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PHARMACOLOGICAL CHARACTERIZATION OF TERPENIC SECONDARY METABOLITES ISOLATED FROM SALVIA SPECIES

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SOMMARIO

Questa tesi di dottorato è incentrata sullo studio di composti di natura terpenica isolati da piante del genere *Salvia* al fine di evidenziare le basi razionali che giustifichino l'utilizzo tradizionale delle piante medicinali.

Alla parte più propriamente sperimentale è stata premessa una rassegna sui principali composti di natura terpenica e le rispettive attività biologiche possedute.

In collaborazione con diversi gruppi di ricerca è stato possibile effettuare un lavoro di screening su otto piante, appartenenti al suddetto genere, che ha permesso di ottenere informazioni interessanti, dal punto di vista farmacologico, su i principali terpenoidi isolati da *Salvia divinorum*, *Salvia jamensis* e *Salvia cinnabarina*.

Per quanto riguarda la *Salvia divinorum*, poiché in letteratura è riportato che le foglie fresche, masticate lentamente, costituiscono un rimedio della tradizione mazteca adoperato per il trattamento della diarrea, nel nostro studio abbiamo valutato l'effetto di un estratto etanolico ottenuto dalle foglie di *Salvia divinorum* (SDE) e del suo principio attivo la salvinorina A (noto diterpenoide clerodano), sulla trasmissione colinergica enterica nell'ileo isolato di cavia. I dati ottenuti mostrano che la *Salvia divinorum* esercita un effetto inibente sulle contrazioni della muscolatura liscia intestinale. Tale effetto è strettamente legato all'attività agonista della salvinorina A, sui recettori κ degli oppioidi, i quali svolgono un ruolo cruciale sul controllo del rilascio di mediatori chimici eccitatori quali l'acetilcolina a livello pregiunzionale.

Particolare attenzione è stata rivolta poi alla *Salvia cinnabarina* e al principale costituente attivo da essa isolato il diterpenoide pimarano, acido 3,4-seico-isopimaric-4(18),7,15-triene-3-oico (CMP1). Il nostro lavoro è stato finalizzato ad investigare i potenziali effetti a livello del Sistema Nervoso Centrale (SNC) del diterpenoide (composto 1). Nei nostri esperimenti condotti in vivo su topi, il composto 1 mostra effetti ansiolitici valutati mediante diversi modelli sperimentali (elevated plus maze e Porsolt test; test di locomozione: open field) possiede inoltre proprietà sedative, in quanto incrementa il tempo di sonno indotto da pentobarbitale. Gli effetti sono stati confrontati con quelli del diazepam, utilizzato come controllo positivo. L'elemento interessante dal punto di vista farmacologico emerso dal nostro studio è rappresentato dal fatto che il composto 1, alla dose ansiolitica, non modifica l'attività motoria spontanea al contrario del diazepam che induce effetti sedativi e nello stesso tempo provoca una riduzione dell'attività motoria. Il diterpenoide in questione ha inoltre

dimostrato, in un ulteriore studio condotto su topi anestetizzati in monitoraggio continuo della pressione, un interessante effetto ipotensivo. Tale effetto, legato ad un meccanismo periferico del composto, ha dimostrato di essere indipendente sia da una sua azione sul sistema nervoso autonomo via stimolazione centrale ,sia dal rilascio di ossido nitrico.

La terza pianta medicinale presa in esame è la *Salvia jamensis*. Nonostante sia poco conosciuta dal mondo scientifico, essa contiene diversi composti di natura terpenica (di- e triterpenoidi), alcuni dei quali possiedono notevoli attività biologiche. Il nostro gruppo ha indagato l'effetto dell'essudato ottenuto da foglie (SDE) e dei principali terpenoidi isolati (composti 1-7), sull'aggregazione piastrinica di ratto in vitro. I dati ottenuti hanno messo in evidenza che l'essudato (SDE) possiede un lieve effetto inibente, concentrazione-dipendente, sull'aggregazione indotta da ADP (3μM). Tale effetto è dovuto alla presenza di due principi attivi dotati di attività contrapposte. In particolare abbiamo visto che l'acido isopimarico (composto 2), diterpenoide pimarano ben conosciuto, possiede un'azione anti-aggregante e concentrazione-dipendente significativa, al contrario il nuovo diterpenoide clerodano (composto 1), trovato sinora solo nelle foglie di *Salvia jamensis*, induce un aumento dell'aggregazione piastrinica indotta da ADP (3μM).

I risultati riportati in questa tesi sono stati in parte presentati a congressi nazionali e divulgati su riviste internazionali di interesse scientifico.

ABSTRACT

Recently most interest has been focused on active compounds extracted from natural sources. This is due to the fact that many of these natural products in plants of medicinal value can offer new sources of drugs.

The genus *Salvia* from the Lamiaceae family has numerous different species - about 900 species- which are extensively distributed in various regions of the world. Many *Salvia* species are well-studied and widely used in traditional medicine. Plants belonging to the genus *Salvia* are of particular interest, due to the wide variety of secondary metabolites produced in these plants, such as flavonoids, tannins and terpenoids.The terpenoid class, extracted from *Salvia*, includes a wide range of mono-, di-, tri-, sesqui- and tetraterpenoids. Many diterpenoids and triterpenoids, isolated from plants of several species of the genus *Salvia*, have been investigated for their pharmacological activities: analgesic; anti-inflammatory; hemostatic; antioxidant; antimicrobial and as an antitumoral remedy. It has been widely shown that many terpenoid compounds have significant anti-inflammatory effects. For this reason, they are potential molecules for the development of new drugs especially designed for the treatment and/or control of chronic inflammatory states such as rheumatism, asthma, inflammatory bowel diseases, atherosclerosis, etc. Some di- and triterpens from *Salvia* ssp are able to influence the central nervous system (CNS).

In this thesis, the pharmacological effects of bioactive diterpenoids and triterpenoids from the *Salvia* species are investigate. In a screening programme on *Salvia* medicinal plants, like *S. divinorum*, *S. cinnabarine* and *S. jamensis*, we have find interesting biological effects.

Following anecdotal reports that extracts of *Salvia divinorum* may possess antidiarrhoeal activity and because κ-opioid receptors may modulate intestinal peristalsis we investigated the effect of *Salvia divinorum*, and its main active ingredient, salvinorin A, on myenteric cholinergic transmission. For this purpose we evaluated the effect of a standardized extract from *Salvia divinorum* leaves (SDE) and of isolated salvinorin A on the contractions elicited either by electrical stimulation or by exogenous acetylcholine in the guinea-pig ileum. These observations strongly support the hypothesis that SDE inhibits the twitch response by acting prejunctionally rather than through a direct action on intestinal smooth muscle. The hallucinogenic herb *Salvia divinorum* exerted inhibitory effects on enteric cholinergic transmission in

the guinea-pig ileum through activation of prejunctional κ-opioid receptors. Salvinorin A may be the chemical constituent responsible for this activity. These results could provide the pharmacological basis underlying its traditional antidiarrhoeal use.

During this PhD project the author has studied the effects in vivo of a new diterpenoid compound 3,4-secoisopimar-4(18),7,15-triene-3-oic acid (CMP 1) from *Salvia cinnabarina*. The possibility that CMP 1, like other isolated diterpenoids, may affect the CNS, was the main aim of these investigations. A putative anxiolytic and antidepressive activity of CMP 1 were studied in the elevated plus-maze test and in the forced swimming test. Furthermore, CMP 1 was administered after a pre-treatment with pentobarbital and its effects on sedative activity was monitored. The CMP 1 was also tested for its effects on spontaneous motor activity (total motility and locomotion) and several models of nociception have been used to examine the potential analgesic effects. These studies demonstrate for the first time that CMP 1 has pronounced CNS depressant properties, manifested as antinociception, sedation and axiolitic effects. In another study we evaluated the effect of CMP1 on arterial blood pressure in anaesthetized rats. On different groups of them treated with the ganglion-blocking agent chlorisondamine, the effect of CMP1 was evaluated before and following an infusion of the nitric oxide synthase inhibitor L-NAME. Intravenous administration of CMP1 led to a fall in mean arterial blood pressure that was not modified by treatment of the rat with chlorisondamine nor with L-NAME. The results demonstrate , for the first time, a hypotensive effect of CMP1 due to a peripheral mechanism but independent of endothelial nitric oxide release.

The chemical constituents of *Salvia x jamensis* J. Compton and their platelet antiaggregating activity in vitro were also investigated. The surface exudate of *Salvia x jamensis* J. Compton, obtained by rinsing the plant material with methylene chloride and terpenic compounds 1-7 isolated have been tested on ADP-induced platelet aggregation. Among all the tested compounds, isopimaric acid **2** showed a significant concentration-dependent antiaggregating activity when ADP (3 μM) was used as agonist on rat platelets. Conversely, a new clerodane derivate 1 increased ADP–induced platelet aggregation.

INTRODUCTION

The *Salvia* genus belongs to the Lamiaceae or Labiatae family (about 200 kinds break into more than 3,000 species) and it is composed of at least 900 species and dozens of *cultivars* consisting of hybrids [1-2]. This is a genus consisting of species with annual, biennial and perennial cycle shrub shape in origin countries. Although species belonging to genus *Salvia* are common throughout the world, they are more concentrated in Europe (in countries bordering the Mediterranean), in South-Eastern Asia, Central and South America and South Africa [2].

The history of the use of several plants of the *Salvia* genus as a medicinal treatment spans several millennia, and both ancient and modern cultures. The name *Salvia* comes from the Latin *salveo* or *salvus*, the verb *salvare*, in reference to the curative properties of the specie that is certainly better known and used: *Salvia officinalis.* The Medical School of Salerno, one of the most famous in the Middle Ages, depositary throughout antiquity of medical knowledge, called this plant *Salvia salvatrix,* that is "Salvia saving"-*salvia salvatrix naturae conciliatrix*- and it was considered to be the miracle herb par excellence [2]. Before and after the Romans, from the Egyptians to medieval pharmacopoeia, *Salvia officinalis* was always appreciated in herbal medicine. It is no coincidence that in the 1700's the Swedish biologist and naturalist Linneaeus (father of binomial nomenclature for the systemic classification of plants and animals) assigned the name *officinalis* to this plant. Today, many of the numerous medicinal properties that have been attributed to it in the past have been confirmed by scientific studies. Therefore, several species of *Salvia* are found in the Pharmacopoeias of numerous countries around the world. In the therapeutic field several drugs isolated from plants of the genus *Salvia* are used to treat various disorders. This is due to its several pharmacological properties [3]. The *Salvia* genus has been found to be a rich source of terpenes, tannins and flavonoids [3]. Terpenes are mainly compounds of plant and animal origin [4]. All terpenes are hydrocarbons having carbon skeletons based on combinations of a C-5 unit, called isoprene unit (figure 1).

Terpenoids are compounds related to terpenes, which may include some oxygen functionality or some rearrangement. However, the two terms are often used interchangeably. Just like terpenes, the terpenoids bind to each other by following the "head-tail" order and, depending on how many units work together to form the bonds,

are divided into: monoterpenoids (2 units); sesquiterpenoids (3 units); diterpenoids (4 units), sesterterpenoids (5 units); triterpenoids (6 units) and tetraterpenoids or carotenoids (8units).

Diterpenoids found in *Salvia* species

Diterpenes or diterpenoids derive from condensation of four isoprene units, but the structural changes involved in the last stages of the biosynthetic pathway lead to a wide variety of complex chemical structures, including acyclic compounds, bi-, tri-and tetra-cyclic.

a) Bi-cyclic diterpenoids.

Bi-cyclic diterpenoids are classified in labdanes and clerodanes types (see Table A). A labdane diterpenoid, 8(17),12E,14-labdatrien-6,19-olide was obtained from the chloroform extract of *Salvia leriaefolia* and its antimicrobial activity against the Gram-positive bacterium Staphylococcus aureus has been reported [5].

The Lamiaceae are a rich source of clerodane diterpenoids. A number of these metabolites have been isolated from *Salvia* species including salvinorin A, divinatorin and salvinicin A, from *Salvia divinorum,* [6]. Salvinorin A has been evaluated for its hallucinogenic [7], anti-inflammatory [8] antinociceptive and hypothermic [9] activities. Salvinorin A, a non-nitrogenous opioid receptor agonist, displays significant binding at the Gαi-coupled κ opioid receptor (KOR) [10]. Anti-diarrhoeic effect is also reported: Salvinorina A is able to inhibit intestinal hypermotility in vitro [11] and in vivo [12]. Its inhibitory effect in the inflamed gut in vivo reveals a functional interaction between salvinorina A binding at KORs and cannabinoid CB(1) receptors [13]. In a recent study, oxadiazole and salvidivin A, a photooxygenation product of salvinorin A, have been identified as the first neoclerodane diterpenes with kappa antagonist activity [14]. A number of clerodane diterpenoids have been obtained from *Salvia* species including splenolide A [15] and salvixalapadiene and isosalvixalapadiene from *Salvia xalapensis* [16]. Salvisplendins A-D were isolated from *Salvia splendens* [17] and have been tested for affinity at human μ, δ and κ opioid receptors [18]. Salvileucalin B, a novel diterpenoid with a neoclerodane skeleton from *Salvia leucantha*, has shown cytotoxic activity in A549 e HT-29 cancer cells [19]. A new diterpenoid with clerodane skeleton from *Salvia jamensis*: 15, 16-epoxy-cleroda-3-en-7α, 10β-dihydroxy-12,17,19,18-diolide was found to increase ADP–induced platelet aggregation in vitro [20].

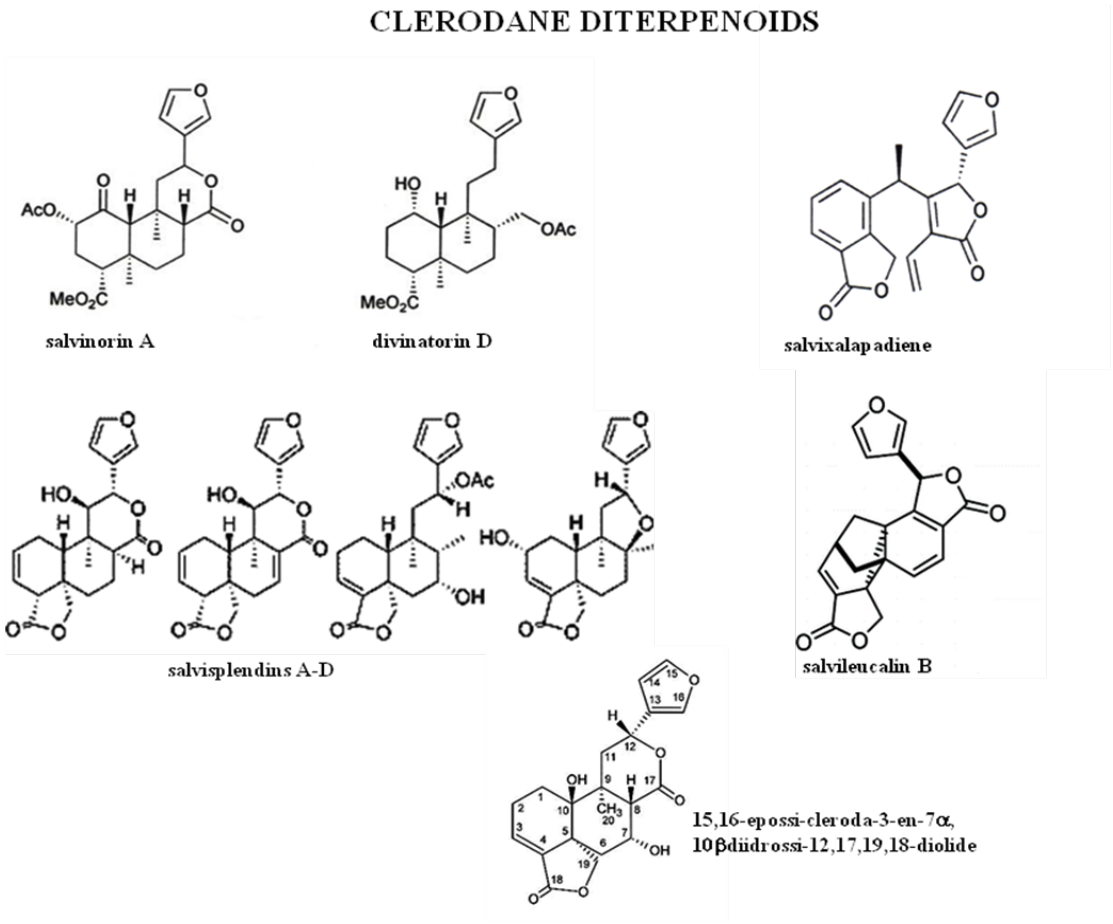

Figure 1: clerodane-type diterpenoids from *Salvia* species.

Table A: Bi-cyclic diterpenoids isolated from Salvia species and their biological activies

Bi-cyclic diterpenoids	Secondary metabolite constituents	Salvia species	Biological activities
Labdane-type	• 8(17),12E,14-labdatrien-6,19-olide	*Salvia leriaefolia*	• antimicrobial activity
Clerodane-type	• salvinorin A, divinatorin e salvinicin A	*Salvia divinorum*	• hallucinogenic, anti-inflammatory, antinociceptive, hypothermic and anti-diarrheic proprieties
	• splenolide A e salvisplendins A-D	*Salvia splendens*	• affinity at human μ, δ and κ opioid receptors
	• salvixalapadiene e isosalvixalapadiene	*Salvia xalapensis*	
	• salvileucalin B	*Salvia leucantha*	• cytotoxic activity in A549 e HT-29 cancer cells
	• 15,16-epossi-cleroda-3-en-7α,10βdiidrossi-12,17,19,18-diolide	*Salvia jamensis*	• increase ADP–induced platelet aggregation in vitro

b) Tri-cyclic diterpenoids.

