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Salograviolide A: A Plant-Derived Sesquiterpene Lactone with Promising Anti-Inflammatory and Anticancer Effects

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1. Introduction

Natural products are chemical substances produced by living organisms of the Biota superdomain, namely plants, animals, fungi and bacteria (G. & C. Merriam Co., 1913). Up to 80% of all drugs discovered prior to 1981 and 50% of all approved drugs between the years 1994 and 2007 are of natural product origin (Harvey, 2008). The contribution of natural products derived from plants, particularly to the well-being of mankind, extends very far back in history. Some of the most famous findings supportive of this fact come from the Middle East region and include documented medical papyri of ancient Egyptians dating back to ca. 1,850 B.C., as well as recent studies revealing their use of medicinal herbs dispensed in grape wine ca. 3,150 B.C. (McGovern et al., 2009). Incidentally, the oldest evidence for the use of herbal medicine to be so far discovered dates back to the prehistoric Neanderthal man who lived also in the Middle East region around 60,000 years ago (Solecki, 1971).

Several world heritages of medicinal plants have also inspired and greatly contributed to the development of modern medicine (Azaizeh et al., 2008). Indeed some of the early drugs were derived from plants. Morphine, for instance, was the first pharmacologically active pure compound to be extracted from a plant over 200 years ago (Jesse et al., 2009). Clinical, pharmacological, and chemical studies have since then led to the identification of a lengthy list of drugs derived from plants covering a wide range of diseases from diabetes, malaria, microbial infections, osteoporosis to inflammation and cancer.

The story of the discovery of plants exhibiting anticancer properties in particular, began almost fifty years ago. With the increase in cancer incidences, there has been an increase of interest in screening for anti-tumor agents from diverse sources including plants. For that purpose, in 1960 the National Cancer Institute (NCI) launched a large-scale screening of 35,000 sample plants. The program resulted among others, in the discovery in 1967 of the best-selling anticancer drug today; Taxol (Cragg, 1998). This breakthrough boosted cancer researchers all over the world and especially those in regions with high diversity to explore the indigenous plants' active ingredients efficacy against cancer. Considering that more than 60% of anticancer drugs available for clinical use today are derived from natural products including plants (Balunas et al., 2005; Newman and Cragg, 2007), one cannot deny that there has been a successful contribution of plants to the fight against cancer (reviewed in Gali-Muhtasib and Bakkar, 2002; Darwiche et al., 2007).

Lebanon falls within the Levantine Uplands center of diversity. Compared to other Mediterranean countries, it stands second after Turkey in its floristic diversity with 2600 plant species distributed around its humble 10452 km² (Nehmeh, 1977). About 311 plants corresponding to 12% of the total plant species are endemic to Lebanon and have been used in part by Lebanese folk medicine practitioners and Lebanese people for preventive and therapeutic purposes (Nehmeh, 1977). As members of the Nature Conservation Center for Sustainable Futures (IBSAR), we have been leading research over the past 10 years for the understanding and evaluation of Lebanese indigenous plant properties against various conditions, especially cancer. The following chapter aims at retracing our adventure with "Salograviolide A" (Sal A), and bringing to light this peculiar molecule that exhibits both anti-inflammatory and anticancer effects.

2. Sesquiterpene lactones in traditional and conventional medicine

Sesquiterpene lactones (SLs) are generally colourless bitter phytochemicals of lipophylic nature. Thousands of molecules are classified in the SLs subfamily of the terpenoids group of plant secondary metabolites. They are predominantly isolated from leaves or flowering heads of plants of the sunflower family Asteraceae and to a limited extent from Umbelliferae and Magnoliaciae (Heywood et al., 1977). The percentage of SLs per plant dry weight often exceeds 1% (Heywood et al., 1977).

The benefits of many plants enriched with SLs have been described in depth in Mediterranean folk literature with emphasis on their laxative values as well as their potential for the treatment of sores, wounds, sprains, fever, pain, headaches, malaria, anaemia, microbial infections, arthritis, cough, bronchitis, diabetes, hypertension and inflammation (Awadallah, 1984; Moukarzel, 1997).

Similarly, scientific literature supports most of the SLs activities attributed to their plants of origin in traditional medicine such as the hypoglycemic (Genta et al., 2010), antibacterial (Bach et al., 2011), antifungal (Vajs et al., 1999), antiplasmodial (Medjroubi et al., 2005), antinociceptive, antipyretic (Akkol et al., 2009) and anti-inflammatory (Al-Saghir et al., 2009) effects.

There are to date around 1500 publications that have reported the anticancer and anti-inflammatory properties of SLs. Three major SLs and/or many of their synthetic derivatives have reached phase I-II cancer clinical trials, namely thapsigargin from *Thapsia garganica* (Apiaceae), artemisinin and artesunate from *Artemisia annua*, and parthenolide from *Tanacetum parthenium* (Fig. 1) (reviewed in Ghantous et al., 2010). These SLs have properties that enable them to target tumor cells and cancer stem cells while sparing normal cells. They also affect different cancers or inflammation conditions. For example, thapsigargin demonstrated promising results against advanced solid tumors (breast, kidney and intestine) while parthenolide had an effect on blood and lymph nodes tumors (reviewed in Ghantous et al., 2010). Artemisinin showed efficacy against lupus nephritis, metastatic breast and colorectal cancer while clinical evidence indicated that artesunate is effective against nonsmall cell lung cancer, metastatic uveal melanoma and laryngeal squamous cell carcinoma (Berger et al., 2005; Christensen et al., 2009; Efferth, 2006; Guzman et al., 2007, as cited in Ghantous et al., 2010). The chemical basis for the observed biological activities of SLs has been reviewed by our group recently (Ghantous et al., 2010).

Centaurea is one of the largest genera of the Asteracae family with almost 250 species (Font et al., 2008). Plants belonging to the *Centaurea* genus are native to Eurasia. They were

introduced to North America around the late 1800's and can now be found all around the world. *Centaurea* extracts have also been used in traditional medicine for their effects as stimulants, diuretics, analgesics, anti-rheumatics, anti-microbial, anti-diabetics and anti-inflammatory (refer to www.ibsar.org). Anecdotally; the genus' name is a dedication to the centaur Chiron who, according to Greek mythology, had discovered the curative properties of these medicinal plants (Nehmeh, 1977). Scientists eventually investigated the medicinal properties traditionally attributed to the *Centaurea* genus and isolated a multitude of SLs in addition to other various types of compounds such as alkaloids, lignans, acetylenes and flavonoids. When entering on PubMed the search terms "Centaurea and sesquiterpene lactones", and adding to them the hits from the search "Centaurea and cancer", 46 results get displayed. We investigated the number, nature and biological activity of SLs in the different species and summarized some of them in Table 1.

