Green Drugs: Anticancer Properties of *Clerodendrum viscosum* and Curcumin Conjugates

By

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A dissertation submitted to the graduate Faculty in Biochemistry in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York.

2013

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Abstract

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This thesis aims to find potential anti-cancer drug from natural sources like plants. (i) The objectives are to find and characterize the anticancer components from the plant *Clerodendrum viscosum*. (ii) the synthesis of curcumin derivatives with increased solubility and amplified bioactivity.

***Clerodendrum viscosum* (Cv) is a traditional medicine plant in India for a long period of time employed to promote wound healing, and recently it has been found have some positive effects on cervical cancer. However, there were no direct**

molecular and biochemical evidences for that. In this thesis, we made aqueous extracts (Cv-ap) from the Cv by ammonium sulfate precipitation. It was found that this extract had bioactivity against Hela cells – a human cervical cancer cell line. Caspase-3 immuno-staining proved that the Cv-ap can induce Hela cells apoptosis. It is also interesting that the Cv-ap can inhibit the movement of the cells at lower concentration without inducing cells apoptosis, which means it may stop the invasion of the cancer cells. MTT assays showed that at lower concentration the Cv-ap may not only inhibit the movement of the cells but also inhibit proliferation of the Hela cells.

For the characterization of the Cv-ap, it was found that it had glycoproteins confirmed by SDS-PAGE staining with the Pro-Q emerald 300, and by binding to the concavalin A column. For further purification, the bio-assay guided method was used to purify the bioactivity components. After Cv-ap was passed through the column, the fraction with the most activity fraction was selected and followed by heating, and finally by precipitation with 66.7% ethanol. In the end we got the

bioactivity component EPHP3 fraction which has a 30 fold increasing activity when compared to the Cv-ap.

My second project aimed to overcome the difficulties associated with the low water/plasma solubility of the potent anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-Alzheimer's active curcumin. We have successfully synthesized a curcumin derivate BSA-curcumin via a one-step successfully synthesized. BSA-curcumin had the high solubility in water and good cytotoxicity to the Hela cells.

In conclusion, my research has characterized and produced novel plant derived components that have anti-cancer activity and may eventually be used in clinical therapies.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisors Professor Krishnaswami S.Raja and Jimmie E Fata, Department of Biology and Chemistry at College of Staten Island, the graduate center of The City University of New York, New York, for his valuable guidance, stimulating inspiration and all the supports throughout my research career.

My grateful thanks are due to my research committee members; Professor Edward J. Kennelly, (Plant sciences, Lehman College (CUNY)), Professor David Bolton, Institute for Basic Research (IBR), Professor Probal Banerjee, (Department of Chemistry, College of Staten Island (CUNY)) for being in my committee and for providing me valuable suggestions throughout my research career.

My special thanks are due to Mr. Edmund Jenkins, Jr, Ms. Shawon Debnath, Dr. Wei Shi, for their endless help and inspiration throughout my research career. I gratefully acknowledge my other colleagues Mr. Jose Saltos, Ms. Xin Yan, Ms. Kirmia for their help and encouragement and also for providing me a friendly atmosphere in the department.

I also acknowledge the help and support of my previous and current undergrads, Mr. Thomas, Mr. Mahamad, Ms. Serena, Ms. Helen

I sincerely acknowledge Professor David Bolton for his help with various analytical techniques. Ms. Shawon Debnath is acknowledged for her help in tubulin experiments towards my thesis.

The source of inspiration and inclination towards my career has been my beloved parents Mr. Zonggeng Sun and Mrs. Chunxiu Jiang who were my strength throughout my career. I wish to thank them for their valuable guidance, support and encouragement.

I especially thank my beautiful wife Mrs. Zhengyi Liang, the love of my life, whose unconditional love, support and

encouragements throughout my career was my life-line to reach my goal. My daughter, Jiawei, the little wonder, helped me to be more focused towards my destiny. I wish to express my sincere thanks to Dr. Rema Balambika advice and support was a valuable gift throughout my career.

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Chapter III

Chapter I

Background Information and Rationale for the Research

Part I

Green Drug Development Using the Ayurvedic Collaboration Approach: *Clerodendrum viscosum*

1. Background Information

A green drug is one which can be directly used after isolation from a renewable resource (plant, plants/other) after purification, one which can be produced with efficient chemical modification of the same or a bio-similar compound. It is estimated that 61% of 877 small molecule chemical entities approved as drugs during the period 1981-2000 can be traced to natural products; however only 5-15% of the ~25000 existing species of higher plants have been mined by western science for medicines [1]. The ancient medical systems such as Ayurveda, Siddha and Unani (ASU) have a 3000 year history and employ a vast number of plants products (most of which have not been explored by western science) [2-4].The fact that a herb is already being used for the treatment of humans by the ASU medical systems implies that it is highly likely that the bioactive components present in the natural product do not adversely interact with the billions of chemicals present the human body (complex biochemical system) (Figure 1).

Some green drug candidates have the Xenohormetic advantage: Xenohormetic green drug candidate sources are based on plants which are widely used by a major fraction of humanity, for example resveratrol or curcumin have co-evolved synergistically with human biochemistry; thousands of years of evolutionary refinement is inbuilt in the green drug development model [5].

In the current thesis the Xenohormetic drug lead we propose to study is *Clerodendrum viscosum* (Cv) and curcumin the primary active ingredient in the Turmeric (the dried powdered rhizomes of Curcuma longa of the ginger family.

The set of green drug candidates based on the ASU medical systems benefit from the Collective Wisdom of the Species Factor (CWS): Ayurvedic green drug leads have had a history ~3000 years of use in humans resulting in a much higher probability of lack of toxicity. The possibility of positive bio-efficacy of an ASU green drug candidate in comparison to other drug development approaches is much higher because it is based on practical clinical use in the human complex biochemical system.

The current “gold standard” strategy in drug development involves the rational design of drug leads/focused smart libraries (keys) that bind/block

specific protein/ other biomolecule binding pockets (lock) based on X-ray crystal structures of therapeutically relevant proteins such as a Kinases. The LoKey approach is based on a strong biochemical foundation which typically involves identifying protein targets, solving the X ray crystal structure of the protein, locating a binding pocket in the protein which if blocked can inhibit it and then designing a smart library of key molecules which fit and block the protein along with the development of a rigorous assay which assesses binding.

At the stage at which a “Key” drug lead (which has been screened and selected *in vitro*) enters the *in vivo* evaluation phase in the mouse complex biochemical system (CBS#1) and further the human complex biochemical system (CBS#2), the drug lead attrition rates are very high. An explanation for this is afforded by Figure 2, the possibility of the drug lead “Key” binding one or more of the millions of other proteins and biomolecules (which span trillions of three dimensional shapes/binding pockets, with numerous functional groups) present in the CBS#1 other than the target protein is very high, this can result in side effects and toxicity. An example serves to illustrate this point: several acrylamide based Kinase inhibitors have the potential to form adducts with off target thiols such as glutathione. Such interactions on many occasions result in unwanted toxicity. These

possibilities are very real; certain cancer drugs which function by targeting tyrosine kinsases cause cardio toxicity, cardiomyocyte dysfunction and/or death [6]. It has been established that the interruption of certain signaling pathways causes this toxic side effect [6].

There is still another layer of complexity which exists: the mouse model complex biochemical system (CBS#1) is different from the human complex biochemical system (CBS#2); because one is dealing with two widely different species, this affords an explanation regarding why many drugs candidates that work well in the mouse model fail to perform in clinical trials. The recent failure of BMS-986094 (hepatitis C drug candidate) cost Bristol-Meyers Squibb $2.5 billion (C&EN Sept 2012). The ten fold decrease in inflation adjusted R&D productivity over the last sixty years could be partly ascribed to the inherant limitation of the Lokey approach.

The current procedure followed by academia and the industry in identifying plant leads and natural products for drugs against disease also has its limtataions, it is an ill-defined process which can take several years and involve screening thousands of samples [7].

Modern science has screened only 5%- 15% of higher plants for drug leads. To screen the rest of the 95% in a random fashion without any

knowledge of the correlation between a particular plant and the pathological conditions it can be used to treat is a Herculean task and represents a very inefficient strategy. Systematic collaborations between experts in alternative medicine and biomedical researchers can however overcome this severe handicap and significantly load the dice in favor of successful green drug discovery. The current study hinges on the hypothesis that an expert in Ayurveda knows the exact correlation between a particular plant and the specific disease it can be employed to treat (Figure 3). A collaborative lead from such an Ayurvedic expert serves as an invaluable screening process and can save decades of random searching and millions of dollars. To use such a lead without a proper collaborative arrangement is nothing less than bio-piracy. In the new drug discovery approach we present here the single herb lead screened/chosen by such an expert for the treatment of a specific pathological condition is investigated using standard drug discovery strategies by evaluating bioactivity of standardized extracts and purified components.

The general drug discovery and development approach proposed above which is referred to as the Ayurvedic collaborative approach (ACA) was evaluated in this benchmark study involving *Clerodendrum viscosum* (Cv) a weed commonly growing in the Indian subcontinent. Nirmalananda

(Ayurvedic expert) has discovered that the roots of Cv has anti-cervical cancer activity and has employed Cv to treat cervical cancer in humans (in India) for several years.

Cervical cancer is the second most common cancer worldwide, with an estimated 275,000 deaths annually [8]. It is estimated that 70% of cervical cancer is caused by the Human papilloma virus (HPV). HPV is one of the most common causes of sexually transmitted disease in both men and women worldwide and is thought to be the most common sexually transmitted viral disease in the United States [31]. Papillomaviruses are ubiquitous and have been detected in a wide variety of animals as well as in humans and are specific for their respective hosts. More than 200 types of HPV have been recognized on the basis of DNA sequence data showing genomic differences. An additional 120 isolates are partially characterized potential new genotypes. Eighty-five HPV genotypes are well characterized [32]. HPVs are grouped to high-risk and low-risk HPV types based on their relationship with the cervical cancer. Low-risk HPV types include types 6, 11, 42, 43, and 44. High-risk HPV types include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70. Included in the high-risk group are some HPV types that are less frequently found in cancers but are often found in squamous intraepithelial lesions [31].

Most HPV infections are benign. HPV was first recognized as the cause of cutaneous warts on the hands and feet (Warts are areas of hypertrophied skin filled with keratin). The link between genital HPV infections and cervical cancer was first demonstrated in the early 1980s by Harold zur Hausen, a German virologist. Since then, the link between HPV and cervical squamous cell carcinoma has become well established. The magnitude of the association between HPV and cervical squamous cell carcinoma is higher than that for the association between smoking and lung cancer [33]. Scientists have identified about 30 HPV types that are spread through sexual contact and infect primarily the cervix, vagina, vulva, penis, and anus. Of these, four are most often found within the malignant cells of cervical cancers, with type 16 accounting for about half of the cases in the United States and Europe and types 18, 31, and 45 accounting for an additional 25 to 30% of cases [34].

Most of cervical cancer can be detected by the Pap test. HPV vaccines have been licensed in countries like the USA, Canada, Australia and the European Union. In the United States, the FDA has approved two vaccines which are the Human Papillomavirus Quadrivalent (Types 6, 11, 16, 18)

Vaccine and the Human Papillomavirus Bivalent (Types 16, 18) Vaccine [9]. Despite the fact that cervical cancer can dramatically be reduced by cervical screenings and vaccinations, in 2009 11,270 women in the United States were diagnosed and approximately 4,070 women died of this disease [10]. The incidence of invasive cervical cancer has currently remained stable in industrialized nations. In developing countries, cervical cancer is the most common cancer and the leading cause of cancer related death among women. It is estimated that 200,000-300,000 women die from cervical cancer every year. Currently, chemotherapy and radiation are two of the major therapies for the treatment of cancer. However, the side-effects of chemotherapy and radiation are unavoidable, due to the indiscriminate killing of all fast-growing cells.

Genus *Clerodendrum* (common name *glorybower*) is a large group of evergreen trees with about 560 taxa. Most of them grow wild in tropical and warm areas of the world. Most of these species grows in tropical Africa, southern Asia, and a few in tropical parts of America such as *clerodendrum paniculatum L* [11]. The term *Clerodendrum* was named by Linnaeus in Species *Plantarum* in 1753 [12]. The name is derived from two Greek words, *keros*, meaning “chance” or “fate” and *Dendron* “a tree” [13].

Adanson changed the Latin name “*Clerodendrum*” to its Greek name “*Clerodendron*” after a decade. The reason behind the name may be related to the belief that some species have healing properties while others are poisonous [14].

Clerodendrum viscosum (Cv), also refered to as *Clerodendrum infortunatum*, is a perennial shrub belonging to the family *lamiaceae*, subfamily *ajugoideae* and sometimes classified under family *verbenaceae* (figure 4). *Clerodendrum* is a common Indian herb that has been used for medical applications for a long period of time; the tender leaf paste has been employed to promote wound healing. The major chemical components from this genus are steroids, flavonoids and phenolic compounds. It has been recently reported that the methanol extract from the leaves of *Clerodendrum onfortunatum* has potential anticancer properties [15]. Ethanolic extract of *Clerodendrum infortunatum L*. has been to exhibit have antioxidant properties [16]. In addition, it has also been reported that saponin isolation from the CV leaves produce analgesia in mice and potentiated the analgesic action of pentazocine and aspirin [17]. However, there are other CV bioactive components that have not been identified.

2. RATIONALE FOR RESEARCH

The primary objective of this research is to produce and employ *clerodendrum viscosum* formulations and molecules present therein as medicines for the prevention or cure of cervical cancer in particular. The first goal of our research is to evaluate whether Cv has a components which can inhibit the growth of the cancer or induce apoptosis of the cancer cells. The promising anticancer components in Cv were evaluated against healthy human cell lines to establish whether the bioactive components exhibit selective cytotoxity only towards cancerous cells. There are three kinds of cell death, apoptosis, necrosis and autophagy. Comparing necrosis, apoptosis is important since they occur as a physiological process to any mild cell injury and simply when cell function is finished or disturbed. They also occur by a predictable and coordinated pathway, so that cellular deletion does not involve inflammation [18][19]. In contrast, necrosis is difficult to prevent and always develops an inflammatory response and leads to death of the surrounding cells [20]. Apoptotic cells develop typical morphological alterations. At early stages of apoptosis, cells have a smaller size, showing a dense cytoplasm with thinner organelles, which is called shrinkage [21]. During the intermediate stage of apoptosis,

the cell's membrane takes part in blebbing, which occurs when a cell is fragmented into apoptotic bodies. In the final stage of apoptosis cells break up and form apoptotic bodies [21]. Apoptosis also induces nuclear alterations, such as nuclear fragmentation and chromatin condensation [22].

Besides morphological alterations, there are other cellular and molecular markers associated to cellular apoptosis. Examples of these other makers are, compounds associated with cell cycle arrest, mitochondrial dysfunction and oxidative damage, alterations in caspase cascade, non-caspase proteases involvement, alterations in the Bcl-2 protein family and alterations in membrane ion channels [18]. In this study we focused on the caspase cascade alterations during the death induced by the Cv. Caspases are families of cysteine aspartate protease that act as the central executers of apoptosis. Caspases exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis [22]. According to their point of entrance into the apoptotic process, caspases can be classified as initiators or effectors. Initiator caspase, includes caspase – 8, -9, and -10 and effector caspase includes caspase -3, -6, and -7. The initiator caspase activates the downstream effector caspase, in a cascade of events that

triggers a controlled and programmed cell death [23]. The caspase-3 activity assay used in this study is highly specific for apoptosis. *Propidium iodide* stains are also used in this research for monitoring the death of cells. *Propidium iodide* (PI) is an intercalating agent and a fluorescent molecule with a molecular mass of 668.4 Da, that can be used to stain cells. When PI is bound to nucleic acids, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm. Excitation energy can be supplied with a xenon or mercury-arc lamp or with a 488 line of an argon-ion laser [24]. PI is membrane impermeable and generally excluded from viable cells, so it can be used to identify dead cells.

