the differences, two different distillation processes were compared. The steam distillation process was described previously⁵. Briefly, a two-ended reservoir flask containing the PV herb was placed above another flask, which boiled water in it to produce steam. The herb was, thus, in the passageway of the rising steam flow. Steam carrying the volatile compounds extracted was condensed in a condenser on the other leg of the setup. The distillate was then collected in 50-ml portions during the whole process.

The setup of the hydro distillation process was similar, except that the PV herb was submerged in the boiling water instead; so, the herb-containing flask was omitted. Steam from the boiling mixture was guided to the other leg of the setup where it was condensed. Then, the distillate was again collected in successive 50-ml portions.

For clarity, both setups for the steam distillation and for the hydro distillation are drawn side-by-side in Fig. 1 for comparison.

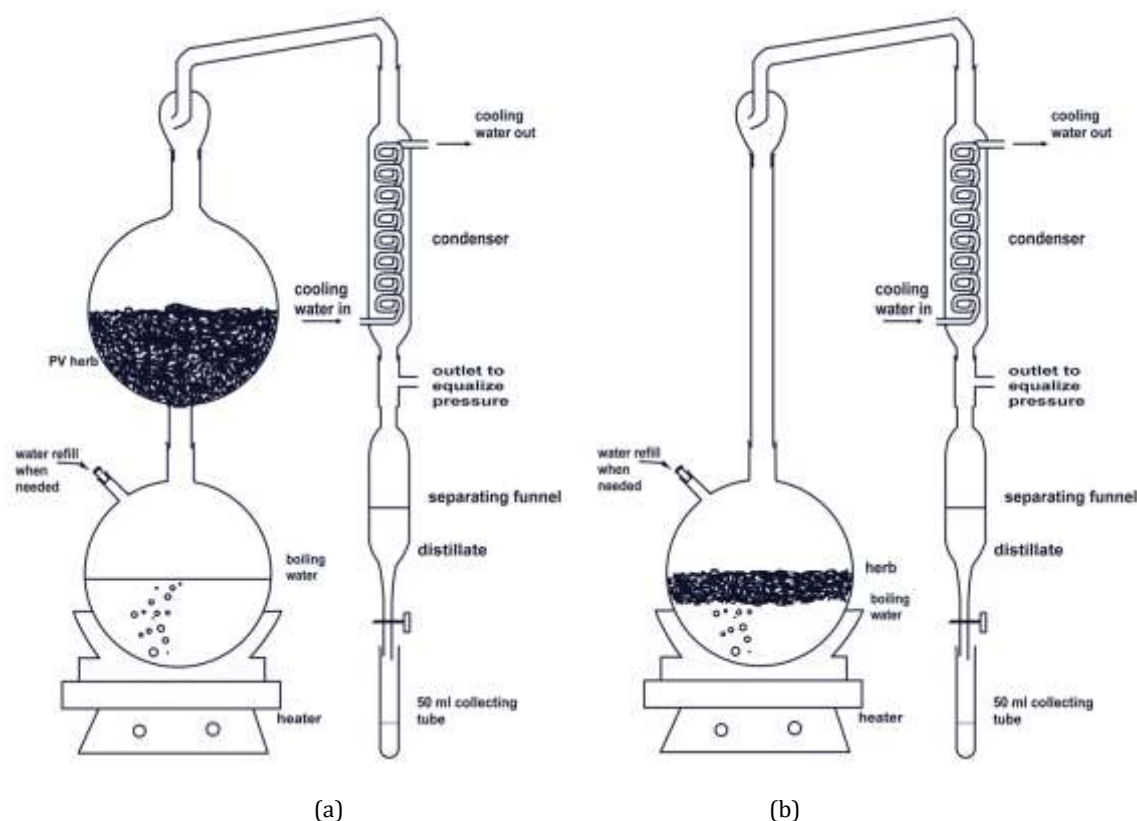


Figure 1: Both setups for (a) the steam distillation and (b) the hydro distillation processes are shown for comparison. The two-ended flask which contained the PV herb in the steam distillation setup was replaced by a long glass tube, to maintain the same potential energy which had to be overcome by the rising distillate-carrying steam. In the hydro distillation setup, the PV herb was submerged in the boiling water.

2.2.2 GC-MS

The GC-MS machine is an Agilent machine, 6390N Network GC System and 5973 Network Mass Selective Detector. The detailed setup parameters of the machine were reported in the previous article⁵.