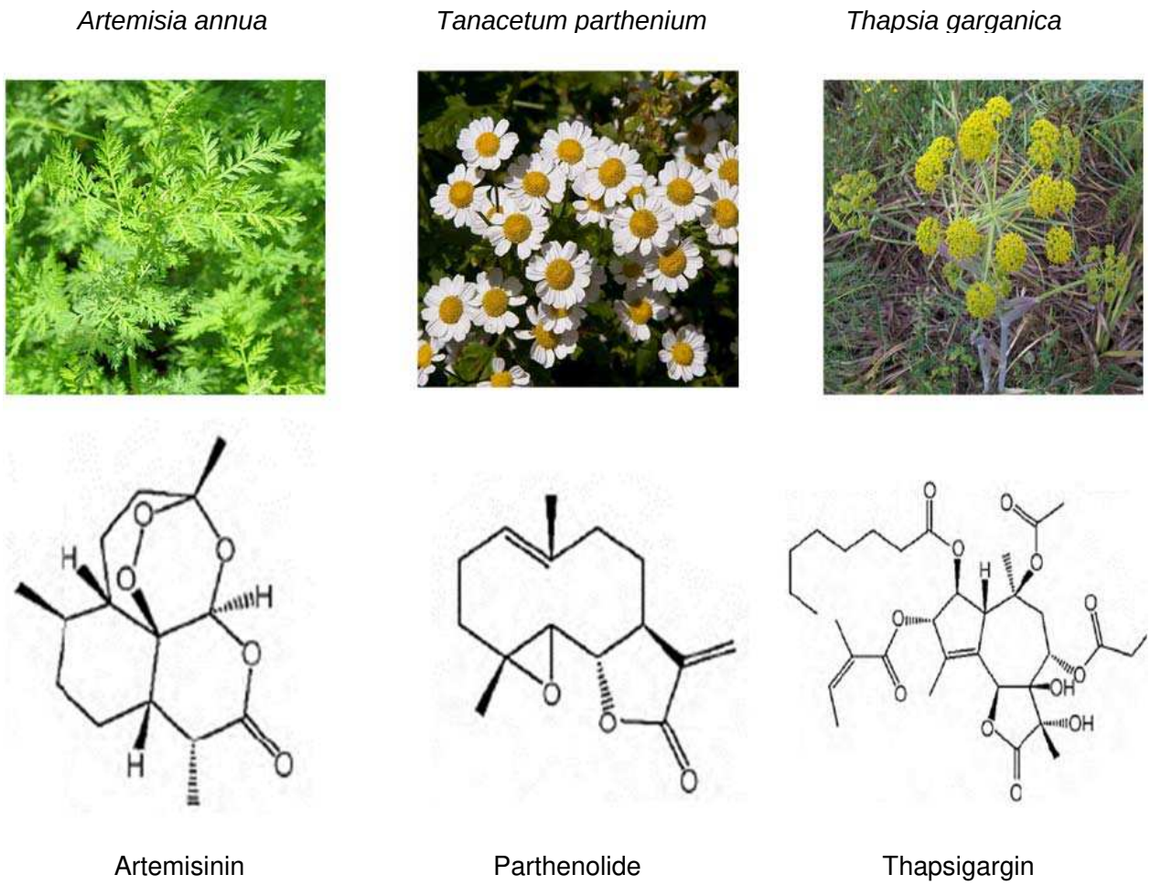


Fig. 1. Illustration of the sesquiterpene lactones that have reached clinical trials, namely thapsigargin extracted from *Thapsia garganica*, artemisinin from *Artemisia annua* L., and parthenolide from *Tanacetum parthenium*. The plants pictures are courtesy of Mr. Luigi Rignanese, Mr. Peter Griffee and Mr. Paul Drobot, respectively.

Table 1 indicates that there are at least 89 SLs in 10 *Centaurea* species. As expected, the amount of the SLs as well as their nature is species-specific. Also, the activities of the represented SLs cover most of those attributed to the plants in traditional medicine. Aside

from Sal A, several articles have reported anticancer properties of SLs extracted from *Centaurea* species (Bruno et al., 2005; Chicca et al., 2011; Csupor-Löffler et al., 2009; El-Najjar et al., 2007; Ghantous et al., 2007; González et al., 1980; Koukoulista et al., 2002; Saroglou et al., 2005).

Plant	Number of SLs	Example of SLs	Activity	Reference
C. bella	26	Repin	Anti-tumor	1, 2
C. musimomum	11	Cynaropicrin	Antiplasmodial Cytotoxic	3, 4
C. napifolia	4	Cnicin	Antibacterial Cytotoxic	1, 5
C. nicolai	5	Kandavanolide	Antifungal	6
C. pullata	10	Melitensin	Antibacterial Antifungal	7
C. scoparia	9	Chlorohyssopifolin	Anti-tumor Antiviral Antimicrobial	8, 9, 10
C. solstitialis	7	Solstitialin A	Hypoglycemic Antiviral Antimicrobial	11, 12, 13, 14
C. spinosa	10	Malacitanolide	Antibacterial Cytotoxic	15
C. sulphurea	3	Sulphurein	-	16
C. tweediei	4	Onopordopicrin	Antibacterial Antifungal Cytotoxic	5, 17, 18

1: Bruno et al., 2005 – 2: Nowak et al., 1993– 3: Cho et al., 2004 – 4: Medjroubi et al., 2005– 5: Bach et al., 2011 – 6: Vajs et al., 1999 – 7: Djeddi et al., 2007, 2008 – 8: González et al., 1980 – 9: Ozçelik et al., 2009 – 10: Youssef et al., 1994, 1998 – 11: Akkol et al., 2011 – 12: Cheng et al., 1992 - 13: Gürbüz et al., 2007 – 14: Ozçelik et al., 2009 – 15: Saroglou et al., 2005 – 16: Lakhal et al., 2010 – 17: Fortuna et al., 2001 – 18: Lonergan et al., 1992.

Table 1. Sesquiterpene lactones of different *Centaurea* species and their biological activities.