The main terpenic constituents of the *Salvia* species are represented by tri-cyclic diterpenoids, which can be classified in abietane and pimarane diterpenoids (table B).

Among the abietane-type, phenolic, quinone and icetaxane diterpenoids have been reported.

It is known that phenolic diterpene compounds, including carnosic acid, carnosol, ferruginol, 7-methoxyrosmanol and galdosol, obtained from the *Salvia* species, exhibit a variety of biological activities. It is demonstrated that carnosic acid and carnosol inhibited pancreatic lipase activity, which is participated in digestion of lipids [21]. Both compounds are activators of the human peroxisome proliferator-activated receptor gamma (PPAR-gamma) and this activation increases the transcription of enzymes involved in primary metabolism, leading to lower levels of fatty acids and glucose in the blood [22]. Carnosic acid and carnosol show pronounced antioxidant and anti-inflammatory effects. A recent study indicated that carnosic acid and carnosol inhibit the formation of pro-inflammatory leukotrienes in intact human polymorphonuclear leukocytes (PMNL) and both potently antagonise intracellular Ca^{2+} mobilisation induced by a chemotactic stimulus. Carnosic acid and carnosol attenuate formation of reactive oxygen species and the secretion of human leukocyte elastase [23]. Some of the phenolic-type diterpenoids show antimicrobial activity: carnosic acid 12-Me ester, isolated from the acetone extract of *Salvia microphylla*, possessed antimicrobial activity against Staphylococcus aureus [24]. Carnosic acid, from *Salvia africana-lutea*, exhibited MICs of 28 μM, against Mycobacterium tuberculosis [25], while carnosol greatly reduced the MICs of various aminoglycosides (potentiated the antimicrobial activity of aminoglycosides) and some other types of antimicrobial agents in vancomycin-resistant enterococci (VRE) [26]. A scientific report indicates that some diterpenoids with abietane-phenolic skeleton have activities on the central nervous system (CNS). 7-methoxyrosmanol competitively inhibited 3H-flumazenil binding to the benzodiazepine receptor and galdosol showed the strongest binding activity to the benzodiazepine receptor [27].

Examination of the *Salvia* species has afforded a series of abietane-quinone type diterpenoids, the secondary metabolites that are widely known and studied. Aethiopinone, salvipisone, 1-oxoaethiopinone and ferruginol, abietane-phenolic type, obtained from *Salvia sclarea*, proved to be bacteriostatic as well as bactericidal for the

cultures of Staphylococcus aureus and Staphylococcus epidermidis strain [28]. Walencka et al (2007) [29] reported that salvipisone and aethiopinone from *Salvia sclarea* showed synergy activity with β-lactam antibiotics through alteration of cell surface hydrophobicity and permeability, but not by changing penicillin-binding protein. Two abietane-quinone type diterpenoids, yunnannin A and danshenol C, were isolated from *Salvia yunnanensis* and were tested for their antitumor activity in T-24, QGY, K562, Me180 and BIU87 cell lines and showed inhibited growth [30]. Examination of the Algerian Salvia species has afforded 7-oxoroyleanone-12-Me ester and inuroyleanol, isolated from *Salvia barrelieri*, which showed potential antioxidant activity. In particular, 7-oxoroyleanone-12-Me ester possessed the highest superoxide anion scavenging activity, while inuroyleanol inhibited lipid peroxidation and showed the highest diphenyl-2-picrylhydrazyl (DPPH) scavenging activity [31].

An increasing number of quinone diterpenoids are being found in *Salvia miltiorrhiza*, which is used in many therapeutic remedies in Chinese traditional medicine. Tanshinones are the major bioactive compounds of this plant and have been well-studied for their biological activities. A recent study has shown the anticancer activity of tanshinones and this effect is associated with their inhibition of HIF-1 accumulation [32]. It as been reported that sibiriquinone A, sibiriquinone B, cryptotanshinone, and dihydrotanshinone I greatly inhibited hypoxia-induced luciferase expression on AGS cells (a human gastric cancer cell line), and on Hep3B cells (a human hepatocarcinoma cell line)[32]. Recently, anti-cancer activities of tanshinone I have been reported. The effect of tanshinone I on the induction of apoptosis in human breast cancer cells (MCF-7 and MDA-MB-231) in vitro has been investigated. Tanshinone I inhibited cell proliferation of MCF-7 and MDA-MB-231 cells in a concentration- and time-dependent manner and significantly induced apoptosis in these cells. The induction of apoptotic cell death was mediated by the activation of caspase 3, the downregulation of the level of the anti-apoptotic protein Bcl-2, and the upregulation of the level of the pro-apoptotic protein Bax [33]. In another study, it has been found that tanshinone I concentration dependently inhibited ICAM-1 and VCAM-1 expressions in human umbilical vein endothelial cells (HUVECs) and pre-treatment with tanshinone I significantly reduced adhesion of either monocyte U937 or MDA-MB-231 cells to HUVECs. In addition, tanshinone I effectively inhibited TNF-alpha-induced production of vascular endothelial growth factor (VEGF) and VEGF-mediated tube formation in HUVECs. Tanshinone I also

inhibited TNF-alpha-induced VEGF production in MDA-MB-231 cells and migration of MDA-MB-231 cells through extracellular matrix. Additionally, a reduction in tumor mass volume and decrease in metastasis incidents by tanshinone I was observed in vivo [34]. Another recent study investigated the anticancer effects of tanshinones on the highly invasive human lung adenocarcinoma cell line CL1-5. Tanshinone I significantly inhibited migration, invasion, and gelatinase activity in macrophage-conditioned medium-stimulated CL1-5 cells in vitro and reduced the tumorigenesis and metastasis in CL1-5-bearing severe combined immunodeficient mice. These effects are mediated, at least in part, through transcriptional activity of interleukin-8, the angiogenic factor involved in cancer metastasis, Ras-mitogen-activated protein kinase, and Rac1 signaling pathways [35]. Unlike tanshinone I, which did not have direct cytotoxicity, tanshinone IIA, a diterpene quinone extracted from *Salvia miltiorrhiza*, has been reported to have apoptosis inducing effects on a large variety of cancer cells. The anti-proliferation and apoptosis inducing effects of tanshinone IIA as well as its influence on cell adhesion to and invasion through the extracellular matrix (ECM) on acute promyelocytic leukemia (APL) NB4 cells in vitro were studied. Tanshinone IIA exhibited induction of apoptosis by activation of caspase-3, downregulation of anti-apoptotic protein bcl-2 and bcl-xl and upregulation of pro-apoptotic protein bax, as well as disruption of the mitochondrial membrane potential [36]. Recently, Liu et al [37] have confirmed the antiproliferation and apoptosis effects of tanshinone IIA on leukemia THP-1 cell lines. It is reported that tanshinone IIA arrests cancer cells in mitosis by disrupting the mitotic spindle and subsequently triggered cells to enter apoptosis through the mitochondria-dependent apoptotic pathway. In comparison with other existing anti-cancer drugs that cause mitotic arrest by interfering with the microtubule structure (such as vincristine or taxol), tanshinone IIA destroyed only the mitotic spindle during the M phase but not the microtubule structure in interphase cells [38]. Kim et al [39] have recently shown that tanshinone IIA completely inhibited osteoclastogenesis, indicating a correlation between *Salvia miltiorrrhiza* extract-inhibitory effect on osteoporosis and its chemical metabolites.

Tanshinone IIA also exerts antioxidant and anti-inflammatory actions in many experimental diseases: Tanshinone IIA protects cardiac myocytes against oxidative stress-triggered damage and apoptosis [40] and reduces macrophage death induced by hydrogen peroxide by up regulating glutathione peroxidise [41]. Tanshinone IIA decreases the transcription level of the microsomal triglyceride transfer protein gene,

suggesting that it inhibits ApoB secretion via a proteasome-dependent pathway [42]. Recently, it has also been reported that tanshinone IIA, tanshinone I and 15,16-dihydrotanshinone, from *Salvia miltiorrhiza*, enhanced the activity of insulin on the tyrosine phosphorylation of the insulin receptor (IR) beta-subunit and the activation of the downstream kinases signalling in vitro [43]. It has been demonstrared that tanshinone IIA not only inhibits the oxidation but also suppresses the inflammation in atherosclerotic lesion [44]. Tanshinone IIA has multiple effects on the inhibition of human aortic smooth muscle cell (HASMC) migration and may offer a therapeutic approach to block HASMC migration [45]. The effects of tanshinone derivatives (tanshinone I, cryptotanshinone, 15,16-dihydrotanshinone I) on prostaglandin (PG) and nitric oxide (NO) metabolism have also been reported. Jeon et al 2008[46] have recently demonstrated that cyclooxygenase-2 (COX-2)-mediated PGE2 production, from lipopolysaccharide-treated RAW 264.7 cells, was inhibited by tanshinone I, cryptotanshinone and 15,16-dihydrotanshinone I, while only cryptotanshinone and 15,16-dihydrotanshinone I inhibited inducible NO synthase (iNOS)-mediated NO synthesis. These results prove that cryptotanshinone is a down-regulator of proinflammatory molecule expression, including COX-2 and iNOS, and inhibits the activation of the transcription factors, such as nuclear transcription factor-kappaB and activator protein-1. Moreover, cryptotanshinone exhibits in vivo anti-inflammatory activity against carrageenan-induced paw edema in rats [46]. The immunomodulatory action of tanshinlactone A, from *Salvia miltiorrhiza*, has been reported [47]. It has been pointed out that tanshinlactone A reduced mitogen-activated protein kinases (MAPK) activation and modulated interleukin-2 (IL-2) and interferon-gamma (IFN-gamma) gene expression.

The vascular effects of tanshinones have been studied. The most abundant lipophilic diterpene quinones obtained from *Salvia miltiorrhiza* and *Salvia przewalskii* roots, tanshinone IIA and cryptotanshinone, inhibited contraction of the isolated porcine coronary artery to the thromboxane A 2 analogue, U46619. Przewaquinone A, a lipophilic diterpene quinone that can be found only in *Salvia przewalskii*, induced a similar but greater inhibitory action on vascular contraction than tanshinone IIA and cryptotanshinone [48]. Sodium tanshinone II-A sulfonate (DS-201), a water-soluble derivative of tanshinone IIA, induced relaxation of the coronary smooth muscle which had been contracted with KCl, and the relaxation was inhibited by iberiotoxin (IbTX), a specific BK(Ca) channel blocker, indicating that the vasodilatation is related to

activation of BK(Ca) [49]. Previous reports have demonstrated that radix *Salvia miltiorrhiza* extract (Danshen) induces the expression of CYP3A in rodents and Yu et al [50] have recently shown that tanshinone IIA and cryptotanshinone are able to activate the CYP3A promoter by binding at human pregnane X receptors (PXR), a family of nuclear receptors.

The activities on the central nervous system (CNS) of some abietane-quinone-type diterpenoids have been reported. It is known that tanshinone IIB (primary active diterpenoids from the roots of *Salvia miltiorrhiza*) is widely used in the treatment of cardiovascular and central nervous system (CNS) diseases [51]. Tanshinone I, tanshinone IIA, cryptotanshinone, and 15, 16-dihydrotanshinone I significantly reversed scopolamine-induced cognitive impairments in mice. Tanshinone I and tanshinone IIA also reversed diazepam-induced cognitive dysfunctions. In addition, cryptotanshinone and 15, 16-dihydrotanshinone I were found to have an inhibitory effect on acetylcholinesterase (AChE) in vitro [52]. Cryptotanshinone also modulates amyloid precursor protein metabolism and attenuates beta-amyloid deposition through upregulating alpha-secretase in vivo and in vitro [53].

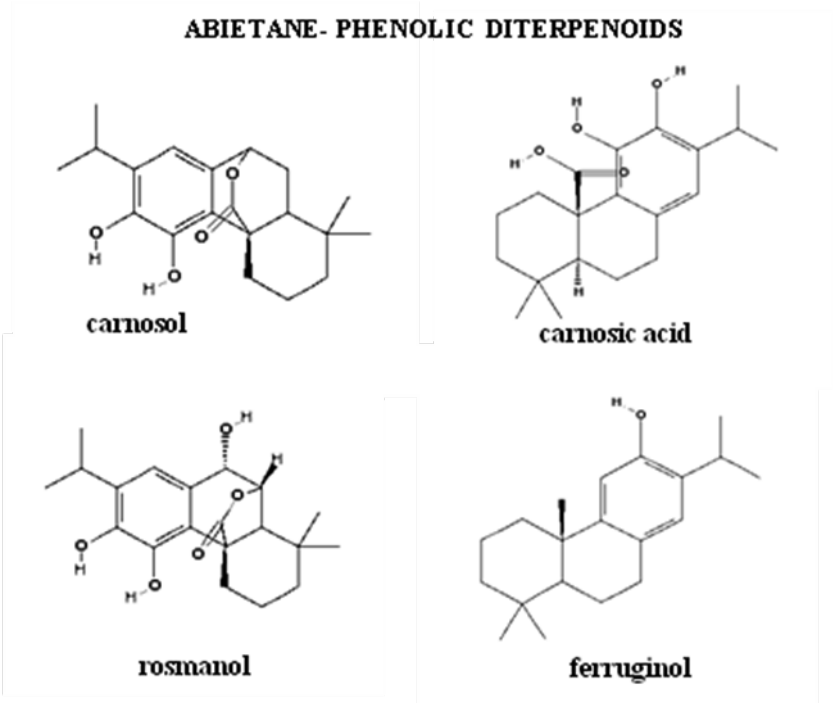

Figure 2: abietane phenolic-type diterpenoids from Salvia species.

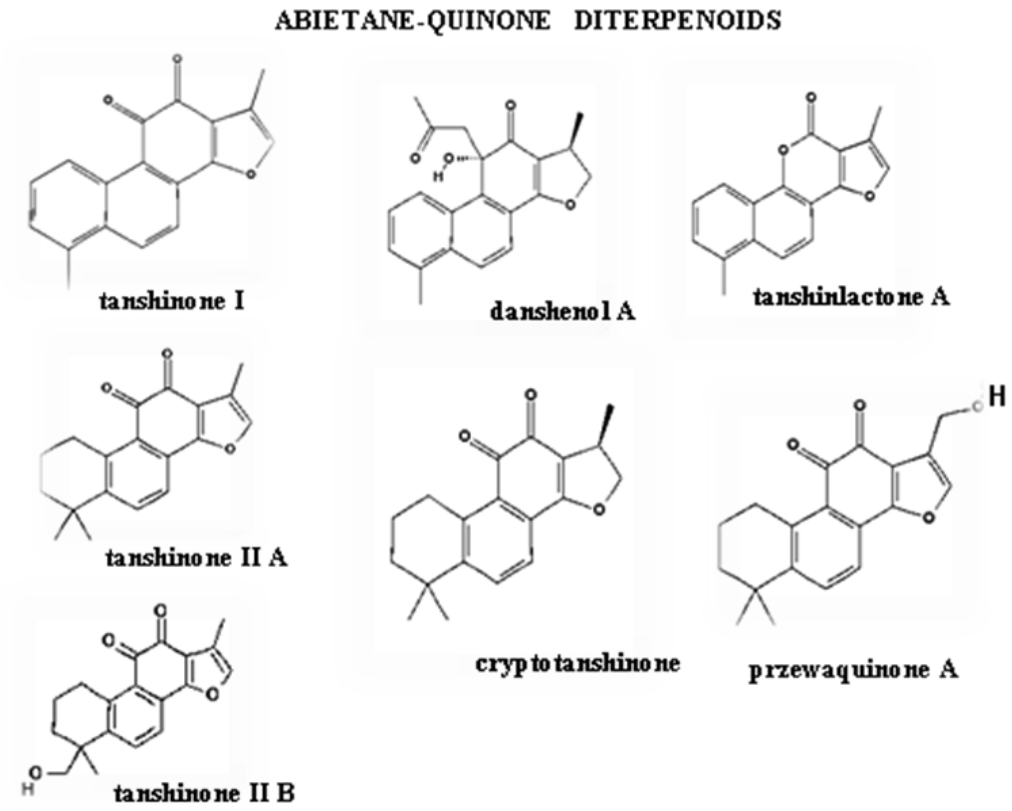

Figure 3: abietane quinone-typr diterpenoids from Salvia species.

Among abietane-type diterpenoids from *Salvia* species, some compounds with icetexane skeleton were found. Lanigerol, from *Salvia lanigera* [54] and brussonol, from *Salvia broussonetii* [55] are an example. 5-epi-icetexone, from *Salvia gilliessi*, was found to exert an antiproliferative effect on cultured epimastigotes of Trypanosoma cruzi (Tulahuen) [56]. New two-icetaxane diterpene, fruticuline A and demethylfruticuline A, were obtained from *Salvia corrugata*. Demethylfruticuline A was shown to be highly bactericidal against Staphylococcus aureus and S. epidermidis and bacteriostatic against Enterococcus faecalis and E. faecium. Fruticuline A manifested bacteriostatic activity against all tested strains [57]. Another group of tri-cyclic diterpenoids is of pimarane-type. From the aerial parts of *Salvia cinnabarina* a diterpenoid with pimarane skeleton, the compound 3,4-secoisopimar-4(18),7,15-triene-3-oic acid was isolated [58]. Further studies have shown that 3,4-secoisopimar-4(18),7,15-triene-3-oic acid reduced intestinal motility in vivo [59], the urinary bladder contractility in vitro [60], both with a mechanism involving calcium channel. It possessed a weak hypotensive activity in vitro [61] and has pronounced CNS depressant properties manifested as sedation and axiolitic effects in vivo [62].

