Chemical and Biological Properties of *Euphorbia ingens* E.Mey

Musiwalo Reuben Ramavhoya

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Supervisors: Dr. S. van Dyk
Prof. J.C. Breytenbach
Co-supervisor: Prof. S.F. Malan

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They can take away your house, rob you of your money, seize your car or fire you from work. They can even steal your wife, but there's one thing that nobody in the world can take away from you – your education.

ABSTRACT

The search for new effective antimicrobial agents is necessary due to the appearance of microbial resistance to antibiotics and occurrence of fatal opportunistic infections associated with the Acquired Immunodeficiency Syndrome (AIDS), cancer and chemotherapy. The isolation of antimicrobial compounds from plants provides a solution to increased demands for new antimicrobial agents to combat infection and overcome the problem with resistance and side effects of the currently available antimicrobial agents (antibiotics).

The aim of this study was to identify extracts from *Euphorbia* species with antimicrobial activity and to isolate and characterise the compound(s) responsible for this activity.

Euphorbia clavaroides Boiss. var. truncate (N.E.Br.) A.C. White was selected for screening based on the antimicrobial activity reported during previous routine screening of species selected from plant families in our laboratory. Due to unavailability of *E. clavaroides* plant material in large quantity, *E. ingens* E.Mey. ex Boiss. was also selected for screening. It is known that plants from the same family may contain the same chemical compounds. Soxhlet extraction was used to prepare extracts of each plant using petroleum ether, dichloromethane, ethyl acetate and ethanol successively. These plant extracts were screened for antimicrobial activity against a range of microorganisms using the disc diffusion and microplate assays. The toxicity evaluation of the prepared extracts was assayed against human epithelial cell lines (HeLa) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The ethyl acetate extract of the fleshy inner part of *E. ingens* showed the most promising antimicrobial activity against Gram-positive bacteria *B. subtilis* and *S. aureus* in both the disc diffusion and MIC assay and was therefore selected for further study. The security index (117,2) against *B. subtilis* of the ethyl acetate extract of the fleshy inner part of *E. ingens* showed that it is relatively safe to use at the concentration of 0,64 mg/ml in cases of *B. subtilis* infections. The ethyl acetate extract of the fleshy inner part was subjected to bioassay-guided fractionation approach using column chromatography. This lead to the isolation of kaempferol which was identified by spectroscopic techniques. A brief literature search indicated that kaempferol possessed weak antimicrobial activity against a wide range of microorganisms with a known MIC

value of 100 μg/ml against *Staphylococcus aureus* as well as toxicity against human cancer cell lines. From bioassay-guided fractionation approach kaempferol showed a weak antimicrobial activity against Gram-positive bacteria *Bacillus subtilis* (2 mm) and *S. aureus* (1 mm). Unfortunately, without structural modification it is not suitable for human usage.

In conclusion, although the compound isolated in this study is a fairly common flavonol, it is the first report of the isolation of kaempferol from *E. ingens*. Biological activity of the compound isolated from *Euphorbia ingens* justifies chemotaxonomic approach used to select species of the same genus.

OPSOMMING

Vanweë die ontstaan van weerstandigheid van mikro-organismes teen antibiotika, vanweë dodelike opportunistiese infeksies wat saam met die verworwe immuniteits-gebreksindroom (VIGS) voorkom asook vanweë die effekte van kanker en chemoterapie is dit altyd nodig om na nuwe effektiewe antimikrobiese middels te soek. Die isolasie van antimikrobiese middels uit plante bied 'n oplossing vir die toenemende behoefte aan nuwe antibiotika om infeksies te beveg en om die probleem van weerstandigheid en newe-effekte van bestaande middels te oorkom.

Die doel van hierdie studie was om ekstrakte van *Euphorbia*-spesies met antimikrobiese aktiwiteit te identifiseer en om die verbinding(s) verantwoordelik vir hierdie aktiwiteit te isoleer.

Euphorbia clavaroides Boiss. var. truncate (N.E.Br.) A.C. White is vir siftingstoetse gekies op grond van antimikrobiese aktiwiteit wat voorheen in roetinetoetse met geselekteerde spesies van plantfamilies in ons laboratorium gevind is. Omdat plantmateriaal van *E. clavaroides* nie in groot hoeveelhede beskikbaar was nie, is *E. ingens* E.Mey. ex Boiss. ook vir sifting gekies. Dit is bekend dat plante van dieselfde familie dieselfde chemiese komponente kan bevat. Van elke plant is Soxhlet-ekstrakte gemaak deur petroleumeter, dichloormetaan, etielasetaat en etanol agtereenvolgens te gebruik. Hierdie ekstrakte is met die plaatdifussie- en mikroplaatmetodes vir aktiwiteit teen 'n reeks mikro-organismes getoets. Evaluering van die toksisiteit van die ekstrakte teenoor menslike epiteelsellyne (HeLa) is gedoen deur 3-(4,5-dimetieltiasool-2-iel)-2,5-difenieltetrasoliumbromied (MTT) te gebruik.

Die etielasetaatekstrak van die vlesige binneste deel van *E. ingens* het in sowel die plaatdifussie- as in die MIK-toets die mees belowende antimikrobiese aktiwiteit teen Gram-positiewe bakterieë *B. subtilis* en *S. aureus* vertoon en was daarom vir verdere studie gekies. Die veiligheidsindeks (117,2) van die etielasetaatekstrak van die vlesige binneste deel van *E. ingens* teenoor *B. subtilis* toon dat dit teen die konsentrasie van 0,64 mg/ml redelik veilig is om vir infeksies deur *B. subtilis* te gebruik. Die genoemde ekstrak is met kolomchromatografie in fraksies geskei terwyl biologiese toetse deurgaans as riglyn vir seleksie van fraksies gebruik is. Dit het tot die isolasie van kaempferol gelei wat met spektroskopiese tegnieke geïdentifiseer is. 'n Vinnige

literatuursoektog het getoon dat kaempferol swak antimikrobiese aktiwiteit teenoor 'n wye reeks mikro-organismes, met 'n bekende MIK van 100 µg/ml teenoor *Staphylococcus aureus,* besit en ook toksisiteit teenoor menslike kankersellyne het. Tydens die fraksioneringsproses gerig deur biologiese toetse is gesien dat kaempferol swak antimikrobiese aktiwiteit teenoor die Gram-positiewe bakterieë *Bacillus subtilis* (2 mm) en *S. aureus* (1 mm) besit. Ongelukkig, sonder strukturele modifikase, is dit nie geskik vir menslike gebruik nie.

Hoewel die verbinding wat tydens hierdie studie geïsoleer is 'n redelike algemene flavonol is, is hierdie die eerste verslag van die isolasie van kaempferol uit *E. ingens*. Die biologiese aktiwiteit van die geïsoleerde verbinding uit *Euphorbia* regverdig die chemotaksonomiese benadering om spesies van dieselfde genus te kies.

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CHAPTER 1

Introduction

1.1 Problem statements and aim of the study

The search for new effective antimicrobial agents is necessary due to the appearance of microbial resistance to antibiotics and occurrence of fatal opportunistic infections associated with the Acquired Immunodeficiency Syndrome (AIDS), cancer and chemotherapy (Penna *et al.*, 2001). Medicinal plants are an important element of the indigenous systems in South Africa as well as in other countries. Today, 80% of the black population depend on traditional medicine to meet daily health requirements, especially in developing countries (Rajasekharan, 2002; WHO, 2002b). South Africa has an abundance of medicinal plants used in the traditional treatment of various diseases on an empirical basis (Hutchings & Van Staden, 1994; Salie *et al.*, 1996; Mcgaw *et al.*, 1997). According to Farnsworth (1994), safety and efficacy of medicinal plants should be studied.

Infectious diseases are the number one cause of death accounting for approximately one-half of all deaths in tropical countries. The mortality rate due to infectious diseases is actually increasing in developing countries in Africa for example Botswana. This increase is attributed to an increase in respiratory tract infections and infectious diseases due to Human Immunodeficiency Virus (HIV)/AIDS. Death from infectious diseases, ranked 5th in 1981 and was reported as the 3rd leading cause of death in 1992 (Pinner *et al.*, 1996).

The development of resistance by microorganisms to many of the commonly used antibiotics provide sufficient impetus for further attempts to search for new antimicrobial agents to combat infection and overcome the problem with resistance and side effects of the currently available antimicrobial agents (antibiotics) (Davis, 1994). Much attention has recently been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine (Essawi & Srour, 2000). Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu *et al.*, 1999).

The following are reasons why the study concentrates on medicinal plants rather than synthetic drugs:

- According to Eloff (1998a), "the possibility exists that natural antimicrobial compounds from plants can inhibit the growth of bacteria by mechanisms different from that of the known antimicrobial compounds (antibiotics)" and
- "Since the discovery of penicillin in 1928, resistance to antibiotics by bacteria has been causing concern within the medical profession". The increased resistance is reported to be due to the extensive use of antibiotics worldwide (Abramson & Givner, 1999).

All these problems mentioned above affect the current South African health budget, and it is paramount that alternative and possibly cheaper avenues be explored in the treatment of these conditions. This situation forced scientists to continue with research to investigate new antimicrobial substances from various sources, like medicinal plants, which are the good sources of novel antimicrobial chemotherapeutic agents (Karaman *et al.*, 2003).

The aim of this study was to identify extracts from *Euphorbia* species with antimicrobial activity and to isolate and characterise the compound(s) responsible for the antimicrobial activity. The bioassay-guided fractionation approach was used to identify active fractions leading to the isolation of pure active compound(s).

To reach the aim of this study the following objectives were proposed:

- To screen extracts of *Euphorbia* species by the disc diffusion and minimum inhibitory concentration assay for antimicrobial activity.
- To isolate compounds by chromatographic techniques.
- To characterise the compounds responsible for antimicrobial activity from active extracts of *Euphorbia ingens* by spectrometric methods.
- To determine the toxicity of the extracts with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by calculating the LD_{50} (Lethal dose) and security index.

CHAPTER 2

Literature review

2.1 Development of antibiotics

Penicillin continues to be effective after more than fifty years since its introduction into clinical use. By 1955, most countries had restricted its use to prescription only, because of the development of resistance. The synthesis of methicillin in the early 1960s and other semisynthetic derivatives alleviated the problem for a while, but resistance soon developed to these compounds as well (Levy, 1984). Some antibiotics introduced during and after World War II also continue to be used. These were developed from the antibacterial effects of a whole series of natural products isolated from species of *Penicillium, Cephalosporium* and *Streptomyces* (Kong *et al.*, 2003).

It is estimated that 10 000 natural antibiotics have been isolated and described, and at least 50 000 to 100 000 analogues have been synthesized (Berdy, 1980; Lancini *et al.*, 1995). Most of the natural antibiotics have been isolated from soil microorganisms through intensive screening (Mathekga & Meyer, 1998). After the discovery of β-lactam antibiotics it was possible to treat infectious diseases that have been untreatable before and sometimes even deadly (Miller, 2000).

2.2 Infectious diseases worldwide

Illness and death from infectious diseases are particularly tragic because they are largely preventable and treatable. In 2002, more than 90% of deaths were from infectious diseases such as lower respiratory tract infections, HIV/AIDS, diarrhoeal diseases, tuberculosis, malaria and measles (table 2.1). Most notably infectious diseases are the leading cause of death in sub-Sahara Africa.

Table 2.1: Death from major infectious diseases during 2002 (WHO, 2002a).

Leading cause of diseases	Death in 2002
Lower respiratory infections	3.9 million
HIV/AIDS	2.9 million
Diarrheal diseases	2.0 million
Tuberculosis	1.6 million
Malaria	1.1 million
Measles	0.7 million

Southern Africa, which is home to 10% of the world's population, accounted for more than 40% of deaths due to infectious diseases (Farmer, 2004). Infectious diseases are still a major cause of illness and death in South Africa (Klugman, 1999).

The major infectious diseases such as malaria, tuberculosis and HIV/AIDS claimed 5,7 million lives in 2001 and caused illness in millions more. The above-mentioned diseases keep people in poverty. The WHO reported early in 2001 that more than 36 million people lived with HIV/AIDS worldwide. In sub-Saharan Africa, AIDS killed more than 2 million people. Two billion people worldwide are carriers of the tuberculosis bacillus, the organism that can lead to active tuberculosis. Malaria kills more than 1 million people per year (WHO, 2002a).

2.3 Microbial resistance

Microbial resistance is a matter of great importance and the inappropriate use of antibiotics is the leading cause of microbial resistance. Since the discovery of antibiotics, bacterial resistance has been seen as the major obstacle to the successful treatment of infectious diseases (Amyes, 2000). The basis of microbial resistance can be classified as follows:

- Transformation of the antibiotic into an inactive form – the resistant strain produces an enzyme capable of chemically transforming antibiotics into an inactive product. For example aminoglycosides are phosphorylated and penicillins and imipenem are hydrolysed by β-lactamases.
- Modification of the cell's target site for the antibiotic – many antibiotics act by inactivating a target protein (receptor). For example, mutants resistant to strepomycin have an alteration in the ribosomal protein, which contributes to the formation of a complex of streptomycin with the ribosomal RNA.
- Modification of the permeability of the microorganism to the antibiotic – antibiotics penetrate cell membranes by one of two major mechanisms, passive diffusion or specific active transport. An alteration in the bacterial membrane may decrease permeation and cause resistance for example to tetracyclines.
- Increased production of biochemical intermediate that is competitively antagonised by the drug in sensitive cells – the sulphonamide antimicrobials exert their antimicrobial action by the competitive antagonism of an essential

metabolic intermediate, *p*-aminobenzoic acid (Hugo & Russell, 1983; Lancini *et al.*, 1995).

Some microorganisms may be naturally resistant. They 'achieve' resistance by mutation or have resistance 'thrust upon them' by transfer of plasmids and other mobile genetic elements (Lancini *et al.*, 1995).

Antimicrobial drug resistance is a great public health problem worldwide (Kunin, 1993; Weinstein, 2001). Among the resistant pathogens, methicillin (oxacillin)-resistant *Staphylococcus aureus* (MRSA) is of great concern because of the predominance of this organism that causes various clinical infections, including those acquired in the community or hospital (Bell *et al.*, 2002; Chambers, 2001; Fridkin *et al.*, 1999; NNIS, 2001; Salmenlinna *et al.*, 2002). Worldwide today, approximately 90% of hospital strains and 50% of community strains are resistant to penicillin (OFPL, 2004). Today, resistance occurs in as many as 80% of all strains of *S. aureus*. In South Africa this organism is a problem in hospitals and communities. The incidence of infections due to methicillin-resistant *S. aureus* in South Africa is alarming, with up to 50% of nosocomial isolates being methicillin-resistant (Klugman, 1999).

S. aureus is also a leading cause of nosocomial infections and is almost always resistant to β-lactams, macrolides and tetracyclines, leaving few alternative drugs (Operation Resistance, 2000). The estimated annual cost of antimicrobial resistance in hospitals due to *S. aureus* is $122 million and to nosocomial infectious is $4.5 billion (Institute of medicine, 1998). Vancomycin is the antibiotic of last resort for treatment of resistant infections and within a few years after its discovery, scientists have found strains of *Streptococcus pneumonia* and *S. aureus* resistant to this antibiotic.

Diseases such as tuberculosis, gonorrhoea, malaria and childhood ear infections are now more difficult to treat than decades ago. In short, antimicrobial resistance increases the severity of disease thus increasing the death rate from certain infections (OFPL, 2004).

2.4 Overview of Traditional Medicine

2.4.1 Role of Traditional Medicine in Africa

Traditional Medicine is a broad term used to define any non-western medicinal practice (Fabricant & Farnsworth, 2001). In China, traditional medicine accounts for around 40% of all health care delivered, and is used to treat roughly 200 million patients annually (WHO, 2002b). The interest in traditional knowledge is more and more widely recognised in development policies, the media and scientific literature. In Africa, traditional healers and remedies made from plants play an important role in the maintenance of the health of millions of people (Rukangira, 2003).

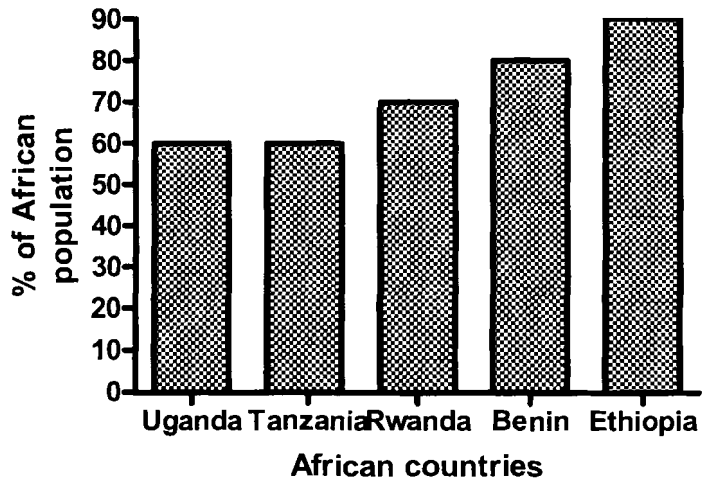


Figure 2.1: Percentage of the population using traditional medicine in African counties.

In many developing countries, the majority of the population continues to use traditional medicine to meet their health care needs. 90% of the Ethiopian population rely on traditional medicine followed by Benin with 80% and Uganda with 60% (figure 2.1). For instance, in Uganda, one traditional medicine practitioner treats between 200-400 patients (WHO, 2002b). To support this, the bar chart (figure 2.1) below indicates the use of traditional medicine for primary health care in some developing African countries, and table 2.2 indicates the number of doctors (western practitioners) and traditional medical practitioners to patients in Africa (Rukangira, 2003; WHO, 2002b). It is clear that in Swaziland 110 patients consult one traditional healer (table 2.2) and 10 000 patients

consult one western doctor. It is estimated that the number of traditional practitioners in Tanzania is 30 000-40 000 in comparison to 600 western doctors (Rukangira, 2003).

Table 2.2: The number of western doctors and traditional medical practitioners to patients in Africa (Cunningham, 1993).

