Anticancerous Efficacy of Betulinic acid: An Immunomodulatory Phytochemical

Aruna Bhatia*, Gurpreet Kaur, Harmandeep Kaur Sekhon

Immunology and Immunotechnology Laboratory, Department of Biotechnology, Punjabi University, Patiala- 147002, Punjab, India

Correspondence: aruna_bhatia@rediffmail.com; +91- 9878263077

Abstract

Cancer is most prevailed of all the diseases and a major cause of mortality. Development of cancer may be due to many factors like exposure to certain chemicals, radiations, unregulated hormones, imbalanced diet, heredity or immune response of body the release of pro-inflammatory cytokines (interferon IFN-γ, tumor necrosis factor TNF-α, interleukin IL-1, IL-4, IL-6, IL-8, IL-10, IL-17, IL-18 and Granulocyte macrophage colony-stimulating factor GM-CSF) as a result of altered immune response play a major role in perpetuation of the disease. The cost and side effects of commonly used anticancer therapies like radiotherapy and chemotherapy raise a need to develop alternative therapeutic agents like immunomodulators. Natural/ medicinal plants and their products/ secondary metabolites are most acceptable, cost effective and safer alternatives to synthetic drugs. Various plant extracts and purified compounds have been evaluated for their biological activities like quercetin, Genistein, Ellagic acid and terpenoid Betulinic acid (Bet A) which induce death in cancer cells. Bet A containing plants and islolated Bet A has been reported not only for its antitumor activity and immunomodulatory property but also for its selective cytotoxicity against variety of cancer types. This review article highlights the mechanism of anticancerous activity of betulinic acid proving it a novel therapeutic agent in treatment of human cancers.

Keywords: Apoptosis, Cancer, Betulinic acid, Mitochondria, Cytokines

1.Introduction

Cancer, also known as malignant neoplasia, involves unregulated cell growth where cells divide and grow uncontrollably, forming malignant tumors, which may invade nearby parts of the body. The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream and can affect any organ of the body. Cancers figure among the leading causes of death worldwide, accounting for 8.2 million deaths in 2012 and it is expected that annual cancer cases will rise from 14 million in 2012 to 22 within the next two decades [1]. There are over 200 different known cancers that affect humans [2].

Diverse factors contribute to the development of cancer including dietary factors, certain infections, exposure to certain chemicals or radiation, lack of physical activity, obesity, hormonal imbalance and environmental pollutants [3]. About 30% of cancer deaths are due to the five leading behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, alcohol use and approximately 5–10% of cancers can be traced directly to inherited genetic defects [4]. Cancer causing viral infections such as HBV/HCV and HPV are responsible for up to 20% of cancer deaths in low- and middle-income countries. Prevention from cancer can be owed to good dietary habits synthetic medication and vaccination to prevent from viral infections [5].

Cancer is usually treated with chemotherapy, radiation therapy and surgery. In chemotherapy, immunosuppressive drugs are given to the patients, and these drugs have a range of side effects like baldness, immunodeficiency, organ damage etc. On the other hand, immunomodulatory plant products have been shown to have less or no side effects at all and this being the reason for preference of natural immunotherapy over conventional therapies by the consumer. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children, the risk of developing cancer generally increases with age [6].

In 1909, Paul Ehrlich proposed that the incidence of cancer would be much greater were it not for the vigilance of our immune defense system in identifying and eliminating nascent tumor cells. This suggestion gave rise to the generally accepted concept that the immune system plays a vital role in the identification and elimination of transformed cells [7-8].

2. Immune System and Tumor immunology

The immune system is a system of biological structures and processes within an organism that protects against disease. To function properly, an immune system must detect a wide array of agent (pathogens), and distinguish them from the organism's own healthy tissue. In many species, the immune system can be classified into subsystems, such as the innate immune system versus the adaptive immune system, or humoral immunity versus cell-mediated immunity. The relation between immune cells and cancer is given in Fig. 1.
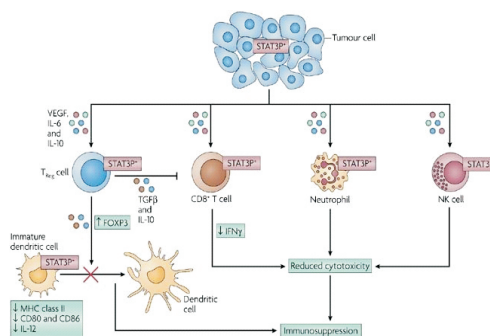

Fig. 1: Immune system and cancer [8]. Signal transducer and activator of transcription 3 (STAT3) activity is increased in tumour-associated regulatory T (TReg) cells. STAT3 signalling in TReg cells can upregulate the expression of forkhead box P3 (FOXP3), transforming growth factor-ß (TGFß) and interleukin-10 (IL-10), which in turn, restrain CD8+ effector T cells, as well as dendritic-cell maturation. Natural killer (NK) cells and neutrophils in the tumour stroma also have persistently activated STAT3, which inhibits the tumour-killing activity of both types of effector cell. IFN-γ ,STAT3P, tyrosine-phosphorylated (activated) STAT3.

An important role of the immune system is to identify and eliminate tumors. The transformed cells of tumors express antigens that are not found on normal cells. To the immune system, these antigens appear foreign, and their presence causes immune cells to attack the transformed tumor cells. Andersen and coworkers (2006) stated that the antigens expressed by tumors have several sources, some are derived from oncogenic viruses like human papillomavirus (HPV), which causes cervical cancer, while others are the organism's own proteins that occur at low levels in normal cells but reach high levels in tumor cells. One example is an enzyme called tyrosinase that when expressed at high levels, transforms certain skin cells (e.g. melanocytes) into tumors called melanomas. A third possible source of tumor antigens are proteins normally important for regulating cell growth and survival, that commonly mutate into cancer inducing molecules called oncogenes [9]. The main response of the immune system to tumors is to destroy the abnormal cells using killer T cells, sometimes with the assistance of helper T cells. Clearly, some tumors evade the immune system and go on to become cancers. Some tumor cells also release products that inhibit the immune response; for e.g. by secreting the cytokine (tumor growth factor-β) TGF-β, which suppresses the activity of macrophages and lymphocytes. Studies on cytokine profiles produced and released by T-helper cells disclosed a functional T-cell dichotomy and provided an explanation for the opposing cellular and humoral responses to various antigens [10]. In principle, two T-helper cell subsets, Th1 and Th2, have been distinguished by their cytokine profiles. Th1 lymphocytes, engaged mainly in generating delayed-type hypersensitivity responses, preferentially produce interleukin-2 (IL-2), interferon IFN-γ and tumor necrosis factor TNF-α, while Th2 lymphocytes, essential in the initiation of antibody responses, release IL-4, IL-5, IL-6 and IL-10. In addition, the activation of one of these subsets results in the suppression of the other. Several *in vitro* studies revealed a decline in the production and/or release of the Th1 cytokines, IFN-γ and IL-2, by mitogen-stimulated mononuclear cells from patients with various malignancies, i.e. colorectal, bladder, renal, prostate, breast, ovarian, cervical, endometrial and other carcinomas, as well as malignant melanoma [11].

