Recent Advances in Chemistry, Biology and Biotechnology of Alkannins and Shikonins

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Abstract: Isohexenylnaphthazarins (IHN), commonly known as Alkannins and Shikonins (A/S), are lipophilic red pigments. They are found in the outer surface of the roots of at least a hundred and fifty species that belong to the genera *Alkanna, Lithospermum, Echium, Onosma, Anchusa* and *Cynoglossum* of the Boraginaceae family. The chiral pairs A/S are potent pharmaceutical substances with a well-established and wide spectrum of wound healing, antimicrobial, anti-inflammatory, antioxidant, anticancer and antithrombotic biological activity.

For organic chemists uninitiated in the chemistry of quinones, the structures of alkannin (1) and shikonin (2) may look misleading simple. However, in spite of great efforts over many years by several research groups worldwide, a much needed viable synthetic route to these enantiomers has remained elusive until very recently.

The value of A/S motivated biotechnologists to develop the world's first manufacturing process utilizing plant cell cultures. The research in this area has provided a wealth of knowledge to the field of biotechnology. In addition, great insights into the biosynthesis of these natural products and to our understanding of plant secondary metabolism in general, has been gained from this work.

The last years there has been extensive scientific research in many areas throughout the disciplines of chemistry and biology and more specifically in cancer chemotherapy and a number of papers have appeared in the literature. Significant research has been conducted on A/S effectiveness on several tumors and on their mechanism of anticancer action. The aim of this paper was to review the recent advances in chemistry, biology, biotechnology and biosynthesis of alkannins and shikonins.

1. INTRODUCTION

The story of the enantiomeric naphthoquinone natural products alkannin (*S*-enantiomer, compound *1*, Fig. **1**) and shikonin (*R*-enantiomer, compound *2*, Fig. **1**) can be traced back many centuries and has been extensively reviewed in a recent paper [1]. Alkannin (A) derivatives are found mainly in Europe in the roots of several plants of the Boraginaceae family, especially *Alkanna tinctoria* Tausch (*A.t.*), while shikonin (S) derivatives predominate in the red pigment extracts mainly from the roots of the plant *Lithospermum erythrorhizon* Sieb et Zucc. (*L.e.*). Biological investigations over the last 30 years have shown that many of the medicinal properties claimed for *A.t.* and *L.e.* in the historical texts, do indeed have a sound scientific basis, attributed mainly to alkannin, shikonin and their isohexenylnaphthazarin (IHN) derivatives.

Thus, both alkannin and shikonin and their derivatives (Table **1**) have been shown to possess strong wound healing, antitumor, antimicrobial, anti-inflammatory and antithrombotic properties. These properties have been extensively described in an exhaustive review published in February 1999 [1]. Since then, there has been extensive scientific research in many areas throughout the disciplines of chemistry and biology and more specifically in cancer chemotherapy and a number of papers have appeared in the literature. Studies approximating the mechanism of their anticancer action have been also reported.

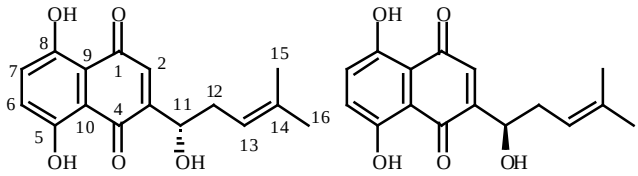

Fig. (1). The chiral pair alkannin (left) and shikonin (right) that possess major biological activity.

A/S and their derivatives are used in several pharmaceutical, cosmetic preparations and as food additives [1,2]. The above isohexenylnaphthazarins are susceptible to several transformations, such as photochemical decomposition, thermal degradation and polymerization [1]. The stability of IHN during processing and storage is crucial to their use in pharmaceuticals and cosmetics.

A/S and their derivatives have been produced by biotechnologists utilizing plant cell cultures. The research in this area has provided a wealth of knowledge to the field of biotechnology. In addition, great insights into the biosyn-

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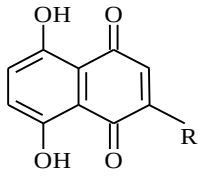
Table 1. Members of the Alkannin/Shikonin family of natural products isolated and identified till date in plant roots and cell cultures



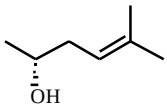
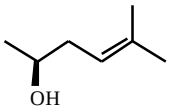
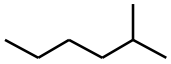
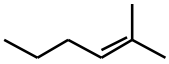
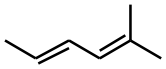
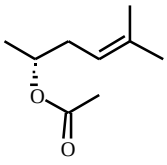
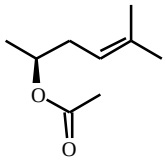
Compound	R	Name
1		Alkannin (also arnebin-4)
2		Shikonin
3		Alkannan
4		Deoxyalkannin, deoxyshikonin or Arnebin-7
5		Anhydroalkannin
6		Acetylalkannin or arnebin-3
7		Acetylshikonin

Table 1. contd….

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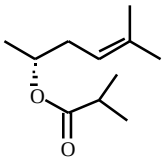
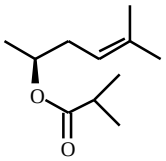
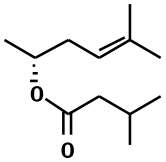
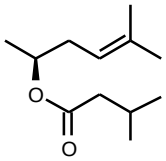
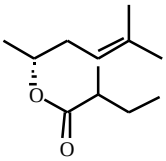
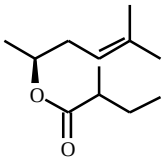
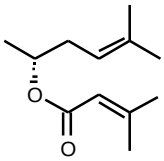
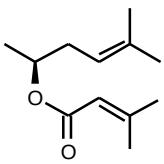
8		Isobutylalkannin
9		Isobutylshikonin
10		Isovalerylalkannin
11		Isovalerylshikonin
12		α-Methylbutyrylalkannin
13		α-Methylbutyrylshikonin
14		β, β-Dimethylacrylalkannin or arnebin-1
15		β, β-Dimethylacrylshikonin

Table 1. contd….

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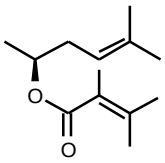
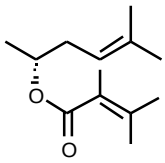
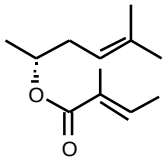
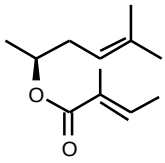
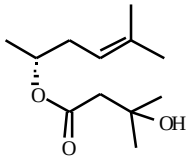
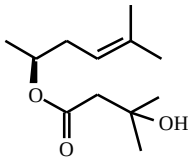
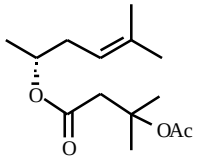
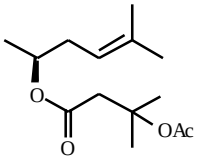
16		Teracrylshikonin
17		Teracrylalkannin
18		Angelylalkannin
19		Angelylshikonin
20		β-Hydroxyisovalerylalkannin
21		β-Hydroxyisovalerylshikonin
22		β-Acetoxyisovalerylalkannin
23		β-Acetoxyisovalerylshikonin

Table 1. contd….

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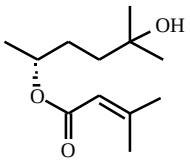
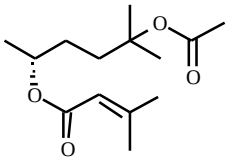
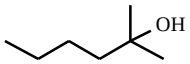
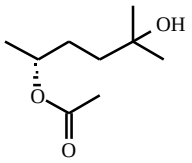
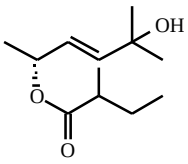
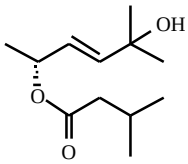
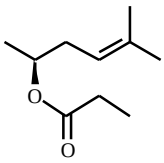
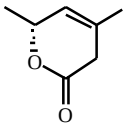
24		Arnebin-2
25		Acetylarnebin-2
26		Arnebin-5
27		Arnebin-6
28		Lithospemidin-A
29		Lithospermidin-B
30		Propionylshikonin
31		-

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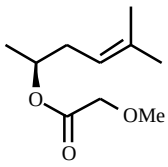
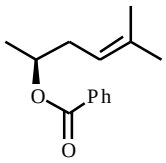
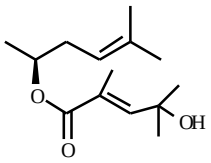
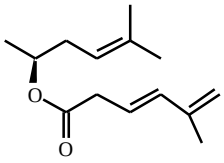
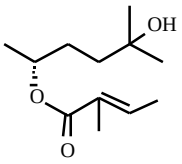
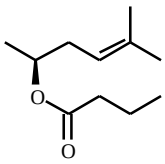
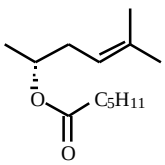
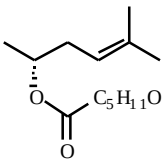
32		1-Methoxyacetylshikonin
33		Benzoylshikonin
34		-
35		2-Methyl-hexa-1′3′-dienoyl shikonin
36		Tiglyl dihydroxyalkannin
37		Butylshikonin
38		-
39		-

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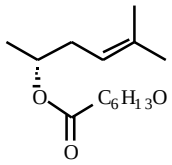
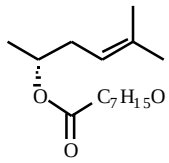
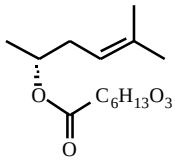
40		-
41		-
42		-

· In many cases no attempt was made to assign the absolute stereochemistry of each derivative, so alleged alkannin derivatives may be shikonin derivatives, or vice versa, or even racemates.

of these natural products and to our understanding of plant secondary metabolism in general, has been gained.

