

APPENDIX 2

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SPOOR AND FISCHER

JOHANNESBURG

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Provisional Patent Specification
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NAME OF INVENTORS : JACOBUS JOHANNES MARION MEYER

ABBEY DANNY MATOME MATHEKGA

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1 BACKGROUND OF THE INVENTION

THIS invention relates to the treatment and control of tuberculosis caused by *Mycobacterium tuberculosis*, as well as to the treatment and control of other bacteria and fungi and in particular to the use of phloroglucinol derivatives for use in such treatment and control.

Tuberculosis (TB) remains a serious health problem in many regions of the world, especially in developing nations. It is a contagious disease and is becoming epidemic in some parts of the world. It is estimated that 30-60% of adults in developing countries are infected with *Mycobacterium tuberculosis*. Approximately 8-10 million individuals develop clinical TB and 3 million die of TB each year (WHO/IUATLD, 1989).

In South Africa, over 3 in every thousand people die of TB, the highest rate in the world, while one out of every 200 people suffers from active tuberculosis. Tuberculosis is the most commonly notified disease in South Africa and the fifth largest cause of death among the black population (South African Tuberculosis Association, 1998).

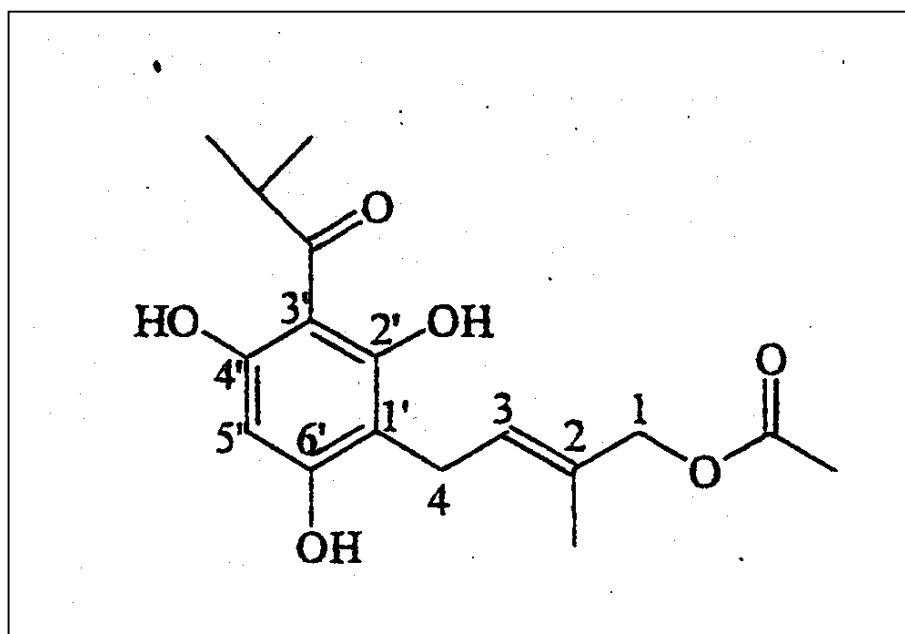
In the United States, the number of TB cases steadily decreased until 1986 when an increase was noted. Since then TB cases have continued to rise. Ten million individuals are infected in the U.S.A., with approximately 26000 new cases of active disease each year (National Jewish Medical and Research Centre, 1994).

Individuals infected with Human Immunodeficiency Virus (HIV) are very susceptible to tuberculosis and often develop this disease before other manifestations of AIDS become apparent (Grange and Davey, 1990). Control of the TB epidemic linked with HIV infection will depend largely on the adequate treatment of TB, and possibly of effective chemoprophylaxis, not just for HIV-infected persons but for communities as well (WHO/IUATLD, 1989).

