Chapter 1

Introduction about the medicinal plants and

Memecylon species
1.1 Introduction

The earth is home to rich and diverse array of living organisms, whose genetic diversity and relationships with one another and with their physical environment constitutes biodiversity. Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times. Herbs are staging a comeback and herbal ‘renaissance’ is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment (Farnsworth, 1988). Although herbs have been priced for their medicinal, flavoring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. Plants have been used as healers and health rejuvenators since time immemorial. Even now, WHO recognizes that medicinal plants plays an important role in health care of about 80 percent of world’s population in developing countries and depend largely on traditional medicines, of which herbal medicines constitutes the most prominent part (Farnsworth et al., 1988). The rest of the 20 percent also depend substantially on the plant-based medicines.

Contrary to the synthetic drugs, medicines of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Fakim et al., 1990). Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Various medicinal plants have been used for years in daily life to treat disease all over the world. Herbal medicine is still the mainstay of about 75-80% of the whole population, mainly in developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and fewer side effects. However, the last few years have seen a major increase in their use in the developed world.

Currently multiple drug resistance has evolved due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and
allergic reactions (Mahomodally et al., 2004). Because of the side effects and the resistance that pathogenic microorganisms build against antibiotics, recently much attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine. The medicinal actions of plants are unique to a particular plant species or group. Thus, there has been a revival of interest in herbal medicines. This is due to increased awareness of the limited ability of synthetic pharmaceutical products to control major diseases and the need to discover new molecular structures as lead compounds from the plant kingdom (Mitscher et al., 1972).

A lot of research work has been done which aim at knowing the different phytochemical constituents of medicinal plants and using them for the treatment of diseases as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become resistant. During recent years, the pace of development of new diseases slowed down while the prevalence of resistance has increased astronomically (Hugo and Russell, 1984). The increase in number of antibiotic resistant bacteria is no longer matched by expansion in the arsenal of agents available to treat infections. Literature reports and ethnobotanical records suggest that plants are the sleeping giants of pharmaceutical industry (Hamburger and Hostettmann, 1991). They may provide novel or lead compounds that may be employed in controlling some infections globally.

India is endowed with a variety of natural resources. All along the west coast, the Western Ghats are sprawling and the entire region is known for its biodiversity, richness and endemism of different species. In India, the Western Ghats mountain region is situated in the southern extending from Kanyakumari to Maharashtra. The Western Ghats are a mountain range that runs almost parallel to the western coast of Indian peninsula. It is a UNESCO World Heritage Site and is one of the eight “hottest hotspots” of biological diversity in the World (Norman et al., 2000). It is sometimes called the Great Escarpment of India (Piotr Migon, 2010). It runs north to south along the western edge of the Deccan Plateau and separates the plateau from a narrow coastal plain called konkan along the Arabian Sea. A total of thirty nine places including national parks, wildlife sanctuaries and reserve forests were designated as World heritage sites- twenty in Kerala, ten in Karnataka, five in Tamilnadu and four in Maharashtra. The range starts near the border of Gujarat and Maharastra, south of
the Tapti river and runs approximately 1,600 km through the states of Maharashtra, Goa, Karnataka, Tamil Nadu and Kerala ending at Kanyakumari at the southern tip of India. These hills cover 160,000 km² and form the catchment area for complex riverine drainage systems that drain almost 40% of India. Western Ghats is one of the 33 recognized ecological sensitive zones in the World. India has four such sensitive zones. The significance of the Western Gants is that along with its rich biodiversity, it also supports a rich environment dependent civilization of several thousand years.

