TERPENES

Editor:
Simone Carradori

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Medicinal Chemistry Lessons From Nature

(Volume 2)

Terpenes

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CONTENTS

FOREWORD

From the dawn of time, men resorted to Nature for all they need. No exception was made for health and, especially, pain. WHO estimated that almost 80% of people count on medicinal plants to take care of their "well-being" and here is the justification for the growing interest in the study of natural products and the development of their derivatives.

Among the wide range of molecules in the rich repository that Nature offers, we need to mention the terpene class, to which a whole volume of this book has been dedicated.

This volume aims to provide the readers with a brief and focused collection of some of the latest advances in the field with particular insight into the development of synthetic derivatives from a parent natural compound with highly promising bioactivity and the design of innovative formulations for possible administration.

Indeed, by scrolling through the volume index, the readers can find exciting novelty on terpenes-related topics in four well-organized chapters, including (1) a detailed overview of the sesquiterpenes polypharmacology; (2) an interesting journey around the cannabinoids world towards the development of new synthetic Δ9-THC derivatives; (3) the design of specific formulations to overcome the volatility issue of small sized terpenes-based essential oils; and (4) an update on the newest generations of endoperoxides endowed with antimalarial activity. Also, the interested audience is strongly encouraged to get more deepen understanding of the presented topics by a large number of selected references present in each chapter.

Notably, every topic dealt with in this volume, and in general in the whole book, fully describes the selected terpene scaffold in all the investigated MedChem and pharmaceutical points of view. Thus, detailed information on the design and synthesis of the compounds, their bioactivity and pharmacokinetics data, along with computational and formulation studies are provided.

The authors, also, discuss how the chemical modification of parent compounds affects biological or enzymatic activity and ADME profile, suggesting how to justify the changes in the activity/ADME data in MedChem terms.

Through the several examples of MedChem strategies to fix the most common issues on terpene derivatives, *e.g*. low potency and poor solubility, the authors drive the young researcher audience to derive general rules that could be useful in different experiments and studies they will perform. For these reasons, I strongly believe the book is addressed to a heterogeneous audience, comprising both expert and beginner MedChem scientists and pharmaceutical technologists and anyone who wants to update their knowledge on this broader and broader field of terpene research under the kind and helpful guidance of the authors, which are widely recognized scientists in Academia.

In the next chapters, the readers will find recurrent concepts that we could summarize with the following keywords: #terpenes; #sesquiterpenes; #medicinalchemistry; #pain; #malaria; #naturalproducts; #optimization; #drugdesign; #bioactivity; #synthesis; #computationalchemistry; #biology; #chemistry; #formulation; and so on.

Good reading and taking notes.

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PREFACE

Natural products are often used in drug development due to their ability to provide unique and chemically diverse structures unmatched by any synthetic chemical collection. Medicinal Chemists have always been inspired by nature because natural products are often perceived as safer and for their capability to interact with biological targets. Indeed, in recent years, there has been emerging research on traditional herbal medicines based on their efficacy in the treatment of diseases for which they have been traditionally applied.

Conversely, natural compounds suffer from several issues such as scarce availability and seasonality, high differences in the production/extraction/isolation, low purity in commercial products from worldwide suppliers, and side effects. Moreover, due to their chemical complexity and the optional presence of different chiral centers, the total synthesis of a natural compound can be also challenging and expensive.

This book series would propose the latest discoveries in the field of compounds inspired by nature and obtained by chemical/enzymatic modification of a natural compound in the search for biologically active molecules for the treatment of human/animal ailments and permit the disposal of a wider arsenal for clinicians. The natural compounds are grouped into three clusters. The chapters are built in the following format: • General background on the (phyto)chemistry of the scaffold; • General background on the pharmacological profile of the scaffold; • Description of the proposed derivatives and their potentialities with respect to the parent compounds (with a particular emphasis on the synthetic approaches and structure-activity relationships); • *In silico* analysis of the crucial interactions with the biological target, when available; • Clinical studies and patent surveys (if available) on the new and proposed structures.

The readership of this book is represented primarily by Academies, Researchers, Specialists in the pharmaceutical field, Industry sector, Contract Research Organizations and hospitals dealing with clinical research.

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CHAPTER 1

Sesquiterpenes: A Terpene Subclass with Multifaceted Bioactivities

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Abstract: Sesquiterpenes are terpene compounds, containing three isoprene units rearranged in a wide variety of structures. They occur widely in nature, not only in plants but also in fungi and marine environments. Owing to peculiar structures and diverse biological activities, they attracted great attention in pharmaceutical, medicinal chemistry and nutraceutical fields. The present chapter collects novel insights into chemistry, distribution in nature and pharmacological properties of sesquiterpenes, focusing especially on caryophyllane, lactone-type, and eremophilane subgroups, due to the growing pharmacological interest. Novel structures and alternative natural sources to be further investigated and exploited have been highlighted too. Moreover, some issues regarding toxicity risk and bioavailability of sesquiterpenes, which can limit their application in practice, have been discussed.

Keywords: Artemisinin, Alantolactone, Arglabin, Anticancer, Antimalarial, Antiinflammatory, Antimigraine, β-Caryophyllene, Capsidiol, Chemopreventive, Eremophilane, α-Humulene, Helenalin, Isopetasin, Parthenolide, Petasin, Terpenes.

INTRODUCTION

Terpenes are a large class of structurally diverse and widely distributed secondary metabolites, derived from a common basic building block, namely five-carbon isoprene unit (C_5H_8), assembled in linear chains or cyclic structures Table **1**. More complex and functionalized terpenes, namely terpenoids, can also occur in nature [1].

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Simone Carradori (Ed.)

Table 1. Classification of terpene subclasses

Terpene Subclass	Isoprene Units	Number of Carbons
Monoterpenes	2	C10
Sesquiterpenes	3	C15
Diterpenes	4	C20
Sestertepenes	5	C25
Triterpenes	6	C30
Tetraterpenes	8	C40

Two major biosynthetic routes, namely the mevalonate (MVA) pathway and 2C-methyl-D-erythritol-4-phosphate (MEP) pathway (or Rohmer pathway), have been reported to be the terpene sources [2, 3]. The MVA pathway leads to the formation of the terpenoid C5 precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP): three molecules of acetyl-CoA are condensed to a 3-hydroxy-3-methylglutaryl-CoA, which is subsequently reduced to MVA, whose phosphorylation and further rearrangements lead to IPP and DMAPP (Fig. **1**) In the MEP (or Rohmer) pathway, 1-deoxy-D-xylulose 5-phosphate, obtained by condensation of pyruvate and glyceraldehyde 3-phosphate, is converted into MEP which further leads to IPP and DMAPP, the basic building blocks of all terpene (Fig. **1**).

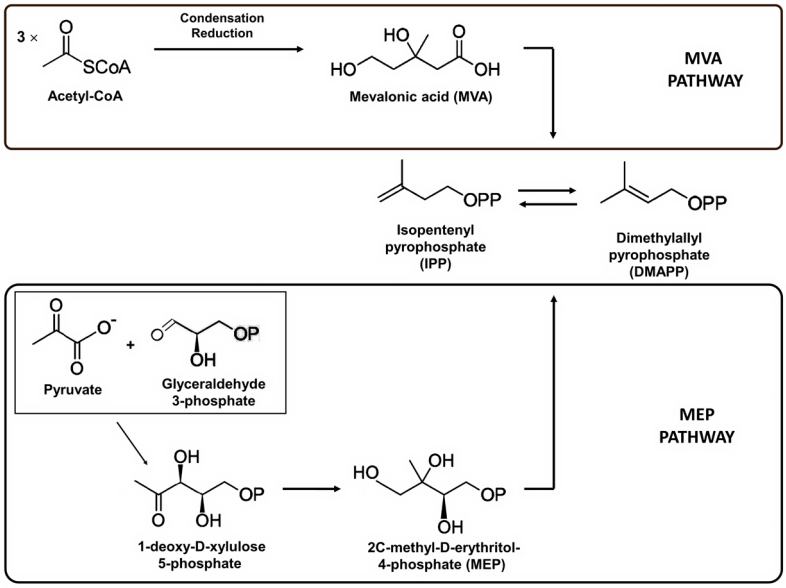

Fig. (1). Biosynthetic pathways of terpenes: MVA or mevalonate pathway and MEP (2C-methyl-D-erythritol-4-phosphate) or Rohmer pathway.

Isoprene directly originates from IPP or DMAPP, while monoterpenes are synthesized from a geranyl pyrophosphate (GPP) precursor (also known as geranyl diphosphate or GDP), produced by the condensation of IPP and DMAPP (Fig. **2**) [4]. GPP and one molecule of IPP can be condensed to farnesyl diphosphate (FPP), which can be further converted into different sesquiterpenes and triterpenes; furthermore, the addition of IPP to FPP leads to geranyl geranyldiphosphate (GGPP), from which diterpenes and tetraterpenes (or carotenoids) arise [4].

Terpenes are produced by a wide variety of plants, fungi and some animals, mediating antagonistic and beneficial interactions among organisms [2]. Particularly, high terpene levels have been found in plant reproductive structures and foliage, where they can act as allelopathic compounds, mediating plant biotic and abiotic interactions [1]. Indeed, some of them, especially volatile compounds, have been exploited by plants as a weapon against herbivores and pathogens; moreover, other compounds can mediate plant metabolic adaptation to climate changes and regulate cell membrane permeability due to their lipophilic nature [5]. For instance, in response to root feeding by caterpillars, corn (*Zea mais* L.) roots release the sesquiterpene β-caryophyllene, which is attractive to entomopathogenic nematodes and stimulates their killing ability against herbivore larvae [6].

The monoterpene ketone pulegone has been reported to be the main environmental defense released by *Mentha pulegium* L., while helivypolides, annuolides and helibisabonols are the most significant allelochemicals produced by sunflower (*Helianthus annuus* L.) [7, 8]. Similarly, monoterpenes and sesquiterpenes contained in the essential oil from *Cinnamomum septentrionale* Hand.-Mazz. produced phytotoxic effects against several species, such as *Taraxacum officinale* L. and *Eucalyptus grandis* L [8].

Another example of allelopathic interaction is the *"Salvia phenomenon"*, characterized by the ability of some *Salvia* species (i.e. *Salvia leucophylla* and *S. apiana*) to form a typical vegetation patterning in the soil in its vicinity, due to the production of monoterpenoids (i.e. camphor, 1,8-cineol, β-pinene, α-pinene and camphene) which hinder the growth of other plants [8]. The phytotoxic effects of *Salvia* spp. have been also ascribed to the presence of di- and triterpene compounds, which include clerodane and neo-clerodane diterpenoids [9]; moreover, a number of phytotoxic diterpenes have been found in both plant and microorganisms [10].

Terpenes have attracted great scientific attention due to their multiple biological properties, thus strengthening the industrial interest in their application as

conservative, antioxidant, flavoring compounds, basic structures for hemisynthesis, along with the research about their possible nutraceutical and pharmacological role [1, 11 - 13]. Owing to the low-level exposure, terpene use is usually recognized as safe; however, some toxicity concerns to be further evaluated have been highlighted for some compounds [14 - 18].

A low yield from natural sources and poor solubility in biological fluids represent the major limits for the use of terpenes. Innovative sources of terpenes have been found in metabolically engineered microbes, thus allowing to improve the production of several monoterpenes, sesquiterpenes, diterpenes and carotenoids (*e.g*. limonene, pinene, sabinene, santalene, bisabolene, sclareol, taxadiene, lycopene, β-carotene and astaxanthin) [19, 20]. On the other hand, suitable pharmaceutical formulations, including nanoemulsions, microcapsules and liposomes, have been evaluated as possible delivery systems to promote bioavailability and stability [21 - 28].

Monoterpenes arise from GPP (Fig. **2**). Table **1**. and occur in nature as acyclic (linear), monocyclic and bicyclic structures, often with an oxygen-containing functional group and are the main components of essential oils. Linalool, β-myrcene, and linalyl acetate are among the most known linear compounds, while limonene, α-terpineol, 1,8-cineol (syn. eucalyptol), terpinen-4-ol, menthol, *cis*-verbenol, eugenol, α-pinene, isoborneol and carvacrol possess cyclic (or bicyclic) structures (Fig. **3**). some of them, co-occur in essential oils being metabolically correlated [29, 30]. For instance, during red wine aging, limonene undergoes biotransformations and chemical rearrangements, leading to α-terpineol and 1,8-cineol generation, which seem to be responsible for the “eucalyptus” aroma of some red wines and to confer healing properties [31]. Different monoterpenes have been highlighted to possess interesting bioactivities, which include antimicrobial, antimutagenic, genoprotective, antioxidant, anti-inflammatory, antiproliferative, penetration enhancing, anxiolytic, myorelaxant and hypotensive ones [13, 31 - 45].

The antimicrobial properties have been ascribed to the ability to interact with phospholipids, due to their high lipophilic nature, thus affecting cell membrane permeability and inducing leakage of the intracellular materials [35]. A modulation of cell membrane permeability seems to be involved in the antimutagenic and genoprotective properties too [31, 32, 35]. The ability of several monoterpenes to interact with the skin phospholipids and to enhance the percutaneous absorption of drugs, and their safe toxicity profile, have strengthened their application as penetration enhancers [32]. Moreover, the activation of transient receptor potential melastatin (TRPM8) ion channels has been found responsible for the analgesic effects of menthol [46], whereas an

increased mucociliary activity and a lowered mucus production contribute to anti-inflammatory and bronchodilator effects of eucalyptol [47].

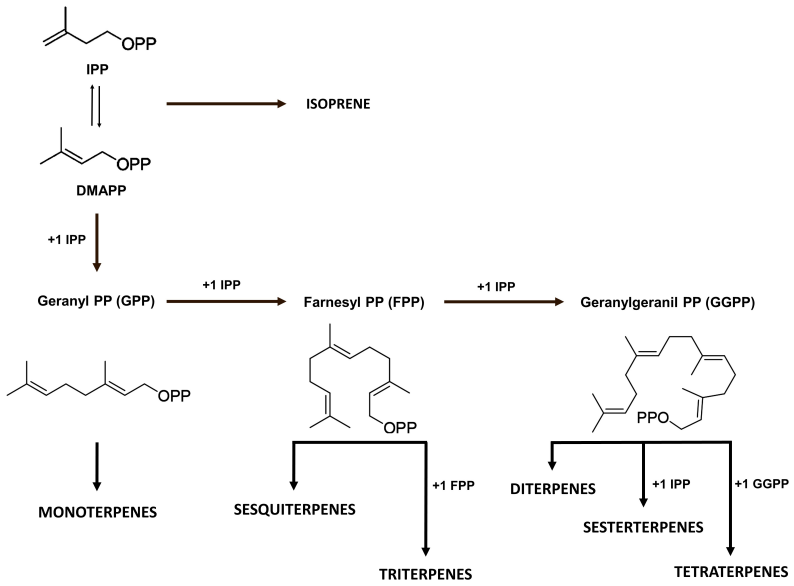

Fig. (2). Biosynthesis of different terpene subclasses from the subunit IPP.

Among the other terpene subclasses, diterpenes are generated from GGPP (Fig. **2**); Table **1** and are widely diffused in nature, being produced by plants, fungi, bacteria, and animals [48]. A number of these compounds have been shown to produce diverse biological effects, thus strengthening the pharmacological interest for future applications and the biotechnological research for alternative sources [48]. For instance, taxanes *e.g*. taxol, (Fig. **3**) and their derivatives have been studied as chemotherapeutic agents [49], while carnosic acid, abietic acid, steviol, and andrographolide (Fig. **3**) displayed antiobesity properties [50]. Remarkable healing properties, including anticancer, antibacterial, genoprotective, anti-inflammatory, antidiabetic, immunomodulatory, and neuroprotective ones, have been reported for other diterpenoids, among which ginkgolides, steviosides, tanshinones, tobacco cembranoids, and abietane, labdane, *ent*-kaurane, isopimarane and seco-isopimarane diterpenes [51 - 65]. Accordingly, coffee bean diterpenes, particularly cafestol and kahweol (Fig. **3**), have been found to produce anti-inflammatory and anticancer effects in preclinical models, although the adverse effects registered at high dosages have suggested the need to define appropriate intake levels [66].

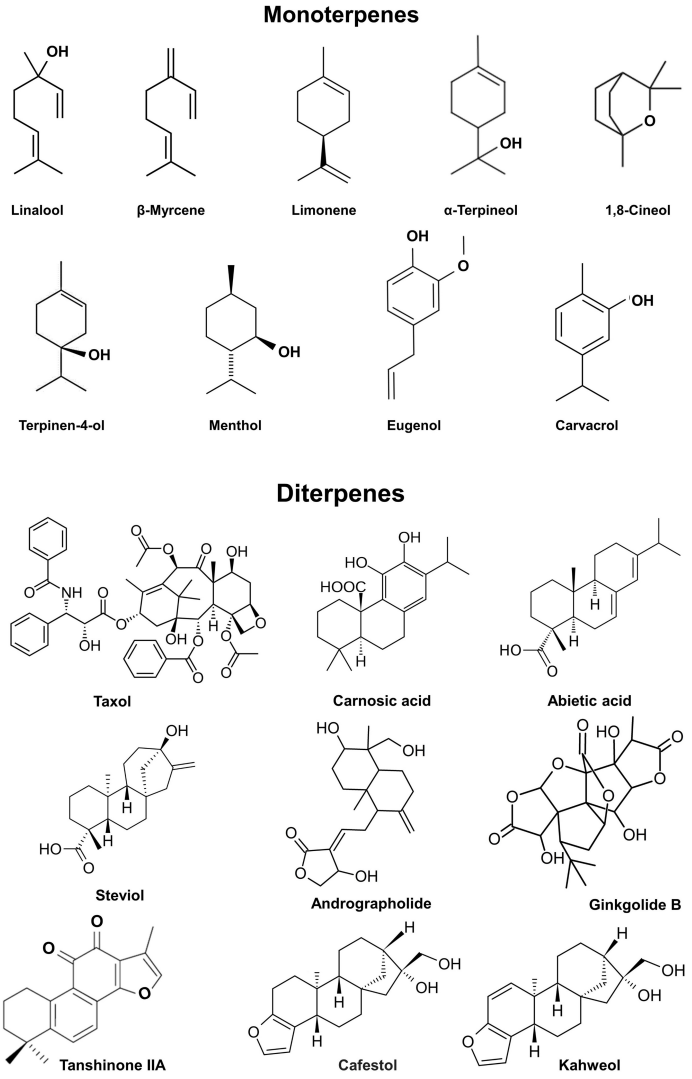

Fig. (3). Chemical structures of major studied monoterpenes and diterpenes.

Triterpenes arise from two molecules of FPP (Fig. **2**); Table **1** and have been identified in leaves, stems, barks, flowers and fruit peels of several plants: licorice (*Glycyrrhiza glabra* L.) roots, centella (*Centella asiatica* L.) leaves, olive (*Olea europea* L.) leaves, *Momordica charantia* L. fruit, avocado (*Persea americana* Mill.) seeds, and horse chestnut (*Aesculus hippocastanum* L.) are examples of known herbal sources of triterpenes [67 - 70]. Owing to their structural diversity, triterpenes are classified as tetra and pentacyclic structures; dammarane-, lanostane- or cycloartane-type compounds are the major subgroups of tetracyclic

triterpenes, while lupane, oleanane and ursane derivatives are pentacyclic triterpenes [67 - 74]. These compounds have shown a plethora of biological activities, which include antidiabetic, cardioprotective, hepatoprotective, anti-inflammatory, antioxidative, anticancer, chemopreventive, and antimicrobial [71]. Some triterpenes, among which 1β-hydroxyaleuritolic acid 3-p-hydroxybenzoate, lupeol, uvaol, β-aescin and glycyrrhizin (Fig. **4**), have been reported to possess antiviral, anti-inflammatory, and immunomodulatory properties, thus suggesting a possible interest against coronavirus infections [75].

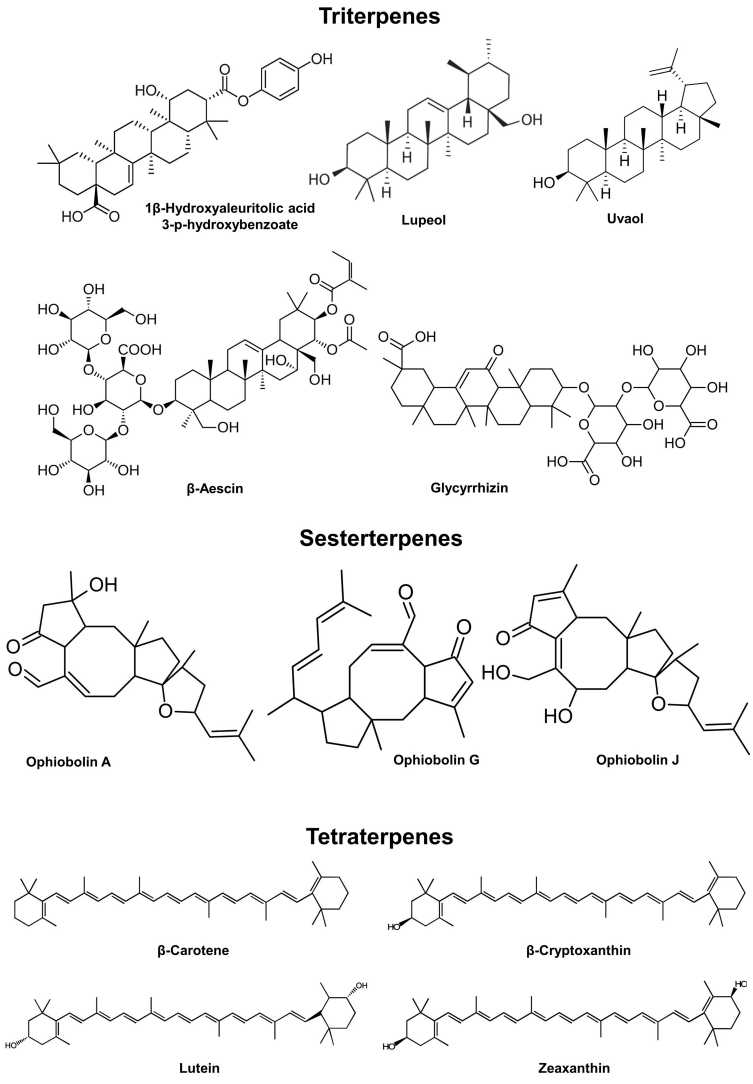

Fig. (4). Chemical structures of major studied triterpenes, sesterterpenes and tetraterpenes.

Sesterterpenes (also named sesterpenes) originate from GGPP and IPP (Fig. **2**) ; Table **1**. and have been mainly found in fungi and marine species [76]. Ophiobolins, which are fungal metabolites, represent the major investigated sesterterpenes for their bioactivities [76]. Ophiobolin A (Fig. **4**) isolated from the pathogenic plant fungus *Ophiobolus miyabeanus*, exhibited remarkable antiproliferative, antibacterial, antiparasitic, antiviral and immunomodulatory effects [77]. Particularly, it produced cytotoxic and pro-apoptotic effects in different cancer cell lines, and reduced tumor size *in vivo* xenograft models of breast cancer [77]. Antiproliferative properties have been also highlighted for other ophiobolins and some hypotheses about the structure-activity relationship have been made [77]. However, more deep studies are required to better defined the anticancer mechanisms of these compounds and their possible usefulness.

Regarding tetraterpenes, also known as carotenoids, they are natural pigments exhibiting yellow, orange, red and purple colors, and contain eight isoprene units with a 40-carbon skeleton Table **1** [78]. Their biosynthesis arises from the condensation of two molecules of GGPP (Fig. **2**) and occur as essential pigments in different photosynthetic organisms, such as bacteria, some species of archaea and fungi, algae, plants, and animals [78]. They are not produced by animals, while can be introduced by food and further modified through metabolic reactions [78, 79]. Particularly, carotenoids which contain unsubstituted β-ionone rings (i.e. α-, β- and γ-carotenes, β-cryptoxanthin; (Fig. **4**) are defined as pro-vitamin A, being retinoid precursors [79 - 81]. In marine environment, these compounds are produced by both autotrophic and non-photosynthetic organisms [79].

Carotenoids exert important physiological functions (i.e. hormones, photo-protectors, antioxidants, color attractants) also in non-photosynthetic organs of plants [78, 82]. Similar roles have been reported in animals, wherein carotenoids act as photo-protectors, antioxidants, enhancers of immunity, and as signals for biotic interactions, both intra- and interspecies [80, 82, 83]. The antioxidant properties have been ascribed to the radical scavenger abilities of carotenoids, which seem to be due to both physical and chemical reactions [79]. Several studies have highlighted an important role of carotenoids in the control of different organ functions and in the preventions and treatment of human disorders, including diabetes, obesity, neurodegeneration, cardiovascular, prostate and eye diseases, and cancer [84 - 92]. For instance, lutein and zeaxanthin (Fig. **4**), the major carotenoids found in human milk, are involved in the visual and cognitive development of infants [93]. Similarly, high dietary intake and blood concentrations of lutein are associated with a lowered risk of coronary heart disease and stroke [94]. Moreover, β-carotene, lutein, and zeaxanthin (Fig. **4**) were found able to protect the retina and lens from photochemical damage induced by light exposure, thus suggesting a potential interest in the prevention of

eye diseases [87]. Beneficial effects of dietary carotenoids, such as lycopene, fucoxanthin, astaxanthin, crocin, and crocetin, have been reported also in preclinical models of neurodegenerative diseases; however, clinical confirmations are needed to support future pharmacological application [95].

The present chapter is focused on the sesquiterpene subgroup and collects novel insights about their chemistry, distribution in nature and pharmacological properties. Some issues regarding toxicity and bioavailability have been discussed too. Owing to the growing pharmacological interest, caryophyllane, lactone-type, and eremophilane sesquiterpenes have been analysed in more detail.

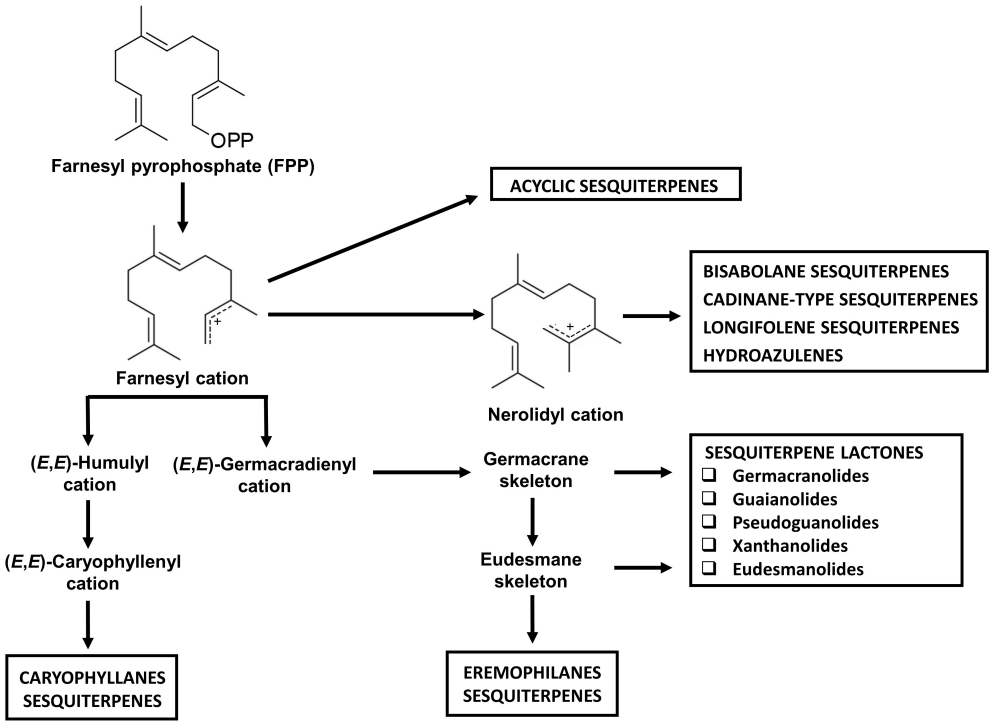

Fig. (5). Biosynthetic pathways of sesquiterpenes.

SESQUITERPENES

Sesquiterpenes are characterized by three isoprene units ($C_{15}H_{24}$) and are widely distributed in nature. Great amount has been found in plants especially in Asteraceae family, where they represent the characteristic constituents [96]. However, they have been reported from several plant families, such as Acanthaceae, Amaranthaceae, Apiaceae, Magnoliaceae and Lamiaceae [97]. A large number of sesquiterpenes have also been identified in marine species (*e.g. Actinocyclus papillatus*, *Sclerodoris tanya*, *Bathydoris hodgsoni*) [98], along with

bacteria (*e.g*. *Streptomyces citreus*, *Streptomyces clavuligerus* and *Roseiflexus castenholzii*) [99], and fungi (*e.g*. *Trichoderma virens*, *Trichothecium roseum Periconia* sp.) [100 - 102]. They originate from the condensation of geranyl pyrophosphate (GPP) with a molecule of 3-isopentenyl pyrophosphate (IPP) to yield a farnesyl pyrophosphate (FPP) which represents their precursor (Fig. **5**)

Indeed, a farnesyl cation is generated by the loss of the diphosphate moiety (OPP) of FPP, whose isomerization, cyclization and rearrangements lead to a wide range of acyclic, monocyclic and ring-fused structures [103].

Acyclic sesquiterpenes, containing a farnesane skeleton, are directly obtained by farnesyl cation, while nerolidyl cation, obtained by farnesyl cation isomerization, is the precursor of bisabolene, cadinane-type, longifolene sesquiterpenes and hydroazulenes [103, 104]. Moreover, different cyclizations and modifications of farnesyl cation leads to (*E*,*E*) humulyl and germacradienyl cations, from which caryophyllane and lactone sesquiterpenes (*e.g*. germacranolides, guaianolides, pseudoguanolides, xanthanolides, eudesmanolides) arise, respectively [105, 106]. Indeed, rearrangements of germacradienyl cation generate a germacrane precursor, whose cyclizations lead to a guaianolide or eudesmane skeleton, from which guanolides and eremophilane sesquiterpenes come from, respectively [106, 107]. Terpene synthases is the enzyme which drives the biosynthesis; afterwards, oxidation, reduction, isomerization, and conjugation reactions determine further modifications of the basic skeletons generating a huge number of different compounds with linear, cyclic, bicyclic, and tricyclic structures, some of which also possess a lactone ring [108].

The unique structure combinations of these secondary metabolites confer them many biological properties, such as insect antifeedant, antiprotozoal, antispasmodic [97], antibacterial, antiviral, cytotoxic, antitumor, anti-inflammatory [109], immunomodulatory, chemopreventive [105], antioxidant [110], anti-ulcer [111], anti-diabetic and lipid-lowering [111]. In the next paragraphs, details about chemistry and natural occurrence of caryophyllane, lactone-type, and eremophilane sesquiterpenes, along with their pharmacological properties are reported.

CARYOPHYLLANE SESQUITERPENES

Chemistry and Distribution in Nature

Caryophyllane sesquiterpenes contain a caryophyllane skeleton, characterized by a dimethylcyclobutane fused with a nine-membered ring, containing a trans-endocyclic (4-5) double bond, whose oxidation generates their epoxide derivatives [105]. In plants, caryophyllane scaffold originates from a

caryophyllenyl cation, obtained by the enzymatic polycyclization of FPP cyclization through the (*E*,*E*)-humulyl carbocation [112].

These compounds widely occur in plants, especially in essential oils, although numerous similar structures have been found in marine species and fungi [105]. Essential oils usually contain mixtures of different sesquiterpenes, especially β-caryophyllene, β-caryophyllene oxide, α-humulene and isocaryophyllene (Fig. **6**), and minor metabolites. β-Caryophyllene (or *trans*-caryophyllene) represents the first compound identified in nature, along with its *cis*-isomer isocaryophyllene (or as γ-caryophyllene), while β-caryophyllene oxide represents its epoxide metabolite [113]. β-Caryophyllene has been found in plant rhizome and wine too [104, 114, 115]. α-Humulene (or α-caryophyllene) is considered an opened-ring isomer of *trans*-caryophyllene [116].

Table 2. Caryophyllane sesquiterpenes identified in nature.

Compounds	Natural Occurrence	Major Sources/Plant Family	Ref.
β-Caryophyllene	Plants	*Scutellaria californica* A. Gray/ Lamiaceae *Eugenia caryophyllata* L./ Myrtaceae *Copaifera langsdorffii* Desf./ Fabaceae *Orthodon dianthera* Maxim./ Lamiaceae *Nepeta curviflora* Boiss./ Lamiaceae *Piper nigrum* L./ Piperaceae *Zingiber nimmonii* (J. Graham) Dalzell/ Zingiberaceae	[105]
β-Caryophyllene oxide	Plants	*Tephrosia persica* Boiss./ Fabaceae *Plinia dermatodes* Urb./ Myrtaceae *Eugenia caryophyllata* L./ Myrtaceae *Eugenia rocana* Britt. et Wils./ Myrtaceae *Syzygium gardneri* Thw./ Myrtaceae *Tagetes patula* L./ Asteraceae *Psidium salutare* (HBK) Berg./ Myrtaceae	[105]

(Table 2) cont.....

Compounds	Natural Occurrence	Major Sources/Plant Family	Ref.
α-Humulene	Plants	*Cachrys alpina* Bieb./ Apiaceae *Callistemon polandii* F.M.Bailey./ Myrtaceae *Helichrysum stoechas* ssp. *barrelieri* var. *spathulatum*/ Asteraceae *Lycopus australis* R. Br./ Lamiaceae *Stachys lanata* K. Koch/ Lamiaceae *Zingiber nimmonii* (J. Graham) Dalzell/ Zingiberaceae	[105]
Isocaryophyllene	Plants	*Baccharis coridifolia* DC./ Asteraceae *Jasminum sambac* L./ Oleaceae *Lantana camara* L./ Verbenaceae *Hypericum heterophyllum* Vent./ Hypericaceae	[105]
Kobusone	Marine species	*Rumphella antipathies*	[117]
Isokobusone	Marine species	*Rumphella antipathies*	[118]
Nanocaryophyllenes A, B	Marine species	*Sinularia nanolobata* V.	[119]
Rumphellatins A, B and C	Marine species	*Rumphella antipathies*	[120 - 122]
Rumphellolides A-F	Marine species	*Rumphella antipathies*	[123]
Sinunorcaryophyllenol	Marine species	*Sinularia* sp.	[124]
Suberosols A-D	Marine species	*Subergiorgia suberosa* P.	[125]
Caryophyllene derivatives	Fungi from marine species	*Ascotricha* sp. *ZJ-M-5*	[126]
Cytosporinols	Fungi	*Cytospora* sp.	[127
Fuscoatrol	Fungi	*Humicola fuscoatra*	[128]
6-Hydroxypunctaporonins	Fungi	*Pestalotiopsis disseminate* T.	[129]
Pestalotiopsins	Fungi	*Pestalotiopsis* spp.	[129 - 131]
Highly oxigenated derivatives[a]	Fungi	*Pestalotiopsis* spp.	[132]
Punctaporonins	Fungi	*Hansfordia sinuosae*	[133]
Punctatins	Fungi	*Poronia punctata*	[134]
Sch 725432, Sch 601253, Sch 601254, and Sch 725434	Fungi	*Chrysosporium pilosum*	[135]
Walleminol, walleminone	Fungi	*Wallemia sebi* J-O	[136]

[a]Pestalotiopsolide A, taedolidol, 6-epitaedolidol.

Owing to the flexibility of the nine-membered ring and the high reactivity of the endocyclic 4,5-double bond [113], caryophyllane skeleton can undergo rearrangements and cyclization reactions, leading to the generation of a number of caryophyllane-like compounds and polycyclic derivatives Table **2** [105].

For instance, rumphellatins, kobusone, isokobusone, sinunorcaryophyllenol and rumphellolides are chloro-containing caryophyllane-type structures (Fig. **6**) [117, 118, 120 - 123]. Suberosols, fuscoatrol A, buddledins and cytosporinols are β-caryophyllene derivatives, while walleminol and walleminone are *cis*-fused isocaryophyllenes (Fig. **6**) [125, 127, 128, 136]. Pestalotiopsins, pestaloporinates, punctaporonin, pestaloporonins, punctatins and trioxygenated caryophyllenes (Sch 601253, Sch 601254, and Sch 725434) are classified as polycyclic highly oxygenated structures [129 - 135].

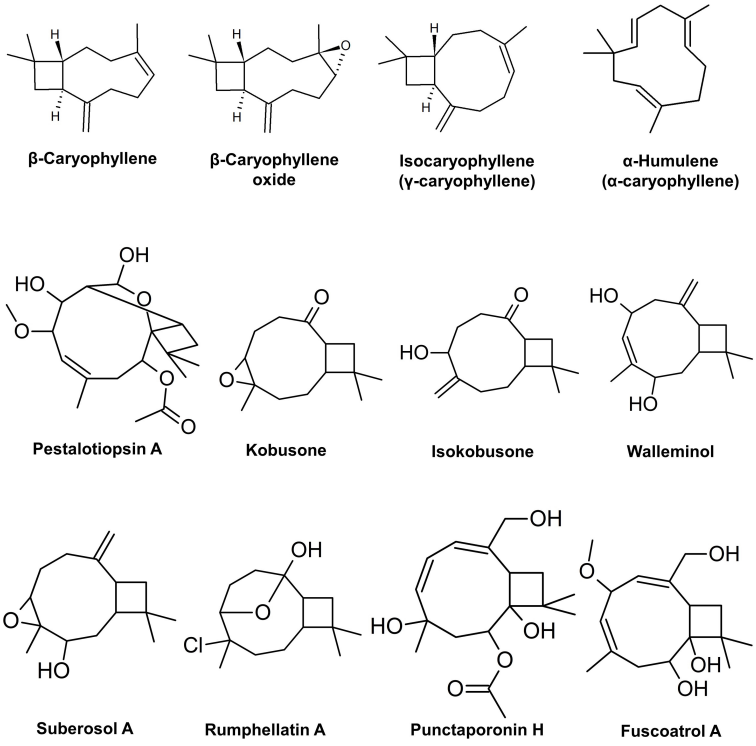

Fig. (6). Examples of caryophyllane sesquiterpene chemical structures.

Rumphellatins, kobusone, isokobusone and rumphellolides have been isolated from a Formosan soft sea coral *Rumphella antipathies* [117, 118, 120, 121, 123], nanonorcaryophyllenes A and B from the Taiwanese soft coral *Sinularia nanolobata* [119], while the norsesquiterpene sinunorcaryophyllenol from

Sinularia sp [124]. Moreover, suberosols A, B, C, and D, along with buddledins C and D were identified in the Taiwanese gorgonian coral *Subergorgia suberosa* [125]. Other compounds (*e.g*. pestalotiopsins, 6-hydroxypunctaporonin, pestaloporonins A-C, and the highly oxidazed caryophyllene derivatives pestalotiopsolide A, taedolidol and 6-epitaedolidol) have been identified in *Pestalotiopis* species, isolated from the bark of various plants [129 - 132, 137, 138]. Pestalotiopsins-like sesquiterpenes were also found in the marine fungus *Ascotricha* sp. ZJ-M-5 [126]. Likewise, the following caryophyllene sesquiterpenoids were isolated from the cultures of endophytic fungi: punctatins from *Poronia punctata*, walleminol and walleminone from *Wallemia sebi*, fuscoatrol A from *Humicola fuscoatra*, Sch 725432, Sch 601253, Sch 601254, and Sch 725434 from *Chrysosporium pilosum*, cytosporinols from *Cytospora* sp., and punctaporonins H–M from *Hansfordia sinuosae* [127, 128, 133 - 136].

Pharmacological Properties

Biological activities of caryophyllane sesquiterpenes have been investigated in different experimental models. Compounds from marine species, including fuscoatrol and rumphellatins A and B showed interesting antimicrobial activities [120, 121, 128], while pestalotiopsins displayed immunosuppresive properties [130]. Different caryophyllane sesquiterpenoids hindered growth and proliferation of cancer cell lines. Particularly, nanocaryophyllene B produced cytotoxic effects in human colon and liver cancer cells, despite a null activity of its *trans*-isomer [119]. Similarly, sesquiterpenes isolated from *Ascotricha*, suberosols and pestalotiopsin A were highly cytotoxic in human leukaemic cells 125,126,139]. Interestingly, the *cis*-pestalotiopsin A was the most potent isomer [139]. By contrast, moderate cancer cytotoxicity was reported for cytosporinols and punctaporonins, while sinunorcaryophyllenol was not cytotoxic [124, 127, 133]. However, no evidence about a possible structure-activity relationship and the mechanisms involved is available.

Caryophyllane sesquiterpenes from plants, including β-caryophyllene, β-caryophyllene oxide, isocaryophyllene and α-humulene attracted a greater attention [105]. A plethora of biological activities, including antibacterial, antifungal, antioxidant, chemopreventive, antiproliferative and anticancer have been highlighted in preclinical models [140 - 142]. α-Humulene and isocaryophyllene displayed a higher antiproliferative power than β-caryophyllene and β-caryophyllene oxide [143, 144], thus suggesting that the *cis*-configuration of caryophyllane skeleton can be responsible for a more potent cytotoxicity [105]. Indeed, highly cytotoxic sesquiterpenes, such as pestalotiopsin A and nanocaryophyllene B, possessed a *cis*-ring [105].

An involvement of apoptotic cell death has been also associated to the antiproliferative activity of caryophyllane sesquiterpenes; particularly, the proapoptotic effects of β-caryophyllene have been associated to the activation of mitochondrial-mediated pathways, DNA fragmentation, down-regulation of anti-apoptotic, up-regulation of pro-apoptotic genes and reduced metastasizing power [105, 145 - 147]. A switch from autophagy to apoptosis has been also reported in glioblastoma cells [148]. However, these effects did not occur at low concentrations of β-caryophyllene, thus suggesting a dose-dependent regulation of apoptosis [149]. Similarly, α-humulene and β-caryophyllene oxide produced proapoptotic effects in different cancer cells [150].

A downregulation of JAK1/STAT3, NF-kB and PI3K/AKT/mTOR/S6K1 signallings has been associated with the proapoptotic effects of caryophyllane sesquiterpenes in cancer cells [145, 149, 151, 152]. Moreover, apoptosis induced by β-caryophyllene has been found associated with a cannabinoid CB2 receptors (CB2R) modulation [148]. Indeed, the compound is known to act as an agonist of CB2R and as a modulator of other targets of endocannabinoidome, such as peroxisome proliferator-activated receptors (PPARs) [153, 154].

An activation of CB2R by β-caryophyllene has been highlighted in different models of inflammatory diseases (such as pain, neurodegeneration, atherosclerosis, anxiety, chronic inflammation, metabolic ailments, arthritis, ulcerative colitis, autoimmune diseases and some types of cancer), and is involved in its anti-inflammatory and antinociceptive effects [105, 155 - 166]. Similarly, anti inflammatory properties along with a modulation of CB2R have been reported for β-caryophyllene oxide and α-humulene, albeit less characterized [167 - 171]. A modulation of different pro-inflammatory pathways, such as iNOS (inducible nitric oxide synthase), TNF-α (tumor necrosis factor-alfa) and NF-κB (nuclear factor-κB), interleukin 1 beta (IL-1β), interleukin-6 (IL-6), cyclooxygenase 1 (COX-1), and cyclooxygenase 2 (COX-2), and redox signallings (*e.g.* Nrf2 and GSH) has been associated with anti-inflammatory effects of these caryophyllane sesquiterpenes [153, 172 - 174]. Furthermore, an inhibition of fatty acid amide hydrolase (FAAH), a further target of endocannabinoidome, has been associated with the chemical features of the caryophyllane scaffold [175]. On the basis of this evidence, the anti-inflammatory activity of these sesquiterpenes, especially β-caryophyllene, could be a result of a multitarget modulation, including FAAH and COX-2 enzymes and CB2Rs [175].

Anti-inflammatory properties along with antioxidant effects also mediated the cytoprotective and chemopreventive activity of these sesquiterpenes in different preclinical models [105, 172, 176 - 185]. Particularly, β-caryophyllene showed to counteract the oxidative, genotoxic and proapoptotic damage induced by

anticancer drugs in epithelial cells [149, 183 - 185]. Comparing the effects in noncancerous cholangiocytes and those in Mz-ChA-1 cholangiocarcinoma cells, the sesquiterpene produced mild genoprotective effects towards DNA-damage induced by doxorubicin, likely due to defective DNA repair systems [149]. This suggested a dual action of β-caryophyllene as cytoprotective in normal cells and chemosensitizer in cancerous ones [149]. The genoprotective properties of β-caryophyllene and β-caryophyllene oxide have been investigated in both bacterial and mammalian cells against different carcinogens and environmental pollutants, such as cigarette smoke and butts, aromatic amins and nitroarenes [105], and resulted to be mediated by desmutagenic and bioantimutagenic mechanisms [34, 186, 187].

Interesting chemosensitizing effects were highlighted for β-caryophyllene, β-caryophyllene oxide and α-humulene in combination studies, in which nontoxic concentrations of the compounds synergistically potentiate the efficacy of different anticancer drugs, such as doxorubicin, sorafenib and paclitaxel [105, 144, 185, 188]. Potentiation of anticancer drug activity by caryophyllane sesquiterpenes has been mainly ascribed to the inhibition of efflux pumps, especially P-glycoprotein (Pgp), MRP1 and MRP2 transporters. A mechanistic study revealed that β-caryophyllene and β-caryophyllene oxide inhibited both function Pgp and expression of Pgp [189]. Moreover, a direct interaction of caryophyllane scaffold in a hydrophobic space next to the nucleotide binding domain of the protein was highlighted by a molecular docking study [189]. Considering that Pgp is codified by *mdr1* gene, which is transcriptionally regulated by STAT3, blocking the activation of STAT3 has been hypothesized to be involved in the modulation of Pgp expression by caryophyllane sesquiterpenes [105]. Owing to the lipophile nature of these compounds, a modulation of membrane permeability, which in turn can interfere with function of membrane transporters, has been also reported [26].

Caryophyllane sesquiterpenes, especially β-caryophyllene, have been reported to modulate glucose metabolism by a CB2R-mediated increase in the insulin secretion in different animal models of diabetes [190]. This effect was found associated with improved levels of antioxidant enzymes, thus confirming the antioxidant potential of β-caryophyllene and suggesting its ability to prevent oxidative stress and related complications of the diabetes [191]. Moreover, it displayed hypolipidemic properties by decreasing the levels of total cholesterol, triglycerides and low-density lipoprotein (LDL), likely through affecting HMG-CoA reductase activity [192 - 194].

The interesting healing properties of caryophyllane sesquiterpenes are limited by their high lipophilicity and poor bioavailability, which can lead to inconstant

biological responses. To overcome this drawback, different pharmaceutical formulations, including nanoparticles, liposomes, and cyclodextrins have been proposed [105], albeit at the moment further studies are needed.

SESQUITERPENE LACTONES

Chemistry and Distribution in Nature

Sesquiterpene lactones (SLs) are chemically distinct from other sesquiterpenoids. Indeed, besides being constituted of three isoprene units arranged by cyclase enzymes in several characteristic ring systems, they possess one or more γ-lactone rings formed by the action of oxidase enzymes, which determines the formation of the characteristic and peculiar structures present in nature (Fig. **7**) [195].

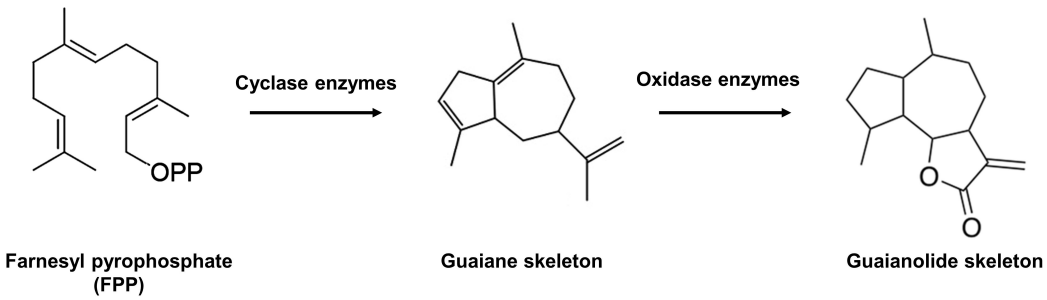

Fig. (7). Biosynthesis of guaianolide-type sesquiterpenes.

According to lactone ring annulations, SLs can be divided into two classes, namely 6,12- (*e.g.*, costunolide, parthenolide, santonin, matricin) and 8,12-olides (*e.g.*, inunolide, alantolactone, thapsigargin, helenalin) (Fig. **8**) [196].

However, SLs differ each other also for the type and position of the substituents, as well as the size of the non-lactone ring. Based on these structural differences, SLs can be divided into several subclasses, among which the major are represented by eudesmanolide with a 6/6 bicyclic structure, guaianolide and pseudoguaianolide both having a 5 and 7 ring pattern, germacranolide with a 10-membered ring, and xanthanolide which presents a non-cyclic carbon chain and a seven-membered ring (Fig. **9**) [197]. There are also several minor types that are described by different authors, namely bisabolenolides, drimanolides, eremophilenolides, fukinanolides, elemanolides, germafurenolides, tutinanolides, and cadinanolides [198].

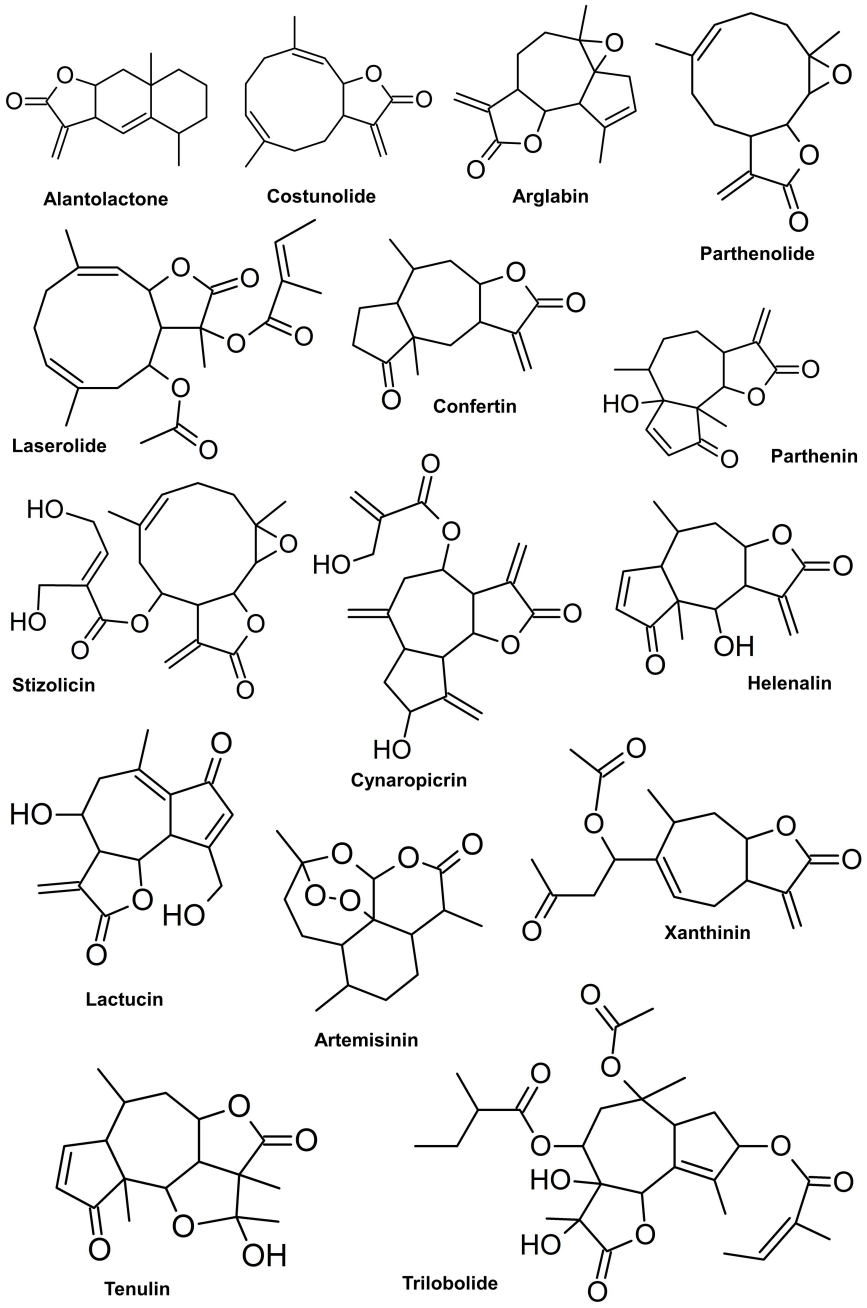

Fig. (8). Examples of sesquiterpene lactone chemical structures.