The aim of this paper is to review the recent advances in chemistry, biology, biotechnology and biosynthesis of A/S and their IHN derivatives. Emphasis is given on their established mechanisms of antitumor action.

2. BIOTECHNOLOGY AND BIOSYNTHESIS OF ALKANNINS/SHIKONINS

Production of alkannin, shikonin and their derivatives from cell tissue cultures and their biosynthesis have been reviewed in our previous paper [1]. Since then, extensive research concerning their production from cell cultures has facilitated the elucidation of several stages in their biosynthesis and will be consicely reported below.

2.1. Production of Alkannins/Shikonins from Cell Tissue Cultures

Shikonin derivatives formation has been observed on the stem of cultured shoots of *L.e* [3], where the effects of various basal media and phytohormones on their formation were investigated. As shown, culturing the shoots on Murashige and Skoog (MS) solid medium, kinetin addition remarkably enhanced shikonin derivatives accumulation in the shoots, while maximum amount of shikonin derivatives was observed in the shoots cultured in phytohormone-free Gamborg B5 liquid medium.

Several cultural factors were recently investigated for shikonin production in *L.e.* cell cultures [4]. Simultaneous analysis of shikimate-derived secondary metabolites in cultured cells of *L.e.* was performed by HPLC [5]. Trying to enhance shikonin production in suspension cultures of *L.e.* cells, low-energy ultrasound was used [6]. As shown, low-energy ultrasound significantly stimulated shikonin biosynthesis in cells, by stimulating two key enzymes for the secondary metabolite biosynthesis in cells, phenylalanine ammonia lyase and p-hydroxybenzoic acid geranyl-transferase.

L.e. cell suspension cultures in M-9 medium produced besides shikonin derivatives, lithospermic acid B, a dimerized caffeic acid ester derivative [7], while in hairy root cultures of *L.e.* a colorless quinone, rhizonone, was observed [8]. Various culture factors for increasing the production of lithospermic acid B were investigated [9]. Thus, blue light showed a stimulatory effect on lithospermic acid B production, strongly inhibiting shikonin production.

In *Onosma paniculatum* cell cultures, the effect of brassinolide on shikonin derivatives formation was studied [10]. As reported, brassinolide with indoloacetic acid (IAA) and 6-benzylaminopurine (BAP) was best for cell growth and shikonin formation. Studies on shikonin derivatives formation were conducted with a BK-39 callus culture of *L.e.*, where a selection of cell aggregates of BK-39 culture on a medium containing p-fluorophenylalanine (PFP) yielded a cell line possessing a higher resistance to the inhibitor than the initial culture [11]. The shikonin derivatives content of PFP-resistant culture was two times higher than that of control.

Finally, the production of biologically active substances by plant cell cultures including *L.e.* and *Macrotomia euchroma*, was investigated in space onboard the orbital station Mir and American Space Shuttle, where the impact of the conditions of space flight on plant cell cultures productivity was investigated. A more pronounced variation of the metabolites (shikonin) output was noted with respect to the ground control, depending upon the properties of the strain and the experimental conditions [12,13].

2.2. Biosynthesis of Alkannins/Shikonins

A lot of work has been conducted in the last years on naphthoquinone production by cell tissue cultures and this gave an impetus for quinone biosynthesis research and related gene expression. Yazaki in 1997 [14] published an extensive review on quinone biosynthesis in *L.e.* Two years later, the same group of Yazaki reviewed the light-induced negative regulation of secondary metabolism of shikonin in *L.e.* cell cultures and characterized the dark-inducible genes (LeDI) isolated from shikonin-producing cells [15], while a more recent review of Yazaki has been reported on root specific production of shikonin in *L.e.* cell cultures [16].

Shikonin is biosynthetically derived from two key precursors, 4-hydroxybenzoate (4HB) and geranyldiphosphate (GPP). Geranyldiphosphate was found to be formed *via* the mevalonate pathway [17]. A 4-coumaroyl-CoA-3-hydroxylase activity was purified from cell cultures of *L.e.*, but was proposed as polyphenol oxidase [18], while 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), a key enzyme of the mevalonate route to isoprenoids, was shown to play a significant role in the regulation of shikonin biosynthesis [19]. Another enzyme, 4-hydroxybenzoate 3-geranyltransferase was found to play a regulatory role in the biosynthesis of shikonin produced in *L.e.* cell cultures [20]. Geranylhydroquinone-3''-hydroxylase was also identified in cell suspension cultures of *L.e.* and was proposed that the enzyme hydroxylates the isoprenoid side chain of geranyl hydroquinone, a known precursor of shikonin [21].

Two cDNAs encoding geranyl diphosphate: 4-hydroxybenzoate-3-geranyltransferase were isolated from *L.e.*, suggesting that they are involved in the biosynthesis of shikonin [22]. Two cDNA clones, LePR1 and LePR2 (*L.e.* pathogenesis-related clones), encoding pathogenesis-related proteins, were isolated from shikonin-producing cells of *L.e.* [23]. Recently [24], *L.e.* dark-inducible genes (LeDIs) were isolated to investigate the regulatory mechanism of shikonin biosynthesis. This was the first report of the gene involved in production of secondary metabolites without affecting biosynthetic enzyme activities.

In another study, the bacterial *ubi*C gene, encoding chorismate pyruvate-lyase (CPL) that converts chorismate to 4-hydroxybenzoate, was expressed in hairy root cultures of *L.e.* [25]. More recently, a cDNA (LEPS-2) encoding a novel cell wall protein was cloned from shikonin-producing callus tissues of *L.e.* The results indicated that expression of the LEPS-2 is closely linked with shikonin biosynthesis and the LEPS-2 protein may be involved in the intra-cell wall trapping of shikonin pigments [26].

Recent developments in plant molecular biology made it possible to produce higher amounts of valuable metabolites by gene manipulation. Genetic engineering of shikonin biosynthesis in cultures of *L.e.* was approached by Heide group. Thus, in 1999 [27], the biosynthetic pathway to 4-hydroxybenzoate (4HB) was modified in *L.e.* hairy root cultures by introduction of the bacterial gene *ubi*C (from *Escherichia coli*) that encodes chorismate pyruvate-lyase, an enzyme that converts chorismate into 4HB and is not normally present in plants. *ubi*C transformation did not lead to a statistically significant increase of shikonin formation, but to an increase of menisdaurin, a nitrile glucoside related to aromatic amino acid metabolism.

The same group [28] manipulated the biosynthetic pathway leading to shikonin by introduction of the bacterial gene *ubi*A (from *Escherichia coli*), that encodes 4-hydroxybenzoate-3-polyprenyltransferase, an enzyme that catalyses a key step in ubiquinone biosynthesis. As suggested, over-expression of *ubi*A alone was not sufficient to increase shikonin formation and further enzymes are involved in the regulation of this pathway. Further research is expected in the near future on metabolic engineering of shikonin biosynthesis.

There are some interesting observations regarding A/S and their biosynthesis that have to be noticed, since these need to be evaluated and further research has to be conducted. Thus, A/S and their derivatives are biosynthesized exclusively in the underground parts of plants (roots) belonging to Boraginaceae family. The only exceptions are shikonin derivatives identified in the branch and trunk of *Jatropha glandulifera* and leaves of *Plagiobotrys arizonicus*, plants that do not belong to Boraginaceae family of plants. Shikonin derivatives were also identified in the aerial part of *Moltkiopsis ciliata* (Forsk.) Jahnst [29].

The second interesting point is that although nature biosynthesizes assymetrically, both enantiomers Alkannin (*S*- enantiomer) and Shikonin (*R*- enantiomer) are present in natural resources, specifically plant roots. It is also noteworthy that both enantiomers A and S and their respective derivatives are formed simultaneously during biogenesis, in the roots of the same plant and its tissue cultures, as have been established [1,30,31], but in different proportions. According to our observations, the A/S enantiomeric ratio differs in relation to geographical distribution, where less or more A or S derivatives are biosynthesized in several species. Thus, we have observed that in Europe and Meditteranean regions alkannin derivatives (*S*- form) are mainly biosynthesized, while moving to Far East the A/S enantiomeric ratio alters and shikonin derivatives (*R*- form) predominate. There are probably geophysical factors owed to geographical region that have to be studied for their effects on A/S enantiomeric ratio. These observations may be attractive to plant biochemists and other relative scientists for conducting further research, in order to elucidate all matters concerning A/S biosynthesis.

