UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Distillation Extraction of *Prunella vulgaris* Volatile Organic Compounds: Characterization and their Anti-tumoral effects

by William Chi Keung Mak

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of Prof. Peter Meier, Dr. Sean Walsh, Prof. Alison Ung, and Prof. Nham Tran

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Certificate of Original Authorship

I, William Chi Keung Mak, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences, Faculty of Science at the University of Technology Sydney.
This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.
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(William Chi Keung MAK)

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Thesis format

The format of this thesis is a Conventional Thesis.

Abstract

This thesis reports on a PhD research project at the University of Technology Sydney. The thesis consists of two parts. The first part reviews the research work of the past three decades on the anti-tumoral and anti-viral activities of the herb Prunella vulgaris L. (PV). The second part explores experimentally the volatile organic compounds (VOCs) of PV. The study includes the characterization of the steam distillation process which extracted the volatile chemical constituents from the herb PV. The characteristics of the VOCs extracted during the distillation process were explored and reported. It was found that VOCs emerged from the herb constantly at approximately the same rates and continuously for a long time. Abundances of typical compounds obtained from the steam distillation process were compared with those obtained from the hydro distillation process and, the dynamics of the extraction process was proposed and verified. It was found that the hydro distillation process is a more efficient extraction method than steam distillation. Then, the pharmacological effect, specifically the anti-tumoral effect, of the VOCs of PV was studied, with a colorimetric assay using a tetrazolium-based reagent to probe the cytotoxicity of the volatile compounds of PV on the cancer cells from the cell line SCC154. The experiments confirmed the cytotoxicity of the VOCs of PV against the cancer cells. As the extraction of the volatile compounds was explored for the potential of drug production, and the manufacturing process took time, the aging effect on the abundances of the volatile compounds was examined. It was found that the cytotoxicity of the herb extracts persisted for up to eight weeks. The effect of the change of the steam flow rate on the abundances of the extracted volatile compounds was also investigated to further explore the dynamics of the extraction process. The findings in the experiments give great insights into the design of a drug manufacture process which makes use of the VOCs from PV.

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List of Abbreviations

CCK = Cell Counting Kit
DNA = deoxyribonucleic acid
ELISA = enzyme-linked immunosorbent assay
GC-MS = Gas Chromatography-Mass Spectrometry
GP1 = glycoprotein 1
HIV = human immunodeficiency virus
HPLC = high performance liquid chromatograph
HSV = herpes simplex virus
IC_{50} = 50% inhibition concentration
NF = nuclear transcriptional factor
PV = Prunella vulgaris
RNA = ribonucleic acid
ROS = Reactive Oxygen Species
SCC = squamous cancer cell
SOD = superoxide dismutase
TLC = thin layer chromatography
TNF = tumour necrosis factor
VOC = Volatile Organic Compounds

List of Publication outcomes

Review articles:

1. MAK, W. C. K. 2021. Review of the Studies on the Anti-tumoral Effect of Prunella vulgaris. *Journal of Biosciences and Medicines,* 9. doi: 10.4236/jbm.2021.912011
2. MAK, W. C. K. & WALSH, S. 2022. The Anti-viral Activity of Prunella Vulgaris: A narrative review. *Integrative Medicine Reports, 1(1).* doi: 10.1089/imr.2022.0045

Articles reporting the experimental findings:

3. MAK, W. C. K. & WALSH, S. 2021. The Characterization of Steam Distillation as an Extraction Method to Extract Volatile Compounds from Prunella Vulgaris and the Investigation of their Anti-tumorous Effect. *Journal of Biosciences and Medicines,* 9. doi: 10.4236/jbm.2021.98011
4. MAK, W. C. K. 2022. Time Depletion Effects on the Volatile Compounds from the Distillation Extracts of Prunella vulgaris and the Dynamics of their Extraction. *Current Drug Research Reviews,* 14**,** 148-56. doi: 10.2174/2589977514666220429104009
5. MAK, W. C. K. 2023. Comparisons between Hydro and Steam Distillation Processes to Extract *Prunella Vulgaris* Volatile Compounds, and their Anti-oxidative Activities. *Journal of Drug Delivery and Therapeutics*, 13(3), 51-57. doi: 10.22270/jddt.v13i3.5955

Chapter 1: Introduction

1.1 Background information

1.1.1 Cancer as a disease

Cancer is a disease which daunted the whole world for centuries. In 2018, it was estimated that there was a total of 18.1 million new cases of cancer around the whole world. In the same year, 9.6 million people died worldwide (Ferlay et al., 2019) from the diseases. According to the most recent report by the World Health Organization, this death toll rose to ten million in 2020 (World Health Organization, 2023). Among all cancer types, the commonest cancer types were breast cancer (2.26 million new cases), lung cancer (2.21 million new cases), and colon and rectum cancer (1.93 million new cases) in 2020. As we used the oral squamous cancer cell line for our experimental work, for a comparison, the number of new cases for oral cancer was 355 thousand. We chose the oral cancer cell line SCC154 (squamous cancer cells) for the experimental work because it was readily available in our research group and used by co-researchers; thus, working on the same cell line might provide results for mutual references. Doctors and researchers in different countries are continuously looking for cures for this disease in different directions.

Cancer evolves when normal body cells undergo mutation. If the immune system fails to check the abnormal development of these cells, a multi-stage process progresses, and the abnormal cells become malignant tumours and invade adjacent tissues. If metastasis happens, the malignancy spreads to other organs. Widespread metastases finally cause fatality.

The major causes of cancer are smoking, alcohol consumption, ultraviolet radiation, viral and bacterial infections (World Health Organization, 2023). Other risk factors include lack of physical exercises, improper diet, and air and water pollution, leading to the

intake of cancer-causing toxins and contaminants. Therefore, the occurrence of cancers can be effectively prevented by avoiding these risk factors.

1.1.2 Cancer treatment in Western medicine

In Western medicine, the commonly used cancer treatment therapies include surgery, chemotherapy, radiation therapy, targeted therapy, immunotherapy, stem cell or bone marrow transplant and hormone therapy (American Cancer Society, 2019, World Health Organization, 2023). However, even with progressively more technological advances emerging, these therapies have their own limitations, and the malignancies remain difficult to control in many cases. Many treatment methods may not be applicable to aged patients. Some cancer patients may refuse to take the surgery, chemotherapy and/or radiation therapy options because of the worry about their severe side effects and prefer 'alternative' treatments. Some cancer patients may not have the choice at all; the patients may be too old or have such weak health constitutions that they may not endure the severe side effects in Western treatment methods. Herbal therapy becomes appropriate to these patients, and serves as an alternative adjuvant treatment to Western medicine (Wang et al., 2018).

1.1.3 Herbal treatment and choice of the target herb

While Western medicine remains the major modality to treat cancer, herbal medicine gradually gains favour as an adjuvant therapy (Wang et al., 2018, Rahman et al., 2021, Qi et al., 2010) to the Western therapies such as chemotherapy and radiotherapy. Previous research efforts showed that many herbs exhibit anti-tumoral activities; examples are *Scutellaria baicalensis* (Cheng et al., 2018)*, Scutellaria barbata* (Sheng et al., 2022)*, Hedyotis diffusa* (Lee et al., 2011). The examples shown here have been extensively researched.

In comparison, relatively less research efforts focused on *Prunella vulgaris* (PV), which also shows good potential to offer anti-tumoral capability (Huang et al., 2015). A simple

literature search using the database Pubmed, using the names of the herbs together with “cancer” or “tumour” or “tumor” as keywords, showed that the numbers of research articles found were 553 (*Scutellaria baicalensis*), 149 (*Scutellaria barbata*), 117 (*Hedyotis diffusa*), and 76 (PV). The number of articles reporting on PV is far less. These numbers indicate the relative interests in the research community and their expectations of the cancer treatment efficacies of these herbs. However, the relatively smaller number of research articles reporting on PV means that there is more room to yield novel research outcomes. Therefore, PV was chosen as the target herb for this project.

Moreover, reports on the research of the capability of the volatile organic compounds (VOCs) of PV to treat cancer were not found in the literature. Thus, it is more promising to yield new discovery by focusing this study on the examination on the potential anti-tumoral effects of the VOCs of PV. Novel discovery is reported in Chapter 5.

1.1.4 The PV plant

PV is a widely distributed low-growing perennial herbaceous plant, which can be found in every continents of the world (Fig. 1.1): in East Asia including China, Japan and Korea; in the Asian sub-continent of India, among the mountainous region of Kashmir; in Europe including Russia, Germany, Poland and Ukraine (Luczaj, 2010, Bomme et al., 2007) in the East, to Britain in the West; in North America including the United States and Canada; in North Africa (Bai et al., 2016, Chen et al., 2011c, Lans et al., 2007). It is widely found in the temperate and tropical regions: in woodlands, ridges, and mountains. Flowering with beautiful purple flowers occurs from April to June, fruiting occurs in late summer from July to October. Traditionally, this herb is widely used in different cultures in folk medicine (Siew et al., 2014, Rahman et al., 2016, Amjad et al., 2017, Kujawska et al., 2017), and also in feeding animals and as veterinary medicine (Wilson and Stine, 1996, Kuriya et al., 2015, French et al., 2018).

Fig. 1.1: A Prunella vulgaris plant

(Source: Wikipedia.org : "Common self-heal (Prunella vulgaris). Keila, Northwestern Estonia" by Ivar Leidus, is licensed under CC BY-SA 3.0 via Wikimedia Commons)

While it grows in the wild, it is also cultivated. For pharmacological use, the spica, which is about 1.5-8 cm long, and 0.8-1.5 cm in diameter, is harvested in summer when it withers and becomes pale brown to reddish brown in color. After harvest, the herb is cleaned to remove any dirt and dried in the sun. This is the form in which the herb is sold and used as medicine in China.

1.1.5 Anti-tumoral activities of PV extracts

One of the mechanisms which accounts for the anti-tumoral activity of PV extract is its anti-oxidative activity. Both PV extract and its main component, rosmarinic acid, were shown to significantly eliminate ROS production and diminish IL-6 release to prevent UVB-caused DNA damage and oxidative stress (Jiao et al., 2018), which is the main cause of skin cancer. Rosmarinic acid also inhibited TNF-α-induced ROS generation, NF-μB activation, and enhanced TNF-α-induced apoptosis (Vostalova et al., 2010). Other chemical constituents which were identified by previous research to have anti-tumoral activity include polysaccharides (Feng et al., 2010b), oleanolic acid (Hwang et al., 2014),

ursolic acid (Li et al., 2019, Jeon et al., 2015, Weng et al., 2014), and hyperoside (Yang et al., 2017b).

Other than melanoma, anti-tumoral effect of PV was also demonstrated for breast cancer (Zhao et al., 2017, Gao et al., 2019), thyroid cancer (Zhang et al., 2019, Yin et al., 2017, Ba and Wang, 2017), hepatocellular carcinoma (Jang et al., 2018, Fan et al., 2018, Su et al., 2016, Wang et al., 2014b, Kim et al., 2012), colon carcinoma (Yi et al., 2017, Fang et al., 2017, Lin et al., 2013, Zheng et al., 2011), lung cancer (Yang et al., 2017b, Feng et al., 2010b, Jia et al., 2009), gastric cancer (Tan et al., 2015), oral carcinoma (Wang et al., 2013), acute leukemia (Woo et al., 2011) , and lymphoma (Liu et al., 2010, Zhang and Wang, 2009, Chen et al., 2009a).

From the literature review, it was noted in a previous article (Golembiovska et al., 2014) that two VOCs in PV, squalene and anethole, were reported to have anti-tumoral effect; however, the description there was very brief.

1.1.6 Anti-viral activities of PV extracts

From the general literature survey found in Ch. 2, numerous works were done to explore the anti-viral activities of PV extracts, especially against the HIV (human immunodeficiency) and HSV (herpes simplex) viruses. A report by the World Health Organization (2023), viral infection is one of the major causes to develop cancer. A survey of previous research findings on the anti-viral activities of PV was undertaken to establish any potential link with the anti-tumoral activities of the herb. This literature review work is reported in Ch. 3.

Again, from the general literature review in Ch. 2, PV has a wide range of pharmacological activities besides its anti-tumoral and anti-viral activities, including anti-inflammatory, antibacterial effects, immune regulation, antihypertensive, hypoglycaemic, lipid-lowering, antioxidant, free radical scavenging, liver protection, sedative, and hypnotic effects. It should be interesting to explore all these activities in

depth. However, this will become an immense task which cannot be fitted into this single research project.

1.1.7 Other issues pertaining to PV

Previous research revealed that the chemical composition of the plant varies from region to region globally. There are various factors which affect the chemical composition of the plant. The chemical composition of the PV extract used in experiments depends on the extraction method too. These variations of the chemical composition subsequently may cause uncertainty and variations in the experimental results in this project because then, the starting material used in the experiments may vary. This issue of variations in chemical composition, together with other general interesting information about PV have been elaborated upon in Appendix 1.

1.2 Aims & objectives of the project

This project can be separated into two parts: The literature review part and the experimental part (See Fig. 1.2).

The literature review summarizes the mechanisms of the therapeutic[1] efficacies of the herb: The anti-tumoral activity and the anti-viral activity of the chemical compounds in PV. The articles in previous research efforts were categorized and analyzed. The results are reported in Chapters 2 & 3. However, since articles, and thus information, on the anti-tumoral activities of the VOCs of PV were not found in the literature, it is not possible to report these activities in Chapter 2.

The second experimental part dealt with two aims:

[1] Note that the prestigious Cambridge dictionary defines the word “therapeutic” as “causing someone to feel happier and more relaxed or to be healthier”. So, this word is used in this project more casually than to limit the meaning so narrowly to a requirement to establish a proof for confirmed clinical use. For a more stringent requirement to confirm suitability for clinical use, as required by the Australian Therapeutic Goods Administration or the Food and Drug Administration of the United States of America, more detailed discussions can be found in Sec. 6.1.3.

- In phytochemistry, the aim was to characterize the VOCs emerged from the PV plant during distillation. The objective was to obtain data on the abundances of the extracted VOCs during the experiments when the experimental independent parameters were varied. However, it was established and emphasized early in the planning stage of the project with the supervisors that the detailed chemical composition of the VOCs of PV was not sought after. The reason was that this work was carried out several times by several research teams previously (Golembiovska et al., 2014, Yang et al., 2013, Morteza-Semnani et al., 2006). Therefore, a detailed study of the phytochemistry (in one of the directions in the conventional sense to study the chemical composition) was not carried out. Attention was given to how the VOCs evolve during the distillation process.
- In the biological part, it was to examine the combined anti-tumoral effect of the VOCs of PV, taken as a whole. So, the null hypothesis was stated as follows:

  "The VOCs of PV were not anti-tumoral."

  To achieve this aim of showing this null hypothesis wrong, the objective was to show the dosage dependence of this anti-tumoral effect. Then, further works were to study how this anti-tumoral effect was affected by the various independent variables, for examples, the collection time of the VOCs during the distillation process, or the effect due to evaporation loss during storage.

1.3 Structure of this thesis

The structure of this thesis is shown in this flowchart:

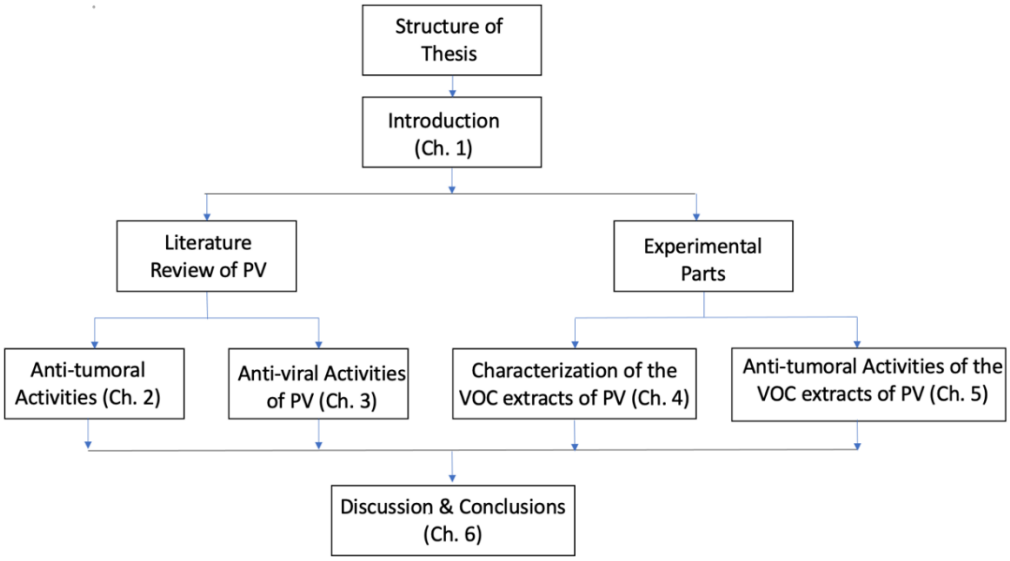


Fig. 1.2: Structure of this thesis

The thesis can be divided into two main parts. The literature review part surveys the previous research articles on the anti-tumoral activities and the anti-viral activities of PV (Chapter 2 & 3). Then, the experimental part reports the experimental works in this research project on the characterization of the VOC extracts of PV and the anti-tumoral activities of these extracts. The reports can be found in Ch. 4 & 5. Then, the general discussions and conclusions can be found in the last chapter, Ch. 6.

1.4 Discussion

Reports in the literature on the use of PV for their anti-tumoral activity were few; none was found on the use of the VOCs of PV for their anti-tumoral activity. Thus, the investigation on the VOCs of PV has great potential to discover new knowledge in this aspect. Consequently, this inspired the investigation of this research study to uncover such potential and thus formed the main theme.

As found in a report by the World Health Organization (World Health Organization, 2023), viral infection is one of the major causes to develop cancer. A survey of previous

research findings on the anti-viral activities of PV was undertaken to establish any potential link with the anti-tumoral activities of the herb. This link was not found however in the literature and was not further pursued in this project.

Moreover, during the execution of this project, the COVID-19 pandemic has attracted global attention. Thus, although the anti-viral activities of PV were not mentioned in this introductory chapter, the anti-viral activities of PV will be elaborated later in Chapter 3. The inclusion of the review of the anti-viral activities of the VOCs of PV in this project is also a preparation to explore future research directions into the potential of using the VOCs of PV to treat viral diseases. This effort proved fruitful in unveiling a promising research direction. Among the five published papers from this project, the paper on the anti-viral activities of PV attracted the second largest number of reads in the research community, highlighting this particular interest in the community.

1.5 Conclusion

This introductory chapter began by giving some background information on cancer and its treatment using Western medicine. Herbal treatment as an adjuvant or alternative method was introduced as it has become more and more acceptable by the health professionals and the community. As PV was chosen as the target herb in this investigation, basic information about this plant was covered, together with a brief description of its antitumoral activities.

Then, an overall view of this thesis was given, by describing the aims & objectives and the structure of the whole thesis.

The next two chapters continue with the results of the literature reviews on the anti-tumoral activities and anti-viral activities of PV.

Chapter 2: Review on Previous Research Efforts on the Pharmacological Effects of PV, with attention to its Anti-tumoral Effect

2.1 Introduction

This chapter reviews the past research efforts on the anti-tumoral effect of the extracts from the PV herb. It gives important background information of what has been achieved in the past to form a solid basis for the subsequent study in this project.

For three decades, there had been a continuous advance in technology to treat cancer. Different test methods emerged in this period. In early days, in vitro tests in cancer research mainly used colorimetric assays to probe cancer cell viability. The most popular choice was the classic MTT assay and related assays based on tetrazolium-based salts. In recent years, more researchers' choice shifted to molecular techniques.

The methodology for the literature review in this chapter involved: defining the scope (see Sec. 2.2.1), setting the inclusion and exclusion criteria for the articles, and categorization according to the research issues (see Secs. 2.2.2 - 2.2.3).

The first article investigating the PV herb was published in 1988 (Lee and Lin, 1988). Since then, different modern techniques in molecular biology, such as proteomic assays and different immunochemical techniques, emerged to elucidate the anti-tumoral molecular mechanisms. This literature review focused on these research efforts.

Using functional tests, research directly monitored cellular functions such as cell viability/apoptosis, proliferation, migration, morphological changes, and anti-oxidative activities, upon herbal treatment. These functional tests included direct microscopic observation, transwell migration and propagation tests, flow cytometry or the use of colorimetric assays.

Finally, in the Discussion section of this chapter, the findings of all the collected articles were summarized. The difficulties in this summarization work were also explained in detail. A table of summary of all the articles surveyed can be found in the Appendix 2.

The findings in this chapter were published in a journal article (Mak, 2021).

Although all the research articles collected were examined, this literature review focused on those which reported on the anti-tumoral and the anti-viral activities. The review concerning the anti-tumoral activities of PV is found in this Chapter, while the review concerning the anti-viral activities is described in the next Chapter.

This review informed of all the currently available techniques which could be adopted in this research project. More discussions on choosing techniques for the experimental part of this project could be found in Sec. 4.2.2 of Chapter 4 and Sec. 5.2.1 of Chapter 5.

2.2 Methods

2.2.1 Scope of the literature review

This chapter of the literature review concentrates on articles relating to the anti-tumoral activities of PV which is discussed in the later part of this chapter; however, Sec. 2.3.1 discusses the research in the PV herb in general (See Fig. 2.1). There are four major ways to research the pharmacological effects of herbs: in vitro methods, in vivo methods, clinical studies, and in silico methods. As the in vitro methods were the most frequently adopted favorite in the past to examine the anti-tumoral activities of PV, this review particularly spent most of the efforts looking at them. The results are given from Sec. 2.3.2 and beyond.

2.2.2 Initial search strategy and categorization

As a preparation step, a preliminary literature search was done using the Pubmed database in early 2019. At this beginning stage, the inclusion criterion was intentionally

broad to include all the articles which might be of interest. So, the broadest general term, "*Prunella vulgaris*", was used as the keyword to search. A categorization of the articles obtained is shown in Fig. 2.1.

Then, a more detailed literature search was done using three databases: Pubmed, Medline and Embase. The two literature searches were carried out about half a year apart, resulting in 80 more articles being found.

2.2.3 Subsequent selection strategy and categorization

Then, to narrow down the attention further to those articles reporting the anti-tumoral activity of PV, the inclusion criterion was restricted by adding words such as "cancer" and "tumor" as search terms. It was then further narrowed down to include only those articles discussing the in vitro methods and excluded other articles which used in vivo methods or clinical studies. This selection process is indicated in Fig. 2.2. The resulting set of articles was then categorized and analyzed to give the results from Sec. 2.3.2, and onward. The popularity of the other investigation methods, including the in-vivo, the in-silico methods, and the clinical studies, are reported in Fig. 2.3.

The articles related to the anti-tumoral activity of PV were again re-categorized, according to the methodologies adopted, proteomic phenomena observed, the proteomic assays used, the molecular entities studied, and the other functional assays used in the articles.

2.3 Literature review findings

2.3.1 Categorization of the research articles related to PV

The initial general search got 206 articles while the detailed search got 286 articles. Categorization of all the articles found in the general search related to PV showed that they could be divided into three broad areas: pharmacological, phytochemical, and agricultural areas (See Fig. 2.1, which shows the distribution of the search results

according to a preliminary literature search using Pubmed with a general keyword "*Prunella vulgaris*").

Other than these three areas, there are also some review papers (Kujawska et al., 2017, Amjad et al., 2017, Wurtele et al., 2012).

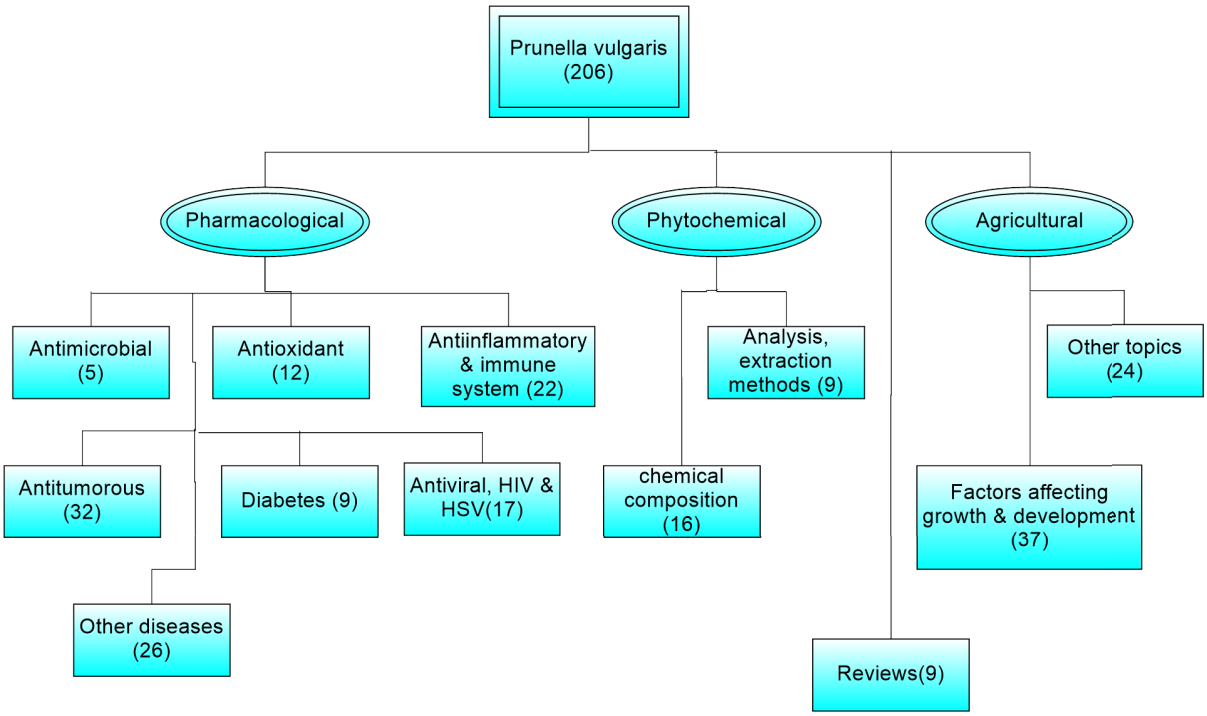


Fig. 2.1: Categorization of all the articles

2.3.1.1 Investigations in the pharmacological area

From Fig. 2.1, under the pharmacological area, the research efforts focused on the sub-categories of:

- anti-microbial activity of PV (Komal et al., 2018, Lu et al., 2013),
- anti-viral activity (Yang et al., 2017a, Zhang et al., 2016) with many articles concentrating on the treatment of herpes simplex (Hassan et al., 2015, Reichling and Schnitzler, 2011, Nolkemper et al., 2006, Chiu et al., 2004) and human

immunodeficiency (HIV) (Oh et al., 2011, Kageyama et al., 2000, Xu et al., 1999, Yamasaki et al., 1998),

- anti-oxidative activity (Zhang et al., 2018b, Lee et al., 2017),
- anti-inflammatory effects (Park et al., 2013, Hwang et al., 2013b),
- the treatment of arthritis (Zaka et al., 2017, Song et al., 2007), and
- the treatment of diabetes (Namgung et al., 2017, Raafat et al., 2016).

There were also investigations on other less popular diseases included anorexia (Woo et al., 2018), dementia (Qu et al., 2017), colitis (Haarberg et al., 2015), hepatitis (Zheng and Zhang, 1990), insomnia (Jeon et al., 2015), gingivitis (Adamkova et al., 2004), hypertriglyceridemia (Skottova et al., 2004), and aging (Zhang et al., 2018b).

2.3.1.2 Investigations in the phytochemical area

In the phytochemical area, the investigations can be divided into two directions: One explored the extraction processes (Tabba et al., 1989), and the other, the analyses of the chemical composition of the PV plant. The use of high-performance liquid chromatography, HPLC, was a favored choice (Yang et al., 2018, Xu et al., 2012, Sun et al., 2008, Yang et al., 2016). Since rosmarinic acid is a major chemical constituent in PV, its determination was the target in many articles (Jirovsky et al., 2007, Qiang et al., 2011). Other important chemical components included polysaccharides (Du et al., 2016, Li et al., 2015a, Li et al., 2015b), triterpenes (Yu et al., 2015, Du et al., 2012), flavonoids and phenolic compounds (Cheung and Zhang, 2008, Feng et al., 2016, Sahin et al., 2011, Gu et al., 2011).

2.3.1.3 Investigations in the agricultural area

In the agricultural area, the interests were mainly on the factors which affect the growth and development of the PV plant, when it was cultivated as a crop. These factors included amount of UV radiation (Chen et al., 2018b), drought (Chen et al., 2016), altitude (Kuriya et al., 2015), and fungal cohabitants (Mariotte et al., 2013, Santos-

Gonzalez et al., 2007). PV was also used to feed livestock. Investigations were done to see how the meat quality was affected when the domesticated livestock was fed with this plant (Kliem et al., 2018, Jirovsky et al., 2007); how honey was affected when PV nectar was used to rear bees (Wilson and Stine, 1996, Kuriya et al., 2015), and how PV plant acted as a veterinary medicine (Lans et al., 2007).

2.3.1.4 Review articles

There were also some review articles of the research on PV. These included ethnobotanical surveys from Singapore (Siew et al., 2014), Eastern Europe (Kujawska et al., 2017), the Indian sub-continent (Amjad et al., 2017), and Japan (Hamada, 1993). Examples of other review articles on PV herb alone are (Markova et al., 1997, Chen et al., 2010, Guo and Chen, 2011, Huang et al., 2015, Wang et al., 2019).

2.3.2 Article selection process for articles on anti-tumoral activity of PV

A PRISMA flowchart showing the selection process for articles related to anti-tumoral activities were shown in Fig. 2.2. After this selection process, the process reduced the number of articles included for detailed examination to 56. A summary of articles included in this review can be found in Appendix 2.

Note that for all the 56 articles found, they reported on research projects which used extraction methods that eliminated all the volatile compounds in the process. Therefore, they did not ever investigate the anti-tumoral activities of the VOCs of PV. They only researched on the non-volatile compounds of PV.

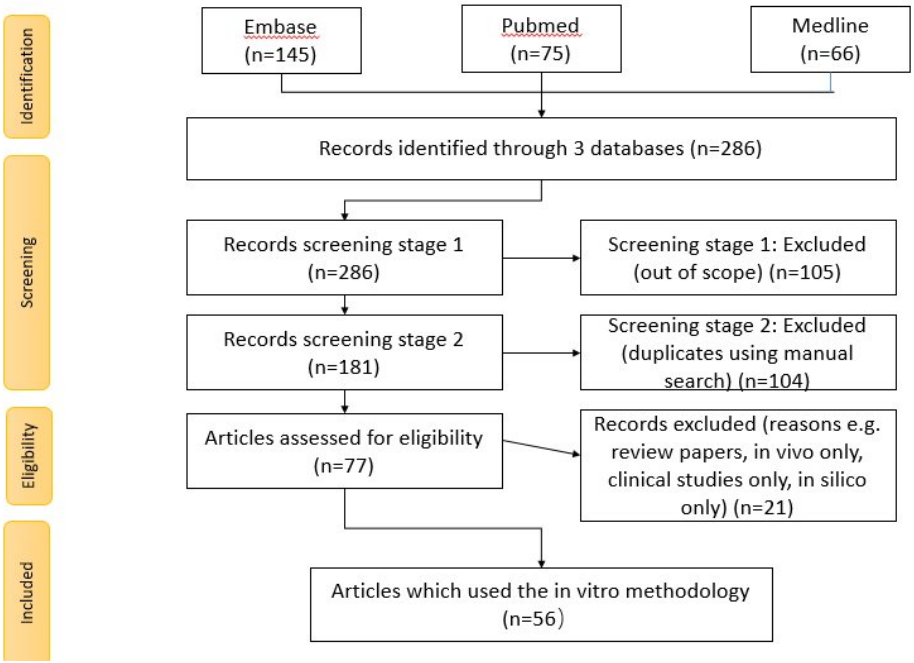


Fig. 2.2: PRISMA flowchart which shows the selection process for articles related to anti-tumoral activities

2.3.3 Popularity of the investigation methodologies

This study concentrated on in vitro methods based on molecular biology, which were the most popular methods chosen in research. Their popularity is shown in Fig. 2.3.

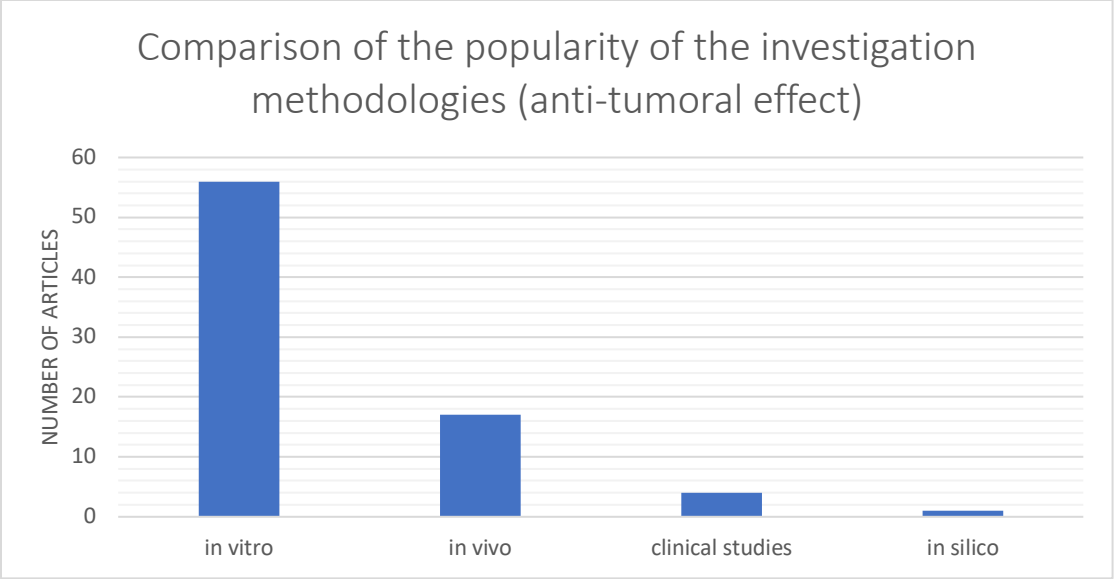


Fig. 2.3: Comparison of the popularity of the investigation methodologies (anti-tumoral effect)

Fig. 2.3 summarized on the number of articles which appeared in the last three decades and used the different research methodologies. In vitro methods accounted for 72% of the articles surveyed. The data shown reflect what was found from the initial literature search from Pubmed.

2.3.4 The Different proteomic phenomena observed, and assays adopted

From the observation of the evolution of the articles which appeared over the years, using the genomic and proteomic approaches became a popular trend which many researchers adopted to elucidate the molecular mechanisms behind the action of herbs (or other drugs) on cancer cells.

Different phenomena were observed experimentally using the in vitro methods. These are summarized in Fig. 2.4.

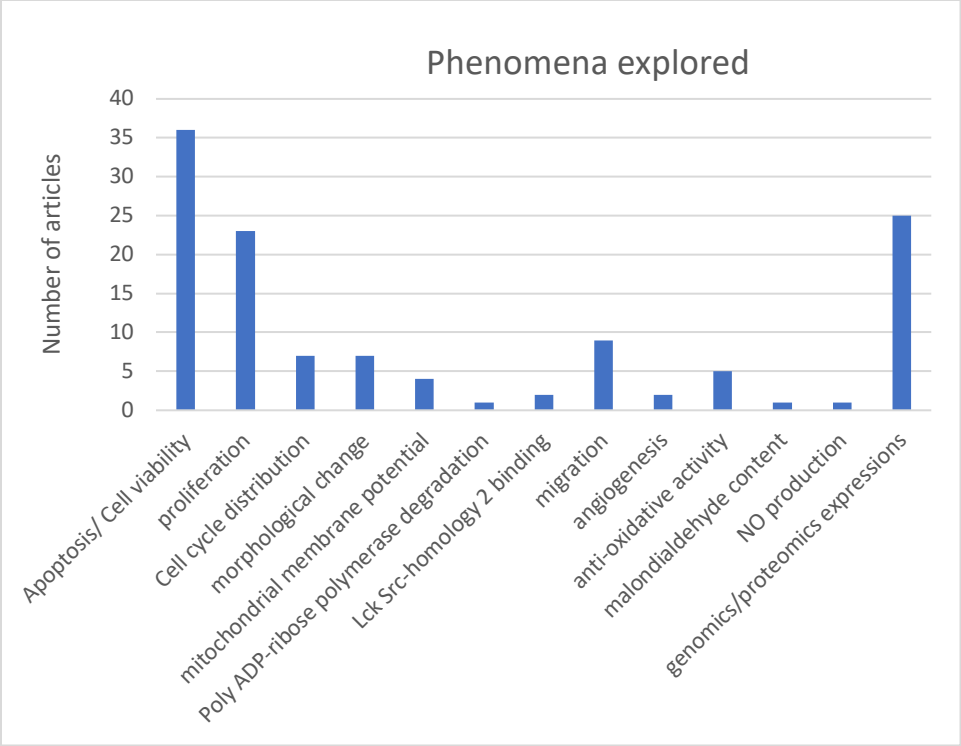


Fig. 2.4: The different cellular phenomena being observed

The most popular phenomenon which was investigated in the past was apoptosis/cell viability of cancer cells. This accounted for 76% of all the articles surveyed. What follows in popularity was the changes in the genomic/proteomic expressions of the treated cells, which were observed in 54% of papers. These changes reflected the action of the herbal treatment on the cancer cells, and this indicated the molecular mechanisms.

Fig. 2.5 displayed the different adopted proteomic assays, and their frequencies of use in the past. These assays measured the cellular entities such as intracellular proteins, enzymes, and intercellular signaling cytokines. The differences in their expressions upon application of PV herbal treatment indicated which genomic pathways were affected, and thus the molecular mechanisms involved.

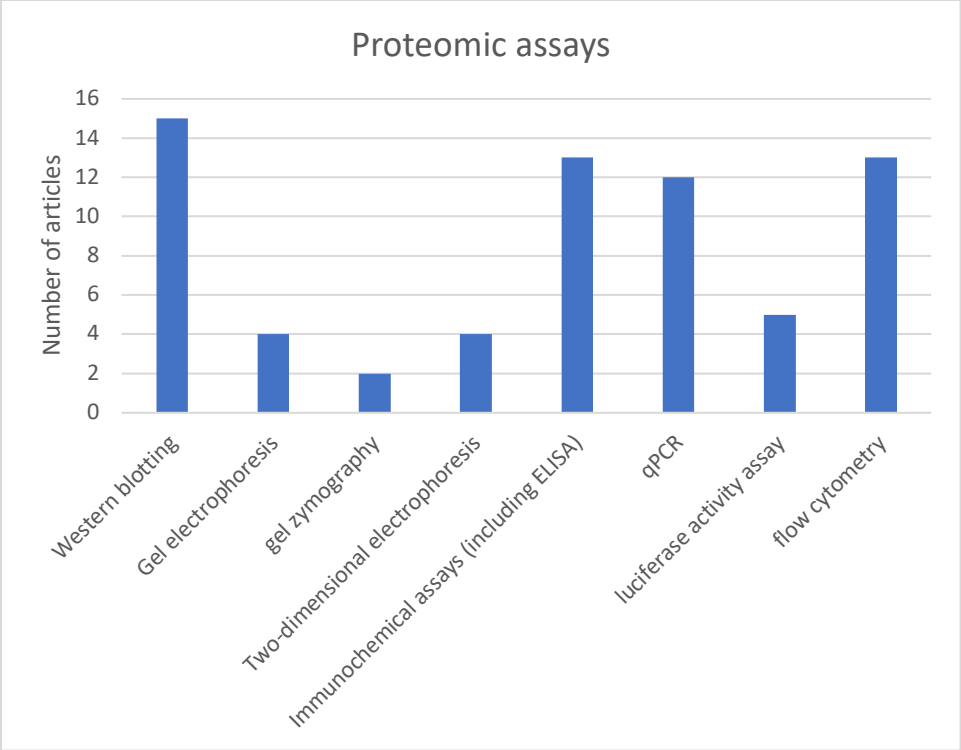


Fig. 2.5: The various proteomic assays used,

based on the articles which were surveyed.

