THE ANALYSIS OF PHARMACOLOGICALLY ACTIVE COMPOUNDS AND BIOMOLECULES IN REAL SAMPLES

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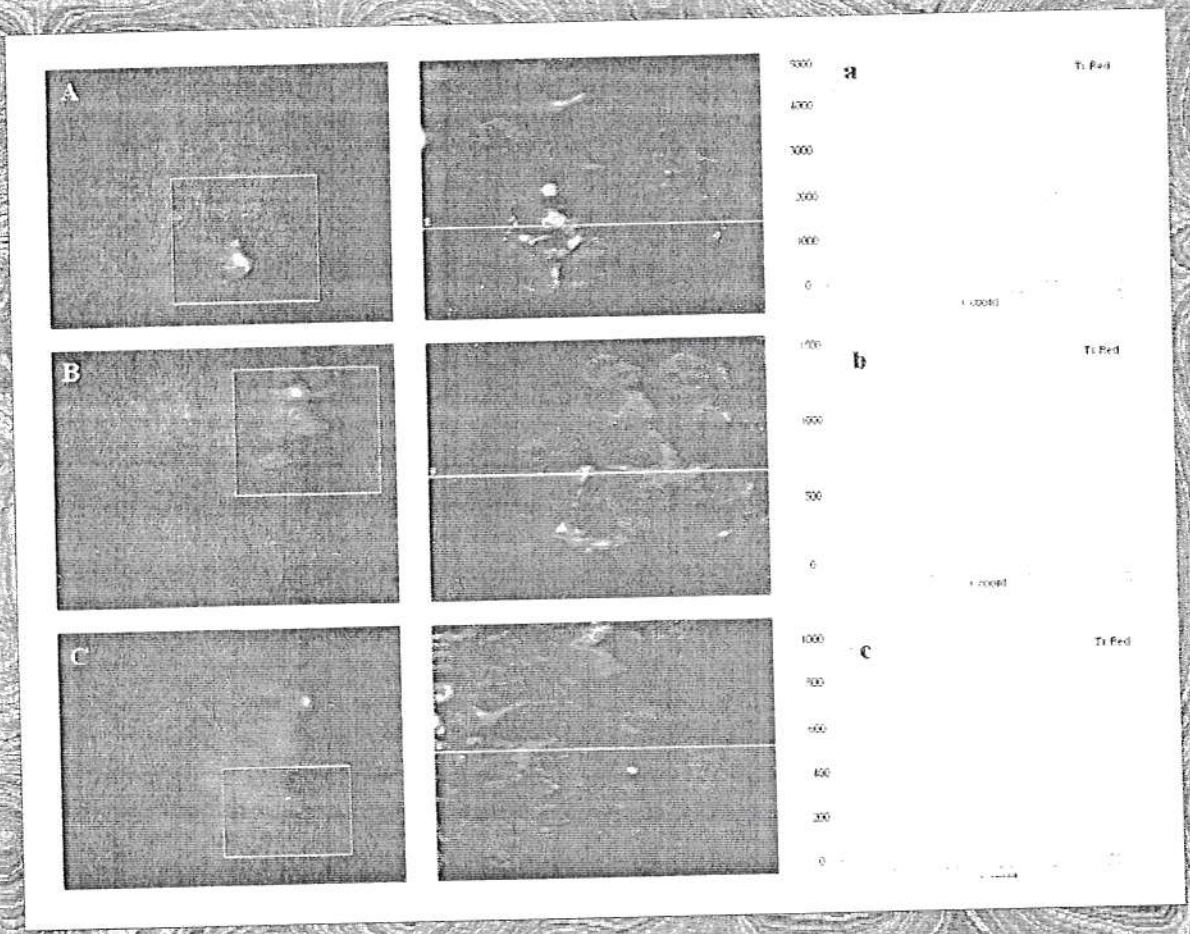

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The Analysis of Pharmacologically Active Compounds and Biomolecules in Real Samples

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4. Analysis of natural products – Sesquiterpene lactones as anti-inflammatory agents

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Abstract. Sesquiterpene lactones (**SQTLs**), a special class of terpenoids, with more than 30 skeleton subtypes, are secondary metabolites from numerous genera of *Asteraceae*, *Lauraceae*, *Apiaceae*, Bursaceaae *Magnoliaceae* and liverworts (*Hepaticae*).

SQTLs are known to possess antimicrobial, antitumor, anti-inflammatory, and allergenic potency, and the effects on the central nervous and cardiovascular system.

The paper presents the methodology in isolation, purification, structure elucidation, as well as structure-activity relationships of **SQTLs**. The applications of two-dimensional nuclear magnetic resonance (2D NMR) experiments, in combination with other spectrometric methods (e.g., chemical ionization mass spectrometry) allow very rapid and straightforward assignment for structure of complex natural products. The examples presented were chosen to illustrate the potential of the 2D NMR methods for structure determination of SQTLs.

The mechanism of anti-inflammatory activity of this group of secondary metabolites is described in detail.

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Introduction

One of the greatest unmet medical needs is the effective treatment of inflammatory and autoimmune diseases. Inflammation is the body's way of dealing with infections and tissue damage, but there is a fine balance between the beneficial effects of inflammation cascades and their potential for long-term tissue destructions. If they are not controlled or resolved, inflammation cascades can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases and psoriasis. Within many inflammation cascades or pathways there often pivotal molecular targets that, when neutralized or antagonized, block the output of the pathway [1]. Historically, at least over the past 20 years in the modern era of the target-based drug discovery, a relatively small number of pivotal targets have been identified that have yielded any successful anti-inflammatory drugs. There are some basic principles that guide the discovery and development of successful therapeutic targets. First, the target should be proximal to the initiation of the disease – not necessarily the very first initiating event but at least close to it. Second, the target should play a pivotal or driving role in the disease process – these targets are often at key regulatory points or rate-limiting steps in pathways (so that blocking such an event stops a whole series of downstream processes). Third, targets should be unique to the disease process – thus giving a desired potent effect on the disease without having unwanted side effects on other physiological processes. Not all of these features need to be present to ensure success but at least one is desirable for an effective drug target (and this is also true in the field of anti-inflammatory drug discovery). Nevertheless, some specific features of immune responses and inflammatory cascades can be exploited to yield good anti-inflammatory targets. For example, many powerful proinflammatory mediators, such as histamine and leukotrienes, are released early in inflammation cascades and blocking their actions has proven to be a successful source of anti-inflammatory drug targets [2]. Many of these are antagonists of endogenous proinflammatory mediators such as prostaglandins, leukotrienes and histamine. These drugs include the histamine H1 receptors antagonists, the enzymes cyclooxygenase 1 and 2 (COX-2 and COX-2) inhibitors, the cytokine tumor necrosis factors-α (TNF-α) and the receptor for the cysteinyl leukotrienes C4 and D4 antagonists. The discovery of these drugs were enable because of new understanding of the pathology of inflammatory diseases and the subsequent discovery of a plethora of these drug targets that have been validated [3].

It is well known that, among synthetic drugs, the physiological anti-inflammatory properties of glucocorticoids have been extensively exploited

in many inflammatory and autoimmune diseases. Corticosteroids are potent anti-inflammatory and immunosuppressive agents but possess major metabolic side effects such as hyperglycemia, decreased carbohydrate tolerance and osteoporosis. On the other hand, conventional non-steroidal anti-inflammatory drugs (NSAIDs) as COX-1 inhibitors may cause gastric damages, which restrict their use, while COX-2 selective NSAIDs, although free of more serious gastrointestinal adverse effects, due to an increased cardiovascular risk have also many limitations as therapeutic agents. Due to this, natural anti-inflammatory products have again become the subject of extensive investigations as potential therapeutics. Namely, as Rudyard Kipling wrote (1910): "Anything green that grew out of the mould was an excellent herb to our fathers of olds..." for centuries, natural products have served as a major source of drugs and many of these, such as morphine, penicillin G, and quinine still remain the cornerstones of modern pharmaceutical care. Their dominant role is evident: in approximately 60% of anticancer compounds and 75% of drugs for infection diseases that are either natural products or natural products derivatives. Natural product substances have historically served as the most significant source of new leads for pharmaceutical development. The rapid developments in this area after the 1980s were attributed to major advancements in molecular biology where mechanism-based assays were available for measuring very specific and selective activities [4].

Natural products represent the most important source of unique chemical substances for evaluation with these new assaying strategies for potential pharmaceutical utility.

Phytochemistry studies have experienced a great deal of change during the last century not only regarding the number of compounds described, but also in the concept of phytochemistry itself. This change has mainly been related to two key points: the methodologies used in phytochemical studies and the questions regarding appearing secondary metabolites and their role. In the middle of XX century the main chromatographic technique available was paper chromatography (PC), taking 48 h for development. The sole method of physical analysis for identification and structure determination of natural products was UV spectroscopy and each spectrum took hours of careful measurements. Melting points and mixed melting points were required with elemental analysis and molecular weight determination. Structure determination was obtained by degradative chemistry and identification of specific moieties of the molecule.

The whole investigation was most time consuming. Within a decade, there were a number of dramatic advances in analytical techniques including thin layer chromatography (TLC) and gas chromatography (GC), infrared

spectroscopy (IR), nuclear magnetic resonance (NMR) and mass spectrometry (MS) that were powerful tools for separation and structure determination. Refinements and introduction of new analytical techniques greatly facilitated natural product research [5,6]. Structure elucidation technology has evolved particularly with the development of high field NMR spectrometry as well as high-resolution technologies in MS.

Most important are the two-dimensional NMR (2D NMR) techniques that have been developed, which allow very rapid and straightforward assignment for structure of complex natural products. Additionally, the technologies of coupled liquid chromatography–mass spectrometry (LC-MS) and similar techniques provide very potent and powerful methodologies for separation and structure elucidation.

Beyond of a continuous flow of information related to the characterization of new structures several phases may be schematically delineated in research of plant secondary metabolites [7]:

1. Determination of the enzymatic steps leading to the common precursors of the metabolic pathway
2. The use of molecular biology to probe the changes in gene expression associated with the plasticity of natural products metabolism
3. The emergence of functional genomics, providing a more accurate picture of the diversity of the genes/enzymes involved in metabolism of secondary metabolites
4. The exploitation of genetic engineering for optimizing the secondary metabolites profiles in plants. The lack of clear understanding of the biosynthesis of the targeted products, the complex interplay between different branches of metabolism and the need for coordinate regulation of multiple gene activities are among the main limiting factors for complete success in engineering biosynthetic pathways leading to plant natural products
5. The explosion of pharmacological and clinical studies supporting the traditional application of plants

The development of phytochemical studies in particular, and of natural products chemistry in a wider sense, would never have been possible without knowledge of the chemical structures of these compounds. Two key issues continuously challenge this goal: (i) to obtain sufficient amounts of pure compounds and (ii) the use of the appropriate tools to elucidate their chemical structures.

