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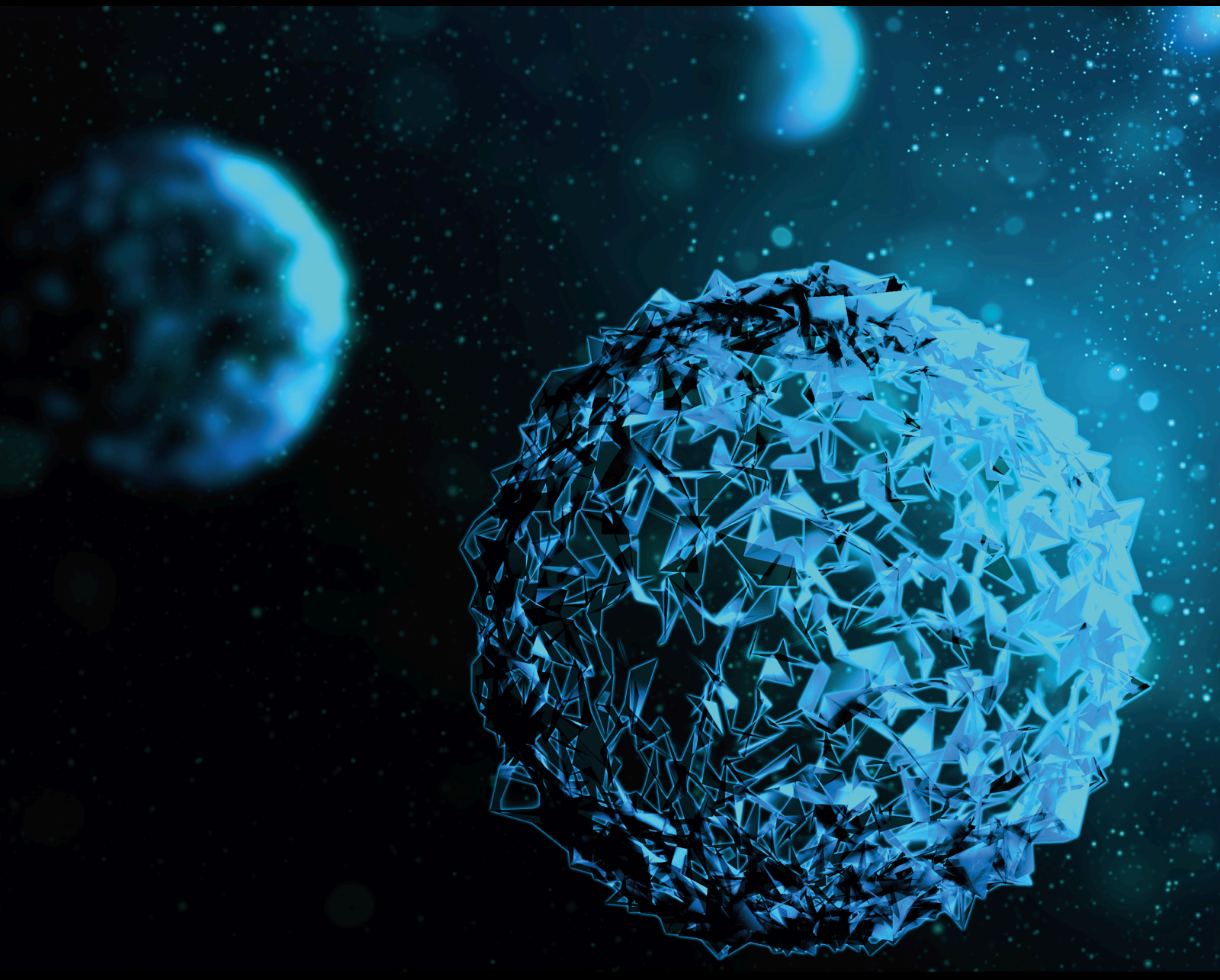
Lead Guest Editor: Yearul Kabir
Guest Editors: Md. Atiar Rahman, Mohammad Nazim, and Chen-Huan Yu



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Review Article

Plant-Produced Monoclonal Antibody as Immunotherapy for Cancer

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Plant-based products have expanded to include cancer immunotherapy, which has made great strides over recent years. Plants are considered inexpensive and facile production platforms for recombinant monoclonal antibody (mAb) due to the latest advancements and diversification of transgenic techniques. Current human biologics, including those based on mAbs produced by fermentation technologies using primarily mammalian cell cultures, have been replaced by plant-produced mAbs, which are cost effective, more scalable, speedy, versatile, and safer. Moreover, the use of animals for antibody production is always a question of ethical unambiguity, and the suitability of animal models for predicting the immunogenicity of therapeutic mAbs in humans and transposition of the immunogenic potential of therapeutic antibodies in animals to the human situation has no scientific rationale. Quite a few plant-based mAbs are approved for the treatment of cancer, ranging from tumors to hematological malignancies. This review focuses on the cutting-edge approaches for using plant-derived mAbs to suppress or prevent cancers. It also discusses the avenues taken to prevent infection by oncogenic viruses, solid tumors, lymphomas, and other cancerous conditions using mAbs. The review emphasizes the use of a plant-derived monoclonal antibody as a premier platform to combat cancer.

1. Introduction

Cancer remains one of the most feared diseases of our time because of its ability to metastasize, our failure to manage and treat the disease properly, and lack of a complete understanding of cancer development mechanisms. Despite the significant improvement in cancer therapies over the last three decades and applying molecular biology techniques, the global burden of cancer continues to increase. It is still one of the most devastating diseases worldwide. Until recently, the basic strategies to treat cancer included chemotherapy, surgery, and radiation therapy, and very recently, targeted therapy either in combination or in sequence has been introduced to reduce, remove, eliminate, or alleviate tumors. Although these strategies provide efficacy and effective actions in making enduring relief in patients with nonmetastatic and early cancers, they are usually unsuccessful in producing long-lasting benefits in patients with late-stage disease, except in certain leukemias, lymphomas, germ cell tumors, and testicular carcinomas. Unfortunately, these multidimensional approaches often face two main problems: development of resistance related to lengthy use and the presence of severe adverse effects made by the overall doses of radiation and the significant impact of cytotoxic agents on healthy cells and physiologic functions [1]. Apart from these, the economic burden from huge expenses for cancer treatments imposes an extreme social burden on a cancer patient and their family members. In this context of increasing cancer prevalence and socioeconomic burden, an alternative cancer-preventing therapeutic option is indispensably urgent. Immunotherapy has blossomed as a treatment option with enormous potential to avert or suppress cancer progression due to its direct influence on malignant cells with superior efficiency to target and attack the cancer cells [2].

However, recent studies on antibody-mediated killing responses against tumor cells and the generation of a huge number of antibodies against antitumor response helped produce monoclonal antibody (mAb) for recognizing specific antigens available on the surface of cancer cells. Despite their advantages and therapeutic potential, some functional limitations, such as the absence of cost-effective manufacturing of mAbs with high quality and purity level and limited scalability of the mammalian production system, impaired their widespread application as therapeutic agents for cancer. Additionally, mAb produced by *in vivo* methods can contain various mouse proteins and other contaminants that could lead to humans harboring pathogens. Therefore, for therapeutic applications, heterogeneous production platforms with affordable costs, scalability, and safety have been ensured using other bioorganisms such as plants, insects, bacteria, and yeast [3]. Among them, plants offer a novel system for the development and production of a monoclonal antibody with the criteria described above of expression paradigms.

2. Materials and Methods

A comprehensive literature work has been pursued to gather relevant information from major science repositories, including Google Scholar, Medline, PubMed, and Science Direct, specifically to classify plant-based monoclonal antibodies and their potential for different forms of cancer. Some articles were cited from other publications or accessed directly through the journal's website. They were considered based on the geographical region of their origin. The literature published in recent years is highly preferred for relevant actions. The keyword combinations for the search were plant-produced monoclonal antibody, monoclonal antibodies for cancer, plant-based monoclonal antibodies, monoclonal antibodies from a plant in cancer, plant products as monoclonal antibodies, etc. Additional information was incorporated by using some other keyword combinations such as plant extract for cancer, medicinal uses of plant-based monoclonal antibodies, and phytomedicinal monoclonal antibody. A total of 53 research articles reporting the action of plant-produced monoclonal antibodies in cancer have been recovered and presented in this review.

2.1. Scopes for the Readers/Audience. This review extends the novice researchers' scope to identify research gaps between the existing line of therapeutics and prospective alternatives but using a highly safer option for cancer treatment with monoclonal antibodies produced from plant bioresources. The decision-makers will be able to have the ways for producing, formulating, and marketing the plant-based mAbs to create easy access for those desiring the category of drugs described above. As a whole, the researchers will be able to focus on the current plant expression systems offering the features beyond the traditional benefits of eukaryotic protein modification and high adaptability, such as low cost and increased safety. An enormous opportunity is expected to develop with novel transient expression vectors that allow mAbs to be produced at unprecedented speed to mitigate potential pandemics. The potentiality for glycoengineering with plants to make mAbs with unique mammalian glycoforms for newer utility in making safe biobetters will also be exposed.

2.2. Why Immune Therapy Is Different: Scientific and Clinical Basis. Cancer is mostly a process of abnormal cell proliferation in an uncontrolled manner, and the cells usually fail to die normally. Cancer cells can invade healthy tissues and organs and eventually extend over the body's parts through the blood and lymphatic systems. In order for altered cells to be established in a host, they should develop ways to avoid eradication by the immune system and respond to treatment. Manipulation of the therapeutic approach can substantially alter the innate immune system, which leads to cell death and, under a suitable physiological environment, adaptive and innate immunity leading to oncolysis while helping long-term memory responses. Thus, immunotherapy for cancer is a treatment that allows a patient's immune system to fight cancer [1].

Conventional chemotherapy desires to rapidly attack the dividing cells within the body through unrestrained, static, indiscriminate, and toxic direct attack on both the malignant and normal cells to destroy the cancer cells more than it destroys the host cells. Recently, it has been reported that this may also target both stromal cells and immune cells [4, 5]. On the contrary, the salient features of immune-based therapy include the breadth of response, specificity, and memory. The tumor immunotherapies usually engage the immune system to identify and eliminate tumor cells, rather than targeting cancerous cells directly, and thus offers a more effective alternative treatment for some cancer patients. Unlike other conventional therapies, immune therapy may take a longer time to get the feedback of treatment because the immune system is mobilized to attack tumors. Sometimes, tumors may even show some pseudoprogression where the tumors grow at first, but eventually, they swell in size due to the infiltration of immune cells into tumors [6]. Most importantly, if immunotherapy can successfully activate the immune memory, it allows the body's defense against a threat to continue even after therapy has been withdrawn and possibly for as long as the patient's entire lifespan [1].

Since immunotherapy is based on the immune system's natural power to identify and remember cancer cells, it can be active against all types of cancer. It has been accepted as the first line of treatment for several cancers. Its efficiency was also confirmed against several types of cancers that were factually resistant to chemotherapy and radiation treatments [6]. Cancer cells share many resemblances with the healthy host cells, and this offers a challenge for achieving high levels of selective cytotoxicity. Chemotherapeutic monoclonal antibodies have appeared as standard therapeutic agents for many cancers in humans in the past decade; they were engineered with the predicted advantage of specificity, thus acting as "targeting missiles" toward cancer cells [7].

2.3. Monoclonal Antibodies: A New Subway to Prevent Cancer. The immune system is a sophisticated host defense system consisting of many biological structures and

processes that identify and destroy harmful disease-causing substances, such as bacteria and viruses. The immune system uses the immunoglobulins (Ig) or antibodies as powerful molecular tools to recognize and neutralize minute quantities of a given target analyte [8]. Recently, the mechanisms of antibody-mediated killing responses against tumor cells that induce consistent, effective, and durable cancer-suppressing activities have been revealed by experimental and clinical studies [3].

From 1940, the scientific understanding of antibodies such as its generation, diversity, and structure and Brunet's clonal selection theory demonstrated that one cell produces one specific antibody [9]. Further, monoclonal antibodies (mAbs) generated using hybridoma technology or fusion of spleen cells from an immunized mouse with immortalized myeloma cells [10, 11] can be used against one specific epitope. Researchers are now designing mAbs that can be targeted at a specific antigen found on cancer cells. These monoclonal antibodies can serve as substituted antibodies that can restore, boost, or imitate the immune system's attack on cancer cells. They are intended to bind to antigens that are usually more abundant on cancer cells' surfaces than on healthy cells' surfaces [12, 13]. For cancer, selecting the right antigen is not always easy, and until now, mAbs have demonstrated to be more suitable against some cancers than against others. As more antigens associated with cancer are found, researchers have created mAbs against more and more cancers, and these mAbs are now under clinical trials on several kinds of cancers. In the last ten years, record numbers of antibody therapeutics have entered into clinical studies and have been approved, and over 570 antibody therapeutics are ongoing in various clinical phases; 62 of them are currently in late-stage clinical studies. Depending on the therapeutic area, the progress of some clinical studies from phase 1 to approval has seen favorable success rates, ranging from 17 to 25% [14].

Even though advances in basic research guided the development of hybridoma technology in 1975, the first mAb used as a therapeutic was approved after eleven years. Since then, the development of mAbs has played a significant role in the pharmaceutical industry [15]. The US Food and Drug Administration (FDA), in the last couple of decades, has approved more than a dozen of mAbs as therapeutics for hematological malignancies and solid tumors [16]. The achievement of antibody therapeutics has inspired pharmaceutical companies to participate in the development of these molecules. Jointly, pharmaceutical industries are presently supporting the clinical trials of more than five hundred seventy mAbs. Ninety percent of those are going through early-stage interventions intended to evaluate the safety (Phase 1) or safety and preliminary efficacy of the molecules in different patient populations, as shown in Figure 1. Among Phase 1 mAbs, most (~70%) are for cancer, reflecting the recent significant increase in the entrance of anticancer antibodies in clinical studies. In contrast, at Phase 2 and end-stage clinical studies, the number of mAbs being planned for the treatment cancer is similar to the number of mAbs being planned for the treatment of noncancer diseases [14].

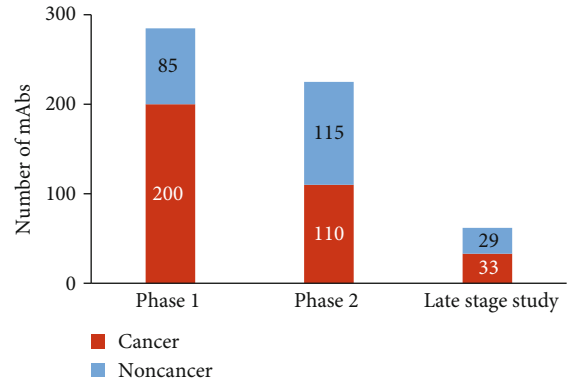

Figure 1: Clinical phases for antibody therapeutics in development. Totals include only antibody therapeutics (biosimilars and Fc fusion proteins were excluded) sponsored by commercial firms [14].

2.4. mAb Structure and Anticancer Mechanism. Antibodies are mostly figured as the typical mammalian serum-type immunoglobulin G (IgG), which contains two identical light chains and two identical heavy chains and linked by disulfide bonds. An antibody is made up of a constant region, the region that has a constant structure for different antigens, and a variable region, the region that changes to different structures depending on differences in antigens. The amino acid terminal sequence domains of the heavy and light variable chains are called V_H and V_L, respectively. In contrast, the corresponding constant sequence domain of each chain is called C_H and C_L. These variable and constant regions are dedicated to preventing pathogens from entering or damaging cells and recruiting various immune-related molecules and cells to disrupt antigen's functions and destroy tumor cells or pathogens through binding with the specific antigen. The antigen-binding fragment (Fab) is the region that binds to Fc, the fragment crystallizable region or the tail region of an antibody, and interacts with cell surface receptors (Fc receptors) and some proteins of the complement system. The Fc regions of IgG bear a highly conserved N-glycosylation site, which is inevitable for Fc receptor-mediated activity [3, 17].

Antibodies are the immunoglobulins classified into five groups: IgM, IgG, IgA, IgD, and IgE, according to their structural, physiochemical, and immunological properties. These five classes have different Fc regions and thus different effects or functions, and some of these have more complex structures than the simplest IgG. For example, IgA (sIgA), the secretory-type, has four heavy chains and four light chains, which assemble as two IgG-like tetramers and a secretory component and a joining chain. The expression of a full-sized IgG in plants requires two genes, whereas the expression of a sIgA requires four genes. In addition to natural antibodies, there are also many smaller antibody derivatives that are functional in terms of antigen binding such as Fab, $F(ab')_2$, minibodies, large single chains, single-chain variable fragments (scFvs), bispecific scFvs, diabodies, camelid antibodies, nanobodies, and antibody fusion proteins as shown in Figure 2. The functional and structural differences between

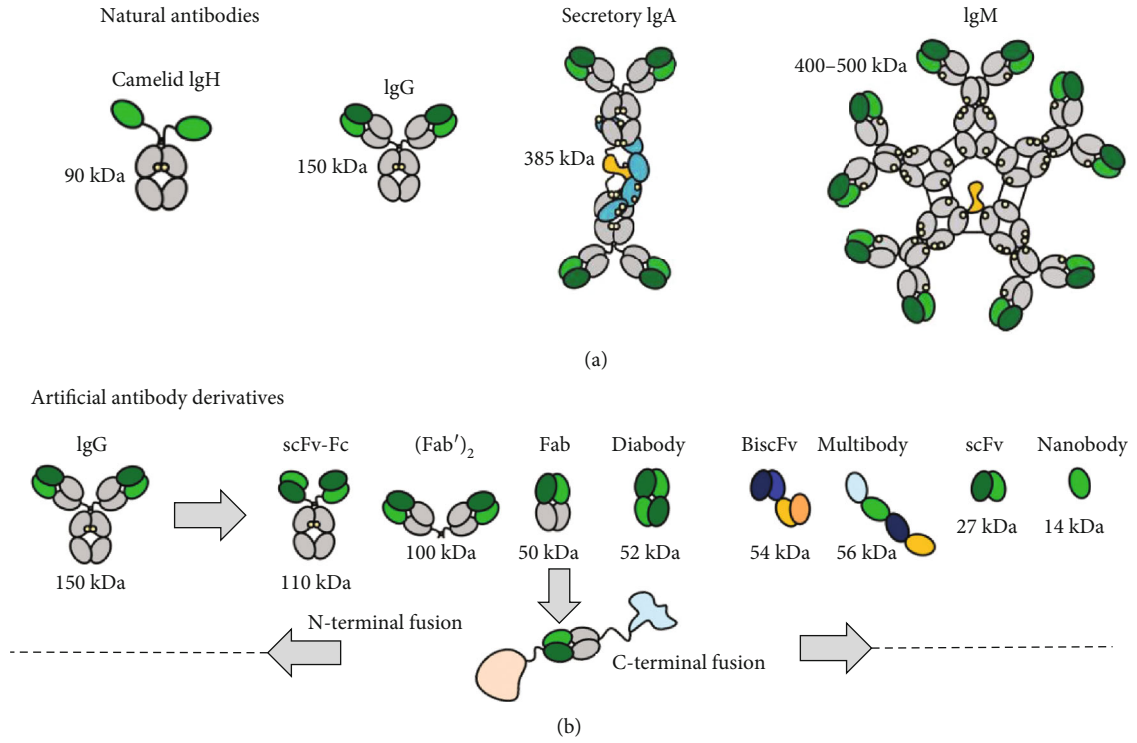

Figure 2: Domain architecture of natural antibodies and some engineered recombinant variants. Domains representing the antigen-binding site are indicated in green and the constant domains in gray [18].

variable domains, constant domains, and the free N- and C-terminal ends of the individual domains have given rise to enormous modifications, derivatives, and combinations [18]. Although antibodies are not a natural production of plants, plants can be engineered to do so by introducing the corresponding immunoglobulin genes. In this way, plants can be instructed to produce antibodies in all kinds of different formats that can target the antigen of choice.

Chemotherapeutic monoclonal antibodies are designed to function in diverse ways. A particular drug may work by more than one means. They can act by directly attacking cancer cells, flagging cancer cells, and thus binding cancer and immune cells, triggering cell-membrane destruction, blocking cell growth, preventing blood vessel growth, blocking immune system inhibitors, delivering radiation treatment, and delivering chemotherapy. They can target cancer cells by binding to cell surface antigens. Cell surface antigens include antigens associated with growth and differentiation, such as cluster of differentiation (CD: e.g., CD20, CD30, CD33, and CD52), epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (HER2), receptor activator of nuclear factor kappa-B ligand (RANKL), vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR), integrins (e.g., $\alpha V\beta 3$ and $\alpha 5\beta 1$), fibroblast activation protein (FAP), and extracellular matrix metalloproteinase inducers (EMMPRIN) [7].

The cancer cell-neutralizing capability of antibodies is considered to be a critical mechanism for immunotherapy. However, certain additional activities, such as antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity, are developing as important contributors to the protection of immunity. Antibody effector functions are due to the effective interactions between their Fc region and Fc receptors of immune cells, including the binding property of mAb to the targeted antigens [3]. In cancer treatment, antibodies have been exploited to eliminate tumor cells by blocking signaling pathways. Recent achievements in monoclonal therapeutic strategy have been attained through technological breakthroughs that enable the creation of modified antibodies that display greater abilities to recruit innate immune killing through antibody Fc glycosylation modification. This posttranslational alteration of the Fc domain of antibodies *in vivo* could accelerate the antibodies' therapeutic efficacy to design monoclonal therapeutics and next-generation vaccines [19].

2.5. Classification of Chemotherapeutic Monoclonal Antibody (CmAb). Monoclonal antibodies (mAbs) are now well known as targeted therapeutics for malignancies, transplant rejection, and infectious and autoimmune diseases, besides a range of new indications. However, the administration of mAbs can cause four kinds of risks: autoimmune diseases, cytokine release syndrome, opportunistic infections, and organ toxicity. Depending on the degree of humanization, which is highly variable, immunogenicity could also lead to adverse effects due to immune-complex formation [20, 21]. Thus, one significant attention of antibody engineering in the past thirty years has been to decrease immunogenicity and enhance the production of antibodies appropriate for human therapeutics. With the understanding of disease biology, recombinant engineering technology, and antibody

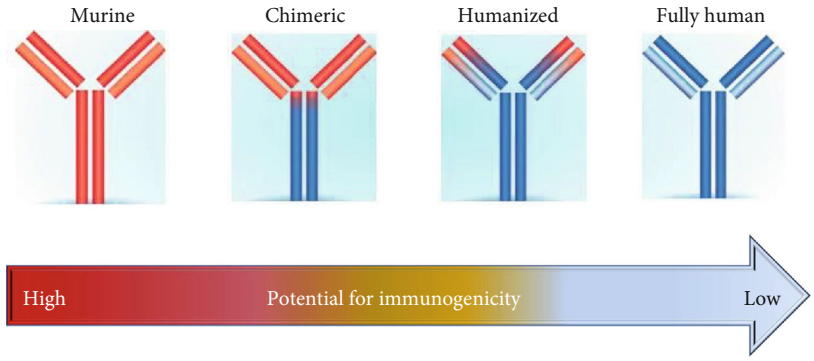

Figure 3: Classification of chemotherapeutic monoclonal antibodies and their corresponding immunogenicity.

mechanisms, a range of novel types of antibody molecules are evolving as promising new generation therapeutics [11].

Advances in genetic engineering technologies have resulted in the development of four main types of CmAbs: murine, chimeric, humanized, and human CmAbs; these were derived exclusively from mouse and were the first to be applied in cancer chemotherapeutics. Chimeric CmAbs typically comprise variable regions gleaned from a murine source, and constant regions (65%) from a human source can also be nonhumanized (chimeric trifunctional CmAbs), such as the rat-mouse hybrid. Humanized CmAbs are predominantly (90%) engineered from a human source except that the Fab portion's complementarity-determining regions are of murine origin [7]. Chimeric trifunctional CmAbs are characterized by a unique capacity to bind with three different cell types: tumor cells, T lymphocyte cells, and accessory cells; thus, they transiently link immune effector cells to tumor cells, which produce cellular cytotoxicity toward the tumor cells [22, 23]. The development of chimeric CmAbs that possess a fully human Fc portion provided considerably less immunogenic and more efficient interaction with human effector cells and the complement system than murine CmAbs. Humanized CmAbs are even less immunogenic than chimeric CmAbs. Human CmAbs, which are 100% human, are engineered from transgenic mice. Compared to chimeric and humanized CmAbs, they have higher affinity values toward human antigens and minimal or no hypersensitivity responses, as explained in Figure 3 [7, 24].

Chemotherapeutic monoclonal antibodies may be conjugated to other forms of cancer therapy, and this conjugation provides a targeted attack on cancer cells and therefore reduced widespread systemic toxicities to healthy cells. There are three types of conjugated CmAbs: radiolabelled CmAbs (linked to radionuclide particles), chemolabelled CmAbs (linked to antineoplastic drugs), and immunotoxin CmAbs (linked to plant and bacterial toxins) [7]. Antibody-drug conjugates (ADCs) are among the fastest-growing groups of cancer therapeutics. More than 60 ADCs are in clinical development for cancer therapy [25].

ADCs characteristically target overexpressed and internalizing antigens on the cancer cell surface and in solid tumors, so that a drug is released very selectively in the tumor microenvironment [26]. A perfect cytotoxic agent should be extremely effective, remain stable while connected to ADCs, destroy the targeted tumor cell upon internalization and release from the ADCs, and uphold its activity in multidrug-resistant tumor cells [27].

2.6. Advantages of Using the Plant for mAb Production. Despite significantly effective therapeutic actions, mAb treatment for cancer has not been established due to the high production expenditures, potential human pathogen contamination, and limited expandability of the mammalian cell-mediated system. Consequently, heterologous production platforms with safety, cost effectiveness, and scalability were established using different organisms such as bacteria, insects, yeast, and plants [3]. Although the existing fermenter-based production of antibodies has biomedical importance, it is costly and tedious and has low yield obtained via the purification process. Plants can be used as promising biofactory systems for the large-scale antibody production to defeat cancer and can bring hope for resource-poor nations to make a move from concept to reality. Some are already approved by the FDA for a clinical trial (Table 1). This is due to the high production capacity, inexpensive large-scale cultivation process, low downstream processing requirements that can be grown under containment conditions, and avoidance of ethical issues associated with transgenic animals [11, 28]. Currently, there are over a dozen FDA-approved mAbs, and as many as 700 therapeutic Abs may be under development. As of 2001, four antibodies expressed in plants had shown the potential to be useful as therapeutics. A chimeric secretory IgG/IgA antibody effective against a surface antigen of *Streptococcus mutans* has been expressed in tobacco and has been demonstrated to be effective against dental caries. Therefore, plants have potential as a virtually unlimited source of mAbs, referred to by some as "plantibodies." Tobacco plants have been used extensively for antibody expression systems [17]. However, several other plants have been used, including potatoes, soybeans, alfalfa, rice, and corn. Antibody formats can be full sized, Fab fragments, single-chain antibody fragments, bispecific scFv fragments, membrane-anchored scFv, or chimeric antibodies. Plant cells, unlike mammalian cell expression systems, can express recombinant secretory IgA (sIgA).

Compared to using mammalian cells in conventional methods, the use of plants for Ab production proposes numerous irreplaceable benefits. Plants are widespread,

Table 1: Examples of plant production systems used for cancer immunotherapy [28].

Malignancy	Antigen/plant system used
Hepatitis B virus-induced hepatocellular carcinoma	HBsAg expressed in transgenic plants
Hepatitis C virus-induced hepatocellular carcinoma	E7 protein expressed in chloroplasts
Non-Hodgkin's lymphoma	Full IgG expressed in TMV-based expression system
Breast cancer	PVX nanoparticles expressing HER2 epitope
Solid tumors	PapMV nanoparticles
Lung melanoma	CPMV nanoparticles
Solid tumors	TMV nanoparticles displaying cRGD

CPMV: cowpea mosaic virus; HBsAg: hepatitis B virus surface antigen; PapMV: papaya mosaic potexvirus; PVX: potato virus X; TMV: tobacco mosaic virus.

plentiful, and develop rapidly; they typically mature after one season of growth. It is possible to bring the product to the market within a short time, which eventually lowers the production cost. Plants also reduce screening costs for bacterial toxins, viruses, and prions because they are less likely to introduce human or animal pathogens than mammalian cells or transgenic animals [29]. It has been reported that plant-based antibodies expressing up to 1% of total soluble protein will cost 0.1% of that of the mammalian cell culture system and up to 2-10% of microbial systems [30]. Plants share a comparable endomembrane system and secretory pathway with human cells, distinct from bacterial and other prokaryotic systems. Although plants produce a reasonably high yield of Abs in a comparatively shorter time, they do not activate immune responses that animal Abs are prone to do after facing foreign/non-self-agents [31].

Like animal cells, plant cells also have posttranslational modification mechanisms that allow them to be considered factories for therapeutic proteins, including antibodies [32]. It has been reported that glycoengineered plants have a considerably higher degree of glycan homogeneity. For example, the plant-derived version of h-13F6, an anti-Ebola virus monoclonal antibody carrying the complex N-glycosylation and lacking a fucose core, showed higher efficacy than the original version originating from mammalian cells [11]. Furthermore, plants are proficient in synthesizing and assembling all types of Ab molecules effectively, for example, the tiniest antigen-binding domains, fragments, and full-length and even multimeric Abs [29]. Therefore, among all kinds of production platforms, the use of plants to produce anticancer mAbs is now attracting attention.

2.7. Posttranslational Glycosylation in Plants. Posttranslational alterations of proteins happening in plant cells resemble those in animal cells. The accurate assembly of complex molecules, such as Abs, is supported by chaperones that facilitate the folding and formation of disulfide bonds. At the same time, the addition of *N*-glycans is accomplished by particular cellular glycosyltransferases [33]. There is a significant difference between mammalian and plant-derived glycoproteins because N-glycan synthesis in the endoplasmic reticulum is almost well preserved in all eukaryotic cells. In contrast, N-glycan processing and O-glycan biosynthesis in the Golgi apparatus are kingdom specific [34]. A major concern is the presence of beta 1,2-xylose and core alpha 1,3-fucose residues on complex N-glycans, as these nonmammalian N-glycan residues may inflame undesirable side effects in humans [34–36]. This highlights the need for the use of glycoengineered plants to remove any potentially antigenic N-glycan structures, if there is, for the production of plant-derived recombinant proteins intended for parenteral human application due to possible immunogenic reactions. Plants' fast, flexible, and easily scalable endogenous N-glycosylation machinery permits the synthesis of complex N-glycans lacking β-1,2-xylose and core α-1,3-fucose. Therefore, the removal or distraction of the genes responsible for integrating these glycan epitopes, i.e., 1,2-xylosyltransferase and core 1,3-fucosyltransferase, offers a sophisticated technique to solve this issue [36]. It is also evident that the glycoengineered plant-derived antibodies, e.g., 2G12 and its Chinese hamster ovary (CHO) cells, have performed and produced counterparts [37]. The feasibility of this strategy was confirmed by the generation of *Arabidopsis thaliana* knockout plants lacking XylT and 1,3-FucT. Without showing any noticeable phenotype under standard growth conditions, these plants were viable and produced proteins carrying complex N-glycans lacking xylose and fucose [36].

The final and most complicated step of human N-glycosylation is terminal sialylation. Several drugs need sialylated oligosaccharides for optimal therapeutic potency, whereas convincing evidence suggests that plants do not have sialylated glycoproteins. Until recently, only mammalian cell-based systems were used for manufacturing, which can accomplish this vital posttranslational modification. The introduction of six proteins from the mammalian sialylation pathway into plants permits the biosynthesis of sialic acid, its activation, its transport into the Golgi apparatus, and finally its transfer onto terminal galactose, which is a milestone in plant glycoengineering [38]. This shows the massive flexibility of plants, allowing them to tolerate mammalian glycosylation and the large extent of conservation between mammals and plants [36]. Overall, current advances in plant glycoengineering permitted the synthesis of "human-like" recombinant glycoproteins with a highly homogeneous glycosylation pattern. Thus, plant expression systems are considered resourceful platforms for the production of mAbs with enhanced desired features [33].

2.8. Production of mAbs in Transgenic Plants. With current progress in genetic engineering, researchers now produce transgenic plants with multiple ideal traits. They are now capable of inserting advantageous/desired genes from a

completely different species or even from a different kingdom into the target plant [39]. These transgenic plants are considered one of the most promising human therapeutic Ab syntheses [11]. The first correctly assembled and functional human antibody, IgG, was successfully produced in transgenic tobacco plants and dates back to almost 30 years ago [40]. This initial success was quickly followed by the successful expression of different antibody formats such as secretory immunoglobulin A (sIgA), Fab fragments, single-chain antibody fragments (scFvs), minibodies, single variable domains, antibody fusion proteins (immune cytokines), scFv-Fc antibodies, and camelid heavy-chain antibodies also in transgenic plants [33, 41]. Twenty years after the successful expression of sIgA antibodies in transgenic plants, the multimeric antibody IgM was recently produced in plants [42].

There are two types of expression strategies based either on the stable transformation of the nuclear genome or on transient expression systems exploiting viral or *Agrobacterium tumefaciens* transfer DNA (T-DNA) expression vectors. Transgenic plants producing correctly assembled whole mAbs were conventionally obtained by cross-pollinating two transgenic lines separately transformed with the antibody heavy-chain (HC) or light-chain (LC) genes. This time-consuming strategy is now replaced by more proficient and fast approaches based on binary vectors containing HC and LC coding sequences in the same T-DNA. Thus, complete IgGs can be produced from transgenic plants in a single transformation event [43]. In the case of transient or epichromosomal transformation, the introduced sequence is not heritable; this is essentially a batch process. This reduces the risk of environmental biosafety issues linked to the propagation of the transgene through seeds or pollen. Generally, large amounts of protein were produced through this approach in a very short period, usually a few days to weeks, which is not achievable via stable transformation [33]. As every plant in each batch should be infiltrated with bacteria, the potential drawback of this rapid method for massive scale production is that this infiltration merely transfers the fermentation costs of bacteria production [44]. The use of transgenic plants can overcome the problem through a well-defined master and working seed banks that can be established. Consecutive batches can be used without further manipulation [45]. These make transgenic plants the most suitable plant-based arrangement for very-large-scale production. Greenhouses or vertical farming units appear to be the most prospective scenarios for the mass production of mAbs in transgenic plants, whereas, due to the absence of containment, an open field approach for the cultivation of transgenic plants may not be able to ensure the complete biosafety for such pharmaceutical products [46].

Other types of production arrangements are plant-based but require cultivation systems similar to those used with mammalian cell cultures, such as aquatic plants, plant cell suspension cultures, and plant tissue cultures such as hairy root culture. These denote an effective method for heterologous protein synthesis in a sterile condition with low contamination risks by human components and pathogens. Additionally, both transformed plant cells and organs can be propagated indefinitely with a simple nutrient requirement. The protein of interest can be secreted into the culture medium, thus providing easy product collection and purification. On the other hand, the low protein yields (in the range of mg per liter of culture) and the complications in setting up large-scale production in bioreactors represent the foremost challenges for the future exploitation of these plant expression platforms [33, 46].

2.9. Plant Selection for Antibody Production. There are numerous plant species, which can be proficiently engineered for mAb production. These include *Nicotiana benthamiana* (a related wild species of tobacco), *Arabidopsis thaliana*, lettuce, potato, and maize; however, a significant amount of antibodies stated in the literature have been expressed in transgenic tobacco (*N. tabacum*) [33]. The high biomass yield and the rapid scale-up by high-volume seed production are the main advantages of tobacco compared to other plant species. The whole tobacco plant biomass (both leaf and stem) can produce the recombinant therapeutic proteins, eventually increasing the upstream production cost effectively [47]. Additionally, tobacco is a nonfood, nonfeed, and well-specified expression system, excluding human pathogen contamination, which can decrease biosafety concerns. The other leafy plant alfalfa (*Medicago sativa*) has a high yield of biomass and a homogeneous glycan structure, making it attractive for antibody production and giving it a comparative advantage over the tobacco plant [48]. Tobacco contains nicotine or other toxic alkaloids that require an additional extraction procedure, and tobacco produces heterogeneously N-glycosylated antibodies. Oxalic acid compounds remain in alfalfa that affect the downstream processing and produce lower amounts of leaf biomass than tobacco, which is the major disadvantage of alfalfa. At the same time, this problem is overcome by the high level of protein in alfalfa leaf tissues, which maximizes the accumulation of recombinant antibodies in plant biomass. However, alfalfa is used as an animal feed though the biosafety concern is not resolved [11]. Some vegetable plants which have comparatively high total soluble protein levels might be advantageous to use for recombinant protein expression. Compared to other plants, the leaf biomass of Chinese cabbage has the highest total soluble protein level, making it a potential candidate bioreactor for the production of recombinant therapeutic proteins [3]. The seeds of legumes and cereal crops such as soybean, rice, and maize have been used for antibody production. Although maize is favored because of its inexpensive, high-quality, large-scale output, it is a wind-pollinated species; thus, it contains the risk of outcrossing to food crops. As seeds have a low level of protease activity due to the high level of protease inhibitors in most of the cases, antibodies expressed in corn seeds are stable for more than three years without losing activity at room temperature [49, 50], whereas the significant drawback of leafy crops is that specific proteins are unstable unless the leaf tissue is frozen or processed because leaves with an active metabolism have high protease activities for degrading particular proteins [51]. Even though rice is a food crop, it has strange advantages over other plants. Transgenic rice seeds have been developed for the production of a delivery vehicle for oral tolerogens [52]. Exciting results have also been

obtained in the small aquatic plant *Lemna minor* (duckweed) [53].

Although the plants show potential advantages in antibody production, they may have allergic reactions to plant proteins which is what human N-glycosylation is incapable of; i.e., their culture parameter becomes uncontrollable, and contamination risks for soil, bacterium, and pollen are less avoidable [11]. Besides, as plants and prokaryotes have differences in codon usage patterns, this can lead to the inefficient expression of prokaryotic proteins in plants [17]. The concern over possible toxin transmission to food crops can be significantly reduced by growing plants in contained spaces such as greenhouses. Although antibodies have been described to have numerous benefits, there are concerns that the purity of food crop strains could be at risk since plants carrying antibodies could contaminate food crops or toxins from pesticides or fertilizers could be transmitted to other plants. Therefore, it has been suggested that the plants that are not used as food for people or feed for livestock should be utilized to produce antibodies [29]. Since each plant species has its own physical and physiological characteristics affecting the expression and glycosylation of recombinant glycoproteins, careful consideration in the selection of plant species is a must for the successful production of antibodies.

3. Conclusion and Future Prospects

The plant-produced monoclonal antibody-based immunotherapy helps the immune system recognize and target cancer cells, and it is hoped that it can be made into a versatile answer to cancer. Recent success and advancement in the designing of monoclonal therapeutics have been achieved through a technological quantum leap that facilitates the generation of modified antibodies capable of exhibiting enhanced potentials, reduced immunogenicity, and reduced widespread systemic toxicities to healthy cells. Enormous progress has been shaped in recent years in the field of plant-made mAbs. Plants could be engineered for introducing expected immunoglobulin genes because they do not naturally produce antibodies. With the aid of new genetic engineering tools, researchers insert the desired gene(s) into a target plant to yield transgenic plants, regarded as one of the most valuable systems to produce human therapeutic antibodies of various ideal traits. For successful production of antibodies, careful consideration to select plant species is a must because every plant species possesses its own physiological and physiochemical characteristics that affect the expression and glycosylation of recombinant glycoproteins. Plants have been chosen as a promising biofactory system to produce antibodies in a large scale because of their high production capacity with affordable cost, higher scalability, reduced screening costs for pathogens, low downstream processing requirements, and easy growth under containment conditions. Many plant-derived mAbs have been produced in large manufacturing scales under the cGMP regulations and have been shown to meet US Food and Drug Administration (FDA) quality standards in identity, purity, and potency. Many of these have shown the proper assembly, effective *in vitro* neutralization, and potent *in vivo* efficacy in animal models. Over the past decades, the FDA has approved more than a dozen mAbs for the treatment of various malignancies, and hundreds of companies have been motivated to be involved in the development of these molecules. Current advancements in genetic engineering, glycoengineering, and other posttranslational modifications have made new strides to provide additional advantages far beyond the traditional benefits of high scalability, economic feasibility, and increased safety, and it is hoped that an excellent platform for the future development of a safer monoclonal antibody is established.

Conflicts of Interest

The authors declare no conflict of interest, financial or otherwise.

Authors' Contributions

MUN designed and wrote the first draft of the manuscript. MAR devised the concept of the review and contributed to the writing and editing of the manuscript. YK contributed to the overall direction of the manuscript, including the general writing and editing processes. All authors read and approved the final manuscript.

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Review Article

Plant-Derived Lectins as Potential Cancer Therapeutics and Diagnostic Tools

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Cancer remains a global health challenge, with high morbidity and mortality, despite the recent advances in diagnosis and treatment. Multiple compounds assessed as novel potential anticancer drugs derive from natural sources, including microorganisms, plants, and animals. Lectins, a group of highly diverse proteins of nonimmune origin with carbohydrate-binding abilities, have been detected in virtually all kingdoms of life. These proteins can interact with free and/or cell surface oligosaccharides and might differentially bind cancer cells, since malignant transformation is tightly associated with altered cell surface glycans. Therefore, lectins could represent a valuable tool for cancer diagnosis and be developed as anticancer therapeutics. Indeed, several plant lectins exert cytotoxic effects mainly by inducing apoptotic and autophagic pathways in malignant cells. This review summarizes the current knowledge regarding the basis for the use of lectins in cancer diagnosis and therapy, providing a few examples of plant-derived carbohydrate-binding proteins with demonstrated antitumor effects.

1. Introduction

Cancer is considered a serious threat to human health globally, constituting the second most frequently diagnosed and deadliest pathology after cardiovascular diseases among ailments of noninfectious etiology [1]. Indeed, the incidence and death rates are steadily rising worldwide, with above 18 million new cases and approximately 10 million deaths caused by malignant diseases [2, 3]. Commonly applied therapeutic options for cancer comprise operation (open surgery or cryoablation), radiotherapy, and chemotherapy [4–6]. These treatment approaches substantially inhibit tumor growth and could even achieve cure, but each has specific advantages and shortcomings.

Chemotherapy is frequently applied for cancer therapy and can be grouped in different categories such as curative (permanent cure following malignant cell elimination), adjuvant (removal of residual undetectable cancer cells following surgery), neoadjuvant (preoperative lesion shrinking), and palliative (symptom alleviation, reduction of complications) types [7, 8]. In general, chemotherapeutics suppress target cells by modulating distinct molecules in various pathways of rapidly growing malignant cells but unfortunately exert deleterious effects on noncancerous cells, with multiple and sometimes serious adverse events [9]. Therefore, developing novel and more selective agents that could target and distinguish malignant cells would likely improve cancer patient prognosis. Meanwhile, lectins display differential binding patterns to cancerous tissues according to the extent of glycosylation and could therefore be employed not only as diagnostic tools but also as anticancer agents [10, 11].

Lectins are ubiquitously found in bacteria, fungi, plants, and animals [12–14]. The term lectin was coined by Boyd and Shapleigh in 1954, to indicate a nonimmunoglobulin protein that binds carbohydrate molecules without modifying them [15]. Lectins are currently considered carbohydrate-

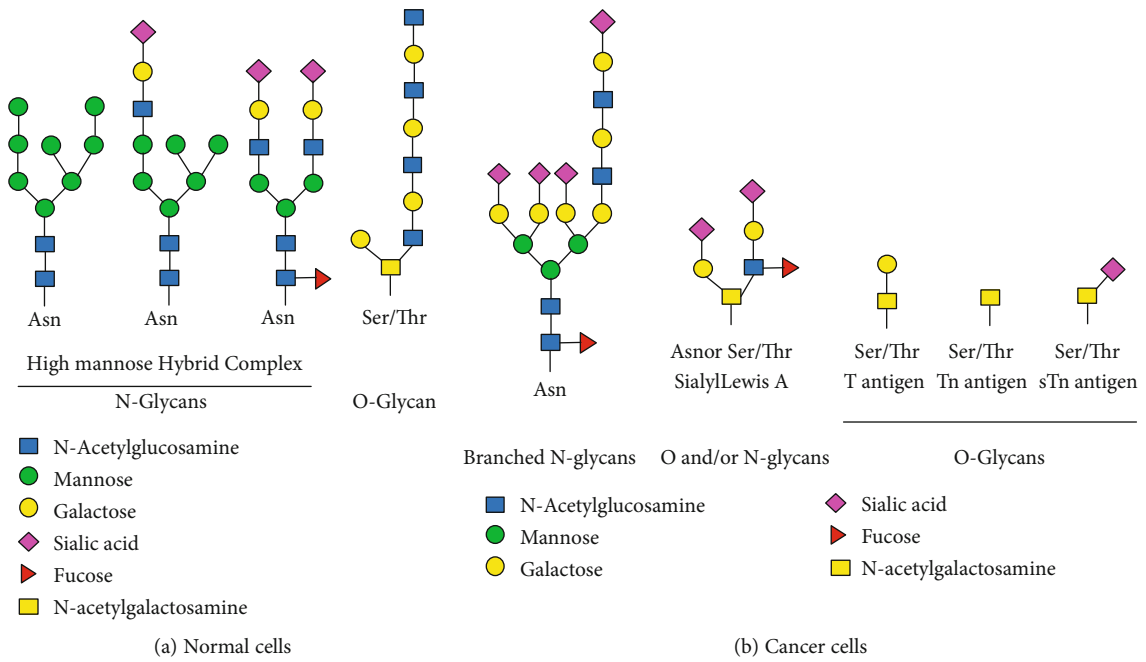


Figure 1: Schematic representation of select N- and O-glycans found in normal and cancer cells. (a) Normal cells have three major types of N-glycans, including high mannose, hybrid, and complex types. The precursor unit is added to the protein through an N-glycosidic bond with the side chain of an asparagine residue that is part of the Asn-X-Ser/Thr consensus sequence. The precursor is trimmed, with additional residues added in the Golgi complex. The first step in O-linked glycosylation involves N-acetylgalactosamine addition to a serine or threonine residue of the polypeptide chain that can proceed with adding other monosaccharides such as galactose, fucose, and sialic acid. (b) Cancer cells have altered glycosylation patterns, comprising either production of new glycans or incomplete synthesis of original glycans. The most common change in N-linked glycoproteins is the production of branched N-glycans; sialyl Lewis A antigen is found in both N- and O-linked, while Tn, sTn, and T antigens are found in O-linked glycoproteins. Glycan structures were adapted from *Essentials of Glycobiology* 3rd edition [29].

binding proteins that reversibly interact with specific saccharides in glycoproteins and glycolipids [16]. Lectins differ by their biophysiochemical properties, inhibiting various organisms, including fungi, viruses, and insects, while also acting as immunomodulatory molecules [17–19]. Additionally, lectins are involved in immune defense, cell migration, cell-to-cell interactions, embryogenesis, organ formation, and inflammation [20, 21].

Lectins protect plants from insects and fungi and are also involved in sugar transport and storage [22]. In addition, some lectins are critical for atmospheric nitrogen fixation [22]. Based on overall structure and the number of carbohydrate binding domains, plant lectins are grouped into hololectins, chimerolectins, superlectins, and merolectins [23]. Additionally, they comprise 12 distinct families that show diverse carbohydrate-binding specificities, including Agaricus bisporus agglutinins, Amaranthins, class V chitinase homologs with lectin activity, Cyanovirins, EEA lectins, GNA lectins, Heveins, Jacalin-related lectins, legume lectins, LysM lectins, Nictaba lectins, and Ricin B lectins [24]. Of these, legume lectins, Ricin B proteins, and GNA-related lectins constitute the most investigated classes, due to remarkable biological functions. Recently, plant lectins have attracted growing attention for selectively and sensitively targeting cell surface glycans, with potential applications in multiple fields [24–27]. This review provides a theoretical basis for applying lectins in cancer diagnosis and therapy, listing a few examples that demonstrate antiproliferative and anticancer activities via autophagy and apoptosis.

2. The Glycocalyx of Eukaryotic Cells

The cell surface in eukaryotes encompasses proteins and carbohydrates, which constitute the glycocalyx [28]. Glycans are very diverse and complex, with various monosaccharides, glycan attachment sites, and bond and branching types [29]. They are mostly linked to the nitrogen of asparagine moieties (N-glycans) or through the oxygen of serine or threonine moieties (O-glycans) on secreted or membrane-bound glycoproteins, with a broad range of structures (Figure 1(a)). Glycans contribute to protein folding, cell-to-cell interactions, pathogen recognition, immune reactions, antigen presentation, and cell adhesion and migration, thereby affecting multiple cellular processes [30–33]. Mature glycoproteins vary N-linked oligosaccharides according to cell type, tissue, and species [34]. All eukaryotic cells share the basic mechanisms of glycoprotein synthesis; however, marked differences exist between malignant and noncancerous cells.

3. Differences in Glycosylation Patterns between Malignant and Noncancerous Cells

Abnormal glycosylation, with either novel glycoforms formed or incompletely synthesized glycans, represents an important hallmark of malignancy [30]. Indeed, altered glycosylation patterns generate multiple biomarkers, some of which are associated with cell malignancy [30]. N-Linked glycoproteins' alterations mostly comprise the generation of branched oligosaccharides. For example, increased amounts of N-acetylglucosaminyltransferase V (MGAT5) induces the β1-6Glc-NAc branching of glycoproteins; this modification contributes to malignancy [35]. Additionally, elevated β1-6Glc-NAc branching of N-linked oligosaccharides promotes sialylation, which is involved in cancer metastasis [36]. It is known that O-linked T, Tn, and sTn antigens are all expressed in multiple malignancies such as breast, lung, colon, cervical, and bladder cancers, but absent in noncancerous cells or tissues (Figure 1(b)) [37]. Many lesions coexpress modified O-glycans, whose upregulation could help predict poor prognosis in some cancers [38, 39]. Altered terminal residues are also related to malignancy, with enzymes catalyzing their addition overexpressed in malignant cells [38]. Examples comprise sialyl Lewis X, sialyl-Tn, Thomas Friedrich (TF), Globo H, Lewis Y, and polysialic acid (PSA) [39]. Overexpression of sialyl- Lewis A/X antigen is shared by many epithelial malignancies (Figure 1(b)); this alteration is associated with loss of ABH blood antigens and poor prognosis [40]. Other glycoproteins and glycolipids show a trend of overproduction in cancer, e.g., gangliosides and mucins. For instance, elevated ganglioside amounts are found in head and neck tumors, neuroblastoma, melanoma, medulloblastoma, lung cancer, and breast cancer [41]. Growing evidence demonstrates that altered glycosylation affects the malignancy potential, tumor immune surveillance, and prognosis and could be employed to develop novel tools for cancer diagnosis and therapy [42].

4. Lectins as Potential Diagnostic Tools

Lectin interactions with target ligands involve a vast array of hydrogen bonds, as well as hydrophobic and nonpolar van der Waals interactions. Small structural alterations of the carbohydrate-binding domains might lead to great differences in ligand binding, with important modifications of biological functions. For instance, PCL and other GNA-related lectins have three putative sugar-binding sites (CBD I, CBD II, and CBD III). Polar amino acids (Gln, Asp, and Tyr) engage in hydrogen bonds with O_2, O_3, and O_4 of mannose while Val interaction with mannose involves hydrophobic interactions. The crystal structure of PLC revealed CBD I as the major mannose-binding domain, while CBD II in which Gln58 and Asp60 are replaced by His58 and Asn60, respectively, no longer interacts with mannose (Figure 2(c)) [43]. Although CBD III has mannose-binding features in other GNA-related lectins, CBD III in PCL instead interacts with other ligands, e.g., sialic acid [44]. These distinct binding features of PCL could explain its biological functions in inhibiting viruses and cancer.

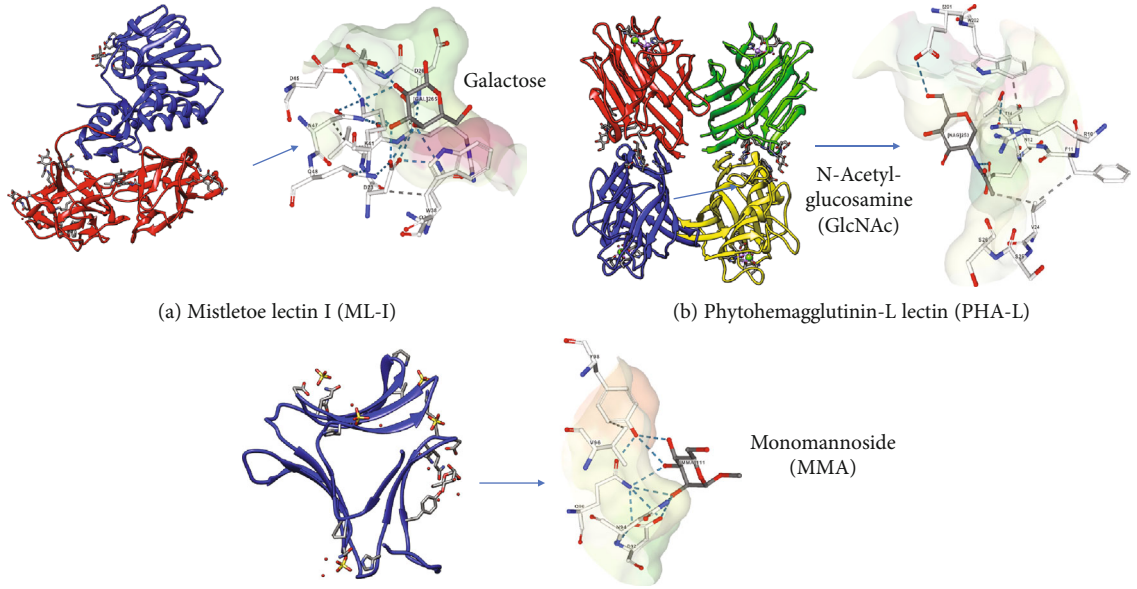
Since various lectins interact with terminal sugars on glycoproteins and glycolipids with high specificity, they could help characterize cell surface alterations in cancer. To this end, wheat germ agglutinin (WGA) was the first lectin shown to agglutinate cancer cells, indicating modified cell surface properties in malignant cells compared with noncancerous ones [45]. Similarly, phytohemagglutinin (PHA-L) produced by *Phaseolus vulgaris*, which is specific to complex-type N-glycans, helps detect modified N-linked carbohydrate core structure or β1-6-GlcNac moiety that is associated with malignancy in colon and pancreatic cancers (Figure 2(b)) [46, 47]. In addition, *Lens culinaris* agglutinin (LCA) from lentil seeds with α1-6 fucose specificity could be employed in early hepatocellular carcinoma (HCC) diagnosis through interaction with the HCC marker α fetoprotein (AFT). Furthermore, the fucosylated α fetoprotein-L3 (AFTL3) isoform shows higher specificity in HCC diagnosis in comparison with total AFT and worsens prognosis [48]. The lectin LCA also interacts with serum thyroglobulin (Tg), a biomarker of thyroid cancer, distinguishing between noncancerous and malignant diseases [49]. Moreover, LCA and *Aleuria aurantia* lectin (AAL) bind to fucosylated prostate-specific antigen (PSA), a commonly employed early diagnostic marker of prostate cancer [50, 51].

Many other plant lectins could help detect malignant tumors. *Wisteria floribunda* agglutinin (WFA), a legume lectin, preferentially interacts with glycans possessing terminal N-acetylgalactosamine. WFA has high affinity to the cancer biomarker L1 cell adhesion molecule (L1CAM) and has been employed for detecting intrahepatic cholangiocarcinoma (CC) [52]. In addition, WFA is lowly specific to another highly glycosylated biomarker, mucin 1 (MUC1) [53]. However, it was suggested that combining both biomarkers would improve diagnostic accuracy and reliability in CC [52].

The current diagnostic biomarker of ovarian cancer is the human cancer antigen CA125; however, serum CA125 amounts are also elevated in some nonmalignant pathologies [54, 55]. Because CA125 is unreliable for diagnosing ovarian cancer, Shewell et al. suggested N-acetylneuraminic acid, which is a major form of glycosylation on cancer cells [56]. Additionally, glycans with terminal N-glycolylneuraminic acid are not detected in considerable amounts in nondiseased tissues in humans. The latter research team engineered the lectin SubB2M based on the B subunit of Shiga toxigenic *E. coli* Subtilase cytotoxin (SubAB) with ameliorated Neu5Gc glycan recognition, which was capable of detecting high serum amounts of Neu5Gc-glycans in patients with ovarian cancer (all stages) versus noncancerous controls [56].

Ricinus communis agglutinin I (RCA-I), which specifically interacts with terminal galactose moieties, binds the membrane glycoprotein POTE ankyrin domain family member F (POTEF) in triple-negative breast cancer (TNBC) cells proportionally to their metastatic abilities [10]. Hence, RCA-1 might help diagnose TNBC and predict the odds of metastasis in TNBC cases.

Recently, the galactose-specific lectins *Aleuria aurantia* lectin (AAL), *Ulex europaeus* I (UEA-I) fucose-binding



(a) Mistletoe lectin I (ML-I)

(b) Phytohemagglutinin-L lectin (PHA-L)

(c) Polygonatum cyrtonema lectin (PCL)

Figure 2: Crystal structures of three representative lectins showing their interactions with the corresponding sugars via carbohydrate-binding domains (CBDs). (a) Mistletoe lectin I (Ricin family) with A chain (blue) and B chain (red) shown as a ribbon in a complex with galactose (protein data bank [PDB]: 1OQL). (b) The tetrameric phytohemagglutinin-L (legume family) with monomers in various colors, in complex with GlcNAc (PBD: 1FAT). (c) *Polygonatum cyrtonema* (GNA-related family) as a monomeric protein in complex with monomannoside (PDB: 3A0D). Dotted lines are hydrogen bonds. The structures were generated with the UCSF Chimera software (Resource for Biocomputing, Visualization, and Informatics (RBVI), USA). Binding of lectins to their respective sugars used the PDB.

lectin, *Agaricus bisporus* agglutinin (ABA), *Maclura pomifera* (MPL), and Phaseolus vulgaris erythroagglutinin (PHA-E) were shown to differentiate between metastatic and nonmetastatic pancreatic cancer cells [57]. The same team demonstrated that genes encoding fucosyltransferases and altering N-linked fucosylation are overexpressed in metastatic pancreatic cancer, providing a possible mechanism by which AAL also blunts motility in metastatic pancreatic cancer. The above examples suggest that lectins might be useful in detecting tumor markers and differentiating between cancer and nonmalignant cells through targeting of specific differentially glycosylated isoforms.

5. Lectins Inhibit Cancer Cells Mostly by Inducing Autophagy and Apoptosis

Understanding how plant lectins exert antiproliferative and cytotoxic effects on cancer cells might help develop novel potent anticancer agents. Previous reports have revealed the ability of plant lectins to bind to tumor cell surface and promote apoptotic and autophagic cell death [27, 58]. Representative lectins are described below, which promote cell death by modulating apoptotic and autophagic signaling pathways (Table 1).

5.1. Ricin B Family of Proteins with Cancer Inhibitory Properties. One of the most toxic plant protein groups is the Ricin B family of ribosome-inactivating proteins (RIPs). RIPs attract increasing interest for their reported therapeutic potential as well as possible utilization in biological warfare and bioterrorism [59]. Plant RIPs comprise two major classes, including types I (single-chain molecules with enzymatic activity) and II (an enzymatic A subunit and one or many B chains with lectin activity) [60]. Since the B subunit has affinity for cell surface sugar-containing molecules, it is involved in the translocation of the toxic A chain into the cytosol where it inhibits ribosomes. RIPs specifically and irreversibly suppress protein synthesis in eukaryotes by enzymatically altering the 28S rRNA of the 60S ribosomal subunit [58]. Plant RIPs comprise ricin, abrin, mistletoe lectins, Korean mistletoe lectin, modeccin, and volkensin, some of which are briefly discussed below [61–64].

