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The Anti-Fungal Properties of Calendula Officinalis on Candida Albicans

A Dissertation submitted in partial fulfilment of the requirements for the degree

Masters of Technology

In Homoeopathy

In The Faculty of Health Sciences

By Marike De Klerk

Supervisor: Dr K Saunders
Co-Supervisor: Dr B R van Olden

At the Technikon Witwatersrand

December 1998
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I hereby declare that the dissertation, which I herewith submit for the research qualification, Masters degree in Homoeopathy, to the Technikon Witwatersrand is, apart from recognised assistance, my own work and has not previously been submitted by me to another institution to obtain a research diploma or degree.
ABSTRACT

This study established the anti-fungal effect of Calendula officinalis on the organism Candida albicans in vitro.

Calendula officinalis was used in the D1, D2, D3 and D4 potencies in a 20% ethanol solution. Two experiments were used. Firstly a disc diffusion method was carried out and compared to results obtained using Nystatin.

It was found that there was inhibitory action shown by the homoeopathic remedy. Although no significant difference was recorded between the remedies, the D1 and D3 potencies seemed to be the most inhibitory, and the D2 and D4 potencies the least inhibitory. None of the potencies had such a marked effect as Nystatin (conventional treatment).

The second experiment was a modification of the broth dilution method. From this method, effects of the different potencies were verified. The D1 and D3 potencies displayed the most inhibition and the D2 and D4 potencies the least inhibitory action.

It can therefore be concluded that a slight anti-fungal action was displayed by Calendula officinalis upon the organism Candida albicans, with the D1 and D3 being most effective and the D2 and D4 potencies the least.

The experiments conducted did not provide a complete explanation of the mode of action of Calendula officinalis and further studies should be conducted in vivo to determine it's true action.
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My family and Attila, for all their help and support.
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Candida albicans is a yeast and is present as a part of the normal population of organisms in the mouth, vagina and skin. (Freeman, 1985) An intact immune system and other normal flora in the mucous membranes prevent it from becoming predominant within the community. Calendula officinalis (Marigold) is an annual herb growing natively in France and Southern European fields. Marigold has been found to be beneficial in the treatment of local skin problems. Its main function is considered to be anti-inflammatory, astringent, vulenary and anti-fungal. (Boericke, 1991) It may be used safely wherever there is an inflammation of skin, whether due to infection or physical damage. (Boericke, 1991) Calendula officinalis can be used as an anti-fungal agent both internally and externally. It is a healthy alternative to conventional anti-fungal agents (e.g. Nystatin, Clotrimazole) that are presently used.

1.1 Candidiasis

Candidiasis is one of the most frequently encountered of the fungal diseases, especially in the category of opportunistic infections. (Freeman, 1995) The species Candida albicans, which is responsible for most infectious processes, is endogenous to man. It is part of the normal flora of the mouth, large intestine and vagina. When body conditions are in equilibrium, this micro-organism is controlled by body defences and other members of the normal flora. If this balance is changed, as in debilitation of defences, especially cell-mediated immunity, overuse of antibiotics or local physiological change, the organism proliferates rapidly and can cause infection (Freeman, 1985). The natural immunity of healthy persons to Candida infection is probably generated early in life when the alimentary tract becomes colonised with Candida albicans. The surface glycoproteins (mannoproteins and glucoproteins) are thought to stimulate both humoral and cellular immunity. Thus, healthy people develop antibodies and delayed hypersensitivity to Candida culture filtrates, which contain glycoprotein and polysaccharide antigens. (Freeman, 1985)

Candidiasis has no geographical limitation, although the cutaneous forms occur more frequently in climates inducing excessive perspiration. In regions of the world where the diet is high in carbohydrates, the intestinal tract contains the yeast more often than in other geographic areas. (Plunkett, 1984) It has been suggested its incidence in the intestinal tract
increases with the age of the person. (Plunkett, 1984) Candidiasis is properly termed an endogenous infection. The mouths of infants are particularly susceptible, but the incidence of cutaneous forms increases with age. There is no hyper susceptibility because of race or skin colour. (Plunkett, 1984)

Candidiasis has various clinical manifestations and may be implicated in dermatophyte infections, nail infections, foot infections, vaginitis and thrush. Infection of the skin occurs principally in moist, warm parts of the body, such as the axilla, intergluteal folds, groin, umbilicus, or inframammary folds, and is likely to result whenever two skin surfaces are kept in contact continuously or for long periods of time without opportunity for aeration and evaporation of sweat. (Plunkett, 1984) It is often associated with obesity, which in really severe forms also furnishes sufficiently deep creases in the neck or beneath overhanging abdominal masses. Often there is chemical irritation, caused in part by the conversion of urea in the perspiration to ammonia by bacterial action, also occurring in diaper rash in infants. Diabetics are especially likely to have candidiasis due to heightened glucose levels in perspiration. (Plunkett, 1984) A careful evaluation of all contributing causes must precede attempts at treatment, and laboratory procedures are mandatory. (Plunkett, 1984) In peripheral locations candidiasis causes rounded lesions, some of which have not yet coalesced. They present an unusual type of scaling, consisting of a circumscribed area of keratinized epidermis attached at the periphery of the lesion, while the central part presents no scaling. Vesicles are common in such borders as well. (Plunkett, 1984) Sometimes, particularly in diabetics seriously out of control, the disease may spread until large areas of the body surface become involved. Intertrigo also occurs between the toes, especially if they are short and thick, furnishing little or no interspaces for aeration, or when tight shoes are worn in hot weather. The same condition is observed in finger webs, particularly in obese women with short thick fingers who have frequent contact with water. (Plunkett, 1984)

Candida albicans is the most common cause of vaginal infection in females. Seventy five percent of women experience at least one episode during their lifetime. It is characterised by a creamy white leucorrhea. (Barnett et al, 1990) This infection may occasionally spread to deeper tissues in the pelvis. The lesions of vulvo-vaginal candidiasis resemble those of thrush, but produce more irritation, severe vulvar itching and a thick vaginal discharge. (Barnett et al., 1984)
Predisposing conditions include the use of oral contraceptives, pregnancy and diabetes mellitus. (Barnett et al., 1990) These cause an increase in glycogen and glucose in the vagina, as well as a decrease in acidity, which stimulates growth of Candida albicans. The mode of transmission is by direct contact with the skin, especially sexual contact. (Barnett et al., 1990) By this means the infection can be transmitted to the male to cause non-gonococcal urethritis. (Barnett et al., 1990)

Infection of the mouth (thrush) occurs mainly in infants, on the buccal mucous membranes. (Plunkett, 1984) They become infected during passage through the vagina, where the fungus exists in a high percentage of women especially in the later months of pregnancy, and often without causing any symptoms. (Plunkett, 1984) Thrush is seldom serious except in weak or premature infants, but it is often stubbornly resistant to treatment because frequent feedings high in carbohydrates are essential at that time of life. The tongue, soft palate, buccal mucosa, and at times all mucous membranes of the mouth and pharynx, are covered by discrete or confluent patches of cream-coloured nonadherent membrane, often painful and easily induced to bleed. (Plunkett, 1984) Oral candidiasis is also troublesome in the aged, especially when chronic disease causes malnutrition or other forms of lowered general resistance. (Plunkett, 1984) Another type of oral candidiasis is termed perleche and is designated by inflammation and some erosion at the corners of the mouth. Candida albicans is often, but not invariably, found in these lesions. (Plunkett, 1984) Growth of buccal candidiasis is enhanced by corticosteroids, high levels of glucose and immunodeficiency. (Adelburg et al., 1989).

Candidiasis may occur in the anal area without intertrigo, especially after broadspectrum antibacterial antibiotics. (Plunkett, 1984) The bacterial flora of the intestinal tract is strongly suppressed by such substances, permitting the unaffected Candida albicans present to migrate from the colon to the perianal area. It has even been stated that these antibiotics directly nourish the fungus. (Plunkett, 1984) Considerable pruritis results from this infection. (Plunkett, 1984)

Paronychia in its acute form consists of a painful and tender swelling of the eponychial tissue, either localised to one edge or all around the base of the nail, and is caused by Candida albicans often with a superinfection of bacteria. (Plunkett, 1984) Only one nail may be involved, but more often several or all of them are affected. The disease occurs more
commonly in fingernails than toenails. (Plunkett, 1984)

People in contact with water or food such as cooks, fruit peelers, laundry workers and dishwashers are especially susceptible. In chronic paronychia there is only slight erythema and little swelling or pain, but tenderness on pressure is common. The eponychium separates from the dorsal surface of the nail, often almost to the point of origin of the nail. (Plunkett, 1984)
Figure 1: Candidiasis: Oral moniliasis or thrush, more common in infancy

Figure 2: Candidiasis: Perleche, cracks in the corners of the mouth
Figure 3: Candidiasis: Severe candidiasis of submammary areas

Figure 4: Candidiasis: Erosion lesions of the web between the third and fourth digits
As opposed to acute candidal infection, a condition called chronic mucocutaneous candidosis (CMC) may be encountered. (Jorizza, 1982) This is an infection of the skin, nails and oral and genital mucosa. Clinically it is typified by the onset, usually within the first year of life, of chronic, occasionally granulomatous, candidal infections. (Jorizza, 1982) Persistent oral thrush with subsequent hypertrophic changes, vulvovaginal infection and cutaneous lesions often involving the trunk, limbs, scalp and parenchymal involvement with accompanying nail dystrophy, are all typical clinical features of CMC. (Jorizza, 1982) Frequently, concomitant dermatophyte infections are present. Traditional anti-candidal and anti-fungal therapies are ineffective. (Jorizza, 1982)

