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Review of the Studies on the Anti-Tumoral Effect of *Prunella vulgaris*

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Abstract

Prunella vulgaris (PV) is a herb which grows widely around the world. It is used in traditional medicine in different continents worldwide. This article reviewed the research studies in the last three decades about the use of this herb in the treatment of cancer. Specifically, this study concentrates on the scientific *in-vitro* methods used, as the *in-vitro* methods were the most preferred methods used in the past. Cell viability/apoptosis, migration, anti-oxidative activities, and the underlying molecular mechanisms were the features which most of the research focused. The aim of this article was to summarize on what molecular mechanisms, which these previous research found responsible for the anti-tumoral effect of PV. The assays to investigate the aforementioned items were organized and displayed, including the proteomic methods which study the underlying molecular mechanisms. By categorizing and organizing these methods, the directions and emphases taken by the research efforts were revealed.

Keywords

Chinese Herbal Medicine, *Prunella vulgaris*, *Xia Ku Cao*, *In Vitro* Methods, Molecular Mechanisms, Functional Assays

1. Introduction

Prunella vulgaris (PV) is a plant which is widely grown around the world. It was used traditionally for medicinal treatment in Eastern Europe, the Indian Subcontinent, North America, and China for many generations. In common, this herb was used as a remedy for reducing fever, wound healing, and sore throat [1].

A preliminary literature review was done with the database, Pubmed, alone to gather initial information. This collected a total of 206 articles. Among these 206

articles, the research on PV can be divided into three broad areas: pharmacological, phytochemical and agricultural areas, apart from some general review papers; for examples, [2] [3] and [4].

In the area of pharmacological research, one interesting topic is to investigate the anti-tumoral effect of PV. This anti-tumoral effect of PV was what the current review focused on. Among the previous research projects, they used different methodologies; namely, *in vivo*, *in vitro* and in silico methods. They probed the antitumoral efficacy of the herb. To achieve our aims, we concentrated on the *in vitro* methods. This enabled the molecular mechanisms behind the anti-tumoral effect of PV to be revealed. Research on the anti-tumoral effect of PV was reviewed before: In an article by Huang *et al.* [5], the review just included the *In vitro* methods as a section in their discussions. Another newer review paper by Wang *et al.* [6] did a review that summarizes the chemical constituents, pharmacological effects and clinical applications of the herb. An even more recent article by Gan *et al.* [7] did a network search to summarize the bioactive ingredients of the herb. It explored the molecular mechanisms of how PV works, which followed the same approach described in the current article; however, it just concentrated on using the herb to treat Hashimoto's thyroiditis.

By concentrating on those articles which used the appropriate *in vitro* methods, a collective review of the responsible molecular mechanisms can be summarized.

2. Methods

To undertake systematically the review, we adhered to the following steps: We defined the limits of the investigation by spelling out what articles were included in this review, outlined the aims of this review. Then, we explained the methodologies to carry out the literature review to select the articles to be analysed.

2.1. Limits of This Investigation

To set the limits of this investigation, the first limit, of course, is to limit the attention only on the pharmacological effects of PV.

A survey of the literature shows that PV is effective to treat a wide variety of diseases. It is difficult to take a deep investigation at every one of the diseases investigated. This investigation concentrated on the anti-tumoral effect; in particular, on the survey of research methods used. Especially, the attention was on those research projects which used *in-vitro* methods based on molecular biology. The *in-vitro* methods were chosen as the most popular research methods used (**Figure 1**).

2.1.1. Aims of This Investigation

Over the past 30 years when the research started working on PV, this survey witnessed a gradual development of technology which the various methods are based on. In earlier years, research projects mainly used colorimetric assays, such as the MTT assay, to characterize the change in the functioning of cells

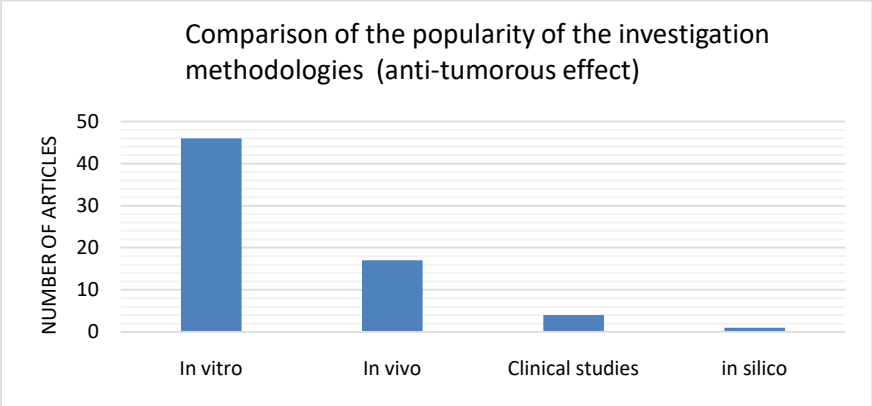

Figure 1. Comparison of the popularity of the methods chosen for research on anti-tumoral effect using PV in the last 3 decades. *In vitro* methods were used in 82% of the articles found. The statistical data used to construct this figure is from the initial literature review using Pubmed.

upon application of the herbal treatment; for example, impact on cell viability, an issue which many investigations probed. In recent years, the choice shifts to proteomic assays, which clarify the molecular mechanisms more clearly.

A major aim of this article was to determine the main molecular mechanisms which the previous research projects focused their efforts on and was responsible for the anti-tumoral effect of PV. Also, what types of assays were chosen to explore these molecular effects.

Then, a secondary aim was to look at the functional tests used to study issues such as cell viability, proliferation, migration, morphological changes of cancer cells upon herbal treatment. These include methods such as the colorimetric assays, and observational methods using microscope or flow cytometry. The functional changes include morphological changes, migration or propagation. Colorimetric assays were the most popular choices.

2.1.2. Approach of the Literature Review

To comprehend a general idea of the scope of studies done on PV in the past, a preliminary literature search was done using a very general term "*Prunella vulgaris*" as the keyword, and the reputable database, Pubmed, was used.

A more detailed literature search was done recently using three databases: Pubmed, Medline and Embase. They were chosen because of their popularity within the research community. This enabled the inclusion of as many relevant articles as possible without omission.