5.1.1. Mistletoe Lectins. Mistletoe lectins (type II RIPs) are arguably the most investigated proteins for cancer treatment. European mistletoe (*Viscum album*) represents a semiparasitic plant growing on multiple trees in Europe, Asia, and North Africa [65]. For centuries, *V. album* extracts have been traditionally applied for treating diverse ailments including seizures, hypertension, wounds, and headaches [66, 67]. The compositions of such preparations vary by host tree, extraction method, and harvest season [68]. Currently, standardized aqueous mistletoe extracts are common in complementary and alternative medicine (CAM) for treating nonmalignant and cancerous lesions in humans, particularly in Europe [69, 70]. Multiple constituents showing cytotoxic

Table 1: Representative plant lectins that induce cell death by modulating apoptotic or autophagic signaling pathways.

Lectin	Sugar(s) bound	Cancer type(s) and/or cancer cell line(s)	Mechanism(s) of cell growth inhibition/target molecule(s) or pathway(s)	References
Mistletoe lectin I (ML-I)	Galactose	Leukemic T and M cells	Apoptosis/activation of caspase-8/FLICE, caspase-9, and caspase-3	[81]
		CT26 cells	Apoptosis/ROS generation and activation of SEK/JNK pathway	[82]
		Glioma (in mice)	Apoptosis/caspase-dependent pathway, activation of NK cells	[83]
Korean mistletoe (KMLC)	Galactose/N-acetylgalactosamine	SK-Hep-1 cells/Hep3B cells	Apoptosis via p21- and p53-independent pathways/activation of Bax and caspase-3, inhibition of Bcl-2	[88]
		Hep3B	Apoptosis/ROS generation and activation of SEK/JNK pathway	[89]
		A253 cells	Apoptosis/inhibition of telomerase activity, decreased phosphorylation of Akt, and activation of caspase-3	[90]
Concanavalin A (Con A)	Mannose/glucose	A375 cells	Apoptosis/caspase-dependent manner	[91]
		HeLa cells	Autophagy/suppressing Pl3K/Akt/mTOR and upregulating MEK/ERK	[92]
		U87 cells	Autophagy/upregulation of BNIP3	[93]
		Hepatoma (in SCID mice)	Antitumor effect	[94]
Dioclea violacea (DVL)	Mannose/glucose	Rat C6 glioma cells	Apoptosis/caspase-3 activation	[95]
		U78 cells	Autophagy/inhibition of Akt, ERK1/2, and TORC1	[96]
Dioclea lasiocarpa lectin (DLL)	Mannose/glucose	A549, MCF-7, PC3, A2780, glioma cell lines	Induction of autophagy/activation of caspase-3	[97]
Dioclea lasiophylla lectin (DlyL)	Mannose	Rat C6 glioma cells	Induction of autophagy/activation of caspase-3	[98]
Bauhinia forficata (BFL)	N-Acetylgalactosamine	MVF7 cells	Apoptosis/inhibition of caspase-9	[99]
Polygonatum cyrtonema lectin (PCL)	Mannose/sialic acid	A375 cells	Apoptosis/caspase-activation, ROS accumulation, and activation of p53 and p38	[100]
		L929 cells	Apoptosis and autophagy/through Ras-Raf and Pl3K-Akt signaling pathways	[101]
		A549 cells	Apoptosis and autophagy/ROS-mediated MAPK and NF-κB signaling pathways	[102]
		MCF-7 cells	Apoptosis/caspase-dependent pathways	[103]
Polygonatum odoratum lectin (POL)	Mannose	A375 cells	Apoptosis/caspase-dependent	[104]
		L929 cells	Apoptosis/caspase dependent	[103]
		A549 cells	Apoptosis and autophagy/inhibition of Akt-NF-κB or Akt-mTOR pathway	[105]
Remusatia vivipara lectin (RVL)	Mannose	MDA-MB-231, MCF-7	Induction of apoptosis	[106]

and immunomodulatory properties, as well as potential anticancer effects, have been detected in mistletoe extracts, including viscotoxins, polysaccharides, lectins, phenolic acids and flavonoids, triterpenes, and polypeptides [71, 72]. However, mistletoe lectins (ML-I, II, and III) are considered the major constituents with antitumoral features [63, 73]. ML-I to III possess cytotoxic (A) and carbohydrate-binding (B) subunits, linked by disulfide bonds to form heterodimers [63]. However, they have differing sugar-binding specificities: ML-I interacts with D-galactose, ML-III with N-acetyl-galactosamine, and ML-II with both (Figure 2(a)) [74]. As RIPs, MLs bind cells via the B subunit, delivering the toxic A subunit into the cytoplasm [62, 75]. Upon internalization, the A subunit suppresses protein synthesis in eukaryotes via hydrolysis of the N-glycosidic bond linking adenine-4324 and G-4325 in 28S rRNA. This rRNA depurination renders the ribosome unable to bind cell factors, consequently blunting protein production [62]. New evidence suggests that the potent cytotoxicity of ML-I in malignant cells is not only due to rRNA depurination but also to the A chain affecting multiple other adenine-containing substrates [76, 77]. It was demonstrated that ML-1 as well as its B chain alone have immunostimulatory features, upregulating cytokines such as TNF-α, interleukin- (IL-) 1, IL-6, and granulocyte macrophage colony-stimulating factor (GM-CSF) upon treatment of peripheral blood mononuclear cells (PBMCs) with ML-I [78]. Such cytokine upregulation is also present in cancer cases treated with mistletoe extracts with adequate ML-I amounts, indicating that mistletoe lectin-carbohydrate interactions induce nonspecific defense pathways that could benefit cancer treatment [78]. The above research team also reported higher amounts of large granular lymphocytes and NK killers upon parenteral injection of rabbits with low doses of ML-I and the related B chain, respectively [79]. NK cells as effector cells contribute to cancer inhibition, by carrying out immune surveillance against primary tumors and preventing cancer spread [80]. However, the major activity of ML-I is its cytostatic and cytotoxic effects on various cancer cell lines. It is widely admitted that ML-I's cytotoxic effects are mediated by apoptosis induction. Treatment of leukemic T and B cells with ML-I results in caspase-8/FLICE, caspase-9, and caspase-3 activation, leading to apoptotic cell death (Figure 3) [81]. Meanwhile, it was determined that ML-I uptake in CT26 mouse colon carcinoma cells is energy dependent, with translocation into the cell occurring by both clathrin-dependent and clathrin-independent mechanisms. After uptake, ML-I is redirected towards the Golgi, undergoes retrograde transport to the ER, and translocates to the cytoplasm in a manner similar to ricin. It was recently shown that ML-I's proapoptotic activity is caspase mediated [82]. On the other hand, the antiproliferative effects of ML-I differ according to the cancer cell lines, which might be related to varying cell glycosylation patterns [82]. Interestingly, it was demonstrated that a mistletoe extract or recombinant ML-I alone could induce NK-cell killing of glioma. Additionally, ML-I enhances the antiglioma effects of T cells as well as animal survival when jointly administered with chemotherapeutics in the mouse model of glioma, suggesting potential adjuvant effects [83]. *V. album* preparations have been assessed clinically for antitumor features, alone or in combination with current treatments; however, their mode of action and therapeutic benefits remain largely undefined. Clinical trials evaluating mistletoe extracts reported a decrease of adverse events due to conventional cancer therapies, with enhanced survival and no adverse interactions with the antitumor agents being applied [84, 85]. Others, however, do not strongly support ML extracts as antitumor products or adjuvant therapeutics in cancer [86].

5.1.2. Korean Mistletoe Lectin. Korean mistletoe lectin, produced by *Viscum album* coloratum (KMLC), interacts with galactose and N-acetylgalactosamine and induces apoptosis in cancer cells while exerting immunomodulatory effects via NK cell induction; KMLC's antitumor properties result from enhanced NK cell cytotoxicity via perforin upregulation [87]. KMLC also induces apoptotic pathways in two hepatoma cancer cells, including SK-Hep-1 (p53 positive) and Hep3B (p53 negative) cells, independent of p53 and p21 signaling cascades. Indeed, this lectin promotes apoptosis by inducing Bax and suppressing Bcl-2, subsequently activating caspase-3 [88]. Others also reported that apoptosis-related cell death in Hep3B cells is caused by ROS generation, low mitochondrial membrane potential, cytochrome c release in the cytosol, and SEK/JNK pathway induction [89]. Further, it was demonstrated that apoptosis is triggered in KMLC-treated A253 cells through suppression of telomerase activity, reduced Akt phosphorylation, and caspase-3 induction [90].

5.2. Legume Lectins with Anticancer Properties. Legume lectins constitute a large group of homologous carbohydrate-binding proteins. They feature two or four equivalent subunits and are specific to multiple sugars, from monosaccharides to complex carbohydrate molecules [107]. They mostly encompass mannose/glucose-specific (e.g., Con A) and galactose/N-acetylgalactosamine-specific (e.g., *Bauhinia forficata* lectin (BFL)) types.

5.2.1. Concanavalin A. Concanavalin A (Con A), a well-known protein with mannose/glucose specificity, was the first lectin purified from Jack bean seeds a century ago. Its antiproliferative effects on human melanoma A375 cells involve caspase-related apoptosis [108]. Additionally, Con A promotes autophagic pathways in HeLa cells through Pl3K/Akt/mTOR pathway suppression and MEK/ERK axis upregulation (Figure 3) [92]. Moreover, low Con A levels activate innate immune cells in the liver and exert inhibitory effects on Colon-26 cancer cells in mouse models [109]. It is admitted that Con A's effects are mediated by internalization and targeting to the mitochondria, resulting in hepatoma cell autophagy; according to treatment time, dose, and frequency, Con A could display anticancer properties in hepatoma-bearing SCID mice [94]. In addition, Con A administration to glioblastoma U87 cells upregulates BNIP3 and autophagy-associated genes, providing a basis for an autophagic mechanism of action for Con A (Figure 3) [93].

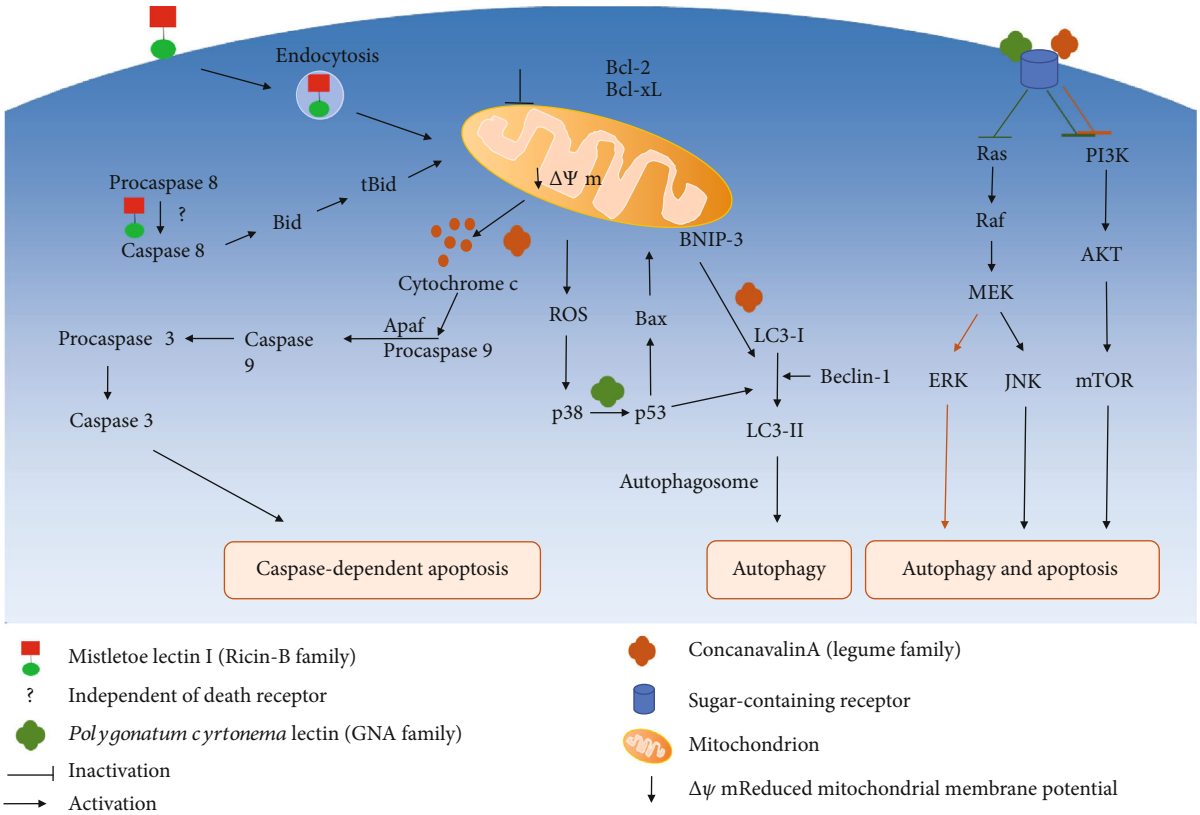


Figure 3: Signaling pathways of selected plant lectins involved in apoptosis and/or autophagy.

5.2.2. Dioclea Violacea Lectin. *Dioclea violacea* lectin (DVL) produced by *Dioclea violacea* seeds is another legume protein showing mannose/glucose specificity and anticancer features [110]. DVL enhances caspase-3 activation and apoptotic cell death in rat glioma C6 cells [95]. Further, DVL was shown to inhibit U87 cells via autophagy enhancement by suppressing effectors such as Akt, ERK1/2, and TORC1, which are overexpressed in cancer [96].

5.2.3. Dioclea lasiocarpa Lectin. *Dioclea lasiocarpa* lectin (DLL) represents a mannose/glucose-specific lectin produced by *Dioclea lasiocarpa* Mart seeds [111]. DLL exerts strong antiproliferative effects on various cancer cells such as A549, MCF-7, PC3, and A2780 cells, of which ovarian cancer A2780 cells were most susceptible with elevated high mannose content on the cell surface [112, 113]. In addition, DLL suppresses glioma cell lines by inducing caspase-3 activation, autophagy, and cell death [97].

5.2.4. Dioclea lasiophylla Lectin. *Dioclea lasiophylla* lectin (DlyL) is a mannose-specific carbohydrate-binding protein obtained from *Dioclea lasiophylla* Mart. Ex Benth seeds. It has a high affinity towards N-glycans of the complex or hybrid types. It was also shown to exert antitumor effects on rat glioma C6 cells, inhibiting cell migration and inducing autophagy and cell death via caspase-3 activation [98].

5.2.5. Legume Lectins from Bauhinia spp. Another legume lectin, *Bauhinia forficata* lectin (BFL), produced by *B. forficata* with specific interaction with GalNac, is toxic to cultured breast cancer MVF7 cells. BFL exerts cytotoxicity via caspase-9 suppression and subsequent G2/M phase arrest [99]. Additionally, BFL inhibits various malignant cells in the NCI-60 panel, with melanoma LOX IMVI cells showing highest susceptibility [114]. Two other lectins, *Bauhinia variegata* lectin (BVL) and *Bauhinia ungulate* lectin (BUL) detected in *Bauhinia* spp., have demonstrated antitumor properties. BLV displays low-micromolar growth suppression of breast cancer MCF7 and hepatoma HepG2 cells [115], while BUL is dose-dependently cytostatic in colon adenocarcinoma HT-29 cells [116].

Mistletoe lectin I triggers caspase activation and apoptosis via a death receptor-independent, but mitochondria-mediated pathway in leukemic T and B cells. ML-1 internalization occurs by endocytosis, which is critical for the lectin's cytotoxicity. This is followed by cytochrome c release and mitochondrial membrane potential reduction, inducing the caspase cascade which causes caspase-associated apoptosis. Treatment with ML-1 also enhances caspase-8 induction, without involving death receptors [82]. Another lectin, *Polygonatum cyrtonema*, triggers both autophagy and apoptosis in cancer. PCL interacts with sugar-containing receptors on cells and induces autophagy by inhibiting the PI3K/AKT/mTOR and Ras-Raf pathways in murine fibrosarcoma L929 cells. Culture of another cancer cell line, A375 cells, in the presence of PCL induces autophagic and apoptotic cell death via mitochondria-associated ROS-p38-p53 pathway. In HeLa cells, Concanavalin A (Con A) suppresses PI3K/AKT/mTOR signaling and

induces the MEK/ERK pathway, resulting in autophagy. In hepatoma cells, Con A triggers autophagy via mitochondria-mediated pathway [92]. Additionally, Con A administration to A375 cells inhibits them through caspase-associated apoptosis [93].

5.3. GNA-Related Lectin Family Members with Antitumoral Features. Galanthus nivalis agglutinin- (GNA-) related lectins constitute a superfamily of strictly mannose-binding proteins active against cancer, viruses, and fungi [117]. The first GNA-related lectin, coined *Galanthus nivalis* lectin (or GNA), was obtained from snowdrop bulbs [118, 119]. GNA-related lectins were previously renamed "monocot mannose-binding lectins". However, after isolating and characterizing many other lectins with GNA domains, they are currently referred to as GNA-related lectins [118].

5.3.1. Polygonatum cyrtonema Lectin. Polygonatum cyrtonema lectin (PCL), a mannose/sialic acid-binding lectin, induces apoptotic and autophagic death in human melanoma A375 cells; apoptosis induction involves Bax and Bcl-2 regulation at the protein level, which remarkably reduces the mitochondrial membrane potential, with subsequent cytochrome c release and caspase activation [100]. Additionally, PCL enhances ROS production as well as p38 and p53 activation, which contribute to autophagy in A375 cells, suggesting that PCL triggers both cell death mechanisms simultaneously [100]. PCL also triggers autophagy and apoptosis in mouse fibrosarcoma L929 cells, via Ras-Raf and Pl3K-Akt signaling suppression (Figure 3) [101]. Recent evidence suggests that PCL enhances autophagic and apoptotic death in A549 cells through ROS-dependent MAPK and NF-κB pathway regulation [102]. Further, PCL and two other prototypic GNA-related lectins, i.e., *Ophiopogon japonicus* lectin (OJL) and *Liparis nervosa* lectin (LNL), exert suppressive effects on MCF-7 cells, enhancing caspase-dependent apoptosis [103].

5.3.2. Polygonatum odoratum Lectin. Polygonatum odoratum lectin (POL) produced by *Polygonatum odoratum* (Mill.) Druce also represents a GNA-related lectin that enhances caspase-associated apoptosis in A375 and L929 cells [103, 104]. POL was demonstrated to simultaneously trigger apoptotic and autophagic death in A549 cells; apoptosis and autophagy were enhanced by the Akt-NF-κB pathway and Akt-mTOR signaling suppression, respectively [105].

5.3.3. Remusatia vivipara Lectin. Remusatia vivipara lectin (RVL) is another mannose-binding protein produced by *Remusatia vivipara* (Araceae), with potent nematicidal activity [120]. RVL shows high affinity to N-linked glycans, but no interactions with O-linked glycans and monosaccharides. It exerts inhibitory and anticell migratory effects on breast cancer MDA-MB-468 and MCF-7 cells, via apoptosis [106].

6. Conclusions and Perspectives

Plant lectins attract wide attention owing to their multifaceted properties in agriculture, blood typing, and diagnosis. In the past decade, a decent number of lectins have been purified with diverse carbohydrate specificities, providing novel directions in lectin research. Currently, lectins are broadly employed in glycobiology research, e.g., for detecting and analyzing glycoproteins, carbohydrate assessment on cells, cell identification and isolation/separation, immunohistochemical analysis, bacterial typing, mapping central neuronal pathways, diagnosis, and tracing [121, 122]. Lectins such as Con A are routinely applied to isolate glycoproteins by affinity chromatography [123]. Lectin-based microarrays help assess the structural alterations of glycans and enable screening and assessment of the glycosylation profiles of therapeutic proteins [124].

In the pharmaceutical field, lectins constitute potential antitumor therapeutics. Specifically, they can differentiate between noncancerous and malignant lesions. Indeed, multiple lectins demonstrate anticancer features in cultured cells and in vivo, with some inhibiting malignant cells via apoptosis and/or autophagy by regulating multiple pathways. Mistletoe extracts are extensively applied for cancer treatment in Europe, with antineoplastic and apoptosis-inducing properties, as well as immunostimulatory and antiangiogenic features [125, 126].

Despite the anticancer features of lectins, there are few drawbacks hindering their development for cancer therapy. A potential issue is toxicity. For example, Con A induces liver failure upon intravenous administration in mouse models [127]. In addition, PHA-L causes nausea, vomiting, and diarrhea following oral treatment [128]. Furthermore, some RIPs (e.g., abrin and ricin) are toxic to mice with low LD50s of 10-13 and 55-65 ng, respectively [129]. Such toxicity could be mitigated by fusing lectins to other proteins for targeted delivery. For example, immunotoxins were designed for selective delivery of a toxin to malignant cells by linking a toxic domain to a specific targeting moiety, e.g., an antibody, or the Fab, Fc, or single-chain variable fragments [130]. Ricin has been widely assessed for such purpose [131]. Another chimeric molecule with antiviral activity, Avaren-Fc, designed by fusing the lectin actinohivin to the Fc region of human immunoglobulin G1, demonstrates potential anticancer activity [132].

Future research aspects of developing lectins as anticancer agents should involve in vitro and in vivo assessments of immunomodulatory and toxic effects on healthy cells. In addition, further understanding of their mechanisms of action and roles in autophagy and apoptosis-induced cell death could provide better targets for cancer therapy in the future.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Effects of Xuefu Zhuyu Decoction on Cell Migration and Ocular Tumor Invasion in *Drosophila*

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Xuefu Zhuyu Decoction (XFZYD), a Traditional Chinese Medicine (TCM) decoction mainly for treating blood stasis syndrome, has been widely investigated and applied in clinic and in laboratory. XFZYD contains 11 herbs and has been identified to promoting blood circulation to remove blood stasis for cardiovascular disease. Meanwhile, blood stasis is directly related to malignant tumor according to TCM basic theory. However, the effects of XFZYD on tumor metastasis and the underlying mechanisms are still largely unknown. Here, we employed well-established *Drosophila* cell migration and tumor invasion models to explore whether XFZYD has the anticancer activity on tumor metastasis *in vivo*. Our work has demonstrated that XFZYD could suppress cell migration and tumor invasion at the moderate concentrations. In addition, XFZYD altered the expression of MMP1, β-integrin, and E-cadherin to impede cell migration. Moreover, XFZYD inhibited ocular tumor invasion presumably by reducing the activity of Notch signaling. Together, these evidences reveal a positive role of XFZYD in suppressing cell migration and tumor metastasis, providing the potential drug targets and key clues for cancer clinical treatment strategies.

1. Introduction

With the increased incidence, cancer has become a worldwide disease and represents one of serious health-care issues for human race [1, 2]. More than 90% fatalities of human cancers are due to the ability of tumor metastasis at late stage, or form discontinuous secondary tumor foci at distant locations, instead of the localized primary tumor growth [3, 4]. Tumor invasion development is an extensive complex progress with multiple steps. Firstly, with the metastatic feature of decrease in adhesive force and increase in superficial charges, cancer cells could separate from the initial tumor. Through releasing numerous proteolytic enzymes and other factors, they possess the abilities of penetrating basement membrane (BM) and destroying cellular matrix, thus infiltrating into peripheral tissue [5]. After cancer enters the vascular and lymphatic vessels, these tumor cells survive and spread across the body through the circulatory system. Finally, they pierce through the vessel wall and settle at a secondary location to expand the metastatic focus [6–8]. A large number of genes and signaling pathways participate in this biological behavior, providing potential drug targets and

valuable therapeutic strategies for clinical cancer treatments [9–12]. Due to the adverse effects and high cost, synthetic chemotherapeutic anticancer drugs have not been as successful as expected in preventing and fighting against cancers in the clinic. Hence, the need to develop effective, safe, and affordable anticancer drugs from alternative and complementary herbal resources is becoming more and more urgent.

Traditional Chinese Medicine (TCM), with ancient application history in China or other regions of Asia, has gained considerable importance in defending against cancer during the last two decades [13–15]. Blood stasis, characterized by blood flow retardation and blood stagnation [16–18], is an essential factor for tumor metastasis during tumorigenesis in the TCM basic theory system [19, 20]. Usually, researchers utilize the hemorheology indexes to evaluate the patients with blood stasis syndrome or the established blood stasis animal models rather than pathological methods [21, 22]. Gao et al. checked the nail fold microcirculation in tumor patients and found that microcirculatory disturbance makes blood flow slow down, whose erythrocyte aggregation, exudation, and hemorrhage in turn promote the generation, development, and metastasis of tumor [23]. And, the association between blood stasis and cancer has received increasing attention in the studies related to TCM and integrative Chinese and Western medicine [24–26]. Thus, promoting blood circulation to remove blood stasis has become one of the major treatment principles for cancer in TCM. While the blood-activating stasis-resolving drugs (BAHRDs) have been well documented in antitumor therapy, their roles in cell migration and tumor metastasis remain controversial. Several reports have indicated that BAHRDs could inhibit tumor cell proliferation, invasion, and metastasis and reduce adverse reactions of radiotherapy and chemotherapy [27–29]. Conversely, other studies suggested that BAHRDs can improve local microcirculation and provide a richer blood supply for tumor growth, thereby promoting tumor metastasis [30]. A possible explanation for the discrepancy is that this category of drugs has multiple active ingredients, which are mainly categorized into the following: flavonoids, terpenoids, alkaloid, and fatty oil [31]. Different compounds and various combinations may trigger different gene expression profiles and signaling pathways, thus producing diverse even opposite effects. Additionally, most work was done *in vitro* or in cultured cancer cell lines that could not adequately recreate the *in vivo* condition.

Xuefu Zhuyu Decoction (XFZYD), a traditional Chinese herbal formula, has been widely investigated and applied in the clinic mainly for treating blood stasis syndrome. It contains 11 herbs (Supplementary Table 1 showing the drug composition and medicinal part, as well as the amount of each herb in one dose XFZYD) [32] and is originated from the well-known medical book *Yi Lin Gai Cuo* (Corrections of Errors Among Physicians) which was written by Wang Qingren in Qing Dynasty [33]. As a representative of blood-activating stasis-resolving recipe, XFZYD is mainly used to treat metabolic syndrome and cardiac-cerebral vascular diseases, including hyperlipidemia, hypertension, coronary heart disease, traumatic brain injury, and ischemic stroke [34–40]. It has been reported that XFZYD displays a significant effect on the pretreatment of the myocardium in sepsis rats by inhibiting myocardial cell apoptosis and antioxidation [41]. In addition, researchers found that XFZYD could protect against retinal ischemic by downregulating HIF-1α and VEGF via a synergistic inhibition of RBP2 and PKM2 [42]. According to the Chinese medical theory, blood stasis is one of main leading causes for malignant tumor formation. With the efficacy in promoting circulation to remove blood stasis, we considered that XFZYD could be potentially used to develop an effective treatment for malignant tumors. However, the effects of XFZYD on tumor metastasis and the underlying mechanisms remain poorly understood.

With less genome redundancy and more genetic tools, *Drosophila melanogaster* serves as an elegant *in vivo* model for the study of tumor metastasis [43–45]. Many genes and signal transduction pathways pivotal for cancer progression were first identified in flies and subsequently verified in mammals. Moreover, several powerful tumor invasion models have been well established in *Drosophila melanogaster*. For instance, the loss of *C-terminal SRC kinase* (*csk*) or cell polarity genes, like *scribbled* (*scrib*), disc large (*dlg*), or *lethal giant larvae* (*lgl*), in the anterior/posterior (A/P) compartment boundary region triggers invasive cell migration phenotype [10, 46, 47]. *Drosophila* is also becoming a potential model for studying ocular tumors [48]. Ferres-Marco et al. found that coupled with overexpression of Delta (the ligand for Notch signal), downregulation of the expression of two epigenetic genes *pipsqueak* (*psp*) and *longitudinal lacking* (*lola*) induces the formation of *Drosophila* eye metastatic tumors during development, which is termed *eyeful* [49]. By using these important *in vivo* models, the purpose of our study was to analyze the effects of XFZYD extract on cell migration and ocular tumor invasion in *Drosophila* and to provide the potential molecular mechanisms for XFZYD in clinical treatment on malignant tumors.

2. Materials and Methods

2.1. Traditional Chinese Herbs. XFZYD is composed of *Bupleurum chinense*, *Paeonia lactiflora*, *Cyathula officinalis*, *Ligusticum chuanxiong*, *Angelica sinensis*, *Prunus persica*, *Glycyrrhiza uralensis*, *Carthamus tinctorius*, *Platycodon grandiflorum*, *Rehmannia glutinosa*, and *Citrus aurantium*. The 11 Chinese herbs in XFZYD were purchased from Beijing Tongrentang Tangshan Chain Store Drug Store Co., Ltd. Their batch numbers and manufacturers are listed in the Supplementary Table 2. All these materials (Supplementary Figure 1a-k) were all authenticated by Prof. Chunyu Tian in the College of Traditional Chinese Medicine, North China University of Science and Technology [50].

2.2. Preparation of Xuefu Zhuyu Decoction. The method of preparing traditional prescription is water extraction. Firstly, one dose of XFZYD (Supplementary Table 1 showing the amount of each herb in one dose) was weighed and soaked overnight [32]. After being soaked for 12 h, the herbs were extracted in boiled double distilled water (ddH_2O) (100 g herbs to 1000 mL ddH_2O) at 60°C for 3 hours. The extract was centrifuged at 4200 × g for 30 min at 25°C, followed by

vacuum filtration with a 0.45 μm filter. The XFZYD extract was stored at -80°C and then freeze dried with a lyophilizer and then reconstituted in ddH_2O in order to obtain a final stock concentration of 0.78 g/mL (Supplementary Figure 1l). XFZYD extract was added directly to regular food from a 0.7800 g/ml aqueous stock to a final concentration of 0.0100, 0.0125, 0.0200, 0.0500, and 0.1000 g/ml. For control food, only water was used.

2.3. Quality Control of Xuefu Zhuyu Decoction. To control the quality of XFZYD, a high-performance liquid chromatography (HPLC) method was performed to establish the fingerprint spectrum. The analyses were performed with Shimadzu LC-20A. The chromatographic column was Agilent Eclipse XDB-C18 (4.6×250 mm, 5μm). The mobile phase flow rate was 1 ml/min, and column temperature was maintained at 25 ± 1°C. The paeoniflorin, ammonium glycyrrhizinate, and naringin were dissolved in methanol as control detected by the HPLC method. Paeoniflorin was eluted with a gradient system consisting of methanol (A) and 0.05 M KH_2PO_4 solution (B) (A : B = 40 : 60). The mobile phase for ammonium glycyrrhizinate is acetonitrile (C) and 0.05% phosphoric acid solution (D). Its elution gradient was 0-8 min (C: 19%, D: 81%), 8-35 min (C: 19%-50%, D: 81%-50%), 35-36 min (C: 50%-100%, D: 50%-0%), and 36-40 min (C: 100%-19%, D: 0%-81%). Naringin was eluted with a gradient system consisting of acetonitrile (E) and water (F) (E : F = 20 : 80).

2.4. Drosophila Strains and Genetics. Flies were kept on a cornmeal and agar medium at 25°C with a 12 h light-dark cycle incubator according to standard protocols unless indicated. *Drosophila* stocks used include the following: w^{1118} (#3605) and $Oregon^{RC}$ (#0005) were obtained from Bloomington *Drosophila* stock center (BDSC); *UAS-scrib-IR* (#27424) was obtained from Vienna *Drosophila* RNAi center (VDRC); *ptc*-GAL4 *UAS*-GFP (*ptc*>GFP) was previously described [51, 52]. *eyeful*/*Cyo* fly is a kind gift of Professor Lei Xue from Tongji University, which has been established by the homologous chromosome recombination (HCR) of *ey*-GAL4 (#8220, BDSC), *UAS*-Delta (#5614, BDSC), and *GS88A8* (previously described [49]) fly strains via the balancer fly stock *Sco*/*Cyo* (#2555, BDSC). The crossing scheme for establishing the *eyeful* model is shown in Supplementary Figure 2. For all fly crossing experiments, healthy unmated male and female parents were randomly assigned to different groups.

2.5. Drosophila Cell Migration Model. To set up the cell migration model, female flies with genotype *ptc*>GFP were crossed to male flies with genotype *UAS-scrib-IR*. These fruit flies mated and laid eggs in one tube, which contains normal food. When the offspring reach the 3rd instar larval stage, the larvae with genotype *ptc*>GFP/*UAS-scrib-IR* were collected and dissected (Supplementary Figure 1n). After fixation, the migrated cells were observed and recorded, which are marked by green fluorescent protein (GFP) in the wing pouch region through a fluorescence microscope. Simultaneously, the migrating cell number was counted and the migrating distance was measured (Supplementary Figure 3). For the XFZYD-treated groups, the offspring were kept on the XFZYD-added medium. Meanwhile, female flies with genotype *ptc*>GFP were crossed to male files of the strain w^{1118} on the regular food, and the progeny larvae with genotype *ptc*>GFP/+ were collected as the control group. For cell migration experiments, all crosses were raised at 25°C for 2 days, then shifted to 29°C for additional 3 days; the 3rd instar larvae were dissected [10].

2.6. Drosophila Ocular Tumor Invasion Model. For ocular tumor invasion assays, female w^{1118} flies and male files with genotype *eyeful*/*Cyo* were crossed. The parental flies mated and laid eggs in one tube, which contains normal food. When the offspring reach the adult stage, the flies with genotype *eyeful*/+ were collected as the model tumor invasion group. Their ocular cells were observed under a stereo microscope, their primary growth and invasion location were recorded (Supplementary Table 3), and the percentage was calculated. For the XFZYD-treated groups, the offspring were kept on the XFZYD-containing medium. Meanwhile, $Oregon^{RC}$ flies which were raised on the regular fly food were used as the control group.

2.7. Immunohistochemistry. Larval discs were dissected and fixed in 40% formaldehyde for 20 min at room temperature (RT). Cold PBS was added to rinse 3 times. After several washes with 0.3% (*v*/*v*) PBST, discs were stained with primary antibodies overnight at 4°C and the following secondary antibodies for 4 h at RT. The primary antibodies used in this study include the following: mouse anti-MMP1 (1 : 200, Developmental Studies Hybridoma Bank, DSHB, 3A6B4), mouse anti-βPS-integrin (1 : 100, DSHB, CF.6G11), and rat anti-E-cadherin (1 : 100, DSHB, DCAD2). The following secondary antibodies were used: goat anti-Mouse-Cyanine3 (Cy3) (1 : 1000, Life technologies, A10521) and goat anti-Rat-Cy3 (1 : 1000, Life technologies, A10522). Vectashield mounting media (Vector Laboratories, H-1500) with DAPI (4′,6-diamidino-2-phenylindole) was used for mounting. The fluorophores of DAPI, GFP, and Cy3 were excited and visualized by a fluorescent inverted microscope system (Olympus, IX51).

2.8. qRT-PCR. TRIzol (Invitrogen) was used to isolate total RNA from ten wing imaginal discs dissected from the third instar larvae or thirty adult heads collected from freshly eclosed flies of indicated genotypes, and qRT-PCR was performed as previously described [53] using the following primers:

(1) For *rp49*: sense—5′-TACAGGCCCAAGATCG TGAA-3′; antisense—5′-TCTCCTTGCGCTTCTT GGA-3′

(2) For *Su(H)*: sense—5′-CTTGCTGCCGGGTCCT TAC-3′; antisense—5′-CTCGCGCATGTACTTC TCCA-3′

2.9. Data and Statistics. All data were verified in at least three independent experiments. Results were presented as bar graphs or scatter plots created with GraphPad Prism 8.0. For statistical significance, one-way ANOVA with Bonferroni's multiple comparison test or chi-squared test was applied. P value less than 0.05 was considered significant and center values as the mean. Error bars indicated standard deviation. P value less than 0.05 was considered significant (ns was not significant; $P \geq 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results

3.1. Xuefu Zhuyu Decoction Preparation and HPLC Analysis. To examine the quality of the Xuefu Zhuyu Decoction (XFZYD), three major compounds were used as quality control standards. When XFZYD high-performance liquid chromatography (HPLC) chromatogram was compared with the standard controls (Supplementary Figure 4a-c), we observed the distinguished peaks for paeoniflorin, ammonium glycyrrhizinate, and naringin in the chemical fingerprints of XFZYD (Figures 1(a)–1(c)). In addition, the content of these three compounds in XFZYD determined by the HPLC method is 0.140%, 0.039%, and 0.220%, respectively. Taken together, these results indicated that the prepared XFZYD consists of the necessary active ingredients and is suitable to carry out the study.

3.2. XFZYD Inhibits Cell Migration in Drosophila Wing Discs. To investigate the XFZYD's suppressive effect on cell migration *in vivo*, we employed a well-established cell migration model in *Drosophila* 3rd instar larval wing imaginal discs [10, 47]. Compared with the control (Figures 2(a), (a')), depletion of the cell polarity gene *scribble* (*scrib*) driven by *patched*-GAL4 (*ptc*-GAL4), which was mediated by RNA interference (RNAi), triggered massive cell migration phenotype (Figures 2(b), (b')). In the *ptc*>GFP/*UAS-scrib-IR*-(*ptc>scrib-IR*-) induced cell migration model, the green fluorescent protein- (GFP-) labeled migrating cells were detached from the anterior/posterior (A/P) compartment boundary and moved toward the posterior part of the wing imaginal discs (Figure 2(b')). To further quantify the cell migration phenotype, we calculated the total number of migrating cells and measured the median or maximum (max) migrating distance in the selected wing pouch region (Supplementary Figure 3) and observed a significant increase in migrating cell number and migrating distance (Figures 2(h)–2(j)).

Next, to test whether there is the inhibitory effect, we prepared feeding media consisting of XFZYD aqueous extract (Supplementary Figure 1m) and raised the *ptc>scrib-IR* flies from the egg stage to the 3rd instar larval stage. Results showed that the loss-of-*scrib*-induced increased migrating cell number was notably suppressed by XFZYD extract at the concentration of 0.0125 g/ml or 0.0200 g/ml, but not that of 0.0100 g/ml or 0.0500 g/ml (Figures 2(b)–2(f), (b')-(f'), (h)). For the max or median migrating distance, XFZYD at the concentration of 0.0125 g/ml showed a stronger inhibitory effect than that of 0.0200 g/ml (Figures 2(i) and 2(j)), while the concentrations of 0.0100 g/ml or 0.0500 g/ml did not perform an obvious inhibitory effect. Intriguingly, we found that XFZYD at the concentration of 0.1000 g/ml could strongly aggravate the *ptc>scrib-IR*-triggered cell migration in cell number, but not in distance (Figures 2(g)–2(j), (g')). Collectively, XFZYD displayed a dose-independent and biphasic effect on the cell migration in the *Drosophila* larval stage. And the concentration of 0.0125 g/ml for XFZYD water extracts was chosen, which significantly suppressed cell migration, to further explore the mechanisms.

3.3. XFZYD Alters MMP1, β-Integrin, and E-Cadherin Expression. To further elucidate the underlying mechanisms of XFZYD in inhibiting cell migration, we analyzed several cell migration-related key factors. Matrix metalloproteinase-1 (MMP1), as a member of the MMPs family, has a direct role in enhancing tumor invasion, whose overexpression indicates a poor prognosis [54–56]. Clearly, inhibition of the well-characterized tumor suppressor E-cadherin's function or expression will lead to cell epithelial-mesenchymal transition, promoting cell migration and metastasis [57]. In addition, as a transmembrane receptor which is composed of α- and β-subunits, integrin adheres to and migrates between cells and is phosphorylated after being subjected to intracellular and extracellular metastasis signals to produce tumor cell migration [58, 59]. In agreement with these previous works, results showed that loss-of-*scrib* under *ptc*-GAL4 control along the A/P boundary could trigger strong epithelial-mesenchymal transition (EMT) like phenotype in the wing pouch region, revealed by upregulated expression of MMP1 (Figures 3(a) and 3(b)) and the major β-subunit of integrin (βPS-integrin) (Figures 3(d) and 3(e)) [60, 61] and downregulation of cell adhesion molecule E-cadherin (Figures 4(a) and 4(b)). And the fluorescence intensity analysis results showed that the altered expression of MMP1, βPS-integrin or E-cadherin is nearly located in the *ptc>scrib-IR* region which was visualized by GFP (Figures 3(b'''), 3(e'''), and Figure 4(b''')), indicating that depletion of *scrib* triggers the increase of MMP1and integrin, or the decrease of E-cadherin in a cell-autonomous manner. Moreover, the upregulation of MMP1 and β-integrin or the reduction of E-cadherin induced by *ptc>scrib-IR* was remarkably ameliorated by XFZYD at the concentration of 0.0125 g/ml (Figures 3(c) and 3(f), and Figure 4(c)). Together, these evidences suggest that the strong inhibitory effect of XFZYD on cell migration may be due to altering the expression of MMP1, β-integrin, and E-cadherin.

3.4. Xuefu Zhuyu Decoction Suppresses Ocular Tumor Invasion. Considering cell migration is a central step for metastasis during tumorigenesis, we introduced a *Drosophila* ocular tumor invasion model, which is named *eyeful* [49], to further analyze the roles of XFZYD in metastasis. In line with a previous study, we found that exogenous ectopic expression of Delta, which functions as the ligand for the Notch signaling pathway, under the control of *ey*-GAL4 in *Drosophila* compound eye, and simultaneous depletion of epigenetic genes *psp* and *lola* leads to overproliferation *in situ* and metastatic tumor formation of *Drosophila* eye tissue cells

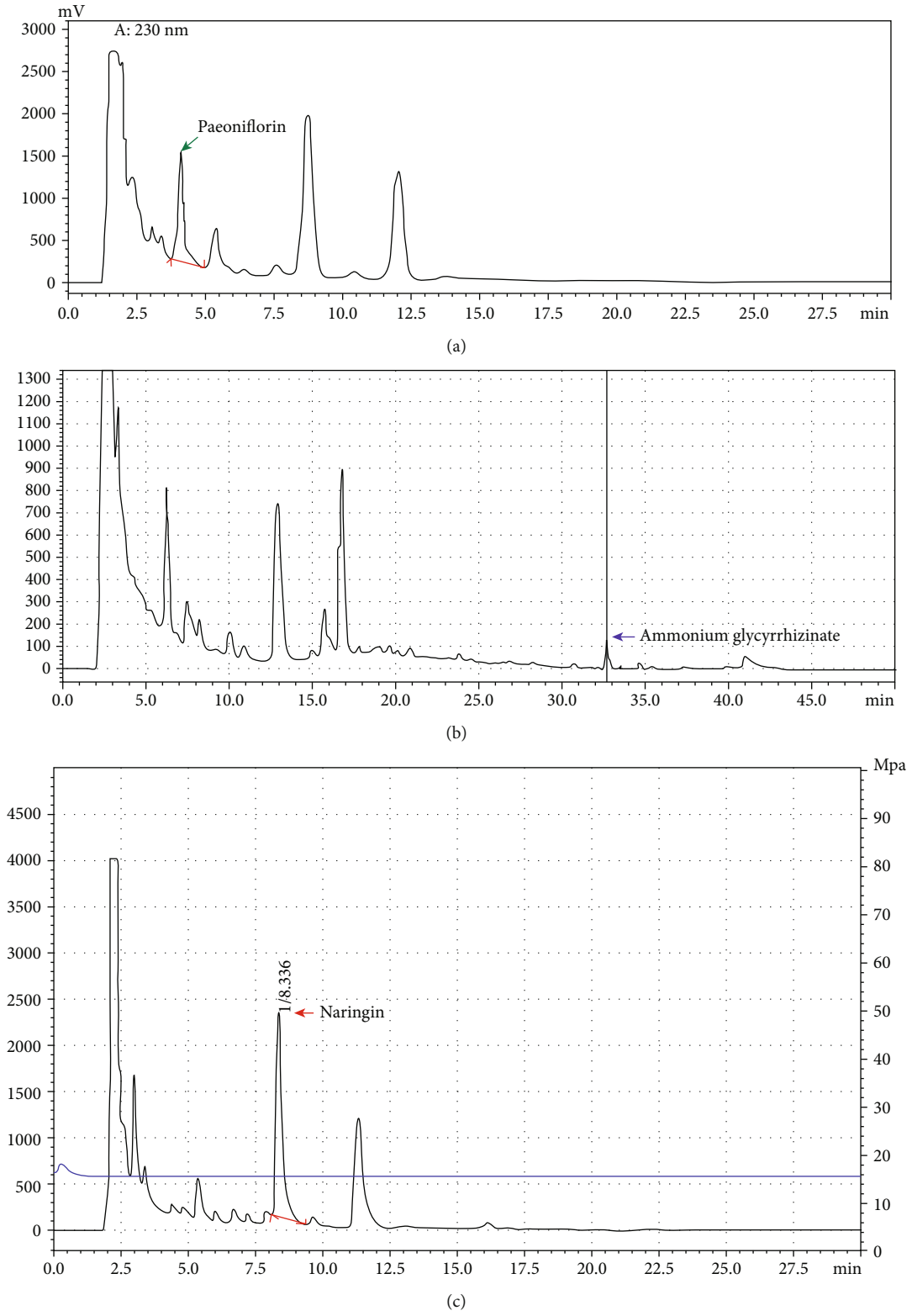

Figure 1: The HPLC chromatogram of XFZYD. Three major compounds (paeoniflorin, ammonium glycyrrhizinate, and naringin) of XFZYD were identified and compared to the standards by the HPLC method. The tested peaks for paeoniflorin (a, 230 nm), ammonium glycyrrhizinate (b, 237 nm), and naringin (c, 283 nm) are indicated with green, blue, and red arrows, respectively.

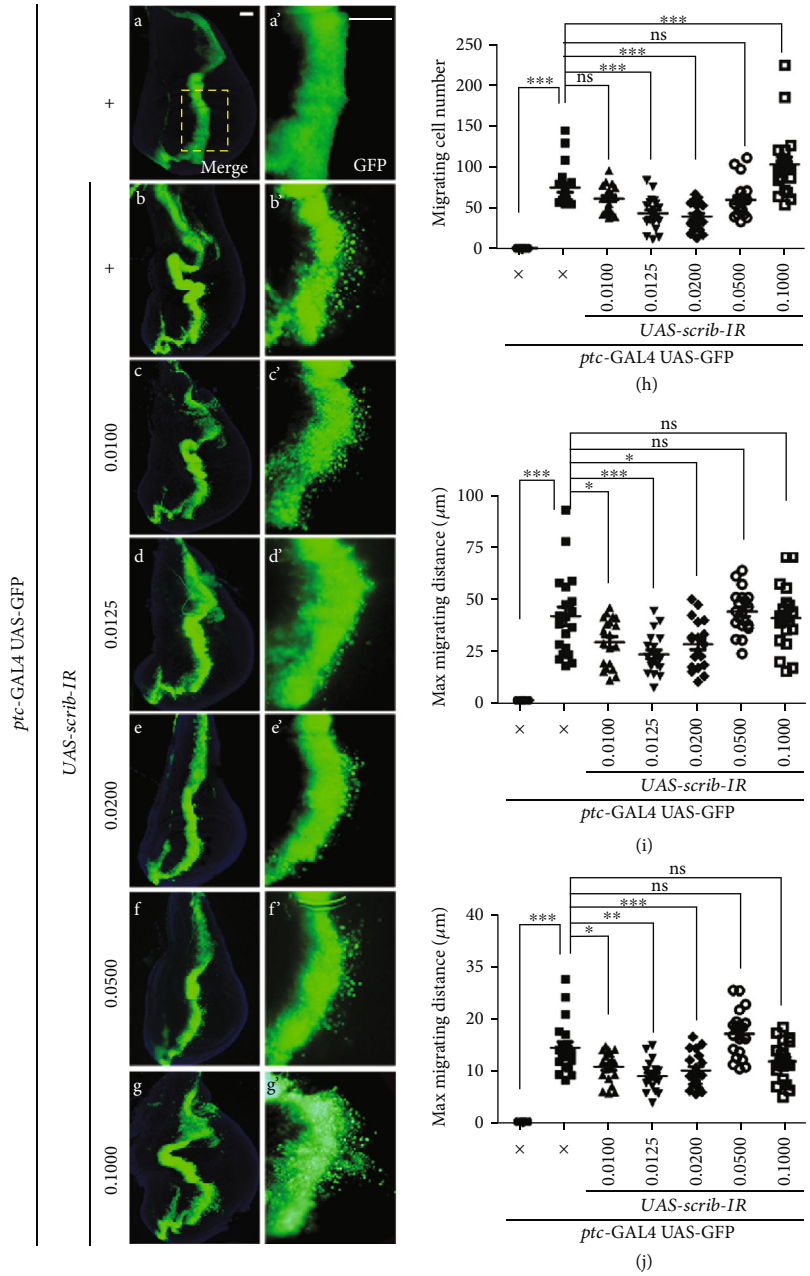

Figure 2: XFZYD suppresses cell migration in *Drosophila* wing discs. (a–g) Fluorescent images of the 3rd instar larval wing discs. Compared with *ptc*-GAL4 *UAS*-GFP control (a and a'), knockdown of *scrib* along the A/P boundary triggered massive cell migration in the wing pouch, marked by green fluorescent protein (GFP) (b and b'). (c–g) and (c'–g') are the representative images of the *ptc>scrib-IR* model when treated with XFZYD at a concentration of 0.0100, 0.0125, 0.0200, 0.0500, or 0.100 g/ml, respectively. Nuclei were labelled with DAPI (4′,6-diamidino-2-phenylindole, blue). Anterior is to the left and dorsal up. (a'–g') are high magnification of the yellow dotted boxed areas in (a–g), respectively. Statistical analysis of the migrating cell number (h), max migrating distance (i), and median migrating distance (j) in indicated groups ($n = 20$ for each genotype). The columns from left to right are (1) *ptc*-GAL4 *UAS*-GFP (*ptc*>GFP)/+, (2) *ptc*>GFP/*UAS-scrib-IR*, and (3–7) *ptc*>GFP/*UAS-scrib-IR* larva treated with XFZYD aqueous extract at different concentrations. Error bars indicate the standard deviation. One-way ANOVA with Bonferroni's multiple comparison test was used to compute P values: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05^{*}$; ns: no significant difference. Scale bar: 50 μm (a–g and a'–g').

(Figures 5(a)–5(h)). Again, 0.0200 g/ml or 0.0500 g/ml XFZYD significantly suppressed the migration rate of the eyeful model (Figure 5(i)), whereas it slightly increased the migration level at the concentration of 0.1000 g/ml (Figure 5(i)). Meanwhile, XFZYD had almost no suppressive effect on *Drosophila* ocular tumor invasion at the concentration of 0.0100 g/ml or 0.0125 g/ml (Figures 5(i)–5(k)). Given that the Notch signal is upregulated in the *eyeful* tumor model, it was hypothesized that XFZYD may inhibit tumor invasion through modulating Notch signaling activity. To verify our assumption, a real-time quantitative PCR (qRT-PCR) assay was performed. In accordance with a previous

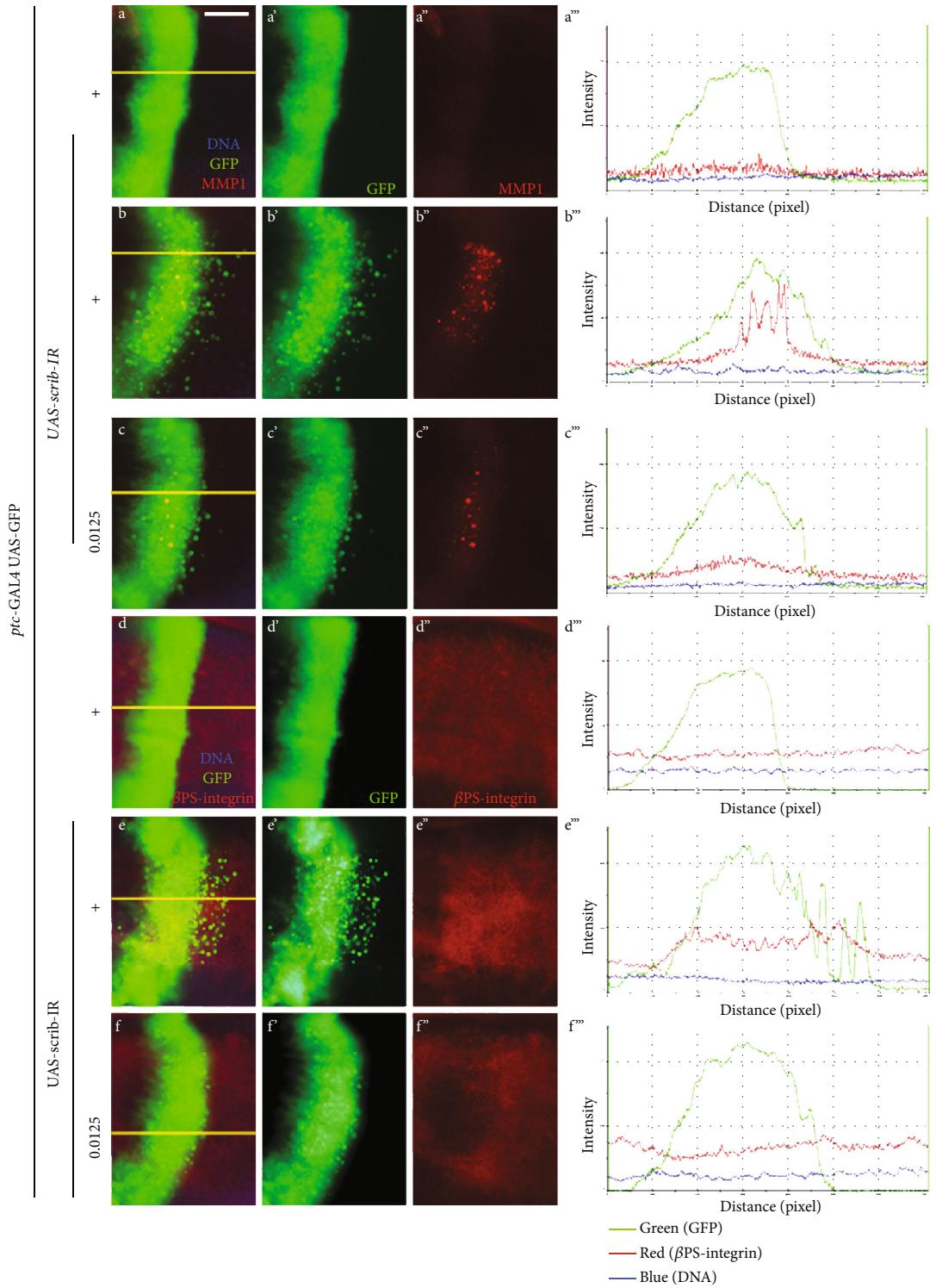

FIGURE 3: XFZYD downregulates MMP1 and β-integrin expression. Merged fluorescence micrographs of the 3rd instar larval wing discs stained with anti-MMP1 (a–c) or anti-βPS-integrin antibody (d–f) are shown. The individual channels detecting only GFP (green, a'–f'), only MMP1 (red, a"–c"), and only βPS-integrin signal (red, d"–f"). Nuclei (DNA) were labelled with DAPI (4',6-diamidino-2-phenylindole, blue). (a"'–f"') Graphs of total intensity sum of each fluorophore with respect to the region of interest (indicated by the yellow solid line) as shown in (a–f), respectively. Fluorescence intensities were measured in pixels using Image-Pro Plus 6.0. Scale bar: 50 μm (a–f).

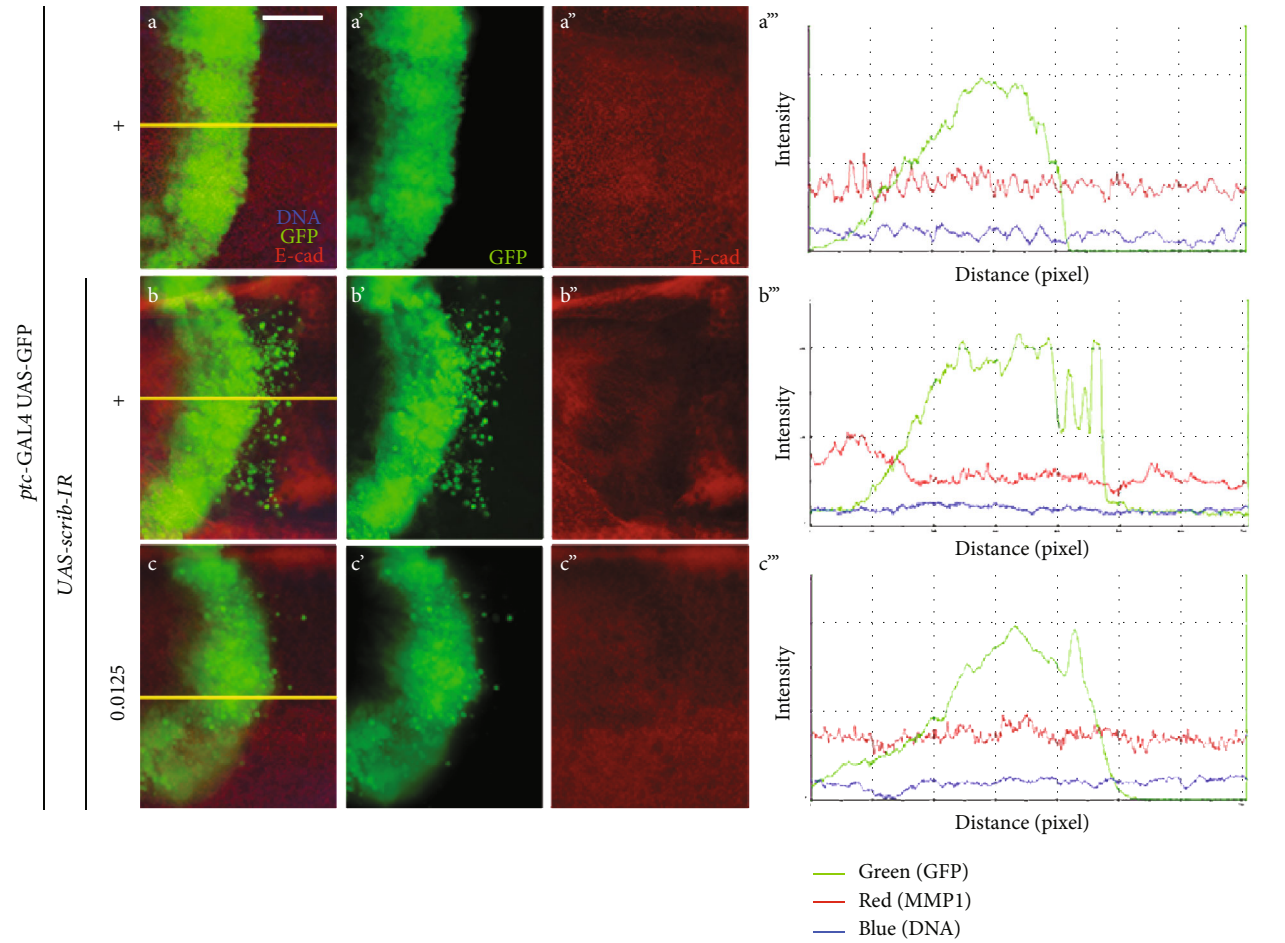

FIGURE 4: XFZYD upregulates E-cadherin expression. Merged fluorescence micrographs of the 3rd instar larval wing discs stained with anti-E-cadherin (cad) antibody (a–c) are shown. The individual channels detecting only GFP (green, a'–c') and only E-cad signal (red, a"–c"). Nuclei were labelled with DAPI (blue). (a"'–c"') Graphs of total intensity sum of each fluorophore with respect to region of interest (indicated by the yellow solid line) as shown in (a–c), respectively. Fluorescence intensities were measured in pixels using Image-Pro Plus 6.0. Scale bar: 50 μm (a–c).

study, we found that the mRNA level of the *Suppressor of Hairless* (*Su(H)*), which acts as the transcription factor in Notch pathway [62], was upregulated in the *eyeful* invasion tumor model (Figure 5(l)). And XFZYD remarkably impeded the *eyeful*-induced elevated transcription level of *Su(H)* at the concentration of 0.0500 g/ml (Figure 5(l)). Hence, we conclude that XFZYD inhibits tumor invasion through downregulating Notch signaling activity.