Isopimaric acid and two new derivates characterised as 14-α-hydroxy-isopimaric acid, 3-β-hydroxy-isopimaric acid from *Salvia jamensis*, have been reported to have platelet antiaggregating activity in vitro [63].

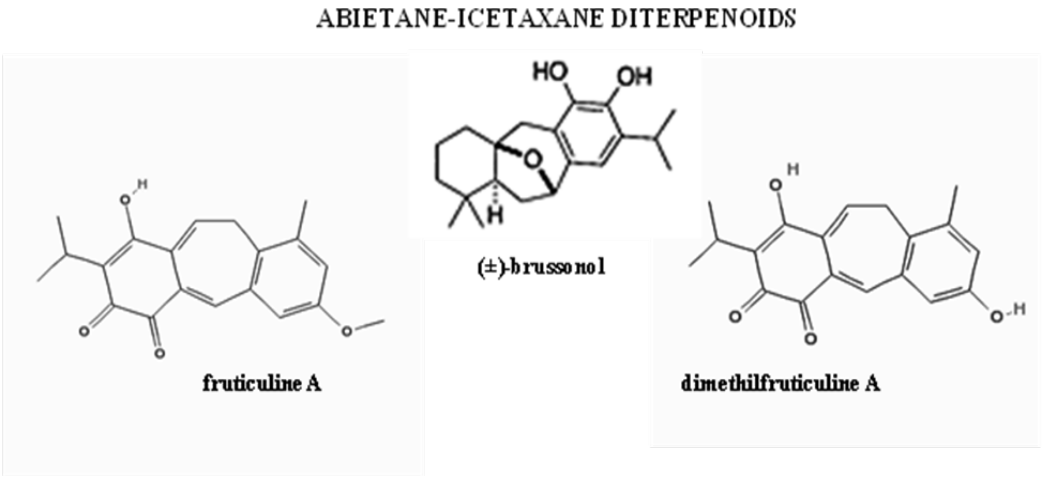

Figure 4: abietane icetaxane-type diterpenoids from Salvia species.

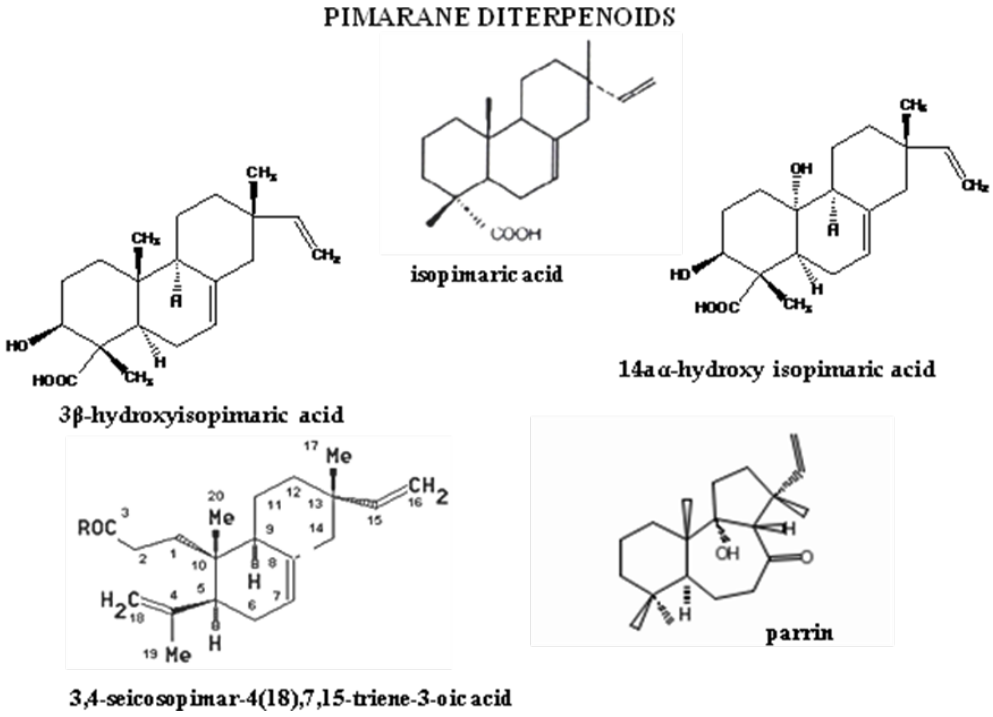

Figure 5: pimarane-type diterpenoids from Salvia species.

Table B: Tri-cyclic diterpenoids isolated from Salvia species and their biological activies

Tri-cyclic diterpenoids	Secondary metabolite constituents	Salvia species	Biological activities
Abietane-Phenolic type	• carnosic acid and carnosol	*Salvia officinalis, Salvia cilicica, Salvia africana-lutea*	• antioxidant, anti-inflammatory effects by antagonism of intracellular Ca(2+) mobilisation induced by a chemotactic stimulus • attenuate formation of reactive oxygen species and the secretion of human leukocyte elastase • antimicrobial activity
	• carnosic acid 12-Me ester	*Salvia microphylla*	• antimicrobial activity
	• ferruginol	*Salvia sclarea*	• antibacterial activity
	• 7-methoxyrosmanol and galdosol	*Salvia officinalis*	• binding activity to the benzodiazepine receptor
Abietane-Quinone type	• aethiopinone, salvipisone • and 1-oxoaethiopinone	*Salvia sclarea*	• bacteriostatic as well as bactericidal for the cultures of S. aureus and S. epidermidis • showed synergy activity with β-lactam antibiotics
	• yunnannin A and danshenol C	*Salvia yunnanensis*	• antitumor activity in T-24, QGY, K562, Me180 and BIU87 cell lines
	• 7-oxoroyleanone-12-Me ester and inuroyleanol	*Salvia barrelieri*	• showed potential antioxidant activity.
	• sibiriquinone A and sibiriquinone B	*Salvia miltiorrhiza*	• antitumor activity in human gastric cancer and human hepatocarcinoma cell lines
	• 15,16-dihydrotanshinone I	*Salvia miltiorrhiza*	• antitumor activity in human gastric cancer and human hepatocarcinoma cell lines • inhibits cyclooxygenase-2 (COX-2)-mediated PGE2 production and inducible NO synthase (iNOS)-mediated NO synthesis • inhibits the activation of the transcription factors, such as nuclear transcription factor-kappaB and activator protein-1 • inhibitory effect on acetylcholinesterase (AChE) in vitro

			• reverses scopolamine-induced cognitive impairments in mice • enhances the activity of insulin on the tyrosine phosphorylation of the insulin receptor (IR) beta-subunit and the activation of the downstream kinases signalling in vitro
	• cryptotanshinone	*Salvia miltiorrhiza*	• antitumor activity in human gastric cancer and human hepatocarcinoma cell lines • inhibits cyclooxygenase-2 (COX-2)-mediated PGE2 production and inducible NO synthase (iNOS)-mediated NO synthesis • inhibits the activation of the transcription factors, such as nuclear transcription factor-kappaB and activator protein-1 • exhibits in vivo anti-inflammatory activity against carrageenan-induced paw edema in rats • inhibits contraction of the isolated porcine coronary artery to the thromboxane A 2 analogue, U46619 • reverses scopolamine-induced cognitive impairments in mice • inhibitory effect on acetylcholinesterase (AChE) in vitro • modulates amyloid precursor protein metabolism and attenuates beta-amyloid deposition through upregulating alpha-secretase *in vivo* and *in vitro* • is able to activate the CYP3A promoter by binding at human pregnane X receptors (PXR), a family of nuclear receptors.
	• przewaquinone A	*Salvia przewalskii*	• inhibitory action on vascular contraction
	• tanshinlactone A	*Salvia miltiorrhiza*	• immunomodulatory action by mitogen-activated protein kinases (MAPK) activation and interleukin-2

			(IL-2) and interferon-gamma (IFN-gamma) gene expression
	• tanshinone I	*Salvia miltiorrhiza*	• antiproliferation activity in human breast cancer cells, MCF-7 and MDA-MB-231 cells, through the regulation of apoptotic pathway • inhibits TNF-alpha-induced production of vascular endothelial growth factor (VEGF) • reduces the tumorigenesis and metastasis in CL1-5, the highly invasive human lung adenocarcinoma cell line, through transcriptional activity of interleukin-8, the angiogenic factor involved in cancer metastasis, Ras-mitogen-activated protein kinase, and Rac1 signaling pathways • inhibits cyclooxygenase-2 (COX-2)-mediated PGE2 production • reverses scopolamine-induced cognitive impairments in mice and diazepam-induced cognitive dysfunctions
	• tanshinone IIA	*Salvia miltiorrhiza*	• anti-proliferation and apoptosis activity on acute promyelocytic leukemia (APL) NB4 cells, by regulation of anti- and pro-apoptotic protein, as well as disruption of the mitochondrial membrane potential • causes mitotic arrest by interfering with the microtubule structure (such as vincristine or taxol) and destroying only the mitotic spindle during the M phase but not the microtubule structure in interphase cells • inhibited osteoclastogenesis • protects cardiac myocytes against oxidative stress-triggered damage and apoptosis and • reduces macrophage death induced by hydrogen peroxide by up regulating glutathione peroxidise

			• inhibits ApoB secretion via a proteasome-dependent pathway • enhances the activity of insulin on the tyrosine phosphorylation of the insulin receptor (IR) beta-subunit and the activation of the downstream kinases signalling in vitro • suppresses the inflammation in atherosclerotic lesion inhibits migration of human aortic smooth muscle cell (HASMC) • reverses scopolamine-induced cognitive impairments in mice and diazepam-induced cognitive dysfunctions • inhibits contraction of the isolated porcine coronary artery to the thromboxane A 2 analogue, U46619 • is able to activate the CYP3A promoter by binding at human pregnane X receptors (PXR), a family of nuclear receptors.
	• tanshinone IIB	*Salvia miltiorrhiza*	• is widely used in treatment of cardiovascular and central nervous system (CNS) diseases
	• sodium tanshinone II-A sulfonate	*Salvia miltiorrhiza*	• induced relaxation of the coronary smooth muscle and the vasodilatation is related to activation of BK(Ca)
Abietane-Icetaxane type	• lanigerol	*Salvia lanigera*	
	• brussonol	*Salvia broussonetii*	
	• 5-epi-icetexone	*Salvia gilliessi*	• antiproliferative effect on cultured epimastigotes of Trypanosoma cruzi
	• fruticuline A	*Salvia corrugata*	• bacteriostatic activity
	• demethylfruticuline A	*Salvia corrugata*	• bactericidal against Staphylococcus aureus and S. epidermidis and bacteriostatic against Enterococcus faecalis and E. faecium

Pimarane-type	• 3,4-secoisopimar-4(18),7,15-triene-3-oic acid	*Salvia cinnabarina*	• reduces intestinal motility in vivo and the urinary bladder contractility in vitro • weak hypotensive activity in vitro • CNS depressant properties manifested as sedation and axiolitic effects in vivo
	• isopimaric acid, 14-α-hydroxy-isopimaric acid, 3-β-hydroxy-isopimaric acid	*Salvia jamensis*	• platelet antiaggregating activity in vitro

Triterpenoids from *Salvia* species

Triterpenoids, resulting from the combination of 6 isoprene units, are classified as oleanane, ursane, lupane, dammarane-type (table C). Many bioactive triterpenoids from *Salvia* species have been documented [3]. Oleanolic acid and ursolic acid, isolated from *Salvia officinalis*, showed antimicrobial activity against vancomycin-resistant enterococci, Streptococcus pneumoniae, and methicillin-resistant Staphylococcus aureus [26]. Ursolic acid, obtained from *Salvia miltiorrhiza*, upregulates eNOS and downregulates Nox4 expression in human endothelial cells [64]. Antiprotease and antimetastatic activity in vitro of beta-ursolic acid isolated from *Salvia officinalis* was also investigated [65]. It has been reported that beta-ursolic acid shows the strongest inhibition activity to urokinase, serine proteases, and cathepsin B, the cysteine protease. These interesting results have indicated a possible anticancer effect of beta-ursolic acid. Therefore, it is reported that beta-ursolic acid inhibits lung colonization of beta16 mouse melanoma cells in vivo [65].

Lupeol, a lupane-type triterpene, has been shown to possess many pharmacological properties including anticancer effects. Lupeol novel antiproliferative and apoptotic potential against prostate cancer has been reported [66]. Lupeol treatment of highly aggressive human metastatic melanoma cells caused G(1)-S phase cell cycle arrest and apoptosis: It is a downregulator of Bcl2 and upregulator of Bax. Lupeol induced the activation of caspase-3 and the cleavage of poly(ADP)ribose polymerase, decreased expression of cyclin D1, cyclin D2, and cdk2; and increased expression of p21 protein [67].

A recent study reports that lupeol attenuates the characteristics of the alterations of allergic airway inflammation in a murine model. Administration of lupeol, in the treatment of bronchial asthma in BALB/c mice immunized with ovalbumin, caused the reduction of cellularity and eosinophils in the bronchoalveolar lavage fluid. Treatment with lupeol also reduced the production of mucus and overall inflammation in the lung, and the levels of Type II cytokines IL-4, IL-5 and IL-13 [68].

Triterpenes also possess cardioprotective effects, which will be beneficial in hypercholesterolemic condition. Lupeol, and its ester lupeol linoleate effects on lipid status and biochemical changes on heart tissue, have been studied on male albino Wistar rats that were fed with high-cholesterol diet. Authors report that triterpenes treatment reduced the above alterations in hypercholesterolemic rats. The

transmembrane enzymes, namely Na(+), K(+)-ATPase, Ca(2+)-ATPase and Mg(2+)-ATPase showed a decrease in their activities. Triterpenes treatment reversed these levels, prevented hypertrophic cardiac histology and restored the normal ultrastructural architecture [69].

In recent studies, lupane-type triterpenoids were investigated for their CNS effects. The acetone, ethanol, butanol and water extracts of the aerial parts of *Salvia sclareoides* were screened for in vitro inhibitory activity of acetylcholinesterase (AChE) and butyrilcholinesterase (BChE), enzymes that play a role in Alzheimer's disease. All extracts inhibited acetylcholinesterase activity, a notable effect, since the standard drug rivastigmine does not inhibit acetylcholinesterase at the same concentration. With regards to butyrilcholinesterase, the acetone extract, where a new lupene triterpenetriol was isolated and characterised as (1beta,3beta)-lup-20(29)-ene-1,3,30-triol, was able to completely inhibit enzyme activity and the butanol and ethanol extracts produced a potent inhibition of BchE [70]. Lupeol, betulin and betulinic acid may also interact with the brain neurotransmitter gamma-amino butyric acid (GABA) receptors in vitro and in vivo [71].

Table C: Triterpenoids isolated from *Salvia* species and their biological activities

Triterpenoids	Secondary metabolite constituents	Salvia species	Biological activities
Oleanane-type	• oleanolic acid	*Salvia officinalis*	• antimicrobial activity against vancomycin-resistant enterococci, Streptococcus pneumoniae, and methicillin-resistant Staphylococcus aureus
Ursane-type	• ursolic acid	*Salvia officinalis* *Salvia miltiorrhiza* *Salvia jamensis*	• antimicrobial activity against vancomycin-resistant enterococci, Streptococcus pneumoniae, and methicillin-resistant Staphylococcus aureus • upregulates eNOS and downregulates Nox4 expression in human endothelial cells
	• beta-ursolic acid	*Salvia officinalis*	• antiprotease and antimetastatic activity in vitro
Lupane-type	• lupeol	*Salvia sclareoides*	• causes G(1)-S phase cell cycle arrest and apoptosis in human metastatic melanoma cells • attenuates the characteristics of the alterations of allergic airway inflammation in a murine model • reduces the alterations produced in hypercholesterolemic rats • in vitro inhibitory activity of acetylcholinesterase (AChE) and butyrilcholinesterase (BChE) • interacts with the brain neurotransmitter gamma-aminobutyric acid (GABA) receptors in vitro and in vivo
	• (1beta,3beta)-lup-20(29)-ene-1,3,30-triol		• is able to inhibit completely the butyrilcholinesterase (BChE) activity
	• betulinic acid	*Salvia jamensis*	• interact with the brain neurotransmitter gamma-aminobutyric acid (GABA) receptors in vitro and in vivo

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CHAPTER 1

The hallucinogenic herb Salvia divinorum and its active ingredient salvinorin A inhibit enteric cholinergic transmission in the guinea-pig ileum.

Salvia divinorum (figure 1.1) belongs to the family *Labiatae* (*Lamiaceae*), commonly referred to as the mint family. Shamanic healers from the Mazatec region in Oaxaca, Mexico, traditionally use the psychoactive herb by chewing fresh leaves or by drinking the juice of its leaves [1-3]. It has also been used traditionally as a medicine for the treatment of a number of diseases including headache, rheumatism, abdominal swelling and diarrhea [1, 4]. More recently, there have been reports of using *Salvia divinorum* recreationally for its hallucinogenic effects. *Salvia divinorum* leaf preparations or concentrated extracts are indeed widely available in western Europe and the USA, notably on Internet sites, [5] and are also used as a marijuana substitute [6, 7]. The hallucinatory effect produced has been reported to be potent and intense, lasting for up to an hour when smoked [6, 8]. Although it is an emerging intoxicant in the USA and Europe [9] very little pharmacological research has been conducted on this plant. The main active constituent of *Salvia divinorum* is salvinorin A (figure 1.2), a neoclerodane diterpene.