2.3.5 Results pertaining to the major aim: molecular mechanisms behind the action of the PV herb

To identify what molecular mechanisms were responsible for the anti-tumoral effect of the PV herbs, the most straightforward approach was to categorize in terms of the entities, such as proteins, genes, or RNAs. Fig. 2.6 depicted the different proteins, cytokines, or enzymes being studied in the articles.

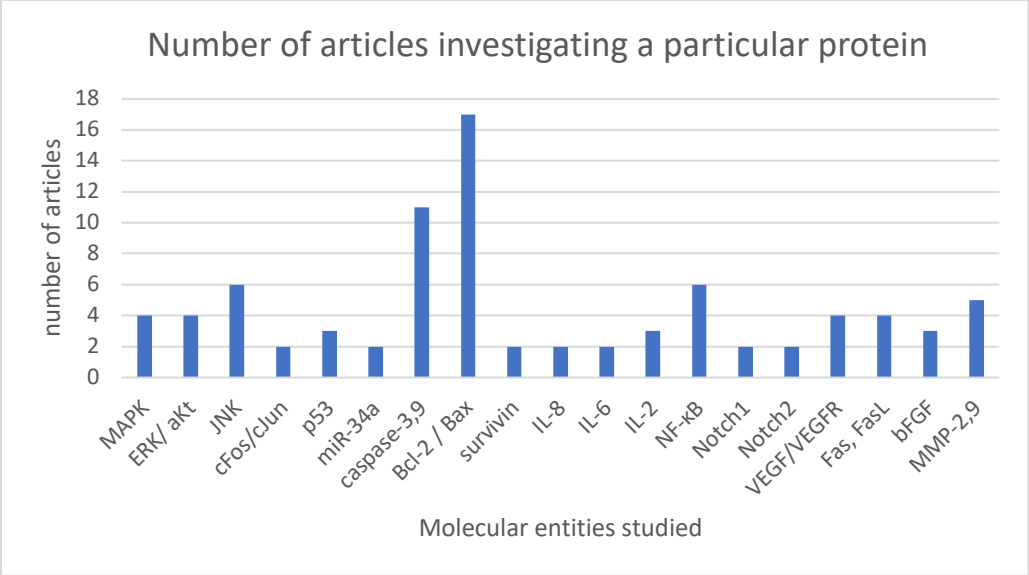


Fig. 2.6: Number of articles investigating a particular protein

Note that for the purpose of clarity of the figure, those entities which were only studied by a single article were not included in this figure. They are CD1 (Fang et al., 2017), CDK4 (Lin et al., 2013), APAF-1 (Zou et al., 1997), AP-1 (Zhang et al., 2018b), cytochrome c (Woo et al., 2011), Bad (Feng et al., 2011), c-myc (Ba and Wang, 2017), iNOS TNF-α (Zhang et al., 2018b), IL-1β (Huang et al., 2013), IL-6 (Zhang et al., 2018b), RANKL/RANK (Xu et al., 2010a), RIPX (Zhao et al., 2008), Stat3 (Zhao et al., 2008), ROD1 (Zhao et al., 2008), IκB-α (Haarberg et al., 2015), Snail (Cho et al., 2015), Notch1 (Fang et al., 2017), Notch2 (Fang et al., 2017), EGFR CAF (Wang et al., 2013), TIMP-1 (Choi et al., 2014),N-cadherin (Cho et al., 2015), β-catenin (Cho et al., 2015), vimentin (Cho et al., 2015).

2.3.5.1 Bcl/Bax

From Fig. 2.6, the Bcl-2 (B-cell lymphoma 2) and its associated family of regulating proteins (Brady and Gil-Gomez, 1998, Bagci et al., 2006, Zhang et al., 2019, Gao et al., 2019) were the most frequently studied. They related to the intrinsic pathway of mitochondria apoptotic activation mechanism. They played significant role in the lymphoma cell growth cycle. Related proteins in this apoptotic mechanism such as Apaf-1, caspases, and cytochrome c were also studied together in many of the articles concerned.

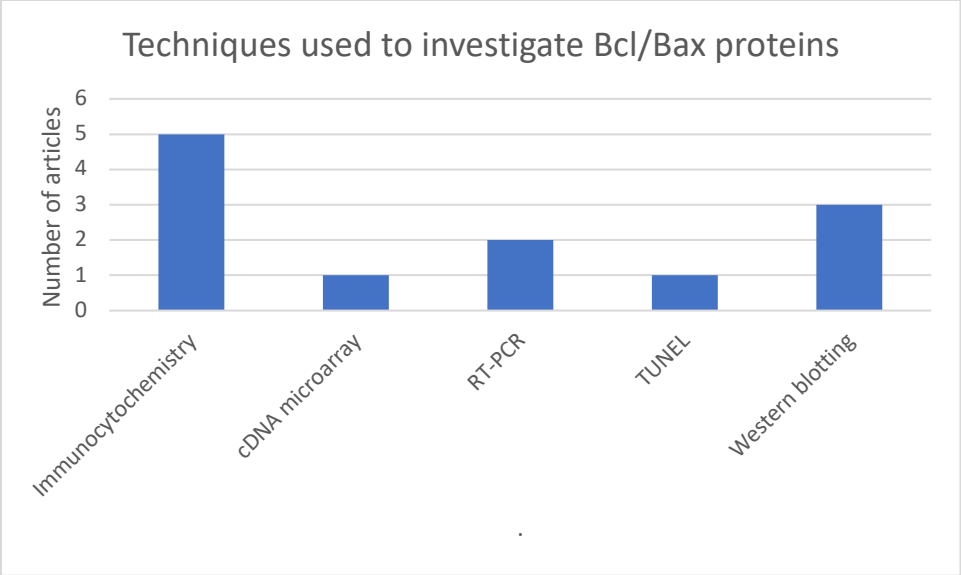


Fig. 2.7: Techniques used to investigate Bcl/Bax proteins

From Fig. 2.7, the most favored methods used were immunocytochemistry methods and Western blotting. All these articles indicated that the treatment of cancer cells by PV herb (or its constituent chemical compounds) did decrease the expression level of the oncogenic Bcl-2 but increase its tumor suppressive counterpart Bax. However, in all the articles surveyed, they did not show the numerical data of their experiments even though their experiments were done quantitatively. The closest to this was, for example, to give a p-value ($p<0.05$) to show that the differential Bcl2/Bax expressions were statistically significant (Yin et al., 2017).

Another example was an article on the effects of the extracts of endophytic fungus found on PV on gastric cancer (Tan et al., 2015). These authors stated that a dosage of 100 mg/kg/day of the extract was effective to decrease Bcl2 and increase Bax levels.

Yet another example (Fu et al., 2012) compared the effects of the treatment using PV herb on B lymphoma Raji cells and on T lymphoma Jurkat cells. With the same dosage, PV extract was more effective on suppressing Raji cell than on suppressing Jurkat cell development ($p<0.05$).

Another article (Gao et al., 2019) which looked at the Bcl2 expression showed that the use of PV extract had effects on the PI3K/AKT signaling pathway. The researchers showed that the expressions of p-PI3K and p-AKT were inhibited but the expressions of PI3K and AKT were unaffected.

2.3.5.2 Caspases-3,9

Caspases are a family of proteases which act to induce programmed cell death (McIlwain et al., 2013, Shalini et al., 2015). The targeted proteins by caspases are attacked and cleaved, and subsequently, this event leads to cell death with abnormal cancerous growth. In many cases, inflammatory responses would be activated. Caspases are classified into different types, among which caspase-3 acts as executioner, and caspase-9 acts as initiator of apoptosis. The chain reaction of caspases in the process of cell death is complex and involves many different proteins, enzymes, and cytokines.

The methods which studied the caspases were similar to those applied to study Bcl-2. However, many research projects used more than one method. For example, fluorometric/colorimetric assay was used in a study by Woo (Woo et al., 2011). Another project used a cDNA microarray, real-time qPCR and immunohistochemical methods to investigate the anti-tumorous effect of PV (as a component in a composite herbal formula) on gastric adenocarcinoma SGC-7901 cells (Zhao et al., 2008).

Among all the articles which studied caspase expressions, they found the expression levels of caspases (including caspases -3,9 and 12) were elevated by the treatment of PV. They pointed out what genomic pathways were involved in the apoptosis process. In a 2017 paper by Yang's team (Yang et al., 2017b), they showed that other than increasing the caspase-3 and -9 levels, hyperosides in PV increased the release of cytochrome c from the mitochondria into the cytosol; this indicated the mitochondrion-mediated apoptosis pathway. This result was also observed in another 2011 article (Zheng et al., 2011).

Another article (Zhang and Wang, 2009) which explored the synergistic effects when PV extract was used together with anti-tumoral drugs paclitaxel and Adriamycin. It showed a dosage of PV extract of 30 μg/mL enhanced the expression of caspase-3.

2.3.5.3 NF-κB

The nuclear factor NF-κB is a transcription factor which controls the production of cytokines (Gilmore, 2006, Brasier, 2006) and is an important participant in the regulation of the inflammatory responses to various kinds of stimuli such as infection or contact with harmful chemicals. Abnormal function or expression of NF-κB is often involved in cancer development and inflammatory diseases. Thus, the monitor of its differential expressions is a useful technique to study the anti-tumorous effect of drugs. TNF- α, the interleukins, and its inhibitors, IκBs which are related entities with the activation of NF-κB are often examined together in research projects.

Like the study of Bcl-2/Bax, immunocytochemical methods and Western blotting remained the most popular methods chosen to study NF-κB. For example, the strategy adopted by Choi et al. (2010) was: the pre-treatment of the cancer cell culture with NF-κB inhibitor (or not), followed by the observation of the downstream expression and activation of MMP-9 after the treatment with the PV herb, which allowed the effect of the PV herb treatment on NF-κB to be indirectly deduced. Another example of the techniques to study the binding of NF-κB to DNA is the electrophoretic mobility shift assay (Wu et al., 2012).

NF-κB is an upstream component of the expressions of many cytokines relating to inflammation and metastasis. So, the studies with NF-κB often included the study of these cytokines: MMP-9 (Choi and Jeong, 2009, Choi et al., 2010, Su et al., 2016), vimentin, N-cadherin, β-catenin (Cho et al., 2015). These studies traced out the molecular signaling pathways clearly.

2.3.5.4 MAPK, ERK, and JNK

MAPK, ERK and JNK belong to the same family of kinases (Orton et al., 2005, Ip and Davis, 1998). They were given different names due to historic reasons. They relate to the Ras-Raf-MEK-ERK genomic pathway and respond to intercellular or external stimulations such as cytokines, UV radiation, osmotic stress and heat stress. They may invoke cellular responses such as proliferation, differentiation, cell cycle progression, mitosis and meiosis, cell survival and apoptosis. Any dysfunctions of these cellular functions may cause the development of cancer. The molecular signaling pathways relating to these kinases can be deduced by examining the multiple proteins: mitochondrial cytochrome c, caspases-3,9 (Yang et al., 2017b), MMP-9, NF-B (Choi and Jeong, 2009, Choi et al., 2010, Su et al., 2016). These articles examined the effect of PV extracts on these entities.

2.3.5.5 MMP-2,9

The metalloproteinases (MMPs) are the cell surface proteins which are involved in the metastasis of cancer cells (Verma and Hansch, 2007, Hessing et al., 2003). Thus, they are frequently examined to explore the anti-tumoral property of drugs when metastasis is concerned. MMPs are proteases responsible for the degradation of extracellular matrix proteins in the process of epithelial-to-mesenchymal transition. Other related cell surface proteins, such as TIMPs, cadherins, catenins, vimentin, were also targets of investigation of metastasis. There were examples which studied on MMP-9 (Choi and Jeong, 2009, Choi et al., 2010, Su et al., 2016) and on MMP-2 (Kim et al., 2012).

2.3.5.6 Growth factors bFGF, VEGF, and IL-8

Growth factors are signaling proteins, cytokines, or hormones among cells (AAronson, 1991, Karkkainen and Petrova, 2000, Florkiewicz et al., 1991). They initiate different cellular processes, after activating cell surface receptors. These cellular processes include cellular growth, proliferation, and tissue remodeling. FGF1 and FGF2 (fibroblast growth factors 1 and 2) function to stimulate endothelial cell organization to form tubular

structure. These studies shed light on angiogenesis, which is an important process in cancer cell development. FGF2 (aka bFGF) helps in human carcinoma-associated fibroblast (CAF) proliferation and migration and protects CAFs from apoptosis and decreases the number of cells in the G_0 phase of cell cycle (Hao et al., 2016). Hao et al. (2016) showed that PV polysaccharides inhibited bFGF expressions. Another article (Xiong-Zhi et al., 2011) showed that sulphated PV polysaccharides competitively bound to the cell binding domain of bFGF, so that PV polysaccharides significantly inhibited the cancer cell proliferation, reduced migration, and increased apoptosis. An anti-tumoral herbal formula which had PV as a component was shown to suppress the expression of EGFR (Wang et al., 2013). There were other articles which studied the growth factors related to the anti-angiogenic effect of PV (Lin et al., 2011, Tan et al., 2015, Wang et al., 2014a).

Other than polysaccharides in PV, studies also showed other chemical constituents in PV, especially rosmarinic acid, suppressed the expression levels of angiogenic growth factor IL-8 (Wang et al., 2014a, Xu et al., 2010a).

2.3.6 Results pertaining to the secondary aim: A Look at the functional tests

2.3.6.1 The use of colorimetric methods

By monitoring the colorimetric (light reflecting) and fluorometric (light emitting) changes of the reagents used, the functional change due to the treatment of drugs on cancer cells can be indicated.

For a long time, the use of the tetrazolium-based salt MTT and its family have been the most popular choice to probe the cell viability. The change of color of the salt monitors the metabolism of the cells. At the same time, to study the anti-tumoral effect of PV, the most frequently chosen cell function (phenomenon) to observe was apoptosis/cell viability. What follows are proliferation, which is closely related to apoptosis/cell viability, and migration. (See Fig. 2.4)

There are three major groups of assays which study cell viability: One is to explore the cell metabolic activity, and the other two looks at the cell membrane permeability and the mitochondrial membrane potential. The use of some dyes to observe these cell functions is the usual tactic, and this is the basis of colorimetric or fluorometric assays. Other than monitoring the cell viability, the use of colorimetric or fluorometric assays can be used to observe other cell functional changes due to the application of drugs; in this study, it is the application of PV herbal treatment.

2.3.6.2 Assays which monitor the cell metabolic activity

When a cell is alive, it undergoes metabolism. So, monitoring metabolic activity shows cell viability. The most frequently used assays are a family of tetrazolium-based salts. Among these, the MTT assay is the most popular. They monitor the NADPH-NADP redox metabolic reactions of living cells. In this process, the MTT dye is reduced to formazan and the color will change from yellow to purple (Mossmann, 1983, Stockert et al., 2018). Other members of this tetrazolium-based family include MTS, XTT and WSTs. However, these assays are often used together with other techniques. For example, Annexin V FITC/PI is often used in conjunction with flow cytometry.

2.3.6.3 Assays which monitor cell membrane permeability

When cells are in ill-health and in the process of death, the cell membrane becomes more permeable. Thus, measuring the leakage of the cell membrane is a way to measure cell viability. An example is the LDH leakage assay (U.S. National Library of Medicine, 2018). LDH (leakage dehydrogenase) is a common enzyme found in cells; thus, its leakage across the cell membrane and its optical observation are indications of the cell membrane permeability.

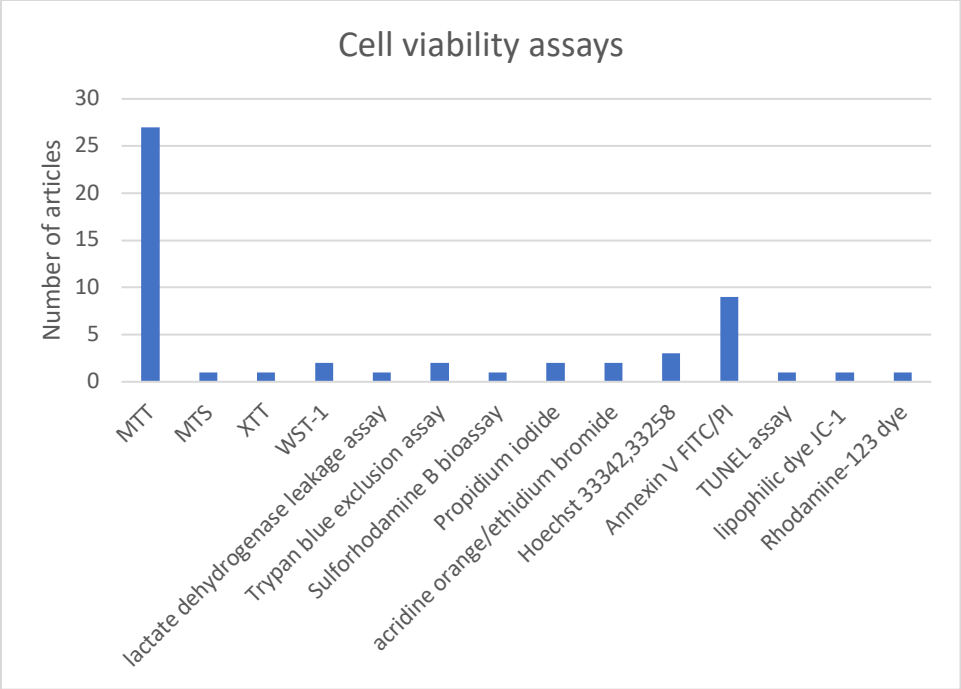


Fig. 2.8: Different assays which test cell viability

The four bars on the left are tetrazolium-based. The six bars in the middle pertain to the group which detects the leakage of cell membrane. The rightmost two bars represent assays which monitor the change in mitochondrial membrane potential.

The second most popular assay, as seen from Fig. 2.8, is Annexin V FITC (Fluorescein isothiocyanate)/PI (Propidium iodide). This assay combines the use of propidium iodide, a DNA staining dye, with annexin V, which is fluorescently labelled by FITC, which binds to phosphatidylserine, a cell surface apoptosis marker. This combination will measure cell apoptosis and necrosis (Vermes et al., 1995). Flow cytometry is often used in combination with Annexin V FITC/PI to monitor apoptosis at early stage of cell cycles.

2.3.6.4 Other functional assays

There are other functional assays which were not colorimetric. They observed cellular phenomena like proliferation, migration, morphological changes, and colony formation

directly using a microscope. However, advanced techniques like flow cytometry are also more frequently used nowadays. The assays in this category are depicted in Fig. 2.9.

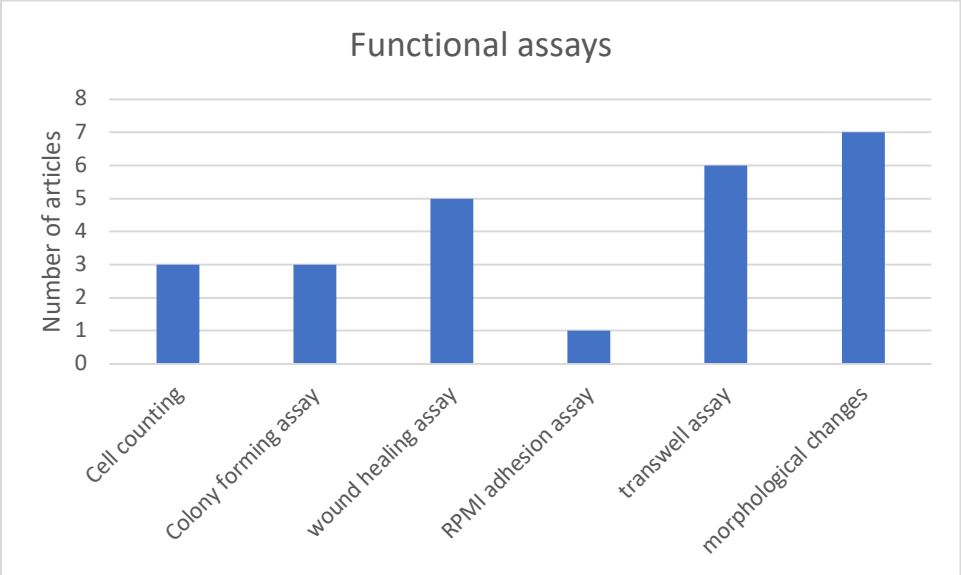


Fig. 2.9: Functional assays which used microscopic observations

2.3.6.5 Assays which observe the anti-oxidative activity

In the micro-environment neighboring cancer cells, the presence of ROS plays an important role in the development, proliferation, and growth of cancer cells. The anti-oxidative activity of drugs contributes to the anti-tumoral effect. Thus, anti-oxidative activity of a drug is often explored by researchers. There are many test methods which can fulfil this purpose, as depicted in Fig. 2.10.

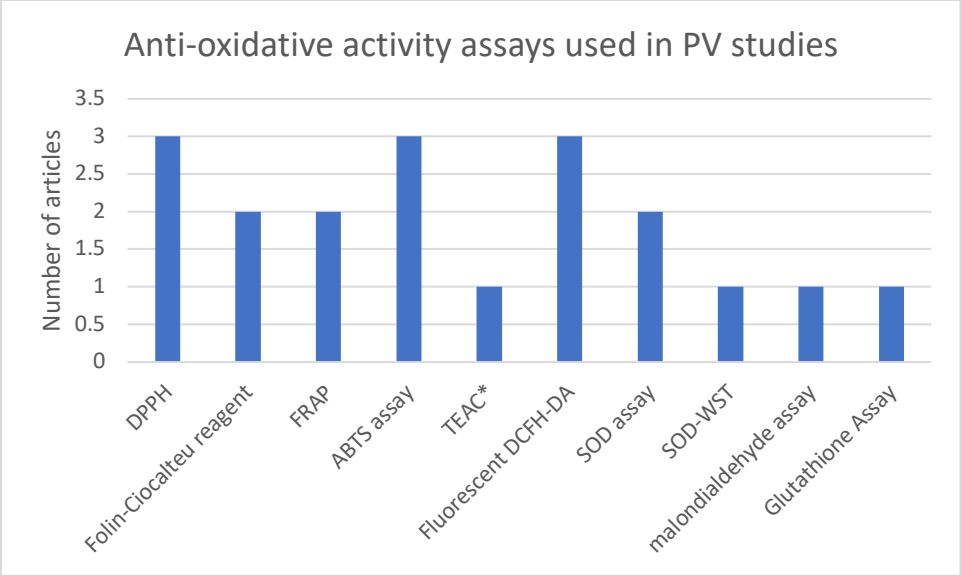


Fig. 2.10: The anti-oxidative activity assays

*: TEAC is a collective name, referring to an assay which may use the DPPH, FRAP, or ABTS reagents but uses Trolox as a standard for comparison.

The six bars on the left in Fig. 2.10 refer to assays which utilize reagents that is a mixture of several chemicals together to act as oxidizing agents or radical traps, to measure the reducing power of the sample drugs under test for their anti-oxidative capabilities. SOD stands for superoxide dismutase, which is an enzyme catalyzing the dismutation of superoxide radicals. Thus, the monitor of SOD activity indicates the oxidative stress of cells. Malondialdehyde is a naturally occurring marker of oxidative stress. Glutathione is another antioxidant naturally found in cells; thus, its measurement also indicates the anti-oxidative state.

2.4 Discussion

2.4.1 Variations which make summarization of results difficult

Examination on the issue of anti-tumorous capability of PV formed the theme of this study. The research efforts in the past three decades were reviewed. All the articles

surveyed showed that PV was effective against cancer in a dosage dependent manner, except four of them. Three of them showed some key constituents of PV had only marginal cytotoxicity against certain cancer cell lines (Lee et al., 2008, Gu et al., 2007a, Lee et al., 1988). One article showed that PV was ineffective for neuroendocrine tumor (Johnbeck et al., 2012). Rosmarinic acid, oleanolic acid, polysaccharides were the most popular choices of PV constituent compounds to research on for their anti-tumoral activity, and they were shown to be effective in various articles.

However, a collective summary of the findings in all the articles concerned is difficult, as explained below:

2.4.1.1 Different starting materials were tested

The first reason is that, in different research projects, researchers were using different starting materials (See Fig. 2.11). These included: the whole PV spica, the PV root, individual chemical constituents of PV, or herbal formulae which had PV as a component. Some articles did not mention whether they started off with the spica of the plant and did the extraction, or they bought the extracts from outside suppliers. So, they are separated into two groups in the figure as the two leftmost bars. Combining these two groups accounts for nearly half of the articles surveyed. Some products manufactured from PV, such as the PV injection drug (Yao et al., 2006, Zhang et al., 2006) and granules (Maimon et al., 2010), were also used as the starting materials. Among the individual chemical compounds in PV, rosmarinic acid (Xu et al., 2010a) and oleanolic acid (Feng et al., 2011) were the most popularly chosen to be examined. For herbal formulae, examples were Ruanjian Sanjie decoction (Zhao et al., 2017) and Wei Chang An (Zhao et al., 2008). Thus, this wide variation of starting materials means comparison cannot be done on an equal basis. It is also difficult to systematically summarize the pharmacological efficacy of PV herb.

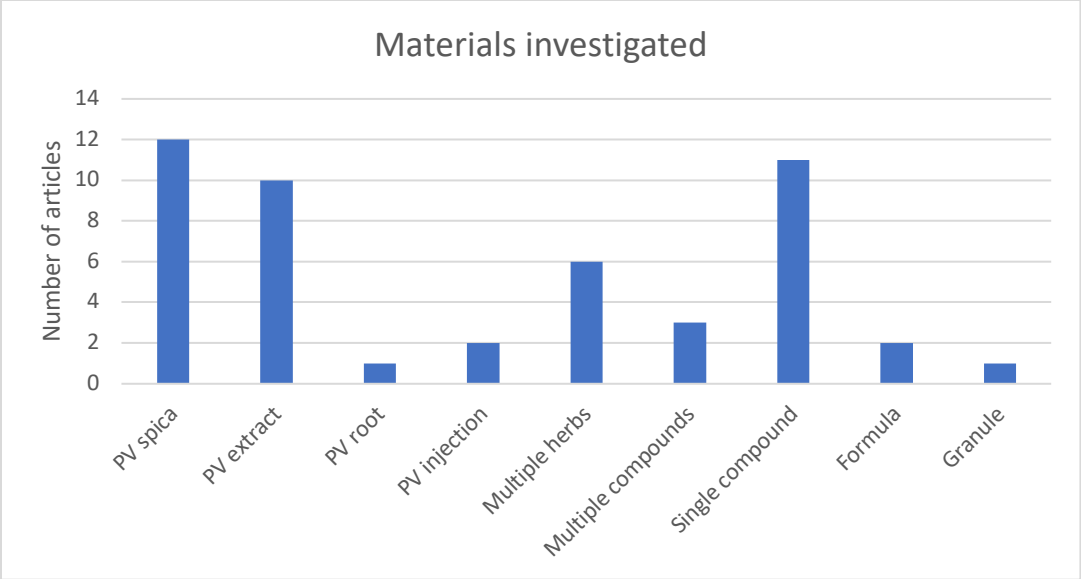


Fig. 2.11: The starting materials used in the investigations

In Fig. 2.11, About half of the articles surveyed used the PV raw herb as the starting material. “Multiple herbs” at the fifth bar means that several herbs (including PV as one of them) were used in the studies to compare their anti-tumoral activities.

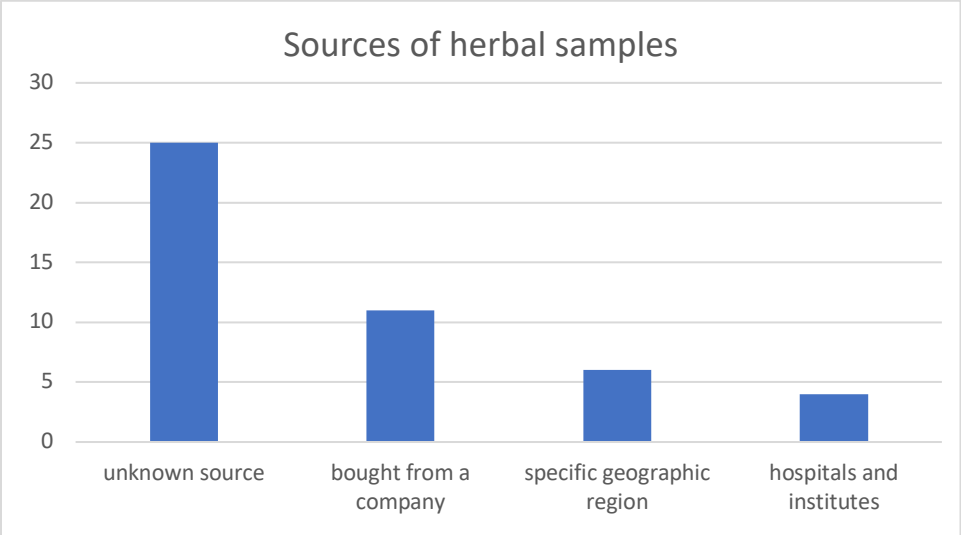


Fig. 2.12: The sources of herbal samples

2.4.1.2 No standard dosage

Even for the half of the articles which used the raw herb PV spica as test material, they did not use a standard dosage. Moreover, they sourced their herbal samples from different sources; some from herbal dispensaries, some from their affiliated institutes, like hospitals. Non-standard sources made it difficult to fix a standard dosage. Fig. 2.12 uncovers a bigger problem in that one-third of the articles did not even mention where they obtained the herbal samples.

2.4.1.3 Lack of information on the geographical sources of PV herb

Also from Fig. 2.12, only 13% of the articles surveyed stated the geographical regions where their samples were sourced. According to some studies (Cho et al., 2015), they showed that PV plants grown in different geographical regions had different profiles of chemical compositions. For example, Yang et al. (2013) showed the chemical compositions of PV from five different producing regions in China were all different. So, the pharmacological effects of the plant differed too. The results from different studies could not be compared against each other if they did not specify the geographic origin. This should at least be indicated in the articles, but most researchers did not. In addition, there were articles (Feng et al., 2010a, Yi et al., 2017), which showed that the variations in water treatment and the harvest time also caused changes in the bioactive components of the plant. This raised the question of how to control the standard quality of the herb used for medical use.

2.4.1.4 No standard extraction and preparation methods

Using PV spica is the most popular choice of the parts of the plant for investigation. However, even among these studies using the same starting material, the extraction methods were different (See Fig. 2.13). The traditional way is to boil the herb in water to obtain a decoction (Cho et al., 2015). The aqueous solution can then be subjected to vacuum evaporation of lyophilization to remove the water content to get a dried

extract. Other researchers might use other solvents. The popular choices included methanol (Ahn et al., 2003) and ethanol (Feng et al., 2010a, Yi et al., 2017), whereas Soxhlet extraction was frequently applied. Even when the same solvent was used, different projects used solvent of different concentrations; for example, some used 70% ethanol, but some used 95% ethanol. Many articles did not even mention the extraction methods at all. As the polarity of the solvents are different, the chemical constituents extracted are different. Thus, the anti-tumoral capabilities of the extracted materials ought to be different also, making the comparison among results from these articles hard to do.

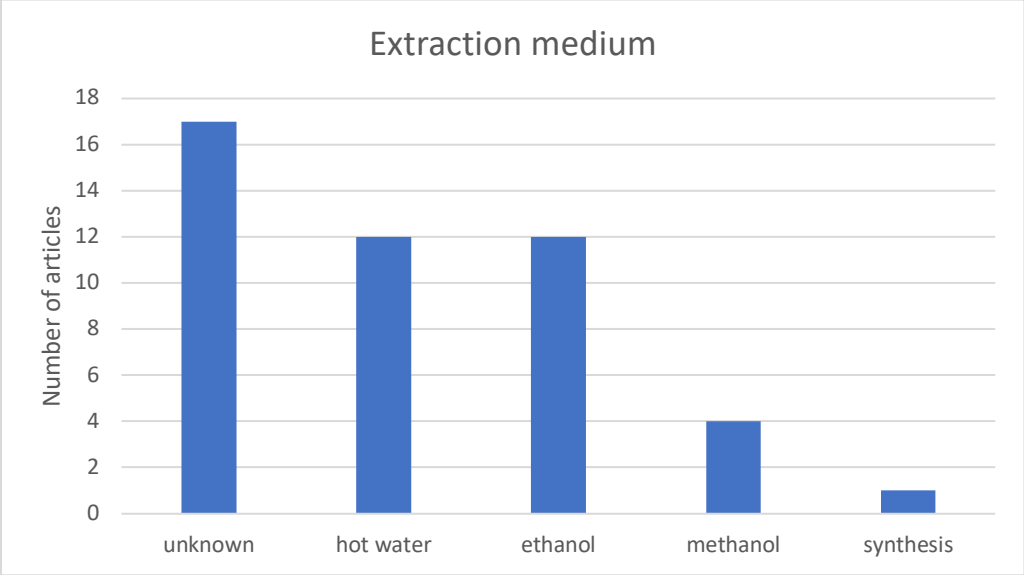


Fig. 2.13: Different extraction media used

2.4.1.5 That different types of tumors were studied made comparison difficult

Another difficulty is that different projects investigated different types of tumors. Thus, comparisons of the anti-tumorous effectiveness of PV herb to cause apoptosis of different types of cancer cells are like comparing an apple with an orange.

Some projects explored the interactive or synergistic effect of using PV herb with Western drugs, like paclitaxel, Adriamycin (Zhang and Wang, 2009) and 5-fluorouracil (Zhao et al., 2008). It is impractical to compare their results with those projects which looked at the effect of using PV alone. As one investigation showed, the inhibitory effect on lymphoma cell proliferation of using PV extract at a dosage of 0.8 μg/mL together with taclitaxel was significantly better than using taclitaxel alone. When PV extract at a dosage of 0.08 μg/mL was used with Adriamycin, improvement of the inhibitory effect was comparably better than the effect of using the Western drug alone. The difference here was ten-fold.

Additionally, one more difficulty in collectively summarizing all the results from these articles is that they did not have a standard way to report their findings. For in-vivo experiments, some researchers chose to report their results in terms of the animal body weight or the tumor weight (Yan et al., 2018) ; the spleen index or the thymus index (Feng et al., 2010b). Some chose to report the animal survival time (Yao et al., 2006). For in vitro experiments, some quoted the percentage improvement when compared with the control. For example, an article by Zhao et al. (2008) showed that the tumor inhibitory rate was improved by 44.32% when the case of using PV herb was compared with the control. The apoptosis index was 9.72% when PV herb was used, whereas it was 2.45% for the control. Another frequently chosen measure was the half maximum inhibitory concentration, IC_{50}. IC_{50} for proliferation was used in some articles (Chen et al., 2009a, Maimon et al., 2010, Zhang et al., 2006). Some used the IC_{50} for apoptosis (Woo et al., 2011, Maimon et al., 2010, Lee et al., 2008). Even more disturbing for the purpose of comparison, some used the unit of molar concentration, but some used weight per unit volume. Table 2.1 showed the closest approach to a direct comparison of the IC_{50} (proliferation) results of six previous studies. In these studies, they chose the same unit, weight per unit volume, as the measure for IC_{50}.

Table 2.1: A comparison of the IC_{50} values for cancer proliferation

articles	(Zhang et al., 2006)	(Chen et al., 2009a)	(Zhang and Wang, 2009)	(Maimon et al., 2010)	(Gao et al., 2019)	(Zhang et al., 2019)
Materials studied	PV injection	PV extract	PV extract	Formula LCS101	Methanol root extract of PV	PV extract
Types of Cancer	Lymphoma (Raji cells)	Lymphoma (Jurkat cells)	Lymphoma (Raji cells)	Breast adenocarcinoma	Breast cancer (MCF-5 cells)	Thyroid cancer (B-CPAP cells)
IC_{50} (proliferation)	0.118 mg/mL	20.23 µg/mL	0.8 µg/mL (used with paclitaxel), 0.08 µg/mL (used with adriamycin)	10 mg/mL	25 µg/mL	1.53 mg/mL

From Table 2.1, the IC_{50} values (proliferation), as quoted from six different articles, varied widely. The materials used for the studies varied from PV extract from the spica or the root, PV injection, to herbal formula which consisted of PV. Note from the data in column 3, the results from the team of Zhang and Wang (2009) showed that when PV extract was used in combination with different Western drugs (paclitaxel and Adriamycin), the IC_{50} values were ten-fold different. Indeed, since the materials being tested were different, or the types of cancer studied were different, the comparisons of results here from different research projects are difficult, and even are irrelevant.

2.4.2 Difficulties in research in Chinese herbal medicine

This review looked at the research efforts in the past three decades, using the in vitro methods to investigate the anti-tumoral effect of the PV herb. By reviewing these articles, the limitations of research on the medicinal use of the Chinese herbs were

revealed. These limitations were often used to attack Chinese medicine as "unscientific". In Western medicine, drug is often a certain chemical compound, or a combination of several compounds. Scientific methodologies derived to study the efficacy of the use of a drug to treat diseases can be easily well-established: Giving the chemical formula of a drug uniquely defines the drug. However, the application of these methodologies on the research of Chinese herbal medicine is not straightforward. For example, a certain herb, say PV, consists of hundreds of different chemical constituents. Different extraction methods extract different sets of these chemical constituents; solvents of different polarities extract chemical compounds of different polarities (Golembiovska et al., 2014, Yang et al., 2013, Morteza-Semnani et al., 2006). Therefore, it is not easy to standardize the starting materials being researched. Adding more complications is that the traditional usage of Chinese medicine involves "formulae" which are composed of several, and sometimes even up to twenty (or more) different herbs to make use of their synergistic effects. Also considering that different dosages of individual herbs are used, the combinations of different starting materials are formidably huge. Adding to the complexity, as different articles pointed out, for example, (Yang et al., 2013), PV plants grown in different geographic regions had different chemical compositions. Moreover, the chemical compositions of herbs also depend on factors such as the drought condition (Phillips et al., 2018), variations in UV-B radiation (Chen et al., 2018b), altitude of the producing area (Kuriya et al., 2015) and others. These factors, when they are all added up, imply wide diversities. These variations pose great difficulties in the identification of the herb. All these considerations together cause standardization of the experiments to enable repeatability immensely difficult.

PV plants grown in different geographical regions have different compositions. Therefore, for research projects, the geographical origins of source materials need to be spelt out; otherwise, this vagueness makes the research unrepeatable. However, sometimes specifying the origins may not be possible, because most suppliers of herbs mix the products which they source from different growing areas.

2.4.3 Types of cancer researched

By categorizing the previous research articles according to what kinds of cancer they spent their efforts, the observation was that researchers believed PV was most effective in treating breast cancer, lymphoma, leukemia, and colon cancer. However, from the wide range of IC_{50} values (Table 2.1) obtained because very different experimental designs were adopted, even for the same cancer type or for the same extraction method, it is not possible to quantify how effective PV is to treat cancer, what dosage is required, or which extraction method is preferable.

