Investigation of the Potential Antibacterial Activity of *Buddleja madagascariensis* Extracts

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SUMMARY

Evidence suggests that *Buddlja Madagascariensis* extracts have antibacterial activity and the leaves can be used to cure asthma, coughs and bronchitis.

The present investigation is aimed at finding the activity present in *Buddlja Madagascariensis* against *Tuberculosis* and identifying the active compounds. Experiments were initiated to assess this possibility using *E.* coli (DC2), *S. aureus* (NCTC 6571) *and M. fortuitum* (NCTC 10394), a model for tuberculosis. The methanol extracts showed some activity against *M. fortuitum*.

The compounds in extraction were separated and purified by HPLC. A series of fractions was achieved. Some of them again showed activity. The fractions then were characterised by mass spectrometry.

On the other hand, column purification in a molecular imprinting approach was taken in parallel with the separation of the components in the extraction. The effect was assessed by comparing groups of peaks from HPLC, one of which was for the crude extracts while the rest were for the fractions from column purification.

Keywords: molecular imprinting; imprinted polymers; antimycobacterial compounds;

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ABBREVIATIONS

HPLC: High Phase Layer Chromatography

TLC: Thin Layer Chromatography

PC: Paper Chromatography

UV: Utraviolet

IR: Infra-red

NMR: Nuclear Magnetic Resonance

MS: Mass Spectral

THF: Tetrahydrofuran

AIBN: 2,2'-Azobis[2-methylpropanenitrile]

MAA: Methacrylic Acid

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INTRODUCTION

Aims and Objects

Tuberculosis, caused by the intracellular pathogen *Mycobacterium Tuberculosis*, is becoming a major health problem in industrialized countries. It kills approximately 2 million people each year. The global epidemic is growing and becoming more dangerous. The serious situation of this illness makes it important to find a relatively cheap and effective source of treatment against, especially in the countries with financial difficulties. (1)

Plants have been used as the source of medicine since ancient times and many of the currently available drugs have been derived directly or indirectly from them. Today 25% of prescription drugs contain a plant-derived chemical or a derivative and interest in developing plants as a likely source of new commercial drugs is resurging. In this study, the possibility of using the chemicals in *Buddlja Madagascariensis* as a way to treat tuberculosis was discussed.

Evidence has suggested that the leaves of *Buddlja Madagascariensis* Lamk. (Loganiaceae), a shrub indigenous to Madagascar, are used in traditional medicine for asthma, coughs and bronchitis. Seeing these facts, there could be compounds with the ability to treat illness of respiration system present in *Buddleja madagascariensis*. The present investigation is aimed at finding an efficient but comparative cheap way to cure tuberculosis by searching for the antibacterial activity in *Buddlja Madagascariensis* against *Mycobacterium Tuberculosis*. Each part of the plant was extracted by a series of solvents. The extractions were assayed and the components in the extraction with antibacterial activity were separated and fractionated. And finally, attempts were made to identify these components by mass spectrometry.

Tuberculosis

An Overview

Tuberculosis, a chronic infectious and zoonotic disease, caused by *Mycobacterium Tuberculosis*, is becoming a global health problem of escalating proportions. According to the report of WHO (World Health Organisation) in 2002, TB kills about 2 million people each year (including persons infected with HIV). More than 8 million people become sick with TB each year. Around 3 million TB cases per year occur in Southeast Asia. Over a quarter of a million TB cases per year occur in Eastern Europe. It is estimated that between 2002 and 2020, approximately 1000 million people will be newly infected, over 150 million people will get sick, and 36 million will die of TB - if control is not further strengthened. (2)

Ninety percent of tuberculosis cases occur in developing countries, where few resources are available to ensure proper treatment and where human immunodeficiency virus (HIV) infection may be common. The concentration of deaths due to tuberculosis in demographically developing nations and mortality rate in the most economically fruitful years of life from 25 to 54 causes substantial losses in productivity and contributes to the impoverishment of third-world countries. (3) The incidence of TB has also increased in developed countries mainly involving HIV-positive individuals, immigrants/expatriate workers from countries in which TB is endemic, the homeless and prison inmates. (4) *Mycobacterium tuberculosis* (*M. tb.*) infection is endemic in Zimbabwe. Since the beginning of the human immunodeficiency virus (HIV-1) epidemic in the late 1980s, the number of tuberculosis (TB) cases significantly increased in the country. In Zimbabwe, and other countries in sub-Saharan Africa, TB is the leading cause of morbidity and mortality among HIV-1 infected individuals. (2)

Almost two thirds of the world's known TB cases are in Asia. In the last 2 years, prevalence of TB increased by 25% in Asia. In Delhi, India, for example, 13% of all TB patients are ill with MDR-TB. The enormous public health and economic ramifications of MDR-TB has lead some international health authorities to refer to this fatal disease as `Ebola with wings'. One-third of the world population suspected to be exposed to this transmittable disease and 10 million new cases per year is expected in this century. (5)

The reemergence and the global epidemic of TB could attribute to the various factors such as the coincidence of tuberculosis and HIV infection, development of multi-drug resistant strains of *Mycobacterium tuberculosis*, (6) and the decline in health care structures and national surveillance. (7)

Because of its association with acquired immune deficiency syndrome (AIDS), tuberculosis now becomes one of the most important infectious diseases in humans. Evidence suggests that HIV-1-infected individuals in Europe and the USA may be more susceptible to new tuberculosis infection to progress to AIDS rapidly. (8)

Mycobacterium tuberculosis

Mycobacterium tuberculosis that leads to tuberculosis is an intracellular pathogen, gram-positive bacterium. The doubling time for *M. tuberculosis* is 12-18 hours, which is unusually slow. There are almost 100 different recognized mycobacterial species and many of them are non-pathogenic to humans. *Mycobacterium tuberculosis* complex (MTC) strains are the majority that are responsible for the mycobacterium infections worldwide. (9)

Spread

TB bacteria are contained in the tiny droplets formed when an infected person with pulmonary or laryngeal TB coughs, laughs, talks or sneezes. These particles can remain suspended in the air for several hours. When another person inhales air containing infectious TB particles, most of the larger particles become lodged in the upper respiratory tract where infection is unlikely to develop. However, some droplets containing tubercle bacilli may reach the lower parts of the lungs where infection begins. (10)

Throughout history, the movement of human populations has spread tuberculosis. Nowadays, modern travel continues to contribute to tuberculosis infection and disease. TB transmission has been found on commercial aircraft, from personnel or passengers

to other personnel and passengers, but the risk of is low. As in other settings, the possibility of transmission is proportional to duration and proximity of contact. Travellers from low incidence to high incidence countries have the risk of acquiring TB infection similar to that of the general populations in the countries they visit, but the risk is higher if they work in health care. Passive diagnosis of symptomatic cases should be facilitated and this can be achieved by placing emphasis on public awareness and education campaigns. For the global traveller *Mycobacterium tuberculosis* (MTB) continues to be a common concern. (11)

Symptoms

General symptoms of active TB infection include weight loss, sweats, fever, chills, fatigue, coughing and loss of appetite. Other symptoms of active TB depend on the site of the infection. The lungs are the most common sites of TB disease and approximately 85% of the TB cases are related to this organism. Patients with TB in lung usually have a productive, prolonged cough and sometimes have chest pain or may cough-up blood. Patients with chest radiographic evidence of TB are considered contagious. Central nervous system and bones and joints are also the parts that can be invaded by the bacterium. (15)

Treatment

Seeing the serious situation of this illness, it's important to find a relatively cheap and effective source of treatment against TB, especially in the countries with financial difficulties.

The most common anti-TB drugs are isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol. The formulation of the essential anti-TB drugs are as followed: Separate drugs: isoniazid, pyrazinamide, ethambutol, streptomycin; Fix-dose combinations: thioacetazone + isoniazid, ethambutol + isoniazid, rifampicin + isoniazid, rifampicin + isoniazid + pyrazinamide. But in the past decade the increasing incidence of tuberculosis worldwide has been facing many challenges. Besides the lack of novel

therapeutics against this pathogen, one of the alarming trends is the growing resistance to current antimycobacterial therapies. Not only strains that are resistant to a single drug have been documented in every country surveyed but also the strains of TB resistant to all major anti-TB drugs has emerged. (17) Much scientific interest has been created in developing new anti-mycobacterial agents to both treat existing drug-resistant strains and shorten the duration of short-course treatment to benefit the patients. (18)

In 1991, an effective but economic TB control method was introduced on a global scale. It's called DOTS (Directly Observed Treatment, Short-course), which is recommended by WHO as the treatment strategy for detection and cure of TB.

Once patients with infectious TB start using microscopy services, health and community workers and trained volunteers will observe patients swallowing the full course of the correct dosage of anti-TB medicines (treatment lasts six to eight months).

By the end of 2000, all 22 of the countries with highest burden with 80% of the world's TB cases had adopted DOTS. Fifty-five percent of the global population had access to DOTS, twice the fraction reported in 1995. In the same year, it is estimated that 27% of TB patients received treatment under DOTS, two and a half times the fraction reported in 1995.

Microbiology

The genus Mycobacterium

The content of this section is about this organism and the ones related to it or applied in this study.

Mycobacterium is a widely distributed bacterium. There are over 80 named species of *mycobacterium* and two main pathogenic species: (1) *Mycobacterium tuberculosis* which is associated with affecting humans and certain mammals. (2) *M. Leprae*, the cause of leprosy, a chronic disease of humans. (19) According to World Health Organisation (WHO), these two diseases are among the main public health problems of many of the developing countries of the world. (20)

The other species that cause disease in man and other animals include: *M. bovis, M. avium intracellulare, M. kansasii, M. fortuitum* complex (including *M. chelonei*), *M. marinum, and M. ulcerans.* (20) Most pathogenic mycobacteria grow unusually slowly. (21)

In microscopy, *Mycobacteria* are seen as slender rod-shaped organisms, which are seen as 2-4 μm long, red, acid-alcohol-fast bacilli in a Ziehl-Neelsen stain. In electron microscopy, a thick cell wall, mesosomes and lipid inclusions can be examined. *Mycobacterium* has an unusually high lipid content, which is responsible for their characteristic resistance to drying in alcohol, acids, alkali, and certain germicides. (21)

The main diseases caused by *Mycobacteria* are tuberculosis (*Mycobacterium tuberculosis*), leprosy (*M. leprae*), systemic infections in AIDS patients (*M. avium*), and nosocomial and opportunistic infections (*M. fortuitum*). It is often difficult to treat mycobacterial infections because of these bacterias' intrinsic resistant ability to most common antibiotics and chemotherapeutic agents. Furthermore, the emergence of multidrug-resistant strains has also become an additional challenge in the control of tuberculosis. (22)

The species Mycobacterium fortuitum

In this study, *Mycobacterium fortuitum* (NCTC 10394) was used as a model substitute for *Mycobacterium tuberculosis* because of its fast growth cycle (3-4 days) and relatively rare human infection. (23)

Micobacterium fortuitum was originally found as an organism, which leads to disseminate skin infection (24). It is one of the rapidly growing species. *Mycobacteria fortuitum* is non-chromogenic. It occasionally causes pulmonary or disseminated disease but is principally responsible for post-injection abscesses and wound infections. (25)

Biological Materials and Methods Applied in This Study

Liquid and solid media:

There are two broad groups of media: liquid and solid. Many liquid media containing different nutrients have been devised and most bacteria can be grown at least one of them. However, liquid media have two disadvantages. In them, the characteristic appearance of growth usually does not exhibit especially and, except when they are designed for a particular biochemical test, they are only limitedly used in identifying species. Also, organisms can't be separated with certainty from mixtures if they are grown in liquid media. These disadvantages can be overcome by using solid media. On solid media the appearances exhibited by the colonies of different bacteria are useful in identification; and solid media are almost necessary in isolation of pure cultures. Organisms can only be grown directly from the body in pure culture occasionally so that solid media are almost always needed for the examination of pathological specimens. (26)

The culture media applied in this study:

Nutrient broth: This is the basis of most media used in the study of the common pathogenic bacteria. Good quality is very important for this media and a poor basal medium cannot be compensated by enrichment. (26)

Nutrient Agar: Nutrient agar is nutrient broth solidified by the addition of agar.