4. Discussion

Cancer has been a worldwide fatal disease that seriously threatens human health. As more than 90% of cancer patients died from tumor migration, suppression of tumor cell migration has emerged as the central target for cancer treatments [3, 4]. *Drosophila melanogaster* is regarded as an excellent model system to study the genetic and molecular mechanisms of tumorigenesis and metastasis [63, 64]. In present study, the *ptc>scrib-IR*-induced cell migration model and *eyeful* ocular tumor invasion model were established and used to investigate the protective effect of XFZYD.

As a well-known TCM herbal formula, XFZYD is categorized into the BAHRDs and has been extensively applied for treating metabolic syndrome and cardiac-cerebral vascular diseases [34–40]. Although blood stasis is also a main pathogenic mechanism for tumor metastasis in the TCM theory, the application of XFZYD in treating malignant tumors is largely unknown. Interestingly, our *in vivo* data showed that XFZYD decreased cell migration and invasion at the low dose (the concentration of 0.0125 g/ml for cell migration and that of 0.05 g/ml for ocular tumor invasion) but increased cell migration and invasion at a high dose (the concentration of 0.1000 g/ml for both). Collectively, the effects of XFZYD on cell migration and tumor invasion in *Drosophila* displayed a dose-independent and biphasic manner, whereas, in a septic shock rat model, XFZYD may reduce myocardial damage and a protective role for the heart structure and function in a dose-dependent manner [41]. Besides, we have noticed that the doses of XFZYD that affect cell migration and tumor invasion are different, which may be due to distinct genetic backgrounds of two fly models. Several previous studies showed that multiple cancer-related factors and signal

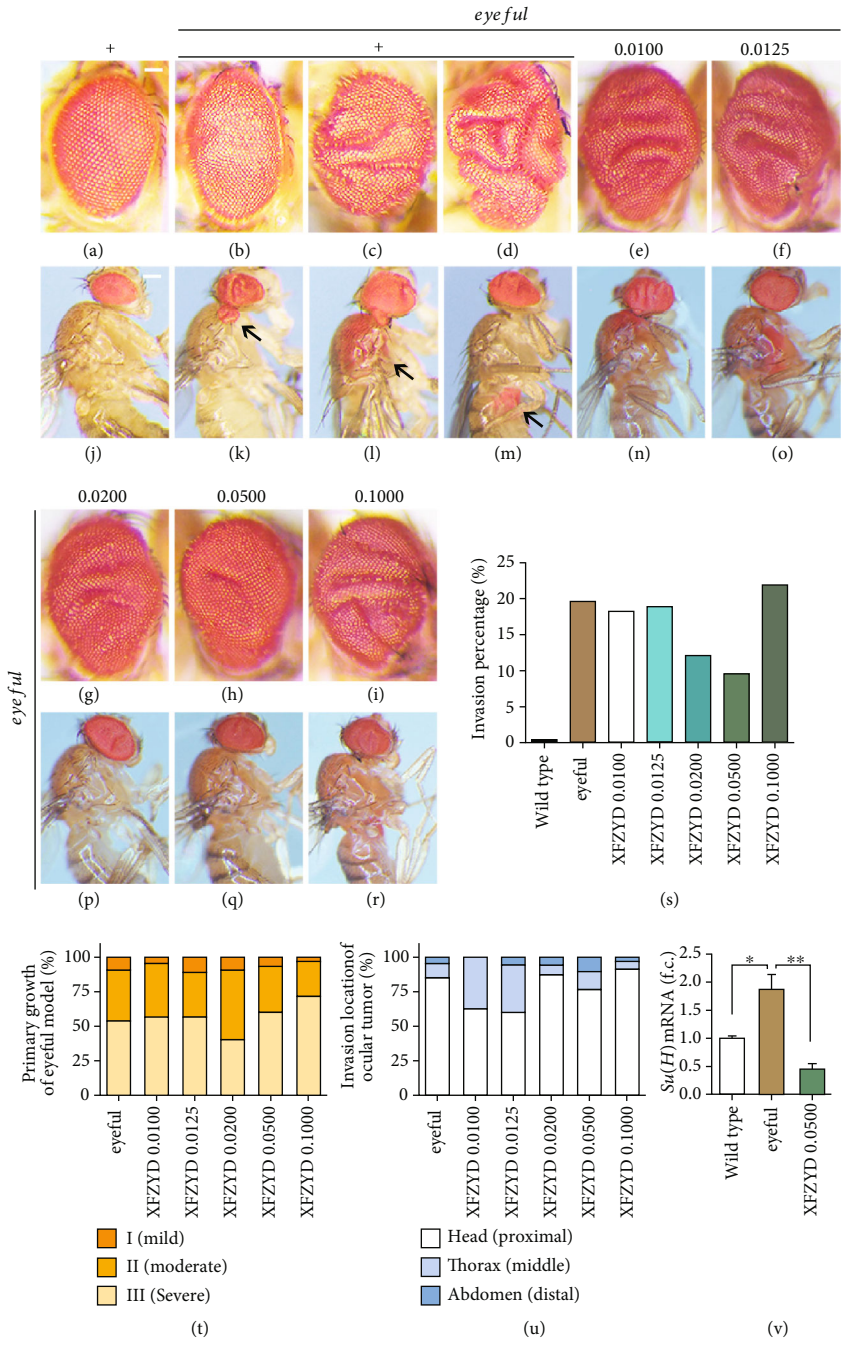

FIGURE 5: XFZYD performs a suppressive effect on *eyeful* ocular tumor invasion. Light micrographs of *Drosophila* adult eye (a–i) and body (j–r) are shown. (a) and (j) are the wild-type controls, and (b–d) and (k–m) are the representative images of the *eyeful* model. When the red compound eye cells are observed outside the eye tissue, the ocular tumor is considered to have migrated. According to the number of folds within *Drosophila* eye, *eyeful* files without migrated tumors are divided into three degrees: I (no obvious fold, mild), II (1-2 folds, moderate), and III (≥3 folds, severe) (b–d). According to the invasion location of the ocular tumor, *eyeful* flies with tumor metastasis are divided into the head (proximal), thorax (middle), and abdomen (distal) subgroups (k–m, the red eye tissue cells are indicated by black arrow). (e–i) and (n–r) are the representative images of the *eyeful* model when treated with XFZYD at a concentration of 0.0100, 0.0125, 0.0200, 0.0500, or 0.100 g/ml, respectively. (s) A quantification of *eyeful* tumor invasion percentage. The number of flies in each group with or without migrated tumors was recorded; then the invasion ratio was calculated. The columns from left to right are (1) wild type (Oregon^{RC}, 0.00%, $n = 65$, 2), *eyeful* (19.57%, $n = 92$, 3), *eyeful*+XFZYD 0.0100 g/ml (18.06%, $n = 72$, 4), *eyeful*+XFZYD 0.0125 g/ml (18.75%, $n = 64$, 5), *eyeful*+XFZYD 0.0200 g/ml (12.07%, $n = 116$, 6), *eyeful*+XFZYD 0.0500 g/ml (9.52%, $n = 84$, 7), and *eyeful*+XFZYD 0.1000 g/ml (21.88%, $n = 96$). A chi-squared test was applied. Stacked bar graphs of primary growth (t) and invasion location (u) for *eyeful* tumor assay are shown (sample size details are shown in Supplementary Table 3). (v) Histogram showing the levels of *Su(H)* mRNA as measured by qRT-PCR. Error bars represent standard deviation from three independent experiments. One-way ANOVA with Bonferroni's multiple comparison test was used to compute P values: $^{**}P < 0.01$ and $^{*}P < 0.05$. Scale bar: 50 μm (a–i) and 150 μm (j–r).

transduction pathways exhibit varied expression levels and activities in the *ptc>scrib-IR* cell migration and *eyeful* tumor models [10, 47–49]. And another reasonable explanation is that the best inhibitory effects of different doses on the two models may be caused by different active ingredients (or different combinations of active ingredients) in XFZYD.

Based on quantifications of daily *Drosophila* food intake [65], it is estimated that flies raised on medium containing 0.0125 g/ml, 0.05 g/ml, or 0.1000 g/ml XFZYD ingest about 0.4 mg/kg body weight of the drug per day [66], which is, respectively, comparable to the treatment dosage of 26.04 g, 104.2 g, or 208.3 g per day for human patients. In the clinic, the patients with coronary heart disease were treated with XFZYD one dose (78 g) a day [32]. Thus, according to the normal dose, taking XFZYD 26.04 g/day for human (equivalent to 0.0125 g/ml for fly) is far below the usual medication dosage (78 g/day). Above all, the data may provide a key clue for the application of XFZYD in cancer clinical treatment.

Notch signaling is an evolutionarily conserved pathway that regulates many cellular processes, including cell proliferation, survival, apoptosis, invasion, angiogenesis, and stem cell self-renewal [67–69]. Recently, Notch signal has been regarded as a central regulator in the induction of EMT, which is important for migration and metastasis of cancer cells [70–72]. In addition, several studies revealed the molecular mechanisms behind Notch-mediated EMT regulation during non-small-cell lung cancer and breast tumorigenesis [73, 74]. Consistently, in this study, XFZYD showed its anticancer activities through inhibiting EMT-mediated cell migration (Figures 3 and 4) and downregulating Notch signal activity (Figure 5) in *Drosophila*. Overexpression of Delta (the ligand in fly) is a very powerful strategy to increase Notch signal by releasing the Notch intracellular domain (NICD), which enters the nucleus and regulates the transcriptional activity of Su(H) [75]. Thus, we would like to examine the subcellular distribution of the NICD protein (tagged by GFP/YFP) and the expression of Notch/Su(H) target genes (*Enhancer of split* (*E (spl)*), *Cut* and *Wingless*) *in vivo* to directly monitor Notch pathway activity for further investigation [76–79].

In conclusion, our work proved that XFZYD has anticancer activity on cell migration and tumor invasion at the moderate concentrations. Moreover, XFZYD could alter the expression of MMP1, integrin, or E-cadherin to suppress cell migration. Finally, it is demonstrated that XFZYD impedes ocular tumor invasion presumably by inhibiting Notch signaling activity. In sum, a positive function of XFZYD in suppressing cell migration and tumor metastasis has been shown, and the results indicated the potential drug targets and provide key clues for cancer clinical treatment strategies.

Abbreviations

TCM: Traditional Chinese medicine
BM: Basement membrane
BAHRDs: Blood-activating stasis-resolving drugs
XFZYD: Xuefu Zhuyu decoction
HPLC: High-performance liquid chromatography
HCR: Homologous chromosomes recombination
MMP1: Matrix metalloproteinase-1
A/P: Anterior/posterior
RNAi: RNA interference
GFP: Green fluorescent protein
EMT: Epithelial-mesenchymal transition
NICD: Notch intracellular domain.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Sitong Wang, Fanwu Wu, and Chenxi Wu conceived the project. Sitong Wang, Fanwu Wu, Bin Ye, Shiping Zhang, Xingjun Wang, Guowang Li, Menglong Zhang, Shuai Wang, Zixue Zhao, and Peng Li performed the experiments. Fanwu Wu, Chunhua Jiang, Xiaojin La, Yongsen Jia, and Hong Chang supervised the study and gave advice. Sitong Wang, Fanwu Wu, Qian Xu, and Chenxi Wu analyzed the data and wrote the manuscript. All authors gave the final approval for publication. Sitong Wang and Fanwu Wu contributed equally to this work.

Acknowledgments

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Supplementary Materials

Supplemental Information includes three tables and four figures. Supplementary Table 1 The formulation of XFZYD (one dose). Supplementary Table 2 Batch numbers and manufacturers of XFZYD. Supplementary Figure 1 Composition of Xuefu Zhuyu Decoction. Supplementary Figure 2 A flow chart for establishing the *eyeful* model. Supplementary Figure 3 Measurement of migrating cell distance. Supplementary Figure 4 The HPLC chromatogram of standard controls. Supplementary Table 3 Ocular tumor's primary growth and invasion location in adult *Drosophila*. *(Supplementary Materials)*

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Research Article

Cauliflower Mosaic Virus TAV, a Plant Virus Protein That Functions like Ribonuclease H1 and is Cytotoxic to Glioma Cells

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Recent comparisons between plant and animal viruses reveal many common principles that underlie how all viruses express their genetic material, amplify their genomes, and link virion assembly with replication. Cauliflower mosaic virus (CaMV) is not infectious for human beings. Here, we show that CaMV transactivator/viroplasmin protein (TAV) shares sequence similarity with and behaves like the human ribonuclease H1 (RNase H1) in reducing DNA/RNA hybrids detected with S9.6 antibody in HEK293T cells. We showed that TAV is clearly expressed in the cytosol and in the nuclei of transiently transfected human cells, similar to its distribution in plants. TAV also showed remarkable cytotoxic effects in U251 human glioma cells in vitro. These characteristics pave the way for future analysis on the use of the plant virus protein TAV, as an alternative to human RNAse H1 during gene therapy in human cells.

1. Introduction

Cancer remains a major public health problem worldwide. Current standards of cancer therapy include resection surgery (if applicable), radiation, chemotherapy, immunotherapy, and/or biological therapy [1]. The development of new therapies, such as targeted gene therapies, may provide an effective and nontoxic method of treating cancer.

A connection between the regression of cancer and viruses has long been theorized and reports of regression (cervical cancer, Burkitt's lymphoma, and Hodgkin's disease) after vaccination or infection with a related virus appeared in the early 20th century. Efforts to treat cancer through vaccination or infection with a virus deliberately started in the mid-20th century.

Plant viruses and vertebrate viruses are believed to exist in two different and nonoverlapping biological niches [2]. To date, plant viruses have not been described as pathogens for vertebrates or humans or known even to infect them [3]. Striking similarities have been observed between a plant pararetrovirus, Cauliflower mosaic virus (CaMV), and animal retroviruses, specifically their replication through productive reverse transcription and their high recombination rate [4]. However, there are also remarkable differences that include the tropism for two different kingdoms, the nature of the CaMV genome (a molecule of circular double-stranded DNA) and its lack of integration into host cell chromosomes [5]. Precisely, for its replication, CaMV is not dependent on the host DNA replication apparatus, in contrast to the geminiviruses (ssDNA plant viruses) that must overcome the lack of DNA replication factors in G_0 cells, similar to the animal DNA tumor viruses such as SV40 and adenovirus [6]. There are just a few examples of plant viruses or plant virus proteins known to interact with human cells. Tobacco mosaic virus has been shown to induce endoplasmic reticulum stress-related autophagy in HeLa cells [7]. Tomato bushy stunt virus P19 has been shown to suppress RNA silencing in animal cells downstream of miRNA maturation [8, 9]. Although the CaMV 35S plant promoter is reported to be active in human enterocyte like cells [10], the expression of CaMV proteins in human cells has not been investigated.

Cauliflower mosaic virus (CaMV) is a DNA plant virus and is the type member of the family Caulimoviridae, which, together with the hepadnaviruses, belong to the group of pararetrovirus, which have a DNA genome but replicate by reverse transcription. The CaMV genome is a circular double-stranded DNA of approximately 8,000 bp and is encapsidated within a 50 nm icosahedral particle. CaMV infects principally plants of the Brassicaceae and Solanaceae families (turnips, cauliflowers, sprouts, and cabbages). Plant viruses replicate within an infected cell, move from cell to cell, and are transported from plant to plant. Once introduced within a host cell, virions migrate to the nuclear envelope, where they decapsidate. The viral genomes then enter the nucleus where all the gaps present in the genome are sealed and the covalently closed DNA then associates with host histones to form a supercoiled minichromosome that does not integrate into the host chromosomes and that is transcribed by the host RNA polymerase II to generate two mRNAs, the polycistronic 35S RNA comprising the entire genome encoding six proteins, and the 19S RNA encoding a single protein, the transactivator/viroplasmin protein (TAV), and the two mRNAs then move to the cytoplasm. In the cytoplasm, TAV is translated from the 19S RNA and aggregates in small inclusion bodies, where it transactivates translation of all other viral proteins from the 35S RNA [11]. TAV is recognized as a multifunctional effector interacting with a broad array of host proteins and either initiates the innate immunity reaction in a nonpermissive host or interferes with it in a permissive host [12–14]. TAV is composed of 520 amino acids that form an alpha-helical motif and is the least conserved protein within the CaMV genome. It contains a nuclear export signal (NES) at the N-terminus, a TAV domain, two nuclear localization signals (NLS), two RNA binding domains, and a putative zinc finger at the C-terminus [15]. In CaMV-infected plants, TAV is a nucleocytoplasmic shuttling protein [16] and nuclear import of TAV by the bipartite NLS and the nonconventional NLSa is likely through the importin alpha pathway. A minor part of TAV import could also occur through interactions between the TAV domain and L13 and L18 and other ribosomal proteins. This process causes TAV retention within the nucleolar, as opposed to the nucleoplasmic compartment [17]. TAV is a translational reinitiation factor that associates with the host translational machinery. This function is mediated by physical interactions between the TAV domain, the initiation factor eIF3 8 (subunit g), L13, L18, and L24 (ribosomal proteins) [16]. The hallmark of the RNase H/caulimovirus nucleic acid binding motif is a stretch of 40 amino acids with 11 highly conserved residues, seven of which are aromatic. Point mutations, insertions, and deletions indicated that the integrity of the motif is important for binding. The similarity between the RNase H and the caulimovirus domain suggests a common interaction with duplex RNAs of these two different groups of proteins [18]. However, little is known about TAV effects in the nonpermissive human host, and the aim of this study is to determine if plant virus proteins may have the same activity in mammalian and plant cells.

2. Materials and Methods

2.1. Computational Methods: Protein-DNA and Protein-Protein Interactions. Interactions between DNA and proteins from CaMV were predicted with DP-bind [19], a freely available program that combines three different tools (support vector machines, kernel logistic regression, and penalized logistic regression) to address the two-class classification problem consisting in recognizing DNA-binding and nonbinding residues.

Prediction considers the input sequence and the Position Specific Scoring Matrix (PSSM) compiled upon PSI-BLAST search for similar sequences. Consensus between two out of the three methods (majority consensus) has been adopted as a prediction criterion. The performance reported for the majority consensus method is 76% accuracy, 76% sensitivity, and 75% specificity [19]. Possible interactions between viral

and human proteins have been inferred by screening the human proteome for sequences sharing similarity with known plant interactors and by analyzing the retrieved proteins in the context of the STRING human interactome [20]. Sequence similarity was searched with BLAST.

2.2. Computational Methods: Modelling of CaMV TAV Protein. The TAV three-dimensional structure is unknown and no template is available for the full-length modelling of the protein. To search for possible structural templates, the CaMV TAV sequence has been launched against Pfam [21], a database containing hidden Markov models for 17929 different protein domains. A 48-residue long domain significantly aligns with Pfam PF01693 (Cauli VI), which includes domains endowed with a three-dimensional structure in the protein database (PDB). Among them, the most similar to TAV's domain (target) is the hybrid domain of the human RNase H1, a specialized enzyme that can specifically resolve long DNA-RNA hybrids that can be used as a template [22]. In particular, we adopted the PDB file 3BSU [23], reporting the structure of the human domain in interaction with double-stranded RNA. The domain has been modelled with Phyre2 [24] on the basis of the hybrid domain of human RNase H1 (PDB code: 3BSU). The secondary structure for P6 protein has been predicted with PSIPRED [25].

2.3. Cell Culture and Transient Transfection. HEK293T cells (human embryonic kidney cell line 293 T cells that contain the SV40 T-antigen) were a gift of the European Brain Research Institute (EBRI) Rita Levi-Montalcini in Rome. HEK293T cells were grown in Dulbecco's modified Eagle's Medium High Glucose (DMEM High, Euroclone) supplemented with 10% fetal bovine serum (Euroclone) and 1% Penicillin/Streptomycin (Life Technologies) and they were cultured at 37°C and 5% CO_2. A human glioblastoma cell line (U251, National Cancer Institute) was maintained in Eagle's Minimum Essential Medium (ATCC, 30-2003) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Thermo Fisher Scientific) at 37°C in a humidified 5% CO_2 atmosphere. CaMV strain CM1841 full-length ORF I, III, IV, V, and VI (corresponding to MP, VAP, CP, EP, and TAV, respectively) were cloned from vector pS10-08 (CaMV strain CM1841 cloned into pBR322) into a mammalian expression vector pcDNA 3.1 (+) (EV), according to the native virus gene sequence (see database Genbank: V00140.1). The full-length ORF VI gene differs from the native sequence by a point mutation A402C (see database Genbank: V00140.1). pcDNA 3.1_P2A EGFP (EGFP) was used to test transient-transfection efficiency. HEK293T cells were seeded on coverslips within the wells of a 6-well plate at a density of 2×10^5 cells/well (for confocal experiments) or in a T75 flask at a density of 3×10^6 cells/flask (for western blot); cells were transiently transfected with 0.5 μg of DNA/well and 8 μg of DNA/flask of each DNA plasmid pcDNA3.1 (EV), pcDNA 3.1 ORF IV (CP), and pcDNA 3.1 ORF VI (TAV) (Genscript) with Polyethylenimine (PEI) 1 : 6 ratio with DNA. Experimental conditions include HEK293T cells transiently transfected with pcDNA 3.1 ORF IV (CP) and pcDNA 3.1 ORF VI (TAV), while controls include HEK293T cells not transfected and transiently transfected with EV and EGFP.

Transfection efficiency was found to be around 90% at 48 hours after transfection. U251 cells were seeded into a 24-well plate at a density of 2×10^4 cells/well; cells were transiently transfected with 0.5 μg of DNA/well of each DNA plasmid with 50 μl of U251 Cell Avalanche transfection reagent mix (EZ Biosystems) 1 : 5 ratio with DNA. Experimental conditions include U251 cells transiently transfected with pcDNA 3.1 (+) ORF I (MP), pcDNA 3.1 ORF III (VAP), pcDNA 3.1 ORF IV (CP), pcDNA 3.1 ORF V (EP), pcDNA 3.1 ORF VI (TAV), and pcDNA 3.1 ORF VI in which the pathogenicity/host-range/avirulence domain of TAV12 (amino acids 2-113) was deleted (TAV deleted); negative controls include U251 cells not transfected, treated with transfection reagent only (media), and transiently transfected with EV and EGFP. Transfection efficiency was tested around 30–50% at 48 hours after transfection.

2.4. Cellular Fractionation. Cell's nucleus and cytoplasm separation was performed as previously described [26], with minor modifications. Nontransfected HEK293T cells and transiently transfected HEK293T cells (EV and TAV), 48 h after transfection, were twice washed in PBS 1X, resuspended in TM5 buffer (10 mM Tris, 5 mM MgCl2), and incubated 1 minute at room temperature and 5 minutes on ice during rotation. Triton X-100 (10% in H_2O) was added and the suspension was passed through a syringe with a 22G needle and centrifuged for 10 minutes at 4°C. The supernatant (cytoplasm proteins) was collected and supplemented with Halt Protease and Phosphatase Inhibitor; then proteins were quantified using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific) and prepared for western blotting. Western blotting of cytosolic proteins was performed using histone H3 (Merck Millipore) for an endogenous nuclear marker, rabbit anti-TAV, and GAPDH-HRP-conjugated antibody (Origene, 2D9) as a control to show the absence of cytoplasm contamination. The pellet was resuspended in TM5 buffer, centrifuged twice for 10 minutes at 4°C, and the supernatant was aspirated to dry the nuclear pellet. Nuclear proteins were extracted using RIPA Buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) supplemented with Halt Protease and Phosphatase Inhibitor and DNase 1 μg/ml (Qiagen), incubated 30 minutes on ice in agitation, and then spun at 17000g for 15 minutes at 4°C. Nuclear proteins (supernatant) were collected and prepared for western blotting that was performed using the GAPDH-HRP-conjugated antibody (Origene, 2D9) for endogenous cytoplasm marker, anti-TAV rabbit polyclonal serum [27, 28], and histone H3 as a control for nuclear contamination.

2.5. Western Blot. HEK293T cells were transiently transfected with EV, CP, and TAV; nontransfected cells were used as a negative control. After 48 h, cells were lysed in RIPA

Buffer with Halt Protease and Phosphatase Inhibitor, incubated 15 minutes on ice, and then spun at 17,000g for 15 minutes at 4°C. The amount of total protein was quantified using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific). The supernatants of each sample were collected and mixed with Bolt LDS Sample Buffer and Bolt Sample Reducing Agent (Thermo Fisher Scientific).

Samples were boiled for 5 minutes, loaded on a Bolt 4–12% Bis-Tris Gel (Thermo Fisher Scientific), and transferred to a PVDF nitrocellulose membrane (Biorad). The blots were blocked in 0.1% Tween-20 and 5% nonfat milk (Biorad) in Tris Buffered saline (Biorad) for 1 h at room temperature, and then membranes were tested for different antibodies: anti-TAV rabbit polyclonal serum (1 : 250) [19, 20] and anti-CP rabbit polyclonal serum (1 : 250, DSMZ AS-0206) overnight at 4°C, followed by the rabbit IgG-heavy and light chain antibody HRP-conjugated (Bethyl). Detection was performed using Clarity Western ECL substrate (Biorad), according to the manufacturer's instructions, and images were acquired using a Chemidoc XRS System (Biorad).

2.6. Plant Material and Infection. B. rapa plants (var. "Nagaoka") were grown in a greenhouse at 20 ± 2°C with a 16 h photoperiod. After 15 days of growth, the plants were infected with an infectious CaMV clone, CaMV strain CM1841 excised from the pS10-08 vector (full-length CaMV strain CM1841 cloned into pBR322) by digestion with SalI. Mechanical inoculation was performed using Paul buffer (phosphate buffer 0.05 M pH 7.0, 5 mM DIECA, 1 mM EDTA, and 5 mM sodium thioglycolate) and celite as an abrasive powder. The typical symptoms of curling and leaf mosaic appeared two weeks after inoculation. Thirty-five days after infection, samples from infected plants were collected for analyses at the ultrastructural level. Noninfected *B. rapa* plants, inoculated with Paul buffer alone, were used as negative controls.

2.7. Tissue Processing for Transmission Electron Microscopy (TEM). Several portions of the infected and uninfected leaf tissue were sampled using an ultrathin blade in the presence of 5% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2). In the same buffer, the HEK293T EV and TAV cells, at 48 h after transfection, were resuspended after centrifugation at 1000g for 5 minutes. Glutaraldehyde fixation was improved at the pressure of 2 bar for 20 min before embedding with an Araldite/TAAB812 Resin kit and mounting on the grids as previously described [29].

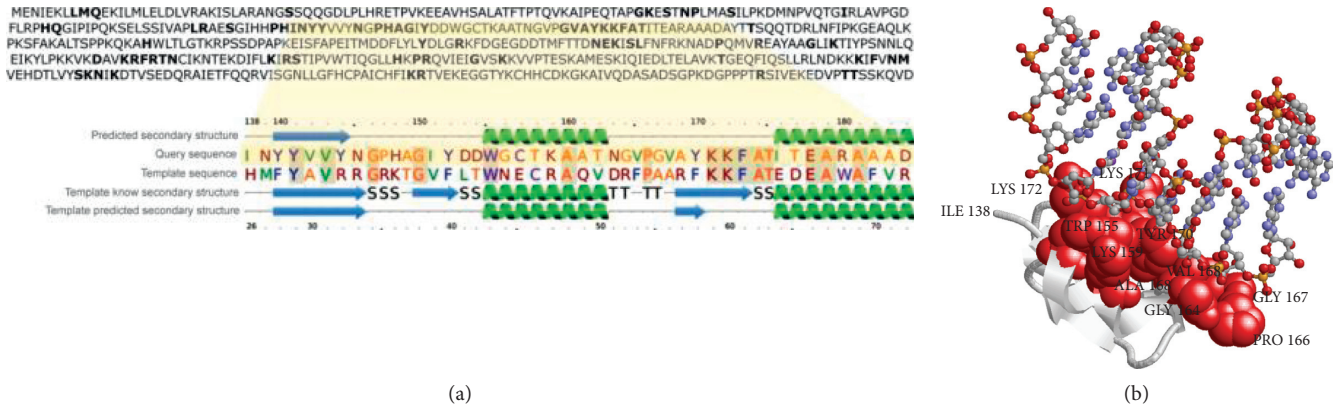
2.8. Immunocytochemical Labelling. Sample sections were processed as previously described [29] on grids for electron microscopy, adding 7% uranyl acetate to the contrasting step. After drying with the filter paper, the grids were examined with a 100 kV PHILIPS CM10 electron microscope.

2.9. HEK293T Cells Staining for Confocal Microscopy Analysis. HEK293T cells were grown on coverslips (0.17 mm thickness round cover glass 18 mm, Warner Instruments) in 6-well plate, and 0.5 μg of DNA (pcDNA3.1, pcDNA 3.1 ORF IV (CP), and pcDNA 3.1 ORF VI (TAV)) with PEI 1 : 6 ratio with DNA was used for each well. Cells were fixed 48 hours after transient transfection in 4% paraformaldehyde (PFA) (Image-IT, Thermo Fisher Scientific) for 15 minutes at room temperature (RT). Afterwards, samples were permeabilized and blocked with 0.3% Triton X-100 and 5% FBS in PBS for 1 hour at RT. Coverslips were incubated with primary antibodies for CP and TAV (1 : 500 in blocking solution) overnight at 4°C, followed by the appropriate secondary antibody goat anti-rabbit Alexa Fluor 594 (Life Technologies, 1 : 600 in blocking solution) for 1 h at RT.

Fluorescence images were acquired on a Carl Zeiss Axioskop 40 Microscope. The following commercially available antibodies were used in immunofluorescence: polyclonal rabbit anti-CaMV IgG (DSMZ, AS-0206) and goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody conjugated to Alexa Fluor 594 (red color; 1 : 600 in blocking solution). TAV antiserum is a noncommercial rabbit anti-TAV polyclonal serum [27, 28].

2.10. Confocal Image Acquisition and Analysis. Confocal images of cell-associated fluorescence were acquired using the Zeiss LSM 800 confocal system (Carl Zeiss Microscopy, Germany). Three laser lines, 405 nm (blue, for nuclei), 488 nm (green, S9.6), and 561 nm (red, TAV), were used in these imaging experiments. Blue, green, and red signals were separated by a quad DAPI/FITC/TRITC/Cy5 dichroic beam splitter and were further acquired using a Gasp detector (Carl Zeiss LSM 800, Germany). A Plan-Apochromat 63x/1.4 Oil DIC objective (Carl Zeiss LSM 800, Germany) was used to visualize multicolored, labelled cell samples. All the parameters used in confocal microscopy were consistent in each experiment, including the laser excitation power, detector, and offset gain. Software Zen Blue (Carl Zeiss Microscopy) was used to generate original images and to collect z-stacks in order to achieve better three-dimensional information about the spatial location of TAV as subcellular localization within the cells (1 μm thickness of sample slices). Negative control samples (nontransfected and EV) were stained with the same conditions as described above. All the images were acquired under the same instrumental settings. To assure the quality of acquired images, we took measurements with the same size of optical sections in three channels (405/488/561 nm). Furthermore, signal/noise ratio was assured by averaging data for every single image acquired. The saturated signal was avoided by using the range. Software Zen Blue was used to measure cell-associated intensity of the green events in nontransfected cells, EV, CP, and TAV samples. Each sample was examined by analyzing a large area [30] or at least 10 different imaging fields. Total intensity of the sample was measured and averaged among all images per set, in order to assure the statistics.

2.11. Cytotoxicity Assay. Cytotoxicity assays using trypan blue were performed on U251 cells not transfected, treated with media only, and transiently transfected with EV, MP, VAP, CP, EP, TAV, and TAV-deleted vectors. The results



(a) (b)

Figure 1: Modelling the 138-184 domain of CaMV TAV protein. (a) TAV sequence is shown, highlighting (in bold) residues putatively interacting with nucleic acids as predicted with DP-bind. Fragment 138-184 is aligned with the hybrid-binding domain of human RNase H1, whose structure is resolved (PDB: 3BSU) and has been adopted as a template. The alignment of structure-derived and computed secondary structures are also reported. (b) The structure of the double-stranded RNA molecule cocrystallized with the human protein is transferred on the modelled domain upon target-template superimposition. Balls and sticks representation is adopted for the RNA molecule. Residues represented with red Van Der Waals spheres are less than 0.5 nm distant from the RNA molecule. Residue marked ILE 138 is the N-terminus of the modelled domain.

come from five biological replicates (technical duplicate of the data for each biological experiment). Cell viability data was acquired 5 days after transient transfection (as described above) using the Trypan Blue Assay reagent with the aid of the Countess II automated cell counter (Thermo Fisher Scientific). Cells were cultured as described above with a change of media after 72 h. The sample sizes of the vitality data differ as not every experimental condition has been analyzed in each experiment. Results are summarized as robust descriptive indices (median and interquartile range) that are not influenced by extreme values.

2.12. Statistical Analyses. The null hypotheses of equality between medians versus the alternative hypothesis of inequality have been inferred by means of the nonparametric Mann–Whitney rank sum test; $\alpha = 0.05$ significance level has been set.

3. Results

3.1. TAV Sequence Similarity with the Human RNase H1. We screened the human proteome for sequences sharing significant similarity with CaMV viral proteins, in order to elucidate the possible effects of viral proteins within human cells. Among CaMV proteins analyzed, TAV is the only one sharing significant similarity with a human protein (see Table S1 in the Supplementary Material for comprehensive analysis). TAV (UniprotKB: P03558) is a transactivator/viroplasmin protein (P6) involved in the translation of polycistronic viral DNA and its domains have been previously described [14, 31, 32]. Figure 1(a) shows the sequence alignment between target (TAV) and the hybrid domain of the human RNase H that we used as template (Protein Data Bank code: 3BSU) and the comparison between their secondary structures as predicted with PSIPRED (for both target and template) or derived from the structure (for the template). Out of 48 residues, 15 are conserved (31.3% sequence identity) [18]. From the structural alignment between template and model (that superimpose with a Root Mean Square Deviation of 0.15 nm), it is possible to infer possible RNA interaction sites for TAV. Figure 1(b) shows the residues in the model with a distance lower than 0.5 nm from the RNA molecule transferred from the template. The hypothesis that the TAV domain can interact with human nucleic acids is corroborated by the fact that 15.2% of TAV residues are predicted to be protein-DNA interaction sites using the program DP-bind (Figure 1(a)). See Table S2 in the Supplementary Material for comprehensive analysis. We further characterized the role of TAV by searching for possible physical interactions of CaMV viral proteins with human proteins. The possible interactors of TAV in the natural host cells, in plants, have been previously reported [14, 33–37]. A clear similarity with human proteins is shown for some of the plant interactors, suggesting that human analogous can conserve the interaction with TAV. See Table S3 in the Supplementary Material for comprehensive analysis.

3.2. Nuclear Expression of TAV in Transiently Transfected HEK293T Cells. In plants, TAV is actively transported into the nucleus through its two importin-α-dependent nuclear localization signals [17]. Since TAV is known to be a nucleocytoplasmic shuttle protein [16], we examined the nuclear expression of TAV in transiently transfected HEK293T cells (TAV-HEK293T cells), which are characterized by a high transfection efficiency and a high level of DNA/RNA hybrids, by using a nuclear separation protocol. Using western blot, we found that TAV is expressed exclusively in TAV-HEK293T cells, both in the cytoplasm and in the nucleus (Figures 2(a) and 2(b)). See Figures S1–S3 in the Supplementary Material for comprehensive image analysis (the quality of the nuclear separation protocol is shown).

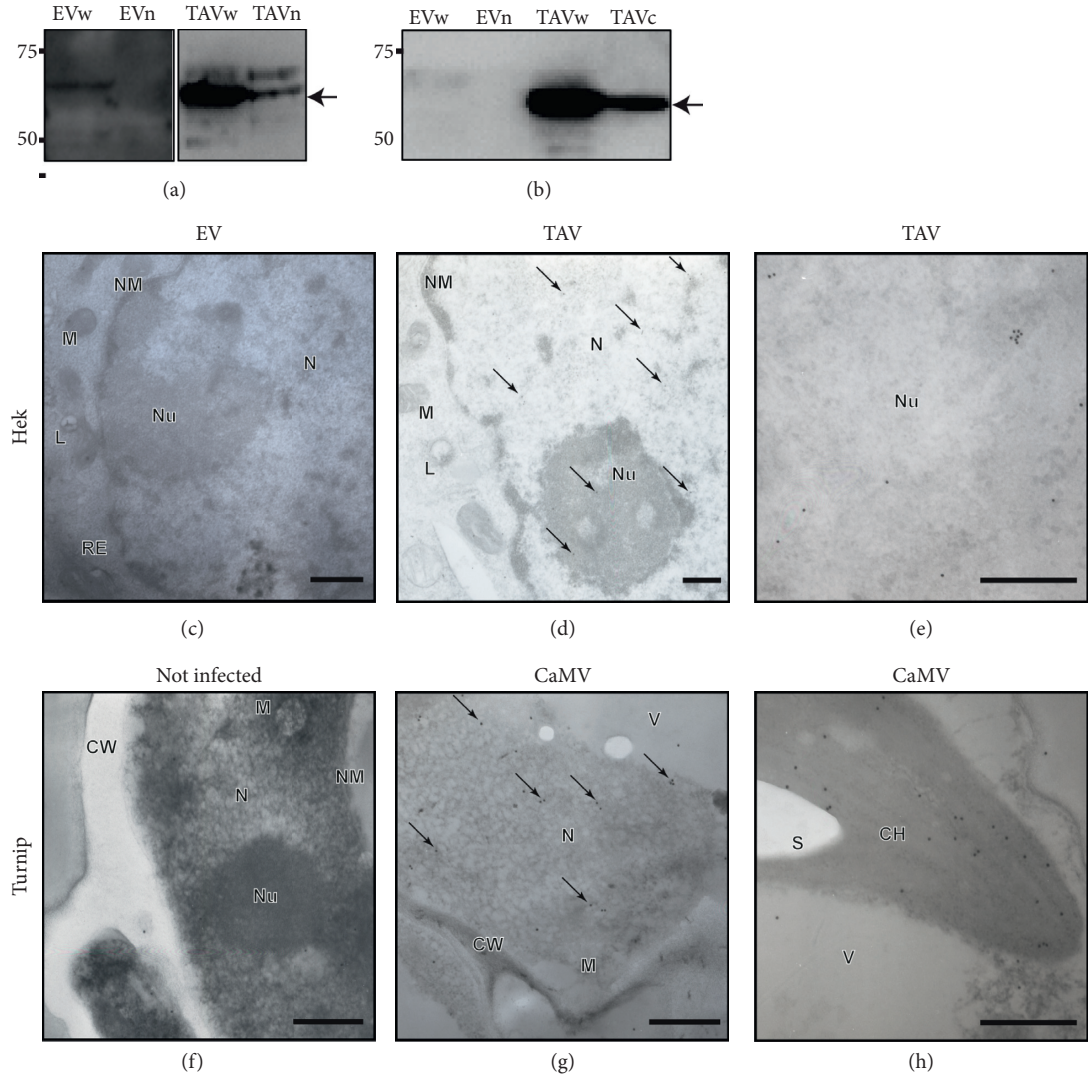
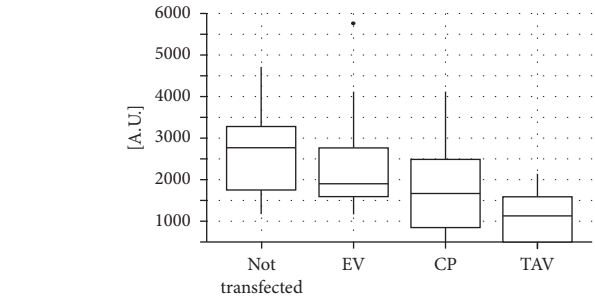


Figure 2: TAV detection with western blot (WB), transmission electron microscopy (TEM), and confocal microscopy. (a) Western blot shows total proteins (w) and nuclear proteins (n) of EV and TAV samples, immunoblotted with anti-TAV polyclonal serum. Arrow indicates TAV band (62 kDa), present in TAV sample only, in both W and N. Since the TAV antibody is a rabbit polyclonal antisera, it showed also a nonspecific band, also in EV sample just above the TAV band. Full-length blots/gels are presented in Supplementary Figure 1. (b) Western blot shows total proteins (w) and cytosolic proteins (c) of each sample, immunoblotted with anti-TAV polyclonal serum. Arrow indicates the TAV band, present in the TAV sample only. Full-length blots/gels are presented in Supplementary Figure 2. (c–h) Immunogold electron microscopy showing localization of CaMV TAV in Hek293T (c, d, e) and turnip cells (f, g, h). Ultrathin sections were immunostained with anti-TAV and gold-labelled secondary antibody. EV (c) and noninfected turnip cells (f) were used as a negative reference. Gold particles are indicated by arrows at low magnification (d, g) and clearly visible at higher magnification (e, h). The bars represent 200 nm. The letter symbols stand for the following: N: nucleus; Nu: nucleolus; NM: nuclear membrane; M: mitochondria; L: lysosome; RE: endoplasmic reticulum; CW: cell wall; CH: chloroplast; V: vacuole; S: starch.

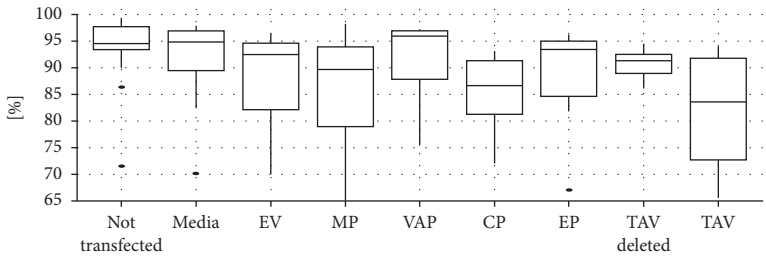
In order to confirm the nuclear expression of TAV, we analyzed EV and TAV- HEK293T cells using transmission electron microscopy (TEM). Only rare gold particles are seen randomly scattered over sections from healthy plants or EV- HEK293T cells indicating the absence of any endogenous substrates for the antibodies used (Figures 2(c) and 2(f)). Specific nuclear localization of gold particles was observed using the TAV antibody for TAV-HEK293T cells or CaMV-infected plant tissue labelling, respectively. TAV was localized intranuclearly both in TAV- HEK293T cells (Figures 2(d) and 2(e)) and in CaMV CM1841-infected turnip leaf cells which showed the typical symptoms of curling and leaf mosaic, used as a positive control (Figures 2(g) and 2(h)). Interestingly, nucleolar gold labelling on TAV-HEK293T samples (Figure 2(d)) and specific labelling of infected chloroplasts (Figure 2(h)) were observed using the antibody against TAV.

3.3. DNA/RNA Hybrids Reduction in TAV-HEK293T Cells. Since TAV protein shows a homology with the mammalian RNase H1, we tested for its effect in reducing DNA/RNA hybrids in TAV-HEK293T cells, using an anti-R-loop antibody, S9.6 Ab [38]. As shown in the boxplots (Figure 3(a)),



	Sample size	Median	IQR
Not transfected	20	2691.611	1483.333
EV	30	2691.611	1483.333
CP	30	1603.285	1610.928
TAV	31	1076.300	1056.532

(a)



	Sample size	Median	IQR
Not transfected	14	94.531	4.294
Media	14	94.909	7.568
EV	8	92.582	12.654
CP	6	89.731	15.014
VAP	6	96.006	9.117
CP	6	86.633	9.998
EP	6	93.477	10.564
TAV deleted	6	91.355	3.739
TAV	14	83.646	19.133

(b)

Figure 3: Quantification of TAV effects in human cells. (a) TAV-induced reduction of DNA/RNA hybrids in HEK293T cells. Boxplots and descriptive statistics of the fluorescence signal data in HEK293T cells. The interquartile ranges show that the cell-associated fluorescence signal values of EV and TAV are well concentrated around their median values, whereas nontransfected and CP values show higher dispersion. A high-fluorescence outlier has been identified for the EV control. (b) TAV-induced cytotoxic effects in U251 cells. Boxplots and descriptive statistics of the cytotoxicity data in U251 cells. Each box shows the first and third quartiles (bottom and top horizontal lines, respectively: box height is the interquartile range, IQR) and the median value (thick horizontal line) and whiskers' (vertical lines) ends are the lowest/highest datum within 1.5 × IQR from the box extremities; all values outside these intervals are considered as outliers (black dots). Tables collect sample size, median, and the interquartile range (IQR, i.e., the difference between the third and first quartiles).

the S9.6 Ab fluorescence signal registered in TAV-HEK293T cells had a median value of 1,076 arbitrary units (AU, i.e., grey levels at 16-bit resolution) which is lower when compared to 2,692, 1,938, and 1,603 AU for non-transfected cells, EV, and CP-HEK293T cells, respectively. Empirical evidence leads one to firmly reject the null hypothesis when comparing TAV-HEK293Tcells with EV-HEK293T cells (P value <0.001); indeed, both graphical and descriptive comparisons point out substantial differences. On the contrary, the difference between the central tendency of CP and EV-HEK293T cells was not statistically significant (P value = 0.061). Taken together, our results suggest that TAV acts like RNase H1 protein in human cells, while the median lower level of CP is probably to be ascribed to its close association with other nucleic acid binding proteins or to the nucleic acid binding of CP through its own predicted motifs (e.g., the zinc finger motif, $CXCX_7HX_{14}H$, described in the PROSITE entry PDOC50158 [39]). See Figure S4 in the Supplementary Material for comprehensive analysis.

3.4. TAV's Cytotoxicity to Human Glioma Cells (U251 Cells). We then sought to investigate whether TAV interferes with cell viability. To this aim, cytotoxicity assays using trypan blue were performed on U251 human glioma cells, which have been transiently transfected with the CaMV proteins analyzed. As shown in the boxplots (Figure 3(b)), the vitality data of TAV-U251 samples reveal the lowest median values. This is more noticeable for the negative controls (83.6% for TAV compared to around 94.5%, 94.9%, and 92.6% for nontransfected cells, media only, and EV, respectively) and VAP and EP (96.0% and 93.5% median vitality, respectively). TAV-deleted median vitality data is 91.4%, therefore comparable to negative controls. Movement protein (MP) and CP median vitality values (89.7% and 86.6%, respectively) are closer to the median TAV one. The interquartile ranges show that the vitality values of nontransfected and TAV-deleted are highly concentrated around their median values, whereas MP and TAV show higher dispersion. Empirical evidence leads one to firmly reject the null hypothesis when comparing TAV with nontransfected (P value <0.001) and media-control values (P value = 0.004); indeed, both graphical and descriptive comparisons point out substantial differences.

4. Discussion

Our results reveal a new and unexpected role for CaMV TAV in reducing DNA/RNA hybrids in HEK293T cells and being cytotoxic for U251 cells. DNA/RNA hybrids are formed continuously throughout the genome during RNA transcription and DNA duplication [40]. Together with the displaced single-strand DNA filament, DNA/RNA hybrids are collectively called R-loops [40]. R-loops are a threat to genome integrity due to their capability to promote DNA double-strand breaks, chromosome rearrangements, and replication fork stalling [40]. R-loops behave as hotspots of

genomic instability in a variety of organisms. Current models suggest that uncontrolled R-loops are a hazard to genome integrity, and the expression of RNA-DNA hybrid-binding proteins in various cancer types is associated with survival [41]. Accordingly, mechanisms that prevent R-loop accumulation (e.g., THO/TREX and BRCA2 [39, 40]) enhance genome stability in human cells [39], whereas the sequestration of hTREX by Kaposi's sarcoma-associated herpesvirus protein ORF 57 leads to R-loop formation and genome instability [40]. Nevertheless, DNA/RNA hybrids exert important physiologic roles in gene regulation, as they control gene expression activation and termination [40]. Moreover, depletion of DNA/RNA hybrids by RNase H1 overexpression has been shown to impair telomere homeostasis in cancer cells that maintain telomere length via the telomerase-independent "alternative lengthening of telomeres" (ALT) pathway [42]. Indeed, these tumor cells need to maintain precise levels of DNA/RNA hybrids to support telomeric homologous recombination (HR) without compromising telomere integrity. Together with our findings, these data suggest that particular sets of cancer cells, such as those carrying active ALT pathway, may be preferentially sensitive to DNA/RNA hybrid depletion and may be suitable targets for TAV/RNase H1 overexpression-based gene therapy.

5. Conclusions

These proof-of-concept data open the door to cross-kingdom use of plant virus proteins in human therapies. Since RNase H-mediated degradation of ASOs, which form an RNA/DNA hybrid once bound to the RNA, is now under clinical investigation for amyotrophic lateral sclerosis among other neurological diseases [43], TAV—a plant virus protein—may become a key player in future gene therapy scenarios in a variety of human diseases, including cancer and neurodegeneration.

Data Availability

All data used in the analysis is available in the main text or the supplementary materials.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Supplementary Table 1: characterization of CaMV proteins and their putative interactions with human proteins and nucleic acids. Together with Figure 1(a) and Supplementary Table 3, these data give a whole overview of possible human proteins interactors with CaMV proteins. Supplementary Table 2: RNA interacting sites in TAV inferred from the modelling procedure. These data complete the model presented in Figure 1(b). Supplementary Table 3: proteins interacting with CaMV TAV in plants and corresponding proteins in humans. Supplementary Figure 1: full-length blot/gel related to Figure 2(a). Supplementary Figure 2: full-length blot/gel related to Figure 2(b). Supplementary Figure 3: western blot detection of GAPDH and Histone H3 as cytoplasmic/nuclear separation controls. Supplementary Figure 4: residues putatively involved in binding DNA and RNA have been predicted with the machine-learning method DP-bind and are presented in this figure. (*Supplementary Materials*)

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Research Article

Berberine Inhibits MDA-MB-231 Cells by Attenuating Their Inflammatory Responses

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Breast cancer initiation is closely associated with cytokines that can change the inflammatory tumor microenvironment. Compounds extracted from plants have been explored for the possibility of cancer treatment in the recent decades. Berberine is an isoquinoline plant alkaloid with remarkable antioxidant and anti-inflammation roles, which is used in ethnic medicines, including traditional Chinese and North American medicine. In the present study, we investigated the effects of berberine on the malignant tumor cell behaviors in a breast cell line, MDA-MB-231. We found that berberine could not influence the cell viability in normal condition but was able to decrease the cancer cell migration capability in a scratch wound model and accordingly prolong the wound healing time. Furthermore, our results demonstrated that berberine inhibited the increased phosphorylation of c-Jun and c-Fos in these scratched cancer cells. With the cotreatment with LPS, which could boost the expression of cytokines in these cancer cells, berberine significantly reduced the increased expression of TNF-α and IL-6. Meanwhile, we found that berberine inhibited the activation of NF-κB by preventing the degradation of IκBα.

1. Introduction

Breast cancer is among the major causes of cancer mortality in the female population worldwide [1]. It accounts for one-third of newly diagnosed cancer in women of the United States [2]. Multiple factors are associated with the occurrence of breast cancer, such as body weight or obesity, diet, ethnicities, and gynecology and obstetrical history. Despite the remarkable progress in the treatment of breast cancer surgically and chemotherapeutically, breast cancer still remains one of the key vicious diseases for women in modern society globally. Also, although scientists have put intensive efforts in this field to reveal the molecular mechanisms of breast cancer, the tumorigenesis of breast cancer on the level of pathophysiological conditions is not well profiled yet. There are emerging evidences implying that activation of certain immune receptors in cancer cells could result in the products of proinflammatory cytokines and chemokines in the tumor microenvironment. The increased inflammatory context further initiates the tumorigenesis [3]. Inflammatory condition also facilitates metastasis. Therefore, regulating the inflammatory response in the tumor microenvironment may amend the current treatment regime of breast cancer.

A great deal of studies has revealed that natural compounds extracted from plants may exert anticancer property [4, 5]. Berberine is an isoquinoline alkaloid derived from herbal materials and has been prescribed for diabetes and infections as a complementary treatment in China [6]. Berberine was found to regulate the LPS-induced inflammatory response in macrophages [7]. In breast cancer, berberine could inhibit cancer cell proliferation and cell migration [8]. Moreover, a recent study reported that in a human triple-negative breast cancer cell line, berberine inhibits not only the tumor outgrowth and metastasis in TNBC but also the NLRP3 inflammasome, which leads to markedly reducing a series of cytokine products, including IL-1, IL-6, and TNF-α [9]. Therefore, berberine may be a potential therapeutic compound for the breast cancer treatment by regulating the inflammatory microenvironment of cancer cells.

In the present study, we used a human breast cancer cell line, MDA-MB-231, to further elaborate the possible ways that berberine affects the inflammation of cancer cells and

the cell behaviors. Our data will provide more information to validate the application of berberine in the treatment of breast cancer.

2. Materials and Methods

2.1. Cells and Treatment. The human breast cell line MDA-MB-231 is maintained in 35 mm dishes with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS). The cultures were incubated at 37°C in a 5% CO_2 atmosphere. A scratch wound was made by using the pipette tip in the dishes followed by changing the medium to remove debris. Berberine chloride was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) as stock solution. LPS (10 μg/L) was added to the cultures for the inflammation study, and berberine was employed 30 min before the addition of LPS. In a scratch model, berberine was added after changing the medium. Photos were taken 24 hours later after scratching, and the wound healing process was recorded and compared among groups.

2.2. Cell Viability. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to examine the cell viability. Generally, breast cancer cells were plated in 96-well plates, and we added MTT into each well at the final concentration of 10% and incubated them for 4 hours in an incubator. Optical densities were probed at 570 nm, and viabilities were expressed as a relative percentage compared to their controls.

2.3. Western Blot. Cultured cancer cells were washed with cold PBS for 3 times, and protein extraction buffer was added to the dishes. With a cell scraper, we collected the total protein and then boiled them for denaturation. The equal amount of total protein was loaded and separated in SDS-PAGE protein gel after determining the protein concentration. The protein was then transferred to a PVDF membrane (Millipore) and blocked with 5% bovine serum albumin (BSA). Primary antibodies of c-jun (Cell Signaling), c-fos (Cell Signaling), NF-κB p65 (Cell Signaling), IκBα (Cell Signaling), and β-actin (Santa Cruz) were incubated with the membranes at cold room temperature overnight, respectively. HRP-conjugated secondary antibodies were used for further 1 hr incubation at room temperature after washing the membranes with TBST. The corresponding bands were developed and visualized by chemiluminescence with ECL solution. The protein expression was presented as a ratio compared to internal control protein (β-actin).

2.4. Scratch Wound Assay. A scratch wound model was then created by using plastic pipette tips (10–20 μL pipette tips) as previously reported by [6]. We washed the dishes immediately after scratching to remove dead cells and other debris generated during scratching. The scratched dishes were then treated with or without berberine. During this time, the wound gaps between groups were repeatedly imaged to measure the distance between two edges of the wounds in each dish.

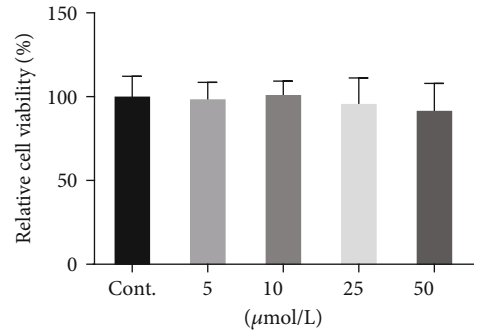

Figure 1: The effects of berberine on the cancer cell viability (24 hours). Data was expressed as mean ± SEM. $n = 7$.

2.5. ELISA. The ELISA assay was performed with kits of TNF-α and IL-6 (Abcam) according to the protocol provided by the manufacturer. MDA-MB-231 cells were washed with cold PBS, and then, lysis buffer was added to extract the total protein of cells. After protein concentration was determined, the same amount of protein was loaded in the well in a duplicate manner. The expressed levels of TNF-α and IL-6 in the cell lysis were normalized with total loading protein.

2.6. Statistical Analysis. All values in the present study were expressed as mean ± SEM. Statistical analysis was performed with Prism 5 software. Multiple-group analysis was done with one-way ANOVA followed by the Newman–Keuls post hoc test. Two-group analysis was done with the t-test. A p value of less than 0.05 was considered statistically significantly different.

3. Results

3.1. Berberine Had No Effects on the Cancer Cell Viability. To test the possible anticancer effects of berberine in these breast cancer cells, we first incubated MDA-MB-231 cells with berberine in a range of concentration (Figure 1) for 24 hr. The doses of the berberine used here were based on the previous report from Sarna et al. [7]. As shown in this figure, berberine failed to show any sign of reduction of the viability of these breast cancer cells even in the highest concentration. These results indicated that berberine might not affect the cell viability in certain condition with these above doses. 25 μmol/L was used for the following experiments based the above results and previous report [7].

3.2. Berberine Inhibited the Wound Healing Process of the Scratched Breast Cancer Cells. Next, we investigated whether berberine could affect the capacity of migration of these cancer cells. We scratched the confluent monolayer cancer cells and monitored the cell migration along the wound edge by photographing the wound in a manner of time course. We found that the cells along the edge started the migration almost immediately after scratching with or without the treatment with berberine. But berberine treatment reduced the migration capacity since the wound gap was much bigger in berberine-treated cells than in the cells without treatment (Figure 2). These results implied that berberine could inhibit

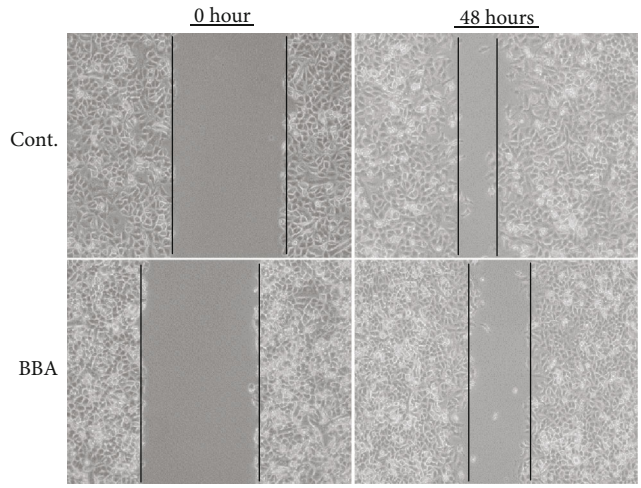

Figure 2: The effects of berberine on the cancer cell migration in a scratch wound model (48 hours). BBA: berberine (25 μmol/L).