CMC is not a single disease entity but rather a final common pathway for multiple predisposing abnormalities of the immune system that range from severe, life-threatening immunodeficiency syndromes to subtle deficiencies, usually of cell-mediated immunity. CMC has the ability to remain cutaneous but also has a systemic spread. (Jorizza, 1982) Systemic disseminated candidosis has become an increasingly important infection in immunocompromised patients and in patients who have undergone surgery, especially those with indwelling catheters. (Jorizza, 1982) Host defence factors that protect against the development of systemic disseminated candidosis have been recently reviewed. While the stratum corneum is generally an effective barrier against most candidal penetration, Candida albicans can penetrate skin. (Jorizza, 1982) Desquamation and inflammation in the stratum corneum aid in eliminating the infection when the barrier is impaired by external factors e.g. maceration. (Jorizza, 1982) Humoral factors form the second line of defence against the organism, with candidal cell wall products activating the alternative complement pathway that produces potent chemotactic agents, which enhance the accumulation of polymorphonuclear cells and macrophages around invading Candida albicans organisms. These defence mechanisms are not in tact in patients with CMC. (Jorizza, 1982)

Pulmonary candidiasis is usually found in association with other pulmonary diseases, such as tuberculosis or coccidiomycosis. (Plunkett, 1984) The incidence of this disease is increasing markedly, because of the extensive use of antibiotics, corticosteroids and antifungal agents. It is often difficult to separate the pulmonary symptoms actually caused by Candida albicans from those of the underlying disease. (Plunkett, 1984)
Candidal endocarditis resembles acute subacute bacterial endocarditis, with evidence of growths on the heart valves. (Plunkett, 1984) Most cases have occurred in "mainliners" addicted to narcotics taken by means of intravenous injections, who do not adequately sterilise the needles and syringes. (Plunkett, 1984) When any of the viscera or the central nervous system is invaded by the elements of the yeast carried in the bloodstream, disseminated candidiasis results. It is usually a terminal event superimposed upon another fatal illness, for example, AIDS. (Plunkett, 1984)

Approximately ten percent of healthy people carry Candida albicans in their mouths and fifteen percent in the rectum. (Braude, 1982) These percentages can increase to forty percent or more in elderly patients and those on antibiotic treatment. Candida albicans is amorphic, meaning it may grow as a mycelium in nature and a yeast in the body. It can undergo rapid transformation from yeast to hyphal phase in vivo, which contributes to the invasion of the organism into the tissue. This explains why the normal carriage sites of Candida albicans are foci for transmission. (Braude, 1982)

The organism can spread from the vagina to rectum, from the vagina to the infant during passage through the birth canal to cause oral thrush in the new-born, from skin to gut via ulceration or catheters. (Braude, 1982) This rapid and efficient spread of the organism is the reason why Candidiasis is such a widespread and complicated infection. (Braude, 1982).

As proof of the seriousness of this disease, studies conducted have shown that it has widespread epidemiology and causes many obstacles in the treatment of a patient. As shown by Fisher-Hoch et al., (1991), Candida albicans has caused many epidemics in hospitals. During the 1980's, the rate of invasive and mucosal candidiasis among hospitalised patients increased significantly, i.e. over the whole decade, the rates of disseminated candidiasis increased eleven fold. (Hammerman et al., 1974) In another study conducted by Dyes et al., (1987), it was shown that Candida albicans infection dramatically decreases the survival rate of patients with bacteraemia. Another complication stated by Desai et al, 1985, is that Candida is one of the frequent opportunistic organisms causing sepsis and death in the patient with burn injury. Overall between twenty and forty deaths attributable to systemic candidiasis are reported to the registrar general each year for England and Wales. (Hammerman et al., 1974)
1.2 **Candida albicans** organism

1.2.1 History and Morphology

In 1839 Langenbeck discovered the yeast by microscopic examination of scrapings from the mouth of an infant suffering from thrush. Gruby (1842, cited by Plunkett, 1974) confirmed this finding, and Robin (1843, cited by Plunkett, 1974) named the organism *Oidium albicans*. Candidiasis is thus a close competitor of favus as the earliest human fungal disease to be discovered. Schamberg (1915, cited by Plunkett, 1974) studied a fatal case of thrush in 1915. In 1927 Castellani (cited by Plunkett, 1974) delineated bronchopulmonary candidiasis, and classified yeast-like fungi according to their fermentation reactions. Joaquim and Polayes reported endocarditis in 1940. (cited by Plunkett, 1974)

1.2.2 Genus classification

The genus *Candida* accommodates a heterogenous collection of asporogenous yeast species which do not qualify for classification in any of the more homogenous genera of the imperfect yeasts. (Lodder, 1974) The genus *Candida* is diagnosed according to the following factors:

i) The cells are globose, cylindrical or elongate, sometimes irregularly shaped, normally not ogival, apiculate or flask-shaped. (Lodder, 1974)

ii) Reproduction normally occurs by multipolar budding. Pseudomycelium formation occurs in all or most strains of all species and varieties. (Lodder, 1974)

iii) The pseudomycelium is often differentiated into pseudohyphae and blastospores.

iv) Chlamydospores may be formed. (Lodder, 1974)

v) True mycelium may also be formed, while athrospores are absent. Ascospores, teliospores or ballistospores are not formed.

vi) Visible pigmentation due to carotenoid pigments is absent. (Lodder, 1974)
vii) Extracellular polysaccharides may be formed and may give a positive iodine reaction.

viii) Alcohol fermentation occurs in many species. (Lodder, 1974)

1.2.3 Species classification

Throughout history the organism has been studied in depth and can now be described as follows:

i) Candida albicans appears as a Gram-positive oval budding yeast measuring 2-4 by 4-6 micrometers and elongated budding cells resembling hyphae i.e. the pseudohyphae. (Freeman, 1985)

ii) On Sabouraud’s agar incubated at room temperature, soft cream-coloured colonies with a yeasty odour develops. Older colonies may have submerged hyphal growth resembling feathers deep in the agar. (Beneke et al., 1970)

iii) The surface growth consists of oval budding cells. The submerged growth consists of a pseudomycelium which is composed of pseudohyphae that form blastospores at the nodes and sometimes chlamydospores terminally. (Freeman, 1985)

iv) The ability to produce chlamydospores is usually sufficient to identify this species. (Beneke et al., 1970) These chlamydospores are large and round with a thick wall. If no chlamydospores are present, sugar fermentation and sugar assimilation are useful for species determination. (Beneke et al., 1970)

v) Candida albicans ferments glucose and maltose, producing acid and gas; produces acid from sucrose; and does not attack lactose. When Candida albicans is suspended in serum and incubated at 37°C, it produces germ tubes which are unique to this particular organism. (Freeman, 1985) Reproduction occurs by means of pinched blastoconidia. These carbohydrate fermentation patterns, together with colonial and morphological characteristics, differentiate Candida albicans from the other species of Candida. (Freeman, 1985)
1.2.4 Virulence factors

_Candida albicans_ is a pleomorphic structure and may exist either in a yeast or mycelial form. (Hugo _et al._, 1987) This provides the organism with the means to propagate deeper into the tissues. Such diversity and morphology is an important property in pathogenicity and is associated with changes in cell wall composition which result in increased pathogenicity. This recently observed phenomenon of high frequency switching has been implicated in providing resistance to anti-fungal agents. (Hugo _et al._, 1987)

Another factor that increases the pathogenicity of the organism is the formation of encapsulated chlamydospores. They are large in size and store reserve nutritional substances. Their thick walls protect them from unfavourable environments. (Braude _et al._, 1982) The organism has special properties for adherence to the host tissue. _Candida albicans_ has a cell surface receptor for binding soluble fibronectin that attaches the organism to an extracellular matrix. (Braude _et al._, 1982)

In addition to this, _Candida albicans_ secretes hydrolytic enzymes, proteinases and phospholipases which assist in tissue invasion. They also play a major role in dissemination of candidal disease. (Desai _et al._, 1992)

1.2.5 Serology

With the increased incidence of opportunistic infections, a serological test for systemic infection has become extremely important. The immuno-diffusion test has therefore been developed. (Braude _et al._, 1982) In this test, which uses the cell sap (S-antigen), precipitin bands form in gel only in cases of systemic candidiasis and long-term chronic mucocutaneous disease. False positive reactions are rare but false negative reactions may occur in cases of allergy or long-term immunosuppressive therapy. (Braude _et al._, 1982)
1.2.6 Histopathology

The organism is not likely to occur in biopsy material unless taken from the pulmonary region, mucous membranes of the alimentary tract or bronchi or endocarditic heart tissue in systemic cases. (Beneke et al., 1974) Hyphae (psuedohyphae), three or four microns in diameter, and blastospores (buds) are usually present in tissue in place of oval to round budding cells. The organism can readily be demonstrated using the Gridley, PAS or silver nitrate stains. (Beneke et al., 1974)
Figure 5: *Candida albicans* on blood agar plates showing cream coloured colonies. Small extensions or "feet" that increase with the age of the colony, are visible. (Baron *et al*, 1997)

![Figure 5](image)

Figure 6: Blood culture of *Candida albicans* showing hyphae and budding cells. (x 1350)

The blastoconidia stain Gram-positive, are round or oval, and measure approximately 3 x 5 micrometers. (Baron *et al*, 1997)

![Figure 6](image)
1.3 Conventional treatment of Candidiasis

1.3.1 Nystatin

The conventional drug of choice in the treatment of muco-cutaneous candidiasis is Nystatin, a polyene antibiotic. (Bevan, 1986) Polyenes are complex molecules with a large conjugated double bond system in a lactone ring linked to an amino acid. Nystatin is produced by Streptomyces noursei. (Bevan, 1986) Nystatin exhibits both fungistatic and fungicidal activity, depending on the drug concentration, the presence of blood, pus or tissue fluid which could reduce activity, and the susceptibility of the fungus. (Bevan, 1986). It is somewhat toxic and is therefore preferably used to treat topical infections (Rose et al, 1987). Nystatin is used mainly to treat candidal skin and vaginal infections, and intestinal infections in very ill, immunocompromised patients. For gastrointestinal infections, it is administered orally. It is not absorbed across the intestinal wall, which means it never enters the blood and remains in the intestine to act locally on the intestinal lining. (Ingraham & Ingraham, 1995) Nystatin is contraindicated where there has been previous hypersensitivity to Nystatin or other ingredients in the formulation. Untoward effects of Nystatin are uncommon. Mild and transitory nausea, vomiting and diarrhoea may occur after oral administration. (Goodman, 1975) Irritation of the skin and mucous membranes does not result from topical application, nor does it have toxic effects on the blood or blood-forming organs. (Goodman, 1975) Since Nystatin has no effect on bacteria, supra-infection does not occur even when large doses are used. (Goodman, 1975) Relief of symptoms occurs 24-72 hours after initiating therapy and treatment generally has to be continued for 14 days. Well-known tradenames for this product include Mycostatin, Canstat and Nystacid. (South African Medicines Formulary 1996.)