The fact that our group has shown [32,33,34] that oligomeric/polymeric A/S and HNQ derivatives are biogenetically formed in plant roots and in shikonin samples prepared by plant tissue cultures, has to motivate other researchers to study the biosynthetic mode of polymeric A/S derivatives.

3. BIOLOGICAL ACTIVITY OF ALKANNINS/ SHIKONINS

Biological investigations over the last 30 years revealed that A/S and their derivatives and also their main natural resources, *Alkanna tinctoria* (*A.t.*) and *Lithospermum erythrorhizon* (*L.e.*) root extracts possess wound healing, antitumor, antimicrobial, anti-inflammatory and anti-thrombotic properties. These biological properties have been extensively reviewed initially in a review article [49] and

afterwards in a recent article published in February 1999 [1]. Since then, there has been extensive scientific research on biological properties of A/S and their derivatives and more specifically on cancer chemotherapy. The major results obtained hitherto are herein concisely reviewed.

It is noteworthy that both A and S exert the same biological properties [1], although enantiomers of chiral drugs differ considerably in their pharmacological and toxicological effects, because they interact with biological macromolecules, the majority of which are stereoselective [31].

3.1. Wound Healing Effects

Root extracts of the natural resources *A.t.* and *L.e.* have been used for the treatment of wounds since ancient times. This medicinal property has been found to have a sound scientific basis and the active ingredients of the root extract of *A.t.* were identified by Papageorgiou [35,36].

Much research has been performed during the last years which established the wound healing activity of *A.t.* and *L.e.* root extracts, as well as of alkannin and shikonin derivatives. Additionally, several ointments containing alkannin and shikonin derivatives have been prepared and evaluated for their effectiveness on wound healing. In recent years, research is focused on determining the precise mode of A/S action during wound healing, tissue repair and regenerative processes, since wound healing is a complicated biological process that involves interaction of multiple cell types, various growth factors, their mediators and the extracellular matrix proteins.

Since 1998, several papers have appeared in the literature. Very recently [37] Shiunko, which is a traditional medicine used clinically for the treatment of wounded skin in Japan and China, has been evaluated for the epithelization of wounded skin compared with povidone iodine and saline. As shown, after cutting wounds on the skin of rats and inoculating with several bacteria, the incidences of wound infection following *Pseudomonas aeruginosa* inoculation were lower in both the Shiunko-treated group (0%) and povidone-iodine-treated one (5%) than the saline treated group (40%). The Shiunko-treated group reported higher percentages of complete epithelization not only on the sterilized wounds (100%) but also on the contaminated wounds (90%), compared with the saline-treated group (60 and 40% respectively), with the povidone iodine not promoting epithelization of wounded skin [37].

The effect of the root extract of *L. erythrorhizon* (*L.e.*), which is the active ingredient of Shiunko, on accelerating wound healing was examined [38] in healing-impaired diabetic (db/db) mice. After applying *L.e.* root extract, wound closure was complete in *L.e.*-treated mice. Capillary vessel number and collagen synthesis increased early in wound healing in *L.e.*-treated wounds. Vascular endothelial growth factor (VEGF)-positive neutrophils infiltrated the wound and the appearance of apoptotic fibroblasts and endothelial cells in the granulation tissue was more advanced than in the controls. The results indicated that the inflammatory phase was shortened and the proliferation and maturation phases were advanced by *L.e.* root extract. Thus, this extract was proposed to help patients with intractable bedsores and other chronic ulcers.

Since the activity of *L.e.* and *A.t.* root extracts was attributed to A/S derivatives, further research was conducted on pure A,S compounds. Thus, shikonin ointments were prepared and presented an enhancing effect on wound healing in experimental burn and open wound healing models in rats, with the 0.1% shikonin concentration being the most effective [39]. Ozaki and coworkers [40] reported a study on biochemical factors and histological changes induced by A and S. As reported, A and S produced a dose-dependent acceleration of the cotton pellet-induced granuloma formation and increased the expression of $CD11b^+$ cells (the membrane antigen of leukocytes) with granulocytes such as macrophages, histiocytes and TGF-β. Acceleration of the proliferation of fibroblasts and collagen fiber in the granulation tissue was also observed [40, 41]. These results indicated that the accelerating effect of the proliferation of granulation tissue produced by A and S can be attributed to an increase in new blood vessel cells, in the expression of $CD11b^+$ cells and also the acceleration in the proliferation of fibroblasts.

The effect of shikonin on granulation tissue formation was biochemically evaluated and compared with carrageenan. Shikonin was shown to enhance the formation of granulation tissue accompanied by the proliferation of capillaries and the increased production of collagen in rats. The mechanism underlying the biological effect of shikonin on granulation tissue formation was proposed to be different from that of carrageenan [42].

To reveal the augmenting effect of shikonin and *L.e.* extract on vascular endothelial growth factor (VEGF) production and neovascularization [43], the researchers investigated murine granulomatous tissue induced by shikonin and *L.e.* extract. Their injection resulted in granulomatous tissue formation and histological observations showed that the pouch was formed in the submuscular connective tissue and necrotic tissue directly facing the cavity and granulomatous tissue developed in the connective tissue. Results indicated that shikonin and extract of Shikon (*Lithospermum erythrorhizon* root) induced neovascularization in granulomatous tissue [43]. Finally, shikonin was also patented as a wound treatment agent [44].

From root plant extracts, focus was given on a shikonin ester, β,β-dimethylacrylshikonin (Arnebin-1, Table **1**) that significantly accelerated healing of wounds with or without hydrocortisone treatment. Arnebin-1 treatment promoted cell proliferation, migration and vessel formation forming a thick granulation tissue and re-epithelialization of wounds. Increased synthesis of collagen, fibronectin and transforming growth factor-β1 (TGFβ1) were observed in arnebin-1 treated wounds. As some forms of TGFβ1 are known to enhance wound healing and to be associated with the wound healing defect in hydrocortisone-treated wounds, the enhanced expression of TGFβ1 at both translational and transcriptional level by arnebin-1, may mediate the enhancement of wound healing [45].

Another pharmaceutical preparation containing a shikonin analog 93/637 (SK) derived from the plant *Arnebia nobilis* was evaluated on normal and hydrocortisone-induced impaired healing in full thickness cutaneous punch wounds in rats. SK treatment (0.1% applied topically as an ointment in polyethylene glycol base) significantly accelerated healing

of wounds, promoted formation of granulation tissue including cell migration and neovascularization, collagenization and reepithelialization. The expression of basic fibroblast growth factor (bFGF) was higher in treated wounds compared to controls and thus the increased expression of bFGF by SK may be responsible for the enhancement of wound healing [46].

Finally, regarding the activity of alkannin derivatives, the olive oil extract of *Alkanna tinctoria* Tausch was tested on partial thickness and burn wounds on rabbits. As shown, complete healing of patrial thickness and olive oil burn wounds occurred in 7 to 10 and 26 days respectively with well-formed dermal-epidermal junctions in both groups [47]. A clinical trial was conducted on HELIXDERM® pharmaceutical ointment and proved its efficacy on patients suffering from indolent ulcers of the leg (Fig. **2**) [48].

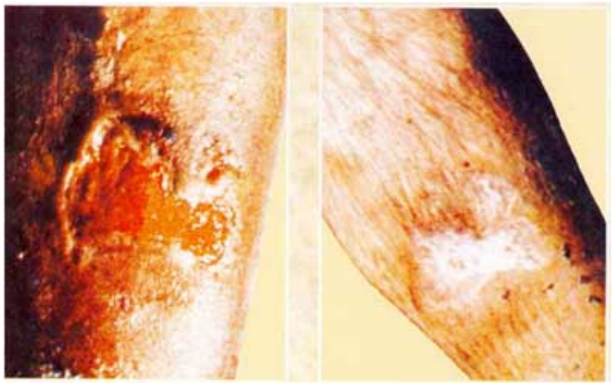


Fig. (2). An indolent ulcer before (left) and after (right) five weeks of treatment with the wound healing ointment HELIXDERM® [48].

3.2. Anti-Inflammatory Effects

The anti-inflammatory activity of A,S and their derivatives, as well as of *A.t.* and *L.e.* root extract has been well established as reviewed in our previous articles [1, 49]. Since then, several articles have been published on their anti-inflammatory effects and mechanism of action. It is well known that tumor necrosis factor alpha (TNF-alpha) contributes to the pathogenesis of both acute and chronic inflammatory diseases. For this reason it has been a target for the development of new anti-inflammatory drugs. Therefore, the effects of shikonin and its derivatives were evaluated on the transcriptional activation of human TNF-alpha promoter in a gene gun-transfected mouse skin system. The crude plant root extract of *Lithospermum erythrorhizon*, as well as derived shikonin derivatives, showed significant dose-dependent inhibition of TNF-alpha promoter activation. Among the tested compounds, shikonin and isobutyryl-shikonin exhibited the highest inhibition. In this study, it was proposed that shikonin derivatives inhibited the transcriptional activation of the human TNF-alpha promoter through interference with the basal transcription machinery [50].