TB therapy has been revolutionized and the present treatment regimes for TB are based on multidrug therapy with usually 3 or 4 antituberculosis drugs. However, the problem of multidrug resistant tubercle bacilli is emerging for various drugs such as isoniazid,

ethambutol, rifampicin and streptomycin, for example (Girling, 1989; Grange and Davey, 1990). Drug-resistant TB is very difficult to treat requiring greater numbers and varieties of medications for a longer period of treatment. The need for new antituberculosis agents is urgent due to the increasing resistance of mycobacteria to these classic antituberculosis drugs. A recent WHO report states that, globally, 2% of all cases of tuberculosis are multidrug resistant-by definition, resistance to rifampicin plus isoniazid (plus/minus other resistances). Such cases can be treated in the USA and other high resource regions but at a great cost (> US\$ 250,000 per case!) and using very long courses of rather toxic drugs, thereby raising serious problems of compliance (WHO, 1997). South Africa is witnessing an explosion in the number of cases of drug-resistant tuberculosis. In some parts of South Africa, 1 in 10 cases of TB is resistant to treatment (*New Scientist*, March 1997). It is essential to have new antituberculosis agents, preferably those that can readily and simply be produced from some local source.

The present invention is directed at the use of phloroglucinol derivatives in the treatment and/or control of tuberculosis caused by *Mycobacterium tuberculosis* and infection caused by other pathogenic bacteria and fungi. In particular, phloroglucinol derivatives of the general Formula 1 have been found to be effective against *Mycobacterium tuberculosis* and infection caused by other pathogenic bacteria and fungi.



The inventors of the present application undertook an extensive research program in order to identify antituberculosis, antibacterial and antifungal agents that can readily and simply be produced from local resources.

Twenty-eight South African medicinal plants used to treat pulmonary diseases were screened by the inventors for activity against drug-resistant and sensitive strains of *M. tuberculosis*. A preliminary screening of acetone and water plant extracts, against a drug-sensitive strain of *M. tuberculosis* H37Rv, was carried out by the agar plate method. Fourteen of the 20 acetone extracts showed inhibitory activity at a concentration of 0.5 mg/ml against this strain. Acetone as well as water extracts of *Cryptocarya latifolia*, *Euclea natalensis*, *Helichrysum caespitium*, *Nidorella anomala* and *Thymus vulgaris* inhibited the growth of *M. tuberculosis*.

the agar plate method a further study was carried out employing the rapid radiometric method to confirm the inhibitory activity. These active acetone extracts were screened against the H37Rv strain as well as a strain resistant to the drugs, isoniazid and rifampin. The minimal inhibitory concentration of *Croton pseudopulchellus*, *Ekebergia capensis*, *Euclea natalensis*, *Nidorella anomala* and *Polygala myrtifolia* was 0.1 mg/ml against the H37Rv strain by the radiometric method. Extracts of *Chenopodium ambrosioides*, *Ekebergia capensis*, *Euclea natalensis*, *Helichrysum caespitium*, *Nidorella anomala* and *Polygala myrtifolia* were active against the resistant strain at 0.1 mg/ml. Eight plants showed activity against both the strains at a concentration of 1.0 mg/ml.

The following procedure was developed by the applicant for the isolation of caespitate from *H. caespitium* and other species in this genus, as well as any other plants that may synthesise caespitate or other compound of formula 1 acylated phloroglucinol derivatives.

2. Identification of plant species

The aerial plant parts of *H. caespitium* were collected near Harrismith and identified at the HGWJ Schweickerdt Herbarium of the University of Pretoria and also at the herbarium of the National Botanical Institute, Pretoria.

2.1 Extraction

Dried aerial plant parts of *H. caespitium* were shaken in acetone for 5 minutes, filtered and concentrated to dryness at reduced pressure on a rotary evaporator.