Not only can an anti-cancer drug induce apoptosis, it can also slow the proliferation of the cancer cells. Mitochondria has essential functions in aerobic cells and interferences in its normal behavior is crucial in determining a cell's fate [25] [26]. Dysfunction in these organelles imbalances the cell redox potential which induced damages in cell components. This damage can lead to the failure of pro-survival mechanisms leading to apoptosis [27]. Mitochondrial signaling cascades not only implicates the activation of programmed cell death, it also controls cellular proliferation. MTT (3-[4, 5- dimethylthiazolyl-2]-2, 5-

diphenyltetrazolium bromide) (yellow) is cleaved by mitochondrial dehydrogenases to formazan crystals (purple) in metabolically active cells. This method was used to detect viable cells or the metabolism in viable cells, so it can be used to measure the proliferation of cells [28].

Cancer cells divide and grow uncontrollably and much faster than normal cells. They can also invade nearby parts of body, by spreading and forming malignant tumors. A hallmark of most cancer cells is the loss of normal cell-to-cell interactions. In contrast to the well-ordered arrangement of cells in healthy tissues, cancer cells fail to make contact with neighboring cells and they grow in a seemingly haphazard fashion. This absence of systematic intercellular relationships, contributes to a cancer cell's ability to invade normal tissues and metastasize to distant parts of the body [29]. Migration or invasion of cancer cells is a factor, which reduces the survival rate in cancer patients [30]. Stopping or slowing the migration of cancer cells is a promising mechanism against cancer. In this research study we will use microscopy to observe the migration of the cancer cells to determine the drug's effects on the migration of cancer cells and compare them to the cells without the drug.

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Part II

Conjugation Strategies Towards Increasing the Water/Plasma Solubility and Bioactivity of Curcumin

1. Background Information

The synthesis of polymeric drugs and bioconjugates is a major growing field of interdisciplinary research with promising application in the development of more efficient routes for the delivery of chemotherapeutics [1-25]. Among the advantages of polymer drug conjugates are improved *in vivo* half-lives and improved targeting due to the Enhanced Permeability and Retention (EPR) effect [21-25]. The efficacy of targeted chemotherapeutics such as Antibody-Drug Conjugates (ADC) for cancer therapy hinges on the covalent attachment of a highly cytotoxic drug to targeting antibodies [1-10]. The typical approach is to produce conjugates of molecules which have been established to be too toxic to be employed as “stand alone” chemo-therapeutic drugs. This approach is fraught with danger, the possibility of the cytotoxic drug cleaving from the antibody in the complex biological milieu is real and consequent adverse side effects to healthy tissue can be catastrophic. An FDA-approved drug derived from this strategy failed in the clinic: Mylotarg® (a recombinant humanized monoclonal antibody covalently linked with the anti-tumor drug

Calicheamicin) was recently withdrawn from use by the FDA (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm216448.htm). Many Chemotherapeutic drugs are associated with side effects associated with high and indiscriminate cytotoxicity. The approach adopted in the current thesis hinges on a fundamentally safer strategy: we propose start with a non-toxic FDA GRAS (Generally Regarded as Safe) Nutraceutical (with promising anti-cancer properties) to produce a BSA-nutraceutical conjugates with amplified bioactivity and minimum side effects to healthy tissue [25]. The cleavage of a payload *in vivo* from the nutraceutical conjugates would not result in adverse side effects because it would result in the release of a harmless FDA approved molecule.

The nutraceutical we have selected for producing conjugates is curcumin [(1E, 6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl) hepta-1,6-diene 3,5-dione] (Scheme1) derived from *Curcuma longa* (Zingiberaceae – Ginger family). Turmeric is the yellow dried powdered rhizome derived from *Curcuma longa*, it has been used in the ancient Indian Ayurvedic system of medicine [26]. Both turmeric and curcumin are generally regarded as safe by the USFDA, over 2000 publications and 18 clinical trials support the tremendous potential of curcumin in treating cancer and several other pathological conditions (figure 5) [26-44]. Epidemiological studies have

correlated the daily intake of Turmeric with a significantly lower incidence of several cancers among the Southeast Asian population in comparison to the rate of occurrence in the United States [26, 29-31].

Cancer is a multistep event encompassing several biochemical and biological processes including increased cell proliferation, angiogenesis, metastasis and proteins involved in these processes, diverse mechanisms regulating the signaling pathways involved in cancer have clearly established that cancer requires a broad spectrum/ diverse treatment. Curcumin has broad spectrum bioactivity against cancer, it works on multiple targets, it activates p53 (cell cycle inhibitor) and inhibits expression of cyclin D1 (cell cycle promoter) and epidermal growth factor receptor (EGF-R) (induces mitosis), nuclear factor NF-kB, activator protein AP-1, STAT-3, specificity protein SP-1, inhibitors of apoptosis IAP and XIAP, Bcl-2, Bcl-xL, and also the angiogenesis-associated proteins, metalloproteinase MMP-9 and vascular endothelial growth factor VEGF [29-30]. Curcumin inhibits proliferation of almost all cancer cells while protecting healthy cells [29]. The exquisite selectivity of curcumin arises

from the fact that it interacts with thioredoxin reductase (TrxR), which is overexpressed in tumor cells. This interaction promotes alkylation of TrxR at its catalytic site, thereby converting it into an NADPH oxidase, which in turn results in increased production of reactive oxygen species in the tumor cells [32]; glutathione levels in tumor cells are generally lower than in normal cells. Curcumin-evokes further inhibition of glutathione and the ensuing superoxide formation causes apoptosis only in the tumor cells [33], The transcription factor NF-kB is constitutively activated in most cancer cells, NF-kB is strongly inhibited by curcumin which triggers apoptosis of these cells [34]. The cell selectivity arises from the fact that curcumin does not induce superoxide injury to the normal cells, which contain higher levels of glutathione [33].

Research investigators and clinicians have been confronted with the poor water/plasma solubility and hence poor bioabsorption and bioavailability of curcumin, this severely limits its application as a drug candidate [35-36]. The glucuronidation of curcumin *in vivo* is another possible reason for the observation of very low plasma level curcumin. [37] To circumvent this problem one can encapsulate curcumin in liposomes/nanoparticles [38-41, 42, 45], improve the aqueous solubility of

the molecule through covalent modification [26-28] and target curcumin to cancer cells [25], in the current proposal we adopt covalent modification and targeting strategies. It should be noted that many of the encapsulation strategies have resulted only in a marginal improvement in bioactivity [42]. A curcumin based drug candidate should be easy to synthesize, have improved water/plasma solubility and amplified bioactivity.

2. RATIONALE FOR RESEARCH

The chemical structure of curcumin having an α,β-diketone unit and two phenolic-OH groups plays a pivotal role in its biological activity. For example, isomerization has been proved to have an influence on antioxidant activity of curcumin [53]. Thus, researchers hope to achieve improved biological activity of curcumin by structural modifications. Numerous studies dealing with the enhanced biological activity of curcumin derivatives and/or analogues can be found in a recent literature-review by Mosley et al. [54]. Although there has been considerable effort to synthesize curcumin derivatives, formulations, chelated conjugates etc. in order to make curcumin more water/plasma soluble, bioavailable and effective, the direct functionalization of curcumin moiety is very rare [55]. The development of a synthetic methodology to produce curcumin

conjugates with water soluble polymers and targeting proteins can potentially enhance the therapeutic efficacy of curcumin. Bovine serum albumin (BSA) is a serum albumin derivate from cow. And it is used as a nutrient in cell culture and less toxicity to the human. Since it is low cost and large produced from bovine blood, we decided use BSA to synthesis the water soluble curcumin.

Hence, we plan to develop convenient method to water soluble curcumin conjugates via the synthesis of novel mono-functional curcumin derivatives in which one of the phenolic groups of curcumin has been chemically modified with NHS groups.

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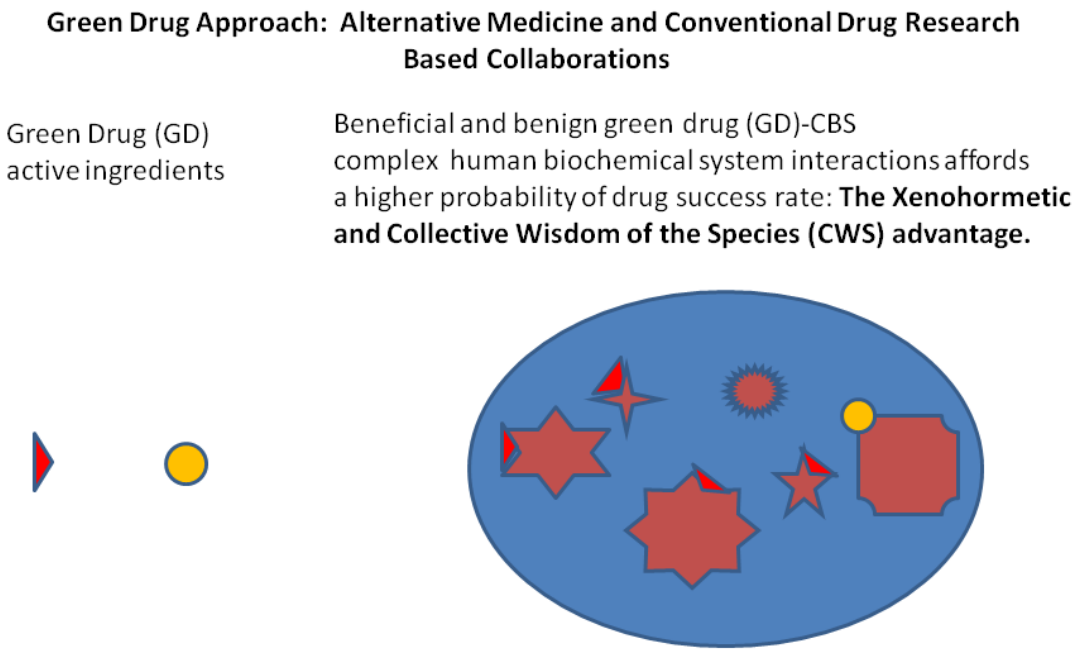

Figure 1. The Big Picture Advantages, the GD strategy has a significantly high likelihood of success compared to the random combinatorial chemistry and the LoKey strategy.

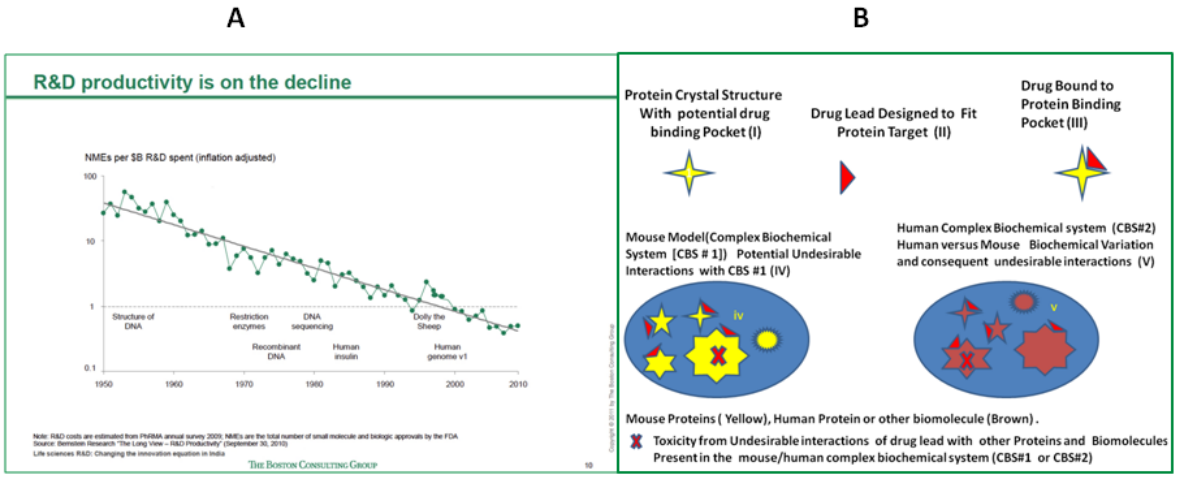

Figure 2. A R&D productivity decline over the sixty years (Life Sciences R&D: Changing the Innovation Equation in Indial 2011 [Courtesy The Boston Consulting Group]). B. Pitfalls in the Current Single Protein binding pocket "Lock" – designer "Key" drug lead approach (LoKey) results from the fundamental assumption that there are no other unfavorable interactions with the mouse/human Complex Biochemical System (CBS): From Steps I –V it is evident that some of the millions of proteins and biomolecules (which span trillions of different shapes/binding pockets with numerous functional groups) in the mouse and human CBS other than the therapeutic target protein/other biomolecule can bind a LoKey drug candidate resulting in toxicity , this affords an explanation for the high drug lead attrition rates.

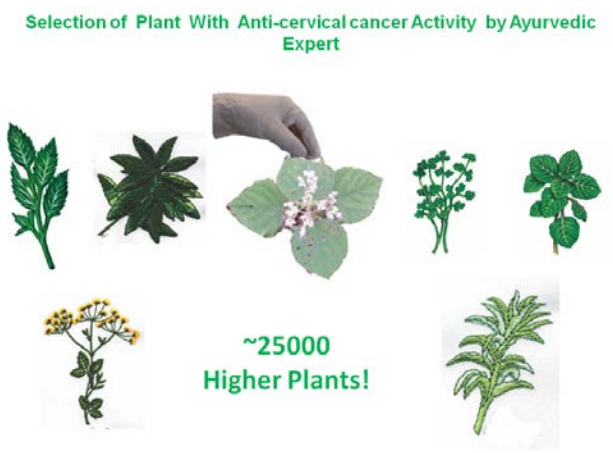

Figure 3 The green drug lead (specific plant source) is chosen from the ~25000 known higher plants based on empirical/practical clinical experience of an Ayurvedic expert in treating a specific pathological condition with the green drug source.

Figure 4. Photograph of Clerodendrum Viscosum / Goddess Saraswathy's plant / Peruvelum (arrow); this plant was photographed at Gokul, Mulayara, Trivandrum, India (June 20th 2008).

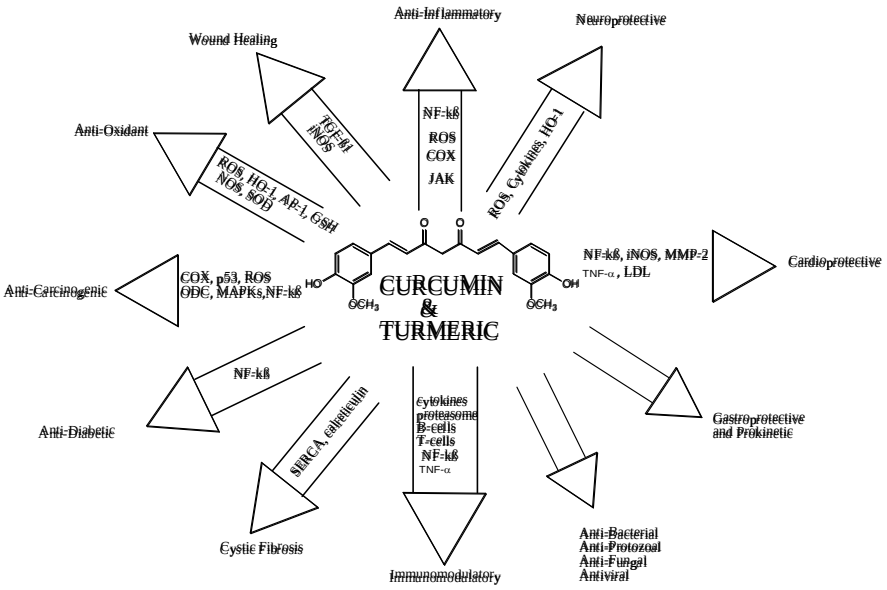

Figure 5. Biological activities of curcumin: Diverse activities of biologic relevance demonstrated in preclinical models and in human studies are shown to illustrate the potential of curcumin to treat human diseases. Cellular mechanisms associated with the effects observed are shown inside the arrows. AP-1, activator protein-1; COX, cyclooxygenase; GSH, glutathione; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; JAK, janus kinase; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinases; MMP, matrix metalloproteinase; NF-κβ, nuclear factor-kappa B; NOS, nitric oxide synthase; ODC, ornithine decarboxylase; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum calcium ATPase; SOD, superoxide dismutase; TGF, transforming growth factor; TNF, tumor necrosis factor. [Adapted from Strimpkos and Sharma].

