ANTI-INFLAMMATORY PROPERTIES AND BIOACTIVE NATURAL PRODUCTS FROM *WITHERINGIA SOLANACEA*, *WITHANIA FRUTESCENS* (SOLANACEAE), AND *VALERIANA OFFICINALIS* (VALERIANACEAE) USING NF-kappaB AS A MOLECULAR TARGET.

Nadia Judith Jacobo-Herrera

A thesis submitted for the degree of Doctor of Philosophy at The School of Pharmacy, University of London

This thesis describes research conducted in the School of Pharmacy, University of London between October 2001 and June 2005 under the supervision of Prof. Michael Heinrich. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

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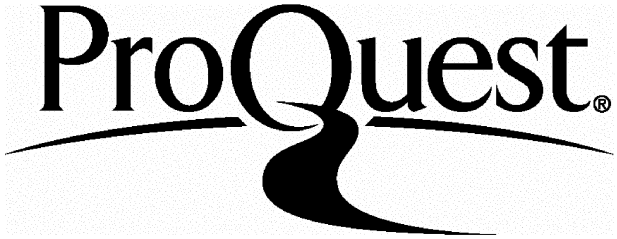

Abstract

Nuclear Factor kappaB (NF-κB) is a transcription factor implicated in the regulation of many pro-inflammatory cytokines and related proteins that participate in a number of inflammatory diseases. The inhibitory activity of NF-κB cascade was evaluated through bioassay-guided fractionation for three medicinal plants, *Witheringia solanacea, Withania frutescens, and Valeriana officinalis*. The selected plants are included in an Anti-Inflammatory Natural Products (AINP) EU-funded project, which attempts to identify new natural products as inhibitors of NF-κB activation.

Six different chromatography techniques were employed in the fractionation of crude extracts and isolation of pure compounds and the latter were subsequently characterised using spectroscopic techniques (NMR and MS experiments). Luciferase–based assays were performed to test the biological activity of fractions and compounds using HeLa cells. These cells were stably transfected with a luciferase reporter gene controlled by the IL-6 promoter (one of the target genes that promotes NF-κB activation), and phorbol 12-myristate-13-acetate was used as a stimulant of NF-κB at 50 ng/ml for 7 h. Three compounds were isolated from an active fraction of *Witheringia solanacea*, which were identified as physalins B, F and D. Physalins B and F inhibited NF-κB at 16 and 8 μM, whereas physalin D was inactive. Subsequent cell cycle assays performed for physalins F and B revealed that both induce apoptosis after 24 h at 10 and 20 μM, respectively. Two compounds were isolated from the chloroform extract of *Withania frutescens*, 5,6-epoxy-1-oxowitha-2, 14, 24-trienolide and withaferin A, though the latter was recovered together with an unidentified compound. 5,6-epoxy-1-oxowitha-2,14,24-trienolide

reduced NF-κB activity at 25 μM, whereas the fraction containing withaferin A reduced NF-κB activity at 25 μg/ml. The EtOAc extract of *Valeriana officinalis* also inhibited the NF-κB cascade at 100 μg/ml, though, of its three compounds isolated, only one, acetylvalerenolic acid, showed inhibitory activity at 100 μg/ml. This study provides molecular evidence for the anti-inflammatory activity of these plants, and some of their compounds isolated as small molecule inhibitors of the NF-κB pathway.

Acknowledgments

I am very thankful to my supervisor Prof. Michael Heinrich for all his help and support during my PhD, and for introducing me with the fascinating world of natural products. I also want to express my gratitude to Dr. Simon Gibbons, my second supervisor, for all his interest towards my work, his help on phytochemistry, and his patience and time to explain me NMR. I thank Dr. Paul Bremner for the NF-κB assays and for reading my dissertation, but especially for his encouragement and friendship during my PhD.

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I am short of words to say how grateful I am to my family for all their love and support.

Para Alejandro, por todo lo que nos espera juntos, con todo mi amor.

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List of biological abbreviations

BAFF/Blys	B-cell-activating factor belonging to the TNF family
BLC	B-lymphocyte chemoattractant
CD40L	CD40 ligand
COX	Cyclo-oxygenase
ELC	Epstein-Barr virus-induced molecule 1 ligand CC chemokine
GM-CSF	Granulocyte macrophague colony-stimulating factor
IκB	NF-κB inhibitory protein
IKK	IκB kinase complex
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Irf3	Gene Irf3
LPS	Lipopolysaccharides
LT	Lymphotoxin
MCP-1	Monocyte chemotactic protein-1
MIP-1α	Macrophage inflammatory protein-1α
NF-κB	Nuclear factor kappaB
NIK	Nuclear factor-inducing kinase
NLS	Nuclear localization signal
NO	Nitric oxide
PLA2	Phospholipase 2
Rel	Reticuloendotheliosis protein family
RHD	Rel-homology-domain
Rxra	Gene Rxra
SDF-1	Stromal cell-derived factor-1α
SLC	Secondary lymphoid tissue chemokine
TF	Transcription factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor

List of chemical and chromatographic abbreviations

ACN	Acetonitrile
$CHCl_3$	Chloroform
DCM	Dichloromethane
EtOAc	Ethyl acetate
Hex	n-Hexane
HPLC	High performance liquid chromatography
MeOH	Methanol
MTT	Tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
PBS	Phosphate-buffered saline
PMA	Phorbol-12-myristate-13-acetate
SPE	Solid phase extraction
TLC	Thin layer chromatography
VLC	Vacuum liquid chromatography

List of spectroscopic abbreviations

COSY	Correlated spectroscopy
DEPT	Distortionless enhancement by polarization transfer
HMBC	Heteronuclear multiple bond coherence
HMQC	Heteronuclear multiple quantum correlation
ES	Electron spray
MS	Mass spectroscopy
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear overhauser effect spectroscopy

CHAPTER 1. INTRODUCTION

1.1. Natural Products

Natural products are the most valuable source for the discovery of novel drugs (Harvey, 2000; Tulp and Bohlin, 2002, 2004). Currently, more than 160,000 compounds have been identified from different kinds of living organisms, including plants, microbes, and marine and terrestrial organisms (Tulp and Bohlin, 2002). However, bioactive compounds have only been obtained from about 10% of the estimated world's biodiversity (Harvey, 2000).

One of the most frequently used approaches in the search for new natural drugs is through traditional knowledge (Tulp and Bohlin, 2004). Medicinal plants have been traditionally used during human history for the treatment of a variety of ailments, and currently it is known that approximately 60% of the world's population uses plants as a first source of medication (Harvery, 2000). Moreover, around 25% of the modern pharmaceutical drugs are of botanical origin (O'Hara *et al.*, 1998; Tulp and Bohlin, 2002). For example, it is estimated that over 60% of the anticancer drugs available on the market are of natural origin (Cragg and Newman, 2005). Some of the more popular commercial natural herbal medicine found nowadays are *Ginkgo biloba* (Ginkgoaceae), mainly used in the treatment of dementia, cerebral deficiencies and circulatory problems; *Hypericum perforatum* (Hypericaceae) which is regularly prescribed for the treatment of depression; *Oenothera biennis* (Onagraceae) used for the relief of atopic eczema and for the symptomatic treatment of breast pain; and *Plantago ovata* (Plantaginaceae) as laxative (De Smet, 1997; O'Hara *et al.*, 1998;

Heinrich *et al.*, 2004). Some examples of important plant-derived drugs are shown in Table 1.

Table 1. Examples of important commercial drugs derived from plants (De Smet, 1997; O'Hara *et al.*, 1998; Heinrich et al., 2004; Heinrich and Teoh, 2004).

Species	Name of the drug	Action/use
Atropa belladonna (Solanaceae)	Atropine	Anticholinergic
Camellia sinensis (Theaceae)	Caffeine	CNS stimulant
Cinchona succirubra (Rubiaceae)	Quinine (chloroquine)	Antimalarial
Colchicum autumnale (Liliaceae)	Colchicine	Antigout
Ephedra sinica (Ephedraceae)	Ephedrine	Sympathomimetic
Papaver somniferum (Papaveraceae)	Codeine, morphine	Analgesics
Pilocarpus jaborandi (Rutaceae)	Pilocarpine	Glaucoma
Salix spp (Salicaceae)	Aspirin	Analgesic
Taxus brevifolia (Taxaceae)	Paclitaxel	Cancer therapy
Galanthus spp, *Narcissus* spp, *Leucojum* spp (Amaryllidaceae)	Galanthamine	Alzheimer's disease

The search and discovery of new natural drugs can be approached in different ways and requires the collaboration of different disciplines. Although the most common approach is mainly based on ethnobotanical research, marine sources and their screening, as well as fungal metabolites seem to be at least as popular (Heinrich, 2003). Ethnobotany is defined as the study of the relationships between humans and plants, the latter used as a source of food, medicine, poisons, construction material, clothing, and ornamental objects (Ford, 1978). Ethnopharmacology on the other hand is the interdisciplinary scientific collaboration of biologically active agents traditionally employed or observed by humans (Bruhn

and Holmstedt, 1981). Ethnopharmacological research usually combines ethnobotanical, anthropological, chemical, and pharmacological studies, seeking among many goals the evaluation and validation of culturally important medicinal plants (Heinrich, 2000; Heinrich and Gibbons, 2001). This discipline is concerned in protecting and conserving biodiversity and its sustainable exploitation, thus to document, catalogue and protect the traditional knowledge (Martin, 1995; Heinrich *et al.*, 2004). Additionally, it is also of ethnopharmacological interest, to find new natural products of commercial value (Martin, 1995). Currently, ethnopharmacological surveys are regulated by a series of international conventions, most notably the Convention on Biological Diversity (also known as the Convention of Rio; see www.biodiv.org/chm/conv.htm). These international conventions were agreed in order to establish the State's rights and responsibilities associated with biodiversity, working in an ethical way, where the benefits are shared with those countries that provide the genetic resources and traditional knowledge. Specifically, Article 8 from the Convention of Rio addresses the rights of the indigenous and local people, the promotion of their knowledge with their approval, and the sharing of the benefits derived from this knowledge (Heinrich and Gibbons, 2001).

Alternatively, bioprospecting is another common approach for the search of new bioactive natural products for commercial gain. This kind of research consists of the collection of biogenic samples from different taxa and the development of new drugs for the international market (Heinrich and Gibbons, 2001; Heinrich *et al.*, 2004). Bioprospecting differs from the ethnopharmacological approach, in that it does not involve traditional knowledge as a core criterion for selecting biogenetic samples, and does not specify the non-commercial or commercial benefit that the people

and/or regions of origin will receive (Heinrich and Gibbons, 2001; Heinrich *et al.*, 2004).

Natural products research is a field of great commitment, in which it is of high priority to develop ethnopharmacological data-bases comprising the right identification of the biological samples, the correct interpretation of their traditional use, exact doses, and most importantly their safety and efficacy (Heinrich and Gibbons, 2001).

1.2. Inflammatory process

The inflammatory process is an essential response in the organism to the invasion of infectious agents, antigen challenge, or physical damage, where plasma molecules and leukocytes interact on the site of infection or on the damaged tissue (Roitt *et al.*, 1998). Among the main indicators of inflammation are the increase in blood supply, vasodilation, microvascular permeability, redness, swelling, and pain (Roitt *et al.*, 1998; Lydyard *et al.*, 2000). The principal mediators of the immune inflammatory response are: inflammatory cytokines, cell adhesion molecules, immunoreceptors, haematopoietic growth factors, acute phase proteins, transcription factors, prostaglandins, tromboxanes and leukotrienes(Bork *et al.*, 1997; Celotti and Laufer, 2001). The inflammatory response can occur as acute or chronic inflammation (Roitt *et al.*, 1998). The acute inflammation is initiated by infection, trauma, or allergy through the release of inflammatory mediators into the tissues, whereas chronic inflammation is provoked when the cell-mediated mechanisms are directed towards the antigens (Roitt *et al.*, 1998; Lydyard *et al.*, 2000).

Chronic inflammatory diseases are of special concern because of their long permanency and subsequent damage to the organism. Specifically asthma, arthritis,

and the inflammatory bowel disease (IBD) are among the most common and widespread chronic inflammatory conditions around the world and their reported cases are increasing every year. For example, asthma is the most common respiratory inflammatory disease in industrialised countries, affecting around 10% of children and 5 to 7% of the adult population (Mallarkey, 1999; Kogevinas *et al.*, 1999; Volmer, 2001). On the other hand, in Western countries rheumatoid arthritis has an important impact, causing disability especially in women between 30 to 50 years (Katz, 1998; Bertolini *et al.*, 2001). Furthermore, in the United Kingdom, IBD affected about 1 in 1500 people during 1999 (Rampton, 1999).

Non-selective non-steroidal anti-inflammatory drugs (NSAIDs) are the most recurrent treatment to relieve chronic pain, although they have important adverse reactions, causing principally gastrointestinal problems such as gastric ulceration and renal damage (Bertolini *et al.*, 2001; Maxwell and Webb, 2005). The evidence that cyclo-oxygenase 2 (COX-2) expression is associated with inflammation, rheumatoid arthritis, seizures and ischemia, have lead to the development of selective- NSAIDs targeting specifically COX-2 (Mamdani *et al.*, 2004; Warner and Mitchell, 2004; Graham *et al.*, 2005; Maxwell and Webb, 2005). After synthesis and transport, COX-1 and COX-2 are located in the endoplasmic reticulum; both catalyse the formation of prostaglandin (PG) H2 from arachidonic acid, which is the first step in prostanoid biosynthesis (Hawkey, 1999; Warner and Mitchell, 2004). COX-1 is involved in the physiological production of prostanoids and COX-2 in the elevated production of prostanoids that occur in sites of disease and inflammation (Warner and Mitchell, 2004). Consequently, COX-2 has been selected as a target for the development of new drug leads with anti-inflammatory effects (selective NSAIDs), while COX-1, as a target for the secondary effects, such as gastrointestinal toxicity

due to location predominantly in the stomach, yielding protective prostaglandins (Hawkey, 1999; Maxwell and Webb, 2005). Despite being a good alternative in the treatment of inflammatory conditions, recently there have been some clinical trials regarding the safety of some of the most widely used NSAIDs, showing some hazards related to the cardiovascular system including myocardial infarctions, strokes, and elevation in blood pressure (Ray *et al.*, 2002; Jüni *et al.*, 2004; Warner and Mitchell, 2004; Graham *et al.*, 2005; Maxwell and Webb, 2005).

Regarding asthma, bronchodilator drugs are widely used, although they only relieve the symptoms and do not stop the inflammatory process (Barnes, 1999). Finally, the lack of knowledge about the development process of IBD has impeded the use to any specific treatment for this condition, although sulfasalazines and corticosteroids are frequently used (Yang *et al.*, 2001). Therefore, the two major goals in the search for new therapeutic alternatives for these three syndromes are, firstly stopping the inflammatory process, and secondly, limiting the side effects in the organism (Barnes and Lim, 1998; Bertolini *et al.*, 2001).

1.3. Transcription factor Nuclear Factor kappa- B (NF-κB)

Nuclear Factor kappa-B (NF-κB) is a transcription factor involved in the inducible expression of a variety of cellular genes that regulate the inflammatory responses (Suto and Ransone, 1997; Hehner *et al.*, 1998). Inhibiting NF-κB is important for counteracting the inflammatory process, particularly for the treatment of chronic inflammatory illnesses (Bork *et al.*, 1997; Lyss *et al.*, 1997). Therefore, the understanding of the NF-κB cascade is an important element in the development of new anti-inflammatory drugs.

Transcription factors (TF) are proteins that activate or repress gene expression by specific interaction with regulatory DNA sequences and by interactions with other proteins (Makarov, 2000; Villard, 2004). TF are localized in the cytoplasmic membrane and the majority of them bind DNA as homo or heterodimers (dimerization domain) (Makarov, 2000; Villard, 2004). After stimulation, TF pass into the nucleus where they selectively bind their cognate sites in the regulatory elements of targeted genes and activate or repress transcription (Makarov, 2000).

Nuclear factor kappa B (NF-κB) was first identified in 1986 as a B cell-specific regulator of κ immunoglobulin light chain gene expression in lymphocytes (Sen and Baltimore, 1986). Since then, NF-κB has been subjected to extensive investigation due to the wide variety of stimuli that activate it and the diversity of genes and biological activities that it regulates. NF-κB is implicated in several aspects of cell regulation such as inflammatory and innate immune responses, apoptosis, proliferation, differentiation and oncogenic transformation (Karin and Delhase, 2000; Yamamoto and Gayor, 2004; Meffert and Baltimore, 2005). NF-κB is found in the cytoplasm of non- stimulated cells interacting with inhibitory IκB proteins (IκB α, IκB β, IκB γ, and IκB ε). These proteins contain 6-7 ankyrin repeats, which are required for association with the nuclear localization signal of NF-κB proteins (Karin *et al.*, 2002; Rangaswami *et al.*, 2004).

NF-κB belongs to the reticuloendotheliosis (Rel) protein family, which in mammals consists of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor 105) and NF-κB2 (p52 and its precursor p100) (Makarov, 2000; Yamamoto and Gaynor, 2004). Table 2 lists the different functions known for the five aforementioned proteins (Karin *et al.*, 2002; Yamamoto and Gaynor, 2004). The

REL family is known to have an N-terminal domain of about 300 amino acids, called Rel-homology-domain (RHD) (Karin *et al.*, 2002). This domain is required for mediating DNA binding, dimerization, and nuclear translocation (Makarov, 2000; Yamamoto and Gaynor, 2004). The RHD contains a nuclear localization signal (NLS) at its carboxyl terminus, and is recognized by the IκB proteins, which is the binding site where the RHD interferes with the function of NLS (Karin *et al.*, 2002).

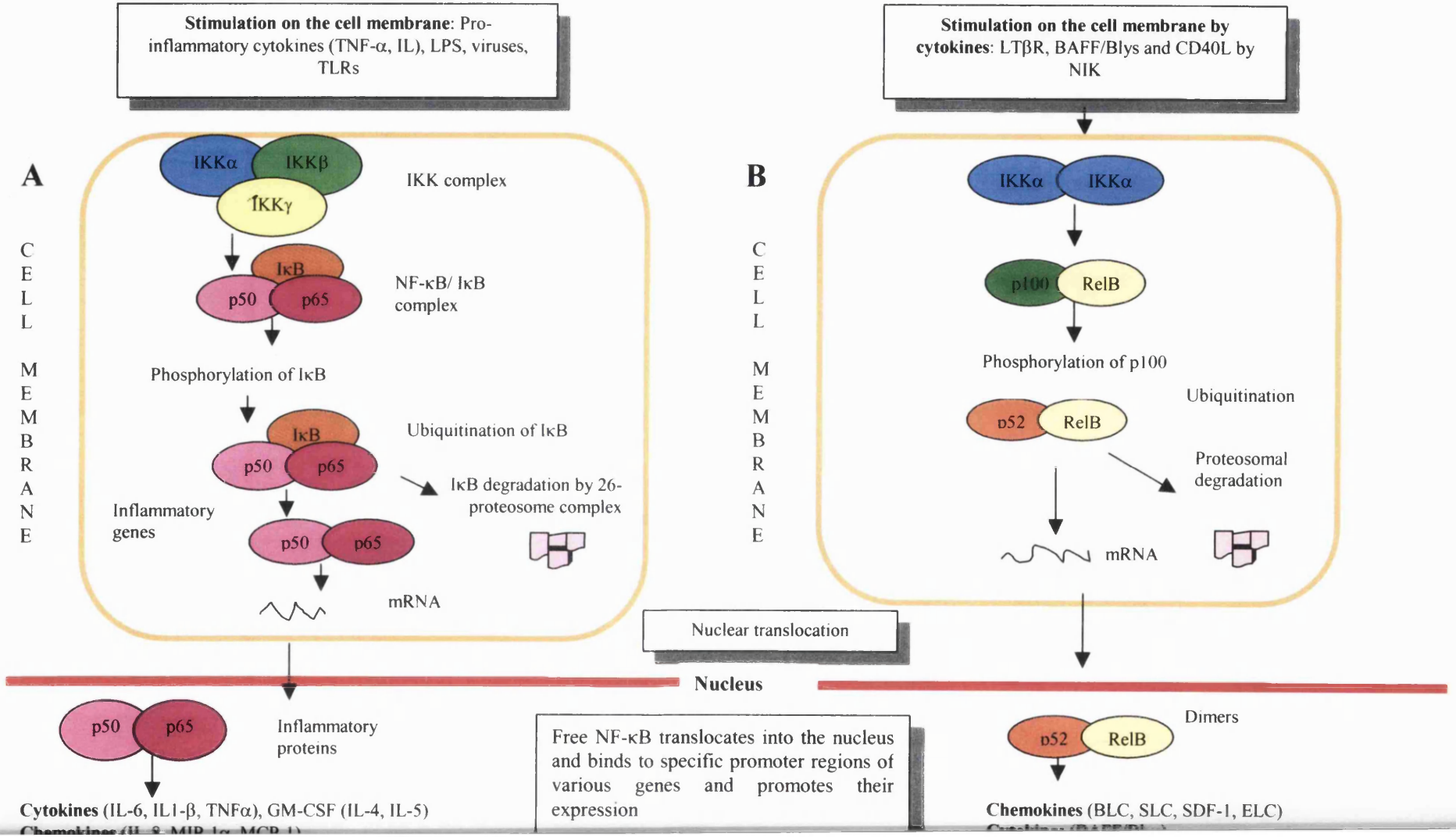
Table 2. Reticuloendotheliosis family/nuclear factor κB proteins present in mammals.

Protein	Function	Reference
RelA (p65)	Classical NF-κB binding pairs by forming dimers with p50. Responsible for most of NF-κB transcriptional activity. Regulated by the canonical pathway	Karin *et al.* (2002); Yamamoto and Gaynor (2004) (2)
RelB	Mostly associated with p100. Release of RelB-p52 dimers through activation of the non-canonical pathway	Karin *et al.* (2002); Yamamoto and Gaynor (2004)
c-Rel	The cellular homologue of the avian retroviral protein, v-Rel	Yamamoto and Gaynor (2004)
p50	Derived from processing of p105. Canonical NF-κB activation pathway	Karin *et al.* (2002); Yamamoto and Gaynor (2004)
p52	Derived from processing of p100. Homodimeric form has repressor function. Transcriptionally active heterodimers with RelA or c-Rel. Non-canonical pathway	Karin *et al.* (2002); Yamamoto and Gaynor (2004)

The activity of NF-κB is regulated by two different pathways based on the stimulants that trigger it and the Rel type proteins affected: 1) the canonical pathway and 2) the non-canonical pathway (Fig. 1). The canonical or classical pathway is fundamental for innate immunity, based on IKKβ-dependent IκB degradation (Senftleben *et al.*, 2001; Lin and Karin, 2003; Bonizzi and Karin, 2004). On the other hand, activation of the non-canonical pathway is involved in lymphoid organ development and adaptive immunity, it is dependent on IKKα homodimers that

targets NF-κB2/p100, which is phosphorylated at two C-terminal sites (Senftleben *et al.*, 2001; Lin and Karin, 2003; Bonizzi and Karin, 2004). Dimers are implicated in the canonical NF-κB activation pathway, which are composed of RelA, c-Rel, and p50, which are found in the cytoplasm inhibited by IκB proteins (Karin *et al.*, 2002; Greten and Karin, 2004; Yamamoto and Gaynor, 2004). This pathway is normally

Fig. 1. Canonical (A) and non-canonical (B) NF-κB pathways (Bremner and Heinrich, 2002; Bonizzi and Karin, 2004).



induced by microbial and viral infections and by pro-inflammatory cytokines (e.g. TNF-α and IL-1), which activate the IκB kinase complex (IKK) (Greten and Karin, 2004; Yamamoto and Gaynor, 2004). After stimulation, IKK is phosphorylated and degraded via the ubiquitination and proteosome-mediated pathway and NF-κB dimers translocate to the nucleus. Within the nucleus, NF-κB allows the transcription of different pro-inflammatory genes (Bremner and Heinrich, 2002; Rangaswami *et al.*, 2004). It has been shown that IKKβ is the dominant kinase involved in the phosphorylation of IκB proteins, playing a crucial role in the regulation of the canonical NF-κB pathway (Yamamoto and Gaynor, 2004).

The second pathway or non-canonical is regulated through the processing of NF-κB2/p100, vital for modulating the level and activity of the RelB-p52 heterodimer complex (Yamamoto and Gaynor, 2004). This pathway is triggered by members of the tumour necrosis factor (TNF) cytokine family, which activate the IKKα catalytic subunit and the nuclear factor-inducing kinase (NIK) (Karin *et al.*, 2002; Yamamoto and Gaynor, 2004). IKKα and NIK together, induce the phosphorylation-dependent proteolytic removal of the IκB-like-C-terminal domain of NF-κB2, allowing RelB-p52 dimers to translocate to the nucleus (Karin *et al.*, 2002).

Although it is probable that each NF-κB dimer has a distinct regulatory function, many target genes are common to several NF-κB factors, which can be classified into four functional categories: 1) immunoregulatory and inflammatory genes, 2) anti-apoptotic genes, 3) genes that positively regulate cell proliferation, and 4) genes that code negative regulators of NF-κB, all of them involved in tumorogenesis (Karin, *et al.*, 2002; Greten and Karin, 2004).

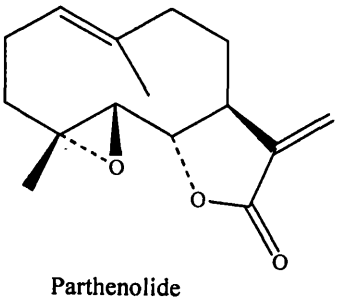
1.4. Natural products as inhibitors of the transcription factor NF-κB

Several medicinal plants have been shown to contain compounds that inhibit NF-κB activity, and therefore mediating the inflammatory process. Currently, there are two groups of interest as potential targets on NF-κB cascade: 1) the IKK complex, and 2) the ubiquitination and subsequent degradation of IκB (Bremner and Heinrich, 2002).

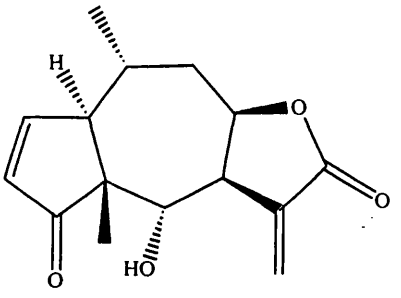
Probably the most well-known natural compounds inhibitors of NF-κB are the sesquiterpene lactones. Parthenolide is a sesquiterpene lactone isolated from *Tanacetum parthenium* (Asteraceae) (Hehner *et al.*, 1998) (Fig. 2). Parthenolide was shown to be an inhibitor of NF-κB in HeLa cells at 5-10 μM concentrations. It was subsequently shown that parthenolide targets the IKK complex, specifically IKKβ by modification of cysteine 179, and that it prevents IκBα and IκBβ from degrading (Hehner et al, 1998; Kwok *et al.*, 2001). From the same botanical family, helenalin (sesquiterpene lactone) was isolated from *Arnica montana* (flower heads) as an inhibitor of NF-κB (Lyss et al, 1997) (Fig. 2). The mechanism of action of helenalin has been studied and it was found that this sesquiterpene lactone acts directly against p65 by alkylation of the NF-κB subunit (Rüngeler at al, 1999).

Curcumin, a phenolic class compound, has been the subject of research interest due its anticancer properties (Fig. 2). Curcumin is found in turmeric, the powdered rhizome of *Curcuma longa* (Zingiberaceae), which has been traditionally used in the treatment of a variety of inflammatory conditions and chronic diseases, and also as a spice (Ammon and Wahl, 1991; Kawamori *et al.*, 1999). Treatment of prostate cancer cells with curcumin suppressed both constitutive (DU145) and inducible (LNCaP) NF-κB activation, and potentate TNF-induced apoptosis (Mukhopadhyay

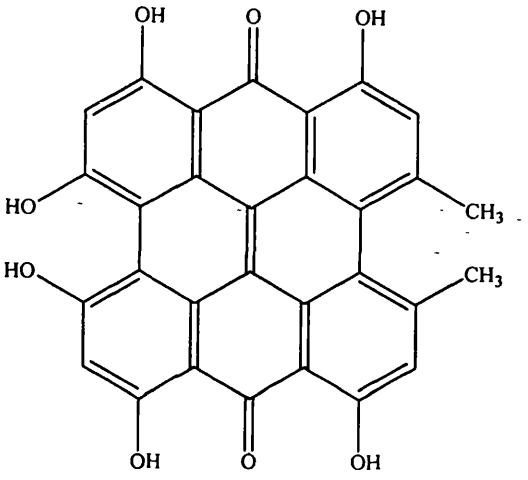
et al., 2001). Moreover, curcumin has shown to inhibit activation of NF-κB and AP-1 in bone marrow cells following both IL-1α and TNF-α stimulation (Xu *et al.*, 1998). Furthermore, *Hypericum perforatum* (Hypericaceae), a popular medicinal plant used for mild to moderate depressive disorders (Barnes *et al.*, 2001) has been the subject of evaluation as an inhibitor of NF-κB. Hypericin, one of the principal active constituents of the plant, has been shown to inhibit NF-κB in HeLa and TC10 cells (Fig. 2). In HeLa cells the inhibitory concentration of hypericin was at 1.98 μM using PMA as stimulant; in the case of TC10 cells, the stimulant used was TNF-α, and the inhibitory concentration was 3.96 μM (Bork *et al.*, 1999). Typical examples of natural products of plant origin that are known to inhibit the NF-κB cascade are given in Table 3 [see Bremner and Heinrich (2002) and Calixto *et al.* (2003) for a review].



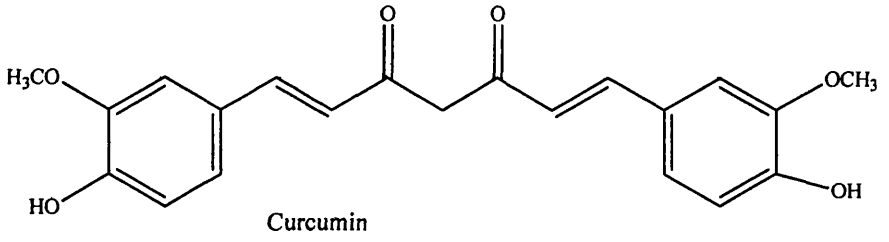
Parthenolide



Helenalin



Hypericin



Curcumin

Fig. 2. Examples of some natural products of natural origin inhibiting the NF-κB cascade.

Table 3. Natural products of plant origin targeting the NF-κB cascade.

Plant species (Family)	Compounds (structural class)	Inhibitory activity	Reference
Sideritis foetens (Lamiaceae)	Andalusol (diterpenoid)	Reduce NO synthesis via iNOS inhibition	Navarro *et al.* (1997, 2001)
Isodon japonicus (Lamiaceae)	Kamebakaurin (kaurane diterpenoid)	Prevents the activation of NF-κB by different stimuli in various cell types by directly targeting the DNA-binding activity of p50	Hwang *et al.* (2001)
Hypoestes rosea (Acanthaceae)	Hypoestoxide (diterpenoid)	Targets IKK; inhibits the production of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in LPS-stimulated peripheral blood mononuclear cells	Ojo-Amaize *et al.* (2001)
Acacia victoriae (Fabaceae)	Triterpenoid saponins	Induce apoptosis and decrease tumour-cell proliferation in Jurkat T cells and breast cancer cells	Mujoo *et al.* (2001)
Celastraceae and Hippocrateaceae	Pristimerin (triterpenoid)	Reduces the induction of iNOS in LPS stimulated RAW264.7 macrophages; cytotoxic in different cell lines	Itokawa *et al.* (1991); Dirsch *et al.* (1997)
Nerium oleander (Apocynaceae)	Oleandrin (cardenolide glycoside)	Inhibition of NF-κB in different cell lines (U937, HeLa, CaOV3, Jurkat); inhibition of IκBα	Manna *et al.* (2000a)
Vitis vinifera (Vitaceae), *Polygonum cuspidatum* (Polygonaceae)	Resveratrol (stilbene)	Inhibitor of TNF-α-, PMA, H_2O_2, okadaic acid, LPS, or ceramide induced NF-κB activation in a number of different cell types	Manna *et al.* (2000b)

1.5. Selection of plants

The research presented here was based on ethnobotanical data; the selection of the plants was done accordingly to their traditional medicinal use for inflammation and related diseases. The goals were to identify and evaluate the anti-inflammatory properties of the selected plants in order to contribute to their chemical and biological knowledge.

Three plants were investigated for the presence of inhibitors of the NF-κB cascade in this study: *Witheringia solanacea*, *Withania frutescens*, and *Valeriana officinalis*. The selection of these plants was based on their traditional medicinal use as anti-inflammatory remedy and/or their inhibitory activity against NF-κB reported in the database records of the AINP project. Both *W. solanacea* and *W. frutescens* are solanaceous plants with potential biological activity, the first one commonly used by different ethnic groups in Central America for the treatment of several anti-inflammatory related diseases, and the second being closely related to *W. somnifera*, which is an extensively studied Asian plant that has proved to have remarkable anti-inflammatory properties. *Valeriana officinalis* on the other hand is a popular European plant mainly employed as a tranquilliser and mild sedative, but that has also been reported as an anti-inflammatory remedy. A description of the main taxonomic diagnostic features, chemical composition, and biological activity of the aforementioned plants is mentioned below.

1.5.1. *Witheringia solanacea* L'Her (Solanaceae)

Economic and medicinal importance of the Solanaceae family

The Solanaceae is a cosmopolitan family with up to 3000 species in some 90 genera of herbs, shrubs and few trees. It is one of the most economically important botanic

families due to its variety of uses: ornamental, food, medicine, and narcotic (Schultes and Raffauf, 1990). The largest genus *Solanum*, contains about 1000 species, and the most important food plant from this family is *Solanum tuberosum*, potato, which lies fourth in world food production (Hawkes *et al.*, 1991). Regarding the importance of the Solanaceae family in modern and traditional medicine, several species can be named for instance *Atropa belladonna* (deadly nightshade), *Hyoscyamus niger* (henbane), *Mandragora officinarum* (mandrake), *Datura* species, *Withania somnifera*, *Nicotiana tabacum* and *N. rustica* (tobacco), among others (Roddick, 1991). The family Solanaceae has its greatest diversity in South and Central America, where *Datura* and *Solanum* spp are of particular importance in traditional medicine (Roddick, 1991). Some solanaceous plants used in traditional medicine are listed in Table 4.

Table 4. Selected solanaceous plants used in traditional medicine.

Continent	Species	Medicinal use	Reference
South/Central America			
	Brugmansia aurea	Treatment of chills, fever and swollen joints, muscle cramps, erysipelas, colds	Schultes and Raffauf (1991)
	Brunfelsia chiricaspi	Fever	Schultes and Raffauf (1991)
	B. grandiflora	Syphilis, rheumatism, diuretic	Schultes and Raffauf (1991);
	Capsicum frutescens	Rheumatism, arthritis, neuralgia, itching, lumbago, spasms, dyspepsia, colic, flatulence, chronic laryngitis, flatulence	Wyk and Wink (2004); Schultes and Raffauf (1991)
	Cestrum latifolium.	Skin diseases	Roddick (1991)
	Datura stramonium	Analgesic, asthma, respiratory disorders	Wyk and Wink (2004)
	Nicotiana tabacum	Enema, ringworm, toothache, rheumatism, ulcers, wounds, baldness, snake bites	Schultes and Raffauf (1991); Roddick (1991)
	Physalis angulata	Gonorrhoea, nephritis, indigestion, diuretic, narcotic, earache	Schultes and Raffauf (1991); Roddick (1991)
	Solanum spp	Eczema, wounds, ulcers, rheumatism, colds, cough, fever, tumours, skin disorders, worms, diuretic, leprosy, worms, burns	Roddick (1991)
Africa			
	Datura fastuosa= *D. metel*	Abortive, hepatopathy, cardiac palpitation, mental diseases, tooth pain, asthma, snakebite, heachache	Roddick (1991); Neuwinger (2000)
	D. stramonium	Ear problems, rheumatism and joint disorders, asthma, cough, headache, abortifacient, epilepsy, gout, wounds, ulcers, schizophrenia, sedative, toothache	Roddick (1991); Neuwinger (2000)
	Physalis angulata	Antihelmintic for children, constipation, pneumonia, skin diseases, hemostatic	Neuwinger (2000)
	Solanum aethiopicum	Sedative, tetanus after abortion, vomiting, colic, indigestion, elephantiasis, aphrodisiac, arterial hypertension, rheumatism	Neuwinger (2000)
	S. dulcamara	Tumours, syphilis, diuretic, expectorant, rheumatism	Roddick (1991)
	S. nigrum	Diseases of mouth, tongue, teeth, stomach-ache, diarrhoea, abortifacient, arterial hypertension, arthritis, asthma, diuretic	Roddick (1991); Neuwinger (2000)
	Withania somnifera	Headache, constipation, stomach-ache, skin rashes and ulcers, analgesic, liver and	Roddick (1991); Neuwinger

		spleen disorders, abortifacient, purgative, aphrodisiac, body pain, otitis, stomach pain, cough, rheumatism, fever, chills, colic, wounds, intestinal pain	(2000)
India			
	Capsicum frutescens	Headache, bronchitis, colds, sores, crocodile bite	Roddick (1991)
	Datura metel	Headache, asthma, leprosy, sores, epilepsy, convulsions, venereal disease, mumps	Roddick (1991)
	Solanum incanum	Abortive, contraception	Roddick (1991)
	Solanum indicum	Fever, flu, respiratory problems, asthma, whooping cough, chest pains	Roddick (1991)
	S. nigrum	Gonorrhoea, dysuria, stomach-ache, hepatitis, inflammation of the spleen and uterus, burns, wounds, skin infections	Roddick (1991); Williamson (2002)
	S. surattense= *S. xanthocarpum*	Earache, cough, bronchitis, asthma, fever, toothache, rheumatism, syphilis, tuberculosis, mumps, chest and muscular pains, diuretic and antiemetic	Roddick (1991); Williamson (2002)
	Withania somnifera	Abortifacient, anodyne, asthma, bactericide, contraceptive, diuretic, sedative, tonic, anti-inflammatory, cold, dropsy, anaemia, fever, hypertension, lumbago, sedative, depurative, dyspepsia	Williamson (2002)
China			
	Atropa belladonna	Convulsions, analgesic	Roddick (1991)
	Datura metel	Cough, asthma, analgesic	Roddick (1991)
	Mandragora caulescens	General pain, stomach problems, tonic	Roddick (1991)
	Physalis alkekengi	Abortive	Roddick (1991)
	Scopolia tangutica	Swellings and pain	Roddick (1991)
	Solanum nigrum	Febrifuge and internal fever	Roddick (1991)
	S. torvum	Antitussive, general pain	Roddick (1991)

Botanic description

The genus *Witheringia* L'Her belongs to the Solanaceae, subfamily Solanoideae, tribe Solaneae (D'Arcy, 1991). It includes 20 species of neotropical herbs, shrubs, and small trees. The genus is related to the genera *Physalis* L., *Margaranthus* Schlecht, *Leucophysalis* Rydb., and *Chamaesaracha* (A. Gray) Benth. The majority of the species are distributed in Mexico, Costa Rica, and Panama (Bohs, 2000).

Witheringia solanacea L'Her (Fig. 3) is widely distributed from southern Mexico through Central America and the Antilles to South America (Bohs, 2000). It is a small shrub; the leaves are entire, elliptic, unequal in size, and paired at the nodes; flowers are often tetramerous and clustered in axillary fascicles due to the lack of obvious peduncles. The corollas are yellowish stellate. The fruit is red, juicy, many seeded, glabrous to very sparsely pubescent (Bohs, 2000).

Fig. 3. Flower and fruits of *Witheringia solanacea* (Solanaceae) (www.cs.umb.edu/~whaber/Monte/Plant/Sola/Wit-sol.html).

Chemical and therapeutic properties of Witheringia

The most important compounds present in the Solanaceae are tropane alkaloids, steroidal alkaloids, polyphenols including benzoic acids, coumarins and flavonoids, steroidal saponins and sapogenins, steroidal lactones, pentacyclic triterpenes, resins, waxes, and essential oils. The alkaloids of the family are of five general structural types: 1) tropane, 2) steroidal, 3) the tobacco alkaloids, 4) terpenoids, and 5) piperidine alkaloids. The tropane alkaloids are the most important therapeutic agents from the Solanaceae including l-hyoscyamine, l-hyoscine (scopolamine), and atropine. The main commercial sources of these type of compounds are *Atropa belladonna, Brugmansia sanguinea, Datura inoxia, D. metel, D. stramonium, Duboisia leichhardtii, D. myoporoides, Hyoscyamus albus, H. muticus, H. niger, H. reticulatus, Scopolia carniolica, and S. japonica* (Roddick, 1991). Moreover, the Solanaceae family produces steroidal alkaloids that can be used as precursors for the production of important steroids in drug therapy such as corticosteroids (used in the treatment of rheumatoid arthritis, inflammatory conditions and skin disorders), sex steroids and anabolic steroids (Roddick, 1991).

Little is known about the chemistry of the genus *Witheringia*, there is only one report on its chemical constituents. Antoun and colleagues (1981) isolated nine physalin type compounds from the roots of *W. coccoloboides* Dammer. Physalins are withanolides modified in the carbocyclic skeleton and in the side chain of the molecule (Glotter, 1991). These compounds are highly oxygenated C/D seco-steroids that commonly possess a 16,24-cyclo-13, 14-seco-ergostane skeleton and are classified as types A and B according to the absence or presence of a C (14)-O-C (27) acetalic linkage, respectively (Fig. 4) (Januário *et al.*, 2002; Makino *et al.*,

2002). The precursor of physalins was suggested to be an unknown withanolide-type compound whose structure is 17α-hydroxy-15-oxowithaphysalin A. The biosynthetic process could be initiated by the oxidative cleavage of the 13, 14 bond leading to 13α,17α-dihydroxy-14-one, in a 9-membered ring system which is stabilized by 14α,17α-hemiketal formation. Apparently, the attack of the oxidizing agent was directed by the 17α-OH group, the result being the α-orientation of the 13-OH in the final product (Glotter, 1991).

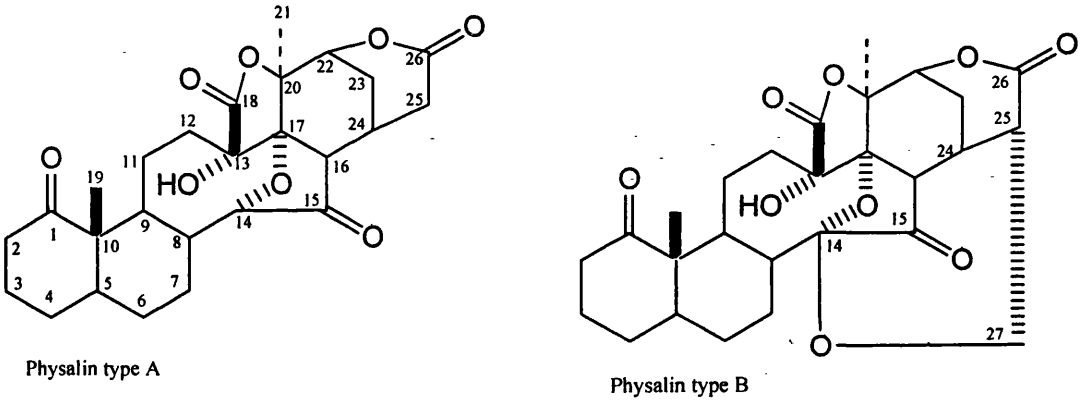


Fig. 4. Skeletons of physalin type A and B.

Biological and pharmacological activity of Witheringia

Antoun *et al.*, (1981) reported that physalin B and 25,26-epidihydrophysalin C isolated from *W. coccoloboides* showed cytotoxic activity in 9KB and 9PS tumour cells (*in vitro*), and physalin B demonstrated moderate activity against 3PS mouse leukaemia (*in vivo*). On the other hand, in a study of nineteen medicinal plants of Panama using radioligand-receptor-binding assays the methanol-dichloromethane extract of the roots and branches of *W. solanacea* inhibited the [^{3}H]-AT II binding (angiotesin II AT_1 receptor) (Caballero-George *et al.*, 2001).

Ethnomedicinal uses of Witheringia solanacea

This species is used in traditional medicine in countries of Central America, where the plant has common names in native languages suggesting the importance of the species in the area. In Panama, *W. solanacea* is known as Tinanguak'Gid and Diguima goi, and it is used as an anti-hypertensive remedy and for general pain (Caballero-George *et al.*, 2001). In Provincia de Pastanza, it is called Simbiu panga in Ketchwa language, and the leaves are used for infected wounds and boils, and for fungal infections of the head (Schultes and Raffauf, 1991). In Mexico the tender leaves of the plant are eaten, and the species is used in the treatment of fungal infections and acne. In Mexico, *W. solanacea* is known in Spanish as hierba cimarrona and in Náhuatl as cuauhta tomatquilit (Basurto-Peña, 1998; Fierro-Álvarez *et al.*, 2000).

1.5.2. *Withania frutescens* Pauquy (Solanaceae)

Botanic description

The genus *Withania* (Pauquy) belongs to the subfamily Solanoideae, tribe Solanaeae (D'Arcy, 1991). Ten species have been described for this genus, most of which are distributed along the Canary Islands, northwest Africa, Spain, Southern Arabia, Pakistan and Nepal (Hepper, 1991). Perennial herbs of semi-woody unarmed shrubs. Leaves alternate or in unequal pairs, margin entire. Flowers are several and occur together in axils on short pedicels, hermaphrodite or unisexual. Calyx campanulate, 5-6 toothed, much enlarged and inflated or surrounding the fruit. Corolla narrowly campanulate, 3-6 lobed, valvate. Stamens inserted at base of corolla tube, ± exserted. Disc annular, crenulated or absent. Ovary 2-loculate, with numerous ovules. Fruit a globose berry. Seeds numerous, compressed, reticulate; embryo near the margin, and incurved or spiral (Hepper, 1991). *Withania frutescens* Pauquy (Fig. 5) is distinguished within the *Withania* genus for having a corolla three to five times longer than the calyx tube, narrow corolla lobes, and pendulous flowers. Its lamina is broadly ovate, cordate at base and equal-sided; calyx lobes triangular. It is distributed in Spain along the coast of Levante and Andalusia, in Algeria, Morocco, and on the Canary Islands (González *et al.*, 1982; Hepper, 1991).

Fig. 5. *Withania frutescens* (Solanaceae) (www.uib.es/depart/dba/botanical/herbaris/geners/Withania/frutescens/).

Ethnomedicinal uses of the genus Withania

Withania frutescens is commonly known in the south of Spain as Paternostra, but no traditional uses have been reported so far (Montilla *et al.*, 1990). Only three species of the genus *Withania* are known to have ethnomedicinal use. The most well known of these species is *W. somnifera*, which is used in the treatment of ulcers, for arthritis, rheumatism, asthma, cough, dropsy, consumption, and senile debility, and treatment of tumours and inflammation (Mishra *et al.*, 2000). In the Canary Island, *W. aristata* is used as spasmolytic and for its cicatrising properties, and *W. coagulans* is largely used in Pakistan, North India and Afghanistan as emetic, and for dyspepsia. The fruits of *W. coagulance* are also commonly used in India as a remedy for skin infections, inflammatory diseases, asthma, liver problems, and as sedative (González *et al.*, 1982; Budhiraja *et al.*, 1977; Rahman *et al.*, 1993; Jayaprakasam *et al.*, 2003).

Principal chemical constituents of the genus Withania

Withanolides are C28 steroidal lactones with an ergostane skeleton, with atoms C-22 and C-26 bridged by a δ-lactone functionality and an oxidized C-1 position (Fig. 6). They occur in the Solanaceae family especially in the genus *Withania*, *Acnistus* (*Dunalia*), *Physalis*, *Jaborosa*, *Datura*, and *Lycium* (Kirson and Glotter, 1981; Glotter, 1991; Ganzera *et al.*, 2003). A list of withanolides isolated from *Withania spp* is shown in Table 5.

Table 5. Withanolides compounds isolated from *Withania* species.

Species	Withanolides	Reference
W. frutescens, *W. aristata*	Withaferin A; 2,3-dihydrowithaferin A, 14-en-withaferin A; 14-en-withaferin A; 14-en-27-desoxywithaferin A; 1,15-dihydroxy-22-with a-5, 22-dienolide; 5-en-17-hydroxywithaferin A; 5-hydroxy-6-chlorowithaferin A (Fig. 6)	González *et al.* (1971, 1982)
W. coagulans	Coagulin; 14, 15β-epoxywithanolide I; 17β-hydroxywithanolide K; withacoagulin (Fig. 6)	Rahman *et al.* (1993); Choudhary *et al.* (1995); Rahman *et al.* (2003).
W. somnifera	Withaferin A; 4-(1-hydroxy-2, 2-dimethylcyclopropanone)-2, 3-dihydrowithaferin A; 2,3-dihydrowithaferin A; 24,25-dihydro-27-desoxywithaferin A; withanolide D; sitoindoside IX, X; withanosides (Fig. 6)	Das *et al.* (1985); Matsuda *et al.* (2001); Furmanowa *et al.* (2001)

Biological and pharmacological activity of Withania

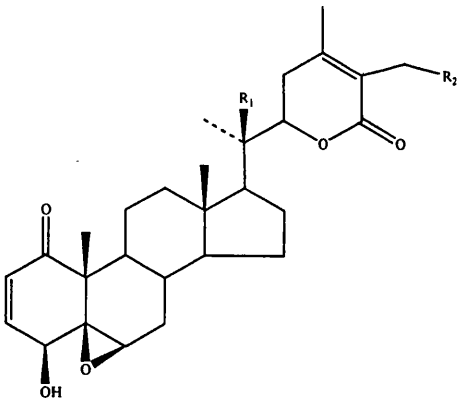
Several *in vivo* experiments have revealed that *W. somnifera* has anti-inflammatory (e.g. Anbalagan and Sadique, 1981, 1984; Sudhir *et al.*, 1986; Begum and Sadique, 1987, 1988; Kulkarni *et al.*, 1991; Al-Hindawi *et al.*, 1992), antioxidant (e.g. Bhattacharya *et al.*, 1997; Panda and Kar, 1997; Gupta *et al.*, 2003), and anti-tumour activities (e.g. Singh *et al.*, 1986; Devi *et al.*, 1992). A recent study also showed that the methanolic extract of *W. somnifera* increased inducible nitric oxide synthase-derived (iNOS) nitric oxide (NO) production in J774 macrophages, possibly through NF-κB transactivation (Iuvone *et al.*, 2003). In addition, withaferin A isolated from *W. somnifera* proved to inhibit NF-κB activation in human umbilical vein endothelial cell (HUVECs) by interference with the ubiquitin-mediated proteosome pathway.