The advances in chromatography and structural elucidation techniques allowed an increase in the number of compounds isolated and characterized

and expanded the study of their ecological and physiological roles: the everlasting questions of 'why?' and 'for what?'

Perhaps the strongest impetus for development of new natural products is the advancement in bioassay technology over the last several years. We now have highly automated, very specific and selective bioassays in which materials, including natural products preparations, can be evaluated quickly and economically. Indeed, advances in bioassay technology have been so great that the availability of substances for evaluation has become more limiting than the ability to carry out those evaluations. Once biological activity has been demonstrated in an appropriate bioassay or primary screen, we now have available, based upon advances in separations and structure elucidation technology, the capability to isolate, purify and determine the chemical structure of the active constituent in a few days or, at most, a few weeks. The advances in separations technology are particularly associated with high performance chromatography methodologies. Most recently, improved methodologies in countercurrent partition chromatography (CCPC) have further expanded the capabilities for separations.

Taking into account the importance of sesquiterpene lactones (SQTLs) as secondary metabolites and their pharmacological properties, the purpose of the current review was to present the examples of advanced techniques for structure elucidation of isolated compounds (SQTLs) from plant material, the potential of 2D NMR methods in structure determination of SQTLs, and to present the methodology used for detecting the anti-inflammatory activity of some well-known SQTLs and its mechanism.

1. Sesquiterpene lactones

1.1. Significance of sesquiterpene lactones

Sesquiterpene lactones are one of the largest biogenetically homogenous groups of natural products known. Currently, the Dictionary of Natural Products holds over 11000 entries on sesquiterpene, of which almost 5000 contain at least one lactone group. SQTLs are a special class of terpenoids with more than 30 skeleton subtypes and several substitutional features [8-10]. They are typical metabolites from the large plant family *Asteraceae*, but are also present in species from *Lauraceae*, *Apiaceae*, *Burseraceae* and *Magnoliaceae* as well as in liverworts (*Hepaticae*). Since the SQTLs show a vast array of biological activities, have ecological importance and are used as taxonomic markers in the family of *Asteraceae*, they have chemical, biological, medicinal and commercial interest. In the early 1960s, major emphasis was on the use of SQTLs as taxonomic markers in systematic

biochemical studies within, first of all the family *Asteraceae*. This provided the impetus for many detailed structural studies of SQTLs in these plant families. In recent years, an increasing number of publications describing the ecological functions of SQTLs have been reported. The first report of the antifeedant properties of SQTLs in *Vernonia* species (*Asteraceae*) upon herbivorous insects created an awareness of a possible ecological role of SQTLs as natural deterrents against insects and mammals, as toxins against plant pathogens and as allelophathic agents in plant-plant interactions [11]. The biological activity of SQTLs, regarding their anti-inflammatory, antimicrobial, antiviral, antitumor effects, have been the scope of numerous investigation performed in the last years [12-25].

The common, as well as specific structural features of SQTLs, responsible for quoted activities are given further in the text (part **1.3.**).

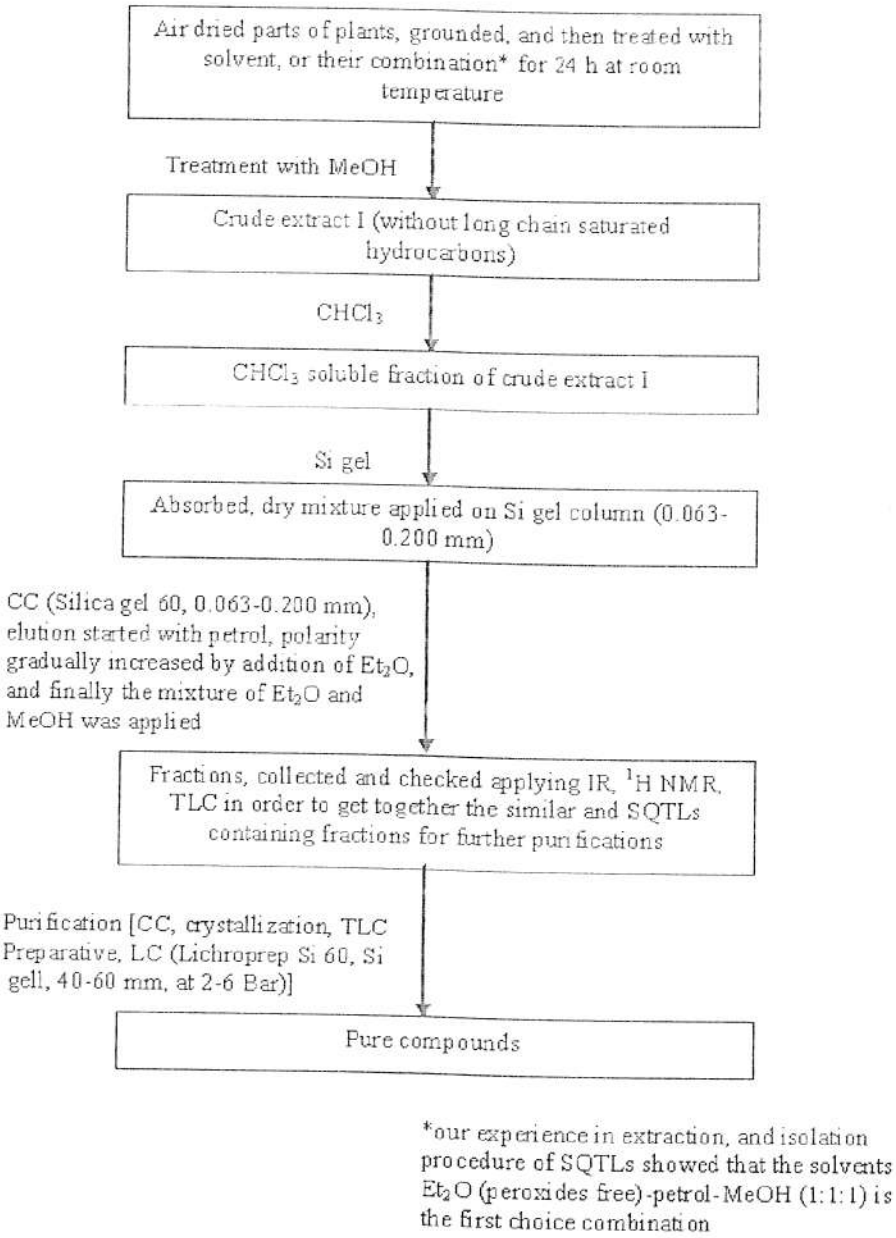
1.2. Structure elucidation of sesquiterpene lactones

According to proven chemotaxonomic importance and various biological activities, structure elucidation plays an important role in all of the studies concerning this class of compounds. The isolation of sesquiterpene from plant material might be performed using simple procedures, namely liquid-liquid extraction (LLE), with different solutions, enabling the isolation of numerous SQTLs from plant material [26-28]. The crude extracts obtained then passed through several steps of purification, applying the combination of repeated column chromatography (CC, with silicagel as adsorbent), dry-column flash chromatography, crystallization, preparative TLC, LC [29-32]. In the structure elucidation of SQTLs, the most widely used method is NMR spectroscopy. ^{1}H NMR spectral data analysis is usually starting point, since it is one of the most informative techniques for this purpose. Several scientific publications, including books [33,34], journals articles [35] and reviews [36,37] have appeared during the last 30 years with the regard to this subject. Furthermore, conformational studies based on optimization of 3D structures as well as analysis of X-ray diffraction data are also accessible as supporting information [38]. Additionally, an expert system has been developed to perform structure elucidation of SQTLs based on ^{13}C NMR data [39]. Finally, the work providing the use of neural networks as a tool for structure elucidation of SQTLs based on NMR spectroscopy appeared in 2004 [40].

The application of 2D NMR experiments, including some new variations of the known techniques (e.g., ^{13}C NMR spectra editing using Heteronuclear Single Quantum Correlation, HSQC) in combination with other spectrometric methods (*e.g.* chemical ionization mass spectrometry, CIMS), enabled complete

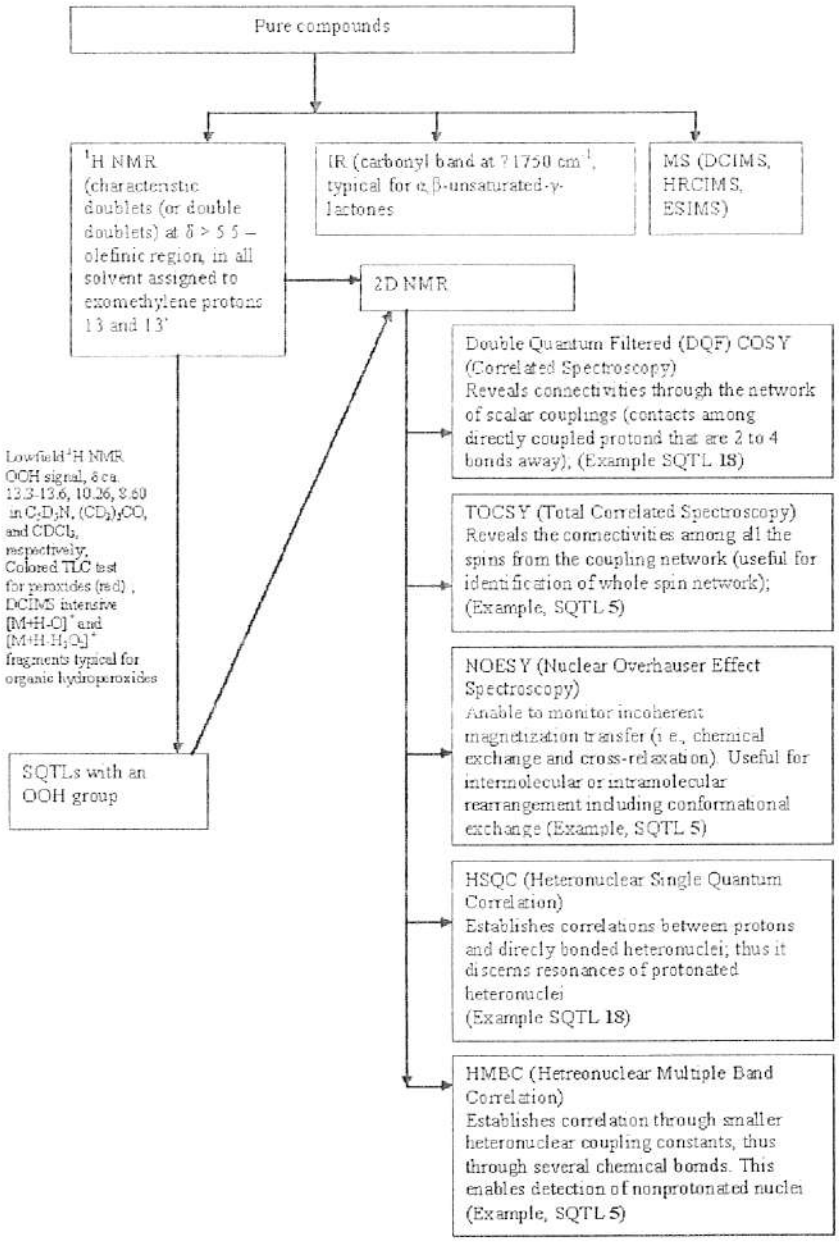
structural assignment and determination of relative stereochemistry of natural products [41,42].