Blood agar: Blood agar is an enrichment media and is widely used in medical bacteriology. During the preparation of the medium, sterile blood is added to sterile nutrient agar that has been melt and cooled to 50°C. The concentration of blood may be varied from 5 *per* cent up to 50 *per* cent for special purposes. The concentration of ten *per* cent is most usually used. Either human or animal blood may be used; horse blood is the commonest one. (26)

Serial dilution methods:

In this method, a series of dilutions of both the standard antibiotic preparation and the test sample are prepared in a fluid nutrient medium. The tubes that contain sample with a serial of dilution concentrations are inoculated with a standard suspension of the test organism with and then incubated. The greatest dilution of the standard and of the sample, by which the growth of organisms is completely inhibited, is noted and from these the potency of the test sample is estimated. The bactericidal level in these tubes may be determined by sub-culturing from each tube and determining which concentration of the drug kills the test organism. The main drawback of the test is that it is laborious, requires strictly aseptic conditions (a chance contamination may completely invalidate the results) and is liable to be influenced by the growth of resistant mutants, which have an advantage in the presence of the antibiotic. Serial dilutions are now mainly used for the assay of antibiotics because the agar diffusion methods are too slow to be applicable. (27)

Agar diffusion methods:

In the bioassay the sample diffuses from a confined source through nutrient agar seeded with the test organism during the incubation. A concentration gradient of the antibiotic is thus set up and this results in a zone of inhibition being formed around the well containing the samples. Once the conditions of temperature, medium, and inoculum size are settled, the size of the zone of inhibition is proportional to the concentration of the antibiotic in the sample at the source. At the end of this bioassay the size of zones produced by a range of dilutions of a standard preparation are plotted and the concentration of antibiotic in various dilutions of the sample that give zones of comparable size are read from the graph. It has been found that the relationship between the square of the zone diameter and the logarithm of the antibiotic with antibiotics of small molecular size concentration is strictly linear. (27)

Medicinal Plants

Introduction to Natural medicine

Since ancient times, natural sources such as vegetable, animal, and mineral substances have been used for health care by 'witch-doctors'. The methods used are not only based on the knowledge and attitudes regarding physical, mental, and social well-being but also on the causes of disease and disability on social, cultural, and religious beliefs as well. Currently, these approaches, which are referred to 'traditional medicine', are still used in allopathic medicine as a major source of some of the useful drugs. Modern research into natural medicines is mainly concentrating on discovering new therapeutic agents and novel and richer sources of traditional natural drugs. (28)

Traditional medicine is cheaper than modern medicine. The high cost of the modern technology increased the cost of modern medicine and due to the higher cost of basic commodities the cost of modern health care has also recently increased. For most of the population in the third world, traditional medicine is more accessible. This is mainly because of a shortage of hospitals and health centres, and the unavailability of the medical and paramedical staff needed to man modern health delivery systems. Traditional medicine is also more acceptable for the people of developing countries than modern medicine. This could be due partly to the inaccessibility of modern medicine but the major reason contribute to the fact that it conflicts with the culture of those regions where the culture has been deeply rooted in people's social lives. (28)

One of the greatest arguments against traditional medicine today is that its efficacy is lack of scientific proof. Most of the claims about the efficacy of traditional medicine are made by the traditional medicine practitioners themselves and many have not been thoroughly investigated through scientific method. Another shortcoming of traditional medicine is the inaccurate diagnosis often given by the practitioners. In the description in the diagnosis of a traditional medicine practitioner, 'stomach trouble' could mean indigestion, an ulcer, or cancer of the stomach. Such imprecision is because the fact that the traditional medical practitioner does not know the pathology of certain diseases. As a result, therefore, he tends to treat the symptom rather than the disease, which can sometimes lead to further complications. Furthermore, their prescriptions lack precise

dosage. Even nowadays, some practitioners are still using such terms as teaspoonfuls while measuring specific dosage. Comparing to modern medical practice, the practitioner of traditional medicine is not hygienic enough in their methods. Traditional medicine has always been regarded with suspicion by modern doctors because there are some intangible aspects or occult practices that cannot be verified scientifically contained in the theory and method of traditional medicine such as witchcraft and the evil aspects which have made traditional medicine discredited. (28)

Medicinal plants

Since ancient times, plants have been one of the main sources of medicine and many of the currently available drugs have been derived directly or indirectly from them. In spite the rise of the pharmaceutical industry and the dominance of the synthetic drugs in western market, over 10,000 species of higher plants have been used in a medical way and over 70% of the world's population still relief from illness depending through this way. Today 25% of prescription drugs contain a plant-derived chemical or a derivative and interest in developing plants as a likely source of new commercial drugs is resurging. (29)

A medicinal plant is any plant with substances that can be used for therapeutic purpose in one or more of its organs or the ones that are precursors for the synthesis of useful drugs.

Traditionally, the plant material is taken by drinking an infusion made with hot water. The availability of material of the species enables hot water extracts to be made with a composition that is similar to the medicine. (30)

An example of a medicinal plant:

GINSENG- *Panax ginseng*

In Chinese, the word ginseng is directly translated as the "essence of man". It is the most valued herb used in China, and is also widely used in other Asian countries such as Japan and Korea. For thousands of years, ginseng has been used by the common people

as a tonic and emergency medicine to rescue dying patients and used by the rich as a rejuvenating and revitalizing agent. (31)

Although the seeds, flowers, leave, and stems of ginseng are available for use, common references to the herb allude to its root or rhizome. In the apothecary or the market, only the root or the rhizome is generally seen. (31)

The main therapeutic usage of ginseng is for revival of dying patients. It is reputed to be effective in treatment of shock, collapse of the cardiovascular system, haemorrhaging, and heart failure. Clinical trials on volunteers showed that ginseng extract can slow down the heart rate and reduce oxygen demand but the mechanism of action is very debatable. (31)

Ginseng is also can be used to treat the mild hyperglycaemia, reinforce sexual desire, reduce the effects of oxygen deprivation of brain tissue to improve mental and neurological performance, treat coughs, headaches, and fevers, etc. (31)

Medicinal plants and modern medicine

Currently, medicinal plants have become a field that is a curious mixture of ancient tradition applied to modern conditions without, in many cases, the benefit of modern science and technology. To be totally effective, it is important for the traditional practices to eventually be coupled with up-to-date scientific method. (32)

A German physician, B. Lehmann, drew a conclusion on the subject of cooperation between empiricism and science: 'Phytomedicines, exactly like other medicines, must stand up to the challenge of modern scientific evaluation. They need no special consideration when it comes to the planning and conduct of the clinical trials intended to prove their safety and efficacy. The distinctive feature of phytotherapy is its origin, namely, the many years of empirical use of plant drugs. Experience gained during this period should be taken into account along with clinical testing, in evaluating the effectiveness of phytomedicines.' (32)

Active substance in medical plants

Active substances refer to the various materials that are contained in medicinal plants and possess a number of activities. The discovery of naturally occurring chemical compounds was initiated by the chemical analyses of medical plants performed in the nineteenth century. It has been revealed that not only do medicinal plants contain compounds with special structures and pronounced properties, but also almost any plant contains some kind of special material. (33)

Currently, much research has been performed on the extraction and purification of the active compounds present in medical plants. For example, the flavonoid and phenylpropide constituents present in the aqueous extraction from *Buddleja* species have been found to have the anti-hepatotoxic activity through testing the isolated compounds. Fourteen extracts from Brazilian traditional medicinal plants applied to treat infectious diseases have also be found and can be potentially used as new effective strategies to treat methicillin-resistant infections. (33)

Some chemical constituents of plants:

Flavonoids

The flavonoids are all structural derivatives from the parent substance flavone, which occurs as a white mealy farina on *Primula* plants. (33)

Some flavonoid classes are more widely distributed than others such as flavones and flavonols which are more universal while some flavonoid such as isoflavones and biflavonyls are found in only a few plant families. (33)

Generally, flavonoids are present in the plants as mixtures and it is very rare to find a single flavonoid component in a plant tissue. In addition, the mixtures often are made up with different flavonoid classes. (33)

Alkaloids:

The alkaloids are the largest single class of secondary plant substances. The most common precursors of alkaloids are amino acids. About 5,500 alkaloid structures are known. There are always one or more nitrogen atoms, usually in combination as part of a cyclic system. Alkaloids are often toxic to man and many have dramatic physiological activities that make them widely used in medicine. They are usually colourless and optically active substances. Most of them present in the plants in the phase of crystalline but in fresh leaf or fruit material there a few of them (*e.g.* nicotine) present as liquids which often impact to the tongue as bitter taste. (33)

Strategies in the search for new active substance in medical plants

The approach adopted to obtain an exploitable pure constituent involves interdisciplinary work in botany, pharmacognosy, pharmacology, chemistry and toxicology. The procedure can be formulated as follows:

(1) Plant material is selected, collected, and botanical identified and prepared.
(2) The prepared materials are extracted with suitable solvents and are preliminarily analysed.
(3) The bioactivity in the extraction is preliminarily analysed by biological and pharmacological screening.
(4) Pure bioactive constituents are separated by chromatographic means.
(5) The structures of the constituents are determined.
(6) The pure compounds are analysed and pharmacological profiled.
(7) Toxicological testing.
(8) Partial or total synthesis.
(9) Derivatives of the constituent are prepared for studying structure-activity relationships.

In this project, the research was concentrated on the steps (1)-(5).

Processing medicinal plants to obtain active substances

Selection of plant material:

The collection of the plant materials can be random but it is more judicious to select the plant materials with certain criteria. By way of illustration, plants used in traditional medicine are more likely to provide pharmacologically compounds, which can be taken as criteria of plant material selection. Ideally, in phytochemical analysis, plant tissues used should be fresh and the material should be plunged into boiling solution within minutes after its collection. Sometimes, the plant under study is not at hand and material may have to be collected from another continent. In such cases, it is important for the freshly picked tissue being stored dry in a plastic bag to remain in good condition for analysis during the several days transport by airmail. Alternatively, plants may be dried before extraction. In this kind of operation, it is essential that the conditions of drying operation are carried out under controlled to avoid too many chemical changes occurring. Drying should be carried out as soon as possible in a good air draft without using high temperatures. After being thoroughly dried, plant can be stored for long periods of time before analysis. It has been proved that the analysis for flavonoids, alkaloids, quinines and terpenoids in the plant tissue that has been kept for many years still can be successfully carried out. (33)

At the stage of plant material selection, there is a very important point that contaminating material from other plants should be get rid of from the plant tissue under study. For example, it is essential to employ plants which are free from disease, i.e., the plants not affected by viral, bacterial or fungal infection. It is very likely for plant materials with contamination to lead to a poor result. Not only may products of microbial synthesis be detected in such plants, but also plant metabolism may be seriously altered by infection and unexpected products could be formed, possibly in large amounts. (33)

Extraction and isolation:

The precise method of extraction naturally depends on the texture and water content of the plant material to be extracted and on the type of substance that is to be isolated. In

general, to achieve a more effective extraction, the plant should be dried and ground first to enlarge the contact surface. (34)

The extraction is always taken using a series of solvents with of gradually increasing polarity in turn to obtain the active compounds with different polarities. Chloroform and ethyl ether are often used for terpenes and steroids while water and ethanol are always for glycosides and amino acid. (34) Alcohol, in any case, is a good all-purpose solvent for preliminary extraction. (33)

Subsequently, if exhaustive extraction is being attempted, it is really necessary for the material to be macerated in a blender and filtered. When isolating substances from green tissue, a successful extraction with alcohol should remove the extra chlorophyll into the solvent. When the tissue debris is completely free of green colour after repeated extraction, it can be assumed that the low molecular weight compounds have been extracted. (33)

The classical chemical procedure for obtaining organic constituents from dried plant tissue (heartwood, dried seeds, root, leaf) is to continuously extract the power of material in a soxhlet apparatus with a range of solvents, starting with ether, petroleum and chloroform (to separate lipids and terpenoids) and then using alcohol and ethyl acetate (for more polar compounds) in turn. This method is useful working on the gram scale. However, separation of the constituents can rarely be completed just in one solvent and the same compounds may be contained in several fractions in varying proportions. (33)

The extract obtained is usually evaporated in a rotary evaporator, which will concentrate bulky solutions down to small volumes under vacuum, at temperatures between 30 and 40°C. (33)

If a single crystalline substance is present, the crystals can be purified by recrystallization and then the material is available for further analysis. In most cases, mixtures of substances will be present in the crystals and it will be then be necessary to redissolve them up in a suitable solvent and separate the constituents by

chromatography. Many compounds also remain in the mother liquor and these will also be subjected to chromatographic fraction. As a standard precaution against degradation of material, concentrated extracts should be stored in the refrigerator. (33)

Separation:

The most important point for any successful programme involving the investigation of plant constituents with biologically active is the availability and choice of chromatographic techniques for the separation of pure substances. (35)

The most important preparative separation techniques employed in the isolation and purification of plant constituents are divided into two groups: solid phase chromatography and liquid-liquid chromatography, according to whether a solid stationary phase (or liquid fixed on a solid support) or an all-liquid partition procedure is used. Among the methods in the former category, column chromatography is very popular and used extensively. This method includes ion-exchange resins, polymeric columns, gel filtration, and chromatography over silica gel or chemically modified silica gel. (35)

Buddleja madagascariensis

Introduction

The genus *Buddleja* L. includes approximately 100 species, which include *B. asiatica* Lour, *B.officinal* Maxim, *B. curviflora* Hook. *and* Arn, *B. davidii* Franchett, *B. madagascarensis* Lam, *B. amaricana* L, *B.globosa* Lam, *B. incana* Ruiz and Pav. *Buddleja* species play a minor role in the ethnopharmacology of several areas of the world where they are indigenous. (36)

Buddleja species are widespread and there are some remarkable similarities in their medical uses including the treatment of skin complaints and particular as eye lotions and the treatment to catarrh and coughs. They are also used as fish poisons and as

diuretics. They can benefit the liver by either increasing biliary flow or by treating cirrhosis.