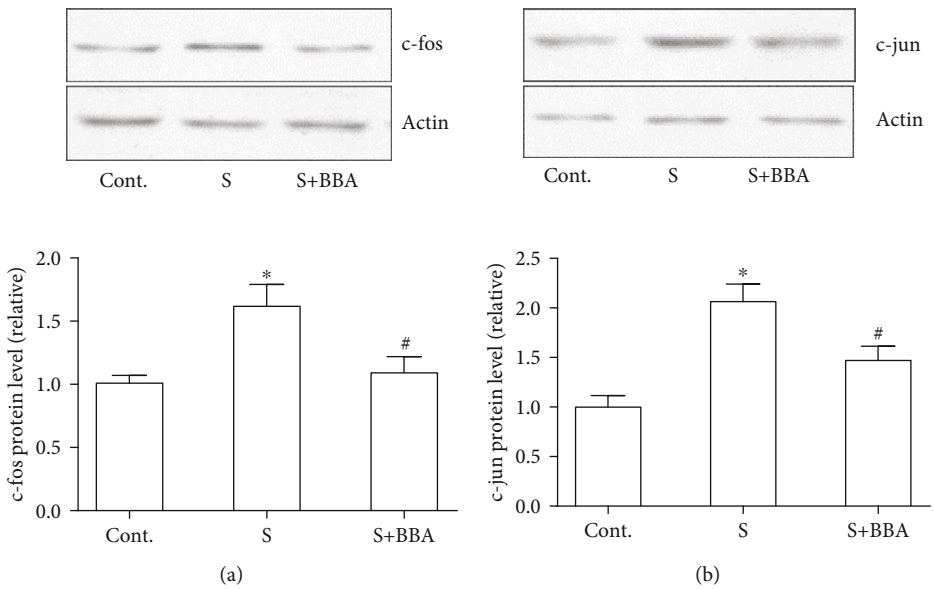

Figure 3: Berberine inhibited the increased expression of c-fos and c-jun in scratched cancer cells. (a) The effect of berberine on the expression of c-fos at 30 min after scratching. (b) The effect of berberine on the expression of c-jun at 30 minutes after scratching. Data was expressed as mean ± SEM. $^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to scratch ($n = 5$). BBA: berberine (25 μmol/L); S: scratch.

the migration of breast cancer cells, which is a feature property of malignancy of metastasis.

3.3. Berberine Inhibited the Activation of c-fos and c-jun in the Cancer Cells after Scratching. We also tried to identify the involved molecular candidates which might trigger the migration after scratching for the purpose of exploring the possible target proteins of berberine. A study has suggested that early response genes were activated in scratched cells [10]. Therefore, we performed western blot to study the expression level of c-fos and c-jun in these scratched breast cancer cells in the early stage of post-scratching (30 minutes). We found that the protein levels of both c-fos and c-jun were boosted after scratching but their expression level inhibited with the cotreatment with berberine (Figures 3(a) and 3(b)).

3.4. Berberine Prevented the LPS-Induced Cytokine Expression. Breast cancer cells could boost the capacity of generating cytokines [11]. To explore the possible effects of berberine on the inflammation in breast cancer cells, LPS was used to treat these cancer cells in the next experiments. With the studies of ELISA, we found that the products of TNF-α and IL-6 in these cells were significantly increased compared to control groups (Figures 4(a) and 4(b)). Interestingly, cotreatment with berberine was able to effectively decrease the expression levels of these two cytokines (Figures 4(a) and 4(b)). These results demonstrated that

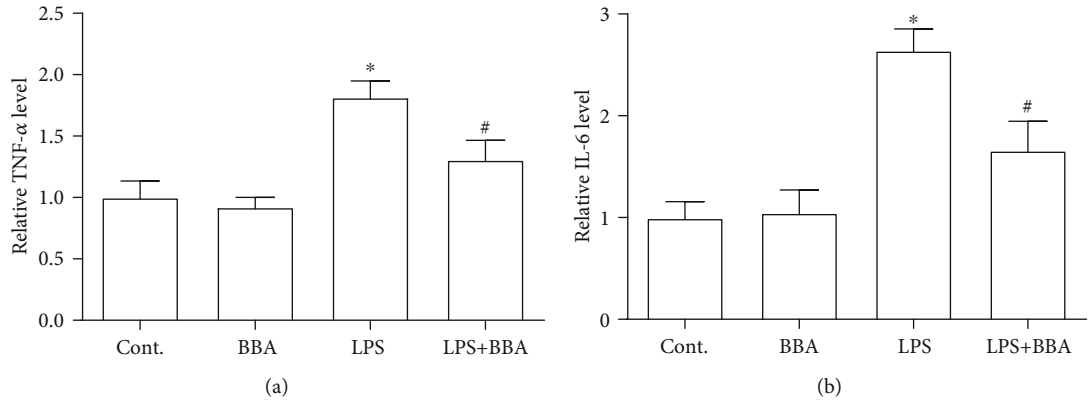

Figure 4: Berberine inhibited the increased expression of TNF-α and IL-6 in cancer cells exposed to LPS. (a) The effect of berberine on the expression of TNF-α at 6 hours after LPS treatment. (b) The effect of berberine on the expression of IL-6 at 6 hours after LPS treatment. Data was expressed as mean ± SEM. $^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to LPS ($n = 7$). BBA: berberine (25 μmol/L).

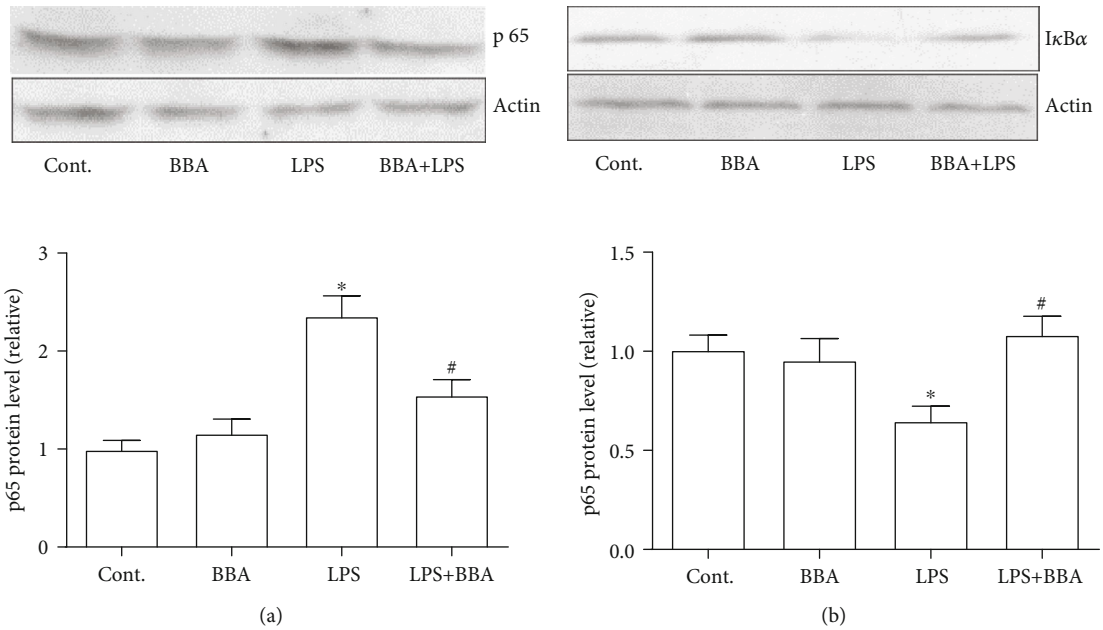

Figure 5: Berberine inhibited the increased expression of NF-κB p65 and prevented the protein loss of IκBα in cancer cells exposed to LPS. (a) The effect of berberine on the expression of NF-κB p65 at 6 hours after LPS treatment. (b) The effect of berberine on the expression of IκBα at 6 hours after LPS treatment. Data was expressed as mean ± SEM. $^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to LPS ($n = 6$). BBA: berberine (25 μmol/L).

berberine might exert anti-inflammation effects on the breast cancer cells.

3.5. Berberine Prevented the Increased Expression of NF-κB p65 and the Degradation of IκBα. Last, we investigated the possible mechanism by which berberine exerted those beneficial effects on breast cancer cells. We performed western blot to measure the protein level of NF-κB p65. We found an increased expression level of p65 in cancer cells exposed to LPS, but this increase could be prevented by berberine (Figure 5(a)). Accordingly, there was reducing expression of IκBα that is the inhibiting protein of NF-κB in the LPS-treated cancer cells. However, the cotreatment with berberine improved the expression level of IκBα (Figure 5(b)). The above data implicated that berberine might reduce the inflammatory response in breast cancer cells by increasing the expression of IκBα, which then led to inhibiting the p65 protein.

4. Discussion

Diagnosis, care, and prognosis of breast cancer have improved significantly during the past decades with the further understanding of their pathogenies and etiology. However, breast cancer is still a major threat to female in middle age, especially in economically developed countries, and represents one of the top biomedical research priorities globally [12]. Although it remains still unknown, extensive efforts

have been devoted to find the initiation process of breast cancer. Inflammatory response in the microenvironment was considered an active player in the initiation, promotion, angiogenesis, and metastasis [13]. Consequently, the boosted expression of cytokine, activated cytokine receptor, and intracellular signaling of NF-κB facilitated the malignant cell behaviors [14]. Therefore, a compound which could target the inflammatory reaction in the tumor microenvironment as a supplementary treatment to the mainstream chemotherapy could influence the outcome and further optimize the treatment regime of breast cancer.

Compounds derived from natural plants have been extensively studied of their application in many fields, including cancers. As mentioned above, berberine was found to possess prominent features in anti-inflammation and antioxidant function. Here, we used this compound to test several hypotheses in the breast cancer cell line. Our data here were in line with previous reports [15]. Furthermore, we found that berberine could inhibit the breast cell migration in a scratch wound injury. Since increased migration capacity is a feature of malignant cell behavior in cancer cells, the role of berberine in the cell migration is a foundation finding in this study. And based on the finding, we explored the possible mechanism in several ways, such as inflammation-related signaling pathway and early response genes. The c-fos and c-jun signaling pathway has been studied for many years of their role in the cell migration [16, 17]. In the present study, we found that scratching caused increased protein expression levels of these two proteins, which could be inhibited by the berberine treatment. These results implied that inhibiting the c-fos and c-jun protein response in the early stage might be one of the possible mechanisms. It has been well established that the NF-κB signaling pathway is the center of inflammation reaction [17, 18]. In the study, we found that berberine inhibits the expression of p65 which was triggered by the LPS stimulation in breast cancer cells. More importantly, we also revealed that the inhibiting component of NF-κB and IκBα was regulated by the berberine treatment. As shown in Figure 5, the expression level of IκBα was significantly decreased in LPS-treated cancer cells, which indicated a desuppression of NF-κB. With the cotreatment with berberine, the degradation of IκBα was prevented and resulted in reduced expression level of p65, accordingly. A previous study also indicated that berberine could inhibit the phosphorylation of NF-κB and c-fos and c-jun proteins and lung cell invasion [19]. In the present study, we found that berberine inhibited the increased expression levels of c-fos and c-jun and the cell migration in the scratch wound model. Combined with the results of the NF-κB signaling pathway mentioned above, we concluded that berberine might affect the NF-κB signaling pathway and exert its beneficial effects on cancer cell migration and inflammatory response.

In a nutshell, our study provided more evidences to support the application of berberine in the treatment of breast cancer cells at cellular and molecular levels. To further confirm the role of berberine, an animal study is warranted for the future.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest to disclose in this study.

Acknowledgments

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Research Article

Pomegranate Seeds Extract Possesses a Protective Effect against Tramadol-Induced Testicular Toxicity in Experimental Rats

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Tramadol is a centrally acting opioid analgesic that is extensively used. The chronic exposure to tramadol induces oxidative stress and toxicity especially for patients consuming it several times a day. Previously, we and others reported that tramadol induces testicular damage in rats. This study was conducted to investigate the possible protective effect of pomegranate seed extract (PgSE) against tramadol-induced testicular damage in adult and adolescent rats. Male rats were orally treated with tramadol or in a combination with PgSE for three weeks. Testes were then dissected and analyzed. Histological and ultrastructural examinations indicated that tramadol induced many structural changes in the testes of adult and adolescent rats including hemorrhage of blood vessels, intercellular spaces, interstitial vacuoles, exfoliation of germ cells in lumen, cell apoptosis, chromatin degeneration of elongated spermatids, and malformation of sperm axonemes. Interestingly, these abnormalities were not observed in tramadol/PgSE cotreated rats. The morphometric analysis revealed that tramadol disrupted collagen metabolism by elevating testicular levels of collagen fibers but that was protected in tramadol/PgSE cotreatment at both ages. In addition, DNA ploidy revealed that S phase of the cell cycle was diminished when adult and adolescent rats were treated with tramadol. However, the S phase had a normal cell population in the cotreated adult rats, but adolescent rats had a lower population than controls. Furthermore, the phytochemistry of PgSE revealed a high content of total polyphenols and total flavonoids within this extract; besides, the DPPH free radical scavenging activity was high. In conclusion, this study indicated that PgSE has a prophylactic effect against tramadol-induced testicular damage in both adult and adolescent ages, although the tramadol toxicity was higher in adolescent age to be completely protected. This prophylactic effect might be due to the high antioxidant compounds within the pomegranate seeds.

1. Introduction

There has been expanding global concerns on the decline of male fertility rates and testicular cancer in the past 3–5 decades [1, 2]. Along with the conventional causes of male infertility [3], exposures to certain analgesic drugs such as opioid narcotics induce oxidative stress and adversely affect male fertility [4–8]. Opioid analgesic drugs are known to be the most effective medication for moderate to serious pain for adult and young patients [9].

Tramadol hydrochloride (tramadol HCl) is one of the centrally acting opioid drugs that is extensively used

parenterally and orally as a pain reliever [10]. It is rapidly absorbed and distributed throughout the body with a higher analgesic effect than the parent drugs [11]. Tramadol contributes to analgesic activity by working as agonists of the μ-opioid receptor and also by inhibiting serotonin and norepinephrine reuptake and therefore enhancing inhibitory effects on pain transmission in the spinal cord [12]. Tramadol is used to reduce pain resulted from posttraumatic, obstetric, osteoarthritis, fibromyalgia, renal, biliary colic, and neuropathic pain [12]. However, multiple tissue toxicities were reported when tramadol was administrated [13–17], especially for patients consuming tramadol medication several times daily to relieve chronic pain. We and others have shown that tramadol induces testicular toxicity in adults [5, 9, 18–20].

Tramadol like other opioids induces oxidative stress by decreasing the antioxidant levels in the body [21–23]. Oxidative stress ensues when the Reactive Oxygen Species (ROS) level and antioxidant defense system are imbalanced, which results in structural alterations of the cell and apoptosis [24, 25]. Recently, natural products have been used for the treatment of oxidative stress [26]. Several plants were suggested, such as pomegranate [27]. The pomegranate (*Punica granatum* L.) belongs to the family Punicaceae. This fruit has long been cultivated and widely consumed as fresh fruit or in beverage form. The edible parts of pomegranate fruit comprise 78% juice and 22% seed [28]. Pomegranate is rich in sugars, vitamins, polysaccharides, polyphenols, and minerals [29–31]. Pomegranate was used as a medicinal plant for long [27, 32, 33]. Its extract was found to increases the antioxidant capacity *in vivo* and *in vitro* [34, 35]. This activity may be related to the diverse phenolic compounds present in pomegranate, including punicalagin isomers, ellagic acid, anthocyanins (3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin), and different flavanols [35–38]. Moreover, pomegranate juice consumption increases significantly sperm quality, spermatogenic cell density, and testosterone level in male rats [39].

Although the incidence that both adult and young patients are treated with tramadol, the risk factors of age-dependent treatment are poorly studied. Thus, the present study aimed to determine the risk level of tramadol treatment on the testicular structure at adult and adolescent ages and to evaluate the possible protective effects of pomegranate seed extract upon coadministration with tramadol at both ages.

2. Materials and Methods

2.1. Animals. This study was carried out on adult (six weeks old; weight 96 ± 19 g) and adolescent (three weeks old; weight 82 ± 20 g) male Wistar rats (*Rattus norvegicus*). Rats were bought from animal colony NRC-Egypt and were housed in a quite nonstressful environment for an acclimation period of one week prior to the study to be stabilized in the new environment. They were allowed for free access to food and water during the experimental period. The maintained lab diet was as standard (protein: 160.4, fat: 36.3, fiber: 41 g/kg, and metabolizable energy: 12.08 MJ). All animal experiments were performed in a humane manner upon approval by the Animal Experiment Committee of National Research Center, Egypt, and Nagoya City University, Japan. Moreover, they were euthanized with carbon dioxide gas to minimize animal suffering.

2.2. Chemicals. Tramadol hydrochloride (tramadol HCl), 225 mg tablets, was obtained from Mina-Pharm, Egypt. The LD_{50} values of tramadol administration orally were estimated to be 300–350 mg/kg body weight for rats [40]. The treated doses used of this drug were calculated as previously reported [41], which were nearly comparable to the human effective therapeutic doses. Tramadol was dissolved in distilled water and given by stomach tube during the experimental period.

2.3. Preparation of Pomegranate Extract (PgSE). Pomegranate seeds were collected, dried, crushed to fine powder, and mixed with 70% ethanol for 24 hours with repeated stirring. Ethanolic extract was obtained by repeating the extraction procedure for 3 successive times. The resulting ethanol extracts were subsequently filtered and concentrated with a vacuum rotary evaporator (Heidolph®.VV2000) under reduced pressure at a temperature of 55°C and then the residues were lyophilized using a vacuum freeze drier (Tilburg, Holland; 145Fm-RB). The lyophilized powder was diluted in distilled water on the day of treatment. The treated doses used for PgSE were as previously reported [42]. Each rat received 40 mg/kg body weight in a total volume of 1 ml by stomach tube during the experimental period.

2.4. Experimental Design. The experimental model of the current study was as shown in Figure S1. Thirty-six male rats were divided according to their age into two main groups: 18 adult rats and 18 adolescent rats. Each group was then subdivided into three groups (six animals per group). The control group received orally 1 ml of 0.9% saline. The treated group received orally tramadol dose of 20, 40, and 80 mg/kg during the 1st, 2nd, and 3rd week, respectively. The cotreated group received orally 40 mg/kg PgSE in addition to the tramadol dose.

2.5. Tissue Collection and Histological Analysis. Animals were sacrificed and testis samples were incised and fixed immediately in 10% phosphate-buffered formalin (pH 7.4) for 48 h. Tissues were then processed routinely for paraffin embedding. Sections with 5 μm thickness were cut and mounted on glass slides. After deparaffinization, slides were stained with hematoxylin and eosin (H&E), examined under bright field light microscopy, and photographed.

2.6. TUNEL Assay. Testicular apoptotic cells were examined by Terminal deoxynucleotidyl transferase dUTP Nick end labeling (TUNEL) assay using Invitrogen Click-iT Plus TUNEL imaging kit from Invitrogen (C10618) following the

manufacturer's protocol. Briefly, testis sections of 5 μm thickness were deparaffinized, fixed in 4% PFA for 15 min, and permeabilized with Proteinase K for another 15 min. The TdT reaction cocktail was added and incubated for 1 h, followed by 30 min incubation with the Click-iT reaction cocktail. The sections were then counterstained using DAPI and observed with an Olympus FV3000 confocal laser scanning microscope. For quantitative analysis, at least 6 sections of each testis from three rats within each group were examined.

2.7. Transmission Electron Microscopy (TEM). Testis samples were cut into small blocks, fixed in 2.5% glutaraldehyde for 3 h at 4°C, and postfixed in 1% osmium tetroxide for one hour at room temperature. The tissues were dehydrated through a graded ethanol series and embedded in Epon 812. The blocks were then cut into ultrathin sections with LKB ultramicrotome and stained with uranyl acetate followed by lead citrate. Sections were examined using a transmission electron microscope (Model JEM-1400 Plus). Flagella with axonemal disorganization were counted in 5 random fields of each group and presented as a percentage.

2.8. Morphometric Analysis of Collagen Fibers. Testis sections (5 μm) were stained with Masson's trichrome for the quantification of collagen fibers according to Drury and Wallington [43]. The area percentage of collagen fibers was measured in four sections of each testis from three rats within each group. The percentage of positive reaction was measured in ten random fields of each section (FOV = 25 mm^2) at high power (400x) using a computer-assisted image analyzer (Qwin Leica image processing analysis system, Cambridge, England). Measurements were made based on the intensity of blue color which represents the collagen density.

2.9. DNA Ploidy Analysis. Testis sections (5 μm) were stained with Feulgen staining for DNA ploidy analysis. The nuclear integrated optical density (OD) which is the cytometric equivalent of DNA content was measured in four sections of each testis from three rats within each group. The intensity of the stain was measured in ten random fields of each section (FOV = 25 mm^2) at high power (400x), using a computer-assisted image analyzer (Qwin Leica image processing analysis system, Cambridge-England). The DNA histograms were classified as previously described based on the amount of DNA relative to normal control [44].

2.10. Phytochemical Analysis of Pomegranate Extract. Pomegranate seeds extract was examined for its total phenolic content, total flavonoids content, and free radical DPPH scavenging activity. The total phenolic content was determined using the Folin-Ciocalteu colorimetric method [45]. Briefly, the extracts were oxidized with Folin-Ciocalteau reagent and then neutralized with aqueous Na_2CO_3 solution. After 40 min in dark, the absorbance was measured at 725 nm. The total phenolic content was determined using a calibration curve prepared and expressed as micrograms of gallic acid equivalents per gram of sample.

The total flavonoid content was quantified as previous [46]. Briefly, the extracts were mixed with sodium nitrite followed by adding aluminum chloride and sodium hydroxide. The optical absorbance was then measured at 510 nm. Total flavonoid was expressed as micrograms of gallic acid equivalents (GAEs) per gram of sample.

The antioxidant activity was determined by DPPH free radical scavenging assay as previous [47]. Briefly, the extracts were mixed with DPPH solution and incubated in the dark at room temperature for 1 h. The absorbance at 517 nm was measured against a blank of pure methanol. The antioxidant activity was determined by means of a calibration curve prepared with Trolox and expressed as micrograms of Trolox equivalent (TE) per unit weight of the sample. Percent of the DPPH free radical effect was calculated by the following equation:

$$\text{DPPH scavenging effect\%} = 100 \times \frac{\left(A_{\text{control}} - A_{\text{sample}}\right)}{A_{\text{control}}}. \quad (1)$$

2.11. Statistical Analysis. The data obtained were compiled and statistically analyzed and expressed as mean ± standard deviation. The data normal distributions were examined by the Shapiro-Wilk test. Differences between groups were compared by ANOVA using the SPSS software (version 16). Tukey post hoc multiple comparison tests of significant differences among groups were determined. The probability value $p < 0.05$ was considered statistically significant.

3. Results

3.1. Histopathological Examination of Testes. The experimental model of the study was shown in Figure S1. Adult and adolescent male rats were treated orally with normal saline (saline-treated controls), tramadol (Tr-treated), or tramadol with pomegranate seed extract (Tr/PgSE cotreated) for 3 weeks period. The tramadol concentration was gradually increased from 20 to 80 mg/kg to simulate the physical dependence of the drug similar to the chronic use in humans to reach the effective doses. Animals were then sacrificed and testes were collected, sectioned, and stained with HE for histological analysis. Results indicated that several testicular abnormalities were induced when tramadol alone was administrated to mice in both adult and adolescent ages (Figure 1). Testicular sections from Tr-treated groups showed hemorrhage of blood vessels, intercellular spaces within seminiferous tubules, interstitial vacuoles, and exfoliation of germ cells in the seminiferous lumen. However, these testicular abnormalities were observed in neither saline-treated controls, PgSE-treated controls, nor Tr/PgSE cotreated groups at both adult and adolescent ages (Figure 1 and Figure S2).

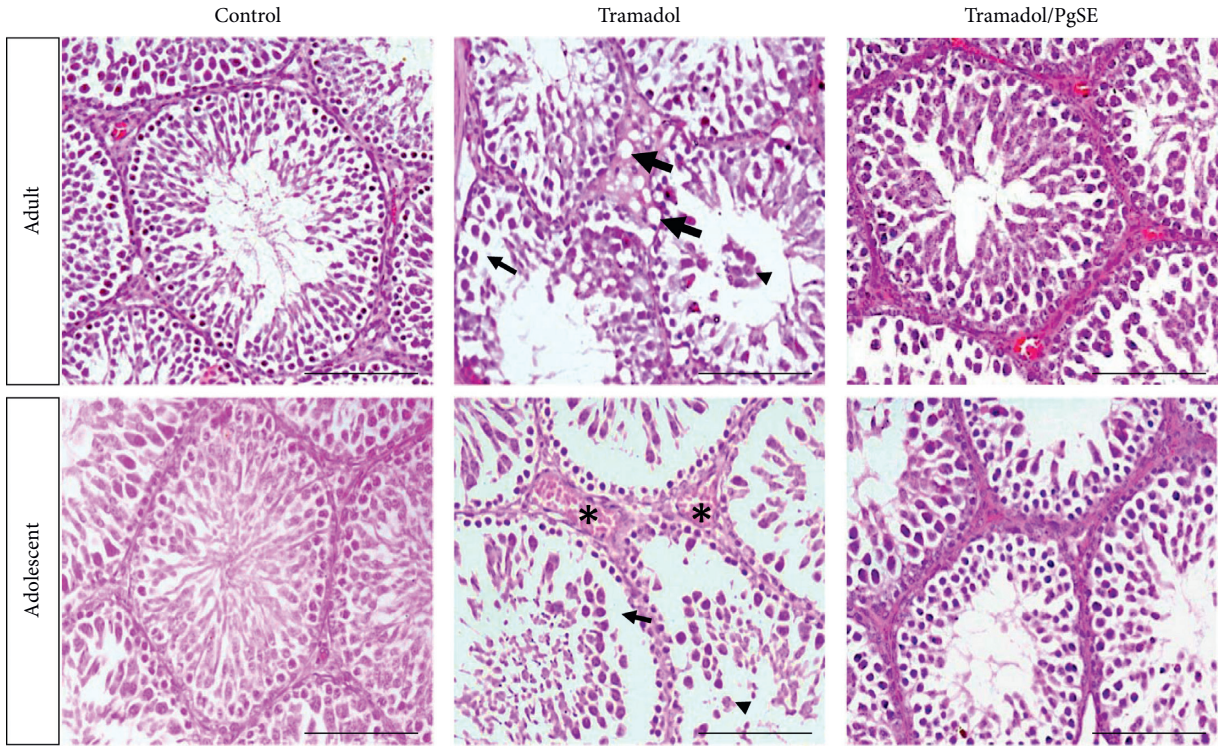

Figure 1: Histopathology of Tr-treated testicular tissues and cotreated with PgSE. Sections of adult and adolescent rat testes were examined via HE staining. Tr-treated group induced intercellular spaces within seminiferous (thin arrow), interstitial vacuoles (thick arrow), exfoliation of germ cells in lumen (arrowhead), and hemorrhage of blood vessels (star). Scale bars: 100 μM.

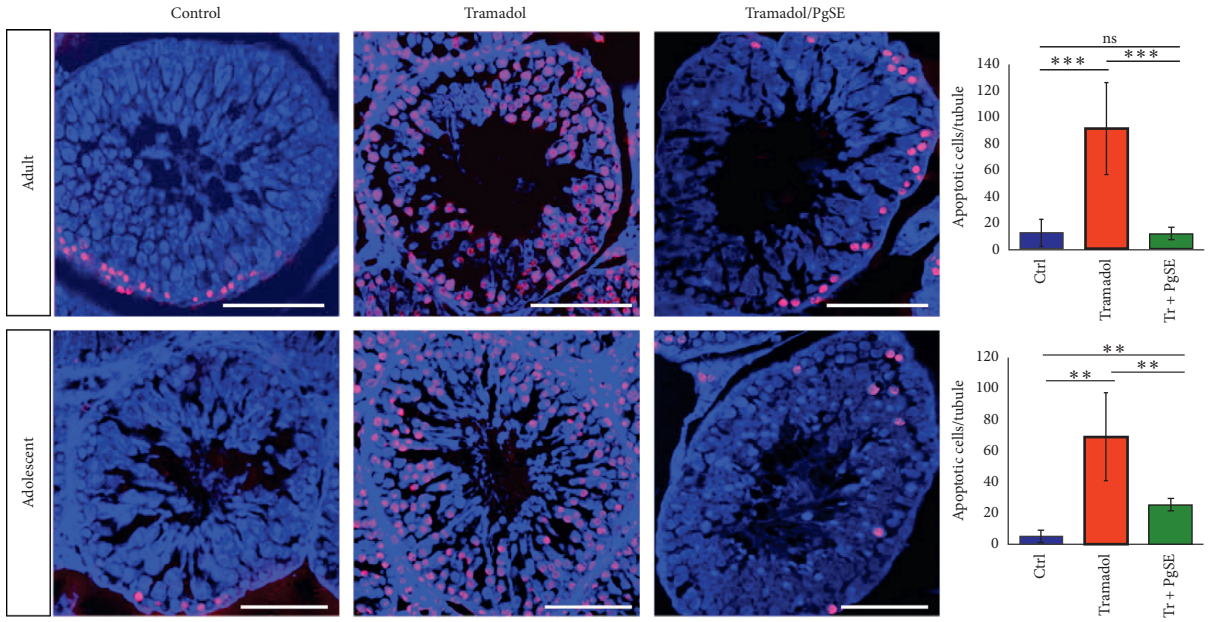

Figure 2: Testicular apoptotic cells of Tr-treated testicular tissues and cotreated with PgSE. Adult and adolescent rat testes were examined via TUNEL assay. TUNEL-positive cells are stained in red. The nuclei were counterstained with DAPI. The quantitative numbers of apoptotic cells were shown on the right. The data represent the mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ mean statistical difference. Scale bars: 100 μM.

3.2. Apoptosis of Testicular Cells. To investigate whether the testicular abnormalities observed by HE were accompanied by apoptosis of the testicular cells, we carried out a Terminal deoxynucleotidyl transferase dUTP Nick end labeling (TUNEL) assay. TUNEL-positive cells were identified and enumerated from histological sections per seminiferous tubule (Figure 2). The TUNEL-positive cells were observed in saline-treated controls, and the numbers of these cells

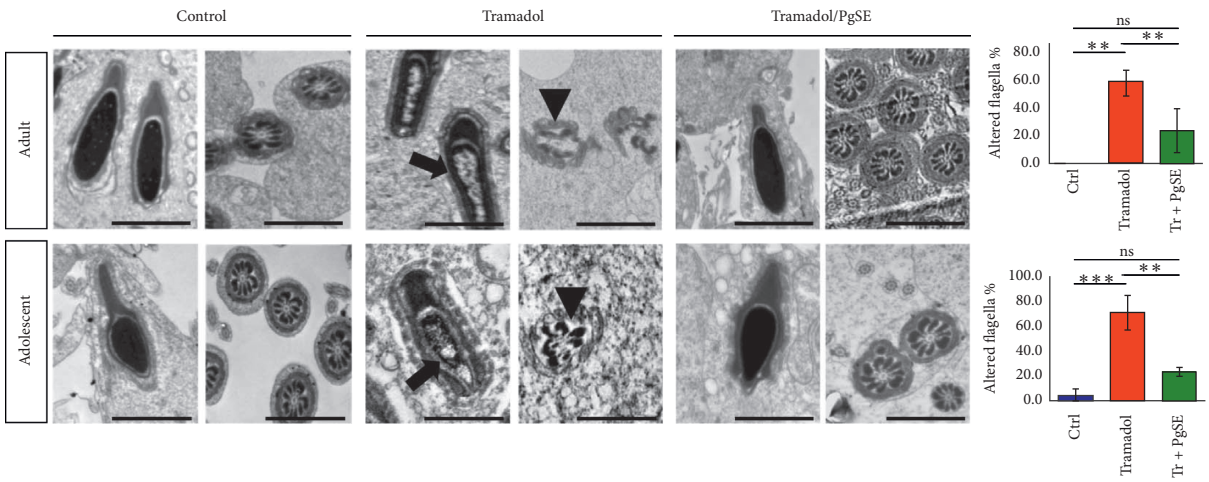

FIGURE 3: Ultrastructural analyses of Tr-treated testicular tissues and cotreated with PgSE. Adult and adolescent rat testes were examined via transmission electron microscopy. The Tr-treated group showed degeneration of elongated spermatid's chromatin (arrow) and malformation of sperm axonemal structure (arrowhead) in both adult and adolescent rats. In the cotreated groups, the abnormal ultrastructure appearances were not detected. The quantitative numbers of altered flagella were shown on the right. The data represent the mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ mean statistical difference. Scale bars: 1 μM.

were significantly increased ($P < 0.001$ and $P < 0.01$) in Tr-treated groups of both adult and adolescent rats, respectively. Significant fewer apoptotic cells were detected in the Tr/PgSE cotreated adults ($P < 0.001$) and Tr/PgSE cotreated adolescents ($P < 0.01$) when compared to Tr-treated groups. The Tr/PgSE cotreated adults showed a nonsignificant difference with saline-treated controls. However, the number of apoptotic cells in Tr/PgSE cotreated adolescents was still statistically different from saline-treated controls ($P < 0.01$), although it was visibly lower than the Tr-treated group. The overall incidence of these results indicated that PgSE could protect against the cell death reprogramming by tramadol administration.

3.3. Ultrastructure Analysis of Testicular Cells. To investigate in-depth the effect of the tramadol and the Tr/PgSE cotreatment on haploid germ cells, the ultrastructure of the testicular tissues was characterized. Testis samples were cut into small blocks, fixed, embedded, ultrathin sectioned, and finally examined using a transmission electron microscope (Figure 3). Results indicated that Tr-treated groups of both adult and adolescent rats had a degeneration in the elongated spermatid's chromatin which appears unraveled in an empty perinuclear area due to shrinkage of the nuclear content comparing to saline-treated controls. In the Tr/PgSE cotreated groups, the elongated spermatids showed a pyriform nucleus with condensed chromatin and completely formed acrosome. The second type of defect we observed is a malformation of sperm axonemal structure in both adult and adolescent rats comparing to saline-treated controls. Cross section of spermatozoa from Tr-treated groups shows complete disruption and disorientation of axonemal structure microfilaments, mitochondrial membrane degeneration, and rapture of plasma membrane sheath. No abnormal ultrastructure phenomena were detected in Tr/PgSE cotreated groups at both ages. This suggests that the chromatin alteration in elongated spermatids and sperm axonemal malformation was related to tramadol toxicity and that could be protected by PgSE cotreatment.

3.4. Morphometric of Collagen Fibers in Testes. The morphometrical analyses of the collagen fibers were examined by staining the testis sections with Masson's trichrome staining. A high degree of collagen fibers deposition surrounding the seminiferous tubules was observed in the testes of Tr-treated groups (Figure 4(a)). Quantification of collagen fibers from adult rat testes revealed that the measured area percentage of saline-treated control, Tr-treated, and Tr/PgSE cotreated groups were 22.97 ± 2.45, 42.82 ± 4.25, and 20.88 ± 2.45, respectively (Figure 4(b)). Quantification of collagen fibers from adolescent rat testes revealed that the measured area percentage of saline-treated control, Tr-treated, and Tr/PgSE cotreated groups were 18.18 ± 1.14, 46.68 ± 3.76, and 19.59 ± 2.08, respectively (Figure 4(b)). Tr-treated groups were significantly higher in collagen fibers ($P < 0.01$) compared to saline-treated control or Tr/PgSE cotreated groups at both adult and adolescent ages. But no statistical difference in collagen fibers area percentage was observed between saline-treated control versus Tr/PgSE cotreated groups in both adult and adolescent rat testes. That unequal collagen distribution indicated a tramadol-based disrupted metabolism.

3.5. DNA Ploidy of Testicular Cells. The DNA ploidy analysis was examined by staining the testis sections with Feulgen staining (Figure 5). The DNA content per nucleus is calculated for all the nuclei from the measured sample to yield a histogram of the cell cycle distribution. The DNA histograms are classified into four cell populations: haploid (<1.5c), diploid (1.5–2.5c), triploid (2.5–3.5c), and tetraploid (3.5–4.5c) based on the amount of DNA. The results indicated that the triploid which represents the population at S

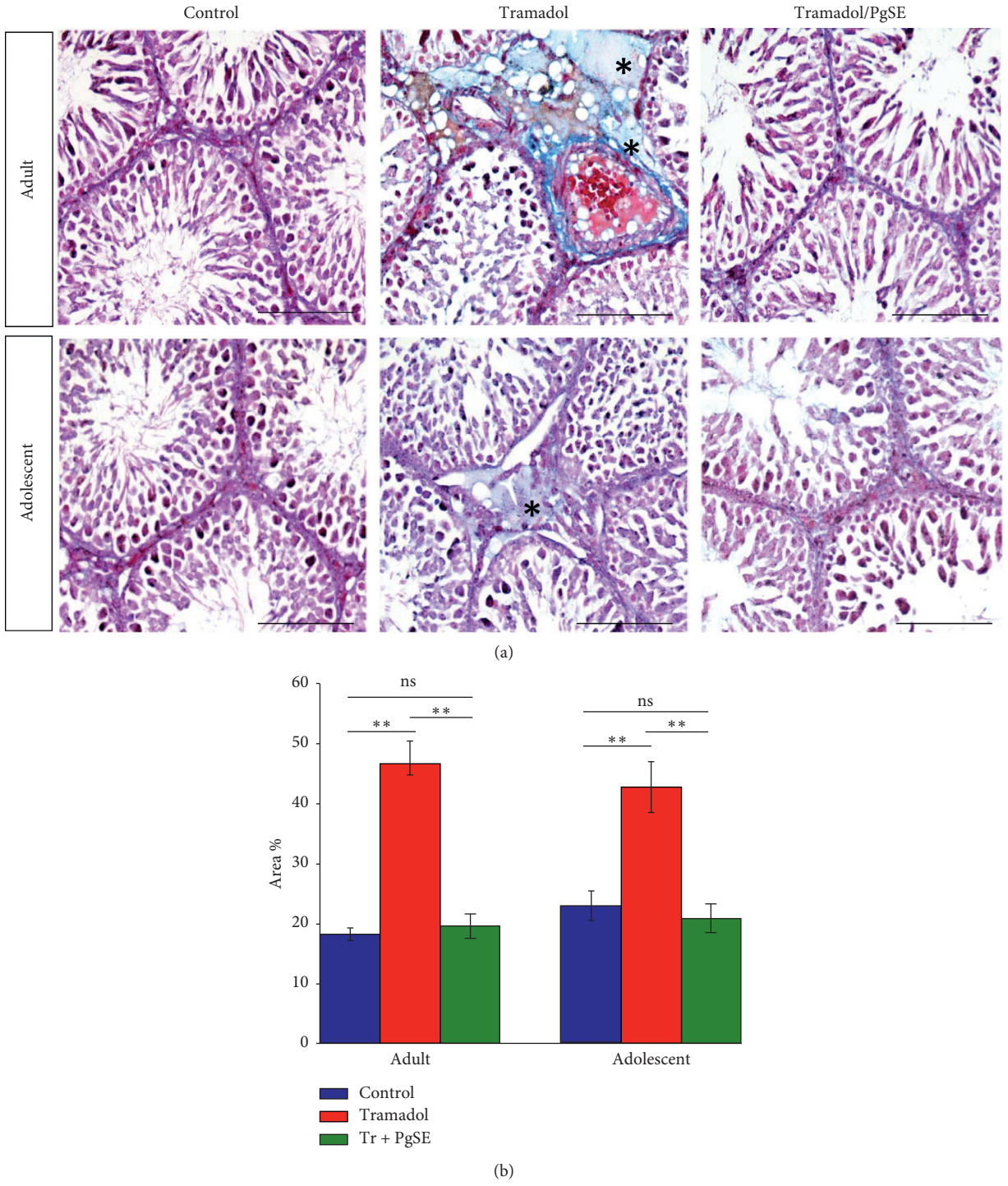

FIGURE 4: Morphometrical analyses of Tr-treated testicular tissues and cotreated with PgSE. (a) Adult and adolescent rat testes were examined via Masson's trichrome staining. The blue color represents collagen density (star). The Tr-treated group showed a significant increase in collagen fibers content in both adult and adolescent rats but not in the cotreated groups. Scale bars: 100 μM. (b) The area percentage of collagen fibers measured in rat testes from each group was shown as bars. Data represent the mean ± SD, $^{**}P < 0.01$. ns: nonsignificant.

phase was diminished in Tr-treated groups as compared with saline-treated control in both adult and adolescent ages. However, the Tr/PgSE cotreated group in adults showed similar S phase distribution to saline-treated control, but in the adolescent, the S phase population was lower than saline-treated control (Figure 5 and Table S1). The overall results indicated that tramadol induces disruption of cell cycle progression and PgSE could protect against that effect in adult rats, but adolescent rats were more sensitive to be completely protected.

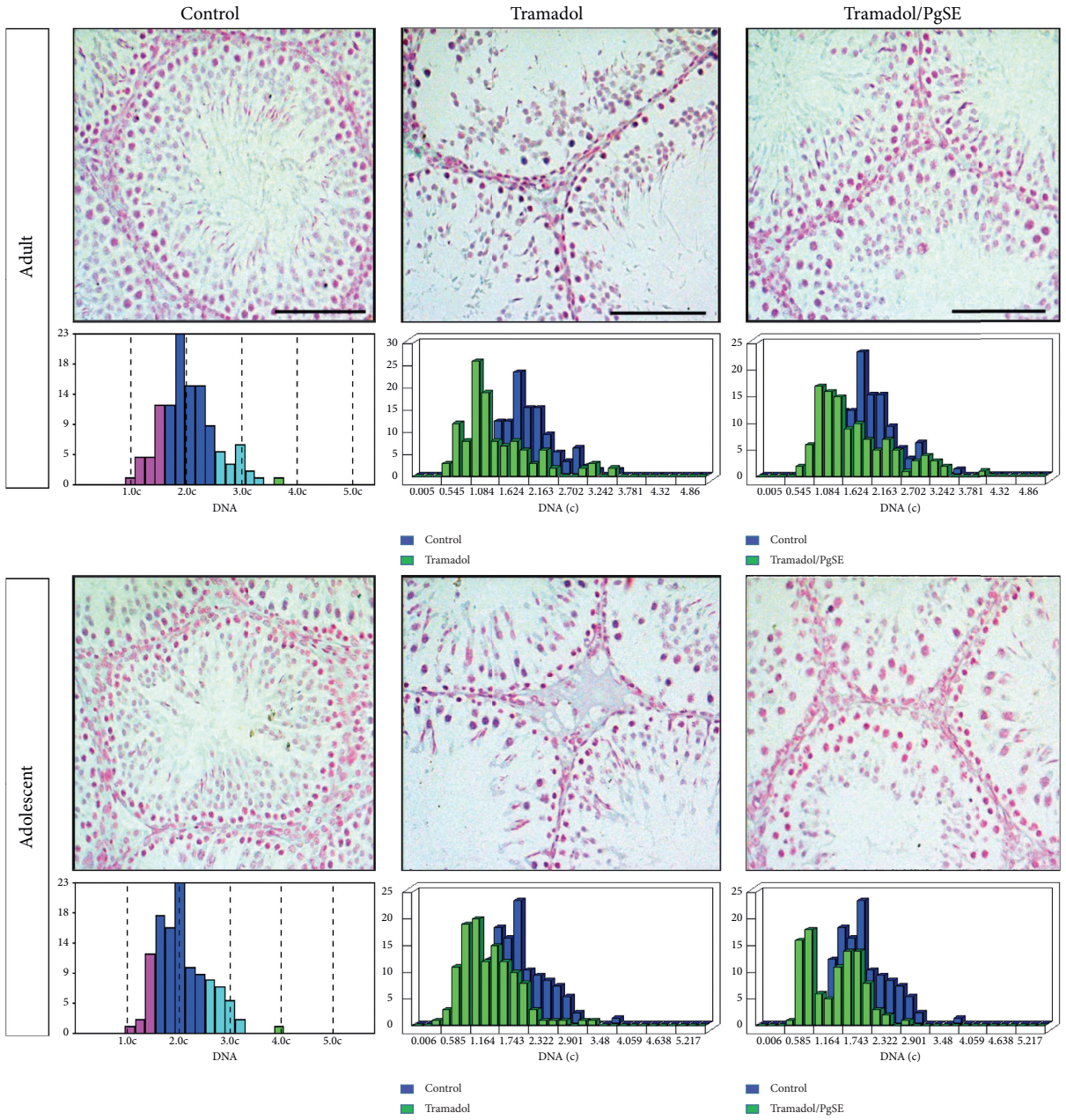

Figure 5: DNA ploidy image cytometry of Tr-treated testicular tissues and cotreated with PgSE. Adult and adolescent rat testes were examined via Feulgen staining. The nuclear integrated optical density (OD) which is the cytometric equivalent of DNA content was measured. The DNA histograms were classified based on the amount of DNA as haploid (<1.5c), diploid (1.5–2.5c), triploid (2.5–3.5c), and tetraploid (3.5–4.5c). The Tr-treated group had diminished the triploid cell population in both ages. Scale bars: 100 μM.

3.6. Phytochemical Analysis of PgSE. To understand the protective effect of PgSE against the tramadol toxicity, we analyzed the phytochemistry of PgSE which represents the antioxidant activities including total phenolic content, total flavonoids content, and free radical DPPH scavenging activity (Figure 6). The PgSE exhibited high total polyphenol content of 83.8 μg/g (expressed as gallic acid equivalent) and high total flavonoid content of 68.8 μg/g (expressed as gallic acid equivalent). The DPPH free radical scavenging of PgSE is 359.1 μg TE/g and the percentage of DPPH inhibition effect was 210.4%.

4. Discussion

One of the main causes of male infertility is the exposure to opioid analgesic drugs that induce oxidative stress [4–9]. Tramadol is one of the opioid drugs that is extensively used as a pain-killer for chronic pain and cancers [10]. However, multiple tissue toxicities were reported for patients consuming tramadol medication several times a day [13–17]. Since tramadol like other opioids induces oxidative stress which alters cell structure causing apoptosis [21–25], the pomegranate was used in the current study as a protective

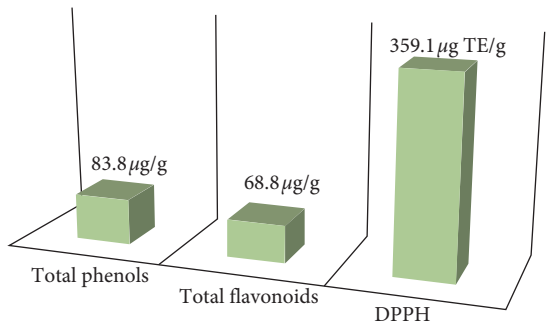

Figure 6: Phytochemistry of pomegranate seeds extract (PgSE). The PgSE was examined for its total phenolic content, total flavonoids content, and free radical DPPH scavenging activity. The phytochemical analyses indicated the presence of a high level of phenols, flavonoids, and DPPH. The measured values were written over the bars.

agent based on its antioxidant activity [34, 35, 48, 49]. Thus, our study was focused on evaluating the toxicity of tramadol on testicular tissue from adult and adolescent ages and testing the protective effect of pomegranate seed extract (PgSE) against this toxicity.

Results revealed that tramadol had adverse effects on testes of both adult and adolescent rats with different ranges on the histopathology, ultrastructure architecture, morphometry, and DNA ploidy. Interestingly, the above-mentioned defects caused by tramadol were prevented when PgSE was coadministered with tramadol. The protective effect could be due to the bioactive compounds that are known to have multiple therapeutic effects such as tannins [50], anthocyanins [51], alkaloids [52], phenolic acids [53], estrogenic flavonoids [54], and conjugated fatty acids [55], which are found vigorously in pomegranate [56].

The histopathology of the adult and adolescent testes showed congested blood vessels, intercellular spaces within seminiferous tubules, interstitial vacuoles, and exfoliation of germ cells in lumen. These results were matched with previous studies [11]. It is known that testicular blood flow is controlled by testosterone, so any defect in Leydig cells alters the size of blood vessels [57]. Previous reports found that opiates including tramadol reduce the serum testosterone levels [58]. The congested blood vessels and interstitial vacuoles observed indicated testicular vascular atrophy and Leydig cell damage has occurred. The protective effect of PgSE shown in the cotreated groups indicated the ability of PgSE to prevent the damage of Leydig cells and restore testosterone to control levels as previously found with pomegranate juice [59]. Besides, the intercellular spaces within seminiferous tubules and exfoliation of germ cells indicated that germ cells may undergo apoptosis or they may lose the Sertoli-germ cell contact being shed into the seminiferous lumen and form intracellular spaces as previously described [60, 61]. In this connection, we examined the testicular cells apoptosis by TUNEL assay. Apoptosis has a critical role in the removal of damaged germ cells to prevent the formation of abnormal sperms. Results indicated that a significant increase of apoptotic cells was detected in testicular sections of both adult and adolescent rats when treated with tramadol. Moreover, a diffuse and defect in the chromatin condensation of elongated spermatids were found. This could be explained by the ability of tramadol to decrease the antioxidant levels in the testis which imbalance the antioxidant defense system leading to cell oxidative stress [9, 21, 23]. Oxidative damage involved inactivation of p53 which, in turn, mediates either DNA repair or apoptosis [62]. Tramadol was found to induce neurotoxic effects through alteration of p53, Bax, and Bcl-2 apoptotic pathway [63]. On the other hand, the cotreated groups have comparable apoptotic cell numbers to controls and normal spermatids' chromatin. Previous reports revealed that extracts from plants with antioxidant activity can reduce the apoptotic of germ cells caused by chemical toxicity [64, 65]. Similarly, pomegranate seed extract prevented germ cell apoptosis, possibly by modulating the ROS/Nrf2/p53 signaling cascade as previously described [66–68].

The ultrastructural examination showed complete degeneration and disorientation of axonemal structure microfilaments. This is predominantly due to the fact that sperms are rich in polyunsaturated fatty acids, rendering them highly susceptible to oxygen-induced damage and, hence, lipid peroxidation. Previous reports indicated that tramadol increases peroxidation of sperm lipids [9, 59], which in particular produces cytotoxic aldehydes [69]. These cytotoxic aldehydes were shown to inhibit a large number of cellular enzymes including glyceraldehyde-3-phosphate dehydrogenase [70], one of the key enzymes for the generation of ATP in mitochondria, and therefore a rapid loss of intracellular ATP occurs. The ATP depletion leads to damage of axonemal structure due to insufficient axonemal protein phosphorylation as previously reported [71, 72]. We found that the cotreatment of PgSE with tramadol could protect against the axonemal damage, and this could be due to the ability of pomegranate to inhibit testicular lipid peroxidation as previously revealed [59].

Additionally, a high degree of collagen fibers deposition in the extracellular matrix surrounding the seminiferous tubules was also observed in the adult and adolescent rat testes treated with tramadol indicating tramadol disrupted collagen metabolism. Nevertheless, the cotreatment of PgSE with tramadol has not revealed a high degree of collagen fibers deposition. Sertoli cells are involved in the deposition of extracellular matrix components such as collagen [73, 74]. Thus, the increased collagen fibers in Tr-treated groups might be a result of impaired Sertoli cell function or due to the activation of fibroblasts by free radicals [75]. PgSE seems to overcome collagen accumulation directly or indirectly via the antioxidant constituents within it. For instance, a report indicated that pomegranate treatment inhibits collagen deposition in CCl_4-induced liver toxicity [76]. This is particularly interesting because an increase in the thickness of the extracellular matrix has been correlated with male infertility [77].

Furthermore, the examination of the DNA ploidy indicated that the S phase population was diminished in the testes of tramadol-treated adult and adolescent rats. S phase is the phase of the cell cycle in which DNA is replicated and represents the proliferation index. This

result means that tramadol might induce oxidative damage of proteins involved in cell cycle progression and ends up with p53 activation which in turn mediates apoptosis [21, 62, 78]. In contrast, the cotreatment of PgSE with tramadol showed a normal S phase distribution; this result could be explained by the evidence that pomegranate polyphenolics induce the cell cycle progression of the UV-induced S phase arrested fibroblast cells as previously identified [79].

Taken together, the presence of free radicals in testis is a normal physiological event; however, the increase in their synthesis stimulates the oxidative stress and DNA damage of cells. All of the above protective effects of pomegranate could be due to the prevention of free radicals generated by tramadol. We specifically tested an extract from pomegranate seeds which are commonly considered an agrowaste, while it has a valuable content of bioactive compounds. We measured the phenolic and flavonoids contents in addition to the free radical DPPH scavenging activity in the seed extract. Phenolic and flavonoid compounds are good electron donors because they hold an aromatic ring bearing at least one hydroxyl group which can directly terminate free radical chain reaction [80, 81]. Likewise, the DPPH free radical scavenging assay is a widely used assay to predict antioxidant activities based on the ability of certain substances to donate a hydrogen atom to the radical [82]. The measured values in PgSE are considered having a high antioxidant activity as compared with previous studies [83–89]. Consequently, this assumes that both total phenols and total flavonoids within PgSE possess a mutual activity to protect against tramadol-induced testicular toxicity. However, the PgSE had a complete protective effect on adults but partially on adolescent rats as revealed by TUNEL assay and DNA ploidy, reflecting that young age patients are more sensitive to analgesic drugs and that should be considered. It is recommended that patients on chronic tramadol therapy should be routinely screened for symptoms of gonadotoxicity.

In brief, our finding hypothesizes that PgSE supplementation may have a potential therapeutic role to overcome the toxic effects resulting from various analgesic drugs on long-term use. Further investigations with other analgesic drugs are necessary to confirm this hypothesis.

5. Conclusions

The chronic exposure to tramadol induces testicular damage in adult and adolescent rats. Histological and ultrastructural examinations revealed that tramadol induced hemorrhage of blood vessels, intercellular spaces, interstitial vacuoles, exfoliation of germ cells in lumen, cell apoptosis, chromatin degeneration of elongated spermatids, and malformation of sperm axonemes. Moreover, tramadol disrupted collagen metabolism and cell cycle progression. All of these adverse effects were protected by the PgSE cotreatment in adults and partially in adolescent rats. The phytochemistry of PgSE showed a high content of antioxidant compounds and high DPPH free radical scavenging activity.

Data Availability

All data used to support the findings of this study are included within the article and the supplementary information files.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Fatma Minisy and Hossam Shawki are the first authors.

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Supplementary Materials

Figure S1: the experimental model used in the current study. Adult and adolescent male Wistar rats were examined as two different groups. Each group was divided into three subgroups as follows: control, Tr-treated, and Tr/PgSE-treated groups. The control group received orally 1 ml of 0.9% saline. The Tr-treated group received orally tramadol dose of 20, 40, and 80 mg/kg during the 1st, 2nd, and 3rd week, respectively. The Tr/PgSE-treated group received orally 40 mg/kg PgSE in addition to the tramadol dose. Tr: tramadol. PgSE: Pomegranate seed extract. Figure S2: histopathology of adult and adolescent rat testicular sections at low magnification. Sections of saline-treated controls, PgSE-treated controls, Tr-treated group, and Tr/PgSE-treated group were examined via HE staining. Scale bars: 100 μM. Table S1: quantitative DNA ploidy of Tr-treated testicular tissues and cotreated with PgSE. (*Supplementary Materials*)

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Research Article

Anticancer Properties of Different Solvent Extracts of *Cucumis melo* L. Seeds and Whole Fruit and Their Metabolite Profiling Using HPLC and GC-MS

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Honeydew melon (*Cucumis melo* L.) is an oval-shaped delicious fruit of one cultivar group of the muskmelon with immense nutritional importance and is extensively consumed by many tropical countries. The effect of various organic solvents on the recovery of phytochemicals from honeydew melon plant fruits and seeds was assessed. Further, High-Performance Liquid Chromatography (HPLC) was used to examine and assess the contents of phenolic acid (gallic acid) and flavonoid (rutin) compounds. The use of gas chromatography–mass spectrometry (GC-MS) analysis explained the presence of volatile phytocompounds in the extracts. The use of organic solvents had a substantial impact on the total dry weight and extract yield. In general, the solvent-extracted constituents remained in the order of methanol>chloroform>distilled water for both honeydew melon seeds and whole fruit. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to assess the cytotoxicity effect against PC3, HCT116, HeLa, and Jurkat cell lines. The chloroform extract exhibited a good cytotoxic activity against all cell lines as compared to other solvent extracts. HPLC analysis revealed the occurrence of gallic acid content of 0.102 ± 0.23 mg/10 mg of dry whole fruit extract, while 10 mg of dry seed extract contained only 0.022 ± 0.12 mg of gallic acid content. Likewise, rutin content was observed to be 0.224 ± 0.31 mg and 0.1916 ± 0.82 mg/10 mg of dry whole fruit and seed extract, respectively. Further, GC-MS analysis revealed the presence of a total of 37 compounds in chloroform extract of whole fruit, while only 14 compounds were found in seed extract. Nevertheless, more examinations are needed to identify and characterize other metabolites from honeydew melon and evaluate their pharmacological importance.

1. Introduction

Natural products play a significant role in the modern medicine. Ever since ancient period, naturally derived plant products are being widely used by mankind for treating several ailments. Nature has existed as a source of almost all drugs for many years, and herb-based products play an important role in primary human health care as the majority (80%) of the global population rely on traditional medical practices [1, 2]. Several drugs used in the current medicinal practices are mainly derived either from natural resources or from their chemical derivatives. Also, plant-derived phytocompounds are having huge demand in the pharmaceutical industry [2]. Currently, the prevailing health issues along with emergence of communicable infections and disorders are a major concern to the world population as they are seriously causing increased mortality rate day by day. About 50% of all mortality arising in nations is because of these diseases [3, 4]. Hence, a search for an effective curative agent is continuous. In this regard, natural products are the best choice of sources to isolate and identify new leads for discovering novel drug molecules. This is due to the fact that natural products are cost-effective, exhibit therapeutic potential with high efficacy, and possess no or negligible toxicity effect. The available traditional information related to the therapeutic importance of plants is the basis for their explorations by the experts [5].

Orange-fleshed honeydew melon, also known as (*Cucumis melo* L.), is an oval-shaped delicious fruit with immense nutritional importance and is extensively consumed by many tropical countries. It is one cultivar group of the muskmelon and belongs to the family, *Cucurbitaceae*. It is produced by crossing orange-fleshed cantaloupe with non-netted, green-fleshed honeydew [6–9]. The distinctive aroma of orange-fleshed honeydew melon fruit is due to the presence of several volatile compounds that are derived biosynthetically from fatty acids, amino acids, carotenoids, and terpenes. The major volatile constituents imparting include phenylethyl alcohol and (*Z*,*Z*)-3,6-nonadien-1-ol. The melon fruit is a good source of vital vitamins, i.e., thiamine, riboflavin, and folic acid; provitamin A; and ascorbic acid [7, 9]. It is stated that different parts of the orange-fleshed honeydew melon fruit possess varied levels of soluble solids, total sugars, sucrose, 5-methyltetrahydrofolic acid, and β-carotene [6]. Studies have revealed that secondary metabolites accumulated in plant species depend on different parameters including plant organs and ecological conditions [10, 11]. Also, the amount of the phytocompounds isolation via different extraction approaches may significantly fluctuate. Further, different solvents used for extracting plant-derived compounds can influence on the yield of phytocompounds [11]. Literature survey shows that meager information is available on phytochemicals and bioactivities of honeydew melon plant's different organs, and there is no comparative studies related to this aspect. Therefore, in this study, the effect of various organic solvents on the recovery of phytochemicals from honeydew melon plant fruits and seeds is assessed. Anticancer activities were assessed to compare between fruit and seed extracts. Further, High-Performance Liquid Chromatography (HPLC) was used to examine and assess the total phenolic content. The use of gas chromatography–mass spectrometry (GC-MS) analysis explained the presence of volatile phytocompounds in the extracts.