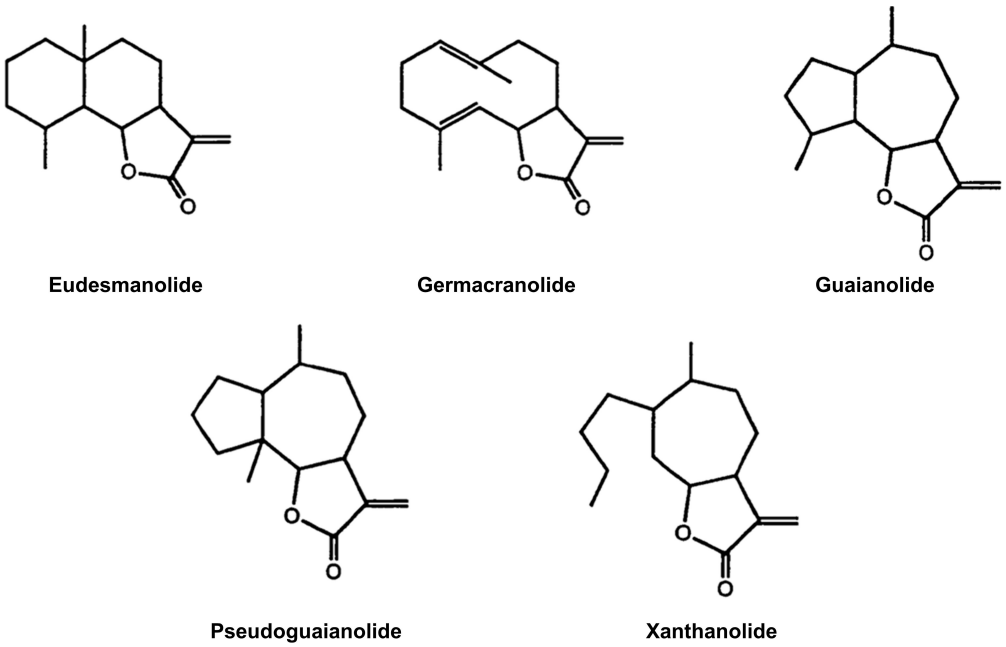

Fig. (9). Structure skeleton of some subclasses of sesquiterpene lactones. Modified from Nunez, 1992 [201].

The presence of the α-methylene-γ-lactone nucleus, the α,β-unsaturated carbonyl group, and other electrophilic sites in the SL structure seems to be important moieties that influence their biological properties. Particularly, the activity displayed by SLs depends on the number of alkylating groups in their structure, being two the optimal number [197]. Both the α-methylene-γ-lactone nucleus and the α,β-unsaturated carbonyl group act as strong alkylating agents that may react with intracellular nucleophiles (*e.g.* thiol groups of proteins, cysteine residue in GSH) by Michael-type addition. Consequently, SLs could impair cell functionality by affecting gene regulation, protein synthesis, and cell metabolism [199].

However, SL bioavailability is limited by their lipophilicity and molecular geometry [199]. Therefore, new synthetic derivatives and delivery systems have been approached by researchers in order to exploit the advantage resulting from the therapeutic application of these compounds [200].

SLs are colourless, bitter, and stable compounds, mainly found in species of the plant kingdom although further structures have been highlighted in marine species and fungi [199]. Particularly, the majority of SLs (more than 90%) has been characterized in Asteraceae family, which also includes common edible plants such as lettuce, chicory, endive, artichokes, salsify, sunflower seed, and dandelion

[202]. Examples of SLs Fig. **8** of biological interest are represented by alantolactone and isoalantolactone from the root of *Inula helenium* L., parthenolide from *Tanacetum parthenium* L. shoots, arglabin from *Artemisia* spp. aerial parts, cynaropicrin from *Cynara scolymus* L. leaves, lactucin, 8-desoxylactucin, and lactucopicrin from *Cichorium intybus* L. and *Lactuca virosa* L. roots, and artemisinin from *Artemisia annua* L. leaves [198, 203 - 209].

SLs are also present in less amount in Lauraceae, Asclepiadaceae, Araliaceae, Annonaceae, and Lamiaceae [109]. Some examples are magnolialide and santamarine from *Laurus nobilis* L. leaves, guatterfriesols A-C from *Guatteria friesiana* (W.A. Rodrigues) Erkens & Maas stem barks, and eudebeiolides A−F from *Salvia plebeia* R. Br. aerial parts [210 - 212]. In Table **3**, some SLs present in the plant kingdom are reported and divided based on their chemical structure.

Table 3. Sesquiterpene lactones present in the plant kingdom.

Compounds	Plant Source/Family	Part of Interest	Ref.
Eudesmanolide-type sesquiterpenes lactones			
Alantolactone Isoalantolactone	*Inula helenium* L./Asteraceae	Root	[203]
Artemargyinins A-F	*Artemisia argyi* Levl. et Vant./Asteraceae	Leaves	[213]
Eudebeiolides A−F Plebeiolide C	*Salvia plebeia* R. Br./Lamiaceae	Aerial parts	[212, 214]
Ivalin Telekin	*Carpesium divaricatum* Siebold. & Zucc./Asteraceae	Whole plant	[215, 216]
Germacranolide-type sesquiterpene lactones			
Costunolide	*Costus speciosus* (J. Koenig) Sm./Costaceae	Rhizome	[217]
Enhydrin Uvedalin Polymatin B	*Smallanthus sonchifolius* (Poepp & Endl.) H. Robinson/Asteraceae	Aerial parts	[218]
Laserolide	*Laser trilobum* (L.) Borkh/Apiaceae	Roots	[219]
Parthenolide	*Tanacetum parthenium* L./*Asteraceae*	Shoots	[204]
Guaianolide-type sesquiterpene lactones			
Arglabin	*Artemisia* spp./Asteraceae	Aerial parts	[205]
Cynaropicrin	*Cynara scolymus* L./*Asteraceae*	Leaves	[206]
Inuviscolide	*Ferula communis* L./Apiaceae *Inula viscosa* (L.) Ait./Asteraceae	Aerial parts and roots Young shoots	[220]
Lactucin 8-desoxylactucin Lactucopicrin	*Cichorium intybus* L. *Lactuca virosa* L./Asteraceae	Roots	[207, 209]

(Table 3) cont.....

Compounds	Plant Source/Family	Part of Interest	Ref.
Thapsigargin	*Thapsia garganica* L. */Apiaceae*	Roots and fruits	[221]
Trilobolide	*Laser trilobum* (L.) Borkh/Apiaceae	Roots	[219]
Pseudoguaionolide-type sesquiterpene lactones			
Hymenin Ambrosanolide Tetraneurin A Parthenin Hysterin Confertdiolide	*Partienium hysterophorus* L./Asteraceae	Whole plant	[222]
Confertin Neoambrosin	*Ambrosia* spp./Asteraceae	Twigs and leaves	[223]
Helenalin	*Arnica* spp. *Helenium* spp./Asteraceae	Flowers	[197]
Mexicanin I	*Gaillardia megapotamica* (Spreng.) Baker/Asteraceae	Aerial parts	[224]
Tenulin	*Helenium amarum* (Raf.) H.Rock/Asteraceae	Leaves and stems	[225, 226]
Xanthanolide-type sesquiterpene lactones			
Pungiolide A, D, E	*Xanthium sibiricum* Patr./Asteraceae	Aerial parts	[227]
Mogolides A, B	*Xanthium mogolium* Kitag/Asteraceae	Aerial parts	[228]
Xanthalongin	*Arnica longifolia* D.C. Eaton/Asteraceae	Flowerheads	[229]
Xanthinin Xanthatin Stizolicin Solstitialin	*Xanthium spinosum* L./Asteraceae	Aerial parts	[230]
Cadinanolide-type sesquiterpene lactones			
Artemisinin	*Artemisia annua* L./Asteraceae	Leaves	[198, 208]
Spicatocadinanolide A	*Pseudoelephantopus spicatus* (Juss.) C.F. Baker/Asteraceae	Aerial parts	[231]

Pharmacological Properties

SLs exhibit a wide range of biological activities, such as antimalarial, antibacterial, antioxidant, antitumor, anti-inflammatory, neuroprotective, hepatoprotective, and immunomodulatory properties [197]. As previously mentioned, the presence of the α-methylene-γ-lactone nucleus and the α,β-unsaturated carbonyl group has a crucial role in almost all the observed biological effects [197].

The antibacterial activity of SLs is mainly due to their lipophilicity. Indeed, they can easily permeate through the cell wall and cell membrane, so disrupting membrane integrity and potential. This leads to leakage of cellular contents, denaturation of cytoplasmic proteins, and inactivation of cellular enzymes with consequently bacterial cell death [232, 233]. SLs seem to possess a higher activity against Gram-positive species respect to Gram-negative ones. Helenalin and alantolactone (Fig. **9**) are examples of SLs with antibacterial activity. Particularly, helenalin showed an inhibitory action against *Mycobacterium tuberculosis* and *Corynebacterium diptheriae*, while alantolactone against *Staphylococcus aureus* [233, 234]. Recently, it has also been showed that alantolactone exerts its antimicrobial effect against *Staphylococcus aureus* also by enhancing its clearance and modulating host immune response [235].

Several SLs have also showed to possess antimalarial activity, being artemisinin the most representative due to its medical application. This compound is a highly oxygenated sesquiterpene, containing a unique 1,2,4-trioxane ring structure, which is responsible for the antimalarial activity. Particularly, artemisinin is activated by reduced heme, a byproduct of hemoglobin endocytosis and catabolism within *Plasmodium* parasite. The cleavage of the endoperoxide bridge generates a free radical that alkylate and damage *Plasmodium* proteins and lipids, leading to death. This suicide activation led to a concomitant 10,000-fold reduction in parasite density in human patients [236]. Artemisinin (Fig. **9**) was found to be superior to conventional antimalarial drugs, such as chloroquine and quinine, and also completely effective in the treatment of chloroquine-resistant *falciparum* malaria [196]. However, recently, *Plasmodium* parasites resistant to artemisinin have been highlighted [236]. Due to the low bioavailability of artemisinin, which limits its effectiveness, several semisynthetic derivatives such as artemether, arteether, and artesunate have been developed [236].

In the last years, many natural SLs have been investigated for their potential antitumor properties, some of which (such as parthenolide, artemisinin and its derivatives) were under clinical evaluations [237 - 239]. *In vitro* and *in vivo* studies showed that these compounds are able to inhibit cell cycle and proliferation, and to induce apoptosis [199]. The exact mechanism of SLs anticancer activity is not well elucidated yet, but it is probably due to their interaction with multiple pathways. Indeed, they act as alkylating agents leading to inhibition of key enzymes and proteins (*e.g.* glutathione, farnesyl protein transferase enzyme); moreover, emerging data suggest that they also determine an overproduction of reactive oxygen species (ROS), so impairing the intracellular redox homeostasis. At last, the induction of apoptosis through the inhibition of STAT3 signalling have been recognized in different cellular and animal models of cancer [197, 199]. Alantolactone, parthenolide, arglabin, costunolide, and

cynaropicrin are examples of SLs with anticancer activity [197]. Particularly, arglabin has been used in the therapy of several cancer types (*e.g*. breast, lung, liver, esophageal tumors) in oncological clinics of Kazakhstan and showed to significantly reduce the tumor volume in esophageal carcinoma patient [240]. Noteworthy, alantolactone and costunolide have also displayed, *in vitro* and *in vivo* studies, to be potent chemosensitizing agents able to reverse the multidrug resistance which often occur during cancer therapy [197].

At last, SLs have shown to be potent anti-inflammatory agents through the inhibition of NF-kB pathway. This protein complex regulates the expression of many key genes involved in inflammation and human cancers, so it represents a promising target for the development of new chemopreventive and chemotherapeutic agents [241]. Some important SLs which have displayed anti-inflammatory activity are alantolactone, arglabin, costunolide, helenalin, and parthenolide [242]. Particularly, mechanistic studies have revealed that costunolide and parthenolide, owing to their α,β-unsaturated carbonyl group, significantly inhibit NF-kB activation by preventing the phosphorylation of IkB, and therefore, sequestering the complex in an inactive form [242]. Conversely, helenalin, which contains an α,β-unsaturated carbonyl group and an α-methylen--δ-lactone ring, seems to exert its effect by direct alkylation of the p65 subunit of NF-kB without inhibition of IkB degradation. It seems that helenalin selectively modifies the p-65 subunit of NF-kB at the nuclear level, therefore inhibiting its DNA binding [242].

EREMOPHYLANE SESQUITERPENES

Chemistry and Distribution in Nature

Eremophilanes are 6-carbon bicyclic sesquiterpenes, containing only two complete isoprene subunits; compounds carrying a 5-membered ring can be included too [243]. They are derived from eudesmane by a methyl shift across the ring junction (Fig. **10**) and are structurally nonconform to the isoprene rule of Wallach, according to which terpenes are multiples of isoprene subunits, arranged head-to-tail [243]. The first eremophilanoid sesquiterpene, namely eremophilone, was isolated in 1932 from the oil of *Eremophila mitchelli* Benth. wood. Other similar structures, some of which in oxygenated forms (*e.g*., eremophilane alcohol, eremophilane acid, eremophilane lactone) have been identified [108].

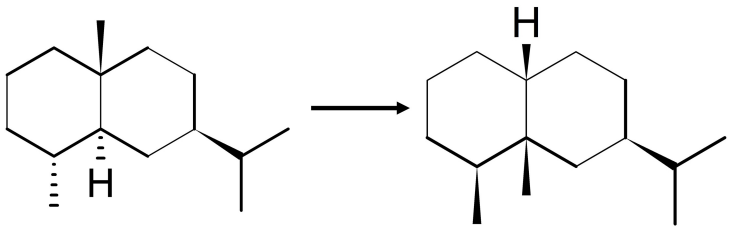

Fig. (10). Conversion of eudesmane in the eremophilane skeleton.

Owing to their carbon framework and stereochemistry, eremophilanoid sesquiterpenes can be classified in seven major subgroups, including bicyclic eremophilanes, furanoeremophilanes, 4-epi-eremophilanes, nootkatanes (or bicyclic 7-epi-eremophilanes), tricyclic 7-epi-eremophilanes, ishwarenes, and noreremophilanes Table **4**.

Table 4. Major groups of eremophilane sesquiterpenes and their occurrence in plant kingdom.

Compounds	Plant source/Family	Part of interest	Ref.
Bicyclic Eremophilanes			
Alloeremophilone	*Eremophila mitchelli* Benth./Scrophulariaceae	Heartwood	[244]
Eremoligenol	*Ligularia fischeri* Ledeb. Turcz./Asteraceae	Root	[245]
Eremophilene	*Petasites albus* L. Gaertn/Asteraceae	Leaves, flower stems and rhizomes	[246]
	Petasites hybridus (L.) G. Gaertn., B. Mey & Scherb/Asteraceae	Leaves, flower stems and rhizomes	[246]
	Petasites japonicus (Siebold & Zucc.) Maxim./Asteraceae	Rhizome	[247]
	Pogostemon cablin Benth (patchouli oil)/Lamiaceae	Leaves	[248]
	Valeriana officinalis L./Valerianaceae	Root	[249]
Eremophilone	*Eremophila mitchelli* Benth./Scrophulariaceae	Root	[244]
Fukinone	*Petasites japonicus* (Siebold & Zucc.) Maxim./Asteraceae	Fresh bud	[246]
Petasin	*Petasites hybridus* (L.) Gaertn., B. Mey & Scherb/Asteraceae	Rhizome	[246]
	Ligularia fischeri Ledeb. Turcz./Asteraceae	Root	[245]
S-petasin	*Petasites hybridus* (L.) Gaertn., B. Mey & Scherb/Asteraceae	Rhizome	[246]

(Table 4) cont.....

Compounds	Plant source/Family	Part of interest	Ref.
PR toxin	*Penicillium roqueforti* Thom/Aspergillaceae	Fungus	[250]
S-Japonin	*Petasites japonicus* (Siebold & Zucc.) Maxim. var. Aichiwasebuki/Asteraceae	Leaves	[246]
Warbugiadione	*Warburgia ugandensis* Sprague/Canellaceae	Heartwood	[251]
Xylarenona A-B	*Xylaria* Hillex ex Schrank/Xylariaceae	Fungus	[252]
Furanoeremophilanes type - Butenolactones			
Bieremoligularolide	*Ligularia muliensis* Hand.-Mazz./Asteraceae	Root	[253]
Eremophilenolide	*Petasites hybridus* (L.) G. Gaertn., B. Mey & Scherb/Asteraceae	Rhizome	[246]
Ligularenolide	*Ligularia sibirica* L. Cass./Asteraceae	Leaves	[254]
Petasitolide A Petasitolide B S-Petasitolide A S-Petasitolide B	*Petasites hybridus* (L.) G. Gaertn., B. Mey & Scherb/Asteraceae	Rhizome	[246]
	Senecio aegyptius *L./Asteraceae*	Flowering plant	[255]
Furanoeremophilanes type - Furanolactones			
Adenostylone	*Adenostyles alliariae* (Gouan) A. Kern./Asteraceae	Rhizome	[256]
Berkeasmin A	*Paraphaeosphaeria* O. E. Erikss./Didymosphaeriaceae	Fungus	[257]
Decompostin	*Psacalium decompositum* (A. Gray) H. Rob & Brettell/Asteraceae	Roots	[258]
Euryopsol	*Euryops* spp./Asteraceae	Resin	[259]
Furanofukinol	*Petasites japonicus* (Siebold & Zucc.) Maxim. var. Aichiwasebuki/Asteraceae	Rhizome	[247]
Furanojaponin	*Petasites japonicus* (Siebold & Zucc.) Maxim. var. Aichiwasebuki/ Asteraceae	Rhizome	[247]
Furanoligularenone	*Ligularia fischeri* Ledeb. Turcz., *Ligularia sibirica* L. Cass., *Ligularia pleurocaulis* Franchet/Asteraceae	Root	[260]
	Senecio nemorensis L. var. Bulgaricus/Asteraceae	Rhizome	[261]
Furanopetasin	*Petasites hybridus* (L.) G. Gaertn., B. Mey & Scherb/Asteraceae.	Rhizome	[246]
Isoadenostylone	*Adenostyles alliariae* (Gouan) A.Kern./Asteraceae	Rhizome	[256]
Ligularone	*Petasites japonicus* (Siebold & Zucc.) Maxim./Asteraceae Aichiwasebuki/Asteraceae	Rhizome	[247]
	Ligularia fischeri Ledeb. Turcz./Asteraceae	Roots	[262]
Nemosenin-A, -B, -C, -D	*Senecio nemorensis* L. var. fuchsii/Asteraceae	Rhizome	[261]
Petasalbin	*Petasites albus* L. Gaertn./Asteraceae	Rhizome	[246]
	Ligularia virgaurea (Maxim.) Mattf./Asteraceae	Roots	[263]
Senemorin	*Senecio nemorensis* L. var. fuchsii/Asteraceae	Rhizome	[261]

(Table 4) cont.....

Compounds	Plant source/Family	Part of interest	Ref.
Warbugin	*Warburgia ugandensis* Sprague/Canellaceae	Heartwood	[251]
Tricyclic 7-epi-Eremophilanes			
α-Ferulene	*Ferula communis* L./Aspiaceae	Latex	[264]
Aristolone	*Aristolochia ringen* Vahl./Ristolochiaceae	Roots	[265]
Calarene	*Acorus calamus* L./Acoraceae	Rhizome	[266]
	Dipterocarpus dyeri Pierre/Dipterocarpaceae	Resin	[267]
	Nardostachys jatamans (D. Don) DC./Valerianaceae	Roots	[268]
	Valeriana jatamansi Jones/Valerianaceae	Roots and rhizome	[269]
Calarenol	*Nardostachys jatamans* (D. Don) DC./Valerianaceae	Roots	[268]
Debilone	*Aristolochia debilis* Siebold. & Zucc./Aristochilaceae	Roots	[270]
Ishwaranes			
3-Ishwarone	*Peperomia scandens* Ruiz & Pavon/Piperaceae	Aerial parts	[271]
Ishwarane	*Aristolochia indica* L./Aristochilaceae	Roots	[270]
	Corallocarpus epigaeus Benth. ex Hook.F./Cucurbitaceae	Roots	[272]
Ishwarol	*Aristolochia indica* L./Aristochilaceae	Roots	[270]
Ishwarone	*Aristolochia indica* L./Aristochilaceae	Roots	[270]
	Corallocarpus epigaeus Benth. ex Hook.F./Cucurbitaceae	Roots	[272]
Noreremophilanes			
Bakkenolide A, B, C, D, E	*Petasites japonicus* (Siebold & Zucc.) Maxim. var. Aichiwasebuki/Asteraceae	Leaves	[273]
	Senecio aegyptius L./Asteraceae	Flowering plant	[255]
Nootkatanes			
Aristolochene	*Aristolochia indica* L./Aristochilaceae	Roots	[270]
Bicyclovetivenol	*Chrysopogon zizanioides* L. Roberty/Poaceae	Roots	[274]
Nootkatene and Nookatone	*Chamaecyparis nootkatensis* (D. Don) Spach/Lauraceae	Heartwood	[275]
	Chrysopogon zizanioides L. Roberty/Poaceae	Roots	[274]
Valencene	*Chamaecyparis nootkatensis* (D. Don) Spach/Lauraceae	Heartwood	[275]
	Chrysopogon zizanioides L. Roberty/Poaceae	Roots	[274]
α-Vetivone β-Vetivenene γ-Vetivenene	*Chrysopogon zizanioides* L. Roberty/Poaceae	Roots	[274]
4-epi-Eremophilanes			

(Table 4) cont.....

Compounds	Plant source/Family	Part of interest	Ref.
Capsidiol	*Capsicum annuum* L./Solanaceae	Fruits	[276]
	Nicotiana tabacum L./Solanaceae	Leaves	[277]

Among eremophilane sesquiterpenes, furanoeremophilanes contain a furan or a modified furan ring, fused onto the bicyclic eremophilane system, and are further classified as butenolactones and furans; moreover, ishwarenes are a small group of tetracyclic sesquiterpenes, related stereochemically to nootkatane [108]. Chemical structures of some eremophilanoid sesquiterpenes are displayed in Fig. **(11)**. Eremophilane sesquiterpenes are produced from several plant species and fungi [278]. They have been identified in about twenty genera of Asteraceae family, mainly *Ligularia*, *Senecio*, *Cacalia* and *Petasites*, being also considered their chemotaxonomic markers [108]. Moreover, eremophilane-like compounds have been found in other plant families, among which Valerianaceae, Lamiaceae and Canellaceae [243]. Some compounds have been detected both in plants and in fungi; for instance, petasol is produced by *Penicillium* spp. and is a typical constituent of *Petasites* spp. too, while mairetolide F has been found both in *Xylaria* spp. and in *Senecio mairetianus*DC [278].

Pharmacological Properties

Along with their unique structural features, eremophilane sesquiterpenes have displayed a number of interesting biological activities, including antimicrobial, antiproliferative, anti-inflammatory and antiallergic [108]. Recently, a modulation of glucose and lipid metabolism, which suggests a possible interest for the treatment of metabolic diseases, has been reported, although further studies are required in confirmation [279, 280].

Antibacterial properties of eremophilane sesquiterpenes have been displayed against both Gram-positive and Gram-negative bacteria, such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* [108]. Particularly, dacrymenone, a compound obtained by fermentation of *Dacrymyces* spp., showed antibacterial and antifungal activities against some filamentous fungi (*e.g*. *Aspergillus ochraceus* and *Cladosporium cladosporioides*) [281]. Antifungal activities have been also reported for capsidiol, an eremophilane sesquiterpene found in many solanaceous species, and its derivatives [277].

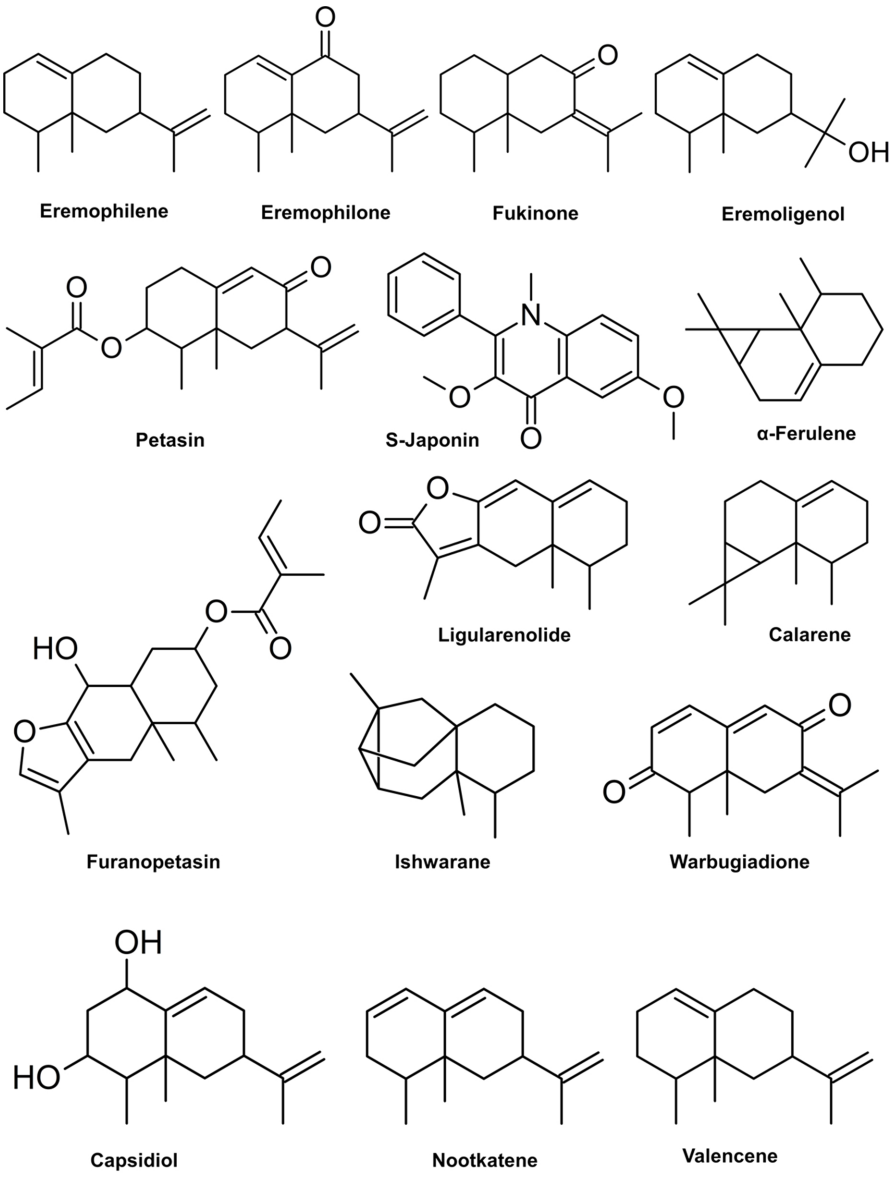

Fig. (11). Examples of eremophilane sesquiterpene chemical structures.

Eremophilane-type sesquiterpenes were also found to possess a broad-spectrum but moderate cytotoxic activity in different human cancer cell lines, including liver (HepG2 and SMMC-7721 cells), lung (A549 cells), ovarian (HO-8910 cells), cervical (HeLa cells), prostate (PC3 cells) and leukemic (HL60, SMMC7721) [198, 282 - 284].

A number of compounds have been highlighted to possess anti-inflammatory properties, being able to affect the release of a variety of inflammatory mediators and key signallings [108]. Particularly, furanoligularenone, butanoeremophilanes, acrememeremophilanes B-F showed inhibitory effects against the nitric oxide (NO) production induced by lipopolysaccharide (LPS) in murine macrophages [260, 277]. Moreover, the compounds were able to affects the LPS-induced expression of iNOS and COX-2 and the activation of the NF-kB pathway [108, 277]. Similarly, culcitiolides B and C, isolated from *Senecio culcitioides* were shown able to inhibit the NF-kB activation [285], while nigriterpene C and periconianones, produced by *Xylaria nigripes* and *Periconia* spp. fungi respectively, inhibited the LPS-induced NO production in murine brain microglial cells [278].

Anti-inflammatory effects were also reported for the extracts (*e.g*. alcoholic and ethyl acetate) of *Senecio* spp., characterized to contain eremophilane sesquiterpenes, in carrageenan-induced rat hind paw oedema assay [108]. Furthermore, an extract from the leaves of *Petasites hybridus* (L.) Gaertn., B. Mey. et Scherb. (namely Ze339), containing petasin, neopetasin, and isopetasin, produced anti-inflammatory effects, by inhibiting the production of IL-8 and eicosanoid LTB4 in allergen-challenged patients [286]. Modulation by Ze339 of pro-inflammatory mediators has been found associated to a reduced activation of STAT-signalling pathways in primary human nasal cells; however, the contribution of petasins was not clarified [287]. Conversely, the contents of petasin and isopetasin did not affected the anti-inflammatory power of some lipophilic extracts from rhizomes of *Petasites hybridus* [288].

Petasin, isopetasin, S-petasin and S-isopetasin were also reported to possess spasmolytic activity, being S-petasin the most potent compound [289]. Nonspecific antispasmodic and antimuscarinic mechanisms have been associated to the relaxant effects of S-petasin and S-isopetasin [290]. The antimuscarinic effects of S-isopetasin have been mainly due to a block of tracheal muscarinic M3 receptors, instead of cardiac muscarinic M2 ones [291]. Moreover, an antagonism of L-type voltage-dependent Ca^{2+} channel activity by S-petasin in vascular smooth muscle cells was demonstrated, thus suggesting a possible interest in the management of hypertension [292]. Depressant effects on the cardiac contractile function, along with antihypertensive effects, have been reported for S-isopetasin too [293]. Among eremophilane sesquiterpenes, fukinone, 2-β-hydroxyfukinone and capsidiol were shown to suppress smooth muscle constriction induced by different agents, likely by inhibiting the Ca^{2+} influx [294, 295]. Myorelaxant effects of eremophilane sesquiterpenes can be usefully exploited to counteract the airway hyperresponsiveness induced by allergens. Indeed, S-petasin has been found able to suppress the increased levels of inflammatory cells (*e.g*.,

lymphocytes, neutrophils, eosinophils) and cytokines, induced by ovalbumin in a murine model of allergic asthma; moreover, it reversed the lowering of IgG2a in serum of treated mice and competitively inhibited the activity of phosphodiesterases (PDEs) 3 and 4 [296]. Similarly, inhibition of the antigen-induced degranulation of β-hexosamidase, LPS-induced iNOS expression and NO production by S-petasin were highlighted in mouse peritoneal macrophages, thus suggesting potential usefulness in the treatment of asthma [297]. On the other hand, the compound inhibited leukotriene synthesis in eosinophils and neutrophils, and the earlier signalling events initiated by G protein-coupled receptors in granulocytes [298, 299]. Although clinical trials have been performed to assess the efficacy of some petasin-based extracts as antiallergy treatments, the true contribution of the pure compounds remains to be clarified [299]. Among eremophilane sesquiterpenes, eremoxylarins A and B (from *Xylariaceous Endophytic* fungus YUA-026) were also revealed to possess immunosuppressive properties by inhibiting calcineurin (a protein phosphatase known to play a key role in the activation of the T-cells of the immune system) without affecting immunophilins [300]. This suggests a possible interest for the development of immunosuppressants and anti-allergic drugs [108]. Owing to the relaxing and anti-inflammatory effects, isolated petasins (and their isomers isopetasins) have been evaluated for the antimigraine effects [301].

Preclinical evidence has suggested that petasin and isopetasin can affect the release of calcitonin gene related peptide (CGRP), thus modulating its nociceptive effects. Moreover, an inhibitory effect by petasin and isopetasin on subtypes calcium conducting transient receptor potential channels TRPA1 and TRPV1 could be involved in the lowered CGRP levels [301]. Moreover, the activation of TRPA1 channels by isopetasin can induce the excitation of neuropeptide-containing nociceptors, with a marked heterologous neuronal desensitization [302]. Such analgesic effects along with the anti-inflammatory and vascular relaxant activities may contribute to the anti-migraine effects of petasins [303]. Petasin-based herbal extracts from *Petasites* spp. have been mostly studied as migraine preventive treatments: Petadolex®, a proprietary CO_2 extract from the root of *P. hybridus* (butterbur), containing ≥15% of a mixture of petasin, isopetasin, neopetasin, was shown to significantly reduce migraine attacks, especially after four months of treatment, in placebo-controlled double-blinded clinical investigations [304, 305]. Despite the recognized clinical efficacy of Petadolex® [304], the occurrence of some cases of liver injury, mainly ascribed to the presence of pyrrolizidine alkaloids, leads to the product withdrawn in Europe due to safety concerns, although the actual mechanisms of the adverse reactions and the role of butterbur extract and petasins remain to be clarified [306, 307]. Recent studies highlighted that petasins are subjected to an extended liver metabolism to petasols, and that the presence of other herbal phytochemicals can

change the metabolic pathway producing different metabolic derivatives: therefore, characterizing represents a key issue to predict the metabolic fate of petasins [308].

Safety and Toxicological Concerns of Sesquiterpenes

Besides the huge range of biological properties ascribed to sesquiterpenes, some safety concerns have been reported, especially for sesquiterpene lactones and eremophilanes. Conversely, caryophyllane sesquiterpenes have been shown to possess low toxicity, and so they are approved as food additives, fragrances, and as cosmetic ingredients [105]. Regarding sesquiterpene lactones, possible toxic effects, including alkylation of protein, nucleic acids, and glutathione, oxidative stress, antagonism of $GABA_A$ and glycine receptors, inhibition of SERCA (Sarco-Endoplasmic Reticulum Calcium ATPase) pumps, epigenetic machinery deregulation, and hypersensitivity induction, have been reported [309]. Contact sensitization and systemic allergic reactions have been also described in humans [310]. These reactions have been mainly ascribed to the ability of sesquiterpene lactones (*e.g.*, thapsigargin) to increase cytoplasmic calcium concentrations, as a consequence of SERCA pump inhibition, thus leading to extensive mast cell degranulation and histamine release [309]. Alkylating sesquiterpene lactones can also induce allergic contact dermatitis by reacting with SH protein residues, through their α-methylene-γ-lactone moiety (*e.g.*, parthenolide), thus forming a carrier-hapten complex (antigen) that is recognized by the immune system and elicits a cell-mediated, delayed type (type IV) hypersensitivity reaction [309].

Recently, several preclinical studies have shown that some sesquiterpenes (*e.g.*, zederone, germacrone) could exert liver toxicity, through the formation of reactive metabolites, with increased reactive oxygen species and impaired antioxidant defenses [311]. Moreover, liver injury has been associated with the use of *P. hybridus* extracts, characterized for the petasin content [306]. On the other hand, preclinical data did not highlight toxicity risks due to the use of petasins (or petasin-based herbal extracts) at therapeutic levels [306]. Considering that terpenes can enter the human body by oral absorption, penetration through the skin, or inhalation, leading to measurable blood concentrations [309], their toxicological investigations are an urgent need in order to ascertain the safety of their use.

CONCLUDING REMARKS

Natural sesquiterpenes are of great interest in pharmacological and medicinal chemistry research due to their unique chemical features and multifaceted bioactivities. Some compounds, such as parthenolide and artemisinin derivatives have highlighted pleiotropic anticancer effects, which have led to their clinical

evaluation; however, standardized methodologies and high-quality studies are needed to achieve convincing results. For other substances, despite their promising properties, some bioavailability and stability issues have limited their application in practice, thus suggesting the need for medicinal chemistry or pharmaceutical interventions in order to overcome these drawbacks, and effectively exploit their pharmacological power. Novel findings have also displayed a potential interest for some sesquiterpenes (*e.g*., β-caryophyllene, S-isopetasin) as antidiabetic and hypolipidemic agents, which could represent an important alternative strategy in the management of dysmetabolic diseases, although future deep investigations are needed. The wide diffusion in the nature of sesquiterpenes enables to approach their extraction by several sources, which include marine species and fungi, along with waste biomass to support pharmacological studies or hemisynthetic processes in a recycling and sustainable approach. Altogether, the collected evidence strengthens the interest for natural sesquiterpenes in the pharmacological, pharmaceutical and medicinal chemistry field.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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CHAPTER 2

From Δ^9-THC to Synthetic Cannabinoids: Multi-Faceted Therapeutic Agents and Versatile Scaffolds for Drug Discovery

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Abstract: *Cannabis sativa* L. has been used for millennia by humans for medicinal, ritual and recreational uses. Commonly known under its dried form (flowers and leaves) as marijuana, this plant produces hundreds of phytomolecules, including phytocannabinoids, terpenes and flavonoids. Over the past decades, it is most abundant and most therapeutically relevant component, (-)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC) has generated considerable interest due to its various therapeutic properties. Most of them result from the interaction with two G-protein coupled receptors named cannabinoid receptors (CB1 and CB2). This chapter gives a broad overview of the main structural investigations performed on the natural scaffold of Δ^9-THC in order to modulate the affinity for the cannabinoid receptors and, potentially, its therapeutic properties. The design of several synthetic cannabinoid derivatives will be presented, and their structure-activity relationships will be analysed.

Keywords: Cannabinoids, Cannabinoid Receptors, Structure-Activity Relationship, Synthesis, Δ^9-THC, Δ^8-THC, Therapeutic Application.

INTRODUCTION

HISTORY OF CANNABIS SATIVA L.

Cannabis sativa L. is considered a very unique plant due to its history, chemistry, pharmacology, toxicology, and deep social impact. *Cannabis sativa* L. belongs to the family of Cannabaceæ which includes only two genera (*Cannabis* and *Humulus*). The various subspecies of *C. sativa* L. identified so far mostly reflect the chemotype or geographical variants of a single taxonomic entity rather than distinct species [1]. It is one of the best characterized plant varieties with an

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inventory of at least 489 natural compounds identified so far [2], comprising many different chemical classes, such as mono- and sesquiterpenes, sugars, steroids, flavonoids, hydrocarbons, nitrogen compounds, and especially cannabinoids, which are terpenophenolic compounds. Marijuana, a drug derived from *Cannabis sativa* L., is an illicit substance made mainly of the dried flowers and leaves of the plant which is sold in the illegal market. Despite the prohibitions, marijuana is the most cultivated, trafficked, and consumed drug in the world. This versatile crop has been used for millennia by humans, not only for recreational or ritual purposes but also for medicinal uses. Whereas its medicinal and psychoactive properties were well known for thousands of years, the elucidation of the mechanisms of actions of cannabis was only established in the late 19th century. Indeed, in 1843 Sir William B. O'Shaughnessy, an Irish physician, was the first to report on the medical use of cannabis, noting that hemp: "*possesses, in small doses, an extraordinary power of stimulating the digestive organs, exciting the cerebral system, of acting also on the generative apparatus*" [3]. The report also noted the ability of hemp oil to alleviate pain and to reduce seizures in infants. Cannabinol **1** was the first compound isolated in 1896 [4] and was initially considered as the active constituent of cannabis. Its chemical structure was fully elucidated only in 1940 (Fig. (**1**) [5, 6] together with the isolation of several other non-cannabinoid natural products, including cannabidiol **2** (CBD) [7]. Finally, the active component of cannabis, (–)-Δ^9-tetrahydrocannabinol **3** (Δ^9-THC), was discovered in 1964 by Gaoni and Mechoulam, who reported its structure elucidation and its partial synthesis [8].

Biogenesis of Phytocannabinoids

More than 100 natural products were isolated from *Cannabis sativa* L. and characterized mostly in the 1960s and 1970s [2, 9 - 11]. These compounds have been divided according to their structure into different classes, including the two predominant Δ^9-THC **3** and CBD **2**, Cannabinol **1** (CBN), but also Δ^8-*trans*-tetrahydrocannabinol **4** (Δ^8-THC), Cannabigerol **5** (CBG) and Cannabichromene **6** (CBC) among others (Fig. **1**).

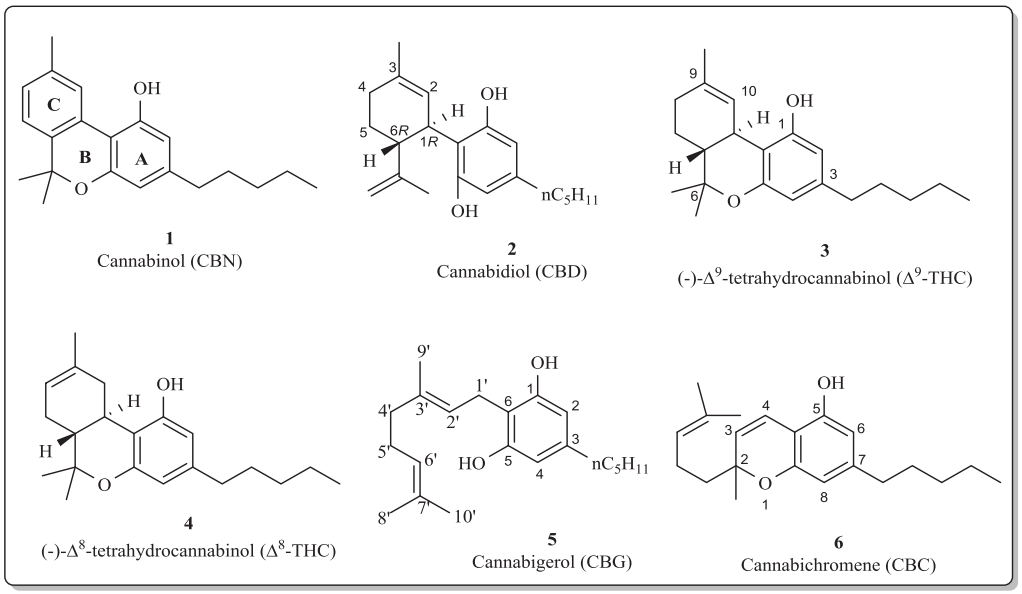
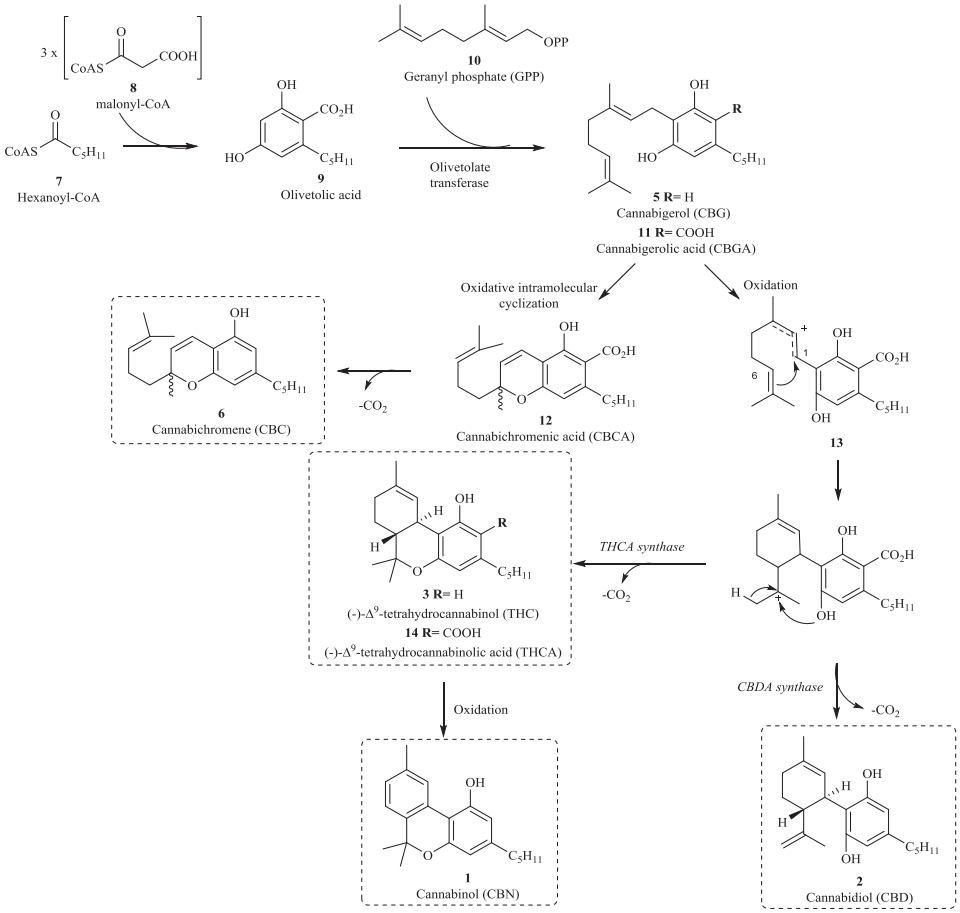


Fig. (1). Main phytocannabinoids isolated in *Cannabis sativa* L.

The biogenesis of phytocannabinoids is summarized in Scheme **(1)** [12, 13]. The natural precursor *n*-hexanoylCoA **7** is transformed into olivetolic acid **9** through the tri-fold addition of malonyl-acetate derived units **8**, followed by cyclization and aromatization. A specific prenyltransferase catalyzes the condensation of **9** and geranyl phosphate (GPP, **10**) [14 - 16] to afford cannabigerolic acid (CBGA, **11**) which gives, after decarboxylation, cannabigerol **5**. It is nowadays generally accepted that the decarboxylation step for CBGA **11** and all the other cannabinoids is non-enzymatic and occurs spontaneously during either the storage, the extraction or the purification of the compounds. The oxidative intramolecular cyclization of **11** leads to the formation of cannabichromenic acid (CBCA, **12**) and, by decarboxylation, cannabichromene **6**. Moreover, the oxidation of CBGA **11** is also leading to the formation of a link between C-1 and C-6 of the prenyl unit, affording cannabidiol **2**, the main constituent of the fiber-type (non-psychotropic) varieties of *C. sativa.* This stereospecific intramolecular cyclization occurs through the cationic intermediate **13** and is catalysed by *CBDA synthase* [17], which has been isolated and characterized [18]. Another enzyme, *THCA synthase*, promotes the attachment of the phenolic oxygen leading to the formation of the tricyclic system of tetrahydrocannabinolic acid (THCA) **14**. As mentioned above, its decarboxylated analog Δ^9-THC **3** is considered as an artifact since its concentration in extracts increases during storage, while simultaneously, the concentration of THCA **14** decreases. Numerous analogues sharing this terpenoid structural framework have been identified: Δ^8-THC **4** shows

isomerization of the double bond, whereas the derivative having the C-cycle completely aromatized corresponds to cannabinol **1**. Δ^9-THC and CBD have received the most attention in both basic science and clinical research, as they are the most abundant and the most therapeutically relevant components. CBD has not yet been approved by the US Food and Drug Administration (FDA), but clinical trials are underway exploring the use of CBD, branded as Epidiolex®, in the treatment of epilepsy and Dravet syndrome, a severe seizure disorder in children [19, 20].



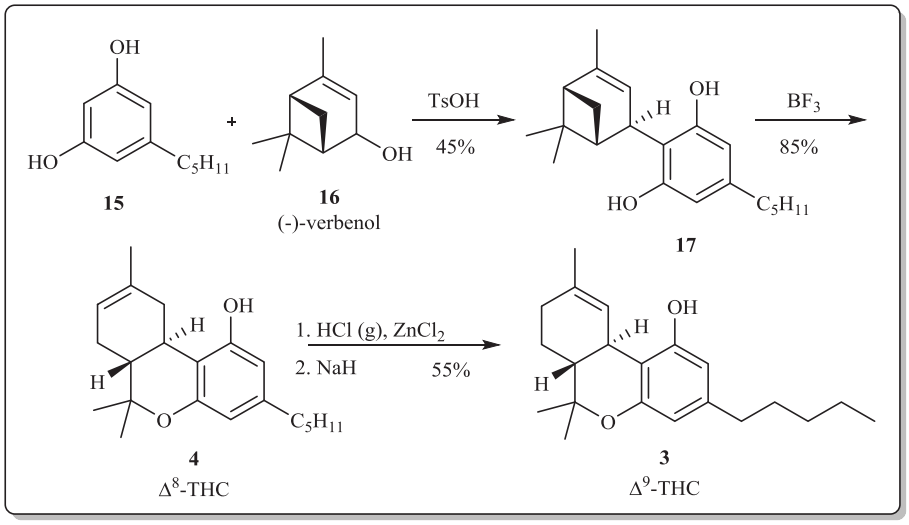
Scheme (1). Biogenesis of the main cannabinoids found in *Cannabis sativa*.

Total Synthesis of Δ^9-THC

The most prominent synthetic pathways to the Δ^9-THC scaffold were recently

reported and classified in two categories: starting from chiral pool terpenoids or concerted approaches [21]. The use of chiral pool materials typically avoids more complex asymmetric transformations and ensures control of the stereochemistry. Therefore, from a synthetic perspective, they could be preferred over asymmetric methods. The first stereoselective synthesis of Δ^8-THC **4** was reported in 1967 by Mechoulam, Braun and Gaoni [22] *via* a Friedel-Crafts alkylation of olivetol **15** with (-)-verbenol **16**, using p-toluenesulfonic acid or boron trifluoride as a catalyst to generate olivetylverbenyl **17** (**Scheme 2**). Repeated treatments with BF_3 afforded Δ^8-THC **4**, which was first chlorinated on the C-ring in order to perform a base-induced elimination which gave its isomer Δ^9-THC **3** [23].

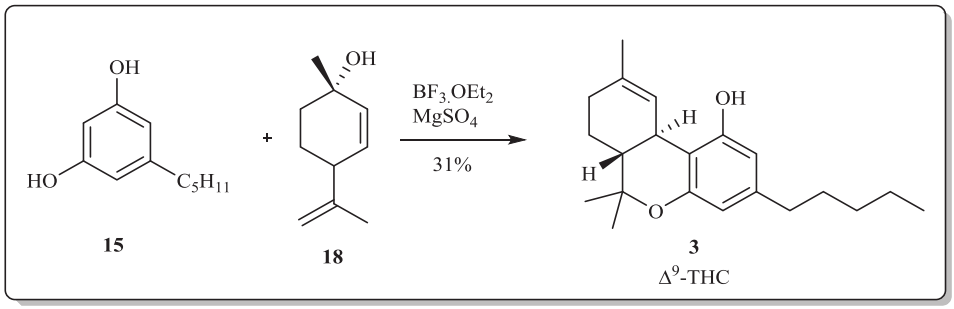
Direct synthesis of Δ^9-THC **3** was then described starting from *p*-mentha-2-8-dien-1-ol **18** in the presence of catalyic amounts of BF_3 and magnesium sulfate as drying agent **Scheme (3**) [24]. Besides the generation of bis-adducts and *iso*-THC derivatives, there was no Δ^8-THC formation observed in these conditions.