3. Salograviolide A: Isolation and chemical characterization

In 1992, Daniewski et al. isolated an unusually hydroxylated SL from the aerial parts of the plant *Centaurea salonitana* Vis. of Bulgarian origin collected in the Greek region Gravia (Fig. 2a). The compound was baptised "Salograviolide A" with the prefix "salo" referring to the

species *salonitana* from which it was isolated, "gravi" in allusion for the region Gravia and finally the suffix "olide" indicating the presence of a lactone group.

The first procedure used for the isolation and characterization of Sal A (Fig. 2b) included drying the plant and grinding it to a powder. This was followed by dividing the dry material (600 g) into three parts that were each soaked in 1 l of methanol (MeOH) for 24 h. The extracts were afterwards combined, evaporated (7.5 g) and dissolved in a mixture of equal amounts of water and chloroform (H_2O-$CHCl_3$ (1:1)). The aqueous layer was extracted three times with chloroform alone and the final step consisted of evaporating the extract and subjecting it to two separate chromatography techniques for optimal purification, thin layer chromatography (TLC) and column chromatography (CC). Infra Red (IR), high resolution Mass Spectrum (MS) and ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy techniques enabled the identification of the structure of Sal A that was later confirmed by Rychlewska et al. (1992) using crystal X-ray diffraction techniques.

Sal A is a 3β-acetoxy-8α, 9β-dihydroxy-lαH, 5αH, 6βH, 7αH-guaian-4(15), 10(14), 11(13)-trien-6, 12-olide with the molecular formula $C_{17}H_{20}O_6$. It is classified according to its carbocyclic skeleton in the guaianolides group, one of the major groups of SLs. Comprised of 15 carbons (15-C) as indicated by the prefix "sesqui", Sal A has 3 isoprene (5-C) units and a lactone group (cyclic ester) (Fig. 2c). The presence of this α-methylene-γ-lactone is thought to be responsible for the biological activity of Sal A because of its ability to react with nucleophiles by a Michael-type addition (Ghantous et al., 2010).

Sal A was subsequently isolated from other *Centaurea* species, namely from *C. nicolai* Bald (Vajs et al., 1999) and from *C. ainetensis* Bois by bioguided fractionation following an extraction scheme adopted from Harborne (1998) (Saliba et al., 2009).

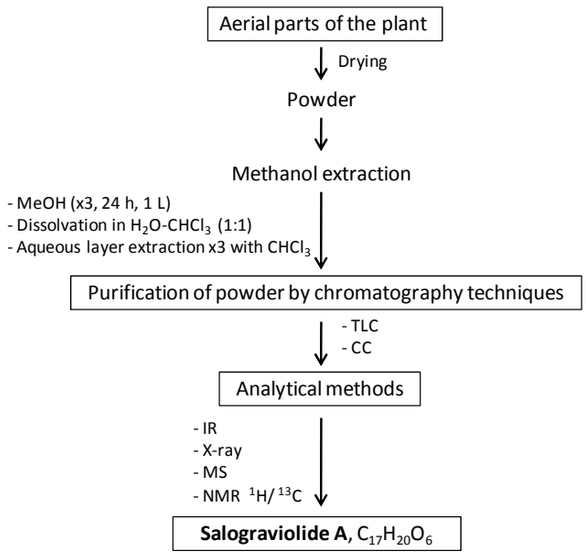
4. IBSAR efforts for bringing Sal A to the forefront

Ibsar, AUB's nature conservation center for sustainable futures, is an interdisciplinary and interfaculty center founded in the year 2002 by AUB faculty. Ibsar's mission is "to promote the conservation and sustainable utilization of biodiversity in arid and Mediterranean regions by providing an open academic platform for innovative research and development", and its vision is "for societies to become guardians and primary beneficiaries of biodiversity in the region" (www.ibsar.org).

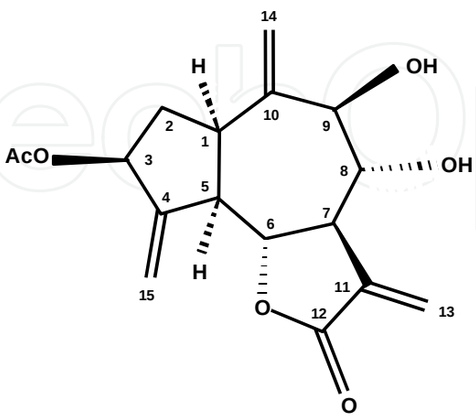
Very early in its establishment, Ibsar recognized that the Lebanese floristic richness also represents an untapped resource for the potential discovery of new therapeutic agents and/or useful dietary supplements. As a result, one of the key program areas in Ibsar has been to integrate traditional knowledge and biotechnology. The objective of this program is to discover useful therapeutic agents that may be hidden in wild Lebanese plants and to develop products attractive to biotechnology industries. Towards this end, plants from the region are collected, extracted and tested for their potential effects on major diseases such as cancer, inflammation, microbial infections, skin diseases and diabetes as well as their value in nutrition and use for general health purposes.

C. ainetensis (Arabic name; Qanturyun Aynata or Shawk al-dardar) whose specimen is deposited at the herbarium of the American University of Beirut (Lebanon), is an endemic plant to Lebanon. It flowers from May to June, has purplish tube of anthers and can only be found growing wild in stony, sterile or bushy places in particular areas in Lebanon, mainly Dayr-ul-Ahmar to Aynata region at elevations of 1200–1800 m above sea level respectively, and in Anti-Lebanon Mountain range above Ayn-Burday at 1250–1300 m (Dinsmore, 1932 as cited in Talhouk et al., 2008).

(a) Left: Photo of the plant *Centaurea salonitana* of Portuguese origin. Right: The aerial part of the plant



(b) Procedure for extraction of Sal A, $C_{17}H_{20}O_6$



(c) Chemical structure of Sal A.

Fig. 2. Illustration of *Centaurea salonitana*, the procedure of Sal A extraction and its chemical structure.

The plant was collected during its flowering season and water extract was obtained from it by decoction. Briefly, the plant was air dried and soaked either entirely or only its ground flower head in hot boiling water for 20-30 min with a ratio of plant material weight to water volume of 1/8 (1 g of plant for every 8 ml of water). The solution was filtered either through 3 mm Whatman filter or through Sterile Gauze sponges 30x30 cm which yielded a residue and a filtrate named the "crude water extract". The resulting aqueous layer was sterilized using 0.2 µm non-pyrogenic sterile-R filter before storing it at -20°C until use.