The ethanol extract of the leaves of *W. frutescens* had an *in vivo* depressant action on the CNS in rats indicated by decreasing exploratory activity and the prolongation of barbiturate-induced sleep (Montilla *et al.*, 1990). The authors

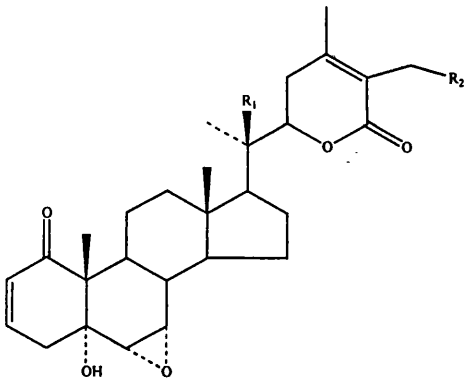
attributed the depressive action on CNS and anti-inflammatory action to withanolides and to alkaloids, respectively. It has been demonstrated that the major components responsible for the pharmacological activities of the genus *Withania* and other solanaceous plants are withanolides, which possess different biological activity such as antimicrobial, antitumor, and immunosuppressive and immunoactivating properties (see Budhiraja and Sudhir, 1987 for a review). Jayaprakasam *et al.* (2003) evaluated three groups of withanolides (withaferin A, physagulin D, and viscosalactone B) from *W. somnifera* as growth inhibitors of four different human tumour cell lines. These authors found that these withanolides gave similar trend in their antiproliferative activity against lung, breast, colon, and CNS human tumour cell lines. They also mentioned that a double bond in withaferin A and α,β-unsaturated ketone functionality and the C-27 hydroxyl contributed significantly to its antiproliferative activity compared to other withaferin A type withanolides.

Withaferin A has shown antibacterial properties against acid-fast bacilli and Gram-positive microorganisms (Rahman *et al.*, 1993), and 3β-hydroxy-2,3-dihydrowithanolide F demonstrated hepatoprotective activity against CCl_4-induced hepatotoxicity in adult albino rats (Budhiraja *et al.*, 1986). In addition, an evaluation of seven withanolides isolated from two *Withania* species revealed that the lactonic ring (E) is necessary for cytostatic activity (González *et al.*, 1982). In this study, the most potent compounds for cytostatic activity were those with a 2-3 double bond in the first ring (A) or a carbonyl and hydroxyl in position 1 and 4, respectively, whereas the presence of an epoxide ring in the 5-6 position or a halogen atom in the second ring (B) was less important.

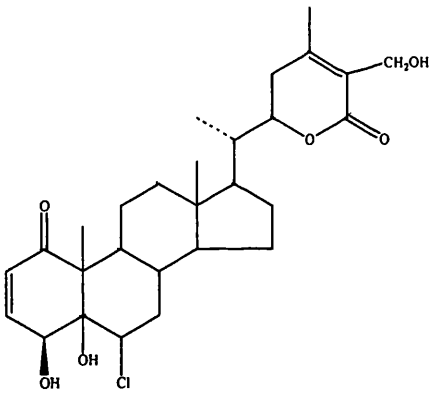
Fig. 6. Examples of withanolides present in *Withania* species.



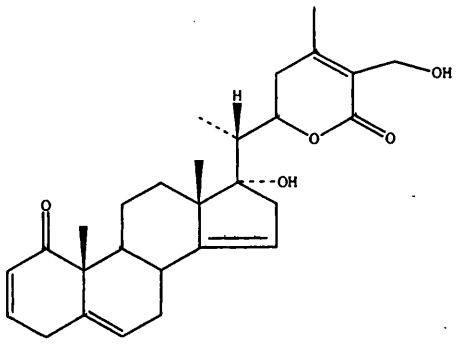
	R1	R2
Withaferin A	H	OH
27-deoxywithaferin A	H	H
2,3-dihydrodeoxywithaferin A	H	H
2,3-dihydrowithaferin A	H	OH



	R1	R2
Withanolide B	H	H
Withanolide A	OH	H
Withanone 17α-OH	H	H
Withanolide T (14α-OH, 17α-OH)	OH	H

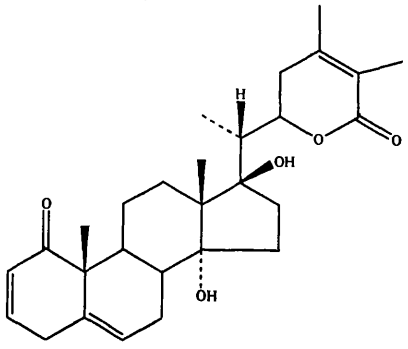


5-hydroxy-6-chlorowithaferin A

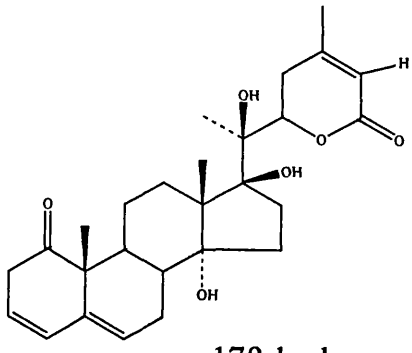


withanolide N

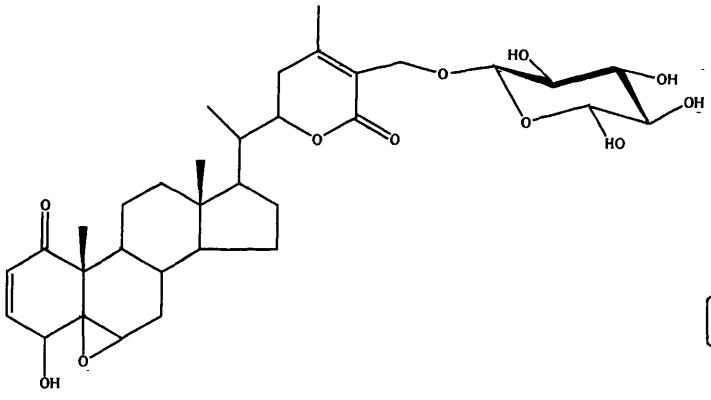
Fig. 6 (continued). Examples of withanolides present in *Withania* species.



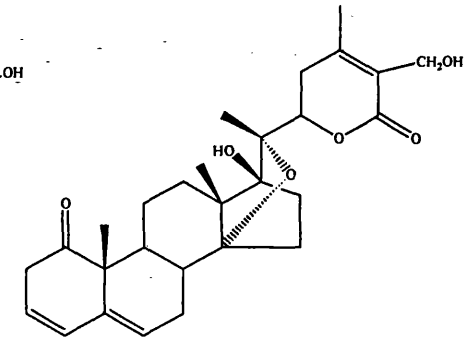
withanolide P



17β-hydroxywithanolide K



Sitoindoside IX



Coagulin

1.5.3 *Valeriana officinalis* L (Valerianaceae)

Botanic description

Valeriana officinalis L. (Valerianaceae) (Fig. 7), commonly named valerian, is a native plant from Europe and temperate zones of Asia, which usually inhabits damp and swampy areas (Schulz *et al.*, 2001). This herb is about 30-150 cm high; with odd-pinnate leaves and indented-dentate leaflets, the lower ones being petiolate and the upper ones sessile and clasping with a white sheath; its rhizome is strongly smelly when dried, with light greyish brown colour, short and cylindrical, with finger-length and round roots; its roots have a light to medium greyish brown colour, are 1-3 mm thick and several centimetres long, and partly covered with coarse longitudinal wrinkles (Gruenwald *et al.*, 1998; Bisset and Wichtl, 2001). *V. officinalis* blooms from late June to August; its flowers are pink to white and grow in terminal cymes; the calyx consists of 10 revolute tips; the corolla is funnel-shaped with a 5-sectioned margin; the tube has a bump at the base with 3 stamens; the ovary is inferior and has 3 chambers; the fruit is ovate-oblong, yellow, indehiscent and with 10-rayed tuft of white hair (Gruenwald *et al.*, 1998; Bisset and Wichtl, 2001; Schulz *et al.*, 2001).

Fig. 7. Flower of *Valeriana officinalis* (Valerianaceae).
(www.ces.ncsu.edu/depts/hort/consumer/factsheets/herbs/Valeriana_officinalis.html;

www.popgeno146uns50.unimaas.nl/~jindsey/commanster/Plants/Flowers/SpFlowers/Valeriana.officinalis.html)

Ethnobotanical uses

The genus *Valeriana* is widely spread all over the word, however only few of them are used in traditional medicine. Interestingly, in Mexico three species are reported with medicinal properties: *Valeriana edulis* is employed for blood pressure, *V. edulis ssp. procera* has been used for bleeding wounds and nervousness (Aguilar *et al.*, 1997; Heinrich and Jacobo, 2003), *V. prinophylla* in the treatment of nervous, and *V. sorbifolia* var. *mexicana* for rheumatism (Aguilar *et al.*, 1997).

Valeriana officinalis is probably one of the most popular medicinal herbs used during human history. The name *Valeriana* means "well being", derived from the Latin word "valere", and *officinalis* indicates the pharmaceutical use of the plant (Morazzoni and Bombardelli, 1995). *V. officinalis* has been used in Europe for different purposes since Greek and Roman times, described by Dioscorides in the first century A.D. as a mild sedative (Morazzoni and Bombardelli, 1995). In Italy in the year 70 B.C. it was used for pain relief in case of stitches in the side, and as stimulant of menstruation (Mayer, 2003), and in 16th Century as a treatment for epilepsy (Spinella, 2001). The most constant mentioned uses of valerian in Europe were from the year 70 B.C. to the middle 18th Century as a remedy for good digestion, cough, diuretic, strangury, kidney problems, gout, dysuria, dropsy, ulcers, flatulence, and animal bites (Mayer, 2003). Since approximately 1539, the uses of this plant were included in nervous conditions such as hysteria, neuropathy, cramps, hypochondria, headache, epilepsy, heart disease, insomnia, and anxiety (Mayer, 2003). Since the 18th Century valerian has been used as a sedative and tranquilliser, and it was very popular during the Second World War as sedative among the

"hysterical and neurotic soldiers" (Dweck, 1997). Table 6 lists some other traditional medicinal uses for *V. officinalis*.

Table 6. Traditional medicinal uses of *V. officinalis*.

Continent	Traditional use	Reference
Europe	To relief pain/sorrow of the lungs and breast	Mayer (2003)
Europe	Urinary tract disorders	Mayer (2003)
Europe	Vaginal yeast infections	Mayer (2003)
Europe	Digestion	Mayer (2003)
Europe	Throat inflammation	Mayer (2003)
Europe	Gum sores	Mayer (2003)
Europe and Central America	Fever, cold, cough	Mayer (2003); Cáceres (1990)
Europe and Central America	Eye diseases, to improve vision	Mayer (2003); Cáceres (1990)
Europe and Central America	Wounds	Mayer (2003)
Central America	Rheumatism	Cáceres (1990)
Central America	Molar tooth ache	Aguilar *et al.* (1994)
Central America	Cardiac problems	Cáceres (1990)

Today, *V. officinalis* is used throughout the world as an antidote for many nervous conditions and during stressful periods in life such as sleeping disorders, mental strain, lack of concentration, excitability, stress, headache, neurasthenia, epilepsy, hysteria, nervous cardiopathy, neuralgia, fainting, nervous stomach cramps, colic, uterine spasticity, and anxiety (Gruenwald *et al.*, 1998; Bisset and Wichtl, 2001). Furthermore, this herb is stated to possess sedative, hypnotic, antispasmodic, carminative and hypotensive properties (Newall *et al.*, 1996; Trease and Evans, 1996). Additionally, valerian is the phytopharmaceutical agent most commonly used by people suffering insomnia (Herrera-Arellano *et al.*, 2001).

Principal chemical constituents in Valeriana

The main chemical compounds of *V. officinalis* are listed in Table 7. These are basically divided into three main groups: alkaloids, mono and sesquiterpenoids, and

valepotriates (Houghton, 1997). Alkaloids (Fig. 8a) are present in small amounts; two examples of them are valerianine and actinidine (Houghton, 1997). The mono and sesquiterpenes (Fig. 8c) on the other hand are the major constituents of the volatile oil of the plant (Houghton, 1997; 1999). The most common monoterpenes are borneol and its isovaleric and acetyl esters (Houghton, 1999). Valerian sesquiterpenes have a two-ring system unique to the family: valerenal type and kessane type (Houghton, 1997). In *V. officinalis*, the valerenal type is represented by valerenic acid, valerenol, and hydroxyvalerenic acid, among others (Bos *et al.*, 1986), whereas the kessane type is represented by the kessyl glycol (Gruenwald *et al.*, 1998; Bisset and Wichtl, 2001). Finally, the valepotriates (valerian epoxy triesters) (Fig. 8b) are distinguished by a monoterpene skeleton very similar to the iridoid skeleton and are responsible for several biological effects (Houghton, 1997, 1999). The most representative valepotriates present in *V. officinalis* are valtrate, acevaltrate, isovaltrate, and dihydrovaltrate (Houghton, 1999; Bisset and Wichtl, 2001).

The usual commercial product of *V. officinalis* consists of yellowish-brown rhizomes, stolons and roots, and contains two sesquiterpenes of the volatile oil: valerenic acid and acetoxy valerenic acid (0.3-0.7 %) (Bisset and Wichtl, 2001; Schulz *et al.*, 2001).

Table 7. Principal secondary metabolites of *V. officinalis.*

Class of Compound	Name	Reference
Alkaloids	Actinidine, skyanthine, valerianine, valerine, α-methylpyrrylketone	Morazzoni and Bombardelli (1995); Houghton (1999)
Volatile oils	Monoterpenes (borneol and its acetyl and isovaleryl esters, camphene, eugenol); sesquiterpenes (valerenic acid with acetoxy and hydroxy derivates, kessyl glycol, □aleranone, valerianol, valerenol, valerenal)	Morazzoni and Bombardelli (1995); Houghton (1999); Bisset and Wichtl (2001)
Valepotriates	valtrates (valtrate, acevaltrate, valechlorine, valeroxyhydrin); dihydrovaltrates (didrovaltrate, deoxydidrovaltrate); isovaltrates (isovaltrate, 7-epideacetylisovaltrate)	Morazzoni and Bombardelli (1995); Houghton (1999); Bisset and Wichtl (2001)

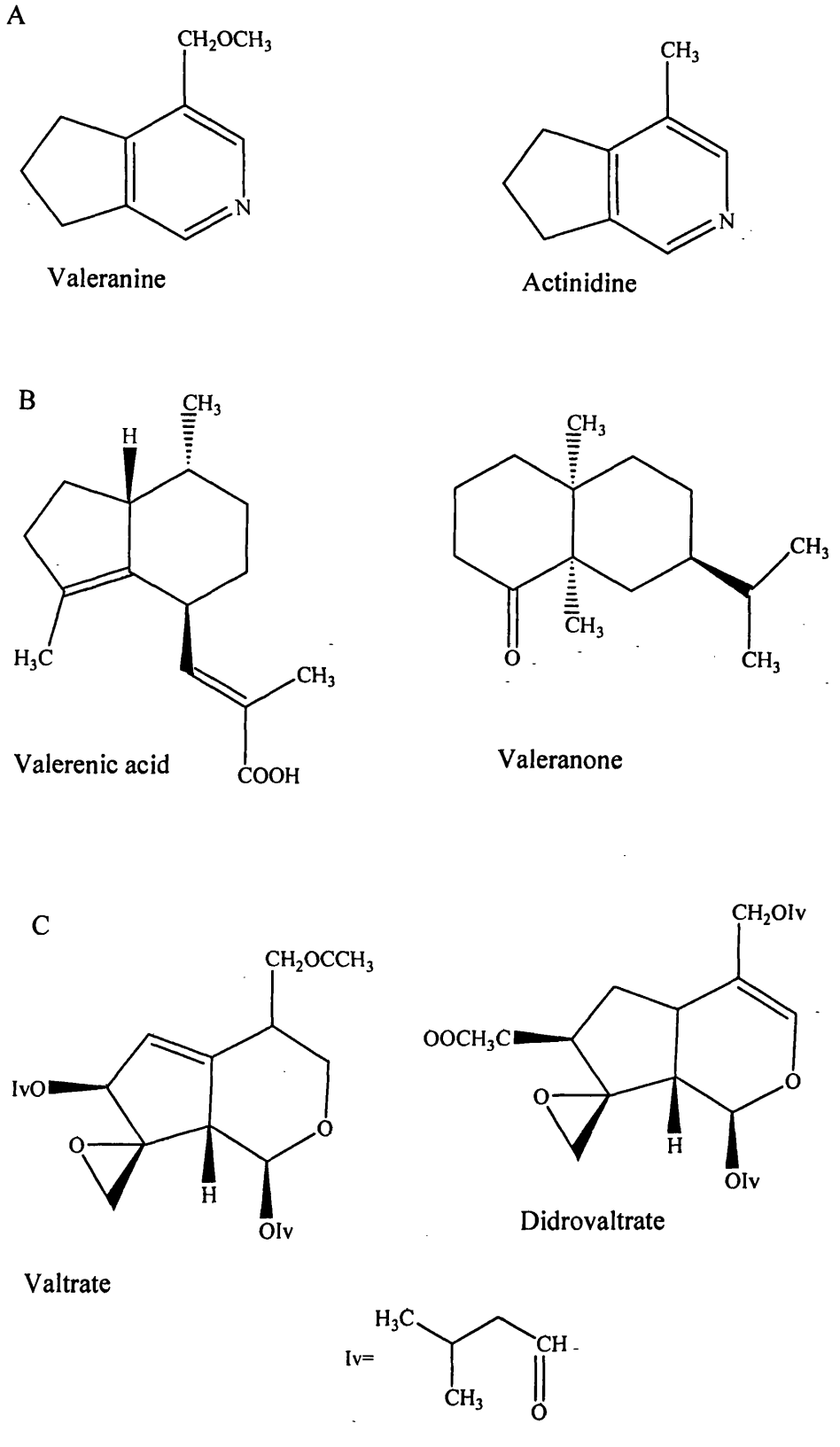


Fig. 8. Examples of the three principal classes of chemical constituents of *V. officinalis* A) Alkaloids, B) sesquiterpenes, C) valepotriates.

Biological and pharmacological activity

The precise pharmacological mode of action of valerian is still unclear, and several pharmacological assays and phytochemical analysis have been performed in order to link biological action with a compound. As result of this, many chemical compounds have been isolated from this plant, suggesting that the two major compounds linked with the effectiveness of the drug are the monoterpene bornyl acetate and the sesquiterpene valerenic acid (Houghton, 1999). Moreover, Bisset and Wichtl (2001) summarised information indicating that valerenic acid and the valepotriates were the principal bioactive agents for *V. officinalis*, which could be used as spasmolytic, muscle-relaxant, central nervous system sedative, tranquilliser, and decrease anxiety. There is some concern regarding the cytotoxicity of valepotriates due their epoxide group and its alkylating potential (Bounthanh *et al.*, 1981; Braun *et al.*, 1982). However, it has been shown that the C5-C6 double bond in some valepotriates is more important in the cytotoxic activity than the epoxde ring and these compounds have failed to show carcinogenic effect *in vivo* in rats (Braun *et al.*, 1982; Houghton, 1999). Table 8 lists compounds isolated from valerian and their biological activity.

Table 8. Biological activity of selected compounds present in *V. officinalis*.

Compound	Action/function	Reference
Valerenic acid	Spasmolytic and muscle relaxant	Birnbaum *et al.* (1978); Bisset and Wichtl (2001)
Valerenic acid and related sesquiterpenes	Inhibit the degradation of the CNS transmitter γ-aminobutyric acid	Bisset and Wichtl (2001)
Valeranone	Mild sedative	Birnbaum *et al.* (1978)
Valerenic acid and kessane types	CNS sedative action (sedative and spasmolytic activity)	Hendriks et al. (1981); Houghton (1999)
Dienes (valtrate or acevaltrate)	Thymoleptics	Bisset and Wichtl (2001)
Mono-unsaturated valepotriates (didrovaltrate)	Tranquillisers	Bisset and Wichtl (2001)
Valepotriates	Decrease anxiety	Houghton (1999)

Side effects and toxicity

Valerian is a safe drug with no toxicity known (Bisset and Wichtl, 2001; Barnes *et al.*, 2002). Gruenwald *et al.* (1998), however, reported that the use of this plant occasionally provokes gastrointestinal complaints and allergies, and in a long term conditions its administration may cause headache, restless stares, sleeplessness, mydriasis and disorders of cardiac function. Due to lack of data the use of this drug is also not recommended during pregnancy, lactation, or in children younger than 3 years old (Schulz *et al.*, 2001).

1.6. Aims

Based on the screening data obtained in the AINP project, *W. solanacea*, *W. frutescens* and *V. officinalis* could yield novel inhibitors acting on the NF-κB cascade. The aim of the present study was therefore to investigate through bioassay-guided fractionation the inhibition of NF-κB activation on HeLa cells of fractions and compounds isolated from the above plants. Moreover, MTT cytotoxicity and HeLa TET-ON-luc assays and determination of nuclear DNA loss (apoptosis) and cell cycle analysis were carried out for the compounds isolated from *W. solanacea*.

CHAPTER 2. MATERIALS AND METHODS

2.1. Plant material

Leaves of *Witheringia solanacea* were collected at Cerro Trinidad, Capira, Panama, and identified by Prof. Mahabir P. Gupta and colleagues at Centro de Investigaciones Farmacognósticas de la Flora Panameña (CIFLORPAN), Panama. Aerial parts of *Withania frutescens* were collected in Rumbla de Bolnuevo, Murcia, Spain, and identified by Prof. Rivera and Prof. Obón de Castro, Universidad de Murcia, Spain. Underground parts of *Valeriana officinalis* were commercially supplied by Potter's Herbal Medicines© (Leyland Mill Lane Wigan, Lancs WN1 2SB, UK). Voucher specimens of *W. solanacea* and *Withania frutescens* are deposited at the herbarium of Centre for Pharmacognosy and Phytotherapy, School of Pharmacy, University of London.

2.2. Extraction of plant material

Two different methods for obtaining crude extracts were employed: 1) Soxhlet extraction and 2) cold maceration. The first technique employs a Soxhlet apparatus with increasing polarity of solvents, where the powdered plant material is placed in a filter paper thimble and the solvent is continuously refluxed. One if it advantages is that it tends to yield larger quantities of crude extract (Heinrich *et al.*, 2004). Cold maceration consists of agitating the ground plant material using a stirrer at room temperature and sequentially increasing the polarity of solvents. The advantage of this extraction technique is that heating is not needed so it helps to avoid degradation of compounds (Heinrich *et al.*, 2004). Increasing polarity of solvents in both

techniques enables distribution of compounds according to their polarity and provides a crude fractionation step before more detailed chromatographic analysis.

2.2.1. Soxhlet extraction

Crude extracts from a total 500 g and 320 g of *W. solanacea* and *W. frutescens*, respectively, were obtained by increasing polarities of the following solvents in a Soxhlet apparatus: n-hexane, chloroform, ethyl acetate, acetone and methanol. These crude extracts were dried down with a Büchi rotapavor R-200 (which was employed throughout this study) and then stored at –20°C until they were used for fractionation.

2.2.2. Cold maceration

The crude extract from 1950 g of *Valeriana officinalis* was obtained following the methodology designed by the research team working on the "New Anti-inflammatory Natural Products from Medicinal Plants using inducible transcription factors and their signalling pathways as molecular targets" (AINP) project (Bremner *et al.*, 2004). Two different crude extracts were obtained at room temperature for this plant. One of these extracts used n-hexane, whereas the other one used ethyl acetate as solvent. The two extracts were filtered in vacuo, dried with a rotavapor, and stored at –20° C.

2.3. Chromatographic techniques

A total of six different preparative and analytical chromatographic techniques were employed for the fractionation of crude extracts and isolation of compounds: **1)** vacuum liquid chromatography (VLC); **2)** solid phase extraction (SPE); **3)** silica gel column chromatography; **4)** Sephadex LH-20 column chromatography; **5)** analytical

and preparative thin layer chromatography (TLC); and **6)** analytical and preparative high performance liquid chromatography (HPLC). Details of each technique are highlighted below.

2.3.1. VLC

Column and adsorbent: Kieselgel 60 PF 254 + 366 silica gel for preparative chromatography MERCK; 60 mm x 120 mm glass column with frit and a Quick fit (24/29) opening at the bottom.

Silica gel was used as the stationary phase for the separation of natural products. Silica gel is a 3-D polymer of tetrahedral units of silicon oxide ($SiO_2 \cdot H_2O$). The silanol groups (SiOH) are the active centers of the surface of silica and can form hydrogen bonds with compounds, therefore the stronger the ability for a compound to form hydrogen bonding, the stronger it will be retained by silica gel. Polar compounds tend to attach to the silica due to interactions with the SiOH groups, usually requiring very polar solvents to recover them. In the case of non-polar compounds, non-polar solvents will be required as eluent (Salituro and Dufresne, 1998).

VLC was first used in the fractionation of non-polar and medium polarity crude extracts. The column was two-thirds filled with dry silica using a beaker and spatula and leaving the vacuum on. This last procedure makes the silica more compact, leaving a firm and flat column for good separation. The top of the column was covered with filter paper and then washed with a non-polar solvent to remove silica fines and to equilibrate the column before the extract was loaded. An alternative method was used by adding equal proportions of the plant extract to silica and a suitable solvent according to the nature of the crude extract. Subsequently this

mixture was evaporated using a rotavapor until a completely dry powder was obtained. This final product was then applied onto the top of the column and then eluted with solvents of increasing polarity (gradient system). Fractions of 100 ml were collected in round bottom flask fitted at the mouth of the column, monitored by TLC, and dried under vacuum on a rotavapor and stored at –20° C.

2.3.2. SPE

Columns and adsorbents: Strata SI-1 Silica (55 μm, 70A) 10 g /60 ml Giga tubes normal column cartridges; Strata C18-E (55 μm, 70A) 10 g/ 50 ml Giga tubes Reverse phase column cartridges.

SPE was employed for fractionation of extracts of compound mixtures. It employs the principle of liquid-solid extraction and utilises adsorbents that are available commercially in the form or pre-packed cartridges, which are disposable (Gailliot, 1998). Columns were mounted on the manifold tank and vacuum adjusted to the desirable flow rate. Fractionation was carried out with a gradient system, changing the polarity of solvents according to the nature of the sample. The solvents used were: acetonitrile, methanol, and water for reverse phase; hexane, ethyl acetate, chloroform, and acetone for normal phase. Columns were equilibrated by washing through with appropriate solvents (n-hexane for normal phase, and distilled water for reverse phase). Up to 400 μg of sample were dissolved in a suitable solvent (same solvent used to start the elution process) and loaded onto the column. The compounds were then eluted with 50 ml of the selected solvent system. Fractions collected were monitored by TLC, dried with a rotavapor and stored at –20° C.

2.3.3. Silica gel column chromatography

Column and adsorbent: Silica gel 60 (0.040-0.063) for column chromatography (MERCK).

Pure compounds were separated from small fractions using silica gel as the stationary phase. The proportion of silica gel used in all preparations was 1:10 (solid material: silica gel). The solvents used were n-hexane-ethyl acetate and chloroform-acetone. Different glass columns were used according to the quantity of sample.

2.3.4. TLC

Column and adsorbent: MERCK Si Gel 60 with F254 indicator on aluminium sheets (20 x 20 cm) analytical TLC plates for normal phase; MERCK Si gel 60 with RP-18 F^{254} indicator on aluminium sheets (20 x 20 cm) analytical TLC plates for reverse phase; MERCK Si gel 60 with $F_{254 + 366}$ indicator on glass plates (20 x 20 cm) preparative TLC plates for normal phase.

Analytical TLC plates were used for detection and monitoring of compounds. Different solvent systems were used in order to localise, identify and, when possible, to quantify the compounds present in the crude extracts and fractions. Simple and multiple developments in different solvent systems were used to improve the separation of compounds (isocratic and gradient systems). The solvent was allowed to migrate up the plate by capillary action and the compounds separated by polarity. Compounds were visualised with UV light at 254 nm and 365 nm, and also visualised by the naked eye, following spraying the plates with 4% vanillin-sulphuric acid and then heating them with a hot-air gun. To measure the migration of the

different compounds that were present in the fractions, Rf values were calculated using the following formula given by Gibbons and Gray (1998):

$$Rf = \frac{\text{Compounds distance from origin (midpoint)}}{\text{Solvent front distance from origin}}$$

The isolation of compounds was carried out with analytical and preparative TLC plates. Analytical TLC was used for samples up to 20 mg, whereas preparative TLC was employed for samples up to 100 mg. The extract was dissolved in a small volume of the appropriate solvent and then applied as a thin line across the plate. The plates were developed in glass tanks for thin layer chromatography, with the solvent system varying depending on the sample. Visible compounds under UV light were marked out with pencil and then scrapped off with a spatula. The powdered silica gel was poured into a small beaker, stirred with 20-50 ml of ethyl acetate (EtOAc) for 15 min, filtered, and dried down with a rotavapor. The recovered compounds were redissolved in the appropriate solvent, dried under oxygen free nitrogen gas, and stored at –20° C. The purity of the compound was confirmed by analytical TLC plates and by nuclear magnetic resonance (NMR) analysis.

2.3.5. Sephadex LH-20

Adsorbent: Sephadex™ LH-20 Amersham Biosciences.

Sephadex is a polysaccharide gel that has glycerin-ether bonds as cross linker and swells in water due to its hydrophilic property (Salituro and Dufresne, 1998). Sephadex LH-20 belongs to the G series, where the number represents the amount of water absorbed by the dry beads upon swelling (e.g. G-25 absorbs 2.5 ml/g of dry bead). Sephadex LH-20 a hydroxypropylated Sephadex G-25, which adds

lipophilicity to the gel and retains its hydrophylicity. This Sephadex is suitable for use with organic solvents, making it a good choice for the fractionation of organic-soluble natural products (Salituro and Dufresne, 1998). Sephadex is a size inclusion chromatography system that was used to separate chlorophylls from crude extracts and to isolate compounds by molecular size. The Sephadex was allowed to swell in solvent ($CHCl_3$, DCM or MeOH) overnight and sonicated for 30 min before pouring it into the column. The slurry was poured into the column leaving the valve of the column open until the gel was completely settled. About 1 ml of swollen gel was used per milligram of extract. The extract or fraction to be separated was dissolved in a small amount of the solvent and applied on the top of the bed and then the mobile phase was added and samples were collected in tubes of approximately 15 ml. For those columns where the elute solution was $CHCl_3$, a tissue paper was placed on the top of the bed to keep it flat. Fractions were monitored by TLC. All samples were dried with a rotavaporator and stored at –20° C.

2.3.6. HPLC

Prepacked analytical HPLC and column: Waters® 600 with 996 photo-diode array photodiode array detector. Waters® radially compressed model 25 mm. Preparative HPLC Waters® Delta Prep 4000 Preparative Chromatography System.

HPLC is widely used for the analysis and isolation of natural products. HPLC is a reliable and reproducible high-resolution technique with efficient, fast separation. The most common stationary phase used is C_{18} and as mobile phase different mixtures of water, acetonitrile and methanol, employing isocratic or gradient systems are used (Heinrich *et al.*, 2004; Stead, 1998).

Analytical HPLC was run using water and acetonitrile as eluent. The injected volume of the extract was 50 µl, and the flow rate was 2.00 ml/min. The gradient program run for this technique was: a total time of 31 min divided in 100% water for 15 min, 100% acetonitrile for 10 min., and 100% water for 11 min. The preparative HPLC was run in a Waters™ Delta Prep 4000 Preparative Chromatography System. The injected volume and concentration of extract was of 2 ml and 40 mg/3 ml, respectively. Other parameters used were: sensitivity 1.00, absorbance: -0.677, wavelength λ 210 nm. The chromatography conditions for the preparative HPLC were the same as in the analytical HPLC procedure. Samples recovered were dried with a rotavapor and/or freeze drier in the case of aqueous samples. The isolated compounds were stored at -20° C.

2.4. Structure elucidation and identification of compounds

The complete structure elucidation of compounds was performed using two different spectroscopic techniques: **1)** Nuclear Magnetic Resonance (NMR), and **2)** Mass Spectroscopy (MS).

2.4.1. Nuclear magnetic resonance

One-dimensional (1D) (^{1}H, ^{13}C and DEPT), and two-dimensional (2D) (HMQC, HMBC, COSY and NOESY) experiments were performed on a Bruker AVANCE 400 and 500 MHz NMR spectrometers. 1D ^{1}H NMR spectrum is used for the determination of each separate signal the chemical shift value, multiplicity, coupling constants and intensity (the number of hydrogens, i.e. integral) (Neri and Tringali, 2001). 1D ^{13}C NMR spectrum on the other hand, gives information regarding the carbon skeleton of the molecule, to establish the number of multiple bonds, and the

number or rings (Neri and Tringali, 2001). DEPT experiment (Distortionless Enhancement by Polarization Transfer) is the most common method to determine carbon multiplicities. With this method is possible to establish the number of protonated carbons (CH, CH_2 and CH_3) (Neri and Tringali, 2001).

The next step in the elucidation of a chemical structure using NMR analysis was the employment of 2D analysis involving proton-carbon and proton-proton correlations. Heteronuclear Multiple Quantum Correlation (HMQC) correlates carbon and proton shifts via one-bond CH coupling (J_{CH}). Heteronuclear Multiple Bond Coherence (HMBC) is used to correlate proton and carbon shifts through both two and three bonds ($^2J_{CH}$ and $^3J_{CH}$). Correlation Spectroscopy (COSY) correlates the proton shifts of the coupling protons of a molecule. Nuclear Overhauser Effect Spectroscopy (NOESY) is a very sensitive analysis for studying the spatial proximity of protons in a molecule, being useful to determine the stereochemistry of the molecule (Breitmaier 1995; Neri and Tringali, 2001).

Chemical shifts values (δ) are reported in parts per million (ppm) relative to appropriate internal standard and coupling constant (*J* values) are given in Hertz. Deuteriated solvents used: δH acetone-d_6: 2.05 ppm; ^{13}C: 30.5, 205.1 ppm; δH chloroform-d_1: 7.27 ppm; ^{13}C: 77.23 ppm; δH methanol-d_4: 3.31, 4.70 ppm; ^{13}C: 49.15 ppm; δH pyridine-d_5: 8.72(H1), 7.21(H2), 7.57 (H3) ppm; ^{13}C: 149.5 (C1), 123.5 (C2), 135.5 (C3) ppm.

2.4.2. Mass spectroscopy

Mass spectra were recorded on VG ZAB-SE instrument (FAB and EIMS) and Finnigan navigator (ESMS), IR spectra on Nicolet 360 FT-IR spectrophotometer.

Positive and negative electron spray (ES) analyses were performed on pure compounds.

2.5. Biological assays

This study used a bioassay-guided fractionation procedure, where inhibitory activity against NF-κB cascade was monitored using the Luciferase assay IL/6, carried out at the Centre for Pharmacognosy and Phytotherapy (CPP), The School of Pharmacy, University of London. In the case of some selected pure compounds, another four tests were performed **1)** NF-κB -dependent luciferase assay TNF-α; **2)** HeLa TET-ON-luc assay; **3)** determination of nuclear DNA loss (apoptosis) and cell cycle analysis; and **4)** cytotoxicity MTT assay. The three first tests were performed at the Department of Cellular Physiology and Immunology, University of Cordoba, Cordoba, Spain by Nieves Márquez and Eduardo Muñoz and the last one **(4)** at the CPP, The School of Pharmacy.

2.5.1. Luciferase assay IL/6 luciferase assay (NF-κB inhibition)

The activity of crude extracts, fractions and compounds as inhibitors targeting the NF-κB cascade was tested with a luciferase assay using HeLa cells. The luciferase assay is a reporter system, which uses the firefly luciferase to determine the level of expression of a transfected gene (Bork *et al.*, 1997). HeLa cells were stably transfected with plasmids containing the interleukin 6 (IL-6) promoter (which is one of the inflammatory cytokines regulated by NF-κB) fused to the gene which codes for luciferase, an enzyme found in fireflies and glow beetles which produces light by adenosine tri-phosphate (ATP) dependent oxidation of the substrate luciferin. After stimulation of NF-κB, the freed NF-κB then binds to the IL-6 promoters and induces

the expression of luciferase (Bremner *et al.* 2004). The presence of luciferase can then be measured as a relative intensity corresponding to NF-κB activation (high intensity) or inhibition (low intensity).

HeLa cells were maintained in DMEM (Invitrogen®) supplemented with 10% foetal bovine serum and antibiotics and were maintained at 37° C in a 5% CO_2 humidified atmosphere and split when confluent (Bremner *et al.*, 2004). Extracts/fractions were dissolved in DMSO or acetone according to their solubility (10 mg/ml) and for pure compounds 5 mM. 10 µl of the sample was added to the wells (1 ml media) to obtain final concentrations of compounds (50 µM) or plant extracts/fractions (100 µg/ml) for 1 h. Each test was replicated three times. Following the 1 h period the stimulant, phorbol-12-myristate-13-acetate (PMA), was added at 50 ng/ml and the plates incubated for 7 h. Positive controls consisted of stimulated cells with PMA, whereas the negative control contains resting cells without stimulation. For the harvesting of cells the medium was removed and cells were washed with PBS and lysed by incubation with lysis buffer [1 M Tris-phosphate 6.25 ml adjusted to pH 7.8 with NaH_2PO_4, 1 M DTT (dithiothreitol) 50 µl, 0.5 M CDTA 1 ml, glycerol 25 ml, Triton X-100 (10%) 5 ml plus 50 ml of purified water store at -20°C] (100 µl per well) for 10-15 min at 4° C. The suspensions were transferred to Eppendorf tubes and centrifuged at 1300 rpm for 15 min at 4° C, and then the supernatant were tested for luciferase activity. The IL-6/Luc assay employed 15 µl of supernatant mixed with 50 µl luciferase assay reagent. Readings were taken with an Anthos Lucy 1 luminometer/photometer, where high light incidence means activation of NF-κB, and low light incidence means inhibition. To be considered active, the luciferase value had to be at least 30% of the positive control (Bremner *et al.*, 2004). Toxicity was evaluated by physiological changes in

the cells under microscope at 40X, and in some cases a more detailed cell viability test was undertaking using the MTT assay (see section 2.5.5.).

2.5.2. Inhibition of the nuclear transcription factor NF-κB

The NF-κB -dependent luciferase assay was used employing the 5.1 cell line. This cell line is a Jurkat-derived clone stably transfected with a plasmid containing the firefly luciferase gene driven by the HIV-LTR promoter (Márquez *et al.* 2004). The latter promoter is highly dependent on NF-κB activation induced by TNFα, and therefore high expression of luciferase activity reflects NF-κB activation through the canonical pathway (Yamamoto and Gaynor, 2004). To determine the NF-κB dependent specific transcription, 5.1 cells were pre-incubated with the different compounds for 30 min and then stimulated with TNFα (2 ng/ml) for 6 h. The cells were then lysed in 25 mM Tris phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 9510 following the instructions of the luciferase assay kit (Promega®) and the protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation was calculated as RLU/μg protein (relative light units) and expressed as a fold induction.

2.5.3. HeLa TET-ON-luc assay

This assay measures if the inhibitory activity of the compounds tested is specific for the NF-κB cascade, or if the compounds are inhibiting the synthesis of the luciferase. The HeLa TET-ON cells ($5x10^5$ cells/ml) were seeded the day before the assay, and then stimulated with doxycycline (2 μg/ml) in the presence or absence of the

compounds for 6 hours. The cells were subsequently washed twice in PBS, lysed, and the luciferase activity measured as described in the aforementioned assay (section 2.5.2). The Hela-Tet-On-Luc cell line was constructed by stable co-transfection of HeLa cells following Tet-On system kit instructions. This cell line contains two plasmids: the pTET-ON codifies constitutively for rtTA protein that in response to doxycycline it gets active and binds to the pTRE2hyg-Luc starting the synthesis of luciferase gene. HeLa-Tet-On-Luc cell line was maintained in DMEM (Gibco BRL) in the presence of 100 μg/ml of Hygromycin (Invitrogen®, Barcelona, Spain) and 100 μg/ml of G418. Cells were maintained in a humid chamber at 37°C under 5% CO_2.

2.5.4. Determination of nuclear DNA loss (apoptosis) and cell cycle analysis

The human T cell leukemia Jurkat cell line is commonly used in the first round of cytotoxicity assays. This cell line was maintained in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum, 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). The cells (10^6) were treated with different concentrations of the compounds for 6 or 24 h. The percentage of cells undergoing apoptosis (subdiploid cells) was determined by ethanol fixation (70%, for 24 h at 4°C). After that, the cells were washed twice with PBS containing 4% glucose and subjected to RNA digestion (Rnase-A, 50 U/ml) and PI (20 μg/ml) staining in PBS for 1h at RT, and analysed by cytofluorimetry. As a consequence, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining allows the determination of the percentage of subdiploid cells (sub-G_0/G_1 fraction).

2.5.5. Cytotoxicity MTT assay (tetrazolium salt 3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolim bromide)

The MTT assay is a standard procedure to measure cytotoxicity (Mosmann, 1983). It is a colorimetric assay based on the conversion of a tetrazolium salt MTT, a pale yellow substrate to formazan, a purple dye. HeLa cells were grown in media to 70-80% confluence, harvested and counted in a haemocytometer. The density was adjusted to $7.5x10^4$ cells/ml in media. 200 µl of cell suspension ($1.5x10^4$ cell/ µl) was added to each test well. Plates were incubated for 24 h at 37° C with 5% CO_2 at 95% humidity. The media was aspirate from each well immediately before the assay began. 100 µl of media containing 1% DMSO was added to each test well (columns 5-11), leaving columns 2 and 3 with normal media (controls). 200 µl of the test solution was added to column 4, and doubling dilutions were performed to column 11. Plates were incubated at 37° C for 3 days. The wells were aspirated of the old media and washed by adding 200 µl of PBSA. 200 µl of MTT solution (0.5 mg/ml) was added to each well and incubated for 2 h at 37° C. The MTT solution was removed and 200 µl of 10% DMSO + 90% isopropanol added to each well and left for 10 min wrapped in Al-foil. The wells were then resuspended with a multichannel pipette. Readings were conducted using the photometric mode of the Anthos Lucy 1 luminometer/photometer at 570 nm.

CHAPTER 3. RESULTS

3.1. *Witheringia solanacea*

3.1.1. Phytochemistry

Extraction of crude extracts

A total of five crude extracts were obtained from 500 g of powdered leaves using a Soxhlet apparatus and increasing the polarity of the solvents from n-hexane to methanol. Of these, only the chloroform extract ($CHCl_3$ extract) was used for further fractionation due to its superior chemical complexity and its considerably higher yield recovered in comparison with the remaining crude extracts (hexane = 12.5 g, $CHCl_3$ = 19 g, ethyl acetate = 2.4 g, acetone = 1.9 g, methanol = 8 g).

Fractionation of crude extract

A flow diagram showing the steps followed for the fractionation of the $CHCl_3$ extract and the isolation of three compounds, plus pictures of the TLC plates, are presented in Figs 9 and 10, respectively. Twelve grams of $CHCl_3$ extract were fractionated by VLC (gradient: Hex-EtOAc, increments of 10%) and 22 fractions of 100 ml each were collected. TLC plates (EtOAc-Hex 8:2) were then employed to observe the compounds present in these fractions, and those that had a similar chemical composition were combined together to give a total of 9 fractions. TLC analysis showed fractions 7, 8 and 9 had the highest number of compounds and also showed a white powder adhered to the round bottom flask after complete evaporation. The chlorophyll present in these fractions was eliminated by adding activated carbon and chloroform and then filtering the resulting mix in a Büchner funnel and evaporating the final filtrate.

Fig. 9. Flow diagram showing the procedure followed for the isolation of compounds from *Witheringia solanacea* using a bioassay-guided fractionation.

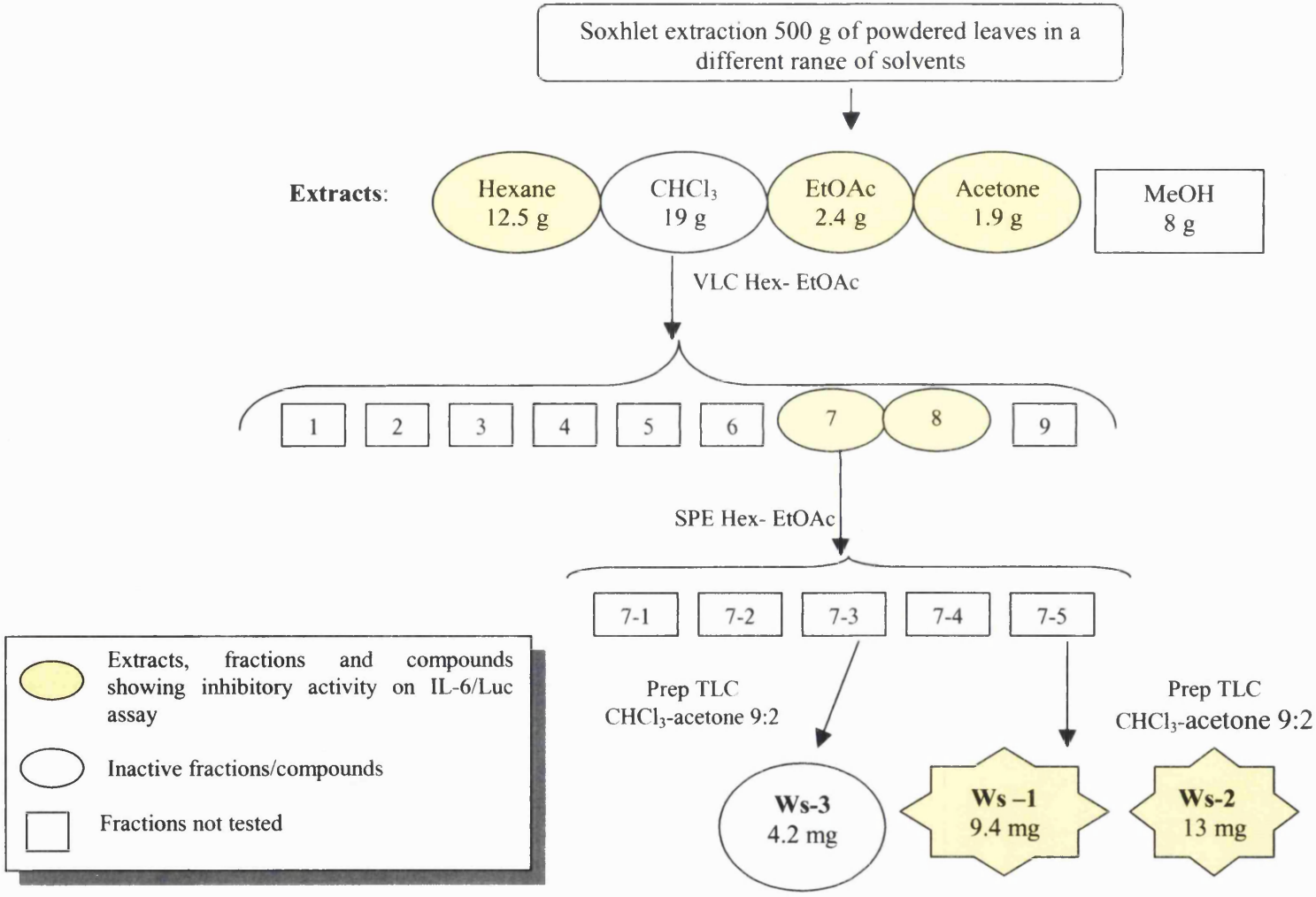
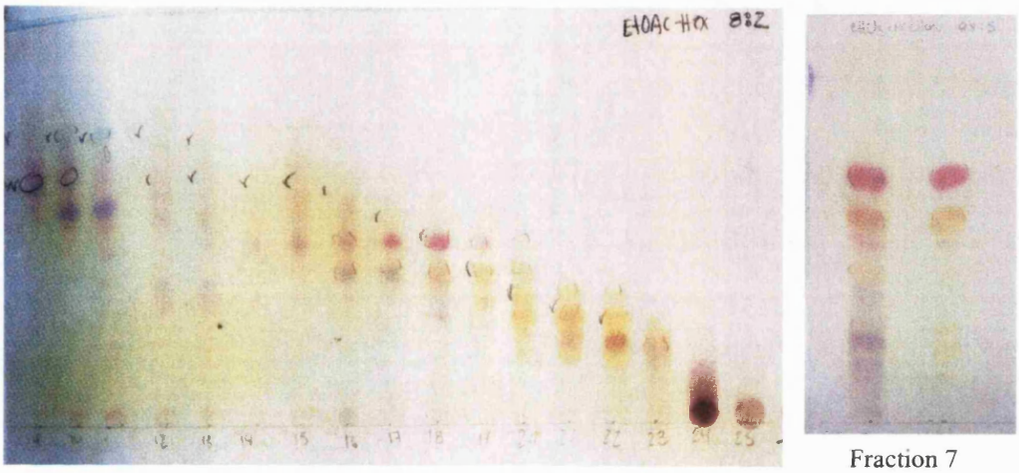
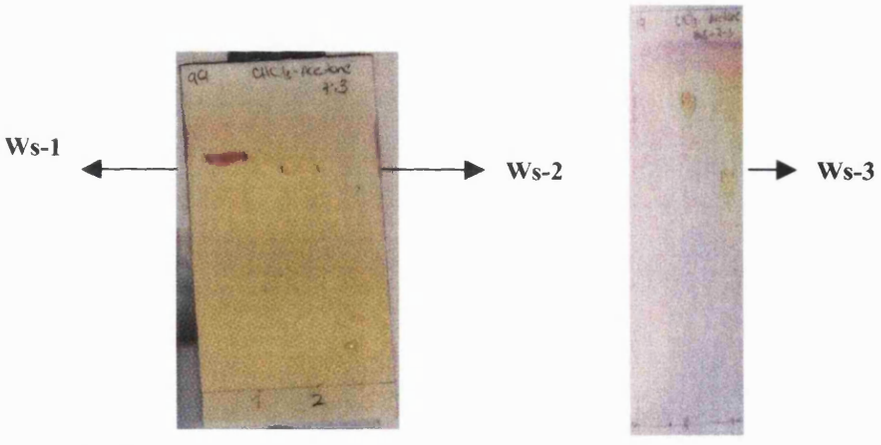


Fig. 10. TLC plates of fractions and compounds obtained from *Witheringia solanacea*. Spray reagent vanillin-H_2SO_4.