Using the usual extraction procedure for isolation of SQTLs [26] in combination column chromatography and preparative TLC, the examination of aerial part of *Anthemis carpatica* Willd. and *A. montana* L., *Asteraceae*, originated from Serbia yielded thirty-one SQTLs of the same guaiadienolide type, all of them exhibiting an exomethyene 11(13)double bond, and one of germacranolide type (**Figures 1,2**). The extraction procedure is shown on the **Scheme 1**.



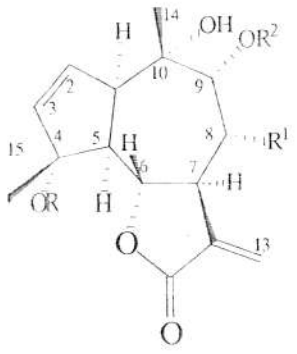
Scheme 1. Procedure of extraction, isolation and purification of SQTLs isolated from aerial parts *A. carpatica* and *A. montana*.

^{1}H and ^{13}C NMR assignment and structure determination of the isolated SQTLs were based on the characteristic chemical shifts and couplings obtained by the first-order analysis combined with 2D NMR measurements, such as Double Quantum Filtered (DQF)COSY Correlated Spectroscopy), TOCSY (Total Correlated Spectroscopy), NOESY (Nuclear Overhouser Effect Spectroscopy), HSQC and HMBC (Heteronuclear Multiple Band Correlation) performed on isolated compounds [32,43]. Schematically, the procedure used in structure elucidation of isolated SQTLs are presented on **Scheme 2**.



Scheme 2. Methods used to elucidate the structure of SQTLs isolated from *A. carpatica* and *A. montana*.

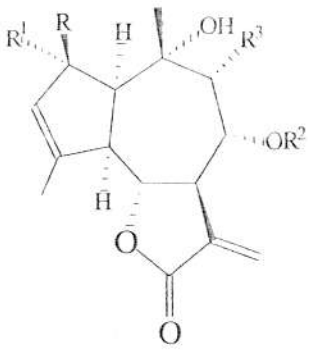
Δ^2-guaiadienolides



	R	R^1	R^2
1^c :	H	H	Ac
$2^{c,m}$:	OH	OAc	H
3^c :	OH	OH	Ac
$4^{c,m}$:	OH	OAc	Ac
$5^{c,m}$:	OH	O-*i*-But	Ac
6^c :	H	O-*i*-But	Ac
7^c :	OH	H	Ac
$8^{c,m}$:	OH	OH	H
9^c :	OH	OTig	Ac
10^m :	OH	OAng	Ac

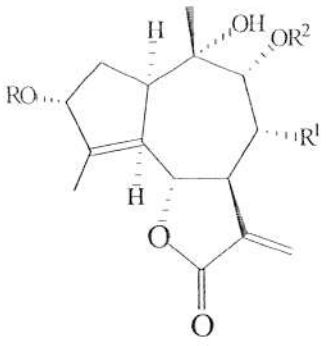
^{c}-SQTLs isolated from *A. carpatica*; ^{m}-SQTLs isolated from *A. montana*; ^{c,m}-SQTLs isolated from the both species

Δ^3-guaiadienolides



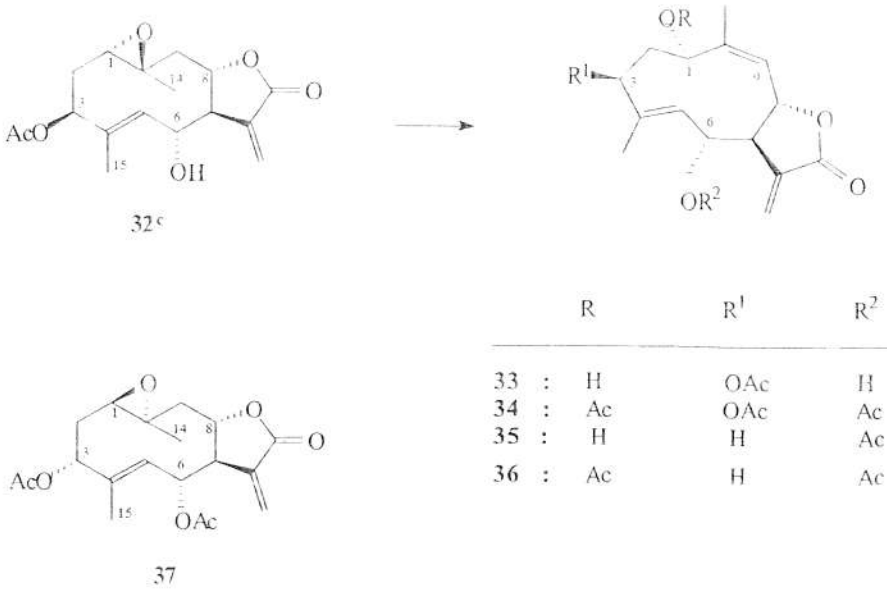
	R	R^1	R^2	R^3
$11^{c,m}$:	H	H	H	OAc
$12^{c,m}$:	H	H	Ac	OH
13^c :	H	H	Ac	OAc
14^c :	H	OOH	*i*-But	OAc
15^c :	H	H	H	H
16^c :	OH	H	H	H
17^c :	H	H	*i*-But	OAc
18^m :	OAc	H	*i*-But	OAc
19^m :	OAc	H	H	OAc
20^m :	OAc	H	Ac	OAc
21^m :	OAc	H	H	O-*i*-But
22^m :	OAc	H	H	O-*i*-Val
23^m :	OAc	H	H	O-2-MeBut

Δ^4-guaiadienolides



	R	R^1	R^2
24^c :	H	H	Ac
$25^{c,m}$:	H	OAc	Ac
26^c :	OH	OAc	Ac
27^c :	H	OAc	H
28^c :	H	OProp	Ac
29^c :	H	O-*i*-But	Ac
30^c :	H	OTig	Ac
31^c :	OH	O-*i*-But	Ac

Figure 1. SQTLs of guaiadienolide type isolated from two *Anthemis sp.* originated from Šara Mountain.



	R	R^1	R^2
33 :	H	OAc	H
34 :	Ac	OAc	Ac
35 :	H	H	Ac
36 :	Ac	H	Ac

Figure 2. New germacranolide isolated from the aerial parts of *A. carpatica*.

The spectral data confirming the structure of lactones (**Figure 1**) were in accordance with the same type of guaiadien-6,12α-olide skeleton (exception was new germacranolide **32**, **Figure 2**, isolated from *A. carpatica*), oxygenated at C-9 and C-10, with 10α-OH and 9α-OAc (or OH). Cumambrin B (**15**), containing no oxygen functionality at C-9, was the exception, as well. The similar frequency of a lactone carbonyl band ($\trianglelefteq$750 cm^{-1}) in all compounds, typical for α,β-unsaturated-γ-lactones, together with two characteristic doublets (or double doublets in some cases) in olefinic region (δ > 5.5 in all solvent used) of their ^{1}H NMR spectra, assigned to exomethylene protons 13 and 13', revealed the exocyclic 11,13-double bond. According to the position of the remaining double bond, all isolated lactones are divided into three groups: Δ^2, Δ^3, and Δ^4. The common ^{1}H NMR spectral characteristic for all isolated SQTLs, as well the specific features for SQTLs containing Δ^2, Δ^3, or Δ^4 double bond are presented in **Figure 3a-d**.

Lactones **2-5**, **7-10**, **14**, **26** and **31** (**Figure 1**), exhibited a hydroperoxy function that was identified on the basis of spectroscopic evidence. All hydroperoxy lactones also exhibited specific peroxide (red) colored TLC test with N,N-dimethyl-*p*-phenylendiammonium dichloride [44]. Direct chemical ionization mass spectrometry (DCIMS) (isobutane) revealed more structural information compared to electrospray ionization (ESI) applied in some cases.

Namely, DCIMS yielded $[M+H]^+$ ions and also abundant fragmentation ions obtained via elimination of neutral molecules, such as $[M+H-H_2O]^+$, $[M+H-AcOH]^+$ and $[M+H-H_2O_2]^+$, from the lactones containing OH, OAc or OOH groups respectively.

