Doctoral Thesis

Biological and phytochemical investigations of Euphorbiaceae from Papua New Guinea

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BIOLOGICAL AND PHYTOCHEMICAL INVESTIGATIONS OF EUPHORBIACEAE FROM PAPUA NEW GUINEA

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Natural Sciences

presented by

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Zurich, 1999
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<td>Acetonitrile</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Cultures Collection</td>
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<tr>
<td>n-BuOH</td>
<td>1-Butanol</td>
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<tr>
<td>c</td>
<td>Concentration</td>
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<tr>
<td>CDCl₃</td>
<td>Deuterated Chloroform</td>
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<td>CHCl₃</td>
<td>Chloroform</td>
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<td>Dichloromethane</td>
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<tr>
<td>COSY</td>
<td>Correlated Spectroscopy</td>
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<tr>
<td>CuSO₄</td>
<td>Copper Sulphate</td>
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<tr>
<td>d</td>
<td>Doublet</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless Enhancement by Polarization Transfer</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<tr>
<td>DQF-COSY</td>
<td>Double Quantum Filtered Correlation Spectroscopy</td>
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<td>EIMS</td>
<td>Electron Impact Mass Spectroscopy</td>
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<tr>
<td>EtOAc</td>
<td>Ethyl Acetate</td>
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<tr>
<td>eV</td>
<td>Electron Volt</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Correlation</td>
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<td>HSQC</td>
<td>Heteronuclear Single Quantum Correlation</td>
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<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>i.d.</td>
<td>Internal Diameter</td>
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<tr>
<td>INT</td>
<td>Iodonitrotetrazolium Chloride</td>
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<tr>
<td>IR</td>
<td>Infrared Spectroscopy</td>
</tr>
<tr>
<td>KBr</td>
<td>Potassium Bromide</td>
</tr>
<tr>
<td>m</td>
<td>Multiplet</td>
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<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
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</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>m.p.</td>
<td>Melting point</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NCI</td>
<td>U.S. National Cancer Institute</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>NP</td>
<td>Normal Phase</td>
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<tr>
<td>OMe</td>
<td>Methoxy</td>
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<td>ppm</td>
<td>Parts Per Million</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PNG</td>
<td>Papua New Guinea</td>
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<tr>
<td>q</td>
<td>Quartet</td>
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<tr>
<td>ROe</td>
<td>Rotating Frame Overhauser Effect</td>
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<td>ROESY</td>
<td>Rotating Frame Overhauser Spectroscopy</td>
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<tr>
<td>RP</td>
<td>Reversed Phase</td>
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<td>s</td>
<td>Singlet</td>
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<tr>
<td>sh</td>
<td>Shoulder</td>
</tr>
<tr>
<td>Si Gel</td>
<td>Silica Gel</td>
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<tr>
<td>t</td>
<td>Triplet</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>UV</td>
<td>Ultraviolet Spectroscopy</td>
</tr>
<tr>
<td>VLC</td>
<td>Vacuum Liquid Chromatography</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Summary

Based on ethnopharmacological literature traditional medicinal plants of Papua New Guinea were collected during two field trips. The collection included the following plant species: *Aleurites moluccana* (Euphorbiaceae), *Desmodium umbellatum* (Papilionaceae), *Euphorbia buxoides* (Euphorbiaceae), *Euphorbia geniculata* (Euphorbiaceae), *Ficus dammaropsis* (Moraceae), *Ficus nodosa* (Moraceae), *Ficus opposita* (Moraceae), *Homalanthus nervosus* (Euphorbiaceae), *Homalanthus novoguineensis* (Euphorbiaceae), *Mangifera minor* (Anacardiaceae), *Securinega melanthesoides* (Euphorbiaceae), and *Scaevola oppositifolia* (Goodeniaceae).

Crude extracts of different polarities were prepared and submitted to a preliminary biological screening. Antifungal activity was determined using *Penicillium oxalicum* and a newly introduced in-house bioassay with *Candida albicans* as test strain. Antibacterial activity was tested against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Mycobacterium fortuitum*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*. Molluscicidal activity was determined using snails of the species *Biomphalaria glabrata*. Cytotoxicity was examined against KB cells and CaCo2 cells, general toxicity against brine shrimps (*Artemia salina*).

Of the investigated plant extracts, plants belonging to the Euphorbiaceae showed the most interesting biological activities. Further phytochemical work was thus focused on two euphorbiaceous plant species: *Euphorbia buxoides* and *Homalanthus nervosus*.

Extraction and further purification of *Euphorbia buxoides* leaves by means of different chromatographic techniques (VLC, HPLC, TLC, open column chromatography, liquid liquid chromatography) led to the isolation of the four euphane-type triterpenoids euphol, corollatadiol, eupha-8,23-dien-3ß-25-diol and eupha-8,25-dien-3ß-24-diol, the latter two as unseparable mixture. Additionally, the benzoquinone α-tocopherolquinone, the fatty alcohol n-octacosanol and the flavonoids hyperoside and kaempferol-3-O-β-D-
glucuronide were isolated. DAD-HPLC analysis allowed furthermore the identification of kaempferol-3-O-glucoside. Hints to the presence of ingenane diterpenes were found with the isolation of a nonadeca-3,5-dien-2-ol-1-yl moiety as possible side chain of an originally present ingenol diterpene. Extraction and purification of Homalanthus nervosus leaves yielded β-sitosterol and the coumarin derivative scoparone. DAD-HPLC analysis allowed the identification of hyperoside and kaempferol-3-O-glucoside as major flavonoids. Structure elucidation of the isolated compounds was carried out by means of 1D and 2D NMR experiments (HMQC, HMBC, DQF-COSY, t-ROESY, TOCSY), as well as mass spectrometry and further spectroscopic methods (UV, IR). This is the first phytochemical report on the constituents of Euphorbia buxoides and Homalanthus nervosus. The constituents isolated from E. buxoides as unseparable mixture, eupha-8,23-dien-3β-25-diol and eupha-8,25-dien-3β-24-diol have been isolated for the first time from natural sources.
Zusammenfassung

Während zwei Feldstudien in Papua Neuguinea wurden, basierend auf ethnopharmakologischen Literaturangaben, Pflanzen der dortigen traditionellen Medizin gesammelt. Die Sammlung beinhaltete die folgenden Pflanzenspezies: \textit{Aleurites moluccana} (Euphorbiaceae), \textit{Desmodium umbellatum} (Papilionaceae), \textit{Euphorbia buxoides} (Euphorbiaceae), \textit{Euphorbia geniculata} (Euphorbiaceae), \textit{Ficus dammaropsis} (Moraceae), \textit{Ficus nodosa} (Moraceae), \textit{Ficus opposita} (Moraceae), \textit{Homalanthus nervosus} (Euphorbiaceae), \textit{Homalanthus novoguineensis} (Euphorbiaceae), \textit{Mangifera minor} (Anacardiaceae), \textit{Securinega melanthesoides} (Euphorbiaceae) und \textit{Scaevola oppositifolia} (Goodeniaceae).


Von den untersuchten Extrakten zeigten Pflanzen aus der Familie der Euphorbiaceen (Wolfsmilchgewächse) die interessantesten biologischen Aktivitäten. Für die weiteren phytochemischen Untersuchungen wurden daher die beiden Euphorbiaceen \textit{Euphorbia buxoides} und \textit{Homalanthus nervosus} ausgewählt.


Die Strukturaufklärung der isolierten Substanzen erfolgte mittels eindimensionaler und zweidimensionaler NMR Experimente (HMQC, HMBC, DQF-COSY, t-ROESY, TOCSY), Massenspektrometrie, sowie weiterer spektroskopischer Methoden (UV, IR).

1 Introduction

1.1 Plants as a resource for medicinal remedies

Since its earliest beginnings higher plants have served humankind as sources of medicinal agents. Today, natural products, their derivatives and their analogs still constitute over 50% of all drugs in clinical use, with higher plant-derived natural products representing ca. 25% of the total (Balandrin et al. 1993). It is estimated that nowadays perhaps 80% of the world's population rely chiefly on traditional medicines for their primary health care needs. It can be presumed that a major part of traditional therapy involves the use of plant extracts or their active principles (Farnsworth et al. 1985).

Natural products continue to play an important role in drug discovery programs of the pharmaceutical industry and other research organizations. An important reason for the use of natural products as a source of lead compounds is the tremendous variety of species found in nature and the resulting molecular diversity of the isolated compounds (Borris 1996). In addition to the biologically active plant-derived secondary metabolites which have found direct medicinal application as drugs, many other bioactive plant compounds are useful as "leads" or model compounds (templates) for drug syntheses or semisyntheses (Balandrin et al. 1993).

Research in the field of chemical and biological properties of natural products yielded drugs for the treatment of many human diseases. Additionally it gave the stimulus for the development of modern synthetic organic chemistry, and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents (Baker et al. 1995).

For example the positive benefits of extracts of Digitalis purpurea and D. lanata (Scrophulariaceae) were recognized long before the active constituents were isolated and characterized structurally. The cardiac glycosides, including digoxin, digitoxin and deslanoside, exert a powerful and selective positive
inotropic action on the cardiac muscle. Digoxin is still produced by mass
cultivation and extraction of *D. purpurea* strains that have been selected for
maximum production of the bioactive glycosides.

Quinidine, isolated from the bark of the *Cinchona pubescens* tree (Rubiaceae),
is an important anti-arrhythmic drug, and was also one of the earliest and most
well known examples of the important role of chirality in drug action. Its
diastereoisomer, quinine, has virtually no cardiac activity, but was recognized
as one of the first antiinfective agents for the treatment of malaria.

Besides their therapeutic effects as remedies against pain and cough, the
opium alkaloids, including morphine and codeine, isolated from *Papaver
somniferum* (Papaveraceae) served as models for the synthesis of opioid
analgesics as well as naloxone, an important analog used to treat and diagnose
opiate addicts. Studies on the mode of action of the opium alkaloids led to the
discovery of "endogenous opioids" and the opiate receptors (Clark 1996).

Recent approvals of several new plant-derived drugs and semi-synthetic and
synthetic drugs based on plant secondary compounds confirm the importance
of natural product research. Taxol, for example, an anticancer taxane
diterpenoid derived from the relatively scarce Pacific or western yew tree, *Taxus
brevifolia* (Taxaceae), has recently been approved for the treatment of
refractory ovarian cancer and breast cancer. Artemisinin, a chinese drug
isolated from *Artemisia annua* (Asteraceae), and several of its derivatives,
newly discovered as effective antimalarial agents, are currently undergoing
intensive clinical testing (Balandrin et al. 1993).

In recent years the advances made in biotechnology led to new features for
plants. Advances in recombinant DNA technology have enabled plant molecular
biologists to transfer a remarkable variety of foreign genes encoding therapeutic
proteins into plant species such as potato and tobacco (Arakawa and Langridge
1998). Recently the first clinical trials of plant vaccines on humans were
described. The gene encoding for an enterotoxin of *Escherichia coli*, causing
serious diarrhea, was introduced into potatoes. After consumption of these
genetically modified potatoes an immune response (production of antibodies for the neutralization of the enterotoxin) could be shown in humans. Especially for third world countries vaccines from genetically modified edible plants could constitute a cheap alternative to the costly vaccination campaigns (Ma et al. 1998); (Tacket et al.1998).

These examples clearly demonstrate that plant-derived products are still important as medicinal agents and models for the design, synthesis, and semisynthesis of novel substances for the treatment of various diseases. Considering that only a very small percentage of the 300'000 to 500'000 plant species existing on earth have been minimally evaluated, nature still provides a large amount of new substances (Clark 1996).

1.1.1 Anticancer agents from plants

Cancer still claims over 6 million lives each year. It consists one of the main causes of death in developed countries.

Over 40 years ago, the U.S. National Cancer Institute (NCI) began a program to explore natural products as a source of anticancer drugs. Since then preclinical screening of extracts led to the isolation of many pure compounds active against cancer cells. Thus the NCI played a major role in the discovery and development of many of the commercial and investigational anticancer agents (Cragg et al. 1993). Of the actually 87 approved anticancer drugs, 62% are of natural origin or were modeled on natural product parents (Cragg et al. 1997). Currently, four plant-derived anticancer drugs are in regular clinical use.
Figure 1.1 shows examples of plant derived anticancer agents which will be discussed subsequently.

![Chemical structures of anticancer agents](image)

**Fig. 1.1: Examples of plant-derived anticancer drugs**

Taxol (Fig. 1.1), a taxane diterpenoid isolated from *Taxus brevifolia* (Taxaceae), was first reported in 1971. At the time of its discovery taxol was only one of a number of promising natural products under pharmacological investigation. It showed significant, but modest *in vivo* activity in two leukemia cell lines (Kingston 1993). Interest in taxol received a significant boost with the finding of Horwitz and coworkers in 1979, that taxol is a mitotic inhibitor, showing the unique mechanism of stabilizing microtubules and inhibiting depolymerization back to tubulin (Schiff et al. 1979). This is in contrast to other antimitotic agents binding to soluble tubulin and inhibiting the polymerization to tubulin.

In 1992 Taxol® (paclitaxel) was approved for marketing as a new drug for treatment of refractory ovarian cancer. Use of paclitaxel has been expanded and includes a greater variety of cancer now. In 1994 a supplemental approval for metastatic breast cancer was given (Suffness and Wall 1995). Recently
Taxotere® (docetaxel), a semisynthetic analogue of Taxol, approximately twice as potent as Taxol in the microtubule assay, has got approval by the Food and Drug Administration (FDA) (Arbuck and Blaylock 1995).

During extensive screening of random plant products by the NCI, a crude extract of *Camptotheca acuminata* (Nyssaceae), a plant growing in China, exhibited anticancer activity. From the extract camptothecin (Fig. 1.1) was isolated and demonstrated significant anticancer activity in a leukemia and a carcinosarcoma cell line. Inhibition of topoisomerase I was identified as the mode of action of camptothecin. The camptothecin analogs topotecan (Hycamtin®) and irinotecan (Campto®) have been approved for human use in the treatment of metastatic ovarian carcinoma (topotecan) or metastatic colorectal cancer (irinotecan) (Pezzuto 1997). Other analogs are undergoing phase I, II and III clinical trying (Eckardt et al. 1995).

Phytochemical and biological investigation of *Catharanthus roseus*, a traditional Chinese medicinal plant, led to the isolation of the so-called Vinca alkaloids vinblastine and vincristine (Fig. 1.1), two important cancer chemotherapeutic agents in current use. These two dimeric indole-indoline alkaloids are used for the treatment of acute childhood leukemia (vincristine), Hodgkin's disease (vinblastine) and metastatic testicular tumors (vinblastine) (Lee 1993).

Podophyllin, a crude resin used topically to treat Condylomata acuminata is obtained from *Podophyllum peltatum* (Berberidaceae), used for years in folk medicine. Among other compounds podophyllin contains the lignan podophyllotoxin. Etoposide and teniposide (Fig. 1.1), two derivatives of podophyllotoxin, were introduced as anticancer drugs in 1983 and 1992 respectively. Their mode of action is the inhibition of topoisomerase II (Clark 1996).

### 1.1.2 Anti-HIV agents from plants

In 1981 acquired immunodeficiency syndrome (AIDS) was recognized as a new clinical entity. By 1984, a newly discovered pathogenic human retrovirus,
human immunodeficiency virus (HIV), was identified as the etiologic agent causing AIDS (Yarchoan and Broder 1988). Subsequently AIDS spread rapidly and has become an infection of worldwide dimension. A second immunodeficiency virus was later isolated from West African patients with AIDS (HIV-2). HIV virus type-2 was found to be similar to HIV-1 in morphology, ultrastructure and genomic organization, but differing at both nucleotide and amino acid levels (Tan et al. 1992). Although HIV-2 is clearly related to immunodeficiency and clinical syndromes similar to AIDS, it is less spread over the world and is supposed to be less pathogenic than HIV-1 (Connor and Ho 1992).

Search for therapeutic agents for the treatment of AIDS started soon after the identification of HIV and has become a worldwide priority in medical research. Besides the search for anticancer drugs, the National Cancer Institute (NCI) also initiated a program to develop therapies for AIDS. An important part of the program was the introduction of a large-scale high throughput screening of plant extracts and pure compounds for anti-HIV activity (Lednicer and Snader 1991). In the following years a variety of chemically different molecules, having been isolated from species distributed across the whole plant kingdom such as algae, pine trees and flowering plants. Compounds included terpenoids, alkaloids, coumarins, flavonoids, xanthones, polyphenols (tannins), polysaccharides and proteins (Ng et al. 1997).

Although a number of antiviral agents are now being considered for the therapy of AIDS, no cure is known to date. Handling of the disease concentrates on three topics nowadays: reduction of the viral load, relief of the clinical symptoms and prolongation of life span. Like other retroviral diseases HIV infection of cells involves a series of critical steps including the binding of virus to cellular receptors, uncoating and internalization of the virus, transcription of the viral RNA into DNA by means of viral enzyme reverse transcriptase, integration of the viral DNA transcript into host chromosomal DNA, and subsequent transcription of viral particles, protein synthesis, assembly of the virus and finally release of the virus (Boyd 1988).
Therapeutic strategies have been focused on virus replication, especially on inhibition of the viral enzymes reverse transcriptase (RT), protease and integrase.

Figure 1.2 shows some plant derived anti-HIV agents, which will be discussed in the next paragraphs.

Glycyrrhizin (Fig. 1.2) isolated from the aqueous extract of liquorice root *Glycyrrhiza glabra* (Fabaceae), inhibited HIV replication in several *in vitro* systems. Its activity was suggested to be due, at least partially, to the inhibition of protein kinase C (PKC) and the viral adsorption process. PKC is supposed to play an important role in binding HIV-1 on the cellular receptor (Ito et al. 1988).

Castanospermine (Fig. 1.2) is a plant alkaloid isolated from seeds of the Australian chestnut tree, *Castanospermum australe* (Fabaceae). It could be shown that castanospermine is able to reduce the virion infectivity as well as the cell-to-cell spread of virus infection by inhibiting cell fusion events. The anti-viral effects of castanospermine may be due to modifications of the envelope glycoprotein that affect the ability of the virus to enter cells after attachment to the CD4 cell receptor (Walker et al. 1987).

Hypericin (Fig. 1.2) and pseudohypericin, two compounds isolated from *Hypericum perforatum* (Guttiferae), showed activity against a variety of retroviruses, including HIV, *in vivo* and *in vitro*. Hypericin and pseudohypericin showed no inhibitory activity on reverse transcriptase, but inhibited the release of reverse transcriptase by stabilizing the structure of the HIV capsid and so preventing the uncoating process. It was observed that the mode of action of hypericin as an antiretroviral agent was related to the effects of light (Hudson et al. 1993). Hypericin excites oxygen to its singlet state (Duran and Song 1986). These reactions may lead to stabilization of the capsid, and result in virions which are inactive because they cannot uncoat (Meruelo et al. 1992).
Isolation of leaves and stems of the Central African plant *Ancistrocladus korupensis* (Ancistrocladaceae) led to the isolation of michellamines A, B, and C (Fig. 1.2), atropisomeric naphtylisoquinoline alkaloid dimers with anti-HIV activity. Michellamines, either as free bases or water-soluble acid salts, are capable of inhibiting the replication and cytopathic effects of HIV-1 and HIV-2. Based on the promising *in vitro* and *in vivo* results, the U.S. National Cancer
Institute has committed michellamine B to preclinical development (Boyd et al. 1994).

Lee et al. isolated suksdorfin (Fig. 1.2) from the fruits of *Lomatium suksdorfii* (Umbelliferae). Suksdorfin inhibited HIV replication in T cells and HIV replication in a monocytic cell line as well as in peripheral blood mononuclear cells (Lee et al. 1994). A derivative of suksdorfin demonstrated potent inhibitory activity and good selectivity against HIV replication *in vitro*. Suksdorfin and its derivative did not affect HIV-1 reverse transcriptase activity. The anti-HIV activity of these compounds seems to be stereoselective, but the exact mechanism of their antiviral activity is not yet clear (Huang et al. 1994).

The bark of *Homalanthus nutans* (Euphorbiaceae), a small tree, is part of the traditional medicine of Samoa. Phytochemical and biological investigations led to the isolation of prostratin (Fig. 1.2), an anti-HIV active phorbol ester. Researchers of the NCI proved that prostratin represents a non-tumor-promoting activator of protein kinase C which strongly inhibits the killing of human host cells *in vitro* by HIV (Gustafson et al. 1992); (Cox 1992; 1993).

Calanolide A (Fig. 1.2), a coumarin derivative isolated from the tree *Calophyllum lanigerum* (Guttiferae), showed inhibitory activity against HIV-1 *in vitro* but was inactive against HIV-2. This non-nucleoside inhibitor of HIV reverse transcriptase, is assumed to bind near the active site of the enzyme and interferes with deoxynucleotide triphosphate (dNTP) (Currens et al. 1996a); (Currens et al. 1996b)

Flavonoids, including tiliroside, quercetin, and kaempferol were found to be weak HIV-1 reverse transcriptase inhibitors. This could be due to their weak intercalating properties, which are enhanced by structural planarity (Tan et al. 1991). Hashimoto et al. tested thirty-eight tea polyphenols for their inhibitory effects against HIV replication in H9 lymphocyte cells. Among these 8-C-ascorbyl (-)-epigallocatechin and theasinensin-D demonstrated relatively potent anti-HIV activity. Structure-activity studies showed that location(s) of the galloyl group(s) is important for retaining anti-HIV activity in epigallocatechins. In the case of the theasinensins it was suggested that biphenyl atropisomerism is also important to the anti-HIV activity (Hashimoto et al. 1996).
Tannins are known to be active in anti-HIV screening assays. The HIV-inhibitory activity of more than 50% of the plant organic extracts has been assigned to polar phases and subsequently traced to tannins (Cardellina II et al. 1993). Kakiuchi and coworkers demonstrated that hydrolyzable tannins inhibited the polymerization catalyzed by the reverse transcriptase from retroviruses. Dimeric ellagitannins were more effective inhibitors than monomeric ellagitannins. Tri- and tetra-galloylglucose were less effective compared to gallotannins with a larger number of galloyl residues. This findings suggested that tannins with a higher molecular weight and a number of functional groups can strongly interact either with nucleotides or with proteins (Kakiuchi et al. 1985).

Extracts of *Echinacea purpurea* (Compositae) and *Panax ginseng* (Araliaceae) were evaluated for their capacity to stimulate cellular immune function in patients with acquired immunodeficiency syndrome. Increasing concentrations of *Echinacea purpurea* and *Panax ginseng* demonstrated an *in vitro* enhancement of cellular immune function in normal subjects and in patients with AIDS. Immunopotentiating herbs such as echinacea and ginseng may be useful therapeutic agents for disorders of cellular immunity such as AIDS. A clinical study is currently underway to evaluate the capacity of echinacea to reduce the viral load and to stimulate natural killer function in HIV-infected patients (See et al. 1997).

Over the last decade, strong efforts have been made in finding strategies for the treatment of acquired immunodeficiency syndrome (HIV) infection, AIDS. The search for new compounds against AIDS is still a unique challenge. Active compounds, if not eventually suited for the treatment or prophylaxis of AIDS per se, may serve as new leads for the synthetic development of selective retroviral inhibitors (Tan et al. 1991).
1.1.3 Antifungal agents from plants

It is estimated that over 120,000 different species of fungi exist, about one hundred of them are pathogenic to humans (Wiesmann et al. 1986). Numerous diseases of serious concern in plants, humans and animals are caused by fungi. Allergies produced by fungal spores also present a big problem. Fungi are ubiquitous and able to use a wide range of substrates as their carbon, nitrogen and energy metabolism (Paxton 1991).

The search for antifungal compounds has become growing importance mainly as a result of an increasing occurrence of systemic mycoses, associated primarily with immunodeficiency diseases (such as AIDS) and an increased use of immunosuppressors. The fungal infections observed in immuno suppressed or HIV infected patients are mainly candidiasis, cryptococcosis and aspergillosis (Rahalison et al. 1991).

Currently three structural classes of compounds find wide therapeutic application for the treatment of disseminated mycoses, namely polyene antibiotics (amphotericin B), flucytosine, and systemic azoles. Amphotericin B was the first clinically used systemic antifungal antibiotic, and after more than 20 years it is still the most effective therapeutic agent for disseminated mycoses (Clark and Hufford 1993). Although the search for antifungal natural compounds has been focused on microorganisms over the last several decades, higher plants appear to be a good basis for antifungal constituents. Agents with antifungal activity are widely distributed among higher plants, but very few have been evaluated for their activity against human pathogenic fungi and scarcely one has been evaluated in animal models of disseminated mycoses (Hufford and Clark 1988).

Ficuseptine (Fig. 1.3), an indolizidine alkaloid, was isolated together with antofine (Fig. 1.3), a phenanthroindolizidine alkaloid, from *Ficus septica* (Moraceae), a traditional remedy used in Papua New Guinea. Both agents showed significant activity against the plant-pathogenic fungus *Penicillium oxalicum* (Baumgartner et al. 1990).
Chemical investigation of *Gentiana macrophylla* (Gentianaceae), a traditional Chinese drug, led to the isolation of new compounds with antifungal activity. 2-Methoxyanofinic acid (Fig. 1.3) and its methyl ester were found to be active against *Cladosporium cucumerinum*. The ester derivative inhibited also the growth of *Candida albicans* (Tan et al. 1996).

Besides the alkaloids vilmorrianone and panicutine (Fig. 1.3), the roots of *Delphinium denudatum* (Ranunculaceae) yielded the new diterpenoid alkaloid, 8-acetyl-heterophyllisine (Fig. 1.3). All three compounds showed antifungal activity against a number of human pathogenic fungi (Atta-ur-Rahman et al. 1997).

Other natural products identified as antifungal compounds include monoterpenes, flavonoids, terpenoids, alkaloids, naphthoquinones and a range of crude plant extracts (Rios et al. 1988).