Even in large doses, Nystatin is relatively innocuous when applied topically, but cutaneous irritation may develop following repeated exposure. Minor gastrointestinal symptoms follow oral administration. Nystatin injected intra-muscularly is painful and may produce haemolytic anaemia through forming complexes with red blood cell sterols. (Bevan, 1986) (South African Medicines Formulary, 1996)
Strains of microbes develop resistance to drugs normally effective against that species. Resistance is a major medical problem because it severely limits the usefulness of many antibiotics and often necessitates the substitution of toxic and less potent drugs where the more acceptable agents are found to be ineffective. (Bevan, 1986) The medical profession is increasingly disturbing the body's natural flora with antibiotics and the natural defense reactions of the body with immunosuppressive drugs and cytotoxic agents causing pulmonary and systemic infections with Candida. This is becoming a significant problem in the hospital medicine of today. (Bevan, 1986) Development of microbial resistance makes it essential to revise periodically the drugs of choice for different infections and to determine the sensitivity of the invading organism in all infections whenever possible. Failure to do this wastes time, money and needlessly exposes the patient to potentially harmful drugs. (Bevan, 1986)

Microbial resistance to antimicrobial drugs is either natural or acquired. The development of microbial resistance is simply an expression of evolution, with survival of the fittest. Microbial multiplication is so rapid that within 3.5 years an average strain of organism could have passed through as many generations as man has gone through in one million years. (Bevan, 1986)

Natural resistance is genetically determined and depends upon the absence of a metabolic process affected by the antibiotic in question. Naturally resistant organisms proliferate as their sensitive brethren are killed. (Bevan, 1986)

Acquired resistance refers to resistance developing in a previously sensitive species. It can arise through mutation, adaptation or through development of multiple drug resistance. By spontaneous mutation a species produces some members which differ from the parent strain. If mutation is favourable, the microbe will survive. (Bevan, 1986) Step-wise increase in resistance is thought to be due to mutations occurring in a number of different genes, each of which is responsible for a slight increase in resistance. One-step increase in resistance is due to mutations occurring in more powerful genes which confer a considerable degree of resistance. (Bevan, 1986)
Adaptation presupposes that organisms contain low concentrations of antimicrobial destructive enzymes, or potential for synthesising such enzymes. These lethal enzyme concentrations are triggered through enzyme induction following antibiotic exposure. (Bevan, 1986)

Multiple drug resistance is the transfer in vivo or in vitro of genetic material coding for resistance from a resistant to a sensitive microbe. It has long been held that clinical and veterinary use of antibiotics leads inexorably to an increase in microbial resistance. (Bevan, 1986)

1.4 Determination of antimicrobial activity of compounds

Antimicrobial susceptibility is a measure of how much of a drug is required to kill or stop the growth of a pathogen. The antimicrobial susceptibility or sensitivity can be determined in the microbiology laboratory by means of susceptibility tests. Two of the test methods used include disc diffusion and broth dilution. (Ingraham & Ingraham, 1995)

i) The disc diffusion method, sometimes-also called the Kirby-Bauer method, tests organism sensitivity using filter paper discs impregnated with known quantities of antimicrobial agents. (Ingraham & Ingraham, 1995) A petri dish is inoculated with the pathogen, and the discs that are impregnated with the antimicrobial agent, are placed on its surface. The drug diffuses from the disc through the gel so the highest concentration exists near the disc and progressively lower concentrations exist further away. (Ingraham & Ingraham, 1995) When the plate has been incubated long enough for the organism to produce confluent growth (24 hours) on the medium, results can be interpreted. Some of the drug discs will be surrounded by a clear halo, indicating anti-microbial inhibition of growth. (Ingraham & Ingraham, 1995) If the microorganisms growth is inhibited by a low drug concentration, the halo will be large. If growth is inhibited only by a high concentration, the halo will be small. (Ingraham & Ingraham, 1995) The size of each halo is measured and compared to susceptibility standards for each drug. Based on the comparisons, the micro-organism is judged to be either sensitive, which indicates high susceptibility, intermediately susceptible, or resistant to the antimicrobial agent. These results can be extremely useful. Knowing
if an organism is sensitive, intermediate or resistant to a given antimicrobial agent is almost always enough information upon which to base a treatment decision. Disc diffusion tests are also relatively easy and inexpensive to perform. This method is the most commonly used type of susceptibility test used in clinical medicine. (Ingraham & Ingraham, 1995)

ii) The broth dilution method tests drug sensitivity by cultivating micro-organisms in liquid cultures with progressively higher concentrations of the antimicrobial agent. (Ingraham & Ingraham, 1995) A series of tubes containing decreasing concentrations of an agent in solution is prepared and inoculated with the organism to be tested. Results can be interpreted when growth occurs (approximately 24 hours) i.e. when some tubes are turbid (cloudy). (Ingraham & Ingraham, 1995) Growth occurs in tubes that contain no or a low concentration of antimicrobial agents, while no growth occurs in the tubes with higher concentrations. The clear test tube with the lowest drug concentration contains the minimum inhibitory concentration (MIC) of that agent. (Ingraham & Ingraham, 1995) To obtain more information, inoculum from the growth-inhibited tubes can be taken and grown on a petri dish. If organisms do not grow in the broth culture but do grow on an agent-free petri dish, the antimicrobial agent inhibited the organism's growth but did not kill it. (Ingraham & Ingraham, 1995) This is called the fungistatic drug concentration. If organisms do not grow on the agent-free petri plate, the organism has been killed. This is called the fungicidal drug concentration. (Ingraham & Ingraham, 1995) The tube with the lowest drug concentration that proves to be fungicidal contains the minimum fungicidal concentration (MBC) of that antimicrobial agent. Determining the MIC and MBC gives valuable information for treatment. The MIC and MBC can be compared to the serum drug levels of the patient to see if inhibitory or bactericidal concentrations are achieved in the patient's body. (Ingraham & Ingraham, 1995) Broth-dilution susceptibility testing is more complicated and expensive than disc-diffusion, but may be helpful in conditions such as when an ill patient is not responding to the treatment, to determine whether the treatment is correct, incorrect or whether the concentration levels of the agent are sufficient. (Ingraham & Ingraham, 1995)
All antimicrobial drugs act by damaging a vital cell structure or inhibiting a vital metabolic function, but different drugs have different targets. There are five principal targets for antimicrobial agents. (Ingraham & Ingraham, 1995)

i) Organisms have a unique cell wall made of peptidoglycans which can withstand the prokaryotic cell's turgor pressure. It is this cell wall that is targeted by antimicrobial drugs. Drugs block peptidoglycan synthesis, killing the bacterial cell, but no human cells. (Ingraham & Ingraham, 1995) Because growing cells that can no longer maintain their cell wall, lyse and die, cell wall-inhibitors causes cell death. Microbes lacking peptidoglycans are naturally resistant to these drugs. (Ingraham & Ingraham, 1995)

ii) Some drugs inhibit microbial growth by damaging components of the plasma membrane, destroying its selective permeability, and allowing vital molecules to leak out. Because all cells in the human body have a plasma membrane, the action of these drugs is not selective and will destroy all human cells along with the microbial cells. (Ingraham & Ingraham, 1995) Different members of this group will damage different molecules which make them selective for certain microbes e.g. drugs that damage ergosterols which are found in fungal plasma membranes will only target these cells. (Ingraham & Ingraham, 1995)

iii) Antimicrobial drugs that interfere with protein synthesis affect the bacterial ribosome. When ribosomal function is disturbed, the synthesis of protein may stop entirely or may be slowed so significantly that normal growth cannot proceed. (Ingraham & Ingraham, 1995)

iv) The drugs in this category inhibit nucleic acid synthesis, the building blocks of DNA, the genetic material present in all cells. All cells, including prokaryotic and eukaryotic cells must manufacture nucleic acids, therefore selective toxicity in this category is relatively limited. (Ingraham & Ingraham, 1995)
v) A major group of antimicrobials acts by interfering with the production of the enzyme co-factor folic acid which has many functions including synthesis of the nitrogenous bases that build DNA. These drugs are selectively toxic to the micro-organism since humans do not synthesise folic acid, but obtain it from their diet. (Ingraham & Ingraham, 1995)

The mode of action of Nystatin is to increase the cell membrane permeability of the yeast, thus provoking leakage of amino acids, sugars and other metabolites from the cytoplasm leading to lysis and cell death. (Rose et al, 1987). It is selectively toxic for fungi because it combines primarily with ergosterol, a membrane sterol produced by fungi, but not humans. (Ingraham & Ingraham, 1995) Resistance to this antibiotic is not usually encountered in clinical practice. However, it is possible to isolate polyene-resistant yeast mutants in the laboratory, including the Candida organism. (Hamilton-Miller, 1972) Used topically the drug is virtually free from toxic or allergic side effects, and can be used safely during pregnancy. (South African Medicines Formulary 1996)

Although Nystatin seems to be the "perfect" drug in the treatment of muco-cutaneous candidiasis, a homoeopath alternative has to be found.

