A STUDY OF ANTICANCER AGENTS DERIVED FROM PLANTS UTILIZED IN TRADITIONAL CHINESE MEDICINE (TCM)

By

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I dedicate my dissertation to my family and many friends.

A special feeling of gratitude to my parents for their encouragement.

I would like to sincerely thank my lovable sister Areej. She has never left my side and I will always appreciate all that she has done.

Special thanks to my small family, my husband and my wonderful daughters, for their love and support.

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ABSTRACT

Cancer is caused by abnormal cellular growth. It has the ability to invade any tissue of the body and can spread from the area or origin throughout the body. Chemotherapy has been used for decades to treat cancer. However, the effectiveness of chemotherapeutic drugs are severely limited by drug resistance which can develop over time and the inherent severe toxic effects of chemotherapeutic drugs to normal tissue. Due to these limitations of cancer therapy, there is an urgent need to develop new anticancer drugs. A revitalization of interest in drug discovery research on natural products derived from plants has occurred in recent years, and the biological activity of these products has attracted the attention of chemists, biochemists, biologists, and microbiologists. As a part of traditional culture in China, medicinal plants from traditional Chinese medicine (TCM) have been used for thousands of years to treat and prevent diseases. Herbal medicine used in TCM plays an integral role in primary health care in East Asia, as it has been used for many generations by the Chinese community. Our findings suggest that extracts from Yao Ethnomedicine and compounds *cis*- and *trans*-suffruticosol D isolated from the seeds of *Paeonia suffruticosa* have promising chemotherapeutic potential for treating cancer. The most promising Yao Ethnomedicinal plants were the extracts from Extracts from *Bidens biternata, Wedelia calendulacea* and *Stephania longa* because of their cytotoxicity and selectivity. Our findings suggest that both *cis*- and *trans*-suffruticosol D have promising chemotherapeutic potential for treating cancer. Our experimental data suggest that both *cis*- and *trans*-suffruticosol D inhibited cancer cells through apoptosis induction.

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INTRODUCTION

Cancer is abnormal cell growth. It has the ability to invade any tissue of the body and can spread from one area to other origins and tissue (Li, Chan, Guo, & Yu, 2007). It is a life-threatening disease and its morbidity has increased rapidly. It is the second leading of cancer death in the United States. Cancer is a multistep process that can be caused by a number of factors that lead to changes in cell growth and tumor metabolism. The energetics regarding amino acid, lipid, glucose metabolism and the formed metabolites including inflammatory cytokines affects cancer cell survival, proliferation, angiogenesis, invasion, metastasis and cell singling pathways (Singh et al., 2015).

In 2018, the American Cancer Society (ACS) estimates that 1,735,350 new cancer cases and 609,640 cancer deaths will occurs in the United States. Cancerous conditions can involve any tissue or organ, such as bone, brain, breast, cervical, larynx, lung, pancreatic, prostate, skin, spine, stomach, uterine or blood (WHO cancer fact sheet, 2018).

Cancer treatment strategies used in clinical practice include surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapy. The most currently used classical anticancer drugs are genotoxins. They mainly cause DNA damage by either directly or indirectly inducing cell apoptosis not only to cancer cells but also to normal tissues (Becker et al., 2014; Roos & Kaina, 2013). Several of the current tumor treatments are based on DNA damage due to the effectiveness of those drugs to treat cancer, including; Bleomycin, Teniposide, Etoposide, Doxorubicin (Bonner et al., 2008).

The main challenge of using these treatments is that they may cause severe side effects to the hematopoietic system. Thus, the number of leukocytes decrease causing immune suppression (Becker et al., 2014; Bodey, 1981; Mackall et al., 1994). Consequently, doses are often reduced or the treatment may be stopped completely in order to avoid severe complications. In addition, these exciting treatments are often insufficient because of the development of multi-drug resistance in cancer cells. Due to these limitations of cancer therapy, there is an urgent need to develop new antitumor drugs as well as to reduce the toxic effects of these treatments on normal cells, while maintaining their activity on cancer tissues (Konkimalla & Efferth, 2008).

A revitalization of interest in drug discovery research on natural products from plants has recently emerged and the biological activity of these products has attracted the attention of chemists, biochemists, biologists, and microbiologists. According to the World Health Organization (WHO), 80% of the world population use traditional medicine to treat different disease including cancer (WHO cancer fact sheet, 2017). About 55% of the new chemotherapy drugs are derived from plants used in traditional medicine (Newman and Cragg, 2012; Nie et al., 2016).

Medicinal plants from traditional Chinese medicine (TCM) have been used for thousands of years in China to treat and prevent diseases. Herbal medicine used in TCM plays an integral role in the primary health care in East Asia, as it has been used for many generations by the Chinese community (Efferth, Li, Konkimalla, & Kaina, 2007). Chinese medicine and Western medicine differ in how they respond to diseases. Chinese medicine analyses and treats the entire body, while Western medicine considers illnesses at their

molecular, cellular and pharmacological levels (Z. F. Chen & H. Liang, 2010; Newman & Cragg, 2007).

In the 20th century, the importance of medicinal herbs has decreased due to the progress of chemically synthesized drugs. Recently, interest in TCM has risen in the western scientific community as numerous drugs have been derived from these medicinal plants for the treatment of several diseases (Attele, Wu, & Yuan, 1999; Deng et al., 2013; Sakamoto et al., 1991).

The discovery of single bioactive compounds from Chinese medicinal plants has produced promising results for novel antitumor compounds. The research shows that compounds from TCM plants can kill cancer cells through several mechanisms (Figure 1) such as enhancing the immune system, inducing cellular apoptosis, reversing multidrug resistance (MDR) and the inhibiting of angiogenesis (Chiu, Yau, & Epstein, 2009; Parekh et al., 2009; Ikezoe et al., 2003). Extracts and compounds from traditional Chinese medicinal plants are considered to be multi-targeting and multi-signaling in cancer treatments with low toxicity compared to the routinely used drugs. An ethane extract of *Hedyotis Diffusa Willd*. (EEHDW) induced apoptosis in a cancer cell by inhibiting cell proliferation due to DNA damage (Lin et al., 2010). Oxymatrine isolated from *Sephora flavescent Ait* demonstrated the ability to induce cell apoptosis and reduce cancer cell metastasis (Ling et al., 2011). An ethane extract from *Scutellaria baicalensis*affected cancer cell lines *in vitro* by different antitumor pathways including the inhibition of cyclooxygenase 2 (COX-2), inhibiting the production of prostaglandin E2 (PGE2)

as well as inhibiting angiogenesis (Wei et al., 2012; Parekh et al.,2009; Efferth et al., 2002). Several TCM extracts and compounds have been reported to reverse multidrug resistance (MDR) (nie et al., 2016) including; *Paris saponin* VII extracted from *Trillium tschonoskii* Maxim (Li et al 2014), germacrone (the main component of *Rhizma curcuma*) (Xie et al., 2014), and quinolones, indoloquinazoline alkaloids derived from *Evodia ruaecarpa* (Adams et al., 2007).

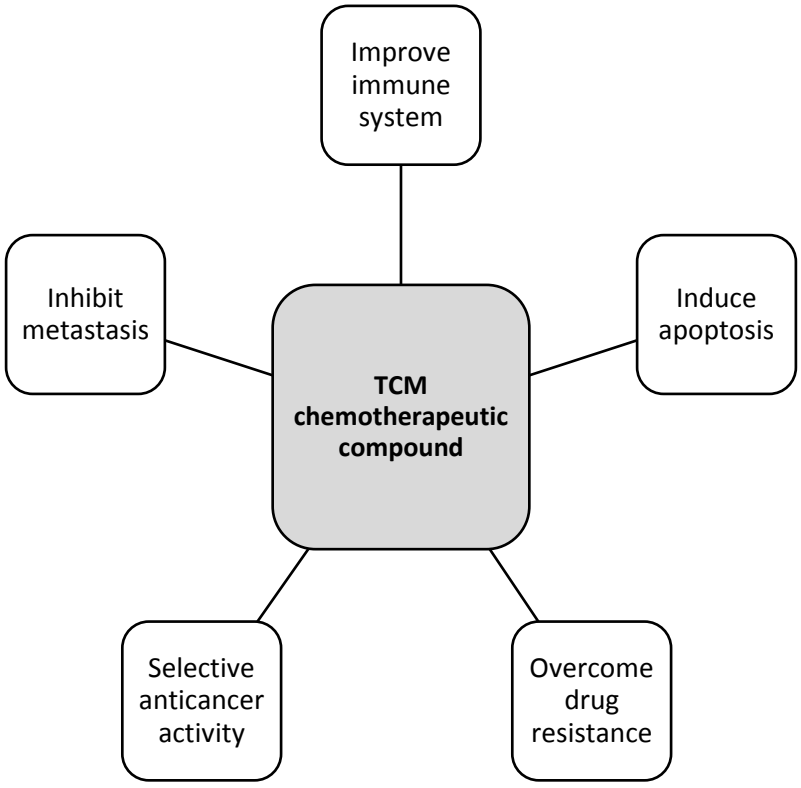

Figure 1. Common anticancer modes of action of TCM compounds

Recently, studies have indicated that herbal extracts derived from TCM may reduce *in vivo* toxicity of tumor chemotherapy. Several clinical studies showed that Chinese plant extracts have the ability to significantly reduce damage to the hematopoietic system during chemotherapy (Lam et al., 2010). In 2014, a study was done on the TCM compound Rocaglmide (Roc-A), derived from the Chinese medicinal plant *Aglaia*. This study showed that Ros-A is a potential chemoprotective agent in many nonmalignant human primary cell lines; however, it maintains the efficacy of chemotherapy against tumor tissue *in vitro* and *in vivo* in a mouse model. Roc-A reduced DNA damage caused by classical cancer chemotherapy via the essential role of the transcription factor p53-upregulating mechanism in normal tissue but not in cancer tissue due to the p53 deficiency in tumor cells (Becker et al., 2014).

Some compounds from TCM target DNA similar to classical anticancer drugs, Cisplantin and other alkylating agents. For example, the compound camptothecin from *Camptotheca acuminate* (happy tree) inhibits DNA ligation (Z. F. Chen & H. Liang, 2010; Pommier, 2006). Several studies suggest that some of the TCM products can enhance the activity of chemotherapy. For instance, compounds isolated from *Asiaticoside* could increase the efficacy of vincristine in cancer treatment by increasing the rate of apoptosis rate (Huang,2004; Z. F. Chen & H. Liang, 2010; Newman & Cragg, 2007). Many TCM compounds showed selective anticancer activity for specific types of cancer. For instance, a study has demonstrated artesunate (ART) isolated from *Artemisia annua*, strongly inhibits colon cancer and leukemia cell lines but does not inhibit lung cancer cell lines (Efferth et al., 2002).

Many compounds isolated from Chinese herbal plants have a selective antitumor activity for only cancer cells, without affecting normal cells (Zhang et al., 2003). It was reported that compounds from *Mylabris phalerlata* have strong cytotoxic activity for human leukemia cells but not normal lymphocytes (Huh et al., 2003). Additionally, the compound triploid induces apoptosis in lung cancer rather than in normal bronchial epithelial cells (Frese et al., 2003; Huh et al., 2003). However, the anticancer mechanisms of many TCM compounds are complicated; the specific mechanisms that kill tumor cells are not fully understood (Ruan, Lai, & Zhou, 2006; Sakamoto et al., 1991).

Although, several studies demonstrate that compounds derived from TCM have important roles in cancer treatment, it is necessary to investigate their safety. Though they are derived from natural sources, they could have toxic effects on normal cells, since it is hard to achieve biological activities without some toxicity (Ruan et al., 2006). The improper use of Chinese herbal medicine can lead to harmful effects to the human body. In the 1990s, several countries in Europe reported many poisoning cases by aristolochic acids from herbal mixtures for weight loss, including the Chinese herb *Airstolochia fangchi* (Nortier et al., 2000).

Although products from natural medicinal herbs in China are consider safe for treatment without serious consequences various studies demonstrate that TCM can lead to direct or indirect toxicity. Products from natural medicinal herbs in China are thought to be safe medicine without serious consequences. However, side effects from TCM treatment can include allergy response and serious microbial disease reactivation including acute viral hepatitis and tuberculosis (Chiu, Yau, & Epstein, 2009). The most common complication

reported in 2002 was liver toxicity that ranged from asymptomatic transaminitis to liver failure from several Chinese medicinal herbs such as *Teucrium chamaedrys*, *Atractylis gummifera*, *Curcuma longa*, Kava, *Callilepsis laureola*, and *Dictamnus dasycarpus* (Chiu et al., 2009; Pak, Esrason, & Wu, 2004). Moreover, a study on the TCM anticancer product PP2A revealed that severe renal lesions can occur through the use of this compound (Chiu et al., 2009). Another complication was reported in two studies on anticancer agents extracted from *Selaginaella doederleinii* (she shang bai), which showed that this product inhibits the DNA polymerase in the human cells causing severe bone marrow suppression (Pan, Lin, & Chen, 2001).

The screening of Chinese herbal medicine products is essential in order to modernize TCMs. Due to their complexity and variability, there is a challenge to separate, identify and analyze their components. The pharmacological evaluation process of these compounds is based on *in vitro* and *in vivo* assays (Littleton, Rogers, & Falcone, 2005).

Classical pharmacological screening includes animal studies to test the efficacy and toxicity of the desired compound (Littleton et al., 2005). However, this method is not flexible for screening TCM compounds as it is known to be slow and expensive with low throughput. To overcome these limitations, high throughput pharmacological screening (HTPS) has been developed a better alternative since it rapidly measures specific biological activities of thousands of compounds. A limitation of HTPS techniques is that they are designed to screen pure compounds.

In the 1990s, the 60 cell panel cancer test was developed by National Cancer Institute (NCI) for the *in vitro* screening of compounds, such as those derived from Chinese plant products, against 60 different human cancer cell lines. The test utilized seven cancer types: colon, brain, lung, leukemia, renal and ovarian cancer cells, including drug-resistant tumor cells. In 1992, prostate and breast tumor cell lines were added to the panel. Up till now, more than 85.000 compounds have been screened using the 60-cell panel lines (Littleton et al., 2005).

The standard cytotoxicity screening tool for TCM extracts and compounds has been Alamar Blue cell viability test. The Alamar Blue test is the ideal *in vitro* test in terms of being rapid, simple, reliable, safe, affordable, efficient and sensitive to measure cell proliferation and cytotoxicity. However, some TCM products act too quickly to be measured by Alamar Blue. Luminescent cell viability assessments can be used to test each products. Its principle is based on the quantification of ATP, which is an indicator of active cells in the culture (O'Brien, Wilson, Orton, & Pognan, 2000).

In the past decade, high content screening (HCS) has been developed to meet the requirements of research in both early drug discovery and biomedical research. HCS involves automated cell biology methods that combine high throughput screening with the ability to collect and view data at both cellular and sub-cellular levels. In recent years, HCS has become the main technique involved in several stages in the drug discovery process, particularly in the post-primary screening stage. HCS incorporates several assays based on the use of different fluorescent probes and marker proteins (Giuliano et al., 1997; Thomas, 2010). Recent studies in drug discovery from TCM have used HCS techniques

to measure different cellular responses that contribute to observed toxicity, including intracellular glutathione, oxidative stress, and mitochondrial damage. They demonstrated that HCS is also highly specific due to the capability to measure nuclear area, nuclei counts, lipid intensity, mitochondrial tetramethyl rhodamine methyl ester (TMRM), glutathione counts and reactive oxygen species (ROS) intensity from more than 300 compounds, which showed low false positives between 0% and 5% with a high true positive rate between 50% and 60% (Xu et al., 2008; Zanella, Lorens, & Link, 2010). Overall, HCS can be an important and efficient automated biological tool that has significantly contributed to the development of drug discovery from Chinese medicinal plants. For instance, a study in 2013 on the potential anticancer TCM compound Panduration A (PA), isolated from *Boesenbergia rotunda*, used HCS to examine the mechanism of inhibitory activities of this compound by measuring oxidative stress induction and the induction of poly ADP-ribose polymerase (PARP) (S. C. Cheah, Lai, Lee, Hadi, & Mustafa, 2013).

The Tennessee Centre for Botanical Medicine Research (TCBMR) was established as result of a partnership between Middle Tennessee State University (MTSU) and the Guangxi Botanical Garden of Medicinal Plants (GBGMP) in Nanning, China. GBGMP has the largest depository in China of more than 7,000 medicinal plants used in TCM and they have developed a novel technique to purify and isolate active pure compounds from these medicinal plants, while the TCBMR specializes in rapid bioactivity screens. In this collaborative study, the *in vitro* anticancer efficacy and safety of novel oligostilbene compounds isolated from *Paeonia suffruticosa* was demonstrated and 16 medicinal plants

that have been historically used in Yao ethnomedicine were evaluated for their potential as sources of new anticancer agents. This study provides evidence that plants used in TCM remain a viable source for new anticancer agents.

In studies described here, the *in vitro* anticancer efficacy and safety of 16 medicinal plants that have been historically used in Yao ethnomedicine was demonstrated, and novel oligostilbene compounds isolated from *Paeonia suffruticosa* were evaluated for their potential as sources of new anticancer agents. This study provides evidence that plants used in TCM are a viable source for new anticancer agents.

FIRST ARTICLE

EVALUATION OF EXTRACTS PREPARED FROM 16 PLANTS USED IN YAO ETHNOMEDICINE AS POTENTIAL ANTICANCER AGENTS

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Abstract

Ethnopharmacological relevance: Medicines of the Yao ethnic group in China are a special branch of traditional Chinese medicine (TCM) and are well documented for use in disease prevention. According to an ethnopharmacological survey, there are 1392 species of medicinal plants that have been documented as Yao ethnomedicines and 104 of these species are used routinely. This study evaluated a partial collection of these 104 core plant species for their potential as anticancer agents.

Material and methods: A literature study of scientific journals and books in the local language was conducted. Based on an ethnopharmacological survey, 16 plant species widely used in Yao ethnomedicine were collected and 64 plant extracts were prepared from these plants. *in vitro* cytotoxicity screening was conducted with a panel of four human cancer cell lines, lung cancer A549, breast cancer BT20 and MCF-7, bone cancer U2OS. The potential toxicity of the extracts was evaluated using two normal human cell lines, human peripheral lung epithelial cells (HPL1A) and human umbilical vein endothelial cells (HUVEC). Additionally, the 10 extracts that demonstrated cytotoxicity in cancer cells with an IC50 of less than 25.0 μg/mL were examined for the ability to induce apoptosis in U2OS cells.

Results: The up-to-date information regarding the traditional uses, pharmacological and biological activities, as well as the chemical constituents of the 16 plants are presented. Extracts from all 16 plants showed cytotoxicity against one to four of the human cancer cell lines and the cytotoxic effects of extracts from *Melaleuca leucadendra, Stephania longa*, *Microsorium fortune* and *Bidens biternata* were demonstrated for the first time.

The highest anticancer potential was observed for extracts prepared from *Melaleuca leucadendra Linn* against all tested cancer cells (BT20, A549, U2OS, and MCF7) with an IC50 range of 3.1–32.7 μg/mL. The selectivity index of the active samples varied from 0.1 to 25, and five extracts from *Bidens biternata, Wedelia calendulacea, Stephania longa* and *Achras zapota* showed significant selectivity against cancer cell lines versus normal cell lines.