Flavonoid and iridoid glycosides appear to be the active secondary metabolites so far isolated which are contained in all the species of this genus. (36)

Phytochemistry of Buddleja

Phytochemical investigation of the genus has been somewhat neglected but some constituents which are present in reasonable quantities, namely iridoid and flavonoid glycosides have been looked into. The leaves and flowers of *Buddleja* species have been found to contain large amounts of flavonoids, iridoids, and phenylpropide glycosides. Fractionation from aqueous extracts of leaves of one of *Buddleja* species (*B. globosa*) that is used in the traditional medicine for the treatment of liver ailments has been found to contain five iridoids, two phenylpropide glycosides, and a flavonoid glycoside. A number of bioactive flavonoids, sesquiterpenoids, iridoids, and steroids have been found in another buddleja specie-*B. cordata subsp. cordata*. (38)

Medical usage of Buddleja madagascariensis

Many of the medical usages of *Buddleja* in folk medicine such as a topical antiseptic and as diuretics are due to the compounds that are known to have biological activity and identical or similar to those found in the genus. (39)

The leaves are used in traditional medicine for asthma, coughs and bronchitis. This is probably due to an expectorant action caused by stimulation of secretion in the respiratory tract. It is known that several terpene and saponins have this effect, while mimengoside B (structure sees Figure1) has been found in the methanol extract of the leaves, which might account for its therapeutic activity. (40)

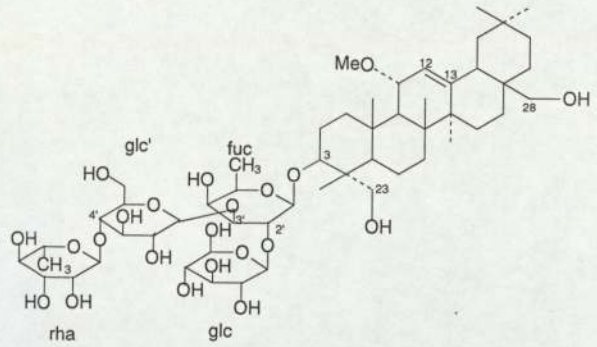


Figure 1: Structure of mimengoside B (adapted from A.M. Emam, A.M. Moussa, R. Faure, R. Elias, G. Balansard, Isolation of B, a trierpenoid saponin from *Buddleja madagascariensis*. Ethnopharmacology) (40)

Seeing the facts mentioned above, there could be compounds with the ability to treat illness of respiration system present in *Buddleja madagascariensis*. Current research is trying to discover the bioactive compounds of anti *Mycobacteria fortuitum* in *madagascariensis*. The same activity has been found in the stem bark of one of the *Buddleja* species-B. cordata subsp. cordata. (41)

Molecularly Imprinted Polymers (MIPs)

Introduction

Molecular imprinting is an emerging technique for the preparing polymeric materials with highly selective and affinitive properties. The method involves complexation in solution of a target compound (template) with functional monomers, through either covalent or non-covalent bonds followed by a polymerisation reaction with an excess of cross-linkers. (42) After the highly crosslinked polymer has formed, the template molecules are removed from the polymer matrix leaving selective binding sites that are complementary to the template in terms of its shape, size and functionality in the

polymer template. Because of these binding sites, MIPs can selectively rebind the template in preference to other closely related structures. So ideally, the molecularly imprinted polymer is able to selectively recognize the template molecule from other components in a complex sample. (43)

In terms of the relationship between the template molecule and the polymerisable sub-units, the methodology for preparing the MIP can be categorized mainly into two methods- covalent imprinting and non-covalent imprinting. (42)

The pre-organized approach (covalent imprinting) involves the formation of covalent bonds between the functional monomers and the template molecules prior to polymerisation. Thus the template molecules need to be chemically modified with the functional monomers, and after polymerisation the template molecule is removed by cleavage of the covalent bonds via which it is attached to the polymer. Upon rebinding of the template to the polymer, the covalent bonds are re-formed. (42) Another methodology is the self-assembly approach (non-covalent imprinting) where relatively weak non-covalent intermolecular interactions, such as electrostatic interactions, hydrogen bonding, π-π bonding and hydrophobic interactions, between the template and the functional monomers serve to get molecular assemblies. Hence the selection of functional monomers is crucial to form high affinity binding sites. (44, 45)

In the majority of recent works, the non-covalent has become the method of choice because of its advantages over covalent method. The most obvious one is that no specific covalent modification of the template molecule is required. In terms of the binding sites that can be exploited, non-covalent imprinting is much more flexible for the range of templates which can be targeted. Additionally, non-covalent imprinting appears to offer significant improved rebinding kinetics and practical flexibility over the covalent imprinting. Furthermore, the non-covalent approach is experimentally simpler to realize than covalent imprinting methods because the complexation step is achieved simply by mixing the template with the functional monomer(s) in a suitable solvent. The template can be removed by being simply washed repeatedly with a suitable solvent or solvent mixture. Seeing these advantages, in this project, non-covalent imprinting was taken as the approach of preparing the MIP. (42)

On the other side, a major drawback of non-covalent systems is the unavoidable heterogeneity of the binding sites obtained from the multitude of complexes formed between the template and the functional monomers. The non-covalent bonding is generally not strong and thus an excess of functional monomer is usually required to favour template-functional monomer complex formation and to maintain its integrity during the polymerisation. As a result, a fraction of the functional monomers are randomly incorporated in the polymer matrix resulting in the formation of non-selective binding sites. (46)

Non-covalent molecular imprinted polymers

A stable pre-polymerisation complex between the template molecule and functional polymerisable units is the foundation of all imprinted polymers formation. In the non-covalent approach, the bond between the template and polymerisable moiety is non-covalent. In the complex, non-covalent interactions, typically, proton transfer events and hydrogen bonds, are the force to the formation of the complex and to maintain the integrity. Figure 2 shows the pre-polymerisation complex stabilised by hydrogen bonds and ionic forces and formed between an amphiphilic template and two monomers. (44, 45)

Figure 2: pre-polymerisation complex between an amphiphilic template and two monomers (adapted from C. J. Allender, K. R. Brain, C. M. Heard, Molecularly Imprinted Polymers-Preparation, Biomedical Application and Technical Challenges.) (42)

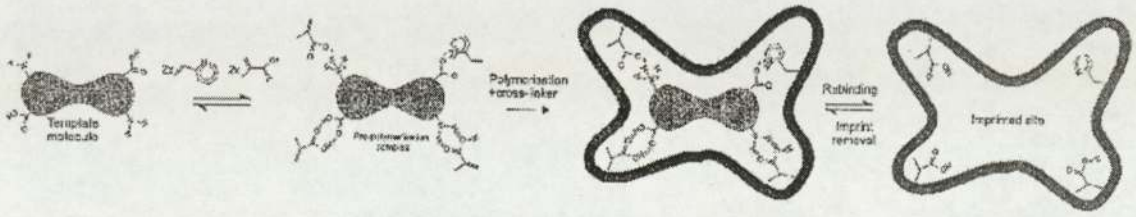
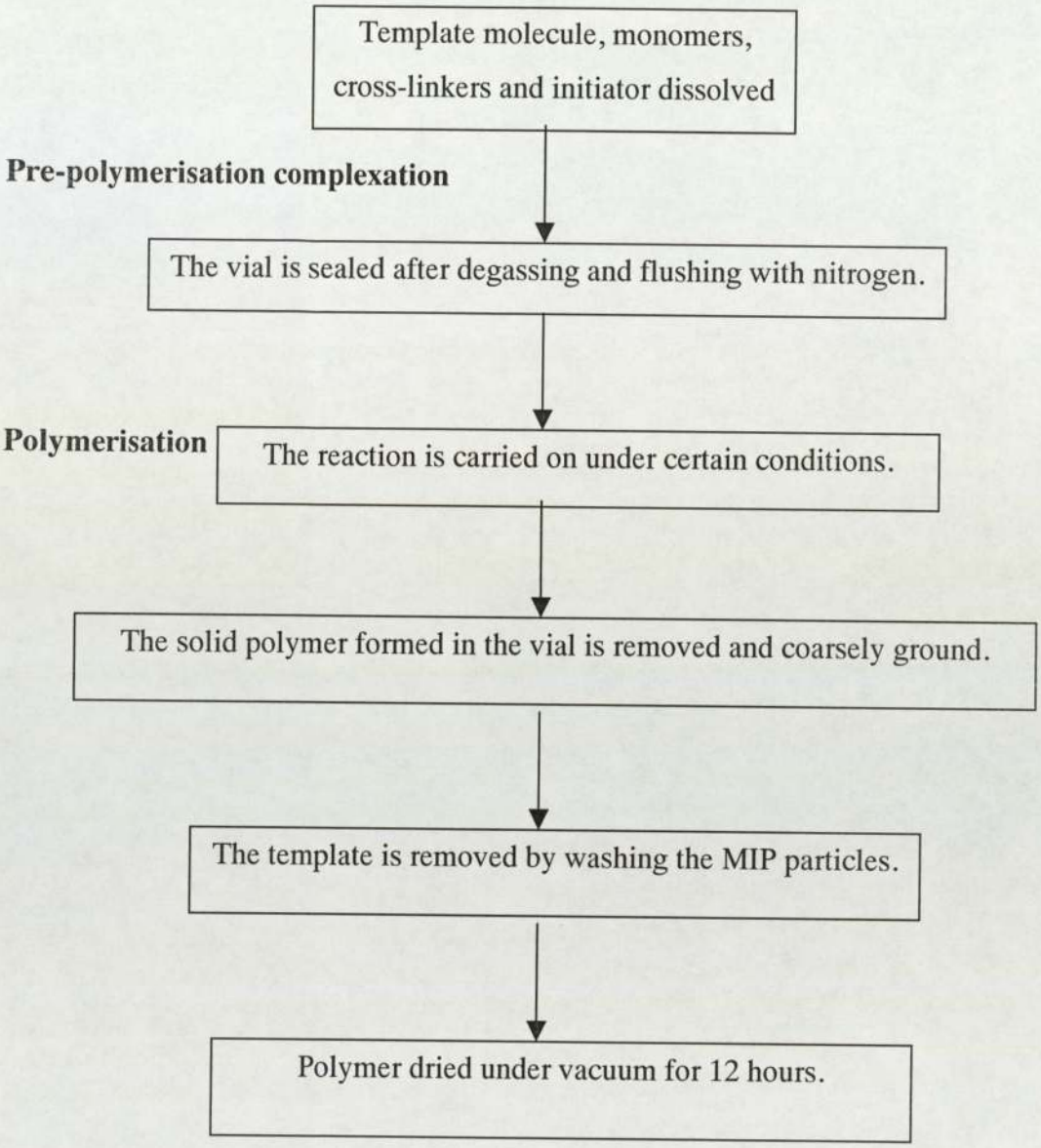


Figure 3: the process of preparing the non-covalent imprinted polymers is summarized below:



The chemistry of non-covalent molecular imprinting:

Template: The template is a molecule that contains a certain level of functionality that can be paired with reciprocating moieties. It has been observed that small, multi-functional templates produce imprinted sites that are less specific. In addition, a complex molecule with a more specific three-dimensional structure leads to a more highly specific imprint than a simple linear geometry.

Generally, non-polar solvents favour the stability of the pre-polymerisation complex. Therefore, the choosing of solvent for a polar template molecule, both imprint

specificity and solubility for the template are considered. If a polar solvent is required to match the solubility of the template, then the complex will be less stable and the imprint less specific. (47)

Cross-linker: The crosslinker provides imprinted polymers with high mechanical stability and chemical inertness. A very high degree of crosslinking (70–90%) is required since the polymer matrix needs a certain rigidity to retain the shape of the specific cavities and preserve the imprinted memory. (48)

In polymer matrix, rigid structure is good for selectivity while flexible structure favours accessibility. Thus the choice of cross-linker is a compromise between rigidity and accessibility. (47, 48, 49) After many different cross-linkers were evaluated, EGMA (ethylene glycol dimethacrylate) has been proved to be the most suitable one for most imprinting process. (48)

Functional monomers: A functional monomer is a twofold molecule which contains the units which undergo the interaction with the template molecule and the polymerisable ones. Methacrylic acid (MAA) has been used extensively as it fulfils the essential criteria for a functional monomer. (42)

Factors that affect the function of molecularly imprinted polymer:

The structure of the polymer matrix is fundamental to MIP function. A high degree of cross-linking, good accessibility and flexibility, sufficient mechanical strength and a degree of thermal stability are all important aspects of MIP function. (47, 48)

Cross-linker: In general, the higher the proportion of cross-linker the greater the selectivity. (49) It is considered that at higher degrees of cross-linking, the polymer chain is less mobile and thus helps to retain the recognition site integrity. (49) 80-85% of cross-linker is used in the majority of studies. (42)

Solvent: In the pre-polymerisation complex, the significant interactions are polar. So the complex is more stable in apolar solvents. (45, 50, 51). Generally, the more polar the porogen, the weaker the resulting recognition effect. (45) Seeing the fact that relatively non-polar organic solvents favour the formation of the non-covalent interactions between the template molecule and the functional monomer, polymerisation has generally been carried out in non-polar solvents such as chloroform, toluene, dichloromethane and benzene. In practical terms, the solubilising power of the solvent must be considered. The final aim is to find a solvent that is as non-polar as possible yet still provides a system in which monomers and template dissolve well. (42) Research has shown that the ideal rebinding condition for a given template should include the solvent used as porogen. (46) The solvents that are most often used to prepare MIPs for SPE applications are chloroform, acetonitrile, dichloromethane and toluene. (45)

Proportion of chemicals: The relative proportion of the constituents of a MIP affects both the morphology and the function of the polymer. It has been concluded that the radio of 1:1(mL:g) of porogen to monomer favours the optimal selectivity and affinity (52).