Before the GC-MS analysis was done, the chemical compounds in the distillate were partitioned from the aqueous medium

into an ethyl acetate medium to prevent damaging the coating material of the gas chromatographer column.

By comparing the mass spectra of the samples, with those of standard known compounds available in the chemistry laboratory, various chemical compounds in the extracts from the distillation were identified. The mass spectrum library of the National Institute of Standards and Technology (NIST08) available inside the machine was also used to aid the identification.

2.2.3 Cell Viability Test

The tetrazolium salt-based colorimetric test kit, the Cell Counting Kit-8 from Sigma-Aldrich was used to probe the metabolic activities of the cancer cells from the cell line SCC154, when the cells were treated with the PV distillate. Because cells are alive when they have metabolic activities. Monitoring the metabolic activities of cells indicates the cell viability.

Detailed procedures to conduct the test were described previously⁵.

2.2.4 Anti-oxidative test

The assessment of the anti-oxidative activity of the PV herb was done using the FC reagent, purchased from Sigma-Aldrich. The FC assay is a popular standardized method in the measurement of anti-oxidative capacity of food products and dietary supplements¹⁰. The procedure to conduct such a test was adopted from what was proposed in an article by Ainsworth & Gillespie¹¹. Briefly, the procedure was as follows: 100 μ L of the distillate was added to a 2-ml microtube. 200 μ L FC reagent was added and then vortexed thoroughly. 800 μ L 700 mM sodium carbonate was added and allowed to incubate at room temperature for 2 hours. 200 μ L of the sample was transferred from the assay tube to a 96-well microplate. Three samples were repeated each, and thus, tests were done in triplicate sets. The absorbance of each well was measured by a microplate reader at around 765 nm.

3. RESULTS AND DISCUSSION

3.1 Results

The results can be divided into two parts: The first part looked at the differences in abundances of PV volatile compounds extracted during the two different distillation processes. This enabled the study of the dynamics of the extraction processes and verified the postulate in the previous article⁵ which explained the dynamics involved. The second part looked at the cell viability of the cancer cells SCC154 after they were treated by the PV distillate from the two different distillation processes.

3.1.1 Abundances of volatile compounds extracted

To compare between the two distillation methods, the settings of both methods should be adjusted to give meaningful comparison. This was done by adjusting the temperature of the heater so that the steam flow rates were the same. This was monitored by the distillate collection rate to about 50 ml every 40 minutes. Four alkanes with different molecular weights were observed: decane, dodecane, tetradecane and hexadecane. They were chosen because their identities were positively identified⁵. They were found to come out from the herb continuously with about constant rates even after the distillation process continued for a long time.

Fig. 2 shows the results.