Nearly all the articles found were included except those articles which used the *in-vivo* methodology only. Studies which reported clinical studies, single case report, ethnobotanical survey and in silico analysis were also excluded. A PRISMA flowchart for articles investigating the anti-tumoral effect is included in **Figure 2**. It is worth to mention that about 40 more articles were discovered in the final search as compared with the initial search done about a year ago. This reflected the enthusiastic research interests on this herb.

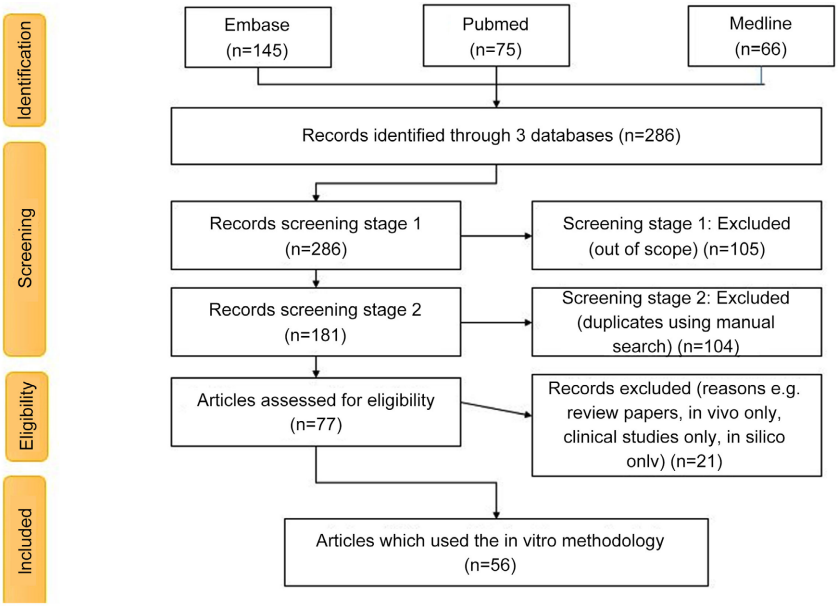

Figure 2. PRISMA flowchart of the final literature survey. This survey included articles which used the *In vitro* methodologies to investigate the anti-tumoral effects of *Prunella vulgaris*. These articles extended from 3 decades ago until recently.

3. Results

The result section is organized into two parts: One pertaining to the first aim, which is to look at what molecular mechanisms were found responsible for the efficacy of PV, another part pertaining to what functional assays were used by previous researchers. At the end of this article in the **Appendix** section, we include a summarizing descriptive table of all the articles included in the review.

To investigate the anti-tumoral effects of PV, different phenomena were observed experimentally. As discussed above, we concentrated on those articles which used the *in-vitro* methods. **Figure 3** summarizes the different phenomena observed.

Apoptosis/cell viability was the most popular phenomenon investigated; also, observing the effects on changes in the genomic/proteomic expressions of the treated cells were also frequently taken, comprising 76% and 54% of all the articles surveyed. The differential genomic/proteomic expressions indicate the molecular mechanisms behind the effects of the herb. This is the main aim of this review.

3.1. Results Pertaining to the Major Aim: A Look at the Molecular Mechanisms Responsible for the Antitumoral Activity of the Herb

As the technology advances throughout these 3 decades, more and more studies were done using the genomic and proteomic approaches. The use of these approaches reveals the molecular mechanisms more clearly behind the anti-tumoral effects of the herb. The main theme of this review is to summarize on the molecular mechanisms.

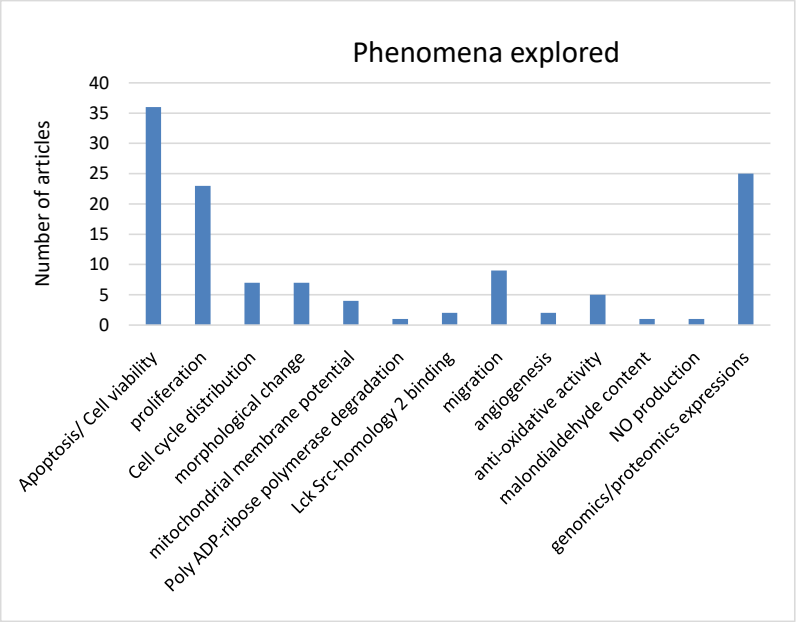

Figure 3. The different cellular phenomena which were observed experimentally in the articles surveyed in this review.

Figure 4 shows the different techniques in genomic and proteomic methodologies. These techniques measure the cellular products such as intracellular proteins, enzymes, and intercellular signaling cytokines. By monitoring the changes in these entities resulting from the treatment of the herb will elicit the genomic expression pathways involved and thus expose the underlying molecular mechanisms.

To elicit what molecular mechanisms which the researchers in the last 3 decades most popularly investigated, several approaches can be taken. In this article, we choose to discuss in terms of the entities, such as proteins, genes, or RNAs being studied to expose the underlying pathways and mechanisms; an approach which we think is the most clarifying (**Figure 5**), as follows:

1) Bcl-2/Bax [8] [9]

From **Figure 5**, it is obvious that the most frequently investigated regulating proteins were the apoptosis regulating Bcl-2 (B-cell lymphoma 2) family, which includes pro-apoptotic members (such as Bax) and anti-apoptotic members (such as Bcl-2). They are involved in the intrinsic pathway of mitochondria-related apoptotic activation mechanism. They have significant role in the lymphoma growth cell cycle, and thus given the most attention. Related proteins in the apoptotic process such as Apaf-1, caspases, and cytochrome c were also tested in other articles.