Country	**Doctor: Patient**	**Traditional medicinal practitioners: Patient**
Botswana		Traditional medicinal practitioners estimated at 2 000 in 1990
Eritrea	Medical doctors estimated at 120 in 1995	
Ethiopia	1 : 33 000	
Kenya	1 : 7 142	1 : 987 (Urban-Mathare)
	1 : 833	1 : 378 (Rural-Kilungu)
Lesotho		Licensed TMPs estimated at 8 579 in 1991
Madagascar	1 : 8 333	
Malawi	1 : 50 000	1 : 138
Mozambique	1 : 50 000	1 : 200
Namibia		1 : 1000 (Katutura) 1 : 500 (Cuvelai) 1 : 300 (Caprivi)
Somalia	1 : 14 285 (Overall) 1 : 2 149 (Mogadishu) 1 : 54 213 (Central region) 1 : 216 539 (Sanaga)	
South Africa	1 : 1 639 (Overall)	1 : 700-1 200 (Venda)
	1:17 400 (Homeland areas)	
Sudan	1 : 11 000	
Swaziland	1 : 10 000	1 : 100
Tanzania	1 : 33 000	1 : 350 - 450 in DSM
Uganda	1 : 25 000	1 : 200 - 400
Zambia	1 : 11 000	
Zimbabwe	1 : 6 250	1 : 234 (urban) 1 : 956 (rural)

In sub-Saharan Africa, one traditional healer treats approximately 500 patients, while one medical doctors treats 40 000 patients (Abdool Karim *et al.*, 2002). It is clear that traditional healers play an influential role in the life of African people and have the potential to serve as crucial components of a comprehensive health care strategy. The demand for traditional medicine increases with the growth of the population of Africa and thus the harvesting of medicinal plants by traditional healers increases.

Traditionally, rural African communities have relied upon the spiritual and practical skills of the traditional medicinal practitioners, whose botanical knowledge of plant species and their ecology and scarcity is invaluable. In contrast to western medicine, which is technically and analytically based, traditional African medicine takes a holistic approach. Good health, disease, success or misfortune are not seen as chance occurrences, but are believed to arise from the actions of individuals and ancestral spirits, according to the balance or imbalance between the individual and the social environment (Rukangira, 2003).

2.4.2 Traditional Medicine in South Africa

The traditional health practitioners in South Africa play a crucial role in providing health care to the majority of the population. The traditional medicines market in South Africa is huge and includes "complementary medicines", which are largely imported traditional and alternative medicines (DoH *et al.*, s.a).

It is estimated that at least 80% of all South Africans especially in rural areas consult traditional healers for their health care needs (DoH *et al*, s.a; Hasslberger, 2004). The South African Traditional Health Council (SATHC) provides the following categories of traditional healers: Inyanga (Herbalist or traditional doctor), Sangoma (Diviner), Ababelekisi (Traditional birth) or Inggabi (Traditional surgeons) (DoH *et al.*, s.a).

South Africa is considered to be a "hotspot" for biodiversity with more than 24 000 indigenous plants occurring within its boundaries. This represents about 10% of the world species, although the land surface of South Africa is less than 1% of the earth. This country also has a long tradition of medicinal use of plants (Coetzee *et al.,* 1999; DoH *et al.*, s.a).

According to Rajasekharan (2002), only 5–10% of the approximate 250 000 species of higher plants have been investigated for the presence of bioactive compounds so far. About 35 000 are used worldwide for medicinal purposes (Kong *et al.*, 2003). The Cape Floral Kingdom alone has nearly 9 000 species and is the most diverse temperate flora on earth, rivalling the tropical rainforests in terms of species richness (Van Wyk *et al.*, 1997).

The demand for medicinal plants is likely to remain buoyant in the future. There are a wide range of aliments and needs that cannot be adequately treated by western medicine. This implies that indigenous medicine is a basic consumer good, essential for the welfare of black households. Zulu medicinal plants are traded and used all over Southern Africa (Mander, 1998). The African traditional medicine market in South Africa has been estimated at R 2.5 million (Killham, s.a.).

2.5 Role of ethnopharmacology in drug development

The development of drugs from plants is a long and arduous process, which involves many disciplines (Grabley & Thiericke, 1999). Ethnopharmacology is a highly diversified approach to drug discovery involving botany, chemistry, biochemistry, pharmacology and other disciplines that contribute to the discovery of natural products with biologic activity (Fabricant & Farnsworth, 2001).

In industrialised countries, it was reported that plants have contributed to more than 7 000 compounds produced by the pharmaceutical industry, including ingredients in heart drugs, laxatives, anticancer agents, hormones, contraceptives, diuretics, antibiotics, decongestants, analgesics, anaesthetics, ulcer treatments and antiparasitic compounds (WWF, 2003). Some medicines, such as the cancer drug taxol (from *Taxus brevifolia*) and the antimalarial quinine from *Cinchona pubescens* are isolated from plants. Other medicinal agents such as pseudoephedrine original derived from *Ephedra* species, menthol and methylsalicylate original derived from *Mentha* species and wintergreen (*Gaultheria procumbens*) respectively are synthesised on an industrial scale (Killham, s.a.).

Plant materials have been used in the treatment of infectious diseases for centuries (Kong *et al.*, 2003). A recent study by Fabricant & Farnsworth (2001) showed that approximately 80% of the plant-derived drugs they studied had an ethnomedical use identical or related to the current use of the active principle.

The goals for using plants as a source of therapeutic agents are:

- To isolate pure compounds for direct use as drugs, for example digoxin, digitoxin, morphine, reserpine, taxol, vinblastine and vincristine;

- To produce compounds that may serve as precursors of bioactive compounds, for example metformin, anbilone, oxycodon, tarotere, teniposide, verapamil, and amiodarone, which are based, respectively, on galegine, Δ^9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin and khellin;
- To use agents as pharmacologic tools, for example lysergic acid diethylamide (LSD), mescaline and yohimine; and
- To use the whole plant or part of it as a herbal remedy, for example cranberry, *Echinacea*, feverfew, garlic, *Gankgo biloba*, St. John's Wort and Saw Palmetto (Fabricant & Farnsworth, 2001).

Although the direct uses of herbal medicine continued to increase, medicinal plants still contribute significantly to prescription drugs. According to Duke (1993), Farnsworth & Bingel (1977), concluded that 25% of prescriptions written in the United States contain plant-derived active ingredients. One in four of all prescription drugs dispensed by western pharmacists are likely to contain ingredients derived from plants (WWF, 2003).

Table 2.3: Plant-derived drugs widely employed in western medicine (Adapted from Farnsworth, 1984).

Acetyldigoxin	Ephedrine	Pseudoephedrine*
Aescin	Hyoscyamine	Quinidine
Ajmalicine	Khellin	Quinine
Allantoin*	Lanatoside C	Rescinnamine
Atropine	Leurocristine	Reserpine
Bromelain	α-Lobeline	Scillarens A & B
Caffeine*	Morphine	Scopolamine
Codeine	Narcotine	Sennosides A & B
Colchicine	Ouabain	Sparteine
Danthron*	Papain	Strychnine
Deserpidine	Papaverine	Tetrahydrocannabinol
Digitoxin	Physostigmine	Theobromine*
Digoxin	Picrotoxin	Theophylline
Tubocurarine	Pilocarpine	Tubocurarine
Emetine	Protoveratrines A & B	Vincaleukoblastine
		Xanthotoxin

*Produced industrially by synthesis.

Many drugs from higher plants have been discovered but less than a 100 of defined structure are in common use today. Less than half of these are accepted as useful drugs in industrialized countries. Table 2.3 lists additional plant-derived drugs that are

either widely used in developed countries, perhaps with medical acceptance as to efficacy or also included in many of the pharmacopoeias of many developing countries. Less than 10 of these well-established drugs mentioned above are produced commercially by synthesis, although laboratory synthesis has been described for most of them (Farnsworth, 1984).

Today, 50% of western drugs are derived from plant material (Robbers *et al.*, 1996). Thirty per cent of the worldwide sales of drugs are based on natural products (Grabley & Thiericke, 1999). Commercially, these plant-derived medicines are worth about US$ 14 billion a year in the United States and US$ 40 billion worldwide. Eisenberg *et al* (1998) indicated that the Americans paid an estimated US$ 21,2 billion for services provided by alternative medicine practitioners.

2.6 Antimicrobial compounds from plants

The active principles in medicinal plants are chemical compounds known as secondary plant products. Some secondary products inhibit bacterial or fungal pathogens (Levetin & McMahon, 2003). Some of these compounds are constitutive, existing in healthy plants in their biologically active forms (Mathekga, 2000). The significance of secondary compounds is defence against predators and pathogens, as allelopathic agents or attractants in pollination and seed dispersion. Major categories of these compounds known for antimicrobial activity are described below.

2.6.1 Phenolic compounds

Phenolic compounds are composed of one or more aromatic benzene rings with one or more hydroxyl groups (C-OH). Although essential oils are classified as terpenes, many volatile chemicals are actually phenolic compounds, such as vanillin from *Vanilla fragrans*, and catechol from *Chrysobalanus icaco* (Armstrong, 2003).

Phenolic compounds such as simple phenol, phenolic acid and tannins are active against microorganisms (Cowan, 1999). The mechanism thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidised compounds, possibly through reaction with sulphydryl group or through more non-specific interactions with proteins (Mason & Wasserman, 1987).

2.6.1.1 Simple phenolic compounds

This group often possesses alcoholic, aldehydic and carboxylic acid groups, and are derivatives of catechol, phloroglucinol, eugenol, vanillin and various phenolic acids such as caffeic and vanillic acid (Trease & Evans, 1983). The common herbs tarragon and thyme both contain caffeic acid (3,4-dihydrocinnamic acid) (**1**), which is effective against viruses, bacteria and fungi (Brantner *et al.*, 1996). Vanillic and caffeic acids completely inhibited both the growth and aflatoxin production of *Aspergillus flavus* and *A. parasiticus* (Wahdan, 1998). Gallic acid (3,4,5-trihydrobenzoic acid) (**2**) and its methyl ester had a clear inhibitory effect on several harmful intestinal bacteria (Ahn *et al.*, 1998), and six other simple phenolic acids were found to be active against a variety of bacteria and moulds (Aziz *et al.*, 1998).

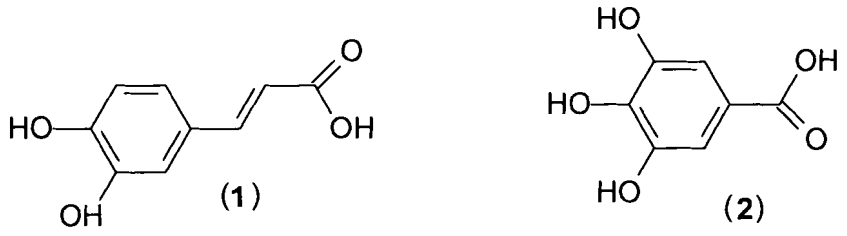


Figure 2.2: Simple phenolic compounds with antimicrobial activity

2.6.1.2 Flavonoids

Flavonoids are 3-ringed phenolic compounds consisting of a benzopyran ring system attached by a single bond to a third ring. The structural basis for all flavoniods is the flavone nucleus (2-phenyl-benzo-γ-pyran) (**3**), but depending on the classification method, the flavonoid group can be divided into several categories based on hydroxylation of the flavonoid nucleus as well as the linked sugar. Flavonoids include water soluble pigments such as anthocyanins (Rauha, 2001).

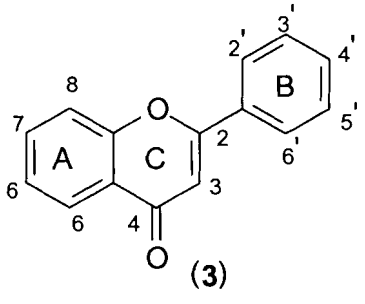


Figure 2.3: Basic structure of the flavone nucleus

Flavonoids are known to be synthesised by plants in response to microbial infections and has been found (*in vitro*) to be effective antimicrobial substances against a wide range of microorganisms (Cowan, 1999; Dixon *et al.*, 1983; Recio *et al.*, 1989). The structure-activity relationships of the antimicrobial activity of flavonoids are contradictory. It has been shown that less polar compounds, for example flavonoids lacking hydroxyl groups on ring B, are more active against microorganisms than those with hydroxyl groups (Chabot *et al.*, 1992). This is supported by the finding that methylation of the flavonoid nucleus increases antibacterial activity against *S. aureus* (Ibewuike *et al.*, 1997).

Catechins (**4**) are flavonoid compounds with a reduced C_3 unit and deserve special mention. These compounds possess antimicrobial activity against *Vibrio cholerae, Streptococcus mutans, Shigella* and other microorganisms in *in vitro* tests. Toda *et al.* (1989) indicated that the antimicrobial activity exerted by green teas was due to a mixture of catechin compounds.

In a basic structure of the flavone nucleus (**3**) (figure 2.3), a free 3',4',5'-trihydroxy ring B and a free 3-OH have been found to be necessary for antimicrobial activity against *S. aureus* and *Proteus vulgaris* (Mori *et al,* 1987). This is supported by the result of Puuponen-Pimiä *et al.* (2001), in which the broadest antimicrobial activity of the tested flavonoids was achieved using myricetin against *Lactobacilli* and *Escherichia coli*.

Various flavonoids and even chalcones were found to be active against fungi. Recently, the investigation of flavans from *Mariscus psilostachys* revealed that (2*S*)-4'-hydroxy-5,7,3'-trimethoxyflavan (**5**) was active in the bioautography assay, but its activity in the dilution assay against *C. albicans* was weak (MIC 50 μg/ml) (Hostettmann *et al.*, 2000). The ethanol-soluble fraction of purple prairie clove yields a flavonoid called petalostemumol (**6**), which showed excellent activity against *Bacillus subtilis* and *S. aureus* and lesser activity against Gram-negative bacteria and *Candida albicans* (Hufford *et al.*, 1993).

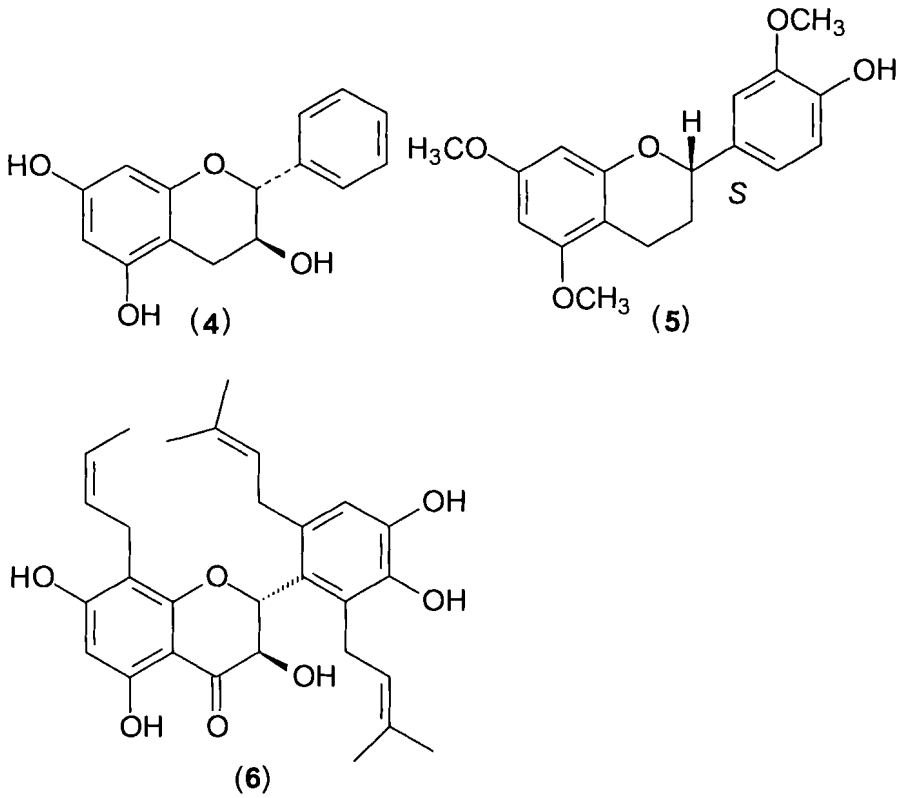


Figure 2.4: Flavonoid compounds with antimicrobial activity

2.6.1.3 Tannins

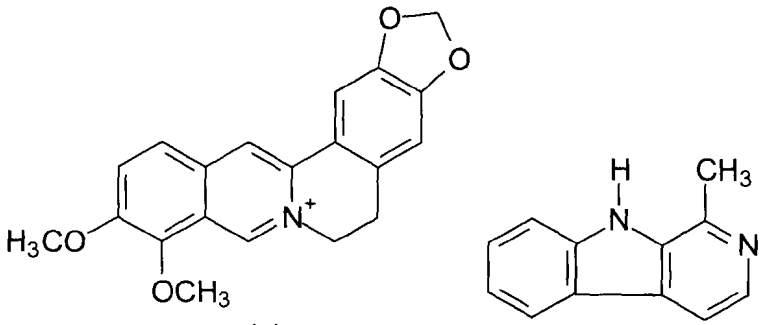
Some phenolic compounds (often combined with glucose) occur as polymers known as tannins. Tannins are naturally occurring plant polyphenols and are soluble in water, dilute alkalis, alcohol, acetone, etc. (Armstrong, 2003; Trease & Evans, 1982). Their main characteristic is that they bind and precipitate proteins. They are composed of a very diverse group of oligomers and polymers (Armstrong, 2003). Tannins are reported to have antibacterial, antifungal and antiviral activity (Nonaka *et al.*, 1990; Scalbert, 1991). According to Cowan (1999), antimicrobial action of tannins may be related to their ability to inactivate microbial adhesions, enzymes, cells envelop transport proteins, etc.

2.6.2 Alkaloids

Many of the earliest isolated pure compounds with biological activity were alkaloids. Alkaloids include those natural compounds that contain nitrogen, usually as part of a cyclic system (Kaufman *et al.*, 1999). The diversity of the phytochemical is impressive, over 35 000 terpenoids, more than 12 000 alkaloids, several thousand

phenylpropanoids and a variety of other compounds have been isolated and their structures elucidation (Facchini, 2003). The first medicinally useful example of an alkaloid was morphine, isolated from the opium poppy, *Papaver somniferum*.