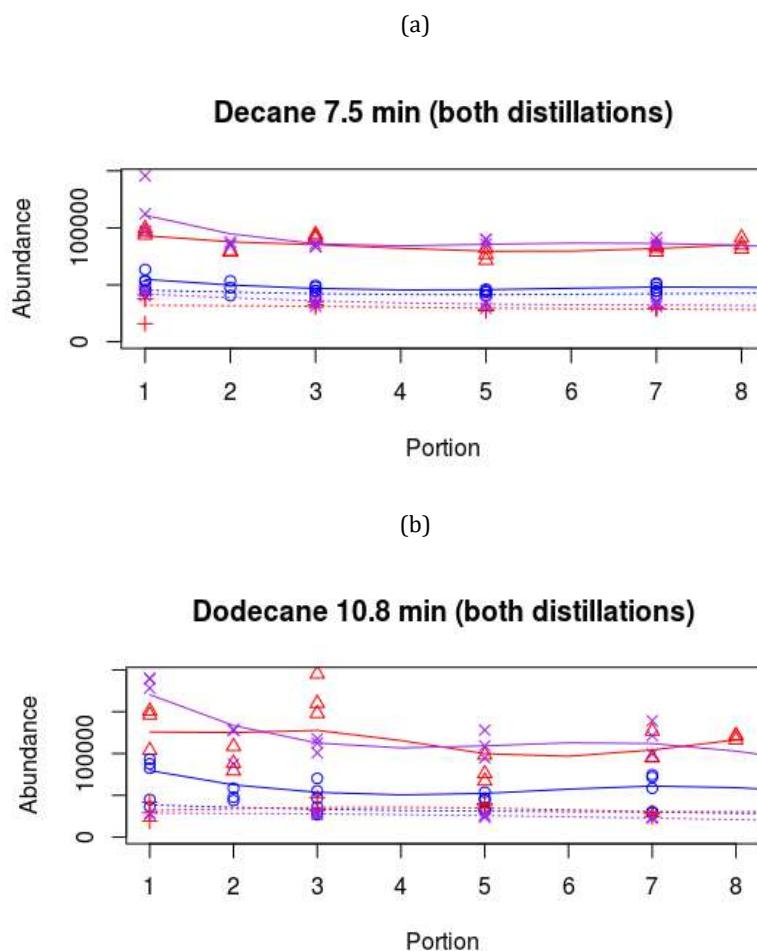


Figure 2 (a), (b): Abundances of alkanes extracted for the first 8 portions of 50 ml each. The dotted lines pertain to steam distillation, and the solid lines pertain to the hydro distillation. Three different amounts of PV herb were used: 15 g (blue), 25 g (red), 35 g (purple). The numbers appear in the sub-titles are the retention time of the compounds in minutes in the GC-MS coil. The smoothing lines were fitted to the data using cubic smoothing splines with a degree of smoothing parameter equal to 0.4.

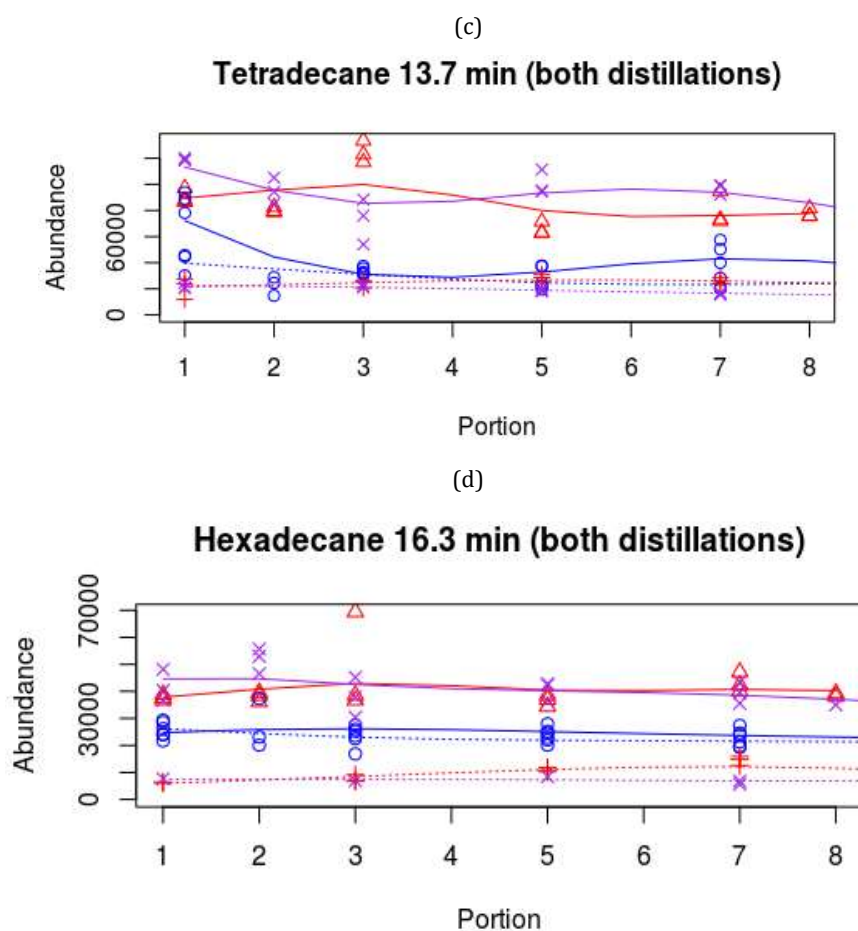


Figure 2 (c), (d): Abundances of alkanes extracted for the first 8 portions of 50 ml each. The dotted lines pertain to steam distillation, and the solid lines pertain to the hydro distillation. Three different amounts of PV herb were used: 15 g (blue), 25 g (red), 35 g (purple). The numbers appear in the sub-titles are the retention time of the compounds in minutes in the GC-MS coil. The smoothing lines were fitted to the data using cubic smoothing splines with a degree of smoothing parameter equal to 0.4.