From **Figure 6**, the most frequent techniques used were immunocytochemical methods, such as ELISA, followed by Western blotting.

In all the articles surveyed, when the Bcl2/Bax expressions were investigated, they did show that PV was effective to decrease the expression of Bcl2 and increase that of Bax. However, even when experiments were done quantitatively,

numerical data were not given in the articles. The closest to this is, for example, to give a p-value ($P < 0.05$) to show that the differential Bcl2/Bax expressions were statistically significant [10].

Another example which reported the effects of the extracts of endophytic fungus from PV to inhibit gastric cancer. The study reported that a dosage of 100 mg/kg/day (this study used an *in-vivo* mice model) treating tumor tissue caused a decrease of Bcl2 and an increase of Bax levels [11].

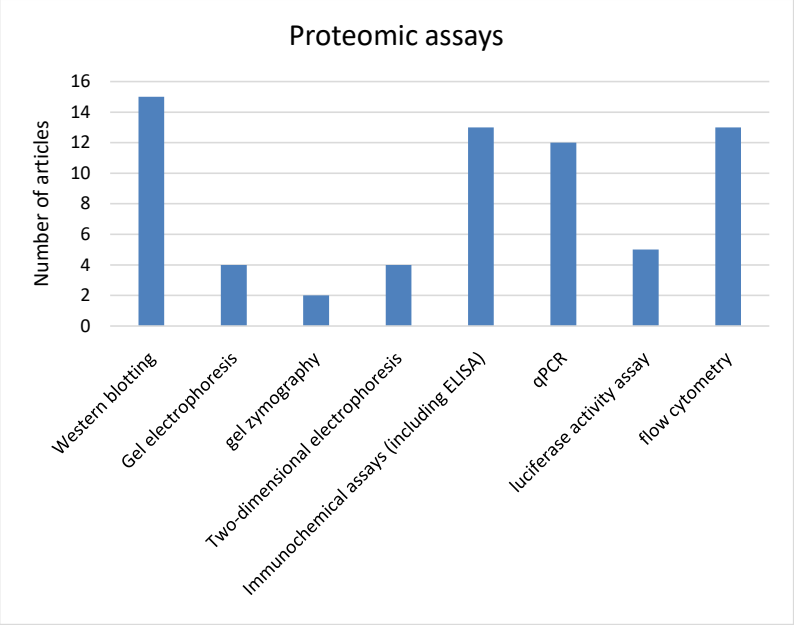

Figure 4. The proteomic assays used in the articles surveyed in this review study, to investigate the underlying molecular mechanisms.

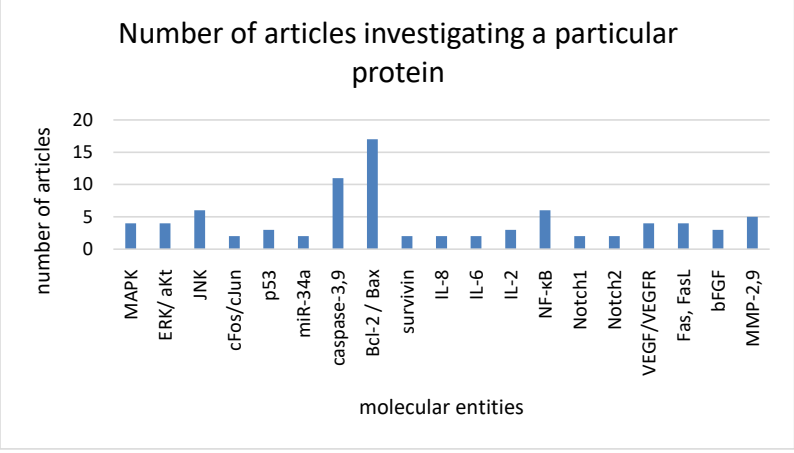

Figure 5. The different proteins being studied in the articles under the survey in this review study. Note that those proteins/mRA which were only studied by one article are not included in this figure for clarity. Those proteins/mRA are listed here: CD1, CDK4, APAF-1, AP-1, cytochrome c, Bad, c-myc, iNOS TNF-α, IL-1β, IL-6, RANKL/RANK, RIPX, Stat3, ROD1, IκB-α, Snail, Notch1, Notch2, EGFR CAF, TIMP-1, N-cadherin, β-catenin, vimentin.

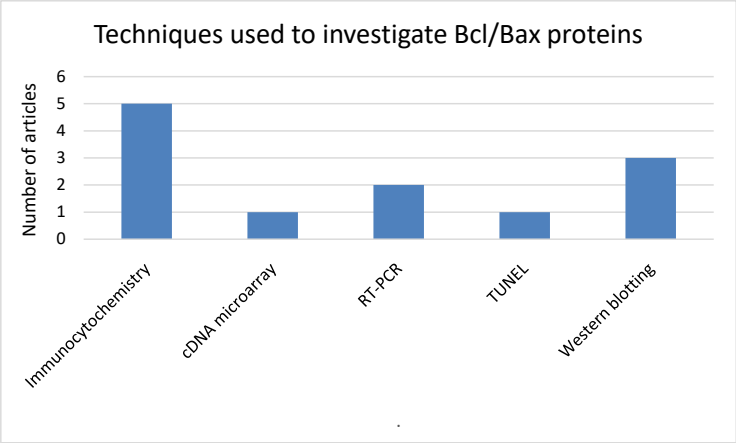

Figure 6. The different techniques adopted by the articles surveyed in this study, and used to investigate the differential expression of Bcl2/Bax due to the use of PV treatment. There were articles which did not explicitly mention what techniques they used.

There was an article [12] which described the use of PV to treat B lymphoma cell line Raji cells and T lymphoma cell line Jurkat cells. It showed that,with the same dosage, the degrees of decrease in the Bcl-2 expression level and the increase in Bax protein expression in Raji cells were more significant than that in Jurkat cells ($P < 0.05$).