In another study, an ethanolic extract of *Arnebia hispidissima* roots and several isolated shikonin derivatives were examined for their anti-inflammatory activity, where models with carrageenan-induced paw edema and complete Freund's adjuvant (CFA)-induced chronic arthritis were conducted in rats. The results indicated that pre-treatment with arnebinone, significantly inhibited the carrageenan-induced paw edema and also suppressed the development of chronic arthritis induced by CFA [51]. Following that study, the same research team investigated the anti-inflammatory activity of the hexane extract of *Arnebia hispidissima* roots and its active constituents, specifically alkannin derivatives (not shikonin as reported in their previous study) [52]. Pre-treatment with cycloarnebin-7 significantly inhibited the carrageenan-induced acute arthritis, while arnebin-1 significantly suppressed the development of chronic arthritis induced by CFA.

Following these studies, research was conducted on examining the active ingredients of root extracts, specifically A,S and derivatives on inhibiting inflammation and their mode of action. Thus, Chen and his co-workers established that shikonin blocks radiolabelled Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) and macrophage inflammatory protein-1 (MIP-1 alpha) binding to human monocytes. Additionally, shikonin blocked RANTES and MIP-1 alpha binding to stable CC chemokine receptor-1 (CCR1) transfected human embryonic kidney (HEK)/293 cells. With that study it was proposed that shikonin in the extracellular compartment inhibits specific chemokine function. Thereby, it selectively inhibits inflammatory responses mediated by CCR1 and thus shikonin is proposed as a useful anti-inflammatory agent for selectively blocking the binding of CCR1 ligands [2,53].

The *in vitro* antioxidant, hydroxyl radical scavenging activity and the *in vivo* anti-inflammatory activity of A and S (that is attributed at least partly to the intervention in free radical processes), has been recently investigated [54]. As shown, both A,S and their parent moiety, naphthazarin (5,8-dihydroxy-1,4-naphtho-quinone), significantly inhibited *in vitro* lipid peroxidation of rat hepatic microsomal membranes, competed with DMSO for free hydroxyl radicals and reduced inflammation in mice very efficiently. It was proposed that the claimed actions of A/S are due to their intervention in free radical processes. Finally, shikonin was found to react directly with reactive oxygen species (ROS) and exhibited antioxidative activity, which in turn exerts anti-inflammatory activity [55]. Shikonin esters have also been patented for their anti-inflammatory and antibacterial activity and the treatment of several inflammatory diseases [56].

3.3. Antimicrobial Effects

Over the last 30 years, the antimicrobial activity of A/S and their derivatives has been investigated by a number of groups. These compounds were found to be active against Gram-positive bacteria, such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Bacillus subtilis*, as described in our previous review [1]. Since 1998, several researchers have investigated the antimicrobial activity of A/S derivatives.

Following a previous study that demonstrated the antimicrobial activity of A/S on methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* (MRSA and MSSA) [57]. Shen and coworkers [58] reported the isolation of alkannin, shikonin and six other A/S derivatives from *Arnebia euchroma* root extract as the active principles

against MRSA and vancomycin-resistant *Enterococci* (VRE). Anti-MRSA activity of these compounds was bactericidal. A/S derivatives were also active against vancomycin-resistant *Enterococcus faecium* (F935) and vancomycin-resistant *Enterococcus faecalis* (CKU-17) [58].

In another study, shikonin was found to exhibit same antibacterial activities against both methicillin-Sensitive *S. aureus* and methicillin-Resistant *S. aureus in vitro*, almost the same as silver sulfadiazine. Afterwards, a shikonin ointment was prepared in PEG 400 and PEG 4000 base (0.1% shikonin) and exhibited an antibacterial effect against *S. aureus* in open wounds in rats, although this activity was affected by serum protein [59]. Additionally, another shikonin preparation demonstrated a high antibacterial activity with respect to *Gram*-positive species, considerably superior to the commercial antimicrobial preparations tested [60]. Recently, shikonin was found to elicit dose-dependent bacteriostatic activity in *Helicobacter pylori* cultures. In this study, shikonin inhibited N-acetylation of 2-aminofluorene in the examined *H. pylori* cytosols and intact cells [61].

An extract of the roots of *Onosma argentatum* was effective on *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* and also shared very high antioxidant activity [62]. Therapeutic antimicrobial preparations containing *Alkanna tinctoria* have been patented [63].

In vitro antifungal activities of several IHN derivatives and *Lithospermum erythrorhizon* root extracts, were investigated by several groups. Thus, shikonin exhibited a stable fungistatic effect to various cultures of *Candida* genus and one culture of *Trichosporon* genus [60]. Propionylshikonin and β-hydroxyisovalerylshikonin, isolated from the roots of *Lithospermum erythrorhizon*, showed both antifungal (*Cladosporium herbarum*) and antiviral (tobacco mosaic virus) activities [64]. Acetylshikonin and β-hydroxyisovalerylshikonin were biologically active against soil-born bacteria and fungi [65]. In another study, extracts of Zicao plant (*Lithospermum erythrorhizon* root) containing shikonin derivatives and *Arnebia euchroma* containing alkannin derivatives, shared anti-*Candida albicans* properties. Also, acetylshikonin inhibited the fungal growth [66]. Following that study, the same group investigated *in vitro* antifungal activities of several naphthoquinones from *L.e.* roots against several fungal pathogens [67]. Shikonin and deoxyshikonin were found to have a much stronger activity than fluconazole against yeast-like fungi (*Candida crusei, Saccharomyces cerevisiae, C. glabrata),* whereas acetylshikonin and β-hydroxyisovaleryl-shikonin lower than the standard. All naphthoquinones tested were found to have a range of activity against the filamentous fungus, *Trichosporon cutaneum* [67]. Finally, the antifungal activity of shikonin among other quinones was tested and proved moderate against *Colletotrichum fragariae* [68].

3.4. Antitumor Activity

A,S and their derivatives have been investigated as potential drug candidates for various aspects of cancer treatment within the last 30 years [1]. Since 1999, further research has been conducted on A/S effectiveness on several tumors and also their mechanism of anticancer action. Recent studies revealed that alkannin and shikonin derivatives exert antineoplastic effects by inhibiting cancer cell growth, inducing apoptosis, inhibiting DNA topoisomerases, possessing antimitogenic action, reducing carcinogenesis and angiogenesis, as have been summarized in recent reviews [1,2].

The root extract of *Onosma echioides* containing A/S derivatives was recently studied on two-stage skin carcinogenesis and on tumor promoter induced markers and oxidative stress in mice. As shown, treatment with *O. echioides* root extract restored the levels of reduced glutathione and cellular protective enzymes. Additionally, malondialdehyde formation and hydrogen peroxide (H_2O_2) content were also reduced significantly. On conducting studies on mouse skin carcinogenesis, that extract showed reduction in the number of tumors/mouse and percentage of tumor bearing mice at the end of the study, owed to A/S derivatives [69].

Very recently [70], the effectiveness of shikonin was shown on treating human bladder transitional cell carcinoma. As established, shikonin affected NAT (N-acetyltransferase) enzyme activity, gene expression (NAT 1 mRNA), acetylated 2-aminofluorene (AF)-DNA adducts formation and formation of NAT Ag-Ab in human bladder tumor T24 cells. Thus, it is proposed as a candidate for the prevention or treatment of transitional cell carcinoma. In another paper [71] the mechanism of shikonin action on ultraviolet B-induced skin carcinogenesis was studied. Furthermore, *Lithospermum* root was patented among other plants for its anticancer effect on mouse leukemia P388 [72]. The papers published since 1999 on antitumor activity of A/S and derivatives and their mechanism of anticancer action, are concisely reviewed below.

Tumor Cell Growth Inhibition and Induction of Apoptosis

Apoptosis, a new therapeutic target of cancer research, is a specific process that leads to programmed cell death [73]. Initiation of apoptosis appears to be a common mechanism of many cytotoxic agents used in chemotherapy. A family of cytosolic proteases, the caspases, plays an essential role in the execution of apoptosis [74].

Shikonin and its derivatives were studied and demonstrated that induced apoptosis. The last years a lot of research is conducted on defining the mechanism by which shikonin induces apoptosis. Thus, shikonin was first observed to induce apoptosis in HL60 human premyelocytic leukemia cell line and induced DNA fragmentation [73]. The increase of apoptotic cells was preceded by the activation of caspase-3 that plays a central role in apoptotic process. Trying to elucidate the mechanism of shikonin inducing apoptosis [75], proposed that the chemical reaction between shikonin and cellular thiols, such as glutathione and protein thiols, induces apoptosis in HL60 cells. Recently, shikonin was found to induce apoptosis for K562 leukemia cells [76].