Chapter II

Water Extract of *Clerodendrum viscosum* induces apoptosis in Hela Cells

1. Introduction

Cervical cancer is the second most common cancer worldwide, and there are about 275,000 deaths annually [1]. In 2009, approximately 11,270 American women were diagnosed with cervical cancer, from which, approximately 4,070 women died from the disease [2]. The incidence of invasive cervical cancer has remained stable in industrialized nations. However, in developing countries, cervical cancer is the most common cancer and the leading cause of the cancer death among women. It is estimated that 200,000-300,000 women die from cervical cancer every year, mostly in the poor countries. Death resulting from cervical cancer is particularly tragic because this type of cancer develops slowly and has a detectable precursor condition, carcinoma in situ (CIS), which is treatable [3].

Currently, chemotherapy is one of the major therapies for cancer. However, the side-effects of chemotherapy are unavoidable, as it indiscriminately kills all fast-growing cells. Natural products are a rich source of compounds that possess anticancer bioactivity. Natural agents and plant products have been used as complementary medicines to reduce the toxicity of the chemotherapeutic drugs [4-6]. Several plant-based components and extracts have been reported to reduce

proliferation and induce the apoptosis of tumor cells [7-9]. Natural products are therefore a promising source of potential medicinal agents for the treatment of cancer.

MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay is a colorimetric assay for measuring the cellular growth and survival [19,20]. The basis of the MTT assay is that the soluble tetrazolium salt (MTT) is converted to an insoluble purple-colored formazan product by the reduced pyridine nucleotides NADH and NADPH in living cells [21]. NADH/NADPH is product of the mitochondria respiration, a basic mitochondrial function and is important to maintain the survival of the cells. Therefore, we can monitor the cell activity by evaluating the activity of the mitochondrial. To evaluate the activity of the respiratory chain in vivo, it is possible to monitor the mitochondrial NADH, FAD, or the cytochrome oxidase oxidation-reduction state [22]. The principle of the MTT assay is that, for most viable cells, mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity [23]. The MTT assay can be used to measure the viable cells without the need for cell counting. The assay is also used to determine the cytotoxicity of the drugs at different concentration. WST (water-

soluble tetrazolium) was developed by introducing positive or negative charges and hydroxyl groups to the phenyl ring of the tetrazolium salt that improve the soluble of the formazan.

Clerodendron viscosum (Cv) is a traditional Indian plant which is used as Herbal composition for cuts, burns and wounds [10]. There are no reports on *Clerodendrum viscosum* in Pubmed (www.ncbi.nlm.nih.gov/pubmed/). In this research, we produced a water soluble extract from the *Clerodendrum viscosum* root and investigated the anti-cancer ability of the Cv extract. The Cv extract was shown to induce apoptosis in Hela cells by the caspase-3 pathway. It is also shown that the Cv extract decreased the proliferation and motility of Hela cells. And the Cv extract could be digested by proteinase *k* and stained by ProQ Emerald 300 (glycoprotein specific stain), suggesting that the Cv extract contained glycoprotein components.

2. Materials and Methods

2.1 Plant Extract Preparation

Clerodendrum viscosum roots were dried in a vacuum oven at 37 °C for 3 hours. 100g of dried root was homogenized with a blender and the mixture was stirred in 1000ml PBS (PH=7.4) at 4 °C for 24 hours.

The residue was removed by centrifugation at 15,000 rpm/min for 15 min and the supernatant was filtered using Fisher ScientificP2filter paper. The filtered fluid is treated with solid ammonium sulfate to 80% saturation and the solution was stirred at 4 °C overnight. The precipitate was collected by centrifugation at 3,000 rpm/min for 15 min and dissolved in deionized water. The solution was centrifuged at the 16,000 rpm for 20 min to remove the insoluble particles, and the supernatant was lyophilized and stored at -20°C.

2.2 Cell Culture

Hela cells were grown in DMEM/F12 (DMEM:Nutrient Mixture F-12) at 37°C in a humidified atmosphere and 5% CO_2. Culture media was supplemented with 10% fetal bovine serum, and 50 U/ml penicillin. Cells were seeded in a 96-well plate with 5,000 cells per well overnight before treatment with plant extracts. Fibroblast cells were grown in ATCC® Primary Cell Solutions™ Media, supplemented with 5ng/ml Rh-FGF-B, 7.5mM L-glutamine, 50ug/ml Ascorbic acid, 1ug/ml Hydrocortisone, 5ug/ml Rh Insulin and 2% Fetal Bovine Serum.

2.3 Detection of Glycoprotein

To investigate the composition of the extract, the extract was dissolved in deionized water and incubated with 20ug/ml proteinase *k* at 37˚C overnight. The resulting fragments and the initial extract was loaded in a non-reducing SDS-PAGE(4-20% gel). Total glycoprotein was detected by staining of gels with ProQ Emerald 300, following manufacturer's instructions (Molecular Probes, Invitrogen). Then the gel was washed twice with 100% methanol for 15 min. Coomassie blue was used to to detect the total protein.

2.4 Binding Evaluation of Concanavalin A (con A)

Con A is a lectin with receptors that can specifically bind α-D-mannosyl and α-D-glucosyl moieties in glycoproteins and glycolipids. The binding can be disrupted by competitive displacement by free glucose. This process could be detected by UV. The assay was used to determine the presence of mannosyl and glucosyl moieties in Cv extract. Con A (0.5 g) was dissolved in 2mL binding buffer (20mM tris pH 7.4, 0.5M NaCl, 1mM $MnCl_2$ and 1mM $CaCl_2$). CV Extract (10mg) was added to the Con A solution and stirred for 30min. Then glucose was added to a final concentration of 0.5M/L and stirred for 10 min. The

formation of Con A-CV aggregates (tyndall scattering observed in the recorded U.V spectra) and their competitive disruption by added glucose was observed by UV spectroscopy (Agilent U.V spectrophotopmeter).

2.5 Determination of Cell Proliferation of the Cv Extracts by WST-1 Assay

Hela cells were seeded in 96-well plates with culture media (DMEM:Nutrient Mixture F-12, 10% fetal bovine serum and 50 U/ml penicillin) for 24 hours at a density of 5,000 cells/well. After 24 hours the nutrient mixture was removed and rinsed with culture media without serum. Culture media without serum (negative control) and culture media without serum plus lyophilized extract were added into the wells. Hela cells were grown under 4 different concentration of extract (1mg/ml, 0.1mg/ml, 0.01mg/ml and 0.001mg/ml) for another 24h. The effect of extract on the proliferation of cells was determined by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay according to the manufacturer's instructions[11].

2.6 Determination of the Cytotoxicity of Cv to the Cervical Cancer Cell Line

The degree to which a substance is able to induce cytotoxicity can be measured using a standard MTT-based in vitro toxicity assay (Sigma, USA). The MTT assay is a colorimetric test that measures the activity of enzymes capable of reducing yellow MTT to purple formazan. Reduction primarily occurs within active mitochondria and therefore the test is a reflection of mitochondrial activity. Since normal mitochondrial activity indicates a healthy normal metabolically active cell, mitochondrial dysfunction points to cell toxicity and can be measured by less formazan production, which equates to less purple absorbance. To recover the insoluble purple formazan from cells a solubilization solution such as acidified ethanol is used. Solubilization of formazan can then be measured and quantified by measuring an absorbance at 570 nm using an automated spectrophotometer. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced and a dose-response curve can be generated.

We initially chose to use HeLa cells to test the effective cytotoxicity of the *Clerodendrum viscosum* extracts. The Hela cell line is a commonly used huma cervical cancer cell line which is often used for medical research involving measurements of cytotoxicity. This study was expanded to six kinds of cells lines. The cells were plated at 5000 cells / well (using a 96-well cell culture plate) and cultured for 24hrs using established HeLa cell culture media (Eagle's MEM with non-essential amino acids and Earle's BSS with 10% FBS). After 24 hours the media was removed and replaced with media alone or media plus lyophilized clerodendrum viscosum (formulation 4, see formulations section) redissolved in media at 3 different concentrations (2.5mg/ml, 5 mg/ml, 10 mg/ml; w/v). Under these conditions cells were cultured for another 24 hours followed by the MTT assay.

2.7 Caspase-3 Immunofluorescence

Hela cells were seeded into the 6-wells chamber ($1x10^5$ cells/well) and allowed to adhere to the plate for 24 hours before treatment with the extracts at three different concentration (2.5, 5.0 and 10. mg/ml). They were fixed with paraformaldehyde solution (4%). Then the wells were washed twice with PBS for 1 min. PBS was removed and the wells were blocked with 5% bovine serum for 1 hour and rinsed with 0.5% triton-x.

The triton-x solution was removed and the chambers were incubated with cleaved caspase-3 antibody (1:200 dilution) (cell signaling) for 1 hour at a room temperature. After washing three times with PBS, Hela cells were incubated with second antibody goat anti-rabbit IgG (1:10000 dilutions) for 1 hour at a room temperature. The samples were observed by confocal microscopy.

2.8 Viability Assay

Propidium iodide (PI) is a fluorescent dye which is used as a nucleic acid stain. Once the dye is bonded to nucleic acid, enhanced red fluorescence is released. PI is membrane impermeable and generally excluded from viable cells, thus reflecting the cell viability. Hela cells and primary fibroblast cells were seeded at a density of 5,000 cells per well in 96-well plates and allowed 24 hours for attachment to occur. Later the cells were treated with duplex serial dilutions of the extract (2.5, 5 and 10mg/ml). After 8 hours of incubation PI was added into the wells at the concentration of 100ug/ml. Finally, cells were observed by living microscope imaging. The cell viability was determined using the formula:

Viability % = (density of the PI staining cells / density of the total cells) X 100

2.9 Cell Tracking and Motility Analysis

To study the effect of the extract on dynamics of Hela cells, time-lapsed microscopy was used to record the movement of Hela cells. Hela cells were seeded at a density of 5,000 cells per well in 96-well plates and incubated for 24 hours to attachment. Later the cells were treated with double serial dilutions of the extract (2.5mg/ml, 1.25mg/ml and 0.625mg/ml). Then the 96-well plate was left in an environmentally controlled chamber supplemented with 5% CO_2. Images were captured every 30 min for 24 hours with a CCD camera controlled by software. The cell movement was analyzed based on captured image frames using celltrack software (the Ohio State University, USA) as previous described [12]. Briefly, we draw the boundary of each cell, and speed, area, deformation, trajectory and detailed tracking of the cells are computed and displayed for analysis.

2.10 Tubulin Staining

Microtubules are important in mitosis and cell division and they have been a new target for the development of new anti-cancer drugs. α,β-Tubulin is the basic unit of the microtubule. As previous results have shown that the Cv can affect the morphology of the cells, we

investigated whether the roots affect the expression of tubulin. Hela cells were seeded into the 8-well chamber ($1x10^5$ cells/well) and allowed to attach for 5 h. After 24 hours the media was removed and replaced with media alone or media plus lyophilized *Clerodendrum viscosum* redissolved in media at 3 different concentrations (2.5mg/ml, 5 mg/ml, 10 mg/ml; w/v). The Cv treated cells were incubated in a humidified environment of 5% CO_2 and 37°C for 1 h. Tubulin was stained by the standard method (see caspase staining).

2.11 Statistical Analysis

The experimental results were expressed as mean ± standard deviation of triple replicates. Where applicable, the data were subjected to one-way analysis of variance (ANOVA). P-value of ≤ 0.05 was regarded as significant.

3. Results:

3.1 Characterization of extract from the roots of *Clerodendrum viscosum*

The Cv extract was collected from the root of *Clerodendrum viscosum* by two steps. The first step involves using a sodium phosphate

buffer(pH=7.4) to make extract from the Cv root; the following step is precipitation of the big hydrophobic components from *Cv* extracts solution with 80% ammonium sulfate (Cv-ap). After dissolving the precipitate with deionized water, it was found to have biological activity that can induce the cancer cells apoptosis. We also found that it contains a protein about 18 kDa (figure 1). The protein disappeared during incubation with proteinase K. (figure 1). The extract also showed a positive result when staining with emerald 300 (figure 1), indicating that the root's extract has glycoproteins. To determine the glycosylation of the extract a lectin based assay was employed. The lectin selected for this study was concavalin A. Concavalin A can specifically bind to certain structures found in various sugars, glycoproteins and glycolipids, mainly internal and non-reducing terminal α-D-mannosyl and α-D-glucosyl groups [13]. The addition of a Con A solution to Cv-AP results in an increased absorbance throughout the U.V spectrum which characteristic of Tyndall scattering resulting from the formation of cross linked aggregates between Con-A and Cv-ap. The Con-A-Cv-ap aggregate was dissolved by the addition of glucose; glucose disrupts the aggregates by competitively displacing Cv-ap (The U.V absorbance decreases throughout the U.V spectrum).

3.2 Cv Induced Cervical Cancer Cells Apoptosis

The morphological change associated with cell death was observed with normal inverted microscope. Hela cells were induced to undergo apoptosis with shrinkage of cells and membrane blebbing after treatment with 10mg/ml roots extract for 8 hours (figure 2A).

It is important to ascertain whether the death of the Hela cells is meditated via activation of apoptotic pathway. The expression of apoptotic related protein Cleaved caspase-3 was examined by immunofluorescence. Cleaved caspase-3 was expressed almost in all the Hela cells after treated with the Cv extract of 10mg/ml. By contrast, insignificant amounts of cleaved caspase-3 were expressed in untreated Hela cells (figure 3).

Moreover, root extract induced cell death was quantified using *propidium iodide* (PI) staining and examined under fluorescence microscope. The presence of red coloration was due to the binding of PI to denatured DNA (figure 2A). Based on counting Hela cells treated with different concentration of root extracts together with untreated Hela cells as negative control, LC_{50} was calculated (figure 2B).

3.3 Inhibition of the Motility of Hela Cells by Cv Extract

Stopping or slowing the cancer cell migration is a very important approach to stop the invasion of a tumor. Therefore, to know whether the

Cv extracts can affect cancer cell motility is essential. For this purpose we used a lower concentration of extract to treat Hela cells to avoid killing cancer cells. Time-lapsed microscopy was used to record the movement of the cells in real time for 24 hours. Three different concentrations (2.5mg/ml, 1.25mg/ml, and 0.625mg/ml) of extract were used to treat Hela cells and all three different concentrations extracts impaired Hela cells motility by 60% compare to the Hela cells without treatment. In addition, we found there were no obvious motility differences among the three different concentration extracts (figure 4). The result shows that the Cv extract slows the motility of the cells instead of inducing the apoptosis of the cells at low concentration of the extract.

3.4 Tubulin Degradation after Treatment with the Cv

The tubulin present in Hela cells underwent a change after treatment with Cv extract (figure 5). Tubulin can be seen in control Hela cells which were not treated with CV. After exposure to 10mg/ml CV extract, the cells round up. The amount of tubulin was diminished and shrinkage was observed. When the cells were treated with different concentrations of CV, the response elicited dose-dependent. Currently we are unable to determine if the change of tubulin is the result of cell shrinkage or caused

by depolymerization. However, the extracts can inhibit cell movement and proliferation by modulating tubulin directly or indirectly.

3.5 Analysis of Cv Extract for Growth Inhibitory Activity against Cervical Cancer Cells

Clerodendrum Viscosum root extracts were examined for cell growth inhibitory properties on Hela cell lines. Cells were treated with increasing concentration of CV extract over varying time period and then analyzed by the WST-1 assay for cell viability. When treated with 1mg/ml of rootsCv extract, the absorbance of Hela cells from day 0 to day 3 were almost identical(figure 6), which demonstrates that the Hela cells growth was completely inhibited at 1mg/ml concentration CV root extract, on day 3, the 1mg/ml root extract inhibited the Hela cells growth by 50%. The root extract showed inhibitory bioactivity even as low as 1ug/ml, in day 3, the 1ug/ml root extract inhibit the cells growth by 20% (figure 6).

3.6 Cytotoxicity of the Cv to Cervical Cancer Cell Lines

To analyze the cytotoxicity of the Cv, MTT assays were performed on human cervical cancer cell lines. IC_{50} of the different cell lines were

calculated (table 1). The extract worked well on all the cervical cancer cell lines. The lowest IC_{50} values were recorded as 2.66±0.38 mg/ml for the C33a cell line (table 1). The data confirmed the hypothesis that the extract has anti-cervical cancer ability.

3.7 CV has Less Effect on the Normal Human Fibroblast Cells

To test the effect of Cv extract on normal cells, we used primary fibroblast cells and Hela cells were used as a control. After treatment with the 10mg/ml CV extract for 8 hr, almost all the Hela cells were dead (PI stained most of the cells); on the contrary, nearly no PI staining was observed in primary fibroblasts indicating that Cv is non-toxic to healthy cells (figure 7).