This review highlights the cross link between cancer and inflammation, the effect of betulinic acid on cancer, mechanism of betulinic acid action and its effects on different cytokines in different types of cancers.

3. Inflammation and Cancer

The link between chronic inflammation and cancer is very well understood. The events in tumorigenesis involve cells that are activated at the cancer microenvironment, tumor infiltrating polymorphonuclears, immune cells including lymphocyte subtypes and peripheral blood mononuclear cells (PBMC), along with tumor-associated macrophages. The immune cells produce inflammatory cytokines which play a crucial role in tumorigenesis. Additional factors, such as gene expression regulated by cytokines, assembling of tumor growth- and transforming factors, accelerated angiogenesis, delayed apoptosis, contribute all to initiation, development and migration of tumor cells. Oxygen radicals originating from the inflammatory area promote cell mutation and cancer proliferation [12]. Tumor cells may over-express pro-inflammatory mediators that in turn activate immune cells for inflammatory cytokines production. Cytokines, including migration inhibitory factor (MIF), TGF-β 1, TNF-α, Interleukin (IL)-6, IL-10, IL-12, IL-17, IL-23 have been reported to be involved in human cancer development. Some cytokines for example IL-5, IL-6, IL-10, IL-22 and growth factors promote tumor development and metastasis, and inhibit apoptosis via activation of signal transducer activator transcription-3 transcription factor. Assessment of the interaction between components in the tumor environment and malignant cells requires a reconsideration of a few topics elucidating the role of chronic inflammation in carcinogenesis, the function of the immune cells

expressed by inflammatory cytokine production, the immunomodulation of cancer cells and the existence of a cross-talk between immune and malignant cells leading to a balance in cytokine production [12].

Breast, head and neck, colon, cervical, and GI tract cancers are among of the common malignancies observed in clinical practice and are among the frequent causes of human death.

Cervical cancers are characterized by the cytokines TGF-β1, IL-4, IL-12p35, and IL-15. A study reported that these were produced by all Cervical Cancer Cell Lines (CCCL) and Normal Primary cervical epithelial cell cultures (NPE). TNF-α, IL-10, IL-5 were present in most NPE, but not in any of the CCCL [13]. In cervical cancer, the most important of all cytokines is IL-10 which has a dual biological role anti-inflammatory and anti-angiogenic effect. These IL-10 molecules are produced by T cells, Monocytes, Macrophages, B cells, Natural killer cells, Eosinophils, Mast cells and Keratinocytes. Th1 cells are their main target. In cervical cancer patients the incidence of IL-10 is found to increase with the severity of the disease [14].

Breast carcinomas are heavily infiltrated by different types of host leukocytes, including primarily T cells, and monocytes that differentiate into tumor-associated macrophages (TAM) at the tumor site. A major TAM-derived inflammatory cytokine shown to be highly expressed in breast carcinomas is TNF-α which is a multifactorial cytokine. Patients with more progressed tumor phenotypes were shown to have significantly higher TNF-α serum concentration [15]. Two other inflammatory cytokines i.e. IL-6 and IL-1, also have a major role in breast carcinoma. Of interest is the fact that the two cytokines (IL-6 and IL-1) and TNF-α are interrelated and may act in an additive manner, suggesting that these three cytokines form a network of related factors that may affect tumor cell progression in a cooperative manner.

Results of one study showed that most resected prostate tissue shows signs of inflammatory response, and a relationship between T-cell infiltration and stromal proliferation can be found. Evidence for the importance of estrogen and pro-inflammatory cytokine interleukin (IL-6, IL-8, IL-15, IL-17) can also be found [16]. Another study support the possibility of prostate cancer cell-induced cytokine production by PBMC, and particularly IL-6, are involved in prostate cancer development [17].

GI tract carcinoma is characterized by TGF- ß, IL-6, IL-8, IL-18, IL-1 ß. IL-6, and IL-8 are associated with regulation transcription. IL-1 ß and IL-6 are stimulators of proinflammatory cytokine production. TGF-ß plays a dual role in the tumor development. In the early phase of carcinogenesis, this cytokine acts as a tumor suppressor, but later it is an inductor of tumor invasion by stimulation of extracellular matrix production, tumor cells proliferation, angiogenesis, and inhibition of host immune functions [18].

One of the major mechanisms by which head and Neck Small Cell Carcinoma (HNSCC) tumors are thought to evade host immune recognition is by modulating the cytokine environment at the tumor site. By secreting cytokines such as IL-6 and IL-10, HNSCC tumor cells promote a Th2-skewed response, which is associated with decreased antitumor efficacy. Along with Th2-skewed cytokines, HNSCC tumors secrete increased levels of immunosuppressive factors such as TGF-β that function to directly inhibit cytotoxic T cell-mediated immunity and recruit immunosuppressive cells, including myeloid-derived suppressor cells (MDSCs) and M2-skewed macrophages, to the tumor site. Once at the tumor site, HNSCC tumor cells harness these immunosuppressive cells for several tumor-promoting functions, including increased growth and angiogenesis. HNSCC cells trigger increased IL-6 production from CD34+ progenitor cells, for example, promoting angiogenesis in the tumor microenvironment. In the 4-NQO mouse model, the premalignant state is also characterized by a significant increase in IL-17A-

secreting Th17 cells in tumor-draining lymph nodes compared to HNSCC-bearing mice [19].

4. Herbalism

The majorly used therapies against cancer like radiotherapy and chemotherapy have various side effects including suppression of required immune response. Also in cancer research, angiogenesis inhibitors were once thought to have potential treatment application to many types of cancer, but this has not been the case in practice [20-21]. Synthetic medicine is being replaced by herbal medicine due to the less or no side effects of the latter. Herbal Medicine or Herbalism is use of plants for medicinal purposes. Plants have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today. Modern medicine recognizes herbalism as a form of alternative medicine. In a 2010 survey of the most common 1000 plant-derived compounds, only 156 had clinical trials published [22]. Preclinical studies (tissue-culture and animal studies) were reported for about one-half of the plant products, while 12% of the plants, although available in the Western market, had "no substantial studies" of their properties. Strong evidence was found that 5 were toxic or allergenic, so that their use ought to be discouraged or forbidden. Nine plants had considerable evidence of therapeutic effect [23].

5. Plants and their Metabolites

Plants are the producers of primary and secondary metabolites. Primary metabolites are compounds that are directly involved in the growth and development of a plant whereas secondary metabolites are compounds produced in other metabolic pathways that, although important, are not essential to the functioning of the plant. However, secondary plant metabolites are useful in the long term, often for defense purposes, and give plants characteristics such as color and are also used in signaling and regulation of primary metabolic pathways. Plant hormones (secondary metabolites), are often used to regulate the metabolic activity within plant cells. Secondary metabolites are categorized as alkaloids, flavonoids, phenols, terpenes and terpenoids, tannins and organic acids etc [24]. Many of these metabolites have been found to have anticancerous, antioxidant or immunotherapeutic efficacy and one of them is triterpenoid betulinic acid which has proven antitumor activity.