All tested extracts induced apoptosis in U2OS cells, and for the first time extracts from *Melaleuca leucadendr*a and *Microsorium fortune* were shown to induce apoptosis.

Conclusion: We demonstrated the in vitro anticancer efficacy and safety of 16 medicinal plants that have been historically used in Yao ethnomedicine. This study provides evidence to assist the clinical practice of Yao ethnomedicine and the development of chemotherapeutic agents from extracts prepared from these plants.

Keywords: Anti-proliferation, Apoptosis, Chemotherapeutic, Cytotoxicity, Ethnomedicine, Selectivity

1. Introduction

Cancer is a life threating disease that causes 63% of deaths worldwide and is known to be the second leading cause of death in western countries according to the World Health Organization (WHO) (Alwan et al., 2010; Kashfi, 2013; Monteiro et al., 2014). Chemotherapy has been used for decades to cure cancer. However, chemotherapeutic drugs are known to exhibit severe toxicity against normal tissues and are affected by the development of drug resistance. Due to these limitations of cancer therapy, there is an urgent need to develop new chemotherapeutic drugs (Efferth and Konkimalla, 2008; Tan et al., 2011). Medicinal plants have been used for many generations and play an integral role in primary health care in East Asia (Efferth et al., 2007). Attention to medicinal plants had begun to decrease due to the progress of chemical synthetic drugs, however, in the past two decades, interests in the plants used in traditional Chinese medicine (TCM) has risen in the western scientific community as new medicines have been derived from medicinal plants for the treatment of several major diseases (Chen and Liang, 2010; Efferth et al., 2007).

Numerous studies on plant extracts or individual bioactive compounds derived from medicinal plants have demonstrated promise for treating cancer. These extracts or compounds can kill cancer cells through several mechanisms, such as enhancing the immune system, inducing cell apoptosis, reversing multidrug resistance (MDR) and inhibiting angiogenesis (Balunas and Kinghorn, 2005; Ruan and Zhou, 2006). However, the anticancer mechanisms of TCM preparations are complicated, and specific mechanisms have not yet been fully determined (Ruan and Zhou, 2006). Although many

studies have demonstrated that compounds derived from TCMs have great promise in cancer treatment, there is an urgent need to investigate their safety. Despite the fact that they are derived from natural sources, TCMs can have toxic effects since it is difficult to achieve biological activities without side effects (Chiu et al., 2009; Ruan and Zhou, 2006). For instance, studies on anticancer agents extracted from *Selaginaella doederleinii* reported that the agents inhibited the DNA polymerase in the human body and caused severe bone marrow suppression (Katsuhiko et al., 1989; Pan et al., 2001). Extracts from *Trypterigium wilfordii* Hook.f. (Thunder God Vine) demonstrated a promising anticancer activity, however, the extracts caused serious side effects when used at high concentration, including headaches, infertility, diarrhea and nausea (Shamon et al., 1997). Therefore, it is essential to evaluate the selectivity of extracts or the major constituents of herbal medicines in cancer cells versus normal cells to better understand their safety when investigating potential anticancer agents.

The Yao ethnic minority is an ancient ethnic group that has a total population of 2.6 million and is mainly distributed in the mountains of the Guangxi, Guangdong and Guizhou provinces in China and Yao ethnomedicine is a special branch of TCM. According to an ethnopharmacological survey, there are 1392 species of medicinal plants that have been used as Yao ethnomedicine and 104 of these species are used routinely. These 104 species, which belong to 84 genera of 50 families, constitute the core of the Yao minority healthcare system (Dai, 2009; Dai and Cui-chang, 1998; Li et al., 2006; Long and Li, 2004). Long and Li (2004) and Li et al. (2006) reported the ethnobotanical

studies on the medicinal plants of Yao medicine, including the herbs that were used for medicinal baths, and recorded 66 species that belong to 61 genera of 43 families.

In this study, we collected a total of 16 plant species, *Garcinia paucinervis, Elephantopus scaber, Evodia lepta, Achras zapota, Stephania longa, Crocosmia crocosmiflora, Pandanus tectorius, Euphorbia hirta, Bidens biternata, Cyperus rotundus, Microsorium fortuni, Bidens pilosa, Wedelia calendulacea, Uncaria macrophylla, Sarcandra glabra* and *Melaleuca leucadendra* from the 104 core species routinely used in Yao medicine from the mountains of Guangxi, China. This effort took two years as some of the plants are very rare and often grow on the mountain cliffs, which makes them extremely difficult to collect. These plants have been used mostly for detoxification, expelling wind and dampness, or promoting and regulating blood circulation (Jia and Li, 2005b). The traditional uses of these plants have been passed down orally from generation to generation by the Yao ethnic minority people or have been recorded in local herbal books. We conducted a literature study of scientific journals as well as books written in the local language and present here the up-to-date information regarding the traditional uses, pharmacological and biological activities as well as the chemical constituents that have been isolated from these 16 plants. For each plant, four fractions (petroleum ether, ethyl acetate, ethanol and water) were prepared and tested against several cancer and normal cells lines to characterize their *in vitro* cytotoxicity and selectivity. This study enhances our understanding of Yao ethnomedicine and provides evidence to ensure its safety and efficacy in clinical practice as well as identifying potential new sources for the development of anticancer agents.

2. Materials and methods

2.1. Plant materials

The plant species were collected throughout the Guangxi Autonomous Region, China. Plants were characterized by Dr. Yimin Zhao and Dr. Xueyan Huang at the Guangxi Botanical Garden of Medicinal Plants (GBGMP). Which part(s) of the plant to collect (e.g. leaves, barks, stems, roots or whole plant) was based on their use in TCM. A summary of the collection date, venue and plant part(s) is shown in Table 1. Voucher specimens were deposited and identified at the GBGM

Table 1. Summary of the collection date, venue and plant part(s) of 16 medicinal plants used in Yao ethnomedicine.

	Name of the plant	Genus	Family	Plant part(s)	Collection date	Collection venue	Vochure #
1	*Garcinia paucinervis* Chun et How	*Garcinia*	*Clusiaceae*	Stem, branch, leaf	Jun 2009	GBGMP	200906001
2	*Elephantopus scaber* Linn.	*Elephantopus*	*Asteraceae*	Branch, leaf	Jun 2010	Jinxiu County, Guangxi	201006002
3	*Evodia lepta* (Spreng.) Merr.	*Evodia*	*Rutaceae*	Branch, leaf	Jun 2010	Jinxiu County, Guangxi	201006004
4	*Achras zapota* Linn.	*Achras*	*Sapotaceae*	Branch, leaf	May 2010	Lipu County, Guangxi	201005007
5	*Stephania longa* Lour.	*Stephania*	*Menispermaceae*	Whole plant	Jul 2012	Nanning city, Guangxi	201207009
6	*Crocosmia crocosmiflora* (Nichols.) N. E. Br.	*Crocosmia*	*Iridaceae*	Whole plant	Jul 2012	Nanning city, Guangxi	201207011
7	*Pandanus tectorius* Sol.	*Pandanus*	*Pandanaceae*	Aerial parts	Oct 2010	Wuzhou city, Guangxi	201010012
8	*Euphorbia hirta* Linn.	*Euphorbia*	*Euphorbiaceae*	Whole plant	Jun 2011	Yulin city, Guangxi	201106014
9	*Bidens biternata* (Lour.) Merr.	*Bidens*	*Asteraceae*	Whole plant	Jun 2010	Jinxiu County, Guangxi	201006016
10	*Cyperus rotundus* L.	*Cyperus*	*Cyperaceae*	Whole plant	Aug 2009	Wuzhou city, Guangxi	200908017
11	*Mosla caualeriei* Levl.	*Mosla*	*Labiatae*	Aerial parts	Aug 2011	Jinxiu County, Guangxi	201108021
12	*Bidens pilosa* L.	*Bidens*	*Asteraceae*	Whole plant	Jul 2011	Nanning city, Guangxi	201107022
13	*Wedelia calendulacea* Less.	*Wedelia*	*Asteraceae*	Whole plant	Jul 2011	Nanning city, Guangxi	201107027
14	*Uncaria macrophylla* Wall.	*Uncaria*	*Rubiaceae*	Branchs, leaves	Jun 2012	Guilin city, Guangxi	201206028
15	*Sarcandra glabra* (Thunb.) Nakai	*Sarcandra*	*Chloranthaceae*	Whole plant	Sep 2011	Lipu County, Guangxi	201109030
16	*Melaleuca leucadendra* Linn.	*Melaleuca*	*Myrtaceae*	Stem	May 2012	Nanning city, Guangxi	201205034

2.2. Preparation of plant extracts

Plant samples were dried and ground into powder using an electric blender. Each dried plant sample was extracted with petroleum ether and reflux. The solubilized material was dried down to generate the petroleum ether fraction (A). The remaining undissolved material was then extracted with ethyl acetate and reflux. The solubilized material was dried down to generate the ethyl acetate fraction (B). The remaining undissolved material was then extracted with ethanol and reflux. The solubilized material was dried down to generate the ethanol fraction (C). The remaining undissolved material was then extracted with water and reflux. The solubilized material was dried down to generate the water fraction (D). Each extract was dried to completeness to ensure there was no residual solvent and then resuspended at a final concentration of 10 mg/mL in dimethyl sulfoxide (DMSO). The extracts were used in cell culture experiments at a final concentration of 100 µg/mL or lower. Numerous studies have shown that DMSO is well tolerated by both cancer and normal cells up to a final concentration of 1% (Gao et al., 2015; Almosnid et al., 2016).

2.3. Cell culture

Four human cancer cell lines, MCF-7 (estrogen receptor-positive human breast adenocarcinoma), BT20 (estrogen receptor-negative human breast adenocarcinoma), U2OS (human osteosarcoma) and A549 (human lung carcinoma) from the American Type Culture Collection (ATCC, Manassas, VA, USA) were used in this study. MCF7 cells were cultured in DMEM medium (ATCC), BT20 and A549 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) and U2OS cells were cultured in

McCoy's 5A medium (ATCC). Two normal cell lines were also tested, HUVEC cells (human umbilical vein endothelial cells) from the ATCC and HPL1A cells (human peripheral lung epithelial cells) obtained from Nagoya University. HUVEC cells were cultured in vascular cell basal medium Kit-BBE (bovine brain extract 0.2%,rh EGF 5 ng/mL, L-glutamine 10 mM, heparin sulfate 0.75 Units/mL, hydrocortisone hemisuccinate 1 µg/mL, fetal bovine serum 2% and ascorbic acid 50 µg/mL) (ATCC) and HPL1A cells were cultured in DMEM/F12 medium (Sigma-Aldrich). All medium contained 1% streptomycin and penicillin (Sigma-Aldrich) and 10% fetal bovine serum (Sigma-Aldrich) and all cells were incubated in a humid atmosphere with 5% CO_2 at 37 °C.

2.4. Anti-proliferation assay

Cells were plated at 4000 cells per well in a 96-well microplate and were incubated overnight to allow attachment to occur. Subsequently, the cells were incubated with medium containing plant extracts at 100 µg/mL. After treatment at 37 °C for 48 h, the cells were stained with resazurin reduction reagent AlarmaBlue (Invitrogen, Frederick, MD, USA) to determine the cytotoxicity of the extracts. The fluorescent intensity was measured using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA) at Ex 555 nm and Em 590 nm. Percent inhibition (%) was calculated using the following formula, and IC_{50} values were calculated using non-linear regression analysis.

$$\frac{\text{Fluorescent intensity of control cells} - \text{fluorescent intensity of tested cells}}{\text{Fluorescent intensity of tested cells} - \text{blank}} \times 100$$

2.5. Apoptosis assay

U2OS cells were seeded at 5000 cells per well in a 96-well plate and incubated at 37 °C overnight. Then cells were treated with extracts at different concentrations (12.5–50 µg/mL) for 18 h. After treatment the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) was used to evaluate apoptosis induction by the extracts in U2OS cells. Briefly, a dye solution containing 1 µL of Hoechst nuclear dye (Sigma-Aldrich), 5 µL of propidium iodide (PI) (BD Biosciences) and 5 µL of FITC Annexin V (BD Biosciences) were added to each well and incubated at 37 °C for 10 min. Immediately following the fluorescent staining, cell images were taken using an Arrayscan VTI high-content screening reader (Thermo Scientific, Waltham, MA, USA). The fluorescent intensity was analyzed using the vHCS Scan software (Thermo Scientific).

2.6. Statistics analysis

The mean values and standard deviation of all the data was determined. A two-tailed *t*-test was used to calculate the statistical significance using Prism GraphPad software (GraphPad Software Inc., La Jolla, CA, USA) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

3. Results

3.1. The traditional use and pharmacological background of 16 plants used in Yao ethnomedicine

We investigated the background information of 16 plants used in Yao ethnomedicine, including their traditional uses in TCM, reported pharmacological and biological activities, and known compounds that have been isolated from each plant. This information is presented in Table 2. *Garcinia paucinervis* is traditionally used in China to treat epigastralgia (Jia and Li, 2005a). Previous studies indicated that the extract and compounds isolated from this plant have cytotoxic effects and induce apoptosis in cancer cells (Gao et al., 2010a; Gao et al., 2010b; Li et al., 2016a, 2016b). *Elephantopus scaber* is traditionally used to treat a broad range of diseases including jaundice, tonsillitis, eczema, cough, enteritis and dysentery (Hiradeve and Rangari, 2014a, 2014b; Cao et al., 1997; Jia and Li, 2005g). Extracts from this plant also showed various bioactivities, such as anticancer, antimicrobial, hepatoprotective, antidiabetic, antioxidant, anti-inflammatory, analgesic, antiplatelet, antiasthmatic and nephroprotective activities (Hiradeve and Rangari, 2014a; Pitchai et al., 2014). *Evodia lepta* is used to treat arthritis, fever, chickenpox, meningitis, infectious hepatitis, epidemic influenza, colds and teaumatic injuries as well as being used as a febrifuge, antipruritic or depurative agent (Gunawardana et al., 1987; Jia and Li, 2005e; Juan and Lee, 1981; Yoon et al., 2013). The extracts from *Evodia lepta* have demonstrated hepatoprotective, anti-inflammatory and antioxidant activities (Bi et al., 2007; Deng et al., 2011; Sichaem et al., 2014; Yoon et al., 2013). In TCM practice, the bark of *Achras zapota* is used as an astringent, antibiotic and

antidiarrheal agent (Nair and Chanda, 2008), the fruit is used to treat pulmonary diseases and diarrhea and the leaves are used to treat diarrhea, colds and coughs (Guangxi Resources Survey Committee, 1993a; Mondal et al., 2012) as well as acute gastroenteritis (Guangxi Resources Survey Committee, 1993b). Extracts from *Achras zapota* have exhibited antibacterial, anti-inflammatory, antipyretic, antidiabetic and antilipidemic activities (Barbalho et al., 2015; Nair, 2008). *Stephania longa* is used in TCM to treat fever, inflammation, dysentery, induce diuresis, reduce edema, improve collateral circulation (State Administration of Traditional Chinese Medicine; Bencao and Ben, 1998; Zhang and Yue, 2005) and as a treatment for bleeding of the bowel (Jia and Li, 2005b). Surprisingly, there have been no reports on *Stephania longa* and no bioactivities have been determined. *Crocosmia crocosmiflora* is traditionally used to treat sores and bruises (Guangxi Resources Survey Committee, 1993d). The extract of this plant possesses antimicrobial and antitumor activities (Masuda et al., 1987; Nagamoto et al., 1988) and several pure compounds have been isolated (Asada et al., 1988). *Pandanus tectorius* is used to treat hepatitis and nephritis (Guangxi Resources Survey Committee, 1993a) and extracts from *Pandanus tectorius* have been shown to possess antidiabetic and antihyperlipidemic activities (Cheng-hui, 2010; Mai et al., 2015; Wu et al., 2014; Zhang et al., 2013). *Euphorbia hirta* is used to treat dysentery, enteritis, dermatitis, eczema, hematuria and diarrhea (State Administration of Traditional Chinese Medicine, 1999; Jia and Li, 2005f). Extracts from *Euphorbia hirta* have shown a broad range of biological activities, including antimicrobial, antidiarrheal, antiallergic, anti-inflammatory, antioxidant, antitumor, antidiabetic, anxiolytic, diuretic and sedative activities (Huang et al., 2012; Jia and Li, 2005f; Kumar et al., 2010; Li et al., 2015). *Bidens biternata* is used

to treat hepatitis, coughs, colds, dysentery, throat swelling and pain and acute appendicitis (Jia and Li, 2005a). Extracts from *Bidens biternata* have demonstrated α-glucosidase inhibitory and antioxidant activities (Jia and Li, 2005a; Ma et al., 2012; Sukumaran et al., 2012). *Cyperus rotundus* is used as an anthelmintic, diuretic and digestive stimulant (Badgujar and Bandivdekar, 2015; Sivapalan, 2013) and has also been used to treat bruises and swelling (Jia and Li, 2005d). Extracts from *Cyperus rotundus* have demonstrated antimicrobial, anti-inflammatory, antidiabetic, antimalarial and antioxidant activities (Badgujar and Bandivdekar, 2015; Singh et al., 2012). *Microsorium fortuni* is used to treat coughs and asthma, and its extracts have shown antioxidative activity (Guangxi Resources Survey Committee, 1993e; Li et al., 2011; Zhou et al., 2009). *Bidens pilosa* has been used to manage snake bites and treat colds in Chinese culture (Bartolome et al., 2013; Jia and Li, 2005c). Its extracts have showed antidiabetic, antihyperglycemic, antioxidant, immunomodulatory, anti-malarial, antimicrobial, antihypertensive, vasodilatory and antiulcerative activities (Horiuchi and Seyama, 2008; Kviecinski et al., 2008). *Wedelia calendulacea* is used to treat several diseases, including diphtheria, diarrhea and jaundice and is also used to treat colds and their symptoms, such as fever and sore throats (Guangxi Resources Survey Committee, 1993c; Mottakin et al., 2004; Nomani et al., 2013). The extracts of *Wedelia calendulacea* have showed broad activities, such as antimicrobial, antitumor, anti-inflammatory, antistress, antiulcerogenic, anticonvulsant, hepatoprotective and androgen suppressing activities (Nomani et al., 2013; Sharma et al., 1989). *Uncaria macrophylla* is used in TCM as a sedative, analgesic, anticonvulsive and hypotensive agent, and has also been used to treat hypertension, epilepsy, preeclampsia, rheumatism and improve psychiatric symptoms (Guangxi Resources Survey Committee, 1993e). The

extracts of *Uncaria macrophylla* have demomstrated cytotoxic, vasodilating, hypnotic, antispasmodic and neuroprotective activities (Sakakibara et al., 1998; Sun et al., 2012; Wang et al., 2010). The whole plant of *Sarcandra glabra* is used for liver protection and the treatment of several diseases, such as pneumonia, dysentery sciatica, inflammation, bone fracture and even cancer (Dai, 2009). The extracts of *Sarcandra glabra* have showed antimicrobial, antioxidant, antitumor, anti-inflammatory and hepatoprotective activities (Jin et al., 2012; Ni et al., 2013). *Melaleuca leucadendr* is traditionally used to treat nasal catarrh, skin lesions and insomnia (Guangxi Resources Survey Committee, 1993f; Chao-jun et al., 2012) and its extracts have showed antioxidant activity (Li et al., 2013; Pino et al., 2010).

