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RESEARCH ARTICLE

Time Depletion Effects on the Volatile Compounds from the Distillation Extracts of *Prunella vulgaris* and the Dynamics of their Extraction

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Abstract: ***Background*:** *Prunella vulgaris* (PV) is a low-growing perennial herb, which can be found in different parts of the world as Asia, Europe and North America. It is traditionally used for medicinal treatment in various cultures in India, China, Japan, Korea, Russia, and Eastern Europe for treating different ailments, such as fever, and healing wounds. In our previous article, we showed the anti-tumorous effect of the volatile organic compounds (VOCs) of PV and characterized the steam distillation process in the extraction of VOCs from PV. This has never been done before as we are aware of. To use the VOCs as drugs, there is a question of how much of the VOCs are lost before the prepared drugs reach the patients. Thus, the first aim of the present article is to try to explore the time depletion effect on the VOCs in the PV extracts. Then, the second aim is to extend the work in the previous paper and further understand the dynamics of the distillation process of PV by changing the steam flow rate in the extraction process.

***Methods*:** To achieve the first aim to explore the aging effect of how much VOCs are depleted after they are extracted, the VOCs were first extracted by the same method as before, *i.e.*, using steam distillation. Then, tubes of the aqueous solution containing the VOCs were then stored in a 5°C refrigerator. They were then taken out for GC-MS analysis according to a preplanned schedule up to 8 weeks after the VOCs were extracted. The chemical composition of the distillate could then be evaluated. This revealed the changes in the abundance of VOCs with aging. At the same time, the cell viability of SCC154 oral squamous cells treated by these herbal solutions, which were at different aging stages, was evaluated using a tetrazolium-based colorimetric reagent, Cell Counting Kit-8. To achieve the second aim of exploring the dynamics of the steam distillation process, the steam flow rate was adjusted by changing the temperature setting of the hot plate. GC-MS was again used to quantify the chemical constituents of the distillates.

***Results*:** By using GC-MS to measure the abundance of volatile compounds at different time points after the distillation process, it was found that the volatile compounds persist for a very long time, or over 8 weeks, which was the longest period of our experiment. The aging of the distillates also did not depreciate much the cell cytotoxicity of the PV distillate on the cancer cells. With respect to the dynamics of the steam distillation process, it was found that, at a low steam flow rate, volatile compounds of lower molecular weight are more efficient to be extracted, while at a high steam flow rate, volatile compounds of higher molecular weight are more efficiently extracted.

***Conclusion*:** Our findings demonstrate that the VOC compounds extracted and present in aqueous form do not deplete much for at least 2 months after the extraction process, neither they exhibit cell cytotoxicity. The experiments on the dynamics of the steam distillation process demonstrate that the mass of herb present in the flow path of the steam has significant effects on the relative amounts of VOCs extracted.

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1. INTRODUCTION

1.1. Background

Prunella vulgaris (PV) is a low-growing perennial herb that can be found in different parts of the world. It belongs to the mint family Lamiaceae, and is used as a medicine in different cultures, including the Chinese, Indian, East European, and Native American. Around Far East Asia, the plant is widely distributed throughout China, Japan, and Korea, and thus, is popularly used in their traditions for treating various conditions, *i.e*., to soothe inflammation, heal wounds, alleviate fever [1, 2], lower hypertension [3], and treat tuberculosis [4] and other minor ailments. Coincidentally, the herb is similarly used in other regions around the world.

The various bioactive components in PV have been extracted in many research projects done previously, using various methods, such as Soxhlet reflux, decoction, ultrasound, and others. Also, various solvents have been used, with the most popular choices including water, ethanol, and methanol. These extraction methods can be followed by fractionation using organic solvents of varying polarities, such as hexane or chloroform [2], so that the bioactive constituents can be further separated. Many studies have concentrated on the identification of the chemical constituents in PV. The compounds identified include campherol, rutin, flavonoids, phenolics, complex carbohydrates, and triterpenoids [2, 5]. Studies show that they have various pharmacological effects; rosmarinic acid, one of the polyphenolic compounds known to be present in abundance in PV, was shown to have anti-inflammatory and anti-tumorous activities [6], while polysaccharides were also shown to exhibit the same [7]. Other abundant phenolic acids studied for their pharmacological activities include ursolic acid, caffeic acid, and oleanolic acid [8]. Other pharmacological activities that the chemical constituents of PV have been shown to exhibit include antimicrobial [9], anti-viral on which the research efforts have been concentrated, anti-HIV [10] and herpes simplex [11], anti-oxidative [12], and anti-diabetic [3] effects; PV has also been found effective for sleeping disorders [13] and some other illnesses.

