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Natural products are the future of anticancer therapy: Preclinical and clinical advancements of *Viscum album* phytometabolites

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Abstract

Cancer is a multifaceted and genomically complex disease. Research over the years has gradually provided a near complete resolution of cancer landscape and it is now known that genetic/epigenetic mutations, inactivation of tumor suppressors, Overexpression of oncogenes, spatio-temporally dysregulated intracellular signaling cascades, epithelial to mesenchymal transition (EMT), metastasis and loss of apoptosis are some of the most extensively studied biological mechanisms that underpin cancer development and progression. Increasingly it is being realized that current therapeutic interventions are becoming ineffective because of tumor heterogeneity and rapidly developing resistance against drugs. Considerable biological activities exerted by bioactive ingredients isolated from natural sources have revolutionized the field of natural product chemistry and rapid developments in preclinical studies are encouraging. *Viscum album* has emerged as a deeply studied natural source with substantial and multifaceted biological activities. In this review we have attempted to provide recent breakthroughs in existing scientific literature with emphasis on targeting of protein network in cancer cells. We partition this review into different sections, highlighting latest information from cell culture studies, preclinical and clinically oriented studies. We summarized how bioactive ingredients of *Viscum album* modulated extrinsic and intrinsic pathways in cancer cells. However, surprisingly, none of the study reported stimulatory effects on TRAIL receptors. The review provided in-depth analysis of how *Viscum album* modulated Endoplasmic Reticulum Stress in cancer cells and how bioactive chemicals tactfully targeted cytoskeletal machinery in cancer cells as evidenced by cell culture studies. It is noteworthy that *Viscum album* has entered into various phases of clinical trials, however, there are still knowledge gaps in our understanding regarding how various bioactive constituents of *Viscum album* modulate intracellular signaling cascades in cancer. Better and deeper comprehension oncogenic signaling cascades will prove to be helful in getting a step closer to individualized medicine.

Key words: TRAIL, Cancer, Apoptosis, Death receptor, Signaling.

Introduction

Data obtained from sequencing and genome-wide expression studies has helped in carving a broader landscape of molecular oncology. Intriguingly, inactivation of tumor suppressor genes, overexpression of oncogenes, genetic/epigenetic mutations (1,2), genomic instability, chromosomal rearrangements, fused oncoproteins (3) and different spatio-temporally dysregulated intracellular signaling cascades have been noted to play key roles in cancer development and progression. It is becoming characteristically more understandable that because of tumor heterogeneity, new layers of intricacy have been added into a complex picture of molecular cancer representing diversified molecular subclasses traversing conventional grade and stage groupings (4,5) . Genome re-sequencing has provided information related to differential mutation loads which may vary by several orders of magnitude (6).

There is an unending list of synthetic and natural agents being tested for remarkable biological activities and natural products have doubtlessly provided many of the lead structures, acting as templates for designing and development of novel compounds with enhanced biological properties.

Some naturally derived molecules have already been shown to be effective against cancer (7) For example, Vinblastine and vincristine, isolated from *Catharanthus roseus* (L.) G. Don (Apocynaceae) , have entered into the existing list of clinically effective agents (7) Camptothecin, isolated from *Camptotheca acuminata Decne*. (Nyssaceae), selectively inhibited topoisomerase I and recent research is focusing on identification of efficient delivery systems for Camptothecin-based-therapeutics. Paclitaxel originally isolated from Taxus brevifolia Nutt. (Taxaceae) was introduced for clinical use in early 1990s (7).

Mistletoe, *Viscum album* L., grows on different plants and particularly on deciduous trees, has been widely used both in traditional and complementary medicine. Various extracts prepared from different parts of the plant have well established biological activities. *Viscum album* extracts and preparations from mistletoes grown on different host plants using different extraction

solvents and processed through various procedures are well documented.

Categorically Mistletoe lectins are characterized as type II ribosome inactivating proteins, consisting of two protein chains linked intermolecularly by Disulphide Bridge. Carbohydrate binding-lectin motifs are present in Chain B of ML1 (32 kDa) reportedly involved in facilitating the attachment to the target cell and translocation of A chain to cytoplasm. Interestingly, isoforms are differentially specific to monosaccharides for example D-galactose (ML I), and N-acetyl-D-galactosamine(ML III) and MLII notably attached to both carbohydrates. Moreover, these compounds, at non-toxic concentrations, transcriptionally enhanced and induced secretion of pro-inflammatory cytokines TNFa, IL-1 and IL-6 (8).