Alkaloids are often toxic to humans and many have dramatic physiological activities. Some are central nervous system depressants such as morphine and scopolamine and some are stimulants such as strychnine and caffeine (Tam, 1990). Berberine (**7**) is an important representative of the alkaloid group. It is potentially effective against plasmodia (Omulokoli *et al.*, 1997). Dicentrine, harmine (**8**) and several related alkaloids were also shown to have bactericidal activity. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine (**7**) and harmane (**8**) is attributed to their ability to intercalate with bacterial DNA (Cowan, 1999, Phillipson *et al.*, 1987).



(7) (8)

Figure 2.5: Alkaloids with antimicrobial activity

2.6.3 Terpenoids and essential oils

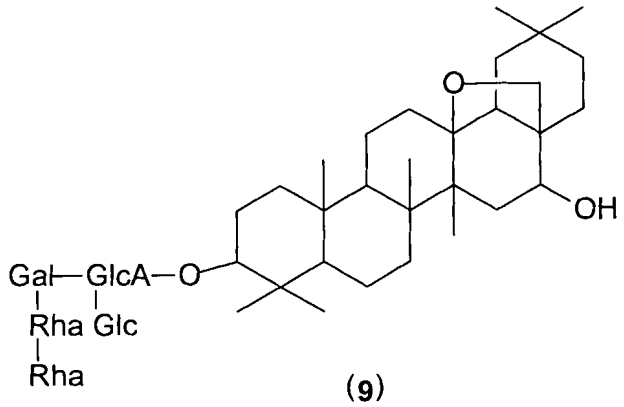
Many natural products, other than alkaloids, show biological activity (Ikan, 1969) against microorganisms. Amongst these are compounds which fall in the general class of terpenes, compounds consisting of 5-carbon units, often called isoprene units, put together in a regular pattern (Cowan, 1999). Terpene hydrocarbons are classified as follows: monoterpenes ($C_{10}H_{16}$), sesquiterpene ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), triterpene ($C_{30}H_{48}$), tetraterpenes ($C_{40}H_{64}$) and polyterpenes $(C_5H_8)_n$ (Ikan, 1969). Terpenes containing 30 carbons or more, and are usually formed by fusion of two smaller terpenes precursors. When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999). Essential oils are an abundant source of terpenoids (Ikan, 1969).

Mono-oxygenated monoterpenoids exhibit antimicrobial effects against a wide range of microorganisms examined, however, most of these compounds are not active at low concentrations. Mono-oxygenated sesquiterpenoids are strong inhibitors of Gram-positive bacteria, yeasts and some fungi, while Gram-negative bacteria are more resistant (Pauli, 2001).

2.6.4 Glycosides

Glycosides are named because of the sugar molecule (*glycol-*) attached to the active component. They are generally categorised by the non-sugar (aglycone) or active component (Levetin & McMahon, 2003). Their solubility and hence extraction method depend on the nature of the aglycone and the number and type of sugar molecules linked to it. The aglycone tends to be soluble in organic solvents, and the sugar part in aqueous and organic solvents. Examples of pharmacologically active glycosides range from the simple phenolic compounds e.g. flavonoids (rutin), antraquinones (sennosides), cardiac glycosides (digoxin) (Ikan, 1969).

Some glycosides are covalently bonded through a C-C bond (Williamson *et al.*, 1996). Several types of glycosides yielding toxic products upon hydrolysis occur in widely unrelated families. The most important glycosides involved in plant poisons are cyanogenic glycosides, saponin glycosides, solanines and mustard oil glycosides (Armstrong, 2003). Triterpene glycosides (saponins) may also exhibit interesting activities. Sakurasaponin (**9**) was isolated from the methanolic leaf extract of *Rapanea melanophloeos* and was found to be active against *Cladosporium cucumerinum*. The activity of compound **9** might be due to the presence 13β,28-exoxy moiety, since it is also absent from other saponins (Hostettmann *et al.*, 2000).



(9)

Figure 2.6: Compound with antimicrobial activity, sakurasaponin

2.7 Family Euphorbiaceae

This family was chosen because it is known to produce biologically active compounds (Cox, 1990). *Euphorbia clavaroides* was selected based on the antimicrobial activity reported during previous routine screening of several families in our laboratory. *Euphorbia clavaroides* particularly the aerial parts possessed interesting antimicrobial activity. Due to the unavailability of *E. clavaroides* plant material in large quantity, *E. ingens* was also selected for screening, as it is known that plants from the same family may contain the same chemical compounds (chemotaxonomy approach) (Christensen & Kharazmi, 2001; Cox, 1990).

The Euphorbiaceae (spurge family) is one of the largest families in the plant kingdom. It comprises of 7 300 species in 263 genera and is of cosmopolitan distribution. *Euphorbia* is the largest genus in this family comprising of 1600 species characterised by the presence of a milky latex (Ahmad & Jassbi, 1998; Ferreira & Ascenso, 1999; Hohmann *et al.*, 1999; Marco *et al.*, 1999; Öksüz *et al.*, 1999; Singla & Pathak, 1990; Vogg *et al.*, 1999). This genus has been subjected to numerous chemical studies (Marco *et al.*, 1999).

2.7.1 Phytochemistry of some *Euphorbia* species

Most *Euphorbia* species produce a milky latex, which yields a wide range of chemicals such as rubber, oil, terpenes, waxes, hydrocarbons, starches, resins, tannins and balsams (Watt & Breyer-Brandwijk, 1962).

Diterpenes euphosalicin and 2 japtrophane diterpenes were isolated from the dichloromethane extract of the fresh plants of *E. salicifolia* (Hohmann *et al.*, 1999).

Table 2.4: Chemical compounds isolated from *Euphorbia* species

Plant species	Chemical compounds
Euphorbia ebracteolata	Casbane diterpenoid (flavonol glycosides)
Euphorbia nicaeensis	Glucocerebrosides
Euphorbia peplis	
Euphorbia villosa	Tri- & tetracyclic diterpenes
Euphorbia nivulia	Diterpenes
Euphorbia stygiana	Pentacyclic triterpenes
Euphorbia ingens	Macrocyclic diterpene alcohol
Euphorbia sessiliflora	Jolkinolide & *ent*-11α-hydroxyabieta-8(14),13(15)-dien-16,12α-olide

Isolation and structural elucidation of four cerebrosides (1-*O*-(β-D-glucopyranosyl)-(2S,3S,4R,8Z)-2-[(2'R)-2'-hydroxytetracosenoilamino]-8-(Z)-octadecene-1,3,4-triol) (table 2.4) from *E. peplis* was reported. These compounds have interesting antifungal and antitubercular activity. The cerebroside mixture showed activity against three different *Candida* species, but it has been indicated that pure cerebroside compounds are not active against *Candida* spp (Cateni *et al.*, 2003).

Euphorbia stygiana was screened for triterpenoids and pentacyclic triterpenes and the following compounds were isolated: D-friedomadeir-14-en-3β-yl acetate, D:C-friedomadeir-7-en-3β-yl acetate, named madeiranyl acetate and isomadeiranyl acetate. Other triterpenes known as D-friedomadeir-14-en-3-one and D:C-friedomadeir-7-en-3-one (table 2.4) were previously isolated from *E. mellifera* (Lima *et al.*, 2003). A kaempferol glycoside (**10**) has been isolated from *Euphorbia ebracteolata*. Kaempferol as an aglycone and glucose, rhamnose and galactose were identified through GC-MS analysis (Liu *et al.*, 2004).

3,7,12-Triacetate-8-nicotinate (**11**) has been separated from the acetone extracts of the latex of *Euphorbia ingens* by combination of adsorption chromatography and Craig-distribution. A number of conversions were synthesised from the original source (**11**) (Opferkuch & Hecker, 1973). The agar dilution method showed that *ent*-11α-hydroxyabieta-8(14),13(15)-dien-16,12α-olide (**12**) isolated from chloroform extract of *Euphorbia sessiliflora* had moderate to strong growth inhibition against *B. cereus, B.*

subtilus, M. flavas, M. catarrhalis, N. sicca and *C. albicans* at a concentration of 12,5 µg/ml (Suffhivaiyakit, 2000).

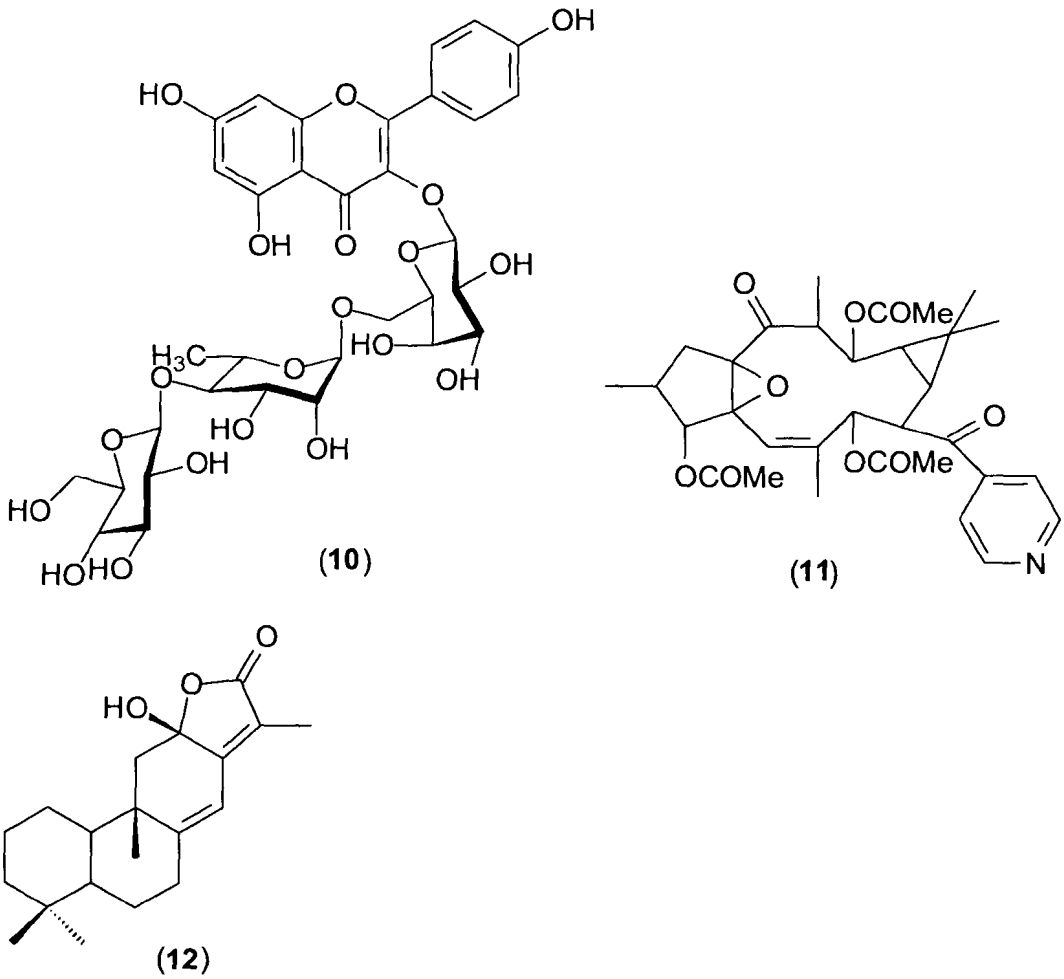


Figure 2.7: Terpenes isolated from *Euphorbia* species

2.7.2 *Euphorbia clavaroides* Boiss. var. truncate (N.E.Br.) A.C. White

2.7.2.1 Botanical description

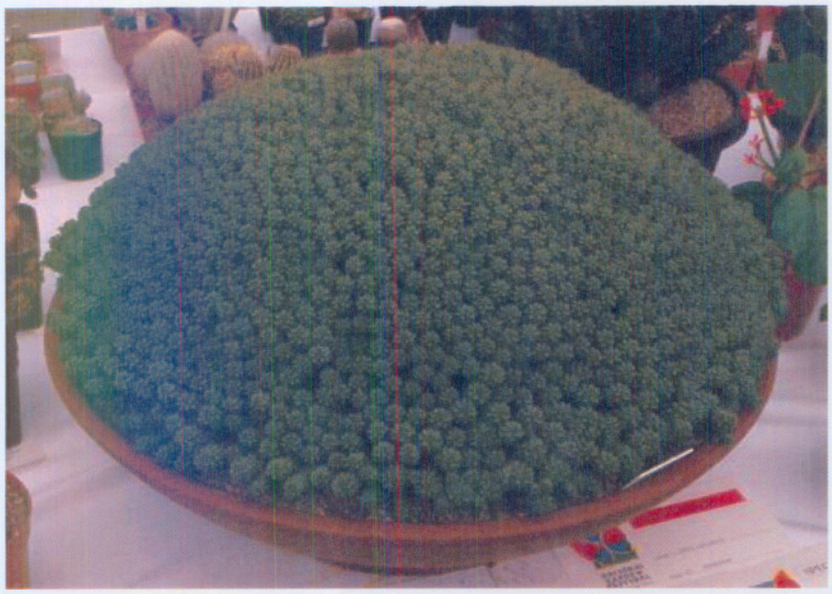


Figure 2.8: *Euphorbia clavaroides* Boiss

A unique species which is widely distributed throughout the Graaff-Reinet district and other parts of the great Karoo, many parts of the Free State, Lesotho, KwaZulu-Natal and Limpopo province. The sessile cyathia are produced at the tips of the branches, thus leaving the truncated habit undisturbed with the main stem underground with the tips of the branches forming a mat or cushion. *Euphorbia clavaroides* is commonly known as *Clavaria*. It is a club-shaped species with short branches which make up this extraordinary euphorbia (Balkema, 1981). The size of the whole plant is 5-10 cm in diameter (Slabý, 2004)

2.7.2.2 Uses and cultural aspect of *Euphorbia clavaroides*

E. clavaroides is used for respiratory disorders such as asthma, bronchitis, catarrh and laryngeal spasm. It has also been used for the treatment of intestinal amoebiasis (Huang, 1997). It is applied by the African in the Mphomalanga to cancerous sores and to warts. In Lesotho, the Sotho makes a lotion for bathing swollen feet from the plant and combine it with *Berkheya* as a leprosy remedy. They also use the latex for making glue (Watt & Breyer-Brandwijk, 1962).

2.7.3 *Euphorbia ingens* E.Mey. ex Boiss.

2.9.3.1 Botanical description

Figure 2.9: *Euphorbia ingens*

Euphorbia ingens commonly known as "naboom", "gewone naboom" (Afrikaans), "mohlohlokgomo", "mokgoto" (Northern Sotho); "unHlonhlo" (Zulu); "Nkondze", "Nkonde (Tswana), Mukonde (Venda) (Joffe, 2001; Roux, 2004). This tree is a true xerophyte, i.e. it prefers a warm area and can survive in areas that go through long periods of drought or are generally very dry (Palgrave, 2002; Palgrave, 1956; Roux, 2004). The name is derived from the Afrikaans 'boom' meaning tree, and 'gnap' from Khoi meaning strong (Balkema, 1981; Esterhuyse *et al.*, 2001).

A succulent tree with a dark green crown that is well rounded and often shaped like hot-air (Roux, 2004). A tree with a massive, many-branched, rounded crown up to 10 m in height (Palgrave, 2002), usually grows on rocky outcrops or in deep sand within bushveld vegetation (Balkema, 1981; Roux, 2004). The branches are usually 4-5° (angled), up to 12 cm in diameter, segmented with parallel sides. Spines paired up to 2 mm long, or absent; spine shields forming separate cushions, often in the hollows of the margin. Inflorescence yellowish green flowers on the ridges (Palgrave, 2002; Roux, 2004). The stem is very brittle, and when broken exudes large quantities of milky sap or latex (Palgrave, 1956). The fruit is a round 3-lobed capsule up to 1,5 cm in diameter which turns red to purple when ripening and appear in August.

E. ingens is distributed throughout Kwazulu-Natal, Swaziland, Limpopo province (particularly Naboomspruit), Gauteng, North West province, Mozambique, Zimbabwe and further in tropical Africa (Balkema, 1981; Roux, 2004).

2.7.3.2 Uses and cultural aspect of *Euphorbia ingens*

The latex of this species is extremely toxic and can cause severe skin irritations. If it comes into contact with eyes it causes temporary or even permanent blindness. It causes severe illness to human and animals if swallowed (Palgrave, 2002; Roux, 2004). The Zulu use it as a drastic purgative in very small dose. The Sotho administers the latex for the cure of dipsomania (Watt & Breyer-Brandwyk, 1962). The Venda and Sotho use it as a cancer remedy. Branches are used as a fish poison in South Africa and Zimbabwe (Roux, 2004). The symptoms of a toxic dose are vomiting and acute abdominal colic with excessive and intractable purgation (Palgrave, 1956).

CHAPTER 3

Biological experiments and results

3.1 Selection of plants

During routine previous screening of species selected from several plant families in our laboratory, *Euphorbia clavaroides* was found to possess antimicrobial activity. Due to the unavailability of *E. clavaroides* plant material in large quantity, *E. ingens* was also selected for screening, as it is known that plants from the same family (section 2.7) may contain the same chemical compounds. Positive screening results lead to the selection of *E. ingens* for further research.

3.2 Collection and storage of plant materials

Fresh or dried plant material can be used as a source for secondary plant components (Eloff, 1998a). In the present study, fresh plant material was used. *Euphorbia clavaroides* was collected from the Potchefstroom area between June and July 2004. *Euphorbia ingens* aerial parts were obtained from Lowland's Nursery Keiroad, South Africa between October and November 2004. *E. clavaroides* was positively identified by Mr. P. Mortimer, the Curator of the Botanical Garden, North-West University (Potchefstroom Campus). Plant materials were stored in a freezer at approximately ± -4°C until time of use to prevent spoilage because of the high water content of these plants.