In another recent study [74] the molecular mechanism by which shikonin triggered human colorectal carcinoma COLO 205 cells undergoing apoptosis was examined. Shikonin-induced apoptotic cell death was accompanied by up-regulation of p27, p53 and Bad and down-regulation of Bcl-2 and Bcl-X_L, while little effect was observed on the levels of Bax protein. It was thus suggested that shikonin-induced apoptosis is triggered by the release of cytochrome C into cytosol, procaspase-9 processing, activation of caspase-3,

degradation of poly-(ADP-ribose) polymerase (PARP) and DNA fragmentation caused through the caspase-activated deoxyribonuclease through the digestion of DNA fragmentation factor (DFF-45).

Shikonin induced apoptotic cell death in some other cancer cells, specifically human hepatoma cell line, SK-Hep-1 [77]. As shown in that study, shikonin generated increased amounts of intracellular reactive oxygen species (ROS) during initiating apoptosis, accompanied by the dissipation of mitochondrial transmembrane potential. Thus, as suggested shikonin-induced apoptosis of SK-Hep-1 cells proceeds through an oxidative-stress mediated pathway.

In a very recent study shikonin inhibited tumor cell growth and induced cell death in various tumor cells, such as human cervical cancer cells, HeLa. Approximating the mechanism of shikonin-inducing apoptosis, it was demonstrated that caspase-3 activity significantly increased after shikonin treatment. Furthermore, reduced expression of inhibitor of caspase-activated deoxyribonuclease, after exposure to shikonin, suggested the resultant activation of caspase-activated deoxyribonuclease, leading to apoptosis [78]. The same group, in another study, approaching the mechanism of shikonin apoptotic induction [79], reported that shikonin was found to inhibit growth of human malignant melanoma A375-S2 cells mediated through up-regulation of p-53 and down-regulation of cyclin-dependent protein kinase 4, while caspase activation was detected in shikonin-induced cell apoptosis. Shikonin-induced DNA damage was reported to activate p53 protein and caspase-9 pathways.

Singh and co-workers reported that shikonin inhibited the growth of human epidermoid carcinoma cells [80]. Inhibition was followed by decreased phosphorylated levels of epidermal growth factor receptor (EGFR), extracellular regulated kinases (ERK 1/2) and protein tyrosine kinases, but increased phosphorylated c-Jun-N-terminal kinases (JNK 1/2) levels. Thus, shikonin treatment was associated with increased intracellular levels of phosphorylated apoptosis-related proteins and decreased levels of proteins associated with proliferation in human epidermoid carcinoma cells.

Another derivative of shikonin, β-hydroxyisovaleryl-shikonin (b-HIVS) (Table **1**), isolated from the roots of *Lithospermum erythrorhizon*, was found to induce apoptosis in HL-60 cells, while the mechanism of its action was approximated. Thus, characteristic features of apoptosis, such as DNA fragmentation, nuclear fragmentation, activation of caspase-3-like activity and activation of MAP kinases, such as ERK2, JNK and p38, were observed after treatment with b-HIVS [81]. Following the above study [82], reported the induction of apoptosis by b-HIVS in human leukemia cells (HL60 and U937) by inhibiting the activity of polo-like kinase 1 (PLK 1) through inhibiting tyrosine kinase activity. Very recently, the same research group reported that b-HIVS suppressed the expression of the gene for tumor necrosis factor receptor-associated protein 1 (TRAP 1), a member of the heat-shock family of proteins [83]. When human leukemia HL60 cells and human lung cancer DMS114 cells were treated with b-HIVS, the amount of TRAP1 in mitochondria decreased during apoptosis. The suppression of the level of TRAP1 by b-HIVS was blocked by N-acetyl-cysteine indicating the involvement of ROS in the regulation of the expression of TRAP1 and induction of apoptosis [83].

Inhibition of Protein Tyrosine Kinases

Growth factors and their membrane associated receptor tyrosine kinases play important roles in both cell proliferation and differentiation in tumor cells. Evidence reveals that oncogenesis results from mutations leading to constitutive activation of growth factor receptors. As such, growth factor related signaling cascades may present novel targets for cancer chemotherapy. The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases lies at the top of one such complex [80]. In that study, shikonin revealed to exert its antiproliferative potential by modulating the EGFR signaling cascade in human cutaneous neoplasm.

By testing A/S derivatives, it was established that b-HIVS most efficiently induced cell-death in two lines of lung cancer cells, NCI-H522 and DMS114, while shikonin was effective against a wide variety of tumor cell lines. b-HIVS and shikonin were found to inhibit protein tyrosine kinase (PTK) activity, suggesting a novel group of PTK inhibitors based on shikonin as the parent compound [84]. Shikonin and its derivatives were thus patented as tyrosine kinase inhibitors having slight adverse effects and a chemical structure different from the conventional ones [85]. Finally, the characteristics of the inhibition of PTK activity from b-HIVS were compared with those of other inhibitors in a recent review [86].

Inhibition of Human Telomerases

Human telomeres are located at the end of eukaryotic chromosomes and telomerase has the ability to maintain telomere length. That enzyme is present in the vast majority of cancer cells, while is largely absent in normal somatic cells and is considered to play an important role in the maintenance of telomeres in cancer cells. Designing drugs with inhibitory activity against telomerase is emerging as an attractive strategy for chemotherapy [87]. Trying to explore new chemotherapeutic agents, shikonin derivatives were examined for their possible inhibitory activity on telomerase. As shown, alkannin, shikonin and several A/S derivatives (mainly acyl ones), except cyclo-derivatives, inhibited telomerase enzyme and presented low cytotoxicity. Therefore, A/S moiety was proposed for the future development of potent telomerase inhibitors [87].

Inhibition of Angiogenesis

The growth of new blood vessels or angiogenesis is important in the growth of solid tumors [88]. Shikonin was proposed as a novel inhibitor of angiogenesis [89]. It revealed to inhibit tumor necrosis factor-alpha-induced and B16-melanoma-induced angiogenesis in mice and normal development angiogenesis in the yolk-sac membranes of chick embryos. The mechanism of this inhibitory effect on angiogenesis was suggested to involve the prevention of network formation by endothelial cells vial blocking integrin αvβ3 expression [89].

Inhibition of DNA Topoisomerases

DNA topoisomerases are a class of enzymes that alter DNA conformation, controlling the topological state of DNA

and are therefore a target of many anticancer drugs [2]. Strong inhibition of topoisomerase I by naturally occurring and synthetic naphthoquinones, including A/S and derivatives, has been reported by our group [90]. This study revealed that naphthoquinones bearing at least one phenolic hydroxyl group are potent inhibitors of topoisomerase I. Their ability to complex divalent zinc was correlated with their inhibitory activity. With this study and with other ones reported in our previous review [1], it is suggested that inhibition of topoisomerase I by A/S derivatives is a secondary mechanism of their anticancer activity.

Inhibition of Cyclooxygenase-2 Transcription

Cyclooxygenase-2 (COX-2) is a recognized target for cancer prevention and possibly treatment. Several plant extracts including *Arnebia euchroma* and its constituents, A/S derivatives, inhibited the stimulation of COX-2 promoter activity [91]. Shikonin was found to inhibit phorbol 12-myristate 13-acetate (PMA)-mediated induction of COX-2 mRNA, protein and prostaglandin E_2 synthesis. It also inhibited PMA-mediated stimulation of extracellular signal-regulated kinase 1/2, which are mitogen-activated protein kinases and activator protein-1 activity.

Antitumor Activity of Several Synthetic A/S Derivatives

Proven the antitumor activities of A/S, several derivatives were synthesized trying to approximate a structure activity relationship and exploring drug candidates more potential than A/S. Haloacetylshikonin derivatives were synthesized and their antitumor activity on L1210 cells was evaluated [92]. A synthetic shikonin analog 93/637 inhibited cellular growth and insulin-like growth factors on prostate cancer cells and suggested a potential therapeutic use [93]. Another naphthoquinone derivative, 2-(hydroxyiminoalkyl)-5,8-dimethoxy-1,4-naphthoquinone–S-33 was found to be a potent anticancer agent [94]. Very recently [95], several alkannin derivatives were synthesized and their cytotoxicities were evaluated against four-human carcinoma cell line (GLC-82, CNE2, Bel-7402, K-562) and were found markedly higher than that of naturally occurring β,β-dimethylacrylalkannin and acetylalkannin.

3.5. Other Biological Effects and Applications of Alkannins/Shikonins

Recent studies revealed that A,S, and *A.t., L.e.* root extracts were found to possess strong antioxidant and radical scavenging activity. Antioxidative activities were tested against several types of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion radical ($^{\cdot}O_2$), hydroxyl radical ($^{\cdot}OH$), tert-butyl peroxyl radical ($BuOO^{\cdot}$), DPPH radical, against copper-induced LDL oxidation, as well as iron-dependent microsomal lipid peroxidation [54,55, 96-100]. Monomeric A, S, polymeric A/S and *A.t., L.e.* root extracts were also shown to exert antioxidant activity in several oil substrates studied [101,102]. As revealed, A and S radical scavenging activity, plays probably an important role in enhancing wound healing and in the anti-inflammatory effect of shikonin, while their reduction to A/S semiquinone radicals may exhibit cytotoxicity, that may be responsible for the antitumor and antibacterial activity of IHN. A tobacco filter containing shikonin and alkannin for free radicals removal has been patented [103].