It has been shown that the internal surface area of the polymer was proportional to the percentage of cross-linker so the quantity of cross-linker is fundamentally important for high selectivity. As the proportion was increased from 10% to 95%, a corresponding increase in selectivity is observed. Below 10% cross-linker, the selectivity was not observed. (53)

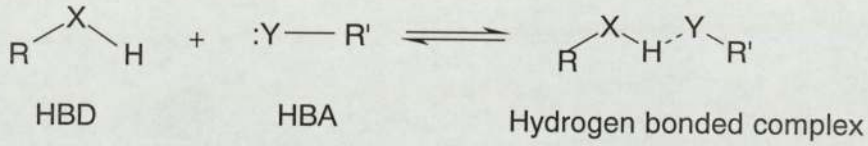
As to the ratio of functional monomer to template, subsequent studies have generally used approximately twice the stoichiometric ratio to one. (42)

The forces and interactions in non-covalent imprinted polymers:

Directional, induction and dispersion forces: these forces exist between a dipole and a system containing an opposite charge. The dipoles can be permanent, induced or temporary. (54)

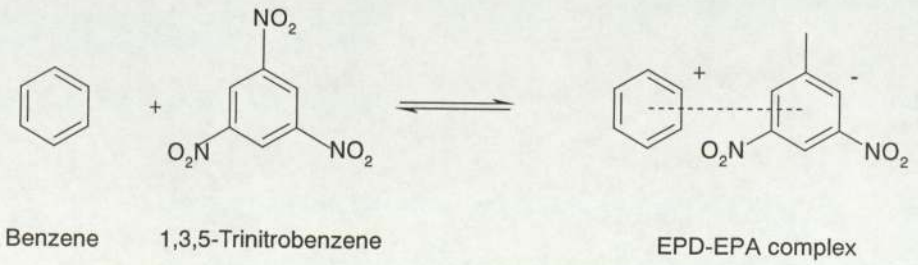
Hydrogen bonding and charge transfer forces: A hydrogen bond is the bond between a covalently bound hydrogen atom and another atom. The bond consists of a hydrogen bond donating moiety (electron acceptor) and a hydrogen bond accepting group (electro donor). (Figure 4) (55)

Figure 4: Hydrogen bond (adapted from C. J. Allender, K. R. Brain, C. M. Heard, Molecularly Imprinted Polymers-Preparation, Biomedical Application and Technical Challenges.) (42)



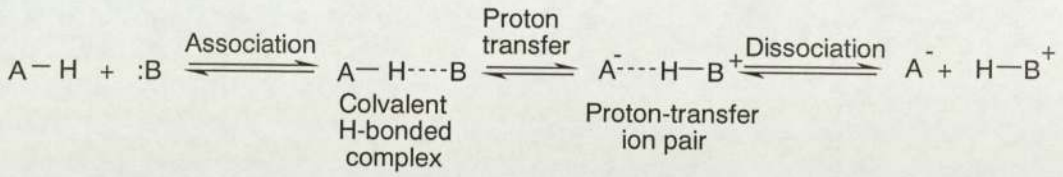
Charge transfer forces are from the movement of electron pairs and protons from one molecule to another. In these forces, both bonding electrons are derived from the same molecule. The π-π and π-σ are the most important charge transfer forces at the pre-polymerisation stage of molecular imprinting. Figure 5 shows an example of these interactions. (55)

Figure 5: π-EPD-π-EPA complex formation (adapted from C. J. Allender, K. R. Brain, C. M. Heard, Molecularly Imprinted Polymers-Preparation, Biomedical Application and Technical Challenges.)(42)



Ionic interactions: Acid-base or proton transfer process (Figure 6) is the most important ionic interaction in the non-covalent molecular imprinting. It is the electrostatic attraction between ions resulted by the removal of an acidic proton by a base. (42)

Figure 6: The proton transfer process (adapted from C. J. Allender, K. R. Brain, C. M. Heard, Molecularly Imprinted Polymers-Preparation, Biomedical Application and Technical Challenges.)



The imprinting process:

Pre-polymerisation complex: In the formation of a stable pre-polymerisation complex, the hydrogen bonding, steric effects and acid/base proton transfer equilibria are the prime interactions. (48)

Polymerisation: The polymerisation process contains three stages: initiation, propagation and termination. First, the initiation stage forms reactive species which is to start the polymerisation. And then, propagation results in the formation of a high molecular weight polymer. Finally, the stable polymeric product is obtained at the termination stage. (42)

Post-polymerisation processing: After being formed, MIPs need to be reduced to a fine material with certain particle size. This is achieved by grinding and sieving processes. (42) Since the particles are not uniform in shape, MIP columns tend to demonstrate relatively poor flow dynamics, which results in poor chromatographic performance. (42)

The final step in the preparation of a MIP is the template removed. A suitable solvent system is taken to remove the template by washing the formed polymer. Methanol or acetonitrile, with the addition of acetic acid, is commonly used in this step. The removing process can be carried in a soxhlet in exhaustive hot extraction or by simple stirring. (42)

The final product of the MIP production process is mainly a fine powder containing a population of vacant binding sites. There is still a small proportion of non-recoverable template trapped deep in the particle matrix. (42)

The interactions in rebinding are the same as those that responsible for the pre-polymerisation complex under the same conditions. Therefore, the conditions that are applied in the pre-polymerisation complex stage are also suitable for template rebinding. (42) Hydrophilic interactions (hydrogen bonding and ionic interactions) are the significant interactions for rebinding and recognition. Selectivity is determined and related with the number of theoretical hydrophilic interactions between the template and monomers. (43) Since the electronic environment of the polarisable group and the environment in which it exists are the main aspects affecting the strength of a hydrogen bond, in a hydrophobic environment, a strongly hydrogen bonding functional monomer always is used to get the maximum affinity for the template. (42)

It is known that the template-MIP rebinding affinity can be controlled by changing the composition of the rebinding solvent. The increase in hydrogen bonding potential of the solvent/mobile phase can deduce the affinity and selectivity (45). This is because of the competition between the template-MIP hydrogen bonding and the solvent molecules-MIP hydrogen bonding is increased. (42)

The MIPs used for Solid Phase Extraction (SPE) are mainly polymerised in the way of at a high temperature (e.g. 60°C). In the most common polymerisation procedures the yield polymer monoliths are then ground and sieved to produce particles of the required dimensions. (45)

An automated protocol which results in savings of time and reagents compared to the monolith procedure has been further optimised. In fact, it is the scaled-down version of

the established monolith procedure: molecularly imprinted polymers were prepared on the bottom surface of chromatographic vials. 60 polymers (50 mg each) were allowed to be synthesized in parallel. Both blank and imprinted polymers were then evaluated by rebinding tests. By this method, an automated way could be found to make the polymer with good applicability for template targets and monomer compositions. In this method, for a larger number of templates, a thermal initiation based system was applied (58).

Chemical intuition is very important in successfully imprinting protocol design. To obtain the desired recognition properties, in the evaluation of this big group of polymers, it is desirable to find a practical technique that can rapidly synthesize the molecularly imprinted polymers (58).

The evaluation is firstly taken on the amount of template in the solvent released from the polymers, and then, on the amount of template rebound to the polymers after an exhaustive extraction. In this step, the calculation of the evaluation is based on a reference, which can be an internal standard or the rebinding to a blank, non-imprinted polymer. Using this method, combinatorial synthesis of a large number of polymers and efficient screening of the various factors influencing the recognition ability of MIPs can be well carried out. (58)

In all free radical polymerisations, it is of extreme importance to removal the oxygen from the pre-polymerisation solution. It has been observed that in the rebinding tests, a much lower rebinding percentage was observed only for the batch that had not been degassed. (58)

MIPs have been used in many areas for selective recognition of particular molecules, including separations, immunoassay, sensors and catalysis/artificial enzymes, but there are few reports about the use of MIPs as separation materials for extracting certain active compound directly from herbs. However, it was still expected that the use of MIPs in this field could be exploited because of such features as the shape, size and functionality selectivity, strong affinity on rebinding target compounds, the significantly low cost for the preparation, and the workability in organic solvent. (56)

MIPs are very suitable as stationary phases in separation science and have already proven their capability in HPLC and thin layer chromatography (TLC) for separating structural analogues. Besides their evident potential as stationary phases in chromatographic techniques, the usefulness of MIPs has also been demonstrated by solid-phase extraction (SPE) in sample pre-treatment and in analyte recognition studies employed as affinity assays. (57) Solid phase extraction (SPE) can be used to isolate and preconcentrate the components in complex samples. The binding agents in the materials routinely used in SPE are usually non-specific based ones. Molecularly imprinted polymers (MIPs) can be used to achieve selective extraction, analogous to those achieved by immuno-based extraction systems, and thus may represent an advance on conventional SPE materials. (58)

The design of molecularly imprinted solid phase extraction (MISPE) methods includes also the choice of the selective solvent. MISPE columns are actually very short chromatographic columns. A good selective solvent should elute the interferents by the first 1–2 ml passing through the MISPE column. The analyte, on the other hand, should not be removed in the same volume. A typical MISPE column is filled with 50 mg of MIP. (59)

One limitation of MIPs applied in SPE is even after extensive washing, there may be leaching of the template from the polymer, and this contaminates the sample. Since MIPs are made with large quantities of template, a small number of imprint molecules may remain in the formed polymer and these may leak later during the process of SPE, which can interfere the result of trace analysis. This has been observed in several cases. This problem has been solved by using a structural analogue as the template when preparing the polymer. The template can be separated in the subsequent chromatographic analysis, and therefore, the interference of the template bleeding can be avoided. However, this approach can only be taken in the situation that a suitable analogue exists but this is often difficult to find. (46)

Since best recognition is often showed in the solvent used as porogen in the polymerisation of the MIP, the same solvent is often also used as the washing solvent, or, in case of selective adsorption, as the extraction solvent. The most widely used organic solvents for the selective washing step are therefore dichloromethane,

acetonitrile and chloroform. (46) It has been generally recognized that solvent used to wash the MIP is extremely important for ensuring a selective extraction. It is also very important that the analyte can be efficiently desorbed in a small volume to achieve a higher recovery. For this reason, the eluent to be used must also be optimised. (46)

Not only can MIPs selectively retain the template, but also can recognize other structurally related compounds. In some cases this can be used for extracting a family of compounds rather than only one compound. For example, Ferrer et al. demonstrated that a MIP prepared with terbutylazine (triazine herbicide) as template was able to selectively retain a family of chlorotriazines and phenylurea herbicides were washed from the MIP after a washing step with dichloromethane. (46)

In this study, a series of MIPs was prepared using a non-covalent imprinting approach. Curcumin, rutin and quercetin were chosen as the representative target compounds because they are the typical members of the large family of flavonoids which are commonly found in medical plants (see Introduction-medical plant) and also widely distributed in *Buddleja* species (see Introduction-*Buddleja madagascariensis*)

Chapter 1

Screening the antibacterial activities of *B. Madagascariensis*

The antibacterial activities in the extractions from *B. Madagascariensis* were detected using the tube dilution method, agar plate method and agar diffusion method. Three kinds of organisms (*E.coli*, *S. aureus* and *M.fortuitum*) were chosen for the test. The preparation of the extractions and the details of the organisms and the test methods are given in Chapter 5 *Tests of the antibacterial activities in B. Madagascariensis*)

Anti M.fortuitum activity in the extractions (1)-(9) of B. madagascariensis

The extractions (1)-(9) of *B. madagascariensis* are laid below:

1. Ethanol Extraction of Leaves
2. Petrol Extraction of Leaves
3. Water Extraction of Leaves (prepared in Chapter5 - Extraction of Antimicrobial Compounds From Buddleja Madagascariensis - Extraction of leaves - Part I:)
4. Ethanol Extraction of Bark
5. Petrol Extraction of Bark
6. Water Extraction of Bark (prepared in Chapter5 - Extraction of Antimicrobial Compounds From Buddleja Madagascariensis - Extraction of bark)
7. Ethanol Extraction of Secondary Bark
8. Petroleum ether Extraction of Secondary Bark
9. Water Extraction of Secondary Bark (prepared in – Chapter5 - Extraction of secondary bark)

The test procedures can be found in Chapter5- *Anti M.fortuitum activity in the extractions (1)-(9) of B. madagascariensis*. The results of these tests are given in Chapter 5 - Table 5.2.

From the results, no anti mycobacterial activity was found for extracts (2)-(9). Extract (1) was active and the MIC was 0.625mg/L.

The different results in these experiments could due to the different *Mycobateria fortuitum* cultures applied in these tests. The culture used in experiment 1 was taken from freezer and used directly after thawing, while the one used in experiments 2-4 was from the warm room at 37°C. The *Mycobateria fortuitum* in these cultures were all in their log phase but the different conditions before applied to use could lead to the various in number of the organisms in these two cultures, which perhaps lead to difference in the results.

The antibacterial activity in the methanol extraction

The procedure can be found in Chapter5- *The antibacterial activity in the methanol extraction.* The results are given in Table5.4

The methanol extract was tested for its antimycobacterial activity three times. An anti *M. fortuitum* effect was found in the third time of experiment. The MIC (minimum inhibition concentration) was the concentration of the extraction in tube 4 (10mg/ml).

The procedure was exactly the same for experiment 2 and 3 but the anti *M. fortuitum* effect was only found in experiment 3. The reason could contribute to the different *Mycobaterium fortuitum* culture applied in these tests (further explanation sees Chapter1: *Anti M. fortuitum activity in the extractions (1)-(9) of B. madagascariensis*)

The antibacterial activity in the chloroform, hexane, dichloromethane extractions

The content in the test tubes and the results are laid in Chapter5- *The antibacterial activity in the chloroform, hexane, dichloromethane extractions*-Table5.5 - 5.10.

From these results no antimicrobial activity was found in these samples.