2. Materials and Methods

2.1. Collection of Honeydew Melon Fruits. Five completely ripened honeydew melon (*Cucumis melo* L.) fruits were collected directly from the agricultural field during the harvesting season (August, 2018). The fruits were then washed to remove the dust particles and then wiped neatly with sterile cotton cloth to remove any traces of water on the surface. Later, the fruits were cut open to obtain the seeds, and one whole fruit was cut in to pieces without removing any contents. The samples were then dried in a hot air oven till they were completely dried at 55°C–65°C and then ground to powder using a blender. All the powdered samples were then weighed and kept in air-tight containers for further use.

2.2. Solvent Extraction of Phytochemicals. The phytochemicals from powdered seeds and whole fruits were extracted using three different solvent systems such as distilled water, chloroform, and methanol to determine the ideal solvent extraction system. Each dried powdered sample weighing 20 g was added into 300 mL solvents such as distilled water, chloroform, and methanol in a conical flask and stirred for 24 h. Later, the solutions were double filtered using Whatman filter No.1 filter paper, and the filtrates were then evaporated completely in incubator at 40°C. After complete evaporation, the extracts were weighed, and the extract yield (%) and total dry weight were determined. Later, the dried extracts were preserved at 4°C in for further use.

2.3. Cytotoxic Assay through MTT Method. The cytotoxic activity of phytoextracts was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reduction assay. The cytotoxic activity of different extracts was determined against human prostate cancer cell line (PC-3), human colon cancer cell line (HCT116), immortalized human T lymphocyte cells (Jurkat), and immortal cell line (HeLa). For MTT assay, cell lines were seeded in 96 well culture plates, at a cell density of 20,000 cells per well (200 μL cell suspension), without the test agent and allowed to grow for about 12 h. The IC_{50} values of different phytoextracts against these cell lines were determined using the concentrations 50, 150, 250, 350, and 450 μg/mL, and the assay was carried out in triplicates. The culture medium without cells was used as medium control, whereas, medium with cells, but without the experimental drug/compound, was used as negative control, and medium with cell lines containing curcumin (15 μM) was used as a positive control. All the plates were incubated for 24 h at 37°C in humidified CO_2 (5%) incubator. After the incubation period, the plates were taken out from incubator, and spent media was removed followed by the addition of MTT reagent to a final concentration of 0.5 mg/mL. The plates were returned to the incubator and incubate for 3 h and then the MTT reagent was removed, and 100 μL of solubilization solution (DMSO) was added.

The absorbance was in an ELISA reader at 570 nm with reference wavelength 630 nm. The IC_{50} values for each extract were determined by using linear regression equation, i.e., $Y = mX + C$. Here, $Y = 50$, m (angular coefficient) and C (linear coefficient) values were derived from the viability graph, by the relationship between extract concentration and absorbance (cell viability).

2.4. High-Performance Liquid Chromatography (HPLC) Analysis of Polyphenols. The analysis was made in isocratic mode with the mobile phase acetonitrile and water in the ratio 7 : 3 with the RP-HPLC C-18 column at a flow rate of 1 mL/min. The standards, gallic acid and rutin (Sigma Aldrich, St. Louis, MO, USA) (0.4 mg/mL), and samples (10 mg/mL) were dissolved in mobile phase, and 20 μL was injected, and the elution was monitored. The spectral data was obtained between 190 and 500 nm. The available standards, their UV spectra, and retention times were compared with the samples to identify and quantify phenolic compounds.

2.5. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. Each solvent extract was subjected to GC-MS analysis using the model instrument, GCMS-QP2010 Ultra (Shimadzu Co., Japan) attached with a capillary column DB-1 (0.25 μm film × 0.25 mm I.d. × 30 m length). Analysis was performed by injecting 1 μL of the sample with a split ratio of 20 : 1, and Helium gas (99.9%) was used as the carrier gas at a flow rate of 1 mL/min. The analysis was performed in the electron impact (EI) mode with 70 eV of ionization energy. The injector temperature was maintained at 250°C (constant). The column oven temperature was set at 50°C (held for 3 min), raised at 10°C per min to 280°C (held for 3 min) and finally held at 300°C for 10 min. The compounds were identified after comparing the spectral configurations obtained with that of available mass spectral database (NIST and WILEY libraries).

2.6. Statistical Analysis. The results were statistically analyzed using GraphPad Prism (version 5.0) statistical software and expressed as the mean ± standard deviation (SD). Multiple comparisons were done through Dunnett's test by comparing the test data with control/untreated data to determine the statistically significant differences. Further, Student's t-test was used to compare the cytotoxicity effect between whole fruit and seed extracts. p value ≤0.05 was considered statistically significant.

3. Results

The use of organic solvents had a substantial impact on the total dry weight and extracts yield. Comparatively, the methanolic whole fruit extract showed with a higher extraction yield of 11.12%, and total dry weight was observed to be 546.01 ± 1.91 μg of dry weight. Likewise, the methanolic seed extract showed the highest extraction yield of 11.62% with 552.02 ± 2.23 μg of total dry weight. The water extract of both seeds and whole fruit yielded the lowest dry weight and yield. In general, the solvent-extracted constituents remained in the order of methanol>chloroform>distilled water for both honeydew melon seeds and whole fruit.

The HPLC analysis used to identify the presence of polyphenols in the chloroform extracts of seeds and whole fruit showed the maximum antiproliferative activity against all the cells. The gallic acid content was found to be 0.102 ± 0.23 mg/10 mg of dry whole fruit extract, while for seeds, it was observed to be 0.022 ± 0.12 mg/10 mg. Likewise, the flavonoid, rutin, was observed to be 0.224 ± 0.31 mg/10 mg of dry whole fruit extract. In the chloroform extract of seeds, it was found to be 0.1916 ± 0.82 mg/10 mg. Further, the GC-MS analysis revealed that the chloroform extract of whole fruit contains a total of 37 compounds, while seed extract contained only 14 compounds. The identified nonvolatile compounds of chloroform whole fruit extracts are given in Table 1 and Figure 1.

To explore the cytotoxicity effect of methanol, chloroform and water extracts of seeds and whole fruit of honeydew melon were determined by MTT assay. The anticancer activity was carried out against different cancer cells, such as PC3, HCT116, HeLa, and Jurkat cell lines, and the results showed a decreased viability (%) with increased concentration of the phytoextracts. The results have shown that different solvent extracts of whole fruit and seeds inhibited different cancer cells in a dose-dependent manner. The methanol and chloroform extracts exhibited a good cytotoxic activity against HeLa cell lines as compared to distilled water extracts (Table 2).

However, almost similar cytotoxic activity has been exhibited against HCT116 by all different solvent extracts tested (Table 3). The chloroform extract of seed has shown a better cytotoxicity against PC3 when compared to the whole fruit; however, distilled water and methanol extracts of whole fruit exhibited better cytotoxicity compared to seed extract (Table 4). The cytotoxicity against Jurkat cell lines was found to be almost similar among all different extracts (Table 5). Both whole fruit (Figure 2) and seed extract extracts (figure not shown) exhibited good cytotoxic activity against different cancer cell lines.

The cytotoxicity was not observed in untreated cell lines; however, all the cell lines treated with curcumin (15 μM) showed the cytotoxicity. The statistical analysis by one-way ANOVA followed by Dunnett's test for multiple comparisons revealed high significance at $p < 0.0001$ in comparison with control/untreated cells against all the different tested cell lines. The seed extracts from all the three solvent systems exhibited anticancer activity against HeLa cell lines; the IC_{50} values were found to be 347.55, 349.43, and 331.33 μg/mL, respectively, for methanol, water, and chloroform extracts. Further, the cytotoxic activity of whole fruit extracts against HeLa cell lines revealed IC_{50} values of 264.27, 332.89, and 257.7 μg/mL, respectively, for methanol, water, and chloroform extracts. The IC_{50} values of all different extracts against different cell lines are shown in Table 6. Further, the cytotoxicity effects between whole fruit and seed extracts were assessed using t-test ($p < 0.05$). The results showed that there was no statistically significant difference between the extracts. Overall, the seed extract was more cytotoxic to HCT116 cell line, and whole fruit extract was more cytotoxic to HeLa, PC3, and Jurkat cell lines.

Table 1: The GC-MS analysis report showing the presence of different metabolites.

Sl. No.	Apex RT	Area	% area	Height	% height	Identification
1	7.83	813202.579	0.31	330482.409	0.34	Ribitol, 1,3:2,4-di-O-benzylidene
2	13.63	415974.127	0.16	164857.783	0.17	Beta-estradiol 3,17-disulfate
3	13.9	898950.604	0.35	262301.786	0.27	Malic acid
4	14.95	356696.389	0.14	121270.499	0.12	Undecane
5	15.1	1003747.7	0.39	324390.461	0.33	2,3-Dihydroxy-2-methylpentanoic acid
6	17.4	810167.007	0.31	280707.991	0.29	Mephaneine
7	18.93	421209.113	0.16	164030.915	0.17	Oxazolam
8	20.81	138570.826	0.05	58502.816	0.06	5-(Hydroxymethyl)-2-(dimethoxymethyl)furan
9	23.23	132501.875	0.05	61695.305	0.06	9-Nonadecene
10	23.43	326043.858	0.13	125731.585	0.13	Tetradecane
11	23.61	740844.025	0.28	291654.173	0.3	Glafenin
12	26.35	4846262.298	1.86	1783633.242	1.81	2,4-Di-*tert*-butylphenol
13	26.6	326311.396	0.13	122525.028	0.12	Lauric acid, methyl ester
14	28.18	845949.533	0.33	331696.785	0.34	2-Dodecanol
15	28.35	1219645.767	0.47	409647.806	0.42	Hexadecane
16	28.56	1134791.726	0.44	411097.895	0.42	Methyl 3,5-dimethoxybenzoate
17	31.22	895920.761	0.34	326795.903	0.33	Myristic acid, methyl ester
18	32.58	356138.721	0.14	154978.494	0.16	Pentadecanoic acid, methyl ester
19	32.64	754986.253	0.29	332653.706	0.34	1-Docosene
20	32.78	2052151.175	0.79	575060.411	0.58	Nonadecane
21	32.96	1498170.836	0.58	564804.031	0.57	N^1-(*tert*-Butyldimethylsilyl)-6-nitrobenzene-1,3-diamine
22	33.36	867260.244	0.33	307142.157	0.31	Methyl 13-methyltetradecanoate
23	34.32	764655.12	0.29	253979.509	0.26	Phthalic acid, butyl isobutyl ester
24	34.66	976392.889	0.38	359128.863	0.36	Palmitic acid, methyl ester
25	34.99	4397282.814	1.69	1756158.358	1.78	(*Z*)-Methyl hexadec-11-enoate
26	35.41	77711514.61	29.89	30214884.99	30.7	Palmitic acid, methyl ester
27	36.09	466517.711	0.18	183204.419	0.19	Hexadecanoic acid
28	36.21	5520414.14	2.12	2061336.773	2.09	Dibutyl phthalate
29	36.74	997875.035	0.38	447963.602	0.46	Palmitic acid, ethyl ester
30	36.81	669635.521	0.26	300464.6	0.31	2-Octadecoxyethanol
31	38.66	71694171.07	27.58	28428235.56	28.88	Linoleic acid, methyl ester
32	38.78	41915737.09	16.12	13833942.29	14.05	Linolenic acid, methyl ester
33	38.87	3935994.727	1.51	1775814.73	1.8	13-Octadecenoic acid, methyl ester
34	39.23	13455425.91	5.18	5116611.537	5.2	Stearic acid, methyl ester
35	39.88	649279.136	0.25	224145.171	0.23	Linoleic acid ethyl ester
36	42.65	1173609.722	0.45	447888.354	0.46	Eicosanoic acid, methyl ester
37	44.62	1960767.037	0.75	1100376.213	1.12	Docosanoic acid, methyl ester

4. Discussion

Naturally derived products are the vital source for developing novel drugs. Studies have shown that phytochemicals possess incredible health benefits and play a significant role in human disease prevention. Phytochemicals like plant secondary metabolites and antioxidants exhibit important therapeutic properties [12–14]. In general, phytocompounds are extracted from different plant sources using many ways including decoction, maceration, Soxhlet extraction, supercritical fluid extraction, microwave-aided extraction, and ultrasound-aided extraction approaches [15]. The maceration technique is widely used at the preliminary investigation level due to its simplicity and easy to use [11, 15]. Hence, maceration was used in the present study to preliminarily examine the bioactive principles and bioactivities.

The organic solvents will have a substantial impact on the total dry weight and extract yield. Many studies have evidently suggested that methanol as the best solvent to be used for recovering higher extractable phytocompounds from honeydew melon, irrespective of the plant parts [11, 16, 17]. Likewise, in the present study also, methanol solvent recovered maximum extractable elements from the muskmelon whole fruit and seed extracts. Thus, the present study

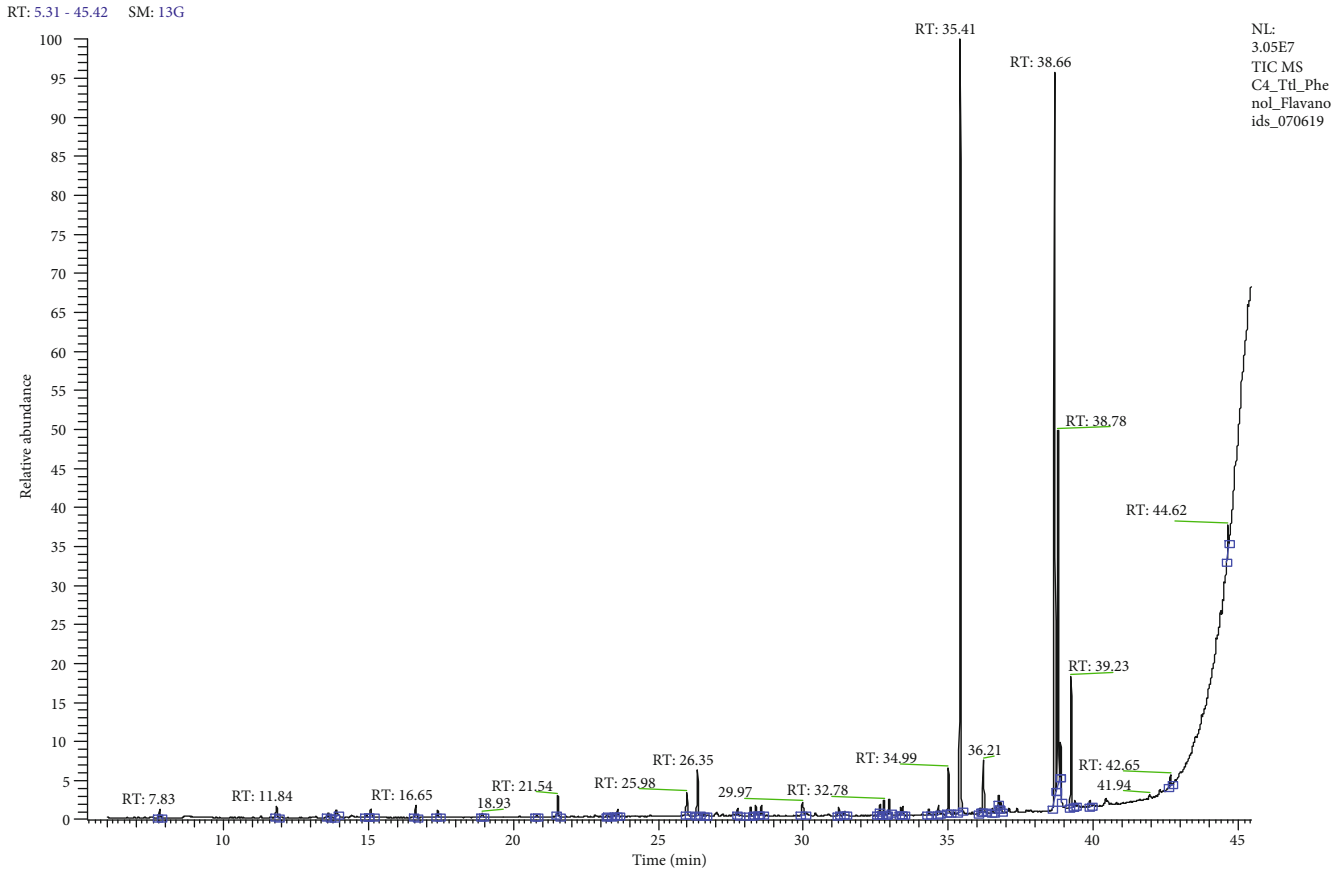

Figure 1: The major phytocompounds detected in the chloroform whole fruit extract of honeydew melon using GC-MS analysis.

Table 2: Effect of phytoextracts on cancer cell line, HeLa.

Drug/phytoextracts	Concentration	Percentage cell viability		
Control/untreated cell lines	—	100 ± 0.005		
Curcumin	15 μM	49.85±0.05****		
		Methanol extract	Distilled water extract	Chloroform extract
Whole fruit	50 μg/mL	89.80±0.007****	91.40±0.006****	86.12±0.004****
Seeds		86.02±0.004****	84.36±0.004****	93.68±0.006****
Whole fruit	150 μg/mL	75.25±0.005****	76.19±0.001****	72.46±0.005****
Seeds		77.95±0.002****	77.95±0.002****	76.08±0.004****
Whole fruit	250 μg/mL	46.27±0.003****	64.18±0.001****	51.35±0.005****
Seeds		63.04±0.003****	61.07±0.005****	56.02±0.02****
Whole fruit	350 μg/mL	32.60±0.007****	54.86±0.004****	32.40±0.005****
Seeds		54.76±0.002****	50.51±0.005****	43.78±0.003****
Whole fruit	450 μg/mL	18.32±0.004****	25.87±0.004****	14.59±0.005****
Seeds		32.09±0.003****	36.12±0.002****	38.01±0.004****

Comparative cytotoxic effect of different solvent extracts of honeydew melon on HeLa cell lines after 24 h of incubation. Data is expressed as the mean ± SD (each treatment, $n = 3$). **** indicates $p < 0.0001$ in comparison with control/untreated cells by one-way ANOVA followed by Dunnett's test for multiple comparisons.

results are in agreement with earlier recorded observations. The presence of substantial variations in the obtained total yield of the extract in different solvents could be correlated to polar nature of the organic solvents evaluated [11, 18].

Previously, researchers have studied cytotoxicity effect of muskmelon fruits and seeds [19, 20]. However, the research efforts were limited to only a single plant parts (seeds, peel, or whole fruit) and to single cancer cells. Moreover, the use

Table 3: Effect of phytoextracts on cancer cell line, HCT116.

Drug/phytoextracts	Concentration	Percentage cell viability		
Control/untreated cell lines	—	100 ± 0.006		
Curcumin	15 μM	50.63±0.003****		
		Methanol extract	Distilled water extract	Chloroform extract
Whole fruit	50 μg/mL	80.15±0.007****	84.27±0.001****	89.25±0.01****
Seeds		80.35±0.004****	83.94±0.006****	80.40±0.007****
Whole fruit	150 μg/mL	70.53±0.003****	66.75±0.004****	73.98±0.003****
Seeds		68.81±0.007****	69.87±0.006****	70.40±0.01****
Whole fruit	250 μg/mL	49.30±0.007****	56.93±0.004****	60.45±0.007****
Seeds		48.50±0.007****	58.46±0.002****	47.37±0.002****
Whole fruit	350 μg/mL	31.25±0.007****	46.25±0.01****	42.01±0.007****
Seeds		37.35±0.003****	47.40±0.01****	30.12±0.01****
Whole fruit	450 μg/mL	28.46±0.003****	16.72±0.002****	27.07±0.004****
Seeds		22.49±0.01****	27.14±0.007****	18.97±0.004****

Comparative cytotoxic effect of different solvent extracts of honeydew melon on HCT116 cell lines after 24 h of incubation. Data is expressed as the mean ± SD (each treatment, $n = 3$). **** indicates $p < 0.0001$ in comparison with control/untreated cells by one-way ANOVA followed by Dunnett's test for multiple comparisons.

Table 4: Effect of honeydew melon phytoextracts on cancer cell line, PC3.

Drug/phytoextracts	Concentration	Percentage cell viability		
Control/untreated cell lines	—	100 ± 0.008		
Curcumin	15 μM	49.08±0.002****		
		Methanol extract	Distilled water extract	Chloroform extract
Whole fruit	50 μg/mL	96.77±0.01****	86.33±0.004****	89.00±0.007****
Seeds		96.89±0.005****	98.32±0.002****	89.56±0.002****
Whole fruit	150 μg/mL	80.47±0.002****	75.77±0.002****	77.45±0.01****
Seeds		91.63±0.005****	90.43±0.009****	75.85±0.01****
Whole fruit	250 μg/mL	70.35±0.004****	62.70±0.007****	65.49±0.001****
Seeds		75.53±0.01****	69.08±0.002****	56.77±0.01****
Whole fruit	350 μg/mL	58.80±0.002****	50.91±0.003****	52.66±0.004****
Seeds		64.38±0.004****	56.65±0.003****	41.75±0.001****
Whole fruit	450 μg/mL	34.66±0.003****	46.62±0.009****	36.81±0.005****
Seeds		44.22±0.006****	39.92±0.01****	31.39±0.007****

Comparative cytotoxic effect of different solvent extracts of honeydew melon on PC3 cell lines after 24 h of incubation. Data is expressed as the mean ± SD (each treatment, $n = 3$). **** indicates $p < 0.0001$ in comparison with control/untreated cells by one-way ANOVA followed by Dunnett's test for multiple comparisons.

of different solvent extracts against different cancer cells is limited. This is a first comparative study to examine the cytotoxicity effect of different solvent extracts of whole fruit and seeds against several cancer cells, which was carried out for the first time. The highest cytotoxicity observed in the extract of whole fruit could be because of higher levels of bioactive compounds present in it. Similarly, it has been documented that biological activities are directly correlated to the amount of active metabolites that occur in plant extracts [5, 8, 11].

Polyphenols are a group of valuable plant secondary metabolites known for their free radical scavenging activity in cells and protects body from many diseases, such as neuronal disease, cardiovascular diseases, cataract, and cancers [5]. Mainly, secondary metabolites of plants include polyphenolics and flavonoids, and they possess superior pharmacological activities including antimicrobial, antioxidant, anti-inflammatory, and anticancer activities [21]. The quantitative analyses of honeydew melon extracts revealed that whole fruit extract possesses the highest gallic acid and rutin contents. This could be attributed to the fact that whole fruit is being exposed to external environmental stresses as compared to seeds; it contains higher levels of polyphenols.

Table 5: Effect of honeydew melon phytoextracts on cancer cell line, Jurkat.

Drug/phytoextracts	Concentration	Percentage cell viability		
Control/untreated cell lines	—	100 ± 0.009		
Curcumin	15 μM	43.10±0.007****		
		Methanol extract	Distilled water extract	Chloroform extract
Whole fruit	50 μg/mL	79.48±0.006****	90.77±0.007****	87.81±0.001****
Seeds		87.45±0.009****	73.63±0.002****	88.62±0.004****
Whole fruit	150 μg/mL	68.27±0.002****	85.03±0.004****	67.74±0.004****
Seeds		71.59±0.002****	66.21±0.002****	74.55±0.001****
Whole fruit	250 μg/mL	46.32±0.004****	69.62±0.006****	52.68±0.004****
Seeds		58.06±0.004****	56.36±0.003****	47.31±0.004****
Whole fruit	350 μg/mL	34.1±0.003****	52.86±0.005****	29.65±0.003****
Seeds		43.99±0.003****	47.04±0.002****	32.61±0.009****
Whole fruit	450 μg/mL	16.68±0.002****	38.08±0.006****	11.55±0.003****
Seeds		27.41±0.008****	28.13±0.005****	16.12±0.008****

Comparative cytotoxic effect of different solvent extracts of honeydew melon on Jurkat cell lines after 24 h of incubation. Data is expressed as the mean ± SD (each treatment, $n = 3$). **** indicates $p < 0.0001$ in comparison with control/untreated cells by one-way ANOVA followed by Dunnett's test for multiple comparisons.

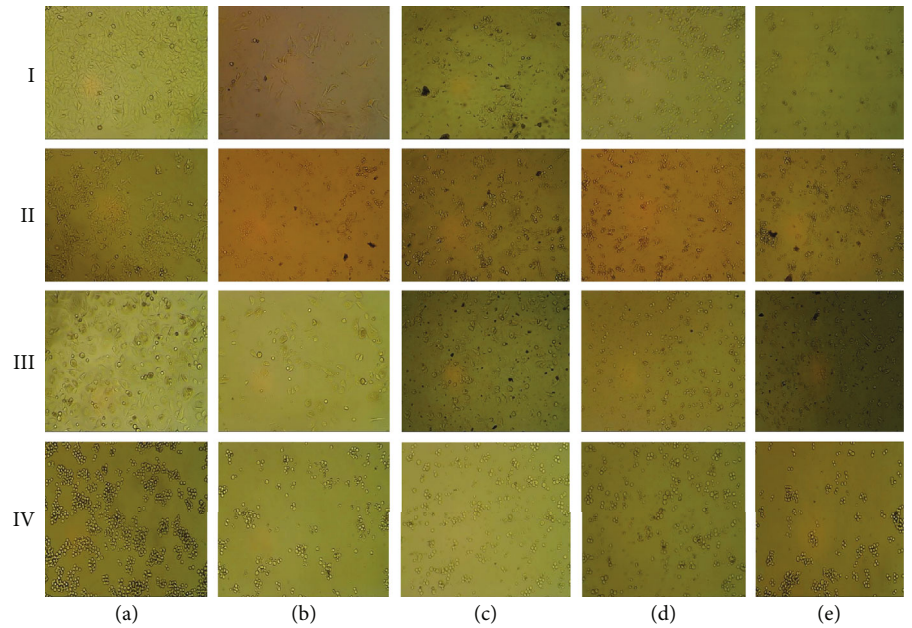

Figure 2: Anticancer activity of honeydew melon whole fruit extract against different cancer cell lines, (a) untreated/control HeLa cell line, (b) treated with curcumin (15 μM), and (c–e) treated with methanol, water, and chloroform seed extracts, respectively, at IC_{50} values. I, II, III, and IV: HeLa, HCT116, PC3, and Jurkat cell lines, respectively.

Similarly, a previous study by Rolim and coworkers have shown that peel extract having higher levels of polyphenols compared to seeds [20].

The biological properties of honeydew melon seed and whole fruit extracts could be due to the occurrence of different phytoconstituents. The literature survey discloses the presence of several nonvolatile compounds from aqueous, methanol, and ethanol extracts of muskmelon seeds and fruit peel around the globe. To the best of our awareness, there is no single report of GC-MS analysis to characterize honeydew melon metabolites. Therefore, this study also included GC-MS analysis to identify the possible bioactive metabolites of honeydew melon extracts. The whole fruit extract was identified with numerous metabolites, including previously known compounds possessing numerous pharmacological properties. In this investigation, the superior anticancer activities exhibited by chloroform extract of whole fruit could be related to the existence of more number of biologically active compounds, such as hexadecanoic acid, undecane, and 2-dodecanol [2, 18, 22]. Further, the presence of other

Table 6: Cytotoxic activity of different phytoextracts against different cancer cell lines and IC_{50} values.

Phytoextracts		IC_{50} values			
Fruit part	Solvent	HeLa	HCT116	PC3	Jurkat
Seed	Methanol	347.55 μg/mL	259.25 μg/mL	436.36 μg/mL	303.54 μg/mL
	Water	349.43 μg/mL	304.42 μg/mL	390.0 μg/mL	293.13 μg/mL
	Chloroform	331.33 μg/mL	246.93 μg/mL	319.8 μg/mL	261.13 μg/mL
Whole fruit	Methanol	264.27 μg/mL	264.86 μg/mL	376.92 μg/mL	243.47 μg/mL
	Water	332.89 μg/mL	277.93 μg/mL	373.09 μg/mL	376.64 μg/mL
	Chloroform	257.70 μg/mL	290.32 μg/mL	341.01 μg/mL	250.21 μg/mL

newly determined compounds in the extract clearly opens a new avenue for the exploration of muskmelon plant in drug discovery and other medicinal applications. Yet, more examinations are needed to identify and characterize other metabolites from muskmelon and evaluate their pharmacological importance.

5. Conclusion

The present study revealed the occurrence of various soluble biologically active phytocompounds in honeydew melon whole fruit and seed extracts using different solvent systems. Overall, the study evidenced the role of phytocompounds occurring in honeydew melon extracts in inhibiting the growth of several types of cancer cells. Also, polyphenol content and yield of the extract varied depending on the solvent types used for the extraction. In the chloroform extract, more soluble metabolites were evidenced, and hence, it exhibited higher anticancer activity as compared to other solvent extracts. These observations clearly support the use of whole fruit for further investigations for treating various diseases, including cancers.

Data Availability

Not applicable.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

Antioxidant and Antiproliferative Potential of Bioactive Molecules Ursolic Acid and Thujone Isolated from *Memecylon edule* and *Elaeagnus indica* and Their Inhibitory Effect on Topoisomerase II by Molecular Docking Approach

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The present study aimed to evaluate the antioxidant and antiproliferative potential of ursolic acid and thujone isolated from leaves of *Elaeagnus indica* and *Memecylon edule* and their inhibitory effect on topoisomerase II using molecular docking study. The isolated ursolic acid and thujone were examined for different types of free radicals scavenging activity, the antiproliferative potential on U-937 and HT-60 cell lines by adopting standard methods. Further, these compounds were docked with the active site of the ATPase region of topoisomerase II. The findings of the research revealed that ursolic acid harbor strong antioxidant and antiproliferative capacity with low IC_{50} values than the thujone in all tested methods. Moreover, ursolic acid shows significant inhibition effect on topoisomerase II with a considerable docking score (−8.0312) and GLIDE energy (−51.86 kca/mol). The present outcome concludes that ursolic acid possesses significant antioxidant and antiproliferative potential, which can be used in the development of novel antioxidant and antiproliferative agents in the future.

1. Introduction

Free radicals are involved in numerous cellular functions including defense mechanisms and cell signaling and are essential for the aerobic metabolism. However, the overproduction of free radicals in cells leads to oxidative stress, consequently causing damage to vital macromolecules, like DNA, lipids, and proteins [1]. Several degenerative related diseases such as cancer, inflammation, atherosclerosis, cataracts, asthma, diabetes mellitus anemia, brain dysfunction, arthritis, liver diseases, and renal problems are predominantly linked to the oxidative stress [2].

Cancer is the malignant illness and the second most leading cause of mortality worldwide. Globally, various types of cancer caused 9.6 million deaths in 2018, approximately 1 in 6 deaths. The people of low- and middle-income countries are highly affected by cancer, due to the change in behavior and diet, and the estimates were around 70% of deaths [3]. In western countries, hormone-dependent cancers such as breast, prostate, and uterine cervix are a common cause of several deaths [4]. Nowadays, different treatments such as chemotherapy, surgery, radiotherapy, and antihormone therapy are used to treat cancer. However, these therapies are expensive and cause an adverse effect on host health. Several

plant-derived compounds like vinblastine, vincristine, taxol, and camptothecin were used in the treatment of cancer [5]. Thus, various research groups around the world have focused on the investigation of plant extract to find a plant-based novel, broad-spectrum, cost-effective, better, and safer anticancer and antioxidant agent from plant materials [6].

Elaeagnus indica (Elaeagnaceae) and *Memecylon edule* (Melastomataceae) are known to possess various ethnobotanical properties and used to treat various ailments in traditional medicine [2, 7, 8]. Both plants were reported to have various biological activities, including larvicidal, antibacterial, anti-inflammatory, analgesic, antioxidant, and anticancer activities, and few compounds were identified [1, 2, 7–17]. However, there is no data available on the antioxidant and antiproliferative properties of the isolated compound thujone (from *Elaeagnus indica*). Even though anticancer and antiproliferative activity of ursolic acid (isolated from *Memecylon edule*) were reported [18–21], there is no data available on growth inhibitory effect of ursolic acid on the human leukemic monocyte lymphoma (U-937) cells, and antioxidant potential of ursolic acid was the least explored. Moreover, there are no previous studies available on the *in silico* inhibition interactions of thujone and ursolic acid with topoisomerase II in detail. Thus, the present investigation aimed to examine the antioxidant and antiproliferative potential of ursolic acid and thujone isolated from leaves of *Elaeagnus indica* and *Memecylon edule* and evaluate their inhibitory effect on the topoisomerase II using molecular docking tools.

2. Materials and Methods

2.1. Plant Materials. Fresh and healthy aerial parts of *Elaeagnus indica* and *Memecylon edule* were collected from different regions of Shervarayan Hills (latitude 11°47′–12°33′ N, longitude 77°02′–78°40′ E, 1300–400 m MSL), Salem District, and Kolli Hills (latitude 10°12′–11°07′ N, longitude 76°–77°17′ E, 900–1100 m MSL), Namakkal District, Tamil Nadu, India, respectively. The nomenclature of collected plant material was authenticated by the Botanical Survey of India (BSI) (*E. indica* reference letter No. BSI/SRC/5/23/2014–15/Tech/1942 and *M. edule* reference letter No. BSI/SRC/5/23/2014–15/Tech./248) Coimbatore, Tamil Nadu, India. Herbarium specimens of collected plants were deposited (*E. indica* specimen No. PU/DBT/NDRL//2010/03 and *M. edule* specimen No. PU/DBT/NDRL//2010/05) in Natural Drug Research Laboratory (NDRL), Department of Biotechnology, Periyar University, Salem, Tamil Nadu, India. The plant materials were washed with the running water prior to sterile distilled water and air-dried at room temperature for 14–21 days. The dried plant materials were pulverized, using an electric grinder, and then sieved through 100-mesh sifter and stored in an airtight container for further use.

2.2. Extraction of Plant Materials. Pulverized plant materials (2 kg) were successively extracted with various organic solvents such as hexane, chloroform, ethyl acetate, acetone, and methanol (1 : 5 solvent ratio) in an increasing polarity manner using a Soxhlet apparatus until the efflux solvents become colorless. The extracts were filtered through Whatman No. 1 filter paper and condensed using a rotary evaporator in vacuum at 40°C which yields greenish crude extracts. These extracts were stored in an airtight container at 4°C until use.

2.3. Isolation of Bioactive Molecules. Based on the preliminary results of phytochemical profile and biological activity [7, 8, 14–17], two extracts (namely acetone extract of *E. indica* and the ethyl acetate extract of *M. edule*) were selected for the isolation of active principles. The activity guided isolation of extract yields two active compounds [15, 17]. These compounds were identified using various spectral studies, like UV, FT-IR, LCMS, ^{1}H, 13C, DEPT-135, HMBC, and HSQC Nuclear Magnetic Resonance [15, 17].

2.4. In Vitro Antioxidant Studies. Antioxidant potential of isolated compounds were examined on different types of free radicals, that is, DPPH, nitric oxide, hydroxyl, and superoxide radical and ferric reducing antioxidant power assay (FRAP) as per the previous standard methods [22–25]. Various concentrations (20–100 μg/mL) of the isolated compounds were used in the radical scavenging potential analysis. Similar concentrations of a natural (ascorbic acid) and synthetic compound butylated hydroxyanisole (BHA) were used as reference molecules in all antioxidant investigations.

2.5. Antiproliferative Activity. The human leukemic monocyte lymphoma (U-937) and human acute promyelocytic leukemia (HT-60) cell lines were acquired from the National Institute of Cell Sciences, Pune, India, and maintained in Minimal Essential Medium (MEM) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS), 3% L-glutamine, 100 IU/mL penicillin *G*, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. The antiproliferative potential of isolated compounds was detected by methyl thiazolyl diphenyl-tetrazolium bromide (MTT) by adopting the method of Srinivasan et al. [16] on U-937 and HT-60 cell lines.

2.6. Molecular Docking Studies. Molecular interactions of ursolic acid and thujone with topoisomerase II (PDB id: 1QZR) were studied using *GLIDE* (*Grid-Based Ligand Docking with Energetics*) [26] software v5.5 developed by Schrödinger executed on Red Hat Enterprise Linux 5. *Maestro* v9.0 used for the preparation of ligands and proteins and docking study was carried out in Graphical User Interface (GUI, Maestro, 2009) workspace. *LigPrep* (Schrödinger suite, 2009) module of v2.3 of Schrödinger Suite 2009 was used to prepare the ligands. The energy minimization of *LigPrep* follows the optimized potential liquid simulations for all-atom force fields. Induced fit docking (IFD) of the prepared ligands with target proteins was done in *Induced Fit Docking* protocol of *GLIDE* v5.5

Table 1: DPPH radical scavenging activity of ursolic acid and thujone.

Concentration (μg/mL)	DPPH radical scavenging activity (% of inhibition)*			
	Ursolic acid	Thujone	Ascorbic acid	BHA
20	11.92 ± 0.53^{a}	19.00 ± 0.33^{a}	53.22 ± 0.27^{a}	65.42 ± 0.44^{a}
40	37.33 ± 0.57^{b}	31.51 ± 0.64^{b}	61.12 ± 0.66^{b}	69.77 ± 0.58^{b}
60	41.36 ± 0.31^{c}	49.05 ± 0.62^{c}	73.68 ± 0.38^{c}	74.08 ± 0.38^{c}
80	55.96 ± 0.54^{d}	63.25 ± 0.82^{d}	79.80 ± 0.27^{d}	79.94 ± 0.17^{d}
100	76.92 ± 0.53^{e}	72.79 ± 0.82^{e}	83.53 ± 0.41^{e}	84.73 ± 0.38^{e}

*The values are mean of triplicates with (±) standard deviation (mean ± S.D; $n = 3$). Different superscript letters (a–e) in column within treatments indicates significant differences (at $p < 0.05$) when subject to Tukey's multiple comparison test.

(Schrödinger Suite 2009). The images of docked complexes and hydrogen bond interactions were analyzed in the *PyMol* Molecular Graphics System. *Ligplot* diagram of docked complexes was obtained from PDBsum server (http://www.ebi.ac.uk/pdbsum) for better clarity.

2.6.1. Preparation of Topoisomerase II. The crystal structure of the 1QZR complex (ATPase region of topoisomerase II from *Saccharomyces cerevisiae*) was retrieved from PDB which contains two identical 418 amino acid length polypeptide chains (A and B) with one (s)-4, 4′-(-1-methyl-1,2-ethanediyl) bis-2,6-piperazinedione (CDX), phosphoaminophosphonic acid-adenylate ester (ANP), and magnesium ion in each chain. Two methods were used in the preparation of protein for docking study. In the first method, all the water molecules, one CDX, one magnesium ion, and one ANP were removed and in the second method the the water molecules present in 1QZR were removed.

2.7. Statistical Analyses. All the analyses used in the present research were carried out in triplicate. Data were represented as the mean ± standard deviation of three quotients. The inhibitory concentrations 50 (IC_{50}) were calculated by the curve fitted method using OriginPro 8 software. The significant difference was obtained from the results of Tukey's test ($p < 0.05$) of ANOVA (SPSS 25.0).

3. Results and Discussion

3β-hydroxyurs-12-en-28-oic acid (ursolic acid, a pentacyclic triterpenoid) and 1-isopropyl-4-methylbicyclo [3.1.0] hexan-3-one (thujone, a monoterpene ketone) were isolated from *Memecylon edule* and *Elaeagnus indica,* respectively, through activity guided isolation methods. Those compounds' isolation and structural elucidation were reported [15, 17]. Previously, these isolated compounds were reported from different parts of several plants [15–18].

3.1. In Vitro Antioxidant Studies. Both the isolated compounds ursolic acid and thujone expressed good to moderate radical scavenging activity in all tested methods in a concentration-dependent manner (Tables 1–5). Ursolic acid exhibited significant free radical scavenging activity on all tested radicals with the lowest IC_{50}/EC_{50} values followed by thujone with sustainable IC_{50} values. Ursolic acid expressed good Fe^{3+} reduction potential in FRAP assay with the least EC_{50} value 18.42 ± 0.03 μg/mL (Table 2) followed by hydroxyl radicals (IC_{50} value 29.69 ± 0.44 μg/mL) (Table 3). NO radical scavenging potential of ursolic acid and thujone is almost similar to high IC_{50} value of 70.40 ± 0.88 µg/mL and 77.68 ± 0.58 μg/mL, respectively, which are 2-fold higher than the positive control ascorbic acid (Table 4). Both the ursolic acid (76.92 %) and thujone (72.79 %) show nearly similar percentage of DPPH radical scavenging potential. However, considerable difference was found in the IC_{50} values (Table 1). Ursolic acid expressed a significantly high superoxide radical scavenging ability with low IC_{50} value of 43.35 ± 0.95 μg/mL, which is lower than both natural and synthetic antioxidant controls, namely, ascorbic acid and BHA, respectively (Table 5). Thujone show the lowest quenching ability on the superoxide radicals with high IC_{50} value of 131.78 ± 1.27 μg/mL (Table 6). The results of the antioxidant potential of thujone revealed that IC_{50} values of all radical scavenging activity were two- to threefold higher than the isolated ursolic acid when compared with reference compounds in most of the tested methods. Maximum antiradical activity was found in FRAP method with of EC_{50} value of 44.58 ± 0.89 μg/mL. The results of the rest of assays expressed considerable radical scavenging potential of thujone.

The present investigation used 5 different assays to assess the antioxidant potential of ursolic acid and thujone. Both ursolic acid and thujone expressed different degree of radical scavenging potential on various tested radicals. The difference in the antioxidant capacity of the tested compounds obtained by the results of FRAP, DPPH radical, hydroxyl radical, nitric oxide radical, and superoxide radical scavenging methods is probably a result of the variation in sensitivity of isolated compounds to the various species of radicals [27]. Moreover, the variation in the reaction media such as lipophilic, hydrophilic, and amphiphilic and nature of radicals and antioxidant molecules have impact on the antiradical potential. For example, DPPH radicals are used to evaluate the antiradical potential of both hydrophilic and hydrophobic antioxidants, whereas nitric oxide assay is used for hydrophilic antioxidants [27]. The difference in the antioxidant potential of ursolic acid and thujone is due to the variation in their structure and functional groups such as free hydroxyl moiety and polarity.

There are no studies/reports on the antioxidant activity of thujone. However, several reports are present on the antioxidant activity of essential oils from various plants, that is, *Salvia officinalis* [28], *Artemisia herba-alba* [29],

Table 2: FRAP activity of ursolic acid and thujone.

Concentration (μg/mL)	FRAP (OD values)*			
	Ursolic acid	Thujone	Ascorbic acid	BHA
20	0.543 ± 0.006^{a}	0.084 ± 0.016^{a}	0.305 ± 0.007^{a}	0.885 ± 0.007^{a}
40	0.827 ± 0.006^{b}	0.366 ± 0.009^{b}	0.593 ± 0.006^{b}	1.360 ± 0.006^{b}
60	1.037 ± 0.006^{c}	0.923 ± 0.023^{c}	0.988 ± 0.010^{c}	2.133 ± 0.011^{c}
80	1.357 ± 0.006^{d}	1.078 ± 0.015^{d}	1.505 ± 0.040^{d}	2.510 ± 0.009^{d}
100	1.427 ± 0.170^{d}	1.451 ± 0.005^{e}	2.134 ± 0.012^{e}	2.993 ± 0.005^{e}

*The values are mean of triplicates with (±) standard deviation (mean ± S.D; $n = 3$). Different superscript letters (a–e) in column within treatments indicate significant differences (at $p < 0.05$) when subject to Tukey's multiple comparison test.

Table 3: Hydroxyl radical scavenging activity of ursolic acid and thujone.

Concentration (μg/mL)	Hydroxyl radical scavenging activity (% of inhibition)*			
	Ursolic acid	Thujone	Ascorbic acid	BHA
20	46.59 ± 0.24^{a}	26.43 ± 0.51^{a}	49.60 ± 0.55^{a}	42.75 ± 0.52^{a}
40	53.48 ± 0.07^{b}	47.02 ± 0.71^{b}	61.40 ± 0.41^{b}	49.65 ± 0.38^{b}
60	60.20 ± 0.33^{c}	53.35 ± 0.47^{c}	76.43 ± 0.34^{c}	60.18 ± 0.75^{c}
80	70.51 ± 0.21^{d}	56.76 ± 0.30^{d}	88.25 ± 0.42^{d}	73.15 ± 0.38^{d}
100	74.62 ± 0.17^{e}	63.34 ± 0.22^{e}	94.06 ± 0.60^{e}	88.55 ± 0.57^{e}

*The values are mean of triplicates with (±) standard deviation (mean ± S.D; $n = 3$). Different superscript letters (a–e) in column within treatments indicates significant differences (at $p < 0.05$) when subject to Tukey's multiple comparison test.

Table 4: Nitric oxide radical scavenging activity of ursolic acid and thujone.

Concentration (μg/mL)	Nitric oxide radical scavenging activity (% of inhibition)*			
	Ursolic acid	Thujone	Ascorbic acid	BHA
20	21.70 ± 0.25^{a}	14.25 ± 0.88^{a}	28.80 ± 1.21^{a}	30.26 ± 0.66^{a}
40	32.86 ± 0.32^{b}	29.61 ± 0.66^{b}	54.53 ± 0.91^{b}	41.23 ± 1.54^{b}
60	40.16 ± 0.34^{c}	39.55 ± 2.16^{c}	65.79 ± 0.96^{c}	53.58 ± 0.67^{c}
80	59.49 ± 0.18^{d}	51.24 ± 0.51^{d}	79.39 ± 1.22^{d}	65.35 ± 1.54^{d}
100	70.50 ± 0.39^{e}	74.12 ± 0.66^{e}	94.15 ± 1.13^{e}	87.28 ± 0.38^{e}

*The values are mean of triplicates with (±) standard deviation (mean ± S.D; $n = 3$). Different superscript letters (a–e) in column within treatments indicates significant differences (at $p < 0.05$) when subject to Tukey's multiple comparison test.

Table 5: Superoxide radical scavenging activity of ursolic acid and thujone.

Concentration (μg/mL)	Superoxide radical scavenging activity (% of inhibition)*			
	Ursolic acid	Thujone	Ascorbic acid	BHA
20	24.11 ± 1.13^{a}	07.95 ± 0.71^{a}	25.31 ± 0.29^{a}	13.04 ± 0.62^{a}
40	48.49 ± 0.41^{b}	$10.95 \pm 0.37^{a,b}$	34.24 ± 0.42^{b}	24.10 ± 0.45^{b}
60	57.71 ± 0.89^{c}	$19.82 \pm 0.58^{a,b}$	44.62 ± 0.54^{c}	37.28 ± 0.17^{c}
80	64.67 ± 0.41^{d}	24.64 ± 0.37^{b}	60.06 ± 0.50^{d}	40.18 ± 0.42^{d}
100	76.91 ± 0.33^{e}	43.41 ± 9.69^{c}	75.56 ± 0.25^{e}	56.35 ± 0.29^{e}

*The values are mean of triplicates with standard deviation (mean ± S.D; $n = 3$). Different superscript letters (a–e) in column within treatments indicates significant differences (at $p < 0.05$) when subject to Tukey's multiple comparison test.

Table 6: Antioxidant activities IC_{50}/EC_{50} values of ursolic acid and thujone.

Assays	IC_{50}/EC_{50} values (μg/mL)#			
	Ursolic acid	Thujone	Ascorbic acid	BHA
DPPH radical	71.86 ± 0.54	60.92 ± 1.61	18.77 ± 0.52	15.48 ± 0.61
FRAP	18.42 ± 0.03	44.58 ± 0.89	33.62 ± 0.61	11.24 ± 0.37
Hydroxyl radical	29.69 ± 0.44	49.47 ± 0.62	20.47 ± 0.86	40.78 ± 0.98
Nitric oxide radical	70.40 ± 0.08	77.68 ± 0.58	36.43 ± 0.71	53.90 ± 0.64
Superoxide radical	43.35 ± 0.95	131.78 ± 1.27	67.03 ± 0.80	90.84 ± 1.76

#The values are mean of triplicates with standard deviation (mean ± S.D; $n = 3$).

Table 7: Antiproliferative activity of ursolic acid and thujone on U-937 and HL-60 cell lines.

Concentration (μmol/mL)	% Cell viability*			
	Ursolic acid		Thujone	
	U-937 CL#	HL-60 CL#	U-937 CL#	HL-60 CL#
03.13	73.15 ± 2.01^{h}	69.64 ± 0.68^{h}	90.17 ± 1.08^{h}	96.33 ± 0.78^{h}
06.25	65.18 ± 1.86^{g}	66.92 ± 1.31^{h}	85.80 ± 0.64^{g}	88.88 ± 0.91^{g}
12.50	59.63 ± 0.67^{f}	56.29 ± 0.75^{g}	78.38 ± 1.64^{f}	83.22 ± 1.10^{f}
25.00	54.69 ± 0.64^{e}	50.44 ± 0.79^{f}	71.38 ± 1.03^{e}	77.48 ± 1.66^{e}
50.00	44.66 ± 1.35^{d}	45.18 ± 0.75^{e}	69.67 ± 0.68^{e}	71.15 ± 1.23^{d}
100.0	32.74 ± 2.58^{c}	39.01 ± 1.17^{d}	64.59 ± 1.20^{d}	56.22 ± 0.70^{c}
250.0	27.67 ± 1.28^{b}	33.37 ± 1.17^{c}	53.85 ± 1.80^{c}	52.66 ± 1.52^{b}
500.0	23.08 ± 1.65^{a}	29.15 ± 0.55^{b}	42.51 ± 0.73^{b}	49.67 ± 0.69^{b}
1000	19.67 ± 0.98^{a}	23.70 ± 1.27^{a}	37.29 ± 1.43^{a}	44.66 ± 1.26^{a}

Control, nil mortality. *The values are mean of triplicates with standard deviation (mean ± S.D; $n = 3$). Different superscript letters (a–i) in column within treatments indicates significant differences (at $p < 0.05$) when subject to Tukey's multiple comparison test, CL#, cell line.

Artemisia japonica, *Artemisia nilagirica* [30], and *Artemisia absinthium* [31], which contains high amount of thujone. Based on the results of the aforementioned studies, thujone exhibited low to moderate antioxidant activities of all tested radicals. Mighri et al. [32] reported that thujone-rich oil from *Artemisia herba-alba* showed the lower inhibition percentage of antioxidant activity than the positive control (BHA) which strengthens the outcome of the present study. Earlier study on the antioxidant potential of ursolic acid shows similar IC_{50} value for the DPPH radicals scavenging activity [33] which supports the findings of the present investigation.

3.2. Antiproliferative Activity. The results of the antiproliferative activity of ursolic acid and thujone expressed notable growth inhibitory effect on both U937 and HL-60 cells in a dose-dependent manner (Table 7). The present study shows ursolic acid harbor higher antiproliferative potential on both U-937 and HL-60 cells than the thujone. Furthermore, the findings of the present investigation revealed that HL-60 cells were more sensitive to ursolic acid than U-937 cells (Figure 1). Ursolic acid possessed a profound inhibitory effect on the proliferation of HL-60 cells (~77% growth inhibition) with the lowest IC_{50} value ($26.83 \pm 3.07\,\mu$mol/mL) followed by U-937 cells (~80% growth inhibition) with considerable IC_{50} value ($36.59 \pm 0.80\,\mu$mol/mL). Thujone shows moderate antiproliferative potential on U-937 cells (~63% growth inhibition) with a high IC_{50} value of $297.42 \pm 1.64\,\mu$mol/mL (Figure 2). The least cytotoxic activity on HL-60 cell line (~56% growth inhibition) with a higher IC_{50} value of $486.15 \pm 2.74\,\mu$mol/mL was noticed in thujone.

The total number of U937 and HL-60 cells was decreased with increased dose of both ursolic acid and thujone. Distinctive morphological changes in the U937 and HL-60 cells occurred with increasing concentration of ursolic acid and thujone treatment (Figures 1 and 2). Typical apoptotic features such as cell shrinkage and membrane blebbing were found in the U937 and HL-60 cells upon the low- to mid- and/or high-dose treatment of ursolic acid and thujone [27]. The treatment of ursolic acid and thujone induced the loss of cellular adhesion, echinoid spikes, and blistering. Moreover, the cells are detached from their basal membrane (anoikis) and lost their contact with adjacent cells upon treatment of ursolic acid and thujone at high dose. The increasing concentration of ursolic acid and thujone induced cell death through the necrosis process which, detected with characteristic morphological changes of necrosis in U937 and HL-60 cells such as membrane bubbling, detached from neighbor cells and evaginations [28]. Similarly, morphological changes were reported in the ursolic acid-treated various cancer cells that support the present findings [18–20, 33–36].

Ursolic acid is reported to have antiproliferative activity on different types of cancers such as lung, colon, breast, renal, prostate, melanoma, and leukemia [18–20, 33–36]. Many reports support the antiproliferative potential of ursolic acid because it might induce the apoptosis process in different mode on various types of cell lines [37, 38]. Previously, the antiproliferative activity of ursolic acid on HL-60 cell line was well-reported. It mainly induced apoptosis in HL-60 leukemia cells accompanied by mediating the release of intracellular calcium ions [39], inhibiting DNA synthesis [40] and blocking the cell cycle process [39, 41], and inducing Atg5-dependent autophagy [42] and mitochondria dependent apoptosis [19] which supports the findings of the present study. Moreover, many reviews described the mode of action of ursolic acid in the control of cancer cells [18–21].

Biswas et al. [43] evaluated the antiproliferative and apoptosis-inducing properties of thujone-rich fraction separated from *Thuja occidentalis* on melanoma (A375) cell line harbor higher IC_{50} ($226.18\,\mu$g/mL) value. Moreover, the thujone-rich fraction displayed least (~14%) cytotoxicity on normal peripheral blood mononuclear cell (PBMC) [44] (Biswas et al., 2010). Similarly, Zolotovich et al. [44] reported that thujone exhibits no cytotoxic effect on HeLa cells at high concentrations ($100\,\mu$g/mL), which supports the outcome of present study. Likewise, Privitera et al. [5] documented that thujone-rich essential oil from *Salvia officinalis* has no cytotoxic effect on the LNCaP cells in all tested concentrations that strengthen the present findings.

3.3. Molecular Docking Studies. DNA topoisomerases are ubiquitous enzymes that unwind DNA molecule which is

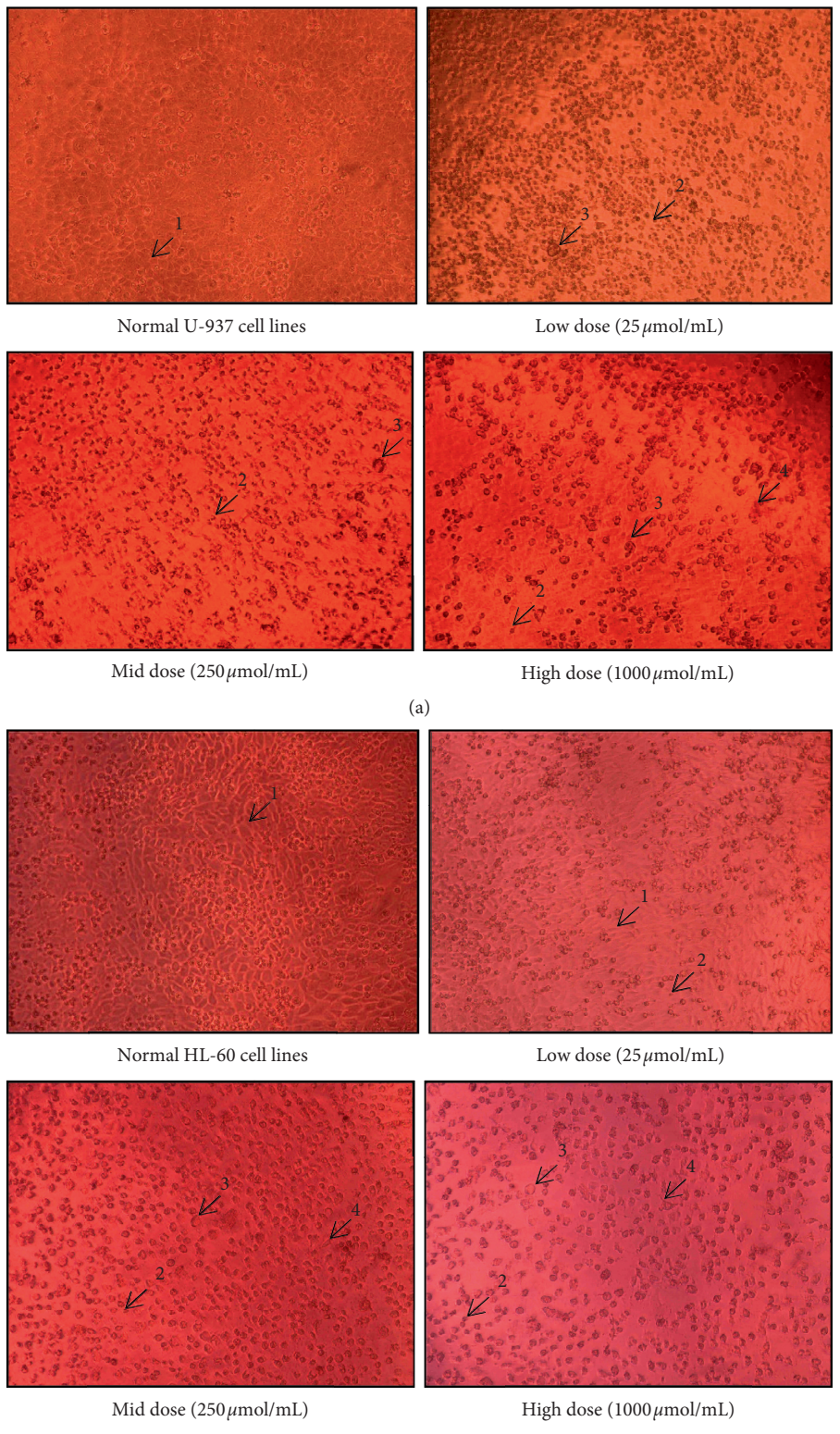

Figure 1: Antiproliferative potential (a and b) of isolated thujone (1-normal cells, 2-cell shrinkage, 3-membrane blebbing, 4-echinoid spikes, and 5-anoikis). (a) Antiproliferative effect of isolated thujone on U-937 cell line. (b) Antiproliferative effect of isolated ursolic acid on HL-60 cell line.