In an article which investigated Bcl-2 [13], the signal transduction pathway was further investigated to trace back to the PI3K/AKT signaling pathway. Using Western blotting, it was shown that PV extract inhibited the expression of p-PI3K and p-AKT but did not affect the expression of PI3K and AKT, and thus, explored the role of the PV extract application as anti-tumoral.

2) **Caspase-3,9** [14] [15]

Caspases are a family of proteases which act as enzymes for programmed cell death, in which targeted proteins are attacked and cleaved. So, they play a key role in inducing cell death in abnormally growing cancer cells. Some of them are involved in the activation of inflammatory responses. Caspases are classified into different types, in which caspase-3 acts as executioner, and caspase-9 as initiator of apoptosis. The process of cell death through the action of caspases is complex, which consistsof complex chain reactions involving multiple proteins, enzymes and cytokines.

For the investigations on the caspases, the methods chosen were about the same as those for Bcl-2. One additional method used was the fluorometric/colorimetric assays [16]. Similarly, the research projects did not depend on a single method. For example, in a project which studied the effect of PV on gastric adenocarcinoma SGC-7901 cells [17], the alternations in the gene expression levels were studied using a cDNA microarray, real-time qPCR and immunohistochemical methods.

In all these previous studies, the caspases-3, 9 and 12 were followed. The expression levels of caspases were significantly increased by the treatment of PV.

In these studies, usually more than one protein was examined. These somehow indicated more clearly which pathways were involved in the apoptosis process. For example, Yang *et al.* [18], reported that, other than an increase in the caspase-3, and -9 levels, hyperoside in PV increased the phosphorylation of p38 MAPK and JNK, disrupted the mitochondrial membrane potential, increased the release of cytochrome c from the mitochondria into the cytosol. These revealed the whole apoptotic pathway through the mitochondrion. Another article [19] also revealed this mitochondrion-mediated apoptotic pathway by following the expressions of multiple proteins.

Another article quantified that a dosage of PV of 30 μg/mL was enough to up-regulate the expression of caspase-3 [20]. In this previous article, the authors, Zhang and Wang, explored the synergistic effects of PV when used together with anti-tumoral drugs paclitaxel and adriamycin. They showed that PV enhanced the effects of these drugs.

3) ***NF-κB*** [21] [22]

The nuclear factor NF-κB is a protein which acts on controlling the transcription of DNA, and the production of cytokines, and thus, is involved in the immune responses of cells to various kinds of external stimuli. So, NF-κB is a key component to regulate inflammatory responses to infection. Its abnormal function or expression is thus implicated in cancer development and other inflammatory diseases. The characterization for its differential expression is used to study the anti-tumoral effects of drugs. Known inducers of NK-κB activation such as the interleukin, TNF-α, and its inhibitors, IκBs, were also included in some studies in the articles found.

While immunocytochemical methods and Western blotting were the main methods chosen to investigate protein expressions relating to NF-κB, additional methods were used to study NF-κB. The effect of the application of the herb on NF-κB expression can be deduced by the successive pretreatment of NF-κB activation inhibitor, IκB, and then, observing the effect on the downstream MMP-9 expression. Also, NF-κB binding activity to DNA can be examined by electrophoretic mobility shift assay [23].

NF-κB situates upstream of the expressions of cytokines related to inflammation, and of proteins related to metastasis and epithelialmesenchymal transition. The studies on these issues concentrated on the investigations on MMP-9 [24] [25] [26], vimentin, N-cadherin, β-catenin [27]. In these studies, the levels of expressions of these proteins will show the molecular signaling pathways which involve NF-κB.

4) MAPK, ERK, and JNK [28] [29]

On the left of the graph in **Figure 5**, the enzymes MAPK (mitogen activated protein kinases), ERK (extracellular signal-regulated kinases), and JNK (c-Jun N-terminal kinases) are shown. Indeed, these enzymes belong to the same family, but were given different names because of historic reasons. These enzymes are related to the Ras-Raf-MEK-ERK genomic pathway, which responds to intercellular or exterior stimulating signals such as cytokines, UV radiation, os-

motic stress, and heat stress. The responses include the regulation of cell functions from proliferation, differentiation, cell cycle progression, division events such as mitosis and meiosis, to cell survival and apoptosis. Disruption in these signaling pathways, such as the malfunction of the proteins in the pathways, can cause cancers.

The articles which reported investigations on these kinases had been described in the paragraphs above looking into those proteins. This means that signaling transduction pathways relating to these kinases were identified by following multiple proteins: mitochondrial cytochrome c, caspase-3,9 [18], MMP-9, NF-κB [24] [26].

Comparing the differential expressions of these enzymes upon the application of herbal treatment showed the genomic pathways which were involved in the anti-tumoral action.

5) MMP-2,9 [30] [31]

When metastasis was the issue studied, cell surface proteins were used as the target of investigation. The most frequently studied proteins were the MMPs (matrix metalloproteinases) and the other related cell surface proteins. MMPs are proteases responsible for the degradation of all kinds of extracellular matrix proteins so that epithelial-to-mesenchymal transition can proceed; cancer cells can then be set free and move to other part of the body. Other cell surface proteins, such as TIMPs, cadherins, catenins, vimentin, were also targets of investigation in metastasis of cancer cells.

The articles which investigated metastasis through the study on MMP-9 were described in [24] [25] and [26]. There was another article on metastasis which studied MMP-2 [32].

These studies showed that the application of the herbal treatment effectively reduced the abnormal expressions of the MMPs.

6) Growth Factors: bFGF, VEGF, and IL-8 [33] [34] [35]

Growth factors are cell signaling proteins, cytokines or hormones which carry signals among cells. They usually function by activating cell surface receptors to initiate a wide variety of cellular processes, including cellular growth, proliferation, tissue remodeling. FGF1 and FGF2 (fibroblast growth factors 1 and 2) have an important function to stimulate endothelial cell organization to form tube-like structure, and thus they involve in angiogenesis, an important process in cancer cell development. It was shown that FGF2 (aka bFGF) promoted human carcinoma-associated fibroblast (CAF) proliferation and migration, protected CAFs from apoptosis and reduced cells in G_0 phase in cell cycle [36]. VEGF (vascular endothelial growth factor) is another important angiogenic factor.