C. ainetensis water extract is claimed in Lebanese folk literature to have anti-inflammatory effects. However, no research paper prior to 2004 investigating this plant's biological activity had ever been published. A screening of 29 plants reported by traditional medicine practitioners to have anti-inflammatory effects led to the validation of this activity for *C. ainetensis* water extract (Talhouk et al., 2008). Moreover, a screening of 109 wild Lebanese plant extracts, including 41 crude water, 34 methanol and 34 chloroform extracts, has resulted in the identification of selective and anti-proliferative bioactivity against several cancer cell lines in four wild Lebanese plant species namely, *Achillea damascene* also known as *Achillea falcata*, *Centaurea ainetensis*, *Onopordum cynarocephalum* and *Ranunculus myosuroudes* (Table 2). Although, *in vitro*, the three other plant extracts showed higher activities than *C. ainetensis*, the latter extract demonstrated the highest tumor growth inhibition ranging from 73 to 79% when tested *in vivo* in a mouse model of colorectal cancer (El-Najjar et al, 2007).

It was also shown later, that *C. ainestensis* water extract was mildly toxic but largely inhibited metastasis of leukemic cells (El-Sabban, unpublished findings).

Finally the extract was tested against the Infectious Bursal Disease (Gumboro) Virus (IBDV) in broilers and showed a mild reduction of IBDV viral antigens in the Bursa of Fabricius as well as a mild reduction in bursal lesions (Barbour, unpublished findings).

In an attempt to unravel the underlying causes for *C. ainetensis* water extract activity, we isolated the bioactive compound Sal A. The plant was subjected to bio-assay guided fractionation which consisted of testing the anti-tumor, anti-inflammatory and cytotoxicity effects of each fraction of the plant extract. Fractions of the crude water extract were inactive; however, Sal A was obtained from a fractionation of the methanol crude extract. Sal A manifested the same biological activities as the crude water extract with greater efficacy at lower concentrations. A parallel study was conducted to assess the effect of both the water crude extract and methanol crude extract of *C. ainetensis* along with 26 other indigenous Lebanese plants against nine microbial species. The results showed that the crude water extract was inactive against all microbial species whereas the methanol extract was effective against 88.8% of the tested microorganisms (Barbour et al., 2004).

Fig. 3 summarizes the acid-base extraction procedure used to fractionate the methanol crude extract and isolate Sal A. First the methanol crude extract was obtained by soaking the dried plant flowers in methanol with w/v of 1/10 for 16 h.

The mixture was then incubated on a shaker for 2 h at 20°C. The extract was filtered and yielded a residue and a filtrate named the "methanol crude extract". For further fractionation, the residue issued from the methanol extraction was soaked in EtOAc mixture in a ratio of 10/1 w/v. It was then separated by filtration into a residue and a filtrate consisting of fat and waxes and numbered I.1. To the crude methanol extract, concentrated H_2SO_4 solution was then added drop-wise till the pH reached 2. Following, a mixture of $CHCl_3$-H_2O (2:1 ratio) was added. The $CHCl_3$ phase enriched with terpenoids and phenols was collected and labeled as I.2. The aqueous layer, on the other hand, was basified to pH 10

Plant	Name in Arabic	Concentration (%)*	Growth Inhibition (%)	Cell line
Achillea damascene	Akhilia zat al-alf waraqah	0.5	65	Breast: Scp2
				Colon/intestine:
		3	85	HCT-116
		3	80	HT-29
		3	70	Mode K
Centaurea ainetensis	Quanturyun Aynata/ Shawk al-dardar	3	60	Breast: Scp2
				Colon/intestine:
		3	60	HCT-116
		3	80	HT-29
		5	50	Mode K
				Skin:
		3	40	PMK
		3	87	SP1
		3	65	308
		3	67	PAM212
		3	40	17
Onopordum cynarocephalum	Aqsun harshafi al ra's	0.1		Breast:
			50	Scp2
		0.5		Colon/intestine:
		0.5	70	HCT-116
		1.5	50	HT-29
			50	Mode K
Ranunculus myosuroudes	Hawdhan			Skin:
		2	36	PMK
		2	98	SP1
		2	64	308
		2	61	PAM212
		2	20	17

Table 2. Representation of the four Lebanese plant extracts with anticancer potentials. The following results were obtained for the water extracts of *A. damascene*, *C. ainetensis* and *O. cynarocephalum* and the methanol extract of *R. myosuroudes*. Cell proliferation and cytotoxicity were determined using the CellTiter 96 Non-Radioactive Cell Proliferation Assay and the CytoTox 96 Non-Radioactive Cytotoxicity Assay (both kits from Promega, Madison, WI) according to the manufacturer's suggestions.
* % = Volume extract/ Volume media

by adding concentrated NH_4OH drop-wise and then resuspended in a $CHCl_3$-MeOH mixture (3:1 ratio) to be later separated into two organic and aqueous layers labeled I.3 containing alkaloids and I.4, respectively. I.1, I.2, I.3 and I.4 were evaporated to dryness under reduced pressure and weighed. A known amount of each subfraction was dissolved in a known volume of suitable solvent for further chromatographic and or/bioassays analysis.

Fraction "I.2" was the only of three other fractions to exhibit activity against cancer cells and in models of inflammation. Therefore TLC, thick layer chromatography and CC were used to further subdivide the I.2 bioactive fraction and yielded six subfractions I.2.1-I.2.6. Additional testing showed that only subfraction I.2.2 exhibited the anti proliferative, anti-inflammatory activity observed with the fraction I.2. This subfraction also maintained its bioactivity after it was purified using Solid Phase Extraction.

Finally, UV, IR, NMR and MS enabled the identification of the bioactive compound Sal A.

In conclusion, three research papers on *C. ainetensis* water extracts and three on Sal A anti-inflammatory and anti-tumor activities have been so far published by Ibsar while a fourth one is in progress (Al-Saghir et al., 2009; Barbour et al., 2004; El-Najjar et al., 2007; Ghantous et al., 2007; Saliba et al., 2009; Talhouk et al., 2008). Prior to our work, there was only one published article on Sal A's biological activity since its isolation (Vajs et al., 1999). Therefore, Ibsar has had a major contribution in identifying Sal A's biological activity and determining its mechanisms of action.