Triterpenes are essential bioactive constituents of mistletoe, however poorly soluble in H_2O. Extraction was noted to be improved by using sodium phosphate with increasing pH. There was a concentration dependent increase in solubility of triterpene acids as evidenced by 77.2 µg/mL of Oleanolic Acid (OA) and 40.1 µg/mL Betulinic Acid (BA) at pH 11.4 (9). 2-hydroxypropyl-b-cyclodextrin as solubilizer has also been used as an effective agent to enhance triterpene isolation from mistletoe (10).

This review summarizes recent advancements made in cell culture, preclinical and clinical studies related to *Viscum album* anticancer activities. We also discuss how apoptosis inducing machinery is modulated by VA derived bioactive components, and how these bioactive components of extracts target MAPK pathway and ER stress pathway in cancer cells. Before specifically focusing on existing literature on signaling pathways, we briefly review how functionally active AKT is negatively regulated by VA.

Cell Culture Studies

Confluence of information suggested that PI3K–AKT pathway played a vital role in growth and survival of cancer cells. AKT, a serine/threonine kinase, is frequently over-expressed in different cancers. Targeting of PI3K/AKT pathway is necessary to effectively inhibit cancer progression. This section deals with the targeting of AKT.

Iscador M , Iscador Qu Spezial and Iscador P are different *Viscum album* extracts tested for efficacy in SCC9 and SCC25 cancer cells. Intriguingly, slightly reduced pAKT were noted in Iscador M and Iscador Qu treated cancer cells (11). Abnobaviscum F®, a European mistletoe extract has been shown to efficiently induce apoptosis via inhibition of pAKT in myeloid leukemia K562 cells (12). Shown in figure 1. AKT was notably dephosphorylated in *Viscum album* var. *coloratum*, VCA treated A253 cancer cells (13).

Next we emphasize on how *Viscum album* controlled apoptotic pathway in cancer cells and is there any role of VA derived bioactive ingredients in stimulating receptor expression of apoptosis inducing ligand (TRAIL) in cancer cells.

Apoptosis

Apoptotic cell death is an extensively studied molecular mechanism and tremendously accumulating experimental evidence has identified an intricate protein network modulating cancer progression. Apoptosis is triggered by binding of FasL, TNFα and TRAIL to their respective death receptors (14). It can also induced as a result of DNA damage by UV irradiation and cytotoxic agents (15, 16). Characteristically, apoptotic cell death is triggered either through an extrinsic pathway operated through ligand-death receptor-interaction or through mitochondrial pathway (intrinsic pathway). DR-mediated activation of caspase is inhibited by cFLIP, which interferes with activation of caspase-8. Functionally active caspase-8 activates its downstream effector caspase-3. Caspase-8 can also proteolytically cleave Bid into a truncated form (tBid) that shuttles into mitochondrion to facilitate cytosolic accumulation of cytochrome-c. Cytosolically accumulated cytochrome c, in the presence of ATP and APAF1 forms a apoptosome that consequently activates caspase-9 (15, 16). There is a delicate stoichiometric ratio of pro- and anti-apoptotic proteins. Bcl-2 and Bcl-XL are anti-apoptotic proteins which protect against mitochondrial transmembrane potential loss that is induced by pro-apoptotic molecules, such as BAX and BAK (15, 16).

Mistletoe lectin I (ML-I) isolated from *Viscum album* L. extract has previously been reported to effectively trigger receptor-independent intrinsic pathway because it rapidly promoted cytosolic accumulation of cytochrome c. Death receptor independent ML-I activity was verified using dominant-negative FADD mutant overexpressing cancer cells that revealed higher apoptotic rate (17). Triterpene acid-containing extracts significantly induced apoptosis through extrinsic and