1.5. Homoeopathic principles

1.5.1 Law of Similars

Homoeopathic remedies are prescribed according to the Law of Similars. Hahnemann formulated this principle in 1796. (Vithoulkas, 1986) He published a paper entitled "An experiment concerning a new principle for determining the medicinal powers of drugs" in Hufeland's Journal. (Vithoulkas, 1986) This "new principle" involved the testing of drugs on healthy human subjects. His conclusion read as follows:

"Every active principle provokes its own kind of disease, as it were, in the human organism. We should imitate nature, for she often cures a chronic disease with another which comes in new. When it is a question of curing a particular disease (especially chronic disease), we should therefore use a drug which is capable of provoking another artificial disease, that
Drug tests on healthy subjects initiate an artificial disease and the symptoms of this should as closely as possible resemble those of the disease being treated. (Vithoulkas, 1986) Drug action resulting in man-made disease is something often encountered since the introduction of chemical drugs. These "diseases" are then explained as side-effects. (Vithoulkas, 1986)

The Law of Similars, first put down in print in 1796, was given its final classic form in Hahnemann's *Organon of Practical Medicine*:

"To achieve a gentle, rapid, certain and lasting cure, always choose a drug capable of provoking a disease similar (homoion pathos) to the one it is to cure."

The Law of Similars is based on comparison between the total pictures presented by two sets of facts. The patient's symptoms are matched against symptoms produced by the action of the drug in healthy subjects, making a phenomenological comparison. (Koehler, 1989)

An example of the implementation of this principle is as follows:

"Suppose your healthy, robust child suddenly develops a high fever, a flushed face, glassy dilated pupils, dry mouth without thirst, a dry raw sore throat, swollen maxillary glands, more prominent on the right side, and a wild kind of delirium causing him to try climb the walls. The allopathic physician interprets these symptoms and signs as evidence of a viral or bacterial infection and takes a culture of the throat in the hope of finding an organism which responds to antibiotics; this approach assumes that the "cause" is the microbe. The homoeopathic practitioner, on the other hand, is relatively uninterested in the nature of the microbe. He sees the symptoms as a manifestation of disturbance on the dynamic plane which can never be "cultured". The homoeopath, therefore, studies carefully the symptoms themselves in their totality. He searches for a substance which reflects as closely as possible the total picture of symptoms. In this example, such a substance is Belladonna; the patient is given Belladonna in a single, minute dose, the fever drops rapidly to normal, and the child falls into a peaceful sleep." (Vithoulkas, 1986)
Another aspect that is addressed in Homoeopathy is that of the vital force. This is explained in reasoning that the human organism is more than the mere sum of its physical components. From this is surmised the presence of an intelligent "vital force" which animates, guides and balances the human on all levels in both health and disease. (Vithoulkas, 1986) The defence mechanism is that aspect of the vital force responding specifically in the diseased state. During disease the vital force reaches the lowest state of activity and a stimulus, a homoeopathic remedy, will stimulate the vital force to bring about cure. (Vithoulkas, 1986)

The vital force is an influence that directs all aspects of life in the organism. It adapts to environmental influences, it animates the emotional life of the individual, it provides thoughts and creativity, and it conducts spiritual inspiration. (Vithoulkas, 1986) Clearly the vital force includes a wide variety of functions, and that aspect of the vital force which establishes balance in states of disease is called the "defence mechanism". It is an integral part of the vital force, but it is only one of the many functions. (Vithoulkas, 1986) The defence mechanism, acting on all three levels of the subject, the mental, physical and emotional levels, can be viewed as a tool of the vital force acting in the context of disease. (Vithoulkas, 1986) In light of the above explanation, it is not expected that this research will have a positive outcome, i.e. the destruction of the Candida albicans organism. The experiments will be conducted on the Candida albicans organism in vitro, and since homoeopathic remedies act upon the above-mentioned vital force within the body to cure disease, it is not expected to have an effect on the organism isolated outside the body.

1.5.3 Potentisation

When the body is in a diseased state, the frequencies within the dynamic plane, or vital force change. The body will thus resonate on a different level than when it is in a "healthy" state. A homoeopathic remedy, prescribed according to the Law of Similars will resonate with the dynamic plane of the patient and stimulate the vital force to restore the frequency to what is "normal" for that patient and bringing about cure. (Vithoulkas, 1986) In order to produce lasting curative results, it is necessary to increase the intensity of the electrodynamic field of the therapeutic agent, or in other words, we must liberate the energy contained within the
substance in such a way as to make it more available to interaction with the dynamic plane of the subject (Vithoulkas, 1986) Hahnemann used the technique of adding kinetic energy to the dilutions through shaking or "succussion". This technique of succussion is described as repetitive hitting of the remedy container on a solid surface or the palm of the hand, so as to add the extra energy. (Vithoulkas, 1986)

Hahnemann also implemented a technique called serial dilutions, where he diluted the substance until not one molecule of the original substance was left, way beyond Avogadro's number. (Vithoulkas, 1986) Hahnemann noted during his early studies that giving a drug in its traditional form was not the best way, since the drug reaction tended to be too powerful causing side-effects, excessive initial aggravation, or it was inadequate because the material had not been suitably prepared. He now ruled out all the above obstacles by reducing the dose to a minimum by means of the dilution process. (Koehler, 1989) Two scales are used in these dilutions, a 1 in 10 or decimal scale where 1 part of dry substance is added to 10 parts of solvent (alcohol/distilled water), or the centisimal or 1 in a 100 scale where 1 part dry substance is added to 100 parts of solvent. (Vithoulkas, 1986)

This combination of succussion and serial dilution Hahnemann called potenisation or dynamisation. (Vithoulkas, 1986) Both dilution and succussion are required to stimulate the dynamic plane. To date, there is no explanation in modern physics or chemistry for this phenomenon. It has been suggested that some new form of energy is released by this technique, where energy which is contained in a limited form in the original substance, is somehow released and transmitted to the molecules of the solvent. (Vithoulkas, 1986) Once the original substance is no longer present, the remaining energy in the solvent can be continually enhanced ad infinitum. The solvent molecules have taken on the dynamic energy of the original substance. (Vithoulkas, 1986) We know from clinical results that the therapeutic energy still retains the "vibrational frequency" of the original substance, but the energy has been defined to such a degree that it is capable of stimulating the dynamic plane of the patient sufficiently to produce a cure. (Vithoulkas, 1986)

The homoeopathic remedy, Calendula officinalis, which will be used throughout this research is in a D1, D2, D3 and D4 potency. A D1 potency is described as a 1 in 10 dilution i.e. 1 part of the active substance to 9 parts of dispensing (20%) alcohol, with 10-100 succussions in
between each potency. The motivation to use only low potencies was because *Calendula* is used to treat cutaneous candidiasis in a topical form, in low potencies. Since the conventional drug, Nystatin, is used only for topical treatment of candidiasis, it was deemed logical to use the remedy in potencies that were comparable to the conventional drug used.

1.5.4 Homoeopathic research

The Law of Similars has been proved by modern researchers such as Endler, (1991). This study was conducted on juvenile frogs at the two-legged stage. Homoeopathic Thyroxine was given to them to determine its effect on their jumping activity and was found to markedly reduce this activity. This is in conflict with the normal pharmacological action of conventional thyroxine, but in the light of the Law of Similars may be explained as a justifiable reaction (Endler, 1991).

The results of an experiment conducted by Koopman *et al*, (1990), investigating the inhibition of *Viscum album* on the proliferation of human fibroblast cell lines, mouse tumour cells and human carcinoma cell lines, stated that there was no cytostatic evidence that tumour or malignant cells were killed. *In vitro* specific anti-tumour effects of the remedy have been described, but *in vivo* effects may have been brought about by a mechanism differing from *in vitro* activity, for instance by stimulation of the immune system.

If during this study the results imply that the homoeopathic remedy has a direct effect on the yeast cells, then it may be assumed that a mode of action of this remedy is by acting directly against the microorganism. If this is not the outcome, it can be assumed that the mode of action lies within a mechanism that is inherent to the human body, i.e. the destruction of the yeast is not directly accomplished by the homeopathic remedy, but *via* the actions of the body itself. (Koopman *et al*, 1990)
1.6 *Calendula officinalis*

The homoeopathic remedy of choice in the symptomatic treatment of muco-cutaneous candidiasis is *Calendula officinalis*. *Calendula officinalis* is regarded homoeopathically as an antiseptic fungicide which implies that it is a substance that prevents growth by either inhibiting or destroying fungi. (Volk *et al*., 1980). This plant has been described as follows: "A most remarkable healing agent, applied locally. Useful for open wounds, parts that will not heal, ulcers etc. Promotes healthy granulations and rapid healing by first intention." (Boericke, 1921)

*Calendula* is a plant of the order Compositae. It is an annual herb with fibrous root. (Volk *et al*., 1980) It has large yellow or orange solitary flower heads. The fresh flowering tops that are used to manufacture the remedy, appear for the greater part of summer and fall and have a distinct odour. A mother tincture is prepared from the macerated flower tops that are steeped in alcohol. (The Homoeopathic Pharmacopoeia, 1954)

1.6.1 Origin of the plant

This plant grows natively in central, eastern and southern Europe. It is cultivated in Mediterranean countries, in the Balkans, Eastern Europe and to a small extent in Germany. Imports of the drug come from Egypt, Poland and Hungary. (Bisset, 1994)

1.6.2 Constituents of the plant

The plant consists of the following constituents:

i) Essential oils including menthone (0.4%), flavonol glycosides and their quercetin derivatives (0.3-1.5%).

ii) A mixture of several haemolytically active bisdesmosidic saponins (2-10%), triterpene alcohols, glucosidic sterols, carotenes and xanthophylls, polyacetylenes

iii) and bitter substances. (Bisset, 1994)
Phytochemical studies have reported three main groups of constituents for Calendula, namely flavonoids, volatile oil and triterpenes. (Anderson et al., 1996) The latter seems to represent the principal group, with many compounds isolated including pentacyclic alcohol, glycosides (saponins) and sterols. Flavonoid constituents may contribute to the anti-inflammatory effect, whilst the reputed antispasmodic effect may be attributed to the volatile oil fraction. In addition, immunostimulant activity has been reported for higher molecular weight polysaccharide components. (Anderson et al., 1996)
Figure 7: *Calendula officinalis* (Marigold)
1.6.3 Pharmacological actions

Preparations of the drug inhibit inflammation and promote the formation of granulation tissue. They are used externally in the form of infusions, tinctures, and ointments as a wound-healing remedy for inflammation of the skin and mucous membranes, for poor healing wounds, bruises, boils and rashes e.g. pharyngitis, dermatitis, leg ulcers. (Bisset, 1994)