There have been several review articles on PV in previous years. Some more recent examples can be mentioned here for a deeper understanding of the herb. An article by Huang *et al*. [14] reviewed the anti-tumorous effect of PV. It discussed the chemical constituents of *P. vulgaris*, summarized some known formulas that contain PV, and discussed *in vitro*, *in vivo*, and clinical studies on the anti-tumorous properties of PV. Another review article by Wang *et al*. [15] also summarized the chemical constituents, pharmacological effects and clinical application of the herb, but the review was not limited to the anti-tumorous aspect but extended to other pharmacological applications. A more recent review by Mir *et al*. [16] focused on the aspects of phytochemistry and therapeutic uses of the plant and its constituent compounds. The latest review article by Mak [17], which can be found by the end of 2021, however, focused the attention on the molecular mechanisms behind the anti-tumorous effect of PV.

However, most of the previous studies have concentrated on the nonvolatile compounds of PV. This is understandable because the traditional method to prepare the herbal treatment is by decoction, which extracts the active ingredients by boiling in water. Boiling the herb in water means that nearly all volatile compounds will be lost in the process. The studies of the volatile compounds in the herb are relatively few due to this historical reason. Moreover, most of the studies have concentrated on the analysis of the chemical composition of PV [18-20]. As the standard procedure of extracting volatile compounds by hydro distillation from herbs is stipulated in the Chinese Pharmacopoeia [21], most of the studies inside China which investigated volatile compounds in herbs followed this standard procedure. The other distillation method, the steam distillation method, was seldom used. Moreover, the studies on the pharmacological activities of the volatile components in herbs, especially in PV, have been rarely carried out. The report of the medicinal use of the volatile compounds in PV was first described in an ancient text [22], which inspired this investigation.

The purpose of this series of investigations is two-folded: the first is a focus on the extraction process. It aimed to study the characteristics of the extraction process when steam distillation was used for the extraction of volatile compounds from PV, how the different volatile compounds come out during the distillation process, and how the different parameters will affect the extraction process. The second was to look at the pharmacological activities of the PV volatile compounds. This article is one of the several articles which report on this series of this investigation.

In another article [23], hereafter referred to as Paper 1 we reported that most of the volatile compounds came out evenly throughout the whole distillation process. Most of the volatile compounds did not show much depletion even after the distillation process went on for a long time. However, there were some less abundant compounds, an example of which was benzaldehyde, which did show gradual depletion, while some rarer compounds, an example of which was caryophyllene oxide, only appeared briefly at the beginning of distillation. One of the interesting findings was that as more herb was used, it did not imply that more volatile compounds would be extracted. There was an optimal amount of herb used which would produce the most abundant amount of volatile compounds. In the setup we used to do the experiment, that optimal amount was about 15 g. We proposed an explanation of this interesting observation. We attribute the decrease in extracted volatile compounds to the resistance of the mass of PV herb hanging above the boiling water in the path of the passage of steam. When more herb was used, the higher was the obstacle imposed by them, which condensed the rising volatile compounds to drop back to the boiling water.

It was also found that the volatile compounds of PV were cytotoxic to the squamous cancer line SCC154 in a dosage-dependent manner. For the cytotoxicity of PV distillates extracted at different times during the distillation process, it was found that the cytotoxicity remained uniform for distillate portions obtained throughout the whole process, but just a little higher for portions obtained in later stages.

1.2. Aims of this Part of the Investigation (Described in this article)

As the VOCs deplete with time, a question arises as when the VOCs are prepared as drugs, how much of them will be lost during the manufacturing process after extraction. Moreover, how many of them will remain when the drugs reach the patients.

In this article, we address this query, which forms the first aim to investigate the time depletion effect on the abundance of the PV volatile compounds: How do the abundance of different compounds decreases with time after extraction?

In our previous study 1, we postulated that the mass of herb in the steam flow path forms an obstacle for the VOCs to be collected in the condensation branch of the experimental setup [23]. Here, we explore this issue further to look at the extraction dynamics by changing the steam flow rate to see the impacts on the abundance of the VOCs collected. This offers an explanation of the effect of the weights of PV herbs used on the extraction of volatile compounds.