Historically the Western Ghats were well covered in dense forests that provided wild foods and natural habitats for native tribal people. The tropical climate complimented by heavy precipitation from southwest monsoon and favorable edaphic factors create an ideal condition for the luxuriant growth of plant life, which can be seen only in few parts of the World. With its rainfall regime, the western slopes of the Ghats have a natural cover of evergreen forest, which changes to moist and then dry deciduous type as one comes to the eastern slopes. The vegetation reaches its highest development towards the southern tip in Kerala with rich tropical rain forests. This area has over 5000 species of flowering plants, 139 mammal species, 508 bird species, 179 amphibian species and 288 freshwater fish species; it is likely that many undiscovered species live in the Western Ghats. At least 325 globally threatened species occur in the Western Ghats (Vijaya, 2005). The plant species known to be from the Western Ghats is about 5000 species out of which 35 percent are endemic. Levels of endemism in this area are high- nearly 2000 species of higher plants, 84 species of fishes, 87 species of amphibians, 89 species of reptiles, 15 species of birds and 12 species of mammals are endemic to the Western Ghats (Daniel, 1997). Three major gradients in the distribution of this diversity, especially for flowering plants, have been recognized (Gadgil, 1996). The first and major one occurs along the north-south direction, species diversity increases as one travels from north to south direction along the Ghats. Southward increase of number of rainy days can be related to this phenomenon. The decrease in rainfall, relates to the decrease in diversity from west to east. The third known gradient is an increase in number of plant species found with the increase in temperature, as one goes from higher elevation hills to lower coastal plains. This heterogeneous condition, which is affluent all along the ranges and regions of the Western Ghats, makes it an ideal ground for the luxurious growth of plants with therapeutic value.
Among the rich and varied plants of Indian forests, the medicinal plants constitute an important source, the use of which for human and veterinary health care has probably continued, in an unbroken tradition for well over 2 millennium. The number of medicinal plants in India, both indigenous and introduced has been estimated to be between 3,000-3,500 species of higher plants. The Glossary of Indian Medicinal Plants has listed around 3,000 plants (Asolkar et al., 1992; Chopra et al., 1956). Among 2500 listed in Ayurvedic Materia Nighantu, 560 have been mentioned in Bhav Prakash Nighantu (Ravikumar, 2001). The Ayurvedic Drug Formulary prepared by Department of Indian System of Medicine and Homoeopathy lists 387 plants (Sarin, 2004). The Unani system of medicine describes 440 plants (Said, 1969) out of which 360 are common to other systems practiced in the country. The number of plants having confirmed therapeutic properties or yielding a clinically useful chemical compound thus lays around 700 species. The occurrence of these medicinal plants availability of raw materials from them is as follows:

- Plants occurring wild in forests, grasslands, aquatic and desert ecosystems, associated with other forms of natural vegetation.
- Plants growing as weed.
- Plants cultivated as ornamentals or as cereal, fruit, vegetable, spice, oil seed, essential oil or other cash crop.
- Plants cultivated as medicinal crop.

In addition, almost 25% of the entire compounds of current prescription drugs were derived originally form plant sources (Balandrin et al., 1985). Of the estimated 25,000 flowering plant species in the World today, only about 10% have been scientifically examined for their medical application, mostly in rudimentary way. Undoubtedly, many more plant-derived medicinal substances await discovery (Akerale et al., 1991). The information on the medical wealth of these examined 10% of plant species is scattered in different sources.

The Western Ghats is very rich in its medicinal wealth. The forests and hills of this region is a treasure house of about 700 medicinal plants. Out of which some are used for traditional and folk medicinal practices. Many are exploited commercially for their active enzymes and their commercial value. Medicinal plant species of Western Ghats represent a variety of life form ranging from lichen, algae, herbs, shrubs, climber and trees, which are annuals to perennials. Moreover these species are
distributed from canopy to understory and are characterized seasonally. The auto-
ecology and syn-ecology of medicinal plant species is complex and their proper
understanding requires a sound knowledge of the ecology, taxonomy and ethno-
botany for these species. Western Ghats with its species diversity is a treasure house
of different kind medicinal plants. The limited knowledge on the varied use of the
medicinal plants, their availability and extent of distribution weakens the ways to
utilize these resources efficiently. Therefore, it is required to bring the information in
various sources into one roof.