E. clavaroides was separated into aerial parts and roots. The aerial parts showed significant antimicrobial activity as it was reported from a routine screening in our laboratory. The total aerial part of *E. ingens* possessed interesting antimicrobial activity against microoragnisms (table 3.2; section 3.4.1). The total aerial part of *E. ingens* was divided into a fleshy inner part and a rind (figure 3.1) to reduce the complexity of the extracts and was also tested for antimicrobial activity (table 3.2 & 3.5). Extracts were prepared from each of the two sections and tested for antimicrobial activity.

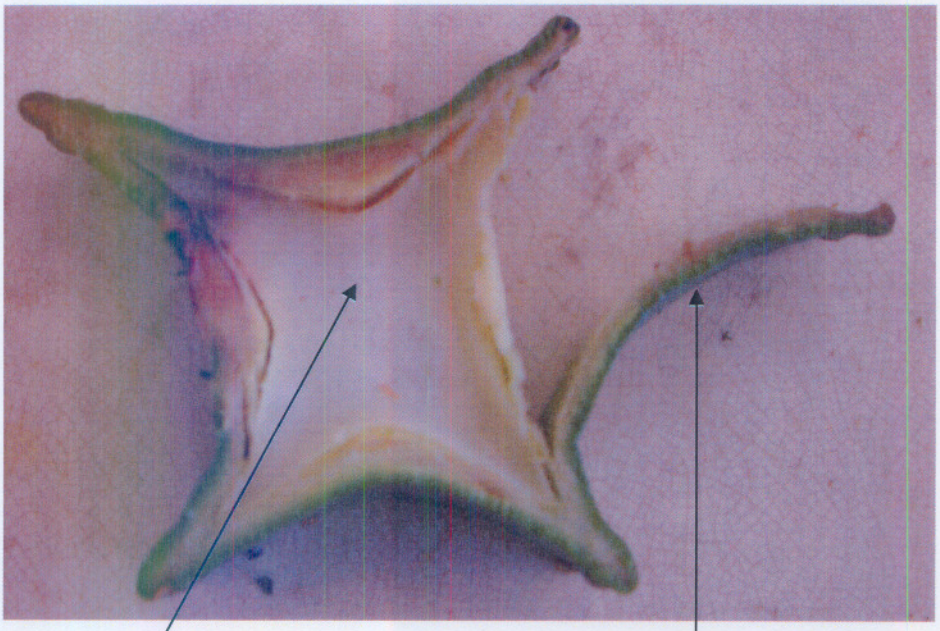


Figure 3.1: Cross section of *E. ingens* aerial part

3.3 Preparation of extracts and solvent extraction

Prior to the extraction, plant material was allowed to thaw for five hours and thereafter chopped into smaller pieces before being used. According to Fransworth (1994), the biggest problem in drug development from plants is to choose the appropriate solvents for extraction. If the type of compounds being isolated is known, selective solvent extraction will make the process safe (Williamson *et al.*, 1996). For the purpose of this study, plant material was extracted using a series of solvents in an increasing order of polarity. Petroleum ether was used as the first solvent to remove fixed oils and waxes. The following solvents were successively used:

- Petroleum ether (PE)
- Dichloromethane (DCM)
- Ethyl acetate (EtOAc)
- Ethanol (EtOH)

Increasing polarity

Soxhlet extraction is a convenient way to prepare crude extracts. The important advantages of soxhlet extraction are that plant material is separated from the extract and that fresh solvent continually flows through the plant material. Furthermore, the temperature of the system is close to the boiling point of the solvent, providing energy in the form of heat that helps to increase the extraction kinetics of the system (Ganzler & Salgo, 1987; Silva *et al.*, 1998).

This method is only suitable for compounds that can withstand high temperatures. This problem can be overcome by boiling at reduced pressure, but this was not used in this study.

The disadvantages of soxhlet extraction are that it requires several hours or days of extraction, the sample is diluted in large volumes of solvent, and losses of compounds occur due to thermal degradation and volatilization because of the heat supplied (Ganzler & Salgo, 1987).

The plant material was extracted for 24-48 hours with each solvent (starting with non-polar solvents), after which the extracts were concentrated using a rotary vacuum evaporator and allowed to dry completely in a fume hood.

3.3.1 Extracts obtained

The percentage (w/w) of the plant extracts were calculated by using the weight of the dried extract per weight of fresh plant material and are summarized in table 3.1.

Table 3.1: Percentage of extracts

Botanical name	Plant parts	Solvents	Fresh plant material (g)	Dry extracts (g)	Percentage yield (%)
E. clavaroides	AP	PE	300	1,02	0,34
		DCM		0,7	0,23
		EtOAc		5,5	1,83
		EtOH		0,9	0,3
E. ingens	Total AP	PE	108,9	0,81	0,74
		DCM		0,28	0,26
		EtOAc		0,69	0,63
		EtOH		0,80	0,74
	CRP	PE	351,8	1,8	0,51
		DCM		1,07	0,30
		EtOAc		6,1	1,73
		EtOH		0,4	0,11
	FIP	PE	508,1	0,17	0,03
		DCM		0,2	0,04
		EtOAc		0,24	0.05
		EtOH		0,37	0,07

AP = Aerial parts, CRP = Rind, FIP = Fleshy inner parts; PE = Petroleum ether, DCM = Dichloromethane, EtOAc = Ethyl acetate EtOH = Ethanol.

3.4 Primary biological screening of plant extracts

In order to find new drugs in plants, it is necessary to screen plant extracts for the presence of novel compounds and to investigate their biological activities (Hostettmann *et al.*, 2000). The primary screening of the selected plants was done by evaluating the plant extracts that possessed antimicrobial activity. This procedure was significant because further studies were conducted on plant extracts which possessed the best antimicrobial activity. The biological assays employed were chosen because of their simplicity, reproducibility, sensitivity and relatively low cost while being rapid and simple at the same time. The following methods were used for determination of antimicrobial activity from plant extracts: the disc diffusion assay (section 3.4.1.1) and the microplate method (section 3.4.1.2). The microplate method was used to calculate minimum inhibitory concentrations (MIC - values) for the extracts.

In order to compare the toxicity of the extracts to its MIC values, the *in vitro* toxicity profile of plant extracts (table 3.6) were determined. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was chosen for its simplicity and ease of determination, as no specialised equipment is required.

3.4.1 Antimicrobial screening assay

The following test organisms were collected from the Department of Microbiology North-West University (Potchefstroom campus) and are commonly used for the primary screening of the extracts. Gram-positive bacteria: *Bacillus subtilis* [ATCC 6633], *Staphylococcus aureus* [ATCC 6538], Gram-negative bacteria: *Escherichia coli* [ATCC 8739], *Pseudomonas aeruginosa* [ATCC 9027] and a Yeast: *Candida albicans* [ATCC 10231]. All this organisms are important nosocomial pathogens widely used in screening test and known to cause resistance to available antibiotics (5.1.1).

3.4.1.1 Disc diffusion assay

The method as described by van der Vijver and Lötter (1979) with a slight modification was used to establish the antimicrobial properties of the crude extracts and isolated compounds. The growth medium containing 16 g/ℓ nutrient broth (Rolab-Merck) and 12 g/ℓ bacteriological agar (Rolab-Merck) was sterilised for 15 minutes at 120°C. Before pouring into petri dishes, it was allowed to cool down enough to hold by hand. The

growth medium was not seeded with the test organisms before pouring, but 100 µl of a 24 hour nutrient broth culture was spread evenly over the solid agar surface.

The dried plant extracts (table 3.1) were reconstituted in 1 ml of acetone. Acetone was chosen because of its high solubility to plant extracts and fast evaporation. Filter paper discs were soaked in these solutions for a few minutes, removed with tweezers and left to air-dry for an hour to allow evaporation of all solvents from the discs before being used in the assay. The discs were placed onto the inoculated agar plates and incubated at 37°C for 24 hours for the bacteria and 48 hours for the yeast. After incubation, the plates were examined for zones of growth inhibition. The zones of inhibition were measured from the end of the disc to the end of the inhibition zone in millimetres (mm). The results of this assay are depicted in table 3.2.

Table 3.2: Antimicrobial activity of screened plant extracts

Botanical name	Plant parts	Solvents	Solution [g/ml]	Test organisms			
				B.s	S.a.	E.c.	P.a.
E. clavaroides	AP	DCM	0,7	1	2	-	-
		EtOAc	5,5	3	2	-	1
		EtOH	0,9	-	1	-	-
E. ingens	Total AP	DCM	0,28	2	5	-	-
		EtOAc	0,69	7	10	1	-
		EtOH	0,80	4	6	-	-
	CRP	DCM	1,07	1	1	-	-
		EtOAc	6,1	2	1	-	1
		EtOH	0,4	-	1	-	-
	FIP	PE	0,17	-	3	-	-
		DCM	0,2	3	4	-	-
		EtOAc	0,24	3	4	1	1
		EtOH	0,37	1	1	-	-

AP = Aerial parts, CRP = Rind, FIP = Fleshy inner parts; B.s = *Bacillus subtilis*, S.a = *Staphylococcus aureus*, E.c = *Escherichia coli* and P.a = *Pseudomona aeruginosa*; PE = Petroleum ether, DCM = Dichloromethane, EtOAc = Ethyl acetate EtOH = Ethanol; Number represent the size of the inhibition zone in mm, Dash represent no inhibition zone.

The size of an inhibition zone is influenced by the concentration of the extracts, diffusion of the active compounds from the filter paper into the agar and the activity of the compounds present in the extract.

After the initial screening of the raw extracts, it was determined that the petroleum ether extracts had no activity with the exception of the petroleum ether extracts of the fleshy

inner part of *E. ingens*. None of the extracts exhibited activity against *Candida albicans* (table 3.2).

During fractionation and isolation process, a number of active fractions were identified. The active compound(s) identified from these fractions could not be determined due to insufficient quantities (table 3.3). The active compound from fraction EFI4X3 was identified as kaempferol (figure 4.1). These fractions (table 3.3) were only tested against *B. subtilis* and *S. aureus* because the extracts of the total aerial part of *E. ingens* showed best inhibition zone against *B. subtilis* and *S. aureus* only. The MIC values of these fractions were not determined because disc diffusion assay was selected as a bio-guided fractionation approach, simple to determine the activity of the fractions in short period. The isolation procedures of these fractions are described in section 4.2.

Table 3.3: Antimicrobial activity of the fractions

Fractions	Test organisms		Fractions	Test organisms	
	B.s	S.a		B.s	S.a
DFI4	0,5	1	EFI1	1	1
DFI5	3	3	EFI4	2	1
DFI6	2	2	EFIX	3	2
DFI52	1	0,5	EFI4X	2	2
ECR2	-	2	EFI4Y	2	2
ECR3	3	4	EFI4X3	2	1
ECR32	2	-	EFIX2	2	-
ECR33	2	-	EFIX3	3	1
ECR34	1	-	EFIX4	3	2
ECR32X	2	-	EFIX5	3	-

B.s = *Bacillus subtilis* & *Staphylococcus aureus;* Number represent the size of the inhibition zone in mm, Dash represent no inhibition zone.

3.4.1.2 Minimum inhibitory concentration determination for plant extracts

Determining the minimum inhibitory concentration with the serial dilution method gives a better indication of antimicrobial activity as problems with diffusion into the agar are eliminated. MIC values were determined by serial dilution of extracts beyond the level where no inhibition of growth of test organisms was observed (Eloff, 1998b). The MIC value was regarded as the lowest concentration of the extracts or compounds inhibiting visible growth of each microorganism.

3.4.1.2.1 Preparation of extracts

The plant extracts (table 3.1) were suspended in 1 ml of H_2O:DMSO (75:25) to prepare the relevant concentrations. The prepared concentrations were variable and ranged from 15,2 mg/ml to 115,2 mg/ml. The concentrations varied because the amount of dried extracts obtained during the preparation of extracts varied (table 3.1).

3.4.1.2.2 Standardisation of microbial culture

Microorganisms were incubated in 50 ml Mueller-Hinton broth (Fluka) and left to grow for 24 hours at 37°C before being used in the test. Tween 80 (500 µl) was added to *B. subtilis* and *C. albicans* before being incubated, in order to break up the colonies, thus producing a more homogenous suspension of microorganisms. After incubation, broth cultures were diluted with sterile Mueller-Hinton broth to contain approximately 10^7 colony forming units/ml. Dilutions were monitored by measuring the absorbance at 500 nm with a spectrophotometer (Miton Roy Spectronic 1201) to ensure that they contain appropriate cell concentrations (table 3.4; Swart, 2000).

Table 3.4: Absorbance values of different microorganisms at 500 nm used to prepare standardised cultures.

Microorganisms	Absorbance (nm)
B. subtilis	0,120
S. aureus	0,030
E. coli	0,120
P. aeruginosa	0,045
C. albicans	0,150

3.4.1.2.3 Preparation of test 96 well microtitre plate

100 µl of the sterile broth was pipetted into all microplate wells. Thereafter, 100 µl of the prepared extracts were added to the first set of wells and two-fold serial dilutions were made from well 1 to 10 in the microplate. In addition, 100 µl of standardised microorganism cultures were added to all the wells except the wells of column 11 (0% growth control). The wells of column 12 contained only broth and microorganims (100% growth control). The plates were then incubated for 24 hours at 37°C. After 24 hours of incubation, 20 µl of 0,2 mg/ml *p*-iodonitrotetrazolium violet [INT] (Sigma) was added to all the wells. With further incubation for 10–30 minutes, bacterial growth was indicated

by a colour change to red. p-INT is a dehydrogenase activity detecting reagent, which is converted into a corresponding intensely coloured formazan by metabolically active microoganisms. The MIC values obtained are depicted in table 3.5.

Table 3.5: The MIC values (mg/ml) determined for crude plant extracts

Botanical name	Plant parts	Solvents	Solution [mg/ml]	Test organisms				
				B.s	S.a	E.c	P.a	C.a
E. clavaroides	AP	PE	64,9	4,06	32,45	8,11	8,11	4,04
		DCM	16,4	2,05	4,10	2,05	2,05	1,04
		EtOAc	27,9	1,74	3,49	0,87	3,49	3,49
		EtOH	23,7	0,74	2,96	0,74	2,96	1,48
E. ingens	Total AP	PE	15,4	-	-	-	-	7,7
		DCM	51,4	25,7	12,85	12,9	-	12,9
		EtOAc	52,7	6,59	6,59	13,2	13,2	26,4
		EtOH	115,2	28,8	7,2	14,4	14,4	28,8
	CRP	DCM	15,2	2,09	7,6	-	-	7,6
		EtOAc	15,8	0,95	1,98	7,6	7,6	7,9
		EtOH	17,6	2,2	-	8,8	-	-
	FIP	DCM	20,2	-	10,1	-	-	-
		EtOAc	20,6	0,64	5,15	10,3	10,3	5,15
		EtOH	20,8	5,2	10,4	-	-	-

AP = Aerial parts, CRP = Rind, FIP = Fleshy inner parts; B.s = *Bacillus subtilis*, S.a = *Staphylococcus aureus,* E.c = *Escherichia coli* and P.a = *Pseudonoma aeruginosa* and C.a = *Candida albicans;* PE = Petroleum ether, DCM = Dichloromethane, EtOAc = Ethyl acetate EtOH = Ethanol; Number represent the MIC values; Dash represents lack of activity.

The ethyl acetate extracts of *Euphorbia ingens* showed a broad spectrum of activity against the range of microorganisms tested. Although the rind section of *E. ingens* had the lowest MIC values, the fleshy inner part was selected for further study as the problem posed by chlorophyll could be eliminated. After the initial screening of the raw extracts, it was determined that the petroleum ether extracts of both the fleshy inner part and rind section had no activity with the exception of the petroleum ether extracts of the aerial parts of both *E. ingens* and *E. clavariodes*.

3.4.2 Toxicity testing

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was first described by Mosmann in 1983. This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable cells.

To measure the amount of formazan product formed, the formazan crystals must be solubilised by the addition of an organic solvent (isopropanol) to produce a homogeneous solution suitable for measurement (Mosmann, 1983). The colour can then be quantified using a simple colorimetric assay. The results were measured by a multiwell scanning spectrophotometer (Labsystems iEMS reader MF). The number of surviving cells is directly proportional to the level of the formazan product formed and thus the absorbance is directly proportional to the number of viable cells.

3.4.2.1 Determination of cell density using regression curve

Cells density determination was estimated by plotting the regression curve (figure 3.2) of absorbance against the number of cells (million). The linear regression curve was used to calculate the coefficient of determination (R^2) at a test wavelength of 560 nm and a reference wavelength of 650 nm.

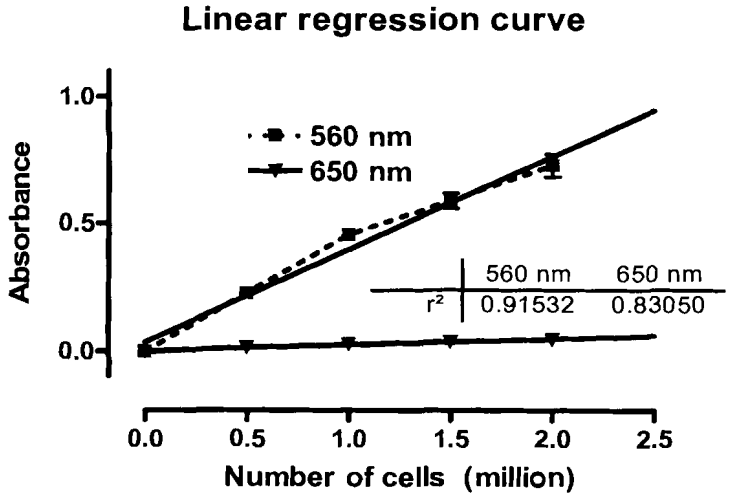


Figure 3.2: A linear regression curve indicating cell viability at different wavelengths.