Fraction 7

Solvent system: EtOAc-Hex 8:2 $CHCl_3$-Acetone 9.5:0.5



Solvent system: $CHCl_3$-Acetone 7:3 $CHCl_3$-Acetone 7:3

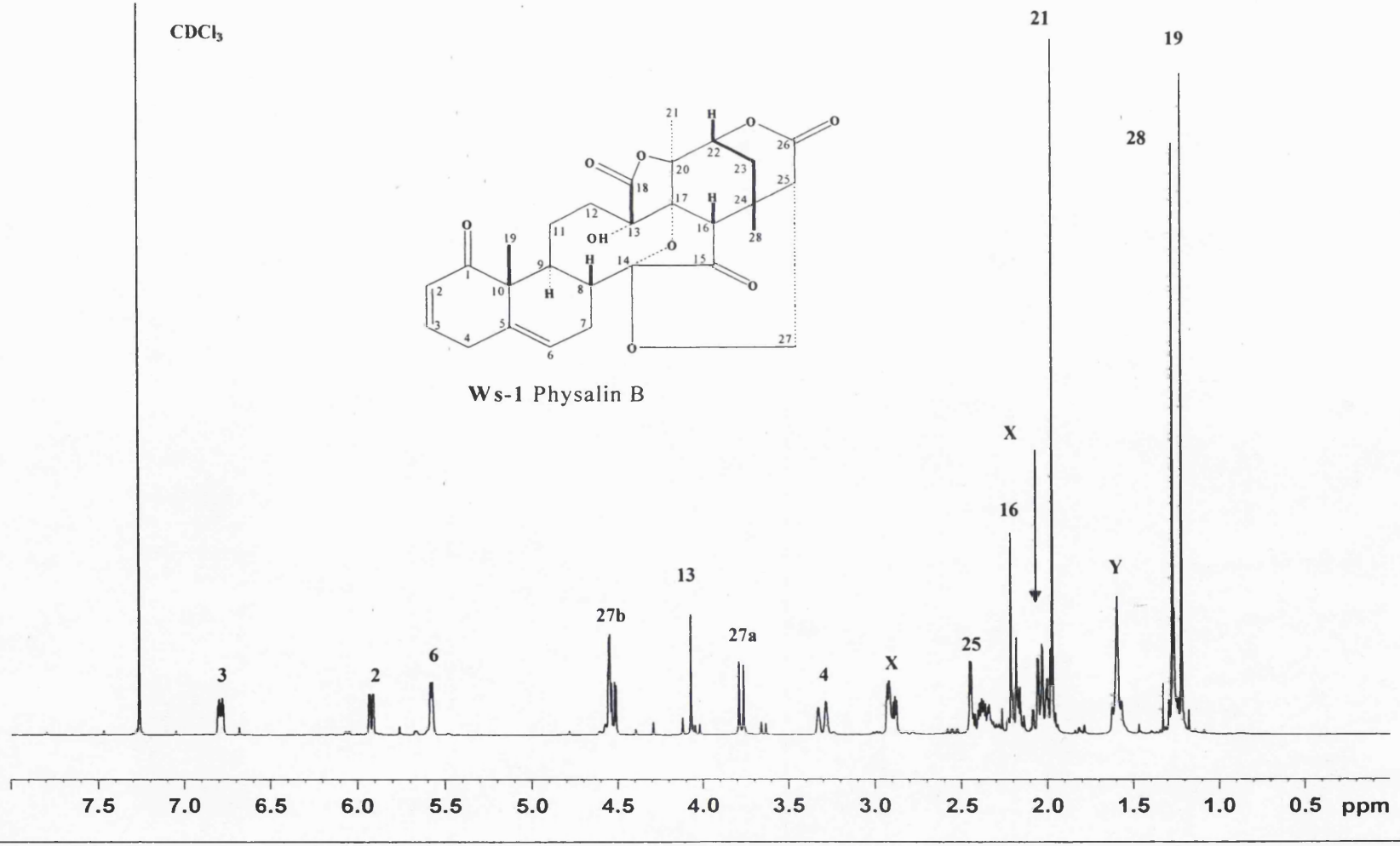
Characterisation of compounds

Three compounds all of the physalin class, were isolated from the $CHCl_3$ extract. The procedure followed for the isolation of each of these physalins was as follows:

Ws-1. This compound was isolated from fraction 7 by VLC (gradient method Hex-EtOAc), followed by SPE (Hex-EtOAc increments of 10%), and then by preparative TLC ($CHCl_3$-Acetone 9:2, double development) (Fig. 9). This compound was recovered as a white powder, soluble in chloroform (9.4 mg). On analytical TLC plates, it was visible under UV light at short wave (254 nm) and after spraying with vanillin-sulphuric acid it turned red ($R_f = 0.79$; Fig. 10).

The ESI mass spectrum showed a molecular ion at ES *m/z* 511.2 [+H], which was consistent with the molecular formula of physalin B ($C_{28}H_{30}O_9$; molecular weight of 510.532 Fig. 11a). The ^1H and ^{13}C NMR spectra corroborated that Ws-1 represented physalin B, which was corroborated with previously published spectral data (Chiang *et al.*, 1992a; Januário *et al.*, 2002) (Fig. 11b; Tables 9 and 10). The ^1H and ^{13}C NMR spectra in chloroform- d_1, showed that this compound had characteristic signals for a double bond at C2-C3 forming a conjugated enone system (δ_H 5.92 and 6.80; δ_C 127.1 and 145.9), a double bond at C5-C6 (δ_C 133.6 and 79.4), a 13-tertiary hydroxyl (δ_H 4.07; δ_C 80.7), a 14-17-oxide bridge (δ_C 107.3 and 207.8), an acetalic carbon at C14 and a methylene at C27 (δ_C 107.3 and 60.4), and three methyl groups at C19, C21 and C28 (δ_H 1.21; 1.97; 1.27, respectively).

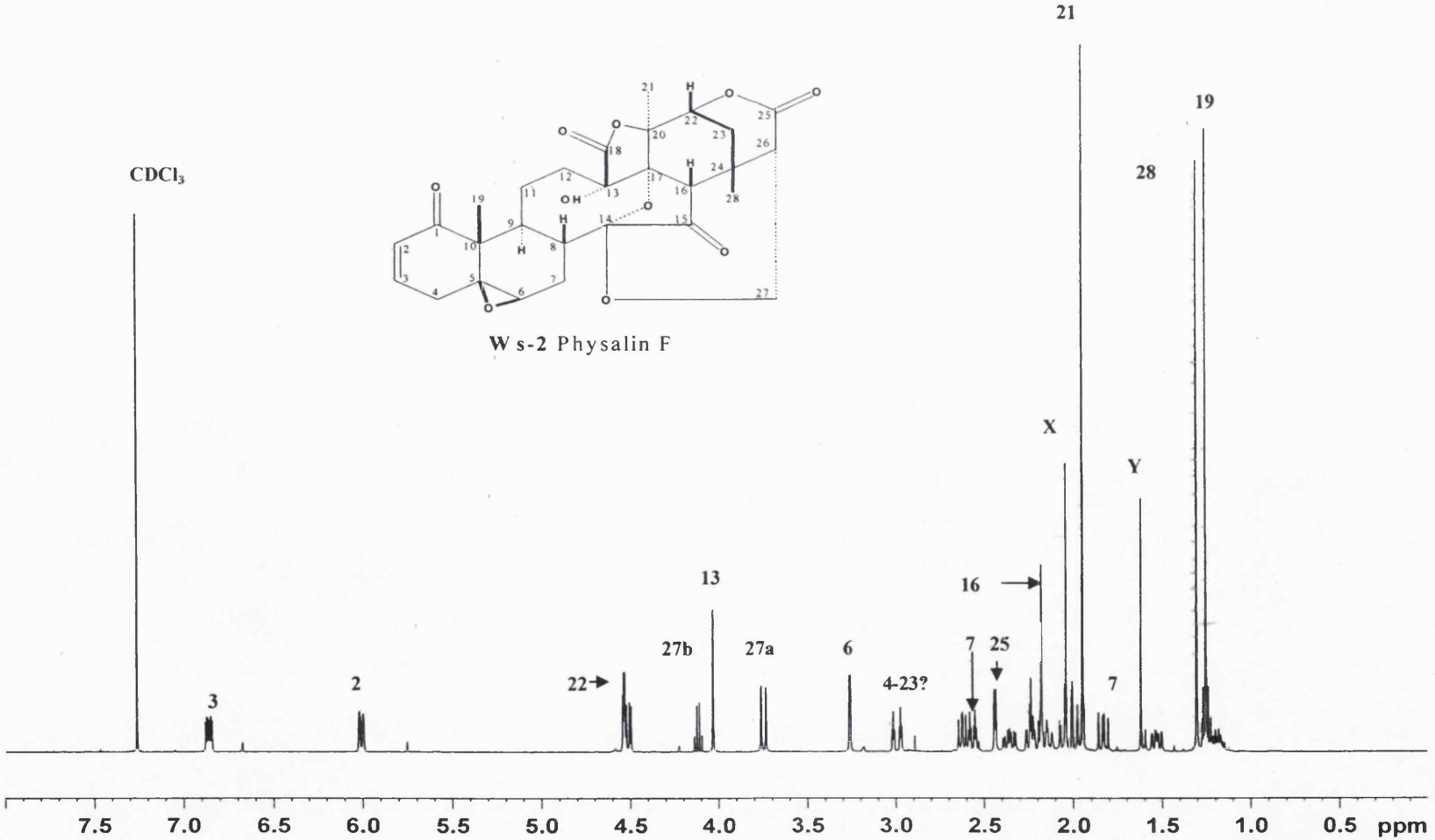
Fig. 11. Structure and ^{1}H NMR spectrum of physalin B (500 MHz, $CDCl_3$). X= unassigned peaks; Y= impurities.



Ws-2. This compound was isolated from fraction 7 by VLC (gradient method Hex-EtOAc), followed by SPE (Hex-EtOAc increments of 10%), and then by preparative TLC ($CHCl_3$-Acetone 9:2, double development) (Fig. 9). A total of 13 mg of this compound was recovered as a white powder soluble in chloroform. On analytical TLC plates, Ws-2 was visible under UV light at short wave (254 nm) and spraying with vanillin-sulphuric acid gave a yellow colour (R_f = 0.67; Fig. 10).

The results of the ^1H and ^{13}C NMR spectra in chloroform-d_1 and their comparison with previously published spectral data (Chiang *et al.*, 1992a; Januário *et al.*, 2002) revealed that Ws-2 represented physalin F (5β, 6β-epoxy physalin B) (Fig. 12b; Tables 9 and 10). The ^1H and ^{13}C NMR spectra showed that this compound contained a double bond at C2-C3 forming a conjugated enone system (δ_H 6.02 and 6.87; δ_C 127.7 and 146.3), at C5- C6 is an epoxide ring (δ_C 61.7; δ_C 64.9), and had a 13-tertiary hydroxyl (δ_H 4.03; δ_C 80.9), a 14-17-oxide bridge (δ_C 107 and 80), an acetalic carbon at C14, and a methylene at C27 (δ_C 107 and 60.7), and three methyl groups at C19, C21 and C28 (δ_H 1.25; 1.94; 1.30, respectively). The molecular formula obtained by ESI mass spectrometry showed an intense ion peak at *m/z* 527 [+H] consistent with the molecular formula $C_{28}H_{30}O_{10}$ (molecular weight = 526.531) further confirming that Ws-2 was physalin F (Fig. 12a).

Fig. 12. Structure and ^{1}H NMR spectrum of physalin F (500 MHz, $CDCl_3$). X= unassigned peaks; Y= impurities.



Ws-3. Isolation of this compound was achieved by VLC fraction 7 (gradient method Hex-EtOAc), SPE (Hex-EtOAc increments of 10%), and finally by preparative TLC ($CHCl_3$-Acetone 9:2, double development) (Fig. 9). Ws-3 was recovered as white a powder (4.2 mg), which was soluble in chloroform. On analytical TLC plates it was visible under UV light at short wave (254 nm) and after spraying with vanillin-sulphuric acid it turned to yellow (R_f = 0.65; $CHCl_3$-Acetone 7:3; Fig. 10).

The ES mass spectroscopy showed an intense ion peak at *m/z* 561 [$-H_2O$][-H], which corresponded to the molecular formula of $C_{28}H_{32}O_{11}$ (molecular weight= 544.547) (Fig. 13a). This characterisation with the ^1H and ^{13}C NMR spectra in chloroform-d_1, and comparison of the resulting data with previously published spectral data (Chiang *et al.*, 1992a; Januário *et al.*, 2002) indicated that this compound was physalin D (5α-OH, 6β-OH physalin B) (Fig. 13b; Table 9 and 10). Physalin D is distinguished by having a 14,17-oxide bridge (δ_C 106.5 and 81.2), a 13-tertiary hydroxyl (δ_H 2.08, 79.6), and a double bond at C2-C3 forming a conjugated enone system (δ_H 6.03, and 6.82; δ_C 126.9 and 143.9, respectively). Moreover, the HMBC analysis showed a two-bond correlation between the hydroxyl hydrogen OH-6 (δ_H 4.31) and C-6 (δ_C 65.4), which was also characteristic for physalin D.

Fig. 13. Structure and ^{1}H NMR spectrum of physalin D (500 MHz), $CDCl_3$. X= unassigned peaks; Y= impurities.

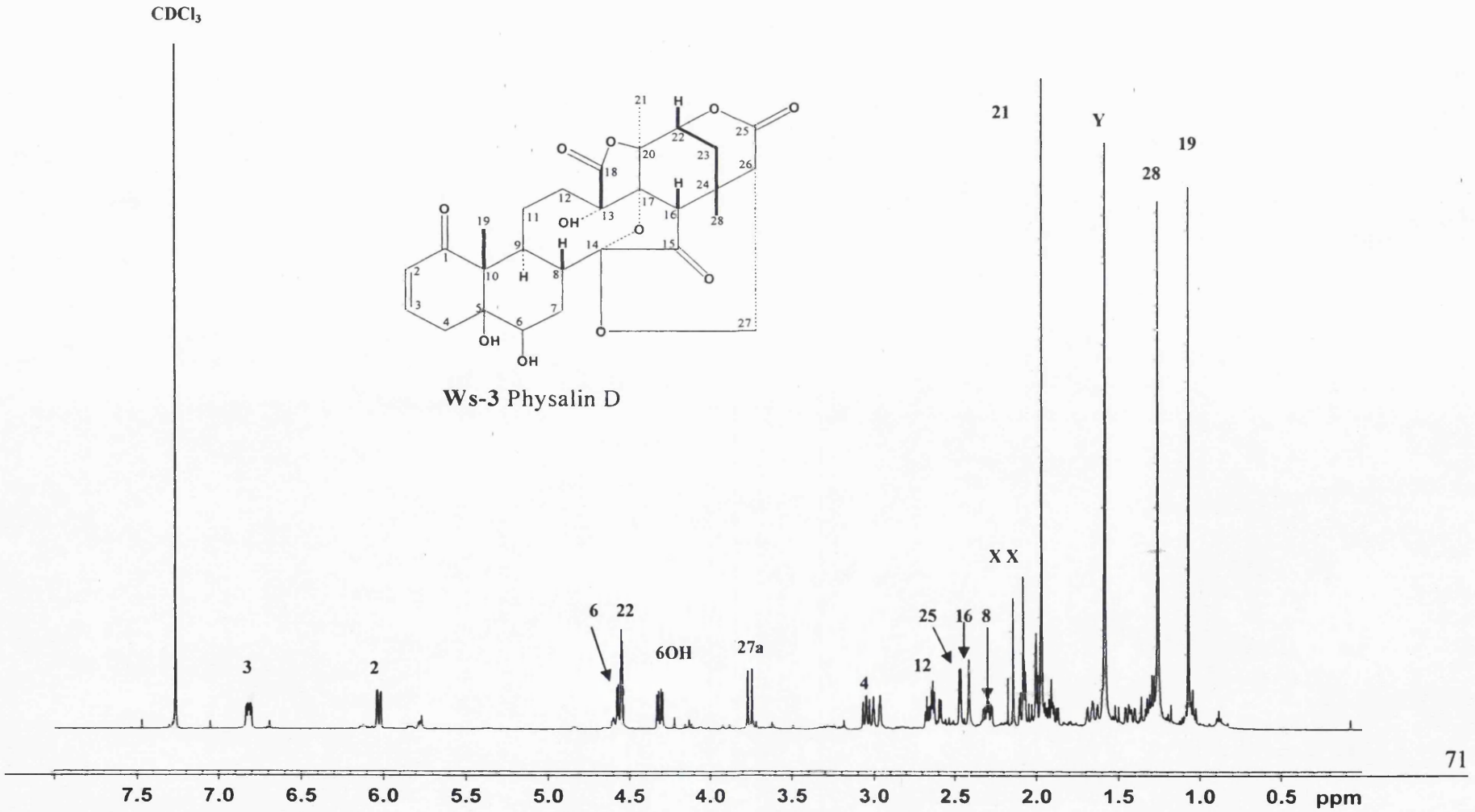


Table 9. ^{13}C NMR (500 MHz) spectral data obtained for physalins B, F, and D in $CDCl_3$. *† = Previously published spectral data. Chemical shifts in δ ppm.

Carbon/	DEPT	Physalin B	Literature*	Physalin F	Literature†	Physalin D	Literature*
1	C	205.5	202.6	205.9	205.8	201.2	204.3
2	CH	127.1	127.1	127.7	127.8	126.9	127.1
3	CH	145.9	146.4	146.3	146.3	143.9	142.8
4	CH_2	21.8	32.5	33.3	33.3	32.9	35.1
5	C	133.6	135.8	61.7	61.7	77.6	76.3
6	CH	124.2	123.6	64.9	64.8	65.4	72.5
7	CH_2	24.5	24.6	24.8	32.9	21	26.6
8	CH	39.6	40.3	37.4	37.4	42.6	38.2
9	CH	32.9	33.3	34.22	34.3	32	29.8
10	C	52.4	52.2	50	49.9	56.3	53.4
11	CH_2	23.9	24.6	23.6	23.5	31.3	24.7
12	CH_2	25.6	25.7	25.7	25.6	31.5	25.7
13	C	80.7	78.4	80.9	79.3	79.6	78.5
14	C	107.3	106.5	107	106.9	106.5	106.8
15	C	207.8	209.6	207.5	207.5	206.8	209.8
16	CH	59.2	54.4	56.2	56	55.8	53.8
17	C	79.4	80.9	80	80	81.2	80.4
18	C	172	172	172.1	172.1	171.4	171.8
19	CH_3	17.6	17	15.6	15.4	8.2	13.2
20	C	79.9	80.5	79.4	80.9	80.6	80.6
21	CH_3	21.1	21.9	21.4	21.4	21.6	21.6
22	CH	76.6	76.5	76.9	77	76.7	76.3
23	CH_2	32.4	31.6	33	24.8	25.9	31.2
24	C	30.9	30.7	31.1	31	31	30.4
25	CH	50.7	49.6	50.8	50.8	50.7	49.3
26	C	166.4	167.5	166.6	166.6	166.2	167.3
27	CH_2	60.4	60.8	60.7	60.6	61	60.4
28	CH_3	26.3	24.6	26.5	26.4	26.4	24.4

* Januário *et al.* (2002).

† Chiang *et al.* (1992a).

Table 10. ^{1}H NMR spectral (500 MHz) data obtained for physalins B, F, and D in $CDCl_3$. *† = Previously published spectral data. Chemical shifts in δppm (spin multiplicity and coupling constant *J* in Hz).

Proton	Physalin B	Literature*	Physalin F	Literature†	Physalin D	Literature*
2	5.92 *dd* (10.0, 2.0)	5.80 *dd* (10.0,2.0)	6.02 *dd* (3.0, 1.0)	5.95 *dd* (10.0, 2.5)	6.03 *dd* (10.5, 2.5)	5.70 *dd* (10.0, 2.0)
3	6.80 *ddd* (7,4.5,2.5)	6.89 *ddd* (10.0, 5.0, 2.0)	6.87 *ddd* (8.5, 5.5, 3.0)	6.83 *dm* (10.0)	6.82 *ddd* (7.5, 5.5, 2.0)	6.62 *ddd* (10.0, 5, 2)
4	3.32 *dd* (22.0, 2.5)	2.89 *dd* (a) (20.0, 5.0)		2.96 (2.2)		1.98 *dd* (20.0, 5.0)
		3.27 *d* (b) (20.0)				3.11 (20)
5					4.21 *s* (OH)	4.25 *s* (OH)
6	5.57 *d* (6.0)	5.59 *d* (6.0)	3.25 *d* (3.5)	3.22 *m*		
6 OH					4.31 *m* (OH)	4.90 *d* (4.0) (OH)
13	4.07 *s* (OH)	6.28 *s* (OH)	4.03 *s*			5.76 *s*
16	2.21 *s*	2.86 *s*	2.17 *s*	2.1	2.40 *s*	2.77 *s*
19	1.21 *s* (Me)	1.09 *s* (Me)	1.25 *s* (Me)	1.27 *s*	1.06 *s* (Me)	1.11 *s*
21	1.97 *s* (Me)	1.78 *s* (Me)	1.94 *s* (Me)	1.93 *s*	1.96 *s* (Me)	1.81 *s*
22		4.56 *dd* (3.0, 2.0)	4.53 *dd* (3.5, 2.5)	4.51 *m*	4.54 *dd* (3.0, 1.5)	4.56 *dd* (3.0, 2.0)
25	2.44 *d* (4.5)	2.88 *d* (4.0)	2.43 *d* (4.5)	2.4		
27	3.76 *d* (13.0)	3.60 *dd* (14.0, 1.0)	3.76 *d* (13.5)	3.72 *d* (12.0)	3.76 *d* (13.5)	3.58 *d* (13.0)
	4.52 *dd* (8.5, 4.5)	4.26 *dd* (14.0, 4.0)	4.12 *dd* (14.5, 7.5)	4.46 *dd* (12.2)		4.25 *dd* (13.0, 4.0)
28	1.27 *s* (Me)	1.16 *s* (Me)	1.30 *s* (Me)	1.22 *s*	1.25 *s* (Me)	1.17 *s*

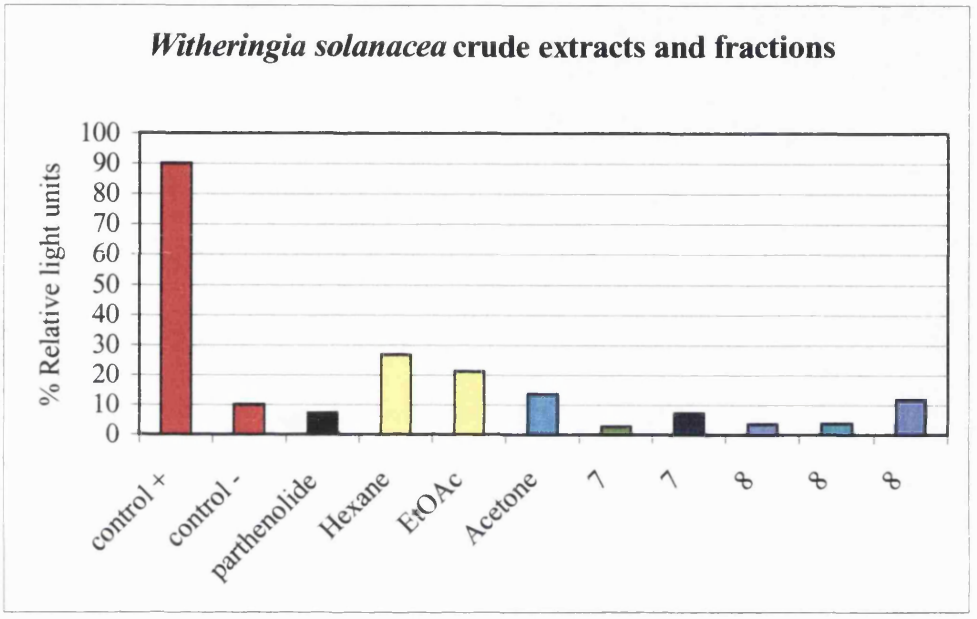
*Januário *et al.* (2002).

†Chiang *et al.* (1992a).

3.1.2. Biological activity

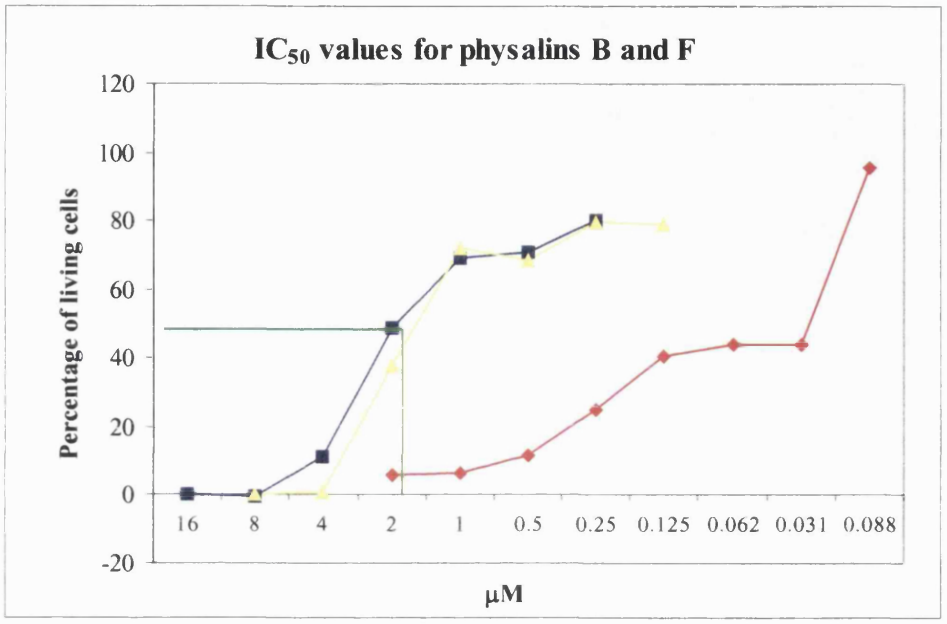
Crude extracts obtained from Soxhlet extraction were initially tested as inhibitors of the NF-κB cascade controlled by the IL-6 promoter (Bremner *et al.*, 2004). Hexane, EtOAc, and acetone extracts showed inhibitory activity at 100 μg/ml, and reduced NF-κB activity to 27%, 21% and 13%, respectively, with no cytotoxicity observed according to the three replicates performed (mean of three replicates) (Fig. 14). Although the $CHCl_3$ extract was 100% cytotoxic at 100 μg/ml, this was fractionated and tested for NF-κB inhibition due to its considerable chemical complexity and high weight. Fractions obtained from the $CHCl_3$ extract by VLC were tested at different concentrations. Fractions 7 and 8 were active at lower concentrations than crude extracts, especially fraction 7 at 12 μg/ml and fraction 8 at 50 μg/ml, reducing NF-κB activity to 3% and 4%, respectively (Fig. 14). No cytotoxicity was observed towards HeLa cells at any of these concentrations.

Fig. 14. Inhibitory activity of crude extracts and fractions obtained from *Witheringia solanacea* against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. Concentrations: ■ 100 ■ 50 ■ 25 ■ 12 ■ 6 μg/ml. ■ Parthenolide (+ control) at 2.5 μg/ml.



The isolated compounds obtained from the powdered leaves of *Witheringia solanacea* (physalins B, F, and D) were tested at 100 μg/ml (equivalent to 195.8 μM, 189.92, and 183.65 for physalins B, F, and D, respectively). The inhibitory activity of physalins B and F could not be established due to a highly cytotoxic effect at this concentration, whereas physalin D did not show inhibitory activity or cytotoxicity. An MTT assay carried out to establish the minimum μM concentration for physalins B and F revealed that these compounds were not cytotoxic on HeLa cells after 72 h of exposure at 2 μM (Fig. 15).

Fig. 15. IC_{50} values (green line) for physalins ■B and ■F at different μM concentrations. ■Doxorubicin (positive control).



Physalins B and F were tested again in the IL-6/Luc assay at different μM concentrations that did not show cytotoxicity towards HeLa cells after 72 h of exposure on the MTT assay. Both compounds were active at 16 and 8 μM, respectively, with no cytotoxicity observed in HeLa cells after 7 h of exposure (Fig. 16) and physalin F was also active at 4 μM (Fig. 16). Several tests carried out with different combinations of the three physalins isolated at 4 μM showed an inhibitory effect only when physalin F was present (Fig. 17).

Fig. 16. Inhibitory activity of physalins B, F, and D against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. ■ Physalins B ■ physalin F ■ physalin D at different μM concentrations. ■ Parthenolide (positive control at 2.5 μg/ml (data not shown).

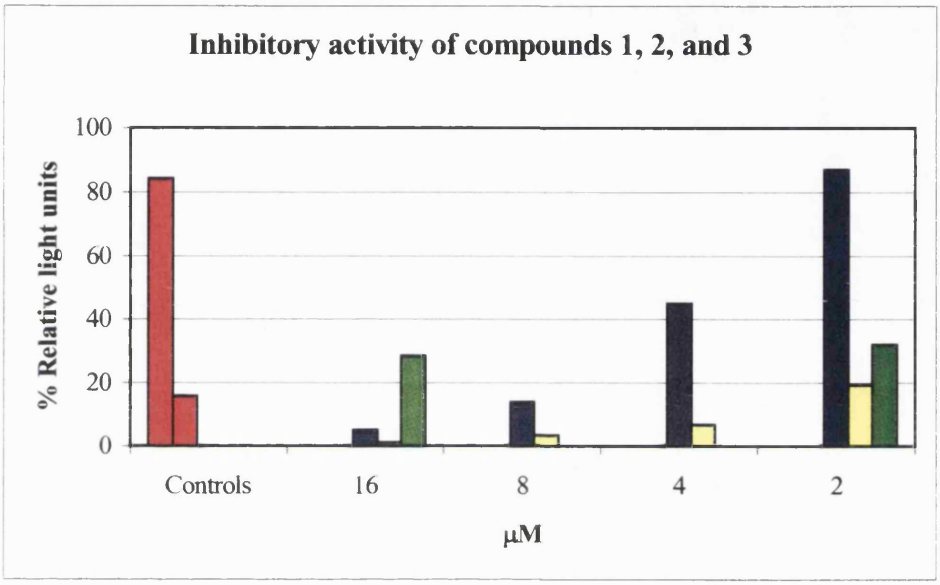
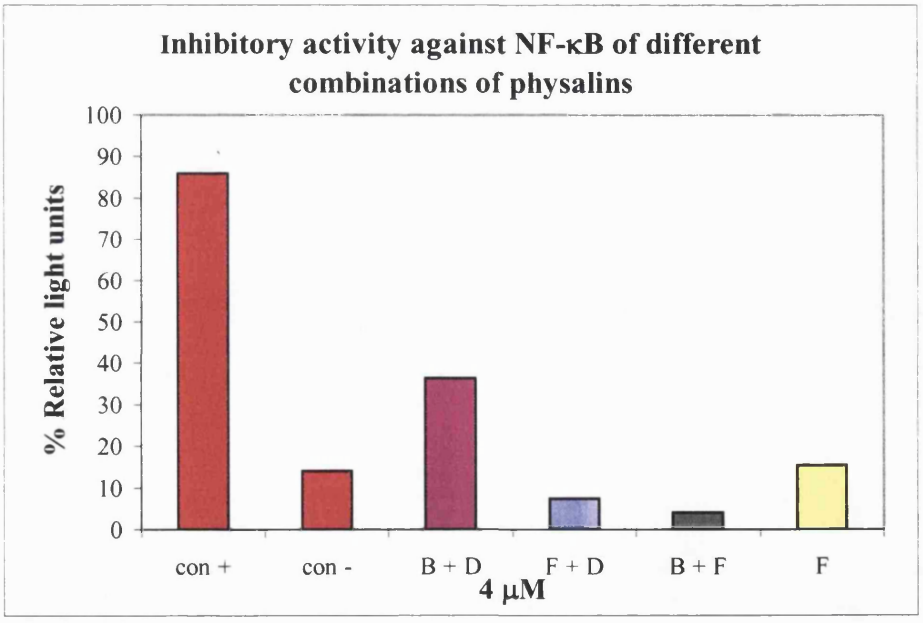
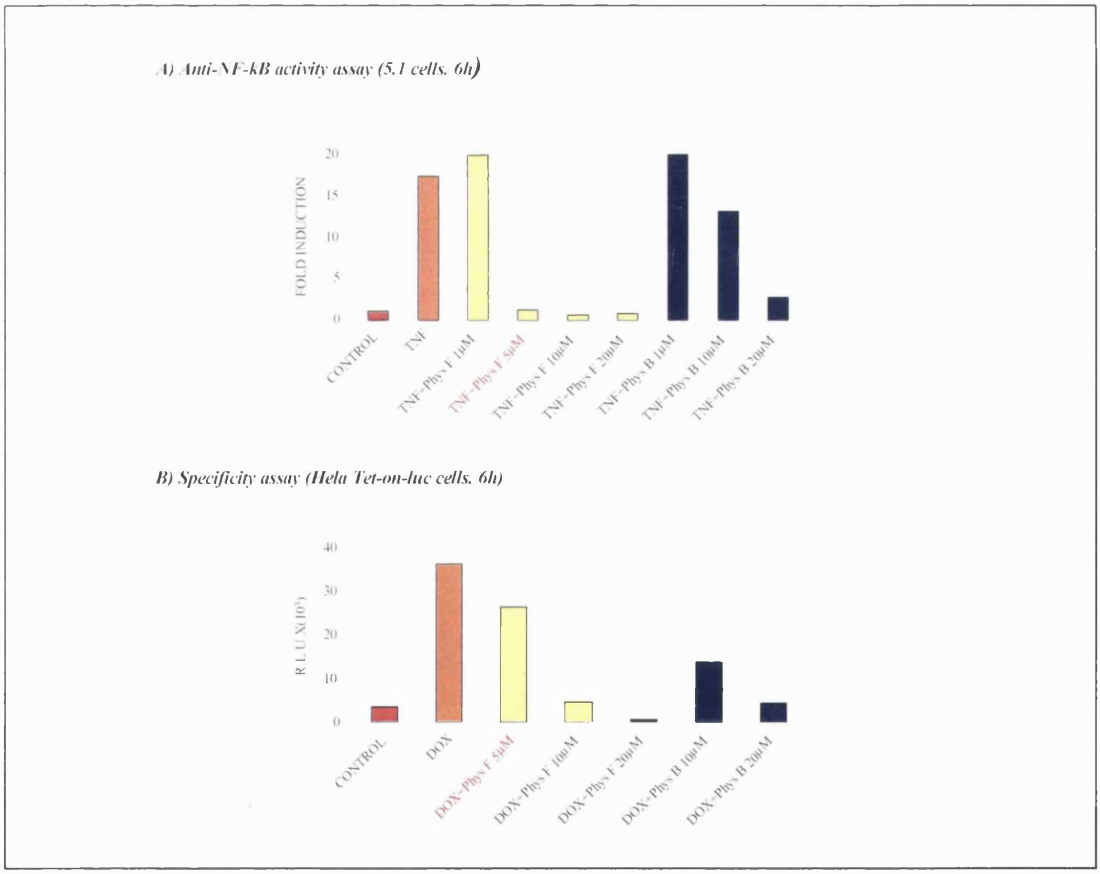


Fig. 17. Inhibitory activity of different combinations of physalins B, D, and F against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. ■ physalins B + D ■ physalins F + D ■ physalins B + F ■ physalin F. Parthenolide (+ control) at 2.5 μg/ml (data not shown).



Evaluation of the effects of physalins B and F on TNFα-induced NF-κB activation using the 5.1 cell line revealed that only physalin F inhibits the luciferase expression through the HIV-1 LTR promoter in TNFα-stimulated cells (Fig. 18). Moreover, the inducible expression of luciferase mediated by doxycycline in Hela-Tet-On-Luc cells was not affected by physalin F at any of the concentrations tested, which suggests that its inhibitory effects were not due to interference with the transcriptional machinery or with the *in vitro* activity of the luciferase enzyme.

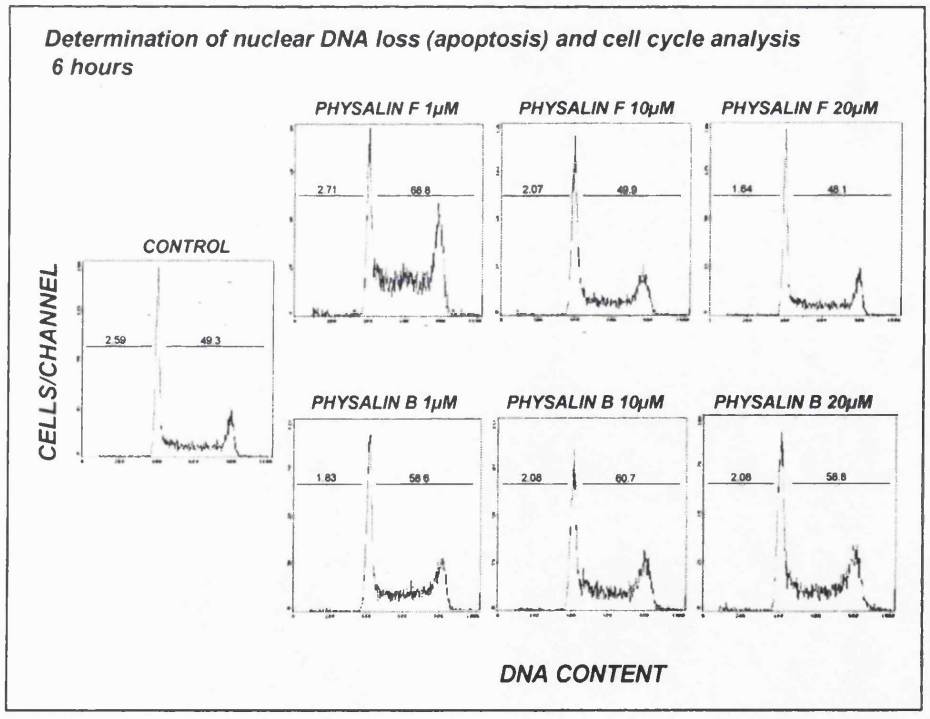
Fig. 18. A) Anti-NF-κB activity of physalins ■ B and ■ F in 5.1 cell line stably transfected with a plasmid containing the firefly luciferase gene driven by the HIV-LTR promoter, using TNF-α as stimulant. ■ Control ■ TNFα. B) Specificity assay on HeLa TET-ON-luc assay stimulated with doxycycline in the presence or absence of the compounds for 6 h.



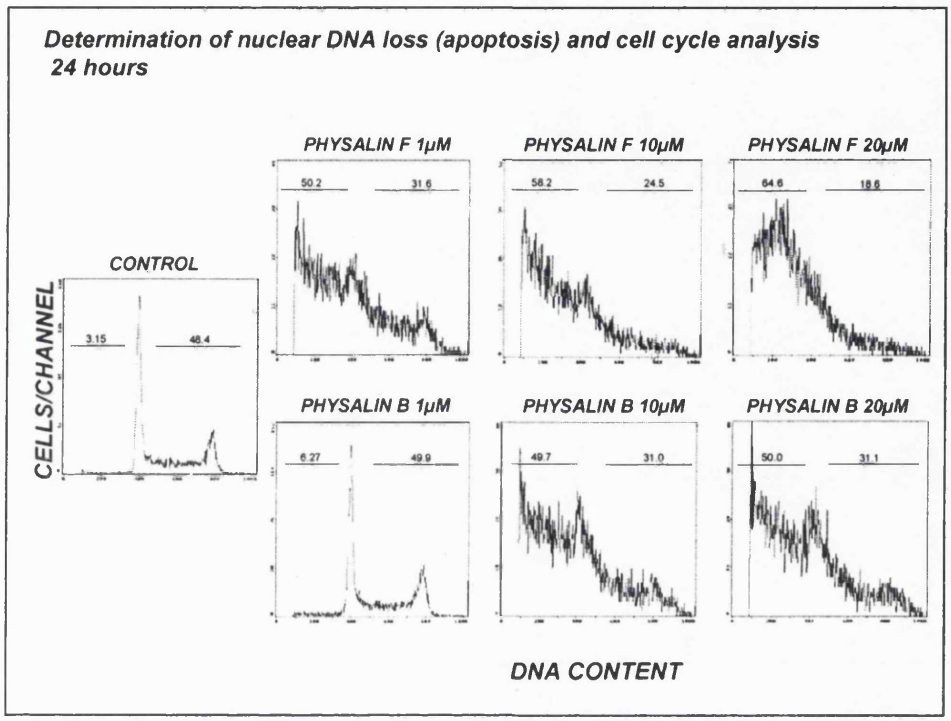
Cell cycle analyses were also carried out for physalins B and F to see whether they induced apoptosis. For this, Jurkat cells were incubated with different compound concentrations for 6 and 24 h, stained with PI and the DNA content analysed by flow cytometry. This method determines the nuclear DNA loss as a marker of apoptosis. Neither physalins F nor B were able to induce apoptosis after 6 h of treatment (Fig. 19), thus supporting that the luciferase inhibitory activity of these compounds were not due to non-specific cytotoxicity. After 24 h of exposure, however, physalin F appear to induce apoptosis of Jurkat cells in a concentration-dependent manner.

Fig. 19. Determination of nuclear DNA loss and cell cycle analysis on human T cell leukaemia Jurkat cell line treated with different concentrations of physalins F and B. A) After 6 h of exposure B) after 24 h of exposure.

A)



B)


Determination of nuclear DNA loss (apoptosis) and cell cycle analysis
24 hours
CONTROL
3.15
48.4
PHYSALIN F 1μM
50.2
31.6
PHYSALIN F 10μM
58.2
24.5
PHYSALIN F 20μM
64.6
18.6
PHYSALIN B 1μM
6.27
49.9
PHYSALIN B 10μM
49.7
31.0
PHYSALIN B 20μM
50.0
31.1
CELLS/CHANNEL
DNA CONTENT

3.2. *Withania frutescens*

3.2.1. Phytochemistry

Extraction of crude extracts

The following amounts of crude extract were recovered from the aerial parts of *Withania frutescens*: hexane (3.7 g), chloroform (5.4 g), and ethyl acetate (2.8 g). Of these crude extracts, the $CHCl_3$ extract was selected for further fractionation due to its positive activity as an inhibitor of the NF-κB cascade (see below).

Fractionation of crude extract

A flow diagram showing the steps followed for the fractionation of the $CHCl_3$ crude extract and isolation of compounds from the aerial parts of *Withania frutescens* is shown in Fig. 20. The $CHCl_3$ extract was fractionated by VLC using a step gradient method (Hex-EtOAc, increments of 10%). Fractions with similar chemical composition were combined and a total of three fractions were obtained (Fractions 1, 2, and 3). Of these, fractions 2 and 3 were selected for further phytochemical examination due the positive biological activity in the first and the complex chemical composition present in the second. A Sephadex LH-20 column was employed to separate chlorophyll from other compounds present in this fraction using dichloromethane as mobile phase. Fractionation of fraction 2 was done by TLC and similar subfractions were mixed together, giving a total of four subfractions. Fraction 3 was fractionated by SPE (DCM-Acetone 4% increments), of which six sub fractions were recovered. All the above fractions and sub fractions were monitored by TLC and sprayed with vanillin-sulphuric acid, followed by heating.

Fig. 20. Bioassay guided fractionation of *Withania frutescens* and isolation of compounds.

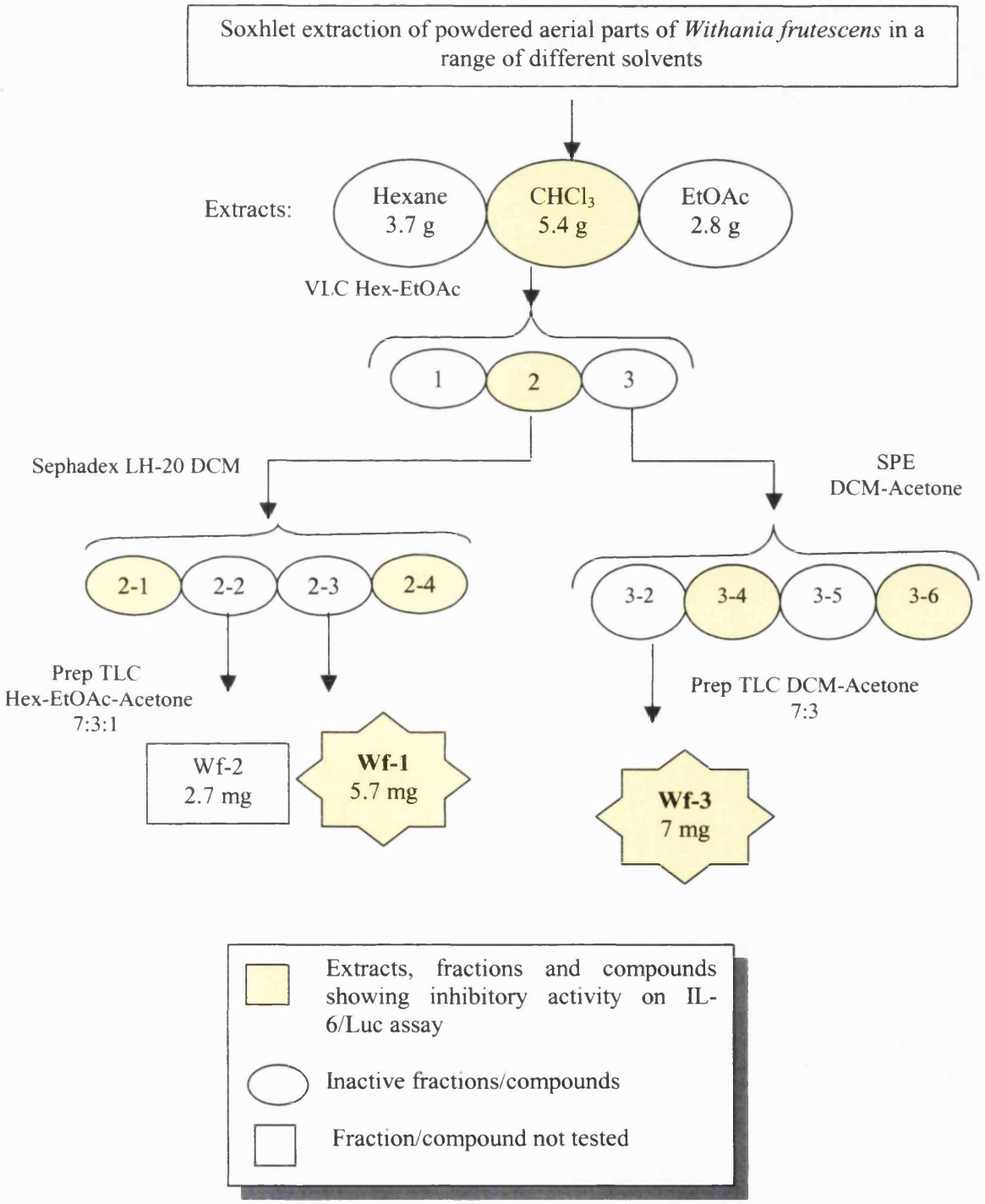
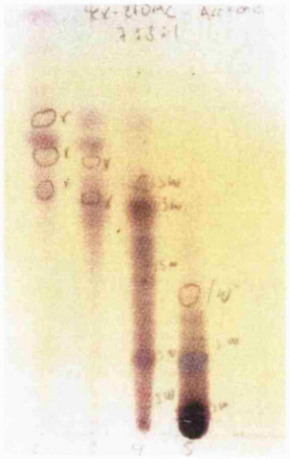
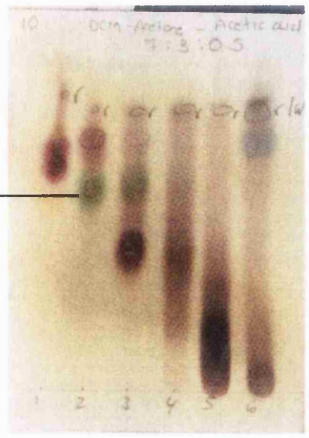


Fig. 21. TLC plates of fractions and compounds obtained from *Withania frutescens*. Spray reagent vanillin-H_2SO_4.