Figure 1.1: *Salvia divinorum*

Salvinorin A represents the only known hallucinogenic terpenoid and has been reported to be the most potent naturally occurring hallucinogen, with an effective dose, when smoked, of 0.2–1 mg in humans [7]. Salvinorin A induces an intense, short-lived hallucinogenic experience qualitatively distinct from that induced by classical

hallucinogens such as lysergic acid diethylamide (LSD), psilocybin and mescaline [7]. Quite recently, it has been reported that salvinorin A has high affinity and selectivity for the cloned κ-opioid receptor [10]. Indeed, it has been suggested, that salvinorin A is a κ-opioid receptor agonist [10–13], although there is no definitive evidence that hallucinations are mediated by κ-opioid receptors. However, the effects of salvinorin A on neural functions are largely unexplored. As a result of anecdotal reports that extracts of *Salvia divinorum* may possess antidiarrhoeal activity and because κ-opioid receptors may modulate intestinal peristalsis [14, 15] the effect of *Salvia divinorum*, and its main active ingredient, salvinorin A, on myenteric cholinergic transmission was investigated. For this purpose the effect of a standardized extract from *Salvia* divinorum leaves (SDE) and of isolated salvinorin A on the contractions elicited either by electrical stimulation or by exogenous acetylcholine in the guinea-pig ileum were evaluated.

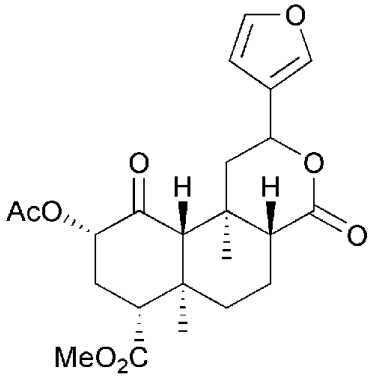

Figure 1.2: Salvinorin A

1.1 MATERIAL AND METHODS

1.1.1 Animal

Male guinea-pigs weighing between 250 and 350 g were purchased from Harlan Italy (S. Pietro al Natisone, UD, Italy), and were maintained under controlled conditions of temperature (24 ± 2 °C) and humidity (60%) until used. The guinea-pigs had free access to water and food. All experiments complied with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

1.1.2 Tissues and treatment procedure

Guinea-pigs were killed by asphyxiation with CO_2 and segments (1–1.5 cm) of the distal ileum lying 5– 15 cm proximal to the ileocaecal valve were quickly removed and flushed of luminal contents and placed in Krebs solution (mmol L^{-1}: NaCl 119, KCl 4.75, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgSO_4$ 1.5 and glucose 11).

The segments were set up (in such a way as to record contractions mainly from the longitudinal axis) in an organ bath containing Krebs (20 mL) equilibrated with 95%O_2, 5%CO_2 at 37°C. The tissues were connected to an isotonic transducer (load 0.5 g) connected to PowerLab system (Ugo Basile, Comerio, Italy). After a minimal 1 h equilibration period, the strips were subjected to electrical field stimulation (EFS, 2.5 Hz for 1 s, 400 mA, 1 ms pulse duration), delivered through electrodes placed around the tissue. Stable and reproducible contractions for a time period of 4 h were obtained with stimulation every 20 s. Contractions were expressed as % of contractions produced by 10^{-5} mol L^{-1} carbachol; this concentration of carbachol produced a maximal contractile effect (100% contraction). After stable control contractions evoked by EFS had been recorded, the contractile responses were observed in the presence of increasing cumulative concentrations of SDE (0.01–300 ng mL^{-1}). The contact time for each concentration was 10 min.

Preliminary experiments showed that this contact time was sufficient for SDE to achieve maximal effect. The effect of SDE was also evaluated after the administration in the bath (contact time 30 min) of N^G-nitro-L-arginine methyl ester (L-NAME, $3x10^{-4}$ mol L^{-1}) (to block NO synthesis), apamin (10^{-7} mol L^{-1}) (a blocker of Ca_2^+-activated K^+ channels which blocks the enteric inhibitory component mediated by ATP or a related purine), yohimbine (10^{-7} mol L^{-1}) (to block a_2-adrenergic receptors), thioperamide (10^{-6} mol L^{-1}) (to block H_3 receptors), methysergide (10^{-6} mol L^{-1}) [to

block 5- hydroxytryptamine (5-HT) receptors], naloxone (10^{-6} mol L^{-1}) (to block opioid receptors), naltrindole ($3x10^{-8}$ mol L^{-1}) (to block δ-opioid receptors), CTOP (10^{-6} mol L^{-1}) (to block μ-opioid receptors) and norbinaltorphimine ($3x10^{-8}$ mol L^{-1}) (to block κ-opioid receptors). In preliminary experiments the effect of tetrodotoxin ($3x10^{-7}$ mol L^{-1}) or atropine (10^{-6} mol L^{-1}) (contact time 10 min) on EFS-induced contractions was evaluated. These concentrations were selected on the basis of previous work. [15–18] The effect of SDE was also evaluated (contact time 10 min) on the contractions produced by exogenous acetylcholine (10^{-6} mol L^{-1}). This concentration of acetylcholine gave a contractile response that was similar in amplitude to that of electrical stimulation. Acetylcholine was left in contact with the tissue for 60 s and then washed out. The interval between each contraction was 10 min. In another set of experiments, the effect of salvinorin A (10^{-12}–10^{-6} mol L^{-1}), the κ-opioid antagonist U- 50488 (10^{-12}–10^{-6} mol L^{-1}) or SDE without salvinorin A (1–100 000 ng mL^{-1}) on EFS- or acetylcholine-induced contractions was also evaluated (contact time: 10 min for each concentration). Salvinorin A was also evaluated in the presence of naloxone (10^{-6} mol L^{-1}), naltrindole ($3x10^{-8}$ mol L^{-1}), CTOP (10^{-6} mol L^{-1}) and nor-binaltorphimine ($3x10^{-8}$ mol L^{-1}).

1.1.3 Drugs

An ethanolic extract (320 g) from dried *Salvia divinorum* leaves (2 kg) was prepared and a portion of this extract (20 g) was standardized to contain 1.6% salvinorin A (SDE) or the equivalent to 0.3% salvinorin A from dried leaves. Briefly, the extract was separated by reverse phase C18 vacuum liquid chromatography (VLC) eluted in a gradient manner using methanol: water (70: 30–100: 0) until no color was observed in the fractions. The fractions containing salvinorin A were combined and all VLC fractions and washings, with the exception of the salvinorin A fractions, were recombined and evaporated to dryness to give an extract without salvinorin A (263 g). The salvinorin A from the crystallizations and column fractions was purified by solvent/solvent partitions (aqueous methanol: hexanes) and recrystallizations (95% ethanol). A 1.6% standardized extract was made from the purified salvinorin A (333 mg) and the extract without salvinorin A (19.78 g). Acetylcholine hydrochloride, carbachol chloride, apamin, yohimbine hydrochloride, methysergide, L-NAME hydrochloride, thioperamide, U-50488, naloxone hydrochloride, tetrodotoxin, were purchased from Sigma (Milan, Italy). Naltrindole, CTOP and nor-binaltorphimine were purchased from Tocris Cookson (Avonmouth, UK). Salvinorin A, naltrindole,

CTOP, nor-binaltorphimine, tetrodotoxin and thioperamide were dissolved in dimethyl sulphoxide (DMSO). SDE and SDE without salvinorin A were dissolved in DMSO (stock solution: 1 mg mL^{-1} and 100 mg mL^{-1}, respectively) and then diluted in distilled water for the lower concentrations. The other drugs were dissolved in distilled water. DMSO (<0.01%) did not modify EFS-induced contractions.

1.1.4 Data analysis and statistics

Results are expressed as mean ± SEM. Nonlinear regression analysis for all concentration response curves were performed (Graph Pad Instat program version 4.01; GraphPad Software, Inc., San Diego, CA, USA). Data were analysed by Two-way ANOVA. A value of $P < 0.05$ was considered significant. The concentrations of SDE, SDE without salvinorin A, salvinorin A or U-50488 that produced 50% inhibition of EFS-induced contractions (IC_{50}) or maximal effect (E_{max}) were used to characterize their potency and efficacy respectively. The IC_{50} and E_{max} values [geometric mean ± 95% confidence limits (CL)] were calculated using the Graph Pad Instat program version 4.01.

1.2 RESULTS

1.2.1 EFS-induced contractions: effect of various antagonists or inhibitors

The EFS (2.5 Hz for 1 s, 400 mA, 0.25 ms pulse duration) of the guinea-pig ileum evoked a contractile response that was 49 ± 5% of the contraction produced by 10^{-4} carbachol (n=7). These EFS-induced contractions were abolished by tetrodotoxin ($3x10^{-7}$ mol L^{-1}) or atropine (10^{-6} mol L^{-1}), thus indicating that these contractions were due to the release of acetylcholine from enteric nerves. EFS-induced contractions were not significantly modified by naloxone (10^{-6} mol L^{-1})(5 ± 3% increase), yohimbine(10^{-7} mol L^{-1})(6 ± 4% increase), L-NAME ($3x10^{-4}$ mol L^{-1}) (22 ± 5% increase), thioperamide (10^{-6} mol L^{-1}) (4 ± 5% increase), naltrindole ($3x10^{-8}$ mol L^{-1}) (12 ± 4% increase), CTOP (10^{-6} mol L^{-1}) (10 ± 5% increase), (n =7–8 experiments for each antagonist used). By contrast, nor-binaltorphimine ($3x10^{-8}$ mol L^{-1}) and apamin (10^{-7} mol L^{-1}) significantly increased EFS-induced contractions (36 ± 5% and 46 ± 4% increase, respectively, $P < 0.05$, n=7–8 experiment for both compounds). Methysergide (10^{-6} mol L^{-1}) produced a transient contractile effect, but did not modify EFS-induced contractions after 30-min contact (data not shown).

1.2.2 EFS-induced contractions: effect of SDE or salvinorin A

The SDE (0.01–300 ng mL^{-1}) as well as SDE without salvinorin A (3–100 000 ng mL^{-1}) decreased, in a concentration- dependent manner, the amplitude of EFS evoked contractions (figure 1.3). Statistical significance was achieved starting from the 3 ng mL^{-1} concentration (SDE) and 10 000 ng mL-1 concentrations. The IC_{50} (95% CL) values were 1.72 ng mL^{-1} (0.93–3.1) for SDE and 7504 ng mL^{-1} (5406–1042) for SDE without salvinorin A. The E_{max} (95% CL) values were 55.89% (48.89–62.88) for SDE and 59.67% (52.19–67.16)% for SDE without salvinorin A. Figure 1.4 shows the effect of SDE on EFS-evoked contractions in the presence of drugs that block main prejunctional receptors (i.e. opioid, a_2-adrenergic and histamine H3 receptors) (figure 1.4 panel A), or in the presence of selective opioid receptor antagonists (figure 1.4 panel B), and in the presence of drugs that block the enteric inhibitory transmission (i.e. L-NAME and apamin) (figure1.4 panel C). Naloxone and nor-binaltorphimine, but not the other drugs, counteracted the inhibitory effect of SDE on EFS-induced contractions. Moreover, the inhibitory effect of SDE on EFS-induced contractions was not modified by methysergide [(% variation, SDE): 0.01 ng mL^{-1}: 1.1 ± 1; 0.03 ng mL^{-1}: 1.90 ± 1; 0.1 ng mL^{-1}:2.61 ± 2; 0.3 ng mL^{-1}: 4.98 ± 3; 1 ng mL^{-1}: 21.24 ± 4; 3 ng

mL^{-1}: 36.49 ± 5; 10 ngmL^{-1}: 53.07 ± 4; 30 ng mL^{-1}: 54.13 ± 5; 100 ng mL^{-1}: 55.13±4; 300 ng mL^{-1} :55.52 ± 5; (% variation, SDE + methysergide) 0.01 ng mL^{-1}: 1.5 ± 4; 0.03 ng mL^{-1}: 2.0±4; 0.1 ng mL^{-1}: 3.0 ± 4; 0.3 ng mL^{-1}: 5.10 ± 5; 1 ng mL^{-1}: 20.04 ± 4; 3 ng mL^{-1}: 31.63 ± 6; 10 ng mL^{-1}: 49.50 ± 5; 30 ng mL^{-1}: 50.82 ± 5; 100 ng mL^{-1}: 52.64±4; 300 ng mL^{-1}: 55.91 ± 5; n = 7–8].

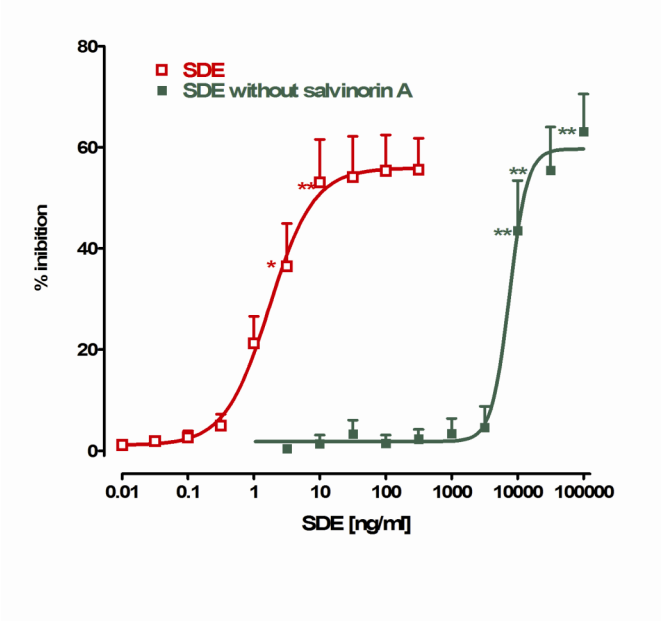

Figure 1.3: Effect of *Salvia divinorum* extract (SDE) with or without salvinorin A on the contractile response produced by electrical field stimulation (EFS) in the isolated guinea-pig ileum. Each point represents the mean of 7 – 8 experiments. Vertical lines show SEM *P < 0.05 and **P < 0.01 vs corresponding control.

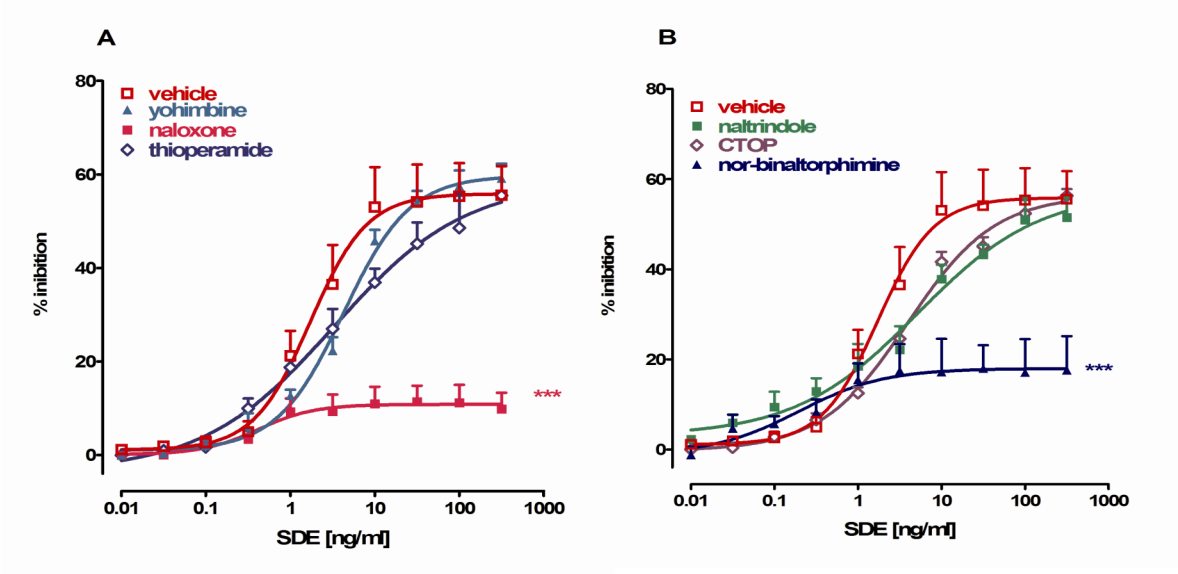

Figure 1.4: Electrical field stimulation (EFS)-induced contractions in the isolated guinea-pig ileum: Effect of *Salvia divinorum* extract (SDE; 0.01–300 ng mL^{-1}) alone (vehicle) or in presence of yohimbine (10^{-6} mol L^{-1}), naloxone (10^{-6} mol L^{-1}), thioperamide (10^{-6} mol L^{-1}) (A), naltrindole ($3x10^{-8}$ mol L^{-1}), CTOP (10^{-6} mol L^{-1}), nor-binaltorphimine ($3x10^{-8}$ mol L^{-1}) (B), NG-nitro-L-arginine methyl ester (LNAME, $3x10^{-4}$ mol L^{-1}) or apamin (10^{-7} mol L^{-1}) (C). Each point represents the mean of 7–8 experiments. Vertical lines show SEM. ***P < 0.001 vs vehicle (significance between curves).

The effect of salvinorin A, the main psychoactive compound in *Salvia divinorum*, on EFS-induced contractions is shown in figure 1.5. Salvinorin A (10^{-12}–10^{-6} mol L^{-1}) decreased, in a concentration-dependent manner, the amplitude of EFS-evoked contractions [IC_{50} (95% CL): $3.9x10^{-9}$ mol L^{-1} ($8.40x10^{-10}$– $1.48x10^{-8}$); E_{max} (95% CL): 68.12 (50.62–87.28) %]. The inhibitory effect of salvinorin A was counteracted by naloxone and nor-binaltorphimine, but not by CTOP or naltrindole. The selective j-opioid receptor agonist U-50488, used as a reference compound, also inhibited EFS-induced contractions [(IC_{50} (95% CL): $1.86x10^{-9}$ mol L^{-1} ($1.48x10^{-9}$–$2.23x10^{-9}$); E_{max} (95% CL): 51.32% (49.47–53.18)].