Chapter 2

Preparation and Testing of Molecularly Imprinted Polymers

The MIPs were made in bulk (as large scale monoliths) or on a small-scale in a matrix of small vials. The preparation procedure was given in Chapter 6. Their nomenclature is given below:

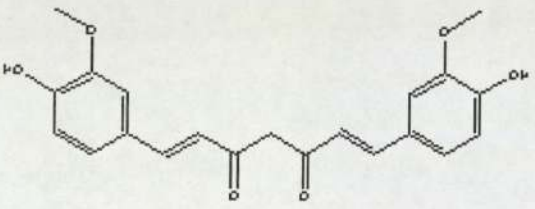
Bulk MIP with Rutin as the template	**Rutin#1**
Two batches of MIPs made in bulk with curcumin as the template	**Curcumin#1, Curcumin#2**
Five batches of MIPs made in bulk with quercetin as the template	**Quercetin#1 - #5**
Small scale matrix synthesis of MIPs with rutin as the template	**Rutin#1'**
Small scale matrix synthesis of MIPs with curcumin as the template	**Curcumin#1'**
Small scale matrix synthesis of MIPs with quercetin as the template	**Quercetin#1', Quercetin#2'**

Polymer preparations

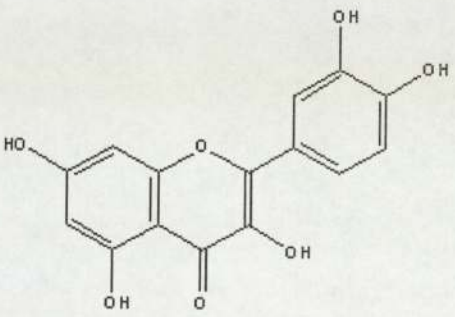
Figure 2.1 molecular structure of the chemicals used in forming the polymers

Templates

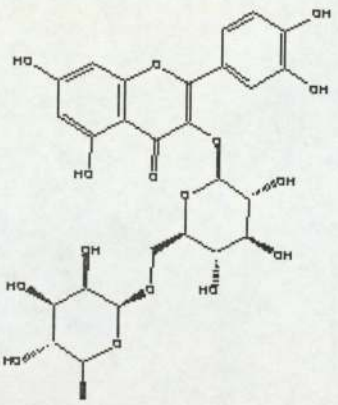
Curcumin



Quercetin

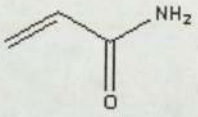


Rutin

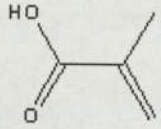


Monomers

Acrylamide

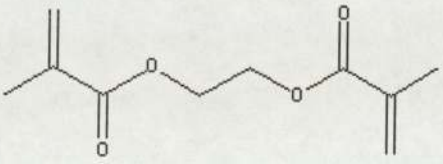


MAA



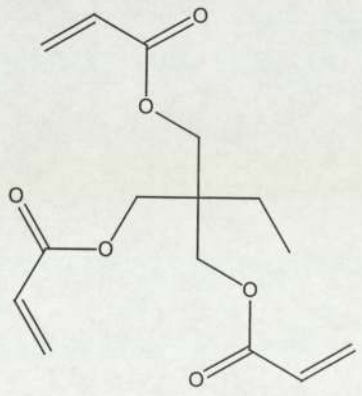
Cross-linker

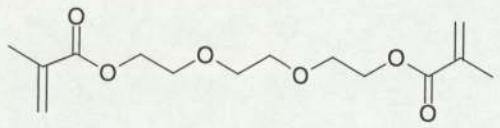
Ethylene Glycol Dimethacrylate



Triethylenglycol-dimethacrylate

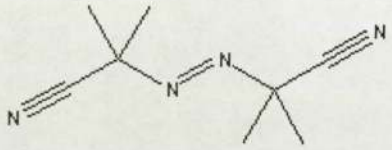
Trimethylopropane Triacrylate





Initiator

AIBN



Generally, it is believed that compounds soluble in polar solvents are not desirable as templates because polar solvents can disturb the non-covalent reactions between the templates and functional monomers. But in this study, quercetin, which has several polar hydroxyl groups and can be dissolved in THF, was used as a template. This was based upon the successful experience of reference 4, in which acrylamide was chosen as the functional monomer because it is favourable for the interaction with quercetin in polar environment. The successful imprinting of quercetin was because the cohesion between quercetin and acrylamide was strong enough to overcome the competing action of the solvent THF. Consequently, compounds soluble in polar solvents still could be imprinted by the non-covalent method in polar solvents as long as the negative action caused by polar solvents was not so strong as to impede the complexation.

The proportion of cross-linker applied in this study in polymer formation was very high especially in the polymers made on a small scale (see Table 6.1 and section Chapter 6 - Preparation and Testing of Molecularly Imprinted Polymer - Polymer preparations) to achieve a high selectivity and good rigidity. On the other side, the proportion of cross-linker was reduced by dilution of the crosslinker with ethyl acrylate

in the formation of Quercetin#4 (the fourth batch of MIPs made in bulk with Quercetin as the template) and Quercetin#5 (see Chapter 6-Table 6.1), and Quercetin#2' (the second batch of MIPs made on a small-scale. see Chapter 6 - Preparation and Testing of Molecularly Imprinted Polymer - Polymer preparations) in order to achieve a looser construction polymer with better accessibility and flexibility.

When extracting the templates, methanol was used for removing the template rutin instead ethanol which had been used in dissolving it (see Chapter 6 - Preparation and Testing of Molecularly Imprinted Polymer - Polymer preparations) and acetone for quercetin instead of THF because these solvents showed better dissolving ability to these templates.

Test of rebinding ability by UV

The results of the rebinding test (procedure see Chapter 6 - Preparation and Testing of Molecularly Imprinted Polymer - Test of rebinding ability by UV) are presented in Table 2.1 and Table 2.2.

In the results of the test of the polymers made in bulk (see Table 2.1), the concentration of stock solution was reduced apparently in Quercetin#5 (from 25mg/L to 19mg/L), which shows the rebinding ability of this polymer. The specific binding amount on this polymer was 120μg/g. It was counted as below: the difference between the concentrations of stock solution of before and after the rebinding plus the volume of the solution and then divided by the mass of the polymer (25mg/l-19mg/L)×4mL /0.2g). There was no apparent reduction of the concentration of stock solution in the test of the other polymers, including Rutin #1(the first batch of MIPs made in bulk with Quercetin as the template), Curcumin#1, #2, Quercetin #1-#3 and Quercetin#4.

In the results of the test of the polymers made in small-scale matrix (see Table2.2), there was no apparent reduction in the concentration of stock solution was observed, which means there is no rebinding ability presents in these polymers.

The proportion of cross-linker applied in this study in polymer formation was very high especially in the polymers made in small-scales (see Table6.1 and section Chapter 6 - Preparation and Testing of Molecularly Imprinted Polymer - Polymer preparations) to achieve a high selectivity and good rigidity for the templates but also can lead to difficulty in templates removing, and further, poor rebinding ability. This could account for the results for Rutin#1(the first batch of MIPs made in bulk with Quercetin as the template), Curcumin#1, #2, Quercetin#1, #2, and #3.

Table2.1: Results of the rebinding ability test of the polymers formed in bulk

Polymers with Various Templates		Rutin#1	Curcumin#1	Curcumin#2	Quercetin#1	Quercetin#2	Quercetin#3	Quercetin#4	Quercetin#5
Size of the Particle of the Polymer (mesh)		60-80	60-80	60-80					
Mass of Polymer (mg)		50	50	50	200	200	200	200	200
Concentration of Stock Solution	Before(mg/L)	50	50	50	25	25	25	25	25
	After(mg/L)	53	51	51	24	24	24	25	19

Table2.2: Results of the rebinding ability test of the polymers formed in small-scale matrix

Polymer with Various Templates		Rutin#1'	Circumin#1'	Quercetin#1'	Quercetin#2'
Mass of Polymer (mg)		32-70	23-64	12-55	17-38
Concentration of Stock Solution	Before(mg/L)	50	50	25	25
	After(mg/L)	49-60	51-67	24-27	24-26

To avoid the problem just mentioned, the proportion of cross-linker was reduced by dilution with the monomer ethyl acrylate (Figure2.2) together used with cross-linker-ethylene glycol dimethacrylate (Figure2.3) in order to achieve a looser construction polymer with better accessibility and flexibility in formation of Quercetin#4 and Quercetin#5 (the fifth batch of MIPs made in bulks with Quercetin as the template. see Chapter-Table 6.1) made in bulks and Quercetin#2' made in small-scale (see Chapter 6 - Preparation and Testing of Molecularly Imprinted Polymer - Polymer preparations).

Figure2.2

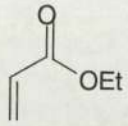
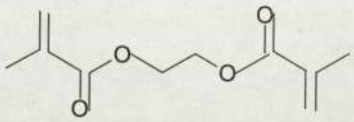


Figure2.3



Rebinding ability was observed in Quercetin#5 (see Table 2.1 and Table 2.2) but not in Quercetin#4. This could due to the proportion of ethyl acrylate to cross-linker (ethylene glycol dimethacrylate) (1:2) in Quercetin#4 was much lower than Quercetin#5 (2:1). It would seem that a better compromise between polymer flexibility and rigidity of cavity shape was achieved in polymer batch Quercetin#5. In batch Quercetin#4, with its lower proportion of ethyl acrylate, the polymer was too rigid to allow access to the binding sites. Quercetin#2' was made with the same chemistry and approximate proportions (see Chapter 6 - Preparation and Testing of Molecularly Imprinted Polymer - Polymer preparations - *Small-scale matrix synthesis of imprinted polymers*- Quercetin#2') as Quercetin#5 but had no rebinding ability observed. This could be attributed to the different situations of the formed polymers and the processes after the polymers were formed. Polymer Quercetin#5 was presented as a sieved powder from a ground monolith whereas Quercetin#2' was used as an intact monolith at the bottom of the vial. Because of the greater surface area exposed in Quercetin#5 to the extraction solvent, the template in the former was more likely to be removed leaving more cavities in the solid. In extraction test, after the stock solution was added, Quercetin#5 was shaken for 3 hours while Quercetin#2' was kept steady for 1 hour. The mixing of polymer and stock solution gave Quercetin#5 a better opportunity to encounter and absorb the test molecules present in solution.

Further rebinding tests on quercetin and a related compound – rutin - were taken with various particle sizes of batch Quercetin#5. The results are given in Table2.2. The concentration of the stock solution apparently was reduced in all the polymers. The rebinding ability was calculated from the quantities given in Table 2.2.

When quercetin was used as the template, polymer Quercetin#5 with a smaller particle size (less than 60mesh) showed a much better rebinding ability (the specific binding amount is 465.1μg/g) than the bigger size (60-20mesh) polymer (the specific binding amount is 79.2μg/g). It also showed a better rebinding ability to rutin (the specific binding amount is 710.5μg/g) than quercetin.

Table2.2: The Results of Further Extraction Tests on Polymer #5

Test Compounds		Quercetin		Rutin
Size of the Particle of the Polymer(m)		60-20	<60	<60
Weighth of the Polymer(mg)		202	51.6	56.3
Concentration of Stock Solution	Before(mg/l)	25	25	100
	After(mg/l)	21	19	90
Specific Binding Amount (μg/g)		79.2	465.1	710.5

Quercetin#1(the first MIP made in bulk with Quercetin as the template) was made with the same chemicals and their proportion as the polymer that had been found exhibiting high selectivity for quercetin and good affinity to its structure analogues in reference 4. There was, however, no recognition of template observed in Quercetin#1. This could be attributed to the template removing processes applied in this study is different from the original one in reference 4. The template was removed from Quercetin #1 by soxhlet extraction using a single solvent while the latter was packed into steel column first and then washed online in the HPLC system with a series of mixed solvents with declining polarity. The method of gradient elution washing which worked well reference 4 wasn't applied to Quercetin#1 in this study because the quoted mixed solvents do not form a suitable azeotrope.

In the polymerisation step nitrogen is always used to exclude the oxygen to avoid radical quenching. In the polymers made in small-scale matrix, it was impractical to use nitrogen and this may have influenced the course of the polymerisation reaction and lead to variable results.

Chapter 3

HPLC Fractionation

The methanol extraction that had been found to have activity against M fortuitum was made into solution. Attempts were made to separate the components by HPLC. The procedure and conditions are seen in Chapter 7 Experimental Relating to Chapter 3 - HPLC Fractionation – Conditions of HPLC.

The retention times and masses of the fractions from the HPLC fractionation are given in Table3.1

Table 3.1 The retention times and weights of the fractions form HPLC

Fraction No.	1	2	3	4 5 6 7	8	9	10	11
Retention Time of the Fractions (min)	5	30	30	from 35 to 40	50	60	88	105
Weight of the Fractions(mg)	2	38	20	10 15 10 19	2	4	1	20

Note:

Eleven main fractions at various retention times were obtained. There were four peaks from 35min to 40min but their retention times could not be determined accurately.

Screening of the Fractions from HPLC

The antibacterial activity in the 11 fractions from HPLC was detected. For the procedure see Chapter - 7 Experimental Relating to Chapter 3 - Screening of the Fractions from HPLC.

In the tube testing, there was no growth of *M. fortuitum* observed in the tubes containing fractions 3, 4, 5, 8 or 10. This could be due to the presence of the compounds with antibiotic activity against *M. fortuitum* in these fractions. The concentrations of

these fractions in these tubes were 1.00, 0.50, 0.75, 0.10, 0.05mg/ml. On the other hand, there was no zones of inhibition was observed in the test against *E.* coli and S. *aureus* in agar diffusion methods, which means there could be no antibiotic activity against *E.* coli and S. *aureus* in these fractions. The following research is concentrated on the anti *M. fortuitum* ability present in this extraction.

Chapter 4

Characterisation of the Compounds in the *Buddlja Madagascariensis* Extraction Samples by Mass Spectrum

The data for the fractions 1, 2, 4, 5, 6, 7, 8, 10 from MS (Electrospray +) are given in Table 4.1.