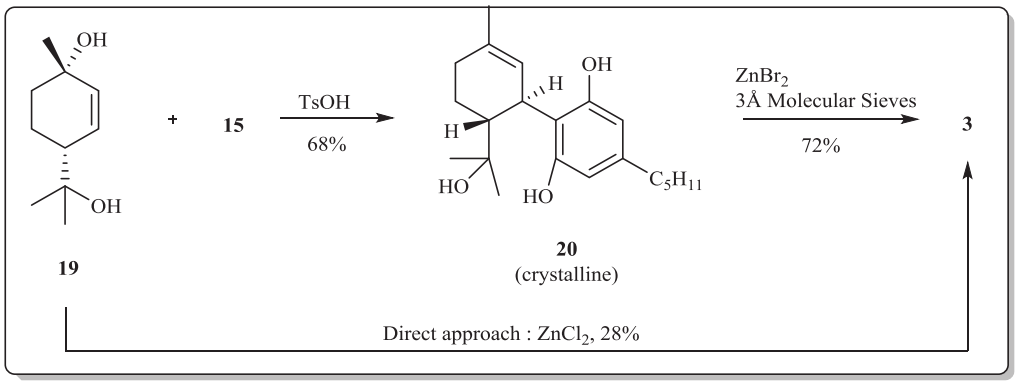
Scheme (2). Stereoselective synthesis of Δ^8-THC according to Mechoulam, Braun and Gaoni [22, 23].

p-Menth-2-ene-1,8-diol **19**, a structurally comparable starting material, was activated in a similar fashion using Brønsted or Lewis acid catalysis, as reported in **Scheme (4)** [25]. Conversions up to 51% were observed and Δ^9-THC **3** was isolated with 28% overall yields by using anhydrous $ZnBr_2$ instead of the earlier reported boron trifluoride etherate [22, 24]. Notably, with this zinc-based Lewis acid, the reactions were successfully conducted on multi-gram scale. Despite the

one-step procedures of both *p*-mentha-2,8-dien-1-ol **18** and *p*-menth-2-ene-1,8-diol **19**, these reactions produced a large variety of side-products which limits large-scale application [26]. The crystallisation of the intermediate (–)-*trans*-6-hydroxy-CBD **20** allowed a cleaner cyclisation reaction with $ZnBr_2$ to form Δ^9-THC **3**, leading to the isolation of the desired product in higher yields. Recently, a new synthetic route for the large-scale preparation of *p*-menth-2-ene-1,8-diol **19** was described [27]: this discovery may contribute to industrial application of **19** in the synthetic preparation of cannabinoids.



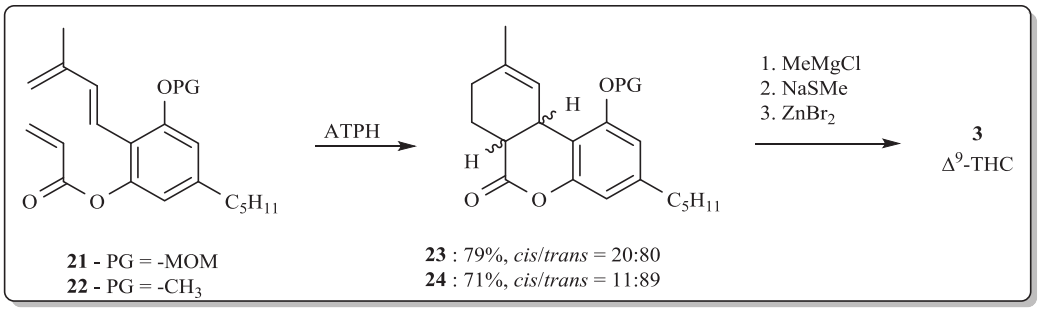
Scheme (3). Straightforward synthesis of Δ^9-THC from *p*-mentha-2,8-dien-1-ol **18** [24].



Scheme (4). Lewis acid-catalyzed synthesis of **3** from *p*-menth-2-ene-1,8-diol **19** [22, 24].

Alternatively to the above presented approaches based on the use of chiral starting materials, more elaborated synthetic pathways using asymmetric catalysts were investigated, affording high levels of enantioselectivity and the access to unnatural THC enantiomers [28]. In 2010, the synthesis of Δ^9-THC was achieved with a late stage *trans*-selective Diels-Alder cyclisation **Scheme (5**) [29]. The method was optimised by using aluminium tris(2,6-diphenylphenoxide) (ATPH) as a Lewis acid catalyst on olefins **21** and **22** [30], which afforded the isomers of

23 and **24** in moderate diastereoselectivity. Intermediate **24** was finally treated with methylmagnesium chloride and cyclized using $ZnBr_2$ to obtain Δ^9-THC **3** [31, 32].



Scheme (5). Synthesis of **3***via* a late-stage *trans*-selective Diels-Alder cyclization [31, 32].

Cannabinoid Receptors

Notwithstanding the well-established traditional medicinal uses of cannabis of Δ^9-THC, the mechanism of action of cannabinoids in humans remained a mystery until recently. The cannabinoid receptors (CB) remained elusive for 30 years after the discovery of Δ^9-THC. Two CB receptors have been identified and cloned to date: CB1 and CB2, which share 40% homology and the heptahelical structure of G-protein coupled receptors (GPCR) [33]. The activation of CB receptors promotes an intracellular cascade of signal pathways which results in the interaction with potassium and calcium channels and several kinases among the others. As shown in Fig. (**2**), either CB1 or CB2 promotes a dose-dependent decrease in cellular cyclic adenosine monophosphate (cAMP) levels and modulation of intracellular Ca^{2+} and K^+ levels [34]. The existence of other cannabinoid receptors has long been pursued, since a number of cannabinoid-like effects persist in CB1/CB2 knockout mice [35]. The recently identified GPR55 has been proposed as third cannabinoid receptor [36, 37] but its role in the pharmacological actions of Δ^9-THC and in the physiological effects of endogenous cannabinoids is still controversial.

Many of the psychoactive effects of Δ^9-THC appear to be mediated by CB1 receptors [38], while non-psychoactive cannabinoids (as CBD) have very low affinity both for CB1 and CB2. Δ^9-THC is the phytocannabinoid showing the highest affinity to CB receptors, with a $K_i \approx 40$ nM [13] for both CB1 and CB2. Δ^8-THC shows almost equivalent potency as Δ^9-THC [39], while all the cannabinoid acid analogues are free of central nervous system (CNS) activity [40]. Noteworthy, CB2 receptors are highly expressed in some cells of the immune system and are believed to play a role in the immune cell function, thus providing a rationale to the immunomodulatory properties of Δ^9-THC [41].

Moreover, CB2 receptor is suspected to be involved in neuroinflammation, atherosclerosis, and bone remodelling [42]. The localization of both CB1 and CB2 on adipocytes, where their activation appears to stimulate lipogenesis, is particularly interesting and may have a clinical utility in the treatment of some forms of anorexia [43]. Interaction with CB receptors has been unambiguously associated to a number of pharmacological effects, the most important being: 1) *psychotropic effects* (euphoria), 2) *antiemetic effect*, 3) *analgesic effect*, 4) *immunomodulation,* 5) *motor effects* (hypokinesia, ataxia, antispasticity). Most likely, the most important potential therapeutic effect associated to the interaction with CB receptors is the analgesic effect, due to the role of CB1 receptors in the transmission of nociceptive information in several key tissues. Δ^9-THC has been estimated to be as potent as morphine in blocking nociceptive stimuli in many animal models [44]; moreover, it can synergistically act with opioid-receptor agonists. Interaction of Δ^9-THC with CB1 receptors on presynaptic nerve terminals in the brain results in the euphoric feelings associated with *Cannabis* use. This effect could be beneficial in the treatment of depression, however further studies are required to clarify the role of the cannabinoid system in this pathology. Other effects of Δ^9-THC in the CNS are ascribable to the presence of cannabinoid receptors in other areas: impairment of cognition and memory (hippocampus) [45]; involuntary movements and partial loss of motor control (basal ganglia and cerebellum) [46]. Since CB1 receptors are not present in the brain region responsible for respiratory and cardiovascular functions, cannabinoid consumption cannot be associated to an increased risk of respiratory or cardiovascular failures, as happens for opiates. The location of CB1 receptors in cholinergic nerve terminals of the gastrointestinal tract accounts for the THC-induced inhibition of digestive-tract motility [47], while the presence of CB1 receptors in the brainstem is responsible of the THC-induced inhibition of emesis [48]. The antiemetic effect of Δ^9-THC has been well established and proposed for treatment of chemotherapy-induced emesis [49] leading to the approval in 2006 of Nabilone (Cesamet®) by FDA for the treatment of the emesis caused by cancer chemotherapy [50]. Moreover, many studies have reported that Δ^9-THC has a stimulatory effect on appetite and food intake, which can be co-adjuvant in cancer anorexia [51]. As a matter of fact, synthetic Δ^9-THC has been marketed as Dronabinol (Marinol®) for the treatment of anorexia in Acquired Immune Deficiency Syndrome (AIDS) patients and chemotherapy-induced nausea and vomiting. This effect could be mediated both by CB1 receptors present in the CNS or in nerve terminals and adipocytes. Table **1** summarizes the characteristics of CB1 and CB2 together with some of their more relevant physiological effects.

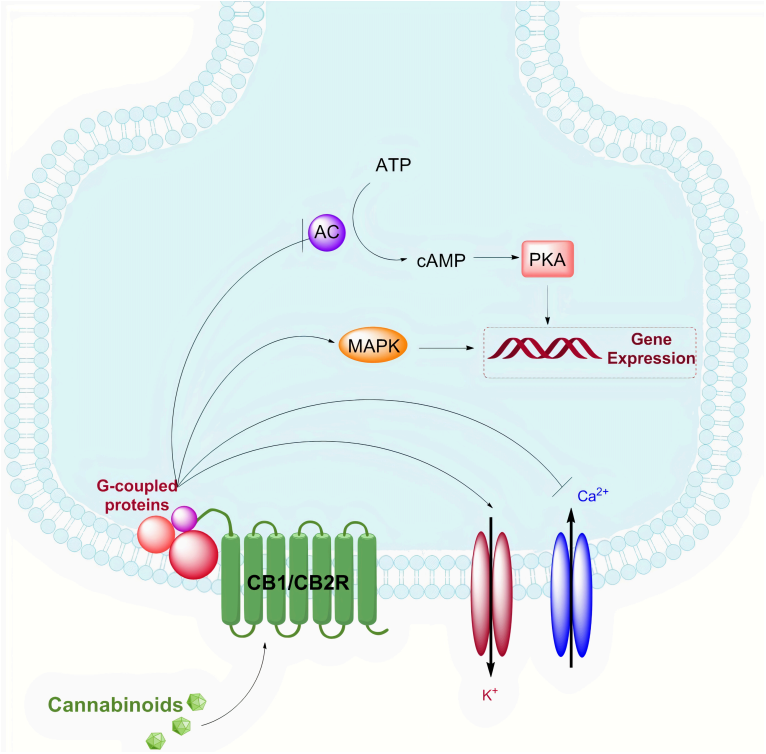


Fig. (2). The intracellular signalling cascade activated by cannabinoid receptors – AC: Adenyl cyclase; ATP: Adenosine triphosphate; cAMP: Cyclic adenosine monophosphate; MAPK: Mitogen-activated protein kinases; PKA: Protein kinase A.

Table 1. The main properties of CB1/CB2 receptors and the effects which they mediate.

	CB1	CB2
Type of receptor	GPCR (G_i-G_o)-protein coupled	
Localization	CNS, adipocytes, kidney, lung, liver	Immune system cells, spleen, CNS, osteo-cells, adipose tissue
Inducibility	Low inducibility	High inducibility
Mediated effects	Psychotropy Antiemesis Analgesy	Immunomodulation Anti-inflammatory

Endocannabinoid System

The discovery of the CB receptors was driven by the desire to understand the pharmacological mechanism of cannabis, underlining that both CB receptors are involved in the extensive signaling pathway known as the endocannabinoid

system. As a matter of fact, the presence of CB GPCRs suggested the existence of endogenous ligands (Fig. **3**) and, since the phytocannabinoids are highly lipophilic, it was assumed that these ligands would likely be lipids.

The identification of anandamide (AEA) **25** (from the Sanskrit word *ananda*, meaning "delight, bliss") by the Mechoulam group in 1992 confirmed the existence of an endogenous ligand for the CB receptors [52, 53]. AEA binding to CB receptors produces similar effects to that of the exogenous phytocannabinoids, inducing hypothermia, analgesia, catalepsy, and appetite stimulation [54, 55]. Furthermore, its tissue distribution is highly similar to that of CB1: the highest levels of AEA were found in the hippocampus and cerebellum and to a lesser degree in the spleen and heart tissue [56]. Soon after the discovery of AEA, the identification of several other endocannabinoids was achieved: 2-arachidonoylglycerol **26** (2-AG), 2-arachidonoyl ethanolamine **27** and 2-arachidonoyl glycerol ether **28** Fig. (**3**) [57 - 60]. Although initially considered an insignificant component of the endocannabinoid system, the role of 2-AG **26** has evolved to that of one of the more important signaling molecules in the brain. Indeed, 2-AG has been linked to the modulation of feeding, hypotension, neuroprotection, cell proliferation, and other interesting central physiological processes [61 - 64]. Due to the highly hydrophobic nature of endocannabinoids, it was initially thought that they were synthesized in the same cells in which receptor binding occurs. However, it has been later suggested that AEA and the other endocannabinoids can travel across the synaptic membrane by either passive diffusion or active transport, although a specific mechanism has yet to be resolved [65 - 68]. Endocannabinoid signalling typically occurs in retrograde fashion, from post- to presynaptic neurons, causing a variety of downstream effects, as summarised in Fig. (**4)**

Once released, endocannabinoids are rapidly deactivated by two enzymes: *Fatty Acid Amide Hydrolase 1* (FAAH) and *Monoacylglycerol Lipase* (MAGL) [69]. The distribution of these enzymes provided additional evidence to the retrograde signaling mechanism, since FAAH is located postsynaptically and MAGL presynaptically. The function of CB2 is less well defined than CB1 in the system and its role seems to be limited to that of an immunomodulatory mediator. CB2 also decreases the production of cAMP but its inhibitory effect on Ca^{2+} and K^{+} channels is limited with respect to CB1 [70]. For a more in-depth discussion of endocannabinoid signaling, see the following references [68, 71 - 75].

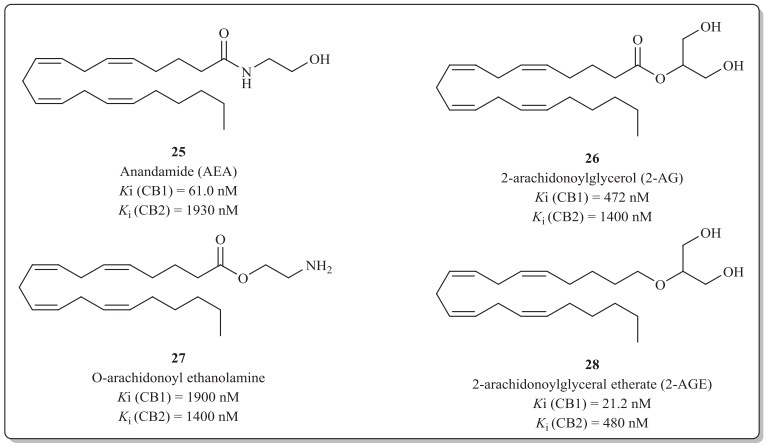


Fig. (3). The main endocannabinoids and their chemical structures.

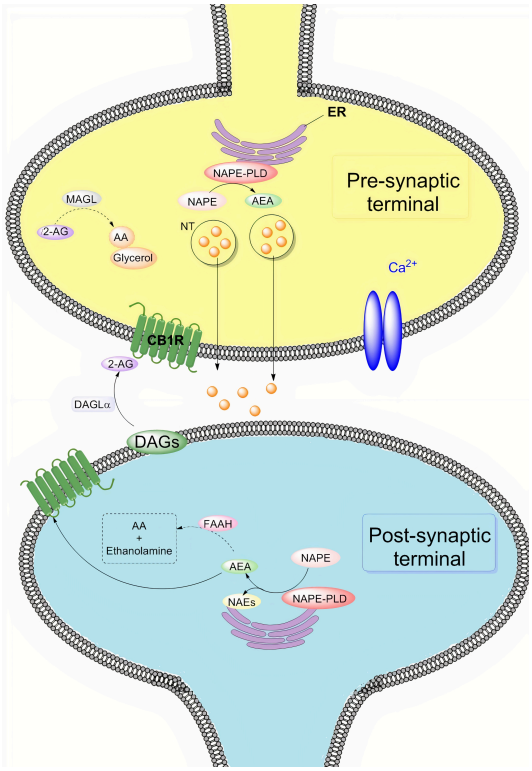


Fig. (4). The retrograde synaptic signalling pathway mediated by endocannabinoid – Dashed arrows indicate inactivation of endocannabinoids – [2-AG: 2-arachidonolglycerol; AA: arachidonic acid; AEA: Anandamide; DAGs: diacylglycerols; DAGL: diacylglycerol lipase- ER, endoplasmic reticulum; MAGL: monoacylglycerol lipase; NAPE: *N*-acyl-phosphatidylethanolamine; NAPE-PLD: NAPE-specific phospholipase-D; NT: neurotransmitter].

Synthetic Cannabinoids

The term "cannabinoids" was first used to refer to the typical C_{21} group of compounds found in *Cannabis sativa.* In the last three decades, this term has been modified in agreement to the rules of pharmacological research to describe: "compounds showing affinity to the two GPCRs known as cannabinoid receptors CB1 and CB2, independently from any structural or biogenetic relationship with the cannabis meroterpenoids" [76]. From the chemical point of view, "cannabinoids" comprise a broad variety of compounds and have been classified into different categories according to their different structures [70]. In this chapter only the derivatives of Δ^9-THC, its isomers and its structurally related synthetic analogues defined as "classical cannabinoids" will be analysed.

INFERRING THE STRUCTURE-ACTIVITY RELATIONSHIP: CLASSICAL CANNABINOIDS DERIVED FROM Δ^9-THC AND Δ^8-THC

The terpenoid structure of Δ^9-THC **3** is characterized by a tricycle in which a central pyran is fused to a benzene ring and a cyclohexene through a *trans* junction Fig. (**5**)., resulting in a slightly V-shaped arrangement of the molecule [77, 78]. As a matter of fact, the C-ring assumes a peculiar flattened chair conformation that directs the C-9 methyl group toward the aromatic portion [79]. According to the more commonly used dibenzopyran numbering, the position of the four alkyl pendants can be identified as: a C-3 *n*-pentyl chain, two geminal methyl groups in C-6 and a CH_3 on the double bond in C-9 [80]. Since Δ^8–THC **4** (Fig. **5**), possessing the double bond between C-8 and C-9, is almost equiactive to the Δ^9-isomer, it was often used as a lead compound for the development of new cannabinoid agents.

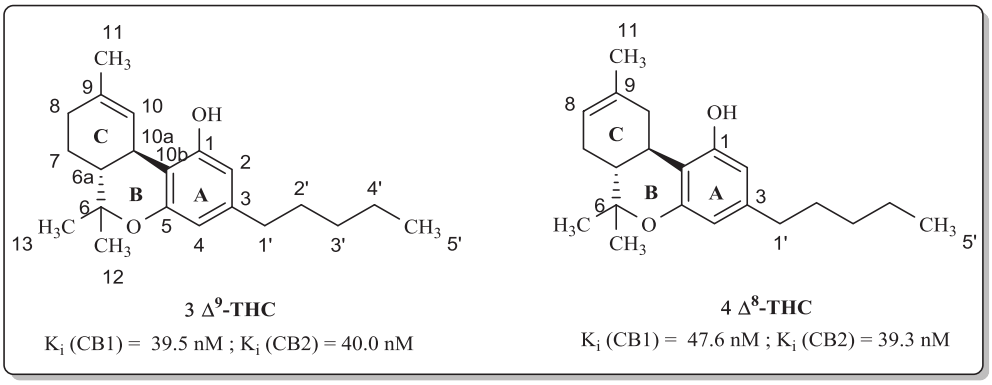


Fig. (5). The dibenzopyran numbering system of Δ^9- and Δ^8-THC and their K_i values on CB receptors.

Together with the OH group in C-1 and the three central cycles themselves, the alkyl substituents constituted the first points prone to chemical modifications to investigate the structure-activity relationship (SAR) of Δ^9-THC. By focusing separately on the key-portions of the molecule, the most important synthetic manipulations which have been performed on Δ^9–THC to modulate its affinity for the CB receptors will be analysed.

Tricycle Scaffold

Several subsequent modifications have been carried out on the central meroterpenoid scaffold: some reference derivatives are reported in Fig. (**6**). The pyran ring was expanded (**29**) [81], substituted with a piperidine (**30**) [82] or even removed (**31**) [83] to generate the so-called "AC-bicycle class of non-classical cannabinoids derivatives" [84]. The double bond can be moved into a different position of the C-cycle [85], saturated or the ring itself could be substituted with a heterocycle like piperidine (**32**) [86] without activity impairment. However, the absolute configuration of the two stereogenic centers C-6a and C-10a is essential since the other stereoisomers are inactive [87]. When the C-ring has been completely saturated, the configuration of the C-9 substituent became crucial for the activity of the cannabinoid derivatives and can affect the conformation of the C-cycle [79, 88]. Most of the modifications to the meroterpenoid portion of the molecule were done before the CB discovery: therefore, the tests were performed on animal models, making the values of the *in vitro* assays not available.

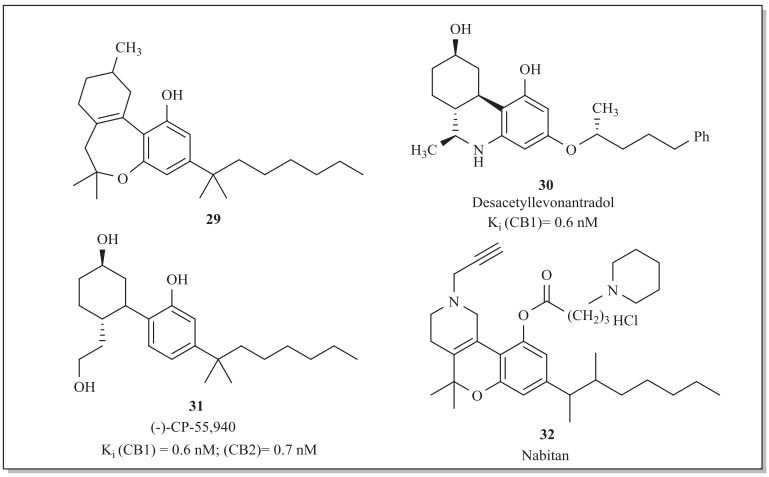


Fig. (6). Reference derivatives with structural modifications on the meroterpenoid scaffold.

The outline of the important SAR for this portion of the molecule is reported in Fig. (**7**)

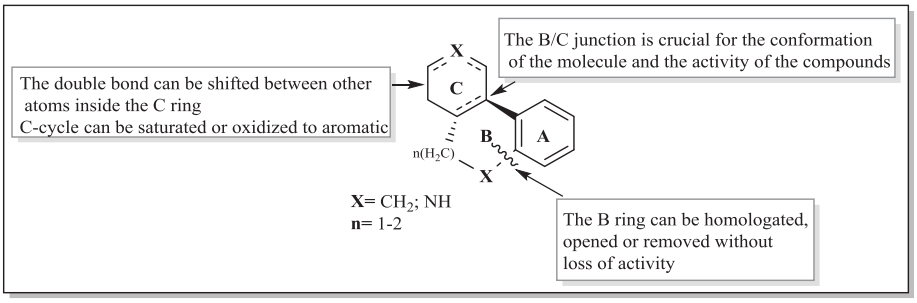


Fig. (7). Summary of the SAR related to the tricycle scaffold.

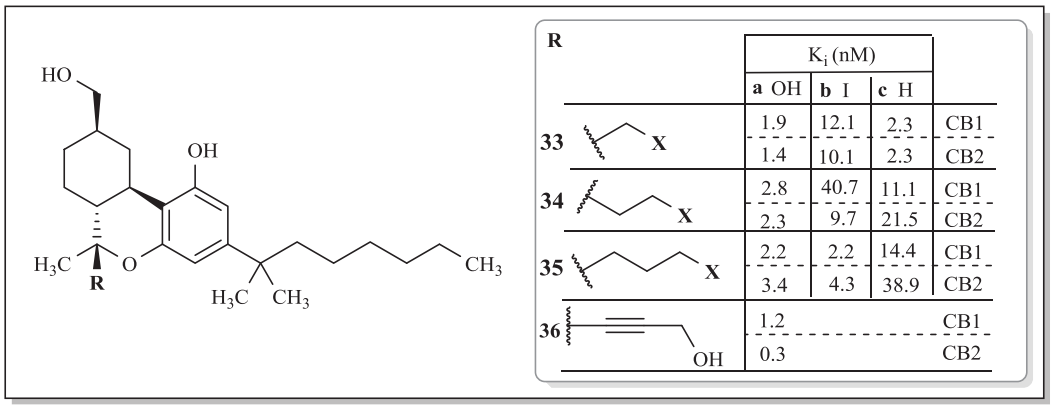


Fig. (8). Modifications to C-6 geminal dimethyl function.

The summary of the important SAR for this portion of the molecule is reported in Fig. (**9**).

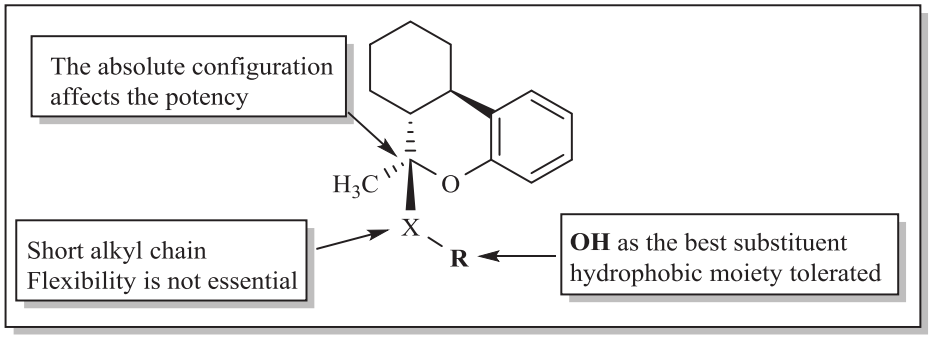


Fig. (9). Summary of the SAR related to the C-6 position.

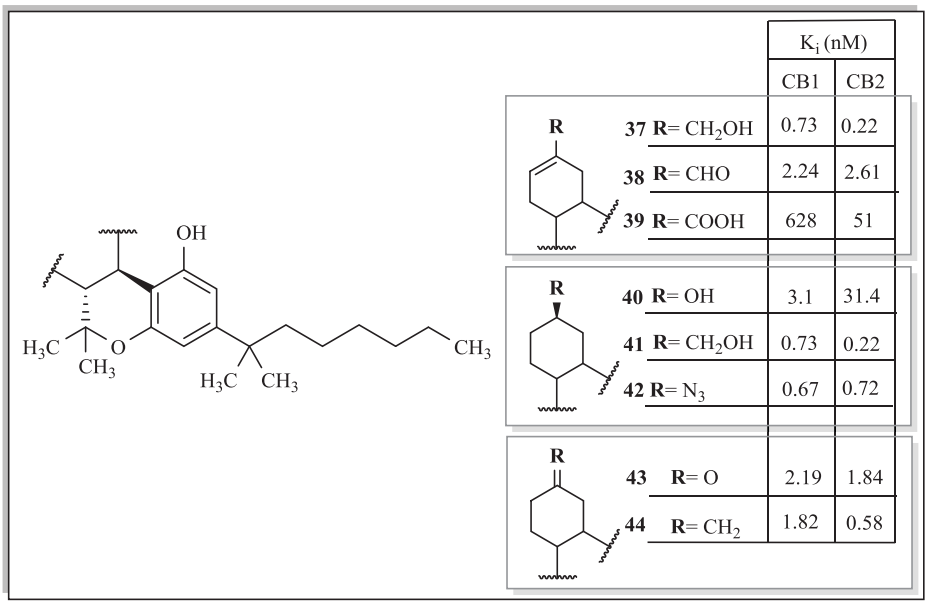
Geminal Dimethyl Function

The development of the non-classical cannabinoid CP-55,940 (**31** in Fig. (**6**)) in 1974 from the investigation performed by Pfizer on the series of AC-bicycle derivatives [89], revealed that by inserting a polar hydroxyl group on the corresponding C-6 of Δ^9-THC, it was possible to increase the potency of the compounds on the CB receptors. The so-called "Southern Aliphatic Hydroxyl" (**SAH**) [90] indeed became an important pharmacophore to develop new classical or non-classical cannabinoids [88]. The SAH has often been varied together with other key-positions of Δ^9–THC, making difficult to interpret how the single SAH modification affected the compounds affinity. By keeping constant one methyl group on C-6 and progressively elongating the hydroxyalkyl chain, Tius *et al.* [91, 92] determined that the length was not affecting the potency of the compounds either on CB1 and CB2 (**33a**-**35a** in Fig. (**8**)). The substitution of the terminal hydroxyl with iodine (**33b**-**35b** in Fig. (**8**)) decreased slightly the K_i values, while a terminal CH_3 was keeping the potency constant (**33c**-**35c** in Fig. (**8**)) [93]. The flexibility of the alkyl chain seemed not to be crucial since derivative **8**, bearing a more rigid alkyne spacer, showed also low nM K_i values on both the cannabinoid receptors [94].

Methyl Group in 9

As shown above for other portions of Δ^9–THC, the C-9 pendant has also been modified in order to investigate its effect on activity. It was demonstrated that the presence of the natural occurring CH_3 group is not fundamental since the desmethyl derivative possessed cannabinoid activity in animal model [95, 96]. The introduction on the C-11 position of a hydroxyl moiety remarkably enhanced the potency of the cannabinoid derivatives on both CB1 and CB2. This modification has been studied since the correspondent 9-OH derivative of Δ^9–THC, which represents one of its major metabolites, was almost equipotent to the parent compound in animal assays [97, 98]. Therefore, in analogy with already done for the C-6 position, this portion of the molecule has been defined as "Northern Aliphatic Hydroxyl" (**NAH**) [90], underlining the importance of this pharmacophore in improving the affinity of synthetic cannabinoids for CB1 and CB2. Taking advantage of the NAH, several C-9 hydroxy or hydroxymethyl derivatives, structurally related to Δ^8–THC, have been prepared, such as the low nanomolar active derivative **37** in Fig. (**10**) [99]. Based on the evidence that two of the C-11 oxidized metabolites of Δ^9–THC maintained weak cannabinoid activities, the insertion in C-9 of an aldehyde function (**38**) [100] or a carboxylic acid moiety (**39**) [101] was investigated. While the aldehyde derivative **38** showed a K_i value in the low nanomolar range, the insertion of the COOH group was detrimental for the potency. Nevertheless, compound **39**, named Ajulemic Acid

(Resunab®), is currently undergoing clinical trials for the treatment of systemic sclerosis [102]. When the double bond of the C-ring was saturated, two types of isomers could be generated with respect to the relative configuration of the substituent at the C-9 position: 9α- and 9β-epimers [84]. The β-conformer, with the C-9 pendant in the equatorial position (as verified for compounds **40**-**42** in Fig. (**10**)), showed to be more potent than the correspondent α-derivative, even though both of them possessed cannabinoid activity [103 - 105]. The explanation for this difference can be attributed to the presence of a critical area in the CB receptor active site, located at the top of the C-ring, which should not be occupied in order to avoid detrimental effects from steric hindrance [79, 106]. A severe potency increment was also evidenced when the C-9 was oxidized to ketone group. The most important compound bearing this modification is Nabilone (Cesamet®) **43**, the only synthetic cannabinoid approved by FDA for therapeutic applications [50]. Therefore, it can be speculated that functional groups capable of accepting hydrogen bonds could be beneficial in this portion of the molecule. As a matter of fact, the affinity value of the C-9 azido derivative **42**, member of a series of highly active photoactivatable probes for cannabinoid receptors, is also able to accept hydrogen bonds and seems to support this hypothesis [107, 108]. However, the ability to accept an H-bond does not seem to be a crucial property of the C-9 substituents since by shifting the double bond between the C-9 and C-11 atoms (**44**), Gareau *et al.* [100] obtained a 10-fold increment in potency on both CB1 and CB2 receptors with respect to the parent compound Δ^8–THC.



		K_i (nM)	
		CB1	CB2
37	**R**= CH_2OH	0.73	0.22
38	**R**= CHO	2.24	2.61
39	**R**= COOH	628	51
40	**R**= OH	3.1	31.4
41	**R**= CH_2OH	0.73	0.22
42	**R**= N_3	0.67	0.72
43	**R**= O	2.19	1.84
44	**R**= CH_2	1.82	0.58

Fig. (10). Chemical modifications on the C-9 position.

The summary of the important SAR regarding the C-9 substituents is reported in Fig. (**11**).

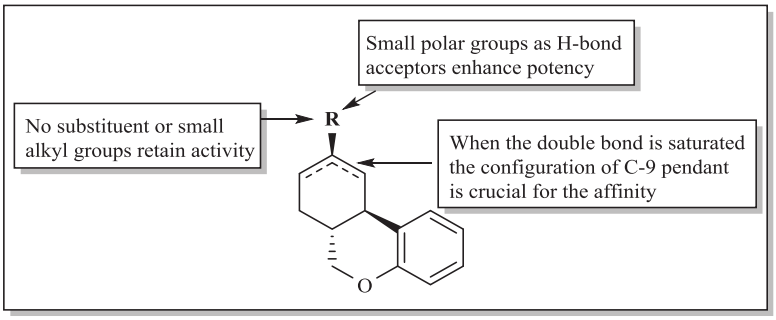


Fig. (11). Outline of the SAR on the C-9 position.

C-1 Hydroxyl Group

From the very first SAR investigation studies, the phenolic hydroxyl moiety has been considered as an essential structural feature for the pharmacological activity of cannabinoids on CB1. As a matter of fact, the **A** and **B** series of 1-deoxy-Δ^8-THC derivatives, reported in Fig. (**12**), showed a drastic loss of affinity for this receptor which confirmed the crucial role of this moiety for the interaction with the receptor [109 - 111]. Nonetheless, the removal of the hydroxyl group conferred at the same time modest to significant selectivity for CB2 to these compounds [112]. Indeed, the deoxy-Δ^8-THC analogue **45** showed no affinity for the CB1 receptor while maintaining almost the same potency of the parent derivative on CB2 (300-fold more potent on CB2 than CB1). Interestingly, by elongating the alkyl side-chain and branching two CH_3 on the C-1' position, affinity for CB1 could be restored, as shown by **46** and **47** [99]. The docking studies, performed on compound **46**, indicated that the orientation of this compound in CB1 active site would have to be inverted relative to that of Δ^9-THC in order to account for the same receptor affinity. In this inverted orientation, the pyran oxygen could form a hydrogen bond interaction with the residue of Lys192, thus being beneficial for the binding to the receptor. At the same time, the longer dimethylheptyl side-chain of **46** can be able to reach a side hydrophobic pocket formed by Val351 and Ile354 which can further stabilize the interaction [113]. Derivative **46** has further been modified, taking advantage of the NAH pharmacophore and obtaining compound **48** which, as expected, showed a considerably higher affinity for both cannabinoid receptors (Fig. **12**) [99].

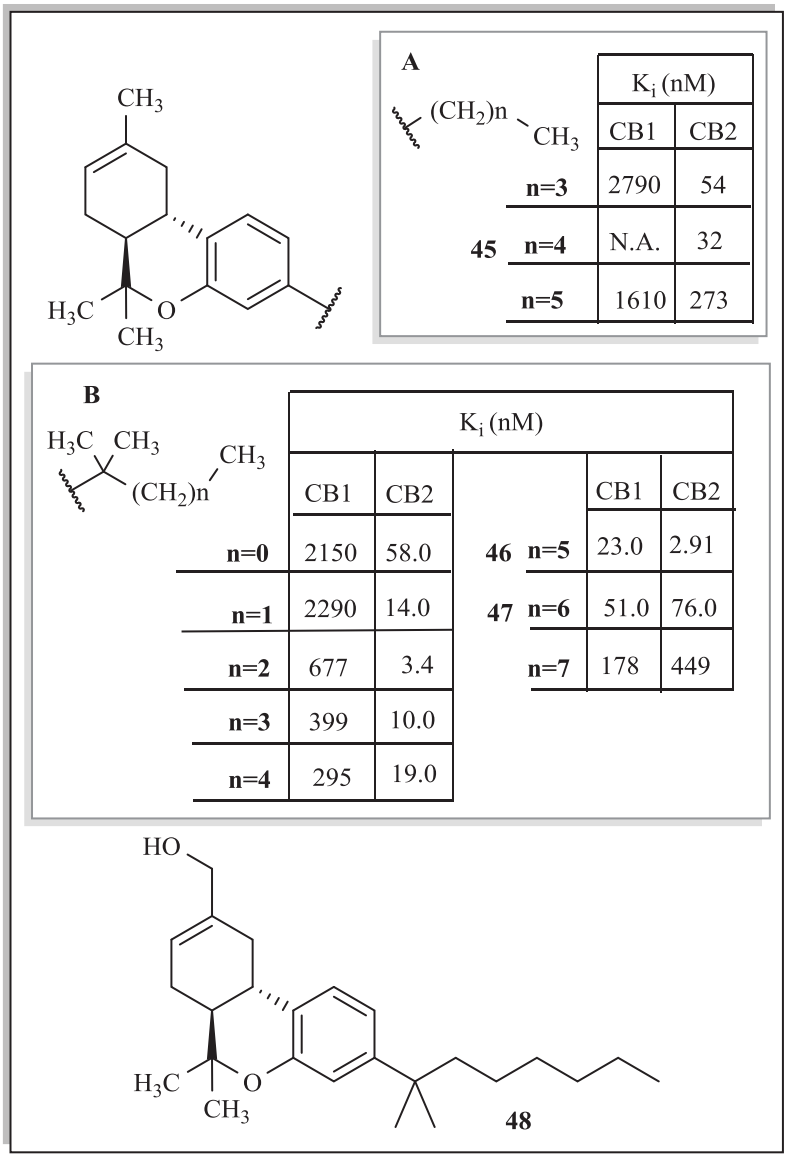


Fig. (12). Chemical modifications of the C-1 hydroxyl group.

The role played by the C-1 hydroxyl function for the formation of a crucial hydrogen bond interaction with CB1 was further confirmed by the affinity values shown by the 1-methoxy series of compounds in Fig. (**9**) [24]. As a matter of fact, the removal of the protic OH moiety led to a complete loss of activity on CB1 for compounds **49** and **50**, while the insertion of the NAH function (carbonyl group

of derivative **51**) maintained a μM K_i value on this receptor. As already verified for the above reported series of 1-deoxy-Δ^8-THC derivatives, the conversion of the phenolic hydroxyl to a methoxy group resulted in approximately 800- and 1000-fold selectivity for the CB2 receptor over CB1 for **49** and **50** respectively, whereas compound **51** showed 5-fold selectivity for CB2 Fig. (**13**).

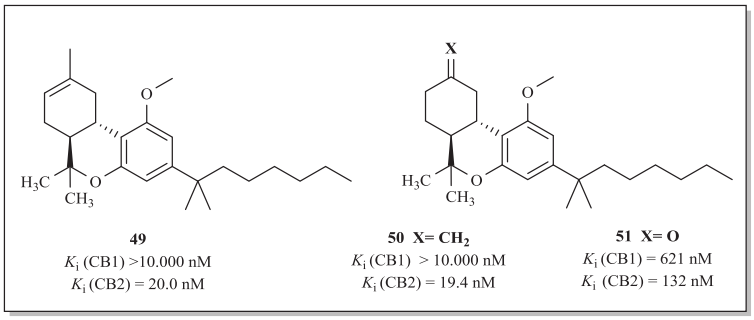
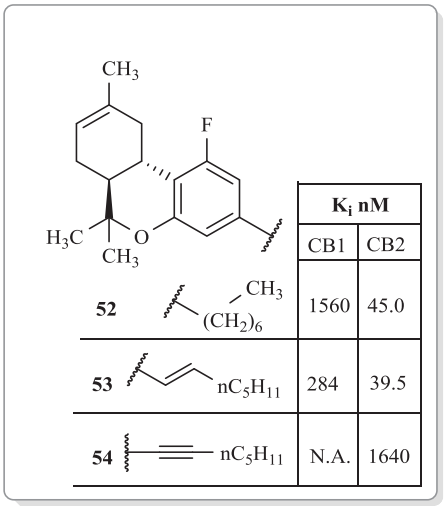


Fig. (13). C-1 methoxy-derivatives (**49-51**).

The substitution of the 1-OH moiety with the strong electron-withdrawing fluorine led again to a severe decrement of potency on CB1. The 1-fluorinated analogues **52-54** in Fig. (**14**) showed little or no activity on this receptor [114]. Interestingly, only a slight selectivity for CB2 over CB1 has been evidenced for these compounds. Probably, the pronounced electronegativity of F drained the delocalized electron cloud of benzene and, as a consequence, their binding interactions with the two cannabinoid receptor subtypes.



		K_i nM	
		CB1	CB2
52	CH_3 / $(CH_2)_6$	1560	45.0
53	nC_5H_{11}	284	39.5
54	nC_5H_{11}	N.A.	1640

Fig. (14). C-1 fluorinated derivatives decorated with various C-3 substituents. N.A.: not active.

Compounds **55** and **56** in Fig. (**15**), analogues of Δ^8- and Δ^9-THC respectively, were generated by incorporating the 1-OH group into a pyran ring [115]. These compounds were both tested before the discovery of the CB receptors, making *in vitro* analysis unavailable since they have not been determined since then. However, intraperitoneal injection of these derivatives in animal models highlighted that **56** possessed a similar potency to Δ^8-THC, whereas almost no activity was detected for **55**. As a matter of fact, the rigid tetracyclic structure of **55** forced the terpenoid scaffold into an almost flat conformation, unfavourable for the binding to both CB receptors. On the contrary, the additional pyran ring in compound **56** did not affect the non-planar shape of the central scaffold. Unfortunately, since the K_i values on the CB receptors are not available, it is not possible to understand which of the two receptor subtypes was producing the effects evidenced in the animal assays.

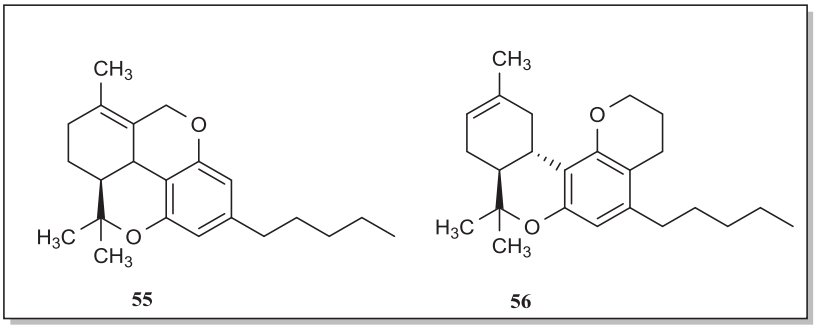


Fig. (15). Δ^8- and Δ^9-THC tetracycle analogues incorporating the 1-OH into a pyran ring.

The summary of the most important SAR for the 1-OH moiety of the molecule mentioned above is reported in Fig. (**16**).

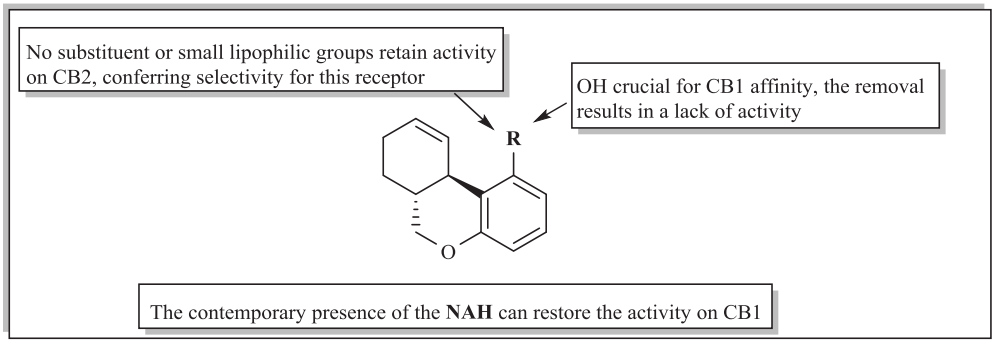


Fig. (16). Summary of the SAR on the C-1 hydroxyl group.

C-3 alkyl chain

The C-3 *n*-pentyl chain represents the most widely modified portion of the Δ^9–THC structure. A remarkable amount of synthetic cannabinoids has been generated by varying the C-3 pendant and the modifications performed in this position clearly had the largest influence on the binding affinity to both CB receptors [13]. The methylene chain was shortened [116] and elongated [117] Fig. (**17A**). in order to determine the best length, revealing that at least 3 carbons are necessary to maintain a good cannabinoid activity, while a potency increment can be achieved by extending the chain to 6-8 carbons (compounds **57**-**59** in Fig. (**17A**).

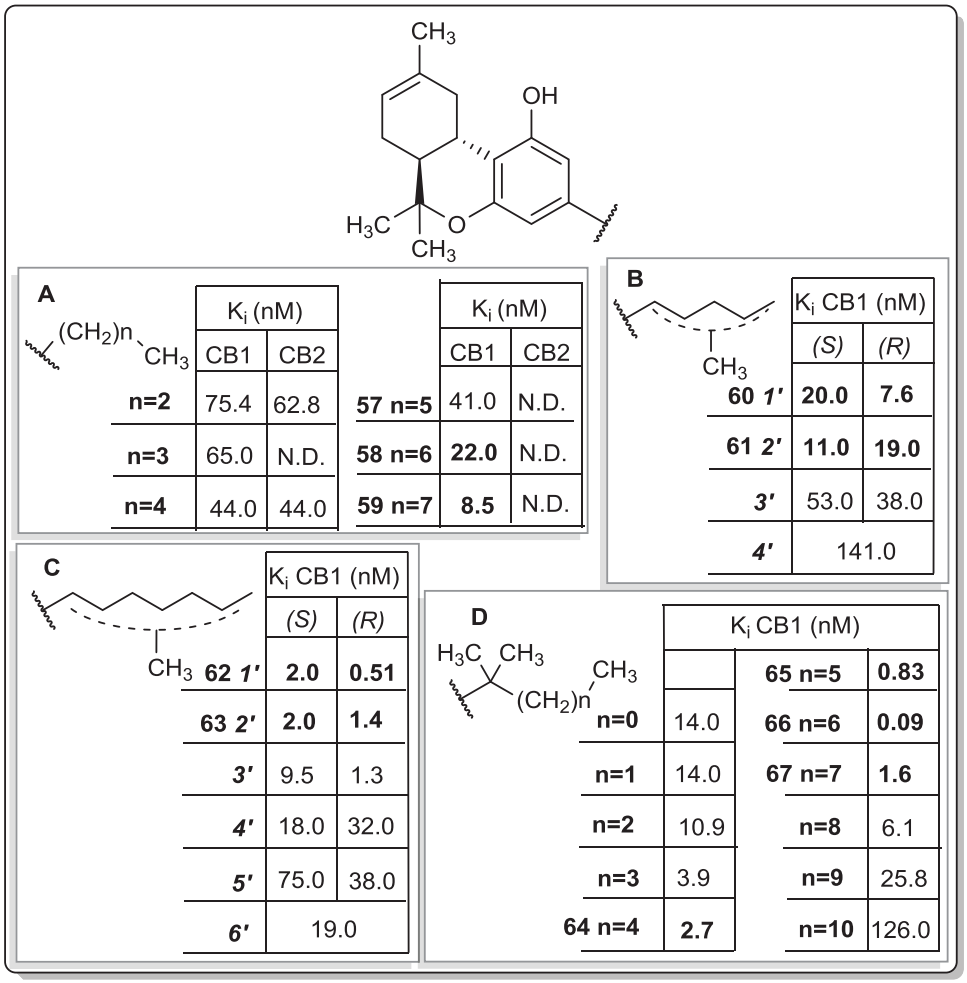


Fig. (17). Chemical modifications to the C-3 alkyl chain.

By branching small non-polar substituents like the methyl group on the C-3 position, the activity on both CB receptors can be drastically improved. As shown by the K_i values of compounds **60**, **61** and **62**, **63** in Fig. (**17A** and **17B**) [118, 119], the best results were obtained by inserting them close to the phenol ring on C-1' and C-2'. Interestingly, the addition of a second CH_3 in C-1' further improved the affinity of the compounds to CB1 receptor, thus making the derivatives bearing 5-8 carbon atoms in the C-3 alkyl chain the most potent (**64**-**67** in Fig. **17D**) [120]. From the analysis of several other studies, it became evident that the geminal C-1',1'-dimethyl function was one of the most extensively utilized C-3 pendant [13], but the insertion in C-1' of bulkier substituents has also been investigated.

As a matter of fact, several products belonging to the series of C-1'-spiro derivatives reported in Fig. (**18**). showed K_i values in the low nanomolar range [121 - 125]. The activity increment was explained by the presence of a subsite in the active site of both CB1 and CB2 receptors at the level of the benzylic side chain carbon that, if occupied, could drive to a tighter interaction among the cannabinoid agent and the receptors [123, 124]. Rings of different size and properties were investigated and all of them appeared to be tolerated inside the pocket. The cycloalkane derivatives **68**-**70** in Fig. (**18**) showed almost the same K_i values on CB1, while the cyclohexane derivative **71** was 10-fold less potent than the other analogues [121 - 123]. Interestingly, moving from the lipophilic cyclopentane derivative **70** to the polar non-protic 5-termed derivatives **72** the potency was retained, meaning that more polar and hydrogen-bond acceptor rings were tolerated too. On the contrary, the insertion of a secondary amine, as verified for the pyrrolidine substituted compound **73** [125], led to a complete loss of activity on both CB receptors. The methylation of the endocyclic nitrogen of **73** to tertiary amine restored the cannabinoid activity on both CB1 and CB2, suggesting that a protic function is not tolerated (derivative **74** in Fig. (**18**).

The insertion of extremely bulky lipophilic pendants directly on the phenyl ring incremented in some instances the activity [126 - 129]. The bornyl derivative **75** in Fig. (**19**) showed low nanomolar K_i values on both cannabinoid receptors [127], while its isomer **76** was almost 10-fold less active on CB1. Therefore, the cavity of the CB receptors could be able to hold hulking moieties and, by modifying the terpenoid scaffold/substituents connection, the activity on the receptors could be modulated. Indeed, a slight difference in potency was obtained by linking the adamantyl residue to different carbon atoms in the series of derivatives **77**-**80** in Fig. (**19**) [126]. Aromatic pendants could also lead to activity increment and, by combining a *p*-substituted phenol moiety with the C-1',1'-geminal dimethyl function, it was possible to obtain derivatives endowed with low nanomolar K_i values on CB1, such as **80** and **81** in Fig. (**19**) [129].