2.4.4 Molecular mechanisms

The molecular mechanisms by which PV is effective to be anti-tumoral noted in Fig. 2.6 showed that Bcl/Bax and caspases were the most popular choices researchers spent their efforts on. This indicated that the intrinsic pathway of mitochondria-related apoptotic activation was believed to be the main molecular mechanism by which PV worked (Zhang et al., 2019, Gao et al., 2019, Yin et al., 2017, Yi et al., 2017, Tan et al., 2015, Fu et al., 2012, Zheng et al., 2011, Woo et al., 2011, Feng et al., 2011, Chen et al., 2009a, Zhao et al., 2008, Zhang et al., 2006).

Other molecular mechanisms which were identified were:

- Lck-dependent Ca^{2+} signaling pathway. Its downstream effectors modulate the IL-2 gene expression, which is related to T-cell activation and inflammatory responses (Ahn et al., 2003)
- MRK/ERK/JNK signaling pathway. This involves the inhibition of NF-κB and MMP activities, and is thus related to metastasis (Choi et al., 2010, Xu et al., 2010b, Xu et al., 2010a, Cho et al., 2015, Su et al., 2016, Yang et al., 2017b)
- PI3K/AKT signaling pathway (Gao et al., 2019).

However, these molecular signaling pathways identified are by no way a complete list. There has not been a dedicated research effort to exhaust all possible molecular mechanisms. Researchers just chose a particular pathway to do their investigations on and did not consider if there might be other pathways involved. This drawback can be remedied by more recent research technologies such as the use of the NGS sequencing technique.

2.5 Conclusion

This review looked at the research efforts in the past three decades on the anti-tumoral effect of the herb, PV; in particular, where the in vitro methods were adopted. The review aimed to identify the molecular mechanisms which the researchers found to be accountable for the anti-tumoral activity of PV by looking at the differential expressions of various proteomic entities. The finding was that researchers concentrated on just a few popular molecular pathways. This implies that there may be some undiscovered molecular pathways yet to be found.

Then, another aim was to look at the functional assays which the researchers used. Researchers studied the cell viability, and other cellular functions such as propagation and tumor development processes such as angiogenesis and metastasis. This review provided a background knowledge for the experimental part of the whole project and helped to choose the appropriate experimental method for this project.

Then, the Discussion section collected and summarized on the results found in those articles about the efficacy of PV herb for its anti-tumoral effect and explained the difficulties in such summarization.

From this chapter, none of the articles found in this literature survey ever touched on the anti-tumoral activities of the VOCs of PV. This is the novel element of the experimental part of this project, which is elaborated in Chapters 4 & 5.

Chapter 3: Review of Research on the Anti-viral Activity of *Prunella vulgaris*

3.1 Introduction

3.1.1 Outline of this chapter

The understanding of the growth and development cycles of viruses enables researchers to adopt specific investigation strategies to study the anti-viral activities of the PV herb. A brief description of the life cycle of virus is given, after a brief description of the herb and a section on the aims and objectives of this chapter.

The article selection process is then explained, and the inclusion and exclusion criteria described.

The results which reported the viruses investigated, the extraction and analysis techniques adopted, and the single or groups of chemical constituents of PV studied follows.

Due to the specific nature of the life cycle of viruses, when researchers sought to elucidate the mechanisms of how drugs exert their anti-viral effects, they study the stages in the life cycle of viruses and when drugs block the development of viruses. Thus, this review categorized the research articles according to the life cycle of viruses where the blockage of infectivity occurred. Categorizations according to other aspects, like the experimental analytic techniques applied, will also be found in the Results section. This chapter will finish by the Discussion and Conclusion sections.

The findings in this chapter were published as a journal article (Mak and Walsh, 2022).

3.1.2 The PV herb

The main theme of this research project was on the study of the anti-tumoral activity of the PV herb. However, the PV herb has more pharmacological effects than anti-tumoral effect alone. In this chapter, the anti-viral effect of the herb was reviewed.

The COVID-19 has plagued the world in the past three years. It is helpful to understand the full potential of the PV herb for it pharmacological use. Thus, this chapter elaborates on the anti-viral activities of PV and helps to explore new research direction other than the anti-tumoral theme selected. Also, a recent report by the World Health Organization (2023) listed viral infection as one of the major causes of cancer, a survey of literature on the anti-viral activities of PV is helpful to understand the link with the anti-tumoral activities of the herb. However, such a link was not found within the research issues of the articles found in the literature. This link was not further investigated in this project.

3.1.3 Aims & objectives

Similar to the aims of the previous chapter review on the anti-tumoral effect, this chapter explores the anti-viral activity mechanisms of PV. To achieve this aim, the objective was to categorize and analyze the articles collected.

The categorization and analyses provide a narrative review of the approaches and focus of researchers demonstrated through a survey of what viruses, chemical constituents of PV, extraction and analytical methods used to conduct their investigations has been reported to date.

The second aim of this review is to reveal what stages in the life cycle of viral development that treatment by PV could effect. This elucidates how the blockage of viral infectivity materializes.

3.2 Brief life cycle of virus

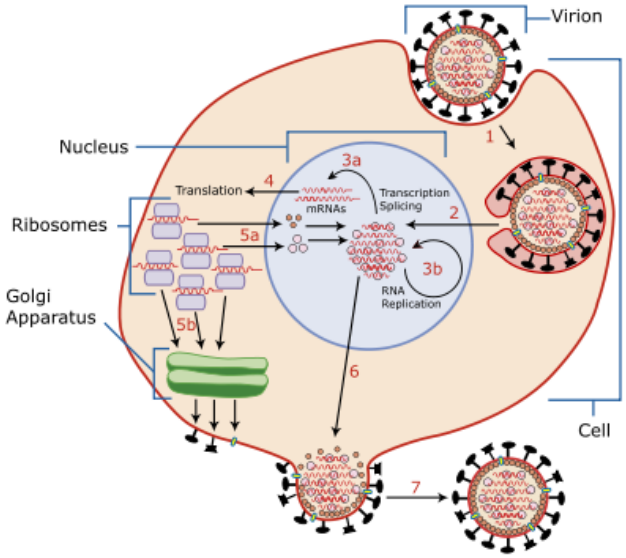


Fig. 3.1: Virus life cycle

(The Permission is granted to copy, distribute and/or modify this image under the terms of the GNU Free Documentation License, Version 1.2 or any later version published by the Free Software Foundation. This file is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported ; title: Virus Life Cycle; author: User: YK Times. Date: 5 March 2007)

Fig. 3.1 shows a picture depicting the life cycle of virus.

Since virus does not show capabilities of self-metabolism and replication, the classification of viruses as a life form continues to be debated (Koonin and Starokadomskyy, 2016). Due to these special characteristics, viruses need to coopt the cellular processes of other life forms to proceed through the stages of its life cycle (Dimmock et al., 2007). It is worthwhile to give a brief description of the life cycle of a virus (Collier et al., 1998):

3.2.1 Attachment

The attack of cells by viruses starts with the attachment of the virus particles to cells. This happens when the surface proteins of a virus join the surface receptors of the host cells. For instance, in the case of SARS-CoV-2 virus, the S proteins of the virus have

specific affinity to the ACE2 entry receptors of the host cells and attach to them. Therefore, this binding is specific and determines host cell tropism.

3.2.2 Viral entry

Moreover, a cellular protease is needed to facilitate the viral entry protein priming. In the case of SARS-CoV-2, the cellular protease is the serine protease TMPRSS2, which performs the S protein priming (Hoffmann et al., 2020) before the membrane fusion between the viral envelope and the host cellular membrane. By endocytosis, the foreign virions are absorbed into the host cells.

3.2.3 Release of viral genomic materials inside the host cells

After entry into the host cells, either viral or host enzymes degrade the viral capsid. Then, the viral genomic materials are released into the nucleus of the host cells.

3.2.4 Replication

There are different types of viruses: double-stranded or single stranded DNA or RNA viruses, whereas RNA viruses can be further classified as positive or negative stranded. Thus, the replication processes vary among these different classes. For retroviruses, such as human immunodeficiency viruses, HIV-1 and HIV-2, a reverse transcription step is needed to translate its RNA genome into the corresponding DNA segment with the help of its reverse transcriptase, before the DNA segment can be inserted into the DNA of the host cell (Freed, 2015). Then, the machinery of the host cell is used to produce a new generation of virions.

3.2.5 Assembly

After new virions are produced, they organize themselves through self-assembly to have complete structures, during which some modifications of the viral protein structures often occur.

3.2.6 Release from the host cells

After the new viruses mature, the final stage is the lysis of the host cell. The cell membrane of the host bursts and the new virions are released. The host cells may be killed, or in the case when the host cell survives, the viral genome may be incorporated into that of the host's chromosome. Then, the viral genome gets replicated when the host cell divides. The viral disease becomes chronic. Herpes zoster is an example (Gilden et al., 2003).

3.3 Methods

3.3.1 Search strategies

As noted in the previous chapter on the review of the anti-tumoral effects of PV, a search for the relevant articles to be included in this review was undertaken using Embase, Medline and Pubmed databases. The keywords used as search terms were very broad, trying to include all the articles which investigated the use of PV for its anti-viral activities. The main keyword used in the search was "*Prunella vulgaris*", together with specific keywords to confine the study to virus by using "viral", "virus", "HIV", "HSV" or "herpes simplex". The latter three keywords were used because HIV and HSV were the most frequently researched viruses in pre-reading. Then, these viruses were so frequently studied that many articles which investigated them used them directly to name the viruses without the use of the general word "virus" in their titles or abstracts of the articles. The "virus" and "viral" keywords were broad terms used to identify articles covering other viruses, and other articles which missed through the specific virus

keywords. There was no limit on the date of publication of articles to include all articles concerned. Then, the screening steps included the removal of duplicated articles from the different databases. Review papers and articles discussing phytochemistry only were also screened out. This whole process is depicted in Fig. 3.2.

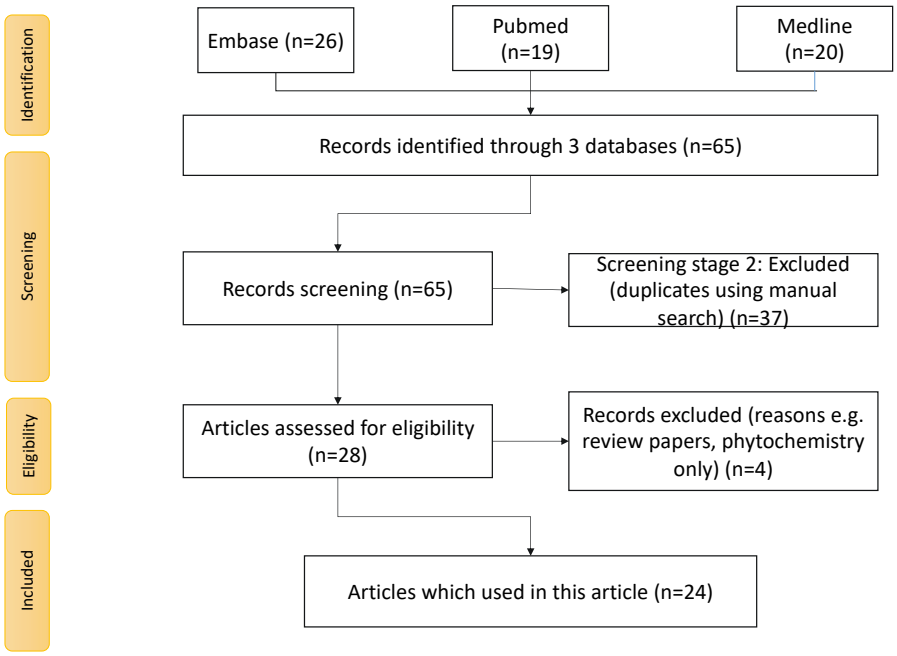


Fig. 3.2: PRISMA flowchart of the literature survey to get a collection of articles on the anti-viral effects of PV

3.3.2 Categorization

The set of 24 articles so collected was then categorized according to the publication dates of the articles, the viruses they investigated, the extraction methods they used, the chemical compounds investigated, the experimental techniques applied to analyze, the stages of the viral cycle targeting, and the use of other functional assays. The results are elaborated in Sec. 3.4.

3.4 Literature review findings

The collected group of 24 articles shows that the research on the anti-viral effects of PV dated back to about three decades ago. The earliest article appeared in 1989 (Tabba et al., 1989). The frequency of articles published during this period was displayed in chronological order in Fig. 3.3. A summary of articles included in this review can be found in the Appendix 3.

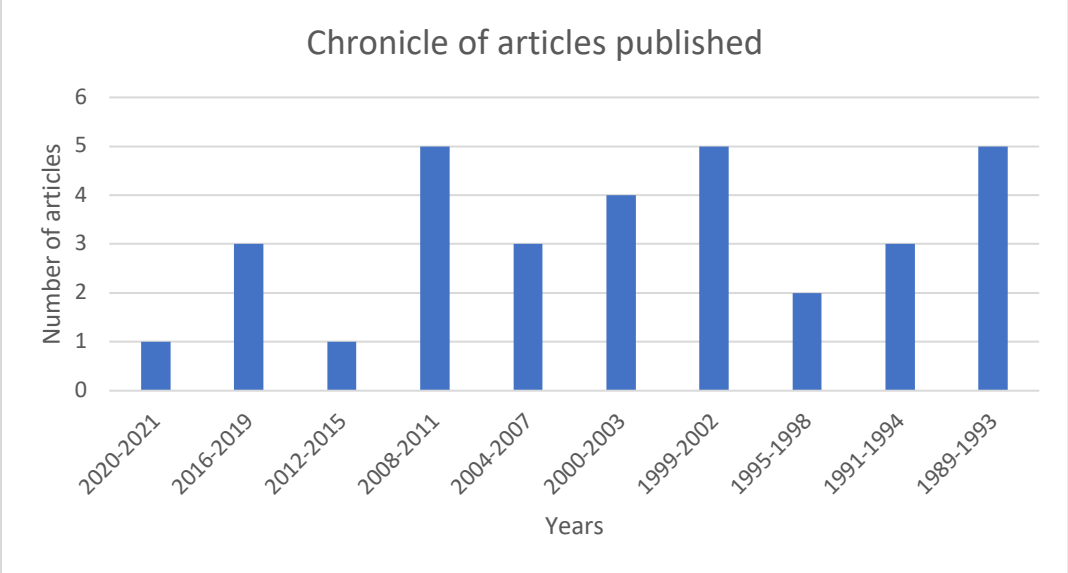


Fig. 3.3: Number of articles appeared in the recent three decades (anti-viral effects)

The number of articles found in the past about thirty years by this survey, which investigated on the anti-viral efficacies of the PV herb. They used different methodologies in their investigation.

3.4.1 Articles screening multiple herbs

Many research articles did not concentrate on the single PV herb, but on multiple herbs to compare their anti-viral efficacies (Li et al., 2019, Yang et al., 2017a, Feng et al., 2012, Tian et al., 2011, Reichling et al., 2008, Nolkemper et al., 2006, Liu et al., 2002, Au et al., 2001, Lam et al., 2000, Yamasaki et al., 1998, Yamasaki et al., 1993, Yao et al., 1992,

Zheng, 1990). More recent articles focused solely on PV or PV extracted compounds alone (Xu et al., 1999, Chiu et al., 2004, Zhang et al., 2007, Brindley et al., 2009, Oh et al., 2011, Zhang et al., 2016, Ao et al., 2021).

3.4.2 Virus investigated

The HIV was the most frequently studied virus with ten out of the total 24 articles; followed by HSV (8 out of 24 articles). These two types of viruses were the focus on research reported to date, because researchers believed in the effectiveness of PV herb in blocking their infectivity. Other viruses which were investigated included influenza (Yang et al., 2017a, Tian et al., 2011), Ebola (Yang et al., 2017a, Zhang et al., 2016), IHNV (infectious hematopoietic necrosis virus) (Li et al., 2019) , equine infectious anemia lentivirus (Brindley et al., 2009), Lassa virus (Yang et al., 2017a), SARS-Covid-2 (Ao et al., 2021). They were reported in one to two studies each, dating from 2009 – 2021. One article (Yang et al., 2017a) covered three viruses: Ebola, Lassa, and avian influenza. See Fig. 3.4.

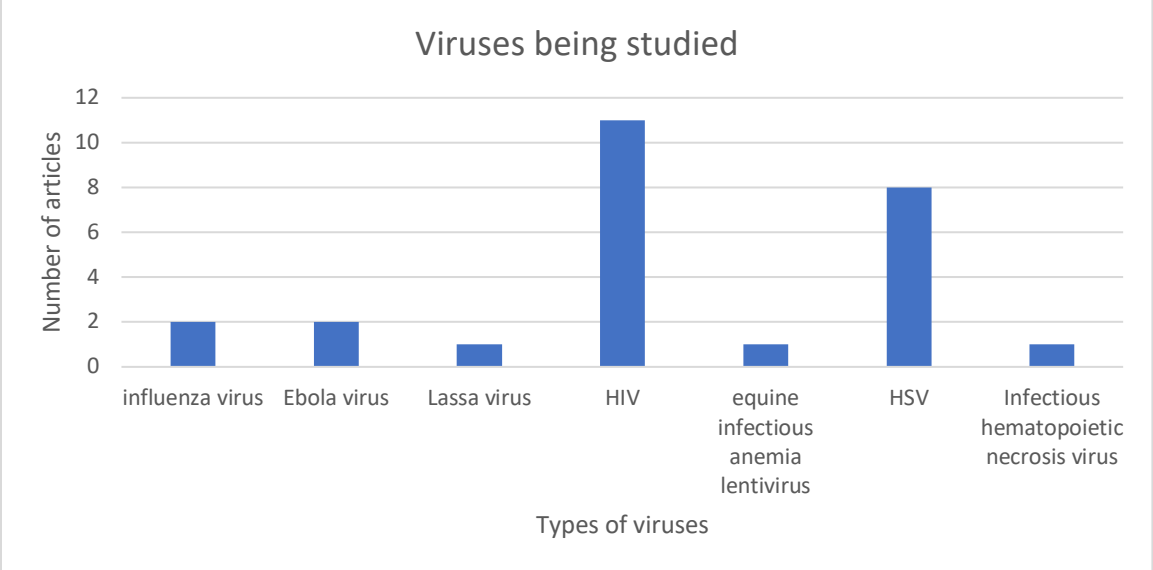


Fig. 3.4: Types of viruses being investigated

For the 24 articles under this survey, they looked at different viruses as shown in this figure. HIV and HSV were two types of viruses which attracted the most attention.

3.4.3 Extraction methods

The most frequently adopted extraction method reported was decoction in water (boiling the herb in hot water to obtain the aqueous extracts) (Brindley et al., 2009, Oh et al., 2011). Ethanol (Reichling et al., 2008, Huang et al., 2009, Zheng, 1990) and methanol (Lam et al., 2000, Au et al., 2001) were the two other frequently chosen solvents. Polysaccharides in PV were sometimes obtained by ethanol precipitation after aqueous extraction was done by boiling in water (Xu et al., 1999, Zhang et al., 2007).

3.4.4 Chemical compounds investigated

Nine articles investigated single chemical compounds from PV (or a group of compounds) and were reported. These individual chemical compounds might be isolated by techniques such as HPLC (Xu et al., 1999). The most frequently investigated compound groups were polysaccharides (Xu et al., 1999, Chiu et al., 2004, Zhang et al., 2007), polyphenols (Au et al., 2001, Liu et al., 2002), essential oils (Reichling and Schnitzler, 2011, Yamasaki et al., 1998), and triterpenoic acids (Li et al., 2019, Ryu et al., 1992). Individual compounds investigated included ursolic acid (Li et al., 2019), and prunellin (Tabba et al., 1989).

3.4.5 Experimental techniques applied

Some popular experimental techniques were adopted in anti-viral research reviewed. Immunochemistry techniques such as the plaque reduction assay (Ryu et al., 1992, Xu et al., 1999, Reichling et al., 2008, Nolkemper et al., 2006, Zhang et al., 2007, Chiu et al., 2004, Reichling and Schnitzler, 2011), flow cytometry (Chiu et al., 2004), and the ELISA techniques (Feng et al., 2012, Au et al., 2001, Kageyama et al., 2000, Zhang et al., 2016) were used. These techniques enable the determination of the virus concentration, and thus, the evaluation of the anti-viral effects upon the herbal treatment.

Strategies specific to viral research included the time-of-addition method; ten articles surveyed (Yao et al., 1992, Xu et al., 1999, Kageyama et al., 2000, Nolkemper et al., 2006, Reichling et al., 2008, Brindley et al., 2009, Oh et al., 2011, Reichling and Schnitzler, 2011, Zhang et al., 2016, Yang et al., 2017a) applied this technique. (See Fig. 3.5.) Pseudotyping (Feng et al., 2012, Zhang et al., 2016, Yang et al., 2017a, Ao et al., 2021) was another commonly applied method in examining the anti-viral effects of herbs.

Measures, such as antigens and virus count, affected cell counts, were also quantified by other conventional experimental techniques such as PCR (Yao et al., 1992), fluorogenic assays (Lam et al., 2000), and luciferase reporter assays (Feng et al., 2012).

Fig. 3.5 summarizes the different techniques employed in the investigations.

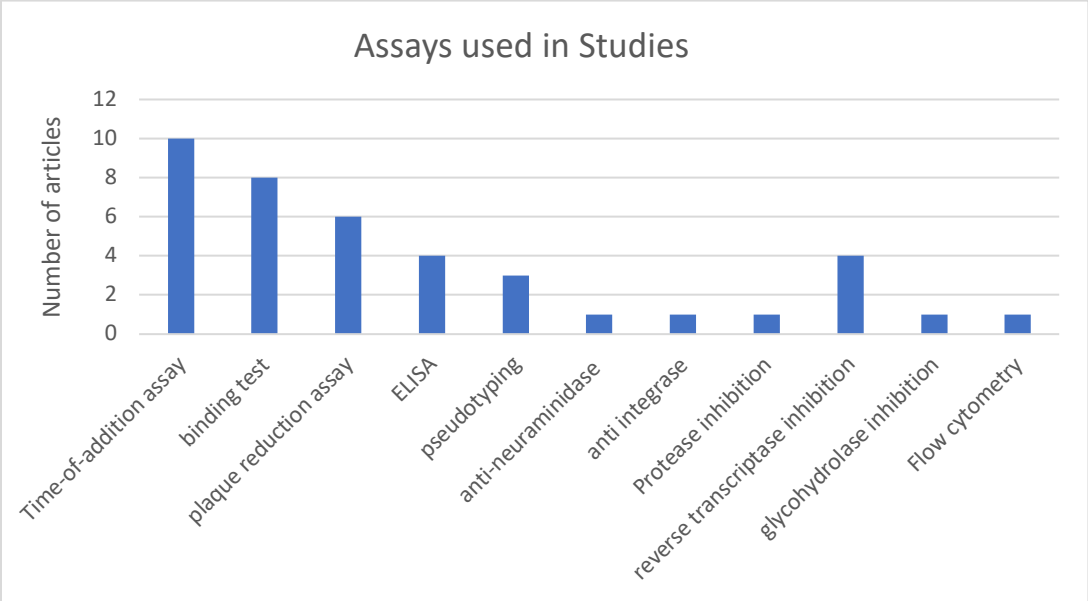


Fig. 3.5: Different techniques employed in the investigation

3.4.6 Categorization of the articles

The anti-viral mechanisms of the drug treatment were usually geared to block viral development and replication at one or more of the viral life cycle stages. This was

reflected in the chosen strategies reported in the literature. So, categorization according to the stages of the viral development cycle targeted was a natural choice.

3.4.6.1 Articles targeting the stages of the viral life cycle

Refer to Fig. 3.1, which is a diagram showing the viral life cycle.

3.4.6.1.1 Attachment and Entry Inhibition

The first step of a viral infection is the attachment of the virions to the host cells. Thus, the prevention of virus entry into the host cells is the main target. The experimental approach to explore this mechanism is to treat the viruses or the target host cells by the herb before they are placed together. This tests whether the characteristics of the surface proteins of the viruses or the host cells alters. The experimental techniques which were applied to explore the attachment and entry inhibition events include the following:

3.4.6.1.1.1 Time-of-addition technique

The stage in the life cycle of a virus that it is blocked from further development can be derived by timing the application of the herbal treatment to the virions or the host cells before they are put together in a medium or after they are mixed to allow infection to occur.

In 2008 and 2011, Reichling et al. (2008, 2011) studied the HSV-1 using treatment with alcoholic extracts of several herbs from the mint family, including PV. They treated cell-free HSV virions and pre-treated uninfected host cells, and they showed that the viral attachment to the host cells was blocked, and the virions inactivated.

Similarly, Oh et. al. (2011) also applied the time-of-addition technique to show that the aqueous extracts of PV blocked the entry of HIV-1 viruses into host cells. They applied

the herbal treatment during the first five hours of treatment to further show that PV extracts also block post-binding events.

Brindley et. al. (2009) studied the equine infectious anemia virus. Using the time-of-addition technique, they showed that the entry of virions into the host cells was forbidden. By pre-treating the host cells with the PV extracts, the infectivity of the viruses can also be reduced. They further demonstrated the synergistic anti-viral effects among the different components in the PV extracts. They used size fractionation to separate the extracts into fractions, and then, applied the treatments using the fractions alone or in combinations.

Nolkemper (2006) conducted a study which surveyed the anti-viral effects of the aqueous extracts on HSV-1 and HSV-2 viruses from six herbs, using a plaque reduction assay on RC-37 cells. They also applied the time-of-addition technique to prove that the PV herb blocked the viral development before the virions attached to the host cells but did not show any effects on the intracellular replication events after entry into the host cells.

Yao et al. (1992) did a similar survey but used four herbs, including PV, on several different cell lines, for HIV virus. When the HIV-1 viruses were pre-incubated with PV extracts, attachment of the virions to the host cells was drastically reduced. Using PCR, they also showed that HIV-1 proviral DNAs were absent in the infected cells. Furthermore, they found that the herb blocked the binding of purified HIV envelope glycoprotein gp120 to the CD4 cell receptors; thus, the viral attachment to the CD4 cells was blocked by the PV herb.

3.4.6.1.1.2 Pseudotyping technique

There were four projects which used the pseudotyping technique (Ao et al., 2021, Yang et al., 2017a, Zhang et al., 2016, Feng et al., 2012). In this technique, artificial viruses were produced by combining viral components with foreign viral envelope proteins. This allows researchers to choose and engineer the expression of the envelope proteins,

sometimes for the purpose of engaging investigation on dangerous viruses but at a reduced risk. There is less danger because the artificial viruses do not possess the genomic materials carrying the information to produce additional envelope proteins, making the original virus replication defective. However, the investigation of the binding between the virions and the host cell receptors can still be carried out.

Yang et al. (2017a) applied the pseudotyping technique to investigate the effectiveness of herbs to inhibit viruses to enter host cells. They studied three separate viruses: Ebola, Lassa and avian influenza. They established that PV acted as entry inhibitor to Ebola virus.

Zhang et al. (2016) also studied Ebola virus. They conducted their study on two recombinant Ebola pseudoviruses; EBOV-GP-V (Ebola glycoprotein pseudotyped HIV-1-based virus) and eGFP-ZEBOV (enhanced green fluorescent protein Zaire Ebola virus), using PV extracts on various cell lines: HUVECs (human umbilical vein endothelial cells), macrophage, VeroE6 cells. As the pseudoviruses were produced by co-transfection with a vector encoding the Gaussian Luciferase gene, the viral infectivity was measured using the luciferase fluorescent property. They also made use of an anti-HIV-1-p24 ELISA assay. Their result showed that the aqueous extracts of PV blocked the attachment event of the GP1 surface protein of the Ebola virus.

Feng et al. (2012) studied the effect of treatment of PV extracts on the CXCR4 and CCR5 receptors of healthy $CD4^+$ cells. They found that the expression levels of these receptors were down regulated. By using ELISA and pseudotyped HIV-luciferase virus to detect the HIV p24 proteins, they showed that PV extracts blocked binding and replication of the virus.

For the SARS-Cov-2 virus which drew a lot of attention in these three years, Ao et al. (2021) also used the pseudotyping technique to demonstrate the inhibitory effects of an aqueous PV extract to interrupt the binding of the viral glycoproteins to the ACE2 receptors of the host cells in a mutant SARS-CoV-2 SP pseudotyped HIV-1-based vector

system. They further demonstrated that the inhibitory effects also appeared for the wild type SARS-CoV-2 virus to infect Vero host cells.

3.4.6.1.2 Replication inhibition

Three enzymes participate in the replication process of viruses; they are integrase, protease, and reverse transcriptase. So, one of the strategies for a drug to achieve anti-viral effect is to inhibit the actions of these enzymes, without which replication cannot proceed.

3.4.6.1.2.1 Integrase inhibition

Integrase is an enzyme which a retrovirus uses to integrate its genomic materials into the genome of the host cell. So, developing drugs which inhibit integrase is a direction to treat diseases caused by retroviruses.

Au et al. (2001) screened both the aqueous and methanolic extracts of 20 herbs for stopping HIV-1 integrase activity. They conducted the screening using a non-radiative ELISA-based integrase assay. They found that the PV extracts demonstrated strong anti-HIV-1 integrase activity. Out of the 20 herbs they surveyed, sixteen aqueous extracts showed at least 60% inhibition of HIV-1 integrase activity and twelve of them even exhibited complete inhibition. They attributed these strong inhibitory effects to tannins or polyphenolics in the extracts. They supported this claim by removing polyphenolics by a column of polyamide resin, and subsequently resulted in the loss of the inhibitory effects.

3.4.6.1.2.2 Protease inhibition

Protease catalyzes the proteolysis which breaks down proteins by cleaving their peptide bonds. This process is used by viruses to form functional units after the host cells help to replicate them. So, inhibiting the activity of protease halts the viral development.

Lam et al. (2000) made use of a procedure which probed and quantified the protease cleavage products. Their procedure included a sequence-specific cleavage of a fluorogenic synthetic peptide substrate, and by HPLC analysis of the cleavage products, they could then quantify the protease enzyme. They used this method to monitor the HIV-1 protease inhibitory activities of the aqueous and methanolic extracts of 31 herbs, including PV. Their results showed that the aqueous extracts of PV exhibited strong protease inhibition.

3.4.6.1.2.3 Reverse transcriptase inhibition

Reverse transcriptase is the enzyme which facilitates the process of reverse transcription for RNA viruses to produce complimentary DNA so that their genomes can be replicated. So, inhibition of the reverse transcriptase activity is an important means to block the viral replication.

By observing the morphological change such as giant cell formation using a light microscope, Kageyama et al. (2000) first evaluated the adsorption inhibitory effect of HIV-1 virions to test cells after treatment with the PV extracts. Using the time-of-addition technique, they further established the stages of action. They found that the suppression of viral infectivity was both effective during and after the viral attachment. The number of viable cells was evaluated using the Trypan blue exclusion method. The production of HIV-1 in the culture medium was assessed by measuring the concentration of HIV-1 p17 by an ELISA assay. The inhibition of the reverse transcriptase activity was evaluated using a radiative assay for reverse transcriptase activity.

3.4.6.2 Articles which used functional assays not specific to the viral life cycle

3.4.6.2.1 Articles which screened multiple herbs for anti-viral potential

There were 15 articles (Li et al., 2019, Reichling et al., 2008, Yao et al., 1992, Yamasaki et al., 1993, Yang et al., 2017a, Feng et al., 2012, Tian et al., 2011, Nolkemper et al., 2006,

Liu et al., 2002, Au et al., 2001, Lam et al., 2000, Yamasaki et al., 1998, Reichling and Schnitzler, 2011, Collins et al., 1997) which screened multiple herbs for anti-viral potential. Many herbs were involved in each study, so, the complexity of their experiments to test the anti-viral efficacy was limited. Usual methods, such as plaque reduction test, flow cytometry, or observation of morphological changes such as the formation of giant infected cells by an optical microscope, were applied.

Tian et al. (2011) did a wide survey of the anti-influenza virus capabilities of the aqueous extracts of 439 herbs, and conducted deeper investigation on five of them, and an in-vivo test on one of them (*Melia Toosendan*) using a mouse model. They showed that PV aqueous extracts (together with four others: *Fragaria indica Andr*., *Liquidambar formosana Hance.*, *Lithospermum erythrorhizon Sieb. et Zucc.*, *Melia toosendan Sieb. et Zucc.*) were effective to suppress viral reproduction and the cytopathic effect.

Yamasaki et al. (1993, 1998) also screened many herbs; 204 and 46 respectively in their articles, for their anti-viral effects against HIV-1 viruses. They found that the aqueous extracts of PV were effective and suppressed reverse transcriptase activity.

Collins et al. (1997) screened the aqueous extracts of 19 herbs to check for their anti-HIV-1. They applied three types of in vitro assays to examine the inhibitory activity of the herbs on the binding of HIV-1 gp120 to the immobilized CD4 receptors, on the HIV-1 reverse transcriptase, and on the protein glycosylation by three glycohydrolases. Their findings were that the anti-viral effects occurred at the attachment stage and at the replication stage.

Zheng (1990) screened 472 herbs with both in vitro methods and clinical studies to see their effects on HSV-1 viral activity. They found that PV was highly effective when used therapeutically and as a preventive measure in clinics. He also reported on nine other highly effective herbs: *Aristolochia debilis, Artemisia anomala, Lindera strychnifolia, Patrinia villosa, Pinus massoniana, Pyrrosia lingua, Rhus chinensis, Sargussum fusiforme* and *Taraxacum mongolicum*.

3.4.6.2.2 Articles which investigated phytochemistry as the main theme

There were nine articles which focused on phytochemistry. One of the research approaches was to focus on specific chemical compounds in PV. Polysaccharides (Xu et al., 1999, Chiu et al., 2004, Zhang et al., 2007) was the most popular choice, followed by triterpenoic acids (Ryu et al., 1992, Ryu et al., 2000), and polyphenols (Au et al., 2001, Liu et al., 2002). These compounds were identified to be effective to block viral infectivity. Some articles focused on finding effective extraction and analysis methods (Yao et al., 1992, Xu et al., 1999, Lam et al., 2000, Liu et al., 2002, Zhang et al., 2007, Reichling et al., 2008, Brindley et al., 2009) of the targeted chemical constituents. They usually resorted to simple experimental methods to investigate the anti-viral activities of the compounds.

Xu et al. (1999) studied and established the anti-viral activities of an PV polysaccharide on HSV-1 and HSV-2 viruses using a plaque reduction assay. Using a time-of-addition approach, they further showed that the anti-viral activities of PV polysaccharides were both at the initial attachment stage and the post entry stage. Pre-incubation of the HSV-1 viruses with the PV polysaccharides reduced infectivity, but pre-treatment of the host Vero cells did not.

Chiu et al. (2004) monitored the expression levels of HSV-1 and HSV-2 antigens in the host Vero cells after treatment with the PV polysaccharides, so that the anti-viral effect of PV polysaccharides could be deduced. They observed using flow cytometry and concluded that the expression levels of the antigens were reduced.

Ryu et al. (1992) used Vero cells to conduct experiments with a plaque reduction assay to identify two triterpenes in PV to possess anti-HSV-1 capability.

Zhang et al. (2007) reported their study on the anti-HSV activities of PV polysaccharides, which were isolated using ethanol precipitation. They used both in vitro and in vivo tests. The in vivo experiments were done on guinea pigs and mice. In the in vitro tests, they found that PV extracts blocked the entry of HSV-1 viruses into the Vero cells. Then,

they also showed, by plaque reduction assay, that a PV polysaccharide had inhibitory effects on both HSV-1 and HSV-2 viruses.

Li et al. (2019) investigated a fish viral pathogen, and showed that PV, and specifically one of its constituents, ursolic acid, had high anti-IHNV efficacy. They used both in-vivo and in vitro tests using epithelioma papulosum cyprinid cells.

Liu et al. (2002) applied a method which used a conformation-specific monoclonal antibody NC-1 to identify small organic compounds that interrupted and thus enabled the investigation of the formation of the glycoprotein gp41 six-helix bundles on the HIV-1 virions. In so doing, they established the inhibitory capability of PV extracts on the attachment event of the virions to the host cells.

Ryu et al. (1992, 2000) established the anti-HSV-1 effects of two triterpenes in PV. Their in vitro tests used plaque reduction test on Vero cells.

3.5 Discussion

3.5.1 Future research directions

In recent decades, research attention focused on the discovery of novel anti-viral effects of traditionally used herbal medicines like PV to treat viral diseases, such as those caused by HIV and HSV. This had strategic research importance, as in recent years, there had been an increased emergence of novel viral diseases. In the US, the CDC (Centre for Disease Control) reported that the incidence and severity of infections caused by the HSV (McQuillan et al., 2018, Kreisel et al., 2021) and the HIV (CDC, 2021) have also increased and are common. Some viruses like the Ebola virus (Zhang et al., 2016, Yang et al., 2017a) and SARS-COV-2 (severe acute respiratory syndrome coronavirus 2) (Shahzad et al., 2020, Din et al., 2020, Wyganowska-Swiatkowska et al., 2020, Ao et al., 2021) are even deadly. Especially, SARS-Covid-2 in the last two years when the pandemic spreads to all populations globally, and new viral variants potentially effecting

transmissibility, reinfection, and evasion of immunity emerge. Besides Western medicine, the use of natural herbal medicine as an adjuvant or alternative treatment agent is a potential option to treat viral diseases. Of particular interest in this review is the anti-viral activities of PV.

Pseudotyping is a prominent technique which is used in anti-viral research. It allows the expression of the viral envelope proteins to be freely controlled, by combining genomic materials of one virus with the viral envelope of another. This allows the binding of the glycoproteins of the target virus with host receptors can be investigated separately. The potential hazard of dangerous viruses can be avoided because of the artificial virus is replication defective. This approach was demonstrated in the research projects by Liu et al., Collins et al. (1997) and Yao et al. (1992). They investigated the effects of treatment of PV extracts on the binding between the HIV gp41 (Liu et al., 2002) and gp120 (Yao et al., 1992, Collins et al., 1997) to their corresponding receptors on the CD4 host cells. For the case of coronavirus SARS-CoV-2, the corresponding spike glycoproteins are the G1 and G2, and the receptors on the host cells are ACE2 receptors. Ao et al. (2021) applied the pseudotyping technique also to investigate the anti-viral effect of PV against the coronavirus and demonstrated the effectiveness. Several other publications discussed the use of different herbs from the Chinese medicine pharmacopoeia to treat Covid-19 (Shahzad et al., 2020, Din et al., 2020). Some potential herbal candidates that are commonly used to treat influenza-like diseases are, for examples, *Chrysanthemum morifoliu, Agastache rugosa, Lonicera japonica, Satis indigotica L.*. Research on these herbs to treat Covid-19 warrants future research efforts.

Proteomic assays which are based on molecular biology technologies, including flow cytometry, PCR, ELISA, western blotting, and other immunochemical techniques, are frequently used (Refer to Fig. 3.5). These techniques are equally applicable in future investigations.