With respect to steam distillation, the results repeated what was found in the previous article⁵. The amounts of volatile compounds extracted reached the optimal when 15 g of PV herb was used. It was postulated that, as more herb was used, the mass of herb in the way of steam flow became more and more obstructive and more volatile compounds were re-condensed and dropped back to the boiling liquid below. This reduced the extraction efficiency.

For the case of hydro distillation, the amounts of distillate obtained were larger through the whole distillation process than steam distillation at the same steam flow rate. This agrees well with the suggestion that the mass of PV herb hanging in the path of the steam flow acts as an obstacle in the case of steam distillation process. Moreover, the amount of 15 g of PV herb was no longer the optimal value to get the maximum amounts of volatile compounds, increasing the amount of herb to 25 g and 35 g extracted more volatile compounds. There was less resistance to the steam flow.

A closer and careful observation of Fig. 2 shows that, however, an increase of herb from 25 g to 35 g, did not increase the amounts of volatile compounds obtained. Their curves twisted together and indicated extraction limit. This seemed to contradict the intuitive suggestion that because the hydro distillation process eliminated the obstacle of a mass of herb in the steam flow path, there was no limit in the extraction efficiency. However, looking more meticulously as the distillation proceeded revealed the reason. Because within the

experimental setup, it was a nearly completely closed space except a tiny opening on the condenser side to allow a balance of pressure inside and outside. This balance opening was, however, not enough if the heating and the steam pressure was immense. The buildup of inside pressure might cause the boiling liquid to gush out or even trigger an explosion. To ensure safety, the heating needed to be limited. A consequence of this was that the convection of the boiling liquid had to be limited and was weak. When only 15 g of herb was used, the convection could still raise some boiling liquid above the herb floating at the surface and carried some herb in the convection current. When 25 g and 35 g of herb were used, the floating layer of herb at the surface was so thick that the weak convection current could not move it, and only bubbling steam could get through. Thus, the situation became similar to that found in steam distillation: the layer of herb at the liquid surface posed an obstacle to the steam flow, and so, saturation of extracted volatile compounds was again observed.

3.1.2 Cancer cell cytotoxicity

Cancer cell cytotoxicity was tested using the Cell Counting Kit-8 purchased from Sigma-Aldrich. The kit provides a means to check the cell metabolic activity through a colorimetric method using the tetrazolium salt WST-8. The procedure followed that provided by the vendor and was described previously⁵. The cancer cell line used was SCC154. Fig. 3 shows the results.

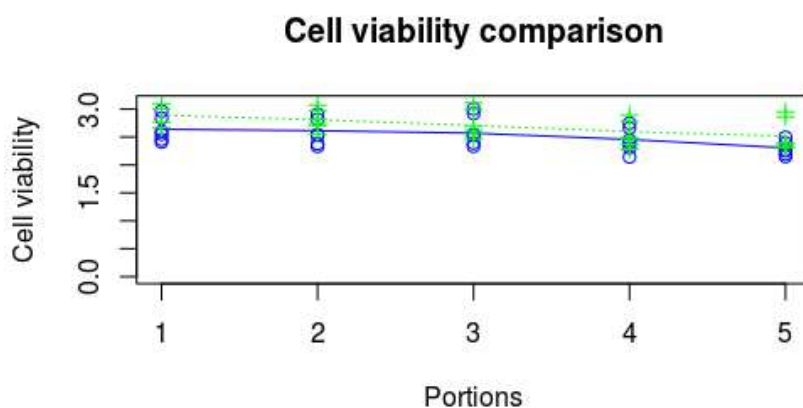


Figure 3: The cell viability of the cancer cells SCC154, treated by distillates from both hydro distillation (blue solid line and points) and steam distillation (green dotted line and points). The weights of herb used were 15 g. Only the first 5 portions collected during the distillation processes were used to plot the curves.

The cell viabilities found in this experiment agreed with what was found previously⁵. By visual observation of the curves, the cell cytotoxicity remained about the same for distillates in different portions obtained at different time during the distillation process, but was a little bit higher for later portions, which were collected later in the distillation processes. However, using the statistical two-way ANOVA method to analyze the differences among the portions gave a p-value of 0.0048. Using the commonly used threshold of $p=0.05$, the null hypothesis was rejected and thus there was significant differences in cell cytotoxicity among the portions.