2.2 Thin layer chromatography

A direct antibacterial bioassay (Dilika and Meyer 1996) on TLC-plates was employed to speedup the activity guided isolation of the antimicrobial compound. *M. tuberculosis* cannot be tested in this way because of its very slow growth rate. The direct antibacterial bioassays of the acetone extract were done on TLC plates (Merck) developed with chloroform-ethylacetate (1:1). After development, the TLC plates were dried and sprayed with a 24 hr old *Staphylococcus aureus* culture in nutrient broth. After 24 hr incubation, the plates were sprayed with an aqueous solution of 2mg/ml p-iodonitrotetrazolium violet to visualise the bacterial cells. The plates were then reincubated at 37⁰C for 2-3 hours.

Four zones of bacterial growth inhibition could be seen on TLC plates sprayed with *S. aureus*. Activity was more pronounced in the R_f 0.57 zone (chloroform-ethylacetate (1:1)) than in the other 3 zones.

2.3 Column chromatography

The crude extract of the plant was dried, its mass determined and resuspended in chloroform. Column chromatography was performed on silica gel 60 using chloroform as eluent. The antibacterial fractions collected were then again tested for antibacterial activity on TLC to detect the fraction containing the active compound of R_f 0.57.

2.4 High performance liquid chromatography

The compound was further purified by HPLC utilising an analytical Phenomenex reverse phase 250x4.60 mm column, at a flow rate of 1.0 ml/min, oven temp. 40⁰C and a wavelength of 206nm. An ethanol-water (50:50) solution was employed as mobile phase. The pure compound was collected from the eluent. The chemical

structure was confirmed by ^1H -, ^{13}C -, COSY-, DEPT- and HETCOR-nmr, MS and crystallography.

3 ANTIBACTERIAL ACTIVITY

The activity of caespitate was examined against ten bacteria by the agar dilution method (Turnbull and Kramer, 1991). Caespitate significantly inhibited the growth of all the Gram-positive bacteria tested at a concentration of between 0.5 and 5 $\mu\text{g/ml}$ (Table 1).

Caespitate had no activity against all the Gram-negative bacteria tested. These results are in accordance with previous reports (Tomas-Barberan, Iniesta-Sanmarin, Tomas-Lorente, and Rumbero, 1990; Dekker, Fourie, Snyckers and Van der Schyf, 1983) of similar antimicrobial activity of related compounds against Gram-negative bacteria. Most bacillus species are regarded as having little or no pathogenic potential, however, both *Bacillus cereus* and *B. subtilis* have been known to act as primary invaders or secondary infectious agents in a number of cases and have been implicated in some cases of food poisoning (Turnbull and Kramer, 1991). *Staphylococcus aureus*, is a human pathogen, whose infections are amongst the most difficult to combat with conventional antibiotics (Tomas-Barberan, Msonthi and Hostettmann, 1988; Tomas-Barberan, Iniesta-Sanmarin, Tomas-Lorente, and Rumbero, 1990).

TABLE 1. Antibacterial activity (MIC) of the crude acetone extract of the aerial parts of *H. caespitium* and caespitate isolated from the extract.

Bacteria	Gram +/-	Crude extract (mg/ml)	Caespitate MIC(μg/ml)
<i>Bacillus cereus</i>	+	1	0.5
<i>B. pumilus</i>	+	1	0.5
<i>B. subtilis</i>	+	1	0.5
<i>Micrococcus kristinae</i>	+	1	0.5
<i>Staphylococcus aureus</i>	+	1	5
<i>Enterobacter cloacae</i>	-	1	na ^b
<i>Escherichia coli</i>	-	1	na
<i>Klebsiella pneumoniae</i>	-	na	na
<i>Pseudomonas aeruginosa</i>	-	1	na
<i>Serratia marcescens</i>	-	na	na

^a Minimum inhibitory concentration

^b Not active

This study provided a probable scientific explanation for the therapeutic potency attributed to *H. caespitium*, claimed by traditional healers in the Free State province of South Africa, for example, during wound treatment in male circumcision rites.