According to Chinese ethnomedicine, a traditional remedy (Chen and Chen, 2009, Chen, 2016b) for influenza-like diseases in the southern province of Guangdong recommends

that the patient inhales the rising steam from a washing basin of freshly boiled PV decoction. This points to the remedial effect of the aromatic or volatile organic compounds (VOCs) in herbs. The investigations in the use of herbal VOCs to treat diseases, however, are relatively few. This is the underlying idea behind this research project. Indeed, there were three reviewed articles (Zheng, 1990, Nolkemper et al., 2006, Reichling and Schnitzler, 2011) reporting the use of the VOCs (essential oils) from PV to treat HSV viral diseases. The use of the VOCs in PV, or other herbs, to treat SARS-CoV-2 has potential to yield useful results.

3.5.2 Summary and comparisons of results

The articles which were reviewed all showed that the PV herb exhibited effective anti-viral activities. The comparisons of their results to identify, for example, what concentrations of the PV extract were required to block the viral development and replication can foster deeper understanding. When the number of articles reporting on a particular virus was small; say just one or two articles, comparison was not valuable because it was not statistically significant. Conversely, there were more articles which reported on HIV and HSV. Comparisons among these articles were then more statistically meaningful. However, a deeper look into these articles revealed that such comparison was difficult. For example, for the ten HIV-related articles, the following was observed:

1) The PV herb used were not the same.
   Previous studies (Chen et al., 2009b, Yang et al., 2013) showed that PV plants originated from different geographic regions had different profiles of their chemical compositions, and thus, their pharmacological activities may differ. Except for Oh et al. (2011), however, all the other articles did not mention of where the PV herb was sourced from. In contrast, Oh et al. (2011) did their experiments using four accessions of PV sourced from different geographic regions in North America. Their article did show that the anti-viral efficacies

were different. Adding a further confounder to PV research, non-viral research articles (Guo et al., 2009b, Guo et al., 2010, Chen et al., 2011b, Chen et al., 2011c, Zhou et al., 2011, Kuriya et al., 2015, Chen et al., 2016, Phillips et al., 2018) have reported that variations in water management, light intensity during growth, the use of fertilizers, altitudes, and time of harvest effected variations in PV's chemical compositions. Consequently, in future research, plant standardization must be addressed, and certainly in the anti-viral research domain (the focus of this chapter). Finally, some articles investigated not the whole herb (and thus did not investigate synergistic effects among the constituent compounds, reflecting the traditional medical practices of using herbs), but rather gave attention to a group of chemical constituents extracted from PV: polyphenolics (Au et al., 2001, Liu et al., 2002), polysaccharides (Xu et al., 1999, Chiu et al., 2004, Zhang et al., 2007), triterpenes (Ryu et al., 1992, Ryu et al., 2000), and studied their corresponding anti-viral activities. The anti-viral numerical measures, such as IC_{50} (50% inhibition concentration), obtained consequently have different meanings.

2) The viruses have different strains.

   Different articles reported the use of different HIV virus strains. This meant comparisons on the anti-viral efficacies were difficult to conclude quantitatively. Oh et al. (2011) used three different infectious molecular clones (corresponding to three different HIV-1 strains), and their experimental results showed that the anti-viral efficacies for these different strains were not the same.

3) Different entities were tested.

   The various articles reported investigations which used cell lines to test the infectivity. However, the cell lines used were different. Moreover, for example, instead of using the herbal extracts directly, Feng et al. (2012) asked human volunteers to consume PV decoction, and fed mice with samples of the herbal decoction, before preparing human blood T-cells and rat sera as a source of the herbal treatment to treat the target cells. In another example, Liu et al. (2002)

did not use target cells to infect at all. They developed a special ELISA kit, based on a monoclonal antibody which could recognize the conformational epitopes on the HIV-1 glycoprotein gp41 six-helix bundles. The formation of these gp41 bundles is essential for the attachment of the HIV-1 virions to the corresponding CXCR4 or CCR5 cell receptors. They used this special ELISA kit to screen potential herbs which exhibit anti-HIV-1 activities.

4) Different anti-viral mechanisms were investigated.
   As viral development is a staged process, research into the anti-viral activity of the herb undertook different test methods to investigate which viral life cycle stages were affected by the herb (such as attachment, entry, or replication). For the articles on HIV, these different methods are highlighted and summarized in Table 3.1.

Table 3.1: The test methods taken to investigate the anti-viral mechanisms

Test methods	Attachment inhibition						Binding (Entry) inhibition	Replication inhibition				
Authors/Years	CXCR4, CCR5 receptors, p24,p120 expression test kits	pre-treatment of target cells	pre-treatment of virions	g41 expression test kit	glycohydrolase assay	time of addition of cells and viruses	time of addition of cells and viruses	p17, p24 antigen expression ELISA kit	Protease inhibition kit	Reverse transcriptase kit	Integrase inhibition kit	time of addition of cells and viruses
Feng et al. 2012	X							X				
Oh et al. 2011		X	X			X	X					X
Liu et al. 2002				X								
Au et al. 2001											X	
Lam et al. 2000									X			
Kageyama et al. 2000		X				X	X	X		X		X
Yamasaki et al. 1998										X		X
Collins et al. 1997	X				X	X				X		
Yamasaki et al. 1993												X
Yao et al. 1992	X	X				X	X	X		X		

This table evidenced that it was impossible to compile a consensus about numerical measures, for example, such as the IC_{50} values for blocking viral infectivity, or reverse transcriptase inhibition, due to the diverse focus of each study.

5) The experimental procedures were different.
   Even when the same test method was adopted, the experimental procedures would differ. For example, to check the effect of PV extracts to inhibit HIV-1 infectivity, the procedure adopted by Oh et al. (2011) used an incubation time of 40 hours, after the extract and the virus were mixed to the media containing the target cells. However, the procedure used by Kageyama et al. (2000) allowed an incubation time of four days, while Yamasaki et al. (1998) used five days.

6) The testing reagents were different.
   For example, when reverse transcriptase assays were used to check the reverse transcriptase inhibition activities, different reaction reagents were used. Yamasaki et al. (1998) and Collins et al. (1997) used non-radioactive ELISA test kits, while Kageyama et al. (2000) used a radioactive kit that contained radioactive [3H]-thymidine triphosphate.

From the list of sources of variations listed above, it becomes clear why no specific conclusion can be drawn on, for example, what concentration of the PV herbal extract is needed to cause the viral infectivity of HIV to reduce to 50%. Generally, however, it was noted that PV extract had a significant effect to inhibit the HIV viral attachment events. Since the inhibitory effect was strong when the virions alone were pre-treated with the extract, while there was virtually no (or little) inhibitory effect when the target cells were pre-treated alone, it suggests the anti-viral effect of PV extract was mainly due to

its effect on the viruses. The experiments demonstrated that PV extract also inhibited post-entry replication events.

More specific outcomes for the anti-viral effects of PV, however, can be noted for HSV. Since the standard plaque reduction test was commonly adopted to check the anti-viral effect against HSV, comparisons among the eight HSV-related articles can be made. The results are shown in Table 3.2.

Table 3.2: The efficacy of inhibitory effect of PV extracts on HSV virus

Authors/Year	Virus	Extract	Efficacy of inhibition
Reichling et al. 2008	HSV-1	20% and 80% ethanolic extracts	IC_{50} = 0.05μg/mL(20% EtOH) & 0.82μg/mL(80% EtOH)
Reichling & Schnitzler 2011	HSV-1	20% and 80% ethanolic extracts	IC_{50} = 0.08μg/mL (20% EtOH) & 0.2μg/mL (80% EtOH)
Zhang et al. 2007	HSV	aqueous extracts	IC_{50} = 18μg/mL, for polysaccharides
Nolkemper 2006	HSV	aqueous extract	IC_{50} = 0.229μg/mL (HSV-1) & 2.114μg/mL (HSV-2)
Chiu, Zhu & Ooi 2004	HSV	aqueous polysaccharide extract	IC_{50} = 20.6μg/mL (HSV-1) & 20.1μg/mL (HSV-2), for polysaccharides
Xu et al. 1999	HSV-1	aqueous extract	IC_{50} = 18μg/mL, for polysaccharides
Ryu et al. 1992	HSV-1	methanol extract, triterpenes	IC_{50} = 30μg/mL (betulinic acid) & 8μg/mL (2α,3α-dihydroxyurs-12-en-28-oic acid)
Zheng et al. 1990	HSV-1	aqueous & alcoholic extract	IC_{50} not given, clinical study

Referring to Table 3.2, the comparison of results showed that the ethanolic extracts of PV exhibited a much stronger inhibitory effect on HSV virus than the aqueous extracts or polysaccharides extracted from PV. The exception was the result reported by Nolkemper et al. (2006), who used fractionation to purify and obtain a potent fraction to treat the infected cells. For the aqueous extracts, otherwise, the IC_{50} value was around 20 μg/mL.

Although Table 3.2 just shows the IC_{50} values reported in the articles, other additional issues; for example, at what stage the herbal extract exerted its action, were also investigated. Because the complexity and diverse focus of these HSV-related articles are like what had been discussed above for the HIV case, the discussion will not be repeated.

3.6 Conclusion

This review explored the research activities in the use of PV herb for the treatment of viral diseases in the past three decades. Strategies and methodologies were systematically categorized and summarized. It was found that the aqueous extracts from PV were the most popular choice for research, followed by alcoholic extracts by ethanol and methanol. These research projects found that effective chemical constituents included polysaccharides, polyphenolics, triterpenoids and essential oils. HIV, HSV and influenza were the viruses most frequently researched. In this project, distillation by using steam to extract the VOCs of PV implied that the chemical compounds extracted will be closer to the aqueous extracts found in these previous projects.

Also, the articles investigated what mechanisms were responsible for the anti-viral activities of the PV herb. In this review, the mechanisms were categorized according to the life cycle of viruses. The blocking mechanisms included blocking at the attachment and entry stage, inhibition of the enzymes: proteases, integrases, glycohydrolases and reverse transcriptases. In this project, as no proteomic method was employed, the molecular mechanism behind the anti-tumoral effect of the VOCs in PV was not targeted.

PV extracts exhibited good anti-viral activities. However, there is difficulty in producing a collective summarized and quantitative analysis from the reviewed articles to the anti-viral effects of PV. The reason is the complexity and diverse focus of investigations reported. This extended to the lack of approaches to standardise and identify the geographic sources and growth conditions of the PV material. This needs to be addressed in future research.

Recommendations for future anti-viral investigations of PV should extend into novel viruses such as SARS-Cov-2 and explore the synergistic effect of multiple compounds

together for efficacy of treatment, reflecting the traditional herbal medical approaches of using herbs such as PV. This might extend into research into other herbal medicinal herbs categorized traditionally in Chinese medicine as “heat clearing”, while the aromatic VOC compounds in herbs have drawn few research attention to date.

Similar to the last chapter, previous research was confined to investigate the activities of non-volatile chemical compounds of PV. Few of them looked at the VOCs. This indicates promising research direction to investigate the anti-viral activities of the VOCs of PV. Consequently, this highlights the importance of the experimental works described in the next two chapters which can indicate any potential difficulties when experimental works are done to investigate the anti-viral activities of the VOCs of PV.

Chapter 4: Characteristics of Extracts from *Prunella vulgaris*, obtained from the Distillation Processes

4.1 Introduction

4.1.1 Outline of this chapter

In this chapter, the experimental study to characterize the VOC extracts from the distillation processes was reported. The outline of this chapter, after this introductory section 4.1, comprises of the following:

- all the materials and apparatus used in the experiments, and the experimental procedures undertaken; see Fig. 4.1.
- the results of the following experiments:
    1. *General characteristics of VOCs,
    2. *Characteristics of rarer volatile compounds,
    3. Concentration of VOCs at different depths inside the distillate,
    4. Volatile compounds in the residue solution,
    5. Time depletion effect,
    6. The effects of changing the stream flow rate,
    7. *Comparisons between the hydro and the steam distillation processes.

*: Steps 1, 2 and 7 pertain to the main aim stipulated in Sec. 4.1.3.1 above.

4.1.2 Opening remarks

In the previous introductory chapters, the discussion was on the literature review of previous research efforts in the past three decades on the therapeutic use of the herb, PV. This plant can widely be found worldwide, from East Asia to the Indian sub-continent, East Europe, to Great Britain in West Europe and to the North American continent (Chen et al., 2009b, Guo et al., 2009a, Golembiovska et al., 2014, Siew et al.,

2014, Kujawska et al., 2017). The use of this herb extended to over thousands of years in different cultures (Matthiolus, 1626, Bai et al., 2016, Amjad et al., 2017), and the use is not only limited to human, but to veterinary use as well (Lans et al., 2007, Kliem et al., 2018). A more detailed discussion of this can be found in the Appendix 1. PV was chosen to be the subject of investigation because research on it in the past, especially for its pharmacological use against cancer, was relatively fewer than other herbs.

In Chapter 2 and 3, previous articles which investigated the anti-tumoral and anti-viral effects of the herb were explored. The traditional preparation method of the herb for medicinal use is by decoction, which means the boiling of the herb in water to extract the water-soluble substances from the plant. The herbal tea is then consumed as medicine by the patient. Similar treatment is applied to animals, although the plant is also sometimes used as animal feed (Kliem et al., 2018) to improve the immunity against diseases.

As the traditional use of the PV herb involving decoction which unavoidably eliminates all the VOCs, very rarely previous research efforts were found on the investigation of these volatile compounds. This inspired the study which formed the experimental part of this project, both in this chapter and the next chapter, Ch. 5.

For the experimental work in this research project, the attention was shifted to the use of the VOCs of PV, which was different from the conventional usage. The extracts from the PV plant, no matter whether they were from decoction, or were extracted using other solvents, such as ethanol or methanol, consisted mainly of non-volatile chemical compounds. Especially in the traditional method of decoction, VOCs unavoidably vaporized away; thus, the extracts from these traditional extraction methods became depleted of any VOCs (However, there is a way to preserve some VOCs in the traditional decoction. For example, when Lamiaceae [mint] is prescribed in a formula, it is usually put into the boiling liquid at the very end). To obtain VOCs specifically, other methods need to be adopted. In this research project, the extraction method chosen is distillation.

There are two different methods of distillation: hydro distillation and steam distillation. There was a standard method recorded in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010), and can be found in Appendix VIB (p. A-78) and p. 359 of this reference. This standard method is hydro distillation. Since the inclusion of this method in the "standard" reference published by the Chinese government, most of the investigations in China on volatile compounds of herbs follow this standard method. Those which based on steam distillation have not been found yet.

Research in Chinese herbs usually adopted the decoction method to prepare the test solution because decoction remained the most frequently used method for preparation of medicine. Consequently, the number of articles on VOCs of PV was relatively few (Gu et al., 2007b, Sun, 2017, Wang et al., 1994, Cai et al., 2005). Most of them studied the chemical composition of the herb. None was found on the investigation of the anti-tumoral activities of the VOCs of PV. This was the idea behind the motivation inspiring this study project.

4.1.3 Aims & objectives

4.1.3.1 Main aim

The aim of the experimental part of this research project was to investigate, firstly, the characteristics of the VOC extracts from PV through the distillation processes; this meant from both the steam distillation process and the hydro distillation process. This work then resulted in the characterization of the distillation processes by obtaining data on the abundances of a small group of VOCs as representatives, extracted during the experiments.

Note that, however, it was agreed early in the planning stage of the project with the supervisors that the detailed chemical composition of the VOCs of PV was not the target of investigation. This was because this research work was done several times by several research teams previously (Golembiovska et al., 2014, Yang et al., 2013, Morteza-

Semnani et al., 2006). Therefore, a detailed study of the phytochemistry in the direction of identifying the chemical composition of the herb (see Fig. 2.1) was not carried out. Attention was given to how the VOCs evolve during the distillation process.

4.1.3.2 Secondary aims

The secondary aims are to examine how the abundances change when the different factors or independent variables change. Experiments were done to look at the distribution of the VOCs inside the distillate, VOCs in the residue solution, the time depletion effect due to storage after extraction, the effect of changing the steam flow rate (thus looking at the extraction dynamics), a comparison between the hydro and the steam distillation processes (so as to clarify and explain the saturation phenomena observed in the abundances of the extracted VOCs).

The purpose of doing these extra experiments was to foster more understanding, which might help to have a better design of a drug manufacture process which used VOCs as drugs.

4.1.4 Perspective and theme of this study

In Chinese medicine, prescription is always given as herbal formulae. So, the combined effect of many chemical compounds in the herbs is used to achieve treatment effects. Additionally, because there have been many research articles which investigated and reported the chemical VOC composition of PV (Morteza-Semnani et al., 2006, Yang et al., 2013, Golembiovska et al., 2014), in this project, identifying exhaustively individual active chemical compounds was not undertaken to repeat what other researchers had done. In this study, only a handful of chemical compounds, such as several alkanes and several rarer compounds such as caryophyllene oxide, were identified and used as samples for the characterization of the extracted VOCs. Attention was concentrated on these compounds because they are more abundant, and thus usually found in experiments, while other rarer compounds might not appear or be discovered in

extractions every time. Thus, these compounds were chosen as representatives to be studied.

In essence, the emphasis was to look at the PV herb as a whole, for its therapeutic effects. This adhered to the authentic Chinese medicine ideas, because traditionally herbal medicine is prepared by decoction of the whole herb, and not extracting a single or a few compounds from herbs in Western medicine. So, an exhaustive identification of all the chemical constituents in the PV herb was not undertaken.

In Chapter 2, a major difficulty in previous research efforts was mentioned that there was no standard or clearly defined starting material. In this study, the most easily accessible part of the plant, the spica of PV, was used as the starting material. This was also the part of the plant commonly used to prepare decoction for treatment.

Then, the second part of this investigation was to look at the pharmacological activities of the VOCs (as a whole) of the herb, which will be discussed in Chapter 5.

4.2 Materials and Methods

4.2.1 Materials

Dried spica of PV was sourced from the Traditional Medicine Clinic of the University of Technology Sydney. The supplier was TDT Australia Pty Ltd., Rydalmere, NSW, and they imported the herb from China. The herb was finely dedicated by a chopper produced by Russell Hobbs (Classic chopper RHMFP2). Rosmarinic acid used as a standard for the identification of the herb was purchased from Sigma-Aldrich, which was the supplier of all the chemicals used subsequently. These included the tetrazolium salt-based test reagent, Cell Counting Kit-8, the antibiotic gentamycin, which was used to prevent microbial contaminants to grow during the storage period of the extracted distillates, and the Folin-Ciocalteu reagent which was used to probe the anti-oxidative activity. The cancer cell line SCC154 was obtained internally from the biology laboratory in the university.

To conduct analysis by gas chromatograph–mass spectrometry (GC-MS), the machine used was manufactured by Agilent™ Technologies (6390N Network GC System and 5973 Network Mass Selective Detector).

4.2.2 Methods

The previous two chapters discussed the findings of reviewing previous research efforts. This review informed of all the currently available techniques which could be applied in this research project. Some examples of techniques which could be applied were identified (Morteza-Semnani et al., 2006, Yang et al., 2013, Golembiovska et al., 2014).

To extract volatile compounds, however, distillation is the most frequently chosen method. Hydro distillation is more popular in both laboratories and in industry than steam distillation. It is the prescribed method in the Chinese Pharmacopoeia (2010). Usually, hydro distillation was used as a tool to obtain the chemical constituents, and the emphasis of the articles was on reporting on the phytochemistry, i.e., the chemical composition (Morteza-Semnani et al., 2006).

However, in this project, steam distillation was chosen initially to explore if it could offer better efficiency of extraction, but extraction using hydro distillation was also done in this project to compare.

Fig. 4.1 shows the flowchart of the experimental procedures undertaken.

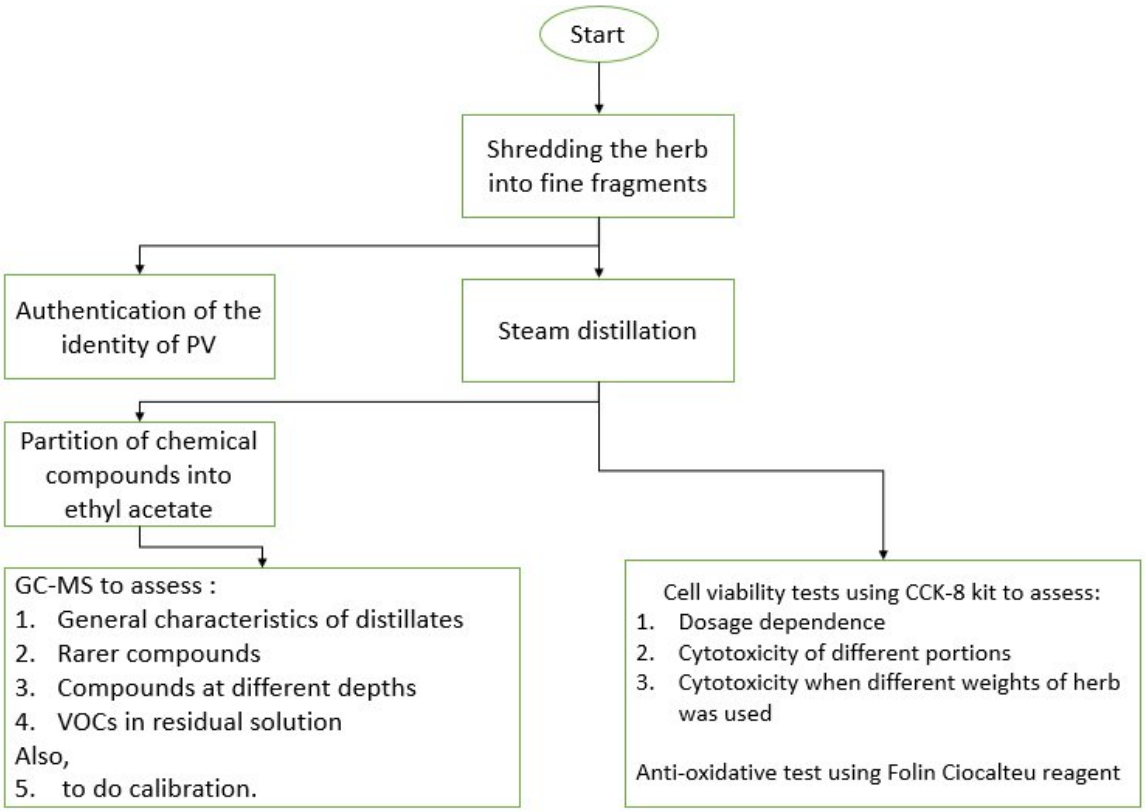


Fig. 4.1: Flowchart showing the experimental procedures undertaken.

CCK-8 is the code assigned by the manufacturer. CCK stands for Cell Counting Kit.

Chromatography is a conventional method to separate and analyze the chemical compositions of herbs. The most popular methods are High-performance liquid chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS). For GC-MS, the mobile phase is always a gas, and GC-MS analyzes compounds that dissolve in gas, so it is perfect for volatile or semi-volatile substances that readily vaporize. Thus, GC-MS is the natural choice in this project. In this project, GC-MS measured the abundances of the volatile chemical compounds in the PV herbs (raw data from the machine is in arbitrary units). The abundances so measured could be calibrated to give absolute concentrations in, e.g. grams per liter. The calibration could be done by comparing the GC-MS measurement readings with those of the standard compounds after the identification was confirmed. However, in this project, we looked at the trends

of the concentration changes with various parameters, e.g., time duration of the distillation process from start. Therefore, determination of the absolute abundances was not necessary. Relative abundances were sufficient.

To test for the cancer cell cytotoxicity, sophisticated techniques such as flow cytometry are also applicable. However, in this research project, tests using colorimetric assay which monitored cell metabolism was chosen. Colorimetric assays are simple and easy to use. They are inexpensive too, while other more sophisticated methods are not accessible to this project. Flow cytometry facility was not accessible.

4.2.2.1 Identification of the herb

In the official reference manual of the Chinese government, the Chinese Pharmacopoiea (Chinese Pharmacopoeia Commission, 2010), the procedure for the identification of the PV herb was stated (p. 359, 2010 edition). This procedure was followed to establish the identity of the herb used in the experiments.

The procedure involved: 5 g of finely chopped PV herb subjected to ultra-sonification in 30 ml of ethanol to extract the chemical compounds. The extracted product was then dried and re-dissolved in ethanol to give the test solution. Thin layer chromatography was applied to the test solution (extracted from the herb by 70% ethanol) to separate the extracted components. Rosmarinic acid was used as a standard to compare with the chemical components from the test solution, as traces on a test paper, with the mobile phase carrier solvent (cyclohexane, ethyl acetate, isopropanol and formic acid in the ratio of 15:3:3.5:0.5) prescribed in the Chinese Pharmacopoeia.

4.2.2.2 Extraction of VOCs by steam distillation

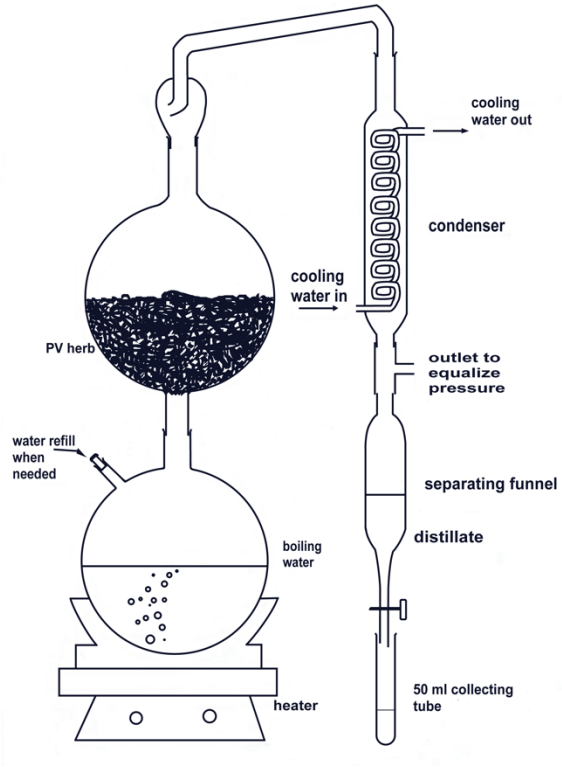


Fig. 4.2: Experimental setup of the steam distillation process

The experimental setup used to conduct the distillation experiments to extract the VOCs from the PV herb is shown in Fig. 4.2. The setup consisted of two arms. On the left, de-ionized water was boiled in a two-necked flask to produce steam. The steam rose to pass through the herb above in a two-ended reservoir flask to extract the VOCs within the herb.

Before doing the steam distillation, the dried herb was shattered to fine pieces using a chopper for five minutes to keep the consistency of the fineness of the fragmented herb.

The steam which carried the VOCs was guided to the right arm of the setup, where the distillate was condensed in a condenser cooled by running tap water. To monitor the abundances of the extracted VOCs during the whole distillation, samples of the distillate was collected at successive time points with equal time intervals among them. To keep

the distillation process consistent and repeatable, the temperature setting of the heater was adjusted to give a constant distillate collection rate of around 50 ml per 40 minutes across different distillation runs. 50-ml distillate portions were successively collected in test tubes 40 minutes apart. In most cases, nine portions were collected, making a total volume of distillate collected equal to 450 ml. As a result, graphs which plotted the abundances of the extracted VOCs measured in each sample portion versus the portion number indeed monitored the whole distillation process at successive time points with equal time intervals during the distillation process.

Different amounts of herb were used in separate distillation runs, ranging from 2 g, 5 g, 15 g, 25 g, 35 g to 45 g. These different quantities were used to assess the dosage effect of herb used. These quantities were chosen recursively after successive analyses and assessment of data obtained, as trends of results were progressively obtained.

4.2.2.3 Preparation of the GC-MS test solution

As the distillate obtained from the distillation process was aqueous, it was incompatible with the GC-MS machine. Water is detrimental to the coating material in the capillary column of the GC-MS machine. So, the chemical compounds dissolved in the distillate were first partitioned into an ethyl acetate medium before the GC-MS analysis. A mixture of 1 ml of the aqueous distillate and 1 ml of ethyl acetate was pipetted into a test tube together. The mixture was vortexed for one minute to mix well and allowed the chemical compounds to be sufficiently partitioned into the ethyl acetate portion. The mixture was allowed to settle into two immiscible layers and the top ethyl acetate layer was pipetted out and delivered into another test tube. Anhydrous potassium sulfate powder was added to the test tube to remove any residual water. The dehydrated solution was then used for the GC-MS analysis, which was done as soon as possible to prevent VOC's vaporization. In case when immediate GC-MS analysis was not practical, the distillates were stored in a refrigerator with a temperature setting at 5^{o}C.

Otherwise, if the chemical compounds had been partitioned into ethyl acetate, the ethyl acetate test solutions were stored at -21°C.

4.2.2.4 GC-MS analysis

The GC-MS machine was an Agilent™ gas chromatography machine in tandem with an Agilent mass spectrometer. The gas chromatography machine was equipped with a capillary column with dimensions of 30 m x 0.25 mm x 0.25 µm. The settings were as follows: 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, at a flow rate of 27.7 mL/min and a pressure of 4.24 psi. The temperature profile of the oven was: Temperature held at 50° C for 3 min; then, ramping up to 250° C at a rate of 10° C/min; holding at 250° C for 3 min; then, ramping up to 280° C, where the temperature was at hold 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 temperatures at the MS source and quad, were at 230 and 150° C respectively.

The identities of the VOCs were established by comparing the mass spectra obtained from the GC-MS runs with the mass spectrum library of the National Institute of Standards and Technology (NIST08), available internally in the software supplied in the GC-MS machine. This identification was further verified by a comparison method. The patterns appeared in the chromatograph of the distillate sample was compared with that of standard compounds available in the university chemistry laboratory. The successful matching of the patterns confirmed the identity.

Fig. 4.3 shows a typical chromatogram from a GC-MS run.

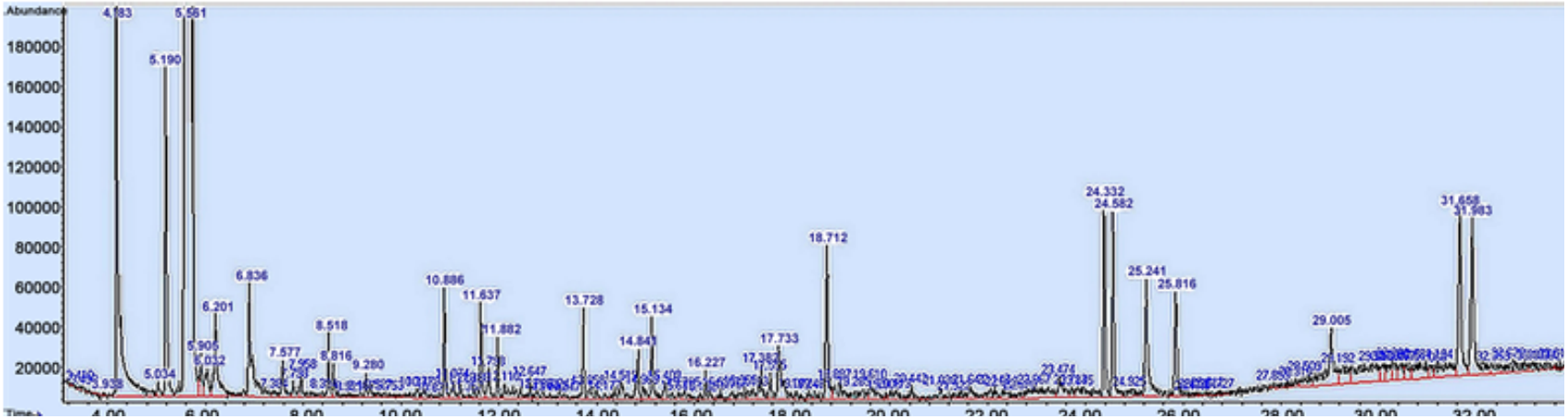


Fig. 4.3: A typical chromatogram from a GC-MS run

The amount of herb used was 15 g. The sample was taken from the fourth portion of a distillation run. The numbers shown at the peaks are the retention time. Retention time of some VOCs discussed in this article are: Furfural (3.46 min), anisole (6.04 min), benzaldehyde (6.85 min), decane (7.48 min), eucalyptol (8.15 min), dodecane (10.8 min), tetradecane (13.84 min), caryophyllene oxide (16.18 min), hexadecane (16.27 min).

4.2.2.5 Calibration using an internal standard

Compounds which were identified in the distillate could be used as internal standard for calibration of the distillates obtained. Dodecane was chosen in the experiments as the internal standard, as it was consistently and stably present in all samples collected and identified in all chromatograms. Dodecane sample, as obtained locally in the laboratory, was used as a reference for comparison. A known amount in weight of dodecane was measured and dissolved in a known amount of ethyl acetate. GC-MS analysis was then done on this dodecane sample with known concentration to obtain a GC-MS measurement of the abundance reading. Then, the dodecane sample was successively diluted and then successive GC-MS measurements done. This enabled a calibration curve to be drawn, which was a plot of abundance readings versus dodecane concentrations.

4.2.2.6 Scientific controls

Scientific controls were needed to minimize the effects of confounding variables in experiments.

Negative control was implemented into the experimental design by using “water” as the test solution. De-ionized water (instead of the distillate samples) was used to go through the whole procedure of preparing the GC-MS test solution by carrying out the step of partitioning into ethyl acetate.

This will provide the noise background level of the GC-MS runs.

4.2.2.7 Statistical analysis

For the distillation experiments covered in this chapter, no statistical analysis could be practically done. Each set of data pertained to one distillation run, which in the experimental design, took eight to twelve hours, which meant one working day to do. To get a meaningful good sample size of, say, 10 (Indeed, each distillation data set had been repeated for three times already, as seen from Fig. 4.6 to Fig. 4.15), ten distillation

runs took ten days for a single line on a graph with statistics. So, for a plot with five lines, this meant 50 days for a single graph. This number doubled to 100 days for the case to make a comparison between the hydro distillation and the steam distillation processes. Therefore, it is easy to understand that it needed two to three years just to obtain enough data to plot all the graphs in this chapter. This is quite impractical.

However, as a compromise for this shortfall, to circumvent the variations due to sampling and measurement errors, three GC-MS test solution samples were prepared repeatedly for each distillation sample. The result is thus three separate GC-MS runs to get three abundance readings for each distillation sample. However, as noted from Sec. 4.2.2.3 and Sec. 4.2.2.4, these procedures to get the abundance readings are time consuming. It took more than three hours to get a set of three abundance readings for a single distillation sample. Therefore, it is obvious to appreciate the time restriction to get even more repetitions of abundance readings to smooth out the sampling and measurement errors.

4.3 Results

The results as reported in this chapter (and the next chapter) were published in the following articles(Mak and Walsh, 2021, Mak, 2022, Mak, 2023).

4.3.1 Identification of the herb

The procedures described above in Sec. 4.2.2.1 established the identification of the herb. Basically, it is a thin-plate chromatography experiment to compare the trace obtained with chemical compounds extracted from the herb with the trace obtained using the reference standard compound, rosmarinic acid. The experiment was repeated four times to obtain a table of retention factors deduced from the experiment runs. (See Table 4.1)

The TLC chromatogram (see Fig. 4.4) shows the two trails left by the phase carrier solution of PV (the right trail) and left by the standard solution of rosmarinic acid (the left trail). A conspicuous blot left by PV is at the same location as the blot left by the standard rosmarinic acid. The presence of rosmarinic acid, and thus the identity of the herb as PV, were confirmed.

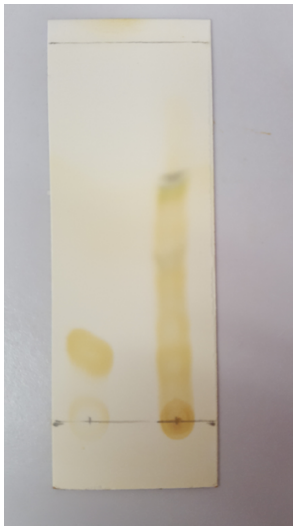


Fig. 4.4: A TLC chromatogram identifying the PV herb.

It clearly shows a blot along the trail left by the phase carrier solution of PV (on the left) is at the similar location (with the same retention factor RF) as the blot left by the standard control solution of rosmarinic acid (on the right). So, the identity of the herb used is verified to be PV.

Table 4.1: Table of retention factors to establish the identity of the herb

Run	R_f (reference sample)	R_f (test sample)
#1	0.18	0.17
#2	0.21	0.21
#3	0.17	0.15
#4	0.31	0.29

4.3.2 Calibration using dodecane as the internal standard

A calibration standard curve was drawn as Fig. 4.5. This calibration curve was used to calibrate distillates used in the biological tests.

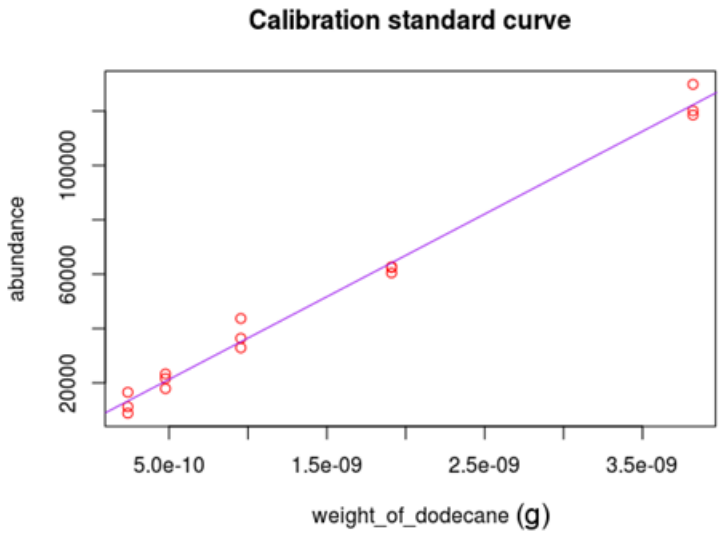


Fig. 4.5: The calibration standard curve

The formula is: $a = c_1 * g + c_o$

where a = abundance found in the GC-MS analysis, given in arbitrary units

g = weight of dodecane in 5 µL of injection distillate fluid.

c_1 = slope of interpolation curve, 3.04345e+13

c_0 = y-intercept, 6.036528e+3

variance σ^2 = 2.25365097e+8

As the abundance of dodecane in the distillate measured by GC-MS is around 20000 a.u. (when 15 g of herb was used). Thus, it is equivalent to have a concentration of around 0.1 ng / µL. In the distillation experiments carried out in this study, we only investigated the change in abundances when various independent variables changed. Therefore, relative abundances were sufficient. The absolute value of abundances in ng/ µL

(weight/volume) was only determined for dodecane as an internal standard. This internal standard, however, was not used to determine the absolute values of abundances of other compounds. If needs arise to determine the absolute values of abundances of other compounds, it can be achieved by comparing the relative abundances of the other compounds with the abundances of dodecane.

4.3.3 Characteristics of the distillates and the residue

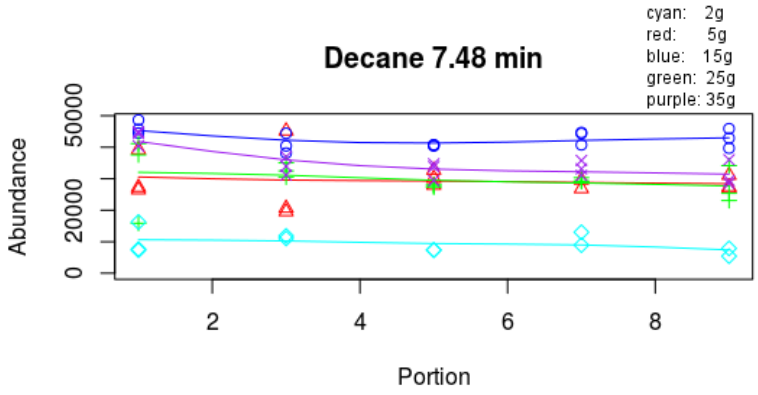
4.3.3.1 General characteristics

Experiments were done in triplicate sets and data were collected on the abundances of different VOCs in the distillates, obtained in different distillation runs. To save run time, GC-MS analyses were done on alternative portions of distillate collected during the distillation runs.