3.4.2.2 Standardisation of the cell culture

The toxicity evaluation of the prepared extracts (table 3.1) were performed on human epithelial cell lines (HeLa) in DMEM medium containing 10% (v/v) of heat inactivated delta fetal bovine serum (FBS) (1:1) and 5 ml streptomycin, respectively. The media was replaced every second day and the cells were trypsinised weekly, and then allowed to reach confluency before being used in the toxicity assays. After washing with phosphate buffer solution (PBS) (2 ml) and trypsinisation with trypsin-EDTA (2 ml), a

single cell suspension was obtained. Cell density was adjusted to 1,5 x 10^6 cell/ml (figure 3.2). An equal volume of the single cell suspension and trypsin-EDTA liquid was mixed and a cell count (cell density) was performed by visualising with a microscope. The stock cell suspension was then diluted to the required volume (1,5 x 10^6 cell/ml) with DMEM culture medium containing FBS and 5 ml streptomycin using equation 3.1.

$$VCR = \frac{CD \times FVSS}{NCSC}$$ **Equation 3.1**

CD = Cell density (1,5 x 10^6 cell/ml)
FVSS = Final volume of single cell + DMEM required to seed
NCSC = Number of counted single cells available
VCR = Volume of cells required
Single cell = Cell suspension + 2 ml of trypsin-EDTA

3.4.2.3 Preparation of the extracts

The prepared extracts (table 3.1) were dissolved in H_2O:DMSO (99:1) in concentrations of 50 mg/ml. Dilutions and an appropriate control (H_2O:DMSO) were made. Dilutions prepared were 10 mg/ml, 2 mg/ml, 0,4 mg/ml and 0,08 mg/ml.

3.4.2.4 Preparation of microtiter plate

The 24-well microtiter plates were used in the toxicity assay. 1000 µl of standardised cell cultures (1,5 x 10^6 cell/ml) were pipetted into wells of column 1 through column 4 and was incubated under humidified conditions at 37°C and 5% CO_2 for 6-7 hours. The culture was aspirated after incubation, 400 µl of DMEM media were added to the wells of column 1 through to 4. 100 µl of each prepared extract (table 3.1) dilution were added to wells of column 2 through to 4 and 100 µl of H_2O:DMSO (99:1) to the wells of column 1 (control). In column 5 (blank), 500 µl of DMEM media were added to every well to obtain the same volume as the rest of the wells. The plates were incubated under humidified conditions at 37°C and 5% CO_2 for 24 hour.

The wells of column 5 served as a 0% growth control (blank control) to give an indication of contamination, while the wells of column 1 served as a 100% cellular

growth control to ensure that normal growth occurs. The assay was conducted in triplicate.

3.4.2.5 Preparation and addition of MTT

The stock solution of MTT (5 mg/ml PBS) was filter sterilised and stored at 4°C until required. After a 24 hour incubation period, 200 µl of the prepared MTT solution (0,25 mg/ml PBS) were added to all wells and the plates were incubated for a further 2 hours to terminate cell growth or MTT cleavage. This was performed in the laminar flow chamber with the light off.

After a further 2 hour incubation period, the MTT supernatant was aspirated from each well. The reaction was stopped and the formazan crystals solubilised with the addition of 250 µl of isopropranol to each well. The plates were shaken to allow the isopropranol to dissolve the formazan crystals completely. 100 µl of the solution were transferred from each well to a 96 well plate. The absorbance of each well was measured at a test wavelength of 560 nm and a reference wavelength of 650 nm using a microplate reader (Labsystems iEMS reader MF). Results were expressed as a percentage cellular viability of the controls, using equation 3.2.

$$\% \text{ Cellular viability} = \frac{\Delta \text{ Absorbance} - \Delta \text{ Blank} \times 100}{\Delta \text{ Control} - \Delta \text{ Blank}} \qquad \textbf{Equation 3.2}$$

Where Δ Control (Mean cell control) = Cell control$_{560}$ – Cell control$_{650}$

Δ Blank = Mean blank$_{560}$ – Mean blank$_{650}$

Δ Absorbance = Absorbance$_{560}$ – Absorbance$_{650}$

The percentage cellular viability was plotted against the log concentration of the extract [mg/ml] by using the Prism 4® program. The LD_{50} values for the different extracts were determined. The concentration response curves were characteristically sigmoidal after logarithmic transformation of the concentration (figure 3.3).

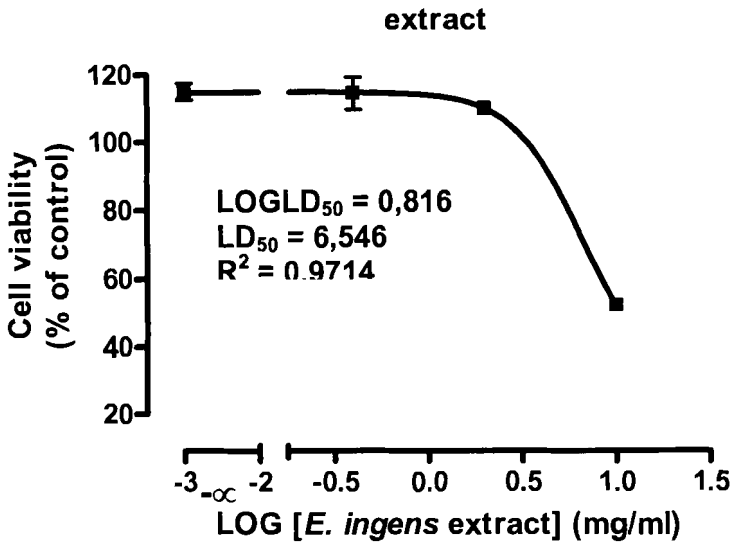


Figure 3.3: Percentage cellular viability plotted of the ethanol extract of *Euphorbia ingens* fleshy inner parts

The LD_{50} values with the coefficient of determination (R^2) of the different extracts are shown in table 3.6.

The security index is used to determine the toxicity of extracts or compounds. A security index greater than 100 (SI >100) is an indication of non toxic versus the activity (MIC values) of the extracts. Equation 3.3 was used to calculate the security index as mentioned in table 3.7 for the comparison of SI with MIC values of the extracts.

$$SI = \frac{LD_{50}}{MIC}$$ **Equation 3.3**

Where SI = security index

MIC values = minimum inhibitory concentrations

LD_{50} (Lethal dose 50%) is the amount of extract given all at once, which causes the death of 50% (one half) of HeLa cell lines.

Table 3.6: Toxicity of the extracts

Botanical name	Plant part	Extracts	LD_{50} (mg/ml)	R^2
E. clavaroides	AP	PE	2,288	0,8673
		DCM	37,42	0,8279
		EtOAc	>500	0,9397
		EtOH	235,1	0,9874
E. ingens	CRP	PE	>500	0,8662
		DCM*	10,01	0,3791
		EtOAc	11,14	0,9549
		EtOH	7,523	0,8857
	FIP	PE*	0,3349	0,09
		DCM	227	0,8475
		EtOAc	75,03	0,7895
		EtOH	6,546	0,9714

AP = Aerial parts, CRP = Rind, FIP = Fleshy inner parts; PE = Petroleum ether, DCM = Dichloromethane, EtOAc = Ethyl acetate EtOH = Ethanol; *was not properly dissolved in 1ml (99% H_2O:1% DMSO).

Table 3.7: Comparison of security index (SI) and MIC values for the extracts

Botanical Name	Pp	Extraction Solvent	Extract [mg/ml]	Test organisms MIC (mg/ml)										LD_{50}(mg/ml)
				B.s		S.a		E.c		P.a		C.a		
				MIC	SI	MIC	SI	MIC	SI	MIC	SI	MIC	SI	
E. clavaroides	AP	PE	64,9	4,06	0,56	32,5	0,07	8,11	0,28	8,11	0,28	4,04	0,57	2,29
		DCM	16,4	2,05	18,2	4,10	9,1	2,05	18,2	2,05	18,2	1,04	36	37,4
		EtOAc	27,9	1,74	>143	3,49	>143	0,87	>143	3,49	>143	3,49	>143	>500
		EtOH	23,7	0,74	317,6	2,96	79,4	0,74	317,6	2,96	79,4	1,48	158,8	235
E. ingens	CP	PE	16,7	-	-	-	-	-	-	-	-	-	-	>500
		DCM	15,2	2,09	4,8	7,6	1,3	-	-	-	-	7,6	1,3	10,01*
		EtOAc	15,8	0,95	11,7	1,98	5,6	7,6	1,5	7,6	1,5	7,9	1,4	11,14
		EtOH	17,6	2,2	3,4	-	-	8,8	0,85	-	-	-	-	7,5
	FP	PE	16,5	-	-	-	-	-	-	-	-	-	-	0,335*
		DCM	20,2	-	-	10,1	22,5	-	-	-	-	-	-	227
		EtOAc	20,6	0,64	117,2	5,15	14,6	10,3	7,3	10,3	7,3	5.15	14,6	75
		EtOH	20,8	5,2	1,26	10,4	0,63	-	-	-	-	-	-	6,55

PE = Petroleum ether, DCM = Dichloromethane, EtOAc = Ethyl acetate, EtOH = Ethanol: AP = Aerial parts, CP = Rind, FP = Fleshy inner parts; B.s = *Bacillus subtilis*, S.a = *Staphylococcus aureus,* E.c = *Escherichia coli,* P.a = *Pseudonoma aeruginosa* and C.a = *Candida albicans*; *was not properly dissolved in 1ml (99% H_2O:1% DMSO).

CHAPTER 4

Isolation of active compound(s) from *Euphorbia ingens*

Extracts selected for study were purified by chromatographic techniques.

4.1 Chromatographic techniques

4.1.1 Thin-layer chromatography (TLC)

Analytical TLC was performed on 0,25 mm thick silica gel aluminium backed sheet (Merk® TLC aluminium sheet gel 60 F_{254}). TLC was employed in the selection of a suitable mobile phase for the isolation of compounds with column chromatography. Preparative TLC was performed on 2,0 mm thick silical gel glass backed sheets (Separation® Pre-coated TLC plate SIL G - 200 UV_{254}). During examination of chromatograms for the detection of the individual compounds only UV-light was used and spraying reagents (5% H_2SO_4 in ethanol) did not detect individual compound(s).

4.1.2 Column chromatography

Column chromatography was performed with glass column of different sizes. The stationary phase used was silica gel (Merk®; 0,063 – 0,2 mm). The plant extracts were dissolved in a small amount of mobile phase and applied to the column bed with a pasteur pipette.

4.1.3 Preparative thin-layer chromatography

Prep-TLC plates were developed in the dichloromethane prior to use to remove dust or contaminants from the plates (silica gel). Fractions were applied in a band across the prep-TLC plate at least 15 mm from the bottom of the plate and within 10 mm from the sides. Plates were developed in dichloromethane:ethyl acetate (1:3) as a mobile phase and the bands were visualised under ultraviolet light (254 and 360 nm), marked and scraped from the glass plate for the extraction of the components. Fractions were extracted from silica gel with acetone as a solvent and concentrated using a rotary vacuum evaporator and allowed to dry completely in a fume hood.

4.2 Isolation of the active compound(s) from *E. ingens*

When undertaking an investigation of a plant to identify the active compounds, it is impossible to isolate all the constituents. Among the hundreds or thousands of different substances, one or a few are responsible for the therapeutic action (or toxicity) (Hostettmann *et al.*, 2000). It is necessary, therefore, to use the bioassay-guided fractionation procedure to identify active fractions and pure active compounds (Hostettmann *et al.*, 2000; Williamson *et al.*, 1996). Bioassay-guided fractionations should be sensitive because the active substances may be present in the plant in very low concentrations (Hostettmann *et al.*, 2000). Disc diffusion assay was used during fractionation procedure to select active fractions leading to pure compound(s) due to the simplicity, reproducibility, sensitivity and relatively low cost while being rapid and simple at the same time.

1,85 kg of fresh plant material of the *E. ingens* fleshy inner part was extracted with each solvent starting with non-polar solvents (petroleum ether, dichloromethane, ethyl acetate and ethanol) (section 3.3). The dichloromethane and ethyl acetate extracts of the fleshy inner parts and ethyl acetate extract of the chlorophyll rich part were chosen because of the interesting activity against Gram-positive bacteria in both the disc diffusion and MIC assay.

The resulting ethyl acetate extract was immediately separated into organic and aqueous phase. These phases were subjected to antimicrobial assay and organic phase showed excellent activity against Gram-positive bacteria *B. subtilis* (3 mm) and *S. aureus* (4 mm) (table 3.2).

The resulting ethyl acetate extract (6,5 g) of *E. ingens* (fleshy inner part) organic phase was fractionated by column chromatography with silica gel as a stationary phase using dichloromethane:ethyl acetate (3:1) as a mobile phase (figure 4.1). Eight fractions were collected based on similarities in TLC: EFI1, EFI2, EFI3, EFI4 and EFIX. These fractions were subjected to antimicrobial activity using the disc diffusion assay. Fraction EFI4 (170,2 mg) showed the best antimicrobial activity when tested, other fractions were slightly activity (table 3.3).

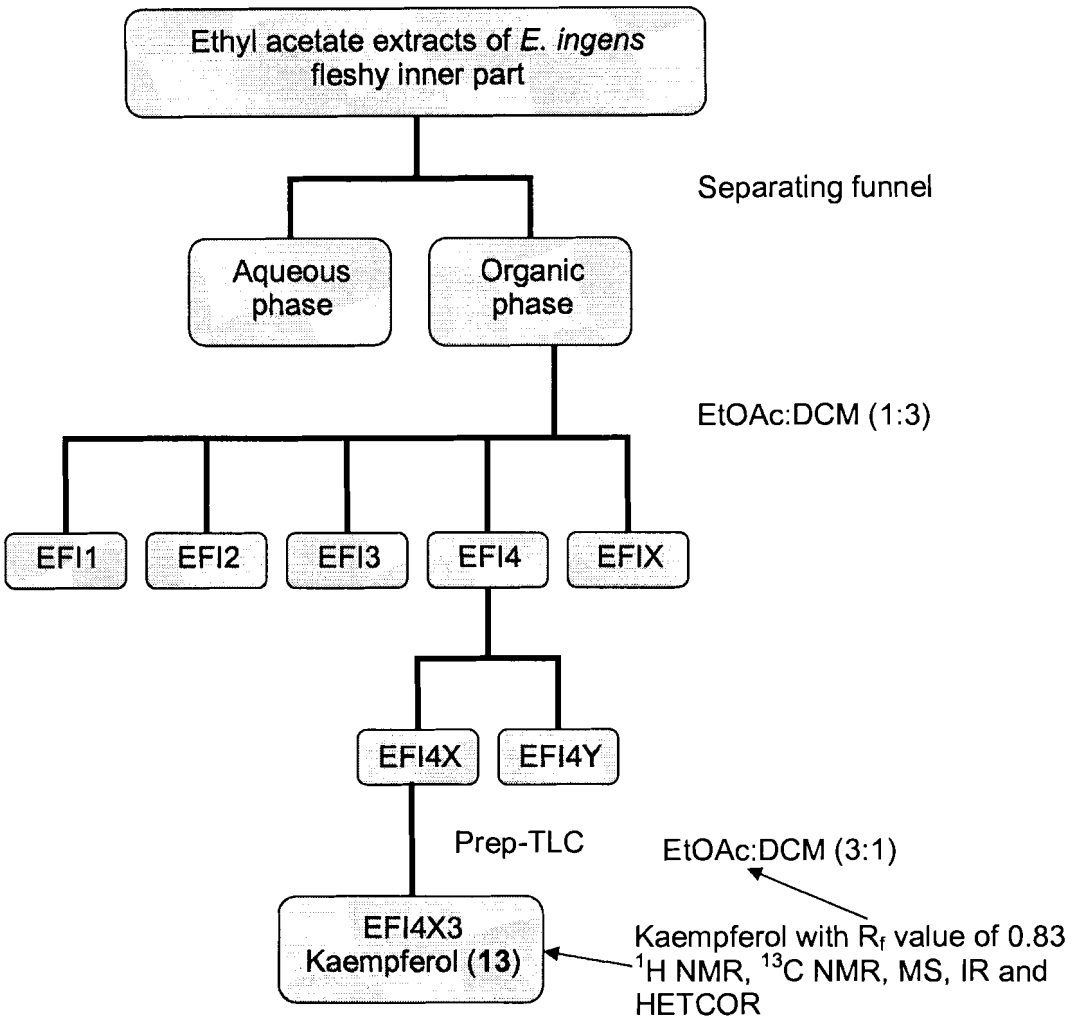


Figure 4.1: Isolation flowchart for the ethyl acetate extract of *E. ingens* fleshy inner part.

Fraction EFI4 was further fractionated into fractions EFI4X and EFI4Y and therefore subjected to the disc diffusion assay. Fraction EFI4X showed activity against Gram-positive microorganisms (table 3.3). EFI4X (159,5 mg) was further purified by prep-TLC plate (section 4.2.3) using dichloromethane:ethyl acetate (1:3) as mobile phase. Only fraction EFI4X3 (26,1 mg) with an R_f value of 0,83 in dichloromethane:ethyl acetate (1:3) was found to be pure.

Fraction EFI4X3 (**13**) was identified as kaempferol by comparing its ^{1}H, ^{13}C-NMR, HETCOR and MS data with that reported in the literature (Lee & Wu, 2001; Lin *et al.*, 2000).

Fractions EFIX (figure 4.1) showed minimum activity against Gram-positive bacteria *B. subtilis* and *S. aureus* (table 3.3). Fraction EFIX was eventually fractionated on a silica gel column once more with dichloromethane:ethyl acetate (1:3) as a mobile phase (figure 4.2). Collected fractions were subjected to antimicrobial activity. Fraction EFIX3 (spectrum **9**) showed activity against Gram-positive bacteria *B. subtilis* and *S. aureus* and EFIX2 (spectrum **8**) showed activity against *B. subtilis* with an R_f value of 0,76 in dichloromethane:ethyl acetate (1:3) (table 3.3). The quantity was not sufficient for further purification or analysis of these fractions, but ^{1}H NMR spectra (spectrum **8** and **9)** were obtained.