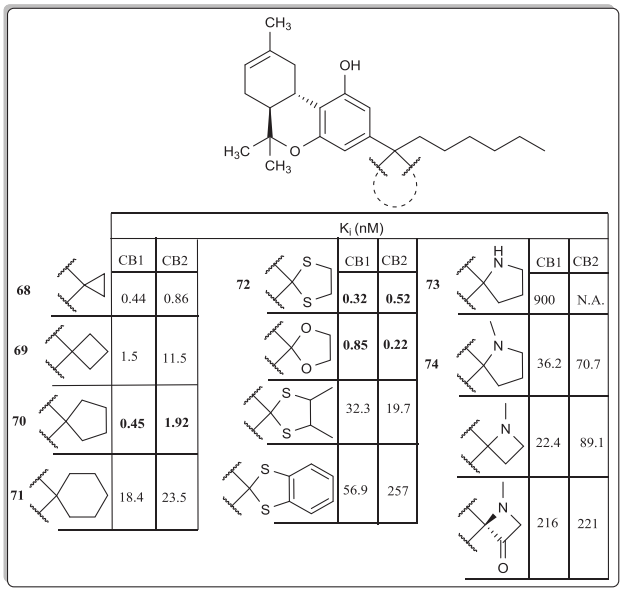


Fig. (18). C-1'–spiro cannabinoid derivatives.

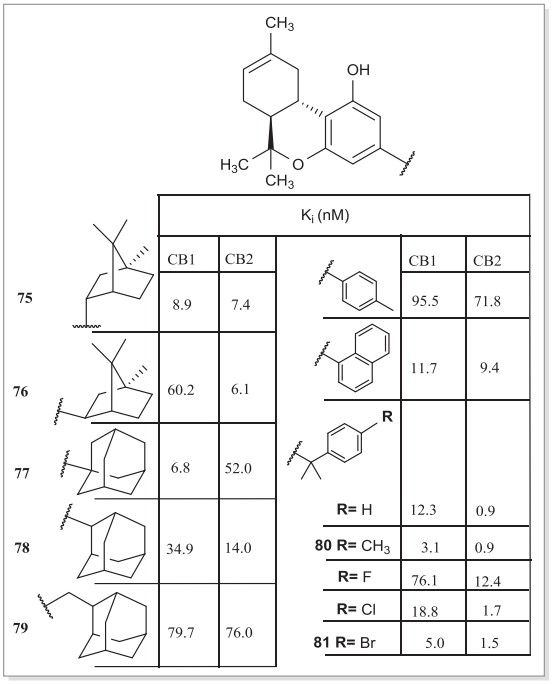


Fig. (19). Δ^8-THC analogues incorporating bulky C-3 lipophilic moieties.

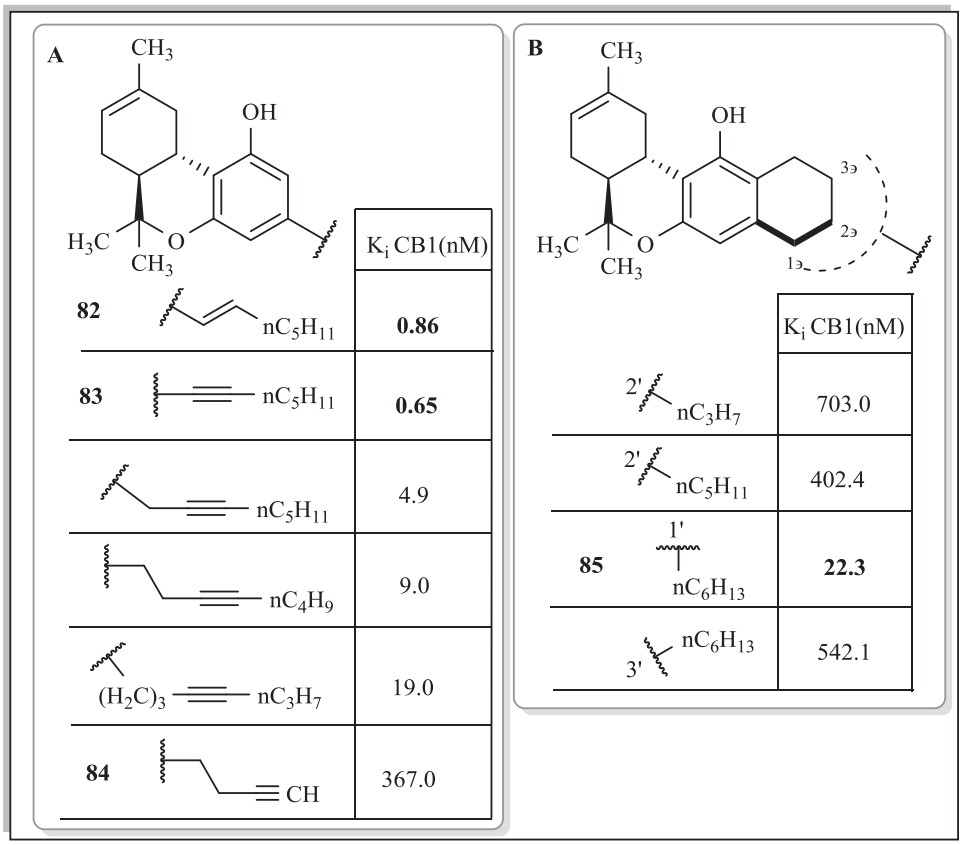


Fig. (20). Δ^8-THC analogues bearing rigid C-3 substituents.

To investigate how the conformation assumed by the C-3 substituents was affecting the activity, restrictions to the alkyl chain flexibility were performed. By saturating two methylene through the insertion of a double [117] or triple bond [124, 130], a potency increment on CB1 was observed (compounds **82** and **83** in Fig. (**20**)). These two derivatives, in which the α-β bond was oxidised to alkene and alkyne respectively, possessed low nM K_i values, probably due to positive interactions of these groups with the CB1 subsite mentioned above. Only when the alkyne function was placed as a terminal position, a detrimental effect on activity was shown (derivative **84** in Fig. (**20**)) [117]. On the contrary, the fusion to the phenyl portion of a cyclohexane ring, which constricted the C-1' and C-2' atoms into a rigid conformation, resulted in a loss of activity on CB1 receptor [118, 131]. Only shifting the alkyl chain toward a "downward orientation", as shown in compound **85**, the affinity for the CB receptors could be restored.

Therefore, restraining the C-3 conformers could be beneficial to enhance the potency of the cannabinoid derivatives and, only when the side chain is forced to orient toward a "lateral" direction, the loss of flexibility could lead to a potency decrement.

Heteroatoms and other functional groups have also been evaluated as potential C-3 pendants. In the series of terminal-substituted derivatives reported in Fig. (**21A**) [132 - 136], the lipophilic non-protic substituents Br (**86**) [132] and CN (**87**) [133] led to a considerable activity increment. The addition of a basic centre, as verified in compounds **89**-**92** [133, 136], improved the potency too. Interestingly, the carboxylic derivative **88** [134] showed a marked selectivity for CB2 being 50-fold more potent on this receptor than on CB1. In the series of derivatives reported in Fig. (**21B**)., the addition of an ester function in β-position of the alkyl chain [137, 138] left the cannabinoids K_i values almost unaffected with respect to the correspondent analogues of Fig. (**21A**).

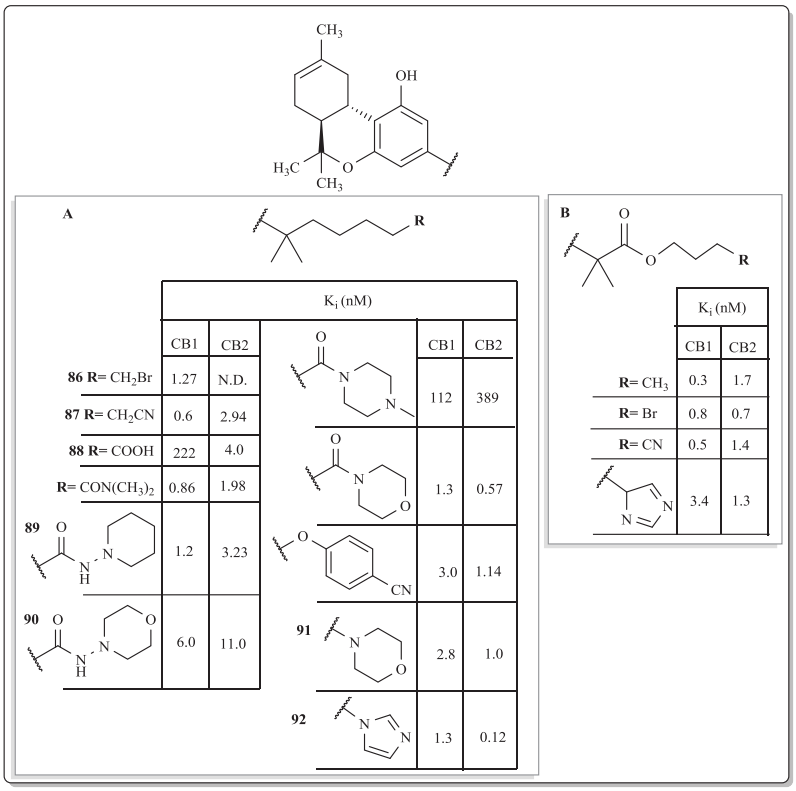


Fig. (21). Δ^8-THC derivatives carrying different terminal substituents on the C-3 side chain.

The summary of the most important above mentioned SAR for the C-3 side-chain is reported in Fig. (**22**). A clear cut relationship among the properties of the various pendants and the activity of the correspondent compounds could not easily be highlighted. In fact, a plethora of substituents possessing different chemical properties were tolerated or were able to improve the potency of the cannabinoid derivatives once placed as C-3 moiety.

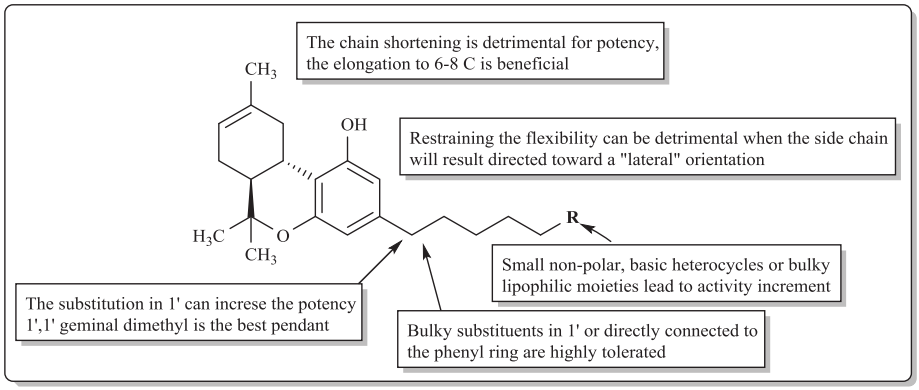


Fig. (22). The main SAR related to the C-3 substituents.

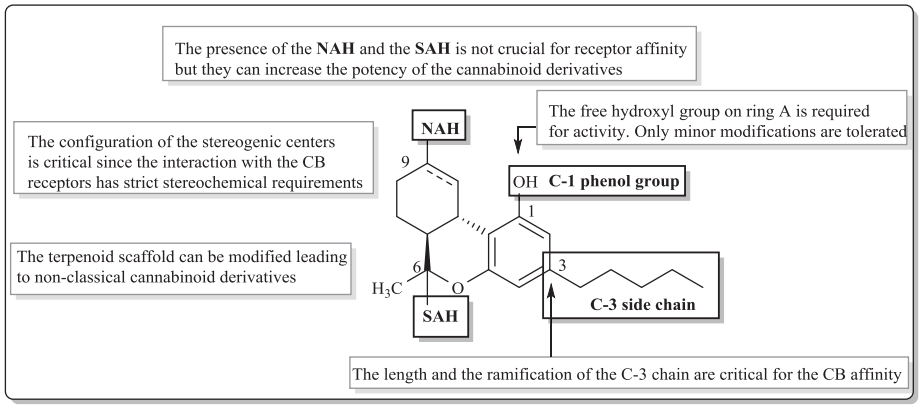


Fig. (23). Summary of the most important SAR related to the crucial positions of Δ^9-THC.

Finally, in Fig. (**23**) a summary of the previously discussed SAR regarding the portions that are crucial for the affinity of the cannabinoid derivatives to the CB receptors is highlighted.

CONCLUSION

The natural occurring Δ^9–THC molecule exemplifies the prototype of an ideal lead compound since the results, which were obtained thanks to several medicinal chemistry efforts for the last three decades, allowed to generate a remarkable amount of synthetic derivatives. These chemical manipulations were mainly done in order to achieve new compounds endowed with less psychoactive effects and higher affinity for the CB receptors. Despite these efforts, Nabilone is still the only synthetic cannabinoid drug in the market since all the other interesting developed derivatives showed psychotropic side-effects, which limited their therapeutic applications. However, the modulation of the cannabinoid receptors remains a promising approach to develop new analgesic drugs or for the treatment of pathological conditions associated with neurodegeneration and chronic pain. This chapter provides therefore a review of the most promising medicinal chemistry investigations which can be used to guide the drug discovery of new cannabinoid agents that could overcome the limitation of the psychotropic side-effects.

LIST OF ABBREVIATIONS

Δ^8-THC: (-)-*trans*-Δ^8-tetrahydrocannabinol

Δ^9-THC (-)-*trans*-Δ^9-tetrahydrocannabinol

µM: micromolar

2-AG: 2-arachidonoylglycerol

AA: arachidonic acid

AC: adenyl cyclase

AEA: anandamide

AIDS: Acquired Immuno Deficiency Syndrome

ATP: adenosine triphosphate

ATPH: aluminium tris(2,6-diphenylphenoxide)

cAMP: cyclic adenosine monophosphate

CB: cannabinoid receptor

CBC: cannabichromene

CBCA: cannabichromenic acid

CBD: cannabidiol

CBDA synthase: cannabidiolic acid synthase

CBG: cannabigerol

CBGA: cannabigerolic acid

CBN: cannabinol

CNS: central nervous system

DAGs: diacylglycerols

DAGL: diacylglycerol lipase

ER: endoplasmic reticulum

FDA: Food and Drug Administration

FAAH: fatty acid amide hydrolase 1

GPCR: G-protein coupled receptor

GPR55: G-protein coupled receptor 55

GPP: geranyl phosphate

Ile: L-isoleucine

Ki: inhibitory constant

Lys: L-lysine

MAGL: monoacylglycerol lipase

MAPK: Mitogen-activated protein kinases

NAH: northern aliphatic hydroxyl

NAPE:*N*-acyl-phosphatidylethanolamine

NAPE-PLD:*N*-acyl-phosphatidylethanolamine

n*-hexanoyl CoA:n*-hexanoyl coenzyme A

nM: nanomolar

NT: neurotransmitter

PKA: protein kinase A

SAH: southern aliphatic hydroxyl

SAR: structure-activity relationship

THCA: tetrahydrocannabinolic acid

Val L-valine

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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Declared none.

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CHAPTER 3

Encapsulation of Essential Oils within Lipid-Based Formulations for Enhanced Antimicrobial Activity

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Abstract: Aromatic plants have been used since ancient times for their medicinal properties, including potent antimicrobial activity. Strong evidence indicates that plant extracts, in general, and essential oils (EOs), in particular, can act as effective antimicrobial agents against a wide spectrum of pathogenic microorganisms. However, their poor water solubility and stability, as well as their high volatility, make the administration of EOs to achieve the desired therapeutic effects particularly challenging. Therefore, these features severely limit the application of EOs in the pharmaceutical field. In this context, nanotechnology-based strategies for developing nano-scaled carriers for the efficient delivery of EOs might offer potential solutions. In particular, considering the lipophilic nature of EOs, lipid-based nanocarriers represent the most suitable vehicles for the effective encapsulation and delivery of EOs. This chapter provides an overview of the different chemical compositions due to various endogenous and/or exogenous factors of a selection of oils and the most recent lipid-based encapsulation strategies to enhance their antimicrobial activity and promote their pharmaceutical application.

Keywords: Antimicrobial Activity, Chemical Composition, Essential Oils, Encapsulation, Liposomes, Microemulsions, Nanoemulsions, Nanostructured Lipid Carriers, Solid Lipid Nanoparticles.

INTRODUCTION

Essential oils (EOs) are very complex natural mixtures that can contain around 60 components at quite different concentrations. These volatile compounds extracted from plants or plant organs like flowers, seeds, buds, leaves, fruits, wood, roots, barks and twigs are responsible for the characteristic flavour and aroma. There are several methods for extracting EOs: by use of liquid carbon dioxide or microwaves, by distillation (*via* steam and/or water) or mechanical methods, such

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as cold pressing. The aromatic chemicals that give the typical essence to each oil are extracted and combined with carrier oil.

EOs can be applied in various cases, including pharmaceutical and health industries but are most commonly used in the practice of aromatherapy; they are also used in a wide range of consumer goods, such as soaps, detergents, toilet products and cosmetics. EOs are obtained from aromatic flora or plants bearing many Angiospermic families, such as Rutaceae, Lamiaceae, Myrtaceae, Asteraceae and Zingiberaceae [1]. All EOs have their own unique smell and potential health benefits, such as for treating insomnia (lavender oil), as an antibiotic and antimicrobial (peppermint and tea tree oils) or as an anti-inflammatory (cumin and rosemary oils). The presence of a variety of diverse constituents in EOs could be responsible for wide spectrum of biological activities of the plant.

EOs are products of an unregulated sector, and the quality and their chemical composition can vary greatly. In this regard, it is very important that an EO is pure and of high quality, that it is free of synthetic additives and that has not been modified during the extraction process from the plant.

The main volatile constituents of EOs are terpenes, organic compounds consisting of multiples of isoprene units (containing five carbon atoms) and linear-chain, aromatic or heterocyclic compounds. Different combinations of the isoprene units originate structurally and functionally different classes of terpenes [2]. When a terpene contains oxygen, it is called a terpenoid.

Generally, hydrocarbons and oxygenated compounds such as alcohols, aldehydes, ketones, acids, esters, and oxides are responsible for odors and the characteristic aroma. The analytical technique useful for determining the chemical composition of EOs is gas chromatography. There are many reports in the literature that have contained useful information about the composition of different EOs [3 - 5]. EOs are complex materials and multi-component systems classified into non-volatile, semi-volatile, and volatile compounds according to their nature. Furthermore, the chemical composition of EOs depends on the place of origin, climatic conditions, and plant species [6]. By the analysis of EO, the following compounds are found in varying proportions, and they are the main groups [7]:

TERPENE HYDROCARBONS

-Monoterpene hydrocarbons: found in almost all EOs and have a structure of 10 carbon atoms and at least one double bond. The 10 carbon atoms are derived from two isoprene units.

-Sesquiterpenes: they consist of 15 carbon atoms and have complex pharmacological actions.
Monoterpenes, diterpenes, and sesquiterpenes are the main groups of terpenes found in spices and herbs; they have notable biological activities such as antimicrobial effects on different pathogens [8, 9].

Oxygenated Compounds

• Phenols: some examples are thymol, eugenol and carvacrol. These components have great antiseptic, antibacterial and disinfectant qualities.
• Alcohols: they are divided into monoterpene and sesquiterpene alcohols, such as linalool, citronellol, terpineol and bisabolol.
• Aldehydes: they have antifungal, anti-inflammatory, disinfectant, and sedative therapeutic properties.
• Ketones: they can be toxic, but they also have some great therapeutic benefits.
• Esters: like linalyl acetate, they are normally very fragrant and tend to be fruity and their therapeutic effects include sedative and antispasmodic activities.
• Ethers: the most common are the phenolic ones, such as the anethole present in anise.
• Oxides: the main therapeutic effect of oxides is that of expectorant, with 1,8-cineole, commonly known as eucalyptol, the best known.

Finally, lactones and coumarins can also be found.

The chemical profile of an EO, even obtained from the same species, may differ according to the geographical source and the harvest season of a particular plant species and also for the same species from different regions [10 - 13]. Genotype, interaction with the environment and agronomic conditions, such as the age of the plant, the degree of maturity of the plant, the harvest time and the composition of the soil, can influence the quali-quantitative composition of EO [11, 14]. Furthermore, the extraction product can vary in quality and/or quantity depending on the type of extraction method chosen [15].

In this regard, Table **1** shows different chemical compositions of a selection of EOs endowed with antimicrobial properties. They have been selected on the base of the formulation studies reported in the following paragraphs.

NANOENCAPSULATION OF ESSENTIAL OILS

EOs represent an important part of the traditional Pharmacopeia [115]. Nowadays, a large number of biological activities have been reported for EOs to prevent and treat human diseases, and, in particular, a lot of evidence exists on their antimicrobial properties [116, 117].

Table 1. Chemical composition of a selection of EOs.

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Peppermint	Lamiaceae	Shiraz, Iran	menthol (53.28%) menthyl acetate (15.0%) menthofuran (11.18%) 1,8-cineole (6.69%)	[16]
		Jazan, (Saudi Arabia)	menthol (36.02%) menthone (24.56%) menthyl acetate (8.95%) menthofuran (6.88%)	[17]
		Puli, Nantou County, Taiwan	menthol (30.35%) menthone (21.12%) trans-carane (10.99%)	[18]
Cardamom (*Elettaria cardamomum*)	Zingiberaceae	Mashhad city (Iran)	1,8-cineole (36.74%) α-terpinyl acetate (33.07%)	[19]
		Plovdiv, Bulgaria	α-terpinyl acetate (39.032%) eucalyptol (31.534%) β-linalool (4.829%) sabinene (4.308%) α-terpineol (4.127%)	[20]
		Jeddah city (KSA)	1,8-cineole (55.4%), α-terpinyl acetate (28.6%) 4-terpineol (3.3%)	[21]
Citronella or ***Cymbopogon nardus***	Poaceae	Kelantan, Malaysia	citronellal (29.6%) 2,6-octadienal, 3,7-dimethyl-(E) (11.0%) cis-2,6-dimethyl-2,6-octadiene (6.9%) propanoic acid 2-methyl-, 3,7-dimethyl-2,6-octadienyl ester, (E) (6.9%)	[22]
		Dragoco (Germany)	citronellal (27.00%) trans-geraniol (22.78%) citronellol (10.09%)	[23]
		Malacca, Malaysia	citronellal (11.35%) z-citral (11.34%) β-myrcene (6.70%) β-trans-ocimene (6.03%) geranyl acetate (3.82%) limonene (3.50%) citronellol (3.22%)	[24]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Eugenia caryophyllata	Myrtaceae	Québec, Canada	eugenol (88.58%) eugenyl acetate (5.62%) β-caryophyllene (1.39%)	[25]
		Ardakan, Iran	eugenol (68.9%) trans-caryophyllene (12.6%) eugenol acetate (12.4%)	[26]
		Madagascar	eugenol (70-88%) eugenyl acetate (4-15%) β-caryophyllene (4-21%)	[27]
Mentha pulegium	Lamiaceae	Ribatejo, Portugal	menthone (35.9%) pulegone (23.2%) neo-menthol (9.2%) 8-hydroxy-4(5)-p-menthen-3-one (2.1%)	[28]
		Algeria	pulegone (70.66%) neo-menthol (11.21%) menthone (2.63%)	[29]
		Sicily, Italy	pulegone (50.6%) piperitenone (27.8%) menthone (6.9%)	[30]
Nigella sativa	Ranunculaceae	Tehran, Iran	trans-anethole (38.3%) p-cymene (14.8%) limonene (4.3%) carvone (4.0%)	[31]
		Tunisia	p-cymene (60.5%) α-thujene (6.9%) γ-terpinene (3.5%) thymoquinone (3.0%) β-pinene (2.4%) carvacrol (2.4%) terpinen-4-ol (2.1%)	[32]
		India	p-cymene (31.4%) thymoquinone (37.6%) thymohydroquinone (3.4%) α-thujene (5.6%)	[33]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Origanum vulgare	Lamiaceae	Saudi Arabia	carvacrol (70.2 ±1.37%) γ-terpinene (5.6 ± 0.11%) p-cymene (4.5 ± 0.42%) trans-sabinene hydrate (3.8 ± 0.07%) thymol (2.2 ±0.12%)	[34]
		Turkey	carvacrol (63.97%) p-cymene (12.63%) linalool (3.67%)	[35]
		Portugal	carvacrol (14.5%) thymol (12.6%) β-fenchyl alcohol (12.8%) δ-terpineol (7.5%) γ -terpinene (11.6%) α-terpinene (3.7%)	[36]
Ridolfia segetum	Apiaceae	Tunisia	dillapiole (47.4%) myristicin (19.2%) α-phellandrene (3%) p-cymen-8-ol (1.2%)	[37]
		Portugal	α-phellandrene (53.0-63.3%) terpinolene (11.9-8.6%) ß-phellandrene (5.5-6.0%) dillapiol (1.9-8.0%)	[38]
		Andalusia	Steam Oil: α-phellandrene (39.4-62.0%) p-cymene (10.4-22.7%) β -ocimene (10.2-11 .7%) terpinolene (7.0-15.6%) Leaf oil: α-phellandrene (61.8%-69.5%) β-ocimene (10.7-12.0%) terpinolene (6.0-10.7%)	[39]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Rosmarinus officinalis	Lamiaceae	Taizhou, China	1,8-cineole (26.54%) α-pinene (20.14%) camphor (12.88%) camphene (11.38%) β-pinene (6.95%)	[40]
		Belgrade	1,8-cineole (43.77%) camphor (12.53%) α-pinene (11.51%) β-pinene (8.16%) camphene (4.55%) β-caryophyllene (3.93%)	[41]
		Ethiopia	1,8-cineole (23.55%) verbenone (18.89%) camphor (15.06%) α-terpineol (6.43%) isoborneol (5.68%) tridecyl acrylate (5.57%) linalool (3.71%) bornyl acetate (3.57%) trans-caryophyllene (3.36%) terpine-4-ol (2.78%) α-pinene (1.40%)	[42]
Lavandula x intermedia	Lamiaceae	Italy	linalool (35.8%) 1,8-cineole (19.8%) α-pinene (8.7%) linalyl acetate (7.5%) myrcene (4.9%)	[43]
		Romania	camphor (32.7%) eucalyptol (26.9%) borneol (7.11%) caryophyllene (4.88%)	[44]
		Turkey	linalool (39.43%) 1,8-cineole (12.08%) camphor (9.21%)	[45]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Eucalyptus globulus	Myrtaceae	Algeria	1,8-cineole (55.29%) spathulenol (7.44%) α-terpineol (5.46%)	[46]
		Tehran	1,8-cineole (76.65%) α-pinene (5.65%) α-terpineol acetate (4.85%) alloaromadendrene (3.98%)	[47]
		Montenegro Coast and East Spanish	1,8-cineole (4.10-50.30%) α-pinene (0.05-17.85%) p-cymene (trace-27.22%) cryptone (0.00-17.80%) spathulenol (0.12-17.00%)	[48]
***Citrus limon* (var. *pompia*)**	Rutaceae	Sardinia, Italy (leaves)	limonene (256.3 mg/ml) geranial (213.8 mg/ml) neral (172.9 mg/ml) cis-β-ocimene (71.7 mg/ml)	[49]
		North-East Sardinia, Italy (favedo)	limonene (803.8 mg/ml) geranial (31.2 mg/ml) neral (24.9 mg/ml) β-myrcene (20.4 mg/ml)	[50]
		North-East Sardinia, Italy	limonene (29.7%) lilalyl acetate (20.9%) geranial (11.1%) linalool (11.0%)	[51]
Artemisia annua	Asteraceae	Bosnia	artemisia ketone (30.7%) camphor (15.8%) artemisia alcohol (6.5%)	[52]
		Hungary	β-selinene (12.27%) (E)-pinocarveol (7.55%) camphor (7.06%) caryophyllene (5.26%) farnesene (4.8%)	[53]
		Bulgaria	α-caryophillene (24.73%) α-cuvebene (13.53%) α-copaene (7.42%) α-selinene (8.21%) artemisia ketone (8.45%) camphor (3.61%)	[54]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Salvia triloba	Lamiaceae	Amman, Jordan	1,8-cineole (45.16%) camphor (11.53%) γ-terpineol (4.40%)	[55]
		South Brazil	α -thujone (20.1%) camphor (12.6%) 1,8-cineole (15.7%), β -pinene (3.95%)	[56]
		Turkey	In hydrodistillation (HD) and microwave-assisted hydrodistillation (MWHD): 1,8-cineole (52.0% and 47.5%) camphor (10.4% and 11.8%) α-pinene (6.0% and 5.2%) β-pinene (3.9% and 3.2%) respectively.	[57]
Trachyspermum ammi	Apiaceae	Iran	thymol (74.2%) p-cymene (16%) γ-terpinene (7.1%)	[58]
		Tehran	thymol (47.05%) γ-terpinene (27.78%) p-cymene (22.06%)	[59]
		Fars	γ -terpinene (48.07%) p-cymene (33.73%) thymol (17.41%)	[60]
Allium sativum	Alliaceae	Egypt	*white-skin cultivar:* diallyl trisulfide (45.76%) diallyl disulfide (15.63%) *purple-skin cultivar:* diallyl trisulfide (58.53%) diallyl disulfide (22.38%)	[61]
		Brazil	diallyl trisulfide (38, 81%) diallyl disulfide (25.23%) methyl allyl trisulfide (12.52%)	[62]
		Spain	By hydrodistillation, industrial steam distillation, and industrial hydrodistillation diallyl sulfide (1.9–9.5%), diallyl disulfide (20.8–27.9%), diallyl trisulfide (16.8–33.4%), allyl methyl disulfide (4.4–8.3%), and allyl methyl trisulfide (14.5–19.2%) respectively	[63]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
***Ferula gumosa* Bioss**	Apiaceae	Iran	sabinene (40.1%) α-pinene (14.3%) β-pinene (14.1%)	[64]
		Tehran	α-pinene (50.1%) β-pinene (18.3%) 3-carene (6.7%) α-thujene (3.3%) sabinene (3.1%)	[65]
		Kashan, Iran	β-pinene (60.84%) α-pinene (9.14%) β-phellandrene (6.94%)	[66]
Curcuma longa	Zingiberaceae	Northern India	Rhyzome EO: ar-turmerone (31.7%) α-turmerone (12.9%) β-turmerone (12.0%) (Z)-β-ocimene (5.5%) Leaves EO: α-phelladrene (9.1%) terpinolene (8.8%) undecanal (7.1%) p-cymene (5.5%)	[67]
		Brazil	α-turmerone (42.6%) β-turmerone (16.0%) ar-turmerone (12.9%)	[68]
		Ecuador	ar-turmerone (45.5%) α-turmerone (13.4%) α-phelladrene (6.3%)	[69]
Cymbopogon flexuosus	Poaceae	China	geranial (29.36%) neral (30.39%) caryophyllene (25.39%)	[70]
		Karnataka, India	citral (64.98%) 1,7-octadien-3-ol (10.97%) dimethyl oxatricyclo nonanone (9.44%) nerol (2.85%) verbenol (1.77%) caryophyllene oxide (0.71%)	[71]
		São Paulo, Brazil	geranial (41.80%) neral (33.25%) geranyl acetate (4.23%)	[72]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Citrus limon	Rutaceae	Cap Bon of Tunisia	limonene (39.74%) β-pinene (25.44%) α-terpineol (7.30%) linalyl acetate (3.01%)	[73]
		Algeria	limonene (61.3%) β-pinene (9.7%) α-citral (4.2%) α-terpinene (3.8%)	[74]
		South of Iran	linalool (30.62%), geraniol (15.91%) α-terpineol (14.52%) linalyl acetate (13.76%)	[75]
Citrus sinensis	Rutaceae	China	Light and cold pressed EO: limonene (60.44%, 85.32%) beta-myrcene (7.60%, 5.11%), respectively	[76]
		Vietnam	HD and SFME extraction: limonene (98.28%, 98.41%) β-myrcene (1.16%, 1.17%) respectively	[77]
		Greater Noida, Uttar Pradesh	EO from leaves: cis-sabinene hydrate (35.1%) l-limonene (30.1%) citral (27.9%) lavendulol (2.5%) perillaldehyde (2.0%) EO from fresh fruit peels: α-pinene (60.80%) verbenone (15.40%)	[78]
***Mentha spicata* (var. *viridis*)**	Lamiaceae	Khartoum, Sudan	D-carvone (64.63%), D-limonene (12.27%) (-)-8-p-menthen-2-yl, acetate trans (2.59%) cyclohexanol,2-methyl-5-(1-methylethenyl) (2.36%) eucalyptol (2.28%)	[79]
		Shamabt, Sudan	carvone (71.98%; 84.81%; 67.62%; 78.33%) from spearmint herb in January, April, August and December respectively	[80]
		Tunisia	carvone (50.47%) 1,8-cineole (9.14%) limonene (4.87%)	[81]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Pelargonium graveolens	Geraniacee	Sidi Thabet North of Tunisia	citronellol (27.53%) geraniol (25.85%) 6-octen-1-ol,3, 7-dimethyl-formate (8.75%) isomenthone (6.22%)	[82]
		Brazil, Egypt, South Africa, China, Reunion Island-Bourbon and Albania	geraniol and citronellol from Brazil (39.8%, 11.4%), Egypt (18.9%. 28.8%), South Africa (38.7%, 10.8%), China (9.4%, 39.3%), Reunion Island-Bourbon (9.6%, 22.0%), Albania (5.5%, 40.0%)	[83]
		Tajikistan	citronellol (37.5%) geraniol (6.0%) caryophyllene oxide (3.7%) menthone (3.1%) linalool (3.0%) β-bourbonene (2.7%)	[84]
Curcuma xanthorrhiza	Zingiberaceae	Malaysia	β-curcumene (17.1%) ar-curcumene (13.2%) camphor (5.4%)	[85]
		Thailand	α-terpinolene (24.86%) p-cymen-7-ol (12.17%)	[86]
		India	EO from flower and leaves: ar-turmerone (31.0% and 46.8%, respectively) cymen-8-ol (26.0%) α-phellandrene (32.6%)	[87]
Matricaria chamomilla	Asteraceae	Bosnia and Herzegovina	β-farnesene (29.8%) α-farnesene (9.3%) α-bisabolol and its oxide (15.7%) chamazulene (6.4%) germacrene D (6.2%) spiroether (5.6%)	[88]
		Iran	α-bisabolone oxide A (35.74%) α-bisabolol oxide A (19.07%) (Z)-β-Farnesene (6.63%) chamazulene (6.46%)	[89]
		Nepal	(E)-β-farnesene (42.2%) α-bisabolol oxide A (22.3%) (E,E)-α-farnesene (8.3%) cisbicycloether (5.0%) α-bisabolol oxide B (4.5%) α-bisabolone oxide A (4.0%)	[90]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Lavandula angustifolia	Lamiaceae	Xinjiang, China	linalyl acetate (28.89%) linalool (24.30%) caryophyllene (7.89%) (E)-3,7-dimethylocta-1,3,6-triene (4.64%) 4-terpineol (4.04%) acetic acid lavandulyl ester (3.49%) borneol (2.60%) eucalyptol (2.05%).	[91]
		Algeria	1,8-cineole (29.4%) camphor (24.6%) borneol (4.1%)	[92]
		Romania	linalool (26.783%) terpinen-4-ol (22.143%) 3-carene (21.668%)	[93]
Thymus daenensis	Lamiaceae	Isfahan	thymol (57.4%) carvacrol (9.8%) β-caryophyllene (6.9%) γ-terpinene (6.7%) p-cymene (6.3%)	[94]
		Isfahan, Iran	thymol (80.24%) γ-terpinene (3.51%) p-cymene (2.15%) carvacrol (1.72%)	[95]
		Isfahan, Iran	carvacrol (37.0%) thymol (12.8%) β-caryophyllene (7.6%) geraniol (5.74%)	[96]
***Satureja khuzistanica* Jamzad**	Lamiaceae	Iran	Wild plants'EO: carvacrol (93.9%); eugenol (1.0%); p-cymene (0.8%); thymol (0.6%). Cultivated plants' EO: carvacrol (80.6%); p-cymene (4.8%); myrcene (1.5%); γ -terpinene (2.1%); terpinene-4-ol (2.1%).	[97]
		Iran	carvacrol (87.16%) p-cymene (6.39%)	[98]
		Central Iran	carvacrol (69.62%) γ-terpinene (9.25%) p-cymene (8.36%)	[99]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Satureja montana	Lamiaceae	Khoramabad, Iran	carvacrol (83.4%) γ-terpinene (9.62%) thymol methyl ether (1.12%) α-terpinene (1.70%)	[100]
		France	carvacrol (53.35%) γ-terpinene (13.54%) p-cymene (13.03%)	[101]
		Montenegro	Sample at 100 m: thymol (24.69%); linalool (15.38%); carvacrol (15.19%) Sample at 500 m: thymol (24.69%); carvacrol (24.46%); linalool (17.94%) Sample at 800 m: linalool (32.58%); cis-sabinene hydrate (23.05%); nerolidol (9.36%)	[102]
Celery (*Apium graveolens*)	Apiaceae	Marrakech	D-limonene (79.15%) α-hydroxypropylbenzene (4.23%) β-selinene (9.25%) β-myrcene (1.93%)	[103]
		India	p-cymene (16.73%) γ–terpinene (13.78%) β-selinene (8.05%) thymol (6.93%) α –terpinyl acetate (5.81%) 1,4-dimethyl-4-acetyl-1-cyclohexene (5.59%) kessane (3.64%) β-pinene (3.08%)	[104]
		Lublin	from whole leaves of leaf EO: limonene (54.04–58.29%), myrcene (19.51–27.65%), 1,2 ethanediol, 1-phenyl (5.62–7.17%), furan, 2-(2-propenyl) (2.25–2.27%), Z-β-ocimene (1.45–1.85%)	[105]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Cuminum cyminum	Umbelliferae	Tunisia	cuminlaldehyde (39.48%) γ-terpinene (15.21%) O-cymene (11.82%) β-pinene (11.13%) 2-caren-10-al (7.93%) trans-carveol (4.49%) myrtenal (3.5%)	[106]
		Iran, Egypt, India, Europe	Iran, Egypt, India, Europe: cuminic aldehyde (41.5%, 29.3%, 23.2%, 22.4%) p-cymene (17.4%, 10.1%, 18.4%, 20.2%) β-pinene (10.7%, 15.7%, 12.6%, 14.1%) respectively.	[107]
		India	cumaldehyde (32.50%) α-pinene (9.68%) sabinene (7.54%) o-cymene (6.55%) β-pinene (6.0%) isopropyl benzaldehyde (4.91%) 3-carene (4.42%) D-limonene (4.41%) α–Terpinyl acetate (3.53%) trans-nerolidol (3.02%)	[108]
Ocimum basilicum	Lamiaceae	Algeria	linalyl acetate (53.89%) linalool (22.52%) eucalyptol (3.29%)	[109]
		Armenia	*O. basilicum* var. purpureum, *O. basilicum* var. thyrsiflora, *O. basilicum x citriodorum:* methyl chavicol (57.3%, 20.00%, 9.45%) trans-α-bergamotene (4.34%, 1.34%, 3.52%) respectively	[110]
		India	methyl cinnamate (70.1%) linalool (17.52%) tau-cadinol (2.59%)	[111]

(Table 1) cont.....

Plant Name from which EOs were Derived	Family of Plants	Origin	Main Components Identified	Refs.
Thymus capitatus	Lamiaceae	Greece	carvacrol (81.8%) linalool (3.5%) (E)-caryophyllene (3.5%)	[112]
		Tunisia	Vegetative Leaves and Flowering Leaves, Flower buds, Flowers Leaves, Post-flowering Leaves and Post-flowers: Carvacrol (65.9%, 83.0%, 71.3%, 80.7%, 77.4%, 70.6%, 69.5%, 68.1%,72.9%,78.6%,65.6%, 78.0%, 61.6%, 63.0%, 70.7%, 68.5%) p-Cymene (16.7%, 5.5%, 8.1%, 5.4%, 7.3%, 8.0%, 8.9%, 8.2%, 5.9%, 5.3%, 8.6%, 4.8%, 13.7%, 15.4%, 9.1%, 10.8%) respectively	[113]
		Libya	Sidi-Alhamry and Abu-Draa: γ-terpinene (16.18%, 0.76%) carvacrol (24.28%, 58.56%) caryophyllene oxide (10.43%, 6.26%)	[114]

Despite their interesting therapeutic potential, the pharmaceutical use of EOs is limited by their volatility, poor water solubility and instability in the presence of heat, light and oxygen. Therefore, the development of strategies to optimize or even enhance the stability and effectiveness of EOs is a current challenge, in order to fully exploit their biological potential. In this context, nanotechnology proved to be a very promising strategy to protect EO integrity from high volatility, limited stability and poor solubility, which can reduce their efficacy and hamper their applications. It has become progressively more evident that formulations containing natural and/or synthetic lipids represent a promising tool for enhancing the chemical stability and water solubility of poorly water-soluble and highly lipophilic compounds. A wide range of different nano-sized delivery systems has been proposed for the encapsulation of EOs. Recently, considering the lipophilic nature of EOs, lipid-based formulations, distinguished into vesicular and non-vesicular systems, have been considered for effective encapsulation and delivery of EOs. In specific, liposomes, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and nano- and microemulsions encapsulating EOs have been developed and investigated for this purpose [7, 118 - 120]. A schematic showing the structure of the different lipid-based formulations used for the EOs encapsulation is reported in Fig. (**1**).

Fig. (1). Schematic showing the structure of the different lipid-based formulations used for the EOs encapsulation.

In recent years, the possible improvement of the antimicrobial activities of formulated EOs was investigated against different pathogens [121 - 123]. When encapsulated in lipid based nanosystems, EOs usually show improved antimicrobial activity, probably due to the ability of lipid and/or phospholipids to interact and easily fuse with the membrane of infectious microorganisms [124], with consequently improved delivery of the active components to the microbial cell. Indeed, the antimicrobial activity of EOs depends on the ability of their active components to disrupt cell walls and cytoplasmic membranes, leading to lysis and leakage of intracellular compounds [125]. Therefore, these carriers have been proposed as therapeutic options for the treatment of infections across the human body or as innovative and promising strategies to reduce the spread of multidrug-resistant pathogens and to eradicate or avoid the development of microbial biofilms [126]. Hence, the following paragraphs will focus on the use of these nanotechnologies to develop EO-based formulations with enhanced antimicrobial properties and better effectiveness for application in the pharmaceutical field.

Liposomes

This paragraph details specifically on encapsulation of EOs into phospholipidic vesicles, also known as liposomes, and reports the methods that are usually used to prepare liposomes incorporating EOs, their characterization in terms of size and encapsulation efficiency, as well as the improvement of their activity, focusing on the antimicrobial properties for pharmaceutical applications.

Liposomes are viewed as attractive delivery vehicles by the pharmaceutical industry. They are nanometric self-closed structures of spherical shape with a diameter ranging from 20 nm to a few thousand nm, capable of entrapping hydrophilic and hydrophobic drugs in their aqueous core and lipid bilayer, respectively. Liposomes can be formed from phospholipids that, in water, are arranged spontaneously in double concentric layers of ~4 nm of thickness, separated by aqueous compartments. Depending on the number of lipid bilayers, which composes the vesicles, and on the base of their size, liposomes can be classified into one of two categories: multilamellar (MLV) and unilamellar vesicles (ULV). MLVs have an onion structure characterized by five or more concentric layers, with a final diameter in the range from 0.4 to 3.5 μm. Unilamellar liposomes, instead, have a single phospholipid bilayer enclosing the aqueous solution. Among ULVs, it is also possible to distinguish some kinds of vesicles for their diameter: small unilamellar vesicles (SUV), with a diameter in the range from 25 to 100 nm, and large unilamellar vesicles (LUV), from 0.1 to 1 μm. The membrane composition and arrangement determine their versatility and ability to carry both hydrophilic and hydrophobic drugs in the aqueous core and lipid bilayer, respectively, as well as the possibility of protecting the vesicular cargo. Liposome properties differ considerably with lipid composition, surface charge, size, and method of preparation. The method used to produce liposomes should achieve a high entrapment efficiency, narrow size distribution and long-term stability. The thin-film hydration method is one of the most widely used for the formulation of liposomes [127], and for this reason, it has been used in many articles cited in this chapter as a reference method. The vesicle size is an acute parameter determining the circulation half-life of liposomes, whereas both size and number of bilayers affect the amount of EO that can be encapsulated in these delivery vehicles. Furthermore, the choice of the liposome components determines the ‘rigidity’ or ‘fluidity’, as well as the surface charge of the bilayer. For instance, unsaturated phosphatidylcholine species from natural sources (egg or soybean phosphatidylcholine) give much more permeable and less stable bilayers, whereas saturated phospholipids with long acyl chains (for example, dipalmitoylphosphatidylcholine) form a rigid and rather impermeable bilayer structure. EOs may affect liposome characteristics such as size, encapsulation efficiency and thermal behavior of lipid bilayers. In particular, several EOs can

decrease the size of liposomes, increase the membrane fluidity and reduce the oxidation of the lipid bilayer. It is reported that EOs cause a higher cohesion packing among the apolar chains of phospholipids in the vesicle membrane [128] or increase their surface curvature. This effect could be explained considering that some EOs components, such as monoterpenes, can be located at the polar head group region of the membrane, and they could increase in various ways the polarity of the membrane environment, forcing the curvature of the vesicles. Many authors also showed that the thermal-oxidative stability of EO-loaded liposomes is higher than plain liposomes. These authors demonstrated that the presence of EO elevates the temperature at which oxidation of liposomes occurs [129]. When EOs are entrapped within the lipid molecules of the vesicle bilayer, they have the ability to increase the membrane fluidity of liposomes since they decrease the phase transition temperature (T_m) of vesicles (or only broaden the peak) and/or modify the enthalpy of the gel-to liquid crystalline transition. EOs can also cause the disappearance of the pre-transition peak characteristic of phospholipid vesicles, which indicates their ability to interact with the lipid bilayer surface and disturb the acyl chain organization within the double-layer [130].

The ability of the EOs components to interact and modify membrane properties and organization of phospholipids of liposome double-layer gives an explanation of their antifungal, antibacterial, anesthesia-potentiating, neuro-protective, antioxidant, and antiparasitic effects. Indeed, the antimicrobial efficacy of EOs is a consequence of their interaction with the membrane of the microorganism. It has been shown that the bioactive components of EOs might attach to the surface of the microbial cell and thereafter penetrate through the phospholipid bilayer of the cell membrane [131]. Their accumulation greatly affects the structural integrity of the microbial membrane, influencing the ion transport processes and causing cell death due to the leakage of critical molecules and ions. The extent of the membrane damage is related to the physicochemical characteristics (such as lipophilicity and water solubility) of the EOs components. In addition, this effect seems to be dependent on the lipidic composition and net surface charge of the microbial membrane [132]. Liposome-based vesicular nanosystems are considered a promising strategy to increase the antimicrobial activity of EOs, as they enable improved delivery of antimicrobials to bacterial cells, thus increasing the efficacy of the therapeutic treatments. Liposomes have the ability to interact with several cell types, and this capacity is involved in their antimicrobial efficacy. Indeed, liposomal phospholipid bilayer easily fuses with bacterial cell membranes and releases high doses of EOs, directly inside microbial cells. Furthermore, liposome composition can be changed to promote the adsorption onto, or fusion with the microbial cell membrane, as well vesicle surface can be changed based on the characteristics of the infectious agent. Arguably, EOs

protection in liposomal nanocarriers, their fusogenicity and versatility properties constitute the biggest advantages of using liposomal carriers to deliver EOs over non-encapsulated EOs [133].

Taken together, the principal investigations published between 2015 and 2020 demonstrate the great potential of liposomal vesicles as carriers for EOs delivery, and stress the potentiality of these lipid-based vehicles for the treatment of bacterial infections and biofilm targeting. The most common and simplest preparation technique used to prepare EOs-liposome based formulations is the thin-film hydration method. The preparation of liposomes by hydration of a lipid film involves the evaporation of the organic solvent under low pressure conditions from a lipid solution, which results in a thin phospholipid film stuck at the bottom of a flask. The hydration of the lipid film by an aqueous buffer aided by mechanical energy, such as vortication, results in the spontaneous formation of multilamellar vesicles (MLVs). The method is simple, but this procedure gives heterogeneous vesicles of large size and thus requires additional steps, such as sonication or extrusion, in order to reduce the size and improve the homogeneity of the vesicle sample. Sonication transforms the MLVs population into SUVs by using ultrasound that provides enough energy to break MLVs. In addition, LUVs may be the desired product of extrusion, which can be obtained whereby the MLVs are forced through polycarbonate filters with defined pore sizes. The lipid bilayers of the vesicles can be disrupted, and if the applied force is strong enough, the MLV is completely torn apart, creating smaller fragments that aggregate as SUV or LUV at the other side of the membrane, with a diameter reflecting the pore size of the membranes. Table **2** reports the methods used to prepare EO-loaded liposomes (multilamellar or unilamellar).

Table 2. Preparation techniques of liposome formulations encapsulating EOs.

Essential Oil	Vesicle Composition and Lamellarity	Preparation Technique	Refs.
***Citrus limon* var. *pompia* Citral**	Lipoid S75 (soybean phospholipids with 70% phosphatidylcholine) unilamellar vesicles	Thin-film hydration method combined with sonication	[51]
***Citrus limon* var. *pompia* Citral**	Lipoid S75 (soybean phospholipids with 70% phosphatidylcholine) unilamellar vesicles	Thin-film hydration method combined with sonication	[134]
***Thymus capitatus* *Citrus limon* var. *pompia* Citral**	Soybean lecithin liposomes, glycerosomes unilamellar vesicles	Thin-film hydration method combined with sonication	[135]
Thyme (*Thymus capitatus*)	Soybean lecithin liposomes, glycerosomes unilamellar vesicle	Thin-film hydration method combined with sonication	[136]

(Table 2) cont.....

Essential Oil	Vesicle Composition and Lamellarity	Preparation Technique	Refs.
Artemisia annua	Soybean lecithin, cholesterol, β-cyclodextrin solid multilamellar liposomes (SLP)	Thin-film hydration method combined with ultrasonication	[137]
Salvia (*Salvia triloba*) Rosemary (*Rosmarinus officinalis*)	P90G (non-hydrogenated soy phosphatidylcholine) and cholesterol multilmellar vesicles	Thin-film hydration method	[138]
Thyme (*Thymus capitatus*)	P90G (non-hydrogenated soy phosphatidylcholine) and cholesterol multilamellar vesicles	Thin-film hydration method combined with ultrasonication	[139]
Tea tree oil (*Melaleuca alternifolia*)	Phosphatidylcholine, cholesterol and tween 80 unilamellar vesicles	Thin-film hydration method combined with sonication	[140]
Trachyspermum copticum	SPC80 (soybean phospholipid containing 75% phosphatidylcholine) and cholesterol multilamellar liposomes	Thin-film hydration method	[141]
Cymbopogon densiflorus	Phosphatidylcholine and cholesterol unilamellar vesicles	Thin-film hydration method combined with ultrasonication	[142]
Cinnamon	Soy lecithin and cholesterol multilamellar vesicles	Thin-film hydration method	[144]
Estragole, eucalyptol, isoeugenol, pulegone, terpineol and thymol	Lipoid S100 (non-hydrogenated soybean) HP-ß-cyclodextrin multilamellar vesicles	Ethanol injection method	[145]

Generally, EOs have the ability to decrease the sizes of plain liposomes produced with the same method. This effect was explained by the capability of EOs to cause higher side-chain packing among the apolar chains of phospholipids in the membrane vesicles [120]. The encapsulation efficiency differs from an EO to another for liposomes prepared by the same method.