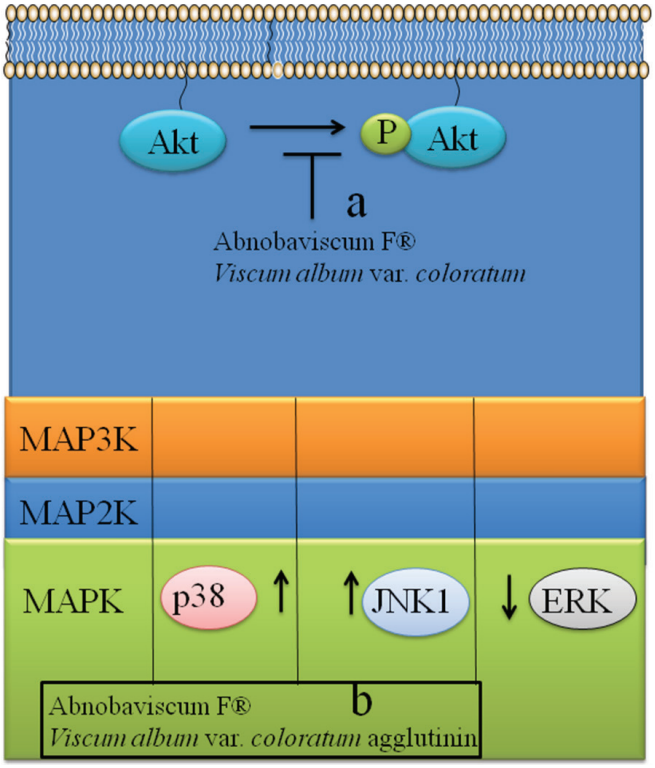

Figure 1. shows (a)conversion of inactive Akt into functionally active form. (a)Abnobaviscum and Viscum album var. coloratum agglutinin inhibited phosphorylation of Akt. (b) Hierarchically organized modulators of MAPK signaling cascade are shown. Abnobaviscum and Viscum album var. coloratum agglutinin notably enhanced p38 and JNK1 activation.

intrinsic pathways (18). Shown in figure 2.

Viscum album agglutinin-I (VAA-I) potently induced apoptotic cell death in chronic granulomatous disease (X-CGD) cells and PLB-985 cells. Moreover, extrinsic and intrinsic pathways were also noted to be considerably enhanced in VAA-I treated cancer cells (19). Shown in figure 2. There was a considerable cytosolic accumulation of cytochrome c in hepatocellular carcinoma cell line (SMMC7721) treated with recombinant VAA-I (20). It is important to note that Abnobaviscum F®, a European mistletoe extract did not modulate DR4 or DR5 levels in myeloid leukemia K562 cells (12). *Viscum album* var. *coloratum*, VCA significantly induced apoptosis in A253 cancer cells via Bax up-regulation and downregulation of Bcl-2 (13).

Oleanolic acid rich triterpene extract from *Viscum album* solubilized by 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD) has previously been shown to effectively kill mouse melanoma B16.F10 cells (et al, 10).

Abnobaviscum F®, a European mistletoe extract increased functionally active caspase-9 in treated leukemic cells. Protein levels of Mcl-1were also significantly reduced however decrease in mRNA was not noted. Surprisingly, mRNA levels of DR4 and DR5 were also not effected (12).

Recent literature suggested that *Viscum album* triggered expression of DR4 or DR5 in HL-60 cells by solubilised triterpene acids present in *Viscum album L.* extracts. Triterpene acids dose-dependently increased DR4 expression, whereas DR5 firstly revealed upregulation and then down-regulation in treated cells (33).

It is clear that VA contributed in re-balancing of pro- and anti-apoptotic proteins but future studies must converge on synergistic administration of VA derived bioactive ingredients with phytochemicals with reported potential to stimulate expression of DR4 or DR5. Furthermore VA may exert cell type specific effects in different cancer subtypes that still needs to be investigated. Multi-pronged approach consisting of TRAIL based therapeutics with VA and phytochemicals having potential to enhance DR4 or DR5 will be helpful in achieving considerable results in preclinical studies.