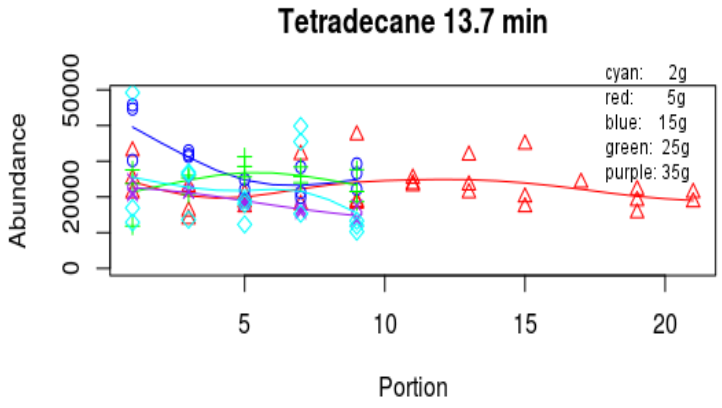
From the GC-MS analyses, some VOCs in PV were identified. These included alkanes such as decane, dodecane and hexadecane, which were reported previously (Morteza-Semnani et al., 2006, Yang et al., 2013). Examples of other chemical compounds identified were eucalyptol, anisole, furfural, benzaldehyde. For the few chemical compounds identified, all were affirmatively identified by comparing their signatures on the chromatograms with those of standards chemical compounds. The only exception was eucalyptol; it was only identified by the analysis software of the GC-MS machine with the library NIST08, because standard eucalyptol was not found in the university chemistry laboratory. As the main aim of this research project was to explore the therapeutic activities of the VOCs of PV as a whole. Moreover, many previous research efforts had accomplished much in identifying chemical compounds in PV already (Wang et al., 1994, Cai et al., 2005, Morteza-Semnani et al., 2006, Gu et al., 2007b, Yang et al., 2013, Sun, 2017). Therefore, this study did not spend more efforts exhaustively on identifying all the chemical composition.

By conducting distillation runs with 2 g, 5 g, 15 g, 25 g and 35 g of PV herb, the dependence of the abundances of VOCs extracted on the amounts of herb used could

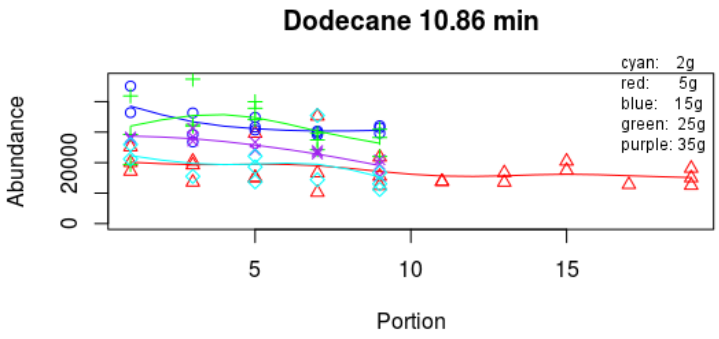
be deduced, and thus, the efficiency of the extraction process be characterized. The results were summarized in Fig. 4.6, showing the abundances of four alkanes. (Note that the abundances shown in the following figures [Figs. 4.6, 4.7, 4.8, 4.10, 4.11, 4.12, 4.13 & 4.15] are in arbitrary units as measured by GC-MS; so, they indicate relative abundances.) They were chosen because their presences in the distillate were confirmed by the comparison of the chromatograms with those obtained with the standard compounds which were obtained in the university chemistry laboratory.



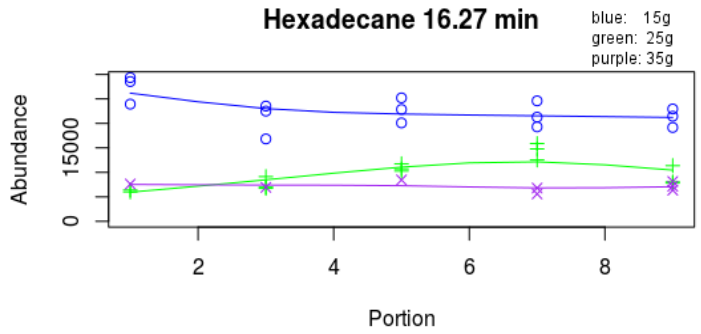
(a) The abundance of decane



(b) The abundance of dodecane



(c) The abundance of tetradecane



(d) The abundance of hexadecane

Fig. 4.6: The abundances of four volatile alkanes

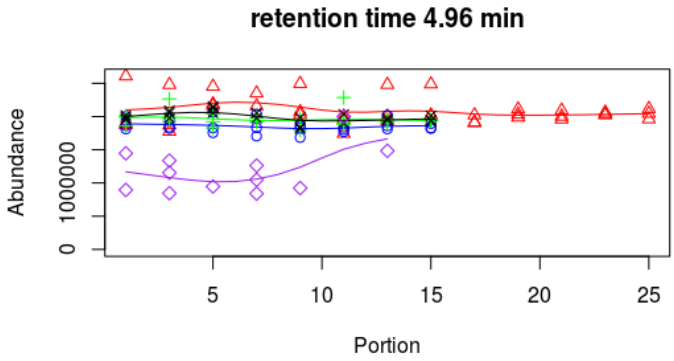
(a) decane, (b) dodecane, (c) tetradecane and (d) hexadecane

The abundances in the diagrams are in arbitrary units.

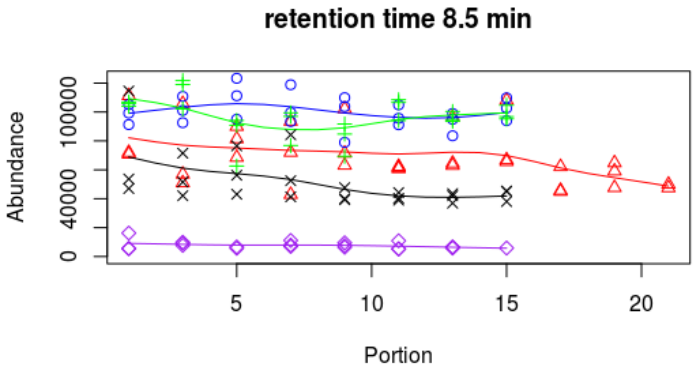
These four alkanes were obtained in successively collected 50-ml portions during the distillation process. Different curves represent cases when different amounts of herb were used: 2 g, 5 g, 15 g, 25 g and 35 g respectively. The numbers appear in the sub-titles are the retention time of the compounds in minutes. The smoothing lines were fitted to the data using cubic smoothing splines with a degree of smoothing parameter equal to 0.4.

From Fig. 4.6, the abundances of the extracted VOCs maintained relatively constant and did not diminish much even after the distillation process proceeded for a long time as practically allowed. This is further checked by runs with relatively small amounts of herb used, 5 g, but were carried out for as long as practical, extending to more than 14 hours (See Figs. 4.6(b) & (c)).

Fig. 4.7 showed the abundances for two more compounds, the identities of which were not confirmed by comparisons of their chromatograms with standard compounds. So, as these compounds were not properly identified, only their retention time in the GC-MS analysis were shown. Comparison of Fig. 4.7 with Fig. 4.6 showed that there was no contradiction with the observations made above.



(a) The abundance of an unidentified compound with retention time of 4.96 minutes. The abundance is in arbitrary units.



(b) The abundances of an unidentified compound with retention time of 8.5 minutes The abundances are in arbitrary units.

Fig. 4.7: The abundances of two volatile compounds (no identification)

For the two compounds shown in Fig. 4.7, only the retention times of the compounds were indicated. Different curves represent cases when different amounts of herb were used: 2 g (colour code: cyan), 5 g (red), 15 g (blue), 25 g (green), 35 g (purple) and 45 g (black) respectively. The smoothing lines were fitted to the data using cubic smoothing splines with a degree of smoothing parameter equal to 0.4.

4.3.3.2 Characteristics of rarer volatile compounds

In the last sub-section, it was demonstrated that the VOCs came out from the distillation process quite steadily without much depletion through the distillation process for as long as practically allowed. There were rarer compounds which deviated from this general statement and showed depletion as distillation proceeded. Rarer VOCs which were identified included benzaldehyde, caryophyllene oxide, eucalyptol, anisole and furfural. The experiments adopted an identification method by comparing the chromatograms of the distillates with those of standard compounds which were obtained in the university chemistry laboratory. The exception was eucalyptol, which was repeatedly reported by the analysis software of the GC-MS machine, but with no comparison with standard compound, because eucalyptol was not available. Fig. 4.8 showed the abundances of benzaldehyde in the successive portions obtained during the distillation process. Benzaldehyde was the most abundant among the rarer compounds identified. The abundances of it did show obvious decline during the whole distillation process. In the figure, two curves were for two amounts of herb used, 25 g and 35 g. When smaller amounts of herb were used, the presence of benzaldehyde was not detected, with the set threshold for detection. This showed its relative scarcity.

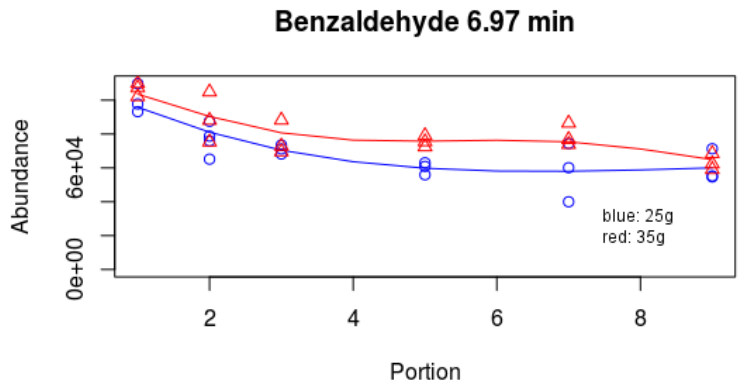
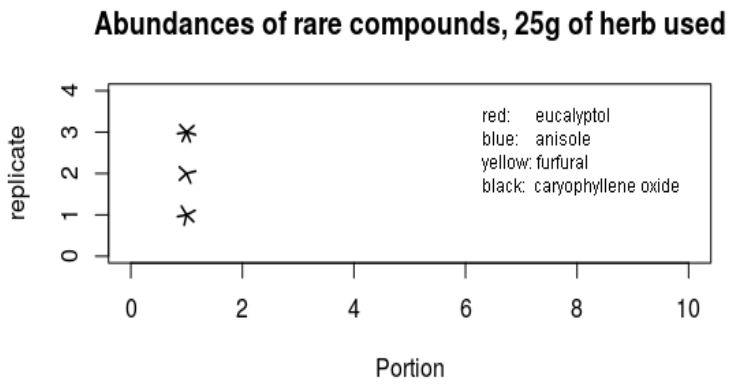


Fig. 4.8: The abundances of benzaldehyde obtained in 50-ml portions

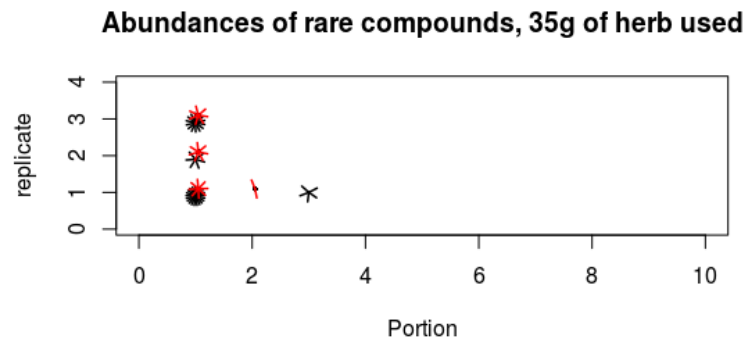
Only two curves (for 25 g and 35 g of herb used) were shown, as benzaldehyde could not be detected when the amount of PV used was below 25 g. Abundances are in arbitrary units.

Other rarer VOCs found were detected even less frequently, so that an alternative format was adopted to show their abundances in graphs, as shown in Fig. 4.9. They were shown as sunflower plots, in which the number of sunflower "petals" was directly proportional to the abundances. Different colors were used to indicate different compounds. None of the rare compounds shown was detected for amounts of PV herb used less than 25 g.

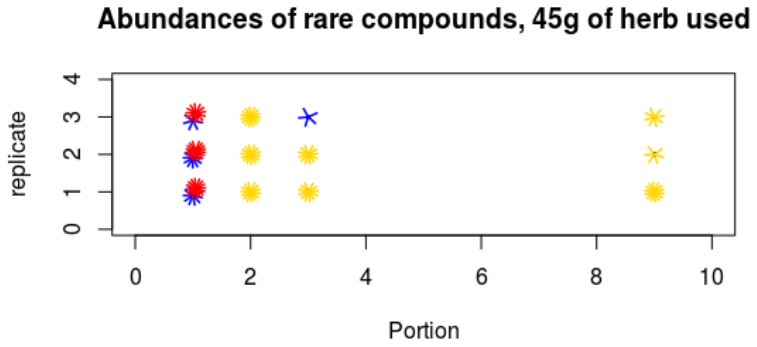
Caryophyllene oxide, anisole and eucalyptol were found only in the first portion at the beginning of the distillation process, except briefly in two instances in the second and the third portions (for caryophyllene oxide), and once in the third portion (for anisole), and briefly in one instance in the second portion (for eucalyptol). Anisole and furfural also appeared only after a large amount of PV herb (45 g) was used. Both mainly only appeared in the first three portions. For furfural, it appeared also, however, briefly in the ninth portion (reason unknown).



(a) Abundances of rare compounds when 25 g of herb was used



(b) Abundances of rare compounds when 35 g of herb was used



(c) Abundances of rare compounds when 45 g of herb was used

Fig. 4.9: The abundances of rarer VOCs, as indicated as sunflower plots

The abundances were proportional to the number of "petals" at each data point (sunflower). The measurements were made in triplicates, expressed as the y-axis. The compounds might not appear in each of the triplicates even though they were sampled from the same portion.

4.3.3.3 The concentration of VOCs at different depths inside the distillate

Samples were taken from the surface, the mid-level (as judged from the graduation marks on the distillate collecting tube) and the bottom of the test tube containing the ninth portion of the distillation which used 15 g of PV herb. Samples were taken at least one hour after the distillate collection. To maintain consistency among the samples taken in triplicates, the distillate in the tube should be allowed to settle well before the

samples were taken. GC-MS analyses were then done on these samples. Fig. 4.10 is a plot of the abundances of VOCs of various molecular weights versus the retention time in the coil of the GC-MS machine for the three depth levels. Since the retention time in the coil is directly proportional to the molecular weights of the compounds which flowed through the GC-MS coil, Fig. 4.10 shows the abundances versus the molecular weights of the VOCs. VOCs with high molecular weights were found mostly at the bottom, indicating that they had higher densities than water. However, this was reverse for VOCs with low molecular weights (small retention time), abundances at the surface were the highest, showing that the VOCs floated to the surface.

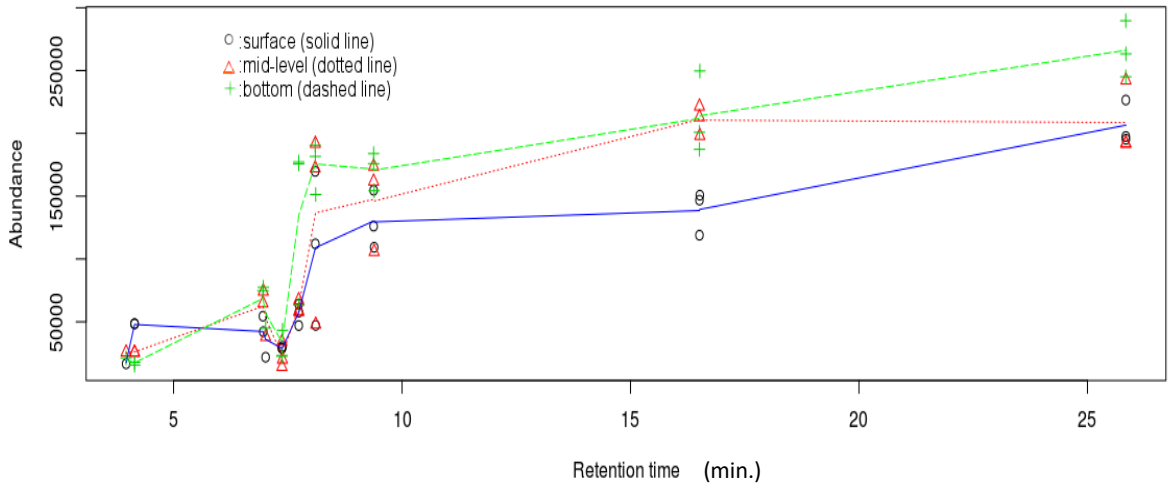


Fig. 4.10: Abundance of various VOCs at three different depth levels

Note that compounds with very different abundances were not chosen to be included in this figure, as they will obscure the whole picture. The abundances are in arbitrary units.

4.3.3.4 Volatile compounds in the residue solution

The residual solution which was left behind in the steam producing flask after the distillation process finished was also subjected to GC-MS analysis after it had settled for more than an hour. It was found that the VOCs in the distillate were also present in the residual solution, but with reduced varieties and abundances. The reductions were also

more prominent for the lighter compounds (with retention time less than ten minutes). This indicated that some VOCs extracted by the rising steam re-condensed and dropped back to the boiling water below before it could be carried over to the collecting arm of the setup. The lighter compounds were more effective to evade this re-condensation and escaped to the other arm of the setup and collected in distillates there.

4.3.3.5 Time depletion effect: Aging of the distillate

After the VOCs are extracted by distillation, there ought to be some time lag between their extraction and their packaging to be encapsulated into a form useful as medicine and deliverable to patients. Thus, it is practically necessary to investigate the time depletion effect, or aging, of the distillate on the chemical changes or loss of VOCs during this time lag period, because this has implication to change the therapeutic efficacy.

Distillate samples collected were analyzed by GC-MS for the abundances of the volatile chemical compounds once every week (and more frequently during the first week).

As the first observation, it was noted that the distillate still gave off fragrant smell even after the distillate had been collected for about two months, showing that some VOCs persisted in the distillate even after such a long time. Fig. 4.11 shows the abundances of four alkanes and benzaldehyde, which were tracked for 45 days since the distillation process started.

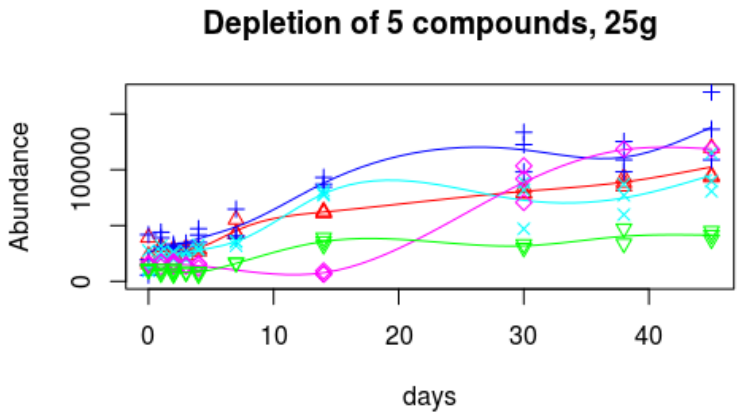
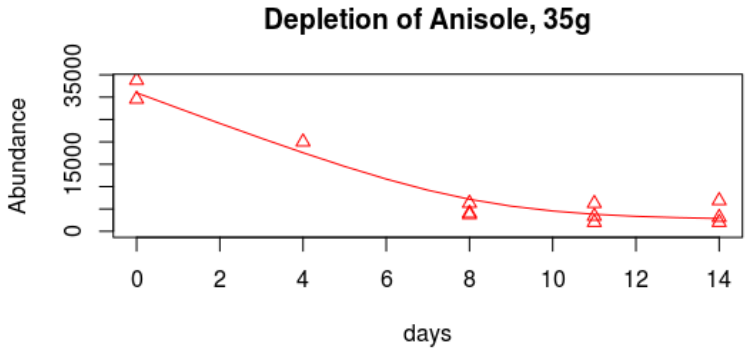


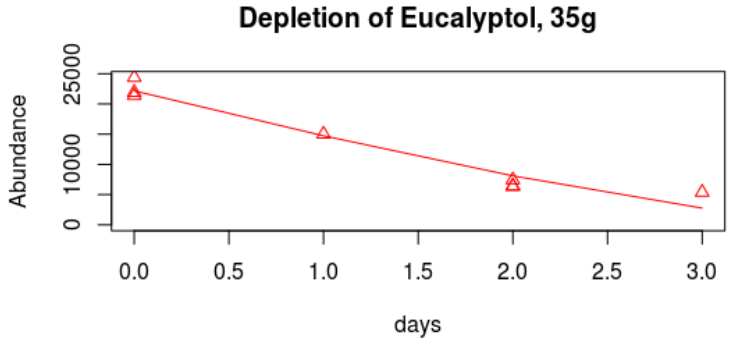
Fig. 4.11: The abundances of four alkanes and benzaldehyde change with storage time in days (Colour code: Decane, red; dodecane, blue; tetradecane, cyan; hexadecane, green; benzaldehyde, magenta). 25 g of PV herb was used as the starting material for the distillation process. The abundances are in arbitrary units.

Unexpectedly, in contrary to what was expected, the abundances of these compounds increased. This shows that these volatile compounds, instead of being dissipated by evaporation, were enriched by unknown chemical processes in the distillate. Microbial growth happened inside the distillates when they were kept in storage if precautionary steps were not undertaken to de-sanitize the distillate before storage. The microbial growth inside the distillates may be the cause of the unknown chemical reactions. However, this is just a speculation. No further investigation in the enrichment of some chemical compounds while in storage was carried out, as the theme of this project is not to study individual chemical compounds, and the chemical reactions among them.

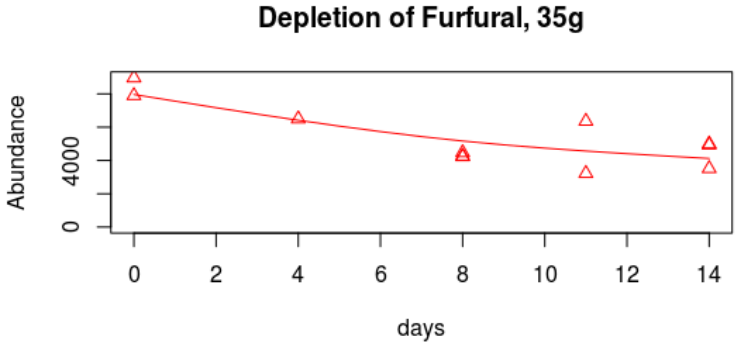
However, some rarer compounds did show depletion as time passed. Three of them were shown in Fig. 4.12: anisole, eucalyptol, and furfural. Eucalyptol dissipated in around three days, whereas anisole and furfural persisted and still were found after about two weeks.



(a) The abundance of eucalyptol with storage time in days



(b) The abundance of eucalyptol with storage time in days



(c) The abundance of eucalyptol with storage time in days

Fig. 4.12: The abundances of three rarer compounds change with time

The abundances are in arbitrary units. The weights shown in the sub-titles of the graphs refer to the weights of the herb used. Note that to collect the data to construct this figure, 35 g PV herb

was used, because these rarer compounds cannot be detected when only 25 g of PV herb was used (the detection threshold at the mass spectrometer was set at 5000 a.u.)

4.3.3.6 The Effects of changing the steam flow rate on the extraction

Earlier discussion reported that the abundances of the VOCs extracted did not increase with the amounts of PV herb used when the weight of herb increased beyond 15 g. So, saturation in the amounts of extracted VOCs occurred when the weights of herb used were above this value. An explanation was postulated as follows: In the experimental setup, the mass of PV herb above the boiling water in the passage of steam flow presented as an obstacle to obstruct the upward flow of the passing steam. Steam with the carried VOCs were re-condensed and dropped back into the boiling liquid below.

To further investigate the dynamics of the extraction process, the steam flow rate was varied by changing the temperature setting of the heater below, which produced steam. The dynamics were then changed, and the steam flow rate increased as more energetic steam was produced by higher heat. The steam flow was then able to overcome the resistance which the mass of herb presented above. The experiment used two temperature settings at the heater: 180^{o}C and 200^{o}C and the results were shown in Fig. 4.13.

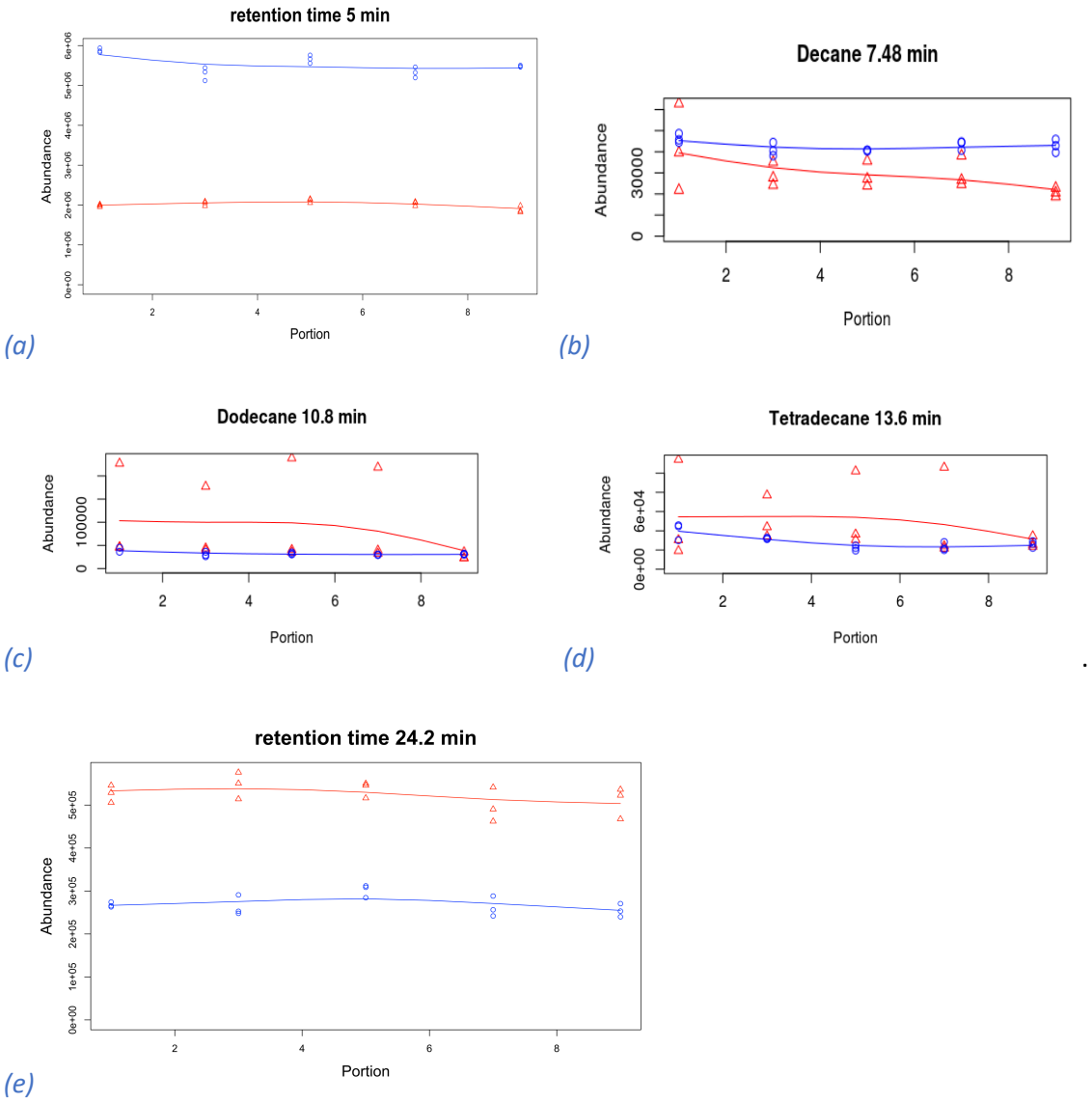


Fig. 4.13: The abundances of VOCs collected for two heater temperature settings. (colour codes of lines: Blue, 180°C; red, 200°C). The five graphs are: (a) for an unidentified compound with retention time of five minutes; (b) for decane; (c) for dodecane; (d) for tetradecane; (e) for an unidentified compound with retention time of 24.2 minutes. The abundances are in arbitrary units.

Fig. 4.13 shows five chemical compounds, among which three alkanes were properly identified: Decane, dodecane and tetradecane. The other two compounds shown in Figs. 4.13 (a) and Fig. 4.13 (e) were represented by their retention time in the GC-MS coil of

5.0 minutes and 24.2 minutes only (shown in the subtitles), because their identities were not positively recognized. 15 g of PV herb was used in this experiment. The retention time of the individual chemical components in the coil directly related to their molecular weights.

Observation can be deduced from the five graphs shown: The smaller steam flow rate (corresponding to lower heating temperature) extracted lighter compounds (corresponding to shorter retention time) more efficiently, while the opposite occurred for larger steam flow rate. The switching point occurred at about a retention time of ten minutes, which roughly corresponded to dodecane.

The following explanation was proposed. When the steam flow was less energetic, the heavier compounds did not gain enough momentum to overcome the obstruction of the mass of PV herb in the steam flow path. They got re-condensed and fell back to the boiling liquid below. When the steam got more energetic because of the higher heating temperature, the heavier compounds got sufficient energy and could thus overcome the obstacle from the mass of overhanging herb. When the heavier compounds had sufficient energy to overcome the resistance, the more resistance which the mass of herb imposed on the lighter compounds would show up. This caused the heavier compounds to be more efficiently extracted at higher steam flow rate.

4.3.3.7 Comparisons between the hydro and the steam distillation processes

Previous discussion proposed that, in the setup of the steam distillation, the mass of herb hanging above the boiling water which produced steam imposed an obstacle in hindering the passage of steam and VOCs through it. The steam and the VOCs got re-condensed and dropped back to the boiling liquid below. This reduced the efficiency of extraction of VOCs from the herb using the steam distillation process. This was proposed to explain the observation of the saturation phenomenon of extracted VOCs when the amount of herb used increased further to beyond 15 g; the thickness of the layer of herb became more and more efficient as obstruction to the rising steam flow.

The purpose of this sub-section was to verify this claim to explain the saturation, using another distillation method, the hydro distillation. It had a different setup as the steam distillation. It did not have a mass of herb hanging above the boiling water in the passageway of the steam flow, thus the obstacle was completely removed. The herb was submerged inside the boiling water.

Usually, hydro distillation was used as a tool to obtain the chemical constituents, and the emphasis of the articles was on reporting on the phytochemistry, i.e., the chemical composition (Morteza-Semnani et al., 2006). No research articles were found to report similar experiments with hydro distillation for the same purpose as what was done in this project.

4.3.3.7.1 Experimental setup

The experimental setup for the hydro distillation process was depicted in Fig. 4.14. To recall for comparison of the difference in the setup, please note that the experimental setup for the steam distillation process can be found in Fig. 4.2. Instead of hanging above the boiling water in another flask, the herb was submerged inside the boiling water and was directly heated. The distillate was again collected in 50-ml portions.

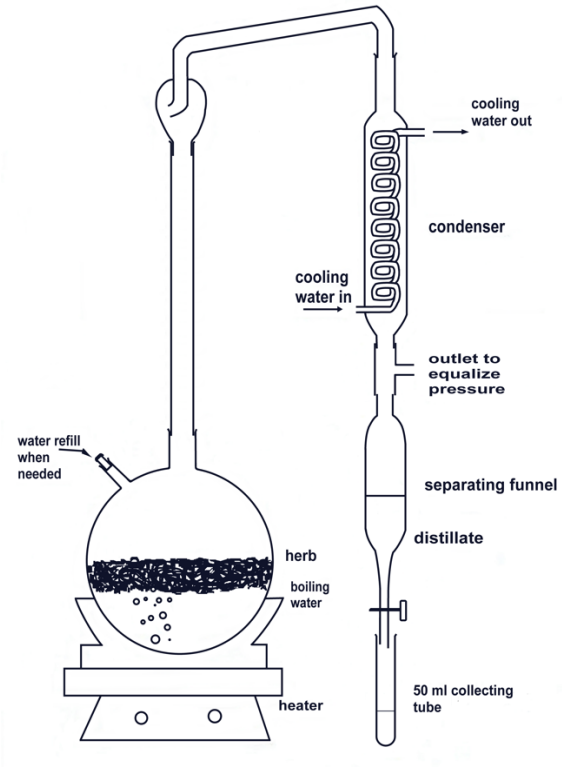


Fig. 4.14: Experimental setup of the hydro distillation process

4.3.3.7.2 The amounts of VOCs extracted through hydro distillation: A comparison

To give meaningful comparison, the heater temperature was adjusted so that the steam flow rate was about the same as that found in the steam distillation experiments described above. So, heater temperature was adjusted so that the distillate was also collected at a rate of about 50-ml every 40 minutes.

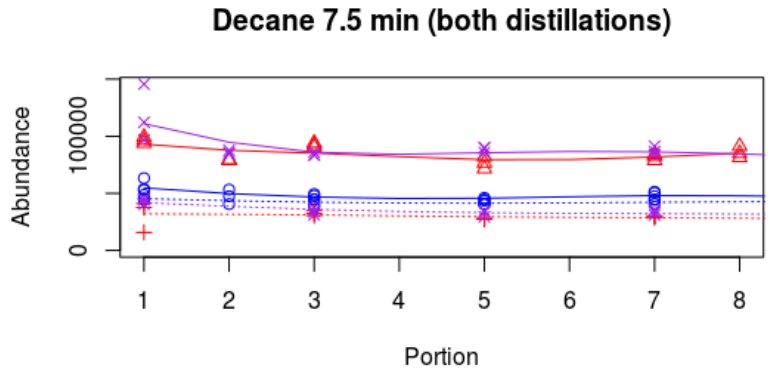
Again, different distillations were done using different amounts of PV herb as starting material: 15 g, 25 g and 35 g. GC-MS analysis was done in triplicates for each portion of the distillates collected. Chemical compounds were identified and data on abundances were monitored and summarized in Fig. 4.15, where the results for both the hydro distillation and steam distillation processes were shown together for comparison. The abundances of four alkanes: Decane, dodecane, tetradecane and hexadecane were shown; they were chosen as they were positively identified. Same as observed from the

results of the steam distillation process, these compounds also came out from the herb continuously with about the same rate for as long as the experiments ran.

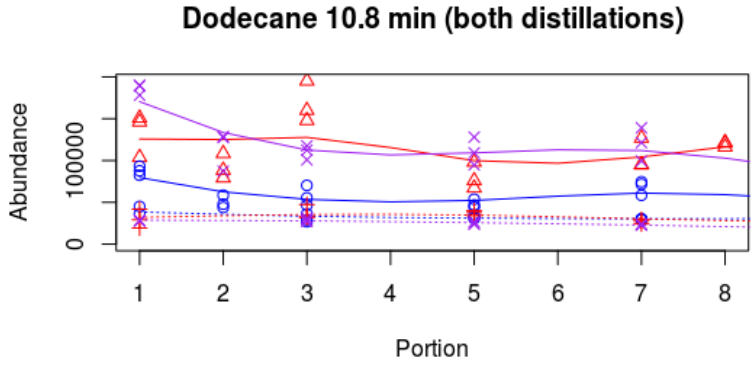
The first observation from these graphs is that the hydro distillation process, in general, extracted more volatile compounds than the steam distillation process, when the same amount of herb was used and for the same portion number. This means that the hydro distillation process is more efficient than steam distillation. This can be easily understood. In the steam distillation setup, the mass of herb hanging in the path of steam flow acts as an obstacle and re-condensed the VOCs-carrying steam. For hydro distillation, the procedure prescribes the removal of this mass of herb in the other flask hanging above the boiling water. With the obstacle removed, thus the extraction efficiency is increased.

Moreover, for hydro distillation, the amount of 15 g of PV herb was no longer the optimal value at which the maximum amounts of volatile compounds were obtained.

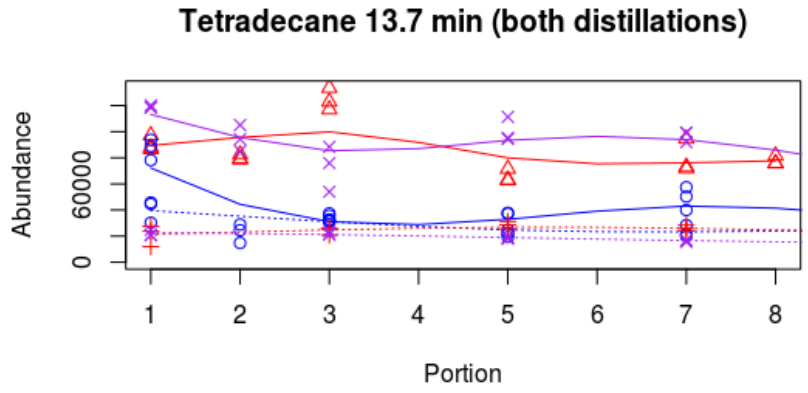
However, a careful examination of Fig. 4.15 showed that when the amount of herb used was increased from 25 g to 35 g (for the hydro distillation process), the amounts of VOCs extracted did not increase, and the two corresponding curves twisted together. Using both 25 g and 35 g of herb as the starting material extracted about the same amounts of VOCs.



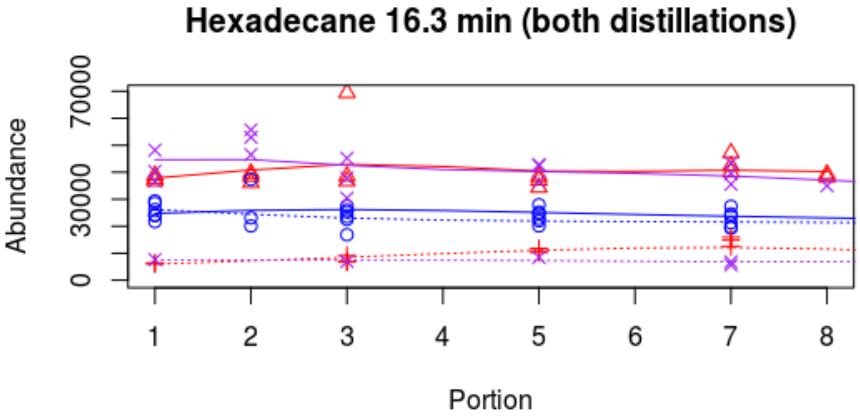
(a) Abundance of decane extracted from both distillation processes



(b) Abundance of dodecane extracted from both distillation processes



(c) Abundance of tetradecane extracted from both distillation processes



(d) Abundance of hexadecane extracted from both distillation processes

Fig. 4.15: Abundances of alkanes extracted from both distillation processes for comparison

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 times 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. The abundances are in arbitrary units.