6. Betulinic acid, a Phytochemical with Antitumor Activity

Betulinic acid is a naturally occurring pentacyclic found in the bark of several species of plants, principally the white birch *(Betula pubescens)* [25] from which it gets its name (Fig. 2), the ber tree *(Ziziphus mauritiana)* [26], selfheal (), the tropical carnivorous plants *Triphyophyllum peltatum* and *Ancistrocladus heyneanus, Diospyros leucomelas,* a member of the persimmon family, *Tetracera boiviniana, rosemary,* and *Pulsatilla chinensis.*

Fig. 2: *Betula alba*

Reduced form of BetA was first isolated from plants in 1788 by Johann Tobias Lowitz and found to be a prominent constituent of the outer-bark of white-barked birch trees. Betulinic acid (Fig. 3) exerts a number of biological activities like anti-inflammatory, anti-malarial, anti-retroviral and anticancerous properties [27].
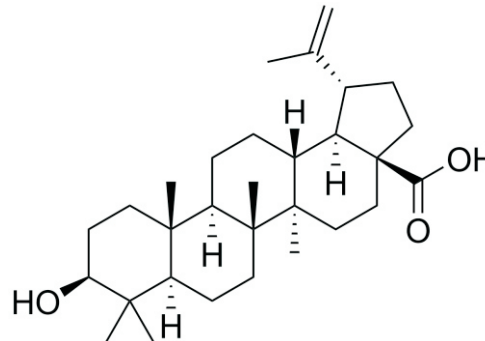

Fig. 3: Structure of Betulinic acid ((3β)-3-Hydroxy-lup-20(29)-en-28-oic acid)

The extracts from *Betula alba* (Betulaceae) leaves and bark which is known to be among the plants richer in betulin and betulinic acid is often used in phytopharmacology as diuretic, antimycotic and anti-inflammatory remedies. Such treatments are being developed alongside companion diagnostic tests to target the right drug to the right patients, based on their individual biology [23].

7. Mechanism of Action of Bet A

Although Betulinic acid is cytotoxic against a variety of cancer types including those with multidrug resistance (MDR) but normal cells and tissue are relatively resistant to betulinic acid [28]. Very little is known about antiproliferative and apoptosis-inducing mechanisms of Betulinic acid. Various studies have been carried out to elucidate the mechanism of Betulinic acid-mediated antitumor activity which includes:

- Induction of apoptosis
- Induction of mitochondrial outer membrane permeabilization
- Generation of reactive oxygen species (ROS)
- Regulation of induced apoptosis by Bcl-2 family proteins
- Modulation of NF-kB activity

7.1. Apoptosis pathways

Apoptosis or programmed cell death is the cell's intrinsic death mechanism which plays a pivotal role in maintaining tissue homeostasis and is highly conserved among different species. Since apoptosis is involved in the regulation of many physiological processes, defective apoptosis signaling may lead to various pathological conditions. A hallmark of human cancers is the evasion of apoptosis [29]. Cancer cells have the tendency to disable the mitochondrial (intrinsic) pathway of apoptosis. Betulinic acid is a natural compound that exhibits potent antitumor activities by triggering the mitochondrial path towards apoptosis [30]. The mitochondrial pathway of apoptosis is shown in Fig. 4.

Apoptosis pathways can be initiated at the level of mitochondria by the release of apoptogenic factors such as cytochrome C, Smac (small mitochondria-derived activator of caspases) or AIF (Inhibitor of Apoptosis Proteins) from the mitochondrial intermembrane space into the cytosol (mitochondrial or intrinsic pathway) [31]. Smac promotes apoptosis by neutralizing IAP-mediated inhibition of caspase-3 and -9 [32]. Alternatively, apoptosis can be triggered by ligation of death receptors (DR) such as CD95 or TRAIL receptors by their cognate ligands, i.e. CD95 ligand or TRAIL (receptor or extrinsic
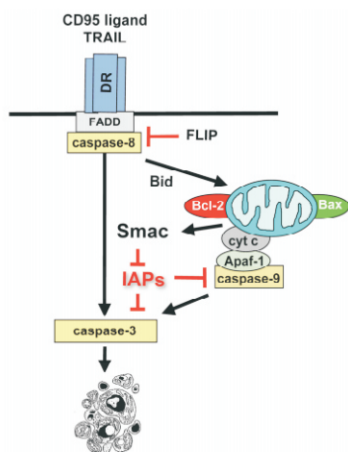
Fig. 4: Mitochondrial pathway of apoptosis [31]. Mitochondrial pathway of apoptosis. The intrinsic (mitochondrial) pathway of apoptosis is linked to mitochondrial outer membrane permeabilization (MOMP), which is regulated by various factors including pro- and antiapoptotic Bcl-2 proteins, reactive oxygen species (ROS), proteins from the mitochondrial permeability transition pore complex (PTPC), ions, sphingolipids and BA. MOMP in turn results in the release of soluble intermembrane proteins from mitochondria into the cytosol such cytochrome c, Smac/DIABLO, AIF and endonuclease G. Cytochrome c and Smac/DIABLO promote activation of caspases, whereas AIF and endonuclease G contribute to caspase-independent chromatolysis.

pathway) [33]. Death receptor stimulation leads to receptor trimerization, recruitment of adaptor FADD (Fas associated protein with Death Domain) and activation of the initiator caspase-8, which propagates the death signal to caspase-3 [34]. The BH3 domain of protein Bid links the receptor to the mitochondrial pathway [35]. Bid is activated by caspase-8-mediated cleavage and translocates to mitochondria to promote cytochrome-c release. Apoptosis can be inhibited at various levels by FLIP (FLICE- like Inhibitory Protein), Bcl-2 or IAPs (Inhibitor of Apoptosis) [36-37]. The intrinsic (mitochondrial) pathway of apoptosis is triggered upon treatment with chemotherapeutic agents or upon radiotherapy as a result of DNA damage or cellular stress response. A pivotal initial step in the activation of the mitochondrial pathway is the permeabilization of the outer mitochondrial membrane [37]. During this process, both the mitochondrial outer and inner membranes are permeabilized, which in turn results in the release of soluble proteins from the mitochondrial intermembrane space into the cytosol, for example cytochrome-c, Smac or AIF [38]. Factors that can directly induce mitochondrial outer membrane permeabilization can act effectively as cytotoxic agents.