The articles which studied the growth factors mainly looked at the anti-angiogenic effect of the PV herb [11] [37] [38]. By competitively binding of the sulfated polysaccharide extracted from PV to the cell binding domain of bFGF, it was shown that PV significantly inhibited the proliferation, and down-regulated migration, increased apoptosis of cancer cells [39], while another article showed that PV had anti-tumoral effect by inhibiting bFGF expressions [36]. An an-

ti-tumoral formula which includes PV was shown to suppress the expression of EGFR [40]. The polysaccharide in PV could inhibit bFGF expression, and thus exerted anti-tumoral effect [36].

There were also articles which followed the suppressive effect of PV chemical constituents, and the most frequent choice, rosmarinic acid, on the expression level of the angiogenic growth factor IL-8 [38] [41].

3.2. Results Pertaining to the Secondary Aim: A Look at the Functional Tests

3.2.1. Using Colorimetric Methods to Check Cell Viability

To fulfil the secondary aim, we started by discussing the functional assays using colorimetric methods. In earlier days, for example in the tests for cell viability, colorimetric methods were the preferred methods, with the use of the tetrazolium salt MTT being the most popular.

As shown in **Figure 3**, most of the articles which investigated the anti-tumoral effects of PV, frequently examined cell functions (phenomenon) which were apoptosis/cell viability, proliferation, and migration.

To examine cell viability, two large groups of assays followed the cell metabolic activity and the cell membrane permeability respectively. To observe these, often some dyes are used. Assays can be further classified as fluorometric assays or colorimetric assays, depending on whether the observed light is due to emission or reflection.

Other than monitoring the cell viability, the use of optical observation is often applied to examine other functional changes (**Figure 3**) due to herbal applications.

1) Cell metabolic activity assays

One commonly used group of assays which examine cell metabolism is based on the tetrazolium dyes. The most popular assay is the MTT assay, which monitors the NADPH-NADP redox metabolic reactions of living cells when they are alive. In the process, the yellow MTT dye is reduced to its purple counterpart formazan [42] [43]. Other closely related tetrazolium dyes include MTS, XTT and WSTs. These are colorimetric assays shown in **Figure 7**. Most of the assays shown there depend on optical observation. However, they are often used in conjunction with other techniques; for example, Annexin V FITC/PI is often used together with flow cytometry.

2) Cell membrane permeability assays

When cells are failing, the cell membrane becomes more permeable. So, measuring the leakage of the cell membrane is a way to measure cell viability. For example, LDH leakage assay is one of these [44], which measures the leakage of a common enzyme, lactate dehydrogenase, across the cell membrane. The middle six bars in **Figure 7** pertain to this group of assays which monitor the cell membrane permeability. They are all dye based. Dependent on the permeability of these dyes across the cell membrane, they can be used to measure the cell viability.

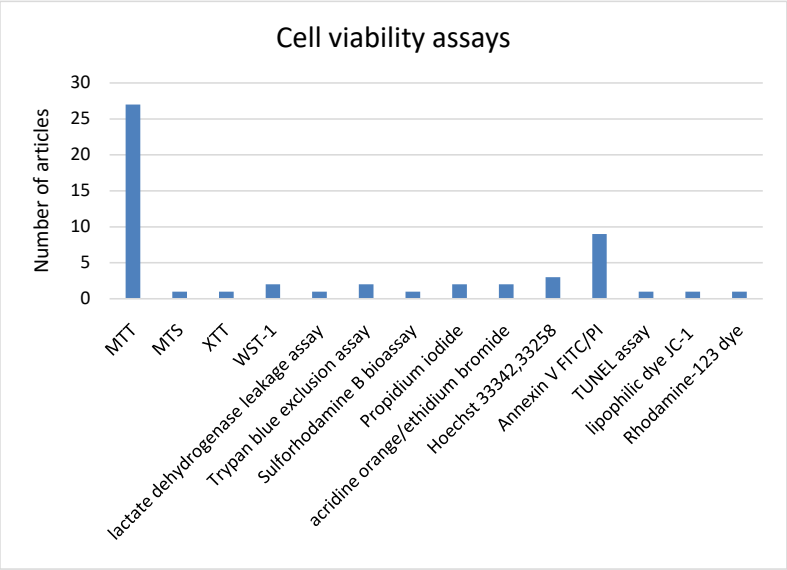

Figure 7. The cell viability can be monitored by different phenomena during apoptosis. The first 4 bars pertain to the tetrazolium dye family of assays. The middle 6 assays detect the leakage of cell membrane. The rightmost 2 bars monitor the change in mitochondrial membrane potential. The data was collected from the articles surveyed in this articles.

Annexin V FITC (Fluorescein Isothiocyanate)/PI (Propidium iodide) was the second most popular assay used in the articles which measure cell viability. It is a combined use of the DNA staining dye, propidium iodide, with annexin V, which is fluorescently labeled by FITC and binds to phosphatidylserine, a marker of apoptosis on cell surface, to measure cell apoptosis and necrosis [45]. This assay is often used together with flow cytometry to monitor early-stage apoptosis and cell cycle.

3.2.2. Other Functional Assays

Other than the colorimetric assays discussed above, it is worthwhile to describe some assays which do not use colorimetric methods, but observe some phenomena, like the cell proliferation, migration, morphological changes, or colony formation, directly under a microscope. The assays in this group are shown in **Figure 8**.

3.2.3. Assays Which Observe the Anti-Oxidative Activity

As the status of the presence of ROS in the tumor environment relates closely with the development, proliferation and growth of cancer cells, researchers frequently probed the anti-oxidative activity of the herb. See **Figure 9**.

In **Figure 9**, the six bars on the left pertain to assays which use reagents that mix chemicals together to behave as oxidizing agents or radical traps. They then measure the reducing power of the samples under test for their anti-oxidative capabilities. SOD refers to superoxide dismutase, an enzyme which cells uses to catalyze dismutation of superoxide radicals. Thus, its measurement indicates the oxidative stress. Malondialdehyde is a naturally occurring marker of oxidative stress, and glutathione is a naturally occurring antioxidant in cells. So, their

measurements give the anti-oxidative state also.