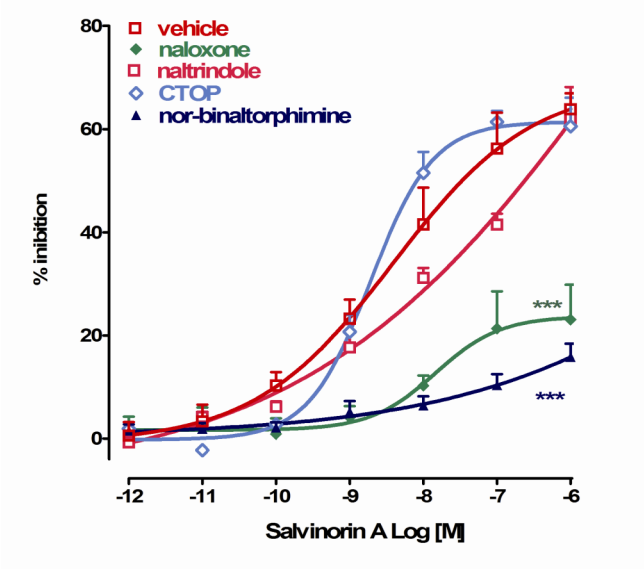

Figure 1.5: Electrical field stimulation (EFS)-induced contractions in the isolated guinea-pig ileum: effect of salvinorin A (10^{-12}-10^{-6} mol L^{-1}) (vehicle) alone or in presence of naloxone (10^{-6} mol L^{-1}), of naltrindole ($3x10^{-8}$ mol L^{-1}), of CTOP (10^{-6} mol L^{-1}) and of nor-binaltorphimine ($3x10^{-8}$ mol L^{-1}). Each point represents the mean of 7–8 experiments. Vertical lines show SEM. ***P < 0.001 vs vehicle (i.e. the naloxone and nor-binaltorphimine concentration–response curves were significantly different from the vehicle curve).

1.2.3 Acetylcholine-induced contractions: effect of SDE or salvinorin A

When the ileum was stimulated with exogenous acetylcholine (10^{-6} mol L^{-1}), both SDE and salvinorin A were without effect [(% inhibition): SDE 0.01 ng mL^{-1}: 1.8 ± 4; SDE 0.03 ng mL^{-1}: 2.3 ± 5; SDE 0.1 ng mL^{-1}: 4.0 ± 4; SDE 0.3 ng mL^{-1}: 8.10 ± 5; SDE 1 ng mL^{-1}: 6.04 ± 4; SDE 3 ng mL^{-1}: 8.53 ± 5; SDE 10 ng mL^{-1}: 8.53 ± 4; SDE 30 ng mL^{-1}: 9.02 ± 5; SDE 100 ng mL^{-1}: 7.74 ± 4; SDE 300 ng mL^{-1}: 8.71 ± 5, P > 0.05, n ¼ 7–8; salvinorin A 10-12 mol L-1: 0.8 ± 4; salvinorin A 10-11 mol L-1: 1.3 ± 4; salvinorin A 10^{-10} mol L^{-1}: 5.0 ± 5; salvinorin A 10^{-9} mol L^{-1}: 6.50 ± 5; salvinorin A 10^{-8} mol L^{-1}: 8.04 ± 5; salvinorin A 10^{-7} mol L^{-1}: 7.53 ± 5; salvinorin A 10^{-6} mol L^{-1}: 8.0 ± 4; P > 0.05, n=7–8]. At the higher concentrations tested neither SDE (300 ng mL^{-1}) nor salvinorin A (10^{-6} mol L-1) significantly modified the concentration–response curve to acetylcholine (10^{-8}– 10^{-4} mol L^{-1}) (data not shown).

1.3 DISCUSSION

Salvia divinorum is a hallucinogenic sage that has been gaining recreational popularity. Although traditionally the herb is mainly used for its psychoactive effects, it is also reportedly used to treat a variety of disorders, including those affecting the digestive tract (e.g. abdominal swelling and diarrhoea) [1, 19]. However, the potential pharmacological activities of this plant related to the central and peripheral nervous system are largely under-researched. In the present study it is shown that SDE, as well as its main active ingredient salvinorin A, inhibited enteric cholinergic transmission in the guinea-pig ileum. These results could provide the pharmacological basis underlying its traditional antidiarrhoeal use. It is shown that SDE inhibited the contractions induced by EFS in the guinea-pig ileum, which are mediated by the release of acetylcholine from myenteric nerves. Moreover, when the ileum was exposed to SDE at concentrations that markedly inhibited the twitch response, the contractile response to exogenous acetylcholine (which is mediated by activation of postjunctional muscarinic receptors) remained unchanged. These observations strongly support the hypothesis that SDE inhibits the twitch response by acting prejunctionally rather than through a direct action on intestinal smooth muscle. Consequently, the mechanism(s) underlying the inhibition of neural function by SDE was/were investigated.

It is well known that opioid peptides exert inhibitory effects on gastrointestinal motor function [20]. Opioid agonists inhibit acetylcholine release from myenteric neurones and attenuate twitch contraction of the longitudinal muscle in response to transmural electrical stimulation [21]. Immunohistochemical studies have revealed that the opioid receptor subtypes μ, δ and κ are present in neural tissues of the guinea-pig enteric nervous system, but not in smooth cells,[22] and their activation results in inhibition of enteric cholinergic transmission [22]. In the present study strong evidence is provided that the inhibitory effect of SDE is mediated by κ-opioid receptors. Indeed the inhibitory effect of SDE on electrically evoked contractions was abolished by the non-selective opioid antagonist naloxone and the selective κ-opioid antagonist nor-binaltrophimine, but not by the μ-opioid antagonist CTOP or by the δ-opioid antagonist naltrindole. These experiments make it possible to exclude a number of factors as potential contributory mechanisms involved in the inhibition of cholinergic transmission by SDE. In particular what may be excluded is the involvement of (a)

other prejunctional systems (i.e. histamine H_3 and α_2- adrenergic receptors) negatively coupled to cholinergic transmission as the inhibitory effect of SDE was not affected by α_2-adrenergic receptor antagonist yohimbine or by the histamine H_3 receptor antagonist thioperamide; (b) enteric inhibitory nerves as apamin (a blocker of Ca_2^+ activated K^+ channels which blocks the enteric inhibitory component mediated by ATP or related purine [23]) or L-NAME (an inhibitor of NO synthase) did not modify the inhibitory effect of SDE on twitch response; (c) 5-HT receptors as the depressant effect of SDE was insensitive to the 5-HT receptor antagonist methysergide. The lack of involvement of 5-HT receptors is relevant in the light of the observation that 5-HT_2A receptors represent the primary molecular target responsible for the actions of classic hallucinogens such as LSD, psilocybin and mescaline [24, 25]. *Salvia divinorum* contains numerous biologically active constituents, including salvinorins, divinatorins and hardwickiic acid. [2] Salvinorin A is believed to be the compound responsible for the hallucinogen properties of *Salvia divinorum*. Salvinorin A has been reported to be the most potent naturally occurring hallucinogen, with an effective dose, when smoked, of 200–1000 g in humans [7, 8]. In the present study strong evidence is provided that the pharmacological activity of SDE on myenteric nerves observed here is mainly the result of salvinorin A content. Indeed, extracts of SDE without salvinorin A were approximately 4500-fold less active than the same extract with salvinorin A, and salvinorin A itself inhibited myenteric cholinergic transmission. Moreover, the main findings reported for SDE were confirmed with salvinorin A. In particular, it was found that salvinorin A: (a) inhibited electrically evoked contractions without affecting the contractions induced by exogenous acetylcholine (which is consistent with a prejunctional site of action); (b) exerted this inhibitory effect possibly through activation of κ-opioid receptors, as the inhibitory effect of this compound (as well as of the κ-opioid agonist U-50488, used as a reference compound) on electrically induced contractions was abolished by the non-selective opioid antagonist naloxone as well as by the selective κ-opioid antagonist nor-binaltrophimine. Consistent with these results, it has been shown that salvinorin A has been found to be a potent agonist of the human κ-opioid receptor expressed in human embryonic kidney 293 (HEK293) cells with an EC_{50} of 1.05 nmol L^{-1} [10]. Moreover, the finding that salvinorin A has similar potency and efficacy as U50488 in inhibiting cholinergic transmission is in accordance with other experiments in isolated cells (i.e. oocytes from Xenopus laevis, Chinese hamster ovary, HEK293) expressing κ-opioid receptors [10–12]. It is

interesting to note that both salvinorin A and U50488 did not produce a total inhibition of the contractions evoked by EFS (E_{max} values in the 50–70% range), which is in line with the ability of U50488 to produce a partial inhibition (maximal inhibition: 55%) of electrically evoked acetylcholine release from guinea-pig myenteric neurones [26]. In preparing this manuscript, it has been noted that salvinorin A produced κ-opioid agonist-like discriminative effects in rhesus monkeys [27] and decreased dopamine levels in the mouse caudate putamen through activation of κ-opioid receptors [28]. In conclusion, it is reported here that the hallucinogenic herb *Salvia divinorum* exerted inhibitory effects on enteric cholinergic transmission in the guinea-pig ileum through activation of prejunctional κ-opioid receptors. Salvinorin A may be the chemical constituent responsible for this activity. These data may justify the traditional use of the herb in the treatment of diarrhoea.

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CHAPTER 2

Effects in vivo of a new diterpenoid from Salvia cinnabarina with anxiolytic and antinociceptive properties.

In a screening programme on *Salvia* medicinal plants (Fam. *Lamiacae*), a crude ethanolic extract from the aerial part of the plant *Salvia cinnabarina* M. Martens et Galeotti (figure 2.1) showed in vitro antispasmodic activity [1]. This led to the isolation of a new diterpenoid of the pimarane skeleton, the compound 3,4-secoisopimar-4(18),7,15-triene-3-oic acid (CMP1) [1] (Figure 2.2), whose complete relative stereochemistry was recently determined by an X-ray diffraction analysis of a single crystal of a suitable crystalline derivate [2]. Further studies have shown that CMP1 reduces intestinal motility in vivo [3], urinary bladder contractility in vitro both with a mechanism involving calcium channel [4] and possesses a weak hypotensive activity [5]. Diterpenoids are chemical compounds found in different plant species of the *Salvia* genus that possess a variety of biological activities including effects on the central nervous system (CNS) [6]. Salvinorin A (*Salvia divinorum*), miltirone, tanshinones IIA and IIB, carnosol and carnosic acid (*Salvia officinalis*) are examples of isolated diterpenes with hallucinogenic, neuroprotective, sedative and hypnotic properties [7-14].

Figure 2.1: *Salvia cinnabarina*

The possibility that CMP1 like other isolated diterpenoids might affect CNS was the main aim of these

investigations. For this reason anxiolytic and antidepressive activity of CMP1 were studied in the elevated plus-maze test and in the forced swimming test. Furthermore, CMP1 was administered after a pre-treatment with pentobarbital and its effects on sedative activity were monitored.

The CMP1 was also tested for its effects on spontaneous motor activity (total motility and locomotion) and several models of nociception have been used to examine the potential analgesic effects. These studies demonstrate for the first time that CMP1 has pronounced CNS depressant properties, manifested as antinociception, sedation and axiolitic effects.

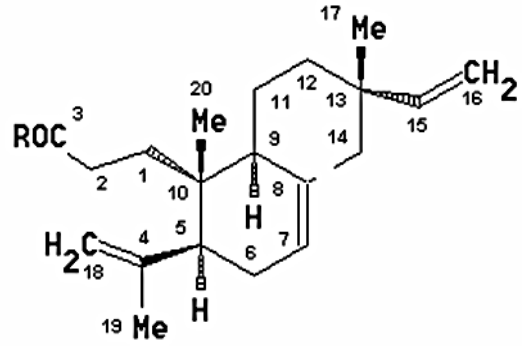

Figure 2.2: CMP1 (3,4-secoisopimar-4(18),7,15-triene-3-oic acid)

2.1 MATERIAL AND METHODS

2.1.1 Animal

Male CD-1 mice (Charles River, Italy) weighing 25–30g were used for all experiments. The mice were housed in colony cages (7 mice in each cage) for at least 1 week prior to experimental sessions under the following conditions: exposed to normal light (from 7.00 a.m. to 7.00 p.m.); temperature 22±1°C; relative humidity 60±10%; and food and water available ad libitum. Animal care and their use conformed to the Italian Law Decree of 27/01/92, No.116.

2.1.2 Elevated plus-maze test

This test has been widely validated to measure anxiety in rodents [15,16]. The apparatus, constructed from black Plexiglas, consisted of two open arms (50cm×10cm each), two enclosed arms (50cm×10cm×40cm each) and a central platform (10cm×10cm), arranged in such a way that the two arms of each type were opposite to each other. The maze was elevated 60cm above the floor. Ten minutes after the i.p. treatment with CMP1 (1, 10, 50, 100mg kg^{-1}) or thirty minutes after the i.p. treatment with diazepam (1 and 2mg kg^{-1}) or vehicles, each animal was placed at the centre of the maze, facing one of the enclosed arms. During the 10 min test period, the number of open arm entries and the time spent therein was recorded [17]. The entry of each animal into an arm with all four paws was considered as end-point. Animal behaviour was observed by using a video camera located above the maze.

2.1.3 Forced swimming test

The test was performed as suggested by Lucki [18] and Porsolt [19] with little modification. The apparatus consisted of a transparent Plexiglas cylinder (50cm×20cm diameter) filled up to 30cm with water at room temperature. CMP1 (1 and 10 mg kg^{-1}) or vehicle were administrated 10 min prior to the test. During the 10 min test, a trained observer recorded immobility or floating considered as when the mice made no further attempts to escape except the movements necessary to keep its head above the water. Reduction in immobility was considered an antidepressant-like action [20].

2.1.4 Pentobarbital-sleeping time

Ten minutes after the i.p. treatment with CMP1 (1 and 10 mg kg^{-1}) or thirty minutes after the i.p. treatment with diazepam (1 and 2 mg kg^{-1}) or vehicles each animal was i.p. injected with pentobarbital (50 mg kg^{-1}) and the time passed for the mouse to be placed on its back was recorded as the sleep latency. Sleeping time was

taken as the period between the loss of the righting reflex and its return. The experiments were carried out in a quiet room where temperature was maintained at 22±24°C during sleeping time.

2.1.5 Spontaneous motor activity

Locomotor activity was recorded with an infrared photocell activity monitor (Ugo Basile, Italy), provided with one array of 15 infrared photocells spaced 2.5 cm apart. Spontaneous motor activity was monitored during 30 min and, contextually, the number of times each animal reared and the time spent in horizontal walking were recorded. Measurements were performed between 10 a.m. and 1 p.m. .The animals were treated with CMP1 (1, 10 ,50, 100 mg kg^{-1}) i.p. immediately before the start of the test.

2.1.6 Motor coordination

Motor coordination was evaluated as previously reported [21] by using a rotarod apparatus (Ugo Basile, Italy) consisting of a bar with a diameter of 3.0 cm, subdivided into five compartments by a disk of 24 cm in diameter. The bar rotated at a constant speed of 16 rev min^{-1}. A preliminary selection of mice was made on the day of experiments excluding those that did not remain on the rotarod bar for two consecutive periods of 45secs each. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 45secs. The performance time was measured before and six more time at 10, 20, 30, 40, 50 and 60 mins after i.p. treatment with CMP1 (10 and 100 mg kg^{-1}) or vehicle.

2.1.7 Hot plate test

The apparatus consisted of a metal plate 25x25 cm (Ugo Basile, Italy) heated to a constant temperature of 48±0.1°C. This low temperature stimulation was used to unravel possible hyperalgesic effects. A plastic cylinder 20 cm diameter, 18 cm high was placed on the plate. The time of latency (s) was recorded from the moment the animal was inserted inside the cylinder up to when it first licked its paws or jerked them off the hot plate. The measurement was terminated if the following occurred: The latency exceeded the cut-off time of 60 secs or if the mouse jumped off the hot plate. Baseline latency was 16–25 secs. For the baseline determination, the hot plate latency was recorded 90, 60 and 30 mins before treatment and at 15, 30, 45, 60, 90 and 120 mins after treatment [22]. In these experiments, CMP1 (10, 50, 100 and 500 mg kg^{-1}, i.p.) or vehicle were injected immediately before the test.

2.1.8 Tail flick test

Tail flick latency was obtained using a tail flick unit (Ugo Basile, Italy), consisting of an infrared radiant light source (100 W, 15 V bulb). A low lamp potential was utilised in order to unravel possible hyperalgesic effects. The light source was focused onto a photocell utilising an aluminium parabolic mirror. The mice were gently hand-restrained with a glove during the trials. Radiant heat was focused 1–2 cm from the tip of the tail, and the latency time (s) the mouse took to flick its tail was recorded. The measurement was terminated if the latency exceeded the cut off time (15 s). Baseline latency was 5–10 secs. Tail flick data were recorded as latency (s) both at 90, 60 and 30 mins before the treatment for baseline determination and at 15, 30, 45, 60, 90 and 120 mins after the treatment [22]. In these experiments, CMP1 (10, 50, 100 and 500 mg kg^{-1}, i.p.) or vehicle were injected immediately before the test.