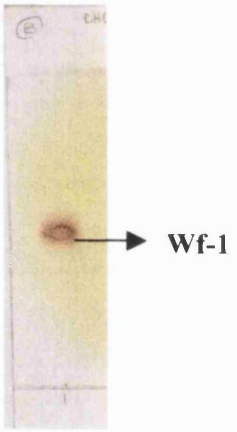


VLC fractions
Solvent
System: Hex-EtOAc-acetone
7:3:1

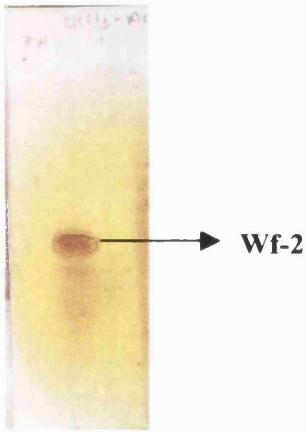


SPE fractions

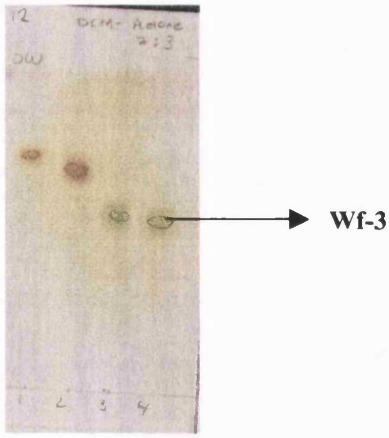
DCM-acetone-acetic acid
7:3:0.5



Solvent system:
$CHCl_3$-acetone 7:3



$CHCl_3$-acetone 8:2



Solvent system: DCM-acetone 7:3

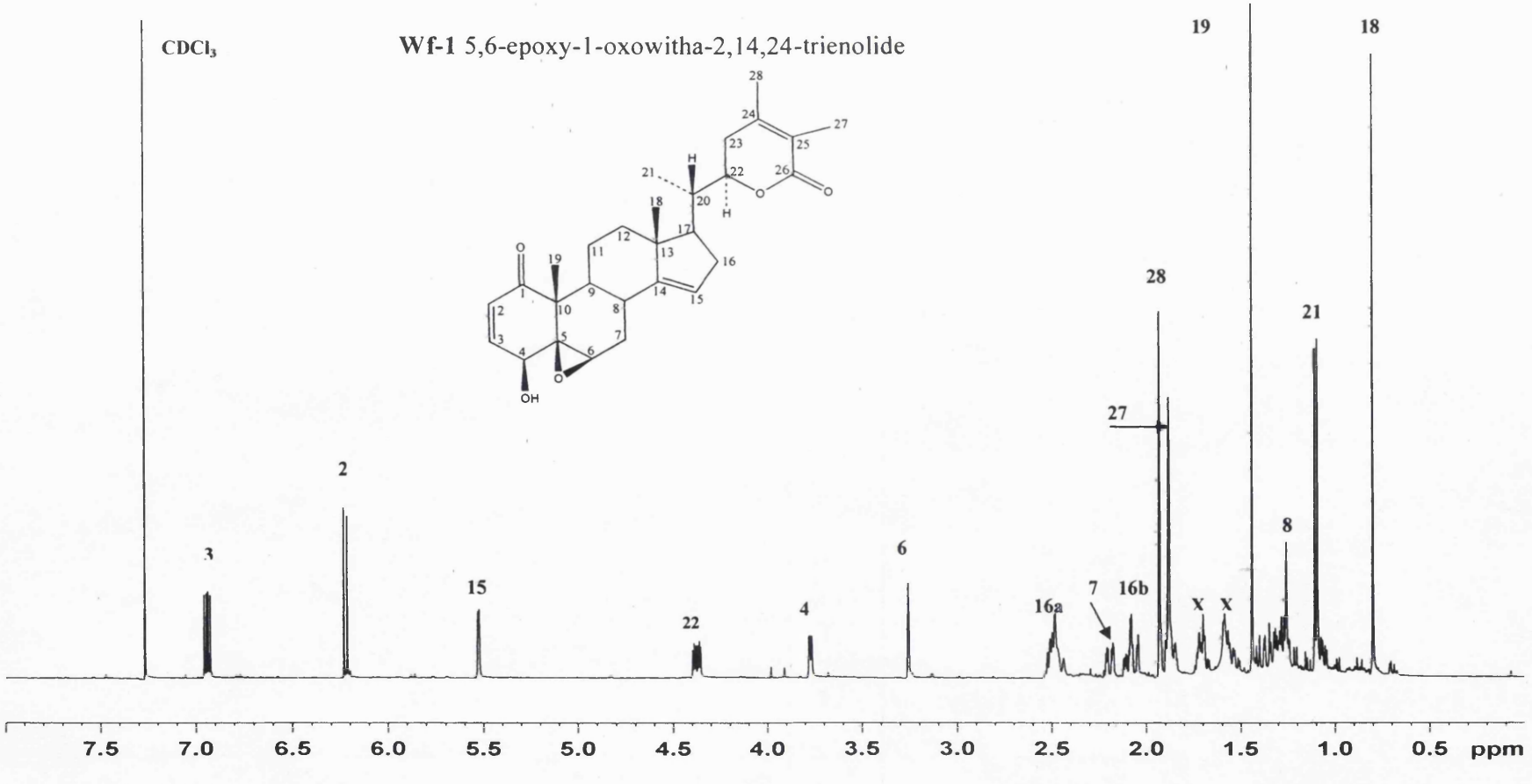
Characterisation of compounds

All the compounds isolated from *W. frutescens* are withanolides. Scanned images of the TLC plates containing the fractions obtained from the $CHCl_3$ extract and the isolated compounds are shown in Fig. 21.

Wf-1. A total of 5.7 mg of this compound was isolated from fraction 2 using preparative TLC (Hex-EtOAc-Acetone 7:3:1, double development) (Fig. 20). Wf-1 was soluble in chloroform and on analytical TLC plates was visible under UV light at short wave (254 nm), and after spraying with vanillin-sulphuric acid it turned pale purple (R_f = 0.46; Fig. 21).

The 1D and 2D NMR analyses indicated that Wf-1 was 5,6-epoxy-1-oxowitha-2,14,24-trienolide. The ES mass spectrum showed an ion peak at *m/z* 453.2627, consistent with the molecular formula $C_{28}H_{36}O_5$ (molecular weight: 452.582; Fig. 22a). Characterisation of this compound was confirmed by comparing the ^1H NMR spectrum obtained (Fig. 22b; Tables 11 and 12) with that reported by Kirson *et al.* (1971). 5,6-epoxy-1-oxowitha-2,14,24-trienolide in comparison to withaferin A had a double bonds in carbons 14 (δ_C 155.5) and 15 (δ_C 124.1, δ_H 5.52 ppm), and five methyl groups at 18 (δ_H 0.79 s), 19 (δ_H 1.43 s), 21 (δ_H 1.10 d), 27 (δ_H 1.87 s) and 28 (δ_H 1.92 s).

Fig. 22. Structure and ^{1}H NMR spectrum of 5,6-epoxy-1-oxowitha-2, 14,24-trienolide (500 MHz, $CDCl_3$). X= unassigned peaks.



Wf-3. The Wf-3 sample contained two compounds, of which only one could be identified. This compound was isolated from fraction 3 by prep TLC (Fig. 20). Wf-3 was colourless and soluble in chloroform (7 mg). By analytical TLC it was visible under UV light at short wave length (254 nm) and after spraying with vanillin-sulphuric acid/heating it turned greenish ($R_f = 0.53$; Fig. 21).

Characterisation of Wf-3 by 1D NMR analysis and comparison of the ^{13}C NMR spectrum obtained from previously published spectral data (Pelletier *et al.*, 1979) (Fig. 23b; Tables 11 and 12) indicated that this compound was withaferin A (5β,6β-epoxy-4β,27-dihydroxy-1-oxo-witha-2,24-dienolide). The ES mass spectrum showed a peak at *m/z* 471.3 [+H] (molecular formula: $C_{28}H_{38}O_6$; molecular weight: 470.59; Fig. 23a). Withaferin A was characterised by having downfield singlets at 202.3 and 167.0 ppm belonging to the carbonyl groups of the α, β unsaturated ketone and the lactone at C-1 and C-26, respectively. The double bond between C-2 and C-3 was assigned to the chemical shifts at 132.3 and 141.8, respectively. The double bond of the α, β-unsaturated lactone appeared at 152.7 and 125.7 ppm. The epoxy ring at carbons 5 and 6 appeared as a singlet at 63.8 ppm, and doublet at 62.4 ppm, respectively. The four methyl groups found in the molecule at δ_C 11.6, 13.3, 17.5 and 20.0 were assigned to C-18, C-21, C-19, and C-28, respectively.

Fig. 23. Structure and ^{1}H NMR spectrum of Withaferin A (500 MHz. $CDCl_3$). X= unassigned peaks; Y= Impurities.

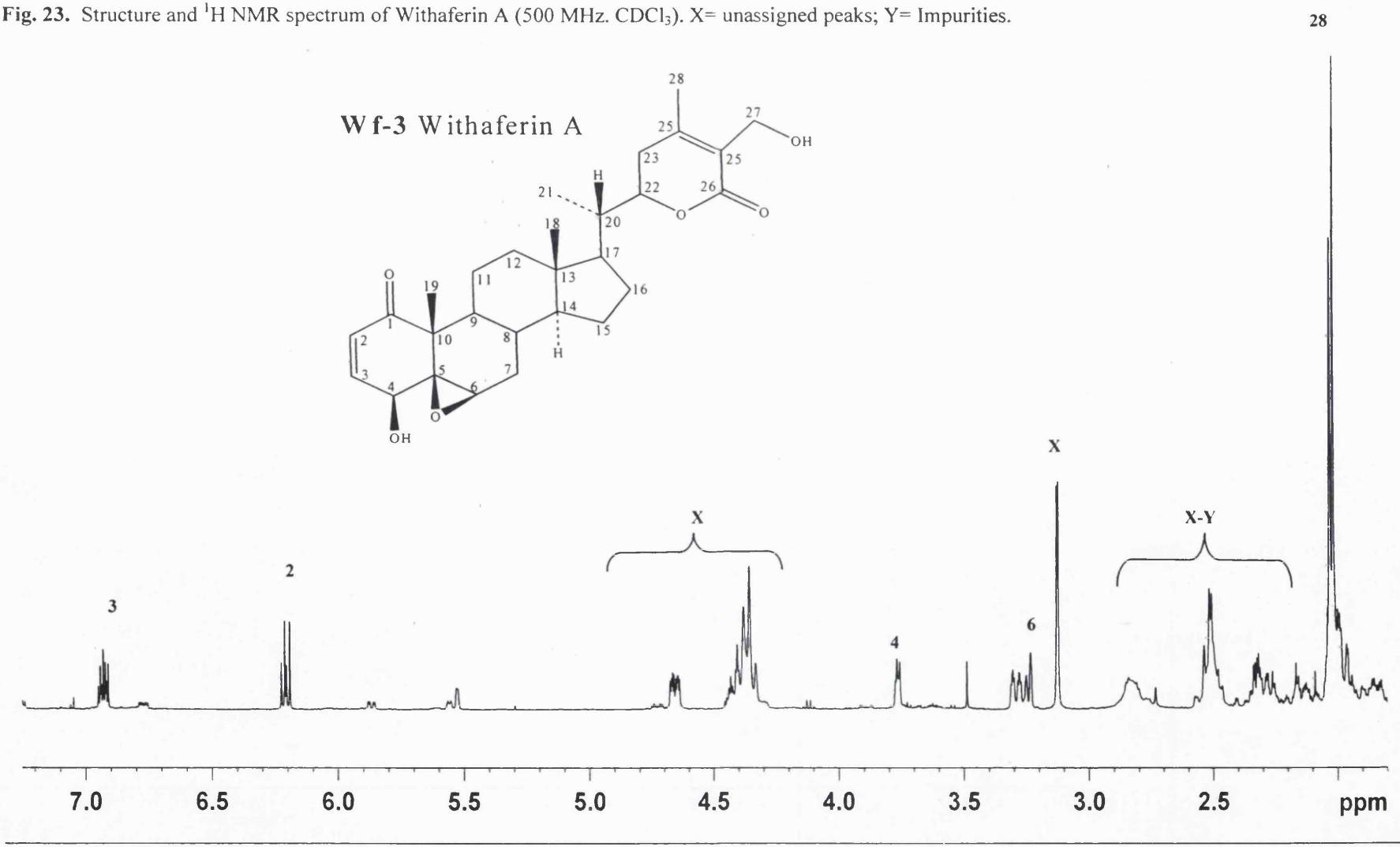


Table 11. ^{13}C NMR (500 MHz) spectral data obtained for withanolides 5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide and withaferin A in $CDCl_3$. † = Previously published spectral data. Chemical shifts in δ ppm.

Carbon	5,6-epoxy-1-oxowitha-2, 14, 24-trienolide	DEPT	Withaferin A	DEPT	Withaferin A†
1	202.0	C	202.3	C	202.3
2	132.6	CH	132.3	CH	132.3
3	141.7	CH	141.8	CH	142.5
4	70.0	CH	69.9	CH	69.8
5	64.1	C	63.8	C	63.9
6	62.4	CH	62.4	CH	61.7
7	31.0	CH_2	29.8	CH_2	29.8
8	28.4	CH	31.2	CH	31.1
9	44.6	CH	44.2	CH	44.0
10	47.9	C	47.7	C	47.8
11	21.7	CH_2	21.8	CH_2	21.8
12	34.4	CH_2	27.3	CH_2	27.2
13	46.8	C	42.6	C	42.5
14	155.5	C	56.1	CH	56.0
15	124.5	CH	24.3	CH_2	24.2
16	32.5	CH_2	39.4	CH_2	39.2
17	57.1	CH	52.0	CH	51.8
18	12.5	CH_3	11.6	CH_3	11.6
19	17.2	CH_3	17.5	CH_3	17.0
20	36.0	CH	38.8	CH	38.7
21	16.3	CH_3	13.3	CH_3	13.3
22	78.5	CH	78.7	CH	78.7
23	31.2	CH_2	29.9	CH_2	29.8
24	148.8	C	152.7	C	153.5
25	122.0	C	125.7	C	125.6
26	166.7	C	167.0	C	167.0
27	20.4	CH_3	57.5	CH_2OH	57.0
28	16.7	CH_3	20.0	CH_3	20.0

† Pelletier *et al.* (1979).

Table 12. ^{1}H NMR spectral (500 MHz) data obtained for withanolides 5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide and withaferin A in $CDCl_3$ and in $CDCl_3$. *‡ = Previously published spectral data. Chemical shifts in δppm (spin multiplicity and coupling constant *J* in Hz).

Proton	5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide	Literature*	Withaferin A	Literature‡
2	6.21 *d* (7.5)	6.23 *d* (10.0)	6.21 *d* (7.0)	3.82 *d* (10.0)
3	6.93 *dd* (9.5, 5.5)	7.00 *dd* (10.0, 6.0)	6.94 *m*	3.05 *dd* (10.0, 6.0)
4	3.76 *d* (5.5)	3.80 *d* (6.0)	3.75 *d* (4.0)	6.25 *d* (.6.0)
6	3.25 *s*	3.26	3.23	6.78
7	2.20 *m*			
15	5.52 *t*	5.68 *t*		
18	0.79 *s* (Me)	0.79 *s*	0.70 *s* (Me)	9.28
19	1.43 *s* (Me)	1.43 *s*	1.41 *s* (Me)	8.58
21	1.10 *d* (7.5) (Me)	1.10 *d* (7.0)	0.99 *d* (6.5)	
27	1.87 *s* (Me)			5.64
28	1.92 *s* (Me)	27 & 28 1.91	2.03 *s* (Me)	7.95

* Kirson *et al.* (1971).

‡ Kupchan *et al.* (1969)

Wf-2. A total of 2.7 mg of Wf-2 was isolated from fraction 2 using preparative TLC (Hex-EtOAc-Acetone 7:3:1, double development) (Fig. 20). This compound was colourless and soluble in chloroform, visible under UV light at short wave (254 nm) on analytical TLC plates, and it turned reddish after spraying with vanillin-sulphuric acid (R_f = 0.46; Fig. 21).

The 1D and 2D NMR analyses suggested that the skeleton of Wf-2 corresponded to a withanolide type compound with 28 carbons, though its complete structure could not be identified (Table 13; Fig. 24a). Comparing the ^{13}C NMR data of this compound with those of withaferin A allowed an assignment of its characteristic functional groups: ketone in C1 (δ_C 216.8), the four methyl groups at C18 (δ_C 17.3, δ_H 1.09), C19 (δ_C 15.7, δ_H 1.25), C21 (δ_C 16.6, δ_H 0.86), and C28 (δ_C 20.2, δ_H 2.04), and the hydroxy-methylene at C27 (δ_C 47.8) (Fig. 24b). This compound also had three double bonds at C5, C16, and C24 (δ_C 169.6, δ_C 124.7, and δ_C 152.3, respectively). In addition, the HMQC spectrum revealed that Wf-2 had a carbon at δ_C 186.8 that corresponded to a proton at δ_H 10.10 whose values were attributable to an aldehyde group, which appeared to be located in ring B according to HMBC correlations (Table 13). This molecule does not have a double bond at C2-C3 in ring A, but instead it appeared to possess two methylene groups (δ_C 34.3 and 31.5).

Fig. 24. Structure and ^{1}H spectrum of withanolide Wf-2 (500 MHz, $CDCl_3$). X= unassigned peaks; Y= impurities.

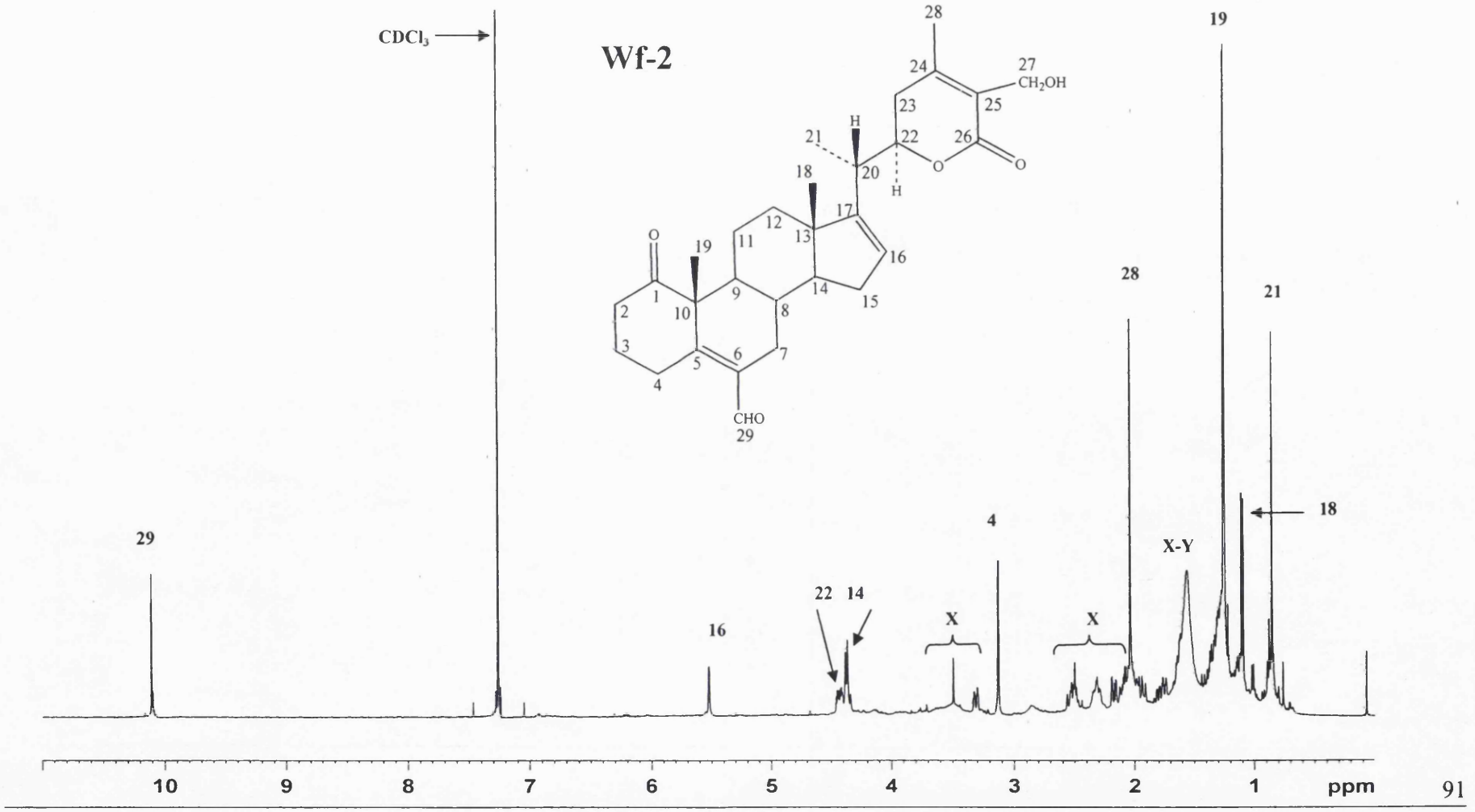


Table 13. ^{1}H and ^{13}C chemical shifts (500 MHz) obtained for withanolide Wf-2 in $CDCl_3$. * = Previously published spectral data. Chemical shifts in δppm (spin multiplicity and coupling constant *J* in Hz; carbons multiplicity based on DEPT experiments). Long-range ^{1}H-^{13}C connectivity detected in HMBC experiments for Wf-2.

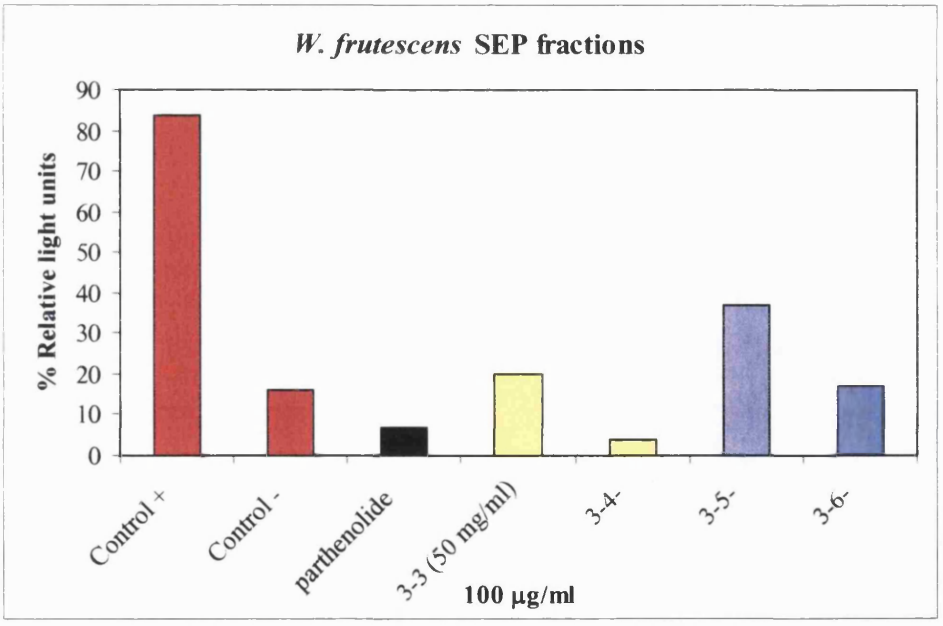
Carbon	Wf-2	DEPT	Proton	HMBC H→C	Withaferin A*
1	216.8	C		H4, 19- C1	202.3
2	34.3?	CH_2	?		132.3
3	31.5?	CH_2	?		142.5
4	39.8	CH_2	3.13 *d* (2.5)	H29-C4	69.8
5	169.6	C		H4, 19-C5	63.9
6	130.2	C		H29-C6	61.7
7	29.9	CH_2	2.85	H19- C7	29.8
8	31.2	CH	2.17 *s*		31.1
9	49.9	CH			44
10	57.4	C			47.8
11	21.6	CH_2			21.8
12	33.3	CH_2		H21-C12	27.2
13	47.5	C		H21-C13	42.5
14	56.5	CH	4.36	H21-C14	56
15	24	CH_2		H8-C15	24.2
16	124.7	CH	5.52 *s*	H18, 21-C16	39.2
17	155.5	C			51.8
18	17.3	CH_3	1.09 *d* (7.0)		11.6
19	15.7	CH_3	1.25 *d* (2.5)		17
20	34.9	CH			38.7
21	16.6	CH_3	0.86 *s*		13.3
22	79.3	CH	4.45 *m*		78.7
23	33.2	CH_2		H28-C23	29.8
24	152.3	C			153.5
25				H28-C25	125.6
26					167
27	47.8	CH_2			57
28	20.2	CH_3	2.04 *s*		20
29	186.8	CHO	10.10 *s*		

* Pelletier *et al.* (1979).

3.2.2. Biological activity

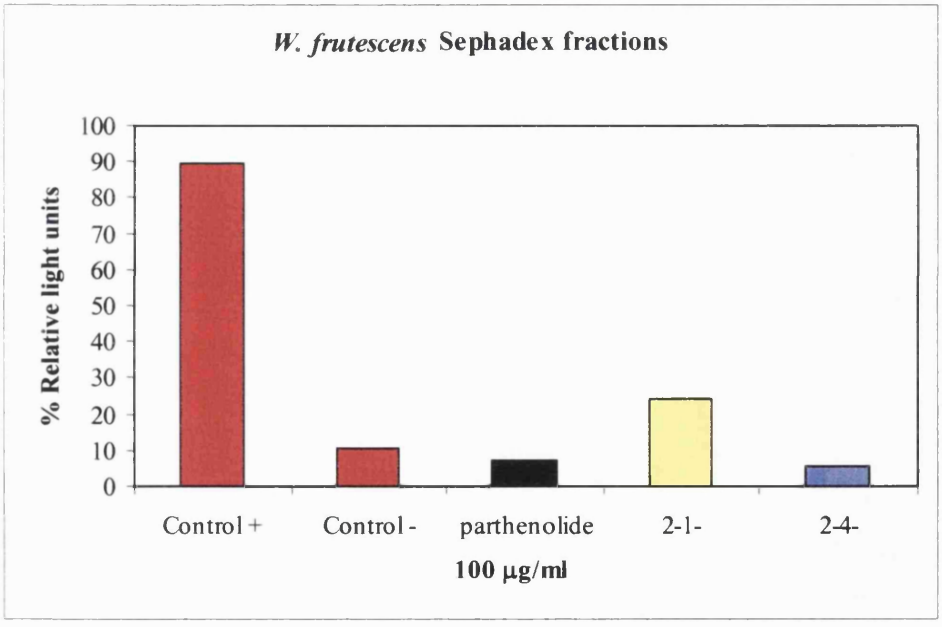
The three crude extracts of *W. frutescens* obtained from Soxhlet extraction were first tested for inhibition of NF-κB in the IL-6/Luc assay at 100 μg/ml. The $CHCl_3$ extract showed a 36% inhibition of NF-κB in IL-6/Luc assay with no cytotoxicity detected according to the mean of three replicates performed (Fig. 25). The $CHCl_3$ extract fractionated by VLC resulted in three large subfractions (fractions 1, 2, and 3; Fig. 20), which were subsequently tested at 100 and 50 μg/ml. Sub-fraction 2 was active at 50 μg/ml (reduced NF-κB activity to 20%), with no cytotoxicity observed (Fig. 25).

Fig. 25. Inhibitory activity of the crude extracts and fractions of *Withania frutescens* against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. Crude extracts and fractions at 100 and 50 μg/ml, respectively. ■ Controls ■ active extract/fraction ■ no active extract. ■ Parthenolide (+ control) at 2.5 μg/ml.



The bioassay-guided fractionation carried out for fraction 2 using a Sephadex® LH-20 column resulted in four subfractions (subfractions 2-1, 2-2, 2-3, and 2-4). Of these, subfractions 2-1 and 2-4 showed a 24% and 5% inhibitory activity at 100 μg/ml, respectively. Sub-fraction 2-4 also showed 50% cytotoxicity on HeLa cells after 7 h of exposure (Fig. 26). Therefore preparative TLC plates were used in order to isolate the possible active compound(s) in these two active fractions; however, their little amount recovered was not enough for NMR analyses or biological assays. 5,6-epoxy-1-oxowitha-2, 14, 24-trienolide (Wf-1), which was isolated from subfraction 2-3, inhibited NF-κB activity at 25 μM to 27% (Fig. 28) and it was observed to show 50% of cytotoxicity towards HeLa cells after 7 h of exposure.

Fig. 26. Inhibitory activity of subfractions of *Withania frutescens* obtained by Sephadex against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. ■ Controls. ■ Parthenolide (+ control) at 2.5 μg/ml.



Fraction 3 on the other hand was fractionated by SPE despite not showing any inhibitory activity at 100 μg/ml. Six subfractions were collected (fractions 3-1, 3-2, 3-3, 3-4, 3-5 and 3-6), of which 3-4 and 3-6 were active at 100 μg/ml, inhibiting 4% and 17% of the positive control, respectively, though subfraction 3-4 showed 50% of cytotoxic on HeLa cells after 7 h of exposure (Fig. 27). The Wf-3 sample, which consists of withaferin A and an unidentified compound (isolated from sub-fraction 3-2), inhibited 16% of the NF-κB activity at 25 μg/ml and showed 50% of cytotoxicity on HeLa cells after 7 h of exposure (Fig. 28).

Fig. 27. Inhibitory activity of subfractions of *Withania frutescens* obtained by SPE from fraction 3-3 against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. ■ Controls ■ active subfractions ■ no active subfractions. ■ Parthenolide (+ control) at 2.5 μg/ml (data not shown).

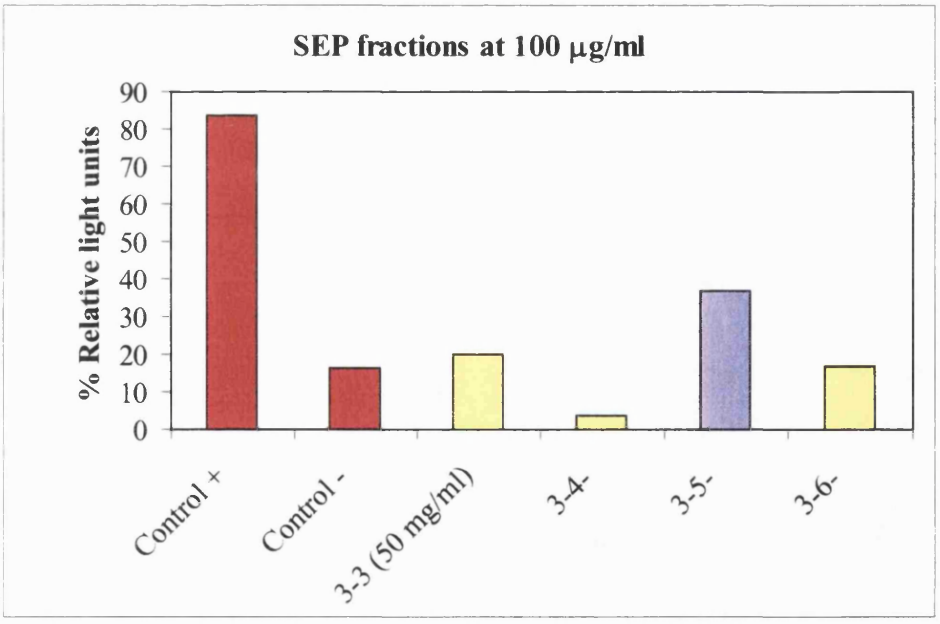
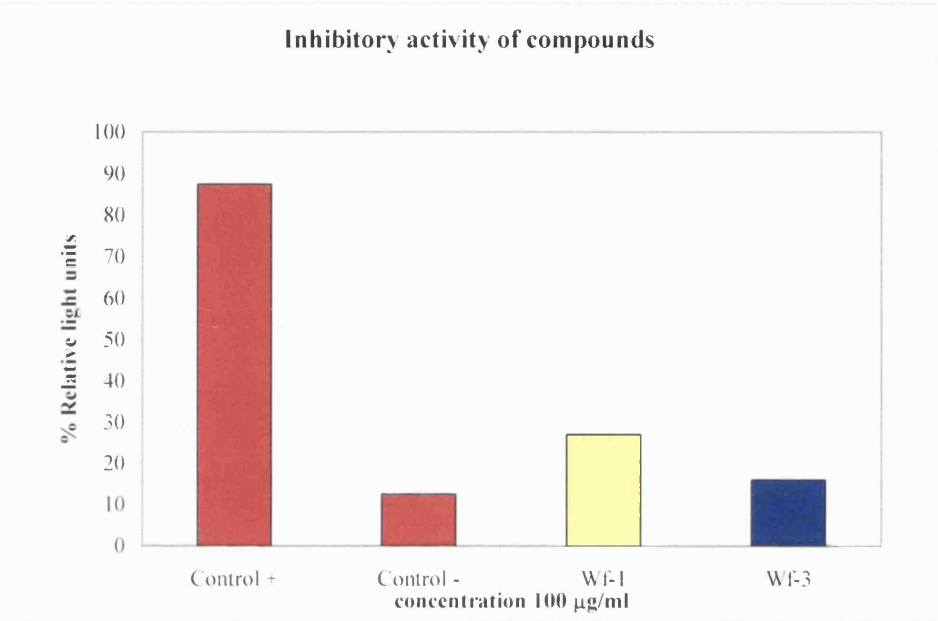


Fig. 28. Inhibitory activity of 5,6-epoxy-1-oxowitha-2, 14, 24-trienolide (Wf-1) and withaferin A (Wf-3) at 25 μM and 25 μg/ml, respectively against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. ■ Controls.



3.3. *Valeriana officinalis*

3.3.1. Phytochemistry

Extraction of underground parts

The EtOAc crude extract recovered from the underground parts of *Valeriana officinalis* (400 g of dry plant in 2000 ml of EtOAc at room temperature) gave a total 9 g of crude extract. This extraction procedure was selected based on a previous plant screening carried out within the AINP project, where the EtOAc showed significant activity as inhibitor of NF-κB in the IL-6/Luc assay.

Fractionation of the crude extract

Fractionation of crude extract was done using two different techniques: Sephadex LH-20 and VLC. For the Sephadex LH-20 technique, four grams of crude extract were dissolved in EtOH and poured onto a Sephadex column, where EtOH was the mobile phase. Fractions of 15 ml were subsequently collected and monitored using TLC and those fractions with a similar chemical composition were mixed together, giving a total number of 15 fractions (Fig. 29). For the VLC technique, 10 g of the EtOAc extract were employed using as mobile phase of 10% increments of Hex-EtOAc. Fractions were collected and monitored by TLC and fractions of similar chemical composition were poured together (Fig. 29).

Fig. 29. Flow diagram showing the methodology followed for the isolation of compounds from *Valeriana officinalis*.

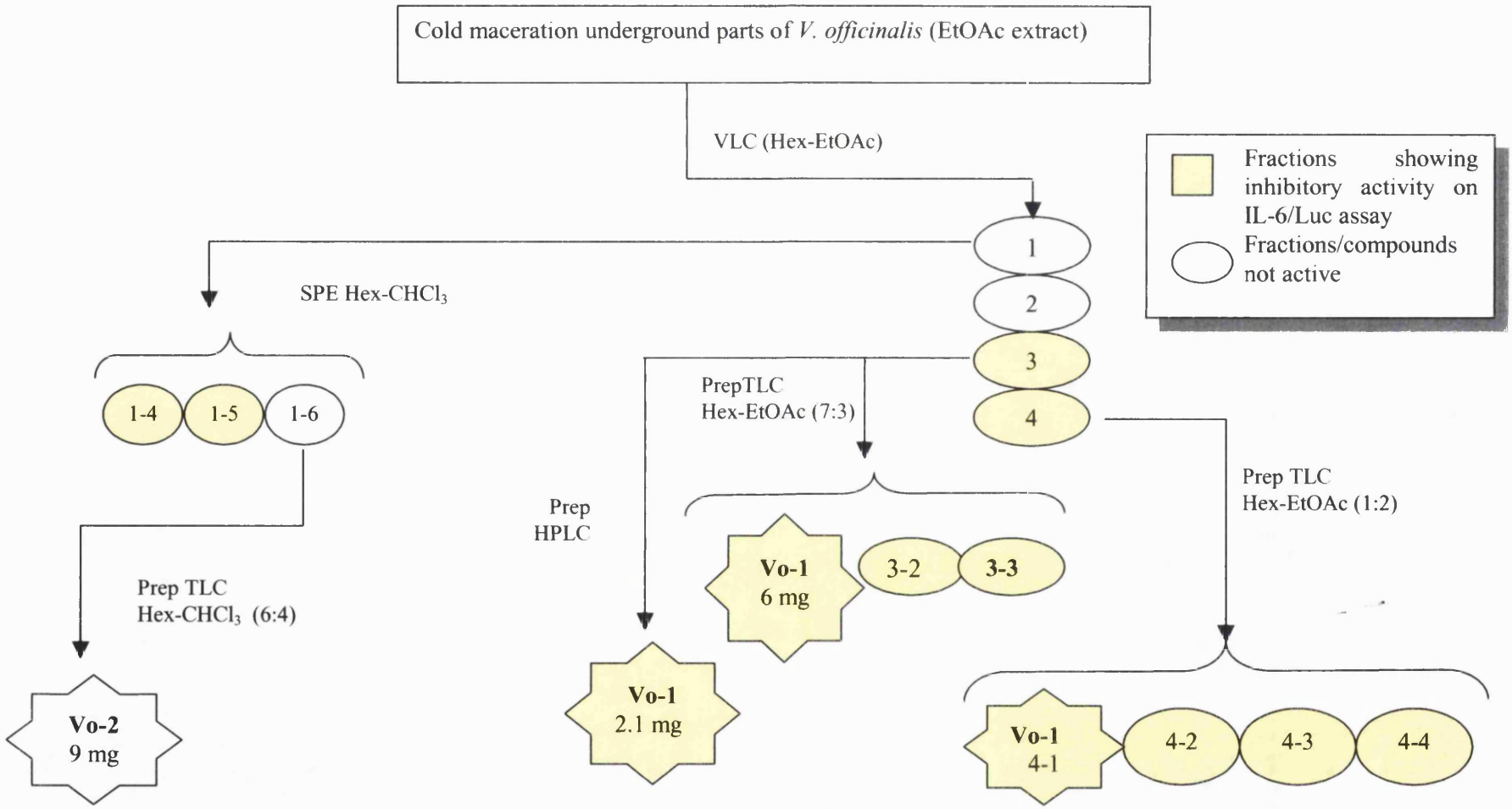


Fig. 29. Continued.

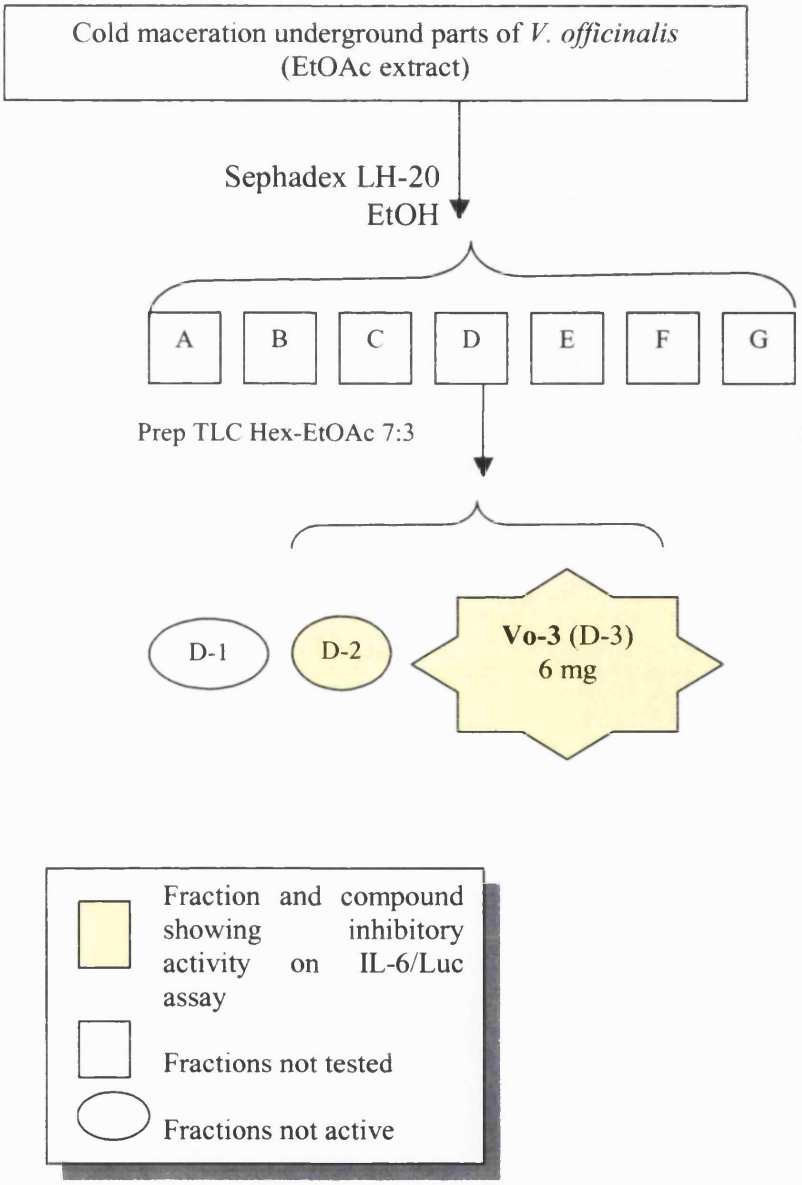
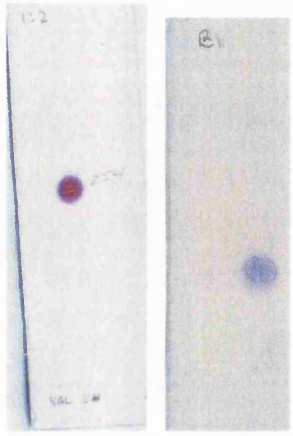


Fig. 30. TLC plates of fractions and compounds obtained from *Valeriana officinalis*. Spray reagent: vanillin-H_2SO_4.

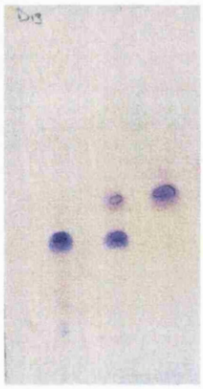


Vo1 **Vo3**

Solvent system: Hex-EtOAc 1:2 1:2

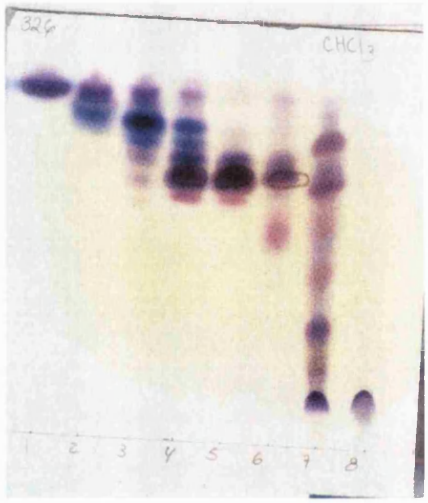
Vo-2

Solvent system: Hex-$CHCl_3$-acetone 1:1



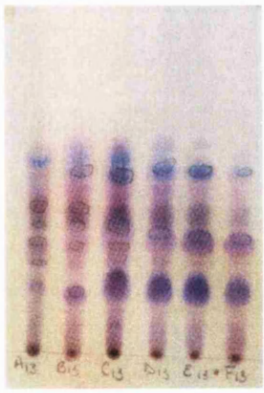
D1 D2 D3 (Vo-3)

Solvent system: Hex-EtOAc 1:2

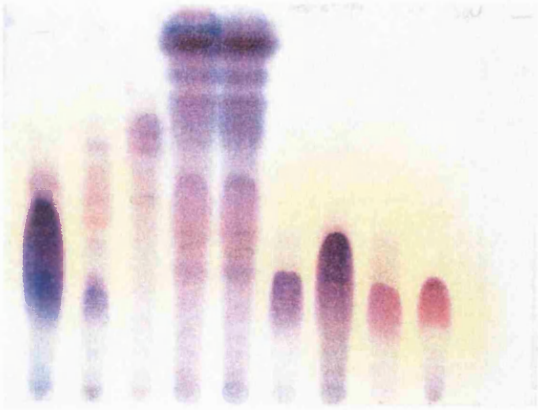


SPE fractions
Solvent system: $CHCl_3$

Sephadex fractions



Solvent system:
Hex-EtOA 7:3



3-1 **3-3** **1-5** **4-2** **Vo-1 (HPLC)**
3-2 **1-4** **4-4** **4-3**
Solvent system: Hex-EtOAc 6:4

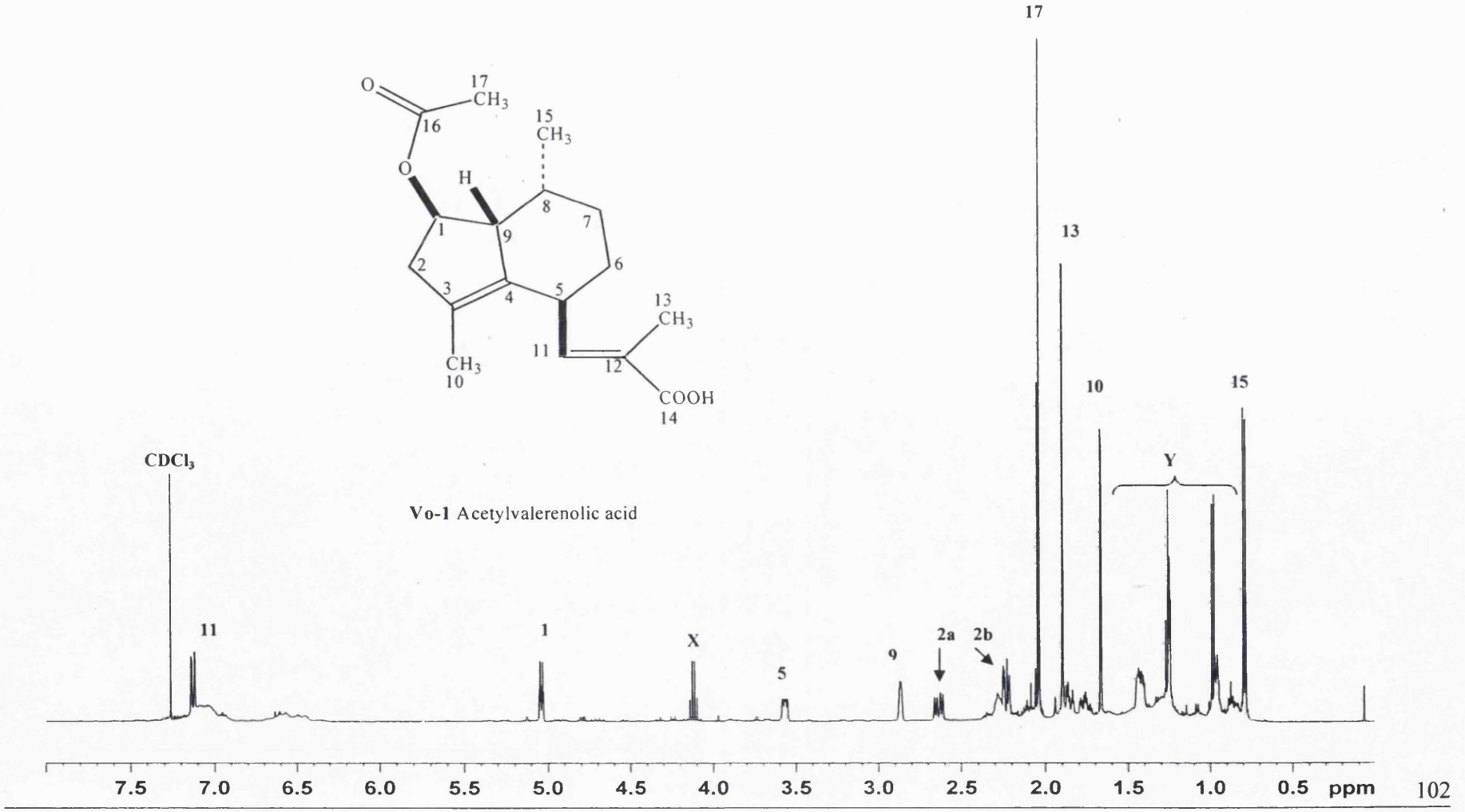
Characterisation of compounds

The following three compounds were isolated from the EtOAc crude extract of *V. officinalis* (Fig. 29).

Vo-1. This compound was isolated from fractions 3 and 4 using preparative TLC (Hex-EtOAc 7:3 and 1:2, 3-1 and 4-1, respectively) (Fig. 29). Six milligrams of the compound were recovered as a yellowish oil, which was visible on a TLC plate under short wave UV light (254 nm) and after spraying with vanillin-sulphuric acid it turned purple (Rf= 0.59; Hex-EtOAc 1:2). Vo-1 was also isolated from fraction 3 by preparative HPLC (gradient method 100% H_20 to 100% ACN) (Fig. 29). In this case, 2.1 mg of the compound were recovered as a yellowish oil, which was visible on TLC plate under UV light short wave (254 nm) and turned purple after spraying with vanillin-sulphuric acid (R_f = 0.3; Fig. 30).

Characterisation of Vo-1 using ^{1}H and ^{13}C NMR spectra in $CDCl_3$ and comparison of the data obtained with those from previously published spectral data (Bos *et al.*, 1986; Dharmaratne *et al.*, 2002) (Fig. 31b; Tables 14 and 15) indicated that it was acetylvalerenolic acid (acetoxyvalerenic acid) (molecular formula: $C_{17}H_{24}O_4$, molecular weight= 292.37; Fig. 31a). Acetylvalerenolic acid is characterised by the presence of an acetoxy group in carbon 1 (δ_C 75.7, δ_H 5.04 dt), a carboxylic acid in carbon 14 (δ_C 172.8), two double bonds in carbons 3 and 11, and three methyl groups at carbons 10, 13, and 15 (δ_C 13.2, 12.1 and 12.7; δ_H 1.66 s, 1.89 d, and 0.78 d), respectively.