7.2. Induction of mitochondrial outer membrane permeabilization by betulinic acid

Betulinic acid has been reported to induce apoptosis via direct mitochondrial perturbations. When added to isolated mitochondria in cell-free systems, betulinic acid induced loss of mitochondrial membrane potential and in intact cells, betulinic acid was shown to trigger cytochrome-C in a caspase-independent and permeability transition pore-dependent manner [39]. In a cell-free system comprising mitochondria, cytosols, and purified nuclei, the mitochondria undergoing betulinic acid-induced permeability transition mediated cytosolic caspase activation and nuclear fragmentation via the release of soluble factors, such as cytochrome-C or AIF (Apoptosis Inducing Factor) [40]. Anti-apoptotic Bcl-2 family of proteins such as Bcl-2 and Bcl-XL inhibited all mitochondrial and

cellular manifestations of apoptosis induced by betulinic acid, indicating that mitochondrial permeability transition is required for these events. Perturbance of mitochondrial function constitutes a central coordinating event in betulinic acid-induced apoptosis leading to caspase activation and apoptotic DNA fragmentation. Mitochondria from cells, which were treated with betulinic acid, induced cleavage of both caspase-8 and caspase-3 in cytosolic extracts.

Cytochrome C, released from mitochondria undergoing betulinic acid-mediated permeability transition, activated caspase-3 but not caspase-8 in a cell-free system [40]. Cleavage of caspase-3 and -8 was preceded by disturbance of mitochondrial membrane potential and by generation of reactive oxygen species. In addition, activation of caspases was restricted to cells that already had lost their mitochondrial membrane potential further suggesting that mitochondrial alterations were involved in betulinic acid induced activation of caspases. Over-expression of Bcl-2 and Bcl- XL conferred resistance to Betulinic acid at the level of mitochondrial dysfunction, protease activation, and nuclear fragmentation indicating that these events occurred downstream of the Bcl-2- or Bcl-XL-controlled checkpoint of apoptosis. These findings suggest that caspase-8 is activated downstream of mitochondria during betulinic acid-induced apoptosis. Activation of the caspase cascade was required for betulinic acid triggered apoptosis. Interestingly, neuroblastoma cells resistant to doxorubicin-mediated apoptosis were still responsive to treatment with betulinic acid [41]. This indicates that betulinic acid may overcome some forms of drug resistance.

7.3. Generation of ROS

Generation of reactive oxygen species (ROS) upon treatment with betulinic acid has been reported to be involved in initiating mitochondrial membrane permeabilization. To this end, ROS generation was detected in cancer cell lines of different origin that were treated with betulinic acid [42-43]. Incubation with antioxidants prior to administration of betulinic acid rescued cells from undergoing apoptosis suggesting that ROS production was involved in mediating cell death. Cleavage of caspase-3 and -8 was preceded by the disturbance of mitochondrial membrane potential and by the generation of reactive oxygen species (ROS) in intact cells treated with BA [40]. Also, ROS generation was linked to activation of pro-apoptotic p38 and SAP/JNK kinases with no change in the phosphorylation of ERK indicating that ROS act upstream of the MAPKs in the signaling pathway of betulinic acid [22].

7.4. Regulation of betulinic acid-induced apoptosis by Bcl-2 family proteins

Proteins of the Bcl-2 family are among the many signal transduction proteins that can act on mitochondria to regulate outer membrane permeabilization [35]. Bcl-2 family proteins comprise both antiapoptotic members, e.g. Bcl-2, Bcl-XL, Mcl-1, as well as pro-apoptotic molecules such as Bax, Bak, Bad and BH3 domain only molecules. Imbalances in the ratio of anti-apoptotic versus proapoptotic Bcl-2 proteins may tip the balance in favor of tumor cell survival instead of cell death [35]. Treatment with betulinic acid resulted in upregulation of the pro-apoptotic Bcl-2 family protein Bax in neuroblastoma, glioblastoma and melanoma cells, whereas Bcl-XS was found at elevated levels in betulinic acid-treated neuroblastoma cells [28, 42, 44]. Expression levels of proapoptotic proteins Bak and Bad were not altered in response to betulinic acid in melanoma cells [45]. While expression levels of anti-apoptotic Bcl-2 remained unchanged upon incubation with betulinic acid in neuroblastoma and squamous cell carcinoma cells, an increase in Bcl-2 protein levels was reported in glioblastoma cells [30]. Also, betulinic acid triggered upregulation of Mcl-1, another anti-apoptotic Bcl-2 family protein, in melanoma cells, whereas no changes in Mcl-1 levels were detected in squamous cell carcinoma cells[30,45] As fas as Bcl-XL is

concerned, no alterations in expression levels were reported upon exposure to betulinic acid in neuroblastoma, glioblastoma or melanoma cells. These findings suggest that betulinic acid regulates Bcl-2 family proteins in a context-dependant manner. Moreover, betulinic acid has been reported to induce apoptosis in a p53- and CD95-independent manner. To this end, apoptosis upon treatment with betulinic acid was not associated with accumulation of wild-type p53 protein. Also, betulinic acid similarly induced apoptosis in p53 mutant and p53 wild-type cell lines and was also active in p53 deficient melanoma cells [46]. Moreover, betulinic acid triggered apoptosis independent of CD95-ligand/receptor interaction [47]

7.5. Modulation of NF-BB activity by Betulinic acid

Betulinic acid has also been reported to modulate activity of the transcription factor nuclear factor- κB (NF-κB), a key regulator of stress-induced transcriptional activation. Betulinic acid was identified as a potent activator of NF-κB in a number of cancer cell lines [48]. Betulinic acid-induced NF-κB activation involved increased IKK activity, phosphorylation of IκBα at serine 32/36 followed by degradation of IκBα and nuclear translocation of the NF-κB subunit p65. Reporter assays confirmed that NF-κB that was activated by betulinic acid is transcriptionally active. Interestingly, inhibition of betulinic acid-induced NF-κB activation by different chemical inhibitors (proteasome inhibitor, antioxidant, IKK inhibitor) also impaired betulinic acid-induced apoptosis. Importantly, specific NF- κB inhibition by transient or stable expression of IκBα super-repressor inhibited betulinic acid induced apoptosis in some neuroblastoma cells, while transient expression of IκBα super-repressor had no influence on betulinic acid-induced apoptosis in other cell lines [48]. These studies indicate that activation of NF-κB by betulinic acid promotes betulinic acid-induced apoptosis in a cell type-specific manner. By comparison, betulinic acid was shown to interfere with NF-κB activation and NF-κB regulated gene expression triggered by carcinogens and inflammatory stimuli [49-50]. These findings may provide a molecular basis for the ability of betulinic acid to suppress inflammation and modulate the immune response and may also point to a context-dependant function of NF-κB in the regulation of betulinic acid-mediated apoptosis [51-52].