4. Discussion

Multiple cell signaling pathways are deregulated in cancer cells which are characterized by uncontrolled cell proliferation and anti-apoptotic features. Apoptosis (programmed cell death) occurs in normal cells if its damage is not repaired. Apoptosis is also a critical protective mechanism against carcinogenesis, eliminating damaged cells or cells proliferating

abnormally in response to carcinogens [14]. Therefore, induction of cell apoptosis selectively in cancer cells is a promising strategy to prevent and treat cancer. In the present research, we established that a standardized extract from Clerodendron viscosum which contains a glycoprotein induces cancer cell apoptosis with the typical morphological alterations: cell shrinkage, blebbing and nuclear condensation.

Cleavage of caspase-3 confirmed apoptosis in Hela cells [15]. Caspase (C: cysteine protease mechanism, aspase: ability to cleave after aspartic Acid) are aspartate-directed cysteine proteases that play a key role in the initiation and execution of apoptosis [15, 24]. The Caspases exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes, a homodimer with each monomer having a large (~17-21 kDa) and a small (~10-13 kDa) subunit [16, 25, 26]. Components of the proteolytic mechanism, including the active site Cys and His residues, are harboured within the large subunit while aspartate (Asp) at the P1 site (P1Asp) is obscured in a cavernous pocket, expressed as S1 site, are derived from both the large as well as small subunits and the S2, S3, and S4 subsites are contributed by the small subunit and thus both the small as well as large subunits determine the active site [27]. The activation of caspases then targets their downstream molecules and

initiates apoptosis. Researches demonstrate that exposure of Hela cells to Cv root extract causes Caspase-3 activation.

Migration/invasion of cancer cells is a factor which reduces the survival rate in cancer patients [17]. Stopping or slowing the migration of cancer cells is a promising mechanism against cancer. In this study, we found that low concentration of root extract alters Hela cells motility. It reduces 60% motility of Hela cells at the concentration of 0.625mg/ml.

We also have studied the effect of Cv extract on other cervical cancer cell lines (Siha, ME-180, Ca Ski, C-33 A, Ms 751). MTT assay was used to measure the IC_{50} of CV on each cervical cancer cell line. The results show that Cv extract had similar potent activity against all the cervical cancer cell lines; the IC_{50} ranged from 2.66mg/ml to 6.6mg/ml (table 1). This demonstrates that Cv extract can inhibit the growth of most of the cervical cancer cell lines and shows great promise for inhibiting the growth of cervical cancer.

Anticancer drugs interfere with the growth of the cancer cells and cause the death of the cancer cells. However, most of the time, the chemotherapeutic drugs are so powerful that they affect the growth of the normal cells, causing indiscriminate cytotoxicity and many side effects; some of them may be very serious. For example, carboplatin (Paraplatin), which is used

for cancers of the ovary, head and neck, and lung, have adverse side effects such as decrease blood cell counts, hair loss, confusion etc [18]. In this research, we used primary human fibroblast cells to test if the Cv extract can induce cell death in healthy cells. The fibroblast cells were unaffected by Cv extract wheras most of the Hela cells were eliminated via apoptotic pathways (figure 7), which demonstrates that Cv extract is a promising potential anti-cancer medicine without the side effects common to most anti-cancer drugs.

In conclusion, this study demonstrates that the roots of Cv contains glycoproteins and strongly inhibit the proliferation of Hela cells. High concentration of the root extract induces the cancer cells apoptosis through the caspase-3 pathway. Downstream molecules need to be evaluated in detail in the future. Even at a low concentration the Cv root extract can slow the movement of cancer cells. We also studied the effect of Cv root extract on normal cells (data not shown), which showed low toxicity to normal cells. These result confirmed the root extract is a promising anticancer agent against human cervical carcinoma cells.

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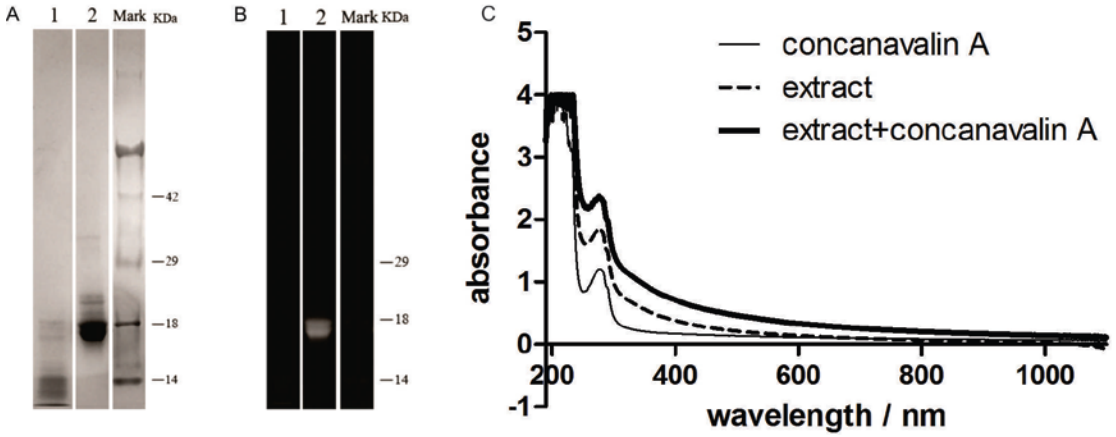

Fig 1. Glycoprotein obtained from the *Clerodendrum viscosum* (Cv). After running a 4-20% polyacrylamide SDS-PAGE, proteins were stained with coomassie blue for total protein A) and Pro-Q emerald 300 dye for glycoprotein. B) 1 is the proteinase K digested aqueous extract of CV. 2 is the aqueous extract of CV. C) effect of conA addition on the UV spectrum of the conA-extract conjugate

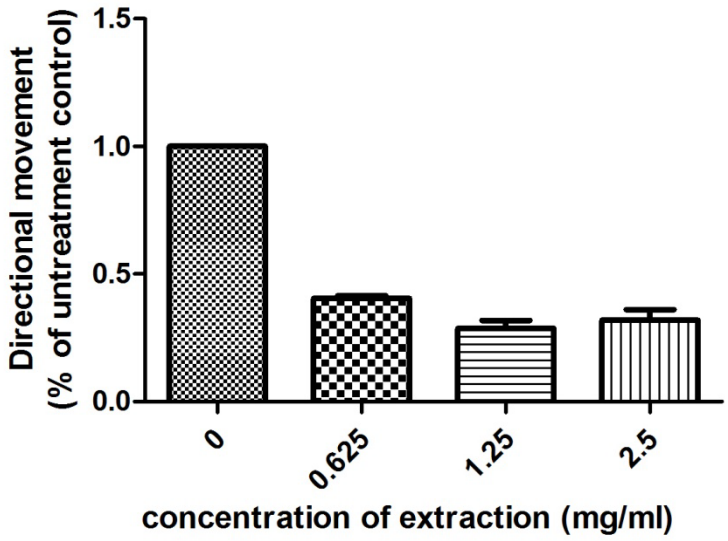

Figure 4. Hela cells were treated with extract(0.625-2.5 mg/ml) for 24 hours. Time-lapse microscopy was used to determine migration of cells at 10 minute intervals. Analysis of individual cell tracks was performed with celltrack software.

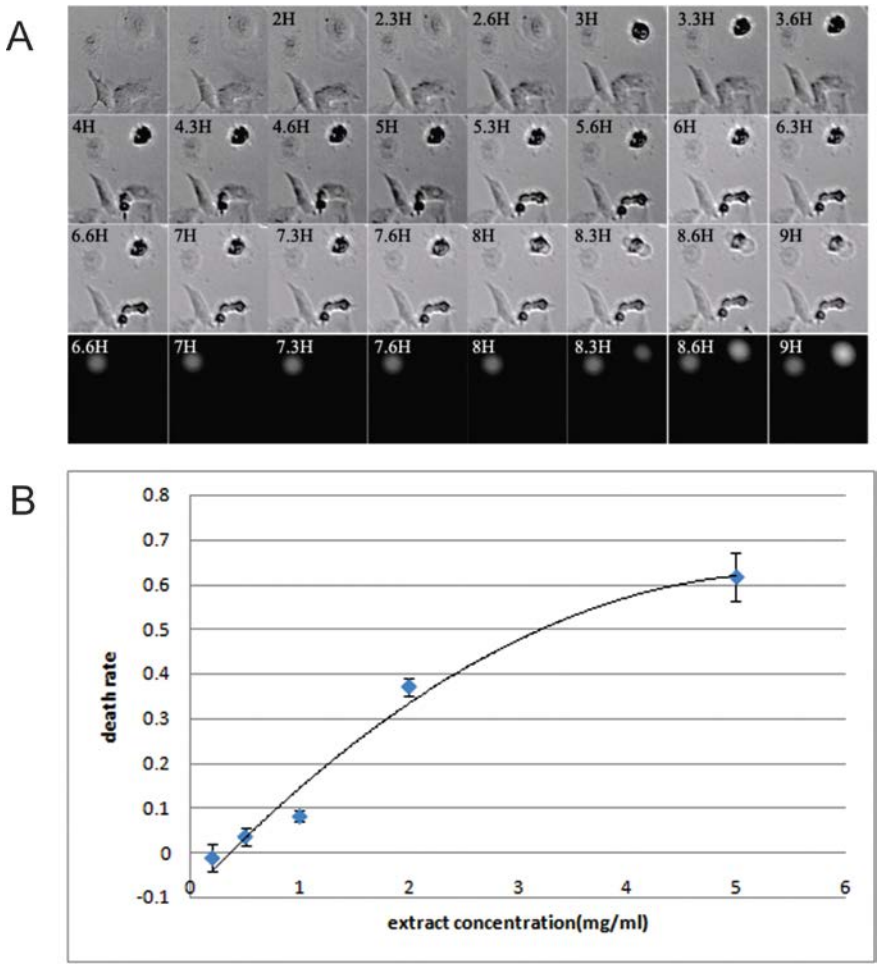

Figure 2. A) Live cell imaging of Hela human cervical cancer cell line (control) for 8 hours following treatment with Cv extract (10 mg/ml). B) Hela cells seeded at 5,000 cell/well in a 96-well plate overnight followed by 24 h incubation with Cv extracts at 0.2 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml and 5 mg/ml respectively. Death measured by Propidium iodide. The LC_{50} = 3.2 mg/ml

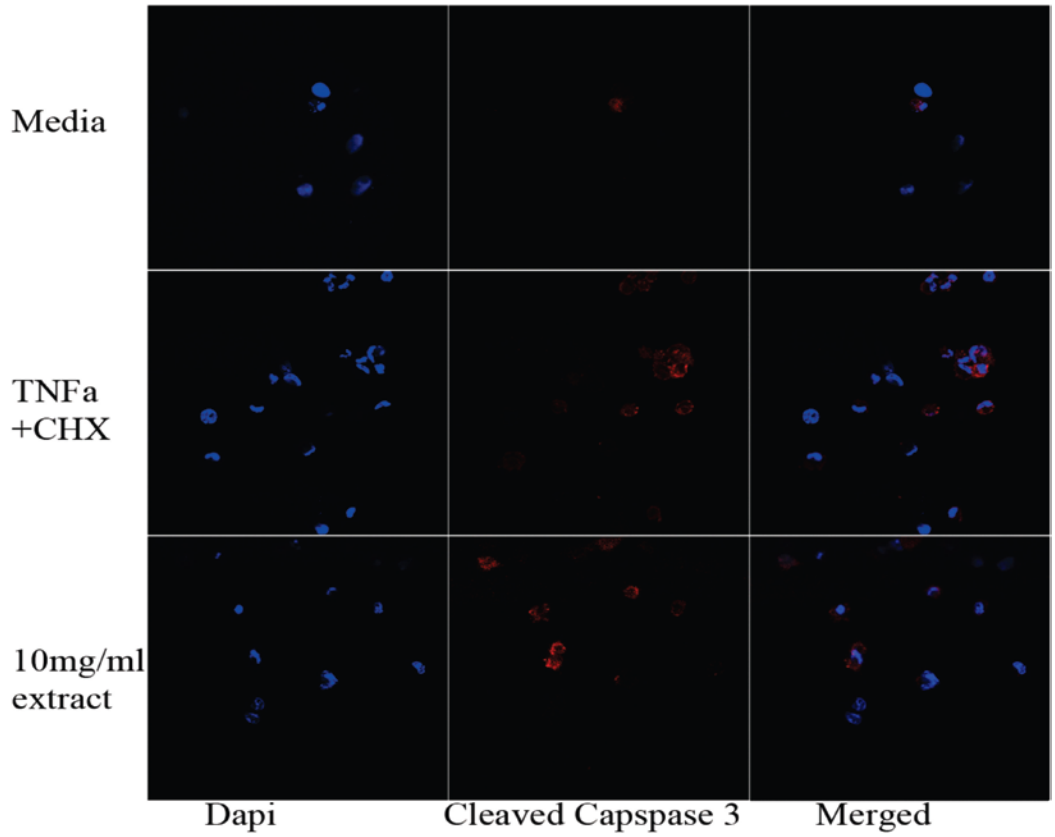

Figure 3. Caspase 3 activation in Hela cell lines following TNFα + CHX treatment (positive control) and treatment with extract.

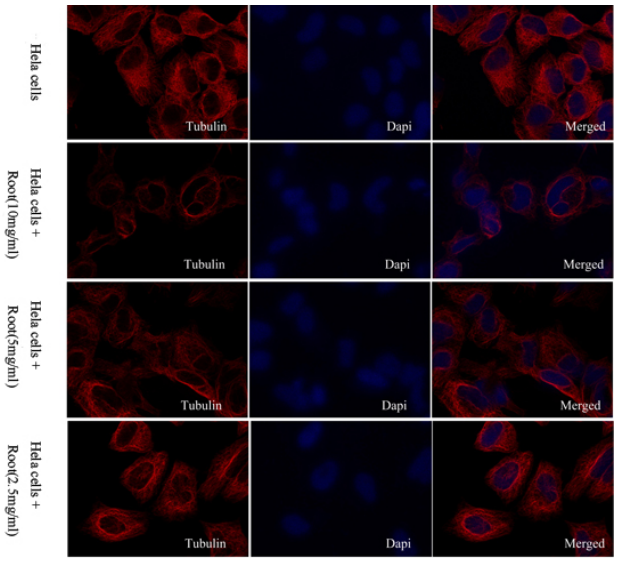

Figure 5. Representation of the tubulin in Hela cells after addition of different concentration of CV extract. At the 10 mg/ml conventration most of the tubulin disappears.

	Hela(IC50) (mg/ml)	siha(IC50) (mg/ml)	me180(IC50) (mg/ml)	caski(IC50) (mg/ml)	c33a(IC50) (mg/ml)	Ms 51(IC50) (mg/ml)
1	5.2	5.6	4.7	2.5	2.4	3.7
2	5.5	5.4	4.1	3.2	2.8	4.2
3	5.7	5.5	4.8	4.6	3.5	4.6
4	5.8	8.7	5.1	4.7	2.8	4.5
5	5.7	7.8	5.5	3.3	1.8	4.4
Median	5.18	6.6	4.84	3.66	2.66	4.28
Standard Deviation	0.24	1.54	0.51	0.95	0.62	0.35

Table 1 Cytotoxicity of the different cervical cancer lines.