Several naphthoquinone derivatives, including shikonin ones, were tested for preventing the growth of human immunodeficiency virus (HIV) [104]. Shikonins and naphthazarin showed weak inhibition. In a more recent study [105] shikonin down-regulated surface expression of CCR5, a primary HIV-1 coreceptor on macrophages, inhibited the replication of a multi drug-resistant strain and pediatric clinical isolates of HIV in human peripheral blood mononuclear cells. It also effectively inhibited the replication of the HIV Ba-L isolate in monocytes/macrophages. It was thus suggested that the anti-HIV and anti-inflammatory activity of shikonin may be related to its interference with chemokine receptor expression and function. Therefore, shikonin is proposed for the development of novel anti-HIV therapeutic agents, as a naturally occurring, low-molecular weight pan-chemokine receptor inhibitor [105].

Shikonin was patented, among other compounds, for down-regulating expression of human papillomavirus (HPV) protein in patients infected with a HP virus [106]. Type 2 diabetes is due to defects in both insulin action and secretion. In an attempt to discover small molecules that stimulate glucose uptake, shikonin was tested [107]. It was shown to stimulate glucose uptake and potentiated insulin-stimulated glucose uptake. Approaching the mechanism, it was proposed that in 3T3-L1 adipocytes, shikonin action was not mediated primarily *via* the insulin receptor/PI3K (phosphatidylinositol 3-kinase) pathway, but rather *via* another distinct tyrosine kinase-dependent pathway leading to glucose uptake involving Akt (serine/threonine protein kinase) phosphorylation.

In another study, roots of *L.e.* as a constituent of Shi-Ka-Ron (a Chinese traditional medicine) showed protective effects to immunosuppressive mice. Thus, it resisted immunosuppression induced by the antitumor agent mitomycin C, by a mechanism correlated with stimulation of the reticuloendothelial system, activation of T cell blastogenesis and NK cell cytotoxicity [108]. In a very recent study [109] shikonin derivatives isolated from *L. canescens* showed immunomodulatory effect on cellular and humoral immunity in Balb/c mice. The effects of A/S and acetyl- and β,β-dimethylacryl-shikonin, isolated from *Macrotomia euchroma*, were studied on vascular reactivity with isolated rat aortic rings [110]. As shown, S,A and their derivatives inhibited agonist-induced relaxation at lower concentrations and induced vasocontraction at higher concentrations, while the effects were endothelium dependent, however through different mechanisms.

Hydroxynaphthoquinones of the A/S series were patented as inhibitors capable of effectively curing autoimmune diseases, such as I type diabetes and chronic arthro-rheumatism [111]. *L.e.* root extract was examined among other plants used in Chinese medicine, for its antiparasitic action *in vitro* against *Trypanosoma cruzi*, the etiologic agent of Chagas disease. As shown, *L.e.* root extract fully inhibited growth of epimastigote form of *T. cruzi* [112]. Finally, *in vitro* metabolism of shikonin was studied [113]. As shown, the main metabolites obtained were dihydroxylated shikonin, 2-OH shikonin and 6-OH or 7-OH shikonin.

Several pharmaceutical and cosmetic preparations containing A,S or *A.t.* and *L.e.* root extracts have been

patented worldwide. Specifically, a hygienic composition of oral cavity (toothpaste, tooth powder, half paste dentifrice, dental ointment, gargling agent, troche or chewing gum) having improved preventing and remedying effect on diseases in the oral cavity, such as dental caries, gingivitis, ulcerative stomatitis, pyorrhea alveolaris, containing shikonin and/or *Lithospermi* Radix, has been patented by Mitsui Petrochemicals Industry [114]. A plaster having high remedying effect on trauma, burn and other skin diseases, containing shikonin or a *Lithospermi* Radix extract has been also patented by Mitsui [115].

A cosmetic having anti-suntan effect and protect skin from sunlight with shikonin has been patented among other compounds by Ichimaru Pharcos [116], while an external agent for skin having excellent effect on suppressing skin inflammation caused by sunburn and pigmentation after sunburn, containing *L.e.* root extract, was also patented by Kose Corp [117].

A face beautifying cosmetic using *A.t.* as functional substance was patented for relieving inflammation, germicide, promoting growth of skin cell, preventing and curing dermatopathies of comedo and acne [118]. Another cosmetic composition that improves skin stains, wrinkles and skin roughening and suppressing allergic dermatitis using *Lithospermum* radix extract was also patented [119]. *L. erythrorhizon* root extract was also patented by Shiseido Co. as an antiaging agent that keeps the skin taut and elastic, possesses elastase inhibitory effect and prevents skin aging [120].

A body protecting cream using *A.t.* oil having curing effect to female pudendal diseases, such as vaginitis, pudendal itching, flushing, edema and papule and preventing wrinkles, was also patented [121]. Alkannin was also proposed as an active ingredient in a bacteriostatic lipstick for bacteriostasis, antiphlogistic action, lip inflammation and virus resistance [122]. A topical composition containing *Lithospermum* extract as skin lightening and conditioning ingredient was proposed by Mitsui Chemicals [123], while shikonin or *Lithospermum* radix extract was patented in a hair cosmetic preparation that gives rich touch to hair by Lion Corp [124]. Recently, a toilet paper containing *L.e.* root extract was patented for healing hemorrhoids [125] in Korea.

In another study, shikonin was tested among other quinones for its inhibitory activity on plant p-hydroxyphenylpyruvate dioxygenase, the target site for triketone herbicides and proved to have good activity [126]. Shikonin release was finally examined from a new cotton fiber with a chitosan coating prepared [127].

As reported above, research on biological properties of A/S has focused exclusively on monomeric A/S and IHN derivatives. Since recent research has detected the presence of oligomeric and polymeric A/S and HNQ in natural resources and the structure of dimeric ones has been determined, there is a great interest to evaluate the biological properties of oligomeric and polymeric A/S and HNQ. This study is crucial and interesting, since both monomeric and polymeric A/S and HNQ are biosynthesized secondarily in the root plants and thus coexist of necessity in the root extracts that comprise the active ingredients for the production of pharmaceutical and cosmetic preparations.

4. CHEMISTRY OF ALKANNINS/SHIKONINS

The chemistry of alkannin, shikonin and their parent naphthoquinone, naphthazarin, has been extensively described in our previous reviews [1,49]. Since then, three shikonin derivatives were isolated and characterized from the roots of *Arnebia hispidissima* (Table **1**): shikonin, butylshikonin and 2'-methyl-hexa-1',3'-dienoylshikonin, the last two esters being new naphthoquinones [128]. Arnebinofuranone was also isolated. The stereochemistry of these derivatives was confirmed by CD spectra. In a more recent study, the hexane extract of *Arnebia hispidissima* (Lehm.) DC. was studied and six alkannin derivatives, not shikonin as above reported, were identified and quantified; specifically arnebin-1 (major one), arnebin-7 (deoxy-alkannin), tiglic ester of dihydroxyalkannin, alkannin, arnebinol and cycloarnebin-7 (Table **1** ; [52]). In this study no attempt was made to assign the absolute configuration of each derivative, so alleged alkannin derivatives may be shikonin derivatives, as proved by CD spectra [128] or racemates. However, the stereochemistry may vary between ester derivatives from the same plant or between plants from different locations, even though both samples were collected from India. Another shikonin derivative, propionylshikonin (Table **1**) was for the first time isolated and identified in nature from the roots of *L. erythrorhizon* [129].

Recently, shikonin derivatives were also determined in the traditional Chinese medicinal preparation "Tzyy-Yun-Gau" containing *Arnebia* radix. In this preparation shikonin, deoxyshikonin, β,β-dimethylacrylshikonin and acetylshikonin were quantified [130]. A pharmacognostical study of the Japanese drug "Nan-Shikon", or Indian "Ratanjot" or "Zicao" in China, root of *Arnebia euchroma* (Royle) Johnston, grown in India, was conducted and established that nine naphthoquinones (β,β-dimethylacrylalkannin, β,β-dimethylacrylshikonin, acetylalkannin, alkannin, shikonin, deoxyshikonin, β,β-dimethylacryl-hydroxyalkannin (arnebin-2), hydroxyalkannan (arnebin-5) and acetyl-hydroxyalkannin (arnebin-6)) were present in the hexane extract [131].

A, S and their IHN derivatives are not stable compounds. They are susceptible to several transformations, such as photochemical decomposition and thermal degradation, as reviewed in our previous paper [1]. The physical stability of five shikonin derivatives (deoxyshikonin, shikonin, acetylshikonin, isobutyrylshikonin, β-hydroxyisovalerylshikonin), isolated from the roots of *L. erythrorhizon*, was tested against heat and light. The thermal degradation of deoxyshikonin ($t_{1/2}$=14.6h, 60°C) and isobutyrylshikonin ($t_{1/2}$=19.3h, 60°C) was greater than the other shikonin derivatives ($t_{1/2}$=40-50h), while light stabilities of the shikonin pigments were similar to each other ($t_{1/2}$=4.2-5.1h, 20000 1x light intensity) [132].