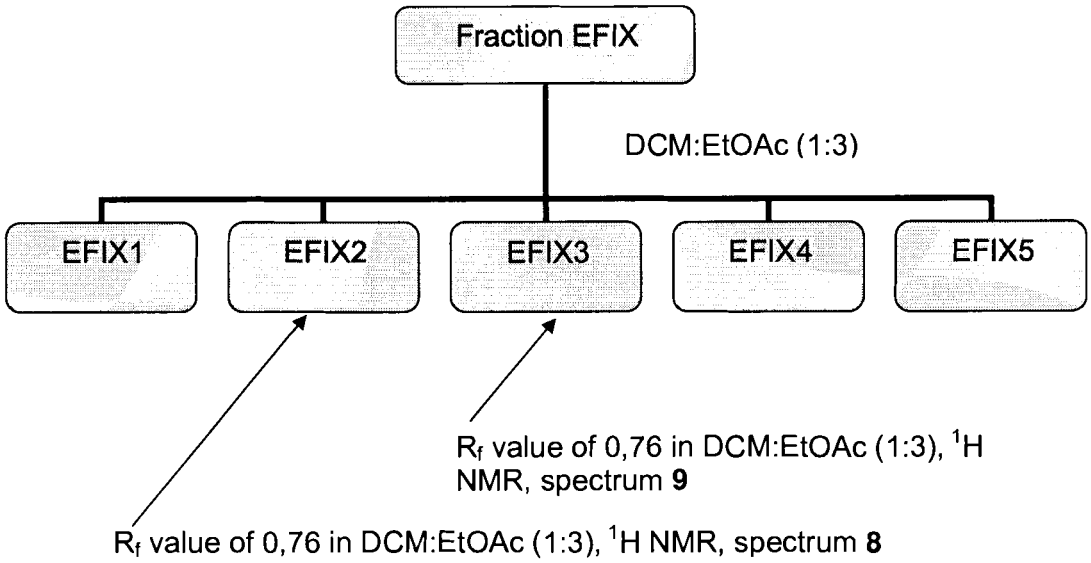


Figure 4.2: Isolation flowchart for the ethyl acetate extract of *E. ingens* fleshy inner part.

The dichloromethane extract (5,7 g) of *Euphorbia ingens* fleshy inner part was fractionated on a silica gel column using petroleum ether:dichloromethane:ethanol (6:1:1) as a mobile phase. Six fractions were collected: DFI1, DFI2, DFI3, DFI4, DFI5 and DFI6. These fractions were subjected to antimicrobial assay and fractions DFI3, DFI4 and DFI5 showed activity against the Gram-positive bacteria *B. subtilis* and *S. aureus* (table 3.3).

Fraction DFI5 (351,5 mg) was further purified by column chromatography with silica gel as a stationary phase using petroleum ether:ethyl acetate (1:3) as a mobile phase. The collected fractions were tested for antimicrobial activity and only fraction DFI52

(spectrum **7**) with an R_f value of 0,71 in petroleum ether:ethyl acetate (1:3) was active against Gram-positive bacteria *B. subtilis* and *S. aureus* (table 3.3). The quantity was not sufficient for further purification or analysis of this fraction, but a ^1H NMR spectrum (spectrum **7**) was obtained.

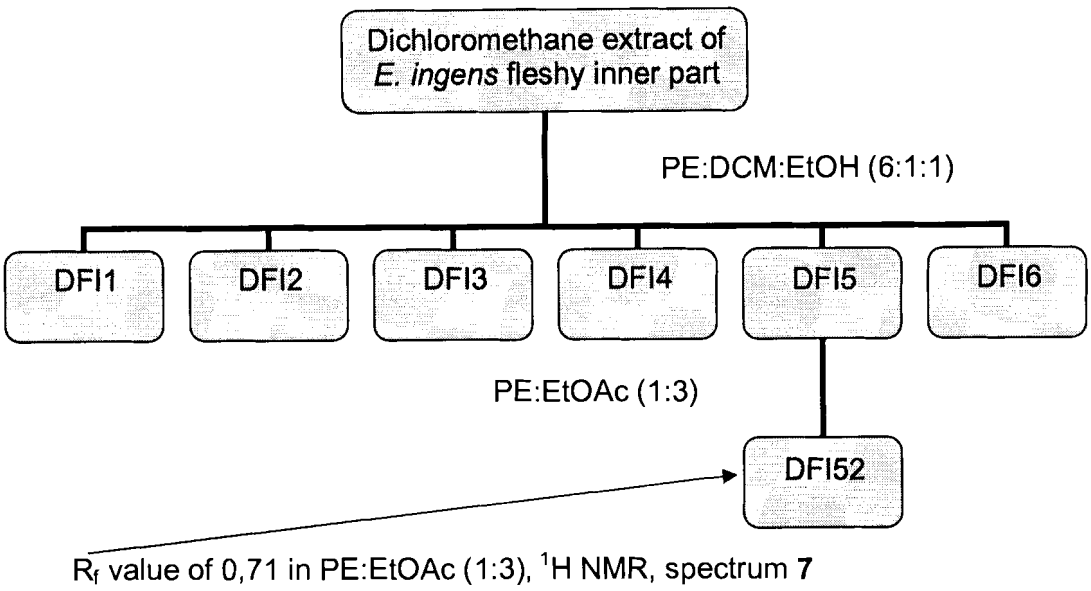


Figure 4.3: Isolation flowchart for the dichloromethane extract of *E. ingens* fleshy inner part.

The ethyl acetate extract (4,4 g) *Euphorbia ingens* rind section was fractionated on a silica gel column using petroleum ether:ethyl acetate (1:2) as a mobile phase. Seven fractions were colleted: ECR1, ECR2, ECR3, ECR4, ECR5, ECR6 and ECR7. Fractions ECR3 (752,1 mg) showed activity against Gram-positive bacteria *B. subtilis* and *S. aureus* (table 3.3). This fraction was further chromatographed on a silica gel using dichloromethane:ethyl acetate (3:1) as a mobile. Because of good resolution in TLC, activity against Gram-positive bacteria *B. subtilis* and *S. aureus* (table 3.3) and sufficient quantity, fraction ECR32 was selected further purification.

Fraction ECR32 (172,3 mg) was further fractionated by column chromatography on silica gel as a stationary phase and dichloromethane:ethyl acetate (1:3) as a mobile phase. The collected fractions were tested for antimicrobial activity and fraction ECR32X showed antimicrobial activity against Gram-positive bacteria *B. subtilis* (table

3.3) with an R_f value of 0,78 in dichloromethane:ethyl acetate (1:3). The quantity was not sufficient for further purification or analysis, but a ^1H NMR spectrum (spectrum **10**) was obtained (figure 4.4).

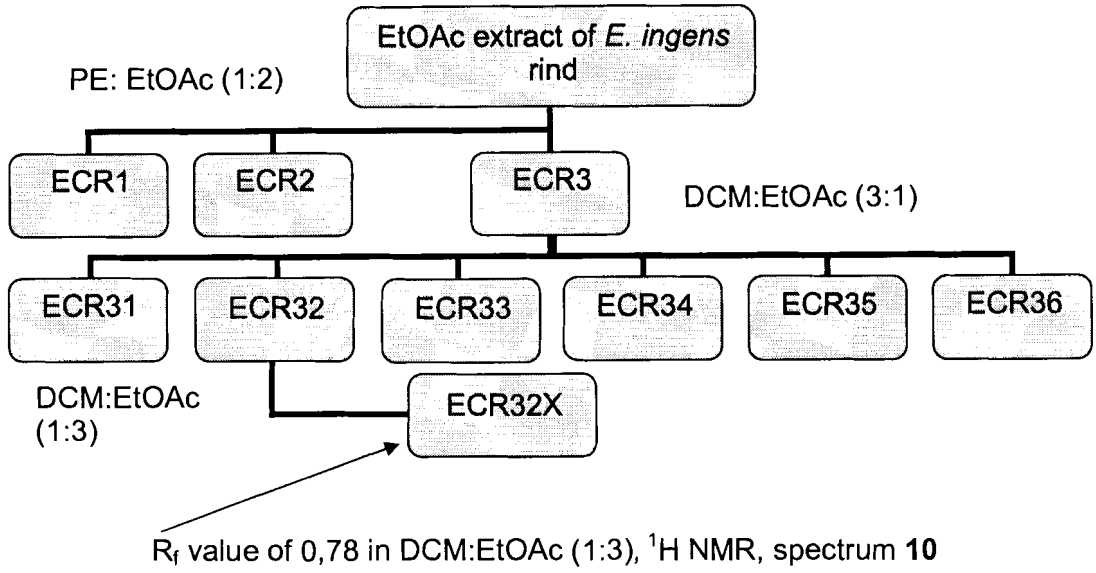


Figure 4.4: Isolation flowchart for the ethyl acetate extract of *E. ingens* rind.

4.3 Characterisation of compound(s) isolated from *E. ingens*

4.3.1 Instrumentation

4.3.1.1 Nuclear magnetic resonance spectroscopy (NMR)

The ^{13}C and ^1H NMR spectra were recorded on a Varian Gemini-300 spectrometer. ^{13}C NMR spetra were recorded at 75,462 MHz while the ^1H NMR spectra were recorded at 300,075 MHz. The chemical shifts are reported in ppm (parts per milliom) relative to tetramethylsilane. The following abbreviations were used to describe the multiplicity of ^1H NMR signals: s = singlet and d = doublet. NMR sample were dissolved in deutirated methanol (CD3OD).

4.3.1.2 Infrared spectroscopy (IR)

The IR spectra were recorded on a Nicolet Magna-IR 550 spectrometer, with the use of KBr pellets.

4.3.1.3 Mass spectroscopy (MS)

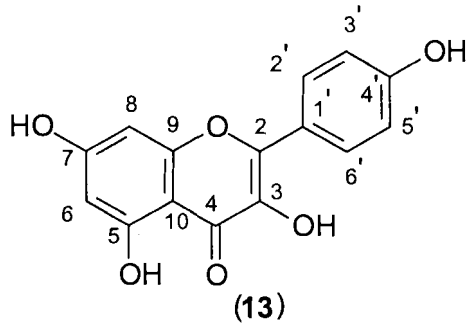
The mass spectra were recorded on an analytical VG 7070E mass spectrometer using fast atomic bombardment (FAB) at 70 eV as ionisation technique.

4.3.1.4 Melting point determination

Melting points (mp) were determined by differential scanning calorimetry (DSC). DSC thermograms were recorded with a shimadzu DSC-50 instrument. Measurement conditions were as follow: sample weight of approximately 1,804 mg, an aluminium crimp cell sample holder, nitrogen gas flow at 40 ml/min and heating rate at 10°C/min.

4.3.2 Characterisation of compound (13)

The physical data of the isolated compound (**13**) corresponded to that described in the literature (Barbera *et al.*, 1986; Lee & Wu, 2001; Lin *et al.*, 2000; Markham *et al.*, 1979; Matthes *et al.*, 1980; Nawwar *et al.*, 1984; Panichayupakaranant & Kaewsuwan, 2004; Wagner *et al.*, 1976). The structure of compound **13** was established as kaempferol (3,4',5,7-tetrahydroxyflavone).



(13)

Figure 4.2: Structure of kaempferol (compound **13**) (Lee & Wu, 2001; Lin *et al.*, 200).

Compound **13**: Yellow powder; mp.= 271,9°C (lit. mp 274-276); R_f = 0,83 (DCM: EtoAc 1:3); $C_{15}H_{10}O_6$; FAB MS *m/z* (%), (spectrum 1): 287 (17), 239 (5), 213 (4), 201 (7), 189 (8), 176 (17), 165 (20), 154 (94), 149 (26), 136 (100), 128 (24), 121 (45), 115 (37), 107 (90); IR (KBr) ν_{max} (spectrum 2; cm^{-1}): 3300, 1660, 1610, 1570, 1520, 1180, 1090, 1010; δ_H (CD_3OD; spectrum 3): 6,165 (d; 1H; J = 2,03 Hz; H-6), 6,364 (s; 1H; J = 2,06 Hz; H-8), 6,889 (d; 2H; J = 9,79 Hz; H-3';H-5'), 8,053 (d; 2H; J = 9,86 Hz; H-2'; H-6'); δ_c (CD_3OD; spectrum 4): 94,5344 (C-8), 99,3281 (C-6), 104,507 (C-10), 116,301 (C-3'; C-5'), 123,695 (C-1'), 130,640 (C-2'; C-6'), 137,030 (C-3), 148,077 (C-2), 158,170 (C-9),

160,406 (C-4'), 162,360 (C-5), 165,514 (C-7), 177,281 (C-4); HETCOR δ_H (CD_3OD, spectrum 5): 6,17 (d; H-6), 6,37 (s; H-8), 6,89 (d; H-3'; H-5'), 8,05 (d; H-2'; H-6'); δ_c 99,3281 (C-6), 94,5344 (C-8), 166,301 (C-3'; C-5'), 130,640 (C-2'; C-6').

CHAPTER 5

Discussion and conclusion

The aim of the study was to identify extracts from *Euphorbia* species with antimicrobial activity and to isolate and characterise the compound(s) responsible for the activity; to evaluate the antimicrobial activity of the plant extracts and isolated compound(s); to evaluate the toxicity of the extracts and compound(s) of interest with the hope to provide new effective antimicrobial agents.

5.1 Selection of plants, extraction and screening of extracts

During routine screening in our laboratory, *Euphorbia clavaroides* tested positively for antimicrobial activity. It is known that plants from the same family (section 3.1 or 2.7) may contain the same chemical compounds. Due to the unavailability of *E. clavaroides* plant material in large quantity, *E. ingens* was selected for screening. The antimicrobial assays used for this screening included the disc diffusion (3.4.1.1) and microplate (3.4.1.2) methods and the toxicity screening was done using the MTT assay.

5.1.1 *In vitro* antimicrobial activity

E. clavaroides extracts showed interesting activity against Gram-positive bacteria *B. subtilis* and *S. aureus* in both the disc diffusion and microplate assay. In the discussion of the results from the two assays actual values are given in brackets with the first number representing the size of the inhibition zone as determined by the disc diffusion assay and the second number represents the MIC value. The dichloromethane extract of *E. clavaroides* showed activity against *B. subtilis* and *S. aureus* and the ethyl acetate extract also showed antimicrobial activity against *B. subtilis* (3 mm & 1,74 mg/ml) and *S. aureus* (2 mm & 3,46 mg/ml) in both the disc diffusion assay and MIC determination (table 3.2 and table 3.5). None of the extracts of *E. clavaroides* showed activity against *E. coli* and *C. albicans* (disc diffusion assay) (table 3.2), but the ethyl acetate and ethanol extracts of this species showed antimicrobial activity against *E. coli* with MIC values of 0,87 and 0,74 mg/ml. The dichloromethane and ethanol extracts possessed antimicrobial activity against *C. albicans* with a concentration of 1,04 and 1,48 mg/ml, respectively (table 3.5). This may indicate that the extracts were not water soluble

enough to diffuse into the agar when tested in the disc diffusion assay, but showed activity when solubilised during the microplate assay.

The extracts of the total aerial part of *E. ingens* showed activity against the same range of microorganisms as *E. clavaroides*. The dichloromethane extract of *E. ingens* aerial part showed activity (5 mm & 12,85 mg/ml) against *S. aureus*, but only slight activity (2 mm & 25,7 mg/ml) against *B. subtilis*. Significant activity was shown by the ethyl acetate extract against *B. subtilis* (7 mm & 6,59 mg/ml) and *S. aureus* (10 mm & 6,59 mg/ml) with slight activity (1 mm & 13,2 mg/ml) against *E. coli*. The ethanol extract of *E. ingens* showed significant activity against the same Gram-positive microorganisms (table 3.2 & 3.5).

The aerial part of *E. ingens* was divided into a fleshy inner part and a rind (figure 3.1) to reduce the complexity of the extracts and was also tested for antimicrobial activity (table 3.2 & 3.5). The ethyl acetate extract of the fleshy inner part of *E. ingens* showed activity against Gram-positive bacteria *B. subtilis* (3 mm & 0,64 mg/ml) and *S. aureus* (4 mm & 5,15 mg/ml). The ethyl acetate extract of the rind possessed activity against Gram-positive bacteria *B. subtilis* (0,95 mg/ml) and *S. aureus* (1,98 mg/ml) in the MIC determination (table 3.5) and weak activity against the same bacteria in the disc diffusion assay (table 3.2). The extracts of both the fleshy inner part and the rind showed the best MIC values against Gram-positive bacteria *B. subtilis* and *S. aureus* (table 3.5).

There is an obvious difference between results obtained with the MIC and disc diffusion assay. For example, all the extracts of *E. clavaroides* showed activity against *C. albicans* and *E. coli* in the MIC assay, yet no activity was recorded with the disc diffusion assay. This difference might be attributed to factors such as water solubility and diffusibility of the antimicrobial compound through the agar matrix. Variation in inoculum size is one of the main sources of error in susceptibility testing (Hawkey & Lewis, 1989). Agar is a natural product, and contains variable concentrations of sulphate ions. This can cause a great impact on the observed zone of inhibition, such that a very potent inhibitor my produce a relatively small "halo" simply because it is unable to diffuse adequately through the medium. The inverse might be true for compounds with low activity, but high water solubility. The concentration of sulphate

ions also plays a role in the zone of inhibition. The higher the concentration of sulphate ions in the agar, the smaller the zone of inhibition will be (Rose & Barron, 1983).

It is important that media used for MIC testing should support the growth of the test organisms and be free from constituents which may influence the activity of the active compounds being tested (Fe^{3+}, Ca^{2+}, Mg^{2+}, thymidine etc.). *P. aeruginosa* strain was susceptible to gentamycin and was markedly dependent on the magnesium and calcium content of the medium (Hawkey & Lewis, 1989).

Infectious diseases remain a major cause of illness and death in South Africa. Among the resistant pathogens, methicillin (oxacillin)-resistant *S. aureus* (MRSA) is of great concern because of the predominance of this organism that causes various clinical infections, including those acquired in the community or hospitals. Recently, MRSA strains with reduced susceptibility to vancomycin have been reported (Hsueh, 2004; Klugman, 1999).

Microorganisms have different susceptibilities towards antimicrobial agents. Many antimicrobial agents have some effects against *Staphylococcus* species *in vitro.* However, the inability of drugs to act in the central necrotic part of the lesions makes it difficult to eradicate infections due to *S. aureus* (Jawetz *et al.*, 1970). The resistance is due to the elaboration of the enzymes β-lactmase which inactivate the antibiotic, for example vancomycin (Lennette *et al.*, 1974). *P. aeruginosa* is resistance to most antimicrobial agents and therefore becomes dominant and important when more susceptible bacteria of the normal flora are suppressed (Jawetz *et al.*, 1970). Patients become susceptible to *P. aeruginosa* infections after prolonged treatment with immunosuppressive agents, for example corticosteroids (Lennette *et al.*, 1974).