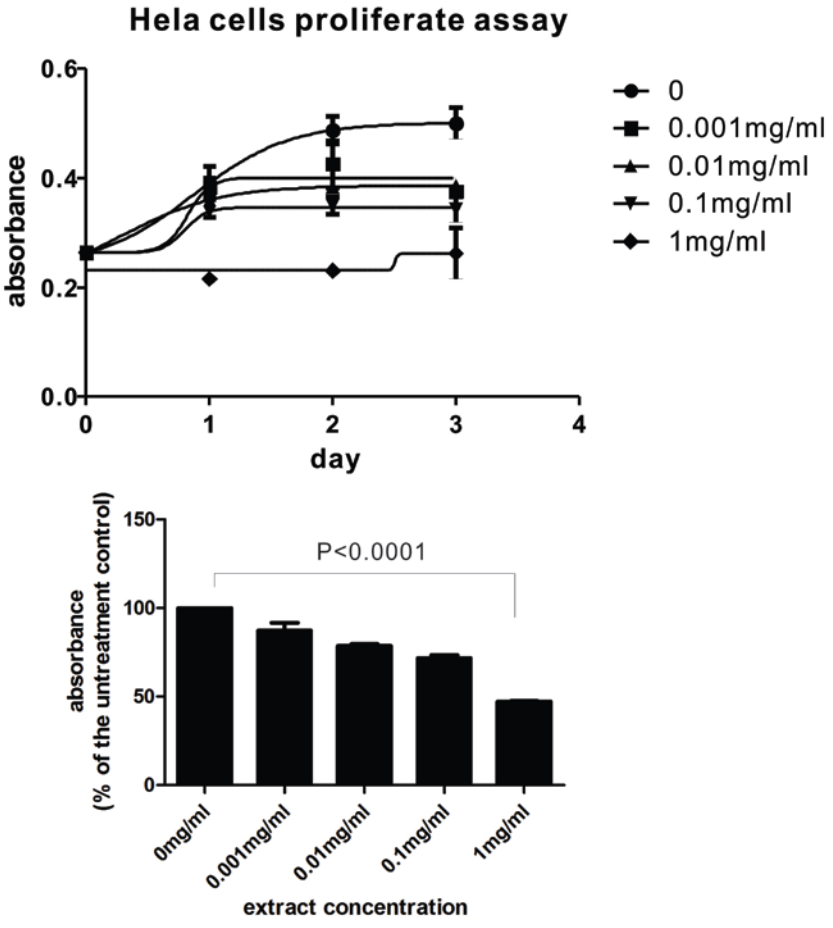

Figure 6. Role of extract in regulate cervical cancer cell proliferation. Growth rates of the cells were determined by WST-1 assay. Top one: Hela cells seeded at 2,000 cells/well in a 96-well plate overnight followed by 1 day, 2 day, and 3day extract incubation (0.001-1 mg/ml) . Bottom graph: cell number after 2 days of incubation at four different extract concentrations. P<0.0001

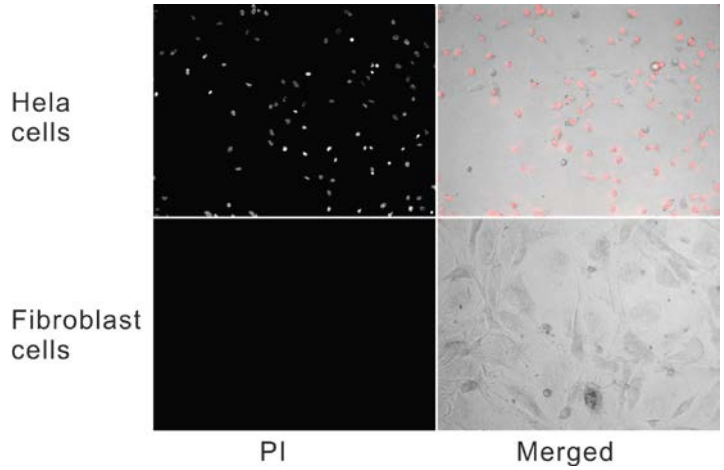

Figure 7. Cv effect on the primary human fibroblast cells. Cv extracts (10 mg/ml) were treated on the fibroblast cells and Hela cells for 8 h and stained with PI.

Chapter III

Purification of *Clerodendrum viscosum*

1. Introduction

Clerodendrum viscosum is a small shrub, it has heart shaped leaves and with “crown shaped” white flowers in terminal panicles which grows as ground cover in India. It is used to be considered as a common weed. Very few publications were reported on *Clerodendrum viscosum* if one searches for research concerning this plant in Pubmed (www.ncbi.nlm.nih.gov/pubmed/). Representative examples of research employing this plant or closely related ones are: (a) “Preliminary study on the antisnake venom activity of alcoholic root extract of *Clerodendrum viscosum* (Vent.) in naja naja venom.”[1] (b) “A neo-clerodane diterpenoid from Clerodendrum viscosum leaves.”[2] (c) “Herbal composition for cuts, burns and wounds.”[3] “The invention provides a novel herbal composition for treatment of cuts, burns and wounds, the composition comprising plant material selected from Utleria solicifolia, Jatropha curcas, *Clerodendrum infortunatum* , and Centella asiatica.”[3] (d) “Sterols of some *Clerodendrum* species (Verbenaceae): occurrence of the 24α- and 24β-epimers of 24-ethylsterols lacking a Δ25-bond.”[4] (e) “Anthelmintic activity of leaves of *Clerodendrum infortunatum*.”[5].

The utility of herbal medicines for cancer treatment and prevention is receiving increasing attention due to the cost and side effects of current

radiation or chemotherapeutic agents used for cancer patients, and the continuing increase in new cancer cases as well as cancer deaths [6 - 8]. Projections indicate that the deaths over the world from cancer will rise to more than 13.1 million in 2030 [9].

This study describes the purification and characterization of the anti-cancer component from the Cv. Many publications have reported plant extract can be used as potential anti-cancer medicine [10 - 12]. Based on our previous study, we found the aqueous extract of Cv had the anticancer properties. Our goal was to purify and identify the anti-cancer components from Cv. We developed two different procedures to separate the active components. One separation method uses ammonium sulfate, the other uses ethanol to make an extract from CV. MTT assay was used to monitor the bioactive components responsible for the anticancer properties using *in vitro* bioassay guided isolation method [13].

2. Materials and Methods

2.1 Isolation of Aqueous Extract by Ammonium Sulfate

2.1.1 Plant Materials and Preparation of Crude Aqueous Extract (Cv-ap).

Roots of the *Clerodendrum viscosum* was collected and stored in alcohol.To prepare the water soluble Cv extract, the roots were dried in a vacuum oven at 37 °C for 3 hours. 100g of dried Cv roots were homogenized with a blender into small pieces, mixed with 1000ml ammonium bicarbonate (0.1M) and stirred at 4 °C overnight. The roots' residue is removed by centrifugation at 15,000 rpm/min for 15 min and the supernatant was filtered using Fisher Scientific P2 filter paper to remove the remaining small particles. Ammonium sulfate was added into the supernatant to 80% saturation and stirred at 4 °C overnight. The precipitate was collected by centrifugation at 3,000 rpm/min for 15 min and the pellet was dissolved in deionized water. The solution (Cv-ap) was centrifuged again at 16,000 rpm for 20 min to remove the insoluble particles, and the supernatant was lyophilized and stored at -20°C. The Cv-ap yield percent was 4.4%.

2.1.2 Chemicals

Ammonium bicarbonate (99%) was obtained from GFS chemicals, Inc. Ammonium sulfate (enzyme grade) was bought from Fisher scientific. α-cyano-4-hydroxycinnamic acid (4-HCCA), Trifluoroacetic acid (TFA), Acetonitrile (ACN), formic acid and isopropanol were purchased from Sigma-Aldrich.

2.1.3 Cell Culture

Hela cells were grown in DMEM/F12 (DMEM:Nutrient Mixture F-12) at 37°C in a humidified atmosphere and 5% CO_2. Culture media was supplemented with 10% fetal bovine serum, 50 U/ml penicillin. Cells were seeded in 96-well plate with 2,000 cells per well overnight before treatment with plant extracts.

2.1.4 Size Exclusion FPLC (faster protein liquid chromatography)

Lyophilized Cv-ap was redissolved in 0.1 M ammonium bicarbonate solution. 1 ml Cv-ap solution was loaded onto a HiPrep superdex 200 column and was separated using an Amersham Biosciences AKTA purifier system (GE Healthcare). The column was equilibrated with 0.1 M ammonium bicarbonate buffer. The fractions were eluted with 0.1M ammonium bicarbonate and collected using a peak fractionation system (figure 2B).

2.1.5 Bioassay Guided Isolation of Bioactivity components from Cv

The fractionation of the anti-cancer components was bioassay guided. For each fractionated extract, cell viability assay was performed to determine its activity. The active fractions were purified and identified (figure 3).

The purification process is shown in figure 3A. First, Cv-ap was redissolved in ammonium bicarbonate (0.1M) and passed through the size exclusion column (superdex 200, Gel Heath). The collected four peaks (P1, P2, P3 and P4) were lyophilized to dryness for further bioactivity testing.

The peak 3 was heated at 95 degree for 1 hr, the resulting precipitate (HPP3) and supernatant (HP3) were collected and lyophilized to dryness. The HP3 was further separated into precipitate (EPHP3) and supernatant (ESHP3) by solvent partition method using 66% ethanol. The subfraction EPHP3 contains the most bioactivity components.

2.1.6 Cell Viability Assay

MTT (3- (4,5-Dimethyl-thiazol-2-yl) - 2, 5-diphenyltetrazolium) assays were used to measure the cell viability. 2,000 Hela cells were seeded in 96 well plates and incubated for 24 h for attachment at 37°C in a humidified atmosphere and 5% CO_2. The cell were rinsed twice with the media without serum and treated the Hela cells with various concentrations of Cv-ap fractions. Hela cells treated with the media only was used as control. The Cv-ap and its fractions were dissolved in the media without the serum to avoid the interference of the components in serum. After incubation for 72 hr at 37°C in a humidified atmosphere and 5% CO_2, 10% MTT solution (5 mg/ml in PBS) was added into the media and incubated for 2 hr at 37°C in a humidified atmosphere and 5% CO_2. MTT solution was removed and 100 µl isopropanol was added to dissolve the purple crystals. Then the absorbance at 570 nm and 690 nm was measured using the microplate reader. The absorbance at 570nm is the purple crystals absorbance and the absorbance at 690 nm is the absorbance of the background. The absorbance of the purple crystals was calculated as (Absorbance (570 nm) – Absorbance (690 nm)). The viability percentage of Hela cells was calculated as 100*(Absorbance of sample/ Absorbance control)).

2.1.7 SDS-PAGE and Silver Staining

An SDS-PAGE gel was run to measure the amount of protein in bioactive fractions. After MTT assays measurement, Cv-ap, P3, HP3 and EPHP3 were found to have bioactivity among the fractions. We run these four samples on 4%-20% gradient SDS-PAGE (PAGE gel INC.)

Protocol:

Sample preparation

1. A concentrated standard 10mg/ml Cv-ap (dissolved in Millipore H_2O) solution was prepared. A range of concentrations of Cv-ap solutions (range from 0.3mg/ml to 5mg/ml) were made by serial dilution of the standard solution.
2. The absorbance readings of 280nm of each dilute solution were recorded with U.V Spectroscope (Agilent 8453)
3. A standard curve of concentration VS absorbance was made by software GraphPad Prism5 (figure 1).
4. The samples were dissolved in deionized water. The concentration of each sample was determined based on the standard curve and the concentration of each sample was adjusted to a concentration of 2.5mg/ml.

SDS-PAGE

1. 4-20% gradient gel (PAGEgel INC) was prepared.
2. 20ul of each sample was mixed with 7ul 4xbuffer (PAGEgel INC) in eppendorf tube and heated for 5 min at 95˚C on the heated plate.
3. The samples were cooled in ice
4. The SDS-PAGE was loaded and run at 150V constant.
5. After the SDS-PAGE gel was done, the power supply was removed for silver staining.

Silver staining

6. The gel was washed in deionized water for 5 minutes. The water was removed and the process was repeated once more.
7. The gel was fixed in 30% ethanol:10% acetic acid solution (25ml) (50ml total, 30ml:15ml:5ml water:ethanol:acetic acid) for 15 minutes. The solution (25ml) was replaced and fixed overnight.
8. The gel was washed in 10% ethanol solution (25ml) for 5 minutes. The solution was replaced (25ml) and washed for another 5 minutes.
9. The gel was washed in deionized water for 5 minutes. The water was removed and the process was repeated once more.
10. Sensitizer Working Solution was prepared by mixing 1 part Silver Stain Sensitizer with 500 parts deionized water (mix 50µL Sensitizer with 25mL water).
11. The gel was incubated in Sensitizer Working Solution for exactly 1 minute, then washed with two changes of deionized water for 1 minute each.
12. Stain Working Solution was prepared by mixing 1 part Silver Stain Enhancer with 50 parts Silver Stain (0.5mL of Enhancer with 25mL Stain).
13. The gel was incubated in Stain Working Solution for 30 minutes. Note: Gel may be incubated in Stain Working Solution for as short as 5 minutes or as long as overnight without affecting stain performance.
14. Developer Working Solution was prepared by mixing 1 part Silver Stain Enhancer with 50 parts Silver Stain Developer (mix 0.5mL of Enhancer with 25mL Developer).

15. A Stop Solution5% acetic acid solution was prepared.
16. The gel was washed quickly with two changes of deionized water for 20 seconds each.
17. The Developer Working Solution was added immediately and incubated until protein bands appear (4-5 minutes). Note: Protein bands will begin to appear within 3 min and then continue to develop.
18. When the desired band intensity was reached, Developer Working Solution was replaced with prepared Stop Solution (5% acetic acid). The gel was washed briefly, then replaced with Stop Solution and incubated for 10 minutes.

2.1.8 Concanavalin A Affinity Chromatography

The presence of a glycoprotein component in Cv-ap was confirmed via glycoprotein and protein specific SDS-PAGE gel analysis and by the fact that it binds to a Concanavalin A (Con A) column (biomolecules that contain mannose and glucose residues bind to Con A). After isolating EPHP3, we further separated it by concavalin A column (histrap 5ml, GE Healthcare) with Amersham Biosciences AKTA purifier system (GE Healthcare). EPHP3 was redissolved in binding buffer (20mM Tris-HCl, 0.5M NaCl, 1mM $MnCl_2$, 1mM $CaCl_2$, pH 7.4). The column was equilibrated with 5 column volume of binding buffer. and 1 ml EPHP3 solution was loaded onto the column. After eluting with 5 column volume of binding buffer, the column was eluted further with stripping buffer buffer (0.5M glucose, 20mM Tris-HCl, 0.5M NaCl, pH 7.4). The eluted fraction was collected and removed the salt by desalting column for future bioactivity tests.

2.1.9 Matrix-assisted Laser Desorption Ionization (MALDI-TOF)

Cv-ap and fractions were redissolved in 0.1%TFA deionized water at the concentration of 2.5mg/ml. The Stainless steel MALDI sample plate was cleaned with methanol and wiped dry followed with water wash and wiping dry. The cleaning was repeated and dried before use. 30 ul of thin layer substrate solution (1 part of saturated 4-HCCA in TAW (0.1%:2:1)(TFA:ACN:water) + 3 parts isopropanol) was applied and spread on the plate. After waiting 5 minutes the excess solution was wiped off. The sample was diluted [1 part into 4 part of matrix solution (saturated 4-HCCA in FWI (3:1:2)(formic acid: water: isopropanol)]. 1 ul sample/matrix mixture was spotted on the plate and waited 5 min to see the crystallization form at the interface of matrix and sample. The excess liquid was removed by vacuum aspiration. Each spot was washed with 1 ul of a ice cold 0.1% TFA solution and the excess liquid was removed by vacuum aspiration. MS experiments were performed using a MALDI- time-of-flight mass spectrometer (Bruker Daltonik, Bremen, Germany). The data was analyzed by software Moverz.

2.1.10 Statistical Analysis

All experimental results were presented as mean. One-way ANOVA with Dunnett's multiple comparison test was used for comparisons among various treatment groups and lipopolysaccharide (LPS) control group. Results were considered statistically significant when $p<0.05$.

2.2 Isolation of the bioactivity molecules by ethanol

2.2.1 Plant Materials

Roots of the Clerodendrum Viscosum were collected and stored in alcohol. The roots were dried in a vacuum oven at 37 °C for 3 hours before use. The purification process is outlined in figure 5. First step: CV roots were homogenized with a blender into small pieces. 85% ethanol was added and stirred for half an hour at room temperature. Second step: after centrifugation at 3,000 rpm for 30 min, the resulting supernatant was filtered and lyophilized (SE8). Third step: the precipitate was dried and then rinsed with acetone to remove the hydrophobic small molecules. Acetone was collected and dried (AC). Fourth step: Deionized water was added to the precipitate from the previous step and stirred overnight. The mixture was centrifuged at 3,000rpm for 30 min. To the resulting supernatant 100% ethanol was added and the final concentration was adjusted to 70% ethanol and stirred overnight. Fifth step: the mixture was centrifuged at 3,000rpm for 30 min, the supernatant was filtered and lyophilized (SE7), the pellet was dissolved in deionized water and lyophilized (PE7).