As antimicrobial agents, liposomes were tested against a large number of Gram-negative and Gram-positive bacteria and fungi, showing generally improved activity in comparison to free EOs, as summarized in Table **3**.

Table 3. Antimicrobial activity of liposome formulations encapsulating EOs.

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Skin and mucosal infections	*Citrus limon* var. *pompia* Citral	liposomes	*Escherichia coli* *Pseudomonas aeruginosa*	*in vitro*	[51]

(Table 3) cont.....

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
			Staphylococcus aureus *Candida albicans*		
Oropharyngeal infections	*Citrus limon* var. *pompia* Citral	liposomes	*Streptococcus mutans*	*in vitro*	[134]
Antimicrobial in caries prevention	*Thymus capitatus* *Citrus limon* var. *pompia* Citral	liposomes glycerosomes penetration enhancer-containing vesicles (PEVs)	*Streptococcus mutans* *Candida albicans*	*in vitro*	[135]
Oral infections	Thyme (*Thymus capitatus*)	liposomes glycerosomes penetration enhancer-containing vesicles (PEVs)	*Streptococcus mutans* *Lactobacillus acidophilus* *Streptococcus sanguinis*	*in vitro*	[136]
Antifungal agents	*Artemisia annua*	liposomes	*Candida norvegensis* *Candida krusei* *Candida tropicalis*	*in vitro*	[138]
	Salvia (*Salvia triloba*) Rosemary (*Rosmarinus officinalis*)	liposomes	*Escherichia coli* *Proteus mirabilis* *Klebsiella pneumoniaee* *Staphylococcus aureus*	*in vitro*	[139]
Antimicrobial agents	Thyme (*Thymus capitatus*)	solid liposomes	not evaluated	-------	[137]
	Tea tree oil (*Melaleuca alternifolia*)	liposomes	*Staphylococcus aureus* *Eschericia coli* *Candida albicans*	*in vitro*	[140]
	Trachyspermum copticum	liposomes	*Escherichia coli* *Staphylococcus aureus*	*in vitro*	[141]
	Cymbopogon densiflorus	liposomes	*Escherichia coli* *Staphylococcus aureus* *Bacillus subtilis*	*in vitro*	[142]
Antibitiotic resistant bacteria and biofilms	Cinnamon	liposomes	methicillin-resistant *Staphylococcus aureus* (MRSA)	*in vitro*	[144]
Preservatives	Estragole, eucalyptol, isoeugenol, pulegone, terpineol and thymol	liposomes	not evaluated	------	[145]

In view of the increasing interest in natural antimicrobial molecules, Usach and Manca's research group, in a recent investigation, screened the ability of *Citrus limon* var. *pompia* (CLP) extract incorporated in vesicular nanocarriers against different bacterial strains and yeasts and the results were compared with those of vesicles loaded with citral, which is one of the most abundant terpenes of *Citrus* EOs [51]. CLP-EO and citral were taken from Sardinia's biodiversity. The authors prepared liposomes containing CLP-EO or raw citral by sonication using soy phosphatidylcholine and focused on the evaluation of their antibacterial activity. The vesicles were small in size (~140 nm), with a polydispersity index (PdI) ~0.31, highly negatively charged (~ -73 mV), and able to incorporate high amounts of EO or citral (entrapment efficiency, EE% ~86%). CLP-EO and citral exhibited antimicrobial activity against all of the assayed microorganisms, with *P. aeruginosa* being the least sensitive. Citral was slightly more effective than CLP-EO in counteracting the growth of *E. coli*, *S. aureus*, and *C. albicans*. The incorporation of citral in vesicles improved its antifungal activity against *C. albicans*. The overall results suggest that CLP-EO and citral can be suitably loaded in liposomes, which are able to facilitate their dermal delivery and interaction with epidermal cells. Afterward the efficacy of the bioactive molecules was increased. Furthermore, encapsulation of EOs within the bilayer of liposomes is advantageous because it reduces the skin-sensitizing properties of some aged EOs, avoiding hypersensitivity reactions and allergic contact dermatitis. Later on, Manca and co-authors [134] proposed a new comparative study that underlined how the incorporation of CLP-EO or citral in phospholipid vesicles enhances the efficacy of the payloads, improving the protection against oxidative stress and accelerates the healing process of wounded mucosa. Liposomes loading 50 mg/ml of citral appeared as the most promising dispersion, since they were also able to inhibit the proliferation of *S. mutans*. Thus, this formulation may represent the starting point to formulate an effective, safe and pleasant mouthwash to control oral hygiene and health. Pinna and co-authors [135] screened the antimicrobial ability of *Thymus capitatus* (TC) EO and CLP extract as raw extracts or incorporated in vesicular nanocarriers against *S. mutans* and *C. albicans* and proposed these formulations for the antimicrobial treatment of oral cavity diseases. TC and CLP extracts were incorporated in different types of phospholipid vesicles, namely liposomes, glycerosomes, and Penetration Enhancer-containing Vesicles (PEVs) aiming at protecting the bioactive components from possible degradation and controlling their release. TC and CLP-loaded liposomes were ~86 and 137 nm, respectively. The addition of glycerol (glycerosomes) or propylene glycol (PG-PEVs) led to an increase in vesicle size, which was more significant for TC essential oil. Regardless of the vesicle composition, the polydispersity index, which is a dimensionless measure of the broadness of the size distribution, was always ≤0.3, thus indicating a

homogeneous distribution of the vesicle size of all the dispersions. The zeta potential of the vesicles was generally highly negative, predicting good physical stability of the vesicle dispersions during storage. On the base of the data obtained in this study, TC essential oil possesses the highest antimicrobial capacity against *S. mutans* and *C. albicans*. CLP extract showed bactericidal properties against *S. mutans*, but it was not effective as a fungicidal compound. All the phospholipid vesicles behaved similarly, suggesting that the transported extract was not the only factor to be considered in the outcomes, but also their components had an important role. Therefore, TC and CLP incorporated in nanocarriers, in particular glycerosomes and PG-PEVs, which behaved similarly against both the bacterial and yeast strains tested, could be promising and safe oral antimicrobial agents in caries prevention. Manconi and co-authors [136] also formulated *Thymus capitatus* EO (mainly composed of carvacrol) in liposomes, glycerosomes and PEVs and proposed these formulations as antibacterial-antioxidant mouthwashes. The oil was mixed with lecithin and water to produce liposomes, or different ratios of water/glycerol and water/propylene glycol (PG) to produce glycerosomes and PG-PEVs, respectively. Formulations appeared as highly biocompatible unilamellar spherical vesicles capable of counteracting oxidative stress and promoting wound repair in keratinocytes, thanks to enhanced uptake of the delivered compounds. These authors showed that the oil had high antimicrobial capacity against cariogenic *S. mutans*, *L. acidophilus*, and commensal *S. sanguinis,* and they referred that the combination of antioxidant and antibacterial activities of thyme EO formulations may be useful for the treatment of oral cavity diseases. PG-PEVs, in particular, showed good stability on storage and optimal antioxidant and inhibitory effect against the most important cariogenic bacteria.

Thyme EO was formulated also in solid liposomes (SLPs), which are newly researched nanocarrier systems characterized by an excellent biocompatibility, bioavailability and drug adaptability. Compared with conventional aqueous liposomes, SLPs have higher stability and longer storage time. Hence, the volatility and instability of EO can be reduced by SLPs encapsulation. SLPs can be obtained via freeze-drying, but this process may result in vesicle destruction because of the ice crystals formation or their subsequent sublimation. Therefore, Lin and co-authors [137] explored the possibility of using β-cyclodextrin as a cryoprotectant to protect the liposomal membrane during the freeze-drying process.

The effect of liposomal inclusion on the *in vitro* antifunginal activity of *Artemisia annua* EO (AEO) was investigated by Bilia and co-authors [138], in order to study the influence of the vesicles composition on the antifunginal activity of unilamellar liposomes incorporating the EO. These authors used different ratios of non-hydrogenated soy phosphatidylcholine (P90G) and cholesterol (CHOL)

(16.5:5, 33:10 and 66:20 mg/ml of vesicular dispersion), loaded with different amounts of AEO (5, 10, 20, 50 mg/ml of vesicular dispersion). EO did not influence the size of liposomes, causing great cohesion packing in the apolar chains of the vesicle bilayers, probably due to the small and lipophilic components of the AEO. Size distribution of AEO-loaded liposomes was affected by the phospholipid concentration, the EO/lipid ratio and cholesterol content. The encapsulation efficiency improved by increasing the lipid (both P90G and CHOL) concentration, but it slightly decreased when 20 mg/ml AEO was added. Instead, the addition of 50 mg/ml AEO prevented the supramolecular organization of lipids, and the bilayer system was not formed. Microbiological studies suggested that the vesicular systems did not alter the antibacterial activity of pure AEO or even improve it. Indeed, they resulted active against *C. krusei* and *C. tropicalis*, which are among the most resistant yeast strains against widely used antifungal agents.

Salvia tribola (S) and *Rosmarinus officinalis* (R) EOs have been loaded in phospholipid vesicles obtaining spherical and stable liposomes [139]. In particular, R and S-loaded liposomes were prepared using the film hydration method employing different amounts of non-hydrogenated soy phosphatidylcholine (P90G) and cholesterol. The optimized preparations exhibited average sizes of about 200 nm (polydispersity index - PdI - was about 0.25) with a zeta potential in the range from −20 mV to −35 mV and good stability on storage. The EE% was around 57% for *S. triloba* and around 65% for *R. officinalis*. In addition to these encouraging physical and chemical properties, Risaliti and co-authors [139] reported that the size of R and S-loaded liposomes decreased with respect to the plain liposomes. This contraction was associated with a significant contribution of the terpenes present in the EOs to the stabilization of the nanocarriers as a consequence of the higher cohesion and packing among the apolar chains of the vesicle membranes. Moreover, formulated EOs exhibited antimicrobial activity, especially against *K. pneumoniae,* which is a pathogen responsible for more than 70% of infections in humans. The antibacterial activity was comparable to that of neomycin. Furthermore, the diameter of inhibition produced in the well diffusion assay by both formulations was larger than that observed for the free EOs. The proposed R and S-loaded liposomes could represent innovative carriers preserving and enhancing the biological properties of *S. triloba* and *R. officinalis* EOs.

The investigation of Yan and Mingqiao [140] promotes the use of formulated EOs as alternative antimicrobials to synthetic chemical substances. Tea tree oil (TTO) was encapsulated into phosphatidylcholine liposomes prepared to employ a thin-film hydration methodology and characterised by dynamic light scattering for size distribution (mean hydrodynamic diameter 75 nm) and transmission electron

microscope for morphology (spherical vesicles). Liposomes not only effectively encapsulated TTO forming a stable suspension (EE% 96.08), but the encapsulation was found to improve the bactericidal effect of the EO on TTO-tolerant strains. Formulated TTO exhibited excellent broad-spectrum antimicrobial activity, superior to free TTO.

The effect of liposomal inclusion on the *in vitro* antibacterial activity of *Trachyspermum copticum* EO was investigated by Tabatabai and co-authors [141]. Vesicles were obtained by the thin-film hydration method from soybean phospholipid containing 75% phosphatidylcholine (SPC80) and cholesterol. These liposomal carriers were examined for their antimicrobial activity against Gram-negative and Gram-positive bacteria by MIC (minimum inhibitory concentration) assay. Results showed that *Trachyspermum copticum* EO can be incorporated in a sufficient amount in the prepared liposomes (EE% 60.78), which successfully demonstrated their antimicrobial activity and their potential suitability for skin disinfection applications or in wound dressing.

Seibert and co-authors [142] developed liposomal vesicles containing phosphatidylcholine, cholesterol and *Cymbopogon densiflorus* leaf EO and evaluated their antimicrobial activity. The EO from *C. densiflorus* is composed mainly of monoterpenoids, lipophilic substances that, it is believed, can interact with the phospholipidic bilayer of the microbial cell membrane, altering its integrity and function. The authors showed, for the first time, the antimicrobial potential of nanostructured systems loaded with *C. densiflorus* EO, encouraging its use in the treatment of microbial infections. In particular, liposomal oil mainly composed of trans-p-mentha-2,8-dien-1-ol, cis-p-mentha-2,8-dien-1-ol, trans--mentha-1,8-dien-2-ol, cis-piperitol, and cis-p-mentha-1,8-dien-2-ol showed improved ability to inhibit microbial growth compared to the free EO. The liposomes were even able to reduce oil cytotoxicity.

Natural compounds in lipid-based nanosystems, have also been investigated as an innovative and promising strategy for overcoming biofilm-related antibiotic resistance. It is urgent to discover new antimicrobial agents that can effectively prevent biofilm formation and avoid its development. It has been well demonstrated that natural products from plants have antimicrobial and chemo-preventive properties in the modulation of biofilm formation. For this reason, encapsulation of EOs in lipid-based nanosystems has been recently highlighted as a promising alternative to conventional antibacterial drugs to face the diffusion of drug-resistant microorganisms [143, 144].

Cui and co-authors [144] designed liposomes to encapsulate cinnamon EO. Its antibacterial properties are attributed to terpenes, which can destroy the microbial

membrane due to their lipophilic characteristics. Nevertheless, the chemical instability of cinnamon oil hinders its application for health purposes, thus, cinnamon oil was encapsulated into liposomes to reduce its chemical instability and improve its antimicrobial activity. Specifically, the effects of different concentrations of cinnamon oil on the viability of the methicillin-resistant *S. aureus* (MRSA) biofilm were evaluated after a 24 h treatment with free or encapsulated EO. Free cinnamon oil reduced the amount of MRSA viable cells by 1.49 logs, while the treatment with liposome containing cinnamon oil reduced this number by 2.45 logs. Microscopic analysis showed reduced thickness and size of MRSA biofilms after treatment with the liposomal formulation. Thus, the improved chemical stability of cinnamon oil after encapsulation into liposomes led to an enhancement of its antibiofilm activity.

Since EOs in cyclodextrins (CDs) could potentially hinder the interaction of phenolic hydroxyl groups of some EO components with the acyl chains of a lipid bilayer, EO-cyclodextrin complexes were included in liposomes and proposed by Hammoud and co-authors [145] for preserving essential oil monoterpenes (eucalyptol, pulegone, terpineol, and thymol) and phenylpropenes (estragole and isoeugenol) and extent their shelf-life and activity. CDs are cyclic oligosaccharides, consisting of (α-1,4)-linked α-D-glucopyranose units with a hydrophilic outer surface (outer protons H1, H2, H4, and H6) and a lipophilic inner cavity (with the inner protons H3 and H5) able to form water-soluble inclusion complexes with a variety of lipophilic poorly soluble molecules, including EOs. In order to ameliorate the formulation and prepare efficient and stable drug-in-cyclodextrin-in-liposome (DCLs) carriers, the authors investigate the intimate interactions of EO/Hydroxypropyl-β-cyclodextrin (HP-β-CD) complexes with liposomes. For this reason, DCLs, prepared by the ethanol injection method, were characterized for particle size, morphology, release kinetics, and storage stability. Regarding the size, the selected HP-β-CD/EO component-inclusion complexes had different effects on the liposome mean dimension. In particular, the entrapment of HP-β-CD/estragole, HP--CD/eucalyptol, and HP-β-CD/terpineol inclusion complexes into the aqueous core of liposomes determined the formation of larger vesicles compared to blank DCLs. Opposite results were obtained with HP-β-CD/isoeugenol, HP--CD/pulegone, and HP-β-CD/thymol complexes. This finding could be explained considering that the aromatic rings of isoeugenol and thymol are completely incorporated within the hydrophobic cavity of HP-β-CD, thereby hindering the interaction of isoeugenol and thymol with the acyl chains of the lipid bilayer. Hence, the effects of isoeugenol and thymol on liposome membrane and particle size are reduced. Furthermore, HP-β-CD could increase the drug-to-lipid mass ratio compared with the conventional incorporation of EO into the lipid phase of liposomes. The high complexation efficiency of EO components into HP-β-CD

obtained by these authors may allow predicting the ability of lipoid S100-DCL to encapsulate a phenylpropene or a monoterpene antimicrobial compound, hence the developed DCL formulations could be proposed as suitable pharmaceutical products able to improve the EO antimicrobial activity.

SOLID LIPID NANOPARTICLES AND NANOSTRUCTURED LIPID CARRIERS

Solid lipid nanoparticles (SLN) are nano-size particles prepared with lipids that remain solid at room and/or human body temperature. The selection of components, production techniques and possible applications were widely reviewed [146]. The use of solid lipids instead of liquid oils is a very attractive idea to achieve controlled drug release because drug mobility in a solid lipid matrix should be considerably lower compared with a liquid one. Several other advantages compete with the solid structure of these carriers, such as an increase in drug stability and high drug payload. Furthermore, large-scale production and eventual sterilization can be implemented easily. The main disadvantage of SLNs is due to the possibility that, during the storage, at least a part of the particles crystallizes in higher energy and more ordered structure. Due to its high degree of order, the number of imperfections in the crystal lattice is reduced, leading to drug expulsion. The second generation of nanoparticles, called nanostructured lipid carriers (NLC), was developed in order to overcome some of the potential limitations associated with SLNs. By creating a less ordered solid lipid matrix, i.e., by blending a solid lipid with a liquid one, a higher active loading of the particles can be achieved. In general, the drug can be located in between the fatty acid chains or in between the lipid layers and also in imperfections of the lipid matrix (*e.g.,* amorphous drug clusters). Therefore, the use of NLC yields an increase in the loading capacity of the active compound in the particles and also avoids or minimizes its expulsion during storage [147]. EOs, mixtures of lipophilic and generally volatile compounds, are ideal candidates to be conveyed in SLNs and NLCs or to be used as liquid components of the same nanoparticles [123]. Table **4** reports the references of some recent and interesting studies about different EOs loaded into these lipid nano-systems, potentially suitable for pharmaceutical application.

Table 4. Antimicrobial activity of solid lipid nanoparticles (SLN) or nanstructured lipid carriers (NLC) formulations encapsulating EOs.

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Not specified	Black cumin (*Nigella sativa*)	SLN	not evaluated	---	[149]

(Table 4) cont.....

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Antimicrobial agent	Clove (*Eugenia caryophyllata*)	SLN	*Salmonella typhi* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Candida albicans*	*in vitro*	[148]
	Turmeric (*Curcuma longa*)	NLC	*Escherichia coli* *Staphylococcus aureus* *Bacillus cereus* *Pseudomonas aeruginosa* *Streptococcus mutans* *Acinetobacter junii* *Candida albicans*	*in vitro*	[151]
Topical delivery	Parsley (*Ridolfia segetum*)	NLC	not evaluated	---	[150]
Anti acne	Java citronella (*Cymbopogon winterianus* Jowitt)	SLN	*Propionibacterium acnes*	*in vitro*	[152]
Wound healing	Mentha (*Mentha pulegium*)	NLC	*Streptococcus pneumoniaee* *Staphylococcus epidermidis* *Staphylococcus aureus* *Listeria monocytogenes* *Escherichia coli* *Pseudomonas aeruginosa* *Bacillus anthracis* *Salmonella typhimurium*	*in vitro* *in vivo*	[153]
	Rosemary (*Rosmarinus officinalis*)	NLC	*Staphylococcus aureus* *Pseudomonas aeruginosa*	*in vitro*	[154]
	Eucalyptus (*Eucalyptus globulus*) Rosemary (*Rosmarinus officinalis*)	NLC	*Staphylococcus aureus* *Streptococcus pyogenes*	*in vitro* *in vivo*	[155]
	Peppermint (*Mentha piperita*)	NLC	*Escherichia coli* *Salmonella typhimurium* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Staphylococcus epidermidis* *Bacillus anthracis* *Staphylococcus pneumoniae* *Listeria monocytogenes*	*in vitro* *in vivo*	[156]
	Lavandula + ferulic acid	NLC	not evaluated	---	[157]

(Table 4) cont.....

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Antifungal agent	Clotrimazol + Rosemary (*Rosmarinus officinalis*) Lavender (*Lavandula x intermedia* Sumian) Oregano (*Origanum vulgare*)	NLC	*Candida albicans* *Candida krusei* *Candida parapsilosis*	*in vitro*	[158]

Microbial resistance to antibiotics is a major problem in the treatment of diseases, so overcoming antimicrobial resistance is an urgent clinical need. Natural products formulated in nanoparticles are promising approaches to reduce microbial resistance. The antimicrobial activity of solid lipid nanoparticles (SLNs) containing *Eugenia caryophyllata* EO against human pathogens was evaluated [148]. A series of formulations was tested for their antimicrobial activity against *S. typhi*, *P. aeruginosa*, *S. aureus* and *C. albicans*. The results indicate that the antimicrobial activity of the EO was remarkably enhanced when it was encapsulated into SLNs. *Nigella sativa* oil was shown to possess antioxidant, anti-inflammatory, anticancer, analgesic and antimicrobial activities. The mixture of lipids used to encapsulate the oil, obtained by supercritical fluid extraction of the seeds, yielded SLNs of low crystallinity [149]. This mixture, which does not form a crystalline matrix, is able to overcome the problem of partial or total drug expulsion encountered with the use of high-purity lipids in the formulation of SLNs. Even *Ridolfia segetum* essential oil (REO), isolated by hydro-distillation from the Portuguese aromatic plant, was used with a dual key function as an active compound and simultaneously as a structuring component of the nanoparticles [150]. The introduction of REO in the lipid nanoparticles yielded a reduced particle size and homogeneous formulation suitable for skin application, with a high entrapment efficiency as well as a good stability profile. NLC was also employed to load turmeric extract (T-NLC) [151]. Turmeric is an indigenous herb in Southern Asia, and it is well recognized for its therapeutic properties. Curcuminoids contained in this herb extract, because of their lipophilic structure, show poor bioactivity in water solution, so their therapeutical use is severely limited. T–NLC showed higher antibacterial activity against rod shape Gram-negative bacteria than free turmeric extract. Java citronella oil (*Cymbopogon winterianus* Jowitt) appears to have the potential for use in topical anti-acne preparations due to its activity against *Propionibacterium acnes*. Unlikely, this use is limited by easy oxidation, high volatility and poor water solubility of the oil. Two preparations were developed, respectively oleogel containing citronella oil and oleogel containing SLNs loaded with citronella oil

[152]. The SLN preparation process caused a change in the composition of the citronella oil. Nevertheless, when the preparations were kept at 40°C for 120 days, the oleogel containing citronella oil-loaded SLNs still remained active against *P. acnes*, whereas the oleogel containing un-encapsulated citronella oil was inactive by day 45. Moreover, the solid lipid matrix provided protection from the volatile oil components and prolonged the release of citronella oil. Gels prepared from *Mentha pulegium* essential oil (MPO) loaded into nanostructured lipid carriers (MPO-NLCs) might hasten the infected wound healing process [153]. Wound repair is a crucial process: tissue regeneration enhancement and infection prevention are key factors to minimize pain, discomfort, and scar formation. The authors evaluated *in vitro* antibacterial activity of MPO-NLCs and *in vivo* wound healing activity of MPO-NLCs in the BALB/c mice model. MPO-NLCs showed high antibacterial activity against three Gram-positive bacteria strains (*S. epidermidis*, *S. aureus* and *L. monocytogenes*) and two Gram-negative bacteria strains (*E. coli* and *P. aeruginosa*) and evidenced potential use for the treatment of infected wounds. The efficiency of topical rosemary essential oil (REO) loaded into NLCs was also investigated *in vitro* and *in vivo*, evaluating their activity in the healing process of infected wounds [154]. REO-NLCs showed antibacterial activity against *S. epidermidis*, *S. aureus*, *L. monocytogenes*, *E. coli* and *P. aeruginosa*. Moreover, REO-NLCs could reduce the rate of tissue bacterial colonization and wound size while they increased vascularization, fibroblast infiltration, re-epithelialization and collagen production. Lipid nanoparticles loaded with rosemary or eucalyptus EOs were able to enhance healing of skin wounds [155]. The antimicrobial activity of nanoparticles was tested against two reference microbial strains: *S. aureus* and *S. pyogenes*. The capability of nanoparticles to promote wound healing *in vivo* was evaluated on a rat burn model. NLCs based on olive oil and loaded with eucalyptus oil showed good wound healing properties toward fibroblasts, associated with antimicrobial properties. Olive oil proved to exert a synergic effect with eucalyptus oil with respect to antimicrobial activity and wound repair promotion. The efficiency of peppermint essential oil (PEO) loaded into nanostructured lipid carriers (PEO-NLCs) was tested *in vitro* and *in vivo* [156]. For *in vitro* studies, PEO and PEO-NLCs were tested for antibacterial activity against *E. coli*, *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *B. anthraci*s, *S. pneumoniaee*, and *L. monocytogenes*. Against all these bacterial strains, they showed similar efficacy. Wound contraction, bacterial count, histological examinations, and molecular analyses were evaluated in infected mice. *In vivo* analysis showed that wound contraction rate, fibroblast infiltration, collagen deposition, and re-epithelialization were increased in PEO and PEO-NLCs-treated animals compared to the control group.

NLCs were also employed for the combined delivery of ferulic acid and Lavandula EO [157]. The co-presence of ferulic acid and Lavandula EO, as compared to synthetic isopropyl myristate-based NLC, increased nanoparticles stability due to higher ordering of lipid chains, as confirmed by morphological and physicochemical studies. The enhanced cytocompatibility was observed when ferulic acid and Lavandula EO were combined in the same carrier, as confirmed by *in vitro* studies on fibroblasts. Furthermore, the combined delivery of ferulic acid and Lavandula EO significantly promoted cell migration with higher effectiveness with respect to the free drug solution and the plain carrier (without the EO). The combined effect of the antioxidant ferulic acid and Lavandula EO, co-delivered in lipid nanoparticles, is effective in promoting cell proliferation and migration, and represents a promising strategy in the treatment of wounds. The increasing development of resistance of *Candida* spp. to traditional drugs represents a great challenge to the medical field for the treatment of skin infections. EOs were recently proposed to increase the effectiveness of pharmacological treatments. Mediterranean essential oil (*Rosmarinus officinalis*, *Lavandula x intermedia* Sumian, *Origanum vulgare* subsp. *hirtum*) lipid nanoparticles were used for clotrimazole delivery, exploring the potential synergistic effect against *Candida* spp [158]. Results of the *in vitro* biosafety on HaCaT (normal cell line) and A431 (tumoral cell line), allowed to select Lavandula and Rosmarinus as anti-proliferative agents to be used as co-adjuvants in the treatment of non-tumoral proliferative dermal diseases. Results of calorimetric studies on biomembrane models confirmed the potential antimicrobial activity of the selected oils due to their interaction with the membrane that improves their permeabilization. Nanoparticles provided a prolonged *in vitro* release of clotrimazole. *In vitro* studies against *C. albicans*, *C. krusei* and *C. parapsilosis*, showed an increase in the antifungal activity of clotrimazole-loaded nanoparticles prepared with Lavandula or Rosmarinus, thus confirming that NLCs containing Mediterranean EOs represent a promising strategy to improve drug activeness against topical candidiasis.

Nanoemulsions and Microemulsions

Nano- and microemulsions are ultrafine isotropic dispersed systems of two non-miscible liquids, generally consisting of an oily phase dispersed in an aqueous one. Most of the physical and pharmaceutical properties of nano- and microemulsions are a consequence of the small size of the dispersed globules [160]. In particular, the large specific surface area of the colloidal dispersion promotes permeation of delivered active compounds across biological membranes, thus resulting in improved bioavailability and pharmacological efficacy. Both these systems are colloidal dispersions characterized by submicrometer-size structures dispersed in a continuous phase. Anyway, while

microemulsions are thermodynamically stable systems, nanoemulsions are thermodynamically unstable but kinetically stable dispersions.

Nanoemulsions consist of very small droplets, exhibiting sizes generally lower than ~300 nm. Like conventional emulsions, nanoemulsions are, from a thermodynamic point of view, in a non-equilibrium state. However, the kinetics of destabilization of nanoemulsions is so slow that they are considered kinetically stable systems. This is mainly due to the very small size of the dispersed globules, resulting in the prevention of droplet flocculation and coalescence during long-term storage, as Brownian motions are able to overcome gravitational separation forces. On the contrary, the formulation of microemulsions corresponds to a thermodynamic equilibrium between all the components (generally water, oils and nonionic or ionic amphiphilic molecules). In this respect, microemulsions are formed spontaneously and may exhibit a wide range of structures, for example, worm-like, bicontinuous sponge-like, liquid crystalline, or hexagonal, spherical swollen micelles. All these different geometrical structures share common nanometric sizes that give them a bluish and translucent aspect.

Nano- and microemulsions are gaining increasing interest for effective delivery of EOs stem of their simplicity and easy fabrication, as well as the limited manufacturing costs. Indeed, the number of research reports investigating the encapsulation of EOs in this type of delivery carrier has markedly increased over the last years, but, at the same time, it has amplified the confusion and mix-up between these two systems. The terms nanoemulsion and microemulsion are frequently interchanged and not used in the proper way [160, 161]. The misconception arises from the fact that, in particular experimental conditions, microemulsions can strongly resemble nanoemulsions, exhibiting a very similar morphology in the form of spherical nano-droplets dispersed in a continuous phase. This often leads to a misinterpretation of the properties and characterization of the generated systems. In quite all of the research articles reported and analysed in this chapter, the formulations investigated are defined as nanoemulsions, even if some of them resemble much more the behavior of microemulsions. However, they will be here reported and commented on according to the classification used by the authors.

Micro- and nanoemulsions of EOs have been extensively investigated for their antifungal, antibacterial and antibiofilm activities. The most recent research reports investigating the potential of micro- and nanoemulsions to boost the application of EOs in the pharmaceutical field are summarized in Table **5**.

Eucalyptus and lemongrass EOs have been formulated as nanoemulsions and tested for their antibacterial and antifungal activity [162, 163]. While they

demonstrated limited antibacterial efficacy against *S. aureus* and *P. aeruginosa*, these nanoemulsions showed interesting *in vitro* antifungal activity. Anyway, while pure and nanoemulsified eucalyptus EO showed the same activity against *C. albicans*, *C. glabrata* and *C. tropicalis*, the nanoemulsion of lemongrass EO proved to have superior ability, compared to pure EO, to reduce the proliferation and the adhesion of pathogenic fungi to solid surfaces, inhibiting the formation of biofilms. Indeed, it was observed, through the microdilution and macrodilution techniques, the potentiation of the antimicrobial activity when the lemongrass EO was nanoencapsulated. In particular, it was evidenced that nanoencapsulation of the EO is able to improve its antimicrobial potential lowering the MIC values against *C. albicans* and *C. grubii*. The biofilm formation of *C. albicans* was inhibited in the same proportion by free oil and nanoemulsion; however, the free oil was tested at concentrations of 1.22 mg/ml and 2.56 mg/ml, while the nanoemulsion was tested at lower concentrations (0.28 and 0.58 mg/ml respectively). Thus, the formulation of the EO as nanoemulsion presented an antibiofilm activity against *C. albicans* 4 times greater than the free oil. Similar results were obtained against *C. grubii*, with a 2-time potentiation of the antibiofilm activity of nanoemulsion compared to pure EO. Based on the interesting results obtained *in vitro*, the antifungal efficacy of lemongrass and eucalyptus nanoemulsions was further tested *in vivo* using a murine model of vulvovaginal candidiasis in BALB/c mice [164]. The nanoemulsions showed a superior activity compared to pure EOs and, more interesting, they showed similar efficacy to a commercial antifungal cream. Indeed, the EOs in their free form did not show any antifungal activity, while their nanoemulsions were able to reduce the fungal load similarly or better than the control animal group treated with miconazole cream. The lemongrass nanoemulsion was also tested against rapidly growing mycobacteria [165]. These pathogens are opportunistic microorganisms that can cause both local and disseminated infections and when in biofilm, they become highly resistant to antimicrobials used in clinical practice. The formulation was tested on 3 strains of rapidly growing mycobacteria in planktonic and sessile forms. Although the nanoemulsion was not able to completely eradicate the mycobacteria as observed with pure EO, it showed interesting bacteriostatic activity probably associated to a slow and prolonged release of the active compounds from the nanoemulsion and consequent inhibition of the mycobacteria growth in a constant and controlled way.

Table 5. Antimicrobial activity of nanoemulsions or microemulsions formulations encapsulating EOs.

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Antibacterial agent	Celery (*Apium graveolens*)	nanoemulsion	*Staphylococcus aureus*	*in vitro*	[104]
	Cumin (*Cuminum cyminum*)	nanoemulsion	*Staphylococcus aureus*	*in vitro*	[108]
	Clove (*Syzygium aromaticum*)	microemulsion	*Staphylococcus aureus*	*in vitro*	[185]
	Lavender (*Lavandula x intermedia*)	nanoemulsion	*Escherichia coli* *Bacillus cereus*	*in vitro*	[43]
	Savory (*Satureja montana*)	nanoemulsion	*Listeria monocytogenes* *Staphylococcus aureus* *Staphylococcus hemolyticus* *Escherichia coli* *Klebsiella pneumoniaee* *Pseudomonas aeruginosa* *Serratia marcescens*	*in vitro*	[198]
Antimicrobial agent	Basil (*Ocimum basilicum*)	nanoemulsion	*Candida albicans* *Candida tropicalis* *Escherichia coli* *Proteus mirabilis* *Staphylococcus aureus*	*in vitro*	[200]
	Eucalyptus (*Eucalyptus globulus*) Peppermint (*Mentha piperita*) Lemongrass (*Cymbopogon citratus*) Garlic (*Allium sativum*) Ginger (*Zingiber officinale*) Dill (*Anethum graveolens*)	nanoemulsion	*Staphylococcus aureus* *Enterococcus faecalis* *Bacillus subtilis* *Pseudomonas aeruginosa* *Klebsiella pneumoniae* *Escherichia coli* *Salmonella typhi* *Candida albicans* *Aspergillus niger*	*in vitro* *in vivo*	[189]
Antimycobaterial agent	Lemongrass (*Cymbopogon flexuosus*)	nanoemulsion	*Mycobacterium fortuitum* *Mycobacterium massiliense* *Mycobacterium abscessus*	*in vitro*	[165]

(Table 5) cont.....

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Antimicrobial and antibiofilm	Eucalyptus (*Eucalyptus globulus*)	nanoemulsion	*Pseudomonas aeruginosa* *Candida albicans* *Candida tropicalis* *Candida glabrata*	*in vitro*	[163]
	Lemongrass (*Cymbopogon flexuosus*)	nanoemulsion	*Candida albicans* *Cryptococcus grubii* *Pseudomonas aeruginosa* *Staphylococcus aureus*	*in vitro*	[162]
	Chamomile (*Matricaria chamomilla*)	nanoemulsion	*Escherichia coli* *Pseudomonas aeruginosa* *Bacillus subtilis* *Staphylococcus aureus* *Streptococcus pyogenes* *Schizosaccharomyces pombe* *Candida albicans* *Candida tropicalis*	*in vitro*	[188]
	Wormwood (*Artemisia annua*)	nanoemulsion	*Escherichia coli* *Pseudomonas aeruginosa* *Bacillus subtilis* *Staphylococcus aureus* *Streptococcus pyogenes* *Schizosaccharomyces pombe* *Candida albicans* *Candida tropicalis* *Candida dubliniensis* *Candida krusei*	*in vitro*	[53]
Antibiofilm	Lavender (*Lavandula angustifolia*) Rosemary (*Rosmarinus officinalis*) Savory (*Satureja khuzestanica*)	nanoemulsion	*Pseudomonas aeruginosa*	*in vitro*	[98]

(Table 5) cont.....

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Vaginal candidiasis	Eucalyptus (*Eucalyptus globulus*) Lemongrass (*Cymbopogon flexuosus*)	nanoemulsion	*Candida albicans*	*in vivo*	[164]
	Geranium (*Pelargonium graveolens*)	nanoemulsion	*Candida albicans* *Candida krusei* *Candida tropicalis* *Candida parapsilosis* *Candida glabrata*	*in vitro*	[83]
	Mentha (*Mentha spicata* var. *viridis*)	nanoemulsion	*Candida albicans* *Candida albicans* *Candida kefyr* *Candida tropicalis*	*in vitro* *in vivo*	[166]
	Clove (*Syzygium aromaticum*)	nanoemulsion	*Candida parapsilosis* *Candida krusei* *Candida albicans*	*in vitro*	[167]
Topical treatment of candidiasis	Clove (*Eugenia caryophyllus*)	nanoemulsion	*Candida albicans* *Candida glabrata*	*in vitro*	[170]
Wound healing	Eucalyptus (*Eucalyptus globulus*)	nanoemulsion	*Staphylococcus aureus*	*in vitro*	[172]
	Clove (*Syzygium aromaticum*)	nanoemulsion	*Staphylococcus aureus* *Escherichia coli* *Pseudomonas aeruginosa* *Klebsiella pneumoniaee*	*in vitro*	[173]
	Orange (*Citrus sinensis*)	nanoemulsion	*Klebsiella pneumoniae* *Pseudomonas aeruginosa* *Escherichia coli* *Staphylococcus aureus*	*in vitro*	[174]
	Tea tree oil (*Melaleuca alternifolia*)	microemulsion	not reported	*in vivo*	[175]
Cutaneous and mucosal leishmaniasis	Orange (*Citrus sinensis*)	nanoemulsion	*Leishmania major* *Leishmania tropica*	*in vitro*	[176]
	Limon (*Citrus limon*)	nanoemulsion	*Leishmania major* *Leishmania tropica*	*in vitro*	[177]
	Clove (*Syzygium aromaticum*)	nanoemulsion	*Leishmania amazonensis* *Leishmania infantum*	*in vitro*	[178]

(Table 5) cont.....

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Bacterial and fungal pneumoniae	Tea tree oil (*Melaleuca alternifolia*)	nanoemulsion	*Klebsiella pneumoniaee* *Escherichia coli* *Acinetobacter baumannii* *Staphylococcus aureus* *Candida albicans*	*in vitro* *in vivo*	[183]
Oral health – mouthwash	*Curcuma xanthorriza*	nanoemulsion	*Streptococcus mutans*	*in vitro*	[187]
	Tea tree oil (*Melaleuca alternifolia*)	microemulsion	*Escherichia coli* *Staphylococcus aureus*	*in vitro*	[199]
Antibiotic resistant bacteria	Cinnamon (*Cinnamomum zeylanicum*) Clove (*Syzygium aromaticum*)	nanoemulsion	*Staphylococcus aureus* (MSSA) *Staphylococcus aureus* (VISA)	*in vitro*	[184]
	Thyme (*Thymus daenensis*)	nanoemulsion	*Acinetobacter baumannii*	*in vitro*	[186]
Photodynamic therapy	Clove (*Syzygium aromaticum*)	nanoemulsion	*Enterococcus faecalis* *Staphylococcus aureus* (MRSA)	*in vitro*	[179]
	Eucalyptus (*Eucalyptus globulus*)	microemulsion	*Staphylococcus aureus* *Staphylococcus epidermidis* *Pseudomonas aeruginosa*	*in vitro*	[180]
Drug delivery	Eucalyptus (*Eucalyptus globulus*)	nanoemulsion (SEDDS)	*Escherichia coli*	*in vitro* *in vivo*	[190]
	Clove (*Eugenia caryophyllus*)	nanoemulsion	*Candida albicans*	*in vitro*	[191]
	Lippia sidoides	microemulsion	*Enterococcus faecalis*	*in vitro*	[192]

(Table 5) cont.....

Application	Essential Oil	Delivery Vehicle	Microorganisms	*In Vitro/In Vivo* Evaluation	Refs.
Nose-to-brain delivery	Thyme (*Thymus vulgaris*) Clove (*Syzygium aromaticum*)	nanoemulsion	*Staphylococcus aureus* *Staphylococcus aureus* (MRSA) *Escherichia coli* *Klebsiella pneumoniaee* (carbapenem-resistant CR-Kp) *Acinetobacter baumanni* (carbapenem-resistant CR-Ab *Pseudomonas aeruginosa* (carbapenem-resistant CR-Pa)	*in vitro*	[197]
Anti-acne	Oregano (*Origanum vulgare*)	nanoemulsion	*Propionibacterium acnes* *Staphylococcus epidermidis*	*in vitro* *in vivo*	[181]
Upper respiratory tract infections	Thyme (*Thymus daenensis*)	nanoemulsion	*Pseudomonas aeruginosa* *Haemophilus influenzae* *Streptococcus pneumoniaee*	*in vitro*	[182]

Interesting antifungal activity was also observed with a nanoemulsion of the EO obtained from *Mentha spicata* var. *viridis* [166]. The naoemulsion was incorporated in a gel made of carbopol 940 to produce an emulgel formulation with texture and viscosity properties acceptable for mucosal administration. The final emulgel was evaluated *in vitro* and *in vivo* for its antifungal activity against selected pathogenic strains of *Candida* spp. The efficacy of the emulgel was compared with a simple gel formulation, containing a coarse emulsion of the EO and with clotrimazole. The emulgel demonstrated a significantly broader zone of growth inhibition than simple gel when studied against *C. albicans* using an agar well diffusion assay; moreover, compared to clotrimazole, the efficacy of the emulgel was around 68% that of the antifungal drug. The efficacy of the formulation was also tested *in vivo* using a vaginal candidiasis mice model. Emulgel was observed to be non-irritant over the mucous membrane and therapeutically more active than simple gel, whereas, in comparison to clotrimazole, emulgel was around 76% efficacious.

Antifungal activity was also observed with nanoemulsions of clove EO [167]. The formulations showed *in vitro* inhibitory effect against *C. parapsilosis*, *C. krusei* and a clinical strain of fluconazole-resistant *C. albicans*, even if the nanoencapsulation partially reduced the activity of the EO, as indicated by the

lower MIC values measured. The reduced antifungal activity may be explained by the addition of coconut oil to clove EO for the preparation of the nanoemulsion. In fact, as reported by Chang *et al.* (2012) [168] and further confirmed by Donsì *et al.* (2012) [169], the addition of low soluble/low volatile oils, such as coconut oil, to increase the physical stability of EO nanoemulsions towards Ostwald ripening, may have a significant influence on the antimicrobial activity of the formulation. In general, increasing the ripening inhibitor level in the lipid phase reduces the antimicrobial efficacy of the nanoemulsions, with a final effect depending on the type of ripening inhibitor used. According to this hypothesis, de Oliveira de Siqueira and co-authors [170] observed an improvement in the antifungal activity of clove EO when in nanoemulsion. In this work, the authors produced different nanoemulsions using an experimental design in order to define the most stable formulation, but no ripening inhibitors were included in the oil phase of the formulations. These nanoemulsions were tested against *C. glabrata* and *C. albicans* by determination of MIC and MFC (minimum fungicidal concentration) values. The reported results showed significant improvement in the activity of clove EO when in nanoemulsion, leading to the reduction of the inhibitory concentration compared to pure EO.

Nanoemulsions of *Pelargonium graveolens* EO were also investigated for the treatment of vaginal candidiasis [83]. A total of 8 *Pelargonium graveolens* EOs from six different countries were investigated. Some differences were observed in the antifungal activity of pure EOs, depending on the climate and growing conditions, which may lead to changes in the chemical profile of EOs extracted from the same species but from different locations. Anyway, more important differences were obtained with the formulation of the EOs, which were used to produce nanoemulsions and the latter inserted in gel formulations. Indeed, the addition of chitosan to thicken the nanoemulsions and promote their mucosal application resulted in the most significant increase of the antifungal activity, as indicated by the lower MIC values reported. The results showed that MIC values obtained with the gel formulations were lower for the most part of fungal strains tested, with an improvement of up to 64 times in antifungal activity against *C. albicans* and *C. glabrata*, when compared to pure EO and to nanoemulsion. *C. krusei* and *C. parapsilosis* also presented high susceptibility for the final formulation, with a reduction in MIC values of up to 32 times for *C. krusei* and 16 times for *C. parapsilosis*. This enhancement of the antifungal activity was attributed to the presence of chitosan, a polycationic polymer, which can improve EO delivery directly to *Candida* cells by allowing an electrostatic interaction between nanoemulsion and microorganisms. Indeed, chitosan possesses antimicrobial property that is mostly associated with the death-proceeding leakage of intracellular content, induced by malfunction and altered permeability of the negatively charged cell membrane, consequent to polymer adsorption [171]. For

this reason, chitosan was also used to prepare polymeric films, including a nanoemulsion of *Eucalyptus globulus* EO, to be used as potential wound dressing materials [172]. Chitosan films showed limited *in vitro* antibacterial activity against *S. aureus*, which was highly potentiated by the presence of the nanoemulsion. In particular, the impregnated films were able to inhibit the proliferation of wound isolated *S. aureus* proportionally to the volume of nanoemulsion included in the final formulation. 5% of nanoemulsion per g of polymer produced a zone of inhibition on solid medium similar to the positive control vancomycin, in the agar disc diffusion method, and shown bactericidal activity in the plate count assay.

To promote the topical application of clove EO, a nanoemulsion of this EO was used to impregnate fast-degradable nanofibers of polyvinyl alcohol prepared by electrospinning [173]. The impregnated nanofibers were tested for their antibacterial activity *in vitro* using a standard method for texture. The bacteria strains (*S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniaee*) were grown to reach defined turbidity, then the bacteria suspensions were incubated for 24h with the nanofibers. After incubation, a sample of the suspension was cultured on nutrient agar plates, then the number of colonies was counted, and the percentage of growth reduction was calculated. Under these conditions, the impregnated nanofibers were quickly degraded and able to completely inhibit the growth of all the different bacterial strains tested. Similar results were also obtained with a nanofibrous mat made of polycaprolactone impregnated with a nanoemulsion of *Citrus sinensis* EO [174] and with a semisolid formulation containing *Melaleuca alternifolia* EO incorporated in bicontinuous microemulsions, which resulted highly effective in the healing process of skin wounds, as it can promote a higher percentage of wound edge contraction when tested on Swiss mice [175]. A formulation analogous to the impregnated fast-degradable nanofibers of polyvinyl alcohol was also tested for the treatment of cutaneous leishmaniasis [176]. The impregnated nanofibers showed interesting leishmanicidal activity against promastigotes of *Leishmania major* and *Leishmania tropica*, which was mainly determined by the EO and partially potentiated by the presence of chitosan. Similar leishmanicidal activity against *Leishmania major* and *Leishmania tropica* was also observed with a nanoemulsion of *Citrus limon* EO [177], whereas nanoemulsions of clove EO resulted in effective against *Leishmania amazonensis* and *Leishmania infantum* in combination with photodynamic therapy [178]. Photodynamic therapy uses light and non-toxic photosensitizers in order to produce cytotoxic reactive oxygen species (ROS), which are capable of killing infectious microorganisms. Anyway, many photosensitizers, such as zinc phthalocyanine (ZnPc), are insoluble in water, therefore, their inclusion in nanoemulsions represents a valid strategy to promote their photobiological activity. Indeed, a clove nanoemulsion was able to maintain ZnPc in its

photoactive monomer form, avoiding its crystallization and sustaining its release over time. The efficacy of ZnPc encapsulated in clove nanoemulsion was greater than the free compound against the promastigote stage of *L. infantum* and *L. amazonensis* and the amastigote stage of *L. amazonensis*. The anti-amastigote activity was also observed in murine RAW 264.7 macrophages infected with *L. amazonensis*, producing a biological effect similar to amphotericin B. Moreover, the formulation showed a more selective photobiological activity against promastigote stages than against murine macrophages. Indeed, the formulation showed a selectivity index (SI = ratio between the CC50 for RAW 264.7 cells and IC50 for parasites) of 5.15 ± 0.60 and 6.74 ± 0.23 for *L. amazonensis* and *L. infantum,* respectively, meaning that the formulation was less toxic to the macrophages than to the parasites. In this work, clove EO did not contribute to the antileishmanial activity, whereas, in a different investigation from the same research group, it was able to potentiate the activity of ZnPc against *E. faecalis* and methicillin-resistant *S. aureus* (MRSA) [179]. Similar results were obtained encapsulating the photosensitizer Toluidine Blue O (TBO) in a microemulsion of eucalyptus EO [180]. Even in this case, the EO contributed to the inhibition of the growth of *P. aeruginosa*. Besides promoting the photobiological activity of TBO, the microemulsion also enabled good penetration of this photosensitizer through the stratum corneum of the skin. Indeed, observations of TBO skin distribution by confocal laser scanning microscopy evidenced the formation of a depot of TBO at a depth of about 200 μm in the porcine ear skin when it was delivered in microemulsion; on the contrary, TBO dispersed in water did not display appreciable penetration into the skin. Therefore, these formulations represent an interesting strategy for the treatment of local infections.

A nanoemulsion of *Origanum vulgare* EO has been proposed as a topical antibacterial formulation for the treatment of acne vulgaris [181]. *Origanum vulgare* EO was selected because it showed the strongest antibacterial activity in a panel of 7 different EOs, comprising oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), lemongrass (*Cymbopogon citratus*), tea tree (*Melaleuca alternifolia*), mentha (*Mentha piperita*), lavender (*Lavandula angustifolia*) and chamomile (*Matricaria recutita*). The antibacterial activity was evaluated *in vitro* on *P. acnes* and *S. epidermidis*, which are two of the major acne-associated bacteria. Based on the results obtained *in vitro* on pure EOs, oregano was formulated as a nanoemulsion and tested *in vivo* in an acne mouse model. To this end, BALB/c mice ears were intradermally infected with *P. acnes*. After two days, nanoformulation or 2% erythromycin was applied epicutanously on mice ears and then the anti-inflammatory and antimicrobial activity against *P. acnes* were measured. Treatment of the acne mouse model with the proposed oregano nanoemulsion resulted in the reduction of inflammation, bacterial load and healing of tissue superior to the reference antibiotic erythromycin. In addition, the

rate of reduction of mice ear thickness, post-treatment, was also superior with the nanoemulsion with respect to erythromycin.

EOs have also been evaluated as therapeutic options for the treatment of respiratory infections. A nanoemulsion of *Thymus daenensis* EO has been investigated for its antimicrobial activity against upper tract respiratory infections (URTIs) [182]. The MIC and MBC (minimum bactericidal concentration) values showed that the conversion of the EO in nanoemulsion improved its antimicrobial activity against a number of URTI generating microorganisms. Analogously, a nanoemulsion of tea tree oil showed good *in vitro* and *in vivo* antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and fungi, including several microbial strains responsible for respiratory infections [183]. In specific, the formulation showed strong *in vitro* antibacterial effects on 4 bacterial strains (*K. pneumoniaee*, *E. coli*, *A. baumannii* and *S. aureus*) and one yeast strain. Based on the *in vitro* activity, the nanoemulsion was tested *in vivo* for the treatment of bacterial and fungi-induced pneumoniae in Sprague-Dawley rats. *C. albicans* and *A. baumannii* were used as microbial models to induce lung injuries. Infected rats were treated with the nanoemulsion administered by aerosolization. The inhaled formulation was able to remarkably attenuate the symptoms of lung injury and inflammation in the infected rats, with an efficacy similar to standard therapies based on fluconazole and penicillin.