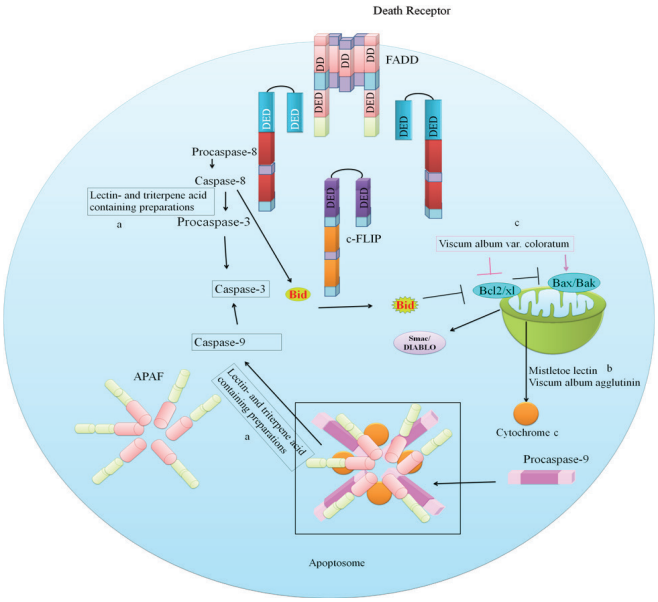

Figure 2. shows intrinsic and extrinsic pathways in cancer cells. (a) Lectin and Triterpene acid-containing extracts functionalized both extrinsic and intrinsic pathways. (b) Mistletoe lectin and *Viscum album* agglutinin enhanced cytosolic accumulation of cytochrome c. (c) *Viscum album* var. coloratum inhibited Bcl2/XL and activated Bax.

Methanolic extract of *Viscum album* induced activation of caspase-3 in C6 rat glioma cells. Moreover, expression levels of 14-3-3 and Hsp27 were also dramatically reduced (21).

Upcoming heading deals with overview of targeting of MAPK pathway by *Viscum album*.

MAPK Pathway

Evolutionarily conserved mitogen-activated protein kinase (MAPK) pathway is an essential signal transduction cascade reportedly involved in modulation of different cellular functions. The three main arms of MAPK pathway, ERK (*extracellular* signal-regulated kinase), p38 and JNK (c-Jun N-terminal kinase), that respond to diverse signals communicated intracellularly through multiple receptors (22, 23). MAPK mediated signal transduction cascade was previously considered simplistically as a linear receptor-to-nucleus pathway, but newer lines of evidence have considerably improved our understanding of role of reversibly phosphorylated kinases in multiple cascades controlled by multiple feedback loops and crosstalks with other linear cascades. It is relevant to mention that VA differentially influenced MAPKs in cancer cells.

Levels of phosphorylated ERK1/2 were reduced in Abnobaviscum F®, a European mistletoe extract treated leukemic cells. However, phosphorylated levels of p38 MAPK and JNK1/2 were notably enhanced (12). Shown in figure 1. JNK1 levels were also enhanced in *Viscum album* L. coloratum agglutinin treated hepatocarcinoma Hep3B cells. Mechanistically, JNK1 overexpressing Hep3B cancer cells demonstrated markedly enhanced apoptotic rate. However, Hep3B cells expressing dominant negative JNK1 did not respond to treatment (24). Shown in figure 1.

Therefore, these kinases are hierarchially organized to tactfully control molecular mechanisms. Expectedly, because of complicated crosstalk, many kinase inhibitors did not show encouraging results in clinical trials.

Next we discuss how ER stress modulators are regulated by *Viscum album* in cancer cells.

ER Stress

Protein misfolding in the Endoplasmic Reticulum is a deeply studied system and mechanistically it has been reported to cause rapid accumulation of misfolded proteins (ER stress) as an alarm that causes activation of unfolded protein response (UPR). Cellular adaptability to ER stress is achievable by the unfolded protein response activation which is an intricate signal transduction cascade reportedly involved in modulation of multifaceted ER responses. When these nano-machines are unable to efficiently manage accumulating unfolded protein load, cells undergo apoptosis (25).

Activating transcription factor 6 (ATF6), an endoplasmic reticulum (ER) proximal unfolded protein response (UPR) protein is functionally inactive in unstressed cells. Immunoglobulin-heavy-chain-binding protein (BIP/GRP78) inhibited ATF6 activation in unstressed

cells (26).

There is a constitutive expression of ATF6 as a 90-kDa protein (p90ATF6) notably embedded in ER. It further is proteolytically processed into 50-kDa protein (p50ATF6) in ER-stressed cells located significantly in nucleus (27).

Abnobaviscum F has been shown to induce ER stress in leukemic cells as evidenced by time dependent increase in GRP78 expression and phosphorylated levels of eIF-2α. However, there was a time dependent decline in levels of p90ATF6, slightly raised levels of p50ATF6 and an increase in CHOP mRNA levels were also reported (12). There are direct pieces of evidence emphasizing on CHOP mediated upregulation of DR4 and DR5 in cancer cells however, astonishingly CHOP did not effect expression levels of these receptors. It needs to be seen if synergistic administration of bioactive ingredients of *viscum album* with other natural agents can stimulate DR4 and DR5 in treated cancer cells.