After the roots were dried in a vacuum oven at 37 °C for 3 hours, CV roots were homogenized with a blender into small pieces. Deionized water was added into the roots and stirred overnight at 4 degree. The mixture was centrifuged at 3,000rpm for 30 min and collected the supernatant. The supernatant was filtered by filter paper and lyophilized (water extract).

2.2.2 MTT Assay

MTT (3- (4,5-Dimethyl-thiazol-2-yl) - 2, 5-diphenyltetrazolium) assays were used to measure the cell viability. 5,000 Hela cells were seeded in 96

well plates and incubated for 24 hr for attachment at 37°C in a humidified atmosphere and 5% CO_2. Then cell were rinsed twice with the media without serum, and the Hela cells were treated with various concentrations of ethanol extract. Hela cells treated with the media only was used as the control. The extracts were dissolved in the media without the serum to avoid the interference of the components in serum. After incubation for 24 hr at 37°C in a humidified atmosphere and 5% CO_2, 10% of MTT solution (5mg/ml in PBS) was added into the media and incubated for 2 hr at 37°C in a humidified atmosphere and 5% CO_2. MTT solution was removed and 100ul isopropanol was added to dissolve the purple crystals. Then the absorbance at 570nm and 690nm was measured using a microplate reader. The absorbance at 570nm is the purple crystals absorbance and the absorbance at 690nm is the absorbance of the background. The absorbance of the purple crystals was calculated as (Absorbance (570nm) – Absorbance (690nm)). The viability percent of Hela cells was calculated as 100*(Absorbance sample/ Absorbance control).

3. Result

3.1 Cell viability results from the Cv-ap and its fractions

. The result of MTT assay of the Cv-ap and its fractions is summarized in table 1. Concentration of IC_{50} was calculated by the MTT assay. The Cv-ap induced 50% of Hela cells death at the concentration of 0.921mg/ml. So we define 1 unit of specific activity as the Cv-ap IC_{50} concentration. The specific activity of the fraction is calculated as Specific activity= (IC_{50} of the fraction / IC_{50} of Cv-ap). The total activity = (specific activity * percentage

yield). Among the downstream fractions (P1, P2, P3 and P4), P3 and P4 have a lower IC_{50} compared to the crude extract Cv-ap which means they have a higher percentage of bioactivity components compared to the Cv-ap. P1 had very little effect on the Hela cells. P3 fraction has is the most potent (lowest IC_{50} value). So we chose P3 for further purification and analysis. The component EPHP3, obtained by the purification of P3 as outlined in figure 3 has the lowest IC50 concentration and highest specific activity (specific activity= 30.7), which means the concentration of active components is 30 times that of the crude extract Cv-ap.

3.2 SDS-PAGE gel Analysis

The concentration of each sample loaded on the gel was measured and determined by UV absorbance. The standard curve was made using Cv-ap. All samples were made at the same concentration (2.5mg/ml) before loading on the gel. Silver staining was used to stain the band (figure 3D). There were three clear bands around 26 kDa in lane Cv-ap, and two clear bands in lane P3, one band in lane EPHP3. We could not see the bands in lane HP3. All the bands are around 30 kDa.

3.3 MALDI-TOF Analysis

To investigate Cv-ap and fractions, we tested them by MALDI-TOF. The MALDI spectrum of Cv-ap (figure 4A) had multiple peaks and the major peak was about 25Kd, and there were additional peaks as 13kDa, 9kDa, and 6kDa. All these peaks were also observed in the MALDI spectrum of P3 (figure 4B). However, the MALDI spectrum of the EPHP3

was a little different compared the spectrum of the Cv-ap and P3 (figure 3C). The MALDI spectrum of the EPHP3 had four peaks at the m/z 6.9KDa, 9.2KDa, 13.5KDa and 25KDa which was 3, 4, 6 and 11 times of the number 2317Da indicating that they are ions of the same protein with varying charges.

3.4 MTT Assay of Ethanol Extract

The MTT assay result ethanol extract is summarized on table 2. The yield percent was calculated by 100* (weight of the extract/ weight of the dry roots). The IC50 was calculated by the MTT assay. There was no IC50 data of PE7 because the PE7 have very little effect on Hela cells. IC50 of SE7 was lowest (1.7mg/ml) among the other ethanol extract, which means the SE7 had high percent of anti-Hela components compared other extracts.

3.5 Cv Ethanol Eextract Effect on Normal Fibroblast Cells

To evaluate whether CV ethanol extract fractions effect/harm normal healthy cells we used the primary fibroblast cells and Hela cells were used as a control. Treatment with the 10mg/ml CV extract for 24 hr resulted in the death of most of the Hela cells (as indicated by PI staining of the cells). On the contrary, the treated primary fibroblast cells exhibited very little apoptosis (there was nearly no PI staining observed) (figure 6).

4. Discussion

4.1 Cv-ap and Its Fractions

Concentrated ammonium sulfate solutions have been shown to alter protein solubility. Therefore ammonium sulfate is widely used to

purify/precipitate proteins from aqueous solutions. The macromolecules including proteins present in the aqueous root extract were precipitated using ammonium sulfate in accordance with figure 1. The fraction Cv-ap is defined as the standardized crude extract. This extract mostly contains macromolecules as indicated by the MALDI-TOF spectrum (figure 4) and SDS-PAGE (figure 3D). The major component in the Cv-ap fraction is approximately 25 kDa (figures 3d and 4).The standard marker used here is normal dual colour protein marker which showed the Cv-ap is around 25 kda. However, in previous SDS-PAGE gel with glycoprotein standard marker, the same Cv-ap showed the molecular weight of about 18 Kda. The reason is because the hydrophilic glycan moieties can obstruct the binding of SDS, and the decreased hydrophobic interaction between the protein and SDS result in an inconsistent charge-to-mass ratio [14].

The SDS-PAGE analysis of Cv-ap and downstream purified fractions (shown in figure 3d) clearly indicates that Cv-ap has very high molecular weight species (smear in the high molecular weight region of the Cv-ap SDS-PAGE gel lane.). P3 had less smear bands at the top and fewer bands around 25-30 kDa and EPHP3 had the least smear bands at the top and only one clear band around 30Kd. It is interesting that there are no bands at the HP3 whereas there are bands in EPHP3. One possible explanation is that although all fractions had the same absorbance

(used for measuring concentration), most of the absorbance come from the small molecules such as small peptides which cannot be seen in the gel. It is likely that the protein around 30 kDa is very dilute in the HP3 sample, the precipitation step which generates the EPHP3 sample selectively concentrates the 30 kDa component (the small molecules were removed by the ethanol precipitation).

Cv-ap and P3 had some small peaks in their MALDI-TOF spectra although they have the similar major peaks as the EPHP3 (figure 4). These small peaks indicated that there were some other compounds in the Cv-ap and P3. EPHP3 had four peaks at the m/z 6.9KDa, 9.2KDa, 13.5KDa and 25KDa which was 3, 4, 6 and 11 times of the number 2317Da indicating that they are ions of the same protein with varying charges indicating that the sample is of fairly well defined purity.

4.2 Ethanol Extract of CV

The ethanol extract purification protocol is outlined in figure 5. In this purification protocol of the CV root, 85% ethanol was used to remove most of the small molecules and the hydrophobic molecules. Acetone was further used to remove the small and hydrophobic molecules. The IC_{50} of SE8 fraction (figure 5) was lower than that of the SE7 which meant that the major bioactive components were in SE7. However, SE7 is composed of molecules that can dissolve in 70-85% ethanol, which meant that bioactive components in SE7 were different

from the compounds in the water extract of Cv-ap (EPHP3 fraction figure 3).

The viability assay on the human primary fibroblast cells with the treatment of ethanol extracts indicated that the SE7 does not harm healthy cells wheras it has potent and selective bioactivity against cervical cancer cells indicating that is a promising therapeutic candidate against cervical cancer.

5. Conclusions

In this study, the Cv extract was further purified and analyzed. The EPHP3 fraction (figure 3) showed 30.7 fold increase in specific activity compared to Cv-ap, which was also supported by the results from SDS-PAGE analysis and MALDI-TOF spectroscopy. However, further research has to be conducted to identify the structure of the bioactive components. These are beyond the scope of this thesis.

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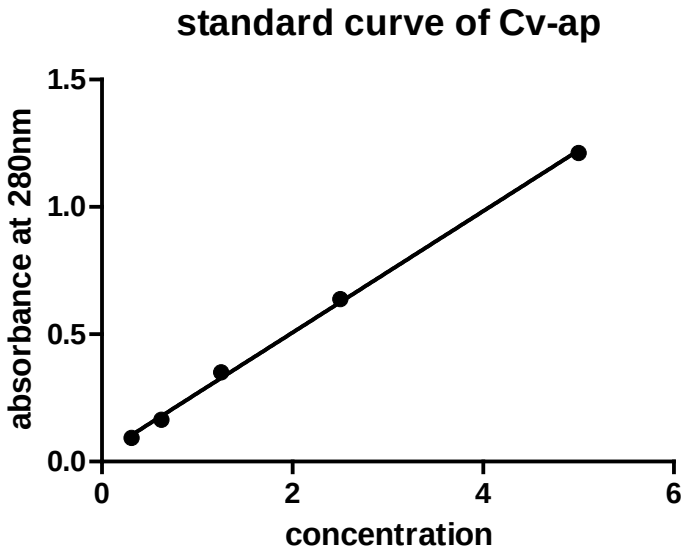

Figure 1 standard curve of Cv-ap

Sample	Concentration of IC_{50}(mg/ml)	specific activity*	yield percent	Total activity
v-AP	0.921	1	1	100%
P1	-	-	2.67%	-
P2	2.828	0.325672	8.27%	2.6933%
P3	0.15	6.14	28.99%	177.98%
P4	0.439	2.09795	11.67%	24.4831%
HP3	0.12	7.675	19.69%	151.12%
ESHP3	1.12	0.822	14.47%	11.90%
EPHP3	0.03	30.7	2.87%	88.11%

Table 1. Summary of the MTT assays of the Cv-ap and its fractions. IC_{50} is calculated by MTT assay. The specific activity is defined as the IC_{50} concentration of Cv-ap.

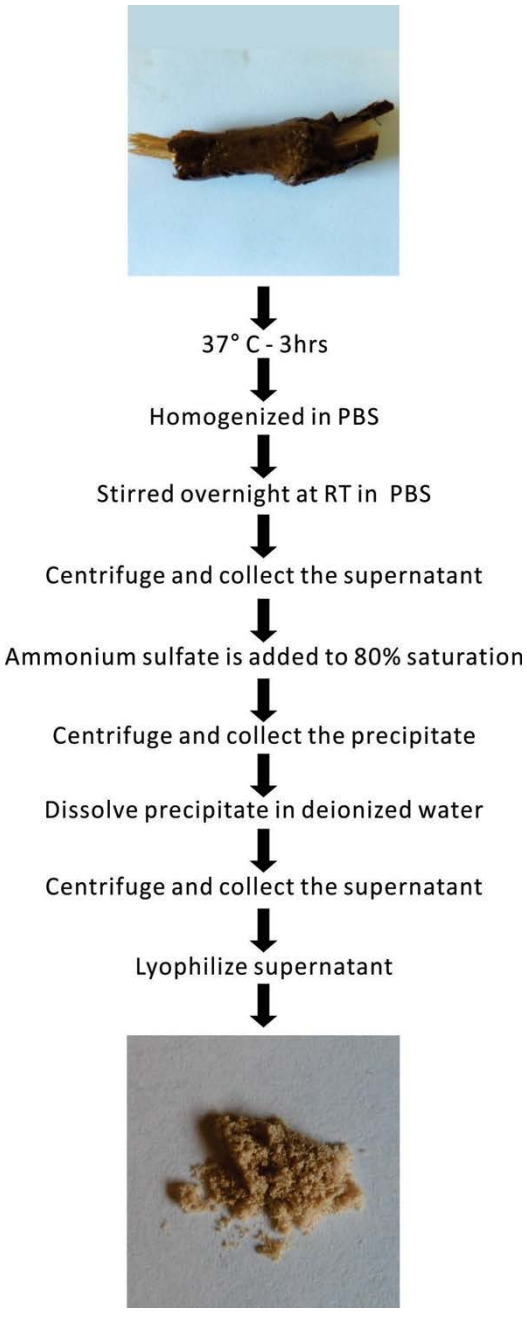

Figure 2 Schematic diagram showing the method of making Cv-ap.

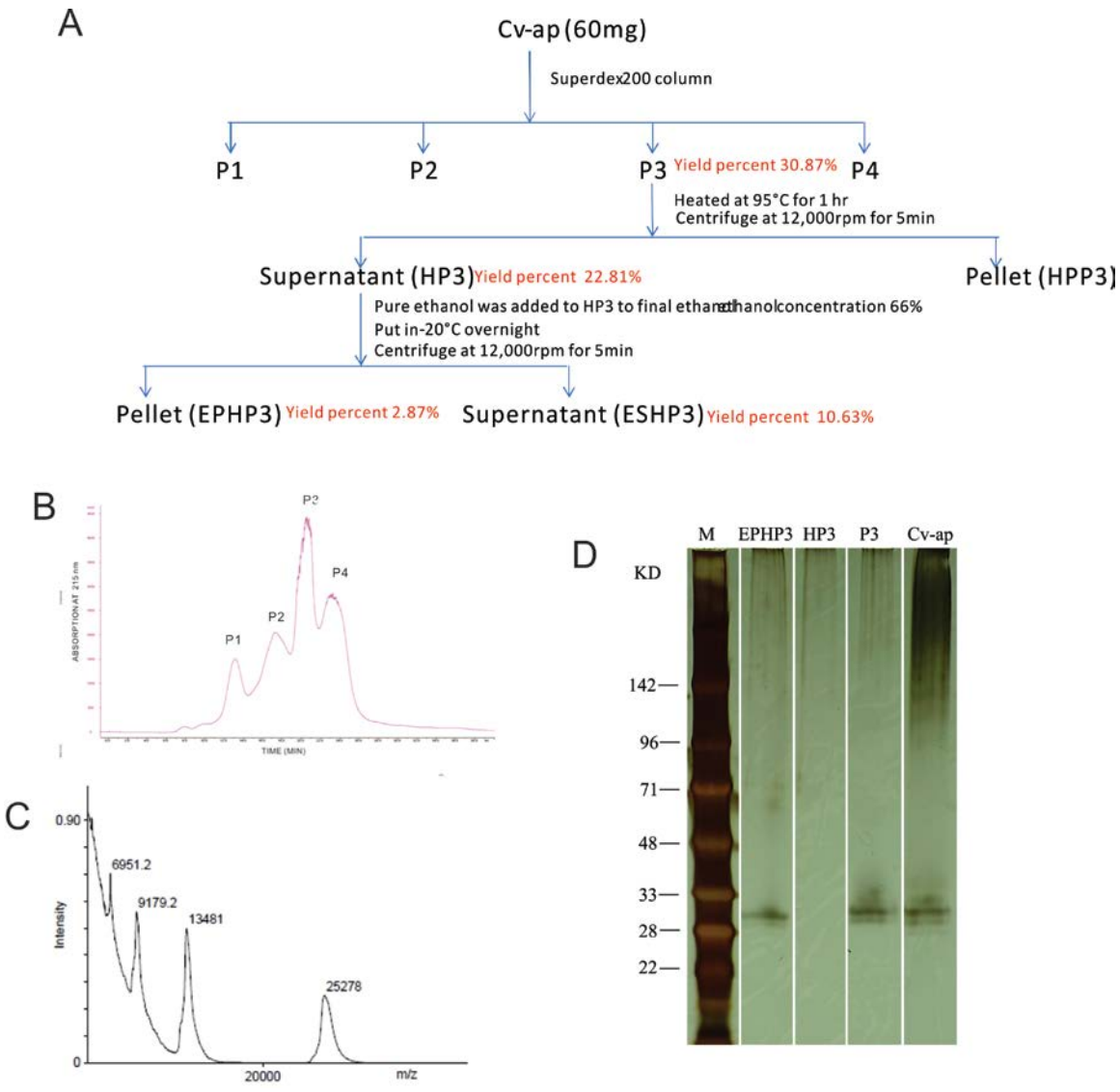

Figure 3. A: Schematic diagram showing the method for the isolation of active fractions from Cv-ap using bioassay guided isolation. B. FPLC file for the separation of Cv-ap by size-exclusion C. MADI-TOF result of the EPHP3 D. Silver staining gel of the Cv-ap and its fractions on 4-20 % gel at different stage of purification