Table 2. Traditional use and pharmacological background of 16 medicinal plants used in Yao ethnomedicine.

	Name of the plant	Known activity in TCM	Known activity of extracts	Known compounds
1	*Garcinia paucinervis* Chun et How	Treatment of epigastralgia (Jia and Li, 2005a).	Cytotoxic and apoptosis-inducing activity (Gao et al., 2010a, 2010b; Li et al., 2016a, 2016b).	Paucinervins A–D, guttiferone I, 30-*epi*-cambogin, cambogin, garcicowin C, formoxanthone A, parvifolixanthone A, 1,3,7-trihydroxy-2-prenylxanthone, guttiferone E, (+)-guttiferone K, jacareubin, nigrolineaxanthone E, cembrene A, parvifoliol F, 2-cyclohexene-c,g,2,6,6-pentamethyl-1-nonanol and vitamin E quinone (Gao et al., 2010a; Li et al., 2016a, 2016b).
2	*Elephantopus scaber* Linn.	Treatment of tonsillitis, conjunctivitis, jaundice, nephritis edema and eczema; used as anti-inflammatory, antipyretic, diuretic, anticough, antibiotic, emollient and tonic agent to treat enteritis and dysentery (Hiradeve and Rangari, 2014a, 2014b; Cao et al., 1997; Jia and Li, 2005g).	Antitumor, antibacterial, antifungal, hepatoprotective, antidiabetic, antioxidant, anti-inflammatory, analgesic, antiplatelet, antiasthamatic and nephroprotective activities (Hiradeve and Rangari, 2014a; Pitchai et al., 2014).	Triterpennoids: friedlin, ursolic acid, betulinieaei, lupeol; sesquiterpenoids: scabertopin, isodeoxyelephantopin, elephantopin, glucozanin C and elescaberin; flavonoids: triein, luteolin and luteolin-7-O-glieoside; sterols: stigmasterol and stitosterol (Hiradeve and Rangari, 2014a, 2014b, 2014a).
3	*Evodia lepta* (Spreng.) Merr.	Antipruritic, depurative and febrifuge agent, treatment of arthritis, fever, chickenpox, epidemic influenza, meningitis, infectious hepatitis, traumatic injuries and colds (Gunawardana et al., 1987; Jia and Li, 2005e; Juan and Lee, 1981; Yoon et al., 2013).	Hepatoprotective, anti-inflammatory and antioxidant activities (Bi et al., 2007; Deng et al., 2011; Sichaem et al., 2014; Yoon et al., 2013).	4,7-dimethoxy-furan quinoline, isoevodionol, dichromene C, dichromene D, leptanoines A–C, melineurine, skimmianine, 7-hydroxydictamnine and leptin A-H (Sichaem et al., 2014; Yoon et al., 2013).

Table 2. (cont.)

4	*Achras zapota* Linn.	Bark: treatment of diarrhea; used as an astringent and antibiotic (Nair and Chanda, 2008). Fruit: treatment of diarrhea and pulmonary diseases. Leaves: treatment of coughs, colds and diarrhea (Guangxi Resources Survey Committee, 1993a; Mondal et al., 2012), treatment of acute gastroenteritis (Guangxi Resources Survey Committee, 1993b).	Antibacterial, anti-inflammatory, anti-pyretic, antidiabetic and antilipidemic activities (Barbalho et al., 2015; Nair and Chanda, 2008).	Lupenyl acetate, oleanolic acid, vitamin, polysaccharide and tannin (Mondal et al., 2012; Nair and Chanda, 2008).
5	*Stephania longa* Lour.	Inducing diuresis, reducing edema, improving collateral circulation; treatment of fever, inflammation and dysentery (State Administration of Traditional Chinese Medicine; Bencao and Ben, 1998; Zhang and Yue, 2005); treatment of bowel bleeding (Jia and Li, 2005b).	Not found.	Alkaloids: stephalonines A-I, stephalonines J and K, stephalonganines A–C, eletefine, norprostephabyssine, isoprostephabyssine, isolonganone, isostephaboline, protostephanine, dehydrostephanine, (-)-stephanine, (-)-isolaureline, R-roemeroline, (+)-pronuciferine, (+)-stepharine, (+)-N-acetylstepharine, (+)-lirioferine and (+)-norlirioferine (Zhang and Yue, 2005).
6	*Crocosmia crocosmiflora* (Nichols.) N. E. Br.	Treatment of bruises and sores (Guangxi Resources Survey Committee, 1993d).	Antimicrobial and anti-tumor activities (Masuda et al., 1987; Nagamoto et al., 1988).	Montbretins A and B, crocosmiosides A-I and tricrozarin A (Asada et al., 1988).
7	*Pandanus tectorius*	Treatment of hepatitis and nephritis (Guangxi Resources Survey Committee, 1993a).	Antidiabetes and antihyperlipidemic activities (Cheng-hui, 2010; Mai et al., 2015; Wu et al., 2014; Zhang et al., 2013).	Geranyl acetate, ethyl cinnamate, 3-Methyl-3-buten-1-yl cinnamate. (Z)-4-hydroxy-3-(4-hydroxy-3-methylbut-2-en-1-yl) benzaldehyde, p-hydroxybenzaldehyde, syringaldehyde, (*E*)-ferulaldehyde, (*E*)-sinapinaldehyde, vanillin and 5-hydroxymethylfurfual (Mai et al., 2015; Wu et al., 2014; Zhang et al., 2013).

Table 2. (cont.)

8	*Euphorbia hirta* Linn.	Treatment of dysentery, enteritis, dermatitis, eczema, hematuria and diarrhea (Committee, 1993; Jia and Li, 2005f).	Antitumor, antibacterial, antifungal, antidiarrheal, antiallergic, anti-inflammatory, antioxidant, antitumor, antidiabetic, anxiolytic, diuretic and sedative activities (Zhe and Fan, 1998; Zhang and Luo, 2005; Huang et al., 2012; Jia and Li, 2005f; Kumar et al., 2010; Li et al., 2015).	Flavonoids: myricitrin, quercitrin, isoquercitrin, afzelin, euphorbianin, quercetin, kaempferol and myricetin; tannins: gallic acid, protocatechuic acid, 3,4-di-O-galloylquinic acid, 5-O-caffeoylquinic acid and euphorbin A-E; triterpenoids: β-amyrin, 24-methylenecycloartenol, cycloartenol, taraxerol and fridedelin (Huang et al., 2012; Kumar et al., 2010; Li et al., 2015).
9	*Bidens biternata* (Lour.) Merr. et Schreff	Treatment of hepatitis, cold, cough, dysentery, throat swelling and pain and acute appendicitis (Jia and Li, 2005a).	ntioxidant and α-glucosidase inhibitory activities (Jia and Li, 2005a; Ma et al., 2012; Sukumaran et al., 2012).	Maritimetin, quercetin, Z-6–0-(6″-propionyl-β-D-glucopyranosyl)-6,7,3′,4′-tetrahydroxyauron, (2″−0-acetyl-6″-P-coumaroyl-β-D-gulcopyranosyl)-P-coumaric acid, triacontanoic acid, stigmasterol, reducing sugar, glycosides, flavonoids, alkaloids, tannins, steroids, terpenoids, coumarins, saponins, anthraquinones, phlobatannins and iridoids (Ma et al., 2012; Sukumaran et al., 2012).
10	*Cyperus rotundus* L.	Used as diuretic, digestive stimulant, anthelmintic, antisaturative, plasma purifier and lactogenic agent (Badgujar and Bandivdekar, 2015; Sivapalan, 2013); treatment of bruises and swelling (Jia and Li, 2005d).	Antitumor, anti-inflammatory, hepatoprotective, cytoprotective, gastroprotective, hypolipidaemic, antidiabetic, antimalarial, antimicrobial and antioxidant activities. (Badgujar and Bandivdekar, 2015; Singh et al., 2012).	Alkaloids, flavonoids, tannins, glycosides, furochromones, monoterpenes, sesquiterpenes, sitosterol, fatty oil, essential oil, glycerol, linolenic, myristic and stearic acids (Sivapalan, 2013; Singh et al., 2012).
11	*Microsorium fortuni* (Moore) Ching	Treatment of coughs and asthma (Guangxi Resources Survey Committee, 1993e; Li et al., 2011; Zhou et al., 2009).	Antioxidant activity (Guangxi Resources Survey Committee, 1993e; Li et al., 2011; Zhou et al., 2009).	Essential oil, β-sitosterol, bergenin, sitosterol β-O-glucopyranoside, pinoresinol, quercetin and quercetin 3-O–β-D-galactoside (Zhou et al., 2009).
12	*Bidens pilosa* L.	Treatment of cold and snake bite (Bartolome et al., 2013; Jia and Li, 2005c).	Antitumor, anti-inflammatory, antidiabetic, antihyperglycemic, antioxidant, immunomodulatory, antimalarial, antibacterial, antifungal,	Aliphatic compounds: heneicosane, docosane, tricosane and myristic acid; flavonoids: sulfuretin, bidenoside A, butein, okanin, aurone (Z)-6-O-(4″,6″-diacetyl-??-D-

Table 2. (cont.)

			antihypertensive, vasodilatory and antiulcerative activities (Horiuchi and Seyama, 2008; Kviecinski et al., 2008).	glucropyranosyl)-6,7,3′,4′-etrahydroxy and apigenin; terpenoids: bicyclogermacrene, germacrene D, d-Muurolene, campestrol and lupeol acetate; phenylpropanoids: eugenol, caffeic acid, chlorogenic acid; aromatic compounds: pyrocatechin, 2-Phenyl-ethanol and gallic acid (Bartolome et al.).
13	*Wedelia calendulacea* Less.	Used as a hepatoprotective agent; treatment of jaundice, diarrhea, diphtheria, pertussis, colds, fevers and sore throats (Guangxi Resources Survey Committee, 1993c; Mottakin et al., 2004; Nomani et al., 2013).	Cytotoxic, antibacterial, antioxidant, anti-inflammatory, analgesic, sedative, antistress, antiulcerogenic, antitumor, antibacterial, antifungal, anticonvulsant, hepatoprotective and androgen suppressing activities (Nomani et al., 2013; Sharma et al., 1989).	carotene, tannin, saponin, phytosterol, isoflavonoids, wedlolactone, bisdesmoside, oleonelic acid saponin (Nomani et al., 2013).
14	*Uncaria macrophylla* Wal1.	Used as sedative, analgesic, anticonvulsive and hypotensive agent; treatment of hypertension, epilepsy, preeclampsia, rheumatism and psychiatric symptoms (Guangxi Resources Survey Committee, 1993e).	Cytotoxic, vasodilating, hypnotic, antispasmodic, antitumor and neuroprotective activities (Sakakibara et al., 1998; Yang et al., 2001; Sun et al., 2012; Wang et al., 2010).	Alkaloids: isorhynchophylline, rhynchophylline, corynoxine, corynoxine B, macrophyllionium, macrophyllines A-B, corynantheidine and dihydrocorynantheine; triterpenes: 3β,6β,19α-trihydroxy-urs-12-en-28-oic acid-24-carboxylic acid methyl ester, 3β,6β,19α-trihydroxy-23-oxo-urs-12-en-28-oic acid, 3β,6β,19α-trihydroxy-urs-12-en-28-oic acid and ursolic acid (Sakakibara et al., 1998; Sun et al., 2012; Wang et al., 2010).
15	*Sarcandra glabra (Thunb.)* Nakai	Treatment of inflammation, bone fracture, cancer; used for liver protection; treatment of pneumonia, dysentery and sciatica (Huang et al., 2006; Dai, 2009).	Antitumor, antioxidant, antimicrobial, anti-inflammatory and hepatoprotective activities (Leng et al., 2010; Jin et al., 2012; Ni et al., 2013).	Sarcandrolides F-J, chloranthalactone A, B, E, F, lupeol, Atractyenolides II, III, shizukanolide A,E,F,24-hydroxylupeol, astibin, 7-

Table 2. (cont.)

			methylnaringenin, 5-hydroxy-7,4'-dimet-hoxy-dihyflavones, isoliquiritin, isofraxidin, fraxin, scopoletin, scoparone and eleutheroside B1 (Jin et al., 2012; Ni et al., 2013).
16	*Melaleuca leucadendra* Linn.	Treatment of nasal catarrh and purulent skin lesions, gout and insomnia (Guangxi Resources Survey Committee, 1993f; Chao-jun et al., 2012).	Antioxidant activity (Li et al., 2013; Pino et al., 2010).	Genistein, betulinic acid, luteolin, rutin, citric acid, trans-cinnamaldehyde, trans-cinnamic acid, vanillin, vanillic acid, salicylic acid, benzoic acid, ursolic acid and essential oils (1,8-cineol, viridiflorol, α-terpineol, α-pinene, and limonene, viridiflorol, globulol, guaiol and α-pinene) (Guangxi Resources Survey Committee, 1993f; Chao-jun et al., 2012; Li et al., 2013; Pino et al., 2010).

* Committee is the abbreviation of Guangxi Resources Survey Committee.

3.2. Antiproliferative activities of 64 extracts prepared from 16 plants

We found that the extracts prepared from 16 plants used in Yao medicine demonstrated antiproliferation activities against multiple cancer cell lines at a concentration of 100 μg/mL (Table 3). Based on the results shown in Table 3, we identified the active fractions for each of the 16 plants that exhibited greater than 90% inhibition to cancer cells. These active fractions were as follows: the petroleum ether fractions of *Garcinia paucinervis*, *Achras zapota* and *Cyperus rotundus*, the ethyl acetate fractions of *Pandanus tectorius, Bidens pilosa, Uncaria macrophylla* and *Sarcandra glabra*, both the petroleum ether and ethyl acetate fractions of *Elephantopus scaber, Evodia lepta, Stephania longa, Euphorbia hirta* and *Wedelia calendulacea*, both the ethyl acetate and ethanol fractions of *Crocosmia crocosmiflora* and *Microsorium fortune* and both the ethyl acetate and water fractions of *Bidens biternata.* Most strikingly, all four fractions (petroleum ether, ethyl acetate, 95% ethanol and water) of *Melaleuca leucadendra* were active. Most of the active ingredients were extracted by petroleum ether (8 out of 16 samples) and ethyl acetate (9 out of 16 samples). In contrast, only two ethanol fractions and one water fraction were shown to be active.

Table 3. Cytotoxicity of 64 extracts from 16 medicinal plants at 100 µg/mL. The heat map indicates the levels of cytotoxicity. Red: high cytotoxicity; Yellow: medium cytotoxicity; Green: little or no cytotoxicity. The values listed are the means from three replicate experiments

	Plant	Fractions	Code	Cancer cell lines				Normal cell lines	
				A549	BT20	MCF7	U2OS	HPL1A	HUVEC
1	*Garcinia paucinervis* Chun et How	Petroleum ether	1A	99.1	99.3	59.0	96.0	89.7	99.0
		Ethyl acetate	1B	57.8	88.7	25.0	3.0	-1.2	82.0
		95% ethanol	1C	0.0	-0.1	-2.0	9.0	-3.1	41.0
		Water	1D	-5.0	0.4	-6.0	1.0	-1.8	9.0
2	*Elephantopus scaber* Linn.	Petroleum ether	2A	-6.0	-0.4	41.0	90.0	71.8	80.0
		Ethyl acetate	2B	83.0	99.8	91.0	92.0	56.3	83.0
		95% ethanol	2C	-5.0	3.4	13.0	16.0	-9.7	5.0
		Water	2D	0.0	5.7	29.0	4.0	13.7	58.0
3	*Evodia lepta* (Spreng.) Merr.	Petroleum ether	3A	21.9	99.9	44.0	95.0	49.3	99.0
		Ethyl acetate	3B	-4.4	22.2	44.0	95.0	65.4	91.0
		95% ethanol	3C	-4.2	1.5	31.0	23.0	18.6	26.0
		Water	3D	-2.1	5.7	27.0	20.0	7.6	20.0
4	*Achras zapota* Linn.	Petroleum ether	4A	-2.5	-4.1	29.0	94.0	44.8	-5.0
		Ethyl acetate	4B	2.7	81.5	10.0	38.0	8.7	59.0
		95% ethanol	4C	-3.0	73.1	20.0	16.0	10.3	78.0
		Water	4D	-2.6	9.6	14.0	9.0	28.2	10.0
5	*Stephania longa* Lour.	Petroleum ether	5A	-1.4	17.9	41.0	91.0	77.5	99.0
		Ethyl acetate	5B	-0.7	9.0	34.0	93.0	81.1	99.0
		95% ethanol	5C	0.6	31.0	11.0	52.0	31.3	85.0
		Water	5D	0.6	1.7	19.0	24.0	12.4	32.0
6	*Crocosmia crocosmiflora* (Nichols.) N. E. Br.	Petroleum ether	6A	-5.0	-3.3	17.0	66.0	27.7	-4.0
		Ethyl acetate	6B	49.7	98.5	38.0	97.0	86.6	49.0
		95% ethanol	6C	65.8	98.4	24.0	73.0	2.8	99.0
		Water	6D	-1.8	1.9	21.0	60.0	49.8	81.0
7	*Pandanus tectorius* Sol.	Petroleum ether	7A	-5.0	3.7	24.0	23.0	-6.8	89.0
		Ethyl acetate	7B	97.2	98.7	24.0	52.0	-3.6	84.0
		95% ethanol	7C	-1.5	2.2	19.0	15.0	42.8	70.0
		Water	7D	-2.4	-0.9	0.0	0.0	5.7	59.0
8	*Euphorbia hirta* Linn.	Petroleum ether	8A	-1.8	-1.7	0.0	98.0	95.4	98.0
		Ethyl acetate	8B	49.8	94.5	12.0	66.0	77.8	65.0
		95% ethanol	8C	0.9	53.7	21.0	28.0	28.2	39.0
		Water	8D	6.4	3.4	40.0	53.0	20.3	79.0
9	*Bidens biternata* (Lour.) Merr. et Schreff	Petroleum ether	9A	-2.3	11.6	30.0	73.0	45.7	92.0
		Ethyl acetate	9B	3.6	99.0	37.0	98.0	85.5	100.0
		95% ethanol	9C	49.9	74.3	17.0	51.0	27.3	92.0
		Water	9D	-1.2	11.5	67.0	95.0	49.2	95.0
10	*Cyperus rotundus* L.	Petroleum ether	10A	-4.2	-2.5	39.0	96.0	47.2	73.0
		Ethyl acetate	10B	0.5	46.8	35.0	99.0	75.3	83.0
		95% ethanol	10C	-0.1	13.5	33.0	7.2	30.4	37.0
		Water	10D	-2.4	9.0	24.4	5.7	27.1	74.0
11	*Microsorium fortuni* (Moore) Ching	Petroleum ether	11A	51.1	68.1	31.0	38.4	-6.0	6.0
		Ethyl acetate	11B	99.3	97.5	25.5	98.4	65.1	99.0
		95% ethanol	11C	48.9	58.0	19.3	97.2	28.2	44.0
		Water	11D	2.3	44.5	17.7	84.4	20.1	83.0
12	*Bidens pilosa* L.	Petroleum ether	12A	0.0	-9.2	26.2	47.5	29.3	98.0
		Ethyl acetate	12B	83.7	96.4	16.5	73.5	86.5	94.0
		95% ethanol	12C	10.0	41.3	28.1	-9.9	-0.9	29.0
		Water	12D	-2.1	41.1	42.3	20.5	18.9	63.0
13	*Wedelia calendulacea* Less.	Petroleum ether	13A	61.9	98.6	34.3	93.2	73.9	19.0
		Ethyl acetate	13B	77.6	95.7	27.0	83.0	75.0	32.0
		95% ethanol	13C	21.1	57.0	45.9	37.2	-3.3	61.0
		Water	13D	30.8	47.0	33.8	-6.0	5.0	68.0
14	*Uncaria macrophylla* Wal1.	Petroleum ether	14A	14.2	26.8	31.4	86.4	74.6	58.0
		Ethyl acetate	14B	73.1	99.6	33.7	59.6	88.0	99.0
		95% ethanol	14C	18.1	67.2	33.9	-6.5	12.8	-2.0
		Water	14D	12.0	60.9	29.0	11.2	15.3	57.0
15	*Sarcandra glabra (Thunb.)* Nakai	Petroleum ether	15A	12.2	-0.3	20.5	21.3	17.2	30.0
		Ethyl acetate	15B	65.0	92.4	12.2	24.6	53.0	88.0
		95% ethanol	15C	32.1	39.7	23.1	-0.7	6.4	45.0
		Water	15D	18.8	29.0	30.9	70.3	11.4	84.0
16	*Melaleuca leucadendra* Linn.	Petroleum ether	16A	98.9	98.0	41.4	18.6	94.8	100.0
		Ethyl acetate	16B	90.8	99.8	90.9	95.1	98.5	100.0
		95% ethanol	16C	67.3	99.6	-4.0	40.3	98.4	99.0
		Water	16D	68.5	98.1	8.6	52.9	92.5	99.0