2. METHODS AND MATERIALS

Briefly speaking, the method used in this investigation was to extract volatile compounds from PV by steam distillation. They were then partitioned from the aqueous medium with ethyl acetate so that the constituent analysis might be done using gas chromatography-mass spectrometry (GC-MS).

2.1. Materials

Dry herb of *Prunella vulgaris* imported from China was used in this project. Rosmarinic acid, which was used as the reference standard to establish the identity of the herb, was bought from Sigma-Aldrich (R4033-10MG, lot # BCBV7877). The GC-MS was done by the machine produced by Agilent Technologies (6390N Network GC System and 5973 Network Mass Selective Detector). To test the cell cytotoxicity of the herb on cancer cells, SCC154, a colorimetric tetrazolium-based reagent, Cell Counting Kit-8 (CCK-8), product number: 96992, purchased from Sigma-Aldrich, was used. To prevent microbial growth in the PV distillate, an antibiotic, gentamycin (part no.: G1272), was also purchased from Sigma-Alrich and used to treat cancer cells in tissue culture.

2.2. Preparation of the PV Distillate

As the experimental procedures have been described in detail in our previous study 1, we shall only describe them briefly here in this article [23].

By boiling deionized water, the steam produced was guided to pass through finely chopped PV herb (25 g) to extract the volatile compounds. The condenser then cooled down the steam, which condensed into the distillate. For the convenience of reference, the experimental setup is shown in Fig. (**1**).

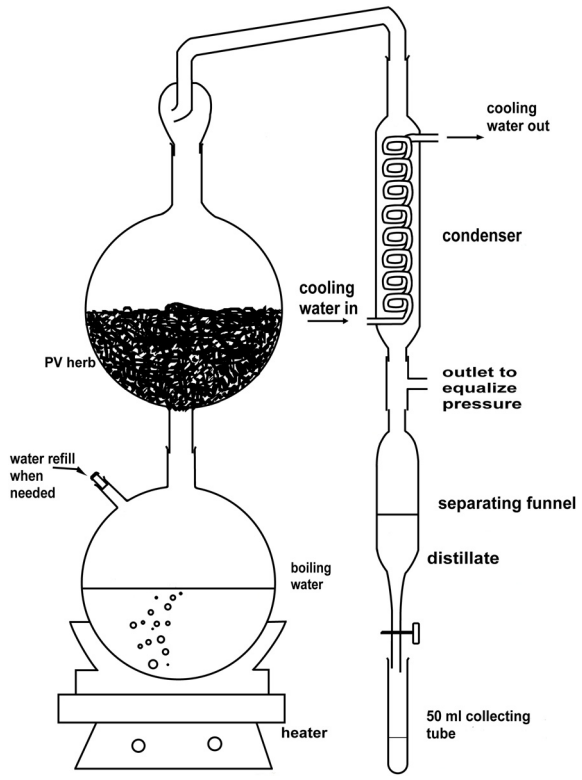

Fig. (1). The experimental setup for the steam distillation process. This figure of the setup has been presented in Paper 1 [23]. It is included here for the convenience of the readers. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

To conduct analysis using GC-MS, the chemical constituents in the PV distillate were partitioned from the aqueous medium with ethyl acetate, because the aqueous medium is detrimental to the capillary column of the GC-MS machine.

Because we were to look at the aging effect of the herbal distillate, it was necessary to consider the bacterial growth problem as the distillate had to be stored for a long time. This precaution is especially necessary for the cell viability test. If the distillate was contaminated by microbes, it would invalidate the results of the cell viability tests because the colorimetric reagent will then not only measure the metabolic activities of the cancer cells.

To circumvent this problem, additional measures were used to prevent microbial growth. Before the distillation process started, all glassware used in the experimental set was flushed with ethanol. Then, the glassware was put inside a 110°C oven to dry and was further sanitized for 2 hours, before being assembled to do the experiment. The distillate was immediately collected in 50 ml test tubes (for GC-MS analysis, 50 ml each) and in cryogenic tubes (for cell cytotoxicity tests, 1 ml each). The different tubes for the two sets of experiments were used to avoid cross-contamination, and because a much smaller amount, 1 ml, was needed to do the cell cytotoxicity tests. The tubes were capped and then stored in a sealed container, which was flushed with ethanol beforehand. This was then stored in a 5°C refrigerator until the pre-planned day when the tests were done.