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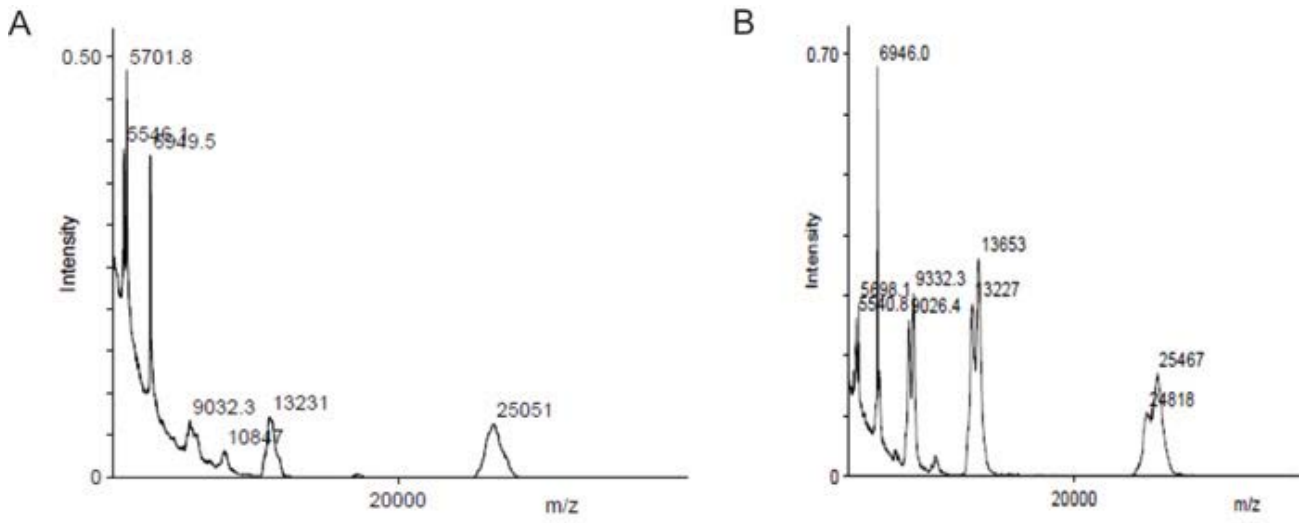

Figure 4. MALDI-TOF mass spectrum of fractions. A. Cv-ap B.P3

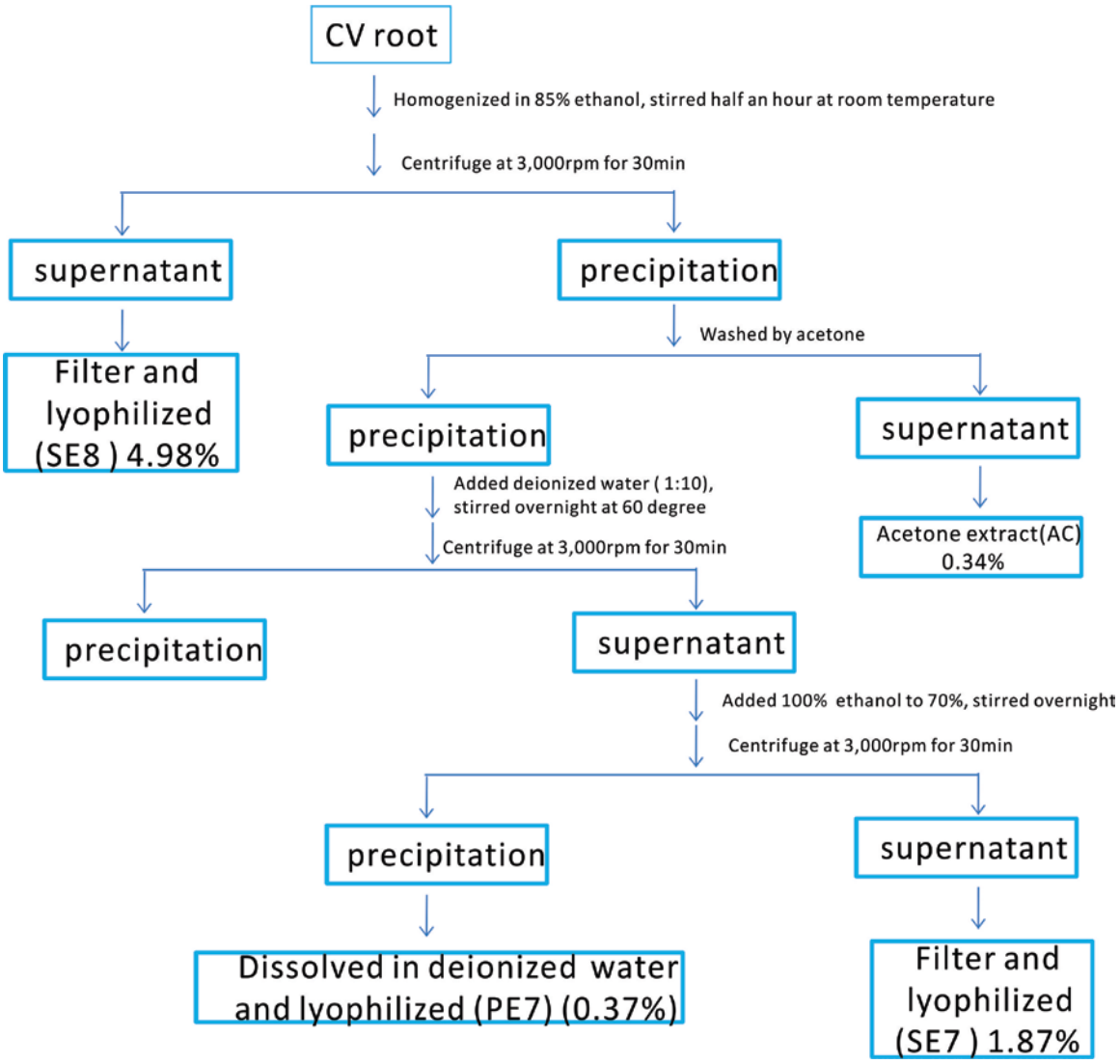

Figure 5 Schematic diagram showing the method of making ethanol extract.

	IC50 (mg/ml)	yield percent
Water extract	3.31	5.33%
Se8	4	4.98%
Se7	1.7	1.87%
Pe7		0.37%

Table 2. Summary of the MTT assays of the ethanol extract. IC50 is calculated by MTT assay.

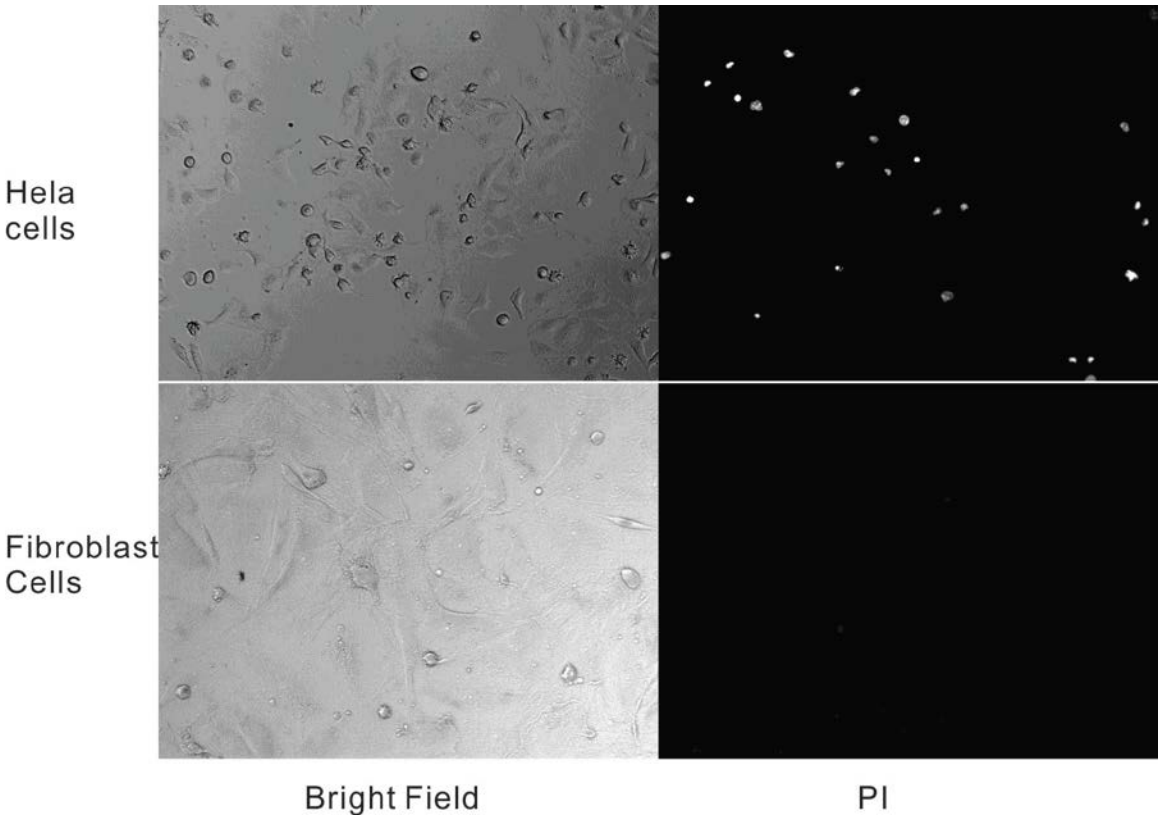

Figure 6. SE7 effect on the primary human fibroblast cells. SE7 (10mg/ml) were treated on the fibroblast cells and Hela cells for 8 hr and stained with the PI.

Chapter IV

Synthesis of the Curcumin Derivates

1.Introduction

Bioconjugation involves the linkage of two or more molecules to form a novel complex having the combined properties of its individual components [1]. The covalent linkage could occur between any kinds of molecules. However, Most of the time, bioconjugation is coupling of small molecules to a protein or protein-protein conjugation. The small moleule we have selected for producing conjugates is curcumin [(1E, 6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl) hepta-1,6-diene 3,5-dione] (figure 2) derived from Curcuma longa (Zingiberaceae – Ginger family) [2].

Curcumin is the principal curcuminoid of Turmeric which is a yellow dried powdered rhizome from Curcuma longa, and has been used in the ancient Indian Ayurvedic system of medicine [3]. Great interesting have been developed in curcumin as it shows the antioxidant [4, 5], anticancer [6, 7], anti-inflammatory [8], anti-Alzheimer's disease activity [9] and antibiotic activity [10]. The safety of eating curcumin has been established in clinical trials. In a trial conducted in Taiwan, volunteers were fed 8 g of curcumin orally without symptom of toxicity. However, only very low concentration of curcumin (1.77 μM) was detected in the plasma; the most possibility of this is that curcumin is relatively hydrophobic that leading to poor water solubility [11, 12]. Glucuronidation of curcumin could be another

explanation for the observation of very low plasma levels of the curcumin [13].

Potential bioactivity and non-toxicity have made curcumin as a good candidate for developing new green drug, however, the poor water/plasma solubility of curcumin limits its potential as a green drug candidate. Several strategies have been developed to overcome this problem: Liposomal formulations of curcumin have been developed, A curcumin-phospholipid complex has recently been reported, which has better hepatoprotective activity compared to free curcumin[14]; Polymeric micelles formulations of curcumin have been developed, the formulation has been evaluated in the mouse model: it was shown via an HPLC assay that the micellar formulation increased the half-life of curcumin 162-fold compared to unformulated curcumin [15]. Nanoparticles formed by random copolymers of N-vinyl-2-pyrrolidone-Nisopropyl acryl amide poly (ethylene glycol) monoacrylate have been loaded with curcumin to produce a water soluble formulation that was termed as “ Nanocurcumin” [16].

The chemical modification of ‘green drug candidates’ using few efficient reactions to produce derivatives with optimized bio-efficacy and pharmacokinetics is another route to green drugs [17]. Most of the encapsulation strategies have resulted only in a marginal improvement in

bioactivity [18]. Here, we adopted covalent modification and targeting strategies. We emphasized on generating a curcumin based drug should be easy to synthesize, have improved water/plasma solubility and amplified bioactivity. Bovine serum albumin (BSA) was chosen because of its low cost, less toxicity and high water solubility.

We developed a synthetic methodology to produce curcumin derivatives BSA-curcumin. At first, the curcumin was covalent attached with N-hydroxysuccinamide (NHS) group to produce the mono-NHS curcumin derivate. Secondly, BSA-curcumin was synthesized by the conjugation process where mono-NHS curcumin was couple to BSA curcumin. The solubility of this conjugate was evaluated. And cell cytotoxicity was anti-cancer bio-ability was measured by MTT assay on Hela cells and was compared with curcumin and its mono-function (alkyne, carboxylic acid) derivates.

2. Material and method

2.1 Chemicals

Curcumin, α-cyano-4-hydroxycinnamic acid (4-HCCA), Trifluoroacetic acid (TFA), Acetonitrile (ACN), formic acid and isopropanol were purchased

from Sigma-Aldrich. Curcumin mono-carboxylic acid, and curcumin mono-alkyne were synthesized in our lab (figure 2).

2.2 Synthesis of BSA-Curcumin Conjugate

BSA (1 ml, 5 mg/ml in sodium bicarbonate buffer, pH 8.5) was incubated with Curcumin mono-NHS (50 mg in 100 µl DMSO) at ice (0° C) for 2 h, then centrifuged at 5000 rpm for 5 mins and finally dialyzed against PBS, pH 7.4 for 24 h to remove unreacted curcumin mono-NHS. The dialyzed product was characterized via FPLC, SDS-PAGE and used for further bioconjugation (figure 1).

Synthetic protocol

Curcumin could be attached to BSA via reaction between lysine groups on the protein surface and novel NHS/sulfo-NHS derivatives of curcumin (scheme 1).

2.3 Size exclusion FPLC

1 ml BSA-curcumin solution in PBS (0.1 M pH 7.4) was loaded onto a HiPrep superdex 200 column and was separated using an Amersham Biosciences AKTA purifier system (GE Healthcare). The column was

equilibrated with 0.1 M ammonium bicarbonate buffer. The fractions were eluted with 0.1 M PBS and collected using a peak fractionation system

2.4 SDS-PAGE

BSA-curcumin conjugate and BSA (used as control) was running on a 4-20% PAGE gel SDS Cassette Gel. The gel was visualized at 365 nm (UV region) using Odyssey imaging system, prior to protein staining with coomassie blue.

2.5 Matrix-assisted Laser Desorption Ionization Time of Flight (MALDI-TOF)

The method has been described previously; briefly, BSA-curcumin and BSA were redissolved in 0.1%TFA deionized water at the concentration of 2.5 mg/ml. BSA was used as control. The sample was diluted [1 part into 4 part of matrix solution (saturated 4-HCCA in FWI (3:1:2)(formic acid: water: isopropanol)]. 1 µl sample/matrix mixture was spotted on the plate and waited for 5 min to allow the crystallization form at the interface of matrix and sample. The excess liquid was removed by vacuum aspiration. Each spot was washed with 1 µl of a ice cold 0.1% TFA solution and the excess liquid was removed by vacuum aspiration. Mass Spectroscopy (MS) experiments were performed using a MALDI- time-of-flight mass

spectrometer (Bruker Daltonik, Bremen, Germany). The data was analyzed by software Moverz.

2.6 MTT assay

Hela cells were plated in a 96-well plate at a density of 5,000 cells/well. Cells were then incubated for 24 h at 37 degree in presence of 5% CO_2 until they were well flattened and distributed evenly in the plates. Cells were washed with media and then treated with various concentrations drug dissolved in media. Next, cells treated with drugs were further incubated for 24 h at 37 degree and in presence of 5% CO_2. MTT solution (5 mg/ml dissolved in PBS) was then added to each well in an amount equal to 10% of the culture media volume. The MTT treated cells were then incubated for 2 h. After the incubation, formazan crystals in each well was dissolved by adding an amount of MTT solubilization solution (isopropanol-HCL) equal to the initial culture media volume. Each well then was carefully mixed to form a homogeneous color by pipetting up and down several times. The plate was covered with aluminum foil and put in a shaker for 15 min. Absorbance of each well was measured at 690 nm and 570 nm.