The extracts that showed greater than 90% inhibition against cancer cells were further tested in lower concentrations (50, 25, 12.5, 6.25 and 3.1 µg/mL) to evaluate their potency. The IC_{50} values were calculated and are listed in Table 4. The greatest antiproliferative activities against cancer cells were observed for the ethyl acetate extract of *Melaleuca leucadendra*, which had a low IC_{50} ranging from 3.1 to 24.0 µg/mL against all four cancer cell lines that were tested. The other extracts of *Melaleuca leucadendra* (petroleum ether, ethanol and water) also showed potent antiproliferative activities with IC_{50} ranging from 4.1 to 32.7 µg/mL. Therefore, *Melaleuca leucadendra* was shown to be the most potent plant species amongst the 16 selected plants. In addition, several extracts, including the petroleum ether extracts of *Garcinia paucinervis* and *Wedelia calendulacea*, the petroleum ether and ethyl acetate extracts of *Stephania longa*, the ethyl acetate extract of *Microsorium fortune*, and the ethyl acetate and water extracts of *Bidens biternata* showed remarkable antiproliferation activity against cancer cells with a low IC_{50} ranging from 3.9 to 10.7 µg/mL. The petroleum ether extract of *Evodia lepta*, the ethyl acetate extracts of *Elephantopus scaber*, *Cyperus rotundus* and *Uncaria macrophylla* demonstrated moderate anti-proliferation activities with IC_{50} values of less than 25.3 µg/mL.

Table 4. IC50 values of the plant extracts that exhibited greater than 90% inhibition against cancer cells at 100 µg/mL. The values listed are the means from three replicate experiments and the standard deviation is given.

Code	Plant	Solvant	Cancer cell lines				Normal cell lines		Selectivity
			A549	BT20	MCF7	U2OS	HPL1A	HUVEC	
1A	*Garcinia paucinervis*	Petroleum ether	9.1± 5.4	8.6 ± 0.6		6.6 ± 1.8	23.9 ± 4.3	11.4 ± 2.0	1.3–3.6
2A	*Elephantopus scaber*	Petroleum ether				26.4 ± 2.4	46.1 ± 4.8	39.6 ± 1.0	1.5–1.7
2B	*Elephantopus scaber*	Ethyl acetate		11.7 ± 3.4	23.3 ± 4.3	12.8 ± 2.4	73.4 ± 1.5	31.0 ± 4.3	1.3–6.3
3A	*Evodia lepta*	Petroleum ether		27.4 ± 2.6		24.2 ± 5.4	> 100	38.8 ± 2.8	1.4–4.1
3B	*Evodia lepta*	Ethyl acetate				39.7 ± 12.6	> 100	12.9 ± 1.6	0.3 – > 2.5
4A	*Achras zapota*	Petroleum ether				49.3 ± 6.6	> 100	> 100	> 2.0
5 A	*Stephania longa*	Petroleum ether				10.7 ± 3.2	23.4 ± 5.5	59.9 ± 4.8	2.2–5.6
5B	*Stephania longa*	Ethyl acetate				10.0 ± 2.2	22.0 ± 3.8	24.4 ± 3.0	2.2–2.4
6B	*Crocosmia crocosmiflora*	Ethyl acetate		47.2 ± 5.0		45.9 ± 6.3	44.9 ± 5.8	>100	1.0 – > 2.2
6C	*Crocosmia crocosmiflora*	95% ethanol		35.1 ± 7.6			> 100	45.6 ± 4.6	1.3–2.8
7B	*Pandanus tectorius*	Ethyl acetate	43.5 ± 2.3	46.6 ± 6.3			> 100	46.9 ± 5.8	1.0–2.3
8A	*Euphorbia hirta*	Petroleum ether				45.0 ± 4.6	14.9 ± 6.8	41.0 ± 4.2	0.3–0.9
8B	*Euphorbia hirta*	Ethyl acetate		52.5 ± 6.3			48.9 ± 9.0	61.9 ± 5.3	0.9–1.2
9B	*Bidens biternata*	Ethyl acetate		27.3 ± 3.0		9.3 ± 3.4	19.5 ± 3.8	29.6 ± 2.5	0.7–3.2
9D	*Bidens biternata*	Water				4.0 ± 3.6	> 100	28.0 ± 2.5	7.0–25.0
10A	*Cyperus rotundus*	Petroleum ether				43.4 ± 4.0	> 100	58.7 ± 1.3	1.4–2.3
10B	*Cyperus rotundus*	Ethyl acetate				25.3 ± 4.3	24.6 ± 2.8	57.8 ± 3.8	1.0–2.3
11B	*Microsorium fortune*	Ethyl acetate	20.8 ± 2.6	5.0 ± 3.1		14.2 ± 1.5	49.8 ± 3.8	23.2 ± 3.8	1.1–10.0
11C	*Microsorium fortune*	95% ethanol				47.1 ± 3.6	> 100	> 100	> 2.1
12B	*Bidens pilosa*	Ethyl acetate		38.9 ± 6.5			32.8 ± 3.0	44.1 ± 4.0	0.8–1.1
13A	*Wedelia calendulacea*	Petroleum ether		6.2 ± 7.3		6.3 ± 3.2	24.4 ± 7.3	> 100	3.9–15.9
13B	*Wedelia calendulacea*	Ethyl acetate		26.3 ± 7.3			39.4 ± 2.8	> 100	1.5–3.8
14B	*Uncaria macrophylla*	Ethyl acetate		13.5 ±0.5			13.9 ± 3.5	53.8 ± 2.6	1.0–4.0
15B	*Sarcandra glabra*	Ethyl acetate		27.1 ± 2.3			83.5 ± 4.8	24.4 ± 2.0	0.9–3.1
16A	*Melaleuca leucadendra*	Petroleum ether	4.1 ± 0.8	31.2 ± 3.2			3.3 ± 1.0	10.7 ± 1.5	0.1–2.6
16B	*Melaleuca leucadendra*	Ethyl acetate	3.1 ± 1.7	24.0 ± 2.6	11.6 ± 4.4	17.2 ± 2.5	6.4 ± 1.5	16.7 ± 0.6	0.3–5.4
16C	*Melaleuca leucadendra*	95% ethanol		32.7 ± 5.8			7.4 ± 1.8	40.0 ± 3.3	0.2–1.2
16D	*Melaleuca leucadendra*	Water		20.7 ± 4.3			16.9 ± 3.0	20.7 ± 3.5	

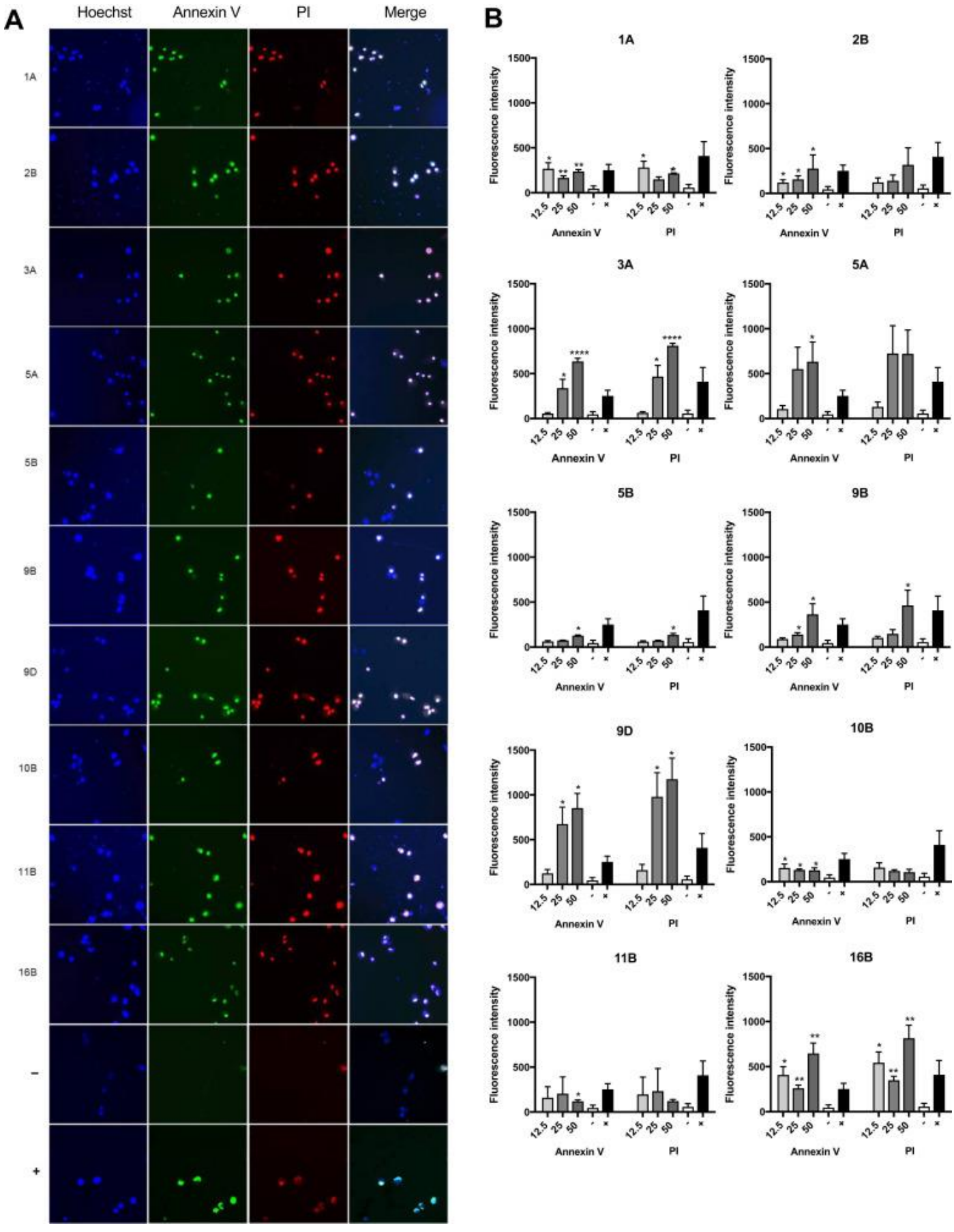
3.3. Evaluating the selectivity of the extracts using normal cell lines

Initially, the cytotoxicity of the extracts was evaluated in the normal cells HUVEC and HPL1A at a high concentration (100 µg/mL) (Table 3). Next, the cytotoxicity of the active extracts which showed greater than 90% inhibition against cancer cells were further tested in lower concentrations (50, 25, 12.5, 6.25 and 3.1 µg/mL) against normal cells to evaluate their safety and their IC_{50} values were determined (Table 4). The IC_{50} of the extracts against normal cells was compared with their IC_{50} against cancer cells, and the selectivity was calculated based on the ratio of the IC_{50} values (Table 4). A selectivity of greater than 1.0 suggests that an extract has a higher IC_{50} against normal cells than cancer cells, i.e. the extract is less toxic to normal cells than cancer cells. Some active extracts, such as the water extract of *Bidens biternata* and the petroleum ether extract of *Wedelia calendulacea*, showed superior selectivity, 7.0–25.0 and 3.9–15.9, respectively. Also, the ethyl acetate extract of *Microsorium fortune*, the petroleum ether extract of *Achras zapota* and the petroleum ether and ethyl acetate extracts of *Stephania longa* showed a good selectivity of greater than 2.0. In contrast, the four extracts of *Melaleuca leucadendra* showed poor selectivity and were generally more toxic to normal cells than cancer cells.

3.4. Determining the ability of the most potent extracts to induce apoptosis in U2OS cells

To determine whether the cytotoxicity of the most potent extracts against cancer cells was caused by apoptosis, an apoptosis assay was conducted in U2OS cells using the 10 extracts that had IC_{50} values of less than 25 µg/mL (Fig. 1). The U2OS cell line was

chosen because the 10 selected active extracts all showed cytotoxicity against U2OS cells. In the apoptosis assay, hoechst nuclear dye was used to indicate the nucleus of each individual cell, PI and FITC-Annexin V double staining indicated late apoptotic cells, while FITC-Annexin V staining alone indicated early apoptotic cells and PI staining alone indicated necrotic cells. After 18-h treatment with various extracts, different stages of apoptosis were observed in the U2OS cells. The ethyl acetate extracts of *Elephantopus scaber*, *Cyperus rotundus* and *Microsorium fortune* showed significant early induction of apoptosis at different concentrations compared with the untreated cells. The apoptotic effects of these three extracts were also dose dependent. The ethyl acetate extract of *Elephantopus scaber* induced apoptosis at 12.5 and 25 µg/mL (*$p < 0.05$), while the ethyl acetate extract of *Microsorium fortune* induced apoptosis only at 50 µg/mL (*$p < 0.05$). Apoptotic effects were observed after treatment with the ethyl acetate extract of *Cyperus rotundus* at all tested concentrations (12.5, 25, and 50 µg/mL) (*$p < 0.05$, or **$p < 0.01$). Moreover, all of the other tested extracts induced late apoptosis at concentrations lower than 25 µg/mL, except for the extract ethyl acetate extract of *Melaleuca leucadendra*, which showed significant late induction of apoptosis at all tested concentrations (12.5, 25, and 50 µg/mL).


A
Hoechst
Annexin V
PI
Merge
1A
2B
3A
5A
5B
9B
9D
10B
11B
16B
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+
B
1A
2B
3A
5A
5B
9B
9D
10B
11B
16B
Fluorescence intensity
1500
1000
500
0
12.5
25
50
-
+
Annexin V
PI

Figure 2. Induction of apoptosis by the 10 most active plant extracts in human bone cancer U2OS cells.

Cells were treated with different concentrations of each extract for 18 h and assessed using Hoechst, propidium iodide (PI) and FITC Annexin V. Cells treated with doxorubicin served as a positive control and cells treated with vehicle only served as a negative control. **A)** Fluorescent cell images by the ArrayScan VTI HCS reader. **B)** Fluorescent intensity of Annexin V and PI as indication of apoptosis induced by different concentrations of each extract in U2OS cells. The values listed are the means from three replicate experiments and the standard deviation is represented by error bars. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4. Discussion

Medicinal plants have been valuable sources for drug development in treating major diseases including cancer (Jeong et al., 2011). Phytochemicals derived from medicinal plants have been used both as safe and effective drugs to prevent the progression of cancer and increase the survival rates in cancer treatment, as well as alternative drugs to reduce the toxic side effects of chemotherapy (Binkhathlan and Lavasanifar, 2013). These phytochemicals are known to act on several cellular targets and have demonstrated various features that meet the need of multi-target drugs to treat cancer (Efferth and Koch, 2011; Ikezoe et al., 2003). They have started to be recognized by the western scientific community and some natural compounds from medicinal plants have undergone clinical trials, such as melatonin and phytoestrogen (Igney and Krammer,

2002). In recent years, novel scientific analyses and screening methods have significantly contributed to the development of drug discovery from medicinal plants (Lam, 2007).

In this study, we for the first time systematically screened and evaluated the collection of Yao Ethnobotanical medicines that have been used for thousands of years. Based on an ethnopharmacological survey, 16 plant species widely used in the medicines of the Yao ethnic group in China were collected and 64 plant extracts were prepared from these plants. The in vitro antitumor effects of these extracts were tested in several cancer and normal cell lines and suggested the presence of potential antitumor activities from various extracts. As a result of screening 102 plant species that have been used in Yao ethnomedicine, 16 plants were identified that showed notable antitumor activities. Among these 16 plants, *Melaleuca leucadendra, Garcinia paucinervis Wedelia calendulacea*, *Stephania longa*, *Microsorium fortune* and *Bidens biternata* showed the most potent antitumor effects against human cancer cell lines. The cytotoxic effects that we found in the extracts of *Garcinia paucinervis* and *Wedelia calendulacea* are consistent with previous findings (Gao et al., 2010b; Li et al., 2016a, 2016b; Mottakin et al., 2004; Sharma et al., 1989). However, the cytotoxic effects of extracts from *Melaleuca leucadendra*, *Stephania longa*, *Microsorium fortune* and *Bidens biternata* were identified for the first time. No bioactivity of *Stephania longa* had been reported previously. In addition, the results indicated that some cancer cell lines were more sensitive than others to these extracts. For example, several plants including *Achras zapota, Stephania longa* and *Cyperus rotundus* only showed antiproliferation activities against the U2OS cell line but not A549, BT20 or MCF7. While the two ethyl acetate extracts of *Elephantopus*

scaber and *Melaleuca leucadendra* were active against both MCF7 and BT20 breast cancer cell lines, 16 extracts showed a specific inhibitory effect on the breast cancer cell line BT20, but not MCF7. Moreover, it is notable that the most active fractions of the tested plants were the petroleum ether (32%) and ethyl acetate (50%) fractions.

Although numerous studies have reported a significant therapeutic effect of agents isolated from medicinal plants, several studies have revealed that serious toxic effects can be caused by natural products (Chiu et al., 2009). Cytotoxicity evaluation of these extracts against normal cells HPL1A and HUVEC demonstrated that most of the extracts had cytotoxicity against normal cells and half of the tested extracts (14 out of 28) showed more cytotoxicity against normal cells than cancer cells. However, five of the extracts showed significant selectivity against cancer cell lines versus normal cell lines, including the water extract of Bidens biternata, the petroleum ether extract of *Wedelia calendulacea*, the petroleum ether and ethyl acetate extracts of *Stephania longa* and the petroleum ether extracts of *Achras zapota*. These results suggest that those five extracts might be better potential candidates than others for developing antitumor agents with high selectivity.