4. Discussion

4.1. Variations Which Make Standardization Difficult

All the articles surveyed showed that PV was effective and possessed anti-tumoral effect in a dosage dependent manner, except four. Three of them [46] [47] [48] showed certain main constituents in PV only had marginal cytotoxicity towards certain cancer cell lines. One article [49], however, showed that PV was ineffective for neuroendocrine tumor. Also, many studies singled out single compounds such as the oleanolic acid, rosmarinic acid and polysaccharides in PV to test its cytotoxic effect and showed that they are effective.

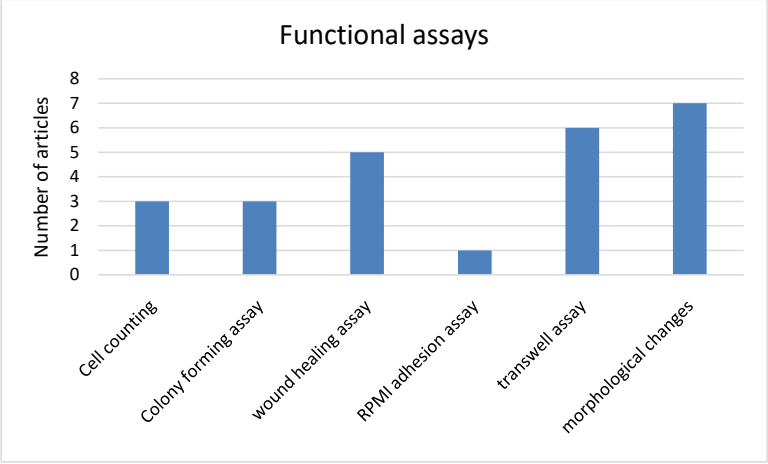

Figure 8. Functional assays used in the articles surveyed in this study, which observe the behaviors of cells under a microscope. The most frequently used functional assays were the wound healing assay and transwell assay.

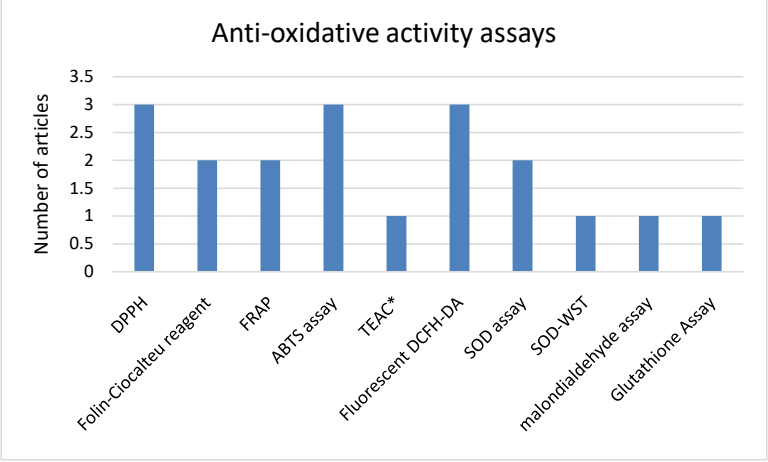

Figure 9. The anti-oxidative activity assays, used in the articles surveyed in this study. *: TEAC is a collective name. It refers to an assay which may use the DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric ion reducing anti-oxidant power), or ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) reagents but uses Trolox as a standard.

However, it is difficult to summarize their findings collectively. There are five-folded reasons for this:

1) Different materials were tested

Different materials were tested in different studies: the PV spica, the PV root, specific compounds which are the constituents found in PV, or the combination of several herbs, including PV as one of them in a herbal formula. In **Figure 10**, the first and the second groups, "PV spica" and "PV extract" are indeed the same, as active compounds need to be extracted from the plant before they can be analyzed. However, some articles did not mention whether they started off with the spica part of the plant, or they bought the extracts from outside sources. Thus, they are separated into two groups in this **Figure 10**. However, combining them, they account for nearly one half of the articles surveyed.

Some researchers focused on just a single target chemical component in PV; for examples, rosmarinic acid [41], and oleanolic acid [50] were the most popular choices. These compounds were previously shown in prior investigations to be anti-tumoral. Some researchers researched on formulae which combined several herbs including PV; for examples, Ruanjian Sanjie decoction [51], Wei Chang An [17].

A large group of 20% of the articles shown in **Figure 10** are comparison papers, which compared the efficacy of multiple herbs or multiple compounds. Two articles [52] and [53] stated that they used PV injection drug, and one article [54] stated that it used PV granules, but they did not mention the methods of preparation of these secondary products from the raw herb. Lacking the preparation method makes their experiments unrepeatable. Similarly, the selection of different materials makes it difficult to summarize systematically the pharmacological efficacy of PV.

Some projects aimed to explore the interacting and comparative effects of using PV together with anti-tumoral Western drugs; for examples, paclitaxel, Adriamycin [20] and 5-fluorouracil [17]. Then, comparison with articles which explored the effect of using PV alone is invalid. An example can be shown by the results in one investigation [20]: They compared the improvements in the inhibitory effect on tumor proliferation when PV extract was used in combination with two Western drugs: taclitaxel and adriamycin, with the effect of using the Western drugs alone. When PV extract at a dosage of 0.8 μg/mL was used with taclitaxel, the improvement on inhibiting the proliferation of lymphoma Raji cells was ten-fold better than the improvement when PV extract at a dosage of 0.08 μg/mL was used with adriamycin (**Table 1**).

2) Lack of a unified measure to show the results

One more hurdle to collectively summarize all their results is that they did not have a unified measure to show their findings. For *in-vivo* experiments, many articles quoted the animal body weight or the tumor weight [55], the spleen index or the thymus index [56], or the animal survival time [52]. For *in-vitro* experiments, some quoted the percentage improvement as compared with the

Table 1. A comparison of the half maximal inhibitory concentration (IC50) results for cancer cell proliferation from 6 articles [13] [20] [53] [54] [57] and [58].

articles	Zhang *et al.* [53]	Chen *et al.* [57]	Zhang & Wang [20]	Maimon *et al.* [54]	Gao *et al.* [13]	Zhang *et al.* [58]
Materials studied	PV injection	PV extract	PV extract	Formula LCS101	Methanol extract of PV root	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