Table 4.1: The data for the fractions 1, 2, 4, 5, 6, 7, 8, 10 from MS (Electrospray +)

Sample Number	Adduct Ion	Actual Peak	Charge	Curve Fitted Mass	Standard Deviation
1	+ Na	585.95, 606.80	28 27	15762.68	0.01
2	+ K	481.10 498.10	27 26	11933.47	0.00
		392,90 437.15	9 8	3184.27	0.14
	+ H + Na		2 1	606.40	0.18
4	+ H2O	459.10 481.15	21 20	9262.82	0.06
		349.05 415.25	6 5	1986.06	0.02
5	+ K	392.80 414.90	17 16	6011.36	0.07
6	+ H	415.25 437.05	20 19	8284.88	0.03
		546.95 585.95	15 14	8189.19	0.04
	+ Na	415.25 437.05	19 18	7453.08	0.10
		391.05 437.05	9 8	3312.78	0.04
	+ H + 2Na	413.40 459.20	9 8	3297.60	0.01
		393.05 413.40	18 17	6228.33	0.08
7	+ H + 2Na	415.00 429.15	27 26	9936.58	0.08
8	+ H + 2Na	307.05 327.05	14 13	3641.17	0.05
10	+H, +Na		13 12	6098.16	0.07
	+Na	537.10 708.40	4 3	2056.26	0.15

The Names and molecular weights and associated references of some components found in *Buddleja* are given in Table 4.2

Table 4.2: Compounds previously reported in *Buddleja*

Compound	Mass	Rerference
vanillic acid	168	61
mannitol	182.2	62
syringin	210.2	62
cyclocolorenone	218	63
buddledone A	221	63
buddledone B	235	63
daidzein	254	61
dihydrobuddledin A	278	63
acacetin	284	63
eriodictyol	286	64
7-hydroxy-7, 9,12-abietrien-14-one	300	65
6- hydroxyluteolin	302	66
sucrose	342	63
2[4'-hydroxyphenyl]-ethyl palmitate	377	67
2[4'-hydroxyphenyl]-ethyl heptadecanoate	391	67
2[4'-hydroxyphenyl]-ethyl stearate	405	67
2[4'-hydroxyphenyl]-ethyl nonadecanoate	419	67
octacosanoic acid	424	61
2[4'-hydroxyphenyl]-ethyl arachidate	433	67
scutellarein 7-glucoside	447	68
pyracanthoside	448	64
aucubin	452	64
betulin acid	456	61
2-(4-hydroxyphenyl) ethanol ester of docosanoic acid	460	69
2[4'-hydroxyphenyl]-ethyl behenate	461	67
glucohesperetin	462	64

ester of 4-hydroxyphenyl alcohol and docosanoic acid	472	69
2[4'-hydroxyphenyl]-ethyl tricosanoate	475	67
O-methylcatalpol	482	64
2-(4-hydroxyphenyl) ethanol ester of lignoceric acid	488	69
2[4'-hydroxyphenyl]-ethyl lignocerate	489	67
ester of lignoceric acid and 4-hydroxyphenyl alcohol	500	69
2[4'-hydroxyphenyl]-ethyl pentacosanoate	503	67
vanilloyl ajugol	511	64
2[4'-hydroxyphenyl]-ethyl hexacosanoate	517	67
6-feruloyl ajugol	537	70
leucoceptoside A	622	64
acteoside	624	70
acteoside isomer	624	70
verbascoside	636	71
plantainoside	638	70
leucosceptoside	638	70
jionoside D	638	70
martynoside	652	70
isomartynoside	652	70
diosmetin 7-O (2",6"-di-O---rhamnopyranosyl)---glucopyranosides	754,	71
Hesperetin	756	71
angoroside C	784	70-
mimengoside B	1104	71
3-O---rhamnopyranosyl-(14)---glucopyranosyl-(13)-[--glucopyranosyl-(12)]---fucopyranosyl-3,23,28-trihydroxyoleane-11,13(18)-diene	1618	62
buddlejasaponin I	1634	62
3-O---rhamnopyranosyl-(14)---glucopyranosyl-(13)-[--glucopyranosyl-(12)]---fucopyranosyl-3,16,23,28-tetrahydroxyoleane-11,13(18)-diene	1676,	62

Comparing the molecular weights in the results from positive electrospray mass spectrometry with the ones of the components reported in references (60)-(71), no compound in this project was found the same as the ones that has been reported before. The molecular weights in the results in this study are very large, showing that there could be long chain of sugars or high molecular mass lipids in these molecules. Further work is required to purify completely and characterise these compounds.

MIP Column Chromatography

The extraction from water layer (See Chapter 7 Experimental Relating to Chapter 3 - HPLC Fractionation – Method) with anti M. fortuitum activity was applied to MIP column chromatography for further separation and purification. For the procedure see Chapter 4 - Characterisation of the Compounds in the *Buddlja Madagascariensis* Extraction Samples by Mass Spectrum - MIP Column Chromatography. Fractions1-70 were obtained and the absorption of UV spectrum ranged between 200nm-900nm of 1, 5, 10, 15, 20, 25, 30, 35, 40 were recorded. Results see Table 4.3

Table 4.3 The absorption of UV spectrum of fraction 1, 5,10, 15, 20, 25, 30, 35, 40

Fractions	1	5	10	15	20	25	30	35	40
λ (nm) range of some main absorbance peaks	Absorbance								
200-269	0.3-0.4		0.1-0.3						
223-276									
285	0.341	0.431		0.276	0.660	0.299	0.291	0.267	0.163
325	0.387	0.443							
410			0.722	0.742	0.476	0.250	0.236		
435						0.211	0.233	0.110	0.092
663			0.298	0.330	0.248	0.145	0.187	0.103	
834									0.103

The main range of absorbance was from 200-663nm (200-325 for fractions 1 and 5, 223-663 for fractions 10, 15, 20, 25, 30, 35 and 223-834 for fraction 40). There were 3-4 main absorbance peaks for each fraction but their ranges were various. The situations of fractions 1 and 5 (both 200-269nm, 285nm, and 325nm) were alike; Fractions 10 (223-276nm, 410nm, 663nm), 15 (223-276nm, 285nm, 410nm, 663nm) and 20 (223-276nm, 285nm, 410nm, 663nm) were alike; 25 (223-276nm, 285nm, 410nm, 435nm, 663nm) and 30 (223-276nm, 285nm, 410nm, 435nm, 663nm) were alike. 35 (223-

276nm, 285nm, 435nm, 663nm) and 40 (223-276nm, 285nm, 435nm, 834nm) were alike. Thus it can be established from these results that through column chromatography the compounds in the extraction were separated. There were different compounds in different fractions. The same compounds may also present in fractions 1 and 5; 10, 15 and 20; 25 and 30; 35 and 40.

The major absorbance was from fraction 1 to 20 (absorbances were mainly from 0.3-0.7) and absorbance reduced after fraction 20(absorbances were mainly from 0.1-0.3). There were only little peaks of absorbance after the fraction 40. It could be told that main part of the compounds in the extraction was out of the column before fraction 20 and there was little extra in the column after fraction 40.

Chapter 5
Experimental Relating to Chapter 1

Plant materials:

B. madagascarensis used in this project was harvested in December and June.

Extraction of Antimicrobial Compounds From Buddleja Madagascariensis

Extraction of leaves:

Part I: Fresh leaves (865g) were removed from each bush of *B. madagascarensis* and the petioles detached. The material was cut into small pieces and was transferred together with the appropriate solvent, in a ratio of 6 parts of solvent to one part of sample, to a blender. The lid was placed on the blender and the contents macerated until all the material was reduced to a fine suspension. The contents of the blender were transferred to a brown Winchester, which was placed in a shaking water bath held at an appropriate temperature for 24h.

The liquid phase was decanted through a glass fibre filter paper attached to a Buchner funnel. The filtrate was then transferred to a round-bottomed flask and attached to a rotary evaporator. The contents of the flask were evaporated to dryness at the appropriate temperature for the chosen solvent and then the sample was kept under 4°C in darkness. The solvents used in order of extraction were, ethanol, petrol and double-distilled water.

Part II: Fresh leaves (151.76g) were made into samples as described in Part I. The solvents used in order of extraction were, methanol, chloroform, hexane, dichloromethane.

Part III: Fresh leaves (166.6g) were extracted with hexane and made into a sample as described in Part I,

Extraction of bark:

The bark (4.67g) was removed from the stems and made into samples as described under 'Extraction of leaves', Part I.

Extraction of secondary bark:

The remaining hard parts of the plant (215g) were cut into small pieces and made into samples in the same way as described in 'Extraction of leaves', Part I.

Screening of the antibacterial activities in B. Madagascariensis

Source of materials:

Nutrient Broth: CM1, Oxoid Ltd, England

Nutrient Agar: CM3, Oxoid Ltd, England

MiddleBrook 7H9: Lot 83583JC, Difco Laboratories, USA

MiddleBrook ADC: Lot 1282257, Becton Dickinson Microbiology Systems, USA

Defibrinated Horse Blood: Batch No.: 00008972, P.C.V.: 39%, E & O Laboratories, Scotland

Organisms used in this experiment were from Dr Peter Lambert's Microbiology Research Laboratory, School of Health and Science, Aston University

E. coli: DC2, S. *aureus*: NCTC 6571, *M fortuitum*: NCTC 10394

Preparation of Agar Plates, Broth and Organisms

Nutrient Agar Plates

Nutrient agar powder (7g) was added to distilled water (250ml) in a conical flask. A Bunsen was used to heat the solution until all the power was dissolved. The conical flask was sealed with aluminium foil and autoclaved. While warm, the agar was aseptically transferred into a series of plate and left to cool and set.

Nutrient Broth

Nutrient broth powder (3.25g) was added to 250ml of distilled water in a conical flask. It was stirred until all had dissolved.

Blood Agar Plates

These were prepared in the same way as the nutrient agar plates except that house blood of (5% in total volume) was added to the agar aseptically which is still warm after autoclaving.

Middlebrook Broth

Middlebrook 7H9 broth (0.97g) was dissolved in distilled water (180ml) in a conical flask. The conical flask was sealed with aluminium foil and autoclaved. The mixture was cooled down to room temperature and Middlebrook ADC Enrichment media (20ml) was added aseptically.

Bacteria cultures preparation:

The 3 test organisms used in the tests were *E.* coli (DC2), S. *aureus* (NCTC 6571) *and M fortuitum* (NCTC 10394).

E. coli/S. *aureus* (one loop) was transferred from agar plate to 10ml nutrient broth media. The culture was incubated under 37°C for 24h, except the one used in plate diffusion, which is incubated for 4 hours to get the log phase.

M fortuitum is prepared by Dr Peter Lambert's Microbiology Research Laboratory, School of Health and Science, Aston University. It was kept in freezer and melt before use.

Protocol for Extract Testing with Organisms

Tube dilution method:

A group of tubes was set up containing media.

Organism stock inoculum (10µl) was added to each of the tubes.

Tube 1 is a control containing only the media.

Tube 2 is a second control containing the media and the inoculum.

Tube 3 is a third control containing the inoculum and the solvent used for dissolving the samples.

The remaining tubes contained doubling dilutions of a sample of the extract i.e.:

a. tube 4 contained undiluted extract
b. tube 5 contained half the concentration in tube 3.
c. tube 6 contained a quarter.

.....

The tubes were incubated at 37°C. 1 day for *E. coli* and *S. aureus*, and 4 days for *M. fortuitum*. The tubes were assessed for the presence of growth.

Agar plate method:

After the incubation, one loop of the containing from each tube was scribed on an agar plate, (Nutrient agar plate for *E. coli* and *S. aureus*, and blood agar plate for.) and incubated (1day for *E. coli* and *S. aureus* and two days for *M. fortuitum*). Then the growth of the organism was assessed. In this study, this method was used as a supplement to tube dilution method for testing the bioactivity presents in the samples. After the incubation, the culture from the test tubes was scribed on a agar plate and incubated. And then the growth of the organism on the media was assessed. If there was no growth observed in test tubes while observed on the agar plate for the same sample, it can be told that this extraction has the inhibition ability but no sterilization ability to the organism.

Agar diffusion method

A dish containing nutrient agar was inoculated with a test organism. The extracts were aseptically placed in holes in the agar. The dish was incubated at 37°C and zones of inhibition were observed. The bioactivity presents in the extracts was analysed according to the diameters of the zones of inhibition.

Anti M. fortuitum activity in the extractions(1)-(9) of B. madagascariensis

The anti *M. fortuitum* activity in the extractions (1)-(9) was tested using the tube dilution method (see Chapter5- *Protocol for Extract Testing with Organisms*). 11 test tubes were set up and each of them contained 1ml nutrient broth media. *M. fortuitum* stock inoculum. Extractions that were dissolved in DMSO were added into each of the tubes. The content in each tube and the concentrations of the extraction are given in Table 5.1. The test was repeated for each extract except 5 and 8. The test on extract 1 was performed four times.

The tubes were incubated at 37°C. After 4 days, the tubes were assessesed for the presence of growth. From the results, the situations were all the same for each experiment except the first time for extraction 1

The results of these tests are given in Table 5.2. For the results of the samples (2)-(9), growth of the organisms was observed in tubes 2-10 and there was no growth in the control 1 that only contained 1ml of media. This means the organism that had been found grow in these tubes was *M. fortuitum* that was added and the media used in these experiments were functioning correctly. From these results, there was no anti *M. fortuitum* activity observed in these extractions.