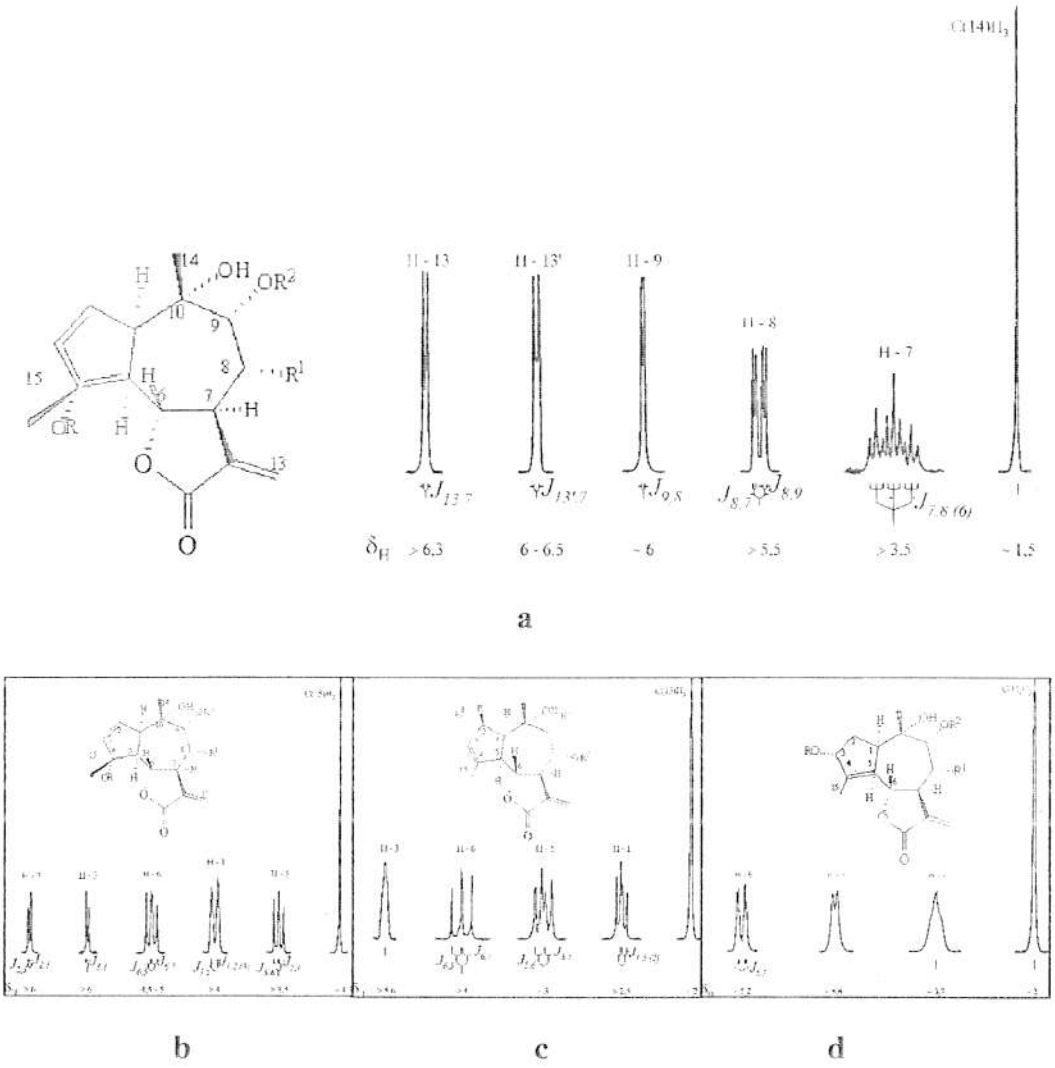


Figure 3. Characteristic chemical shifts in ^1H (C_5D_5N) NMR spectra and couplings assigned to SQTLs isolated from *A. carpatica* and *A. montana*; a) common for isolated SQTLs; b) common for Δ^2-lactones; c) common for Δ^3-lactones; and d) common for Δ^4-lactones.

In structural determination, a special attention was paid to lactone **18**. As far as the ^1H NMR spectra are concerned, this lactone exhibited rather broad resonances at room temperature, which was due to a conformational exchange at intermediate rate (on the NMR time scale). Such a behavior, not typical for guaianolides, is observed more frequently among the conformatioanlly mobile germacranolides. The existence of conformational exchange was confirmed by a low-temperature NMR ($CDCl_3$) study (**Figures 4,5**). At -57 ^0C, most of the broad multiples split into pairs of sharp, well-resolved signals, connected by positive cross peaks in NOESY (**Figure 6**). These data gave evidence of existence of two conformers, **18A** and **18B**, with **18B** slightly predominating (intensity ratio of about 1:1.16). In systems

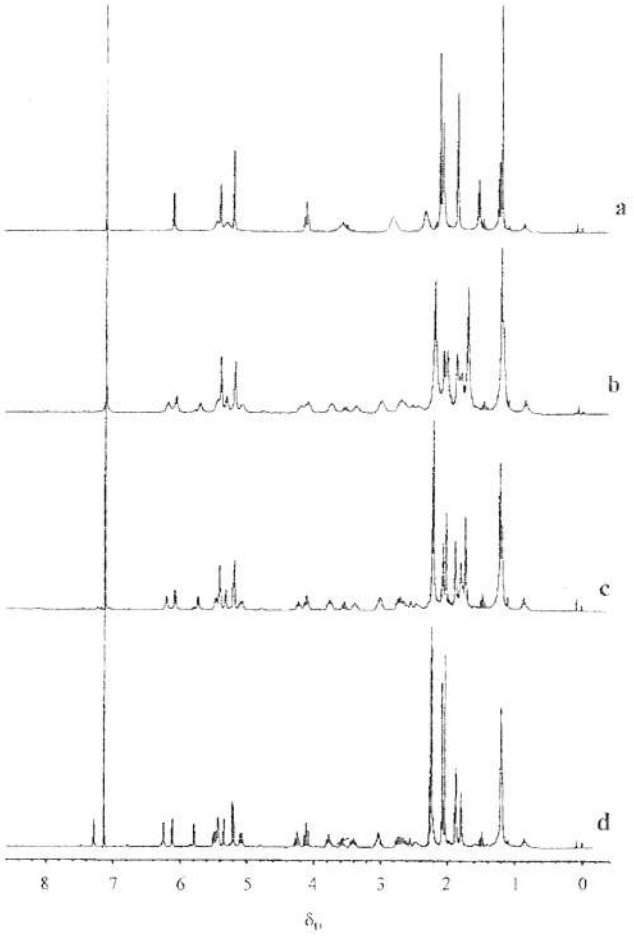
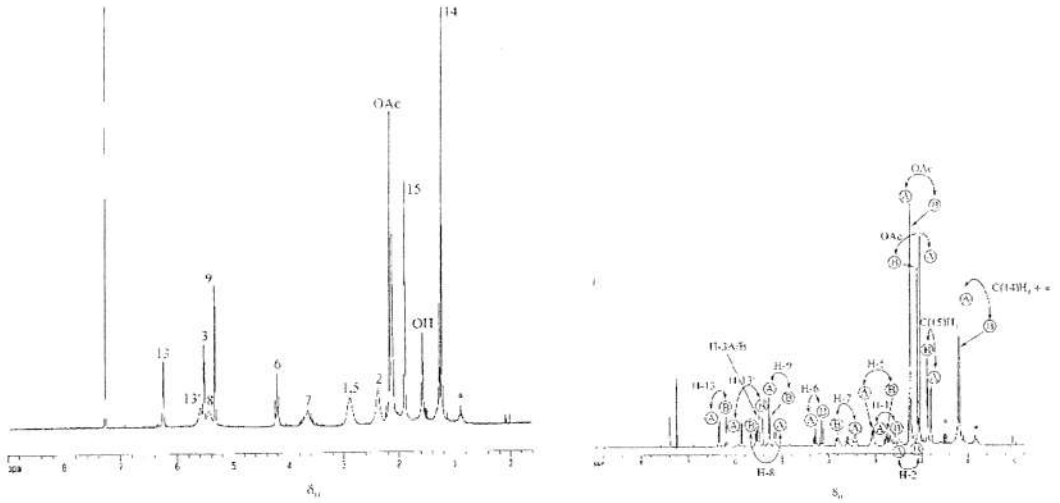
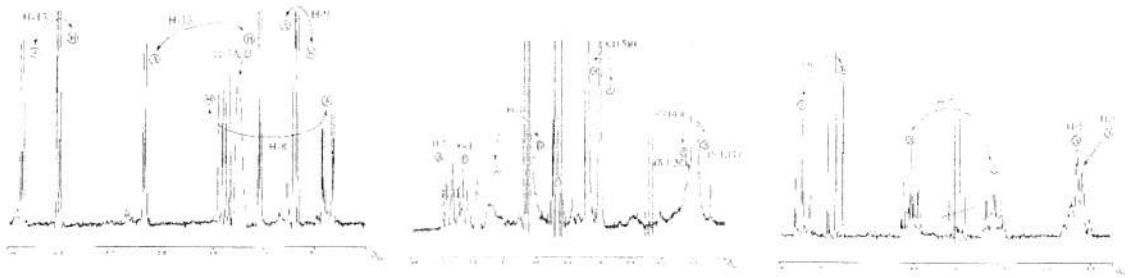


Figure 4. Temperature influence on ^{1}H(300MHz)NMR spectra of lactones **18**: a) 24 ^{0}C; b) -19 ^{0}C; c) -30 ^{0}C; and d) -57 ^{0}C.

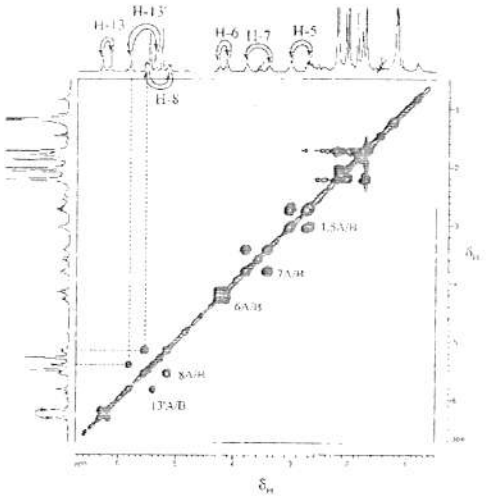


a **b**

Figure 5. ^{1}H NMR spectra of lactone **18** in $CDCl_3$ performed at (**a**) 24 ^{0}C and (**b**) -57 ^{0}C; * - impurities.



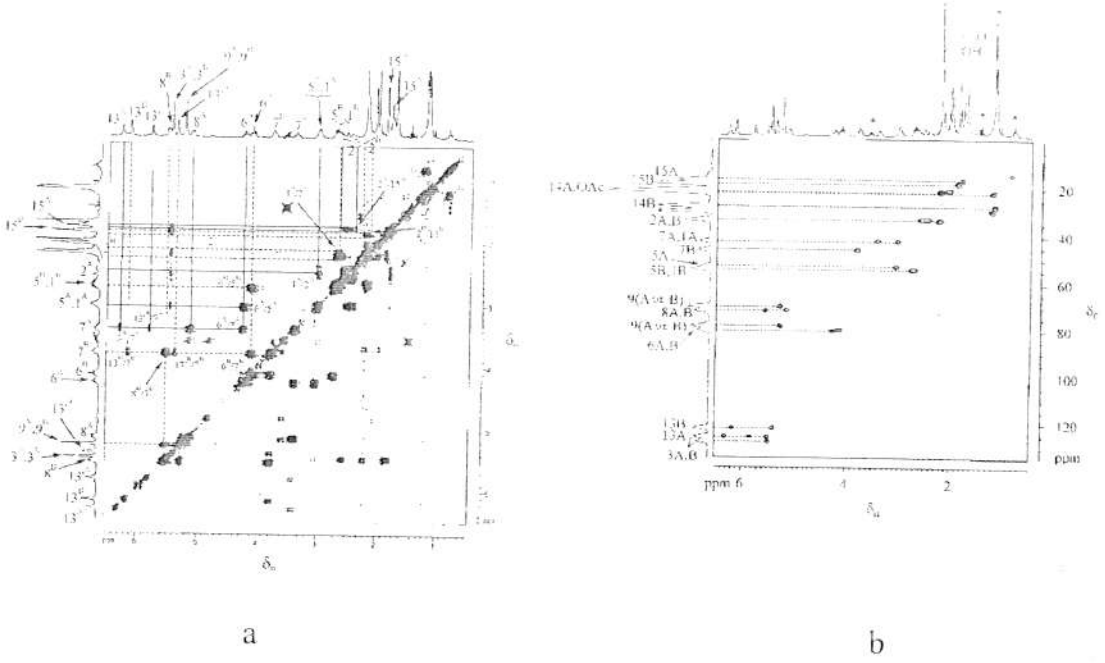
a



b

Figure 6. (**a**) Expansion of the low-temperature (-57 ^{0}C) ^{1}H NMR spectrum of lactone **18** in $CDCl_3$; (**b**) Positive correlations due to a slow conformational exchange in lactone **18**.

mutually exchanging at a slow rate, such as 18A $\rightleftharpoons$ 18B, at low temperature, each conformers exhibits a separate correlation network in DQF COSY and also HSQC (**Figure 7a**, and **Figure 7b**, respectively). The first-order analysis of the low-temperature ^{1}H NMR spectrum (**Figures 5a, 5b** and **6a**) in combination with scalar H,H-coupling networks in **18A** and **18B**, unambiguously assigned in DQF COSY (**Figure 7a**), as well as ^{13}C data measured in HSQC (**Figure 7b**) enabled identification of compound **18** as 9α-acetoxycumambrin A. These NMR data were in accordance with different (distorted chair) conformation of 7-membered ring in lactones **18A** and **18B**, with (pseudo)axial and (pseudo)equatorial 10β-methyl, respectively. Lactone **19** exhibited the same conformational exchange. A more detailed conformational analysis of these lactones, involving low-temperature NMR measurements in different solvents, as well as the evaluation of the conformations by the PM3 semiempirical method is presented in reference [45].



a b

Figure 7. DQF COSY (**a**) and HSQC (**b**) of lactone 18 in $CDCl_3$ at -30 ^{0}C.