It is well known that cancer cells have the ability to escape from apoptosis, therefore targeting apoptosis by cancer agents is a critical strategy for effective cancer treatments. We chose the top 10 cytotoxic extracts that had IC50 values of less than 25 µg/mL against cancer cells and investigated their capability to induce apoptosis in U2OS bone cancer cells by Annexin V/PI double staining. We confirmed that the cytotoxicity of all 10 extracts were due to apoptosis. Particularly, the ethyl acetate extracts from

Elephantopus scaber, *Cyperus rotundus*, *Microsorium fortune* and *Melaleuca leucadendra* against U2OS cell lines induced apoptosis in a concentration-dependent manner. For the first time extracts from *Microsorium fortune* and *Melaleuca leucadendra* were demonstrated to be able to induce apoptosis. Previously, only antioxidant activity has been observed in extracts prepared from these two plants (Li et al., 2011, 2013).

In summary, this study examined the anticancer efficacy and safety of 16 medicinal plants that have been historically used in Yao ethnomedicine, hence providing evidence to assist their use in clinical practice. Also, we identified the potential of some of these plants as anticancer agents. Taking into consideration both cytotoxicity and selectivity, we showed that the anticancer activities of extracts from *Bidens biternata*, *Wedelia calendulacea* and *Stephania longa* should be considered for further investigation.

Declaration of conflicting interests

The authors declared no conflicts of interest.

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SECOND ARTICLE

IN VITRO ANTITUMOR EFFECTS OF TWO NOVEL OLIGOSTILBENES, *CIS*- AND *TRANS*-SUFFRUTICOSOL D, ISOLATED FROM *PAEONIA SUFFRUTICOSA* SEEDS

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Abstract

Naturally derived stilbenes have been shown to elicit cytotoxic, anti-steroidal, anti-mutagenic, anti-oxidative**,** anti-inflammatory, and anti-tumor bioactivities. Previous phytochemical studies revealed that the seeds of *Paeonia suffruticosa* are rich in natural stilbenes. In this study the anti-tumor effects and mechanism of action of the oligostilbene isomers, *cis*- and *trans*-suffruticosol D, isolated from the seeds of *P. suffruticosa* were examined. *cis*- and *trans*-suffruticosol D exhibited remarkable cytotoxicity against the human cancer cell lines A549 (lung), BT20 (breast), MCF7 (breast), and U2OS (osteosarcoma), but showed significantly less toxicity to the normal human cell line HMEC. We also demonstrated that *cis*- and *trans*-suffruticosol D exerted their anti-tumor effects by provoking oxidative stress, stimulating apoptosis, decreasing the mitochondrial membrane potential, inhibiting cell motility, and blocking the NF-κB pathway in human lung cancer cells. In addition, we evaluated their respective bioefficacy and found that *trans*-suffruticosol D is more potent than *cis*-suffruticosol D. Collectively, our results suggest that *cis*- and *trans*-suffruticosol D could be promising chemotherapeutic agents against cancer.

Keywords

Paeonia suffruticosa, *cis*- and *trans*-suffruticosol D, cytotoxicity, apoptosis, oxidative stress, cell motility

1. Introduction

Current cancer medications are costly and often cause serious side effects. The US National Cancer Institute began investigating anti-tumor plant extracts in the 1960s (Y. Cai, Luo, Sun, & Corke, 2004; Monks et al., 2002), and the premise that natural compounds obtained from therapeutic plants could produce anti-cancer medications has henceforth been of great research interest. Traditional Chinese Medicines (TCMs) using dried plants or plant extracts have provided low cost diet and pharmaceutical therapies for thousands of years and experimental and clinical studies have proven that more than 400 plant species used in TCMs as anti-cancer herbal medications are significantly effective in the prevention or treatment of various cancers (Hang and Goge, 1998; Ji et al., 1999; Xu et al., 2000; Bo et al., 2002; Cai et al., 2004; Parekh et al., 2009). However, much work remains to be done to determine the effectiveness of the individual compounds present in the TCMs.

Paeonia suffruticosa, or *Paeoniaceae,* is a widely utilized Chinese medicinal plant within the *Paeonia* genus. This genus comprises approximately 35 species that are classified into three groups: *Oneapia, Paeonia,* and *Moutan* (He et al., 2010). The *Cortex Moutan* (root cortex) of *Paeonia* has been recorded by China's Pharmacopoeia as a significant source of herbal medicine (Z.-F. Chen & H. Liang, 2010). Extracts of *Paeonia* have been shown to posses cytotoxic, antitumor, anti-inflammatory and anti-oxidative activities (He et al., 2010). Previous photochemical research on *Paeonia* identified more than 260 bioactive compounds, including phenols, monoterpenoidglucosides, paeonols, flavonoids, tannins, steroids, triterpenoids and stilbenes (He et al., 2013). A more recent

study showed that the seeds of *Paeonia* contain considerable quantities of stilbenes compared to the other compounds (He et al., 2013).

Stilbenes are a class of polyphenols widely found in plants that contain a 1.2-diphenylethylene nucleus in their structure (Tao et al., 2009). Stilbenes have aroused a lot of interests due to their anti-tumor, anti-steroidal, anti-mutagenic, anti-oxidative and anti-inflammatory bioactivities (T. Cai & Cai, 2011; Yuk et al., 2013). One well-known example of the stilbenes is resveratrol, and its anti-tumor activity has been intensively studied. Several *in vivo* and *in vitro* studies have shown that resveratrol inhibits the growth of cancer cells and effects various molecular targets associated with cancer progression such as the Wnt signaling pathway, nuclear factor-kappa B (NF-κB), and the MAPK/ERK pathway in different types of cancer (Shukla & Singh 2011; Whitlock & Baek, 2012).

Previously, two novel stilbenes, *cis*- and *trans*-suffruticosol D, were extracted from the seeds of *Paeonia* (He et al., 2010). The two chemicals have similar structures as the mass fragmentation pattern of *trans*-suffruticosol D was very similar to *cis*-suffruticosol D, with *cis*-suffruticosol D varying only from *trans*-suffruticosol D in its olefinic hydrogen signal (Fig. 3). In this study, we investigated the antitumor activities of *cis*- and *trans*-suffruticosol D and examined how these two chemicals act against cancer cells *in vitro*.

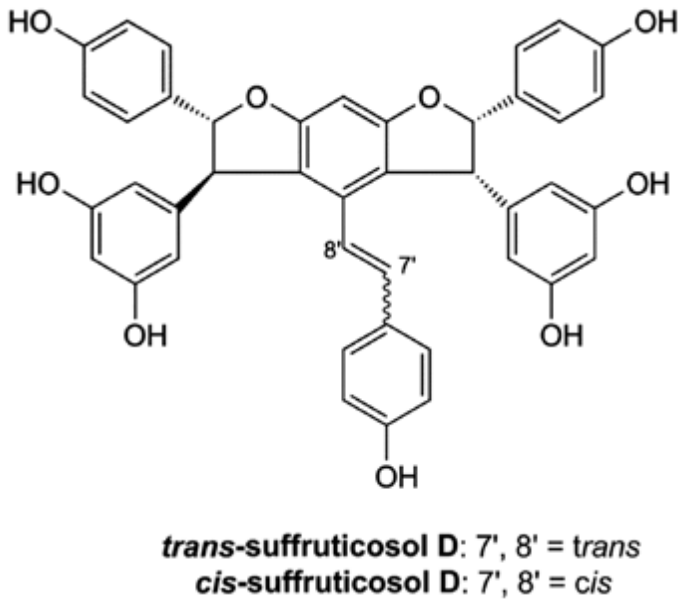


Figure 3. Chemical structures of *cis*- and *trans*-suffruticosol D

2. Methods and Materials

2.1. Plant material and compound isolation

The seeds of *P. suffruticosa* were collected in Tongling, Anhui province, P. R. China, and identified in September 2012. A voucher specimen (2012001) has been deposited in the Seed Resource Bank of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. *cis*- and *trans*- suffruticosol D were extracted and isolated from the dried seeds of *P. suffruticosa* (1.2 kg) as described previously (He et al., 2010a). Compounds were re-suspended in dimethyl sulfoxide

(DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to yield the concentration of 10 mM and stored at 4°C.

2.2. Cell culture

Four human cancer cell lines including A549 (lung carcinoma), BT20 (*estrogen* receptor-negative human breast adenocarcinoma), MCF-7 (*estrogen* receptor-positive human breast adenocarcinoma) and U2OS (human osteosarcoma) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). An A549 cell line that stably expresses Green Fluorescent Protein (GFP) was purchased from Cell BioLabs Inc. (San Diego, CA, USA). A549, A549-GFP and BT20 cells were cultured in RPMI 1640 media (Sigma-Aldrich), MCF7 cells were cultured in DMEM medium (ATCC), and U20S cells were cultured in McCoy's 5A medium (ATCC). As a control, HPL1A cells (human peripheral lung epithelial cells) were obtained from Nagoya University and cultured in DMEM/F12 medium (Sigma-Aldrich); HMEC cells (primary human mammary breast epithelial cells) were purchased from ATCC and cultured in McCoy's 5A medium. All medium contained 10% FBS (Sigma-Aldrich) and 1% streptomycin and penicillin (Sigma-Aldrich). These cells were incubated in a humid environment with 5% CO_2 at 37°C.

2.3. Cell proliferation assay

The resazurin reduction reagent AlamarBlue (Invitrogen, Frederick, MD, USA) was used to evaluate the cytotoxicity of the compounds. Cells were plated at a density of 5×10^3 cells per well in 96-well microplates with 100 μL culture medium, and were allowed to

attach for 16 h prior to treatment. Next, all the medium was replaced with medium containing the *cis*- or *trans*-suffruticosol D compounds at seven different concentrations: 320, 100, 32, 10, 3.2, 1.0, and 0.32 μM. 1% DMSO was used as vehicle control. The cells were placed in an incubator for 48 h at 37°C. Cells that were treated with medium containing vehicle only serve as negative control. Subsequently, AlamarBlue solution was added to the medium and the cells were incubated in the CO_2 incubator for 1 h. The fluorescent intensity change of the dye was measured at Ex 555 nm and Em 590 nm using a plate reader (Molecular Devices, Sunnyvale, CA, USA). The cytotoxicity was examined by determining by IC_{50}, the dose that inhibited 50% of cell growth, using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). For the N-acetyl-L-cysteine (NAC) attenuation assay, the cells were treated with different concentrations of *cis*- or *trans*-suffruticosol D followed by incubation with or without 10 mM NAC (Sigma-Aldrich) for 48 h, and then cell viability was assessed by AlamarBlue assay.

2.4. Apoptosis assay

The FlowCellect Annexin Red Kit (EMD Millipore, Billerica, MA, USA) was used to determine the apoptosis rate in A549 cells according to the manufacturer's instructions. Briefly, A549 cells were plated in 96-well plates. After a 24-h treatment with *cis*- or *trans*-suffruticosol D at concentrations of 100, 32, and 10 μM, the floating and attached cells were collected for analysis. The cells were centrifuged at 700 × g for 7 min and were resuspended in 100 μL Assay Buffer (EMD Millipore). Afterwards, the cells were stained with annexin-V for 15 min and 7-amino-actinomycin D (7-AAD) for 5 min, and

examined with a Guava EasyCyte Flow Cytometer (EMD Millipore). Data was analyzed using Guava InCyte software.

2.5. Apoptosis antibody array

The Human Apoptosis Antibody Array Kit (RayBiotech, Inc., Norcross, GA. USA) was used to evaluate apoptotic protein expression according to the manufacturer's instructions. A549 cells were plated at 8,000 cells/well intensity in a 96-well plate and then treated with *cis* or *trans*-suffruticosol D at a concentration of 50 μM for 6 h. The cells were lysed in lysis buffer with protease inhibitors. The cell lysates were concentrated using a protein concentration column (EMD Millipore) to a total protein concentration of 2 mg/mL. The samples were then diluted10 fold with assay buffer and incubated with an array membrane for 2 h at room temperature, and washed with washing buffer for five times. Subsequently, the cocktail of biotin-conjugated antibody mix was added to the membrane and incubated overnight at 4°C. The samples were then incubated with HRP-conjugated streptavidin for 2 h at room temperature and chemiluminescence substrate was used to detect the signal. Image Studio software (LI-COR Biotechnology, Lincoln, NE, USA) was used to quantify the intensity of each array dot and then normalized to the internal control.

2.6. Oxidative stress assay

The Hitkit oxidative stress kit (Thermo Scientific, Waltham, MA, USA) was used to determine the generation of reactive oxygen species (ROS) according to the manufacturer's instructions. Briefly, A549 cells were treated with *cis*- or *trans*-

suffruticosol D for 24 h, fixed with warm 37% formaldehyde and stained with Hoechst and dihydroethidium (DHE) dye for 30 min at 37°C with 5% CO_2. Doxorubicin (DOX) at 1 μM concentration was used as a positive control and cells treated with vehicle only were used as negative control. ROS generation in the nuclei was indicated by the production of the fluorescent ethidium, and assessed by measuring the nuclear fluorescent intensity using an ArrayScan VTI High-content screening (HCS) reader (Thermo Scientific). Images were acquired and data was analyzed by vHCS Scan software.

2.7. Cell motility assay

A 96-well collagen plate (Corning, Corning, NY, USA) was coated with blue fluorescent beads (Life Technologies, Eugene, OR, USA) as follows. The beads were centrifuged for 1 min at 14,000 g and washed twice with PBS, then 75 μL beads were added to each well of the 96-well collagen plate and incubated for 1 hour at 37°C. The cells were seeded on the lawn of fluorescent beads and the sizes of the tracks generated by migrating cells were measured. After the plate was washed 5 times with PBS, A549-GFP cells were seeded at 500 cells/well in the coated plate and incubated for 1 h at 37°C. Subsequently the cells were treated with different concentrations of *cis*- or *trans*-suffruticosol D in medium containing 10% FBS for 18 h. Cells treated with serum-free medium serve as the negative control and cells treated with medium containing 10% FBS serve as the positive control. Cell tracks were imaged using an Arrayscan VTI HCS reader (Thermo Scientific) and the data was analyzed by vHCS Scan software. The mean of the full track

2.8. Multi-parameter cytotoxicity assay

HCS analysis was used to measure nuclear morphology, cell membrane permeability, and mitochondrial membrane potential changes, the three parameters associated with cytotoxicity. A549 cells were treated with different concentrations of *cis*- or *trans*-suffruticosol D for 24 h. The cells were then fixed and stained with a warm solution containing Hoechst dye, Membrane Permeability Dye, and Mitochondrial membrane Potential Dye (Thermo Scientific). Cells were imaged using an Arrayscan VTI HCS reader (Thermo Scientific). Data on nuclear size, cell permeability, and mitochondria membrane potential were collected and analyzed using vHCS Scan software.

2.9. Western blotting analysis

A549 cells were treated with 50 μM of *cis*- or *trans*-suffruticosol D for 3 hours then incubated with 10 ng/mL of TNF-α for 30 min. Cells treated with the NF-κB inhibitor Bay11-7082 (10 μM) (Sigma-Aldrich) were used as a positive control, and cells treated with vehicle only were used as a negative control. After treatment, the cells were lysed using M-PER mammalian protein extraction reagent (Thermo Scientific) containing proteinase and phosphatase inhibitors (Sigma-Aldrich) and centrifuged at 13,000 rpm for 5 min at 4^{O}C. A Pierce BCA protein assay kit (Thermo Scientific) was used to determined protein concentrations. Proteins were separated on a 4-20% Tris Glycine gel (Thermo Scientific), and electrophoretically transferred to a PVDF membrane. The following primary antibodies were used: phosphorylated-NF-κB p65, NF-κB p65 (Cell Signaling Technology, Danver, MA, USA) and actin (Santa Cruz Biotechnology, Dallas,

TX, USA). The membrane was incubated with the primary antibodies at a 1:1000 concentration at 4^{o}C overnight. After washing with 1 X PBS for 5 times, the membrane was incubated for 2 hours at room temperature with HRP linked anti-rabbit IgG secondary antibodies. Membranes were developed with chemiluinescent substrates (Thermo Scientific) and scanned with a chemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

2.10. NF-κB nuclear translocation assay

A Multiplexed NF-κB activation HCS Kit (Thermo Scientific) was used to assess NF-κB nuclear translocation. A549 cells were pre-treated with different concentrations of *cis*- or *trans*-suffruticosol D for 4 h, then 10 ng/mL of TNF-α (Sigma-Aldrich) was added to the cells for an additional 30 min. After treatment, cells were fixed and permeabilized prior to detection. NF-κB distribution was detected by adding NF-κB p65 primary antibodies and then staining with a secondary antibody conjugated with DyLight 549 and Hoechst dye (Thermo Scientific). Cells treated with medium containing only the vehicle were used as negative control, and cells treated with 25 ng/mL TNF-α were used as a positive control. Cells were imaged using an Arrayscan VTI HCS reader (Thermo Scientific). Data on the mean difference of NF-κB fluorescent intensity between the nuclear and cytoplasmic areas were collected and analyzed by vHCS Scan software.

3. Results

3.1. Cytotoxicity of *cis*- and *trans*-suffruticosol D in lung, breast, and bone cancer cells

After 48 h treatment, both *cis*- and *trans*-suffruticosol D showed significant cytotoxic effects against A549 (lung), BT20 (breast), MCF7 (breast) and U2OS (osteosarcoma) cancer cell lines. IC_{50} values for *cis*- and *trans*-suffruticosol D against these cancer cells ranged from 9.93 to 46.79 μM as shown in Table 1. Interestingly, we observed that *trans*-suffruticosol D had lower IC_{50} values (9.93 - 15.84 μM) than *cis*-suffruticosol D (13.42 - 46.79 μM) in all four cancer cell lines. In addition, both *cis*- and *trans*-suffruticosol D showed notably weaker cytotoxicity against normal breast epithelial cells HMEC (IC_{50} values of 146.3 and 269.5 μM, respectively). The selectivity of *cis*- and *trans*-suffruticosol D ranged from 9.2 - 14.7 fold and from 5.8 – 20 fold, respectively (Table 5).