On the other hand, in the result of the first time for extraction (1), there was no growth in tubes 3, 4, 5 and the situations for the rest tubes were all the same as in the other experiments, which means the anti *M. fortuitum* activity could present in this extraction and the MIC (minimum inhibition concentration) was the concentration of this extraction in tube 5 (1.25mg/ml). This experiment was repeated another three times but

the results were different from the first time- there was growth in every tube except control 1. This means there was no anti *M. fortuitum* activity observed in this extraction.

Table 5.1: antibacterial ability of *Buddleja madagascariensis* extracts with *Mybacterium fortuitum*

Tube Number	Concentration of the Extraction (mg/mL)	DMSO Present (µl)	Volume of the Organism Stock Solution Added (µL)
1(Control 1)	0	0	0
2(Control 2)	0	50	10
3	5	10	10
4	2.5	10	10
5	1.25	10	10
6	0.625	10	10
7	0.318	10	10
8	0.159	10	10
9	0.080	10	10
10	0.040	10	10

Table5.2: Presence of the growth of the organisms in test tubes

Extraction number			(1)				(2)		(3)		(5)	(6)		(7)		(8)	(9)	
Test Number			1	2	3	4	1	2	1	2	1	1	2	1	2	1	1	2
Results	Tube Number	1	N	N														
		2	Y	Y														
		3	N	Y														
		4	N	Y														
		5	N	Y														
		6	Y	Y														
		7	Y	Y														
		8	Y	Y														
		9	Y	Y														
		10	Y	Y														

Note:

1. N= No Growth Y= Growth

2. In these tables, samples of extracts were mentioned as numbers as below:

Ethanol Extraction of Leaves - (1)

Petrol Extraction of Leaves - (2)

Water Extraction of Leaves - (3) (prepared in Chapter5 - Extraction of Antimicrobial Compounds From Buddleja Madagascariensis - Extraction of leaves - Part I:)

Ethanol Extraction of Bark - (4)

Petrol Extraction of Bark - (5)

Water Extraction of Bark - (6) (prepared in Chapter5 - Extraction of Antimicrobial Compounds From Buddleja Madagascariensis - Extraction of bark)

Ethanol Extraction of Secondary Bark - (7)

Petroleum ether Extraction of Secondary Bark - (8)

Water Extraction of Secondary Bark - (9) (prepared in – Chapter5 - Extraction of secondary bark)

The antibacterial activity in the methanol extraction

The antibacterial (including *E. coli*, *S.* aureus and *M. fortuitum*) activity in the methanol extraction (prepared in Chapter5 - *Extraction of Antimicrobial Compounds From Buddleja Madagascariensis* - Extraction of Leaves - Part Π) was tested using the tube dilution method (Chapter 5 - Protocol for Extract Testing with Organisms) and agar plate method (Chapter 5 - Protocol for Extract Testing with Organisms). The antibacterial activities against these three organisms were tested first using the tube dilution method and then using the agar plate method. The test against each organism was repeated for three times. And then agar plate method was used for further detection on the growth of the organisms in the culture. The content in the test tubes is given in Table 5.3. The results of the test are given in Table 5.4

Table5.3: Tube contents in the antibacterial screening of the methanol extraction from *Buddleja madagascariensis*

Test Number	1						2						3					
Tube Number	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Media (mL)	10																	
Organism (µL)	0	10					0	10					0	10				
Extraction Concentration (mg/ml)	0	0	2.5	5	10	20	0	0	5	10	20	40	0	0	5	10	20	40

Table5.4: Presence of growth of the organisms in the antibacterial screening of the methanol extraction from *Buddleja madagascariensis*

Test Times			1						2						3					
Tube Number			1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Results	a	M	N	Y					N	Y										
		P																		
	b	M																		
		P																		
	c	M													N	Y	N	N	Y	Y
		P													N	Y	N	N	Y	Y

Note :

1. a= *E.coli* b= *S. aureus* c= *M.fortuitum*
2. M= Media P= Plate
3. N= No Growth Y= Growth

From the results given in the table, it can be seen that for the first two times of experiment, growth of all the three organisms (mentioned as a, b, c in the table) was observed in the media (M in the table) except control 1 in the tube dilution method, which means there was no activity against these organisms presents in this sample.

The anti M. *fortuitum* effect was showed in the third time of experiment that can be told from the table: no growth observed in tube 3 and 4. The MIC (minimum inhibition concentration) was the concentration of the extraction in tube 4(10mg/ml).

The antibacterial activity in the chloroform, hexane, dichloromethane extractions

The antibacterial (including *E.coli*, *S.* aureus and *M. fortuitum*) activities in the chloroform extraction, hexane extraction and dichloromethane extraction (prepared in Chapter5 - *Extraction of Antimicrobial Compounds From Buddleja Madagascariensis* - Extraction of Leaves - Part II) were tested in tube dilution method (see Chapter 5 - *Extraction of Antimicrobial Compounds From Buddleja Madagascariensis* - Extraction of Leaves) and agar plate method (see Chapter5 - Protocol for Extract Testing with Organisms).

The content in the test tubes and the results are given in Table 5.5 - 5.10.

Table5.5: Tube contents in the antibacterial screening of the chloroform extraction from *Buddleja madagascariensis*

Test No.	1				2				
Tube Number	1	2	3	4	1	2	3	4	5
Media(mL)	1								
DMSO(μL)	500								
Organism (μL)	0	10			0	10			
Extract concentration (mg/mL)	0			9.07	0			0.95	1.90

Table5.6: Presence of growth of the organisms in the antibacterial screening of the chloroform extraction from *Buddleja madagascariensis*

Test No.			1				2				
Tube Number			1	2	3	4	1	2	3	4	5
Results	a	M	N	Y			N	Y			
		P									
	b	M						Y			
		P									
	c	M						Y	Y	N	Y
		P						Y	Y	Y	N

Note :

1. a= *E. coli* b= *S. aureus* c= *M. Fortuitum*
2. M= Media P= Plate
3. N= No Growth Y= Growth

Table5.7: Tube contents in the antibacterial screening of the hexane extract from *Buddleja madagascariensis*

Test No.	1				2				
Tube Number	1	2	3	4	1	2	3	4	5
Media(mL)	1								
DMSO(μL)	500								
Organism(μL)	0	10			0	10			
Extract concentration (mg/mLl)	0			8.53	0			1	2

Table5.8: Presence of growth of the organisms in the antibacterial screening of the hexane extract from *Buddleja madagascariensis*

Test No.			1				2				
Tube Number			1	2	3	4	1	2	3	4	5
Results	a	M	N	Y			N	Y			
		P									
	b	M						Y			
		P									
	c	M						Y	Y	Y	Y
		P						Y	Y	N	Y

Note :

1. a= *E. coli* b= *S. aureus* c= *M. fortuitum*
2. M= Media P= Plate
3. N= No Growth Y= Growth

Table5.9: Tube contents in the antibacterial screening of the dichloromethane extract from *Buddleja madagascariensis*

Test No.	1				2				
Tube Number	1	2	3	4	1	2	3	4	5
Media(ml)	1								
DMSO(µl)	500								
Organism(µl)	0	10			0	10			
Concentration of Extraction (mg/ml)	0			8.53	0			1	2

Table5.10: Presence of growth of the organisms in the antibacterial screening of the dichloromethane extract from *Buddleja madagascariensis*

Test No.			1				2				
Tube Number			1	2	3	4	1	2	3	4	5
Results	a	M	N	Y			N	Y			
		P									
	b	M						Y			
		P									
	c	M						Y	Y	Y	Y
		P						Y	Y	N	Y

Note :

1. a= *E.coli* b= *S. aureus* c= *M. fortuitum*
2. M= Media P= Plate
3. N= No Growth Y= Growth

Chapter 6

Experimental Relating to Chapter2

Preparation and Testing of Molecularly Imprinted Polymers

Polymer preparations

Bulk synthesis of imprinted polymers

Template, monomer, the stirrer bar and solvent were added to a flask. After the chemicals were dissolved in the solvent initiator and cross-linker were added. (The quantities of the chemicals and their molecular structures are given in table 6.1 and Chapter 2-Figure 2.1). The flask was fitted with septum and clamped on a stirrer hotplate and the mixture was stirred rapidly. The flask was evacuated using a vacuum line/needle combination and was filled with argon using an argon balloon/needle combination. The above evacuation/filling procedure was repeated a further 4 times. The flask was fitted with the argon balloon and was clamped in an oil bath and stirred at 60°C for 48hours. The flask was allowed to cool to room temperature. The resulting solid polymer was removed into a mortar and ground into power. It was washed with solvent to remove the template using sinter funnel and a buchner flask and sucked to dryness. In the template extraction process methanol was used for rutin and acetone for quercetin because these two templates showed good solubility in these solvents. Curcumin was removed by THF. The extraction was performed in a soxhlet for 2 days and then polymer was dried under vacuum for 24 hours.

The dried polymer was ground again and then was separated into several groups with different sizes: 20-60mesh, 60-80mesh, less than 80mesh after sieving for 5 hours.

Table 6.1: The Chemicals used in forming polymers

Starting Materials / Names of Polymer Batches		Quantities of Chemicals							
		Rutin#1	Curcumin#1	Curcumin#2	Quercetin#1	Quercetin#2	Quercetin#3	Quercetin#4	Quercetin#5
Template	Rutin	207mg (3.4e-4mol)							
	Curcumin		115mg (3.1e-4mol)	102mg (2.8e-4mol)					
	Quercetin				348mg (1.2e-3mol)	347mg (1.1e-3mol)	376mg (1.2e-3mol)	348mg (1.2e-3mol)	348mg (1.2e-3mol)
Monomer	Methacrylic Acid	305mg (3.5e-3mol)	69mg (8.0e-4mol)	55mg (6.4e-4mol)					
	Acrylamide				370mg (5.2e-3mol)	367mg (5.2e-3mol)	359mg (5.0e-3mol)	370mg (5.2e-3mol)	370mg (5.2e-3mol)
Cross-linker	Trimethylopropane Triacrylate	20mL (7.4e-2mol)				5.0e-2mol			
	Triethylenglycol-dimethacrylate			20ml (7.6e-2mol)			10g (3.5e-2mol)		
	Ethylene Glycol Dimethacrylate		11ml (8.3e-2mol)		10g (5.0e-2mol)			8g (4.0e-2mol)	5g (2.5e-2mol)
Initiator	AIBN	100mg (6.1e-4mol)	153mg (9.3e-4mol)	155mg (9.4e-4mol)	42mg (2.6e-4mol)	34mg (2.1e-4mol)	44mg (2.7e-4mol)	42mg (2.6e-4mol)	42mg (2.6e-4mol)
Solvent	THF		11mL	11mL	11mL	11mL	11mL	11mL	11mL
	Ethanol	30mL							
Other	Ethyl Acrylate							2g (2.0e-2mol)	5g (5.0e-2mol)

Small-scale matrix synthesis of imprinted polymers

90 4ml vials were weighted and placed in 15×6 matrix (Table6.2).

Table6.2: The matrix in polymer formation

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A															
B															
C															
D															
E															
F															

Rutin#1' (The batch of small scale polymers with rutin as the template)

The following solutions were made up each in ethanol. The total volume of each solution was 10mL. The quantity of each compound is given below:

cross-linker 1: trimethylopropane triacrylate: 4.679g (1.6e-2mol).

cross-linker 2: ethylene glycol dimethacrylate: 5.01g (2.5e-2mol).

cross-linker 3: triethylenglycol-dimethacrylate: 5.01g (1.8e-2mol).

Rutin : 0.50g (8.2e-4mol) and AIBN: 0.42g (2.6e-3mol).

methyacrylic acid 1: 0.065g (7.5e-4mol).

methyacrylic acid 2: 0.132g (1.5e-3mol).

methyacrylic acid 3: 0.197g (2.3e-3mol).

methyacrylic acid 4: 0.266g (3.0e-3mol).

methyacrylic acid 5: 0.327g (3.8e-3mol).

methyacrylic acid 6: 0.386g (4.5e-3mol).

methyacrylic acid 7: 0.462g (5.4e-3mol).

methyacrylic acid 8: 0.525g (6.1e-3mol).

methyacrylic acid 9: 0.585g (6.8e-3mol).

methyacrylic acid 10: 0.657g (7.6e-3mol).

methyacrylic acid 11: 0.713g (8.3e-3mol).

methyacrylic acid 12: 0.789g (9.2e-3mol)

methyacrylic acid 13: 0.844g (9.8e-3mol).

methyacrylic acid 14: 0.907g (10.5e-3mol).

methyacrylic acid 15: 0.972g (11.3e-3mol).

Cross-linker solution (100μL), Rutin and AIBN combined solution (50μL), and methycrylic acid solution (50μL) was added to each vial. The quantity of each compound in each vial is given below.

In each vial: Rutin (4.1e-6mol) and AIBN (1.3e-5mol)

1-5: cross-linker1 (1.6e-4mol)

6-10: cross-linker 2 (2.5e-4mol)

11-15:cross-linker 3(1.8e-4mol)

A1-F1: methyacrylic acid 1(3.8e-6mol)

A2-F2: methyacrylic acid 2(7.5e-6mol)

A3-F3: methyacrylic acid 3(1.2e-5mol)

A4-F4: methyacrylic acid 4(1.5e-5mol)

A5-F5: methyacrylic acid 5(1.9e-5mol)

A6-F6: methyacrylic acid 6(2.3e-5mol)

A7-F7: methyacrylic acid 7(2.7e-5mol)

A8-F8: methyacrylic acid 8(3.1e-5mol)

A9-F9: methyacrylic acid 9(3.4e-5mol)

A10-F10: methyacrylic acid 10(3.8e-5mol)

A11-F11: methyacrylic acid 11(4.2e-5mol)

A12-F12: methyacrylic acid 12 (4.6e-5mol)

A13-F13: methyacrylic acid 13 (4.9e-5mol)

A14-F14: methyacrylic acid 14 (5.3e-5mol)

A15-F15: methyacrylic acid 15 (5.7e-5mol)

Curcumin#1' (The batch of small scale polymers with curcumin as the template)

The following solutions were made up each in ethanol. The total volume of each solution was 10mL.

cross-linker 1: trimethylopropane triacrylate: 5.44g (1.8e-2mol).

cross-linker 2: ethylene glycol dimethacrylate: 5.01g (2.5e-2mol).