3. Result and Discussion

3.1 Characterization of BSA-curcumin by FPLC and SDS-PAGE

BSA-curcumin was analysed by running it through the FPLC using a HiPrep superdex 200 column. 0.1 M PBS was used as running buffer. BSA-curcumin have absorbance at 280 nm and 430 nm while BSA only have strong absorbance at 280 nm (figure 3). The reason is that BSA has a strong absorbance peak at 280nm and curcumin has a strong absorbance peak at 430nm. This demonstrates that the conjugation is occurred because the curcumin is hard to be dissolved in water. The FPLC result also proved that the BSA-curcumin is soluble in water compared to the insoluble of the curcumin. It should be noted that the solubility study outlined above measures the direct solubility of the compounds in water and closely models “real life” conditions, in contrast to other experiments in which the compounds are first dissolved in other solvents followed by dilution in water.

BSA-curcumin and BSA were run in SDS-PAGE, and BSA-curcumin was observed at 365 nm because curcumin shows fluorescence emission at 365 nm. On the contrary, the BSA itself has no fluorescence and can only be seen under the visible light as stained by commassie blue(figure 3).

3.2 Characterization of BSA-curcumin by MALDI-TOF

MALDI-TOF method was used to determine the average number of surface lysine residue modifications of the BSA. Results indicate that on an average BSA was modified with 4~5 curcumin (Figure 4). The formation of the conjugation was confirmed via FPLC and SDS-PAGE (figure 3).

3.3 BSA-curcumin effect on Hela cells

To investigate the cytotoxicity of the BSA-curcumin, we performed MTT assay on Hela cells with BSA-curcumin and curcumin derivate. To close the “real life” (aqueous environment), we dissolved BSA-curcumin, curcumin, Curcumin mono-carboxylic acid, and curcumin mono-alkyne directly in media with various concentrations (10mg/ml, 5mg/ml 2.5mg/ml and 1.25mg/ml). The result showed that except BSA-curcumin, curcumin and its derivatives had no bioactivity against Hela cells (figure 5). The reason was curcumin and its derivatives were insoluble in water and cannot inhibit the growth of Hela cells. On the contrary, BSA-curcumin was easily dissolved in water, which facilitated the curcumin groups to bind to Hela cells and inhibit the growth of Hela cells. At the concentration of 2.5 mg/ml

(~36.6uM) of BSA-curcumin, only 25% Hela cells survived, while curcumin have less inhibit effect.

4. Conclusion

The BSA-curcumin conjugate displaying curcumin in a polyvalent pattern is freely soluble in water and shows cytotoxicity against Hela cells, human cervical cancer cells, at micro molar concentrations. This data indicated that the BSA-curcumin conjugate can be a as potential drug against cancer.

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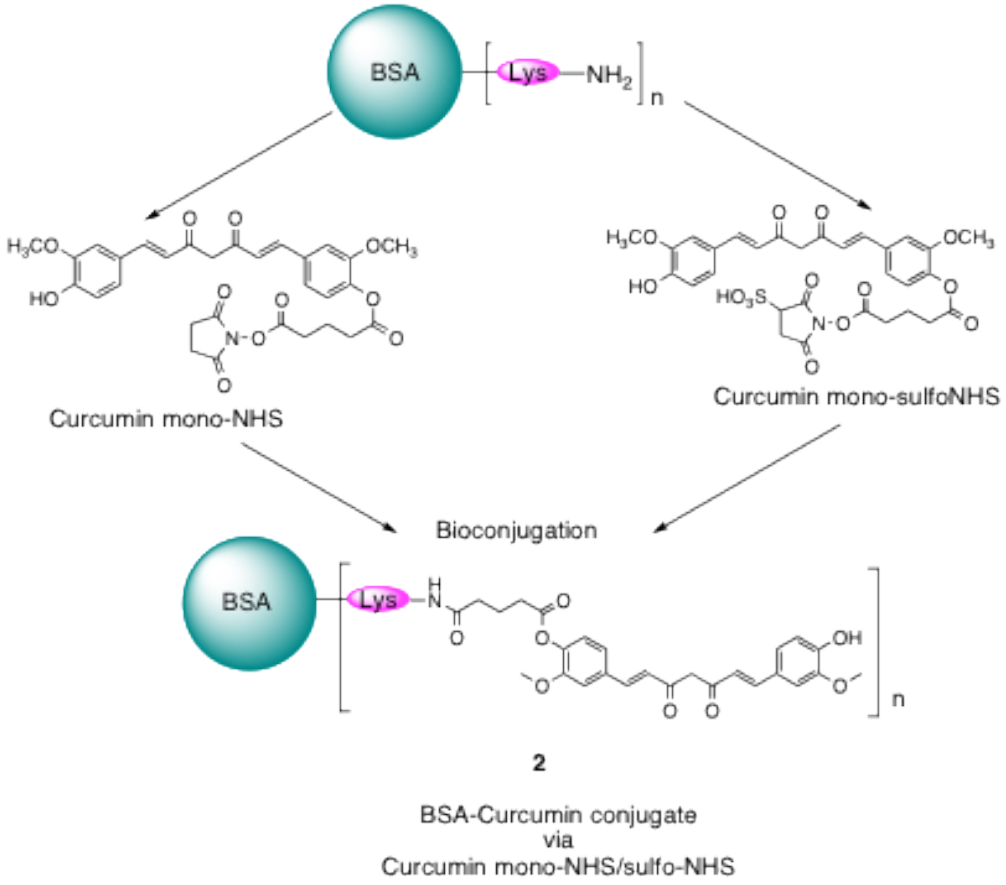


Figure 1. Synthesis of BSA-curcumin conjugate via NHS chemistry.

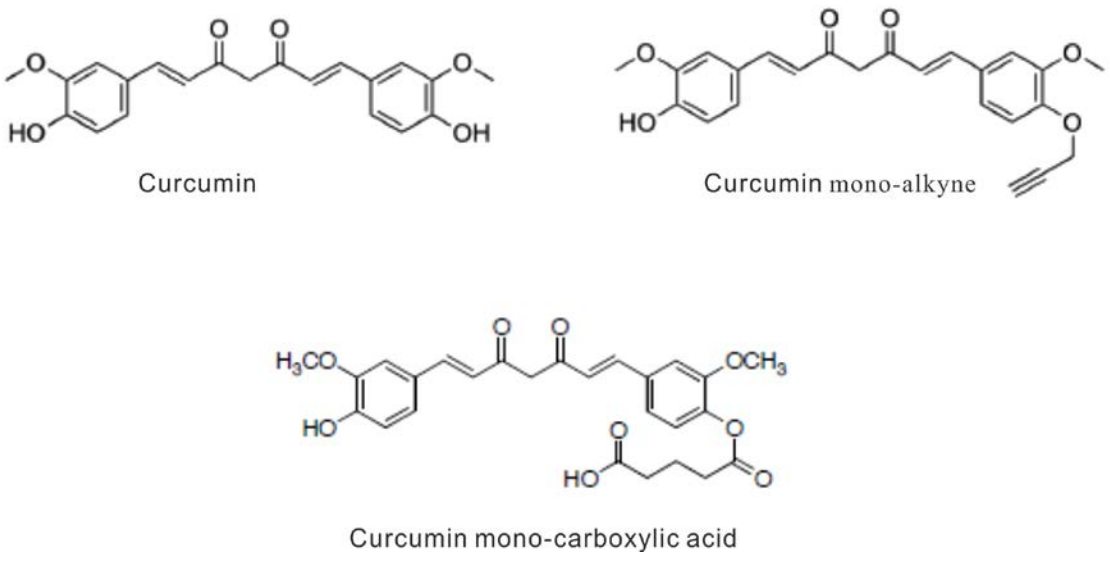
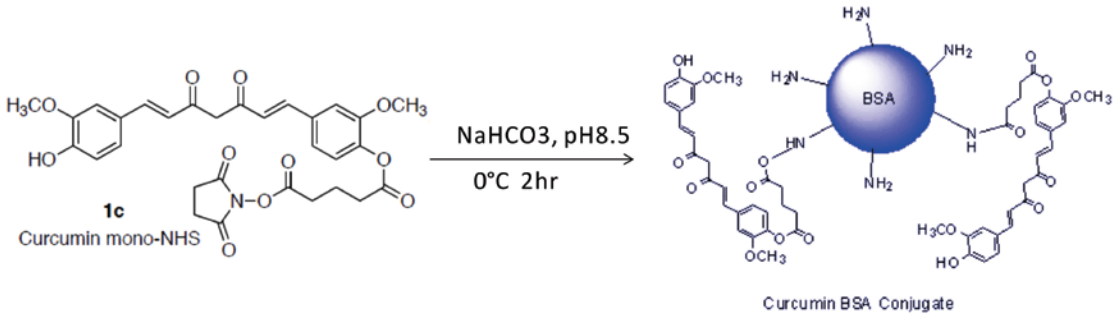


Figure 2. chemical structure of curcumin and its derivates



Scheme 1. Schematic representation to synthesize BSA-curcumin

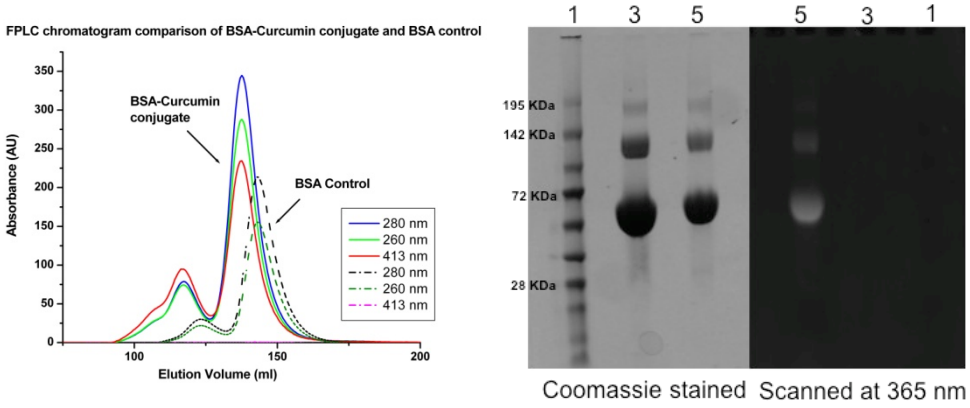


Figure 3. (Left) FPLC chromatogram of BSA-curcumin conjugate (solid line) and BSA control (dashed line) sample. (Right) Photographs of SDS-PAGE of BSA control sample (lane 3) and BSA-curcumin conjugate (lane 5).

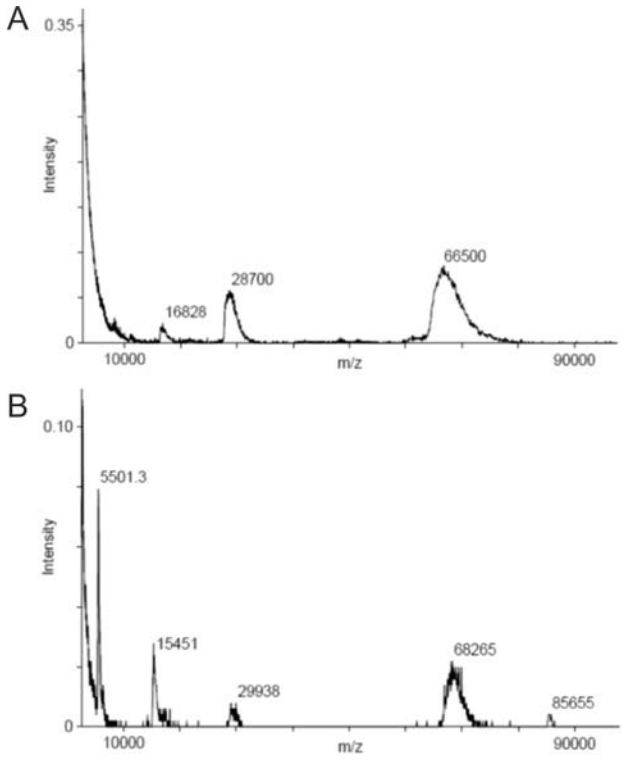


Figure 4. MALDI-TOF spectrum of BSA (A) and BSA-curcumin (B).

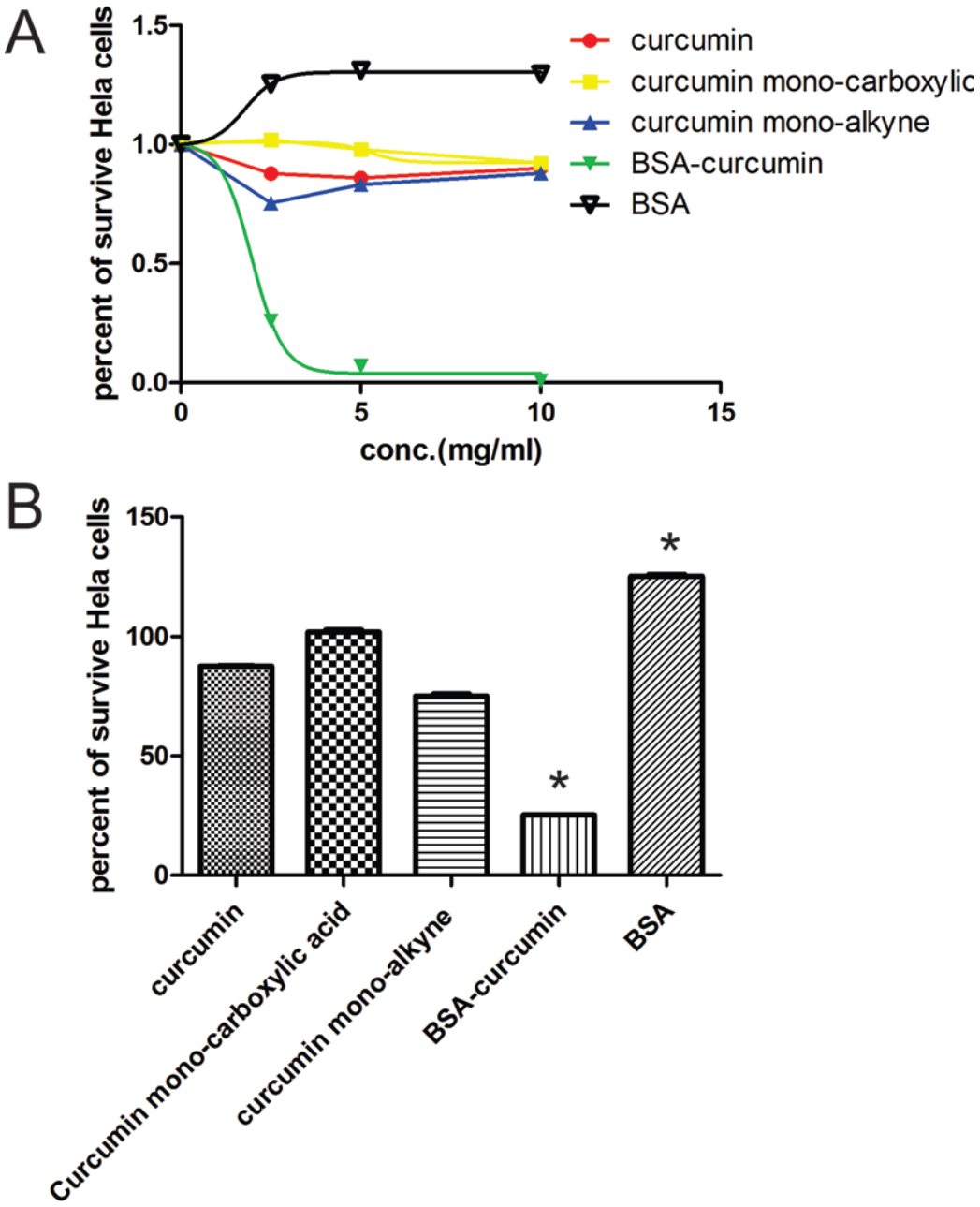


Figure 5. MTT results of Curcumin derivate. A. Curve of the MTT result of curcumin and its derivates at different concentration. B. The MTT result of curcumin and its derivates at 2.5mg/ml. *means the P<0.001.

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