Out of the large variety of species available in the Western Ghats, about 50
species hold a very high value in the folk and herbal health forms for the treatment of
different forms of ailments. The most common plants like the *Mimosa pudica*,
*Hibiscus angulosus*, Leucasaspera, *Phyllanthus neruri*, *Calotropis gigantean*,
*Tridax pro cumbens*, *Parthenium hysterophorus* are all found to have cure for many
major ailments like jaundice, asthma, piles, bronchial and blood disorders. Plants like
*Anona squamosal*, *bucanania lanzan*, *Semecarpus anacardium*, *Dioscorea bulbifera*
and *Aphanamixis polystachya* are recommended for various forms of tumor. Plant
parts of pepper and cinnamon when mixed together make up a very strong formula for
curing migraine. Frequent doses of medicinal plant extracts of *Rhincanthus nasuta*,
*Momordica dioica*, *Cinnamomum zeylanicum*, *Ophiorhizza mungos* relieves cancer
patients, *Aegle marmelos* for treatment of chronic constipation or intestinal blockage,
*Bacopa monnieri* as an anti-anxiety drug and for enhancing cognitive properties
which has shown positive effects on brain, *Celastrus paniculatus* tonic for treating
gout and rheumatism, *Holarrhena pubescens* bark and roots for the treatment of
diarrhea and dysentery, *Centella asiatica* for treating fever and ulcer, *Atalantia
monophylla* to treat chronic rheumatism and paralysis, *Dodonaea viscosa* for healing
wounds and *Euphorbia hirta limn* is used to cure bronchial affection and asthma
(Sukumaran and Raj, 2010). The spread of knowledge on the natural wealth is more
important for a country like India, at a time when the synthetic drugs are stealing the
economy rates. In this regard, efforts are being made by systematic screening of the
plants to discover novel antimicrobial, antioxidant and analgesic compounds.

Most of the medicinal plants are found to occupy forest types like deciduous
forests, evergreen forests and they are found in fallow lands and wayside. It can be
noted that the plants that were very common in the area when they were first studied
have got into the IUCN Red List over the years. *Rauwolfia serpentine*, *Saracaasosa*,
Gymnema sylvestre, Gloriosa superba, and Strychnos nux-bomicae are included in the list which are very rich in their medicinal strength but are in the verge of extinction. The Western Ghats also hosts many medicinal plants that are endemic to the area. Appropriate conservation strategies have to be implemented immediately to protect the fragile habitats of many such medicinal plants.

*M. terminale Dalz*, belonging to the family Melastomataceae, is a small erect shrub found exclusively in Western Ghats of Karnataka, India. All parts of this plant are being used by traditional healers in this region for curing various ailments such as dysentery, fever, diabetes, diarrhea, piles and haemoptysis (Krishnamurthy and Asha, 2010). However, no information is available regarding the systematic study of pharmacological activity of *M. terminale Dalz*. In this regard, the present study was carried out to find out the novelty of different extract of *M. terminale Dalz* against the different pharmacological activity.

### 1.2 Review of literature

A well-known objective for studying medicinal plants is the discovery of new bioactive components which could be promising drugs. The Memecylon plants have been instrumental in the discovery of medicinal natural products. A member of the Melastomataceae family, Memecylon has been described as Iron wood tree. It is a small and semi evergreen shrub or tree bearing several umbellate cymes. The flowers are inflorescence long peduncled umbels, sub-terminal and axillary; flowers are blue in color. Fruit and seeds are berry type and having diameter of 0.4-0.5 cm, globose. The genus Memecylon comprises of about 300 species in the world, of which 30 species have been reported from India and they are distributed in different habitats like semi evergreen, evergreen, deciduous and mountain shoals with a wide range of altitude from the sea level (Krishnamurthy and Asha, 2010). Memecylon species is a small evergreen tree native to India, especially in the Deccan Plateau, including most places of Karnataka, Andhra Pradesh and parts of Tamil Nadu and dry parts of Sri Lanka. Some of the more well-known Memecylon genus used in the traditional medicine is as follows: *M. umbellatum Burm.*, *M. edule*, *M. malabaricum*, *M. ovatum*, *M. talbotianum Brandis*, *M. agastya malaianum* and *M. terminale Dalz*. The focus of this work is providing information on the structure and pharmacological activities of novel compounds isolated and identified from *M. terminale Dalz*. In this review of
literature, we summarized more recent pharmacological studies on *Memecylon* species in several areas with particular emphasis on bioactivities related to anticancer activities, antioxidant activities, antimicrobial activities and other biological activities. Those studies reporting on bioactivities of specific compounds form *Memecylon* species feature many of the alkaloids and phenolic compounds.