The same method was applied for all lactones shown, whose structures were completely resolved. The lactone **32** was exception, because of germacranolide type skeleton of among the rest isolated lactones of guianadienolides type. During the extensive, long-lasting 2D NMR measurements, lactone **32** was transformed into lactone **33**, which instead of 1,10-epoxide ring, has an additional double bond in position 9 (**Figure 2**). The combination of ^{1}H and 2D NMR experiments, the comparative analysis with the literature data regarding the known structure of lactones **35**, **36** and **37** [31, and reference therein], as well as the structural data of the prepared triacetate derivative **34**, enabled the structure determination of lactone **32** as (*E*)-1α,10β-epoxy-3β-acetoxy-6α-hydroxygermacra-4,11(13)-dien-12,8α-olide [31].

The rapid structural determination of both major and minor components of SQTLs applying the combination of LC-MS and LC-NMR demonstrated the power of structure-guided screening as a complementary method to assay-guided screening, providing at an early stage possibility to distinguish novel rather than known or analogues of plant constituents. Thus, this combination of high-performance separations techniques with structurally informative spectroscopic methods (MS and NMR) could allow extracts to be screened not just for biological, but at the same time for structural classes [46]. Recently, total structure elucidation of sesquiterpene lactones, eremantholide C and two of its analogues, known for their biological activity, was given by Heleno *et al.* [47]. The detailed analysis of those results,

correlated to some computational calculations (molecular mechanics), led to unequivocal structural assignment with the determination of all multiplicities and measurements of all $^1H/^1H$couplings constants.

1.3. Structure – activity relationships of sesquiterpene lactones

The common structural feature of SQTLs is the γ-lactone function. The majority of sesquitrpene lactones contain the α-methylen-γ-lactone ring (A), with an α-oriented H-7. Despite the great diversity of sesquiterpene skeletons, the number of carbocyclic ring types of SQTLs is relatively small (**Figure 8**), which is explained by a common cyclodecadiene precursor, such as germacrene A obtained by the cyclisation of *(E,E)*-farnesyl pyrophosphate (**Figure 9**). An individual plant species generally yields a limited number of skeleton types, with oxidative variations on these skeletons. In general, having a broad geographical distribution, a given species may exhibit considerable intraspecific variations on its sesquiterpene structures.

The activities are mediated chemically by α, β-unsaturated carbonyl structures, such as an α-methylene-γ-lactone, an α, β-unsaturated cyclopentenone or a conjugated ester [48]. These structure functionalities (**a**) react with nucleophiles, especially cysteine sulfhydryl groups (**b**), by a Michael-type addition. Therefore, exposed thiol groups, such as cysteine residues in proteins, appear to be the primary targets of sesquiterpene lactones (**Figure 10**). The covalent binding to free sulfhydryl groups in proteins can inhibit a large number of biological processes such as the neutrophil migration, lysosomal rupture and enzymatic activity [49]. The differences in activity between individual SQTLs may be explained by different numbers of alkylating structural functionalities. However, other factors such as lipophilicity, molecular geometry, and the chemical environment of the target sulfhydryl, affecting the steric accessibility of Michael addition sites, may also influence the activity of sesquiterpene lactones [50-53]. Although, the primary request for pharmacological activity of SQTLs is the presence of simple α-methylene-γ-lactone structure, it has been proved that this structural feature caused minimal anti-inflammatory activity. It is obvious that other steric requisites must be fulfilled. In fact, the type of structure that SQTL has is important for the activity, the derivatives of pseudoguaianolide and germacranolide skeletal types being the most active.

Quite surprisingly, despite quite a number of reports on SQTLs bioactivity, only very few systematic studies on structure-activity relationships (SAR) have been carry out. Although many sesquiterpene lactones-containing plants have been used in traditional medicine of all cultures for many centuries and continue to be utilized also in modern phytotherapy, therapeutic use of SQTLs as pure

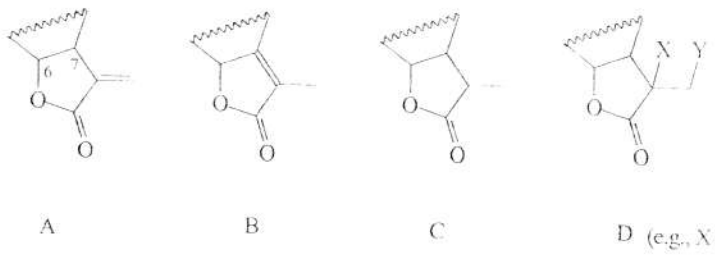


Figure 8. Carbocyclic ring types of sesquiterpene lactones.

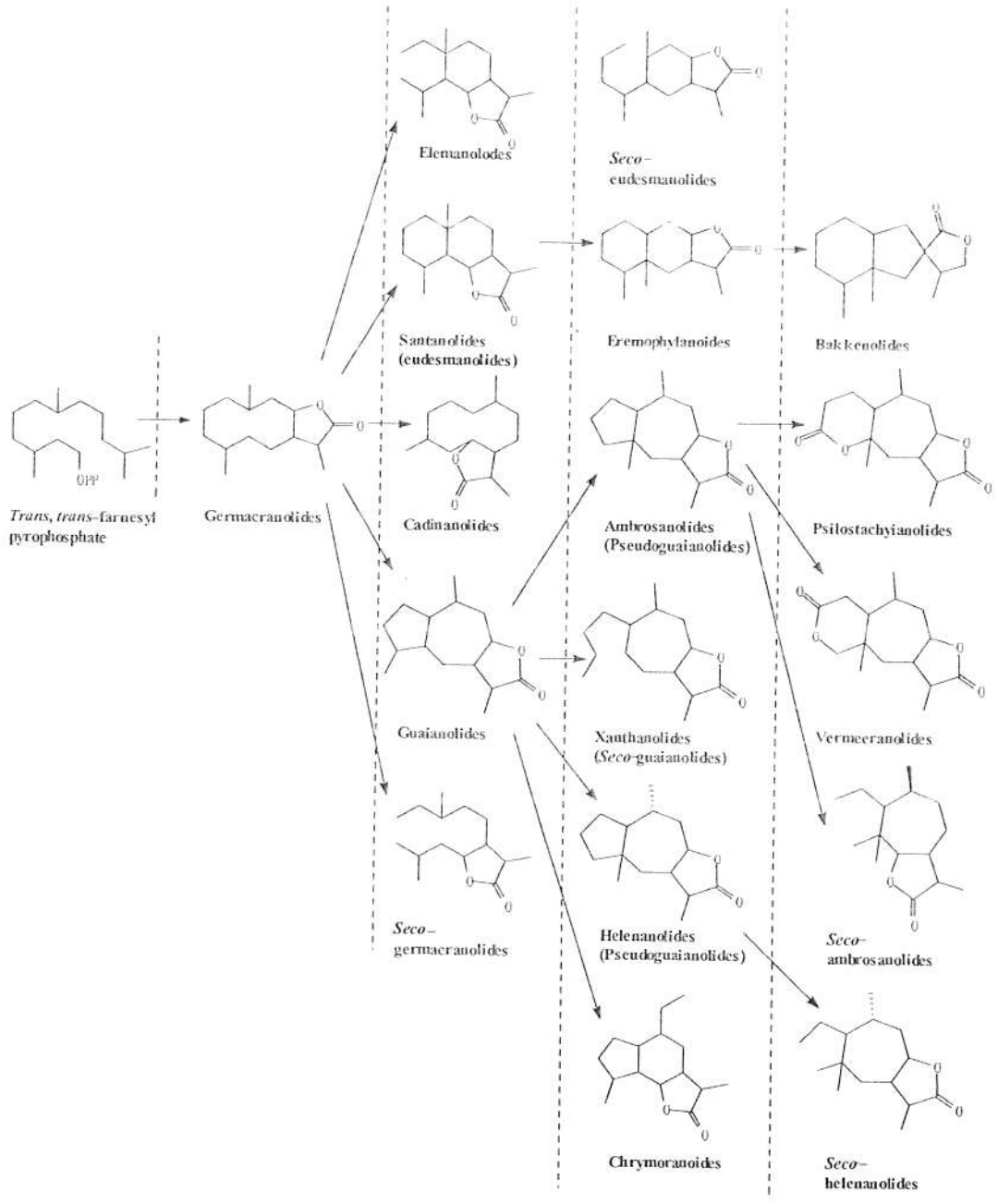


Figure 9. The major skeletal types of SQTLs and their biogenesis.

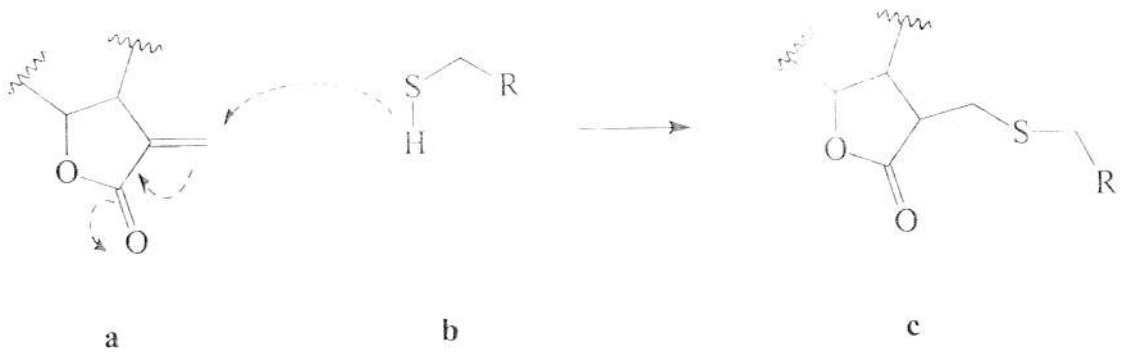


Figure 10. The structure functionalities of SQTLs that react with nucleophiles, especially cysteine sulfhydryl groups, by a Michael-type addition.

chemicals is restricted to very few examples (i. e. artemisinin, helenin [28, 54]). This is mainly due to a lack of knowledge on the structural requirements for selectivity with respect to a shown biological activity. Hence, besides the structural demands, the geometry of entire molecules is important for the expression of biological activity.