Fig. 1.3: Examples of plant-derived antifungal agents
1.2 Strategies in the search of new natural compounds

To study medicinal plants it is first of all necessary to know which plant to select and what type of biological activity to look for. The selection criteria of plants, which potentially contain new biological agents, is based on five principle approaches: the random, the taxonomic, the phytochemical, the ethnomedical and the information-managed approach. In the random approach all available species are collected, irrespective of prior knowledge and experience. In the taxonomic approach, plants of a specific genus or family are deemed to be of interest, and sought from diverse locations. The phytochemical (chemo-taxonomic) approach is based on a particular compound type, which is of biological interest. Plants anticipated to produce related compounds are collected. Taxonomic and the phytochemical approach are closely related and can not be clearly divided from each other. In the ethnomedical approach, credence is given to information on the medicinal use of the plant. Based on this information, the plant is collected and evaluated (Cordell et al. 1991). Much of the traditional medicine clearly reflects real medicinal properties. Indeed, 74% of our plant-derived major medicines were discovered by following up empirical use (Soejarto and Farnsworth 1989); (Gentry 1993). Field studies on the use of traditional medicines should be an important additional tool in the selection of plants for further studies. Most of the medicinal plants used are insufficiently described concerning their phytochemical composition and their biological properties (Verpoorte 1989). Information-managed plant selection collates taxonomic, ethnomedical, biological and phytochemical information to afford a list of plants for specific collection. The information is compiled through computerized databases such as NAPRALERT (Natural Products Alert), a specialized relational database on natural products, based on systematic literature searches (Farnsworth 1993). A sixth “approach” is serendipity, where during the course of pursuing a particular bioactivity or ethnomedical use another pertinent bioactivity is discovered (Cordell et al. 1993). For each of the approaches, the most rational method of
proceeding involves evaluating the collected plant material in a range of bioassays (Cordell et al. 1991). Depending on the aim of the study, either a specific assay for a certain activity or a general screening is performed. Active fractions are subjected to bioassay-guided fractionation procedures for procurement of the active principle(s).

Besides the use of plants in the medical field as source for pure, chemically defined active principles, they also find application as active extracts containing a broad range of constituents (infusions, essential oils, tinctures, extracts) (Hamburger and Hostettmann 1991).

Figure 1.4 shows a general working off scheme to obtain active principles from plants.

Fig. 1.4: General procedure for obtaining active principles from plants
1.3 Candida albicans

The genus Candida, with more than 150 species, is placed taxonomically with the yeastlike organisms of the Fungi Imperfecti. Most of the human pathogenic species belong to this class of fungi. They are 2.5 to 6 μm ovoid cells that reproduce by budding. Within the genus Candida, one species is used for the commercial production of food supplements for animals (C. alboreoa); others are found as natural inhabitants of the skin and mucous membranes of humans (C. albicans and C. stellatoidea). At least ten species are known to be regularly pathogenic, among them C. albicans, C. stellatoidea, C. tropicalis, C. parapsilosis, C. pseudotropicalis, C. guilliermondii, and C. krusei (Wiesmann et al. 1986); (Saltarelli 1989); (Polis and Kovacs 1997).

As mentioned above Candida albicans and to a lesser extent other species of Candida are found frequently as part of the normal flora of humans. Alteration of the normal physiological or immune state, e.g. by AIDS, immunosuppressive therapy, diabetes or pregnancy can lead to a strong growth of C. albicans and subsequently to chronic superficial or severe systemic disease. Candida can cause lesions in a wide variety of anatomical sites: mouth, bronchi, lungs, intestines, vagina, skin, heart, meninges, bones and joints. Mostly the mucous membranes and to a lesser extent the outer skin and inner organs are affected. Candida albicans is responsible for nearly 90% of human Candida infections. Infections caused by Candida are, with exception of the neonatal candidiasis, endogenous infections (Wiesmann et al. 1986).

Probable anticandidal compounds were found among the steroids, terpenes and flavonoids (Caceres et al. 1991); (Rios et al. 1987).
2  Aim of the present work

The present work is part of the research project "Phytochemical and biological study of plants used in the traditional medicine of Papua New Guinea", supported by the Swiss National Science Foundation. Papua New Guinea (PNG) is an area of high biodiversity, holding a tremendous richness of as yet uninvestigated plant species. Still today indigenous people in PNG rely mainly on their herbal traditional medicine.

Holdsworth and coworkers have published over 40 papers, e.g. (Holdsworth 1974); (Holdsworth et al. 1983); (Holdsworth 1989); (Holdsworth 1992), about the occurrence, local and botanical names as well as on the traditional uses of medicinal plants and plant parts from PNG. Based on these literature studies plants were collected. Former works on the same research project dealt with the phytochemical and pharmacological investigations on three Ficus species (Baumgartner 1991), followed by phytochemical and biological investigations on Piper aduncum (Orjala 1993), Dillenia papuana (Nick 1995), Macaranga pleiostemon and Securinega melanthesoides (Schütz 1997).

The present study was divided into three parts: First a new bioassay for detection of activity against Candida albicans was developed. The aim of this part of the work was to extend our in-house bioassays in order to have an easily performable assay against a facultatively human pathogenic yeast.

The second part of the study comprised the biological screening of 13 selected plants. The plants were collected during two field trips in 1988 and in 1991 in different areas of Papua New Guinea. The objective of the screening was to evaluate the most interesting plants for further phytochemical investigations. Based on the screening results and on literature searches phytochemical investigations of selected plants were performed in the third part of the study, focusing on members of the plant family Euphorbiaceae. The Euphorbiaceae represent a very large and botanically as well as phytochemically interesting plant family. Both plants chosen, Euphorbia buxoides and Homalanthus
*nervosus*, are endemic plants of PNG and have not yet been described phytochemically. The third part of the study describes extraction, isolation and structure elucidation of compounds isolated from both plants.
New Guinea, the world's second largest island, located barely south of the
equator, to the north of Australia, is divided in two parts: the western half,
Indonesia's province of Irian Jaya and the eastern half, Papua New Guinea,
independent since 1975 (Wheeler and Murray 1993). The state of Papua New
Guinea consists of the mainland, the former colonies Papua and New Guinea
and different islands, for example Manus, New Britain and New Ireland, the two
northeast islands of the Solomon group (Bougainville and Buka), as well as the
Trobriand islands and the D'Entrecasteaux islands.

Fig. 3.1: Map of Papua New Guinea

The population of PNG, about 4.2 million, is divided into more than 700
linguistic groups, whose customs differ almost as much as their languages.
More than a third of the population live in the Highland provinces. Politically,
four regional groupings, reflecting cultural and historical links, have developed:
Papuans from the south, Highlanders, New Guineans from the north and
Islanders.
Jorge de Meneses, a Portuguese sailor, is considered as the European discoverer of Papua New Guinea, being the first who entered the island in 1527. He named it „Ilhas dos papuas“, island of the curly-haired. In the 17th century the Dutch asserted a claim on the western territory of the island (Indonesia today). In the 19th century the island was divided into three sections – the western half became Dutch New Guinea, the south-eastern section was British New Guinea and the north-eastern section became German New Guinea. In 1906 British New Guinea became Papua and administration was taken over by newly independent Australia. In World War I Australia took over the German territory. During World War II all the northern islands and most of the north coast fell to the Japanese. After the War the eastern part of the island became the Territory of Papua & New Guinea, administered by the Australian. In 1975 Papua New Guinea became its full independence (Lipscomb et al. 1998).

The geography of Papua New Guinea is very interesting and varied: The island shows a mountainous central backbone with peaks over 4000 m high descending sharply to coastal lowlands. This central range becomes wider, higher and more fragmented the closer it gets to the border with Irian Jaya. The larger of the Highlands valleys formed have grass cover rather than the rainforest common at lower altitudes. The lowland areas are notable for their large areas of swamp and grassland. In places, the central mountains descend right to the sea while in other regions broad expanses of mangrove swamps fringe the coast. In the western region there is an endless expanse of flat grassland, sparsely populated, annually flooded and teeming with wildlife. Great rivers flow from the mountains down to the sea. The Fly and the Sepik rivers are the two largest: the Sepik flowing into the sea in the north, the Fly in the south. Both are among the world’s mightiest rivers in terms of annual water flow. PNG is almost entirely surrounded by huge coral reefs.

Papua New Guinea is recognized as an area of high biodiversity. The native flora is very manifold, comprising 15'000 to 20'000 species of vascular plants
and 2000 species of ferns and fern allies. There are no endemic plant families, but 93 endemic genera have been identified (Osborne 1995). The different climatic influences give rise to diverse botanical regions. Along the coasts, in the deltas of the big rivers and in sheltered bays, big mangroves occur. The swamps in the flood areas of the big rivers are characterized by many sago palms (*Metroxylon sagu*), playing an important role as food-stuff for native people. The tropical rainforest, found in lower regions is extended up to 1000 m altitude. More than 100 different tree species constitute the tropical rainforest. In the dryer low areas the tropical rainforest passes over to the so-called monsoon forest or stepp grassland. Between 1000 and 3000 m mixed alpine forest is found. In contrast to the lower tropical rainforest the alpine forest is characterized by a major occurrence of palms and liana. At a height of 2500 to 3000 m foggy forests and mossy forests are found. With the exception of some needle trees, up to 30 m high, the ever moisty, cold foggy forest consists of trees with hardly 15 m altitude. Everything is covered by moss and lichens. Above the foggy forest there is a zone of high alpine forest, up to 4000 m, consisting mainly of short needle trees. Alpine grass meadows, interspersed with shrubs, are extended to the mountain peaks. In contrast to Australia, oaks and rhododendron, with about 48 different species, can be found in PNG. Pines and antarctic beech thrive around the 3500 m mark and above this are predominantly sparse meadows and lichens. In the 1970s and 80s there were large afforestation projects in the Highlands. PNG has become known in recent times for its enormous variety of orchids - there are about 3000 species. Most of PNG is forested, with slash-and-burn cultivation widespread, even in step areas. The tropical rainforest, predominantly in coastal areas rises up to 1000 m. In the south are extensive savannas (Wesemann 1990).
4 Biological assays

4.1 Antimicrobial assays

Despite the worldwide availability and use of antibiotics a continuing search for new anti-infective agents remains indispensable. Infectious diseases are still an important cause of death. The combination of the genetic versatility of microbes and the widespread overuse of antibiotics has led to increasing clinical resistance of previously sensitive microorganisms and the emergence of previously uncommon infections (Mitscher and Rao 1984).

The principle of antimicrobial testing of plant extracts is based on the observation of the growth reduction of microorganisms after contact with plant tissues of extracts to be tested. The test conditions must be chosen the way that the plant extract or pure compound is in contact with the cell wall of the microorganisms. The assay must guarantee normal growth of the microorganisms when no antimicrobial agents are present. The currently available methods can be divided into three groups, including diffusion, dilution and bioautographic methods (Vanden Berghe and Vlietinck 1991), which will be presented subsequently.

The diffusion technique consists in putting a reservoir (e.g. discs, holes or cylinders) into an inoculated medium and, after incubation, measuring the diameter of the bacteria free zone around the reservoir (inhibition diameter). Advantages of the diffusion method are the small size of the sample used in the screening and the possibility of testing five or six compounds per plate against a single microorganism. This method is not suitable for lipophilic samples and for determining the minimal inhibition concentration (MIC) of a sample (Rios et al. 1988).

In the dilution method test samples are mixed with a suitable medium, which has previously been inoculated with the test organism. After incubation, growth of the microorganism is determined by direct visual or turbidimetric comparison
of the test culture with a control culture without test compound or by plating out both test and control cultures. Principally the dilution method is used to determine the MIC of extracts or pure compounds (Rios et al. 1988).

Bioautography is a general method for detection of antimicrobial activity on paper or thin-layer chromatograms (Betina 1973). It is considered as the most efficacious assay for the detection of antimicrobial compounds, because it allows the localization of the activity even in a mixture and therefore it permits a target-directed isolation of active constituents (Rahalison et al. 1991).

Previously chromatographed compounds or extracts are diffused to inoculated agar. After incubation zones of inhibition are visualized by appropriate stains, i.e. a tetrazolium salt. Bacteria with intact metabolism convert the dehydrogenase-activity-detecting tetrazolium salt into the corresponding colored formazan. Antibacterial compounds appear as clear spots against a colored background (Betina 1973); (Hamburger and Cordell 1987).

In order to cover a broad range of different microorganisms our in-house antimicrobial assays were performed with gram positive bacteria, namely *Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus* and *Staphylococcus epidermidis*, as well as the gram negative bacteria *Escherichia coli*, *Mycobacterium fortuitum* and *Pseudomonas aeruginosa*. To determine antifungal activity the plant-pathogenic mold *Penicillium oxalicum* and the yeast *Candida albicans* were used. Biological screening on extracts were carried out applying the diffusion method, with paper discs as reservoir.

It should be pointed out that all available testing methods give only an idea of the presence or absence of substances with antimicrobial activity in the extract. The potency of the active ingredients can only be determined on pure compounds using a standardized methodology.
4.2 Molluscicidal assay

Schistosomiasis (bilharzia) is a parasitic disease endemic throughout South America, Africa and the far East, affecting more than 250 million people in over 76 countries (Maillard 1993).

The reproduction cycle of schistosomiasis involves a stage implicating aquatic snails, in which the parasite multiplies into thousands of cercariae. The cercariae, after leaving the snails, can penetrate the skin of humans who come into contact with the water source. After penetration of the skin, they change gradually into the mature trematodes, also known as schistosomes. The schistosomes mate and lay eggs, which are excreted with faeces or urine. These eggs enter water and produce miracidia, which locate snails of the appropriate species and the cycle begins again. Destroying the snails that harbor the developing schistosome larvae is one way to interrupt the parasite's life cycle and prevent human infection (Marston and Hostettman 1985).

Several groups of compounds present in various plants have been tested for their molluscicidal activity. Thus saponins, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpene lactones, terpenoids and phorbol esters have been found to be poisonous to snails at acceptable doses ranging from <1-100 ppm (Singh et al. 1996).

_Biomphalaria glabrata_, one of the intermediate hosts of schistosomiasis, was used as bioassay organism for the plant screening in the present study.
4.3 *Artemia salina* toxicity assay

Since most active plant principles are toxic at elevated doses, a possible approach for developing an effective general bioassay might be simply to screen for substances that are toxic to zoologic systems. *Artemia salina*, a small crustacean commonly known as "brine shrimp", shows a high sensitivity against a broad range of compounds. The advantages of being rapid (24 hours following introduction of shrimp into the sample), inexpensive and simple has promoted the "brine shrimp" test as suitable in-house bioassay.

In 1956 Michael et al. published the first article on the use of *Artemia salina* as bioassay organism, evaluating the toxicity of different insecticides towards brine shrimp. Vanhaecke and coworkers published in 1981 a proposal to introduce the brine shrimp bioassay as standard bioassay for the marine environment. In 1982 Meyer et al. published an article introducing the brine shrimp as a general bioassay for active plant constituents. Some authors reported on the correlation between brine shrimp lethality and cytotoxicity (Meyer et al. 1982); (Anderson et al. 1991); (McLaughlin et al. 1993); (Solis et al. 1993). These findings could not be confirmed by other authors. Ferrigni et al. described the brine shrimp assay as not specific for antitumor activity or cytotoxicity (Ferrigni et al. 1984). Cepleanu showed that the correlation published by other authors was slightly biased by the fact that mainly known cytotoxic compounds or extracts were tested. It was shown that brine shrimp toxicity could indicate other types of activity besides cytotoxicity, including fungicidal, insecticidal or mollusccidal activities (Cepleanu 1993).

Nowadays the brine shrimp assay is used worldwide to screen the general toxicity of crude extracts and natural compounds.
4.4 Cytotoxicity assays

Cancer is one of the main causes of death in most industrial countries. Over 100 different types of cancer exist. An ideal drug against cancer should therefore be able to kill a variety of subtypes or subpopulations of tumor cells without harming normal tissue. Although it is known that cytotoxicity is neither necessary nor sufficient for antitumor activity, testing compounds for their cytotoxic activity against cancer cell lines can be a reference to antitumor activity. Most research programs dealing with the isolation and identification of potential antitumor compounds from plants still rely on cytotoxicity bioassays. The procedure usually involves the treatment of tumor cell lines with various concentrations of the test substance, and assessing cell growth after 48 or 72 hours of incubation (Suffness and Pezzuto 1991).

Our in-house cytotoxicity assays were performed using two different cell lines, namely CaCo-2 cells and KB cells. CaCo-2 cells are established as a human intestinal cell line isolated from a primary colon tumor by Fogh and Orfeo (Fogh and Orfeo 1977). They consist of fully differentiated colonic cancer cells and have also been used as in vitro model system to study small intestine transport physiology and biochemistry (Laboisse 1989); (Rutzky and Moyer 1990). The KB cell line, an epidermoid human carcinoma from the nasopharynx isolated by Eagle in 1955 (Eagle 1955), has been used by the National Cancer Institute (NCI) as an antitumor assay for screening plant extracts for the last decades (Perdue Jr. 1982). However, the KB cell line (including the KB cell line from the American Type Culture Collection, ATCC) has been found to be contaminated with HeLa cells (Nelson-Rees and Flandermeyer 1976). Considerations on using other in vitro cytotoxicity assays should therefore be made.
5 The family Euphorbiaceae

5.1 Distribution and systematics

The family Euphorbiaceae is the sixth largest and one of the most diversified families of angiosperms, consisting of about 300 genera and over 8000 species (Radcliffe-Smith 1986); (Webster 1987). The largest genus is *Euphorbia* consisting of over 1600 species followed by the genus *Croton* with nearly 700 species. Thirteen other genera contain over 100 species. These include for example *Phyllanthus* (480 species), *Acalypha* (430 species), *Glochidion* (280 species), *Macaranga* (240 species), *Manihot* (160 species), *Jatropha* (150 species) and *Tragia* (140 species). The Euphorbiaceae display an extraordinary range of growth forms, ranging from large desert succulents to trees and even small herbaceous types (Evans and Taylor 1983).

The family Euphorbiaceae has provided many problems for botanists and taxonomists due to the great variation of forms exhibited. Several systematists studied the classification of the Euphorbiaceae in the last 180 years. The first major milestone in the history of the taxonomy of the Euphorbiaceae was the classification of Jussieu (1824), who identified the major series of genera that (after much later revision) correspond roughly to the current subfamilies. Afterwards Müller (1866) provided the first detailed classification of the family into subfamilies, tribes and subtribes. In 1931 Pax and Hoffmann recognized four subfamilies of very different size, the Phyllanthoideae with 65 genera, the Crotonoideae with 209, the Porantheroideae with 34, and the Ricinocarpoideae with 5 (Pax and Hoffmann 1931). In all of the classifications of the Euphorbiaceae proposed before 1975, the major criteria were drawn from details of gross morphology observable with the naked eye or a dissecting lens (Webster 1994 a).

Webster presented in 1975 a classification, grouping the 300 genera of Euphorbiaceae into 52 tribes in the following five subfamilies: Phyllanthoideae,
Oldfieldioideae, Acalyphoideae, Crotonoideae and Euphorbioideae, with several of the tribes divided into subtribes (Webster 1975). In 1994 Webster published a revised classification, suggesting five subfamilies, 49 tribes and 317 genera (Webster 1994 b). Although the taxonomic classification of Webster from 1994 is considered the actual systematic classification, critical remarks showed the difficulties in the classification of infrafamiliar relationships in the Euphorbiaceae (Gilbert 1994). It can thus be assumed, that the classification of the Euphorbiaceae has not yet been accomplished nor will be for the next future.

Although present worldwide, the family Euphorbiaceae is a predominantly tropical family. There are only a few exclusively extratropical genera, e.g. Crotonopsis (North America), Mercurialis (temperate and warm temperate Eurasia), Seidelia (South Africa), Dysopsis (temperate and Andean South America). Only one genus, the genus Euphorbia, is cosmopolitan. In Papua New Guinea there are only two endemic genera, namely Annesijoa and Neomphalea (Radcliffe-Smith 1986).

Characteristic for the family Euphorbiaceae are the so called cyathia; mostly greenish-yellow, single flower type formations, which represent inflorescences. Although looking like a hermaphrodite flower, male and female flowers are separate. The male flowers consist of a single petiolate stamen. They are arranged around a single, female flower, consisting of a three-celled ovary, protruding from the cyathium. The fruit is composed by a small capsule, made up of three fruitlets or "coccæ" (Euphorbiaceae are therefore also known as Tricoccae), which split explosively to release the seed (Neuwinger 1996).
5.2 Medicinal and economic uses

Many species of the Euphorbiaceae are used because of their medicinal, toxic or economically interesting properties. Medicinal purposes for euphorbiaceous plants range from treatment of tumors, migraine, parasite infestations, bacterial infections, venereal diseases, skin conditions, purgatives to the use as abortifacients (Evans 1986). In 1966 Farnsworth published a review on antitumor effects of traditionally used plants, mentioning 12 species of Euphorbiaceae with antitumor activity, including *Acalypha phleidos*, *Croton monanthogynos*, *Euphorbia amygdaloides*, and *Macaranga triloba* (Farnsworth 1966). In a survey on the medicinal use of plants Hartwell mentioned 26 different active genera of Euphorbiaceae for the treatment of tumors, growths and warts (Hartwell 1969). Several Euphorbiaceae are used traditionally as remedies against parasite infections. *Macaranga kilimandscharica* and *Ormocarpum trichocarpum* are used against bilharziosis. *Mercurialis annua* and *Acalypha indica* are traditionally used as anthelmintics and as remedies against scabies (Watt and Breyer-Brandwijk 1962). Bacterial infections such as lepra are treated by natives in Polynesia with a wood decoction of *Excoecaria agallocha* or leaves of *Homalanthus populneus* (Zepernick 1972). Many euphorbiaceous plants are reported as traditional remedies against venereal diseases. *Jatropha curcas* is used against syphilis, *Phyllanthus virgatus* and *Aleurites moluccana* are used against gonorrhea (Zepernick 1972); (Neuwinger 1996). Traditional uses of euphorbiaceous plants as abortifacients or purgatives are widespread. Leaves of *Croton lobatus* are reported to act as abortifacient (Neuwinger 1996). The most drastic of all purgatives known comes from the seeds of *Croton tiglium*. It is now generally out of use, being to toxic. Causing violent evacuation in minutest doses, it may also cause sloughing of the intestinal lining (Radcliffe-Smith 1986).
Besides the medicinal uses of Euphorbiaceae, different species of this family have been noted for their toxicological effects, for example induction of inflammation of skin and mucous membranes, conjunctivitis, and strong purgative activity. Additionally some species are used as fish poisons and as ingredients of arrow poisons. *Ricinus communis* (castor oil plant) is employed in medicine as a cathartic and in industry in the manufacturing processes of greases and other lubricants. In the tanning industry it is used to preserve both the flexibility and the impermeability of leather; and it is also used in the production of soaps, glycerin, paints, enamels, varnishes, dyes, plastics, rubber, linoleum, polishes, waxes, carbon-paper, and crayons. Probably the best known economic plant of the Euphorbiaceae is the rubber tree, *Hevea brasiliensis*, which is the main source of natural rubber. Manioc, cassava, or tapioca plant, *Manihot esculenta*, is source of a staple foodstuff for poorer people in many tropical countries. It is a cultigen which is originated in South America and from there it has been introduced into every part of the worlds tropics. A serious drawback of cassava cultivation is that it exhausts the soil in which it grows (Radcliffe-Smith 1986).

5.3 Chemical constituents

The diversity of this plant family is not only exhibited by its morphological variety but also by its secondary metabolism. Thus the chemistry of the Euphorbiaceae is among the most diverse and interesting of flowering plant families. Thousands of compounds from many different chemical classes have been reported from members of the Euphorbiaceae. A predominance of chemical work concerns the large genus *Euphorbia*, which has been studied in greater detail than most other genera of the family (Seigler 1994).

Most genera contain a characteristic *milky latex* which consists of mineral salts, proteins, amino acids, terpenes and caoutchouc. The composition of the latices shows a big chemical heterogeneity and is mainly responsible for the
toxic effects and biological activities (Hegnauer 1989); (Ponsinet and Ourisson 1965).

**Triterpenes** are present in most species. The major triterpenes are compounds related to cycloartenol and tetracyclic triterpenes, as for example boeticol (Fig. 5.1) (Ferreira et al. 1995) and securinegens (Fig. 5.1) (Schütz et al. 1998). Also pentacyclic triterpenes, like kamaladiolacetate (Fig. 5.1), isolated from a *Mallotus* species (Nair and Rao 1993), often occur. Additionally cucurbitacines and cucurbitacin-related compounds have been reported from several Euphorbiaceae species (Hegnauer 1989).

![Diagram of triterpenoids](image)

**Fig. 5.1:** Examples of triterpenoids isolated from the Euphorbiaceae

Representatives of different classes of **alkaloids** have been isolated from a number of Euphorbiaceae, in particular from the genera *Croton*, *Phyllanthus* and *Securinega* (Rizk 1987). Benzylisoquinoline alkaloids (aporphine, crotonosine) have been isolated from *Croton linearis* (Yamaguchi 1970)
(Fig. 5.2). Securinine alkaloids, a small group of compounds only occur in the subfamily Phyllanthoideae, for example virosecurinine (Fig. 5.2) from Securinega virosa (Yamaguchi 1970). Imidazole alkaloids have been isolated from the genera Glochidion and Alchornea. Alkaloids derived from nicotinic acid such as ricine (isolated from Ricinus communis) were also found (Rizk and El-Missiry 1986).

![Virosecurinine and (+)-Crotonosine](image)

**Fig. 5.2:** Examples of alkaloids isolated from the family Euphorbiaceae

A large variety of **diterpenes** occur in the Euphorbiaceae. They may be divided into compounds derived from monocyclic precursors and their derivatives (including cocarcinogenic diterpenes), kaurene and related compounds as well as labdane and clerodane derivatives (Seigler 1994). Extraction of a Cluytia species led to the secolabdane diterpene saudinolide (Fig. 5.3) (Mossa et al. 1996). A clerodane diterpenoid, cromiargyne (Fig. 5.3), has been isolated from Croton hemiargyreus (Amaral and Barnes 1998). Many genera contain **phorbol esters**, tri- or tetracyclic diterpene esters, with three different structure subtypes, known as tigliane, daphnane and ingenane (Evans 1991). Recently phytochemical investigations on Euphorbia semipertfoliata led to the isolation of new jatrophane polyesters and 4-deoxyphorbol diesters (Fig. 5.3) (Appendino et al. 1998).
The family Euphorbiaceae is rich in flavonoids, particularly flavones and flavonols. They occur as O- and C-glycosides and their methyl ethers. The two common flavonols kaempferol and quercetin (and their glycosides) are widespread in different genera of the family (Rizk 1987). Tannins occur as well as coumarins and cyanogenic glycosides (Seigler 1994). Long-chain fatty alcohols (particularly n-octacosanol and n-hexacosanol) and hydrocarbons have been identified from different genera. Especially the genus Euphorbia yielded a considerable amount of hydrocarbons and alcohols (Rizk 1987).
6 The genus *Euphorbia*

Etymologically *Euphorbia* is said to be named after the physician Euphorbius. King Juba from Mauretania dedicated this plant to his physician Euphorbius who had used it first as a medicinal plant (Benigni et al. 1962).