4 ANTIFUNGAL ACTIVITY

The growth of six fungi, *Aspergillus niger*, *A. flavus*, *Cladosporium cladosporioides*, *C. cucumerium*, *C. sphaerospermum* and *Phytophthora capsici*, were significantly inhibited at very low MIC's by caespitate (Table 2). *A. flavus* and *A. niger* are some of the most important fungi responsible for human systemic infections. These organisms were inhibited at 1.0 μ g/ml. It is generally agreed that at least one acidic hydroxyl group and a certain degree of lipophilicity are required for biological activity compound (Tomas-Barberan, Iniesta-Sanmarin, Tomas-Lorente, and Rumero, 1990).

Lipophilicity is important because many antifungal metabolites exert their toxicity by some membrane associated phenomenon, and it is known that acidic hydroxyl groups may act by uncoupling oxidative phosphorylation. In this case the antifungal caespitate isolated from *H. caespitium* bears three acidic hydroxyls (phenolic hydroxyls) and lipophilicity (3'-isobutrylphenyl and but-2-enyl acetate residues). On the other hand, antibacterial activity, against Gram-positive bacteria seems to be related to the presence of phenolic hydroxyls (phenol itself is a well known antibacterial compound (Tomas-Barberan, Iniesta-Sanmarin, Tomas- Lorente, and Rumbero, 1990).

TABLE 2. Antifungal activity of the crude acetone extract of the aerial parts of *Helichrysum caespitium* and caespitate isolated from the extract.

MIC ^a		
Fungal species	Crude Extract mg/ml	Caespitate mg/ml
<i>Aspergillus flavus</i>	1.0	1.0
<i>A. niger</i>	0.01	1.0
<i>Cladosporium cladosporioides</i>	0.01	5.0
<i>C. cucumerium</i>	0.01	0.5
<i>C. sphaerospermum</i>	0.01	0.5
<i>Phytophthora capsici</i>	1.0	1.0

^a Minimum inhibition concentration.

5 ANTITUBERCULOSIS ACTIVITY

The effect of caespitate on the growth of the sensitive strain (H37Rv) and resistant strains of *Mycobacterium tuberculosis* as determined by the radiometric method are set out in Table 3. The results show that caespitate controls the *Mycobacterium tuberculosis* bacterium effectively. As far as the applicant has been able to establish, caespitate has never been synthesised in a laboratory.

TABLE 3. Inhibition of *Mycobacterium tuberculosis* strains by caespitate

<i>Mycobacterium tuberculosis</i> strains	MIC (mg/ml)	DGI ^a values of plant extracts (mg/ml)	DGI values of the control vial (mg/ml)
H37 sensitive strain	0.1	7.33 ± 4.93	25 ± 4
2 drug resistant strain (res. to Isoniazid and rifampicin).	0.1	7 ± 2	26 ± 3.2
3 drug resistant strain (res. to streptomycin, isoniazid and ethambutol),	0.1	3 ± 1.73	17.33 ± 3.05
4 drug resistant strain (res. to streptomycin, isoniazid, rifampicin and ethambutol).	0.1	8.66 ± 1.52	23 ± 3.5
5 drug resistant strain.(res to isoniazid, streptomycin, rifampicin, thiacetazone and cycloserine).	0.1	8.3 ± 2.88	23.3 ± 3.51
6 drug resistant strain (res. to isoniazid, rifampicin, ethionamide, terizidone, thiacetazone and ofloxacin).	0.1	10 ± 3.60	27 ± 5.56
7 drug resistant strain.(res to isoniazid, steptomycin, ethambutol, kanamycin, rifampicin, and ethionamide)	0.1	10.3 ± 2.52	26.33 ± 7.09

^a DGI values are means ± s.d.

It is believed that caespitate and related acylated phloroglucinol derivatives are viable alternatives to conventional drugs in the treatment and control of tuberculosis in humans.

DATED THIS _____ DAY OF _____ 1999

SPOOR AND FISHER

APPLICANT'S PATENT ATTORNEYS

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