2.3. GC-MS

An Agilent gas chromatography machine in tandem with an Agilent mass spectrometer were used to do the GC-MS analyses. The settings of the gas chromatography machine were as follows: capillary column having dimensions of 30 m x 0.25 mm x 0.25 µm; split mode injection; helium was used as the carrier gas at a flow rate of 1.2 mL/min under a pressure of 6.57 psi and an average velocity of 31 cm/s. At the inlet, the heater setting was at 250° C, a flow rate of 27.7 mL/min and a pressure of 4.24 psi. The temperature profile of the oven was as follows: temperature held at 50° C for 3 min; then ramping up to 250° C at a rate of 10° C/min, and being held at 250° C for 3 min; and then ramping up to 280° C, where the temperature was held for a further 5 min, thus, making the total oven time of 34 minutes.

The mass spectrometer was set at electron impact mode, with a scan range between 30-500 amu, with a data rate of 20 Hz, and the detector set point at 280° C. The temperature at the MS source and quad was set at 230 and 150° C, respectively.

2.4. Cell Viability Test

The squamous cancer cell line, SCC154, was utilized to investigate the anti-tumorous effect of the PV distillate. The CCK-8 kit, a test reagent based on tetrazolium salt, was used for colorimetric assays to reflect the cell viability of the cancer cells upon treatment with the PV distillate. The absorbance at 450 nm, proportional to the cell viability, was measured.

The procedure described in the 'Product Information' [24] of the CCK-8 kit was followed in the cell viability test. Briefly speaking, 100 µl of medium with cells at a concentration of 5000 cells/well was delivered into the wells of a 96-well tissue culture plate. The plate was pre-incubated in an incubator for 24 hours, with the ambient temperature set at 37°C and 5% CO_2. Then, 10 µl of the PV distillate was added into the medium, and then the plate was further incubated for 48 hours. CCK-8 test reagent was added to each well, and incubation was continued for another 4 hours. Finally, the absorbances at 450 nm were measured. Experiments were repeated, and data were taken in triplicate sets.

To further circumvent the problem that there may be microbial growth during the prolonged period of storage of the PV herbal distillate, an antibiotic, gentamycin, was added to the tissue culture. A dosage of 2 µl/ml was adopted according to the recommendation of the vendor (recommended dosage: 1 µl/ml to 5 µl/ml).

3. RESULTS

3.1 Time Depletion Effect

After the distillate samples were obtained, they were stored in test tubes, sealed with lids, in a refrigerator set at a temperature of 5°C. Then, these samples were tested once every week (or more frequently during the first week) by GC-MS for the abundance of chemical compounds and by the colorimetric test kit for cancer cell viability.

3.1.1. Change in Abundance of Chemical Components

It was noted that the distillate still gave off some fragrant smell after it had been extracted for more than two months, indicating that some volatile compounds dissipated very slowly. From the abundances found experimentally by GC-MS, many volatile compounds persisted for more than two months, which was the longest period that we explored by experiments. Fig. (**2**) shows the abundance of 4 alkanes and benzaldehyde, which we have positively identified. Their abundance was tracked for 45 days after the distillation extraction process.

Unexpectedly, instead of observing a decrease in their abundance with time, their abundance increased. This shows that the volatile compounds, instead of dissipation by evaporation, were enriched in abundance due to some unknown chemical processes in the distillate, leading to their production.

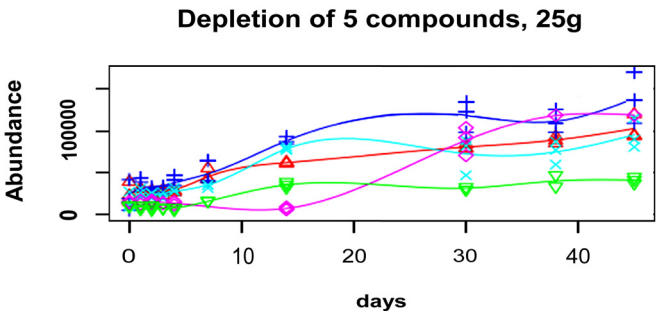

Fig. (2). The change in abundance of 4 alkanes and benzaldehyde with time. (Color code: decane, red; dodecane, blue; tetradecane, cyan; hexadecane, green; benzaldehyde, magenta). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

However, some rarer compounds did show depletion with time. Three of them were identified positively: anisole, eucalyptol, and furfural. They could not be found when less than 25 g of PV herbs were used and when the detection threshold of the mass spectrometer was set at 5000 (a.u. of the machine). Thus, 35 g of PV herb was used (Fig. **3**). When eucalyptol disappeared in around 3 days, anisole and furfural dissipated in around 2 weeks.