EOs possess high activity also against antibiotic resistant pathogens. For example, clove and cinnamon EOs showed effective *in vitro* antimicrobial activity against methicillin-sensitive *S. aureus* (MSSA) and vancomycin intermediate *S. aureus* (VISA) strains, when formulated as nanoemulsion [184]. The formulations showed lower MIC values over bulk oils with 83-166 fold improvement and an effect dependent on the surfactant used to produce the nanoemulsions. Moreover, the formulations showed potent activity not only on the bacterial cells in their planktonic form, but also on their biofilms. SEM analysis visually demonstrated alterations in the morphology of MSSA and VISA when exposed to the formulation. Treated cells showed distorted margins and irregular morphology. These damages and alterations to the integrity of the cytoplasmic membrane caused by the nanoemulsion were responsible of nuclear material release and cell death. Similar results were observed with nanoemulsions of celery, cumin seed and thyme EOs as well as with a microemulsion of clove EO tested for their antibacterial properties through the agar well diffusion assay and membrane permeability assay against *S. aureus* [104, 108, 185, 186]. In all these works, it has been observed that in interaction with pathogen cells, nanoemulsions and microemulsions caused destabilization of membrane permeability and alteration of its function, determining lysis, considerable cytoplasmic leakage and cell death.

Different EOs proved to be effective against microbial biofilms. They are heterogeneous microbial communities consisting of microcolonies of bacterial or fungal cells, which develop immersed in a self-produced extracellular matrix, giving rise to complex three-dimensional structures. The biofilm matrix is mainly composed of exopolysaccharides, proteins, lipids and nucleic acids, which are responsible for the defence mechanism, preventing the penetration of antimicrobial agents. After the biofilm is formed, the microorganisms usually become more resistant to antimicrobials. Anyway, different nano- and microemulsions of EOs have shown high effectiveness in the formation and maturation of microbial biofilms. This high efficacy has been explained by Ghaderi and co-authors [98] considering the Laplace pressure existing in micro- and nanoemulsions, which is responsible for the high chemical potential of the dispersed phase, which provides the driving force for mass transfer and effective penetration of the active compounds within the extracellular polymeric substances in the biofilms. These authors observed that nanoemulsification of EOs greatly enhanced the antibacterial activity against *P. aeruginosa* PAO1. They formulated 3 different EOs (*Lavandula angustifolia*, *Rosmarinus officinalis* and *Satureja khuzistanica*) and observed improved antibacterial and anti-biofilm activity of nanoemulsions compared to bulk oils. Indeed, nanoemulsions were able to efficiently inhibit biofilm formation and eradicate established biofilms, when used in sub-lethal concentrations. Similar efficacy was also observed with a nanoemulsion of *Curcuma xanthorrhiza* EO, which was tested for its antimicrobial activity on *Streptococcus mutans* biofilms [187]. In dentistry, *S. mutans* is a representative cause of dental caries among oral bacteria. Adhesive glucan, a metabolite of *S. mutans*, adheres to the surface of the teeth and aggregates various bacteria to form a biofilm, which results in dental caries. Therefore the *S. mutans* biofilm model is useful to simulate the efficacy of mouthwash. The *Curcuma xanthorrhiza* EO nanoemulsion showed stronger antimicrobial activity than commercial Cool Mint Listerine® against *S. mutans* biofilm in the mouthwash simulation, showing potential as an anti-biofilm agent by effectively inhibiting biofilm formation. Confocal laser scanning microscopy (CLSM) analysis was performed for structural and quantitative analysis of the biofilm after live/dead staining of the bacterial cells. CLSM analysis evidenced that the nanoemulsion treatment effectively damaged the bacterial cells within the biofilm and inhibited its maturation. In the same way, the effects of *Artemisia annua* EO on mature *Candida* spp. biofilms and its antimicrobial activity were studied by comparing a nanoemulsion formulation with a conventional emulsion [53]. The nanoemulsion showed the best ability to deliver the EO to the internal water phase of unilamellar liposomes, used as a cellular model for studying the intracellular delivery of *Artemisia annua* components from different formulations. These results explained the higher antibacterial and antifungal activity of the

nanoemulsion compared to the conventional emulsion. Indeed, the nanoemulsion showed stronger antimicrobial activity at lower concentrations against 5 bacterial strains and 5 fungal strains. Moreover, it was able to reduce the metabolic activity of mature biofilm-attached *Candida* species. Similar results were obtained by the same research group with chamomile EO formulated in nanoemulsion [188].

The antimicrobial activity of EOs should be potentiated by blending different oils in the same formulation. This approach was used by Osonwa and co-authors [189] that tested a nanoemulsion containing 9 different oils (not all of them were EOs) for its antimicrobial activity against different bacterial and fungal strains. The formulation showed good *in vitro* activity, particularly against *A. niger* and *S. aureus*, therefore, these strains were used to test the antimicrobial efficacy of the nanoemulsion in Wistar rats after oral administration. The assay was performed by treating the previously infected animals with the formulation or with a blend of the oils; ketoconazole and ciprofloxacin were used as positive controls. The formulation showed similar activity to ketoconazole and only slightly lower activity than ciprofloxacin. No improvement was given by the formulation compared to blended oils.

In a different approach, the antimicrobial activity of EOs can be exploited to potentiate that of antibiotics. In this sense, a nanoemulsion of *Eucalyptus globulus* EO was used to deliver two different drugs, namely neomycin and thioctic acid [190]. The formulation was developed as a self-nanoemulsifying drug delivery system (SEDDS) to be administered by the oral route for the treatment of hepatic coma, a clinical condition with a poor prognosis, that can be improved reducing the growth of colonic urea-splitting bacteria. The formulation was optimized using the Quality by Design technique, with a three-factor, three-level Box–Behnken statistical design and tested for its antimicrobial activity, which resulted in being mainly determined by the content of the antibiotic neomycin. However, the results showed that the presence of eucalyptus oil might have aided in potentiating the antimicrobial activity of the formulation. In a similar approach, a nanoemulsion of clove EO was used as a delivery system of ketoconazole (KTZ) [191]. The EO was selected on the base of solubility studies of KTZ in different oils. Even in this case, the presence of clove EO in the formulation seems to optimize the fungicidal activity of KTZ. Another research group proposed the use of a microemulsion of *Lippia sidoides*, popularly known as "rosemary pepper", for the delivery of chlorhexidine digluconate (CHX) to be used for disinfection of dental root canals [192]. Dental intracanal disinfection is crucial to achieving the success of endodontic treatment, avoiding the maintenance of endodontic infections. CHX can act as an irrigating agent for it, however, it can cause tissue irritation at high concentrations. Therefore, the combination of CHX with other antimicrobial agents can be useful to obtain

synergistic antibacterial effects, enabling the reduction of their doses and, in this way, making it possible to administer drugs more safely and with minimal adverse effects. The microemulsions showed antimicrobial effects against *Enterococcus faecalis* similar to a commercial gel of CHX conventionally used for this application. The *E. faecalis* bacterium was selected for this study, since it is generally considered the main agent of secondary and persistent root canal infections. This microorganism has a high capacity for penetrating dentinal tubules and can survive in harsh environments, such as extreme alkaline pH and high concentrations of salts, in addition to being resistant to many antimicrobial agents. Therefore, it is extremely important to the ability of the formulation to affect the substantivity of CHX. Indeed, the positive ions released by CHX can adsorb on dentin and prevent microbial colonization on its surface for some time past the actual period of application of the drug. The rate of CHX impregnation in tissues depends on the solubility of the drug in the dissolving medium and the number of available CHX molecules to interact with the dentin. In this study, it was observed that the microemulsions increased the availability of CHX, affecting its release pattern, and, in this way, they were able to have been more impregnated to the dentin blocks, which resulted in greater substantivity to the tissues in the root dentin model. Therefore, EO-based microemulsions showed great potential for the administration of drugs for the disinfection of root canals.

Gram-negative bacteria are more resistant to EOs than Gram-positive ones due to differences in the cell wall structures [193]. Formulating EOs as micro- and nanoemulsions aids in delivering active compounds to microbial cells, and, in this way, they can enhance their antimicrobial activity. Anyway, Gram-positive bacteria still remain more resistant than Gram-negative bacteria, even when treated with formulated EOs. While this effect is generally observed, in some cases, it has been reported that inactive compounds can be provided with some antimicrobial activity, when in nanoemulsions. This effect has been observed by Gundel and co-authors [162] with lemongrass EO. The free oil showed no activity against *P. aeruginosa*, while the nanoemulsion showed potential bactericidal activity. Similar results were also obtained by Garzoli and co-authors [43]. They formulated *Lavandula x intermedia* EO and its hydrolate in nanoemulsions and tested the prepared formulations against *E. coli* and *B. cereus*. Lower MIC values were observed for the nanoemulsified EO against both bacterial strains, and, even more, interesting, some activity was observed with the formulated hydrolate, which resulted in completely inactive when tested as bulk material. The improvement in biological activity observed with nanoemulsions is usually attributed to the small size and large curvature of the droplets of the dispersed phase. Nanoemulsions, because of the reduced dimensions, are expected to have better interaction with the biological membranes of microorganisms, since the driving force for the mass transport process, i.e., the concentration difference of

the antimicrobial in the vicinity of the oil droplet and in the bulk phase, is much higher due to the Laplace effect. However, other factors may also influence the antimicrobial activity, such as the surfactants used, the physical-chemical characteristics of the formulation, the microbial strains tested and the EO composition [169, 193 - 196]. In some cases, these additional factors can produce a reduction in the antimicrobial activity of the formulation compared to pure EO [197]. Rinaldi and co-authors [198] reported slightly decreased activity for clove and thyme EOs in nanoemulsion, whereas the same formulation was able to potentiate the activity of *Satureja montana* EO, but no explanation was provided by the authors for the different behavior. A reduction of the antimicrobial activity was also observed with a microemulsion of tea tree oil (TTO) stabilized by polysorbate 80 [199]. *In vitro* experiments on the antimicrobial properties of the colloidal system against *E. coli* and *S. aureus*, revealed that the TTO encapsulation led to a significant loss of biocidal activity. This effect was attributed to two different phenomena: (1) the electrostatic repulsion between the TTO-containing colloidal particles and the bacterial outer surface and (2) the preferential solubilization of the EO in the hydrophobic core of the dispersed structures. This study provides important information for improving the effectiveness of EOs-containing mouthwashes stabilized by polysorbates 80, because the presence of the surfactant may negatively affect the antimicrobial efficacy of the EO.

Overall, these results highlight the need for accurate characterization of the formulations. In particular, it is essential to assess whether the formulation process may have significantly affected the composition of the EO, because there is a direct correlation between chemical composition and biological properties of EOs. However, analyzing the present literature, it can be seen that this issue is often ignored. Only a few reports investigate the composition of the EO after the encapsulation process, and most of them use harsh methods which may further modify the EO composition [200], whereas mild and non-destructive analytical methods should be preferred [43, 196] for this purpose.

CONCLUDING REMARKS

EOs certainly, represent a potent alternative or adjuvant to traditional antibacterial and antifungal treatments in the pharmaceutical field; they also represent a powerful tool to tackle the problem of antibiotic resistance. Their encapsulation in lipid-based delivery vehicles can effectively enhance their use, improving penetration of EOs across biological membranes, chemical stability and dispersion in aqueous fluids. By the way, many efforts should be made for precise and complete characterization of the delivery vehicles. In particular, the effect of the encapsulation process on the composition of the selected EOs should be

routinely evaluated in order to try to find better correlations with the antimicrobial activity. Indeed, some preparation techniques may cause a significant loss or degradation of some of the EO components, and consequently, a modification of the antimicrobial activity may be expected, as there is a direct correlation between chemical composition, structure and biological properties of EOs. On the other hand, in some cases, a reduced biological activity may not be caused by the loss of active compounds of the EO, but it can be due to the excipients used to prepare the formulation or to the strategies used to stabilize the delivery vehicle, which fail to release the EO or release it at an improper rate. When these events happen, it is necessary to redesign the formulation.

Based on these considerations, in our opinion, a proper characterization of the composition of EOs before and after their formulation and their monitoring over time represent an essential requirement for a correct analysis and interpretation of the experimental results and, consequently, for the development of highly effective formulations, which can really improve and make feasible the use of EOs in the pharmaceutical field as antimicrobial agents.

CONSENT OF PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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CHAPTER 4

Antimalarial Endoperoxides: from Natural Sesquiterpene Drugs to a Rising Generation of Synthetic Congeners

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Abstract: Malaria is a vector-borne tropical disease caused by protozoans belonging to the genus *Plasmodium*, which has been scourging mankind for hundreds of millions of years. Despite the masterful progress in preventing disease transmission and reducing morbidity and fatal outcomes, malaria is on the rise again. Global concerns are focused on the spread of resistance to current drugs in the management of severe or ultimately lethal *P. falciparum* infection. To fully exploit the potential of existing agents and overcome their critical drawbacks, novel synthetic and formulation approaches have been explored. In this field, the clinical value of the natural drug artemisinin (ART) and its derivatives have been firmly established, and ART combination therapies (ACTs) have been recommended as first-line treatment against infection caused by chloroquine-resistant (CQR) *P. falciparum* strains. Over time, however, ART treatment options have become inadequate, and strict demand for new and effective agents has emerged. In this chapter, the medicinal chemistry aspects of artemisinins will be discussed, covering their unique mode of action and their structural features in relation to stability, pharmacokinetic profile, and antiplasmodial activity. Beyond ACT strategies, significant classes of compounds obtained through both ART covalent bitherapy and dimerization approaches will be presented as well. Furthermore, a special section will focus on the most recent endoperoxide-based synthetic antimalarials as new powerful and cost-effective alternatives to the "golden drug". It is expected that reported results will provide a strong incentive for further studies, and that unceasing research efforts will succeed in reaching the eventual eradication of this endemic plague.

Keywords: Antimalarial drugs, Artemisinin (ART), Artemisinin combination therapy (ACT), Chloroquine (CQ), Covalent bitherapy, Endoperoxides, Iron(II)protoporphyrin IX, Malaria, Molecular hybridization, Multidrug-resistant, Ozonides, Protozoan, *Plasmodium* spp., Sesquiterpene, 1,2,4,5-tetraoxanes, 1,2,4-trioxanes, 1,2,4-trioxolanes, World Health Organization (WHO).

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INTRODUCTION

Malaria is a devasting, vector-borne parasitosis caused by ancient unicellular protozoans of the genus *Plasmodium,* members of the large Apicomplexa taxon. This plague is endemic in tropical and sub-tropical regions, affecting approximately 40% of the world's population. Although malaria ranks fourth among the major human infectious diseases, after pneumococcal acute respiratory infections, acquired immunodeficiency syndrome (AIDS) and tuberculosis (TBC), it is recognized that *Plasmodium* spp. represent the deadliest parasite species throughout the history of mankind [1]. The reasons for parasite survival and continued infection in the human race are the results of an intricate evolutive interplay between hosts, pathogens, and infected vectors, deeply connected with climatic and socio-economic variables.

Over time, an impressive advance in antiplasmodial prophylaxis, chemotherapy, and transmission control through national malaria campaigns has been made, culminating in a sensible decline in disease incidence and associated mortality in many areas of the world in the last decade [2]. According to the World Health Organization (WHO), in 2019, malaria affected 229 million people, with 22 million fewer cases than in 2010 [3]. Unfortunately, in recent years the decrease in malaria burden has stagnated, owing to the persistence of critical conditions in endemic regions that undermine the success of therapeutic/prophylactic protocols and vector containment programs. However, the main causative factor for this *debacle* resides in the great genetic variability of the etiologic agent, leading to a highly adaptive response under widespread drug pressure.

The case of parasite drug resistance is ideally exemplified by the first-line antimalarial agent chloroquine (CQ); introduced in 1950s, this very effective and remarkably cheap drug was the most widely used in the 4-aminoquinolines class for the treatment of uncomplicated *Plasmodium falciparum* malaria till the emergence, over the course of 30 years, of chloroquine-resistant *P. falciparum* (CRPF) strains. The main mechanism of resistance envisions subsequent mutations in the gene *Pfcrt*, which encodes for the parasite CQ resistance transporter (*Pf*CRT) protein, whose amplification leads to an enhancement in the extrusion of the xenobiotic from the digestive vacuole (DV) of the protozoan [4, 5]. Moreover, the degree of resistance can be modulated by polymorphisms in the *P. falciparum* multidrug resistance-1 (*Pf*MDR1) protein, an ABC transporter that also regulates the flux of CQ across the DV membrane [5, 6].

An important contributor to resistance is the elimination time of the dispensed agent from the body; by administering drugs with short half-lives, the window of selection (*i.e.*, the time during which antimalarial drugs persist at sub-therapeutic

concentrations) for drug tolerance and resistance are minimized. Accordingly, at least till 2008, there was no evidence for clinically relevant resistance of *Plasmodium* parasites to artemisinin, a superior drug for the treatment of multidrug-resistant *falciparum* malaria, possessing a very short half-life (~ 1-3 h). The approach of ART co-formulation with a second, longer-acting antimalarial, commonly termed artemisinin combination therapy (ACT), has emerged as a means to confer greater protection against the development of drug-resistant mutants, preserving the effectiveness of ART and the partner agent in the time.

Furthermore, present *Plasmodium* species are the result of a hundred million years of apicomplexan evolutionary adaptation to increasingly elaborate host innate and acquired immunity, and consequently display a high degree of antigen variability to escape such defenses, and to arrange alternative invasion pathways through the generation of functionally redundant ligands for human cell receptors [7, 8]. This scenario emphasizes the need to broaden the range of therapeutic targets and the variety of replacement agents in order to overcome the current protocols' drawbacks and delay antimalarial resistance for the longer-term goal of malaria elimination. Recent advances in our understanding of biology and genomics of malaria parasites may provide information for putative novel structures to be targeted, and help in designing new generations of anti-malarial drugs based on unexplored chemotypes and acting with different mechanisms. On these bases, plentiful strategies for anti-malarial drug discovery are currently inquired, and progress in high throughput screening and computer-aided technologies offers exciting opportunities for developing suited candidates.

A mention of the general aspects of the vector-borne disease could not be presented here for brevity's sake, and readers are then referred to the overwhelming literature existing on the topic [9 - 20]. However, a brief excursus on the antimalarial drugs currently in use will introduce the special focus on the ART "miracle molecule" and its congeners.

THE NATURE-DERIVED MAINSTAYS OF ANTIMALARIAL THERAPY

The existing antimalarial therapeutic arsenal owes a great tribute to nature since most of the curative molecules derive from medicinal plants, fungi, and microorganisms. The therapeutic effect of herbal medicines traditionally used by local communities was confirmed and defined by time, and natural agents such as quinine, artemisinin, febrifugine, and lapachol have been the cornerstone of antimalarial treatment for thousands of years. Again, antimalarial screening of natural products from fungal and microbial sources, of both terrestrial and marine provenience, has revealed a wide potential in view of their chemical diversity [21, 22].

Further optimization of clinical effectiveness has been achieved through modification of structural and physical-chemical properties of these molecules, leading to a wide range of therapeutic optional remedies, which are distinct in chemistry, mechanisms of plasmocidal action, pharmacokinetic profiles, and toxicity issues [23 - 25]. The existing drugs can thus be grouped into major chemical and mechanistic classes, *i.e.*, arylaminoalcohols (quinine **1**, mefloquine **2**, halofantrine **3**, lumefantrine **4**, (Fig. **1**), quinoline derivatives, such as chloroquine (CQ) **5**, amodiaquine **6**, primaquine **7** (Fig. **2**) naphthoquinones, antifolates, antimicrobials, and spiroindolones (compounds **8-21**, Fig. **(3**), sesquiterpene lactone endoperoxides (artemisinins) and related synthetic tri/tetraoxanes and trioxolanes.

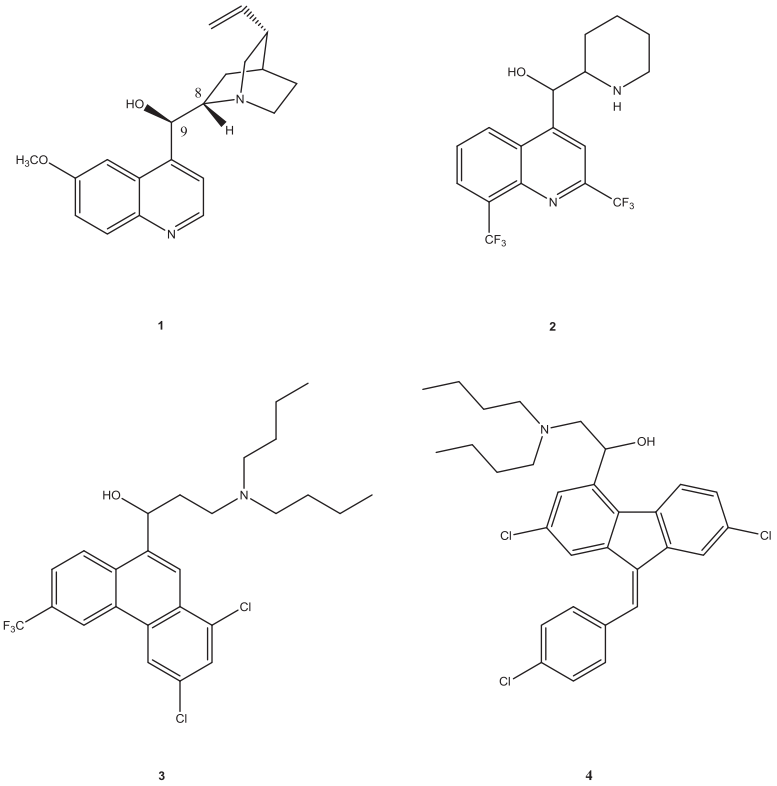
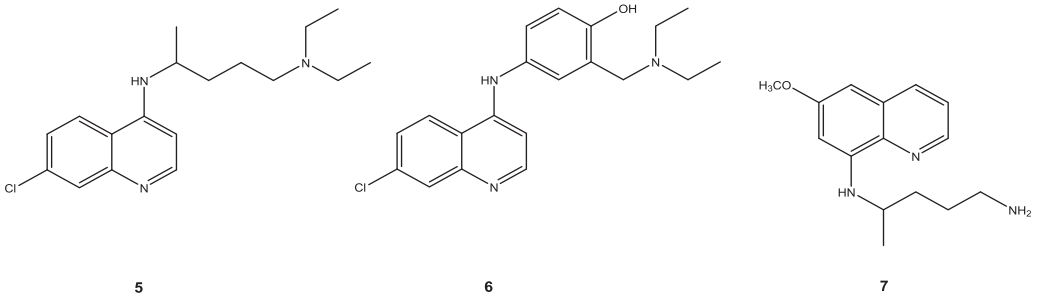


Fig. (1). Antimalarial arylaminoalcohol: quinine **1** and its congeners **2-4** obtained through quinuclidine nucleus disruption.

Critical drawbacks in current therapy are represented by the limited spectrum of activity of traditional agents and the occurrence of *Plasmodium* resistance, which have requested novel approaches, above all, the formulation of drug combinations (artemisinin-based or not artemisinin-based) [26], the exploitation of covalent

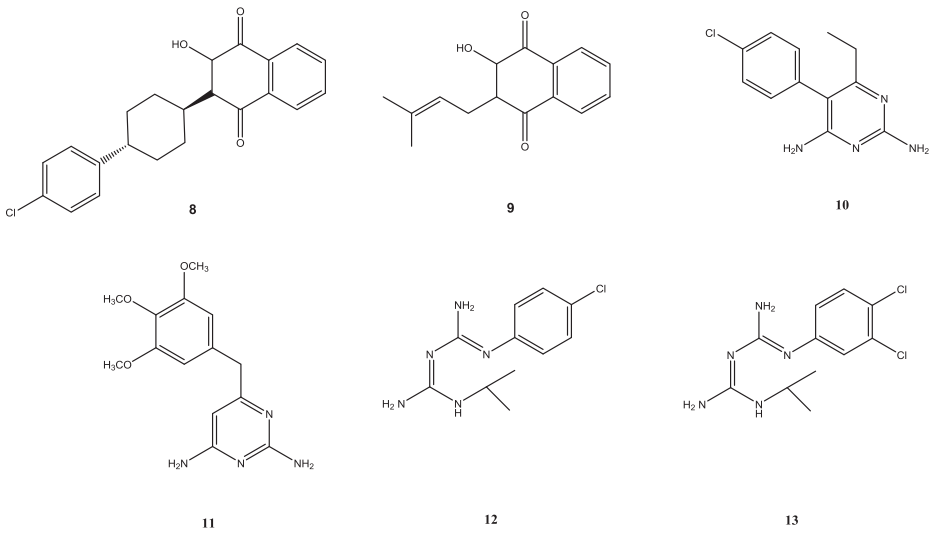
bitherapy [27], and the use of drug resistance reversers [28]. Despite this, there is still an urgent need of molecules targeting the multiple life stages, particularly the asymptomatic liver phase and the gametogenic blood stage, for all the species of human malaria. Moreover, in severe *falciparum* malaria, there is a strict demand for drugs preventing the parasite maturation to the cytoadherent pathological stage, which is primarily responsible for the life-threatening complications.

In this challenging fight, there is the consciousness that a number of unprecedented parasite proteins may be targeted by drugs built on new chemical entities, disclosing a new therapeutic era for the control and elimination of this plague.



5 6 7

Fig. (2). The antimalarial quinoline family: 4-amino- (**5**, **6**) and 8-amino- (**7**) derivatives in antimalarial use.



8 9 10

11 12 13

(Fig. 3) contd.....

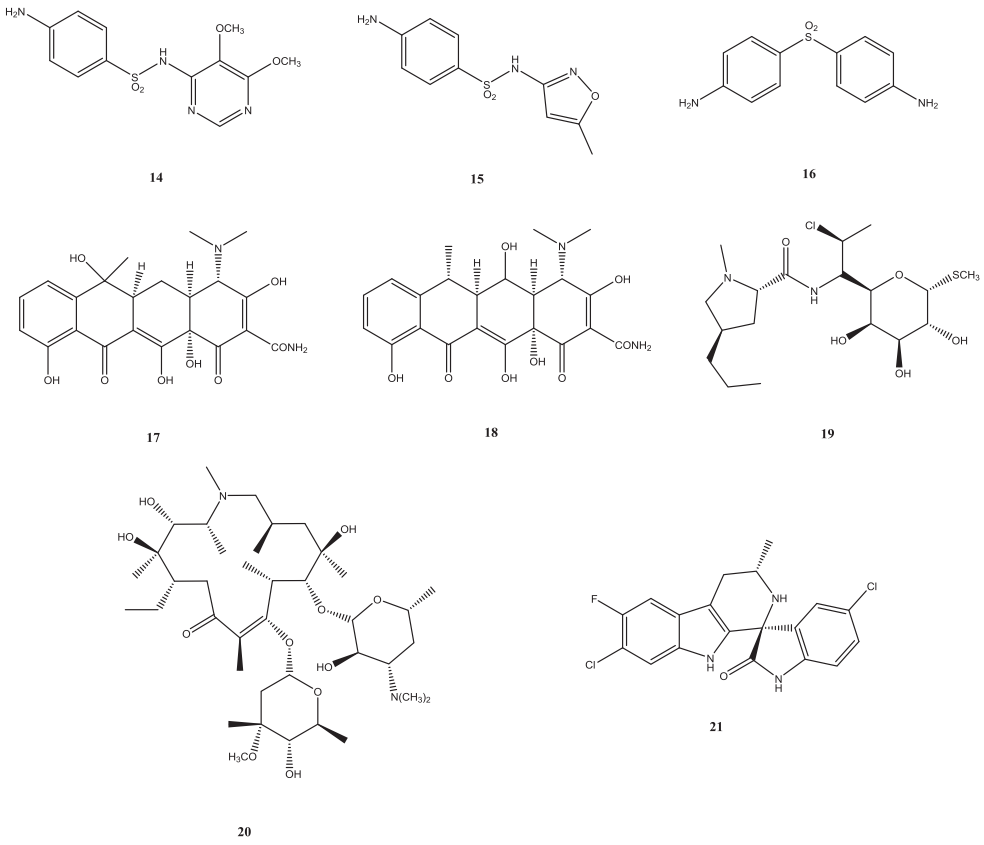


Fig. (3). Miscellaneous antimalarial agents, including atovaquone **8** and lapachol **9**, antifolates (pyrimethamine **10**, trimethoprim **11**, proguanil **12**, chlorproguanil **13**, sulfadoxine **14**, sulfamethoxazole **15**, dapsone **16**), antimicrobials (tetracycline **17**, doxycycline **18**, clindamycin **19**, azithromycin **20**), and the spiroindolone cipargamin **21**, a spirotetrahydro-β-carboline derivative.

In the following sections of this chapter, the importance of the prototype of plant-derived sesquiterpene lactone endoperoxide, namely artemisinin, will be emphasized in regard to its pharmacological and medicinal chemistry aspects. The key role of this natural drug and its combinations with auxiliary agents in the current antimalarial regimens, the potential of semisynthetic derivatives, codrugs, hybrids and next-generation synthetic analogs, derived from the most recent rational approaches holding promise for new drug development, will be covered here.

SESQUITERPENE LACTONE ENDOPEROXIDES: A NATURAL SOURCE OF 1,2,4-TRIOXANE-CONTAINING ANTIMALARIAL DRUGS

The Class of Sesquiterpene Lactones

With over 10,000 elucidated structures, sesquiterpenes or sesquiterpenoids constitute the largest family of terpenoids. This group of lipophilic compounds is widely present as secondary metabolites in plants, fungi, insects, bacteria, marine algae and invertebrates [29 - 31], where they play an important role in communication and defence, acting as attractants, deterrents, and antifeedants [32, 33].

Sesquiterpenes differ from mono-, di-, and tri-terpenes since they derive from the combination of three isoprene (C5) units: their 15-carbon atom precursor, the ubiquitous farnesyl pyrophosphate, undergoes programmed carbocation cascade reactions, providing a variety of sesquiterpene frameworks, which are often furtherly regio- and stereo-selectively subjected to hydroxylation or epoxidation reactions [34]. Sesquiterpenes exist in a wide variety of structures, including linear, monocyclic, bicyclic, and tricyclic hydrocarbon backbones.

Approximately 50% of this large class is represented by sesquiterpene lactones, containing at least one, generally pentacyclic (γ), lactone ring in a linear or annular skeleton. Over 5000 different structures of sesquiterpene lactones, mainly isolated as primary active constituents from the Asteraceae (Compositae) family, but also occurring in Apiaceae, Illiciaceae, Magnoliaceae, Solanaceae, and Euphorbiaceae [35], have been elucidated, uncovering an enormous chemical heterogeneity [36]. Suffice is to say that, based on their skeletal arrangement, cyclic sesquiterpene lactones are grouped into seven major classes: germacranolides and heliangolides (10-membered ring), eudesmanolides and eremophilanolides (6-6 bicyclic compounds), guaianolides, pseudoguaianolides, and δ-lactone-featuring hypocretenolides (5-7 bicyclic compounds) (Fig. **4**) [37].

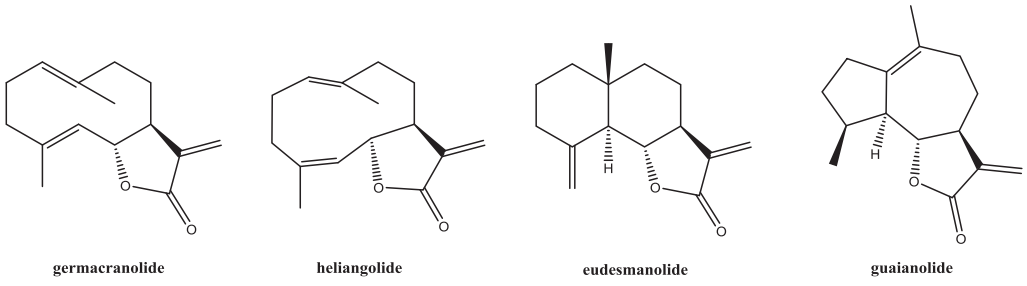


Fig. (4). Main classes of sesquiterpene lactones, exemplifying the most common arrangement of open or fused cycles.

Because of their unmatched structural diversity, sesquiterpene lactones display an impressive biological spectrum, including anti-inflammatory, anticancer, antidiabetic, antimicrobial, antiviral, antimalarial, and insecticidal activities, largely exploited in traditional medicine [38 - 41]. The lactone ring is prevalently fused to the remaining skeleton in a *trans* configuration [42, 43].

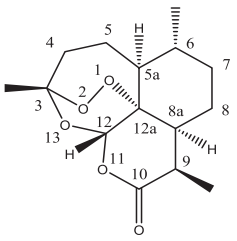
The major responsible group for the biological effects of these lactones is the *α-β*-unsaturated carbonyl group, which selectively acts as a strong alkylating agent in Michael-type addition reactions with intracellular nucleophiles (*i.e.*, cysteine residues of regulatory proteins) [44], leading to a disruption of their biological functions.

Sesquiterpenes containing not-activated lactone rings have been isolated from plants, and their unusual structures have been elucidated. These include the highly neurotoxic β-lactone anisatin [45], and δ-lactones, such as floridanolides[REMOVED HYPERLINK FIELD] [46], wedelolides [47], and the antimalarial drug artemisinin. This latter is furtherly clustered in the family of sesquiterpene lactone endoperoxides, a rare group of natural derivatives containing stably arranged alkyl peroxide rings [48, 49].

Sesquiterpene Lactone Endoperoxides: the Artemisinin Family

The Discovery of the Nobel Molecule

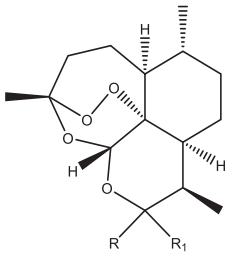
One of the most substantial advances in malaria chemotherapy has been the discovery of artemisinin **22** (Fig. **5**). The sesquiterpene drug is present in meaningful quantity in leaves (particularly the glandular trichomes), stems, and flowering buds of the shrub *Artemisia annua* L. (qinghao or sweet wormwood), a Chinese medicinal plant of the tribe Anthemideae (Asteraceae), whose traditional use dates back to 168 B.C. The adoption of qinghao to treat periodic fevers and malaria was mentioned by Ge Hong in a *Handbook of Prescriptions for Emergencies*, in full Eastern Jin Dynasty (317-420 A.D.).



22

Fig. (5). The molecule of artemisinin (ART) **22** with its skeleton numbering.

However, it was only during the Cultural Revolution that the Chinese government, in an effort to support North Vietnam troops fighting in malaria-plagued areas, started the antimalarial "Project 523", which should have brought in 1972 Prof. Youyou Tu and her research team [50] to first isolate seven bioactive sesquiterpene compounds from the ethereal extracts of aerial parts of *A. annua*; among them, **22** (quinghaosu, meaning "principle from qinghao") was found to possess the most potent antimalarial properties. Having the best on the difficult task of determining the complex structure of the new molecule, the related dihydroartemisinin (DHA) **23**, and its key derivatives, the lipophilic β-artemether **24** and the water-soluble sodium salt of the α-conFig.d artesunic acid **25** (sodium artesunate), were then prepared by the Chinese team as more versatile agents (Fig. **6**). Subsequent clinical studies established the unprecedented ART antimalarial efficacy, and Prof. Tu finally received the Nobel Prize in 2015 for her inestimable work.



22 artemisinin R = R_1 = O
23 dyhydroartemisinin R = H, R_1 = OH
24 artemether R = H, R_1 = β–OCH_3
25 artesunic acid R = H, R_1 = α–$OCO(CH_2)_2COOH$

Fig. (6). The fab four.

The Bioactivity Profile of Artemisinin

Since then, ART and its derivatives have been successfully used as first-line drugs in the cure of *falciparum* malaria, proving effective also against parasite strains resistant to CQ, and still represent the most outstanding part of the protocols for severe disease treatment [51]. These agents are characterized by fast-acting and low nanomolar activity, directed against the broadest range of parasite developmental stages [52, 53]. Artemisinins have been shown to possess non-malarial activities as well, particularly anti-cancer effects mediated by oxidative stress [54 - 56].

Unlike conventional antimalarials, that target *Plasmodium* mature stages, ART derivatives rapidly clear circulating rings, reducing parasitaemia and preventing the consequences of the cytoadherence phenomenon. In addition, they possess gametocidal properties, which contribute to the blocking of disease transmission. The toxicity of artemisinins is negligible, as only reversible effects on

erythropoiesis and allergic reactions are observed in humans, although rare but significant toxicity has been reported [57].

The Production of Artemisinin

In view of its therapeutic relevance, there is a high demand for ART in the international market, increasing dramatically each year. The ART yield from *A. annua* is a serious limitation to meet the current need since it ranges from 0.01 to a maximum of 2% of the dry weight of the shrub tissue. Alternative strategies, including plant breeding technologies [58, 59], biotechnological approaches [60], and total and semi-syntheses [61 - 63], have been investigated to enhance ART production and availability of this too expensive compound. The possibility of engineering the ART production can benefit from a thorough understanding of its biosynthetic route.

The anabolic cascade belongs to the isoprenoid metabolite pathway, and it originates from the common biosynthetic precursor isopentenyl diphosphate, formed via either the cytosolic mevalonate route or the plastid-localized mevalonate-independent pathway [64]. Among the different key enzymes involved in the biosynthesis, amorpha-4,11-diene synthase, a sesquiterpene cyclase which catalyzes the annulation of farnesyl diphosphate to amorpha-4,1--diene, has been postulated as the main regulatory switch for the final assemblage of dihydroartemisinic acid (DHAA), the precursor of ART **22**. The last non-enzymatic step is the conversion of DHAA to **22** through a ROS-mediated photo-oxidative reaction involving highly reactive allylic hydroperoxides as intermediates [65, 66]. Synthetic approaches to **22** are not economically feasible because of costly terpene-based starting materials and long reaction sequences, envisioning redundant protecting group strategies. A relatively straightforward enantioselective total synthesis of **22**, starting from the cheap cyclohexenone and claiming only five pots, was reported by Zhu and Cook in 2012 [67]; nevertheless, very unlikely the chemical routes will address the shortage problem, or supplant the extraction from *A. annua* as the favored method of supply.

Uncovering Artemisinin Structure and Key Determinants for Activity

From the structural point of view, **22** belongs to the amorphene sub-group of *seco*-cadinanes. The amorphane/cadinane group of bicyclic sesquiterpenes is by far the largest class of sesquiterpenes found in *A. annua*, which incorporate the characteristic decaline (cadinane or cadalane) scaffold resulting from the C-1/C-6, C-5/C-10 cyclization of farnesyl pyrophosphate. Amorphane sesquiterpenes differ from their cadinane counterparts, characterized by a *trans*-decalin ring junction (1α,6β), for the presence of a *cis*-arranged scaffold (1β,6β) (Fig. **7**). The prefix "*seco*" indicates that carbon-carbon bond cleavage has occurred, in this case

between C-4 and C-5, accompanying formation of the 1,2,4-trioxane ring in the final biosynthetic phase of **22** [68, 69].

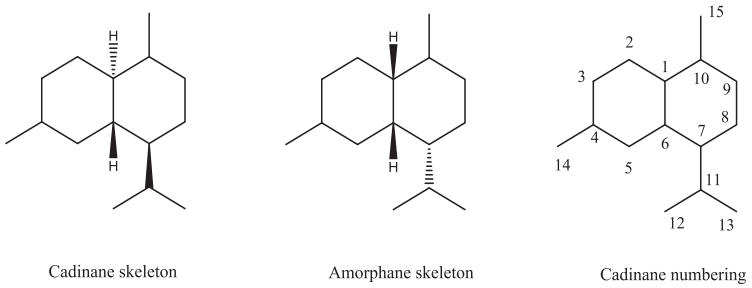
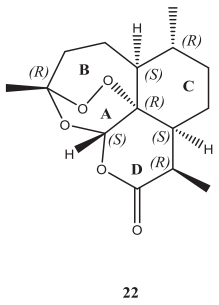


Fig. (7). Bicyclic sesquiterpenes: amorphane/cadinanes scaffolds.

The structure of this enchanting molecule consists of four substituted rings fused together (Fig. **8**): a 1,2,4-trioxane ring (A), adopting a boat conformation in the solid state, a 1,2-dioxaepane ring (B), a cyclohexane ring (C), and a δ-lactone ring in the *trans*-conformation (D).



22

Fig. (8). Labeling system and absolute configuration of (+)artemisinin.

Furthermore, **22** possesses unique perketal and acetal functionalities, very appealing to organic chemists, which represent the most sensitive elements in an unusually stable sesquiterpene lactone skeleton bearing an endoperoxide bridge. Due to the presence of 7 asymmetric carbon atoms in the molecule, theoretically, $2^7 = 128$ diastereomers could be possible. ART **22** is a dextrorotary compound ($[\alpha]_D$ admitted range: +75°/+78° in ethanol as testing item of identification) [70] with absolute configuration at chiral centres 3*R*, 5a*S*, 6*R*, 8a*S*, 9*R*, 12*S*, and 12a*R*,

corresponding definitely to (3*R*,5a*S*,6*R*,8a*S*,9*R*,12*S*,12a*R)*-3,6,--trimethyloctahydro-3*H*-3,12-epoxy [1, 2]dioxepino[4,3-*i*]isochromen-10(12*H*)-one.

ART suffers from poor solubility, either in lipid or water, although it displays a certain degree of lipophilicity and amphiphilic character that are considered crucial for membrane cell permeation.

Extensive SAR studies have established which are the structural determinants for ART bioactivity, clarifying in the meantime that the intact molecular architecture of **22** is not necessary for the maintenance of the antimalarial activity [71 - 73]. Neither the peroxide function nor the 1,2,4-trioxane ring alone is sufficient to confer antimalarial activity, but they are accepted as an essential part of the pharmacophore when assembled within the scaffold. Accordingly, derivatives where the endoperoxide-carrying ring is opened, or the endoperoxide bridge is broken or substituted, are completely devoid of activity, as exemplified by 2-deoxyartemisinin (or deoxartemisinin) **26** (Fig. **9**)., the reduced form of the drug containing only an ether bridge.

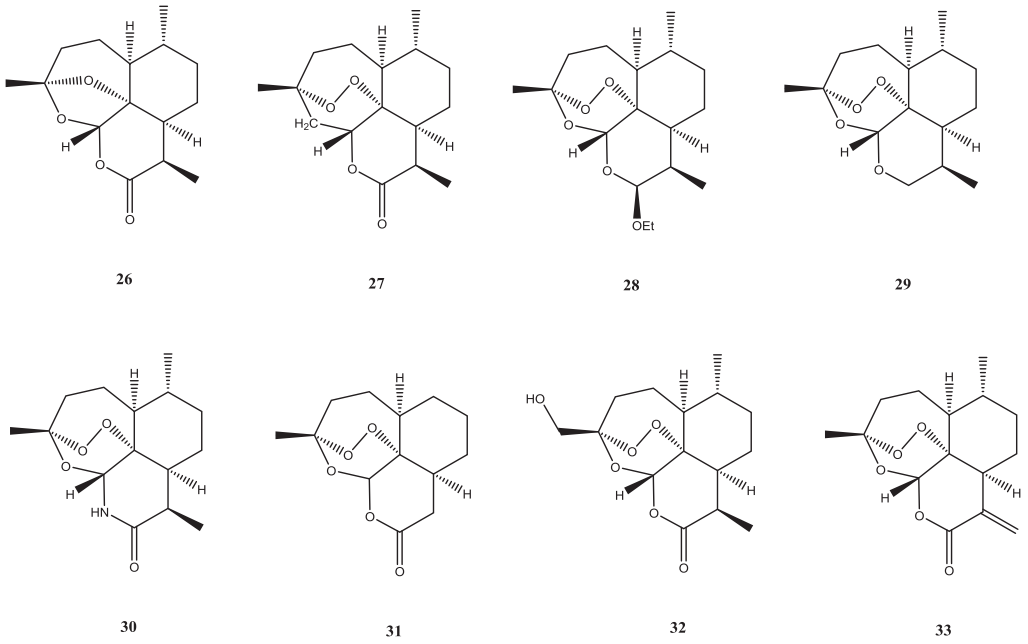


Fig. (9). 2-Deoxartemisinin **26**, (+)-13-carba-artemisinin **27**, β-arteether **28**, 10-deoxoartemisinin **29**, 11-az--artemisinin **30**, (±)-6,9-desmethylartemisinin **31**, (+)-3-hydroxy-methylartemisinin **32**, and the natural sesquiterpene artemisitene **33**.

Interestingly, the non peroxidic oxygen atom may be replaced by a methylene unit to give the still, albeit less potent 13-carba-artemisinin **27**, characterized by a 1,2-dioxane ring. Again, the 1,2-dioxaepane (B) and cyclohexane (C) rings are not required for activity. As far as the importance of acid-labile functions is concerned, the perketal moiety has been found essential for good activity.

The six-membered lactone ring is the most versatile, since the lactone carbonyl in C-10 can be reduced to give DHA **23**, whose schizonticidal potency *in vitro* is up to twice that of the parent compound. The significance of **23** preparation, disclosing the stability of the peroxide unit under certain chemical conditions such as $NaBH_4$, $LiAlH_4$, or di(isobutyl)aluminum hydride (DIBAL) as common reducing agents, is fundamental as it opened the way to the synthesis of the first generation of ART derivatives, grouped in lipid- and water-soluble drugs, which address the problem of the low ART solubility in both fractions. Derivatization, however, does not eliminate potential instability, because β-artemether **24** and β-arteether **28** still feature acetal groups, and artesunic acid **25** an acetal ester bond, at their C-10 centers.

Complete C-10 deoxygenation resulted in the remarkably effective 10-deoxoartemisinin **29**, showing nanomolar activities against both chloroquine-sensitive (CQS) and chloroquine-resistant (CQR) *P. falciparum* strains superior to that of **22**, and approximately similar effect *in vivo* against *P. berghei*.

The lactone ring can be bioisosterically replaced by a lactam counterpart, to afford 11-aza-artemisinin **30**, without affecting the antimalarial efficacy. The SARs of more extended modifications at the C-10 and C-11 positions of **22** will be presented afterwards.

As far as peripheral methyl groups are concerned, (±)-6,9-desmethylartemisinin **31**, prepared by Avery *et al*. starting from pyrrolidinocyclohexene and *cis*-1,--dichloro-2-butene, was shown to maintain significant antimalarial activity against resistant strains of *P. falciparum* [74].

If the removal of both **22** methyl substituents at C-6 and C-9 is still compatible with antiplasmodial activity of the resulting derivative, the C-3 methyl group has been suggested to be a crucial determinant to establish lipophilic interactions with putative proteins of the parasite, as evidenced by biological results on fully synthetic derivatives [75, 76]. Analogs of **22** substituted at C-3 were found to be less active than those substituted at C-9 with ethyl and propyl groups (maximal activity), whilst (+)-3-hydroxy-methylartemisinin **32** was found completely inactive.

In addition, desaturation of the C-9−CH_3 single bond is naturally present in the sesquiterpene endoperoxide artemisitene **33,** isolated as a minor constituent in an American variant of *A. annua by* Acton *et al.* in 1985 [77]. Although its antimalarial profile is inferior with regard to **22**, artemisitene can be used for derivatization, in view of the presence of the α,β-unsaturated lactone trap (Michael acceptor).

The Mechanism of Plasmocidal Action

Although the ART mechanism of action is an intensely debated and still controversial subject, there is general concordance that the endoperoxide linkage is the key determinant for the antimalarial activity of ART and its derivatives. According to the widely accepted "C-radical hypothesis", heme represents both the activator and the target of ART [78]: the peroxide bridge is reductively-cleaved in a heme-dependent process involving the redox-active metal center of the iron(II)-protoporphyrin IX unit, released during the parasite digestion of hemoglobin. Heme reacts with ART much more efficiently than the other iron(II)-containing species, such as free ferrous iron or ferrous sulfide. Evidence that ART reductive activation can only occur if heme is not trapped inside hemoglobin clearly links activity with the unique heme detoxification pathway of malarial parasites, *i.e.*, hemoglobin digestion, explaining the high ART specificity towards *Plasmodium spp*. Following an electron transfer from the low-valent iron(II)-heme to the antibonding σ* LUMO orbital of the peroxide bond, short-lived alkoxy-radicals are formed which, after a thermodynamically favored intramolecular rearrangement, give rise to primary or secondary carbon-centered radicals. These, in turn, alkylate heme to generate non-polimerizable covalent adducts, highly toxic for the parasite, as previously discussed, or target a number of sensitive macromolecular *Plasmodium* proteins [79 - 81], such as *Pf*CRT, *Pf*MDR1, and the translationally controlled tumor protein (TCTP) [82], disrupting many essential pathways and leading to parasite death. Consistent with this mechanism, ART and its derivatives actually represent bioprecursors, that absolutely require endoperoxide group cleavage for drug activation and subsequent antiplasmodial activity in the heme-rich environment specific to infected erythrocytes.

The alternative hypothesis envisions a dual-acting role of mitochondria in the ART specific action: due to its lipophilic nature, the drug reaches the mitochondria membrane, where it is activated by some unknown factors, possibly the components of electron transport chains, with the local generation of free radicals; the dramatic increase in ROS generation causes mitochondrial membrane depolarization, impairment of organelle normal functions, and eventually leads to cellular dysfunction and apoptosis [83, 84]. Existing data demonstrated that the

intrinsic difference between malarial and mammalian mitochondria is the basis of ART specificity. However, the mitochondria-based model cannot exclude the influence of heme on ART activation, as heme is mainly synthesized in mitochondria, and its role as a catalytic source of ROS is well defined [78].

Controversial evidence regards the implication of *Pf*ATP6, the *P. falciparum* orthologue of human sarco-endoplasmic reticulum Ca^{2+}-dependent ATPase (SERCA), as a target. The hypothesis implies that, upon activation by catalytic iron, ART radicals may bind to *Pf*ATP6 through specific interactions, which is followed by irreversible protein damage [81, 85 - 87].

Several computational studies, based on molecular docking and unconventional quantitative structure-activity relationship (QSAR) analyses, were used to predict the antimalarial activity of artemisinins with unknown activity [88]. Molecular docking simulations were used to probe the interactions between artemisinins and hemin. Based on the putative bioactive conformations obtained in the selection, 3D-QSAR models were generated and shown to have good predictive accuracy. A good correlation was found between antimalarial activity and binding energy deriving from electrostatic interactions involving the peroxy group of the analogues and the Fe^{2+} in hemin. The binding mode is beneficial to the electron transfer from iron to the peroxy group, which may lead to the rupture of the bond and the formation of free radicals [89]. An interesting computational study suggests that the iron-ART adduct inhibits *Pf*ATP6 through an allosteric mechanism [90].

Resistance to Artemisinin

In view of its fast clinical response and safety profile, ART has encountered global application to treat uncomplicated and severe *P. falciparum* malaria, and blood stage *P. vivax* infections. However, antimalarial ART monotherapy was soon considered inappropriate for more than one reason, including the frequency of recrudescence cases, due to short plasma half-life, and the potential insurgence of resistant parasites.

The mechanism of parasite resistance to ART is debated: according to *in vitro* experiments, it would be related to observed polymorphisms in the gene encoding *Pf*ATP6 [91]. ART resistance across several Countries in Southeast Asia is associated with mutations in *kelch13* gene (*Pfk13)* sequences encoding the β-propeller and BTB/POZ domains, which lead to increased parasite survival rates in response to DHA *in vitro*, and long parasite clearance half-lives in response to ART treatment *in vivo* [92 - 94].

Artemisinin Derivatives and Analogs

In an effort to improve ART properties in terms of efficacy, resistance susceptibility, selectivity indices, and particularly pharmacokinetic issues, several cognate drugs have been developed and admitted to clinic use. The synthetic elaboration of these derivatives, starting from the historical artemether, arteether, artesunate, to include artelinic acid, and the most recent analogs artemisone and artemiside, has allowed for in-depth knowledge of their chemical properties, as well as the establishment of key SARs.