The general mechanism of SQTLs activity is alkylation of biological nucleophiles by α,β-unsaturated carbonyl structures in a Michael-type addition. Covalent binding of SQTLs to free sulfhydryl groups in proteins may interfere with the functions of these macromolecules. Consequently, SQTLs inhibit a large number of enzymes involved in key biological processes, such as DNA and RNA synthesis, protein synthesis, purine synthesis, glycolysis, citric acid cycle, and the mitochondrial electron transport chain.

In 1997 Beekman *et al.* gave an evidence of structure-citotoxicity relationships of some helenanolide-type (10α-methylpseudoguaianolide) SQTLs [53]. Determination of the influence of substitution patterns on the toxicity of the investigated helenanolides to a cloned Ehrlich ascites tumor cell line EN2 showed that lipophilicity and steric effects on the accessibility of the reactive sites might be responsible for intensity of biological activity. Although the target molecules affected by the sesquiterpene lactones in the cell line used in this study are not known, it is clearly demonstrated that differences in substitution pattern and molecular geometry of sesquiterpene lactones, affecting both the steric accessibility of Michael addition sites and lipophilicity, should be considered in the interpretation of their biological activity. The investigated SQTLs were esters of helenalin (**Figure 11**), the acetate (**39**), isobutyrate (**40**), which were more active of helenalin itself (**38**). Less activity was observed at lactones with larger acyl group – tiglate (**41**) and isovalerate (**42**). Estars of mexicanin I (**43**), acetate (**44**) and isovalerate (**45**) showed the same pattern of activity. In contrast, cytotoxicity within

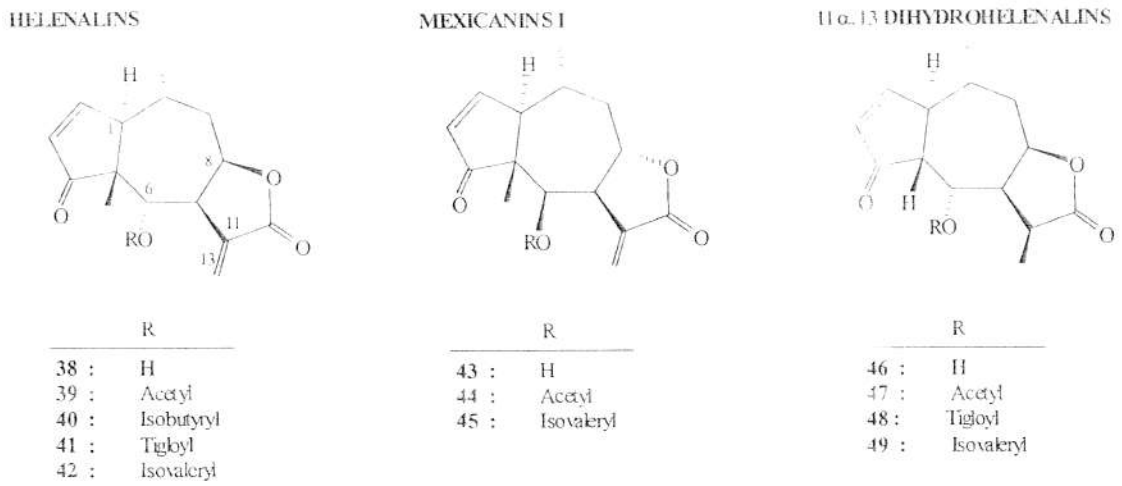


Figure 11. The SQTLs of helenanolide-type used in structure-citotoxicity relationship.

series of 11α,13-dihydrohelenalin (**46-49**) esters was shown to be directly related to the size and lipophilicity of the ester side chain. The investigation of structure-activity relationships of SQTLs, parthenolide (**50**) and their natural analogues (**Figure 12**), as well as synthetic ones (not shown, for structure see cited reference), synthesized in order to examine anti-hepatitis C virus (HCV) effects, showed that spatial arrangement of the terpanoid skeleton fused with α-methilene-γ-lactone moiety is required. In addition, the results suggest that exo-methylene lactone functionality may be partly responsible for anti-HCV activity of parthenolide and its analogues [4]. Anti-HCV activities of partehnolide (**50**), costunolide (**51**), dehydrocostus lactone (**52**), helenalin (**53**), alantolactone (**54**), epoxy(4,5α)-4,5-dihydrosantonin (**55**), 4(5) α-epoxy-4,5-dihydrosantonin (**56**) and artemisinin (**57**) was significant. Although the presence of epoxide moiety is of significance in anti-inflammatory activity [55], parthenolide and costunolide showed similar anti-HCV activity, indicating that the epoxide moiety of parthenolide played a less important role in the anti-HCV activity.

Described as constituents of many traditionally used medicinal plants with anti-inflammatory properties, various studies have been constructed to investigate how these natural compounds exert their anti-inflammatory effect. SQTLs modulate many inflammatory process, such as the exocytosis of cathepsin G and acid phosphatase from the azurophilic granules of rat polymorphonuclear leukocytes, the release of histamine from mast cells and serotonine from blood platelets, and the exocytosis of elastase from human neutrophils [49,56-58]. They inhibit the 5-lipoxygenase and leukotriene C4 synthase in human blood cells [59]. SQTLs possess pro-apoptotic effects that can be desirable in eliminating nonfunctional cells in tissues under inflammation [60]. *In vivo* their anti-inflammatory activity was proven in the

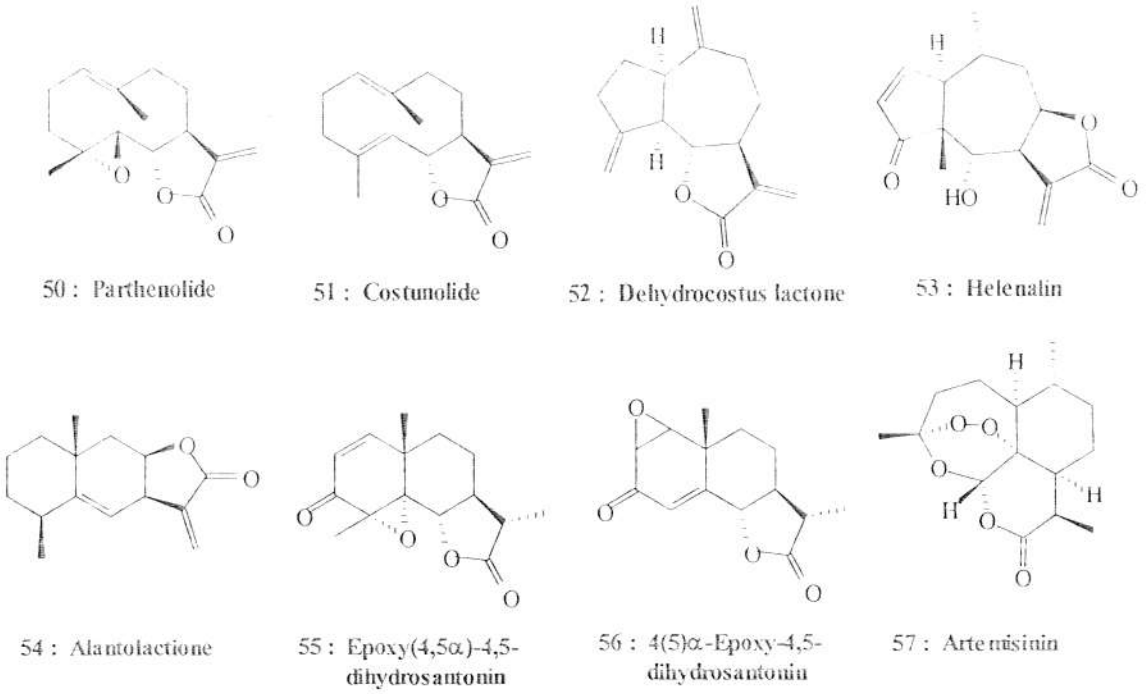


Figure 12. SQTLs with anti-HCV activity.

rat paw and mouse ear edema [58,61]. Besides, it was demonstrated that binding of the transcriptional nuclear factor of activated T-cells (NFAT) to DNA and activator protein 1 (AP-1) was prevented by SQTLs. Recently, it was shown that SQTLs inhibit the central transcription factor NF-κB [12,62-64]. These transcription factors play a pivotal role in controlling the expression of multiple inflammatory and immune genes involved in toxic shock, asthma, rheumatoid arthritis, or cancer [65]. Structural studies identified exomethylene group in conjugation with lactone function as the decisive structural feature of SQTLs for their inhibitory activity. Because of the central role in regulating inflammatory responces, a pharmacological inhibition of NF-κB activation *in vivo* might be beneficial in the treatment of inflammation. The investigation of the structure-activity relationship of SQTLs of all major skeletal classes revealed that a strong NF-κB inhibitory activity correlates with the methylene lactone and conjugated keto or aldehyde functions, but not conjugated ester groups. Lipophilicity did not influence NF-κB inhibitory activity of this set of compounds. Further, the study, in which investigated compounds are with known mode of the action in the NF-κB cascade, regarding the molecular geometry and the chemical environment that might influence the inhibitory activity, indicated that topological and structure-coding parameters contribute to the NF-κB inhibitory activity of SQTLs with rigid skeleton (furanoheliangolides and guaianolides), whereas in the cases of flexible skeletons (germacranolides)

inhibition could be mostly determined by reactivity-coding parameters (number or type of α,β-unsaturated carbonyl structure functionalities). Good correlations were only obtained for individual subgroups of SQTLs and not for the SQTLs entire class of compounds The potential compound with strong NF-κB inhibiting activity should posses two α,β-unsaturated carbonyl groups and an acyl moiety near exocyclic methylene group. All these results indicate that might be hardly possible to separate the wanted therapeutic effects from the unwanted side effects such as cytotoxicity [48,66]. Nowadays, the structure-activity relationship investigations have been performed using the counterpropagation neural network (CPGNN) model to predict NF-κB inhibitory potency of known SQTLs [67,68]. Combining the local radial distribution function, π-electronegativity and hydrogen-binding potential, the mentioned investigations provided an evidence, that besides α-methylene-γ-lactone moiety involved in the proposed reaction with cystein-38 of the p65 subunit and the exocyclic carbon atom, SQTLs with more α,β-unsaturated carbonyl units in molecule showed the higher NF-κB inhibitory activity. The constructed structural model can contribute to the search and optimization of lead structures for the therapeutically used cytokine suppressing remedies valuable for the treatment of various inflammation diseases. As anti-inflammatory compounds, SQTLs also inhibit serotonin release. The developed computational structural models allowed the specification of structural features necessary for either serotonin or NF-κB inhibiting activity. For the both activity, an intact γ-lactone with an exomethylene group and an oxygen group adjacent to this exomethylene group are essential structural features. Contrary to request for NF-κB inhibitory activity, additional α,β-unsaturated systems have no strong impact on serotonin release inhibition.