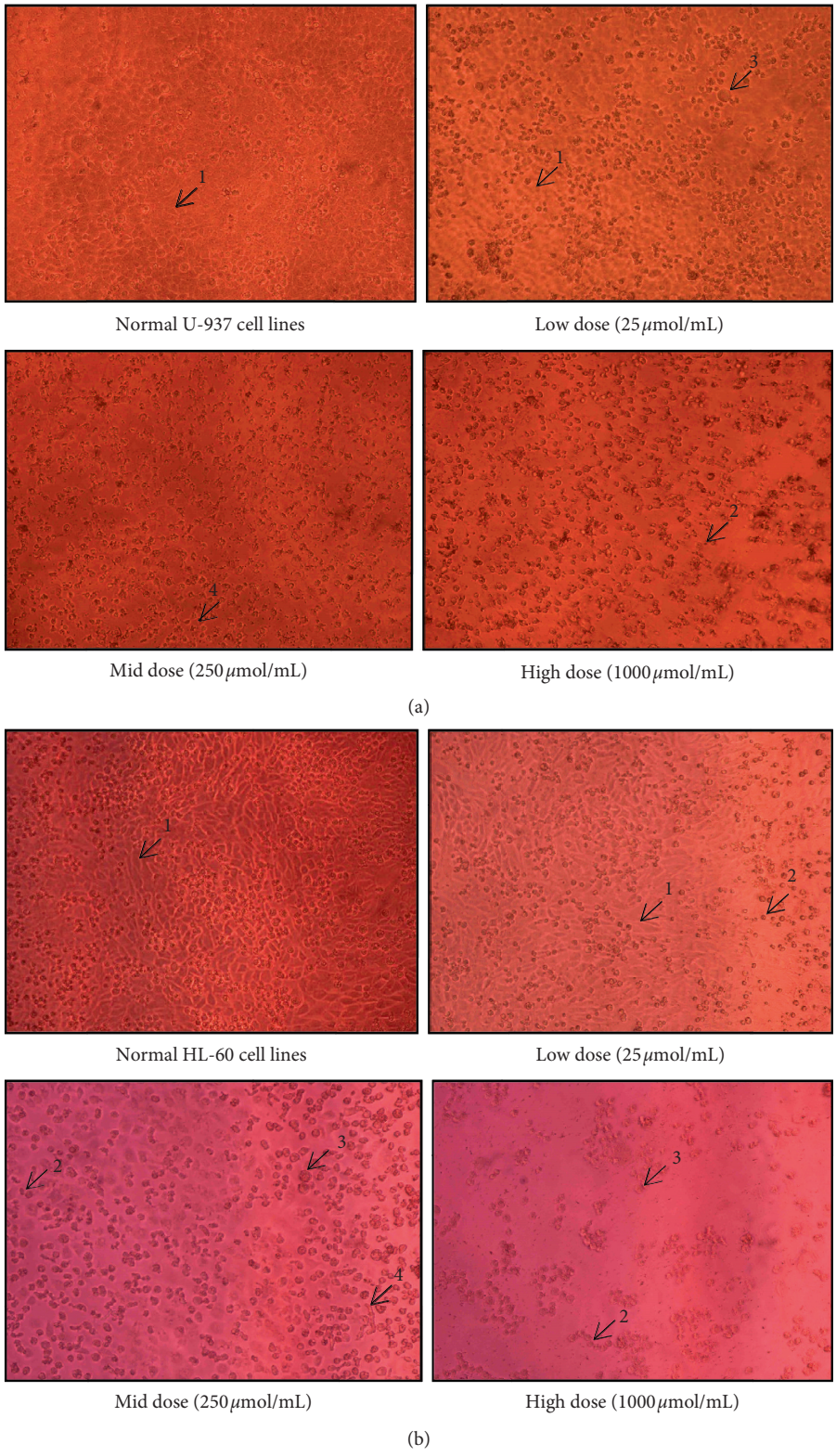

Figure 2: Antiproliferative potential (a and b) of isolated ursolic acid (1-normal cells, 2-cell shrinkage, 3-membrane blebbing, and 4-echinoid spikes). (a) Antiproliferative effect of isolated ursolic acid on U-937 cell line. (b) Antiproliferative effect of isolated ursolic acid on HL-60 cell line.

necessary for various DNA dependent biological processes [45]. Generally, two types of topoisomerases are predominantly found in cells, namely, type I and type II. Type I topoisomerases break single strand of duplex DNA that create a gate for the transition of the another DNA strand, whereas type II topoisomerases cut the double strands of

Table 8: Docking results of ursolic acid and thujone with topoisomerase II (1QZR).

Compounds	Docking score	GLIDE energy (kcal/mol)	Interactions D–H···A	D···A* distance (Å)
Target protein: 1QZR (single chain)				
Ursolic acid	−4.2933	−37.82	(O–H···O) ASN142 PHE362 (N–H···O) (O–H···O) THR27	2.79 2.93 2.85
Thujone	−5.4444	−16.964	GLN365 (N–H···O) TYR28 (O–H···O) GLN365 (N–H···N)	2.98 3.05 3.15
CDX (cocrystallized ligand)	−7.0166	−35.33	(O–H···O) THR363 (N–H···O) THR363 (N–H···O) PHE362 (O–H···O) PHE362 PHE362 (N–H···O)	3.30 3.16 3.32 2.80 3.46
Target protein: 1QZR (double chain)				
Ursolic acid	−8.0312	−51.86	(N–H···O) ARG77A THR195B (O–H···O)	3.36 2.86
Thujone	−8.1810	−27.38	TYR144A (O–H···O) TYR28B (O–H···O) (O–H···O) THR27A	2.94 2.93
CDX (cocrystallized ligand)	−12.105	−56.86	GLN365A (O–H···N) GLN365A (O–H···N)	3.24 2.74 2.73

*D, donor; A, acceptor.

DNA and transit another duplex DNA through the gate and then both type I and type II topoisomerases religate the broken DNA strands [45]. Both the type I and type II topoisomerases are involved in the maintenance of the DNA topology. Inhibition of either type I or type II has significant impact on the DNA replication and other biological process. Expression level of topoisomerase II is often elevated in cancer cells [46]. Therefore, topoisomerase II is an attractive target for various antitumor and antimicrobial drugs [47]. The clinically successful/approved anticancer drugs which target the topoisomerase II induce topoisomerase II poisoning that leads to the arrest of replication and formation of duplex DNA break as the result cells undergo apoptosis [46]. Previous studies revealed that terpenes and terpenoids are potential inhibitor of topoisomerase II [47, 48] and they prefer to bind at the ATPase domain due to the hydrophobic nature of binding site [49]. Ursolic acid and thujone are lipophilic in nature. Thus, in the present study, topoisomerase II ATPase region was selected as a target.

Ursolic acid and thujone possess a significant binding affinity with topoisomerase II. The H-bond interactions between the ligands and proteins are N–H···O and O–H···O type. Besides the H-bond interactions, van der Waals and hydrophobic interactions with the ATPase region residues of topoisomerase II were noticed. The results of ursolic acid and thujone were compared with the docked pattern of CDX (cocrystallized ligand). The GLIDE energy of ligands, H-bond interactions along with distance, and docking score are given in Table 8. Ursolic acid showed H-bond interactions with the active site Thr27, Asn142, and Phe362 residues of a single chain of topoisomerase II (Figure 3) along with other nonbonded interactions with a sustainable docking score (−4.29) and GLIDE energy (−37.82 kcal/mol).

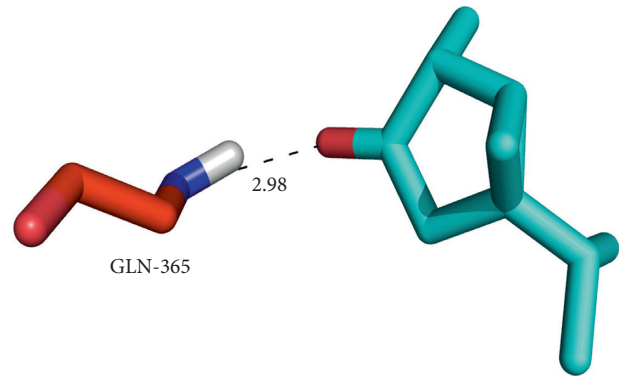

Figure 3: Binding of thujone at the interface of topoisomerase II (chain A) and the corresponding interactions with the residues.

Moreover, while ursolic acid docked with both A and B chains displayed H-bond interactions with the Arg77a and Thr195b residues with notable GLIDE energy (−51.86 kcal/mol) and docking score (−8.0312) (Figure 4), thujone expressed H-bond interaction with the Gln365 residue (Figure 5) when docked with a single chain of topoisomerase II along with hydrophobic interactions with a docking score of −5.44 and GLIDE energy −16.96 kcal/mol. While docking, the thujone with two chains of topoisomerase II displayed interactions with Tyr144a and Tyr28b residues (Figure 6) with a considerable docking score (−8.18) and GLIDE energy (−27.38 kcal/mol). The docking results clearly show that ursolic acid is a strong inhibitor of 1QZR and energetically similar to the CDX. Hence, the antiproliferative activity of ursolic acid might be persistent via the findings of molecular docking studies with respect to the inhibition of topoisomerase II.

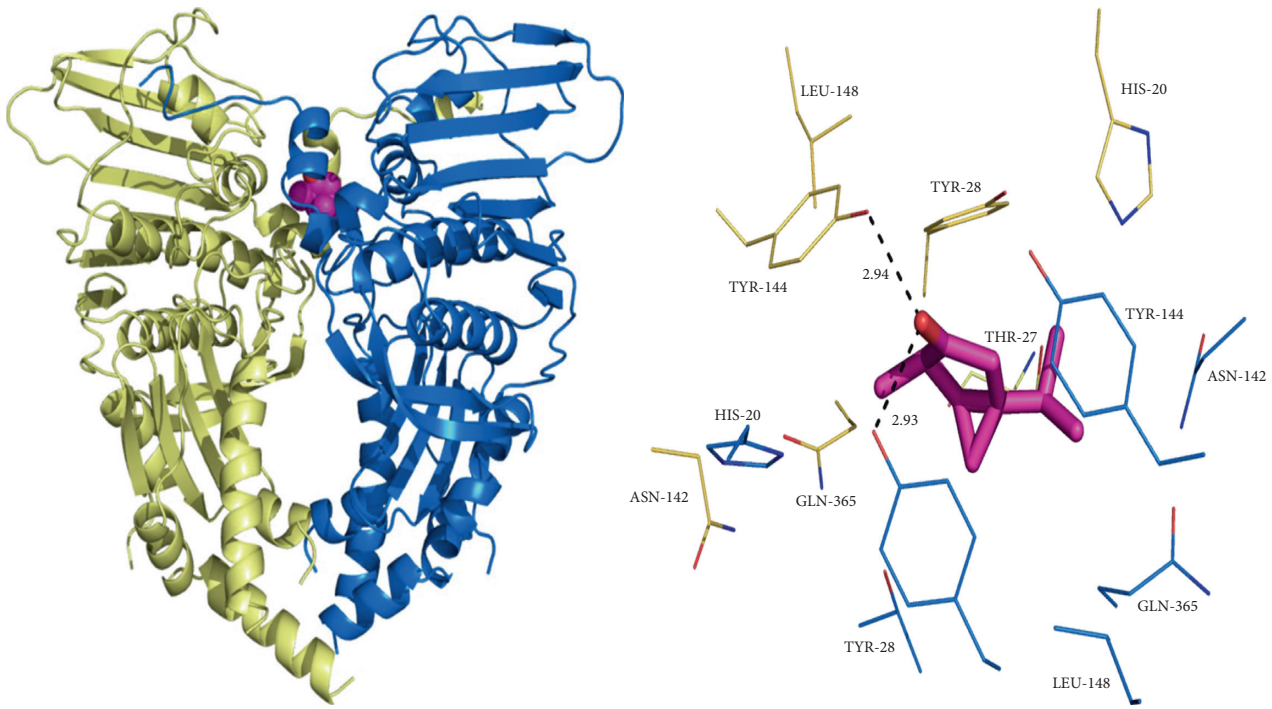

Figure 4: Binding of thujone at the interface of topoisomerase II (double chain) and the corresponding interactions with the residues.

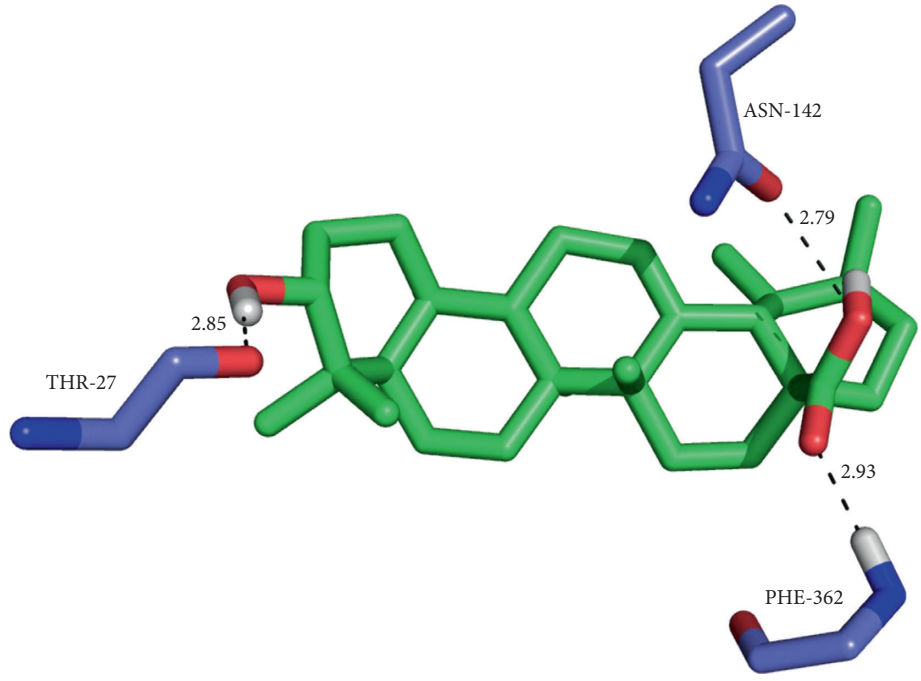

Figure 5: The interactions exhibited by ursolic acid with the active residues of topoisomerase II (chain A) and the corresponding interactions with the residues.

There are no detailed data available on the docking studies of ursolic acid and thujone with topoisomerase II. Even though earlier reports revealed that ursolic acid has significant inhibition potential on topoisomerase II in *in vitro* and *in vivo* assays [50], no detailed data is available on their *in silico* interactions. Two docking studies report the binding energy of ursolic acid (used as control) with topoisomerase II ATPase site (1QZR) but do not show the interaction between them. Those studies reported −21.2 kcal/mol [48] and −57.7 kJ/mol [51] (equivalent to −13.79 kcal/mol) as a binding energy of ursolic acid with topoisomerase II which is 2.44- and 3.76-fold higher than the present study, respectively; and the possible reason for the difference in binding energy is variations in the protein preparation and docking platform. This is the firsthand report of the *in silico* interactions of ursolic acid and thujone

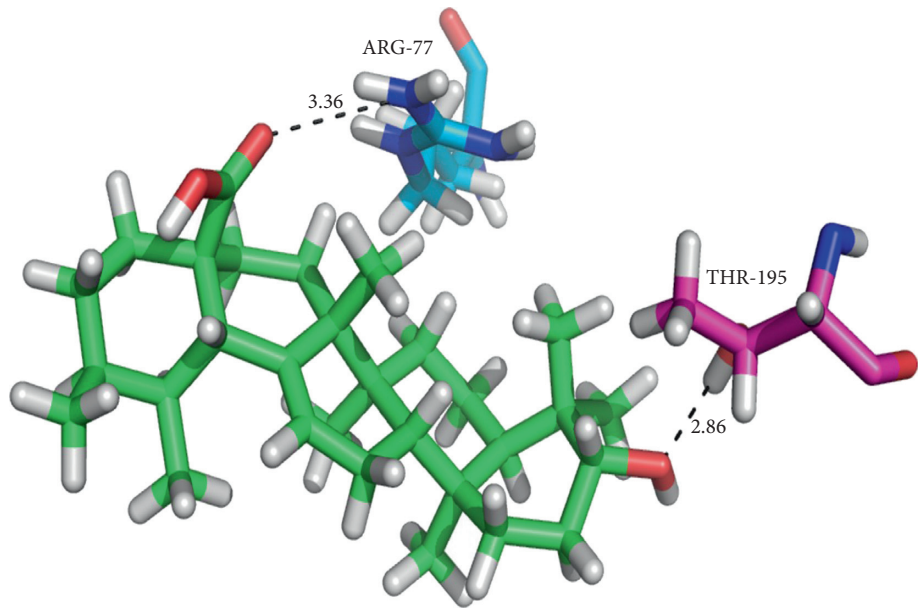

Figure 6: The binding of ursolic acid at the interface of topoisomerase II (double chain) and the corresponding interactions with the residues.

at the ATPase region of topoisomerase II. The antiproliferative activity of ursolic acid is supported by the docking results which may be achieved due to the inhibition of topoisomerase II.

4. Conclusion

Overall outcome of this investigation concludes that ursolic acid isolated from *M. edule* harbors significant antioxidant and antiproliferative potential with low IC_{50} values than the thujone isolated from *E. indica*. HL-60 cells are more sensitive to ursolic acid than the U-936 cells. The outcome of docking results shows that the ursolic acid has a strong affinity with the ATPase active site of topoisomerase II. Ursolic acid might be used as a good molecular template in the discovery of novel and highly potent antioxidant and antiproliferative agents.

Data Availability

No data were used to support this study.

Disclosure

R. Srinivasan, A. Aruna, M. S. Shivakumar, and Jong Suk Lee are co-first authors.

Conflicts of Interest

The authors declare that they do not have any conflicts of interest.

Acknowledgments

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Review Article

Herbal Active Ingredients: An Emerging Potential for the Prevention and Treatment of Papillary Thyroid Carcinoma

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Papillary thyroid carcinoma (PTC) is the most common subtype of differentiated thyroid cancers in Asian coastal cities, where the patients have increased risk of potentially high or excessive iodine intake. Given the high metastasis and recurrence of patients with $BRAF^{V600E}$ mutation, the mortality rate of thyroid cancer has recently shown an upward trend. A variety of therapies, including surgery, radiotherapy, and chemotherapy, have been used to treat thyroid cancer, but these therapies still have limitations, including postoperative complications, drug resistance, poor efficacy, or serious side effects. Recent studies have shown the potential of active ingredients derived from herbal medicine in inhibiting PTC via various cell signaling pathways. Some plant-derived compounds, such as apigenin, genistein, and curcumin, are also known to prevent and treat PTC. This article summarizes the recent advances in the structure-functional impact of anti-PTC active ingredients and their effects on PTC cells and tumor microenvironments with an emphasis on their challenges from basic research to clinical practice.

1. Introduction

Thyroid cancer is the most common endocrine neoplasms accounting for 5.0% of head and neck cancers [1]. Studies have shown that approximately 95% of thyroid cancers originate from thyroid follicular epithelial cells, including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), and anaplastic thyroid cancer (ATC); in addition, a small amount is medullary thyroid carcinoma (MTC) originated from parafollicular cells in the thyroid gland [2, 3]. Among those subtypes, approximately 70% to 80% of all types of thyroid cancers is PTC. Epidemiological studies have revealed that PTC prevalence has increased at an average annual rate of nearly 4% in recent years, and most patients with thyroid cancer suffer from PTC, which is also the main driver of the increased incidence of thyroid cancer [4–7]. PTC has become one of the seven major causes of new malignant tumors among women, and almost all thyroid cancers among children are classified as PTC [8, 9]. Among patients with PTC, the accompanying cervical lymph node metastasis rate reaches 5.4% to 13% after surgery [10–12]. The most common clinical therapies being used for managing PTC include surgery, chemotherapy, and physiotherapy, all of which are hindered by recurrence and metastasis.

With irregular living habits (such as sleep deprivation and long-term high calorie diet intaking) and environmental factors (such as electromagnetic radiation), endocrine disorders including thyroid dysfunction become more and more common to human beings [13, 14]. Both genetic and environmental factors act on thyroid cells and ultimately lead to the transformation of normal thyroid cells into tumor cells. During PTC pathogenesis, some critical genes (including BRAF, RET, KRAS, and PI3KCA) through mutation or chromosomal translocation continuously activate their dependent downstream signaling pathways, such as

mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT, nuclear factor-κB (NF-κB), and Notch-1, and thereby lead to cellular proliferation, migration, invasion, and angiogenesis [15–17]. Recently, emerging clinical trials and experimental researches also demonstrated that some noncoding RNA expressions, such as miRs-21, -34b, -221/222, lncRNA ATB, lncRNA H19, lncRNA HOXA-AS2, circITCH, and circZFR, showed significant association with aggressive clinicopathologic feature in PTC, including tumor size, lymphovascular invasion, lymph node metastases, and presence of $BRAF^{V600E}$ mutation [17–21]. Despite the advances in tumorigenesis, metastasis, and therapy, the underlying mechanism of PTC remains unclear. Therefore, further studies on the pathogenesis, prevention, and treatment of PTC in the pharmaceutical circle should be conducted.

Traditional herbal medicine has an important position in PTC prevention and treatment in Asian countries for a long history. Many active ingredients derived from food and herbs could prevent the development of PTC. Characterized by their mildness and long-lasting action, limited side effects, long-term use, and multitarget regulation, these herbal active ingredients provide many advantages and cannot be replaced by western medicine [22–24]. They not only inhibit the proliferation and promote the apoptosis of PTC cells by regulating critical signal pathways but also improve the immunity of patients and decrease stress response [25–27] as shown in Table 1. This study discusses the role of phytochemicals in thyroid signaling modulation and their possible beneficial or unfavorable effects on the risk of thyroid cancer.

2. Tannins (Phenolic Acids)

On the basis of their chemical structure, tannins can be categorized into hydrolyzable, condensed, and complex tannins. Condensed tannins manifest numerous pharmacological effects, such as antioxidant, antitumor, antihuman immunodeficiency virus, anti-inflammatory, and antimicrobial properties, and are widely found in many medicinal plants and dietary sources, including fruits, nuts, grains, spices, and beverages [58, 59]. Similarly, hydrolyzable tannins have a variety of pharmacological effects, such as antiviral, antibacterial, antitumor, hypolipidemic, and antioxidant properties, and serve as pharmacodynamic bases of many commonly used medicinal plants [60–64].

Epigallocatechin-3-gallate (EGCG), which is the major catechin in tea, shows remarkable protective effects against several chronic inflammatory and cardiovascular diseases, such as cancer, obesity, diabetes, myocardial ischemia, bronchitis, and asthma [65–70]. EGCG exerts chemopreventive effects on various tumors and selectively inhibits various cancer cell proliferation, metastasis, and invasion via regulating VEGF, MAPK, PI3K, and Wnt pathways [71–73]. Wu et al. treated human PTC cell lines (TT and TPC-1) and the ATC cell line (ARO) with EGCG at concentrations of 10~200 μM and observed that EGCG concentration-dependently inhibited the proliferation of these PTC cells and made the cell cycle arrest at the S phase. EGCG also induces the apoptosis of both PTC and ATC cells by inhibiting the EGFR-dependent ERK pathway. In addition, it could inhibit growth and angiogenesis but induce the apoptosis of PTC xenograft tumors in nude mice [28]. De Amicis et al. demonstrated that treatment with EGCG at the doses of 10–60 mM inhibited the proliferation of PTC (FB-2) and FTC (WRO) cell lines through suppressing the phosphorylation of AKT and ERK1/2; furthermore, EGCG reduced cell motility and migration by modulating cell adhesion, reorganizing the actin cytoskeleton, increasing E-cadherin expression, and suppressing SNAIL, ZEB, TWIST, Vimentin, N-cadherin, and α5-integrin, thereby indicating that EGCG inhibited the proliferation and epithelial-to-mesenchymal transition (EMT) of PTC cells [29].

Resveratrol is a polyphenolic phytoalexin with antioxidant and chemopreventive activities [74]. This material has a wide spectrum of targets, including COX2, Sirt1, p53, and miR-17/miR-20b [75], and can inhibit multiple cellular signaling pathways, which were associated with carcinogenesis and progression [76]. Plenty of studies over the past decades have shown that resveratrol downregulated thyroid cancer stem cell markers (including aldehyde dehydrogenases (ALDH), SOX2, OCT4, and NANOG), decreased proliferation and invasiveness, induced apoptosis, reduced ALDH-associated cancer cell stemness, and upregulated thyroid differentiation markers TTF-1 and NIS, which contributed to radioiodine uptake in the treatment of aggressive thyroid cancers [30]. Notably, it was more effective on the redifferentiation of PTC than that of ATC with a high CSC content [22, 30].

Punicalagin is a large polyphenol compound extracted from pomegranates and is classified as an ellagitannin, a family of hydrolyzable tannins [77]. Punicalagin not only induces the cell death of the PTC cell line BCPAP by triggering ATM-mediated DNA damage response [31] but also leads to the G0/G1 phase arrest and senescence-associated secretory phenotype by triggering NF-κB activation [32].

Curcumin is a natural polyphenol extracted from *Rhizoma curcumae longae*, which is the main component of *Curcuma longa*. Curcumin is one of the best-selling natural edible pigments all over the world and is widely used as a food additive approved by the World Health Organization and most countries. It had various chemopreventive properties, such as antioxidant, antitumoral, antiviral, anti-inflammatory, antihepatotoxic, antidiabetic, hypolipidemic, and neuroprotective properties [78–82]. Several studies have also revealed that curcumin induced PTC cell BCPAP apoptosis and cell arrested at the G2/M phase with the concentration increased involving in multitargeting mechanisms, including the activation of reactive oxygen species (ROS)-independent DNA damage by recruiting ATM-mediated Chk2-Cdc25C-Cdc2 pathway [33], the activation of endoplasmic reticulum (ER) stress by disrupting intracellular calcium homeostasis [34], the inhibition of the β-catenin pathway [35], and the modulation of the mitochondrial Bcl-2/Bax pathway [36]. Furthermore, curcumin inhibits invasion and metastasis in PTC cells by upregulating E-cadherin expression and downregulating matrix

Table 1: Anti-PTC mechanisms of herbal active ingredients.

Phytochemicals	Cell lines/patient	Dose (μM)	Mechanisms	References
EGCG	TPC-1, ARO	10~200	Induce apoptosis via inhibiting EGFR/RAS/ERK pathway	[28]
	FB-2, WRO	10~60	Inhibit EMT	[29]
Resveratrol	TPC-1, BCPAP	5~50	Induce apoptosis and differentiation of CSC	[22, 30]
Punicalagin	BCPAP	12.5~100	Induce cell death by triggering ATM-mediated DNA damage; inhibit senescent growth via NF-κB pathway.	[31, 32]
Curcumin	TPC-1, BCPAP, K1	12.5–50	Induce apoptosis via (1) induction of ROS-independent DNA damage by triggering an ATM-activated Chk2-Cdc25C-Cdc2 pathway;	[33]
			(2) activation of ER stress by disruption of intracellular calcium homeostasis;	[34]
			(3) inhibition of β-catenin pathway;	[35]
			(4) modulation of mitochondrial Bcl-2/Bax pathway.	[36]
	BCPAP	12.5~50	Inhibit invasion and metastasis via (1) upregulating E-cadherin and downregulating MMP-9;	[37]
			(2) reversing EMT by inhibiting TGF-β1/Smad2/3 pathway.	[38]
Apigenin	BCPAP	12.5~100	Arrest the cell growth in G_2/M phase; induce autophagy via ROS-mediated DNA damage.	[39]
	PCCl3 with $BRAF^{V600E}$, primary TPC cells	20	Synergistic effects with akt inhibitor	[40]
Quercetin	BCPAP	50–75	Induce apoptosis via inhibiting Hsp90 and caspase-3/parp pathways	[41, 42]
Myricetin	SNU-790	25~50	Induce apoptosis via inhibiting the caspase-dependent mitochondrial pathway	[43]
Icariin	SW579, TPC1	20–50	Induce apoptosis via downregulation of miR-625-3p and inactivation of PI3K/Akt and MEK/ERK signaling pathways	[44]
Flavokawain B	ARO, WRO, TPC-1	3.5–25	Induce autophagy via regulating AMPK/mTOR pathway	[45]
Genistein	BHP10-3, BCPAP, IHH4	9.5–300	Inhibit β-catenin and EMT	[46]
Silibinin	TPC-1	10–100	Suppress migration and MMP-9 expression via ERK pathway	[47]
Ginsenoside Rg1	IHH-4, BCPAP	5–40	Inhibit cell malignancies by upregulating Cx31	[48]
Ginsenoside Rg3	TPC-1, BCPAP	6.25–100	Inhibit invasion and metastasis via reducing rho GTPase	[49]
Capsaicin	BCPAP	25–100	Inhibit invasion and metastasis via activation of TRPV1 and subsequently regulating EMT	[50]
Berberine	TPC-1	10–160	Induce apoptosis, G_0/G_1 cell cycle arrest and migration via PI3K/Akt and MAPK pathways	[51]
Paclitaxel	PTC patient with SCC component	Weekly 80 mg/m^2	The response rate was 67% and the clinical benefit rate was 100%	[52]
Pseudolaric acid B	SW1579	1.25–5	Induce G_2/M cell cycle arrest by activating autophagy by decreasing nuclear p53 expression	[53]
Shikonin	8505c, 8305c, FTC133, BCPAP, C643, TPC-1, IHH4, K1, HTori-3	3–6	Induce apoptosis via suppression of ERK/Akt and DNMT1, and activation of p16/Rb and caspase-3-dependent mitochondrial pathways;	[54–56]
			Inhibit migration and invasion by suppressing EMT and expression of slug and MMP-2, -9, and -14.	[54]
Allicin	HTh-7	10	Activating autophagy via inactivation of akt and S6 pathways	[57]

EGCG: Epigallocatechin-3-gallate; ATM: ataxia telangiectasia-mutated; ROS: reactive oxygen species; EMT: epithelial-to-mesenchymal transition; CSC: cancer stem cell; ER: endoplasmic reticulum; PTC: papillary thyroid carcinoma; SCC: squamous cell carcinoma; EGFR: epidermal growth factor receptor.

metalloproteinase-9 (MMP-9) [37]. Curcumin can also inhibit TGF-β1-induced EMT via the downregulating phosphorylation of Smad2/3, which in turn inhibits the metastasis of human PTC BCPAP cells [38]. Hypoxia plays a crucial role in tumor metastasis, which is the leading cause of death in patients with PTC [83]. Curcumin significantly reduces the production of hypoxia-induced ROS and the binding capacity of HIF-1α to its downstream oncogenes

and weakens the migration of PTC cells under hypoxic conditions [84]. Meanwhile, when combined with sorafenib, curcumin significantly inhibits the apoptosis of FTC133 cells via PI3K/AKT and ERK pathways; moreover, compared with chemotherapy drugs, curcumin has lower cytotoxic effects on normal cells [85]. When cotreated with other natural extracts such as spirulina and Boswellia, curcumin can effectively reduce the size of benign thyroid nodules and restore thyroid hormonal dysfunction, thereby preventing the progress of PTC canceration [86].

3. Flavonoids

Flavonoids are a group of phenolic antioxidants with strong biological activity that have been widely used in pharmaceutical and food additives. Some flavonoids, such as soy genistein, naringenin, phloretin, and chrysin, are structurally similar to estrogen and have little or weak estrogen-like effects [87]. These phytoestrogens can affect not only thyroid hormone synthesis but also thyroid hormone metabolism [88–90]. Therefore, the beneficial or adverse effects of flavonoids depend on their target tissue and their daily consumption. However, an excessive intake of phytoestrogens, especially soy isoflavones, can undo any benefits of flavonoids and interfere in the iodination of human thyroid hormones [91, 92].

Many studies have shown that the estrogenic potencies of these compounds depend mainly on the presence/absence of bicyclic and hydroxyl structures. (1) The B ring position of flavonoids affects their estrogen-like activity, and the strongest activity is present on the 3 position; (2) the hydroxyl groups on the 5 position of the A ring increase the activity; (3) the hydroxyl groups on the 5′ position of the B ring reduce the activity; (4) the conjugated double bond on the 2 and 3 positions of the C ring greatly enhances the activity; and (5) both glycosyl and isopropenyl inhibit the activity. Moreover, different flavonoids perform selective functions during the estrogen receptor subtype stimulation [87, 93].

Apigenin and quercetin are flavonoids that are most commonly found in a variety of fruits, vegetables, and herbs [94, 95]. Treatment with apigenin at concentrations of 12.5~100 μM can inhibit the proliferation of BCPAP cells arrested in the G2/M phase and induce autophagy via ROS-mediated DNA damage [39]. Moreover, combining with apigenin and AKT inhibitors enhances the antitumor effects of radioiodine in both $BRAF^{V600E}$-expressed rat thyroid cells and primary cultured PTC cells from $TR\beta^{PV/PV}$ mice [40]. Unlike those of apigenin, the effects of quercetin on thyroid cells have been disputed. Some studies showed that 1.25~20 μM of quercetin inhibited normal thyroid cell growth in association with the inhibition of the insulin-induced PI3K/AKT pathway. Moreover, quercetin decreases the expression of thyroid-stimulating-hormone-modulated thyroid-restricted gene sodium/iodide symporter (NIS) [96, 97]. By contrast, treatment with 50~75 μM quercetin shows an excellent anticancer activity by inducing S phase arrest and apoptosis via Hsp90 and Caspase-3/PARP pathways in BCPAP cells [41, 42]. Similarly, myricetin, of which the B ring presents one more hydroxyl group at the 5′ position compared with quercetin, dependently induces apoptosis and DNA condensation of SNU-790 PTC cells, which also involves caspase-dependent mitochondrial dysfunction [43].

Icariin is the main active ingredient of *Epimedium davidii* Franch. and has gained much attention because of its erectogenic and neurotrophic effects [98]. Recently, many studies have demonstrated the application of icariin on hormone-dependent neoplasia and in the treatment of prostatic, ovarian, and thyroid cancers [44, 99–101]. Icariin can inhibit cell proliferation, migration, and invasion via downregulating miR-625-3p and suppressing PI3K/AKT and ERK pathways in both SW579 and TPC1 cells [39].

Flavokawain B is a hepatotoxic constituent extracted from kava root [102] and shows potent cytotoxicity by inducing ROS-mediated apoptotic and autophagic cell death in various tumor cells [103, 104]. This material also inhibits cell viability, migration, and invasion and causes autophagy via the activation of the AMPK/mTOR pathway in thyroid cancer ARO, WRO, and TPC-1 cells [45].

Genistein is the main active ingredient of *Leguminosae*. This isoflavone inhibits the invasion and metastasis of the PTC-derived BHP10-3 cell (with RET/PTC 1 rearrangement), BCPAP, and IHH4 (with $BRAF^{V600E}$ mutation) by inhibiting β-catenin and EMT [46]. However, genistein upregulates most thyroid transcript signals, except for thyroid peroxidase, in zebrafish embryos, thereby indicating potential disruptions [105].

Silibinin is a natural hepatoprotective drug and has excellent antioxidant and anticancer properties. It also induces apoptosis, autophagy, makes cell cycle arrest, and inhibits onco-miRNAs which is involved in the PTC tumorigenesis [106]. Previous studies showed that it suppressed cell migration and MMP-9 expression by regulating the ERK pathway in thyroid cancer cells [47].

4. Saponins

Saponins are steroid or triterpenoid glycosides commonly found in plants. Extensive studies have shown that saponins have various pharmacological effects, including hypoglycemic, antitumor, anti-inflammatory, immunomodulatory, and vasoprotective properties, and thus they have been widely used for preventing and treating cardiovascular and immunodeficiency diseases [107]. Ginsenosides are by far the most investigated group of saponins with a triterpenoid dammarane skeleton and are the main active ingredients of the ginseng genus (*Panax ginseng* C. A. Mey. *Panax notoginseng* (Burk.) F. H. Chen and *Panax quinquefolium* L.) in *Araliaceae* and *Gynostemma pentaphyllum* (Thunb) Makino. in *Cucurbitaceae* [108, 109]. At high concentrations (>100 μM), ginsenosides exert cytotoxic and haemolytic effects, while treatment at low concentrations (10~100 μM) inhibits the proliferation of PTC cells, thereby indicating its multidirectional effects on cancer cells [110]. Despite similarities in the structure of dexamethasone, both 20(S)-protopanaxadiol (PPD)-type ginsenosides (such as Rb1, Rb2, Rc, Rd, Rh2, and Compound K) and

20(S)-protopanaxatriol (PPT)-type ginsenosides (such as Re, Rf, Rg1, and Rh1) do not have any effects on glucocorticoid receptor transactivation or transrepression [111]. However, they exert synergistic anti-inflammatory effects when in combination with glucocorticoids at the low doses [112]. Previous studies show that PPT-type ginsenosides (Rg1 and Rg3) can reduce the proliferation, migration, and invasion of PTC cells by upregulating Cx31 and inhibiting Rho GTPase to an alternate cytoskeleton [48, 49]. Furthermore, Rg3 remarkably reduces the expression of the VEGF-C protein in TPC-1, BCPAP, C643, and Ocut-2c cells and inhibits lymph node metastasis in mice [49]. Structure-activity relationships elucidate the association between chemical structures and the anticancer activities of a series of ginsenosides. As shown in Table 2, the anticancer activities of ginsenosides generally take the descending order of CK > Rg1≈20(S)-Rh2≈20(S)-Rg3 > PPT≈PPD, thereby suggesting that the ginsenoside with less polar chemical structures has stronger cytotoxic effects on PTC cells [50, 51].

5. Other Bioactivities

Capsaicin (CAP), a major active component of chili peppers, selectively binds to transient receptor potential vanilloid type 1 (TRPV1). CAP (25–100 μM) dose-dependently suppresses the migration, invasion, and adhesion of BCPAP cells by activating TRPV1 and subsequently regulating EMT [52].

Berberine is an isoquinoline alkaloid mainly isolated from *Coptis chinensis* Franch. and *Berberis wilsonae* Hemsl. et Wils. Numerous studies have shown that it had multiple pharmacological effects, including antimicrobial, hypoglycemic, anti-inflammatory, antifibrotic, and antineoplastic properties. Berberine can also induce mitochondrial apoptosis, trigger G0/G1 cell cycle arrest, and suppress the migration of TPC-1 cells via inhibiting PI3K/AKT and MAPK pathways [113].

Paclitaxel is a tetracyclic diterpenoid derived from the dried roots, branches, leaves, and barks of *Taxus chinensis*. Paclitaxel is a first-line chemotherapeutic agent for PTC patients with a squamous cell carcinoma component, of which response rates are 67% and the clinical benefit rate (PR + SD) is 100%. Therefore, weekly paclitaxel may be applied as effective adjuvant chemotherapy after surgery [114].

Pseudolaric acid B (PAB) is a diterpenoid acid extracted from the root barks or barks near the roots of *Pseudolarix amabilis* (Nelson) Rehd. (*Pinaceae*) and is known as an antitubulin therapeutic agent that can suppress microtubule assembly to inhibit the proliferation of cancer cells [53, 115]. PAB can also inhibit the proliferation, invasion, and migration of SW579 cells by preventing the regulation of Bcl-2 and Beclin-1 expression but decreases the expression of nuclear p53 and induces G_2/M cell cycle arrest by increasing the ratio of autophagic cells [54].

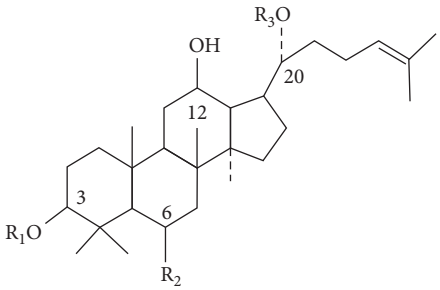
Shikonin is a natural bioactive naphthoquinone derived from *Lithospermum erythrorhizon* Sieb. et Zucc (also called Zi Cao in Asia). It has been recently considered as a natural food additive and antitumor agent for breast cancer, leukemia, hepatoma, and nonsmall cell lung cancer [55, 56, 116]. Previous studies reveal that shikonin dramatically inhibits the migration and invasion of PTC cells via suppressing EMT and inhibiting the expressions of slug and MMP-2, -9, and -14. Furthermore, shikonin induces PTC cell apoptosis by targeting several signaling pathways, suppressing ERK/AKT and DNMT1, and activating p16/Rb and caspase-3-dependent mitochondrial pathways [57, 117, 118]. The oral administration of 2.0 mg/kg shikonin does not cause any liver injury in mice, thereby indicating its safety [117].

Allicin, as another well-known natural food additive, is mainly isolated from garlic. Allicin inhibits the proliferation of cancer cells, induces cell apoptosis, and increases the intracellular levels of ROS [57, 118]. It can also improve multidrug resistance in thyroid cancer cells via inducing autophagy but inactivating AKT and S6 pathways [119].

6. Conclusions and Outlook

Although the problems of overdiagnosis and overtreatment now seem to be acknowledged, how to acquire survival benefit for PTC patients becomes to be a basic challenge [120]. Therefore, it is obviously important to explore new mild strategies to prevent and treat PTC. Phytochemicals have received much attention over the past three decades as potential sources of new candidates for cancer chemoprevention and treatment. Given the fact that many available anticancer drugs are derived from plant substances (e.g., taxol, vinblastine, homoharringtonine, β-elemene, indole-3-carbinol) as prototypes, this review focuses on herbal active ingredients with high potentials for the prevention and treatment of PTC. The benefits of these ingredients in PTC prevention and treatment have been well investigated, but their underlying mechanism and direct molecular targets remain unclear. Due to the latent toxicological, low bioavailability, foreseeable multidrug resistance, and deficient clinical trials, the extensive assessment of those untapped natural compounds on humanized immune system mouse models and achievable doses and drug delivery systems compatible for human studies still need to be further explored. Moreover, the development and progression of tumors are strongly associated with the physiological and pathological characteristics of the tumor microenvironment (TME) [121], in which hypoxia, chronic inflammation, oxidative stress, and acidosis contribute to cancer progression, including immune escape, angiogenesis, and metastasis. Given their multihydroxy structures, most phytochemicals with antioxidant and anti-inflammatory potentials can be preponderant on the improvement of TME profiles. According to the different features of cell subsets in TME, the patients could derive benefits from the intervention of multidrug combination. However, the effectiveness of herbal active ingredients on TME has been rarely reported. The application of those strategies might promote the clinical translation of these herbal active ingredients for PTC prevention and treatment.

Table 2: The structure-activity relationship of ginsenosides.

Basic structure	Compounds	R1	R2	R3	IC_{50} (μM)[a]
	Protopanaxadiol type (PPD)				
	Rb_1	-Glc^2-Glc	-H	-Glc^6-Glc	>200
	Rb_2	-Glc^2-Glc	-H	-Glc^6-Ara (p)	>200
	Rc	-Glc^2-Glc	-H	-Glc^6-Ara (f)	>200
	Rd	-Glc^2-Glc	-H	-Glc	>200
	Rg_3	-Glc^2-Glc	-H	-H: 20 (S)	50
	Rh_2	-Glc	-H	-H: 20 (S)	45
	CK	-Glc	-H	-Glc^6-Ara (p)	10
	PPD	-H	-H	-H	80
	Protopanaxatriol type (PPT)				
	Re	-Glc^2-Rha	-OH	-Glc	>200
	Rf	-Glc^2-Glc	-OH	-H	>200
	Rg_1	-Glc	-OH	-Glc	40
	Rg_2 (S)	-Glc^2-Rha	-OH	-H: 20 (S)	>200
	Rh_1	-Glc	-OH	-H	>200
	Rg_2 (R)	-Glc^2-Rha	-OH	-H: 20 (R)	>200
	PPT	-H	-OH	-H	70

a: inhibition of thyroid cancer cells (for 48 h).

Conflicts of Interest

The authors declare no conflicts of interests.

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Review Article

Phytochemicals as Potential Anticancer Drugs: Time to Ponder Nature's Bounty

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Medicinal plants have been used from the beginning of human civilization, which is mostly evident from the ancient script and traditional herbal medicine recipe. Despite the historically enriched demonstration about the use of plant as therapeutics, the pharmaceutical industries lack interest on phytochemical research compared with synthetic drug. Mostly, the absence of information about plant-based medicinal therapeutics is responsible to draw the attention of researchers to think about natural products as potential drug for detrimental diseases, such as cancer. This review will cover about clinically successful plant-based anticancer drugs and underappreciated, but potential, drugs to bridge the information gap between plant biologists and clinical researchers. Additionally, unprecedented advancement of synthetic chemistry, omics study to pin point the target genes/proteins, and efficient drug delivery system have made it easier for researchers to develop a phytochemical as an efficient anticancer drug.

1. Introduction

Undoubtedly, we are living in a time when cancer is epidemic and one of the medical challenges of this century. Statistically, it is a devastating number to deal. According to the Cancer Research UK, 17 million new cases had been reported, and among them, 9.6 million patients ended up to death in 2018. If it continues in such a rate, by 2040, there will be 27.5 million new cancer patient each year. At the same time, if we wonder, how far we came to cure cancer—forget about that—because our understanding about cancer is like looking at the White House from the international space station, where we certainly do not realize the color or the power of the stuff we are looking at. However, if we take a step back before meddling our mind with thousands of target genes, proteins, and possible or probable drugs, the fundamental idea of different types of cancer is "uncontrolled cell division" [1]. In short, cell division is a rudimentary process from the very beginning of the existence of life in the universe. Symmetric cell division leads to proliferation and asymmetric cell division is an informative step for differentiation. It has been reported that uncontrolled symmetric cell division is the major factor for causing cancer [2]. It is quite reasonable to aim anticancer drugs to control the cell cycle machineries [3]. In recent years, along with the effort of traditional anticancer drug discovery approaches, which is time-consuming and expensive, there is a search for anticancer drug from plant-derived bioactive compounds. Furthermore, drug discovery and development from plant-based compounds are also inexpensive compared with conventional synthetic compounds [4].

The plants are visibly an efficient provider of food and shelter, but the role of plants as a source of medicine is underappreciated. Human civilization is using plant as a source of food, shelter, and medicine for almost same time [5]. The contribution of plant as medicine was neglected due to the lack of precise biochemical and pharmacological mechanisms. Surprisingly, in nature, plants are continuously and extensively exposed to natural pollutants, carcinogens, and toxic metals [6–9] compared with human. Apart from the classic crown gall disease [10], the example of uncontrolled cell division in plant is insignificant compared with the animal system. At the same time, plants

synthesize divergent of secondary metabolites, mostly used for their defense and response to environmental cues, such as biotic and abiotic stresses [11–14]. In most of the cases, we have limited idea how plants tightly regulate their cell cycle machineries endogenously even after enormous exposure to hazardous components. Till date, several plant-derived compounds such as taxol [15], vinblastine [16], topotecan [17], and many more have been used as anticancer drugs successfully in clinical studies.

In this review, the existing successful plant-based anticancer drugs will be explored first and then the future direction of this emerging area and how the advancement of drug delivery system and cell type-specific production of anticancer drugs will uplift plant-based compounds as anticancer agents will be discussed.

2. Plant-Based Cancer Treatment

2.1. Looking Back to the Past to Find the Future. The most ancient text described the use of plant material as medicine was found in the Sumerian clay slab from Nagpur, India, and written approximately 5000 years ago. It contains information about using recent days' popular poppy, henbane, and mandrake as therapeutics [18]. Next, oldest evidence of medicinal plant was demonstrated in ancient Chinese literature written by Emperor Shen Nung circa 2500 BC [19, 20]. In a continuous historical effort, Theophrastus, known as "the father of botany," first established the botanical science, documented in his book "*De Causis Plantarum*," and classified several hundred medicinal plants [21, 22]. In addition, historically prominent Greek physician, pharmacologist, and botanist Pedanius Dioscorides wrote a 5-volume book, "*De Materia Medica*," on the medicinal use of plant [23]. His book and research were enormously successful, and he was hired by Roman army as a physician. The legacy of Roman Empire on medicinal plant study was further carried by Muslim scholars during the Islamic Golden Age. For instance, Islamic scholar Ibn Baitar described more than thousand medicinal plants in his book, "*Liber Magnae Collectionis Simplicum Alimentorum Et Medicamentorum*" [24]. The knowledge of medicinal plants from ancient literature and text came to light through Carl Linnaeus's classification system, described in his book "*Species Plantarum*" [25].

In the early 19th century, the advent of advanced synthetic chemistry helps us to decipher the mechanism, isolation and synthesis of active compounds from popular medicinal plants, such as poppy, ipecacuanha, strychnos, quinine, and pomegranate [26]. Despite the enriched history and success of medicinal plants, during the late 19th and early 20th centuries, the research on medicinal plant did not progress as it was supposed to. The reluctance of pharmaceutical industries about plant-based components caused a significant shift of focus from plant to synthetic chemistry on drug development [27].

Fortunately, the gear has been shifted in recent years. In 2015, the Nobel Prize in physiology and medicine was awarded to Tu Youyou for her discovery of artemisinin and dihydroartemisinin as antimalarial drug and highlight of the importance of plant-based components as a potentially powerful source of drug discovery. During the Vietnam War, Ho Chi Minh urged to develop antimalarial drugs for his soldiers. Tu Youyou became the part of that project to find the antimalarial treatment, and she screened over 2,000 traditional herbal medicines and discovered *Artemisia annua* recipe [28].

The success story of antimalarial drug based on traditional herbal medicine is not an isolated story; rather, it is a tiny part of plant-based arsenal as potential drugs. As a result, the significant effort on finding therapeutic agents for cancer treatment has been focused on plant-based compounds by the National Cancer Institute (NCI), USA. NCI-initiated Cancer Moonshot^{SM} project, aimed to accelerate the cancer research by making more cancer therapeutics available for patients, is focused on phytochemicals. As a part of this project, they have established a repertoire of natural products, and their purified chemical components to make them available for researchers to find new anticancer drugs.

Till date, several plant-based compounds have been reported for their anticancer activity, and among them, a good number of compounds is clinically successful as well. I have tried to summarize some important phytochemicals as potential anticancer drugs in Table 1 and Section 2.2.

2.2. Success Stories of Medicinal Plant-Based Anticancer Drug. Till date, more than thousand plants species have been identified with noteworthy anticancer potential [90, 91]. The isolation of the vinca alkaloids, vinblastine [92] from the Madagascar periwinkle, and *Catharanthus roseus* G. Don. (Apocynaceae) is one of the major examples of anticancer medication. This along with vincristine and other cancer chemotherapeutic drugs are used for the treatment of a range of cancers such as leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma [30, 91]. The discovery of paclitaxel (Taxol) [93] from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae), is another major success story in natural product drug discovery. Utilization of various parts of *Taxus brevifolia* from which paclitaxel was discovered and other *Taxus* species (e.g., *Taxus Canadensis* Marshall and *Taxus baccata* L.) by several Native American Tribes kindle the idea of indigenous knowledge-based medicinal plants [30, 91]. Another potent plant-acquired active compound, Homoharringtonine [94], was extracted from the Chinese tree *Cephalotaxus harringtonia* var. drupacea (Sieb and Zucc.) (Cephalotaxaceae) and has been used successfully for a long time in China in a racemic mixture with harringtonine for the treatment of acute myelogenous leukemia [30]. Elliptinium, a derivative of ellipticine, isolated from a Fijian medicinal plant *Bleekeria vitensis* A. C. Sm., is shipped to France for the treatment of breast cancer [30, 91]. These events represent only the surface of the success story of plant-based anticancer drug discovery with a promise to find more in the near future [91].

Table 1: Known phytochemicals, their source, and therapeutic use.

Phytochemical	Source	Therapeutic use	Reference
5-Fluorouracil	*Withania somnifera*	Human cervical cancer cell	[29]
Vindesine	*Catharanthus roseus*	Leukemias, testicular, breast and lung cancer	[30]
Vincristine	*Catharanthus roseus*	Lymphocytic leukemia	[30]
Vinblastine	*Catharanthus roseus*	Lymphocytic leukemia	[30]
Colchicine	*Colchicum autumnale*	Multiple solid tumors	[31]
Larotaxel	*Taxus baccata*	Breast, bladder, and pancreatic cancer	[32]
Cabazitaxel	*Taxus baccata*	Prostate cancer	[33]
Paclitaxel	*Taxus brevifolia*	Breast and ovarian cancer	[30]
Bullatacin	*Annona squamosa*	Liver cancer	[34]
Bryophyllin A	*Bryophyllum pinnatum*	Cervical cancer	[35]
Harmine	*Peganum harmala*	Breast cancer	[36]
Artemisinin	*Artemisia annua*	Liver, breast, and pancreatic cancer	[37]
Tannins	*Debregeasia saeneb*	Internal tumors	[38]
Theabrownin	*Camellia sinensis*	Lung cancer	[39]
Solamargine	*Solanum nigrum*	Breast, liver, lung, and skin cancer	[40]
Psoralidin	*Psoralea corylifolia*	Stomach and prostate cancer	[41]
Xanthatin	*Xanthium strumarium*	Lymphocytic leukemia and liver cancer	[42]
Thymoquinone	*Nigella sativa*	Colon, prostate, breast, and pancreas cancer	[43]
Kaempferol galactoside	*Bauhinia variegata*	Breast, lung, and liver cancer	[43]
Withaferin A, D	*Withania somnifera*	Breast, cervix, prostate, and colon cancer	[44]
Ginger	*Zingiber officinale*	Ovary, cervix, colon, liver, and urinary caner	[45]
Silibinin	*Sylibum marianum*	Lung, liver, skin, colon, and prostate cancer	[46]
Luteolin	*Capsicum annuum*	Colorectal cancer	[47]
Colchicine	*Colchicum autumnale*	Hodgkin's lymphoma, chronic granulocytic leukemia	[48]
Skimmianine	*Aegle marmelos*	Liver cancer	[49]
Boswellic acid	*Boswellia serrata*	Prostate cancer	[50]
Silymarin	*Sylibum marianum*	Colorectal cancer and colon cancer	[51]
Curcumin	*Curcuma longa*	Colon adenocarcinoma	[52]
Podophyllotoxin	*Podophyllum peltatum*	Non-small-cell lung carcinoma	[53]
Andrographolide	*Andrographis paniculata*	Colon cancer	[47]
Podophyllotoxin	*Podophyllum hexandrum*	Breast, ovary, lung, liver, bladder, and testis cancer	[54]
Betulinic acid	*Betula utilis*	Melanomas	[55]
Panaxadiol	*Panax ginseng*	Human colon cancer	[56]
Gossypol	*Gossypium hirsutum*	Colorectal cancer	[57]
Chrysin	*Passiflora caerulea*	Colorectal cancer	[58]
Plumbagin	*Plumbago zeylanica*	Liver, fibrosarcoma, leukemia, and breast cancer	[59]
6-Shogaol	*Zingiber officinale*	Ovary cancer	[60]
Curcumin	*Curcuma longa*	Breast, lung, colon, prostate esophagus, liver, and skin cancer	[61]
Ursolic acid	*Oldenlandia diffusa*	Lungs, ovary, uterus, stomach, liver, colon, rectum, and brain cancer	[62]
Isoliquiritigenin	*Glycyrrhiza uralensis*	Human lung cancer	[63]
Punarnavine	*Boerrhavia diffusa*	Malignant melanoma cancer	[64]
Procyanidins	*Vitis vinifera*	Human colon cancer	[65]
Resveratrol	*Polygonum cuspidatum*	Colorectal, skin, and liver cancer	[66]
Damnacanthal	*Morinda citrifolia*	Lung cancer, sarcomas	[67]
Gossypol	*Gossypium hirsutum*	Breast, stomach, liver, prostate, and bladder cancer	[68]
Niazinine A	*Moringa oliefera*	Blood cancer	[69]
Amooranin	*Amoora rohituka*	Lymphocytic leukemia	[70]
Betulinic acid	*Ziziphus rugosa*	Cytotoxicity against human melanoma cells	[71]
Asiatic acid	*Centella asiatica*	Melanoma, glioblastoma, breast cancer	[72]
Gallic acid	*Leea indica*	Ehrlich ascites carcinoma	[73]
Combretastatins	*Combretum caffrum*	Colon, leukemia, and lung cancer	[74]
Lycopene	*Solanum lycopersicum*	Prostate and colon cancer	[75]
Plumbagin	*Plumbago zeylanica*	Blood and skin cancer	[76]
Cannabinoid	*Cannabis sativa*	Lung, pancreas, breast, prostate, and colorectal cancer	[77]
Silymarin	*Sylibum marianum*	Colorectal cancer	[78]
Tylophorine	*Tylophora indica*	Breast cancer	[74]
Saffron	*Saffron crocus*	Liver, lung cancer and pancreatic cancer	[79]
nab-paclitaxel	*Taxus brevifolia*	Ovarian and breast cancer	[80]
Cyanidin	*Vitis vinifera*	Colon cancer	[81]
Actein	*Actaea racemosa*	Liver and breast cancer	[82]

Table 1: Continued.

Phytochemical	Source	Therapeutic use	Reference
Betulinic acid	*Betula Sp.*	Human melanoma xenografts and leukemia	[30]
Allin	*Allium sativum*	Carcinoma of human mammary gland	[83]
Neferine	*Nelumbo nucifera*	Liver cancer	[84]
Calcaelin	*Calvatia caelata*	Breast and spleen cancer cells	[85]
Lentinan	*Lentinus edodes*	Sarcoma-180 in mice	[86]
Schizophyllan	*Schizophyllum commune*	Head and neck cancer	[87]
Apigenin	*Matricaria chamomilla*	Colorectal cancer	[88]
Vitex	*Vitex agnus-castu*	Human uterine, ovarian, cervical, and breast cancer	[89]

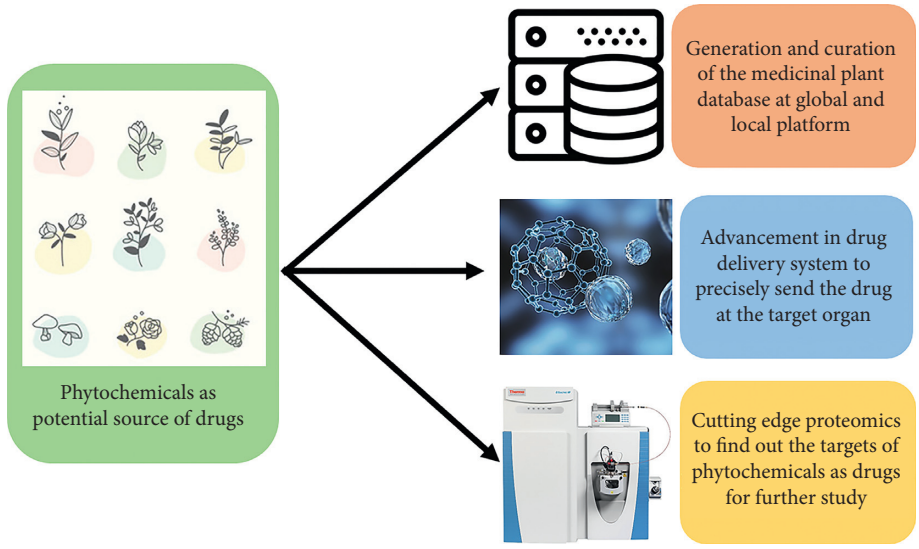

Figure 1: Phytochemicals can be used as drugs based on medicinal plant database, omics study to find the target, and efficient drug delivery system.

2.3. Toddler's Step Leads to the Unknown Horizon. The question is whether long-term ignorance of natural products for drug development can fit into the multibillion-dollar pharmaceutical industry? The answer certainly depends on multiple factors. However, the bright side is that the recent advancement of multiple medicinal plant database from local and global researchers, cutting edge omics technique to accelerate to find the drug targets and unprecedented improvement on drug delivery system (Figure 1).

Over the last decade, along with the effort of Moonshot[SM] project, local scientists from different parts of the work tried to curate the information of traditional herbal practice, preparation, recipe, and their ailments [12, 95–97]. Furthermore, to find out the target of potential phytochemicals is easier than ever, because of advancement of genomics, proteomics, transcriptomics, and metabolomics in recent years [91, 98–100]. The final frontier of using natural products as cancer treatment depends on the efficient drug delivery system. Fortunately, the advent of nanotechnology for drug delivery system has fast-forwarded this sector over the last few years [101, 102].

3. Conclusions

If we assume the research avenue of phytochemicals as potential cancer therapeutics as an image of a pyramid, this review has demonstrated a piece of stone from that pyramid. However, the idea of pushing natural products' research on drug discovery and development requires constant update and well-documented literature. This review paper will take the reader from the ancient history of herbal medicinal practice to the modern day's isolation, purification, identification, biosynthesis, in vitro or in vivo study, drug development, efficient delivery of drugs, and therapeutic trial.