6.1 Occurrence

*Euphorbia*, or spurge, is the largest genus of Euphorbiaceae, with about 1600 species known; about 10 species are endemic in Papua New Guinea (Airy Shaw 1980). Although more common in tropical areas, *Euphorbia* is the only genus of Euphorbiaceae that occurs all over the world. Its appearance ranges from herbs and shrubs to trees and cacti type. The genus *Euphorbia* is characterised by the presence of milky latex, being more or less toxic (Singla and Pathak 1990).

6.2 Uses

The latex of a considerable number of species of *Euphorbia* is used as an ingredient in *arrow poison* (e.g. *E. candelabrum*, *E. venefica*) and has two functions: Increase of cohesivity and irritation of the arrow wound to favour absorption of the poison (Watt and Breyer-Brandwijk 1962). Some species (e.g. *E. hamata*, *E. obesa*) are useful as *fodder*. Other species serve as source for *honey* (e.g. *E. triangularis*). An irritant principle can get into the honey, causing a burning taste in mouth and esophagus (Watt and Breyer-Brandwijk 1962). Plant latices of *Euphorbia* have been used as *fish poison* (e.g. *E. dendroides*), *insecticides*, and as *ordeal poison*. The latices of *E. tetragona* and *E. triangularis* have been utilized for manufacture of inferior *rubber*, as the basis of chewing gum and in confectionery trade, indicating that they are non-
irritant. Latices of *E. ingens*, *E. tirucalli* and *E. triangularis* have been suggested as possible source of rubber.

*Euphorbia* species have found wide application as medicinal plants including the use for the treatment of skin diseases, gonorrhoea, migraine, cough, dysentery, intestinal parasites and wart cures (Kinghorn and Evans 1975); (Singla and Pathak 1990); (Usher 1974).

### 6.3 Phytochemical and biological investigations

The genus *Euphorbia* has been subject of intensive phytochemical investigations, first because it comprises a big number of different species and second because of its highly irritant principles, the diterpenes of the phorbol ester type. The following substance classes have so far been reported from the genus *Euphorbia:*

*Euphorbia* is a genus rich in triterpenoids. Many reports on the isolation of cycloartenol derived triterpenoids and euphane-type triterpenoids have been published (Singla and Pathak 1990). Euphol has been isolated as major compound from many *Euphorbia* species (Gewali et al. 1990); (Ferreira et al. 1995). Numerous phytochemical investigations on diterpenoids, especially on the phorbol esters have been published, reporting on the isolation as well as on biological activity studies (Shi and Jia 1997). Diterpene esters based on all three structural types (tigliane, daphnane, ingenane) have been identified. Ingenane type diterpenoids have hitherto only been isolated from *Euphorbia* and *Elaeophorbia* species. Besides the tri- or tetracyclic diterpenes of the phorbol type, diterpenoids with novel carbon frameworks have been isolated (Jakupovic et al. 1998). *E. hirta* was found to contain the alkaloid xanthorhamnin (Ueda and Hsu 1949). An other alkaloid was isolated from *E. atoto* (Beecham et al. 1968). Flavonoids have been isolated, as well as coumarins and different sterols (Singla and Pathak 1990).
6.4 Euphorbia buxoides

Lit.: (Frohne and Jensen 1985); (Webster 1994 b)

6.4.1 Botany and systematics

*Euphorbia buxoides* is an evergreen shrub or tree up to 7 m with stiffly erected branches, endemic in Papua New Guinea. The plant presents inflorescences in form of cyathia. No fruits are known. Nearly all of the collections of *Euphorbia buxoides* were stated to be from cultivated plants. Thus the species was thought to be a particular morphological selection of *E. plumerioides* which has been vegetatively propagated (Airy Shaw 1980). Forster published a revision of the genus *Euphorbia plumerioides*, indicating that *E. buxoides* is a species on its own, belonging to the complex of *E. plumerioides* and allied species (Forster 1994).
6.4.2 Traditional uses

In the Eastern Highlands of PNG leaves of *Euphorbia buxoides* are buried inside bamboo stems together with leaves of a *Garcinia* species to prevent sickness (Holdsworth 1989). Crushed leaves of *E. buxoides* are used as a fish poison (Forster 1994). In the Southern Highlands the bark is used as poison and antidote (Holdsworth 1974). The latex finds application as a ropy adhesive (Kremnitz 1988). In the Highlands *E. buxoides* is cultivated as a hedge plant (Radcliffe-Smith 1986).
7 The genus Homalanthus

Synonym: Omalanthus, Carumbium

7.1 Occurrence and systematics

Homalanthus is a genus of about 20 to 25 species. Its distribution is from South Asia through Malaysia to Australia and Polynesia (Esser 1996). About ten species are endemic in New Guinea (Airy Shaw 1980).

*Homalanthus* was first mentioned as *Duania* by Noronha (1790), then as *Carumbium* by Reinwardt (1823) and finally as *Omalanthus* by Jussieu (1824). Neither *Duania* nor *Carumbium* were validly published. *Carumbium* was later validated by Reinwardt (1825) but this detailed description had been preceded by Jussieu 1824. Müller (1866) published the first revision of the genus *Homalanthus*. The second and latest revision was done by Pax and Hoffmann (1912). Since then several contributions to the systematics of *Homalanthus* were presented (Webster 1975); (Airy Shaw 1980); (Esser 1997).

The name of Jussieu's genus *Omalanthus* is based on the greek words *homalos* (= equal, even) and *anthos* (= flower), referring to the flattened male flowers of the genus. A few years later, Reichenbach (1828) corrected the original spelling to *Homalanthus*. Since then both spellings have been in use and several publications recommending the use of one or the other name have been published (Esser 1994; 1996; 1997).

All species of *Homalanthus* are trees. Similar to *Macaranga*, *Homalanthus* species are ant-plants. Mackay and Whalen (1991) described a remarkably low leaf damage of *H. novoguineensis* and *H. populifolius* with ant attraction by the foliar nectaries. *Homalanthus* shows unusual variation schemes. The leaves of all species are not only morphologically but even in their masses almost constant through the genus, all species are therefore not to be distinguished vegetatively (Esser 1994).
7.2 Uses

Bark and leaves of *H. populifolius* are used in some areas as black dye for staining basketwood (Usher 1974). *H. fastuosus* serves as fish poison (Burkill 1935). *Homalanthus* species find application as medicinal plants for the treatment of fever, diarrhea and gonorrhoea (*H. nutans, H. populifolius*). Leaves of some *Homalanthus* species serve as cataplasms (Sterly 1970) or as a curative of sores and boils (Holdsworth and Kerenga 1987); (Burkill 1935). The diluted sap of the underskin of *H. populneus* is drunk by pregnant women in Papua New Guinea to induce labor and ease the child delivery (Holdsworth 1993).

7.3 Phytochemical and biological investigations

The genus *Homalanthus* has not been intensively studied phytochemically. In 1969 Cambie and Parnell isolated a C\textsubscript{30} ketone from the wood of *H. polyandrus*. Phytochemical investigations on *Homalanthus nutans*, a traditional medicinal plant from Samoa, yielded the diterpene prostratin (Fig. 7.1) (Gustafson et al. 1992).

![Prostratin](image)

**Fig. 7.1: Structure of prostratin**

The isolation of prostratin marked an important step. In contrast to most other diterpenes of the phorbol type, prostratin showed no tumor promoting activity, but a potent cytoprotective effect in human lymphocytic cells infected with HIV-1, HIV-2 and drug-resistant HIV-1 strains. The anti-HIV activity appeared to
be due to the ability of binding and activating protein kinase C (Beutler et al. 1995).

7.4 Homalanthus nervosus

Synonym: H. vernicosus, H. deltoideus (Esser 1997)

Lit: (Frohne and Jensen 1985); (Webster 1994 b)
7.4.1 Botany and occurrence

*Homalanthus nervosus* is a shrub or a tree up to 21 m tall, found especially on ridges and slopes, in mossy, mountain forests in the highlands of Papua New Guinea, at 1400 - 2400 m altitude (Esser 1997). The leaves present a characteristic thickish, somewhat dull appearance, with a green undersurface (Airy Shaw 1980).

7.4.2 Traditional uses

Leaves of *Homalanthus nervosus* are used in PNG to treat boils and sores. *Polgai* leaves (unidentified) are placed under glowing wood ashes until soft. The juice is squeezed onto a sore and bandaged with leaves of *H. nervosus* (Holdsworth and Rali 1989). The stem sap also finds application in the treatment of boils and sores. The affected skin area is treated with stem sap and then covered with a *kambali* (unidentified) leaf (Holdsworth and Rali 1989). In the Simbu Highlands the juice from heated young leaves and flowers of *H. nervosus* is applied to ulcers and sores. Although painful, the treatment is said to be effective (Holdsworth and Kerenga 1987).
8 Triterpenoids

8.1 General remarks

Triterpenes are widespread non-steroidal secondary metabolites in terrestrial and marine flora and fauna, having been isolated even from rocks and fossils (Mahato et al. 1992); (Dev and Nagasampagi 1989). More than 4000 natural triterpenoids have been isolated and more than forty skeletal types have been identified (Hill 1993).

In the plant kingdom, triterpenoids are ubiquitous among the Dicotyledones and may be found in any part of the plant. Among the families producing considerable amounts of triterpenoids are Apocynaceae, Araliaceae, Betulaceae, Cactaceae, Caryophyllaceae, Cucurbitaceae, Ericaceae, Lecythidaceae, Leguminosae, Moraceae, and Myrtaceae. Angiosperm exsudates are exceptionally rich sources of certain types of triterpenoids; resins from Anacardiaceae, Burseraceae, Dipterocarpaceae, and latex from Euphorbiaceae are only a few examples. Triterpene alkaloids are widely distributed in the family Buxaceae. A variety of fungi, lichens, bryophyta, and ferns are also known to produce triterpenoids. Isolation of triterpenoids have been reported from marine blue-green, green, and red algae (Dev and Nagasampagi 1989).

Triterpenoids occur in nature either as free aglycones or as glycosides. In the case of glycosides sometimes cleavage of the sugar moieties by acid or enzymatic hydrolysis may be necessary before isolation and purification of the triterpenoid moiety. The usual method of acid hydrolysis of glycosides often leads to artifacts and many of the triterpenoids known today are artifacts (Mahato et al. 1992).

The majority of triterpenes shows the conventional skeleton arising biosynthetically from the cyclization of squalene-2,3-epoxide to fused polycyclic products (Harrison 1988). More unusual are the incompletely cyclized
compounds, or those exhibiting cyclization within the chain, or others with two consecutive cyclizations rather than with cyclization beginning at one end (Mahato and Sen 1997). Cyclic triterpenoids can be divided into two major categories, the tetracyclic and pentacyclic compounds; two minor groups are the compounds with tricyclic and double bicyclic systems (Fig. 8.1).

Fig. 8.1: Simplified biosynthesis scheme of triterpenoids

In photosynthetic organisms (higher plants, algae), cycloartenol is the first cyclized triterpene (from squalene); in nonphotosynthetic organisms (fungi, animals), it is lanosterol. Isolation studies have shown that ursolic acid, β-amyrin, and friedelin are the most frequently occurring triterpenoids (Dev and Nagasampagi 1989).

Although triterpenoids are widely distributed and, due to their structural diversity many different biological activities have been reported, application of triterpenes as successful therapeutic agents is limited (Mahato and Sen 1997). Pentacyclic
triterpenoids, for example boswellic acids isolated from *Boswellia serrata* (Burseraceae) as well as glycyrrhizin and glycyrrhetinic acid isolated from different *Glycyrrhiza* species (Leguminosae), were investigated for their anti-inflammatory activities in different *in vitro* and *in vivo* test systems, including inhibition of 5-lipoxygenase, and led to interesting results which need further studies (Safayhi and Sailer 1997). Triterpenoids isolated from different ferns were found to possess anti-tumor-promoting activities (Konoshima et al. 1996). Some triterpenes possess anti-HIV activity, as for example the quassinoids (biosynthetically degraded triterpenoids), isolated from different *Brucea* species (Okano et al. 1996). Further investigations of triterpenoids on potential anti-HIV activity were published. Quéré and coworkers published a work on triterpenes as potential inhibitors of the active protease of HIV-1 (Quéré et al. 1996). Phytochemical work on seeds of horse chestnut tree *Aesculus hippocastanum* (Hippocastanaceae), yielded triterpene oligoglycosides, namely escins and isoescins. Biological investigations showed that escins possess hypoglycemic as well as antiinflammatory activity (Yoshikawa et al. 1998).
8.2 Euphane/tirucallane type triterpenoids

Cyclisation of squalene-2,3-epoxide to the chair-chair-chair-boat conformation leads to the dammarane type triterpenoids (Fig. 8.2). Backbone rearrangement of the initial cyclisation product yields tirucallanes and euphanes (Fig. 8.2) (Connolly and Hill 1991).

![Fig. 8.2: Dammarane, tirucallane and euphane type triterpenoids](image)

Euphanes and their C-20 epimers tirucallanes are stereochemical variants of lanosterol. They represent a group of approximately 70 known C$_{30}$ triterpenoids, having mostly a tirucallane configuration at C-20. All compounds of this class showed unsaturation emanating at C-8. Euphanes and tirucallanes have been found in the latices of different Euphorbia species. Resins from trees of Burseraceae and Anacardiaceae families are also rich sources of euphanes and tirucallanes. Euphorbol (Fig. 8.3), isolated from the latex of several Euphorbia species, including Euphorbia maddeni (Euphorbiaceae) (Sahai et al. 1981) is an example of a C$_{31}$ triterpene that belong to this class (Dev and Nagasampagi 1989).
Euphane type triterpenoids have been isolated from different plant families. Phytochemical investigations on sunflowers, *Helianthus annuus* (Compositae) led to the isolation of eupha-7,24-dien-3β-y1 acetate (Fig. 8.3) (Akihisa et al. 1996). Bioactivity-guided fractionation of extracts of *Schinus molle*, a tropical tree belonging to the Anacardiaceae, yielded hydroxylanosta-dienoic acids (Fig. 8.3), euphane triterpenoids with moderate Angiotensin-Converting-Enzyme-inhibitory activity (Olafsson et al. 1997). An other representative of this genus, *Schinus terebinthefolius* contained euphane triterpenoids with specific inhibition activity against phospholipase A₂ (Jain et al. 1995). Recently cyclic lactone euphane triterpenoids, (e.g. GR 133686) (Fig. 8.3), isolated from leaves of *Lantana camara* (Lamiaceae), commonly known as wilde sage, were found to be potent inhibitors of human α-thrombin (Weir et al. 1998). Glycoside euphane-type triterpenes have also been found in leaves and fruits of *Rhoiptelea chiliantha* (Rhoipteleaceae) (Jiang et al. 1997).

![Fig. 8.3: Examples of euphane type triterpenoids](image-url)
9 Phorbol esters

"Phorbol esters" or "phorbols" is a term generally used to denote a family of highly toxic tri- or tetracyclic diterpene alcohols based on three distinct carbon skeletons known as tigliane, daphnane and ingenane. These compounds are widely distributed in many genera of the families Euphorbiaceae and Thymeleaceae, being mainly present in the milky latex at an amount of 0.2 to 1 (-1.5)% of the dry latex (Neuwinger 1996). In the plant latex they occur as esters, mainly as O-acyl esters or, more rarely as ortho-esters of the above named skeletons (Evans 1991).

Phorbol esters are thought to be biosynthesized via the classical acetate-mevalonate pathway typical for terpenoid compounds. Figure 9.1. illustrates a simplified biosynthesis scheme, which will be explained in continuation.

Fig. 9.1: Simplified biosynthesis scheme of phorbol ester type diterpenoids
An appropriate tetrapienyl diphosphate, e.g. geranyl-geranyl diphosphate, is considered to be the parent molecule of this class of compounds. Cyclization through a „head-to-tail“ condensation of the tetrapienyl diphosphate precursors leads to cembrane, which is transformed to a casbane skeleton. Further cyclization of casbane leads to the lathyranes and subsequently to the tiglianes, daphnanes and ingenanes (Adolf and Hecker 1977); (Schmidt 1987).

The first tigliane found was the tetracyclic diterpene phorbol (Fig. 9.2), consisting of a seven-, a six- and a five-membered ring as well as a three-membered cyclopropane ring. The five- and seven-membered as well as the seven- and six-membered rings are trans linked, the six- and three-membered rings are cis linked. Six oxygen functions are present. The ester functions are located at positions C_{12}-OH, C_{13}-OH and on a primary hydroxyl group at C_{20}. The most common tigliane diterpenoids are based on 12-deoxyphorbol and 12-deoxy-16-hydroxyphorbol. Although the term „phorbols“ is loosely mentioned for diterpenes based on the three skeletal types, the correct use of the term describes only tigliane type diterpenoids derived from phorbol (Evans 1986).

Daphnanes, for example daphnetoxin (Fig. 9.2), are tricyclic diterpenes, resembling tigliane in structure. The cyclopropane ring of tigliane has opened in the daphnanes to give an isopropylene side chain at C_{13} and a hydroxyl group at C_{14}. Daphnanes predominantly occur naturally as ortho-esters but can also be found in the O-acyl form (Evans and Soper 1978).

Ingenanes, including ingenol (Fig. 9.2), are tetracyclic diterpenes structurally related to phorbol because they possess a five-membered ring connected to a seven-membered ring. They differ from phorbol in a seven-membered ring attached via C_{10} to the quaternary center at C_{9} by a keto bridge. The 12,13 diglycol group of phorbol is absent. Ingenol esters have been isolated as monoesters (esterified at C_{3}, C_{5} or C_{20}) or diesters (esterified at both C_{3} and C_{20}). It is of taxonomic interest that ingenane-type diterpenes have only been found in the species *Euphorbia* and *Elaeophorbia* (Evans and Soper 1978).
Phorbols have intensively been studied for their toxicological and tumor-promoting (cocarcinogenic) activity. Biological activities found, include irritant and inflammatory action on skin and mucous membranes (Evans and Soper 1978). The latex containing phorbol diterpenes can cause kerato-conjunctivitis and uveitis up to blindness and loss of the eye (Evans and Kinghorn 1975). Cell proliferation and hyperplasia were observed in mice after phorbol ester administration (Blumberg 1980). The action of mutagens is enhanced by phorbol esters (comutagenic activity), probably by inhibition of the DNA repair mechanism (Soper and Evans 1977). Phorbol esters are especially known for their cocarcinogenic or tumor-promoting activity. Tumor-promoting agents do not themselves elicit tumors but they promote tumor growth following exposure
to a subcarcinogenic dose of a carcinogen (Evans 1986); (Evans and Taylor 1983). It is suggested that phorbol esters act by stimulating protein kinase C with different substrate specificities (Aitken 1987). Several phorbols have the paradoxical biological property to show tumor-promoting and tumor-inhibiting, antileukemic activity (Adolf and Hecker 1977). Prostratin, a relatively polar 12-deoxyphorbol was found to have anti-HIV activity. Although prostratin is structurally similar to known tumor-promoting phorbol esters, it doesn’t appear to have tumor-promoting activity itself (Gustafson et al. 1992). Phorbol esters are known to be extremely toxic to fish. The molluscicidal activity is moderate (Jurberg et al. 1995).

The isolation and identification of phorbol esters initiated intense research onto pro-inflammatory and tumor-promoting activities as well as in the understanding of cancer and inflammation. Therefore manyfold studies on structure-activity relationship have been made for phorbol esters. Some observations concerning structural features which are necessary for production of inflammation and tumor-promoting activity can be made: The diterpene alcohols themselves are biologically inactive. The ester function is essential for any biological activity. Further structural requirements are the presence of a free unsaturated primary OH group at C_{20} as well as the presence of polar centers at C_3 and C_9 (Evans and Soper 1978). In addition the trans connection (4ß, 10α) of the five- and seven-membered rings is a structural element absolutely essential for biological activity (Fürstenberger and Hecker 1972). Lipophilicity is also an important determinant of activity, suggesting that lipophilicity could increase tumor promoting activity (Gustafson et al. 1992).

Phorbol esters are difficult to isolate, many of the esterified forms of these diterpenes being highly unstable. Hydrolysis or transesterification reactions are known to occur during separation. Configurational changes may occur if crystallisation is attempted (Evans and Taylor 1983). Furthermore the compounds are sensitive to light, oxygen, acid and alkaline conditions (Evans and Kinghorn 1973). Tetradecanoylphorbolacetate (TPA), originally isolated from the seed oil of *Croton tiglium* by Hecker and coworkers (Hoppe et al. 1967)
was the first pure tumor-promoting agent isolated and is still the most widely used compound of a series of similar esters for tumor-promotion related studies (Evans 1991). Phorbol esters are a very interesting group of natural products, not only for their varied biological activities, but also as tools in investigations of the mechanism of carcinogenesis and irritancy.
10 Methodology of isolation procedures

Plants accumulate an enormous variety of organic substances. Methods are needed for separation, purification and identification of the different constituents present in plants. The choice of the appropriate technique is crucial for the successful isolation and identification of pure compounds. Ideally, plants should be extracted in the fresh state, just after having been collected. Since this is not feasible in most cases, plants are normally dried before further phytochemical work.

10.1 Extraction procedures

In the present phytochemical work the dry, ground plant material was extracted with different solvents of increasing polarity. The extraction with different solvents of increasing polarity leads to a first crude separation, yielding extracts, containing compounds of different polarities. *Euphorbia buxoides* was extracted at room temperature with hexane, dichloromethane methanol and a methanol-water (4:1) mixture, using a forced-flow extraction method. For *Homalanthus nervosus* percolation at ambient temperature was performed, using petroleum ether, dichloromethane, ethyl acetate, methanol and a methanol-water (4:1) mixture as extraction solvents.

10.2 Liquid-liquid partition

Liquid-liquid partition is a method based on the separation of molecules as a result of their differential solubility between two or more non-miscible phases. The advantage of liquid-liquid distribution lies in the absence of solid supports (Nyiredy et al. 1990). Kupchan et al. (1973) described a multiple step liquid-liquid partition technique. A slightly modified separation procedure was used to
further purify the methanol extract of *Euphorbia buxoides*. Crude fractionation of the hexane extract of the same plant was also done by liquid-liquid partition according to Evans and Taylor (Evans and Taylor 1983).

10.3 Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC), a so-called “planar” or “flat-bed” chromatography, is an easily applicable chromatographic method with good separation results. A coated thin-layer plate serves as stationary phase. The sample mixture is placed on the coated plate and developed in a closed chamber with a variable eluent. The components of the sample migrate at different rates during movement of the mobile phase on the stationary phase (Sherma 1996). Depending on the stationary phase, separation is based on adsorption and/or distribution processes. In special cases ion exchange effects or sieve effects can be involved. Thin-layer chromatography has found wide application for separation of substances as well as for their qualitative and quantitative analysis. Preparative TLC separation methods require minimal financial outlay and employ the most basic equipment (Hostettmann et al. 1986).

In the present study TLC (using silica gel and RP-modified silica gel as stationary phase) was used for optimization of mobile phases for VLC, HPLC and open columns, for monitoring chromatographic separations, for antimicrobial screenings and for preparative separations.
10.4 Open column chromatography

The classical open column chromatography is a method often used in natural products chemistry for separation and purification. Column chromatography is easy to perform and allows an efficient and rapid separation of mixtures. The method works by gravity and no additional pressure is required. Open column chromatography is suited for small as well as for large sample amounts. In this work several open column chromatographies were performed, using silica gel or magnesium silicate as stationary phases. Separations were obtained using either an isocratic solvent mixture or eluting gradually with solvents of increasing polarity.

10.5 Vacuum liquid chromatography (VLC)

Vacuum liquid chromatography (VLC) (Fig. 10.1) is a simple and rapid method for a crude separation of extracts. The technique is essentially a preparative layer chromatography run as column, the flow of which is activated by vacuum (Coll and Bowden 1986). The apparatus consists of a sintered glass funnel, packed with TLC sorbent material. Uniform packing of the column is important for good results. This is achieved by tapping the funnel during packing and by applying a vacuum from below the funnel. After packing the column is conditioned with the initial solvent. The sample is placed on the top of the packed column either in dissolved form, or as a powder. Separation is achieved by elution with appropriate solvent mixtures, starting usually with solvents of low polarity and gradually increasing the polarity. The flow of the solvent mixtures is maintained by vacuum (Hostettmann et al. 1986). Although usually silica gel is used as stationary phase, VLC can be performed with reversed-phase sorbents.
VLC has proven to be a useful tool for crude separation of mixtures of natural products as well as mixtures resulting from synthetic operations (Pelletier et al. 1986).

**Fig. 10.1: Vacuum liquid chromatography (VLC) set up**

10.6 **High performance liquid chromatography (HPLC)**

High performance liquid chromatography (HPLC) has become one of the leading technologies in natural products chemistry. While analytical HPLC is used for identification and quantitation of sample mixtures, the aim of semipreparative HPLC is to isolate pure compounds.

Determining an appropriate mobile phase is crucial for a successful application of HPLC. Usually TLC analysis of the sample is performed for finding a suited mobile phase. The applications of HPLC are vast; many different columns are available. Besides normal silica gel and reversed-phase columns, columns for specific separation problems, including chiral phases, polymeric phases, ion exchange and ion exclusion columns are available.
Normal-phase and reversed-phase semipreparative HPLC was done using both, ultraviolet (UV) detection and refraction index (RI) detection. Identification of common flavonoids was performed with analytical reversed-phase HPLC using a diode array detector.
11 Methodology of structure elucidation

Once a compound has been isolated and purified, it is necessary to determine the class of compound and finally the particular structure. This is achieved by combination of different structural informations obtained from numerous sources. A wide range of spectroscopic techniques is nowadays available to get structural information necessary for the identification of unknown compounds. These techniques include nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) spectroscopy, infrared (IR) spectroscopy and optical rotatory dispersion.