7.6. Additional Anticancer Effects of Betulinic acid

Betulinic acid has also been reported to inhibit aminopeptidase N, an enzyme involved in the regulation of angiogenesis and over-expressed in several cancers [52-54]. Betulinic acid was reported to exert anti-angiogenic effects by inhibiting growth factor-induced *in vitro* angiogenesis by affecting mitochondrial functions [53]. Further, the anti-angiogenic activity of betulinic acid was attributed to activation of selective proteasome-dependent degradation of the transcription factors, specificity protein 1 (Sp1), Sp3, and Sp4, which regulate vascular endothelial growth (VEGF) expression [55]. Bet A decreases the Sp1 Level in Lung Adenocarcinoma by Modulating Its Post-Translational Modification. A previous study showed that BA inhibits the growth of prostate cancer by degrading Sp1 protein, but it is still not clear how Sp1 is degraded [56]. *In vitro* cytotoxic activity and *in vivo* anti cancerous activity of BA was evaluated in athymic nude mice bearing MCF-7 breast adenocarcinoma xenografts and a significant reduction in the tumor size was reported along with decreased angiogenesis, proliferation and invasion in treated animals [57]. Experimental results confirmed that the complexation model inhibited the growth of MCF-7 cells in a dose-dependent manner, arrested cell cycle in the G2/M phase and induced apoptosis via the mitochondria transduction pathway. Gene and protein analyses showed that the complexation model significantly inhibited Bcl-2 expression and promoted Bax expression, causing caspase-3 and caspase-9 cascade activation. These findings corroborated evidence on microencapsulated BA as an apoptosis inducer in MCF-7 cells. Thus, sour jujube fruits may have potential use as a breast cancer chemotherapeutic agent [58]. Also, Bet A Decreases Cyclin A2

Expression by Suppressing Sp1 Expression in Lung Cancer Formation [59]. Compared to betulinic acid, 20, 29-dihydro-betulinic acid derivatives were claimed to posses better anti-angiogenic properties as betulinic acid [60]. Also, betulinic acid was shown to inhibit the catalytic activity of topoisomerase-I [61-62]. While betulinic acid was found to reduce expression of p21 protein in melanoma cells, an increase of p21 protein was observed upon treatment with Betulinic acid in glioblastoma cells. Betulin and 28-O-propynoylbetulin inhibited cell proliferation in a concentration-dependent manner. The cell cycle analysis revealed an increase of the sub-G1 cell fraction (representing dead cells) after incubation of cells with betulin and 28-O-propynoylbetulin. The observed cytotoxic effects were more pronounced for 28-O-propynoylbetulin. Activity of caspase-3 in 28-O-propynoylbetulin treated cells was nearly 2-fold greater compared to cells incubated with betulin [63]. Whether betulinic acid-mediated cell cycle changes are linked to its antitumor activity remains to be addressed in future studies. Besides its potent antitumor activity *in vitro*, betulinic acid also suppressed tumor growth in several animal models of human cancer. In a xenograft mouse model of ovarian cancer administration of betulinic acid significantly increased the survival time [23]. Also *in vivo*, betulinic acid cooperated with chemotherapeutic agents such as vincristin to reduce lung metastasis in a metastatic melanoma model, hence showing the synergistic effect [64]. Of note, no systemic toxicities or weights loss were observed in betulinic acid-treated mice even at high systemic doses of betulinic acid [46]. Pharmacokinetic studies in mice bearing melanomaxenografts demonstrated that betulinic acid was well absorbed and distributed with highest concentrations found within the tumor [65-66]. Phase I/II studies of 3-o-(3',3'-dimethylsuccinyl) betulinic acid (bevirimat) in patients with human immunodeficiency virus (HIV) infection demonstrated that single oral doses of bevirimat were well tolerated and that plasma concentrations ranged from 8 to 58 g/ml [67-69]. This indicates that plasma levels of betulinic acid could be achieved after oral administration in humans that correspond to concentrations, which were found to exert antitumor activity *in vitro*. Furthermore, betulinic acid was reported to harbor anticarcinogenic properties that could be exploited in cancer prevention settings. To this end, betulinic acid was shown already more than a decade ago to inhibit tumor formation in mouse skin two-stage carcinogenesis [69]. Betulinic acid is currently under evaluation as a topical agent in a phase I/II clinical trial for the treatment of dysplastic nevi with the potential to transform into melanoma and it is also found to have anti-HIV properties [64]. The antitumor cytotoxicity of Bet A has been extensively studied in cancer cell lines and the effective dose on different cancer cell lines is given in Table 1.

Table 1. *In vitro* cytotoxic effect of betulinic acid on human cancer cell lines

Cancer type	ED_{50} (µg/ml)
Melanoma	1.1-4.8 [70]
Neuroblastoma	2-10 [23]
Medulloblastoma	3-15 [71]
Glioblastoma	5-16 [71]
Head & neck cancer	8.0 [22]
Ovarian carcinoma	1.8-4.5 [72]
Cervix carcinoma	1.8 [72]
Lung carcinoma	1.5-4.2 [72]
Leukemia	2-15 [40]

8. Betulinic acid and Cytokines

Betulinic acid exerts different effect on different types of cytokines involved in the carcinomas. One study indicates that betulinic acid is a modest TNF-α inducer and also an enhancer of mitogen-induced TNF-α production. In contrast to betulin, betulinic acid is a modulator of cytokine production by Th1/Th2 cell subpopulations which slightly enhances IL-10 formation and inhibits IFN-γ production, reducing IFN-γ /IL-10 ratio from 3.6 to 2.6 [73]. Studies carried out by Yoon and coworkers have shown that TNF-α-induced intracellular ROS was markedly decreased by pretreatment with BA. Furthermore, BA significantly inhibited the translocation and transcriptional activity of NF-kB increased by TNF-α.These results suggested a vascular protective role of BA via inhibition of ROS and NF-kB activation [74]. Another study suggested that betulinic acid inhibits IL-6 production by preventing NF-kB nuclear translocation involving ERK- MAPKs as cross talks occur between MAPK and NF-kB pathways [75]. Also, The anti-inflammatory mechanism of betulinic acid was examined by detecting the levels of cyclooxygenase-2 (COX-2), nitric oxide (NO), TNF-α, IL-1β and it was suggested that betulinic acid possessed anti-inflammatory activities [76]. Also it was suggested that betulinic acid inhibited TGF-β production [77]. Jana and coworkers showed that Bet A inhibited IL-17 and IFN-γ production in a dose-dependent manner. Besides inhibiting encephalitogenic T cell cytokines and reducing NO generation, Bet A induced tissue-damaging ROS generation within Central Nervous System. Bet A inhibits IL-17 and IFN- γ in a dose dependent manner [78]. *In vitro*, Bet A inhibits ethanol-induced activation of hepatic stellate cells, as a antioxidant, suppressing of ROS, TNF-α and TGF-β production as well as NF-κB/IκB transduction signaling and TNF-α-induced intracellular ROS generation is markedly decreased by pretreatment with BA[79].