This seemed to contradict the intuitive reasoning that, because the obstacle of the mass of herb in the steam path had been removed. The use of more herbs should lead to the extraction of more VOCs. However, through careful observation as the experiment proceeded, the reason was clarified. The experimental setup dictated that the inner space within the apparatus where steam flowed through was nearly a complete closed space except a tiny opening on the condenser side which facilitated a balance of pressure inside and outside. However, this tiny opening was too small to balance the pressure if the heating was immense to produce a high steam vapor pressure. So, to ensure safety, the heating needed to be limited so that the convection of the boiling water was weak. When only 15 g of herb was used, the convection of boiling water still had enough energy to carry the herb into the convection current. However, when 25 g or 35 g of herb were used, the convection current was no longer energetic enough to move the mass of herb floating at the surface. Bubbles of steam could only burst through it. So, the same situation as in the case of steam distillation occurred. A mass of herb existed in the steam flow path, not inside the flask hanging above in the steam distillation case, but at the boiling water surface. So, saturation of extracted VOCs occurred again. The implication of this observation was the need of a good compromise in the drug manufacture process design in how much herb used, how intense the water heating, and the diameters of the flasks and tubing.

Finally, because the abundances measured by GC-MS depend on the design and the settings (Sec. 4.2.2.4) of the GC-MS machine, comparison with results from previous hydro distillation studies by other researchers is not possible. Another reason was that previous research works which used hydro distillation for the same purpose as this project were not found in the literature.

4.4 Discussion

The results of this chapter and those of the next chapter are closely related. Considering them together and linking up of corresponding figures give useful information on the

design of a drug manufacturing process using the VOCs as the materials, such as what the optimal point during a distillation process to collect the required VOCs. Therefore, it should be more informative and productive to engage a combined discussion of both chapters together. This is the natural choice. This discussion will thus be postponed to the last chapter, Chapter 6, after results of the biological part of the experiments were reported in the next chapter.

However, there is one issue which does not link with the biological experimental part and thus, can be brought up and discussed here instead. It is the issue about the identification of the herb. Different papers on the phytochemistry of PV reported different lists of the chemical constituents; the chemical compositions reported were not fixed (Golembiovska et al., 2014, Yang et al., 2013, Morteza-Semnani et al., 2006). Therefore, there is no formal or authoritative unique combination of chemical compounds to give the formal and authoritative definition of what a "real" PV plant is.

Thus, in many research articles (Jiao et al., 2018, Zhao et al., 2017, Fang et al., 2017, Cohen, et al., 2017), the researchers just ignored this shortcoming and omitted the herb identification procedures all together and depended on the sources from which they bought the herb. Some researchers resorted to authority by quoting a judgement by an expert or a professor (Chen et al., 2012). In the identification procedure prescribed by the Chinese Pharmacopoeia (2010), it just used the identification of a single compound, rosmarinic acid for the identification of the whole herb. This is a flaw obviously because rosmarinic acid is not only found in PV but in many other plants as well. However, this highlights the difficulty described above.

4.5 Conclusion

This chapter reported the experiments which were conducted to study the characteristics of the VOC extracts from the distillation of the PV herb. It was found that the VOCs continued to evolve even after the distillation process had been in progress for a long time. Depletion was only observed for rarer compounds.

When depletion with respect to the aging of the VOCs during storage after the distillation processes finished was concerned, quite unexpectedly, some compounds (alkanes in the experiments) did show enrichment instead. This points to the fact that internal chemical reactions might have taken place in the distillate during storage.

Some rarer VOCs did show depletion with aging of the distillate though.

A comparison of the VOCs extracted by hydro distillation with those extracted by steam distillation indicated that the hydro distillation was more efficient. Moreover, this part of the experiments verified the postulate suggested to explain the saturation phenomenon of extracted VOCs, when more herb was used. This saturation phenomenon reduced extraction efficiency in steam distillation. However, this problem of efficiency reduction remained in hydro distillation.

Chapter 5: Anti-tumoral Activity of the Volatile Organic Compounds of *Prunella vulgaris*

5.1 Introduction

5.1.1 Outline of this chapter

This chapter reported the materials and procedures, aiming to explore the cytotoxicity of the VOCs of PV on cancer cells from the SCC154 cell line. To study the aging effect of the distillate on the cytotoxic efficacy, extra steps were needed to prevent microbial growth to interfere with the test results. The methods of doing so were described in the next section.

Then, the results will then be reported on the following issues:

1. *Dosage dependence of cell cytotoxicity;
2. Cell cytotoxicity of different distillate portions obtained at even time intervals of during distillation;
3. Cytotoxicity of distillate when different weights of herb were used in distillation;
4. Time depletion effect (Aging of the distillate);
5. Cytotoxicity of VOCs extracted from Hydro Distillation: A comparison with steam distillation;
6. Anti-oxidative activity of the PV distillate.

*: item 1 addressed the main aim stipulated in Sec. 5.1.3.1.

5.1.2 Opening remarks

Previous chapter reported the experiments on the characterization of the VOCs extracts of PV during distillation. This chapter switched the attention to report on the biological experiments. This set of experiments established the efficacy of the VOCs of PV for their anti-tumoral activity.

In the past decades, the interest in the study of the PV herb for its anti-tumoral efficacy persisted, so that articles which discussed this issue emerged continually (Govind, 2011, Sarangi and Padhi, 2014, Huang et al., 2015, Zhou et al., 2017, Bijauliya et al., 2017, Jiao et al., 2018, Mak, 2021). The chemical constituents of PV which were identified to be responsible for the anti-tumoral activities were, for examples, polysaccharides (Hao et al., 2016, Wang et al., 2014b, Feng et al., 2010b) and triterpenoic acids (Bai et al., 2015, Lee et al., 2008, Gu et al., 2007a). This topic was discussed in detail in Chapter 2, so, they will not be further discussed it here. Note that these compounds were non-volatile.

This chapter reports the biological experiments to elucidate the cancer cell cytotoxicity of the VOCs of PC.

5.1.3 Aims & objectives

5.1.3.1 Main aim

This study explored the therapeutic[2] efficacy of the VOCs of the herb, and specifically, the aim concentrated on establishing the efficacy of the anti-tumoral activity of all the VOCs as a whole cooperatively of PV. To achieve this aim, the objective of the experiment was to establish the fact of the dosage dependence of the anti-tumoral effects of the VOCs of PV. It is necessary to point out, however, that because this project is limited to use in vitro method alone, the finding is preliminary and its needs further research based on, say, in vivo experiments and clinical studies to conclude more firmly. See Sec. 6.1.4 for further discussion.

[2] Note that the prestigious Cambridge dictionary defines the word “therapeutic” as “causing someone to feel happier and more relaxed or to be healthier”. So, this word is used in this project more casually than to limit the meaning so narrowly to a requirement to establish a proof for confirmed clinical use. For a more stringent requirement to confirm suitability for clinical use, as required by the Australian Therapeutic Goods Administration or the Food and Drug Administration of the United States of America, more detailed discussions can be found in Sec. 6.1.3.

5.1.3.2 Secondary aims

Further works were to study how this anti-tumoral effect is affected by the various independent factors; for examples, the collection time to get the VOCs during distillation, how this effect varied when the VOCs continuously vaporized during storage after extraction (the aging effect of the distillate), how this effect varied when different initial weights of herb were used, what the differences of this anti-tumoral effect were when hydro distillation was compared with steam distillation. These experiments aided better understanding, and thus could facilitate a better design of drug manufacture processes which use VOCs as drugs.

The final part of the experiments explored the anti-oxidative activity of the VOCs. Besides playing a key role in an inflammation process, the presence, and their oxidative activities of reactive oxidative species (ROS) play an important part in the micro-environment of tumor development. The examination of the anti-oxidative effect of the herb (Hwang et al., 2013a, Choia et al., 2016, Ahn et al., 2018) is useful to understand the etiology of diseases, including cancer. So, the aim was to establish the efficacy of this anti-oxidative activity of the VOCs of PV.

5.1.4 Perspective and theme of this study

This chapter concentrated on reporting the experiments and the findings around the anti-tumoral activity of the VOCs of PV. In this aspect, the most popular approach to explore anti-tumoral activities of drugs by research in the past was adopted. This approach probed the effect on cell viability of the cancer cells due to the treatment of the herb using colorimetric assays.

The theme of this project did not identify all individual chemical constituents of the PV herb. The study looked at the cooperative effect of all the VOCs in PV herb as a whole.

5.2 Materials and methods

5.2.1 Cell viability tests

There are different assays which can be used to probe the cell viability of cancer cells when they are treated with drugs under test conditions. Colorimetric assays (Mossmann, 1983) were the first methods used for this purpose; however, it remains a favorite used by researchers. The reasons are that they are simple, convenient, and rapid to use and their low costs.

Among the colorimetric assays to probe the cell viability, those based on tetrazolium-based salts (Stockert et al., 2018) were the most popular. The assay used in this study, the Cell Counting Kit-8 (CCK-8), is one of them. The test kit was purchased from Sigma-Aldrich (product id: 96992). The cancer cell line used was the oral squamous cancer cell line SSC154, which was obtained internally from the university biology laboratory. The cancer cells were kept in a DMEM medium supplemented with 10% (v/v) FBS (fetal bovine serum) in a humidified incubator with the inside atmosphere with 5% CO_2 and at a temperature of 37^{o}C.

The cell viability test was carried out according to the procedure supplied by the vendor, which was packaged as part of the product. To summarize, 100 µl of cancer cell suspension which was diluted to a concentration of 5000 cells per well was dispensed to the wells of a 96-well tissue culture plate. This was done in triplicate sets and then the cells in the plate were pre-incubated for 24 hours according to the same conditions stated above. After incubation, 10 µl of various concentrations of the PV distillate was added to the medium in the wells. The concentrations of the PV distillate used to treat the cells were the full-strength for all tests, except for the test of dosage dependence which is to be discussed next. The plate of test samples was further incubated for 48 hours. CCK-8 reagent was then taken out from the refrigerator and allowed to thaw at room temperature for half an hour. Thawed CCK-8 was then added to each well.

Incubation was allowed for another 3 hours. Finally, the absorbance at 450 nm, which reflects the cell viability, was measured using a microplate reader.

Thus, the outcomes are the optical density (od) as measured by the microplate reader (in arbitrary units). By varying the different independent variables, such as the strengths of the distillate, the distillate portions, weights of herb used in distillation, storage period lengths, different diagrams can then be drawn to show the dependency of the cell viability with the changes in these independent variables.

5.2.1.1 Specific to the dosage dependence of the cell viability

Full strength distillates from distillation using 25 g of PV herb were used in the experiments. From the results described in the last chapter, some rarer VOCs were not detected if smaller amounts of herb were used. So, 25 g was the optimal quantity to include all the chemical constituents which might be detrimental to the cancer cell viability. To explore the dosage dependence, the distillate was diluted with deionized water to concentrations of 1, 0.5, 0.1, 0.01 & 0.001 v/v of the full-strength solution; so, the complete set consisted of four sets of three wells each. In additions, some wells were reserved to conduct the positive and negative controls.

5.2.1.2 Prevention of microbial growth

In exploring the aging effect of the PV herb, the distillate needed to be stored for a lengthy period before the actual tests were carried out. It was then imperative to ensure that any microbial contamination did not interfere and spoil the experimental outcomes. Bacteria and other microbes were not allowed to grow. This was important in the cell viability tests since the colorimetric reagent did not only measure the metabolic activities of the cancer cells but also those of the microbes also. To circumvent this problem, additional precautionary steps needed to be implemented to eliminate this interfering factor.

To facilitate this, before the experimental setup was assembled, all the glassware was washed and then flushed with ethanol.

Then the apparatus was dried and, at the same time, sanitized inside a 110^{o}C oven for two hours. When the distillate was later collected, it was collected using cryogenic tubes of 1 ml each for the cell cytotoxicity tests, instead of using the 50 ml tubes for the GC-MS analysis. The use of different sets of tubes of different volume for the two sets of experiments (chemical and biological) was to avoid crossed contamination and also because a much smaller amount of distillate was needed to do the cell cytotoxicity tests. The tubes aimed for the biological tests were then capped and stored in a sealed container which was flushed with ethanol beforehand. The tubes will then be stored in a refrigerator which was set at a temperature of 5^{0}C until they were retrieved to do the tests on a later date.

Additionally, to further prevent any microbial growth during the storage of the distillate, an antibiotic gentamycin (part number: G1272) was purchased from Sigma-Aldrich and added to the cancer cell culture medium one day before the experiment (The vendor specifies that the antibiotic product is stable for five days at 37^{o}C). The dosage of the antibiotic solution to treat the culture medium was adjusted to 2 µl/ml according to the supplier's recommendation (1 to 5 µl/ml).

5.2.1.3 Scientific controls

Both positive and negative control measures were incorporated in the experiments to minimize the effects of confounding variables. By assigning some wells on the tissue culture test plate to contain cell culture medium without adding the PV distillate provided the negative controls. By assigning some wells on the test plate to contain medium with no cancer cell loaded (which is similar to the case when all the cancer cells were dead) provided the positive control.

5.2.2 Anti-oxidative test

Anti-oxidative activity of the VOCs from PV was examined using the Folin-Ciocalteu (FC) reagent which was purchased from Aldrich-Sigma. Folin-Ciocalteu reagent is a popular standardized method in the food industry to measure the anti-oxidative activities of food products and diet supplements (Prior et al., 2005). This study adopted the procedure according to an article by Ainsworth & Gillespie (Ainsworth and Gillespie, 2007). Briefly speaking, the procedure consisted of the following steps: 100 µL of the distillate was added to a 2-ml microtube. Then, 200 µL FC reagent was added. The mixture was vortexed thoroughly before 800 µL 700 mM sodium carbonate solution was added. The whole mixture was allowed to incubate at room temperature for two hours. 200 µL of the sample was transferred from the assay tube to a 96-well microplate. Samples were taken and tested in triplicates. The absorbance of each well, which reflects the anti-oxidative activity, was measured by a microplate reader in the wavelength range around 765 nm (in arbitrary units).

5.2.3 Statistical analysis

The sets of data collected (absorbance measurements) were analyzed statistically by the ANOVA method. The online facility

https://www.socscistatistics.com/tests/anova/default2.aspx

was utilized to do the analyses of data collected. The usual significance level threshold, $p = 0.05$, was chosen throughout all the experiments.

5.3 Results

The results as reported in this chapter (and the last chapter) were published in the following articles (Mak and Walsh, 2021, Mak, 2022, Mak, 2023).

5.3.1 Cell viability tests

5.3.1.1 Dosage dependence

The procedure described above in sub-section 5.2.1.1 was followed to examine the cell viability on the application of PV distillate.

Fig. 5.1 showed the result on the dosage dependence. The od shown on the y-axis was in arbitrary unit and showed the cell viability. To statistically analyze the significance of the result, one-way ANOVA was applied. The null hypothesis was that the cancer cell cytotoxicity was independent to the strength of the PV solution used to treat, which meant there was no dosage dependence. The ANOVA analysis gave an f-ratio value of 36.5 with the corresponding p-value of less than 0.00001. Thus, with the significance threshold chosen as the usual 0.05, the conclusion was that the null hypothesis was rejected and the cell cytotoxicity by the PV distillate on the SCC154 cancer cells was dosage dependent. However, as seen from Fig. 5.1, the difference in the cell viability due to the treatment with PV solutions of different concentrations was only slight.

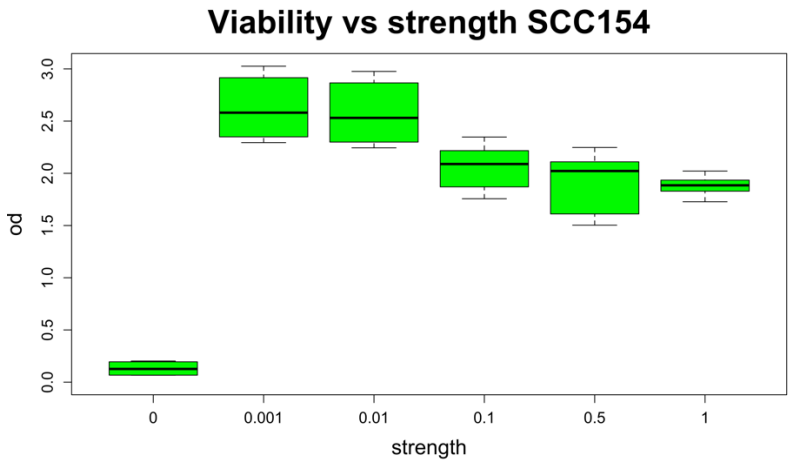


Fig. 5.1: Cell viability plotted against the strength of the PV distillate

(od stands for optical density)

Different strengths were obtained by repeatedly diluting the full-strength solution with appropriate amounts of de-ionized water (thus, concentrations from 1, 0.5, 0.1, 0.01 to 0.001 v/v). Cell viability was indicated by the optical density, indicating the absorbance. The full-strength distillate was from distillation using 25 g of herb. Portion 1 was used. From this description, the "strengths" on the x-axis are relative concentrations as compared with the full-strength solution. The p-value for this set of data was, p<0.00001, showing that the dosage dependence of cell viability was significant. The usual significance threshold (p<0.05) was used to reject the null hypothesis.

Note that for the case of the distillate strength = 0.001 (obtained by diluting the full-strength solution with 999 times, v/v, of de-ionized water), the strength was so weak that the data there can be regarded as the negative control data. For the case where the strength = "0", the value of 0 does not really mean the strength; it was assigned just to provide a place in the graph to express the positive control data. No cancer cells were loaded into the tissue culture medium; thus, it was similar to the situation that all cancer cells were killed.

5.3.1.2 Cell cytotoxicity of different distillate portions

Experiments were also carried out using distillate portions obtained successively during distillation using 25 g of herb. (An explanation of the meaning of the term "portions" can be found in Sec. 4.2.2.2). 10 µl of distillate samples from different portions were added to the cell culture medium in each well. The procedure described in sub-section 5.2.1 was followed. The result is shown in Fig. 5.2. Again, one-way ANOVA analysis was again applied to explore the statistical significance. The usual significance level was chosen, p = 0.05.

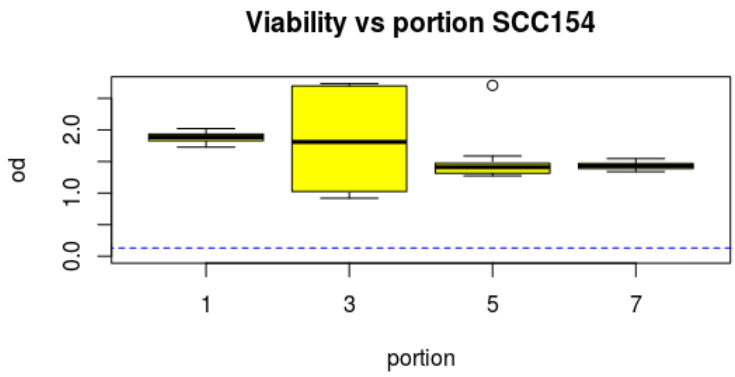


Fig. 5.2: Cell viability of cells taken at different time points

(at about 40 minutes intervals; od stands for optical density)

This figure shows the cell viability of SCC154 cells treated with different portions of distillate. The bar and error bars show the spreads of the data points as calculated by the charting software, R. 25 g of herb was used to do the distillation. The blue dotted line indicates the background level (average) obtained by the positive control. The value for the negative control can be inferred from Fig. 5.1. Using ANOVA analysis, the p-value is 0.001329, which is less than 0.05, the usually chosen significant value, which is also used here. The optical density indicating the cell viability shown on the y-axis is of arbitrary unit.

The null hypothesis was that different distillate portions obtained during the distillation process caused no difference in their cancer cell cytotoxicity. The f-ratio value obtained by ANOVA analysis was 5.825 and the corresponding p-value was 0.001329. Thus, the null hypothesis was rejected; different distillate portions did cause different cell cytotoxicity. Since more volatile compounds depleted during the early stage of the distillation process, the result hinted that the anti-tumoral activity was due to compounds which had lower volatility, although no effort was spent to identify them. Also, Fig. 5.2 shows that the differences in cell viability among different distillate portions were only slight, the differences in cell cytotoxicity due to distillates collected during the whole distillation process were only small.

5.3.1.3 Cell cytotoxicity of distillate when different weights of herb were used

Different weights of PV herb were used as the starting material for different distillation runs. The full-strength distillates of such distillations were used to treat cancer cells. The procedure described in sub-section 5.2.1 was followed to evaluate the cell viability, which was measured using the CCK-8 test kit. Fig. 5.3 shows the result.

One-way ANOVA analysis was used to explore the statistical significance of the result. The null hypothesis was that there were no differences in cytotoxicity due to distillates obtained from distillations using different weights of herb to start with. The statistical analysis gave an f-ratio value of 110.66 and a p-value of less than 0.00001. For the significant threshold value of 0.05, this meant that the null hypothesis was rejected and the differences of cancer cell cytotoxicity due to the treatment using different weights of herb were significant. By comparing the cell viability as observed from Fig.5.3 with the abundance curves in Fig. 4.6 and 4.7, they were consistent with each other. In Fig. 5.3, the cell viability was the least when the distillate was obtained from 15 g of herb. On the other hand, from Fig.4.6 and 4.7, the abundances of VOCs were the highest for the case when 15 g of herb was used. This comparison further supported the statement that the VOCs of PV was cytotoxic to cancer cells SCC154 in a dosage dependent manner. However, although the conclusion of the least cancer cell viability when 15 g of PV was used was obtained by both a visual observation of Fig. 5.3 and by statistical calculations that the sample means of the optical densities for the 5 g herb group and for the 15 g herb group were 1.5005 and 1.479 g respectively, further ANOVA analysis using these two groups of data alone gave a p-value of 0.47995. This means that the difference in cell viability between these two groups (5 g and 15 g herb used) was not significant (significance threshold taken as p = 0.05).

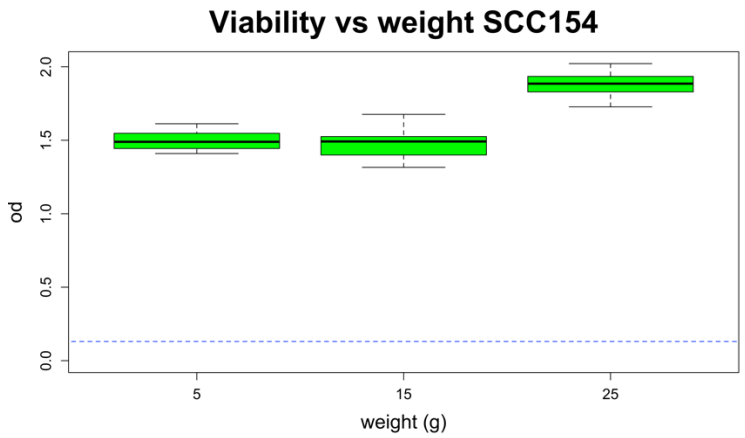


Fig. 5.3: Cell viability of cells treated when different initial weights of herb were used

(od stands for optical density)

This figure shows the cell viability of SCC154 cells treated with distillates prepared from different amounts (loading) of PV herb used in the different distillations.

Portion 1 in each distillation was used. ANOVA analysis gave a p-value of less than 0.00001. The optical density shown on the y-axis is of arbitrary unit. The usual significance threshold ($p<0.05$) was used to reject the null hypothesis.

The blue dotted line indicates the background level (average) obtained by the positive control. The value for the negative control can be inferred from Fig. 5.1.

5.3.1.4 Time depletion effect (Aging of the distillate)

After the VOCs were extracted from the herb by distillation, there should be unavoidably some time lag between their extraction, their packaging in sealed form in the manufacturing process, and then being supplied as medicine to patients. Thus, there is a question of whether there is any change in the therapeutic efficacies of the VOCs due to this time delay.

This was the aim of this part of the investigation.

After the distillation process, the distillate samples were sealed and stored in 1-ml cryogenic tubes at 5^{o}C. Then, cell viability tests using the CCK-8 test kit on the SCC154 cancer cells were performed once a week after the distillation process, using the stored distillate.

This was done for eight consecutive weeks, which should be sufficiently long time for any drug manufacturing procedures. The results were shown in Fig. 5.4.

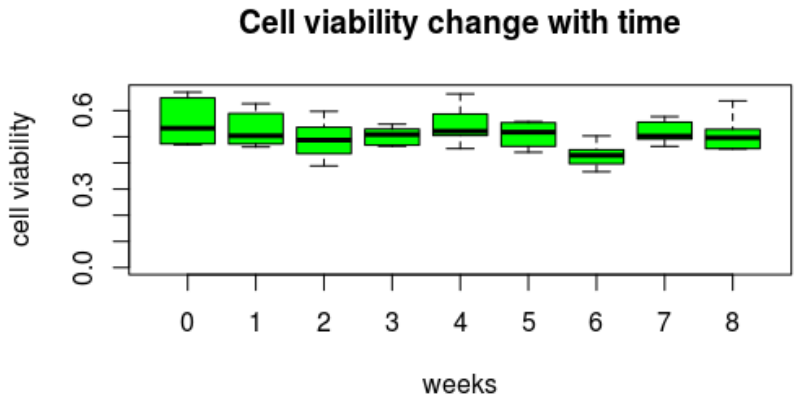


Fig. 5.4: The cell viability changes with time during storage

The storage time was up to eight weeks after VOCs extraction. Portion 1 was used and 25 g of herb was used. The usual significance threshold (p = 0.05) was used to reject the null hypothesis.

The cell viability did not change much for the whole period of eight weeks of the experiment. Using one-way ANOVA to statistically check the results, using the null hypothesis that there were no significant differences in the cell viability data for the whole period of eight weeks, it gave a p-value of 0.0749. When the usual threshold p-value of 0.05 was adopted, the null hypothesis was not rejected: The cell cytotoxicity due to the VOCs in PV did not change during this period. This observation implied that the cell cytotoxicity of the PV VOCs was due to those less volatile compounds, as those

more volatile compounds dissipated more quickly in days, as reported in the last chapter.

5.3.1.5 Cell cytotoxicity of VOCs extracted from hydro distillation

In the last chapter, the last sub-section discussed the characteristics of extracted VOCs obtained from the hydro distillation process.

For completeness, this sub-section examined the anti-tumoral activity of the distillates obtained from this alternative distillation process. The CCK-8 kit was again adopted to monitor the cell cytotoxicity of PV herb on cancer cells from the cell line SCC154. Fig. 5.5 showed the results which compared the cell viability due to the distillates from both distillation methods.

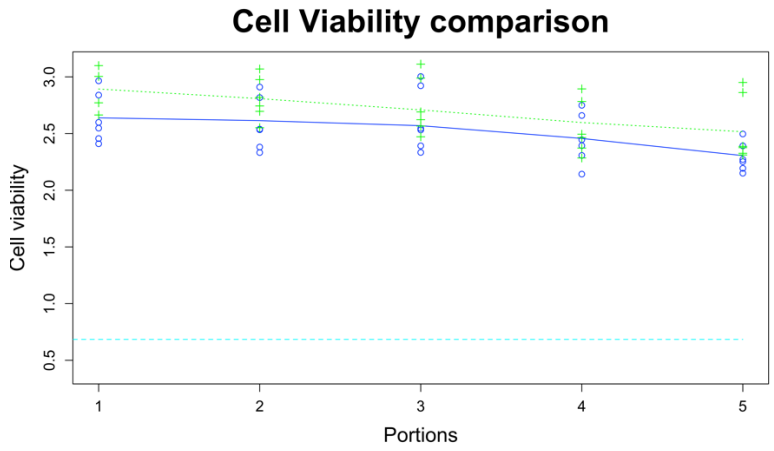


Fig. 5.5: The cell viability of the cancer cells SCC154, treated by distillates from both distillation processes

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 five portions collected during the distillation *processes were used to plot the curves. The cyan dotted line indicates the background level (average) obtained by the positive control. The usual significance threshold ($p<0.05$) was used to reject the null hypothesis.*

The graph showed the same pictures as reported earlier in this chapter. Although the cell viability remained approximately the same for distillates taken from the portions of both distillation processes, the cell cytotoxicity due to portions collected later in the processes was slightly higher. Statistical two-way ANOVA test was used to analyze the differences among the portions gave a p-value of 0.0048. The null hypothesis was that there was no difference in cell cytotoxicity due to distillates from different portions. If the conventional threshold value of p=0.05 was again applied, this null hypothesis was rejected. There were significant differences in cell cytotoxicity among samples of distillate taken from different portions. This agreed with the results in sub-section 5.3.1.2.

Another observation was that the cell cytotoxicity due to distillates from the hydro distillation process was higher. This result agreed very well with what was concluded previously. The previous chapter showed that the hydro distillation process was more efficient to extract more VOCs. Cell cytotoxicity due to products obtained from hydro distillation being higher meant that the cell cytotoxicity of the VOCs from PV herb on cancer SCC154 cells behaved in a dosage dependent manner.

To check the statistical significance, the two-way ANOVA method was applied to the differences in cell cytotoxicity due to distillates from the two different distillation methods. The null hypothesis was that there was no difference in cell cytotoxicity due to distillates from the two distillation methods. The p-value calculated was 0.0061, concluding that the null hypothesis was rejected. This confirmed that there was a significant difference in the anti-tumoral effects of the distillates obtained from hydro distillation and from steam distillation. Distillate from hydro distillation was more potent.

5.3.2 Anti-oxidative activity of the PV distillate

As the ROS plays an important role in the cell environment for the development of cancer, many previous research activities explored the anti-oxidative effectiveness of

drugs as part of their research of anti-tumoral effects of drugs (Liu and Ng, 2000, Vostalova et al., 2010, Hwang et al., 2013a).

This study used Folin-Ciocalteu reagent to perform such a task to check the anti-oxidative capability of PV distillate. Only distillate from hydro distillation was tested.

The measurement of the absorbance in the wavelength range around 765 nm showed that there was no peaking of absorbance at the wavelength 765 nm. The measurement curve remained flat in this wavelength region. See Fig. 5.6. Using one-way ANOVA analysis to check statistically, the p-value was 0.994. Thus, the null hypothesis that there was no difference in the absorbance was accepted. The usual significance threshold (p = 0.05) was used to reject the null hypothesis.

The PV VOCs did not exhibit anti-oxidative activity.

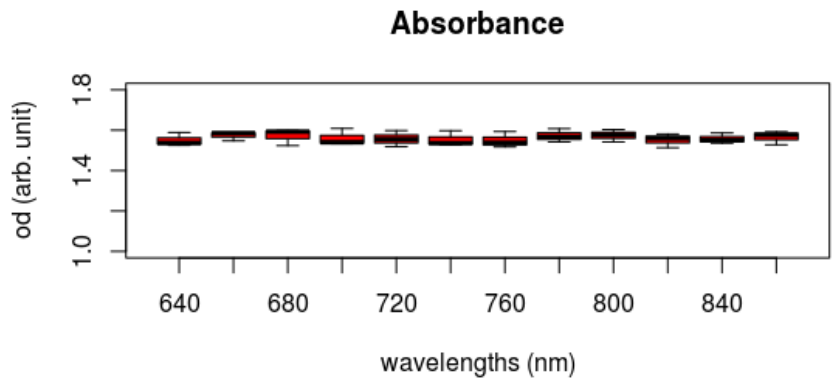


Fig. 5.6: Absorbance measurements around 765 nm in Folin-Ciocalteu test

The absorbance, which reflects the anti-oxidative activity, was measured by a microplate reader in the wavelength range around 765 nm. The weight of herb used to extract PV distillate were 15 g. The first portion of distillate was used. The usual significance threshold (p = 0.05) was used to reject the null hypothesis.

5.4 Discussion

The results of this chapter and those of the last chapter are closely related. Considering them together and linking up of corresponding figures give useful information on the

design of a drug manufacturing process using the VOCs as the materials, such as what the optimal point during a distillation process to collect the required VOCs. Therefore, it should be more informative and productive to engage a combined discussion of both chapters together. This will thus be postponed to the next chapter, Chapter 6.

5.5 Conclusion

This chapter reported the biological part of the experiments in this project. The experiments used the distillates containing the VOCs of PV to treat cancer cells from the cell line SCC154. Experiments demonstrated that the VOCs from PV were cytotoxic to the cancer cells in a dosage dependent manner.

The first experiment to achieve this objective of demonstrating the dosage dependence was by varying the strengths of the distillate used to treat the cancer cells.

The second experiment to support this objective was to monitor the different cancer cell cytotoxicity measurement values when the initial amount of herb used for distillation varied.

Then, the third experiment to provide further support for dosage dependence was to compare the cancer cell cytotoxicity of the distillates from the hydro and the steam distillation processes.

The part of experiments which examined the anti-oxidative efficacy of the VOCs of PV showed that they exhibited no anti-oxidative activity. This meant that the anti-tumoral effect was not related to the anti-oxidative effect.

Finally, discussion resulted from this experimental part will be postponed to the next and the last chapter, Chapter 6.

Chapter 6: General discussion and conclusions

6.1 Discussion

6.1.1 Literature review part

Very detailed discussions pertaining to the literature review part were undertaken and can be found at the ends of both Chapter 2 & 3. To reduce excessive redundancies, only a brief discussion will be carried out here, for completeness of this chapter.

6.1.1.1 No unified standard approach

There were difficulties to collectively summarize how effective the anti-tumoral effect of PV by doing, say, a meta-analysis on all the articles collected. The reason was that there was not a unified standard approach which the researchers designed their experiments, and there was not a unified metric to quantify the anti-tumoral effect.

Moreover, different research projects worked on different materials: Many researchers used the whole PV spica, but others used various chemical constituents of PV, such as the polysaccharides, and the triterpenoids, or specific promising chemical compounds such as rosmarinic acid and oleonolic acid, which are key constituents of PVs.

The researchers also investigated different types of cancer and used different extraction methods and different solvents such as ethanol, methanol and water. Thus, different chemical components, in fact, were obtained. The detailed discussion on the lack of a unified approach in research can be found in sub-section 2.4.1. This difficulty in summarization was also demonstrated in Table 2.1.

6.1.1.2 Difficulties in the scientific research in Chinese medicine

The lack of standardization of the starting materials, and the numerous possible variations in the experimental designs for research work in Chinese medicine impose a

great difficulty to the research in Chinese herbal medicine. This sort of difficulties is faced by all researchers who investigate into Chinese herbal medicine, and it is not easy to overcome. It also causes problems in the repeatability of the experimental works and the overall summarization of all results among the different studies in the literature. The discussion of this problem pertaining to the anti-tumoral effect of PV was detailed in Sub-section 2.4.1. This lack of standard approach and lack of well-defined starting material impose similar problems in the research of anti-viral activities of PV too. The detailed discussion of this problem pertaining to anti-viral activities of PV can be found in Sub-section 3.5.2.

Indeed, these difficulties reflect the different paradigms of the authentic Chinese medicine ideas and the Western therapeutic scientific research. The traditional Chinese medicine ideas cannot easily fit into the framework of Western scientific research.

6.1.1.3 Relevance of the review of the anti-viral activity of VOCs of PV

The original theme of the project was on the anti-tumoral activity of VOCs of PV. Then, COVID-19 pandemic emerged at around the beginning of 2020. The pandemic attracted global attention, and research on this newly emerged virus picked up its momentum. The inclusion of the review of the anti-viral activities of the VOCs of PV in this project is a preparation to explore future research direction into the potential of using the VOCs of PV to treat viral, especially influenza-like diseases. In Chinese ethnomedicine, a traditional remedy (Chen and Chen, 2009, Chen, 2016b) in the southern Chinese province of Guangdong uses the rising steam from a boiling decoction of PV to treat such diseases by the patient inhaling the steam. This provides a support from the literature for such a research potential to yield fruitful outcome.

6.1.2 Experimental part

The last two chapters reported the experimental findings of the chemical and biological experiments. Here, the implications of those findings will be discussed, specifically on

the drug manufacture process when the VOCs of PV used as the materials to produce medicine, together with other miscellaneous issues.

6.1.2.1 Implications to the design of a good drug manufacture process using VOCs as drug

A combination and comparison of the findings from the chemical distillation experiments and from the biological cancer cell cytotoxicity experiments enable the identification of the optimal point during the distillation process to collect the distillate which has the highest potency. Also, an optimal heating temperature (which dictates the steam flow rate) can be used to selectively extract certain target chemical compounds.

6.1.2.1.1 Stable extracted abundances and stable cell cytotoxicity

From the last two chapters, the findings were:

- The VOCs came out from the herb continuously with nearly constant rates even after the process had been carried on for a long period of time; as long as 14 hours in my experiments.
- The cancer cell cytotoxicity due to distillates sampled at different time points during the distillation process maintained at about the same level, but a little bit higher for distillates collected at later time.
- The cancer cell cytotoxicity of the distillates also maintained at about the same level during the whole storage period of up to eight weeks in the experiments.

Since the cell cytotoxicity to cancer cells was not much different, whether the distillates were obtained from whatever stages of the distillation process, the experimental results hinted that the cell cytotoxicity was due to the less volatile components of the VOCs, because, in Chapter 4, it is reported there that lighter VOCs were observed to deplete early in the distillation process. The same conclusion was also hinted by the fact that the cell cytotoxicity remained about the same, independent to the length of the storage

period (up to eight weeks in this experiment). The lighter VOCs were expected to vaporize faster than the heavier ones and the heavier ones are easier to preserve.

These collectively imply more relaxed requirements for the design of a drug manufacture process. The manufacture process may be then less costly.

6.1.2.1.2 The existence of an optimal amount of herb used

An observation was that there was an optimal amount of herb used which gave the maximum abundance of VOCs. In the experiments, this amount was about 15 g of PV herb used for both the hydro and steam distillation cases. A drawback of the experimental setup was that the cross-section of the steam flow path was small (from ~3 cm across for tubing to ~15 cm across for flasks). This limited the flow of VOCs and impeded their collection. This dictated the optimal amount of herb used to be 15 g. For a different setup, this optimal amount of herb used will thus be different. It is not hard to understand and speculate that as the cross sections of the different parts in the steam path are larger, this optimal value will then be larger. So, for the design of a good manufacture process, a careful examination of the apparatus setup is needed.

6.1.2.1.3 Preferentially select or enhance some chemical components in the extract

Another experiment studied the effect of changing the steam flow rate by changing the heater temperature. The following observation was noted: The smaller steam flow rate (corresponding to lower heating temperature) extracted lighter compounds (corresponding to shorter retention time in the GC-MS coil) more efficiently; while the opposite, vis-à-vis, heavier compounds were extracted more efficiently by using larger steam flow rates. The switching point occurred for compounds with about a retention time of ten minutes in the coil of the GC-MS machine. For alkanes, this roughly corresponded to dodecane. What this implied in the drug manufacture process was that the change of the steam flow rate could be used to preferentially select or enhance the collection of some chemical components in the extract.

6.1.2.2 Anti-oxidative activity of VOCs of PV

This study used the Folin-Ciocalteu reagent to check the anti-oxidative activity of the PV distillate. The finding was that the PV distillate did not exhibit any anti-oxidative activity. From previous research (Hwang et al., 2013a, Wang et al., 2019), the anti-oxidative activity of PV extract was mostly attributed to the phenolic contents of higher molecular weights, which were selectively difficult for distillation to extract. This also meant the VOCs of PV did not consist of phenolics.

6.1.3 Limitations of this study

This study successfully confirmed the anti-tumoral effect of the VOCs in PV.

More sophisticated techniques like flow cytometry used with Annexin V FITC/PI, immunochemistry, and others, which were described in Chapter 2 to confirm the anti-tumoral activity of the VOCs, were unavailable and thus inaccessible to this project due to administrative and funding reasons; so, the use of a tetrazolium salt-based colorimetric assay, CCK-8, was the only technique adopted to quantify the cell cytotoxicity. However, colorimetric assays were frequently used in the past to examine the cancer cell cytotoxicity. Some examples are given here (Bai et al., 2015, Cho et al., 2015, Wang et al., 2014a, Fu et al., 2012, Woo et al., 2011).