11.1 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) has become an indispensable tool for all natural scientists. The main field of application for NMR spectroscopy is the structure elucidation of molecules. The structural information available through pulsed NMR is very manifold: Number and information about the number of nuclei present in a molecule (signal intensity), information about connectivities between entities and information about the fine structure in spectra, arising from the coupling between neighbored nuclei. Various other interactions such as the nuclear Overhauser effect (nOE), depending on the relationship between nuclei, yield additional information about the spectroscopic structure.

In natural products chemistry NMR spectroscopy deals mainly with proton (1H) and carbon (13C) nuclei, since the resonances of these nuclei are the most important for structure elucidation of organic compounds. NMR is a method using nuclei, which have a nonzero spin quantum number (e.g. 1H, 13C, 15N, 31P). When a sample is placed into a strong magnetic field, nuclei that have a nonzero spin quantum number are able to absorb energy from the radio frequency range of the electromagnetic spectrum and subsequently occupy a
specific energy state. NMR is based on disturbing this state of equilibrium by introducing a second field at appropriate radio frequency. The response of the system to the disturbance generates the nuclear magnetic resonance spectrum (Byrne 1993). In Fourier transform NMR spectroscopy, the entire spectrum of interesting frequencies is stimulated by a pulse of radio frequency energy and the response of the system is measured as a function of time using a digital recorder. The frequency spectrum is generated mathematically using a Fourier transformation, which converts the time domain data into the classical frequency domain spectrum (Sanders and Hunter 1993).

11.1.1 One-dimensional (1D) NMR experiments

One-dimensional (1D) NMR spectra, usually $^1$H and $^{13}$C NMR experiments, show the signal frequencies in the x-axis. The y-axis shows the signal intensities. Almost all NMR studies begin with the recording of a $^1$H spectrum, the $^1$H nucleus being the most easily observed and the most studied. The chemical shift of each signal characterizes the environment of the proton from which it arises. It is seldom possible to analyze completely the coupling networks from the $^1$H spectrum without taking further spectra. The $^{13}$C spectrum, among other spectra, provides important structural information, since it arises from the nuclei that form the backbone of the molecule, in contrast to the $^1$H nuclei that are at the periphery. The DEPT (Distortionless Enhancement by Polarization Transfer) experiment is the most commonly used method for determining the number of hydrogens bonded to each carbon atom. This method involves transfer of magnetization from protons to their directly attached carbons. The DEPT-135 (using a final $^1$H pulse angle of 135°) shows all protonated carbon signals with CH$_3$ and CH resonances being positive, while CH$_2$ signals are negative (Byrne 1993). The DEPT-90 (using a final $^1$H pulse angle of 90°) shows positive CH resonances while CH$_3$ and CH$_2$ signals are not visible. The inverse-gated $^{13}$C decoupling experiment uses the fact that Nuclear Overhauser effect (NOE)
builds up slowly. The decoupler is always switched off during the relaxation delay. The coupling immediately vanishes if the decoupler is turned on, so decoupling is only performed during detection. In inverse-gated $^{13}$C spectra the signal intensity is proportional to the number of $^{13}$C atoms present in the molecule (Zerbe 1996). This experiment is used for quantitative measurements of the Overhauser effect and for quantitative $^{13}$C NMR spectroscopy where the Overhauser effect has to be suppressed. The resulting signals of the protonated carbon atoms all have nearly the same height (Braun et al. 1996). In the present study inverse-gated $^{13}$C NMR allowed the assignment of the carbon atoms in an unseparable mixture of two similar compounds. Because it was not a 1:1 mixture, the $^{13}$C signals of the respective compounds showed different height levels and could therefore be assigned easily.

11.1.2 Two-dimensional (2D) NMR experiments

A standard 1D NMR experiment shows two dimensions. The abscissa corresponds to the frequency axis; the ordinate shows the signal intensities. A 2D NMR spectrum shows two frequency axis, the abscissa and the ordinate. The signal intensities correspond to the third dimension. The two frequency domains are usually called the direct ($F_2$; abscissa) and the indirect ($F_1$; ordinate) frequency domain (Zerbe 1996). 2D NMR spectra are obtained by recording a series of 1D NMR spectra. These individual spectra differ only by a time increment, which is introduced within the pulse sequence (Braun et al. 1996). In the present study a series of 2D NMR experiments, which will be presented subsequently, were used.

Homonuclear correlated experiments contain frequencies of the same nuclei in both dimensions (e.g. $^1$H-$^1$H correlations). DQF-COSY (double-quantum filtered proton correlation spectroscopy) shows vicinal/geminal protons correlated via scalar coupling. TOCSY (total correlation spectroscopy), an experiment with multiple proton relay, shows correlations of all protons within the same spin system via scalar coupling. The nuclear Overhauser effect (NOE) permits the
identification of those nuclei within a molecule that are close in space (dipolar coupling). This allows the investigation of the geometrical structure of molecules (Friebolin 1992). The disadvantage of the NOE is that it changes its sign depending on the molecular correlation time. T-ROESY (transverse rotating-frame Overhauser effect spectroscopy) experiments are based on determining the NOE in the rotating frame under spin-lock conditions. As a result the sign is always positive. One disadvantage of normal ROESY is that TOCSY correlations may also occur (Braun et al. 1996). This problem has been greatly diminished by measuring transverse cross-relaxation in the rotating frame (Hwang and Shaka 1992).

Heteronuclear correlated experiments contain frequencies of different nuclei (e.g. ^1H-^13C correlations). HMQC (heteronuclear multiple quantum coherence) is a form of an inverse heteronuclear ^1H detection technique showing correlations of directly bonded protons and carbons. While HMQC performs the H,C correlation via the ^13C chemical shift evolution of a double quantum coherence, this can also be achieved by the HSQC (heteronuclear single quantum coherence) method (Braun et al. 1996). HMBC (heteronuclear multiple bond correlation) leads to the detection of proton, carbon correlations via long-range (^2J, ^3J) couplings.

11.2 Mass spectrometry

Mass spectrometry (MS) plays an essential role in chemistry. It can provide an accurate molecular weight of an unknown compound and may yield a complex fragmentation pattern which is often characteristic for that particular compound. MS consists of degrading trace amounts of an organic compound by generation of gas-phase positive or negative ions and subsequent separation of these ions. The fragmentation pattern according to the mass is recorded (Bloor and Porter 1993). The sample vapor diffuses into the low-pressure system of the mass spectrometer where it is ionized with sufficient energy to cause fragmentation of
the chemical bond. The resulting positively charged ions (or in some techniques negative) are accelerated in a magnetic field which disperses and allows relative abundance measurements of ions to give mass-to charge ratio (Harborne 1984).

Electron Impact Positive-Ion (EI+) MS is the most commonly used MS method in natural products chemistry. The sample is bombarded with electrons from a glowing filament at low source pressure to produce a positively charged molecular radical ion of high internal energy, which usually fragments to positively charged fragment ions, neutral fragments and radicals. The relative abundance of these fragments and positively charged radicals, together with their isotope ions form a characteristic EI+ spectrum fragmentation pattern for a given organic molecule under defined conditions (Bloor and Porter 1993).

Fast Atom Bombardment (FAB) MS is a method involving the suspension of a target compound in a liquid matrix that is then bombarded by fast argon or xenon atoms (6 to 10 keV energy). Bombardment with fast atoms or ions displaces positive (M + H)+ or negative (M - H)- ions from the surface. (M + Na)+ and (M + K)+ may also be observed as well as other signals related to the matrix (Bloor and Porter 1993).

Electrospray Ionization (ESI) represents an alternative ionization method for large involatile analytes, operating at almost atmospheric pressure. The sample is injected into a flowing liquid stream, frequently methanol-water or acetonitrile-water (acidified with acetic acid), which protonates basic sites on the sample molecule. Application of a high electric field disrupts the liquid surface and produces highly charged droplets (positively or negatively charged). Solvent is evaporated and ions or ion clusters are generated (Bloor and Porter 1993).
11.3 UV/VIS spectroscopy

UV/VIS spectroscopy deals with the interaction of electromagnetic radiation with a compound in a wavelength range of 200 to 800 nm (ultraviolet: 200-380 nm; visible: 380-800 nm). Thereby electrons are excited. The frequency of the absorbed radiation correlates with the structure of the test compound. Molecular absorption depends on the electronic structure of a molecule and is mostly limited to conjugated systems. The value of UV and visible spectra in identifying unknown constituents is obviously related to the complexity of the spectrum and to the positions of the wavelength maxima. UV/VIS spectroscopy is rather used for quantitative analytical work than for structure elucidation of unknown compounds (Harborne, 1984).

11.3.1 Diode array detection (DAD)

Diode array detection (DAD) is a special form of UV detection adding a third dimension – the wavelength – to time and absorption. DAD finds wide application as detection system coupled with analytical HPLC. The continual acquisition of absorbance data across the UV visible portion of the electromagnetic spectrum allows the simultaneous use of more than two wavelengths for detection as well as the complete application of available detector information to the analytical problem. Thus peak homogeneity, peak purity and peak identity can be investigated based on the comparison of peak spectra with reference spectra (Sievert and Drouen 1993); (Wickham 1993). The simultaneous record of chromatograms obtained at different wavelengths and the measurement of the UV spectrum of each eluted compound makes HPLC-DAD coupling a method of choice for the analysis of complex mixtures. An identification of the peaks in the chromatogram is possible by comparing their retention times and UV spectra with those of authentic samples (Hostettmann et al. 1984).
In the present study a method was developed using DAD-HPLC for the identification of some of the most common plant phenolics.

11.4 Infrared spectroscopy

Infrared (IR) spectroscopy is the measurement of the vibrational energy changes of a molecule incited by electromagnetic radiation of specific wavelength (1-1000 μm). The covalently bonded atoms of a molecule keep continuously oscillating about an equilibrium position, due to vibrational energy. The movements of these vibrations oscillate with frequencies that comprise the infrared spectral region. When exposed to infrared radiation, energy is absorbed by the molecule, which results in an increase of vibrational motion. The frequency of the absorbed infrared radiation is correlated with the structure of the compound (Cooper and Knutson 1995).

11.5 Optical rotation

Optical activity of a compound is related to the asymmetry of a molecule, that means to its chirality. Optically active materials absorb right- and left-handed circularly polarized light to a different extent. The optical rotation depends on concentration, temperature, path length, and wavelength (Bloemendal and Curtis Johnson 1995). All these factors are taken into consideration by expressing the optical rotation as specific optical rotation (α). The specific rotation is a characteristic property of any chiral compound.
12 Results and discussion

12.1 Introduction of a *Candida* bioassay

In order improve our in-house bioassays we introduced an antifungal assay, using *Candida albicans*, an optional pathogenic yeast, as test germ. Existing test procedures were the basis for the development of the assay (Rahalison et al. 1994). *Candida* overnight-suspensions, using Sabouraud liquid medium, were prepared. Two different agars were compared, namely Müller-Hinton agar and malt extract agar. The yeast grew on both agars, but malt extract agar showed better growing conditions than Müller-Hinton agar. The use of malt extract agar is therefore suggested for performing the *C. albicans* bioassay.

Four different screening methods were performed and compared, using both miconazole and amphotericin B as reference agents (applied amounts 0.01 to 5 μg respectively).

1) **Disc diffusion assay:** Solutions of miconazole and amphotericin B were applied in different concentrations (applied amount 0.01 to 5 μg) on special paper discs. 5 ml of sterile malt extract agar were inoculated with 50 μl of an over-night *Candida* suspension. The inoculated agar was poured over an agar plate and the paper discs were put on it. The inoculated plates were then incubated over night at 37 °C.

2) **Hole plate assay:** 5 ml of sterile malt extract agar were inoculated with 50 μl of an over-night *Candida* suspension. The inoculated agar was poured over an agar plate. As soon as the agar had become solid, holes of 6 mm in diameter were punched into the agar. Solutions of miconazole and amphotericin B respectively were applied in different concentrations into the
holes and filled up to 100 μl with sterile water. The inoculated plates were incubated over night at 37 °C.

3) TLC stick assay: Alumina TLC silica plates were cut into 1 cm broad strips. Different concentrations of miconazole respectively amphotericin B were applied as spots on the TLC plates. 5 ml of sterile malt extract agar were inoculated with 50 μl of an over-night *Candida* suspension. The inoculated agar was poured over an agar plate. As soon as the agar had become solid, the TLC plates were stuck horizontally into the inoculated agar plates, so that the applied spots were at least half covered with agar. The inoculated plates were incubated over night at 37 °C.

4) Agar overlay assay: Different concentrations of miconazole and amphotericin B were applied as spots on an aluminum TLC silica gel plate. The TLC plates were placed in plastic Petri dishes (10 x 10 cm). 10 ml of sterile malt extract agar were inoculated with 100 μl of an over-night *Candida* suspension. The inoculated agar was poured onto the TLC plate. The inoculated plates were incubated over night at 37 °C. For detection the TLC plates were sprayed with an aqueous solution of a tetrazolium salt and incubated again at 37 °C for 30 minutes.

The results of the performed assays are summarized in Table 12.1. Diameters of the active zones of inhibition are presented. For the TLC stick assay, the radius of the inhibition zone is indicated.

<table>
<thead>
<tr>
<th></th>
<th>Disc diffusion assay</th>
<th>Hole plate assay</th>
<th>TLC stick assay</th>
<th>Agar overlay assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Miconazole</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 μg</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>0.1 μg</td>
<td>5 mm</td>
<td>6 mm</td>
<td>/</td>
<td>4 mm</td>
</tr>
<tr>
<td>1 μg</td>
<td>9 mm</td>
<td>10 mm</td>
<td>3 mm</td>
<td>9 mm</td>
</tr>
<tr>
<td>5 μg</td>
<td>11 mm</td>
<td>11 mm</td>
<td>4 mm</td>
<td>13 mm</td>
</tr>
<tr>
<td><strong>Amphotericin B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 μg</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>0.1 μg</td>
<td>0.5 mm</td>
<td>7 mm</td>
<td>2 mm</td>
<td>6 mm</td>
</tr>
<tr>
<td>1 μg</td>
<td>1 mm</td>
<td>8 mm</td>
<td>4 mm</td>
<td>13 mm</td>
</tr>
<tr>
<td>5 μg</td>
<td>1.5 mm</td>
<td>9 mm</td>
<td>5 mm</td>
<td>18 mm</td>
</tr>
</tbody>
</table>

*Table 12.1: Results of the preliminary antifungal assays*
The results showed, that the TLC stick assay is not suited for a general bioassay against *Candida albicans*, showing a much lower detection limit than the other assays. For the other three assays the detection limits were similar, between 0.01 and 0.1 μg for both miconazole and amphotericin B. Disc diffusion assay and hole plate assay allow an easy interpretation, because the active zones are visible as brown spots on the whitish inoculated plates. The agar overlay is not easily visible, the white color of the TLC plate being very similar to the whitish colored *Candida* suspension. In order to improve the detection of inhibition zones two different tetrazolium salts were tested, namely p-iodonitrotetrazolium chloride (INT) and methylthiazoyltetrazolium chloride (MTT). Both tetrazolium salts were tested using aqueous solutions of 0.25% and 0.5% (m/V). Both concentrations gave about the same stains, the colorless INT turning into the purple colored corresponding formazan and the yellow colored MTT turning into a dark blue colored formazan. MTT gave a more homogenous and better visible coloration, suggesting the use of a 0.25% aqueous solution of MTT for performing the agar overlay test.

The main problem with the methods tested is the solubility and the diffusion. Samples to be tested should easily diffuse into the inoculated agar in order to show inhibiting activity. Hydrophilic compounds easily diffuse into the agar, whereas it is more difficult for lipophilic compounds. Especially in the hole diffusion assay lipophilic substances might precipitate, when sterile water is added to fill the holes.

We keep in mind that these assays can give especially false negative results, due to the solubility and to the fact, that active compounds might be present in very small amount in the sample to be tested and may therefore not be detected. But they represent good tools for screening activity against *Candida albicans*. 
12.2 Preliminary biological screening

The 13 plants used for a preliminary biological screening were collected in Papua New Guinea during three field trips in 1989, 1991 and in 1992. Results of the preliminary biological screening are summarized in Table 12.2.

Molluscicidal activity was determined using extract concentrations of 100 ppm. The number of dead snails after 24 hours versus total number of snails was pointed out. Antifungal as well as antibacterial activity was determined using the paper disc diffusion technique, applying 500 µg of the corresponding extract on the paper discs. For the antifungal activity assays Penicillium oxalicum and Candida albicans were used as test germs. Antibacterial activity was determined using the gram positive bacteria Bacillus subtilis, Bacillus cereus, Micrococcus luteus and Staphylococcus epidermidis and the gram negative germs Escherichia coli, Mycobacterium fortuitum and Pseudomonas aeruginosa. The brine shrimp lethality assay was carried out with concentrations of 500 ppm of each extract. The percentage of dead shrimps after 24 hours was determined. Cytotoxicity was screened with two cell lines, KB cells and CaCo2 cells, using concentrations of 50 ppm for each extract. After 72 h of incubation cell growth was determined by protein determination.

Molluscicidal activity was found only in extracts of plants of the family Euphorbiaceae, namely in the butanol extract of Aleurites moluccana and in all three extracts of Euphorbia buxoides. No antifungal activity was found, neither against Penicillium oxalicum nor against Candida albicans. Antibacterial activity was predominantly restricted to members of the Euphorbiaceae with the exception of the DCM/MeOH extract of Mangifera minor (Anacardiaceae) showing moderate activity against the gram positive bacterium Staphylococcus epidermidis. Both members of the genus Euphorbia tested showed activity. Especially the methanol extract of E. buxoides showed activities against E. coli, M. luteus and S. epidermidis. E. geniculata was only active against S. epidermidis, showing good activity, but a diffuse inhibition zone, indicating that
the antibacterial activity was not completely bactericidal. The methanolic extracts of members of the genus *Homalanthus* tested demonstrated moderate activities against *M. fortuitum* (*H. nervosus*) and *S. epidermidis* (*H. nervosus* and *H. novoguineensis*). The butanol extract of *Securinega melanthesoides* demonstrated moderate activity against the gram negative germ *Escherichia coli* and the gram positive germ *S. epidermidis* and good activity against the gram positive bacterium *Micrococcus luteus*.

The general toxicity assay using brine shrimps revealed a slight activity of 11% of dead shrimps for the butanol extract of *Mangifera minor*. The DCM/MeOH extract of *A. moluccana* showed good, but not uniform activity against *Artemia salina*. The test was repeated three times, testing always four charges. Activity was found in all charges, but diverging between 20% and 60%. The butanol extract in contrast showed an uniform activity of 34% of dead shrimps after 24 hours. *E. buxoides* showed activity with all three extracts tested, ranging between 20% and 32%. All other extracts tested exhibited no activity.

The cytotoxicity assays showed different results. Against the KB cell line two extracts, namely the butanol extracts of *M. minor* and *E. buxoides*, showed remarkable activity. The assay for determining activity against CaCo2 cells yielded a higher extent of activity, with 11 of the 41 tested extracts being active.

The interpretation of biological screening results is always related to the question about their significance. It has to be pointed out, that the screening results can only give an overview whether interesting active compounds are present or not. A sample to be tested may comprise 30–40 assayable compounds and other compounds may be present under detection limit (Cordell 1995). Thus the amount of the active compound within a test sample is decisive for a potential positive result. Another important problem with bioassays is the solubility of the compounds in a sample. Using the paper disc agar diffusion method hydrophilic compounds can easily diffuse into the agar, whereas lipophilic compounds have difficulties to diffuse into the media. Furthermore the diffusion technique does not allow the concentration of the test compound or extract which reaches the microorganism to be known (Cole 1994). Other
factors influencing the results of a biological screening may not be obvious and can be due to synergistic effects, chemical changes during extraction and manipulation of extracts, and the canceling of activity by certain concentrations of substances (Ghisalberti 1993).

With regard to the biological activities obtained in the screening *Euphorbia buxoides* seemed to be the most interesting plant. The genus *Euphorbia* although having been intensively studied phytochemically, nevertheless represents a very interesting and manifold genus until today. These indications, together with the fact, that no preceding phytochemical work had been done on this plant, led to the choice of *Euphorbia buxoides* for further phytochemical investigations. Focusing on plants of the family Euphorbiaceae occurring in Papua New Guinea, another member of this family, *Homalanthus nervosus*, was chosen for continuing studies. The genus has not been intensively studied, but some phytochemical and biological studies deal with promising compounds. Prostratin, an interesting anti-HIV active and non-tumor promoting phorbol ester has been isolated from a member of the genus *Homalanthus*. (see chapter 7). Preliminary TLC screening of *H. nervosus* and *H. novoguineensis* indicated that they probably contain similar compounds. *H. nervosus* was finally chosen, considering that this species, in contrast to *H. novoguineensis*, is endemic in Papua New Guinea.
### Table 12.2: Results of the preliminary biological screening

<table>
<thead>
<tr>
<th></th>
<th>Mollusc activity</th>
<th>Antifungal activity</th>
<th>Antibacterial activity</th>
<th>Brine shrimp lethality assay/ Cytotoxic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 ppm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mangifera minor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM/MeOH</td>
<td>0/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-BuOH</td>
<td>0/2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>0/2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Euphorbia buxoides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>2/2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM</td>
<td>1/2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>1/2</td>
<td>-</td>
<td></td>
<td></td>
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<tr>
<td><strong>Euphorbia geniculata</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM/MeOH</td>
<td>0/2</td>
<td>-</td>
<td></td>
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<tr>
<td>n-BuOH</td>
<td>0/2</td>
<td>-</td>
<td></td>
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</tr>
<tr>
<td>H₂O</td>
<td>0/2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Homalanthus nervosus</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>0/2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>MeOH</td>
<td>0/2</td>
<td>-</td>
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</tr>
</tbody>
</table>

**Antibacterial activity:**
- No activity (-)
- Inhibition zone ≤ 1.5 mm, corresponding to 0.5 μg CA resp. TC (chloramphenicol resp. tetracycline hydrochloride); (+) : Inhibition zone ≤ 1.5 mm, diffuse;
- Inhibition zone 2 - 5 mm, corresponding to 5 μg CA resp. TC; (++): Inhibition zone 2 - 5 mm, diffuse

**Cytotoxicity assays:**
- No activity (-);
- 50% of cell inhibition (+)
Table 12.2 (continued)

<table>
<thead>
<tr>
<th>Mollusc activity</th>
<th>Antifungal activity</th>
<th>Antibacterial activity</th>
<th>Brine shrimp lethality assay/ Cytotoxic activity</th>
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<td></td>
<td>P. C. oxal. alb.</td>
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<td>E. coli</td>
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<td>500 εg</td>
<td>500 εg</td>
<td>500 εg</td>
</tr>
<tr>
<td>Securinega melanthesoides</td>
<td>(Euphorbiaceae)</td>
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<tr>
<td>n-BuOH</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂O</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Scaevola oppositifolia - leaves</td>
<td>(Goodeniaceae)</td>
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<td>-</td>
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</tr>
<tr>
<td>H₂O</td>
<td>0/2</td>
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<td>-</td>
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<td>Scaevola oppositifolia - stems</td>
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<tr>
<td>H₂O</td>
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<tr>
<td>H₂O</td>
<td>0/2</td>
<td>-</td>
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</tr>
<tr>
<td>Ficus opposita</td>
<td>(Moraceae)</td>
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<td></td>
</tr>
<tr>
<td>n-BuOH</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂O</td>
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</tr>
<tr>
<td>Desmodium umbellatum</td>
<td>(Papilionaceae)</td>
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</tr>
<tr>
<td>n-BuOH</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂O</td>
<td>0/2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Antibacterial assays: - : no activity; + : inhibition zone ≤ 1.5 mm, corresponding to 0.5 μg CA (chloramphenicol) resp. TC (tetracycline hydrochloride); (+) : inhibition zone 1.5 mm, diffuse; ++ : inhibition zone 2 - 5 mm, corresponding to 5 μg CA resp. TC; (++) : inhibition zone 2 - 5 mm, diffuse

Cytotoxicity assays: - : no activity; + : 50 % of cell inhibition
12.3 Isolation of compounds from *Euphorbia buxoides*

### 12.3.1 Extraction

1.4 kg of air-dried leaves of *Euphorbia buxoides* were powdered and mixed with quartz sand. Continuous extraction with three solvents of increasing polarity, namely hexane, dichloromethane and methanol was performed, by means of percolation at ambient temperature. Solvents were changed after 5 days of extraction, allowing maceration over night with the new solvent. The solvents were evaporated in vacuo, at temperatures not exceeding 40 °C, yielding the corresponding hexane, dichloromethane and methanol extract.

### 12.3.2 Fractionation of the hexane extract

The isolation procedure, including sample amounts, separation techniques and mobile phases used is given in Figure 12.1.

The hexane extract was further separated by means of liquid-liquid partition between hexane and methanol/water (4:1) yielding a hexane fraction and a methanol/water fraction. The non-polar hexane fraction was evaporated to dryness in vacuo, resulting in 35 g of hexane fraction. The methanol/water fraction was partly evaporated to remove the methanol. The remaining water fraction was partitioned against diethyl ether. The ether-phase was washed by partition against 1% sodium carbonate solution to remove pigments and acidic material. After elimination of residual water by shaking with anhydrous sodium sulfate, the ether-phase was evaporated in vacuo below 40 °C.

The hexane fraction, resulting from the liquid-liquid partition of the hexane extract, was further separated using VLC with a stepwise solvent gradient (hexane-> ethyl acetate), yielding 16 fractions. Fraction 10 was subjected to
VLC, yielding high amounts of pure euphol. The purity was controlled by TLC and ¹H-NMR. Fraction 11 was subjected to VLC. Subfraction 11.13 seemed to contain compounds with similar structure, proven by TLC and ¹H-NMR analysis. Final purification was done by normal phase HPLC, yielding 2 mg of a mixture of the two closely related compounds, eupha-8,23-dien-3β-25-diol (euphane 1) and eupha-8,25-dien-3β-24-diol (euphane 2). Because of the small amount isolated, complete identification was done after isolation and structure elucidation of the same mixture isolated from the dichloromethane extract.