2.1.9 Formalin test

In the formalin test, 20 µl of a 1% solution of formalin in saline was injected subcutaneously into the dorsal surface of the right hind paw of the mouse, using a microsyringe with a 27-gauge needle. The formalin injection produced a distinct biphasic response consisting of licking or biting of the paw. The early phase occurred from 0 to 10 mins after the formalin injection and the late phase from 15 to 40 min. The mouse was placed in a Plexiglas cage (30×14×12 cm), utilised as an observation chamber, 1 h before administering the formalin injections. The total amount of time (s) that the animal spent licking or biting the paw after the formalin injection, at intervals of 5 mins was recorded for a period of 40 min [22]. Ten minutes after the i.p. treatment with CMP1 (1, 10 and 50 mg kg^{-1}) or thirty minutes after the i.p. treatment with diazepam (2 mg kg^{-1}) or vehicles, each animal was treated with formalin and the licking activity recorded for forty minutes.

2.1.10 Toxicity

Three groups of CD-1 female were treated p.o. with CMP1 at doses of 300, 500 and 2000 mg kg^{-1} following the guidelines [23] OECD GUIDELINE FOR TESTING OF CHEMICALS Acute Oral Toxicity – Acute Toxic Class OECD/OCDE 423 adopted 17th December 2001.

2.1.11 Drugs and treatment procedure

Extraction of CMP1 from leaf surface constituents of fresh aerial parts of *Salvia cinnabarina* was performed as previously described [1] (HPLC purity grade 96%). The sodium salt (prepared by reaction with an equivalent quantity of NaOH in

MeOH solution and evaporation to dryness) was dissolved (5 mg) in distilled water. On each test day, an aliquot of drug solution were freshly dissolved in PBS and then i.p. injected in a volume of 35 mg kg^{-1}. Diazepam solutions were prepared in distilled water added with Tween 80 (2 drops every 10 ml) and dispersed by ultrasound. Injections were given i.p. at a volume of 10 ml kg^{-1}.

2.1.12 Data analysis and statistics

Experimental data were analysed using analysis of variance (ANOVA) followed by Bonferroni's test procedure for verifying the significance between two selected means. For all the experiments, statistical significance was always assumed to be $p<0.05$.

2.2 RESULTS

2.2a Elevated plus-maze test

The results of these experiments are reported in figure 2.3. CMP1 was administered at doses of 1, 10, 50 and 100 kg^{-1} i.p. 10 mins before the test. Diazepam was administered at doses of 1 and 2 mg kg^{-1} i.p. 30 mins before the test. Data are reported as a percentage of the vehicle-induced effects. No behavioural differences were observed after the administration of CMP1 or diazepam vehicle. CMP1 administered at a dose of 1 mg kg^{-1} did not change the time spent in the open arms or the number of entries into the open arms compared to confront to vehicle-treated animals (figure 2.3). When administered at the dose of 10 mg kg^{-1}, CMP1 was able to increase both the time spent in the open arms and the number of entries into the open arms (figure 2.3). Diazepam administered at a dose of 1 mg kg^{-1} was able to increase the number of entries into the open arms only, while at a dose of 2 mg kg^{-1} ,diazepam increased both the time and the number of entries into the open arms (figure 2.3).

2.2b Forced swimming test

CMP1 administered at doses of 1 and 10 mg kg^{-1} i.p. did not change the time of floating compared to vehicle-treated animals (data not shown).

2.2c Pentobarbital-sleeping time

In these experiments the treatment with CMP1 (1 and 10 mg kg^{-1}) or with diazepam (1 and 2 mg kg^{-1}) significantly increased the sleeping time induced by pentobarbital (figure 2.4). The increase in sleeping time observed after CMP1 or diazepam administrations were in the same order of magnitude, since no differences were found between CMP1 and diazepam treatments. The effects observed after CMP1 or diazepam administrations were independent of the dosage used and for the sake of simplicity, only the results obtained after highest dose administration were reported.

2.2d Spontaneous motor activity

The horizontal motor activity and the vertical motor activity were recorded after CMP1 (1, 10, 50 and 100 mg kg^{-1}) administration. CMP1 administered at the lowest doses (1 and 10 mg kg^{-1}) did not change the locomotor activity count of mice, in contrast to diazepam (2 mg kg^{-1}) which reduced spontaneous motor activity (figure 2.5). When administered at a dose of 100 mg kg^{-1} i.p, CMP1 strongly reduced the locomotor activity count of mice.

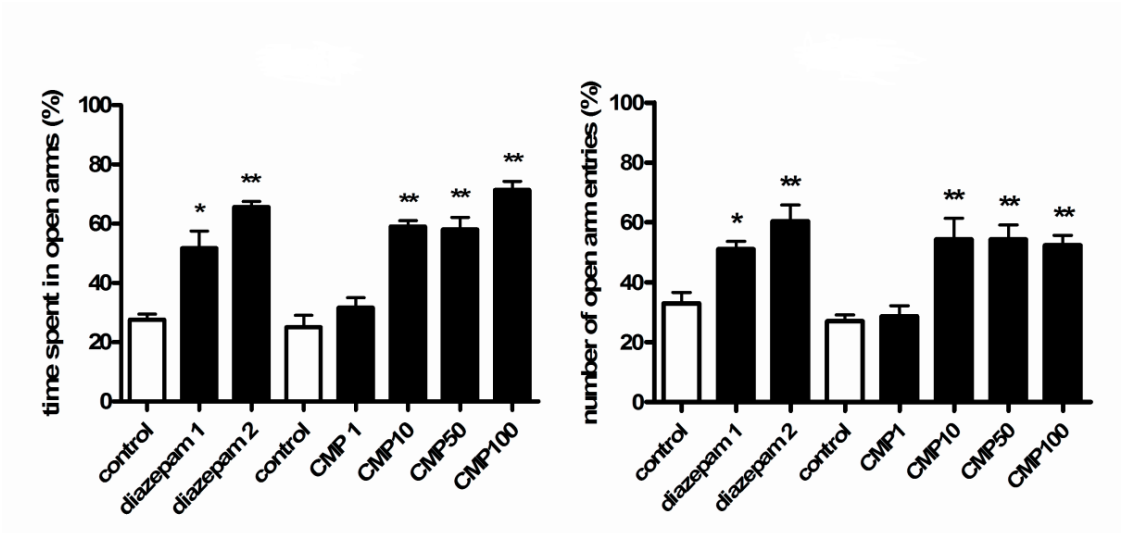

Figure 2.3. CMP1 effects in the plus maze test. TOA, time spent in the open arms; NEOA, number of entries in the open arms. Animals were treated i.p. 10 min before the test with CMP1 (1, 10, 50 and 100 mg kg^{-1}) or 30 min before the test with diazepam (D, 1 and 2 mg kg^{-1}). [TOA] Vehicle (D) value was 27.92 ± 2.78% and Vehicle (CMP) value is 23.5± 4.52%; [NEOA] Vehicle (D) value was 36.76 ± 2.48% and Vehicle (CMP) value is 27.5± 2.28%. * $p<0.05$, ** $p<0.01$ *vs* vehicle. (N= 12-15).

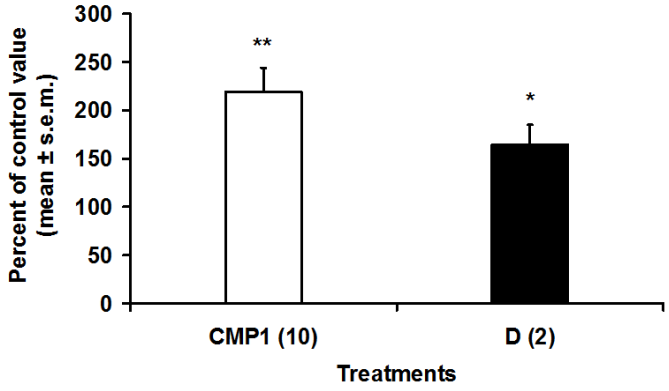

Figure 2.4. CMP1 effects on the sleep induced by pentobarbital (50 mg kg-1, i.p.). Animals were treated i.p. 10 mins before the test with CMP1 (10 mg kg^{-1}) or 30 mins before the test with diazepam (D, 2 mg kg^{-1}). Data are reported as a percentage of the vehicle-induced effects (CMP1vehicle-treated animals: 1011±158 secs; D vehicle-treated animals: 1755±277 sec). * is for $p<0.05$ and ** is for $p<0.01$ *vs* vehicle-treated animals. N, 15-20.

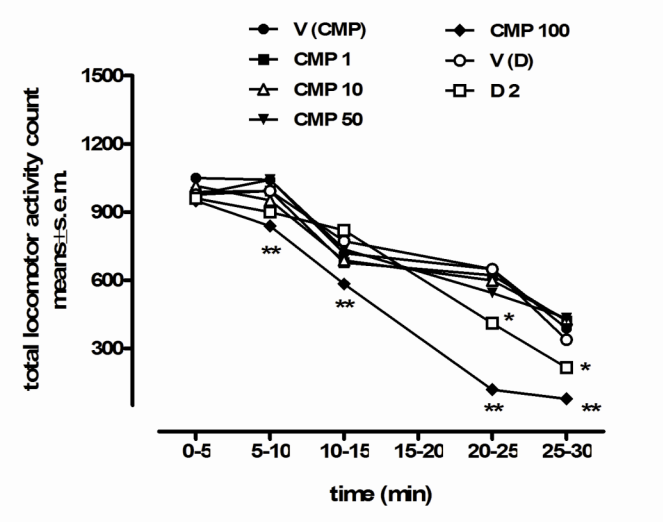

Figure 2.5 CMP1 effect on total spontaneous motor activity. Animals were treated i.p. 10 min before the test with CMP1 (1, 10, 50 and 10 mg kg^{-1}) or 30 min before the test with diazepam (D, 2 mg kg^{-1}). Results are expressed as mean ± s.e.m. * $p<0.05$, ** $p<0.01$ *vs* vehicle. (N= 12-15).

2.2e Motor coordination

Despite the doses used (1, 10 and 100 mg kg^{-1}, i.p.) CMP1 did not change the motor coordination of mice (data not shown).

2.2f Hot plate test

The nociceptive latency evoked by thermal stimuli applied by the hot plate are reported in figure 2.6. In animals treated with CMP1 at dose of 10, 50, 100 and 500 mg kg^{-1} there was no significant difference compared to vehicle-treated animals. However, a slight reduction in time latency was observed after CMP1 administration at doses of 10 and 50 mg kg^{-1} (figure 2.6). Furthermore, a slight increase in time latency was recorded after CMP1 administration at dose of 500 mg kg^{-1} (figure 2.6).

2.2g Tail flick test

The results obtained in the tail flick test are reported in figure 2.6. In animals treated with CMP1 at dose of 10, 50, and 100 mg kg^{-1} there was no significant difference compared to vehicle-treated animals. When CMP1 was administered at a dose of 500 mg kg^{-1} a strong increase in the latency of the mice to respond was observed (figure 2.7).

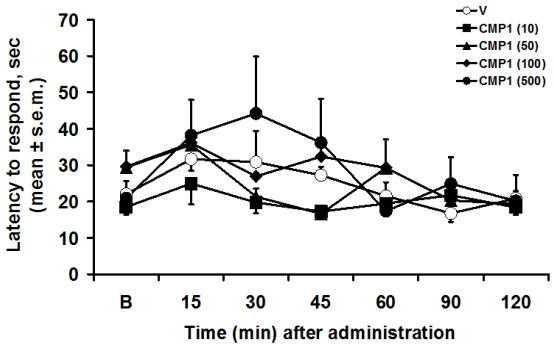

Figure 2.6. CMP1 effects in the hot plate test. Animals were treated with CMP1 (10, 50, 100 and 500 mg kg^{-1}) i.p. immediately before the test starts. V is for vehicle-treated animals. N, 8.

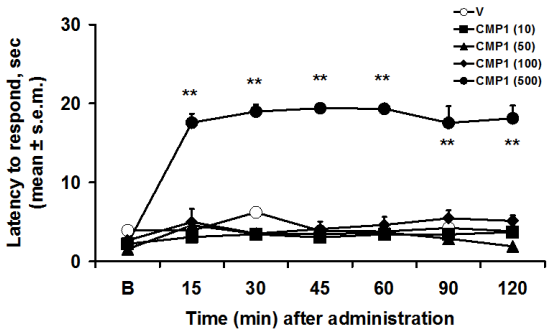

Figure 2.7. CMP1 effects in the tail flick test. Animals were treated with CMP1 (10, 50, 100 and 500 mg kg^{-1}) i.p. immediately before the test starts. V is for vehicle-treated animals. ** is for $p<0.01$ *vs* vehicle-treated animals. N, 8.

2.2h Formalin test

In these experiments, CMP1 administered at doses of 10 and 50 mg kg^{-1} i.p. was able to reduce the early phase of the formalin test (figure 2.8, panel A). On the contrary, CMP1 did not change the licking activity recorded in the late phase of the formalin test. In these experiments, the effects of diazepam were also investigated. Diazepam administered at the dose of 2 mg kg^{-1} was able to reduce the late phase of the formalin test showing a slight but not significant difference in the early phase compared to vehicle-treated animals (figure 2.8, panel B).

2.2i Toxicity

The CMP1 toxicity evaluated following the guidelines OECD/OCDE 423 adopted 17th December 2001 gave a LD50 >500 mg kg^{-1}.

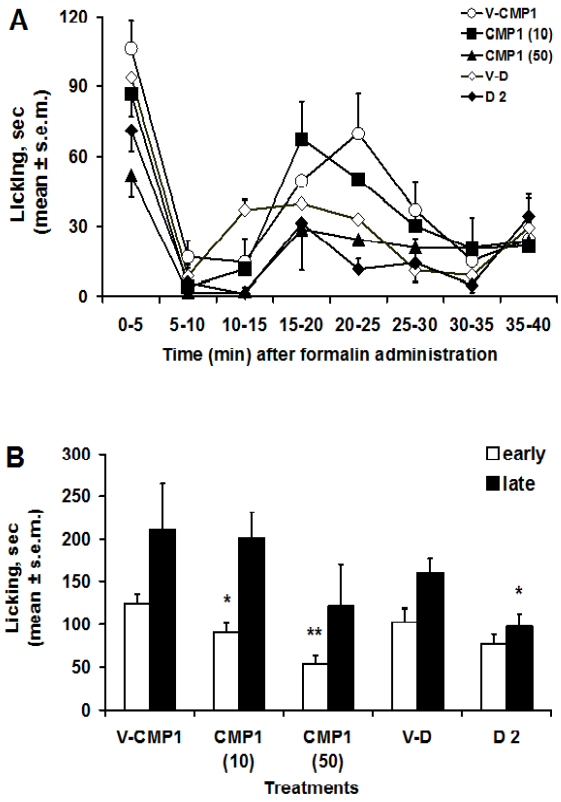

Figure 2.8. CMP1 effects in the formalin test. In the panel A, the licking activity recorded from 0 to 40 mins after formalin injection is reported. In the panel B both the early and the late phase are reported. CMP1 (10 and 50 mg kg^{-1}, i.p.) was administered 10 mins before formalin. Diazepam (D, 2 mg kg^{-1}, i.p.) was administered 30 mins before formalin. V-CMP1, CMP1 vehicle-treated animals. V-D, diazepam vehicle-treated animals. * is for $p<0.05$ and ** is for $p<0.01$ *vs* vehicle-treated animals. N, 8-9.

2.3 DISCUSSION

It is known that plants have played an important part in the development of new drugs and it is estimated that at least 25% of the molecules currently used as prescribed drugs originate, directly or indirectly, from plant sources [24]. There are numerous traditional medicinal plants with a reputation for affecting CNS functions and activities. Among these plants, *Salvia* species are prominent for their reputed beneficial effect on CNS disorders. Their crude or semipurified extracts show an efficacy that is the result of the combined action of several types of compounds so that the final effect is often of weak or mild intensity. Efforts directed to obtain the active principle(s) from plants of genus *Salvia* have led to identifying a class of compounds of diterpenoid structures. In this context, this work has aimed to investigate the potential central effects of the CMP1, the major diterpenoid constituent of *Salvia cinnabarina*. These results demonstrate for the first time that CMP1 has pronounced CNS depressant properties, manifested as sedation, axiolitic effects and antinociception.

In these experiments, CMP1 showed anxiolitic effect evaluated either as the time spent inside and as the number of entries into the open arms in the plus maze paradigms. Similar effects were observed after diazepam administration at a dose of 2 mg kg^{-1}, since the lowest dose of diazepam (1 mg kg^{-1}) did not modify the number of entries into the open arms. Furthermore, these findings demonstrated sedative properties for CMP1 since it increases the time of sleep after pentobarbital injections already at the lowest dose (1 mg kg^{-1}). In these last experiments CMP1 increases pentobarbital-induced sleep as diazepam did. However, it is well-known that diazepam-induced sedative effects are paralleled by a reduction in spontaneous motor activity [25]. These experiments furthermore, have shown that CMP1 did not change the spontaneous motor activity at sedative doses. These findings are of interest, since sedative effects should arise without affecting locomotor activity.

These anxiolitic and sedative properties were also observed using polar extracts from other species of *Salvia*. The hydroalcoholic extract of *Salvia reuterana Boiss* increased the percentage of time-spent and the percentage of arm entries in the open arms of the elevated plus-maze [26]. In the same way, the hydroalcoholic extract of *Salvia elegans* increased the percentage of time spent and the percentage of arm entries into the open arms of the elevated plus-maze, and also increased increase the

time the mice spent in the illuminated side of the light-dark test [27]. However, the hydroalcoholic extract of *Salvia elegans* also increased the immobility time of mice subjected to the forced swimming test [27]. These finding were confirmed by Mora [28] who found a hydroalcoholic extract from the leaves of *Salvia elegans* able to increase at a dose of 12.5 mg kg^{-1} the exploration of the open arms of the pus maze and with all dose used (3.12, 12.5, 25 and 50 mg kg^{-1}) a reduction of immobility in the forced swimming test. These anxiolitic and anti-depressive effects were similar to those observed after diazepam (1 mg kg^{-1}) and fluoxetin (10 mg kg^{-1}) respectively [28].