One more observation is that the cell cytotoxicity of the distillate from the hydro distillation was higher than that obtained from the steam distillation. This result complied with what was found previously: Hydro distillation extracted more volatile compounds; the volatile compounds were more concentrated and thus the cell cytotoxicity was higher. Again,

applying the two-way ANOVA analysis on the difference in cytotoxicity between hydro and steam distillation, it gave a p-value of 0.0061. Thus, the null hypothesis was also rejected, and there is a significant difference in anti-tumoral activities of the distillates obtained from hydro and steam distillation.

3.1.3 Anti-oxidative activity of the PV distillate

FC reagent was used to check the anti-oxidative capability of the PV herbal distillate, according to the procedures described earlier.

However, measurement of the absorbance of the treated PV distillate at 765 nm by a microplate reader showed that there was no peaking at this wavelength. The measurement curve remained flat in the vicinity of 765 nm (Fig. 4). Using one-way ANOVA analysis gave a p-value of 0.994. This means that the null hypothesis was accepted. The PV herbal distillate did not show anti-oxidative activity.

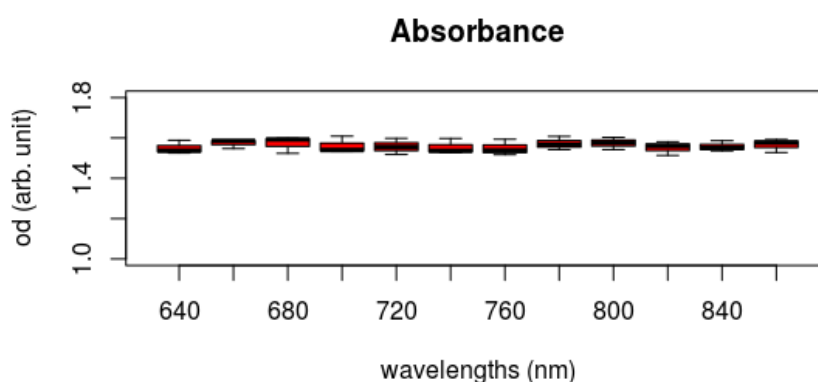


Figure 4: Absorbance measurements around 765 nm in Folin-Ciocalteu test

3.2 DISCUSSION

The main aim in the study of this article is to demonstrate the dynamics of the steam distillation of PV herb is as postulated in the previous article⁵. The mass of herb hanging above the boiling water in the path of steam flow was an obstacle to hamper the efficiency of the extraction of volatile compounds. The hydro distillation is a process which eliminates this obstacle. This study demonstrates that the hydro distillation process achieved a higher efficiency than the steam distillation process; more volatile compounds were obtained at the same steam flow rate. On the other hand, looking at the different

cancer cell cytotoxicity due to treatments using distillates from the two distillation processes revealed that the distillate from hydro distillate was more potent. This, once again, demonstrated the cancer cell cytotoxicity was dosage dependent because the distillate from hydro distillation was more concentrated in volatile compounds. An unexpected observation in this study, however, was that the volatile compounds extracted did not increase without limit when more and more herb was used for hydro distillation. Careful observation disclosed that the mass of floating herb on the surface of the boiling water again presented as an obstacle to the steam flow. The heating was kept mild to assure safety and

thus the convection of the boiling liquid underneath was not strong enough to move the floating herb on top when the layer was thick.

The observations above have implications to the production process, when we apply the results found here to the manufacture of medicine which makes use of the PV volatile compounds. The hydro distillation is a more efficient method than steam distillation. This is convenient because the current practice of producing drugs, say, in granule form, is by boiling the herb submerged in water inside a big tank. So, hydro distillation becomes just a small additional step to collect the steam with volatile compounds above the boiling liquid. On the other hand, the use of steam distillation implies a different setup of equipment and thus additional cost for drug manufacture. An additional advantage is that hydro distillation extracts more volatile compounds at the same steam flow rate, and thus more efficient.