Table 5. IC50 values of *cis*- and *trans*-suffruticosol D in selected cancer and normal cell lines

IC_{50} (μM)	***trans*-SD**	***cis*-SD**
A549	11.9±1.2	17.1±1.0
BT20	9.9±3.8	13.4±2.5
MCF-7	15.8±1.6	46.8±3.3
U2OS	11.3±2.3	24.6±4.4
HPL1A	78.3±6.1	177.5±9.3
HMEC	146.3±2.7	269.5±2.2

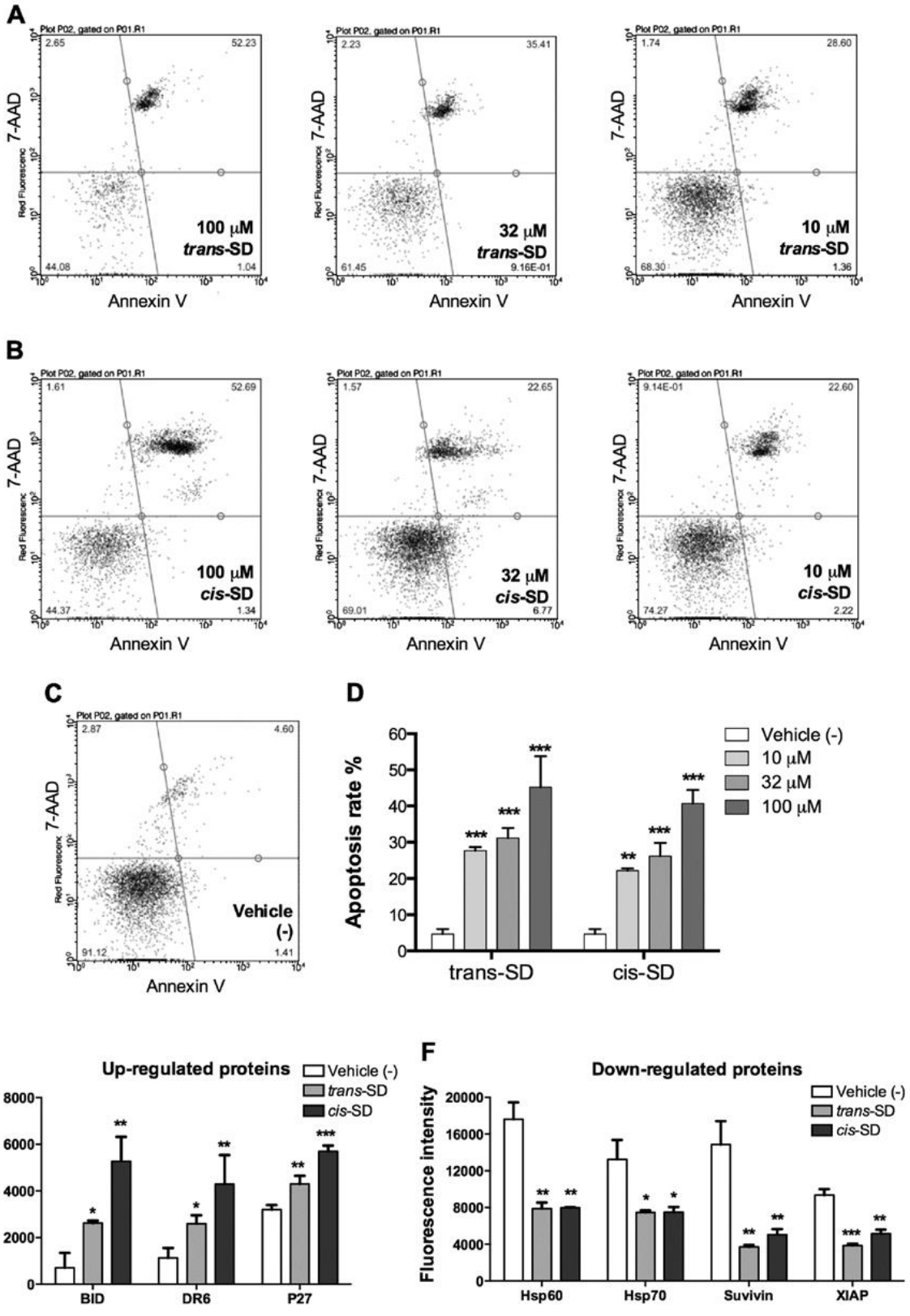
Cells were treated with various concentrations of *cis*- or *trans*-suffruticosol D for 48 h, and the viability of cells was evaluated with the AlamarBlue dye. Data are expressed as mean ± SD, and experiments were performed in triplicates

3.2. *cis*- and *trans*-suffruticosol D induce apoptosis in A549 lung cancer cells

To find out whether these cytotoxic properties were due to apoptosis, we conducted an apoptosis assay using A549 cells treated with *cis*- or *trans*-suffruticosol D. Following 24 h treatment, both compounds showed significant apoptosis induction at a wide range of concentrations compared with the non-treated cells (*P<0.05, **P<0.01, or ***P<0.001) and the apoptotic effects were concentration-dependent (Fig. 4A-D). *trans*-suffruticosol D induced 30.1%, 39.8%, and 41.9% of A549 cells into apoptosis at concentrations of 10, 32, and 100 μM, respectively. *cis*-suffruticosol D induced 22.2%,

27.1%, and 45.3% of A549 cells into apoptosis at concentrations of 10, 32, and 100 μM, respectively.

Next, we performed an apoptotic protein array analysis to investigate the effect of *cis*- and *trans*-suffruticosol D on apoptotic proteins. Two proteins from the inhibitor of apoptosis proteins family (IAPs), X-linked inhibitor of apoptosis protein (XIAP) and survivin, as well as the heat shock proteins Hsp60 and Hsp70, showed significant down regulation after treatment by *cis*- and *trans*-suffruticosol D (Fig. 4E). Meanwhile, death receptor 6 (DR6), also known as Tumor necrosis factor receptor superfamily member 21 (TNFRSF21), the cycline dependent kinase inhibitor 1B (p27), and the BH3 interacting-domain death agonist (BID), were up-regulated by both *cis*- and *trans*-suffruticosol D (Fig. 4F).


A
Plot P02, gated on P01.R1
2.65
52.23
44.08
1.04
100 μM
trans-SD
7-AAD
Annexin V
2.23
35.41
61.45
9.16E-01
32 μM
trans-SD
1.74
28.60
68.30
1.36
10 μM
trans-SD
B
1.61
52.69
44.37
1.34
100 μM
cis-SD
1.57
22.65
69.01
6.77
32 μM
cis-SD
9.14E-01
22.60
74.27
2.22
10 μM
cis-SD
C
2.87
4.60
91.12
1.41
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Apoptosis rate %
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Up-regulated proteins
Vehicle (-)
trans-SD
cis-SD
Fluorescence intensity
BID
DR6
P27
F
Down-regulated proteins
Hsp60
Hsp70
Suvivin
XIAP

Figure 4. Induction of apoptosis by *cis*- and *trans*-suffruticosol D in A549 cells. After 24-h treatment by *cis*- or *trans*-suffruticosol D, A549 cells were stained with Annexin V/7-AAD, and the percentage of apoptotic cells was assessed by flow cytometry. Cells treated with the vehicle only served as a negative control. (A-C) Annexin V/7-AAD double staining of A549 cells treated with various concentrations of *cis*- or *trans*-suffruticosol D. (D) Percentage of apoptotic cells induced by *cis*- or *trans*-suffruticosol D. (E and F) Effect of *cis*- or *trans*- suffruticosol D on key regulatory proteins of apoptosis. The error bars indicate the standard deviation from three experiments. *P≤0.05; **P≤0.01; ***P≤0.001.

3.3. *cis*- and *trans*-suffruticosol D induce ROS generation in A549 lung cancer cells

We examined the cellular ROS levels in A549 cells to determine whether *cis*- and *trans*-suffruticosol D induced oxidative stress. As shown in Fig. 5A, both *cis*- and *trans*-suffruticosol D converted non-fluorescent DHE to fluorescent ethidium, which binds to DNA, suggesting they induced ROS generation in A549 cells. Quantitative data showed both compounds significantly induced ROS generation in a concentration-dependent manner (**P<0.01, ***P<0.001, or ****P<0.0001). After treatment for 24 h, *trans*-suffruticosol increased the ROS levels by 32.8%, 34.6%, and 87.2% at concentrations of 10, 32, and 100 μM, respectively, while *cis*-suffruticosol increased the ROS levels by 32.8%, 55.6%, and 73.1% at concentrations of 10, 32, and 100 μM, respectively, in A549 cells (Fig. 5B). To further investigate whether the cytotoxicity induced by *cis*- and *trans*-suffruticosol D was associated with ROS levels, we co-treated A549 cells with the

antioxidant N-acetyl-L-cysteine (NAC) and different concentrations of *cis*- or *trans*-suffruticosol D for 48 h. We observed that 10 mM NAC attenuated the cell death induced by *cis*- or *trans*-suffruticosol D in A549 cells at all tested concentrations (Fig. 5C).

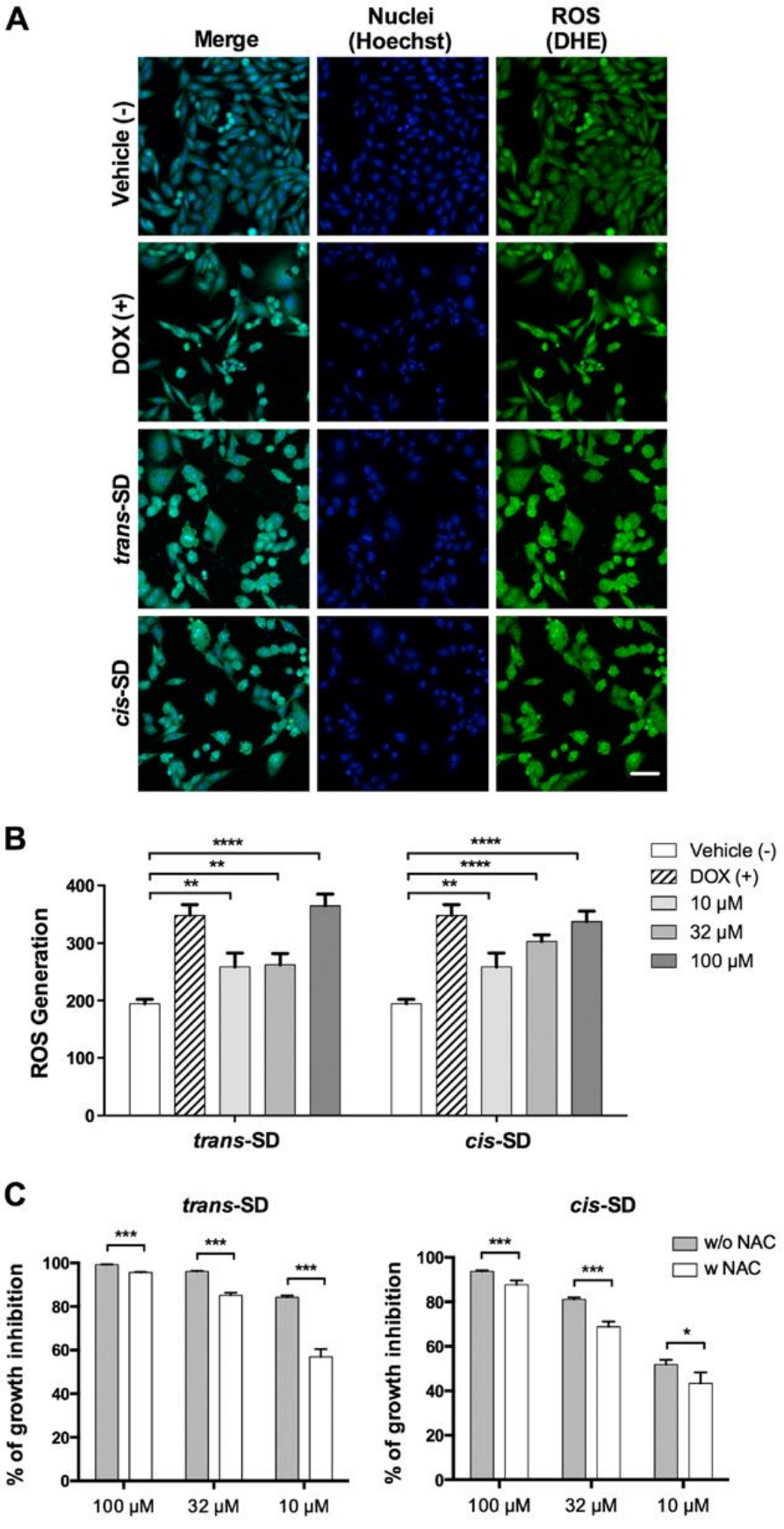
A
Merge
Nuclei (Hoechst)
ROS (DHE)
Vehicle (-)
DOX (+)
trans-SD
cis-SD
B
ROS Generation
400
300
200
100
0
****
**
**
****
****
**
trans-SD
cis-SD
Vehicle (-)
DOX (+)
10 μM
32 μM
100 μM
C
trans-SD
cis-SD
% of growth inhibition
100
80
60
40
20
0
***
***
***
***
***
*
100 μM
32 μM
10 μM
w/o NAC
w NAC

Figure 5. Induction of oxidative stress by *cis*- and *trans*-suffruticosol D in A549 cells. A549 cells were treated with various concentrations of *cis*- or *trans*suffruticosol D for 24 h, then stained with Hoechst and DHE dye. Cells treated with doxorubicin served as a positive control, and cells treated with vehicle only served as a negative control. The ROS levels were measured by the fluorescent intensity of DHE that was converted to ethidium bromide. (A) Fluorescent cell images by the HCS reader. Scale bar, 100 μm. (B) ROS levels in A549 cells treated with various concentrations of *cis*- or *trans*-suffruticosol D. (C) The anti-oxidant NAC attenuated the cell death of A549 cells induced by *cis*- or *trans*-suffruticosol D. The error bars indicate the standard deviation from three experiments. *P≤0.05; **P≤0.01; ***P≤0.001.

3.4. *cis*- and *trans*-suffruticosol D inhibit motility of A549 lung cancer cells

To test if *cis*- and *trans*-suffruticosol D affected cancer cell motility, we measured the size of the tracks generated by migrating cells after treatment, which is proportional to the magnitude of cell movement. As shown in Fig. 6A, A549 cells treated with *cis*- or *trans*-suffruticosol D in serum-containing medium showed less motility activity evidenced by a smaller track area per cell than the untreated cells. Both *cis*- and *trans*-suffruticosol D significantly inhibited cell movement at all the concentrations that were tested in A549 cells (***P<0.001 or ****P<0.0001) (Fig. 6B). *trans*-suffruticosol D decreased the A549 cell motility by 40.7%, 40.7%, and 54.9% at concentrations of 10, 32 and 100 μM, respectively, while *cis*-suffruticosol D decreased the A549 cell motility by 42.3%, 42.0%, and 50.4% at concentrations of 10, 32 and 100 μM, respectively.

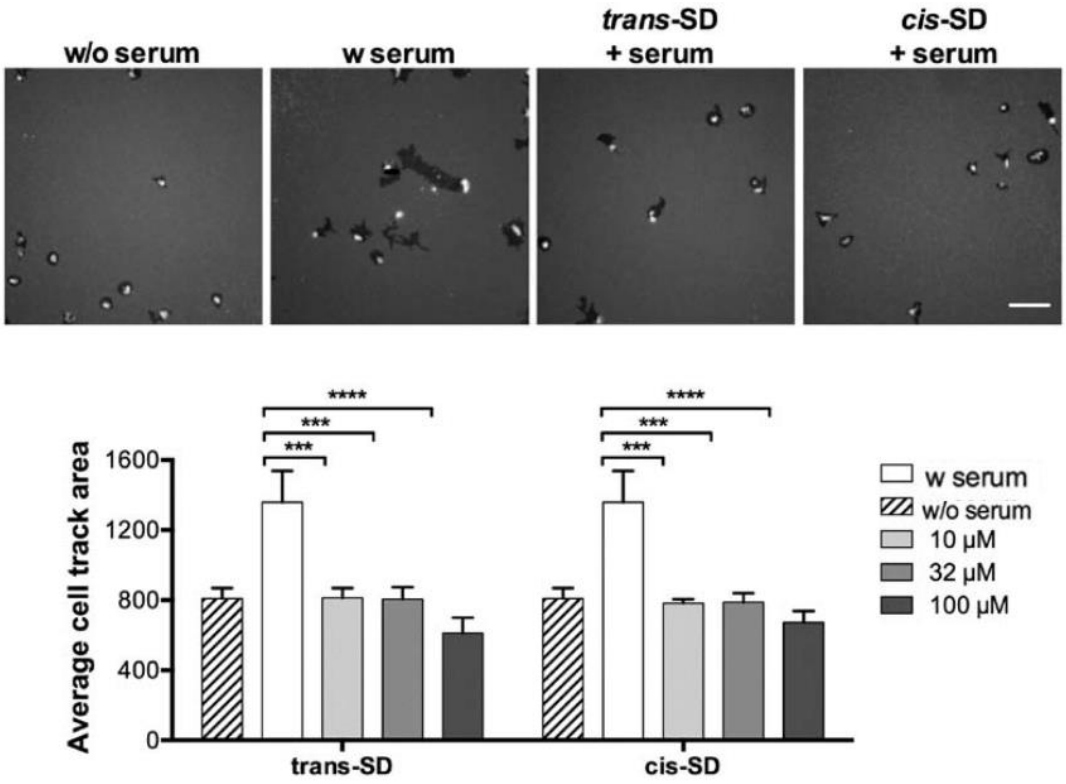

Figure 6. Cell motility changes induced by *cis*- and *trans*-suffruticosol D in A549 cells. A549-GFP cells were seeded in a 96-well plate with a monolayer of fluorescent beads. After treatment with *cis*- or *trans*-suffruticosol D for 18 h, individual cell movement was evaluated by measuring the fluorescent track area. Cells treated with serum-free medium served as a negative control and cells treated with medium containing 10% serum served as a positive control. (A) Fluorescent track area showing the movement of the cells. Scale bar, 200 μm. (B) Measurement of the cell track areas of cells treated with various concentrations of *cis*- or *trans*-suffruticosol D. The error bars indicate the standard deviation from three experiments. *P≤0.05; **P≤0.01; ***P≤0.001.