Recently, polymerization of A/S and their derivatives was studied for the first time by Papageorgiou research team [133]. Size Exclusion Chromatography (SEC) was used for qualitative and quantitative analysis of monomeric, oligomeric and polymeric IHN derivatives [32]. In that study, the purity and degree of polymerization was determined in several *Alkanna tinctoria* root samples and commercial samples of A and S. As shown, the presence of polymeric/oligomeric IHN is indigenous to Boraginaceous roots,

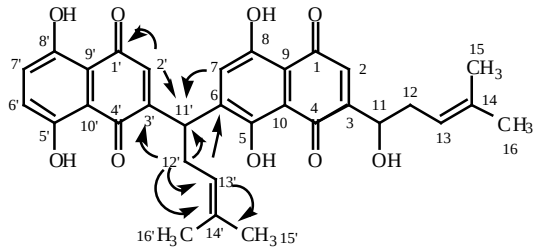


Fig. (3). Structure proposed for the most abundant dimeric A/S isolated by A/S commercial samples. (Spyros, A.; Assimopoulou, A.N.; Papageorgiou, V.P. Structure determination of oligomeric alkannin and shikonin derivatives, *Biomed. Chromatogr*., **2005,** 19, 498) [© 2005. Copyright John Wiley & Sons Limited. *Reproduced with permission*]

probably as a result of A/S biosynthesis. In a recent study, Papageorgiou group investigated the influence of several variables processing and storage on A/S polymerization [33]. Temperature and solvent polarity increased significantly the concentration of hydroxynaphthoquinone oligomers/ polymers, while light and air exposure conditions tested did not significantly affect polymerization. Very recently the structure of the most abundant dimeric IHN, isolated by SEC from alkannin and shikonin commercial samples, was proposed by Papageorgiou group (Fig. **3**; [134]). This structure was determined by one- and two- dimensional NMR spectroscopy and as proposed coupling of the side chain of one naphthoquinone unit with the aromatic ring of a second naphthoquinone leads to dimer formation.

Additionally, the influence of alkaline media was investigated on IHN polymerization [34]. As shown, during IHN esters hydrolysis, oligomeric derivatives were formed and it was proposed that oligomerization/polymerization of IHN in alkaline media proceeds through the intermediate formation of semiquinones (Fig. **4**), while after acidification,

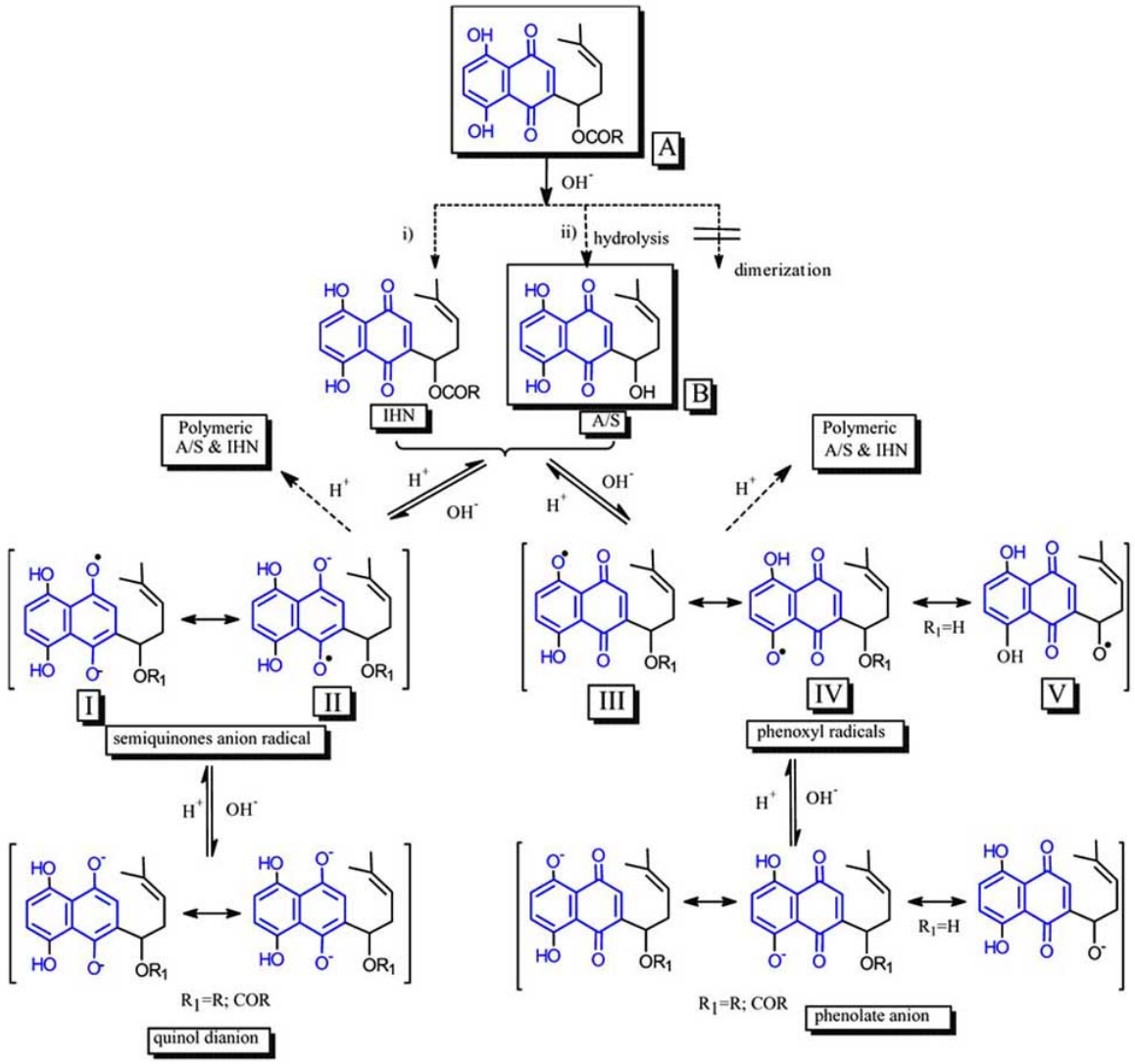


Fig. (4). Mechanism of hydrolysis of IHN esters and effect of alkaline media on A/S. (Assimopoulou, A.N.; Papageorgiou, V.P. *Biomed. Chromatogr*. Study on isohexenylnaphthazarins polymerization in alkaline media. **2004b,** *18*, 508) [© 2004. Copyright John Wiley & Sons Limited. *Reproduced with permission*].