Moreover, albeit the availability of just a simple colorimetric technique, the anti-tumoral activity was supported by three separate experiments of different designs in this study.

Secondly, it is of great value if the mechanisms behind the anti-tumoral activity can be sorted out; for example, in the original research plan, new generation sequencing (NGS) was planned to find out the differential non-coding RNA expressions of the cancer cells after the treatment by the distillate. However, because of a lack of support funding, this

plan was finally aborted. Consequently, the work looking at the anti-tumoral mechanisms was not done.

The third limitation was that the analysis of the chemical composition of the VOCs of PV was only done using Gas chromatography – Mass spectroscopy (GC-MS). Other techniques, like Nuclear magnetic resonance (NMR) spectroscopy or supercritical fluid chromatography (SFC), were not available to this study to further clarify the chemical composition of the VOCs of PV. (This was also one of the reasons why this project did not attempt to identify the chemical composition of the VOCs, as what some phytochemistry studies do.) Thus, an interesting follow-up investigation to further identify the chemical compounds responsible for the anti-tumoral activity could not be carried out. Subsequently, the investigation to clarify the synergistic effect of these anti-cancerous compounds also could not be pursued.

The fourth limitation was that only oral squamous cancer cells from the cell line SCC154 were used in this project. Therefore, the anti-tumoral activities of the VOCs of PV against other cancer types were not explored in this study. This was an availability issue also.

The fifth limitation was that the experimental part of this project was confined to the use of in vitro method only. From the procedures undertaken by government authorities like the Australian Therapeutic Goods Administration or the Food and Drug Administration of the United States of America, to ensure the safety and effectiveness of pharmaceuticals, these authorities do not depend on experimental results from in vitro experiments alone, which are only regarded as preliminary evidence to approve drugs. More experimental evidence needs to be provided from, firstly, in vivo experiments, secondly, clinical trials among a small sample, and thirdly, among a much bigger population. These procedures may take as long as a decade before drugs are granted approval for the marketing and sale to the public and can be of clinical use. This process is designed to protect public health by ensuring that drugs meet rigorous standards for safety and efficacy. From this discussion, the conclusion in this study that

the VOCs of PV exhibit anti-tumoral activities should be regarded with such attitude that it is a very preliminary result and further in-depth studies are essential to confirm its feasibility to be used as drugs.

The sixth limitation was that thorough statistical analysis was not done for the characterization of the distillation process. For the distillation experiments carried out in this project, no statistical analysis could be practically done, because each distillation took one day to finish in the experimental design. Therefore, to repeat the distillation runs for a statistically meaningful number of times needs prohibitively long time. This is quite impractical. Detailed discussion can be found in Sec. 4.2.2.7.

The seventh limitation relates to the variations in the chemical compositions of the herb originated from different geographical areas. See Secs. A1.1 & A1.2 for detailed discussion. It is helpful to understand how these geographical variations may affect the experimental findings in this study. However, due to limited source to obtain the herb, this exploration is not practical. Similar limitation exists to use different extraction methods to obtain the VOCs to explore how the use of different extraction methods impacts on the experimental results.

A final remark is that a shortcoming in the design of the scientific controls in Ch. 5 may not exclude the possibility that the low concentration of PV distillates may increase cancer cell proliferation/growth, while high concentrations of PV distillates inhibit cancer cell growth. Indeed, this possibility is ruled out by looking at Fig. 5.1, where the cell viability was plotted against the strength of the PV distillate. Comparing the viability for distillate of $1/1000^{th}$ strength (which means one part of the original distillate was diluted by 999 parts of de-ionized water, v/v) with that of $1/100^{th}$ strength shows that they are not too much different and the viability for distillate of $1/1000^{th}$ strength is just a little bit higher. It did not increase cancer cell proliferation/growth profoundly.

6.1.4 Future research directions

This study targeted the investigation of the VOCs of PV focusing on their anti-tumoral activity. However, other therapeutic efficacies of the VOCs of PV are equally important and useful to investigate. The anti-viral activity of the VOCs is a good direction. The original findings of the present study do not indicate that the VOCs of PV have significant anti-tumoral effects. By doing a review on the anti-viral effects, it is realized that investigation on the anti-viral effects of the VOCs of PV should provide more fruitful research outcomes. Anti-viral investigations of the VOCs of PV can extend to novel viruses such as SARS-CoV-2. This is currently a hot issue which attracts immense attention and interest in the research community, because of the ongoing Covid-19 pandemic.

Further suggestion for future research direction is to investigate therapeutic efficacies of the VOCs of other herbs, which are traditionally used to treat influenza-like diseases. They are good candidates to yield fruitful results. Some examples are *Chrysanthemum morifoliu, Agastache rugosa, Lonicera japonica,* and *Satis indigotica L.*

In this study, the theme was to purposefully avoid identifying individual chemical constituents (except just a few) inside the PV herb. However, identifying them and finding out the optimal ratios among them to achieve the best combined effect will provide a better understanding of the synergistic effect among them.

About the study on the use of VOCs for the manufacture of drugs, the present study is indeed insufficient, and can only be viewed as preliminary. Much further work is needed to expose further issues involved in the drug manufacturing design. For example, the encapsulation of the VOCs (Perinelli et al., 2020, Kose et al., 2021) by such methods, such as incorporating them into polymeric micro- or nanocapsules or inclusion complexes with cyclodextrins, is an interesting issue. This should be undertaken for the practical use of the VOCs as medicine.

Relating to this encapsulation issue, it may be interesting to mention two prior research articles (Nolkemper et al., 2006, Reichling and Schnitzler, 2011) which reported the

incorporation of the VOCs from PV into fat to form creamy medicaments, which could be applied to treat HSV-infected skin. Cream made this way by incorporating the VOCs of PV can be applied to treat skin tumors. This way of application to treatment of tumors was never mentioned before and thus further investigation is worthwhile.

Finally, a research team reported their studies on nanoparticle formation from acid-base reactions in an air flow tube environment (Chen et al., 2018a, Chee et al., 2019). The flow tube environment in their setup had very similar situation to the steam flow environment in my distillation setup. Their experiments and simulations found that the concentrations and sizes of nanoparticles formed, the humidity inside the flow tube, and the acid/base ratios were highly inter-dependent. The implication of their findings is that the acidity of the water used in the distillation process of this project should have great impact on the compositions of the extracted VOCs. It is impractical to expect drug manufacturers to use deionized water in their drug manufacturing processes. Therefore, further investigation is warranted to examine the effect of the quality of the water used, its hardness or acidity, on the contents of the extracted VOCs. However, the research of that team considered just two to three chemical compounds while there were hundreds of chemical constituents involved in this project (see Sec. A1.1). The number of possible chemical reactions is numerous and thus the situation is much more complex. The investigation is much more involved.

6.2 Conclusions

This project can be divided into two parts: The literature review part and the experimental part (See Fig. 1.2).

The literature review part is divided further into two subordinate parts: anti-tumoral activity and anti-viral activity of PV herb (non-volatile compounds).

The experimental part is also divided into two subordinate parts: distillation process to extract VOCs and anti-tumoral activity of the VOCs of PV.

The four subordinate parts are treated separately as below.

6.2.1 Literature review of past research articles

Detailed conclusions for the literature review part of this project were given at the ends of Ch. 2 & 3 respectively for the anti-tumoral and the anti-viral activities of the herb. So, it is repeated here briefly only for completeness.

To build up the background knowledge before reporting the experimental work, the historic research works in the past three decades on the PV herb were reviewed. Nearly all these works were on the studies of non-volatile chemical constituents of the herb, however. The areas of study included the chemical composition, the extraction methods, the use in agriculture, and the therapeutic activities. Attention was paid to the therapeutic activities, especially, the anti-tumoral activities and the anti-viral activities.

6.2.1.1 Articles on the anti-tumoral effects of PV extracts

In those articles which appeared in the past three decades and discussed the anti-tumoral activity, the most frequently investigated tumor types were breast cancer (Xu et al., 2010a), colon cancer (Fang et al., 2017), lung cancer (Jang et al., 2018), lymphoma (Liu et al., 2010), and leukaemia (Woo et al., 2011). Only one of the articles found showed that PV extract was ineffective to treat human neuroendocrine tumours (Johnbeck et al., 2012), while all others showed effectiveness.

For the molecular mechanisms behind the anti-tumoral activities of PV, four molecular pathways were the most popularly researched on (See Sec. 2.4.3), while the most frequently studied pathway was the intrinsic pathway of mitochondria-related apoptotic activation (Zhang et al., 2019). It was believed to be the main mechanism by which PV exerted its effectiveness.

6.2.1.2 Articles on the anti-viral effects of PV extracts

For the anti-viral activities of the PV herb, Human Immunodeficiency virus (HIV) and Herpes Simplex virus (HSV) were the most frequently researched on (See Fig. 3.4). The

mechanisms by which the PV extracts blocked viral infectivity were attributed to blocking the viral entry (Ao et al., 2021) into cells and blocking viral replications (Brindley et al., 2009). Investigating techniques included the time-of-addition (Oh et al., 2011), pseudotyping (Ao et al., 2021), and various immunochemistry techniques (Wang et al., 2014b).

6.2.2 Experimental part

In the past, very rarely research was done on the VOCs of PV. This was scarcely found and reported in the literature. This inspired the motivation to study the VOCs of the PV herb for their pharmacological activities in this project, but the study was confined to their anti-tumoral activities.

6.2.2.1 For the phytochemistry part (Distillation process)

Chapter 4 started with the recapitulation of the aims of this subordinate part of the experimental study.

The main aim of this subordinate part was to investigate the characteristics of the VOC extracts from PV through distillation by collecting and analyzing data on the abundances of the VOCs of a few representative volatile chemical constituents extracted from PV. In so doing, the distillation processes were also characterized.

The secondary aim was to get more information by looking at the changes in the VOCs as various independent factors varied, such as the distribution of VOCs inside the distillate, or how their abundances changed due to aging while in storage. This set of information fosters more understanding of the distillation and storage process, thus aiding the good design of a drug manufacture process, which targets to use VOCs as drugs.

6.2.2.1.1 Achieving the main aim

A few volatile chemical constituents of PV were identified by GC-MS to aid achieving this aim. These included several alkanes, and some rarer compounds such as caryophyllene oxide, anisole, furfural, and eucalyptol. Data of their abundances were sampled at time intervals of about 40 minutes (so, obtaining collection portions). These data of abundances showed that (See Fig. 4.6 to Fig. 4.9, and the description in Secs. 4.3.3.1 & 4.3.3.2 for details) the VOCs emerged at about relatively constant rates for the more abundant chemical compounds, and these emerging rates maintained even when the distillation continued for a very long time (up to 14 hours, to be practical to the laboratory conditions).

However, rarer compounds did show exhaustion as distillation proceeded. They depleted within one to several hours as distillation proceeded.

6.2.2.1.2 Achieving the secondary aim

Conclusions of the experiments can be summarized as follows:

- Inside the distillate, heavier volatile chemical compounds sank to the bottom and lighter volatile compounds floated at the top of the liquid (as expected from intuition). See Fig. 4.10.
- VOCs were found in the residual solution which was left behind in the steam producing flask, but with reduced varieties and abundances, especially for the compounds of smaller molecular weights. These VOCs in the residual solution resulted from the re-condensed rising steam dropping back to the boiling water below (Sec. 4.3.3.4).
- After the distillate was stored for a prolonged period, rarer compounds showed depletion within from about 3 days to two weeks. (See Fig. 4.12). However, more abundant compounds such as the alkanes did show enrichment. (See Fig. 4.11). The enrichment might be due to some unknown chemical reactions occurring inside the distillate in storage. However, this enrichment phenomenon was not

further investigated, because the theme of this research project purposefully avoided the study of individual chemical constituents.

- The dynamics of the steam flow and extraction were studied by changing the heater temperature so that the steam energy was changed. It was shown (See Fig. 4.13) that less energetic steam extracted lighter volatile compounds more efficiently, while more energetic steam extracted heavier volatile compounds more efficiently. An explanation of this observation was offered in Sec 4.3.3.6.
- Comparisons of the abundances of the extracted VOCs from the two distillation processes showed that the hydro distillation process was a more efficient process than steam distillation (see Sec. 4.3.3.7), resulting in more abundant amounts of VOCs being extracted. It was discovered in this set of comparison experiments that saturation of the abundances of extracted VOCs when the amount of herb used increased also occurred in hydro distillation. This set of comparison experiments also facilitated an explanation of the saturation phenomena observed in both the hydro distillation and steam distillation processes.

6.2.2.2 For Anti-tumoral/biological effect

To recapitulate the aims of this subordinate part of experimental study, which forms Chapter 5:

The main aim was to explore the pharmacological effects and, specifically, the anti-tumoral effect of the VOCs of PV. As decoction with water is the traditional way to prepare drugs for patients in Chinese Medicine, the VOCs in herbs are usually ignored, because when herbs are boiled in water, the volatile components inside the herbs will be lost. Because of the same reason, the VOCs in herbs are also usually ignored in research of the therapeutic effects of herbs. For the few research projects which study the VOCs of PV, often only the chemical identities of these volatile components were worked on. To satisfy this aim, the objective was to verify the dosage dependence of the

anti-tumoral effect of the VOCs of PV against the cancer cells from the oral squamous cancer cell line SCC154.

Another aim was to characterize the cancer cell cytotoxicity of the PV's VOCs versus different experimental parameters, such as the portion number, the initial weights of PV herb used for distillation, and the storage periods in weeks when the distillates were kept in storage. This study would aid in the design of a drug manufacture process which extracts VOCs from herb.

6.2.2.2.1 Achieving the first aim

In the biology part of the experiments, the dosage dependence of the anti-tumoral effect of the VOCs of PV was verified by three different experiments.

The first one was to treat the cancer with distillates of different concentrations and then observe the cancer cell cytotoxicity using a colorimetric reagent, Cell Counting Kit-8, which monitors cell metabolism. As every living cell undergoes metabolism, monitoring the metabolism also monitors cell viability. Distillates of different strengths were obtained by diluting the full-strength distillate with de-ionized water. Experiments showed that the cancer cell viability increased with the dilution of the distillate. This established the dosage dependence. See Fig. 5.1 and Sec. 5.3.1.1. This provided the first evidence.

Then, as a second support for the dosage dependence, another experiment was done by treating the cancer cells with distillates obtained using different weights of herb as the initial materials, from 5 g, 15 g, to 25 g. It was found that (see Fig. 5.3 and Sec. 5.3.1.3) the distillate when 15 g of herb used was the most cytotoxic to cancer cells. An experiment described in Chapter 4 (Sec. 4.3.3.1) showed that the most abundant amounts of VOCs were obtained when 15 g of herb used as initial material (see Fig. 4.6). This provided the second evidence.

Then, as a third support for the dosage dependence, yet there was another experiment which compared the cancer cell cytotoxicity due to the treatments by the distillate from the hydro distillation process with that from the steam distillation. The result from this experiment was that the distillate from the hydro distillation process was more cell cytotoxic to the cancer cells. See Fig. 5.5 and the description in Sec. 5.3.1.5. This provided the third evidence to support the dosage dependence, because Chapter 4 (Sec. 4.3.3.7.2) reported that the hydro distillation was a more efficient process than the steam distillation process to extract more abundant amounts of VOCs.

6.2.2.2.2 Achieving secondary aims

Experiments related to the secondary aims (see Sec. 5.1.3.2) gave the following results:

- The cancer cell cytotoxicity due to distillates gathered at later portions (time points) of the distillation process was higher than those collected at the beginning (see Sec. 5.3.1.2), but the differences are indeed not much.
- It was shown in Sec. 5.3.1.4 that the aging of the distillate did not lead the anti-tumoral efficacy to deteriorate.
- There was an optimal amount of herb used, leading to the highest cancer cell cytotoxicity (see Sec. 5.3.1.3). This optimal amount was 15 g (specific to the experimental setup), and this observation provided a support for the dosage dependence for the anti-tumoral effect of the VOCs of PV.
- Distillates obtained from the hydro distillation were more cell cytotoxic. This provided yet another support for the dosage dependence.
- The VOCs extracted from PV were not anti-oxidative.

These results gave information about harvesting VOCs to be used as drugs from the distillation processes. This information should aid understanding, and thus facilitate better design in drug manufacture process when distillation is chosen to be the extraction process.

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Appendix 1: General characteristics of PV

This appendix section outlines some general characteristics of PV, which are indirectly relevant to the main theme of this research project.

By looking at the chemical composition of the plant, it was discovered that it varies from region to region globally. Therefore, the factors which affected its composition, growth and development are examined. PV is a widely utilized herb with a long history of human use, both as a medicinal plant, and for agriculture, including feed for domestic livestock or as ethnoveterinary medicine by different cultures. The ethnopharmacological applications in Europe, Indian Sub-continent, and China are discussed. The widespread distribution of the herb globally highlights its easy accessibility to be used as medicine.

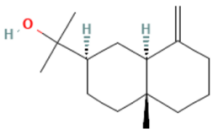
Other miscellaneous issues such as the pharmacological applications of the plant besides its anti-tumoral activities, its extraction methods are also discussed.

A1.1 Chemical constituents of PV

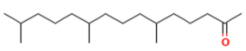
Many previous phytochemical studies revealed and reported the bioactive chemical constituents of PV (Wang et al., 2019, Zhang et al., 2018a, Zhou et al., 2017, Ba and Wang, 2017, Raafat et al., 2016, Gu et al., 2013, Chen et al., 2012c, Sahin et al., 2011, Feng et al., 2010b, Qi et al., 2009). Compounds identified included triterpenoids (Yu et al., 2015), sterols (Bai et al., 2015), flavonoids (Fazal et al., 2016), coumarins (Wang et al., 2019), phenylpropanoids (Wang et al., 2019), polysaccharides (Bai et al., 2016) and volatile oils (Yang et al., 2013). Triterpenoids and polysaccharides had been shown to exhibit anti-tumoral and anti-microbial activities (Li et al., 2019).

It should be highlighted that this study concentrated on the volatile organic compounds (VOCs) of PV. Previous research showed the amounts of VOCs in PV were very small (Golembiovska et al., 2014) and identification of individual volatile compounds in the

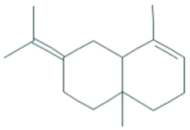
herb was challenging. Most of the articles reporting the VOCs of PV concentrated on identifying the hundreds of chemical constituents in the plant (Golembiovska et al., 2014, Yang et al., 2013, Morteza-Semnani et al., 2006). VOCs identified include monoterprene, sesquiterpene and aliphatic hydrocarbons, aliphatic alcohols, aldehydes, esters, ketones and acids (Golembiovska et al., 2014). It is important to note that the lists of compounds identified by different groups of researchers were not quite the same. For example, selinenol, eudesmadiene, dicubenol, spathulenol, and germacrene were the major constituents found in one article (Morteza-Semnani et al., 2006) , while hexahydrofarnesyl acetone, santalene, bourbonene, geranyl acetone, caryophyllene oxide, phytol, isobutyl phthalate, bergamotene and nonanal were identified in another (Yang et al., 2013). See Fig. A1.1 for the chemical structures of several volatile compounds in PV. Note that in both examples, the flower aerial part of the plant was used to extract the chemical compounds. It was important to specify the part of the plant being used as the chemical composition of each part was different (Chen et al., 2010, Wang et al., 2011, Chen et al., 2012c). Also, when different extraction methods were used, the resulting chemical composition extracted would vary. For example, one used hydro distillation (Morteza-Semnani et al., 2006), but another used headspace solid-phase microextraction in another (Yang et al., 2013); what they found were very different. There were other factors that caused variations in the chemical composition of the herb. Yang et al. (2013) showed that PV plants grown in different geographical regions had variations in the chemical composition of the extract. They sourced their plant samples from five provinces of China: Jiangsu, Fujian, Guangxi, Hunan and Zhejiang. See Table 2 in their article, where they listed the different chemical compositions in the extracts from different provinces.



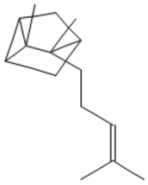
(a) β-selinenol (National Center for Biotechnology Information, 2024b), molecular formula: $C_{15}H_{26}O$



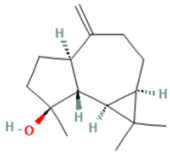
(e) hexahydrofarnesyl acetone (National Center for Biotechnology Information, 2024a), molecular formula: $C_{18}H_{36}O$



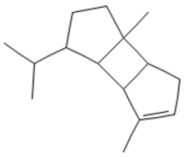
(b) eudesmadiene (National Center for Biotechnology Information, 2024h) , molecular formula: $C_{15}H_{24}$



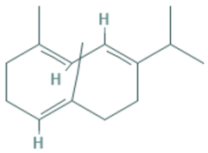
(f) santalene (National Center for Biotechnology Information, 2024d), molecular formula: $C_{15}H_{24}$



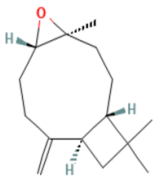
(c) spathulenol (National Center for Biotechnology Information, 2024c), molecular formula: $C_{15}H_{24}O$



(g) bourbolene (National Center for Biotechnology Information, 2024e), molecular formula: $C_{15}H_{24}$



(d) germacrene C (National Center for Biotechnology Information, 2024g), molecular formula: $C_{15}H_{24}$



(h)caryophyllene oxide (National Center for Biotechnology Information, 2024f), molecular formula: $C_{15}H_{24}O$

Fig. A1.1: Chemical structures of eight major essential oil constituents of PV

as reported by Morteza-Semnani et al. (2006) (left column, a to d) and Yang et al. (2013)(right column, e to h), four from each team. Note that they found different compounds in their projects. Note that these two teams found completely different compounds. This highlights the difficulty with the identification of the herb. Only caryophyllene oxide was found by this project, however.

(courtesy: National Centre for Biotechnology Information, National Library of Medicine)

A1.2 Factors affecting the growth and development of the PV plant

As PV is a commonly used herb in Chinese medicine, to secure a stable and economical supply, agricultural cultivation of the herb as a domesticized crop is desirable (Chen et al., 2013). As its natural habitats are in woodland and mountains, cultivation of this plant as a crop in its natural habitats will not compete with land for other food crops

usually grown in plains of lower altitude. Thus, the factors which affect the quality and yield in the cultivation of the PV plant need to be examined more closely. Chen et al. (2011) showed that the mineral contents in different parts of PV plant were significantly different between cultivated and wild species.

The factors which cause variations in the chemical composition of the plant include the differences in harvest time (Chen et al., 2012b, Chen et al., 2012c), whether the plant is cultivated or is a wild species (Chen et al., 2011a), water stress (Guo et al., 2009b, Chen et al., 2016, Phillips et al., 2018), latitude variations (Winn and Gross, 1993), altitude variations (Kuriya et al., 2015), variations in light intensity (Zhou et al., 2011), soil fertilization condition (Veresoglou et al., 2012, Chen et al., 2016), variations in level of herbicide application (Olszyk et al., 2013), storage period and grading standards (Chen et al., 2012a), levels of UV-B radiation intensity and stages of growth of the plant (Chen et al., 2018b). Among these factors, drought stress imposes the strongest limit in the photosynthetic process in the plant (Chen et al., 2016) while the use of fertilizers improves it. In general, the increase in soil water supply has a better effectiveness in encouraging the growth of PV seedlings than a better supply of nutrients through fertilization. By monitoring the rosmarinic acid content, which is the main constituent of PV, it was found that a more abundant rosmarinic acid content was from spica samples harvested from early May to late June (Chen et al., 2016).

In North America, by analysing the samples collected from Michigan in the north to South Carolina in the south, genetic variations, flowering timing variations, and flower number variations were found (Kuriya et al., 2015, Winn and Gross, 1993). This agreed with the findings in China (Yang et al., 2013). These geographic variations also related to the local pollinator variation (Kuriya et al., 2015); so, the geographic variations might not be eliminated, and products might not be standardised by agricultural technology. This posed difficulties in standardizing the chemical compositions when producing drugs.

A1.3 Ethnopharmacological uses

This section describes the traditional uses of the herb in different cultures.

A1.3.1 Europe and West Asia

PV grows in East Europe and West Asia (Kujawska et al., 2017, Golembiovska et al., 2014, Golembiovska and Tsurkan, 2013). PV as an herbal drug was popular during the 17th century in Europe. Hot water infusion was used as a cure to assuage sores in the mouth and throat (Matthiolus, 1626). Traditional use also included remedies to reduce fever and promote wound healing. These remedies were still used in modern time (Grieve, 1974). However, in modern use, the herb is often packaged in different dosage forms: capsules, tablets, and spray. It is used alone or used in multi-drug form containing PV and other herbs. PV is used by some local communities as a kind of vegetable to make soup, tea, or mix in salad for the purpose as a panacea (Bai et al., 2016).

In a study on the archival data of ethnomedical knowledge available from the Polish Lithuanian-Belarusian borderland (Kujawska et al., 2017), 153 local herbal plants were registered. PV was one of the herbs in the register. See Fig. A.1.2.

Fig. A1.2: The locations of the survey of medicinal plants in the region of Polish-Lithuania-Belarusian borderland.

The locations of the survey of medicinal plants in the region of Polish-Lithuania-Belarusian borderland [courtesy: (Kujawska et al., 2017)].

PV is a frequently used herb there. In the region of Grauzyszki, PV is consumed as a refreshing drink and a substitute for tobacco. For medical use, it is used to treat sore throat and headache in the regions of Giby and Polany.

However, whilst PV is not found in some other East European countries like Ukraine or Poland, it was reported to be found and used in Great Britain and West Europe (Phillips et al., 2018) with similar indications.

A1.3.2 Indian sub-continent

In a quantitative ethnobotanical survey of the herbal plants in Toli Peer National Park (See Fig. A1.3) of the provinces of Azad Jammu and Kashmir, Pakistan (Amjad et al., 2017), researchers interviewed local people and recorded on their traditional use of the local herbs to treat various kinds of diseases. They surveyed 121 different native plants and compiled a documentation of indigenous pharmacological ethnobotanical knowledge in that community.

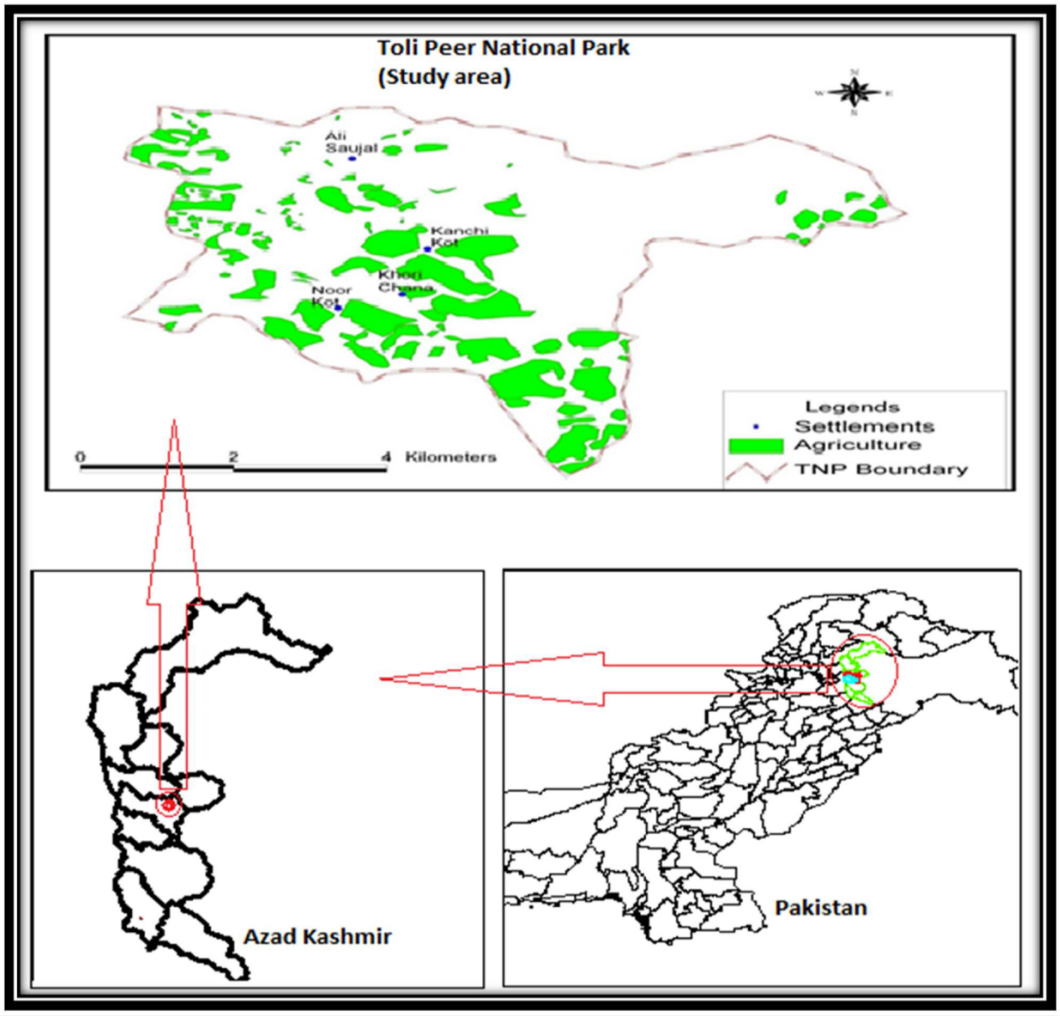


Fig. A1.3: The location of the ethnobotanical survey in Azad Kashmir, Pakistan [courtesy: (Amjad et al., 2017)]

Among the 121 plants under the survey, PV was among the most frequently used herb.

The indigenous people consumed the seeds of PV plant as food. Local people popularly believed that its consumption was effective to be laxative, antipyretic, tonic, diuretic. Also, it treated inflammation, heart disease, difficulty in breathing and eyesight weakness.

A1.3.3 China

PV is found widely south of the Yellow River, and in the southern part of China along the Yangtze River, mainly in the provinces of Anhui, Henan, Jiangsu, Hunan, Zhejiang, Fujian and Guangxi (Yang et al., 2013). See Fig. A1.4.

In China, the use of PV as medicine dated back to at least two thousand years ago. The earliest record in Chinese medical literature, which reported its use, was found in the ancient text, Shen Nong Ben Cao Jing (神農本草經) which was compiled during this period (Sun, 2006). PV and its use also appeared in later texts such as Compendium of Materia Medica (Li, 1979), Tai Ping Sheng Hui Fang (太平聖惠方)(Wang, 1958) and Supplements of the Compendium of Materia Medica (Zhao, 1983).

Fig. A1.4: Distribution of PV growing regions in China.

The colours of the dots indicate abundance of the plant. [Courtesy: (Bai et al., 2016)]

As explained before, the chemical compositions and the abundances of individual compounds of PV herb from different geographical origins are different. Thus, they also have different pharmacological efficacy. This warrants deeper investigative efforts. A detailed comparison of the chemical compositions among PV plants from the different Chinese provinces was given, for example, by Yang et al. (Yang et al., 2013).

The applications of this herb were described as promotion of wound healing, treatment of sore throat, swollen thyroid gland, dermal allergy, dermatosis, jaundice, fever, hepatitis (Guo and Chen, 2011). In Chinese medicine terms, however, PV possesses the ability to clear heat, soften and resolve hard mass; thus, heal sores and abscess. However, in Chinese medicine, herbs are usually used in combinations to form medicinal formulae for the synergistic effect. Although few but with number increasing, PV appears alone in some over-the-counter drugs, such as PV cream, and PV oral liquid (Chinese Pharmacopoeia Commission, 2010). They are commonly used for the treatment of lumps around the neck, mastitis and hyperplasia of the mammary glands, scrofula, goitre, headache, dizziness, and diseases of the lymphatic system (Bai et al., 2016).

Similar to the European and Pakistani traditions, PV is also consumed as food in China. It may appears in the forms of herbal tea, or mixed with glutinous rice as steamed rice cakes, for daily consumption by local people in the lower Yangtze River basin and the Guangdong province (Li, 1979, Li and Yao, 1990). This tradition is also followed in some of the Southeast Asian countries. In the southern province of Guangdong in south China, during summer, people consume a tea with PV as a main ingredient to clear “heat” (Bai et al., 2016).

A.1.3.3.1 Examples of uses in China

A1.3.3.1.1 Beverages

Two beverage drinks which can be bought at convenience stores, for ordinary consumers are shown in Fig. A1.5. There is a box of *xiasangju* powder, packaged into 20 sachets in a box. Each sachet is emptied into a cup of hot water to make a beverage drink. *Xiasangju* stands for *xiakucao* (PV), *sangye* (mulberry leave) and *juhua* (chrysanthemum). This product is a combined traditional formula of three "heat clearing" herbs, and so, it is good for people feeling hotness and vexation during summertime.

Another product is a bottled beverage of PV. Its target market is thus the same as soft drinks or fruit juice, and the indication is like the *xiasangju* powder. Note that the manufacturer is Coca Cola Company, and thus, it shows the complete adaptation to modern time of old traditional Chinese beverages.

Fig. A1.5: Two kinds of PV beverages.

A1.3.3.1.2 Traditional Chinese medicine formulae

Two traditional Chinese Medicine formulae are shown here (Yang, 2013) as examples:

1. *Nei Xiao Luo Li Wan* (Scrofula-reducing Pill from within, 内消瘰癧丸)

   This formula is made up of 17 herbs, including *xiakucao,* PV. Its indication is to treat scrofula, goitre, hard lump on the skin, cyst.

   For completeness, the 17 herbs are: *xiakucao, haizao, zhike, jiegeng, beimu, haigeke, qingyan, xuanshen, shengdihuang, tianhuafen, danggui, lianqiao, bailian,dahuang, mangxiao, bohe, gan cao.*

2. *Niu Bang Jie Ji Tang* (Niu Bang Zi Decoction to expel evil from the flesh, 牛蒡解肌湯)

   This second formula is made up of nine herbs. Its indication is to cool the blood and reduce swelling. It treats localized abscesses mostly found on the head, face or neck, carbuncles, tonsillitus.

   The nine herbs are: *xiakucao, niubangzi, bohe, jingjie, lianqiao, shanzhizi, mudanpi, xuanshen, shihu.*

A1.4 Other uses of the PV herb

A1.4.1 Agricultural uses

Although the main attention on PV is its use as a medicinal herb, it is also used in aquaculture and agriculture. In domesticated animal grazing or in fishery, PV is used as dietary supplement, which also has the effect as an immunostimulant to elicit a vaccination effect to control diseases (Park and Choi, 2014, Ringo et al., 2010). There were articles which showed the immunomodulatory activities of polysaccharides from PV (Li et al., 2015a, Li et al., 2015b). In agriculture, it is used in both North America and United Kingdom as a pasture to graze lambs, with the effect to increase the fatty acid contents in the meat (Kliem et al., 2018). As PV is a flowering plant, its flowers and

nectar are utilised to feed bumblebees to produce honey in North America (Kuriya et al., 2015).

A1.4.2 Ethnoveterinary medicine

In the field of ethnoveterinary medicine, PV is used to treat animals with deep wounds, broken horn, or cuts. The treatment consists of a wash, which is made with an infusion of the aerial parts of PV in boiling water (Lans et al., 2007). This tradition is not only followed in Europe, but among red Indians in North America too.

A1.5 Other miscellaneous issues

A1.5.1 Contraindications of PV

Because of the 'cold' nature of the PV herb, it is not advisable to take PV for long term. Also, it is not suitable for patients who have weak body build ('cold' nature according to Chinese Medicine theory) or in poor health (Chen, 2016a). Long term consumption causes deterioration of patient's health. According to Western medicine, an explanation is that because of the anti-inflammatory capability of PV herb, long term consumption will weaken the immune system of a patient.

A1.5.2 Endophytic fungi

It was discovered that not only the PV plant is valuable for medical use, the endophytic fungi found in the plant also possess therapeutic potential, which is derived from the active metabolites from these fungi. The use of fungi offers several advantages: wider scope of resources which facilitate sustainable utilisation, convenient mass production process, and easy quality control (Tan et al., 2015, Mariotte et al., 2013, Diaz et al., 2012).

A1.6 Extraction methods

The usual preparation method of medicinal herbs in traditional Chinese medicine is by decoction, i.e., the boiling in hot water. It is the most popular extraction method in research projects too. The other frequently used extraction medium in laboratory is ethanol, followed by methanol. Other popular extraction methods in laboratory include different chromatographic methods, such as column chromatography and High-performance liquid chromatography (HPLC).

However, as this study concentrated on VOCs, we adopted distillation as the method of extraction. To extract the VOCs from herb, the commonest method used is hydro distillation, as this method was prescribed in an official manual published by the Chinese government (Chinese Pharmacopoeia Commission, 2010). However, this study adopted another distillation method, steam distillation as well, which enabled the comparison between the two.

A1.7 Pharmacological activities discovered in modern research

From recent research in the last three decades, PV extracts from the whole spica or its individual chemical constituents, were demonstrated to possess anti-tumoral capability (Feng et al., 2010b) , and showed antimicrobial (Komal et al., 2018), antiviral (Oh et al., 2011), antioxidant (Hwang et al., 2013a), immunomodulatory (Li et al., 2015a, Li et al., 2015b), anti-hypertensive (Zhang et al., 2018a, Bai et al., 2016), anti-inflammatory (Choia et al., 2016, Park et al., 2013) and hypoglycaemic (Raafat et al., 2016) activities. As inflammation played an important role in such diseases as Alzheimer's disease, heart disease, cancer, and diabetes, PV was also indicated to treat these diseases (Lee et al., 2017, Wu et al., 2012, Lee et al., 2008).