A combination of allantoin and Calendula extract applied to surgically-induced skin wounds in rats has been reported to stimulate physiological regeneration and epithelisation. This effect was attributed to a more intensive metabolism of glycoproteins, nucleoproteins and collagen proteins during the regenerative period in the tissues. Allantoin applied on its own was found to exert a much weaker action. (Kioucek-Popova, 1982, cited by Anderson et al., 1996)

The essential oil has been shown to have Trichomonacidal action. (Gracza, 1987, cited by Bisset G. N., 1994). More recent studies in rats have again demonstrated the antiphlogistic effect (on carrageenan- and prostaglandin E-induced inflammation) and inhibition of leukocyte infiltration (Shipochliew et al., 1983, cited by Bisset G. N., 1994). An uterotonic effect by is exhibited by the aqueous extracts (Shipochliew et al., 1981, cited by Bisset, 1994). An antiphlogistic and choleretic action is exhibited by the flavonoid extract (Isakowa, 1980, cited by Bisset, 1994) and a bactericidal effect against Staphylococcus aureus can also be observed. (Dumenil, 1980, cited by Bisset, 1994)

In animal experiments, isolated constituents, (e.g. calendulosides) showed an anti-hyperlipidaemic effect and also a certain action on the central nervous system. (Lutomski, 1983, cited by Bisset, 1994)

Anti-inflammatory, antibacterial and anti-viral activities have been reported for Calendula. Weak anti-inflammatory activity in rats (carrageenan-induced oedema) has been reported, with effectiveness in treating burn oedemas and acute lymphoedema. (Peyroux et al., 1981, cited by Anderson et al., 1996) Activity against lymphoedema was primarily attributed to an enhancement of macrophage proteolytic activity. (Lutomski, 1983, cited by Bisset, 1994)
In-vitro cytotoxic activity and in-vivo anti-tumour activity (against mouse Ehrlich carcinoma) have been documented for Calendula extracts. (Boucard-Maitre et al., 1988, cited by Anderson et al., 1996)

A proprietary cream preparation containing various plant extracts, including Calendula, has been reported to reduce pain associated with post-mastectomy lymphoedema, although there was no significant clinical difference in the reduction of oedema between control and experimental groups. (Casley-Smith, 1983, cited by Anderson, 1996)

Calendula tincture 20% has been reported to be useful in the treatment of chronic suppurative otitis. (Wagner et al., 1985, cited by Anderson et al., 1996) Calendula extracts have been used to accelerate healing and to reduce inflammation. (Fleischner, 1985, cited by Anderson et al., 1996)

Calendula is traditionally reputed to affect the menstrual cycle. An uterotonic effect (in vitro) has been reported, and the triterpenoid constituents are reported to be effective as spermatocides and as antiblastocyst and abortion agents. In view of the lack of toxicity data, the use of Calendula is best avoided during pregnancy and lactation. (Anderson et al., 1996)

1.6.4 Indications for use

The most popular form of this medication for topical treatment is in a cream base. Two to five grams of the mother tincture is mixed on a slab with a wooden spatula with one hundred grams of a cream base such as aqueous cream. The cream should be applied copiously to the affected skin area and rubbed in thoroughly. The treatment should be repeated at least twice per day (The Homoeopathic Pharmacopoeia 1954)
1.7 The aims of the study

Candidal infections are commonly treated homoeopathically and show rapid response, especially vaginal candidiasis. (Boericke, 1989) Treatment of candidiasis occurs in three forms, firstly symptomatic remedies repeated often in the case of an acute attack using low potencies, secondly constitutional remedies in cases where the patient has a history of repeated candidiasis and thirdly nosodal treatment where the organism itself, homoeopathically prepared, is used to combat the infection. (Jouanny, 1991). In this study the aim is to determine whether Calendula officinalis, a known antiseptic, is effective in the inhibition or destruction of the organism Candida albicans, using low potencies in vitro.

This study is only a stepping stone for further experimentation of the actual action of Calendula officinalis on the organism and the in vivo effects of this compound.

If there is inhibition of cell growth in the laboratory, then it may be assumed that the medication had an effect on the yeast directly. It can also not be ruled out that the remedy may have an additional effect on the organism, caused by the stimulation of the body's own immune system. If there is no inhibition of cell growth, then it may be assumed that the medication works solely by means of this unknown mechanism in the body to combat the infection.

Because the action of Calendula officinalis has not been scientifically determined, and no examination of possible anti-fungal effects has been conducted on a yeast such as Candida albicans, this study will be important in determining these factors and thereby clarifying the action of this homoeopathic remedy.
CHAPTER 2: Materials and methods

2.1. Bacterial samples

Twenty clinical isolates of Candida albicans were obtained from the South African Institute of Medical Research, Johannesburg. These isolates were maintained aerobically at 37° C. (Labcon, Labex, Orange Grove) on fresh Sabouraud's dextrose agar plates (Biolab Diagnostic Pty. Ltd., Midrand).

Each strain was also freeze-dried in triplicate to ensure the longevity of the strains. This was performed using the LSL Sercfroid freeze-drying system. (Lyolab II, Zone Industrielle, Switzerland), according to manufacturer's instructions. Briefly the Candida albicans organism was cultured in serum broth (SAIMR, Johannesburg), for twenty-four hours until sufficient growth could be observed. One millilitre of the inoculum was pipetted into each vaccine vial. The vials were placed into the freezing bath for one hour. Thereafter the vials were removed and placed onto the shelves of the vacuum chamber where the vials were pneumatically sealed with rubber sleeve stoppers. Whenever isolates were required, vials were revived by adding one millilitre sterile distilled water.

2.2 Calendula officinalis

2.2.1 Preparation of potencies

The homoeopathic remedies were manufactured according to the Hahnemanian method by Natura Pharmacy in Pretoria. The Calendula D1 potency was manufactured by adding 5.0 ml of the mother tincture in 55% ethanol to 50ml of 20% ethanol, and succussing ten times by hand. The Calendula D2 potency was manufactured by adding 5ml of D1 potency in 73% ethanol to 50ml 20% ethanol, and succussing ten times by hand. The Calendula D3 potency was manufactured by adding 5 ml of D2 in 73% ethanol to 50ml of 20% ethanol, and succussing ten times by hand. The Calendula D4 potency was manufactured by adding 5ml of D3 potency in 73% ethanol to 50ml of 20% ethanol, and succussing ten times by hand.
2.2.2 Determination of the effects of ethanol on *Candida albicans*

The effects of ethanol, in which the medicine was manufactured, on *Candida albicans* was determined. Sabouraud's dextrose agar plates (SAIMR, Johannesburg), were inoculated from the *Candida albicans* nutrient broth cultures, which has been incubated for 24 hours at 37°C. Discs impregnated with ethanol of different concentrations were placed upon the agar plates. The agar plates were incubated at 37°C for 24 hours. The zones, or areas of clearing around the discs were regarded as an indication of inhibition by the ethanol. These results were recorded and analysed, and are displayed in Table 1.1. The 20% ethanol was found to have the least inhibitory effect on the organism, and was therefore regarded to be most suitable for the manufacturing of the remedies. The inhibitory effect the remedies have on the organism could therefore be solely attributed to the active ingredients found in *Calendula officinalis*.

2.3 Determination of the effects of *Calendula officinalis* and Nystatin on *Candida albicans*

2.3.1 Disc diffusion

Medicated discs were prepared by adding 1ml of *Calendula officinalis* remedy to one hundred filter paper discs. It was assumed that each disc absorbed 0.01 ml of the remedy. The bottles were stored in a dark cabinet.

Sabouraud's agar plates, divided into five segments, were inoculated from the *Candida albicans* nutrient broth cultures. (Unipath Limited, England) that were incubated for 24 hours at 37°C. The 24-hour broth was vortexed for 3 seconds.(Helena Labs, U. K.) to ensure even distribution. The above process was repeated for each strain in triplicate.

A medicated disc was placed into each segment. Four segments contained discs of the D1, D2, D3 and D4 *Calendula* potencies respectively. The fifth segment contained a Nystatin medicated disc, for the purpose of comparison.

The seeded plates were incubated for 24 hours at 37°C.

Zones of clearance formed after the 24 hours, and indicated inhibition of growth.
Measuring of the zone was performed by means of callipers (High Precision callipers, Mitutoyo)

2.4 Determination of the effect of Calendula officinalis as an anti-fungal agent against Candida albicans.

2.4.1 Determination of a standard curve

A standard curve was determined for the optical density (540nm) of the cultures against colony forming units per millilitre (cfu/ml). 10ml of nutrient broth were inoculated with five of the C. albicans strains. The tubes were incubated at 37°C for 24 hours.

After 24 hours the broth was vortexed and a serial dilution prepared for each strain. (10^0 - 10^8)

Optical density readings (540nm) (Jenway 6100 spectrophotometer, Jenway Ltd., England) were taken of each test tube in the respective serial dilutions. Calibration occurred against distilled water.

To determine the colony forming units per millilitre, 0.1 ml of each tube in the serial dilutions was pipetted onto a fresh Sabouraud's agar plate. The inoculum was smeared onto the plate to ensure even distribution of colonies.

The agar plates were incubated for 24 hours at 37°C. The colonies were counted and converted to cfu/ml for each concentration. OD 540nm readings were plotted against cfu/ml to produce a standard curve. This was then used to interpret OD540nm readings obtained in the broth cultures experiment.

2.4.2 Broth cultures

Candida albicans strains were inoculated into six test tubes each containing 4.5ml nutrient broth. 0.5 ml of D1 Calendula potency was added to the first test tube. This was repeated for potencies D2, D3 and D4 each in a respective test tube.
A fifth test tube served as a control, with no added remedy to the inoculated broth, to
determine the maximum growth possible for Candida albicans under the growth conditions.
A sixth test tube containing no organisms or remedy, served to indicate any contamination of
the broth itself. All test tubes were incubated for 24 hours at 37°C.

The above process was repeated with twenty strains, each strain in triplicate.