After TLC analysis subfraction 11.8 showed a major spot well separated from minor compounds. Thus preparative TLC on silica gel plates was used for isolation. The plates were developed with a mixture of hexane/chloroform/methanol (8:1:1). Purity of the isolate, α-tocopherol-quinone, was confirmed by TLC and ¹H-NMR investigations.

Further separation of the diethyl ether fraction, resulting from the liquid-liquid partition of the hexane extract, was done by means of an open column, with Florisil® (magnesium silicate) as stationary phase. Elution with a stepwise solvent gradient (hexane -> ethyl acetate -> acetone) yielded 47 fractions which where reduced to 6 fractions after TLC control. Fraction 1 was subjected to another open column, using a solvent mixture of diethyl ether/ethyl acetate/hexane (1:1:1). This open column chromatography yielded the euphane triterpene corollatadiol.
Fig. 12.1: Isolation scheme of the hexane extract of *Euphorbia buxoides*
12.3.3 Fractionation of the dichloromethane extract

The isolation procedure, including sample amounts, separation techniques and mobile phases used is given in Figure 12.2. Crude separation of the dichloromethane extract was carried out dividing the dichloromethane extract into 8 equal parts. Each of them was subjected to VLC using the same stepwise solvent gradient (hexane -> dichloromethane -> acetone) and collecting similar fractions. The fractions from the 8 VLCs were controlled by TLC. Identical fractions were combined, affording 24 fractions. Fraction 7 was subjected to an open column using silica gel and a solvent mixture of hexane/isopropanol/methanol (96:2.5:0.5), yielding 12 subfractions. Final purification of fraction 7.5 by HPLC led to the isolation of \textit{n-octacosanol}. Subfraction 7.9 was purified by HPLC using a mixture of hexane/ethyl acetate (9:1). The resulting residue seemed to be a pure compound following TLC and \textit{H}-NMR investigations. After further NMR investigations (inverse-gated \textit{13C}, 2D experiments) it was found to be an unseparable mixture of two closely related euphane triterpenoids, \textit{eupha-8,23-dien-3β-25-diol (euphane 1)} and \textit{eupha-8,25-dien-3β-24-diol (euphane 2)}, already isolated from the hexane extract. Fraction 9, exhibiting strong antibacterial and cytotoxic activity against KB cells, was further separated by RP18-VLC, using a stepwise solvent gradient (acetonitrile -> water). From the resulting 16 subfractions, 9.6 was purified using RP-HPLC. The isolated structure was identified as \textit{nonadeca-3,5-dien-2-ol-1-yl}. 
Dichloromethane extract

65 g

VLC silica gel
Hexane -> DCM -> Acetone

Fraction 7
910 mg
Open column silica gel
Hexane/isopropanol/MeOH
96 : 2.5 : 0.5

Fraction 7.5
18 mg
HPLC Lichrosorb® Si60
250 x 8 mm, 5 μm
Hexane/EtOAc 4:1
n-Octacosanol
8.7 mg

Fraction 7.9
21 mg
HPLC Lichrosorb® Si60
250 x 8 mm, 5 μm
Hexane/EtOAc 9:1
Euphane1 / Euphane 2
9 mg

Fraction 9
230 mg
VLC RP18
H₂O -> ACN

Fraction 9.6
29 mg
HPLC Spherisorb® ODS2
250 x 8 mm, 5 μm
MeOH/ACN/H₂O 25:5:10
Nonadeca-3,5-dien-2-ol-1-yl
8.7 mg

Fig. 12.2: Isolation scheme of the dichloromethane extract of Euphorbia buxoides
12.3.4 Fractionation of the methanol extract

The isolation procedure, including sample amounts, separation techniques and mobile phases used is given in Figure 12.3.

Crude separation of the methanol extract was performed by liquid-liquid partition between butanol and water. The butanol extract was further separated by liquid-liquid partition, affording four fractions of different polarities, a hexane, a chloroform, a butanol and a water fraction. The butanol fraction was subjected to VLC, using a stepwise solvent gradient (dichloromethane -> ethyl acetate-> methanol). From the resulting 16 fractions, fraction 4 was further separated by RP18-VLC, affording 10 subfractions. Hyperoside precipitated from subfraction 6. Subfraction 8 was subjected to analytical HPLC. The obtained spectra showed the presence of kaempferol-3-O-glucoside and hyperoside. Another major compound, not corresponding to kaempferol-3-O-glucoside, or hyperoside nor to the other common flavonoids tested, was found to be present in the same subfraction. Separation using RP18 HPLC afforded kaempferol-3-O-β-D-glucuronide.
Fig. 12.3: Isolation scheme of the methanol extract of *Euphorbia buxoides*
12.3.5 DAD-HPLC analysis of the methanol extract

TLC control of subfraction 8 of the methanol extract indicated the presence of flavonoids (see 12.3.4.). Therefore DAD-HPLC analysis of subfraction 8 was performed, in order to identify the major compounds of this subfraction. The retention times and the corresponding UV spectra of the peaks were compared with authentic samples of common flavonoids. Spiking the samples with the dissolved reference standards yielded hyperoside and kaempferol-3-O-glucoside to be major compounds of the methanol extract. Another major compound showed a UV spectrum typical for flavonoids but did not correspond in its retention time to any of the tested reference flavonoids and was therefore isolated, yielding kaempferol-3-O-β-D-glucuronide.

The DAD-HPLC spectra are shown in Figures 12.4 and 12.5.
Fig. 12.4: DAD-HPLC spectrum of chosen reference flavonoids at three different wavelengths (350, 280, 250 nm)
Fig. 12.5: DAD-HPLC spectrum of the methanol extract of *Euphorbia buxoides*
12.4 Structure elucidation of the compounds from *Euphorbia buxoides*

12.4.1 Euphol

![Structure of euphol (euphane-8,24-3β-ol)](image)

The molecular formula of euphol was determined as C$_{30}$H$_{50}$O, (m/z 426.4) from its EIMS and $^{13}$C NMR data. IR transmission bands (Fig. 12.7) at 2944 and 1455 cm$^{-1}$ suggested the presence of methyl groups, transmission bands at 3340, 1374, 1216, 1094 and 1023 cm$^{-1}$ pointed to the occurrence of a secondary alcohol.

The $^{13}$C NMR spectrum (Fig. 12.8) presented 30 C signals, eight of them being resonances for methyl groups. The $^1$H NMR spectrum (Fig. 12.9) showed singlet signals for five methyl groups on quarternary carbons ($\delta_H$ 0.78, 0.82, 0.88, 0.98, 1.02 each s), one doublet for 3H at $\delta_H$ 0.87 and two vinylic methyl groups at $\delta_H$ 1.62 (s) and 1.70 (s). The $^{13}$C NMR resonances at $\delta$ 133.61 (s) and 134.09 (s) are in accordance with the presence of a $\Delta^8,9$ double bond in a tetracyclic triterpenoid (Gewali et al. 1990). The $^1$H NMR showed a double doublet at $\delta_H$ 3.25 ppm, corresponding to a carbon doublet at $\delta_C$ 78.99 ppm, as seen from the HMQC spectrum. These findings indicated the presence of a hydroxyl group. The distribution of the methyl groups, the occurrence of a $\Delta^8,9$
double bond and the presence of a hydroxyl group suggested the presence of a tetracyclic triterpenoid with a lanostane or an euphane/tirucallane skeleton. The lanostane- euphane/tirucallane skeleton was further verified using HMBC measurements, which allowed the assignments of all proton and carbon resonances of the skeleton, as seen in Table 12.3. The structure of the side chain was determined by HMBC (Fig. 12.10) combined with COSY experiments. \(^1\)H-\(^1\)H-DQF-COSY measurement showed direct correlations between H-20 and H\(_3\)-21. Together with HMBC correlations found between C-22 and H\(_3\)-21, the partial structure of the segment C-21 to C-22 was elucidated. Chemical shifts indicated the two methyl groups C-26 and C-27 to be bonded to the carbon at \(\delta_c 130.76\), belonging to the double bond. This assumption could be confirmed by HMBC correlations.

The El mass spectrum (Fig.12.11) showed a mass peak at \(m/z 426\) and characteristic fragments at \(m/z 411, 393, 189\) and \(109\) (Fig. 12.12).

Tetracyclic triterpenes of the euphane type are distinguished from the lanostanes by inversion of the stereochemistry of carbons 13, 14, and 17 (Bartlett et al. 1990). Therefore final differentiation of the structure was achieved by elucidating the stereochemistry. ROes were observed between H-3 and H\(_3\)-28, H-3 and H\(_2\)-23, indicating them to be all on the same side (\(\alpha\)) of the molecule, while interactions between H\(_3\)-19 and H\(_3\)-29, H\(_3\)-19 and H\(_3\)-30, H-17 and H\(_3\)-30 revealed them to be on the opposite side (\(\beta\)).

Comparison of the obtained \(^1\)H NMR and \(^13\)C NMR data with literature values (Gewali et al. 1990) showed conformity. The isolated compound was established as euphol, having been isolated before from several \textit{Euphorbia} species (Gewali et al. 1990); (Knight 1973).
Fig. 12.8: $^{13}$C/ DEPT 135/ DEPT 90 NMR spectra (75.5 MHz, CDCl$_3$) of euphol
Fig. 12.9: $^1$H NMR spectrum (300 MHz, CDCl$_3$) of euphol
Fig. 12.10: HMBC spectrum (H/C: 300/75.5 MHz, CDCl₃) of euphol
Table 12.3: $^1$H (300 MHz, CDCl$_3$) and $^{13}$C (75.5 MHz, CDCl$_3$) NMR spectral data of euphol

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H (δ ppm, J Hz, multiplicities)</th>
<th>$^{13}$C (δ ppm, multiplicities)</th>
<th>HMBC (C -&gt; H) correlations</th>
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<tr>
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<tr>
<td>8</td>
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<td>133.61 s</td>
<td>H-7, H$_3$-30</td>
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<tr>
<td>9</td>
<td>---</td>
<td>134.09 s</td>
<td>H$_3$-19</td>
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<td>37.31 s</td>
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<tr>
<td>23</td>
<td>5.11 (t; 7.03, 12.88) (1H)</td>
<td>125.24 d</td>
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<tr>
<td>24</td>
<td>---</td>
<td>130.76 s</td>
<td>H$_2$-26, H$_3$-27</td>
</tr>
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<td>25</td>
<td>1.62 (s) (3H)</td>
<td>17.61 g</td>
<td>H$_3$-27</td>
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<tr>
<td>26</td>
<td>1.70 (s) (3H)</td>
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<td>27</td>
<td>1.02 (s) (3H)</td>
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<td>H-5, H$_3$-29</td>
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<td>28</td>
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<td>29</td>
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<td>24.44 g</td>
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</table>

* Signal pattern is unclear due to overlapping

_b: Signal not assigned due to overlapping

c: Multiplicities determined by DEPT sequences
Fig 12.11: EI mass spectrum of euphol (characteristic fragments are marked)
Fig. 12.12: Postulated EI mass fragmentation pattern of euphol
The molecular formula of corollatadiol was determined to be $C_{31}H_{52}O_2$ ($m/z$ 456.4) from its $^{13}$C NMR and EIMS data. Comparison of the fragmentation pattern of the MS and the $^{13}$C NMR data (Table 12.4) suggested the present molecule to be structurally related to the previously isolated euphol. The IR spectrum presented the same transmission bands as euphol, but a much stronger absorption at 3430 cm$^{-1}$, indicating the presence of more than one hydroxyl group. The $^1$H NMR spectrum (Fig. 12.14) showed signals corresponding to a double bond and an extra methyl group. The signal at $\delta_H$ 4.94 indicated the presence of a terminal methylene group. HMBC correlations from the terminal methylene group to a methyl group ($\delta_C$ 14.11) allowed the assignment of positions C-26 and C-27. A signal found at $\delta_C$ 139.36 indicated the occurrence of a quaternary carbon nucleus next to a double bond and was therefore defined as C-25. HMBC correlations were seen between position C-25 and an additional methyl group ($\delta_H$ 4.94). This methyl group showed also long range correlations to C-23 and to a quaternary carbon at $\delta_C$ 82.85, leading to
the structure fragment C-23 to C-26/C-27. The chemical shift value of C-24 ($\delta_c$ 82.85) indicated the presence of a neighbored hydroxyl group. Comparison of the $^{13}$C NMR data with those obtained for euphol, showed conformity regarding the skeleton. Characteristic signals were found for the five methyl groups located in the skeleton ($C_3$-18 15.52, $C_3$-19 20.13, $C_3$-28 28.04, $C_3$-29 15.65, $C_3$-30 24.34) as well as the signals at $\delta_c$ 78.97 (C-3) and $\delta_c$ 133.49 and 134.36 ($\Delta^8,9$ double bond). The structure was identified as corollatadiol (24-methyl-eupha-8,25-dien-3ß,24-diol). The $^1$H and $^{13}$C data (see Table 12.4) as well as the MS data were identical to those reported in literature (Piatak and Reimann 1972). This compound has been isolated before from Euphorbia corollata (Piatak and Reimann 1972).
Table 12.4: $^1$H (300 MHz, CDCl$_3$) and $^{13}$C (75.5 MHz, CDCl$_3$) NMR spectral data of corollatadiol

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H (δ ppm, J Hz, multiplicities)</th>
<th>$^{13}$C (δ ppm, multiplicities$^c$)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.22-1.23 (m') (2H)</td>
<td>35.24 t</td>
</tr>
<tr>
<td>2</td>
<td>1.66 (m') (2H)</td>
<td>27.91 t</td>
</tr>
<tr>
<td>3</td>
<td>3.25 (m) (1H)</td>
<td>078.97 d</td>
</tr>
<tr>
<td>4</td>
<td>--</td>
<td>38.92 s</td>
</tr>
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</tr>
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<td>8</td>
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<td>133.49 s</td>
</tr>
<tr>
<td>9</td>
<td>--</td>
<td>134.36 s</td>
</tr>
<tr>
<td>10</td>
<td>--</td>
<td>38.45 s</td>
</tr>
<tr>
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<td>1.91-1.94 (m') (2H)</td>
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</tr>
<tr>
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<td>30.95 t</td>
</tr>
<tr>
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<td>--</td>
<td>44.10 s</td>
</tr>
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<td>--</td>
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</tr>
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</tr>
<tr>
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<td>18</td>
<td>0.81 (s) (3H)</td>
<td>15.52 q</td>
</tr>
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<td>19</td>
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<td>36.09 d</td>
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<td>b</td>
<td>18.76 q</td>
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<td>b</td>
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</tr>
<tr>
<td>23</td>
<td>0.85 (m) (2H)</td>
<td>22.68 t</td>
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<tr>
<td>24</td>
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<td>82.85 s</td>
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<tr>
<td>25</td>
<td>--</td>
<td>139.36 s</td>
</tr>
<tr>
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<td>14.11 q</td>
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<td>28.04 q</td>
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<td>0.86 (s) (3H)</td>
<td>15.65 q</td>
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<td>30</td>
<td>0.89 (s) (3H)</td>
<td>24.44 q</td>
</tr>
<tr>
<td>31</td>
<td>1.34 (s) (3H)</td>
<td>24.34 q</td>
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</tbody>
</table>

*: Signal pattern is unclear due to overlapping
b: Signal not assigned due to overlapping
c: Multiplicities determined by DEPT sequences
Fig. 12.14: $^1$H NMR spectrum (300MHz, CDCl$_3$) of corollatadiol
Compounds 1 and 2 were isolated as an unseparable mixture giving a sharp peak in the HPLC and one spot in the TLC and thus first thought to be a single substance. The molecular formula $\text{C}_{30}\text{H}_{50}\text{O}_2$ ($m/z$ 442.4) was deduced from the $^{13}\text{C}$ NMR data and the EIMS. The mass spectrum (Fig. 12.17) showed again a similar fragmentation pattern as euphol, indicating the presence of a tetracyclic triterpenoid. Comparison of the obtained $^1\text{H}$ and $^{13}\text{C}$ NMR data confirmed the ring system, substituted with a hydroxyl group at position C-3, a double bond between C-8 and C-9, five methyl groups and an additional side chain (Tables 12.5 and 12.6). The remaining signals were ascribed to the side chain, suggesting the presence of an exocyclic double bond ($\delta_c$ 130.84, 134.35) within the side chain, a quaternary carbon substituted with a hydroxyl group ($\delta_c$ 82.29), a carbon doublet substituted with a hydroxyl group ($\delta_c$ 90.13), and a terminal methylene group ($\delta_c$ 114.26). The euphane/tirucallane-type skeleton was confirmed by recording two-dimensional correlation spectra, especially HMBC and DQF-COSY. Comparison of the $^1\text{H}$ integrals in the $^1\text{H}$ NMR spectrum demonstrated that not all of the signals attributed to the side chain, belonged to the same structure. The number of C atoms found also indicated
the presence of more than one substance. It was thus postulated, that the isolated compound was not a single compound, but a mixture of two different compounds with the same skeleton and closely related side chains.

An inverse-gated $^{13}$C NMR experiment (Fig. 12.20) was performed in order to determine which signals belonged to one structure and which to the other one. Evaluation of the $^{13}$C integrals allowed a good assignment of the signals to the corresponding side chain structures, since all $^{13}$C signals which belong to a structure showed about the same height and the two compounds were mixed in a 3:4 ratio.

Structure 1 was attributed the exocyclic double-bond and the quaternary carbon substituted with a hydroxyl group. Final structure elucidation was done by interpretation of two-dimensional NMR correlations, especially HMBC (Fig. 20.21). Correlations between C-25 and H-23, C-22 and H-24, as well as C-24 to $\text{H}_3$-26/27 allowed the correct assignment of the double bond. The prominent methyl signal at $\delta$H 1.35 (6H, s) indicated the presence of two similar tertiary methyl groups. HMBC correlations between both methyl groups and correlations between these methyl groups and the signal corresponding to C-25 allowed the determination of the side chain fragments from C-23 to C-26/27. The rest of the side chain could be elucidated by interpretation of the correlations resulting from the DQF-COSY (Fig. 12.22), HMQC and HMBC experiments.

The inverse-gated $^{13}$C NMR also allowed the attribution of the signals belonging to the side chain of structure 2, the hydrogen bearing carbon doublet ($\delta_c$ 90.13), the terminal methylene group ($\delta_c$ 114.26), a quaternary carbon in a double bond ($\delta_c$ 145.92), two methylene signals ($\delta_c$ 27.29; $\delta_c$ 30.81) and a methyl group ($\delta_c$ 15.63). HMBC correlations between C-24 and H$_2$-27, as well as the HMQC experiment allowed the correct assignment of structure 2.

Relative stereochemistry was determined by means of t-ROESY. ROes were observed between H$_3$-19 and H-17, H-20 and H$_3$-30, H$_3$-29 and H$_3$-19, as well as H$_3$-19 and H$_3$-30, indicating them to be all on the same side ($\beta$), while ROe
interactions between H₃-18 and H₃-21 revealed them to be on the opposite side (α).

The IR spectrum (Fig. 12.23) showed similar transmission bands as corollatadiol, with bands at 3391, 1376, 1216 and 1024 cm⁻¹ confirming the presence of hydroxyl groups and bands at 2932 and 1456 cm⁻¹ pointing to the presence of methyl groups. A strong transmission band at 757 cm⁻¹ indicated the presence of double bonds.

The two structures were established as eupha-8,23-dien-3β-25-diol (1) and eupha-8,25-dien-3β-24-diol (2), compounds that have not been isolated before.
Table 12.5: $^1$H (300 MHz, CDCl$_3$) and $^{13}$C (75.5 MHz, CDCl$_3$) NMR spectral data of eupha-8,23-dien-3β-25-diol (1)

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H (δ ppm, J Hz, multiplicities)</th>
<th>$^{13}$C (δ ppm, multiplicities$^b$)</th>
<th>HMBC (C→H) correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.20-1.22 (m*), 1.77-1.79 (m*) (2H)</td>
<td>35.24 (t)</td>
<td>H$_2$-2, H$_3$-19</td>
</tr>
<tr>
<td>2</td>
<td>1.39-1.42 (m*)/1.75-1.82 (m*) (2H)</td>
<td>27.91 (t)</td>
<td>H$_2$-1</td>
</tr>
<tr>
<td>3</td>
<td>3.25 (dd; 4.6, 11.4) (1H)</td>
<td>78.99 (d)</td>
<td>H$_3$-28, H$_3$-29</td>
</tr>
<tr>
<td>4</td>
<td>---</td>
<td>38.93 (s)</td>
<td>H-5, H$_3$-28, H$_3$-29</td>
</tr>
<tr>
<td>5</td>
<td>1.13 (m*) (1H)</td>
<td>50.96 (d)</td>
<td>H$_3$-19, H$_2$-28, H$_3$-29</td>
</tr>
<tr>
<td>6</td>
<td>1.42-1.46 (m*)/1.69-1.71 (m*) (2H)</td>
<td>18.92 (t)</td>
<td>H-5</td>
</tr>
<tr>
<td>7</td>
<td>2.10 (m*) (2H)</td>
<td>27.66 (t)</td>
<td>H-5</td>
</tr>
<tr>
<td>8</td>
<td>---</td>
<td>133.49 (s)</td>
<td>H$_3$-30</td>
</tr>
<tr>
<td>9</td>
<td>---</td>
<td>134.03 (s)</td>
<td>H$_3$-19</td>
</tr>
<tr>
<td>10</td>
<td>---</td>
<td>37.27 (s)</td>
<td>H-5, H$_3$-19</td>
</tr>
<tr>
<td>11</td>
<td>1.91-1.99 (m*) (2H)</td>
<td>21.47 (t)</td>
<td>H-5, H$_3$-19</td>
</tr>
<tr>
<td>12</td>
<td>1.71 (m*) (2H)</td>
<td>30.96 (t)</td>
<td>H$_3$-18, H$_3$-30</td>
</tr>
<tr>
<td>13</td>
<td>---</td>
<td>44.16 (s)</td>
<td>H$_3$-18, H$_3$-30</td>
</tr>
<tr>
<td>14</td>
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<td>50.01 (s)</td>
<td>H$_3$-18, H$_3$-30</td>
</tr>
<tr>
<td>15</td>
<td>1.27 (m*) (2H)</td>
<td>29.73 (t)</td>
<td>H$_3$-18, H$_3$-21</td>
</tr>
<tr>
<td>16</td>
<td>1.39-1.42 (m*)/1.75-1.82 (m*) (2H)</td>
<td>27.91 (t)</td>
<td>H$_3$-18, H$_3$-21</td>
</tr>
<tr>
<td>17</td>
<td>1.58 (m*) (1H)</td>
<td>49.60 (d)</td>
<td>H$_3$-18, H$_3$-21</td>
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<tr>
<td>18</td>
<td>0.80 (s) (3H)</td>
<td>15.52 (q)</td>
<td>H$_3$-18, H$_3$-21</td>
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<td>19</td>
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<td>20.14 (q)</td>
<td>H$_2$-1</td>
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<td>36.09 (d)</td>
<td>H$_3$-21</td>
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<td>19.13 (q)</td>
<td>H$_2$-22</td>
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<td>130.84 (d)</td>
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<td>134.35 (d)</td>
<td>H$_3$-22, H$_3$-26, H$_3$-27</td>
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<tr>
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<td>82.29 (s)</td>
<td>H-23, H$_3$-26, H$_3$-27</td>
</tr>
<tr>
<td>26</td>
<td>1.35 (s) (3H)</td>
<td>24.35 (q)</td>
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<td>27</td>
<td>1.35 (s) (3H)</td>
<td>24.35 (q)</td>
<td>H$_3$-27</td>
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<tr>
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<td>28.04 (q)</td>
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</tr>
<tr>
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<td>15.78 (q)</td>
<td>H-3, H-5, H$_3$-28</td>
</tr>
<tr>
<td>30</td>
<td>0.89 (s) (3H)</td>
<td>24.44 (q)</td>
<td>H-3, H-5, H$_3$-28</td>
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</table>

$^a$: Signal pattern is unclear due to overlapping

$^b$: Multiplicities determined by DEPT sequences
Table 12.6: $^1$H (300 MHz, CDCl$_3$) and $^{13}$C (75.5 MHz, CDCl$_3$) NMR spectral data of eupha-8,25-dien-3ß-24-diol (2)

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H (δ ppm, J Hz multiplicities)</th>
<th>$^{13}$C (δ ppm, multiplicities$^b$)</th>
<th>HMBC (C -&gt; H) correlations</th>
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</thead>
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<td>1</td>
<td>1.20-1.22 (m*)/1.77-1.79 (m*) (2H)</td>
<td>35.24 t</td>
<td>H$_2$-2, H$_2$-19</td>
</tr>
<tr>
<td>2</td>
<td>1.39-1.42 (m*)/1.75-1.82 (m*) (2H)</td>
<td>27.91 t</td>
<td>H$_3$-1</td>
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<tr>
<td>3</td>
<td>3.25 (dd; 4.6, 11.4) (1H)</td>
<td>78.99 d</td>
<td>H$_2$-28, H$_3$-29</td>
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<tr>
<td>4</td>
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<td>38.93 s</td>
<td>H$_5$, H$_3$-28, H$_3$-29</td>
</tr>
<tr>
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<td>50.96 d</td>
<td>H$_2$-19, H$_3$-28, H$_3$-29</td>
</tr>
<tr>
<td>6</td>
<td>1.42-1.46 (m*)/1.69-1.71 (m*) (2H)</td>
<td>18.92 t</td>
<td>H$_5$</td>
</tr>
<tr>
<td>7</td>
<td>2.10 (m) (2H)</td>
<td>27.66 t</td>
<td>H$_3$-30</td>
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<tr>
<td>8</td>
<td>---</td>
<td>133.49 s</td>
<td>H$_3$-19</td>
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<td>37.27 s</td>
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<td>1.91-1.99 (m*) (2H)</td>
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<td>49.60 d</td>
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<td>0.85 (m*) (3H)</td>
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<td>15.78 q</td>
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<tr>
<td>30</td>
<td>0.89 (s) (3H)</td>
<td>24.44 q</td>
<td></td>
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</table>

$^a$: Signal pattern is unclear due to overlapping

$^b$: Multiplicities determined by DEPT sequences
Fig. 12.17: El mass spectrum of eupha-8,23-dien-3β-25-diol (1) / eupha-8,25-dien-3β-24-diol (2)
Fig. 12.18: $^1$H NMR spectrum (300MHz, CDCl$_3$) of eupa-8,23-dien-3β,25-diol (1) / eupa-8,25-dien-3β,24-diol (2)
Fig. 12.19: 13C NMR spectrum (75.5 MHz, CDCl₃) of eupha-8,23-dien-3ß-25-diol (1) / eupha-8,25-dien-3ß-24-diol (2)
Fig. 12.20: Inverse-gated $^{13}$C NMR spectrum (75.5 MHz, CDCl$_3$) of eupha-8,23-dien-3ß-25-diol (1) / eupha-8,25-dien-3ß-24-diol (2)
Fig. 12.21: HMBC spectrum (H/C: 300/75.5 MHz, CDCl₃) of eupha-8,23-dien-3ß-25-diol (1) and eupha-8,23-
dien-3ß-24-diol (2)
Fig. 12.22: ¹H-¹H DQF-COSY spectrum (300MHz, CDCl₃) of eupha-8,23-dien-3β,25-diol (1) and eupha-8,25-dien-3β-24-diol (2)
Fig. 12.23: IR spectrum of eupha-8,23-dien-3β,25-diol (1) and eupha-8,25-dien-3β,24-diol (2)
12.4.4 α-Tocopherolquinone

The compound was obtained as yellow oil. The molecular formula was determined as $C_{29}H_{50}O_3$ ($m/z$ 446.3) from the $^{13}$C-NMR data and EIMS measurements. The IR spectrum (Fig. 12.25) showed bands at 3501 cm$^{-1}$, corresponding to a hydroxyl group and at 1643 cm$^{-1}$, pointing to an α,β-unsaturated ketone. Transmission bands appearing at 2927 and 2868 cm$^{-1}$ indicated the presence of methyl groups. The UV absorption spectrum showed a maximum at $\lambda_{\text{max}}$ 262 nm, which is typical for a quinone function (De Pascual et al. 1987). $^{13}$C-NMR resonances (Table 12.7, Fig. 12.26) found at $\delta_c$ 187.65, 140.17, 140.41, 187.23, 140.52, 144.42, all of them being singlets, pointed to a fully substituted benzoquinone. The absence of quinonoid proton signals in the $^1$H NMR (Fig. 12.27), together with the occurrence of three methyl singlets at $\delta_h$ 2.01, 2.01 and 2.04 confirmed a tetra-alkyl benzoquinone structure (Rasool et al. 1991). Further signals were observed for a quaternary carbon at $\delta_c$ 72.51, bearing a hydrogen group, five methyl groups $\delta_c$ 1.24, 0.85, 0.83, 0.87, 0.87 and eleven methylene groups. The specific assignment of most carbon resonances from the side chain appeared difficult at first glance because of their structural similarity. Two-dimensional NMR spectra, mainly HMBC, HSQC and DQF-COSY allowed the structure determination. The methylene group at $\delta_c$ 21.28 showed HMBC correlations (Fig. 12.28) to the methyl protons bonded to
the benzoquinone structure. This indicated the methylene group to be
neighbored to the benzoquinone moiety. DQF-COSY revealed correlations
between H2-1' and H2-2', indicating the corresponding carbon atoms to be
directly bonded. The hydroxyl bearing quaternary carbon C-3' showed long
range correlations to H2-1', H2-2', H2-4' and to a methyl group, leading to its
position between C-2' and C-4'. The methylene signals and the two doublets at
δc 32.75 respectively pointed to similar structure fragments occurring twice
within the molecule. HMBC long range correlations between both methyl groups
at C-15' revealed two similar methyl groups bond to the same carbon atom.
Long range HMBC correlations between C-15' and Me-15' confirmed that both
methylene groups were bound to the doublet C-15'.