5. Salograviolide A: Overview of biological activities

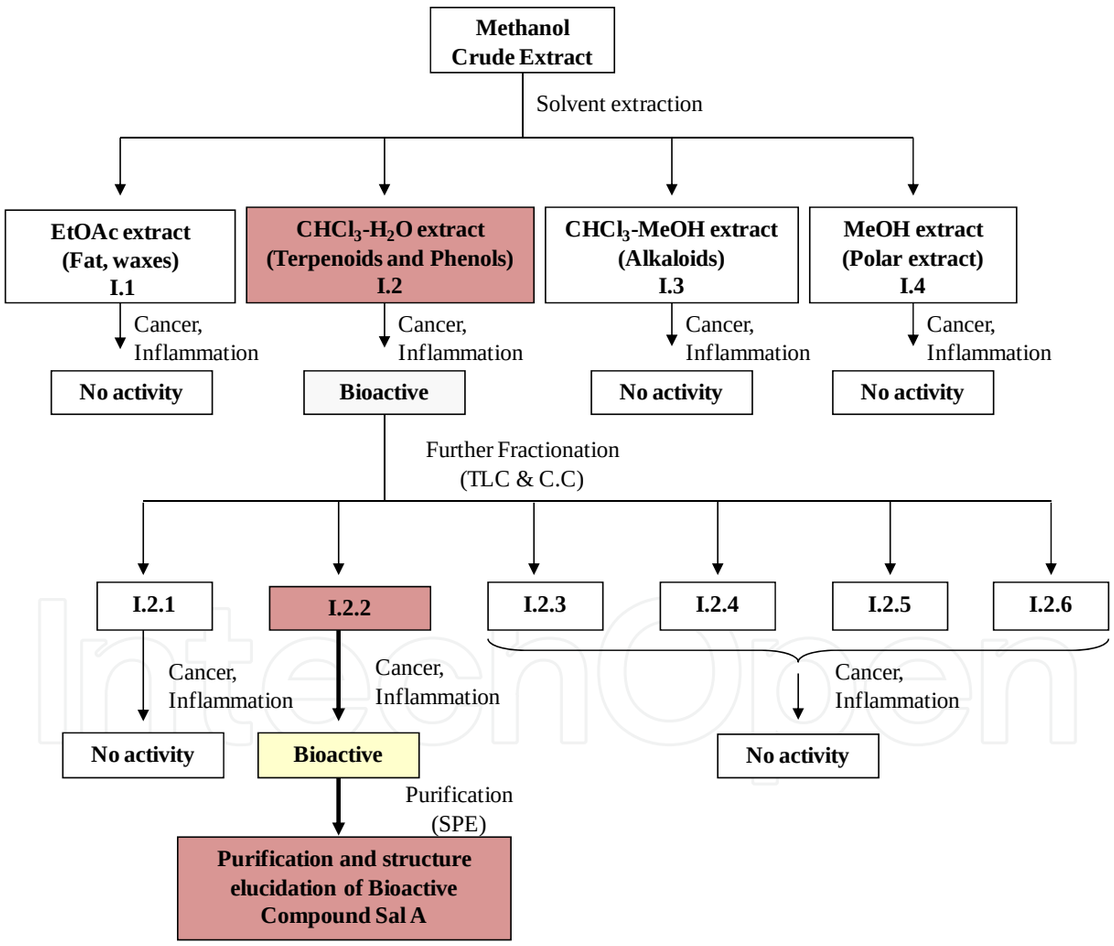
To date, *C. ainetensis* water extract was shown to have antifungal, antibacterial, anti-viral, anticancer and anti-inflammatory activities. Sal A on the other hand, was only shown to have antifungal, anticancer and anti-inflammatory properties. The group of Vajs et al. discovered in 1999 the antifungal activity of Sal A while Ibsar faculty revealed the two others. It is unfortunate that no study has so far assessed whether Sal A is responsible for the antimicrobial and antiviral potentials exhibited by *C. ainetensis* water extract.

Following its isolation from *C. nicolai,* Sal A was tested against seven fungal species: *Aspergillus niger, A. ochraceus, Penicillium ochrochloron, Cladosporium cladosporoides, Fusarium tricinctum, Phomopsis helianthi* and *Trichoderma viride* (Vajs et al., 1999). Each strain was inoculated in the center of a plate with or without Sal A addition to the nutritional agar media. After incubation at 20°C for three weeks, the percentage of fungi inhibition was determined by comparing the diameter of each fungal strain colony inoculated in the presence of Sal A to that of the control inoculated without Sal A. The results showed that Sal A inhibited all the fungi strains except *Trichoderma viride*. Subsequently the use of different concentrations of Sal A enabled the determination of the Minimum Inhibitory Concentration (MICs) to inhibit the mycelial growth of the respective fungal species.

To test for the inflammation potential of *C. ainetensis* water extract, the pro-inflammatory cytokine interleukin-6 (IL-6) was chemically induced in mammary epithelial cells (CID-9 and Scp2) by treatment with endotoxin (ET)). The ability of the extract to reverse or prevent IL-6 production was then assessed and the results demonstrated that *C. ainetensis* water extract inhibited IL-6 in a dose-dependent manner (Talhouk et al., 2008). It was also shown that the extract reversed chemically induced paw edema signs in ET-pretreated Sprague-Dawley rats as well as thermal hyperalgesia in rats subjected to the hot plate test. Sal A was isolated from the water extracts and shown to be responsible for its observed bioactivity. In

Kigndom	Plantae
Subkingdom	Tracheobionta
Division	Magnioliophyta
Class	Magnoliopside
Subclass	Asteridae
Order	Asterales
Family	Asteraceae
Genus	*Centaurea* L.
Species	*ainetensis*

(a) *Centaurea ainetensis* and its taxonomy



(b) The bioguided fractionation procedure

Fig. 3. Illustration of the indigenous Lebanese plant *Centaurea ainetensis* and the bioguided fractionation procedure that enabled the isolation of Salograviolide A. The plant picture is courtesy of Mr. Khaled Sleem.

parallel, the anti-inflammatory property of Sal A was demonstrated in a murine intestinal epithelial cell model (Mode K cells) treated with IL-1 and in *vivo* in a rat model of colonic inflammation induced by rectal injection of iodoacetoamide (Al-Saghir et al., 2009). The results showed that, similarly to *C. ainetensis* water extract, Sal A significantly reduced inflammatory cytokines and acted as a preventive agent to reduce inflammation.
C. ainetensis water extract and Sal A were also tested against different types of cancers (El-Najjar et al., 2007; Ghantous et al., 2007). *In vitro*, they were both non cytotoxic to normal primary murine keratinocytes while they preferentially inhibited neoplastic benign tumors and squamous cell carcinoma growth in a dose-dependent manner. The selective antiproliferative effects were confirmed in leukemic cells at the metastatically invasive stages as well as in human colon carcinomas. Sal A was also tested in combination with another sesquiterpene lactone, the Iso-seco-tanapartholide (TNP) extracted from *Achillea damascene,* against human colon cancer cell lines (Gali-Muhtasib, unpublished findings). The study demonstrated a synergistic apoptotic effect of both sesquiterpene lactones that failed to induce apoptosis when tested alone at the same low concentrations. Finally *in vivo*, the intraperitoneal water extract injection in Balb/c mice before chemically inducing colon cancer reduced drastically the mean size of aberrant crypt foci which is indicative of the extract's cancer preventive activity.