Conflicts of Interest

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

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Research Article

Induction of Endoplasmic Reticulum Stress Pathway by Green Tea Epigallocatechin-3-Gallate (EGCG) in Colorectal Cancer Cells: Activation of PERK/p-eIF2α/ATF4 and IRE1α

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Epigallocatechin-3-gallate (EGCG) is the most abundant bioactive polyphenolic compound among the green tea constituents and has been identified as a potential anticancer agent in colorectal cancer (CRC) studies. This study was aimed to determine the mechanism of actions of EGCG when targeting the endoplasmic reticulum (ER) stress pathway in CRC. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed on HT-29 cell line and normal cell line (3T3) to determine the EGCG toxicity. Next, western blot was done to observe the expression of the related proteins for the ER stress pathway. The Caspase 3/7 assay was performed to determine the apoptosis induced by EGCG. The results demonstrated that EGCG treatment was toxic to the HT-29 cell line. EGCG induced ER stress in HT-29 by upregulating immunoglobulin-binding (BiP), PKR-like endoplasmic reticulum kinase (PERK), phosphorylation of eukaryotic initiation factor 2 alpha subunit (eIF2α), activating transcription 4 (ATF4), and inositol-requiring kinase 1 alpha (IRE1α). Apoptosis was induced in HT-29 cells after the EGCG treatment, as shown by the Caspase 3/7 activity. This study indicates that green tea EGCG has the potential to inhibit colorectal cancer cells through the induction of ER stress.

1. Introduction

Green tea has long been a part of human life, and its earliest consumption can be dated back from 500,000 years ago. Asian ancestors, particularly from China, Japan, India, and Thailand, had been using green tea for healing purposes [1]. There are studies showing the antiproliferative, antimutagenic, antioxidant, antibacterial, antiviral, anticancer, and chemopreventive effects of green tea [2, 3]. From previous studies, it is found that epigallocatechin-3-gallate (EGCG) (Figure 1) is the most bioactive and abundant polyphenolic compound in green tea [3, 4].

EGCG has always achieved a milestone in the quest for cancer therapy. It has been observed to suppress breast cancer [5–7], prostate cancer [8–10], lung cancer [11–13], pancreatic cancer [14–16], and liver cancer [17, 18]. All of this anticancer activity by EGCG has previously been demonstrated that EGCG is the most effective cancer chemopreventive polyphenol in green tea [19]. Rady et al. reviewed that all of these anticancer effects by EGCG work by apoptosis induction, control in cell proliferation, and/or inhibition of angiogenesis [20]. These mechanisms have previously been suggested by Min and Kwon, and they also added inhibition in metastasis; tumorigenesis is also an important mechanism adapted by EGCG to unveil their anticancer properties [21].

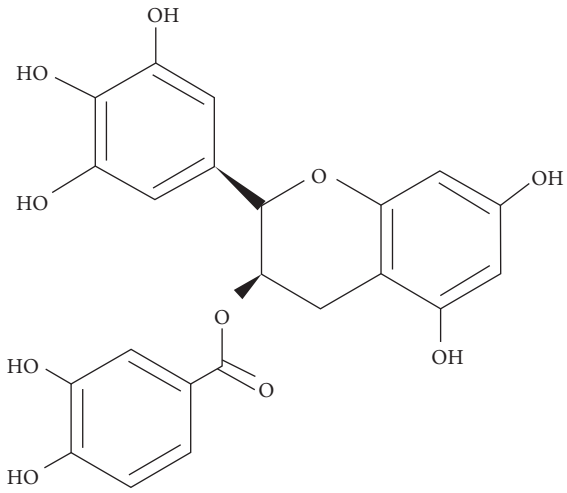


Figure 1: Chemical structure of EGCG compound (ChemSketch Software, Advanced Chemistry Development Labs).

The therapeutic effects of EGCG are indeed unexceptional in colorectal cancer studies too. It has been shown to work in several mechanisms: inhibition of stem cells of colorectal cancer (CRC) via suppression of the Wnt/β-catenin pathway [22], impeding CRC sphere formation [23], inhibition of CRC proliferation [24], inhibition of VEGF signaling [25], and degradation of proteins [26]. The possible molecular mechanisms of EGCG in CRC involve various molecules and signaling pathways [27].

Targeting endoplasmic reticulum (ER) stress and its rescue system and unfolded protein response (UPR) is a promising field for cancer treatments. The UPR system is mediated by three key mediators, namely, pancreatic ER kinase- (PKR-) like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6), where all are located in the membranes of ER [28]. In a resting mode, these three transmembrane proteins bind to the main chaperone, immunoglobulin-binding protein (BiP) [29]. Following an ER stress, these proteins will dissociate from BiP and activate their respective downstream cascades system [30]. The upregulation of UPR sensor proteins is often regarded as the indicator of the incidence of ER stress in cancer [31]. Prolonged ER stress may cause dysfunctional UPR, which leads cells to enter the death mode [32].

The increase in fatal rates due to colorectal cancer (CRC) is now affecting the world's population. Despite numerous studies had successfully demonstrated EGCG as an anticancer agent, none of these studies showed a clear understanding of the role of EGCG in the ER stress pathway, particularly in colorectal cancer. This study was primarily performed to introduce EGCG in combating CRC and as a safer alternative to the chemotherapy that is continuously posing side effects to the CRC patients. Hence, this study was done in order to elucidate the mechanism of EGCG actions in colorectal cancer cells via ER stress pathways.

2. Materials and Methods

2.1. Cell Culture. Cancer cell line used throughout this study was the human colorectal adenocarcinoma cell line (HT-29). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, 1X high glucose—Life Technologies, CA, USA) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin-streptomycin antibiotics. The cells were maintained in an incubator with the setting of 5% CO_2/95%O_2 at 37°C. Meanwhile, normal cell line used was Embryonic Fibroblast Cell Line (designated as 3T3), also originated from ATCC. These cells were grown in similar supplementation as HT-29.

2.2. Cytotoxicity Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay has been performed to assess cell proliferation activity and cytotoxicity in HT-29 cells and 3T3 cells, when treated with different concentrations of EGCG extracted from green tea (Cat. #: E4143, Sigma, Missouri USA). Firstly, cells were seeded on 96-well plates overnight. After achieving confluency around 70%–80%, the HT-29 cells were treated with EGCG from 0 to 1000 (0, 125, 250, 500, and 1000) μM for 24 h, 48 h, and 72 h incubation periods; meanwhile, 3T3 cells were treated with EGCG for 72 h incubation period.

2.3. Cell Treatments for Protein Expression Analysis. HT-29 cells were first seeded on 30 mm tissue culture treated Petri dish at 8×10^5 overnight. The cells were then treated with EGCG for 24 h, 48 h, and 72 h incubation period. Protein harvesting was performed once each treatment period ended.

2.4. Protein Extraction. RIPA lysis buffer (Merck, MO, USA) containing 1% of protease inhibitor EDTA-free (Merck, MO, USA) and 1% of phosphatase inhibitor (Merck, MO, USA) was added to each 30 mm Petri dish containing the cells. The cells were then incubated on ice for about 5 minutes. After that, the cells were gently scraped using a cell scraper and were collected into a 2 mL microtube. The cells were further incubated on ice for 15 minutes. After that, it was centrifuged (10,000 rpm) at 4°C for 10 minutes. The supernatant was then collected. The quantification of proteins was immediately performed by using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, MA, USA).

2.5. Western Blot. The protein samples were separated by SDS-PAGE at 110 V for 50 minutes. Transfer of samples from gel to the PVDF membrane was done by wet transfer with a transfer buffer (1X), loaded with ice packs, at 60 V for 2 hours. After 2 hours, membranes were dried for an hour at room temperature (RT) before being blocked with 5% BSA in TBS-T buffer for 1 hour and was gently rocked on a shaker at RT. Incubation with the primary antibody was performed in TBS-T + 5% BSA overnight in 4°C chiller with a dilution of 1 : 1000. The next day, each membrane was washed three times with TBS-T for 5 minutes. The HRP-conjugated secondary antibody was diluted at 1 : 2500 in TBS-T + 5% BSA, and then the blots were incubated for 1 h at RT and washed with TBS-T [33, 34].

Antibodies used throughout this study include BiP (Cat. #: 3177 Cell Signaling Technology), PERK (Cat. #: 5683),

p-eIF2α (Cat. #: 9721), ATF4 (Cat. #: 11815), and IRE1α (Cat. #: 3294), which were all purchased from Cell Signaling Technology, MA, USA, and GAPDH (Cat. #: SC25778 Santa Cruz Biotechnology, TX, USA) and anti-rabbit IgG HRP-linked (Cat. #: 7074 Cell Signaling Technology, MA, USA).

2.6. Protein Detection. After incubation with the secondary antibody, the membrane was washed three times. Next, Luminata™ Forte Western HRP substrate was used for protein detection. The substrate was added onto the membrane and was let to incubate at room temperature for 2 minutes. Later, the membrane was viewed in a gel doc. The band's intensity was quantified using Image Studio Lite Software, Version 5.2. All the bands were normalized with the loading control GAPDH.

2.7. Caspase 3/7 Assay. EGCG treatments on the cells for the Caspase 3/7 assay (Promega, WI, USA) were based on the concentrations of IC_{50} values, respectively, for each treatment period. A volume of 100 μL Caspase 3/7 reagent was added to each well containing the cells. After this addition, the plate was put on a shaker at 300–500 rpm for at least 30 seconds. When the mixture was well blended, the plate was incubated at RT for 1 hour. The samples were finally measured with luminescence setting. The gain adjustment was performed prior to the luminescence reading.

2.8. Statistical Analysis. The protein expression and Caspase 3/7 results were analyzed using GraphPad software (San Diego, CA, USA). The independent *t*-test was applied to compare the differences in the mean between treated samples and control samples. Differences were only considered as statistically significant when $P < 0.05$.

3. Results

3.1. EGCG Inhibited Colorectal Cancer Cell Growth. As shown in Figure 2, the number of viable cells was reduced after 24 h with the increasing concentration of EGCG. A similar observation was seen following 48 and 72 h of treatment. The viability of the cells was also affected by the duration of EGCG exposure. Thus, the toxicity of EGCG depends on the dose and duration of exposure. This clearly showed the toxicity of the EGCG towards the colorectal cancer cells in a dose-dependent manner.

The EGCG treatment does not only reduce the percentage of HT-29 viable cells by a dose-dependent manner but also works in a time-dependent manner. The comparison of the trends amongst all the incubation time concluded that the longest hour of incubation; 72 h always caused the lowest percentage of viable cells as compared to shorter treatment periods (Figure 2). The inhibitory concentrations of EGCG at 50% of HT-29 population (IC_{50}) were 262.5 μM, 190.3 μM, and 88.1 μM for 24 h, 48 h, and 72 h, respectively.

Since the study has demonstrated the toxicity of EGCG at inhibiting the growth of colorectal cancer cell lines, its toxicity has also been tested on the normal cell line, 3T3 (Figure 3). This embryonic fibroblast cell line (3T3) has shown that the EGCG was not toxic to normal healthy cells, given the treatment at any concentration even at the highest concentration of EGCG (1000 μM).

3.2. EGCG Dissociated Chaperone Protein, BiP from UPR Protein Complexes. There was no significant increment of BiP expression ($P > 0.05$) following EGCG treatment after 24 and 48 h of incubation toward the HT-29 cell line (Figure 4). However, after 72 h of incubation, the expression of BiP was significantly increased ($P < 0.001$) when compared to the respective control. Hence, this indicated the occurrence of ER stress in colorectal cancer cells treated with EGCG as the expression of BiP is associated with ER stress activation.

3.3. EGCG Increased PERK Expression and Its Downstream Molecules p-eIF2α and ATF4. This study demonstrated that EGCG treatment on the HT-29 cell line has activated PERK and its downstream molecules p-eIF2α and ATF4. PERK expression was significantly increased ($P < 0.01$) after a 48 h incubation in EGCG-treated cells when compared to the respective control (Figure 4). However, the PERK expression was significantly decreased ($P < 0.05$) after 72 h which showed the transient expression of PERK in HT-29 cells, as shown in Figure 4.

PERK's downstream target, the phosphorylated eIF2α (p-eIF2α) expression, was significantly increased ($P < 0.01$) after EGCG treatment at 24 h of incubation when compared to the respective control (Figure 4). In addition, another downstream molecule of PERK, ATF4 expression, was significantly increased only after 48 h ($P < 0.01$) and 72 h ($P < 0.01$) of incubations with EGCG (Figure 4).

Overall, these results demonstrated that all the expressions of PERK and its downstream molecules p-eIF2α, and ATF4 were upregulated by EGCG. PERK induction is required for the activation of downstream UPR molecules. The activation of these molecules indicates the occurrence of ER stress in colorectal cancer cell lines, HT-29.

3.4. EGCG Upregulated IRE1α in HT-29 Cell Lines. In this study, treatment with EGCG on colorectal cancer cells (HT-29) has caused upregulation of IRE1α expressions at all incubation times: 24 h, 48 h, and 72 h (Figure 4). The IRE1α expressions were significantly increased at 24 h ($P < 0.01$), 48 h ($P < 0.01$), and 72 h ($P < 0.05$) when compared to their respective controls (Figure 4). The elevation of IRE1α expression was associated with the occurrence of ER stress.

3.5. EGCG Increased Caspase 3/7 Activity in HT-29 Cell Lines. As shown in Figure 5, treatment with EGCG for 24 h on HT-29 had significantly increased ($P < 0.001$) Caspase 3/7 activity when compared to the control. In addition, after 48 h and 72 h of incubations, the Caspase 3/7 activity was also markedly increased (Figure 5). Activated Caspase 3/7 promotes apoptosis and inhibits the normal physiological function of cancer cells. Therefore, the EGCG activated ER-stress protein and thereby induced apoptosis.

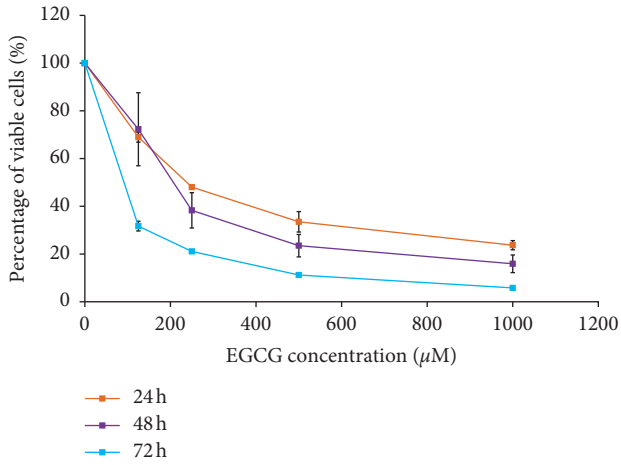


Figure 2: Percentage of HT-29 viable cells. EGCG treatments were given to HT-29 cells at 24 h, 48 h, and 72 h incubation times. The MTT assay was then performed at each incubation time. The results are expressed as mean percentage ± standard error of the mean (SEM) ($n = 3$).

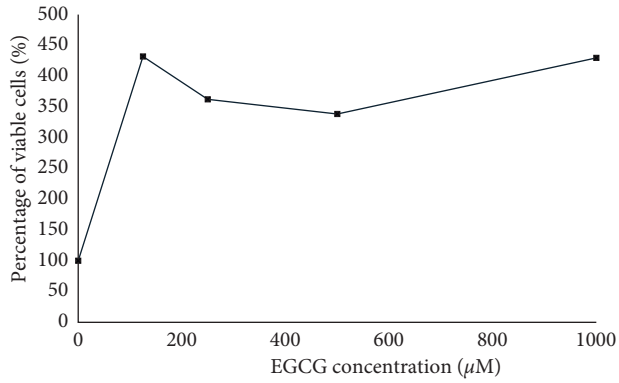
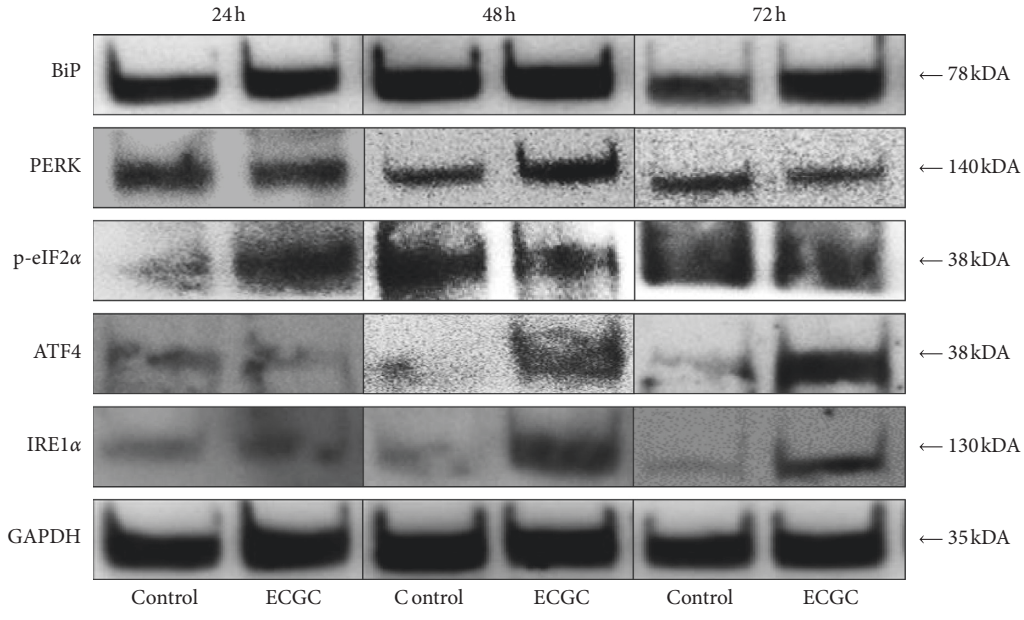
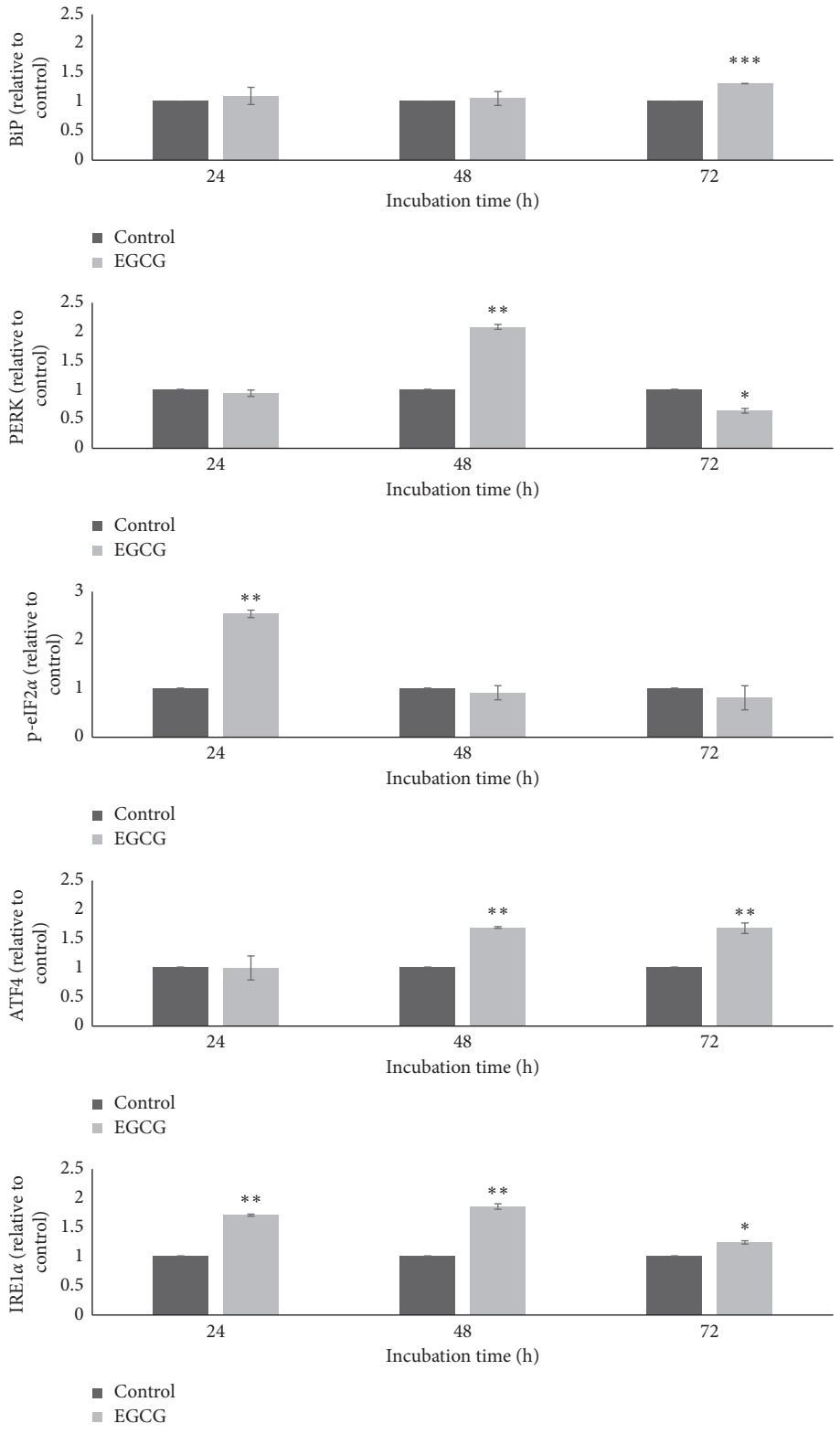


Figure 3: Percentage of viable cells (normal cells 3T3), given EGCG treatment at 72 h incubation time. The results are expressed as percentage ± SEM. The experiments were performed three times independently ($n = 3$).



(a)

Figure 4: Continued.



(b)

Figure 4: Expression of BiP, PERK, p-eIF2α, ATF4, and IRE1α after EGCG treatment. GAPDH was used as the loading control. The densitometry results are from three independent experiments and are expressed as mean ± SEM ($n = 3$) normalized to GAPDH, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, relative to their respective controls at each incubation time.

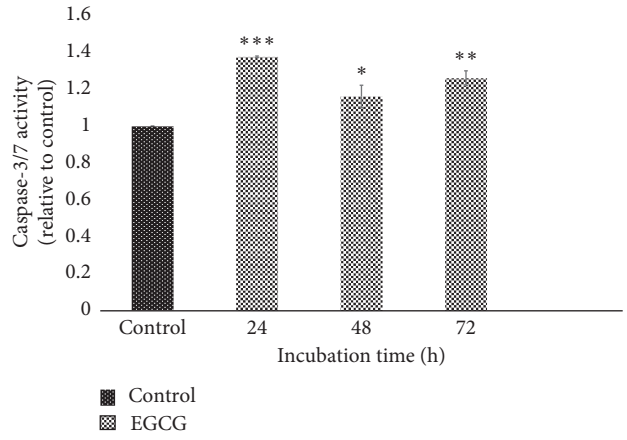


Figure 5: Caspase 3/7 activities at 24 h ($IC_{50} = 262.5\,\mu M$), 48 h ($IC_{50} = 190.3\,\mu M$), and 72 h ($IC_{50} = 88.1\,\mu M$) of incubation times. EGCG concentrations used are based on respective IC_{50} for different incubation times. Caspase 3/7 activity at different times was measured in relative to the control sample. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

4. Discussion

The exploration of EGCG as an anticancer has a wide spectrum of research areas ranging from the molecular level to clinical trials. Hence, the cytotoxicity of EGCG is the first area that needs to be tackled before going further into the focused research area [35]. This study has demonstrated the toxic effect of EGCG on colorectal cancer cells similar to previous studies and does not have cytotoxic effects on normal cells.

In the prospect of colorectal cancer treatment, a number of researches have shown that EGCG absolutely does have cytotoxic effects on the colorectal cancer cells either at growth inhibition or as the cancer chemopreventive mechanism [19, 23, 24, 36]. Mechanism of chemoprevention works by preventing the establishment of cancer cells in the human body [19]. The main purpose of this study is to provide elucidation on the mechanism of actions of green tea EGCG in colorectal cancer (CRC), particularly via the endoplasmic reticulum (ER) stress pathway.

The occurrence of ER stress requires the cells to adapt to the survival mode or failure to maintain this survival which will lead the cells to undergo apoptosis [37]. The pathways activated throughout survival to cell death modes are classified under the unfolded protein response (UPR) mechanism. Due to extravagated intrinsic or extrinsic factors, these UPR pathways become markers for the severity of the ER stress [38].

Exploiting ER stress as part of cancer therapy seems a promising strategy; especially pharmacological agents are used as ER stress inducers [39]. A prolonged period of ER stress will cause cytotoxicity to the cells and hence this leads to apoptosis [40]. From this study, it was revealed that the treatment of EGCG on colorectal cancer cell lines, HT-29, had induced the expressions of UPR-related proteins which are BiP, PERK and its downstream targets (p-eIF2α and ATF4), and IRE1α (Figure 4).

Furthermore, the upregulation of BiP expressions indicates that the first step of the UPR mechanism had indeed occurred which was the dissociation of BiP from the UPR main axes. This dissociation also means that the three UPR arm proteins are expected to be activated as well [41]. This study also demonstrated the induction of ER stress by EGCG that caused marked BiP upregulation. Several pharmacological drug treatments had shown enhanced expressions of BiP which indicated the response of ER stress [38]. These studies are indeed in line with our findings in terms of BiP activation upon ER stress induction.

We had also demonstrated the activation of PERK as well as its downstream targets, p-eIF2α and ATF4, and IRE1α protein-induced ER stress after EGCG treatment. PERK and IRE1α have similar mechanisms of activation due to their homologous secondary structure, and both of these proteins are also dependent on the BiP association [42, 43]. Furthermore, it is anticipated that the mechanism of ER stress sensors is specifically adapted to favor the particular needs of the organisms [44]. Hence, it can be concluded that the mechanism of EGCG actions as the ER stress inducer in colorectal cancer cells had followed this homolog structural theory.

From this study, we also demonstrated that EGCG treatment had induced apoptosis of HT-29 cells via Caspase 3/7 activity. This study revealed that EGCG has the potential to induce robust Caspase 3/7 activity. Recently, the Caspase 3 activity induced by EGCG treatments was also observed in bladder cancer cells SW780 [45] and chondrosarcoma cells [46], as well as in diabetic mice [47], indicating that EGCG plays a role in apoptosis via Caspase 3 activity. In general, polyphenols such as EGCG induced apoptosis in a caspase-dependent manner [48–50]. However, EGCG has also shown its apoptotic activities via caspase-independent pathways [51, 52]. Various findings including our study confirmed that EGCG has apoptotic properties in cancer via both caspase-dependent and caspase-independent mechanisms. This study highlighted caspase 3/7-dependent apoptosis by EGCG in colorectal cancer cells, and to validate this finding, further studies such as caspase 3 and 7 genes knockout study should be carried out in the future. Though this study primarily showed the caspase-dependent pathway upon ER stress induction by EGCG, caspase-independent assays should also be investigated to explore the other potentials of EGCG as an ER stress inducer in colorectal cancer cells.

As mentioned previously, prolonged or chronic ER stress and UPR activation will lead the cells to enter death mode. Based on our findings, due to the ER stress induced by EGCG, HT-29 cells had entered apoptosis mode via Caspase 3/7 activity. Furthermore, key players of cell death resulted from ER stress are dependent on UPR sensors as well. For instance, activation of PERK/p-eIF2α/ATF4 will initiate proapoptotic signaling pathways [53, 54]. This study demonstrated that EGCG treatment on HT-29 cells has activated PERK and its downstream molecules (p-eIF2α and ATF4) and IRE1α which can trigger ER stress-induced apoptosis. Collectively, it can be concluded that EGCG has induced ER stress in CRC which eventually leads to apoptosis. Figure 6 briefly illustrates the mechanism of action of EGCG as an ER stress inducer in CRC.

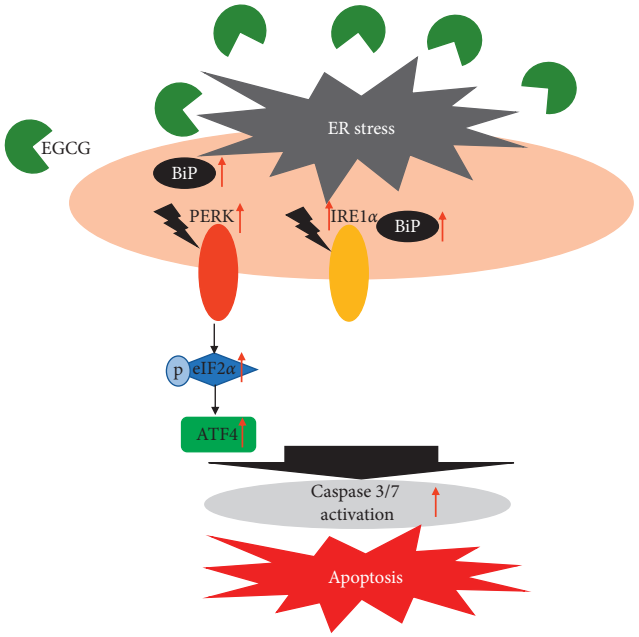


Figure 6: Mechanism of action of EGCG in colorectal cancer (CRC). ER stress is induced by EGCG and activates UPR proteins, PERK (with its downstream targets eIF2α and ATF4), and IRE1α. Caspase 3/7 activity is also enhanced, indicating apoptosis occurrence in the colorectal cancer cells.

5. Conclusions

From this study, the mechanism of actions of green tea EGCG in colorectal cancer was elucidated via the induction of the ER stress particularly through PERK/p-eIF2α/ATF4 and IRE1α pathways, which eventually lead to apoptosis. In addition, this present study has discovered the potential of EGCG in targeted colorectal cancer therapy. The breakthrough from this side will surely help in combating the rising of CRC incidences globally.

Abbreviations

EGCG: Epigallocatechin-3-gallate
DFO: Desferrioxamine
CRC: Colorectal cancer
ER: Endoplasmic reticulum
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
RT: Room temperature
BiP: Immunoglobulin-binding protein
PERK: PKR-like endoplasmic reticulum kinase
p-eIF2α: Phosphorylation of eukaryotic initiation factor 2 alpha subunit
ATF4: Activating transcription 4
IRE1α: Inositol-requiring kinase 1 alpha
UPR: Unfolded protein response
VEGF: Vascular endothelial growth factor
DMEM: Dulbecco's modified Eagle medium
RIPA: Radioimmunoprecipitation assay
EDTA: Ethylenediaminetetraacetic acid
BCA: Bicinchoninic acid
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS-T: Tris-buffered saline Tween
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Review Article

The Application of Nanotechnology in the Codelivery of Active Constituents of Plants and Chemotherapeutics for Overcoming Physiological Barriers during Antitumor Treatment

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Antitumor therapy using a combination of drugs has shown increased clinical efficacy. Active constituents derived from plants can offer several advantages, such as high efficiacy, low toxicity, extensive effects, and multiple targets. At present, the combination of plants' active constituents and chemotherapeutic drugs has attracted increased attention. Nanodrug delivery systems (NDDSs) have been widely used in tumor-targeted therapy because of their efficacy of delivering antitumor drugs. The *in vivo* process of tumor-targeted NDDSs has several steps. They include blood circulation, tumor accumulation and penetration, target cell internalization and uptake, and drug release and drug response. In each step, NDDSs encounter multiple barriers that prevent their effective delivery to target sites. Studies have been performed to find alternative strategies to overcome these barriers. We reviewed the recent progress of codelivery of active constituents of plants and chemotherapeutics using NDDSs. Progress into transversing the physiological barriers for more effective *in vivo* antitumor delivery will be discussed in this review.

1. Introduction

Cancer is one of the most deadly diseases that endangers human health. Chemotherapy is currently the major treatment strategy for treating cancers and preventing postsurgical recurrence. However, multidrug resistance (MDR) in tumor cells and serious adverse effects have hindered chemotherapy [1]. To address these issues, studies have been performed to investigate the effects of drug combinations for cancer treatment. The combination of active constituents of plants with first-line chemotherapy drugs has shown good efficacy in reversing tumor chemoresistance, enhancing curative effects, and reducing adverse reactions. Combination treatment of active constituents of plants with chemotherapy drugs for tumor therapy has recently become very popular [2–4]. However, direct administration of free drugs has several disadvantages, such as short duration in blood circulation and nonselectivity for tumor tissue and tumor cells. This reduces efficacy while increasing adverse reactions due to nonspecific targeting of healthy tissue. To solve this problem, several strategies have been developed. Nanodrug delivery systems (NDDSs) have demonstrated potential advantages for cancer therapy. The most common carriers of NDDSs include liposomes, nanoparticles, micelles, and polymers. They can effectively increase the duration of drugs in systemic circulation, improve pharmacokinetics, and promote drug tumor targeting and tumor accumulation. All these substantially increase the curative effects while reducing toxicity [5, 6]. Intravenous administration of NDDSs results in a series of complex *in*

vivo delivery processes, which includes blood circulation, tumor targeting, tumor accumulation, tumor tissue penetration, tumor cell internalization, and intracellular transport. Several specific drug delivery barriers exist, with each directly affecting efficacy. In order to improve drug efficacy and reduce adverse reactions of NDDSs, researchers have developed several exceptional delivery strategies to overcome these barriers. In this review, the physiological basis of designing tumor-targeted drug delivery systems to overcome these physiological barriers will be discussed.

2. Tumor Pathophysiology

The pathophysiological features of the tumor are the basis for designing tumor-targeting drug delivery systems [7]. One of the important physiological features of tumor tissues is their enhanced permeability and retention effect (EPR effect) to nanoparticles. Tumors that reach greater than $2\,mm^3$ are highly dependent on nutrients and oxygen that are supplied by tumor blood vessels. Tumor and lymph angiogenesis start to develop when tumor blood vessels are unable to meet the requirements of the rapidly growing tumor [8]. Blood vessels that have recently formed through neovascularization have enhanced permeability, lack a smooth muscle layer, and has dysfunctional angiotensin receptors. In addition, lymph vessels in the center of tumor tissues are usually dysfunctional, which results in lymphatic obstruction and retention of macromolecular substances like lipid particles. The high selective permeability and retention in tumor tissues are termed the EPR effect [9]. The EPR effect is the basis for designing passive tumor targeting NDDSs [10].

Additionally, unlike normal cells, tumor cells grow in an uncontrolled and invasive manner. In order to infinitely proliferate, tumor cells have increased expression of certain receptors. These include the folate receptor (FR) [11], integrin receptor, transferrin receptor (TfR), somatostatin receptor, vasoactive intestinal peptide receptor, and cholecystokinin receptor. In addition, several specific receptors are expressed on the surface of tumor blood vessels, such as vascular endothelial growth factor (VEGF) receptor [12], integrin $\alpha v\beta 3$ [13], and E-Selectin [14]. Many of these receptors that are overexpressed are common in tumor tissue and tumor blood vessels. The active targeting mechanism of NDDS relies on these specific receptors to bind specifically to tumors. However, long-term administration of antitumor drugs induces P-glycoprotein (P-gp) overexpression in tumor cells. P-gp functions to expel antitumor drugs from tumor cells, thus reducing the intracellular drug concentration, which in-turn reduces antitumor efficacy and makes tumor cells resistant to chemotherapy. This process is termed MDR [15]. MDR has been identified in almost all human tumor cells.

Cancer stem cells (CSCs) in tumor tissues [16] have the ability to self-renew, multiply, and differentiate. They can also stimulate the growth of new tumors [17]. Even though they exist in limited numbers, CSCs play a significant role in the development, progression, metastasis, and recurrence of tumors. Conventional chemotherapy or radiotherapy induces tumor cell death to reduce tumor cell numbers and prevent the rapid growth of tumors [18]. However, CSCs are not sensitive to conventional chemotherapy or radiotherapy and are not completely eliminated [19, 20]. CSCs are one of the main reasons for tumor recurrence.

Tumor cells have a very high metabolic rate and produce high levels of acid leading to an acidic environment in tumor tissues. The extracellular pH in tumor tissues is approximately 6.0–6.5. In addition, the reduced blood supply to the central area of the tumor leads to local hypoxia to increase the acidic environment [21]. Tumor tissues also have several physiological features that include high interstitial fluid pressure, specific enzymes, and oxidative stress [22].

3. Types of Tumor-Targeted NDDSs for Plant Chemotherapeutic Drugs

Currently, the most commonly used NDDSs include liposomes, nanoparticles, polymeric micelle, and products of polymer-drug conjugates. The major structural features and drug-carrying mechanisms of these NDDSs are listed in Table 1.

3.1. Liposome. Liposomes are lipid nanovesicles formed by lipid bilayers [23–25]. The diameter of a liposome is approximately 90–200 nm. The center of the liposome consists of a hydrophilic internal aqueous phase. The internal aqueous phase and lipid bilayer of the liposome could be used to carry a variety of cargos. For instance, hydrophilic drugs could be packed into the internal aqueous phase, while hydrophobic drugs could be packed into the lipid membrane. Additionally, amphiprotic drugs could be packed in the aqueous phase and phospholipid membrane. Furthermore, antibodies and polypeptides could be used to modify the surface of liposomes to make them to target various organs or tumors [23]. Liposomes continuously release their loaded drugs slowly and hence have the ability to change the distribution and pharmacokinetic properties of the drugs, thereby reducing their toxic effects [24]. Long circulating liposomes will add significant benefit for long-term drug delivery but needs to be further optimized.

In general, the surface of liposomes is modified by hydrophilic macromolecules, such as polyethylene glycol (PEG), to reduce their recognition by opsonin in the blood and to reduce phagocytosis by the reticuloendothelial system (RES) which then increases the drug duration in blood circulation [25, 26]. There is a difference in water solubility between antitumor active constituents of plants and chemotherapeutic agents. This makes liposomes the preferred carrier for *in vivo* delivery of such drugs. To date, numerous studies have used liposomes as nanocarriers for combined antitumor drug therapy using active constituents of plants and chemotherapeutic agents. Hu et al. [27] developed a liposome using distearoylsn-glycero-3-phosphoethanolamine-*N*-(methoxy(polyethylene glycol)-2000) (DSPE-PEG 2000), which cocarried temozolomide (TMZ) and quercetin (QUE) for the treatment of drug-resistant U87 glioma cells. Transmission electron microscopy demonstrated that

Table 1: Types and characteristics of nanodrug delivery systems.

Type	Structure	Drug loading	Advantages	Limitations
Liposomes	Lipid bilayer	Physical entrapment	Great biocompatibility, no immunogenicity	Low stability, hydrophilic drug easily leaks out
Nanoparticles	Nanosphere-/nanocapsule-/ polymer-based nanoparticles with a lipophilic core	Physical encapsulation/ chemical bonds	High drug-loading capability	—
Polymeric micelles	Core-shell structure formed by self-assembly	Physical packing/ chemical bonding	Easy to prepare, increased stability of hydrophobic drugs	Low stability, depolymerizes after dilution
Polymer-drug conjugates	Conjugation of drugs with biodegradable polymers	Chemical bond	Increased drug solubility, high drug-loading capability	Hydrolyzed easily

nanoliposomes loaded with TMZ and QUE had reduced diameters. *In vitro* studies demonstrated that this liposome could favor cellular uptake of drugs and thus effectively reduce the drug dose without reducing efficacy.

3.2. Nanoparticles. Nanoparticles are colloidal particles made from natural or synthetic high molecular polymers as carriers. The drugs are attached to the carrier material by physical entrapment, absorption, or chemical covalent binding. The natural high molecular polymers mainly include heparin [28], chitosan [29], gelatin [30], and albumin [31], while synthetic high molecular polymers are mainly polylactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA), and polycaprolactone (PCL). Nanoparticles can be easily modified to increase their targeting capability. Compared with liposomes, nanoparticles have several advantages, such as better physical stability and higher drug-loading capability. In addition, they are easy to prepare, have certain degrees of sustained release function, and are suitable for packing insoluble drugs. Currently, nanoparticles have been used as carriers for targeting and controlled drug release and are administered by intravenous injection or topical administration. Xu et al. [32] developed a PLGA nanoparticle for the codelivery of docetaxel and gambogic acid. They identified the best ratio of docetaxel with gambogic acid, which was then packed into PLGA nanoparticles. Cell apoptosis and immunoblotting demonstrated that this codelivery nanoparticle could downregulate the expression of P-gp to increase cell apoptosis and thus effectively inhibit MDR of tumor cells.

3.3. Polymeric Micelles. Polymeric micelles are flexible spherical particles that are synthesized from the self-assembly of two amphiphilic block copolymers at the appropriate temperature and concentration. Polymers could be AB-type diblock copolymers or ABA-type triblock copolymers. Micelles formed using the triblock copolymer are more stable. Polymeric micelles have a hydrophobic core that is used to pack drugs that have poor water solubility or hydrophobic drugs. This increases the stability of the packed drugs and prevents their rapid degradation [33]. The hydrophilic outer shell forms a protective barrier to prevent the binding of micelles with plasma proteins. This prevents phagocytosis of the micelle by RES and thereby increases the half-life of the drug [34].

Yao et al. [35] designed a micelle that could codeliver paclitaxel (PTX) and curcumin (CUR) and demonstrated that it had a superior drug-loading capacity of up to 35% (w/w) and was able to synergistically induce anticancer effects. Sarisozen et al. [36] developed a mixed micelle using TF-polyethylene glycol polyethylene (TF-PEG-PE) that actively targeted TF to improve tumor targeting. Fang et al. [37] developed a magnetic micelle for the codelivery of doxorubicin and CUR. This codelivery system targeted lactoferrin and prolonged retention of the drugs at the tumor sites to efficiently suppress cancer growth compared with delivery of either drug alone.

3.4. Polymer-Drug Conjugates. In contrast to other drug-delivery systems, polymer-drug conjugates are drugs conjugated to a polymer via covalent bonding. This drug-delivery system has several advantages: (1) it increases the water solubility of hydrophobic drugs; (2) the covalent bonds could be modified (such as having a pH-sensitive linker, enzyme sensitive linker, or light sensitive linker). This enables the release of drugs to different sites [38]; (3) conjugation of drugs with polymers could increase their half-life; and (4) the increase in molecular weight could prevent EPR effect, thus favoring drug accumulation in solid tumors. Hence, these conjugates could overcome the differences in water solubility, selectivity, and stability between the antitumor active constituents of plants and chemotherapeutic drugs. The most commonly used polymers include polysaccharides, PEG, poly amino acids (PAA), and polypeptides [39].

Xue et al. [40] constructed a self-assembled prodrug nanoparticle that was conjugated to cabazitaxel and citronellol via a disulfide bond. This nanoparticle was redox-sensitive to high concentrations of glutathione (GSH) within tumor cells. Zhang et al. [41] designed a PEG-doxorubicin (DOX)-CUR prodrug nanoparticle for codelivery of DOX and CUR. Schiff-base reaction was used to conjugate DOX to PEG and then was conjugated to CUR in the nanoparticle to obtain PEG-DOX-CUR nanoparticles (NPs). The nanoparticle was acid-sensitive and hence could release DOX and CUR when it reached the tumor.

4. Strategies to Overcome the Physiological Barriers of NDDSs

To effectively deliver drugs to target tissues and sites, NDDSs need to overcome a number of physiological and physical barriers. These include the blood, tissue, and cellular and intracellular transportation barriers. Each barrier could directly affect the final efficacy of the antitumor drugs. In order to overcome these barriers, researchers have developed a series of strategies and methods (see Figure 1 and Table 2).

4.1. Blood Circulation. After intravenous injection, NDDSs enter the blood circulation and encounter a number of obstacles: (1) degradation of the nanoparticles or drugs by the various enzymes in the plasma [95]; (2) opsonization of the nanoparticles and subsequent phagocytosis and clearing by RES [96, 97]; and (3) absorption of the nanoparticles by plasma proteins leading to the aggregation and subsequent hematotoxicity, retention in the pulmonary capillaries, or phagocytosis by RES [98]. To overcome these barriers, NDDS needs to avoid interactions with charged proteins in the blood or avoid phagocytosis. To overcome the limitations of NDDS in blood circulation, numerous studies have focused on modifying the surface of these nanoparticles. PEG coated to the surface of nanoparticles increases the half-life of nanoparticles [99]. It works by inhibiting the formation of the hydrophilic shells around NDDSs [100], which prevents plasma proteins from interacting with the nanoparticle and hence prevents RES. Yu et al. [42] developed a PEG-modified long-circulating liposome that concurrently packed QUE (p-gp inhibitor) and Adriamycin (AMD/DOX). This increased the blood concentration of AMD and half-life of AMD in the plasma. PEG-modified nanoparticles, PEG-modified grafted polymers, PEG-modified polymeric micelles, and PEG-modified dendrimers all have been shown to increase the half-life of drugs in blood circulation [43–47, 101].

However, several studies also observed that continuous injection of PEG-modified nanoparticles could induce immune responses and thus lead to accelerated blood clearance (ABC) [102, 103]. Hence, the design of drug-delivery systems with biological characteristics to evade the immune system has garnered increased attention [104]. Several studies have demonstrated that nanoparticles coated with endogenous substances like red blood cell membranes (RBC-NP) could avoid phagocytosis by macrophages via immunomodulatory proteins (such as CD47) on their surface and hence extend their half-lives [105].

4.2. Tumor Accumulation and Penetration. The low efficiency of drug accumulation in tumor tissues is one of the hurdles in antitumor NDDS therapy. NDDS accumulation in tumors is mainly via EPR. However, the dense extracellular matrix and the extremely high interstitial fluid pressure in tumors significantly prevent drugs from entering the deep tissues of the tumor. Because of this, the majority of the NDDS are distributed in blood vessels around the tumors. This results in lower drug distribution into the actual tumor, reduced efficacy, and hence an incomplete elimination and subsequent recurrence [27, 106]. Several studies have demonstrated that nanodrugs with smaller diameters have increased efficacy because they can penetrate easier and deeper into tumor tissues [33, 51].

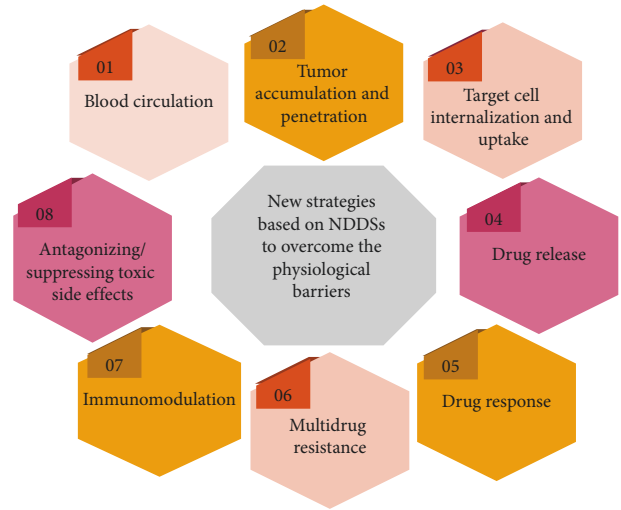

Figure 1: Development of new strategies based on NPs technology for drug delivery to overcome the transport barriers.

Numerous studies have demonstrated that several specific receptors are expressed on the surface of tumor blood vessels compared with normal blood vessels. These include endothelial cell surface-specific receptors integrinαv $\beta 3$ [107] and nucleolin [108]. NDDSs that are modified on the surface to express ligands to these receptors could target both tumor blood vessels and tumor cells. Targeting of tumor blood vessels could increase the retention of the NDDSs near the tumor, which eventually increases the distribution in tumor tissues [93]. For example, RGD peptide is a short peptide containing arginine, glycine, and aspartic acid (Arg-Gly-Asp) and is the ligand for integrin $\alpha v\beta 3$. Jiang et al. [48] developed an RGD-modified PTX and CUR coloaded liposomes and demonstrated better antitumor efficacy compared with unmodified liposomes. As a P-gp inhibitor, CUR could inhibit multidrug resistance, while the combined application of CUR and PTX had a synergistic effect. These RGD-modified nanoparticles have shown high aggregation and deep permeability at tumor sites [48–50]. Hence, targeting common receptors on tumor blood vessels and tumor cells is an effective strategy to improve the accumulation and penetration of nanoparticles into tumor tissues [52, 62, 109].

4.3. Target Cell Internalization and Uptake. After penetrating deep into tumor tissue, NDDS must be internalized by the tumor cells to exert into antitumor effects. Codelivery of active constituents of plants and chemotherapy drugs could promote NDDSs internalization by receptor-mediated endocytosis. In addition, P-gp inhibitors could be used to overcome the drug resistance in tumor cells [110–113].

Table 2: The strategies of overcoming the transport barriers of NDDSs to codeliver two different drugs.

Delivery barriers	Physiological basis	Strategies	Feature	Nanocarrier type	Nanocarrier composition	Drug	Ref
(1) Blood circulation	(1) The mononuclear phagocyte system (MPS)	(1) Hydrophilic polymer nanoparticles	PEG-modified	Liposome	DSPE-PEG 2000	Adriamycin + quercetin	[42]
			PEG-modified	Micelles	mPEG-PCL	Doxorubicin + curcumin	[43]
			PEG-modified	Nanoparticles	PEG-b-PLL	Doxorubicin + triptolide	[44]
	(2) Electrostatic interaction	(2) RBC cloak nanoparticles	PEG-MSN	Mesoporous silica nanoparticle (MSN)	PEG-MSN	Paclitaxel + curcumin	[45]
			PEG-modified	Lipid-coated polymeric nanoparticle	PEG2k-DSPE/PLGA	Doxorubicin + curcumin	[46]
			PEG-MNPs	Magnetic nanocomposite	Fe3O4/HAPA/β-CD	Doxorubicin + curcumin	[47]
(2) Drug accumulation and penetrtion	(1) ECM	(1) EPR effect	RGD peptide-targeted	Liposome	DSPE-PEG 2000	Paclitaxel + curcumin	[48]
	(2) Vascular endothelial barrier	(2) Vascular targeting for accumulation	RGD peptide-targeted	Mesoporous silica nanoparticle	PAA-chitosan	Topotecan + quercetin	[49]
			RGD peptide-targeted	Lipid-coated nanoparticles	DSPE-PEG-NHS, PLGA	Sorafenib + quercetin	[50]
	(3) Thick stroma		EPR	Liposome	SPC : Chol : DOPE/EPG	Doxorubicin + biochanin A	[51]
	(4) Proteolytic enzymes in the tumor	(3) Antivascular targeting by inhibiting tumor angiogenesis	EPR	Lipid-polymer hybrid nanoparticles (Lpns)	DOPE/EPG	Cisplatin + curcumin	[33]
			Smaller and compacted scale nanoparticles	Self-assembled nanoparticle	PEG-VES	Sorafenib + curcumin	[52]
(3) Drug internalization into the targeted cells	(1) Electric interaction needed to stride over the cell membrane to enter inside the cells	(1) P-gp inhibitors combining nanoparticles	P-gp inhibitor (curcumin)	Amphiphilic polymeric micelle	PEG(2k)-PLA(5k)	Doxorubicin + curcumin	[53]
			P-gp inhibitor (tetrandrine)	Lipid polymer hybrid nanosystem	MAL-PEG-DSPE, PLGA	Paclitaxel + Tetrandrine	[54]
		(2) Receptor-targeted nanocarriers	Transferrin-targeted, pH-sensitive	Polymer-drug conjugate	Tf-PEG-CUR	Doxorubicin + curcumin	[55]
			FA-targeted	Planetary ball-milled (Pbm) nanoparticles	FA-PCL-PEG	Docetaxel + resveratrol	[56]
			FA-targeted	Lipid nanoparticles	GMS-TPGS-SA-FA	Paclitaxel + curcumin	[57]
			GLUT1-targeted	Polymeric micelles	PEG2000–DSPE	Doxorubicin + curcumin	[58]
			Transferrin-targeted	Polymeric micelles	PEG-PE	Paclitaxel + curcumin	[36]
(4) Drug release	(1) Acidic environment and specific enzymes present in CSCs in the tumor	(1) PH-sensitive nanoparticles	pH-sensitive	Micellar nanoparticles	DSPE-PEG-imine-MTX	Methotrexate + curcumin	[59]
			pH-sensitive	Nanoparticles	PEG-lipid/PAA/CaCO3	Doxorubicin + curcumin	[60]
		(2) Enzyme-sensitive nanparticles	pH-sensitive, CSCs-targeted	Micellar system	PPBV	Paclitaxel + curcumin	[61]
			pH-sensitive	Nanoparticle	TPGS-PAE	Doxorubicin + curcumin	[62]
			pH-sensitive	Copolymer	PCL-St-POX	Terminator + curcumin	[63]
			Pe-targeted, EPR	Peptosome	PePm/PS	Pe + curcumin	[64]
		(3) Temperature-responsive	Thermosensitive copolymer	Nanogels	Heparin-pluronic F127 (Hep-F127)	Cisplatin + curcumin	[65]
			Near-infrared (NIR)-responsive	Gold nanocages	Biotin-PEG-SH	Doxorubicin + quercetin	[66]

Table 2: Continued.

Delivery barriers	Physiological basis	Strategies	Feature	Nanocarrier type	Nanocarrier composition	Drug	Ref
(5) Drug response	(1) Based on summarized pathophysiological basis	(1) Directly target and kill CSCs	pH-sensitive, CSCs-targeted	Polymeric nanoparticle	mPEG-PLGA-pglu	Doxorubicin + curcumin	[67]
			pH-sensitive, CSCs-targeted	Core-shell nanoparticle	VES-g-e-PLL/γ-PGA-Dopa	Doxorubicin + curcumin	[68]
				Nanoliposomes	mPEG2000-DSPE, DOPA	Cisplatin + curcumin	[69]
				Phytosome	Quercetin and phospholipid (lecithin)	Doxorubicin + quercetin	[70]
				Lipid nanoparticles	Glyceryl distearate,triglycerides medium-chain, soybean lecithin/polyoxyl 40 hydrogenated castor oil, glycerin	Doxorubicin + curcumin	[71]
				Polymeric micelles	PCL-b-ABPA-b-POEGMEA	Platinum drugs + curcumin	[72]
				Nanoparticle	mPEG-PCL	Temozolomide + resveratrol	[73]
				Liposomal	Egg sphingomyelin/ cholesterol/PEG2000 ceramide	Quercetin + vincristine	[74]
		(2) Synergistic combination of two or more drugs	Combination of chemotherapeutic and plants extracts	Polymeric micelles	PGS2000/PEG2000-DSPE	Doxorubicin + curcumin	[75]
				Lipid nanoparticles	PEG-DSPE	Etoposide + curcumin	[76]
				Lipid-polymer hybrid nanoparticles (Lpns)	DSPE-mPEG$_{5000}$/DSPE-PEG$_{5000}$ FITC	Paclitaxel + triptolide	[77]
				Liposomes	Egg phosphatidylcholine/ DSPE-PEG	Doxorubicin + resveratrol	[78]
				Nanoemulsion (NE)	PEG$_{400}$-DOCA, HP-beta-CD	Pemetrexed + quercetin	[79]
				Bottlebrush copolymer-Based micelle	PEG-PNB-TC	Paclitaxel + curcumin	[35]
				Lipid-polymeric nanocarriers	PLGA, PEG$_{2000}$-DSPE	Vincristine + quercetin	[80]
				Niosomes	Tween-60: cholesterol:DPPC : DOTAP : DSPE-PEG2000	Doxorubicin, quercetin + sirna	[81]
				Polymer-lipid nanoparticles	DSPE-PEG2000, POPC,DOPAC	Paclitaxel + curcumin	[82]
		(3) Multifunctional targeted delivery	pH-responsive, CRGDK-targeted, EPR	Nanoparticles	CRGDK-PEG-PCL	Doxorubicin + curcumin	[83]
			Magnetic-guided targeting, T7-mediated targeting	Nanoparticles	PLGA-PEG-T7	Paclitaxel + curcumin	[84]
			Lactoferrin- (Lf-) tethered magnetic-targeted	Magnetic micelle	PVA/PAA	Doxorubicin + curcumin	[37]
			pH-responsive, Folate receptor-targeted	Nanoplates	PEG methacrylate, PEG	Daunorubicin + curcumin	[85]
			EGFR peptide (GE11)-targeted, pH-sensitive, EPR	Prodrug nps	PLGA-PEG-Mal, PLGA-PEG-NH2, PEG-NH2	Docetaxel + curcumin	[86]
			Magnetic targeting, biotin receptors-targeted	Magnetic nanoparticles	Biotin-PEG-PCDA	Paclitaxel + curcumin	[87]

Table 2: Continued.

Delivery barriers	Physiological basis	Strategies	Feature	Nanocarrier type	Nanocarrier composition	Drug	Ref
(6) Mutidrug resistance (MDR)	(1) Based on summarized pathophysiological basis	(1) Reverse transporter-mediated MDR (Inhibition of P-p, LRP, MRPs,BCRP)	PEGylation	Prodrug nanopaticle	PEG-curcumin	Docetaxel + curcumin	[88]
			PAMAM dendrimer	Copolymer nanopaticle	PEG-PAMAM	Paclitaxel + Borneol	[89]
			PEGylated	Liposome	DSPE-mPEG2000, PC	Paclitaxel + resveratrol	[90]
		2) Reverse apoptosis gene-mediated MDR	EPR	PLGA-lipid nanoparticles	DSPE-PEG2000, PLGA	Docetaxel + gambogic acid	[32]
			Anisamide- (AA-) targeted	Nanoparticles	PLGA, CHO-hyd-PEG-AA	Doxorubicin + resveratrol	[91]
(7) Immunoregulation	(1) Oxidative and enzymatic environment	(1) Anti-inflammatory effects	CD44-targeted	Nanohydrogel	FA-HA	Rapamycin + quercetin	[92]
			PEGylated	Long-circulating liposomes	DPPC, PEG-2000-DSPE	Docetaxel + curcumin	[93]
(8) Antagonize/reduce toxicity and side effects	(1) Reactive oxygen species (ROS) environment	1) ROS-sensitive nanoparticles	ROS-cavenger: curcumin	Polymeric micelles	mPEG-PCL	Docetaxel + curcumin	[94]

DSPE-PEG: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol); PLGA: poly(lactic-co-glycolic acid); mPEG-PCL: methoxy poly(ethylene glycol)-poly caprolactone; PEG-b-PLL: poly(ethylene glycol)-b-poly(L-lysine); HAPA: hydroxyapatite; β-CD: β-cyclodextrin; PEIIPDI-PEA: branched polyethylenimine-isophorone diisocyanate-poly(L-lactide)-PEI; PAA: polyacrylic acid; DSPE-PEG-NHS: distearoyl-L-a-phosphatidylethanolamine-polyethylene glycol-N-hydroxysuccinimide; SPC: soy phosphatidylcholine, Chol: cholesterol, EPG: egg phosphatidylglycerol, and DOPE: 1,2-dioleoyl-sn-glycero-3 phosphoethanolamine; PEG-VES: polyethylene glycol derivative of vitamin E succinate; MAL: maleimide; Tf: transferrin; FA: folate; GMS-TPGS-SA-FA: glyceryl monostearate-D-alpha tocopherol acid polyethylene glycol succinate-stearic acid and folate; PEG2000–PE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; PPBV: poly(ethylene glycol)-benzoic imine-poly(gamma-benzyl-l-aspartate)-b-poly(1-vinylimidazole) block copolymer; TPGS-PAE: d-alpha-tocopheryl polyethylene glycol 1000-block-poly(beta-amino ester); PCL-St-POX: poly caprolactone-starch-poly(2-ethyl 2-oxazoline); PePm: TFIIATVEGVLLFLILVVVVGILIKRRGPLGVRGC, PS: peptosomes; mPEG-PLGA-PGlu: monomethoxy (polyethylene glycol)-b-P (D,L-lactic-coglycolic acid)-b-P (L-glutamic acid); VES-g-e-PLL: RRR-a-tocopheryl succinate-grafted-e-polylysine conjugate; **γ**-PGA-Dopa: poly-**γ**-glutamic acid-dopamine; DOPA: 1,2-dioleoyl-sn-glycerol-3-phosphate; PABPA: 3-((tert-Butoxycarbonyl) amino)propyl acrylate; POEGMEA: polymerisation of oligo(ethyleneglycol)methyl ether acrylate; DSPE-PEG5000-FITC: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[(polyethylene glycol)-5000]-fluorescein isothiocyanate; DOCA: deoxycholic acid, HP-beta-CD: 2-hydroxypropyl-beta-cyclodextrin; PEG-PNB-TC: polyethylene glycol-polynorbornene-thiocresol; DPPC: 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine phospholipid, DOTAP: 1, 2-dioleoyl-3-trimethylammonium-propane; POPC: 1-palmitoyl-2oleoyl-sn-glycero-3-phosphocholine; CRGDK: Cys-Arg-Gly-Asp-Lys; T7: sequence HAIYPRH.