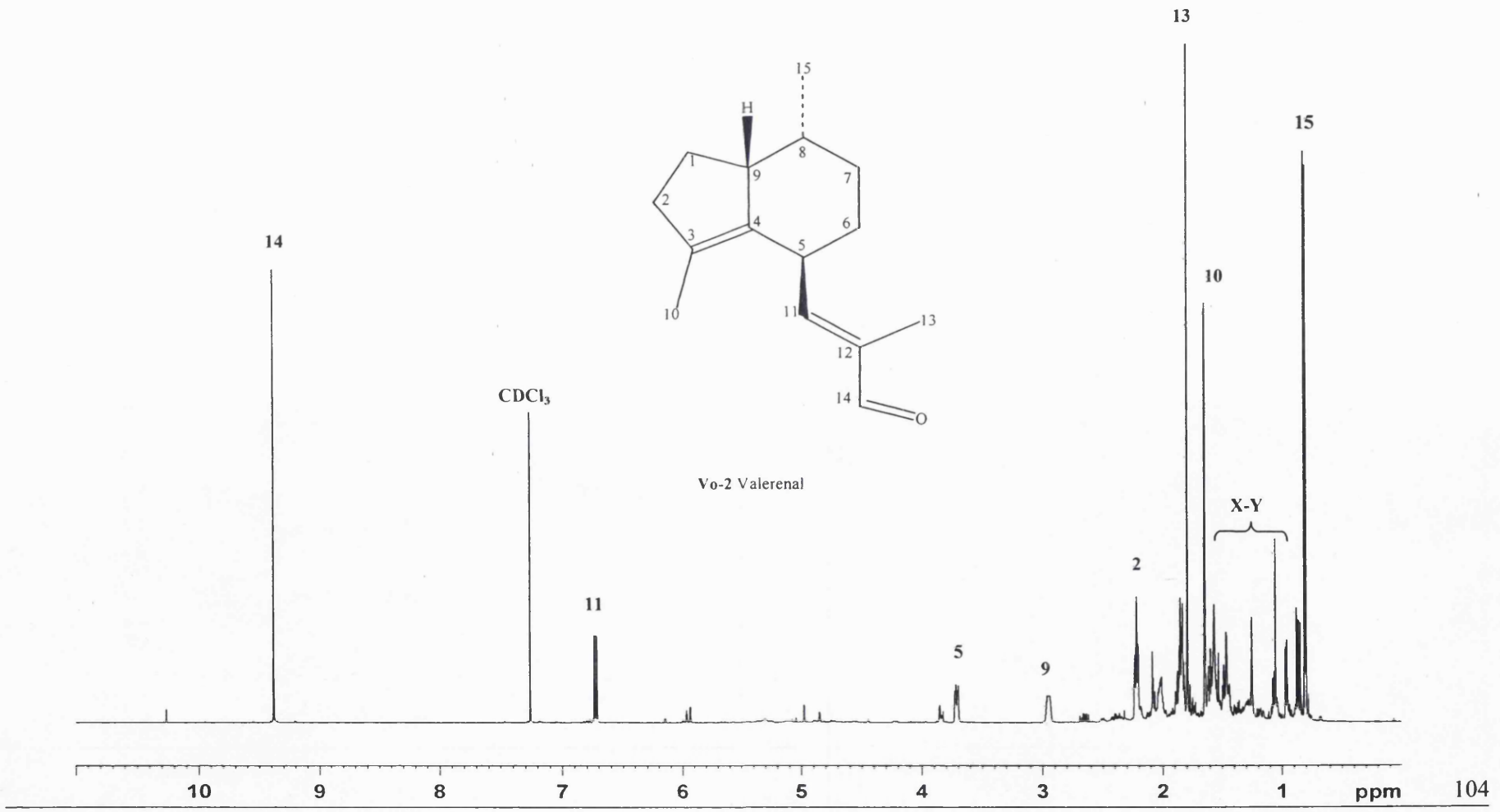
Fig. 31. Structure and ^{1}H spectrum of acetylvalerenolic acid (500 MHz, $CDCl_3$). X= unassigned peaks; Y= impurities.



Vo-2. Vo-2 was isolated separating the fraction 1 by VLC, followed by SPE (Hex-$CHCl_3$ increments of 5%), and then by preparative TLC (Hex-$CHCl_3$ 6:4, double development) (Fig. 29). Nine milligrams of this compound were recovered after preparative TLC in the form of yellowish oil soluble in chloroform. On analytical TLC plates, the compound was visible under UV light at short wave (254 nm) and spraying with vanillin-sulphuric acid showed it up as a purple colour (R_f = 0.65; Fig. 30).

Characterisation of the Vo-2 by 1D and 2D NMR spectra and comparison of the data with data from previously published spectral data (Bos *et al.*, 1986; Dharmaratne *et al.*, 2002) (Fig. 32b; Tables 14 and 15) revealed that was the sesquiterpene valerenal ($C_{15}H_{22}O$, molecular weight= 218.33; Fig. 32a). This compound is mainly characterised by having an aldehyde group in carbon 14 (δ_C 196.0, δ_H 9.38 s), two double bonds at carbons 3 and 11 (δ_C 131.9, and δ_C 155.8, respectively), and three methyl groups at carbons 10 (δ_C 12.0, δ_H 1.64 s), 13 (δ_C 9.2, δ_H 1.79 d), and 15 (δ_C 13.5, δ_H 0.80 d).

Fig. 32. Structure and ^{1}H spectrum of valerenal (500 MHz, $CDCl_3$). X= unassigned peaks; Y= impurities.



Vo-3. Vo-3 was obtained using first a Sephadex column eluted with EtOH and then separating one of its fractions by preparative TLC (Hex-EtOAc 7:3). A total of 6 mg of Vo-3 was recovered as white powder. On analytical TLC plates, Vo-3 was visible under UV light at short wave (254 nm) and after spraying with vanillin-sulphuric acid it turned purple (R_f = 0.52; Fig. 30).

The 1D and 2D spectra of Vo-3 and subsequent comparison of their resulting data with those from previously published spectral data (Bos *et al.*, 1986; Dharmaratne *et al.*, 2002) indicated that this compound was the sesquiterpene valerenic acid (molecular formula $C_{15}H_{22}O_2$, molecular weight = 234.33; Tables 14 and 15; Fig. 33a,b). Valerenic acid was distinguished by having a unique ring system, known as the valerenic acid skeleton. Moreover, on the five-membered ring it possessed two CH_2 groups (δ_C 24.7, δ_C 37.4), a double bond in position 3-4 (δ_C 131.2 and δ_C 133.1), and a methyl group (δ_C 13.5, δ_H 1.63 *s*). In the six-membered ring valerenic acid also had a carboxylic acid side chain (δ_C 173.5), a double bond in carbons 11-12 (δ_C 146.3, δ_C 125.0, respectively), and three methyl groups at carbon 10, 13 and 15 (δ_C 13.5, δ_H 1.63 s; δ_C 12.0, δ_H 1.88 d; δ_C 12.1, δ_H 0.76, respectively).

Fig. 33. Structure and ^{1}H spectrum of valerenic acid (500 MHz, $CDCl_3$). X= unassigned peaks; Y= impurities.

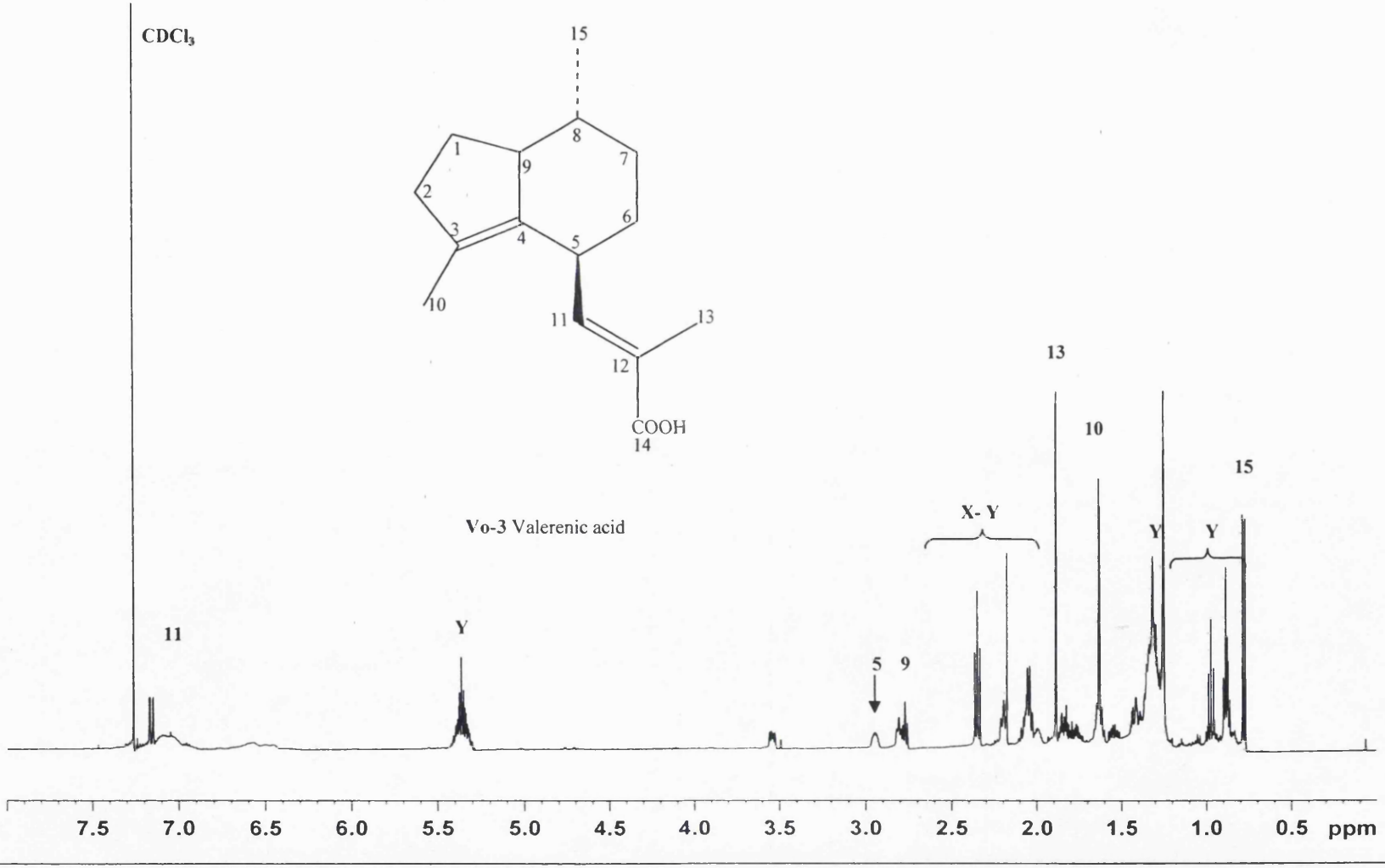


Table 14. ^{13}C NMR (500 MHz) spectral data obtained for sesquiterpenoids valerenal, acetylvalerenolic acid and valerenic acid in $CDCl_3$. *† = Previously published spectral data. Chemical shifts in δ ppm.

Carbon	Valerenal	Literature*	Acetylvalerenolic acid	Literature*	Valerenic Acid	Literature†
1	24.5	24.4	75.7	75.7	24.7	24.9
2	37.4	37.3	44.6	44.6	37.4	37.8
3	131.9	131.8	128.7	128.6	131.3	131.6
4	132.5	132.5	131.7	131.6	133.1	133.5
5	34.7	34.6	34.4	34.4	34.6	35.0
6	25.3	25.3	25.4	25.4	25.6	25.7
7	28.8	28.7	28.5	28.5	28.8	29.2
8	33.0	32.9	31.1	31.2	33.00	33.4
9	47.5	47.4	54.7	54.7	47.4	47.8
10	12.0	11.9	13.2	13.2	13.5	13.9
11	155.8	155.7	145.0	145	146.3	146.6
12	137.4	137.3	125.8	125.9	125.1	125.7
13	9.2	9.1	12.1	12	12.0	12.4
14	196.0	195.5	172.8	173.7	173.5	174.8
15	13.4	13.4	12.7	12.7	12.1	12.3
16 C=O acetate			171.2	171.2		
17 Me acetate			21.3	21.3		

* Bos *et al.* (1986).

† Dharmaratne *et al.* (2002).

Table 15. ^{1}H NMR spectral (500 MHz) data obtained for sesquiterpenoids valerenal, acetylvaleronelic acid anc valerenic acid D in $CDCl_3$.

*† = Previously published spectral data. Chemical shifts in δppm (spin multiplicity and coupling constant *J* in Hz).

Proton	Valerenal	Literature *	Acetylvalerenolic acid	Literature *	Valerenic acid	Literature †
1			5.04 *dt* (2.5, 4.5, 7.5) 2.64 (a)	5.04 *br* d (7.1)		1.80 *m*
2	2.23 *m*		2.23 (b)			
5	3.71 *dd* (4.0, 9.5)	3.71 *dm* (9.4 *d*)	3.56 *dd* (5.0, 9.5)	3.55 *brd* (9.6)	3.54 *q* (5.0, 9.5)	2.57 *dd* (5.5, 9.5)
9	2.96 *m*	2.94 *m*	2.86 *d* (2.0)	2.86 *s*	2.95 *m*	1.85 *m*
10	1.64	0.81 *d* (6.8)	1.66 *s*	1.66 *d* (1.8)	1.63 *s*	1.66 *s*
					7.16 *dd* (1.0, 9.5)	7.16 *d* (9.7)
11	6.73 *dq* (9.5, 1.5)	6.73 *dq* (9.4, 1.3)	7.12 *dd* (1.5, 10.0)	7.14 *ddd* (1.5, 9.6)		
13	1.79 *d* (1.0)	1.79 *d* (1.3)	1.89 *d* (1.0)	1.90 *d* (1.5)	1.88 *d* (1.0)	1.92 *s*
14	9.38 *s*	9.37 *s*				
COOH						12.29 *brs*
15	0.80 *d* (7.0)	1.65 *m*	0.78 *d* (7.0)	0.80 *d* (6.9)	0.76 *d* (6.5)	0.81 *d* (7.0)
17 Me acetate			2.03 *s*	2.04 *s*		

* Bos *et al.* (1986).

† Dharmaratne *et al.* (2002).

3.2.2. Biological activity

The bioassay-guided fractionation of *Valeriana officinalis* using different chromatography techniques yielded a number of fractions that showed inhibitory activity at 100 μg/ml on the IL-6/Luciferase assay stimulated by PMA. The EtOAc extract was active at 100 μg/ml (Fig. 34). Fractionation of the EtOAc extract by VLC resulted in four large fractions (fractions 1, 2, 3, and 4) (Fig. 29). At 100 μg/ml the subfractions 1-4 and 1-5, which were obtained from fraction 1, inhibited NF-κB to 26% and 13%, respectively. At the same concentration fractions 3 and 4 on the other hand reduced NF-κB activity to 3% and 7%, respectively (Fig. 34) though the first one was 50% cytotoxic after 7 h of exposure on HeLa cells. Further fractionation of subfractions 3 and 4 by preparative TLC resulted in three and four subfractions, respectively. All these subfractions were active at 100 μg/ml and no cytotoxicity was observed after 7 h of exposure (Fig. 35). The EtOAc extract fractionated by Sephadex column and preparative TLC yielded two subfractions, D-2 and D-3 (Vo3) (Fig. 29). Subfraction D-2 and compound Vo3 reduced NF-κB activity to 14% and 25% at 100 μg/ml, respectively and no cytotoxicity was observed (Fig. 36).

Three separate samples of Vo-1 were isolated using two different chromatographic methods (Fig. 29). Two of these samples were obtained from fraction 3 by preparative TLC and preparative HPLC, whereas the remaining sample was recovered from fraction 4 using preparative TLC. The three samples of compound Vo-1 showed inhibitory activity at 100 μg/ml (which is equivalent to 342.03 μM) (Fig. 35). The samples of the compound Vo-1 obtained by preparative TLC 3-1 and 4-1 reduced NF-κB activity to 4% and 27%, respectively, and did not

show cytotoxicity towards HeLa cells after 7 h of exposure. The sample of Vo-1 recovered by preparative HPLC on the other hand reduced NF-κB activity to 19% but showed 50% of cytotoxicity towards HeLa cells after 7 h of exposure.

Fig. 34. Inhibitory activity of fractions obtained from *Valeriana officinalis* against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. Fractions obtained by VLC ■ EtOAc extract ■ Controls. ■ Parthenolide (+ control) at 2.5 μg/ml.

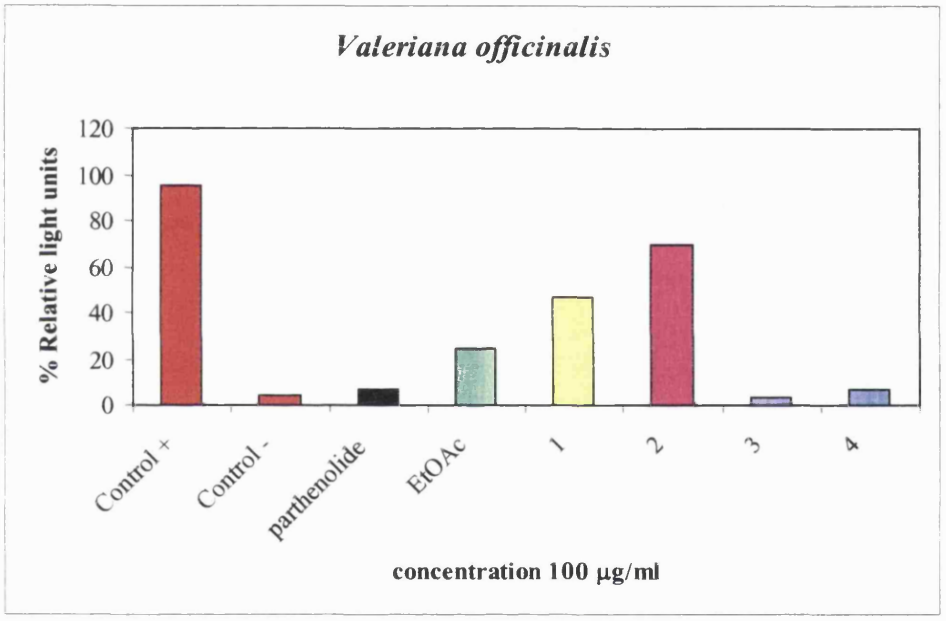


Fig. 35. Inhibitory activity of subfractions of *Valeriana officinalis* and Vo-1 against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. Subfractions obtained by preparative TLC from fractions 3 and 4 at 100 μg/ml. ■Controls ■active subfractions ■Vo-1. ■ Parthenolide (+control) at 2.5 μg/ml.

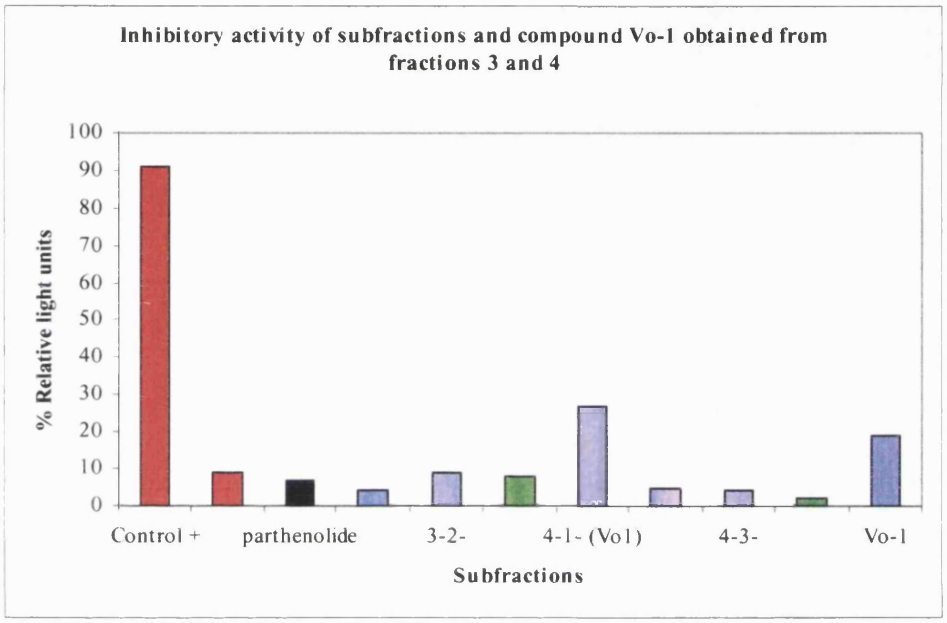
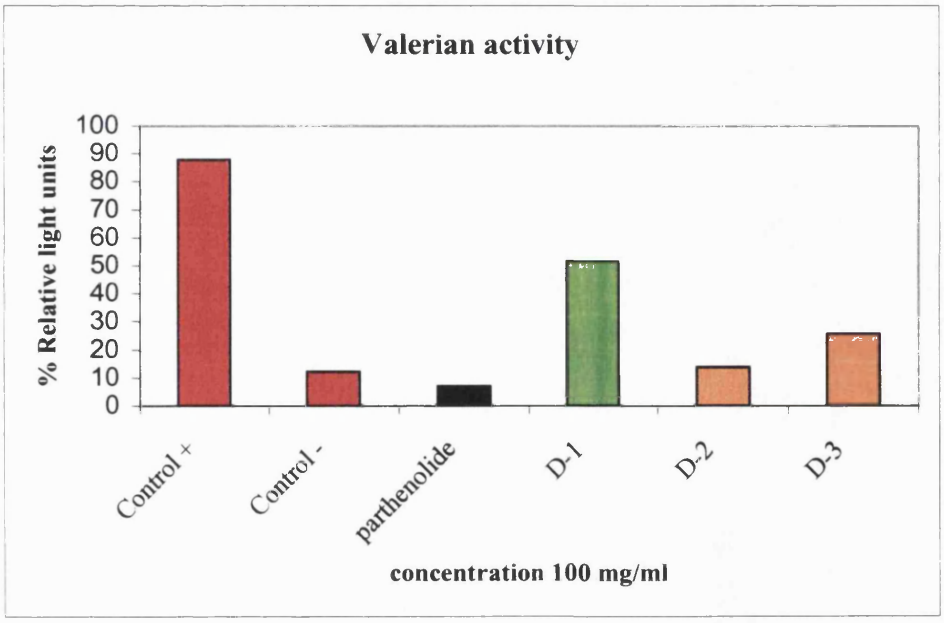


Fig. 36. Inhibitory activity of subractions of *Valeriana officinalis* obtained by Sephadex against NF-κB in HeLa cells stably transfected with a luciferase gene controlled by the IL-6 promoter, using PMA as stimulant at 50 ng/ml. Subfractions obtained by Sephadex column from EtOAc crude extract. ■Controls ■ active subfractions ■ no active subfraction. ■ Parthenolide at 2.5 μg/ml.



CHAPTER 4. DISCUSSION

4.1. *Witheringia solanacea*

4.1.1. Phytochemistry

The procedure most frequently used for the separation of physalins consists of a hot or cold extraction with ethanol, the crude extract is then treated with $CHCl_3$ and partitioned with hexane, and the isolation is completed using silica gel eluted with benzene-EtOAc (e.g. Row *et al.*, 1978a, b; Chiang *et al.*, 1992a, b; Antoun *et al.*, 1981). In this study, the crude extracts recovered from the powdered leaves of *Witheringia solanacea*, which led to the isolation of physalins B, F, and D, were instead obtained using the Soxhlet extraction method. This technique has the advantage of recovering larger amounts of extract and allows the separation of the compounds by polarity, which helps to follow the biological activity of the plant more easily. The Soxhlet extraction, however, requires a prolonged heating of the plant material, and this factor should be considered when the recovered extracts are tested for biological activity due to the possible degradation or decomposition of compounds by overheating.

Since the isolated compounds of *Witheringia solanacea* were obtained from leaves, the content of chlorophyll in the $CHCl_3$ extract was high. Therefore, a Sephadex column eluted with $CHCl_3$ was used first to eliminate the chlorophyll from the $CHCl_3$ extract; however, most of the compounds came out together with the chlorophyll due to the compounds present in the extract being so of high molecular weight. A VLC column was then used, which allowed a better separation by polarity, although traces of chlorophyll were still present in some of the fractions.

The complete removal of chlorophyll from the fractions recovered was finally achieved using a mix of activated carbon and chloroform, which was confirmed by comparing the chemical composition of the fractions before and after cleaning using TLC plates. After this, isolation of the three physalins was straightforward by SPE and preparative TLC as they are visible UV light and therefore easy to detect on the TLC plate.

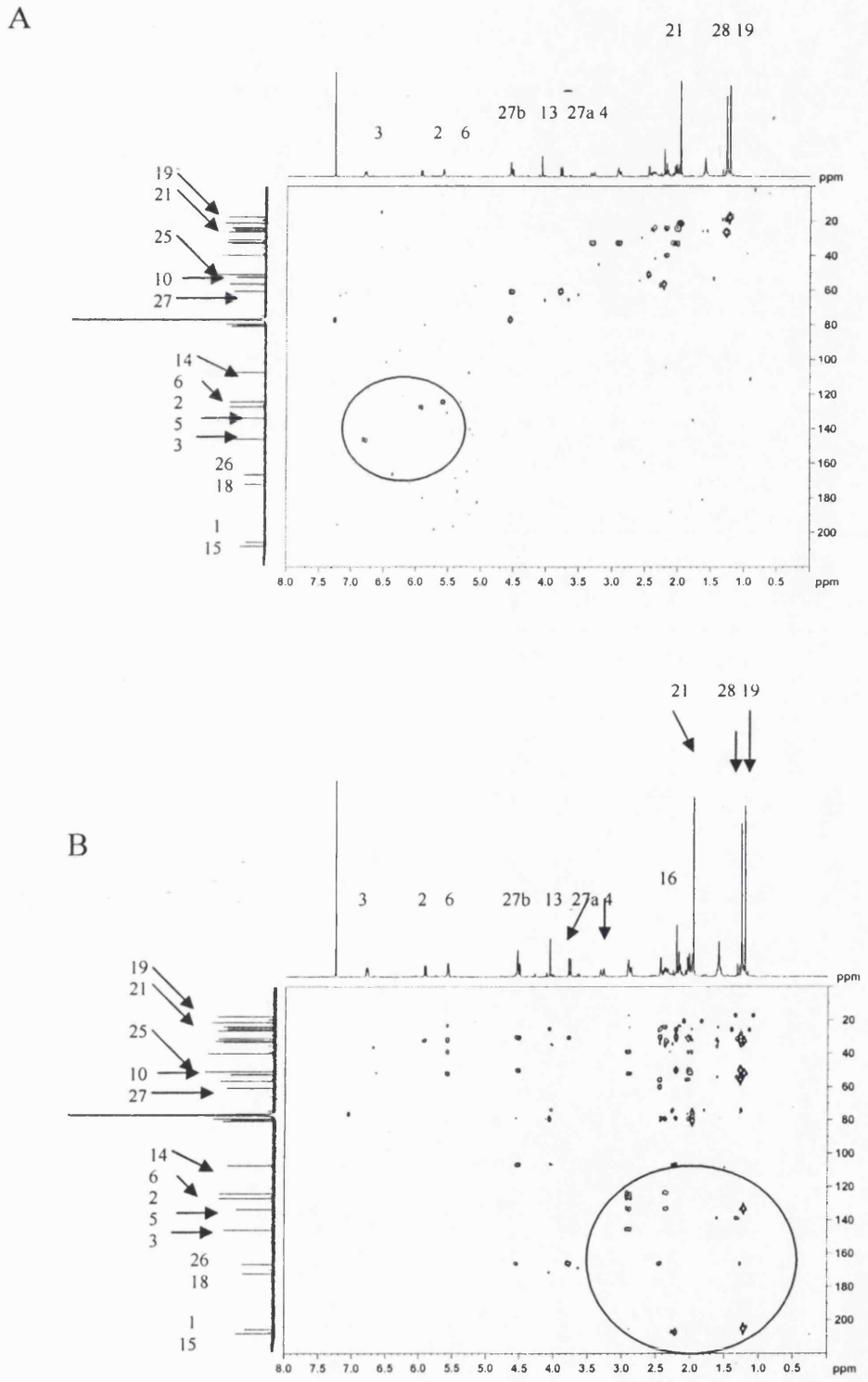
The three physalins isolated from the leaves of *Witheringia solanacea*, physalins B, F and D, have also been isolated from a number of members of the Solanaceae family, including several *Physalis* species, *Brachistus stramoniifolius* and *Witheringia coccoloboides* (see biological activity section). Physalins are highly oxygenated C/D steroids that were firstly reported in *Physalis* species of the Solanaceae family (Matsuura *et al.*, 1970), and subsequently found in other genera of the same family such as *Deprea* (Luis *et al.*, 1994), and *Saracha* (Ripperger and Kamperdick, 1998). Physalins B, F and D belong to the B type physalins, which are characterized by having a C14-O-C27 acetalic linkage (Makino *et al.*, 2002) (see section 1.5.1.).

The structure elucidation of the physalins was done by comparing their ^{1}H and ^{13}C spectra, carrying out HMQC and HMBC analyses, and with the help of previously reported spectral data. The only difference in the structure of physalins B, F, and D is located at position C5-C6 at the A/B ring moiety. Since the chemical shifts values in these physalins are very similar, the following explanation of the elucidation of the molecules only mentions the values obtained for physalin B. In ring A, physalins B, F and D possess a conjugated enone system, which is confirmed by a doublet at δ 5.92 (proton 2) and a doublet of triplets at δ 6.80 (proton 3), corresponding to the α and β protons of the enone system, respectively. In the low

field region are an olefinic proton at δ 5.57 (proton 6), and a singlet that corresponds to a hydroxy group at δ 4.07 (proton 13). At higher field are observed three tertiary methyl singlets at δ 1.21, δ 1.97 and δ 1.27 that corresponded to protons 19, 21 and 28, respectively. These characteristic signals can be compared to those described by Matsuura *et al.* (1970), where physalin B was described for the first time. The NMR data also allowed the identification of other common functional groups present in physalins B, F and D: the methylene oxide bridge between C14 and C25 (C15 δ 207.8; C27 δ 60.4, Ha, δ 3.76 *d J*= 13.0, Hb δ 4.52 *dd*, *J*= 8.5, 4.5), a tertiary hydroxyl at C13 (δ 80.7, δ4.07 s), a ketone at δ 205.5, and a δ-lactone ring at δ 166.4. These values correspond to those previously reported by Row *et al.* (1978b), Antoun *et al.* (1981), Chiang *et al.* (1992a, b), and Makino *et al.* (1995).

As regards the structural differences among physalins B, D, and F, the first can be distinguished by having a double bond in position C5-C6 (δ 133.6, quaternary carbon; and δ 124.2, H δ 5.57 *d J*= 6), which was detected in this study with the HMQC and HMBC analyses (Fig. 37 and 38). Physalin F (Fig. 38) on the other hand has an epoxy ring, which was possible to identify by comparing its resonance to those of physalin B and D. In physalin F, H6 is detected at δ 3.25, indicating that C6 has an oxygen function (in this compound), whereas physalin D (Fig. 38) has two bond correlation between the hydroxyl hydrogen OH-6 (δ 4.31 *m*) and C6 (δ 65.4), which was corroborated by HMQC and HMBC data (Fig. 37). In physalin D, the resonance at C5 at δ 77.6 and C6 at δ 65.4 indicated the presence of carbons bearing oxygen (Fig. 37).

Fig. 37. A) HMQC and B) HMBC correlations for physalin B. 500 MHz NMR, solvent $CDCl_3$.



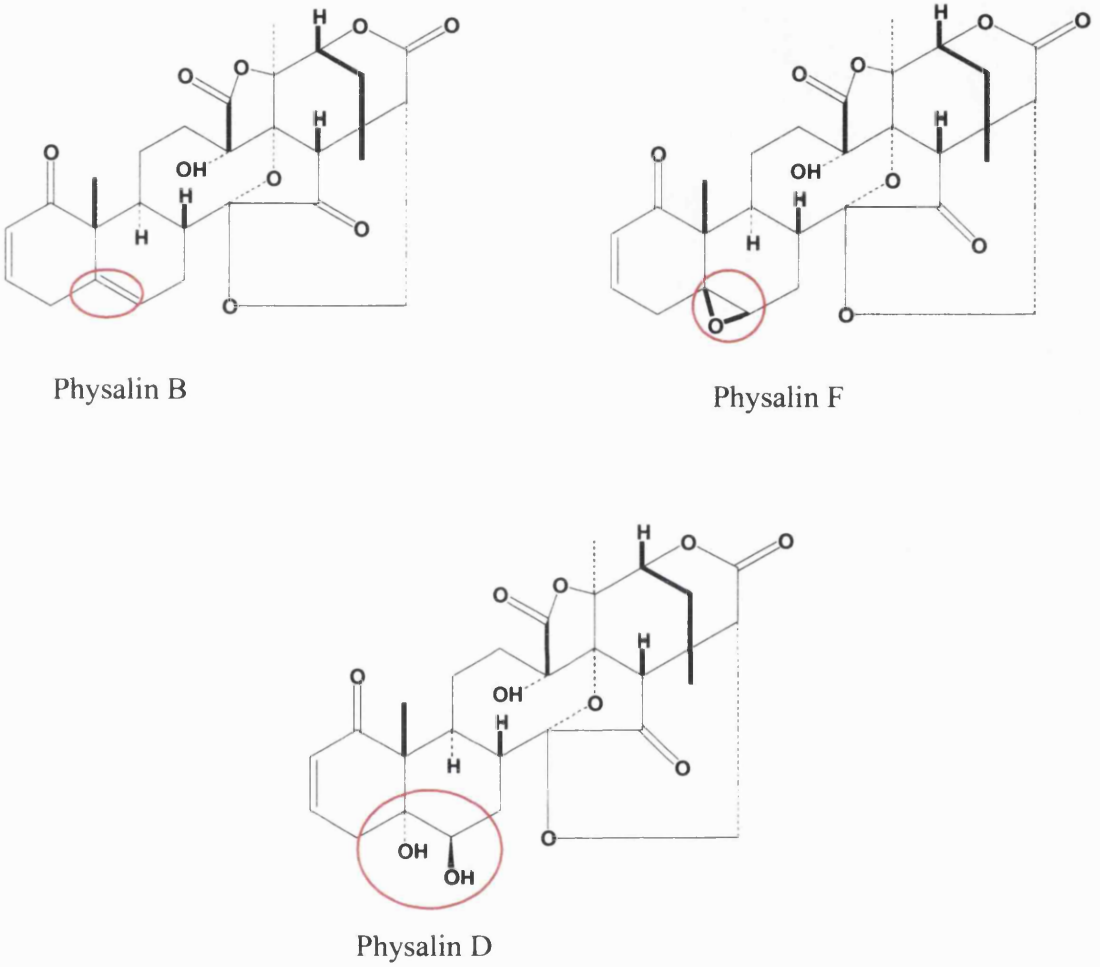


Fig. 38. Physalins B, F and D isolated from *Witheringia solanacea*.

4.1.2. Biological activity

In this study, physalins B and F were found to be highly cytotoxic against HeLa cells after 7 h at different μg/ml concentrations, whereas physalin D was found to be inactive. Owing to the fact that NF-κB regulates the expression of anti-apoptotic genes (Meffert and Baltimore, 2005), it was necessary to identify if the cells were

dying because of the toxic effect of the compounds or due to apoptosis (programmed cell death). The IC_{50} values for physalins B and F both were of 2 μM after 72 h, obtained by MTT assays. At this concentration no cytotoxicity was observed on HeLa cells, which means that the cellular death detected at higher concentrations was occurring due to toxic effects and not because of apoptosis. The aforementioned concentration was therefore used within a concentration gradient for further IL-6/luciferase analyses, which revealed that the maximum inhibitory activity against NF-κB was between 8 to 16 μM, with no inhibitory activity or cytotoxicity at lower concentrations.

Several physalins including the ones isolated herein have previously been evaluated for cytotoxicity and anti-tumour activities, as well as for antimycobacterial properties. For instance, a minimum non-cytotoxic activity has been found for physalins B, C, F, and H against HeLa cells after 72 h, which were of 0.32 μg/ml (647.07 nM), 0.32 μg/ml (626.79 nM), 0.35 μg/ml (664.72 nM), and 1.42 μg/ml, respectively (Fig. 39) (Makino *et al.*, 1995; Kawai *et al.*, 2002). In particular, based on a study using 73 physalins from *Physalis* plants, Kawai *et al.* (2002) concluded that the presence of a conjugated 2-en-1-one moiety at ring A was essential for a high cytotoxic activity of some physalins (among them physalins B, D, and F) against HeLa cells, whereas those physalins with the 2,3-saturated derivatives and the isomeric 3-en-1-one had lower cytotoxicity. Thus, the cytotoxicity found in two of the physalins isolated in the present study could probably be related to the presence of the above functional group.

Cytotoxic activity of physalins B, F and H has also been reported for the solanaceous plant *Brachistus stramoniifolius* against different human cancer cell lines (Fang *et al.*, 2003). In this study, physalin B was found to be highly cytotoxic at an

IC_{50} of 9.6 μM against KB (human nasopharyngeal carcinoma) and LNCaP (hormone-dependent human prostate cancer) cell lines. Moreover, Chiang *et al.* (1992b) found that physalin F showed cytotoxicity *in vitro* by DEA and MTT assays on eight cancer cell lines and three animal cancer lines, whereas physalin D was inactive. These authors attributed the activity of physalin F to the functional epoxy group located at carbons 5 and 6, region where physalin D instead has a 5α-OH and 6β-OH groups. Chiang *et al.* (1992a) subsequently evaluated the effect of physalins B and F on various human leukaemia cells *in vitro*, in which physalin F was the most active. Physalin B and 25,26-epidihydrophysalin C have also been reported to have cytotoxic activity in 9KB nasopharyngeal carcinoma and 9PS mouse lymphocytic leukemia tumour cells *in vitro* (Antoun *et al.*, 1981). These two compounds also possess the conjugated 2-en-1-one moiety at ring A and a double bond in C5-C6, though 25,26-epidihydrophysalin C lacks a C14-O-C27 acetalic linkage.

The results obtained in the present study represent the first report of inhibitory activity of physalin compounds against NF-κB. The inhibitory activity of physalins B (**1**) and F (**2**) against NF-κB (under stimulation with PMA and TNF-α) is also probably related to a double bond in the first one and an epoxy ring in the second, both at positions 5 and 6. This structure-activity relationship was inferred after testing different combinations of the three physalins to see whether the inhibitory activity improved/decreased with the addition/deletion of any of them. The conjugated 2-en-1-one moiety at ring A on the other hand apparently does not have any influence in the anti-inflammatory activity observed in our models. The results of the HIV-1-LTR-Luc assays suggest that the inhibitory activity of physalin F (**2**) is through the NF-κB canonical pathway, which is triggered among others by TNFα (Greten and Karin, 2004).

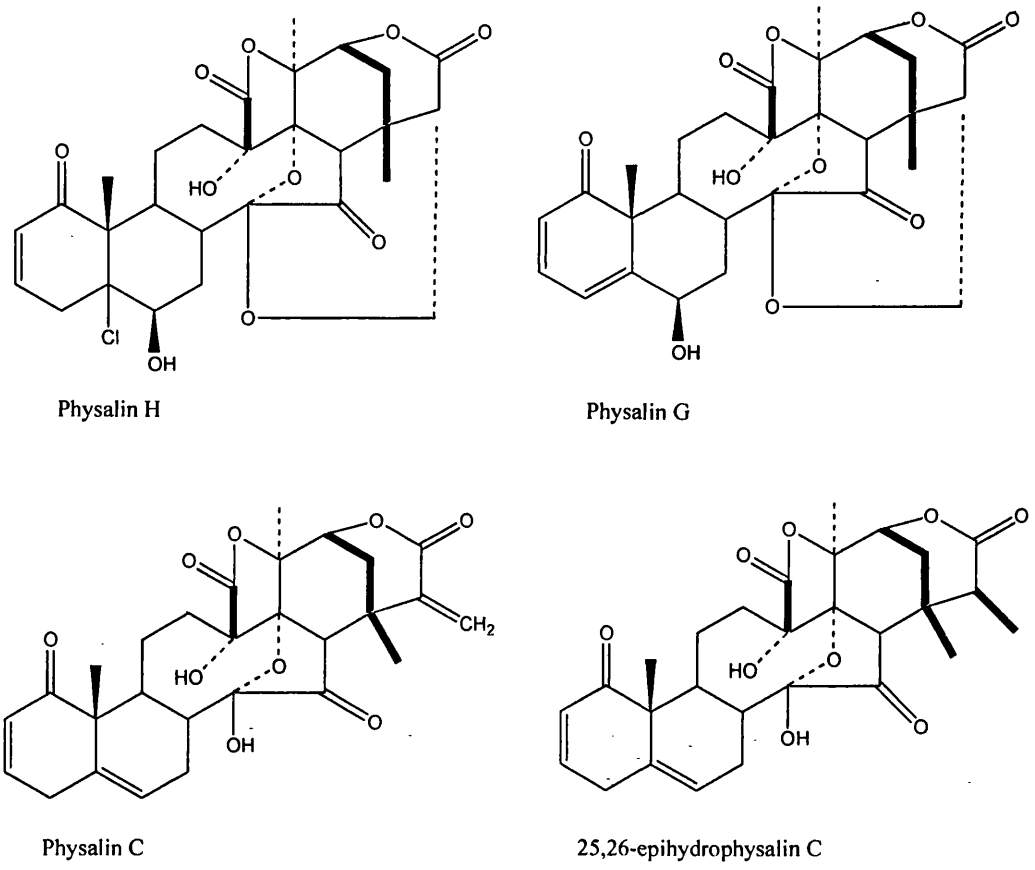


Fig. 39. Examples of physalins with biological activity.

Despite physalin D not being active against NF-κB, previous work demonstrated that it has an important antimycobacterial activity (Januário *et al.*, 2002). In this study, a fraction containing physalins B, F and D obtained from crude extract of *Physalis angulata* exhibited a minimum inhibitory concentration (MIC) value of 32 μg/ml against *Mycobacterium tuberculosis* H_{37} Rv strain. However, further isolation and testing of physalins B and D revealed that the latter had the lowest MIC value, thus suggesting that physalin D plays the most important role in the antimycobacterial activity displayed.

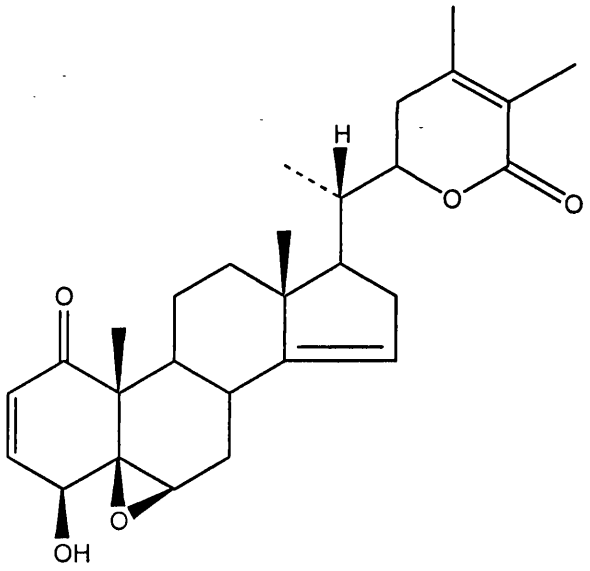
Among the most investigated compounds that inhibit NF-κB activation are the sesquiterpene lactones. For instance, the sesquiterpene lactone parthenolide has been reported to inhibit NF-κB in HeLa cells at concentrations of 5-10 μM using luciferase as a reporter gene (Hehner et al, 1998; Kwok *et al.*, 2001). Nonetheless, a recent study demonstrated that sesquiterpene lactones directly attack the enzyme luciferase, which could give in false positive results for inhibition of NF-κB, though the enzyme β-galactosidase remains unaffected (Lindenmeyer *et al.*, 2004). Thus, these findings highlight the importance of using more than one 'reporter enzyme' when measuring NF-κB inhibition. The mechanism of action of sesquiterpene lactones as inhibitors of NF-κB has been suggested to be due to the reactivity of α, β- unsaturated carbonyl groups, reacting via a Michael-type reaction (Rüngeler et al, 1998, 1999). Interestingly, physalins B, F and D also have a δ-lactone functional group, though not extracyclic, no structure-activity relationship was found for it in this study.

4.2. *Withania frutescens*

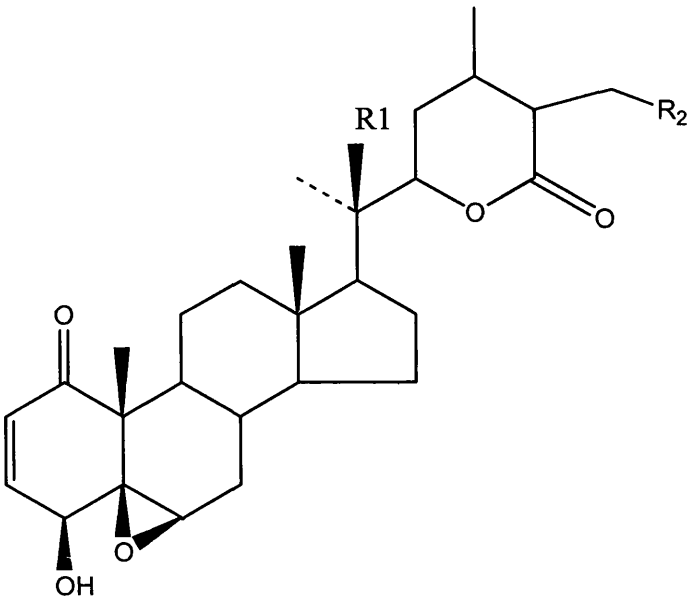
4.2.1. Phytochemistry

In the chemical composition of *Withania frutescens* reported by González *et al.* (1971, 1974b) the extraction procedure for the six withanolides isolated used hot ethanol, the resulting extract was then diluted with water and extracted again with EtOAc, and a silica gel column was finally carried out using benzene-EtOAc. Of the extractions of *Withania frutescens* recovered in the present study using the Soxhlet apparatus, the $CHCl_3$ extract was the only one that inhibited the NF-κB activation and therefore it was further fractionated until three withanolides were obtained.

Three withanolides were isolated from the aerial parts of *Withania frutescens*: 5,6-epoxy-1-oxowitha-2,14,24-trienolide (Wf-1), withaferin A (Wf-3), and a withanolide compound with an aldehyde functional group whose elucidation could not be completed (Wf-2). Withanolides are natural steroids usually present in solanaceous plants. They are characterised by having an ergostane skeleton, in which carbons 22 and 26 are oxidized in order to form a δ-lactone ring, known as the withanolide skeleton (Glotter, 1991). Withaferin A and 5,6-epoxy-1-oxowitha-2,14,24-trienolide belong to the group of withanolides with a 5β, 6β-epoxy-4β-hydroxy-2-en-1-one (C5 δ 64.0, C6 δ 62.4, H6 δ 3.25) pattern in rings A and B, and an unsaturated δ-lactone in the side chain (C26 δ 166.7) (Fig. 40). Among other compounds from this group found in solanaceous plants are 27-deoxywithaferin A, withanolide D, 27-hydroxywithanolide D, and withangulatin (Glotter, 1991). Moreover, withaferin A was isolated together with other compounds that could not be identified, and therefore, its elucidation was confirmed by comparison of its ^{13}C spectrum with the one reported by Pelletier *et al.* (1979) for withaferin A.



Wf-1 5,6-epoxy-1-oxowitha-2,14,24-trienolide



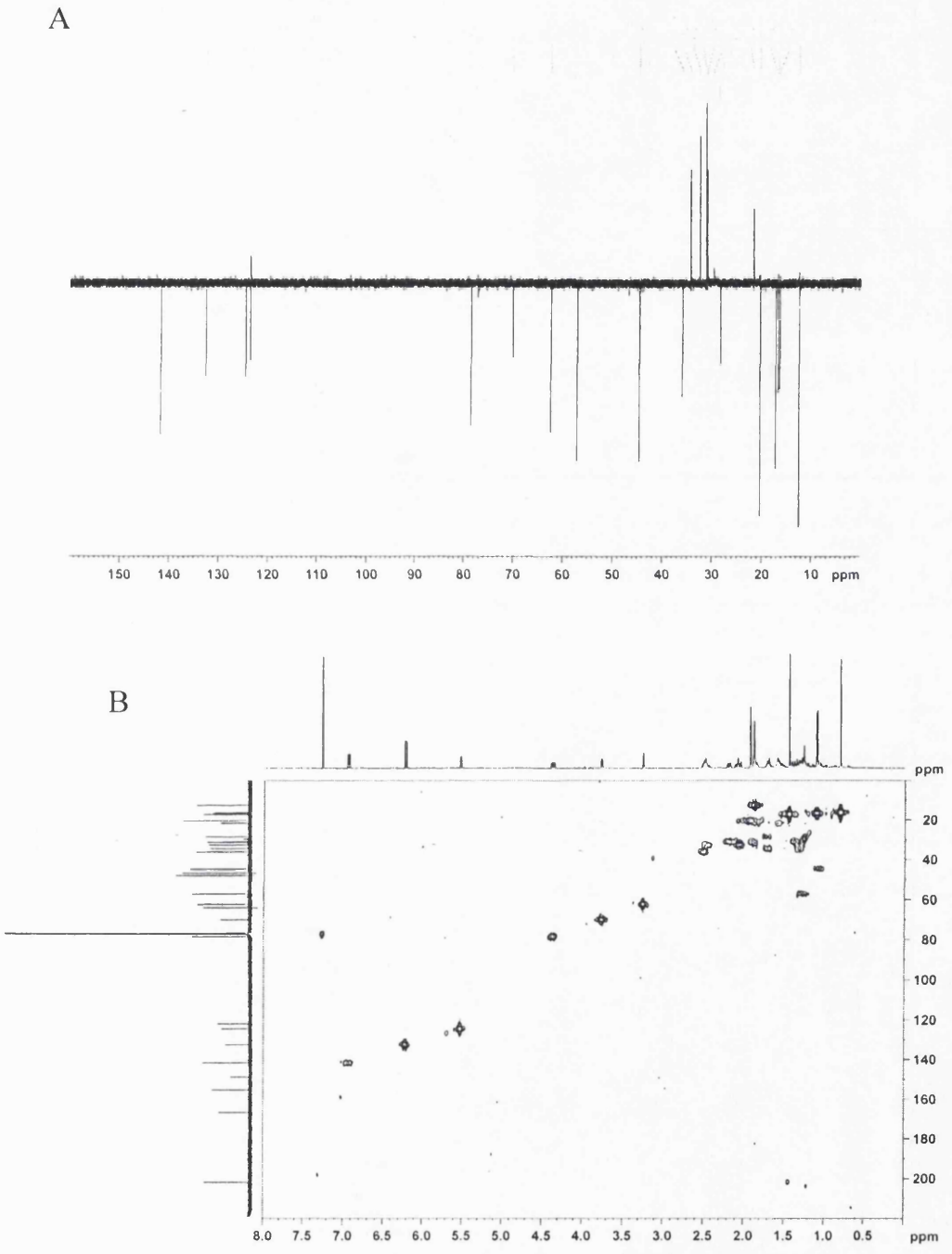
(a) 27-deoxywithaferin A R1=R1=H
(b) withaferin A R1=H, R2=OH
(c) 27-hydroxywithanolide D R1=R2=OH
(d) withanolide D R1=OH, R2=H

Fig. 40. Examples of 5β,6β-epoxy-4β-hydroxy-2-en-1-one type withanolides.