The results indicated the present structure to be α-tocopherylquinone.
The EIMS showed bands at m/z 221, 205, 178, and 121 (Fig. 12.29),
characteristic for a benzoquinone moiety (Fig. 12.30) (Mahmood et al. 1984).
Comparison of the obtained 1H-NMR and 13C-NMR data with literature values
(Rasool et al. 1991) allowed the identification of α-tocopherolquinone. α-
Tocopherolquinone has been isolated from several plants (De Pascual et al.
1987); (Mahmood et al. 1984), but no description of any isolation from an
euphorbiaceous plant has been found.
Table 12.7: $^1$H (500MHz, CDCl$_3$) and $^{13}$C (125 MHz, CDCl$_3$) NMR spectral data of α-tocopherolquinone

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H (δ ppm, $^J$ Hz, multiplicities)</th>
<th>$^{13}$C (δ ppm, multiplicities)$^b$</th>
<th>HMBC (C→H) correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>187.65 $s$</td>
<td>$\text{H}_2-1'$, $\text{Me}-2$, $\text{Me}-3$</td>
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<td>140.17 $s$</td>
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<td>4</td>
<td>-</td>
<td>187.23 $s$</td>
<td>$\text{Me}-3$, $\text{Me}-5$</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>140.52 $s$</td>
<td>$\text{H}_2-1'$, $\text{Me}-5$</td>
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<tr>
<td>6</td>
<td>-</td>
<td>144.42 $s$</td>
<td>$\text{Me}-5$, $\text{H}_2-1'$, $\text{H}_2-2'$</td>
</tr>
<tr>
<td>1$'$</td>
<td>2.55 (m) (2H)</td>
<td>21.28 $t$</td>
<td>$\text{Me}-5$, $\text{H}_2-2'$, $\text{Me}-3'$</td>
</tr>
<tr>
<td>2$'$</td>
<td>1.50 (m) (2H)</td>
<td>40.17 $t$</td>
<td>$\text{H}_2-1'$, $\text{Me}-5$, $\text{H}_2-4'$, $\text{Me}-3'$</td>
</tr>
<tr>
<td>3$'$</td>
<td>-</td>
<td>72.51 $s$</td>
<td>$\text{Me}-2',\text{Me}-2'$, $\text{H}_2-4'$, $\text{Me}-3'$</td>
</tr>
<tr>
<td>4$'$</td>
<td>1.48 (m) (2H)</td>
<td>42.23 $t$</td>
<td>$\text{Me}-2'$, $\text{Me}-3'$</td>
</tr>
<tr>
<td>5$'$</td>
<td>1.27 (m) (2H)</td>
<td>21.38 $t$</td>
<td>$\text{H}_2-4'$</td>
</tr>
<tr>
<td>6$'$</td>
<td>1.07–1.13 (m*) (2H)</td>
<td>37.58 $t$</td>
<td>$\text{H}_2-4'$, $\text{H}_2-8'$, $\text{Me}-7'$</td>
</tr>
<tr>
<td>7$'$</td>
<td>1.34–1.35 (m*) (1H)</td>
<td>32.75 $d$</td>
<td>$\text{H}_2-8'$, $\text{H}_2-9'$, $\text{Me}-7'$</td>
</tr>
<tr>
<td>8$'$</td>
<td>1.07–1.09 (m*) (2H)</td>
<td>37.41 $t$</td>
<td>$\text{H}_2-9'$, $\text{H}_2-10'$, $\text{Me}-7'$</td>
</tr>
<tr>
<td>9$'$</td>
<td>1.27–1.28 (m*) (2H)</td>
<td>24.46 $t$</td>
<td>$\text{H}_2-10'$</td>
</tr>
<tr>
<td>10$'$</td>
<td>1.07–1.09 (m*) (2H)</td>
<td>37.41 $t$</td>
<td>$\text{H}_2-12'$, $\text{Me}-11'$</td>
</tr>
<tr>
<td>11$'$</td>
<td>1.34–1.35 (m*) (1H)</td>
<td>32.75 $d$</td>
<td>$\text{H}_2-9'$, $\text{H}_2-13'$, $\text{Me}-11'$</td>
</tr>
<tr>
<td>12$'$</td>
<td>1.07–1.09 (m*) (2H)</td>
<td>37.26 $t$</td>
<td>$\text{H}_2-10'$, $\text{H}_2-14'$, $\text{Me}-11'$</td>
</tr>
<tr>
<td>13$'$</td>
<td>1.15 (m) (2H)</td>
<td>24.76 $t$</td>
<td>$\text{H}-11'$, $\text{H}_2-12'$, $\text{H}-15'$</td>
</tr>
<tr>
<td>14$'$</td>
<td>1.14 (m) (2H)</td>
<td>39.34 $t$</td>
<td>$\text{H}-15'$, $\text{Me}-15'$</td>
</tr>
<tr>
<td>15$'$</td>
<td>1.51 (m) (1H)</td>
<td>27.94 $d$</td>
<td>$\text{Me}-15'$</td>
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<td>12.26$q$</td>
<td>$\text{Me}-3$</td>
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<td>2.01 (s) (3H)</td>
<td>12.33$q$</td>
<td>$\text{Me}-2$</td>
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<td>Me-5</td>
<td>2.04 (s) (3H)</td>
<td>11.93$q$</td>
<td>$\text{Me}-3'$</td>
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<tr>
<td>Me-3$'$</td>
<td>1.24 (s) (3H)</td>
<td>26.49 $q$</td>
<td>$\text{H}_2-2'$, $\text{H}_2-4'$</td>
</tr>
<tr>
<td>Me-7$'$</td>
<td>0.85 (s) (3H)</td>
<td>19.68$q$</td>
<td>$\text{H}-7'$, $\text{H}_2-6'$, $\text{H}_2-8'$</td>
</tr>
<tr>
<td>Me-11$'$</td>
<td>0.83 (s) (3H)</td>
<td>19.72$q$</td>
<td>$\text{H}-11'$, $\text{H}_2-10'$, $\text{H}_2-12'$</td>
</tr>
<tr>
<td>Me-15$'$</td>
<td>0.87 (d; 6.58) (3H)</td>
<td>22.68$q$</td>
<td>$\text{H}-15'$, $\text{H}_2-14'$, $\text{Me}-15'$</td>
</tr>
<tr>
<td>Me-15$'$</td>
<td>0.87 (d; 6.63) (3H)</td>
<td>22.59$q$</td>
<td>$\text{H}-15'$, $\text{H}_2-14'$, $\text{Me}-15'$</td>
</tr>
</tbody>
</table>

$^a$: Signal pattern is unclear due to overlapping

$^b$: Multiplicities determined by DEPT sequences

$^c$,$^d$: These assignments may be interchanged
Fig. 12.25: IR spectrum of α-tocopherolquinone
Fig. 12.26: DEPT 90/DEPT 135/13C NMR spectrum (75.5 MHz, CDCl₃) of α-tocopheroquinone
Fig. 12.28: Part of the HMBC spectrum (H/C: 500/125 MHz, CDCl₃) of α-tocopherolquinone.
Fig. 12.29: El mass spectrum of α-tocopherolquinone (corresponding fragmentation pattern see Fig.12.30)
Fig. 12.30: Characteristic mass fragments of α-tocopherolquinone (EIMS)
12.4.5  

**n-octacosanol**

<table>
<thead>
<tr>
<th>Octacosanol</th>
<th>CH₃-(CH₂)₂₇-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₂₉H₅₈O</td>
<td></td>
</tr>
<tr>
<td>MW: 410</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 12.31: Structure of n-octacosanol**

The $^{13}$C NMR data of *n*-octacosanol showed only one methyl group ($\delta_{C}$ 14.10), all other signals were methylene groups, one of them ($\delta_{C}$ 63.11) neighbored to a hydroxyl function. These findings indicated the presence of a fatty alcohol, which was confirmed by the EIMS. The resulting spectrum demonstrated fragmentation patterns typical for a long-chain alcohol of this type exhibiting a peak at 392 (M-H₂O) and masses differing by 14 mass units (-CH₂) and increasing in intensity toward the lower end of the spectrum. Two-dimensional NMR measurements, namely HMQC, HMBC and DQF-COSY allowed the correct assignment of the methylene groups. The length of the methylene chain was determined by means of the integrals resulting from the $^1$H NMR spectrum. Interpretation of these integrals indicated the presence of 25 methylene groups at $\delta_{H}$ 1.27, leading to the final formula CH₃-(CH₂)₂₇-OH.

The isolated fatty alcohol was identified as *n*-octacosanol, a substance that has been isolated from different plants, including several species of *Euphorbia*, e.g. *E. corollata* (Piatak and Reimann 1970) and *E. myrsinitis* (Aynehchi et al. 1972).
Table 12.8: $^1$H (300 MHz, CDCl$_3$) and $^{13}$C (75.5 MHz, CDCl$_3$) NMR spectral data of $n$-octacosanol

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H (δ ppm, J Hz, multiplicities)</th>
<th>$^{13}$C (δ ppm, multiplicities$^b$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.65 (t; 6.5, 13.3) (2H)</td>
<td>63.11 t</td>
</tr>
<tr>
<td>2</td>
<td>1.58 (m) (2H)</td>
<td>32.82 t</td>
</tr>
<tr>
<td>3</td>
<td>1.27 (m$^a$) (2H)</td>
<td>25.74 t</td>
</tr>
<tr>
<td>4-25</td>
<td>1.27 (m$^a$) (44H)</td>
<td>29.71 t</td>
</tr>
<tr>
<td>26</td>
<td>1.27 (m$^a$) (2H)</td>
<td>31.92 t</td>
</tr>
<tr>
<td>27</td>
<td>1.27 (m$^a$) (2H)</td>
<td>22.68 t</td>
</tr>
<tr>
<td>28</td>
<td>0.89 (t; 6.4, 13.3) (3H)</td>
<td>14.10 q</td>
</tr>
</tbody>
</table>

$^a$: Signal pattern is unclear due to overlapping

$^b$: Multiplicities determined by DEPT sequences
Fig. 12.32: $^1$H NMR spectrum (300 MHz, CDCl₃) and $^{13}$C NMR spectrum (75.5 MHz, CDCl₃) of octacosanol
Fig. 12.33: El mass spectrum of \( n \)-octacosanol
12.4.6 Hints to the presence of ingenane esters

Ingenol esters are the most common irritants of the genus *Euphorbia*. They have been isolated as either monoesters (esterified at C-3, C-5 or C-29) or diesters (esterified at both C-3 and C-20) (Evans and Taylor 1983). In the present study no ingenane esters nor other phorbol type diterpenoids could be isolated, but hints to the presence of ingenane diterpenoids were found. A compound was isolated and its structure elucidated. The structure was determined as nonadeca-3,5-dien-2-ol-1-yl. It exhibited biological activities against bacteria and KB cells and its mass spectrum showed fragmentation patterns typical for ingenane esters. It was thus postulated that the original ingenane ester had decomposed and that the side chain had been isolated.

12.4.6.1 Structure elucidation of nonadeca-3,5-dien-2-ol-1-yl

In the $^{13}$C NMR spectrum (Fig. 12.35) of the isolated compound 19 carbon signals could be clearly assigned, one of them belonging to a methyl group ($\delta_c$ 14.39), one doublet bearing a hydroxyl group ($\delta_c$ 73.38) and four signals indicating the presence of two double bonds ($\delta_c$ 132.94, 129.35, 126.53, 137.30). The rest of the carbon signals were methylene groups, indicating the presence of an unsaturated fatty alcohol. Structure elucidation was performed by NMR experiments, mainly DQF-COSY and HMBC. Combination of direct COSY correlations (Fig. 12.37) and long range HMBC correlations (Fig. 12.38) allowed the determination of the structure fragment C-1 to C-7. Due to
overlapping, complete assignment of $^1$H signals corresponding to methylene groups was not possible. The $^{13}$C NMR spectrum presented twelve methylene signals, suggesting a molecular formula of C_{19}H_{35}O with a molecular weight of 279. The EI mass spectrum showed a prominent peak at $m/z$ 278 [M-R]$^+$ and fragmentation patterns typical for long chain methylene groups (splitting off of $m/z$ 14 corresponding to a methylene group).

Table 12.9: $^1$H (300 MHz, MeOD-d$_4$) and $^{13}$C (75.5 MHz, MeOD-d$_4$) NMR spectral data of nonadeca-3,5-dien-2-ol-1-yl

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H (δ ppm, J Hz, multiplicities)</th>
<th>$^{13}$C (δ ppm, multiplicities$^b$)</th>
<th>HMBC (C $\rightarrow$ H) correlations</th>
<th>DQF-COSY (H$\rightarrow$H) correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.50 (d; 6.1) (2H)</td>
<td>38.43 t</td>
<td>H-4, H-3</td>
<td>H-2</td>
</tr>
<tr>
<td>2</td>
<td>4.07 (d; 6.5) (1H)</td>
<td>73.38 d</td>
<td>H-5, H-2</td>
<td>H-3, H$_2$-1</td>
</tr>
<tr>
<td>3</td>
<td>5.61 (dd; 6.6, 15.0) (1H)</td>
<td>137.30 d</td>
<td>H-6, H-5, H-2</td>
<td>H-4, H-2</td>
</tr>
<tr>
<td>4</td>
<td>6.49 (m) (1H)</td>
<td>126.53 d</td>
<td>H-4, H-3, H$_2$-7</td>
<td>H-4, H$_2$-7</td>
</tr>
<tr>
<td>5</td>
<td>5.98 (d; 6.52) (1H)</td>
<td>129.35 d</td>
<td>H-6, H-4</td>
<td>H-5, H$_2$-7</td>
</tr>
<tr>
<td>6</td>
<td>5.41 (m') (1H)</td>
<td>132.94 d</td>
<td>H-5, H$_2$-7</td>
<td>H-6</td>
</tr>
<tr>
<td>7</td>
<td>2.19 (d; 6.9) (2H)</td>
<td>28.59 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.34 (m') (2H)</td>
<td>23.59 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.34 (m') (2H)</td>
<td>30.71 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.34 (m') (1H)</td>
<td>30.55 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.34 (m') (2H)</td>
<td>30.42 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.34 (m') (2H)</td>
<td>30.29 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.34 (m') (2H)</td>
<td>30.12 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.34 (m') (2H)</td>
<td>26.54 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.34 (m') (2H)</td>
<td>26.53 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1.34 (m') (2H)</td>
<td>26.29 t</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.34 (m') (2H)</td>
<td>32.56 t</td>
<td></td>
<td>H$_{-}$-19</td>
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<tr>
<td>18</td>
<td>1.34 (m') (2H)</td>
<td>23.70 t</td>
<td></td>
<td>H$_{-}$-19</td>
</tr>
<tr>
<td>19</td>
<td>0.91 (s) (3H)</td>
<td>14.39 q</td>
<td></td>
<td>H$_{-}$-18</td>
</tr>
</tbody>
</table>

$^a$: Signal pattern is unclear due to overlapping

$^b$: Multiplicities determined by DEPT sequences

The isolated compound showed activity against bacteria and KB cells. It was assumed that it was a side chain, split off from an ingenane ester. No additional NMR signals indicating the presence of an ingenane skeleton were found.

Literature (Evans and Taylor 1983) revealed that diterpenes of the ingenane group show typical fragmentation patterns. The ingenane skeleton manifests at
$m/z$ 294 ($C_{20}H_{22}O_{2}$) as an intense fragment ion. The base peak which occurs at $m/z$ 121 has the composition $C_8H_9O$, and differentiates ingenols from the phorbols. The mass spectrum showed supplementary fragmentation peaks, present at a low amount, including fragment ions at $m/z$ 294 and 121.

The IR spectrum of ingenol esters shows characteristic absorbances for the tertiary OH group at C-4 (3420-3430 cm$^{-1}$), ester functions (1740-1750 cm$^{-1}$), the $\beta,\gamma$-unsaturated ketone at C-9 (1705-1715 cm$^{-1}$), and the olefinic region (1640 cm$^{-1}$).

The IR spectrum of the isolated compound showed additional bands that could not be attributed to the isolated side chain structure. Transmission bands were found at 3436 cm$^{-1}$ (OH groups), at 2930 cm$^{-1}$ and 2857 cm$^{-1}$ (methyl groups), 1714 cm$^{-1}$ (unsaturated ketone). At 1740-1750 cm$^{-1}$ transmission bands were not found, indicating that no ester functions were present.

The findings of the mass spectrum and the IR spectrum together with biological activity gave rise to the assumption, that the isolated structure was a side chain from a ingenane type diterpenoid. The diterpene skeleton was probably split off and degradated during extraction and/or isolation. The NMR spectra revealed only the presence of the isolated side chain. No additional signals, indicating the presence of an ingenane skeleton were found. The more sensitive mass spectrum revealed traces of a possible ingenane skeleton.

It is known that ingenane esters, like other diterpene esters of the phorbol type are unstable and easily decompose under heat, light, oxygen, acid and alkaline conditions (Evans and Soper 1978). It is thus very probable, that ingenane esters were originally present in the plant and decomposed during drying of the leaves, extraction or isolation procedures.
Fig. 12.35: $^{13}$C NMR spectrum (75.5 MHz, MeOH-d$_4$) of nonadeca-3,5-dien-2-ol-1-yl
Fig. 12.36: $^1$H NMR spectrum (300MHz, MeOH-d$_4$) of nonadeca-3,5-dien-2-ol-1-yl
Fig. 12.37: Part of the DQF-COSY (1H/1H, 300MHz, MeOH-d$_4$) of nonadeca-3,5-dien-2-ol-1-yl
Fig. 12.38: HMBC spectrum (H/C; 300/75.5 MHz; CDCl₃) of nonadeca-3,5-dien-2-ol-1-yl
The isolated compound showed a strong yellow-orange fluorescent spot on TLC plate after spraying with 2-aminoethyl diphenylborinate, suggesting the presence of a kaempferol derivative. The UV spectrum (MeOH) showed major absorptions at \( \lambda \) 263, 298 (sh) and 352 nm indicating it to be a flavonol derivative. In the IR spectrum bands at 3370 and 1360 cm\(^{-1}\) pointed to the presence of hydroxyl groups (Fig. 12.40). The bands found at 1732 and 1658 cm\(^{-1}\) were attributed to the flavonol carbonylic function in the flavonol structure and to a supplementary carbonylic group. Identification of the structure was based mainly on NMR studies. The \(^1\)H NMR spectrum (Fig. 12.41) showed six aromatic proton signals between 6 and 8 ppm. The signals for ring A indicated a 5,7 substitution, showing chemical shifts of \( \delta_h \) 6.17 (\( d, J = 2.07 \text{ Hz, H-6} \) and \( \delta_h \) 6.37 (\( d, J = 2.08 \text{ Hz, H-8} \)), as well as HMBC correlations between C-6 and H-8 and C-8 and H-6. The other four aromatic proton signals were attributed to ring C. Multiplicities and shift values pointed to the presence of a kaempferol aglycone moiety. The anomeric proton showed a coupling constant of \( J = 7.53 \text{ Hz} \) \( \delta_h \) 5.30, \( d \), revealing the sugar to be \( \beta \)-linked. \(^{13}\)C-NMR data (Fig. 12.42, Table 12.10), in combination with long range HMBC correlations revealed the sugar moiety to be glucuronic acid.
The ESI mass spectrum revealed a molecular ion \([M - H]^-\) at \(m/z\) 461, confirming the presence of kaempferol with glucuronic acid as sugar moiety. Comparison of the \(^1H\) and \(^{13}C\) NMR data with literature values (Imperato 1996) confirmed the isolated structure to be kaempferol-3-\(\beta\)-D-glucuronide.

**Table 12.10:** \(^1H\) (300MHz, MeOD-d\(_4\)) and \(^{13}C\) NMR (75.5 MHz, MeOD-d\(_4\)) spectral data of kaempferol-3-\(\beta\)-D-glucuronide

<table>
<thead>
<tr>
<th>Carbon</th>
<th>(^1H) (δ ppm, J Hz, multiplicities)</th>
<th>(^{13}C) (δ ppm, multiplicities(^b))</th>
<th>HMBC (C-&gt; H) correlations</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>159.22 s</td>
<td>H-2', H-6'</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>135.34 s</td>
<td>H-1&quot;</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>179.28 s</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>163.04 s</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6.17 (d; 2.07) (1H)</td>
<td>99.95 d</td>
<td>H-8</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>166.03 s</td>
<td>H-6, H-8</td>
</tr>
<tr>
<td>8</td>
<td>6.37 (d; 2.08) (1H)</td>
<td>94.82 d</td>
<td>H-6</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
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</tr>
<tr>
<td>10</td>
<td>-</td>
<td>105.66 s</td>
<td>H-6, H-8</td>
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<tr>
<td>1'</td>
<td>-</td>
<td>122.51 s</td>
<td>H-3', H-5'</td>
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<td>H-6'</td>
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<tr>
<td>3'</td>
<td>6.82 (m) (1H)</td>
<td>116.10 d</td>
<td>H-5'</td>
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<tr>
<td>4'</td>
<td>-</td>
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<td>H-2', H-6'</td>
</tr>
<tr>
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<td>6.85 (m) (1H)</td>
<td>116.10 d</td>
<td>H-3'</td>
</tr>
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<td>8.01 (m) (1H)</td>
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<td>H-2'</td>
</tr>
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<td>1&quot;</td>
<td>5.30 (d; 7.53) (1H)</td>
<td>104.35 d</td>
<td>H-2&quot;</td>
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<tr>
<td>2&quot;</td>
<td>3.43-3.62 (m(a)) (1H)</td>
<td>75.44 d</td>
<td>H-4&quot;</td>
</tr>
<tr>
<td>3&quot;</td>
<td>3.43-3.62 (m(a)) (1H)</td>
<td>77.53 d</td>
<td>H-4&quot;</td>
</tr>
<tr>
<td>4&quot;</td>
<td>3.43-3.62 (m(a)) (1H)</td>
<td>72.85 d</td>
<td>H-2&quot;, H-3&quot;</td>
</tr>
<tr>
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<td>3.43-3.62 (m(a)) (1H)</td>
<td>77.06 d</td>
<td>H-3&quot;</td>
</tr>
<tr>
<td>6&quot;</td>
<td>-</td>
<td>171.92 s</td>
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</tr>
</tbody>
</table>

\(^a\): Signal pattern is unclear due to overlapping

\(^b\): Multiplicities determined by DEPT sequences
Fig. 12.40: IR spectrum of kaempferol-3-O-β-D-glucuronide
Fig. 12.41: $^1$H-NMR spectrum of kaempferol-3-O-$\beta$-D-glucuronide

Fig. 12.42: $^{13}$C/ DEPT135-NMR spectrum of kaempferol-3-O-$\beta$-D-glucuronide
12.4.8 Hyperoside

**Hyperoside** (Quercetin-3-O-β-D-galactoside)

C_{21}H_{20}O_{12}

MW: 464

![Structure of hyperoside](image)

Fig 12.43: Structure of hyperoside (quercetin-3-O-β-D-galactoside)

The compound precipitated from methanol as yellow crystals. The $^1$H NMR (Fig. 12.44) and $^{13}$C NMR spectra (Fig. 12.45) resembled the spectra of kaempferol-3-O-β-D-glucuronide, suggesting the presence of another flavonoid glycoside. The signals corresponding to ring C showed different shift values comparing to kaempferol-3-O-β-glucuronide. A missing $^1$H signal for H-3 together with the signal at $\delta_H 8.12$ (dd, 1H) pointed to a C-3', C-4' substitution of ring C. The singlets found in the $^{13}$C NMR spectrum at $\delta_C 146.58$ and $\delta_C 147.36$ confirmed ring C to be substituted at positions C-3' and C-4', indicating the aglycone moiety to be quercetin. The structure of quercetin for the aglycone moiety could be confirmed by interpreting the HMBC correlations and by comparison with values of the reference compound (El-Mousallamy 1998). The anomeric proton showed a coupling constant of $J = 7.27$ Hz ($\delta_H 6.03$, d), revealing the sugar to be β-linked. The remaining six carbon signals were attributed to the sugar moiety. Shift values and multiplicities obtained from the $^1$H NMR and $^{13}$C NMR spectra together with HMBC long range correlations pointed to galactose as sugar moiety. Comparison of the $^1$H NMR and $^{13}$C NMR data with data obtained by measuring the reference compound hyperoside allowed the identification of
the compound as quercetin-3-O-β-D-galactoside (hyperoside). DAD analytical HPLC had revealed the presence of hyperoside as a major compound present in the methanol extract of *Euphorbia buxoides*.