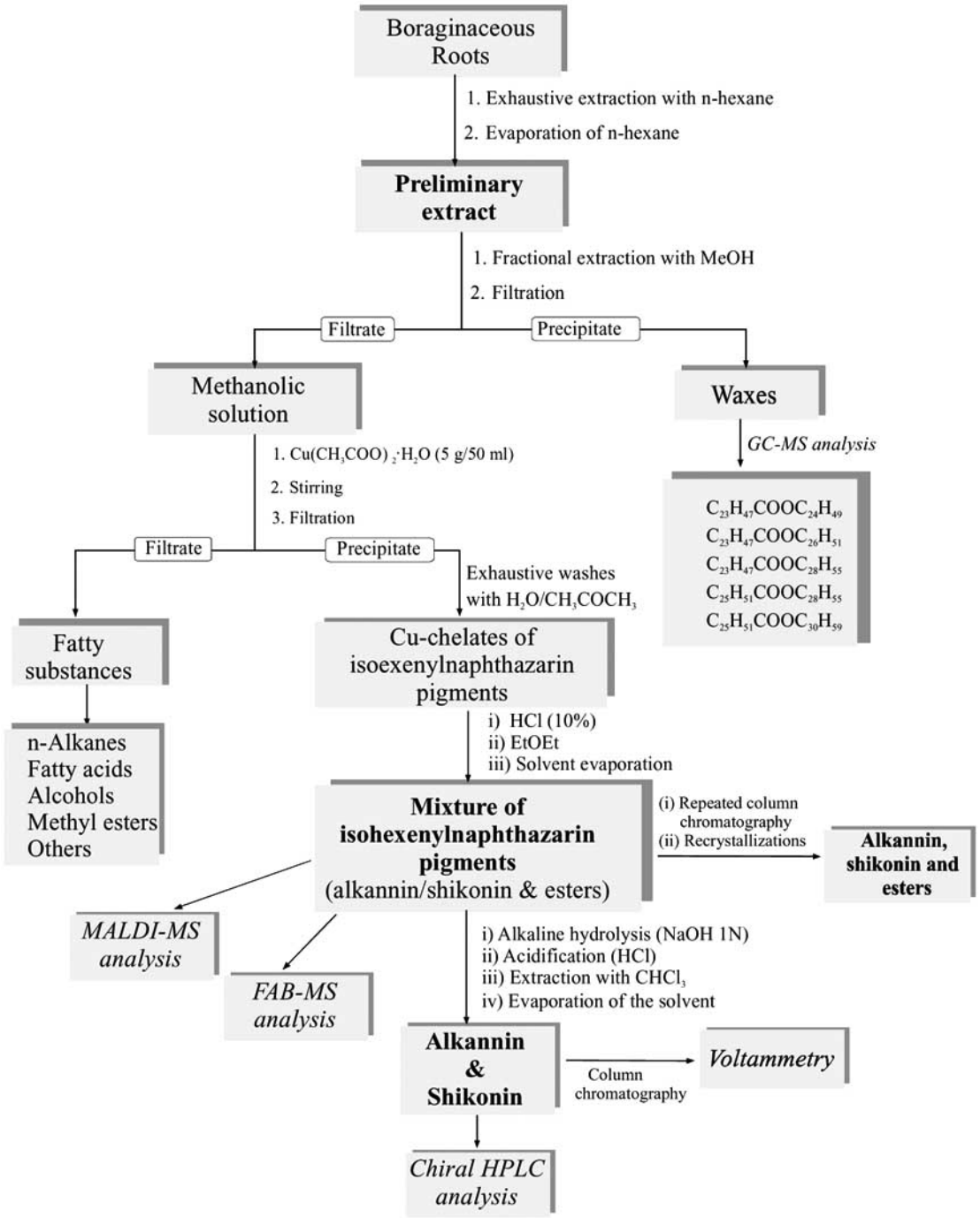


Fig. (5). Analytical procedure for the isolation of a mixture of IHN pigments and pure A/S and their esters from Boraginaceous roots.

coupling of semiquinones with phenoxyl radicals results in oligomeric IHNs.

Finally, in order to increase A/S solubility, stability and decrease intense coloration and achieve controlled release, several drug delivery systems were prepared by Papageorgiou group and specifically: shikonin-containing microcapsules [135], alkannin-containing microcapsules [136] and inclusion complexes with cyclodextrins [137].

The enantiomeric A/S ratio was determined in several commercial samples of alkannin, shikonin and *Alkanna* roots by chiral HPLC [31]. A/S commercial samples consisted of both A/S in different proportions, either these were produced by IHN esters hydrolysis or biotechnologically. When *Alkanna* root samples were examined, a mixture of IHN pigments isolated from roots according to Fig. (**5**), was hydrolyzed to A/S and the total A/S enantiomeric ratio was estimated by chiral HPLC. As shown, in each of the *Alkanna* root samples tested, alkannin derivatives predominated. Light, temperature and air did not influence the A/S enantiomeric ratio on A/S commercial samples. That ratio remained the same when A/S where microencapsulated and encapsulated in cyclodextrins.

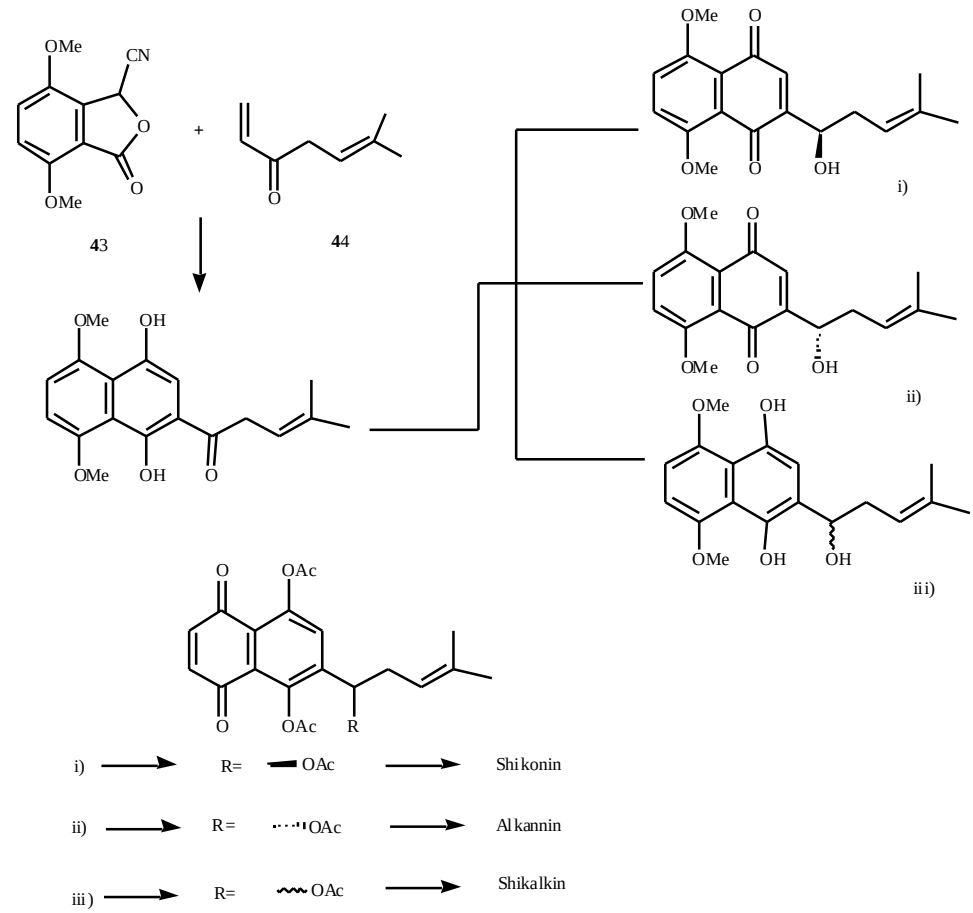


Fig. (6). Synthesis of alkannin, shikonin and shikalkin [139].

The structure of A/S may look misleading simple. However, in spite of great efforts over many years by several research groups worldwide [1], a commercially viable synthetic route to these enantiomers has remained elusive till very recently, where Nicolaou group reported highly efficient and one of the most elegant total syntheses of both alkannin and shikonin [138].

Recently, a short and convergent approach for the synthesis of A,S and racemic shikalkin was presented [139]. A Hauser type annulation of cyanophthalide *43* with enone *44* (Fig. **6**) afforded the complete aromatic system in one step with concomitant attachment of the entire side chain. Subsequently, Corey's oxazaborolidine mediated asymmetric reduction of the above advanced intermediate, leads to the required isomer in high enantiomeric excess. Finally, a selective and high yielding deprotection protocol furnished A/S as pure crystalline precipitates. The total chemical yield of all operations described in Fig. (**6**) from intermediates *i, ii* and *iii* up to the final pure crystalline products, was 75 to 80%.

Analytical methods for the determination and identification of A/S and their derivatives ever reported, have been recently reviewed [140].

CONCLUSION

The chiral pair A/S are potent pharmaceutical substances with a well-established and wide spectrum of wound healing, antimicrobial, anti-inflammatory, antioxidant, anticancer, radical scavenging and antithrombotic biological activity. The ancient medicinal properties claimed for *A.t.* and *L.e.* roots have been confirmed by scientific experimentation within the last 30 years. The clinical application of preparation that contain ester derivatives of A/S for the treatment of burns, wounds and ulcers is, perhaps, the most dramatic development. The last years, research on A/S derivatives has revealed their effectiveness on various aspects of cancer treatment and has approached their mechanisms of action. Significant developments are to be expected in this area in the future. A wide range of biological properties and other applications have been established for A, S and their derivatives. Further developments in other therapeutic areas are expected in the near future.

Recent developments in biotechnology have facilitated the commercial production of A/S by plant tissue cultures and gave impetus on understanding their biosynthesis and plant secondary metabolism in general.

There are several noticeable and interesting observations on A/S chemistry, biosynthesis and biological properties. Thus, these are biosynthesized in the underground parts of plants belonging mainly to Boraginaceae family. Both enantiomers, A and S are observed in natural resources and formed by plant tissue cultures. Additionally, both A and S are formed at the same time during biogenesis in the same plant. Although enantiomers of chiral drugs differ consider-

ably in their pharmacological effects, A and S exert the same biological properties. Furthermore, it is noteworthy that A and S derivatives exhibit opposite biological properties, such as anticancer and antimicrobial with wound healing activity, classifying them to a very rare pair of enantiomeric natural products. It was proposed that anticancer and antimicrobial activities are attributed to the A/S semiquinones formed, while wound healing one to A/S intact moiety.

Since oligomeric and polymeric A/S have been shown to be biosynthesized instantaneously with monomeric ones, research has to be extended to the biological properties of oligomeric and polymeric A/S derivatives and also their biogenesis.

LIST OF ABBREVIATIONS USED

A	=	alkannin
A/S	=	alkannin and shikonin
A.t.	=	*Alkanna tinctoria*
b-HIVS	=	β-hydroxyisovalerylshikonin
CD	=	circular dichroism
DMSO	=	dimethylsulfoxide
DPPH	=	1-diphenyl-2-picryl-hydrazyl
GPC	=	Gel Permeation Chromatography
HNQ	=	hydroxynaphthoquinone
IHN	=	isohexenylnaphthazarin
L.e.	=	*Lithospermum erythrorhizon*
PEG	=	polyethyleneglycol
RP	=	Reversed Phase
S	=	shikonin
SEC	=	Size Exclusion Chromatography
SFE	=	Supercritical Fluid Extraction

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