5,6-epoxy-1-oxowitha-2,14,24-trienolide has been isolated from *W. somnifera* and *W. aristata* (González *et al.*, 1974a; Kirson *et al.*, 1971), but it has not been reported for *W. frutescens* and the ^{13}C NMR data included in this work is the first presented. The additional double bond at C14-C15 (C14 δ 155.5; C15 δ 124.5, H15 δ δ 5.52 *t*) was assigned according to the long range H→C HMBC correlations. The methyl groups at positions 19 and 28 correspond to that of Wf-3 (H19 δ 1.43; H28 δ 1.92 s, respectively), whereas signals for the 18 and 21 methyl protons (H18 δ 0.79 *s*, H21 δ 1.08 *d* *J*=7.0, respectively) are shifted downfield compared to withaferin A, leading to the assumption that a double bond is in ring D. Compared to withaferin A, 5,6-epoxy-1-oxowitha-2,14,24-trienolide differs at C27 by having a

methyl group at δ 1.87 *s* and δ 17.7 ppm, which could be determined by DEPT and HMQC analyses (Fig. 41).

Fig. 41. A) DEPT analysis and B) HMQC correlations for 5,6-epoxy-1-oxowitha-2,14,24-trienolide. 500 MHz, solvent $CDCl_3$.



The unidentified compound isolated (Wf-2) also has a typical withanolide structure consisting of 28 carbons. Fig. 42 illustrates a proposed structure based on the available spectroscopic data. In ring A, the classic double bond at C2-C3 is not present in this compound, but instead it appears to have two methylene groups (δ_C 34.3 and 31.5), whose peaks appear as CH_2 in DEPT analysis. Additionally, Wf-2 has a third double bond at C16-C17 (δ 124.7 and δ 155.5, respectively), which was assigned using HMBC correlations (Fig. 42). Moreover, in the ^1H and ^{13}C spectra it had a characteristic signal for an aldehyde group (δ_H 10.10, δ_C 186.8), which according to the H→C long correlations is situated in ring B as a side chain (Fig. 42). The rest of the molecule is the same as withaferin A, and the carbons were assigned comparing the spectral data of the two compounds.

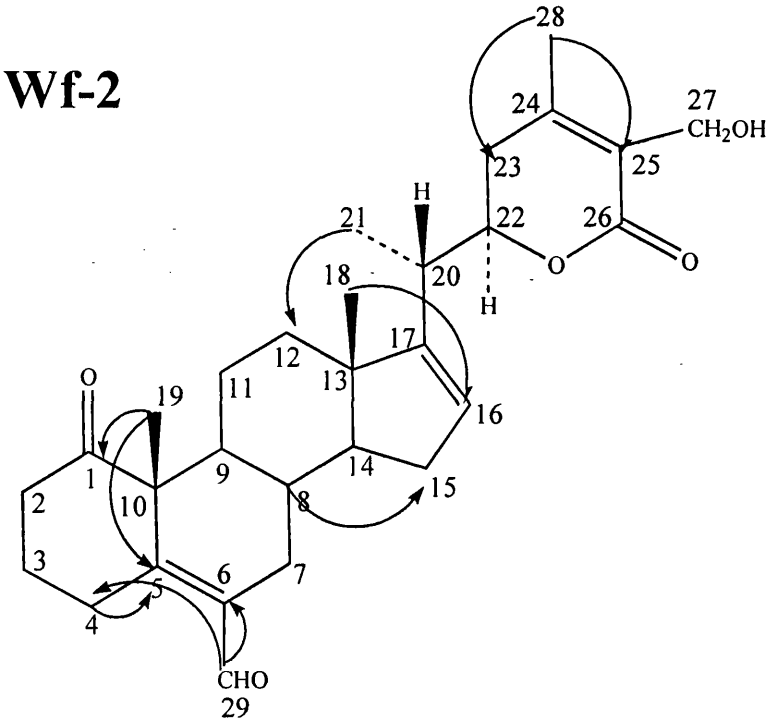


Fig. 42. Selected HMBC correlations for withanolide Wf-2.

4.2.2. Biological activity

Withanolides have been found to be the active compounds of some species of *Withania* and to have cytotoxic, anti-inflammatory, and antibacterial properties (Budhiraja and Sudhir, 1987). For instance, an Asian species of *Withania*, *W. somnifera*, is one of the most important remedies used for anti-inflammatory conditions in Indian Ayurvedic medicine, and its phytochemistry has been investigated extensively (see, Kirson *et al.*, 1971, Abraham *et al.*, 1975 and Ali *et al.*, 1997 for a review of the topic). However, the anti-inflammatory properties of the related European species *W. frutescens* had not been assessed before the present study despite the fact that its chemical composition is very similar to that of *W. somnifera* (González *et al.*, 1971; González *et al.*, 1974b).

The $CHCl_3$ extract obtained from the aerial parts of *W. frutescens* and two of its withanolides isolated were inhibitors of NF-κB controlled by the IL-6 promoter. Moreover, of the three withanolides isolated, 5,6-epoxy-1-oxowitha-2, 14, 24-trienolide (Wf-1) and withaferin A (Wf-3) showed an inhibitory activity against NF-κB in the luciferase assay at 25 μM and 25 μg/ml, respectively, though both were cytotoxic against HeLa cells after 7 h of exposure. These inhibitory effects are in agreement with Mohan *et al.*'s (2004) study, where the root extract of *W. somnifera* (at 50 μg/ml) and its HPLC-purified withaferin A (at a non-cyctotoxic concentration of 1μM) inhibited TNF-α induced NF-κB activation in human umbilical vein endothelial cells (HUVEC). The results obtained in my study thus support withaferin A as an effective anti-inflammatory compound through inhibition of NF-κB, in this case using a different promoter (IL-6). This inhibitory activity against NF-κB was previously reported for the methanolic extract of *W. somnifera* (Iuvone *et al.*, 2003).

The authors suggested that *W. somnifera* increased iNOS-derived NO production in J774 macrophages *in vitro* through NF-κB transactivation. This is explained due to the fact that several genes involved in the immune response are regulated at transcriptional level by NF-κB.

The anti-inflammatory activity of withaferin A has also been proven in a *in vivo* study (Sethi *et al.*, 1970), where the authors mentioned that the anti-inflammatory effects of this compound seem to be more potent than hydrocortisone in adjuvant induced arthritis syndrome in rats. A similar result was observed with withanolide 3β-hydroxy-2, 3-dihydro-withanolide F, which has an effect in sub-acute inflammation at 10 mg/kg and therefore more potent than phenylbutazone and hydrocortisone (Budhiraja *et al.*, 1984).

Mohan *et al.* (2004) proposed that the mechanism of action of withaferin A as inhibitor of NF-κB in HUVEC occurs by interference with ubiquitin-mediated proteosome pathway, which was observed by the increment in the levels of poly-ubiquitinated proteins. As regards the present study on *W. frutescens*, it is not possible to say at this stage what mechanism of NF-κB inhibitory activity can be derived from the IL-6/Luc assay. There is evidence, however, that shows that withaferin A has mitotic activity at metaphase on HeLa, H. Ep, and EAC cells (Shohat *et al.*, 1967; Palyi *et al.*, 1969; Shohat *et al.*, 1970). A cell cycle analysis with the two identified withanolides (Wf-1 and Wf-3) is then necessary to evaluate whether these compounds could induce apoptosis, and to understand whether their inhibition of NF-κB activity is related to cellular death.

The anti-tumour activity of withaferin A has been extensively demonstrated to be associated with cytotoxic effects (Shohat *et al.*, 1967; Kupchan *et al.*, 1969; Fuska *et al.*, 1984). Several functional groups have been proposed to be responsible

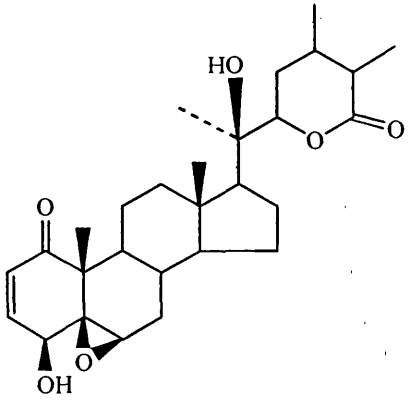
for the cytotoxic activity of withanolides. For instance, Jayaprakasam *et al.* (2003) showed that withaferin A and its acetyl derivate decreased the growth of human tumour cell lines at doses in a nM range, on different cancer cell lines, whereas 27-deoxy-24, 25-dihydrowithaferin A was active at higher concentrations (μM). In this study, it was noticed that the bulky groups attached at C27 were related to the reduction of antiproliferative activity, with an OH group at C27 helping to maintain this activity.

Other studies have suggested the α, β-unsaturated ketone groups as being responsible for cytotoxicity through their interaction with SH groups, which are present in enzymes or other nucleophile groups of enzymes as alkylating agents, or through to the acylation of the lactone (Moncrief and Heller, 1967; Fujita and Nagao, 1977; Fuska *et al.*, 1984). In particular, Fuska *et al.* (1984) inferred that the cytotoxicity of withaferin A and nine synthetic derivates on P388 cells is to be due to the presence of a double bond at position C2-3, since those derivates lacking this double bond were less active. Moreover, other withanolides isolated from a related solanaceous plant, *Discopodium penninervium*, were found to have cytotoxic and immunosuppressive *in vitro* activity on different cancer cell lines when a 16 α-oxygenation pattern was present (Habtemariam, 1997).

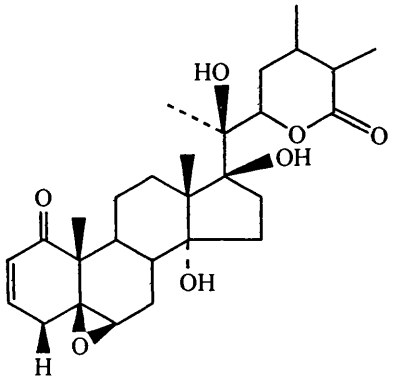
Das *et al.* (1985) on the other hand reported antitumor activity both *in vivo* and *in vitro* for withanolide D isolated from *W. somnifera.* These authors suggested that, as opposed to withaferin A, which inhibits protein synthesis, the mechanism of action of withanolide D is through the stimulation of protein synthesis. Finally, an investigation of the relationship between chemical structure and antitumor activity of several analogues of withaferin A (withanolide D, E, and 4β-hydroxy-withanolide E;

Fig. 43) revealed that the α, β-unsaturated ketone but also the epoxide ring were necessary for an antitumor activity (Yoshida *et al.*, 1979).

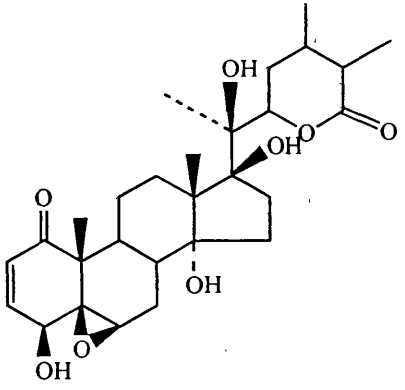
As regards the present study, of the functional groups mentioned above, withaferin A and 5,6-epoxy-1-oxowitha-2, 14, 24-trienolide isolated from *W. frutescens* both have the α, β-unsaturated ketone and the epoxide ring. Thus, it is probable that the cytotoxicity of these compounds could also be due to its anti-tumour activity. Further investigation using MTT assays will help to understand whether the inhibitory NF-κB activity of these two compounds is related to their cytotoxicity as suggested by the anti-tumour activity displayed by withaferin A (Fuska *et al.*, 1984).



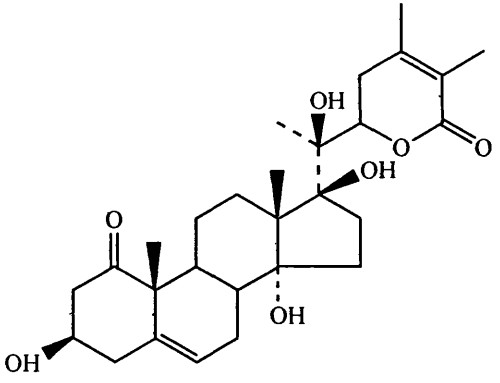
Withanolide D



Withanolide E



4β-hydroxywithanolide E



2,3-dihydro-3β-hydroxy-withanolide F

Fig. 43. Examples of withanolides with biological activity.

4.3. *Valeriana officinalis*

4.3.1. Phytochemistry

The chemical composition of *Valeriana officinalis* is well documented, and the chromatographic methods used in the isolation of its main chemical constituents have been developed precisely according to the chemical group of study (e.g. Hazelhoff *et al.*, 1979; Hazelhoff *et al.*, 1981; Bos *et al.*, 2002; Goppel and Franz, 2004). Moreover, the general crude extract preparation is included in the European Pharmacopoeia. In my study, only the EtOAc extract was prepared and tested for biological activity due to evidence that it had previously shown a positive activity as an inhibitor of NF–κB (pers. commun. AINP project). Nevertheless, the isolation of compounds from the active fractions of this plant was rather difficult due to their inconsistent activity.

Several works have reported the susceptibility of *Valeriana officinalis* to environmental changes, which would explain the difficulty in the isolation of its compounds and the biological activity instability of the latter. For instance, the collecting time has been shown to play an important role in the chemical composition of valerians from one population to another (Houghton, 1999). Also, physical and chemical factors such as exposure to light, heat, humidity, and chemical agents are of great relevance in the alteration and degradation of valerian compounds (e.g. Popov and Handjieva, 1979; Bos *et al.*, 2002; Gopper and Franz, 2004). In addition, the content of sesquiterpenic acids can be highly affected by environmental and storage conditions (e.g. moisture, temperature; Goppel and Franz, 2004), whereas valepotriates are sensitive to heat and are rapidly decomposed under acidic or alkaline conditions in water and in alcoholic solutions (Bounthanh *et al.*, 1983; Bos

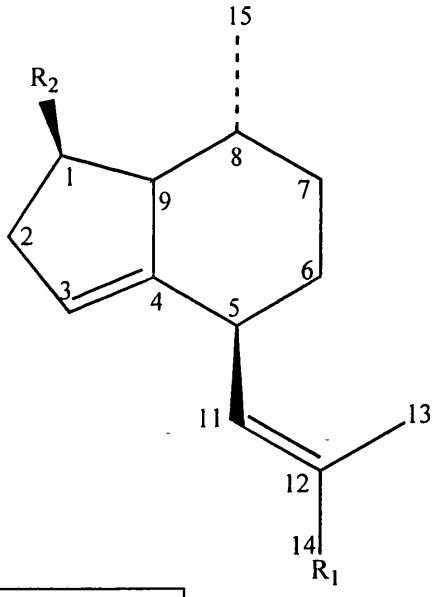
et al., 2002), and alkaloids are highly soluble in water, which makes them more difficult to isolate (Torssell and Wahlberg, 1967).

The compounds isolated in this work were sesquiterpenes, which are the main compounds present in the volatile oil and are more likely to be found in EtOAc extracts due to their nature and polarity. Of these sesquiterpenes, acetylvalerenolic acid was found in most of the active fractions. In this respect, it is known that the concentration of sesquiterpene acids present in dry valerian plants could increase/decrease before extraction depending on the storage and environmental conditions. For example, Goppel and Franz (2004) showed that an increment in temperature increased the possibility of finding hydroxyvalerenic acid since it is the degradation product of acetoxyvalerenic acid, which is more sensitive to changes in the environment and storage conditions.

Three valerenane sesquiterpenoids, acetylvalerenolic acid (Vo-1), valerenal (Vo-2), and valerenic acid (Vo-3), were obtained using different chromatographic techniques, and their isolation was completed using preparative TLC (Hex-EtOAc). Acetylvalerenolic acid and valerenic acid were first isolated from *V. officinalis* by Stoll and Seebeck (1957) from an ether extract and followed by silica gel fractionation using petroleum ether and EtOAc. Bos *et al.* (1986) also isolated nine valerenane sesquiterpenoids extracting the roots of valerian with CH_2Cl_2 and a silica gel column using as an eluent different concentrations of petroleum ether.

Despite the characteristic, unpleasant smell of isovaleric acid being detected during the fractionation process of *V. officinalis*, no valepotriates were isolated from the EtOAc extract. This can be explained by the low stability and high degradation of valepotriates (Bos *et al.*, 2002), which were probably lost during fractionation of the EtOAc extract.

The acetylvalerenolic acid (Vo-1), valerenal (Vo-2), and valerenic acid (Vo-3) all possess the same 5 and 6-membered rings (Fig. 44). The ^{13}C spectra of valerenic acid and valerenal only differ at position 14, where in the first there is an acid at δ 173.3, whilst in the second there is an aldehyde group (δ_C 196.0, δ_H 9.38 *s*). Acetylvalerenolic acid on the other hand is distinguished by having an acetoxy group in carbon 1 (C1 δ 75.72, δ_H 5.04 *dt J*= 2.5, 4.5, 7.5; C16 C=O acetate δ 171.2, C17 Me acetate δ 21.3; H17 δ 2.03 *s*). Since these compounds are very well known, their characterisation could be made comparing their 1D and 2D analyses with previously reported spectroscopic data (Birnbaum *et al.*, 1978; Bos *et al.*, 1986; Dharmaratne *et al.*, 2002).



	R1	R2	
1	CHO	H	Valerenal
2	COOH	H	Valerenic acid
3	COOH	OAc	Acetoxyvalerenic acid

Fig. 44. Structures of valerenal, valerenic acid, and acetoxyvalerenic acid.

4.3.2. Biological activity

The interest for studying valerian as inhibitor of NF-κB emerged from the knowledge of its traditional use as an anti-inflammatory remedy in Europe and Central America (Mayer, 2003; Cáceres, 1990). However, the reputation of this plant in modern phytotherapy is mainly due to its pharmacological properties as tranquilliser and mild sedative, though the active principle for these effects still remains a matter of great controversy.

The EtOAc extract of *V. officinalis* obtained in this study inhibited NF-κB at 100 μg/ml, with the most active fractions being those with a high polarity. Of these active fractions, three sesquiterpenes were isolated, acetylvalerenolic acid, valerenal and valerenic acid. The purified acetylvalerenolic acid and valerenic acid were both inhibitors of NF-κB at 100 μg/ml and no cytotoxicity was observed, though this concentration is higher than the clinical concentration (10 μM). Interestingly, it has also been proposed that the tranquilliser and mild sedative properties of *V. officinalis* lie on the sesquiterpenes present in the volatile oil, which are mainly a combination of valerenic acid, acetoxyvalerenic acid, and hydroxyvalerenic acid (Houghton, 1999; Boyadzhiev, *et al.*, 2004). Since aqueous extracts of valerian seem to have stronger sedative and sleep-inducing effects than other kind of extracts (Houghton, 1999), a IL-6/Luc test with this extract would help to know whether it also increased NF-κB inhibition.

The subfractions where the acetylvalerenolic acid and valerenic acid were recovered generally had a higher inhibitory activity (subfractions: 3 to 26%; acetylvalerenolic acid: 4 to 27%; valerenic acid: 25%). This suggests that a synergistic or additive effect could be involved in the inhibitory NF-κB activity of

Cytotoxicity was found in the IL-6/Luc assay of some fractions of *V. officinalis*; however, when the purified compounds were individually tested they were not cytotoxic. Since the above fractions contain a mixture of compounds, it is possible that valepotriates and other compounds that are present are responsible for this cytoxicity. As regards valepotriates, Bounthanh *et al.* (1983) demonstrated that valtrate, dihydrovaltrate, and deoxido-didrovaltrate have a cytotoxic effect on cell growth and viability on rat hepatic cells (HTC strain), with valtrate being the most active. In any case, further MTT cytotoxic assays should be performed on the crude extracts, fractions, and compounds obtained from *V. officinalis* in order to establish their minimum non-cytotoxic concentration.

CONCLUDING REMARKS

This study provides molecular evidence for the anti-inflammatory activity of *Witheringia solanacea* by targeting the NF-κB pathway. Moreover, bio-assay-guided fractionation of this plant resulted in the isolation of physalins D, F, and B, of which the last two inhibited NF-κB activation and also appear to induce apoptosis.

The $CHCl_3$ extract of *Withania frutescens* on the other hand inhibited NF-κB activation at 100 μg/ml. The withanolides 5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide and withaferin A isolated from the $CHCl_3$ extract also inhibited NF-κB promoted by PMA.

Valeriana officinalis, a plant commonly used as mild sedative and to induce sleep, also has anti-inflammatory properties as inhibitor of NF-κB. Fractions containing valerenane sesquiterpenoids were more active than the purified sesquiterpenoids acetylvalerenolic acid and valerenic acid, thus suggesting they have a synergistic or additive effect.

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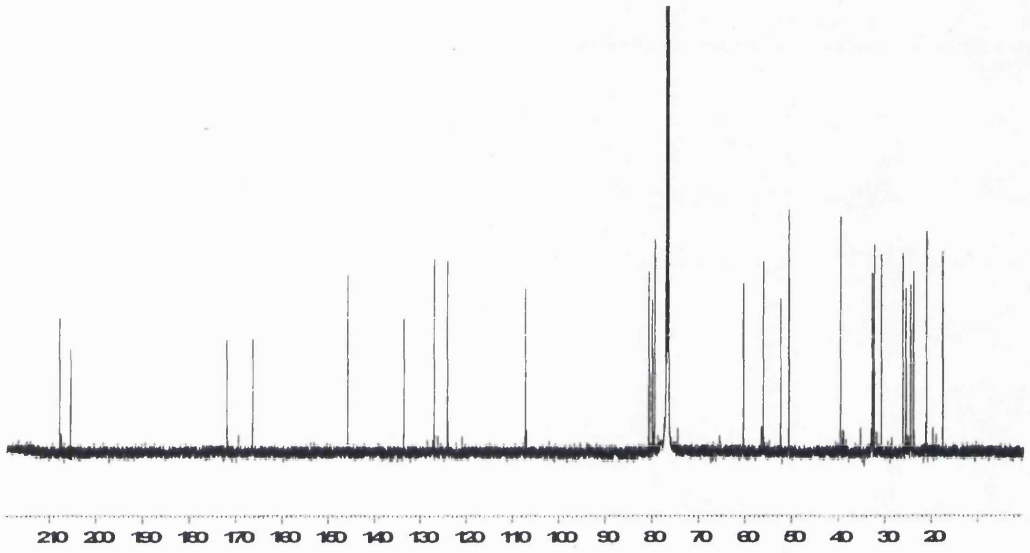
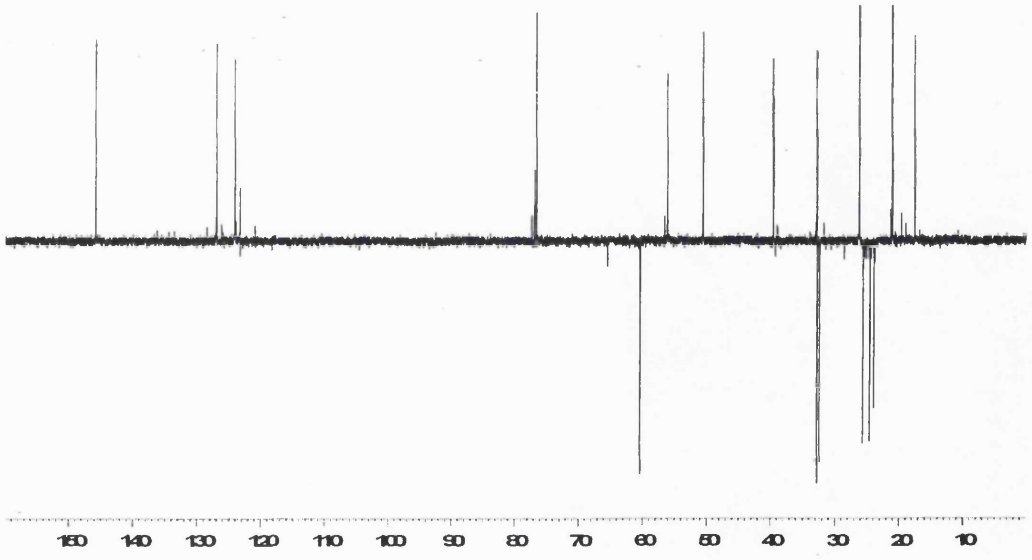
Appendix

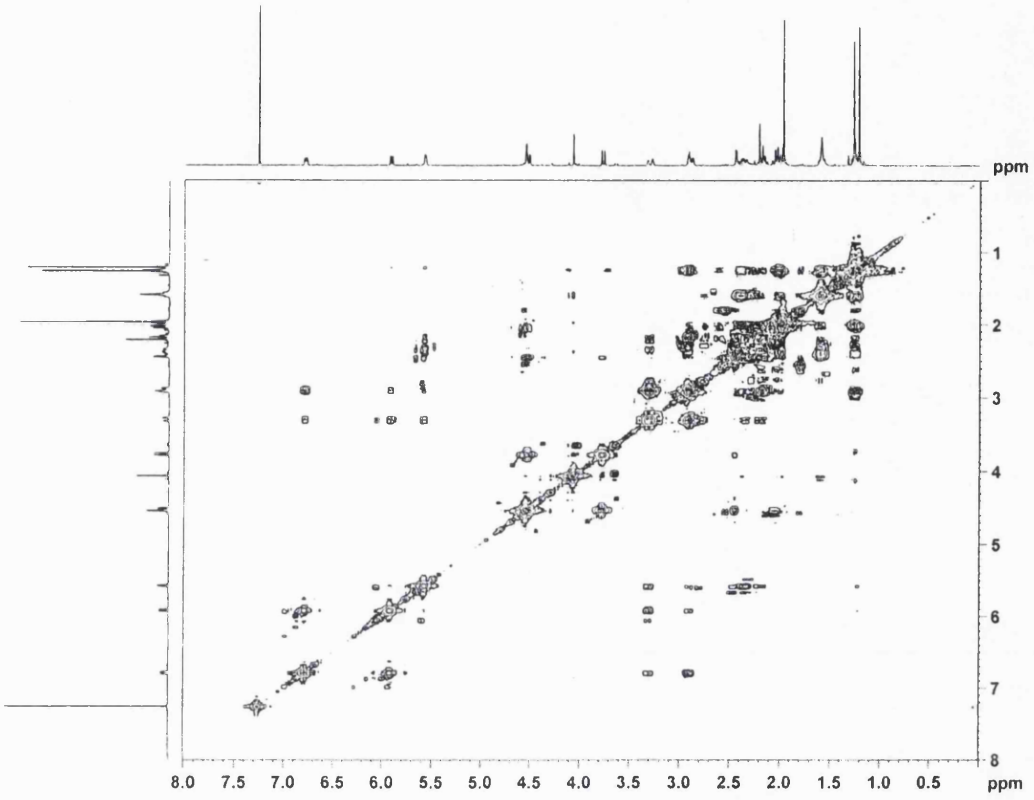
^{13}C NMR (500 MHz, $CDCl_3$) spectrum for physalin B (Wf-1).



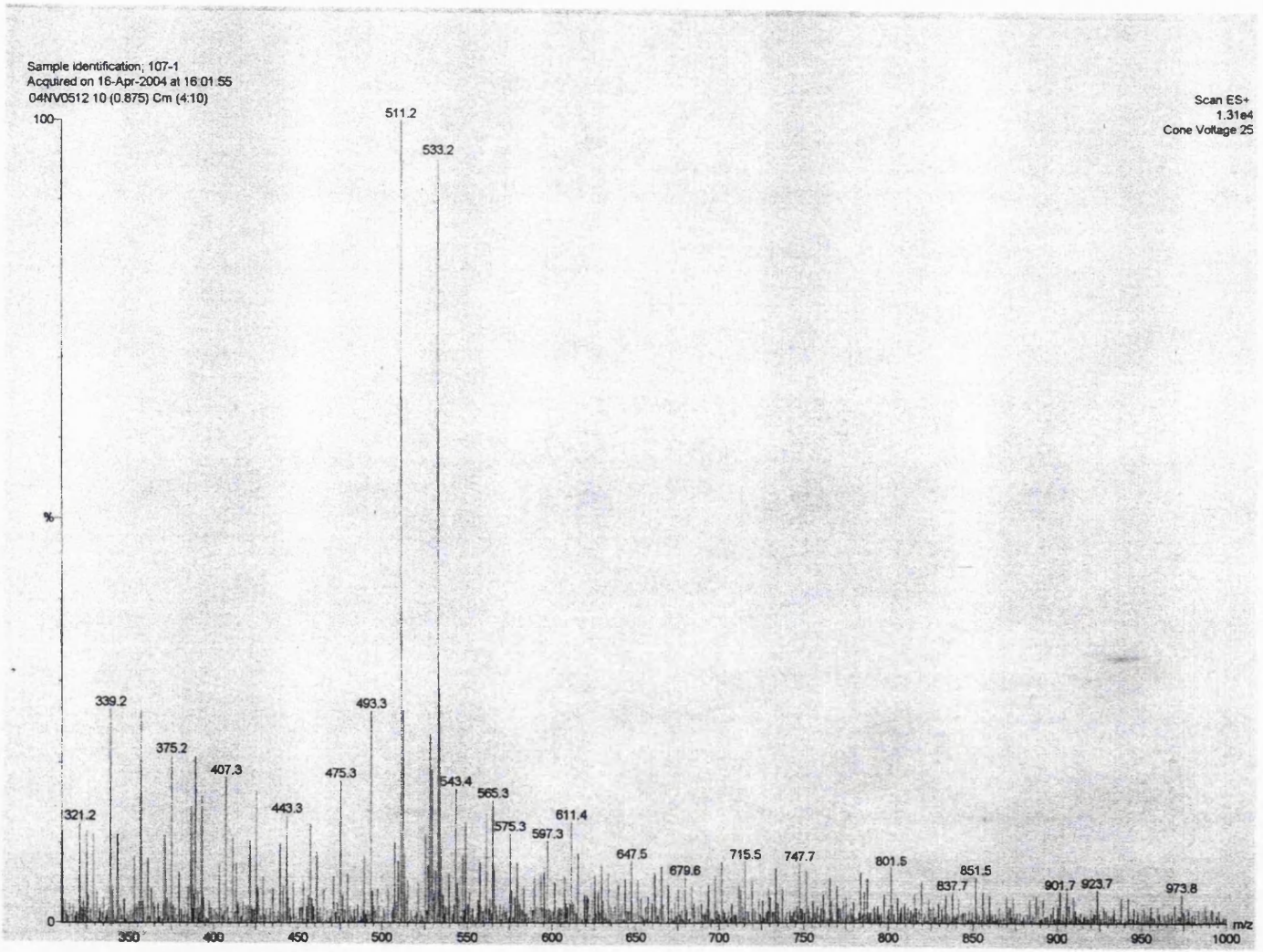
DEPT NMR (500 MHz, $CDCl_3$) spectrum for physalin B (Ws-1).



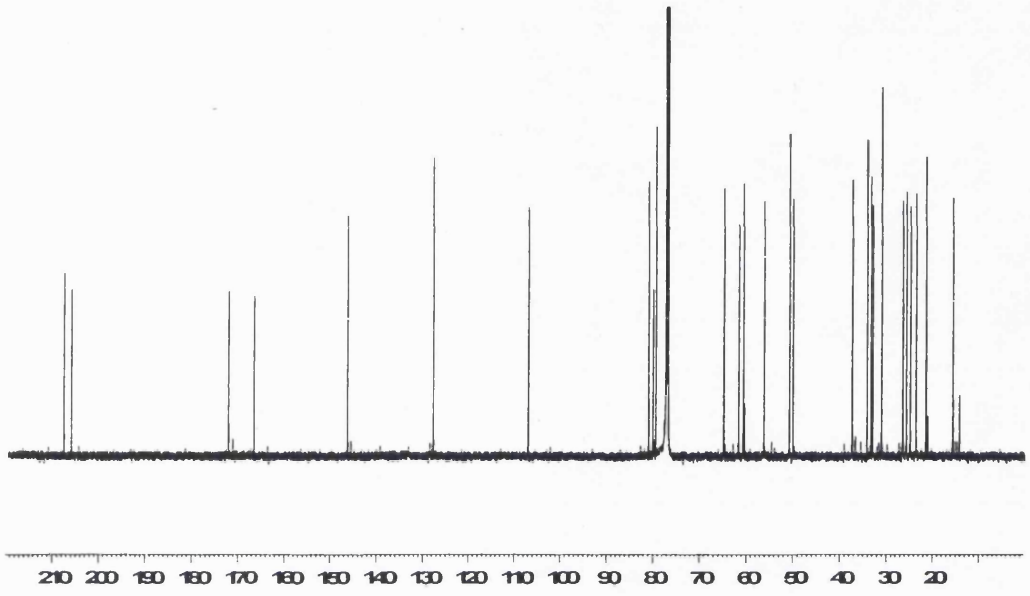
COSY NMR (500 MHz, $CDCl_3$) spectrum for physalin B (Ws-1).



MS for physalin B (Ws-1). Positive electron spray (ES+).



^{13}C NMR (500 MHz, $CDCl_3$) spectrum for physalin F (Ws-2).



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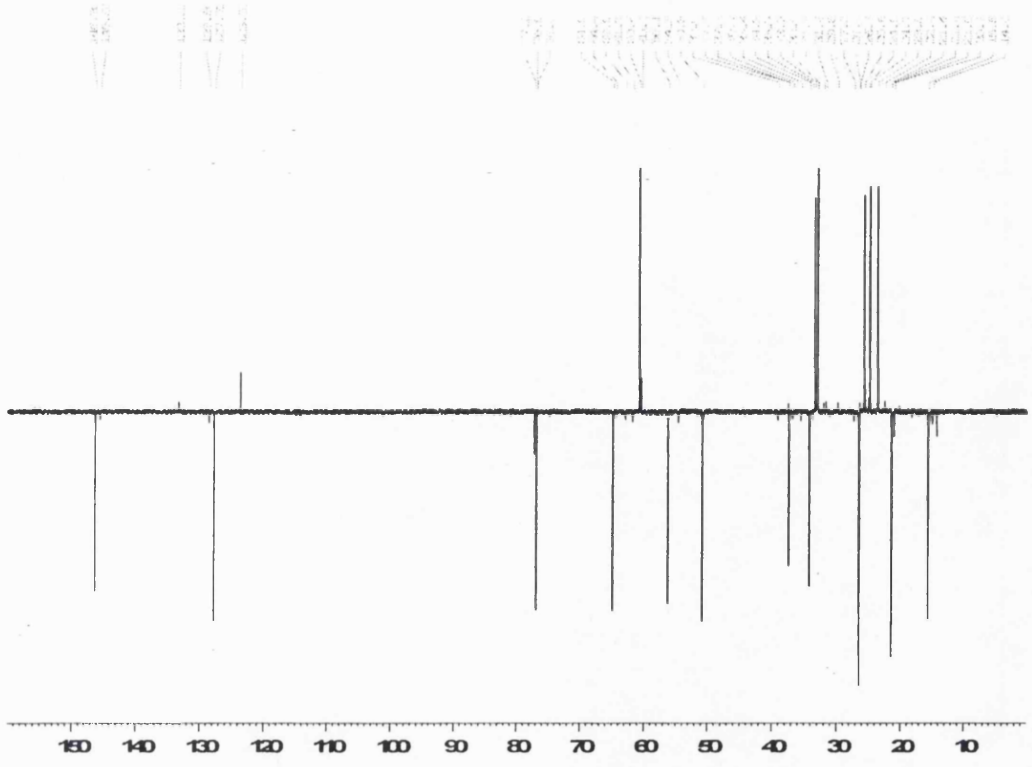
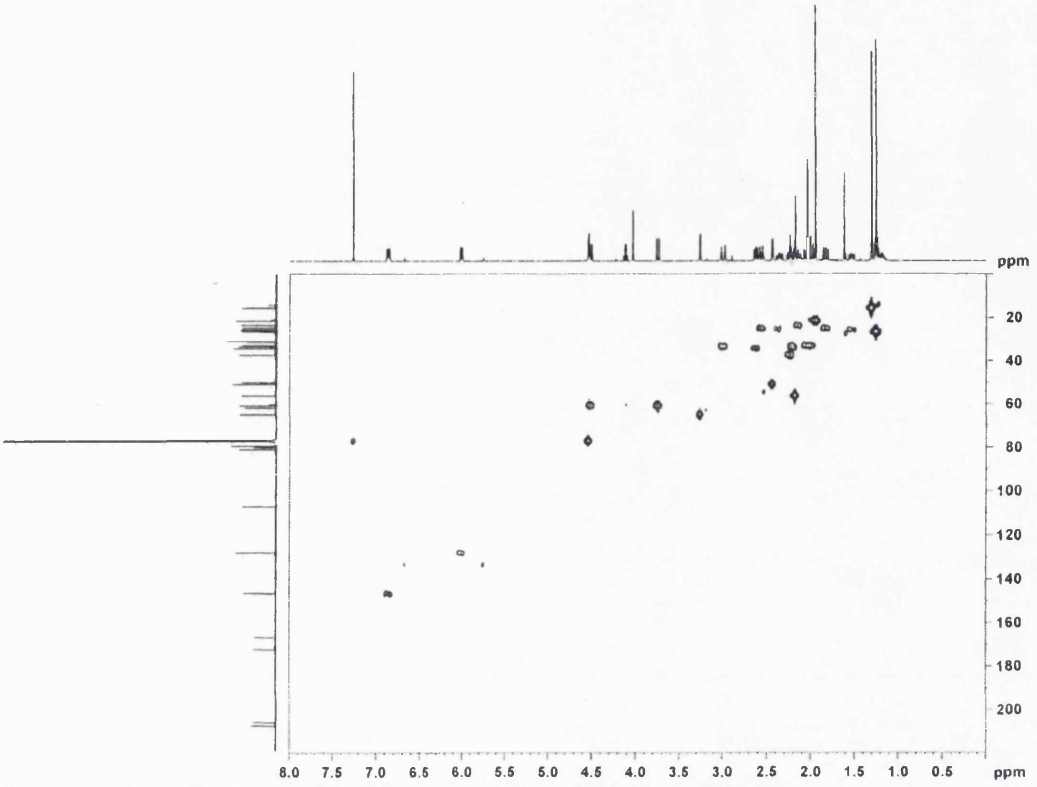
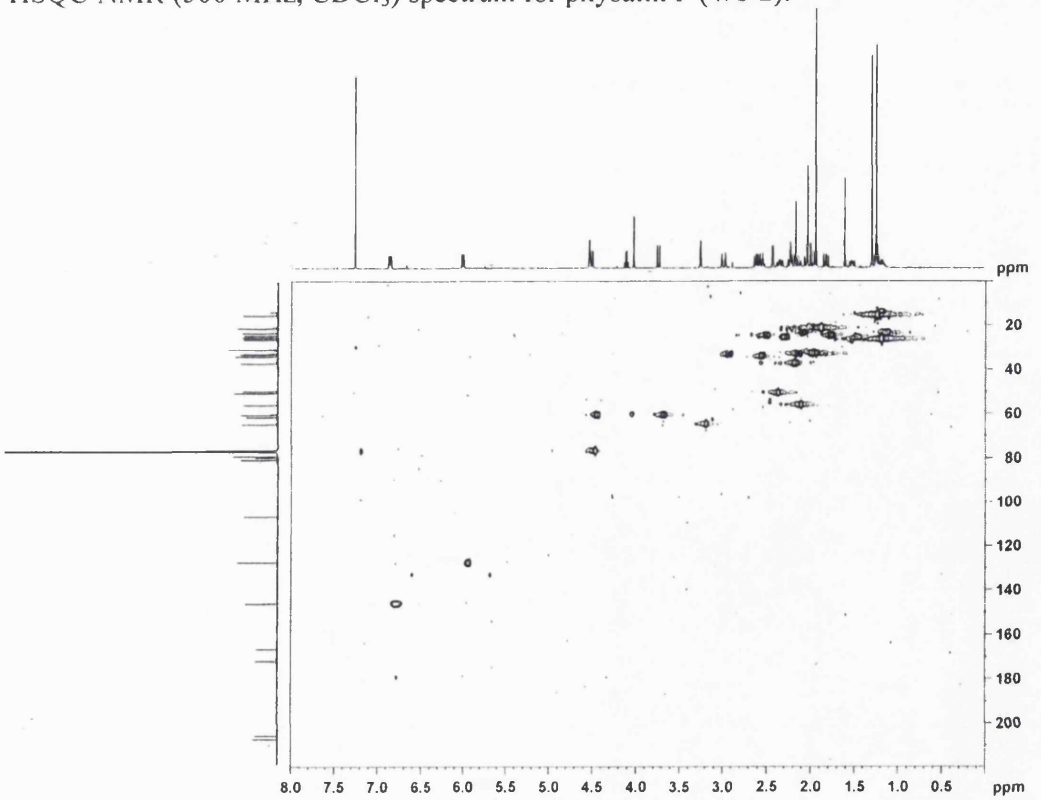


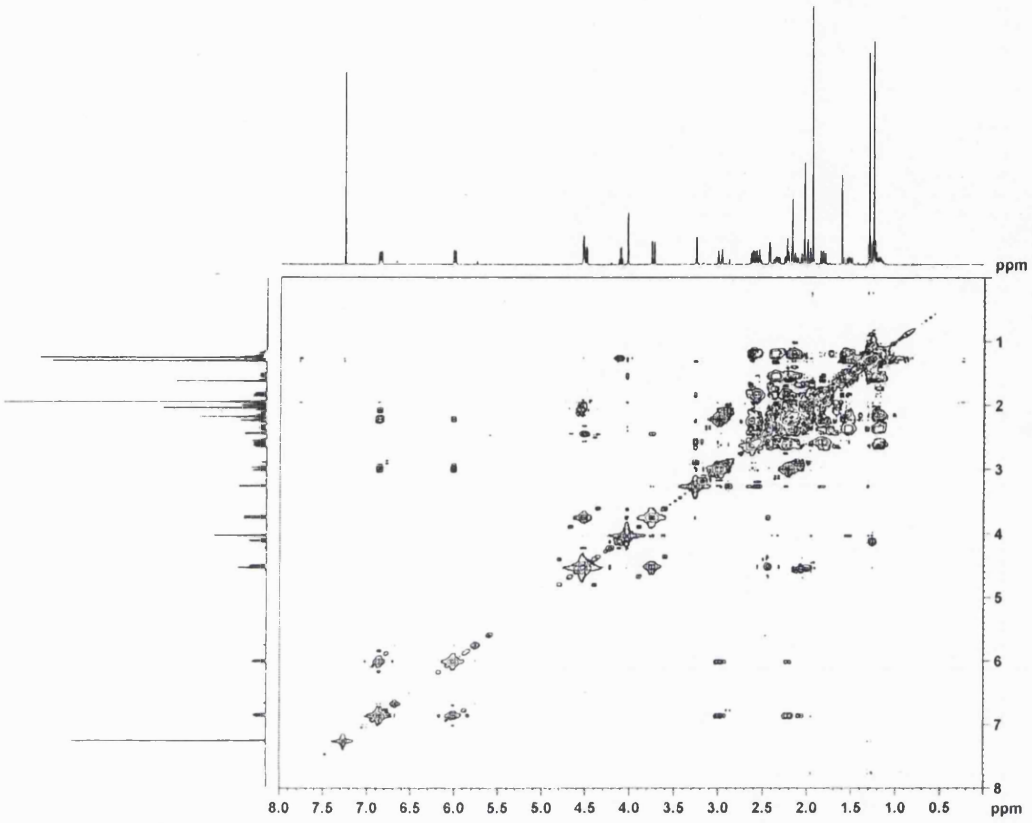
HMQC NMR (500 MHz, $CDCl_3$) spectrum for physalin F (Ws-2).



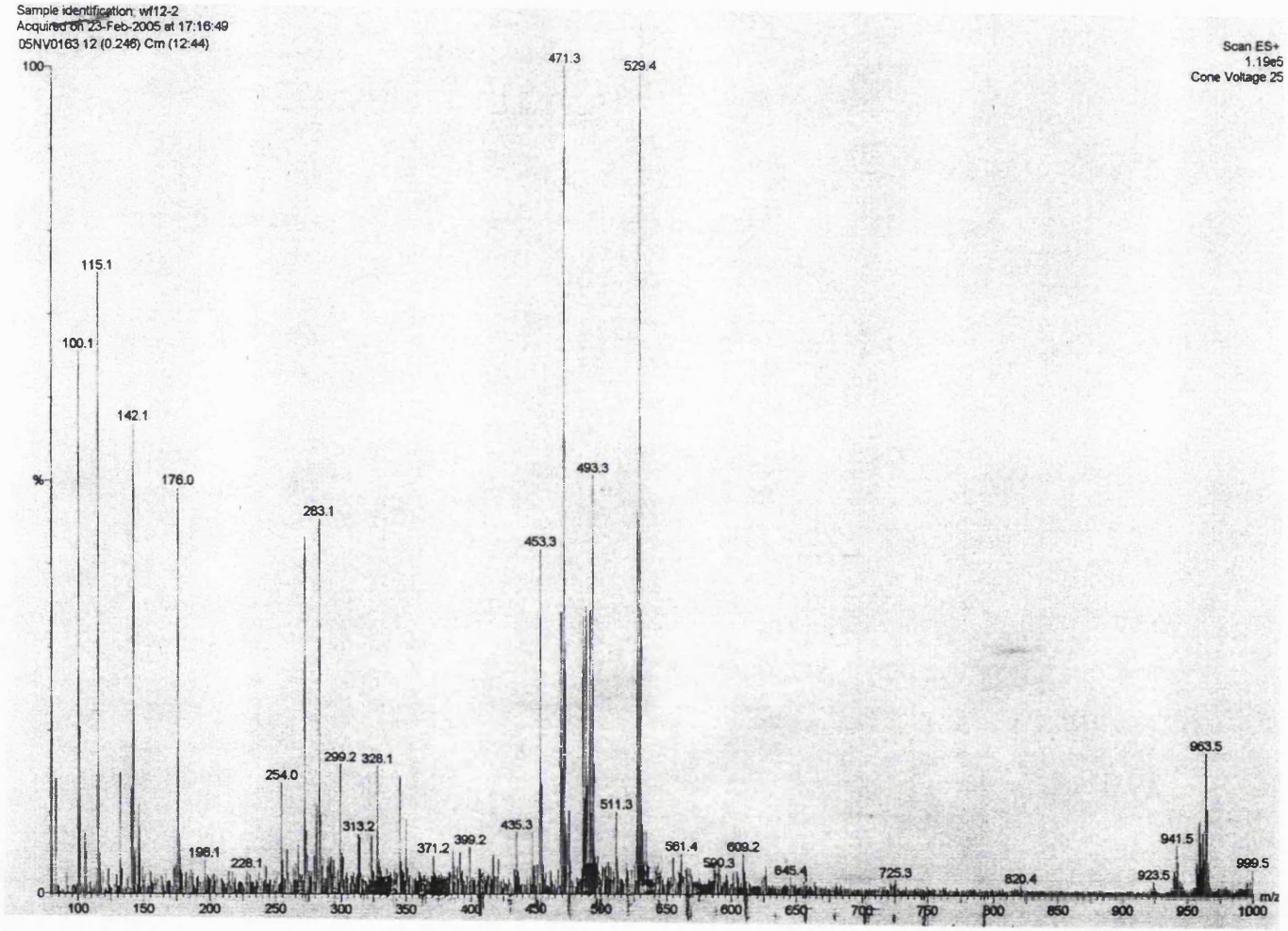
HSQC NMR (500 MHz, $CDCl_3$) spectrum for physalin F (Ws-2).