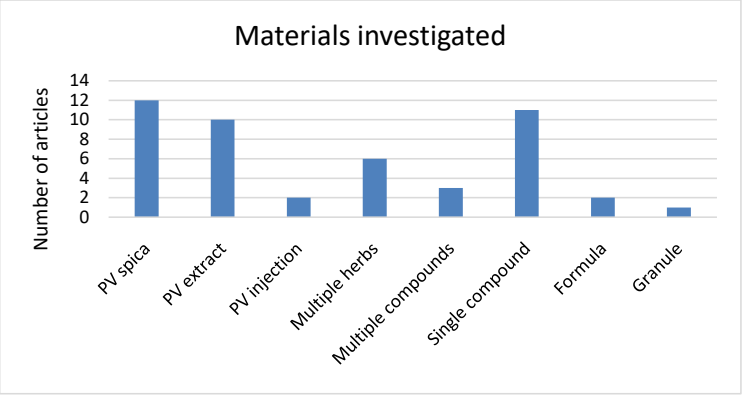

Figure 10. The sample materials used in the articles surveyed in this study. Half of all the articles surveyed used the extract of the raw herb, *Prunella vulgaris* spica, itself.

control; for example, Zhao *et al.* [17] quoted an improvement of the tumor inhibitory rate of 44.32% when compared with the control, and the apoptosis index of 9.72% when using the herb, as compared with 2.45% of the control. A more frequent used measure is the half maximal inhibitory concentration, IC_{50}. Some used the IC_{50} for proliferation; for examples, [53] [54] [57], and some used the IC_{50} for apoptosis; for examples, [16] [46] [54]. However, even more disturbing for comparison is that some used the unit of molar concentration, but some used weight per unit volume. Refer to **Table 1**, it demonstrates the closest we can get to make a direct comparison of the results from six of the previous studies. These projects chose the same unit, weight per unit volume, as the measure to report the IC_{50}.

It can be seen that the IC_{50} results obtained in these studies varied widely. The wide difference is understandable when different forms of the PV drug were used, for example, PV injection, or using a herbal formula which consisted of more than one herb. The results would be different also if the extract of the PV root was used instead of the commonly used spica. From the results of Zhang and Wang [20] when PV extract was used in combination with different Western drugs, the IC_{50} values might be ten-fold different. Also, for different cancer

types, the IC_{50} values were different. All these factors contribute to highlight why the IC_{50} numbers shown in **Table 1** can be a thousand-fold different.

3) Lack of information on the geographical sources of herb

Also from **Figure 11**, only 13% of the articles surveyed stated the geographical regions where their samples were sourced from. According to some studies [59] [60] [61], they showed that PV plants grown in different geographical regions had different profiles of chemical compositions. For example, an article [59] showed the chemical compositions of PV from 5 different producing regions in China were all different. So, the pharmacological effects of the plant from different producing regions will not be the same. The results from different studied regions cannot, indeed, be compared against each other if they do not specify the place of origin, where the plant was grown and harvested. This important information should at least be indicated, but most researchers did not care. In addition, there were articles [62] [63] which showed that the variations in water treatment and the harvest time also caused changes in the bioactive components of the plant. This raises the question of how to control the standard quality of the herb used for medical use.

4) No standard dosage

Even about half of the articles which used the raw herb, the PV spica, as the material to be tested, did not use a standard dosage. Moreover, the authors sourced their herbal samples from different sources; some from herbal dispensaries, some from their affiliated institutes, like hospitals. No standard sources made it difficult to fix a standard dosage. From **Figure 11**, it reveals a bigger problem that about one-third of the articles did not even mention where they obtained the herbal samples.

5) No standard extraction and preparation methods

There was not a standard extraction method. From **Figure 12**, different articles used different methods for extraction. The most frequently used method was by decoction, which is the traditional method of preparing herbal medicinal tea by boiling the herb with water; for example, in the project by Cho *et al.* [27]. The dried extract was then obtained by vacuum evaporation or lyophilization. This accounts for about one-quarter of the articles surveyed. The other frequently used solvents were ethanol [64] [65], and methanol [66]. Depending on the polarity of the solvent, the chemical components which were extracted would then be different. However, even many articles did not mention the extraction method at all; some may be because they used over-the-counter forms such as extracts, herbal injection or granules, so that the extraction methods were unknown. One more complication is that even if articles used the same solvent, the solvent were at different concentrations (for example, some used 70% ethanol and some used 95% ethanol), or at different extraction temperatures. These variations affected the profile of the chemical compositions of the extracts too.

About the extraction techniques used, other than by decoction, many of the studies used the reflux methods, like the Soxhlet method. A few used silica gel chromatography or sonification.

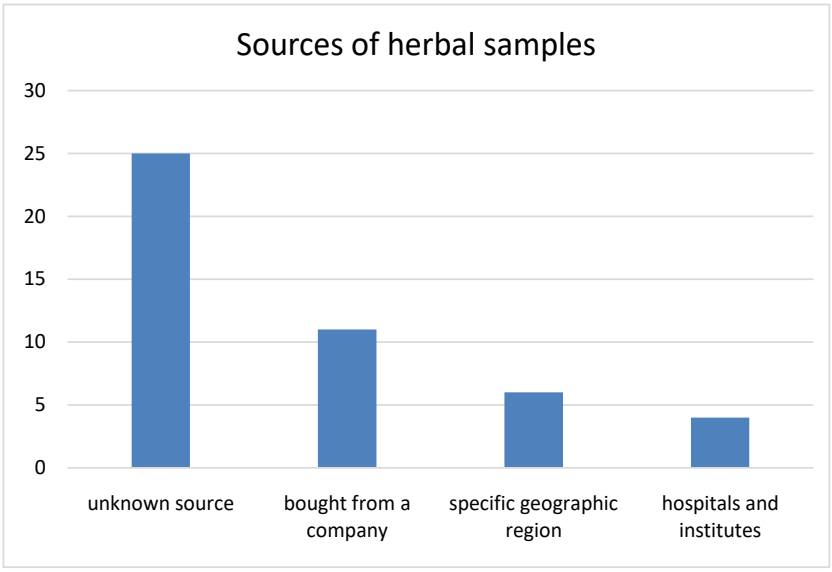

Figure 11. The sources of herbal samples among the articles surveyed. Among them, most investigations just bought their herbal samples from companies such as herbal dispensary.