6. Salograviolide A and inflammation: Mechanisms and targets

Natural products derivatives have contributed over the last 25 years to approximately one fourth of the anti-inflammatory drugs used in the clinic (Newman & Cragg, 2007). In 2008, 18 additional natural product derived drugs were being tested at different clinical stages (Harvey, 2008). Terpenoids including sesquiterpene lactones have also reached clinical trials as potential anti-inflammatory agents. For instance, andrographolide; a labdane diterpenoid derived from the plant *Andrographis paniculata* of the Acanthacaea family, reached phase II clinical trials for rheumatoid arthritis. The sesquiterpene lactone parthenolide that made it to phase I cancer clinical trials, has also reached phase II and III clinical trials for the treatment of allergic contact dermatitis (refer to http://www.clinicaltrials.gov/).
Drugs with anti-inflammatory activities have common targets and modes of action that lead to the downregulation of the signs of inflammation as well as the chemical mediators controlling the mechanisms of inflammation. Elements of the complement system, angiogenic factors, prostaglandins, cytokines such as interleukins and matrix metalloproteinases (MMPs) are some of the most common inflammatory mediators.
Prostaglandins play a role in the modulation of blood flow. They are derived from the arachidonic acid due to the action of two prostaglandins H synthases isoforms known as the cyclooxygenases 1 and 2 (COX-1 and COX-2). COX-1 is constitutively expressed in different tissues while COX-2 is induced by inflammatory stimuli and is therefore thought to be the only isoform involved in propagating the inflammatory response (Larsen & Henson, 1983).
IL-6 secreted by the macrophages releases proteinases and elastases which bind to IL-6 receptor and generate signals implicated in humoral inflammation (Heinrich et al., 2003). IL-1, on the other hand, induces the mobilization of the arachidonic acid and its metabolism into prostaglandins thus contributing to cellular inflammation. It was also shown that IL-1 induces the synthesis of COX-2 through the activation of the nuclear factor Kappa B (NF-κB) transcription factor (Larsen & Henson, 1983). NF-κB is a key modulator of inflammation and is implicated in inflammation-induced tumor formation as well (Karin & Greten, 2005). It is

known to promote the expression of target genes of the inflammation response such as interleukins, COX-2, and inducible nitric-oxide synthase (iNOS) (Mazor et al., 2000).
Finally, components of the MMP family have been reported to act in wound healing and embryogenesis (Mainardi et al., 1991). Gelatinase A or MMP-2 (72 KDa) and gelatinase B or MMP-9 (92 KDa) have been identified as pro-inflammatory agents. They enable the digestion of components of the basement membrane; a function that is referred to as gelatinolysis (Birkedal-Hansen et al., 1993).
Sal A and *C. ainetensis* water extract were shown to modulate some of these major players of the inflammatory response. They both inhibit IL-6 expression (Talhouk et al., 2008) and IL-1-induced COX-2 expression by interfering with their synthesis (Al-Saghir et al., 2009). Only the effect of the water extract on the expression levels of the gelatinases A and B was assessed. The results indicate that the water extract decreased the expression of both proteins with preferential inhibition of gelatinase B 9 h post treatment with endotoxin (Talhouk et al., 2008). Similarly, only the effect of Sal A on the NF-κB signaling was investigated. NF-κB is composed of the two subunits p50 and p65 and is only active after translocation of the subunits into the nucleus and their dimerization. In normal conditions, NF-κB is inactive due to its retention in the cytoplasm by the inhibitor of NF-κB (IκB). Pro-inflammatory stimuli such as IL-1 cause the phosphorylation of the IκB by the inhibitor of NFκB- β (IKK). The phosphorylation in return leads to IκB degradation enabling thus the translocation to the cytoplasm and the dimerization of the NF-κB subunits, their binding to target DNA sequences, initiation of transcription and activation of the NF-κB transduction pathway (Baeuerele & Baltimore, 1996).
It was demonstrated that Sal A inhibits the NF-κB activation by two mechanisms. The first involved the stabilization of IκB, since combination treatment of Sal A with IL-1 decreased the degradation of IκB observed in response to treatment with IL-1 alone. The second involved inhibiting NF-κB translocation and binding to DNA, since the incubation of cells with Sal A after IL-1 addition (hence after IκB degradation) still caused a reduction in the activity of NF-κB (Fig. 4) (Al-Saghir et al., 2009).

7. Salograviolide A and cancer: Mechanisms and targets

The mechanism behind the anticancer activity of sesquiterpene lactones has been extensively investigated. For example, parthenolide was shown to induce cell cycle arrest, promote cell differentiation and trigger apoptosis (Pajak et al., 2008). The molecular basis for parthenolide activity in cancer cells include among others, inhibiting NF-κB and stimulating apoptosis by accumulation of reactive oxygen species (ROS) as well as by regulating the levels of anti-apoptotic and pro-apoptotic proteins (Pajak et al., 2008). Interestingly, parthenolide showed a synergistic effect when used in combination with paclitaxel and increased apoptosis in breast cancer cells (Patel et al., 2000).
As mentioned earlier, *C. ainetensis* crude extract and Sal A were tested against skin, colon and blood cancer cell lines and showed selective antineoplastic effects with no cytotoxicity to normal cells. At the cellular level, *C. ainetensis* crude extract induced G_0/G_1 cell cycle arrest in neoplastic epidermal cells while Sal A increased the population in Pre-G_1. In accordance with this finding, the levels of cyclin D1 whose activity is required for the G_1/S transition were reduced (Li et al., 2011). On the other hand, the levels of the tumor suppressors p16 and p21 which are associated with greater susceptibility to chemotherapy

and with calcium induced differentiation in keratinocytes, respectively were increased (Hochhauser, 1997; Di Cunto et al., 1998, as cited in Ghantous et al., 2007). Moreover, p21 proteins were differentially regulated: they were upregulated in the presence of the crude extract which was consistent with the observed G_0/G_1 cell cycle arrest, whereas their upregulation in the presence of Sal A was found to be transient. This transient upregulation of p21 has been reported as critical for its role in differentiation (Di Cunto et al., 1998).