However, the observation that mild heating (and thus a weak convection liquid current) does not eliminate the obstacle of unmoved herb in the steam flow path, which limits the extraction efficiency. So, the design of the manufacture process should keep the heating of the decoction mixture high enough to achieve a better extraction efficiency, by ensuring the convection current carries the herb with it. However, in a previous paper⁶, it was shown that the composition of the extracted volatile compounds did change with the heating temperature. So, there needs further investigation in the process design to get the optimal settings. Previous studies^{5,6} also pointed to the fact that, as far as anti-tumoral activity is concerned, there are only very relaxed requirements on when the volatile compounds are taken, because volatile compounds taken at different time during the distillation process or taken many days afterward do not differ much in their potency. This offers much convenience to the design of the manufacture process.

The investigation into the anti-oxidative activity of PV volatile compounds showed that they did not consist of any anti-oxidative components. Many previous studies^{12,13,14,15} showed that non-volatile compounds in PV possess anti-oxidative property. So, just the distillation did not extract these anti-oxidative compounds as they were not volatile. Particularly, phenolics such as hydroxycinnamic acids, flavonoids, anthocyanins, tannins¹¹ were not present in the distillate, and they were not responsible for the anti-tumoral property of the distillate.

One issue which may warrant further investigation is on the encapsulation of the VOCs extracted. Some research efforts were reported previously, such as the use of polymer-based microcapsules^{16,17}. Practical applications were reported^{18,19} to treat herpes simplex viral disease using the VOCs from PV. Cream, which locked the VOCs from loss, was made by incorporating the essential oils from PV, and then applied topically on infected skin areas.

Finally, unlike Western pharmacology which identifies and singles out a particular phytochemical compound to be used as a drug, the practice of Chinese medicine is to make use of the synergistic effects of all constituent compounds in an herb or a combination of many herbs being used together. So, we just looked at the anti-tumoral activity of the volatile compounds in the distillate as a whole and intentionally avoid identifying individuals. So, identification of the individual anti-tumorous volatile PV compounds may be another direction of future works.

4. CONCLUSION

In this study, we compared the two distillation processes: the hydro distillation and the steam distillation. The main aim of

this comparison is to verify a suggestion in a previous article⁵ which explained the dynamics of the steam distillation process. In that article, we found that the amounts of volatile compounds extracted did not increase with the amount of PV herb used when the amount of herb was more than 15 g. In fact, the amounts of volatile compounds obtained decreased as more herb was used. We postulated that as the mass of herb was put in the path of steam flow, it became an obstacle which re-condensed the volatile compounds, and they dropped back into the boiling water below. To test whether this postulate was valid, we removed this obstacle in the steam path by using hydro distillation. In this setup, the herb was submerged into the boiling water, and thus the obstacle in the steam path was removed. What we found experimentally validated this postulate. At the same steam flow rate, the amounts of volatile compounds extracted by hydro distillation were more than the corresponding amounts extracted by steam distillation. However, unexpectedly, the amounts of volatile compounds extracted still saturated and could not increase further when the amount of herb used was more than 25 g. Careful observation helped to discover that when the heating was mild to ensure safety, the convection of the boiling liquid was not strong enough to move the floating herb at the surface when it was thick, and thus, the obstacle of an immovable layer of herb in the steam path still existed.

Then, we compare the cancer cell cytotoxicity due to the two different distillation processes. We found that the distillate from hydro distillation was more cytotoxic to the cancer cells. This, once again, showed that the PV herb is cytotoxic to cancer cells, SCC154, in a dosage dependent manner. This is because we showed above that hydro distillation extracted more volatile compounds than steam distillation, and thus, the distillate was more concentrated.

The second part of this study looked at the anti-oxidative activity of PV herb, with the use of FC reagent. Disappointedly, no anti-oxidative activity was observed. This means that anti-oxidative compounds such as phenolics^{11,20} were not present in the distillate.

Statements and Declarations

- There exist no competing interests, or conflict of interest, which may inappropriately influence the author's actions and the integrity of the research reported.
- There are no financial interests, which may be gained or lost from publication of the article. There is no financial gain, such as consulting fees or other remuneration from the publication of the article.
- The university which the author affiliated to has no financial gain or loss from the publication of the article.

List of Abbreviations

PV: *Prunella vulgaris*

VOC: volatile organic compound

GC-MS: gas chromatography – mass spectrometry

ROS: reactive oxygen species

NIST: National Institute of Standards and Technology

ANOVA: Analysis of variance

Significance statement

This article experimentally shows that the hydro distillation process is more efficient than the steam distillation. The limiting factor for the extraction efficiency exists in both,

however. The VOCs from PV don't show anti-oxidative activities.

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