3.1.2. Change in Cell Viability with Time

After the volatile compounds are extracted as distillates from the PV herb, unavoidably, there is a time lag between the distillation process and the encapsulation of the volatile compounds for medical use by patients. During this time lag, the questions are how well the volatile compounds are preserved and how well their pharmacological efficacies are preserved. These questions need to be clarified to prepare the volatile compounds as medicine.

With the use of the tetrazolium salt-based CCK-8 test kit on a cancer cell line, SCC154, the cell cytotoxicity was found to change due to the PV herb during a period of 8 weeks (which amounts to about two months, providing sufficient time for the preparation of medicine). The results are shown in Fig. (**4**).

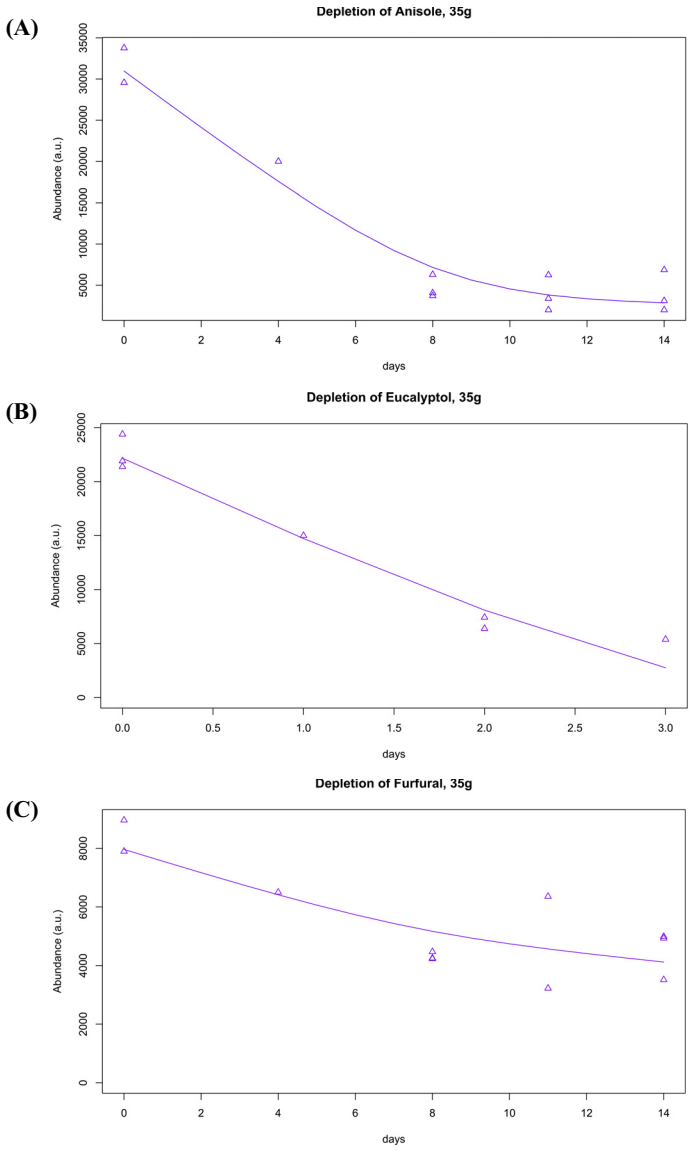

Fig. (3). The change in abundance of 3 rarer compounds found in PV distillate with time. The weights shown in the subtitles of the graph refer to the weights of the herb used.