After incubation, optical density readings (540nm) were taken from each test tube of the
respective twenty strains. OD540nm readings were then converted to cfu/ml with the standard
curve prepared earlier.

2.5 Statistics

A statistical analysis of all results was performed. Means and standard errors were calculated
for all the experiments and one and two way analysis of variance tests were carried out where
appropriate to determine the true significance of the results.
CHAPTER 3: Results

All statistical analysis referred to in this chapter can be found in Appendix D. A P-value of P<0.05 was taken as the level of significance.

3.1 Determination of the effects of ethanol on Candida albicans

According to the results obtained during this experiment, it was clear that the 20% ethanol had the least inhibitory effect upon the Candida albicans organism. (Table 1.1) As can be seen from Table 1.1 this percentage of ethanol served to produce a zone of clearing with only one of the strains tested.

Distilled water, which served as a control during this experiment, also produced a zone of clearing with the same strain. It was not considered as a medium for preparation, as it would not serve as a preservation agent for the Calendula officinalis.

The 35% ethanol had similar results to that of the 20% ethanol, and could therefore also have been considered as an option for the medicating base. Previous research conducted on this matter (Anderson et al., 1996) concurred with the decision that it would be preferable to use the 20% ethanol concentration. 20% ethanol was then used in the preparation of all the homoeopathic potencies used in these experiments.

It can thus be concluded that any inhibitory effect observed during the experimentations was as a result of the Calendula officinalis potencies alone, and not as a result of the medicating base.
Table 1.1: Results of the determination of the effects of ethanol on *Candida albicans*

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* + represents presence of inhibitory zones

* - represents absence of inhibitory zones

* Results are expressed as means of n=3
3.2 Determination of the effects of Calendula officinalis and Nystatin on Candida albicans

3.2.1 Disc diffusion

The results of the experiments conducted to determine the effect of Calendula officinalis and Nystatin on Candida albicans are presented in Table 1.2.

From the Figure 8 it can be seen that there is a significant difference between all the Calendula officinalis potencies and the control, Nystatin. Nystatin was shown to have a powerful fungicidal effect upon Candida albicans, as was anticipated. Nystatin was much more effective in destroying the organism Candida albicans, and as a result formed zones twice to eight times as large as the zones formed by the Calendula officinalis remedies.

There was a significant difference between the D2, D3 and D4 potencies. No significant difference was calculated between D1 and D3, which seemed to be statistically very similar.

It can be seen according to the results (Figure 8) that the D2 potency had the biggest inhibitory effect on the organism, forming the largest zones of clearing amongst all the potencies. D4 was also, but slightly less so than D2. These two potencies ranked the highest in effectiveness of inhibition of the Candida albicans organism.

Although there was no significant difference between D1 and D3, it can be observed from Figure 8 that D1 was more inhibitory than D3. This difference was in fact so small that it was not significantly different.
Table 1.2: Results of the disc diffusion experiments to determine the effect of *Calendula officinalis* and Nystatin upon *Candida albicans*.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Zones of clearance in mm (+- SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nystatin</td>
<td>7.378(0.201)</td>
</tr>
<tr>
<td>Calendula officinalis D1</td>
<td>0.551(0.122)</td>
</tr>
<tr>
<td>Calendula officinalis D2</td>
<td>1.380(0.185)</td>
</tr>
<tr>
<td>Calendula officinalis D3</td>
<td>0.421(0.110)</td>
</tr>
<tr>
<td>Calendula officinalis D4</td>
<td>0.966(0.160)</td>
</tr>
</tbody>
</table>

* Results are expressed as means of n=20 strains.
Figure 8: Graphic display of the results of the disc diffusion experiments to determine the effect of *Calendula officinalis* and Nystatin upon *Candida albicans*.
During experimentations of this kind using allopathic substances, it is found that the effectiveness of inhibition will increase with an increase in concentration of the inhibitory substance. The pattern followed by the potencies of *Calendula officinalis*, in respect to the role that concentration of the inhibitory agent plays in the effectiveness of inhibition, did not follow this expected pattern.

A graph representing this phenomenon will then present as follows:

![Graph representation of the relationship between the concentration of a medication and inhibiting action](image1)

**Figure 9:** Graphic representation of the relationship between the concentration of a medication and inhibiting action

Using the homoeopathically prepared potencies this phenomenon was not observed. With an increase in concentration the substance did become more inhibitory. The results of this experiment presented graphically, appear as follows:

![Graph representation of the relationship between the concentration of *Calendula officinalis* remedies and their inhibitory action](image2)

**Figure 10:** Graphic representation of the relationship between the concentration of *Calendula officinalis* remedies and their inhibitory action.
3.3 Determination of the effects of *Calendula officinalis* as an anti-fungal agent against *Candida albicans*

3.3.1 Determination of a standard curve

The results of the experiments conducted to determine the standard curve, are presented in Figure 11.

The experiment was conducted with concentrations of each of five strains of *Candida albicans*. For each concentration an OD540nm reading and determination of colony forming units per millilitre was made.

The results were plotted on a graph, and a straight-line curve obtained.

The purpose of this standard curve was to determine the effects of the medications (*Calendula officinalis* potencies) on the *Candida albicans* organism in colony forming units per millilitre. For example, if an optical density reading of X was obtained during the broth cultures, it can be related to a colony forming units per millilitre reading with the use of the straight-line curve.
Figure 11: Graphic display of the results of the experimentations of the serial dilutions to determine the standard curve, with cfu/ml against OD540nm.

(Equation of regression: \( y = 200.1789 \times \text{OD540nm} - 1.9124 \))
3.3.2 Broth cultures

The results of the experiments conducted to determine the effect of Calendula officinalis as an anti-fungal agent against Candida albicans, are presented in Table 1.3.

There is no significant difference between the readings of each of the potencies and the broth with maximum growth without medication, implying that their effectiveness of inhibition was almost equal. It can be said that since the cfu/ml readings i.e. the amount of organisms that were not killed by the medication, were very similar to that of the unmedicated test tube, the medication seemed to have a very slight inhibitory effect on the organism.

In Figure 12, a histogram, the mean values of the various potencies and the broth without the medication are presented against the cfu/ml. As was expected the readings of the test tube containing no medication were highest. D3 and D1 gave almost equal readings, although D1 was a fraction lower, indicating that it had a slightly bigger inhibitory effect. Once again D2 and D4 had almost equal readings, although D2 gave a fractionally lower reading, indicating more inhibition of the organism.

Once again the results did not follow the expected pattern, as described in the disc diffusion experiment. It was expected that the degree of inhibition would increase with an increase in concentration of the medication, as is observed during similar experiments with conventional drugs. During this experiment this could not be observed, since the readings did not decrease with a decrease in concentration. Rather each remedy had a reading of random order.
Table 1.3: Results of the broth culture experiment to determine the effect of *Calendula officinalis* as an anti-fungal agent against *Candida albicans* in colony-forming units per millilitre (cfu/ml).

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Inhibition of organism in cfu/ml. (+-SE)</th>
</tr>
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<tbody>
<tr>
<td>Unmedicated broth</td>
<td>$1.57 \times 10^2$ (9.86 $\times 10^1$)</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> D1</td>
<td>$1.44 \times 10^2$ (7.59 $\times 10^1$)</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> D2</td>
<td>$1.27 \times 10^2$ (8.43 $\times 10^1$)</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> D3</td>
<td>$1.45 \times 10^2$ (8.12 $\times 10^1$)</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> D4</td>
<td>$1.29 \times 10^2$ (6.81 $\times 10^1$)</td>
</tr>
</tbody>
</table>

* Results are expressed as means of n=20 strains.
Figure 12: Graphic display of the results of the broth cultures experiment to determine the effect of *Calendula officinalis* as an anti-fungal agent upon the organism *Candida albicans*.
CHAPTER 4: Discussion

4.1 Determination of the effects of ethanol on Candida albicans

According to Ingraham & Ingraham (1995), an alcohol can be described as a compound with a hydroxyl (OH-) group, that kills by disrupting the lipids in cell membranes and by denaturing proteins. Ethanol is widely used as an antiseptic, and is most effective in a 50-70% solution in water.

Taking into consideration the above information, it was of the utmost importance to determine what percentage of ethanol would not destroy the yeast Candida albicans. This served to ensure that the inhibitory effects observed during the experiments were solely due to inhibition by the homoeopathic preparation Calendula officinalis, and that there was no contribution offered by the ethanol.

During the experiments it was found that of the whole spectrum of ethanol concentrations, ranging from 96% to 0%, the 20% ethanol concentration, i.e. 20 parts of alcohol in 100 parts of distilled water, had the least effect on the organism itself. Only in a very few instances were zones formed, implying that only a very slight degree of inhibition was caused by the 20% ethanol. Distilled water also exhibited a low percentage of inhibition, and would therefore also have been suitable for a dilution medium, but because of the undesirability of this medium’s preservation status, it was decided that the 20% ethanol would be the preferred medium for the manufacturing of the homoeopathic preparation of Calendula officinalis.

4.2 Comparison of the effects obtained using the two methodologies.

The process of potentisation contributes to an essential part of the philosophy of Homoeopathy. (Vithoulkas, 1984) Potentisation represents both serial dilutions and succussions. Hahnemann, the father of Homoeopathy, implemented the technique of dilutions. He diluted the substance until not one molecule of the original substance remained, but only the energy inherent to this particular substance. (Vithoulkas, 1984) Two scales are used for these dilutions, the centisimal and the decimal. The centisimal scale represents a 1 in 100
Along with this process, succussion was also implemented. Succussion implies the liberating of the energy contained in the substance in such a way as to make it more available for interaction with the dynamic plane of the organism. When considering this process of potentisation, it becomes evident that each potency of a remedy represents a whole different level of energy and dilution, and can therefore almost be considered as a different medication in a range of medications manufactured from the same source. (Hahnemann, 1982)

This explanation would then throw more light on the peculiar patterns observed during this experiment. As a rule we would expect the number of organisms to decrease with an increase in the concentration of an inhibiting substance. This phenomenon was not observed at all during the broth cultures experiment, and a graph of abnormal presentation was observed.