Future studies must converge on identification of allosteric sites on UPR components and regulators that fine-tune UPR. High throughput assays for drug discovery and characteristically defining phytochemicals mediated effects on the pathway as a global network will also be helpful in improving our knowledge.

Cytoskeletal Proteins

Intermediate filaments (IFs) are nucleoskeletal and cytoskeletal structures reportedly involved in provision of stress-coping resilience and mechanical support to cells. These IFs modulate intracellular communication and are functionally active in cancer cells.

Mostly, myosins belong to class II and work synchronously with actin, making up the major contractile proteins of skeletal, smooth and cardiac muscle, in which the sliding crossbridges connect thin actin filaments with thick myosin filaments. Non-muscle myosin II (NM II), an actin binding protein is activated by kinases via phosphorylation of its heavy and light chains (28).

Viscum album agglutinin-I (VAA-I) has previously been reported to induce breakdown of cytoskeletally associated proteins in leukemic PLB-985D cells. Caspase-3 mediated degradation of gelsolin was noted in VAA-I treated leukemic PLB-985D cells. It had also been convincingly revealed that VAA-I induced cleavage of lamin B, paxillin and vimentin . Shown in figure 3. Moreover, native form of NMHC-IIA (c. 200 kDa) and proteolytically processed forms of F95 and F60 were detected in VAA-I treated PLB-985D leukemic cells (29).

In-vivo studies

Viscum Album aqous extract Isorel® (Novipharm GmbH, Austria) has shown immunomodulation, tumor growth inhibition and metastases reduction in tumor bearing mice (30). ISCADOR, fermented plant extract has been shown to effectively inhibit tumor growth in nude mice subcutaneously implanted with LNT-229 GBM cells. It is noteworthy that intratumorally injected (20 µL, 100 µg/mL) of ISCADOR Q exerted considerable tumor growth retarding effects as compared to subcutaneously injected ISCADOR Q on the contralateral body site (31). Anti-tumor effects of the mistletoe extracts were enhanced when combined with solubilized triterpenoids. Treating xenografted mice with this combination resulted in considerably enhanced anti-tumor effects with 6 temporary remissions and 2 complete remissions. Significantly reduced CD31-positive tumor blood vessels was also noted in treated xenografted mice (32). Mean survival was prolonged in Pre-B ALL NALM-6 cells bearing SCID mice treated with Triterpene acid-containing extracts (18). Triterpene acids in VAE inhibited tumor growth in NOD/SCID/IL2rg mice bearing systemic HL-60 cells and consequently, tumour weight was significantly reduced. Tolerability of the administered VAE concentrations was impressive with no evidence of toxicity measured by autopsy and weight including histology of spleen, liver and bone marrow (33). *Viscum album* L. var. coloratum coated with a biodegradable polymer, notably inhibited growth of mouse melanoma in vivo. Orally administered enteric-coated mistletoe (430 mg/kg/day) significantly reduced tumor volume (5728.7 mm^3) on 14th day (34).

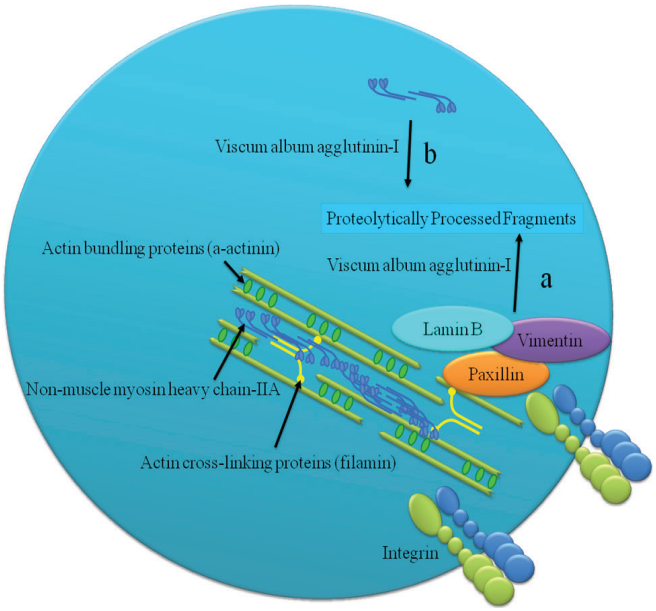

Figure 3. shows structural network in cancer cells. (a) VA agglutinin proteolytically processed Lamin B, Vimentin and Paxillin. (b) Non-muscle myosin heavy chain-IIa was also proteolytically processed by VA agglutinin.