The first generation of derivatives obtained through modification at the C-10 position of the sesquiterpene scaffold was essentially designed to ameliorate bioavailability. Reduction of the lactone carbonyl led to the corresponding lactol, dihydroartemisinin (DHA) or artenimol **23**, as a couple of epimeric hemiacetals at C-10. Derivative **23** is three-to five fold more active *in vitro* than related compounds, but is highly neurotoxic in humans at high doses, particularly the β-hemiacetalic adduct. Compound **23** is suitable for derivatization with either lipophilic or hydrophilic functionalities derivatives, to afford compounds which *in vivo* are mainly converted, albeit to different extents, to the neurotoxic metabolite **23**.

Structure-activity studies on derivatives indicate that the degree of residual neuronal toxicity is influenced by stereoisomerism and substitutions at the 10 position of the ART backbone, whilst the endoperoxide is a necessary but not sufficient determinant of neurotoxicity [95].

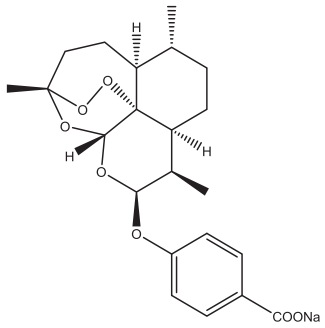
The lipophilic ether derivatives β-artemether **24** and β-arteether **28** are well absorbed on intramuscular administration, whilst the hydrophilic artesunate, *i.e.*, the sodium salt of the lactol hemi-succinate derivative (artesunic acid **25**), is suitable for intravenous and suppository routes [96]. When administered intramuscularly, owing to the 'depot' effect of their oily formulations, the lipid-soluble **24** and **28** are released slowly and can cross the blood-brain barrier before being completely metabolized to artenimol [97]. On the contrary, sodium artesunate, designed to be intravenously administered, is largely hydrolyzed in plasma and the liver following entrance into the body. Despite this event, sodium artesunate is actually the drug of choice among the group. Arteether may exist as the C-10 β-epimeric form (β-arteether or artemotil **28**) or α/β-arteether, a mixture of α- and β-diastereomers at a ratio of 30:70, respectively. Either **28** or α/β-arteether can be used in the treatment of severe *falciparum* malaria, without a statistically significant difference between cure rates [98].

In general, the oral formulations of these drugs are not completely absorbed, and their bioavailability is low, because of extensive first-pass metabolism in the liver.

In addition, the development of resistance has been documented, and therefore their use as monotherapies is not recommended.

Although characterized by superior antimalarial activity and increased lipid-permeability/water solubility compared to the lead compound, these agents are still affected by metabolic instability, short half-lives, and questionable neurotoxicity, since they represent pro-drugs of **23** [99]. Therefore, the emphasis had to be placed on the development of derivatives incapable of providing this active plasma metabolite.

In this regard, sodium artelinate (Fig. **10**). was shown to address most of the drawbacks of its precursors [100]. This water-soluble agent, namely the sodium salt of artelinic acid **34**, the β-hydroxymethylbenzoate ether of **23**, was expected to be resistant to liver metabolism, starting from the speculation that the benzoate moiety would increase the steric hindrance of the drug, making the ether bond less accessible to oxidative enzymes. Furthermore, its electron withdrawing effect on the ether bond would further increase metabolic stability. Actually, sodium artelinate was found to possess a much longer plasma half-life than **24**, **28** and sodium artesunate. Despite controversial results, the molecule has been withdrawn because of neurotoxicity concerns [101].

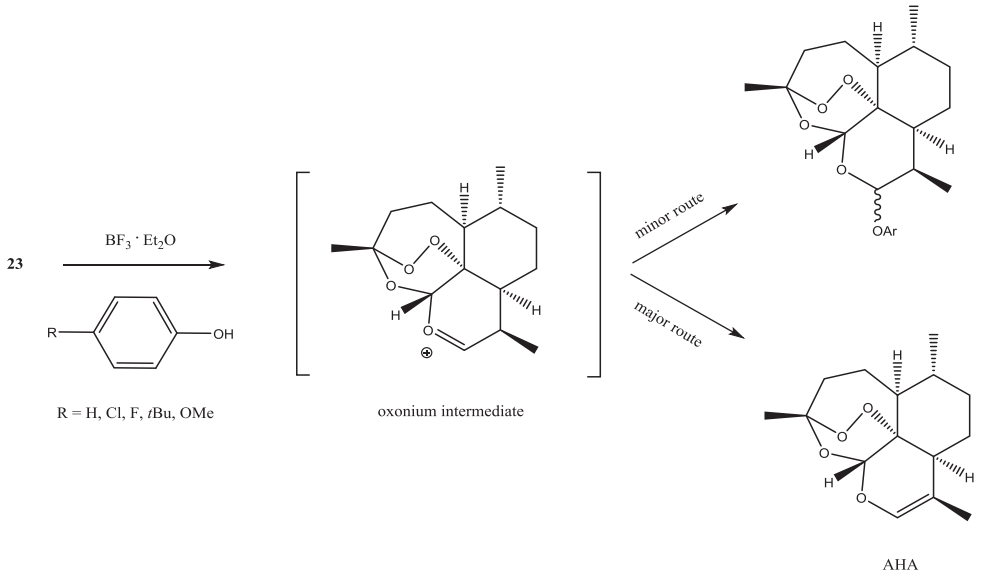


34

Fig. (10). The sodium salt of artelinic acid **34**.

A similar approach has been pursued by O'Neill's group with the elegant incorporation of an ether-linked phenyl ring to block oxidative formation of **23***in vivo* [102]. Several synthetic approaches have been previously investigated to couple **23** with various phenols: by using boron trifluoride diethyl etherate catalys

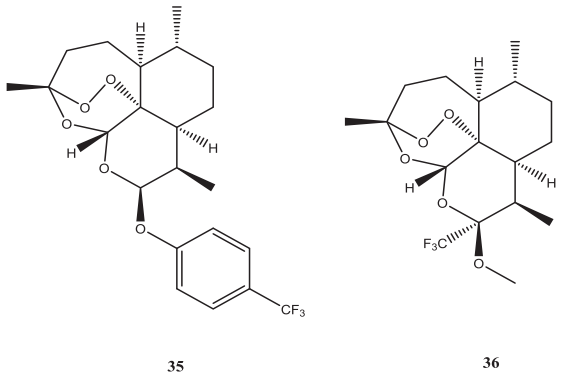
sis, the major product obtained was the anhydro derivative (AHA) in high yields. This suggests the involvement of an oxonium ion Scheme (**1**). intermediate.



Scheme (1). Classical synthetic route to C-10 phenoxy derivatives.

By exploring the use of TMSOTf-$AgClO_4$ catalysis in DHA-phenol coupling reactions, good chemical yields and stereoselectivity in favor of the β-isomers were obtained. When tested *in vitro* against *P. falciparum* (HB3 and K1 strains), all of the phenoxy-derivatives displayed IC_{50} values in the low nanomolar range, comparable to that of **24**. The *p*-trifluoromethyl-phenoxy derivative **35** (Fig. **11**), chosen for further biological evaluation on a rodent *P. berghei* model, disclosed an outstanding *in vivo* antimalarial activity, equal to that of **23** and superior to that of **24**. Further studies conducted by the WHO also demonstrated that **35** is orally active in mice with an ED_{50} of 2.7 mg/kg and an ED_{90} of 5.4 mg/Kg. Metabolic studies on **34** assessed that the *p*-trifluoromethyl group on the phenyl ring actually blocks oxidative de-arylation.

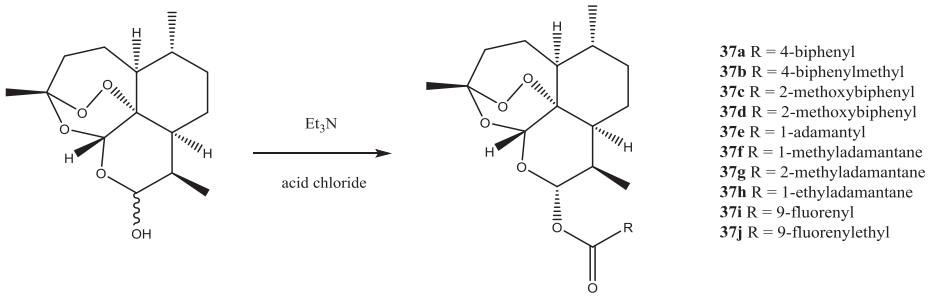
In a related approach, the introduction of a trifluoromethyl group in C-10 to improve hydrolytic stability of the acetal functionality of **24** was exploited, leading to candidate **36**, which was found ≈ 33 times more stable than **24** in simulated stomach *milieu* and more active after intraperitoneal (ip) administration in mice (ED_{50} value of 1.25 mg/Kg *vs.* ED_{50} = 2.5 mg/Kg for **24**) [103].



35 **36**

Fig. (11). Compounds **35** and **36** as more stable C-10 derivatives of **24**.

Ester derivatives of **23** at the C-10 position, incorporating biphenyl, adamantyl, and fluorenyl groups as privileged lipophilic appendages, have been prepared via acid chloride as the α-isomers (compounds **37a-j** in Scheme **2.**), and tested for their antimalarial activity towards multi-drug resistant *P. yoeli nigeriensis* by oral route in a murine model [104]. Several compounds in the series displayed a better efficacy profile than β-arteether **28** and artesunic acid **25**. In particular, ester **37i** was found to be more than twice as active as **28** and **25**. It must be considered, however, that whilst an increase in lipophilicity generally improves antimalarial activity, it also enhances toxicity [105].

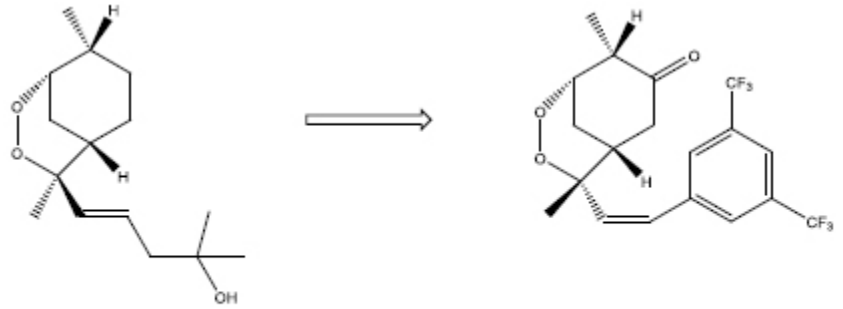


23 **37a-j**

Scheme (2). Preparation of lipophilic esters of **23**.

The approaches to replace oxygen at the C-10 position with a carbon atom, to give carba-analogues (the so-called 10-deoxoartemisinins), have been developed as well, with the aim to enhance hydrolytic stability and reduce toxicity. Starting

from aldehyde **38**, obtained after the brilliant synthetic strategy of Ziffer *et al*. [106], 10-deoxoartemisinins amine derivatives **39a-d**, or esters **40a-c** containing water-soluble carboxylic groups Scheme (**3**). have been prepared [107]. Some of the hydrophilic esters have been found to be about 25 times more potent than **22** against CQR clone (W-2) and 20 times towards the sensitive strain (Ghana) of *P. falciparum*. Comprehensive literature on carba-analogs is available, with some other relevant series reported [54].

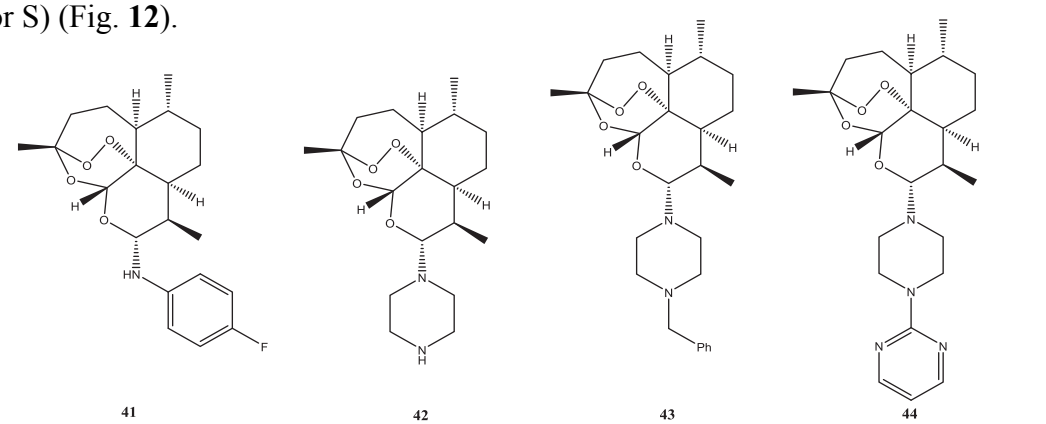


118
(IC_{50} = 17 nM)

119
(IC_{50} = 71 nM)

Scheme (3). Chemical approach to artemisinin C-10 carba-analogues.

An enhancement in activity was obtained by the replacement of oxygen at C-10 with a different heteroatom, such as nitrogen, which disclosed the class of 10-amino-artemisinins. This includes 10-aryl-amino derivatives, and 10-piperazine, 10-morpholine, and 10-thiomorpholine analogs, bearing a six-membered aliphatic ring incorporating the C-10-linked nitrogen atom and a second heteroatom (N, O, or S) (Fig. **12**).



41 **42** **43** **44**

(Fig. 12) contd.....

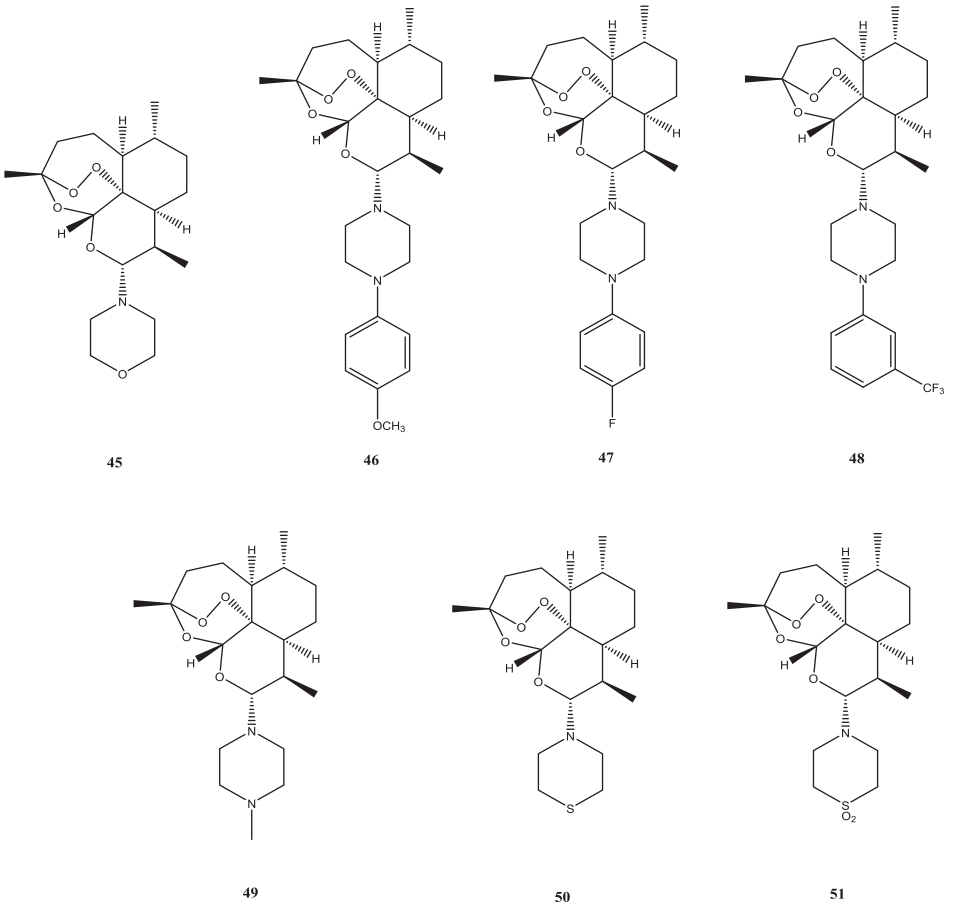


Fig. (12). 10-amino-artemisinins.

10-Arylaminoartemisinins have been designed as structural analogs of arylglycosylamines to have acceptable stability at pH 4, thus resulting in more stability than artesunate. Among the 10-arylamino congeners prepared so far, the *p*-fluorophenylamino derivative **41** was shown to be about 13 times more efficient than artesunate *in vivo* against *P. berghei* N strain after subcutaneous (sc) administration, and highly active by the same route towards CQR *P. yoelii* NS strain. However, activities upon oral administration are of the same order of magnitude as artesunate, to indicate that at lower pH, as in the case of glycosylamines, protonation of the basic nitrogen actually occurs, leading to hydrolysis to **23** [108].

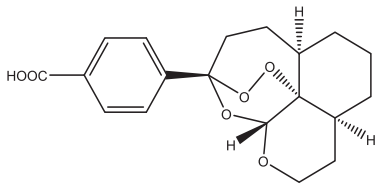
A number of 10-piperazine derivatives (compounds **42**-**44**, **46**-**49**) and the 10-morpholine bioisostere **45** have been prepared to start from the trimethylsilyl ether of 23: subsequent treatment with bromotrimethylsilane and then an excess of amine gives the 10 α-configurated amino compounds exclusively. In the murine malaria models, the activity of **43**-**45** against CQS *P. berghei* and CQR *P. yoelii* was shown to be notably high, and superior to those of ART, artesunate, and any other peroxide-containing compound. However, the potent anti-malarial effect of piperazine-substituted analogs **43** and **44** was suggested to be disconnected from the ability to alkylate heme, since they were found relatively unreactive towards free Fe(II). In the reactions involving free Fe(II), the authors hypothesized that the piperazine forms a complex with the ferrous ion, and the amine-iron complex blocks the access of further iron to the peroxide bridge [109]. This conclusion was countered by evidence reporting that these derivatives are indeed potent heme-alkylating agents [110].

The thiomorpholine series has attained relevant results with artemiside **50** and its *S*,*S*-dioxide analog artemisone **51** [111]. In comparison to sodium artesunate, **51** displays higher activity, especially against multidrug-resistant *Plasmodium* parasites, and is more stable towards hydrolysis to **23**, despite the presence of the C-10 aminal group [112]. In animal experiments, **51** was about two to five times more efficient than artesunate [113].

In view of their low-nanomolar activities against both drug-sensitive and mutant asexual parasites, and lack of neurotoxicity, **50** and **51** represent promising candidates for further development within combination therapies. Quite interestingly, they maintain a significant efficacy against both early-stage and mature gametocytes. Since these latter are regarded as metabolically hypoactive stages, devoid of hemoglobin digesting pathways, it has been suggested that heme is not required for activation of these 10-amino-artemisinins [114].

In addition to C-10 modifications, chemical derivatization at different positions, such as C-3, C-4, C-9, and O-11, was investigated as well.

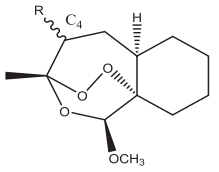
C-3 modified analogs, mainly obtained by total synthesis, did not display a significant activity profile in comparison to cognate leads; based on its good aqueous solubility, compound **52** (Fig. **13**). was tested orally in *P. berghei*-infected mice against the congener artelinic acid **33**, but it was found to be less potent (ED_{50} value of 15 mg/Kg *vs.* ED_{50} = 9.6 mg/Kg for artelinic acid) [115].



52

Fig. (13). The C-3 modified artelinic acid congener **52**.

Compounds **53a-c** (Fig. **14**). were designed by Posner's group to get insights into the role of C-4 radicals in the ART plasmocidal mechanism. Indeed, the introduction of substituents, such as the benzyl group in **53b**, which favor radical formation at C-4, resulted in better antimalarial activity, while potency was decreased by different groups leading to a major stabilization of the C-4 radical (**53c**). The role of stereochemistry at C-4 was also assessed for better activity, based on the observation that the 1,5-H shift is favored in C-4 β-epimers [116].

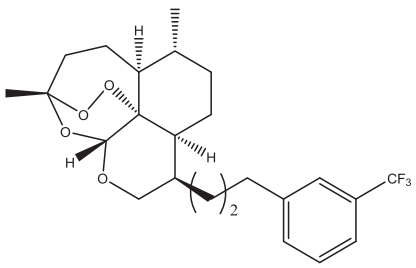


53a R = H
53b R = CH_2Ph
53c R= Ph

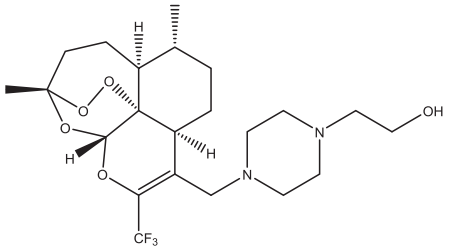
53a-c

Fig. (14). C-4-substituted artemisinin congeners **53a-c**.

The available C-9 modified analogs (Fig. **15**). come from two different assembling procedures: the C-9 β-aralkyl derivatives (such as **54**) were prepared through Michael addition to artemisitene **32** by different donors, whilst Δ^9-anhydro-compounds (*cf.***55**) were obtained from 16-bromo-10-trifluoromethyl-anhydrodihydroartemisinin through direct derivatization. Compounds in the first series were found remarkably active in a *P. berghei*-infected rodent model, with compound **54** displaying the most significant activity (ED_{50} value of 1.25 mg/Kg *vs.* ED_{50} = 2.4 mg/Kg for sodium artesunate) [117]. On the other side, in the related group **55** has emerged as a highly potent antimalarial compound *in vivo* in a mice model, dramatically effective in reducing parasitemia (100% by day 4, after both oral and sc route, at 10 mg/Kg dosing), more potent than artesunate [118].



54

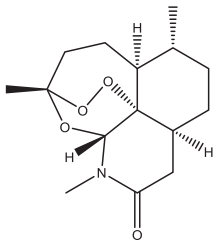


55

Fig. (15). Artemisinin C-9 derivatives.

Again, the bioisosteric replacement of the 11-positioned oxygen atom with nitrogen paved the way for the class of 11-aza-artemisinins. These analogs are very attractive since they incorporate a lactam unit which is more stable under acidic and basic conditions than the ART lactone. 11-Aza-artemisinin **30** is readily obtained from **22** and aqueous ammonia according to literature protocols [119], however, only a limited number of derivatives can be made at the nitrogen position, due to their amide nature.

N-methyl-11-aza-9-desmethylartemisinin **56** (Fig. **16**). exhibited an almost five-fold increase in activity compared to **22** when tested *in vitro*. No significant improvement in potency was observed in the related *N*-alkyl, *N*-phenyl, and *N*-phenethyl series reported in the same study [120].

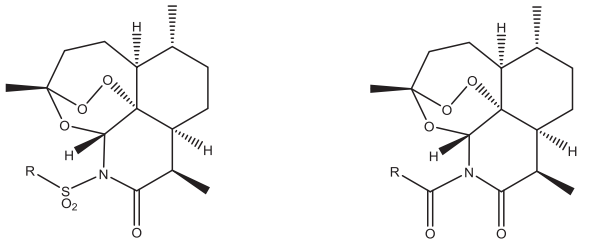


56

Fig. (16). The simplest 11-aza-artemisinin analog.

A series of *N*-sulfonyl and *N*-carbonyl-11-aza-artemisinins, bearing electron-withdrawing groups whose inductive effects would influence both the thermal

stability and the overall physicochemical properties of the endoperoxide derivatives, was made synthetically accessible by Haynes' group (compounds **57a-e** and **58a**,**b** in (Fig. **17**) [121, 122].



57a-e **58a,b**

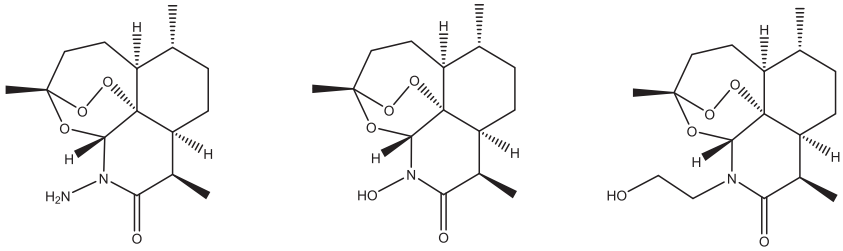
57a: R = CH_3CH_2
57b: R = $CH_3(CH_2)_6CH_2$
57c: R = 3'-$O_2NC_6H_4$
57d: R = 2'-naphthyl
57e: R = 8'-quinolinyl

58a: R = 4'-$O_2NC_6H_4$
58b: R = 3'-$O_2NC_6H_4$

Fig. (17). *N*-sulfonyl and *N*-carbonyl-11-aza-artemisinins.

11-Aza-artemisinin **30** itself was screened against W2 (CQR) and D6 (CQS) strains, with respective IC_{50} values of 1.73 and 2.60 ng/mL. The carbonyl derivatives **58** were generally more active as antimalarials than arylsulfonyl counterparts **57**, which however exhibited good activities, being similar to artesunate against the drug-sensitive 3D7 clone and the multidrug-resistant K1 strain of *P. falciparum*. Despite its good antimalarial activity, compound **57b** was found to be highly cytotoxic, due to its significant lipophilicity. Quite interestingly, the remaining compounds exhibited improved solubility in water, inability to provide **23** either by hydrolysis or metabolism, and also enhanced thermal stabilities, which may be ascribed to remote inductive effects raising the (homolytic) bond dissociation energy of the peroxide bond. This would be a good requisite for storage conditions in the endemic areas.

ART modifications with amine or hydrazine groups at this position have led to new 11-aza derivatives (compounds **59-61** in Fig. **1**) with reactive functionalities, which have been exploited to generate a wide panel of compounds, characterized *in vivo* by interesting activity against multidrug-resistant malaria [123].



59 **60** **61**

Fig. (18). *N*-Amino-11-aza-artemisinin **59**, *N*-hydroxy-11-aza-artemisin **60**, and *N*-hydroxyethyl-11--za-artemisin **61**.

Artemisinin Combination Therapy

The strategy to combine an antimalarial drug with a partner agent presents major challenges, particularly in endemic areas. These include the right choice of the best-suited drug combinations, in terms of synergic efficacy and pharmacokinetic profiles, cost of combination agents, and compliance.

Artemisinin combination therapy (ACT) was suggested as early as 1986 as a tool to overcome treatment failures and mutually preserve coupled drugs from the risk of resistance development [124 - 126]. The rationale behind this approach resides in associating the ART fast removal of a large fraction of parasites with the persistent action of a second drug, characterized by an independent mechanism of action and prolonged half-life, on the residual parasite biomass. Since the publication of the first edition of *The guidelines for the treatment of malaria* in 2006, WHO has highly recommended ACT as the first-line treatment for CQR *P. falciparum* malaria cases in endemic areas. The WHO-endorsed combinations include artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine/pyrimethamine, artemether-lumefantrine, and DHA-piperaquine [127]. Artesunate-pyronaridine (Pyramax®) is the only ACT for the treatment of acute *P. vivax* malaria.

Unfortunately, since its first appearance in 2008 in Western Cambodia [128], resistance to ART has been detected in many other countries, and the phenomenon was shown to accelerate parasite resistance to partner drugs [129, 130]. For this reason, very recently, the ACT concept has been expanded with the introduction of a second, long-lasting auxiliary drug in the so-called triple artemisinin-based combination therapy (TACT) [131]. In analogy with therapeutic protocols in use for treating multi-drug resistant infections such as AIDS or TBC, it was reasoned that the combination of more agents, with different targets and

resistance mechanisms, could reduce the probability for resistance to emerge to any of these components. Of course, a correct TACT approach should not prescind from the appropriate choice and dosage of each individual component of the combination in terms of synergic efficacy, half-lives and pharmacokinetic profiles, potential drug-drug interactions, safety, and tolerability.

Artemisinin Molecular Hybrids

Over the past decade, molecular hybridization or covalent bitherapy turned out to be a powerful approach in medicinal chemistry to overcome the limitations of multi-component therapeutic regimens. The strategy involves the assemblage of two or more molecules, acting by different mechanisms on the same or distinct targets, into a single new chemical entity containing covalently linked pharmacophores. The underlying rationale goes beyond the simple grouping of singular components to reach the synergic biological effect, because the hybrid compound expresses the potential of unprecedented properties with respect to the precursors [132, 133]. Although of general application, for instance, in the therapy of diseases such as cancer and AIDS, the hybridization approach is comparatively new in the field of antimalarial drug discovery. At best, it is possible to combine into a single agent all the desired multistage antiplasmodial activities, to redesign a given drug if the toxicophore and the pharmacophore fragments of the molecule are not overlapped, or to arrange the nature and the extension of the linker between the two moieties of the hybrid to probe the accessibility and the relative proximity of the reputed cellular targets. A number of potential advantages of hybrids over ACTs have been suggested, including mutual protection of each pharmacophoric moiety against drug resistance development, enhancement of solubility/stability of the more inadequate partner drug, and/or of the entire hybrid molecule, resulting in better bioavailability, a decrease of synthetic and formulation costs, improvement of selectivity profile with reduction of undesired drug-drug interactions and adverse side effects, and amelioration of patient compliance. However, the real advantages of the hybrid over the separate pharmacophores should always be verified. The hybridization approach has been exploited on several current antimalarial drugs, in search of novel bioactive agents with distinct pharmacological profiles [134 - 136].

The relevance of artemisinins in present clinical protocols against CQR malaria strains led to the exploration of several ART-based hybrid compounds with the potential of a superior antimalarial activity in comparison to component drugs. In most cases, they are simple conjugates, in which the pharmacophores for each target are separated by a metabolically stable fragment, that is not present in either of the individual drugs, or *cleavage conjugates*, containing a linker unit designed to be metabolized to release the drugs at each independent target. Further, *in the*

*so-called fused hybrid*s, the linker is minimized in such a way that the pharmacophores are essentially touching [137]. Lastly, *merged hybrids* take advantage of the structural resemblance of the starting compounds to give rise to smaller molecules in which the two scaffolds are closely intermingled (Fig. **19**).

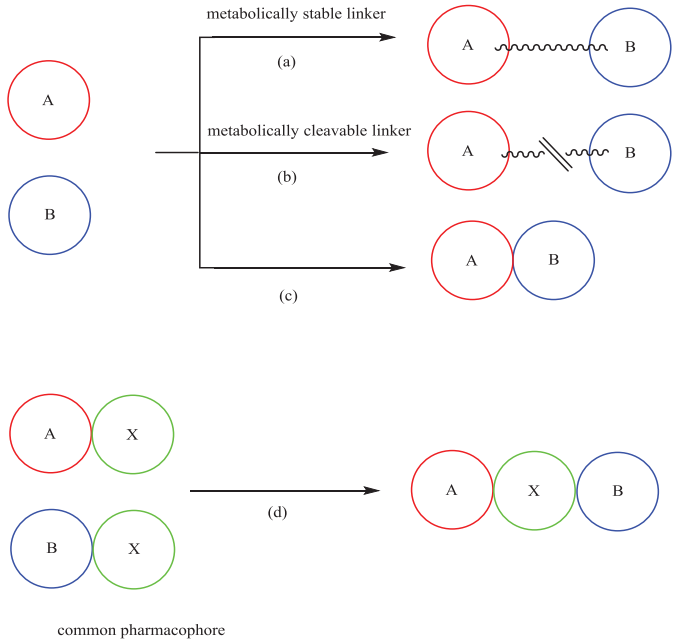
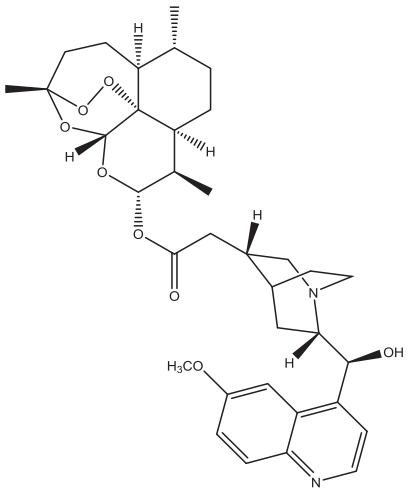


Fig. (19). Different hybridization approaches (a) conjugate hybrid; (b) cleavage conjugate hybrid; (c) fused hybrid; (d) merged hybrid.

As previously discussed, currently used artemisinins are known to undergo *in vivo* enzymatic, oxidative dealkylation and are easily hydrolysed into **23**, which represents their principal metabolite and the supposed causative agent of neurotoxicity [138]; although endowed with potent antimalarial activity, **23** is characterized by high chemical and thermal instability, as well as poor solubility. Since the hemiacetalic nature of the C-10 adduct accounts for the chemical, stereochemical [139], and metabolic lability of the molecule, to address these drawbacks, different ART-derived hybrids have been developed with the common strategy to transform the lactol at C-10 into a more stable functionality, through both direct or spacer-mediated covalent linkage to the partner motif.

The hybrid by definition, *i.e.*, the ART-quinine conjugate **62**, was assembled by coupling **23** to a carboxylic acid derivative of quinine **1**, obtained through modification of the non-essential vinyl moiety of the quinuclidine nucleus, thus introducing an acetal ester connection (Fig. **20**). The logic underneath was that coupling the lipophilic, fast-acting, but quickly cleared ART to the slow-acting, relatively polar quinine derivative might increase the half-life of the ART moiety. The hybrid displayed significant activity *in vitro* against both CQS (3D7; IC_{50} = 0.008 μM) and CQR (FcB1; IC_{50} = 0.009 μM) strains of *P. falciparum*, resulting in more potent than **22** and **1** tested as individual drugs on the same strains, and about 3-fold superior compared to a 1:1 mixture (on a molecular basis) of these two drugs [140]. This suggested that the actions of both quinine and ART components were preserved.



62

Fig. (20). The artemisinin-quinine hybrid **62**.

Molecular interactions and binding affinity of the ART-quinine conjugate (and some related hybrids) with iron(II)-protoporphyrin-IX as a putative target have been evaluated *in silico* [141]. The model involves a close interaction between heme iron (II) and the endoperoxide oxygen couple of the ART moiety, with the more negatively charged O-2 preferred over the sterically hindered O-1. These results are in agreement with docking studies performed by Shukla *et al*. [142].

Coming soon after for significance, the artemisinin-chloroquine (ART-CQ) hybrid has been developed. From a conceptual point of view, this molecule falls into the class of trioxaquines, conjugates obtained by covalent attachment of a 1,2,4-trioxane pharmacophore to an aminoquinoline moiety. They were designed by Meunier and co-workers as dual-mode twin drugs, in which the trioxane alkylating properties are combined with the quinoline ability to easily penetrate within infected erythrocytes and inhibit the β-hematin polymerization [143, 144]. The result is the fast removal of the bulk of parasite load by the ART pharmacophore, sustained by the quinoline moiety clearance of the survival parasite, until complete plasmocidal effect is achieved.

The CQ nucleus, truncated in its 4-alkylamino chain, has been linked to either **23**, **25**, or artelinic acid **33**, and the resulting classes screened for their antiplasmodial activity in comparison to individual components.

In the first group of conjugates (compounds **63-68** in Fig. **(1)**, the linkage of the alkylamino quinoline appendage enables the stabilization of the **23** C-10 hemiacetalic adduct in the form of an ether derivative (acetal), more stable *in vivo* than an ester, adding in the meantime a basic nitrogen functionality, suitable for salt formation.

When tested *in vitro* on *P. falciparum* CQS D10 and CQR Dd2 strains, compounds **63**, **64**, and **68** in the form of oxalate salts, and **66** as both free base and salt, were found equipotent to CQ **5** against the D10 strain, and more potent than CQ against the CQR Dd2 strain, with IC_{50} in the two-digit nanomolar range [145]. In general, the oxalates were found to be more active than their free base hybrids, presumably due to their higher aqueous solubility in the testing medium. An optimum chain length of 2/3 carbon atoms has been identified, with or without an extra methyl substituent. Hybrid **66** and its oxalate salt were the most active ones against the Dd2 strain, being 9- and 7-fold more active than **5**, respectively (17.12 nM; 20.76 nM *vs* 157.9 nM). However, despite an increase in half-life with respect to **23**, hybrids were less active than the reference drug, irrespective of the *P. falciparum* strain. *In vivo* hybrids **66** and **63** displayed potent anti-malarial efficacy against *P. vinckei*, with ED_{50} values of 1.1 mg/kg by ip route, and 12 mg/Kg *per os* for hybrid **66**, and 1.4 mg/kg and 16 mg/Kg by ip and oral route, respectively (compound **63**). Long-term monitoring of parasitaemia showed that hybrids **63** and **66** are completely curative in *P. vinckei* infected mice (without recrudescence) *via* both ip (15 mg/kg) and oral (50 mg/kg) routes, with no visible sign of toxicity at higher doses (up to 50 mg/kg) [146].

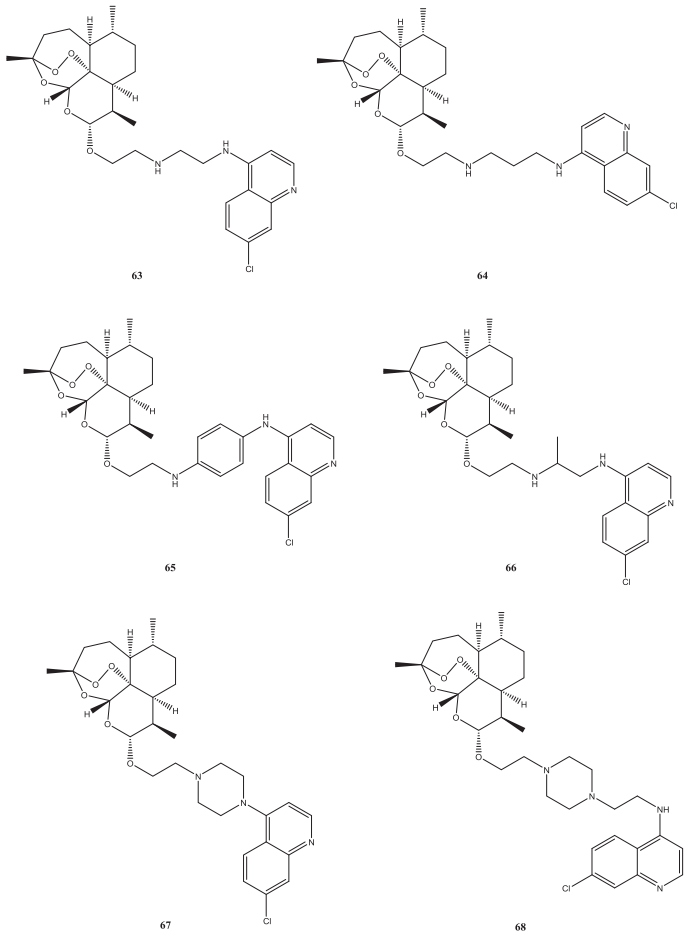
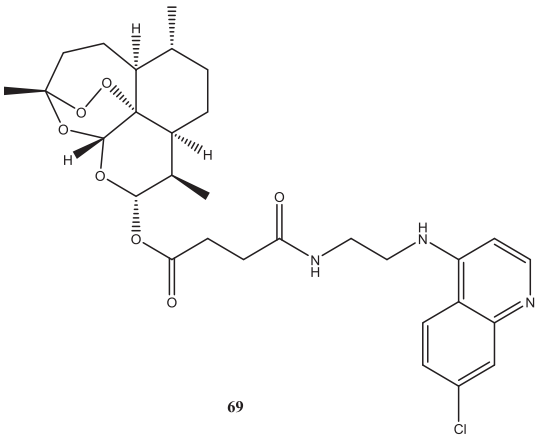


Fig. (21). DHA-CQ conjugates **63-68**.

The artesunic acid conjugate **69** reported in Fig. **(1)** is an artemisinin-based trioxaquine possessing potent antiplasmodial activity *in vitro* against CQS (D6) (IC50, 6.89 ng/mL) and CQR (W2) (IC50, 3.62 ng/mL) *P. falciparum* strains, corroborated by a remarkable *in vivo* effect against blood stage rodent malaria parasite (ED50 and ED90 of 5.5 and 13.5 mg/kg, respectively) [147]. When tested in a human cerebral malaria (CM) mice model, the hybrid displayed a higher efficacy compared to individual precursors alone (artesunate and 4,7-dichlor--quinoline) and quinine **1** chosen as controls. The very encouraging post-treatment survival data in the trioxaquine-treated group compared to that receiving iv artesunate alone was suggested to depend upon the contribution of the quinoline

pharmacophore in sequestering the ART-related partner in the DV, thus extending its half-life [148].



69

Fig. (22). Structure of *N*-(7-chloroquinolin-4-ylamino)-ethyl-artesunate-19-carbossammide **69**.

The strictly related study by Tsogoeva and co-workers reported about the smart synthesis and biological evaluation of artesunate/DHA-quinoline and -isoquinoline hybrids, including the previously reported conjugate **69** (Fig. **23**) [149]. This outstanding work investigated SARs of linker-units, generated through copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry (for triazole derivatives **70-78**), classical coupling reactions (for esters and amides **79-81** and **69**), and a novel rearrangement of the *in situ* formed tertiary amide to the secondary amide (for the alkyne-tagged compound **82**). All the investigated hybrids displayed potent activities in the nanomolar to picomolar range against the *P. falciparum* wild-type strain 3D7 and the two multidrug-resistant strains Dd2 and K1. In the artesunate derivatives **79**, **80**, and **69**, the gradual replacement of H-bond acceptor oxygen atoms by H-bond donor nitrogens in the linker units led, as expected, to a remarkable increase in activity, with the most outstanding hybrid **69** showing EC_{50} values of 2.7 nM, 1.0 nM and 780 pM against 3D7, Dd2, and K1 strains, respectively. Furthermore, compound **80** was found to exceptionally suppress parasitemia in a *P. berghei* infected-mice model, upon both sc and oral administration, being superior to artesunate.

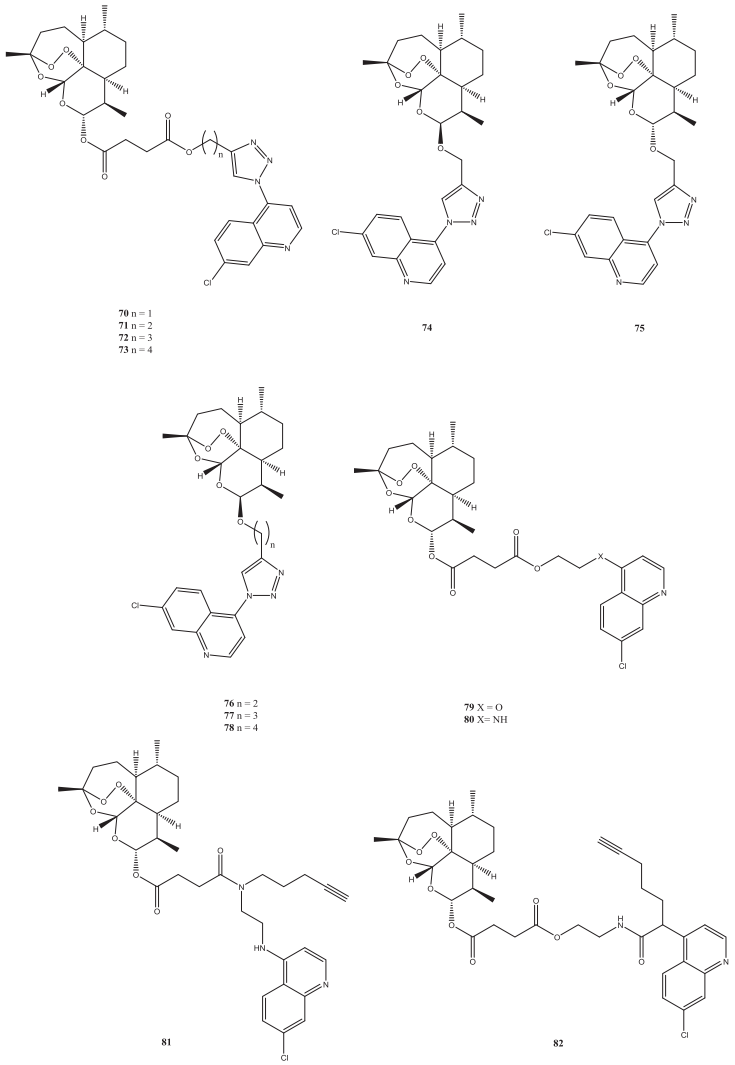
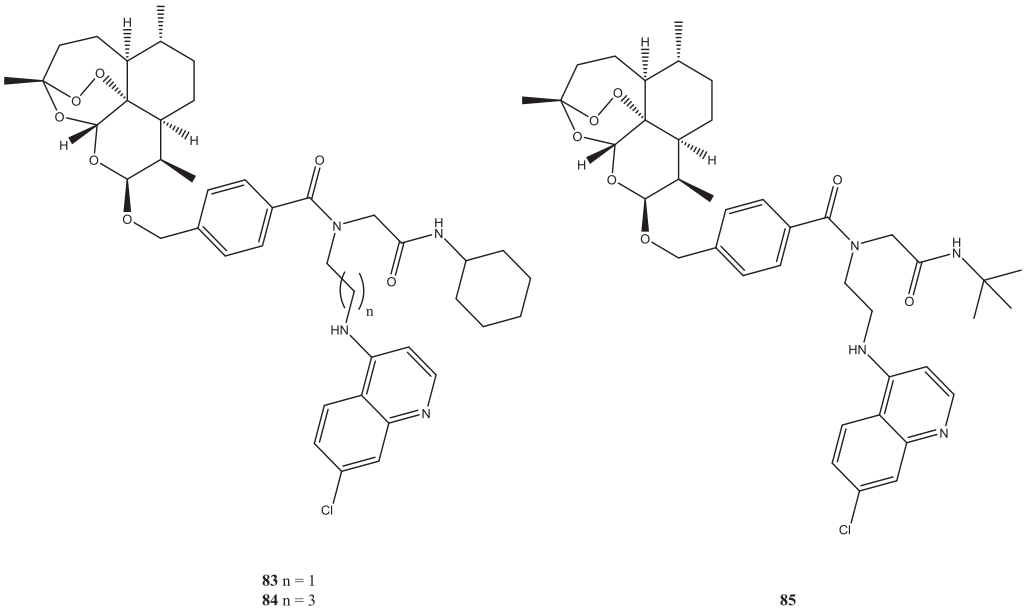


Fig. (23). Exploring the linker spatial and electronic requirements in potent artesunate/DHA quinoline hybrids.

Finally, using a chemical proteomics approach, the authors selected the highly active alkyne-tagged hybrids **81** and **82** to identify a set of parasite proteins, such as *Pf*ATP6 (responsible for ART action) and the 40S ribosomal protein machinery (classically recognized for quinoline effect), to support the hypothesis that hybrids act by multiple modes of action to overcome resistance.

Conjugates embodying artelinic acid **33** (compounds **83-85** in Fig. **24** were built employing an Ugi four-component condensation based on the one-step combination of **33**, the 4-amino-quinoline amine, paraformaldehyde, and cyclohexyl or *tert*-butyl isocyanide as the isocyanide component amide. The reported compounds displayed an excellent *in vitro* antiplasmodial activity against the CQS D10 and CQR K1 isolates of *P. falciparum*, which resulted in comparable to that of **5** used as a control [150].



83 n = 1
84 n = 3

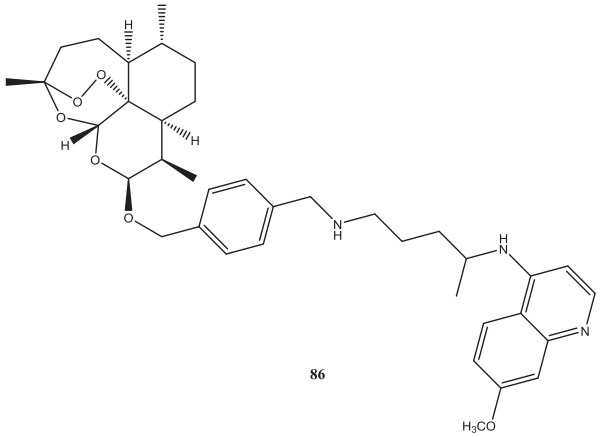
85

Fig. (24). Artelinic acid-chloroquine hybrids **83-85**.

Furthermore, biological experiments were conducted to disclose the prevailing mechanism by which the hybrids are able to prevent hemoglobin endocytosis by the parasite, to assess if their mechanism of action is more similar to that of CQ or ART: in fact, whereas CQ is thought to block the fusion of hemoglobin transport vesicles to the DV, ART rather reduces the amount of hemoglobin-filled endocytic vesicles. The obtained results suggested a more ART-like action for the hybrid compounds.

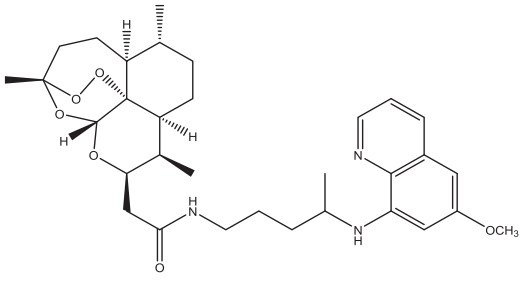
The prototype of the liver schizonticidal drug, primaquine **7**, was selected as a partner drug in *a few* ART-based hybrids. The underlying rationale is to take advantage of **7** abilities to eliminate the parasite in its liver and sexual stages and to disrupt disease transmission [151]. Conjugate **86** illustrated in Fig. (**25).** was obtained starting from artelinic aldehyde, in turn, prepared from **33**, followed by reductive amination using **7** and $NaBH_3CN$. Again, the bioisosteric replacement

of an oxygen atom with a CH_2 unit, which converts the canonical lactol ring of **23** in a tetrahydropyrane nucleus, allows the most versatile functionalizations in position 10, as illustrated for hybrid **87** in Fig. (**26).**, which contains a deoxy-acetyl artesunic acid moiety. *In vitro* evaluation of reported hybrids disclosed that they are actually superior to **7** against *P. falciparum* W2 strains (IC_{50} = 0.0125 and 0.0091 μM, respectively). Furthermore, the activity of both compounds against cultured *P. falciparum* was comparable to that of **22** (50% inhibitory concentration [IC_{50}], ~10 nM). More interestingly, the conjugates displayed enhanced *in vitro* activities against liver-stage *P. berghei* compared to their parent drugs.



86

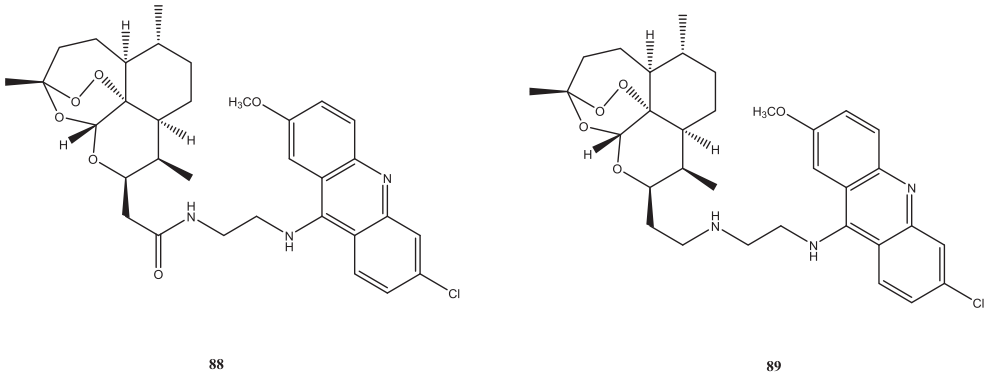
Fig. (25). Artelinic acid-primaquine conjugate **86**, reduced at the CONH linker moiety.