Appendix 2: A summary of articles included in the review of Chapter 2

Author/ Year/ Location	Primary objective	Study design	Cancer/ Cell lines targetted/ animal model	Proteins/ Genes targetted	Results
Lee et al. 1988	To screen 36 herbs (with PV as one of them) to study their antimutagenic activity	To do the extraction, the crude herbs were put in hot water for 2 h to get the aqueous extract, and then lyophilized. The antimutagenic activities of the extract was characterized by a salmonella/liver microsomal test system.	N/A	N/A	The antimutagenic capabilities of the 36 herbs were categorized. Pteris multifida showed the highest antimutagenic effect against picrolonic acid induced mutation.
Lee H. & Lin J.Y. 1988	To characterize the cytotoxicity of 3 herbs (with PV as one of them) against 6 different cancer cell lines.	The cell was treated by the methanol extract of PV, which was fractionated with hexane, CHCl3 and water. Different chemical components was separated by column chromatography on silica gel. The cell viability was measured by cell counting using a hemacytometer.	P-388, L-1210, A-549, KB, HCT-8, MCF-7	N/A	The ursolic acid in PV showed the highest cytotoxicity against P-388 and L-1210, and A-549 cell lines.
Ahn S.C. et al. 2003	To characterize rosmarinic acid as an inhibitor against Lck Src-homology 2 binding.	The inhibition effect of rosmarinic acid to inhibit cytokine expressions was characterized by immunochemical methods.	Jurkat cells, hmTpY324	IL-2	Rosmarinic acid from PV inhibited IL-2 gene expression, inhibited intracellular [Ca2+]l increase. Rosmarinic acid had the potential to inhibit Lck SH2 domain binding to cognate ligands, and thus modulate IL-2 gene expression.
Yao Z.H. et al. 2006	To investigate the anti-tumor effect of the PV extract	In vivo method using 5 groups of BALB/C mice, with 10 mice each. The mice were injected intraperitoneally with PV extract, while the control groups were injected with saline. Tumor growth and survival time were measured by DNA gel electrophoresis and the TUNEL method.	T-lymphoma EL-4 cells.		
Zhang K.J. et al. 2006	To investigate the anti-lymphoma effect of PV.	The cell viability was tested by MTT assay. The cellular morphology was observed by the use of MTT with Giemas staining under a microscope. Immunocytochemical methods were used to study the proteomics.	Raji cells	Bcl-2/Bax	PV suppressed Raji cancer cell proliferation.
Gu X.J. et al. 2007	To establish the structures of the 11 chemical constituents isolated from PV, including 3 oleanane-skeleton triterpenoid saponins.	The structures of the chemical components were investigated by spectroscopic analysis, IR, HR-ESI-MS, and NMR. The compounds were also tested for their inhibition activity against tumor growth by MTT assay.	SMMC-7721, MCF7, HeLa	N/A	Only 1 triterpenoid saponin was found to have marginal inhibition activity against tumor cells.
Park S.H. et al. 2007	To investigate the structure-activity relationship of rosemarinic acid as an antagonist for the p56lck SH2 domain.	To synthesize several analogs of rosmarinic acid. The structures were purified by HPLC and identified by NMR. The synthesized compounds were tested for in vitro binding activity for the SH2 domain by using a competitive assay based on ELISA. T-cell inhibitory activity was measured by using the blocking of IL-2 gene activation, through the use of luciferase activity assay.	Jurkat cells	IL-2	The identification of several rosmarinic acid analogs with a more potent T-cell inhibitory activity than that of rosmarinic acid, to be used as antagonists for the SH2 domain, which can be regarded as novel anti-tumor candidates

Lee I.K. et al. 2008	To isolate the chemical compounds in PV. The compounds were evaluated for their cytotoxicity against cancer cell lines.	The compounds were extracted by methanol and separated by column chromatography. The isolated compounds were tested for their cytotoxicity against cancer cell lines using the sulforhodamin B bioassay.	A549, SK-OV-3, SK-MEL-2, HCT15.	N/A	One compound isolated was identified to have moderate cytotoxic activity against the cancer cell lines tested.
Zhao A.G. et al. 2008	To investigate the gene expression changes due to the use of a Chinese herbal formula, Wei Chang An, in gastric cancer cell line SGC-7901	The gastric adenocarcinoma cells were grafted onto nude mice, which were divided into 3 groups, with one of them as a control group which received saline. One group received the herbal formula injection; another group received 5-FU. After the mice were sacrificed, cancer samples were taken. The gene expression profiles were measured by using a cDNA microarray and then RT-qPCR. The treatment groups were also compared with TUNEL, and immunochemical methods.	gastric adenocarcinoma	Stat3, RIPX, ROD1, Bcl-2	The herbal formula could induce cancer cell apoptosis and suppressed proliferation. It down-regulated Stat3, RIPX, ROD1 and Bcl-2.
Chen C., Wu G., Zhang M. 2009	To study the effect of PV extract on the Jurkat human T lymphoma cell line.	The cell proliferation and apoptosis were determined by MTT assay and flow cytometry. DNA fragmentation was observed by gel electrophoresis. Western blotting was used to study the proteomics.	lymphoma/ Jurkat cells	Bcl/Bax	The PV extract significantly inhibited the proliferation of Jurkat cells. Bcl-2 was down-regulated but Bax was up-regulated.
Choi J.H. & Jeong H.G 2009	To study the effect of PV aqueous extract on lung metastasis of melanoma cells.	In vivo, C57BL/6 mice were used to observe the inhibition effect of PV on the number of lung metastatic colonization. In vitro, luciferase activity assay were used to study the expression level of MMP-9 through the mediation of NF-κB. Wound healing assay was also used to examine cell migration.	melanoma/ HT-1080 cells	MMP-9, NF-κB	Cell migration was shown to be significantly inhibited by PV aqueous extract, and it was mediated by the suppression of MMP-9 activity and through the inhibition of NF-κB.
Han E.H. et al. 2009	To study the immunostimulatory and antitumor activities of PV in murine macrophage RAW 264.7 cells	PV extract was obtained by submerging the spica in hot water for 5h. Cell cytotoxicity was assayed by WST-1 reagent. Production of NO was measured by Griess reagent. Cytokine production was quantified by sandwich immunoassays, RT-qPCR, Western blotting and luciferase activity assay.	RAW 264.7 cells	TNF-α, IL-1β, IL-6, NF-κB, MAPK	PV aqueous extract stimulates macrophage activation through activation of NF-κB and MAPK
Zhang,M.Z. et al. 2009, China	To analyse the proteomics change after treatment by PV	Two dimensional electrophoresis and mass spectrometry were used. Cell proliferation by MTT assay.	Lymphoma/ Raji cells	Multiple proteins were identified	The PV extract inhibited the growth of Raji cells
Zhang M.Z. et al. 2009	To analyse the proteomics change after treatment by PV	Two dimensional electrophoresis and mass spectrometry were used. Cell proliferation by MTT assay.	Lymphoma/ Jurkat cells	Multiple proteins were identified	The PV extract inhibited the growth of Jurkat cells
Zhang, M.Z. & Wang X.Q. 2009, China	To investigate the effects of the extract of PV combined with chemotherapeutic agents on the proliferation of lymphoma cells	Raji cells were treated with PV extract combined with paclitaxel and adriamycin. Cell viability was tested by MTT assay. The cell cycle and apoptosis were studied by flow cytometry, proteins study by immunochemistry.	Lymphoma/ Raji cells	Survivin, caspase-3	The PV extract inhibited the growth of Raji cells. The inhibitory effects of using the combination with chemotherapeutic agents were significantly higher.

Cheng, W.W. et al. 2010, China	To investigate the effects on the growth and proliferation of breast cancer MCF-7 cells by 9 kinds of herbs.	9 kinds of herbs were compared. MTT and trypan-blue staining assay was used. Morphological changes of cells were observed.	Breast cancer/ MCF-7	N/A	The cold herbs including PV inhibited the growth and proliferation of MCF-7 cells, while the hot herbs promoted. However, the herbs at the tested concentrations howed no cytotoxicity.
Choi J.H. et al. 2010	To examine the inhibitory effects of tumor cell migration by aqueous extract of PV	Both in vivo and in vitro assays were used. Expression levels of MMP-9, NF-KB, ERK1/2, mRNA and transcription activities	Melanoma/ B16-F1 cells, B16-F10/ mice	MMP-9, NF-κB, ERK1/2	The administration of PV extract inhibited MMP-9 expression and activity. It also reduced metastasis and tumor cell growth.
Feng,L. et al. 2010a, China	To examine the chemopreventive effects by 60% ethanol extract of PV to decrease morbidity and mortality of non-small cell lung cancer	Both in vivo and in vitro assays were used. Apoptosis was studied by MTT assays and by Annexin V-FITC kit. Cell cycle analysis by propidium iodide, and then flow cytometry. PV extract was done with reflux in 60% ethanol, 30% ethanol and water.	non-small cell lung cancer/ SPC-A-1 cells/ A/J mice	N/A	The 60% ethanol extract showed the strongest anti-proliferative activity as compared with the 30% ethanol extract
Feng,L. et al. 2010b, China	To examine the antioxidative effects of the 60% ethanol extract of PV. The inhibitory effect on tumor growth was also studied.	ABTS, TEAC, DPPH, and FRAP assay methods were used to study the anti-oxidative effect. C57BL/6 mice was used in in vivo test to study tumor growth. SOD activity and malondialdehyde contents in mouse serum were also examined to explore the antioxidative effect.	tumor in anterior limbs of C57BL/6 mice.	N/A	60% ethanol extract showed strong antioxidative effects both in vivo and in vitro tests. The extracts also showed inhibitive effect on tumor growth in mice. The authors attributed the effects to the phenolic contents in PV.
Feng, L. et al., 2010, China	Two polysaccharides from the aqueous extract of PV were examined to explore their anti-tumor effects.	Two polysaccharides were isolated from the aqueous extract of PV and purified through ethanol precipitation. Anti-tumor effect was explored by in vivo test, and characterised by the thymus index and the spleen index; so, immunomodulation effects were also studied.	lung adenocarcinoma/ C57BL/6 mice	N/A	The polysaccharides of PV had anti-lung adenocarcinoma activities and immunomdulation effects. They increased the thymus index and the spleen index in tumor-bearing mice.
Liu X.K., Wang L. & Zhang M.Z., 2010, China	To characterise the effect of PV on the cell proliferation and apoptosis of Raji cells. The underlying mechanisms were also studied.	MTT and FCM assays were used to measure cell proliferation and apoptosis. Western blotting was used to determine the phosphorylation of JNK, c-Jun, and expressions of caspase-3.	Lymphoma/ Raji cells	JNK, c-Jun, caspase-3	PV decreased the proliferation rate of the cancer cells. Phosphorylation of JNK and c-Jun was increased. The expression level of caspase-3 was oncreased by PV.
Xu,Y., et al., 2010, China	To study the effect of rosmarinic acid (RA) from PV on the bone metastasis from breast carcinoma.	Western blotting and real-time qPCR were used to determine the mechanisms.	breast cancer/ MDAMB-231BO cancer cells, ST-2 murine bone marrow stromal cells	RANKL/RANK/osteoprotegerin pathway, IL-8	RA significantly inhibited migration of bone-homing cancer cells, increased alkaline phosphatase activity. The pathway affected was mainly the RANKL/RANK/osteoprotegerin and the expression of IL-8 was suppressed.

Xu Y. et al. 2010, China	To study the anti-invasion activity of rosmarinic acid (ra) from PV on colon carcinoma cells.	In vitro, the investigation was done using the wound healing assay, the adhesion assay and the Transwell assay. In vivo, the anti-tumor effect was measured by the tumor weight. Western blotting and qPCR was used to study the molecular mechanisms.	Colon carcinoma Ls174-T cells/ mice model	MMP-2,9, ERK	ra inhibited migration, adhesion, and invasion dose-dependently. It also decreased the level of reactive oxygen species, repressed the expression of MMP-2,9 through the ERK pathway. Ra reduced the tumor weight in test mice,
Feng L. et al. 2011, China	To study the effect of oleanolic acid (oa) from PV on lung adenocarcinoma	oa isolated from PV ethanol extract was identified by HPLC, HPTLC and LC-MS. Cell viability was tested by MTT assay; apoptosis was further studied by acridine orange-ethidium bromide fluorescence detection. Protein expressions were investigated by immunocytochemistry assays.	lung adenocarcinoma/ SPC-A-1 cells	Bax, Bad, Bcl-2	Effects on cell viability by oa indicate no differences from ethanol extract of PV. Oa significantly increased cell apoptosis. Expressions of Bax and Bad were increased, but that of Bcl was decreased.
Lin W. et al. 2011, China	To investigate the anti-angiogenic effects of PV	In vitro, the proliferation wa studied by migration and tube formation assays of HUVECs. In vivo, the chicken embryo chorioallantoic membrane assay was used.	human umbilical vein endothelial cells (HUVECs)/ HT-29 colon carcinoma cells	VEGF-A, VEGFR-2	The ethanol extract of PV inhibited the migration and tube formation of HUVECs. It also decreased the expression of VEGF-A in HT-29 cells, and the expressions of VEGF-A and VEGFR-2 in HUVECs.
Woo H.J. et al. 2011, Korea	To evaluate the apoptotic effect of an acid from PV on leukemia Jurkat cells	Cell viability was assessed by MTT assay. Flow cytometry was used to measure mitochondrial membrane potential, apoptosis and cell cycle. Mitochondrial cytochrome c and caspases were determined by Western blotting. Caspase-12 and caspase-3 activities were assayed using the fluorometric and colorimetric assay kits.	Leukemia/ Jurkat cells	Bcl-2,cytochrome c, caspase-3,7,8,9	Treatment caused cytotoxicity and apoptotic DNA fragmentation, mitochondrial membrane potential loss, mitochondrial cytochrome c release, activation of caspase-3,7,8,9 and PARP degradation.

Zheng L. et al, 2011	To investigate the effects of the ethanol extract on colon carcinoma cancer cells.	The inhibition of cell growth by observing the morphological cell changes. Western blotting was used to measure the expression levels of proteins and flow cytometry was to evaluate mitochondrial membrane potential changes.	colon carcinoma/ HT-29 cells	Bcl-2/Bax,	The ethanol extract of PV reduced cell viability, loss of plasma membrane asymmetry, loss of mitochondrial membrane potential, activation of caspase-3 and caspase-9, increase the ratio of Bax to Bcl-2.
Fu X.R., Sun,Z.C. & Zhang M. 2012	To investigate the effects of PV on the proliferation of lymphoma Raji cells and Jurkat cells, and find the mechanisms.	The cell proliferaton apotosis, and viability were examined by MTT assay, gel electrophoresis and flow cytometry. Western blotting was used to detect the changes in expression levels of proteins.	leukemia/ Jurkat cells, Raji cells	Bcl-2, Bax	The PV extract inhibited the proliferation of cancer cells and promoted apoptosis. It increased the ratio of Bax/Bcl-2.
Johnbeck C.B. et al. 2012	To monitor the treatment response in neuroendocrine tumors treated with PV with the proliferation-detecting tracer 18F-FLT	In vivo test was done as human neuroendocrine xenografts in mice. Treatment with PV extract was followed by FLT PET scan and CT scan, which defined the tumor volume.	neuroendocrine tumors	N/A	PV does NOT have any effect on cell proliferation measured with 18F-FLT tracer.
Kim S.H. et al. 2012	To study the mechanism of action of PV aqueous exttract to affect cell migration and invasion of liver cancer.	Tumor cell viability, migration and invasion were studied by measuring the activities and transcription of metalloproteases.	liver hepatocarcinoma cell	MMP-2,9, p53	PV suppressed migration through attenuation of the transcription levels of MMP-2,9
Hwang Y.J. et al. 2013	To investigate the antioxidant and anticancer activities of an ethanol PV extract.	The extraction was by ethanol and then fractionated to produce hexane, butanol, chloroform and water fractions. The antioxidant activities were analyzed by the Folin-Ciocalteu, DPPH, FRAP, ABTS and SOD. The cell cytotoxicity was assessed by MTT assay. The expression of genes was investigated by RT-PCR.	HepG2, HT29, A549, MKN45 and HeLa cells.	p53, Bax, Fas	The PV ethanol showed stronger cytotoxic effects than the solvent fractions. The ethanol extract and the water fraction showed stronger antioxidant action than the other solvent fractions. They also significantly increased the expression of p53, Bax, and Fas
Lin, W. et al. 2013	To investigate the mechanisms of action of PV against colon carcinoma.	The proliferation of cells was studied by MTT and colony formation assays. The cell cycle was determined using fluorescence-activated cell sorting with propidium iodide staining. RT-PCR and western blotting were used to measure mRNA and protein expressions.	Colon carcinoma/ HT-29 cells	CDK4, cyclin D1	PV ethanol extract inhibited HT-29 viability and survival. It blocked the G1/S cell cycle progression and reduced the expression of cyclin D1 and CDK4.

Liu, Z. et al. 2013	To investigate the distribution characteristics of TCM syndromes and the related herbal prescriptions for malignant tumors	To analyse a database of 136 cancer patients using a cluster an frequency analysis.	N/A	N/A	Two patterns of TCM syndromes were the most frequently made diagnosis: Deficiency of Qi and Yin and then the internal accumulation of toxic heat.
Wang Y. et al. 2013	To investigate the anti-tumor effect of a herbal formula, Antitumor B.	In vivo test using A/J mice. The herbal formula was delivered through diet. The serum was analysed to determine the expression levels of EGFR and phosphorylated EGFR	oral squamous cell carcinoma	EGFR	The herbal formula inhibited oral carcinoma development by reduction of cell proliferation. The expressions of EGFR and phosphorylated EGFR were down-regulated.
Lou H. et al. 2014	To show the cytotoxicity of a new diterpenoid, Vulgarisin, discovered in PV	To elucidate the structure of the fused tetracyclic ring skeleton of vulgarisin, and to show the cytotoxicity of it.	lung carcinoma/ A549	N/A	Vulgarisin showed weak cytotoxicity against lung carcinoma A549 cells.
Wang P. et al. 2014	To explore the effects of PV extracts on human adenocarcinoma, and to elucidate the mechanism at the proteomic level.	The effect of PV on cell proliferation was explored by MTT assay. Proteins were isolated by two-dimensional electrophoresis and then silver staining was used to acquire the proteomic maps, which was then analyzed by mass spectrometry and Western blotting.	Lung adenocarcinoma/ A549 cells	A wide range of proteins were found.	Differential expressions of a wide range of proteins were found. This imples multiple targets and multiple pathways were affected by the treatment of PV extracts.
Wang Y. et al. 2014	To study the effects of sulfated polysaccharide from PV on the expression of angiogenic factors, and angiogenesis in hepatocellular carcinoma	ELISA assay was used. In vivo, the microvessel density in carcinoma tissue sections was calculated, analyzed.	hepatocellular carcinoma/ HepG2 cells	bFGF, VEGF and IL-8	The sulfated polysaccharide from PV inhibited bFGF, but did not affected the VEGF and IL-8. In vivo results showed the polysaccharide reduced the microvessel density in tumor tissue sections.
Zhao L. et al. 2014	A clinical study with a randomized control trial to investigate the efficacy of a Chinese herbal formula to treat gastric cancer	A randomized control trial was conducted based on 93 patients, who were treated with a Chinese herbal formula with PV as a component. Kaplan-Meier method was used to assess the differences in survival time. Cox proportional hazards regression analysis was to identify independent prognostic factors.	gastric cancer with peritoneal metastasis	N/A	The Chinese herbal formula was effective and improve the prognosis of the gastric cancer patients.
Bai Y.B. et al. 2015	To study the chemical constituents from PV and the antitumor activities	Silica gel, reverse-phase octadecylsilyl, sephadex LH-20 chromatographic methods and HPLC were used to isolate and purify the compounds. MS and NMR spectroscopic methods were used to determine their structures. The cytotoxicity was evaluated by MTT assay.	breast cancer/ MCF-7, MDA-MB231, MCF-10A cells	N/A	Three compounds isolated were found to have significant inhibitory effects on the cancer cells.

Cho I.H. et al 2015	To explore the effects of PV aqueous extract on epithelial-mesenchymal-transition	The extraction was done by boiling in water. Vacuum evaporation and lyophilization were applied to get the dry residue. Proliferation was done with MTT assay. Colony formation was detected by crystal violet staining. Western blotting and immuno-precipitation techniques were used to characterize the proteins. Cell migration assay and transwell assay were used to measure the cell migration and invasion.	MDA-MB-231, SKOV-3	vimentin, β-catenin, N-cadherin, NF-κB	PV aqueous extract markedly inhibited the cell migration and invasion. It produced changes in the expressions of EMT markers. The NF-κB/Snail signaling pathway was identified as the mediating pathway.
Lee D.H. et al. 2015	To report a single case study on a female patient with stage III breast cancer. To report the effect of using pharmacopuncture with PV on the treatment outcome.	The whole healing process of using pharmacopuncture , herbal medicine together with chemotherapy was described	breast cancer	N/A	A complete resolution was confirmed on PET-CT after about 1 year of treatment.
Peng Z., Shen H. & Gu J. 2015	To report a randomized control trial study of using a Chinese herbal formula, Xiaopijian, together with auricular acupuncture, on 91 female patients. To compare the difference in efficacy between the combined therapy of auricular acupuncture and using herbal formula, and the use of Xiaopijian alone.	The 91 patients were divided into two groups of 46 and 45. Treatment with the herbal formula Xiaopijian alone was applied to one group, and treatment with both the herbal formula and auricular acupuncture was applied to another group. Treatment outcomes were compared after 3 sessions, with each session lasted one month.	breast hyperplasia	N/A	The combined therapy achieved superior efficacy on breast hyperplasia, as compared with using the herbal formula alone.
Hao J. et al. 2016	To study the effects of polysaccharide from PV on breast carcinoma associated fibroblasts.	Cell viability was assessed by MTT assay. Cell migration was assessed by wound healing assay and transwell migration assay. Cell apoptosis and cell cycle distribution were detected by flow cytometry. RT-PCR and ELISA were used to detect the expression levels of the fibroblast growth factor bFGF.	breast carcinoma/ SKBr-3 cells	bFGF	Polysaccharide in PV inhibited the growth of the fibroblasts, inducing apoptosis and arresting cell cycle. It also inhibited migration.
Li C. et al. 2016	To report on the preparation method of PV polysaccharide-zinc complex by a facile method, and to explore its antiproliferative effect on hepatocellular carcinoma. The underlying mechanisms were also investigated.	The polysaccharide was extracted with hot water, fractionated and purified using fast flow columns DEAE-sepharose and Sephadex G-100. The polysaccharide-zinc complex was characterized by atomic absorption spectrophotometry, conductivity, SEM and FT-IR. The use of observation of morphological changes, chromatin condensation was used to detect the inhibition on proliferation. Cell viability and apoptosis was measured by MTT assay, flow cytometry, the Hoechst 33258 detection kit and annexin V-FITC kit. Cell cycle arrest was analyzed by flow cytometry.	Liver hepatocarcinoma/ HepG2 cells.	caspase-3 and -9	The PV polysaccharide-zinc complex was found to inhibit cancer cell proliferation, to cause apoptosis, and effect cell cycle arrest.

Su Y.C. et al. 2016	To elucidate the molecular mechanism underlying the suppression of MMP-9, inhibition of cell invasion and migration.	To investigate the differential expression levels of VEGF, MMP-9, AP-1, NF-κB, IκB by proteomic assays.	hepatocellular carcinoma/ Huh-7 and HA22T cells	VEGF, MMP-9, AP-1, NF-κB, IκB	
Ba Y., Wang Y. 2017	To establish the method to determine the chlorogenic and caffeic acid contents in PV, and to investigate their antiproliferative effect.	HPLC was used to separate the chemical components of PV. Cell viability was assessed by MTT assay. Morphological changes were observed by inverted phase contrast microscope. Western blotting was used to detect the expression level of c-myc.	thyroid cancer/ K1 cells	c-myc	PV extract inhibited the growth and proliferation of the cancer cells. It significantly lowered the expression of c-myc protein.
Cohen, Z. et al. 2017	To compare, among 27 herbs surveyed, their sensitivity to induce ROS-mediated cytotoxicity in cancer cells by the herbs.	The inducing effects of the herbs to cause ROS-mediated cytotoxicity were measured by XTT assay. To distinguish between ROS-dependent and ROS-independent cytotoxic effects of herbs, the ROS scavenger pyruvate was also added to the medium to block the ROS-inducing effect.	10 different cancer cell lines: A549, MCF7, MDA-MB-231, PC-3, DU-145, T24, PANC-1, SK-N-BE, 526mel and 624mel.	N/A	Out of the 27 herbs tested, 10 exhibited cytotoxic effect, 7 of which the cytotoxic effect was ROS-mediated, and 3 of which were ROS independent.
Fang Y. et al. 2017	To explore the antitumor effect of PV extract, and to study the roles of multiple oncogenes, and the microRNA miR-34a	Cell proliferation and viability were studied by MTT assay, and flow cytometry with annexin V/PI staining analysis. Colony formation assay was used to observe the formation of colonies. To examine the role of miR-34a in mediating the expression levels of the oncogenes, cells were treated with miR-34a inhibitor first, before they were examined by RT-qPCR and Western blotting to detect the expression levels of the oncogenes.	Colon carcinoma/ HCT-8 cells	miR-34a, Notch1, Notch2, Bcl-2.	
Yang Y. et al. 2017	To examine the cytotoxic effect of hyperoside in PV on non-small cell lung cancer cells and to study its underlying mechanism	The cytotoxicity was detected by MTT assay. Cell apoptosis and mitochondrial membrane potential were determined by flow cytometry with annexin V FITC/PI fluorometric test kit. Western blotting was used to identify the expression levels of associated proteins, and phosphorylation of MAPK. Western blotting was used to study the levels of the proteins, cytochrome c, caspase-3,9.	Non-small cell lung cancer/ A549 cells	p38 MAPK, JNK, cytochrome c, caspase-3,9.	The hyperoside in PV significantly inhibited the viability of A549 cells, increased the protein phosphorylation of p38 MAPK and JNK, reduced the mitochondrial membrane potential and triggered the release o cytochrome c , raised the levels of caspase-3 and -9.
Yin D.T. et al. 2017	To study the apoptotic effect of PV on well-differentiated thyroid carcinoma cells, and to elucidate the underlying mechanism.	The cell apoptosis was studied by the cell counting kit-8 assay. Morphological changes were observed by Hoechst 33342 and acridine orange/ethidium bromide staining. DNA gel electrophoresis was used to detect the ladder pattern of DNA fragmentation. RT-qPCR was used to measure the expression levels of Bcl-2/Bax and caspase-3.	well-differentiated thyroid carcinoma/ TPC-1 and FTC-133 cell lines.	Bcl, BAX, caspase-3	The PV extract induced apoptosis. It significantly increased the level of expression of BAX and caspase-3, but decreased that of Bcl-2.

Zhao X. et al. 2017	To demonstrate the antitumor effect of a Chinese herbal formula, Ruanjian Sanjie decoction.	The study used an in vivo mice model, using Swiss albino mice and breast cancer xenografts in nude mice. The body weight loss, immune function toxicity or myelosuppression was measured. In vitro, cell viability was assessed by MTT assay. The caspase activities were measured by Caspase-Glo 3/7 assay and Caspase-Glo 9 assay kits. The nuclear morphology was observed by Hoechst 33258 staining method. Cell apoptosis was assessed by flow cytometry with annexin V-FITC/PI staining. RT-qPCR was used to measure the expression levels of Bcl-2 and survivin, which were further studied by Western blotting.	breast cancer, Ehrlich ascites carcinoma/ MDA-MB-231 cells and MCF-7 cells	Bcl-2, survivin	The herbal formula exhibited effective antitumor effect. Administration of the formula in combination with 5-Fu or doxorubicin is more effective than using the chemotherapeutic treatment alone. RT-qPCR and western blotting results both revealed that the formula inhibited the expression of Bcl-2 and survivin proteins.
Zhou Y.M. et al. 2017	To isolate and purify the polar chemical compounds from PV. To explore the cytotoxicity of these compounds on cell lines.	The isolation and purification were done using silica gel, reverse-phase octadecylsilyl, and Sephadex LH-20 chromatographic methods. MCI and HPLC were then used. MS and NMR were used to elucidate the structures of the compounds. Cytotoxicity was measured by MTT assay.	breast cancer/ MCF-7, MDA-MB-231 and MCF-10A cell lines.	N/A	A total of 12 compounds were isolated. 4 of them showed significant anti-tumor effect on cancer cells.
Ahn E.Y. et al. 2018	To explore the efficiency of the 3 herbs selected (including PV) as reducing agent in the biofabrication of gold nanoparticles.	The efficiency in biofabrication was evaluated by measuring surface plasmon resonance at 530 nm, and high-resolution X-ray diffraction analysis. The anti-oxidative power was assessed using DPPH, ABTS, and the total phenolic content was found by Folin-Ciocalteu's reagent. Cell cytotoxicity was evaluated by WST assay.	HT-29, PANC-1, MDA-MB-231	N/A	PV demonstrated to be an efficient reducing agent for biofabrication of gold nanoparticles, and the PV-AuNP complex exerted the highest cell cytotoxicity.
Fan, Y. et al. 2018	To explore and compare the anti-tumor effect of 6 different herbs (PV being one of them).	Soxhlet extraction method was used with a equal volume mix of ethanol and water, and a petroleum ether-ethyl acetate mix respectively. Cell viability was evaluated by a cell counting kit-8 and MTT assay. Cell apoptosis was analyzed by the annexin V-FITC/PI kit. Cell cycle analysis was done by flow cytometry with PI. DNA fragmentation was detected by DNA gel electrophoresis	hepatoma/ BEL7404, HepG2, HepaRG, Huh-7 cell lines	N/A	Amana edulis from the water/ethanol system was found to show the highest inhibition rate on hepatoma
Jang, H.J. et al. 2018	To report a single case study on a female patient with stage IV hepatocellular carcinoma and lung metastases. To report the effect of using pharmacopuncture with PV and together with chemotherapeutic drug sorafenib on the treatment outcome.	The whole healing process of using pharmacopuncture , herbal medicine together with chemotherapy was described	hepatocellular carcinoma	N/A	The 8 week treatment showed that the size of the metastatic nodules decreased and the sorafenib-associated side-effects improved. The combined treatment approach was a promising method.

Zhao, J. et al. 2018	To explore the efficacy and safety of PV combined with taxane for the treatment of 424 patients with breast cancer.	It was a randomized control trial involving 424 cancer patients with breast cancer. The patients were divided into two groups. The outcome was evaluated by the Miller and Payne system and further evaluated by the Common Terminology Criteria for Adverse Event version and Kaplan-Meier curves. Estrogen receptor status was also measured.	Breast cancer	N/A	Results showed that the combined use of PV with taxane was a safe and effective treatment method for patients with breast cancer.

Appendix 3: A summary of articles included in the review of Chapter 3

Author/Year	Primary objectives	Study design	Number of herbs investigated	Material investigated	Extraction method	Viruses targeted	Mechanisms investigated
Ao et al. 2021	To demonstrate the inhibitory effect of the aqueous PV extract to block the viral entry.	By using a SARS-CoV-2 spike glycoprotein mutant D614G pseudotyped HIV-1 based vector system, to demonstrate its infectivity to ACE2-expressing cells, and the ability of the aqueous PV extract to block viral entry. Comparison of the efficacy of blocking viral binding to the cell ACE2 receptors with another compound, Suramin.	1	whole herb	aqueous extract	SARS-Cov-2	targeting receptor binding and entry blocking
Li et al. 2019	To identify a potent herb and its constituent which can be used as anti-IHNV drug	Screening of 32 herbs for their anti-IHNV activities. Identify PV and its main constituent, ursolic acid, to be the most potent. In-vivo tests were also applied using ursolic acid on rainbow trout.	32	ursolic acid	aqueous extract	infectious hematopoietic necrosis virus	targeting replication, cytopathic effect
Yang et al. 2017	Adopt a screening protocol which was based on pseudotyping to discover entry inhibitors against several types of viruses.	Use of a pseudotyping platform using time-of-addition technique to check the anti-viral activites of several herbs, including PV.	7	whole herb	ethanol extract	Ebola, avian influenza, Lassa	screening for entry inhibitors
Zhang et al. 2016	To provide evidence that the aqueous extract of PV has anti-Ebola virus activity	Pseudotyping, time-of-addition techniques with ELISA, luciferase assays were used in various cell lines.	1	whole herb	aqueous extract	Ebola virus	targeting entry blocking

Author/Year	Primary objectives	Study design	Number of herbs investigated	Material investigated	Extraction method	Viruses targeted	Mechanisms investigated
Feng et al. 2012	To screen 16 herbs for their anti-HIV activities, and to investigate the effect of PV extracts on CXCR4 and CCR5 receptors on healthy CD4+ T cells.	Pseudotyped HIV-luciferase reporter assay was used to identify the anti-HIV mechanisms. HIV-1 replication was evaluated using ELISA. Also, in vivo test was adopted.	16	whole herb	aqueous extract	HIV-1	targeting receptor and entry blocking
Reichling and Schnitzler 2011	To establish the antiviral efficacy of the alcoholic extracts of 3 Lamiaceae herbs, including PV.	The antiviral efficacy was established using a plaque reduction assay. Time-of-addition technique was applied. Preliminary clinical studies were also done.	3	volatile oils and alcoholic extract	alcoholic extract	HSV-1	targeting virus attachment
Tian et al. 2011	Evaluate the anti-neuraminidase activities of 439 herbs	Both in-vitro and in-vivo methods were used to screen the anti-viral effects of the herbs, and more detailed study was done on 5 most promising herbs, and in-vivo test was done with Melia toosendan.	439	whole herb	aqueous extract	Avian influenza A	targeting reproduction
Oh et al. 2011	To establish the inhibitory effect of the aqueous PV extracts against HIV-1 infectivity.	Time-of-addition technique, comparison between aqueous extracts and ethanol extracts, immunostaining, SDS PAGE	1	whole herb	aqueous extract, 95% ethanol Soxhlet extraction	HIV-1	blocking entry, and targeting post-binding events

Author/Year	Primary objectives	Study design	Number of herbs investigated	Material investigated	Extraction method	Viruses targeted	Mechanisms investigated
Brindley et al. 2009	To characterize the anti-lentiviral activities of both aqueous and ethanol extracts.	Time-of-addition technique was applied. Fractionation was used to demonstrate the synergistic anti-viral activities of the separate fractions. Immunostaining was used.	1	whole herb	aqueous extract, ethanol extract	equine infectious anemia lentivirus	blocking binding and entry
Reichling et al. 2008	To characterize the inhibitory activities of the ethanol extracts of 4 Lamiaceae herbs against HSV.	Several Lamiaceae plants were phytochemically characterised before the reduction of HSV infectivity and inhibitory activity were tested using plaque reduction test.	4	whole herb	ethanol extract, phenolics	HSV	entry inhibition
Zhang et al. 2007	To characterize the chemical nature, the mode of action, and the in-vitro and in-vivo anti-HSV activities of the polysaccharide from PV	The polysaccharide from PV was first isolated by ethanol precipitation, dialysis, CTAB precipitation, and gel exclusion chromatography. Then, the anti-viral activity was then characterized using a plaque reduction assay. The in-vivo tests were done using a skin lesion model in guinea pigs, and a genital infection model in mice.	1	polysaccharide	aqueous extract, ethanol precipitation	HSV	target virus directly pre-entry, block binding and entry
Nolkemper et al. 2006	To screen the anti-HSV activities of 6 herbs using a plaque reduction assay, and establish the mode of action of the herbs.	The screening of the anti-viral activities was done using a plaque reduction assay. The stage of anti-viral action was established using the time-of-addition technique.	6	whole herb	aqueous extract	HSV	targeting pre-adsorption events

Author/Year	Primary objectives	Study design	Number of herbs investigated	Material investigated	Extraction method	Viruses targeted	Mechanisms investigated
Chiu, Zhu & Ooi 2004	To establish the antiviral efficacy of a polysaccharide fraction from PV.	A polysaccharide fraction from PV was separated. Then, the expressions of HSV-1 and HSV-2 antigens were investigated with flow cytometry.	1	polysaccharide	aqueous extract	HSV	expression of HSV antigens in Vero cells
Liu et al. 2002	To identify the inhibitors of the HIV-1 gp41 six-helix bundle formation in the PV and Rhizoma cibotte extracts.	To screen for the inhibitors of the gp41 six-helix bundle formation using the antibody NC-1. Then, the potent inhibitory constituents were isolated by passing the extracts through polyamide resin min-columns.	9	polyphenol	aqueous extract	HIV-1	inhibit binding and entry
Au et al. 2001	To compare the integrase inhibitory activities between the aqueous and methanol extracts of 20 herbs	Use of a non-radioactive ELISA-based HIV-1 integrase assay to screen. The potent effective constituents were identified.	20	polyphenols, tannins	aqueous extract, methanol extract	HIV-1	integrase activity
Lam et al. 2000	To compare the protease inhibitory activities between the aqueous and methanol extracts of 31 herbs	The activity of recombinant HIV-1 protease was determined by sequence-specific cleavage of the fluorogenic substrate or by HPLC analysis of the cleavage products.	31	whole herb	aqueous extract, methanol extract	HIV-1	protease inhibition

Author/Year	Primary objectives	Study design	Number of herbs investigated	Material investigated	Extraction method	Viruses targeted	Mechanisms investigated
Kageyama et al. 2000	To investigate the anti-HIV-1 mechanisms of PV	The adsorption inhibition was evaluated by observing the morphological change such as giant cell formation using a microscope. It was further assessed by the time-of-addition technique, and the number of viable cells was calculated using the Trypan blue exclusion method. The production of HIV-1 virions was assessed in the culture medium by the measurement of the concentration of HIV-1 p17 by ELISA. The inhibition of the reverse transcriptase activity was evaluated using a radiative assay for reverse transcriptase activity.	1	whole herb	aqueous extract	HIV-1	pre-entry and post-entry events, inhibition of reverse transcriptase
Xu et al. 1999	To isolate and characterize of an anti-HSV polysaccharide from PV	The water soluble polysaccharide was isolated using hot water, then ethanol precipitation and gel permeation column chromatography. The antiviral effect was characterized using a plaque reduction assay. The stage of action was identified using the time-of-addition technique.	1	polysaccharide	aqueous extract, ethanol precipitation, further extraction by butanol and methanol	HSV	targeting binding

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Yamasaki et al. 1998	To screen the antiviral activities of 46 herbs	The extracts of the herbs were obtained using acetone, ethanol, and 70% aqueous ethanol, and then, by vacuum evaporation. MT-4 cells were infected and treated by the herbal extracts. The viable cells were identified by trypan blue exclusion method. The suppression of HIV-1 induced giant cell formation was observed under a microscope. The inhibition of the reverse transcriptase activity was evaluated using a non-radiative assay for reverse transcriptase activity.	46	whole herb	acetone, ethanol, and 70% ethanol aqueous extract	HIV-1	inhibition of reverse transcriptase, inhibit giant cell formation and virus replication
Collins et al. 1997	To screen the antiviral activities of the aqueous extracts of 19 herbs	Using a series of in-vitro assays, the extracts were tested for inhibition of the interaction between HIV-1 gp120 and immobilized CD4 receptors, inhibition of recombinant HIV-1 reverse transcriptase and for inhibition of three glycohydrolase enzymes that contribute to vital protein glycosylation.	19	whole herb	aqueous extract	HIV-1	inhibit the interaction between HIV-1 gp 120 and CD4 receptor, inhibit reverse transcriptase and inhibition of three glycohydrolase enzymes

Author/Year	Primary objectives	Study design	Number of herbs investigated	Material investigated	Extraction method	Viruses targeted	Mechanisms investigated
Yamasaki et al. 1993	To screen the antiviral activities of 204 herbs and to evaluate their anti-HIV-1 activities	The use of MT-4 cells to evaluate the HIV-1 induced cytopathogenicity. Both hot water and cold water extracts from the herbs were used and compared.	204	whole herb	aqueous extract	HIV-1	cytopathic effect, suppress replication
Yao et al. 1992	To screen the antiviral activities of 4 herbs, and to establish that PV showed the most significant inhibitory efficacy	The extracts were obtained by sequential precipitation, followed by reverse-phase and gel permeation HPLC separations. The time-of addition technique was used to identify pre-treatment effect of the HIV-1 virus with the PV extracts. PCR analysis was also used to confirm the inhibition of infectivity by the herbal extracts.	4	whole herb	aqueous extract	HIV-1	inhibit replication, prevent viral attachment to the CD4 receptor
Ryu et al. 1992	To establish the antiviral activities of two triterpenes in PV	Plaque reduction assay was used to estimate the anti-viral activity. Two potent anti-viral triterpene constituents in PV, butulinic acid and 2α,3α-dihydroxyurs-12-en-28-oic acid, were identified by observing the spectroscopic properties.	1	2 triterpenoic acids	aqueous extract	HSV-1	block entry and post-entry events
Zheng 1990	To evaluate the antiviral effect of 472 traditional Chinese medicine herbs	The aqueous and alcoholic extracts of the herbs were screened qualitatively initially, and then repeated screenings were done quantitatively.	472	whole herb	aqueous and alcohol extracts	HSV-1	cytopathic effect