Specific receptors that are highly expressed in tumors and on the surface of tumor endothelial cells include transferrin receptor, folate (FA) receptor, integrin receptor, somatostatin receptor, lectin receptor, and epidermal growth factor receptor (EGFR). NDDSs modified with different ligands or antibodies which is so-called active targeting NDDSs have shown better tumor-targeting and drug-delivery capability both *in vivo* and *in vitro*. TF and FA are very commonly used malignant tumor-targeting ligands, as most tumor cells express high levels of TfR or FR on their surface, while the expression of TfR and FR in normal tissues are much lower [114–116]. Cui et al. [55] used transferrin-modified nanoparticles for the codelivery of DOX and CUR. They first synthesized a pH-sensitive Tf-PEG-CUR prodrug and then packed DOX into the Tf-PEG-CUR NPs to obtain the Tf-PEG-CUR/DOX nanoparticle. This nanoparticle had active tumor-targeting features and could release their payload into tumor sites specifically because of its pH sensitivity in tumor tissue. Singh et al. [56] conjugated planetary ball-milled (PBM) nanoparticles and loaded it with resveratrol and DTXto FA for the treatment of prostate cancer. They found that Fa-modified DTX nanoparticles had increased cytotoxicity and hence could reduce the concentration of free drug by 28 folds. In addition, when DTX-resistant prostate cancer (PCa) cells were treated using this nanoparticle, there was a reversal of multidrug resistance in these cancer cells [57]. In addition, anti-GLUT1 antibody (GLUT1) has been used to modify polymeric micelles coloaded with CUR and doxorubicin and have shown increased efficacy in human colorectal cancer cell HCT-166 both *in vitro* and *in vivo* [58].

P-gp is a highly expressed multidrug transporter that could lead to MDR and chemotherapy failure in a number of cancers [15]. Numerous studies have demonstrated that the active constituents of plants, such as CUR, QUE, and triptolide, could inhibit the expression of P-gp gene and protein [117–121]. Thus, codelivery of such drugs with chemotherapeutic drugs could reverse MDR, increase chemotherapy sensitivity, and decrease adverse effects [53, 54]. Compared with chitosan-conjugated PLGA nanoparticles [122, 123], chitosan and anti-P-gp antibody-conjugated PLGA nanoparticles have demonstrated enhanced cell internalization. Hence, NDDS-targeting P-gp has provided an effective method to overcome drug resistance and increase drug internalization.

4.4. Drug Release. Drug release is the last step in drug delivery. Only released drugs from these nanoparticles can exert antitumor effects. Studies have taken advantage of the differences between the tumor microenvironment and the normal environment to figure out ways to release drugs from these nanoparticles. This has mainly been via dissociation of chemical bonds or structures that are sensitive to the tumor microenvironment [124, 125]. There are three common strategies for designing NDDS to promote drug release, i.e., pH-sensitive NDDSs, enzyme-sensitive NDDSs, and temperature-sensitive NDDSs.

The pH in the extracellular tumor tissues (~pH 6.5) and endosomes/lysosomes (pH 4.5–6.0) are lower compared with normal tissues and blood (pH 7.4). This difference in pH could be used to design pH-responsive nanocarriers [126]. Acetal, hydrazine, imine, and esters are unstable at low pH and could be used to construct pH-responsive NDDSs. Xie et al. [59] utilized Schiff-base reaction to link methotrexate(MTX) with (DSPE-PEG2000) using a pH-sensitive imine linkage to obtain a pH-sensitive prodrug, i.e., DSPE-PEG-Imine-MTX. This prodrug could self-assemble to micellar nanoparticles (MTX-Imine-M) in aqueous solutions and could pack CUR into its core via hydrophobic interaction to form MTX-Imine-M-CUR nanoparticles. The active form of MTX is released more efficiently when the pH is 5.0 compared with 7.4. These nanoparticles are more efficacious and have lower toxicity profiles. In addition, pH-sensitive inorganic substances and polymers have been used to create nanoparticles to release drugs into tumor cells. pH-responsive nanocarriers, such as liposomes, nanoparticles, nanogels, polymer-drug conjugates, and micelle, have been designed and reported to have good efficacy and function [127]. Inorganic substances like calcium phosphate, chitosan, closed mesoporous silica nanoparticle pores (MSNPs), and poly(styrene-co-*N*,*N*′-dimethylaminoethyl methacrylate nanoparticles (P(St-co-DMAEMA))complexes and mPEG-b-PMaIPG (methoxy-polyethylene glycols (PEG)-b-poly (d-galactopyranose)) nanoparticles have been demonstrated to be sensitive to relatively small pH changes and have good drug release and relatively high antitumor activity [44, 49, 60, 62, 63]. Yang et al. [61] synthesized the pH-sensitive polymer (poly(ethylene glycol)-benzoic imine-poly(gamma-benzyl-l-aspartate)-b-poly(1-vinylimidazole) block copolymer (PPBV) and developed a multistage pH-responsive micelle system for the codelivery of PTX and CUR for the treatment of breast cancer CSCs. This multistage pH-responsive micelle system could intelligently convert the charges on the surface from neutralities to cations, reduce its diameter size to favor long-term blood circulation into tumor blood vessels, and promote tumor cell uptake and tumor permeability. All this enhanced the treatment efficacy of the nanoparticle for combination therapy using PTX and CUR.

Enzyme-responsive NDDS utilizes the overexpression of various enzymes in tumors to develop NDDS that contain substrates that could be specifically degraded by such enzymes [128]. Various proteases, such as matrix metalloproteases (MMPs) [129, 130] in tumor tissues and cathepsin in lysosomes of tumor cells [131, 132], are overexpressed in tumors. Li et al. [64] developed a protein NDDS that could intelligently respond in the tumor microenvironment. Based on the microenvironment, i.e., enzymes on the tumor surface (MMPs), pH, and high tumor GSH concentrations, the nanoparticle can respond and efficiently target tumors and also reduce metastatic rates.

Temperature-sensitive NDDS regulates drug release based on changes in temperature. Due to severe inflammatory reactions, the internal temperature of most solid tumors is generally higher compared with surrounding normal tissues. Temperature-sensitive drug-delivery systems

could enhance the release of drugs in tumors, i.e., after adding the active tumor targeting feature, the temperature-sensitive targeting NDDS could be constructed [133]. Nguyen et al. [65] synthesized a heat-responsive Hep-F127 polymer for the codelivery of cisplatin (CDDP) and nano curcumin complex/pack to form a dual drug-delivery system. This nanoparticle had antiproliferative effects and tumor inhibition on MCF-7 cells and xenograft transplantation models. Zhang et al. [66] developed a near-infrared-responsive gold nanocagesusing biotin PEG thiol (biotin-PEG-SH) to codeliver doxorubicin and QUE for the treatment of breast cancer. This system had the feature of rapid drug release upon radiation of near-infrared rays and high cytotoxicity for MCF-7/ADR cells.

4.5. Drug Response

4.5.1. Synergistic Antitumor Effects. Using a single chemotherapeutic drug for antitumor treatment has several limitations, such as inducing drug resistance, high toxicity, and low therapeutic index [75, 134]. Drug combinations for the treatment of cancers have become more favorable. Active constituents of plants have multiantitumor effects. They can synergistically inhibit tumor-cell proliferation by enhancing tumor-cell apoptosis, induce cell autophagy, enhance oxidative stress, improve the sensitivity, and increase cell cycle arrest when used in conjunction with chemotherapy drugs [69, 134–138]. Investigators have developed novel combinatory approaches using cationic PEGylatedniosome-encapsulated nanoparticles. These have demonstrated synergistic effects in gastric, prostate, and breast cancer cells [81]. Delivering nanoparticles (functional polymeric micelles, polyamidoamine dendritic polymers, and copolymers) that carry active constituents of plants and chemotherapeutic drugs is more efficacious compared with monotherapy [33, 35, 69–80, 82, 106].

4.5.2. CSC-Targeting System. Although CSCs account for only a small portion of tumor cells, they have the capacity to self-renew, differentiate, and maintain tumor growth [139, 140]. CSCs can induce recurrence, metastasis, and resistance to antitumor drugs [141], which subsequently leads to chemotherapy failure. As the "drug pumps," ABCG2 is highly expressed in CSCs. They pump drugs out of cells to prevent tumor killing. CSCs are considered the key to eradicating tumors. Signaling pathways and specific markers in CSCs could be the ideal targets for CSC-targeting NDDSs [142]. The active constituents of plants, such as CUR, can inhibit several signaling pathways, such as the Wnt/β-catenin, Notch, and Hedgehog pathways [143] and thus effectively inhibit the self-renewal of CSCs. Combining CSC-targeting therapy with conventional chemotherapy drugs could result in synergistic antitumor activity [61, 68]. For instance, pH-sensitive nanoparticles coloaded with CUR and DOX (CURDOX-NPs) prepared using monomethoxy (polyethylene glycol)-b-P (D, L-lactic-co-glycolic acid)-b-P (L-glutamic acid) polymer (mPEG-PLGA-PGlu) have shown better breast cancer-inhibitory effects compared with monotherapy [67].

4.5.3. Multifunctional Targeting Drug Delivery Systems. To further increase the targeting of NDDS to tumor tissues, several multifunctional targeting drug delivery systems using different modifications to overcome multiple barriers simultaneously have been developed [86, 87]. Nanoplatform for combinational therapy using PEG, PCEC (poly("-caprolactone)-b-poly(ethylene glycol)-b-poly("-caprolactone)) and CRGDK (cell-penetrating peptide (Cys-Arg-Gly-Asp-Lys, CRGDK)) has been developed. These can coload DOX/CUR, target the tumor, and respond to intracellular acidic environments. The synergistic antitumor effects of these nanoparticles have the following four aspects: (1) increased stability in blood circulation; (2) passive targeting due to EPR; (3) active targeting by recognition of CRGDK to neuropilin-1 receptor; and (4) high stability and low drug leakage under physiological pH, while the acidic environment dissociates the prodrugs to release DOX and CUR into the cells [83]. Based on the expression of Rf in the blood-brain barrier and glioma cells, human TfR ligand T7 (sequence: HAIYPRH)-modified magnetic PLGA nanoparticle (MNP/T7PLGA NPs) target tumors and release PTX and CUR. This system provides a dual-targeting strategy, i.e., ligand-mediated targeting and magnetic-guided targeting [37, 84]. Saha et al. [85] used the high-temperature solvothermal technique to manufacture $Eu:Gd_2O_3$ triangular nanoplates. Using this method, nanoparticles are conjugated on its surface to FA, which is the targeting ligand and is then loaded with daunorubicin and CUR via ester bonds. The acidity in tumor tissues induces esterolysis to release the chemotherapeutic drugs into the tumor.

To enhance the antitumor effects of NDDSs, combination drug administration, CSCs-targeting drug delivery system, and multifunctional targeting drug delivery systems have been widely used to achieve additive or synergistic antitumor effects. This approach is a new promising method for efficient tumor targeting.

4.6. Multidrug Resistance. MDR occurs when tumor cells are resistant to one or a series of chemotherapeutic drugs with different structures and mechanisms. MDR is an important reason for chemotherapy failure in clinical practice [144, 145]. The mechanisms of MDR are very complex and include inherent cellular or changes in tumor microenvironments. The complexity of the mechanisms involving MDR brings about challenges to overcome tumor drug resistance [146, 147]. One of the advantages of using nanocarriers to codeliver chemotherapy drugs and active constituents of plants is their capability to reverse MDR [32, 91, 148](seen Figure 2). Rejinold et al. [88] investigated using CUR as the nanocarrier to load PEG-doxorubicin hydrochloride for HCT-8/DOX-resistant cells to increase the *in vivo* and *in vitro* antitumor efficacy. *In vitro* anti-MDR experiments have shown that PEG CRC/DOX NPs had a higher antimetastatic and antiproliferative effect on MDR cancer cells while normal fibroblasts were unaffected. In addition, PEG CRC/DOX NPs have longer blood circulation times compared with CRC NPs. Previous studies have used NDDS coloaded with PTX and active constituents of plants

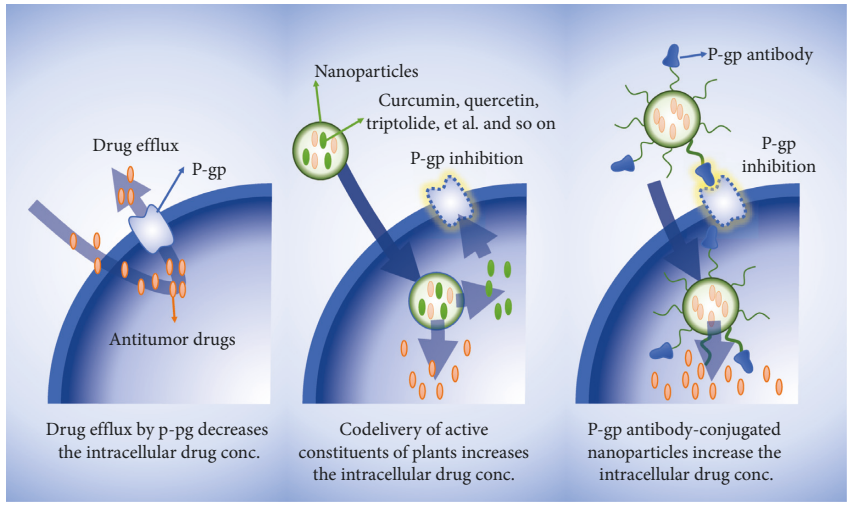

Figure 2: NDDS-mediated P-gp inhibition is an effective method to reverse MDR and increase drug internalization.

(such as baicalin and borneol) for anti-MDR. These *in vitro* experiments have shown that such combinations enhanced the concentration of PTX in MCF-7/Tax and A2780/PTX cells, as well as increased cellular drug and cytotoxic effects [89, 90].

4.7. Immunomodulation. The body's immune system has a significant influence on tumor development and progression. The tumor can modulate the immunocompetence of the body to recognize and kill tumor cells [149]. Quagliariello et al. [92] demonstrated that coadministration of rapamycin and QUE could reduce the levels of IL-8, IL-6, and IL-19, suggesting that such combinations could modulate the body's immune system and thus enhance the tumor-killing capability. In addition, such combinations could downregulate VEGF, MMP2, and MMP9 levels, suggesting they could inhibit tumor metastasis. Sesarman et al. [93] developed a long term circulating liposome that could pack CUR and PTX (LCL-CURC-DOX). This liposome could significantly increase the cytokine rations of IL-12/IL-4, IL12/IL-1α, IL-12/IL-1β, IFN-γ/IL-6, IFN-γ/IL-1α, and IFN-γ/IL-1β by 1.18–3.14-fold, ($P < 0.05$), thus favoring the balance of Th1 and Th2 cells to stimulate antitumor effects in the tumor microenvironment.

4.8. Antagonizing/Supressing Toxic Side Effects. Chemotherapy drugs damage normal tissues and cells in the body. Using plants (crude drugs) in combination with chemotherapy drugs could affect tumor tissues via multiple targets and pathways thereby reducing the dose of chemotherapy drugs and hence decrease drug toxicity. In addition, some plants (crude drugs) could also suppress the toxic side effects of chemotherapy. Hence, the combined application of such plants could increase the safety of chemotherapy. Guo et al. [150] combined andrographolide, the active constituent of the plant *Andrographis panicula*, with bleomycin and found that it not only enhanced the antitumor effects but also reduced the toxic effects of bleomycin on the body. In addition, the combined application of andrographolide and bleomycin effectively reduced pulmonary fibrosis induced by bleomycin, which was manifested by the activation of superoxide dismutase, and inhibition of malondialdehyde and hydroxyproline. Such combinations also suppressed cytokine expression. Zhang et al. [94] developed a complex polymeric micelle system that copacked Adriamycin and CUR (CPMDC) and investigated their protective effects on Adriamycin-induced cardiac toxicity. Pharmacokinetics and tissue distribution showed that CPMDC increased DOX accumulation in tumors but decreased the levels of the toxic metabolite doxorubicin in heart tissue compared with DOX alone.

5. Future Prospects

With the continuous advancements in the understanding of the mechanisms of tumor development and progression, strategies using combination drug therapy have demonstrated significant advantages for cancer treatment. The development of nanotechnology further provides broad application prospects. In this review, we summarized novel strategies and methods in developing NDDSs for codelivery of active constituents of plants and chemotherapy drugs to overcome barriers to drug delivery. NDDSs must circumvent all these barriers before successful and effective antitumor activity is observed. Any problems in these delivery systems could lead to tumor treatment failure. Hence, NDDSs should be appropriately designed based on the physiological process in the body to overcome these barriers. However, the preparation of multifunctional and intelligent tumor-targeted NDDSs is very complex, and hence reduces the drugability of NDDSs. Currently, NDDSs for codelivery still faces a lot of challenges regarding its formulation, design, synthesis, and assessment. More studies are needed to further investigate the pathological features of tumors. This could help in developing multifunctional NDDSs that response to the pathological features of the body. The

combination of pharmaceuticals, medicine, chemistry, and materials science will help in the development of NDDSs.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

In Vitro Evaluation of the Potential Use of Propolis as a Multitarget Therapeutic Product: Physicochemical Properties, Chemical Composition, and Immunomodulatory, Antibacterial, and Anticancer Properties

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Propolis is a resin that honeybees produce by mixing saliva and beeswax with exudate gathered from botanical sources. The present *in vitro* study investigated the potential use of propolis as a multitarget therapeutic product and the physicochemical properties, chemical composition, and immunomodulatory, antioxidant, antibacterial, and anticancer properties of a propolis extract from the northern Morocco region (PNM). Pinocembrin, chrysin, and quercetin were the main phenolic compounds of PNM as measured in HPLC. The PNM showed significant inhibitory effects against all tested Gram-positive and Gram-negative strains and showed high antioxidant activities by scavenging free radicals with IC50 (DPPH = 0.02, ABTS = 0.04, and FRAP = 0.04 mg/ml). In addition, PNM induced a dose-dependent cytostatic effect in MCF-7, HCT, and THP-1 cell lines at noncytotoxic concentrations with IC50 values of 479.22, 108.88, and 50.54 μg/ml, respectively. The production of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) was decreased in a dose-dependent manner in LPS-stimulated human peripheral blood mononuclear cells (PBMNCs), whereas the production of the anti-inflammatory interleukin-10 (IL-10) was increased in a dose-dependent manner reaching 15-fold compared to the levels measured in untreated PBMNCs. Overall, the results showed that the traditionally known multitarget therapeutic properties of the PNM seem to be mediated, at least in part, through cytostatic, antibacterial, and immunomodulatory effects.

1. Introduction

For centuries, bee products have been used in all traditional medical systems. Honey and propolis (also known as bee glue) have been the subject of extensive studies for their health-promoting properties. Propolis is a resinous substance collected by bees from their surrounding plants [1, 2]. Hence, it contains a variety of phytochemicals that are produced by plants through primary and secondary pathways. Bees use propolis as an insulating material and as a sealant for unwanted open spaces in the hive. Due to its high antimicrobial properties, it plays an essential role in the hive defense [2]. In recent years, propolis has attracted much attention as a valuable or potential substance used in medicine and cosmetic products. It is known to exhibit valuable therapeutic and biological activities. These include, but not limited to, anticancer, antitumor, antioxidant, antimicrobial, antiulcer, and antifungal properties. It was also

reported to have hepatoprotective, cardioprotective, antihypertensive, hypoglycemic, and hypolipidemic activities [3, 4].

The chemical composition of propolis is quite complex. More than 300 phytochemicals have been identified so far in propolis. These include polyphenols, phenolic aldehydes, sesquiterpene quinines, coumarins, amino acids, steroids, and inorganic compounds. The chemical composition of propolis depends on the collecting time, location, and plant source. As a result, biological activities of propolis gathered at various times and from different phytogeographical areas vary greatly [3–5]. For example, propolis samples collected from temperate areas contain flavonoids pinocembrin, chrysin, ferulic acid, cinnamic acid, and caffeic acid [6, 7], whereas propolis from tropical regions is rich in prenylated derivatives of benzophenones, *p*-coumaric acid, lignans, and diterpenes [8]. Many of the propolis active components exhibited *in vitro* and *in vivo* anti-inflammatory effects through affecting common and/or distinct anti-inflammatory pathways. One example is the pathway that involves the Toll-like receptors (TLRs), which recognize various microbial receptors called pathogen-associated molecular patterns (PAMPs). Consequently, proinflammatory cytokines are released through the activation of NF-kB and other transcriptional factors [9]. This response is mediated by B and T cells and results in pathogen-specific adaptive immunity [10]. Propolis-derived neovestitol, an isoflavonoid, inhibited nitric oxide (NO) production and reduced proinflammatory cytokine levels from lipopolysaccharide- (LPS-) stimulated macrophage cell line RAW264.7 [11]. Propolis-derived caffeic acid, phenethyl ester, quercetin, and hesperidin strongly reduced DNA synthesis and the production of IL-1, IL-12, IL-2, and IL-4 and enhance the production of transforming growth factor-β (TGF-β) from T cells [12]. In addition, apigenin, galangin, and pinocembrin, isolated from propolis collected in southern Brazil [13], were found to modulate the production of proinflammatory *in vitro*. Apigenin decreased the mRNA levels of IL-1, IL-6, and TNF-α in human THP-1-derived macrophages [14]. The levels of these cytokines were also significantly reduced by pinocembrin in macrophage cell line RAW264.7, whereas IL-10 was significantly increased [15]. In the same RAW264.7 line, the level of IL-6 and TNF-γ cytokines was clearly inhibited by galangin [16]. *In vivo,* propolis administration to C57BL/6 mice for 14 days led to reduced production of IL-1, IL-6, IL-2, IL-10, and IFN-γ by spleen cells [17]. In addition, ethanolic extract of Brazilian propolis reduced the expression of IL-17 in collagen-induced arthritis in mice [18]. Thus, bee propolis and its constituents can be considered as potential natural anti-inflammatory agents that act by modulating immune responses.

Besides the anti-inflammatory effects, propolis-derived flavonoids exhibit powerful antioxidant activities and are capable of scavenging free radicals and thereby protecting the cell membrane against lipid peroxidation. In addition, propolis extracts were found to protect the liver in rats against carbon tetrachloride (CCl) injury. It seems that propolis exerts these hepatoprotective effect through the inhibition of phase I enzymes and the induction of phase II enzymes [19].

Many studies have been conducted on the propolis of Morocco and have shown the importance of some of its biological activity [20, 21]. However, there are no detailed studies on the action mechanisms of these activities, especially anticancer and anti-inflammation activities. Therefore, we investigated here the anti-inflammatory, antioxidant, antibacterial, and cytostatic effects on cancer cell lines of a propolis extract from the northern Morocco region. Dose-dependent cytostatic effects of propolis were evaluated in all three tested cancer cell lines. Propolis extracts also suppressed the TNF-α and IL-6 production in a dose-dependent manner in LPS-stimulated human peripheral blood mononuclear cells. Dose-dependent cytostatic effects of propolis were seen in all three cell lines. Propolis extracts suppressed the TNF-α and IL-6 production in a dose-dependent manner in LPS-stimulated PBMNCs, reaching control levels at 250 μg/ml. Propolis increased the production of the anti-inflammatory IL-10 in a dose-dependent manner.

2. Materials and Methods

2.1. Chemicals, Reagents, and Equipment

2.1.1. Chemicals and Reagents. 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), sulfuric acid, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide ($K_3[Fe(CN)_6]$), sodium carbonate, caffeic acid, *p*-coumaric acid, quercetin, gallic acid, cinnamic acid, naringenin, pinocembrin, galangin, rutin, pinobanksin, and chlorogenic acid were purchased from Sigma-Aldrich, Germany. Trichloroacetic acid, trisodium phosphate (Na_3PO_4), potassium dihydrogen phosphate (KH_2PO_4), and dipotassium hydrogen phosphate anhydrous (K_2HPO_4) were purchased from VWR, Leuven, Belgium, and chrysin was purchased from AbCam, UK. Ferulic acid was purchased from Acros Organics, USA. Iron (III) chloride was purchased from Buchs, Switzerland. Ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}$) was purchased from Pronalab, Lisbon, Portugal. Folin-Ciocalteu's phenol and AlCl3 were purchased from Panreac Quımica, Montcada i Reixac, Barcelona, Spain. Ascorbic acid, cell culture medium (DMEM and RPMI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich, USA. TNF-α, IL-6, and IL-10 kits were purchased from R&D Systems, Inc., USA. Mueller Hinton broth and Mueller Hinton agar were purchased from Biokar Diagnostics (Beauvais, France).

2.1.2. Equipment. Specmate UV-VIS spectrophotometer (CLS-4048), pH meter (WTW Inolab pH 720), HPLC (Hitachi, Chrom-master; Japan), and microplate reader (Tecan Infinite M200; Tecan, Austria) were used.

2.2. Collection of Propolis Sample. Propolis was collected by scratching from the northern Morocco region (PNM); this region is known for olive trees in addition to other trees such as *Pinus*, *Quercus*, *Rosmarinus*, *Juniperus*, *Lavandula,* and *Pistacia*. The propolis sample was stored at room temperature (22–24°C) in airtight plastic containers until analysis.

2.3. Physicochemical and Antioxidant Content of Propolis Sample. In the present work, wax, balsam, and resin contents were measured in the PNM according to methods described by Papotti et al. [22]. The ash content in the sample was determined according to the method described by Imtara et al. [23]. The pH was measured according to the method described by Dias et al. [24]. The moisture content of samples was determined according to the method described by AOAC [25].

In order to determine the total antioxidant content, the total phenol, flavones, and flavonols contents were determined according to the method described by Imtara et al. [26]. The results of total phenol are expressed as the mg gallic acid/g of propolis, and for flavones and flavonols contents, the results are expressed as mg quercetin/g of propolis.

2.4. Antioxidant Activity of Propolis Sample. The total antioxidant capacity (TAA) was estimated by the phosphomolybdenum method according to the procedure described by Prieto et al. [27]. Total antioxidant capacity contents are expressed as mg of ascorbic acid equivalent per g of the propolis mass (mg AA/g).

The ability of sample for scavenging of free radical was determined by three methods: the scavenging activity of DPPH radical was measured according to the method described by Brand-Williams et al. [28], the scavenging activity ABTS radical was carried out according to the method of Miguel [29], and the reducing power was determined according to the method of Oyaizu [30]. The results of each test are expressed by IC50 value (concentration of samples is able to scavenge 50% of free radicals).

2.5. Determination of Phenolic Compounds by RP-HPLC Analysis. Hundred mg of PNM was extracted by sonication at 50°C using 70% ethanol (10 mL) for 30 min. The solution was cooled to room temperature and the volume was made up to 10 ml with 70% ethanol in volumetric flask, followed by centrifugation at 3500 rpm. The centrifuged supernatant was filtered through HPLC syringe filter (0.45 μ) before injection to HPLC. Pure compounds that are used as standards included caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, chlorogenic acid, rutin, quercetin, cinnamic acid, naringenin, pinocembrin, chrysin, galangin, and pinobanksin. Phenolic compounds of propolis were identified by comparing their retention times with those of pure standards. The results were obtained in mg/g of propolis.

2.6. Antibacterial Activity of Propolis Sample. The bacterial strains used in the present work were isolated at the University Hospital Hassan II and at the Microbiology Laboratory of the FMP, Fez. The *E. coli* BLSE (ATB: 87) BGN, *E. coli* (ATB: 57) B6N, *E. coli* (ATB: 97) BGM, and *Pseudomonas aeruginosa* strains are Gram-negative bacilli, and the *Streptococcus faecalis* and *Staphylococcus aureus* strains are Gram-positive cocci bacteria. The ability of propolis sample to inhibit bacterial growth was determined by qualitative and quantitative tests.

The agar diffusion assay was performed by Kirby-Bauer method [31]. With some modification, Mueller Hinton agar plates are inoculated by swabbing from the standardized suspensions (10^8 cfu/mL). Then, Whatman paper disks (6 mm) are deposited on the surface of the preinoculated agar. Then, the disks are impregnated with 10 μl of propolis extract. All plates were incubated at 37°C for 24 hours. After incubation, the diameters of the inhibition zones were measured.

The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) tests were determined by microdilution method according to NCCLS standards in microplate (96-well plates) [32, 33]. With modification, ten concentrations of PNM were prepared in sterile tubes by successive dilutions 1/2 in hydroethanol (70%). A volume of 10 μl of each concentration of this dilution series was mixed in microplate wells with 170 μl of Mueller Hinton broth and 20 μl of bacterial inoculums with a final microbial concentration 5×10^5 CFU/ml. The final volume was 200 μl and the concentration of ethanol in each well does not exceed 3.5%. The same percentage of ethanol was used as a negative control. After the plates are incubated at 37°C for 20 h, 40 μl of triphenyltetrazolium chloride was added to each well. Then, the microplate was incubated for 2 hours. After incubation, the MIC is the lowest concentration that does not produce a red color [34]. To determine the MBCs, a portion from each well in which the concentration is ≥ MIC was subcultured on Muller–Hinton agar (MHA) and incubated at 37°C for 24 h. The MBC is defined as the lowest concentration of the extracts at which inoculated bacteria were 99.9% killed [35].

2.7. Cytotoxic and Cytostatic Effects of Propolis Sample

2.7.1. Cell Culture. The human monocytic cell line THP-1 (ATCC 202-TIB) was purchased from American Type Culture Collection (Manassas, VA, USA). These cells are known to express various monocytes receptors and have been widely used as a model system for macrophage research [36, 37]. Cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% vol/vol inactivated fetal calf serum (FCS), 1% nonessential amino acids, 1% glutamine, 100 U/mL penicillin, and 10 μg/ml streptomycin and kept in a humidified atmosphere of 5% CO_2 at 37°C. Human colorectal carcinoma cell line HCT-116 (ATCC® CCL-247™) and breast cancer cell line MCF-7 (ATCC® HTB-22™) were grown in DMEM-5671 with a high glucose content (4.5 g/l), supplemented with 10% vol/vol inactivated fetal calf serum (FCS), 1% nonessential amino acids, 1% glutamine, 100 U/ml penicillin, and 10 μg/ml

streptomycin. The pH of the media for growing cells was maintained at 7.4 under 5% CO_2 at 37°C.

2.7.2. Cytotoxic and Cytostatic Effects in Monoculture System. For the cytotoxic and cytostatic assays, 20,000 cells/100 μl and 5,000 cells/100 μl media were seeded in 96-microtiter plates, respectively. Twenty-four hours later, cells were incubated with increasing concentrations of the PNM (0–1000 μg/ml of culture media) for 24 hours and 72 hours for cytotoxic and cytostatic assays, respectively. Then, cell viability was measured using the MTT assay. Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells (control) [38].

2.7.3. MTT Assay. MTT (tetrazolium dye) is widely used to measure the viability and/or the metabolic state of cultured cells [19]. Twenty-four hours after cell seeding, cells were treated with varying concentrations of PNM (0–1000 μg/ml of culture media) for 24 hours at 37°C. Cells were then washed in phosphate buffered saline, incubated in serum-free RPMI to which MTT (500 μg/mL) was added to each well (100 μL), and incubated for a further four hours. After the removal of the medium, the cells were incubated for 15 minutes with 100 μl of acidic isopropanol (0.08 N HCl) to dissolve the formazan crystals. The absorbance of the dissolved MTT formazan was measured at 570 nm in an ELISA reader. Viability was defined as the ratio (expressed as a percentage) of absorbance of propolis-treated cells to untreated control cells.

2.8. Anti-Inflammatory Activity of Propolis Sample

2.8.1. Isolation of Peripheral Blood Mononuclear Cells (PBMNCs). Blood samples were taken from 16 healthy volunteer students from the Arab America University (Jenin, Palestine), aged 19–21 years (7 males and 9 females).

Blood samples were withdrawn in heparin tube after filling consent and a written questionnaire. Venous blood (15 mL) was processed immediately after collection. Peripheral blood mononuclear cells (PBMNCs) were isolated using gradient centrifugation in Histopaque-1077 solution (Sigma-Aldrich) [37]. Separated PBMNCs were incubated in RPMI-1640 media supplemented with sodium bicarbonate, L-glutamine-penicillin-streptomycin solution (200 mM L-glutamine, 10,000 U penicillin, and 10 mg streptomycin/mL in 0.9% NaCl), and 10% vol/vol FCS. Isolated PBMNCs were seeded at a cell density of 1×10^6 cells/ml in 24-well plates and exposed to propolis extract (125 μg/mL and 250 μg/ml) in a fresh serum-free medium in the absence and presence of *Escherichia coli* serotype O127 : B8 (5 μg/mL). Cells were maintained at 37°C for 4, 6, and 20 hours at 5% CO_2 and the levels of secreted IL-6, TNF-α, and IL-10 were determined as described below.

2.8.2. Immunoassay for Cytokines. The anti-inflammatory activities were assessed by investigating propolis ability to alter the production of tumor necrosis factor-α (TNF-α) and the cytokines interleukin-6 (IL-6), and IL-10 in human peripheral blood mononuclear cells (PBMNCs) was co-stimulated with lipopolysaccharide (5 μgLPS/mL). The amounts of secreted TNF-α, IL-6, and IL-10 were measured using a commercial ELISA kits (R&D Systems, Minneapolis, MN, USA). The absorbance at 450 nm was read by a microplate reader (model 680; Bio-Rad Laboratories, Mississauga, ON, Canada) with the wavelength correction being set at 550 nm. The amounts of TNF-α, IL-6, and IL-10 were calculated with the help of a standard curve, which was constructed using serial dilutions of cytokine standards provided with the kit.

3. Results and Discussion

3.1. Physicochemical Properties of Propolis Sample. Propolis is a resin that honeybees produce by mixing saliva and beeswax with exudates gathered from botanical sources. The amount of each of these compounds is often used as an indication of propolis quality, which depends on the phytogeographic and climatic conditions around the beehive [39, 40]. In the present work, the physicochemical properties of PNM showed that the concentrations of wax, resin, and balsam were $20.31 \pm 1.03\%$, $59.01 \pm 0.12\%$, and $16.4 \pm 0.01\%$, respectively (Table 1). These values are within the range accepted for propolis by the Brazilian legislation [41] and were similar to those found in the Italian and Moroccan propolis [21, 22]. Moreover, high moisture in propolis is indicative of inadequate storage and manipulation conditions [42]. The moisture of the studied propolis sample was $1.01 \pm 0.01\%$; this value is within the limit established by the Brazilian legislation (not more than 8%) [41]. The pH was somewhat acidic (5.1 ± 0.11), and it was similar to propolis pH found in other studies [43, 44].

With regard to the ash content, this analysis can identify a possible adulteration of the material through the presence of impurities [15]. The value of ash content in the PNM was $4.87 \pm 0.01\%$ which is lower than the upper limit which was defined as a quality standard in the propolis [41].

3.2. Bioactive Compounds and Pharmacological Activities of the Propolis Sample. The concentration and the type of bioactive compounds of propolis depend on many factors such as plant species, season of propolis harvesting, and geographical location of beehive collected [45]. Table 2 shows the values for the antioxidant content and antioxidant activity of the ethanol extract of PNM. The values of phenol and of flavone and flavonol were 141.46 ± 1.67 mg GAE/g, and 98.33 ± 1.19 mg QE/g, respectively. The total antioxidant capacity of the ethanol extract of PNM was 94.76 ± 1.91 mg AAE/g. These results are within the range reported in other studies [21, 46]. The capacity of the propolis samples to scavenge free radicals was evaluated and the results were expressed as IC50 mg/ml (Table 2). The results show a strong free radical scavenging with IC50 being 0.02 ± 0.002 for DPPH assay, 0.04 ± 0.001 for ABTS assay, and the same value for FRAP assay. The values of the IC50

Table 1: Determination of physicochemical properties of PNM.

Sample	Wax (%)	Resin (%)	Balsam (%)	Moisture (%)	Ash (%)	pH
Moroccan propolis	20.31 ± 1.03	59.01 ± 0.12	1.11 ± 0.01	1.01 ± 0.01	4.87 ± 0.01	5.1 ± 0.11

Table 2: Determination of the content of total phenols, flavone, and flavonol and antioxidant activity by TAA, DPPH, ABTS, and FRAP of PNM.

Sample	Phenols (mg GAE/g)	Flavone and flavonol (mg QE/g)	TAA (mgAAE/g)	DPPH IC50 (mg/ml)	ABTS IC50 (mg/ml)	FRAP IC50 (mg/ml)
Moroccan propolis	141.46 ± 1.67	98.33 ± 1.19	94.76 ± 1.91	0.02 ± 0.002	0.04 ± 0.001	0.04 ± 0.001

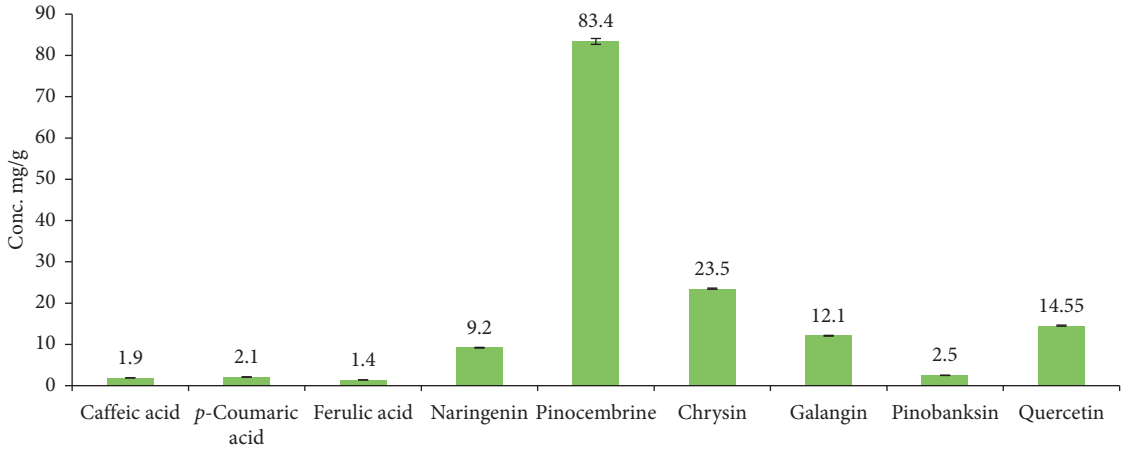

Figure 1: The concentration of phenolic compounds (mg/g) in PNM analyzed by HPLC, as described in Methods.

are within the range reported in other propolis samples [46–48].

The pharmacologic properties of the phenolic compounds of propolis are documented in numerous scientific papers. These include, but are not limited to, anticancer, anti-inflammatory, antibacterial, and antioxidant activities [6, 49]. The PNM was analyzed via HPLC under the same chromatographic conditions for the determination of the phenolic compounds. The analysis identified nine phenolic compounds in propolis sample with different concentrations: caffeic acid, *p*-coumaric acid, ferulic acid, naringenin, pinocembrin, chrysin, galangin, pinobanksin, and quercetin (Figure 1). The major constituent in the sample was pinocembrin with concentration being 83.4 ± 0.71 mg/g of propolis. No detectable amounts of gallic acid, chlorogenic acid, and rutin were found in the propolis sample. The results of bioactive compounds identified in this article are in agreement with other studies, which have found that propolis samples collected in different countries contain many phenolic and flavonoid components at different concentrations such as caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, chlorogenic acid, rutin, quercetin, cinnamic acid, naringenin, pinocembrin, chrysin, CAPE, galangin, apigenin, kaempferol, chrysin, cinnamyl caffeate, and aromatic acids [50, 51]. Many studies documented the antimicrobial activity of these compounds against large number of bacteria [46, 52, 53].

The diameters of bacterial growth inhibited by disc agar diffusion method of the propolis sample are shown in Figure 2. Comparing the six strains studied, the highest activity of PNM was observed on *S. faecalis* and *S. aureus* with zone inhibition values of 33.5 ± 1.57 and 30.2 ± 1.02 mm, respectively. The lowest activity was observed on *P. aeruginosa* with zone inhibition value of 17.54 ± 1.76 mm. The determination of the zone inhibitions of PNM on different bacteria strains showed that Gram-positive strains are more sensitive than Gram-negative bacteria. This is consistent with many studies [54, 55].

In the present work, the minimum inhibitory concentration (MIC) is the lowest concentration of propolis, at which no bacterial growth was observed [46]. The MIC and MBC values for PNM on studied strains were 90–625 and 90–1250 μg/mL, respectively (Figure 3). As found in the disc diffusion method, *S. faecalis* and *S. aureus* were the most sensitive and *P. aeruginosa* was the most resistant. The values of MICs reported in this study are lower than those reported in propolis from Canada [56], but similar to the results of MICs reported by other studies [46, 54].

3.3. Cytotoxic and Cytostatic Effects of Propolis in Monocultures of Cells from the Human THP-1, HCT-116, and MCF-7 Cell Lines. The research for new natural anticancer drugs is one of the main objectives of scientific research. As

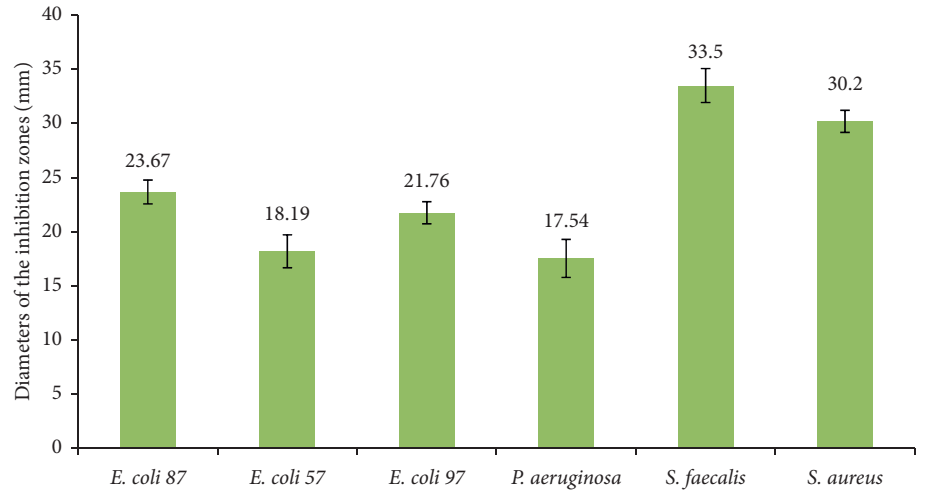

Figure 2: Antibacterial activity of PNM as detected by using the disc diffusion method. Whatman paper disks (6 mm) were impregnated with 10 μl of propolis. The plates were incubated at 37°C for 24 hours subsequent to measuring the diameters of the inhibition zones in mm. The bacteria stains tested are indicated in the x-axis.

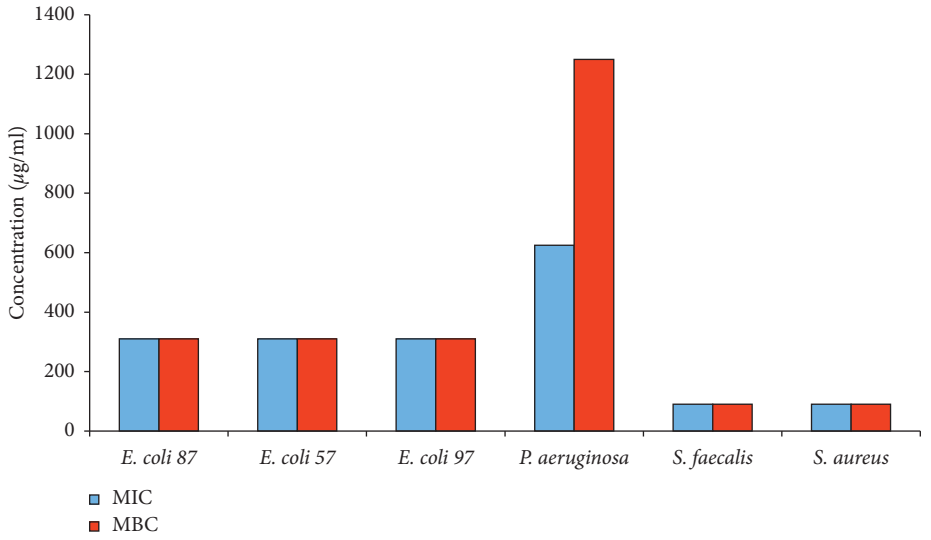

Figure 3: PNM minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for six distinct bacteria strains as indicated in the x-axis.

part of this work, we assessed here the capability of PNM to exert antiproliferative effects (cytostatic effects) in cancer cells at noncytotoxic concentrations.

No significant cytotoxic effects were observed in HCT cells at all PNM concentrations (up to 1000 μg/ml) (Figure 4). Significant cytotoxic effects (LD50: lethal dose) were observed in MCF-7 cells and THP-1 at concentration higher than 494 and 385 μg/ml, respectively (Table 3, Figure 4). Many reports suggest that cytotoxicity may largely vary in different samples of propolis. Szliszka et al. [57] reported that 50 g/mL ethanolic extract of propolis from southern Poland exhibited 25% cytotoxicity in prostate cancer cells. Seda Vatansever et al. [58] showed that ethanolic extract of propolis at a concentration of 125 g/mL is cytotoxic in MCF-7 cell line and reported differences in cytotoxic effects of seven different ethanolic extracts of propolis samples collected from the same location. Compared to these results, PNM exhibited relatively low grade of cytotoxic effects.

Dose-dependent cytostatic effects were measured in MCF-7 (IC50 of 108.9 μg/ml), HCT (IC50 of 279.2 μg/ml), and THP-1 (IC50 of 50.5 μg/ml) cells with PNM concentrations higher than 30 μg/ml and 100 μg/ml, respectively [58]. Many reports have indicated that different types of honey and propolis extracts significantly inhibit cell growth and reduce the differentiation or proliferation of cells from various tumor cell lines [58]. For example, Imtara et al. characterized the phenolic compounds of twelve honey samples collected from different locations in Palestine and Morocco to evaluate their cytotoxic and cytostatic effects on cells from the human colorectal carcinoma cell line HCT-116 and breast cancer cell line MCF-7. They found a

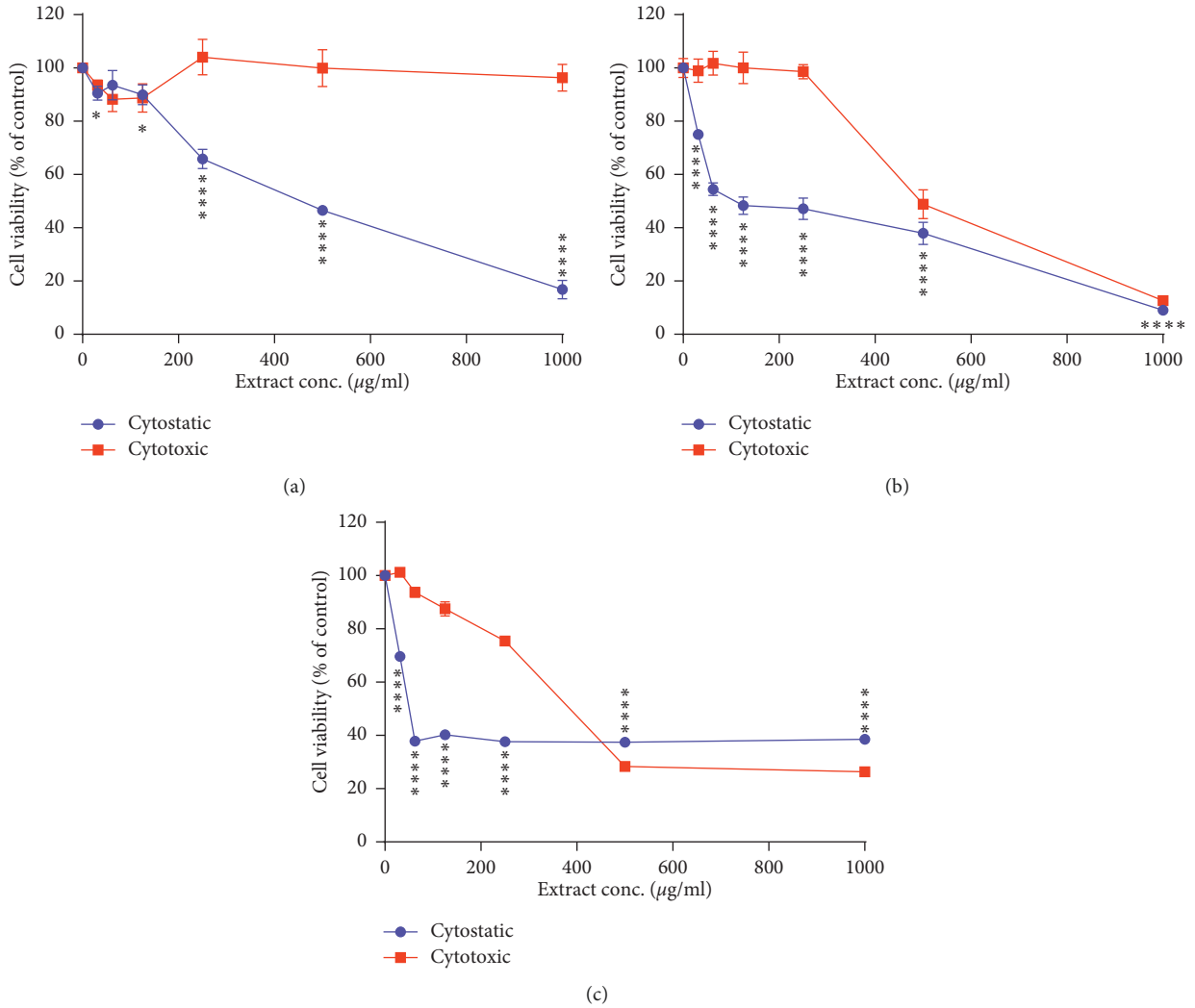

Figure 4: Cytostatic and cytotoxic activities of PNM in cells from the (a) HCT-116, (b) MCF-7, and (c) THP-1 cell lines. 20,000 cells/100 μl and 5,000 cells/100 μl media were seeded in 96-microtiter plates for the cytotoxic and cytostatic assays, respectively, and incubated with PEB (0–1000 μg/ml of culture media) for 24 hours and 72 hours for cytotoxic and cytostatic assays, respectively. $(^{*})p < 0.05$; $(^{***})p < 0.001$.

Table 3: IC50 values (cytostatic) and DL50 values (cytotoxic) of the PNM were measured in three types of cells using the MTT test.

	HCT-116	MCF-7	THP-1
IC50 (μg/ml)	479.22	108.88	50.54
LD 50 (μg/ml)	—	493.97	385.11

significant cytostatic effect after treatment of HCT cells as well as a strong correlation was observed between cytostatic activity of MCF cells and antioxidant content (phenols, flavonoids, and flavonol). Furthermore, a strong negative correlation was detected between the cytostatic activity in HCT cells and the contents of syringic acid and tannic acid. These results indicate that the traditionally known anticancer effects of honey might be mediated in part through cytostatic effects [38]. Another study investigated the anticancer effects of Indian ethanolic extract of propolis on four different cancer cell lines and demonstrated lower cytotoxicity effect of PNM evaluated by MTT assay (i.e., 250 g/mL) as compared to reported value [57, 58], which can be attributed to its different geographical origin. Apoptosis is an important phenomenon in chemotherapeutic agent which induced killing of cancer cells. Apoptosis induction is one of the mechanisms proposed for the therapeutic effects of propolis [59, 60]. Results obtained indicated that the mode of action of ethanolic extract of propolis is by inducing apoptosis, since DNA fragmentation is evidenced by the TUNEL assay. Seda Vatansever et al. have shown induction of caspases in MCF-7 cells [58]. Szliszka et al. discussed augmentation of TRAIL-induced apoptotic death in prostate cancer cells due to ethanolic extract of propolis [57]. The mechanisms by which our PNM exhibits its dose-dependent cytostatic effects need to be investigated.

3.4. Effect of Propolis Extracts on Proinflammatory Anti-Inflammatory Cytokines Production in PBMNCs. The PBMC cellular model includes T and B cells (~80%), natural killer cells (~10%), and monocytes (~10%). These blood cells play

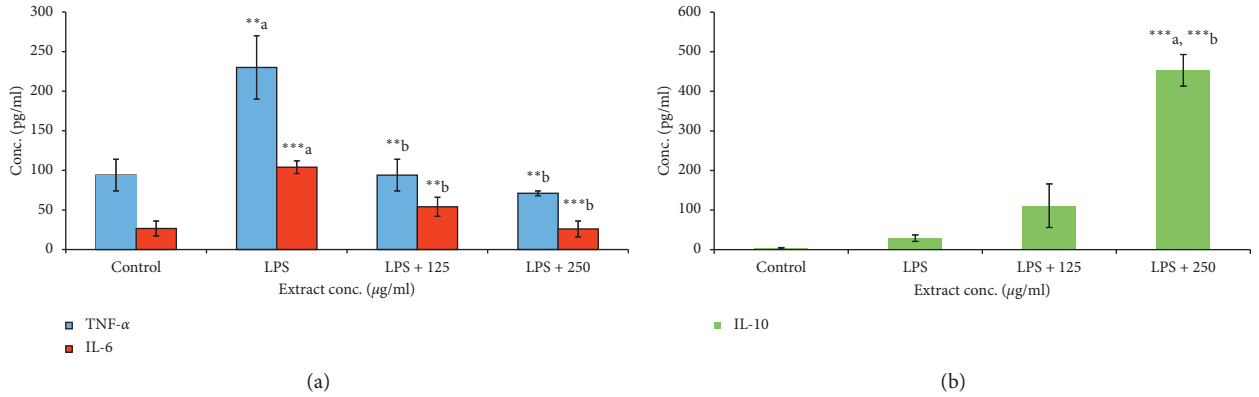

Figure 5: Dose-dependent inhibition of LPS-mediated production of TNF-α: IL-6 (a), and IL-10 (b) by PNM in PBMNCs. The bar heights represent the concentration means ± SD. [a]Comparison between control group and all groups. [b]Comparison between LPS group and all groups. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

an important role in the adaptive immune response [61]. Several studies have found that propolis has immunological activities [62]. For example, Brazilian green propolis exhibited antioxidant and anti-inflammatory activities in LPS-stimulated macrophage cell line J774A.1 through the inhibition of the production of reactive oxygen species, nitric oxide, and proinflammatory cytokines (e.g., TNF-α, IL-1, and IL-6) [13, 63]. In the present study, PNM on its own showed no effect on the production levels of the proinflammatory cytokines TNF-α and IL-6 as well as the anti-inflammatory cytokine IL-10 in PBMNCs when compared to untreated control cells (data not shown). However, when PBMNCs were stimulated with LPS, the PNM significantly inhibited the secretion of TNF-α and IL-6 compared with LPS alone. Treatment with PNM completely inhibited the TNF-α and IL-6 secretion reaching control levels at extract concentration of 250 μg/ml (Figure 5(a)). These results are in agreement with previous finding obtained in macrophage cell lines in which propolis compounds were found to have direct regulatory action on cytokine production. For example, neovestitol, an isoflavonoid from propolis, had an immune modulatory effect on LPS-stimulated cells from the macrophage cell line RAW264.7, where it clearly inhibited the production of nitric oxide (NO) proinflammatory cytokine. In Th1- and Th2-type T cells, propolis extracts and propolis active compounds caffeic acid, phenethyl ester, quercetin, and hesperidin strongly reduced the production of IL-1, IL-12, IL-2, and IL-4 and enhanced the production of transforming growth factor-β1 (TGF-β1) [13]. Zhang et al. reported that propolis-derived apigenin decreased the mRNA levels of IL-1, IL-6, and TNF-α in human THP-1-derived macrophages [16]. The levels of these proinflammatory cytokines were also significantly decreased by pinocembrin in macrophages (RAW264.7) [17]. In the same RAW264.7 line, the level of IL-6 and TNF-α cytokines was clearly reduced by propolis-derived galangin [18]. *In vivo* studies showed that propolis administration to C57BL/6 mice for 14 days led to the inhibition in the production of IL-1, IL-6, IL-2, IL-10, and IFN-γ by spleen cells [64]. In addition, ethanolic extract of Brazilian propolis reduced the expression of IL-17 in collagen-induced arthritis in mice [65].

IL-10 is a potent anti-inflammatory mediator; it reduces and terminates the ongoing inflammatory process [66, 67]. The effect of PNM on the secretion of the anti-inflammatory IL-10 cytokines by PBMNCs was measured by adding the extracts on their own and in combination with 10 μg/mL LPS. LPS treatment of PBMNCs cells induced the secretion of a low level of IL-10 (30 ± 8 pg/ml). Compared to LPS-stimulated cells, treatment with propolis increased the secretion of IL-10 by 3.7-fold and 15-fold at propolis concentration of 125 μg/mL and 250 μg/mL (Figure 5(b)), respectively. Previous reports have clearly shown the antagonist effect of IL-10 on the secretion of proinflammatory cytokines [19, 68], suggesting that propolis-mediated inhibition of the LPS-induced secretion and mRNA expression of IL-6 and TNF-α may pass through the induction of IL-10 production. Several inflammatory diseases share the dual characteristic of a very low blood level of IL-10 and a high blood level of TNF-α. Furthermore, injection of the recombinant form of IL-10 decreased the blood concentrations of TNF-α that has proven beneficial for such diseases [19, 68, 69]. The ability of propolis extract to modulate both the proinflammatory and anti-inflammatory cytokines in LPS-activated PBMNCs represents an additional argument for the suggestion that it is an alternative or a complement that may help in the treatment and/or prevention of inflammatory diseases. Thus, our results support previous findings that suggest that propolis can be considered as potential natural anti-inflammatory agents that act by modulating the production of immune mediators.

4. Conclusion

Taken collectively, our results show that traditionally known anticancer effects of the PNM seem to be mediated in part through cytostatic effects and immunomodulatory effects. These include the inhibition of proinflammatory cytokines and stimulation of the anti-inflammatory cytokine in LPS-activated PBMNCs. The results of this study show that propolis from Morocco has important therapeutic activities

especially in suppressing the TNF-α and IL-6 production and increasing the production of the anti-inflammatory IL-10. These multitarget actions including antibacterial, antioxidant, cytostatic, and immunomodulatory actions of the Moroccan propolis seem to be as a result of the presence of several active compounds which may act in a synergistic pathway. Our findings suggest the potential application of PNM in the pharmaceutical industry as well as in health foods and nutritional supplements.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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