3.5. *cis*- and *trans*-suffruticosol D decreased mitochondrial membrane potential in A549 cells

To determine the cytotoxic effect of *cis*- and *trans*-suffruticosol D in human lung cancer cells, we measured three cell health parameters, nuclear morphology, cell membrane permeability and mitochondrial membrane potential changes, using a HCS reader. As shown in Fig. 7, in the mitochondrial potential channel, untreated A549 cells exhibited bright fluorescent intensity, indicating intact mitochondrial membranes. In comparison, in cells treated with *cis*- or *trans*-suffruticosol D the fluorescent intensity of the dye was significantly decreased at all tested concentrations, indicating that *cis*- and *trans*-suffruticosol D induced a significant decrease of the mitochondrial membrane potential in A549 cells (***$P<0.001$). We also observed nuclei shrinkage and increased cell membrane permeability in cells treated with a high-concentration (100 μM) of *trans*-suffruticosol D (*$P<0.05$ or **$P<0.01$). However, no significant change was detected in nuclear size and cell membrane permeability in cells treated with *cis*-suffruticosol D**.**

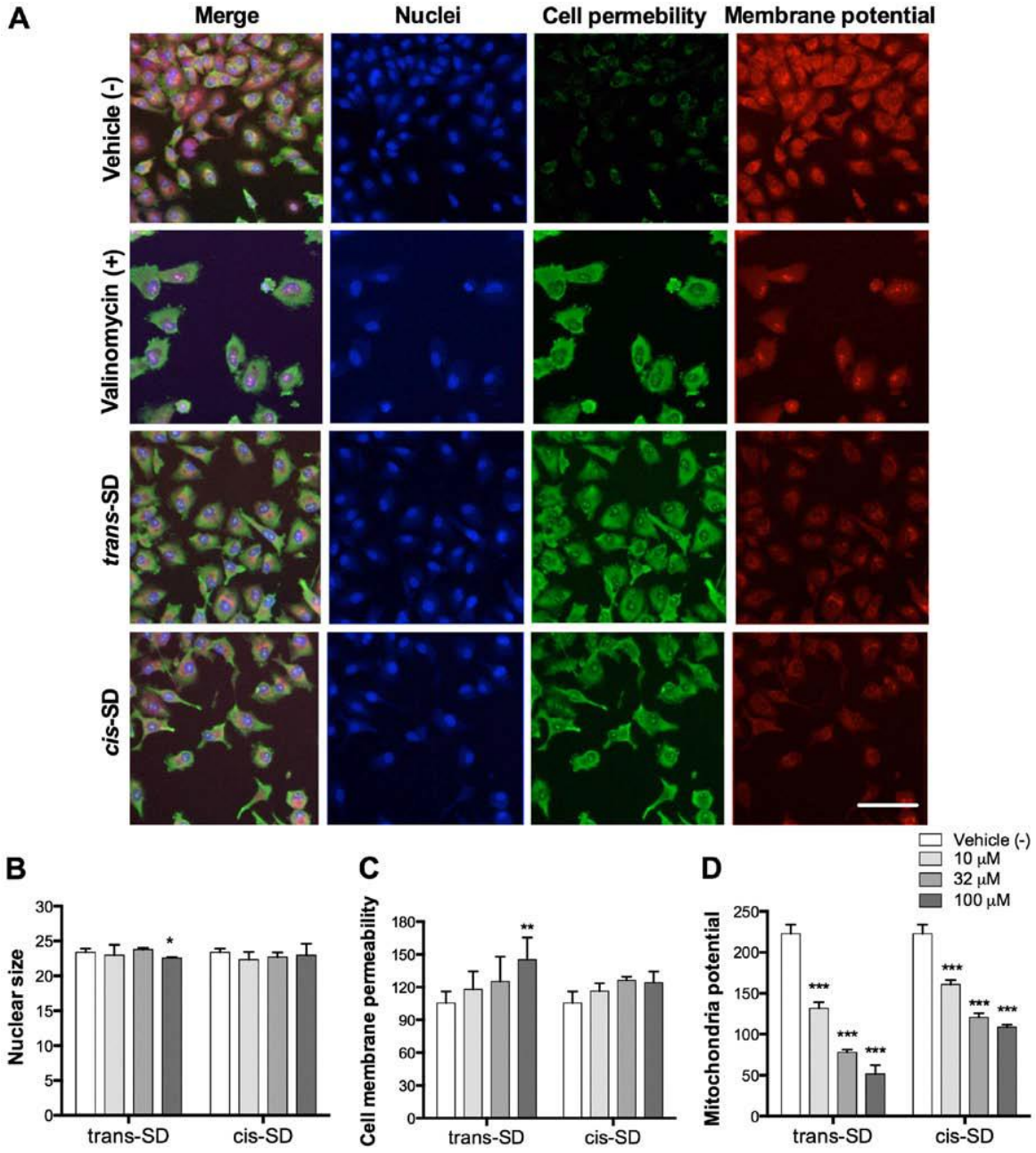
A
Merge
Nuclei
Cell permebility
Membrane potential
Vehicle (-)
Valinomycin (+)
trans-SD
cis-SD
B
Nuclear size
trans-SD
cis-SD
C
Cell membrane permeability
trans-SD
cis-SD
D
Vehicle (-)
10 μM
32 μM
100 μM
Mitochondria potential
trans-SD
cis-SD

Figure 7. Multi-parameter cytotoxicity induced by *cis*- and *trans*-suffruticosol D in A549 cells. A549 cells were treated with various concentrations of *cis*- or *trans*-suffruticosol D for 24 h, then stained with three dyes simultaneously Hoechst, cell permeability dye and mitochondrial membrane potential dye). Cells treated with vehicle only served as a negative control and cells treated with 10 μM valinomycin served as a positive control. (A) Fluorescent cell images by HCS reader. Scale bar, 100 μm. (B-D) Evaluation of nuclear size, cell permeability and mitochondrial membrane potential of cells treated with *cis*- or *trans*suffruticosol D. The error bars indicate the standard deviation from three experiments. *P≤0.05; **P≤0.01; ***P≤0.001.

3.6. *cis*- and *trans*-suffruticosol D inhibit TNF-α-induced NF-κB activation

We performed western blot analysis to examine the effects of *cis*- and *trans*-suffruticosol D on the expression of NF-κB in A549 cells. As shown in Fig. 8A, upon TNF-α stimulation, overexpression of phosphorylated NF-κB p65 was detected, and the overexpression was significantly inhibited by *cis*- and *trans*-suffruticosol D. In *trans*-suffruticosol D treated cells, the expression of phosphorylated NF-κB p65 was almost completely blocked, and in *cis*-suffruticosol D treated cells, the expression of phosphorylated NF-κB p65 was blocked as effectively as the blockage caused by the Bay11-7082 inhibitor control.

Next, we used HCS analysis to test whether *cis*- or *trans*-suffruticosol D could block NF-κB nuclear translocation in A549 cells. As shown in Fig. 8B, NF-κB fluorescent staining remained in the cytoplasmic area and no fluorescence was detected in the nuclear area in

non-treated cells, however, in cells treated with TNF-α the NF-κB fluorescent staining was detected in the nuclear area, indicating that NF-κB was translocated from the cytoplasm to the nucleus. In A549 cells treated with *cis*- or *trans*-suffruticosol D, NF-κB fluorescent staining remained in the cytoplasm, suggesting that NF-κB translocation to the nucleus was blocked. Treatment with *trans*-suffruticosol D at all the tested concentrations, caused a significant inhibition of NF-κB activation (***$P<0.001$) (Fig. 8C). In contrast, treatment with *cis*-suffruticosol D only caused a significant inhibition of NF-κB at 100 μM **(*****$P<0.001$).

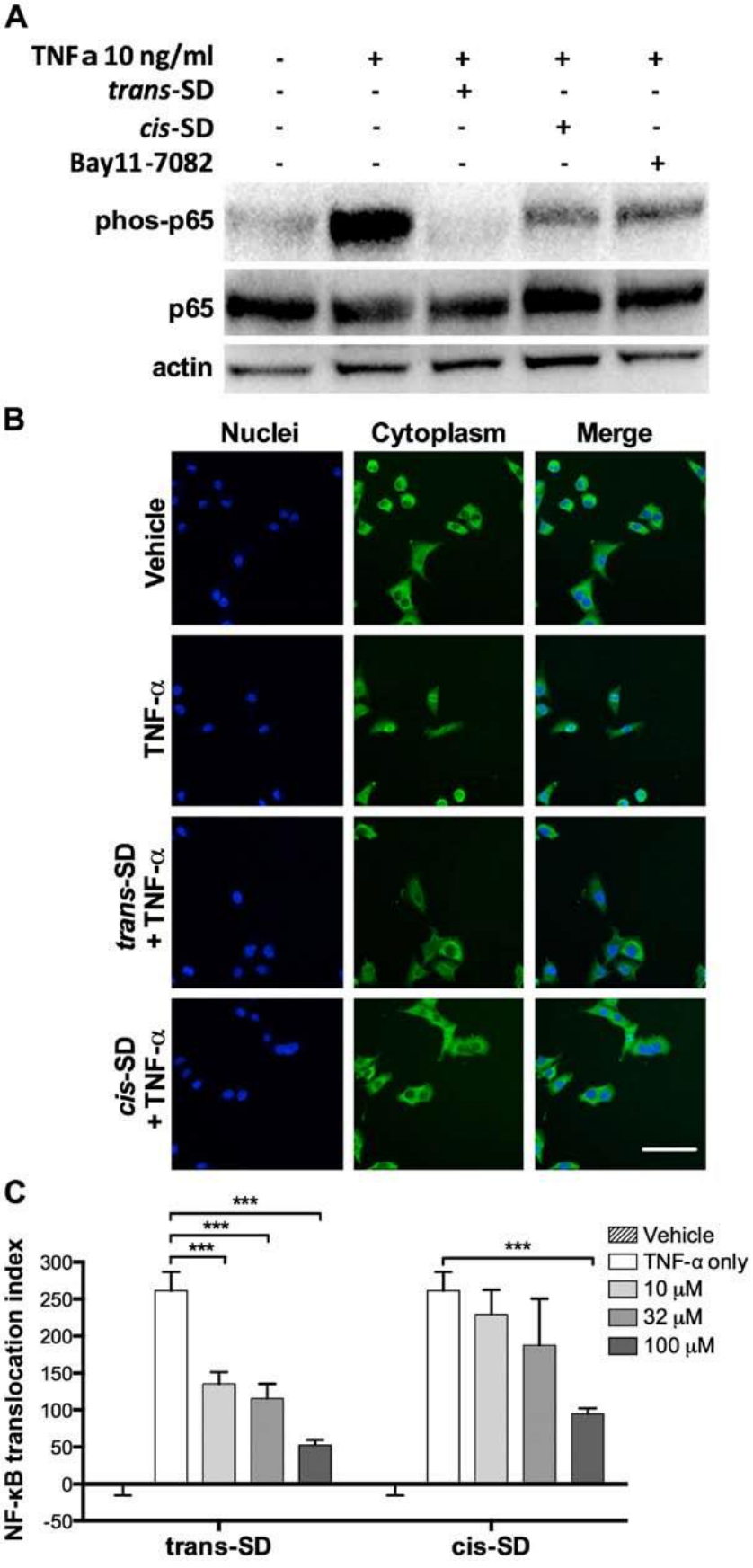
A
TNFa 10 ng/ml - + + + +
trans-SD - - + - -
cis-SD - - - + -
Bay11-7082 - - - - +
phos-p65
p65
actin
B
Nuclei
Cytoplasm
Merge
Vehicle
TNF-α
trans-SD + TNF-α
cis-SD + TNF-α
C
NF-κB translocation index
300
250
200
150
100
50
0
-50
***
***
***
***
Vehicle
TNF-α only
10 μM
32 μM
100 μM
trans-SD
cis-SD

Figure 8. Inhibition of NF-κB translocation by *cis*- and *trans*-suffruticosol D in A549 cells. A549 cells were treated with various concentrations of *cis*- or *trans*-suffruticosol D for 4 h and then stimulated with 25 ng/ml TNF-α for 30 min. Cells treated with TNF-α alone or the vehicle only served as controls. The NF-κB translocation index was measured by the fluorescent intensity difference between the nucleus and cytoplasm. (A) Western blot analysis of the expression of phosphorylated-NF-κB p65 and total NF-κB p65. (B) Fluorescent cell images by the HCS reader. Scale bar, 100 μm. (C) Evaluation of the NF-κB translocation index in A549 cells. The error bars indicate the standard deviation from three experiments. *P≤0.05; **P≤0.01; ***P≤0.001.

4. Discussion

Oligostilbenes have been widely considered to be valuable resources of anti-tumor agents. Previously, two novel oligostilbenes, *cis*- and *trans*-suffruticosol D, were extracted from the seeds of *P. suffruticosa*, but their anti-tumor activities were not determined. In this study, we found that both of these oligostilbenes exhibited remarkable anti-proliferation activities against several types of cancer cell lines, and their cytotoxicity effects and related mechanisms were investigated.

trans-suffruticosol D exhibited lower IC_{50} values (9.93 - 20.8 μM) than *cis*-suffruticosol D (13.42 - 46.79 μM) in all of the cancer cell lines that were tested, indicating that *trans*-suffruticosol D is more cytotoxic than its *cis* isomer. Consistent with this conclusion, *trans*-suffruticosol D had stronger effects than *cis*-suffruticosol D on three cytotoxicity parameters, changes in nuclear size, cell membrane permeability and mitochondrial

transmembrane potential. *trans*-suffruticosol D also showed higher inhibition activity of NF-κB activation than *cis*-suffruticosol D. These observations are consistent with a previous report, which showed that *trans*-resveratrol had stronger cytotoxicity than its *cis*-isomer (Pettit et al., 2002). In addition, both chemicals showed selective cytotoxicity against cancer cell lines versus a normal cell line.

Cancer cells usually develop the ability to escape apoptosis, or programmed cell death, which is a homeostatic mechanism to maintain cell populations in the body (Kasibhatla & Tseng, 2003). Hence, targeting apoptotic induction has become an important strategy of anti-cancer therapies. It is commonly known that there are two apoptotic pathways, the extrinsic, or the death receptor pathway, and the intrinsic, or the mitochondrial pathway. Previous studies have shown that mitochondria play a critical role in apoptosis, especially in the intrinsic apoptosis pathways (S.-C. Cheah et al., 2011; Ly, Grubb, & Lawen, 2003; TEDESCHI, 1980). Mitochondria are the main source of ROS inside the cell, and increases in ROS production can damage the mitochondrial membrane and subsequently lead to the release of pro-apoptotic proteins and cytochrome c, thus activating the apoptotic pathway (Kannan & Jain, 2000; Ozben, 2007; Sosa, 2013). In this study, we found that *cis*- and *trans*-suffruticosol D induced apoptosis in A549 lung cancer cells after 24 h treatment in a concentration-dependent manner. Both oligostilbenes significantly decreased the mitochondrial membrane potential in lung cancer cells, suggesting they might induce the mitochondrial apoptosis pathway. Since both chemicals significantly increased cellular ROS levels in lung cancer cells and their cytotoxicity was associated with ROS levels as shown by the NAC attenuation assay, it can be speculated

that the excessive ROS induced by *cis*- and *trans*-suffruticosol D act as an apoptosis mediator by damaging the mitochondrial membrane, causing the release of the mitochodria's contents, which eventually leads to apoptosis. In addition, *cis*- and *trans*-suffruticosol D affected the expression of several key regulators involved in apoptosis; XIAP, survivin, Hsp60 and Hsp70 were down regulated, while BID, DR6 and p27 were up regulated.

XIAP and survivin are known apoptosis inhibitors (Suzuki, Y., *et. al.,* 2001, Pavlidou, A., *et, al.,* 2014) that prevent apoptosis by inhibiting caspases-3, -7, and -9 (Schimmer, *et al*., 2006, Ryan, B. M., *et. al.,* 2009). Down regulation of XIAP or survivin has been demonstrated to inhibit the progression of cancer and increase the sensitivity of cancer cells to chemo reagents (Hu, Y., et. al.,2003, He, X., et al.,2012, Oost, T. K., et. al., 2004, Mita, A. C., et. al., 2008). Heat shock proteins Hsp60 and Hsp70 are chaperones that play essential roles in tumor cell survival and proliferation due to their ability to block both the intrinsic and extrinsic apoptosis pathways (Cappello, F., et. al., 2008, Murphy, M. E. 2013). BID is a pro-apoptotic member of the Bcl-2 protein family, and is a mediator of mitochondrial damage induced by caspase-8 (Luo et al., 1998). p27, the cycline dependent kinase inhibitor, controls the cell cycle progression at G1 by preventing the activation of cyclin E-Cdk2 or cyclin D1-Cdk4 complexes (Yamamoto, H., et. al., 1999, Nickeleit, I., et. al., 2007). DR6, also known as TNFRSF21, is a member of the death receptor family, which induces apoptosis in mammalian cells and its apoptotic function is inhibited by survivin (Kasof et al., 2001). Down regulation of XIAP, survivin, Hsp60 and

Hsp70, as well as up-regulation of BID, DR6 and p27 by *cis*- and *trans*-suffruticosol D at least partially contribute to the apoptotic effect of *cis*- and *trans*-suffruticosol D.

Tumor cells have the ability to migrate to surrounding tissues and organs through reorganization of the actin cytoskeleton (Yamazaki et al., 2005; Olson & Sahai, 2009). Most of the fatality from tumors occurs when cells move from the initial organs where they originated (Wells et al., 2013). Therefore, control of cancer cell motility and migration is an essential issue in cancer treatment and represents a new opportunity for a potential tumor therapy (Levin, 2005). *cis*- and *trans*-suffruticosol D significantly inhibited the mobility of lung cancer cells after treatment for 18 h at all the concentrations that were tested. Therefore, both chemicals exhibit therapeutic potential as an inhibitor of cancer cell mobility.

The NF-κB pathway is known to control cell growth and survival, and the transcription factor NF-κB has been found to be permanently activated in various tumors (S.-C. Cheah et al., 2011; Monika et al., 2014). Activation of NF-κB in cancer cells is often associated with drug resistance as both radio- and chemo- therapies induce constitutive activation of the NF-κB pathway (Jin et al., 2008). Therefore a compound's ability to block the NF-κB pathway is important for the efficacy of cancer therapy (Monika et al., 2014; Nakanishi & Toi, 2005). In this study, we evaluated *cis*- and *trans*-suffruticosol D for their abilities to inhibit TNF-α induced NF-κB activation in lung cancer cells. After 4 h treatment both chemicals significantly blocked NF-κB p65 phosphorylation as well as NF-κB p65 translocation from the nucleus to the cytoplasm, suggesting they might act as an inhibitor of the NF-κB pathway. Since NF-κB affects the transcription of a number of anti-

apoptotic proteins, including cellular inhibitor of apoptosis proteins (cIAP)s, XIAP, bcl-2, bcl-XL, FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) etc., blocking NF-κB nuclear translocation decreases the expression of anti-apoptotic proteins and subsequently promotes apoptosis. In addition, several studies have shown that an increase of ROS can block the NF-κB pathway by the inhibition of cytokines, such as TNF and IL-1 (Reuter et al., 2010). Because *cis*- and *trans*-suffruticosol D increased ROS generation in lung cancer cells, the block in the NF-κB pathway may be associated with the inhibition of the inducer cytokines by excessive ROS.

In conclusion, this study provides evidence that *cis*- and *trans*-suffruticosol D have promising antitumor activities. Both compounds selectively inhibited the growth of various cancer cells, induced apoptosis in A549 lung cancer cells, as well as inhibited A549 cell movement. The induction of apoptosis may be associated with ROS generation and inhibition of the NF-κB pathway. Collectively, our results suggest a potential mechanism for the cytotoxicity of *cis*- and *trans*-suffruticosol D. As shown in Fig. 9, in A549 lung cancer cells, *cis*- and *trans*-suffruticosol D trigger oxidative stress, which in turn leads to mitochondrial damage, blocks NF-κB activation and ultimately triggers apoptosis. Our findings suggest that both *cis*- and *trans*-suffruticosol D have promising chemotherapeutic potential for treating cancer.