The graph can be presented as follows:

Figure 13: Graphic display of the individual actions of each of the Calendula officinalis potencies in the broth cultures experiment.
As explained above, this peculiarity could be explained by the assumption that every different potency, i.e. the D1, D2, D3 and D4 potencies, acted as individual medications with their own mode and degree of action of inhibition.

A good example of this can be illustrated by means of the actions of Hepar Sulfuris. This remedy is commonly used in conditions of suppurations e.g. boils, abscesses and septic wounds. A higher potency of this remedy, i.e. a 9CH, will promote the absorption of the pus, while a lower potency, i.e. a 4CH will promote the formation of pus to improve drainage.

Another example of this is mentioned in Jouanny, (1984). According to this author, Silicae made from rock crystal or quartz can be used to stop suppuration in a high potency and to drain pus in a low potency.

These suggested potencies is not governed by any laws, and is prescribed according to the above principle solely based on experience gained by these various authors during the prescription of these remedies. It can therefore not be assumed that every patient will react the same way when treated with these remedies and certain individuals may need different potencies of the remedy to achieve favourable results. These principles can therefore only be used as a guide-line in prescription.

To illustrate the diversity of potencies used in different references by different authors it will be useful to look at the potencies suggested in the treatment of breast tumours. The most suitable remedy for pathology of this kind is Conium maculatum, the Poison Hemlock. According to Boericke, (1921) the most favourable potencies will be the higher potencies given infrequently. According to Jouanny, (1984) a 7CH or 9CH potency must be prescribed every day upon awakening. A few other examples of this contradicting kind can be quoted from various sources.

This then illustrates the diversity in choice of the most effective potency for certain ailments. According to Vithoulkas, (1980) there are no set rules when choosing a suitable potency, and experience and observation plays a very large role. Vithoulkas also states that the selection of a potency is secondary in importance to remedy selection. “The Law of Similars is the primary law of cure, and the process of potentisation is merely an accessory factor. If the
correct remedy is selected, then it will act curatively in any potency, even though a correct potency will act more gently for the comfort of the patient; conversely, an incorrect remedy can be either inactive or disruptive to a case, regardless of what potency is given.”

In the experiments it was noted that some potencies were more effective than others. The diversity in the choice of a remedy for a certain condition will show that certain potencies has a definite more favourable effect in curing in certain individuals than another potency. This “perfect” potency, matching the resonance of the disease, can only be determined through experience and trials, since it has to be determined according to the individual patient treated. Since this study was conducted in laboratory conditions and not in a human subject, no real conclusions can be drawn relating to the potencies that was more anti-fungal than the others. If this study was conducted in humans the outcome may have been totally different. This once again reinforces the suggestion that in order to understand the action of Calendula officinalis as an anti-fungal agent properly, this study has to be repeated in vivo.

From the broth cultures and disc-diffusion experiments a correlation can be made concerning the inhibitory actions of the various potencies.

A graph representing the results obtained during the disc-diffusion experiment is presented for comparison with the broth cultures experiment:
Figure 14: Graphic display of the individual actions of each of the *Calendula officinalis* potencies in the disc diffusion experiment.

During the broth cultures experiment the D2 potency seemed to exhibit the largest inhibitory action. It had the lowest OD540nm reading, implying that it inhibited the most organisms i.e. the least organisms alive. The D4 potency was also effective in inhibition, but less so than the D2 potency. The D1 and D3 potencies were found to be the least effective i.e. the highest OD540nm readings, with the D1 potency the least effective inhibiting the least organisms.

During the disc diffusion experiment the D2 potency formed the largest zone of clearing of all the potencies. This implies that it had the largest inhibitory effect upon the yeast, excluding that of the conventional drug Nystatin. Once again the D4 potency proved to be inhibitory in its action but less so than the D2 potency. The D1 and D3 potencies were the least inhibitory forming the smallest zones of clearing, with the D1 potency once again the least inhibitory with the smallest zone of clearing.

There is thus a definite correlation between the results of the two experiments, deeming the D2 potency the most effective in inhibition, followed by the D4 potency. The D1 potency proved to be the least effective overall, with the D3 potency just a fraction more inhibitory than the D1 potency.
4.3 Determination of the effects of *Calendula officinalis* and Nystatin on *Candida albicans*

During the experiments it was evident that the conventional treatment, Nystatin, was much more effective in inhibiting the organism *Candida albicans*. The zones formed by the Nystatin, were much larger than those produced by the *Calendula officinalis*, and conformed to the standard zone size according to Baker (1980), of 7.8 mm. The zones formed by the homoeopathic preparation were three to four times smaller than those of the conventional drug. Although these zones were small, they cannot be neglected as not significant, for an inhibitory effect was noted.

Antibiotic resistance first appeared soon after antibiotics came into common use. The ways in which an organism can change genetically to become resistant to an antibiotic can be explained according to the following three statements given by researchers (Ingraham & Ingraham, 1995):

1) The target in the microbial cell changes so that it remains capable of fulfilling its vital cellular function and is no longer sensitive to the antibiotic.

2) The microbial cell becomes able to exclude the antibiotic or to pump it out of the cell after it enters.

3) The cell becomes able to destroy the antibiotic chemically.

In the light of the above statements, we can now debate the suitability of conventional treatments such as Nystatin in candidiasis, even though it appeared to be more effective in destroying the organism than its homoeopathic counterpart. Unlike antibiotic treatments that have to enter the cell to destroy it, homoeopathy acts on a whole different plane.

According to Vithoulkas, 1986, our bodies i.e. the cells our bodies are made up of, resonate at a certain frequency within the dynamic plane. During a diseased state this frequency will change. When a homoeopathic remedy is prescribed according to the Law of Similars, i.e. a remedy picture that will duplicate the disease picture, the vibrations of this homoeopathic
remedy will stimulate the vital force, the body's inherent "Healer", to restore the frequency to what is "normal" for that particular patient and bring about cure.

Yearly more antibiotic resistant strains of micro-organisms are emerging. This can be explained in light of the frequent and unnecessary prescription of these drugs by the medical fraternity. The microorganisms are not always isolated during the infection and subsequently the wrong antibiotics are often prescribed, creating the opportunity for these micro-organisms to develop resistance. Another complication is that the patients do not complete the course of antibiotics prescribed for them, only taking it for a few days until they feel better, therefore only destroying a certain percentage of the organisms, leaving the rest of the micro-organisms with the chance to develop resistance. (Ingraham & Ingraham, 1995)

We are also acquiring antibiotics by consuming meat that came from livestock treated with these antibiotics. As explained previously, this creates the opportunity for micro-organisms to develop resistance. (Ingraham & Ingraham, 1995)

The medical field is steadily running out of antibiotic generations that can be used to treat infections, and are experiencing lots of frustration with infections that are just not responding to treatments previously known to be effective. (Ingraham & Ingraham, 1995) Now may therefore be the right time to start viewing other treatments such as homoeopathy as an alternative in the treatment of these diseases.

As previously explained, the way in which homoeopathy is believed to work i.e. the mode of action, differs greatly from those of conventional medicines. It is thought that the medication will act upon the vital force governing the human body, and not the diseased cell itself. This may be the reason why the homoeopathic preparation seemed to be inferior in its action of inhibition of the organism. Nystatin acts by damaging the plasma membrane and allowing the cell contents to leak out. (Goodman, 1975) This then demonstrates the direct cellular action of Nystatin opposed to the indirect action of the homoeopathic preparation on the vital force.

It is therefore important, in further research, to conduct this experiment in vivo, i.e. in a living human being. It is necessary to observe what effect the remedies will have upon the micro-organism, Candida albicans, when the action of the vital force comes into play.
Homoeopathy is known to be a slow, deep-acting treatment. (Boericke, 1921) It strives by considering the patient as a wholistic being, to bring about a permanent cure. This process of treating the patient as the sum of different parts, is called constitutional treatment. (Koehler, 1989) This is also very important to consider during further research on this topic. Candidiasis cannot be treated only by targeting the organism itself, but by treating the patient as a whole, considering all the presenting symptoms.

Another speculation might be that since the homoeopathic remedy acts upon the vital force, which governs the health of the human body, the body may prefer a slower, more gentle approach to cure than the aggressive action employed by their conventional counterparts.

4.4 Determination of the effect of *Calendula officinalis* as an anti-fungal agent against *Candida albicans*

According to David Hoffmann, (1996), *Calendula* is believed to be most effective in its herbal form i.e. the mother tincture. In spite of this statement *Calendula officinalis* is also considered as an effective anti-fungal agent in homoeopathic potencies up to the third potency, according to Boericke, (1921). For this research, homoeopathic potencies D1, D2, D3 and D4 were used, thus excluding the mother tincture. *Calendula officinalis* mother tincture could only be manufactured in 63% ethanol, which as can be seen from our results would have exhibited anti-fungal activity.

During the experiments with the broth cultures it was observed that the homoeopathic remedy, *Calendula officinalis*, did not have a marked effect upon the organism *Candida albicans*. Although no statistical significance was noted between the unmedicated broth culture and the *Calendula* medicated broth cultures, it can be observed from Figure 12 that the homoeopathic remedy did have a slight inhibitory action.

Once again the lack of inhibitory action of the homoeopathic preparation is debatable. As explained in the disc-diffusion experiment, it can once again be employed that the indirect effect that the remedy has on the body’s vital force, may play a significant role. The remedy will act upon the dynamic plane to correct vibrations that has been disturbed by the diseased state of the human being. The vital force will then govern the body to bring about cure. It is
therefore evident that only a slight inhibitory action could have been expected from the remedy's direct action upon the yeast, *Candida albicans*. This should be considered in future research on this topic. An *in-vivo* study might prove very helpful to establish the true ability of *Calendula officinalis* to fight candidiasis.

This experiment conducted in a laboratory, and not in a human being, may limit the action of the homoeopathic remedy, since the curing success of a remedy depends upon its prescription for the totality of symptoms. (Jouanny, 1991) None of this was considered and it is therefore difficult to speculate on the true suitability or unsuitability of this remedy in the treatment of candidiasis.