**1.3 Ethnobotanical uses of Memecylon Species**

Many general traditional uses of *Memecylon* genus including the treatment for curing various ailments such as dysentery, fever, piles and haemoptysis, cough, sedative, bruises have been documented (Krishnamurty and Asha, 2010). The *Memecylon* species leaves are used to treat eye troubles, gonorrhea, leucorrhea and wounds (Anonymous, 1998; Dhar *et al.*, 1968; Puratchikodi and Nagalakshmi, 2007), treatment of bone fracture and herpes (Rajakumar and Shivanna, 2009), diabetes (Grover *et al.*, 2002; Ayyanar *et al.*, 2008; Akanksha and Maurya, 2009), skin diseases (Karuppasamy, 2007), and snake bite (Kshirsagar and Singh, 2001).

**1.4 Medicinal uses of selected Memecylon genus**

**1.4.1 Memecylon umbellatum burm F**

*M. umbellatum* Burm. F. is an ethnomedicinal plant used traditionally for treating various diseases. The infusion of leaves is used in the treatment of gonorrhea and leucorrhoea (Nadkarni, 1976; Kirtikar and Basu, 1991). The paste of leaves is used in the treatment of herpes (Maruthi *et al.*, 2000). The decoction of the roots is used for the treatment of menorrhagia and the preparation from the bark for the treatment of bruises (Nadkarni, 1976). The seeds are used to cure cough and sedative (Balakrishna, 2004). The leaf powder is used for the treatment of diabetes (Ayyanar *et al.*, 2008). It has showed hypoglycemic effect in normal and alloxan diabietic mice (Amalraj and Ignacimuthu, 1998).

**1.4.2 Memecylon malabaricum**

It has considerable reputation for its traditional use in the treatment of diabetes, various bacterial infections, inflammatory and skin disorders including herpes, and chicken pox. The root has ebolic properties and it is being used as a root ebolic like ergot (Kiran *et al.*, 2004; Ambasta, 2000). A decoction of the flowers and
twigs has been used to treat skin diseases (Anon, 1962). It was found that the leaves of *M. malabaricum* have been traditionally used in the treatment of psoriasis by traditional soddja healers in the Malabar region of Karnataka, India. So far this plant has not been scientifically evaluated for antipsoriatic activity.

1.4.3 *Memecylon talbotianum Brandis*

*M. talbotianum Brandis* is used as good antibacterial agent and good antioxidant (Shailasree *et al.*, 2013). Detailed studies carried out on this plant are not available in the literature.

1.4.4 *Memecylon edule Roxb*

The leaves of *M. edule Roxb* used as anti-inflammatory, analgesic and antioxidant agent in Thailand (Somsak, 2009). Traditionally, leaves of *M. edule Roxb* have been used to treat leucorrhrea, gonorrhrea, wound and gastrointestinal ailments (Elavazhagan and Arunachalam, 2011). The *M. edule Roxb* plant extracts have proved to be a good anticancer agent on gastric carcinoma cell lines (Naidu *et al.*, 2013).

1.5 Phytochemistry

The phytochemistry of *Memecylon* genus has been extensively studied since early 1990s (Amalraj and Ignacimuthu, 1998). One of the earlier phytochemical reports was published in 1998 (Amalraj and Ignacimuthu, 1998). The most prominent compounds in *Memecylon* genus are tannins, alkaloids, octocosonoic acid, cerotic acid, ethyl palmitate, palmitic acid, butyric acid, flavonoids, steroids, terpenoids, tannins, phenolic groups, phenols, quercetin, saponins, steroids and glycosides (Suresh and Harinath, 2010; Ravindra and Achur, 2013; Vivek *et al.*, 2014; Harinath *et al.*, 2014; Sivu *et al.*, 2013; Subban *et al.*, 2011; Sangai *et al.*, 2012). Most of these compounds were identified, which demonstrates the intense focus of early research on the *Memecylon* genus. The above mentioned compounds also include pharmacologically significant compounds found in *Memecylon* species that have been previously reported in other plants.
1.5.1 Occurrence of compounds across *Memecylon* species