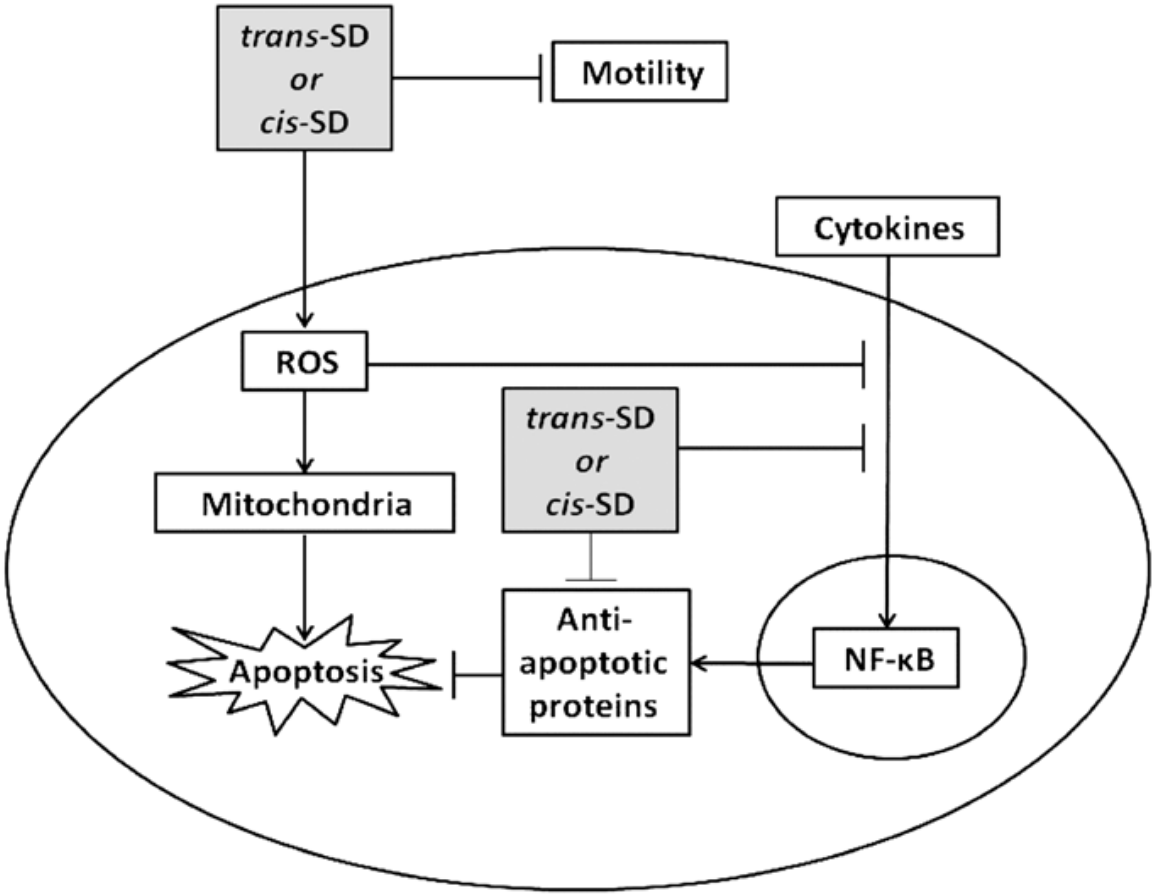

Figure 9. Proposed cytotoxicity mechanism of *cis*- and *trans*-suffruticosol D.

5. Acknowledgements

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OVERALL CONCLUSION

Cancer treatments are often limited due to the severe side effect associated with this treatment. Natural products from TCM have undergone many years of clinical trials and screening. Recently, compounds derived from TCM have demonstrated various features that meet the need for multi-target drugs. Therefore, they have started to be recognized by the western scientific community.

The new scientific analyses and screening methods of herbal products have significantly contributed to the development of drug discovery from Chinese medicinal plants. Although numerous studies have reported a significant therapeutic effect of products isolated from medicinal plants, several studies have revealed that serious toxic effects can be caused by these natural products. As the drug discovery process and development is continued, potential drugs from TCM research will contribute to the treatment of many diseases including cancer.

The present study describes the *in vitro* anticancer activity of extracts and pure compounds isolated from Chinese medicinal plants and evaluate their toxicity on other cell types (HUVEC and HPL1A). 104 plants that historically have been used for thousands of years in Yao Ethnomedicine were screened and 64 extracts isolated from the 16 most promising plants were evaluated for chemotherapeutic potential and toxicity in different cancer and normal cell lines.

Results from this studies showed that the highest anticancer potential was observed from extracts isolated from *Melaleuca leucadendra, Garcinia paucinervis, Wedelia calendulacea, Stephania longa, Microsorium fortune* and *Bidens biternata.*
This study for the first time identifies the cytotoxic effect of extracts prepared from *Melaleuca leucadendra, Stephania longa, Microsorium fortune* and *Bidens biternata*. The cancer cell lines were employed in these studies differed in their sensitivities to the extracts. For instance, *Elephantopus scaber* and *Melaleuca leucadendra* showed antiproliferation activities against breast cancer cells (BT20 and MCF7) while *Cyperus rotundus Achras zapota,* and *Stephania longa* were active only against the U2OS cell line. However, 16 extracts inhibited BT20 but not MCF7 cells.

Apoptosis induction is essential target in cancer therapy. The top 10 extracts that have an IC_{50} lower than 25 µg/mL were able to induce apoptosis in U2OS bone cancer cells. Extracts from *Microsorium fortune* and *Melaleuca leucadendra* are known to have antioxidant activity (Li et al., 2011). However in this study, we provide evidence for for their ability to also induce apoptosis.

The results indicated that five extracts, including the water extract of *Bidens biternata*, the petroleum ether extract of *Wedelia calendulacea,* the petroleum ether and ethyl acetate extracts of *Stephania longa,* and the petroleum ether extracts of *Achras zapota* should be considered for further investigation due to their selective properties against cancer versus normal cell lines. Further studies are necessary to evaluate their mechanisms of action, and the isolation of active antitumor compounds will essential to validate their potential as chemotherapeutic agents.

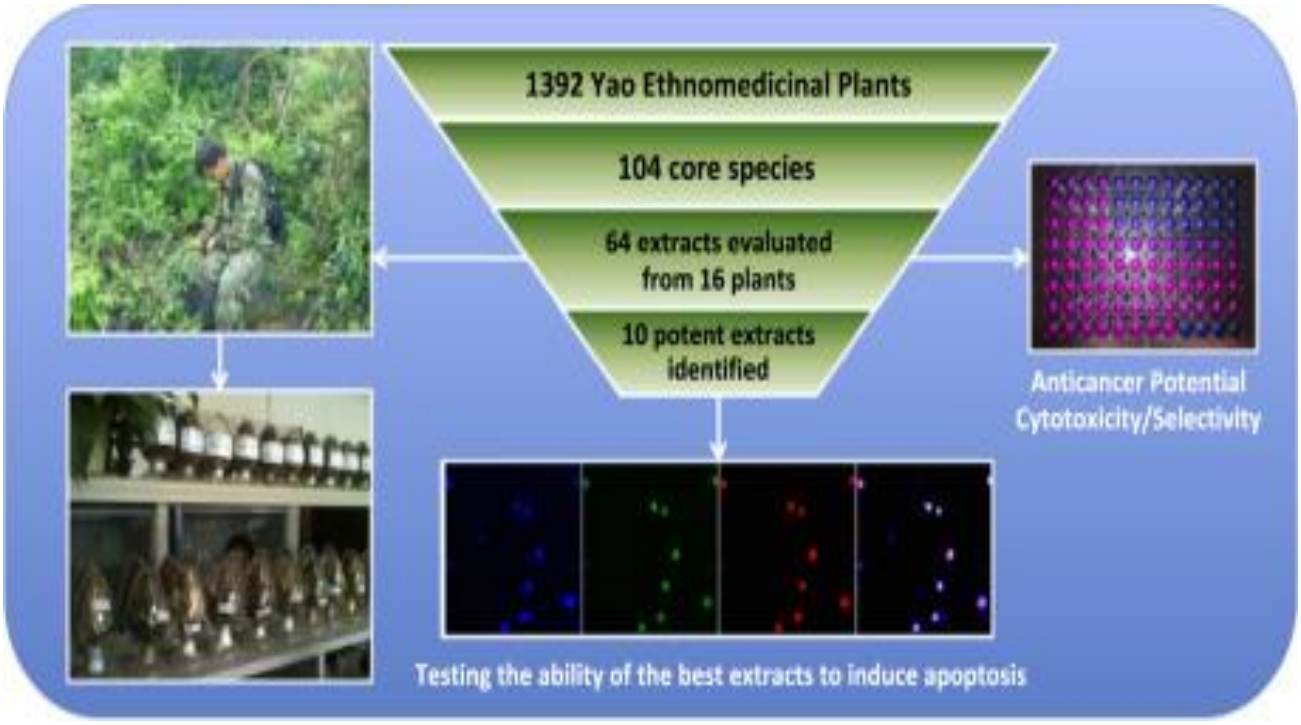

Figure 10. Evaluation of extracts isolated from 16 plants used in Yao ethnomedicine for their anticancer activities.

A more in depth study was performed on pure compounds isolated from the Chinese medicinal plant *P. suffruticos*. In particular, several novel oligostilbene compounds have been isolated from plants known to treat cancer in TCM, but their activities have never been determined. Many compounds isolated from Chinese medicinal plant *Paeonia suffruticosa* have exhibited potential anticancer activity with low toxicity. The oligostilbenes are promising compounds for use as an antitumor therapy with low selectivity. The two novel oligostilbenes, *cis*- and *trans*-suffruticosol D from seeds of *P. suffruticos* were investigated for their antitumor activities, their cytotoxicity effects and related mechanisms.

Both tested compounds (*cis*- and *trans*-suffruticosol D) were able to induce apoptosis through the mitochondrial pathway (intrinsic pathway) by increasing the ROS level causing damage to the mitochondrial membrane and the release of the pro-apoptotic protein which eventually leads to apoptosis. Both *cis*- and *trans*-suffruticosol D downregulate essential proteins that contributed to the apoptotic effect of *cis*- and *trans*-suffruticosol D such as XIAP, survivin, Heat shock proteins Hsp60 and Hsp70 and upregulate pro-apoptotic BID, death receptor DR6 and the cyclin-dependent kinase inhibitor p27.

Most of the mortality from cancer due to the cell motility and migration from the initial organs to surrounding tissues and organs through actin cytoskeleton reorganization (Yamazaki et al., 2005; Olson et al., 2009; Wells et al., 2013). Hence, metastatic control is essential to target for cancer treatment. The NF-κB pathway is known to control cell growth and the drug resistance in cancer treatment associated with continued activation

of NF-κB in cancer cells (Monika et al., 2014). Both chemicals *cis*- and *trans*-suffruticosol D showed to promising therapeutic agent as inhibitor cancer cell motility and NF-kB Inhibitors. Our finding from this result suggested that *trans*-suffruticosol D is more cytotoxic than its *cis*-isomer. Consistent with this conclusion in previous studies *trans*- resveratrol showed stronger cytotoxicity than its *cis* - isomer had stronger cytotoxicity than its *cis* - isomer.

In conclusion, these results indicate the potential use of traditional Chinese medicinal herbs as antitumor agents. Therefore, plants still remain an essential source of drugs for cancer treatment and can lead to development of novel anticancer agents. However, further studies are required to evaluate their mechanisms of action and the isolation of active antitumor compounds from promising extracts is necessary.

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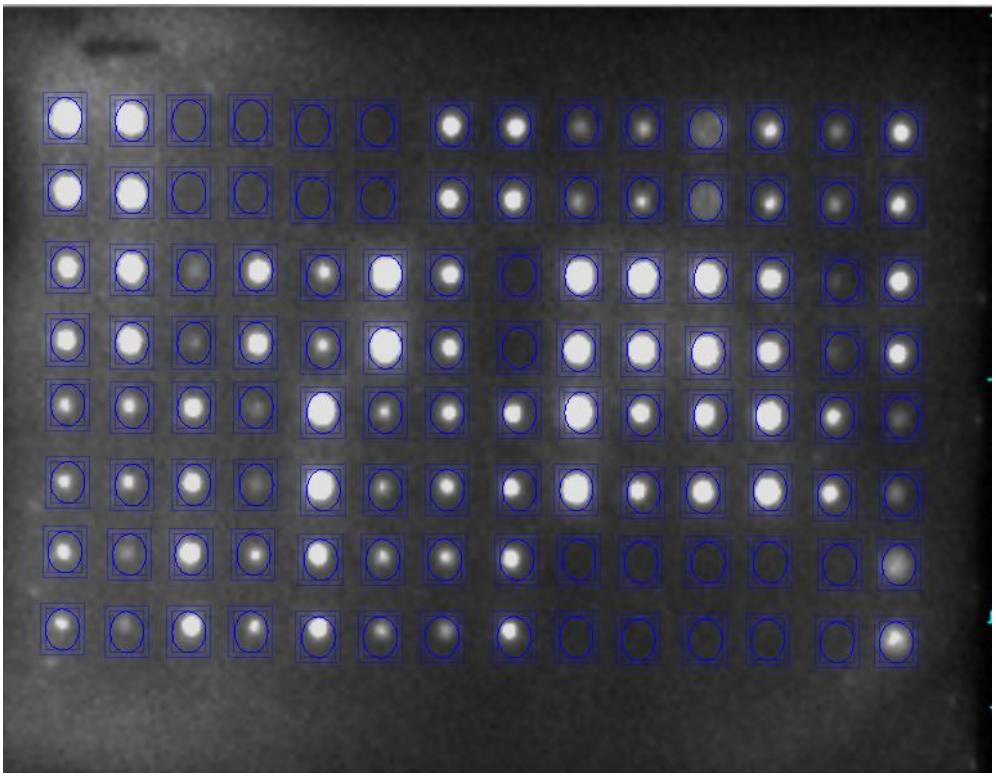
APPENDICES

APPENDIX A: EFFECT OF *CIS*- AND *TRANS*-SUFFRUTICOSOL D ON KEY REGULATORY PROTEINS

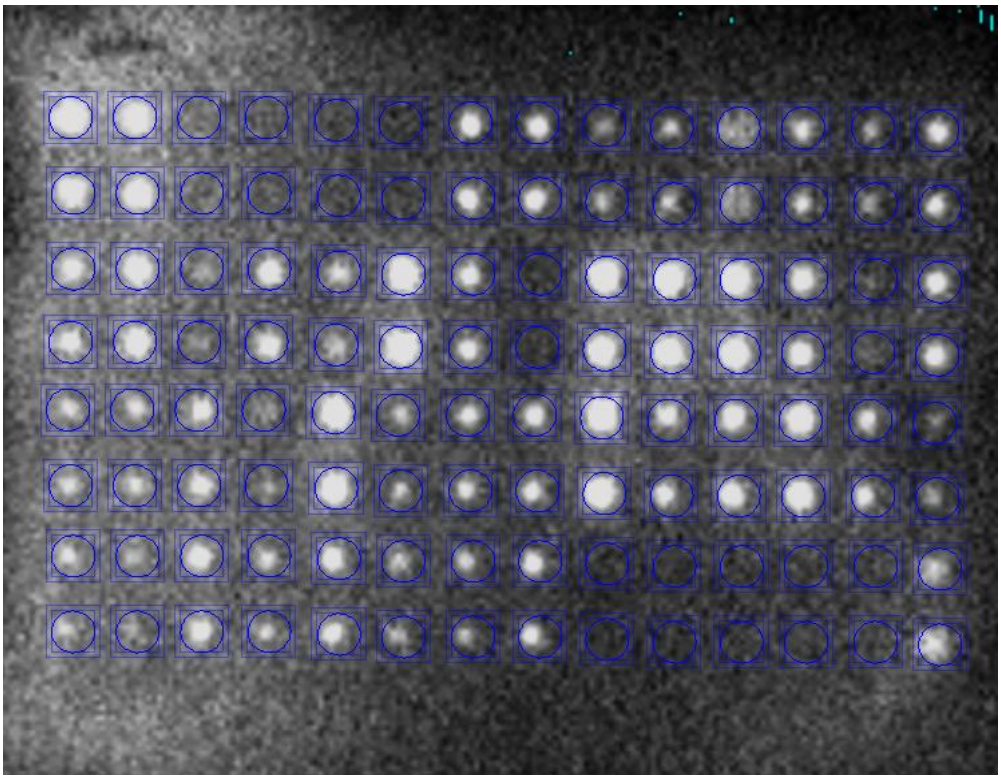
A

Each antibody is spotted in duplicate vertically		A	B	C	D	E	F	G	H	I	J	K	L	M	N
	1	POS	POS	NEG	NEG	BLANK	BLANK	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase-3	Caspase-8
	2														
	3	CD40 (TNFRSF5)	CD40 Ligand (TNFSF5)	cIAP-2	CytoC	DR6 (TNFRSF21)	Fas (Apo-1)	Fas Ligand (TNFSF6)	BLANK	HSP27	HSP60	HSP70	HTRA2	IGF-1	IGF-2
	4														
	5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1 R	livin	p21	p27	p53	SMAC	Survivin	TNF RI (TNFRSF1A)
	6														
	7	TNF RII (TNFRSF1B)	TNF alpha	TNF beta	TRAIL R1 (TNFRSF10A)	TRAIL R2 (TNFRSF10B)	TRAIL R3 (TNFRSF10C)	TRAIL R4 (TNFRSF10D)	XIAP	BLANK	BLANK	NEG	NEG	NEG	POS
	8														

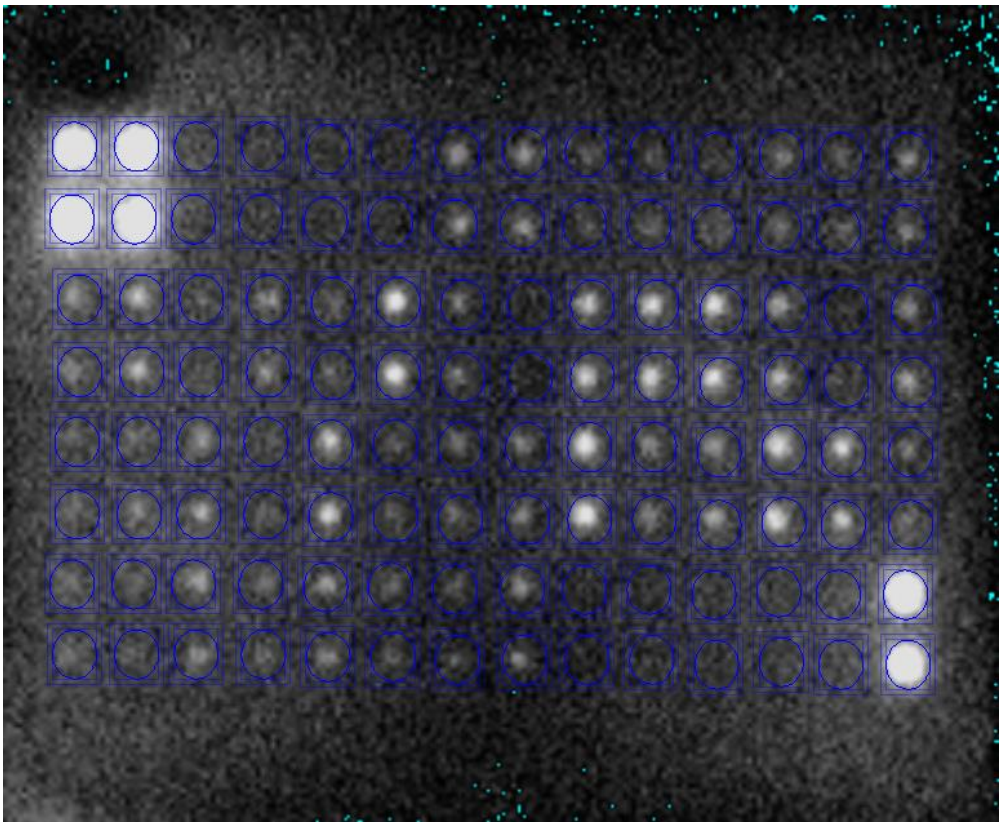
B



C



D



Supplement Figure 1. Apoptotic protein profiling in A549 cell line treated with *cis*- and *trans*-suffruticosol D: Cell lysates from A549 cell treated with *cis*- and *trans*-suffruticosol D and incubated overnight with RayBio Human Apoptosis Antibodies Array slide. (A) Array map. (B) Array incubated with cell lysates from A549 cell treated with *trans*-suffruticosol D. (C) Array incubated with cell lysates from A549 cell treated with *cis*-suffruticosol D. (D) Array incubated with control cell lysate from untreated A549 cell.