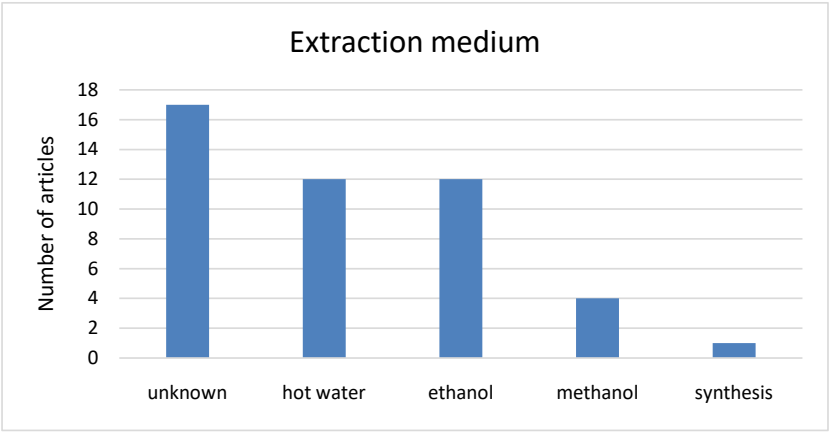

Figure 12. The extraction methods used. Most investigations used the traditional method which was used to prepare herbal medicine for patients.

In summary, from the discussion above, the approaches taken in these previous studies to obtain their results varied; were piecemeal and unorganized. It is thus impossible to summarize meaningful collective answers to many questions on the anti-tumoral effects of PV, like what the most effective chemical components in PV are, what method for extraction is the best, and many other queries.

4.2. Limitations in the Pharmacological Research of Chinese Herbs

This article discussed the use of the herb, PV, for the treatment of tumor. Especially, it analyzed the *in-vitro* methods used in the past 3 decades. By looking in-depth into these articles, certain limitations in the pharmacological research of Chinese herbs were exposed. In traditional Chinese herbal medicine, a whole

plant, or part of it; say, the root or the spica is used as the drug. In Western medicine, drugs are just a certain chemical compound found in a plant, or the combination of individual compounds. Then, it is easy to identify uniquely what material is investigated. Giving the chemical molecular formula of the compound(s) uniquely identifies the drug. In herbal medicine, it is not so straightforward. As pointed out in different articles, for example [59], a certain herb, such as PV, has different chemical components for plants grown in different geographic regions. The composition also depends on other factors such as the drought condition [67], variations in UV-B radiation [68] and altitude of producing area [69]. These variations of different factors weaken the quality of the research, as these various factors affect the morphology of the plant; then, the identity of what material is investigated becomes ambiguous. This also poses difficulty in the identification of herbs. So, many researchers just resorted to authorities, for example, by saying a certain professor verified the identity of the herb. Some researchers simply omitted the identification step of the herbs they used completely. This is far from ideal.

If the geographical origins are not explicitly spelt out, the ambiguity weakens the quality of the research. However, sometimes this is unavoidable, as most herb suppliers mix the products they source from different growing areas. It is thus not always possible to state the geographical origin.

From the categorization of all these previous research studies, it highlights that researchers believe that PV is most effective in treating breast cancer, lymphoma, leukemia and colon cancer. However, as indicated in **Table 1**, by looking at the wide range of IC_{50} values, even for the same cancer type or for the same extraction method, it is not feasible to conclude how effective the PV herb can be, what the effective dosage is, or which extraction method is the best.

For the main aim of this paper to identify what molecular mechanisms are responsible for the anti-tumoral effect of the PV herb, Bcl/Bax and caspases are the most popular proteins which most researchers concentrated their attention on. This highlights that they regarded the intrinsic pathway of mitochondria-related apoptotic activation [10] [11] [12] [13] [16] [17] [19] [50] [53] [57] [58] [65] as what the herbal treatment of PV affects most.

Other molecular pathways identified were:

- Lck-dependent Ca^{2+} signaling pathway and its downstream effectors which finally modulate IL-2 gene expression, relating to T cell activation, and thus to inflammatory response [66].
- MRK/ERK/JNK signaling pathway. This relates to the inhibition of NF-κB and MMP activity, and thus to metastasis [18] [24] [26] [27] [41] [70].
- PI3K/AKT signaling pathway [13].

However, the concentration of research efforts in just a few genomic pathways is too limiting, and points to the limitation of these conventional research methodologies. Researchers needed to target and specify the pathway which the research concentrated on. Thus, these traditional methodologies may miss many

other possible pathways. This drawback should be remedied by newer technologies such as the use of the NGS sequencing technique.

5. Conclusions

This article reviewed the research efforts in the anti-tumor effect of the herb, PV, in the past 3 decades. Specifically, this article emphasized on the *in-vitro* methods employed. This article tried to identify the molecular mechanisms which the previous research efforts on the PV herb concentrated on and found to be responsible for the anti-tumoral effect of the herb. Then, we followed the types of assays to explore them. These chiefly pointed to proteomic assays, which are still widely used.

The main issues of cancer investigation by the previous works were cell viability/apoptosis, migration, and anti-oxidative activity. However, most research efforts concentrated on the cell viability. Different assays which investigated the different phenomena that appeared during the apoptosis process were organized and disclosed. This overview provides a good summary and understanding of the methodologies employed.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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Appendix

A descriptive table summarizing the articles surveyed in this review.

Author/ Year	Primary objective	Study design	Cancer/Cell lines targetted/animal model	Proteins/ Genes targetted
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
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
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
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
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
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
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

Continued

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
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
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
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
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/ Raji cells	Multiple proteins were identified
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
Zhang, M.Z. & Wang X.Q. 2009	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
Cheng, W.W. *et al.* 2010	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
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

Continued

Feng,L. *et al.* 2010a	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
Feng,L. *et al.* 2010b	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
Liu X.K., Wang L. & Zhang M.Z., 2010	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
Xu,Y., *et al.*, 2010	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
Xu Y. *et al.* 2010	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
Feng L. *et al.* 2011	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
Lin W. *et al.* 2011	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
Woo H.J. *et al.* 2011	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

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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,
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
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
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
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
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
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.
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
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

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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
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
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
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
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

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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.
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
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
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
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

Continued

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