The ethyl acetate extract of the fleshy inner part of *E. ingens* extracts showed the best antimicrobial activity against Gram-positive bacteria *B. subtilis* and *S. aureus* in both the disc diffusion and microplate assay.

5.1.2 *In vitro* toxicity

The toxicity of the extracts was assayed against human epithelial cell lines (HeLa). Each extract has its own inherent toxicity. The overall toxicity (table 3.6) for the different

extracts ranged from 0,3349->500 mg/ml as determined against HeLa cells. For the extracts and compounds to be relatively safe when used against infectious diseases in human, the calculated security index of the extracts should be greater than 100.

The security index was calculated for each microorganism. The evaluation of the security index (SI) values of all the extracts showed that those of *E. clavaroides* were considered not to be safe when tested against HeLa cells. This is due to the fact that the calculated SI values of the extracts were less than 100, with the exception of that of ethanol .However, the ethanol extract of *E. clavaroides* was considered safe when tested against *B. subtilis*, *E. coli* and *C. albicans* with a MIC values ranging from 0,74-2,96 mg/ml and a SI values ranging from 317,6-158,8.

None of the rind extracts were considered safe when comparing the MIC value and SI value. However, the ethyl acetate extract showed a LD_{50} value of 11,14 mg/ml against HeLa cells with the MIC values ranging from 0,95-7,9 mg/ml against test organisms (table 3.7).

The extracts of *E. ingens* fleshy inner parts were considered not to be safe with the exception of the ethyl acetate extract of the fleshy inner part with a SI value of 117,2 and a MIC value of 0,64 mg/ml (table 3.7) against *B. subtilis*. The ethyl acetate extract of the fleshy inner part of *E. ingens* showed antimicrobial activity with both the disc diffusion and MIC assay (table 3.2 & 3.5).

From the results of the microbiological tests and toxicity test, further purification was conducted on the ethyl acetate extract of the fleshy inner part of *E. ingens* with the aim of isolating antimicrobial compound(s) with the best security index.

5.2 Isolation and characterisation of active compound(s)

The ethyl acetate extract of the fleshy inner part of *E. ingens* showed the best activity against Gram-positive microorganisms. This extract was subjected to column chromatography (figure 4.1) and kaempferol was isolated from this extract and found to be pure. Kaempferol possess antimicrobial activity against *S. aureus* (Arima *et al.*, 2002). The isolation of kaempferol was not surprising as *Euphorbia ingens* belongs to the family Euphorbiaceae that is known to be rich in flavonols and its glycosides.

The dichloromethane and ethyl acetate extracts of the fleshy inner part and the ethyl acetate extract of the rind of *E. ingens* showed the best antimicrobial activity against Gram-positive bacteria *B. subtilis* and *S. aureus* in both the disc diffusion and MIC assay. These extracts were subjected to column chromatography (figure 4.1, 4.2, 4.3 & 4.4). Fractions ECR32X from the ethyl acetate extract of the rind and EFIX2 from the ethyl acetate extract of the fleshy inner part showed no activity against *S. aureus* and possessed antimicrobial activity against *B. subtilis* (2 mm). Fractions DFI52 from the dichloromethane extract of the fleshy inner part, EFI4X3 and EFIX3 from the ethyl acetate extract of the fleshy inner part possessed antimicrobial activity against Gram-positive bacteria *B. subtilis* and *S. aureus* (table 3.3). The fraction EFI4X3 was identified as kaempferol **13**. The other fractions such as DFI52, ECR32X, EFIX2 and EFIX3 were not identified due to insufficient quantity.

5.2.1 Characterisation of active fractions and compound(s)

The molecular formula of the compound **13** was established as $C_{15}H_{10}O_6$ by the MS $[M]^+$ at *m/z* 287. Because of poor ionization of the compound **13**, fast atomic bombardment (FAB) ionization of MS was used to determine the molecular weight of this compound **13**. The ^1H NMR spectrum (**3**) revealed four aromatic proton signals, located at 6,165 (d; 1H; J = 2,03 Hz; H-6), 6,364 (s; 1H; J = 2,06 Hz; H-8), 6,889 (d; 2H; J = 9,79 Hz; H-3';H-5'), 8,053 (d; 2H; J = 9,86 Hz; H-2'; H-6'). The ^{13}C NMR revealed 15 carbon signals (spectrum **6**). Correlation of proton signals with carbon signals using HETCOR (spectrum **5**) helped in the identification of compound **13**. This compound was identified as kaempferol (**13**) by comparing its MS (spectrum **1**), NMR (spectra **3**, **4**, **5** & **6**) and IR (spectrum **2**) data with those reported in the literature (Barbera *et* al., 1986; Lee & Wu, 2001; Lin *et al.*, 2000; Markham *et al.*, 1979; Matthes *et al.*, 1980; Nawwar *et al.*, 1984; Panichayupakaranant & Kaewsuwan, 2004; Wagner & Chari, 1976).

The ^1H NMR of this compound (**13**) showed a singlet proton at δ_H = 6,364 (s, 1H, J = 2,06 Hz) (spectrum **3**) and the cause of this effect is not known. This signal represents the proton on C-8 and should show *meta* coupling with the proton on C-6.

Another four active fractions DFI52, ECR32X, EFIX2 and EFIX3 were only analysed by ^{1}H NMR. A broad overview of these spectra revealed four aromatic proton signals comparable to that of compound **13** and other flavonols.

Flavonols are three 3-hydroxy derivatives of flavones. The simplest of the flavonols, 3',4'-dihydroxyflavonol (**14**) gives a spectrum containing only thirteen carbon signals. The introduction of hydroxyl (3-OH) at C-3 caused significant chemical shifts in the signals relating to C-2 and C-3. The C-2 signal shifts upfield from δ_c = 164,16 ppm to about 151 ppm and the C-3 signal downfield from δ_c = 105,37 ppm to about 132 ppm (Ternia & Markham, 1976). Generally, the resonance appearing at δ_c = 140,0 – 151,2 ppm corresponds to C-2 and δ_c = 133,5 – 140.0 ppm to C-3 (Agrawal, 1989). The only other carbons notably affected by the introduction of the 3-OH group are C-2', C-5' and C-6'. These carbons are represented by signals at δ_c = 121,72; 116,71 and 116,32 ppm. The C-5' signal is readily identified as that at δ_c = 116,71 ppm by its lack of *meta*-proton coupling in the proton coupling spectrum. The C-6' shifts downfield by ca 2.3 ppm because of the introduction of 3-OH (figure 5.1) (Ternia & Markham, 1976).

Even though the compounds of these fractions were not identified due to insufficient quantity, the pattern seems to be consistent with that of flavonols and that of kaempferol (**13**). Signals on spectra (**7**, **8**, **9** & **10**) in the aliphatic region could be hidden by impurities. These compounds could then be kaempferol or its derivatives.

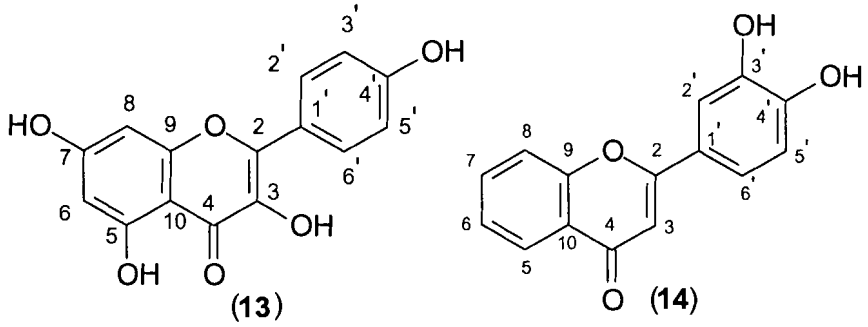


Figure 5.1: Structure of kaempferol (**13**) and 3',4'-dihydroxyflavonol (**14**)

5.3 Biological activities of kaempferol

Keampferol was the only compound with antimicrobial activity isolated from *E. ingens* extracts. As the biological activity of this compound is well documented (Arima *et al.*,

2002; Cai & Wu, 1996; Duke, 1998; Ilić *et al.*, 2004; Salvador *et al.*, 2004), no further tests were conducted on this compound. From the literature it is reported that kaempferol exhibits antimicrobial activity against a series of microorganisms. MIC values for the specific microorganisms is given in brackets *Porphyromonas gingivalis* (20 μg/ml), *P. intermedia* (20 μg/ml), *S. mutans* (2500 μg/ml), *A. viscosus* (1250 μg/ml) (Cai & Wu, 1996), *Herpes simplex virus type* (15.90 mm) (Ilić *et al.*, 2004), *S. aureus* (100 μg/ml), *S. aureus penicilinase* (500 μg/ml), *S. mutans* (100-500 μg/ml), *S. sorbrinus* (50 μg/ml), *C. glabrata* and *C. krusei* (500 μg/ml), *Trichophyton rubrum* (500 μg/ml) (Salvador *et al.*, 2004) and *Salmonella enteritidis* (400 μg/ml), and *B. cereus* (800. μg/ml) (Arima *et al.*, 2002).

The antimicrobial activity of kaempferol can be attributed to the hydroxyl group (OH-7) at C-7 (Cai & Wu (1996). Kaempferol showed a weak antimicrobial activity as compared with the known activity of both cloxacillin and gentamicin with MIC values of 0,01-1,0 mg/ml (Lateef *et al.* 2004) against *S. aureus* for cloxacillin and 0,008 mg/ml (Samie *et al.*, 2005) against *B. subtilis* for gentamicin.

Kaempferol is also known to possess high free radical-scavenging activity (Farkas *et al.*, 2004; Mikamo *et al.*, 2000).

Microorganisms (section 3.4.1) used for screening were collected from (section 3.4.1) the Department of Microbiology North-West University (Potchefstroom campus) and no hospital strains were collected because of the weak antimicrobial activity of the extracts and kaempferol as compared to the compounds available in the market for example cloxacillin and gentamicin etc. It was therefore not considered worth while to test against resistant strains of microorganisms.

5.4 Conclusion

As seen from the results (chapter 3 & 4), the aim of the study was successfully achieved. *Euphorbia ingens* plant extracts showed variable activity against a broad spectrum of microorganisms.

Kaempferol was isolated from the ethyl acetate extract of the fleshy inner part of *E. ingens*. This was not surprising because *Euphorbia ingens* belongs to the genus of

Euphorbia known to contain flavonols and its glycosides (Liu *et al.*, 2004). The isolation of kaempferol was reported from other *Euphorbia* species, for example *E. lathyris* and *E. armena* etc as well as from a number of other families, for example *Rhododendron* species, *Podophyllum hexandrum* etc (Duke, 1998; Lili, 1998). This study is the first to report the isolation of kaempferol from *E. ingens*. A brief literature search indicated that kaempferol possess weak antimicrobial activity against a wide range of microorganisms and toxicity against human cancer cell lines (Kajiya, 2001; Mutoh *et al.*, 2000). One of the microorganism posing a problem with drug resistance in South African hospitals is *S. aureus*, but kaempferol showed weak activity against this organism with a MIC value of 100 μg/ml (Arima *et al.*, 2002). Unfortunately, without structural modification it is not suitable for human usage. The security index (117,2) against *B. subtilis* of the ethyl acetate extract of the fleshy inner part of *E. ingens* showed that it is relatively safe to use at the concentration of 0,64 mg/ml in cases of *B. subtilis* infections.

From the evaluation of the MIC and disc diffusion results, it is clear that kaempferol is not the only compound responsible for the antimicrobial activity of *E. ingens* plant extracts (table 3.2 & 3.5). I recommend that, studies should be conducted to identify the other compounds responsible for the antimicrobial activity. Possible synergisms among its phytochemicals should also be considered.

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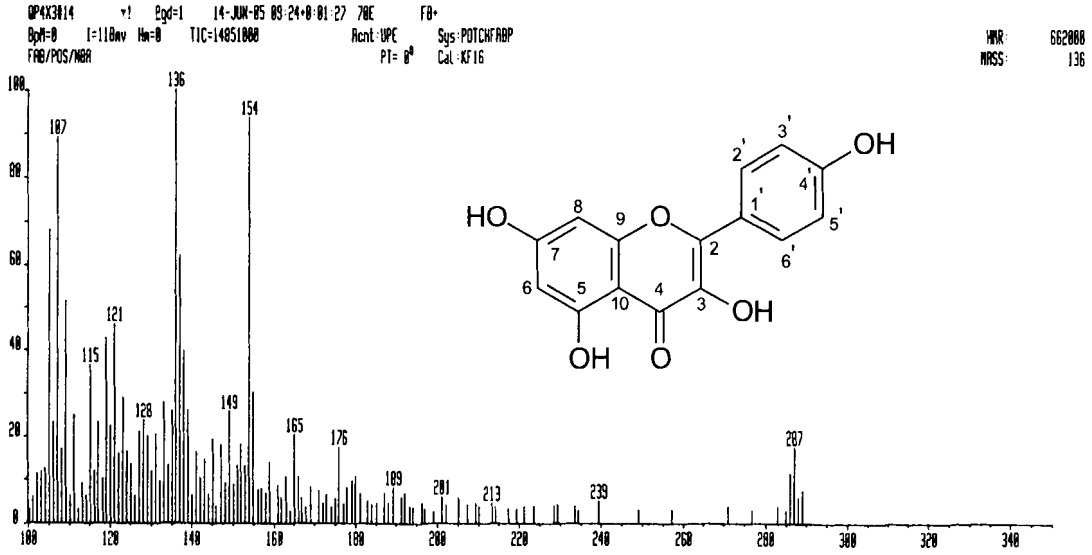
Spectrum 1



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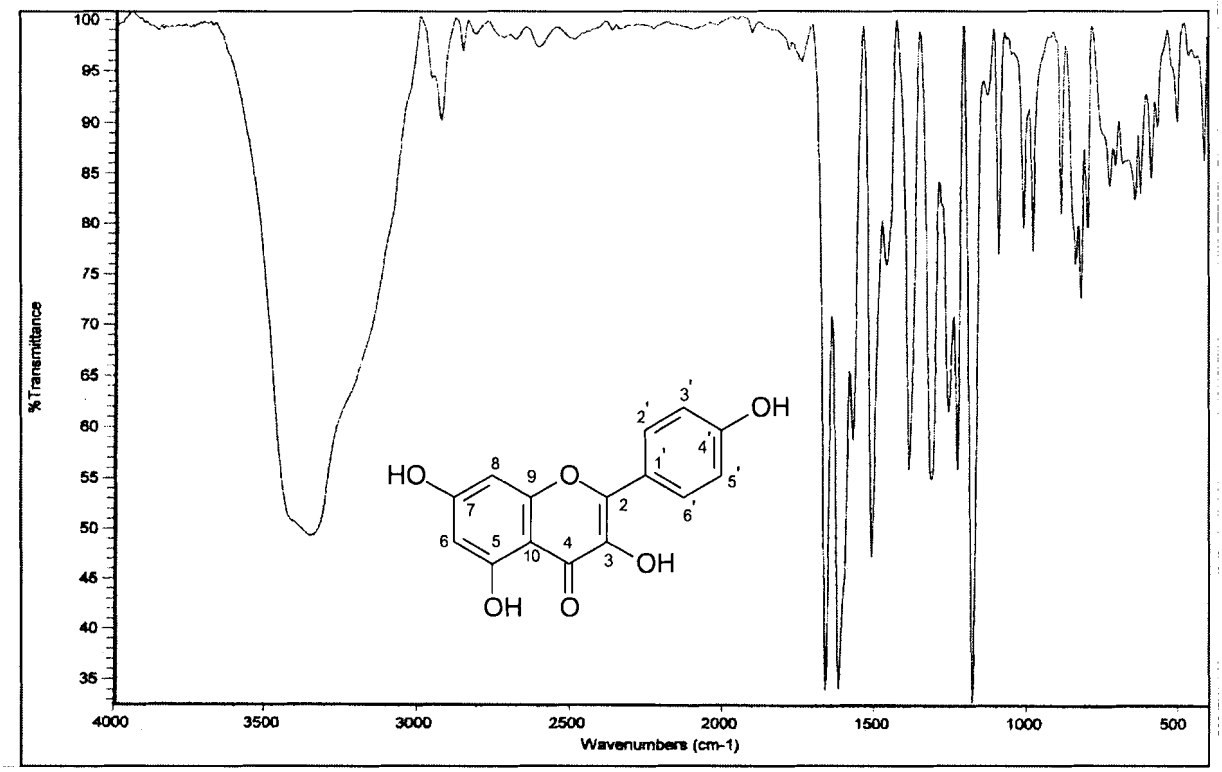