The antinociceptive activities of diterpenoid from *Salvia* species are already reported. Salvinorin A, the active constituent of *Salvia divinorum* induces profound hallucinations and binding studies suggest that the biological activity of salvinorin A involves the kappa-opioid receptor. Functional studies confirm the involvement of kappa-opioid receptor in the mechanism of salvinorina A effects. Salvinorin A increased tail-flick latencies and the pretreatment with the kappa-opioid receptor antagonist nor-binaltorphimine attenuated the salvinorin A induced increase in tail-flick latency whereas the mu-opioid receptor antagonist beta-funaltrexamine and the delta-opioid receptor antagonist naltrindole were ineffective [29]. These observations were further demonstrated by Ansonoff [30], who found the intracerebroventricular injection of salvinorin A able to produce antinociception in wild-type mice but not in a novel strain of kappa-opioid receptor knockout mice. Furthermore, salvinorin A reduces rectal body temperature in a similar way to conventional kappa-opioid receptor agonists, in a genotype-dependent manner. In the experiments, we cannot exclude an involvment of opioid receptor in the mechanism of CMP1-induced antinociceptive effects, since CMP1 was able to change the response to thermal stimuli in the tail flick test and CMP1 reduced the early phase of the formalin test. However, the increase of latencies obtained in the tail flick test was observed only at the highest dose tested and no effects were observed in the hot plate test after CMP1 administration. It is well known that opioids are also able to increase the response to thermal stimuli in the hot plate test and opioids are able to reduce both the early and the late phase of the formalin test [31]. From all the above findings, further mechanisms other than opioid receptors involvement might explain antinociceptive effects induced by CMP1 in mice.

CMP1 was isolated from *Salvia cinnabarina*, but other *Salvia* species are reported to induce antinociception in animals. Water extracts of *Salvia africana-lutea L*. significantly reduced the writhes induced by acetic acid administration and significantly delayed the reaction time of mice to thermal stimulation produced by the hot plate [32]. The crude acetone and methanol extracts of *Salvia aegyptiaca L.* caused dose-related inhibition of acetic acid-induced abdominal constriction and significantly reduced formalin-induced pain. It also significantly increased the reaction time in the hot-plate test [33]. The above findings and our data further suggest *Salvia* species as a useful tool for the identification and the development of new analgesic drugs.

These findings further suggest that in the extracts from various *Salvia* species CMP1 isolated to date only from *Salvia cinnabarina*, is one of the active compounds with anxiolitic and antinociceptive activity. The interesting activities of CMP1 lead towards the identification of more effective derivatives. Our findings also indicate that CMP1 may be appropriate for the treatment of psychiatric ailments or pain treatment, also considering that the pharmacological activity is exerted without affecting spontaneous motor activity.

Further in vitro and in vivo studies are necessary to determine structure-activity relationships and the exact mechanism/s of action.

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CHAPTER 3

Effect of a Diterpenoid from Salvia cinnabarina on Arterial Blood Pressure in Rats.

The genus Salvia is known for its wide spectrum of pharmacological properties, such as antioxidant, antifungal, antinociceptive, anti-bacterial, antiinflammatory [1-3].

Salvia cinnabarina is an American species in the subgenus Calosphace, section Incarnate [4] containing a high amount of oxygenated monoterpenes (particularly linalool) and oxygenated sesquiterpenes [5]. The new secoiso-pimarane diterpenoid, namely3,4-seicosopimar-4(18),7,15-triene-3-oic acid (CMP1), recently isolated from the leaf exudates of aerial parts of S. cinnabarina , has been shown to have intestinal spasmolytic activity in vitro, with an aspecific mechanism [6]. In vivo, CMP1 inhibits mouse intestinal motility with a mechanism involving L-type Ca2+ channels [7]. Furthermore, CMP1 inhibits rat bladder contractility in vitro with the partial involvement of nitric oxide [8]. To further characterize the pharma-cological profile of CMP1, in the present study we have investigated the effect of CMP1 on rat arterial blood pressure.

3.1 MATERIAL AND METHODS

3.1.1 Animal

Male Wistar rats (200–250 g; Harlan Nossan), housed under conditions of constant temperature (22– 24 °C) and humidity (50 ± 10%) under a 24 h light– dark cycle with food and water freely available, were used for the experiments. All animal experiments complied with the Italian D.L. no. 116 of 27 January 1992 and Associated Guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ ECC).

3.1.2 Drugs.

Urethane, N^{ω}-nitro L-arginine methyl ester (L- NAME) and heparin were purchased from Sigma (Milan, Italy). Chlorisondamine was purchased from Tocris (UK). Extraction, isolation and structural characterization of CMP1 were performed as described previously (Romussi et al., 2001). HPLC analysis indicated that CMP1 was 96% pure.

3.1.3 Measurement of arterial blood pressure.

Male Wistar rats were anaesthetized with urethane (sol 10% w/v i.p.) and placed supine on an operating table. The right jugular vein was cannulated for drug administration, the left carotid artery was cannulated with a cannula containing heparinized saline (5 U/mL) and connected to a pressure transducer (Basile, Comerio (VA), Italy) for continuous monitoring of arterial blood pressure. Acquisition data were performed by a computerized system PowerLab (ADInstruments, v 3.4.3). After surgery, the arterial blood pressure was allowed to stabilize for about 30 min. The effect of three different doses (3, 10 and 30 mg/kg i.v.) of CMP1 was tested in different groups of animals. Each dose was administered on three consecutive times, for 20 min each, in the same animals. Subsequently, a single administration of CMP1 (3 mg/kg i.v.) was chosen, after the preliminary experiments in which each dose was administered for three consecutive times in the same animal.

3.1.4 Ganglion-blockade experiments.

To eliminate any influence of autonomic nervous system activation on changes in mean arterial blood pressure (MABP) induced by CMP1, different groups of animals were pretreated with the irreversible ganglion-blocking agent chlorisondamine at a dose of 2.5 mg/kg i.p., before administering CMP1 (3 mg/kg i.v.) and changes in blood pressure were evaluated.

3.1.5 Role of nitric oxide.

The role of nitric oxide was assessed by infusing L-NAME (0.3mg/kg/min i.v.), through a butterfly needle inserted into the caudal vein, in ganglion blocked rats and changes in blood pressure induced by CMP1 (3 mg/kg i.v.) were evaluated and compared with values obtained before the infusion. The dose of L-NAME chosen was able to restore blood pressure in ganglion-blocked rats to the normal value.

3.1.6 Statistical analysis.

Changes in MABP have been evaluated as the difference from the basal value or as the percentage change from the baseline, when groups with different basal blood pressure were compared. The results obtained are expressed mean ± SEM and analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's or Dunnett's test, as appropriate. When requested, Student's t-test was used. A value of $p < 0.05$ was considered statistically significant.

3.2 RESULTS AND DISCUSSION

Intravenous administration of CMP1 (3–10 mg/kg i.v.) into anaesthetized rats caused a transient dose-dependent fall in MABP. The dose of 3 mg/kg i.v. caused a fall of 14.75 ± 1.44 mmHg; there was no tachyphylaxis after repeated administrations of 20 min each into the same animal and so this dose was chosen for all successive experiments (Fig. 3.1).

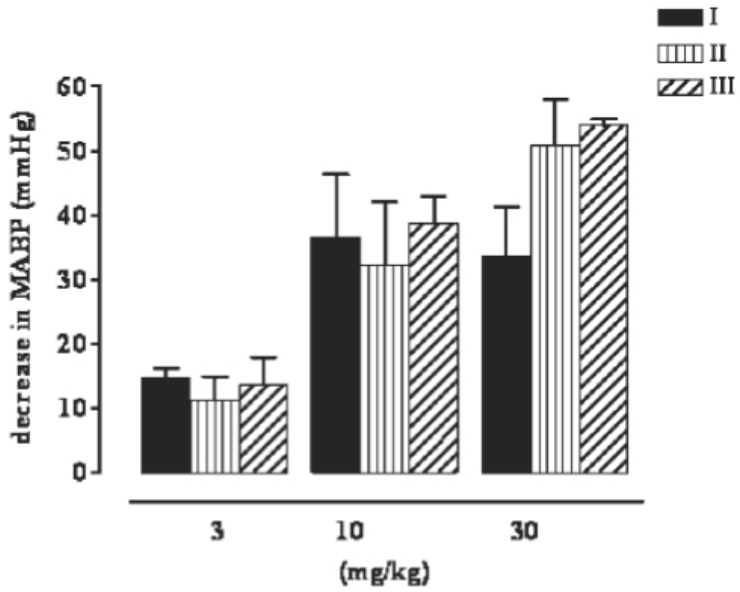

Figure 3.1. Decrease in MABP induced by CMP1 (3, 10 and 30 mg/ kg i.v.) in anaesthetized rats. Each dose of CMP1 was administered in different groups of rats and repeated into the same animals three consecutive times at 20 min intervals (n = 5).

Hypotension induced by CMP1 (3 mg/kg i.v.) was associated with a fall in heart rate from 443 ± 24 to 264 ± 44 beats per minute (bpm) ($p < 0.01$; $n = 9$). To differentiate between a central and a peripheral effect of CMP1, experiments were performed in groups of rats treated with the irreversible ganglion-blocking agent chlorisondamine (2.5 mg/kg i.p.). After treatment with chlorisondamine, the MABP value was 54.64 ± 1.59 mmHg ($n = 11$), significantly different from the control value ($p < 0.0001$); however, the hypotensive effect of CMP1, evaluated as the percentage (%) change in MABP, was not different from the control animals (21.50 ± 6.76% vs 16.00 ± 2.38%), suggesting that it does not involve the autonomic nervous system activation deriving from a central effect of CMP1. Interestingly, in ganglion-blocked

rats the hypotensive effect of CMP1 was followed by a slight but significant hypertension (10.75 ± 2.14%; n = 4) that was not evident before ganglion-blockade. Several studies describing the effect of L-NAME on changes in MABP induced by drugs have been performed in normal animals, in which the substantial increase in MABP and vascular resistance above physiological values, due to nitric oxide inhibition, might activate a reflex response masking the real effect of drugs. For this reason, to investigate on the possible role of nitric oxide on changes in blood pressure induced by CMP1, experiments were performed on ganglion-blocked rats, in which an infusion of L-NAME (0.3 mg/kg/min i.v.) restored blood pressure to the normal value. Under these conditions, no inhibition of CMP1-induced hypotension was observed, ruling out the involvement of nitric oxide (Fig. 3.2A).

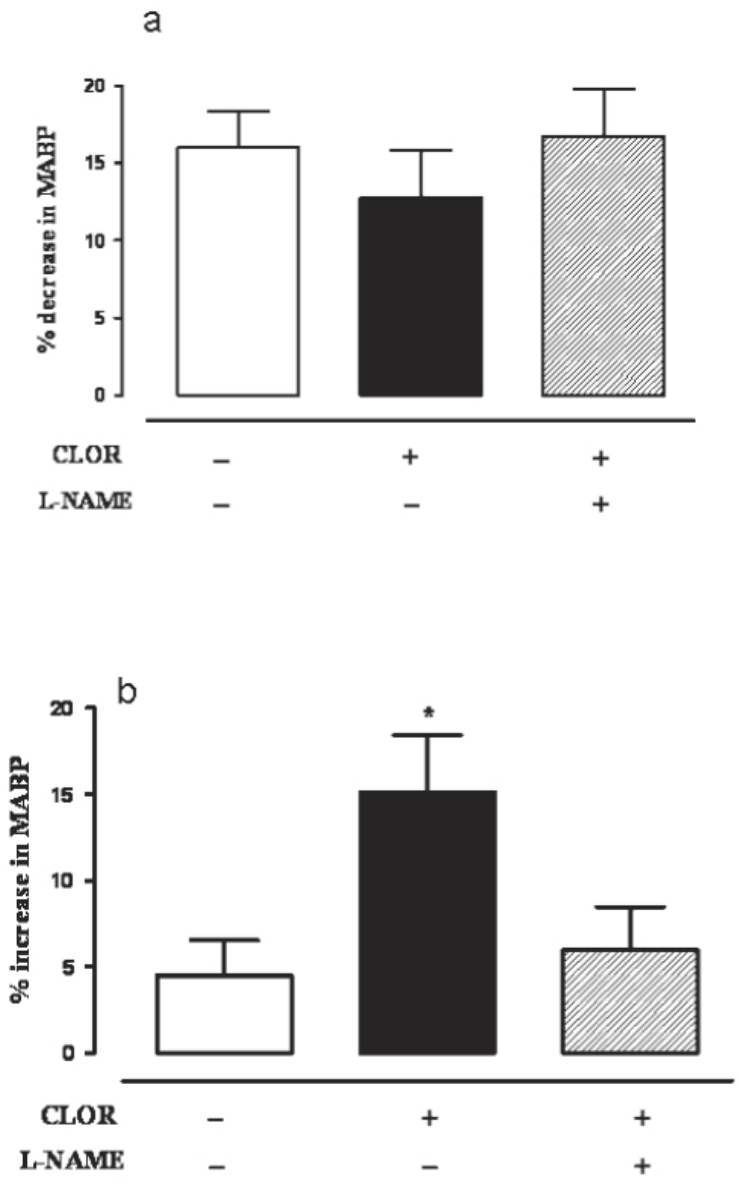

Figure 3.2. Change in MABP induced by CMP1 in ganglion-blocked rats and effect of L-NAME infusion. (a) Decrease in MABP induced by CMP1 (3 mg/kg i.v.) before (white bar), after treatment with the ganglion-blocking agent chlorisondamine (2.5 mg/kg i.p. solid bar) and in ganglion-blocked rats treated with an infusion of L-NAME (0.3 mg/kg/min i.v. hatched bar). (b) Increase in MABP induced by CMP1 (3 mg/kg i.v.) before (white bar), after treatment with ganglion-blocking agent chlorisondamine (2.5 mg/kg i.p. solid bar) and in ganglion-blocked rats treated with an infusion of L-NAME (0.3 mg/kg/min i.v. hatched bar) (n = 4–8, * $p < 0.01$ vs before treatment with chlorisondamine and vs after L-NAME infusion).

This finding, together with the observation that in ganglion-blocked rats CMP1-induced hypotension was not different from the value obtained in normal, non-ganglion-blocked rats, suggests that the vasodilator mechanism is due to a peripheral action but independent from nitric oxide release. Interestingly, following L-NAME infusion, hypertension that was evident only after chlorisondamine treatment was abolished (Fig. 3.2B).

In conclusion, this is the first work demonstrating an in vivo hypotensive effect of CMP1 due to a direct peripheral action but independent of nitric oxide release. Our findings further contribute to delineate the pharmacological profile of this natural compound.

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CHAPTER 4

Platelet antiaggregating activity and chemical constituents of Salvia jamensis J. Compton

A phytochemical study has been carried out on the surface exudate of *Salvia x jamensis* (figure 4.1) which showed a significant platelet antiaggregating activity. Platelet aggregation inhibitors are widely used in thromboembolic diseases. Since currently available compounds have various limitations, it is desirable to find new types of platelet antiaggregating agents, which prevent the initiation of thrombus formation.

Figure 4.1: *Salvia jamensis*

The surface exudate of *Salvia x jamensis* J. Compton, obtained by rinsing the plant material with methylene chloride and terpenic compounds isolated have been tested on ADP-induced platelet aggregation. The known compounds isopimaric acid (compound **2)**, 14-α-hydroxy-isopimaric acid (compound **3)**, 3-β-hydroxy-isopimaric acid (compound **4)**, 3β-dihydrosalviacoccin (compound **5)**, betulinic acid (compound **6)**, ursolic acid (compound **7)** (figure 4.2) were isolated together with the new diterpene (compound **1)** (figure 4.3).

The structure of compound **1** was determined as 15, 16-epoxy-cleroda-3-en-7α, 10β-dihydroxy-12,17, 19,18-diolide on the basis of spectroscopic data analysis.

Among all tested compounds, **2** showed a significant concentration-dependent antiaggregating activity when ADP (3 μM) was used as agonist on rat platelets. Conversely, **1** increased ADP–induced platelet aggregation.