9. Derivatives

A major inconvenience for the future clinical development of betulinic acid and analogues resides in their poor solubility in aqueous media such as blood serum and polar solvents used for bioassays. To circumvent this problem of hydrosolubility and to enhance pharmacological properties, many derivatives were synthesized and evaluated for cytotoxic activity. In addition to betulinic acid, a variety of betulinic acid derivatives were developed with the aim to increase the anticancer potency and to improve the pharmacokinetic properties. For example, replacing the cyano group with a methoxycarbonyl was reported to markedly enhance the apoptosis-inducing activities of betulinic acid[66]. Further, C-3 modified Betulinic acid derivatives proved to have better *in vivo* anti-tumor efficacy as compared to betulinic acid in vivo against human colon cancer and also displayed favorable pharmacokinetic properties [67]. Moreover, 17-carboxylic acid modified 23- hydroxy betulinic acid ester derivatives demonstrated for cytotoxic activity on five cancer cell lines *in vitro*: all tested compounds showed higher cytotoxic activity as compared to 23-hydroxy betulinic acid and betulinic acid *in vitro* and also in vivo [68]. One study showed C-20 modifications involve the loss of cytotoxicity and demonstrated the importance of the presence of the - COOH group, since compounds substituted at this position, such as lupeol and methyl betulinate, were less active on human melanoma than betulinic acid. Also, some C-28 amino acids and C-3 phthalates derivatives exhibited higher cytotoxic activity against cancer cell lines with improved selective toxicity and water solubility. 28-O-β-D-glucopyranoside glucoside did not exhibit any significant *in vitro* activity on human melanoma (MEL-2) and human colorectal adenocarcinoma (HT-29) cell lines, which confirms the importance of the carboxylic acid function to preserve the cytotoxicity. Gauthier et al. synthesized a series of 3-O-glycosides of betulinic acid which exhibited a strongly potent *in vitro* anticancer activity against human cancer cell lines [69]. A recent study has shown that ten new 3,4-seco Bet A derivatives were designed and synthesized. Among them, compounds 7-15 exhibited enhanced chemopreventive ability in an *in vitro* short-

term 12-O-tetradecanoylphorbol-13-acetate (TPA) induced Epstein-Barr virus early antigen (EBV-EA) activation assay. The most potent compound displayed 100% inhibition at $1 \times 10(3)$ mol ratio/TPA [80].

10. Conclusion

Betulinic acid is a phytochemical which shows potent anticancer activity. The mechanism behind the anticancerous activity of Bet A is induction of apoptosis in cancer cells by its immunomodulatory activity through the cytokine release and generation of ROS. In addition, alterations in some enzymes and expression of certain other proteins may also contribute to its anticancerous activity.

Conflicts of Interest

The author declared no competing interests.

References

[1].Website: HO cancer fact sheet 2014.

[2].How many different types of cancer are there : Cancer Research UK : Cancer Help UK

[3].Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharankan ST, Lai OS, Sung B, Aggarwal BB. Cancer is a preventable disease that requires major lifestyle changes. Pharm Res 2008; 25: 2097-116.

[4].Heredity and Cancer. American Cancer Society.

[5].Kushi LH, Doyle C, McCullough M, Kushi LH, Doyle C, McCullough M, Rock CL, Demark-Wahnefried W, Bandera EV, Gapstur S, Patel AV, Andrews K, Gansler T. American Cancer Society Guidelines on nutrition and physical activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity. CA Cancer J Clin 2012; 62: 30–67.

[6]. Website: Openclinical.net

[7].Oki K. Dzivenu., and Jill O'Donnell-Tormey,. Cancer and the immune system: The Vital Connection. A publication from Cancer Research Institute. 2003.

[8].Hua Y., Marcin K. and Drew P. Crosstalk between cancer and immune cells. Nature Reviews Immunology 2007; 7:41-51.

[9].Andersen MH, Schrama D, Thor Straten P, Becker JC. "Cytotoxic T cells", the Journal of Investigative Dermatology 2006; 126: 32-41.

[10].Abbas, A.K., Murphy, K.M. and Sher, A. Functional diversity of helper T lymphocytes. Nature 1996; 787-793.

[11]. Chechlińska M., Duma A., Świerkowska K., Kamińska J. and Steffen J. Sera Of Lung Cancer Patients Affect The Release Of Th1, Th2 And Monocyte-Derived Cytokines, And The Expression Of IL2R-α By Normal, Stimulated Mononuclear Cells. Cellular & Molecular Biology Letters. 2004; Volume 9, pp. 69 – 81.

[12].Djaldetti M, Bessler H. Modulators affecting the immune dialogue between human immune and colon cancer cells. World J Gastrointest Oncol 2014; 6: 129-138.

[13].Hazelbag S, Fleuren GJ, Baelde JJ, Schuuring E, Kenter GG, Gorter A. 2001.Cytokine profile of cervical cancer cells. Gynecol Oncol 2014; 83(2):235-243.

[14].Vinuselvi. P., AbiramiVeena. R., Vani. V., Sanjay Prasad. YING YANG Effect of IL-10 in the incidence of Cervical Cancer. Advanced Biotech 2008; 26-29.

[15]. Ben-Baruch A. Host microenvironment in breast cancer development: Inflammatory cells, cytokines and chemokines in breast cancerprogression: reciprocal tumor–microenvironment interactions. Breast Cancer Res 2003; 5:31-36.

[16].Djavana B., Eckersberger E., Espinosaa G., Kramer G., Handisuryab A., Leec C., Marberger M., Lepor H., Georg E. Complex Mechanisms in Prostatic Inflammatory Response. European Urology Supplements 2009; 8:872–878.

[17]. Salman H, Ori Y, Bergman M, Djaldetti M, Bessler H. Human prostate cancer cells induce inflammatory cytokine secretion by peripheral blood mononuclear cells. Biomed Pharmacother 2012; 66:330-333.

[18]. Diakowska D. Cytokines Association with Clinical and Pathological Changes in Esophageal Squamous Cell Carcinoma Disease Markers. 2013; Vol 35; 6, 883–893.

[19]. Sara D. Johnson I, Anna-Maria A, De Costa and M. Rita I. Young. Effect of premalignant and tumor microenvironment on immune cell cytokine production in head and neck cancer. Cancers 2014; 6:756-770.

[20]. Cravotto G, Boffa L, Genzini L, Garella D. phytotherapeutics: an evaluation of the potential of 1000 plants. J Clin Pharm Ther 2010; 35: 11-48.

[21].Tan Y, Yu R, Pezzuto JM. Betulinic acid-induced programmed cell death in human melanoma cells involves mitogen-activated protein kinase activation. Clinical Cancer Research 2003; 9: 2866-75

[22]. Thurnher, D.; Turhani, D.; Pelzmann, M.; Wannemacher, B.; Knerer, B.; Formanek, M.; Wacheck, V.; Selzer, E. Betulinic acid: a new cytotoxic compound against malignant head and neck cancer cells. Head Neck 2003; 25, 732-74

[23]. Fulda, S.; Friesen, C.; Los, M.; Scaffidi, C.; Mier, W.; Benedict, M.; Nunez, G.; Krammer, P.H.; Peter, M. E.; Debatin, K. M. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. Cancer Res. 1997; 57: 4956-4964.

[24]. Introduction: Biochemistry, Physiology and Ecological Functions of Secondary Metabolites. Annual Plant Reviews Volume 40: Biochemistry of Plant Secondary Metabolism, Second Edition. 26 Mar 2010.

[25]. Raisova M, Hossini AM, Eberle J, et al. The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. The Journal of Investigative Dermatology 2001; 117: 333–340.