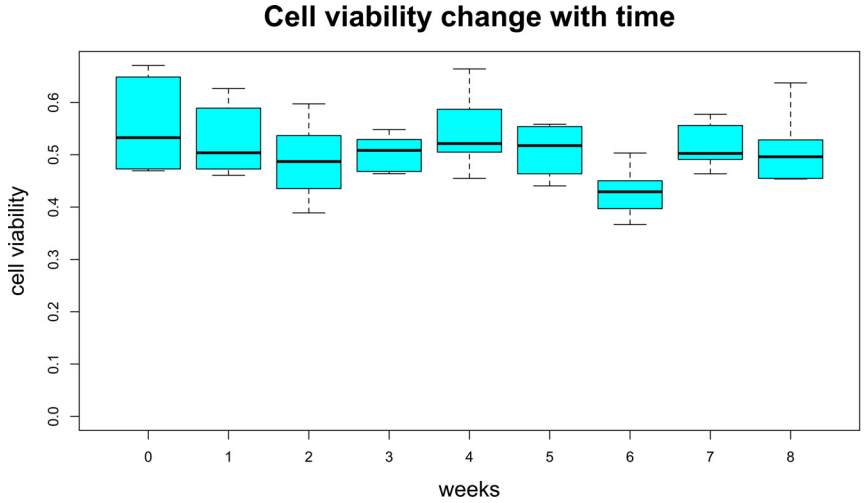

Fig. (4). The cell viability changed with time for 8 weeks after extraction for SCC154 cells. Portion 1 collected during the distillation process was sampled, when 25 g of the herb was used. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

It can be seen that the cell cytotoxicity of the volatile compounds in the PV herb on the cancer cell did not change much for nearly two months after extraction. Using ANOVA to analyze this set of data provided a p-value of 0.0749. If we adopt the usual significant p-value chosen to reject the null hypothesis as 0.05, then the null hypothesis, which means there is no significant difference in the cell viability data during the 8 weeks, is accepted. This also implies that the cell cytotoxicity of the PV volatile compounds is due to those less volatile components, but not due to those volatile compounds, which dissipate quickly in days, as found and reported above.

3.2. The Effects of Steam Flow Rate on the Extraction of Volatile Compounds in PV

In the previous article, we reported that the amount of volatile compounds extracted did not increase monotonically with the amount (weight) of PV herb used [23]. We attributed this observation to the presence of a mass of PV herb in the path of steam above the boiling water in the setup of the steam distillation process. This presented an obstacle to the flow of the volatile compounds to be collected later in the collection arm of the setup.

Here, we further clarify the mechanism by which the mass of PV herb presented as an obstacle, affecting the amount of volatile compounds collected. This was done by changing the steam flow rate by changing the temperature setting of the heater, which boiled water to produce steam. This changed the dynamics of the steam flow; the more energetic steam stream overcame the resistance of the mass of PV herb in the steam path. Experimentally, the heater temperature settings were set at two values, 180^{o}c and 200^{o}C. Fig. (**5**). presents these results.

In Fig. (**5**), we look at three alkanes that were positively identified: decane, dodecane, and tetradecane. The retention time in the GC-MS coil indicates the molecular weights of the constituent compounds; the heavier compound results in a longer retention time. To supplement the results of these three alkanes, we have also included the curves for the other two compounds, which have not been positively identified; we have only named them by their retention time: one lighter compound at a retention time of 5.0 minutes and another heavier compound at 24.2 minutes.

These five graphs indicate a nice trend. The smaller steam flow rate (corresponding to lower heater temperature) extracts lighter compounds more efficiently, while the opposite occurs for higher steam flow rates. The turning point occurred at around a retention time of about 10 minutes, where dodecane locates. We postulate an explanation for this, as follows: when the steam flow is less energetic, the heavier compounds do not attain sufficient energy to overcome the resistance of the mass of PV herb in the steam path, and thus fall back to the boiling water. When the steam gets more energetic (with a higher flow rate), the heavier compounds get sufficient energy and thus can pass through. As the heavier compounds get an equal footing to get enough kinetic energy to overcome the resistance of the obstructing mass of herb, the more resistance which the mass of herb imposes on the lighter compounds shows up, and thus we observe that heavier compounds are more efficiently extracted at a higher steam flow rate.

4. DISCUSSION

We have investigated two issues in this article with respect to the study of the steam distillation process to extract volatile compounds in PV. By tracking the abundance of PV volatile compounds over many days after the distillation process, we found that many volatile compounds in PV preserved for a very long time, even for up to 8 weeks after the distillation finished. The biological tests using the CCK-8 kit, which characterizes the cell viability of a cancer cell line, SCC154, upon treatment with the distillate, showed that the cytotoxicity of the PV distillate did not reduce and lasted for as long as 6 weeks. This has a good implication for the preparation of PV volatile compounds as anti-tumorous medicine; the manufacturing process can be made much simpler, and the cost of the process can be reduced.