**Table 12.11:** $^1$H (300 MHz, pyridine-d$_5$) and $^{13}$C (75.5 MHz, pyridine-d$_5$) NMR spectral data of hyperoside (quercetin-3-O-β-D-galactoside)

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H (δ ppm, J Hz, multiplicities)</th>
<th>$^{13}$C (δ ppm, multiplicities$^b$)</th>
<th>HMBC (C→H) correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>157.91 s</td>
<td>H-5', H-6'</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>135.72 s</td>
<td>H-1''</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>178.92 s</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>162.54 s</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6.67 (d; 2.0) (1H)</td>
<td>99.75 d</td>
<td>H-6</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>165.80 s</td>
<td>H-8</td>
</tr>
<tr>
<td>8</td>
<td>6.62 (d; 2.0) (1H)</td>
<td>94.61 d</td>
<td>H-6, H-8</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>157.66 s</td>
<td>H-8</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>105.25 s</td>
<td>H-6, H-8</td>
</tr>
<tr>
<td>1'</td>
<td>-</td>
<td>122.44 s</td>
<td>H-2''</td>
</tr>
<tr>
<td>2'</td>
<td>8.40 (d; 2.0) (1H)</td>
<td>117.82 d</td>
<td>H-6'</td>
</tr>
<tr>
<td>3'</td>
<td>-</td>
<td>146.58 s</td>
<td>H-5'</td>
</tr>
<tr>
<td>4'</td>
<td>-</td>
<td>147.36 s</td>
<td>H-2'</td>
</tr>
<tr>
<td>5'</td>
<td>7.26</td>
<td>116.29 d</td>
<td></td>
</tr>
<tr>
<td>6'</td>
<td>8.12 (dd; 2.0, 8.4) (1H)</td>
<td>122.97 d</td>
<td>H-5'</td>
</tr>
<tr>
<td>1''</td>
<td>6.03 (d; 7.27) (1H)</td>
<td>105.52 d</td>
<td>H-5''</td>
</tr>
<tr>
<td>2''</td>
<td>4.74 – 4.80 (m') (1H)</td>
<td>73.32 d</td>
<td>H-2''</td>
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<tr>
<td>3''</td>
<td>4.28 (m') (1H)</td>
<td>75.37 d</td>
<td>H-4'', H-5''</td>
</tr>
<tr>
<td>4''</td>
<td>4.56 (m') (1H)</td>
<td>69.76 d</td>
<td>H-5''</td>
</tr>
<tr>
<td>5''</td>
<td>4.14 (m') (1H)</td>
<td>77.65 d</td>
<td>H-3'', H-6''</td>
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<tr>
<td>6''</td>
<td>4.35 – 4.37 (m') (2H)</td>
<td>61.88 t</td>
<td>H-5''</td>
</tr>
</tbody>
</table>

$^a$: Signal pattern is unclear due to overlapping

$^b$: Multiplicities determined by DEPT sequences

$^c$: Reference compound, Rotichrom® CHR
Fig. 12.44: \( ^1\)H NMR spectrum (300MHz, pyridine-d5) of hyperoside.
Fig. 12.45: DEPT 90/DEPT 135/13C NMR spectrum (75.5 MHz, pyridine-d$_5$) of hyperoside
Fig. 12.46: IR spectrum of hyperoside
12.5 Isolation of compounds from *Homalanthus nervosus*

12.5.1 Extraction

The isolation procedure, including sample amounts, separation techniques and mobile phases used is given in Figure 12.47.

1.5 kg of air-dried leaves of *Homalanthus nervosus* were powdered and mixed with quartz sand. The extraction was performed using a forced-flow percolation at ambient temperature with solvents of increasing polarity, namely petroleum ether, dichloromethane, ethyl acetate, methanol and methanol-water (4:1). Solvents were changed after 4 days of extraction, allowing maceration over night with the new solvent. The solvents were evaporated in vacuo, at temperatures not exceeding 40 °C.

12.5.2 Fractionation of the petroleum ether extract

The petroleum ether extract was subjected to VLC, using a stepwise solvent gradient (hexane -> ethyl acetate -> methanol), affording 12 fractions. Final purification of fraction 4 by means of normal-phase HPLC (hexane/ethyl acetate 4:1) yielded ß-stigmasterol.

12.5.3 Fractionation of the dichloromethane extract

Separation of the dichloromethane extract was performed using VLC with a stepwise solvent gradient (hexane -> ethyl acetate -> methanol) resulting in 16 fractions. Fraction 11 showed fluorescence on the TLC plate, both at UV λ 245 nm and 365 nm and was chosen for further separation. RP18-VLC separation was performed using a stepwise solvent gradient (water -> methanol).
Final purification by HPLC (hexane/ethyl acetate 8:2) led to the coumarin derivative scoparone.

12.5.4 DAD-HPLC analysis of the ethyl acetate extract

TLC studies performed with the ethyl acetate extract indicated the presence of flavonoids. A diluted sample of the ethyl acetate extract was subjected to DAD analytical RP18 HPLC, in order to determine whether the major compounds present corresponded to common flavonoids. The retention times and the corresponding UV spectra of the peaks were compared. Spiking the samples with the dissolved reference standards confirmed the findings that hyperoside and kaempferol-3-O-β-glucoside represented the major compounds in the ethyl acetate extract.

12.5.5 DAD-HPLC analysis of the methanol extract

A small portion of the methanol extract was partitioned against water and butanol. The resulting butanol phase, containing all the flavonoids, was diluted and subjected to analytical RP18 HPLC with DAD detection. Comparison of the retention times, the corresponding UV spectra and spiking the sample with reference compounds afforded again hyperoside and kaempferol-3-O-β-glucoside as major components.
Leaves of *Homalanthus nervosus*

1.5 kg

forced flow percolation at ambient temperature

PE -> DCM -> EtOAc -> MeOH -> MeOH / H2O (4:1)

---

**Petroleum ether extract**
32.6 g

VLC Silica gel
Hexane -> EtOAc -> MeOH

Fraction 4
368 mg

**Dichloromethane extract**
25.4 g

VLC Silica gel
Hexane -> EtOAc -> MeOH

Fraction 11
251 mg

HPLC Lichrosorb® Si60
Hexane/EtOAc 4:1

**Ethyl acetate extract**
33.1 g

VLC Silica gel
Hexane -> EtOAc -> MeOH

Analytical HPLC RP18 with DAD coupling
Isopropanol/THF:ACN:H2O

Fraction 11.6
69 mg

Hyperoside
Kaempferol-3-glucoside

Scopolamine
2.4 mg

**Methanol extract**
864.9 g

VLC RP18
H2O -> MeOH

**Methanol/water extract (4:1)**
54.7 g

liquid-liquid partition (10 g)

Water phase
Butanol phase

Hyperoside
Kaempferol-3-O-β-glucoside

---

Fig. 12.47: Isolation scheme of *Homalanthus nervosus*
12.6 Structure elucidation of the compounds from *Homalanthus nervosus*

12.6.1 Scoparone

![Structure of scoparone](image)

From the $^{13}$C NMR (Fig. 12.49) and the MS (Fig. 12.50) data a molecular formula of $C_{11}H_{10}O_4$ ($m/z$ 206.0) could be determined. Strong fluorescence detected by TLC at UV 254 nm and 365 nm indicated the presence of a coumarin derivative. The $^1$H NMR spectrum (Fig. 12.49) showed no signals up to 3.8 ppm, pointing to an unsaturated structure, without aliphatic methyl or methylene groups. Two strong singlets at $\delta_H$ 3.93 and 3.96 revealed the presence of two methoxy groups. The IR spectrum showed transmission bands at 1615, 1559, 1518, 1140 and 1006 cm$^{-1}$ indicating an aromatic structure. The band at 1728 cm$^{-1}$ was attributed to a carbonylic function. Bands at 2920 and 2851 cm$^{-1}$ were assigned to methyl groups. Comparison of the $^{13}$C and $^1$H NMR data (Table 12.12) with literature data (Reinecke et al. 1995) confirmed the structure of scoparone (6,7-dimethoxycoumarin). This compound has been reported from several natural products, including the euphorbiaceous plant *Jatropha curcas* (Talapatra and Mandal 1993).
Table 12.12: $^1$H (300 MHz, CDCl$_3$) and $^{13}$C (75.5 MHz, CDCl$_3$) NMR spectral data of scoparone

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H ($\delta$ ppm, J Hz, multiplicities)</th>
<th>$^{13}$C* ($\delta$ ppm, multiplicities$^b$)</th>
<th>$^1$H (DMSO) Lit. values$^b$</th>
<th>$^{13}$C (DMSO) Lit. values$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.30 (d; 9.5) (1H)</td>
<td>161.40 s</td>
<td>-</td>
<td>160.5 s</td>
</tr>
<tr>
<td>3</td>
<td>7.63 (d; 9.5) (1H)</td>
<td>113.58 d</td>
<td>6.30 (d; 9.5) (1H)</td>
<td>112.6 d</td>
</tr>
<tr>
<td>4</td>
<td>6.86 (s) (1H)</td>
<td>146.36 s</td>
<td>-</td>
<td>145.8 s</td>
</tr>
<tr>
<td>5</td>
<td>7.63 (d; 9.5) (1H)</td>
<td>107.98 d</td>
<td>7.21 (s) (1H)</td>
<td>108.8 d</td>
</tr>
<tr>
<td>6</td>
<td>6.86 (s) (1H)</td>
<td>152.87 s</td>
<td>-</td>
<td>152.5 s</td>
</tr>
<tr>
<td>7</td>
<td>7.63 (d; 9.5) (1H)</td>
<td>100.04 d</td>
<td>7.02 (s) (1H)</td>
<td>99.9 d</td>
</tr>
<tr>
<td>8</td>
<td>6.86 (s) (1H)</td>
<td>150.05 s</td>
<td>-</td>
<td>149.4 s</td>
</tr>
<tr>
<td>9</td>
<td>7.63 (d; 9.5) (1H)</td>
<td>111.44 s</td>
<td>-</td>
<td>111.1 s</td>
</tr>
<tr>
<td>10</td>
<td>6-OMe 3.93 (s) (3H)</td>
<td>56.38 q</td>
<td>3.83 (s) (3H)</td>
<td>55.8 q</td>
</tr>
<tr>
<td>7-OMe</td>
<td>7.63 (d; 9.5) (1H)</td>
<td>56.38 q</td>
<td>3.89 (s) (3H)</td>
<td>56.1 q</td>
</tr>
</tbody>
</table>

$^*$ Multiplicities determined by DEPT sequence

$^b$ Literature values (Reinecke et al. 1995)
Fig. 12.49: 1H NMR spectrum (300 MHz, CDCl₃) and 13C NMR spectrum (75.5 MHz, CDCl₃) of scoparone
The results of the present investigations demonstrated that *Euphorbia buxoides* accumulates different triterpenoids of the euphane type. Euphol was found to occur in a high amount, in accordance to the literature, where euphol is mentioned as major compound in other *Euphorbia* species (Dev and Nagasampagi 1989). Corollatadiol and $\beta$-octacosanol were previously found in *Euphorbia corollata* (Piatak and Reimann 1972). Two new euphane-type triterpenoids, eupha-8,23-dien-3\(\beta\)-25-diol and eupha-8,25-dien-3\(\beta\)-24-diol could be isolated. The isolated triterpenoids showed no biological activity.

Phorbol esters, present in many Euphorbiaceae, could not be isolated from the two investigated plant species. It is supposed that phorbol esters have decomposed and could therefore not be isolated. The isolation and identification of a biologically active nonadecadien-ol-yl moiety from *E. buxoides*, as a possible side chain of ingenol nonadecadienolyl, pointed to decomposure of the originally present diterpene esters. The isolation of phorbol esters is known from literature to be difficult (Evans and Taylor 1983). Many of the esterified forms of these diterpenes are unstable compounds and break down during isolation procedures. Hydrolysis or transesterifications are known to occur during chromatographic separations. Furthermore, the compounds are sensitive to heat, light, oxygen, acid and alkaline conditions (Evans and Taylor 1983). Another possible reason for the lack of phorbol esters is the fact, that phorbol esters occur mainly in the latex at very low doses (0.2-1% of the dry latex) (Neuwinger 1996).

A main reason for the choice of *E. buxoides* and *H. novoguineensis* for the present study was the activity found in the preliminary biological screening. However, the isolated structures showed almost no biological activity. It is known from literature, that in dealing with plant extracts, generally the activity of approximately 50% of the samples will reconfirm on recollection, and the activity of about 50% of the samples will be lost during the process of bioassay-directed
fractionation (Pezzuto 1997). Antimicrobial activity of the methanol extract of *E. buxoides* could clearly be attributed to polyphenolic compounds. Most bioassays are sensitive to interferences from plant metabolites such as tannins, etc. (Hamburger and Hostettmann 1991). The loss of biological activity during fractionation can be due to lack of chemical stability or to synergistic effects of different compounds.

The present study represents a contribution to the phytochemical investigation of plants from Papua New Guinea, focused on species belonging to the plant family Euphorbiaceae, namely *Euphorbia buxoides* and *Homalanthus nervosus*. 
14 Materials and methods

14.1 Extraction of plant material

14.1.1 Plant material


During March and April 1991 the following plant species were collected: *Euphorbia buxoides* (Euphorbiaceae) Southern Highlands province, *Homalanthus nervosus* (Euphorbiaceae) Southern Highlands province, *Homalanthus novoguineensis* (Euphorbiaceae) Morobe province and *Securinega melanthesoides* (Euphorbiaceae) Central province. *Scaevola oppositifolia* (Goodeniaceae) was collected 1992 in the Morobe province. Collected amounts ranged from 0.5 to 4 kg dry weight. Voucher specimens were deposited at the Rijksherbarium, University of Leiden (The Netherlands).

14.1.2 Preparation of extracts for biological screening

20 g of dried and powdered plant material were extracted three times by Polytron® with 90 ml of a mixture of DCM/MeOH (2:1) during 5 minutes. The solution was filtered and evaporated to dryness in vacuo at 30 °C to give the DCM/MeOH extract. The residual plant material was further extracted by
Polytron® with 90 ml of MeOH 70%. MeOH of the filtrates was evaporated in vacuo. The remaining aqueous extract was shaken out three times with water-saturated butanol. The butanol and water fractions were evaporated to dryness in vacuo at 30 °C to give the butanol and the water extracts.

For *Euphorbia buxoides*, *Homalanthus nervosus* and *Homalanthus novoguineensis* 20 g of dried and powdered plant material were extracted successively with hexane, dichloromethane and methanol (for *H. novoguineensis* also with ethyl acetate and a mixture of methanol/water 4:1) by percolation (1000 ml of each solvent) at ambient temperature. The solvents were removed in vacuo at 30 °C affording three, respectively five, extracts of increasing polarity, a hexane extract, a dichloromethane extract and a methanol extract (for *H. novoguineensis* additionally ethyl acetate and methanol/water extracts).

The resulting 41 extracts were subjected to biological screenings (see 12.2).

Mill: Culatti, Zürich, CH
Polytron®: Kinematica GmbH, Luzern, CH
Rotavapor: R 114, Büchi AG, Flawil, CH
Percolation column: 29 x 2.5 cm i.d. (internal diameter)

### 14.1.3 Extraction of *Euphorbia buxoides* leaves

Leaves of *Euphorbia buxoides* A.R. Smith were collected in 1991, near Mendi in the Southern Highlands province, Papua New Guinea. A voucher specimen has been deposited at the Rijksherbarium (ETH 91/16 2-04-91), University of Leiden. A quantity of 1.4 kg of dried leaves was powdered and continuously extracted with hexane, dichloromethane and methanol using percolation at ambient temperature.

Mill: Retsch, Schieritz & Hauenstein AG, Arlesheim, CH
Percolation column: 150 x 9 cm i.d.
14.1.4 Extraction of *Homalanthus nervosus* leaves

Leaves of *Homalanthus nervosus* J.J. Sm. were collected in 1991, near Mendi in the Southern Highlands province, Papua New Guinea. A voucher specimen has been deposited at the Rijksherbarium (ETH 91/14 A,B,C 1-04-91), University of Leiden.
A quantity of 1.5 kg of dried leaves was powdered and continuously extracted by forced-flow percolation at ambient temperature with petroleum ether, dichloro-methane, ethyl acetate, methanol and methanol/water (4:1).

Mill: Retsch, Schieritz & Hauenstein AG, Arlesheim, CH
Percolation column: 46 x 10 cm i.d., Büchi AG, Flawil, CH

14.2 Biological Assays

14.2.1 Bioassay for activity against *Candida albicans*

Test organism: *Candida albicans* H29 ATCC 26790

Cultures were stored at \(-20\,^\circ\text{C}\) as suspensions in skim milk. The suspensions were prepared by spreading out *Candida albicans* cultures onto a malt extract agar plate and incubating it over night at \(37\,^\circ\text{C}\). A 1% suspension of skim milk in sterile water was applied to a cryo tube. A sample of *Candida albicans* culture from the inoculated agar plate was given into the cryo tube and the suspension mixed. Aliquots of 30 \(\mu\text{l}\) were given into cryo tubes and subsequently deep-frozen at \(-20\,^\circ\text{C}\).

Overnight suspensions were prepared using Sabouraud liquid medium. Test tubes were filled with 5 ml of Sabouraud liquid medium and autoclaved for 20 minutes at \(121\,^\circ\text{C}\). After cooling, 25 \(\mu\text{l}\) of the thawed skim milk *Candida* suspension were added and the test tubes incubated over night at \(37\,^\circ\text{C}\).
Media:
- Skim milk powder, OXOID, Unipath LTD, Basingstoke, Hampshire, England
- Sabouraud liquid medium, OXOID, Unipath LTD, Basingstoke, Hampshire, England

Agar:
- Mueller-Hinton agar, OXOID, Unipath LTD, Basingstoke, Hampshire, England
- Malt extract agar, OXOID, Unipath LTD, Basingstoke, Hampshire, England

References:
- Miconazole: Sigma, Chemical co., St. Louis, USA
- Amphotericin B: Sigma, Chemical co., St. Louis, USA

Spraying reagents:
- MTT Fluka, Chemie AG, Buchs, CH
- INT Fluka, Chemie AG, Buchs, CH

Mixer:
Vortex, Inotech, Dottikon, CH

Autoclav:
Type 23, Melag, Cobaco AG, Fehraltdorf, CH

Incubator:
Salvis Thermocenter, Reussbühl, CH

Gilson pipetman:
Gilson Medical Electronics, Villiers-le-Bel, France

Micropipettes:
Treff AG, Degersheim, CH

Cryo tubes:
Nalge Nunc International, Roskilde, Denmark

Petri dishes:
9 cm i.d., Sarstedt, Sevelen, CH

Blank paper discs:
Unipath LTD, Basingstoke, Hampshire, England

TLC sheets:
Silica gel 60 F_{254}, aluminum sheets, Merck, Dietikon, CH
14.2.2 Paper disc assay for antifungal activity

Test organisms:  
*Penicillium oxalicum* CBS 219.30  
*Candida albicans* H29 ATCC 26790

Strains of *P. oxalicum* were maintained on solid, autoclaved slant agar cultures (39 g/l) at -20 °C. Conidia were transferred with a platinum loop to 5 ml of sterilized broth. The inoculated broth was mixed and spread out with the platinum loop onto a sterile agar plate containing 10 ml of solid agar. Paper filter discs containing 500 µg of plant extracts were placed on the inoculated agar. The agar plates were incubated for three days in a moist atmosphere at room temperature. Antifungal activity was determined by measuring the radius of the clear zones of inhibition surrounded by greenish colored fungus. As positive control 1 µg of miconazole was used.

Media:  
Potato dextrose agar, Merck AG, Dietikon, CH (slant agar)  
Czapek-Dox broth, Difco Laboratories, Detroit, USA (liquid medium)

Agar:  
Malt extract agar, OXOID, Unipath Ltd., Basingstoke, Hampshire, UK (solid agar)

Petri dishes:  
9 cm i.d., Sarstedt, Sevelen, CH

Blank paper discs:  
Unipath LTD, Basingstoke, Hampshire, UK

Miconazole:  
Sigma, St. Louis, USA

Overnight cultures of *Candida albicans* were prepared as described in 14.2.1. 50 µl of the overnight culture were mixed with 5 ml of sterilized agar and the suspension poured onto a sterile agar plate containing 10 ml of solid agar. 500 µg of plant extract were applied on paper filter discs. The paper discs were placed on the inoculated agar plates. The petri dishes were incubated at 37 °C over night. The zones of inhibition were determined by measuring the radius of the zone without yeast growth. For better visualization the agar plates were sprayed with a 0.25% aqueous solution of MTT and incubated for another 30
minutes. Zones of inhibition then could be determined as clear zones against the violet colored background. As positive control 1 μg of miconazole was used.

Media: Sabouraud liquid medium, OXOID, Unipath Ltd., Basingstoke, Hampshire, UK

Agar: Malt extract agar, OXOID, Unipath Ltd., Basingstoke, Hampshire, UK (solid agar)

Petri dishes: 9 cm i.d., Sarstedt, Sevelen, CH

Blank paper discs: Unipath LTD, Basingstoke, Hampshire, UK

Miconazole: Sigma, St. Louis, USA

Spraying reagent: MTT, Fluka, Chemie AG, Buchs, CH

Mixer: Vortex, Inotech, Dottikon, CH

Autoclav: Type 23, Melag, Cobaco AG, Fehraltdorf, CH

Incubator: Salvis Thermocenter, Reussbühl, CH

Gilson pipetman: Gilson Medical Electronics, Villiers-le-Bel, France

Micropipettes: Treff AG, Degersheim, CH

14.2.3 Paper disc assay for antibacterial activity

<table>
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<th>Test organism</th>
<th>ATCC</th>
</tr>
</thead>
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<td>Bacillus cereus</td>
<td>10702</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>6633</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>25922</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>9341</td>
</tr>
<tr>
<td>Mycobacterium fortuitum</td>
<td>from human material</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>27853</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>1228</td>
</tr>
</tbody>
</table>

Bacterial cultures were stored at −20 °C as suspensions in skim milk. Overnight cultures were prepared adding 25 μl of skim milk bacterial suspension to 5 ml of sterilized nutrient broth and incubating the suspension at 37 °C over night. For *M. fortuitum* 100 μl of skim milk suspension were used. 50 μl of overnight cultures were mixed with 5 ml of sterilized agar. The suspension was poured over a sterile agar plate containing 10 ml of solid agar. Paper filter discs
containing 500 µl of plant extract were placed on the inoculated agar plates. The petri dishes were incubated at 37 °C over night. For *M. fortuitum* the incubation time was prolonged to three days. The zones of inhibition were determined by measuring the radius of the zone without or with only diffuse bacterial growth. For better visualization the agar plates were sprayed with a 0.25% aqueous solution of MTT and incubated for 30 minutes. Zones of inhibition then could be determined as clear zones against the violet colored background. As positive control 2 µg of chloramphenicol and 2 µg of tetracycline hydrochloride were used.

**Nutrient broth:** BBL nutrient broth, Becton & Dickinson Co. Cockeysville, USA

**Agar:** Mueller-Hinton agar, OXOID, Unipath LTD, Basingstoke, Hampshire, England

**References:** Chloramphenicol, Siegfried AG, Zofingen, CH  
Tetracycline hydrochloride, Fluka Chemie AG, Buchs, CH

**Spraying reagent:** MTT, Fluka, Chemie AG, Buchs, CH

**Mixer:** Vortex, Inotech, Dottikon, CH

**Autoclav:** Typ 23, Melag, Cobaco AG, Fehraltdorf, CH

**Incubator:** Salvis Thermocenter, Reussbühl, CH

**Gilson pipetman:** Gilson Medical Electronics, Villiers-le-Bel, France

**Micropipettes:** Treff AG, Degersheim, CH

**Petri dishes:** 9 cm i.d., Sarstedt, Sevelen, CH

**Blank paper discs:** Unipath LTD, Basingstoke, Hampshire, England
14.2.4 Assay for molluscicidal activity

Test organism: *Biomphalaria glabrata*

Snails of the species *Biomphalaria glabrata* were bred in an aquarium with continuous water circulation. The test was performed following the rapid screening procedure described by Marston and Hostettmann (Marston and Hostettmann 1991) with slight modifications. Two snails of uniform size (shell diameter 8-10 mm) were placed in distilled water solutions of the plant extracts (with 0.4% EtOH and 0.2% polyethylene glycol as solubilizers), at a concentration of 100 ppm. After 24 hours the snails were placed on a petri dish and the heart beat of the snails was observed under the binocular microscope in order to determine whether the snails were dead or alive. An aqueous solution of CuSO₄ (100 μg/ml) was used as positive control. A negative control was performed using an aqueous solution of distilled water with the solubilizers.