Curcumin: 0.48g (1.3e-3mol) and AIBN: 0.45g (2.7e-3mol)

methyacrylic acid 1: 0.24g(2.8e-3mol).

methyacrylic acid 2: 0.36g(4.2e-3mol).

methyacrylic acid 3: 0.48g(5.6e-3mol).

90 4ml vials were weighed. Cross-linker solution (100μL), Curcumin and AIBN combined solution (50μL) and methycrylic acid solution (50μL) were added to each vial. The quantity of each compound is given below:

In each vial, Curcumin (6.5e-6mol) and AIBN (1.4e-5mol)

A1-C15: cross-linker 1 (1.8e-4mol)

D1-F15: cross-linker 2 (2.5e-4mol)

A1-F5: methyacrylic acid 1(1.4e-5mol)

A6-F10: methycrylic acid 2(2.1e-5mol)

A11-F15: methyacrylic acid 3(2.8e-5mol)

Quercetin#1' (The batch of small scale polymers with quercetin as the template)

The following solutions were made up each in ethanol. The total volume of each solution was 10mL.

cross-linker 1: trimethylopropane triacrylate; 5g (1.7e-2mol).

cross-linker 2: ethylene glycol dimethacrylate: 5g (2.5e-2mol).

cross-linker 3: triethylenglycol-dimethacrylate: 5g (1.7e-2mol).

Quercetin: 0.30g (9.9e-4mol) & AIBN: 0.40g (2.4e-3mol).

methyacrylic acid 1: 0.066g(7.7e-4mol).

methyacrylic acid 2: 0.131g(1.5e-3mol).

methyacrylic acid 3: 0.195g(2.3e-3mol).

methyacrylic acid 4: 0.264g(3.1e-3mol).

methyacrylic acid 5: 0.325g(3.8e-3mol).

methyacrylic acid 6: 0.392g(4.6e-3mol).

methyacrylic acid 7: 0.456g(5.3e-3mol).

methyacrylic acid 8: 0.520g(6.0e-3mol).

methyacrylic acid 9: 0.586g(6.8e-3mol).

methyacrylic acid 10: 0.650g(7.5e-3mol).

methyacrylic acid 11: 0.717g(8.3e-3mol).

methyacrylic acid 12: 0.786g(9.1e-3mol)

methyacrylic acid 13: 0.846g(9.8e-3mol).

methyacrylic acid 14: 0.915g(1.1e-2mol).

methyacrylic acid 15: 0.978g(1.1e-2mol).

Cross-linker solution (100μL), quercetin and AIBN combined solution (50μ L), and methycrylic acid solution(50μ L) were added to each vial. Details as below:

In each vial, quercetin (5.0e-6mol) and AIBN (1.2e-5mol)

1-5: cross-linker1 (1.7e-4mol)

6-10: cross-linker2 (2.5e-4mol)

11-15:cross-linker3 (1.7e-4mol)

A1-F1: methyacrylic acid 1 (3.9e-6mol)

A2-F2: methyacrylic acid 2 (7.5e-6mol)

A3-F3: methyacrylic acid 3 (1.2e-5mol)

A4-F4: methyacrylic acid 4 (1.6-5mol)

A5-F5: methyacrylic acid 5 (1.9-5mol)

A6-F6: methyacrylic acid 6 (2.3-5mol)

A7-F7: methyacrylic acid 7 (2.7-5mol)

A8-F8: methyacrylic acid 8 (3.0-5mol)

A9-F9: methyacrylic acid 9 (3.4-5mol)

A10-F10: methyacrylic acid 10 (3.8-5mol)

A11-F11: methyacrylic acid 11 (4.2-5mol)

A12-F12: methyacrylic acid 12 (4.6-5mol)

A13-F13: methyacrylic acid 13 (4.9-5mol)

A14-F14: methyacrylic acid 14 (5.5-5mol)

A15-F15: methyacrylic acid 15 (5.5-5mol)

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Quercetin#2' (The batch of small scale polymers with quercetin as the template)

The following solutions were made up each in 10mL THF.

Cross-linker 1: Triethylenglycol-dimethacrylate: 5.44g (1.9e-2mol).

Cross-linker 2: Ethylene glycol dimethacrylate: 5g (2.5e-2mol).

Ethyl Acrylate: 5g (5.0e-2mol).

Quercetin : 0.3g (9.9e-4mol) & AIBN: 0.4g (2.4e-3mol).

Acrylamide: 0.315g (4.4e-3mol)

90 4mL vials were weighted. Rutin and AIBN combined solution, cross-linker, ethyl acrylate and acrylamide solution were added to each vial. Details are as below:

Quercetin and AIBN combined solution (50μL) was added to each vial. The amount of Quercetin and AIBN was 5.0e-6mol and 1.2e-5mol.

The amount of acrylamide applied in each vial is laid down below:

A1-F1 and A9-F9: 30μL (1.3e-5mol) A2-F2 and A10-F10: 40μL (1.8e-5mol)

A3-F3 and A11-F11: 50μL (2.2e-5mol) A4-F4 and A12-F12: 60μL (2.6e-5mol)

A5-F5 and A13-F13: 70μL (3.1e-5mol) A6-F6 and A14-F14: 80μL (3.5e-5mol)

A7-F7 and A15-F15: 90μL (4.0e-5mol) A8-F8: 100μL (4.4e-5mol)

cross-linker applied in each vial is as below:

1-8: cross-linker 1 9-15: cross-linker 2

The volume of ethyl acrylate and cross-linker solution in each vial is as below:

A: 65μL and 35μL B: 60μL and 40μL C: 55μL and 45μL D: 50μl and 50μL

E: 45μL and 55μL F: 40μL and 60μL

Table 6.3: The amount of ethyl acrylate and cross-linker in each vial is given in the table below. The unit in this table is mole.

	A	B	C	D	E	F
ethyl acrylate	3.3e-4	3.0e-4	2.8e-4	2.5e-4	2.3e-4	2.0e-4
cross-linker 1 (1-8)	6.7e-5	7.6e-5	8.6e-5	9.5e-5	1.0e-4	1.1e-4
cross-linker 2 (9-15)	8.8e-5	1.0e-4	1.1e-4	1.3e-4	1.4e-4	1.5e-4

The vials were capped and heated at 70°C till the polymers were formed. The polymers were washed (9×3mL, stand for 10minutes for each time) with appropriate

solvent used before as the solvent to form the solution and dried in vacuum overnight. The vials were weighted again to get the net weight of the polymers.

Test of rebinding ability by UV

The maximal absorbance wavelength of template was fixed and standard graph was made according to three standard solutions.

Conditions:

Rutin as template: λ_{max} = 358nm

standard solutions(in methanol): 100mg/L, 50mg/L, 25mg/L

stock solution(in methanol): 50mg/L

Curcumin as template:

λ_{max} = 358nm

standard solutions(in methanol): 100mg/L, 50mg/L, 25mg/L

stock solution(in methanol): 50mg/L

Quercetin as template:

λ_{max} = 368nm

standard solutions(in methanol): 25mg/L, 12.5mg/L, 5mg/L

stock solution(in methanol): 25mg/L

Procedure:

The polymers with rutin as the template: The polymer was transferred into a little vial, 4mL pure methanol was added and kept standing for 1 hour. After that, the solution was transferred into cuvettes and inspected by UV to make sure that the removing is complete. Further removing had to be taken until there was very few

template molecular presents in the solution (Concentration<=3mg/L). Stock solution was added to the vial containing 50mg polymer (kept standing for 1 hour and then inspected by UV.

The polymers with curcumin as the template: The same procedure was taken as described in the test for the polymer with rutin template.

The polymers with quercetin as the template: The same procedure was taken as described in the test for the polymer with rutin template.

According to the results (see table 2.1), quercetin#5 was picked out for further test. 202mg with the size 60-20 mesh and 51.6mg with the size <60mesh were put into two little vials. 4ml stock solution was added to each vial and the vials were shaken for 3 hours. The solution was inspected by UV.

The extracting ability of quercetin#5 on other compounds was investigated. Rutin was chosen for this test.

Conditions: λ_{max} = 358nm.

standard solutions (rutin in methanol): 100mg/L, 50mg/L, 25mg/L

stock solution (rutin in methanol): 100mg/L

205.5mg with the size 60-20mesh and 56.3mg with the size <60mesh were put into two little vials. 4ml stock solution was added to each vial and the vials were shaken for 3 hours. The solution was inspected by UV.

Chapter 7

Experimental Relating to Chapter 3

HPLC Fractionation

Conditions of HPLC

Instrumentation:

Liquid Chromatography: Model: LC-6A, SHIMADZU CORPORATION

Column: C18 150 × 4.60mm, Phenomenex

Printer: Attenuation: 10

Conditions:

λ_{max}: 270nm

T.flow: 0.5mL/min

SolventA: water-acetic acid (100:1, v/v) SolventB: methanol

Time function of the gradient elution: Concentration of B: 0-5min 0-30%

5-90min 30-100%

Injection volume: 150μL

Methods

The methanol extraction (2.4g) that had been found to have activity against *M. fortuitum* was dissolved in double distilled water (220mL) and chloroform (220mL) in a separating funnel. The mixture was shaken and kept standing until it had separated into two layers. They were transferred into two flasks separately. The solvents were rotary evaporated in vacuum.

The extraction from the water layer was made into solution (10mg/L) in methanol and was centrifuged. The supernatant was applied to HPLC (Conditions are the same as Chapter 7 - HPLC Fractionation - Conditions of HPLC). Fractions at different retention

times were achieved after 20 injections by combining the fractionated output from the combination of twenty injections. The solvent in each fraction was evaporated in vacuum. The weights of each fraction were recorded (See Chapter 3 - HPLC Fractionation).

Screening of the Fractions from HPLC

The bioactivities against *E. coli*, S. *aureus* and *M. fortuitum* of the 11 fractions from HPLC were screened.

Each fraction was dissolved in 100μL double distilled water for further assessment. 11 test tubes containing 2mL nutrient broth media, 4μL *M. fortuitum* and 10μL of fractions 1-11 were set up. They were incubated for 4 days under 37°C.

The bioactivities against *E. coli* and S. *aureus* were tested in agar diffusion methods. Agar plates were set up, 1000μL of culture was spread on one plate and 10μl sample was placed in each well. There were two wells in each plate. The dishes were incubated at 37°C for 1 day and zones of inhibition were observed. (For the results see Screening of the Fractions from HPLC)

Chapter 8

Experimental Relating to Chapter 4

Identification of the Compounds in the Samples

The compounds in the fractions 1-11 from the purification for the methanol extraction by HPLC (see Chapter 7 - Experimental Relating to Chapter 3) were identified by mass spectrum (Electrospray +). Results of fractions 1, 2, 4, 5, 6, 7, 8, 10 were recorded (See Chapter 4 - Identification of the Compounds in the Samples by Mass Spectrum).

MIP Column Chromatography

A glass column (d: 1.6cm) was slurry packed with polymer quercetin#5 (the fourth batch of MIPs made in bulk with Quercetin as the template, 4g), which had been found to have influence on some flavonoids (Chapter 6 - Experimental Relating to Chapter 2 - Test of rebinding ability by UV) using methanol. Methanol (50mL)was pumped through the column to equilibrate the column. The height of the column was 8.0cm.

The water extract (see Chapter 7 - Experimental Relating to Chapter 3 - HPLC Fractionation - Methods) (100mg) was dissolved in methanol (10mL) centrifuged. The supernatant was transferred to round-bottomed flask and the solvent was rotary evaporated. The sample was made into solution (50mg/mL) in methanol and solution (1mL) was submitted to the column. Methanol was used as eluant. The flow-rate was adjusted to 8mL/min. After 4mL solvent left the column, fractions (4mL for each) were collected and the absorptions were checked by UV spectrum at 270nm till no more compounds from the extract were present in the solvent. Fractions1-70 were obtained and the results of UV spectrum of sample 1, 5, 10, 15, 20, 25, 30, 35, 40 were recorded. The wavelength of the UV spectrum ranged from 200nm to 900nm. (Results see Chapter 4 - Identification of the Compounds in the Samples by Mass Spectrum - MIP Column Chromatography).

CONCLUSION

Bioactivity against *Mycobacterium tuberculosis* has been shown to present in the methanol extract of leaves of *Buddleja madagascariensis*. The MIC (minimum inhibition concentration) was 10mg/mL. No bioactivity anti *E.* coli and *S. aureus* was observed in the extractions of *Buddleja madagascariensis*.

According to the results from mass spectra, the components in the methanol extract with bioactivity were not found in previous published work connected with bio-prospecting in *Buddleja*. Their identities are to be confirmed by further detection.

After preparing polymer monoliths, both on a large scale and on a small scale, the latter by a semi-automated process, an imprinted polymer was found which exhibited a small ability to extract quercetin. When applied to column chromatography it also showed effects on separation and purification to the components.

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