In the exploration of the effects of the steam flow rate in the steam distillation process on the abundance of different volatile compounds, a big limitation of the efficiency of the steam distillation process in extraction has been observed. The mass of herb above the boiling water in the path of steam imposed an obstacle for the passage of volatile compounds. This leads to the result that if more herb is used, it does not imply that more volatile compounds will be collected. The volatile compounds can most efficiently be extracted via an optimal amount (weight) of the herb. This, however, depends on the exact setup of the apparatus used in the process; for example, the cross-sectional area of the steam passage path, like the size of the flask, the flask neck, the tubing, and so on. In our experimental setup, the optimal amount of herb used was found to be 15 g.

This leads to the topic which we shall further report on in another article, *i.e*., a comparison of the steam distillation process with another distillation process, the hydro distillation process, in which the herb is soaked inside the boiling water, and thus does not present as an obstacle in the path of steam that carries the volatile compounds.

Finally, I would like to mention some prior works on aerosol nanoparticle interactions, having similar situations as in the steam flow environment in our distillation setup. Chen and Chee *et al*. [25, 26] studied nanoparticle formation from acid-base reactions in an air flow tube environment. They used both experimental and modelling techniques to find that the concentrations and sizes of nanoparticles formed, the humidity inside the flow tube, and the acid/base ratios were highly inter-dependent. This shows that the acidity of the water which we use in the distillation process will have significant impact on the compositions of the VOCs which we collect. Indeed, previous studies [27-29] show that the