A major consideration of the comparison of identified components in *Memecylon* species is that the chemical composition can vary based on its geographical and seasonal collections. This has specifically been shown with *M. umbellatum*, and should not be discounted with other species. However, *Memecylon* also contains a large population of “common” compounds that occur across a variety of species (Mary et al., 2005). It is useful to compare and contrast all of these factors when searching past phytochemical analyses of the *Memecylon* genus. Table 1.1 lists most of the compounds reported in *Memecylon*, including structures discovered before, as well as the species and plant from which they have been isolated.

*M. umbellatum* has been reported to have chemical compounds such as umbelactone (4-hydroxymethyl-3-methyl-3-methyl-but-2-ane-4, 7-oxide), amyrin, sitosterol, tartaric acid, malic acid, oleanolic acid, ursolic acid (Asolkar et al., 1956), and tannins (Killedar and More, 2010). The umbellactone, sitosterol, glycoside, olenalic and ursolic acids were isolated from the *Memecylon* species (Rastogi and Mehrotra, 1993). The compounds such as cerotic acid, octacosonoic acid, ethyl palmitic acid and butyric acid have been isolated from methanolic extract of *M. umbellatum* (Himanshu et al., 2009).

1.5.2 Pharmacological activity

The pharmacological studies have generally focused on the fractions of either a plant species or a “crude drug”, considered as a preparation from either a single or a mixture of *Memecylon* plants. A number of studies, however, have focused on the bioactivity of specific isolated compounds. The information in this section is organized by pharmacological activity with reference to the crude extract or isolated compounds. Among these plant crude extracts, *M. umbllumatum* has been the most widely studied followed by *M. malabaricum*.

1.5.3 Genotoxicity

Mutagenicity testing has now been recognized as an essential part in the evaluation of the genetic toxicity of plant extracts. Genotoxic activity of methanolic extracts of *M. umbellatum* leaves was determined by chromosomal aberration test and micronucleus assay (Prashanth et al., 2010). The extract treated showed aberration frequency that is higher than those of the normal at all-time intervals. The extract
showed the dose related response. As the dose increased, the mitotic index value decreases. Further, in micronucleus assay, the extract showed dose dependent response as well as time dependency. The methanolic extract of the plant has a good suppression capacity to inhibit the proliferation activity of the bone marrow cells (Prashanth et al., 2010).

1.5.4 Anticancer activity

*M. umbellatum* extract has been examined to find out the potency of different extracts on selected cancer cell lines (Ravindra and Achur, 2013). The cytotoxic effect of crude methanol and the chloroform extract on Dalton’s lymphoma ascites cell (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines was assayed by trypan blue method. The methanolic extract showed good inhibition activity as compared to chloroform extract. The antiproliferative and apoptogenic activity of ethyl acetate extracts of *M. edule* on gastric cancer cells lines have been studied (Naidu et al., 2013). Preliminary screening for cytotoxicity studies of ethyl acetate extract of plant showed significant inhibition on the growth of gastric cancer cells in a dose dependent manner (IC$_{50}$-50 µg and IC$_{80}$-100 µg/ml). Aqueous and hydroalcoholic extracts of *Memecylon malbaricum* leaves showed good *in vitro* antipsoriatic activity in HaCaT cells (*M. malbaricum* water extract, IC$_{50}$-185.8±14.70 µg/ml; *M. malbarucu* hydroalcoholic extract, IC$_{50}$-57.4±3.11 µg/ml), lipooxygenase inhibition assay (*M. malbaricum* water extract, IC$_{50}$-134.61±8.07 µg/ml) and thymidine phosphorylase inhibition assays (Sangai et al., 2012).