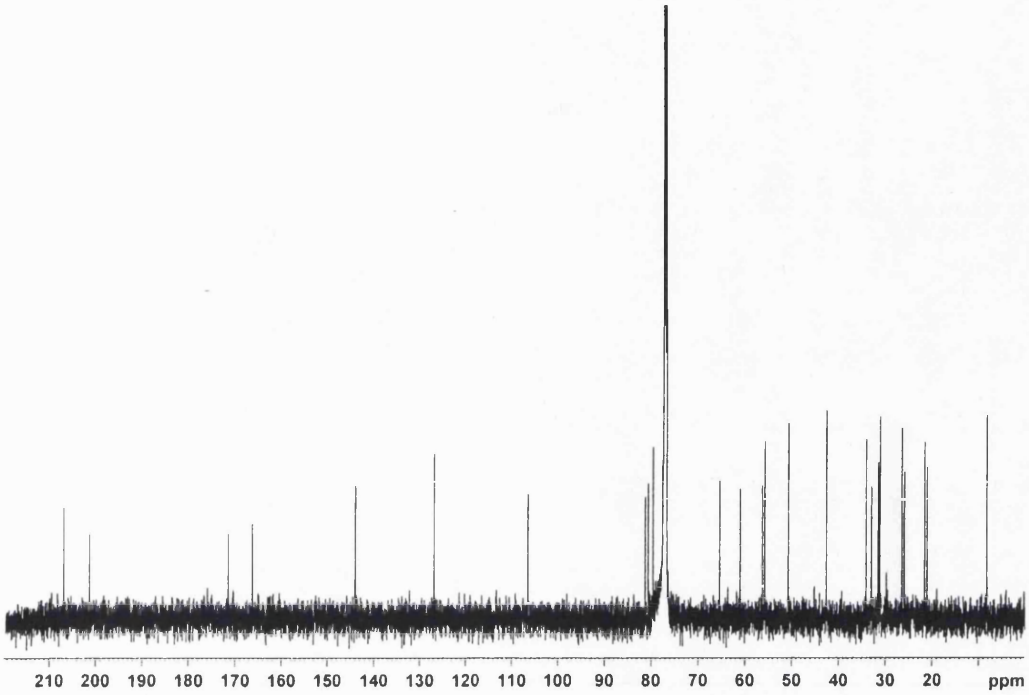
COSY NMR (500 MHz, $CDCl_3$) for physalin F (Ws-2).



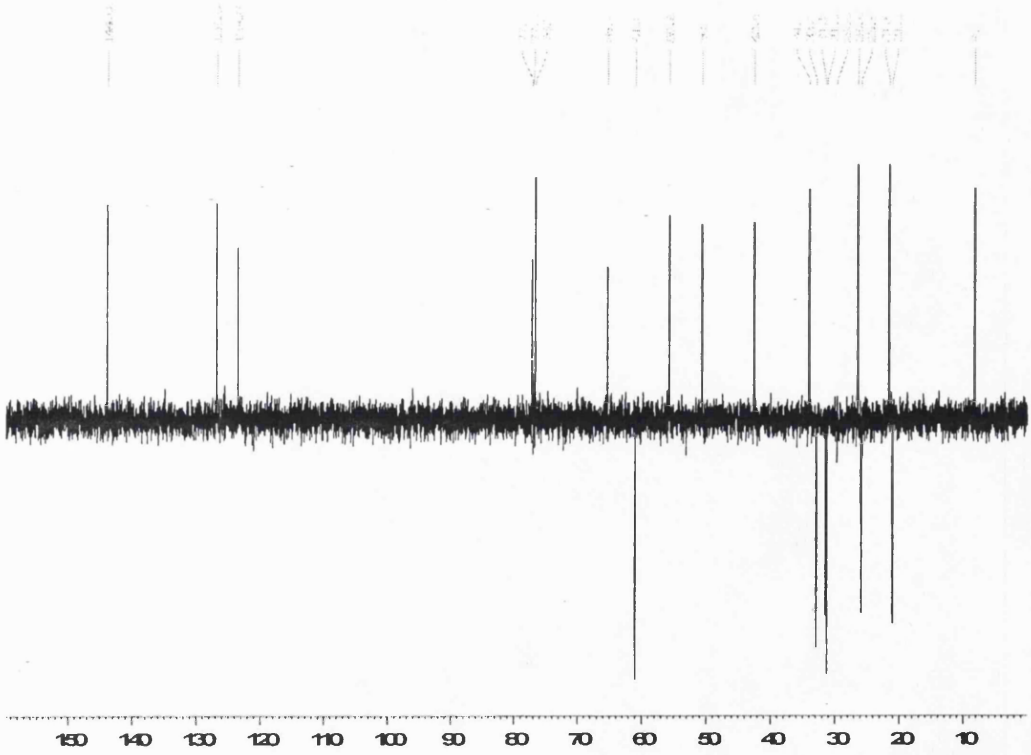
MS for physalin F (Ws-2). Positive electron spray (ES+).



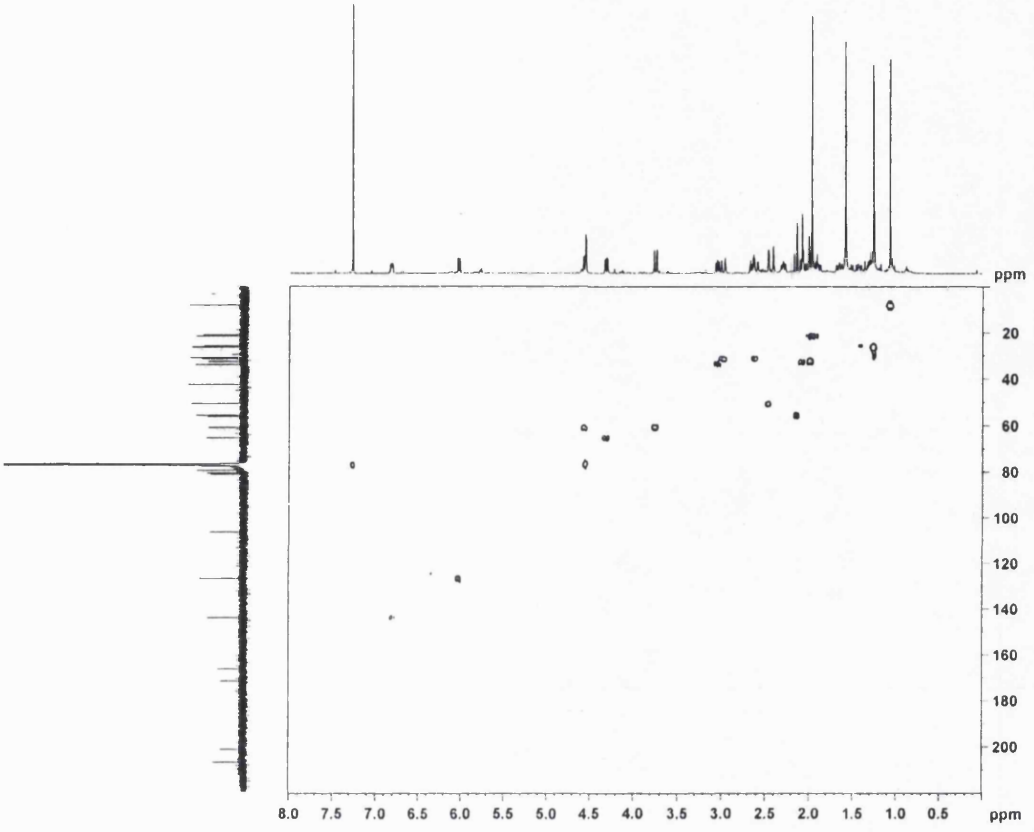
^{13}C NMR (500 MHz. $CDCl_3$) spectrum for physalin D (Ws-3).



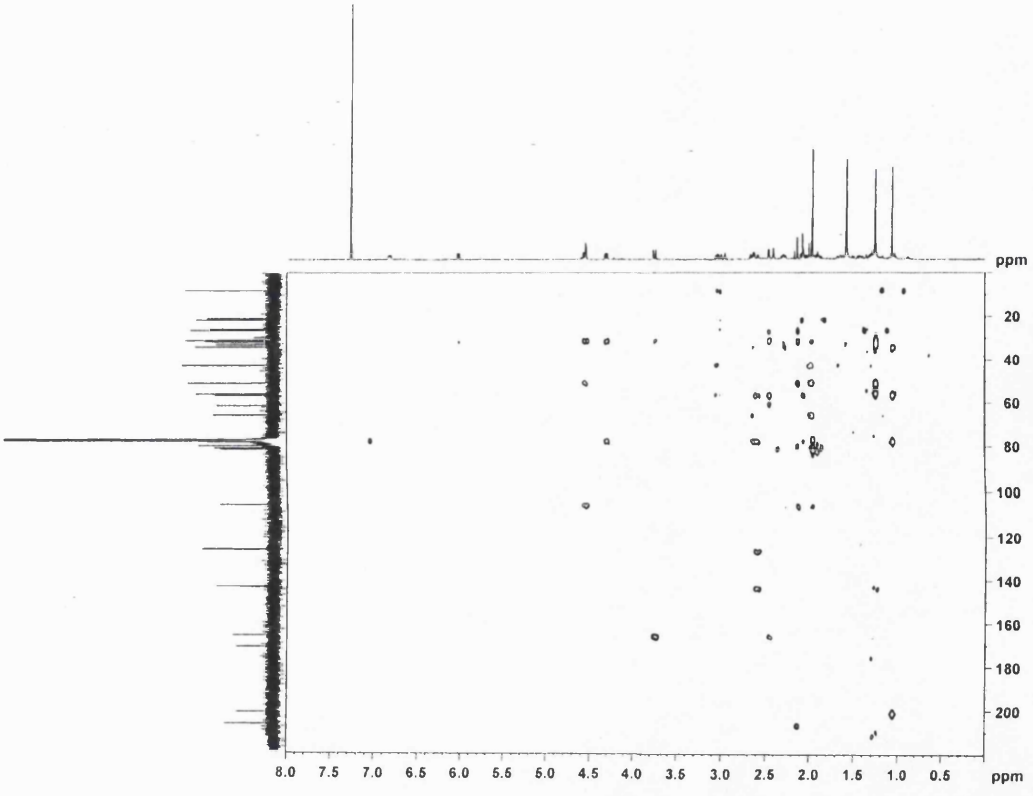
DEPT NMR (500 MHz, $CDCl_3$) spectrum for physalin D (Ws-3).



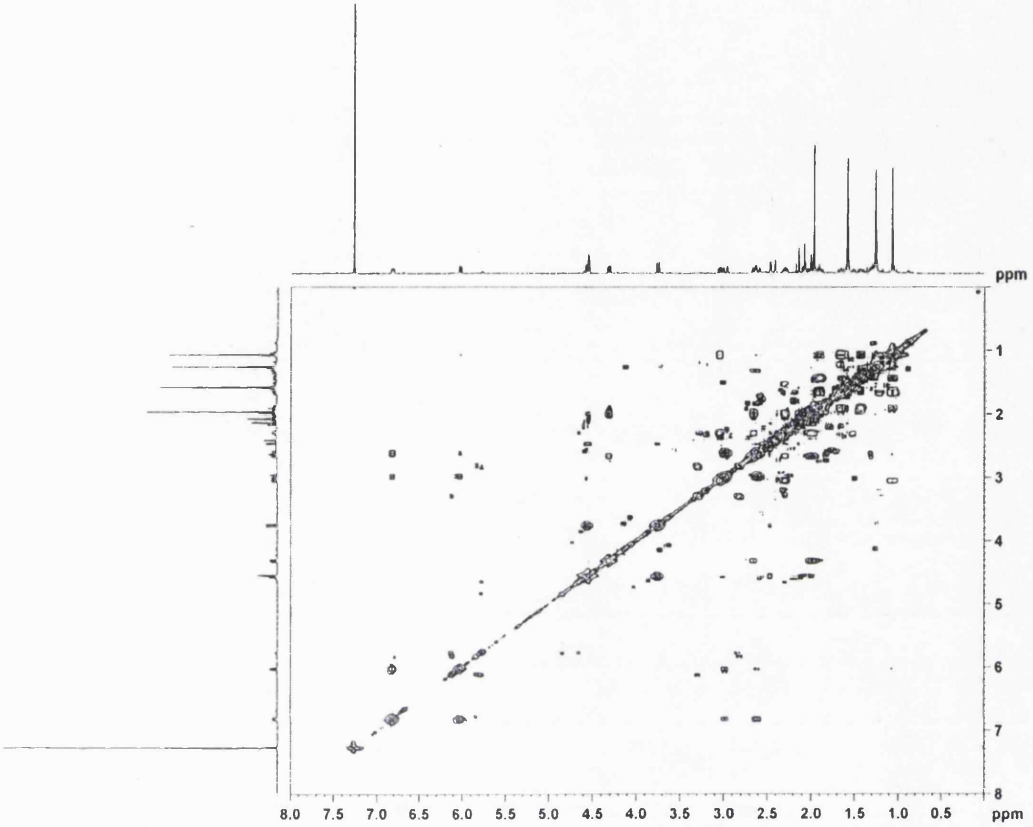
HMQC NMR (500 MHz, $CDCl_3$) spectrum for physalin D (WS-3).



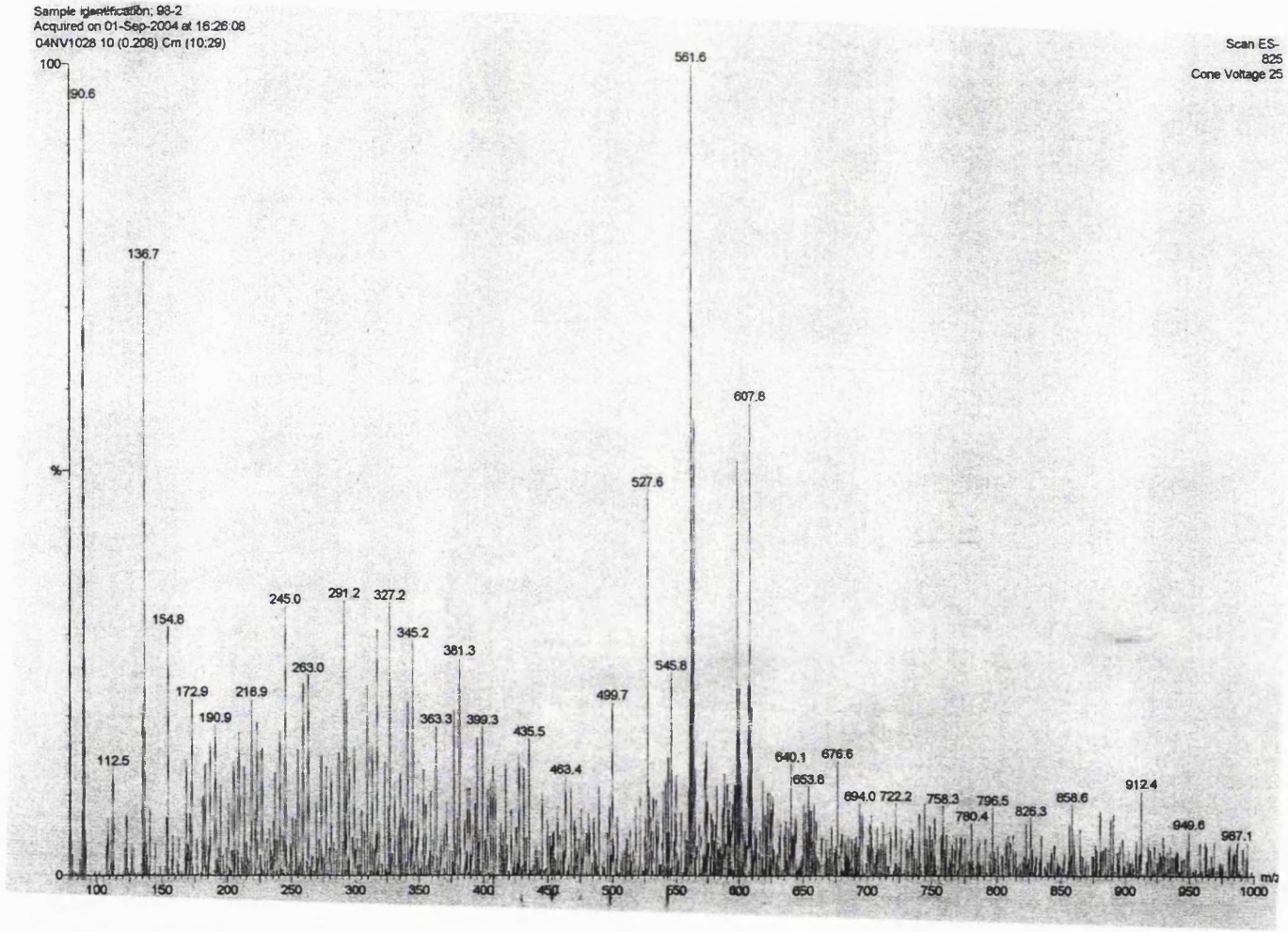
HMBC NMR (500 MHz, CDCl3) spectrum for physalin D (WS-3).



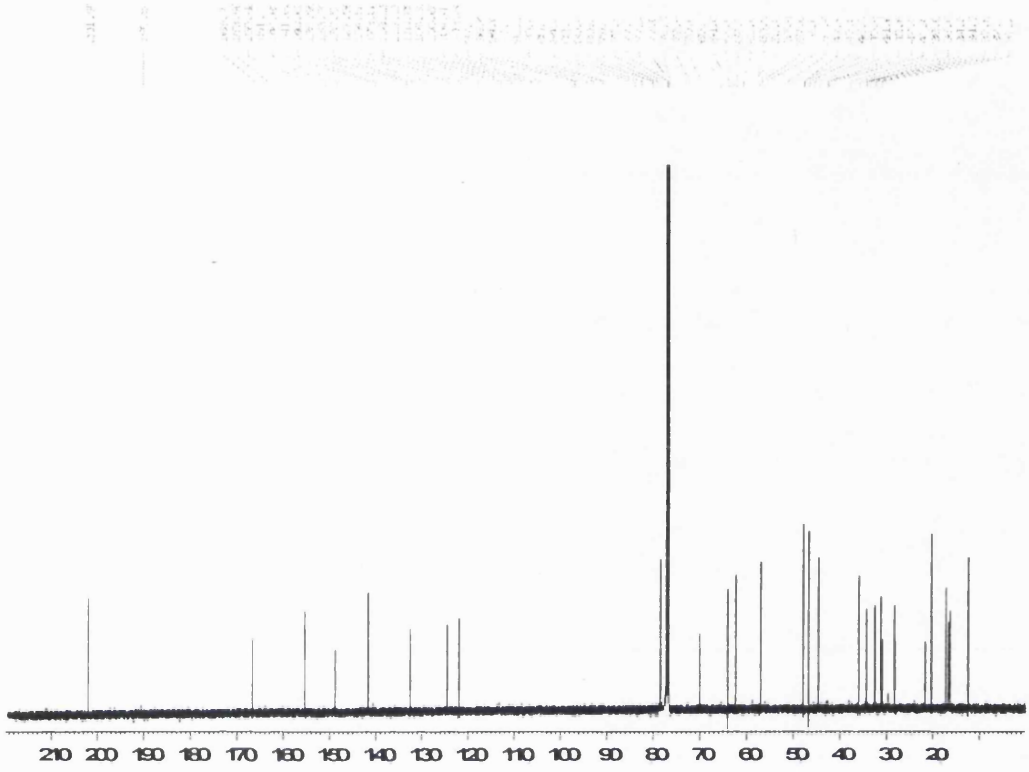
COSY NMR (500 MHz, $CDCl_3$) spectrum for physalin D (Ws-2).



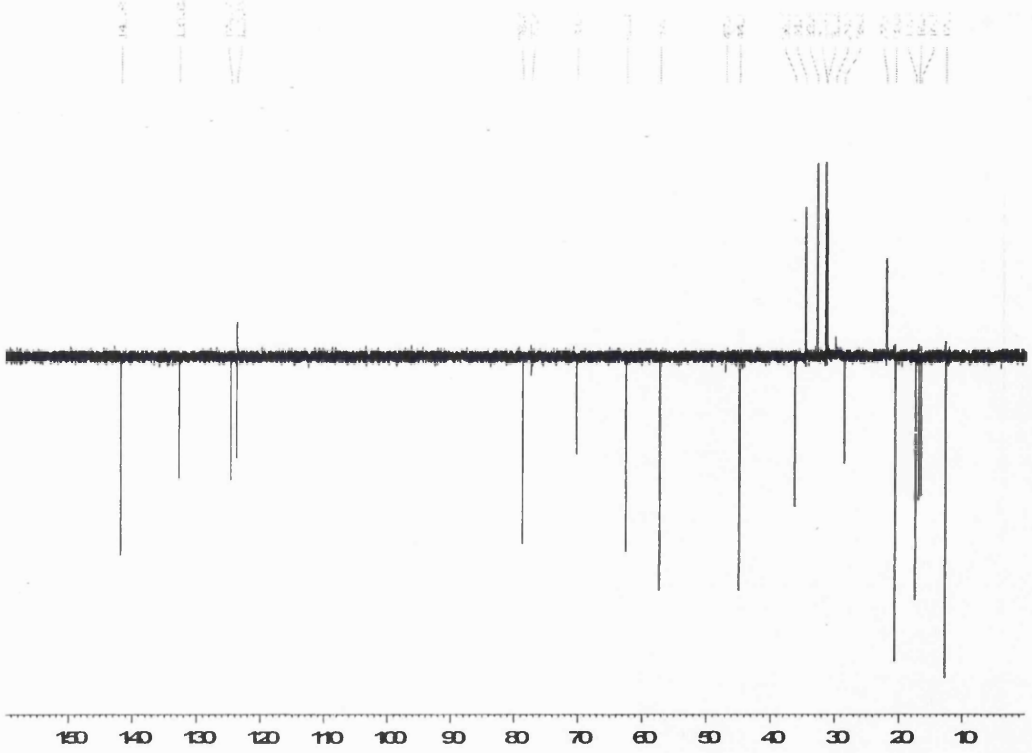
MS for physalin D (Ws-3). Positive elctron spray (ES+).



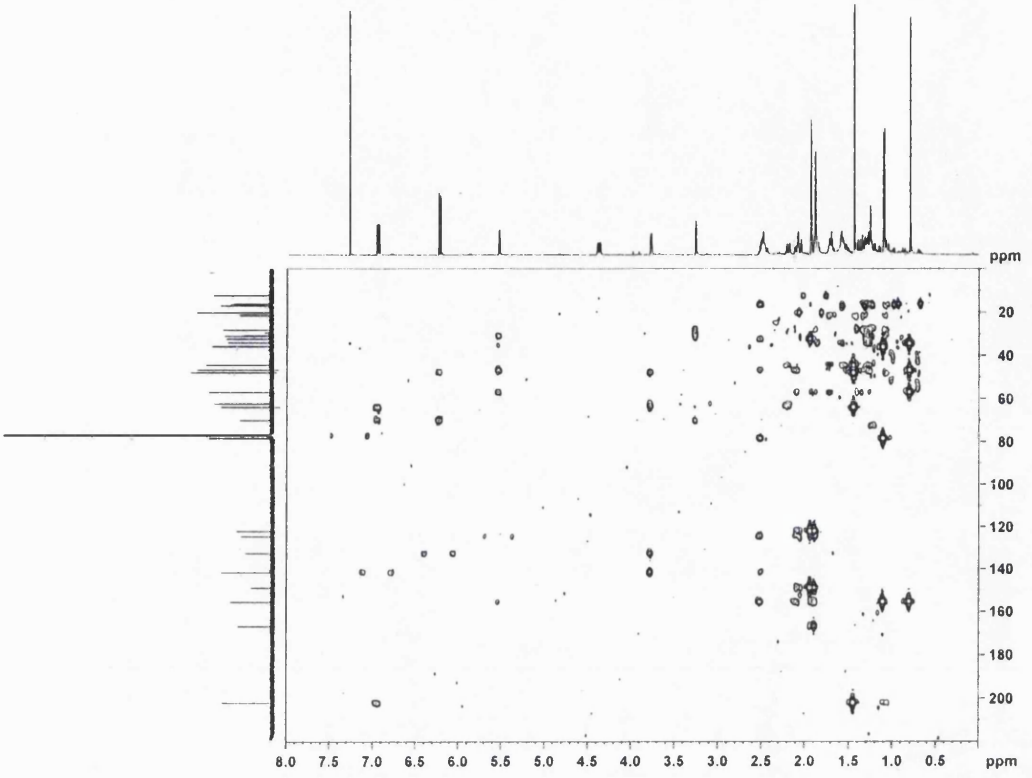
^{13}C NMR (500 MHz, $CDCl_3$) spectrum for 5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide (Wf-1).



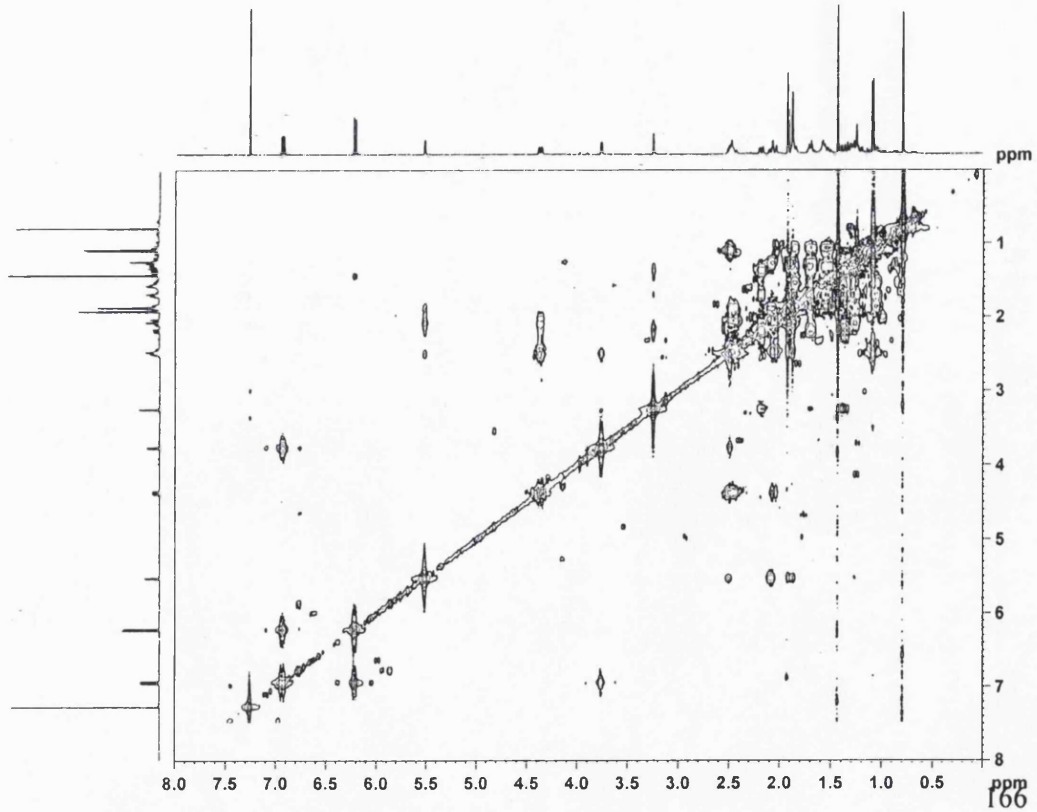
DEPT NMR (500 MHz, $CDCl_3$) spectrum for 5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide (Wf-1).



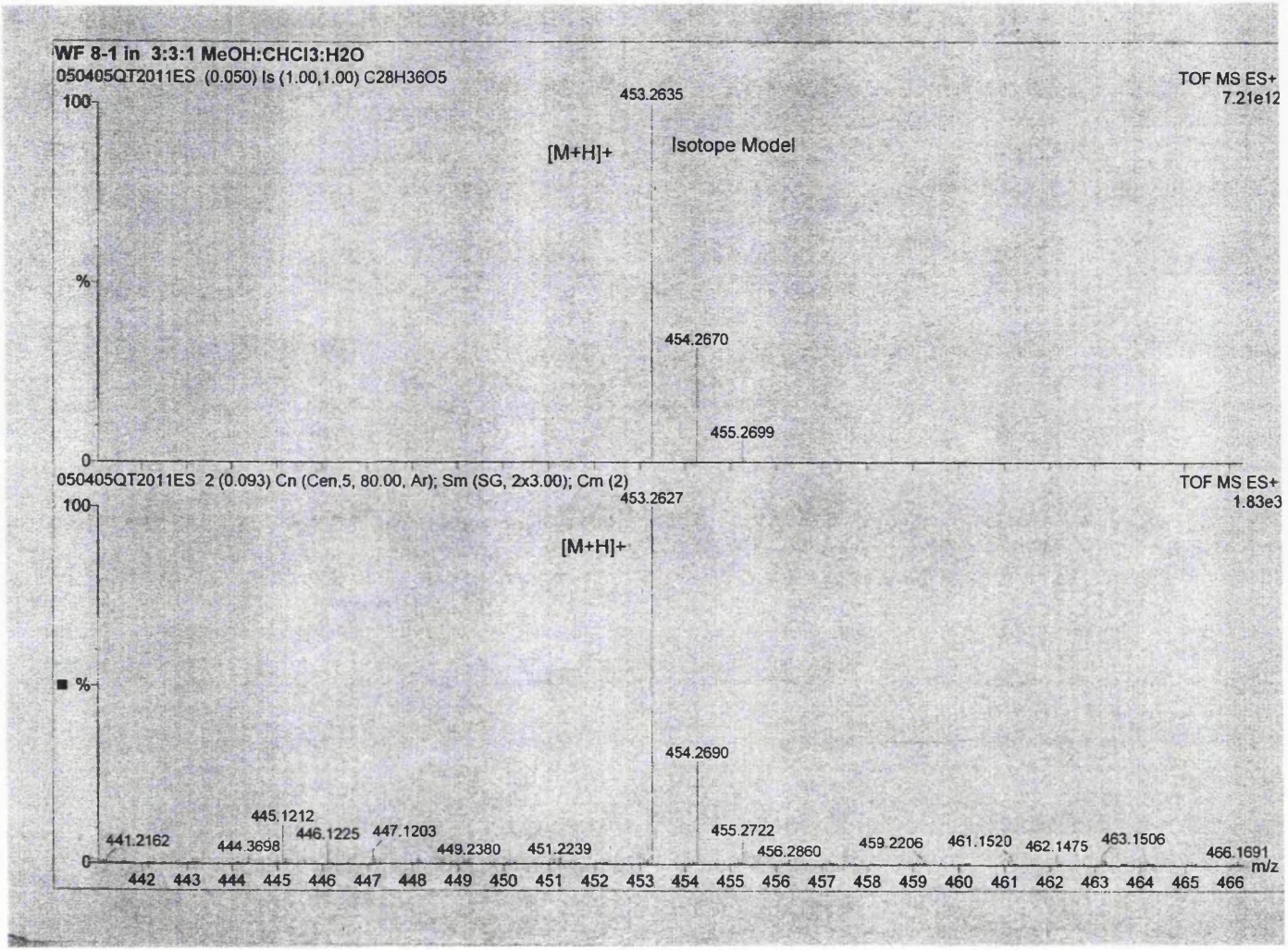
HMBC NMR (500 MHz, $CDCl_3$) for 5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide (Wf-1).



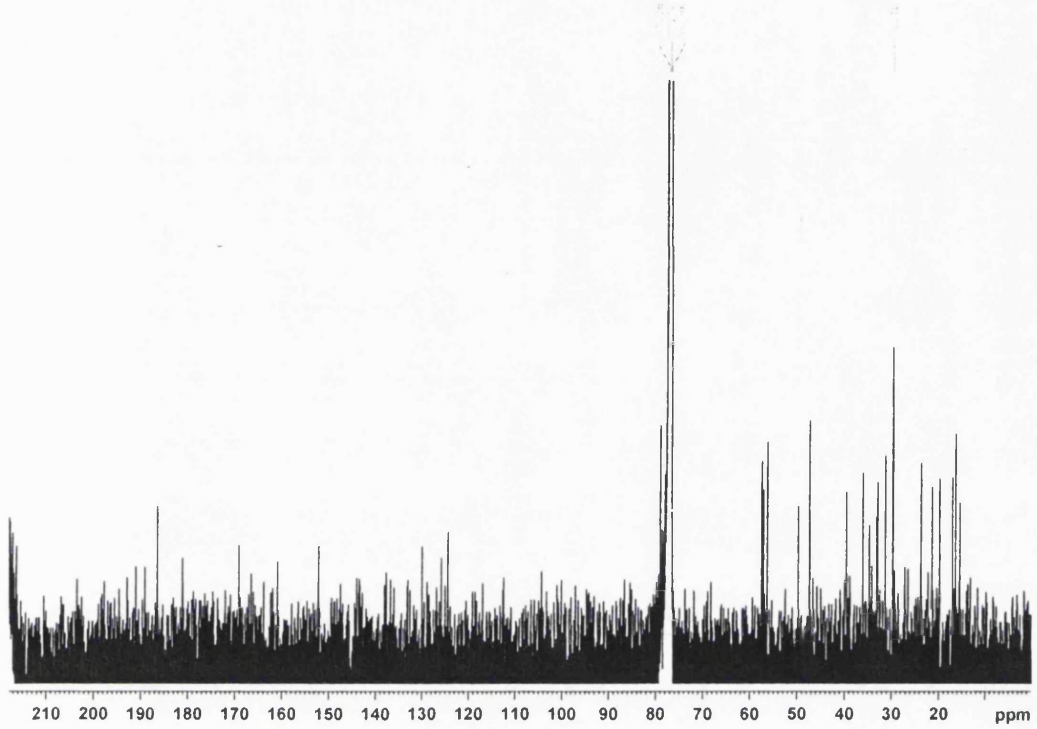
COSY NMR (500 MHz, $CDCl_3$) for 5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide (Wf-1).



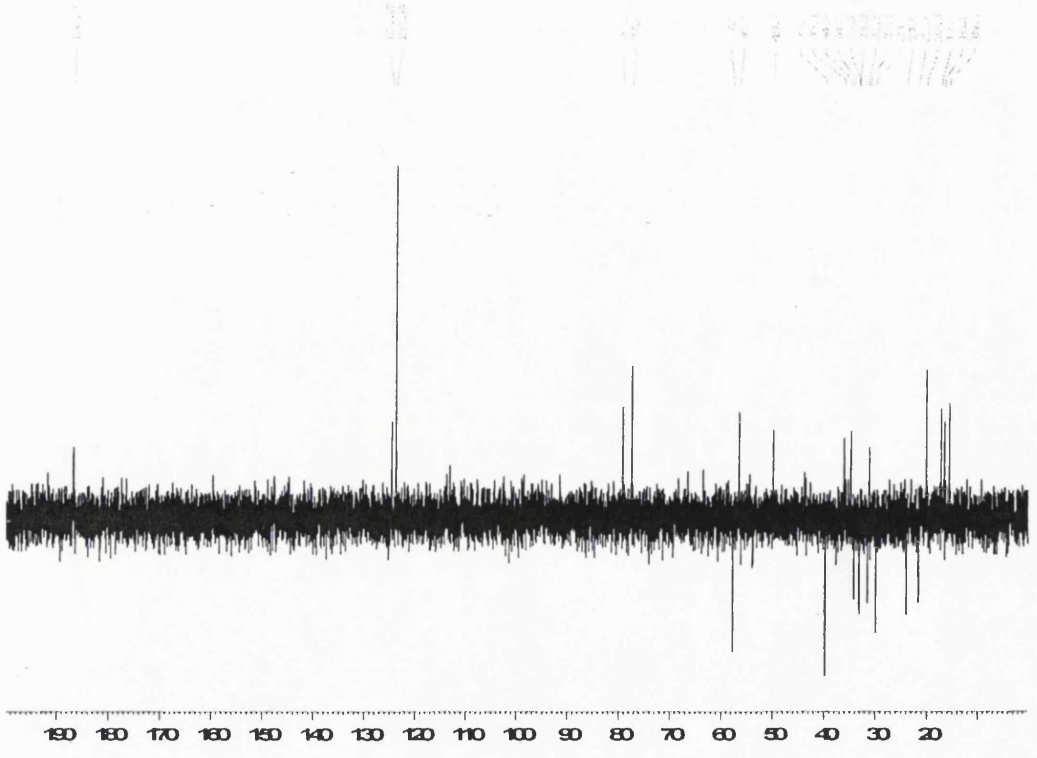
MS for 5, 6-epoxy-1-oxowitha-2, 14, 24-trienolide (Wf-1). Positive electron spray (ES+).



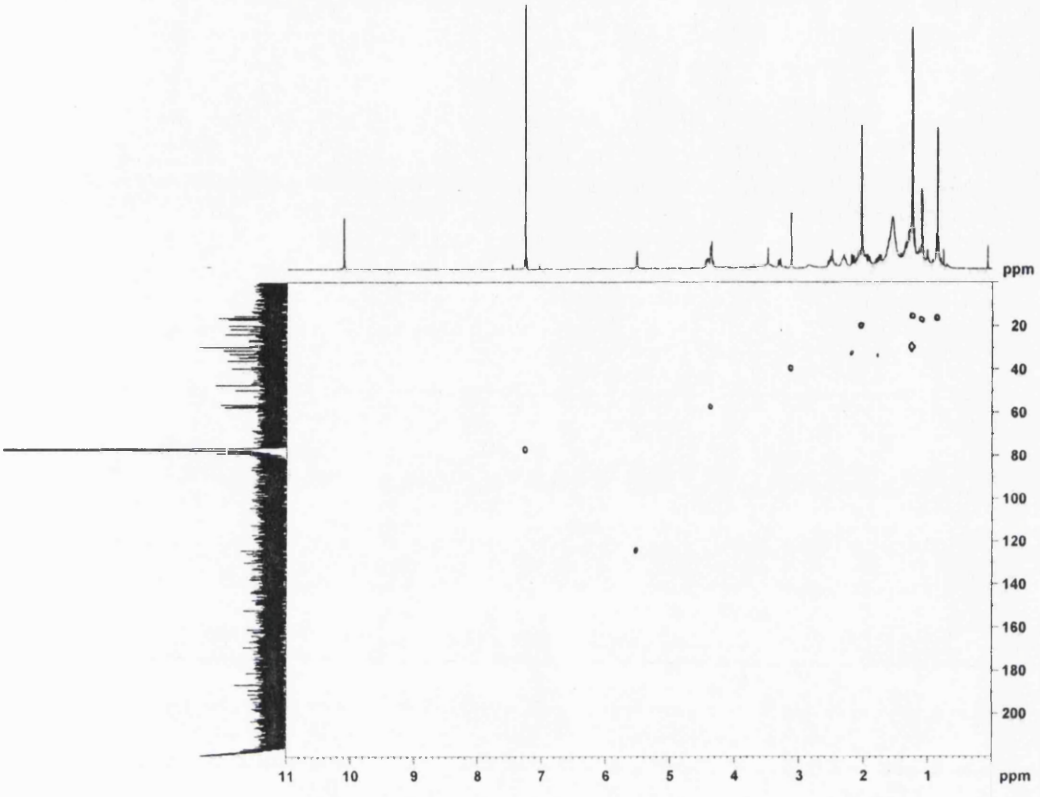
^{13}C NMR (500 MHz, $CDCl_3$) spectrum for Ws-2.



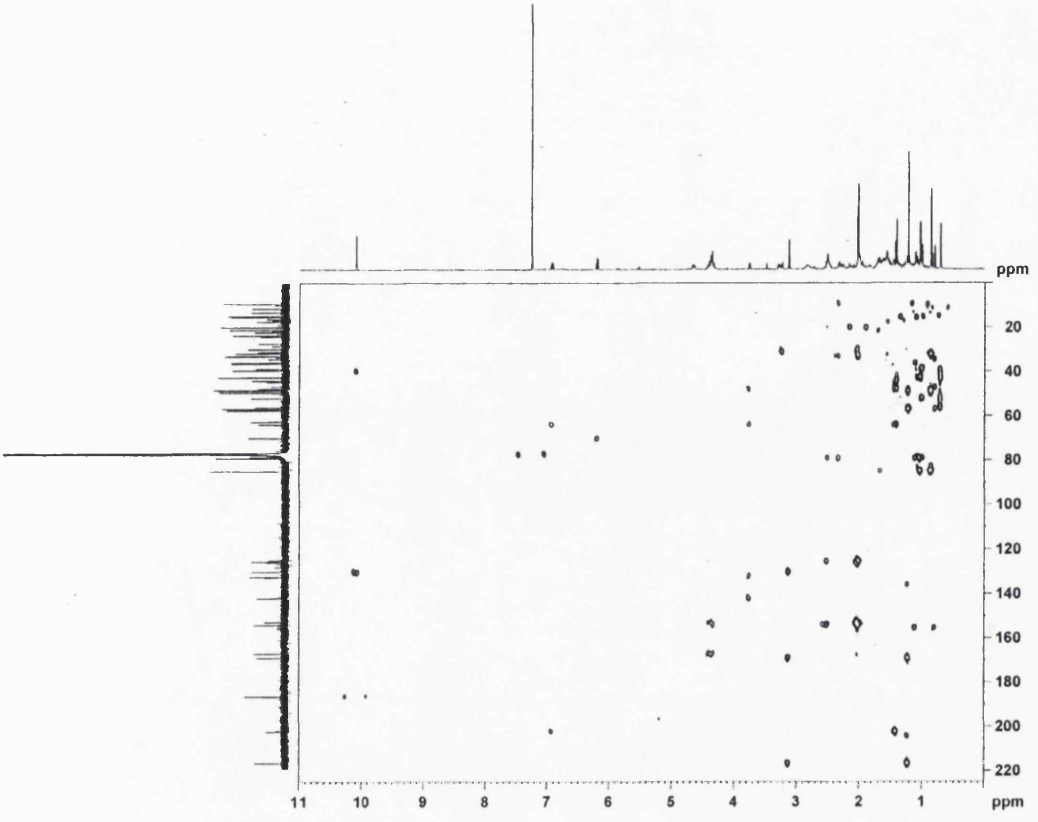
DEPT NMR (500 MHz, $CDCl_3$) spectrum for Ws-2.



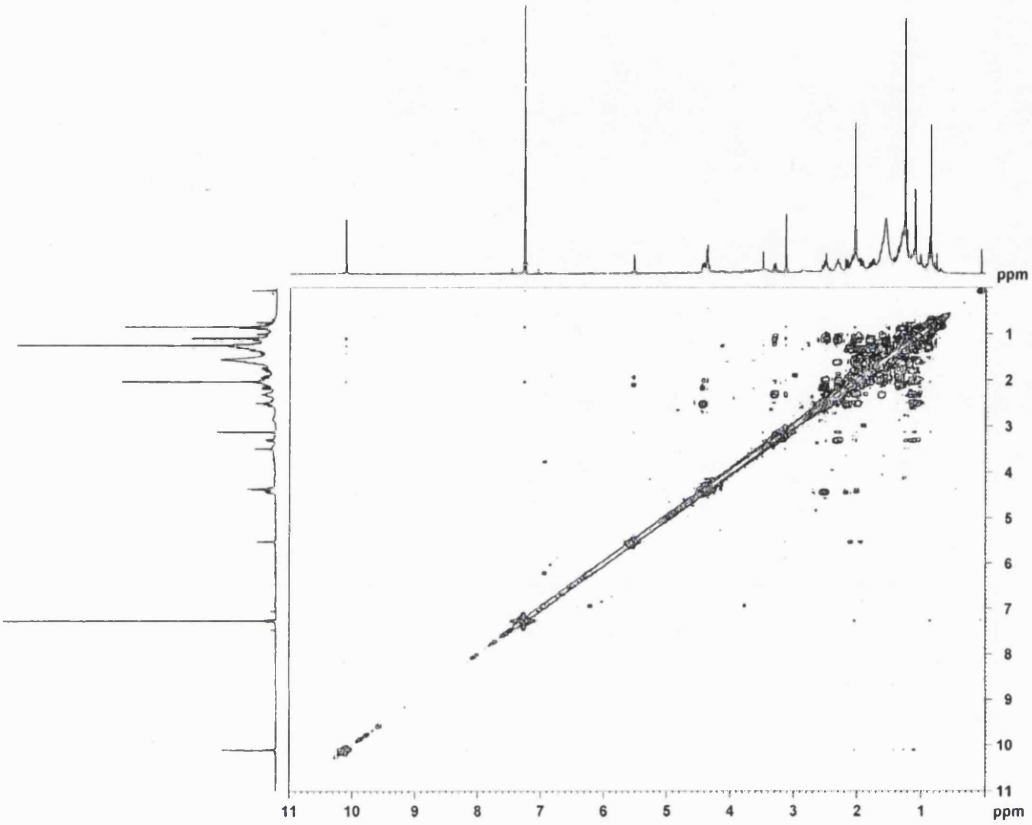
HMQC NMR (500 MHz, $CDCl_3$) spectrum for Ws-2.



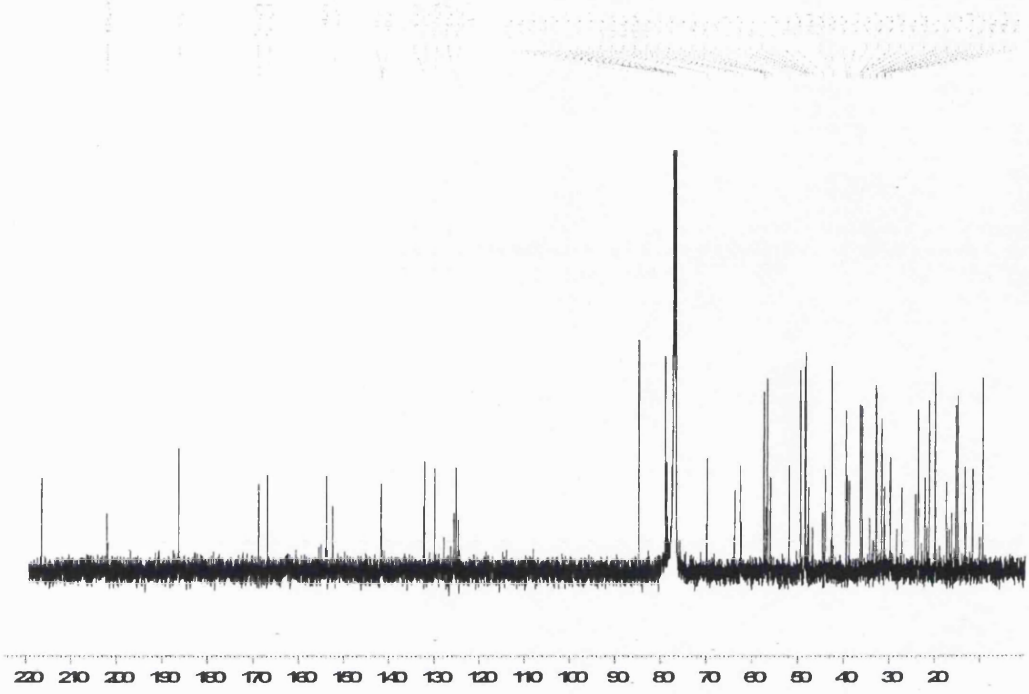
HMBC NMR (500 MHz, $CDCl_3$) spectrum for Ws-2.



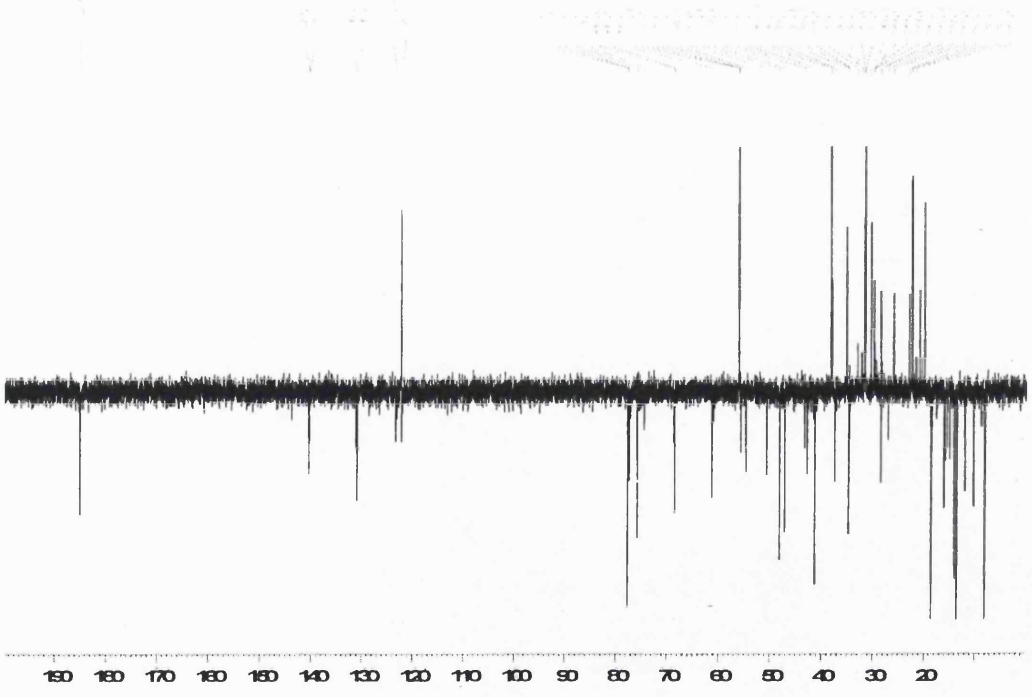
COSY NMR (500 MHz, $CDCl_3$) spectrum for Ws-2.



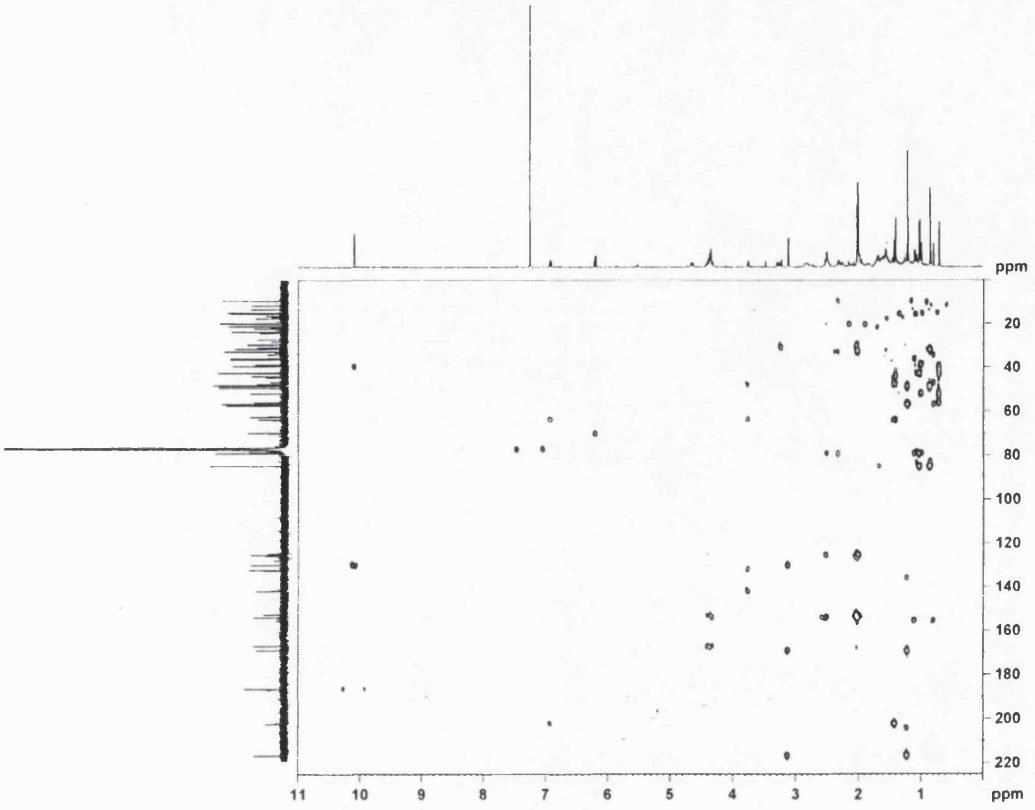
^{13}C NMR (500 MHz, $CDCl_3$) spectrum for withaferin A (Wf-3).