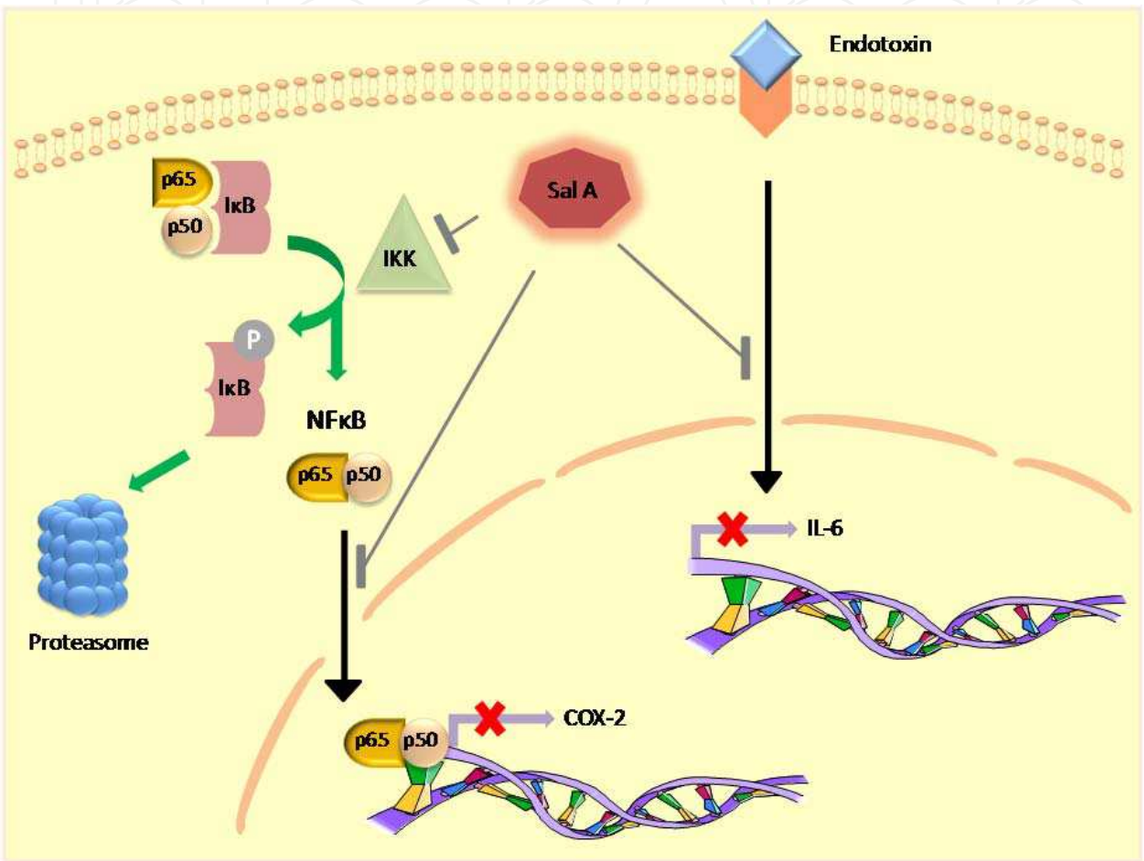


Fig. 4. Anti-inflammatory cascade triggered by Sal A. Details of the mechanism are explained in section 6 above.

In addition to cell cycle modulation, characteristic signs of apoptosis such as the partial and complete condensation of the chromatin were noted in the presence of Sal A. Furthermore, the ratio of the pro-apoptotic protein Bax to the anti-apoptotic protein Bcl-2 was found to be elevated. Bax and Bcl-2 have counteracting roles regarding the mitochondrial membrane permeabilization and thus the high ratio of Bax to Bcl-2 reflects the permeabilization of the mitochondrial membrane to release factors such as cytochrome c and the apoptosis inducing factor that will induce cell death. In conjunction, a considerable amount of ROS accumulated in the cells in the presence of Sal A. In fact, the accumulation of ROS was even shown to precede the growth inhibition and indicated an oxidant role of Sal A in these cells. Finally, the crude extract and Sal A had a contradictory regulatory effect on NF-κB. The crude plant extract decreased in a dose-dependent manner the binding of NF-κB to the DNA without

affecting the expression level of IκB, whereas Sal A increased it. It is worth noting that in cancer, the role of NF-κB activation or inhibition is in itself conflicting. NF-κB can be best described as a double edged sword for its activation can promote tumorigenesis (by inducing inflammatory mediators) or promote differentiation and thus inhibit tumorigenesis (Seitz et al., 1998, as cited in Ghantous et al., 2007).

In addition, the crude extract was assessed in human colon cancers and showed both an increase in p21 protein level of expression and in the ratio of Bax to Bcl-2 proteins. In colon cancer, cyclin B1 levels were decreased by the extract, while they were found to be unaffected by neither Sal A nor the extract in skin cancer. Cyclin B1 decrease is important for the exit from mitosis and for the cytokinesis (Takizawa & Morgan, 2000, as cited in El-Najjar et al., 2007). Another protein which was differentially modulated in skin *vs* colon cancer is p53. In colon cancer, the crude extract increased the expression levels of the p53 protein, while the levels were unaffected in skin cancer.