The investigation of the link between anti-inflammatory and cytotoxicity effects of SQTLs, revealed hypothesis that SQTLs bearing either an α,β-unsaturated cyclopentanone or an α-methylene-γ-lactone induced different forms of cell death. Whereas the cyclopentanone SQTLs induced typical apoptosis, the α-methylene-γ-lactone SQTLs-induced cell death lacked partly classical signs of apoptosis, such as DNA fragmentation, but showing striking characteristics regarding following: i) strong induction of the phagocytic response of macrophages by rapid and strong phosphatidylserine exposure on target cells; and ii) a transient increase in TNF-α levels. Thus, possible physiological consequences of external treatment using this type of SQTLs may be a transient proinflammatory response. Improved corpse cell clearance might contribute to final resolution of inflammation [69].

2. Inflammation

2.1. Inflammation

Inflammation is a normal and essential response to any noxious stimulus, which threatens the host and may vary from localized response to a more generalized one. It is clinically defined as a pathophysiological process characterized by redness, oedema, fever, pain, and loss of function. The inflammatory response can be divided into a series of overlapping stages: acute vascular, acute cellular, chronic cellular and resolution. The inflammatory process is designed to provide a rapid mechanism by which the host can respond to the invasion of foreign materials and return to homeostatic equilibrium. Excessive or inadequate activation of the system can have serious effects, as can cause the failure of inactivation mechanisms.

Neutrophil granulocytes are the cornerstone of innate and humoral immunity in the host defence system [70]. Superoxide anions and enzymes, proteases such as elastase or collagenase, are releases from neutrophils and are crucial products in the phagocytosis of microorganisma [71]. However, the excess of enzymatic release can provoke the destruction of healthy tissue and contribute to inflammatory process. Due to this, inhibitors of neutrophil enzymes release might produce an anti-inflammatory effect.

Products of biosynthetic cascade of arachidonic acid have also been recognized to contribute to a variety of inflammatory diseases. Arachidonic acid liberated from phospholipids by various stimuli can be metabolized by COX pathway to prostaglandins (PGs) and tromboxane A2 or by lipoxygenase (LOX) pathway to leukotrienes (LTs). Some of both PGs and LTs are known to be potent proinflammatory compounds, i.e. to mediate inflammatory processes. It was found that anti-inflammatory action of aspirin and other NSAIDs is a consequence of blocking the biosynthesis of PGs [72].

Finally, most studies suggest that excessive production of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and the potent chemoattractant IL-8 are important in pathophysilogy of many inflammatory diseases [73,74]. These cytokines are all dependent on the transcription factor NF-κB, one of the key regulators of genes involved in the immune and inflammatory response. NF-κB is activated when its inhibitor, IκB, is phosphorylated by IκB Kinase (IKK), then ubiquitinated and degraded [75]. The free NF-κB translocates to the nucleus, binds to promoters of NF-κB-dependent genes, and facilitates their transcription. It is known that NF-κB regulates the transcription of a number of inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-8 and TNF-α, as well as genes encoding COX-2, NO-synthase, immunoreceptors, cell adhesion molecules, hematopoetic growth

factor and growth factor receptors [76,77]. Because of that, NF-κB may be an important target for new anti-inflammatory approaches for treating inflammatory diseases.

2.2. Mechanism of anti-inflammatory activity of sesquiterpene lactones

SQTLs, as active compounds of a variety of traditionally used medicinal plants from the *Asteraceae* family, exhibited a considerable anti-inflammatory activity in different inflammation models. They inhibit inflammatory oedema induced by cotton pellet granuloma, complete Freund's adjuvant, 4-beta-phorbol 12 myristate 13-acetate, formalin, and carrageenan [21,23,78-81]. Additionally, using the acetic acid-induced writhings model it was demonstrated the antinociceptive effect of SQTLs (e.g. parthenolide, costunolide, dehydrocostus lactone, budlein A) [21,82-84]. Furthermore, Recio *et al.* [22], demonstrated the concomitant inhibition by different SQTLs of inflammatory oedema and leukocyte migration to ear skin challenged with 12-O-tetradecanoylphorbol-acetate. All mentioned studies, as well as many others clearly demonstrated the potential of SQTLa to modulate many inflammatory processes.

It was shown that SQTLs may inhibit phospholipase A2, an enzyme that mediates release of arachidonic acid from cell membrane phospholipids, and consequently further synthesis of PGs and LTs [85]. They also inhibit release of proteases from activated nautrophils, as well as some of the proinflammatory cytokines and these effects are also recognized as anti-inflammatory ones [86-88]. However, these results have not sufficiently explained the molecular mechanism by which SQTLs exert anti-inflammatory effect.

At present, it is thought that inhibition of activation of transcription factor NF-κB, a central regulator of the immune response, by SQTLs is a key molecular mechanism of their action, although this mechanism is not yet completely elucidated, too.

The transcription factor NF-κB promotes the expression of over 150 target genes in response to inflammatory stimulators [89,90]. In mammalian cells, NF-κB is composed of a homo- or heterodimer of various DNA-binding subunits. In most cell types, NF-κB is composed of a p50 and p65 subunit and is retained in the cytoplasm bound to the inhibitory subunit IκB in its inactive form. Constitutive NF-κB activity in the cell nucleus can only be detected in certain neurons, some of the cells of monocyte/macrophage lineage and B cells. Stimulations of the cells including inflammatory cytokines, or other pathogenic agents leads to the intracellular generation of the reactive oxygen intermediates (ROIs), as a key event and results in the

activation of NF-κB. Phosphorylation and ubiquitinylating of IκB (existing in two major forms, termed IκB-α and IκB-β) by activating IκB-kinase complex (IKK) release active NF-κB [66]; this induced degradation of IκB proteins unmasks the nuclear localization sequences of the DNA-binding subunits of the NF-κB dimmer and allows NF-κB to enter the nucleus, to bind to its DNA sequence, and to induce transcription. As mentioned above, NF-κB regulates the transcription of various inflammatory cytokines. Among its many different biological activities, NF-κB seems to play an important role in cell killing. Recently, it has been shown that NF-κB counteract the induction of the apoptosis by the cytokine TNF-α, ionizing radiation, and the cancer chemotherapeutic agent daunorubicine [64].

SQTLs have been recognized as specific inhibitors of transcription factor NF-κB. They prevent the activation of NF-κB by different stimuli such as phorbol esters, tumor necrosis factor-α, ligation of the T-cell receptor, and hydrogen peroxide in various cell types. Treatment cells with SQTLs prevented the induced degradation of IκB-α and IκB-β by these stimuli, suggesting that they interfere with a rather common step in the activation of the NF-κB. SQTLs do not interfere with the generation of ROIs following the stimulation of cells, they do not directly act on the DNA-binding subunits of NF-κB, also the IκB subunits seems not to be a direct target for the SQTLs [64]. It is currently not clear which of so far identified members of the signaling cascade responsible for NF-κB activiation, is the target of SQTLs. Furthermore, the number of molecules inhibited by SQTLs remains unknown. The SQTLs display a high degree of specificity for their inhibiting activity, since they do not influence the activity of other transcription factors such as AP-1, RBP-Jκ, and Oct-1. Nor the SQTLs did not impair the activity of the T-cell kinases $p59^{fyn}$ and $p60^{src}$. Results of investigation presented by Hehner *et al.* [64] have shown that the SQTLs do not interfere in a nonspecific manner with transcription factors of signaling molecules. A potential target specificity of SQTLs might well be explained by considering the fact that the combination of the reactive Michael-acceptor system together with the oxygen-substituted isoprenoide rings forms a pattern of potential noncovalent binding sites (*e.g.* hydrogen bonds). These binding sites would allow the SQTLs to interact with complementary sites on the surface of the target molecule(s). The relative positions of SQTLs in the downstream signaling cascade of inflammatory conditions are schematically displayed in **Figure 13**. SQTLs lacking in exomethylene group in conjugation with the lactone function display no activity on NF-kB. SQTLs have no direct or indirect anti-oxidant properties. Parthenolide (**50**) and helenalin (**38**) have been shown to be active NF-kB inhibitors. The mechanism that provides inhibition of DNA binding of NF-κB is probably alkylation p65 at Cys38, at

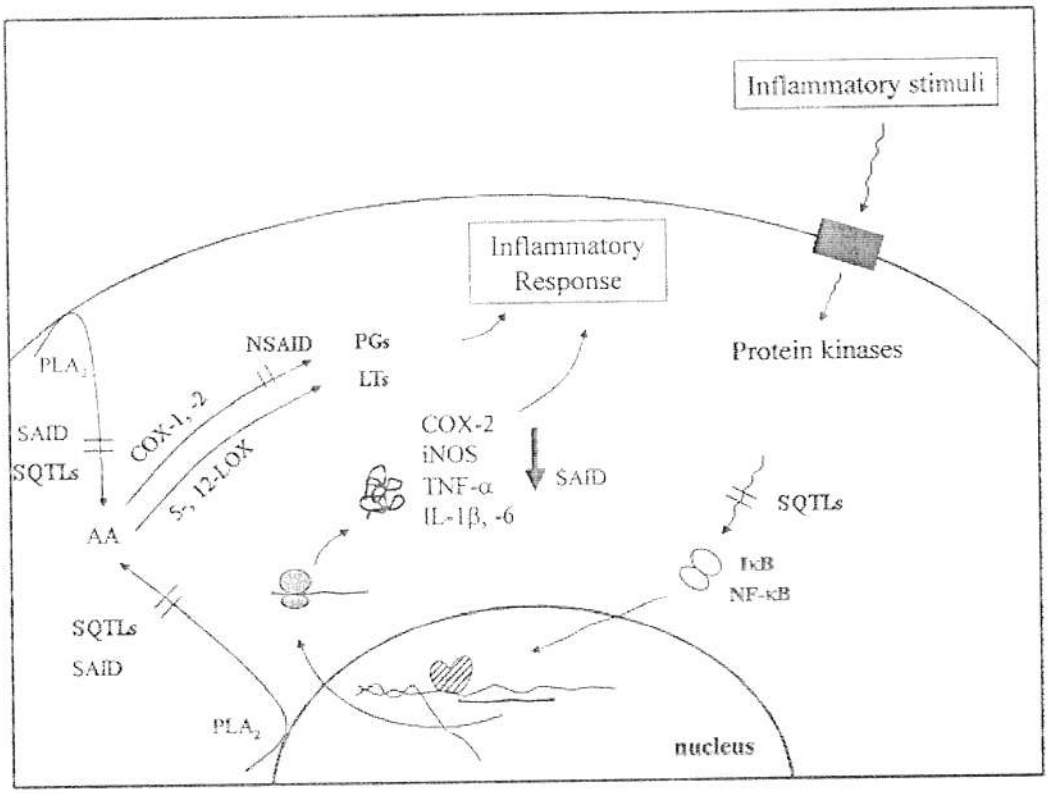


Figure 13. The proposed action mechanism of SQTLs. Sesquiterpene lactones (SQTLs), nonsteroidal anti-inflammatory drug (NSAID), steroidal anti-inflammatory drug (SAID), "=" and "↓" denote enzyme inhibition and down regulation of the expression, respectively (Abbreviations used: PLA_2 – phospholipase A_2; AA – arachidonic acid; LTs – leukotrienes; PGs – prostaglandins; LOX – lipoxigenase; COX – cyclooxigenase; iNOS – inducible nitric oxide synthase; TNF-α – tumor necrosis factor-α; IL-1β – interleukin 1β; NF-κB – nuclear transcription factor κB; IκB – inhibitor-κB).