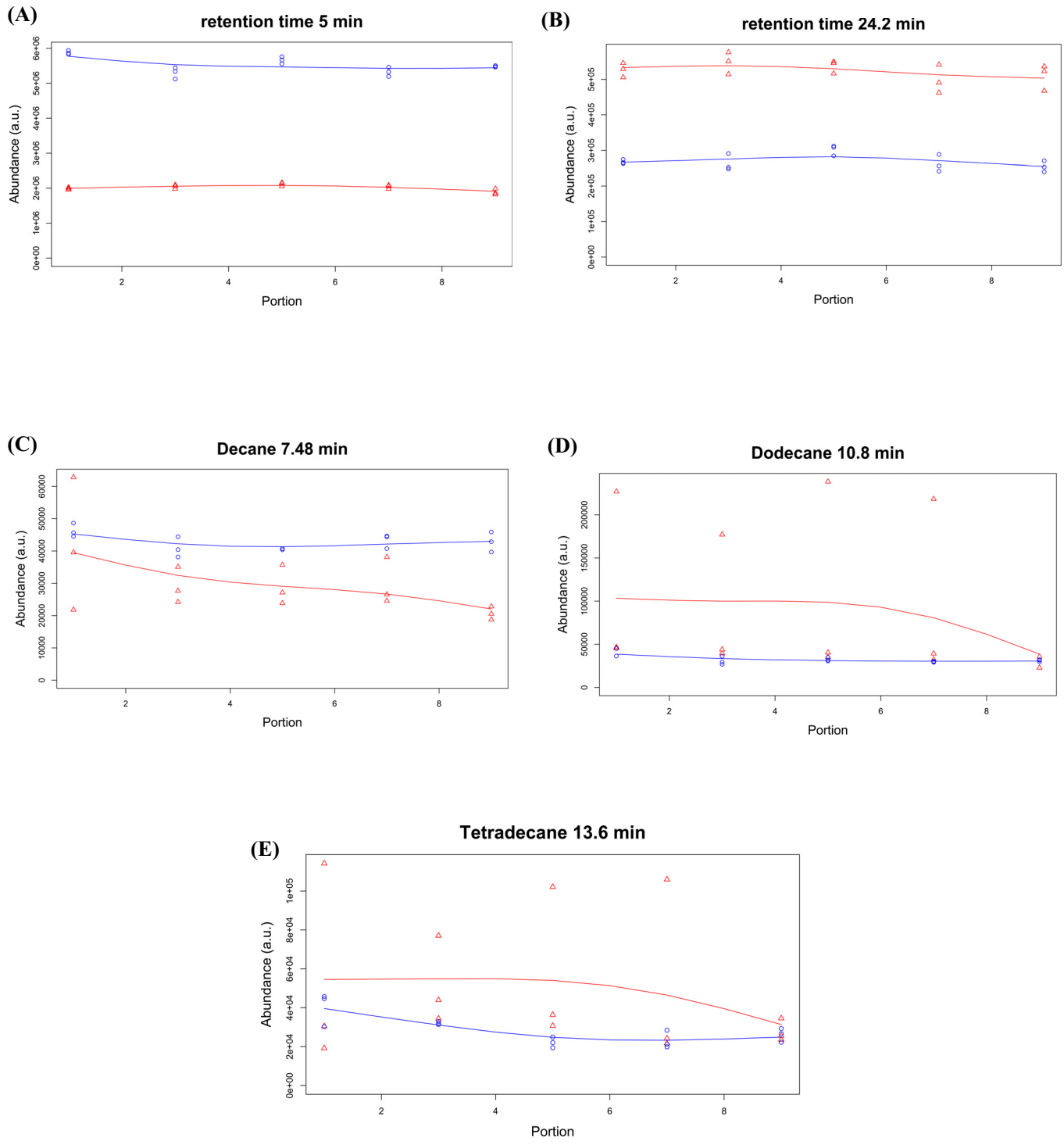

Fig. (5). The abundance of volatile compounds collected during the distillation process, as the steam flow rate was changed by changing the heater setting (Color code: blue, 180°C; red, 200°C). Experimentally, 15 g of PV herb was used. The time shown in the subtitles of each graph is the retention time in the GC-MS coil of the individual chemical components, which reflects the molecular weights. Those compounds which could not be positively identified are titled with the retention time within the capillary coil only. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

water quality and management greatly affect the chemical constituents of the PV plant. So, it is quite natural to see that the quality of the water, its hardness or acidity, will have a great impact on the contents of the VOCs being collected in the distillation process. This points to a direction for further investigation of this impact. Since our aim was to investigate the feasibility of the therapeutic use of the VOCs in PV, it is impractical to expect drug manufacturers to use deionized water in their manufacturing process, implying the investigation as necessary. However, as the PV species includes hundreds of chemical constituents [19, 20], with new constituents being discovered as new research results emerge, using similar experimental and computational modelling approaches as employed earlier [25, 26] will cause very complicated problems, as hundreds of volatile chemical compounds will be involved and needed to be considered. Reactions among single acid-base pairs were considered in those earlier investigations. Moreover, the idea of changing the VOCs collection efficiency by changing the acidity of the water used may not be desirable because the chemical composition of the VOCs being collected and used as medicine may be changed.

CONCLUSION

In this article, we have reported two aspects of this project on the study of the volatile compounds in PV: the effects of the aging of the distillate after the extraction process, and the effects of changing the steam flow rate in the steam distillation process on the abundance of the volatile compounds extracted.

Quite unexpectedly, the volatile compounds did not dissipate and evaporated away quickly after the distillation process finished. They persisted for a long time, extending for up to 8 weeks, which was the longest period that we observed experimentally. It was found that chemical reactions took place in the liquid even when the samples were stored in a refrigerator set at 5^{o}C, and these chemical changes enriched some compounds. However, agreeing more with intuition, it was observed that some rarer compounds dissipated quickly within days.

When the steam flow rate was changed, the mechanism of extracting volatile compounds by steam distillation was explored. When the steam flow rate was slow, the steam flow could not impart sufficient energy to the heavier volatile compounds so they were less efficiently collected. On the other hand, when the steam flow rate was higher, sufficient energy was imparted to all volatile compounds. The higher resistance imposed by the mass of herbs against lighter compounds enhanced the collection efficiency of heavier compounds.

LIST OF ABBREVIATIONS

PV	=	*Prunella vulgaris*
VOC	=	Volatile Organic Compounds
GC-MS	=	Gas Chromatography-Mass Spectrometry
CCK	=	Cell Counting Kit
SCC	=	Squamous Cancer Cells

AUTHORS' CONTRBUTIONS

The author contributed to all facets in the completion of the article: Conceptualization, data curation, analysis, investigation, methodology, project administration, writing, review & editing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The author declares no conflicts of interest, financial or otherwise.

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