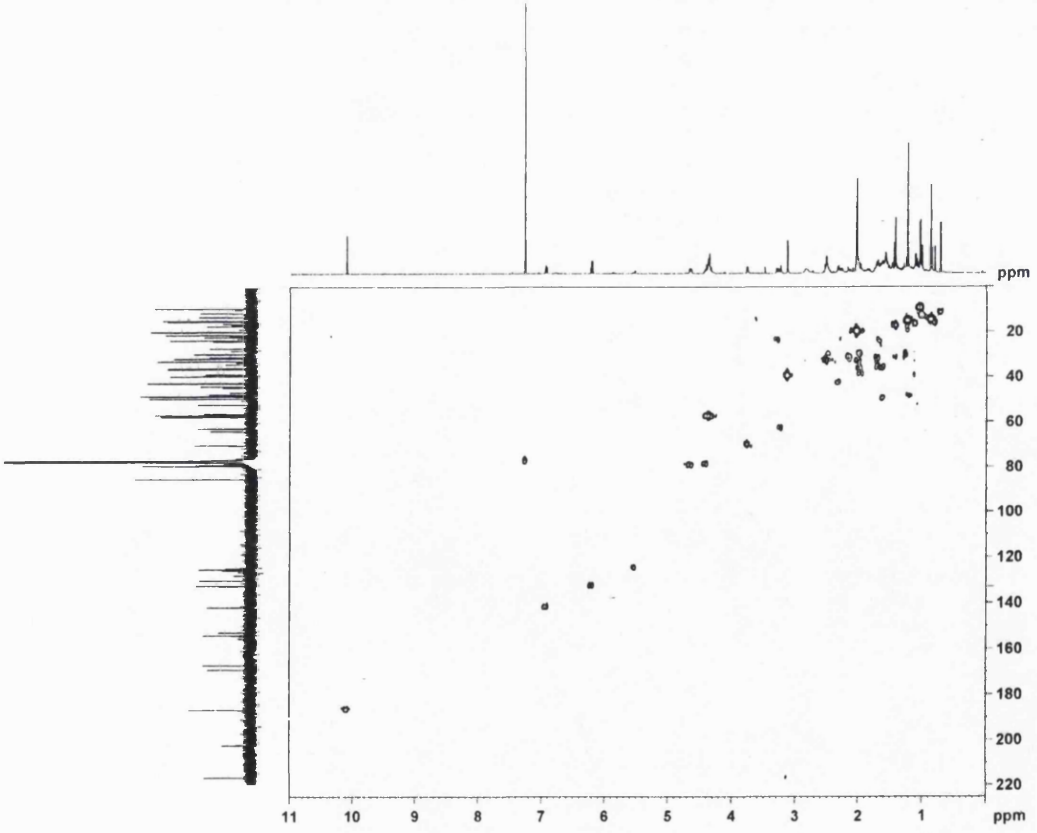
DEPT NMR (500 MHz, $CDCl_3$) spectrum for withaferin A (Wf-3).



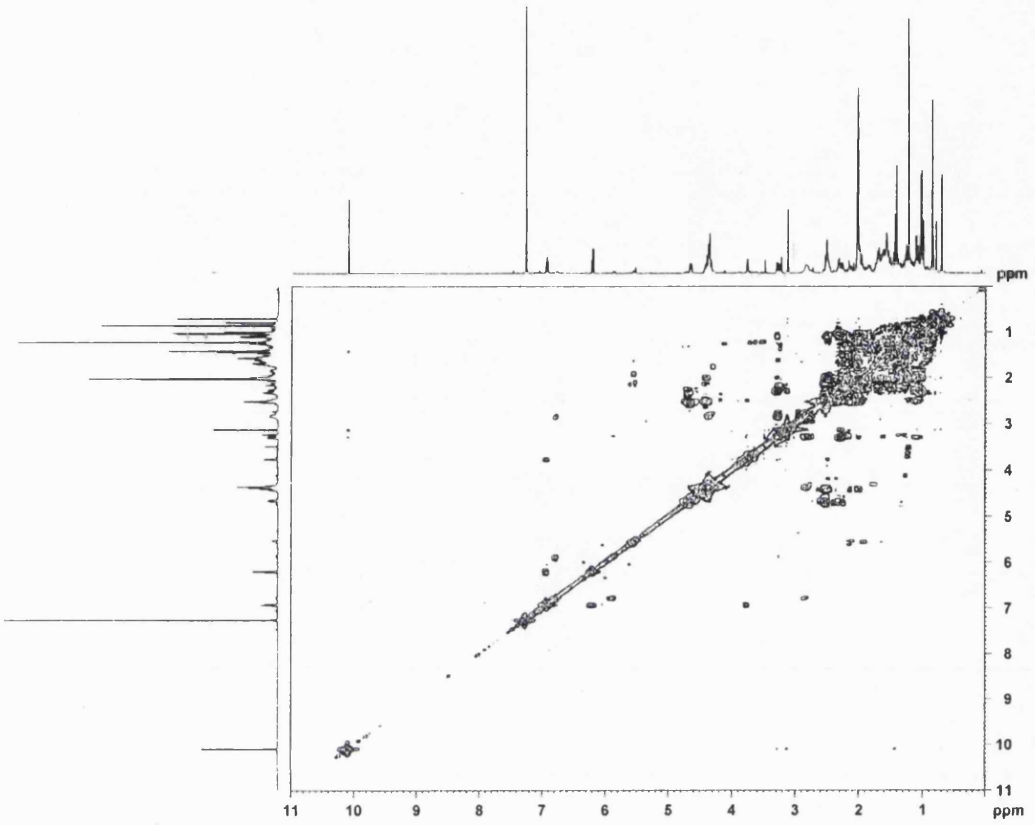
HMQC NMR (500 MHz, $CDCl_3$) spectrum for withaferin A (Wf-3).



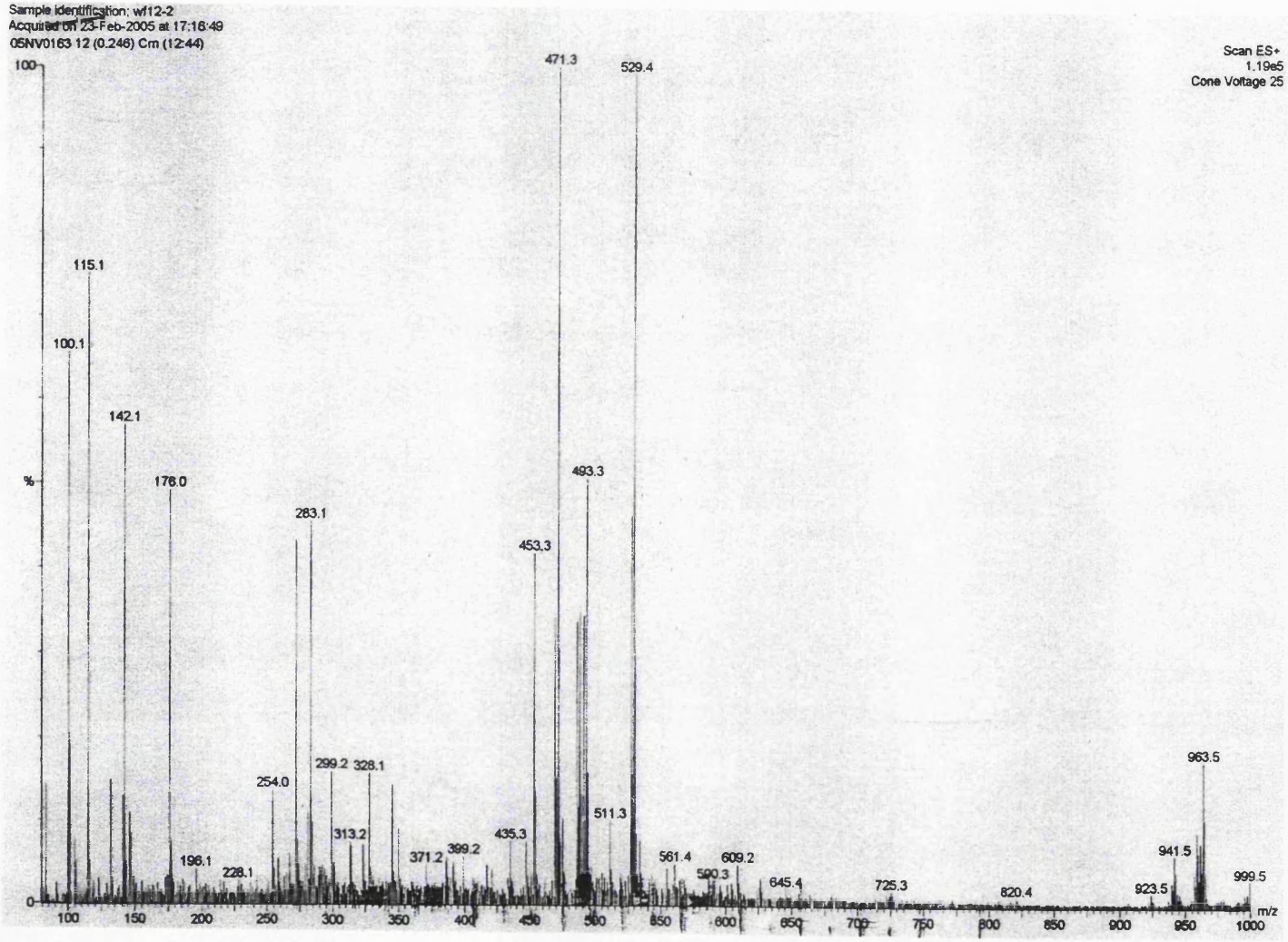
HMBC NMR (500 MHz, $CDCl_3$) spectrum for withaferin A (Wf-3).



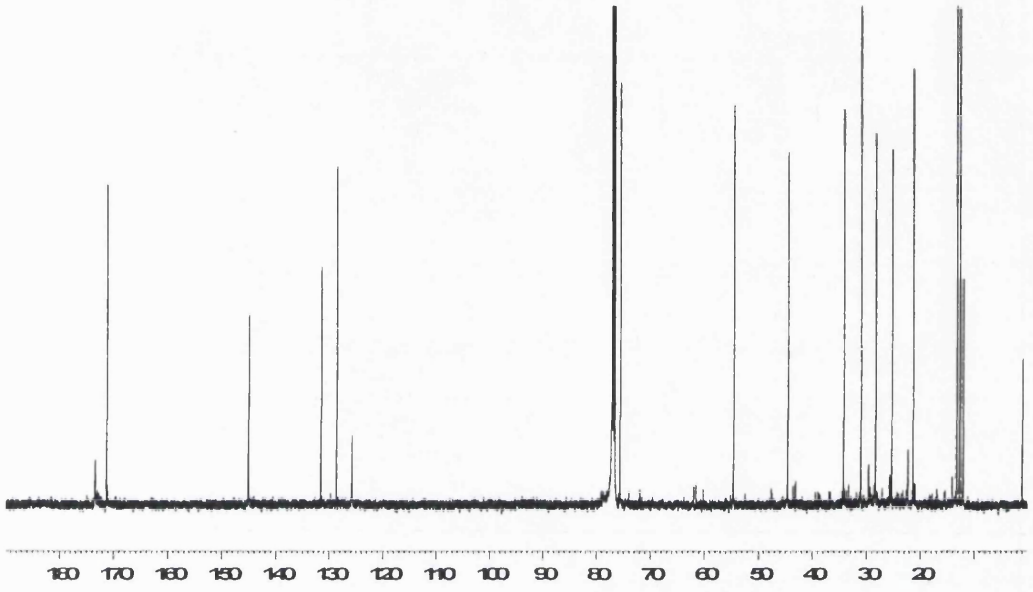
COSY NMR (500 MHz, $CDCl_3$) spectrum for withaferin A (Wf-3).



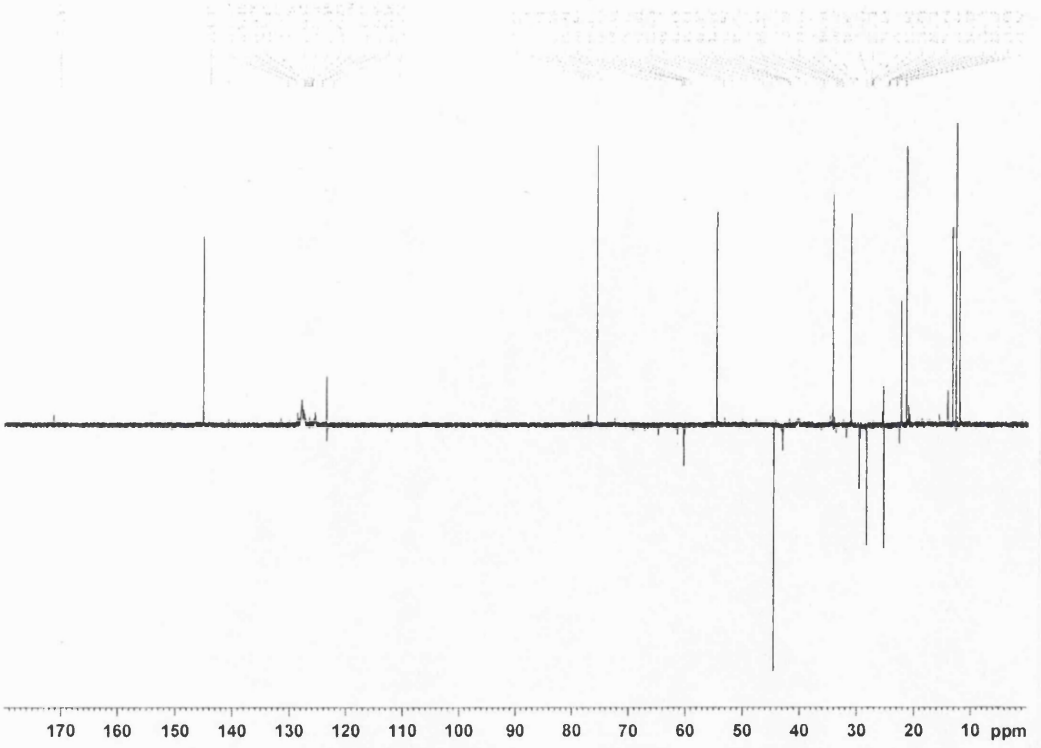
MS for withaferin A (Wf-3). Positive elctron spray (ES+).



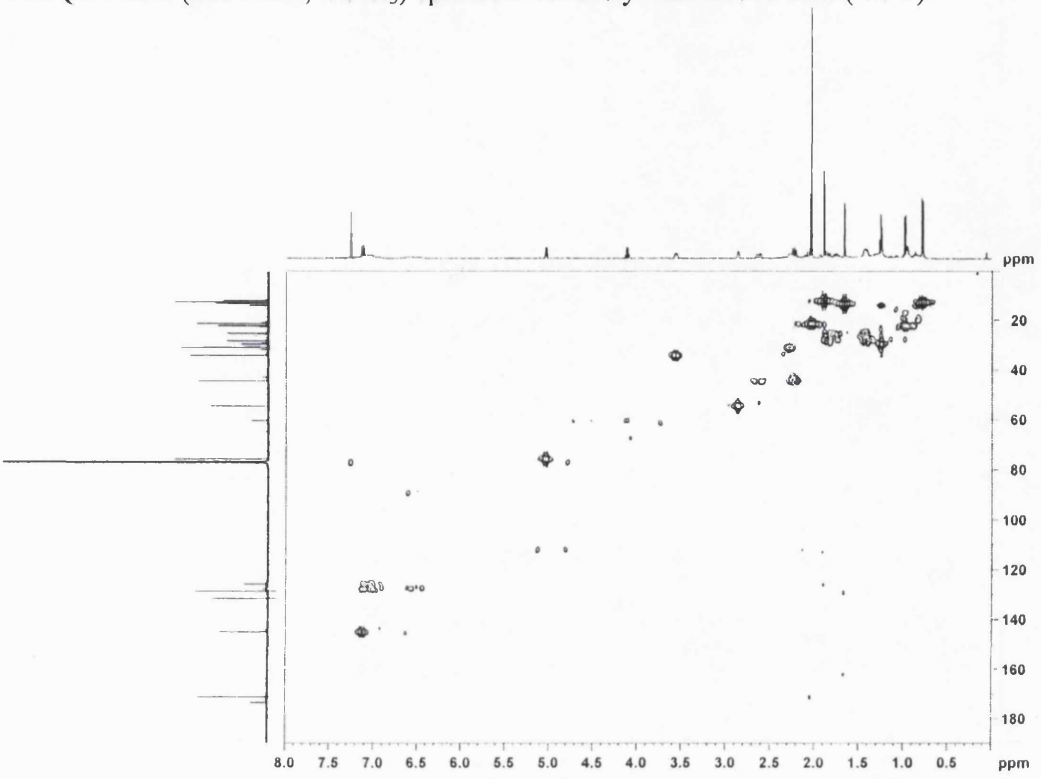
^{13}C NMR (500 MHz, $CDCl_3$) spectrum for acetylvalerenolic acid (Vo-1).



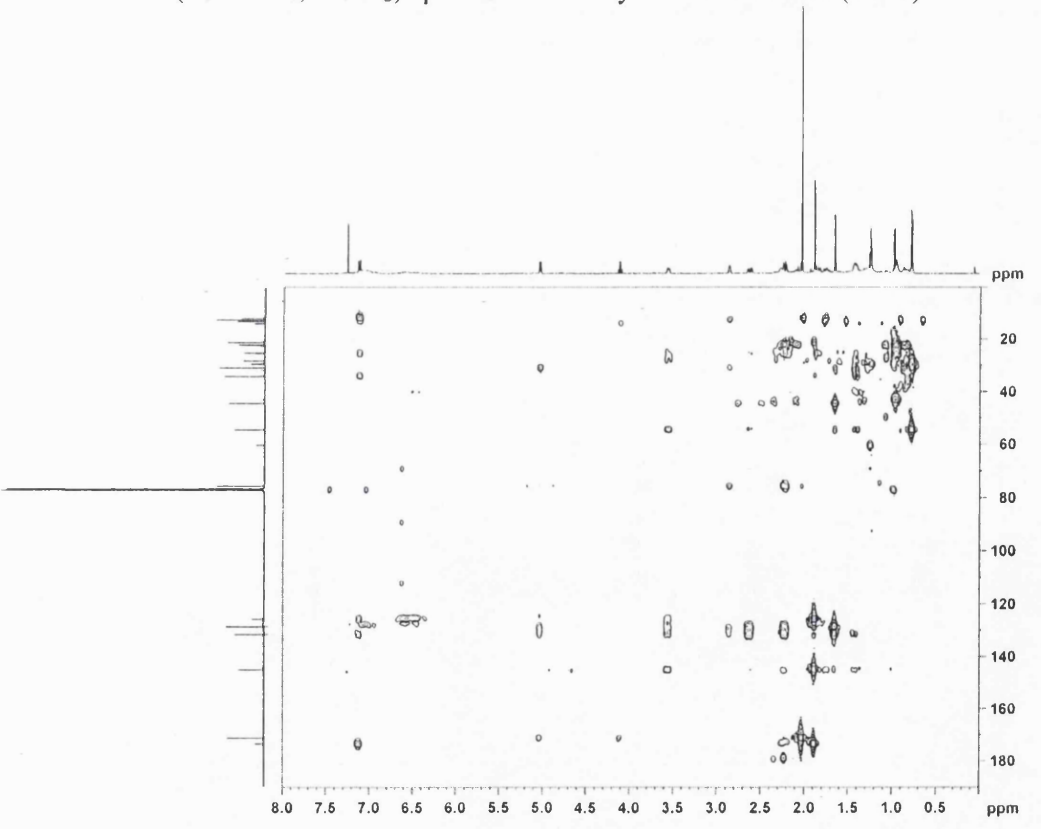
DEPT NMR (500 MHz, $CDCl_3$) spectrum for acetylvalerenolic acid (Vo-1).



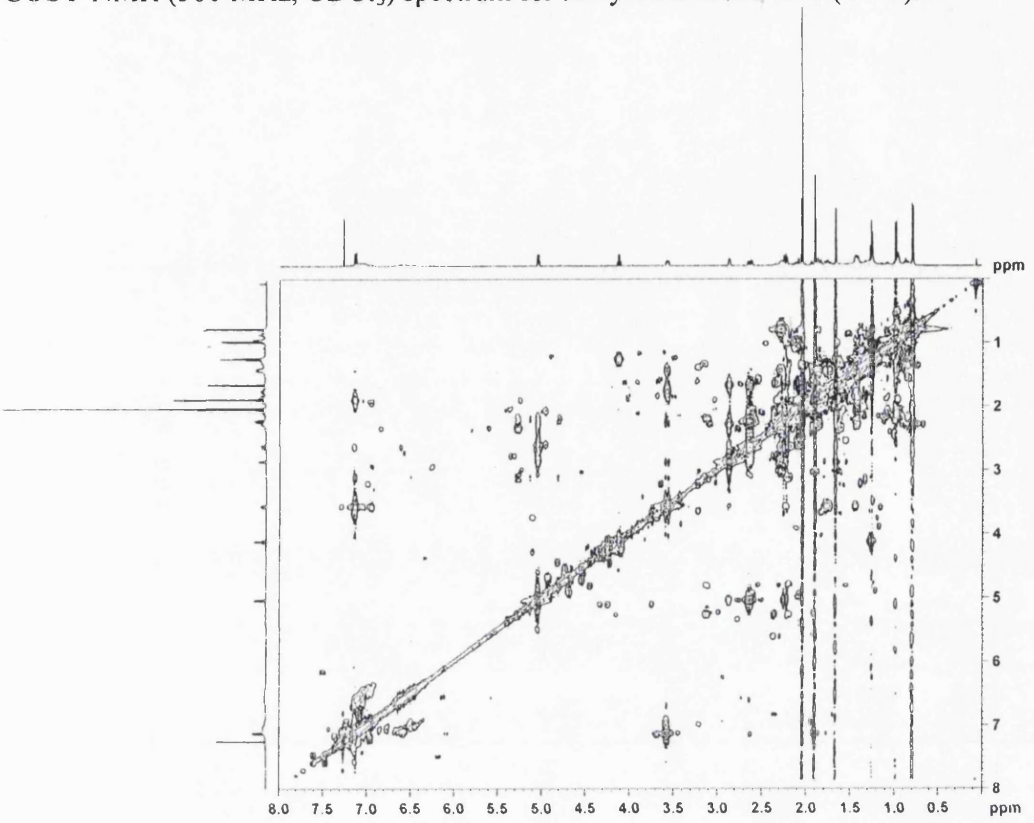
HMQC NMR (500 MHz, $CDCl_3$) spectrum for acetylvalerenolic acid (Vo-1).



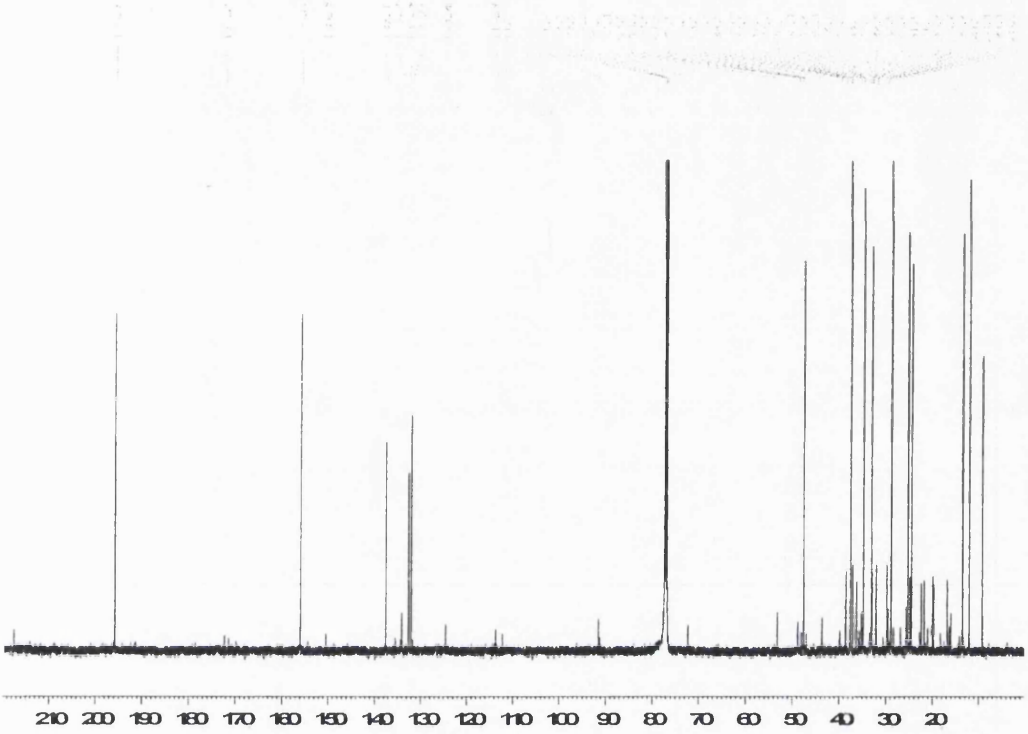
HMBC NMR (500 MHz, $CDCl_3$) spectrum for acetylvalerenolic acid (Vo-1).



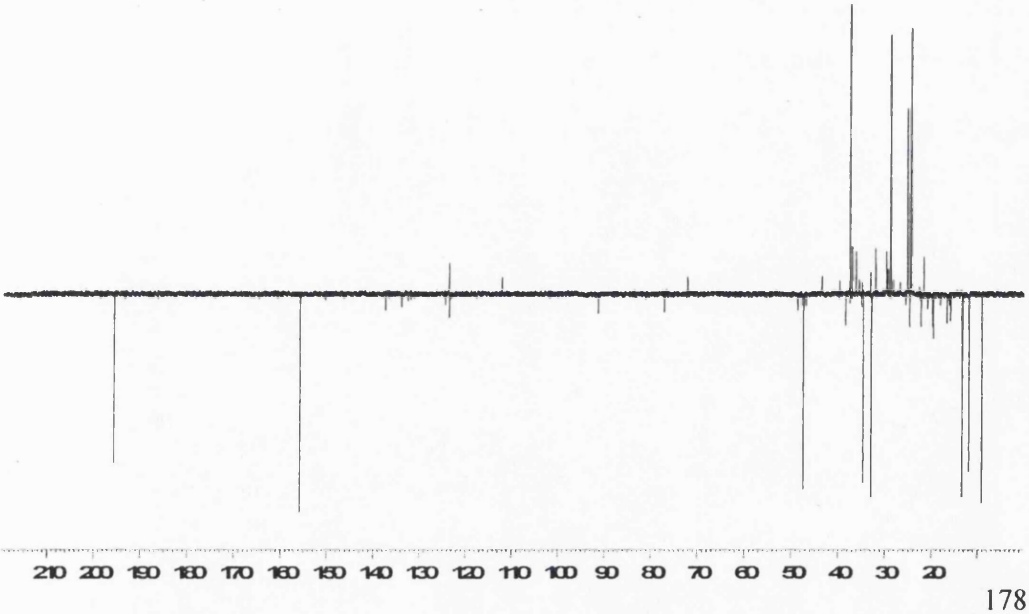
COSY NMR (500 MHz, $CDCl_3$) spectrum for acetylvalerenolic acid (Vo-1).



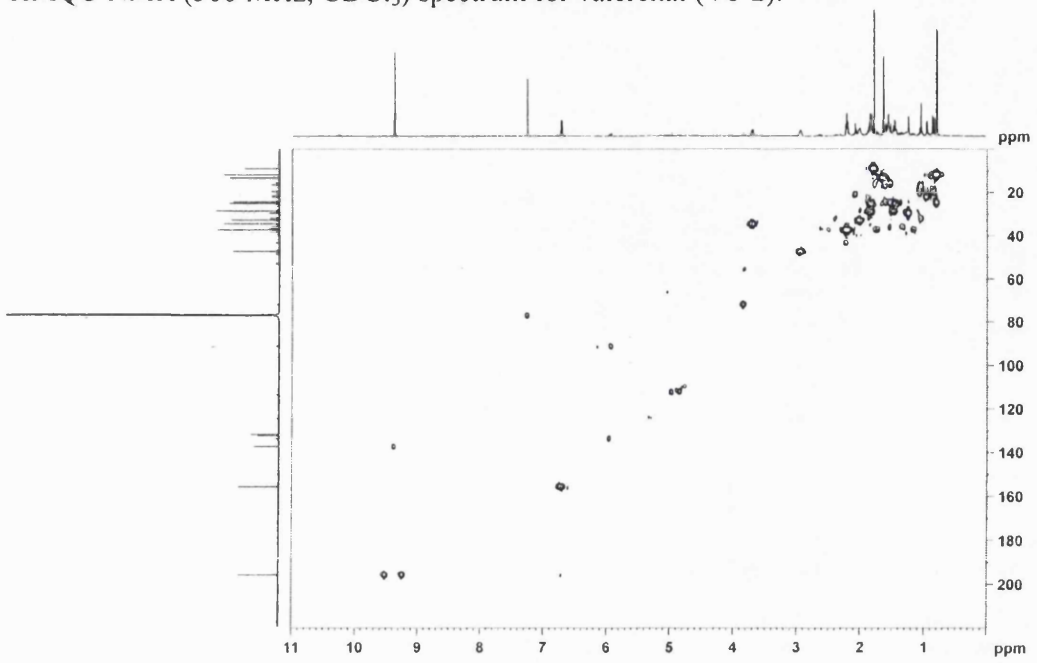
^{13}C NMR (500 MHz, $CDCl_3$) spectrum for valerenal (Vo-2).



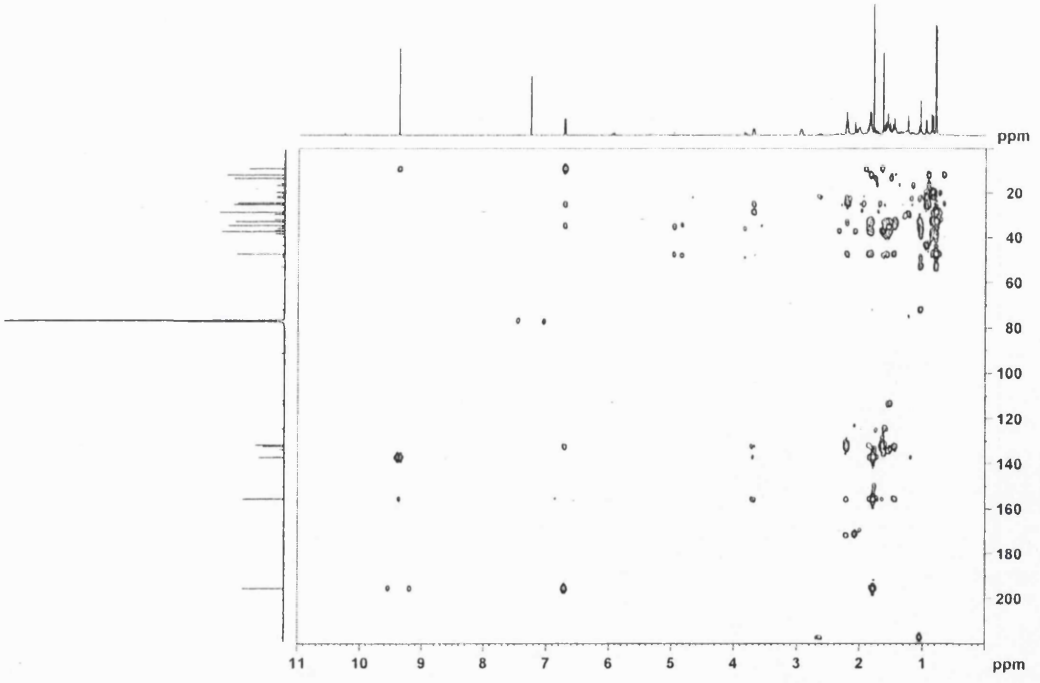
DEPT NMR (500 MHz, $CDCl_3$) spectrum for valerenal (Vo-2).



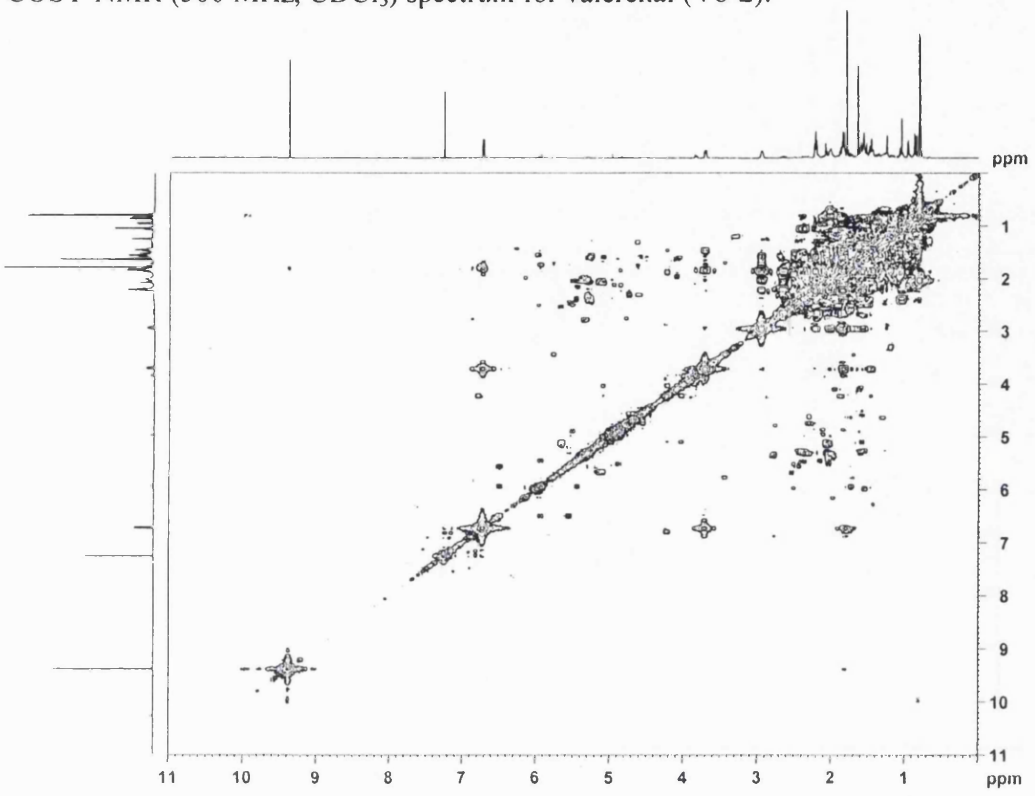
HMQC NMR (500 MHz, $CDCl_3$) spectrum for valerenal (Vo-2).



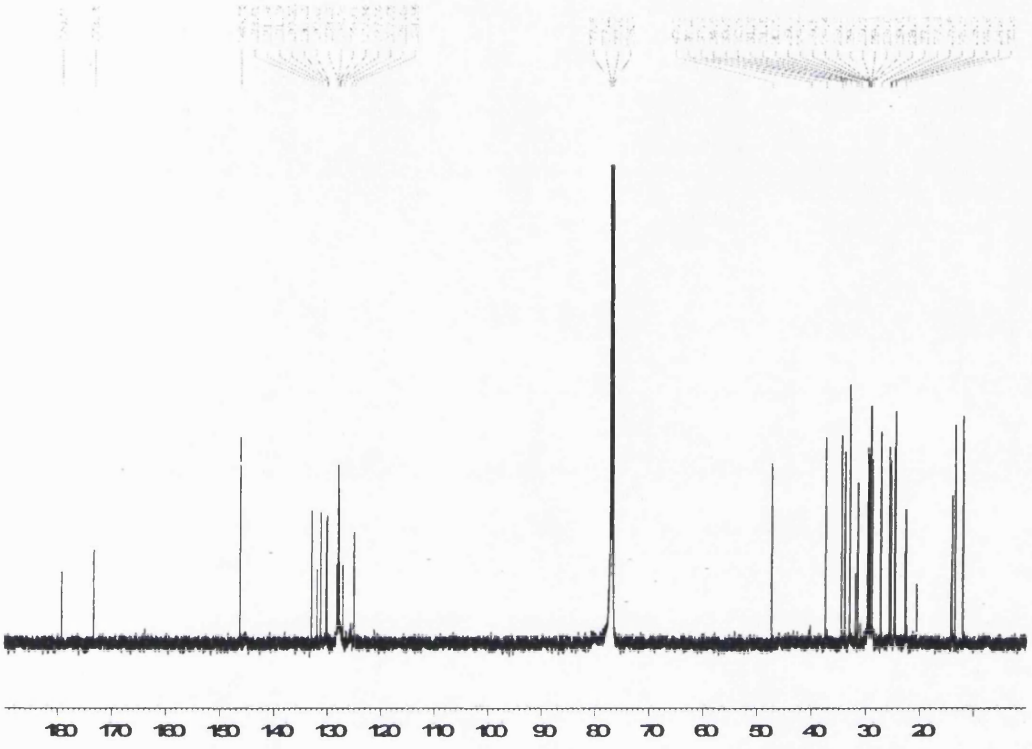
HMBC NMR (500 MHz, $CDCl_3$) spectrum for valerenal (Vo-2).



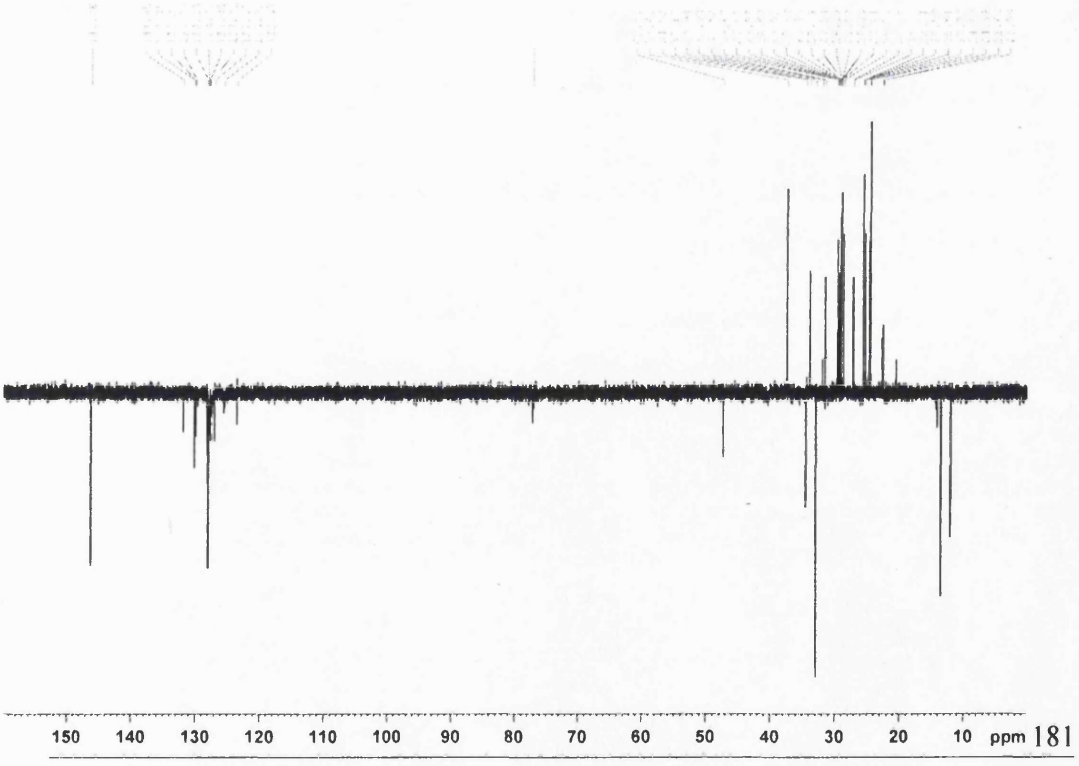
COSY NMR (500 MHz, $CDCl_3$) spectrum for valerenal (Vo-2).



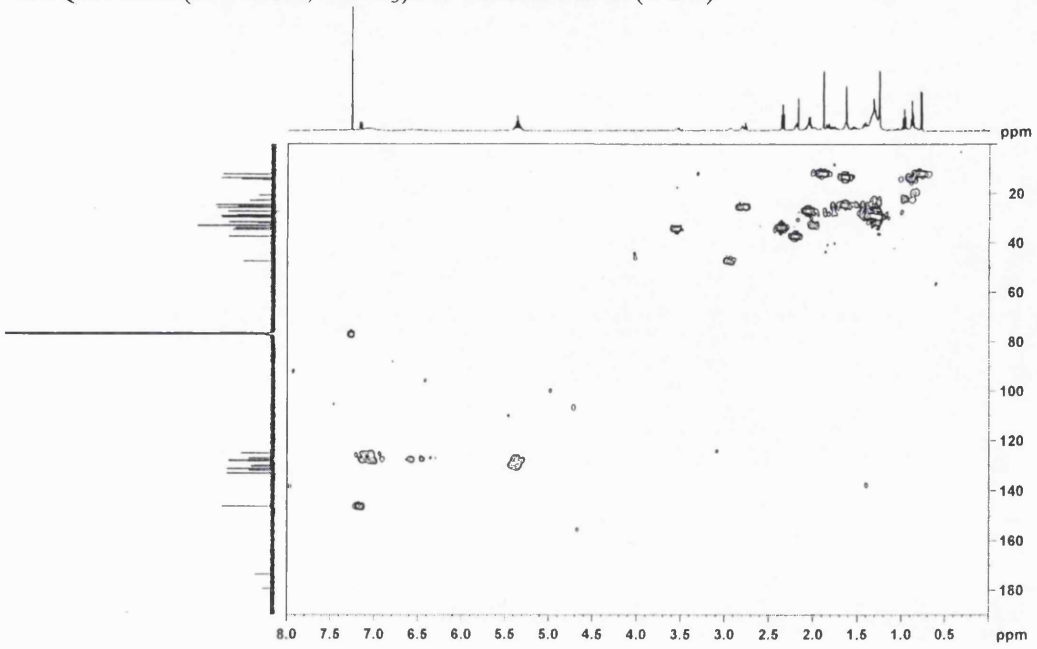
^{13}C NMR (500 MHz, $CDCl_3$) for valerenic acid (Vo-3).



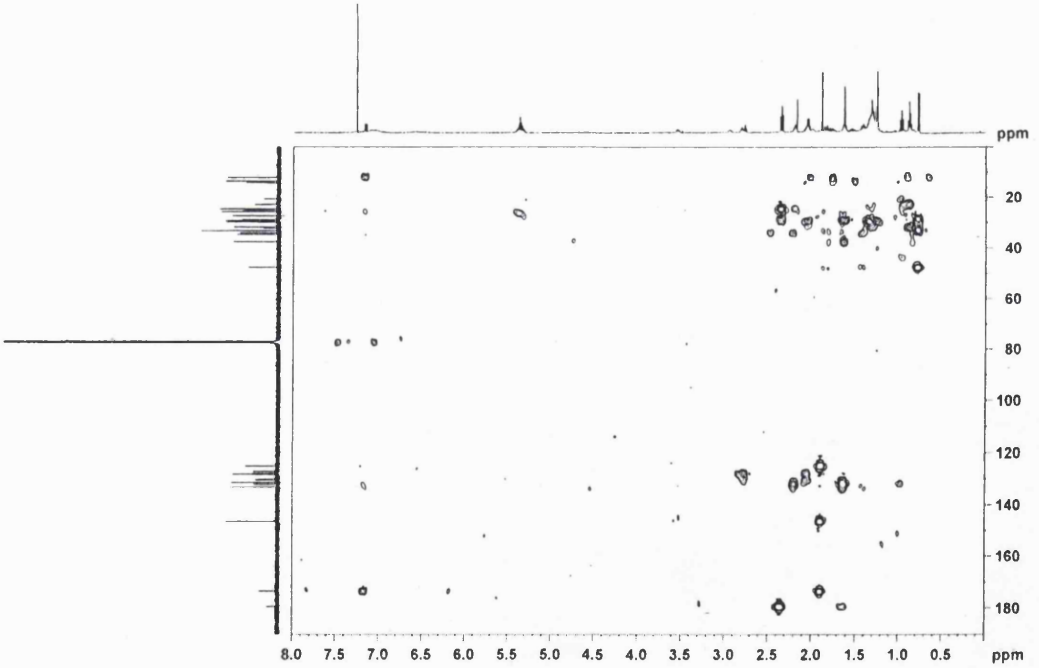
DEPT NMR (500 MHz, $CDCl_3$) for valerenic acid (Vo-3).



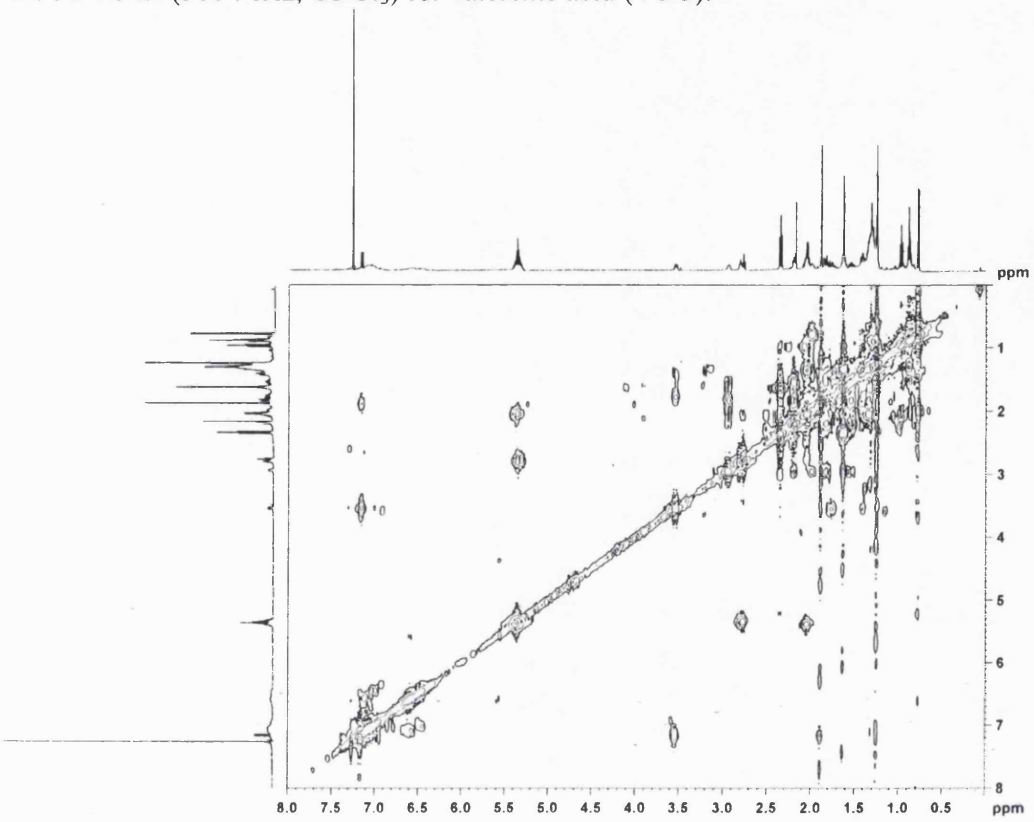
HMQC NMR (500 MHz, $CDCl_3$) for valerenic acid (Vo-3).



HMBC NMR (500 MHz, $CDCl_3$) for valerenic acid (Vo-3).



COSY NMR (500 MHz, $CDCl_3$) for valerenic acid (Vo-3).



List of publications (published and submitted):

- Heinrich M, Jacobo-Herrera N. Mexikanischer Baldrian (*Valeriana edulis* ssp. *procera*). **Zeitschrift für Phytotherapie** 2003; 24: 83-86.
- Jacobo-Herrera NJ, Bremner P, Márquez N, Gupta MP, Gibbons S, Muñoz E, Heinrich M. Physalins from *Witheringia solanacea* L'Her (Solanaceae) as modulators of the NF-κB cascade. Submitted to the Journal of Natural Products.

Conferences:

- NF-κB inhibitors from *Valeriana officinalis* L., an important European medicinal plant. Poster. 7th Conference of the International Society for Ethnopharmacology and 29th Conference of the South African Association of Botanists, Pretoria, South Africa. January 2003.
- NF-κB inhibitors from *Valeriana officinalis* L Poster. 51st Annual Congress of the Society for Medicinal Plant Research. Kiel, Germany. September 2003.
- Searching for anti-inflammatory compounds in *Witheringia solanacea* L'Her (Solanaceae). Poster. International Congress on Natural Products Research, Phoenix, Arizona, U. S. A. August 2004.
- Searching for anti-inflammatory properties in *Witheringia solanacea* L'Her (Solanaceae). Oral presentation. British Pharmaceutical Conference, Manchester, U. K. September 2004.
- Searching for anti-inflammatory properties in *Witheringia solanacea* L'Her (Solanaceae). Oral presentation. PhD Research Day, The School of Pharmacy, University of London, U. K. October 2004.