Patient-derived xenograft (PDX) models are also being used in molecular oncology research to improve our comprehension of individualized medicine (35). Next we summarize most recent advancements made in evaluating the clinical efficacy of VA.

Clinical Trials

Intratumourally injected *Viscum album* L extract in a stage IIIC colon cancer patient having a high-grade dysplasia colon adenoma induced significant regression of colon adenoma (36). There was a notable decrease in tumor size in adenoid cystic carcinoma patient intratumourally injected with high-dose of VAE over a 10-month period (37). Randomized Study was conducted in Osteosarcoma Patients to evaluate Postrelapse Disease-Free Survival with Adjuvant Oral Etoposide or Mistletoe. Results revealed that median PRDSF was 39 months in the Viscum group and 4 months in the Etoposide treated group (38).

Mistletoe in combination with Gemcitabine has been studied for efficacy in patients with advanced solid cancers. Results reveald that patients combinatorially treated with gemcitabine and mistletoe had drug tolerability and treatment compliance was high. Maximumally tolerated dosage for gemcitabine was1380 mg/m2 weekly on day one and eight of a 3-week cycle when co-administered with mistletoe 250 mg daily (39). Locally advanced or metastatic pancreatic cancer patients subcutaneously injected with *Viscum album* L. extract displayed a differential response as evidenced by median Overall Survival (OS) of 6.6 versus 3.2 months within subgroup of 'good' prognosis, and 3.4 versus 2.0 months within the 'poor' prognosis subgroup (40). Patient of cutaneous squamous cell carcinoma was noted to be clinically responsive to high-dose peri-lesional *Viscum album* L. extract injections (41). It has recently been reported that frequency of adverse drug reactions (ADRs) because of intratumorally injected mistletoe was 3 times and 5 times higher than what had earlier been noted in subcutaneously and intravenously administered mistletoe. However, most frequently noted ADRs were immune related and changes in body temperature. Intensity of ADRs ranged from mild (83.8%) to moderate (14.9%) (42).

Conclusion

Although it is well known that cancers show intratumour heterogeneity, we are far away from comprehensive information of communications among subpopulations within tumours. Emerging scientific findings are emphasizing on community based behaviour of cancer cells, and increasing attention is now being directed towards the cooperative behaviour of subclones that can influence drug resistance and progression of disease.

Data obtained through cell culture, pre-clinical and clinical studies is emphasizing on an essential role of *Viscum album* and its bioactive ingredients in regulation of protein network in different cancers. Although tremendous advancements have been made but findings obtained from PhaseII and Phase III will provide more detailed understanding of clinical efficacy and most relevant subgroup of patients responsive to different therapeutic interventions. Moreover, Pharmacokinetics and bioavailability of the chemicals are some of the important facets which need futher research. Overview of the published experimentally verified data although has provided a better understanding of the mechanisms reported to modulate protein netwok in cancer cells, still there are knowledge gaps.

Therapeutics have also been shown to induce stromal, immunological and vascular changes in the tumour microenvironment which may contribute in development of resistance and tumor recurrence (43). Dormant disseminated tumor cells (DTCs) have been reported to show hallmark features including temporary growth arrest, considerable survival and are therapeutically resistant to different drugs (44). Necessarily, molecular therapeutics directed towards niches that harbor dormant DTCs will be helpful in improving the clinical outcome (45).

Different signalling cascades are still insufficiently studied, we still do not have clear knowledge of evidence of modulation of Death receptors by ingredients isolated from *Viscum album.* miRNA mediated post-transcriptional regulation of genes is a well established area of research (46), however whether or not bioactive ingredients isolated from *Viscum album* considerably regulate expression of different miRNAs is an outstanding question that needs detailed research. Interdisciplinary approaches are necessary for the development of anticancer- drugs with notable efficacy and fewer off-target effects.

Other articles in this theme issue include references (47-58).

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