In leukemic cells, similarly to what was shown with the other two types of cancers, the extract induced pre-G_1 cell cycle arrest, increased the levels of p53 and p21 as well as the ratio of Bax to Bcl-2, and decreased cyclin D1 levels. In addition, the secretion of the vascular endothelial growth factor (VEGF) was significantly decreased by Sal A and the crude extract, adding VEGF to the list of targets of Sal A (El-Sabban, unpublished findings). Finally, Sal A was tested in combination with TNP against human colon cancer cell lines (Gali-Muhtasib, unpublished findings). At low concentrations, Sal A and TNP induced G_2/M cell cycle arrest when tested separately. At the same low concentrations, the combination of Sal A and TNP synergistically inhibited tumor growth and triggered apoptosis. ROS, which increased by a factor of 25 upon combination treatment, were found to be responsible for the synergistic-induced cell death. The pretreatment of the colorectal cancer cells with the antioxidant N-acetyl-L-cysteine (NAC), which diminishes the intracellular ROS, reversed the synergistic anti-proliferative effect and protected the cells from apoptosis and therefore confirmed the implication of ROS in the induction of apoptosis. Moreover, p38 kinase, the extracellular signal regulated kinase (ERK) and the c-Jun N-terminal kinase (JNK) of the mitogen-activated protein kinases (MAPK) pathway were found to be phosphorylated and thus induced after the combination treatment. Literature strongly advocates for the MAPK pathway implication in ROS induced cell death (Zhang et al., 2003). Using specific inhibitors of both ERK (usually involved in mitogenic signals and cellular proliferation), and of p38 (associated with stress along with JNK and commonly known as the stress activated protein kinases (Lewis et al., 1998)) abolished the apoptotic synergistic effect of the combination treatment and emphasized the pathway's association to cell death. Further studies with Sal A and TNP suggested a cross-talk between ROS, JNK and Bcl-2 family in the induction of apoptosis. ROS accumulation was found to induce Bax relocalization to the mitochondrial membrane on the one hand and to decrease the ani-apoptotic Bcl-2 expression levels on the other (Gali-Muhtasib, unpublished findings). Bcl-2 decrease was also associated with a maximal JNK activation which suggested that JNK might play a role in further inactivation of Bcl-2. In accordance with our findings several studies have reported the involvement of JNK but not ERK nor p38 in the phosphorylation and inactivation of Bcl-2 (Srivasta et al., 1999).

Taken together these results indicate a promising chemotherapeutic effect for using Sal A alone or in combination against various cancer types.

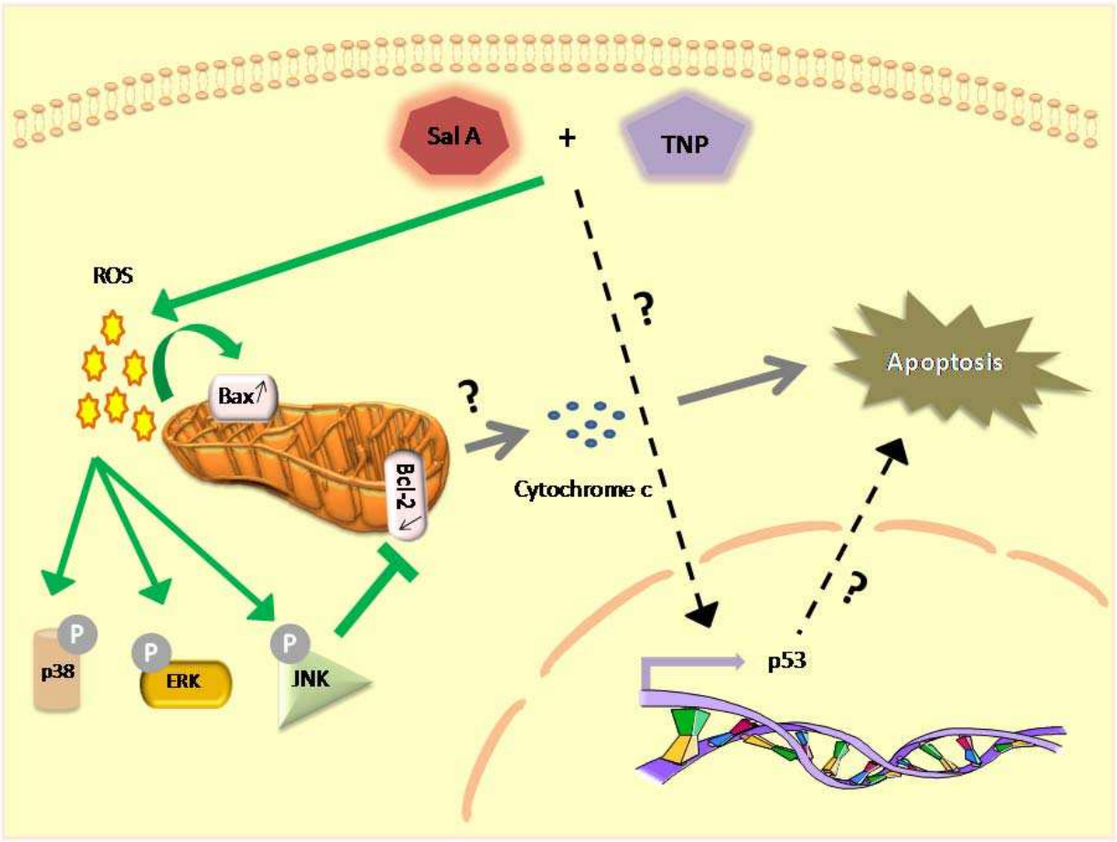


Fig. 5. Anticancer cascade triggered by the combination of Sal A and TNP in colon cancer cells. The combination caused increase in ROS and activation of MAPK molecules, ERK, JNK and p38 leading to apoptosis. It is still unclear whether apoptosis is associated with the modulation of p21, p53, and cyclin B1 proteins or the release of cytochrome c from mitochondria.

8. Conclusion and future direction

Cancer progression is characterized by seven hallmarks: sustaining proliferative signaling, insensitivity to growth suppressors, evading apoptosis, acquisition of replicative immortality, induction of angiogenesis, activation of invasion and metastasis and finally chronic inflammation (Colotta F. Et al., 2009).

In a nutshell, the evidence that has been collected so far by multiple investigators suggests that Sal A is a promising compound for cancer drug discovery for it acts at least on three cancer hallmarks: it upregulates tumor suppressors, triggers apoptosis and downregulates the mediators of inflammation. Its selectivity toward tumor cells and ability to target multiple pathways involved in inflammation and cancer imply that this compound is unlikely to have a single target that is responsible for its biological activities. Although a large body of information supports the role of Sal A against cancer and inflammation, there are yet no studies assessing its toxicology profiles or its absorption, distribution and metabolism in animals and humans. Such studies are warranted to better determine its potential for future applications in the clinical setting whether alone or in combination with standard clinical drugs.

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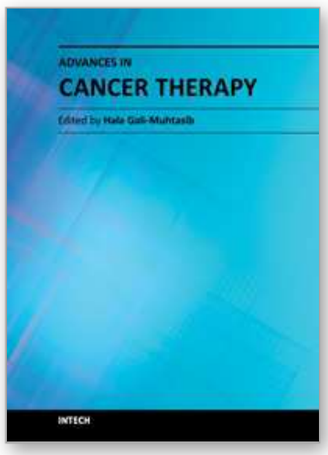

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