87

Fig. (26). Hybridization of primaquine and deoxy-acetyl artesunic acid (compound **87**).

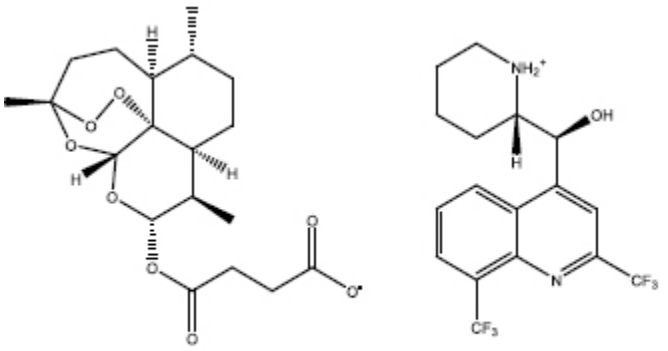
The same approach has guided the design of ART/quinacrine hybrids (Fig. **27**)., prepared by joining the 9-aminoacridine unit of quinacrine (also known as mepacrine) with a metabolically stable ART analogue, through a C-10 carba-linkage and the interposition of an alkyl-diamine linker of appropriate length [152, 153]. When tested *in vitro* against *P. falciparum* strains, all the hybrids were found active in the nanomolar range: the most potent compounds were **88**, derived from deoxy-acetyl artesunate (IC_{50} = 5.96 nM against CQS 3D7 strains), and **89** (3D7, IC_{50} = 12.52 nM; CQR K1 isolates, IC_{50} = 14.34 nM), both characterized by an ethylene spacer. Despite the presence of the basic polyamine spacer, which should favor the accumulation within the acidic DV through an ion-trapping mechanism, the hybrids are not superior to **24**, suggesting that other targets outside the DV, such as *Pf*ATP6, may be more important for this class of chimeric molecules.



88 **89**

Fig. (27). Artemisinin-quinacrine hybrids.

Artesunate is the common anionic component of two hybrid salts, namely mefloquine-artesunate (MEFAS) **90** and primaquine-artesunate (PRIMAS) **91** (Fig. **28**), incorporating mefloquine **2** and primaquine **7** in the protonated forms as respective counterions.



90

Fig. (28). MEFAS and PRIMAS salts (**90** and **91**, respectively).

When tested for antimalarial activity, **90** was proven quite promising against CQR (W2) and CQS (3D7) *P. falciparum* parasites, resulting in at least five times more potent than **2** alone, more active than artesunate against 3D7 and equally effective against W2, and superior to different combinations of artesunate and **2**. Furthermore, **90** was found *in vitro* more effective than its components taken alone in blocking the final steps of *P. falciparum* gametocyte maturation. Additionally, a curative action in *P. berghei* experimentally infected mice was assessed, with no recrudescence observed in the long period. By altering the pH gradient across the parasite DV, this hybrid salt is suggested to have a dual mode of action, *i.e.*, the endoplasmic reticulum and DV. Because of its ability to target both asexual parasites and gametocytes, the low toxicity and cheap production costs, MEFAS represents a valuable alternative anti-malarial drug in endemic zones [154].

Developed with the aim to reduce primaquine toxicity, PRIMAS **91** was found *in vivo* and *in vitro* more active and less toxic than the individual drugs [155, 156].

The molecular hybridization approach was successfully exploited by linking artesunic acid or its deoxy-acetyl derivative to the quinazoline nucleus, considering that the latter scaffold is one of the most studied in medicinal chemistry, and, more importantly, is contained in febrifugine. This naturally occurring alkaloid was isolated 60 years ago from the Chinese plant aseru (*Dichroa febrifuga* Lour), and has been used as an antimalarial agent in traditional Chinese medicine for over 2000 years. The five novel ART-quinazoline hybrids reported in the study (compounds **92-96** in Fig. **29**. exhibit excellent antimalarial activity against the *P. falciparum* 3D7 strain, with EC_{50} values within the nanomolar range (EC_{50} = 1.4–39.9 nM). The most active compounds are compounds **92** (EC_{50} = 3.8 nM) and **95** (EC_{50} = 1.4 nM), which are superior to **25**

(EC_{50} = 9.7 nM). Remarkably, hybrid **95** is even more active than **23** (EC_{50} = 2.4 nM) and **5** (EC_{50} = 9.8 nM). An analysis of the structure-activity relationships of ART-quinazoline conjugates disclosed that the C-10 acetal linkage seems to be beneficial for antimalarial activity. Furthermore, an opportunely functionalized aromatic subunit needs to be encompassed, whilst the secondary amine group of the 4-anilino-quinazoline moiety should be left untouched, worth a decrease in activity [157].

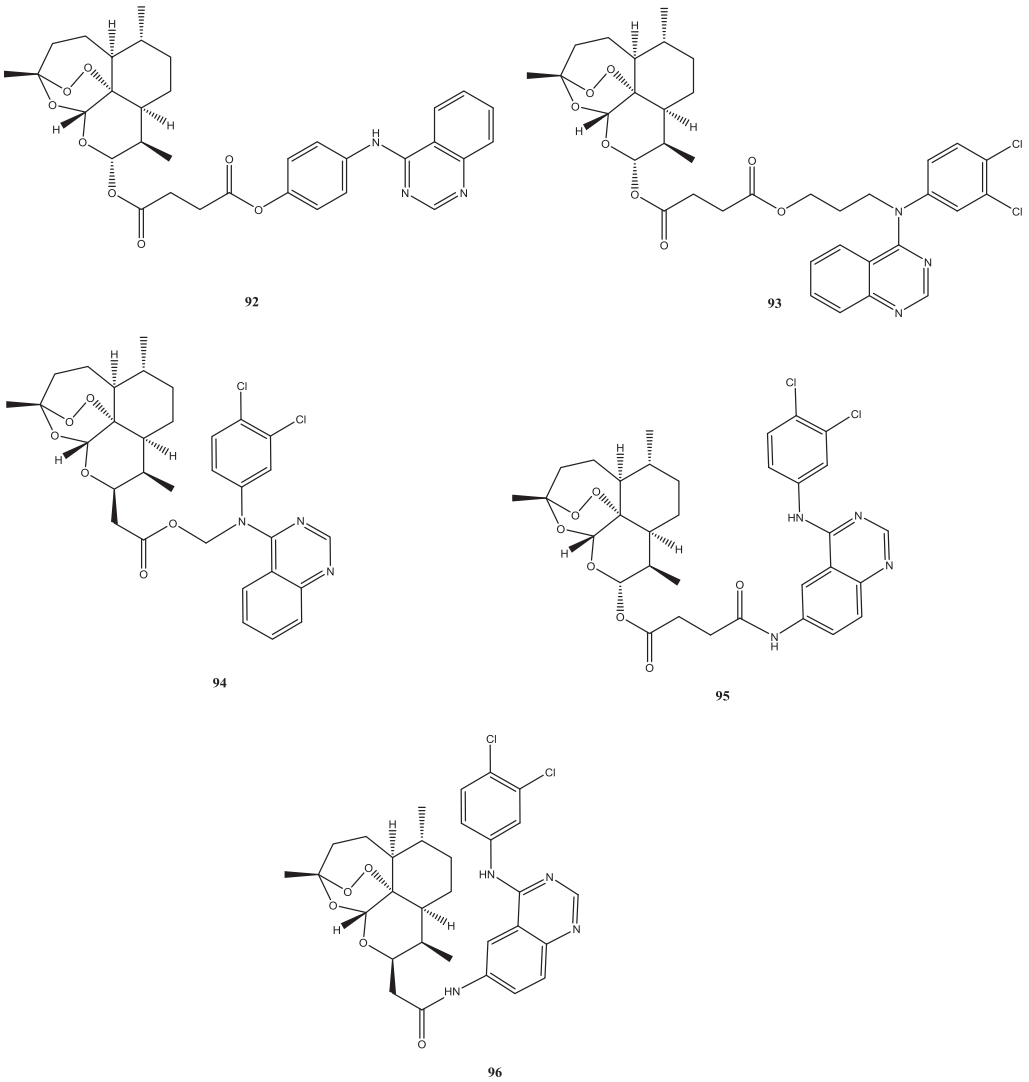
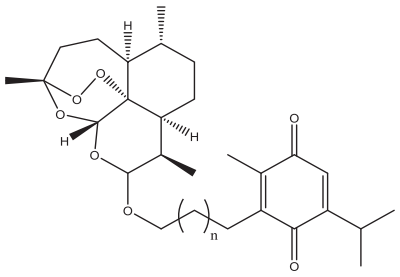


Fig. (29). Molecular hybrids of artesunic acid **25** and its deoxy-acetyl congeners with quinazoline.

On account of its wide range of pharmacological applications, thymoquinone, the main constituent of the volatile oil of *Nigella sativa* (black seed), is emerging as a promising natural drug. Several ART-thymoquinone hybrids were assembled and tested *in vitro* against *P. falciparum* 3D7 strains and compared to their parent compounds **25**/**23** and thymoquinone, as well as the standard drug **5** [158]. All hybrids exhibited excellent antimalarial activities, with EC_{50} values within the nanomolar range (3.7-54 nM) combined with a low toxicity/high selectivity profile. Ether-linked **97b** (Fig. **30**). was proven to be the most effective one (EC_{50} = 3.7 nM), superior to **25** (EC_{50} = 8.2 nM in this study) and **5** (EC_{50} = 9.8 nM), and comparable to **23** (EC_{50} = 2.4 nM).

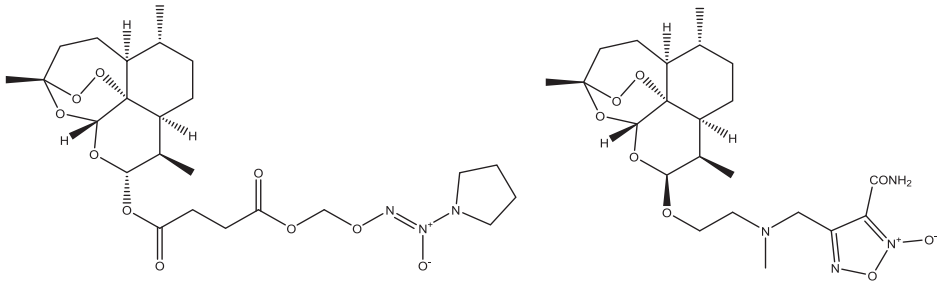


97a n = 1
97b n =2

Fig. (30). Potent artemisinin-thymoquinone hybrids.

An elegant application of covalent bitherapy involving artemisinin was exploited with the rational design of nitric oxide (NO)-donor hybrid drugs, obtained by joining the scaffolds of **25** or **23** with NO-donor moieties, to treat cerebral malaria [159] (compounds **98** and **99** in (Fig. **31**). The basis for this original approach is the finding that low availability of NO, consequent to the NO-scavenging effects by high concentrations of free oxyhemoglobin derived from malaria-related hemolysis, plays an important role in the pathogenesis of human and murine experimental cerebral malaria, and that the neurological syndrome and the associated cerebrovascular dysfunction can be prevented by administration of NO-donors. The *in vitro* and *in vivo* antiplasmodial activity of hybrid compounds **98** and **99** towards a transgenic *P. berghei* ANKA (PbA) clone expressing the green fluorescent protein (GFP) as a tag (PbA-GFP) was in the low nanomolar range, comparable to that of artesunate. Furthermore, hybrid **99** was found to behave as a good vasodilator agent at low micromolar concentration. It contains the NO-donor 1,2,5-oxadiazole-2-oxide (furoxan) substructure present in CAS 1609 (4-hydroxymethyl-3-furoxancarboxamide), an *in vivo* effective, long-lasting vasodilator agent. Following administration of hybrid **99,** mice with experimental

cerebral malaria showed a survival rate of 51.6%, which was markedly higher compared to the survival rate in the group of artemether-treated mice (27.5%). Thus, great potential can be envisaged for this new class of compounds, which retain the rapid parasite killing activity of the parent and the cerebrovascular flow restoring properties of the NO-donor moieties.



98 **99**

Fig. (31). Conjugates **98** and **99**, built by linking **25** or **23**, respectively, with NO-donor moieties.

In light of the role of *Plasmodium* cysteine proteases, such as falcipains and other papain-family proteases, in the host (hemo)globin digestion [160] in the DV, and capitalizing on the known inhibitory effect of chalcones on cysteine proteases, DHA[REMOVED HYPERLINK FIELD]-chalcone hybrids were synthesized to be tested *in vitro* as antimalarial agents (Fig. **32**) [161]. Conjugates were prepared through the esterification of substituted chalcones with the C-10 hemiacetal **23** group, using either 1,1'-carbonyldiimidazole as a coupling reagent, or oxalyl chloride as activation reagent. The hybrid compounds were all found to be active against CQS (3D7) and CQR (W2) *P. falciparum* strains, with IC_{50} values in the nanomolar range against both strains (1.9-10.7 nM, and 1.6-10.6 nM, respectively). The antiplasmodial activity was increased by the presence of electron donating, oxygenated aryl or furan scaffolds as B ring of the chalcone moiety, independently of the substituent position. Accordingly, compounds **100-102** were proven to be almost equipotent to **23**, and 2-3 times more active than artesunate against the 3D7 and W2 strains; furthermore, they were more than forty-fold more effective than **5** against the W2 strain of the malaria parasite. The chemical binding of chalcone and DHA pharmacophores into hybrids resulted in no significant advantage compared to 23 alone or in 1:1 molar ratio combinations of the two agents, suggesting an antagonistic rather than a synergistic effect, probably related to their high log P values, low solubility and poor absorption levels. Nevertheless, all the esters showed remarkable selectivity indices in targeting intraerythrocytic *P. falciparum* parasites compared to mammalian cells, and increased thermal stability with respect to **23**.

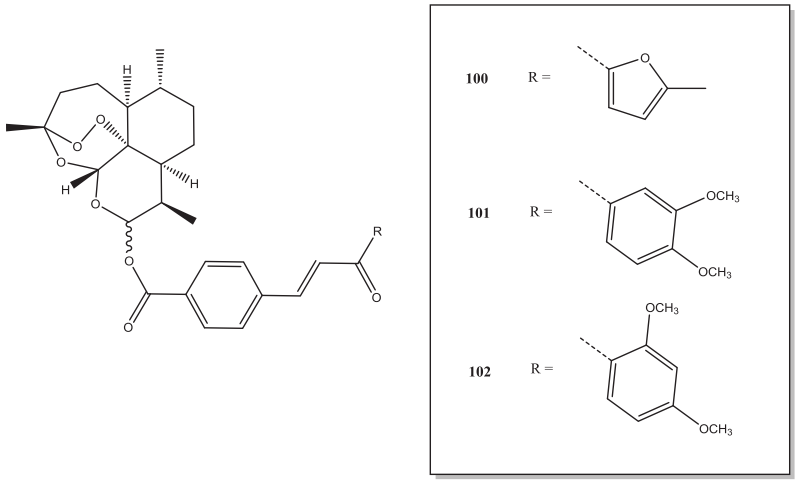


Fig. (32). DHA-chalcone hybrids.

Much interest resides in hybrid compounds incorporating a (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine moiety coupled to an ART scaffold *via* ester linkage (Fig. **33**) [162].

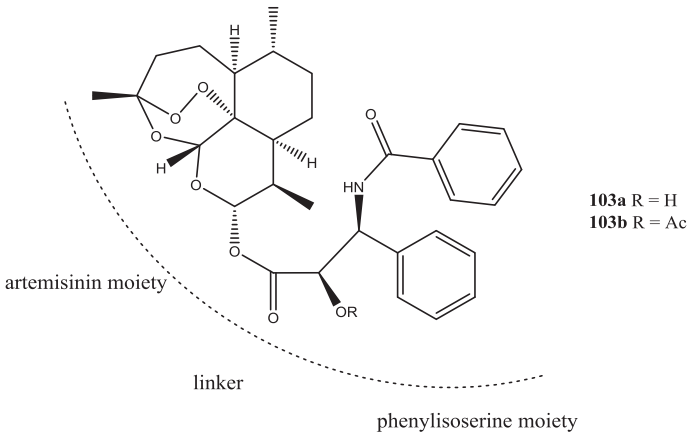


Fig. (33). DHA hybrids incorporating a paclitaxel fragment.

The rationale motivating the designed compounds is that (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine is a structural component of the antimicrotubular drug paclitaxel (a taxol), which owns antimalarial efficacy in addition to its strong well-known antitumor activity [163]. Hybrids **103a** and **103b** differ only for the presence or absence of acetylation of the isoserine hydroxyl group. Tested compounds showed *in vitro* a quite similar antiplasmodial profile, resulting equipotent to **23** against CQR W2 strain and approximately 3-4 times more active than the same drug against the multidrug-resistant K1 *P. falciparum* isolate. Thus, a potential synergistic interaction between the ART and the isoserine pharmacophores for antiplasmodial activity was suggested.

Artemisinin Dimers and Trimers

The acknowledged connection between the endoperoxide structural requisite and ART anti-malarial activity has prompted researchers to exploit the pharmacophore duplication approach, connecting two identical entities through spacers of convenient length and flexibility, to improve the pharmacological effect. The rationale to dimerize ART originates from several considerations: (i) covalently linked pharmacophores are simultaneously uptaken into the cells, thus giving rise to multiple copies of the drug available at the target level, which corresponds to a concentration enhancement, particularly useful for a short half-life agent such as ART; (ii) the bivalent dimer may establish binding interaction with independent recognition sites of a receptor or multivalent binding to different protein targets; (iii) binding affinities are likely enhanced in respect to a monomeric moiety.

Based on these issues, over the last 20 years, the ART dimerization approach has been widely investigated in medicinal chemistry to design systems that could be more stable under metabolic conditions, and consequently less prone to give recrudescence phenomena and toxic events compared to monomers.

The efficacy of ART dimers is strictly dependent upon the correct distance between the two ART moieties, and consequently, the linkers' features, such as length and conformational flexibility, play a crucial role. An outstanding review on synthetic approaches to ART dimers, containing a large variety of both symmetric and non-symmetric linker units, is currently in press [164]. Apart from the length and flexibility of the spacer arm, in these adducts, the stereochemistry at the C-10 position is crucial for dimer activity, and accordingly, different attachment points of linkers to the ART scaffold have been explored.

Novel ART hybrids, including also dimers, were obtained by linking ART and triazine pharmacophores (compounds **104-109** in Fig. (**35**) with the adoption of microwave synthetic techniques. Notably, under radiation conditions, the ART endoperoxide bridge was found preserved [165].

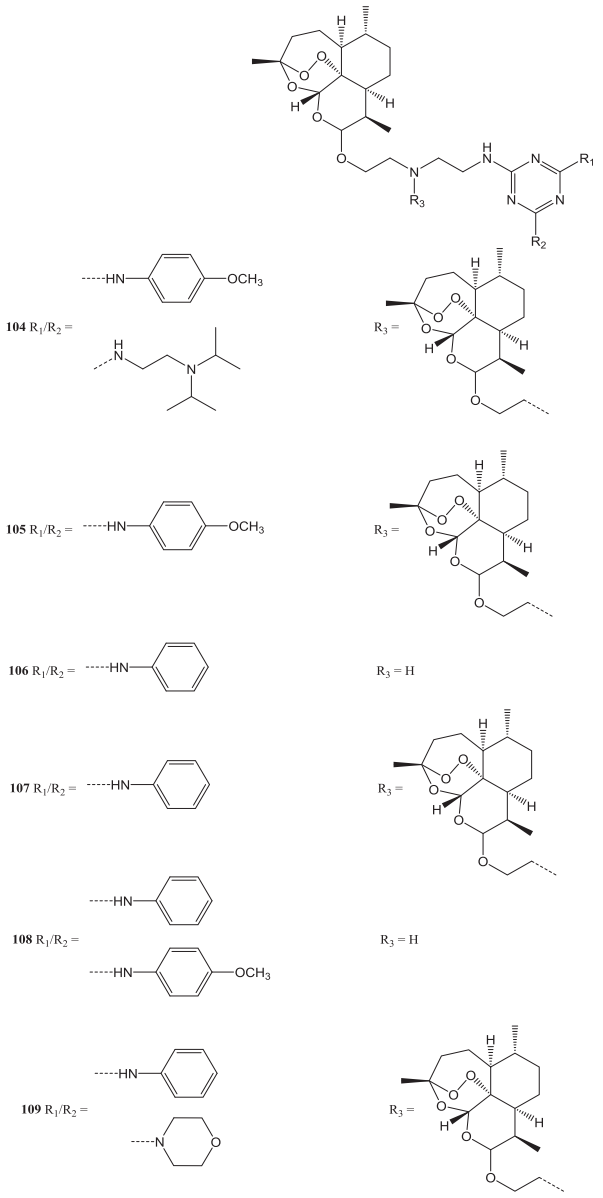
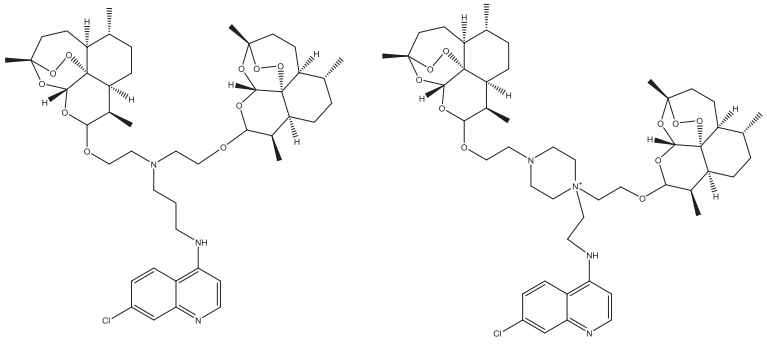


Fig. (34). Triazine conjugates with monomeric and dimeric artemisinin scaffolds.

The 1,3,5-triazine moiety is a common structure present in antifolate drugs (such as cycloguanil), which interfere in the tetrahydrofolate (THF) biosynthetic pathway by inhibiting dihydrofolate reductase (DHFR). In each reported compound, the triazine nucleus is substituted in positions 2,4,6 with three different groups; the one including an ethylendiamine arm is the common linker

unit to the single (hybrid monomers) or doubled (hybrid dimers) ART scaffold. All synthesized compounds were screened against the *P. falciparum* multidrug-sensitive NF54 strain, and the CQ- and mefloquine-resistant Dd2 strain. With regards to the NF54 strain, which is the strain of choice for testing gametocidal activity, all hybrid monomers and dimers were found to be less potent than both **23** and artesunate, although they were up to six thousand-folds more active than pyrimethamine. No synthesized compound showed better activity than the equimolar combination of **23** and pyrimethamine. This observation suggests that the triazine moiety may exert an antagonistic effect on the ART gametocydal action. On the opposite, dimers **105**, **107**, and **109** displayed potencies comparable to those of **23**, artesunate and the DHA-pyrimethamine equimolar mixture towards the Dd2 strain. However, all of the investigated hybrids were more potent than pyrimethamine against the same isolate. Again, **105** and **109** displayed a high level of selective toxicity towards the parasitic cells. More significantly, dimeric adducts proved to be slightly more active than their corresponding hybrid monomers against both the NF54 and Dd2 strains, probably as the result of more ART pharmacophore units reaching the site of action.

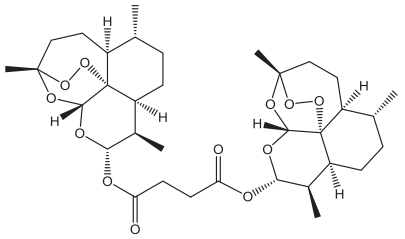
As prosecution of his pioneering work on ART-quinoline hybrids, Lombard and his group developed DHA-4-aminoquinoline dimers **110** and **111** (Fig. **35**)., which feature a diaminopropane arm connecting 4-amino-7-chloro-quinoline to the two ethyl ether linked-DHA scaffolds directly, like in hybrid-dimer **110**, or through interposition of a piperazine ring (compound **111).** Dimeric hybrids **110** and **111** displayed low nanomolar *in vitro* antimalarial activity against the *P. falciparum 3D7 strain*, similar to that previously observed against D10 and Dd2 strains, in comparison with **23** and **5** as standards. Further investigated in a *P. vinckei-infected mice model,* both compounds were shown to decrease parasitemia to extremely low levels [166, 167].



110 **111**

Fig. (35). DHA-aminoquinoline dimers **110** and **111** reported by Lombard's group.

An interesting series of ART dimers and trimers was masterly synthesized and screened for activity against the *P. falciparum* 3D7 strain, in comparison with the single counterparts artesunate and **23** (compounds **112-117** in Fig. (**36**) [168]. Although the antiplasmodial effectiveness of hybrids **112** and **113** (IC_{50} value of 2.6 nM for both) was higher than their parent compound **25** (IC_{50} = 9.0 nM), it was slightly lower when compared to **23** (IC_{50} = 2.5 nM). Interestingly, dimers **112** and **113** were considerably more effective than trimers **114** and **115** (IC_{50} of 12.8 nM for both) against the malaria parasite 3D7 strain. Chemically, they were obtained starting by either ART-derived acids or alcohols, in some cases with the interposition of spacer units with amine functions, linked by means of ester-, ether or amide bonds.



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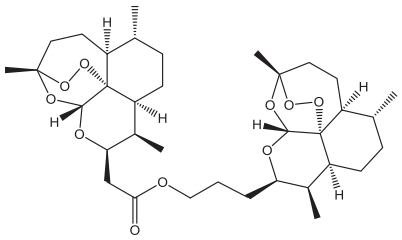

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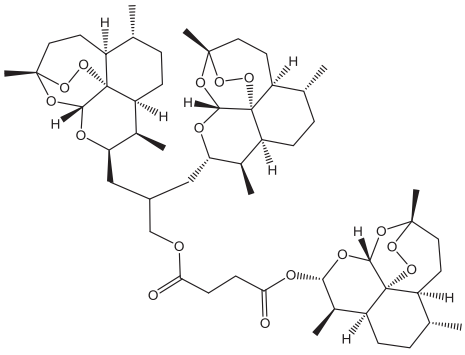

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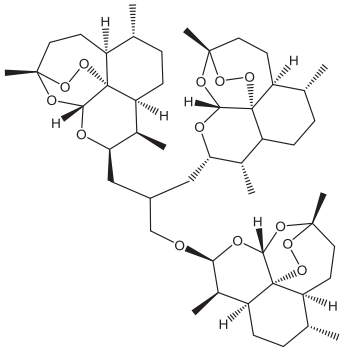

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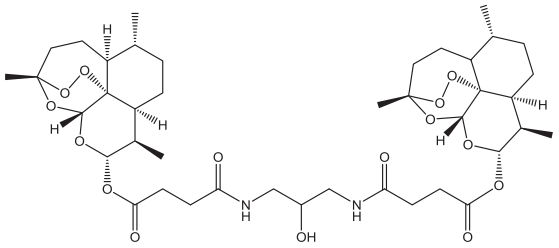

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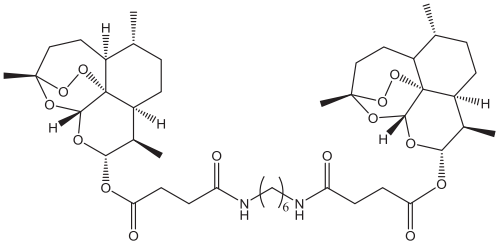


117

Fig. (36). Artemisinin dimers and trimers.

Beyond dimers and trimers, also ART tetramers, based on the rationale of adding as many peroxide moieties as possible to extend antimalarial activity, have been developed and thoroughly described [169].

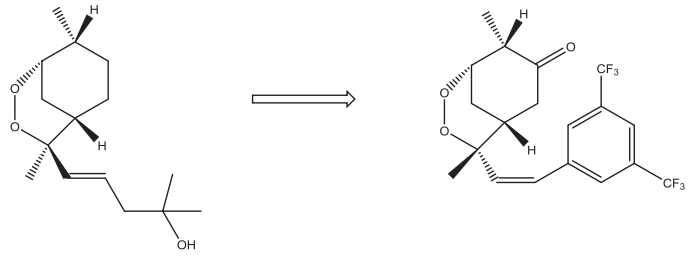
1,2-DIOXANE-, 1,2-DIOXOLANE-, 1,2,4-TRIOXANE-, 1,2,4-TRIOXOLANE-, AND 1,2,4,5-TETRAOXANE-BASED SYNTHETIC ENDOPEROXIDES AS ANTIMALARIAL CANDIDATES

One of the most unquestionable innovations in malaria chemotherapy has been the development of synthetic endoperoxide-containing drugs.

The definite statement that the crucial pharmacophoric moiety in the natural drug is represented by the 1,2,4-trioxane annular system has driven research into related endoperoxides, particularly 1,2,4-trioxane-, 1,2,4-trioxolane-, and 1,2,4,5-tetraoxan-based scaffolds, for developing novel antimalarial agents to be used against resistant parasites [170, 171]. Synthetic endoperoxides have been widely explored in an effort to preserve the potential of the iron(II)-catalyzed hemolytic cleavage of the peroxide bond in return for simpler structures and more cost-effective synthetic strategies in respect to artemisinins.

Dioxanes and Dioxolanes

The finding that yingzhaousu A **118**, a phytochemical endoperoxide isolated from *Artabotrys uncinatus*, owns good antimalarial activity despite the simplified 1,2-dioxane skeleton, has prompted the investigation of synthetic 1,2-dioxanes (exemplified by arteflene **119**, Ro-42-1611) and 1,2-dioxolanes, which all feature an endoperoxide bond constrained within a rigid, albeit simpler, skeleton. 1,2-dioxane **119** was progressed by Hoffmann-LaRoche as a stable analogue of yingzhaosu A Scheme (**4**), and although its suppressive activity is comparable to CQ in uncomplicated malaria, its development was discontinued after Phase III trials owing to high recrudescent rates, and long synthetic protocols [172].

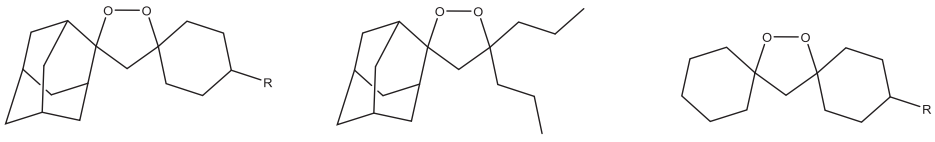


118
(IC_{50} = 17 nM)

119
(IC_{50} = 71 nM)

Scheme (4). Development of 1,2-dioxane-based arteflene **119** from the natural product yingzhaousu A **118**.

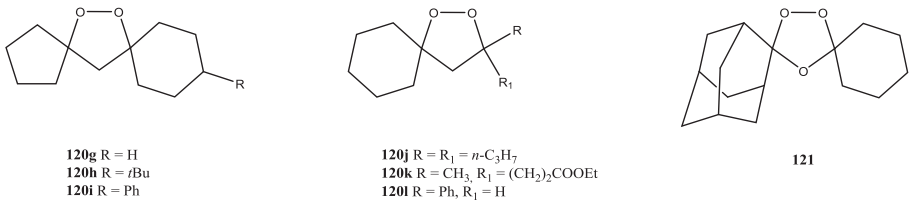
A dispiro-1,2-dioxolane series (compounds **120a-l** in Fig. **37** was synthesized by the Vennerstrom's group via peroxycarbenium ion annulations with alkenes; the most active 1,2-dioxolane **120f** was found more than 1000-fold less effective than dispiro-1,2,4-trioxolane **121** (Fig. **37**). and **22** against the CQR K1 and CQS NF54 strains of *P. falciparum* [173]. Furthermore, *in vivo*, only **120f** exhibited a reduction in parasitemia that exceeded 50% in a *P. berghei* ANKA strain-infected murine model.



120a R = H
120b R = Ph

120c

120d R = H
120e R = *t*Bu
120f R = Ph



120g R = H
120h R = *t*Bu
120i R = Ph

120j R = R_1 = *n*-C_3H_7
120k R = CH_3, R_1 = $(CH_2)_2COOEt$
120l R = Ph, R_1 = H

121

Fig. (37). Synthetic dispiro-1,2-dioxolanes **120a-l**, and dispiro-1,2,4-trioxolane **121** strictly related to **120a**.

The reported 1,2-dioxanes and 1,2-dioxolanes are typically one order of magnitude less potent than their poly-oxygenated counterparts; it has been suggested that the observed preference of these compounds to undergo a reduction of the peroxide bond by a two-electron pathway may enhance the pool of inactive species (the diol forms). Such behavior was observed for both arteflene **119** [174, 175] and the achiral 1,2-dioxolane **120a**, containing the oxygenated ring flanked by a spiroadamantane cage and a spirocyclohexane [173], in a model mimicking Fe(II)-catalysed decomposition of the peroxide unit. Furthermore, biopharmaceutical profile of 1,2-dioxanes and 1,2-dioxolanes is conditioned by short half-lives, due to instability, and poor physical-chemical properties. In this respect, 1,2,4-trioxanes, 1,2,4-trioxolanes, and 1,2,4,5-tetraoxanes hold greater promise.

TRIOXANES AND TRIOXOLANES

Trioxanes

Several synthetic 1,2,4-trioxanes have been investigated, many of which displayed low nanomolar antiplasmodial activity *in vitro* (compounds **122a-i** in Fig. (**35**). These compounds, featuring tricyclic or bicyclic scaffolds as proof that certain ART rings are not essential for activity, have greatly contributed to detail SARs [116, 176] and plasmocidal mechanism of action of endoperoxides. Classes of simpler spirocyclic 1,2,4-trioxanes, including a geraniol derivative, have been investigated for their ability to suppress parasitemia, with the 2-spiroadamantyl derivatives proven to be the most effective in regard to antimalarial activity. Despite this, interest in them has vanished, probably due to the greater large-scale synthetic viability and drug potential of 1,2,4-trioxolanes and 1,2,4,5-tetraoxans.

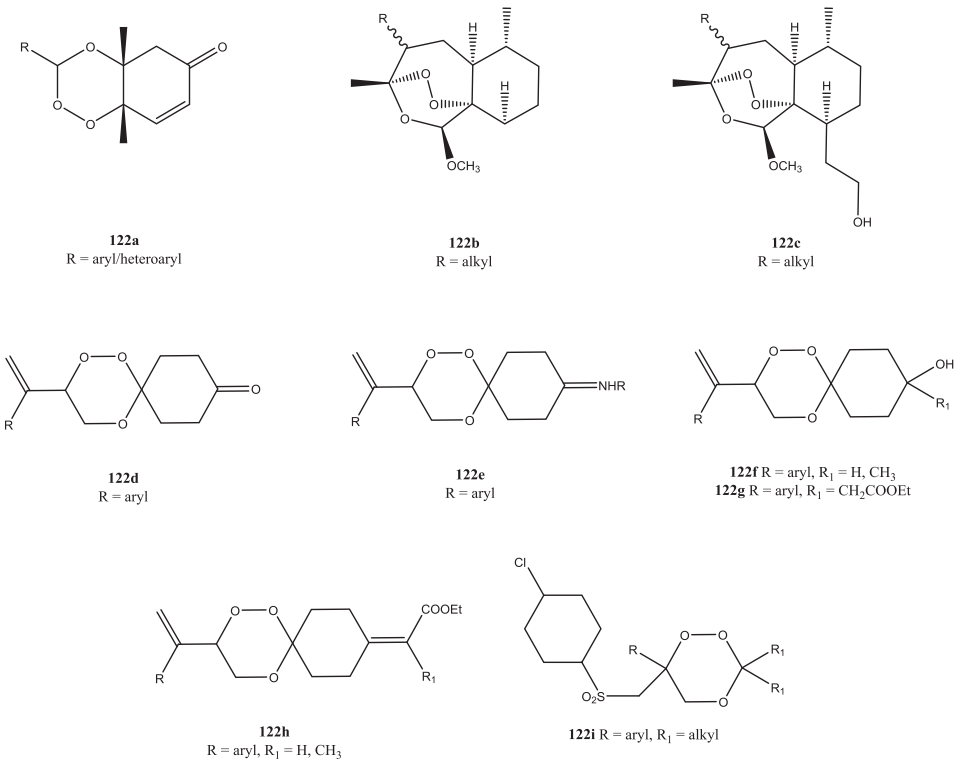
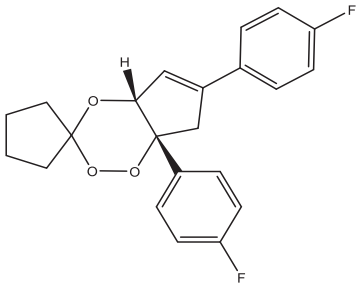


Fig. 38. The most relevant 1,2,3-trioxane antimalarial compounds representing structural simplification of artemisinin.

However, the class of fenozans, spirocyclic compounds containing a *cis*-fuse--ciclopentene-1,2,4-trioxane scaffold, deserves to be mentioned. Although sharing very little structural similarity with **22**, the difluorinated fenozan B07 **123** (Fig. **39**) has evidenced potent *in vitro* and *in vivo* antimalarial activity, which has been linked to the formation of primary carbon-centered radical species [177, 178].

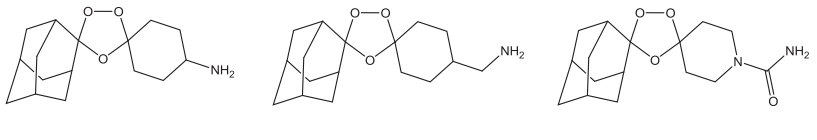


123

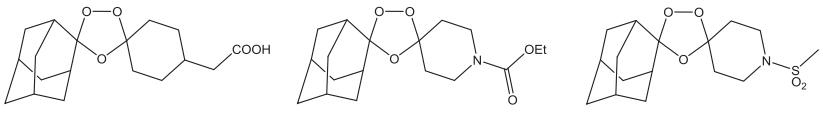
Fig. 39. The structure of fenozan B07 **123**.

Trioxolanes

1,2,4-Trioxolanes or ozonides (OZs) are a well-known class of intermediate organic compounds in the ozonolytic transposition of olefins into carbonyls. Since the surprising discovery of their excellent antimalarial activity, 1,2,4-trioxolanes have been the subject of wide interest, and thanks to the unceasing work of Vennerstrom's group, a huge number of OZs have been developed using the convenient Griesbaum co-ozonolysis reaction of suitable methyl oximes and ketones, and subsequent functionalization steps [179, 180]. In the dispiro-1,2,--trioxolane series (compounds **124a-k** in Fig. (**40**), the presence of an adamantane nucleus, connected to the 1,2,4-trioxolane ring through a spiro-perketalic carbon, and a spirocyclohexyl group on the other side have been found essential for antimalarial activity [181 - 183]. In the most active compounds, the lipophilic adamantane scaffold is counterbalanced by polar functional groups, preferably basic in nature [184]. Most OZ compounds have been shown to be more active than **24** and artesunate both *in vitro* and *in vivo*, and are able to target multiple stages of the parasite cycle.



124a **124b** **124c**



124d **124e** **124f**

(Fig. 40) contd.....

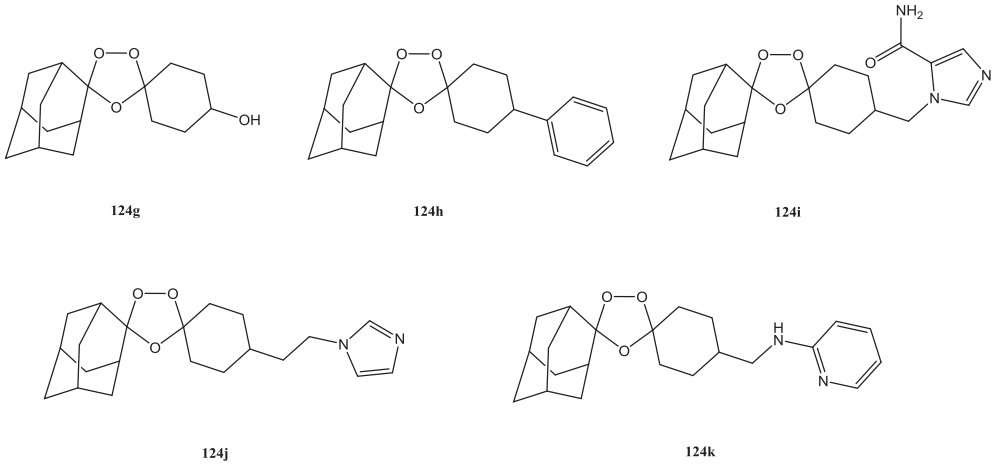


Fig. 40. Dispiro-1,2,4-trioxolane-based antimalarials **124a-k**.

The most promising candidates in the ozonide series are OZ277 **125** (arterolane), OZ339 **126**, and OZ439 **127** (artefenomel) (Fig. **41**). With regard to arterolane **125**, the trioxolane peroxide bond and the cyclohexyl group substituent in 8' are *cis*-configurated; despite the 8'-*cis* and 8'-*trans* diastereomers have been shown to be equipotent *in vitro*, **125** was found 40 times more active *in vivo* in respect to its 8'-*trans* diastereoisomer, owing to a longer half-life and greater oral bioavailability. Studies on conformational equilibria of the two diastereoisomers have found a rationale for this different behavior: the 8'-*cis* substituent favors a conformer in which the peroxide bond adopts an axial position, which is sterically hindered and less exposed to degradation *in vivo* [185]. Ozonide **125** has been registered in India in combination with piperaquine and was recently approved in seven African Countries [186].

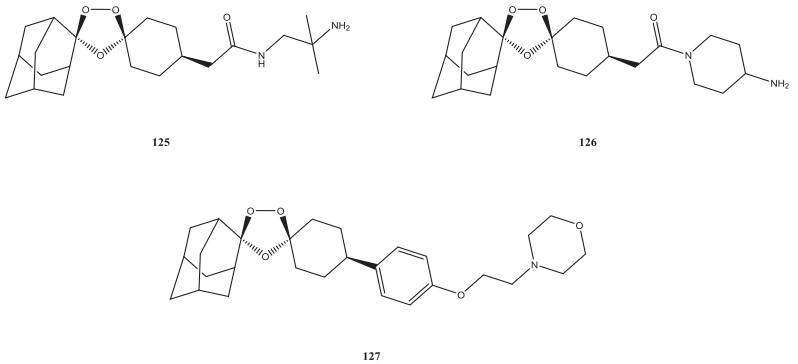


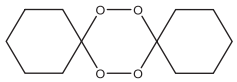
Fig. (41). Dispiro-1,2,4-trioxolanes OZ277 **125** (arterolane), OZ339 **126**, and OZ439 **127** (artefenomel).

As far as OZ339 **126** is concerned, the 1,2,4-trioxolane was tested in a comparative *P. berghei*-infected mice study including **125**, with artesunate as control. This potent ozonide was found to possess the highest antimalarial efficacy, with excellent survival time, due to a better pharmacokinetic profile with respect to **125** [182].

The most encouraging drug-like molecule is artefenomel **127**, obtained by replacement of the 8'-*cis*-positioned cyclohexyl ring with an 8'-aryl nucleus. This modification resulted in slower elimination compared with ART derivatives and the first-generation ozonide **125**, making the candidate adapt for single-dose treatment. 1,2,4-Trioxolane **127** is a fast-acting inhibitor of all asexual erythrocytic *P. falciparum* stages; furthermore, it maintains strong antimalarial activity in infections carrying the *Pfk13* propeller mutations, which are strongly related to ART resistance [187].

Tetraoxanes

Finally, the development of antimalarial candidates containing the 1,2,4,5-tetraoxane ring has been actively pursued. Largely used in industrial production of macrocyclic hydrocarbons, in the early 1990s, symmetrical dispiro-1,2,4,--tetraoxanes as simple as **128**, containing the 1,2,4,5-tetraoxacyclohexane core (Fig. **42**), were discovered to possess impressive *in vitro* antimalarial activity [188]. Due to achirality and inherent thermodynamic stability as well [189], **128** has been chosen as the key scaffold of a series of both symmetric and asymmetric disubstituted derivatives. An expected advantage of these compounds is they can be easily synthesized by acid-catalyzed peroxidation of widely available cyclic ketones, followed by subsequent incorporation of polar functionalities, *via* reductive amination and amide bond formation.



128

Fig. (42). The simplest dispiro-1,2,4,5-tetraoxane with potent antimalarial activity.

In the mixed dicyclohexylidene compounds (compounds **129a-d**, **130a-c**, **131a-f**, and **132a-d** in Fig. (**43**), the aim was to achieve the minimal amphiphilic structures to minimize the effect of steric effects on antimalarial mechanism. This series is characterized by similar *in vitro* nanomolar activities against both CQS and CQR *P. falciparum* strains, disregarding the nature of the substituent (neutral, polar, or basic) [190 - 192].

Again, the incorporation of a spiroadamantyl substituent, compared to other groups, resulted in superior effectiveness against both CQS and CQR *P. falciparum* isolates. In order to augment water solubility and optimize the biopharmaceutical profiles of these candidates, the highly lipophilic nature of the adamantyl scaffold was finely tuned by adding polar groups at the other end, such as the sulfonamide unit (compounds **133a-c**), or mainly basic appendages at the C-9' position (compounds **134a**,**b**) [193].

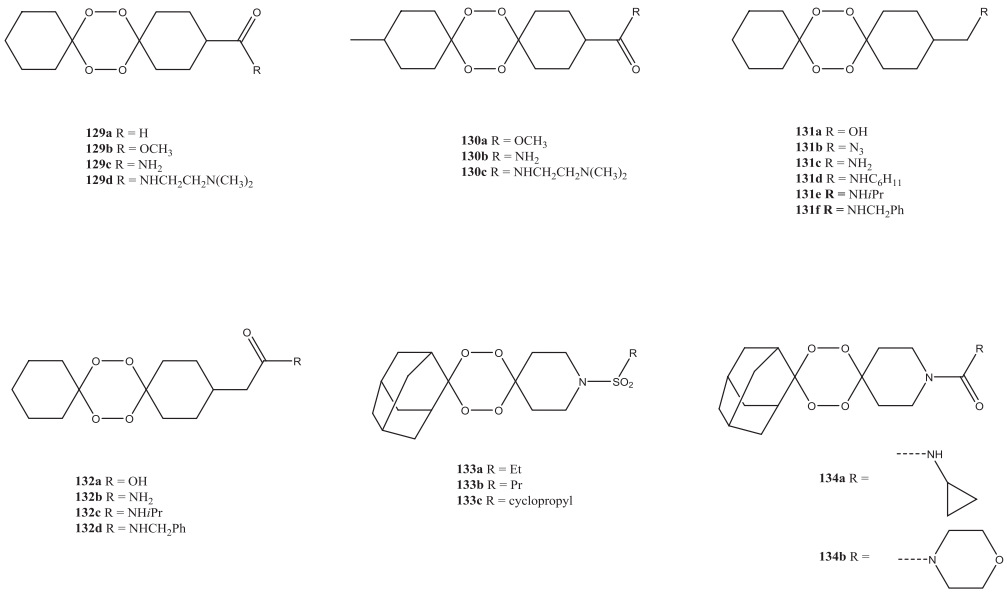
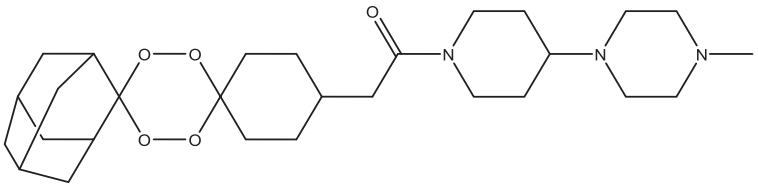


Fig. (43). Mixed amphiphilic tetraoxanes.

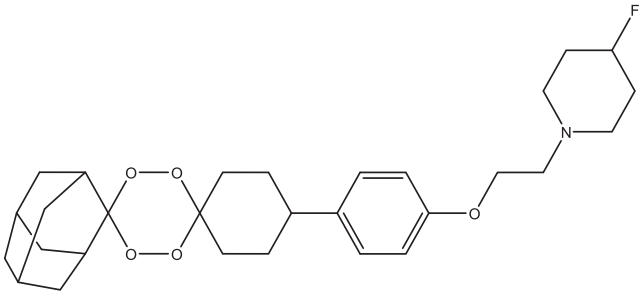
Through endless research, O'Neill's group developed other promising candidates [194, 195]. RKA182 **135** (Fig. **44**). was reported as a metabolically stable and potent derivative, being superior to artemether **24** and artesunate, and comparable to artemisone **51**, in both *in vitro* and *in vivo* assays. Unfortunately, it failed to reach the requirements of a single-dose treatment, and its full preclinical development has been discontinued.



135

Fig. (44). The molecule of RKA182.

From a wide library of **135** analogs developed by the same group in search of more balanced ADME properties, no candidate emerged in the amide-linked series. Finally, SAR studies on a new class of aryloxy-containing tetraoxanes, led to the discovery of E209 **136** (Fig. **45**). as the front-runner molecule in regard to curing rates in the *P. berghei* model and initial Drug Metabolism and PharmacoKinetics (DMPK) investigation. E209 displays potent nanomolar activity *in vitro* against multiple strains of *P. falciparum* and *P. vivax* (mean IC_{50} range 2.9/14.0 nM), and complete *P. falciparum* clearance *in vivo* achievable with a single oral dose of 30 mg/kg, with a calculated ED_{90} of 11.6 mg/kg (*cf.* ED_{90} of 10 mg/kg for artesunate, following four consecutive daily doses, corresponding to total 50 mg/kg) [196].



136

Fig. (45). The molecule of E209.

Further, chimeric peroxide compounds with other drugs, preferably aminoquinolines (trioxaquine derivatives), have been widely investigated and thoroughly reviewed [197].

The alkylation of heme by synthetic peroxides has been assessed, and their alkylation ability correlates well with their antimalarial efficacy. Most probably, however, they do not fit into a unique mechanism of action; single compounds could target parasites at multiple sites, or exert their antiplasmodial activity by causing oxidative stress [198].

CONCLUSION

The golden age of antimalarial therapy has been marked by the discovery and application of artemisinin as a new curative agent towards the most severe *P. falciparum* infection presentations. This natural endoperoxide-containing antimalarial has captured a great deal of attention since its introduction, due to a combination of outstanding potency, safety, and unique mechanism of action. Nevertheless, acknowledged drawbacks, mainly concerning the pharmacokinetic profile and the risk of emerging resistant parasites, have initially oriented the scientific community towards the structural modification and simplification of this truly fascinating drug, and the use of combination therapies to provide a prolonged, synergistic activity and avoid resistance. Synthetic approaches have also allowed for in-depth knowledge of chemical properties of artemisinin-related compounds, as well as the establishment of fundamental SARs. Research interest culminated in the discovery of more effective drugs, offering advantages in terms of chemical stability, synthetic feasibility and costs. The most promising therapeutic option is by far represented by the plethora of endoperoxide-based synthetic antimalarials, with more than a few currently under clinical evaluation. In view of their structural diversity and synthetic feasibility, in the near future, we should expect that endoperoxide scaffolds (1,2,4-trioxane-, 1,2,4-trioxolane- and 1,2,4,5-teraoxane-based) and their chemical analogs, including chimeric molecules, will represent the richer source of drug-like candidates for a cogent, safe, and cost-effective treatment of malaria. This developmental strategy would benefit from molecular design and *in silico* optimization studies, as well as from bioavailability and toxicity assays.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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SUBJECT INDEX

A

B

C

D

E

F

G

H

I

L

M

N

O

P

Q

R

S

T

V

W

Z

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