the same time the p50 subunit remained unchanged. Although a slight inhibition of IκB degradation of SQTLs was also detected, it is likely that this effect is secondary to the alkylation of p65 [66,91,92]. The investigation of the SQTLs structure indicated that the majority of potent NF-κB inhibitors possess two reactive centers in form of α-methylene-γ-lactone group and an α,β- or $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl group [48]. The recent X-ray structure of the p65 homodimer [93], which showed that cystein 38 is located within DNA binding domain 1 (L1) and another cystein, Cys 120, found in a proximal loop (E' region), supported the hypothesis that these two cystein residues (Cys38 and Cys120) are the targets for alkylation by SQTLs. Bifunctional compounds can crosslink these two residues, thereby inhibiting the DNA binding. Inhibition of NF-κB DNA binding by monofunctional SQTLs requires alkylation of these cysteine residues by two molecules of SQTLs. As Lyß *et al.* indicated [91], helenalin (**38**) directly interferes with

NF-κB DNA binding due to its alkylating activity. Helenalin selectively modifies the p65 subunit of the transcription factor, thereby inhibiting its DNA binding. Unlike antioxidans, such as acetylsalicylic acid, helenalin can inactivate the active NF-κB complex. This property is crucial for the treatment of inflammation, where previously activated NF-κB is sustaining the process of inflammation and needs to be inactivated. Also, helenalin is known to inhibit cytochrome *P*450 enzymes. Isoforms of cytochrome *P*450 families are expressed in human myeloid leukemia cell lines and the inhibition of cytochrome *P*450 enzymes by this lactone may be mediated via inhibitory effect on NF-κB activity [20]. Neither the lipophilicity nor the molecular geometry influenced SQTLs NF-κB inhibition activity.

Anti-inflammatory properties of SQTLs was investigated using iNOS and COX-2 expression, and NF-κB activation as a molecular targets, taking into account that activation of NF-κB is involved in iNOS and COX-2 expression [25]. SQTLs inhibit iNOS and COX-2 expression through inactivation NF-κB.

2.3. Parthenolide as sesquiterpene lactone with potent anti-inflammatory activity

Parthenolide, one of the major SQTLs with germacranolide skeleton found in feverfew (*Tanacetum parthenium* (L.) Schultz-Bip., *Asteraceae*), *T. larvatum* (Gris.) Kanitz, an endemic plant in Montenegro [94,95] and Mexican Indian medicinal plants [12], has attracted considerable attention because of its anti-inflammatory effects [82,96]. In traditional medicine, the parthenolide-containing feverfew has been used orally or as infusion in conditions like arthritis and migraine [97] and Mexican Indians used the herbs known to contain parthenolide for the treatment of skin infections and infections of other organs. The specific mechanisms by which feverfew and/or parthenolide may inhibit proinflammatory signaling pathways *in vivo* have not been completely defined. *In vitro* studies suggest that parthenolide can inhibit IKK [64,,98,99] and can directly inactivate NF-κB [12,55,92,98], but there is controversy as to whether either or both of these mechanisms are important *in vivo* [100]. Both of these actions, independently or together, could contribute to net inhibition of NF-κB–dependent proinflammatory gene expression in the CF lung [101]. Investigation of molecular basis of the anti-inflammatory activity of partehnolide revealed that it is potent inhibitor of the proinflammatory transcription factor NF-κB.

By inhibition NF-κB activity, parthenolide interferes with various aspects of inflammatory reaction, such as the production of proinflammatory cytokines and the production of inducible nitric-synthase. Like others SQTLs,

parthenolide inhibits NF-κB most probably alkylating p65 at Cys38. Althogh it was observed that parthenolide slightly inhibits IκBdegradation, the amount of remaining IκB-α was too low to explain potent NF-κB inhibition. The fact that inhibition of NF-κB activation by parthenolide occurs at last step of the transduction pathway, by inhibition of its DNA binding, makes parthenolide very interesting NF-κB inhibitor. Interestingly, Kwok *et al.* [99] suggested that parthenolide-mediated NF-κB inhibition is consequence of direct binding to IκB kinase β (IKKβ), known for its crucial role in cytokine-mediating signaling. The results are supported by the finding that the peptide containing Cys179 was modified by parthenolide, indicating that IKKβ is the direct target mediating the anti-inflammatory activity of parthenolide.

Besides anti-inflammatory properties, recent studies revealed that parthenolide has also anti-microbial and anti-cancer activities, which may depend on a wide range of parthenolide-stimulated intracellular signals [102]. Parthenolid was proven to have ability to inhibit as well STATs (the signal transducer and activator of transcription) proteins action, responsible for cell proliferation, transformation, apoptosis, differentiation, fetal development, inflammation and immune response. Although parthenolide can suppress the activity of the NF-κB family, which is known to affect apoptosis, Anderson *et al.* speculated that apoptosis induced by parthenolide did not go through mechanism that involves inhibition of NF-κB activity [103]. Based on literature data, parthenolide may either induce or protect from cell death [16]. It seems that in normal cell parthenolide protects cell from apoptosis, whereas in cancer cells it supports immune system- or anti-cancer drug-induced cell death. At low doses, parthenolide functions as an antioxidant that can reduce the oxidative stress generated through the TCR (T cell receptor) signalling pathway. In contrast, at high doses, parhenolide by itself induces reactive oxygen ($\cdot O_2^-$) and causes oxidative-stress-mediated apoptosis.

Parthenolide exhibits ability to inhibit COX-2 as well as the proinflammatory cytokines in macrophages, suggesting that blocking the hyperalgesyc and attenuating the edema response might be useful in the treatment of inflammatory pain [23]. Also, the beneficial effects of parthenolide in myocardial reperfusion injury were associated with inhibition of IKK activity, enhanced stability of IκB-α, and inhibition of nuclear translocation of NF-κB [100].

Additionally, parthenolide significantly inhibits interleuikn-12 (IL-12) production in lipopolysaccharide-activated macrophages in a dose-dependant manner [14]. This inhibition was, at least in part, due to the down-regulation of NF-κB binding to the p40-κB sequence. Pharmacological control of IL-12 might be a key therapeutic strategy for modulating immunological diseases

dominated by type-1 cytokine responses (type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and acute graft-versus-host). IL-12 exerts multiple biological activities mainly through T and natural killer cells by inducing their production of interferon-γ, which augments their cytotoxicity, and by enhancing their proliferation potential. Besides, its production is critical for the development of T-helper type-1 cells and the initiation of cell-mediated immune responses. Also, parthenolide is a potent inhibitor IL-4 mediated inflammatory disease [13]. IL-4 is a key T helper (Th) cell 2 cytokine involved in regulation of antybody production, hematopoesis, differentiation of Th2 cells, and development of effector T cell responses. Anti-inflammatory effect of parthenolide might be partly due to ability to suppress IL-4 mRNA expression levels and to impair IL-4 promoter activity through two NF-κB acting sites. Parthenolide was shown to modulate chemokine interleukin-8 (IL-8) gene expression. The most proximal mechanism of inhibition appears to involve inhibition of IκB-α degradation [15].

Due to such a mechanism of action, parthenolide might be an ideal target-based anti-inflammatory drug. Except allergenic potential, it has a very favourable safety profile as shown in clinical studies with the parthenolide-containing feverfew. Moreover, in contrast to conventional NSAIDs, the most often used anti-inflammatory drugs with significant ulcerogenic activity that is the cause of serious gastrointestinal adverse effects, parthenolide produces gastroprotective effects. Tournier *et al.* [104] demonstrated dose-dependent inhibition of the formation of ethanol-induced ulcers in rats when parthenolide was given as a 30 min pretreatment in a dose of 100 mg/kg p.o. Complete restoration of mucosal sulphydryl content to normal and 91% protection of the gastric mucosa was achieved from a much higher dose (400 mg/kg p.o.). Similarly, Petrovic *et al.* [105] showed that the chloroform extract of *T. lavratum* [94,95], one of the parthenolide-containing plant, protected rats against indomethacin-induced gastric lesions when given in an anti-inflammatory dose (200 mg/kg p.o.) concomitantly with the NSAID. Besides gastric protection, the extract was enhanced anti-inflammatory effect of indomethacin suggesting possibility of combining two drugs whose net effect is enhancement of their favourable therapeutic effects and diminution in adverse ones.

Having in mind all these data, clinical studies with parthenolide as potential anti-inflammatory agent would be desirable and justified.

Summary

Procedures for isolation and purification of SQTLs from plant material and crude extracts are multistep, relatively simple, and they include repeated

column chromatography, dry-column flash chromatography, crystallization preparative thin layer chromatography and preparative liquid chromatography.

For the structure elucidation of SQTLs NMR spectroscopy (^{1}H NMR, ^{13}C NMR, 2D NMR) is the most widely used technique, although IR spectroscopy, mass spectrometry and X-ray diffraction (optimization of 3D structures) are in frequent use.

The main request for pharmacological activity of SQTLs is the presence of α,β-unsaturated carbonyl structures in molecule (α-methylene-γ-lactone, and α,β-unsaturated cyclopentenon, or their conjugated esters). The general mechanism of SQTLs action is alkylation of biological nucleophyles (sulfhydryl groups in proteins and enzymes) by α,β-unsaturated carbonyl structures in Michael-type addition.

Considerable anti-inflammatory effect of SQTLs could be explained by their ability to inhibit phospholipase A_2, and activation of transcription factor NF-κB, as well as to inhibit proteases and proinflammatory cytokines released from the activated neutrophils.

For parthenolide, *in vitro* studies suggest that both the inhibition of IKK and directly inactivation of NF-κB could be involved in its anti-inflammatory effect.

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