[26]. Mishra T., Khullar M., and Bhatia A. Anticancer Potential of Aqueous Ethanol Seed Extract of Ziziphus mauritiana against Cancer Cell Lines and Ehrlich Ascites Carcinoma Evid Based Complement Alternat Med 2011; 1-11.

[27]. Chowdhury AR, Mandal S, Mittra B, Sharma S, Mukhopadhyay S, Majumder HK. Betulinic acid, a potent inhibitor of eukaryotic topoisomerase I: identification of the inhibitory step, the major functional group responsible and development of more potent derivatives. Medical Science Monitor. 2002; 8: 254-265.

[28]. Hanahan, D.; Weinberg, R. A. The hallmarks of cancer. Cell 2000; 100: 57 -70.

[29]. Galluzzi, L.; Larochette, N.; Zamzami, N.; Kroemer, G. Mitochondria as therapeutic targets for cancer chemotherapy. Oncogene 2006; 25, 4812-4830.

[30].Saelens, X.; Festjens, N.; Vande Walle, L.; van Gurp, M.; van Loo, G.; Vandenabeele, P. Toxic proteins released from mitochondria in cell death. Oncogene 2004; 23: 2861-2874.

[31]. Walczak, H.; Krammer, P. H. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp. Cell Res 2000; 256: 58-66.

[32]. Potze L, Mullauer FB, Colak S, Kessler JH and Medema JP. Betulinic acid-induced mitochondria-dependent cell death is counterbalanced by an autophagic salvage response. Cell Death and Disease 2014; 5.

[33]. Reiner T, Parrondo R, de las Pozas A, Palenzuela D, Perez-Stable C. betulinic acid selectively increases protein degradation and enhances prostate cancer-specific apoptosis: possible role for inhibition of deubiquitinase activity. PLos ONE 2013; 8: 1-11.

[34].Adams, J. M.; Cory, S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 2007; 26: 1324-1337.

[35]. Salvesen, G. S.; Duckett, C. S. IAP proteins: blocking the road to death's door. Nat. Rev. Mol. Cell Biol 2002; 3: 401-410

[36]. Fulda S., Kroemer G. Targeting mitochondrial apoptosis by betulinic acid in human cancers. Drug Discovery Today 2009; 14:885-89

[37]. Micheau, O. Cellular FLICE-inhibitory protein: an attractive therapeutic target? Expert Opin. Ther. Targets 2003; 7: 559-573.

[38].Green, D. R.; Kroemer, G. The pathophysiology of mitochondrial cell death. Science 2004; 305: 626-629.

[39]. Selzer, E. Thallinger, C. Hoeller, C. Oberkleiner, P. Wacheck, V. Pehamberger, H. Jansen, B. Betulinic acid-induced Mcl-1 expression in human melanoma--mode of action and functional significance. Mol. Med 2002; 8: 877-884.

[40]. Ehrhardt, H.; Fulda, S.; Fuhrer, M.; Debatin, K. M.; Jeremias, I. Betulinic acid-induced apoptosis in leukemia cells. Leukemia 2004; 18:1406-1412.

[41]. Kasperczyk, H.; La Ferla-Bruhl, K.; Westhoff, M. A.; Behrend, L.; Zwacka, R. M.; Debatin, K. M.; Fulda, S. Betulinic acid as new activator of NF-kappaB: molecular mechanisms and implications for cancer therapy. Oncogene 2005; 24: 6945-6956.

[42]. Takada, Y.; Aggarwal, B. B. Betulinic acid suppresses carcinogen-induced NF-kappa B activation through inhibition of I kappa B alpha kinase and p65 phosphorylation: abrogation of cyclooxygenase-2 and matrix metalloprotease-9. J. Immunol 2003; 171: 3278-3286.

[43].Galluzzi, L.; Larochette, N.; Zamzami, N.; Kroemer, G. Mitochondria as therapeutic targets for cancer chemotherapy. Oncogene 2006; 25: 4812-4830.

[44]. Rabi, T. Shukla, S. Gupta, S. Betulinic acid suppresses constitutive and TNFalpha-induced NF-kappaB activation and induces apoptosis in human prostate carcinoma PC-3 cells. Mol. Carcinog 2008; 47: 964–973.

[45]. Deeb, D. Gao, X. Dulchavsky, SA. Gautam, SC. CDDO-me induces apoptosis and inhibits Akt, mTOR and NFkappaB signaling proteins in prostate cancer cells. Anticancer Res 2007; 27: 3035–3044.

[46]. Rieber M, Strasberg Rieber M. Induction of p53 without increase in p21WAF1 in betulinic acid-mediated cell death is preferential for human metastatic melanoma. DNA and Cell Biology 1998; 17: 399–406

[47]. Deeb D, Gao X, Dulchavsky, SA. Gautam SC. CDDO-Me inhibits proliferation, induces apoptosis, downregulates Akt, mTOR, NF-kappaB and NF-kappaB-regulated antiapoptotic and proangiogenic proteins in TRAMP prostate cancer cells. J Exp Ther Oncol 2008; 7: 31–39

[48]. Kwon HJ, Shim JS, Kim JH, Cho HY, Yum YN, Kim SH, Yu J. Betulinic acid inhibits growth factor-induced *in vitro* angiogenesis via the modulation of mitochondrial function in endothelial cells. Jpn. J. Cancer Res 2002; 93: 417-425

[49]. Melzig MF, Bormann H. Betulinic acid inhibits aminopeptidase N activity. Planta Med 1998; 64: 655-657.

[50]. Sjostrom H, Noren O, Olsen J. Structure and function of aminopeptidase N. Adv Exp Med Biol 2000; 477: 25-34.

[51]. Chintharlapalli S, Papineni S, Ramaiah SK, Safe S. Betulinic acid inhibits prostate cancer growth through inhibition of specificity protein transcription factors. Cancer Res 2007; 67: 2816-2823.

[52]. Mukherjee R, Jaggi M, Rajendran P, Srivastava SK, Siddiqui MJA, Vardhan A, Burman AC. Synthesis of 3-O-acyl/3-benzylidene/3-hydrazone/3-hydrazine/17- carboxyacryloyl ester derivatives of betulinic acid as anti-angiogenic agents. Bioorg Med Chem Lett 2004; 14: 3169-3172.

[53]. Syrovets, T. Buchele. B, Gedig, E. Slupsky, JR. Simmet, T. Acetyl-boswellic acids are novel catalytic inhibitors of human topoisomerases I and II alpha. Mol. Pharmacol. 2000; 58: 71–81.

[54]. Sawada N, Kataoka K, Kondo K, Arimochi H, Fujino H, Takahashi Y, Miyoshi T, Kuwahara T, Monden Y, Ohnishi Y. Betulinic acid augments the inhibitory effects of vincristine on growth and lung metastasis of B16F10 melanoma cells in mice. Br. J. Cancer 2004; 90: 1672-1678.