Reference: CuSO₄, Fluka Chemie AG, Buchs, CH
Binocular microscope: Wild, Heerbrugg, CH

14.2.5 Brine shrimp toxicity assay

Test organism: Larvae of *Artemia salina*

Sample preparation:
Samples were prepared by dissolving 15 mg of the crude plant extracts in 200 μl DMSO (solubilizer) and adding artificial sea water (Artemia salt 32 g/l) up to 1 ml (solution A). 100 μl of solution A were diluted in artificial sea water up to 3 ml to give a final test concentration of 500 ppm.
Hatching the shrimps:
A modified assay was performed, based on the brine shrimp lethality assay proposed by McLaughlin (McLaughlin 1991). A glass tank (35 x 15 x 6 cm) was filled with artificial sea water and divided into two compartments by means of a perforated aluminum foil, in order to give one compartment illuminated by daylight, while the other compartment was darkened. Eggs of *Artemia salina* were sprinkled into the dark compartment and allowed to hatch and mature during two days. When hatched and matured the phototropic larvae (nauplii) moved through the perforated aluminum foil to the illuminated part of the glass tank.

Assay:
The hatched larvae were attracted with a lamp. 20-30 shrimps were transferred to each sample vial by pipette. The sample vials were controlled under the microscope, to assure that no dead shrimps or eggs were transferred. Podophyllotoxin was used as positive control at a dose level of 100 ppm. The negative control was done using the artificial sea water solution containing the solubilizer. After 24 hours at ambient temperature, the number of dead and alive shrimps was determined. Every sample, as well as the positive control and the negative control, were tested in quadruplicate. In the case of dead shrimps present in the negative control, percent values were corrected using the Abbott's formula (Abbott 1925):

\[
\frac{\text{\% Mortality}_{\text{Sample}} - \text{\% Mortality}_{\text{Control}}}{100 - \text{\% Mortality}_{\text{Control}}}
\]

Artemia salt: Artificial sea water, Dohse Aquaristik, Bonn, Germany
*Artemia salina* eggs: Dohse Aquaristik, Bonn, Germany
Binocular microscope: Wild, Heerbrugg, CH
Podophyllotoxin: Fluka Chemie AG, CH
14.2.6 Cytotoxicity assays

14.2.6.1 KB and CaCo2 cell toxicity assays

Test cell lines:  
<table>
<thead>
<tr>
<th></th>
<th>KB</th>
<th>ATCC CCL 17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CaCo2</td>
<td>ATCC HTB-37</td>
</tr>
</tbody>
</table>

Cytotoxicity of the plant extracts was assessed using two cell lines, the KB cell line, a human nasopharyngal carcinoma cell line, and the CaCo2 cell line, a human colon adenocarcinoma cell line. Both cell lines were prepared identically, except the use of different media for each cell line.

Preparation of cells:
For the KB cell assay the medium was prepared as follows: Eagle's Minimum Essential Medium (EMEM) was mixed with foetal bovine serum (FBS), non-essential amino acids (MEM) and L-glutamine. Penicillin, streptomycin, and amphotericin B were added as antimicrobial agents.
For the CaCo2 cell assay the medium was prepared as follows: Dulbecco's Modified Eagle Medium was mixed with foetal bovine serum (FBS) and non-essential amino acids. No antimicrobial agents were added.

Cell cultures were stored at −196 °C as cell suspensions in medium with DMSO and higher serum content as cryoprotectants. The thawed cells were transferred to a culture flask (75 cm²) containing 20 ml of medium and were allowed to grow. When about 80% of confluent cells had grown, the culture medium was aseptically decanted and the cell cultures were washed twice with phosphate buffered saline (PBS). After addition of 2 ml trypsin/EDTA (37 °C) to the cells and incubation (5% CO₂, 95% relative humidity, 37 °C), the cells detached. The cell suspension was drawn to 4 ml of medium in a glass tube and resuspended. The cells were separated by centrifugation. 10 ml of medium were added to the washed cells. After resuspension, 1 ml of cell suspension was poured to 25 ml
of medium in a culture flask (75 cm²). The cells attached to the surface of the culture flask within 24 hours (incubation in 5% CO₂, 95% relative humidity, 37 °C).

Preparation of samples:
2 mg of plant extracts were solved in 2 ml of an aqueous solution, using 10% DMSO as solubilizer. The solutions were filtrated through a 0.2 μm sterile/pyrogen-free filter. The sterile extract solutions (having a concentration of 1000 ppm) (solutions A) were used for both cytotoxicity assays. For the assays the solutions were diluted to give a final concentration of 50 ppm. The solubilizer DMSO was diluted to a final concentration of 0.5%.

Assay:
37.5 μl of solution A were added to 712.5 μl of cell suspension in tissue culture plates in order to obtain 750 μl suspension with 50 ppm of plant extract. Cell growing was observed after 72 hours under the microscope. Podophyllotoxin was used as positive control, at a dose level of 50 ppm. For the negative control, aqueous solutions containing the solubilizer were tested.

Media:

for KB cells: Eagle's Minimum Essential Medium (EMEM) with Earle's salts and sodium bicarbonate, Gibco BRL, Life Technologies Ltd., Basel, CH

for CaCo2 cells: Dulbecco's Modified Eagle Medium with GlutaMAX®, Gibco BRL, Life Technologies Ltd., Basel, CH

Serum: Foetal bovine serum (FBS), Gibco BRL, Life Technologies Ltd., Basel, CH

Amino acids: Non-essential amino acids (MEM) without L-glutamine, Gibco BRL, Life Technologies Ltd., Basel, CH
<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsine/EDTA</td>
<td>Mixture of 0.05% trypsin and 0.02% EDTA in salt solution, Gibco BRL, Life Technologies Ltd., Basel, CH</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>200 mM, Gibco BRL, Life Technologies Ltd., Basel, CH</td>
</tr>
<tr>
<td>Streptomycin/Penicillin</td>
<td>10,000 IU/ml / 10,000 µg/ml, Gibco BRL, Life Technologies Ltd., Basel, CH</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium, Gibco BRL, Life Technologies Ltd., Basel, CH</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Gibco BRL, Life Technologies Ltd., Basel, CH</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>Fluka Chemie AG, Buchs, CH</td>
</tr>
<tr>
<td>Sterile syringes</td>
<td>Becton &amp; Dickinson Co., Heidelberg, Germany</td>
</tr>
<tr>
<td>Disposable filter holders</td>
<td>Schleicher &amp; Schüll, Riehen, CH</td>
</tr>
<tr>
<td>Cell culture flasks</td>
<td>75 cm², TPP, Winiger AG, Wohlen, CH</td>
</tr>
<tr>
<td>Multiwell plates</td>
<td>24-multiwell tissue culture treated plates, Becton &amp; Dickinson Co., New Jersey, USA</td>
</tr>
<tr>
<td>Microscope</td>
<td>Leitz Fluover FU, Leica AG, Glattbrugg, CH</td>
</tr>
</tbody>
</table>
14.3 Separation methods

14.3.1 Liquid-liquid partition

System A: (see 12.3.2.)
Two-phase system: hexane (750 ml) and methanol/water 4:1 (750 ml)
Separation funnel: 2 l
Sample amount: 69 g

System B: (see 12.3.2.)
Two-phase system: water (250 ml) and saturated diethyl ether (250 ml)
Separation funnel: 1 l

System C: (see 12.3.2.)
Two-phase system: 1% Na₂CO₃ (250 ml) and diethyl ether (250 ml)
Separation funnel: 1 l

System D: (D1: see 12.3.4.; D2: 12.5.5.)
Two-phase system: water-saturated butanol (D1: 750 ml; D2: 150 ml)
and water (D1: 750 ml; D2: 150 ml)
Separation funnel: D1: 2 l; D2: 500 ml
Sample amount: D1: 226 g; D2: 10 g

14.3.2 Vacuum liquid chromatography (VLC)

VLC columns were dry-packed. After packing vacuum was applied and the column was conditioned with the first solvent used for VLC separation. For normal-phase VLC extracts or fractions were applied on inert Celite® as preabsorbent and drawn onto the packed VLC column under vacuum. For reversed-phase VLC, fractions were applied on the VLC column in solved form.
Columns: 200 x 70 mm i.d. (see 12.3.2; 12.3.3; 12.5.2; 12.5.3)
200 x 40 mm i.d. (see 12.3.2)
200 x 30 mm i.d. (see 12.3.4; 12.5.3)
100 x 20 mm i.d. (see 12.3.3; 12.3.4; 12.5.3)

**Normal-phase VLC:**

Stationary phase: Silica gel CC 60, particle size 40 – 60 μm, Chromagel, Chemie Brunschwig AG, Basel, CH

Mobile phases: Hexane -> EtOAc step gradient (see 12.3.2)
Hexane -> DCM -> acetone step gradient (see 12.3.3)
DCM -> EtOAc -> MeOH step gradient (see 12.3.4)
Hexane -> EtOAc -> MeOH step gradient (see 12.5.2; 12.5.3)

Detection of eluates: TLC monitoring
Sample amounts: 1.6 – 10 g

**Reversed-phase VLC:**

Preabsorbent: Celite® 535, Fluka Chemie AG, Buchs, CH

Stationary phase: RSiL C18 HL, diameter 0.015–0.035 mm, Chemie Uetikon, Dr. Glaser AG, Basel, CH

Mobile phases: Water -> ACN step gradient (see 12.3.3; 12.3.4)
Water -> MeOH step gradient (see 12.5.3)

Detection of eluates: TLC monitoring
Sample amount: 0.25 – 1.6 g
14.3.3 High performance liquid chromatography (HPLC)

**Normal-phase HPLC:**

**Columns:**
- Vertex columns (Knauer, Berlin, Germany)
  - 250 x 8 mm (see 12.3.2; 12.3.3)
  - 250 x 16 mm (see 12.5.3)

**Stationary phase:** LiChrosorb Si 60, 5 µm

**System A:** (see 12.3.2; 12.3.3)

**Mobile phases:**
- Hexane/EtOAc 9:1 (12.3.2)
- Hexane/EtOAc 4:1 (12.3.3)

**Flow rate:** 2 ml/min

**Pump:** Waters 590 (Waters Inc., Milford, MA, USA)

**Injector:** Rheodyne 7125 (Rheodyne, Cotati, CA, USA)

**Detector:** Knauer Differential Refractometer (Knauer, Berlin, Germany)

**Recorder:** Knauer Strip Chart Recorder (Knauer, Berlin, Germany)

**System B:** (see 12.3.3; 12.5.2; 12.5.3)

**Mobile phase:** Hexane/EtOAc 4:1

**Flow rates:**
- 2 ml/min (12.3.3)
- 4 ml/min (12.5.2; 12.5.3)

**Pump:** Merck L-6200A Intelligent Pump (Merck, Darmstadt, Germany)

**Injector:** Rheodyne 7125 (Rheodyne, Cotati, CA, USA)

**Detector:** Merck L-4250 UV-VIS Detector (detection wavelength 254 nm, 12.5.2; 275 nm, 12.5.3)

**Recorder:** Merck D-2500 Chromato-Integrator (Merck, Darmstadt, Germany)
**Reversed-phase HPLC:**

**Columns:** Vertex columns (Knauer, Berlin, Germany)
- 250 x 8 mm (see 12.3.3)
- 250 x 16 mm (see 12.3.4)

**System A:** (see 12.3.3)
- **Stationary phase:** Spherisorb ODS2 (RP18), 5 μm
- **Mobile phase:** MeOH/ACN/water 25:5:10
- **Flow rate:** 2 ml/min
- **Pump:** Waters 590 (Waters Inc., Milford, MA, USA)
- **Injector:** Rheodyne 7125 (Rheodyne, Cotati, CA, USA)
- **Detector:** Knauer Differential Refractometer (Knauer, Berlin, Germany)
- **Recorder:** Knauer Strip Chart Recorder (Knauer, Berlin, Germany)

**System B:** (see 12.3.4)
- **Stationary phase:** Nucleosil (RP18), 5 μm
- **Mobile phase:** A= Isopropanol/THF (25:65), B= ACN, C= 0.5% o-H₃PO₄ in water

**Gradient:**

<table>
<thead>
<tr>
<th>time (min)</th>
<th>A%</th>
<th>B%</th>
<th>C%</th>
</tr>
</thead>
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<tr>
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<td>15</td>
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</tr>
<tr>
<td>40.01</td>
<td>15</td>
<td>5</td>
<td>80</td>
</tr>
</tbody>
</table>

**Flow rate:** 4 ml/min
Pump: Merck L-6200A Intelligent Pump (Merck, Darmstadt, Germany)
Injector: Rheodyne 7125 (Rheodyne, Cotati, CA, USA)
Detector: Merck L-4250 UV-VIS Detector (detection wavelength 280 nm, 12.5.2; 275 nm, 12.5.3)
Recorder: Merck D-2500 Chromato-Integrator (Merck, Darmstadt, Germany)

14.3.4 Preparative thin layer chromatography (preparative TLC)

(see 12.4.4)

TLC plates were prewashed, developing them in methanol.
Stationary phase: TLC aluminum sheets, 20 x 20 cm, silica gel 60 F<sub>254</sub>, Merck, Dietikon, CH
Linomat: Type IV, CAMAG AG, Muttenz, CH
Applied amount: 5 mg (in 100 μl)
Mobile phase: Hexane/Chloroform/MeOH 8:1:1
Development distance: 18 cm
R<sub>f</sub> value: 0.34 (α-tocopherolquinone)

14.3.5 Open column chromatography

System A: (see 12.3.2)
Column: 60 x 2.5 cm i.d.
Stationary phase: Florisil®, magnesium silicate, particle size (0.150-0.250 mm), Merck AG, Dietikon, CH
Mobile phase: Hexane -> EtOAc -> Acetone step gradient
Detection of eluates: TLC monitoring
Sample amount: 680 mg
168

System B: (see 12.3.2)
Column: 55 x 1.7 cm i.d.
Stationary phase: Florisil®, magnesium silicate, particle size (0.150-0.250 mm), Merck AG, Dietikon, CH
Mobile phase: Diethyl ether/ EtOAc/Hexane 1:1:1
Detection of eluates: TLC monitoring
Sample amount: 141 mg

System C: (see 12.3.3)
Column: 60 x 2.5 cm i.d.
Stationary phase: Silica gel CC 60 HF254, particle size 40-63 μm, Merck AG, Dietikon, CH
Mobile phase: Hexane/Isopropanol/MeOH 96:2.5:0.5
Detection of eluates: TLC monitoring
Sample amount: 910 mg

14.3.6 DAD High performance liquid chromatography (DAD-HPLC)

DAD-HPLC analysis was performed according to Hasler (1990). Reference compounds were injected as single compounds and as mixtures. Samples to be tested were solved in methanol and prepurified through 0.45 μm filters before injection.

References: Roth AG, Reinach, CH
Filters: Nylon syringe filters, Scientific resources Inc., NJ, USA
HPLC work station: Hewlett Packard HP 79994A Analytical Workstation, 1090 LC, 1040 DAD, Waldbronn, Germany
Column: Vertex columns, 100 x 4 mm i.d., Knauer, Berlin, Germany
Stationary phase: Spherisorb S3 ODS2, 3 μm, Phase separations, Queensferry, UK
Mobile phase: A= Isopropanol/THF (25:65), B= ACN, C= 0.5% o-H_3PO_4 in water

<table>
<thead>
<tr>
<th>time (min)</th>
<th>A %</th>
<th>B %</th>
<th>C %</th>
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<tr>
<td>35.00</td>
<td>0.0</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>35.01</td>
<td>15.0</td>
<td>1.5</td>
<td>83.5</td>
</tr>
</tbody>
</table>

Flow rate: 1 ml/min
Injection volume: 1 ml
Detection wavelength: \( \lambda = 350 \text{ nm}, 280 \text{ nm}, 250 \text{ nm} \)
14.4 Spectroscopic and physical methods

14.4.1 NMR spectroscopy

Spectra were recorded on the following spectrometers:
Bruker AMX-300 (300.13 MHz for $^1$H; 75.47 MHz for $^{13}$C), Spectrospin,
Fällanden, CH ($^1$H, $^{13}$C, DQF-COSY, t-ROESY, TOCSY, HMQC, HMBC)
Bruker DRX-500 (500.13 MHz for $^1$H; 125.77 MHz for $^{13}$C), Spectrospin,
Fällanden, CH ($^1$H, $^{13}$C, HSQC, HMBC)

The following resonances of solvents (Dr. Glaser AG, Basel, CH; CIL
Cambridge Isotope Laboratories Inc., Andover, MA, USA) were used as internal
references:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$^1$H-NMR</th>
<th>$^{13}$C-NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDCl$_3$</td>
<td>7.25 ppm</td>
<td>77.2 ppm</td>
</tr>
<tr>
<td>MeOH-d$_4$</td>
<td>3.35 ppm</td>
<td>49.0 ppm</td>
</tr>
<tr>
<td>Pyridine-d$_5$</td>
<td>8.71, 7.55, 7.19 ppm</td>
<td>149.9, 135.5, 123.5 ppm</td>
</tr>
</tbody>
</table>

14.4.2 Mass spectrometry

EI mass spectra were taken on a Hitachi-Perkin-Elmer-RMUGM mass
spectrometer using an ionisation power of 70 eV. ESI mass spectra were
recorded on a Finnigan TSQ 7000 at 70 keV.

14.4.3 Infrared spectroscopy

Perkin-Elmer 2000 FT-infrared spectrometer (Perkin Elmer Corp., Norwalk, CT,
USA), using KBr as a matrix.
Scan mode: Transmission  Resolution: 4.00
Number of scans: 12    Cycles: 1
14.4.4 Ultraviolet spectroscopy

UV spectra were recorded on a Kontron-Uvikon 930 spectrophotometer (Kontron instruments AG, Zürich, CH).

14.4.5 Optical rotation

Optical rotation was measured on a Perkin-Elmer 241 polarimeter (Perkin-Elmer Corp., Norwalk, CT, USA), at a wavelength of 589.5 nm (sodium D line).
15 Physical data of isolates

15.1 Euphol

$[\alpha]_D^{20} +33^\circ$ (c = 0.001; CDCl$_3$); IR$_{\text{max}}$ (KBr): 3340, 2944, 1455, 1374, 1216, 1094, 1023, 758 cm$^{-1}$; EIMS m/z (rel. int., %) 426 [M$^+$] (33), 411 (100), 393 (32), 271 (5), 259 (9), 229 (7), 215 (9), 189 (12), 173 (8), 147 (11), 135 (14), 121 (16), 109 (27), 95 (21), 81 (14), 69 (48), 55 (21), 41 (23); $^1$H NMR (300 MHz, CDCl$_3$) and $^{13}$C NMR (75.5 MHz CDCl$_3$) see Table 12.3.

15.2 Corollatadiol

$[\alpha]_D^{20} +20^\circ$ (c = 0.001; CDCl$_3$); IR$_{\text{max}}$ (KBr): 3430, 2927, 2856, 1710, 1673, 1457, 1376, 1027, 757 cm$^{-1}$; EIMS m/z (rel. int., %) 456 [M$^+$], 440 (17), 424 (32), 409 (60), 391 (21), 327 (20), 311 (24), 187 (43), 173 (37), 121 (67), 109 (100), 95 (73), 69 (60), 55 (39), 43 (31); $^1$H NMR (300 MHz, CDCl$_3$) and $^{13}$C NMR (75.5 MHz CDCl$_3$) see Table 12.4.

15.3 Eupha-8,23-dien-3β-25-diol / Eupha-8,25-dien-3β-24-diol

$[\alpha]_D^{20} +27^\circ$ (c = 0.001; CDCl$_3$); IR$_{\text{max}}$ (KBr): 3391, 2932, 1710, 1652, 1456, 1376, 1024, 757, 667 cm$^{-1}$; EIMS m/z (rel. int., %) 442 [M$^+$] (14), 438 (31), 423 (29), 409 (26), 341 (22), 327 (60), 273 (19), 187 (42), 147 (44), 135 (64), 121 (73), 109 (87), 95 (61), 81 (40), 69 (49), 55 (53), 43 (100); $^1$H NMR (300 MHz, CDCl$_3$) and $^{13}$C NMR (75.5 MHz CDCl$_3$) of eupha-8,23-dien-3β-25-diol (1) see Table 12.5, $^1$H NMR (300 MHz, CDCl$_3$) and $^{13}$C NMR (75.5 MHz, CDCl$_3$) of eupha-8,25-dien-3β-24-diol (2) see Table 12.6.
15.4 \( \alpha \)-Tocopherolquinone

\[ \alpha \]_D^{20} + 3.14° (c = 1.6; CDCl\textsubscript{3}); IR\textsubscript{\text{max}} (KBr): 3501, 2927, 2868, 1643, 1463, 1376, 1307, 715 cm\textsuperscript{-1}; EIMS m/z (rel. int., %) 446 [M*] (10), 430 (28), 402 (6), 237 (48), 221 (100), 205 (14), 195 (33), 178 (67), 165 (50), 150 (22), 137 (26), 121 (15), 95 (17), 69 (13), 55 (9); \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) and \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) see Table 12.7.

15.5 \textit{n}-Octacosanol

\[ \alpha \]_D^{20} -5.6° (c = 0.001; CDCl\textsubscript{3}); m.p. 80-82 °C; IR\textsubscript{\text{max}} (KBr): 2917, 2849, 1472, 1462, 729, 718 cm\textsuperscript{-1}; EIMS m/z (rel. int., %) 410 [M*] (<1), 392 (7), 364 (5), 196 (4), 181 (5), 167 (9), 153 (11), 139 (16), 125 (32), 111 (60), 97 (100), 83 (65), 69 (63), 57 (66), 43 (31), 18 (32); \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) and \textsuperscript{13}C NMR (75.5 MHz, CDCl\textsubscript{3}) see Table 12.8.

15.6 Nonadeca-3,5-dien-2-ol-1-yl

\[ \alpha \]_D^{20} + 6° (c = 0.001; MeOH); IR\textsubscript{\text{max}} (KBr): 3436, 2930, 2857, 1714, 1384, 1029, 725, 667 cm\textsuperscript{-1}; EIMS m/z (rel. int., %) 294 (<1), 278 [M*] (46), 223 (11), 207 (16), 179 (17), 171 (35), 153 (34), 135 (38), 121 (41), 109 (55), 93 (97), 81 (100), 67 (76), 55 (70), 43 (48); \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) and \textsuperscript{13}C NMR (75.5 MHz, CDCl\textsubscript{3}) see Table 12.9.

15.7 Kaempferol-3-O-\( \beta \)-D-glucuronide

\[ \alpha \]_D^{20} -20.6° (c = 0.01; MeOH); IR\textsubscript{\text{max}} (KBr): 3370, 1732, 1658, 1607, 1505, 1442, 1360, 1209, 1180, 1088, 1018, 890, 843, 514 cm\textsuperscript{-1}; UV \textit{\lambda}_{\text{max}}^{\text{MeOH}} 263 nm, 298 nm (sh), 352 nm (c=0.01 MeOH); ESIMS m/z 462 [M*]; \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) and \textsuperscript{13}C NMR (75.5 MHz, CDCl\textsubscript{3}) see Table 12.10.
15.8  Hyperoside

$[\alpha]^{20}_D - 67^\circ$ (c = 0.001; MeOH); $\lambda_{\text{max}}^{\text{MeOH}}$ (KBr): 3380, 1713, 1652, 1608, 1505, 1448, 1354, 1204, 1088, 1035, 763 cm$^{-1}$; UV $\lambda_{\text{max}}^{\text{MeOH}}$ 256 nm, 298 nm, 356 nm (c = 0.01 MeOH); EIMS $m/z$ 464 [M$^+$]; $^1$H NMR (300 MHz, CDCl$_3$) and $^{13}$C NMR (75.5 MHz, CDCl$_3$) see Table 12.11.

15.9  Scoparone

$[\alpha]^{20}_D + 5.0^\circ$ (c = 0.01; CDCl$_3$); $\lambda_{\text{max}}$ (KBr): 2920, 2851, 1728, 1615, 1559, 1518, 1448, 1281, 1249, 1140, 1006, 847, 814 cm$^{-1}$; EIMS $m/z$ (rel. int., %) 206 [M$^+$] (100), 191 (36), 178 (12), 163 (25), 149 (8), 135 (11), 107 (9), 97 (7), 79 (11), 69 (15); $^1$H NMR (300 MHz, CDCl$_3$) and $^{13}$C NMR (75.5 MHz, CDCl$_3$) see Table 12.12.


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List of Publications and Presentations

Publications

Volken, C.; Çalis, I.; Heilmann, J.; Rali, T.; Sticher O.
Euphane-type triterpenoids from the leaves of *Euphorbia buxoides*
In preparation.

Schmitt, J.; Schühly, W.; Volken, C.; Sticher, O.
Multiple Screening of Medicinal Plants from Oaxaca, Mexico: Ethnobotany
and Bioassays as a Basis for Phytochemical Investigations

Poster Presentation

Volken, C.; Çalis, I.; Rali, T.; Sticher, O.
New Euphane-type triterpenoids from *Euphorbia buxoides*
46th Annual Congress of the Society for Medicinal Plant Research (GA),
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1969 Born on February 21 in Biel (BE) as daughter of Othmar and Maria Pilar Volken-Pradal

1975-1980 Primary school in Oberwil (BL)

1980-1984 Progymnasium Oberwil (BL)

1984-1987 Gymnasium Oberwil (BL), Matura Typus B

1987-1993 Study of Pharmacy at the University Basel, Department of Pharmacy

1987-1988 Scientific basic course
1988-1989 Pharmaceutical basic course
1989-1990 Practical training at the Pharmacie du Landeron, Le Landeron (NE)
1991-1993 Pharmaceutical specialist course
1993 Diploma in pharmacy (Eidgenössisches Staatsexamen)

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