1.5.4 Anti-inflammatory activity

Few *Memecylon* plants have been used to treat numerous inflammatory disorders, usually prepared from bark and root of *M. edule* Roxb. Anti-inflammatory activity of different extracts of *M. edule* Roxb was studied by stimulation of interleukin-10 and ethyl-phenylpropiolate (EPP) induced mouse ear edema (Somsak et al., 2009). The ethanolic extract of the plant showed best activity (69.7%), followed by methanolic extract (57.3%) and hexane extract (14.1%). EPP-induced mouse ear edema results indicate a significant reduction of the edema after application of the ethanolic extract (50.2%) comparable to positive control dexamethasone (62.4%) (Somsak et al., 2009).
1.5.5 Antioxidant activity

Recent studies have revealed that certain *Memecylon* species contains phenolic, flavonoids and tannins with antioxidant properties which are likely to be responsible for some of the *Memecylon* species pharmacological effects (Suresh and Harinath, 2010). These compounds have been previously identified in other family plants (Mary et al., 2005). The leaves extract of *M. umbellatum*, *M. talbotianum* and *M. malabaricum* has been tested in vitro for antioxidant activity. The analysis included DPPH radical scavenging assay, ABTS radical scavenging assay and hydroxyl radical scavenging assay, which determines the free radical scavenging activity of different extract of *Memecylon* species. The DPPH radical scavenging assay showed that methanolic extract of *M. umbellatum*, *M. talbotianum* and *M. malabaricum* have good ability to scavenge the free radicals. The methanolic extract of the plants also inhibited the thiobarbituric acid free radicals (Suresh and Harinath, 2010; Sivu et al., 2013; Subban et al., 2011). A comparison study of methnolic extract of leaves from *M. umbellatum* and *M. talbotianum* (Shailasree et al., 2013) found that the DPPH free-radical scavenging activity of both were in the 50 μg/ml range, with *M. umbellatum* having greater activity than *M. talbotianum*. No phenolics were quantified in the study, but the authors concluded that polyphenol and flavonoids were the reason for good antioxidant activity.

Among the 32 plants screened for their ability to scavenge DPPH radical, the *M. heynanum Benth* (IC_{50}-15.30 μg/ml) followed by *M. sisparensis Gamble* (IC_{50}-40.06 μg/ml) and *M. sessile Benth* (IC_{50}-40.10 μg/ml) showed good activity whereas the remaining species of *Memecylon* failed to show good and comparable activity to that of the standard Quercetin (IC_{50}-10.16 μg/ml) (Sivu et al., 2013). In this study, the active principles have not been identified, but it has been suggested that the activity may be useful toward the prevention reactive oxygen species produced during many biological process.

1.5.6 Antimicrobial activity

Although *Memecylon* species is not used extensively to treat bacterial infections in traditional medicine, some reports evaluating antibacterial activity have appeared (Vivek et al., 2014). An extract of *M. malbaricum* was observed to inhibit the growth of *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumonia* when compared to the extract of *M.*
talboltianum (Vivek et al., 2014). One use of *M. malbaricum* in traditional medicine which may be related to its antibacterial activity is in the treatment of dysentery and diarrhea. The bacterial inhibitory activity of extracts could be attributed to the presence of phytochemical constituents that are mainly phenolic constituents. The *M. umbellatum* leaves extracts were tested for antimicrobial activities and the result showed good activity against a panel of microorganisms especially against *Streptococcus aureus* and *Bacillus subtilis* (Subban et al., 2011).

Overall, *Memecylon* genus has proven to be a very valuable for the discovery and utilization of medicinal natural products, particularly phenolics, alkaloids and tannins. The collected information provides a means to understand the latest developments in the pharmacological and phytochemistry of these plants. The potential for development of leads from *Memecylon* continues to grow, particularly in the area of inflammatory, antioxidant, antiviral and diabetic related conditions. In view of this, the present study on the *M. terminale Dalz* plant extracts, isolation and characterization of pure compounds from extracts and their biological activities has been undertaken to systematically evaluate its pharmacological potential.
Table 1.1: List of the compounds reported in Memecylon, including structures discovered before, as well as the species and plant from which they have been isolated.

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<tr>
<th>Sl. No.</th>
<th>Compound name</th>
<th>Compound structure</th>
<th>Plant from which they have been isolated</th>
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