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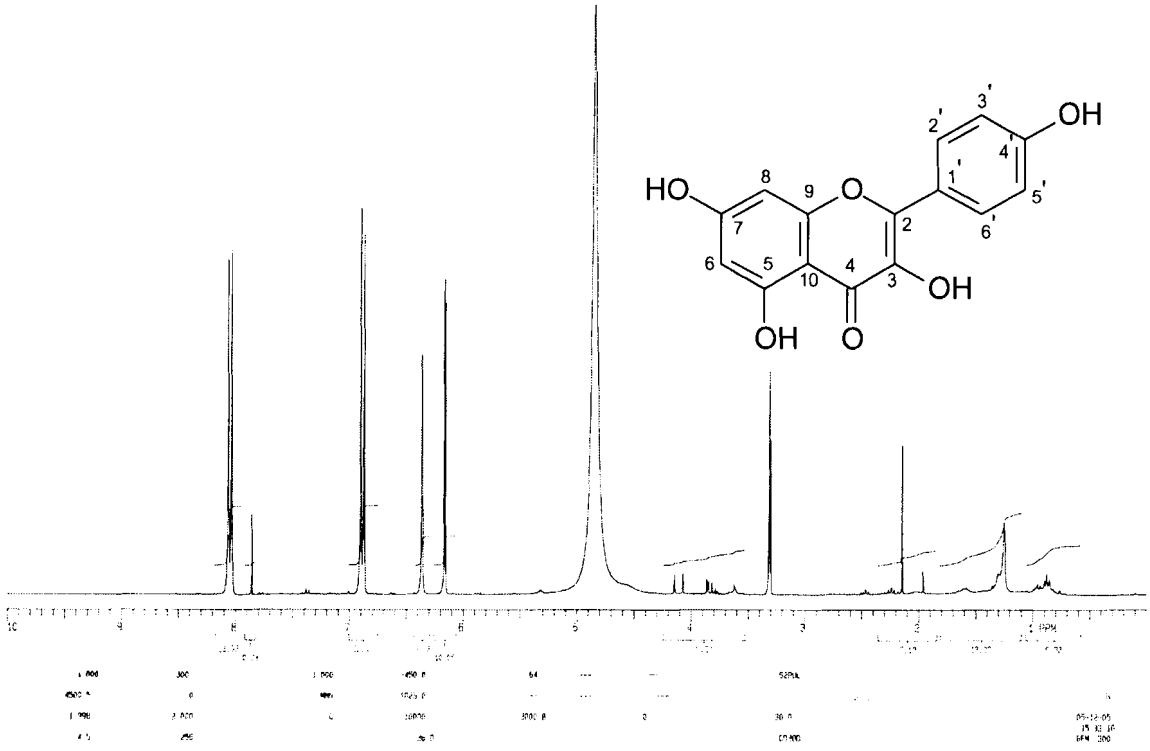

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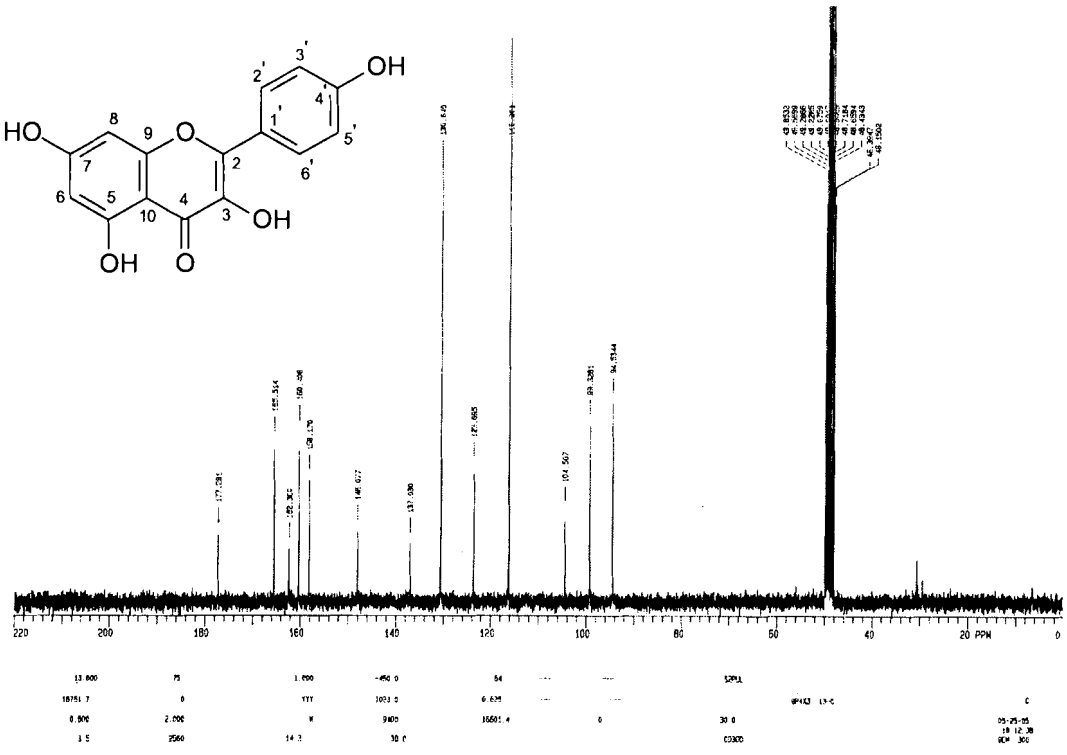

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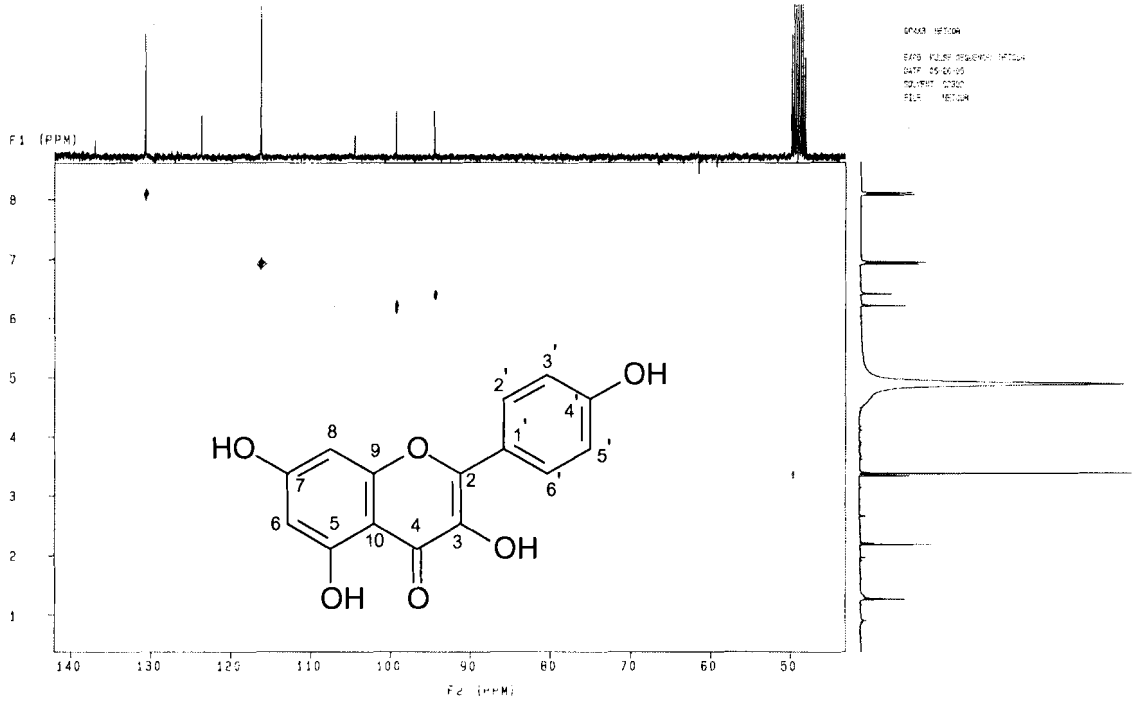

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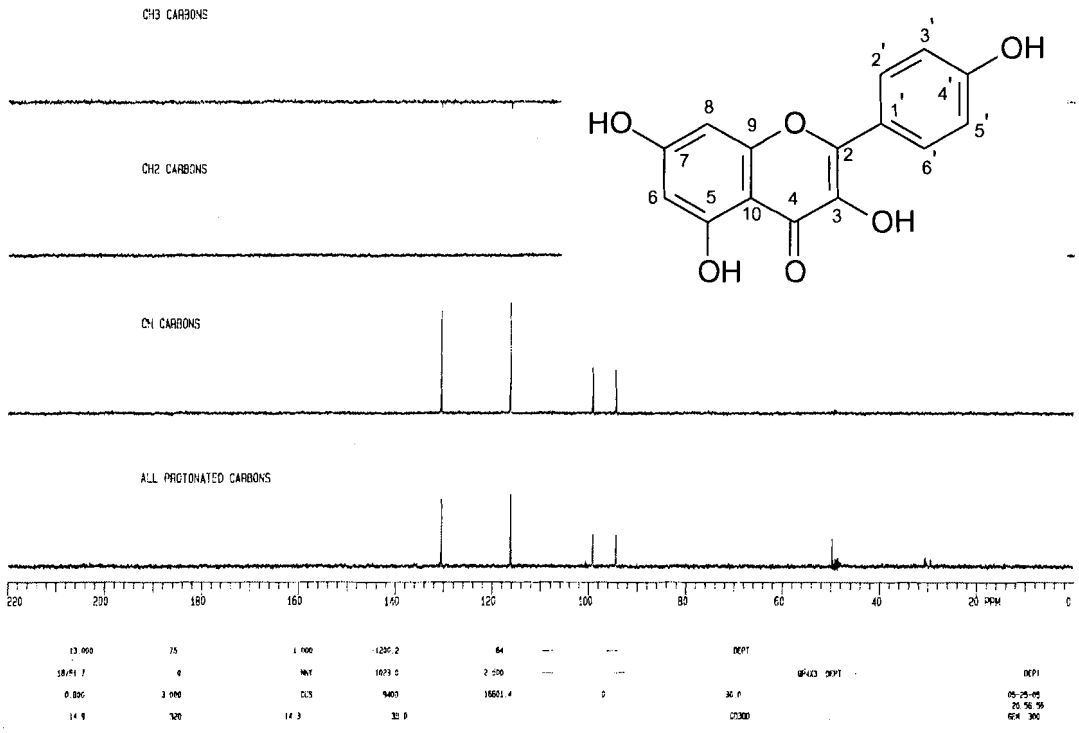

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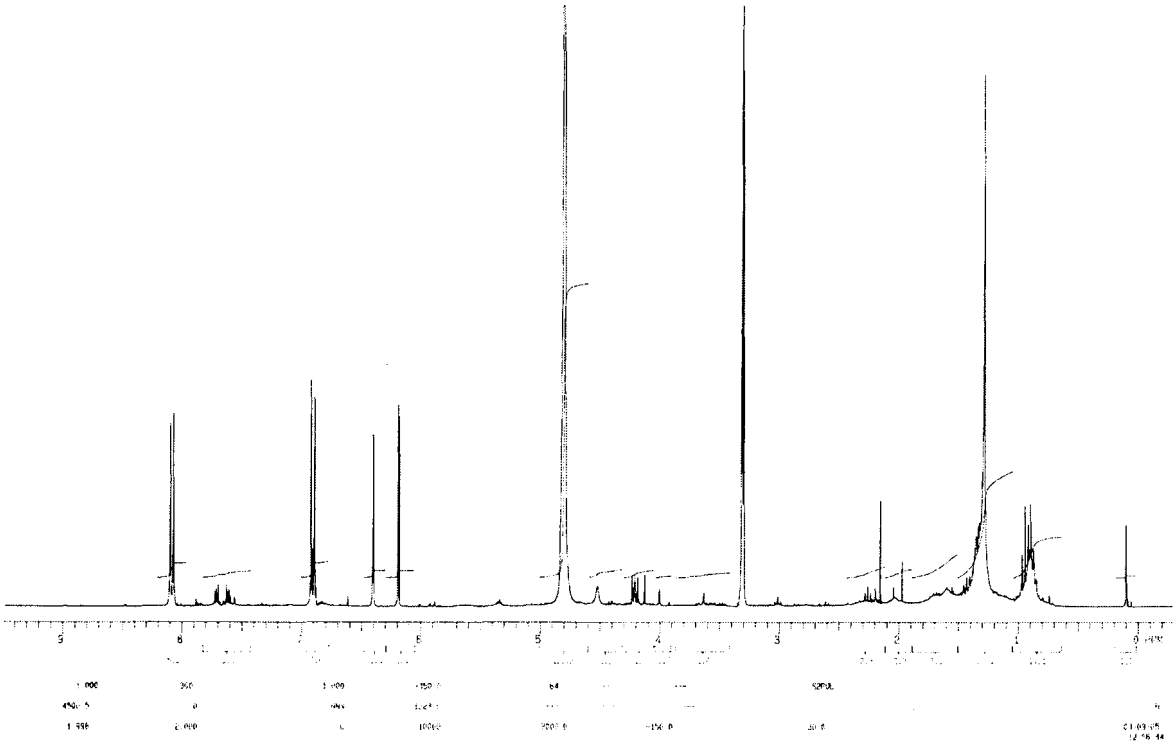

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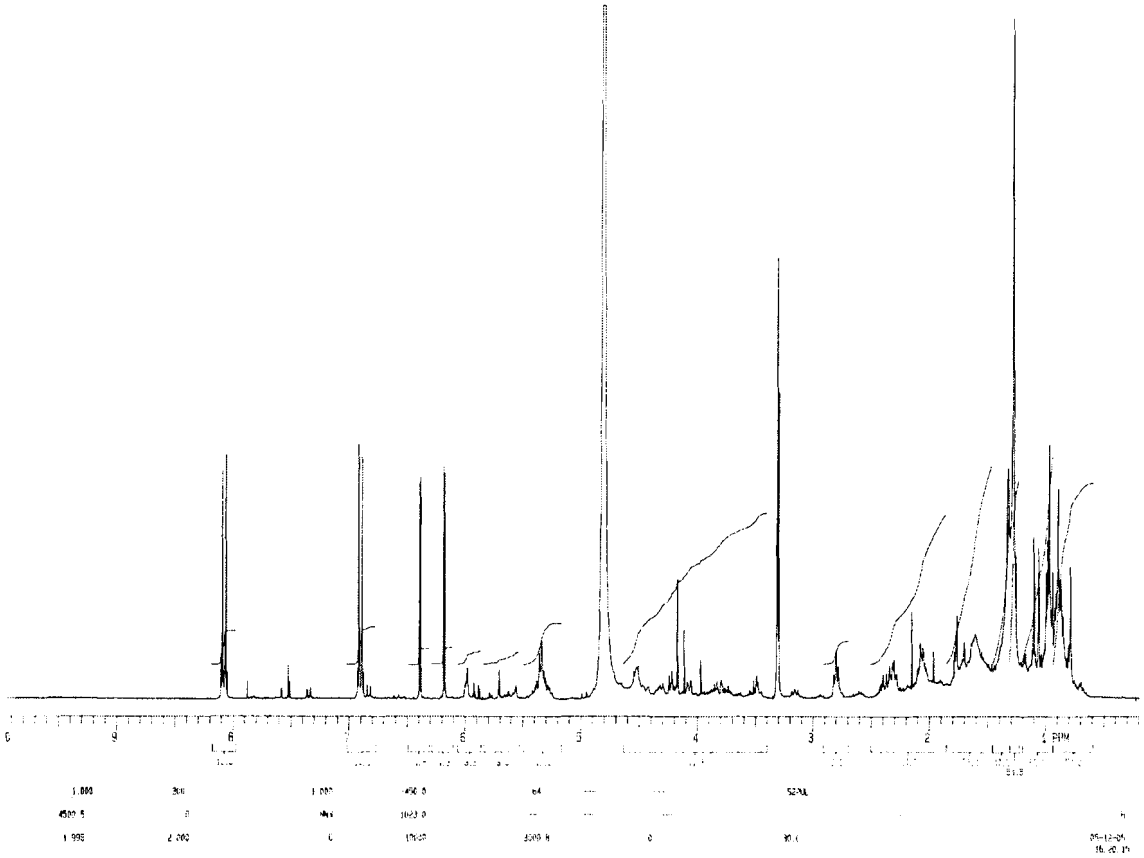

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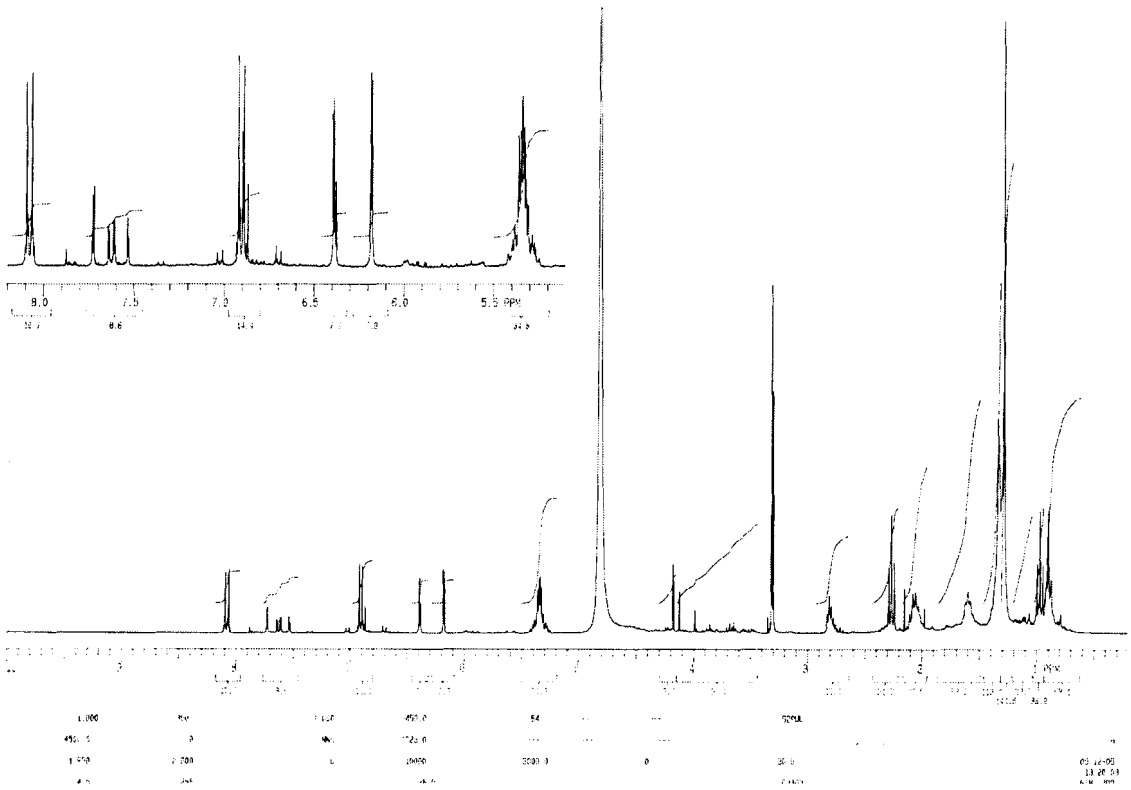

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