This experiment was conducted to determine the effectiveness of *Calendula officinalis* as a topical anti-fungal agent. This includes the use of *Calendula* in a cream base for skin affections, as a mouthwash for oral thrush, and as a douche/ovule in vaginal thrush. It was therefore compared to its medical counterpart Nystatin. According to Murphy, (1993), *Calendula* can be used for the following conditions:

i) inflammation of tissues after surgery

ii) inflammation of wounds

iii) general injuries

iv) complications from surgery, and more specific and to prevent or arrest gangrene.

v) complications from surgery with inflammation

vi) complications from surgery of wounds

vii) dissecting wounds

viii) wounds with granulations

ix) offensive wounds

x) old neglected wounds with pain

xi) reopening of old wounds

xii) septic wounds, slow to heal, maggoty wounds

xiii) and finally ulcers of the female genitalia
The beneficial action of *Calendula officinalis* to control the growth of micro-organisms in abnormal skin conditions, can be seen from the above. It can even be used for septic, offensive and maggoty wounds. (Murphy, 1993)

It is noted that there are no indications for the use of *Calendula officinalis* for the treatment of candidiasis in Murphy’s Repertory, neither in any other reliable *Materia Medica*. This may lead to the conclusion that *Calendula* will therefore not be successful in the treatment of candidiasis. This statement is incorrect.

The use of *Calendula* lies within its antiseptic action used as a complementary remedy assisting other remedies. When the suggested treatment of systemic candidiasis is investigated, remedies such as *Helonia* (Unicorn-root), *Medorrhinum* (the gonorrhoeal virus), *Sepia* (inky juice of cuttle-fish), and *Thuja* (Arbor vitae) are encountered. (Murphy, 1993)

These are the remedies that are used according to the Law of Similars, because of their exhibition of similar symptoms, in the effective treatment of candidiasis. These remedies are used in conjunction with a local application such as *Calendula officinalis* when topical, oral or vaginal manifestations can be observed.

*Calendula* may be prescribed for the treatment of slow-healing or deep wounds in high potencies as a similimum (Jouanny, 1991), but no conscientious homoeopath will endeavour the treatment of such a condition with only the use of *Calendula* and no constitutional remedy. (Jouanny, 1993)

It is always essential, according to Jouanny, (1993), to treat wholistically as well as according to the symptomatology. Candidiasis can therefore only be successfully eradicated with the treatment with the similimum. Vithoulkas, (1984), also stated that a person has to considered as a whole and a prescription must include all the presenting symptoms of the patient in his present diseased state. If a patient is only treated with a symptomatic remedy, the predisposition of that patient for that specific disease will never be eliminated and will recur often e.g. a patient with candidiasis that is not treated with the similimum will have recurring candidiasis and even though the topical treatment will afford relief of the disease in its skin manifestation, recurrence will often occur with exacerbations. (Vithoulkas, 1984)
In this research the anti-fungal activity of *Calendula* was only measured by means of *in vitro* studies. This then once again brings to our attention the fact that homoeopathic action centres upon the workings of the vital force in the body. Because human specimens were not used for the experiments, the law of wholistic consideration and treatment was not considered.

Candidiasis is endogenous to man. The inflammatory reactions of the skin are normally mild and superficial. The sites affected are principally mucosa where *Candida albicans* is normally present in health. Although from the above statement it can be deduced that candidal skin disease are normally mild with slow progression, some forms of systemic candidiasis can be life-threatening with rapid progression. Once again this emphasises the conclusion that candidiasis cannot be treated solely with *Calendula* in either low or high potency if the similimum is not reached.

Since the homoeopathic treatment of systemic candidiasis has not been researched fully, it is debatable whether the treatment of this disease with homoeopathy will be just as effective as the medical treatment.

This sheds light on the results obtained during the research reflecting the fact that *Calendula* was not as effective as Nystatin in the destruction of the fungus *Candida albicans*. It can now be speculated that the absence of a constitutional treatment and an *in vitro* study, excluding the vital force, contributes to the inferior anti-fungal action of *Calendula* in comparison to Nystatin.

It is now necessary to determine whether *Calendula* used in higher potencies as a similimum in a patient diagnosed with systemic candidiasis, will prove to be effective in the treatment of this condition. This would involve not using *Calendula* as a complementary remedy, but as the sole remedy for the constitutional treatment. This was not investigated during this study and may prove of great value in any further studies.

*Calendula officinalis* is considered to be an effective antiseptic agent, and even though no actual scientific studies have been conducted previously, it has been used throughout centuries to combat infections. According to Jouanny, (1991), *Calendula* clearly has antiseptic, analgesic and healing action and can therefore be used to treat fungal infections. During this
research many questions have been raised and the actual action of this homoeopathic remedy seems to be vague and requires further research. Results were recorded that differ greatly from what is usually accepted in a microbiological field.

It was important to note that unlike conventional drugs that exhibit a straight line graph relationship when concentration of organisms is plotted against the concentration of the drug, the homoeopathic remedy acted totally differently, opening the debate as to whether each different potency exhibits a different action. It may be interesting in future studies, to use increasing potencies i.e. D5 and upwards, to observe any patterns.

From a homoeopathic point of view it was also important to determine whether the action of the homoeopathic preparation centred upon the action of the actual substance within the remedy or if the vital force contributed to the anti-fungal activity. No definite evidence was obtained in this matter, but it can be said that a more significant effect would have been expected if the anti-fungal activity lay within the actual substance. Only a small degree of inhibition was observed and it can therefore be debated that there was probable action by the vital force.

From the readings obtained in this research it is evident that it is of the utmost importance to conduct a repeat study in-\textit{vivo}. Only then will it be possible to clear up the discrepancies relating to the vital force and its action and the importance of constitutional prescribing.


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## APPENDIX A

### Disc-diffusion readings in (mm)

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CHAPTER 5: References


Braude A. I., Davis C. E., Fierer J., 1982, Microbiology, W. B. Saunders company, Philadelphia; pg. 643-669.

APPENDIX B

Results of the broth cultures experiment to determine the effect of *Calendula officinalis* as an anti-fungal agent upon the organism *Candida albicans* in cfu/ml.

<table>
<thead>
<tr>
<th>Strain</th>
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<th>D2</th>
<th>D3</th>
<th>D4</th>
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## APPENDIX C

Spectrophotometer (OD) and cfu/ml readings for the determination of the standard curve

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APPENDIX D

Monday, December 08, 1997, 09:56:04

One Way Analysis of Variance

Normality Test: Failed (P = 0.0001)

Test execution ended by user request, ANOVA on Ranks begun

Monday, December 08, 1997, 09:56:04

Kruskal-Wallis One Way Analysis of Variance on Ranks

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
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<td>D1</td>
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<tr>
<td>D2</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>D3</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>D4</td>
<td>20</td>
<td>0</td>
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</tbody>
</table>

| Group | Median | 25%            | 75%            |
|-------|--------|----------------|
| D1    | 3.30E-001 | 0.00E+000  | 1.13E+000 |
| D2    | 1.25E+000 | 9.25E-001  | 1.74E+000 |
| D3    | 1.00E-001 | 0.00E+000  | 9.00E-001 |
| D4    | 8.24E-001 | 3.40E-001  | 1.45E+000 |

H = 2.12E+001 with 3 degrees of freedom. (P < 0.0001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 9.72E-005)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method):

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<th>Comparison</th>
<th>Diff of Ranks</th>
<th>p</th>
<th>q</th>
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<tbody>
<tr>
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<td>4</td>
<td>5.92E+000</td>
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<td>D2 vs D1</td>
<td>4.93E+002</td>
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<td>6.31E+000</td>
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<tr>
<td>D2 vs D4</td>
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<td>2</td>
<td>4.40E+000</td>
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<tr>
<td>D4 vs D3</td>
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<td>4.94E+000</td>
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<td>D1 vs D3</td>
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<td>2.35E+000</td>
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Comparison P<0.05
D2 vs D3 Yes
D2 vs D1 Yes
D2 vs D4 Yes
D4 vs D3 Yes
D4 vs D1 Yes
D1 vs D3 No
One Way Analysis of Variance

Normality Test: Failed (P = 0.0213)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

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</tr>
<tr>
<td>D2</td>
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<td>0</td>
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<tr>
<td>D4</td>
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</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Median 25%</th>
<th>75%</th>
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<td>8.07E+000</td>
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<tr>
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<td>1.13E+000</td>
</tr>
<tr>
<td>D2</td>
<td>1.25E+000</td>
<td>1.74E+000</td>
</tr>
<tr>
<td>D3</td>
<td>1.00E-001</td>
<td>9.00E-001</td>
</tr>
<tr>
<td>D4</td>
<td>8.24E-001</td>
<td>1.45E+000</td>
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</table>

H = 6.13E+001 with 4 degrees of freedom. (P = <0.0001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 1.58E-012)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunnett's Method):

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<th>Diff of Ranks p</th>
<th>q'</th>
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<td>D4 vs N</td>
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<tr>
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Comparison | P<0.05 | Yes
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<tbody>
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<tr>
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<tr>
<td>D4 vs N</td>
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<tr>
<td>D2 vs N</td>
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</table>
One Way Analysis of Variance

Normality Test: Passed \( (P = 0.2272) \)

Equal Variance Test: Passed \( (P = 0.4202) \)

Group N Missing
unmed 19 0
d1 20 0
d2 20 0
d3 20 0
d4 20 0

Group Mean Std Dev SEM
unmed 157.9 43.0 9.86
dl 144.5 34.0 7.59
d2 128.0 37.7 8.44
d3 145.7 36.3 8.13
d4 130.0 30.5 6.82

Power of performed test with alpha = 0.0500: 0.3699

The power of the performed test (0.3699) is below the desired power of 0.8000.
You should interpret the negative findings cautiously.

Source of Variance DF SS MS
Between Treatments 4 11974.2 2993.5
Residual 94 124997.4 1329.8
Total 98 136971.6

Source of Variance F P
Between Treatments 2.25 0.0693
Residual
Total

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference \( (P = 0.0693) \).