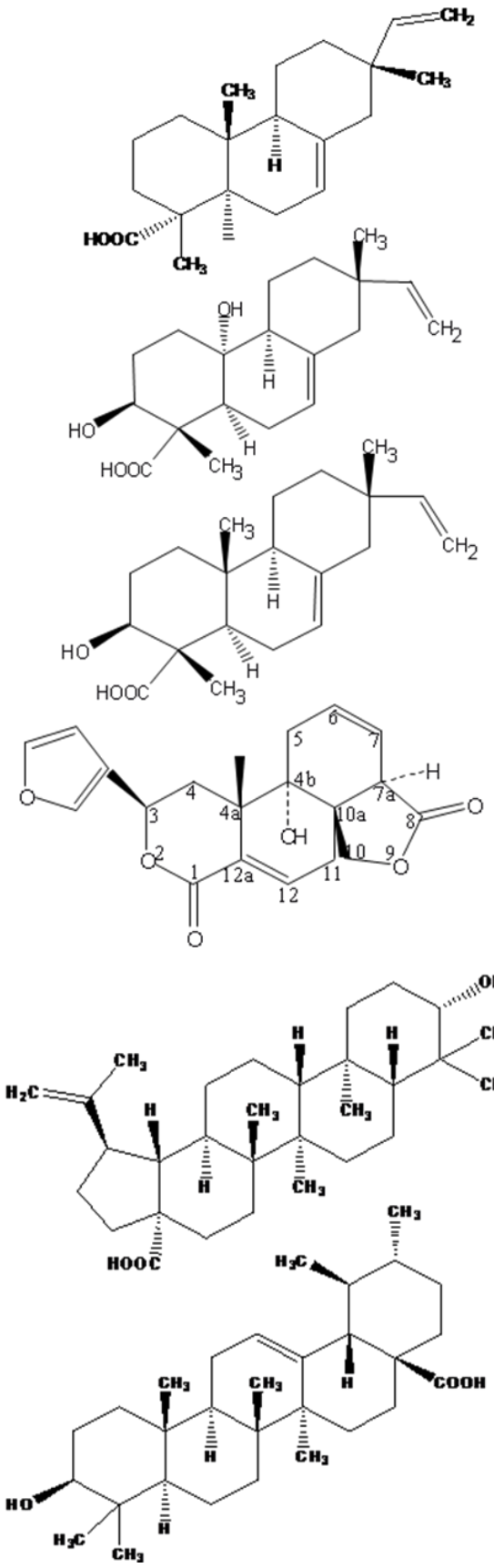
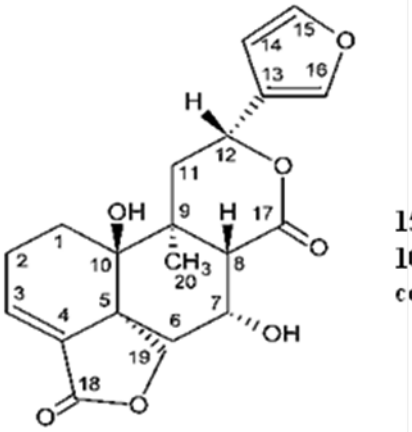


Figure 4.2: known diterpenoids and triterpenoids isolated from *Salvia jamensis*



15, 16-epoxy-cleroda-3-en-7α, 10β-dihydroxy-12,17, 19,18-diolide compound 1

Figure 4.3: new diterpene isolated from *Salvia jamensis* (compound **1)**

4.3 MATERIAL AND METHODS

4.1.1 Plant Material

Aerial parts of *S. x jamensis* J. Compton were obtained from the collections of *Salvia* species established and available in the Istituto Sperimentale per la Floricoltura (Sanremo, Italy) and Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga. Italy). Dr. Gemma Bramley has identified the species and a voucher specimen is deposited in Kew Herbarium (K).

4.1.2 Extraction and isolation

For the isolation of leaf surface constituents, fresh aerial parts (3.1 kg) were immersed in CH_2Cl_2 for 20 sec. After filtration the extraction solvent was removed under reduced pressure. The exudate (18 g) was chromatographed in portions of 1.5 g on Sephadex LH-20 columns (60 x 3 cm) using $CHCl_3$/MeOH (7:3) as eluent to give in order of elution fractions (3g) with waxy compounds (170 mL), fractions (3g) with very crude compound **7** (from 170 mL to 220 mL) and fractions (12g) with mixture of compounds **1**-**7 (**from 220 mL to 290). These last fraction groups were chromatographed in portions of 4 g on silica gel columns (40 x 4 cm) eluting with mixtures of *n*-hexane- $CHCl_3$ [50:50 (4.1 L), 40:50 (2.1 L), 33:67 (1.0 L), 25:75 (1.0 L), 10:90 (11.0 L], then with $CHCl_3$ (8.3 L), and then with $CHCl_3$-MeOH [95:5 (3.4 L)]. Elution with *n*-hexane-$CHCl_3$ 40:50 (from 0.2 L to 2.1 L) and with with *n*-hexane-$CHCl_3$ 33:67 and 25:75 afforded fractions with compound **2** (crystallized from MeOH: 2.05 g). Elution with with *n*-hexane-$CHCl_3$ 10:90 afforded first fractions with compound **6** (from 1.0 to 1.4 L; crystallized from EtOH: 0.2 g), then fractions with compound **7** (from 1.6 to 2.7 L; crystallized from EtOH: 1.4 g), then fractions with compound **5** (from 2.7 to 3.0 L; crystallized from $CHCl_3$/MeOH: 0.14 g), then with compound **1** (from 3.8 to 11.0 L; crystallized from $CHCl_3$/MeOH: 0.58 g). Elution with $CHCl_3$ afforded fractions with **3** (from 3.5 to 6.0 L; crystallized from MeOH/H_2O: 0.25 g). Elution with $CHCl_3$-MeOH 95:5 afforded fractions with compound **4** (from 1.1 to 2.0 L, crystallised from MeOH/H_2O: 50 mg).

4.1.3 Animal

Male Wistar rats (Harlan - Nossan) weighing 200-250 g, housed under conditions of constant temperature (22 – 24 °C) and humidity (50 ± 10 %) under a 24 h light–dark cycle with food and water freely available, were used for all experiments. All animal experiments complied with the Italian D.L. no. 116 of 27

January 1992 and associates guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

4.1.4 Platelet preparation and aggregation

Blood was withdrawn by cardiac puncture from male Wistar rats, slightly anaesthetised with enflurane, and anticoagulated with 3.8% (w/v) trisodium citrate (1:9 v/v). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described [1]. Platelet rich plasma (PRP) was prepared by centrifugation of the anticoagulated blood at 250 x g for 10 min. The Platelet count in PRP was adjusted to $3x10^5$ platelets μl^{-1} with autologous PPP. To prepare washed platelets suspension (WPS), PRP was diluted with an equal volume of citric acid/ sodium citrate buffer and centrifuged at 650 x g for 10 minutes. The platelet pellet was suspended in calcium-free Tyrode's buffer, centrifuged to 650 x g for 10 mins and then resuspended in a volume of calcium-free Tyrode's buffer to obtain the final platelet concentration of $3x10^5$ platelets μl^{-1}. Platelet aggregation was monitored in an Elvi 840 light transmission aggregometer by measuring changes in turbidity of 0.25 ml PRP warmed at 37°C and under continuous stirring. ADP (3-10 μM) was used as aggregating agent. After preliminary experiments, the concentration of 3 μM ADP was chosen and used throughout the study.

To evaluate the effect of compounds **1-7** on ADP-induced platelet aggregation, 5 μl of compounds tested, were added to PRP 5 minutes before the ADP (3 or 10 μM) at the final concentrations ranging between 2 x 10^{-4} and 10^{-3} M. As a control, an equal volume of DMSO (dimethyl-sulfoxide) was added to PRP.

4.1.5 Data analysis and statistics

Ex vivo data of platelet aggregation are presented as % aggregation relative to PPP or suspension buffer. For statistical analysis, a computerized statistical package was used (11). Data were analyzed by one-way ANOVA followed by Dunnett's test vs. vehicle (n=10) for multiple comparisons. Vehicle (DMSO) value was 27.92 ± 2.78 %. All results are expressed as means ± s.e.m. Values of $p< 0.05$ and $p< 0.01$ were considered significant.

4.3 RESULTS

4.2.1 Effects of exudate from aerial part of Salvia x jamensis and of compounds 1-7 on ADP(3 μM) -induced platelet aggregation

The surface exudate of *Salvia x jamensis* J. Compton, obtained by rinsing the plant material with methylene chloride, showed, at concentration of 100 – 500 μg/ml, a concentration – dependent trend toward a platelet antiaggregating activity (figure 3.4).

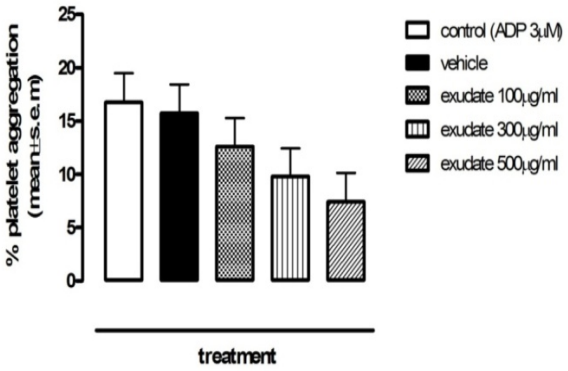


Figure 4.4: Effects of exudate from aerial part of *Salvia x jamensis* on ADP (3 μM) -induced platelet aggregation. Results are expressed as mean ± s.e.m. and represent aggregation (%) in response to ADP 3μM alone (control) or in presence of DMSO (vehicle) or exudate of *Salvia x jamensis* (exudate 100-500 μg mL^{-1}). (n=10)

Compound **2** (HPLC analysis: one single peak) at concentrations of 6 x 10^{-4} and – 1 x 10^{-3} M inhibited significantly ADP (3 μM) – induced platelet aggregation. Compounds **3 – 4** did not modify platelet aggregation in a significant manner. On the contrary, compound **1** (HPLC analysis: one single peak) increased at the highest concentration ADP- induced platelet aggregation (Table 3.A and figure 4.5).

	% platelet aggregation		
Compound	(2 x 10^{-4}) M	(6 x 10^{-4}) M	(1 x 10^{-3}) M
1	48.10 ± 14.06	29.83 ± 7.15	62.67 ± 8.88 **
2	18.36 ± 2.89	5.38 ± 3.11 **	2.29 ± 1.35 **
3	33.58 ± 9.01	21.02 ± 4.66	16.71 ± 4.03
4	46.66 ± 3.34	34.57 ± 5.14	22.38 ± 3.72

Table 4.A: Results are expressed as mean ± s.e.m. and represent aggregation (%) in response to ADP 3μM. In brackets are reported concentrations (M) of compounds tested. Vehicle (DMSO) value was 27.92 ± 2.78%. ** $p< 0.01$ one way ANOVA followed by Dunnett's test vs vehicle (n=10)

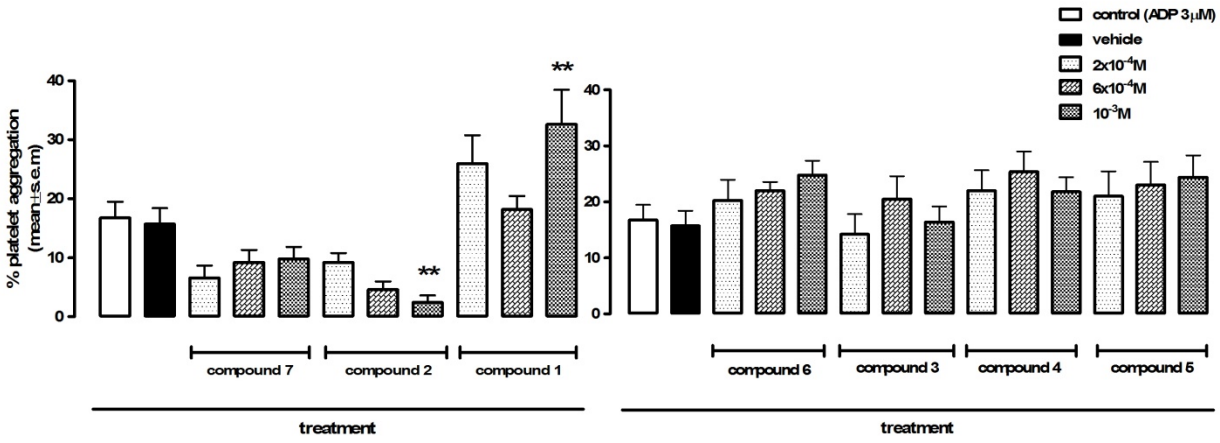


Figure 4.5: Effects of compounds **1-7** from aerial part of *Salvia x jamensis* on ADP (3 µM) -induced platelet aggregation. Results are expressed as mean ± s.e.m. and represent aggregation (%) in response to ADP 3 µM alone (control) or in presence of DMSO (vehicle) or compounds **1-7** (2 x 10^{-4}; 6 x 10^{-4}; 1 x 10^{-3} M). ** $p < 0.01$ and one way ANOVA followed by Dunnett's test vs. vehicle (n=10).

4.2.2 Effects of exudate from aerial part of Salvia x jamensis and compounds 1-7 on ADP(10 μM) -induced platelet aggregation

The exudate of *Salvia x jamensis* J. Compton did not show, at a concentration of 100 – 500 μg/ml, significant effects on ADP(10 μM) -induced platelet aggregation (figure 4.6).

In compounds 1-7 only compound 2, at concentrations of 1 x 10^{-3}M, showed inhibitory effect on ADP (10 μM) -induced platelet aggregation (5.931±) (figure 4.7).

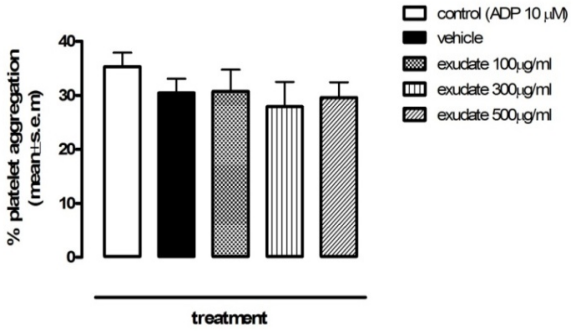


Figure 4.6: Effects of exudate from aerial part of *Salvia x jamensis* on ADP (10 μM) -induced platelet aggregation. Results are expressed as mean ± s.e.m. and represent aggregation (%) in response to ADP 10 μM alone (control) or in presence of DMSO (vehicle) or exudate of *Salvia x jamensis* (exudate 100-500 μg mL^{-1}). (n=10).

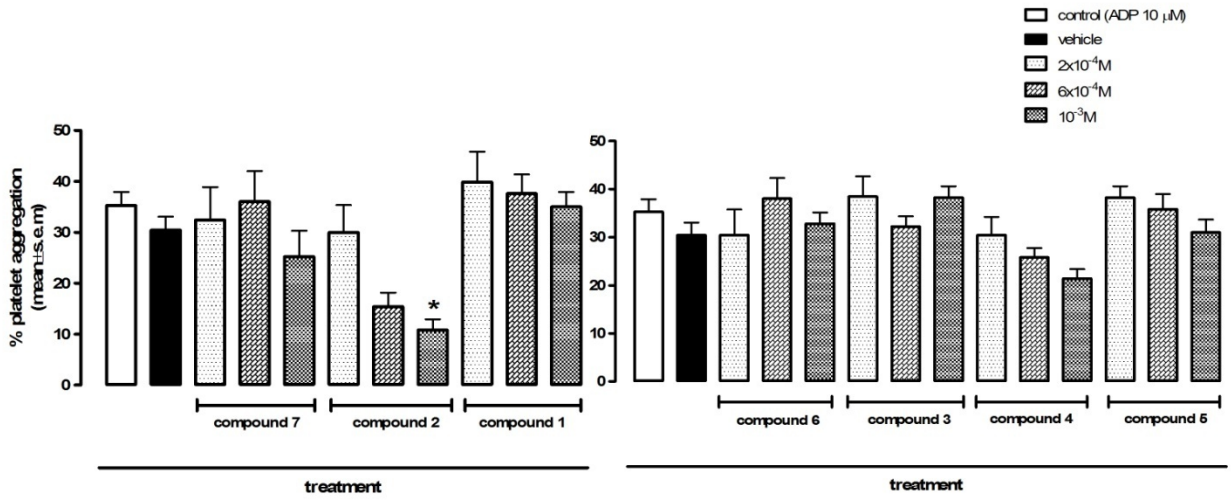


Figure 4.7: Effects of compounds **1-7** from aerial part of Salvia x jamensis on ADP (10 μM) -induced platelet aggregation. Results are expressed as mean ± s.e.m. and represent aggregation (%) in response to ADP 10 μM alone (control) or in presence of DMSO (vehicle) or compounds **1-7** (2 x 10^{-4}; 6 x 10^{-4}; 1 x 10^{-3} M). * $p < 0.05$ one way ANOVA followed by Dunnett's test vs. vehicle (n=10).

4.3 DISCUSSION

Platelet aggregation is a complex process. It is generally held that platelet activation is mainly mediated through the adhesiveness of platelets to the site of injury and through the action of endogenous agonists such as ADP, collagen, and thrombin, followed by the release of TXA2, which acts as an amplifying factor in the platelet aggregation [2-3](Jackson et al., 2003; Farndale et al., 2004). It is well established that platelets form aggregates in response to a number of stimuli, readily demonstrated in vitro using the aggregometer [4], and in vivo using radiolabelled platelets [5]. Intravenous administrations of ADP induce accumulation of platelet microaggregates which display spontaneous disaggregation [5]. In vitro ADP, at high concentrations, induces irreversible formation of platelet aggregates [6].

It is well known that some natural compounds show a wide variety of pharmacological effects on platelet aggregation [7-9] In previous papers the inhibition of platelet aggregation by diterpenes of abietane groups and by triterpenoids was also described , so what is examined here are the possible effects of exudate from aerial part of *Salvia x jamensis* and compounds **1-7** on ADP (3 or 10 μM) -induced platelet aggregation.

The observed trend toward the platelet anti-aggregating effect of the surface exudate of *Salvia x jamensis* is likely due to the presence of the active compound **2;** however, compound **3 (**HPLC analysis indicated that the compound was 97% pure) shows an inhibitory trend, although not significant. It is worth noting that compound **3** in comparison with **2** differs as to the presence in the molecules of a hydroxyl group. This suggests that reduced activity could be due to a reduced lipophylicity. Compound **4 (**HPLC analysis: one single peak), that is an isomer of **3**, loses the observed biological activity. Compounds **5 – 7** were completely inactive (data not shown). The inactivity of compound **7** on ADP – induced platelet aggregation has already been described [10]. All these results suggest that pimarane, isopimarane and abietane derivatives may be useful for the semisynthesis of new compounds with platelet antiaggregating activity.

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