[55]. Shin YG, Cho KH, Chung SM, Graham J, Das Gupta TK, Pezzuto JM. Determination of betulinic acid in mouse blood, tumor and tissue homogenates by liquid chromatography-electrospray mass spectrometry. J Chromatogr B Biomed Sci Appl 1999; 732: 331-336

[56]. Chintharlapalli S, Papineni S, Ramaiah SK, Safe S. Betulinic acid inhibits prostate cancer growth through inhibition of specificity protein transcription factors. Cancer Res 2007; 67:2816–2823.

[57]. Archana AD, Yogita PP, Archana AN. Anticancer activity of BA on MCF-7 tumors in nude mice. Indian J Exp Biology 2013; 51: 485-489.

[58]. Yan-Fang S, Chuan-Kui S, Helmut V, Frank U, Zong-Suo L. Apoptosis of human breast cancer cells induced by microencapsulated Betulinic acid from sour jujube fruits through the mitochondria transduction pathway. Food Chemistry 2013; 138: 1998–2007.

[59]. Tsung-IH, Mei-Chun W, Szu-Yu C, Shih-Ting H, Yu-Min Y, Wu-Chou S, Wen-Chang C, Jan-Jong H. Betulinic Acid Decreases Specificity Protein 1 (Sp1) Level via Increasing the Sumoylation of Sp1 to Inhibit Lung Cancer Growth. Molecular Pharmacology 2012; 82: 1115-1128.

[60]. Udeani GO, Zhao GM, Geun Shin Y, Cooke BP, Graham J, Beecher CW, Kinghorn AD, Pezzuto JM. Pharmacokinetics and tissue distribution of betulinic acid in CD-1 mice. Biopharm. Drug Dispos 1999; 20: 379-383.

[61]. Smith, P. F.; Ogundele, A.; Forrest, A.; Wilton, J.; Salzwedel, K.; Doto, J.; Allaway, G. P.; Martin, D. E. Phase I and II study of the safety, virologic effect, and pharmacokinetics/pharmacodynamics of single-dose 3-o-(3',3'-dimethylsuccinyl)betulinic acid (bevirimat) against human immunodeficiency virus infection. Antimicrob Agents Chemother 2007; 51: 3574-3581.

[62]. Martin DE, Blum R, Doto J, Galbraith H, Ballow C. Multiple-dose pharmacokinetics and safety of bevirimat, a novel inhibitor of HIV maturation, in healthy volunteers. Clin Pharmacokinet 2007; 46: 589-598.

[63]. Arkadiusz O, Anna K, Ewa C, Ewa B, Paulina SB, Jan K, Zofia D. Influence of betulin and 28-O propynoylbetulin on proliferation and apoptosis of human melanoma cells (G-361). Postepy Hig Med Dosw 2014; 68: 191-197.[64]. Cichewicz RH, Kouzi SA. Chemistry, biological activity, and

chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. Med Res Rev2004; 24: 90-114

[65].   Yasukawa K, Takido M, Matsumoto T, Takeuchi M, Nakagawa S. Sterol and triterpene derivatives from plants inhibit the effects of a tumor promoter, and sitosterol and betulinic acid inhibit tumor formation in mouse skin two-stage carcinogenesis. Oncology 1991; 48 72-76

[66].   Liby K, Honda T, Williams CR, Risingsong R, Royce DB, Suh N, Dinkova-Kostova AT, Stephenson KK, Talalay P, Sundararajan C, Gribble GW, Sporn MB. Novel semisynthetic analogues of betulinic acid with diverse cytoprotective, antiproliferative, and proapoptotic activities. Mol. Cancer Ther 2007; 6: 2113-2119.

[67]. Rajendran P, Jaggi M, Singh MK, Mukherjee R, Burman AC. Pharmacological evaluation of C-3 modified Betulinic acid derivatives with potent anticancer activity. Invest. New Drugs 2008; 26: 25-34.

[68]. Bi Y, Xu J, Wu X, Ye W, Yuan S, Zhang L. Synthesis and cytotoxic activity of 17- carboxylic acid modified 23-hydroxy betulinic acid ester derivatives. Bioorg Med Chem Lett 2007; 17: 1475-1478.

[69].   Gauthier C, Legault J, Lebrun M, Dufour P, Pichette A. Glycosidation of lupane-type triterpenoids as potent *in vitro* cytotoxic agents. Bioorganic & Medicinal Chemistry 2006; 14: 6713- 25.

[70]. Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA, Beecher CW, Fong HH, Kinghorn AD, Brown DM, et al. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med 1995; 1: 1046-1051.

[71]. Fulda, S.; Jeremias, I.; Steiner, H. H.; Pietsch, T.; Debatin, K. M. Betulinic acid: a new cytotoxic agent against malignant brain-tumor cells. Int J Cancer 1999; 82: 435-441

[72]. Zuco V, Supino R, Righetti SC, et al. Selective cytotoxicity of betulinic acid

on tumor cell lines, but not on normal cells. Cancer Letters 2002; 175: 17–25.

[73]. Zdzisińska B, Rzeski W, Paduch R, Szuster-Ciesielska A, Kaczor J, Wejksza K, Kandefer-Szersze M. Differential effect of betulin and betulinic acid on cytokine production in human whole blood cell cultures. Pol J Pharmacol 2003; 55:235-238.

[74]. Yoon JJ, Lee YJ, Kim JS, Kang DG, Lee HS. protective role of betulinic acid on tnf-alpha-induced cell adhesion molecules in vascular endothelial cells. Biochem Biophys Res Commun 2009; 391:96-101

[75]. Viji V, Shobha B, Kavitha SK, Ratheesh M, Kripa K, Helen A. Betulinic acid isolated from Bacopa monniera (L.) Wettst suppresses lipopolysaccharide stimulated interleukin-6 production through modulation of nuclear factor-kappaB in peripheral blood mononuclear cells. Int Immunopharmacol 2010; 10:843-849.

[76]   Tsai JC, Peng WH, Chiu TH, Lai SC, Lee CY. Anti-inflammatory effects of Scoparia dulcis L. and betulinic acid.Am J Chin Med 2011; 39:943-56.

[77]. Szuster-Ciesielska A, Plewka K, Kandefer-Szerszeń M. Betulin, betulinic acid and butein are inhibitors of acetaldehyde-induced activation of liver stellate cells. Pharmacol Rep 2011; 63:1109-1123.

[78]. Blaževski J, Petković F, Momčilović M, Paschke R, Goran N, Marija K, Mos- Tarica S, Djordje M. Betulinic acid regulates generation of neuroinflammatory mediators responsible for tissue destruction in multiple sclerosis *in vitro*. Acta Pharmacologica Sinica 2013; 34: 424–431.

[79].   Jine Yi, Wei Xia, Jianping Wu, Liyun Yuan, Jing Wu, Di Tu, Jun Fang, Zhuliang Tan. Betulinic acid prevents alcohol-induced liver damage by improving the antioxidant system in mice. J Vet Sci 2014; 15: 141–148.

[80]. Hung HY, Nakagawa-Goto K, Tokuda H, Iida A, Suzuki N, Bori ID, Qian K, Lee KH. A-ring modified betulinic acid derivatives as potent cancer preventive agents. Bioorg Med Chem Lett 2014; 24:1005-1008.